GENETIC MARKERS FOR BEER-SPOILAGE BY LACTOBACILLI AND PEDIOCOCCI

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By

Monique Haakensen

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ABSTRACT

The brewing industry has considerable economic impact worldwide; therefore, demand exists for a better understanding of the organisms that cause beer-spoilage. Low nutrient levels, depleted oxygen levels, high alcohol levels, and the presence of antimicrobial hop compounds all play a role in making beer an inhospitable environment for most microorganisms. Nonetheless, there are bacteria that are resistant to all of these selective pressures. The most common beer-spoilage bacteria are the Gram-positive lactic acid bacteria *Lactobacillus* and *Pediococcus*. It is currently believed that hop-resistance is the key factor(s) permitting *Lactobacillus* and *Pediococcus* bacteria to grow in beer. However, it is likely that in addition, ethanol-tolerance and the ability to acquire nutrients also play roles in the beer-spoilage ability of *Lactobacillus* and *Pediococcus* isolates. The ability of *Lactobacillus* and *Pediococcus* to grow in beer was assessed and correlated to the presence of previously described beer-spoilage related genes, as well as with the presence of novel genes identified in this study. Molecular and culture-based techniques for detection and differentiation between *Lactobacillus* and *Pediococcus* isolates that can and cannot grow in beer were established and described in detail. Interestingly, beer-spoilage related proteins were often found to share homology with multi-drug transporters. As such, the presence of these beer-spoilage associated genes was also compared to the ability of isolates to grow in the presence of a variety of antibiotics and, unexpectedly, beer-spoiling bacteria were found to be more susceptible to antibiotics than were non beer-spoiling isolates of the same genus. Additionally, it was found that isolates of *Lactobacillus* and *Pediococcus* that can grow in beer do not group phylogenetically. In order to fully appreciate the relationship of speciation with beer-spoilage, phylogenetic and whole genome/proteome studies were conducted to clarify the taxonomy of the *Lactobacillus* and *Pediococcus* genera. Through the research in this thesis, a greater understanding of the mechanism(s) enabling bacteria to grow in beer has been gained and taxonomy of the genera *Lactobacillus* and *Pediococcus* has been clarified.

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LIST OF ABBREVIATIONS

ABC ..... ATP-binding cassette
ATCC ..... American Type Culture Collection
ATP ..... adenosine tri-phosphate
BLAST ..... basic local alignment search tool
BU ..... bitterness units
CFU ..... colony forming units
COG ..... cluster of orthologous groups
Ct ..... cycle threshold
FAM ..... fluorescein
HGA ..... hop-gradient agar
HGA+E ..... HGA plus ethanol
LAB ..... lactic acid bacteria
LSM ..... lactic acid bacteria susceptibility test medium
MDR ..... multidrug resistance
MIC ..... minimum inhibitory concentration
ML ..... maximum likelihood
MLSA ..... multilocus sequence analysis
mMRS or MMRS ..... modified MRS (Tween 80™ omitted)
MP ..... maximum parsimony
MRS ..... de Man, Rogosa, Sharpe
MSA ..... multiple sequence alignment
NCBI ..... National Centre for Biotechnology Information
NR ..... non-ropy
NSERC ..... Natural Sciences and Engineering Research Council (of Canada)
PCR ..... polymerase chain reaction
R ..... ropy
RDPII ..... ribosomal database project II
rPCR ..... real-time PCR
UPGMA ..... unweighted pair group method of arithmetic means

CHAPTER 1

LITERATURE REVIEW, INTRODUCTION, AND OBJECTIVES

Literature Review and Introduction

1.1 General overview

Because each chapter of this thesis is presented as a published manuscript or manuscript in preparation, the relevant literature review and introduction are provided within each chapter. The purpose of this general literature review/introduction chapter is therefore to give a brief overview of the subject matter that will be covered in the context of the thesis as whole. As this chapter is written from the perspective of the research environment at the onset of the thesis work, chapters of this thesis are referred to where results are necessary to clarify the continuity and progression of the work.

1.2 Beer-spoilage bacteria

The brewing industry has considerable economic impact worldwide. Therefore, demand exists for better understanding of the organisms that cause spoilage and fiscal losses. Only certain bacteria can survive the antibacterial hop-compounds, acidic pH, low levels of nutrients and oxygen, and high alcohol levels found in beer [1, 2]. While many aspects of various beers have been studied in hopes of determining the causes of susceptibility to spoilage, the most reliable measure is alcohol content and hop-concentration [3]. However, it is likely that in addition to hop-resistance and ethanol-tolerance, the ability to acquire nutrients may also play a role in the beer-spoilage ability of *Lactobacillus* and *Pediococcus* isolates. Figure 1.1 shows a schematic representation of this concept and the interplay of factors that may be required by a bacterium to grow in beer.

Despite the presence of multiple inhibitory pressures in beer, some bacteria have emerged that are resistant to all of the selective pressures, including hop-compounds. These so called "beer-spoilage bacteria" fall into four main categories: wort spoilers, acetic acid bacteria, anaerobic Gram-negative bacteria, and the Gram-positive lactic acid bacteria (LAB). The most commonly occurring beer-spoilers belong to the LAB genera *Lactobacillus* and *Pediococcus* [1, 2].

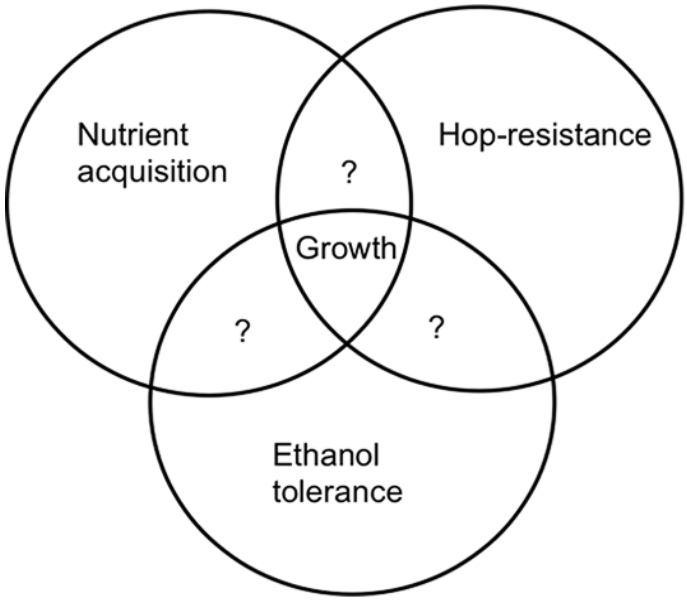


Figure 1.1 - Hypothesized basis for ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer. A combination of hop-resistance, ethanol-resistance, and nutrient acquisition mechanisms are required to permit an organism to grow in beer. A combination of only two categories would result in an uncertain phenotype, as indicated by the question marks.

1.2.1 Beer-spoilage *Lactobacillus* and *Pediococcus*

The genera *Lactobacillus* and *Pediococcus* contain organisms that are Gram-positive, usually non-motile, non spore-forming, and facultatively anaerobic, and that are found in a wide range of environments. The bacteria used in this study grow readily in an oxygen-reduced environment in de Man-Rogosa-Sharpe (MRS) broth at 30°C [4-6].

During the brewing process, one mole of glucose is broken down into two moles of ethanol and two moles of carbon dioxide. However, in the presence of LAB, glucose is metabolized into one mole of ethanol and one mole of lactic acid, producing not only an off-taste, but also lower ethanol and CO_2 yields. Some LAB can also produce diacetyl which, like lactic acid, confers an abnormal smell and taste to the beer. While some isolates of *Lactobacillus* and *Pediococcus* are beneficial in numerous settings (e.g., starter cultures for yogurt and cheeses, immunomodulators/stimulators, probiotics, and possible anti-ulcer treatments [7]), their presence in breweries is ultimately detrimental to the brewing industry, with the exception of the unique production method used for lambic beers [2].

1.3 Antimicrobial mechanism of hop-compounds

There are six traditional reasons for the addition of hop-compounds during the brewing process: (i) to impart bitterness and hoppy character, (ii) to act as a filter-aid, (iii) to assist in the sterilization of wort, (iv) to promote foam lacing, (v) to enhance and stabilize beer foam, and (vi) to confer antibacterial properties to beer [8]. The active antimicrobial form of the hop-compounds is iso-α-acid in the *trans* form of the isomer (i.e., *trans*-isohumulone). Simpson [9] and Sami *et al.* [10] have both reported that moderate *trans*-isohumulone concentrations can exert a bacteriostatic effect on both *Lactobacillus spp.* and *Pediococcus spp.*, with growth of the bacteria resuming after the organisms are transferred to fresh medium. The *trans*-isohumulone acts by penetrating the cell membrane disrupting the ionic flow and thus destroying the trans-membrane pH gradient [1, 10]. It is important to note that while some papers claim that there is no relationship between resistance to *trans*-isohumulone and bacterial ability to spoil beer, it is the concentration of undissociated acid that must be determined (calculated based on concentration and pH) in order to establish the correlation [3, 11].

1.4 Hop-resistance

Mechanisms of hop-resistance have the common feature that they must permit the organism to maintain a trans-membrane pH gradient in the presence of hops. While beer-spoilage LAB constitutively express hop-resistance, they are not invariably able to grow in beer – a further induction is required for growth [12]. This is achieved through the passaging and growth of the organism in medium containing a sub-inhibitory concentration of hops before being subcultured to beer. It may be that this induction step is required to provide the cell with a stimulus that causes the cell to increase the transcription of a gene or genes involved in hop-resistance. Resistance to the hop compound *trans*-isohumulone by a given LAB could be accomplished by one or more of the mechanisms discussed below.

1.4.1 Enzymatic inactivation of *trans*-isohumulone

While there are currently no known hop-resistance mechanisms of this type, it must remain as a consideration that bacteria may be capable of inactivating hop-compounds by enzymatic means.

1.4.2 Target alteration and/or inhibition of influx

It is through the cell membrane that *trans*-isohumulone enters the bacterial cell, and as such, it is possible that altered lipid composition could change the permeability, or a switch to extra long-chain fatty acids could prevent the entrance of *trans*-isohumulone into the cytoplasm. These two mechanisms of target alteration have not been investigated beyond the initial report [1]. Inhibition of influx could also be accomplished by the formation of an impermeable barrier around the bacterial cell. Some LAB are capable of producing exopolysaccharide. In the brewing industry, the ability to produce this exopolysaccharide is referred to as a "ropy" phenotype [13]. A ropy phenotype results in a substantial increase of viscosity of liquid culture and wet looking colonies when grown on solid agar. While it is known that many beer and wine spoilage organisms possess this phenotype, studies have not been performed to establish a correlation between its presence and the ability to grow in beer. While it is likely that a ropy biofilm would permit bacteria to establish a permanent infection that may persist during cleaning of brewing equipment, it is unknown whether the rope could act as a physical barrier to entry of the cell and thereby confer hop-resistance.

1.4.3 Active efflux

In this scenario of hop-resistance, *trans*-isohumulone is allowed to enter the cell and is then actively pumped out of the cell before a large amount of damage is caused. A known example of this mechanism is the product of the multi-drug resistance gene *horA*. The *horA* gene is thought to confer hop-resistance to the bacterium, having previously been found in isolates of *Lactobacillus brevis* [10], *Lactobacillus casei* [14], and *Lactobacillus lindneri* [14], and more recently in isolates of *Lactobacillus paracollinoides* [15] and *Pediococcus damnosus* [16]. The *horA* gene codes for what appears to be a trans-membrane protein with an ATP-binding cassette (ABC) domain consensus sequence [10]. The HorA protein shares significant homology with known ABC multi-drug resistance (ABC MDR) type transporters which are primary-type multidrug transporters. It is hypothesized that HorA acts to restore the pH gradient by transporting *trans*-isohumulone out of the cell [1].

1.4.4 Over expression of H+ ATPase

It is possible that the over expression of existing H+ ATPases to pump out protons released by *trans*-isohumulone could also confer resistance to hop-compounds. In this situation, uncharged *trans*-isohumulones enter the cytoplasm where they dissociate and remain in the cell, but their activity is counteracted by pumping protons out of the cell to maintain a trans-membrane pH gradient. This method would be unlikely to result in sufficient levels of resistance, however, as it would require large amounts of energy to be expended by the bacterial cell while living in a low-nutrient environment.

1.4.5 Divalent cation transporters

It has been hypothesized that divalent cation transporters could counteract the activity of mobile-carrier ionophores (i.e., the activity of *trans*-isohumulone) by transporting cations such as magnesium into the bacterial cell [17]. The *hitA* gene (hop-inducible cation transporter) was discovered in *L. brevis* by Hayashi *et al.* [17]. The *hitA* gene has a significant level of similarity to the natural resistance-associated macrophage protein family of divalent-cation transporters which are secondary type multi-drug transporters. Hayashi *et al.* [17] also demonstrated with Northern hybridization that transcription of this gene is induced by the presence of hop bitter acids. It is unknown whether *hitA* is plasmid- or chromosomally located. The *hitA* gene

previously was only described in *L. brevis* and prior to this thesis work, there were no publications that elaborate upon the prevalence of the gene or whether it correlates with the ability of organisms to grow in beer.

1.4.6 Genes of interest with unknown function(s)

In addition to those genes described above which have inferred function based on homology, there are genes that may have a role in hop-resistance in LAB, but possess no significant percentage of similarity to any other known gene. Two such genes that may play a role in the ability of bacteria to grow in beer are the *horC* and *ORF5* genes that code for proteins of unknown function (i.e., they have no homology to known proteins). In this thesis, *horC* and *ORF5* genes were selected for Polymerase Chain Reaction (PCR) screening for association with beer-spoilage based on their hypothetical membrane localization, similarity to other membrane proteins, and their potential correlation with hop-resistance as suggested previously by Suzuki *et al.* [18, 19]. Independent studies identified two unique plasmids that both harbored *horC* and *ORF5* [18, 20], with *horC* corresponding to *ORF2* and *ORF9* as described by Suzuki *et al.* [18] and Fuji *et al.* [20], respectively, while *ORF5* corresponds to *ORF5* and *ORF2*, respectively, in the same two articles.

1.5 Antimicrobial resistance of *Pediococcus* isolates

As pediococci are also used as beneficial microbes in the context of food microbiology and animal husbandry (e.g., wine, cheese, and yogurt industries as well as for the production of silage), the emergence of hop-resistant *Pediococcus* isolates in the brewing industry is of broader interest. These isolates frequently harbour one or more ABC MDR genes, suggesting that resistance to hop-compounds may also confer resistance to other antimicrobial compounds [10].

Antimicrobial susceptibility testing of *Pediococcus* isolates has been attempted by several methods, many of which are performed using some variety of agar diffusion [21-23, 25]. More recently, dilution methods have been preferred over diffusion tests as the former allow for determination of minimum inhibitory concentrations (MICs) which is a more accurate indicator of resistance [21, 22]. However, as many pediococci have special nutritional requirements for growth, conventional antimicrobial-sensitivity testing media have been shown to be ineffective for testing of *Pediococcus* isolates for antimicrobial resistance [21, 22, 24]. The enriched media

that are commonly used to permit growth of pediococci might be inhibitory to some of the antimicrobial compounds under investigation. For these reasons, LAB susceptibility test broth medium (LSM; [22]), is now considered the new testing standard when assessing the antimicrobial resistance spectra of LAB. Despite the effectiveness of LSM having been shown for two species of *Pediococcus*, namely, *P. acidilactici*, and *P. pentosaceus* [22], it has not been used to study the prevalence and spectrum of antimicrobial resistance among other members of the *Pediococcus* genus. Additionally, the use of antimicrobial compounds by some industries to combat *Pediococcus* contaminants (e.g., hop-compounds, Penicillin, Virginiamycin) is long-standing, yet knowledge about the resistance of pediococci to antimicrobial agents is minimal [24].

1.6 Historical methods for detection of beer-spoilage bacteria

The approaches used to detect beer-spoilage bacteria can be divided into two types of methodologies. These are methods for detecting the whole organism (or its metabolic by-products) and methods for detecting microbial DNA. A number of classical methodologies have been used or proposed to detect beer-spoilage organisms. A comprehensive description and comparison of such methodologies is provided by Priest and Campbell [2]. Included are direct methods of detecting bacterial growth in beer, such as cell counts through plating or microscopy, and indirect methods, such as pH change of the media and ATP bioluminescence. Currently, methods for detection of whole organisms are slow (taking >30 days) and the more rapid methods for detecting microbial DNA lack sensitivity and/or specificity [10, 13-20].

1.7 Phylogenetics

Phylogenetics is the area of systematics which examines the evolutionary relationships among living organisms based on their genetics. The study of these relationships is pursued in an effort to clarify taxonomic classifications and the causes for such relationships. The evolutionary history of organisms is usually represented as tree-like diagrams that depict an estimated degree of the evolutionary relationships among molecules (i.e., DNA), organisms, or both. The main idea of phylogenetics is that a set of organisms descends from a single ancestor and the more closely related a group of organisms is the more related to each other they are compared to members of other phylogenetic groups.

In order for phylogenetic analyses to be performed, three basic assumptions must be made. These are that every organism is related to a common ancestor, there is a bifurcating pattern, and changes in characteristics (e.g., DNA or protein sequence) occur over time. As numerous different types of data and sizes of datasets exist, the type of phylogenetic algorithm to be used for each particular situation should be determined on a case-by-case basis. However, data selection and the alignment of this data has greater influence over the output tree than the method of phylogenetic inference itself. Although the outputs from different methods of phylogenetic inference will possess a degree of variation in comparison to one another, there cannot be confidence in any of the outputs unless there is confidence in the input data. Considering DNA or amino acid sequences as the input data, care must be taken that the region of sequence to be used contains sufficient amounts of variation and conservation, and that the dataset does not contain inappropriate sequences such as sequences present in reverse orientation. The length of sequence chosen for the dataset must also be taken into consideration, as the sequence must be of sufficient length to be informative. The same is true for the size of the dataset, for example, comparison of three sequences will provide less information than a phylogenetic tree of the same loci containing 50 sequences.

In order to construct a phylogenetic tree, the sequences must first be aligned. To align sequences, an appropriate multiple sequence alignment (MSA) algorithm must be used. For example, for protein sequences, an amino acid substitution matrix must be used that is appropriate for the organism(s) in question. One must also be mindful to manually inspect a MSA before use for phylogenetic inference. As many MSA programs assume amino acid input by default, they do not take into account the coding frame of nucleic acid sequences, thereby introducing gaps and creating alignments which would not likely be encountered in reality due to creation of stop codons and frame shifts. Ultimately, the type and size of the dataset will determine the reliability and practicality of the phylogenetic algorithms to be used. There are advantages and disadvantages to all methods and programs utilized for phylogenetic analysis.

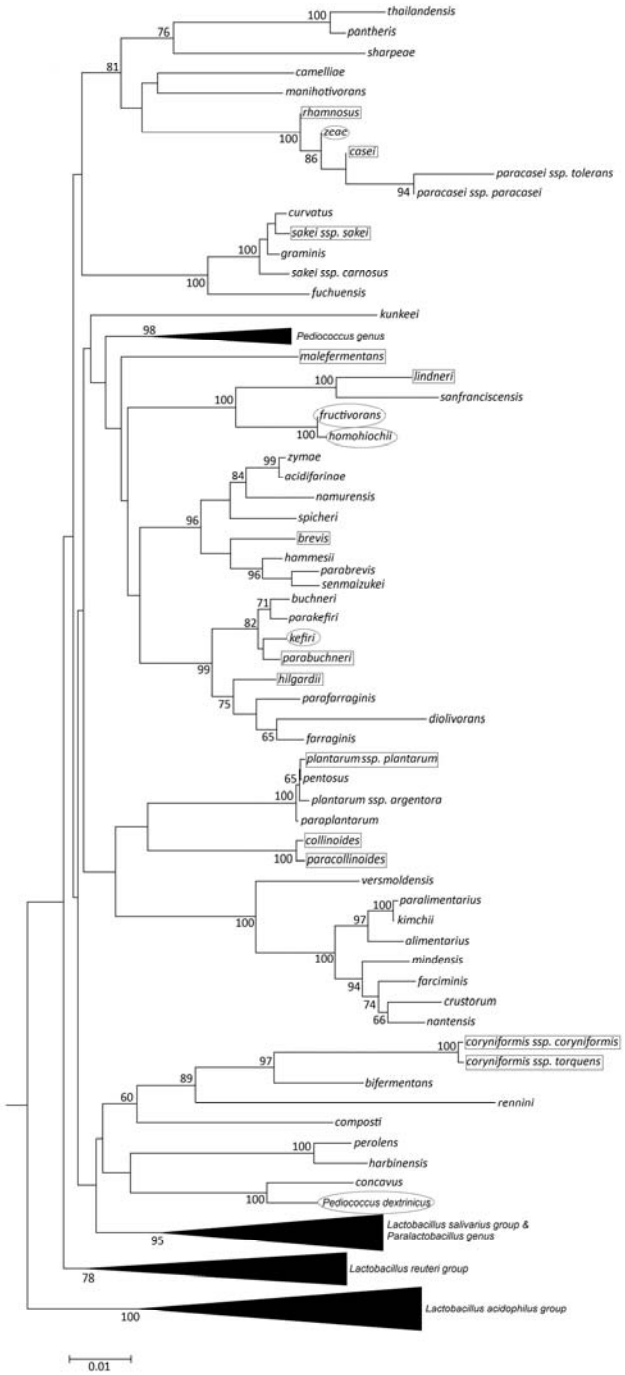
The three main methods of phylogenetic inference are evolutionary distance, maximum parsimony (MP), and maximum likelihood (ML); however, the method of phylogenetic inference must be properly chosen according to the dataset [29]. For example, evolutionary distance methods rely explicitly on a measure of genetic distance between the sequences being classified and, therefore, require a MSA. Conversely, MP methods assume a constant rate of evolution

and, therefore are not appropriate for use with genes or protein sequences that are believed to be under the influence of external selective pressures [30].

With the development of molecular tools such as 16S rRNA gene sequencing, new groups of bacteria have been identified and genera have been created from species formerly considered to be lactobacilli (e.g., *Carnobacterium*, *Leuconostoc*, *Oenococcus*, and *Weissella* [6, 31, 32]). With further genetic characterization, whole genome sequencing, and the curation of genetic databases, a more accurate classification of bacteria currently placed in the genus *Lactobacillus* is possible in order to reflect the evolutionary relationships between species. It has been speculated that the wide range of phenotypic diversity seen amongst *Lactobacillus* isolates is due to an exceptionally high level of genome degradation and horizontal gene transfer [33-36]. As such, using phenotypic categorization for bacteria within this genus will not work. Although 16S rRNA gene sequencing is used extensively in bacterial systematics, there is a growing call from the scientific community to strengthen, or refute, the conclusions drawn from the study of only one gene. As such, multilocus sequence analysis (MLSA) is the preferred method to assess the relationship of bacterial isolates, species, and genera. In addition to the full-length 16S rRNA gene, portions of several housekeeping genes may be analyzed. The most commonly used being portions of the *cpn60* (552 bp), *recA* (531 bp), *pheS* (455 bp), and *rpoA* (533 bp) genes and the corresponding protein sequences. The usefulness of these five regions in assessing phylogenetic relationships has previously been shown [37-46]. Data obtained through a MLSA approach creates a solid representation of the phylogenetic relationships among the bacterial species analyzed.

1.8 Taxonomic status of the genus *Lactobacillus*

Members of the genus *Lactobacillus* are extremely varied in phenotype, G-C content, morphology, and 16S rRNA gene percent identity. The number of species within the genus *Lactobacillus* has been growing exponentially, currently encompassing 121 validly described species and an additional nine subspecies [47] (a complete list is provided in Chapter 11). Moreover, the genus *Lactobacillus* is polyphyletic, encompassing the genera *Paralactobacillus* and *Pediococcus*. A phylogenetic tree of the genus is provided in the following pages, but due to the large size of the genus, the tree had to be presented by major clades (Figures 1.2-1.5). In these figures, the discrepancy between ability of lactobacilli to spoil beer and phylogeny is



(expanded in Figure 1.6)

(expanded in Figure 1.5)
(expanded in Figure 1.4)
(expanded in Figure 1.3)

Figure 1.2 - Phylogenetic tree of the *Lactobacillus* genus (the clades representing the *L. acidophilus, L. reuteri*, and *L. salivarius* groups as well as the genera *Pediococcus* and *Paralactobacillus* are collapsed due to space limitation). Species outlined by a box contain at least one isolate that is known to spoil beer. Conversely, all isolates of a species outlined by an ellipse have so far been found unable to grow in beer. Species without any outline have yet to be tested for ability of isolates to grow in beer. Bar indicates 1% divergence.

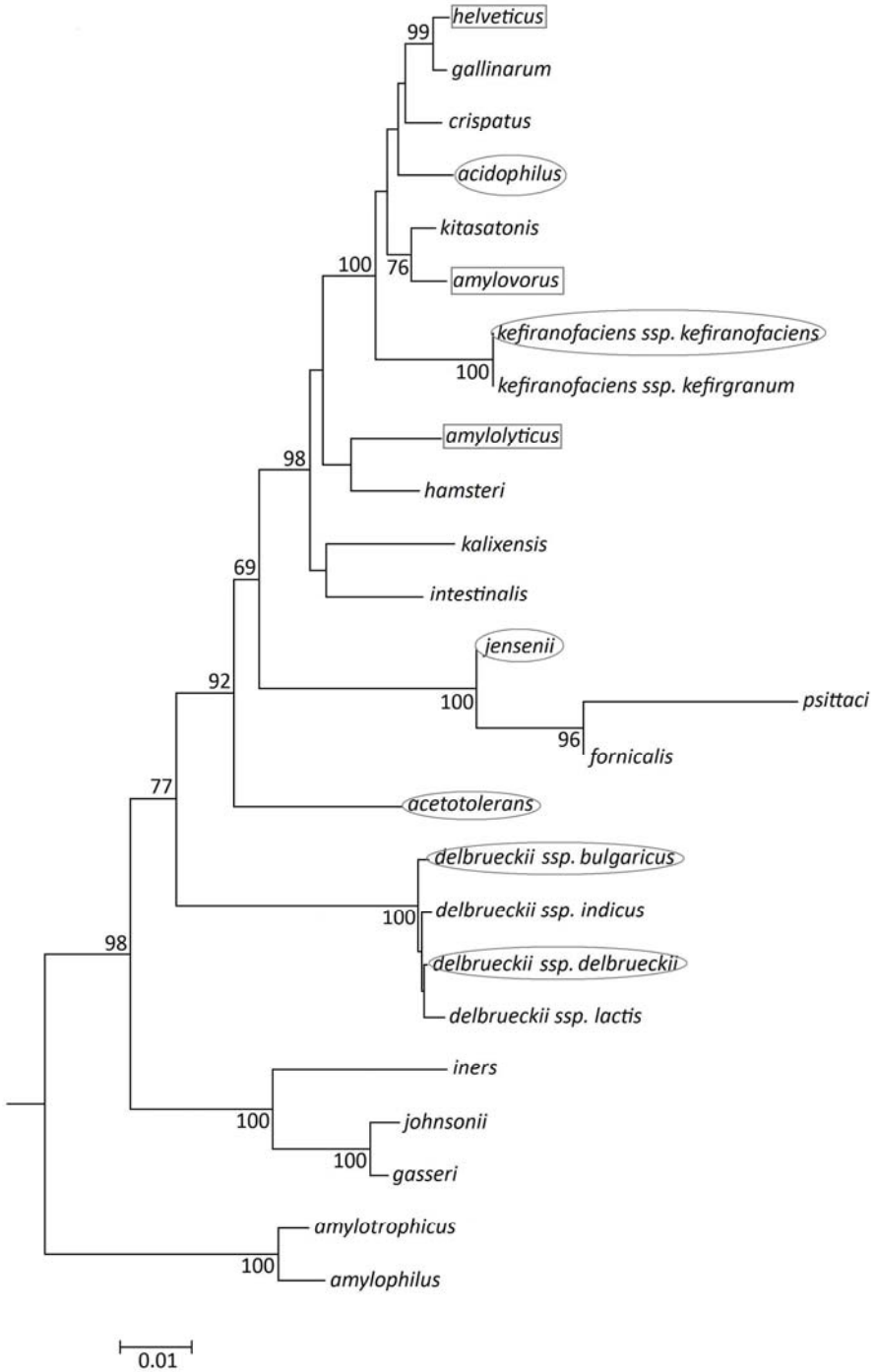


Figure 1.3 - Phylogenetic tree of the *Lactobacillus acidophilus* group (expanded from Figure 1.2). Species outlined by a box contain at least one isolate that is known to spoil beer. Conversely, all isolates of a species outlined by an ellipse have so far been found unable to grow in beer. Species without any outline have yet to be tested for ability of isolates to grow in beer. Bar indicates 1% divergence.

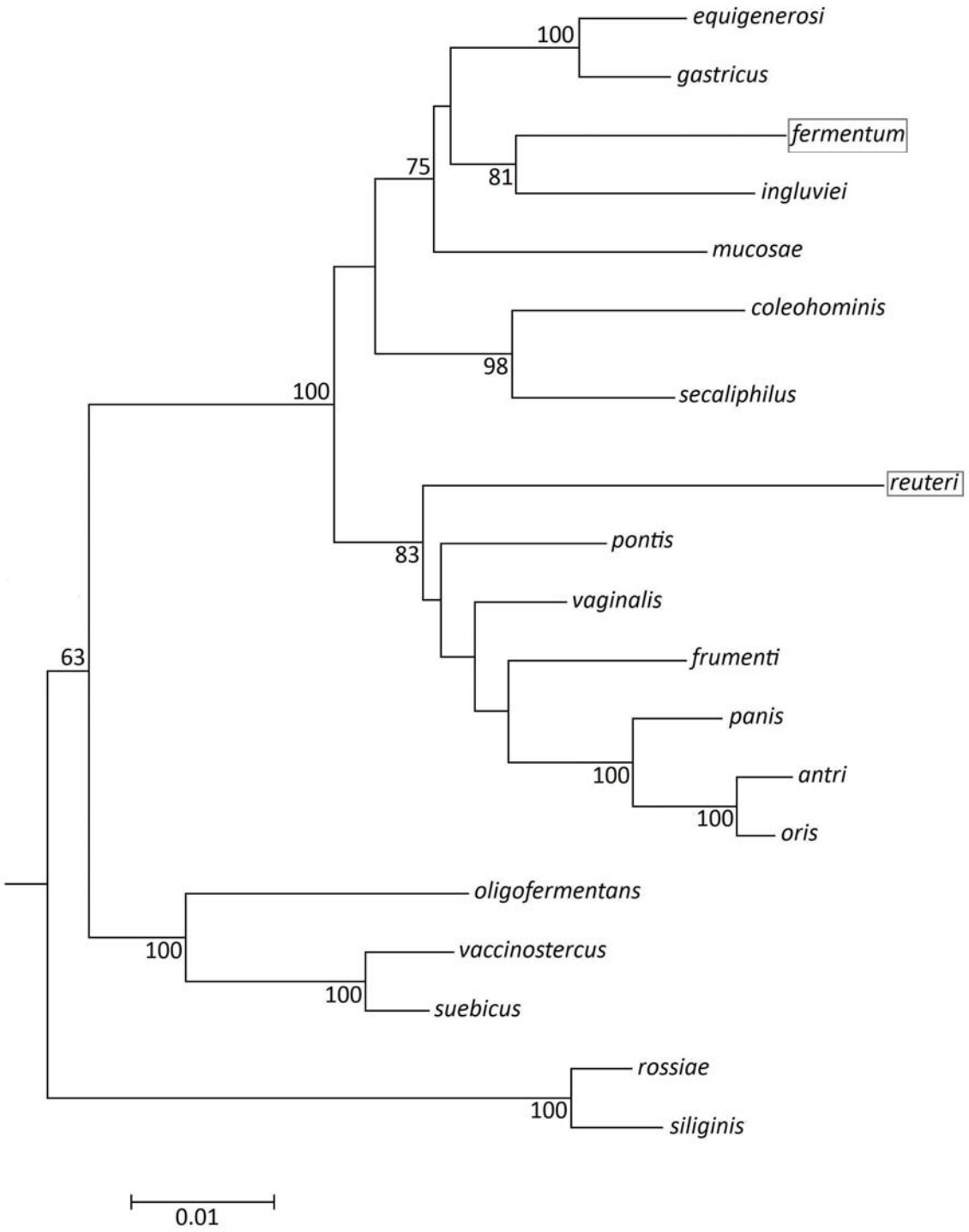


Figure 1.4 - Phylogenetic tree of the *Lactobacillus reuteri* group (expanded from Figure 1.2). Species outlined by a box contain at least one isolate that is known to spoil beer. Species without any outline have yet to be tested for ability of isolates to grow in beer. Bar indicates 1% divergence.

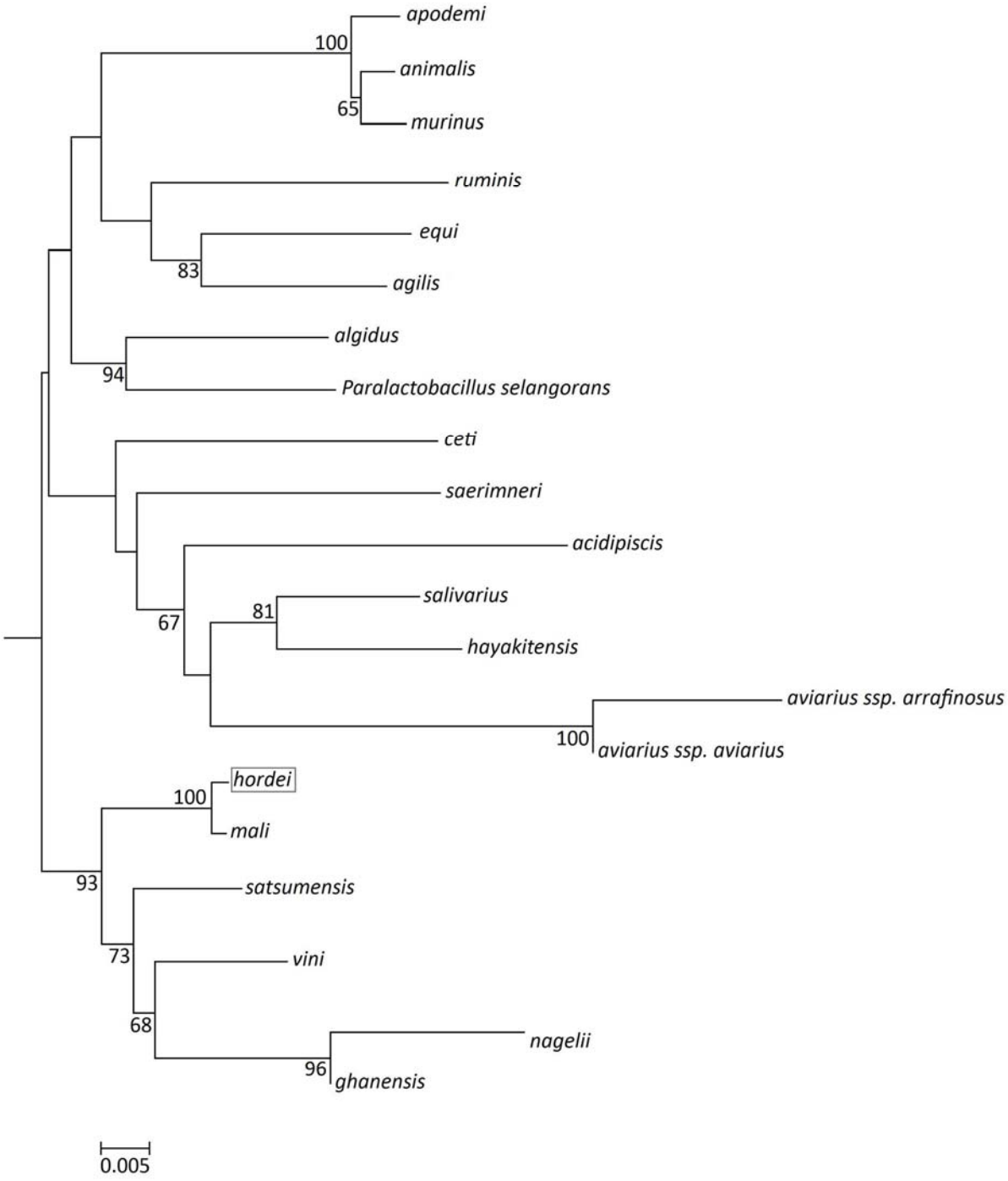


Figure 1.5 - Phylogenetic tree of the *Lactobacillus salivarius* group (expanded from Figure 1.2). Species outlined by a box contain at least one isolate that is known to spoil beer. Species without any outline have yet to be tested for ability of isolates to grow in beer. Bar indicates 0.5% divergence.

highlighted, with the ability of most organisms to grow in beer determined in Chapter 2. While the ability of many of these isolates to spoil beer was elucidated through the work in the following chapters, it is important to make note of the range and discontinuity of beer-spoiling species to appreciate the diversity of these beer-spoiling microbes.

As the number of *Lactobacillus* species has increased, the definition of the genus has become increasingly diffuse. There are no criteria for the inclusion or exclusion of new species within the genus *Lactobacillus*, leading to confusing situations where new genera (such as *Paralactobacillus* [48]) can be described that actually belong amid the current span of lactobacilli (See Chapter 11). Also, some phenotypic markers can undergo lateral gene transfer, making their use in nomenclature unstable. As such, the use of phenotypic properties for the classification of *Lactobacillus* isolates has resulted in a confusing classification scheme that has contributed to the present disorder. In its current state, the *Lactobacillus* genus is extremely heterogeneous and there is a need for a stable system of nomenclature to ensure that all members of the genus can be clearly identified, facilitating further classification and study.

1.9 Taxonomic status of the genus *Pediococcus*

At the onset of this study, the genus *Pediococcus* consisted of 8 species, including *Pediococcus acidilactici, Pediococcus claussenii* [37], *Pediococcus damnosus, Pediococcus dextrinicus*, *Pediococcus inopinatus, Pediococcus parvulus, Pediococcus pentosaceus,* and *Pediococcus urinaeequi*. Since beginning this thesis work in 2004, five additional species have been described, namely*, Pediococcus argentinicus* [49], *Pediococcus cellicola* [50]*, Pediococcus ethanolidurans* [51], *Pediococcus siamensis* [52], and *Pediococcus stilesii* [53], and *P. urinaeequi* was reclassified to the genus *Aerococcus* [54]. At the point of assembling the thesis (April 25, 2009), the genus *Pediococcus* consisted of 11 species as the species *P. dextrinicus* was reassigned to the genus *Lactobacillus* as a result of the work described in Chapter 9. For the purposes of this introduction, however, the genus *Pediococcus* will be discussed in the context of containing all currently validly described pediococci, and also including *P. dextrinicus*.

Pediococci are homofermentative and produce DL-lactate from glucose; the exceptions being *P. claussenii* and *P. dextrinicus* which produce only L(+)-lactic acid [53]. The *Pediococcus* genus forms a distinct taxonomic group, with the exception of *P. dextrinicus* which is a distant outlier to the genus, instead falling phylogenetically within the *Lactobacillus* genus

(Figure 1.2). A phylogenetic tree in Figure 1.6 expands the compressed *Pediococcus* clade from Figure 1.2 and highlights the ability of isolates to grow in beer. As with the lactobacilli, the ability of many of these isolates to spoil beer was elucidated through the work in the following chapters, but is presented here as it is important to make note of this feature to appreciate the diversity of these beer-spoiling microbes.

The species *P. dextrinicus* was included within the genus *Pediococcus* based upon morphology, cell wall composition, homofermentative lactic acid production, and nutritional requirements [55]. It should be noted, however, that many of these characteristics are also shared by other LAB, including the related *Aerococcus*, *Lactobacillus*, and *Tetragenococcus* genera [6, 32, 56]. In Chapter 9, the species *P. dextrinicus* is reclassified as *Lactobacillus dextrinicus*, resulting in a more cohesive taxonomy of the genus *Pediococcus*.

1.10 Whole genome analysis

The phylogenetic trees in Figures 1.2-1.6 make it apparent that the phenotype of being able to grow in beer does not abide by the boundaries of speciation or follow the same evolutionary path as predicted by housekeeping genes. Historically, taxonomic analyses have been performed using a diverse and often arbitrary selection of morphological and phenotypic characteristics. These characteristics are now considered unsuitable for generating reliable and consistent taxonomies, as there is no rational basis for choosing which morphological or phenotypic characteristics should be examined, and the extent that individual phenotype or small collection of phenotypes consistently represent true phylogeny is generally considered to be minimal. This situation holds true in the context of brewing microbiology as shown by the diverse taxonomy of beer-spoilage *Lactobacillus* and *Pediococcus* species (Figures 1.2-1.6).

While 16S rRNA gene sequence analysis and MLSA have proven to be effective tools for phylogenetics, one deficiency inherent in these techniques is that only a small amount of information is used to represent the entire organism. This practice has largely been accepted due to restraints such as the cost, time, and complexity involved in genome sequencing. However, there are now hundreds of sequenced genomes that are available in publicly accessible databases. As a result, there is the opportunity to explore the use of whole genomes in analyzing evolutionary relationships. As more genomes have become publicly available, numerous different approaches to determining genomic relatedness have been attempted, including

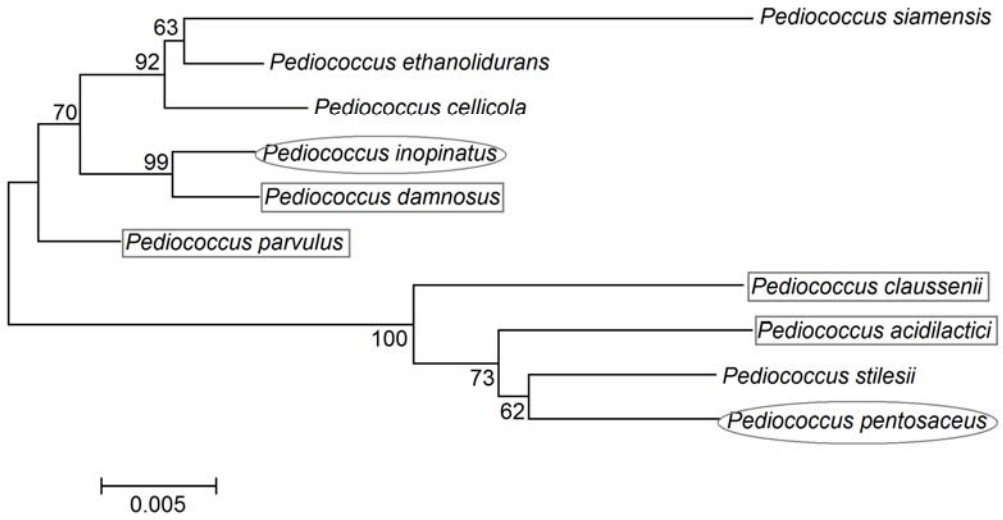


Figure 1.6 - Phylogenetic tree of the *Pediococcus* genus (expanded from Figure 1.2). *P. dextrinicus* is not included in this phylogenetic tree due to being a distant outlier, and instead can be found within the *Lactobacillus* genus in Figure 1.1. Species outlined by a box contain at least one isolate that is known to spoil beer. Conversely, all isolates of a species outlined by an ellipse have so far been found unable to grow in beer. Species without any outline have yet to be tested for ability of isolates to grow in beer. Since the creation of this phylogenetic tree, an additional *Pediococcus* species has been described, *P. argentinicus* [49]. Bar indicates 0.5% divergence.

dinucleotide frequencies, [57-59], genomic G+C content [45], codon usage [60, 61], gene order [62], and amino acid k-mer composition [63]. These methods, as well as a number of others, are reviewed by Coenye and colleagues [64]. Earlier, Coenye *et al.* [61] performed a comparison of some of these methods and showed that the phylogenetic trees derived from these characteristics are usually quite consistent with each other, as well as with the tree derived from comparing 16S rRNA gene sequences. As these comparisons were performed on relatively small, related groups of bacteria, it remains unclear whether these results generalize to all organisms or even to all bacteria.

Another approach to whole-genome phylogenetics is the comparison of gene content. This technique involves the identification of predicted orthologues in pairs of organisms and then assigning a "distance" between that pair based on the putative number of shared genes. This technique was originally proposed by Snel *et al.* [65] and has subsequently been revisited with larger groups of organisms [66, 67]. Compared to other whole-genome techniques for phylogeny, this method seems particularly attractive, as differences in gene content among organisms are readily explicable both in terms of their evolutionary meaning (adaptation to its environment) and the mechanisms behind the evolutionary events (gene duplication, gene loss, horizontal gene transfer).

Some of the most useful information from an evolutionary standpoint is genome content or protein composition. For this reason, several methods have been developed to compare protein profiles of organisms. One such method that has been developed is Clusters of Orthologous Groups (COGs) of proteins [68]. This method classifies proteins into COGs based on sequence similarity via a best-hit gapped-BLAST approach [69]. The end result is clusters of proteins that are supposed orthologs and therefore are proposed to have the same or similar functions. Unfortunately, the COG database currently consists of a list of COGs that were developed in 2003 with the use of 66 genome sequences [70]. The COG database website states that a newer version is said to be in development, which will include 261 genomes (http://ncbi.nlm.nih.gov/COG/; accessed April 5th, 2009). However, this is less than half of the microbial genome sequences available through NCBI's Entrez Genome Project Database which, as of April 5th, 2009, consisted of 862 sequenced microbial genomes. On this same date, the NCBI database also indicates that an additional 1565 bacterial genomes are currently being sequenced. With the already overwhelming amount of genomic information available, coupled

with the exponential growth rate of genetic information, the ability to efficiently include all genomic information in a comparative COG analysis is going to be increasingly difficult. The creation of COGs is time-consuming and labor-intensive, requiring a large amount of manual analysis. This is so much so that including all of the presently available genome sequences in the development of COGs would appear to be unfeasible. Therefore, COGs have been created for smaller groups of organisms [33, 71, 72], but these COGs cannot be applied for analysis or comparisons of other organisms. In light of this problem, COGs should only be used for comparison of entire protein profiles of organisms that were originally used (or very closely related to those used) to create the given COG database.

1.11 General hypotheses and objectives

There were seven hypotheses providing the foundation for this thesis research. (i) Ability for lactobacilli and pediococci to grow in beer is multifactorial and requires factors such as ethanol-tolerance and nutrient acquisition in addition to hop-resistance. (ii) The presence of specific genes can be used to predict the ability of lactobacilli and pediococci to grow in beer. (iii) Phyla can be detected and identified using conserved regions of the 16S rRNA gene. (iv) The *horA* gene exists outside of the brewery environment and that additional, as of yet unknown, beer-spoilage associated genes must exist. (v) The presence of antimicrobial compounds should have some correlation to the presence of beer-spoilage associated genes, hop-resistance, and ability to grow in beer. (vi) Insights can be gained into the genetic basis of beer-spoilage by developing a better understanding of the taxonomy of lactobacilli and pediococci. (vii) Lastly, genomes and/or proteomes can be grouped and added/subtracted from one another in order to determine core and unique proteomes which can then be associated to phenotypic information.

From these hypotheses, general objectives were developed. While specific objectives are given in the brief introduction to each Chapter, the objectives as presented here are intended to be an overview of the goals of this thesis as a whole. There were four general objectives. Figure 1.7 shows a schematic diagram of how the components of the thesis tie together. The four general objectives and the research directions taken in each case were as follows.

My first objective was to develop a PCR-based method to detect beer-spoiling bacteria. Here I focused on developing an internal positive PCR control, determining which isolates can

grow in beer (for statistical analysis), testing for the presence of currently known putative beer-spoilage associated genes, and identifying additional beer-spoilage associated genes. Chapters 2 through 6 relate to this objective (Figure 1.7).

My second objective was to develop a method to detect beer-spoiling bacteria that is independent of genetic background. This involved investigating the effects and usefulness of varying concentrations of hop-compounds, ethanol, nutrients (as hypothesized in Figure 1.1), and using an agar or broth medium to yield the most accurate detection method. Chapter 7 relates to this objective (Figure 1.7).

My third objective was to gain insights into the implications of the uses of antimicrobial compounds (i.e., hop-compounds) on beer-spoiling bacteria. Here I compared the presence of genes putatively associated with hop-resistance with degree of resistance to antibiotics and I statistically determined whether it is plausible that genes associated with hop-resistance also confer resistance to other antibiotics. Chapter 8 relates to this objective (Figure 1.7).

Lastly, my fourth objective was to contribute to the taxonomic understanding of lactobacilli and pediococci that are able to spoil beer. Here I conducted a phylogenetic analysis of the *Lactobacillus* and *Pediococcus* genera, identified and reclassified an inappropriately named species, and suggested reorganization or restructuring where needed in order to clarify the relationships of species within these and neighboring genera. Additionally, genomic and proteomic relatedness of lactobacilli in comparison to 16S rRNA gene similarity and other genera were investigated and a novel method for elucidation of genes putatively related to phenotypic groups is explored using proteomic comparisons. Chapters 9 through 11 relate to this objective (Figure 1.7).

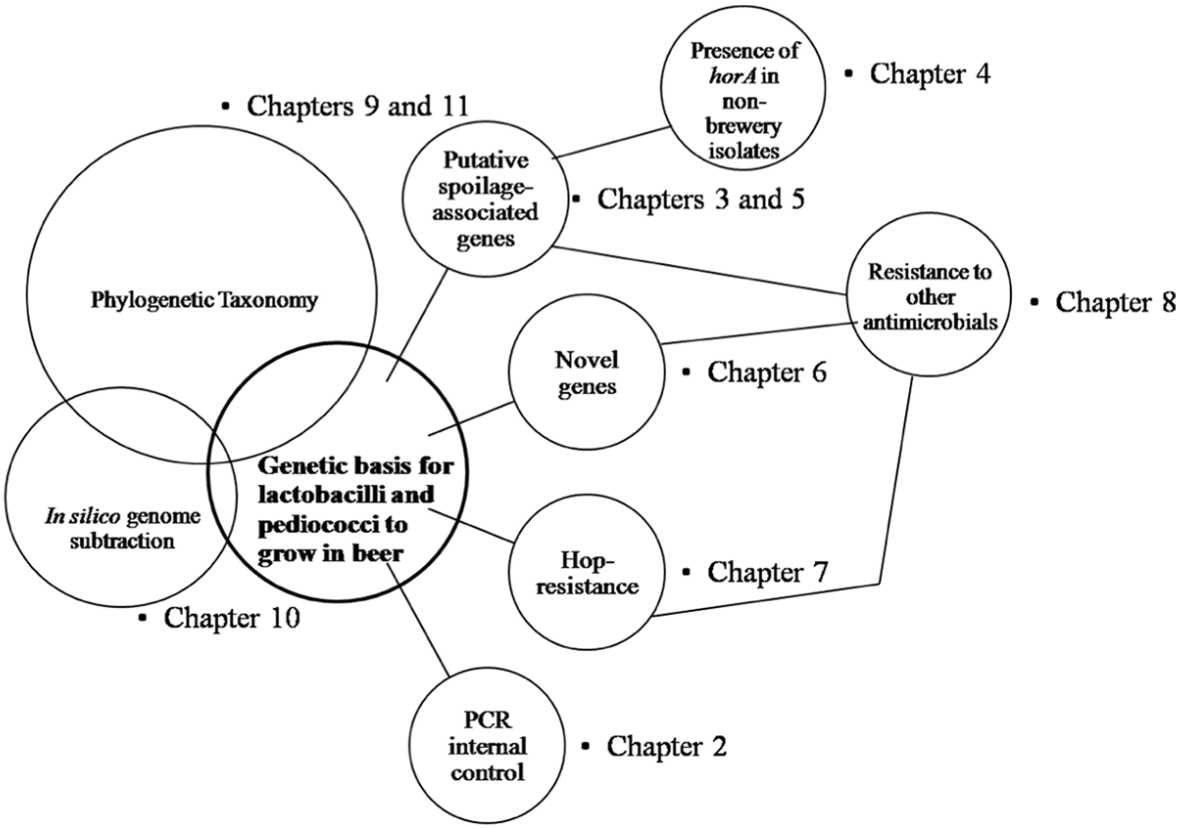


Figure 1.7 - Schematic overview of the thesis objectives, showing how contents of the various Chapters are interconnected.

1.12 References

1. Sakamoto K, Konings WN: **Beer spoilage bacteria and hop resistance.** Int J Food Microbiol 2003, **89**:105-124.

2. Priest FG, Campbell I: *In Brewing microbiology, 3rd ed. Kluwer, New York, N.Y.* ; 2002.

3. Fernandez JL, Simpson PJ: **Measurement and prediction of the susceptibility of lager beer to spoilage by lactic acid bacteria.** J Appl Microbiol 1995, **78**:419-425.

4. Weiss N: **The genera *Pediococcus* and *Aerococcus*.** In *The Prokaryotes.* Edited by Balows A, Trüper HH, Dworkin M, Harder W, Schleifer K-. New York: Springer; 1992:1502-1507.

5. Kandler O, Weiss N: **Genus *Lactobacillus*.** In *Bergey's Manual of Systematic Bacteriology. Volume 2.* Edited by Sneath PHA, Mair NS, Sharpe ME. Baltimore, MD: Willams and Wilkins; 1986:1209-1234.

6. Hammes WP, Hertel C: **The Genera *Lactobacillus* and *Carnobacterium* .** In *The Prokaryotes.* Edited by Balows A, Trüper HH, Dworkin M, Harder W, Schleifer K-. New York: Springer; 2006:320-403.

7. Chabot S, Yu H, De Leseleuc L, Cloutier D, Van Calsteren M, Lessard M, Roy D, Lacroix M, Oth D: **Exopolysaccharides from *Lactobacillus rhamnosus* RW-9595M stilmulate TNF, IL-6 and IL-12 in human and mouse cultured immunocompetent cells, and IFN-gamma in mouse splenocytes.** Lait 2001, **81**:683-691.

8. Hughes PS, Simpson WJ: **Production and composition of hop products.** MBAA Tech Quart 1993, **4**:146-152.

9. Simpson WJ: **Ionophoric action of *trans*-isohumulone of *Lactobacillus brevis.*** J Gen Microbiol 1993, **139**:1041-1045.

10. Sami M, Yamashita H, Hirono T, Kadokura H, Kitamoto K, Yoda K, Yamasaki M: **Hop-resistant *Lactobacillus brevis* contains a novel plasmid harboring a multidrug resistance-like gene.** J Ferment and Bioeng 1997, **84**:1-6.

11. Simpson WJ, Smith AR: **Factors affecting antibacterial activity of hop compounds and their derivatives.** J Appl Bacteriol 1992, **72**:327-334.

12. Fernandez JL, Simpson WJ: **A cost-effective solution to control of microbiological stability?** EBC congress, Analysis and Process Management 1995, 713-721.

13. Gindreau E, Walling E, Lonvaud-Funel A: **Direct polymerase chain reaction detection of ropy *Pediococcus damnosus* strains in wine.** J Appl Microbiol 2001, **90**:535-542.

14. Sami M, Yamashita H, Kadokura H, Kitamoto K, Yoda K, Yamasaki M: **A new and rapid method for determination of beer-spoilage ability of lactobacilli.** J Am Soc Brew Chem 1997, **55**:137-140.

15. Suzuki K, Ozaki K, Yamashita H: **Genetic marker for differentiating beer-spoilage ability of *Lactobacillus paracollinoides* strains.** J Appl Microbiol 2004, **97**:712-718.

16. Suzuki K, Sami M, Iijima K, Ozaki K, Yamashita H: **Characterization of *horA* and its flanking regions of *Pediococcus damnosus* ABBC478 and development of more specific and sensitive *horA* PCR method.** Lett Appl Microbiol 2006, **42**:392-399.

17. Hayashi N, Ito M, Horiike S, Taguchi H: **Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus brevis* strains capable of growing in beer.** Appl Microbiol Biotechnol 2001, **55**:596-603.

18. Suzuki K, Ozaki K, Yamashita H: **Comparative analysis of conserved genetic markers and adjacent DNA regions identified in beer-spoilage lactic acid bacteria.** Lett Appl Microbiol 2004, **39**(3):240-245.

19. Suzuki K, Iijima K, Ozaki K, Yamashita H: **Isolation of a hop-sensitive variant of *Lactobacillus lindneri* and identification of genetic markers for beer spoilage ability of lactic acid bacteria.** Appl Environ Microbiol 2005, **71**(9):5089-5097.

20. Fujii T, Nakashima K, Hayashi N: **Random amplified polymorphic DNA-PCR based cloning of markers to identify the beer-spoilage strains of *Lactobacillus brevis, Pediococcus damnosus, Lactobacillus collinoides* and *Lactobacillus coryniformis*.** J Appl Microbiol 2005, **98**:1209-1220.

21. Klare I, Konstabel C, Muller-Bertling S, Reissbrodt R, Huys G, Vancanneyt M, Swings J, Herman G, Witte W: **Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria.** Appl Environ Microbiol 2005, **71**(12):8982-8986.

22. Klare I, Konstabel C, Werner G, Huys G, Vankerckhoven V, Kahlmeter G, Hildebrandt B, Muller-Bertling S, Witte W, Goossens H: **Antimicrobial susceptibilities of *Lactobacillus, Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use.** J Antimicrob Chemother 2007, **59**(5):900-912.

23. Danielsen M, Simpson PJ, O'Connor EB, Ross RP, Stanton C: **Susceptibility of *Pediococcus* spp. to antimicrobial agents.** J Appl Microbiol 2007, **102**(2):384-389.

24. Ammor MS, Belén FA, Mayo B: **Antibiotic resistance in non-enterococcal lactic acid bacteria and *Bifidobacteria*.** Food Microbiol, 2007, **24**(6):559-570.

25. Tankovic J, Leclercq R, Duval J: **Antimicrobial susceptibility of Pediococcus spp. and genetic basis of macrolide resistance in Pediococcus acidilactici HM3020.** Antimicrob Agents Chemother 1993, **37**(4):789-792.

26. Muyzer G, de Waal EC, UIitterlinden, AG: **Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA.** Appl Environ Micrbiol 1993, **59**:695-700.

27. Relman DA: ***In* Diagnostic Molecular Microbiology: Principles and Applications.** Edited by Persing DH, Smith TF, Tenover FC, White TJ. Washington, DC: American Society for Microbiology; 1991:489-495.

28. Sami M, Suzuki K, Sakamoto K, Kadokura H, Kitamoto K, Yoda K: **A plasmid pRH45 of *Lactobacillus brevis* confers hop resistance.** J Gen Appl Microbiol 1998, **44**:361-363.

29. Kolaczkowski B, Thornton JW: **Performance of maximum parsimony and likelihood phylogenetics when evolution is heterogeneous.** Nature 2004, **431**:980-984.

30. Felsenstein J: **Cases in which parsimony and compatibility methods will be positively misleading.** Syst Zool 1978, **27**:401-410.

31. Felis GE, Dellaglio F: **Taxonomy of lactobacilli and *Bifidobacteria*.** Curr Issues Intestinal Microbiol 2007, **8**:44-61.

32. Stiles ME, Holzapfel WH: **Lactic acid bacteria of foods and their current taxonomy.** Int J Food Microbiol 1997, **36**:1-29.

33. Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S, Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker D, Hughes J, Goh Y, Benson A, Baldwin K, Lee J, Diaz-Muniz I, Dosti B, Smeianov V, Wechter W, Barabote R, Lorca G, Altermann E, BarrangouR., Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O'sullivan D, Steele J, Unlu G, Saeier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B, Mills D: **Comparative genomics of the lactic acid bacteria.** Proc Natl Acad Sci USA 2006, **103**:15611-15616.

34. Claesson MJ, van Sinderen D, O'Toole PW: **The genus *Lactobacillus* - a genomic basis for understanding its diversity.** FEMS Microbiol Lett 2007, **269**:22-28.

35. Makarova KS, Koonin EV: **Evolutionary Genomics of Lactic Acid Bacteria.** J Bacteriol 2007, **189**:1199-1208.

36. Nicolas P, Bessieres P, Ehrlich SD, Maguin E, van de Guchte M: **Extensive horizontal transfer of core genome genes between two *Lactobacillus* species found in the gastrointestinal tract.** BMC Evol Biol 2007, **7**:141-155.

37. Dobson CM, Deneer H, Lee S, Hemmingsen S, Glaze S, Ziola B: **Phylogenetic analysis of the genus *Pediococcus*, including *Pediococcus claussenii* sp. nov., a novel lactic acid bacterium isolated from beer.** Int J Syst Evol Microbiol 2002, **52**(Pt 6):2003-2010.

38. Dobson CM: **Phylogenetic analysis of *Lactobacillus* and *Pediococcus* based on the 16S rRNA gene, 16S-23S rRNA interspacer region, and heat shock 60 protein.** 2001 M.Sc. thesis. University of Saskatchewan, Saskatoon, Canada.

39. Eisen JA: **The RecA protein as a model molecule for molecular systematic studies of bacteria: Comparison of trees of RecAs and 16S rRNAs from the same species.** J Mol Evol 1995, **41**:1105-1123.

40. Felis GE, Dellaglio F, Mizzi L, Torriani S: **Comparative sequence analysis of a *recA* gene fragment brings new evidence for a change in the taxonomy of the *Lactobacillus casei* group.** Int J Syst Evol Microbiol 2001, **51**:2113-2117.

41. Jian W, Zhu L, Dong X: **New approach to phylogenetic analysis of the genus *Bifidobacterium* based on partial *hsp60* gene sequences.** Int J Syst Evol Microbiol 2001, **51**:1633-1638.

42. Kwok AYC, Chow AW: **Phylogenetic study of *Staphylococcus* and *Macrococcus* species based on partial *hsp60* gene sequences.** Int J Syst Evol Microbiol 2003, **53**:87-92.

43. Lloyed AT, Sharp PM: **Evolution of the *recA* gene and the molecular phylogeny of bacteria.** J Mol Evol 1993, **37**:399-407.

44. Naser SM, Thompson FL, Hoste B, Gevers D, Dawyndt P, Vancanneyt M, Swings J: **Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes.** Microbiology 2005, **151**:2141-2150.

45. Vandamme P, Pot B, Gillis M, deVos P, Kersters K, Swings J: **Polyphasic taxonomy, a consensus approach to bacterial systematics.** Microbiol Rev 1996, **60**:407-438.

46. Naser SM, Dawyndt P, Hoste B, Gevers D, Vandemeulebroecke K, Cleenwerck I, Vancanneyt M, Swings J: **Identification of lactobacilli by *pheS* and *rpoA* gene sequence analysis.** Int J Syst Evol Microbiol 2007, **57**:2777-2789.

47. Euzeby JP: **List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet (http://www.bacterio.net).** Int J Syst Bacteriol 1997, **47**:590-592.

48. Leisner JJ, Vancanneyt M, Goris J, Christensen H: **Description of *Paralactobacillus selangorensis* gen. nov., sp. nov., a new lactic acid bacterium isolated from chili bo, a Malaysian food ingredient.** Int J Syst Evol Microbiol 2000, **50**:19-24.

49. De Bruyne K, Franz CMAP, Vancanneyt M, Schleifer KH, Mozzi F, Font de Valdez G, de Vuyst L, Vandamme P: ***Pediococcus argentinicus* sp. nov. from Argentinean fermented wheat flour and identification of *Pediococcus* species by *pheS*, *rpoA*, and *atpA* sequence analysis.** Int J Syst Evol Microbiol 2008, **58**:2909-2916.

50. Zhang B, Tong H, Dong X: ***Pediococcus cellicola*** **sp. nov., a novel lactic acid coccus isolated from a distilled-spirit-fermenting cellar.** Int J Syst Evol Microbiol 2005, **55**:2167-2170.

51. Liu L, Zhang B, Tong H, Dong X: ***Pediococcus ethanolidurans*** **sp. nov., isolated from the walls of a distilled-spirit-fermenting cellar.** Int J Syst Evol Microbiol 2006, **56**:2405-2408.

52. Tanasupawat S, Pakdeeto A, Thawai C, Yukphan P, Okada S: **Identification of a lactic acid bacteria from fermented tea leaves (miang) in Thailand and proposals of *Lactobacillus thailandensis* sp. nov., *Lactobacillus camelliae* sp. nov., and *Pediococcus siamensis* sp. nov.** J Gen Appl Microbiol 2007, **53**:7-15.

53. Franz CMAP, Vancanneyt M, Vandemeulebroecke K, DeWachter M, Cleenwerck I, Hoste B, Schillinger U, Holzapfel WH, Swings J: ***Pediococcus stilesii*** **sp. nov., isolated from maize grains.** Int J Syst Evol Microbiol 2006, **56**:329-333.

54. Felis GE, Torriani S, Dellaglio F: **Reclassification of *Pediococcus urinaeequi* (ex Mees 1934) Garvie 1988 as *Aerococcus urinaeequi* comb. nov.** Int J Syst Evol Microbiol 2005, **55**:1325-1327.

55. Coster E, White R: **Further studies of the genus *Pediococcus*.** J Gen Microbiol 1964, **37**:15-31.

56. Simpson WJ, Taguchi H: **The genus *Pediococcus*, with notes on the genera *Tetragenococcus* and *Aerococcus*.** In *The Genera of Lactic Acid Bacteria.* Edited by Wood BJ, Holzapfel WH. London: Blackie Academic & Professional; 1995:125-172.

57. van Passel MWJ, Kuramae EE, Luyf ACN, Bart A, Boekhout T: **The reach of the genome signature in prokaryotes.** BMC Evol Biol 2006, **6**:84.

58. Karlin S, Burge C: **Dinucleotide relative abundance extremes: a genomic signature.** Trends Genet 1995, **11**:283-290.

59. Karlin S, Mrazek J, Campbell AM: **Compositional biases of bacterial genomes and evolutionary implications.** J Bacteriol 1997, **129**:3899-3913.

60. Wright F: **The 'effective number of codons' used in a gene.** Gene 1990, **87**:23-29.

61. Coenye T, Vandamme P: **Extracting phylogenetic information from whole-genome sequencing projects: the lactic acid bacteria as a test case.** Microbiology 2003, **149**:3507-3517.

62. Suyama M, Bork P: **Evolution of prokaryotic gene order: genome rearrangements in closely related species.** Trends Genet 2001, **17**:10-13.

63. Qi J, Bin W, Bai-lin H: **Whole proteome prokaryote phylogeny without sequence alignment: a K-string composition approach.** J Mol Evol 2004, **58**:1-11.

64. Coenye T, Gevers D, Van De Peer Y, Vandamme P, Swings J: **Towards a prokaryotic genomic taxonomy.** FEMS Microbiol Rev 2005, **25**:147.

65. Snel B, Bork P, Huynen MA: **Genome phylogeny based on gene content.** Nature Genetics 1999, **21**:108-110.

66. House CH, Fitz-Gibbon ST: **Using homolog groups to create a whole-genomic tree of free-living organisms: an update.** J Mol Evol 2002, **54**:539-547.

67. Henz SR, Huson DH, Auch AF, Nieselt-Struwe K, Schuster SC: **Whole-genome prokaryotic phylogeny.** Bioinformatics 2005, **21**:2329-2335.

68. Tatusov RL, Koonin EV, Lipman DJ: **A genomic perspective on protein families.** Science 1997, **278**:631-637.

69. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: **Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.** Nucleic Acids Res 1997, **25**:3389-3402.

70. Tatusov RL, Galperin MY, Natale DA, Koonin EV: **The COG database: a tool for genome-scale analysis of protein functions and evolution.** Nucl Acids Res 2000, **28**:33.

71. Makarova K, Sorokin AV, Novichkov PS, Wolf YI, Koonin EV: **Clusters of orthologous genes for 41 archaeal genomes and implications for evolutionary genomics of archaea.** Biology Direct 2007, **2**:33.

72. Mulkidjanian AY, Koonin EV, Makarova KS, Mekhedov SL, Sorokin A, Wolf YI, Dudresne A, Partensky F, Burd H, Kaznadzey D, Haselkorn R, Galperin MY: **The *cyanobacterial* genome core and the origin of photosynthesis.** Proc Natl Acad Sci USA 2006, **103**:13126.

2. REAL-TIME PCR DETECTION OF BACTERIA BELONGING TO THE *FIRMICUTES* PHYLUM

Author contributions:

Monique Haakensen conceived the study, conducted the experiments and bioinformatic analyses, and wrote the manuscript.

Melissa Dobson generated most of the *Lactobacillus* and *Pediococcus* 16S rRNA gene sequences and created the initial multiple sequence alignment.

Harry Deneer provided scientific input regarding PCR experiments.

Barry Ziola conceived the study, edited the manuscript, and is the holder of the research grant used to fund the study.

BRIEF INTRODUCTION TO CHAPTER 2

In order to effectively detect beer-spoilage associated genes by PCR, a positive internal control is needed. The *Lactobacillus* and *Pediococcus* genera belong to the Phylum *Firmicutes* which also contains nearly all beer-spoiling bacterial genera. It is estimated that bacteria from the *Firmicutes* Phylum are responsible for >90% of beer-spoilage incidents. There is currently no method that allows for detection of *Firmicutes* as a group. A multiple sequence alignment of *Lactobacillus* and *Pediococcus* 16S rRNA gene sequences was used to identify a putative *Firmicutes*-specific region of this gene. The region identified was then evaluated by *in silico* and *in vitro* methods to determine the range and specificity of the *Firmicutes*-specific real-time PCR probe.

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Erratum: in this manuscript the sequence of the *Firmicutes* probe is incorrectly given as CTTGCTGCCTCCCGTAG and should be CTGCTGCCTCCCGTAG.

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Real-time PCR detection of bacteria belonging to the *Firmicutes* Phylum

M. Haakensen, C.M. Dobson [1], H. Deneer, B. Ziola *

Department of Pathology and Laboratory Medicine, College of Medicine, 2841 Royal University Hospital, 103 Hospital Drive, Saskatoon SK, Canada S7N 0W8

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ABSTRACT

Members of the bacterial Phylum *Firmicutes* occupy a wide range of habitats and can be either beneficial or detrimental in diverse settings, including food- and beverage-related industries. *Firmicutes* are responsible for the vast majority of beer-spoilage incidents and, as such, they have a substantial financial impact in the brewing industry. Rapid detection and identification of a bacterium as a *Firmicutes* is difficult due to widespread genetic transfer and genome reduction resulting in phenotypic diversity in these bacteria. Here we describe a real-time multiplex PCR to detect and differentiate *Firmicutes* associated with beer-spoilage from non-*Firmicutes* bacteria that may be present as benign environmental contaminants. A region of the 16S rRNA gene was identified and predicted to be highly conserved amongst, and essentially specific for, *Firmicutes*. A real-time PCR assay using a hydrolysis probe targeting this region of the 16S rRNA gene was experimentally shown to detect ten genera of *Firmicutes* known to be beer spoilers, but does not cross-react with eleven of twelve non-*Firmicutes* genera which can periodically appear in beer. Only one non-*Firmicutes* species, *Zymomonas mobilis*, weakly reacted with the *Firmicutes* probe. This rPCR assay has a standard curve that is linear over six orders of magnitude of DNA, with a quantitation limit of DNA from <10 bacteria. When used to detect bacteria present in beer, the assay was able to detect 50–100 colony forming units (CFU) of *Firmicutes* directly from 2.5 cm membranes used to filter 100 ml of contaminated beer. Through incorporation of a 4.7 cm filter and an overnight pre-enrichment incubation, the sensitivity was increased to 2.5–10 CFU per package of beer (341 ml). When multiplexed with a second hydrolysis probe targeting a universal region of the 16S rRNA gene, the assay reliably differentiates between *Firmicutes* and non-*Firmicutes* bacteria found in breweries.

1. Introduction

The bacterial Phylum *Firmicutes* contains the three Classes *Bacilli*, *Clostridia*, and *Mollicutes* which include a total of 235 genera and all species of lactic acid bacteria. Members of this Phylum are highly diverse in morphology, physiology, and Gram-staining characteristics (Sneath et al., 1986), making phenotypic properties unsuitable for detection or identification of *Firmicutes*. *Firmicutes* occupy a wide variety of habitats, and can be either useful or problematic in various food- and beverage-related industries (Bjorkroth et al., 1996; Fernandez et al., 1996; Llauberes et al., 1990; Sakamoto and Konings, 2003; Stiles and Holzapfel, 1997), in the fuel alcohol industry (Skinner and Leathers, 2004), and in human and animal health (Carr et al., 2002). It is believed that numerous industrial applications of *Firmicutes* remain to be exploited (Teusink and Smid, 2006).

Firmicutes are responsible for the majority of beer-spoilage incidents and, as such, have a substantial financial impact for brewers. Current detection methods for beer-spoilage bacteria rely largely upon species-specific identification (DiMichele and Lewis, 1993; Asano et al., 2008), which can miss certain subspecies, as of yet undescribed species, or isolates possessing variant phenotypic properties. A method by which all *Firmicutes* within a sample could be detected would greatly improve the turn-around time and effectiveness of quality control decision making in breweries. However, due to widespread genetic transfer, there are no reliable biochemical methods which can identify bacteria as belonging to *Firmicutes* (Makarova et al., 2006). Inclusion of bacteria in the Phylum *Firmicutes* is based on 16S rRNA gene sequence as this is the only *Firmicutes* gene that has been shown to ascribe to a molecular clock (Makarova et al., 2006). Attempts thus far at generating *Firmicutes*-specific (or lactic acid bacteria-specific) PCR primers are predicted to amplify numerous species from other phyla, and are lacking in depth, range, and specificity (Neeley et al., 2005). Furthermore, current methods of detecting *Firmicutes* rely upon post-PCR techniques such as differential gradient gel electrophoresis which are time consuming and subject to human interpretation (Lopez et al., 2003; Walter et al., 2001). As such, a more rapid, precise, and reproducible screening method for *Firmicutes* is desirable.

Here we describe a *Firmicutes* real-time PCR (rPCR) detection system. By using a single set of universal eubacterial PCR primers (Muyzer et al., 1993; Relman, 1991) along with hydrolysis probes that recognize opposite strands of the PCR-amplified DNA, we assembled a

* Corresponding author. Tel.: +1 306 966 4330; fax: +1 306 966 8049.
E-mail address: b.ziola@usask.ca (B. Ziola).
[1] Current address: CanBiocin, #1015-8308 114 Street, Edmonton AB, Canada T6G 2E1.

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Table 1
In silico prediction of probe and primer binding specificities

Name	Sequence	Location[a]	Beer-spoilage *Firmicutes*[b]	All *Firmicutes*	All other bacteria
8F	AGAGTTTGATCCTGGCTCAG	8–27	117[c] (100%)	443[d] (99.1%)	985[e] (97.3%)
534R	ATTACCGCGGCTGCTGG	534–518	348[f] (100%)	1328[g] (99.1%)	3767[h] (98.5%)
357R probe	CTTGCTGCCTCCCGTAG	341–357	348[f] (100%)	1328[g] (99.1%)	3764[i] (98.5%)
Firmicutes probe	CTGATGGAGCAACGCCGCGT	386–405	348[f] (100%)	1248[g] (93.1%)	1093[h] (28.6%)

[a] 16S rRNA gene location is based on *Escherichia coli* numbering.
[b] Contains the genera *Bacillus*, *Enterococcus*, *Lactobacillus*, *Megasphaera*, *Pectinatus*, *Pediococcus*, *Selenomonas*, *Streptococcus*, and *Zymophilus*.
[c,d,e,f,g,h,i] Number of 16S rRNA gene sequences of "good" quality for type strain, cultured isolates in the RDPII as of April 2, 2008. These regions of the 16S rRNA gene had 117, 447, 1012, 348, 1340, 3824, and 3823 respective sequences from which to search.

multiplex PCR containing a *Firmicutes* probe with a second probe targeted to a universal region of the 16S rRNA gene (i.e., a non-competitive internal control). Impetus for development of the *Firmicutes* rPCR was the need to rapidly detect potential spoilage bacteria in the brewery environment, with the assay nonetheless being applicable to any setting in which rapid and accurate detection of a *Firmicutes* microbe is desired. As filtration of beer is a step commonly used for concentration of contaminating microbes in the brewery (Dreier and Kleesiek, 2006; Priest and Campbell, 2003; Reid et al., 1990; Roche et al., 1990; Satokari et al., 1998), the *Firmicutes* rPCR was assessed not only using quantified DNA, but also using known numbers of spoilage *Firmicutes* filtered from artificially inoculated, commercially available beer.

2. Materials and methods

2.1. Development of the Firmicutes probe

A Multiple Sequence Alignment (MSA) of the first three variable regions of the 16S rRNA gene (Neefs et al., 1993) was constructed as described by Dobson (2001). The MSA consisted of 30 lactobacilli and pediococci species (135 isolates), four *Staphylococcus aureus* isolates, and one *Leuconostoc mesenteroides* isolate. A consensus sequence of the MSA was generated using GeneDoc software (Nicholas et al., 1997), and was inspected for highly conserved regions. The *Firmicutes* probe was designed to one such region of the consensus sequence and paired for use with a set of universal eubacterial primers 8F and 534R (Muyzer et al., 1993; Relman, 1991) and eubacterial probe 357R, the reverse sequence of a previously described primer (Muyzer et al., 1993). The sequences of PCR primers and probes are given in Table 1.

Table 2
Multiple sequence alignment of consensus sequences

	Genera	Consensus sequence from MSAs[a]	Number of sequences[b]
	Firmicutesprobe	CTGATGGAGCAACGCCGCGTGA	
Firmicutes			
	Bacillus	CTGACGGAGCAACGCCGCGTGA	113
	Enterococcus	CTGACCGAGCAACGCCGCGTGA	36
	Lactobacillus	CTGATGGAGCAACGCCGCGTGA	103
	Leuconostoc	CTGATGGAGCAACGCCGCGTGA	15
	Megasphaera	CTGACGGAGCAACGCCGCGTGA	2
	Pectinatus	CTGACGGAGCAACGCCGCGTGA	2
	Pediococcus	CTGATGGAGCAACGCCGCGTGA	6
	Selenomonas	CTGACGGAGCAACGCCGCGTGA	9
	Streptococcus	CTGACCGAGCAACGCCGCGTGA	66
	Zymophilus	CTGACGGAGCAACGCCGCGTGA	1
Non-Firmicutes			
	Acetobacter	CTGATCCAGCAATGCCGCGTGT	13
	Acinetobacter	CTGATCCAGGCATGCCGCGTGT	18
	Alcaligenes	CTGATCCAGGCATNCCGCGTGN	4
	Citrobacter	CTGATGCAGGCATGCCGCGTGT	9
	Enterobacter	CTGATGCAGGCATGCCGCGTGT	10
	Gluconobacter	CTGATCCAGCAATGCCGCGTGT	4
	Klebsiella	CTGATGCAGGCATGCCGCGTGT	11
	Micrococcus	CTGATGCAGGCATGCCGCGTGA	4
	Obesumbacterium	CTGATGCAGGCATGCCGCGTGT	1
	Proteus	CTGATGCAGGCATGCCGCGTGT	2
	Pseudomonas	CTGATGCAGGCATGCCGCGTGT	83
	Zymomonas	CTGATCCAGCAATGCCGCGTGA	2

[a]Nucleotides highlighted in grey indicate a mismatch that does not affect binding of the *Firmicutes* probe, while those highlighted in black indicate mismatches that prevent the *Firmicutes* probe from binding. The sequence shown corresponds to bases 386–407 of the *E. coli* 16S rRNA gene.
[b]Number of type-strain sequences of "good" quality, accessible through the RDPII database and used to create the MSA and consensus sequence.

2.2. In silico testing

The range and specificity of the *Firmicutes* probe and universal eubacterial primers were tested *in silico* using the Ribosomal Database Project II (RDPII) probematch tool (Cole et al., 2007) with default parameters for type strain 16S rRNA gene sequences and allowing for a maximum of 2 bp to be mismatched. The matches were divided into the categories beer-spoilage *Firmicutes* (Priest and Campbell, 2003), all *Firmicutes*, and all other bacteria (Table 1).

To determine the specificity of the putative *Firmicutes*-specific region, the corresponding region of the 16S rRNA gene for all known beer-spoilage associated genera (*Firmicutes* and non-*Firmicutes*) were assembled from the RDPII, limiting the search to type isolates with "good" sequences. The number of type-strain sequences satisfying these requirements for each genera are listed in Table 2. MSAs were created from these sequences using ClustalX 1.81 on default settings (Thomson et al., 1997). Consensus sequences were created from the MSAs using the European Molecular Biology Software Suite v2.2.0 "cons" program (Rice et al., 2000), using the threshold settings of "required number of identities at a position" equal to one greater than half the number of sequences in the MSA, and "threshold above which the consensus is given in uppercase" equal to three quarters the number of sequences in the MSA. The resulting consensus sequences were then aligned using the ClustalX 1.81 software on default settings (Thomson et al., 1997) (Table 2).

Cross-reactivity of the primers and probes with brewer's yeast (*Saccharomyces cerevisiae*) was assessed using the NCBI GenBank BLAST search tool for short, nearly exact matches on all primers and probes. The eubacterial primers 8F and 534R were predicted to create an amplicon (approximately 526 bp) from the yeast 18S rRNA gene, while the 357R probe was predicted to bind weakly (due to mismatches) to a region within this amplicon. Most importantly, the *Firmicutes* probe was not predicted to bind to any region of the *S. cerevisiae* genome.

2.3. rPCR parameters

The *Firmicutes* probe was labeled with a 5′ 6-FAM (fluorescein) and a 3′ Black Hole Quencher I molecule (Integrated DNA Technologies, Coralville IA). PCR amplification of the 16S rRNA gene was performed with the forward and reverse primers 8F and 534R (Muyzer et al., 1993; Relman, 1991). A universal eubacterial probe (357R probe) was designed using the primer sequence 341 as described by Muyzer et al. (1993), however, the sequence was used in reverse complement to bind to the strand opposite of the strand recognized by the *Firmicutes* probe (Table 1). The 357R probe was labeled with a 5′ Cy3 and 3′ Black

Table 3
Detection of *Firmicutes* associated with brewery contamination

	Beer-spoilage Genera[a]	Species Tested[b]	rPCR results
Firmicutes			
	Bacillus	3 spp	+ (all spp)
	Enterococcus	*faecalis*	+
	Lactobacillus	23 spp	+ (all spp)
	Leuconostoc	*mesenteroides*	+
	Megasphaera	*cerevisiae*	+
	Pectinatus	2 spp	+ (both spp)
	Pediococcus	7 spp	+ (all spp)
	Selenomonas	*lacticifex*	+
	Streptococcus	*viridans*	+
	Zymophilus	2 spp	+ (both spp)
Non-Firmicutes			
	Acetobacter	*aceti*	–
	Acinetobacter	*calcoaceticus*	–
	Alcaligenes	*faecalis*	–
	Citrobacter	*freundii*	–
	Enterobacter	*agglomerans*	–
	Gluconobacter	*oxydans*	–
	Klebsiella	*pneumoniae*	–
	Micrococcus	*luteus*	–
	Obesumbacterium	*proteus*	–
	Proteus	*mirabilis*	–
	Pseudomonas	*aeruginosa*	–
	Zymomonas	*mobilis*	+

[a] As listed by Priest and Campbell (2003).
[b] A comprehensive list of the species and origins of isolates tested is provided in the table provided as Supplementary data.

Hole Quencher II molecule (Integrated DNA Technologies, Coralville, IA).

DNA extractions were performed using 10 µl of culture with 100 µl of the BioRad Instagene DNA Matrix Kit (BioRad, Mississauga, ON), as directed by the manufacturer. Cultures were grown in de Man Rogosa Sharpe (MRS) broth at 30 °C as described in Haakensen et al. (2007), except for anaerobes which were grown as described by Chaban et al. (2005). At the final step, 90 µl of supernatant were removed and stored at −20 °C. Each reaction contained 2 U of Invitrogen Platinum® Taq DNA polymerase, 1× PCR buffer (Invitrogen, Burlington ON), 1.5 mM of $MgCl_2$, 0.2 mM of each of the four deoxynucleotide triphosphates, 0.4 µM of primers 8F/534R and 0.2 µM of each of the *Firmicutes* and 357R probes. Template DNA was added (2.5 µl) and the volume was brought to 25 µl with water. The rPCR program consisted of a denaturation step of 5 min at 95 °C, followed by amplification cycles of 95 °C for 15 s, 52 °C for 30 s, 72 °C for 30 s. Amplification and monitoring of fluorescence after each cycle was performed in a Cepheid Smart-Cycler I (Cepheid, Sunnyvale CA). Cycle threshold (Ct) values of 30 and 10 fluorescence units for FAM and Cy3, respectively, were used as cut-offs for determining positive and negative results. If the threshold value had not been reached by 45 cycles of amplification, the sample was deemed to be negative for that rPCR-target. Binding specificity of the *Firmicutes* probe and multiplex rPCR system was confirmed with at least one species belonging to each genus known to be associated with beer-spoilage (Priest and Campbell, 2003) (Table 3). A comprehensive list of the species and origins of isolates tested is provided in the Supplementary data. The multiplex PCR was also tested on *S. cerevisiae* DNA to confirm *in silico* predictions. rPCR results were confirmed by agarose gel electrophoresis.

To ensure that beer components (possibly including live or dead *S. cerevisiae* or free-floating DNA from bacteria or yeast) did not produce false-positive results or increased background fluorescence in the rPCR, four different tests were performed using a pasteurized 5% v/v alcohol beer, pH 4.8, containing an average of 11.0 bitterness units. The first test was to use beer instead of template DNA in the PCR reaction. The second and third tests involved centrifuging 341 ml of beer (the volume of one standard size bottle) for 20 min at 10,000 *g*. The supernatant was removed, and the pelleted material was either directly used as the template DNA in a PCR reaction, or subjected to the Puregene Genomic DNA purification kit (Inter-Medico, Markham ON), using the DNA extraction protocol for 0.5 ml of yeast culture. Lastly, 341 ml of beer was filtered through 0.45 µm HVLP type Durapore filters (Millipore, Billerica MA). The filter was placed in a tube with MRS media and vortexed at top speed for 30 s. The filter was removed and the DNA extraction protocol was performed on the MRS media.

2.4. rPCR standard curve

A standard curve was constructed using serially diluted DNA from *Pediococcus claussenii* ATCC BAA-344^T (American Type Culture Collection; see Supplementary Data). The OD_{260} of the DNA was 0.046, giving a concentration of 2.3 µg/ml. The DNA was diluted in 10-fold increments in water to a final concentration of 2.3 pg/ml and 2.5 µl of each dilution was used per PCR reaction. DNA was amplified as per the conditions described above. The cycle number at which the fluorescence produced crossed the threshold (threshold cycle, Ct) were plotted against the Log_{10} fg DNA per PCR reaction and the standard curve was constructed. The correlation coefficient (R^2) was calculated as previously described (Higuchi et al., 1993).

2.5. Determining threshold detection limit

The threshold detection limit for bacteria artificially inoculated into beer was determined by membrane filtration and the number of colony forming units (CFU) in each case was determined by plating on MRS agar. Serially diluted *P. claussenii* ATCC BAA-344^T was inoculated into 100 ml of beer (commercially available pasteurized 5% v/v alcohol beer, pH 4.8, containing an average of 11.0 bitterness units). Artificially contaminated and control (uncontaminated) beer were passed through 0.45 µm Durapore membrane filters (Type HV, 2.5 cm; Millipore, Billerica MA) using a vacuum manifold. DNA was extracted from the filter membranes using the PureGene DNA Purification System DNA (Gentra Systems, Minneapolis MN). Filters were placed in a 1.5 ml microfuge tube, filtration-side to the inside, and 300 µl of cell suspension solution was added. Tubes were vigorously vortexed and then centrifuged for 5 min at 16,000 *g* in an angle rotor centrifuge at room temperature. After the filter was then removed from the side of the tube, the bacterial pellet was processed according to the manufacturer's protocol for Gram-positive organisms.

Alternatively, an overnight incubation (16 h) in MRS broth after filtration, but prior to DNA extraction, was used as a pre-enrichment step. *Lactobacillus brevis* CCC1202 (Molson Coors Culture Collection; see Supplementary data) as well as *P. claussenii* ATCC BAA-344^T were used and tests were performed on both 100 ml and 341 ml volumes of beer. For the 341 ml volume (equivalent to a package unit of beer), a larger diameter filter membrane (4.7 cm) was used to allow for a more rapid filtration. At the beginning of the DNA extraction procedure, the MRS broth was vigorously vortexed with the filter and, after the filter was removed, centrifugation was used to produce a bacterial pellet. The resuspended pellet was then transferred to a 1.5 ml microfuge tube and the DNA extraction procedure was continued.

3. Results

3.1. Predictions

In silico assessment of the *Firmicutes* probe predicted that the probe would bind to 100% of beer-spoilage *Firmicutes* (Priest and Campbell, 2003), and 92.9% of all *Firmicutes* (Table 1). Of the *Firmicutes* species that were not predicted to bind to the probe, 95% are from the Classes *Mollicutes* and *Clostridia*, namely, the families *Eubacteriaceae* and *Syntrophomonadaceae* which do not contain any

known beer-spoilage organisms. When allowing for a 2 bp mismatch, approximately 94% of all non-*Firmicutes* species that might match the probe belong to the Phylum *Actinobacteria*. However, all of these potentially reactive microbes had 16S rRNA genes mismatched to the *Firmicutes* probe at bases seven and thirteen, namely, those bases apparently critical for effective binding of the probe (Table 2).

3.2. Experimental confirmation

Experimental testing of isolates from 22 beer-spoilage associated genera confirmed *in silico* predictions of binding for the *Firmicutes* probe (Table 3). Simultaneously, the universal eubacterial primers amplified any bacterial DNA present, which was confirmed by the Cy3 fluorescence signal given by the universal 357R probe. Only one of the twelve non-*Firmicutes* beer-spoilage genera (*Zymomonas*) produced a weakly positive result (Universal probe Cy3 Ct-value of 21.4 and *Firmicutes* probe FAM Ct-value of 35.7 using approximately 5 ng of DNA in the reaction) and this species was one of the few non-*Firmicutes* that were predicted by the RDPII search to be capable of binding the probe. The much lower Ct-value for the *Firmicutes* probe compared to the Universal probe suggests inferior binding of the *Firmicutes* probe, likely due to mismatches. When *S. cerevisiae* DNA was directly used as the rPCR template, the universal 16S rRNA primers amplified a segment of the yeast 18S rRNA gene. However, the amplicon produced only reacted weakly with the 357R probe (Ct-value of 29.8 with approximately 12 ng of DNA in the reaction) and, as expected, did not react with the *Firmicutes* probe. In the tests run to determine if beer components produce false-positive results or increased background fluorescence in the rPCR, negative results were obtained for both the 357R probe and *Firmicutes* probe. Most importantly, the *Firmicutes*-specific rPCR accurately detected all *Firmicutes* tested in this study (Table 3 and Supplementary data).

3.3. Standard curve and threshold detection limit in beer

Using *P. claussenii* as a representative *Firmicutes*, a standard curve was constructed to test the accuracy of the *Firmicutes* rPCR. Based on serial DNA dilution experiments, as little as 5.75 fg of DNA could be reproducibly detected (Fig. 1). The linearity range of the rPCR covered six orders of magnitude and produced a high R^2 value of >0.993. In consensus with published data (Dreier and Kleesiek, 2006), and based upon the average size of known *Lactobacillus* and *Pediococcus*

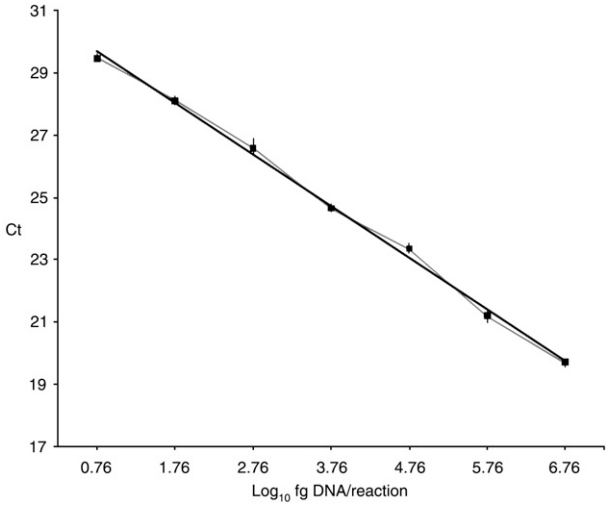

Fig. 1. Standard curve showing average of three trials (with range indicated by bars) using serially diluted DNA from *P. claussenii* ATCC BAA-344^T. The straight line corresponds to the regression of Log_{10} fg DNA/reaction; R^2>0.993.

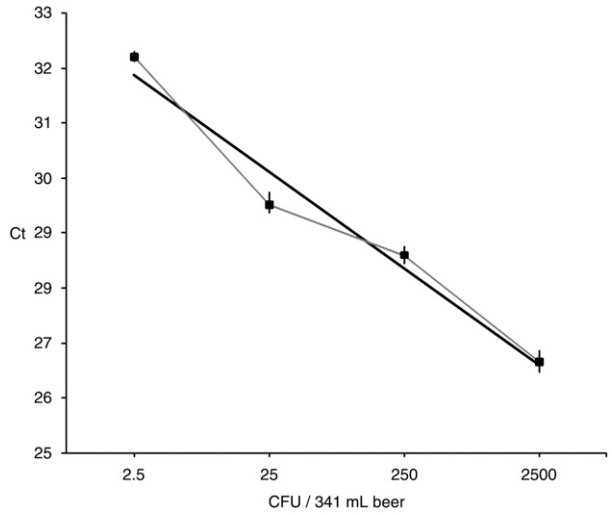

Fig. 2. Standard curve showing average of three trials (with range indicated by bars) using DNA extracted from overnight cultures grown from filters of *P. claussenii* ATCC BAA-344^T serially diluted and artificially inoculated into beer. The straight line corresponds to the regression of CFU/341 ml of beer (one standard bottle); R^2>0.966.

genomes, 5.75 fg of DNA corresponds to DNA from fewer than 10 bacterial cells. However, when DNA was directly extracted from bacteria trapped on filters in the artificially spiked beer procedure, the detection limit for *P. claussenii* ATCC BAA-344^T using the rPCR assay was 50–100 CFU per 100 ml of beer. Incorporation of an overnight pre-enrichment incubation of the filter allowed for a greater than 30-fold increase in sensitivity, reducing the detectable number of bacteria detectable to 2.5–10 CFU when 100 or 341 ml of beer were filtered (Fig. 2). This far exceeds the 50 CFU/250 ml set as a standard to aim for when doing microbiological assessment in breweries by Reid et al. (1990).

4. Discussion

The *Firmicutes* detection system uses a set of universal eubacterial primers and probe, together with a novel *Firmicutes* probe, all targeting the 16S rRNA gene. To our knowledge, this is the first description of a DNA-based detection system that accomplishes Phylum-specific identification through the use of a single probe. As discussed by Reid et al. (1990), there is surprisingly little published on the standards required for microbiological stability for beer, but the only safe standard should be zero microorganisms. Inspite of this desired target, a more realistic goal of detecting contaminated beer with more than 50 CFU per 250 ml was set (Reid et al., 1990). In combination with filtration concentration of bacterial cells, the *Firmicutes* rPCR gives brewers a screening assay which can detect as few as 2.5–10 CFU of *Firmicutes* per packaged 341 ml bottle or can of beer (Figs. 1 and 2). This detection sensitivity is some 300-fold better than methods previously described for detecting *Firmicutes* in beer by PCR (Bischoff et al., 2001) or *in situ* hybridization techniques (Yasuhara et al., 2001), and comparable to the detection limit accomplished by chemiluminescence which requires the production of bacteria-specific monoclonal antibodies and specialized visualization equipment (March et al., 2005). In addition to the sensitivity achieved, the *Firmicutes* rPCR is unique in that simultaneous detection of all bacteria associated with post-wort beer-spoilage is accomplished.

Deployment of this *Firmicutes* detection system allows the user to concurrently obtain two critical pieces of information. The PCR primers amplify a portion of the 16S rRNA gene from all bacteria present in a sample, as reported by the universal probe, while the *Firmicutes*-specific probe reports whether there is a *Firmicutes* bacterium present in the sample. As such, the rPCR indicates whether *Firmicutes* bacteria are

present, or whether any bacteria are present at all. Because the vast majority (>90%) of beer-spoilage incidents are caused by *Firmicutes*, the timely detection and differentiation of these organisms from non-*Firmicutes* (which are unlikely to grow in beer) by brewery quality control laboratories is important. *S. cerevisiae* DNA directly added to the PCR reaction produced an amplicon of the yeast 18S rRNA gene, but only weak fluorescence was seen with the universal 357R probe and no cross-reactivity occurred with the *Firmicutes* probe. Moreover, all tests for cross-reactivity of this PCR system with beer and "free" DNA in beer were negative. As such, background yeast DNA present in beer does not appear to pose a problem for the assay.

While several of the consensus sequences for *Firmicutes* genera have mismatches at bases five and six (highlighted in grey in Table 2), these mismatches do not sufficiently alter the binding affinity of the probe to affect the reporting of a *Firmicutes* bacterium. Since the probe functions as a reporter molecule during the 72 °C elongation step of the rPCR, only three key mismatches are apparently required for destabilization of the probe (highlighted in black in Table 2). These mismatches appear to require periodic spacing along the span of the probe in order to prevent binding. To prevent binding, mismatches are required at bases seven and thirteen, with at least one additional mismatch at either base eleven and/or twenty-two. The *Firmicutes* rPCR accurately detected all *Firmicutes* tested in this study (Table 3), and all 42 species from the ten beer-spoilage associated *Firmicutes* genera were rPCR-positive (Supplementary data). The Class *Mollicutes* contained the majority of the *Firmicutes* species that were not predicted to bind to the *Firmicutes* probe, however, there are no species within this Class known to be capable of growing in beer. The Class *Clostridia* contained the second greatest number of *Firmicutes* species not predicted to bind to the *Firmicutes* probe, however, *Acidaminococcaceae*, the only family within *Clostridia* known to contain beer-spoilage genera (i.e., *Megasphaera*, *Pectinatus*, *Selenomonas* and *Zymophilus*) was predicted and confirmed experimentally to bind the *Firmicutes* probe.

While the majority of non-*Firmicutes* predicted to bind the probe by *in silico* analysis belonged to the Class *Actinobacteria*, the only genera of this class known to be capable of growing in beer is *Micrococcus*. When tested empirically, *Micrococcus* was rPCR-negative. All of the *Actinobacteria* (including *Micrococcus*) that were predicted to be similar to the probe within a 2 bp mismatch had a mismatch at bases seven and thirteen, both of which appear to be critical in the binding of the probe (Table 2).

The *Firmicutes* rPCR described here is the first method by which a bacterium can be accurately identified as belonging to the *Firmicutes* Phylum, thus providing a key piece of information for the triaging of quality control decision making within the brewery setting. This rPCR can be used as a stand-alone detection system (i.e., a screening assay) for determining the presence of *Firmicutes versus* non-*Firmicutes* bacteria in a sample, thus serving as a starting point for species identification. Alternatively, the *Firmicutes* rPCR can serve as an internal control when incorporated with primers targeted to genes of interest (e.g., hop-resistance genes relevant to beer-spoilage by *Firmicutes*; Haakensen et al., 2007, 2008) in a multiplex rPCR. With such a multiplex PCR, the *Firmicutes* probe would confirm the presence of a *Firmicutes* bacterium, thereby allowing both positive and negative results for genes of interest to be accepted with certainty. Overall, the *Firmicutes* rPCR effectively addresses the issue of rapid detection, with concurrent accurate identification of *Firmicutes* in breweries as well as in other settings where confirmation of the presence of a *Firmicutes* bacterium is of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijfoodmicro.2008.04.002.

References

Asano, S., Suzuki, K., Ozaki, K., Kuriyama, H., Yamashita, H., Kitagawa, H., 2008. Application of multiplex PCR to the detection of beer-spoilage bacteria. Journal of the American Society of Brewing Chemists 66, 37–42.

Bischoff, E., Bohak, I., Back, W., Leibhards, S., 2001. Rapid detection of beer-spoilage bacteria by PCR and universal primers. Monatsschrift fur Brauwissenschaft 54, 4–5.

Bjorkroth, J., Ridell, J., Korkeala, H., 1996. Characterization of Lactobacillus sake strains associating with production of ropy slime by randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) patterns. International Journal of Food Microbiology 31, 59–68.

Carr, F.J., Chill, D., Maida, N., 2002. The lactic acid bacteria: a literature survey. Critical Reviews Microbiology 28, 281–370.

Chaban, B., Deneer, H., Dowgiert, T., Hymers, J., Ziola, B., 2005. The Flagellin Gene and Protein from the Brewing Spoilage Bacteria *Pectinatus cerevisiiphilus* and *Pectinatus frisingensis*, vol. 51, pp. 863–874.

Cole, J.R., Chai, C., Farris, R.J., Wang, Q., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Bandela, A.M., Cardenas, E., Garrity, G.M., Tiedje, J.M., 2007. The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. Nucleic Acids Research 35, D169–D172 (Database issue).

DiMichele, L.J., Lewis, M.J., 1993. Rapid, species-specific detection of lactic acid bacteria from beer using the polymerase chain reaction. Journal of the American Society of Brewing Chemists 51, 63–66.

Dobson, C.M. 2001. Phylogenetic analysis of Lactobacillus and Pediococcus based on the 16 S rRNA gene, 16S-23S rRNA interspacer regions, and heat shock 60 protein. M.S. thesis. University of Saskatchewan, Saskatoon SK, Canada, 2001.

Dreier, J., Kleesiek, K., 2006. Rapid test for foreign yeasts and slow growing beer-spoilage organisms during beer production. Brauwelt International 4, 102–103.

Fernandez, K., Duenas, M., Irastorza, A., Bilbao, A., Del Campo, G., 1996. Characterization and DNA plasmid analysis of ropy *Pediococcus* spp. strains isolated from Basque country ciders. Journal of Food Protection 59, 35–40.

Haakensen, M.C., Butt, L., Chaban, B., Deneer, H., Dowgiert, T., Ziola, B., 2007. A horA-specific real-time PCR for detection of beer-spoilage lactic acid bacteria. Journal of the American Society of Brewing Chemists 65, 157–165.

Haakensen, M., Schubert, A., Ziola, B., 2008. Multiplex PCR for putative Lactobacillus and Pediococcus beer-spoilage genes and ability of gene presence to predict growth in beer. Journal of the American Society of Brewing Chemists 66, 63–70.

Higuchi, R., Fockler, C., Dollinger, G., Watson, R., 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology 11, 1026–1030.

Llauberes, R.M., Richard, B., Lonvaud, A., Dubourdieu, D., Fournet, B., 1990. Structure of an exocellular beta-D-glucan from *Pediococcus* sp., a wine lactic bacteria. Carbohydrate Research 203, 103–107.

Lopez, I., Ruiz-Larrea, F., Cocolin, L., Orr, E., Phister, T., Marshall, M., VanderGheynst, J., Mills, D.A., 2003. Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69, 6801–6807.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D.M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.-., Diaz-Muniz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'sullivan, D., Steele, J., Unlu, G., Saeier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B., Mills, D., 2006. Comparative genomics of the lactic acid bacteria. Proceedings of the National Academy of Science USA 103, 15611–15616.

March, C., Manclus, J.J., Abad, A., Navarro, A., Montoya, A., 2005. Rapid detection and counting of viable beer-spoilage lactic acid bacteria using a monoclonal chemiluminescence enzyme immunoassay and a CCD camera. Journal of Immunological Methods 303, 92–104.

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59, 695–700.

Neefs, J.M., Van de Peer, Y., De Rijk, P., Chapelle, S., De Wachter, R., 1993. Compilation of small ribosomal subunit RNA structures. Nucleic Acids Research 21, 3025–3049.

Neeley, E.T., Phister, T.G., Mills, D.A., 2005. Differential real-time PCR assay for enumeration of lactic acid bacteria in wine. Applied and Environmental Microbiology 71, 8954–8957.

Nicholas, K.B., Nicholas, H.B.J., Deerfield, D.W.I., 1997. Genedoc: Analysis and Visualization of Genetic Variation. . EMBNEWS [Online: http://www.psc.edu/biomed/genedoc 4:14].

Priest, F.G., Campbell, I., 2003. In: Priest, F.G., Campbell, I. (Eds.), Brewing microbiology, 3rd ed. Kluwer Academic, New York, N.Y.

Reid, G.C., Hwang, A., Meisel, R.H., 1990. The sterile filtration and packaging of beer into polyethylene terephthalate containers. Journal of the American Society of Brewing Chemists 48, 85–91.

Relman, D.A., 1991. In: Persing, D.H., Smith, T.F., Tenover, F.C., White, T.J. (Eds.), In diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, DC, pp. 489–495.

Rice, P., Longden, I., Bleasby, A., 2000. EMBOSS: the European Molecular Biology Open Software Suite. Trends Genetics 16, 276–277.

Roche, K.L., Meier, P.M., Levy, R.V., 1990. Retention of beer spoilage microorganisms by polyvinylidene fluoride microporous membranes with various retention ratings. Journal of the American Society of Brewing Chemists 51, 4–9.

Sakamoto, K., Konings, W.N., 2003. Beer spoilage bacteria and hop resistance. International Journal of Food Microbiology 89, 105–124.

Satokari, R., Juvonen, R., Mallison, K., von Wright, A., Haikara, A., 1998. Detection of beer spoilage bacteria *Megasphaera* and *Pectinatus* by polymerase chain reaction and colorimetric microplate hybridization. International Journal of Food Microbiology 45, 119–127.

Skinner, K.A., Leathers, T.D., 2004. Bacterial contaminants of fuel ethanol production. Journal of Industrial Microbiology and Biotechnology 31, 401–408.

Bergey's manual of systematic bacteriology. In: Sneath, P.H.A., Mair, M.E., Sharpe, M.E., Holt, J.G. (Eds.), Gram-positive Bacteria other than Actinomycetes, vol. 2. Williams and Wilkins, Baltimore, MD.

Stiles, M.E., Holzapfel, W.H., 1997. Lactic acid bacteria of foods and their current taxonomy. International Journal of Food Microbiology 36, 1–29.

Teusink, B., Smid, E.J., 2006. Modelling strategies for the industrial exploitation of lactic acid bacteria. Nature Reviews Microbiology 4, 46–56.

Thomson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25, 4876–4882.

Walter, J., Hertel, C., Tannock, G.W., Lis, C.M., Munro, K., Hammes, W.P., 2001. Detection of *Lactobacillus, Pediococcus, Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 67, 2578–2585.

Yasuhara, T., Yuuki, T., Kagami, N., 2001. Novel quantitative method for detection of *Pectinatus* using rRNA targeted fluorescence probes. Journal of the American Society of Brewing Chemists 59, 117–121.

3. A *horA*-SPECIFIC REAL-TIME PCR FOR DETECTION OF BEER-SPOILAGE LACTIC ACID BACTERIA

Author contributions:

Monique Haakensen performed most of the bacterial growth experiments, designed PCR primer h198F2 and *horA* rPCR probe, carried out rPCR experiments, conducted statistical analyses, and drafted the manuscript.

Leah Butt performed some of the lactobacilli growth experiments.

Bonnie Chaban conceived the study and designed PCR primers h198R, h297F, and h297R.

Harry Deneer provided scientific input regarding PCR experiments.

Barry Ziola conceived the study, edited the manuscript, and is the holder of the research grant used to fund the study.

BRIEF INTRODUCTION TO CHAPTER 3

At the onset of the research for this thesis, it was widely believed that hop-resistance could be attributed solely to the presence of the antimicrobial resistance gene *horA*. However, previous studies were limited in scope and did not examine a variety of *Lactobacillus* and *Pediococcus* species from different environmental origins, including non-beer spoiling isolates. The purpose of this study was to determine the potential of the *horA* gene to be used as a genetic marker for assessing the beer-spoilage potential of lactobacilli and pediococci in the brewing industry. In order to achieve this goal, it was also necessary to perform a large scale study on the ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer. A method for assessing ability of these bacteria to grow in beer was developed and 133 isolates were tested for their ability to grow in beer. This growth data serves as a basis for statistical analysis used for work in Chapters 5, 6, and 7.

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horA-Specific Real-Time PCR for Detection of Beer-Spoilage Lactic Acid Bacteria

M. C. Haakensen, L. Butt, B. Chaban,[1] H. Deneer, and B. Ziola,[2] *Department of Pathology, University of Saskatchewan, Saskatoon, SK, Canada;* **and T. Dowgiert,** *Molson Coors Brewing Company, Golden, CO*

ABSTRACT

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Beer-spoilage bacteria have long been a problem for brewers. Among the most problematic beer spoilers are several species of the Gram-positive genera *Lactobacillus* and *Pediococcus.* Current methods of detecting and identifying these organisms are time-consuming and do not differentiate between bacteria capable of spoiling beer and benign bacteria. The *horA*-specific real-time polymerase chain reaction (rPCR) described here identifies beer-spoilage organisms based not on their identity, but on the presence of a gene that we show to be highly correlated with the ability of an organism to grow in beer. The *horA* hop-resistance gene has been shown to be associated with beer spoilage by isolates from four *Lactobacillus* spp. and one *Pediococcus* sp. We document the presence of the *horA* gene in one additional genus and 11 additional species, with many of these bacteria commonly found as beer spoilers. The use of *horA*-specific rPCR allows for a substantial reduction in the time required for detection of potential beer spoilage bacteria and efficiently discriminates between those organisms that have the *horA* gene (highly likely to spoil beer) and those organisms that do not have the gene (much less likely to spoil beer).

Keywords: Beer spoilage, Hop resistance, *horA,* Lactic acid bacteria, Real-time PCR

RESUMEN

Las bacterias dañinas a la cerveza han sido un problema para cerveceros por mucho tiempo. Entre la más problemática organismos dañinas a la cerveza son varias especies Gram-positiva del género *Lactobacillus* y *Pediococcus*. Los métodos actuales de detectar y de identificar estos organismos son desperdiciadores de tiempo y no distinguen entre las bacterias capaces de deteriorar la cerveza y bacterias benignas. La *horA*-específica reacción en cadena de la polimerasa en tiempo real (rPCR) descrita aquí identifica los organismos dañinos a la cerveza basada no en su identidad, sino en la presencia de un gene que demostremos para ser correlacionados fuertemente con la capacidad de un organismo de crecer en cerveza. El gene *horA* de lúpulo-resistencia se ha demostrado para ser asociado con la deterioración de cerveza por los aislados de cuatro especies de *Lactobacillus* y una especie de *Pediococcus*. Documentamos la presencia del gene *horA* en un género adicional y 11 especies adicionales, con muchas de estas bacterias encontradas comúnmente como bacterias dañinas a la cerveza. El uso del rPCR *horA*-específico permite una reducción substancial en el tiempo requerido para la detección de las bacterias con potencial a deteriorar la cerveza y discrimina eficientemente entre esos organismos que tengan el gene *horA* (altamente probable estropear la cerveza) y esos organismos que no tienen el gene (mucho menos probable estropear la cerveza).

Palabras claves: Bacterias ácido-lácticas, Deterioración de la cerveza, *horA*, PCR en tiempo real, Resistencia de lúpulo

Spoilage of beer by microorganisms is a significant problem for the brewing industry. Although most microorganisms fail to grow in beer due to the presence of hop compounds, ethanol, reduced oxygen, and low nutrient levels, some organisms possess this ability (11,18). The most common beer spoilers are lactic acid bacteria (LAB), namely selected species within the genera *Lactobacillus* and *Pediococcus* (12). Bitter acid compounds derived from hop extract α-acids (i.e., *trans*-isohumulone) act as mobile carrier protonophores to dissipate the transmembrane pH gradient of potentially harmful microbes (18). Many beer-spoilage bacteria have developed mechanisms by which they can maintain or restore the transmembrane pH gradient when grown in the presence of hop compounds (19).

The *horA* gene has been associated with the beer-spoilage ability of some isolates of *Lactobacillus brevis* (16), *Lactobacillus casei* (17), *Lactobacillus lindneri* (17), *Lactobacillus paracollinoides* (23), and *Pediococcus damnosus* (24). *horA* is believed to function by allowing the organism to maintain a pH gradient in the presence of hops (13,14). The strength of the correlation between the presence of *horA* and the ability of bacteria to grow in beer has not yet been documented. Should a significant correlation exist, the presence of *horA* would serve as a predictor of the beer-spoilage potential of bacteria found in breweries.

Lactobacilli and pediococci occupy a variety of niches and can be either beneficial or detrimental to various food- and beverage-related industries (3,7,9,12). It is unknown whether *horA* is unique to bacteria associated with the brewing industry or whether this gene is also present, perhaps with a different phenotypic importance, in bacteria found in other settings. There presently is no information available as to the distribution or presence of *horA* in LAB found in nonbrewery environments.

Sami et al (17) described a polymerase chain reaction (PCR) designed to amplify a portion of the ATP-binding cassette (ABC) region of *horA*. This region is highly conserved in multiple drug-resistance genes in many bacteria (15) and, therefore, is not specific to *horA*. The *horA* PCR primers described by Sami et al (17) are not optimal, and a low PCR cycle number must be used to avoid nonspecific amplification of non-*horA,* ABC-containing genes. In addition, the primers have an undesirably high melting temperature (>70°C) caused by high GC content (24). In 2006, Suzuki et al (24) attempted to improve on the *horA* primers of Sami et al (17). The primers designed by Suzuki et al (24) span a 543-bp region of *horA* that has 246 bp in common with the region amplified by Sami et al (17). In fact, 71% of the region amplified by Sami et al (17) also was amplified by Suzuki et al (24), and the improved specificity was more likely caused by a greater stringency implemented by the PCR amplification program than by the sequence of the targeted region. Because a highly sensitive and specific *horA*-PCR is necessary to establish the distribution of *horA* across multiple genera and environments, we have designed primers and a hydrolysis probe for real-time PCR (rPCR) amplification of a specific (non-ABC) region of *horA*.

EXPERIMENTAL

Bacteria

Bacterial strains used are listed in Table I. All cultures were grown in 15-mL capped tubes containing de Man, Rogosa, Sharpe (MRS) broth as described previously (4). Two beers were used in

[1] Current address: Department of Microbiology and Immunology, Queen's University, Kingston, ON K7L 3N6, Canada.
[2] Corresponding author. Department of Pathology, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada. E-mail: b.ziola@usask.ca; Phone: +1.306.966.4330; Fax: +1.306.966.8049.

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growth experiments. Beer 1 was a filter-sterilized 4% vol/vol alcohol beer, pH 4.2, containing an average of 9.8 bitterness units (BU; determined using ASBC Methods [1]). Beer 2 was a pasteurized 5% vol/vol alcohol beer, pH 3.8, containing an average of 11.0 BU.

Bacteria were induced to grow in the presence of hops through serial passaging in double-strength modified MRS (2×MMRS, with Tween 80 excluded [20]) combined with incremental concentrations of beer. Initially, tubes containing 50% beer 2 and 50% 2×MMRS (50/50 medium) were inoculated in duplicate with 100 µL of log-phase culture from the initial MRS culture. All bacterial strains, except *Lactobacillus fermentum* (ATCC 14931^T), were capable of growing (i.e., visible turbidity compared with control

TABLE I
Bacterial Strains, Presence of *horA* Gene, and Ability to Grow in Beer

Isolate[a]	Origin	*horA*[b]	Growth (days)[c]	
			Beer 1	Beer 2
Lactobacillus acetotolerans				
ATCC 43578^T	Rice vinegar	–	–	–
Lactobacillus acidophilus				
ATCC 521	Unknown	–	–	–
ATCC 4356^T	Human	–	–	–
CCC B1209	Brewery	–	–	–
Lactobacillus amylovorus				
ATCC 33198[d]	Hog intestine	–	–	–
ATCC 33620^T	Corn silage	–	–	–
Field isolate[e]	Unknown	–	–	–
Ingledew I1[f]	Fuel alcohol	–	–	–
Ingledew I2	Fuel alcohol	+/+	+ (2)	+ (3)
T-13[g]	Poultry	–	–	–
Lactobacillus brevis				
ATCC 4006	Unknown	–	–	–
ATCC 8007	Kefir grains	+	–	–
ATCC 14869^T	Human feces	–	–	–
BSO 31[h]	Brewery	+/+	+ (5)	+ (5)
CCC 96S1L	Brewery	+/+	+ (5)	+ (5)
CCC 96S2AL	Brewery	+/+	+ (5)	+ (5)
CCC B1202	Brewery	+/+	+ (5)	+ (5)
CCC B1203	Brewery	+/+	+ (5)	+ (5)
CCC B1206	Brewery	+/+	+ (5)	+ (5)
CCC B1204	Brewery	+/+	+ (5)	+ (5)
CCC B1300	Brewery	+/+	+ (2)	+ (3)
ETS.1	Wine	–	–	–
ETS.2	Wine	–	–	–
Lactobacillus casei				
ATCC 334[g]	Cheese	–	–	–
ATCC 4913[g]	Unknown	–	–	–
ATCC 25598^T	Milking machine	–/–	+ (8)	+ (11)
CCC 95G1L	Brewery	–/–	+ (8)	+ (10)
CCC 95G2L	Brewery	–/+	+ (5)	+ (5)
CCC B9657	Brewery	–/+	+ (9)	+ (9)
CCC B1205	Brewery	–/+	+ (2)	+ (2)
CCC B1241	Brewery	–/+	+ (27)	–
Ingledew I3	Fuel alcohol	–	–	–
Ingledew 18C	Fuel alcohol	+/+	+ (27)	–
Lactobacillus delbrueckii				
ATCC 4797	Corn mash	–	–	–
ATCC 9649^T	Sour grain mash	–	–	–
ATCC 11842^T	Bulgarian yogurt	–	–	–
ATCC 12315^T	Cheese	–	–	–
CCC 95G3L	Brewery	–	–	–
CCC B1044	Brewery	–	–	–

(*continued on next page*)

[a] Isolate identity as determined by C. M. Dobson (5), with type strains indicated. ATCC = American Type Culture Collection, Manassas, VA; CCC = Coors Brewing Company, Golden CO; BSO = beer spoilage organism; ETS = ETS Laboratories (T. Arvik), St. Helena, CA; and Molson = Molson Breweries of Canada Limited, Montreal, PQ, Canada.
[b] Determined by *horA* real-time polymerase chain reaction. For bacterial isolates capable of growing in beer, this was recorded as pre- or post-growth in beer.
[c] + = visible turbidity in beer and upon subsequent subculture to 85% beer 2 and 15% double-strength modified de Man, Rogosa, Sharpe medium (85/15 medium); – = no visible turbidity in beer and not capable of growing upon subculture to 85/15 medium; static = no visible turbidity in beer, but capable of growing upon subculture to 85/15 medium. Numbers in parentheses indicate the number of days required to attain visible growth in beer.
[d] A. Estrada, College of Agriculture, University of Saskatchewan, Saskatoon, SK, Canada.
[e] D. Korber, College of Agriculture, University of Saskatchewan, Saskatoon, SK, Canada.
[f] W. M. Ingledew, College of Agriculture, University of Saskatchewan, Saskatoon, SK, Canada.
[g] G. Reid, Lawson Research Institute, London, ON, Canada.
[h] B. Kirsop, Institute for Biotechnology, Cambridge, England.
[i] R. Wheatcroft, Agriculture and Agri-food Canada, Guelph, ON, Canada.
[j] Ropy (R) phenotype.
[k] Nonropy (NR) phenotype.
[l] K. Fernandez, Gipuzko, Spain.

tubes) in this concentration of beer. Once visible growth was attained in 50/50 medium (18–72 hr), the bacteria were passaged into duplicate tubes containing 85% beer 2 and 15% 2×MMRS (85/15 medium). Once visible growth occurred (18–168 hr), the bacteria were inoculated in duplicate into tubes of beer 1 and beer 2. Inoculated beer was incubated at 30°C.

Once turbidity was seen in beer, 500 µL from each tube was inoculated into fresh 85/15 medium to confirm that turbidity was the result of bacterial growth. This step also was performed on those tubes of beer that did not produce visible turbidity after 21 days to ensure that no viable cells persisted in stasis. Those tubes of beer showing no visible turbidity after 21 days of incubation but show-

TABLE I
(***continued from preceding page***)

Isolate[a]	Origin	*horA*[b]	Growth (days)[c]	
			Beer 1	**Beer 2**
CCC B1240	Brewery	–	–	–
CCC B1262	Brewery	–	–	–
Lactobacillus ferintoshensis				
ATCC 11307	Brewery	–	–	–
Lactobacillus fermentum				
ATCC 9338[g]	Unknown	–/–	+ (9)	+ (11)
ATCC 14931[T]	Fermented beets	–	–	–
ATCC 14932[g]	Saliva	–	–	–
Lactobacillus fructivorans				
ATCC 8288[T]	Unknown	–	–	–
Lactobacillus helviticus				
ATCC 15009[T]	Cheese	–	–	–
CCC B1186	Brewery	+/+	+ (6)	+ (static)
Lactobacillus hilgardii				
ATCC 8290[T]	Wine	–	–	–
ATCC 27305	Wine	–/–	+ (4)	+ (8)
ATCC 27306	Wine	–	–	–
Lactobacillus homohiochii				
ATCC 15434[T]	Spoiled sake	–	–	–
Lactobacillus jensenii				
ATCC 25258[T]	Human	+	–	–
Lactobacillus kefiri				
ATCC 35411[T]	Kefir grains	–	–	–
Lactobacillus kefirgranum				
ATCC 51647[T]	Kefir grains	–	–	–
Lactobacillus kefironofaciens				
ATCC 43761[T]	Kefir grains	–	–	–
Lactobacillus paracollinoides				
ATCC 8291	Brewery	–/–	+ (7)	+ (11)
Lactobacillus plantarum				
ATCC 8014	Unknown	–	–	–
ATCC 8041	Corn silage	+/+	+ (2)	+ (3)
ATCC 11305	Brewery	–	–	–
ATCC 12706	Cured meat	–	–	–
ATCC 14431[g]	Grass silage	–	–	–
ATCC 14917[T]	Pickled cabbage	–	–	–
BSO 92	Brewery	+/+	+ (2)	+ (2)
CCC 96M2BL	Brewery	+/+	+ (12)	+ (12)
CCC B1301	Brewery	+/+	+ (5)	+ (12)
Lactobacillus reuteri				
ATCC 19371	Silage	–	–	–
ATCC 25744	Plants	–	–	–
ATCC 31282	Unknown	–/–	+ (13)	+ (13)
ATCC 43200	Cucumbers	–	–	–
RC-14[g]	Unknown	–	–	–
Lactobacillus rhamnonsus				
ATCC 7469[T]	Unknown	–	–	–
ATCC 7469a[g]	Derived from ATCC 7469	–	–	–
ATCC 8530[g]	Unknown	–/–	+ (7)	+ (10)
ATCC 15820	Corn liquor	–	–	–
ATCC 21052[g]	Human feces	+	–	–
Lactobacillus sakei				
ATCC 15521[T]	Moto	–	–	–
ATCC 15578	Moto	+/+	+ (6)	+ (7)
Lactobacillus zeae				
ATCC 393	Cheese	–	–	–
Lactobacillus unspeciated				
ATCC 4005	Tomato pulp	–	–	–
ATCC 27054	Apple juice	–	–	–
ATCC 27304	Wine must	+/+	+ (3)	+ (3)
CCC L86	Brewery	+/+	+ (4)	+ (11)

(*continued on next page*)

ing growth when subcultured to 85/15 medium were incubated for an additional 3 weeks, after which they were again subcultured to 85/15 medium to determine whether cells were in stasis or growing at a slow rate in beer.

Isolates that grew in 85/15 medium when subcultured from beer also were subcultured into a second set of tubes containing beer. This was done to ensure that growth or stasis seen in the initial beer cultures was not due to carryover of 2×MMRS nutrients from the preceding growth of bacteria in 85/15 medium. This second set of beer cultures was assessed for bacterial growth as before. Bacterial isolates producing visible turbidity in the second subculture in beer (and subsequent subculture from beer to 85/15 medium) were considered hop-resistant and capable of growing in beer. Isolates producing no visible turbidity in the second subculture of beer (or

TABLE I
(*continued from preceding page*)

Isolate[a]	Origin	*horA*[b]	Growth (days)[c]	
			Beer 1	Beer 2
Leuconostoc mesenteroides				
CCC 98G3	Brewery	+/+	+ (11)	+ (18)
Oenococcus oeni				
ETS.10	Wine	–	–	–
Pediococcus acidilactici				
ATCC 8042	Brewery	+/+	+ (8)	–
ATCC 12697	Unknown	–	–	–
ATCC 25740	Plant	–	–	–
BSO 54	Brewery	–	–	–
BSO 77[h]	Brewery	–	–	–
Molson B77b	Brewery	–	–	–
Pac 1.0[i]	Unknown	–	–	–
Pediococcus claussenii				
CCC B962A	Brewery	–/–	+ (9)	+ (10)
CCC B1056R[j]	Brewery	+/+	+ (14)	+ (14)
CCC B1056NR[k]	Brewery	+/+	+ (13)	–
CCC B1098R	Brewery	–/–	+ (7)	+ (7)
CCC B1098NR	Brewery	+/+	+ (8)	+ (13)
CCC B1099R	Brewery	–/–	+ (12)	+ (14)
CCC B1099NR	Brewery	–/–	+ (7)	+ (7)
CCC B1100	Brewery	–/–	+ (8)	+ (static)
CCC B1208	Brewery	–	–	–
CCC B1260R	Brewery	–/–	+ (8)	+ (13)
CCC B1260NR	Brewery	–/–	+ (6)	+ (6)
ATCC BAA-344^{T}R	Brewery	+/+	+ (8)	+ (8)
ATCC BAA-344^{T}NR	Brewery	+/+	+ (6)	+ (6)
Pediococcus damnosus				
ATCC 11308	Brewery	–/–	+ (27)	–
ATCC 25248	Brewery	–	–	–
ATCC 25249	Brewery	+	–	–
ATCC 25249a	Brewery	–	–	–
ATCC 29358^T	Brewery	–	–	–
Molson B48	Brewery	+/+	+ (7)	+ (7)
Molson 49	Brewery	+/+	+ (10)	+ (13)
Molson B76	Brewery	+/+	+ (11)	+ (12)
Pediococcus dextrinicus				
ATCC 33087^T	Silage	–	–	–
Pediococcus inoptinatus				
ATCC 49902^T	Brewery	–	–	–
Pediococcus parvulus				
ATCC 43013	Wine	–/–	+ (13)	+ (13)
ETS.3	Wine	–	–	–
ETS.4	Wine	–	–	–
ETS.5	Wine	–	–	–
ETS.6	Wine	–	–	–
ETS.7	Wine	–	–	–
ETS.8	Wine	–	–	–
ETS.9	Wine	–	–	–
ETS.11	Wine	–	–	–
ETS.12	Wine	–	–	–
ETS.13	Wine	–	–	–
ETS.14	Wine	–	–	–
Spain 2.6R[l]	Cider	–	–	–
Spain 2.6NR[l]	Cider	–	–	–
Pediococcus pentosaceus				
ATCC 8081	Milk	–	–	–
ATCC 10791	Cucumber	–	–	–
ATCC 11309	Unknown	–	–	–
ATCC 29723	Horse urine	–	–	–
ATCC 33314	Sake mash	–	–	–
ATCC 33316^T	Brewery	–	–	–

upon subsequent subculture to 85/15) were considered hop-sensitive and incapable of growth or stasis in beer.

DNA Extractions

DNA was extracted from bacteria grown in MRS medium prior to exposure to beer. DNA also was isolated from bacteria growing in 85/15 medium after the second subculture in beer. DNA extractions were performed using 10 µL of culture with 100 µL of the Instagene DNA matrix kit (BioRad, Mississauga, ON, Canada), as directed by the manufacturer. At the final step, 90 µL of supernatant was removed and stored at –20°C. Each DNA extraction was confirmed by PCR using primers to housekeeping genes (16S rRNA gene or *cpn60*), as described previously (6), to ensure that DNA was present and intact. The identity of all organisms capable of growing in beer was confirmed after growth in beer by sequencing the first three variable regions of the 16S rRNA gene.

Primer Design

The h198F2/R primers and corresponding hydrolysis probe (Fig. 1) were designed to a specific region of *horA* gene, based on consideration of rPCR requirements (Invitrogen, Burlington, ON, Canada). The hydrolysis probe (h198probe) was labeled with a 5′ FAM reporter molecule and 3′ Black Hole Quencher (Sigma Genosys, Oakville, ON, Canada).

Primer set h297 was designed to a conserved sequence of the ABC region of *horA*, with the forward primer identical to primer LbHC-1 described by Sami et al (17). A new reverse primer (binding to bases 1621–1601 of *L. brevis horA*; GenBank Accession No. AB005752) was designed to obviate the problems of self-complementarity and high melting temperature inherent in the Sami et al (17) reverse primer LbHC-2. The h297 primer set spanned a region 87% identical to the region amplified by the primers described earlier by Sami et al (17) and 45% identical to the region amplified by the primers recently described by Suzuki et al (24).

rPCR

Each reaction contained 2 U of Invitrogen Platinum *Taq* DNA polymerase, 1× PCR buffer (Invitrogen), 1.5m*M* $MgCl_2$, 0.2m*M* each of the four deoxynucleotide triphosphates, 0.4µ*M* h198F2/R primers, and 0.2µ*M* probe. Template DNA was added (2.5 µL), and the volume was brought to 25 µL with water. The rPCR program consisted of a denaturation step of 5 min at 95°C; followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec; and a final extension step of 8 min at 72°C. Fluorescence after each cycle of amplification was measured in a Smart-Cycler I (Cepheid, Sunnyvale, CA). A cycle threshold (Ct) value of 30 fluorescence units was used as a cut-off for determining positive and negative results. A Ct value of zero indicates that the threshold had not been crossed after 40 cycles of rPCR amplification, and the sample was deemed to be *horA* rPCR negative. rPCR amplification was verified by agarose gel electrophoresis on 1.0% agarose gels containing ethidium bromide at 0.5 mg/mL. Sequencing and sequence analysis were performed as described previously (3).

Determining Threshold Detection Limit

Pediococcus claussenii ATCC BAA-344^T was used to determine the threshold detection limit of the *horA* rPCR. Serial dilutions of bacteria were inoculated into 100 mL of beer 2. The number of CFU inoculated was determined by streaking for isolated colonies on MRS agar. Artificially contaminated and control beers were passed through 0.45-µm Durapore membrane filters (type HV, 20 mm) using a vacuum manifold. DNA was extracted from the filter membranes using the PureGene DNA purification system (Gentra Systems, Minneapolis, MN). Membranes were placed in a 1.5-mL capped microfuge tube, and 300 µL of cell suspension solution was added. After vortexing for 5 min, tubes were centrifuged for 5 min at 16,000 × *g* in an angle rotor microfuge at room temperature. The filter then was removed from the side of the tube, and the bacterial pellet was processed according to the manufacturer's protocol for Gram-positive organisms.

Determining Potential Cross-Reactivity

The h198 primer set was tested against all bacterial genera known to be capable of growth in beer (11). A nucleotide BLAST search was used to determine the potential cross-reactivity of the h198 primers with all known DNA sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank database.

RESULTS AND DISCUSSION

PCR Detection of *horA*

We examined two PCR primer sets for their ability to discriminate between bacterial isolates that possess the *horA* gene and those that do not. Despite optimization of the reverse primer in the h297 primer set, false-positive and inconclusive PCR results still were obtained (data not shown). In contrast to the h297 primer set, our second primer set (h198) was designed to amplify a region of the *horA* gene that does not share significant identity with genes in the NCBI GenBank database (initially determined in May 2003 and still valid as of November 2006), including potential homologous genes (Fig. 1). The h198 primer set did not amplify any nonspecific (i.e., non-*horA*) DNA from any of the bacteria tested (data not shown). All nucleotide comparisons with the NCBI GenBank database supported the prediction that the h198 primers would be unable to bind to any known DNA sequences (bacterial or fungal) other than *horA* (data not shown). The h198 primer set

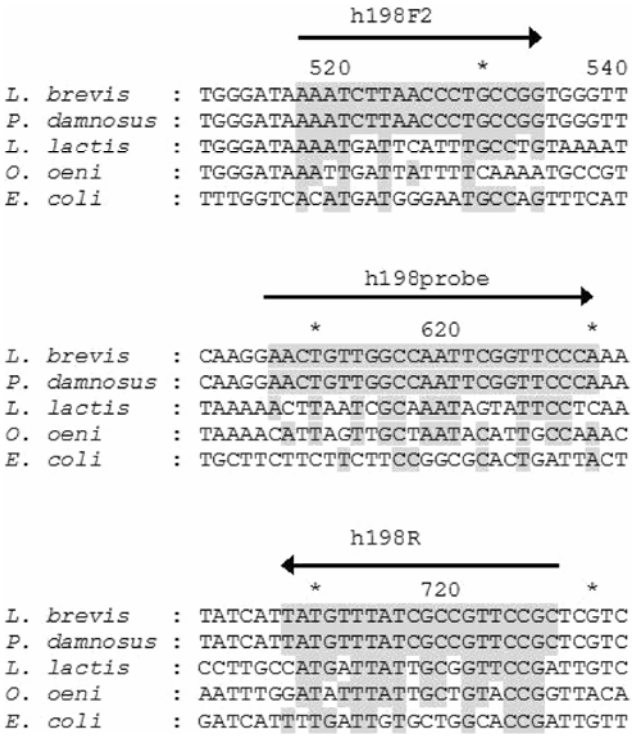

Fig. 1. Location of the h198 real-time polymerase chain reaction primers and hydrolysis probe on the *horA* gene and comparison with homologous genes. Shading indicates consensus with the *horA* gene. Numbering is from the start codon of *Lactobacillus brevis horA* sequence AB005752. *L. brevis* (*horA* gene AB005752); *Pediococcus damnosus* (*horA* gene AB218963); *Lactococcus lactis* (*lmrA* gene U63741); *Oenococcus oeni* (*omrA* gene AY249862); *Escherichia coli* (*msbA* gene Z11796).

reproducibly identified those isolates that possessed the *horA* gene and produced clean negative results, as shown by rPCR Ct values (Fig. 2A) and agarose gel electrophoresis of rPCR-amplified product (Fig. 2B). Use of the h198 primer set with a hydrolysis probe (i.e., *horA* rPCR) (Fig. 1) allowed detection of the *horA* gene in 100–200 CFU of LAB per 100 mL of beer in under 2 hr. This detection sensitivity was sufficiently low to bypass the need for a lengthy growth enrichment step for beer-spoilage LAB prior to detection, allowing detection of spoilage bacteria in a time frame that would enable more effective brewery quality control decisions to be made.

Presence and Distribution of *horA* in LAB Species

In all, 135 LAB isolates were screened for the presence of the *horA* gene and the ability to grow in beer (Table I). Our analysis included isolates from 22 *Lactobacillus* spp., four putative new *Lactobacillus* spp., one *Leuconostoc* sp., one *Oenococcus* sp., and seven *Pediococcus* spp. Previously, *horA* was detected in isolates of *L. brevis* (16), *L. casei* (17), *L. lindneri* (22), *L. paracollinoides* (22), and *P. damnosus* (24), findings that were confirmed here, except for *L. paracollinoides* (Table I). In addition, we detected *horA* in isolates of *Lactobacillus amylovorus*, *Lactobacillus helviticus*, *Lactobacillus jensenii*, *Lactobacillus plantarum*, *Lactobacillus rham-*

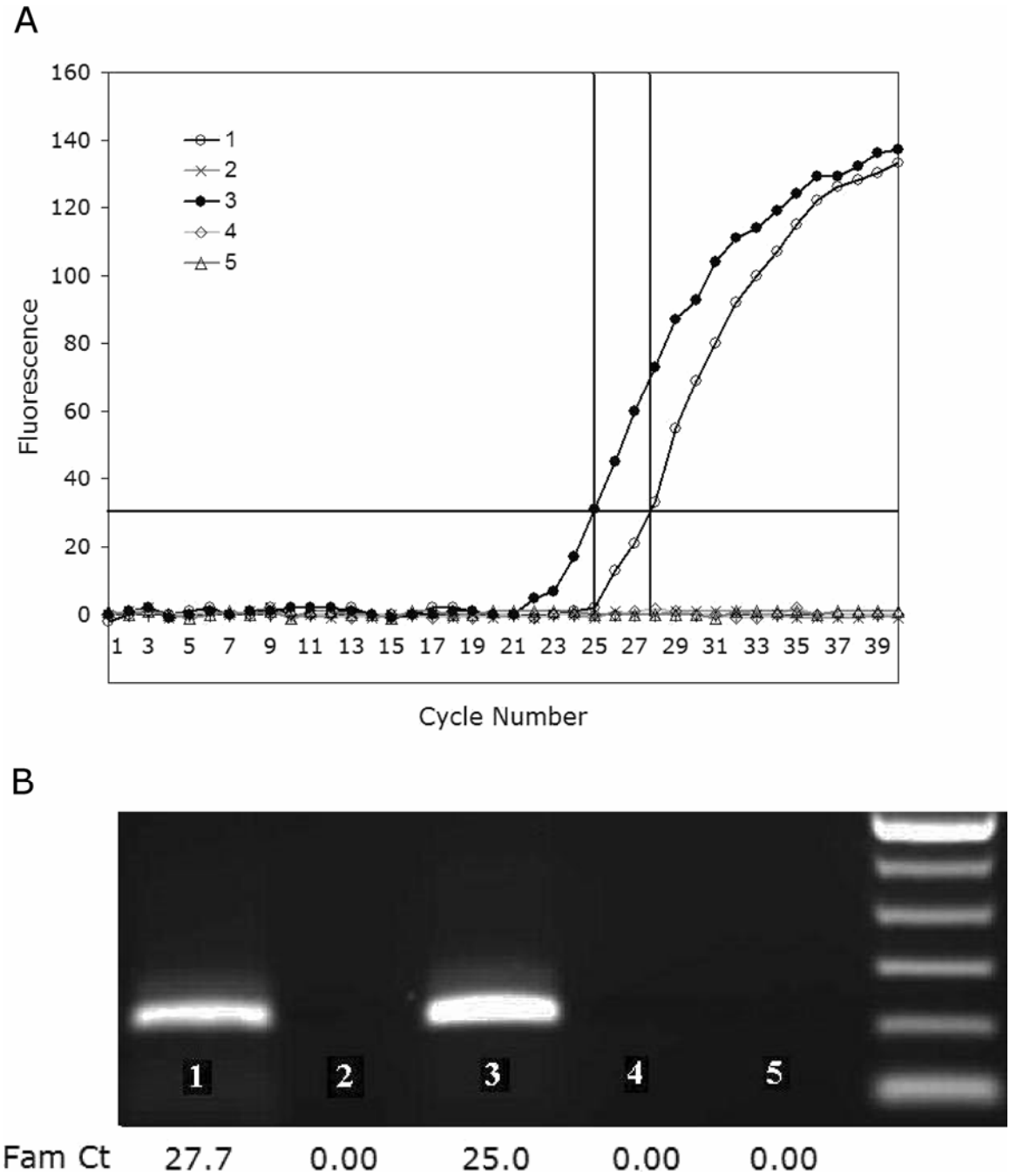

Fig. 2. *horA* real-time polymerase chain reaction (rPCR). **A,** Fluorescence graph for a *horA* rPCR. Vertical lines indicate the cycle during which isolates crossed the assigned threshold (Ct) value of 30. 1 = *Pediococcus claussenii* ATCC BAA-344^T NR; 2 = *Pediococcus damnosus* ATCC 29538^T; 3 = *Lactobacillus brevis* CCC B1202; 4 = *Lactobacillus brevis* ATCC 14869^T; 5 = negative control containing all *horA* rPCR reagents but no DNA template. **B,** Agarose gel electrophoresis of *horA* rPCR products corresponding to the samples in **A**. The right lane contains a 100-bp DNA ladder, with 100–600 bp shown.

nosus, *Lactobacillus sakei*, two putative new *Lactobacillus* spp., *Leuconostoc mesenteroides*, *Pediococcus acidilactici*, and *P. claussenii* (Tables I and II; identities of all isolates containing *horA* were confirmed by sequencing of the 16S rRNA gene after bacterial growth in beer). As such, *horA* now has been detected in isolates from three genera of LAB, including 16 different species.

Interestingly, four *L. casei* isolates were initially *horA* rPCR negative after growth in MMRS media; however, after subsequent passage in beer, they were positive for *horA*. This suggests that previous studies (17,24) may have incorrectly identified some bacterial isolates as being *horA* negative by not screening for the presence of the *horA* gene after growth in beer. If true, this means there is a stronger association between the presence of *horA* and the ability of an organism to grow in beer than previously was appreciated (17,22,24). Because *horA* is located on a plasmid, continual passaging in laboratory media can lead to loss of the plasmid (22, 24,25). Therefore, it is important to look for the gene after a small or extremely small subset of bacteria harboring the plasmid have been amplified preferentially by growth in a beer environment. Support for this conclusion is provided by our finding that a significant correlation exists between the origin of a LAB isolate (brewery versus nonbrewery) and the presence of the *horA* gene (χ^2 $P <$ 0.0001). This finding supports the idea that the *horA* gene is positively selected for the brewery environment, reaffirming that the presence of *horA* enhances the beer-spoilage potential of a LAB. Thus, supposedly harmless LAB introduced into a brewery could become devastating beer spoilers should even a few bacteria among the original population harbor the *horA* gene.

Previously, the *horA* gene had been found only in LAB derived from brewing environments. A question remains—where did the *horA* gene originate? If *horA* only exists in LAB isolated from breweries, the role of *horA* may be very specific to resistance in this environment, indicating a gene duplication event as its evolutionary origin. In an attempt to answer this question, we looked for the *horA* gene in LAB isolated from other environments. Not surprisingly, we found the *horA* gene in LAB isolated from diverse environments (Table I). There were eight isolates of *Lactobacillus* of nonbrewery origin that possessed the *horA* gene (Tables I and II). The isolates were *Lactobacillus* spp. ATCC 27304, *L. amylovorus* Ingledew I2, *L. brevis* ATCC 8007, *L. casei* Ingledew 18C, *L. jensenii* ATCC 25258^T, *L. plantarum* ATCC 8041, *L. rhamnosus* ATCC 21052, and *L. sakei* ATCC 15578 (Table I). This finding has three major implications. The first is that there are selective pressures in some nonbrewery environments that result in bacteria maintaining the *horA* plasmid. The second is that *horA* may have come into the brewery from another environmental source. Last, and alternatively, it is possible that *horA*-harboring LAB may have originated in a brewery and spread elsewhere.

We have found *horA* in the isolates *L. amylovorus* Ingledew I2 and *L. casei* Ingledew 18C, both of which originally were isolated from yeast cultures used to produce fuel alcohol. Both of these bacteria were rapid growers at high alcohol concentrations. Finding *horA* in LAB detected in the fuel alcohol process may indicate that attempts at using hop compounds as antimicrobial agents in the fuel alcohol industry will have to take into account emergence of bacteria with the *horA* gene. Moreover, because *horA* has been associated with wide-spectrum antibiotic resistance (15), it is possible that *horA* may function as a resistance mechanism to antibiotics already used in the fuel alcohol industry to combat LAB contamination, e.g., penicillin G (2,21) and virginiamycin (8). Overall, this means that spread of LAB harboring *horA* through the fuel alcohol industry would have a major economic impact due to lowered alcohol production (10). Further heightening this concern, we have found that *horA* is present not only in LAB from many diverse environments but also in LAB isolated from widespread geographic locations such as Canada, Japan, and the United States (Table I).

Sequence Homology

We sequenced the region of the *horA* gene spanned by the h198 primers for 15 isolates of various origins, locations, and beer-spoil-

TABLE II
***horA* and Ability to Grow in Beer**

Bacteria	*horA* –[a]		*horA* +[a]		*P*[b]	PPV[c]	NPV[d]	Sens.[e]	Spec.[f]
	Growth +	Growth –	Growth +	Growth –					
Beer 1									
All (n = 135)	16	83	32	4	<0.0005	0.89	0.84	0.67	0.95
Lactobacillus (n = 83)	7	51	22	3	<0.0005	0.88	0.88	0.76	0.94
Pediococcus (n = 50)	9	31	9	1	<0.0005	0.90	0.78	0.50	0.97
All nonbrewery origin (n = 64)	3	54	4	3	<0.0005	0.57	0.95	0.57	0.95
All brewery origin (n = 54)	10	16	27	1	<0.0005	0.96	0.62	0.71	0.94
Brewery origin *Lactobacillus* (n = 26)	2	7	17	0	<0.0005	1.00	0.78	0.89	1.00
Brewery origin *Pediococcus* (n = 27)	8	9	9	1	<0.013	0.90	0.53	0.53	0.89
Leuconostoc (n = 1)	0	0	1	0	…	…	…	…	…
Oenococcus (n = 1)	0	1	0	0	…	…	…	…	…
Beer 2									
All (n = 135)	15	84	28	8	<0.0005	0.78	0.85	0.65	0.91
Lactobacillus (n = 83)	7	51	20	5	<0.0005	0.80	0.88	0.74	0.91
Pediococcus (n = 50)	8	32	7	3	<0.001	0.70	0.80	0.47	0.91
All nonbrewery origin (n = 64)	3	54	3	4	<0.0005	0.43	0.95	0.50	0.93
All brewery origin (n = 54)	9	17	24	4	<0.0005	0.86	0.65	0.73	0.81
Brewery origin *Lactobacillus* (n = 26)	2	7	16	1	<0.0005	0.94	0.78	0.89	0.88
Brewery origin *Pediococcus* (n = 27)	8	9	7	3	<0.074	0.53	0.70	0.47	0.75
Leuconostoc (n = 1)	0	0	1	0	…	…	…	…	…
Oenococcus (n = 1)	0	1	0	0	…	…	…	…	…

[a] Determined by *horA* real-time polymerase chain reaction (rPCR). Growth + = visible turbidity (or stasis) in beer and growth upon subsequent subculture to 85% beer 2 and 15% double-strength modified de Man, Rogosa, Sharpe medium (85/15 medium); growth – = no visible turbidity in beer and not capable of growing upon subculture to 85/15 medium.
[b] χ^2 analysis of the correlation of a *horA* rPCR-positive result with growth in beer and a negative result with no growth in beer.
[c] Positive predictive value = the probability that an isolate with a *horA* rPCR-positive result will spoil beer.
[d] Negative predictive value = the probability that an isolate with a *horA* rPCR-negative result will not spoil beer.
[e] Sensitivity = the ability of *horA* rPCR to detect organisms capable of spoiling beer.
[f] Specificity = the accuracy of *horA* rPCR in detecting only organisms capable of spoiling beer.

age ability and compared the sequences with the GenBank entries for *horA* (*L. brevis* AB005752, *L. paracollinoides* AB178589, and *P. damnosus* AB218963). The *horA* region sequenced was identical for nine of our isolates (*L. brevis* BSO 31, *L. brevis* CCC B1202, *L. brevis* CCC B1300, *L. jensenii* ATCC 25258^T, *L. rhamnosus* ATCC 21052, *L. rhamnosus* ATCC 7469^T, *P. claussenii* CCC B1056, *P. claussenii* ATCC BAA-344^T, and *P. damnosus* ATCC 25249). *L. amylovorus* Ingledew I2, *L. casei* Ingledew 18C, *L. plantarum* ATCC 8041, *Lactobacillus* sp. ATCC 27304, and *Leuconostoc mesenteroides* CCC 98G3 all possessed the same 1-bp mismatch, whereas *L. brevis* ATCC 8007 had four unique mismatches. No correlation was found between the presence of base-pair changes and the inability of a *horA*-positive isolate to grow in beer. This level of conservation suggests that the *horA* region spanned by the h198 primers codes for a portion of *horA* containing a structure critical to the function of the protein. Because the nucleic acid sequence in the h198 region is not divergent in the wobble base (third position of the coding frame), this indicates a very recent and common origin for the *horA* gene. Because the evolutionary divergence of *horA* differs from the evolutionary trees of these species, horizontal transfer of *horA* must be occurring freely among LAB.

Correlation of *horA* with Ability of An Isolate to Grow in Beer

When the 135 bacterial isolates were assessed for the ability to grow in beer, we found three distinct growth patterns. The first category was composed of those bacteria capable of growing in both beer 1 and beer 2, indicating that these organisms possessed the highest hop resistance. Organisms in the second category were capable of growing in beer 1 but were either static or unable to grow in beer 2, showing a lower resistance to hop compounds. The third category consisted of organisms that produced no visible turbidity when incubated in beer. They were not capable of returning to active growth when removed from beer and inoculated into a permissive nutrient media.

The *horA* rPCR had a very high specificity for detecting organisms capable of growing in beer (Table II). When *horA* was detected in a LAB, there was an 84% probability the organism would be able to grow in beer 1 and a 78% probability the organism would be able to grow in beer 2 ($P < 0.0005$ in both cases). The overall sensitivity for *horA* rPCR detection of organisms capable of spoilage in beer 1 was moderate (67%), despite a low false-positive (*horA* positive and inability to grow in beer) incidence. This reflected a situation where the proportion of isolates that were *horA* negative and unable to grow in beer was low compared with the number of isolates that were *horA* negative and able to grow in beer. For beer 2, the opposite situation created a similar moderate sensitivity (65%).

Thus, *horA* rPCR was highly accurate in identifying LAB capable of beer spoilage. With positive predictive values (PPV) of 88% for beer 1 and 80% for beer 2, there is a high probability that a *horA*-positive lactobacilli isolate would be capable of causing beer spoilage. The higher negative predictive value (NPV) of *Lactobacillus* compared with *Pediococcus* spp. (88% versus 78 or 80%) indicates that a smaller proportion of *horA*-negative *Lactobacillus* isolates were capable of growing in beer than were *horA*-negative *Pediococcus* isolates (Table II). This differential effect was magnified in *Lactobacillus* and *Pediococcus* isolates of brewery origin (NPV of 78% versus 53 or 70%).

This analysis indicates that, compared with the lactobacilli, a greater proportion of *Pediococcus* isolates possess *horA*-independent hop-resistance mechanisms. Thus, although *horA* is a highly accurate predictor of beer-spoilage ability in *Pediococcus* spp., it must be emphasized that half of the isolates of *Pediococcus* capable of growing in beer did not possess the *horA* gene (Table II). These isolates will be the focus of future studies with the aim of identifying novel hop-resistance associated genes and determining whether there is one or more mechanisms shared by these *horA*-independent hop-resistant *Pediococcus* isolates.

Correlation of the *horA* Gene and Rate of Growth in Beer

All organisms capable of growing in beer were assessed for the number of days required for visible turbidity to be seen in both beer 1 and beer 2 (Table I). A *t* test for independent samples showed that an isolate will grow significantly faster in beer 2 if it possesses the *horA* gene ($P < 0.0025$). The contribution of the *horA* gene to the beer-spoilage virulence of an organism was masked in beer 1 by isolates that were capable of growing in beer 1 but not in beer 2 ($P < 0.l445$). These findings, plus the observation that some *horA*-negative LAB were either incapable of growing in or grew at a much slower rate in higher hop, higher ethanol beer, supports the contention that *horA* contributes to the virulence of an organism by allowing it to grow faster in a higher hop, higher ethanol beer environment.

Although there was no significant correlation between the distribution of the *horA* gene and genus (*Lactobacillus* versus *Pediococcus*), *Lactobacillus* isolates did grow significantly faster than *Pediococcus* isolates (beer 1, $P < 0.0415$; beer 2, $P < 0.0035$). This observation, coupled with our finding that 100% of *horA* rPCR-positive brewery-origin *Lactobacillus* isolates grew in beer 1 (94% for beer 2), reinforces the fact that the *horA* rPCR accurately detected lactobacilli capable of rapidly causing beer spoilage. Although *Pediococcus* spp. did not grow as quickly as *Lactobacillus* spp. in this study, the *horA* rPCR also was effective at identifying pediococci with a high probability of spoiling beer. *Pediococcus* isolates that tested *horA*-positive had a 90% chance of spoiling low hop, low ethanol beer and a 70% chance of spoiling a higher hop, higher ethanol beer, with a mean of 10 days until visible turbidity was attained. As such, the *horA* rPCR is an effective tool for specifically and rapidly identifying LAB with high beer-spoilage potential.

CONCLUSIONS

We have described an rPCR for the specific detection of the *horA* gene that is more efficient at detection of the *horA* gene than previously described PCR methods. Eleven new species in three genera of LAB were found to harbor the *horA* gene, and *horA* was found to have widespread environmental and geographic distribution. The presence of *horA* was assessed in relation to the ability of 135 LAB isolates to grow in two types of beer, and it was determined that *horA* is a significant predictor of beer-spoilage capability. By specifically targeting organisms capable of beer spoilage through deployment of the *horA* rPCR described here, brewery quality control laboratories will be able to make rapid, accurate predictions regarding the potential beer-spoilage outcome of contamination by a LAB.

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LITERATURE CITED

1. American Society of Brewing Chemists. *Methods of Analysis*, 7th ed. Beer-23A Bitterness units, -23B Iso-alpha-acids. The Society, St. Paul, MN, 1976.
2. Bayrock, D. P., Thomas, K. C., and Ingledew, W. M. Control of *Lactobacillus* contaminants in continuous fuel ethanol fermentations by

constant pulsed addition of penicillin G. *Appl. Microbiol. Biotechnol.* 62:498-502, 2003.
3. Bjorkroth, J., Ridell, J., and Korkeala, H. Characterization of *Lactobacillus sake* strains associating with production of ropy slime by randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) patterns. *Int. J. Food Microbiol*. 31:59-68, 1996.
4. Chaban, B., Deneer, H., Dowgiert, T., Hymers, J., and Ziola, B. The flagellin gene and protein from the brewing spoilage bacteria *Pectinatus cerevisiiphilus* and *Pectinatus frisingensis*. *Can. J. Microbiol*. 51: 863-874, 2005.
5. Dobson, C. M. Phylogenetic analysis of *Lactobacillus* and *Pediococcus* based on the 16S rRNA gene, 16S-23S rRNA interspacer region, and heat shock 60 protein. M.S. thesis. University of Saskatchewan, Saskatoon SK, Canada, 2001.
6. Dobson, C. M., Deneer, H., Lee, S., Hemmingsen, S., Glaze, S., and Ziola, B. Phylogenetic analysis of the genus *Pediococcus*, including *Pediococcus claussenii* sp. nov., a novel lactic acid bacterium isolated from beer. *Int. J. Syst. Evol. Microbiol.* 52:2003-2010, 2002.
7. Fernandez, K., Duenas, M., Irastorza, A, Bilbao, A., and Del Campo, G. Characterization and DNA plasmid analysis of ropy *Pediococcus* spp. strains isolated from Basque Country ciders. *J. Food Prot.* 59:35-40, 1995.
8. Hynes, S. H., Kjargaard, D. M., Thomas, K. C., and Ingledew, W. B. Use of virginiamycin to control growth of lactic acid bacteria during alcohol fermentation. *J. Ind. Microbiol. Biotechnol*. 18:284-291, 1997.
9. Llaubères, R.-M., Richard, B., Lonvaud, A., Dubourdieu, D., and Foumet, B. Structure of an exocellular polysaccharide β-D-glucan from *Pediococcus* sp., a wine lactic acid bacteria. *Carbohydr. Res.* 203: 103-107, 1990.
10. Narendranath, N. V., Hynes, S. H., Thomas, K. C., and Ingledew, W. M. Effects of lactobacilli on yeast-catalyzed ethanol fermentations. *Appl. Environ. Microbiol.* 63:4158-4163, 1997.
11. Priest, F. G., and Campbell, I. *Brewing Microbiology,* 3rd ed. F. G. Priest and I. Campbell, eds. Kluwer Academic/Plenum Publishers, New York, 2003.
12. Sakamoto, K., and Konings, W. N. Beer spoilage bacteria and hop resistance. *Int. J. Food Microbiol.* 89:105-124, 2003.
13. Sakamoto, K., Margolles, A., van Veen, H. W., and Konings, W. N. Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-binding cassette multidrug transporter *horA*. *J. Bacteriol*. 183:5371-5375, 2001.
14. Sakamoto, K., van Veen, H. W., Saito, H., Kobayashi, H., and Konings, W. N. Membrane-bound ATPase contributes to hop resistance of *Lactobacillus brevis*. *Appl. Environ. Microbiol.* 68:5374-5378, 2002.
15. Sami, M., Suzuki, K., Kanta, S., Kadokura, H., Kitamoto, K., and Yoda, K. A plasmid pRH45 of *Lactobacillus brevis* confers hop resistance. *J. Gen. Appl. Microbiol.* 44:361-363, 1998.
16. Sami, M., Yamashita, H., Hirono, T., Kadokura, H., Kitamoto, K., Yoda, K., and Yamasaki, M. Hop-resistant *Lactobacillus brevis* contains a novel plasmid harboring a multidrug resistance-like gene. *J. Ferment. Bioeng.* 84:1-6, 1997.
17. Sami, M., Yamashita, H., Kadokura, H., Kitamoto, K., Yoda, K., and Yamasaki, M. A new and rapid method for determination of beer-spoilage ability of lactobacilli. *J. Am. Soc. Brew. Chem.* 55:137-140, 1997.
18. Simpson, W. J. Ionophoric action of *trans*-isohumulone on *Lactobacillus brevis*. *J. Gen. Microbiol.* 139:1041-1045, 1993.
19. Simpson, W. J., and Fernandez, J. L. Mechanism of resistance of lactic acid bacteria to *trans*-isohumulone. *J. Am. Soc. Brew. Chem.* 52:9-11, 1994.
20. Simpson, W. J., and Smith, A. R. W. Factors affecting antibacterial activity of hop compounds and their derivatives. *J. Appl. Bacteriol.* 72: 327-334, 1992.
21. Skinner, K. A., and Leathers, T. D. Bacterial contaminants of fuel ethanol production. *J. Ind. Microbiol. Biotechnol.* 31:401-408, 2004.
22. Suzuki, K., Kazumaru, I., Kazutaka, O., and Kiroshi, Y. Isolation of a hop-sensitive variant of *Lactobacillus lindneri* and identification of genetic markers for beer spoilage ability of lactic acid bacteria. *Appl. Environ. Microbiol*. 71:5089-5097, 2005.
23. Suzuki, K., Ozaki, K., and Yamashita, H. Genetic marker for differentiating beer-spoilage ability of *Lactobacillus paracollinoides* strains. *J. Appl. Microbiol.* 97:712-718, 2004.
24. Suzuki, K., Sami, M., Ijiima, K., Ozaki, K., and Yamashita, H. Characterization of *horA* and its flanking regions of *Pediococcus damnosus* ABBC478 and development of more specific and sensitive *horA* PCR method. *Lett. Appl. Microbiol.* 42:392-399, 2006.
25. Suzuki, K., Sami, M., Kadokura, H., Nakajuma, H., and Kitamoto, K., Biochemical characterization of *horA*-independent hop resistance mechanism in *Lactobacillus brevis*. *Int. J. Food Microbiol.* 76:223-230, 2002.

The pH values for beer 1 and 2 were corrected on page 158 on October 28, 2008.

4. IDENTIFICATION OF NOVEL *horA*-HARBOURING BACTERIA CAPABLE OF SPOILING BEER

Author contributions:

Monique Haakensen conceived the study, performed the experiments, and drafted the manuscript.

Barry Ziola conceived the study, edited the manuscript, and is the holder of the research grant used to fund the study.

BRIEF INTRODUCTION TO CHAPTER 4

Through the research conducted in Chapter 3, the *horA* gene was found to be distributed sporadically across various species from three genera and from diverse environmental origins. As such, it is of interest to determine the possible origin(s) of *horA*. The presence of *horA* in isolates of various environmental origins lends to the theory that there may be *horA*-harbouring bacteria present in the general and ubiquitous environment. A donation of spoiled home-brewed beer was obtained, the *horA* gene assayed for by the PCR methodology described in Chapter 3, and the contaminating bacteria cultured and identified through sequencing of the 16S rRNA gene. The finding of *horA* in bacteria of non-brewery origin which were additionally found to be capable of growing in beer suggests an environmental origin for the gene and raises the possibility that *horA* may play a role in resistance to compounds other than hops. That new genera were found to habour the *horA* gene highlights the threat to breweries for the potential of emerging beer-spoilage bacteria.

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NOTE / NOTE

Identification of novel *horA*-harbouring bacteria capable of spoiling beer

Monique Haakensen and Barry Ziola

Abstract: An ATP-binding cassette (ABC) multi-drug resistance (MDR) gene was found in 4 Gram-positive bacterial isolates of environmental origin and found capable of spoiling beer. The bacteria isolated were *Bacillus cereus*, *Bacillus licheniformis*, *Paenibacillus humicus*, and *Staphylococcus epidermidis*; all of which were previously unappreciated as beer-spoilage bacteria. The MDR gene found in these bacteria has less than 37% similarity to known ABC MDR proteins described for *Bacillus* and *Staphylococcus*, and this is the first finding of an ABC MDR gene in the genus *Paenibacillus*. The sequenced region of the gene was translated and compared phylogenetically with the closest GenBank matches of the respective species and the closest GenBank matches overall. The ABC MDR proteins from these isolates were found to cluster among known sequences of HorA, sharing 99.5% identity within the sequenced region. In the beer-spoilage-associated genera *Lactobacillus* and *Pediococcus*, the presence of the MDR gene *horA* correlates with the ability to grow in beer. As the unique *horA*-harbouring isolates described here are capable of growing in beer, it is likely that the presence of the *horA* gene likewise confers hop resistance to these organisms.

Key words: beer-spoilage bacteria, *Firmicutes*, *horA*.

Résumé : Un gène de résistance multiple aux drogues (MDR) à cassette ABC (ATP binding cassette) a été trouvé chez quatre isolats bactériens Gram-positifs d'origine environnementale, capables d'altérer la bière. Les bactéries isolées étaient *Bacillus cereus*, *Bacillus licheniformis*, *Paenibacillus humicus* et *Staphylococcus epidermidis*, lesquelles avaient été sous-estimées en regard de leur potentiel d'altération de la bière. Le gène MDR de ces bactéries possède moins de 37 % de similarité avec les protéines MDR ABC connues chez *Bacillus* et *Staphylococcus*. Il est aussi le premier gène MDR ABC identifié chez le genre *Paenibacillus*. La région séquencée du gène a été traduite et comparée d'un point de vue phylogénique aux séquences les plus apparentées de ces espèces respectives dans GenBank, ainsi qu'aux séquences les plus apparentées en général dans GenBank. Les protéines MDR ABC de ces isolats s'agrègent avec les séquences connues de HorA, partageant 99,5 % d'identité à l'intérieur de la région séquencée. Chez les genres *Lactobacillus* et *Pediococcus*, qui sont associés à l'altération de la bière, la présence du gène MDR *horA* est corrélée avec leur capacité de croître dans la bière. Puisque les isolats uniques comportant *horA* décrits ici sont capables de pousser dans la bière, il est probable que la présence du gène *horA* leur confère aussi la résistance au houblon.

Mots-clés : bactéries d'altération de la bière, *Firmicutes*, *horA*.

[Traduit par la Rédaction]

Efflux pumps of the ATP-binding cassette (ABC) multi-drug resistance (MDR) type are commonly used by Gram-positive organisms to counter the activity of antimicrobial compounds. Here we report the finding of a new ABC MDR gene for the genera *Bacillus*, *Paenibacillus*, and *Staphylococcus*. Phylogenetic analysis of the novel gene indicated that it is homologous to the hop resistance gene *horA* (Sami et al. 1997). The finding of *horA* is of consequence to the brewing industry, as it has been shown that the presence of *horA* is highly correlated to the ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer (Haakensen et al. 2007, 2008). Like other *horA*-positive isolates identified to date, these 3 genera are Gram-positive and belong to the highly diverse phylum *Firmicutes*. Bacteria of this phylum are known to cause more than 90% of beer-spoilage incidents (Sakamoto and Konings 2003). While *Bacillus* and *Staphylococcus* isolates are sometimes found in beer, they were not previously believed to be capable of growth owing to the presence of hop compounds (Campbell 2001; Priest and Campbell 2002), and *Paenibacillus* isolates have never been associated with beer spoilage. *Bacillus* and *Paenibacillus* species are responsible for numerous food-

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M. Haakensen and B. Ziola.[1] Department of Pathology and Laboratory Medicine, College of Medicine, University of Saskatchewan, 2841 Royal University Hospital, 103 Hospital Drive, Saskatoon, SK S7N 0W8, Canada.

[1]Corresponding author (e-mail: barry.ziola@usask.ca).

poisoning incidents, are capable of withstanding temperature fluctuations, and are readily introduced to grain-based foods owing to their natural occurrence in soil (Pirttijärvi et al. 2000; Granum 2001), while *Staphylococcus* species are implicated in a variety of human and animal diseases. Thus, each of the *horA*-positive bacteria identified here is ubiquitous and can be easily transported into the brewery environment. Since these bacteria were isolated as environmental contaminants of 2 types of spoiled home-brewed beer, their carriage of *horA* is a novel finding not only of relevance to the brewing industry but also to other fermentation industries and possibly to human health and animal husbandry.

Two varieties of spoiled home-brewed beer (one light and one dark, originating from 2 different kit manufacturers) were cultured on de Man, Rogosa, Sharpe (MRS) agar plates containing 10 ppm actidione to inhibit growth of yeast. Incubation was at 30 °C in a candle jar. Four morphologically distinct colonies were picked and inoculated into MRS broth at 30 °C. Bacterial DNA was extracted as described previously (Haakensen et al. 2007). The first 3 variable regions of the 16S rRNA gene were amplified by polymerase chain reaction (PCR) and sequenced as described by Dobson et al. (2002). The NCBI GenBank Basic Local Alignment Search Tool (Altschul et al. 1990) was used to determine the identity of each isolate, and in all cases the best GenBank matches had an E-value of zero. The identities found as the best match for each novel isolate were *Bacillus cereus*, *Bacillus licheniformis*, *Paenibacillus humicus*, and *Staphylococcus epidermidis* (isolate Nos. MH1–4, respectively). The sequenced region of the 16S rRNA gene amplified from each isolate was deposited in GenBank under the accession Nos. EU091076, EU091078, EU091079, and EU091077, respectively.

Each of the 4 isolates was capable of growing in commercially available beer. Two beers were used in growth experiments. Beer 1 was a filter-sterilized 4% (*v/v*) alcohol beer, pH 5.2, containing an average of 9.8 bitterness units. Beer 2 was a pasteurized 5% (*v/v*) alcohol beer, pH 4.8, containing an average of 11.0 bitterness units. Cultures were grown in 15 mL capped tubes containing modified de Man, Rogosa, Sharpe (MMRS) broth containing incremental amounts of beer before being subcultured into Beer 1 and Beer 2. The incremental concentrations of beer used were 50% Beer 2 : 50% 2× MMRS, followed by 85% Beer 2 : 15% 2× MMRS (85:15 medium). Bacteria were grown at 30 °C.

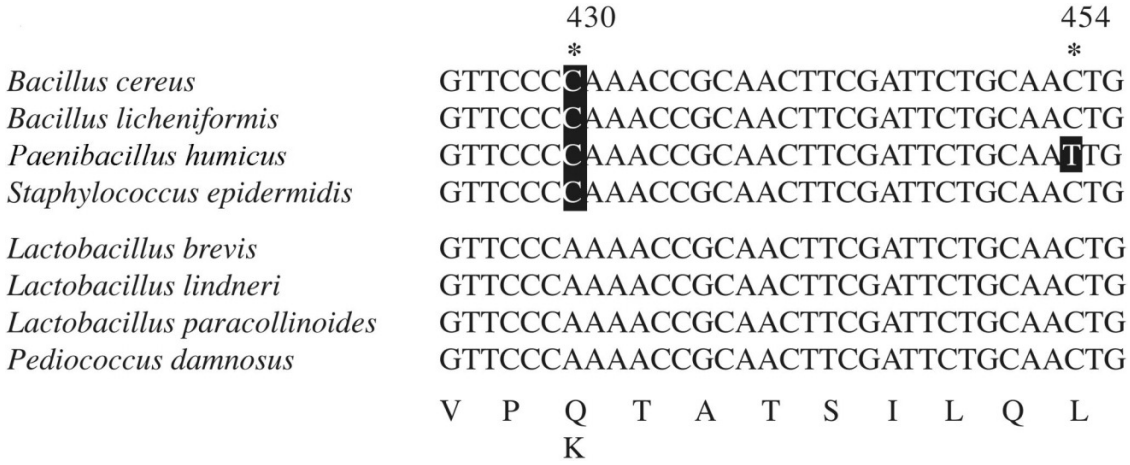
In the brewing industry, the onset of turbidity is synonymous with bacterial growth; therefore, turbidity is used as an indicator of product spoilage by bacteria. As such, once turbidity could be seen in beer, 500 μL from each tube of beer was inoculated into fresh tubes of 85:15 medium to confirm that turbidity was the result of bacterial growth. This step was also performed on control tubes of beer, which did not produce visible turbidity after 21 days, to ensure that there was no bacterial contamination present in the commercially available beer being used in the growth assay. The novel isolates grew in 85:15 medium when subcultured from the initial beer cultures. The bacteria were then subcultured from the first beer cultures into a second set of tubes containing beer. This was done to ensure that growth seen in the initial beer cultures was not due to a carryover of MMRS nutrients from the preceding growth of bacteria in 85:15 medium. This second set of beer cultures was then also assessed for bacterial growth in the same manner as the first set. Bacterial isolates producing visible turbidity in the second culture in beer (and subsequent growth upon subculture to 85:15 medium) were considered to be hop resistant and capable of growing in beer. All 4 novel isolates tested were capable of growing in both Beer 1 and Beer 2. Of the 4 isolates, *B. licheniformis* grew the fastest in beer, producing visible turbidity in only 14 days. Both *B. cereus* and *S. epidermidis* grew in 18 days, while *P. humicus* took 20 days to produce visible turbidity in beer.

As the novel isolates were capable of growing in both home-made and commercially available beer, the isolates were tested for the presence of the *horA* gene, which has previously been shown to correlate with the ability of related bacterial genera to grow in beer (Haakensen et al. 2007, 2008). The *horA* gene was tested for by PCR pre- and post-growth in beer as described previously (Haakensen et al. 2007, 2008). For each isolate, *horA* PCR amplicons were produced and then sequenced to determine similarity within the amplified region (Fig. 1). *horA* PCR amplicons for pre- and post-growth samples of each isolate were found to be identical. The sequenced region corresponded to the PCR-targeted bases 318–521 (coding for amino acids 106–173) of the *horA* gene, including most of the region between the second and third transmembrane helices that is believed to be important to substrate binding specificity (Priest and Campbell 2002). The NCBI GenBank database was screened for genes and proteins with homology to the ABC MDR gene amplified from these bacteria; however, the only significant matches were to *horA* or HorA from known beer-spoilage bacteria in the genera *Lactobacillus* and *Pediococcus*. The sequences of the *horA* homologous amplicons were deposited in GenBank under the accession Nos. EU091080 (*B. cereus*), EU091082 (*B. licheniformis*), EU091083 (*P. humicus*), and EU091081 (*S. epidermidis*).

It should be noted that 16S rRNA gene and *horA* PCR amplicons were sequenced from each organism originally isolated from spoiled beer and again after the same organism had been grown in commercial beer to ensure that the amplified *horA* homologous gene had indeed originated from each of the novel bacteria and not arisen because of PCR contamination by DNA from another *horA*-positive organism. The *horA* gene was PCR amplified in multiplex with the 16S rRNA gene as previously described (Haakensen et al. 2007), and both amplicons were visualized by agarose gel electrophoresis, isolated, and sequenced from both directions. As the *horA* and 16S rRNA genes were amplified in multiplex, the amplified *horA* gene could only originate from the organism identified by the corresponding 16S rRNA gene sequence. For each of the 4 novel isolates, the *horA* and 16S rRNA amplicons for the organism isolated from spoiled beer and for organism after growth in commercial beer had sequences identical to the sequences now deposited in GenBank. As *horA* was previously shown to be highly correlated with the ability of related genera to grow in beer (Haakensen et al. 2007, 2008), the clear association of *horA* with each of these 4 novel beer-spoilage isolates strongly suggests that the presence of *horA* is also associated with hop resistance in these bacteria.

Sequences corresponding to the *horA* gene of known beer-

Fig. 1. Multiple sequence alignment of bases 424–456 of *horA* from the 4 novel *horA*-harbouring bacteria and GenBank *horA* sequences (numbering starting from the start codon of *Lactobacillus brevis horA* gene AB005752). *Lactobacillus brevis*, AB005752; *Lactobacillus lindneri*, AB167898; *Lactobacillus paracollinoides*, AB178589; *Pediococcus damnosus*, AB218963.



spoilage bacteria and that were accessible through GenBank were compared with the novel ABC MDR sequences obtained from the 4 unique isolates. The GenBank search revealed that there are no currently known *Bacillus* or *Staphylococcus* genes yielding proteins with >37% identity with *horA* and that no ABC MDR gene has been previously reported for any *Paenibacillus* species. Interestingly, the *Paenibacillus* isolate described here was identified as *P. humicus*, a newly described species, about which little is yet known, and it is possible that other *P. humicus* isolates may also possess ABC MDR genes. The ABC MDR sequences amplified from *B. cereus*, *B. licheniformis*, *P. humicus*, and *S. epidermidis* in this study were compared with GenBank sequences for *horA* from known beer-spoilage bacteria and with the closest GenBank matches for ABC MDR genes from the respective species. These sequences were compiled and used to create a multiple sequence alignment using ClustalX (Thompson et al. 1997). The alignment was truncated using the GeneDoc program (Nicholas et al. 1997).

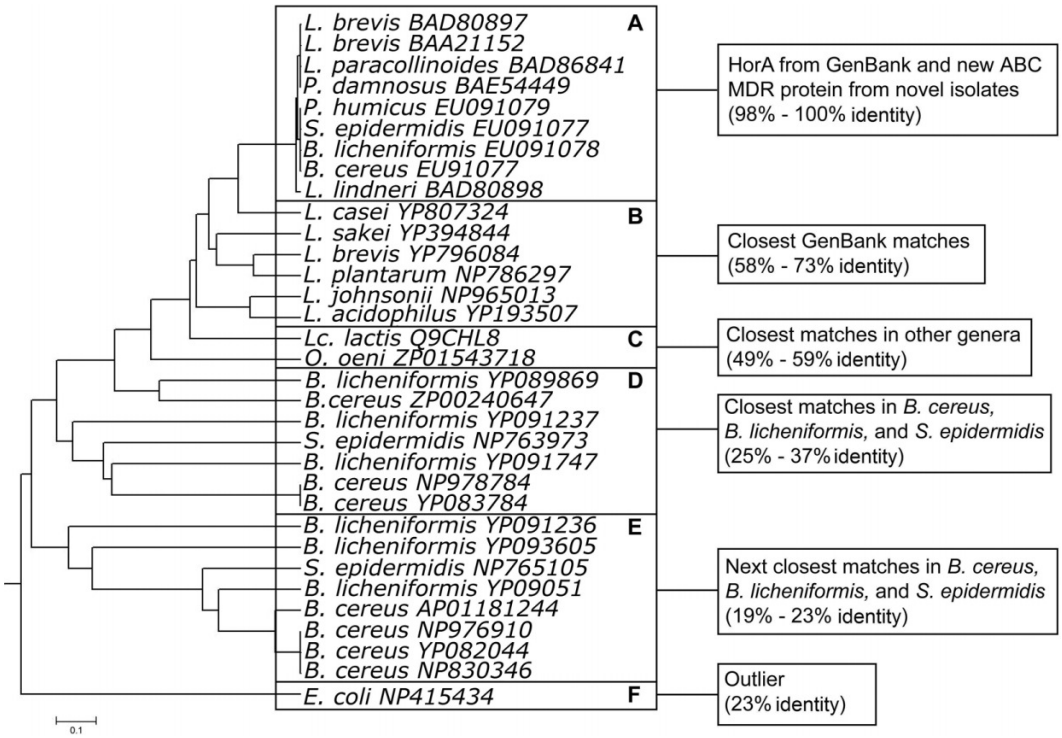
The 203 bp region from the ABC MDR gene of *B. cereus*, *B. licheniformis*, *P. humicus*, and *S. epidermidis* that we analyzed had <37% identity with known ABC MDR genes of these genera, yet shared >99% identity with *horA* from beer-spoilage bacteria. The multiple sequence alignment in Fig. 1 shows the region of the *horA* amplicon from the novel isolates containing base changes in comparison with known *horA* genes. The sequenced region of each of the 4 novel isolates possessed a single base change at position 430, resulting in an amino acid change from lysine to glutamine. The *P. humicus* isolate also possessed a null mutation at base 454. This exceptionally high level of conservation is indicative of lateral transfer of genetic material and the existence of environmental selective pressure(s) for *horA* among Gram-positive bacteria.

Phylogenetic analysis was performed to confirm the identity of the ABC MDR gene amplified from the 4 novel isolates in this study. Figure 2 is a phylogenetic tree of the protein sequence corresponding to the PCR-amplified region of the ABC MDR gene. The phylogenetic tree was created using the Unweighted Pair Group Method with Arithmetic mean algorithm in the MEGA2 software program (Kumar et al. 2001). While the tree in Fig. 2 was created from a truncated portion of the ABC MDR proteins, the branching of this tree is identical to the phylogenetic trees created when full-protein or DNA sequences of the same GenBank sequences are used with bootstrap replicates set at 1000 (data not shown). That is, when only GenBank sequences are used (i.e., the full-length protein or DNA sequences of the GenBank sequences included in Fig. 2, but not our newly identified sequences), the resulting tree has the same branching pattern. This goodness-of-fit test indicates that the 203 bp region sequenced from the ABC MDR gene of our newly identified beer-spoilage isolates is appropriate for determining the identity of the gene. Tree structure and groupings are also identical when various phylogenetic analysis methods are used; i.e., Neighbor-Joining, Minimum Evolution, or Maximum Parsimony (data not shown). The phylogenetic clades outlined in Fig. 2 show that the sequenced region of the ABC MDR gene found in our *B. cereus*, *B. licheniformis*, *P. humicus*, and *S. epidermidis* isolates is >99% similar to HorA and <37% similar to any known ABC MDR proteins in the respective species. Information from the goodness-of-fit test and the high percentage of identity between the sequenced region of the ABD MDR gene from our novel beer-spoilage isolates and *horA* together indicate it is extremely likely that the ABC MDR gene found in the 4 novel isolates is, in fact, *horA*.

The new finding of *horA* in isolates belonging to the genera *Bacillus*, *Paenibacillus*, and *Staphylococcus* suggests that *horA* exists in bacteria in environments beyond the brewery. The finding of *horA* in lactobacilli originating from human vaginal flora or feces or from various fermentations (corn silage, fuel ethanol, kefir, moto, and wine) provides support for this suggestion (Haakensen et al. 2007), while the free exchange of genetic material among *Firmicutes* provides an impetus for investigating whether *horA* is present in a wider range of bacterial genera and environments.

In summary, our findings have 2 major implications. First, novel bacteria with the potential to spoil beer exist and, since these bacteria are not currently being tested for as

Fig. 2. Phylogenetic tree of amino acids 106–173 from HorA, the corresponding region of the new ATP-binding cassette (ABC) multi-drug resistance (MDR) protein from the 4 novel isolates, and the corresponding region of the closest GenBank protein matches. (A) HorA sequences from GenBank and new ABC MDR protein from the 4 novel isolates (98%–100% identity). (B) Closest GenBank matches; all are putative ABC MDR proteins identified through genome sequencing projects (58%–73% identity to HorA). (C) Closest matches to proteins from other genera (49%–59% identity to HorA): *L. lactis* (*Lactococcus lactis* LmrA protein), *O. oeni* (*Oenococcus oeni* OmrA protein). (D) Closest matches within *Bacillus cereus*, *Bacillus licheniformis*, and *Staphylococcus epidermidis* (25%–37% identity to HorA). (E) Next closest matches within *B. cereus*, *B. licheniformis*, and *S. epidermidis* (19%–23% identity to HorA). (F) Outlier (23% identity to HorA), *E. coli* (*Escherichia coli* MsbA protein). Bar = 0.1 difference in 100 amino acids.



beer-spoilage organisms, present a threat to the brewing industry. Second, the *horA* MDR gene that is thought to be significant only in the context of brewing spoilage bacteria is also found in a range of ubiquitous bacteria that may play a role not only in fermentation industries but also in animal and human health.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. **215**: 403–410. PMID:2231712.

Campbell, I. 2001. Food fermentations. *In* Food microbiology. *Edited by* M.P. Doyle, L.R. Beuchat, and T.J. Montville. ASM Press, Washington, D.C. pp. 742–744.

Dobson, C.M., Deneer, H., Lee, S., Hemmingsen, S., Glaze, S., and Ziola, B. 2002. Phylogenetic analysis of the genus *Pediococcus*, including *Pediococcus claussenii* sp. nov., a novel lactic acid bacterium isolated from beer. Int. J. Syst. Evol. Microbiol. **52**: 2003–2010. doi:10.1099/ijs.0.02191-0. PMID:12508860.

Granum, P.E. 2001. Foodborne pathogenic bacteria. *In* Food microbiology. *Edited by* M.P. Doyle, L.R. Beuchat, and T.J. Montville. ASM Press, Washington, D.C. pp. 373–381.

Haakensen, M.C., Butt, L., Chaban, B., Deneer, H., Ziola, B., and Dowgiert, T. 2007. A *horA*-specific real-time PCR for detection of beer-spoilage lactic acid bacteria. J. Am. Soc. Brew. Chem. **65**: 157–165.

Haakensen, M., Schubert, A., and Ziola, B. 2008. Multiplex PCR for putative *Lactobacillus* and *Pediococcus* beer-spoilage genes and ability of gene presence to predict growth in beer. J. Am. Soc. Brew. Chem. **66**: In press.

Kumar, S., Tamura, K., Jakobsen, I.B., and Nei, M. 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics, **17**: 1244–1245. doi:10.1093/bioinformatics/17.12.1244. PMID:11751241.

Nicholas, K.B., Nicholas, H.B., Jr., and Deerfield, D.W., II. 1997. Genedoc: analysis and visualization of genetic variation EMBNEW. News, **4**: 14.

Pirttijärvi, T.S.M., Andersson, M.A., and Salkinoja-Salonen, M.S. 2000. Properties of *Bacillus cereus* and other bacilli contaminating biomaterial-based industrial processes. Int. J. Food Microbiol. **60**: 231–239. doi:10.1016/S0168-1605(00)00313-5. PMID: 11016612.

Priest, F.G., and Campbell, I. 2002. Brewing microbiology. 3rd ed. Kluwer Academic Publishers, N.Y.

Sakamoto, K., and Konings, W.N. 2003. Beer spoilage bacteria and

hop resistance. Int. J. Food Microbiol. **89**: 105–124. doi:10.1016/S0168-1605(03)00153-3. PMID:14623377.

Sami, M., Yamashita, H., Hirono, T., Kadokura, H., Kitamoto, K., Yoda, K., and Yamasaki, M. 1997. Hop-resistant *Lactobacillus brevis* contains a novel plasmid harbouring a multidrug resistance-like gene. J. Ferment. Bioeng. **84**: 1–6. doi:10.1016/S0922-338X(97)82778-X.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25**: 4876–4882. doi:10.1093/nar/25.24.4876. PMID:9396791.

5. MULTIPLEX PCR FOR PUTATIVE *LACTOBACILLUS* AND *PEDIOCOCCUS* BEER-SPOILAGE GENES AND ABILITY OF GENE PRESENCE TO PREDICT GROWTH IN BEER

Author contributions:

Monique Haakensen conceived the study, designed and standardized the experiments, and drafted the manuscript.

Alison Schubert performed the experiments.

Barry Ziola conceived the study, edited the manuscript, and is the holder of the research grant used to fund the study.

BRIEF INTRODUCTION TO CHAPTER 5

In Chapter 3, it was found that the *horA* gene was not present in all lactobacilli or pediococci that were capable of growing in beer. Meanwhile, literature in this subject area had begun to suggest several additional genes which might putatively be involved in the ability of some *Lactobacillus* isolates to grow in beer. Therefore, a multiplexed PCR assay was developed to test for three putative beer-spoilage genes in addition to *horA* (i.e., *horC*, *hitA*, and *ORF5*), while using the 16S rRNA gene as an internal positive control. This multiplex PCR assay was used to screen bacteria whose ability to spoil beer was previously determined in Chapter 3. Statistical analyses were then used to determine which gene(s) is the most accurate genetic marker(s) for differentiating between lactobacilli and pediococci that can grow in beer and those which pose little threat to the brewing industry.

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Multiplex PCR for Putative *Lactobacillus* and *Pediococcus* Beer-Spoilage Genes and Ability of Gene Presence to Predict Growth in Beer

Monique Haakensen, Alison Schubert, and Barry Ziola,[1] *Department of Pathology and Laboratory Medicine, University of Saskatchewan, Saskatoon, SK S7N 0W8, Canada*

ABSTRACT

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Current methods of detecting *Lactobacillus* and *Pediococcus* isolates found in beer are time-consuming and do not differentiate between benign bacteria and those bacteria capable of growing in beer. Four putative beer spoilage-associated genes (*hitA*, *horA*, *horC*, and ORF5) have been suggested but have never been statistically correlated with the ability to grow in beer. We have designed a multiplex PCR to detect these putative spoilage-associated genes that includes the 16S rRNA gene as an internal control. In all, 133 *Lactobacillus* and *Pediococcus* isolates were screened using this multiplex PCR, and the results were compared with the ability of the isolates to grow in beer. We found that only *horA* was predictive of an organism's ability to grow in beer. Although *hitA* and *horC* were not predictive of an organism's ability to grow in beer, the presence of *hitA*, *horC*, or both in addition to *horA* was indicative of the ability to grow rapidly in beer. Statistical modeling based on our data indicates that assaying for the presence of *horA* is highly accurate in predicting the beer-spoilage potential of *Lactobacillus* and *Pediococcus* isolates. This multiplex PCR substantially reduces the time required to determine whether a *Lactobacillus* or *Pediococcus* isolate has a high probability of causing beer spoilage.

Keywords: Beer-spoilage genes, *horA*, *Lactobacillus*, Multiplex PCR, *Pediococcus*

RESUMEN

Los métodos actuales de detección de aislados de *Lactobacillus* y *Pediococcus* encontrado en la cerveza consumen mucho tiempo y no diferencian entre bacterias benignas y las bacterias capaces de crecer en la cerveza. Cuatro putativo genes asociados con la deterioración de la cerveza (*hitA*, *horA*, *horC*, y ORF5) se han sugerido, pero nunca han sido estadísticamente correlacionadas con la capacidad de crecer en la cerveza. Hemos diseñado un PCR múltiplex para la detección de estos putativo genes asociado con la deterioración de la cerveza que incluye el gen 16S rRNA como un control interno. En total, 133 aislados de *Lactobacillus* y *Pediococcus* fueron seleccionados utilizando este PCR múltiplex, y los resultados se compararon con la capacidad de los aislamientos de crecer en la cerveza. Se encontró que sólo *horA* fue predictivo de la capacidad de un organismo para crecer en la cerveza. Aunque *hitA* y *horC* no fueron predictivos de la capacidad de un organismo para crecer en la cerveza, la presencia de *hitA*, *horC*, o ambos, además con *horA* era indicativa de la capacidad de crecer rápidamente en la cerveza. Modelación estadística basada en nuestros datos indican que analizaron para detectar la presencia de *horA* es sumamente preciso en la predicción de la potencial de los aislamientos de *Lactobacillus* y *Pediococcus* para dañar cerveza. Este PCR múltiplex reduce sustancialmente el tiempo necesario para determinar si una aislado de *Lactobacillus* o *Pediococcus* tiene una alta probabilidad de dañar la cerveza.

Palabras claves: Genes asociados con deterioración de la cerveza, *horA*, *Lactobacillus*, PCR múltiplex, *Pediococcus*

Spoilage of beer by bacteria is a significant problem for the brewing industry. Although most gram-positive bacteria fail to grow in beer due to the presence of hop compounds, the presence of a specific resistance-associated gene or genes is believed to be associated with growth in beer (6,13). The most common beer-spoilers are lactic acid bacteria (LAB), i.e., select isolates within the gram-positive genera *Lactobacillus* and *Pediococcus* (5,6). These beer-spoilage LAB are able to withstand the bitter acid compounds (e.g., *trans*-isohumulone) derived from hop-extract α-acids that act as mobile carrier protonophores, effectively dissipating the cell's trans-membrane pH gradient (12).

Although not all *Lactobacillus* and *Pediococcus* isolates can grow in beer, some isolates of these genera have developed mechanisms that confer resistance to hop compounds (13), thus allowing growth in beer. The ability to grow in beer is not restricted by the boundaries of speciation (2) and, as such, spoilage-specific genetic markers must be identified. Although several genes have been claimed to be involved in hop resistance (1,3,7–11,14–17), isolates used in these studies often have been derived from a single source (i.e., one brewery), and supporting statistical evidence has not been provided. As such, the objective of the present study was to determine whether the presence of putative beer-spoilage genes can be used to predict the ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer. To accomplish this goal, the putative beer-spoilage genes chosen as targets for multiplex PCR were *hitA* (3), *horA* (11) *horC*, and ORF5 (1,15).

The *horA* and *hitA* genes code for primary- and secondary-type multidrug transporters, respectively. The *horA* gene has homology to ATP-binding cassette-type multidrug resistance genes, using an ATP-binding transporter to export *trans*-isohumulone, preventing its accumulation in the intracellular space (7–11). It has been suggested that *hitA* is an integral membrane protein that functions as a divalent cation proton motive force transport system, counteracting the activity of *trans*-isohumulone (3). The *horC* and ORF5 genes code for proteins of unknown function with no homology to known proteins. *horC* and ORF5 were selected based on their hypothetical membrane localization, similarity to other membrane proteins, and potential correlation with hop resistance, as suggested previously by Suzuki et al (14,16). Independent studies identified two unique plasmids that both harbored *horC* and ORF5 (1,15), with *horC* corresponding to ORF2 and ORF9, as described by Suzuki et al (15) and Fuji et al (1), respectively, whereas ORF5 corresponds to ORF5 and ORF2, respectively, in the same two articles.

In this paper we describe a multiplex PCR that simultaneously detects these four putative spoilage-associated genes, as well as the 16S rRNA gene used as an internal positive control. This multiplex PCR was used to screen 133 *Lactobacillus* and *Pediococcus* isolates. Statistical analyses were used to delineate the relative roles these four genes play in the ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer.

EXPERIMENTAL

Bacteria

The 133 isolates analyzed in this study were from diverse origins and included 83 lactobacilli and 50 pediococci, comprising 22

[1] Corresponding author. E-mail: b.ziola@usask.ca; Phone: +1.306.966.4330; Fax: +1.306.966.8049.

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known *Lactobacillus* spp., 4 putative new *Lactobacillus* spp., and 7 *Pediococcus* spp. (Table I). Growth of the bacterial isolates in two different kinds of beer involved adaptation of the bacteria to grow in beer using modified MRS medium (de Man, Rogosa, Sharpe medium modified by the omission of Tween 80) supplemented with incremental concentrations of beer (2). The identities of the isolates were confirmed pre- and postgrowth in beer by sequencing of the 16S rRNA gene as previously described (2). The ability of the 133 isolates to grow in beer was reported previously (2), and the results are incorporated into Table I for direct comparison with the results on presence or absence of each of the four putative beer-spoilage genes. Beer 1 was a filter-sterilized 4% (vol/vol) alcohol beer, aver-

TABLE I
Bacterial Strains, Presence of Genes, and Ability to Grow in Beer

		PCR Result[b]					Growth[c]	
Isolate[a]	**Origin**	***hitA***	***horA***	***horC***	**ORF5**	**16S**	**Beer 1**	**Beer 2**
Lactobacillus acetotolerans								
ATCC 43578^T	Rice vinegar	–	–	–	–	+	–	–
L. acidophilus								
ATCC 521	Unknown	–	–	–	–	+	–	–
ATCC 4356^T	Human	+	–	–	–	+	–	–
CCC B1209	Brewery	–	–	–	–	+	–	–
L. amylovorus								
ATCC 33198[d]	Hog intestine	–	–	–	–	+	–	–
ATCC 33620^T	Corn silage	–	–	–	–	+	–	–
Field isolate[e]	Unknown	–	–	–	–	+	–	–
Ingledew I1[f]	Fuel alcohol	+	–	–	–	+	–	–
Ingledew I2	Fuel alcohol	+/+	+/+	–/+	–/–	+/+	+ (2)	+ (3)
T-13[g]	Poultry	–	–	–	–	+	–	–
L. brevis								
ATCC 4006	Unknown	–	–	–	–	+	–	–
ATCC 8007	Kefir grains	–	+(T)[h]	–	–	+	–	–
ATCC 14869^T	Human feces	–	–	–	–	+	–	–
BSO 31[i]	Brewery	+/+	+/+	+/+	+/+	+/+	+ (5)	+ (5)
CCC 96S1L	Brewery	–/+	+/+	–/+	–/–	+/+	+ (5)	+ (5)
CCC 96S2AL	Brewery	–/+	+/+	–/+	–/–	+/+	+ (5)	+ (5)
CCC B1202	Brewery	+/+	+/+	+/+	+/+	+/+	+ (5)	+ (5)
CCC B1203	Brewery	+/+	+/+	+/+	+/+	+/+	+ (5)	+ (5)
CCC B1204	Brewery	+/+	+/+	+/+	+/+	+/+	+ (5)	+ (5)
CCC B1206	Brewery	+/+	+/+	+/+	+/+	+/+	+ (5)	+ (5)
CCC B1300	Brewery	–/–	+/+	–/–	+/+	+/+	+ (2)	+ (3)
ETS.1	Wine	–	–	–	–	+	–	–
ETS.2	Wine	–	–	–	–	+	–	–
L. casei								
ATCC 334[g]	Cheese	–	–	–	–	+	–	–
ATCC 4913[g]	Unknown	–	–	–	+	+	–	–
ATCC 25598^T	Milking machine	–/–	–/–	–/–	–/–	+/+	+ (8)	+ (11)
CCC 95G1L	Brewery	+/+	–/–	–/+	–/–	+/+	+ (8)	+ (10)
CCC 95G2L	Brewery	–/–	–/+	–/–	+/+	+/+	+ (5)	+ (5)
CCC B9657	Brewery	–/–	–/+	–/–	–/–	+/+	+ (9)	+ (9)
CCC B1205	Brewery	–/+	–/+	–/+	–/+	+/+	+ (2)	+ (2)
CCC B1241	Brewery	–/–	–/+	–/–	–/–	+/+	+ (27)	–
Ingledew I3	Fuel alcohol	–	–	–	–	+	–	–
Ingledew 18C	Fuel alcohol	–/–	+/+	–/–	–/–	+/+	+ (27)	–
L. delbrueckii								
ATCC 4797	Corn mash	–	–	–	–	+	–	–
ATCC 9649^T	Sour grain mash	–	–	–	–	+	–	–
ATCC 11842^T	Bulgarian yogurt	+	–	–	–	+	–	–
ATCC 12315^T	Cheese	+	–	–	–	+	–	–
CCC 95G3L	Brewery	–	–	–	–	+	–	–

(*continued on next page*)

[a] Isolate identity as determined by Haakensen et al (2), with type strains indicated. ATCC = American Type Culture Collection, Manassas, VA; BSO = Beer Spoilage Organism; CCC = Coors Brewing Company, Golden, CO; DSM = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; ETS = ETS Laboratories (T. Arvik), St. Helena, CA; Molson = Molson Breweries of Canada Limited, Montreal, PQ, Canada; R = ropy phenotype; and NR = nonropy phenotype.
[b] For bacterial isolates capable of growing in beer, the presence of genes was recorded as pre- or postgrowth in beer.
[c] + = visible turbidity in beer and growth upon subsequent subculture to 85/15 medium; – = no visible turbidity in beer and not capable of growing upon subculture to 85/15 medium; and S = static, no visible turbidity in beer, but capable of growing upon subculture to 85/15 medium. The number in parentheses following a + indicates the number of days required to attain visible growth in beer.
[d] A. Estrada, College of Agriculture and Bioresources, University of Saskatchewan, Saskatoon, SK, Canada.
[e] D. Korber, College of Agriculture and Bioresources, University of Saskatchewan, Saskatoon, SK, Canada.
[f] W. M. Ingledew, College of Agriculture and Bioresources, University of Saskatchewan, Saskatoon, SK, Canada.
[g] G. Reid, Lawson Research Institute, London, ON, Canada.
[h] *horA* was sequenced, and the gene was found to be truncated by approx. 700 bp.
[i] B. Kirsop, Institute for Biotechnology, Cambridge, England.
[j] R. Wheatcroft, Agriculture and Agri-food Canada, Guelph, ON, Canada.
[k] K. Fernandez, Gipuzko, Spain.

aging 9.8 bitterness units (BU) and pH 4.2, whereas beer 2 was a pasteurized 5% (vol/vol) alcohol beer, averaging 11 BU and pH 3.8.

PCR Primers

A region of the bacterial 16S rRNA gene was amplified using the PCR primers 386F (*unpublished*) and 534R (4). The *horA*-specific primers h198F2 and h198R are as previously described (2). When designing PCR primer pairs specific to *hitA*, *horC*, and ORF5, attributes of each primer set were kept relatively similar (e.g., melting temperature and percent G+C content similar to that of the 16S rRNA gene and *horA* primers) to optimize their ability to function under a single set of PCR conditions. Primer pairs were

TABLE I
(*continued from preceding page*)

		PCR Result[b]					Growth[c]	
Isolate[a]	**Origin**	***hitA***	***horA***	***horC***	**ORF5**	**16S**	**Beer 1**	**Beer 2**
CCC B1044	Brewery	–	–	–	–	+	–	–
CCC B1240	Brewery	–	–	–	–	+	–	–
CCC B1262	Brewery	–	–	–	–	+	–	–
L. ferintoshensis								
ATCC 11307	Brewery	–	–	–	+	+	–	–
L. fermentum								
ATCC 9338[g]	Unknown	–/–	–/–	–/–	–/–	+/+	+ (9)	+ (11)
ATCC 14931[T]	Fermented beets	–	–	–	–	+	–	–
ATCC 14932[g]	Saliva	–	–	–	–	+	–	–
L. fructivorans								
ATCC 8288[T]	Unknown	–	–	–	–	+	–	–
L. helviticus								
ATCC 15009[T]	Cheese	–	–	–	–	+	–	–
CCC B1186	Brewery	–/–	+/+	–/–	–/–	+/+	+ (6)	+ (S)
L. hilgardii								
ATCC 8290[T]	Wine	–	–	–	–	+	–	–
ATCC 27305	Wine	–/–	–/–	–/–	–/–	+/+	+ (4)	+ (8)
ATCC 27306	Wine	–	–	–	–	+	–	–
L. homohiochii								
ATCC 15434[T]	Spoiled sake	–	–	–	–	+	–	–
L. jensenii								
ATCC 25258[T]	Human	+	+(T)	–	–	+	–	–
L. kefiri								
ATCC 35411[T]	Kefir grains	+	–	–	–	+	–	–
L. kefirgranum								
ATCC 51647[T]	Kefir grains	–	–	–	–	+	–	–
L. kefironofaciens								
ATCC 43761[T]	Kefir grains	–	–	–	–	+	–	–
L. paracollinoides								
ATCC 8291	Brewery	–/–	–/–	–/–	–/–	+/+	+ (7)	+ (11)
L. plantarum								
ATCC 8014	Unknown	–	–	–	–	+	–	–
ATCC 8041	Corn silage	+/+	+/+	+/+	+/+	+/+	+ (2)	+ (3)
ATCC 11305	Brewery	–	–	–	–	+	–	–
ATCC 12706	Cured meat	–	–	–	–	+	–	–
ATCC 14431[g]	Grass silage	–	–	–	+	+	–	–
ATCC 14917[T]	Pickled cabbage	–	–	–	–	+	–	–
BSO 92	Brewery	+/+	+/+	+/+	+/+	+/+	+ (2)	+ (2)
CCC 96M2BL	Brewery	+/+	+/+	+/+	–/–	+/+	+ (12)	+ (12)
CCC B1301	Brewery	–/–	+/+	–/–	–/–	+/+	+ (5)	+ (12)
L. reuteri								
ATCC 19371	Silage	–	–	–	–	+	–	–
ATCC 25744	Plants	–	–	–	–	+	–	–
ATCC 31282	Unknown	–/–	–/–	–/–	–/–	+/+	+ (13)	+ (13)
ATCC 43200	Cucumbers	–	–	–	+	+	–	–
RC-14[g]	Unknown	–	–	–	–	+	–	–
L. rhamnonsus								
ATCC 7469[T]	Unknown	+	–	–	–	+	–	–
ATCC 7469a[g]	Derived from ATCC 7469	–	–	–	–	+	–	–
ATCC 8530[g]	Unknown	–/–	–/–	–/–	–/–	+/+	+ (7)	+ (10)
ATCC 15820	Corn liquor	–	–	–	–	+	–	–
ATCC 21052[g]	Human feces	–	+(T)	–	–	+	–	–
L. sakei								
ATCC 15521[T]	Moto	+	–	–	–	+	–	–
ATCC 15578	Moto	+/+	+/+	–/+	–/–	+/+	+ (6)	+ (7)
L. zeae								
ATCC 393	Cheese	–	–	–	–	+	–	–
Lactobacillus unspeciated								
ATCC 4005	Tomato pulp	–	–	–	–	+	–	–

(*continued on next page*)

designed to produce amplicons with 15–30 bp differences to allow for differentiation upon visualization by 2% agarose gel electrophoresis. Specificity of the primers was confirmed in silico and by sequencing the amplicons of randomly selected isolates. The five primer pairs used in this multiplex PCR are described in Table II.

Multiplex PCR

DNA extractions were performed as previously described (2). Each multiplex PCR contained 2 U of Invitrogen Platinum *Taq* DNA polymerase, 1× PCR buffer (Invitrogen, Burlington, ON, Canada), 1.5 m*M* $MgCl_2$, 0.2 m*M* each of the four deoxynucleotide triphosphates, 0.2 μ*M* each primer (except for the 16S rRNA gene

TABLE I
(***continued from preceding page***)

Isolate[a]	Origin	PCR Result[b]					Growth[c]	
		hitA	***horA***	***horC***	**ORF5**	**16S**	**Beer 1**	**Beer 2**
ATCC 27054	Apple juice	–	–	–	–	+	–	–
ATCC 27304	Wine must	+/+	+/+	+/+	–/+	+/+	+ (3)	+ (3)
CCC L86	Brewery	–/–	+/+	+/+	+/+	+/+	+ (4)	+ (11)
Pediococcus acidilactici								
ATCC 8042	Brewery	–/–	+/+	–/–	–/–	+/+	+ (8)	–
ATCC 12697	Unknown	–	–	–	–	+	–	–
ATCC 25740	Plant	–	–	–	–	+	–	–
BSO 54	Brewery	–	–	–	–	+	–	–
BSO 77[i]	Brewery	–	–	–	–	+	–	–
Molson B77b	Brewery	–	–	–	–	+	–	–
Pac 1.0[j]	Unknown	–	–	–	–	+	–	–
P. claussenii								
CCC B962A	Brewery	–/–	–/–	+/+	–/–	+/+	+ (9)	+ (10)
CCC B1056R	Brewery	–/–	+/+	–/–	–/–	+/+	+ (14)	+ (14)
CCC B1056NR	Brewery	–/–	+/+	–/–	–/–	+/+	+ (13)	–
CCC B1098R	Brewery	–/–	–/–	–/–	–/–	+/+	+ (7)	+ (7)
CCC B1098NR	Brewery	–/–	+/+	–/–	–/–	+/+	+ (8)	+ (13)
CCC B1099R	Brewery	–/–	–/–	–/–	–/–	+/+	+ (12)	+ (14)
CCC B1099NR	Brewery	–/–	–/–	–/–	–/–	+/+	+ (7)	+ (7)
CCC B1100	Brewery	–/–	–/–	–/+	–/+	+/+	+ (8)	+ (S)
CCC B1208	Brewery	–	–	–	–	+	–	–
CCC B1260R	Brewery	–/–	–/–	–/–	–/–	+/+	+ (8)	+ (13)
CCC B1260NR	Brewery	–/–	–/–	–/–	–/–	+/+	+ (6)	+ (6)
ATCC BAA-344^T R	Brewery	–/–	+/+	–/–	–/–	+/+	+ (8)	+ (8)
ATCC BAA-344^T NR	Brewery	–/–	+/+	–/–	–/–	+/+	+ (6)	+ (6)
P. damnosus								
ATCC 11308	Brewery	–/–	–/–	–/–	–/–	+/+	+ (27)	–
ATCC 25248	Brewery	–	–	–	–	+	–	–
ATCC 25249	Brewery	–	+(T)	–		+	–	–
ATCC 25249a	Brewery	–	–	–	+	+	–	–
ATCC 29358^T	Brewery	–	–	+	+	+	–	–
Molson B48	Brewery	+/+	+/+	+/+	+/+	+/+	+ (7)	+ (7)
Molson 49	Brewery	–/–	+/+	+/–	+/–	+/+	+ (10)	+ (13)
Molson B76	Brewery	+/–	+/+	+/–	+/–	+/+	+ (11)	+ (12)
P. dextrinicus								
ATCC 33087^T	Silage	–	–	–	–	+	–	–
P. inopinatus								
ATCC 49902^T	Brewery	–	–	–	–	+	–	–
P. parvulus								
ATCC 43013	Wine	–/–	–/–	–/–	–/–	+/+	+ (13)	+ (13)
ETS.3	Wine	–	–	–	–	+	–	–
ETS.4	Wine	–	–	–	–	+	–	–
ETS.5	Wine	–	–	–	–	+	–	–
ETS.6	Wine	–	–	–	–	+	–	–
ETS.7	Wine	–	–	–	–	+	–	–
ETS.8	Wine	–	–	–	–	+	–	–
ETS.9	Wine	–	–	–	–	+	–	–
ETS.11	Wine	–	–	–	–	+	–	–
ETS.12	Wine	–	–	–	–	+	–	–
ETS.13	Wine	–	–	–	–	+	–	–
ETS.14	Wine	–	–	–	–	+	–	–
Spain 2.6R[k]	Cider	–	–	–	–	+	–	–
Spain 2.6NR[k]	Cider	–	–	–	–	+	–	–
P. pentosaceus								
ATCC 8081	Milk	–	–	–	–	+	–	–
ATCC 10791	Cucumber	+	–	–	–	+	–	–
ATCC 11309	Unknown	–	–	–	–	+	–	–
ATCC 29723	Horse urine	–	–	–	+	+	–	–
ATCC 33314	Sake mash	–	–	–	–	+	–	–
ATCC 33316^T	Brewery	–	–	–	–	+	–	–

primers, which were at 0.1 μ*M*), and 1 μL of bacterial DNA. Water was added to bring the total volume to 25 μL. The PCR program consisted of an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 94°C for 45 sec, 52°C for 45 sec, and 72°C for 50 sec and a final extension step of 72°C for 5 min. Amplicons were detected by electrophoresis in 2.0% agarose gels containing ethidium bromide.

Statistics

Statistical analysis of data was performed using SPSS for Windows (version 12.0, SPSS Inc., Munich). For both binary logistic regression and *t* test for independent samples, the confidence interval and level of significance were set at 95% and $P = 0.05$, respectively. Binary logistic regression models were calculated for *Lactobacillus, Pediococcus,* and *Lactobacillus* and *Pediococcus* isolates combined, with the ability to grow in beer 1 and beer 2 as outcome variables. For binary logistic regression models, multiplex PCR results for the *hitA, horA, horC*, and ORF5 genes were included as covariates (0 = PCR negative and 1 = PCR positive). Binary logistic regression was performed using the backward stepwise (likelihood ratio) method and forward stepwise method, with the same results produced (data not shown). All binary logistic regression likelihood ratio χ^2 values had $P < 0.00025$.

RESULTS AND DISCUSSION

Gene Detection

The multiplex PCR protocol described here not only detects the presence of *hitA*, *horA*, *horC*, and ORF5 but also provides an internal control indicating the presence of bacterial DNA in the test sample through incorporation of primers that amplify a portion of the 16S rRNA gene. Because the PCR amplicon for each gene has a different length, a bacterial DNA sample should always have a minimum of one band (i.e., the 16S rRNA control amplicon) and may include as many as four additional bands when the PCR reaction is analyzed by agarose gel electrophoresis (Fig. 1). Table I provides the multiplex PCR results for each of the isolates analyzed.

To ensure that the h198F2/h198R primers were successful in detecting the *horA* gene whenever present, the 16 *horA*-negative, growth-positive isolates were subjected to PCR by additional PCR primer set combinations. All PCR primers were designed to be specific to *horA*, and the 16 *horA*-negative, growth-positive isolates were negative for all combinations of primer sets to *horA* (data not shown). The additional primer sets used included the previously described h297F/R primer set (2) and a primer set designed to a multiple sequence alignment of all GenBank *horA* sequences to span the full length of the *horA* gene (forward primer horAFullF located 110- to 96-bp upstream of the start codon and reverse primer horAFullR located at the 3′ end of the *horA* gene at bases 1,745–1,728). The horAFullF/R primers also were used in respective combinations with the h198F2/R primers to produce shorter, overlapping amplicons for DNA sequencing. The *horA* gene of each of the four *horA*-positive, growth-negative isolates (*L. brevis* ATCC 8007, *L. jensenii* ATCC 25258^T, *L. rhamnosus* ATCC 21052, and *P. damnosus* ATCC 25249; GenBank accession nos. EU223373, EU223372, EU223374, and EU223375, respectively) were sequenced and were found to be truncated by approx. 700 bp corresponding to the 3′ end of *horA* sequences deposited in GenBank. Despite this truncation, the sequenced regions of *horA* from these isolates were 97.9–99.7% identical compared with deposited *horA* sequences, and all coded for phenylalanine instead of serine in amino acid position 75.

Interestingly, four isolates were initially *horA* PCR negative prior to growth in beer but, after subsequent passage in beer, were positive for *horA*. This same phenomenon was observed three times for each *hitA* and ORF5, and six times for *horC*. Conversely, one isolate (*P. damnosus* Molson B76) was PCR positive for all four genes prior to growth in beer but, after passage in beer, was positive only for *horA* (Table I), and a second isolate (*P. damnosus* Molson B49) was PCR positive for *horA*, *horC*, and ORF5 prior to growth in beer but was only *horA* PCR positive after growth in beer. These findings suggest that previous studies may have incorrectly identified some bacterial isolates as being positive or negative for genes of interest because they did not screen for their presence after growth in beer (or a similarly appropriate adaptation step). Because the four genes of interest are plasmid localized, continuous passage of the bacteria in laboratory media can lead to gene loss. Therefore, it is important to screen for genes of interest after a small subset of bacteria harboring advantageous plasmids has been preferentially amplified by growth in a beer environment.

It has been suggested previously that *horC* and ORF5 are jointly plasmid localized (1,15). However, our data show that the presence of *horC* and ORF5 did not correlate (Table III). Not only were there numerous instances in which *horC* and ORF5 occurred independently of one another, but there were five isolates that demonstrated preferential selection for *horC* (i.e., *horC* PCR-negative pregrowth in beer or *horC* PCR-positive postgrowth in beer) while failing to maintain the presence of ORF5 (i.e., ORF5 PCR-positive pregrowth in beer or ORF5 PCR-negative postgrowth in beer) (Table I). This finding suggests that, although *horC* and ORF5 sometimes may be located on the same plasmid, these two genes can occur independently.

The widespread lateral gene transfer that can occur among LAB is demonstrated by the fact that a small or extremely small subset of bacteria harboring advantageous plasmids can be preferentially amplified by growth in a beer environment, as well as the additional

TABLE II
Locations and Sequences of PCR Primers

Target	Amplicon (bp)	PCR Primer[a]	Sequence
horA	210	h198F2	AAATCTTAACCCTGCCGG
		h198R	GCGGAACGGCGATAAACATA
hitA	179	28F	AGCGTAGCAGAAGAACCTAAG
		207R	CAATTACCAGGATCCATGTACC
16S rRNA	148	386F	CTACGGGAGGCAGCAAG
		534R	ATTACCGCGGCTGCTGG
ORF5	117	154F	GTACGGATCGTGGTAAACG
		270R	GACCATTTGTCTACAAGGCAG
horC	94	46F	CTTGTTGGAGCAATTATTGG
		139R	CGTTGACAAGTGCTACAGG

[a] With the exception of primers h198F2 and h198R, located at positions 318 and 521, respectively, the number in the primer name refers to its position within the gene. For each primer set, F and R indicate forward and reverse.

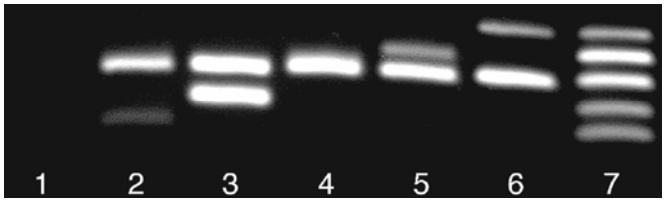

Fig. 1. Agarose gel electrophoresis of multiplex PCR for putative spoilage-associated genes. PCR-positive genes are listed from the bottom to top for each lane. Lane 1, negative control (no added DNA). Lane 2, *Pediococcus claussenii* CCC B962A–*horC* and 16S rRNA. Lane 3, *P. parvulus* ETS.4–ORF5 and 16S rRNA. Lane 4, *Lactobacillus brevis* ATCC 14869–16S rRNA only. Lane 5, *L. delbrueckii* ATCC 12315^T–16S rRNA and *hitA*. Lane 6, *P. claussenii* CCC B1056R–16S rRNA and *horA*. Lane 7, *L. brevis* CCC B1202–*horC*, ORF5, 16S rRNA, *hitA*, and *horA*.

finding that *horC* and ORF5 can be located either separately or together on the same plasmid. Not only does this reflect the genetic diversity and promiscuity of genetic material exhibited by *Lactobacillus* and *Pediococcus* isolates, it also reinforces the need for appropriate subculturing techniques to effectively maintain the presence of genes of interest for the purposes of quality control testing.

Correlation of Genes with Growth in Beer and Isolate Origin

Analysis of the distribution of each of the four putative beer-spoilage genes with respect to whether an isolate was a member of *Lactobacillus* or *Pediococcus* and whether an isolate could grow in beer is presented in Tables III and IV. The χ^2 analysis indicated that each of the four genes correlated with ability of lactobacilli to grow in beer (Table IV). In contrast, for pediococci, only the presence of *horA* was highly correlated with growth in beer, whereas ORF5 was negatively correlated with the ability to grow in beer (i.e., the presence of ORF5 was predictive of the inability of *Pediococcus* isolates to grow in beer). Because the number of lactobacilli exceeded that of the pediococci isolates studied (83 and 50, respectively), three of the four genes (ORF5 being the exception) were significantly correlated with growth in beer when the lactobacilli and pediococci data were combined (Table IV). This correlation is explained by the overlap in the occurrence of genes shown in Table III. In the majority of cases, when the *hitA*, *horC*, or ORF5 genes were found in bacteria able to grow in beer, *horA* also was present (15/16, 16/19, and 13/14 instances, respectively). This may explain why the *hitA*, *horC*, and ORF5 genes previously were erroneously thought to be predictive of the ability of an organism to grow in beer (3,6,8,14,16,17). For this reason, it was necessary that binary logistic regression analyses be performed to determine the actual contribution of each gene to the overall predictive model. Previous studies on *hitA*, *horC*, and ORF5 either failed to concurrently screen for the presence of *horA* or used a set of isolates originating from a single source, thereby skewing interpretation of the importance of a given gene. Moreover, statistical analyses were not performed in previous studies.

To determine whether a correlation exists between the origin of a LAB isolate and the presence of the genes of interest (Table I), a χ^2 test was used to compare the presence of putative spoilage-associated genes in brewery and nonbrewery isolates. Of *hitA*, *horA*, *horC*, and ORF5, only the presence of *horA* was significantly correlated with brewery origin for both *Lactobacillus* and *Pediococcus* isolates ($P < 0.0001$). This suggests that, of the four genes, only *horA* is positively selected for in the brewery environment, reaffirming that the presence of *horA* enhances the beer-spoilage potential of a LAB. Thus, environmental LAB introduced into a brewery could become vicious beer spoilers should even a few bacteria among the original population harbor the *horA* gene.

Predictive Abilities

To determine the actual contribution of each gene to the ability of an organism to grow in beer, binary logistic regression analyses were performed. The results of these analyses (Table V) indicate that only *horA* is a significant predictor of the ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer. When the *Lactobacillus* and *Pediococcus* data were grouped for binary logistic regression analysis, *horA* and *horC* both were retained by the statistical model (data not shown). However, *horC* showed a contribution to the ability to grow in beer only when lactobacilli and pediococci data were combined; this was due to the three *horA*-negative, *horC*-positive isolates (two pediococci and one lactobacilli) that were capable of growing in beer, which created what we believe is a statistical anomaly. The inclusion of *horC* as a predictor in the binary logistic model actually caused a decrease in the odds ratio and only a small increase (<1.5%; data not shown) in the overall ability of the model to predict growth in beer. Although *horC* previously has been shown to confer some level of hop resistance (14), we found that this gene rarely occurred in the absence of *horA*. Meanwhile, *horA* was present in many growth-positive isolates that were *horC* negative. These factors show that *horC* is a much less effective marker than *horA* for the prediction of growth in beer; therefore, *horC* was discarded from the predictive model.

TABLE III
Presence of *hitA*, *horA*, *horC*, and ORF5 Genes and Bacterial Growth in Beer[a]

	Growth +			Growth –		
Genes Present	***Lactobacillus* spp.**	***Pediococcus* spp.**	**All**	***Lactobacillus* spp.**	***Pediococcus* spp.**	**All**
horA only	5	8	13	2	1	3
horA and *hitA*	0	0	0	1	0	1
horA and ORF5	2	0	2	0	0	0
horA, *hitA*, and *horC*	5	0	5	0	0	0
horA, *horC*, and ORF5	1	0	1	0	0	0
horA, *hitA*, *horC*, and ORF5	9	1	10	0	0	0
hitA only	0	0	0	7	1	8
horC only	0	1	1	0	0	0
ORF5 only	0	0	0	4	12	16
horC and ORF5	0	1	1	0	1	1
horC and *hitA*	1	0	1	0	0	0
No known genes	7	6	13	40	17	57

[a] Growth in beer defined as ability to grow in beer 1, or beer 1 and beer 2.

TABLE IV
χ^2 Correlation of Putative Beer Spoilage-Associated Genes with Growth in Beer

	Lactobacillus		***Pediococcus***		**All Bacteria**	
Gene	**Beer 1**	**Beer 2**	**Beer 1**	**Beer 2**	**Beer 1**	**Beer 2**
hitA	$P < 0.005$	$P < 0.005$	NS[a]	NS	$P < 0.005$	$P < 0.005$
horA	$P < 0.005$	$P < 0.005$	$P < 0.005$	$P < 0.005$	$P < 0.005$	$P < 0.005$
horC	$P < 0.005$	$P < 0.005$	NS	$P < 0.05$	$P < 0.005$	$P < 0.005$
ORF5	$P < 0.005$	$P < 0.005$	($P < 0.05$)[b]	($P < 0.05$)[b]	NS	NS

[a] Not significant ($P > 0.05$).
[b] Negatively correlated (i.e., presence of ORF5 is predictive of inability to grow in beer).

The binary logistic regression models in Table V report the predictive abilities generated based on the various groupings analyzed. The models show that *horA* detection has a significant capability for predicting the ability of an isolate to grow in beer. A *horA*-positive PCR result is 88.6% accurate in predicting that an organism will grow in beer (i.e., 31 of 35 *horA*-positive isolates grew in beer) (Table III). Conversely, a *horA*-negative PCR result is 83.7% accurate in predicting that an isolate will not be capable of growing in beer. The lower negative predictive ability was due to the 16 isolates in this study that were *horA*-negative yet capable of growing in beer (Table IV). Because 13 of these 16 isolates did not possess any known spoilage-associated genes, there must be other as yet undefined genetic mechanisms that allow bacterial growth in beer.

The odds ratios given in Table V are the ratio of the probability of growth in beer for *horA*-positive isolates to the probability of growth in beer for a *horA*-negative group. These ratios were normalized to one and, therefore, can be expressed as "times more likely." For example, a *horA*-positive *Lactobacillus* isolate is 53.4 times more likely to grow in beer 1 than a *horA*-negative *Lactobacillus* isolate. As indicated by the predictive abilities and odds ratios in Table V, the ability of *horA* to predict growth was lower for the pediococci than for the lactobacilli, implying that the genetic basis for the ability to grow in beer is currently less well defined for pediococci compared with lactobacilli.

Although *horA* is, overall, >80% accurate in predicting the ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer, Nagelkerke's R^2 values for binary logistic regression confirmed the idea that there must be other mechanisms associated with these bacteria and their ability to grow in beer. As shown by the R^2 values in Table V, *horA* alone could account for only 23–59% of the variability seen in the ability of an organism to grow in beer, depending on the type of beer and the genus of the isolate involved. It must be stressed that these additional mechanisms are not represented by the *hitA*, *horC*, or ORF5 genes, because binary logistic regression analysis found no significant relationship between the presence of these genes and the ability to grow in beer. As such, correlation of these three genes with the ability of a bacterium to grow in beer (i.e., as shown by the χ^2 analyses in Table IV) results from the presence (irrespective of function) of these genes in bacteria also found to possess *horA*. Although *hitA*, *horC*, and ORF5 may somehow play an as yet undetermined role in hop resistance through synergy with *horA* or by other mechanisms (e.g., nutrient acquisition or ethanol resistance), the presence of these three genes cannot be used to predict the ability of an isolate to grow in beer.

Growth Rate in Beer

A *t* test for independent samples was performed to determine whether an organism's growth rate in beer was affected by the presence of any of the four genes (Table VI). Although the presence of *horA* predicted growth in beer, the additional presence of *hitA* or *horC* was associated with an average growth rate in beer that was more than doubled. Because *hitA* and *horC* occurred together in 15 of 16 instances in which either gene was found in the same isolate with *horA*, it was impossible to say whether *hitA*, *horC*, or both genes contributed to the increased growth rate seen in *horA*+/*hitA*+/*horC*+ isolates. Another possibility is that *hitA* and *horC* act as surrogate markers for as yet unknown genes present in these *horA*+/*hitA*+/*horC*+ isolates that actually are responsible for the increased growth rate in beer. Although *hitA* and *horC* were not accurate predictors of the ability to grow in beer by themselves, when multiplexed with *horA* they could serve to identify bacteria able to rapidly spoil beer.

CONCLUSIONS

The described multiplex PCR was effective in detecting the presence of a bacterium in beer and differentiating between *hitA*, *horA*, *horC*, ORF5, and 16S rRNA genes, producing five distinguishable bands (Fig. 1). As emphasized by the R^2 values and odds ratios in

TABLE V
Binary Logistic Regression of Putative Beer Spoilage-Associated Genes and Growth in Beer[a]

Bacterium	R^{2b}	Odds Ratio[c]	Predictive Ability of *horA* (%)[d]		
			Will Grow	Will Not Grow	Overall
Lactobacillus spp.					
Beer 1	0.59	53.4	88.0	87.9	88.0
Beer 2	0.50	29.1	80.0	87.9	85.5
Pediococcus spp.					
Beer 1	0.38	31.0	90.0	77.5	80.0
Beer 2	0.23	9.3	70.0	80.0	78.0
All bacteria					
Beer 1	0.50	39.7	88.6	83.7	85.7
Beer 2	0.40	18.7	77.1	84.7	82.7

[a] All putative spoilage-associated genes (*hitA*, *horA*, *horC*, and ORF5) were included in the analyses, but only *horA* was statistically significant.
[b] Nagelkerke's R^2 value indicates how much of the ability to grow in beer is accounted for by the independent variable *horA*.
[c] Ratio of the probability of growth with the presence of *horA* to the probability of growth in the absence of *horA*. All values were significant at $P < 0.0005$, except for *Pediococcus* in beer 2, which was significant at $P < 0.005$.
[d] Model's ability to predict growth in beer. Only *horA* was significant in each analysis, indicating the ability of *horA* to predict growth in beer.

TABLE VI
Days Required for Growth in Beer in Relation to Genes Present[a]

Genes Present	Beer 1			Beer 2		
	N^b	Mean	Range	N^b	Mean	Range
horA only	15	10.6	2–27	11	9.5	3–14
horA, *horC*, *hitA*	15	4.7	2–12	15	4.9	2–12

[a] The ORF5 gene was excluded as a variable because it had no correlation with the ability to grow in beer. Also, no *horA*+/*horC*–/*hitA*+ and only one *horA*+/*horC*+/*hitA*– growth-positive isolate was found. For beer 1 and beer 2, $P < 0.005$ and 0.0005, respectively, for independent sample *t* tests comparing growth-positive isolates from respective categories.
[b] Number of isolates possessing the gene or genes and capable of growing in beer.

Table V, there currently is a better understanding of the genetic basis for growth in beer for *Lactobacillus* isolates than for *Pediococcus* isolates. Although it is evident that there are other, as yet unknown, mechanisms involved in the ability to grow in beer (Tables IV and V), of the currently known putative beer-spoilage associated genes the only significant predictor of the ability to grow in beer was *horA* (Table V). However, the presence of *hitA*, *horC*, or both in addition to *horA* was predictive of increased growth rate in beer (Table VI).

We suggest that brewery quality control laboratories should, at a minimum, routinely screen lactobacilli and pediococci found in beer for spoilage potential using this multiplex PCR directed to the *horA* and 16S rRNA genes. In addition, although *hitA* and *horC* are not accurate predictors of the ability to grow in beer, incorporation of either gene into a multiplex PCR with *horA* could serve to identify isolates able to grow rapidly in beer (Table VI), thus providing a second crucial piece of information. Until the remaining gene or genes associated with bacterial beer spoilage are defined, the suggested *horA*, *hitA* or *horC*, and 16S rRNA gene multiplex PCR represents the best test available for quickly assessing whether a given *Lactobacillus* or *Pediococcus* isolate is capable of not only growing in, but also rapidly growing in, and spoiling beer.

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LITERATURE CITED

1. Fujii, T., Nakashima, K., and Hayashi, N. Random amplified polymorphic DNA-PCR based cloning of markers to identify the beer-spoilage strains of *Lactobacillus brevis, Pediococcus damnosus, Lactobacillus collinoides* and *Lactobacillus coryniformis. J. Appl. Microbiol.* 98:1209-1220, 2005.
2. Haakensen, M. C., Butt, L., Chaban, B., Deneer, H., Ziola, B., and Dowgiert, T. A *horA*-specific real-time PCR for detection of beer-spoilage lactic acid bacteria. *J. Am. Soc. Brew. Chem.* 65:157-165, 2007.
3. Hayashi, N., Ito, M., Horiike, S., and Taguchi, H. Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus brevis* strains capable of growing in beer. *Appl. Microbiol. Biotechnol.* 55:596-603, 2001.
4. Muyzer, G., de Waal, E. C., and Uitterlinden, A. G. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59:695-700, 1993.
5. Priest, F. G., and Campbell, I. *Brewing Microbiology*, 3rd ed. F. G. Priest and I. Campbell, eds. Kluwer Academic Publishers, New York, 2003.
6. Sakamoto, K., and Konings, W. N. Beer spoilage bacteria and hop resistance. *Int. J. Food Microbiol.* 89:105-124, 2003.
7. Sakamoto, K., Margolles, A., van Veen, H. W., and Konings, W. N. Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-binding cassette multidrug transporter HorA. *J. Bacteriol.* 183:5371-5375, 2001.
8. Sakamoto, K., van Veen, H. W., Saito, H., Kobayashi, H., and Konings, W. N. Membrane-bound ATPase contributes to hop resistance of *Lactobacillus brevis. Appl. Environ. Microbiol.* 68:5374-5378, 2002.
9. Sami, M., Suzuki, K., Sakamoto, K., Kadokura, H., Kitamoto, K., and Yoda, H. A plasmid pRH45 of *Lactobacillus brevis* confers hop resistance. *J. Gen. Appl. Microbiol.* 44:361-363, 1998.
10. Sami, M., Yamashita, H., Hirono, T., Kadokura, H., Kitamoto, K., Yoda, K., and Yamasaki, M. Hop-resistant *Lactobacillus brevis* contains a novel plasmid harboring a multidrug resistance-like gene. *J. Ferment. Bioeng.* 84:1-6, 1997.
11. Sami, M., Yamashita, H., Kadokura, H., Kitamoto, K., Yoda, K., and Yamasaki, M. A new and rapid method for determination of beer-spoilage ability of lactobacilli. *J. Am. Soc. Brew. Chem.* 55:137-140, 1997.
12. Simpson, W. J. Ionophoric action of *trans*-isohumulone of *Lactobacillus brevis. J. Gen. Microbiol.* 139:1041-1045, 1993.
13. Simpson, W. J., and Fernandez, J. L. Mechanism of resistance of lactic acid bacteria to *trans*-isohumulone. *J. Am. Soc. Brew. Chem.* 52:9-11, 1994.
14. Suzuki, K., Iijima, K., Ozaki, K., and Yamashita, H. Isolation of a hop-sensitive variant of *Lactobacillus lindneri* and identification of genetic markers for beer spoilage ability of lactic acid bacteria. *Appl. Environ. Microbiol.* 71:5089-5097, 2005.
15. Suzuki, K., Ozaki, K., and Yamashita, H. Comparative analysis of conserved genetic markers and adjacent DNA regions identified in beer-spoilage lactic acid bacteria. *Lett. Appl. Microbiol.* 39:240-245, 2004.
16. Suzuki, K., Ozaki, K., and Yamashita, H. Genetic marker for differentiating beer-spoilage ability of *Lactobacillus paracollinoides* strains. *J. Appl. Microbiol.* 97:712-718, 2004.
17. Suzuki, K., Sami, M., Kadokura, H., Nakajima, H., and Kitamoto, K. Biochemical characterization of horA-independent hop resistance mechanism in *Lactobacillus brevis. Int. J. Food Microbiol.* 76:223-230, 2002.

6. DISCOVERY AND EVALUATION OF NOVEL ATP-BINDING CASSETTE TYPE MULTI-DRUG RESISTANCE GENES TO PREDICT GROWTH OF *PEDIOCOCCUS* ISOLATES IN BEER

Author contributions:

Monique Haakensen conceived the study, designed and standardized the experiments, and drafted the manuscript.

Vanessa Pittet assisted with design and standardization of the experiments.

Kendra Morrow performed some of the PCR experiments.

Alison Schubert performed some of the PCR experiments.

Janet Ferguson performed some of the PCR experiments.

Barry Ziola conceived the study, edited the manuscript, and is the holder of the research grant used to fund the study.

BRIEF INTRODUCTION TO CHAPTER 6

In Chapters 3 and 5, it was found that the correlation of putative beer-spoilage associated genes with ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer is not absolute. This observation was especially pronounced for *Pediococcus*. As such, a search was undertaken to find additional beer-spoilage associated genes. A directed approach to gene discovery was used, targeting the bacterial isolates which were PCR-negative for all known previously described beer-spoilage genes (i.e., *horA*, *horC*, *hitA*, and *ORF5*), but capable of growing in beer. As the strongest correlation found in Chapter 5 was with the ABC MDR gene *horA*, a highly conserved region of ABC MDR genes was used as the target to search for novel beer-spoilage associated genes by using degenerate PCR primers. Once ABC MDR genes were found in the unique beer-spoilage isolates, the PCR amplicons were sequenced and specific PCR primers are designed to screen a broad selection of lactobacilli and pediococci as was done in Chapters 3 and 5. Novel ABC MDR genes found to correlate with beer-spoilage were sequenced *de novo* and characterized through bioinformatic analysis.

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Ability of Novel ATP-binding Cassette Multidrug Resistance Genes to Predict Growth of *Pediococcus* Isolates in Beer

Monique Haakensen, Vanessa Pittet, Kendra Morrow, Alison Schubert, Janet Ferguson, and Barry Ziola,[1]
Department of Pathology and Laboratory Medicine, College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada

ABSTRACT

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We have recently shown that the *horA* gene is highly accurate for determining the beer-spoilage potential of lactobacilli isolates but not as good for predicting the beer-spoilage ability of pediococci isolates. Our goal in this study was to identify genetic markers for assessing the beer-spoilage potential of *Pediococcus* isolates. *Lactobacillus* and *Pediococcus* isolates negative for the putative beer-spoilage associated genes *hitA*, *horA*, *horC*, and *ORF5*, yet capable of growing in beer, were screened using degenerate PCR primers designed to the ATP-binding cassette region of multidrug resistance (ABC MDR) genes, and amplicons were sequenced to reveal possible identity and function. Six novel ABC MDR genes were found. Specific PCR primers were designed to each gene and used to screen 84 *Lactobacillus* and 48 *Pediococcus* isolates. Three genes had no correlation with hop resistance or ability to grow in beer. Another gene correlated with hop resistance but only in isolates incapable of growing in beer. The remaining two genes, *bsrA* and *bsrB* (beer-spoilage related), were highly correlated with the beer-spoilage ability and hop resistance of *Pediococcus* isolates. Although sharing a low percent identity with one another or other known proteins, both BsrA and BsrB contained conserved motifs typical of ABC MDR-type proteins. The *bsrA* and *bsrB* genes were not found in any *Lactobacillus* isolates, regardless of whether they were able to grow in beer, making them the first genetic markers capable of differentiating between beer-spoilage lactobacilli and pediococci.

RESUMEN

Recientemente hemos demostrado que el gen de *horA* da alta precisión para la determinación del deterioro potencial de cerveza de aislados de lactobacilos, pero no tan buena para la predicción de la capacidad de aislados de pediococci para dañar la cerveza. Nuestro objetivo en este estudio fue identificar los marcadores genéticos para evaluar el potencial a deteriorar la cerveza de aislados de *Pediococcus*. *Lactobacillus* y *Pediococcus* cepas negativas para genes asociados con el deterioración de la cerveza *hitA*, *horA*, *horC*, y *ORF5*, pero capaz de crecer en la cerveza, se proyectaron utilizando cebadores degenerados de PCR diseñados para la ATP vinculantes casete región de multirresistencia (MDR ABC) los genes, y amplicones fueron secuenciados para revelar la identidad y la posible función. Seis nuevos genes ABC MDR se encontraron. Cebadores específicos de PCR fueron diseñados para cada gen y la utiliza para la inspección de 84 *Lactobacillus* y 48 *Pediococcus* aislados. Tres genes que no tenían correlación con la resistencia de lúpulo o la capacidad para crecer en la cerveza. Otro gen correlaciona con la resistencia de lúpulo, pero sólo en aislados incapaces de crecer en la cerveza. Los otros dos genes, *bsrA* y *bsrB* (relacionados con el deterioro de cerveza), fueron altamente correlacionados con la capacidad de deteriorar la cerveza y con la resistencia de lúpulo de *Pediococcus* aislados. A pesar de compartir un bajo por ciento de identidad entre sí o con otras proteínas conocidas, tanto BsrA y BsrB figura conserva motivos típicos de la MDR-tipo ABC proteínas. Los genes de *bsrA* y *bsrB* no se encuentran en ningún *Lactobacillus* aislados, independientemente de si eran capaces de crecer en la cerveza, por lo que los primeros marcadores genéticos capaces de diferenciar entre lactobacilos y pediococci con capacidad de dañar la cerveza.

[1] Corresponding author. E-mail: b.ziola@usask.ca; Phone: +1.306.966.4330; Fax: +1.306.966.8049.

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Spoilage of beer by *Lactobacillus* and *Pediococcus* bacteria is a significant problem for the brewing industry (15). Although most gram-positive bacteria fail to grow in beer due to the presence of hop compounds, ethanol, reduced oxygen, and low nutrient levels, some lactobacilli and pediococci possess mechanisms that allow them to overcome these selective pressures (13,16). Although the *horA* gene is highly accurate for determining the beer-spoilage potential of *Lactobacillus* isolates, we have recently shown that it is not as accurate at predicting the beer-spoilage ability of pediococci (5,8). As such, our goal was to identify genetic markers that can be used for accurate differentiation of beer-spoilage *Pediococcus* isolates from pediococci that do not pose a threat as beer-spoilage organisms. In contrast to the random approaches to gene discovery (e.g., randomly amplified polymorphic DNA PCR) that were used to discover the putatively beer-spoilage associated genes *hitA*, *horB*, *horC*, and *ORF5* (3,9), we chose to use a directed approach targeting 13 bacterial isolates (6 lactobacilli and 7 pediococci) that are capable of growing in beer yet are PCR-negative for all putative beer-spoilage associated genes (i.e., *hitA*, *horA*, *horC*, and *ORF5*) (8). These isolates presumably contain undefined genetic elements that permit bacterial growth in beer.

Currently, the best known marker for the beer-spoilage ability of lactobacilli is the ATP-binding cassette multidrug resistance (ABC MDR) gene *horA* (5,8). Although the association of *horA* with ability of pediococci to grow in beer is much weaker, the genus *Pediococcus* does fall within the multiphyletic genus *Lactobacillus*, making it likely that similar genetic mechanisms are used by both genera to facilitate growth in beer. As such, we used the DNA sequences of ABC MDR-type proteins that are similar to HorA as our starting point in the design of degenerate PCR primers to search for novel beer-spoilage genetic markers. Here, we report the finding of six novel ABC MDR genes within pediococci, two of which are new genetic markers that accurately reflect the ability of pediococci to grow in beer.

MATERIALS AND METHODS

Bacterial Growth Conditions and DNA Extraction

Bacterial growth conditions and verification of the ability of isolates to grow in beer were performed as described previously (5,8). DNA extractions were performed using 10 µL of bacterial culture with 100 µL of a DNA kit (Instagene DNA Matrix kit, BioRad), as directed by the manufacturer. At the final step, 90 µL of supernatant was removed and stored at –20°C.

Design of Degenerate PCR Primers

The beer-spoilage related protein HorA was used to query the GenBank database using the basic local alignment search tool (BLAST) (1). The protein sequence was used as a query to ensure the highest degree of functional similarity and that the nucleotide

sequences corresponding to the top 10 matches (i.e., score >500 when using protein query) were downloaded. The best matches to HorA also putatively coded for ABC MDR- or ABC amino acid transporter-type proteins, and all but one of the sequences downloaded were identified through genome-sequencing projects. The nucleotide sequences used to create degenerate PCR primers are described in Table I.

A multiple sequence alignment (MSA) of nucleotide sequences corresponding to the top 10 protein matches (Table I) and the *horA* gene itself was created using ClustalX 1.81 software and its default settings (17). A consensus sequence was then created from the MSA using the European Molecular Biology Software Suite v2.2.0 "cons" program (14) and threshold settings of "required number of identities at a position" equal to the number of sequences in the MSA and "threshold above which the consensus is given in uppercase" equal to the first whole integer greater than three-quarters of the number of sequences in the MSA. The resulting consensus sequence was then aligned with the sequences originally used in the MSA, again using ClustalX software. The MSA, now also containing the consensus sequence, was visually scanned for regions of high percent identity that might also be capable of functioning as PCR primers. The degenerate PCR primers created in this way correspond to bases 1127–1142 (forward primer) and 1533–1516 (reverse primer) of the *horA* gene (described in Table I). Inosine bases were used to reduce the overall degeneracy of the PCR primers while allowing for a broader range of binding specificity.

TABLE I
Nucleotide Sequences Used in Designing Degenerate PCR Primers for Detecting ATP-binding Cassette Multidrug Resistance Genes[a]

Bacterium	Strain	GenBank Accession No.	Nucleotide Location
Lactobacillus			
acidophilus	NCFM	YP_193507	581970–583727
brevis	ATCC 367	YP_796084	1953654–1955423
casei	ATCC 334	YP_807324	2119775–2121559
johnsonii	NCC 533	NP_965013	1076997–1078748
plantarum	WCFS1	NP_786297	2639403–2641160
reuteri	100-23	ZP_01274735	12372–14093
reuteri	ATCC 55730	EU038268	Draft[b]
reuteri	F275	YP_001270631	18960–20681
sakei	23K	YP_394844	226827–228578
Pediococcus pentosaceus	ATCC 25745	YP_805121	1645664–1647442

[a] Resultant forward and reverse degenerate primers are CIGG(C/T)GG(C/T)GGIAA(A/G)TC and CTIGCIGTIGCTTCATC, respectively, with an expected amplicon size of 380–406 bp.
[b] Draft genome; contigs not available in assembled format.

Each degenerate primer PCR tube contained 1 U of Platinum *Taq* DNA polymerase (Invitrogen), 1× PCR buffer (Invitrogen), 1.5 m*M* $MgCl_2$, 0.2 m*M* each of the four deoxynucleotide triphosphates, and 0.2 μ*M* each of the PCR primer. Template DNA was added (2.5 μL), and the final volume was brought to 25 μL with water. The PCR program consisted of an initial denaturation step of 4 min at 95°C, followed by 40 cycles of 95°C for 45 sec, 42°C for 45 sec, and 72°C for 60 sec and a final extension step of 5 min at 72°C.

Design of Gene-Specific PCR Primers

Amplicons from the degenerate PCR were sequenced and used to create a MSA with ClustalX 1.81 software and its default settings (17). The MSA was then visualized using the GeneDoc software program (12). DNA regions were identified in the MSA that contained sufficient numbers of polymorphisms to create PCR primers specific to each novel gene (Table II). These specific PCR primers were designed to function in multiplex with PCR primers that amplify a portion of the 16S rRNA gene (6). The amplification of the 16S rRNA gene, thus, served as an internal control to confirm the presence of bacterial DNA in PCRs that were negative for the novel ABC MDR genes. The target-specific PCRs were identical to the degenerate PCRs; however, the primers for the 16S rRNA gene were used at 0.1 μ*M*, and a different PCR program was used. The target-specific PCR consisted of a denaturation step of 4 min at 95°C, followed by 30 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec and a final extension step of 2 min at 72°C.

PCR Product Purification and Sequencing

Amplicons from both degenerate and target-specific PCR were visualized by electrophoresis on 1.0% agarose gels containing 0.5 mg of ethidium bromide per mL. DNA sequencing was performed at the Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, SK. Sequencing results were checked manually for base calling errors and were queried with BLAST for similar sequences (1). Bubble PCR (also known as genome walking) was used to obtain the full sequences of the beer-spoilage related genes *bsrA* (*ABC1*) and *ABC2* (2). A minimum 50-bp overlap of amplicons was used for gene sequence assembly. The full-length *bsrB* (*ABC3*) gene was obtained from unreleased *P. claussenii* ATCC BAA-344^T genome sequence data available in our laboratory. Subsequent to using bubble PCR to obtain the sequences of *bsrA* and *ABC2*, these two genes were also confirmed in the *P. claus-*

TABLE II
Gene-Specific PCR Primers

Target Gene	Amplicon Size (bp)	PCR Primer	
		Forward	Reverse
bsrA	529	TACTCACTCCCAAGAGGTTG	GTCATTCGTGCGTTCAGT
	1,857[a]	GTTGTCGATTAGTCAAAATAGG	TTTGAAGTGATTCCCACAATTGC
bsrB	299	AACTAGATTCTATGAAGTTACGTCTGG	AAATTCTTTGCTTTTGACCGCCTG
	2,047[a]	GATTGACTTTAGAATCTATTGTGTC	CTTTCTCACTCGCAATTGGTG
ABC2	373	CAGCTGGGATGCTTGGTCAG	ACATACCCGATTTGTGACCGCCAA
	1,778[a]	GGATACTATACATCAATATCTCG[b]	GTGGGCTTGGCATTAGG
ABC4	165	CCTGATAGCGGCCACATTTTGATCG	CTTCATCTGTATAGTTGCGCGTCG
ABC5	131	TCTTGAATCGTTTAACTTGACGGAC	CTCATCCGAATACTGACCGCTG
ABC6	209	CCAACATTCAAGATATCCAGCTGAC	CTGTATCCAATTGTTTAGGCATTTCTTCC
16S rRNA[c]	148	CTGATGGAGCAACGCCGCGT	ATTACCGCGGCTGCTGG
16S rRNA[d]	526	AGAGTTTGATCCTGGCTCAG	ATTACCGCGGCTGCTGG

[a] Full-length gene and some surrounding DNA sequence.
[b] Located 20-bp upstream of the start codon.
[c] Used in multiplex with *bsrA* PCR primers.
[d] Used in multiplex with all PCR primer sets, except for degenerate PCR primers and those specific to *bsrA*.

senii genome sequence data. PCR amplification (i.e., for sequencing) of the full-length *bsrA* (*ABC1*), *bsrB* (*ABC3*), and *ABC2* genes in all positive isolates was done using the same PCR program as the degenerate PCR program but with an annealing temperature of 60°C and the specific primers indicated in Table II.

Analysis of Novel ABC MDR Proteins

The DNA and corresponding protein sequences of amplicons were used to query the NCBI GenBank database with BLAST. Analysis of complete gene sequences was performed using PSortB software to predict subcellular localization (4). Transmembrane helices were predicted using TMpred (10). The components of Figure 1 were created using the conserved domain database (CDD) available through the NCBI BLASTx program (11). The query sequences were used to determine specific hits within the CDD, and these hits were used to determine the superfamilies and, ultimately, the multidomain architecture of the query protein.

Statistical Analyses

Statistical analysis was performed using SPSS for Windows (version 16.0, SPSS Inc.). For both binary logistic regression and *t* test for independent samples, the confidence interval and level of significance were set at 95% and $P = 0.05$, respectively. Binary logistic regression models were calculated for *Lactobacillus* isolates, *Pediococcus* isolates, and all bacteria (i.e., *Lactobacillus* and *Pediococcus* isolates combined), with the ability to grow in beer 1 (filter-sterilized 4% [vol/vol] alcohol beer, pH 4.2, averaging 9.8 BU) and beer 2 (pasteurized 5% [vol/vol] alcohol beer, pH 3.8, averaging 11 BU) as outcome variables. Assessment of the ability of isolates to grow in beer and the presence of previously described putative beer-spoilage associated genes (i.e., *hitA*, *horA*, *horC*, and *ORF5*) were reported previously by Haakensen et al (5,8).

RESULTS AND DISCUSSION

Our goal was to find genetic markers that can be used for accurate differentiation of beer-spoilage *Pediococcus* isolates from pediococci that do not pose a threat as beer-spoilage organisms. In contrast to other studies to date (3,9), we took a directed approach, using degenerate PCR primers to the ABC region of MDR genes to screen 13 unique bacterial isolates (6 lactobacilli and 7 pediococci) (Table III) that were capable of growing in beer yet PCR-negative for all currently known putative beer-spoilage associated genes (i.e., *hitA*, *horA*, *horC*, and *ORF5*) (Table III) (full data is presented in Haakensen et al [8]). When the *horA* gene was used as a query in the GenBank database, 10 similar genes (i.e., shar-

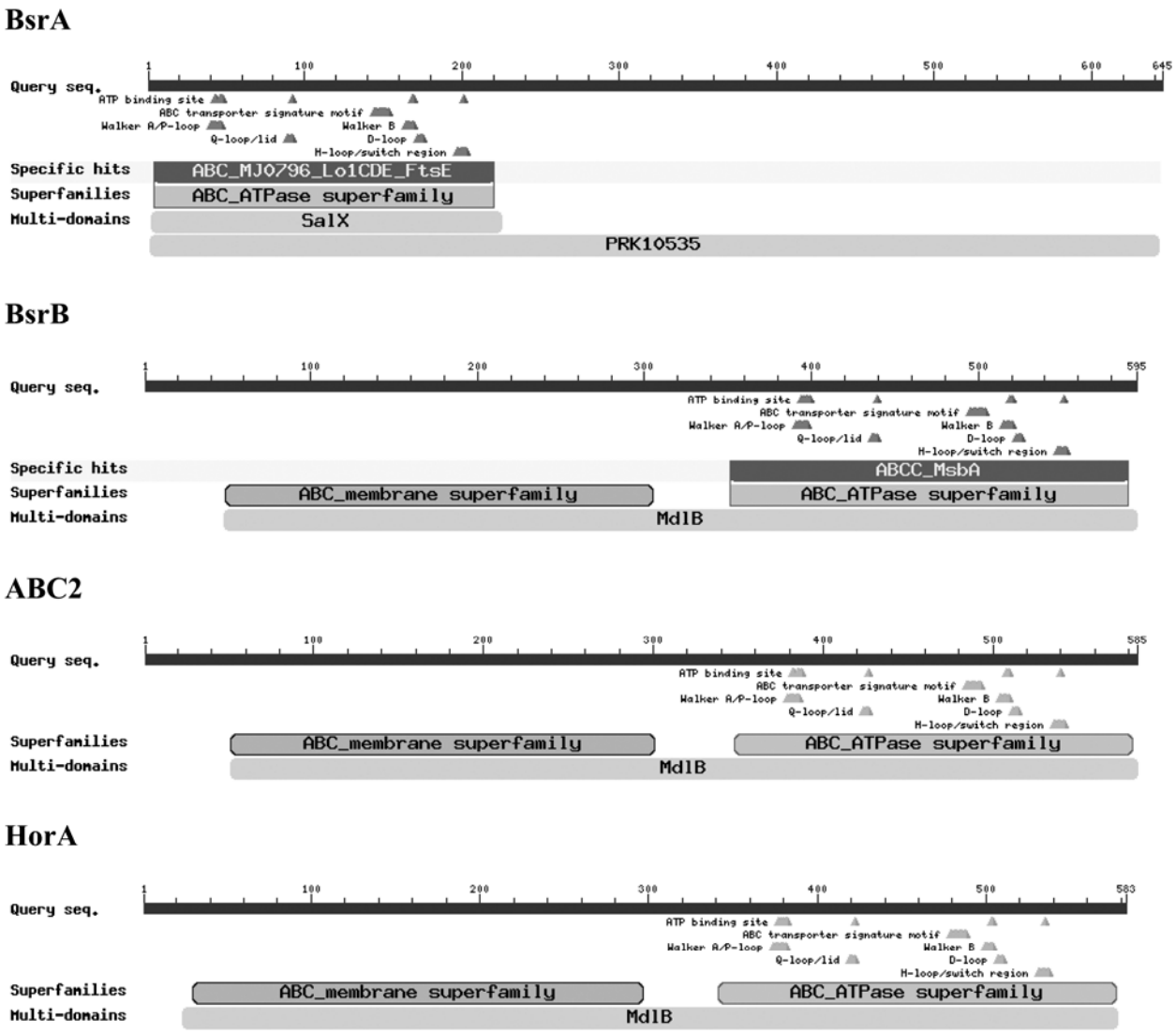

Fig. 1. Schematic diagrams of the conserved domains present in the novel ATP-binding cassette multidrug resistance proteins BsrA, BsrB, and ABC2. For comparison, the conserved domains of HorA are also provided.

ing a high percent identity but not *horA* homologous) were returned that had a score >500 at the protein level (Table I). Degenerate PCR primers were then designed from MSAs of the ABC region of these genes (forward and reverse degenerate primers are described in Table I).

Degenerate PCR amplification resulted in the discovery of six novel ABC MDR genes (Table III), all of which share homology with ABC MDR-type transporters. These genes were named *ABC1* through *ABC6*. The PCR amplicons obtained by degenerate PCR all were 380–406 bp, which was the expected length based on the

TABLE III
Isolates Used for Initial Screening with Degenerate PCR Primers Designed to Target ATP-binding Cassette Multidrug Resistance (ABC MDR) Genes[a]

Bacterium	Strain[b]	Origin	ABC MDR Gene[c]	Gene-Specific PCR					
				bsrA[d]	*bsrB*[d]	*ABC2*	*ABC4*	*ABC5*	*ABC6*
Pediococcus									
claussenii	CCC B1098R	Brewery	*ABC3*	+	+	–	–	–	–
claussenii	CCC B1099NR	Brewery	*ABC1*	+	+	–	–	–	–
claussenii	CCC B1099R	Brewery	*ABC1*	+	+	–	–	–	–
claussenii	CCC B1260NR	Brewery	*ABC1*	+	+	–	–	–	–
claussenii	CCC B1260R	Brewery	*ABC1*	+	+	–	–	–	–
damnosus	ATCC 11308	Brewery	None found	–	–	–	–	–	–
parvulus	ATCC 43013	Wine	*ABC1*	+	–	–	–	–	–
Lactobacillus									
casei	ATCC 25598	Milking machine	*ABC2*	–	–	+	+	+	–
fermentum	ATCC 9338	Unknown	*ABC4*	–	–	–	+	–	–
hilgardii	ATCC 27305	Wine	*ABC5*	–	–	–	–	–	+
paracollinoides	ATCC 8291	Brewery	None found	–	–	–	–	–	–
reuteri	ATCC 31282	Unknown	*ABC6*	–	–	–	–	+	–
rhamnosus	ATCC 8530	Unknown	*ABC2*	–	–	+	–	–	–

[a] All isolates were capable of growing in beer, yet were PCR-negative for previously described putative spoilage-associated genes (i.e., *hitA*, *horA*, *horC,* and *ORF5*).
[b] R = ropy; NR = nonropy.
[c] Novel ABC MDR genes identified using degenerate PCR; each number indicates a unique gene.
[d] Results of screening using gene-specific PCR multiplexed with a 16S rRNA gene internal control.

TABLE IV
Presence of Novel ATP-binding Cassette Multidrug Resistance Genes with Respect to Genus, Species, and Ability to Grow in Beer[a]

Bacterium	Can Grow in Beer							Cannot Grow in Beer						
	No.[b]	*bsrA*	*bsrB*	*ABC2*	*ABC4*	*ABC5*	*ABC6*	No.[b]	*bsrA*	*bsrB*	*ABC2*	*ABC4*	*ABC5*	*ABC6*
Lactobacillus														
acetotolerans	0	–	–	–	–	–	–	1	0	0	1	0	0	0
acidophilus	0	–	–	–	–	–	–	3	0	0	2	0	0	0
amylovorus	1	0	1	0	0	0	0	5	0	0	1	0	0	0
brevis	8	0	0	5	3	1	0	5	0	0	0	1	0	0
casei	7[c]	0	0	1	7	1	0	3	0	0	0	3	0	0
delbrueckii	0	–	–	–	–	–	–	8	0	0	1	0	0	0
dextrinicus	0	–	–	–	–	–	–	1	0	0	1	0	0	0
ferintoshensis	0	–	–	–	–	–	–	1	0	0	0	0	0	0
fermentum	1	0	0	0	1	0	0	2	0	0	1	0	0	0
fructivorans	0	–	–	–	–	–	–	1	0	0	0	1	0	0
helveticus	1	0	0	1	0	0	0	1	0	0	1	0	0	0
hilgardii	1	0	0	0	0	0	1	2	0	0	0	0	0	1
homohiochii	0	–	–	–	–	–	–	1	0	0	0	1	0	0
jensenii	0	–	–	–	–	–	–	1	0	0	0	0	0	0
kefiri	0	–	–	–	–	–	–	1	0	0	0	1	0	0
kefirgranum	0	–	–	–	–	–	–	1	0	0	0	1	0	0
kefironofaciens	0	–	–	–	–	–	–	1	0	0	0	0	0	0
paracollinoides	1	0	0	0	0	0	0	0	–	–	–	–	–	–
planatarum	4	0	0	4	1	0	0	5	0	0	5	1	0	0
reuteri	1	0	0	1	0	1	0	4	0	0	0	0	2	0
rhamnosus	1	0	0	1	0	0	0	4	0	0	2	1	0	0
sakei	1	0	0	0	0	0	0	1	0	0	1	1	0	0
zeae	0	–	–	–	–	–	–	1	0	0	0	0	0	0
Unspeciated	2	0	0	1	1	1	0	2	0	0	0	1	1	0
Pediococcus														
acidilactici	1	1	1	0	0	1	0	6	0	0	0	0	1	0
claussenii	11	11	11	0	2	0	0	1	0	0	1	0	0	0
damnosus	4[d]	0	0	0	0	2	0	4	0	0	1	0	3	0
inopinatus	0	–	–	–	–	–	–	1	0	0	0	0	1	0
parvulus	1	1	1	0	0	1	0	13	0	0	1	7	4	0
pentosaceus	0	–	–	–	–	–	–	6	0	0	2	0	5	0

[a] Gene-specific PCR primers were used.
[b] Total number of isolates for the category (can or cannot grow in beer) for the given species.
[c] Two isolates grew only in beer 1 (growth took 27 days).
[d] One isolate grew only in beer 1 (growth took 27 days).

MSA of ABC MDR genes with high percent identity with *horA*. The DNA and corresponding protein sequences of amplicons obtained from degenerate PCR were used to query the NCBI GenBank database with BLAST. Although distinct from one another (<65% identity at the protein level), all six amplicons contained motifs characteristic of ABC MDR-type genes and had a best match to putative MDR genes that were found through *Lactobacillus* and *Pediococcus* genome-sequencing projects. It was then possible to create a MSA of these six PCR amplicons from which specific PCR primer sets were designed for each of the six novel genes that would function in multiplex with the 16S rRNA gene as an internal control (Table II). Using these specific PCR primers, 132 bacteria (84 lactobacilli and 48 pediococci) were screened for the presence of each of the six novel genes. The presence of each gene with respect to species and ability to grow in beer is shown in Table IV.

The presence of each novel ABC MDR gene was compared with existing data regarding the ability of isolates to grow in beer (5,8) and also to the ability of the isolates to grow in broth medium containing hop compounds and on hop-gradient agar (HGA) and hop-gradient agar plus ethanol (HGA+E) plates (Table V) (7). No correlations were found for novel ABC MDR genes *ABC4*, *ABC5*, and *ABC6*. As such, the coding regions of *ABC4*, *ABC5*, and *ABC6* were not sequenced in full, and these genes retained their original designations. The partial sequences obtained for *ABC4*, *ABC5*, and *ABC6* were deposited in GenBank under accession numbers FJ434143, FJ434144, and FJ434145, respectively. Interestingly, although the *ABC2* gene correlated with the ability of *Pediococcus* isolates to grow in the presence of hops in broth medium or on HGA plates, the presence of *ABC2* only weakly correlated with the ability of pediococci to grow in beer and did not correlate with the ability to grow on HGA+E plates (Table V). Thus, the added selection pressure of ethanol altered the association between *ABC2* and hop resistance. Even more interesting was the finding that the ability to resist the antimicrobial effects of hop compounds on HGA plates and in broth medium was associated with pediococci that are unable to grow in beer (Table V). Consequently, the *ABC2* gene was not given a *bsr* designation, because the gene showed no association with the ability of bacteria to grow in beer. The finding of multiple ABC MDR-type genes that were not correlated with the ability of either lactobacilli or pediococci to grow in beer (i.e., *ABC2*, *ABC4*, *ABC5*, and *ABC6*) reiterates the need to use gene-specific primers to target beer-spoilage associated genes such as *horA* (5). Specifically, using PCR primers designed to the ABC region of MDR genes would likely result in a high number of false positives (i.e., PCR-positive but unable to grow in beer).

The *ABC2* gene is the first example we know of where a gene has been correlated to the ability to resist the effects of hop compounds but not to the ability to grow in beer. *ABC2*-possessing isolates correlated with resistance to the effects of hop compounds only in the absence of ethanol; therefore, it is possible that *ABC2* may be capable of providing resistance to the effects of hop compounds to pediococci but only under high-nutrient or low-stress conditions (i.e., not under the highly selective pressures of beer). The *ABC2* gene is 1,755 bp long (GenBank accession no. FJ434142), coding for a protein that is 100% identical (98% identical at the nucleic acid level) to a hypothetical protein found within the full genome sequence of *Lactobacillus plantarum* strain WCFS1 (GenBank accession no. CAD65153). Like the BsrB (described below) and HorA proteins, ABC2 protein contains motifs toward the C terminal that are typical of ABC MDR-type proteins (Fig. 1). Bioinformatic analysis predicted that the ABC2 protein is located within the cytoplasmic membrane and possesses five putative transmembrane helices.

Chi-square analyses (Table V) showed that the novel ABC MDR genes *ABC1* and *ABC3* were highly correlated with the ability of *Pediococcus* isolates to grow in beer and also with resistance to the effects of hop compounds as tested for by broth medium, HGA plates, and HGA+E plates (Table V). These two genes were renamed *bsrA* and *bsrB*, respectively. The *bsrA* and *bsrB* genes were found only in *Pediococcus* isolates that were capable of growing in beer, including 1 isolate of *P. acidilactici* and 11 isolates of *P. claussenii*. The full lengths of the *bsrA* and *bsrB* genes were sequenced in all isolates that were PCR-positive for the genes, and each gene was 100% identical in all isolates carrying these genes. *bsrA* was additionally found in one isolate of *P. parvulus* that could grow in beer. Neither *bsrA* nor *bsrB* were found in any *Lactobacillus* isolates, whether or not they were able to grow in beer.

The *bsrA* gene is 1,935 bp long, coding for a protein of 645 amino acids (GenBank accession no. FJ434141). *bsrA* has an atypical AAG start codon, and as is characteristic of non-ATG start codons, sequencing of the DNA region upstream of the *bsrA* gene did not reveal a Shine-Dalgarno promoter region. Both of these findings suggest that *bsrA* may reside within an operon of genes. The BsrA protein shares low percent identity with proteins in the NCBI GenBank database, having >70% identity with only a single putative protein found in the full genome sequence of *L. casei* (GenBank accession no. YP_807324). In contrast to HorA and BsrB, the ABC MDR motifs in BsrA were found in the N terminal of the protein (Fig. 1). Bioinformatic analysis predicted that the BsrA protein is located within the cytoplasmic membrane and, depending on the predictive model used, possesses from four to six putative transmembrane helices.

The *bsrB* gene is 1,758 bp long (586 amino acids) and uses an ATG start codon (GenBank accession no. FJ434140). The BsrB protein shares only 53% identity with its closest GenBank match (found in *Leuconostoc citrum*; GenBank accession no. ACA82389) yet con-

TABLE V
Chi-square Correlation of Novel ATP-binding Cassette Multidrug Resistance Genes with the Ability of *Pediococcus* Isolates to Resist the Antimicrobial Effects of Hop Compounds and Grow in Beer[a]

	Growth		Hop Resistance[b]		
Gene	**Beer 1**	**Beer 2**	**HGA+E**	**HGA**	**Broth**
bsrA	<0.0005	<0.0005	<0.0005	<0.0005	<0.034
bsrB	<0.0005	<0.0005	<0.0005	<0.0005	<0.005
*ABC2*all[c]	<0.036	<0.056	NS	<0.051	<0.001
*ABC2*nonbeer[d]	NA	NA	NS	<0.008	<0.001
ABC4	NS	NS	NS	NS	NS
ABC5	NS	NS	NS	NS	NS

[a] NS = not significant ($P > 0.1$); NA = not applicable. *ABC6* was not included because it was found only in *Lactobacillus* isolates (not correlated to growth in beer).
[b] Resistance to effects of hop compounds as described in Haakensen et al (7). HGA = hop-gradient agar plate; HGA+E = hop-gradient agar plate plus ethanol; broth = serial dilution of hop compounds in broth medium in 96-microwell plate format.
[c] All *Pediococcus* isolates were included in the analyses.
[d] Only *Pediococcus* isolates unable to grow in beer were included in the analyses.

tains motifs toward the C terminal that are typical of ABC MDR-type proteins (Fig. 1). Based on percent identity, BsrB is more similar to HorA than either protein is to BsrA. Bioinformatic analyses predicted that BsrB is located within the cytoplasmic membrane and possesses five putative transmembrane helices.

PCR targeting of *bsrA* or *bsrB* provided substantially better accuracy in differentiating between beer-spoilage pediococci and nonspoilage pediococci than did targeting *horA* (Table VI). However, *bsrA* is found in a greater number of *Pediococcus* beer-spoilage isolates, including all of the isolates containing *bsrB*. As such, statistical analysis was only performed using *bsrA*. Binary logistic regression analyses were used to determine the ability of the presence of *bsrA* to predict the growth of isolates in beer. This was calculated for *bsrA* alone and also with the presence of the known beer-spoilage-associated gene *horA* taken into account, as if the two genes were assayed for in multiplex (Table VI).

The *bsrA* and *bsrB* genes (initially called *ABC1* and *ABC3*, respectively) are the first examples of genes that we are aware of that differentiate between lactobacilli and pediococci that are able to grow in beer. Both *bsrA* and *bsrB* have been fully sequenced and contain motifs indicative of their coding for ABC MDR-type proteins (Fig. 1). The *bsrA* and *bsrB* genes were fully sequenced in all isolates that were PCR-positive by gene-specific PCR, and each gene was 100% identical at the nucleic acid level. Using a multiplex PCR directed to both *bsrA* and *horA* dramatically increases the predictive ability that can be achieved versus the use of either gene alone, resulting in 85.7% (*Lactobacillus*) to 94.0% (*Pediococcus*) accuracy in differentiating between bacteria that will and will not grow in beer (Table VI).

CONCLUSIONS

Degenerate PCR primers were effectively designed from a MSA of ABC MDR genes from *Lactobacillus* and *Pediococcus* isolates possessing a high percent identity with *horA*. These degenerate PCR primers identified six novel genes homologous to known ABC MDR-type transporters. Of the six novel genes identified, three (*ABC4, ABC5*, and *ABC6*) showed no correlation to the ability of isolates to grow in beer or to resistance to the antimicrobial effects of hop compounds. Although the presence of *ABC2* did not correlate with the ability to grow in beer, the unexpected correlation found between hop resistance and the presence of *ABC2* suggests that the ability to resist the antimicrobial effects of hop compounds alone is insufficient to permit growth in beer. Rather, we propose that resistance to multiple factors, including resistance to ethanol, is also necessary. As such, testing for specific genes is required, because there are numerous ABC MDR-type genes that may be present in any given *Lactobacillus* or *Pediococcus* isolate, regardless of their ability to grow in beer. Despite testing of 79 lactobacilli, both *bsrA* and *bsrB* were only found in *Pediococcus* isolates and, therefore, may also serve as an indicator of genus identity. Both *bsrA* and *bsrB* were strongly correlated with hop resistance in both the presence and absence of ethanol ($P < 0.0005$). Screening of pediococci for the presence of *bsrA* generated a large increase in sensitivity over the use of *horA*; however, the use of *horA* and *bsrA* together in a multiplex PCR provided the best overall predictive ability for the beer-spoilage potential of both *Lactobacillus* and *Pediococcus* contaminants.

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LITERATURE CITED

1. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410, 1990.
2. Chaban, B., Deneer, H., Dowgiert, T., Hymers, J., and Ziola, B. The flagellin gene and protein from the brewing spoilage bacteria *Pectinatus cerevisiiphilus* and *Pectinatus frisingensis*. *Can. J. Microbiol*. 51: 863-874, 2005.
3. Fujii, T., Nakashima, K., and Hayashi, N. Random amplified polymorphic DNA-PCR based cloning of markers to identify the beer-spoilage strains of *Lactobacillus brevis*, *Pediococcus damnosus*, *Lactobacillus collinoides* and *Lactobacillus coryniformis*. *J. Appl. Microbiol*. 98:1209-1220, 2005.
4. Gardy, J. L., Laird, M. R., Chen, F., Rey, S., Walsh, C. J., Ester, M., and Brinkman, F. S. L. PSORTb v.2.0: Expanded prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis. *Bioinformatics* 21:617-623, 2005.
5. Haakensen, M. C., Butt, L., Chaban, B., Deneer, H., Ziola, B., and Dowgiert, T. A *horA*-specific real-time PCR for detection of beer-spoilage lactic acid bacteria. *J. Am. Soc. Brew. Chem*. 65:157-165, 2007.

TABLE VI
Binary Logistic Regression Analyses of *bsrA* and *horA* Genes and Growth in Beer[a]

	Predictive Accuracy of *bsrA* (*horA*) (%)[b]					Predictive Accuracy of *horA* + *bsrA* Together (%)[c]				
Bacterium	R^2	**Odds Ratio**	**Will Grow**	**Will Not Grow**	**Overall**	R^2	**Odds Ratio**	**Will Grow**	**Will Not Grow**	**Overall**
Lactobacillus										
Beer 1	NA	NA	NA (88.0)	NA (87.9)	(88.0)	0.60	55.9	88.5	87.9	88.1
Beer 2	NA	NA	NA (80.0)	NA (87.9)	(85.5)	0.52	30.6	80.8	87.9	85.7
Pediococcus										
Beer 1	0.70	102.0	100 (90.0)	86.1 (77.5)	89.8 (80.0)	0.79	248.0	94.1	93.9	94.0
Beer 2	0.55	44.0	84.6 (70.0)	88.9 (80.0)	87.8 (78.0)	0.73	149.3	82.4	97.0	92.0
All bacteria										
Beer 1	0.27	39.0	100 (88.6)	71.1 (83.7)	73.9 (85.7)	0.67	88.8	90.7	90.1	90.3
Beer 2	0.17	15.3	84.6 (77.1)	73.6 (84.7)	74.6 (82.7)	0.59	45.4	81.4	91.2	88.1

[a] Nagelkerke's R^2 value indicates how much of the ability to grow in beer is accounted for by the independent variable (i.e., either *bsrA* or *bsrA* + *horA*). Odds ratio = ratio of the probability of growth with the presence of *bsrA* or *bsrA* + *horA* to the probability of growth in the absence of *bsrA* or *bsrA* + *horA* ($P < 0.0005$).

[b] The *bsrA* gene was included in analyses along with previously described putative spoilage-associated genes *hitA*, *horC*, and *ORF5* but not *horA*. Only *bsrA* was significant. Percentages for predictive ability of *horA* were reported previously (8) and are given in parentheses. NA = not applicable (*bsrA* was not found in any *Lactobacillus* isolates).

[c] The *bsrA* gene was included in analyses along with previously described putative spoilage-associated genes (*hitA*, *horA*, *horC*, and *ORF5*). *horA* and *bsrA* were statistically significant for pediococci. For lactobacilli, only *horA* was statistically significant, but for comparison, *bsrA* was added by the "enter" method of binary logistic regression.

6. Haakensen, M., Dobson, C. M., Deneer, H., and Ziola, B. Real-time PCR detection of bacteria belonging to the *Firmicutes* phylum. *Int. J. Food Microbiol.* 125:236-241, 2008.
7. Haakensen, M., Schubert, A., and Ziola, B. Broth and agar hop-gradient plates used to evaluate the beer-spoilage potential of *Lactobacillus* and *Pediococcus* isolates. *Int. J. Food Microbiol.* 130:56-60, 2009.
8. Haakensen, M., Schubert, A., and Ziola, B. Multiplex PCR for putative *Lactobacillus* and *Pediococcus* beer-spoilage genes and ability of gene presence to predict growth in beer. *J. Am. Soc. Brew. Chem*. 66: 63-70, 2008.
9. Hayashi, N., Ito, M., Horiike, S., and Taguchi, H. Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus brevis* strains capable of growing in beer. *Appl. Microbiol. Biotechnol*. 55:596-603, 2001.
10. Hofmann, K., and Stoffel, W. TMbase—A database of membrane spanning protein segments. *Biol. Chem. Hoppe-Seyler* 47:166, 1993.
11. Marchler-Bauer, A., Anderson, J. B., Derbyshire, M. K., DeWeese-Scott, C., Gonzales, N. R., Gwadz, M., Hao, L., He, S., Hurwitz, D. I., Jackson, J. D., Ke, Z., Krylov, D., Lanczycki, C. J., Liebert, C. A., Liu, C., Lu, F., Lu, S., Marchler, G. H., Mullokandov, M., Song, J. S., Thanki, N., Yamashita, R. A., Yin, J. J., Zhang, D., and Bryant, S. H. CDD: A conserved domain database for interactive domain family analysis. *Nucleic Acids Res*. 35:D237-D240, 2007.
12. Nicholas, K. B., Nicholas, H. B. J., and Deerfield, D. W. I. GeneDoc: Analysis and visualization of genetic variation. EMBNEW News 4:14, 1997.
13. Priest, F. G., and Campbell, I. In: *Brewing Microbiology*, 3rd ed. Kluwer, New York, 2002.
14. Rice, P., Longden, I., and Bleasby, A. EMBOSS: The European molecular biology open software suite. *Trends Genet*. 16:276-277, 2000.
15. Sakamoto, K., and Konings, W. N. Beer spoilage bacteria and hop resistance. *Int. J. Food Microbiol*. 89:105-124, 2003.
16. Simpson, W. J. Ionophoric action of *trans*-isohumulone of *Lactobacillus brevis. J. Gen. Microbiol.* 139:1041-1045, 1993.
17. Thomson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876-4882, 1997.

7. BROTH AND AGAR HOP-GRADIENT PLATES USED TO EVALUATE THE BEER-SPOILAGE POTENTIAL OF *LACTOBACILLUS* AND *PEDIOCOCCUS* ISOLATES

Author contributions:

Monique Haakensen conceived the study, designed and standardized the experiments, performed confirmatory replicates of some experiments, and drafted the manuscript.

Alison Schubert performed the experiments.

Barry Ziola conceived the study, edited the manuscript, and is the holder of the research grant used to fund the study.

BRIEF INTRODUCTION TO CHAPTER 7

While the PCR-based detection methods described in Chapters 2, 3, 5, and 6 are very rapid and efficient, their correlations with ability of bacteria to spoil beer are not absolute. Moreover, the finding of bacteria of non-brewery origin which harbour the *horA* gene (Chapters 3 and 4) highlighted the need for a rapid method to detect beer-spoilage bacteria that is independent of the bacteria's genetic background. As hop-compounds are believed to be the most limiting factor in the ability of bacteria to grow in beer, the goal here was to develop a method for direct measurement of hop-resistance. Several other possible growth-inhibiting factors were also investigated, including phase of media (broth *vs* agar), concentration of media, and the presence of ethanol.

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Broth and agar hop-gradient plates used to evaluate the beer-spoilage potential of *Lactobacillus* and *Pediococcus* isolates

M. Haakensen, A. Schubert, B. Ziola *

Department of Pathology and Laboratory Medicine, Room 2841 Royal University Hospital, 103 Hospital Drive, University of Saskatchewan, Saskatoon, SK Canada S7N 0W8

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ABSTRACT

Identification of the beer-spoilage *Lactobacillus* and *Pediococcus* bacteria has largely taken two approaches; identification of spoilage-associated genes or identification of specific species of bacteria regardless of ability to grow in beer. The problem with these two approaches is that they are either overly inclusive (i.e., detect all bacteria of a given species regardless of spoilage potential) or overly selective (i.e., rely upon individual, putative spoilage-associated genes). Our goal was to design a method to assess the ability of *Lactobacillus* and *Pediococcus* to spoil beer that is independent of speciation or genetic background. In searching for a method by which to differentiate between beer-spoilage bacteria and bacteria that cannot grow in beer, we explored the ability of lactobacilli and pediococci isolates to grow in the presence of varying concentrations of hop-compounds and ethanol in broth medium versus on agar medium. The best method for differentiating between bacteria that can grow in beer and bacteria that do not pose a threat as beer-spoilage organisms was found to be a hop-gradient agar plate containing ethanol. This hop-gradient agar plate technique provides a rapid and simple solution to the dilemma of assessing the ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer, and provides new insights into the different strategies used by these bacteria to survive under the stringent conditions of beer.

1. Introduction

Spoilage of beer by bacteria of the genera *Lactobacillus* and *Pediococcus* is a significant problem for the brewing industry, since these bacteria account for the majority of spoilage incidents (Sakamoto and Konings, 2003; Simpson and Fernandez, 1994). While not all *Lactobacillus* and *Pediococcus* isolates can grow in beer, some isolates have developed mechanisms that confer resistance to hop-compounds (Simpson and Fernandez, 1994), thus apparently facilitating growth in beer. Specifically, these bacteria are able to resist the action of bitter acid compounds (e.g., *trans*-isohumulone) derived from hop-extract iso-alpha-acids which act as mobile carrier protonophores, effectively dissipating the cell's trans-membrane pH gradient (Simpson, 1993).

Bacterial ability to grow in beer does not abide by speciation boundaries; therefore, assaying for spoilage-specific genetic markers is currently the most accurate method for detecting *Lactobacillus* and *Pediococcus* beer-spoilage bacteria (Haakensen et al., 2007, 2008; Haakensen and Zoila, 2008). The known beer spoilage-specific genetic markers for these bacteria are *hitA* (Hayashi et al. 2001), *horA* (Haakensen et al., 2007, 2008; Sami et al., 1997), and *horC* (Fujii et al. 2005; Suzuki et al. 2004), with *hitA* and *horC* recently being shown to be less well associated with ability to spoil beer (Haakensen et al., 2008). Another beer-spoilage related gene, *bsrA*, was recently found to be a marker for predicting beer-spoilage ability of *Pediococcus* isolates (Haakensen and Zoila, 2008). *bsrA* and *bsrB* are both primary-type ATP-binding cassette multi-drug resistance genes, while the *hitA* gene codes for a secondary-type divalent transporter. The *horC* gene codes for a protein of unknown function, with little percent identity to any other protein. Correlation of these genes with ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer is still not absolute; therefore, other, as of yet unknown, genetic mechanism(s) must exist which enable bacterial growth in beer. As well, it must be kept in mind that, due to the extensive horizontal gene transfer exhibited by lactic acid bacteria (Makarova et al. 2006), there also remains the possibility of additional genes emerging which would allow a *Lactobacillus* or *Pediococcus* isolate to grow in beer. Assessing beer-spoilage ability of *Lactobacillus* and *Pediococcus* isolates independent of bacterial genetic makeup is thus essential. While this can obviously be done by testing for direct growth in beer, results are only obtained after several days to several weeks.

To resolve this dilemma, we explored approaches for more rapid assessment of the ability of lactobacilli and pediococci to grow in beer. Concentration-gradient agar plates were originally developed to study antibacterial resistance (Bryson and Szybalski, 1952; Hunt and Sandham, 1969). We customized the technique to deal with aspects specific to assessing ability of bacteria to grow in beer. Bacterial growth in broth medium at different concentrations of modified MRS medium (mMRS; Simpson and Smith, 1992) and hop-compounds was

* Corresponding author. Dept. of Pathology and Laboratory Medicine, College of Medicine, 2841 Royal University Hospital, 103 Hospital Drive, Saskatoon SK Canada S7N 0W8. Tel.: +1 306 966 4330; fax: +1 306 966 8049.
E-mail address: b.ziola@usask.ca (B. Ziola).

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Table 1
Bacterial species used and ability to grow in beer in relation to hops-resistance assessed by broth, HGA, and HGA+E testing

Species	Number of isolates		Proportion of isolates correctly identified by		
	Tested	That grow in beer	Broth	HGA	HGA+E
L. acetotolerans	1	0	0/1	1/1	1/1
L. acidophilus	3	0	0/3	2/3	2/3
L. amylovorous	6	1	4/6	6/6	6/6
L. brevis[a]	13	8	10/12	12/13	13/13
L. casei[a]	11	8	8/10	9/11	11/11
L. delbrueckii	8	0	3/8	6/8	7/8
L. dextrinicus[b]	1	0	1/1	1/1	1/1
L. fermentum[a]	3	1	2/2	2/3	3/3
L. fructivorans	1	0	0/1	1/1	1/1
L. helveticus	2	1	1/2	1/2	2/2
L. hilgardii	3	1	1/3	3/3	3/3
L. homohiochii	1	0	1/1	1/1	1/1
L. jensenii	1	0	1/1	1/1	1/1
L. kefiri	1	0	1/1	1/1	1/1
L. kefirgranum	1	0	1/1	1/1	1/1
L. kefironofaciens	1	0	1/1	1/1	1/1
L. parabuchneri	1	0	0/1	0/1	0/1
L. paracollinoides	1	1	0/1	0/1	1/1
L. plantarum	9	4	5/9	4/9	7/9
L. reuteri	5	1	3/5	4/5	5/5
L. rhamnosus	5	1	1/5	3/5	5/5
L. sakei	2	1	0/2	2/2	2/2
L. zeae	1	0	1/1	1/1	1/1
Possible new *Lactobacillus* species	4	2	3/4	3/4	4/4
P. acidilactici[a]	7	1	3/6	6/7	7/7
P. claussenii	13	12	10/13	13/13	13/13
P. damnosus	8	4[c]	4/8	7/8	7/8
P. inopinatus	1	0	1/1	1/1	1/1
P. parvulus	8	1	6/8	8/8	8/8
P. pentosaceus[a]	6	0	3/5	4/6	5/6

[a] One isolate would not grow in the broth 96 microwell plates.
[b] Formerly *P. dextrinicus* (Haakensen et al., in press).
[c] One isolate could only grow in Beer 1 and growth took 27 days.

compared to growth on mMRS agar containing a gradient of hop-compounds. The effect of ethanol as an additional selective pressure was also assessed with both approaches. Through this study, we have not only defined an efficient method for differentiating *Lactobacillus* and *Pediococcus* isolates that can and cannot grow in beer, but have also gained further insights into the physiological basis of ability of these bacteria to grow in beer.

2. Materials and methods

2.1. Bacterial growth in beer

A list of the bacterial species tested is provided in Table 1, with the isolates comprising 85 lactobacilli (23 species and 4 possibly new species) and 43 pediococci (6 species). Included were 64 isolates available from the American Type Culture Collection (see the Supplementary Supplementary material). Parameters for induction of bacteria to grow in beer were as described by Haakensen et al. (2007). In brief, assessment of bacterial isolate growth in beer required adaptation of the bacteria using modified mMRS broth supplemented with incremental concentrations of beer. Identities of the isolates were confirmed pre- and post-growth in beer by sequencing the 16S rRNA gene (Haakensen et al., 2007). Beer 1 was a filter-sterilized 4% v/v alcohol beer, pH 4.2 and averaging 9.8 bitterness units (BU), while Beer 2 was a pasteurized 5% v/v alcohol beer, pH 3.8 and averaging 11 BU. Bacteria capable of growing in either beer were considered to be beer-spoilers. Prior to testing for hop-resistance as described in Sections 2.2 and 2.3, bacteria were initially grown in 50% 2× mMRS broth and 50% Beer 2 as described by Haakensen et al. (2007). Bacteria were then grown at 30 °C for 16–24 h in 15% 2× mMRS broth and 85% Beer 2.

2.2. Hop-compounds in broth microwell plates

Ninety-six well round-bottomed microwell plates (Corning Incorporated Life Sciences, Lowell MA, USA) were used. mMRS broth was tested at 1/8×, 1/4×, 1/2×, 1×, and 2× strength. Each well was inoculated with 50 µl of bacteria diluted to a concentration of 4000–6000 colony forming units (CFU)/ml (chosen so that no visible pellet was produced in the wells due to the inoculate itself). The number of CFU was confirmed by colony counts in parallel on two MRS agar plates. Isohop® isomerized hop extract (28–32% w/w iso-alpha-acids in an aqueous solution of potassium salts; John I. Haas Inc., Washington, DC) was added to the wells in 50 µl of the same strength mMRS broth. Based upon the calculation provided on the Isohop® website (http://www.barthhaasgroup.com) for converting ml/hl of Isohop® to BU, the range of BU originally tested was from 0.6 BU to 240 BU. Later, this range was narrowed to 3–21 BU. This narrowed range of BU was then tested with and without addition of ethanol to 3, 5, and 7% v/v. The plates were sealed with parafilm and incubated for 48 h in a candle jar at 30 °C.

2.3. Hop-gradient agar plates

A gradient of hop-compounds was created by pouring 30 ml of mMRS broth containing 1.5% agar and hop extract into a square petri dish with grid (10×10×1.5 cm; FisherSci, Ottawa ON) inclined 10.5°. Once the agar had solidified, the plate was set onto a flat surface and an additional 30 ml of mMRS broth containing 1.5% agar and no hop extract was added. A schematic diagram of an agar hop-gradient plate is provided in Fig. 1. When ethanol was included, ethanol was added to the same concentration in both the bottom and top agar layers. Control plates were made by the same two-step procedure, minus addition of hop extract and ethanol. Plates were stored upside down in a sealed container at 4 °C until used (plates stored up to 10 days gave comparable results). As with the broth cultures in microwell plates, a range of BU and ethanol were tested.

An equal volume mixture of induced bacterial culture and agar medium was poured onto a sterile microscope slide. Before the agar

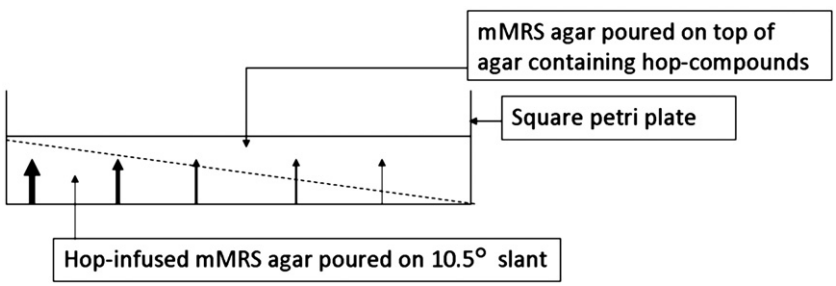

Fig. 1. Schematic diagram of a hop-gradient agar plate. Thickness of vertical arrows is representative of the amount of hop-compounds that diffuse through the top layer of agar to the surface of the agar where bacteria are grown.

Table 2
Comparison of broth, HGA, and HGA+E testing methods for ability to predict growth of *Lactobacillus* and *Pediococcus* isolates to grow in beer

Organism		Broth	HGA	HGA+E
Lactobacillus	Will grow	22/28 (78.6%)	22/30 (73.3%)	30/30 (100%)
	Will not grow	26/54 (48.5%)	43/55 (78.2%)	50/55 (90.9%)
	Overall	48/82 (58.5%)[c]	66/85 (77.6%)[b]	80/85 (94.1%)[a]
Pediococcus	Will grow	15/18 (83.3%)	17/18 (94.4%)	17/18 (94.4%)
	Will not grow	12/23 (52.2%)	22/25 (88.0%)	24/25 (96.0%)
	Overall	27/42 (64.3%)[c]	39/43 (90.7%)[a]	41/43 (95.3%)[a]
Lactobacillus and *Pediococcus*	Will grow	37/46 (80.4%)	39/48 (81.3%)	47/48 (98.0%)
	Will not grow	38/77 (49.4%)	65/80 (81.3%)	74/80 (92.5%)
	Overall	75/124 (60.5%)[a]	105/128 (82.0%)[a]	122/128 (95.3%)[a]

[a] All Chi-square and independent samples *t*-test *P*-values are <0.0005.
[b] Chi-square $P<0.0005$ and independent samples *t*-test $0.005<P<0.0005$.
[c] Chi-square $0.05<P<0.005$ and independent samples *t*-test $0.05<P<0.005$.

set, the long thin edge of a second microscope slide was dipped into the mixture and pressed gently onto a gradient plate traversing the length of the hop-gradient, thus creating a solid line of consistent bacterial inoculate. Multiple plates can be successively stamped using a given mixture and slide if so desired (e.g., a hop-gradient agar [HGA] plate, a HGA plus ethanol [HGA+E] plate, and a control agar plate). Plates containing ethanol were sealed with metal tape to prevent ethanol evaporation and all plates were inverted during incubation in a candle jar for 36 h at 30 °C.

2.4. Statistical analysis

Statistical analyses were performed using SPSS for Windows (Version 15.0, SPSS Inc., Munich, Germany). For Chi-square analysis, *t*-test for independent samples, and *t*-test for paired samples, the confidence interval and level of significance were 95% and $P=0.05$, respectively. Statistical analyses were conducted for *Lactobacillus*, *Pediococcus*, and *Lactobacillus* and *Pediococcus* isolates combined, with growth in beer as the dependant variable.

3. Results

3.1. Broth microwell plates

Bacterial growth in broth microwell plates was reproducibly indicated by the presence of a cell pellet. Though many beer spoilage bacteria grew at concentrations of hop-compounds >21 BU, it was found that the greatest resolution in growth differences between beer-spoilage bacteria and non-beer spoilage bacteria could be obtained by using a concentration range of approximately 3 BU–21 BU, in increments of 3 BU. mMRS broth was used at various concentrations up to 2× to determine whether nutrient availability affected growth of the bacteria. Bacterial growth in 1/4× mMRS broth gave the best association with ability to grow in beer (most significant *P* value); consequently, this strength of mMRS broth was used along with a range of hop-compound concentrations, tested both with and without 5% v/v ethanol added. When growth in 1/4× mMRS broth was compared to growth in full strength mMRS broth, a significant difference was observed (paired samples *t*-test, $P<0.0005$), indicating that reduction of nutrients in the assay affects the ability of isolates to grow in the presence of hop-compounds. With full strength mMRS broth, many non-beer-spoilage bacteria were able to grow in the presence of high BU. On the other hand, reducing the amount of nutrients to 1/8× mMRS broth resulted in many bacteria being unable to grow within 48 h.

When bacteria were grown in 1/4× mMRS broth with and without 5% v/v ethanol, a significant difference in growth was observed (paired samples *t*-test, $P<0.0005$), allowing for more accurate identification of isolates capable of growing in beer. With ethanol present, predictability for growth in beer was significantly better, with an independent samples *t*-test giving values ranging from $P<0.029$ to $P<0.008$, depending on the genus and type of beer used. However, the actual predictive ability of this assay was only 58.5% for *Lactobacillus* and 54.3% for *Pediococcus* (Table 2). A cross-tabulation report (Table 3a) showed that a set cut-off point for accurate differentiation of beer-spoilage bacteria from non-spoilage bacteria was not readily identifiable. Moreover, there were five isolates that could not grow in 1/4× mMRS broth +5% v/v ethanol (Tables 1 and 3a).

3.2. Hop-gradient agar plates

Bacterial growth on HGA plates could be reproducibly measured after a 36 h incubation. After testing a range of concentrations of hop-compounds in the agar base layer, it was determined that, for the lot of Isohop® extract used, 9 BU was optimal for differentiating between beer-spoilers and non-beer spoilers (independent samples *t*-test, $P<0.003$–$P<0.0005$, depending on genus). A cross-tabulation report of ability to grow in beer versus distance of growth along the hop-gradient was used to identify a cut-off point for differentiation of

Table 3
Minimum inhibitory concentration and distance of growth for *Lactobacillus* and *Pediococcus* isolates when assessed by broth, HGA, and HGA+E testing[a]

Broth										
Growth in beer	Minimum BU inhibitory concentration									Total
	0[b]	3	6	9	12	15	18	21	No growth[c]	
–	1	9	13	15	11	7	10	11	3	80
+	0	1	5	3	11	8	6	12	2	48

HGA													
Growth in beer	Distance of growth on plate (cm)												Total
	0.50	1.00	1.50	2.00	2.50	3.00	3.50	4.00	4.50	5.00	5.50	6.00	
–	7	26	5	21	5	4	0	2	0	3	0	7	80
+	1	2	0	5	0	1	1	4	0	0	1	33	48

HGA+E													
Growth in beer	Distance of growth on plate (cm)												Total
	0.50	1.00	1.50	2.00	2.50	3.00	3.50	4.00	4.50	5.00	5.50	6.00	
–	12	33	7	20	2	2	0	1	0	2	0	1	80
+	1	0	0	0	0	4	1	4	0	11	0	27	48

[a] Numbers given under each concentration or distance of growth on a plate indicate the number of isolates with that respective cut-off. The vertical dashed line indicates the cut-off point used to predict beer-spoilage ability of isolates.
[b] Control well containing only 1/4× mMRS broth and 5% ethanol, but no hop-compounds.
[c] Isolates did not grow in any wells containing hop-compounds and also did not grow in the control wells.

isolates capable of growing in beer. It was difficult to determine an appropriate cut-off point, however, due to the surprising number of beer-spoilage isolates that could not grow far along the hop-gradient (Fig. 2, Table 3b). A cut-off point of growth halfway along the hop-gradient (distance ≥ 3 cm) yielded a Chi-square correlation of $P<0.0005$ with ability to grow in beer for both *Lactobacillus* and *Pediococcus* isolates (Table 2). Results obtained with HGA plates were significantly better than results obtained with broth microwell plates (paired samples *t*-test; $P<0.0005$). Predictive abilities of this assay for bacterial growth in beer were 77.6% for *Lactobacillus* and 90.7% for *Pediococcus* isolates (Table 2).

3.3. Hop-gradient agar plates containing ethanol

In preliminary tests using agar plates without hop compounds present, it was found that ethanol concentrations above 5% v/v increasingly inhibited growth. Using HGA plates containing 9 BU in the bottom layer and incorporating ethanol to a final concentration of 5% v/v in both agar layers (HGA+E plates) allowed reproducible bacterial growth after 36 h. Beer-spoilers and non-beer spoilers grew significantly different distances along the HGA+E plates (independent samples *t*-test, $P<0.0005$). A cross-tabulation report of ability to grow in beer versus distance of growth along the hop-gradient allowed for easy identification of a cut-off point which differentiated isolates capable of growing in beer (e.g., ≥3 cm; Table 3c). Many of the beer-spoilage bacteria which could not grow 3 cm along the BU gradient in HGA plates could grow ≥3 cm on the HGA+E plates. In contrast, non-beer spoilers grew either the same or shorter distance than on HGA plates. Using a cut-off of ≥3 cm growth gave a Chi-square correlation of $P<0.0005$ with ability to grow in beer (Table 2). Addition of 5% v/v ethanol to the HGA plates increased the predictive abilities to 94.1% for *Lactobacillus* and 95.3% for *Pediococcus* (Table 2). This is significantly better at predicting ability to grow in beer than results obtained with HGA plates that did not contain ethanol (paired samples *t*-test, $P<0.0025$).

4. Discussion

Our goal was to design a method that is independent of speciation or genetic background that can differentiate between isolates of *Lactobacillus* and *Pediococcus* that can and cannot grow in beer. While developing this assay, we explored the differences between mMRS broth and agar medium, and the effect of adding various concentrations of hop-compounds and ethanol to both types of medium. The ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer was found to be more complex than has been previously appreciated. Ability to resist the antimicrobial effects of hop-compounds appears to be multi-factorial and may vary even with a single isolate, depending upon other selective pressures that are present. Therefore, when evaluating ability of an isolate to grow in beer, determining hop-resistance alone is insufficient; rather, hop-resistance must be assessed in the context of selective pressures such as nutrient availability and presence of ethanol.

When searching for the optimal combination of medium and hop-compounds, we found that adding ethanol to both broth and agar medium provided greater accuracy for differentiation of beer-spoilage and non-beer-spoilage bacteria (broth, data not shown; agar, Tables 1 and 2). Moreover, we found that the state of medium used (i.e., broth vs. agar) produced very different results with respect to ability of isolates to grow in the presence of hop-compounds (Tables 1 and 2). While the reason for this is not fully known, it is possible that bacteria have ready access to nutrients when grown in broth medium because broth medium does not mimic a beer environment with much lower nutrient levels as well as agar medium does (i.e., nutrients in agar medium diffuse slowly and become increasingly depleted in the area of colony growth). Although hop-resistance in mMRS broth did correlate with ability to grow in beer (Table 2), the percentage of isolates correctly identified as being capable of growing (or not growing) in beer was low (Tables 1 and 3a). This reflects that fact that many bacteria not able to grow in beer had a hop-resistance profile in mMRS broth similar to bacteria able to grow in beer (Table 3a). The differences in hop-resistance seen between the two medium states may be explained by nutrients being more readily available to bacteria growing in liquid than when growing on a solid medium.

Despite poor predictive abilities (Tables 1 and 3a), a strong association between hop-resistance and ability to grow in beer was evident when using broth medium (Table 2). This may explain why full-strength broth has been used for testing of hop-resistance and characterization of beer-spoilage "hop-resistance" genes (Behr et al., 2006; Fujii et al., 2005; Hayashi et al., 2001; Iijima et al., 2006; Sakamoto and Konings, 2003; Sami et al., 1997; Suzuki et al., 2005). However, we found that hop-resistance in 1/4× mMRS broth was only 58.5–64.3% accurate in differentiating between beer-spoilers and non-spoilers (Table 2). This together with finding that the discrimination accuracy was less if ethanol was not added or mMRS broth at greater than 1/4× strength was used (data not shown) strongly suggests that

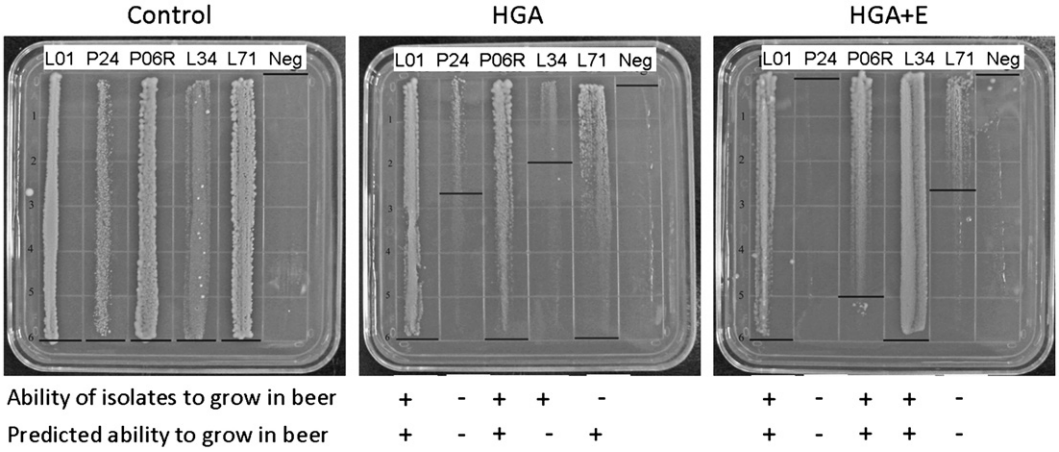

Fig. 2. Comparison of results obtained from hop-gradient agar (HGA), hop-gradient agar plus ethanol (HGA+E), and control plates. The lowest concentration of the hop-gradient is at the top of the picture with concentration increasing towards the bottom of the picture. Horizontal bars indicate the recorded distance of growth along the plate. Isolates used are: L01 — *Lactobacillus brevis* CCCB1202; P24 — *Pediococcus pentosaceus* ATCC 33314^T; P06R — *Pediococcus claussenii* (ropy strain) ATCC BAA-344^T; L34 — *Lactobacillus helveticus* CCCB1186; L71 — *Lactobacillus plantarum* ATCC 14917^T; Neg — negative control containing only agar and no bacterial inoculum.

broth medium is not a good environment for testing of bacterial hop-resistance.

HGA plates showed a stronger correlation with ability of isolates to grow in beer than did broth medium (Tables 1 and 2). Several concentrations of hop-extract were tested in order to optimize the assay; however, despite testing numerous concentrations, there were several isolates of *Lactobacillus* and *Pediococcus* that would not fit into the predictive model. Consequently, ethanol was incorporated as an additional selective pressure when using HGA plates. The presence of ethanol in addition to hop-compounds (i.e., HGA+E plates) provided a much higher accuracy in differentiating between those bacteria that could grow in beer and those which could not (Fig. 2 and Table 2).

Nine non-beer-spoilage isolates (seven lactobacilli and two pediococci) were capable of growing at high BU levels on HGA plates, but only at low BU levels on HGA+E plates The presence of ethanol in the HGA+E plates provides an additional selective pressure, thus making it more difficult for some isolates to grow in the presence of hop-compounds. Unexpectedly, eight beer-spoilage lactobacilli that were hop-sensitive (i.e., growth of <3 cm) on HGA plates were highly resistant to hop-compounds on HGA+E plates (i.e., growth of 5–6 cm). This finding appears to be novel, as we are not aware of any previous link being made between presence of ethanol and bacterial hop-resistance. Although the mechanism of action requires further investigation, it is possible that the reaction of some *Lactobacillus* isolates to the presence of ethanol concurrently helps protect the bacteria against the antimicrobial effects of hop-compounds.

The HGA+E plates described here are very effective at differentiating between lactobacilli and pediococci that can grow in beer and those that cannot. Of non-beer spoiling bacteria, only five lactobacilli and one pediococci (i.e., 9.1% and 4.0%, respectively, of bacteria tested) were able to grow ≥3 cm along the hop-gradient of HGA+E plates. This suggests that there must be selective pressures in addition to hop-compounds and ethanol that are responsible for inhibiting the growth of these organisms in beer. Alternatively, the low nutrient level of beer may cause greater stress in these bacteria, making them more susceptible to the combined antimicrobial effects of hop-compounds and ethanol. More importantly, 100% of lactobacilli that could grow in beer were correctly identified by using the HGA+E plates and, in the case of pediococci, only one isolate that could grow in beer was missed (however, this isolate could only grow in low alcohol, low BU beer and took 27 days to grow). With an overall accuracy of >95% in differentiating between beer-spoilage and non-beer spoilage isolates, HGA+E plates are a reliable method for determining the beer-spoilage potential of contaminating bacteria in a manner which is independent of both genetic background and speciation. Novel *Lactobacillus* and *Pediococcus* beer-spoilage strains and/or species can be detected by using HGA+E plates.

The HGA+E plate assay can easily be performed using other preparations or lots of hop-compounds by setting up a series of plates with a range of concentrations of hop-compounds in the bottom agar layer. By using bacterial standards selected from the list provided as supplementary material (specifically, isolates available through Type Culture Collections), the concentration of any hop-extract which produces results comparable to those recorded in the supplementary material can be determined. These bacterial standards should then be included alongside undefined brewery isolates in subsequent screening to ensure inter-test reproducibility of results. Although Fig. 2 only six isolates tested per plate, we regularly stamped 12 isolates per plate with good separation between lines of bacterial growth.

By evaluating the ability of broth medium, HGA plates, and HGA+E plates to predict the ability of lactobacilli and pediococci to grow in beer, we have gained new insights into the basis for ability of these bacteria to grow in and spoil beer. BU, ethanol, and nutrient levels all apparently play a role. The combined selective pressures present in the HGA+E plates correctly predicted the ability of isolates to grow in beer more with >95% accuracy in 36 h. Not only are the HGA+E plates highly accurate, the results obtained are independent of bacterial genetic background and speciation, thereby allowing for detection of potentially novel lactobacilli and pediococci beer spoilage isolates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijfoodmicro.2009.01.001.

References

Behr, J., Ganzle, M.G., Vogel, R.F., 2006. Characterization of a highly hop-resistant *Lactobacillus brevis* strain lacking hop transport. Applied and Environmental Microbiology 72, 6483–6492.

Bryson, V., Szybalksi, W., 1952. Microbial selection. Science 116, 43–51.

Fujii, T., Nakashima, K., Hayashi, N., 2005. Random amplified polymorphic DNA-PCR based cloning of markers to identify the beer-spoilage strains of *Lactobacillus brevis*, *Pediococcus damnosus*, *Lactobacillus collinoides* and *Lactobacillus coryniformis*. Journal of Applied Microbiology 98, 1209–1220.

Haakensen, M., Ziola, B., 2008. *bsrA*, a genetic marker for beer-spoilage ability of *Pediococcus* isolates. Presented as Poster #67 at the World Brewing Congress, Honolulu, Hawaii. August 2–6, 2008.

Haakensen, M.C., Butt, L., Chaban, B., Deneer, H., Dowgiert, T., Ziola, B., 2007. A *horA*-specific real-time PCR for detection of beer-spoilage lactic acid bacteria. Journal of the American Society of Brewing Chemists 65, 157–165.

Haakensen, M., Schubert, A., Ziola, B., 2008. Multiplex PCR for putative *Lactobacillus* and *Pediococcus* beer-spoilage genes and ability of gene presence to predict growth in beer. Journal of the American Society of Brewing Chemists 66, 63–70.

Haakensen, M., Dobson, C.M., Hill, J.E., and Ziola, B. in press. Reclassification of *Pediococcus dextrinicus* (Coster and White 1964) Back 1978 (Approved Lists 1980) as *Lactobacillus dextrinicus* comb. nov., and emended description of the genus *Lactobacillus*. International Journal of Systematic and Evolutionary Microbiology. Accepted July 1, 2008.

Hayashi, N., Ito, M., Horiike, S., Taguchi, H., 2001. Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus brevis* strains capable of growing in beer. Applied Microbiology and Biotechnology 55, 596–603.

Hunt, D.E., Sandham, H.J., 1969. Improved agar gradient plate technique. Applied Microbiology 17, 329–330.

Iijima, K., Suzuki, K., Ozaki, K., Yamashita, H., 2006. *horC* confers beer-spoilage ability on hop-sensitive *Lactobacillus brevis* ABBC45^{cc}. Journal of Applied Microbiology 100, 1282–1288.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D.M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.-., Diaz-Muniz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'sullivan, D., Steele, J., Unlu, G., Saeier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B., Mills, D., 2006. Comparative genomics of the lactic acid bacteria. Proceedings of the National Academy of Science United States of America 103, 15,611–15,616.

Sakamoto, K., Konings, W.N., 2003. Beer spoilage bacteria and hop resistance. International Journal of Food Microbiology 89, 105–124.

Sami, M., Yamashita, H., Kadokura, H., Kitamoto, K., Yoda, K., Yamasaki, M., 1997. A new and rapid method for determination of beer-spoilage ability of lactobacilli. Journal of the American Society of Brewing Chemists 55, 137–140.

Simpson, W.J., 1993. Ionophoric action of trans-isohumulone of *Lactobacillus brevis*. Journal of General Microbiology 139, 1041–1045.

Simpson, W.J., Smith, A.R.W., 1992. Factors affecting antibacterial activity of hop compounds and their derivatives. Journal of Applied Bacteriology 72, 327–334.

Simpson, W.J., Fernandez, J.L., 1994. Mechanism of resistance of lactic acid bacteria to trans-isohumulone. Journal of the American Society of Brewing Chemists 52, 9–11.

Suzuki, K., Ozaki, K., Yamashita, H., 2004. Comparative analysis of conserved genetic markers and adjacent DNA regions identified in beer-spoilage lactic acid bacteria. Letters in Applied Microbiology 39, 240–245.

Suzuki, K., Iijima, K., Ozaki, K., Yamashita, H., 2005. Isolation of a hop-sensitive variant of *Lactobacillus lindneri* and identification of genetic markers for beer spoilage ability of lactic acid bacteria. Applied and Environmental Microbiology 71, 5089–5097.

8. SUSCEPTIBILITY OF *PEDIOCOCCUS* ISOLATES TO ANTIMICROBIAL COMPOUNDS IN RELATION TO HOP-RESISTANCE AND BEER-SPOILAGE

Author contributions:

Monique Haakensen conceived the study, participated in the design, performed laboratory work, and drafted parts of the manuscript.

David M. Vickers performed statistical analysis and drafted parts of the manuscript.

Barry Ziola conceived the study, participated its design and coordination, edited the manuscript, and is the holder of the research grant used to fund the study.

BRIEF INTRODUCTION TO CHAPTER 8

In Chapters 3, 5, and 6, the PCR-based techniques of detecting beer-spoilage bacteria were all targeted to genes of putative multi-drug transporters. This led to the question of whether hop-resistance and/or beer-spoilage associated genes might also be associated with resistance to antimicrobial compounds other than hops. While pediococci do not commonly cause human infections, the plasmid localization of the *horA* gene is cause for concern as it may be capable of transferring to related pathogenic bacterial genera. As such, antibiotics commonly used to combat human bacterial infections were of particular interest in this study. As there is limited research regarding the antimicrobial susceptibilities of the genus *Pediococcus*, this study focused on isolates which were nearly equally distributed as to ability to grow in beer and to resist hop compounds. The study included 29 isolates from 6 species of *Pediococcus*, making it the most comprehensive antimicrobial resistance study of the genus to date. Antimicrobial susceptibility profiles are compared to the ability of isolates to grow in beer (Chapter 3), resist hop-compounds (Chapter 7), and presence of hop-resistance and beer-spoilage associated genes (Chapters 3, 5, and 6).

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Susceptibility of *Pediococcus* isolates to antimicrobial compounds in relation to hop-resistance and beer-spoilage

Monique Haakensen[1], David M. Vickers [2], Barry Ziola[1§]

[1] Department of Pathology and Laboratory Medicine, Room 2841 Royal University Hospital, 103 Hospital Drive, University of Saskatchewan, Saskatoon, SK Canada S7N 0W8

[2] Department of Computer Science, 110 Science Place, University of Saskatchewan, Saskatoon, SK, Canada S7N 5C9

[§]Corresponding author

Email addresses:

MH: m.haakensen@usask.ca

DMV: david.vickers@usask.ca

BZ: b.ziola@usask.ca

Abstract

Background

Though important in the context of food microbiology and as potential pathogens in immuno-compromised humans, bacterial isolates belonging to the genus *Pediococcus* are best known for their association with contamination of ethanol fermentation processes (beer, wine, or fuel ethanol). Use of antimicrobial compounds (e.g., hop-compounds, Penicillin) by some industries to combat *Pediococcus* contaminants is long-standing, yet knowledge about the resistance of pediococci to antimicrobial agents is minimal. Here we examined *Pediococcus* isolates to determine whether antibiotic resistance is associated with resistance to hops, presence of genes known to correlate with beer spoilage, or with ability to grow in beer.

Results

Lactic acid bacteria susceptibility test broth medium (LSM) used in combination with commercially available GPN3F antimicrobial susceptibility plates was an effective method for assessing antimicrobial susceptibility of *Pediococcus* isolates. We report the finding of Vancomycin-susceptible *Pediococcus* isolates from four species. Interestingly, we found that hop-resistant, beer-spoilage, and beer-spoilage gene-harbouring isolates had a tendency to be more susceptible, rather than more resistant, to antimicrobial compounds.

Conclusions

Our findings indicate that the mechanisms involved in conferring hop-resistance or ability to spoil beer by *Pediococcus* isolates are not associated with resistance to antibiotics commonly used for treatment of human infections. Also, Vancomycin-resistance was found to be isolate-specific and not intrinsic to the genus as previously believed.

Background

Isolates from the genus *Pediococcus* are particularly problematic for the brewing industry where hop-compounds are used to provide flavour to beer. Hop-compounds are antimicrobial in that they dissipate the trans-membrane pH gradient of microbes, thereby inhibiting growth and potential spoilage of product [1]. As pediococci are also used as beneficial microbes in the context of food microbiology and animal husbandry (e.g., wine, cheese, and yogurt industries as well as for the production of silage), the emergence of hop-resistant *Pediococcus* isolates in the brewing industry is of broader interest. These isolates frequently harbour one or more ATP-binding cassette type multidrug resistance (ABC MDR) genes, suggesting that resistance to hop-compounds may also confer resistance to other antimicrobial compounds [2]. We have previously shown that several genes can be correlated with ability of *Pediococcus* isolates to grow in beer and to resist the antimicrobial activity of hop-compounds [3-5]. These are the ABC MDR genes *ABC2*, *bsrA*, *bsrB*, [6] and *horA* [2], a putative divalent cation transporter known as *hitA* [7], and *horC* which codes for a protein possessing little homology to any known protein [8, 9].

Because, many pediococci possess special growth requirements, conventional antimicrobial-sensitivity testing media have been demonstrated to be unsuitable for testing of *Pediococcus* isolates for antimicrobial resistance [10-12]. However, enriched media that permits growth of pediococci may inhibit the antimicrobial activity of some compounds under investigation. Previously, antimicrobial susceptibility testing of *Pediococcus* isolates has been attempted by several methods, many of which are performed using some variety of agar diffusion [10, 11, 13, 14]. However, more recently, dilution methods have been preferred over diffusion tests as the former allow for determination of minimum inhibitory concentrations (MICs), which is a more reliable and reproducible indicator of resistance [10, 11]. For these

reasons, lactic acid bacteria susceptibility test broth medium (LSM), which was recently developed by Klare *et al.* [11], should be considered the new testing standard for assessing the antimicrobial resistance spectra of lactic acid bacteria. Despite this medium being shown to be very effective for establishing antimicrobial susceptibilities of two species of *Pediococcus*, namely, *P. acidilactici*, and *P. pentosaceus* [10], it previously has not been used to study the prevalence, and spectrum, of antimicrobial resistance among other members of the genus.

Overall, the use of antimicrobial compounds by industries such as animal husbandry, brewing, and fuel ethanol to combat *Pediococcus* contaminants (e.g., hop-compounds, Penicillin, and Virginiamycin which is structurally similar to Synercid) is long-standing. However, knowledge about the resistance of pediococci to antimicrobial agents is minimal [12]. As such, the focus of this research was to determine whether the use of antimicrobial hop-compounds in the brewing industry is associated with an increase in the overall antimicrobial resistance of *Pediococcus* isolates. Here we report on the testing of isolates from six species of the genus *Pediococcus* against 17 antimicrobial compounds using LSM broth in commercially available Sensititre GPN3F Gram-positive MIC plates (TREK Diagnostic Systems, Cleveland OH).

Results

Antimicrobial susceptibility testing

Twenty-nine isolates, including six species of the *Pediococcus* genus were tested. Distribution of isolates by species and their ability to grow in beer is given in Table 1. Antimicrobial resistance testing was reproducible and the LSM by itself (containing no antimicrobial compounds) was permissive to the rapid growth of all *Pediococcus* isolates tested. All isolates used in this study were capable of producing visible

turbidity in LSM broth after an incubation period of 24 hours. Isolates were cultured for a period of 48 hours in GPN3F plates so as to allow formation of larger bacterial pellets and thus a more accurate determination of the MIC for a given antibiotic. All control wells in the GPN3F plates produced appropriate results. Eight of the 29 isolates were randomly selected and tested in duplicate by the same method, and no variance in MICs was observed. The antimicrobial compounds and dilutions tested by the GPN3F antimicrobial susceptibility plates are listed in Additional file 1.

Distribution of MIC by species, isolate, and ropy phenotype

Resistance to the 17 antimicrobial compounds and hop-compounds was determined, and the antimicrobial compounds to which resistant isolates of *Pediococcus* were found are given in Additional file 1. For the majority of the 29 isolates tested, a moderate degree of susceptibility was shown to each of the antibiotics and a MIC value could be determined. However, for two of the antibiotics (i.e., Vancomycin and Ciprofloxacin), the majority of isolates (72% and 52% , respectively) grew in the presence of the antibiotic at all concentrations tested. Additionally, 48% of isolates were hop-resistant. When *Pediococcus claussenii* and *Pediococcus parvulus* were assessed on the basis of ropy (i.e., exopolysaccharide-producing) phenotype, there was no significant difference found among the MICs for each antibiotic [Additional files 1 and 2].

Analysis of antimicrobial resistance according to *Pediococcus* species demonstrated that just over half of the antibiotics (9/17) had significantly different MICs for different species (Table 2 and Additional files 1 and 2). The non-parametric Kruskal-Wallis *H*-test was used to test for equality in population medians. This test is an extension of the Mann-Whitney *U*-test which is designed to examine whether two

samples of observations come from the same distribution. Unfortunately, *post-hoc* analyses to determine which of the six species had significantly different MICs for each antibiotic was not possible due to the low number of isolates per species. However, when *P. claussenii* isolates were compared to isolates of the other species combined, *P. claussenii* had significantly lower MICs (Mann-Whitney *U*-test, $p < 0.05$) for all antimicrobial compounds tested, except for Erythromycin, Clindamycin, Daptomycin, and Vancomycin (data not shown).

Distribution of MIC by presence of genes associated with beer-spoilage and/or hop-resistance

Whether any of the beer-spoilage and/or hop resistance-correlated genes *ABC2*, *bsrA, bsrB, hitA, horA,* and *horC* were associated with any of the antimicrobial MICs was determined [Additional file 2]. Of these six genes, *hitA, horC,* and *ABC2*, did not occur with sufficient frequency to be analyzed statistically. The *bsrA*, *bsrB*, and *horA* genes unexpectedly demonstrated significant associations not with antibiotic resistance, but with susceptibility to antimicrobial compounds (*bsrA* and *bsrB* with Ampicillin, Levofloxacin, Penicillin, Ciprofloxacin, Gatifloxacin, and Oxacillin + 2% NaCl; *horA* with Erythromycin).

Distribution of MIC by hop-resistance phenotype

Fourteen of the 29 isolates (48.3%) were deemed resistant to hop-compounds as tested by the hop-gradient agar plate with ethanol method. When the isolates categorized according to susceptibility or resistance to hop-compounds had their MICs compared using the Mann-Whitney *U*-test, 29.4% (5/17) of the antimicrobial compounds had significantly lower MICs for the hop-resistant isolates (Table 3). Of these five antimicrobials, only Ciprofloxacin showed a significant correlation with

hop-resistance. Unexpectedly, the correlation was a negative one (Spearman's ρ = -0.47, $p < 0.01$), since as the MIC for Ciprofloxacin increased, the probability of an isolate's growth in the presence of hop-compounds decreased.

Distribution of MIC by ability to grow in beer

Of the 29 *Pediococcus* isolates tested, 13 (44.8%) were capable of growing in beer. The results of testing for an association between antibiotic susceptibility and growth in beer are given in Table 4. Based on a Mann-Whitney *U*-test, eight of the 17 antibiotics tested demonstrated a significantly *lower* MIC in those isolates that could grow in beer.

Discussion

The finding of *Pediococcus* isolates that showed only moderate resistance to Vancomycin is discordant with other studies to date which have consistently reported the genus *Pediococcus* to be intrinsically Vancomycin-resistant [10, 12-14]. The isolates that were not resistant to all concentrations of Vancomycin tested were from the species *P. acidilactici* (N=1), *P.C claussenii* (Ropy, N=1; Non-ropy, N=3), *P. damnosus* (N=1), and *P. parvulus* (Non-ropy, N=2), suggesting that the phenomenon is not the product of a clonal event. It has previously been shown that intrinsic Vancomycin resistance in *P. pentosaceus* is due to a modified peptidoglycan precursor ending in D-Ala-D-lactate [15]. While this may also be the mechanism used by other Vancomycin-resistant pediococci, it is likely that the eight susceptible isolates do not possess this mechanism. Because media previously used for *Pediococcus* antimicrobial susceptibility testing have since been shown to be inappropriate for such testing (11), it is possible that the earlier finding of intrinsic

Pediococcus Vancomycin-resistance was an artifact of the testing medium used, rather than reflective of pediococci genetic content.

The ropy phenotype did not associate with resistance to any of the antimicrobial compounds tested. This was an unexpected result as the ropy phenotype acts to create a biofilm which is expected to act as a physical barrier for the bacteria, putatively protecting them from the antimicrobial compounds. Why no associations were found is unclear. It may be that the type of exopolysaccharide matrix produced by these isolates did not result in a sufficiently dense matrix so as to inhibit the passage of antimicrobial compounds. Alternatively, the amount of energy expended on the production of exopolysaccharide may have caused a decreased ability to grow in the presence of the antimicrobial compounds, despite the partial antimicrobial barrier created by the exopolysaccharide.

Of particular interest to the brewing industry is the presence in pediococci of hop-resistance or beer-spoilage correlated genes (*ABC2*, *bsrA, bsrB, hitA, horA,* and *horC*). Of these six genes, only *horA* has been conclusively shown to function as a multidrug transporter, however, the *ABC2, bsrA,* and *bsrB* genes are highly similar to known ABC MDR genes, and the *hitA* gene is similar to divalent cation transporters. As such, all six of these beer-spoilage or hop-resistance correlated genes were assessed for associations with antimicrobial resistance. The genes *hitA, horC*, and *ABC2* did not occur with sufficient frequency to determine statistical correlation [Additional file 2]. It is important to note that, as was found for ability to grow in beer, the *bsrA, bsrB,* and *horA* genes did not demonstrate significant associations with resistance to any of the antibiotics tested, but rather with susceptibility.

When MIC was compared to ability of isolates to grow in beer, eight of the 17 antibiotics that we tested surprisingly demonstrated a significantly lower MIC in

isolates that could grow in beer. The eight antibiotics included Synercid, Ampicillin, Levofloxacin, Penicillin, Ciprofloxacin, Sulfamethoxazole/Trimethoprim, Gatifloxacin, and Oxacillin + 2% NaCl. This suggests that, despite repeated exposure to antimicrobial hop-compounds in the brewery setting, *Pediococcus* isolates capable of growing in the beer tend to be more susceptible to commonly used antimicrobial compounds than are isolates which cannot grow in beer. It is possible that this association may actually be independent of the presence of hop-compounds, instead being an indication of the environment encountered within the brewery environment by the beer-spoilage isolates. Although beer-spoilage bacteria must originate from outside the brewery, isolates capable of growing in beer have presumably become highly acclimatized or especially adapted to grow in the beer environment. Ideally, beer will not contain any wild yeasts or bacteria and, as such, contaminating pediococci are growing in an environment that does not contain a plethora of antimicrobial compounds naturally created by other organisms living in the same environment. Based on this scenario, *Pediococcus* isolates entering the brewery environment from outside sources (e.g., plant materials such as hop cones or barley) would possess mechanisms of resistance against multiple antimicrobial compounds. However, upon entering the brewery environment which should be free of other competing microbes, the pediococci would encounter no selective pressures other than hop-compounds and thus fail to maintain the genetic mechanisms for antimicrobial resistance.

It is curious to note that the *bsrA* and *bsrB* genes, hop-resistance, and beer-spoilage are all significantly negatively-associated with resistance to Ciprofloxacin. Moreover, although *horA* is strongly correlated to ability to grow in beer, this gene does not show any association (negative or otherwise) with Ciprofloxacin resistance.

While the underlying mechanism for this association with lowered resistance to Ciprofloxacin is unknown, it strongly suggests that hop-resistance, and in turn beer-spoilage, is linked to the presence of the *bsrA* and *bsrB* genes, while the *horA* gene may simply be correlated by chance to ability of *Pediococcus* isolates to spoil beer. That is to say, because the *bsrA* and *bsrB* genes (like the beer-spoilage phenotype) are negatively correlated to ciprofloxacin resistance, while the *horA* gene is not, the *bsrA* and *bsrB* genes are likely more closely associated with beer-spoilage than is the *horA* gene.

Conclusions

Testing the susceptibility of *Pediococcus* isolates to antimicrobial compounds was effective using LSM in GPN3F antimicrobial susceptibility testing plates. In contrast with previous studies, we found *Pediococcus* isolates that are not intrinsically resistant to Vancomycin. A significant negative association was identified between resistance to Ciprofloxacin and the presence of the *bsrA* and *bsrB* genes as well as the hop-resistant and beer-spoilage phenotypes. Significantly lower MICs to antimicrobial compounds were found in isolates that were hop-resistant and/or capable of growing in beer. Similarly, the presence of genes previously correlated with beer-spoilage (i.e., *bsrA*, *bsrB*, and *horA*) was also found to be associated with significantly lower MICs to several of the antimicrobial compounds tested. These results suggest that the ongoing use of the antimicrobial hop-compounds in the brewing industry and the phenomenon of hop-resistance mediated by ATP-binding cassette type multi-drug transporters is not associated with the emergence of greater antimicrobial resistance in beer-spoilage pediococci.

Methods

Bacterial growth in beer

A list of the bacterial species tested is provided in Table 1, with the isolates comprising 29 pediococci (six species) and including six ropy (exopolysaccharide producing) strains. Speciation of bacterial strains was determined (or in the case of culture collection strains, confirmed) by sequencing of the first three variable regions of the 16S rRNA gene as previously described [4]. Parameters for induction of bacteria to grow in beer were as described by Haakensen *et al.* [4]. In brief, assessment of bacterial isolate growth in beer required adaptation of the bacteria using modified mMRS broth (MRS medium with Tween 80^{TM} omitted [4]) supplemented with incremental concentrations of beer. Beer 1 was a filter-sterilized 4% v/v alcohol beer, pH 4.2 and averaging 9.8 bitterness units, while Beer 2 was a pasteurized 5% v/v alcohol beer, pH 3.8 and averaging 11 bitterness units. Bacteria capable of growing in either beer were considered to be beer-spoilers. Prior to testing for hop-resistance as described in Sections 2.2 and 2.3, bacteria were initially grown in 50% 2x mMRS and 50% Beer 2 as described by Haakensen *et al.* [4]. Bacteria were then grown at 30°C for 16-24 hours in 15% 2x mMRS and 85% Beer 2.

Ability of bacteria to resist hop-compounds

All bacterial isolates were tested for resistance to hop-compounds by the hop-gradient mMRS agar plate containing ethanol method as described by Haakensen *et al.* [5]. The ability of each isolate to grow on the hop-gradient mMRS agar plate containing ethanol is provided in Additional file 2.

Presence of beer-spoilage related genes

All bacterial isolates were tested for the presence of the putative beer-spoilage associated genes *ABC2*, *bsrA*, *bsrB*, *hitA*, *horA*, and *horC* as previously described by Haakensen *et al.* [3, 4, 6]. The presence or absence of these genes in each isolate is recorded in Additional file 2. Only *bsrA*, *bsrB*, and *horA* occurred with sufficient frequency for use in subsequent statistical analyses.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using LSM and Sensititre GPN3F Gram-positive MIC plate (TREK Diagnostic Systems, Cleveland OH). Additional file 1 provides a list of antimicrobial compounds and concentration ranges tested. The GPN3F plates contained vacuum-dried antimicrobial compounds which were rehydrated when LSM containing the bacterial inoculate was added. Bacteria were diluted to approximately 10^3-10^4 cfu/ml in LSM (confirmed by colony counting on MRS agar plates) and 100 µl were inoculated into each well of a Sensititre GPN3F plate. Bacteria were grown for 48 hours in a candle jar at 30°C. The MICs (µg/ml) were determined based on appearance of visible bacterial pellets in the bottom of wells.

Statistical analysis

Non-parametric Mann-Whitney *U* (when testing for a difference between 2 independent samples) or Kruskal-Wallis *H* (in the case of > 2 independent samples) tests were used to compare the MICs for the 17 antibiotics to determine whether antibiotic resistance had an association with resistance to hops, presence of known genes associated with hop-resistance, antibiotic-resistance, as well as with the ability of *Pediococcus* isolates to grow in beer.

For some of the analyses, the indicator (categorical) variable of resistance or susceptibility to hop-compounds was created as described by Haakensen *et al.* [5].

Specifically, if a *Pediococcus* isolate was observed to have positive growth (> 3 cm) on hop-gradient agar with ethanol plates, then that isolate was categorized as 'hop-resistant'. For this indicator variable, Fisher's exact test and Spearman's correlation coefficient ρ were used for the comparison of gene presence and antibiotic resistance, respectively, with the hop-resistance indicator variable. All tests of significance were performed at $\alpha = 0.05$ using SPSS Statistical Software for Windows (SPSS Inc., Chicago, IL, version 14.0).

Authors' contributions

MH conceived the study, participated in the design, performed laboratory work, and drafted parts of the manuscript. DMV performed statistical analysis and drafted parts of the manuscript. BZ conceived the study, participated in its design and coordination, edited the manuscript, and is the holder of the research grand used to fund the study. All authors have read and approved the final manuscript.

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References

1. Simpson WJ: **Ionophoric action of *trans*-isohumulone of *Lactobacillus brevis*.** J Gen Microbiol 1993, **139**:1041-1045.

2. Sami M, Yamashita H, Hirono T, Kadokura H, Kitamoto K, Yoda K, Yamasaki M: **Hop-resistant *Lactobacillus brevis* contains a novel plasmid harboring a multidrug resistance-like gene.** J Ferment Bioeng 1997, **84**:1-6.

3. Haakensen M, Schubert A, Ziola B: **Multiplex PCR for putative *Lactobacillus* and *Pediococcus* beer-spoilage genes and ability of gene presence to predict growth in beer.** J Am Soc Brew Chem 2008, **66**(2):63-70.

4. Haakensen MC, Butt L, Chaban B, Deneer H, Ziola B, Dowgiert T: **A *horA*-specific real-time PCR for detection of beer-spoilage lactic acid bacteria.** J Am Soc Brew Chem 2007, **65**(3):157-165.

5. Haakensen M, Shubert A, Ziola B: **Broth and agar hop-gradient plates used to evaluate the beer-spoilage potential of *Lactobacillus* and *Pediococcus* isolates.** Int J Food Microbiol 2009, **130**(1): 56-60.

6. Haakensen M, Pittet V, Morrow K, Schubert A, Ferguson J, Ziola B: **Ability of novel ATP-binding cassette multidrug resistance genes to predict growth of *Pediococcus* isolates in beer.** J Am Soc Brew Chem 2009, **67**(3):170-176.

7. Hayashi N, Ito M, Horiike S, Taguchi H: **Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus brevis* strains capable of growing in beer.** Appl Microbiol Biotechnol 2001, **55**(5):596-603.

8. Iijima K, Suzuki K, Ozaki K, Yamashita H: ***horC* confers beer-spoilage ability on hop-sensitive Lactobacillus brevis ABBC45cc.** J Appl Microbiol 2006, **100**(6):1282.

9. Fujii T, Nakashima K, Hayashi N: **Random amplified polymorphic DNA-PCR based cloning of markers to identify the beer-spoilage strains of *Lactobacillus brevis*, *Pediococcus damnosus*, *Lactobacillus collinoides* and *Lactobacillus coryniformis*.** J Appl Microbiol 2005, **98**(5):1209-1220.

10. Klare I, Konstabel C, Werner G, Huys G, Vankerckhoven V, Kahlmeter G, Hildebrandt B, Muller-Bertling S, Witte W, Goossens H: **Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use.** J Antimicrob Chemother 2007, **59**(5):900-912.

11. Klare I, Konstabel C, Muller-Bertling S, Reissbrodt R, Huys G, Vancanneyt M, Swings J, Herman G, Witte W: **Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria.** Appl Environ Microbiol 2005, **71**(12):8982-8986.

12. Ammor MS, Belén FA, Mayo B: **Antibiotic resistance in non-enterococcal lactic acid bacteria and *Bifidobacteria*.** Food Microbiol, 2007, **24**(6):559-570.

13. Danielsen M, Simpson PJ, O'Connor EB, Ross RP, Stanton C: **Susceptibility of *Pediococcus* spp. to antimicrobial agents.** J Appl Microbiol 2007, **102**(2):384-389.

14. Tankovic J, Leclercq R, Duval J: **Antimicrobial susceptibility of *Pediococcus* spp. and genetic basis of macrolide resistance in *Pediococcus acidilactici* HM3020.** Antimicrob Agents Chemother 1993, **37**(4):789-792.

15. Billot-Klein D, Gutmann L, Sable S, Guittet E, van Heijenoort J: **Modification of peptidoglycan precursors is a common feature of the low-level vancomycin resistant VANB-type *Enterococcus* D366 and of the naturally glycopeptide-resistant species *Lactobacillus casei, Pediococcus pentosaceus, Leuconostoc mesenteroides,* and *Enterococcus gallinarum*.** J Bacteriol 1994, **176**:2398-2406.

Tables

Table 1 - *Pediococcus* isolates.

Species	N	Origin			Growth in Beer[a]	
		Brewery	Other[b]	Unknown	+	-
acidilactici	6	4	1	1	1	5
claussenii	12	12	0	0	11	1
ropy[c]	(5)	(5)	(0)	(0)	(5)	(0)
non-ropy[d]	(7)	(7)	(0)	(0)	(6)	(1)
damnosus	1	1	0	0	0	1
inopinatus	1	1	0	0	0	1
parvulus	5	0	5	0	1	4
ropy	(1)	(0)	(1)	(0)	(0)	(1)
non-ropy	(4)	(0)	(4)	(0)	(1)	(3)
pentosaceus	4	1	2	1	0	4
Total	**29**	**19**	**8**	**2**	**13**	**16**

[a] Previously reported by Haakensen *et al.* [3, 4].
[b] Isolates of known non-brewery origin, specific origins are provided in Additional file 2.
[c,d] Isolates positive and negative for exopolysaccharide rope production, respectively.

Table 2 – Antimicrobial compounds having significantly different MICs among the six *Pediococcus* species.

Antimicrobial compound	*p*-value[a]
Ampicillin	< 0.02
Ceftriaxone	< 0.02
Ciprofloxacin	< 0.02
Daptomycin	< 0.02
Gatifloxacin	< 0.01
Gentamicin	< 0.05
Levofloxacin	< 0.01
Penicillin	< 0.02
Synercid	< 0.05

[a] *p*-value corresponds to the *H*-test statistic as derived from the non-parametric Kruskal-Wallis *H*-test which tests for equality in population medians where there are three or more groups.

Table 3 – Antimicrobial compounds having significantly lower MICs in hop-resistant isolates[a].

Antimicrobial compound	Median and Distribution of MIC (µg/ml)		*p*-value[b]
	Hop-resistant	Hop-sensitive	
Ampicillin	0.25 (0.12-4)	1 (0.12-4)	< 0.05
Ciprofloxacin	2 (0.5-NR[c])	4 (0.5-NR)	< 0.05
Gatifloxacin	1 (0.5-8)	4 (1-NR)	< 0.05
Penicillin	0.12 (0.06-NR)	2 (0.06-NR)	< 0.02
Rifampin	0.5 (0.5-2)	1 (0.5-NR)	< 0.05

[a] Hop-resistance is as determined by the hop-gradient agar plate with ethanol method.
[b] *p*-value corresponds to *U*-test statistic as derived from the non-parametric Mann-Whitney *U*-test which is designed to examine whether two samples of observations came from the same distribution.
[c] NR; MIC not reached, isolate could grow at highest concentration of antibiotic tested.

Table 4 – Antimicrobial compounds having significantly lower MICs in isolates able to grow in beer.

Antimicrobial compound	Median and Distribution of MIC (µg/ml)		*p*-value[a]
	Grow in Beer	Cannot grow in beer	
Ampicillin	0.25 (0.12-4)	2 (0.12-4)	< 0.01
Ciprofloxacin	2 (0.5-NR[b])	4 (0.5-NR)	< 0.01
Gatifloxacin	1 (0.25-8)	4 (1-NR)	< 0.01
Levofloxacin	2 (0.5-NR)	16 (1-NR)	< 0.05
Oxacillin + 2% NaCl	0.25 (0.25-4)	1 (0.25-NR)	< 0.02
Penicillin	0.12 (0.12-NR)	1 (0.06-NR)	< 0.01
Synercid	0.5 (0.12-1)	1 (0.25-2)	< 0.05
Trimethoprim/ Sulfamethoxazole	0.5/9.5 (0.5/9.5-NR)	R (0.5/9.5-NR)	< 0.05

[a] *p*-value corresponds to *U*-test statistic as derived from the non-parametric Mann-Whitney *U*-test which is designed to examine whether two samples of observations came from the same distribution.

[b] NR; MIC not reached, isolate could grow at highest concentration of antibiotic tested.

Additional Files

Additional File 1

File format: DOC

Title: Range of minimum inhibitory concentrations of antimicrobial compounds summarized by species.

Description: The data provided indicate the range of concentrations tested for each antibiotic and the range of MICs obtained for each *Pediococcus* species.

Additional File 2

File format: XLS

Title: Isolate and antibiotic MIC information

Description: Information regarding the isolates used in the study, and the MICs obtained for each antibiotic by each isolate.

9. RECLASSIFICATION OF *PEDIOCOCCUS DEXTRINICUS* (COSTER AND WHITE 1964) BACK 1978 (APPROVED LISTS 1980) AS *LACTOBACILLUS DEXTRINICUS* COMB. NOV., AND EMENDED DESCRIPTION OF THE GENUS *LACTOBACILLUS*

Author contributions:

Monique Haakensen conceived the study, participated in the design, performed laboratory work, and drafted parts of the manuscript.

C. Melissa Dobson conceived the study, participated in the design, and edited the manuscript.

Janet E. Hill helped to draft the manuscript and provided scientific input.

Barry Ziola conceived the study, participated its design and coordination, edited the manuscript, and is the holder of the research grant used to fund the study

BRIEF INTRODUCTION TO CHAPTER 9

Through the data obtained in the growth experiments of Chapter 3, it became clear that the ability of bacteria to grow in beer does not abide by speciation. The genus *Pediococcus* contains several species which were found to be able to grow in beer (Chapter 3) and also to resist hop-compounds (Chapter 7). Most of the species within the genus *Pediococcus* form a single taxonomic unit, however, the species *Pediococcus dextrinicus* is a distant phylogenetic outlier. This type of nomenclatural inconsistency can make the study of organisms within a genus extremely difficult. As such, there was a need for reclassification of the species *P. dextrinicus* to more accurately reflect its taxonomic positioning. It was hoped that through clarification of taxonomy, the beer-spoilage pediococci can be accurately and efficiently studied. Here a comprehensive phylogenetic study of the genus *Pediococcus* is presented along with phylogenetic and phenotypic evidence supporting the reclassification of *P. dextrinicus* to the genus *Lactobacillus*.

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Reclassification of *Pediococcus dextrinicus* (Coster and White 1964) Back 1978 (Approved Lists 1980) as *Lactobacillus dextrinicus* comb. nov., and emended description of the genus *Lactobacillus*

Monique Haakensen,[1] C. Melissa Dobson,[2] Janet E. Hill[3] and Barry Ziola[1]

Correspondence
Barry Ziola
b.ziola@usask.ca

[1]Department of Pathology and Laboratory Medicine, University of Saskatchewan, 2841 Royal University Hospital, 103 Hospital Drive, Saskatoon SK S7N 0W8, Canada

[2]CanBiocin, #1015 – 8308 114 Street, Edmonton AB T6G 2E1, Canada

[3]Department of Veterinary Microbiology, University of Saskatchewan, 52 Campus Drive, Saskatoon SK S7N 5B4, Canada

The taxonomic status of *Pediococcus dextrinicus* is described and transfer of the species to the genus *Lactobacillus*, with the name *Lactobacillus dextrinicus* comb. nov., is proposed. This reclassification is supported by multilocus sequence analysis of the 16S rRNA gene and Cpn60, PheS, RecA and RpoA proteins. The mode of cell division and existing phenotypic information also show that *P. dextrinicus* does not belong to the genus *Pediococcus*, but rather to the genus *Lactobacillus*. As such, we propose that *Pediococcus dextrinicus* is reclassified as *Lactobacillus dextrinicus* comb. nov. (type strain ATCC 33087^T=DSM 20335^T=JCM 5887^T=LMG 11485^T=NCDO 1561^T).

The genus *Pediococcus* currently consists of 13 species (correct at the time of writing), including *Pediococcus acidilactici*, *Pediococcus claussenii* (Dobson *et al.*, 2002), *Pediococcus cellicola* (Zhang *et al.*, 2005), *Pediococcus damnosus*, *Pediococcus dextrinicus*, *Pediococcus ethanolidurans* (Liu *et al.*, 2006), *Pediococcus inopinatus*, *Pediococcus parvulus*, *Pediococcus pentosaceus*, *Pediococcus siamensis* (Tanasupawat *et al.*, 2007) and *Pediococcus stilesii* (Franz *et al.*, 2006). Two additional species originally classified as members of the genus *Pediococcus* have been transferred to other genera. Pediococci are homofermentative and produce DL-lactate from glucose; the exceptions being *P. claussenii* and *P. dextrinicus* which produce only (+)-L-lactic acid (Dobson *et al.*, 2002; Franz *et al.*, 2006; Pederson, 1949).

The species *P. dextrinicus* was included within the genus *Pediococcus* based upon morphology, cell-wall composition, homofermentative lactic acid production and nutritional requirements (Coster & White, 1964). It should be noted, however, that many of these characteristics are also shared by other lactic acid bacteria, including the related genera *Aerococcus*, *Lactobacillus* and *Tetragenococcus*. Gunther & White (1961a) first introduced the term 'Group III' for what is now *P. dextrinicus*, but unlike *Pediococcus* Groups I and II, Group III was referred to as the 'Possible Group III' as it was less well defined and differed from Groups I and II (true pediococci) in many phenotypic properties. Group III organisms most resembled *Pediococcus halophilus* (now reclassified as *Tetragenococcus halophilus*), except for salt tolerance. Gunther & White (1961a) went further to say 'it is felt that insufficient evidence is at present available on which to base the establishment of this group at specific rank'. The original descriptions of *P. dextrinicus* (originally referred to as 'Group III *Pediococcus*' by Gunther & White, 1961a, b, and later as '*Pediococcus cerevisiae* subsp. *dextrinicus*' by Coster & White, 1964), describe *P. dextrinicus* as being very different from all other *Pediococcus* isolates and their findings indicated that *P. dextrinicus* may in fact be more closely related to members of the genus *Staphylococcus* (Coster & White, 1964). It was mentioned that *P. dextrinicus* strains 'are not related antigenically to *P. cerevisiae* (Group I) and *P. parvulus* (Group II). They may possibly represent a separate serological group' (Gunther & White, 1961b). In 1964, Coster & White noticed that 'Group III (now *P. dextrinicus*) extracts did not react with antisera prepared against strains other than group III', yet antisera produced to isolates found in Groups I and II show extensive cross-reactivity with bacteria in both Groups. Serological data thus indicate that *P. dextrinicus* does not group with other pediococci.

At that point, the genus *Pediococcus* was too diverse to exclude *P. dextrinicus* as the genus still contained bacteria

Abbreviation: MLSA, multilocus sequence analysis.

that have since been reclassified to the genera *Aerococcus* and *Tetragenococcus*. As such, *P. dextrinicus* was elevated to species status not based upon properties that included it within the genus *Pediococcus*, but based on the fact that it could not be included with any of the other species that had arisen from the original grouping of '*Pediococcus cerevisiae*' (Back, 1978). In addition to the serological data presented by Gunther & White (1961b) and Coster & White (1964), several phenotypic properties also distinguish *P. dextrinicus* from other pediococci, including the lack of acid production from growth on trehalose, production of CO_2 from gluconate, lack of growth at pH 4.5 and the ability to hydrolyse starch and dextrin (Dellaglio & Torriani, 2006; Franz *et al.*, 2006; Holzapfel *et al.*, 2006; Simpson & Taguchi, 1995; Weiss, 1992). The phenotypic differences between *P. dextrinicus* and other pediococci are summarized in Table 1. For comparative purposes, the genera *Aerococcus* and *Tetragenococcus* are included in Table 1 as these bacteria were previously classified as pediococci. While *P. dextrinicus* can be excluded from the genus *Pediococcus* based upon the phenotypic properties in Table 1, these data also show that there are no phenotypic properties which can distinguish *P. dextrinicus* from members of the genus *Lactobacillus*.

Although the mode of division of *P. dextrinicus* has been listed as conforming to that of the genus *Pediococcus*, there is no published microscopy work documenting this and, interestingly, the original description of *P. dextrinicus* did not include the mode of division (Gunther & White, 1961a, b; Coster & White, 1964). It was not until *P. dextrinicus* was elevated to species status by Back (1978) that the description of the organism was expanded to include 'occurring predominantly in pairs and tetrads, occasionally in short chains and irregular clusters'. This ability to form chains differentiates *P. dextrinicus* from the descriptions of all other species of the genus *Pediococcus* (Simpson & Taguchi, 1995). Clarification of the mode of division of *P. dextrinicus* is clearly necessary for a proper description of this bacteria.

In addition to these differences in phenotype and mode of division, it has been shown based upon 16S rRNA gene, *cpn60* gene and protein and 16S–23S rRNA interspacer sequences that *P. dextrinicus* is phylogenetically distant to other pediococci, belonging instead within the genus *Lactobacillus* (Collins *et al.*, 1991; Dobson *et al.*, 2002; Franz *et al.*, 2006). Despite the extensive documentation showing that *P. dextrinicus* are not pediococci, no formal reclassification of *P. dextrinicus* has been proposed. As such, the aim of the present study is the evaluation and clarification of the taxonomic position of this species.

Table 1. Phenotypic characteristics that differentiate *P. dextrinicus* from related genera, but not from the genus *Lactobacillus*

Taxa: 1, *Aerococcus*; 2, *Lactobacillus*; 3, *P. dextrinicus*; 4, *Pediococcus*; 5, *Tetragenococcus*. +, Positive; −, negative; +/−, genus includes species that are both positive and negative. Data adapted from Dellaglio & Torriani (2006), Franz *et al.* (2006), Hammes & Hertel (2006), Simpson & Taguchi (1995) and Weiss (1992).

Characteristic	1*	2	3	4†	5*
Production of acid from:					
Starch	−	+/−	+	−	−
Dextrin	+	+/−	+	−	−
Trehalose	+	+/−	−	+	+
Mannose	+	+/−	−	+	+
Gas from gluconate	−	+/−	+	−	−
Configuration of lactate	(+)-L	D, (+)-L, DL	(+)-L	DL‡	(+)-L
Growth in/at:					
6.5 % NaCl	+	+/−	−	+§	+
pH 4.5	−	+/−	−	+	−

*The genera *Aerococcus* and *Tetragenococcus* both contain isolates that were once classified as *Pediococcus* species.
†Includes current species of the genus *Pediococcus*, except for *P. dextrinicus*.
‡All except *P. claussenii* which produces only (+)-L lactate.
§All except *P. damnosus*.

Mode of division

We compared Gram-stains of *P. dextrinicus* with other species of the genus *Pediococcus* by counting the number of cells present as singles, pairs, tetrads and clusters (Table 2). There were no statistically significant differences found for the cell arrangements between: fields of view, isolates of the same species, growth phase or medium (*t*-test for

Table 2. Results of cell division

Species*	Cell arrangement†			
	Single	Pair	Tetrad‡	Cluster§
P. acidilactici	2	12	76	0
P. claussenii	2	2	96	0
P. dextrinicus	13	20	0	67
P. inopinatus	4	4	92	0
P. parvulus	12	20	68	0
P. pentosaceus	4	56	40	0

*Isolates used: *P. acidilactici* ATCC 8042, ATCC 25740; *P. claussenii* ATCC BAA-344^T, CCC B1099; *P. dextrinicus* ATCC 33087^T, ATCC 700477; *P. inopinatus* ATCC 49902^T; *P. parvulus* ATCC 43013, Spain 2.6; *P. pentosaceus* ATCC 33314, ATCC 8081.
†There was no statistically significant difference found between: fields of view, isolates of the same species, growth phase or type of medium (*t*-test; all *P*-values >0.05); consequently, numbers given are the mean number determined by counting 100 bacteria in each of five separate fields of view, from colonies taken from agar plates, and also at both log- and plateau-phase from broth medium.
‡Symmetrical groups of four cells with two perpendicular lines of division.
§Three or more bacteria that were not symmetrical. Clumps of >10 bacteria were frequently observed.

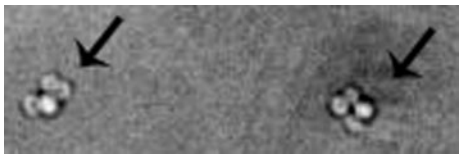


Fig. 1. Clusters of four cells of *P. dextrinicus* ATCC 33087^T produced after division from a single cell. Image taken by phase-contrast microscope.

independent samples; all *P*-values >0.05). In this assessment of cell division, clusters were defined as groups of three or more cells that were not symmetrical and tetrads were defined as symmetrical groups of four cells (Simpson, 1994). This microscopy work strongly indicated that *P. dextrinicus* does not divide using a process like that of other pediococci. To confirm this, single cells of *P. claussenii* ATCC BAA-344^T and *P. dextrinicus* ATCC 33087^T were followed through a minimum of two cycles of division (division from one to four cells) using phase-contrast microscopy. Cells of *P. claussenii* ATCC BAA-344^T divided in two perpendicular directions on a single plane and, once a tetrad was achieved, the four cells split into two doublets along the first line of division. In contrast, cells resulting from the two cell divisions of *P. dextrinicus* were not perpendicular to each other nor were they in a single plane. Moreover, *P. dextrinicus* cells did not separate upon becoming a group of four, but continued to divide, creating large irregular clusters, many of which contained >10 cells. Fig. 1 shows the result of division from one to four cells for two *P. dextrinicus* cells, while Fig. 2 represents *P. dextrinicus* cell division schematically compared with that given in the genus description for *Pediococcus* (Simpson, 1994). While these results suggest a different arrangement and mode of division for *P. dextrinicus* as compared with other pediococci (i.e. non-perpendicular cell arrangement and non-division after tetrad formation but formation of larger clumps), the exact mode of division needs to be further examined, including studies using scanning electron microscopy.

Multilocus sequence analysis

Although 16S rRNA gene sequences are commonly used to elucidate phylogenetic relationships (Woese, 1987), using 16S rRNA gene sequences to infer phylogeny has been criticized as it assumes that one molecule can reflect organismal evolutionary history (Fox *et al.*, 1992). As such, instead of using just the 16S rRNA gene to infer phylogeny, we used a multilocus sequence analysis (MLSA) to assess the relationship of *P. dextrinicus* to other species of the genus *Pediococcus*, species of the genus *Lactobacillus* and species of related genera. In addition to the full-length 16S rRNA gene, we also analysed portions of the following conserved genes: *cpn60* (552 bp), *recA* (531 bp), *pheS* (455 bp) and *rpoA* (533 bp). The usefulness of these five regions in assessing phylogenetic relationships has been shown previously (Dobson *et al.*, 2002; Eisen, 1995; Felis *et al.*, 2001; Jian *et al.*, 2001; Kwok & Chow, 2003; Lloyd & Sharp, 1993; Naser *et al.*, 2005, 2007; Vandamme *et al.*, 1996). We believe that the congruent data obtained through our MLSA approach creates a solid representation of the phylogenetic relationships among the bacterial species analysed, thus clarifying the taxonomic position of *P. dextrinicus*.

Phylogenetic trees were constructed using sequences from, in most cases, the type strains of two representatives from

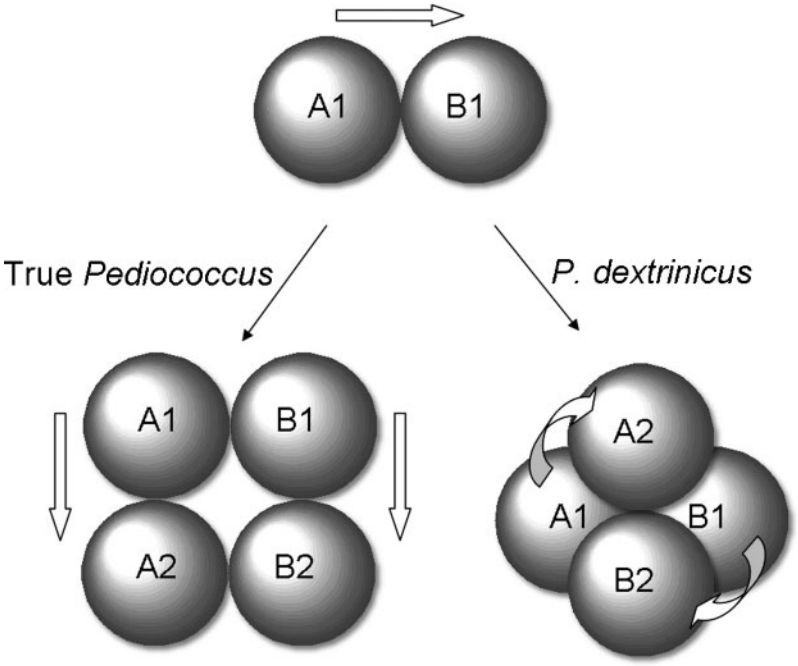


Fig. 2. Schematic representation of cell division from one to four cells for *P. dextrinicus* ATCC 33087^T compared with true pediococci as followed by phase-contrast microscopy. The division of true pediococci is in two perpendicular directions in a single plane at right angles, while the division of *P. dextrinicus* cells is not in two perpendicular directions at right angles, nor is it in a single plane.

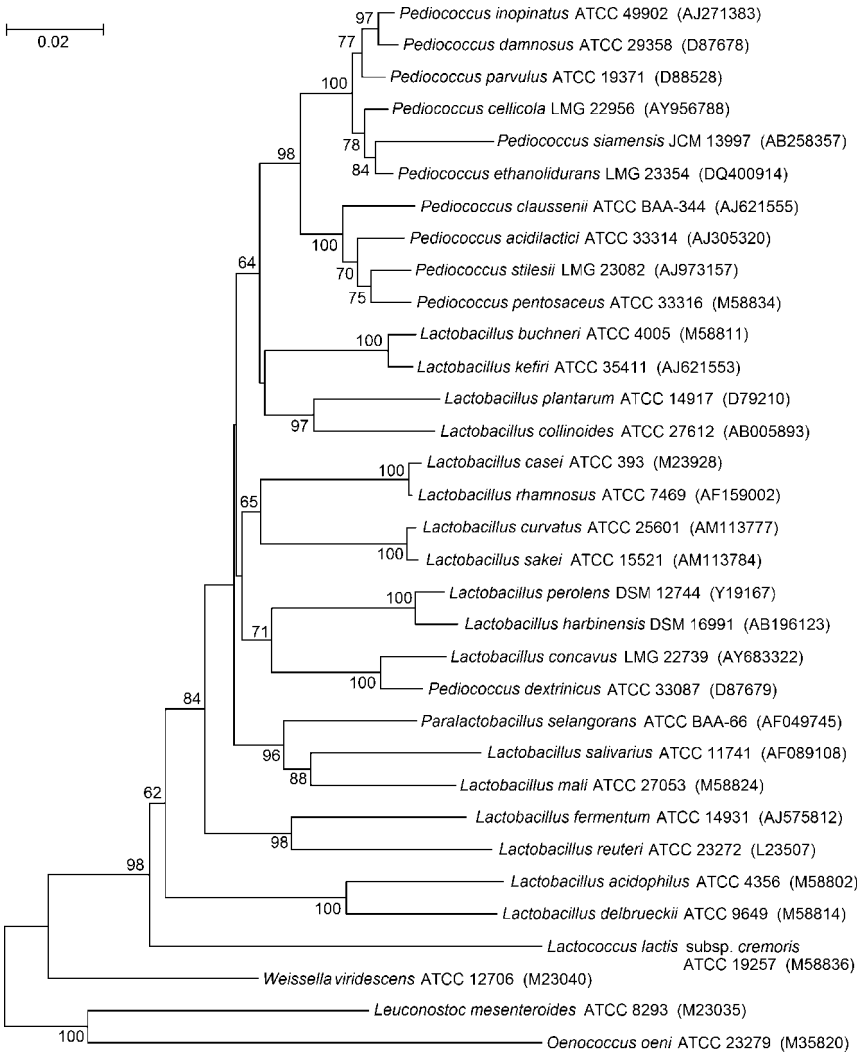
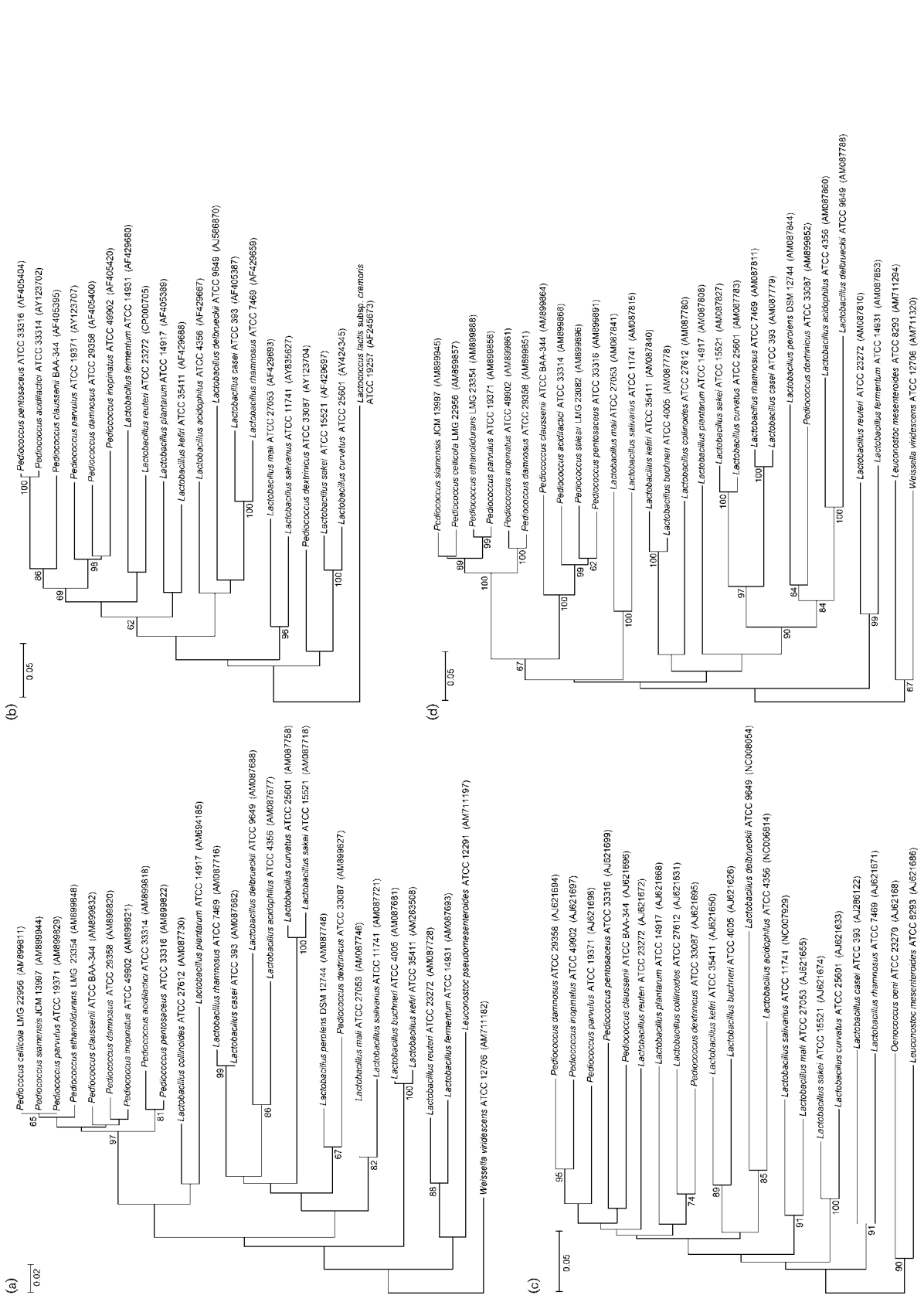


Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences illustrating the evolutionary relationship of *Pediococcus dextrinicus* with all other species of the genus *Pediococcus* and species that are representative of the major clades of the genus *Lactobacillus*. GenBank accession numbers are given in parentheses. Except for *Lactobacillus fermentum*, *P. acidilacti* and *P. ethanolidurans*, all strains are type strains. Bootstrap values >60 % are given at nodes as a percentage of 1000 replicates. Bar, 2 % divergence.

each of the seven groups of the genus *Lactobacillus* as described in *The Prokaryotes* (Hammes & Hertel, 2006). By using two representatives from each of these groups, the overall structure of the 16S rRNA gene tree remains the same as that shown in other publications regarding the current taxonomy of the genus *Lactobacillus* (Collins *et al.*, 1991; Hammes & Hertel, 2006; Holzapfel *et al.*, 2001; Klein *et al.*, 1998; Naser *et al.*, 2007; Stiles & Holzapfel, 1997). The best GenBank matches to *P. dextrinicus* (i.e. *Lactobacillus concavus*, *Lactobacillus harbinensis* and *Lactobacillus perolens*) were also included in the MLSA where sequences were available. The type strain sequences of the closely related genera *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Paralactobacillus* and *Weissella* are included as outliers, again, where sequences were available.

Sequences were aligned with CLUSTAL_X (Thompson *et al.*, 1997) and 16S rRNA gene alignments were visualized and

Fig. 4. Phylogenetic trees illustrating the evolutionary relationship of *Pediococcus dextrinicus* with all other species of the genus *Pediococcus* and species that are representative of the major clades of the genus *Lactobacillus*. GenBank accession numbers are given in parentheses. The strains used are the same as those in Fig. 3. Bootstrap values >60 % are given at nodes as a percentage of 1000 replicates. (a) PheS protein; (b) Cpn60 protein; (c) RecA protein; (d) RpoA protein. Bars, 2 % divergence (a); 5 % divergence (b, c, d).


(a)
0.02
Pediococcus cellicola LMG 22956 (AM899811)
Pediococcus siamensis JCM 13967 (AM899944)
Pediococcus parvulus ATCC 19371 (AM899829)
Pediococcus ethanolidurans LMG 23354 (AM899848)
Pediococcus clausenii ATCC BAA-344 (AM899832)
Pediococcus damnosus ATCC 29358 (AM899820)
Pediococcus inopinatus ATCC 49902 (AM899821)
Pediococcus acidilactici ATCC 33314 (AM899818)
Pediococcus pentosaceus ATCC 33316 (AM899822)
Lactobacillus collinoides ATCC 27612 (AM087730)
Lactobacillus plantarum ATCC 14917 (AM894185)
Lactobacillus rhamnosus ATCC 7469 (AM087716)
Lactobacillus casei ATCC 393 (AM087682)
Lactobacillus delbrueckii ATCC 9649 (AM087688)
Lactobacillus acidophilus ATCC 4356 (AM087677)
Lactobacillus curvatus ATCC 25601 (AM087758)
Lactobacillus sakei ATCC 15521 (AM087718)
Lactobacillus perolens DSM 12744 (AM087748)
Pediococcus dextrinicus ATCC 33087 (AM899827)
Lactobacillus mali ATCC 27053 (AM087746)
Lactobacillus salivarius ATCC 11741 (AM087721)
Lactobacillus buchneri ATCC 4005 (AM087681)
Lactobacillus kefiri ATCC 35411 (AM283508)
Lactobacillus reuteri ATCC 23272 (AM087728)
Lactobacillus fermentum ATCC 14931 (AM087683)
Leuconostoc pseudomesenteroides ATCC 12291 (AM711197)
Weissella viridescens ATCC 12706 (AM711182)
(b)
0.05
Pediococcus pentosaceus ATCC 33316 (AF405404)
Pediococcus acidilactici ATCC 33314 (AY123702)
Pediococcus clausenii BAA-344 (AF405395)
Pediococcus parvulus ATCC 19371 (AY123707)
Pediococcus damnosus ATCC 29358 (AF405400)
Pediococcus inopinatus ATCC 49902 (AF405420)
Lactobacillus fermentum ATCC 14931 (AF429680)
Lactobacillus reuteri ATCC 23272 (CP000705)
Lactobacillus plantarum ATCC 14917 (AF405389)
Lactobacillus kefiri ATCC 35411 (AF429688)
Lactobacillus acidophilus ATCC 4356 (AF429667)
Lactobacillus delbrueckii ATCC 9649 (AJ586870)
Lactobacillus casei ATCC 393 (AF405387)
Lactobacillus rhamnosus ATCC 7469 (AF429659)
Lactobacillus mali ATCC 27053 (AF429683)
Lactobacillus salivarius ATCC 11741 (AY835627)
Pediococcus dextrinicus ATCC 33087 (AY123704)
Lactobacillus sakei ATCC 15521 (AF429687)
Lactobacillus curvatus ATCC 25601 (AY424345)
Lactococcus lactis subsp. cremoris ATCC 19257 (AF245673)
(c)
0.05
Pediococcus damnosus ATCC 29358 (AJ621694)
Pediococcus inopinatus ATCC 49902 (AJ621697)
Pediococcus parvulus ATCC 19371 (AJ621698)
Pediococcus pentosaceus ATCC 33316 (AJ621699)
Pediococcus clausenii ATCC BAA-344 (AJ621696)
Lactobacillus reuteri ATCC 23272 (AJ621672)
Lactobacillus plantarum ATCC 14917 (AJ621668)
Lactobacillus collinoides ATCC 27612 (AJ621631)
Pediococcus dextrinicus ATCC 33087 (AJ621695)
Lactobacillus kefiri ATCC 35411 (AJ621650)
Lactobacillus buchneri ATCC 4005 (AJ621626)
Lactobacillus delbrueckii ATCC 9649 (NC008054)
Lactobacillus acidophilus ATCC 4356 (NC006814)
Lactobacillus salivarius ATCC 11741 (NC007929)
Lactobacillus mali ATCC 27053 (AJ621655)
Lactobacillus sakei ATCC 15521 (AJ621674)
Lactobacillus curvatus ATCC 25601 (AJ621633)
Lactobacillus casei ATCC 393 (AJ286122)
Lactobacillus rhamnosus ATCC 7469 (AJ621671)
Oenococcus oeni ATCC 23279 (AJ62168)
Leuconostoc mesenteroides ATCC 8293 (AJ621686)
(d)
0.05
Pediococcus siamensis JCM 13967 (AM899945)
Pediococcus cellicola LMG 22956 (AM899857)
Pediococcus ethanolidurans LMG 23354 (AM899868)
Pediococcus parvulus ATCC 19371 (AM899856)
Pediococcus inopinatus ATCC 49902 (AM899861)
Pediococcus damnosus ATCC 29358 (AM899851)
Pediococcus clausenii ATCC BAA-344 (AM899864)
Pediococcus acidilactici ATCC 33314 (AM899866)
Pediococcus stilesii LMG 23082 (AM899896)
Pediococcus pentosaceus ATCC 33316 (AM899891)
Lactobacillus mali ATCC 27053 (AM087841)
Lactobacillus salivarius ATCC 11741 (AM087815)
Lactobacillus kefiri ATCC 35411 (AM087840)
Lactobacillus buchneri ATCC 4005 (AM087778)
Lactobacillus collinoides ATCC 27612 (AM087780)
Lactobacillus plantarum ATCC 14917 (AM087806)
Lactobacillus sakei ATCC 15521 (AM087827)
Lactobacillus curvatus ATCC 25601 (AM087783)
Lactobacillus rhamnosus ATCC 7469 (AM087811)
Lactobacillus casei ATCC 393 (AM087779)
Lactobacillus perolens DSM 12744 (AM087844)
Pediococcus dextrinicus ATCC 33087 (AM899852)
Lactobacillus acidophilus ATCC 4356 (AM087860)
Lactobacillus delbrueckii ATCC 9649 (AM087788)
Lactobacillus reuteri ATCC 23272 (AM087810)
Lactobacillus fermentum ATCC 14931 (AM087853)
Leuconostoc mesenteroides ATCC 8293 (AM711294)
Weissella viridescens ATCC 12706 (AM711320)

manually edited using the GeneDoc software program (Nicholas *et al.*, 1997) to conform with structural information and inferred locations of conserved and variable regions (Neefs *et al.*, 1993). Tree topology was evaluated using minimum evolution, maximum-parsimony, neighbour-joining and unweighted pair group method of arithmetic means (UPGMA) algorithms, with all methods producing similar overall topologies (data not shown). Fig. 3 and Fig. 4a–d were inferred using the neighbour-joining maximum composite likelihood method (Saitou & Nei, 1987) for the 16S rRNA gene and the PheS, Cpn60, RecA and RpoA proteins and are representative of the topology constructed by all four algorithms. Phylogenetic trees produced from DNA and translated protein sequences also produced similar overall topologies (data not shown). All phylogenetic trees were visualized and produced using MEGA version 4.0 (Tamura *et al.*, 2007). The percentage of replicate trees in which the associated taxa clustered together at levels >60 % in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985).

P. dextrinicus consistently branches distant to other species of the genus *Pediococcus* for all five genetic loci (Fig. 3, Fig. 4a–d), grouping instead with members of the genus *Lactobacillus*. Based upon the 16S rRNA gene sequence analysis, the species most similar to *P. dextrinicus* are *Lactobacillus concavus*, *Lactobacillus harbinensis* and *Lactobacillus perolens*. This similarity is reinforced by the fact that the 16S rRNA gene of these *Lactobacillus* species shares 93–97 % similarity with that of *P. dextrinicus*, while other members of the genus *Pediococcus* share only 90–92 % sequence similarity with *P. dextrinicus*. In contrast, the other ten species of the genus *Pediococcus* exhibit 93–97 % inter-species similarity for the 16S rRNA gene.

Using 16S rRNA gene sequences, Collins *et al.* (1991) and Franz *et al.* (2006) both found, as did we (Fig. 3), that *P. dextrinicus* forms a distinct line of descent compared to the rest of the species in the genus *Pediococcus*. Here we have supported this finding with the application of a MLSA using PheS, Cpn60, RecA and RpoA sequences (Fig. 4a–d). This MLSA phylogenetic information, in conjunction with evidence that *P. dextrinicus* does not divide in two perpendicular directions in a single plane (Table 2; Figs 1 and 2) as do true pediococci (Table 2; Fig. 2), reinforces the phenotypic evidence (Table 1) indicating that this species does not belong within the genus *Pediococcus*, but rather does belong within the closely related genus *Lactobacillus*. We therefore propose that *Pediococcus dextrinicus* be reclassified as *Lactobacillus dextrinicus* comb. nov.

Emended description of the genus *Lactobacillus* Beijerinck 1901

This description of the genus *Lactobacillus* is based on that of Beijerinck (1901) as given by Kandler & Weiss (1986), with the addition that cells may be rods, cocci or coccobacilli in shape.

Description of *Lactobacillus dextrinicus* comb. nov.

Lactobacillus dextrinicus (dex.tri′ni.cus. N.L. n. *dextrinum* dextrin; L. suff. *-icus* suffix used with the sense of belonging to; N.L. masc. adj. *dextrinicus* related to dextrin).

Basonym: *Pediococcus dextrinicus* (Coster and White 1964) Back 1978 (Approved Lists 1980).

Gram-positive, non-motile, non-spore-forming, facultative anaerobes. Cells are spherical, never elongated and may occur singly, in pairs or clusters and rarely in chains. Clusters of four may be observed, but not as the result of division in two perpendicular directions at right angles. Division does not occur in a single plane. Catalase-negative. Only (+)-L-lactic acid is produced [from maltose, dextrin and starch, but not from ribose, arabinose, xylose, mannitol, trehalose (10–90 % of strains), melezitose]; no ammonia is produced from arginine. Cells grow at pH 7.0, but not pH 4.5 (Dellaglio & Torriani, 2006; Franz *et al.*, 2006; Garvie, 1986; Stiles & Holzapfel, 1997). The optimal temperature for growth is 32 °C (Cai *et al.*, 1999), weak growth is observed at 43–45 °C, but not above 45 °C (Franz *et al.*, 2006). G+C content of the DNA is 40–41 mol% (Cai *et al.*, 1999).

The type strain is ATCC 33087^T (=DSM 20335^T=JCM 5887^T=LMG 11485^T=NCDO 1561^T).

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References

Back, W. (1978). Elevation of *Pediococcus cerevisiae* subsp. *dextrinicus* Coster and White to species status [*Pediococcus dextrinicus* (Coster and White) comb. nov.]. *Int J Syst Bacteriol* **28**, 523–527.

Beijerinck, M. W. (1901). Sur les ferments lactiques de l'industrie. *Arch Néer Sci Exactes Natur (Section 2)* **6**, 212–243.

Cai, Y., Kumai, S., Ogawa, M., Benno, Y. & Nakase, T. (1999). Characterization and identification of *Pediococcus* species isolated from forage crops and their application for silage preparation. *Appl Environ Microbiol* **65**, 2901–2906.

Collins, M. D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J. A. E., Martinez-Murcia, A., Phillips, B. A., Williams, A. M. & Wallbanks, S. (1991). Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiol Lett* **77**, 5–12.

Coster, E. & White, H. R. (1964). Further studies of the genus *Pediococcus*. *J Gen Microbiol* **37**, 15–31.

Dellaglio, F. & Torriani, S. (2006). DNA-DNA homology, physiological characteristics and distribution of lactic-acid bacteria isolated from maize silage. *J Appl Bacteriol* **60**, 83–92.

Dobson, C. M., Deneer, H., Lee, S., Hemmingsen, S., Glaze, S. & Ziola, B. (2002). Phylogenetic analysis of the genus *Pediococcus*, including *Pediococcus claussenii* sp. nov., a novel lactic acid bacterium isolated from beer. *Int J Syst Evol Microbiol* **52**, 2003–2010.

Eisen, J. A. (1995). The RecA protein as a model molecule for molecular systematic studies of bacteria: comparison of trees of RecAs and 16S rRNAs from the same species. *J Mol Evol* **41**, 1105–1123.

Felis, G. E., Dellaglio, F., Mizzi, L. & Torriani, S. (2001). Comparative sequence analysis of a *recA* gene fragment brings new evidence for a change in the taxonomy of the *Lactobacillus casei* group. *Int J Syst Evol Microbiol* **51**, 2113–2117.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P., Jr (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42**, 166–170.

Franz, C. M. A. P., Vancanneyt, M., Vandemeulebroecke, K., DeWachter, M., Cleenwerck, I., Hoste, B., Schillinger, U., Holzapfel, W. H. & Swings, J. (2006). *Pediococcus stilesii* sp. nov., isolated from maize grains. *Int J Syst Evol Microbiol* **56**, 329–333.

Garvie, E. I. (1986). Genus *Pediococcus*. In *Bergey's Manual of Systematic Bacteriology*, pp. 1075–1079. Edited by P. H. A. Sneath, M. E. Mair, M. E. Sharpe & J. G. Holt. Baltimore, MD: Williams and Wilkins.

Gunther, H. L. & White, H. R. (1961a). The cultural and physiological characters of the pediococci. *J Gen Microbiol* **26**, 185–197.

Gunther, H. L. & White, H. R. (1961b). Serological characters of the pediococci. *J Gen Microbiol* **26**, 199–205.

Hammes, W. P. & Hertel, C. (2006). The genera *Lactobacillus* and *Carnobacterium*. In *The Prokaryotes: a Handbook on the Biology of Bacteria*, pp. 320–403. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York, NY: Springer.

Holzapfel, W. H., Haberer, P., Geisen, R., Bjorkroth, J. & Schillinger, U. (2001). Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am J Clin Nutr* **73**, 365S–373S.

Holzapfel, W. H., Franz, C. M. A. P., Ludwig, W., Back, W. & Dicks, L. M. T. (2006). The genera *Pediococcus* and *Tetragenococcus*. In *The Prokaryotes: a Handbook on the Biology of Bacteria*, pp. 229–266. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York, NY: Springer.

Jian, W., Zhu, L. & Dong, X. (2001). New approach to phylogenetic analysis of the genus *Bifidobacterium* based on partial *hsp60* gene sequences. *Int J Syst Evol Microbiol* **51**, 1633–1638.

Kandler, O. & Weiss, N. (1986). Regular nonsporing Gram-positive rods. In *Bergey's Manual of Systematic Bacteriology*, vol 2, pp. 1208–1234. Edited by P. H. Sneath, N. Mair, M. E. Sharpe & J. G. Holt. Baltimore, MD: William and Wilkins.

Klein, G., Pack, A., Bonaparte, C. & Reuter, G. (1998). Taxonomy and physiology of probiotic lactic acid bacteria. *Int J Food Microbiol* **41**, 103–125.

Kwok, A. Y. C. & Chow, A. W. (2003). Phylogenetic study of *Staphylococcus* and *Micrococcus* species based on partial *hsp60* gene sequences. *Int J Syst Evol Microbiol* **53**, 87–92.

Liu, L., Zhang, B., Tong, H. & Dong, X. (2006). *Pediococcus ethanolidurans* sp. nov., isolated from the walls of a distilled-spirit-fermenting cellar. *Int J Syst Evol Microbiol* **56**, 2405–2408.

Lloyd, A. T. & Sharp, P. M. (1993). Evolution of the *recA* gene and the molecular phylogeny of bacteria. *J Mol Evol* **37**, 399–407.

Naser, S. M., Thompson, F. L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M. & Swings, J. (2005). Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. *Microbiology* **151**, 2141–2150.

Naser, S. M., Dawyndt, P., Hoste, B., Gevers, D., Vandemeulebroecke, K., Cleenwerck, I., Vancanneyt, M. & Swings, J. (2007). Identification of lactobacilli by *pheS* and *rpoA* gene sequence analyses. *Int J Syst Evol Microbiol* **57**, 2777–2789.

Neefs, J. M., Van de Peer, Y., De Rijk, P., Chapelle, S. & De Wachter, R. (1993). Compilation of small ribosomal subunit RNA structures. *Nucleic Acids Res* **21**, 3025–3049.

Nicholas, K. B., Nicholas, H. B., Jr & Deerfield, D. W., II (1997). GeneDoc: analysis and visualization of genetic variation. *EMBnet News* **4** (2), 1–4. http://www.embnet.org/download/embnetnews/embnet_news_4_2.pdf

Pederson, C. S. (1949). The genus *Pediococcus. Bacteriol Rev* **13**, 225–232.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Simpson, W. J. (1994). Comments on the mode of division of *Pediococcus* spp. *Lett Appl Microbiol* **18**, 69–70.

Simpson, W. J. & Taguchi, H. (1995). The genus *Pediococcus*, with notes on the genera *Tetragenococcus* and *Aerococcus*. In *The Genera of Lactic Acid Bacteria*, pp. 125–172. Edited by B. J. Wood & W. H. Holzapfel. London: Blackie Academic & Professional.

Stiles, M. E. & Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *Int J Food Microbiol* **36**, 1–29.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Tanasupawat, S., Pakdeeto, A., Thawai, D., Yukphan, P. & Okada, S. (2007). Identification of lactic acid bacteria from fermented tea leaves (miang) in Thailand and proposals of *Lactobacillus thailandensis* sp. nov., *Lactobacillus camelliae* sp. nov., and *Pediococcus siamensis* sp. nov. *J Gen Appl Microbiol* **53**, 7–15.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Vandamme, P., Pot, B., Gillis, M., deVos, P., Kersters, K. & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Rev* **60**, 407–438.

Weiss, N. (1992). The genera *Pediococcus* and *Aerococcus*. In *The Prokaryotes*, pp. 1502–1507. Edited by A. Balows, H. H. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York, NY: Springer.

Woese, C. R. (1987). Bacterial evolution. *Microbiol Rev* **51**, 221–271.

Zhang, B., Tong, H. & Dong, X. (2005). *Pediococcus cellicola* sp. nov., a novel lactic acid coccus isolated from a distilled-spirit-fermenting cellar. *Int J Syst Evol Microbiol* **55**, 2167–2170.

10. TOWARDS A GENOMIC UNDERSTANDING OF PROKARYOTIC PHYLOGENY

Author contributions:

Monique Haakensen* conceived the study, participated in the design, performed statistical analyses and biological interpretation, and drafted the manuscript.

Brett Trost* participated in the design and coordination of the study, developed the software programming, performed computational analyses, and drafted parts of the manuscript.

Vanessa Pittet helped to draft the manuscript, assembled data, and provided scientific input regarding biological interpretation.

Anthony Kusalik participated in the design and coordination of the study, edited the manuscript, and is the holder of a research grant partially used to fund the study.

Barry Ziola conceived the study, participated in the design and coordination of the study, edited the manuscript, and is the holder of a research grant partially used to fund the study.

BRIEF INTRODUCTION TO CHAPTER 10

To be able to better examine the discrepancies seen between phylogeny and phenotype, a method was developed by which whole genomic or proteomic similarity can be determined. Several possible applications of our whole genome comparison method are presented. These include the identification of putative phenotype-related genes, elucidation of core genomes for groups of bacteria, and analysis of proteome similarity for phylogenetic studies.

*Both authors contributed equally, as such, some of the content of this chapter and also an in depth presentation of the algorithms developed are also included in the thesis of Brett Trost at the University of Saskatchewan. Permission for this interdisciplinary overlap in thesis content has been obtained from the College of Graduate Studies and Research at the University of Saskatchewan.

Towards a genomic understanding of Prokaryotic phylogeny

Monique Haakensen[1]*, Brett Trost[2]*, Vanessa Pittet[1], Anthony Kusalik[2], Barry Ziola[1§]

[1]Department of Pathology and Laboratory Medicine, University of Saskatchewan, 2841 Royal University Hospital, 103 Hospital Drive, Saskatoon, Saskatchewan, Canada S7N 0W8
[2]Department of Computer Science, University of Saskatchewan, 176 Thorvaldson Building, 110 Science Place, Saskatoon, Saskatchewan, Canada S7N 5C9

*These authors contributed equally to this work

[§]Corresponding author

Email addresses:

MH: m.haakensen@usask.ca

BT: brett.trost@usask.ca

VP: v.pittet@usask.ca

TK: kusalik@cs.usask.ca

BZ: b.ziola@usask.ca

Forward

The data being used as a "whole" proteome for each isolate consists of a combination of known proteins and translated open reading frames (ORFs) predicted by application of gene-finding algorithms to sequenced genomes. Therefore, a "whole proteome" may contain predicted and hypothetical proteins in addition to known proteins. As such, for many of the isolates in this study the majority of the entries in the "whole proteome" are predicted proteins. Throughout this chapter, the term “proteome” refers to the whole proteome of an isolate, including confirmed and hypothetic proteomic complement.

Abstract

Background

With the increasing availability of whole genome sequences, it is becoming ever more important to develop and evaluate methods by which these sequences can be analyzed and compared with existing phylogenetic information. We developed several new tools, implementing a pairwise best bidirectional Basic Local Alignment Search Tool algorithm to obtain information regarding the relatedness of prokaryotic genomes. Analyses are also compared to the 16S rRNA gene to shed insights on the accuracy of current phylogenetic methods.

Results

Pairwise comparisons of genomes yielded a large amount of easily manipulated data. This data was successfully used to identify candidate proteins for a test phenotype, and also to establish "core" and "unique" proteomes for groups of organisms. By comparing proteomic similarity on the premises of open reading frames and amount of proteome that is in common between organisms, phenotypic similarity may also be inferred for sets of organisms. Phylogeny of the 16S rRNA gene is largely in agreement with phylogeny as constructed by whole proteomic similarity. However, there are several discrepancies in the branching of the two dendrograms, which suggest large scale genomic change can occur with only a small concurrent shift in 16S rRNA gene similarity.

Conclusions

The discrepancies found between 16S rRNA phylogeny and whole proteomic similarity suggests that outlier species may be best studied outside of the context of the genus to which they have been assigned. Creation of "unique" and "core" proteomes confers the ability to determine where species have been misidentified or misclassified, and this is supported by 16S rRNA gene similarity. Putative phenotype-related genes/proteins can be suggested based on subtraction of proteomes of bacterial isolates that are grouped based on phenotypic properties.

Background

Phylogenetics

Historically, taxonomic analyses have been performed using a diverse and often arbitrary selection of morphological and phenotypic characteristics. These phenotypic characteristics are today considered unsuitable for generating reliable and consistent taxonomies for prokaryotes, as there is no rational basis for choosing which morphological or phenotypic characteristics should be examined. Moreover, the extent that individual phenotypes or small collection of phenotypes consistently represent true phylogeny is generally considered to be minimal. The unsuitability of phenotypic factors, along with the advent of DNA sequencing, has led to 16S rRNA gene sequence comparisons becoming the gold standard technique for taxonomic analyses, now otherwise known as "phylogenetic" analyses [1]. Over time, the trend has moved towards using a greater number of genes to infer phylogenetic relationships. This is in part due to the increasing ease and reduced cost associated with DNA sequencing, but also due to criticisms of whether a single gene could possibly infer whole genomic content. This method of inferring phylogeny based on a number of genes is called a multi-locus sequence analysis (MLSA), which attempts to infer phylogenetic relationships by comparing the sequences of several universally conserved housekeeping genes.

Genomic and proteomic comparisons

While 16S rRNA gene sequence analysis and MLSA have proven to be effective tools for phylogenetics, the major deficiency inherent in these techniques is that only a small amount of information is used to represent the entire organism. This practice has largely been accepted due to restraints such as the cost, time, and complexity involved in genome sequencing. However, there are now numerous sequenced genomes that are available in publicly accessible databases. As a result, there is the opportunity to explore the use of whole genomes in analyzing evolutionary relationships. As more genomes have become publicly available, numerous different approaches to determining genomic relatedness have been attempted. One prominent example of a whole-genome similarity measure is the frequency of each possible dinucleotide. These frequencies have been found to be similar in closely related organisms and dissimilar in more distantly related organisms, and therefore constitute a "genomic signature" [2]. Even before many genomes were available, dinucleotide frequencies in different organisms were characterized and compared using what sequence data was available at the time [2]. More recently, Passel *et al.* [3] evaluated the use of this

genome signature for phylogenetics using a large number of prokaryotic genome sequences. Using a calculation called δ*, which represents the average difference in abundance for all dinucleotides in two genomes [4], they showed that intra-species distances are generally much smaller than inter-species (but intra-genus) distances. Interestingly, they also observe an inverse relationship between percent identity of the 16S rRNA gene and δ*, although the strength of this relationship appears to be quite modest and, in fact, is not precisely quantified.

Many other whole-genomic approaches to taxonomy have been explored. A genome's G+C content has been found to be highly similar in related species and less similar in more divergent species [5]. Similar patterns have been discovered for codon usage [6, 7], gene order [8], and amino acid k-mer composition [9]. These methods, as well as a number of others, are reviewed by Coenye and colleagues [10]. Earlier, Coenye *et al.* [7] performed a comparison of some of these methods and showed that the phylogenetic trees derived from these characteristics are usually quite consistent with each other, as well as with the tree derived from comparing 16S rRNA gene sequences. As these comparisons were performed on relatively small, related groups of bacteria, it remains unclear whether these results generalize to all organisms or even to all bacteria.

Another approach to whole-genome phylogenetics is the comparison of gene content. This technique works by predicting orthologues in pairs of organisms and then assigning a "distance" between that pair based on the putative number of shared genes. This technique was originally proposed by Snel *et al.* [11] and has subsequently been revisited with larger groups of organisms [12, 13]. Compared to other whole-genome techniques for phylogeny, this method seems particularly attractive, as differences in gene content among organisms are readily explicable both in terms of their evolutionary meaning (adaptation to its environment) and the mechanisms behind the evolutionary events (gene duplication, gene loss, horizontal gene transfer). In contrast, differences in G+C content, dinucleotide frequencies, gene order, and k-mer composition have no obvious functional or evolutionary interpretation, despite containing a phylogenetic signal. As such, gene content comparisons have more appeal from an evolutionary and functional perspective than other whole-genomic similarity approaches to phylogeny, and also give similar results to phylogenetic studies based on 16S rRNA gene analysis [7]. We therefore favour the use of gene content comparisons by means of

proteomic complement as a supplement to the more traditional approaches to phylogenetic analysis (e.g., analysis of the 16S rRNA gene and MLSA).

Clusters of Orthologous Groups (COGs)

The most commonly used method for gene content comparison is that of Clusters of Orthologous Groups (COGs) of proteins [14]. This method classifies proteins into COGs based on a best-hit gapped-BLAST (Basic Local Alignment Search Tool) approach [15]. The end result is clusters of proteins that are supposed orthologs and are therefore proposed to have the similar or identical functions. These clusters are then used for various applications, such as comparison of protein profiles of organisms or to putatively assign function to predicted open reading frames during genome annotation. Despite the various uses of COGs, one major downfall is that the COG database is not up to date. The database currently consists of a list of COGs that were developed in 2003 with the use of 66 genome sequences [16]. The COG database website states that a newer version is in development, which will include 261 genomes (http://ncbi.nlm.nih.gov/COG/; accessed April 26th, 2009). However, this is less than half of the microbial genome sequences available through the National Centre for Biotechnology Information (NCBI) Entrez Genome Project Database which, as of the same date, consisted of 862 sequenced microbial genomes. The NCBI database also indicates that an additional 1,565 bacterial genomes are currently being sequenced. With the already overwhelming amount of genomic information available, coupled with the exponential growth rate of genetic information, the inclusion of genomic information for all of these organisms in a comparative COG analysis would be difficult. The creation of COGs is time-consuming and labour-intensive, requiring a large amount of manual analysis even for smaller datasets such as the 66 genomes. This therefore makes it unfeasible to include all of the presently available genome sequences in the development of COGs. For feasibility, the proteomes of a subset of genome sequences is used to create the COGs. However, one problem with this is that the subset of organisms used will determine the profile of COGs, and therefore the placement of a protein into a COG greatly depends on which organisms are used to create the database. Because of this, organisms that were not part of the set used or closely related to the set used to create the COGs will have a lower number of proteins that are able to fit and be placed into a COG. As such, the number of proteins belonging to COG groups is low for proteomes of organisms not included in the creation of the COG database. As is shown here in Table 1, using a wide range of genera as an example, as little as 42.7% (and only as much as 81.5%) of the whole proteomic complement of their genomes may be

matched to preexisting COGs. In light of this problem, COGs should only be used for comparing the entire protein profiles of the organisms which were originally used (or very closely related to those used) to create a given COG database.

Because of these shortcomings, COGs have been created for smaller groups of organisms, such as lactic acid bacteria, archaea, and cyanobacteria (LaCOGs, arCOGs, and CyOGs, respectively), for the analysis of particular phenotypes or traits that these bacteria possess [17-19]. This enables the use of COGs for analyses of these organisms despite the lack of representation of these groups in the presently available COG set. Although very useful, the creation of COGs for even these small groups is difficult and time-consuming, requiring manual curation and expertise in the area of COG production. Consequently, the use of COGs for proteomic content comparison is limited, particularly when wanting to perform an analysis of organisms that are not represented in the currently existing COG set or when a comparison of all bacteria with currently sequenced genomes is desired.

In light of the problems outlined above regarding existing genome and proteome comparison methods, we sought to develop a computer program that would allow for the differences and similarities between genomes/proteomes to be identified on the level of individual comparisons (e.g., isolate *versus* isolate) or comparisons of groups of bacteria (e.g., a group of isolates comprising a species, genus, or particular phenotype *versus* a second group). Through identification of the differences between proteomes (instead of the similarities), the creation of dendrograms from the resulting data is simplified and can be compared with conventional phylogenetic methods such as 16S rRNA gene phylogeny. We also show how 'core' and 'unique' proteomes can be identified for groups of organisms. All of these methods are tied together to explore the genomic concept of prokaryotic phylogeny and how well it is reflected in standing phylogeny based on 16S rRNA gene analysis.

Results and Discussion

Measure of proteomic similarity

Our proposed measure of proteomic similarity has two main advantages over other published methods of genomic comparison. First, since larger measures represent greater dissimilarity rather than greater similarity, it can be used more naturally for linkage methods such as

Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Second, it fits naturally within the purpose of phenotypic subtraction, since proteins are being "subtracted" from one organism (or group of organisms) to reveal proteins that are unique to the second organism (or group of organisms)

An E-value cutoff was selected mathematically as described in the materials and methods section. To ensure that this E-value cutoff of 10^{-13} would be appropriate for the different types of comparisons being run in our analyses, proteomes were selected randomly for intra-species, intra-genus (but inter-species), and inter-genus comparisons over a range of E-values spanning from zero to 10^{-180}. These are provided as Figures 2A, 2B, and 2C, respectively, and show the number of unique proteins for each comparison for each E-value threshold used. Briefly, the purpose of these three separate figures (Figure 2A-2C) is to give three separate examples (on each) of how the number of proteins that are reported to be in one isolate but not another isolate of the same species/genus/different genus vary with the choice of E-value threshold. The three lines on any single graph are not mean to be compared with one another. The difference among the three plots on any one of the graphs is mostly attributed to the proteome size of each species. Further discussion of selection of E-value and interpretation of Figures 2A-2C is provided in the M.Sc. thesis of Brett Trost [20].

Identification of orthologous proteins and selection of candidate groups for "shape" phenotype of *Lactobacillus*

The *Lactobacillus* genus is polyphyletic, with the *Pediococcus* genus residing at its centre (Chapters 9 and 11). Lactobacilli are mostly rod-shaped bacteria while the pediococci are always cocci. It is possible that the *Pediococcus* genus has lost a gene or genes involved in producing the rod shape of the *Lactobacillus* cells. Because the *Lactobacillus* genus is known to have an accelerated rate of genomic reduction and gene loss (i.e., genes not being used are rapidly lost) [17, 18], we hypothesized that by performing a subtraction of the orthologous groups that were present in all *Lactobacillus* isolates from the proteins in *Pediococcus* isolates, candidate proteins involved in determining the rod-shape of *Lactobacillus* could be identified. Using this approach, nine candidate protein groups were identified using an E-value cutoff of 10^{-13} (Table 2).

While some candidate groups are certainly not involved in cell shape, all protein groups are *Lactobacillus*-specific in that they do not occur in the *Pediococcus* isolate. Figure 1A shows

an example of a graph of putatively orthologous proteins, and an enlarged section of this figure is shown in Figure 1B. In Figure 1B, the interactive components of the graph can be seen. These include a protein ID number, organism information, a description and keywords for the protein in question, and also GO numbers which link to the gene ontology website, thus providing a user-friendly interface through which researchers can gain additional up-to-date information about the candidate group of interest. Figure 1A has also been included in an electronic format as Additional File 2 where the interactive features of the graph may be explored using an internet connection. Because of the type of information that has been included in the graph, one can readily identify that candidate groups eight and nine are likely to be of interest to the rod cell-shape phenotype in question (Table 2). A feature that may be of particular interest is that putative and uncharacterized proteins also result from this comparison. Candidate protein groups two, three, and four have only "putative" functions, while candidate groups five and six are only known as hypothetical proteins. This type of phenotype-driven genomic analysis may prove to be useful in elucidation of novel biochemical and/or metabolic pathways that may be involved in a phenotype of interest.

Identification of core and unique genomes for species and genera

We sought to determine whether identification of orthologous proteins could provide information regarding the genomic content of a taxonomic unit. Here we applied the same approach as was used to identify putative phenotype-related proteins, but rather than grouping based on a phenotype, isolates were grouped based on genus or species assignment. The protein content for each genome in a group was compared and proteins that were present in each isolate of a group (as determined by best bidirectional BLAST hit with an E-value threshold of 10^{-13}) were assigned to the "core" proteome category (Table 3). A connected components graph was created for each comparison. We refer to each connected component as a "protein set". For some orthologues and/or paralogues, multiple copies of proteins exist within one set making the total "number of proteins" larger than the "number of protein sets". This may relate to certain protein sets being of particular importance to an organism's function. In an extension of the construction of core proteomes, the core proteomes for given taxonomic groups (genera or species) were subtracted from one another to determine which protein sets occurred in all of the isolates of one species, but did not occur in any of the isolates in the other species in question, thus determining a "unique" proteome (Table 3). As was found with the core proteomes, some of the protein groups in the unique proteome

contained more than one protein from each isolate. The unique proteome can be regarded as the protein complement that makes the species or genus distinct from other taxonomic units. The DNA sequences corresponding to the ORFs in the unique proteome would therefore be good candidates for group-specific identification methods such as a species- or genus-specific PCR method.

An interesting application of the calculation of a "unique" proteome may be to identify species that contain erroneously named isolates. For example, *Bacillus cereus* and *Bacillus thuringensis* contain a very small number of proteins within their unique proteome in comparison to other species (Table 3). This suggests that there may be isolates that were placed within other species of the genus that should actually be named as one of these two species. A look at the 16S rRNA gene of the *Bacillus* isolates used in this study confirms that some of the *B. cereus* and *B. thuringensis* isolates in fact have 99-100% identity with the opposite species, and a lower percent identity with the species to which they are currently assigned. As it can be difficult to resolve speciation using only the 16S rRNA gene, the approach of using the unique proteome may well assist in the proper naming of isolates that are difficult to speciate.

When the core and unique proteomes are being calculated, it is also possible to search for additional information regarding the characteristics of the proteomes in question. Some examples of data are given in Table 4, including 'singlet' proteins (i.e., proteins found only within one isolate), average proteome size, and average number of groups and proteins within an isolate.

Comparison of genomic content with percent 16S rRNA gene similarity

Phylogenetic studies currently use the 16S rRNA gene sequence as the "gold standard" for taxonomic classification of prokaryotes. It is therefore of interest to determine how the percent identity of the 16S rRNA gene correlates with proteomic similarity (which could also be interpreted as a measure of phenotypic relatedness). Cut-offs currently used for the 16S rRNA gene are 99% identity for a species and 90-94% identity for a genus (depending on the group in question). In Figure 3, the pairwise number of different proteins between isolates *versus* percent identity of the 16S rRNA gene is plotted for the 16 genera used in this study. The corresponding numerical values corresponding to the range of 16S rRNA gene percent

identity and range of differences in pairwise unique proteins are given in Table 5. Pairwise comparisons of each proteome were performed intra-genus, but excluding intra-species comparisons as this would introduce a bias for genera where multiple isolates have had genomes sequenced for a single species. As there appeared to be confusion in the nomenclature of several organisms (i.e., two isolates given different species names, but sharing >99% 16S rRNA gene identity), a conservative cutoff of 99.5% 16S rRNA gene identity was used rather than the given species naming. A scatter plot of 16S rRNA gene percent identity (x-axis) *versus* number of unique proteins (y-axis) was generated for each genus on the same graph. Several unexpected observations arose from this data plot. The most obvious anomaly is that the genera *Clostridium* and *Lactobacillus* extend well beyond the lower boundary of 90% identity of the 16S rRNA gene, with the *Clostridium* genus containing two distinct clusters. A second observation is that there is no specific range of proteomic diversity for a genus. Despite the fact that genera are created using cut-offs of 16S rRNA gene similarity, there does not appear to be a corresponding range of proteomic similarity.

While other studies have reported a supposed correlation between genomic similarity and identity of the 16S rRNA gene, no statistical correlation has been reported. A substantial review of this topic is given by Rosello-Mora and Amann [21]. Due to this and also the unexpected observation discussed earlier that no specific range of proteomic diversity was evident for genera, the slope (y) and correlation coefficient (R^2) were calculated from a best-fit linear line for each genus to attempt to shed additional insight as to the relationship between the 16S rRNA gene and proteomic similarity (Table 5). It was found that the slope of the best-fit lines varied greatly between genera. Perhaps surprisingly, little or no correlation was found between the similarity of organisms based on 16S rRNA gene identity and proteomic similarity for most genera. Specifically, if $R^2 > 0.5$ is interpreted as indicating similarity, then only 4 of 14 groups had a correlation between 16S rRNA gene identity and similarity of bacterial proteome. Together, these findings indicate that the genomes of the various genera undergo different rates of evolution relative to evolution of the 16S rRNA gene and that whole genome evolution is under different (or additional) selective pressures compared to that of the 16S rRNA gene.

Finally, and even more interesting, genera that are known to be intracellular or have life cycles that are highly dependant on their host organisms (i.e., *Neisseria* and *Rickettsia*) have

an inverse association between proteomic similarity and 16S rRNA gene, indicating that their genome is under a completely different selective pressure than is seen by the exertion of normal evolutionary pressures that affect housekeeping genes. This unexpected finding may someday prove useful in gaining an understanding of the different evolutionary pressures that bacteria face. As evolutionary pressures experienced by organisms will differ based on their environmental niche and life cycle, we will expect to see different patterns of association between 16S rRNA gene identity and proteomic content emerge as a greater number of genome sequences become available.

Construction of dendrogram from unique proteomes

With the unexpected findings from the comparisons of the 16S rRNA gene and of the proteome in mind, we sought to determine whether the phylogeny as determined by the 16S rRNA gene was in fact representative of organism similarity based on proteomic content. A dendrogram was constructed based on differences in the protein content of pairs of organisms. In light of the different evolutionary rates seen between the 16S rRNA gene and the proteome as discussed for Figure 3 and Table 5, it was reassuring to see that the taxonomies determined by both methods produced a number of robust clades corresponding to the respective genera. Due to the size of the images, the full proteome-based dendrogram is provided in Figure 4 (expanded in Additional File 3) and the corresponding 16S rRNA gene phylogenetic tree in Figure 5. Also shown in the dendrogram in Additional File 3 is the number of protein differences between branches on the tree. That genera group into similar taxonomic units in both these two trees suggests that despite being under different evolutionary influences, the 16S rRNA gene and the proteome generally evolve in similar courses, creating similar evolutionary paths or relationships.

While the two trees were quite similar (i.e., most genera in the analyses formed distinct taxonomic units), there were several discrepancies between the taxonomy as determined by the 16S rRNA gene and that of the proteomic dendrogram. Most noticeably, some genera did not group based on higher taxonomic classifications (e.g., in the proteomic dendrogram *Rickettsia* groups within the *Firmicutes* Phylum although it is taxonomically designated as *Proteobacteria*). The following are descriptions (alphabetically by genus) where isolates grouped differently in the proteome dendrogram constructed by proteomic similarity compared to that inferred by 16S rRNA gene phylogeny. *Bacillus*: two distant clades were

formed; the first clade formed a distinct unit comprised of *B. anthracis, B. cereus*, *B. thuringensis,* and *B. weihenstephanensis*, with the second clade containing the remaining species examined and residing more closely to *Brucella* and several *Clostridium* species. *Clostridium*: *C. beijerinckii*, *C. thermocellum*, and *C. difficile* branched separately and distinctly from their genus, from each other, and from all other genera, while *C. acetobutylicum, C. kluyveri,* and *C. phytofermentans* formed a distinct clade distant from the core *Clostridium* genus. *Mycobacterium*: *M. leprae* was extremely distant (residing near the *Rickettsia* genus) from all other *Mycobacterium* species which otherwise formed a distinct taxonomic unit. *Lactobacillus*: *L. plantarum* and *L. casei* branched separately and distinctly from their genus and from each other.

Several explanations may account for circumstances in which proteomic similarity and 16S rRNA similarity are incongruous. Possible scenarios are that these proteomic outlier species may inhabit different environments than other members of their designated genus. However, it is certain that these outlier species have a drastically different protein complement than their nearest neighbouring species as based on 16S rRNA gene sequence phylogeny. In turn, this suggests a great difference in phenotypic and metabolic potential would exist between these outlier isolates and the isolates forming their respective genus.

Conclusions

The ability to create "core" and "unique" proteomes confers the ability to determine where species are either divergent due to accelerated rates of genomic evolution compared to 16S rRNA gene evolution or have been misidentified or misclassified, which can also be supported by 16S rRNA gene similarity. Perhaps more importantly, however, putative phenotype-related genes/proteins can be suggested based on subtraction of proteomes of bacterial isolates that are grouped based on phenotypic properties. Here we used this approach to suggest genes putatively involved in conferring the rod shape to *Lactobacillus* isolates in contrast to their closest phylogenetic neighbours, the cocci *Pediococcus*.

Phylogeny of the 16S rRNA gene is largely in agreement with phylogeny as constructed by whole proteomic similarity. However, there are several discrepancies suggesting that large scale genomic change can occur with only a small shift in 16S rRNA gene similarity. This implies that certain proteomic outlier species may be best studied outside of the context of the genus to which they have been assigned. Alternatively, these species may warrant further study to determine whether they may be more accurately renamed as novel genera in the future.

Methods

Proteomes used

The following criteria were used to select bacterial genera to be used in this study: A) there were more than two species with sequenced genomes and B) at least two of these species has at least two isolates with a sequenced genome so that intra-species comparisons could also be conducted. These criteria resulted in the selection of a total of 16 genera, comprising 107 species and 214 isolates. Table 3 gives a summary of the number of isolates and species used per genus, while the Supplementary Data file 1 provides information regarding each isolate (i.e., genus, species, isolate identity, size of proteome, and total size in base pairs). The proteomes for these bacterial isolates were downloaded on November 28th, 2008 from the European Bioinformatics Institute website at http://www.ebi.ac.uk/genomes/bacteria.html.

Selection of BLAST E-value cutoff

The following is the logical argument we used for choosing a particular BLAST E-value cutoff. Suppose that the number of proteins encoded by the organism with the largest proteome in a given comparison is n_p. Also, let n_o denote the number of organisms involved in this comparison. For each pair of organisms, there will be at most $n_p \times n_p = n_p^2$ pairwise comparisons between proteins. The number of pairs of organisms that must be compared (note that comparisons must be performed in both directions) is $n_o \times (n_o - 1) \approx n_o^2$. Thus, the total number of protein-protein comparisons that must be performed will be bounded above by $n_p^2 n_o^2$. The expected number of spurious matches M will be equal to the number of comparisons performed, multiplied by the probability of a spurious match in each comparison. Let P be the probability of a spurious match. Then:

$$M = P n_p^2 n_o^2$$

How can we derive a value for P? The E-value, which we will denote simply as E in this section, represents for a particular match the number of random matches attaining a score equal to or better than the score actually obtained that would occur given the size of the database. While E does not represent a probability, P can be derived from it: since the probability of finding no random matches with a score greater than or equal to the score actually obtained is e^{-E}, where e is the base of the natural logarithm, then the chance of obtaining one or more such match is $P = 1 - e^{-E}$ [22]. Since P is nearly identical to the E-value

when the E-value is less than 0.01, E can reasonably be used as a proxy for P. As such, the expected number of spurious matches M as a function of E is:

$$M = En_p^2 n_o^2$$

By rearranging, we can get an equation that expresses the E-value threshold that should be chosen in terms of n_p, n_o, and the desired value for the expected number of spurious matches M:

$$\mathrm{E} = \frac{M}{n_p^2 n_o^2}$$

For a given comparison, one will know the values of n_p and n_o, and the value of M that is chosen may depend on the particular application. For simplicity's sake, however, it would be convenient to choose a single E-value that is appropriate for all comparisons done for this thesis. The largest bacterial genome examined in this study is that of *Burkholderia xenovorans*, which encodes $8951 \approx 10^5$ proteins. Thus, a conservative value for n_p would be 10^5, and an estimate for the greatest number of pairwise comparisons that would take place between two bacteria is $n_p^2 = 10^{10}$. Furthermore, the largest group analysis done in this manuscript includes approximately 30 organisms (i.e., the *Streptococcus* genus). Then $n_o = 30$, and the number of comparisons between pairs of organisms is approximately $n_o^2 = 900$. Then the total number of pairwise protein comparisons would be $n_p^2 n_o^2 = 10^{10} \times 900 \approx 10^{13}$. If we wanted the expected number of matches that should occur by chance to be 1 (a single spurious match should have little effect in any of our analyses), then we should choose the E-value as follows:

$$\mathrm{E} = \frac{1}{10^{10} \times 10^3} = 10^{-13}$$

This E-value is always rather conservative, given that most comparisons involve fewer than 30 organisms, and that all of the bacterial proteomes in fact have fewer than 10^5 proteins. Thus, the actual number of expected spurious matches for all comparisons is, in fact, less than 1.

Identification of orthologous proteins

We chose to take a very simple method to identify groups of orthologous proteins. First, the BLAST [22] was used to determine best bidirectional matches between each possible pair of proteins in each possible pair of organisms. A graph was then created wherein each vertex represents a protein. Two vertices are connected by an edge if the protein represented by

each is the other protein's best BLAST hit, and if the E-value for the hits in both directions is less than 10^{-13}. Identification of orthologous groups was then performed by finding the connected components of the graph (i.e., sets of vertices for which there is a path from any vertex to any other vertex). Each vertex had the following information associated with it: the protein's accession number, the source organism, a description and keywords for the protein in question, and a gene ontology (GO) term(s), each of which provides a standardized description of the protein's cellular location, molecular role, or biological process [23].

Finding candidate proteins for phenotypes

To identify proteins which may be involved in rod cell shape of *Lactobacillus* versus the cocci *Pediococcus* cell shape, we used the ortholog detection procedure described above. For this comparison, the proteomes of 15 isolates from the *Lactobacillus* genus (*L. acidophilus* NCFM, *L. brevis* ATCC 367, *L. casei* ATCC 334, *L. casei* BL23*, L. delbrueckii* ATCC 11842, *L. delbrueckii* ATCC BAA-365, *L. fermentum* IFO 3956, *L. gasseri* ATCC 33323, *L. helveticus* DPC 4571, *L. reuteri* F275, *L. johnsonii* 533, *L. plantarum* WCFS1, *L. reuteri* 100-23, *L. reuteri* F275, *L. sakei* 23K, and *L. salivarius* UCC118), as well as the sole isolate of *Pediococcus* whose whole genome has been sequenced to date (*P. pentosaceus* ATCC 25745). Those connected components of the graph that contained at least one protein from all of the *Lactobacillus* isolates, and did not contain any proteins from *P. pentosaceus*, were considered candidate proteins for the cell shape phenotype.

Identifying "core" and "unique" proteomes of groups

We have applied the term "core" proteome to refer to the proteins that are present in every isolate of a predetermined group (e.g., a genus or species). To find the core proteome for a bacterial genus or species, the orthologue identification procedure explained above was performed within each genus or species using all available isolates from the genus. The core proteome was then determined by finding connected components of the graph containing proteins from all of the isolates from that genus or species. The number of these connected components was then counted to determine the size of the core proteome. In an extension of this concept, we use the term "unique" proteome to refer to those proteins that are present in all members of the selected group and not found in any isolate of any other group in the comparison.

Comparison of proteomic content with 16S rRNA gene similarity

Measure of proteomic similarity was compared to 16S rRNA gene similarity by several methods. The 16S rRNA gene was obtained from each sequenced genome used in this study and the RDPII tool was used to align sequences based on known conserved and variable regions according to the rRNA's secondary structure [24]. The percent identity of the 16S rRNA gene was calculated to the nearest 0.01% in a pairwise fashion. A phylogenetic tree of the 16S rRNA gene was created by the UPGMA method and visualized using the MEGA software package [25].

Proteomic dendrogram

For each pair of organisms A and B, we determined the number of proteins encoded by the genome of organism A that are not encoded by the genome of organism B. This was done by identifying orthologues in A and B as described above, then looking for groups containing only proteins from organism A, and no proteins from organism B. The same procedure was then used to find the number of proteins unique to organism B. For the purposes of creating a dendrogram, the "distance" between organisms A and B was defined to be the average of the number of proteins unique to organism A and the number of proteins unique to organism B. This number is given on the branch node in Additional File 3. By this calculation, pairs of organisms with a smaller "distance" have more similar protein complements. These distances are then used to create a phylogenetic tree using the UPGMA method and the dendrogram was visualized using the MEGA software program [25].

Authors' contributions

Monique Haakensen conceived the study, participated in the design and coordination of the study, performed statistical analyses and biological interpretation, and drafted parts of the manuscript.

Brett Trost participated in the design and coordination of the study, developed the software programming, performed computational analyses, and drafted parts of the manuscript.

Vanessa Pittet helped to draft the manuscript, assembled data, and provided scientific input regarding biological interpretation.

Anthony Kusalik participated in the design and coordination of the study, edited the manuscript, and is the holder of a research grant partially used to fund the study.

Barry Ziola conceived the study, participated in the design and coordination of the study, edited the manuscript, and is the holder of a research grant partially used to fund the study.

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Figures

Figure 1A - Graph of MreB-like protein "candidate protein group 8" corresponding to Table 2. The dotted outline indicates the region enlarged in Figure 1B.

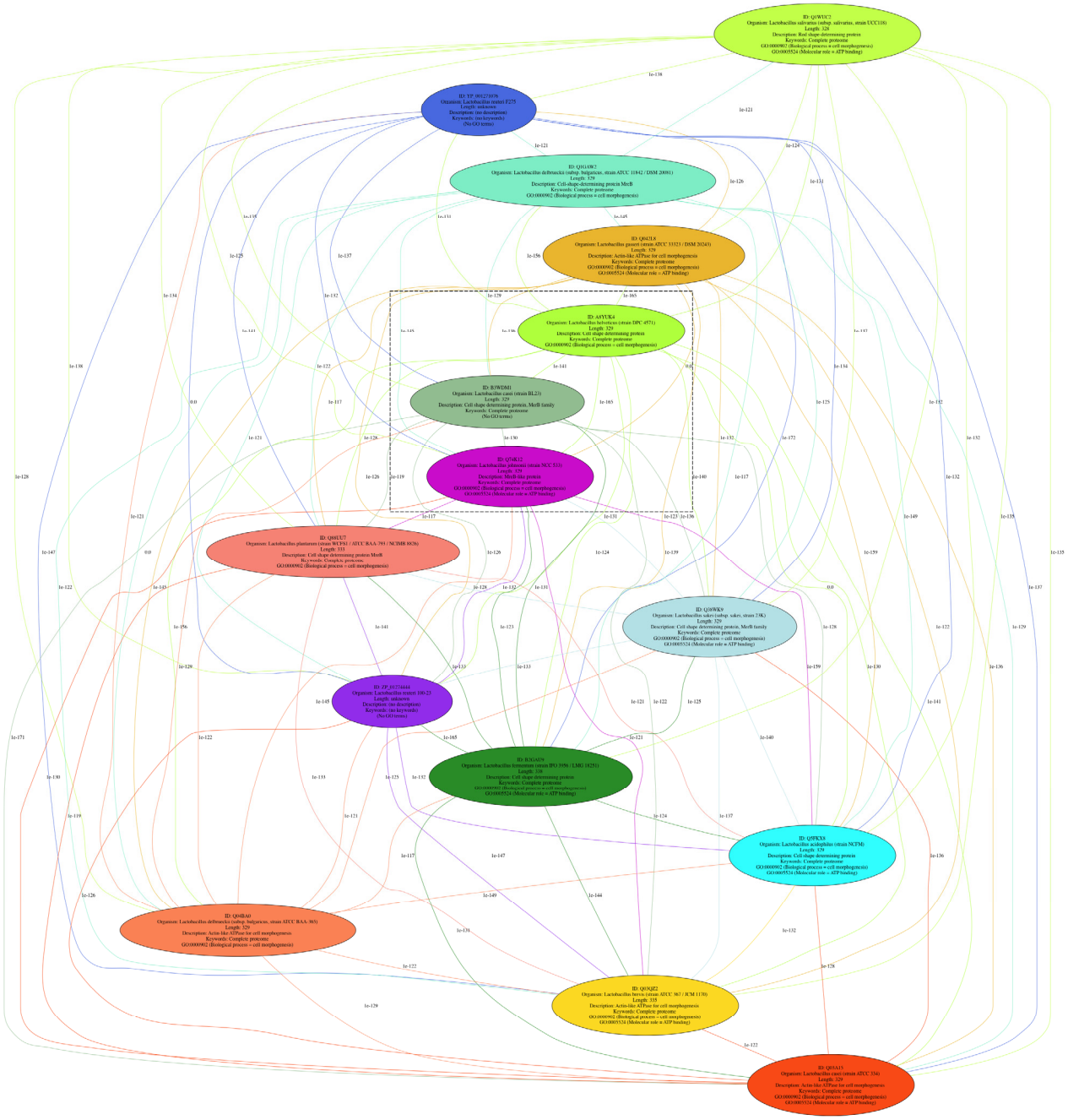


Figure 1B - Close-up of outlined area from graph in Figure 1A for "candidate protein group 8" showing provided data and examples of hyperlink options which are accessible through Additional File 2.

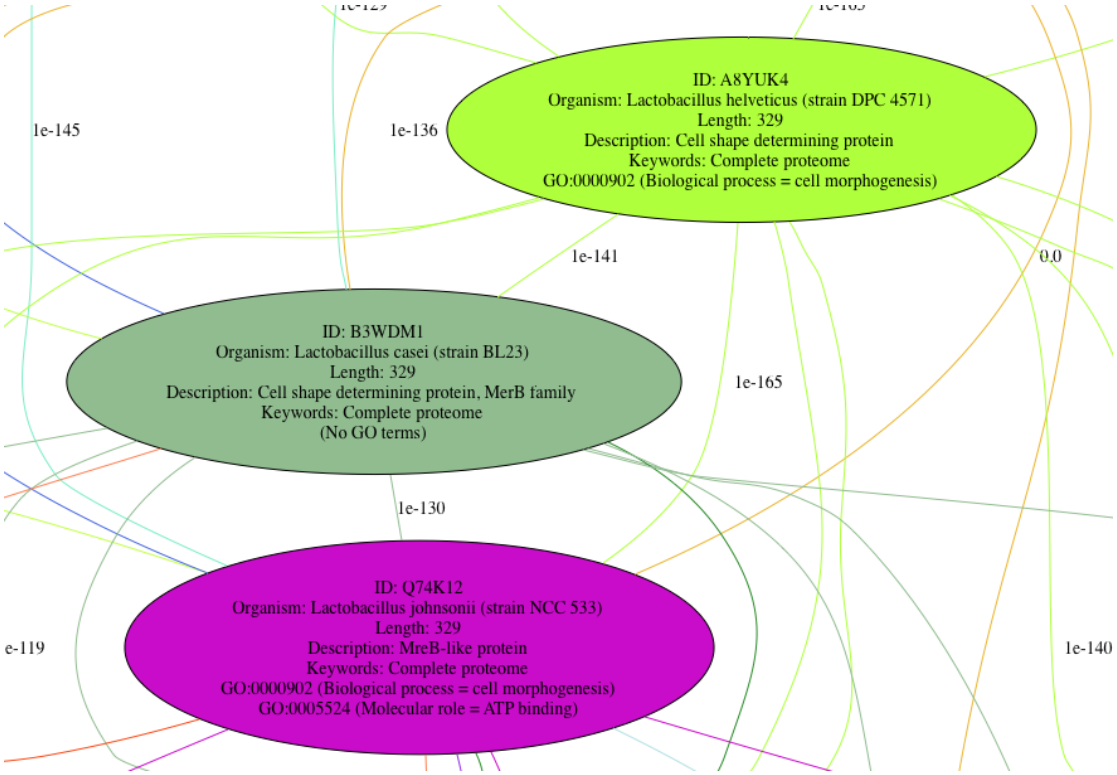
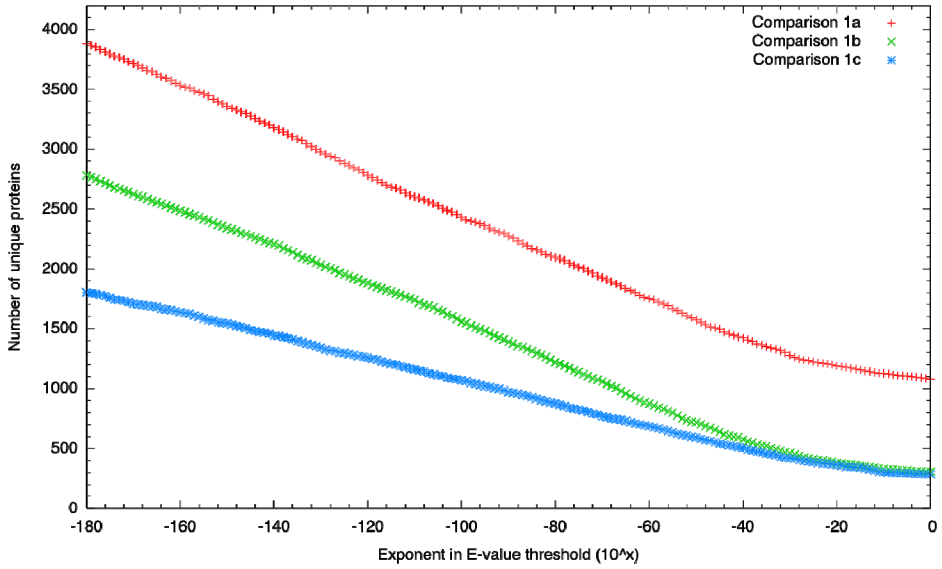
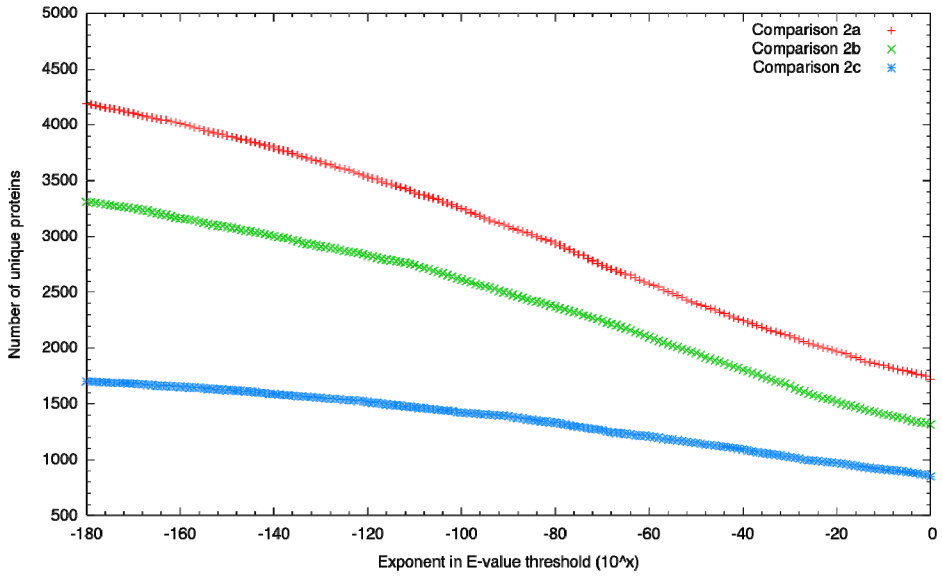


Figure 2 – Evaluation of E-values used as cutoff for selection of bidirectional best BLAST hits. 2A, Intra-species; 2B, intra-genus (inter-species); and 2C, inter-genus comparisons are made between randomly selected pairs of organisms.

2A – Intra-species, comparison 1a - *Pseudomonas putida* GB-1 *vs Pseudomonas putida* KT2440; comparison 1b – *Staphylococcus aureus* COL *vs Staphylococcus aureus* JH1; comparison 1c – *Xanthomonas campestris* 8004 *vs Xanthomonas campestris* B100.



2B – Inter-species, intra-genus, comparison 2a – *Burkholderia mallei* ATCC 23344 *vs* *Burkholderia xenovorans* LB400; comparison 2b – *Vibrio cholerae* ATCC 39315 *vs Vibrio fischeri* ATCC 700601; comparison 2c – *Streptococcus pyogenes* MGAS2096 *vs* *Streptococcus thermophilus* ATCC BAA-250.



2C – Inter-genus, comparison 3a – *Bacillus anthracis* Ames ancestor *vs Shigella flexneri* ATCC 700930; comparison 3b – *Mycobacterium tuberculosis* ATCC 25177 *vs Neisseria meningitidis* 053442; comparison 3c – *Yersinia enterocolitica* 8081 *vs Clostridium tetani* E88.

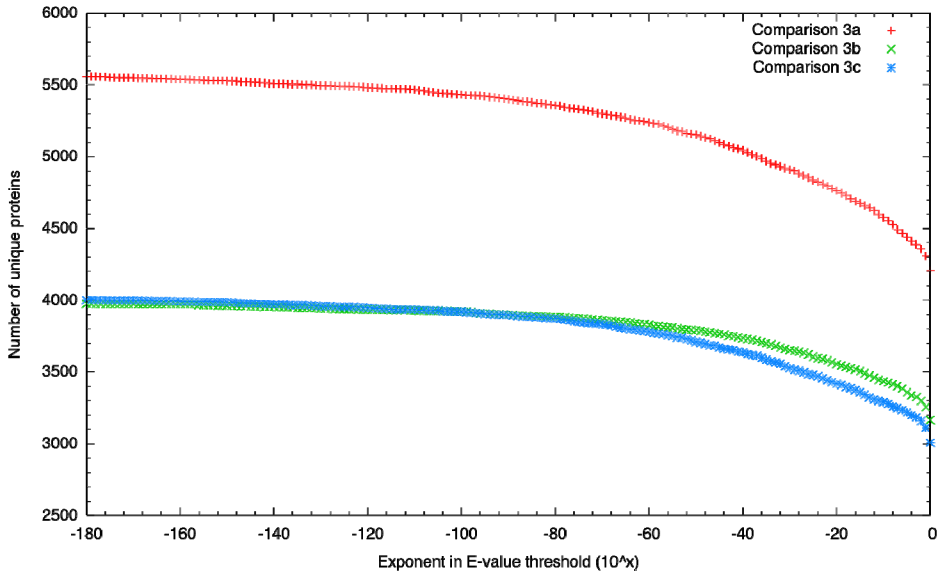


Figure 3 – Percent identity of the 16S rRNA gene *versus* number of differences between proteomes. Pairwise comparisons of each genome were performed intra-genera but excluding intra-species comparisons. The slope (y) and correlation coefficient (R^2) are given for the best fit line for each genus and are also presented in Table 5.

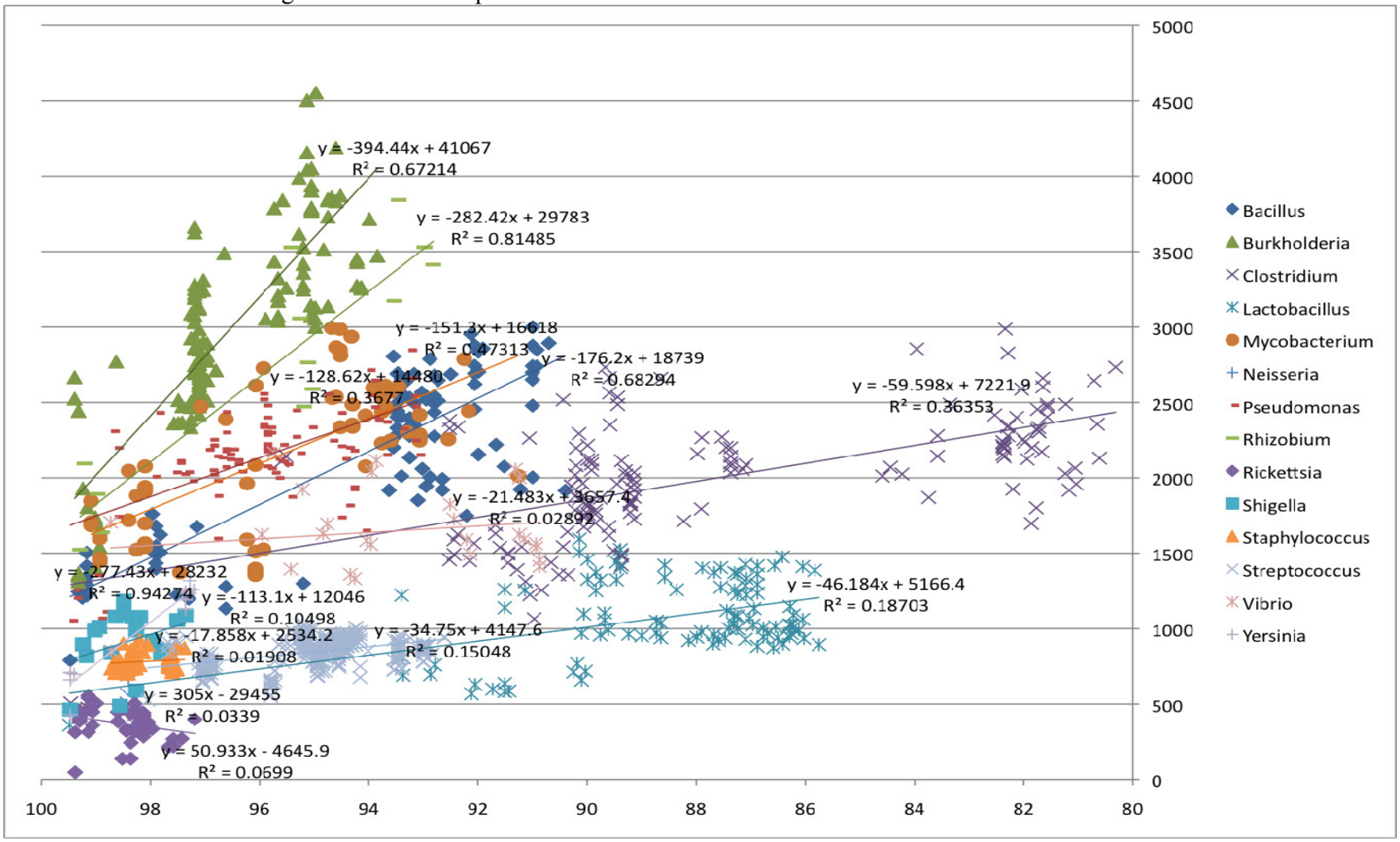


Figure 4 – Dendrogram constructed from unique proteome of all isolates included in comparison with clades compressed from Additional File 3. Clades shaded in black indicate genera that branch as a single group. Clades shaded in grey indicate genera that branch inconsistently as compared to 16S rRNA gene sequence phylogeny. Bar indicates 400 unique proteins in pairwise comparison between proteomes.

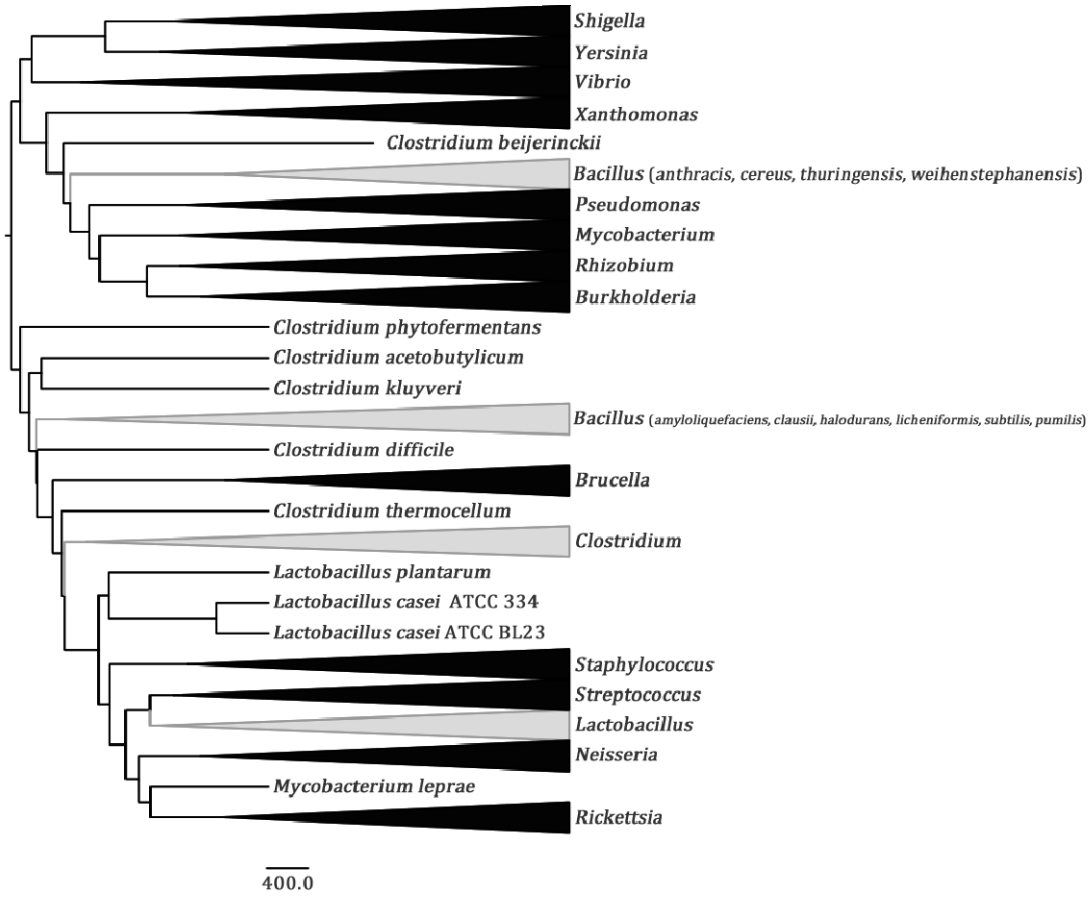
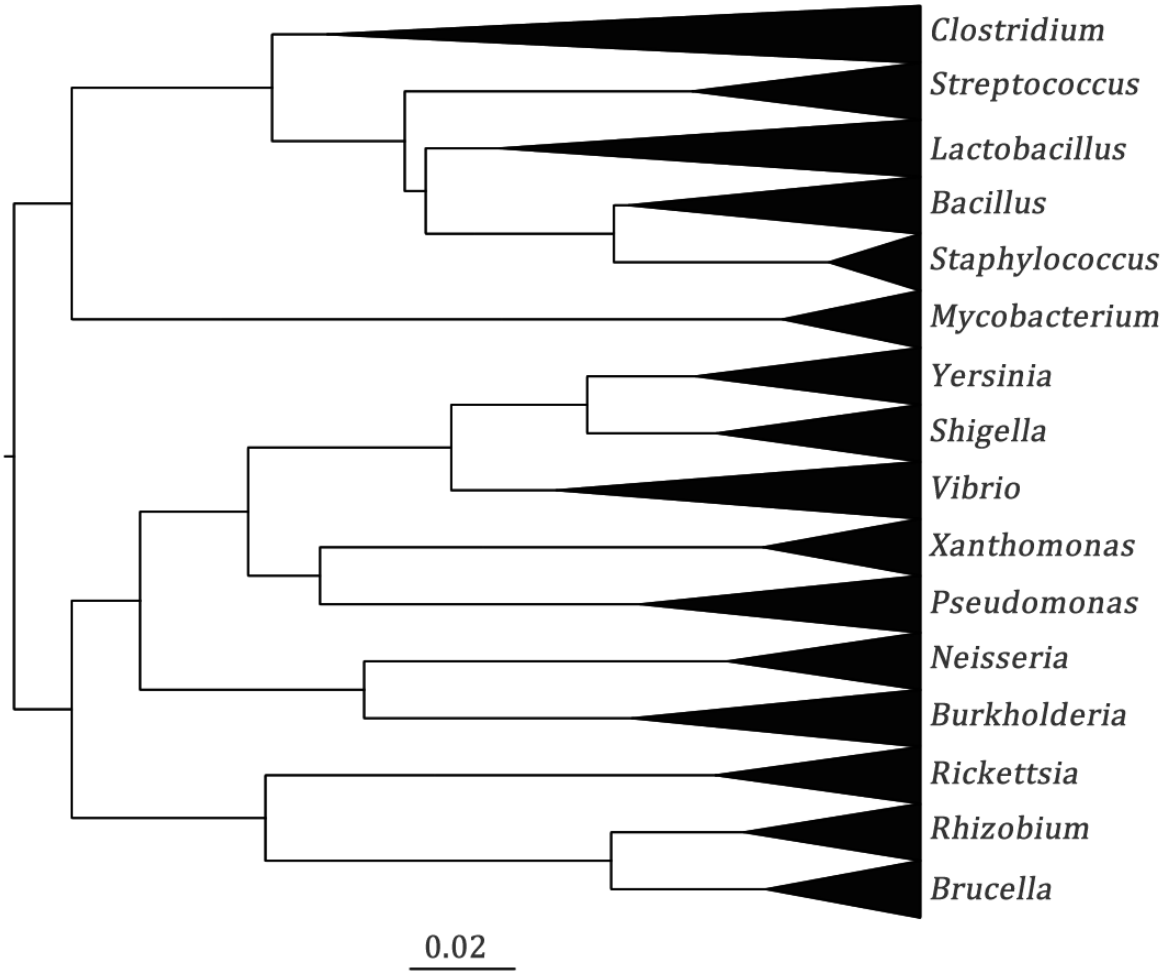


Figure 5 – Phylogenetic tree of the 16S rRNA gene of all isolates included in the proteomic comparison in Figure 4. Bar indicates 2% divergence.



Tables

Table 1 - Percentage of proteome covered by the COG database as of January 2009.

Genus	% of proteome in COG database	Size of Proteome
Bacillus	59.7 – 74.6	4163 – 6115
Brucella	71.1 – 81.4	3066 – 3419
Burkholderia	60.7 – 78.1	5295 – 7307
Clostridium	64.8 – 73.3	2702 – 4092
Lactobacillus	52.0 – 79.3	1721 – 3044
Mycobacterium	42.7 – 72.3	3954 – 4235
Neisseria	57.6 – 74.7	2042 – 2741
Pseudomonas	73.1 – 81.0	5242 – 6371
Rhizobium	74.8 – 77.9	6089 – 7337
Rickettsia	56.1 – 81.5	1382 – 1513
Shigella	73.9 – 79.0	4390 – 4852
Staphylococcus	66.9 – 77.4	2601 – 2971
Streptococcus	67.2 – 74.7	1803 – 2295
Vibrio	62.5 – 76.0	3981 – 5196
Xanthomonas	62.4 – 73.7	4253 – 5048
Yersinia	72.0 – 79.5	4204 – 4491

Table 2 - Candidate protein groups for "rod cell shape" created by subtracting *Pediococcus pentosaceus* from all *Lactobacillus* genomes.

Candidate group	Description of protein
1	Orotate phosphoribosyltransferase
2	Putative uncharacterized protein, Putative endopeptidase La, Ion-like protease with PDZ domain, Hypothetical lipoprotein
3	Putative chromosome partitioning protein ParB, DNA-binding protein, Effector of nucleotide occlusion (Noc)
4	Putative hydrocarbon binding protein, Putative uncharacterized protein
5	Putative uncharacterized protein
6	Putative secreted uncharacterized protein
7	Cell division protein SepF, YlmF, Putative uncharacterized protein,
8	Rod-shape (cell-shape) determining protein MreB, MreB-like protein, Actin-like ATPase for cell morphogenesis
9	Rod-shape (cell-shape) determining protein MreB, MreB-like protein, Actin-like ATPase for cell morphogenesis

Table 3 - Core and unique proteomes

	Number of isolates (species)	Core proteome[a]		Unique proteome[b]	
		Number of protein sets[c]	Total protein sets[d]	Number of protein sets	Total protein sets
Bacillus	16 (10)	1342	25382		
anthracis	3	4941	14823	168	504
cereus	4	2881	11631	2	8
thuringensis	2	4255	8510	4	8
Brucella	8 (5)	2234	17988		
abortus	3	2699	8097	2	6
suis	2	3025	6050	5	10
Burkholderia	19 (10)	2319	47606		
ambifaria	2	5908	11218	198	396
cenocepacia	3	3987	18224	168	504
mallei	4	3623	14512	18	72
pseudomallei	4	4972	19912	45	180
Clostridium	19 (10)	543	14289		
botulinum	8	1514	12446	10	87
perfringes	3	2110	6333	298	896
Lactobacillus	15 (12)	518	8998		
casei	2	2355	4710	593	1186
delbrueckii	2	1372	2744	222	444
reuteri	2	1402	2804	120	240
Mycobacterium	14 (11)	1125	16725		
bovis	2	3822	7644	36	72
tuberculosis	3	3724	11172	26	78
Neisseria	6 (2)	1371	8276		
gonorrhoeae	2	1795	3590	229	458
meningitidis	4	1547	6219	75	318
Pseudomonas	15 (7)	1936	31392		
aeruginosa	3	4959	14898	571	1717
fluorescens	2	4206	8412	142	284
putida	4	3799	15264	69	276
syringae	3	3894	11712	290	874
Rhizobium	7 (4)	2250	14308		
etli	2	4700	9400	251	502
leguminosarum	2	3678	7356	99	198
Rickettsia	11 (8)	686	7574		
bellii	2	1277	2554	219	438
rickettsii	2	1221	2442	93	186
Shigella	7 (4)	2328	16645		
boydnii	1	3170	6340	95	190
flexneri	3	3255	9788	130	395
Staphylococcus	18 (4)	1407	25733		
aureus	14	1917	27118	157	2292
epidermidis	2	2080	4160	131	262

Table 3 continued

	Number of isolates (species)	Core proteome		Unique proteome	
		Number of protein sets	Total protein sets	Number of protein sets	Total protein sets
Streptococcus	31 (9)	735	23727		
agalactiae	3	1688	5070	156	468
pneumoniae	6	1543	9403	150	929
pyogenes	13	1348	17723	49	709
suis	2	1971	3942	336	672
thermophilus	3	1359	4082	145	435
Vibrio	8 (5)	2193	17898		
cholerae	2	3384	6768	425	850
fischeri	2	3380	6760	447	894
vulnificus	2	3882	7764	321	642
Xanthomonas	8 (3)	2666	21488		
campestris	4	3376	13543	49	200
oryzae	3	3276	9863	299	922
Yersinia	12 (3)	2431	29596		
pestis	7	2986	21052	21	152
pseudotuberculosis	4	3424	13731	21	89

[a] Proteins that were found to be present in all isolates of the given genus or species.

[b] Proteins that were found to be present in all isolates of the given species, but not present in any other isolate of different species within the respective genus.

[c] Number of protein sets identified.

[d] Number of proteins present in the protein sets.

Table 4 – Characteristics of proteomes

Genus	Found in only 1 isolate[a]	Found in >1 but < all isolates[b]		Total in analysis[c]		Average proteins per proteome[d]	
	sets[e]	sets	proteins[f]	sets	proteins	sets	proteins
Bacillus	7736	5962	42952	15040	76070	940	4754
Brucella	567	1177	6243	3978	24798	497	3100
Burkholderia	14286	9406	62056	26011	12398	1369	6524
Clostridium	10938	5692	39844	17173	65071	904	3425
Lactobacillus	5058	3279	16645	8855	30701	590	2047
Mycobacterium	7762	6395	38970	15282	63457	1092	4533
Neisseria	1269	866	2764	3506	12309	584	2052
Pseudomonas	7597	6799	41020	16332	80009	1089	5334
Rhizobium	7953	4246	14562	14449	36823	2064	6137
Rickettsia	1340	1008	4446	3034	13360	276	1215
Shigella	2673	2042	85457	7043	27775	1006	3968
Staphylococcus	2644	2010	18923	6061	47300	337	2628
Streptococcus	3838	3756	32347	8329	59912	269	1933
Vibrio	5461	3472	11937	11126	35296	1391	4412
Xanthomonas	3899	2553	9542	9118	34929	1140	4366
Yersinia	2806	2305	16057	7542	48459	629	4038

[a] The number of sets and the total number of proteins in these sets was the same.

[b] Number of proteins that were could in more than one but less than all isolates of a genus.

[c] Total number of proteins that were found in the analysis of each genus.

[d] Averages per proteome in a genus.

[e] Number of proteins sets identified.

[f] Number of proteins found in the protein sets.

Table 5 - % identity of 16S rRNA gene *versus* proteomic similarity. Graph containing data points for all pairwise comparisons is available as Supplementary Data file 5.

Genus	# isolates	# comparisons	Range 16S rRNA gene % identity[a]	Range # different proteins	Slope	R^2
Bacillus	16	120	90.4-100	248-3000	-176.20	0.6829
Brucella	8	28	99.9-100	154-454	ND[b]	ND
Burkholderia	19	171	93.8-100	337-4554	-394.44	0.6721
Clostridium	19	171	80.3-100	141-2987	-59.598	0.3635
Lactobacillus	15	105	85.8-100	235-1595	-46.184	0.1870
Mycobacterium	14	91	91.3-100	87-2994	-151.30	0.4731
Neisseria	6	15	98.4-100	206-753	+305.00	0.0339
Pseudomonas	15	105	93.1-100	383-2847	-128.62	0.3677
Rhizobium	6	15	92.8-99.9	1296-3843	-282.42	0.8149
Rickettsia	11	55	97.2-100	48-556	+50.933	0.0699
Shigella	7	21	97.4-99.7	463-1185	-113.10	0.1050
Staphylococcus	18	153	97.4-100	49-923	-17.858	0.0191
Streptococcus	31	465	92.6-100	84-1028	-34.750	0.1505
Vibrio	8	28	90.9-99.8	396-2167	-21.483	0.0289
Xanthomonas	8	28	99.8-100	201-1653	ND	ND
Yersinia	12	66	97.2-100	216-1319	-27.433	0.9427

[a] Isolates sharing ≥99.5% identity of the 16S rRNA gene were not used in the calculation of slope or R^2.

[b] ND, Not determined; despite having different species names, all isolates with sequenced genomes within this genus shared >99.5% identity of the 16S rRNA gene.

Additional files

Additional file 1 – Isolates used in this study. Genus, species, isolate identity, size of proteome, and total size of genome in base pairs is given.

Additional file 2 – Interactive graph of "candidate protein group 8" for Mre-like protein. The same graph as printed in Figure 1A but in electronic format to allow the reader to explore the built-in interactive features.

Additional file 3 – Proteome dendrogram constructed from pairwise comparisons of the proteomes with clades expanded from Figure 4.

References

1. Woese CR: **Bacterial evolution.** Microbiol Mol Biol Rev 1987, **51**(2):221-271.

2. Karlin S, Burge C: **Dinucleotide relative abundance extremes: a genomic signature.** Trends Genet 1995, **11**(7):283-290.

3. van Passel MWJ, Kuramae EE, Luyf ACN, Bart A, Boekhout T: **The reach of the genome signature in prokaryotes.** BMC Evol Biol 2006, **6**:84.

4. Karlin S, Mrazek J, Campbell AM: **Compositional biases of bacterial genomes and evolutionary implications.** J Bacteriol 1997, **129**(12):3899-3913.

5. Vandamme P, Pot B, Gillis M, deVos P, Kersters K, Swings J: **Polyphasic taxonomy, a consensus approach to bacterial systematics.** Microbiol Rev 1996, **60**(2):407-438.

6. Wright F: **The 'effective number of codons' used in a gene.** Gene 1990, **87**(1):23-29.

7. Coenye T, Vandamme P: **Extracting phylogenetic information from whole-genome sequencing projects: the lactic acid bacteria as a test case.** Microbiology 2003, **149**(12):3507-3517.

8. Suyama M, Bork P: **Evolution of prokaryotic gene order: genome rearrangements in closely related species.** Trends Genet 2001, **17**(1):10-13.

9. Qi J, Bin W, Bai-lin H: **Whole proteome prokaryote phylogeny without sequence alignment: a K-string composition approach.** J Mol Evol 2004, **58**(1):1-11.

10. Coenye T, Gevers D, Van De Peer Y, Vandamme P, Swings J: **Towards a prokaryotic genomic taxonomy.** FEMS Microbiol Rev 2005, **25**(2):147.

11. Snel B, Bork P, Huynen MA: **Genome phylogeny based on gene content.** Nature Genetics 1999, **21**:108-110.

12. House CH, Fitz-Gibbon ST: **Using homolog groups to create a whole-genomic tree of free-living organisms: an update.** J Mol Evol 2002, **54**(4):539-547.

13. Henz SR, Huson DH, Auch AF, Nieselt-Struwe K, Schuster SC: **Whole-genome prokaryotic phylogeny.** Bioinformatics 2005, **21**(10):2329-2335.

14. Tatusov RL, Koonin EV, Lipman DJ: **A genomic perspective on protein families.** Science 1997, **278**(24):631-637.

15. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: **Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.** Nucleic Acids Res 1997, **25**(17):3389-3402.

16. Tatusov RL, Galperin MY, Natale DA, Koonin EV: **The COG database: a tool for genome-scale analysis of protein functions and evolution.** Nucl Acids Res 2000, **28**(1):33.

17. Makarova K, Sorokin AV, Novichkov PS, Wolf YI, Koonin EV: **Clusters of orthologous genes for 41 archaeal genomes and implications for evolutionary genomics of archaea.** Biology Direct 2007, **2**:33.

18. Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S, Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker D, Hughes J, Goh Y, Benson A, Baldwin K, Lee J-, Diaz-Muniz I, Dosti B, Smeianov V, Wechter W, Barabote R, Lorca G, Altermann E, BarrangouR., Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O'sullivan D, Steele J, Unlu G, Saeier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B, Mills D: **Comparative genomics of the lactic acid bacteria.** Proc Natl Acad Sci USA 2006, **103**:15611-15616.

19. Mulkidjanian AY, Koonin EV, Makarova KS, Mekhedov SL, Sorokin A, Wolf YI, Dudresne A, Partensky F, Burd H, Kaznadzey D, Haselkorn R, Galperin MY: **The cyanobacterial genome core and the origin of photosynthesis.** Proc Natl Acad Sci USA 2006, **103**:13126.

20. Trost B: **Creation, evaluation, and use of GenoSub, a program for identifying protein-phenotype relationships and comparing protein content in groups of organisms.** *University of Saskatchewan, Saskatoon, Saskatchewan.* 2009
21. Rosselló-Mora R, Amann R: **The species concept for Prokaryotes.** FEMS Microbiol Rev 2001, **25**:39-61.

22. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** J Mol Biol 1990, **215**(3):403-410.

23. The Gene Ontology Consortium: **Gene Ontology: tool for the unification of biology.** Nature Genet 2000, **25**:25-29.

24. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh TM, Garrity GM, Tiedje JM: **The Ribosomal Database Project: improved alignments and new tools for rRNA analysis.** Nucl Acids Res 2009, **37(database issue)**:D141-D145.

25. Tamura K, Dudley J, Nei M, Kumar S: **MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0.** Mol Biol Evol 2007, **24**(8):1596-1599.

11. DISSECTION OF THE GENUS *LACTOBACILLUS* WITH RECLASSIFICATION OF 23 SPECIES TO THE GENUS *PARALACTOBACILLUS*, 21 SPECIES TO THE GENUS *JENSENELLA* GEN. NOV., 19 SPECIES TO THE GENUS *ORLAEA* GEN. NOV., AND EMENDED DESCRIPTION OF THE GENERA *LACTOBACILLUS* AND *PARALACTOBACILLUS*

Author contributions:

Monique Haakensen conceived the study, participated in the design and coordination of experiments, collected data, conducted phylogenetic analyses, and drafted parts of the manuscript.

Brett Trost performed all genomic analyses, and drafted parts of the manuscript.

Vanessa Pittet provided scientific input, constructed figures, and drafted parts of the manuscript.

C. Melissa Dobson conceived of parts of the study and undertook some of the early experiments.

Anthony Kusalik participated in the design and coordination of the study, edited the manuscript, and is the holder of the research grant partially used to fund the study.

Barry Ziola conceived the study, participated in its design and coordination, edited the manuscript, and is the holder of the research grant partially used to fund the study.

BRIEF INTRODUCTION TO CHAPTER 11

The phylogenetic and taxonomic status of the *Lactobacillus* genus is in a state of disarray. The whole genomic analyses performed in Chapter 10 convincingly showed that the genus *Lactobacillus* contains a greater range of 16S rRNA gene percent identity than most other genera. As was discussed in the brief introduction to Chapter 9, systematic nomenclature is key to enabling the efficient study of groups of organisms. Here the genus *Lactobacillus* is examined more closely in the context of its nearest phylogenetic neighbors and possible subdivisions are recommended on the basis of whole genome and proteome comparisons, 16S rRNA gene percent identity, and multi locus sequence analysis of four additional conserved housekeeping genes.

Manuscript in preparation.

Dissection of the genus *Lactobacillus* with reclassification of 23 species to the genus *Paralactobacillus*, 21 species to the genus *Jensenella* gen. nov., 19 species to the genus *Orlaea* gen. nov., and emended description of the genera *Lactobacillus* and *Paralactobacillus*

Monique Haakensen[1], Brett Trost[2], Vanessa Pittet[1], C. Melissa Dobson[1*], Anthony Kusalik[2], and Barry Ziola[1§]

[1]Department of Pathology and Laboratory Medicine, University of Saskatchewan, 2841 Royal University Hospital, 103 Hospital Drive, Saskatoon, Saskatchewan, Canada S7N 0W8

[2]Department of Computer Science, University of Saskatchewan, 176 Thorvaldson Building, 110 Science Place, Saskatoon, Saskatchewan, Canada S7N 5C9

*Current address: MD consulting, 215 MacEwan Road, Edmonton, Alberta, Canada T6W 1R2

[§]Corresponding author

Email addresses:

MH: m.haakensen@usask.ca

BT: brett.trost@usask.ca

VP: v.pittet@usask.ca

CMD: melissa.dobson@telus.net

TK: kusalik@cs.usask.ca

BZ: b.ziola@usask.ca

Abstract

Background

Current taxonomy of the genus *Lactobacillus* is generally regarded as unsatisfactory, in part due to this genus being polyphyletic by encompassing the genera *Paralactobacillus* and *Pediococcus*. Historical methods of classifying species within the *Lactobacillus* genus are largely based on phenotypic and morphologic attributes, which do not take into account analyses of the ribosomal RNA gene or other genetic loci that are conserved amongst prokaryotes. Moreover, no systematic rationale exists to support the classification and naming of new species, or the study of existing subsets of *Lactobacillus* species. The aim of this study was to apply a polyphasic approach to evaluate the coherence of *Lactobacillus* as a genus. For all currently defined *Lactobacillus*, *Paralactobacillus*, and *Pediococcus* species, we present phylogenetic analyses of the 16S rRNA gene, and the *cpn60*, *recA*, *rpoA*, and *pheS* genes and respective protein coding sequences, along with genomic analyses and existing phenotypic information. To provide insight into the intra- and inter-genus relationships of *Lactobacillus* compared to non-polyphyletic genera, we have conducted phylogenetic analyses and evaluated the percent identity of the 16S rRNA gene of the phylogenetically neighbouring genera *Aerococcus, Carnobacterium, Enterococcus, Lactococcus, Leuconostoc*, *Oenococcus*, *Streptococcus, Vagococcus*, and *Weissella*.

Results

Based on a multi-locus sequence analysis, 16S rRNA gene similarity, G-C content, and whole genome and proteome analyses, the *Lactobacillus* genus was found to be more heterogeneous than closely related genera. This polyphasic analyses identified distinct subgroups of *Lactobacillus* which represent new or emerging genera. Lastly, percent identity of the 16S rRNA gene was found to reflect the genomic similarity of lactobacilli, indicating that sequence analysis of this gene alone is sufficient for appropriate allocation of novel bacterial species to specific subgroups, including the new genera described here.

Conclusions

The genus *Lactobacillus* should be divided into at least four genera. The first three are the phylogenetic clades traditionally referred to as the *Lactobacillus acidophilus, Lactobacillus reuteri*, and *Lactobacillus salivarius* groups, and the fourth group comprises all other *Lactobacillus* species (including the *Lactobacillus casei*, *Lactobacillus sakei,* and

Lactobacillus plantarum clades). Renaming of these distinct phylogenetic units is proposed as follows: the *L. acidophilus* group becomes *Jensenella* gen. nov.; the *L. reuteri* group becomes *Orlaea* gen. nov.; the *L. salivarius* group joins the existing *Paralactobacillus* genus; and all other *Lactobacillus* species remain as *Lactobacillus*.

Background

Members of the genus *Lactobacillus* are extremely varied in phenotype, G-C content, morphology, and percent identity of the 16S rRNA gene. The number of *Lactobacillus* species is growing rapidly (Figure 1), currently encompassing 121 validly described species and an additional nine subspecies [1]. The current taxonomic location of the genus *Lactobacillus* in relation to some of its nearest phylogenetic neighboring genera is provided in Figure 2, based on the 16S rRNA gene sequences of type strains for all currently validly described species for each included genera. The *Lactobacillus* genus is polyphyletic, encompassing the *Paralactobacillus* and *Pediococcus* genera, which contain one and eleven species, respectively [1]. As the number of *Lactobacillus* species has increased, the definition of the genus has become even more diffuse. Moreover, there are no criteria for the inclusion or exclusion of new species within the genus *Lactobacillus* [2, 3]. This leads to confusing situations where new genera can be described that reside amid the current span of lactobacilli (such as occurred with *Paralactobacillus*) [4]. The *Lactobacillus* genus is extremely heterogeneous, having varying phenotype, G-C content, and morphology (Tables 1-4). As such, we believe there is a need for a stable system of nomenclature to ensure that all members of the genus can be clearly identified, facilitating further classification and study.

With the widespread usage of molecular tools such as 16S rRNA gene sequencing, new groups of bacteria have been identified and genera have been created from species formerly considered to be lactobacilli (e.g., *Carnobacterium*, *Leuconostoc*, *Oenococcus*, and *Weissella* [2, 9, 10]). Here, using further genetic characterisation and whole genome analyses, a more accurate presentation of the genus *Lactobacillus* is provided in order to reflect the evolutionary relationships among species. Although 16S rRNA gene sequencing is used extensively in bacterial systematics, there is a growing call from the scientific community to strengthen, or refute, the conclusions drawn from analyses based on only one gene [11-13]. And, while confidence is again growing in the phylogenetic placement of organisms based

upon 16S rRNA gene sequencing [14], we have taken a more comprehensive phylogenetic approach using several conserved genes to determine the most appropriate phylogeny. Taking this concept even further, the genomic and proteomic taxonomy of the *Lactobacillus* genus is also explored.

First described by Beijerinck in 1901, the *Lactobacillus* genus was subsequently organised into groups based upon phenotypic characteristics and has since undergone many regroupings, subdivisions, and reclassifications [2, 3]. Initially, optimal growth temperatures and hexose fermentation pathways were used to form subgroups of lactobacilli by Orla-Jensen [2, 3]. Later, obligate *vs*. facultative and homo- *vs*. hetero-fermentation potential were used as classification criteria [3]. As phenotypic markers may undergo lateral gene transfer, their use for the classification of *Lactobacillus* isolates has resulted in a confusing classification scheme that has contributed to the present disorder [5-8]. Currently, new species of *Lactobacillus* are being described at an increasing rate (Figure 1). This may be due in part to the variation within the genus and partly due to the development of molecular tools such as 16S rRNA gene sequencing which allow for a more rapid identification of bacteria. Despite classification based upon 16S rRNA gene sequencing being the gold standard for phylogenetic classification, there is disorder within the *Lactobacillus* genus as descriptions of new species often fail to make use of the entire genus in the underlying analyses, probably owing to the large size and diversity of the genus. The relation of the breadth and depth of *Lactobacillus* to other genera (including genera which now contain species previously described as *Lactobacillus*) is provided by 16S rRNA gene sequence phylogeny in Figure 2. In the current taxonomic standing, the genera *Paralactobacillus* and *Pediococcus* fall within the *Lactobacillus* genus, yet *Lactobacillus catenaformis* and *Lactobacillus vitulinus* are clearly distant to all other *Lactobacillus* species. A closer look at the *Lactobacillus, Paralactobacillus*, and *Pediococcus* genera is provided in Figures 3-6.

Described in 2000 by Leisner et al [4], the *Paralactobacillus* genus contains only a single species, *Paralactobacillus selangorensis*. At the time of its identification, *P. selangorensis* was assigned to its own genus based upon noted phylogenetic and 16S rRNA gene differences in comparison to the *Lactobacillus acidophilus* and *Lactobacillus casei* groups. While it is true that *P. selangorensis* is distant from these two subgroups of lactobacilli, the *Lactobacillus salivarius* and *Lactobacillus reuteri* subgroups were not considered in the phylogenetic

assignment and description of the *Paralactobacillus* genus [4]. Figures 3 and 6 show the phylogenetic location of *Paralactobacillus* in relation to *Lactobacillus*.

The genus *Pediococcus* was described in 1884 by Balcke and is currently comprised of 11 species [15-17]. The pediococci are distributed between two closely related clades which are phylogenetically located deep within the genus *Lactobacillus,* with nearest neighbours being *Lactobacillus kunkeei* and *Lactobacillus malefermentans*. More generally, the genus *Pediococcus* lies between the *Lactobacillus sakei* and *Lactobacillus brevis* clades (Figure 3). The *Pediococcus* genus has grown relatively slowly compared to *Lactobacillus*, probably owing to the fact that pediococci were originally described as being spherical, in contrast to the variety of shapes that have been permitted in *Lactobacillus*. However, with an increasing number of irregular shaped, curved, coccoid, and cocci lactobacilli being described [3, 18-26], it no longer is appropriate to differentiate pediococci from lactobacilli based upon shape. Consequently, the only characteristic that can currently differentiate pediococci from lactobacilli is that *Pediococcus* species divide in two perpendicular directions in a single plane [18]. The phylogenetic placement of the *Pediococcus* genus within the *Lactobacillus* genus is shown in Figure 3.

Results and Discussion

General findings

As phylogenetic trees are dynamic constructs, they change based on discovery of additional species and genera. That is, additional sequence data either support or refute the *status quo*. While previous taxonomic studies of *Lactobacillus* isolates have encountered difficulty in resolving clades, the rapidly increasing number of validly described species, coupled with the increasing availability of genetic information, has allowed us to assemble concrete evidence in support of a new taxonomic division of *Lactobacillus*. We believe that the currently available data provide strong support for the reclassification of a large number of isolates currently found in *Lactobacillus,* as well as reassessment of the taxonomic independence of the genera *Paralactobacillus* and *Pediococcus,* which currently reside amidst a diverse range of *Lactobacillus* species.

A comprehensive examination of all currently validly described *Lactobacillus*, *Paralactobacillus*, and *Pediococcus* isolates is presented through examination of 16S rRNA gene sequences as well as *cpn60*, *recA*, *rpoA*, and *pheS* gene sequences, where available. To provide quantitative assessment of the phylogenetic trees, we also compare the intra- and inter- genus 16S rRNA gene percent identity of the *Lactobacillus* genus to that of type-strains of all species from the related genera *Aerococcus, Carnobacterium, Enterococcus, Lactococcus, Leuconostoc*, *Oenococcus*, *Paralactobacillus, Pediococcus, Streptococcus, Vagococcus*, and *Weissella* (which were also included in the phylogenetic supertree; see Figure 2). Moreover, multi-locus sequence analysis (MLSA)-based taxonomy is compared to whole genome relatedness through the use of a dendrogram created from numbers of unique proteins between pairs of sequenced genomes.

Multi-locus sequence analysis (MLSA)

Taxonomic consistency of the MLSA was considered necessary in determining the potential subdivision of the genus *Lactobacillus* into more coherent genera. While some loci may be better suited than others for strain differentiation or establishing the relationship of very closely related strains or species [27, 28], it is important to stress that phylogenetic trees created by each genetic loci produced a number of stable and robust clades that were common to all loci. When *cpn60*, *pheS, recA*, or *rpoA* phylogenetic trees were created, the resulting clades confirmed the 16S rRNA gene phylogenetic tree (Figures 2-6). Although the bifurcation patterns varied slightly for the five phylogenetic trees, a set of stable and robust clades was readily distinguishable with all genetic loci used. These phylogenetic trees suggest that the *Lactobacillus* genus be divided into four groups (Figure 3). The clades traditionally referred to as the *L. acidophilus, L. reuteri*, and *L. salivarius* clades (expanded in Figures 4-6, respectively) form distinct lineages compared to all other species of *Lactobacillus* that surround the *Pediococcus* genus (Figure 3).

Similarity of the 16S rRNA gene

The percent identity of the 16S rRNA gene was calculated to ascertain whether cutoffs could be established for the differentiation of clades. The percent identity of the 16S rRNA gene for the *Lactobacillus* genus was compared to that of eleven phylogenetically related genera (Table 1), which are also shown in the phylogenetic supertree (Figure 2). The 16S rRNA

gene of every type strain isolate was included in the analysis and it was found that a minimum of 89% identity of the 16S rRNA gene existed between any two isolates within each of the eleven genera (Table 1). In stark contrast, when all type strains of the current *Lactobacillus* genus were analyzed in the same way, the minimum percent identity of the 16S rRNA gene between two isolates was 71% (see legends, Tables 1 and 2). Although the minimum level of identity can be increased to 83% by removing the distant outlier species *Lactobacillus catenaformis* and *Lactobacillus vitulinus*, the cohesiveness of the currently accepted *Lactobacillus* species remains much worse than what is found between species of other closely related genera (Table 1). By determining the percent identity of the 16S rRNA gene for the major clades of the *Lactobacillus* genus, it was found that the *L. acidophilus*, *L. reuteri*, and *L. salivarius* clades shared less than 90% identity with each other or with the remaining lactobacilli. Through removing these clades (which each possess >90% intra-clade 16s rRNA gene identity), the percent identity of the remaining "core" *Lactobacillus* group (i.e., comprised of subgroups I-VII; Figure 3) increased from 82% to 88% (Table 2). However, it should be noted that for the remaining lactobacilli subgroups, some inter-group pairs of isolates share greater 16S rRNA gene percent identity than do some intra-group pairs of isolates (Table 2). We interpret this to mean that a greater number of species will be required over time to appropriately divide these subgroupings (Figure 3, I-VII).

Whole genome analysis

Although MLSA agreed with the 16S rRNA gene phylogeny, there is always a concern as to whether the chosen loci reflect the actual genomic content (and phenotype) of the organisms in question. We sought to resolve this problem through whole genome analysis. The genome sequences available for *Lactobacillus* and *Pediococcus* isolates were obtained (there is not yet a genome sequence available for *Paralactobacillus*), as were available genome sequences of the related genera *Lactococcus, Leuconostoc, Oenococcus,* and *Streptococcus*. The number of unique proteins between any two isolates was calculated in a pairwise fashion and used to create a dendrogram showing the relatedness of organisms on a genomic level (Figure 7). Visualizing the genera in this way shows a greater diversity among *Lactobacillus* species, yet the groupings and divisions shown here are consistent with those based on MLSA and 16S rRNA gene analyses. Although the taxonomy is slightly different from that of the MLSA, the major groupings remain consistent, with the *L. acidophilus* and *L. reuteri* groups forming distinct clades. Even though only one sequenced genome was available, the *L.*

salivarius genome clearly branches independently. *L. sakei* is placed closest to the *Pediococcus* genus, with *L. brevis* as the nearest *Lactobacillus* relative. Interestingly, the *L. plantarum* and *L. casei* genomes are separated from all other *Lactobacillus* by the *Leuconostoc*, *Oenococcus*, and *Streptococcus* genera, and *L. casei* is placed nearest to *Lactococcus*. These observed divisions between the "remaining lactobacilli" set the stage for future subdivisions of *Lactobacillus* through beginning to mirror subgroupings I-VII in Figure 3.

Phenotypic similarity

A number of phenotypic and biochemical characteristics have been used over the years to create subgroups within the genus *Lactobacillus*. As in previous studies of the *Lactobacillus* genus, the traits considered here were metabolism (obligately homofermentative, facultatively heterofermentative, or obligately heterofermentative), G-C content, lactic acid isomer, and peptidoglycan type [2, 3, 29]. In order to determine whether these traits followed the evolution of the organisms as suggested by MLSA, several basic characteristics are provided alongside the species as divided based upon MLSA and 16S rRNA similarity (Tables 3-6). While our subdivisions allow for more concise descriptions of each group than is currently the case for *Lactobacillus*, there is still a great diversity in phenotype. This strongly suggests that PCR sequencing-based techniques or genome sequence-based techniques, and not phenotypic information, be used as the standard for the classification of these organisms.

G-C content

Interestingly, the range of G-C content did not improve greatly with the regroupings and remains much wider than in other genera (e.g., *Streptococcus*). As G-C content is a factor that is heavily weighted in the description of new species, it is disconcerting that such a wide range exists within a single genus. One possibility is that this may be a reflection of the plasmid content and widespread horizontal gene transfer in lactobacilli. We explored this by examining sequenced genomes and calculating the G-C content with and without plasmids, but no difference was found (data not shown). A second possibility is that the G-C range is an adaptation of the bacteria to their environment as was shown to be the case for *L. delbrueckii* subsp. *bulgaricus* [30]. The coding sequences of all sequenced *Lactobacillus* and

Pediococcus genomes were subjected to a similar analysis, comparing the 3rd coding position to the 1st and 2nd coding positions. In all genomes that had aberrantly high or low G-C contents, it is the 3rd coding position that appears to influence the phenomenon (Table 7). For the sequenced genomes of *Lactobacillus* species, the range of G-C content is 19.7%. However, by observing only the 1st and 2nd coding positions, this range drops to 7.9%. In contrast, the G-C range in the 3rd coding position is very high at 43.5%. When the genus *Lactobacillus* is analyzed according to our proposed groupings, the greatest G-C range for any group is 13.4%, with 4.6% variation in the 1st and 2nd bases, but 39.7% in the wobble base (Table 7). As a greater number of genomes become sequenced, it will be possible to further analyze this phenomenon and perhaps compare G-C content shift at the 3rd position with environmental factors.

This variability in G-C content raises concern as to the accuracy of using G-C content and DNA-DNA hybridization experiments to determine the relatedness of different species. As we have shown that G-C content is greatly influenced by the wobble base in a coding sequence, an alteration of G-C content will not necessarily reflect a change in proteome content or phenotypic similarity. Rather, two strains of a given species that exist in different environments may show a marked variation in G-C content due to changes in wobble base preference.

Proposed division of the genus *Lactobacillus*

Through combining information from the MLSA, 16S rRNA gene similarity analyses, and whole genome analyses, it is apparent that the *Lactobacillus* genus should be divided. To ensure robustness of divisions, thereby avoiding unsupported and haphazard creation of new genera divisions, all cutoffs were determined by choosing stable branch points (maintained the same sub-branches with every gene used), possessed a high bootstrap value, and contained a relatively large number of species (>10 species). Moreover, intra-group pairs of isolates had to share a greater 16S rRNA gene identity than inter-group pairs, with 90% identity being a useful cutoff value. We propose that this would be best accomplished by creating two new genera from the *L. acidophilus* and *L. reuteri* clades and transferring the *L. salivarius* clade to the existing *Paralactobacillus* genus (Figure 8). While the remaining lactobacilli can be subdivided into seven subgroups, retaining the *Pediococcus* genus at its centre, these criteria, did not allow any of these subgroups to be split into separate genera.

The percent identity of the 16S rRNA gene was compared intra- and inter-clade to determine whether subdivisions would contribute to a greater coherence in classification of species that currently belong to the genus *Lactobacillus*. The *L. acidophilus*, *L. reuteri*, and *L. salivarius* groups possessed a cutoff of 91-92% intra- and inter-clade identity of the 16S rRNA gene (Table 1). The lower percent identity in the remaining seven lactobacilli subgroupings prompted further investigation and it was found that there were several subgroups that were stable, but contained relatively few species (Figure 3). With the exception of *L. catenaformis* and *L. vitulinis*, the remaining *Lactobacillus* species have a minimum 88% intra-group identity (Table 2). Table 2 also shows that some inter-clade pairs of isolates share less 16S rRNA gene percent identity than intra-clade pairs of isolates. These seven clades may eventually prove to be better described as independent genera as a greater number of species becomes identified in the different clades. Table 8 summarizes some of the phenotypic and genotypic differences for the new genera proposed here.

Pediococcus

The genus *Pediococcus* falls within the core group of *Lactobacillus* species, with its 16S rRNA gene placing it closest to the *Lactobacillus* "singlets" *L. kunkeei* and *L. malefermentans,* between subgroups I and II. Sharing 88-94% identity of the 16S rRNA gene with the core *Lactobacillus* group, the pediococci are indistinguishable from the lactobacilli by genetic methods. However, all species of *Pediococcus* possess a unique mode of division that readily differentiates them from even the cocci lactobacilli [18]. As the purpose of the suggested subdivisions and reclassifications here are to add clarity to the taxonomic relationships of the bacteria, it is difficult to decide whether reclassification of *Pediococcus* as *Lactobacillus* is necessary. Ultimately, we believe that the seven core *Lactobacillus* subgroups will continue to be divided as a greater number of species, sequences, and genomes become available for analysis. As such, with *Pediococcus* retaining its status as an independent genus, this could be regarded as the first of such subdivisions.

Species retained in the genus *Lactobacillus* (subgroups I-VII)

Fifty-nine species and four subspecies are retained within the *Lactobacillus* genus. As just discussed, we propose that the *Pediococcus* genus comprised of eleven species retain its status as an independent genus, yet remain at the centre of the *Lactobacillus* genus. The

species left within the *Lactobacillus* genus have an intra-genus 16S rRNA percent identity of 88-99%, leaving this as the most divergent of the genera proposed here (Table 3). The *Lactobacillus* genus further subdivides into seven subgroups that are phylogenetically distinct, but each contains a small number of species that cannot be differentiated based upon percentage of 16S rRNA gene identity. These subgroups incorporate all species except for four which exist as "singlet" outliers. In general, subgroups I-IV are most similar to one another, but distant to subgroups V-VII which are each distinct from one another (Figure 3).

Lactobacillus subgroup Ia and Ib

Traditionally referred to as the *L. casei* and *L. sakei* groups respectively, the *Lactobacillus* subgroups Ia and Ib together contain 13 species and 2 subspecies. While phylogenetically distinct from one another, subgroups Ia and Ib are closely related, sharing 91-94% inter-group and 91-99% intra-group identity of the 16S rRNA gene. Despite their similarity based on MLSA, there is little in regards to phenotypic properties that are consistent among either of these groups. Subgroup Ia possesses a G-C content ranging from 45-53%, while the G-C content of subgroup Ib is substantially lower, ranging from 41-44%. Two species (*Lactobacillus curvatus* and *Lactobacillus graminis*) in subgroup Ib deviate from the normal rod shape by possessing a slightly curved morphology.

Lactobacillus kunkeei and *Lactobacillus malefermentans*

Despite residing at the centre of the genus, *L. kunkeei* and *L. malefermentans* are outliers to all clades of *Lactobacillus*. These two *Lactobacillus* species exist as phylogenetic singlets on either side of the *Pediococcus* genus. Interestingly, these two species only share 90-93% identity of the 16S rRNA gene with other lactobacilli, but share as much as 94% identity with the *Pediococcus* genus.

Lactobacillus subgroup II

Subgroup II contains four species in the clade nearest to the *Pediococcus* genus. All species currently within this clade produce a DL lactic acid isomer; however, this may change as a greater number of species are discovered in this subgroup. Subgroup II has a relatively low G-C content, ranging from 35-41%.

Lactobacillus subgroups IIIa and IIIb

Subgroup III, containing the *L. brevis* subgroup, forms two distinct branches. Like subgroup I, these branches could not be differentiated from one another based on inter-group 16S rRNA percent identity. There are eight species in each of subgroup IIIa and subgroup IIIb. Subgroup IIIa has the highest G-C content of any group at 44-53%. The G-C content of subgroup IIIb is slightly lower, ranging from 39-46%.

Lactobacillus subgroup IV

Subgroup IV is created from both subspecies of *L. plantarum* and its four nearest phylogenetic neighbors. Although the 16S rRNA phylogeny suggests that this subgroup should be comprised of two clades, these species had insufficient numbers of sequences for MLSA to conclusively resolve this branching. Members of subgroup IV tend to have a midrange G-C content, spanning from 41-47%.

Lactobacillus subgroup V

This is the only subgroup where all members produce the Lys-D-Asp form of peptidoglycan. Subgroup V possesses a relatively low G-C content, ranging from 33-41%. Subgroup V is formed of 10 core members, with *L. versmoldensis* and *L. nodensis* included as outliers. Being the largest of the subgroups, this clade may be the next genus to be subdivided from the *Lactobacillus* genus. There is already a clear intra-group vs. inter-group cutoff for percent identity of the 16S rRNA gene emerging at 93-94% (Table 2), which is comparable to the division between *Lactococcus* and *Streptococcus* (Table 1). At this point, we have chosen to allow the clade to grow further before dividing it from the *Lactobacillus* genus in order to better clarify taxonomic positioning.

Lactobacillus subgroup VI

Despite containing only four species, subgroup VI possesses three different cell morphologies. Species within this subgroup are bacilli (*Lactobacillus composti* and *Lactobacillus rennini* [31, 32]), coccoid (*Lactobacillus coryniformis* [22]), or tapered (*Lactobacillus bifermentans* [21]). Currently, all members within this group are facultatively heterofermentative and produce the DL lactic acid isomer. However, as with cell

morphology, there are three different varieties of peptidoglycan produced. This subgroup has a tight range of G-C content, ranging only from 45-48%.

Lactobacillus subgroup VII

Subgroup VII contains four species, including *Lactobacillus dextrinicus,* which was only recently transferred to the genus *Lactobacillus* from the genus *Pediococcus* [18]. Members of this subgroup may be rod shaped, bent, or cocci [18, 20]. There is a very wide range of G-C content, spanning from 39-54%. This group appears to have two distinct clades, separating *Lactobacillus perolans* and *Lactobacillus harbinensis* from *L. dextrinicus* and *Lactobacillus concavus*, although additional species will be needed in order to substantiate this division.

Lactobacillus caternaformis and *Lactobacillus vitulinus*

L. catenaformis and *L. vitulinus* have long been recognized as distant outliers to the genus *Lactobacillus* [2, 9, 15, 29]. This distant relationship can be easily seen in Figure 2; however, there has yet to be a formal request for reclassification. Based upon 16S rRNA gene similarity, these two species should be reassigned to the Family *Erysipelotrichaceae*, but being only 85% identical to one another, are not sufficiently similar to be considered a single genus. Proper reclassification of these two species will require adequate attention be paid to the Family *Erysipelotrichaceae* which is not within the scope of this manuscript. As such, formal reclassification of these species is not requested at this time.

Lactobacillus acidophilus group

Twenty-one species and four subspecies belong to the *L. acidophilus* group (Table 4). The taxonomy of species belonging to the *L. acidophilus* group is given in Figure 4, with its relationship to the other clades shown in Figure 3. Historically, members from this group that were included in Orla and Jensen's taxonomy were all classified as "Streptobacteria" and "Thermobacteria" (but not "Betabacteria") [2, 9, 29]. Most currently described members are bacilli, with the exception of *Lactobacillus crispatus,* which is a curled rod [23]. All members produce Lys-D-Asp type peptidoglycan. Metabolism may be either obligately homofermentative or facultatively heterofermentative. L, D, or DL lactic acid isomers may be produced. The G-C content generally ranges from 33-41%, however, several species appear

to have experienced dramatic shifts in G-C content compared to the other members of this group. These are *L. delbrueckii* (49-51%), *L. amylophilus* (44-46%), and *L. amylotrophicus* (44%). Members of the *L. acidophilus* group possess 91-99% intra-genus identity of the 16S rRNA gene. *Lactobacillus delbrueckii* subsp. *delbrueckii* is the type-strain of the genus.

Lactobacillus reuteri group

There are 19 *Lactobacillus* species belong to the *L. reuteri* group (Table 4). The taxonomy of the *L. reuteri* group is shown in Figure 5, with its relationship to the other clades given in Figure 3. Historically, members of this group that were included in Orla and Jensen's taxonomy were all classified as "Betabacteria" [2, 9, 29]. The G-C content ranges from 35-56%, indicating the diverse array of genetic transfer or a wide variety of habitats that may exist for this genus. Despite a diverse G-C content, members of this genus share 91-99% identity of the 16S rRNA gene. Cell morphology may be spherical, oval, bent rod, or bacillus. All members produce the DL lactic acid isomer and all but *Lactobacillus secaliphilus* are obligately heterofermentative [33]. Unlike members of any other *Lactobacillus* group, many species within the *L. reuteri* group produce Orn-D-Asp type peptidoglycan, and several other unique forms of peptidoglycan are also formed (e.g., L-Orn-D-Asp, L-Lys-D-Glu-L-Ala, and Lys-Ser-Ala).

Lactobacillus salivarius group and *Paralactobacillus*

The genus *Paralactobacillus* was described in 2000 [4]. However, the creation of this genus neglected to include an analysis of the entire *Lactobacillus* genus. Specifically, the entire clade surrounding *L. salivarius* was missed from the comparative phylogenetic representation of *Lactobacillus*. By including the entire *Lactobacillus* genus, we have found that *Paralactobacillus selangorans*, in fact, falls within the middle of the *L. salivarius* subgroup of the genus *Lactobacillus*. The description of *Paralactobacillus* was correct, however, in that this genus falls distantly from other groups of *Lactobacillus,* having 92-99% intra-group 16S rRNA gene identity, but <92% identity shared with other groups and genera (Table 1). In a historical context, there were no members of this group that were included in Orla and Jensen's taxonomy where members of *Lactobacillus* were classified as "Betabacteria", "Streptobacteria", or "Thermobacteria" [2, 9, 29].

We propose that 19 species and one subspecies belonging to the *L. salivarius* group are transferred from the genus *Lactobacillus* to the genus *Paralactobacillus*. A list of species transferred to *Paralactobacillus* is given in Table 6, and phylogeny is shown in Figure 6, with relationship to other *Lactobacillus* shown in Figure 3. The G-C content ranges from 32-47%. Species may be cocci or bacilli. A variety of different forms of peptidoglycan may be formed. L or DL lactic acid isomer is made. Metabolism may be facultatively heterofermentative or obligately heterofermentative.

Conclusions

Division of the genus *Lactobacillus* into two new genera and the reclassification of additional isolates to the genus *Paralactobacillus* allows for a better understanding of the taxonomic relationships that exist among these bacteria. Although phenotypic characteristics of isolates do not follow phylogenetic taxonomy, through whole genomic analyses (i.e, the organism's whole phenotype) we have shown that distinct taxonomic units can be delineated based upon degrees of genomic similarity. As a greater number of species, sequences for the *cpn60*, *recA*, *rpoA*, and *pheS* genes, and whole genome sequences become available, further resolution of *Lactobacillus* taxonomic relationships will possible. With our proposed changes to the genus *Lactobacillus*, we have created a classification scheme using 16S rRNA gene percent identity, MLSA, and whole proteomic phylogeny, from which other divisions can be made and new *Lactobacillus* isolates clearly categorized.

Description of the genus *Jensenella* gen. nov.

Jensenella gen. nov. (Jensen'.ella N.L.fem.dim.n. *Jensenella* after the Danish microbiologist S. Orla-Jensen). The following description is adapted from the description for the genus *Lactobacillus* as found in Bergey's Manual of Systematic Bacteriology [3]. Generally rod shape but may also be curled rods, chain formation common. May be motile or non-motile. When motile, by peritrichous flagella. Nonsporing. Gram-positive. Some strains exhibit bipolar bodies, internal granulations or a barred appearance with the Gram-reaction or methylene blue stain. Peptidoglycan is Lys-D-Asp type. Fermentative metabolism, obligately saccharoclastic. They may be facultatively or obligately heterofermentative, and produce a D, L, or DL lactic acid isomer from glucose. At least half of end product carbon is lactate. Lactate is usually not fermented. Additional products may be acetate, ethanol, CO_2, formate or succinate. Volatile acids with more than two carbon atoms are not produced.

Microaerophilic; surface growth on solid media generally enhanced by anaerobiosis or reduced oxygen pressure and 5-10% CO_2; some are anaerobes on isolation. Nitrate reduction is highly unusual; if present, only when terminal pH is poised above 6.0. Gelatin not liquefied. Casein not digested but small amounts of soluble nitrogen produced by most strains. Indole and H_2S not produced. Catalase and cytochrome negative (porphyrins absent); however, a few strains decompose peroxide by a pseudocatalase; benzidine reaction negative. Pigment production rare; if present, yellow or orange-to-rust or brick red. Complex nutritional requirements for amino acids, peptides, nucleic acid derivatives, vitamins, salts, fatty acids or fatty acid esters and fermentable carbohydrates. Nutritional requirements are generally characteristic for each species, often for particular strains only. Growth temperature range 2-53°C; optimum generally 30-40°C. Aciduric, optimal pH usually 5.5-6.2; growth generally occurs at 5.0 or less; the growth rate is often reduced at neutral or initially alkaline reactions. Pathogenicity is rare. Members of *Jensenella* possess 91-99% intra-genus identity of the 16S rRNA gene. G-C content of the DNA ranges from 33-51%. Type species: *Lactobacillus acidophilus* (Moro 1900) Hansen and Mocquot 1970 (Approved Lists 1980) [34, 35], which now becomes *Jensenella acidophilus*, comb. nov. Species transferred from *Lactobacillus* to *Jensenella* are given in Table 4, with spelling of some species altered to reflect the feminine form of the name *Jensenella*.

Description of the genus *Orlaea* gen. nov.

Orlaea gen. nov. (Or.la'.e.a N.L. fem. n. *Orlaea* after the Danish microbiologist S. Orla-Jensen). The following description is adapted from the description for the genus *Lactobacillus* as found in Bergey's Manual of Systematic Bacteriology [3]. Cell morphology may be spherical, oval, bent rod, or bacilli; chain formation common. May be motile or non-motile. When motile, by peritrichous flagella. Nonsporing. Gram-positive. Some strains exhibit bipolar bodies, internal granulations or a barred appearance with the Gram-reaction or methylene blue stain. Peptidoglycan may be Lys-D-Asp, mDpm, L-Orn-D-Asp, Lys-Ser-Ala, L-Lys-D-Asp, L-Lys-D-Glu-L-Ala, or DAP, but the most frequently occurring form is Orn-D-Asp. Fermentative metabolism, obligately saccharoclastic. Most members are obligately heterofermentative, although some may be facultatively heterofermentative. DL lactic acid isomer is produced from glucose, although it is possible that only an L isomer may be produced for some species. At least half of end product carbon is lactate. Lactate is usually not fermented. Additional products may be acetate, ethanol, CO_2, formate or succinate. Volatile acids with more than two carbon atoms are not produced. Microaerophilic; surface

growth on solid media generally enhanced by anaerobiosis or reduced oxygen pressure and 5-10% CO_2; some are anaerobes on isolation. Nitrate reduction is highly unusual; if present, only when terminal pH is poised above 6.0. Gelatin not liquefied. Casein not digested but small amounts of soluble nitrogen produced by most strains. Indole and H_2S not produced. Catalase and cytochrome negative (porphyrins absent); however, a few strains decompose peroxide by a pseudocatalase; benzidine reaction negative. Pigment production rare; if present, yellow or orange-to-rust or brick red. Complex nutritional requirements for amino acids, peptides, nucleic acid derivatives, vitamins, salts, fatty acids or fatty acid esters and fermentable carbohydrates. Nutritional requirements are generally characteristic for each species, often for particular strains only. Growth temperature range 2-53°C; optimum generally 30-40°C. Aciduric, optimal pH usually 5.5-6.2; growth generally occurs at 5.0 or less; the growth rate is often reduced at neutral or initially alkaline reactions. Pathogenicity is rare. Members of the *Orlaea* genus share 91-99% identity of the 16S rRNA gene. The G-C content of the DNA ranges from 35-56%. Type species: *Lactobacillus reuteri* Kandler et al 1982 (Validation List 1982) [36], which now becomes *Orlaea reuteri* comb. nov. Species transferred from *Lactobacillus* to *Orlaea* are given in Table 5, with spelling of some species altered to reflect the feminine form of the name *Orlaea*.

Emended description of the genus *Paralactobacillus*.

The following description is adapted from the description for the genus *Lactobacillus* as found in Bergey's Manual of Systematic Bacteriology [3], and the description of the genus *Paralactobacillus* as described by Leisner et al [4]. Cells may be cocci or bacilli; chain formation common. May be motile or non-motile. When motile, by peritrichous flagella. Nonsporing. Gram-positive. Some strains exhibit bipolar bodies, internal granulations or a barred appearance with the Gram-reaction or methylene blue stain. Peptidoglycan may be Lys-D-Asp, L-Lys-D-Asp, DAP, or mDpm type. Fermentative metabolism, obligately saccharoclastic. May be homofermentative or facultatively heterofermentative, and produce L or DL lactic acid from glucose. At least half of end product carbon is lactate. Lactate is usually not fermented. Additional products may be acetate, ethanol, CO_2, formate or succinate. Volatile acids with more than two carbon atoms are not produced. Microaerophilic; surface growth on solid media generally enhanced by anaerobiosis or reduced oxygen pressure and 5-10% CO_2; some are anaerobes on isolation. Nitrate reduction is highly unusual; if present, only when terminal pH is poised above 6.0. Gelatin not

liquefied. Casein not digested but small amounts of soluble nitrogen produced by most strains. Indole and H_2S not produced. Catalase and cytochrome negative (porphyrins absent); however, a few strains decompose peroxide by a pseudocatalase; benzidine reaction negative. Pigment production rare; if present, yellow or orange-to-rust or brick red. Complex nutritional requirements for amino acids, peptides, nucleic acid derivatives, vitamins, salts, fatty acids or fatty acid esters and fermentable carbohydrates. Nutritional requirements are generally characteristic for each species, often for particular strains only. Growth temperature range 2-53°C; optimum generally 30-40°C. Aciduric, optimal pH usually 5.5-6.2; growth generally occurs at 5.0 or less; the growth rate is often reduced at neutral or initially alkaline reactions. Pathogenicity is rare. Members of this genus have 92-99% intra-group 16S rRNA gene identity. The G-C content of the DNA ranges from 32-47%. Type species: *Lactobacillus salivarius* Rogosa et al 1953 (Approved Lists 1980) emend. Li et al 2006 [37], which now becomes *Paralactobacillus salivarius*, comb. nov. Species transferred from *Lactobacillus* to *Paralactobacillus* are given in Table 6.

Emended description of the genus *Lactobacillus*.

The following description is adapted from the description for the genus *Lactobacillus* as found in Bergey's Manual of Systematic Bacteriology [3]. Cells, varying from long and slender, sometimes bent rods to short, often coryneform coccobacilli or cocci; chain formation common. May be motile or non-motile. When motile, by peritrichous flagella. Nonsporing. Gram-positive. Some strains exhibit bipolar bodies, internal granulations or a barred appearance with the Gram-reaction or methylene blue stain. Fermentative metabolism, obligately saccharoclastic. At least half of end product carbon is lactate. Lactate is usually not fermented. Additional products may be acetate, ethanol, CO_2, formate or succinate. Volatile acids with more than two carbon atoms are not produced. Microaerophilic; surface growth on solid media generally enhanced by anaerobiosis or reduced oxygen pressure and 5-10% CO_2; some are anaerobes on isolation. Nitrate reduction is highly unusual; if present, only when terminal pH is poised above 6.0. Gelatin not liquefied. Casein not digested but small amounts of soluble nitrogen produced by most strains. Indole and H_2S not produced. Catalase and cytochrome negative (porphyrins absent); however, a few strains decompose peroxide by a pseudocatalase; benzidine reaction negative. Pigment production rare; if present, yellow or orange-to-rust or brick red. Complex nutritional requirements for amino acids, peptides, nucleic acid derivatives, vitamins, salts, fatty acids or fatty acid esters and fermentable carbohydrates. Nutritional

requirements are generally characteristic for each species, often for particular strains only. Growth temperature range 2-53°C; optimum generally 30-40°C. Aciduric, optimal pH usually 5.5-6.2; growth generally occurs at 5.0 or less; the growth rate is often reduced at neutral or initially alkaline reactions. Pathogenicity is rare. Members of this genus have 88-99% intra-group 16S rRNA gene identity, with the exception of the distant outliers *L. vitulinus* and *L. catenaformis* which possess 74-78% and 71-76% identity to other members of the genus, respectively. The G-C content of the DNA ranges from 33-54%, with the exception of the outlier *L. catenaformis* which has a G-C content ranging from 31-33%. Type species: *Lactobacillus brevis* (Orla-Jensen, 1919) Bergey et al. 1934, (Approved Lists 1980) [35]. Species remaining within the *Lactobacillus* genus are given in Table 3.

Methods

Multi-locus sequence analysis (MLSA)

The majority of the 16S rRNA gene was used (1329 bases included in the 16S rRNA dataset), as well as portions of the *cpn60* (552 bp), *pheS* (455 bp), *recA* (531 bp), and *rpoA* (533 bp) genes. The regions of the 16S rRNA, *cpn60*, *pheS, recA*, and *rpoA* genes that we used have previously been shown to be useful in assessing phylogenetic relationships [12, 18, 28, 38-44]. Independent phylogenetic analyses were performed for each gene and, when applicable, the corresponding protein sequence for all type isolates of *Lactobacillus*, *Paralactobacillus,* and *Pediococcus* as well as related genera. Sequences for *pheS, recA*, and *rpoA* genes were obtained from GenBank [45], *cpn60* sequences were downloaded from the cpnDB [42], and 16S rRNA gene sequences were obtained from the Ribosomal Database Project [46]. The 16S rRNA gene sequence was available for all type strains, but not all of the other loci have been sequenced for each type isolate. As such, sequences could not be concatenated to form a phylogenetic tree using genetic information of all loci; rather, trees were constructed for each locus and the nodes supported by the loci are indicated on the phylogenetic tree of the 16S rRNA gene. Each instance where there are loci missing and which loci were available is indicated in the caption of the figures. Type strain identifiers and GenBank accession numbers of *Lactobacillus*, *Paralactobacillus*, and *Pediococcus* sequences used in the analysis are given in the Supplementary Material. Protein coding sequences were aligned with Clustal X version 2.0 [47] and 16S rRNA gene alignments were created by downloading sequences from the Ribosomal Database Project website that were pre-aligned based on secondary

structure [46]. All phylogenetic trees were produced and visualized using Molecular Evolutionary Genetic Analysis (MEGA) version 4.0 [48]. The evolutionary history was inferred using the Maximum Likelihood Neighbor-Joining method [49] within the MEGA Program. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches in cases where this percentage is greater than 60% (Felsenstein, 1985).

In addition to the MLSA analyses, a phylogenetic "supertree" was created using the 16S rRNA gene sequence (minimum 1230 bp length) of all type strain isolates of *Lactobacillus* and the phylogenetically neighbouring genera (Figure 2). In addition to the intertwined *Lactobacillus*, *Paralactobacillus*, and *Pediococcus* genera, the 16S rRNA gene sequence for all type-strain isolates for species of the genera *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* were included to provide a visual representation of their phylogenetic relationships to the genus *Lactobacillus*. A list of type strains for the species of these genera is available in the List of Prokaryotic Names with Standing in Nomenclature [1].

Calculation of percent identity of the 16S rRNA gene

The percent identity of the 16S rRNA gene was calculated to the nearest whole number in a pairwise fashion between every isolate in the study using the "Statistics Report" option available in the GeneDoc software application [50]. A comparison of the percent identity of the 16S rRNA gene for *Lactobacillus* groups with the intra- and inter-genus percent identities of phylogenetically related genera is given in Table 1 and a focused study of the core *Lactobacillus* subgroups is given in Table 2.

Whole genome analysis

The phylogeny of the *Lactobacillus* isolates with sequenced genomes, as well as isolates from neighbouring genera having sequenced genomes, were analyzed using a whole genome approach. This approach groups organisms based on gene content, and is similar to the methodology proposed by Snel *et al.* [51]. For each pair of organisms A and B, we determined the number of proteins encoded by the genome of organism A that are not encoded by the genome of organism B. This was done by performing pairwise BLAST alignments [52] between all possible pairs of proteins in the two organisms. A given protein

was considered to be similar to the other protein if the e-value between the two proteins was less than 10^{-25}. Proteins that were present in one organism but not the other were considered "unique". For the purposes of creating a dendrogram, the "distance" between organisms A and B was defined to be the average of the number of proteins unique to organism A and the number of proteins unique to organism B. Thus, pairs of organisms with a smaller "distance" have more similar protein complements. These distances were then used to create a phylogenetic tree using the unweighted pair group method with arithmetic mean (UPGMA) linkage technique [53].

Calculation of G-C content from whole genomes

FASTA formatted files containing open reading frames for each *Lactobacillus* and *Pediococcus* isolate with a sequenced genome were downloaded from the Integr8 website (www.ebi.ac.uk/integr8/; [54]). The G-C content in codon positions 1 and 2 (combined), position 3, and overall (all three codon positions combined) were calculated for each isolate.

Authors' contributions

Monique Haakensen conceived the study, participated in the design and coordination of experiments, collected data, conducted phylogenetic analyses, and drafted parts of the manuscript.

Brett Trost performed all genomic analyses and drafted parts of the manuscript.

Vanessa Pittet provided scientific input, constructed figures, and drafted parts of the manuscript.

C. Melissa Dobson conceived of parts of the study and undertook some of the early experiments.

Anthony Kusalik participated in the design and coordination of the study, edited the manuscript, and is the holder of the research grant partially used to fund the study.

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References

1. Euzéby JP: **List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet (http://www.bacterio.net).** *Int J Syst Bacteriol* 1997, **47**:590-592.

2. Hammes WP, Hertel C: **The Genera *Lactobacillus* and *Carnobacterium* .** In *The Prokaryotes.* Edited by Balows A, Trüper HH, Dworkin M, Harder W, Schleifer K-. New York: Springer; 2006:320-403.

3. Kandler O, Weiss N: **Genus *Lactobacillus*.** In *Bergey's Manual of Systematic Bacteriology. Volume 2.* Edited by Sneath PHA, Mair NS, Sharpe ME. Baltimore, MD: Willams and Wilkins; 1986:1209-1234.

4. Leisner JJ, Vancanneyt M, Goris J, Christensen H: **Description of *Paralactobacillus selangorensis* gen. nov., sp. nov., a new lactic acid bacterium isolated from chili bo, a Malaysian food ingredient.** *Int J Syst Evol Microbiol* 2000, **50**:19-24.

5. Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S, Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker D, Hughes J, Goh Y, Benson A, Baldwin K, Lee J-, Diaz-Muniz I, Dosti B, Smeianov V, Wechter W, Barabote R, Lorca G, Altermann E, Barrangou R, Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O'sullivan D, Steele J, Unlu G, Saeier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B, Mills D: **Comparative genomics of the lactic acid bacteria.** *Proc Natl Acad Sci USA* 2006, **103**:15611-15616.

6. Claesson MJ, van Sinderen D, O'Toole PW: **The genus *Lactobacillus* - a genomic basis for understanding its diversity.** *FEMS Microbiol Lett* 2007, **269**:22-28.

7. Makarova KS, Koonin EV: **Evolutionary Genomics of Lactic Acid Bacteria.** *J Bacteriol* 2007, **189**(4):1199-1208.

8. Nicolas P, Bessieres P, Ehrlich SD, Maguin E, van de Guchte M: **Extensive horizontal transfer of core genome genes between two *Lactobacillus* species found in the gastrointestinal tract.** *BMC Evol Biol* 2007, **7**:141-155.

9. Felis GE, Dellaglio F: **Taxonomy of lactobacilli and *Bifidobacteria*.** *Curr Issues Intestinal Microbiol* 2007, **8**:44-61.

10. Stiles ME, Holzapfel WH: **Lactic acid bacteria of foods and their current taxonomy.** *Int J Food Microbiol* 1997, **36**(1):1-29.

11. Fox GE, Wisotzkey JD, Jurtshuk P,Jr: **How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity.** *Int J Syst Bacteriol* 1992, **42**(1):166-170.

12. Eisen JA: **The RecA protein as a model molecule for molecular systematic studies of bacteria: Comparison of trees of RecAs and 16S rRNAs from the same species.** *J Mol Evol* 1995, **41**(6):1105-1123.

13. Case RJ, Boucher Y, Dahllof I, Holmstrom C, Doolittle WF, Kjelleberg S: **Use of 16S rRNA and *rpoB* Genes as Molecular Markers for Microbial Ecology Studies.** *Appl Environ Mi*crobiol 2007, **73**(1):278-288.

14. Tringe SG, Hugenholtz P: **A renaissance for the pioneering 16S rRNA gene.** *Curr Opin Microbiol* 2008, **11**(15):442-446.

15. Garvie EI: **Genus *Pediococcus*.** In *Bergey's Manual of Systematic Bacteriology*. Edited by Sneath PHA, Mair ME, Sharpe ME, Holt JG. Baltimore, MD: Williams and Wilkins; 1986:1075-1079.

16. Pederson CS: **The genus *Pediococcus*.** *Bacteriology Reviews* 1949, **13**(4):225-232.

17. Coster E, White R: **Further studies of the genus *Pediococcus*.** *J Gen Microbiol* 1964, **37**:15-31.

18. Haakensen M, Dobson CM, Hill JE, Ziola B: **Reclassification of *Pediococcus dextrinicus* (Coster and White 1964) Back 1978 (Approved Lists 1980) as *Lactobacillus dextrinicus* comb. nov., and emended description of the genus *Lactobacillus*.** *Int J Syst Evol Microbiol* 2009, **59**:615-621.

19. Cato EP, Moore WEC, Johnson JL: **Synonomy of strains "*Lactobacillus acidophilus*" Group A2 (Johnson et al. 1980) with the type strain of *Lactobacillus crispatus* (Brygoo and Aladame 1953) Moore and Holdeman 1970.** *Int J Syst Bacteriol* 1983, **33**(2):426-428.

20. Tong H, Dong X: ***Lactobacillus concavus* sp. nov., isolated from the walls of a distilled spirit fermenting cellar in China.** *Int J Syst Evol Microbiol* 2005, **55**:2199-2202.

21. Kandler O, Schillinger U, Weiss N: ***Lactobacillus bifermentans* sp. nov., nom. rev., an organism forming CO2 and H2 from lactic acid.** *Syst Appl Microbiol* 1983, **4**:408-412.

22. Fujii T, Nakashima K, Hayashi N: **Random amplified polymorphic DNA-PCR based cloning of markers to identify the beer-spoilage strains of *Lactobacillus brevis*, *Pediococcus damnosus*, *Lactobacillus collinoides* and *Lactobacillus coryniformis*.** *J Appl Microbiol* 2005, **98**(5):1209-1220.

23. Cato EP, Moore WEC, Johnson JL: **Synonymy of strains of "*Lactobacillus acidophilus*" group A2 (Johnson *et al*. 1980) with the type strain of *Lactobacillus crispatus* (Brygoo and Aladame 1953) Moore and Holdeman 1970.** *Int J Syst Bacteriol* 1983, **33**:426-428.

24. Endo A, Roos S, Satoh E, Morita H, Okada S: ***Lactobacillus equigenerosi* sp. nov., a coccoid species isolated from faeces of thoroughbred racehorses.** *Int J Syst Evol Microbiol* 2008, **58**:914-918.

25. Roos S, Engstrand L, Jonsson H: ***Lactobacillus gastricus* sp. nov., *Lactobacillus antri* sp. nov., *Lactobacillus kalixensis* sp. nov. and *Lactobacillus ultunensis* sp. nov., isolated from human stomach mucosa.** *Int J Syst Evol Microbiol* 2005, **55**:77-82.

26. Fujisawa T, Shirasaka S, Watabe J, Mitsuoka T: ***Lactobacillus aviarius* sp. nov.: a new species isolated from the intestines of chickens.** *Syst Appl Microbiol* 1984, **5**(4):414-420.

27. Claesson MJ, van Sinderen D, O'Toole PW: ***Lactobacillus*** **phylogenomics - towards a reclassification of the genus.** *Int J Syst Evol Microbiol* 2008, **58**:2945-2954.

28. Naser SM, Dawyndt P, Hoste B, Gevers D, Vandemeulebroecke K, Cleenwerck I, Vancanneyt M, Swings J: **Identification of lactobacilli by *pheS* and *rpoA* gene sequence analysis.** *Int J Syst Evol Microbiol* 2007, **57**:2777-2789.

29. Hammes WP, Hertel C: **The genus *Lactobacillus*.** In *The genera of Lactic Acid Bacteria. Volume 2.* 2nd edition. Edited by Wood BJB, Holzapfel WH. Springer; 1995:19-55.

30. van de Guchte M, Penauld S, Grimaldi C, Barbe V, Bryson K, Nicolas P, Robert C, Oztas S, Mangenot S, Couloux A, Loux V, Dervyn R, Bossy R, Bolotin A, Batto JA, Walunas T, Gibrat JF, Bessieres P, Weissenbach J, Ehrlich SD, Magiun E: **The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution.** *Proc Natl Acad Sci USA* 2006, **103**(24):9274-9279.

31. Chenoll E, Macian MC, Aznar R: ***Lactobacillus rennini*** **sp. nov., isolated from rennin and associated with cheese spoilage.** *Int J Syst Evol Microbiol* 2006, **56**:449-452.

32. Endo A, Okada S: ***Lactobacillus composti*** **sp. nov., a lactic acid bacterium isolated from a compost of distilled shochu residue.** *Int J Syst Evol Microbiol* 2007, **57**:870-872.

33. Ehrmann MA, Brandt M, Stolz P, Vogel RF, Korakli M: ***Lactobacillus secaliphilus*** **sp. nov., isolated from type II sourdough fermentation.** *Int J Syst Evol Microbiol* 2007, **57**:745-750.

34. Hansen PA, Mocquot G: ***Lactobacillus acidophilus*** **(Moro) comb. nov.** *Int J Syst Bacteriol* 1970, **20**:325-327.

35. Skerman VBD, McGowan V, Sneath PHA(editors): **Approved lists of bacterial names.** *Int J Syst Bacteriol* 1980, **30**:225-420.

36. Kandler O, Stetter KO, Kohl R: ***Lactobacillus reuteri*** **sp. nov., a new species of heterofermentative lactobacilli.** *Zentralbl Mikrobiol Parasitenkd Infektionskr Hyg Abt 1 Orig* 1980, **C1**:264-269.

37. Li Y, Raftis E, Canchaya C, Fitzgerald GF, van Sinderen D, O'Toole PW: **Polyphasic analysis indicates that *Lactobacillus salivarius* subsp. *salivarius* and *Lactobacillus salivarius* subsp. *salicinius* do not merit separate subspecies status.** *Int J Syst Evol Microbiol* 2006, **56**:2397-2403.

38. Dobson CM, Deneer H, Lee S, Hemmingsen S, Glaze S, Ziola B: **Phylogenetic analysis of the genus *Pediococcus*, including *Pediococcus claussenii* sp. nov., a novel lactic acid bacterium isolated from beer.** *Int J Syst Evol Microbiol* 2002, **52**(Pt 6):2003-2010.

39. Felis GE, Dellaglio F, Mizzi L, Torriani S: **Comparative sequence analysis of a *recA* gene fragment brings new evidence for a change in the taxonomy of the *Lactobacillus casei* group.** Int J Syst Evol Microbiol 2001, **51**(6):2113-2117.

40. Jian W, Zhu L, Dong X: **New approach to phylogenetic analysis of the genus *Bifidobacterium* based on partial *hsp60* gene sequences.** *Int J Syst Evol Microbiol* 2001, **51**(5):1633-1638.

41. Kwok AYC, Chow AW: **Phylogenetic study of *Staphylococcus* and *Macrococcus* species based on partial *hsp60* gene sequences.** *Int J Syst Evol Microbio*l 2003, **53**(1):87-92.

42. Hill JE, Penny SL, Crowell KG, Goh S, Hemmingsen SM: **cpnDB: A Chaperonin Sequence Database.** *Genome Res* 2004, **14**(8):1669-1675.

43. Lloyed AT, Sharp PM: **Evolution of the *recA* gene and the molecular phylogeny of bacteria.** *J Mol Evol* 1993, **37**(4):399-407.

44. Vandamme P, Pot B, Gillis M, deVos P, Kersters K, Swings J: **Polyphasic taxonomy, a consensus approach to bacterial systematics.** *Microbiol Rev* 1996, **60**(2):407-438.

45. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL: **GenBank.** *Nucl Acids Res* 2008, **36**(database issue):D25-D30.

46. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh TM, Garrity GM, Tiedje JM: **The Ribosomal Database Project: improved alignments and new tools for rRNA analysis.** *Nucl Acids Res* 2009, **37(database issue)**:D141-D145.

47. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG: **Clustal W and Clustal X version 2.0.** *Bioinformatics* 2007, **23**:2947-2948.

48. Tamura K, Dudley J, Nei M, Kumar S: **MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0.** *Mol Biol Evol* 2007, **24**(8):1596-1599.

49. Saitou N, Nei M: **The neighbor-joining method: A new method for reconstructing phylogenetic trees.** *Mol Biol Evol* 1987, **4**(4):406-425.

50. Nicholas KB, Nicholas HBJ, Deerfield DWI: **Genedoc: Analysis and Visualization of Genetic Variation.** EMBNEW NEWS [Online] http://www psc edu/biomed/genedoc 1997, **4**:14.

51. Snel B, Bork P, Huynen MA: **Genome phylogeny based on gene content.** *Nature Genetics* 1999, **21**:108-110.

52. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** *J Mol Biol* 1990, **215**(3):403-410.

53. Michener CD, Sokal RR: **A quantitative approach to a problem in classification.** *Evolution* 1957, **11**:130-162.

54. Kersey P, Bower L, Morris L, Horne A, Petryszak R, Kanz C, Kanapin A, Das U, Michoud K, Phillips B, Gattiker A, Kulikova T, Faruque N, Duggan K, Mclaren P, Reimholz B, Duret L, Reuter G, Reuter I, Apweiler R: **In-tegr8 and Genome Reviews: integrated views of complete genomes and proteomes.** *Nucl Acids Res* 2005, **33(Database issue)**:D297-302).

Figure Legends

Figure 1 - Cumulative number of currently accepted, validly described *Lactobacillus* species by year identified [1].

Figure 2 - Phylogenetic tree of the 16S rRNA gene of *Lactobacillus* and neighbouring bacterial genera.
This phylogenetic tree comprises a total of 277 type strains. The length of the base of each triangle represents the number of species within the genus. The edge length of each triangle corresponds with the scale bar indicating 2% divergence.

Figure 3 - Phylogenetic tree of the genus *Lactobacillus*.
Lactobacillus/Paralactobacillus/Pediococcus branch expanded from Figure 2. Bootstrap values are given for branches where this value is greater than 60%. Branches supported by trees created by the same method using the Cpn60 (◆), PheS (●), RecA (■), and RpoA (▲) proteins are indicated where appropriate. Groups I-VII correspond to putative subdivisions given in Table 2. a – only 16S rRNA gene sequence available; b – *cpn60* and *recA* sequences not available; c – *recA* sequence not available; d – *cpn60* sequence not available; e – *cpn60*, *recA*, and *rpoA* sequences not available; f – *cpn60*, *recA*, and *pheS* sequences not available. Bar indicates 1% divergence.

Figure 4 - Phylogenetic tree of the *L. acidophilus* group (*Jensenella* gen. nov.) expanded from Figure 3.
Branch nodes with bootstrap values greater than 60% are given. Branches supported by trees created by the same method using the Cpn60 (◆), PheS (●), RecA (■), and RpoA (▲) proteins are indicated where appropriate. a – *cpn60* and *recA* sequences not available; b – *recA* sequence not available; c – *cpn60* sequence not available; d – *cpn60*, *recA*, and *rpoA* sequences not available; e - *cpn60*, *rpoA*, and *pheS* sequences not available. Bar indicates 1% divergence.

Figure 5 - Phylogenetic tree of the *L. reuteri* group (*Orlaea* gen. nov.) expanded from Figure 3.
Branch nodes with bootstrap values greater than 60% are given. Branches supported by trees created by the same method using the Cpn60 (◆), PheS (●), RecA (■), and RpoA (▲) proteins are indicated where appropriate. a – only 16S rRNA gene sequence available; b –

cpn60 and *recA* sequences not available; c – *recA* sequence not available; d – *cpn60* sequence not available. Bar indicates 1% divergence.

Figure 6 - Phylogenetic tree of the *L. salivarius* group (*Paralactobacillus*) expanded from Figure 3.

Branch nodes with bootstrap values greater than 60% are given. Branches supported by trees created by the same method using the Cpn60 (◆), PheS (●), RecA (■), and RpoA (▲) proteins are indicated where appropriate. a – only 16S rRNA gene sequence available; b – *cpn60* sequence not available; c – *cpn60*, *recA*, and *rpoA* sequences not available; d – *cpn60* and *recA* sequences not available; e – *pheS* and *rpoA* sequences not available; f – *recA* sequence not available. Bar indicates 0.05% divergence.

Figure 7 - Dendrogram of all *Lactobacillus* and *Pediococcus* isolates with sequenced genomes as of March 10th, 2009.

Representative species of the *Lactococcus*, *Leuconostoc*, *Oenococcus*, and *Streptococcus* genera are added to assist with spatial orientation. Scale represents the average number of unique open reading frames present between two genomes (see Methods). Bar indicates 200 pairwise differences.

Figure 8 - Phylogenetic tree of the 16S rRNA gene of the genus *Lactobacillus*, proposed new genera *Jensenella* and *Orlaea*, emended genus *Paralactobacillus* and neighbouring bacterial genera.

This phylogenetic tree comprises a total of 275 type strains. The length of the base of each triangle represents the number of species within the genus. The edge length of each triangle corresponds with the scale bar indicating 2% divergence.

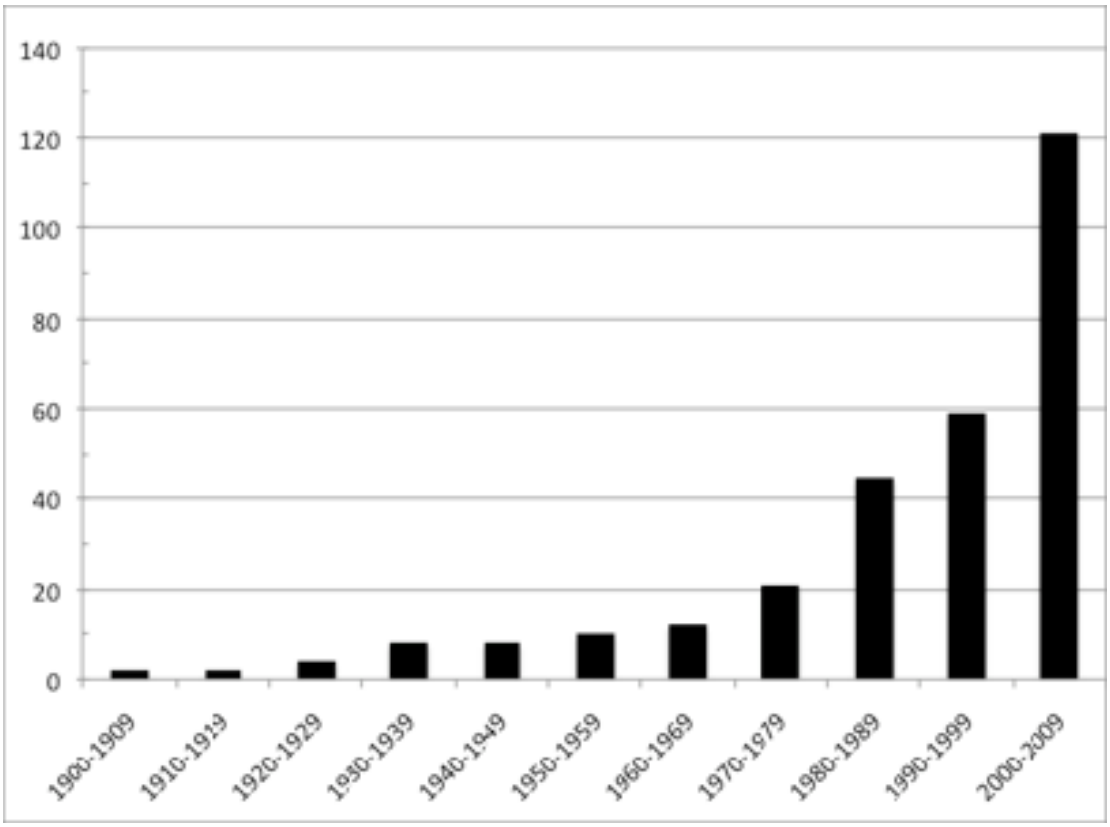
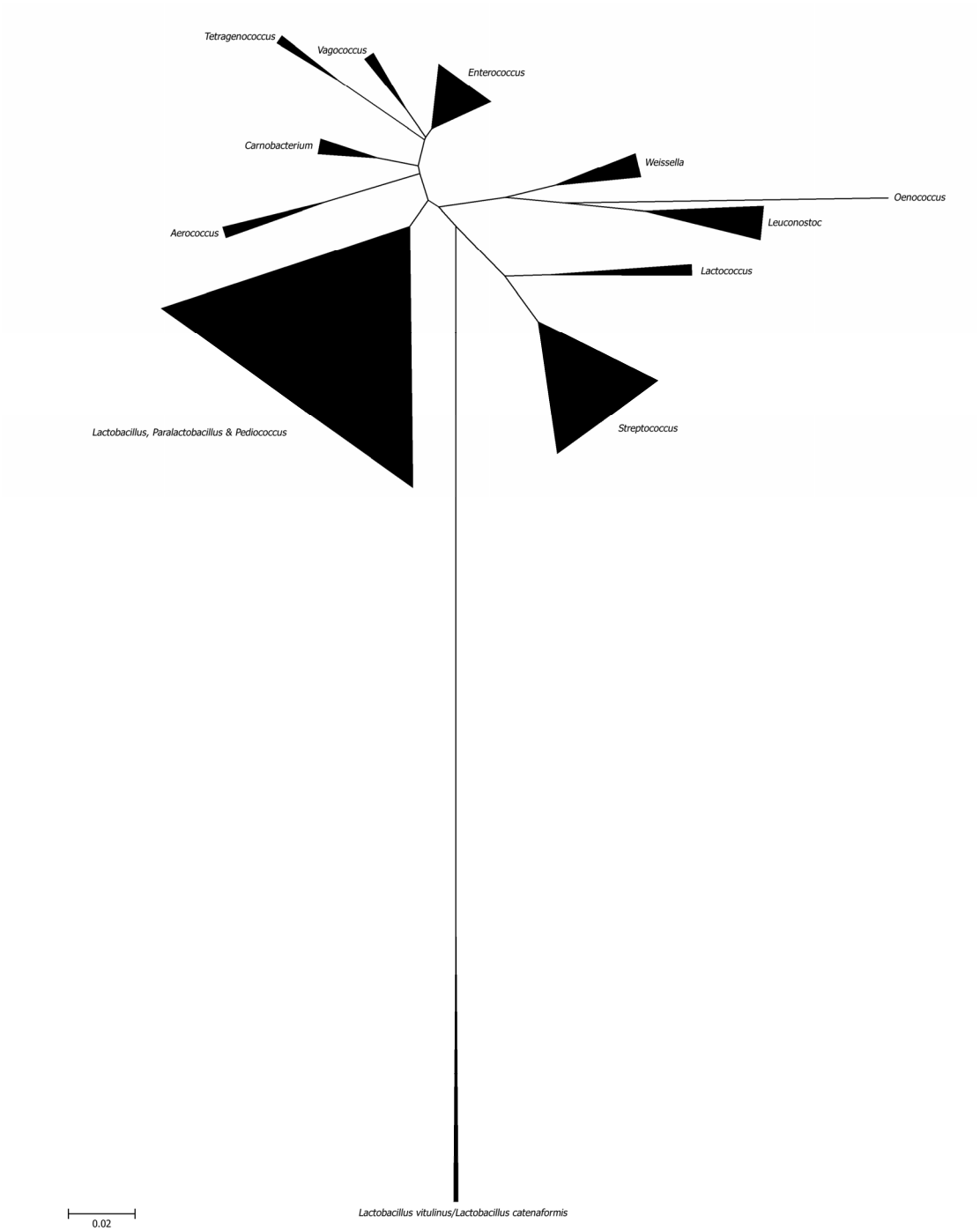


Figure 1 - Cumulative number of currently accepted, validly described *Lactobacillus* species by year identified [1].



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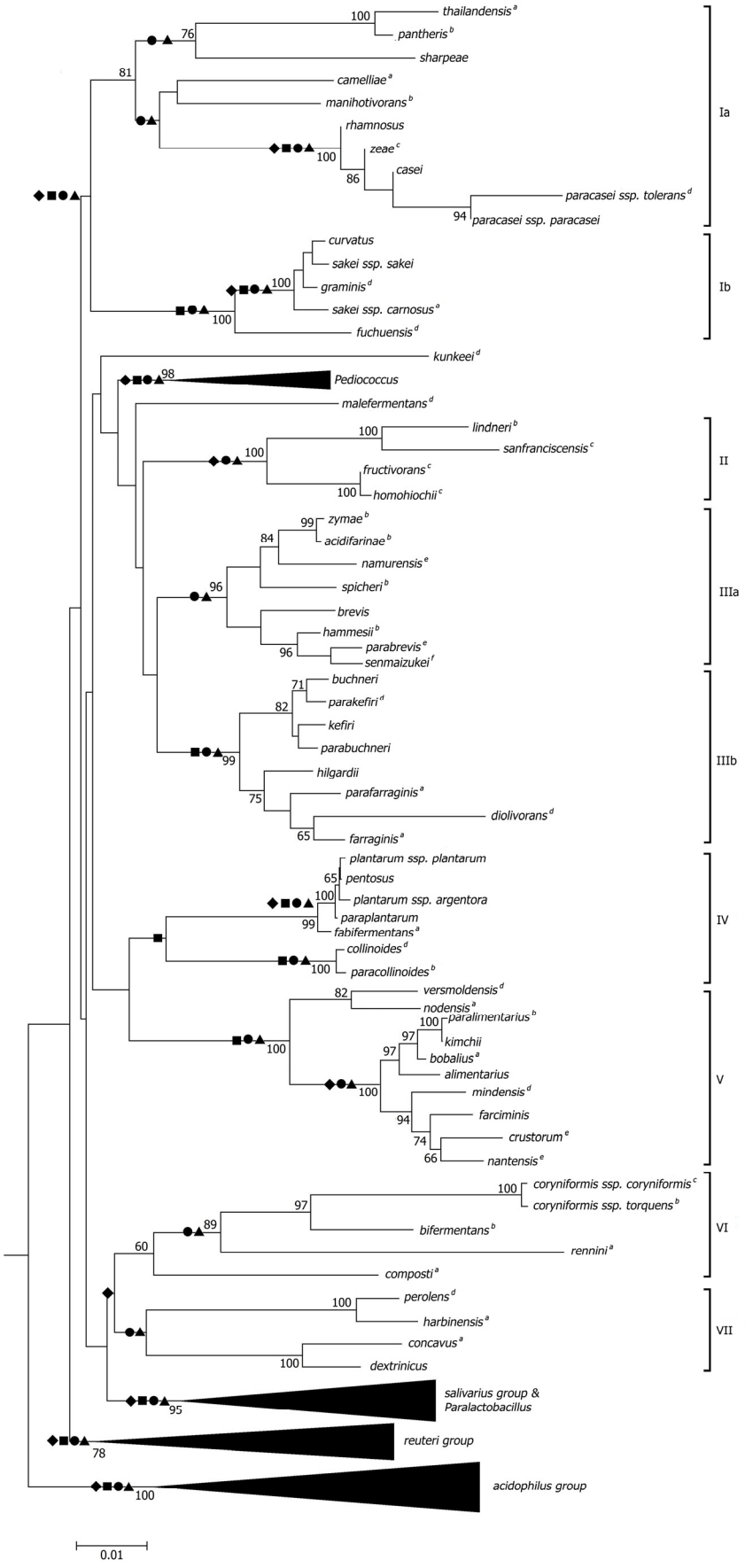

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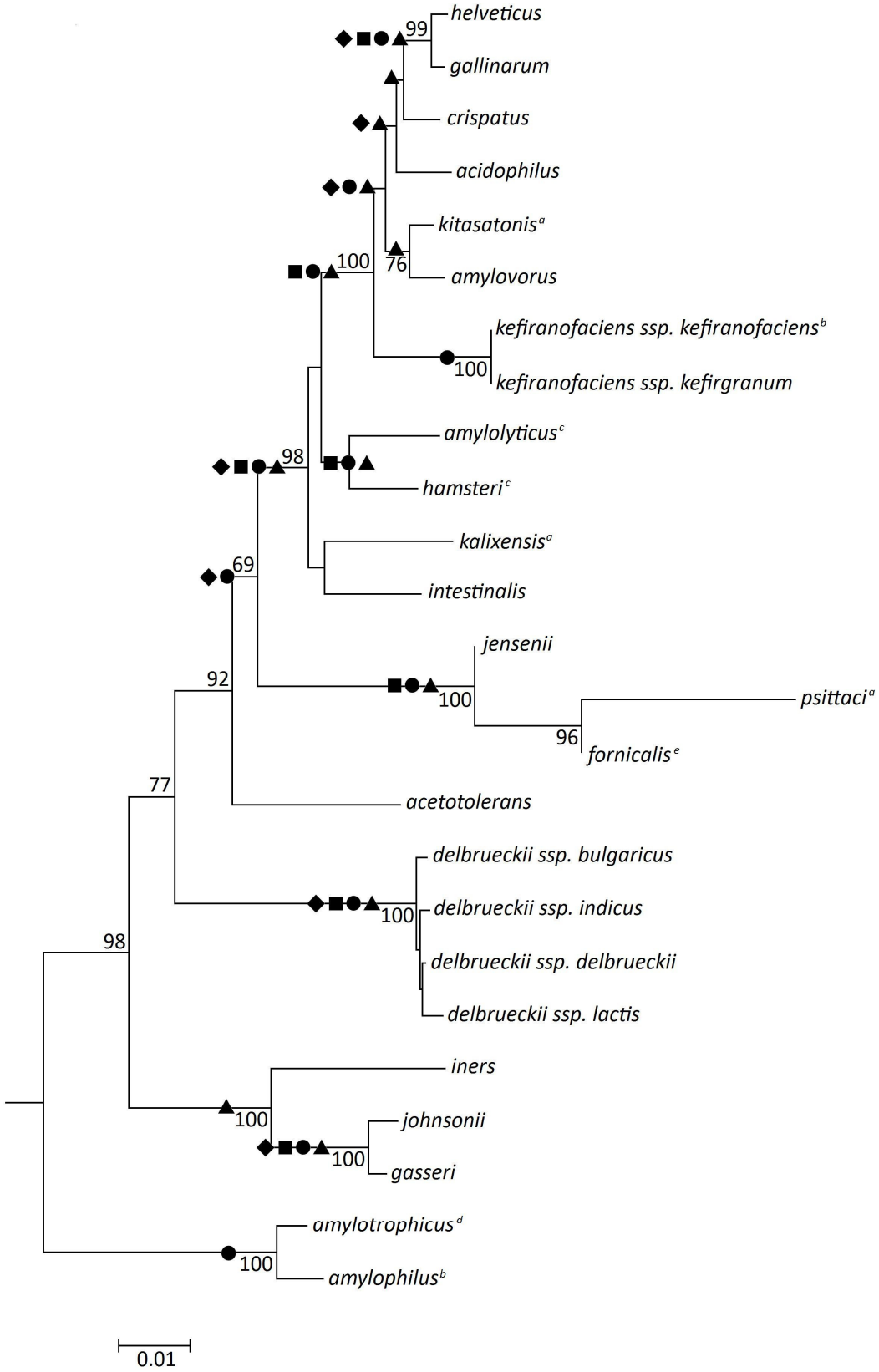

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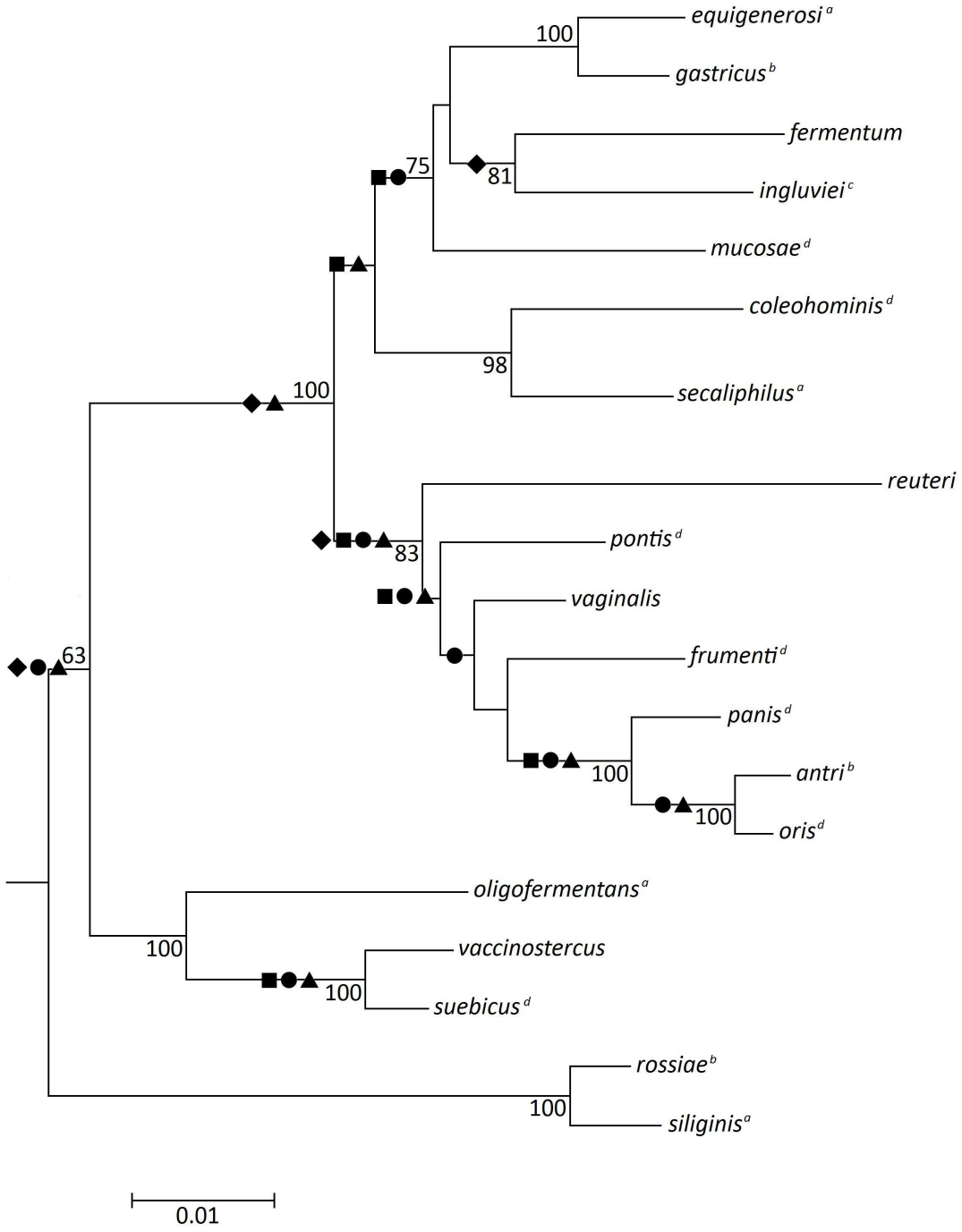

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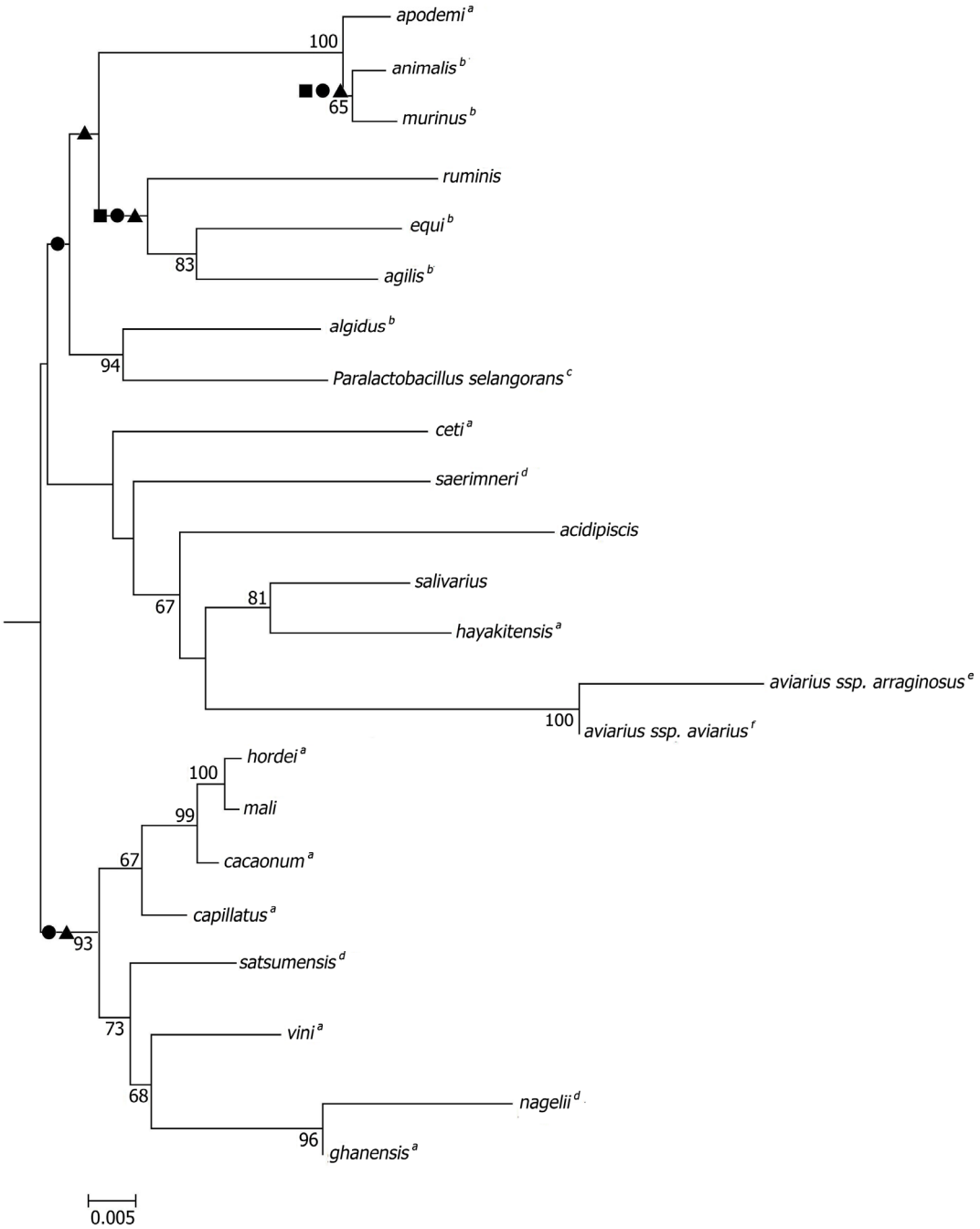

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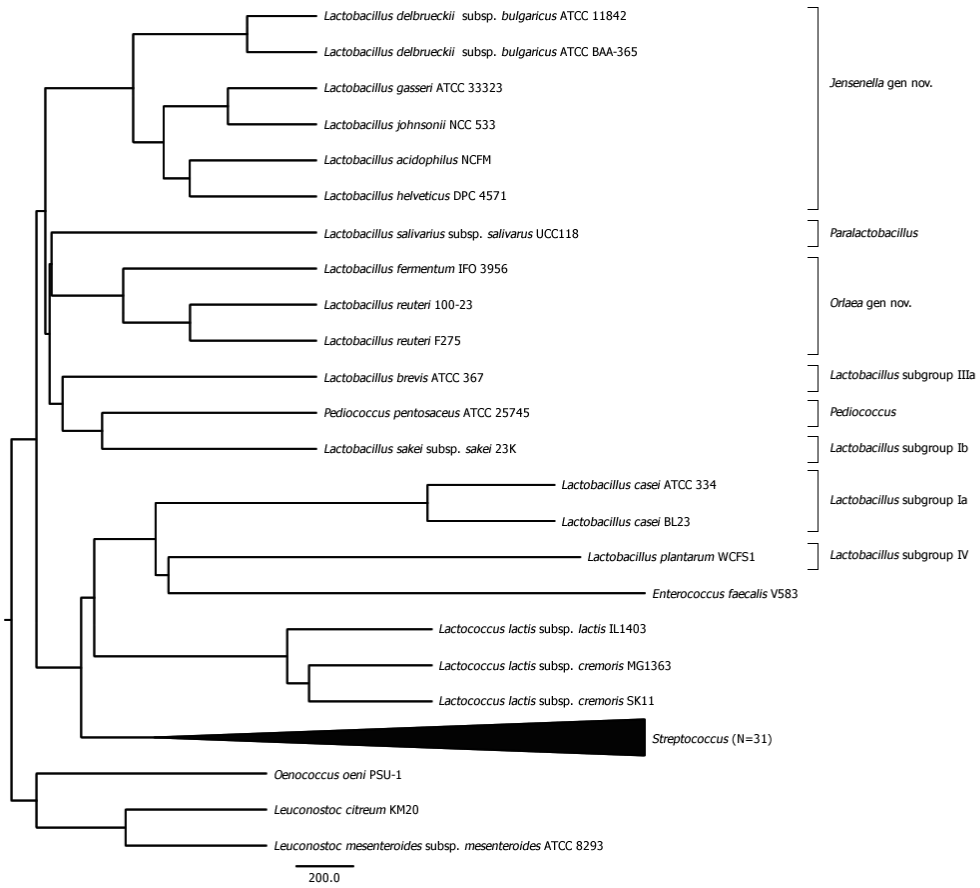

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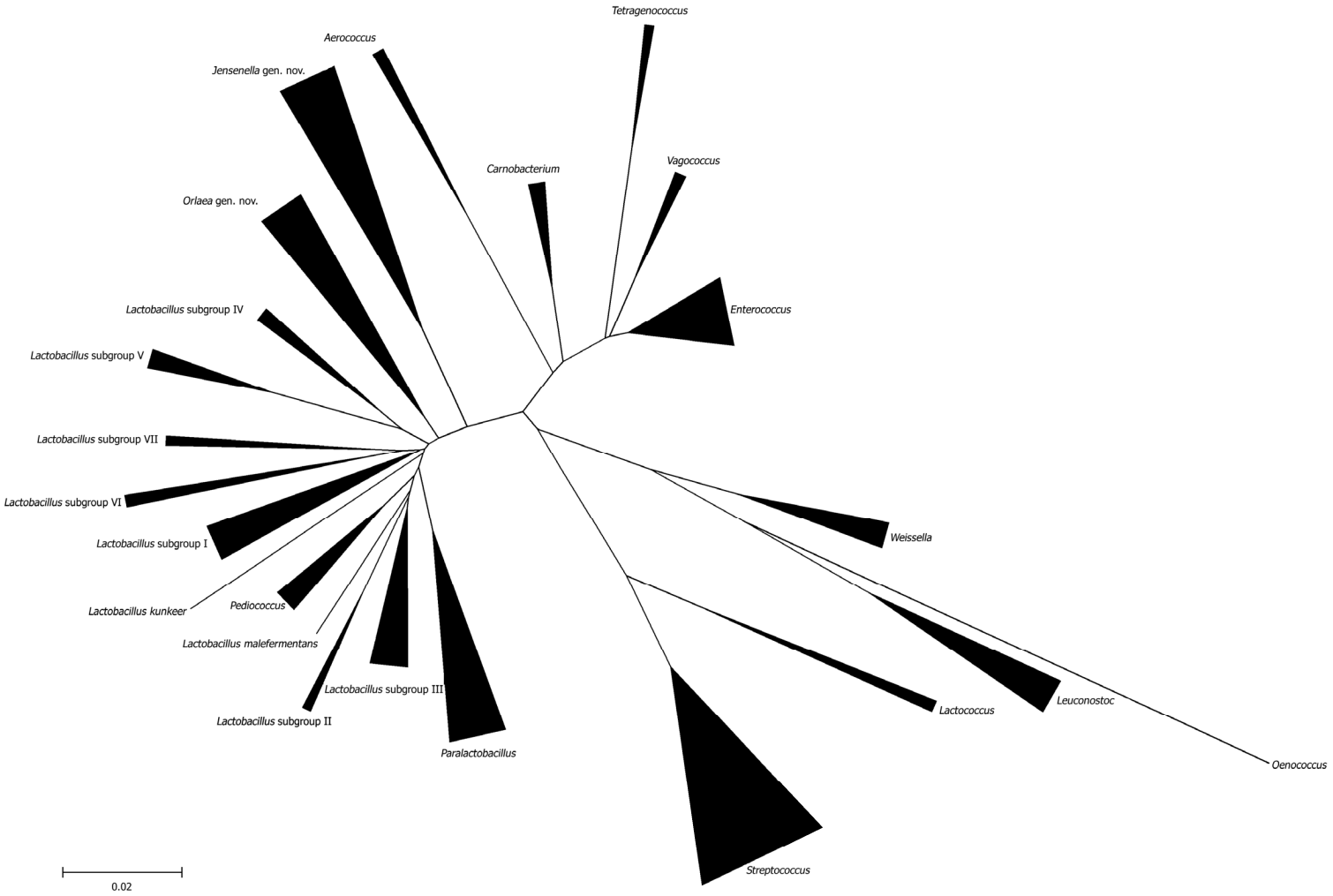

Figure 8 - Phylogenetic tree of the 16S rRNA gene of the genus *Lactobacillus*, proposed new genera *Jensenella* and *Orlaea*, emended genus *Paralactobacillus* and neighbouring bacterial genera.

Tables

Table 1 - 16S rRNA gene percent identity.

The core *Lactobacillus* group is comprised of isolates found in subgroups I-VII in Figure 3 Members of the genus *Lactobacillus* as it stands prior to division share 83-99% identity within the 16S rRNA gene. By removing the *L. acidophilus*, *L. reuteri*, and *L. salivarius* groups, this range tightens to 88-99%. Note that the *L. salivarius* group includes *Paralactobacillus selangorensis*. Cells indicate range of inter-group or inter-genus % identity while the cells on the diagonal (in bold) indicate intra-group or intra-genus range of % identity; n/c, no calculation as only a single species exists.

	Genus or Group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Core *Lactobacillus*	**88-99**														
2	*L. acidophilus* Group	84-90	**91-99**													
3	*L. reuteri* Group	84-91	85-90	**91-99**												
4	*L. salivarius* Group	83-92	85-89	86-92	**92-99**											
5	*Pediococcus*	88-94	86-90	88-94	84-94	**93-97**										
6	*Aerococcus*	85-89	84-87	84-88	85-89	85-88	**92-95**									
7	*Carnobacterium*	84-92	82-89	82-91	84-91	85-88	85-91	**95-98**								
8	*Enterococcus*	85-91	85-88	85-90	81-92	87-91	87-91	91-94	**92-99**							
9	*Lactococcus*	82-86	82-85	81-86	82-87	82-86	83-85	84-86	85-87	**91-97**						
10	*Leuconostoc*	80-87	82-86	81-86	82-86	81-85	83-85	82-85	83-87	79-83	**90-99**					
11	*Oenococcus*	78-81	80-81	79-81	78-81	78-80	78-80	77-81	80-82	79-81	82-84	**n/c**				
12	*Streptococcus*	78-86	78-86	77-86	77-87	78-87	78-86	80-88	80-89	83-92	80-84	79-81	**89-99**			
13	*Tetragenococcus*	85-89	85-87	83-87	86-88	85-87	87-89	89-91	91-94	83-84	83-85	79-80	82-86	**95-97**		
14	*Vagococcus*	84-89	83-88	82-91	85-91	86-91	86-91	90-94	90-96	83-88	80-86	78-81	80-88	87-93	**95-99**	
15	*Weissella*	84-90	84-88	84-90	84-90	84-90	84-88	85-90	85-90	82-85	84-90	82-83	80-87	84-88	85-88	**93-98**

Table 2 - Percent identity of the 16S rRNA gene using *Lactobacillus* isolates that fall into the category of "core *Lactobacillus*", using the groupings as designated in Table 1

The core *Lactobacillus* subgroups correspond to those shown in Figure 3. Cells indicate range of inter-group % identity while the cells on the diagonal (in bold) indicate intra-group range of % identity; n/c, no calculation as only a single species exists.

	***Lactobacillus* subgroup**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	Ia	**91-99**										
2	Ib	91-94	**95-99**									
3	*L. kunkeei*	90-91	91-92	**n/c**								
4	*L. malefermentans*	90-92	92-93	91	**n/c**							
5	II	88-93	89-92	90-91	92-93	**93-98**						
6	IIIa	90-93	91-93	91-92	93-95	91-95	**95-99**					
7	IIIb	89-93	90-92	90-92	92-94	90-94	93-95	**94-99**				
8	IV	89-93	91-93	91-92	93-94	90-92	92-95	90-94	**94-99**			
9	V	91-94	89-93	89-90	91-92	89-91	90-93	88-92	90-93	**94-99**		
10	VI	88-92	89-93	90-92	89-92	89-91	89-92	88-91	89-93	88-90	**91-99**	
11	VII	89-93	90-93	90-91	91-92	89-91	91-92	90-92	91-92	89-91	89-92	**92-98**

Table 3 - Core *Lactobacillus* subgroups corresponding to Figure 3.

Subgroup	Species	Metabolism	%G-C content	Lactic Acid Isomer	Peptidoglycan
Ia					
	camelliae	A	51-52	L	ND
	casei subsp. *casei*	B	45-47	L	Lys-D-Asp
	manihotivorans	A	48-49	L	Lys-D-Asp
	pantheris	A	52-53	D	mDpm
	paracasei subsp. *paracasei*	B	45-47	L	Lys-D-Asp
	paracasei subsp. *tolerans*	B	45-47	L	Lys-D-Asp
	rhamnosus	B	45-47	L	Lys-D-Asp
	sharpeae	A	53	L	DAP
	thailandensis	A	49	DL	ND
	zeae	B	48-49	L	Lys-D-Asp
Ib					
	curvatus	B	42-44	DL	Lys-D-Asp
	fuchuensis	B	41-42	L	ND
	graminis	B	41-43	DL	Lys-D-Asp
	sakei subsp. *carnosus*	B	42-44	DL	ND
	sakei subsp. *sakei*	B	42-44	DL	ND
II					
	fructivorans	C	38-41	DL	Lys-D-Asp
	homohiochii	B	35-38	DL	Lys-D-Asp
	lindneri	C	35	DL	Lys-D-Asp
	sanfranciscensis	C	36-38	DL	Lys-Ala
IIIa					
	acidifarinae	C	51	DL	ND
	brevis	C	44-47	DL	Lys-D-Asp
	hammesii	B	52-53	DL	L-Lys-D-Asp
	namurensis	C	52	DL	ND
	parabrevis	C	49	DL	ND
	senmazukei	B	46	DL	L-Lys-D-Asp
	spicheri	B	55	DL	Lys-D-Asp
	zymae	C	53-54	DL	ND
IIIb					
	buchneri	C	44-46	DL	Lys-D-Asp
	diolivorans	C	40	ND	ND
	farraginis	B	40-41	DL	no mDAP
	hilgardii	C	39-41	DL	Lys-D-Asp
	kefiri	C	41-42	DL	Lys-D-Asp
	parabuchneri	C	44	ND	Lys-D-Asp
	parafarraginis	B	40	DL	no mDAP
	parakefiri	C	41-42	L	Lys-D-Asp
IV					
	fabifermentans	ND	45	DL	ND
	paraplantarum	B	44-45	DL	DAP
	pentosus	B	46-47	DL	DAP

Table 3 continued

Subgroup	Species	Metabolism	%G-C content	Lactic Acid Isomer	Peptidoglycan
	plantarum subsp. *argentoratensis*	B	44-46	DL	DAP
	plantrum subsp. *plantarum*	B	44-46	DL	DAP
	collinoides	C	46	DL	Lys-D-Asp
	paracollinoides	C	44-45	D	ND
V					
	alimentarius	B	36-37	L-DL	Lys-D-Asp
	bobalius	B	33-35	DL	L-Lys-D-Asp
	crustorum	A	35-36	L	ND
	farciminis	A	34-36	L	Lys-D-Asp
	kimchii	B	35	DL	Lys-D-Asp
	mindensis	A	37-38	DL	Lys-D-Asp
	nantensis	B	38-39	DL	ND
	nodensis	A	41	DL	L-Lys-D-Asp
	paralimentarius	B	37-38	ND	Lys-D-Asp
	versmoldensis	A	40-41	L	ND
VI					
	bifermentans	B	45	DL	Lys-D-Asp
	composti	B	48	DL	no mDAP
	coryniformis subsp. *coryniformis*	B	45	DL	Lys-D-Asp
	coryniformis subsp. *torquens*	B	45	DL	Lys-D-Asp
	rennini	B	ND	DL	L-Lys-D-Asp
VII					
	concavus	A	46-47	DL	mDAP
	dextrinicus	ND	39-40	L	ND
	perolens	B	49-53	L	Lys-D-Asp
	harbinensis	B	53-54	L	ND
single					
	kunkeei	C	ND	L	Lys-D-Asp
	malefermentans	C	41-42	ND	Lys-D-Asp
Distant to *Lactobacillus*					
	catenaformis	A	31-33	D	Lys-Ala
	vitulinus	A	34-37	D	mDAP

[a] Type of glucose fermentation, following the conventions of Hammes and Vogel [29] and Hammes and Hertel [2], metabolism is indicated with A, B, and C capital letters for obligately homofermentative (A), facultatively heterofermentative (B), and obligately heterofermentative (C), respectively.

Table 4 - *L. acidophilus* group (*Jensenella*), corresponding to Figure 4.

Species	New Spelling	Metabolism[a]	%G-C content	Lactic Acid Isomer	Peptidoglycan
acetotolerans	NC	B	35-37	DL	Lys-D-Asp
acidophilus	*acidophila*	A	34-37	DL	Lys-D-Asp
amylolyticus	*amylolytica*	A	39	DL	Lys-D-Asp
amylophilus	*amylophila*	A	44-46	L	Lys-D-Asp
amylotrophicus	*amylotrophica*	A	44	L	Lys-D-Asp
amylovorus	*amylovora*	A	40-41	DL	Lys-D-Asp
crispatus	*crispata*	A	35-38	DL	Lys-D-Asp
delbrueckii subsp. *bulgaricus*	*delbrueckii* subsp. *bulgarica*	A	49-51	D	Lys-D-Asp
delbrueckii subsp. *delbrueckii*	NC	A	49-51	D	Lys-D-Asp
delbrueckii subsp. *indicus*	*delbrueckii* subsp. *indica*	A	ND	D	Lys-D-Asp
delbrueckii subsp. *lactis*	NC	A	49-51	D	Lys-D-Asp
fornicalis	NC	B	37	DL	Lys-D-Asp
gallinarum	NC	A	36-37	DL	Lys-D-Asp
gasseri	NC	A	33-35	DL	Lys-D-Asp
hamsteri	NC	B	33-35	DL	Lys-D-Asp
helveticus	*helvetica*	A	38-40	DL	Lys-D-Asp
iners	NC	A	34-35	L	Lys-D-Asp
intestinalis	NC	B	33-35	DL	Lys-D-Asp
jensenii	NC	B	35-37	D	Lys-D-Asp
johnsonii	NC	A	33-35	DL	Lys-D-Asp
kalixensis	NC	A	35-36	DL	Lys-D-Asp
kefiranofaciens subsp. *kefiranofaciens*	NC	A	34-38	DL	Lys-D-Asp
kefiranofaciens subsp. *kefirgranum*	NC	A	34-38	DL	Lys-D-Asp
kitasatonis	NC	B	37-40	DL	ND
psittaci	NC	ND	ND	ND	Lys-D-Asp

NC, no change; ND, no data

[a] Type of glucose fermentation, following the conventions of Hammes and Vogel [29] and Hammes and Hertel [2], metabolism is indicated with A and B capital letters for obligately homofermentative (A), facultatively heterofermentative (B), respectively.

Table 5 - Species belonging to the *L. reuteri* group (*Orlaea*), corresponding to Figure 5.

Species	New spelling	Metabolism[a]	%G-C content	Lactic Acid Isomer	Peptidoglycan
antri	NC	C	44-45	DL	Lys-D-Asp
*coleohominis**	NC	C	ND	DL	mDpm
equigenerosi	NC	ND	ND	ND	ND
fermentum	NC	C	52-54	DL	Orn-D-Asp
frumenti	NC	C	43-44	L	Lys-D-Asp
gastricus	*gastrica*	C	41-42	DL	L-Orn-D-Asp
ingluviei	NC	C	49-50	ND	ND
mucosae	NC	C	46-47	DL	Orn-D-Asp
oligofermentans	NC	C	35-40	DL	ND
oris	NC	C	49-51	DL	Orn-D-Asp
panis	NC	C	49-51	DL	Orn-D-Asp
pontis	NC	C	53-56	DL	Orn-D-Asp
reuteri	NC	C	40-42	DL	Lys-D-Asp
rossiae	NC	C	44-45	DL	Lys-Ser-Ala
secaliphilus	*secaliphila*	B	48	DL	L-Lys-D-Asp
siliginis	NC	C	44-45	ND	L-Lys-D-Glu-L-Ala
suebicus	*suebica*	C	40-41	DL	DAP
vaccinostercus	*vaccinosterca*	C	36-37	ND	DAP
vaginalis	NC	C	38-41	ND	Orn-D-Asp

NC, No change; ND, No data.

[a] Type of glucose fermentation, following the conventions of Hammes and Vogel [29]and Hammes and Hertel [2], metabolism is indicated with A, B, and C capital letters for obligately homofermentative (A), facultatively heterofermentative (B), and obligately heterofermentative (C), respectively.

Table 6 - Species belonging to *L. salivarius* group (*Paralactobacillus*), corresponding to Figure 6.

Species	Metabolism[a]	%G-C content	Lactic Acid Isomer	Peptidoglycan
apodemi	B	39	L	L-Lys-D-Asp
acidipiscis	B	38-41	L	Lys-D-Asp
agilis	B	43-44	L	DAP
algidus	B	36-37	L	DAP
animalis	A	41-44	L	Lys-D-Asp
aviarius subsp. *araffinosus*	A	39-43	DL	Lys-D-Asp
aviarius subsp. *aviarius*	A	39-43	DL	Lys-D-Asp
cacaonum	ND	45	L	ND
capillatus	ND	38	DL	mDpm
ceti	ND	ND	ND	ND
equi	A	38-39	DL	ND
ghanensis	ND	37-41	DL	DAP
hayakitensis	A	34-35	L	Lys-D-Asp
hordei	ND	ND	ND	ND
mali	A	32-34	L	DAP
murinus	B	43-44	L	Lys-D-Asp
nagelii	A	ND	DL	mDpm
ruminis	A	44-47	L	DAP
saerimneri	A	42-43	DL	DAP
salivarius	A	34-36	L	Lys-D-Asp
satsumensis	A	39-41	L	DAP
vini	B	39-40	DL	L-Lys-D-Asp
Paralactobacillus selangorensis	A	46	DL	ND

ND, No data

[a] Type of glucose fermentation, following the conventions of Hammes and Vogel [29] and Hammes and Hertel [2], metabolism is indicated with A, B, and C capital letters for obligately homofermentative (A), facultatively heterofermentative (B), and obligately heterofermentative (C), respectively. *P. selangorensis* info taken from Leisner et al [4].

Table 7 - Percent G-C content analysis of sequenced *Lactobacillus* and *Pediococcus* genomes.

Isolate	Positions 1+2[a]	Position 3[b]	Overall[c]
"core" *Lactobacillus*			
L. brevis ATCC 367	46.6	47.8	47.0
L. casei ATCC 334	46.7	48.7	47.3
L. casei BL23	46.5	47.9	47.0
L. plantarum ATCCBAA-793	45.7	45.1	45.5
L. sakei 23K	44.0	37.7	41.9
P. pentosaceus ATCC 25745	42.1	29.5	37.9
L. acidophilus group (*Orlaea*)			
L. acidophilus NCFM	40.3	25.0	35.2
L. delbrueckii subsp. *bulgaricus* ATCC BAA-365	44.9	64.7	51.5
L. delbrueckii subsp. *bulgaricus* ATCC 11842	45.0	64.9	51.6
L. gasseri ATCC 33323	40.5	25.7	35.5
L. helveticus DPC 4571	40.8	31.6	37.7
L. johnsonii NCC 533	40.3	24.3	35.0
L. reuteri group (*Jensenella*)			
L. fermentum LMG 18251	47.1	64.8	53.0
L. reuteri DSM 20016	43.3	32.0	39.5
L. reuteri JCM 1112	43.3	32.0	39.6
L. salivarius group (*Paralactobacillus*)			
L. salivarius UCC118	39.2	21.4	33.3

[a] Average % G-C content for nucleotide positions 1 and 2 of codons within the predicted ORFs of the indicated genome.

[b] Average % G-C content for nucleotide position 3 of codons within the predicted ORFs of the indicated genome.

[c] Average % G-C content for all nucleotides (1, 2, and 3) within predicted ORFs of the indicated genome.

Table 8 – Summary of characteristics for proposed genera

Genus	Metabolism[a]	%G-C content	Lactic Acid Isomer	Peptidoglycan	Cell Morphology
Jensenella	A or B	33-51	L, D, or DL	Lys-D-Asp	bacilli, curled rod
Lactobacillus	A or B or C	34-54	L, D, or DL	DAP, L-Lys-D-Asp, Lys-Ala, Lys-D-Asp, mDpm, no mDAP	bacilli, bent rod, coccobacilli, cocci
Orlaea	B[b] or C	35-56	L[b] or DL	DAP, L-Orn-D-Asp, L-Lys-D-Asp, L-Lys-D-Glu-L-Ala, Lys-D-Asp, Lys-Ser-Ala, mDpm	cocci, oval, bent rod, or bacilli
Paralactobacillus	A or B	32-47	L or DL	DAP, Lys-D-Asp, L-Lys-D-Asp, mDpm*	bacilli
Pediococcus	A	37-42	L or DL	Lys-D-Asp	cocci[c]

[a] Type of glucose fermentation, following the conventions of Hammes and Vogel [29]and Hammes and Hertel [2], metabolism is indicated with A, B, and C capital letters for obligately homofermentative (A), facultatively heterofermentative (B), and obligately heterofermentative (C), respectively.

[b] Only one species.

[c] Dividing in two perpendicular directions on a single plane.

CHAPTER 12

DISCUSSION, CONCLUSIONS, AND FUTURE WORK

12.1 General Discussion

Specific discussion regarding the details and findings pertinent to the individual manuscripts has been given throughout the thesis in Chapters 2-11. The intention of this final chapter is to discuss the work of the thesis as a whole, with the content of the Chapters relating to one another as depicted in Figure 1.7. Through the course of this thesis, several diverse approaches have been applied to investigate the genetic basis for *Lactobacillus* and *Pediococcus* bacteria to grow in beer. The work in this thesis began when only a single putative beer-spoilage (or hop-resistance) associated gene (i.e., *horA*) was known and progressed to the present situation with this thesis where seven putative beer-spoilage related genes have been investigated in considerable detail (i.e., *ABC2, bsrA, bsrB, hitA, horA, horC,* and *ORF5*). Despite the extensive search for beer-spoilage associated genes, none of the genes identified has an absolute correlation to the ability of bacteria to grow in beer. Even when all seven putative beer-spoilage associated genes were taken into account together, there remained several isolates that could grow in beer for which a genetic basis could not be identified to account for this ability. Rather, a scenario such as that outlined in Figure 1.1 began to emerge, where hop-resistance alone was not sufficient to permit growth in beer, but additional factors such as ethanol-resistance and nutrient acquisition are also required.

Through this research, findings and developments were made that have impacted industries and research beyond the brewing industry. For example, the *Firmicutes*-specific probe developed in Chapter 2 has additionally generated interest for use in the field of probiotics and intestinal health. While Chapters 3-6 were focused to the detection of beer-spoiling lactobacilli and pediococci, the finding of multiple genes coding for MDR transporters led to a greater understanding of the use and application of hop-compounds as antibiotics in the brewing industry and the relation of this use to broader antimicrobial resistance of pediococci (Chapter 8). Furthermore, the lack of correlation between speciation and ability to grow in beer initiated the development of a detection system for these organisms that was independent of genetic background (Chapter 7), a detailed study into the taxonomy of *Lactobacillus* and *Pediococcus*

(Chapters 11 and 9, respectively), and an additional study regarding the genomics of the *Lactobacillus* genus as compared to other genera (Chapter 10).

When examined independently, it may not at first be obvious how topics such as antibiotic resistance, phylogenetics, and genomic analyses tie into the study of beer-spoilage. However, when taken as a whole, in the context of the genetic studies in the earlier chapters, a larger picture begins to emerge as depicted in Figure 1.7. By taking the information in this thesis *en toto*, the interconnectedness of these seemingly disparate areas of study becomes apparent as each sector of analysis provides support and strength to the others and leads towards future areas of research for this field of study. In this context, beer-spoilage is no longer "just" about beer. It has come to be about the bacteria that cause this spoilage, how they might interact with their environment, how they have evolved to arrive at this point, and the impact they may exert on other environments in the future. These findings as presented and discussed in the preceding 10 Chapters can be divided into five major areas as outlined below.

12.2 Beer-spoilage associated genes

Chapters 2-5 were focused on the finding of putative beer-spoilage or hop-resistance related genes, development of efficient methods to detect these genes, and subsequent correlation of their presence with ability of isolates to grow in beer. There were multiple findings that arose from this work, with the first finding being that it is possible to identify molecular signatures at the taxonomic level of Phylum that can be applied for detection of specific groups of bacteria (Chapter 2). Additional work regarding this type of detection method is being conducted in Dr. Barry Ziola's laboratory by a graduate student Vanessa Pittet. In order to further triage and resolve the identity of organisms, a real-time PCR probe is being developed to a region of the 16S rRNA gene (nearby that found to be *Firmicutes*-specific) that can be used to differentiate between Gram-positive and Gram-negative *Firmicutes*.

Using the newly developed probe for detection of all *Firmicutes* as an internal control, it was then possible to begin gene-specific detection. Beginning with the known gene *horA*, it was shown that the correlation of *horA* with ability of organisms is not absolute, and is better correlated to *Lactobacillus* than to *Pediococcus* beer-spoilage isolates (Chapter 3). Moreover, the *horA* gene was also found to be present in bacteria of non-brewery origin, which may pose a threat in the form of potential new beer-spoiling organisms as additional bacteria were found

from genera traditionally thought of as non-beer spoilers but harbour the *horA* gene and are capable of spoiling beer (Chapter 4). The search for putative beer-spoilage related genes was then expanded to three additional genes suggested in literature to play a role in beer-spoilage (i.e., *hitA*, *horC*, and *ORF5*). However, it is shown in Chapter 5 that detection of these three genes does not add to the strength of identifying beer-spoiling bacteria already possible by detection of the *horA* gene alone. Interestingly, Chapter 5 also showed that the presence of the *hitA* gene in addition to *horA* may result in organisms being capable of growing more quickly in beer and it may be of future interest to determine whether this correlation was by chance, or whether there is a cumulative or synergistic effect experienced by the bacterium due to the presence of both the *hitA* and *horA* genes. Ideally, this would be assessed through reciprocal cloning of the genes into isolates that were lacking one or the other gene. However, there is currently no commercially available *Pediococcus* or *Lactobacillus* cloning system, and gram-negative bacteria that are used as hosts for cloning vectors are unsuitable for this work as gram-negative bacteria are intrinsically resistant to hop-compounds.

The correlation between bacterial ability to grow in beer and the presence of putative beer-spoilage related genes (i.e., *hitA*, *horA, horC*, and *ORF5*) was not absolute (Chapters 3 and 5). It was therefore necessary to search for additional putative spoilage-associated genes. Directed gene discovery using MSAs to design degenerate PCR primers against *horA*-like ABC MDR genes was found to be an effective method for identification of putative markers of beer-spoilage (Chapter 6). *Lactobacillus* and *Pediococcus* isolates were found to contain a variety of ABC MDR type genes (Chapter 6). And, of the six novel ABC MDR type genes found, only the *bsrA* and *bsrB* genes were found to correlate with beer-spoilage, with the *bsrA* gene being specific to beer-spoiling *Pediococcus* isolates (Chapter 6). Interestingly, the *ABC2* gene was found to correlate with hop-resistance in isolates which could not grow in beer, suggesting that in contrast to previously accepted belief, the ability to grow in the presence of hop-compounds is not sufficient to permit bacterial growth in beer (Chapter 6). However, even with the finding of additional beer-spoilage and hop-resistance associated genes, there remain isolates which can grow in beer for which there is no known genetic explanation for their ability to do so. As such, there must be as of yet unidentified genes involved in ability of organisms to grow in beer (Chapters 3, 5, and 6), it is also likely that new mechanisms of hop-resistance or ability to grow in beer will evolve over time.

12.3 Culture-dependent method for detecting beer-spoilage bacteria

As Chapters 2-6 found that the correlation between ability of organisms to grow in beer and known genes was not absolute, it was clear that a method to detect beer-spoiling bacteria was needed that would function independently of genetic background or speciation. In Chapter 7, various aspects of ability to grow in beer are explored, such as nutrient availability, presence of ethanol, and concentration of hop-compounds. It was found that nutrient availability and presence of ethanol affect an isolate's ability to grow in the presence of hop-compounds. By using a combination of ethanol and agar-medium with a concentration gradient of hop-compounds, beer-spoiling *Lactobacillus* and *Pediococcus* bacteria can be detected and differentiated from non-beer spoiling isolates (Chapter 7). Although these factors can be used to differentiate beer-spoiling and non-beer spoiling isolates, little is known regarding their interplay or genetic basis. Further work is needed to determine the range of ethanol resistance and/or tolerance possessed by these bacteria, as well as the mechanisms used to acquire the micronutrients needed for their growth.

12.4 Correlation of beer-spoilage associated genes to antimicrobial resistance

The findings from Chapters 3-6 suggested that the gene(s) involved in hop-resistance and/or ability to grow in beer are homologous to multi-drug resistance genes. Because little information is available regarding the antimicrobial resistances of the *Pediococcus* genus, this was chosen to be the focus of the study described in Chapter 8. The ability of each isolate was compared to the presence of known beer-spoilage associated genes (as discussed in Chapters 3-6). It was found that the genetic mechanisms involved in conferring hop-resistance or ability to spoil beer by *Pediococcus* isolates are not associated with resistance to antibiotics commonly used for treatment of human infections (Chapter 8). However, Vancomycin-resistance was found to be isolate-specific for pediococci and not intrinsic to the genus as previously believed (Chapter 8). In the future, it would be ideal if a larger scale study on a greater number of isolates and species of *Pediococcus* could be conducted and the beer-spoilage associated genes (i.e., those discussed in Chapters 2-6) in question also be cloned into a suitable Gram-positive host using appropriate vector system for reasons as discussed in section 12.2.

12.5 Taxonomic status of the *Pediococcus* and *Lactobacillus* genera

It was found through the work in Chapters 3-6 that the ability to spoil beer does not abide by the boundaries of speciation. It is hypothesized that if a better understanding of the taxonomy of the beer-spoiling genera can be attained, a better understanding of the mechanisms of beer-spoilage can be gained in the future. Being smaller, the genus *Pediococcus* was investigated first. *Pediococcus dextrinicus* was reassigned to the genus *Lactobacillus* as *Lactobacillus dextrinicus* comb. nov. to reflect its phylogenetic relationships and mode of cell division, thereby creating a more cohesive group of species in the genus *Pediococcus* (Chapter 9). Based upon multiple genetic criterion, it is suggested that the *Lactobacillus* genus be subdivided into four groups, one remaining as *Lactobacillus*, a second moving to the *Paralactobacillus* genus, and the third and fourth being *Jensenella* gen. nov. and *Orlaea* gen nov. (Chapter 11). Moreover, seven subdivisions are suggested for the species remaining as *Lactobacillus* isolates which may represent emerging genera (Chapter 11). As phylogenetic trees are dynamic constructs that change with time, the taxonomy of these genera, particularly the revised genus *Lactobacillus,* will also continue to change over time and will need to be reevaluated as new species are described. As whole genome sequencing becomes more accessible, genomic taxonomy may prove to be a useful tool for the understanding of taxonomy from a combined phylogenetic and phenotypic perspective.

12.6 Genomic and proteomic analysis of the *Lactobacillus* and *Pediococcus* genera

To aid with the phylogenetic assessment in Chapters 9 and 11, methods for assessing genomic and proteomic similarity between groups of organisms were developed. These methods of proteomic comparison were also developed with the thought in mind that phenotype-driven genomic subtractions could be performed by the same or similar methodologies. For simplicity, in Figure 1.7 this is referred to as *in silico* genome subtraction. A method is described by which groups of organisms can be compared and "core" and "unique" proteomes can be determined. In Chapter 10, this is first applied to the concept of a species or genus. Interestingly, 16S rRNA gene percent identity and proteomic similarity did not always highly correlate. This is particularly interesting in the context of the debate over whether a single gene (i.e., the 16S rRNA gene) can represent the relatedness of prokaryotic organisms. For some genera, the intra-genus comparisons of 16S rRNA gene similarity with proteomic similarity indicated little

correlation between the two parameters, suggesting that different evolutionary pressures are influencing them. However, only a limited number of discrepancies were found between the 16S rRNA gene phylogeny and whole proteomic phylogeny when viewed as a dendrogram (Chapter 10). Other notable findings were that the *Clostridium* and *Lactobacillus* genera contain a wider range of 16S rRNA gene diversity and deserve further examination to determine whether reclassification or subdivision is warranted (Chapter 10). And, in contrast to having a previously described "accelerated rate" of genomic degradation and lateral gene transfer (Chapter 1), the *Lactobacillus* genus was found to contain a lower amount of proteomic diversity than several other genera (Chapter 10). Work is currently underway to expand the whole proteomic dendrogram from Chapter 10 to include all sequenced prokaryotic genomes.

The whole genome sequencing of *Pediococcus claussenii* ATCC BAA-344^T is currently underway in the laboratories of Dr. Barry Ziola and Dr. Tony Kusalik, with draft contigs already assembled. This will be the first genome of a beer-spoiling bacterium to be sequenced. It is hoped that once this genome sequence is completed, it may be subtracted from the existing *Lactobacillus* and *Pediococcus* genomes (which have been determined to be incapable of growing in beer) in order to elucidate additional genetic mechanisms involved in hop-resistance, ethanol-resistance, nutrient acquisition, and ultimately, beer-spoilage, thus clarifying unresolved issues from Chapters 3-7.

12.7 Concluding remarks

The scientific landscape has rapidly evolved during the time course of this thesis. In the brewing industry, methods for detecting spoiling lactobacilli and pediococci have changed from culture-based techniques to genetic techniques, and then merged with more specific and precise culture-based techniques. Similarly, the "gold standard" for phylogenetics has evolved from 16S rRNA gene sequencing, to MLSA, to whole genomic analyses. This, of course, is not meant to be an end point, but a step along the way to continually emerging technologies and a building block for ideas yet to come. Along this same trend, I hope that the interdisciplinary approach taken in this thesis is also indicative of things to come. It has been through reaching out and learning from other disciplines (e.g., computer sciences, bioinformatics, and statistics) that it has been possible to bring together the seemingly disparate components depicted in Figure 1.7 into a cohesive body of research.

Appendix Ch2-1

Supplementary Data

Bacterial Isolates Tested

Isolate[a]	Origin	*Firmicutes*[b]	*Firmicutes* Probe[c]	357R probe[d]
Acetobacter aceti				
BSO 7	Brewery	-	-	+
BSO 8	Brewery	-	-	+
Acinetobacter calcoaceticus				
RUH 40	Human	-	-	+
Alcaligenes faecalis				
RUH 44	Human	-	-	+
Bacillus subtilis				
RUH 44	Human	+	+	+
Bacillus cereus				
MH 1	Spoiled home-brewed beer	+	+	+
Bacillus licheniformis				
MH 2	Spoiled home-brewed beer	+	+	+
Citrobacter freundii				
RUH 46	Human	-	-	+
Enterobacter agglomerans				
Ingledew[e] 127	Brewery	-	-	+
Enterococcus faecalis				
RUH 39	Human	+	+	+
Gluconobacter oxydans				
ATCC 19357	Brewery	-	-	+
Klebsiella pneumoniae				
RUH 47	Human	-	-	+
Lactobacillus acetotolerans				
ATCC 43578^T	Rice vinegar	+	+	+
Lactobacillus acidophilus				
ATCC 521	Unknown	+	+	+
ATCC 4356^T	Human	+	+	+
CCC B1209	Brewery	+	+	+
Lactobacillus amylovorus				
ATCC 33198[f]	Hog intestine	+	+	+
ATCC 33620^T	Corn silage	+	+	+
Field isolate[g]	Unknown	+	+	+
Ingledew I1	Fuel alcohol	+	+	+
Ingledew I2	Fuel alcohol	+	+	+
T-13[h]	Poultry	+	+	+

Lactobacillus brevis				
ATCC 4006	Unknown	+	+	+
ATCC 8007	Kefir grains	+	+	+
ATCC 14869[T]	Human feces	+	+	+
BSO 31[i]	Brewery	+	+	+
CCC 96S1L	Brewery	+	+	+
CCC 96S2AL	Brewery	+	+	+
CCC B1202	Brewery	+	+	+
CCC B1203	Brewery	+	+	+
CCC B1206	Brewery	+	+	+
CCC B1204	Brewery	+	+	+
CCC B1300	Brewery	+	+	+
ETS.1	Wine	+	+	+
ETS.2	Wine	+	+	+
Lactobacillus casei				
ATCC 334[h]	Cheese	+	+	+
ATCC 4913[h]	Unknown	+	+	+
ATCC 25598[T]	Milking machine	+	+	+
CCC 95G1L	Brewery	+	+	+
CCC 95G2L	Brewery	+	+	+
CCC B9657	Brewery	+	+	+
CCC B1205	Brewery	+	+	+
CCC B1241	Brewery	+	+	+
Ingledew I3	Fuel alcohol	+	+	+
Ingledew 18C	Fuel alcohol	+	+	+
Lactobacillus delbrueckii				
ATCC 4797	Corn mash	+	+	+
ATCC 9649[T]	Sour grain mash	+	+	+
ATCC 11842[T]	Bulgarian yogurt	+	+	+
ATCC 12315[T]	Cheese	+	+	+
CCC 95G3L	Brewery	+	+	+
CCC B1044	Brewery	+	+	+
CCC B1240	Brewery	+	+	+
CCC B1262	Brewery	+	+	+
Lactobacillus ferintoshensis				
ATCC 11307	Brewery	+	+	+
Lactobacillus fermentum				
ATCC 9338[h]	Unknown	+	+	+
ATCC 14931[T]	Fermented beets	+	+	+
ATCC 14932[h]	Saliva	+	+	+
Lactobacillus fructivorans				
ATCC 8288[T]	Unknown	+	+	+
Lactobacillus helviticus				
ATCC 15009[T]	Cheese	+	+	+
CCC B1186	Brewery	+	+	+

Lactobacillus hilgardii				
ATCC 8290[T]	Wine	+	+	+
ATCC 27305	Wine	+	+	+
ATCC 27306	Wine	+	+	+
Lactobacillus homohiochii				
ATCC 15434[T]	Spoiled sake	+	+	+
Lactobacillus jensenii				
ATCC 25258[T]	Human	+	+	+
Lactobacillus kefiri				
ATCC 35411[T]	Kefir grains	+	+	+
Lactobacillus kefirgranum				
ATCC 51647[T]	Kefir grains	+	+	+
Lactobacillus kefironofaciens				
ATCC 43761[T]	Kefir grains	+	+	+
Lactobacillus paracollinoides				
ATCC 8291	Brewery	+	+	+
Lactobacillus plantarum				
ATCC 8014	Unknown	+	+	+
ATCC 8041	Corn silage	+	+	+
ATCC 11305	Brewery	+	+	+
ATCC 12706	Cured meat	+	+	+
ATCC 14431[h]	Grass silage	+	+	+
ATCC 14917[T]	Pickled cabbage	+	+	+
BSO 92	Brewery	+	+	+
CCC 96M2BL	Brewery	+	+	+
CCC B1301	Brewery	+	+	+
Lactobacillus reuteri				
ATCC 19371	Silage	+	+	+
ATCC 25744	Plants	+	+	+
ATCC 31282	Unknown	+	+	+
ATCC 43200	Cucumbers	+	+	+
RC-14[h]	Unknown	+	+	+
Lactobacillus rhamnonsus				
ATCC 7469[T]	Unknown	+	+	+
ATCC 7469a[h]	Derived from ATCC 7469	+	+	+
ATCC 8530[h]	Unknown	+	+	+
ATCC 15820	Corn liquor	+	+	+
ATCC 21052[h]	Human feces	+	+	+
Lactobacillus sakei				
ATCC 15521[T]	Moto	+	+	+
ATCC 15578	Moto	+	+	+
Lactobacillus zeae				
ATCC 393	Cheese	+	+	+

Lactobacillus unspeciated				
ATCC 4005	Tomato pulp	+	+	+
ATCC 27054	Apple juice	+	+	+
ATCC 27304	Wine must	+	+	+
CCC L86	Brewery	+	+	+
Leuconostoc mesenteroides				
CCC 98G3	Brewery	+	+	+
Megasphaera cerevisiae				
CCC B1027	Brewery	+	+	+
Micrococcus luteus				
RUH 41	Human	-	-	+
Obesumbacterium proteus				
ATCC 12841^{T}	Brewery	-	-	+
Pectinatus cerevisiiphilus				
ATCC 29359^{T}	Brewery	+	+	+
DSM 20466	Brewery	+	+	+
Pectinatus frisingensis				
ATCC 33332^{T}	Brewery	+	+	+
DSM 20465	Brewery	+	+	+
Pediococcus acidilactici				
ATCC 8042	Brewery	+	+	+
ATCC 12697	Unknown	+	+	+
ATCC 25740	Plant	+	+	+
BSO 54	Brewery	+	+	+
BSO 77^{i}	Brewery	+	+	+
Molson B77b	Brewery	+	+	+
Pac 1.0^{j}	Unknown	+	+	+
Pediococcus claussenii				
CCC B962A	Brewery	+	+	+
CCC B1056R^{k}	Brewery	+	+	+
CCC B1056NR^{l}	Brewery	+	+	+
CCC B1098R	Brewery	+	+	+
CCC B1098NR	Brewery	+	+	+
CCC B1099R	Brewery	+	+	+
CCC B1099NR	Brewery	+	+	+
CCC B1100	Brewery	+	+	+
CCC B1208	Brewery	+	+	+
CCC B1260R	Brewery	+	+	+
CCC B1260NR	Brewery	+	+	+
ATCC BAA-344^{T}R	Brewery	+	+	+
ATCC BAA-344^{T}NR	Brewery	+	+	+
Pediococcus damnosus				
ATCC 11308	Brewery	+	+	+
ATCC 25248	Brewery	+	+	+

ATCC 25249	Brewery	+	+	+
ATCC 25249a	Brewery	+	+	+
ATCC 29358^{T}	Brewery	+	+	+
Molson B48	Brewery	+	+	+
Molson 49	Brewery	+	+	+
Molson B76	Brewery	+	+	+
Pediococcus dextrinicus				
ATCC 33087^{T}	Silage	+	+	+
Pediococcus inopinatus				
ATCC 49902^{T}	Brewery	+	+	+
Pediococcus parvulus				
ATCC 43013	Wine	+	+	+
ETS.3	Wine	+	+	+
ETS.4	Wine	+	+	+
ETS.5	Wine	+	+	+
ETS.6	Wine	+	+	+
ETS.7	Wine	+	+	+
ETS.8	Wine	+	+	+
ETS.9	Wine	+	+	+
ETS.11	Wine	+	+	+
ETS.12	Wine	+	+	+
ETS.13	Wine	+	+	+
ETS.14	Wine	+	+	+
Spain 2.6R^{m}	Cider	+	+	+
Spain 2.6NR^{m}	Cider	+	+	+
Pediococcus pentosaceus				
ATCC 8081	Milk	+	+	+
ATCC 10791	Cucumber	+	+	+
ATCC 11309	Unknown	+	+	+
ATCC 29723	Horse urine	+	+	+
ATCC 33314	Sake mash	+	+	+
ATCC 33316^{T}	Brewery	+	+	+
Proteus mirabilis				
RUH 48	Human	-	-	+
Pseudomonas aeruginosa				
RUH 42	Human	-	-	+
Selenomonas lacticifex	Brewery			
DSM 20575^{T}		+	+	+
Streptococcus viridans				
RUH 45	Human	+	+	+
Zymomonas mobilis				
BSO 57	Brewery	-	+	+
ATCC 29501	Unknown	-	+	+

Zymophilus paucinovorans DSM 20756[T]	Brewery	+	+	+
Zymophilus raffinosivorans DSM 20765[T]	Brewery	+	+	+

[a] Isolate identity as determined by CM Dobson (2001) with type-strains indicated. ATCC = American Type Culture Collection, Manassas, VA; BSO = Beer Spoilage Organism; CCC = Coors Brewing Company, Golden, CO; DSM = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; ETS = ETS Laboratories (T. Arvik), St. Helena, CA; MH = Monique Haakensen, Saskatoon, Saskatchewan, Canada; Molson = Molson Breweries of Canada Limited, Montreal, PQ, Canada; and RUH = Royal University Hospital, Saskatoon, Saskatchewan, Canada.
[b] Whether the isolate belongs to the Phylum *Firmicutes*.
[c] FAM fluorescence signal crossed threshold of 30 fluorescence units.
[d] Cy3 fluorescence signal crossed threshold of 10 fluorescence units.
[e, f, g,] Dr. W.M. Ingledew, Dr. A. Estrada, and Dr. D. Korber, respectively, College of Agriculture, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.
[h] Dr. G. Reid, Lawson Research Institute, London,ON.
[i] Dr. B. Kirsop, Institute for Biotechnology, Cambridge, England.
[j] Dr. R. Wheatcroft, Agriculture and Agri-food Canada, Guelph, Ontario, Canada.
[k] Ropy (R) phenotype.
[l] Non-ropy (NR) phenotype.
[m] Dr. K. Fernandez, Gipuzko, Spain

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Supplementary data

Isolates tested which are available from public culture collections.

Species	Isolate	Growth			
		Broth (BU)	HGA (cm)	HGA+E (cm)	In beer
Lactobacillus					
acetotolerans	ATCC 43578	12	1	1	-
acidophilus	ATCC 521	21	1	1	-
acidophilus	ATCC 4356	21	6	4	-
amylovorous	ATCC 33198	21	1	1	-
amylovorous	ATCC 33620	3	2	1.5	-
brevis	ATCC 4006	6	1.5	0.5	-
brevis	ATCC 8007	15	3	1	-
brevis	ATCC 14869	12	3	1	-
casei	ATCC 334	9	1	1	-
casei	ATCC 4913	15	1	1	-
casei	ATCC 25598	9	1	3	+
delbrueckii	ATCC 4797	18	0.5	0.5	-
delbrueckii	ATCC 9649	12	1.5	0.5	-
delbrueckii	ATCC 11842	9	1	1	-
delbrueckii	ATCC 12315	6	2	1	-
dextrinicus	ATCC 33087	9	2.5	2	-
fermentum	ATCC 9338	12	1	3.5	+
fermentum	ATCC 14931	3	1	0.5	-
fermentum	ATCC 14932	No growth	0.5	0.5	-
fructivorans	ATCC 8288	18	2	1	-
helveticus	ATCC 15009	18	1	1	-
hilgardii	ATCC 8290	6	2	1	-
hilgardii	ATCC 27305	6	6	6	+
hilgardii	ATCC 27306	21	1	1	-
homohiochii	ATCC 15434	3	1	1	-
jensenii	ATCC 25258	6	1	1	-
kefiranofaciens	ATCC 43761	9	1	0.5	-
kefirgranum	ATCC 51646	6	2	1.5	-
kefiri	ATCC 35411	3	2	1.5	-
parabuchneri	ATCC 11307	21	6	6	-
paracollinoides	ATCC 8291	6	2	5	+
plantarum	ATCC 8014	18	6	3	-
plantarum	ATCC 8041	18	4	4	+
plantarum	ATCC 11305	21	4	2	-
plantarum	ATCC 12706	18	6	3	-
plantarum	ATCC 14431	6	3	2	-
plantarum	ATCC 14917	21	5	2.5	-
reuteri	ATCC 19371	9	1.5	1	-
reuteri	ATCC 25744	15	4	1	-

reuteri	ATCC 31282	6	2	3	+
reuteri	ATCC 43200	25	1	2	-
rhamnosus	ATCC 7469	12	6	2	-
rhamnosus	ATCC 8530	9	2	6	+
rhamnosus	ATCC 15820	15	6	2	-
rhamnosus	ATCC 21052	18	2	1	-
sakei	ATCC 15521	18	2	2	-
sakei	ATCC 15578	9	6	6	+
zeae	ATCC 393	6	1	2	-
Pediococcus					
acidilactici	ATCC 8042	15	3.5	3	+
acidilactici	ATCC 12697	No growth	0.5	0.5	-
acidilactici	ATCC 25740	12	3	1.5	-
claussenii	ATCC BAA-344	21	6	5	+
damnosus	ATCC 11308	12	0.5	0.5	+
damnosus	ATCC 25248	15	1	1	-
damnosus	ATCC 25249	21	0.5	0.5	-
damnosus	ATCC 29358	18	2.5	2.5	-
inopinatus	ATCC 49902	12	1.5	1	-
parvulus	ATCC 43013	6	4	4	+
pentosaceus	ATCC 8081	9	2.5	2	-
pentosaceus	ATCC 10791	12	5	2	-
pentosaceus	ATCC 11309	12	5	5	-
pentosaceus	ATCC 29723	9	1	1	-
pentosaceus	ATCC 33314	No growth	2	2	-
pentosaceus	ATCC 33316	3	2.5	0.5	-

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Additional File 1 - Range of minimum inhibitory concentrations of antimicrobial compounds[a] summarized by species.

Antimicrobial compound	Range tested (μg/ml)	Range observed for *Pediococcus* species (N)							
		acidilactici(6)	*claussenii*		*damnosus*(1)	*inopinatus*(1)	*parvulus*		*pentosaceus*(4)
			ropy[b](5)	non-ropy[c](7)			ropy(1)	non-ropy(4)	
Ampicillin	0.12-16	0.12-4	0.12-0.25	0.12-4	0.25	4	0.25	0.12-4	1-4
Ceftriaxone	8-64	8-NR[d]	8	8-NR	8	8	8	8	8-64
Ciprofloxacin	0.5-2	NR	0.5-NR	0.5-NR	0.5	NR	NR	2-NR	2-NR
Clindamycin	0.12-2	0.12-0.5	0.12	0.12-1	0.12	0.12	0.12	0.12	0.12
Daptomycin	0.25-8	0.5-2	0.25-0.5	0.25	0.25	1	0.5	0.25-0.5	0.25-2
Erythromycin	0.25-4	0.25	0.25-0.5	0.25-4	0.25	0.25	0.25	0.25	0.25
Gatifloxacin	1-8	1-8	1	2	1	8	1	1-4	8-NR
Gentamicin[e]	2-16, 500	2-8	2	2-NR	2	4	2	2	2-4
Levofloxacin	0.25-8	2-NR	0.5-2	2-4	1	NR	4	1-NR	4-NR
Linezolid	0.5-8	0.5-4	0.5-2	0.5-4	1	2	1	0.5-2	0.5-4
Oxacillin+2%NaCl	0.25-8	0.25-2	0.25	0.25-4	0.25	4	0.25	0.25-NR	0.25-8
Penicillin	0.06-8	0.25-NR	0.06-0.25	0.06-NR	0.06	2	0.25	0.06-4	0.5-2
Rifampin	0.5-4	0.5-4	0.12-1	0.5-2	0.5	2	2	0.5-4	0.5-4
Streptomycin[f]	1000	1000	1000	1000	1000	1000	1000	1000	1000
Synercid	0.12-4	0.12-2	0.12-0.5	0.12-1	0.25	2	0.5	0.12-1	0.25-2
Tetracycline	2-16	8-NR	2-8	2-NR	2	16	16	2-16	2-NR
Trimethoprim/ Sulfamethoxazole[g]	0.5/9.5-4/76	0.5-NR	0.5/9.5-NR	0.5/9.5-NR	0.5/9.5	NR	4/76	0.5/9.5-NR	4/76-NR
Vancomycin	1-128	4-NR	16-NR	4-NR	16	NR	NR	8-NR	NR

[a] Dilutions were predetermined by the GPN3F antibiotic plate format.

[b],[c] Isolates positive and negative for exopolysaccharide rope production, respectively

[d] NR; MIC not reached, isolate could grow at highest concentration of antibiotic tested.

[e] A range of 2-16 μg/ml was tested as well as a concentration of 500 μg/ml.

[f] A single concentration of 1000 μg/ml was tested.

[g] Recorded as concentration of Trimethroprim / concentration of Sulfamethoxazole.

Appendix Ch8-2

Isolate ID Abbreviations: NR - non-ropy; R - ropy

Concentration of antimicrobial compounds are given in microgram per ml, with NR indicating that an MIC was Not Reached for that combination of antimicrobial compound and isolate

ID	Genus	Species	Origin	Ability to G	Ropy	ABC2	bsrA	bsrB	hitA	horA	horC	Hop-gradie	Hop-gradient agar + ethanol cut	Ampicillin
BSO 77	Pediococcus	acidilactici	Brewery	no growth	Negative	Negative	Negative	Negative	Negative	Negative	Negative	1.5	Neg	2
BSO 54	Pediococcus	acidilactici	Brewery	no growth	Negative	Negative	Negative	Negative	Negative	Negative	Negative	2	Neg	2
Moslon B77b	Pediococcus	acidilactici	Brewery	no growth	Negative	Negative	Negative	Negative	Negative	Negative	Negative	2	Neg	4
ATCC 8042	Pediococcus	acidilactici	Brewery	+	Negative	Negative	+	+	Negative	+	Negative	3	Pos	2
ATCC 25740	Pediococcus	acidilactici	Plant	no growth	Negative	Negative	Negative	Negative	Negative	Negative	Negative	1.5	Neg	0.12
Pac 1.0	Pediococcus	acidilactici	Unknown	no growth	Negative	Negative	Negative	Negative	Negative	Negative	Negative	1	Neg	1
Molson B71R	Pediococcus	claussenii	Brewery	+	+	Negative	+	+	Negative	+	Negative	4	Pos	0.25
CCC B1056R	Pediococcus	claussenii	Brewery	+	+	Negative	+	+	Negative	+	Negative	5	Pos	0.25
CCC B1098R	Pediococcus	claussenii	Brewery	+	+	Negative	+	+	Negative	Negative	Negative	5	Pos	0.25
CCC B1099R	Pediococcus	claussenii	Brewery	+	+	Negative	+	+	Negative	Negative	Negative	5	Pos	0.12
CCC B1260R	Pediococcus	claussenii	Brewery	+	+	Negative	+	+	Negative	Negative	Negative	6	Pos	0.12
CCC B1208	Pediococcus	claussenii	Brewery	no growth	Negative	+	Negative	Negative	Negative	Negative	Negative	2	Neg	0.12
Molson B71R	Pediococcus	claussenii	Brewery	+	Negative	Negative	+	+	Negative	+	Negative	5	Pos	0.25
CCC B1056NR	Pediococcus	claussenii	Brewery	+	Negative	Negative	+	+	Negative	+	Negative	6	Pos	4
CCC B1098NR	Pediococcus	claussenii	Brewery	+	Negative	Negative	+	+	Negative	+	Negative	5	Pos	0.12
CCC B1099NR	Pediococcus	claussenii	Brewery	+	Negative	Negative	+	+	Negative	Negative	Negative	5	Pos	0.12
CCC B1100	Pediococcus	claussenii	Brewery	+	Negative	Negative	+	+	Negative	Negative	+	5	Pos	0.25
CCC B1260NR	Pediococcus	claussenii	Brewery	+	Negative	Negative	+	+	Negative	Negative	Negative	6	Pos	0.12
ATCC 29358	Pediococcus	damnosus	Brewery	no growth	Negative	Negative	Negative	Negative	Negative	Negative	+	2.5	Neg	0.25
ATCC 49902	Pediococcus	inoptinatus	Brewery	no growth	Negative	Negative	Negative	Negative	Negative	Negative	Negative	1	Neg	4
Spain 2.6R	Pediococcus	parvulus	Cider	no growth	+	Negative	Negative	Negative	Negative	Negative	Negative	1	Neg	0.25
ATCC 43013	Pediococcus	parvulus	Wine	+	Negative	Negative	+	Negative	Negative	Negative	Negative	4	Pos	0.12
ETS.6	Pediococcus	parvulus	Wine	no growth	Negative	Negative	Negative	Negative	Negative	Negative	Negative	0.5	Neg	0.5
ETS.8	Pediococcus	parvulus	Wine	no growth	Negative	Negative	Negative	Negative	Negative	Negative	Negative	0.5	Neg	0.25
ETS.14	Pediococcus	parvulus	Wine	no growth	Negative	Negative	Negative	Negative	Negative	Negative	Negative	0.5	Neg	2
ATCC 33316	Pediococcus	pentosaceus	Brewery	no growth	Negative	Negative	Negative	Negative	Negative	Negative	Negative	0.5	Neg	4
ATCC 10791	Pediococcus	pentosaceus	Cucumber	no growth	Negative	+	Negative	Negative	+	Negative	Negative	2	Neg	1
ATCC 29723	Pediococcus	pentosaceus	Horse urine	no growth	Negative	Negative	Negative	Negative	Negative	Negative	Negative	1	Neg	4
ATCC 11309	Pediococcus	pentosaceus	Unknown	no growth	Negative	Negative	Negative	Negative	Negative	Negative	Negative	5	Pos	4

Ceftriaxon	Ciprofloxa	Clindamyc	Daptomyci	Erythromy	Gatifloxaci	Gentamici	Levofloxac	Linezolid	Oxacillin +	Penicillin	Rifampin	Syndercid	Tetracyclin	Triometho	Vancomycin
32	NR	0.12	2	0.25	4	8	NR	2	1	0.5	0.5	1	8	NR	NR
32	NR	0.12	2	0.25	4	4	NR	2	0.25	0.5	0.5	1	16	NR	NR
NR	NR	0.5	1	0.25	4	2	NR	4	2	NR	4	2	NR	NR	NR
64	NR	0.12	2	0.25	8	8	NR	4	0.25	0.5	0.5	1	NR	NR	NR
8	NR	0.12	0.5	0.25	1	2	4	0.5	0.25	0.25	0.5	0.12	8	0.5	4
64	NR	0.12	2	0.25	1	2	2	4	2	1	0.5	0.5	2	4	NR
8	2	0.12	0.5	0.25	1	2	2	1	0.25	0.06	1	0.5	4	0.5	NR
8	0.5	0.12	0.25	0.5	1	2	2	0.5	0.25	0.12	0.5	0.25	4	0.5	NR
8	2	0.12	0.5	0.25	1	2	2	2	0.25	0.12	0.5	0.12	4	1	NR
8	2	0.12	0.25	0.25	1	2	2	2	0.25	0.25	0.5	0.12	2	2	16
8	NR	0.12	0.5	0.25	1	2	0.5	1	0.25	0.06	0.5	0.25	8	0.5	NR
8	2	0.12	0.5	0.25	1	2	2	1	0.25	0.12	0.5	0.25	4	0.5	NR
8	2	0.12	0.5	0.25	1	2	2	0.5	0.25	0.06	1	0.25	2	0.5	NR
NR	NR	1	1	0.25	4	2	NR	4	4	NR	2	1	NR	NR	NR
8	0.5	0.12	0.25	0.25	0.25	2	2	0.5	0.25	0.12	0.5	0.12	2	NR	4
8	1	0.12	0.5	0.25	0.25	2	2	2	0.25	0.12	0.5	0.12	2	4	128
8	2	0.12	1	0.25	1	2	2	1	0.25	0.12	0.5	0.5	8	0.5	NR
8	2	0.12	0.25	0.25	1	2	2	0.5	0.25	0.12	0.5	0.25	2	0.5	4
8	0.5	0.12	0.25	0.25	1	2	1	1	0.25	0.06	0.5	0.25	2	0.5	16
8	NR	0.12	1	0.25	8	4	NR	2	4	2	2	2	16	NR	NR
8	NR	0.12	0.5	0.25	1	2	4	1	0.25	0.25	2	0.5	16	4	NR
8	2	0.12	0.25	0.25	1	2	4	1	0.25	0.06	0.5	0.12	8	0.5	32
8	NR	0.12	0.25	0.25	2	2	8	2	0.25	2	1	0.12	8	NR	NR
8	NR	0.12	0.25	0.25	1	2	1	0.5	0.25	0.12	0.5	0.25	2	0.5	8
8	NR	0.12	0.5	0.25	4	2	NR	2	NR	4	4	1	16	NR	NR
32	NR	0.12	1	0.25	8	2	NR	4	8	2	4	2	NR	NR	NR
8	2	0.12	0.25	0.25	NR	2	4	0.5	0.25	0.5	2	0.25	2	4	NR
64	NR	0.12	2	0.25	NR	4	NR	4	8	2	2	2	NR	NR	NR
64	NR	0.12	2	0.25	8	4	NR	4	8	2	0.5	2	NR	NR	NR

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Genus	Species	Isolate	# ORFs	# bp
Bacillus	*amyloliquefaciens*	FZB42	3692	3,918,589
Bacillus	*anthracis*	Ames ancestor	5590	5,227,419
Bacillus	*anthracis*	Ames, isolate Porton	5313	5,227,293
Bacillus	*anthracis*	Sterne	5288	5,228,663
Bacillus	*cereus*	ATCC 10987	5821	5,224,283
Bacillus	*cereus*	ATCC 14579 / DSM 31	5240	5,411,809
Bacillus	*cereus*	ZK / E33L	5638	5,300,915
Bacillus	*cereus*	subsp. cytotoxis, strain NVH 391-98	3840	4,087,024
Bacillus	*clausii*	KSM-K16	4082	4,303,871
Bacillus	*halodurans*	C-125 / ATCC BAA-125 / JCM 9153 / FERM 7344 / DSM 18197	4006	4,202,352
Bacillus	*licheniformis*	DSM 13 / ATCC 14580, sub_strain Novozymes	4162	4,222,597
Bacillus	*pumilus*	SAFR-032	3675	3,704,465
Bacillus	*subtilis*	168	4112	4,215,606
Bacillus	*thuringiensis*	Al Hakam	4792	5,257,091
Bacillus	*thuringiensis*	konkukian (strain 97-27)	5169	5,237,682
Bacillus	*weihenstephanensis*	KBAB4	5650	5,262,775
Brucella	*abortus*	biovar 1, strain 9-941	3077	3,286,445
Brucella	*abortus*	2308	3022	3,278,307
Brucella	*abortus*	S19	2993	3,283,936
Brucella	*canis*	ATCC 23365 / NCTC 10854	3238	3,312,769
Brucella	*melitensis*	biovar 1, strain NCTC 10094 / ATCC 23456 / 16M	3178	3,294,931
Brucella	*ovis*	ATCC 25840 / 63/290 / NCTC 10512	2820	3,275,590
Brucella	*suis*	biovar 1, strain 1330	3256	3,315,175
Brucella	*suis*	ATCC 23445 / NCTC 10510	3214	3,324,607
Burkholderia	*ambifaria*	AMMD / ATCC BAA-244	6607	7,484,986
Burkholderia	*ambifaria*	MC40-6	6690	7,340,944
Burkholderia	*cenocepacia*	AU 1054	6450	7,279,116
Burkholderia	*cenocepacia*	HI2424	6898	7,537,983
Burkholderia	*cenocepacia*	MC0-3	6986	7,971,389
Burkholderia	*cepacia*	J2315 / LMG 16656	6993	7,963,121

Burkholderia	*mallei*	ATCC 23344	4797	5,835,527
Burkholderia	*mallei*	NCTC 10229	5309	5,742,303
Burkholderia	*mallei*	NCTC 10247	5619	5,848,380
Burkholderia	*mallei*	SAVP1	4981	5,232,401
Burkholderia	*phymatum*	DSM 17167 / STM815	7461	6,176,561
Burkholderia	*phytofirmans*	DSM 17436 / PsJN	7197	8,093,536
Burkholderia	*pseudomallei*	1106a	7138	7,089,249
Burkholderia	*pseudomallei*	1710b	6329	7,308,054
Burkholderia	*pseudomallei*	668	7215	7,040,403
Burkholderia	*pseudomallei*	K96243	5717	7,247,547
Burkholderia	*thailandensis*	E264 / ATCC 700388 / DSM 13276 / CIP 106301	5561	6,723,972
Burkholderia	*vietnamiensis*	R1808 / G4 / LMG 22486) / LMG 22486	7409	7,305,580
Burkholderia	*xenovorans*	LB400	8591	9,731,138
Clostridium	*acetobutylicum*	DSM 792 / JCM 1419 / LMG 5710 / ATCC 824 / VKM B-1787	3847	3,940,880
Clostridium	*beijerinckii*	ATCC 51743 / NCIMB 8052	5003	6,000,632
Clostridium	*botulinum*	ATCC 19397 / Type A	3547	3,863,450
Clostridium	*botulinum*	Alaska E43 / type E3	3255	3,659,644
Clostridium	*botulinum*	Eklund 17B / type B	3525	3,800,327
Clostridium	*botulinum*	Hall / ATCC 3502 / NCTC 13319 / Type A, sub_strain Los Alamos	3401	3,760,560
Clostridium	*botulinum*	Hall / ATCC 3502 / NCTC 13319 / Type A, sub_strain Sanger	3590	3,886,916
Clostridium	*botulinum*	Langeland / NCTC 10281 / Type F	3657	3,995,387
Clostridium	*botulinum*	Loch Maree / Type A3	3982	3,992,906
Clostridium	*botulinum*	Okra / Type B1	3850	3,958,233
Clostridium	*difficile*	630	3712	4,290,252
Clostridium	*kluyveri*	ATCC 8527 / DSM 555 / NCIMB 10680	3828	3,964,618
Clostridium	*novyi*	NT	2305	2,547,720
Clostridium	*perfringens*	13 / Type A	2721	3,031,430
Clostridium	*perfringens*	ATCC 13124 / NCTC 8237 / Type A	2873	3,256,683
Clostridium	*perfringens*	SM101 / Type A	2568	2,897,393
Clostridium	*phytofermentans*	ATCC 700394 / DSM 18823 / ISDg	3891	4,847,594

Clostridium	*tetani*	Massachusetts / E88	2414	2,799,251
Clostridium	*thermocellum*	ATCC 27405 / DSM 1237	3102	3,843,301
Lactobacillus	*acidophilus*	NCFM	1859	1,993,560
Lactobacillus	*brevis*	ATCC 367 / JCM 1170	2201	2,291,220
Lactobacillus	*casei*	ATCC 334	2708	2,895,264
Lactobacillus	*casei*	BL23	2999	3,079,196
Lactobacillus	*delbrueckii*	subsp. bulgaricus, strain ATCC 11842 / DSM 20081	1519	1,864,998
Lactobacillus	*delbrueckii*	subsp. bulgaricus, strain ATCC BAA-365	1682	1,856,951
Lactobacillus	*fermentum*	IFO 3956 / LMG 18251	1818	2,098,685
Lactobacillus	*gasseri*	ATCC 33323 / DSM 20243	1694	1,894,360
Lactobacillus	*helveticus*	DPC 4571	1580	2,080,931
Lactobacillus	*johnsonii*	NCC 533	1809	1,992,676
Lactobacillus	*plantarum*	WCFS1 / ATCC BAA-793 / NCIMB 8826	3051	3,308,274
Lactobacillus	*reuteri*	100-23	1972	2,174,299
Lactobacillus	*reuteri*	F275	1939	1,999,618
Lactobacillus	*sakei*	subsp. sakei, strain 23K	1872	1,884,661
Lactobacillus	*salivarius*	subsp. salivarius, strain UCC118	1998	1,827,111
Lactococcus	*lactis*	subsp. cremoris, strain MG1363	2384	2,529,478
Lactococcus	*lactis*	subsp. cremoris, strain SK11	2442	2,438,589
Lactococcus	*lactis*	subsp. lactis, strain IL1403	2225	2,365,589
Leuconostoc	*citreum*	KM20	1812	1,796,284
Leuconostoc	*mesenteroides*	subsp. mesenteroides, strain ATCC 8293 / NCDO 523	2002	2,038,396
Mycobacterium	*abscessus*	ATCC 19977 / DSM 44196	4939	5,067,172
Mycobacterium	*avium*	104	5040	5,475,491
Mycobacterium	*bovis*	AF2122/97 / ATCC BAA-935	3911	4,345,492
Mycobacterium	*bovis*	BCG / Pasteur 1173P2	3891	4,374,522
Mycobacterium	*gilvum*	ATCC 700033 / PYR-GCK / PYR-GCK	5499	5,619,607
Mycobacterium	*leprae*	TN	1603	3,268,203
Mycobacterium	*marinum*	ATCC BAA-535 / M	5418	6,636,827
Mycobacterium	*paratuberculosis*	ATCC BAA-968 / K-10	4316	4,829,781
Mycobacterium	*smegmatis*	ATCC 700084 / mc(2 / mc(2)155)	6597	6,988,209
Mycobacterium	*tuberculosis*	ATCC 25177 / H37Ra	3990	6,988,209

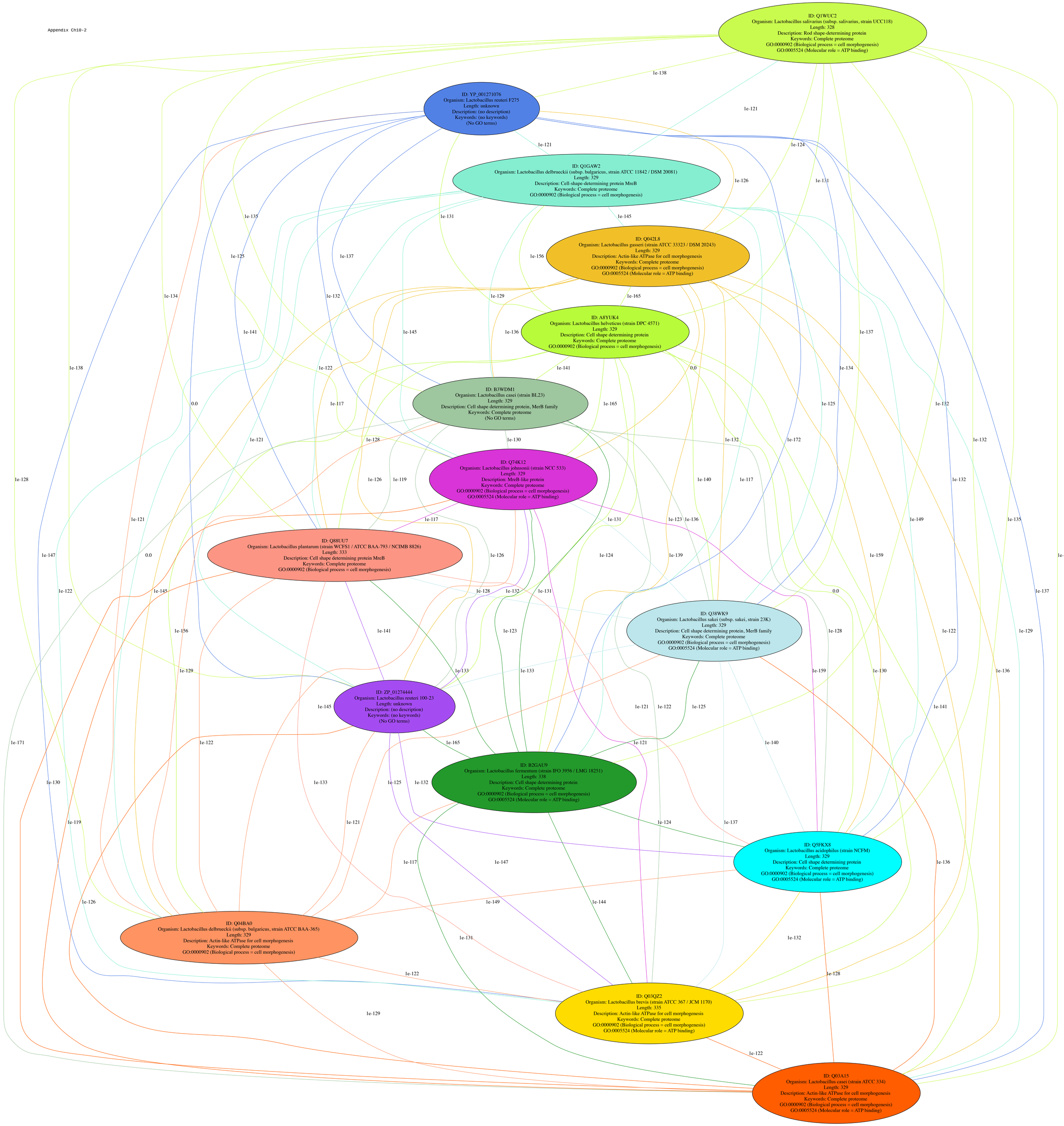
Mycobacterium	*tuberculosis*	ATCC 25618 / H37Rv	3949	6,988,209
Mycobacterium	*tuberculosis*	Oshkosh / CDC 1551	4196	4,403,837
Mycobacterium	*ulcerans*	Agy99	4206	5,631,606
Mycobacterium	*vanbaalenii*	DSM 7251 / PYR-1	5902	6,491,865
Neisseria	*gonorrhoeae*	ATCC 700825 / FA 1090	1963	2,153,922
Neisseria	*gonorrhoeae*	NCCP11945	2595	2,232,025
Neisseria	*meningitidis*	serogroup C / , serovar 2a, strain ATCC 700532 / FAM18	1865	2,194,961
Neisseria	*meningitidis*	serogroup C, strain 053442	1998	2,153,416
Neisseria	*meningitidis*	A (serogroup A, serovar 4A, strain Z2491)	1887	2,184,406
Neisseria	*meningitidis*	B (serogroup B, strain MC58)	2001	2,272,360
Pseudomonas	*aeruginosa*	LMG 12228 / ATCC 15692 / PRS 101 / 1C / PAO1	5558	6,264,404
Pseudomonas	*aeruginosa*	PA7	6246	6,588,339
Pseudomonas	*aeruginosa*	UCBPP-PA14	5886	6,537,648
Pseudomonas	*entomophila*	L48	5126	5,888,780
Pseudomonas	*fluorescens*	Pf-5 / ATCC BAA-477	6137	7,074,893
Pseudomonas	*fluorescens*	PfO-1	5728	6,438,405
Pseudomonas	*mendocina*	ymp	4563	5,072,807
Pseudomonas	*putida*	F1 / ATCC 700007	5245	5,959,964
Pseudomonas	*putida*	GB-1	5396	6,078,430
Pseudomonas	*putida*	KT2440	5313	6,181,863
Pseudomonas	*putida*	W619	5179	5,774,330
Pseudomonas	*stutzeri*	A1501	4093	4,567,418
Pseudomonas	*syringae*	pathovar phaseolicola, strain 1448A / Race	5044	5,928,787
Pseudomonas	*syringae*	pathovar syringae, strain B728a	5071	6,093,698
Pseudomonas	*syringae*	tomato (strain DC3000)	5424	6,397,126
Rhizobium	*etli*	CFN 42 / ATCC 51251	5921	4,381,608
Rhizobium	*etli*	CIAT 652	6050	4,513,324
Rhizobium	*leguminosarum*	bv. trifolii WSM2304	4320	4,537,948
Rhizobium	*leguminosarum*	bv. viciae (strain 3841)	7109	5,057,142
Rhizobium	*loti*	MAFF303099	7255	7,036,071
Rhizobium	*meliloti*	1021	6168	3,654,135
Rickettsia	*akari*	Hartford	1257	1,231,060

Rickettsia	*bellii*	OSU 85-389	1443	1,528,980
Rickettsia	*bellii*	RML369-C	1400	1,522,076
Rickettsia	*canadensis*	McKiel	1091	1,159,772
Rickettsia	*conorii*	ATCC VR-613 / Malish 7	1372	1,268,755
Rickettsia	*felis*	ATCC VR-1525 / URRWXCal2	1428	1,485,148
Rickettsia	*massiliae*	Mtu5	969	1,360,898
Rickettsia	*prowazekii*	Madrid E	834	1,111,523
Rickettsia	*rickettsii*	Iowa	1384	1,268,175
Rickettsia	*rickettsii*	Sheila Smith	1345	1,257,710
Rickettsia	*typhi*	Wilmington / ATCC VR-144	837	1,111,496
Shigella	*boydii*	serovar 18, strain CDC 3083-94 / BS512	4140	4,615,997
Shigella	*boydii*	serovar 4, strain Sb227	3937	4,519,823
Shigella	*dysenteriae*	serovar 1, strain Sd97 / Sd197	3890	4,369,232
Shigella	*flexneri*	serovar 2a, strain 2457T / ATCC 700930	3786	4,599,354
Shigella	*flexneri*	serovar 2a, strain 301	4102	4,607,203
Shigella	*flexneri*	serovar 5b, strain 8401	3867	4,574,284
Shigella	*sonnei*	Ss046	4053	4,825,265
Staphylococcus	*aureus*	COL	2679	2,809,422
Staphylococcus	*aureus*	JH1	2761	2,906,507
Staphylococcus	*aureus*	JH9	2708	2,906,700
Staphylococcus	*aureus*	MRSA252	2639	2,902,619
Staphylococcus	*aureus*	MSSA476	2602	2,799,802
Staphylococcus	*aureus*	MW2	2660	2,820,462
Staphylococcus	*aureus*	Mu3 / ATCC 700698	2684	2,880,168
Staphylococcus	*aureus*	Mu50 / ATCC 700699	2714	2,878,529
Staphylococcus	*aureus*	N315	2580	2,814,816
Staphylococcus	*aureus*	NCTC 8325	2890	2,821,361
Staphylococcus	*aureus*	Newman	2578	2,878,897
Staphylococcus	*aureus*	USA300 / TCH1516	2688	2,872,915
Staphylococcus	*aureus*	USA300	2607	2,872,769
Staphylococcus	*aureus*	bovine RF122 / ET3-1 / RF122	2513	2,742,531
Staphylococcus	*epidermidis*	ATCC 12228	2461	2,499,279
Staphylococcus	*epidermidis*	ATCC 35984 / RP62A	2492	2,616,530
Staphylococcus	*haemolyticus*	JCSC1435	2640	2,685,015

Staphylococcus	*saprophyticus*	subsp. saprophyticus, strain ATCC 15305 / DSM 20229	2404	2,516,575
Streptococcus	*agalactiae*	III (serovar III, strain NEM316)	1999	2,211,485
Streptococcus	*agalactiae*	Ia (serovar Ia, strain A909 / CDC SS700 / ATCC 27591)	1983	2,127,839
Streptococcus	*agalactiae*	V (serovar V, strain 2603 V/R / ATCC BAA-611)	2105	2,160,267
Streptococcus	*equi*	subsp. zooepidemicus, strain MGCS10565	1861	2,024,171
Streptococcus	*gordonii*	Challis / ATCC 35105 / CH1 / DL1 / V288	2050	2,196,662
Streptococcus	*mutans*	serovar c, strain ATCC 700610 / UA159	1951	2,030,921
Streptococcus	*pneumoniae*	serovar 19F, strain G54	2106	2,078,953
Streptococcus	*pneumoniae*	serovar 2, strain D39 / NCTC 7466	1918	2,046,115
Streptococcus	*pneumoniae*	ATCC BAA-255 / R6	2030	2,038,615
Streptococcus	*pneumoniae*	CGSP14	2193	2,209,198
Streptococcus	*pneumoniae*	Hungary19A-6	2152	2,245,615
Streptococcus	*pneumoniae*	TIGR4 / ATCC BAA-334	2109	2,160,842
Streptococcus	*pyogenes*	serovar M12, strain MGAS2096	1886	1,860,355
Streptococcus	*pyogenes*	serovar M12, strain MGAS9429	1868	1,836,467
Streptococcus	*pyogenes*	serovar M2, strain MGAS10270	1964	1,928,252
Streptococcus	*pyogenes*	serovar M4, strain MGAS10750	1964	1,937,111
Streptococcus	*pyogenes*	serovar M5, strain Manfredo	1736	1,841,271
Streptococcus	*pyogenes*	M1 (serovar M1, strain MGAS5005 / ATCC BAA-947)	1840	1,838,554
Streptococcus	*pyogenes*	M1 (serovar M1, strain SF370 / ATCC	1691	1,852,441
Streptococcus	*pyogenes*	M18 (serovar M18, strain MGAS8232)	1835	1,895,017
Streptococcus	*pyogenes*	M28 (serovar M28, strain MGAS6180)	1884	1,897,573
Streptococcus	*pyogenes*	M3 (serovar M3, strain ATCC BAA-595 / MGAS315)	1858	1,900,521
Streptococcus	*pyogenes*	M3 (serovar M3, strain SSI-1)	1852	1,894,275
Streptococcus	*pyogenes*	M6 (serovar M6, strain ATCC BAA-946 / MGAS10394)	1879	1,899,877
Streptococcus	*pyogenes*	NZ131	1700	1,815,785
Streptococcus	*sanguinis*	SK36	2269	2,388,435
Streptococcus	*suis*	05ZYH33	2179	2,096,309

Streptococcus	*suis*	98HAH33	2179	2,095,698
Streptococcus	*thermophilus*	ATCC BAA-250 / LMG 18311	1577	1,796,846
Streptococcus	*thermophilus*	ATCC BAA-491 / LMD-9	1704	1,856,368
Streptococcus	*thermophilus*	CNRZ 1066	1590	1,796,226
Vibrio	*cholerae*	serovar O1, strain ATCC 39315 / El Tor Inaba N16961	3784	4,033,464
Vibrio	*cholerae*	serovar O1, strain ATCC 39541 / Ogawa 395 / O395	3772	4,132,319
Vibrio	*fischeri*	ATCC 700601 / ES114	3814	4,227,869
Vibrio	*fischeri*	MJ11	4034	4,323,877
Vibrio	*harveyi*	ATCC BAA-1116 / BB120	5608	5,969,369
Vibrio	*parahaemolyticus*	serovar O3:K6, strain RIMD 2210633	4821	5,165,770
Vibrio	*vulnificus*	CMCP6	4473	5,126,797
Vibrio	*vulnificus*	YJ016	4990	5,211,578
Xanthomonas	*axonopodis*	pathovar citri, strain 306	4354	5,175,554
Xanthomonas	*campestris*	pathovar campestris, strain 8004	4239	5,148,708
Xanthomonas	*campestris*	pathovar campestris, strain B100	4410	5,079,002
Xanthomonas	*campestris*	pathovar vesicatoria, strain 85-10	4628	5,178,466
Xanthomonas	*campestris*	campestris (strain ATCC 33913 / NCPPB 528 / LMG 568)	4127	5,076,188
Xanthomonas	*oryzae*	pathovar oryzae, strain MAFF 311018	4204	4,940,217
Xanthomonas	*oryzae*	pathovar oryzae, strain PXO99A	4587	5,240,075
Xanthomonas	*oryzae*	oryzae (strain KXO85 / KACC10331)	4380	4,941,439
Yersinia	*enterocolitica*	serovar O:8 / biotype 1B, strain 8081	4021	4,615,899
Yersinia	*pestis*	biovar Mediaevalis, strain 91001	4013	4,595,065
Yersinia	*pestis*	biovar Mediaevalis, strain KIM5	3968	4,600,755
Yersinia	*pestis*	biovar Orientalis, strain CO-92	3908	4,653,728
Yersinia	*pestis*	Pestoides F	3942	4,517,345
Yersinia	*pestis*	bv. (strain Antiqua)	4135	4,702,289
Yersinia	*pestis*	bv. Antiqua (strain Angola)	3821	4,504,254
Yersinia	*pestis*	bv. Antiqua (strain Nepal516)	3946	4,534,590
Yersinia	*pseudotuberculosis*	serovar I, strain IP32953	4016	4,744,671
Yersinia	*pseudotuberculosis*	serovar IB, strain PB1/+	4213	4,695,619
Yersinia	*pseudotuberculosis*	serovar O:1b, strain IP 31758	4305	4,723,306

Yersinia	*pseudotuberculosis*	serovar O:3, strain YPIII	4171	4,689,441


ID: Q1WUC2
Organism: Lactobacillus salivarius (subsp. salivarius, strain UCC118)
Length: 328
Description: Rod shape-determining protein
Keywords: Complete proteome
GO:0000902 (Biological process = cell morphogenesis)
GO:0005524 (Molecular role = ATP binding)
ID: YP_001271076
Organism: Lactobacillus reuteri F275
Length: unknown
Description: (no description)
Keywords: (no keywords)
(No GO terms)
ID: Q1GAW2
Organism: Lactobacillus delbrueckii (subsp. bulgaricus, strain ATCC 11842 / DSM 20081)
Length: 329
Description: Cell-shape-determining protein MreB
Keywords: Complete proteome
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ID: Q042L8
Organism: Lactobacillus gasseri (strain ATCC 33323 / DSM 20243)
Length: 329
Description: Actin-like ATPase for cell morphogenesis
Keywords: Complete proteome
GO:0000902 (Biological process = cell morphogenesis)
GO:0005524 (Molecular role = ATP binding)
ID: A8YUK4
Organism: Lactobacillus helveticus (strain DPC 4571)
Length: 329
Description: Cell shape determining protein
Keywords: Complete proteome
GO:0000902 (Biological process = cell morphogenesis)
ID: B3WDM1
Organism: Lactobacillus casei (strain BL23)
Length: 329
Description: Cell shape determining protein, MerB family
Keywords: Complete proteome
(No GO terms)
ID: Q74K12
Organism: Lactobacillus johnsonii (strain NCC 533)
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Description: MreB-like protein
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ID: Q88UU7
Organism: Lactobacillus plantarum (strain WCFS1 / ATCC BAA-793 / NCIMB 8826)
Length: 333
Description: Cell shape determining protein MreB
Keywords: Complete proteome
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ID: Q38WK9
Organism: Lactobacillus sakei (subsp. sakei, strain 23K)
Length: 329
Description: Cell shape determining protein, MerB family
Keywords: Complete proteome
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GO:0005524 (Molecular role = ATP binding)
ID: ZP_01274444
Organism: Lactobacillus reuteri 100-23
Length: unknown
Description: (no description)
Keywords: (no keywords)
(No GO terms)
ID: B2GAU9
Organism: Lactobacillus fermentum (strain IFO 3956 / LMG 18251)
Length: 338
Description: Cell shape determining protein
Keywords: Complete proteome
GO:0000902 (Biological process = cell morphogenesis)
GO:0005524 (Molecular role = ATP binding)
ID: Q5FKX8
Organism: Lactobacillus acidophilus (strain NCFM)
Length: 329
Description: Cell shape determining protein
Keywords: Complete proteome
GO:0000902 (Biological process = cell morphogenesis)
GO:0005524 (Molecular role = ATP binding)
ID: Q04BA0
Organism: Lactobacillus delbrueckii (subsp. bulgaricus, strain ATCC BAA-365)
Length: 329
Description: Actin-like ATPase for cell morphogenesis
Keywords: Complete proteome
GO:0000902 (Biological process = cell morphogenesis)
ID: Q03QZ2
Organism: Lactobacillus brevis (strain ATCC 367 / JCM 1170)
Length: 335
Description: Actin-like ATPase for cell morphogenesis
Keywords: Complete proteome
GO:0000902 (Biological process = cell morphogenesis)
GO:0005524 (Molecular role = ATP binding)
ID: Q03A15
Organism: Lactobacillus casei (strain ATCC 334)
Length: 329
Description: Actin-like ATPase for cell morphogenesis
Keywords: Complete proteome
GO:0000902 (Biological process = cell morphogenesis)
GO:0005524 (Molecular role = ATP binding)


Appendix Ch10-3Appendix Ch11-1
Burkholderia pseudomallei (strain 1106a)
Burkholderia pseudomallei (strain 668)
Burkholderia mallei (strain ATCC 23344)
Burkholderia mallei (strain SAVP1)
Burkholderia mallei (strain NCTC 10229)
Burkholderia mallei (strain NCTC 10247)
Burkholderia thailandensis (strain E264 / ATCC 700388 / DSM 13276 / CIP 106301)
Burkholderia pseudomallei (strain 1710b)
Burkholderia pseudomallei (strain K96243)
Burkholderia vietnamiensis (strain R1808 / G4 / LMG 22486) / LMG 22486)
Burkholderia ambifaria (strain AMMD / ATCC BAA-244)
Burkholderia ambifaria (strain MC40-6)
Burkholderia cepacia (strain J2315 / LMG 16656)
Burkholderia cenocepacia (strain MCO-3)
Burkholderia cenocepacia (strain AU 1054)
Burkholderia cenocepacia (strain HI2424)
Burkholderia xenovorans (strain LB400)
Burkholderia phymatum (strain DSM 17167 / STM815)
Burkholderia phytofirmans (strain DSM 17436 / PsJN)
Mycobacterium abscessus (strain ATCC 19977 / DSM 44196)
Mycobacterium avium (strain 104)
Mycobacterium paratuberculosis (strain ATCC BAA-968 / K-10)
Mycobacterium tuberculosis (strain Oshkosh / CDC 1551)
Mycobacterium bovis (strain AF2122/97 / ATCC BAA-935)
Mycobacterium bovis (strain BCG / Pasteur 1173P2)
Mycobacterium tuberculosis (strain ATCC 25177 / H37Ra)
Mycobacterium tuberculosis (strain ATCC 25618 / H37Rv)
Mycobacterium marinum (strain ATCC BAA-535 / M)
Mycobacterium ulcerans (strain Agy99)
Mycobacterium smegmatis (strain ATCC 700084 / mc(2) / mc(2)155))
Mycobacterium gilvum (strain ATCC 700033 / PYR-GCK / PYR-GCK)
Mycobacterium vanbaalenii (strain DSM 7251 / PYR-1)
Pseudomonas syringae tomato (strain DC3000)
Pseudomonas syringae (pathovar phaseolicola, strain 1448A / Race 6)
Pseudomonas syringae (pathovar syringae, strain B728a)
Pseudomonas entomophila (strain L48)
Pseudomonas putida (strain W619)
Pseudomonas putida (strain GB-1)
Pseudomonas putida (strain F1 / ATCC 700007)
Pseudomonas putida (strain KT2440)
Pseudomonas fluorescens (strain Pf-5 / ATCC BAA-477)
Pseudomonas fluorescens (strain PfO-1)
Pseudomonas aeruginosa (strain PA7)
Pseudomonas aeruginosa (strain LMG 12228 / ATCC 15692 / PRS 101 / 1C / PAO1)
Pseudomonas aeruginosa (strain UCBPP-PA14)
Pseudomonas mendocina (strain ymp)
Pseudomonas stutzeri (strain A1501)
Bacillus cereus (subsp. cytotoxis, strain NVH 391-98)
Bacillus cereus (strain ATCC 10987)
Bacillus weihenstephanensis (strain KBAB4)
Bacillus cereus (strain ATCC 14579 / DSM 31)
Bacillus anthracis (strain Sterne)
Bacillus anthracis (strain Ames ancestor)
Bacillus anthracis (strain Ames, isolate Porton)
Bacillus cereus (strain ZK / E33L)
Bacillus thuringiensis (strain Al Hakam)
Bacillus thuringiensis konkukian (strain 97-27)
Clostridium beijerinckii (strain ATCC 51743 / NCIMB 8052)
Xanthomonas campestris (pathovar campestris, strain B100)
Xanthomonas campestris (pathovar campestris, strain 8004)
Xanthomonas campestris campestris (strain ATCC 33913 / NCPPB 528 / LMG 568)
Xanthomonas axonopodis (pathovar citri, strain 306)
Xanthomonas campestris (pathovar vesicatoria, strain 85-10)
Xanthomonas oryzae (pathovar oryzae, strain PXO99A)
Xanthomonas oryzae (pathovar oryzae, strain MAFF 311018)
Xanthomonas oryzae oryzae (strain KACC10331)
Clostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)
Clostridium acetobutylicum (strain DSM 792 / JCM 1419 / LMG 5710 / ATCC 824 / VKM B-1787)
Clostridium kluyveri (strain ATCC 8527 / DSM 555 / NCIMB 10680)
Bacillus licheniformis (strain DSM 13 / ATCC 14580, sub_strain Novozymes)
Bacillus pumilus (strain SAFR-032)
Bacillus amyloliquefaciens (strain FZB42)
Bacillus subtilis (strain 168)
Bacillus clausii (strain KSM-K16)
Bacillus halodurans (strain C-125 / ATCC BAA-125 / JCM 9153 / FERM 7344 / DSM 18197)
Clostridium difficile (strain 630)
Brucella ovis (strain ATCC 25840 / 63/290 / NCTC 10512)
Brucella melitensis (biovar 1, strain NCTC 10094 / ATCC 23456 / 16M)
Brucella suis (biovar 1, strain 1330)
Brucella canis (strain ATCC 23365 / NCTC 10854)
Brucella suis (strain ATCC 23445 / NCTC 10510)
Brucella abortus (strain S19)
Brucella abortus (biovar 1, strain 9-941)
Brucella abortus (strain 2308)
Clostridium thermocellum (strain ATCC 27405 / DSM 1237)
Staphylococcus aureus (strain USA300 / TCH1516)
Staphylococcus aureus (strain COL)
Staphylococcus aureus (strain NCTC 8325)
Staphylococcus aureus (strain bovine RF122 / ET3-1 / RF122)
Staphylococcus aureus (strain MRSA252)
Staphylococcus aureus (strain JH1)
Staphylococcus aureus (strain JH9)
Staphylococcus aureus (strain N315)
Staphylococcus aureus (strain Mu3 / ATCC 700698)
Staphylococcus aureus (strain Mu50 / ATCC 700699)
Staphylococcus aureus (strain MSSA476)
Staphylococcus aureus (strain MW2)
Staphylococcus aureus (strain Newman)
Staphylococcus aureus (strain USA300)
Staphylococcus haemolyticus (strain JCSC1435)
Staphylococcus saprophyticus (subsp. saprophyticus, strain ATCC 15305 / DSM 20229)
Staphylococcus epidermidis (strain ATCC 12228)
Staphylococcus epidermidis (strain ATCC 35984 / RP62A)
Streptococcus suis (strain 05ZYH33)
Streptococcus suis (strain 98HAH33)
Streptococcus mutans (serovar c, strain ATCC 700610 / UA159)
Streptococcus thermophilus (strain ATCC BAA-491 / LMD-9)
Streptococcus thermophilus (strain ATCC BAA-250 / LMG 18311)
Streptococcus thermophilus (strain CNRZ 1066)
Streptococcus agalactiae V (serovar V, strain 2603 V/R / ATCC BAA-611)
Streptococcus agalactiae III (serovar III, strain NEM316)
Streptococcus agalactiae Ia (serovar Ia, strain A909 / CDC SS700 / ATCC 27591)
Streptococcus equi (subsp. zooepidemicus, strain MGCS10565)
Streptococcus pyogenes M6 (serovar M6, strain ATCC BAA-946 / MGAS10394)
Streptococcus pyogenes M3 (serovar M3, strain ATCC BAA-595 / MGAS315)
Streptococcus pyogenes M3 (serovar M3, strain SSI-1)
Streptococcus pyogenes (serovar M5, strain Manfredo)
Streptococcus pyogenes M18 (serovar M18, strain MGAS8232)
Streptococcus pyogenes (serovar M4, strain MGAS10750)
Streptococcus pyogenes (serovar M2, strain MGAS10270)
Streptococcus pyogenes M28 (serovar M28, strain MGAS6180)
Streptococcus pyogenes (serovar M12, strain MGAS2096)
Streptococcus pyogenes (serovar M12, strain MGAS9429)
Streptococcus pyogenes M1 (serovar M1, strain MGAS5005 / ATCC BAA-947)
Streptococcus pyogenes M1 (serovar M1, strain SF370 / ATCC 700294)
Streptococcus pyogenes NZ131
Streptococcus pneumoniae (strain Hungary19A-6)
Streptococcus pneumoniae (strain CGSP14)
Streptococcus pneumoniae (strain TIGR4 / ATCC BAA-334)
Streptococcus pneumoniae (serovar 19F, strain G54)
Streptococcus pneumoniae (serovar 2, strain D39 / NCTC 7466)
Streptococcus pneumoniae (strain ATCC BAA-255 / R6)
Streptococcus gordonii (strain Challis / ATCC 35105 / CH1 / DL1 / V288)
Streptococcus sanguinis (strain SK36)
Lactobacillus brevis (strain ATCC 367 / JCM 1170)
Lactobacillus sakei (subsp. sakei, strain 23K)
Lactobacillus delbrueckii (subsp. bulgaricus, strain ATCC 11842 / DSM 20081)
Lactobacillus delbrueckii (subsp. bulgaricus, strain ATCC BAA-365)
Lactobacillus gasseri (strain ATCC 33323 / DSM 20243)
Lactobacillus johnsonii (strain NCC 533)
Lactobacillus acidophilus (strain NCFM)
Lactobacillus helveticus (strain DPC 4571)
Lactobacillus salivarius (subsp. salivarius, strain UCC118)
Lactobacillus fermentum (strain IFO 3956 / LMG 18251)
Lactobacillus reuteri 100-23
Lactobacillus reuteri F275
Neisseria gonorrhoeae (strain NCCP11945)
Neisseria gonorrhoeae (strain ATCC 700825 / FA 1090)
Neisseria meningitidis B (serogroup B, strain MC58)
Neisseria meningitidis (serogroup C, strain 053442)
Neisseria meningitidis (serogroup C / , serovar 2a, strain ATCC 700532 / FAM18)
Neisseria meningitidis A (serogroup A, serovar 4A, strain Z2491)
Mycobacterium leprae (strain TN)
Rickettsia bellii (strain OSU 85-389)
Rickettsia bellii (strain RML369-C)
Rickettsia conorii (strain ATCC VR-613 / Malish 7)
Rickettsia rickettsii (strain Iowa)
Rickettsia rickettsii (strain Sheila Smith)
Rickettsia felis (strain ATCC VR-1525 / URRWXCal2)
Rickettsia akari (strain Hartford)
Rickettsia canadensis (strain McKiel)
Rickettsia massiliae (strain Mtu5)
Rickettsia prowazekii (strain Madrid E)
Rickettsia typhi (strain Wilmington / ATCC VR-144)
Lactobacillus plantarum (strain WCFS1 / ATCC BAA-793 / NCIMB 8826)
Lactobacillus casei (strain ATCC 334)
Lactobacillus casei (strain BL23)
Clostridium botulinum (strain Loch Maree / Type A3)
Clostridium botulinum (strain Okra / Type B1)
Clostridium botulinum (strain Langeland / NCTC 10281 / Type F)
Clostridium botulinum (strain Hall / ATCC 3502 / NCTC 13319 / Type A, sub_strain Sanger)
Clostridium botulinum (strain ATCC 19397 / Type A)
Clostridium botulinum (strain Hall / ATCC 3502 / NCTC 13319 / Type A, sub_strain Los Alamos)
Clostridium botulinum (strain Alaska E43 / type E3)
Clostridium botulinum (strain Eklund 17B / type B)
Clostridium perfringens (strain SM101 / Type A)
Clostridium perfringens (strain 13 / Type A)
Clostridium perfringens (strain ATCC 13124 / NCTC 8237 / Type A)
Clostridium novyi (strain NT)
Clostridium tetani (strain Massachusetts / E88)
Shigella dysenteriae (serovar 1, strain Sd97 / Sd197)
Shigella sonnei (strain Ss046)
Shigella flexneri (serovar 5b, strain 8401)
Shigella flexneri (serovar 2a, strain 2457T / ATCC 700930)
Shigella flexneri (serovar 2a, strain 301)
Shigella boydii (serovar 18, strain CDC 3083-94 / BS512)
Shigella boydii (serovar 4, strain Sb227)
Yersinia enterocolitica (serovar O:8 / biotype 1B, strain 8081)
Yersinia pseudotuberculosis (serovar O:1b, strain IP 31758)
Yersinia pseudotuberculosis (serovar O:3, strain YPIII)
Yersinia pseudotuberculosis (serovar I, strain IP32953)
Yersinia pseudotuberculosis (serovar IB, strain PB1/+)
Yersinia pestis bv. Antiqua (strain Angola)
Yersinia pestis (biovar Mediaevalis, strain KIM5)
Yersinia pestis (biovar Mediaevalis, strain 91001)
Yersinia pestis (biovar Orientalis, strain CO-92)
Yersinia pestis (strain Pestoides F)
Yersinia pestis bv. (strain Antiqua)
Yersinia pestis bv. Antiqua (strain Nepal516)
Vibrio harveyi (strain ATCC BAA-1116 / BB120)
Vibrio fischeri (strain ATCC 700601 / ES114)
Vibrio fischeri (strain MJ11)
Vibrio parahaemolyticus (serovar O3:K6, strain RIMD 2210633)
Vibrio cholerae (serovar O1, strain ATCC 39315 / El Tor Inaba N16961)
Vibrio cholerae (serovar O1, strain ATCC 39541 / Ogawa 395 / O395)
Vibrio vulnificus (strain CMCP6)
Vibrio vulnificus (strain YJ016)
Rhizobium loti (strain MAFF303099)
Rhizobium meliloti (strain 1021)
Rhizobium leguminosarum bv. viciae (strain 3841)
Rhizobium leguminosarum bv. trifolii WSM2304
Rhizobium etli (strain CFN 42 / ATCC 51251)
Rhizobium etli (strain CIAT 652)
824.0

Additional files

Additional file 1 – List of *Lactobacillus, Paralactobacillus,* and *Pediococcus* species and GenBank accession numbers for corresponding DNA sequences used in analyses

Lactobacillus, Pediococcus, and *Paralactobaillus* species as of March 31, 2009

species	reference	year	Type strain	16S rRNA gene	*cpn60*	*pheS*	*recA*	*rpoA*
Lactobacillus								
acetotolerans	[1]	1986	ATCC 43578	M58801	AF429666	AM087733	AJ621615	AM263315
acidifarinae	[2]	2005	LMG 22200	AJ632158	-	AM087757	-	AM087830
acidipiscis	[3]	2000	JCM 10692	AB023836	AJ621719	AM168426	AJ621616	AM087849
acidophilus	[4]	(1900) 1970	ATCC 4356	M58802	AF429667	AM087677	NC006814	AM087860
agilis	[5]	1982	LMG 9186	M58803	-	AM087734	AJ621617	AM087831
algidus	[6]	2000	JCM 10491	AB033209	-	AM263504	AJ621618	AM263511
alimentarius	[7]	1983	ATCC 29643	M58804	AY424318	AM263509	AJ621619	AM087832
amylolyticus	[8]	1999	LMG 18796	Y17361	-	AM087724	AJ621620	AM087822
amylophilus	[9]	1981	ATCC 49845	M58806	AY691260	AM087735	-	AM087833
amylotrophicus	[10]	2006	LMG 11400	AM236149	-	AM236139	-	-
amylovorus	[11, 12]	1981	ATCC 33620	M58805	AF429669	AM087678	AJ621622	AM087774
animalis	[13]	1983	ATCC 35046	M58807	-	AM087679	AJ621623	AM087775
antri	[14]	2005	LMG 22111	AY253659	-	AM263502	-	AM087776
apodemi	[15]	2006	DSM 16634	AJ871178	-	-	-	-
aviarius subsp. *araffinosus*	[16]	1986	ATCC 43235	AB289043	AY691263	-	AJ621624	-
aviarius subsp. *aviarius*	[16]	1985	ATCC 43234	AB326355	AY691261	AM087737	-	AM087835
bifermentans	[17]	1983	ATCC 35409	M58809	-	AM087738	-	AM087862
bobalius	[18]	2008	DSM19674	AY681134	-	-	-	-
brevis	[4]	(1919) 1934	ATCC 14869	M58810	AF405388	AM087680	DQ080023	AM087777
buchneri	[4]	(1903) 1923	ATCC 4005	M58811	AF429673	AM087681	AJ621626	AM087778
cacaonum	[19]	2009	DSM 21116	AM905389	-	AM922295	-	-

camelliae	[20]	2007	BCC 21233	AB257864	-	-	-	-
capillatus	[21]	2008	DSM 19910	AB365967	-	-	-	-
casei	[4, 22, 23]	(1916) 1971	ATCC 334	M23928	AF405387	AM087682	AJ286122	AM157776
catenaformis	[4]	(1935) 1970	ATCC 25536	AJ621549	-	-	AJ621629	-
ceti	[24]	2008		AM292799	-	-	-	-
coleohominis	[25]	2001	DMS 14060	AM113776	-	AM087683	AJ621630	AM087852
collinoides	[26]	1972	ATCC 27612	AB005893	-	AM087730	AJ621631	AM087780
composti	[27]	2007	DSM 18527	AB268118	-	-	-	-
concavus	[28]	2005	LMG 22739	AY683322	-	-	-	-
coryniformis subsp. *coryniformis*	[4]	1965	ATCC 25602	AB289063	AY424321	AM087684	-	AM087869
coryniformis subsp. *torquens*	[4]	1965	ATCC 25600	AB289065	-	AM087865	-	AM087781
crispatus	[29]	(1953) 1970 1983	ATCC 33820	Y17362	AY562570	AM087686	AJ621632	AM087782
crustorum	[30]	2007	LMG 23699	AM285450	-	AM285025	-	-
curvatus	[31, 32]	(1903) 1965 1996	ATCC 25601	AM113777	AY424345	AM087758	AJ621633	AM087783
delbrueckii subsp. *bulgaricus*	[4, 33]	(1919) 1984	ATCC 11842	AY0501	AJ586869	AM087688	NC008054	AM087785
delbrueckii subsp. *delbrueckii*	[4, 33]	(1896) 1901	ATCC 9649	M58814	AJ586868	AM087689	AJ586863	AM087786
delbrueckii subsp. *indicus*	[34]	2005	LMG 22083	AY421720	AJ586872	AM087690	AJ586867	AM087787
delbrueckii subsp. *lactis*	[4, 33]	(1919) 1984	ATCC 12315	M58823	AJ586870	AM087691	AJ586865	AM087788
dextrinicus	[35]	(1964) 1978 2009	ATCC 33087	D87679	AY123704	AM899827	AJ621695	AM899852
diolivorans	[36]	2002	LMG 19667	AB289097	-	AM087763	AJ621635	AM087850
equi	[37]	2002	ATCC BAA261	AB048833	-	AM087740	AJ621637	AM087837
equigenerosi	[38]	2008	DSM 18793	AB288049	-	-	-	-

fabifermentans	[19]	2009	DSM21115	AM905388	AM905388	-	-	AM922294
farciminis	[7]	1983	ATCC 29644	M58817	AY424323	AM087729	AJ621638	AM087867
farraginis	[39]	2007	DSM 18382	AB262731	-	-	-	-
fermentum	[40]	(1901) 2004	ATCC 14931	AJ575812	AF429680	AM087693	AJ579534	AM087853
fornicalis	[41]	2000	ATCC 700934	Y18654	-	-	AJ621639	-
fructivorans	[42, 43]	1934	ATCC 8288	M58818	AF429681	AM087770	-	AM087861
frumenti	[44]	2000	LMG 19473	AJ250074	-	AM087741	AJ621640	AM087838
fuchuensis	[45]	2002	DSM 14340	AB063479	-	AM087766	AJ621641	AM087866
gallinarum	[12]	1992	ATCC 33199	AJ417737	AY691262	AM087694	AJ621642	AM087789
gasseri	[46]	1980	ATCC 33323	M58820	EF571590	AM087695	AJ621643	AM087790
gastricus	[47]	2005	LMG 22113	AY253568	-	AM087696	-	AM087854
ghanensis	[48]	2007	DSM 18630	DQ523489	-	-	-	-
graminis	[49]	1989	ATCC 51150	AM113778	-	AM087742	AJ621644	AM263514
hammesii	[50]	2005	DSM 16381	AJ632219	-	AM087767	-	AM263512
hamsteri	[51]	1988	ATCC 43851	AJ306298	-	AM087743	AJ621646	AM087863
harbinensis	[52]	2006	DSM 16991	AB196123	-	-	-	-
hayakitensis	[53]	2007	DSM 18933	AB267406	-	-	-	-
helveticus	[54]	(1919) 1925	ATCC 15009	AM113779	AF429683	AM087697	AJ621645	AM087791
hilgardii	[55]	1936	ATCC 8290	M58821	AF429684	AM087698	AJ621647	AM087792
homohiochii	[56]	1957	ATCC 15434	AM113780	AF429685	AM087771	-	AM087848
hordei	[57]	2008		EU075840	-	-	-	-
iners	[58]	1999	DSM 13335	AY526083	AY608422	AM087699	AJ621653	AM087793
ingluviei	[59, 60]	2003	LMG 20380	AB289169	AJ621722	AM087731	-	AM087855
intestinalis	[61]	1990	ATCC 49335	AJ306299	AY691264	AM087700	AJ621654	AM087798
jensenii	[62]	1970	ATCC 25258	AF243176	AY608421	AM087744	AJ621648	AM087839
johnsonii	[12]	1992	ATCC 3320	AJ002515	EF571589	AM087701	NCC05362	AM087794
kalixensis	[47]	2005	DSM 16043	AY253657	-	AM087702	-	AM087795
kefiranofaciens subsp. *kefiranofaciens*	[63, 64]	(1988) 2004	ATCC 43761	AM113781	AF429691	AM087745	-	AM087847
kefiranofaciens subsp. *kefirgranum*	[63, 64]	(1994) 2004	ATCC 51647	AM113782	AF429690	AM087703	AJ621649	AM087796
kefiri	[65]	1983	ATCC 35411	AJ621553	AF429688	AM263598	AJ621650	AM087840

kimchii	[66]	2001	ATCC BAA131	AF173986	AY571674	AM087705	AJ621651	AM087797
kitasatonis	[67]	2003	JCM 1039	AB107638	-	AM263506	-	AM263517
kunkeei	[68]	1998	ATCC 700308	Y11374	-	AM087773	AJ621652	AM087864
lindneri	[69]	1997	DSM 20690	X95421	-	AM087704	-	AM087799
malefermentans	[70]	1989	ATCC 49373	AM113783	-	AM263505	-	AM263516
mali	[71]	1970	ATCC 27053	M58824	AF429693	AM087746	AJ621655	AM087841
manihotivorans	[72]	1998	DSM 13343	AF000162	-	AM087732	-	AM087842
mindensis	[73]	2003	DSM 14500	AJ313530	-	AM087706	AJ621656	AM087800
mucosae	[74]	2000	DSM 13345	AF126738	-	AM087707	AJ621657	AM087856
murinus	[75]	1982	ATCC 35020	AB326349	-	AM087760	AJ621658	AM087801
nagelii	[76]	2000	ATCC 700692	AB289206	-	AM087708	-	AM087802
namurensis	[77]	2007	LMG 23583	AM259118	-	AM259121	-	-
nantensis	[78]	2006	DSM 16982	AY690834	-	AM285024	-	-
nodensis	[79]	2009	DSM 19682	AB332024	-	-	-	-
oligofermentans	[80]	2005	DSM 15707	AY733084	-	-	-	-
oris	[81]	1988	ATCC 49062	X94229	-	AM087709	AJ621659	AM087803
panis	[82]	1996	DSM 6035	X94230	-	AM087725	AJ621660	AM087823
pantheris	[83]	2002	LMG 21017	AF413523	-	AM087747	-	AM087843
parabrevis	[84]	2006	ATCC 53295	AM158249	-	AM159098	-	-
parabuchneri	[85]	1988	ATCC 49374	AF275311	AF429638	AM087726	AJ621661	AM087824
paracasei subsp. *paracasei*	[22, 23, 86]	1989	ATCC 25302	D79212	AF424339	AM087710	AJ621664	AM087804
paracasei subsp. *tolerans*	[22, 23, 86]	(1965) 1989	ATCC 25599	AB289229	-	AM087711	AJ621663	AM087805
paracollinoides	[87]	2004	DSM 15502	AJ86665	-	AM087764	-	AM263515
parafarraginis	[39]	2007	DSM 18390	AB262734	-	-	-	-
parakefiri	[88]	1994	ATCC 51648	AY026750	-	AM263510	AJ621665	AM087851
paralimentarius	[89]	1999	DSM 13238	AJ417500	-	AM087712	-	AM087806
paraplantarum	[90]	1996	ATCC 700211	AJ306297	AY424357	AM087727	AJ286120	AM087825
pentosus	[91]	1987	ATCC 8041	D79211	AF429695	AM087713	AJ621666	AM087826
perolens	[52, 92]	1999	DSM 12744	Y19167	-	AM087748	AJ621667	AM087844
plantarum subsp. *argentoratensis*	[93, 94]	2005	DSM 16365	AJ640078	AJ640081	AM694185	AJ640079	AM694186
plantrum subsp. *plantarum*	[4, 95]	(1919) 1923	ATCC 14917	D79210	AF405389	AM087714	AJ621668	AM087808

pontis	[95]	1994	ATCC 51518	AJ422032	-	AM087715	AJ621669	AM087809
psittaci	[96]	2001	DSM 15354	AB289268	-	AM087749	AJ621670	AM087845
rennini	[97]	2006	DSM 20253	AJ576007	-	-	-	-
reuteri	[98]	1982	ATCC 23272	L23507	CP000705	AM087728	AJ621672	AM087810
rhamnosus	[23, 86]	(1968) 1989	ATCC 7469	EF495247	AF429659	AM087716	AJ621671	AM087811
rossiae	[99]	2005	ATCC BAA822	AJ564009	-	AM087768	-	AM087858
ruminis	[100]	1973	ATCC 27780	M58828	AY691314	AM087756	AJ621673	AM087812
saerimneri	[101]	2004	DSM 16049	AY255802	-	AM087717	-	AM087813
sakei subsp. *carnosus*	[31]	1996	LMG 17302	AY204889	-	AM087718	-	AM087828
sakei subsp. *sakei*	[4, 31]	1934	ATCC 15521	AM113784	AF429697	AM087719	AJ621674	AM087827
salivarius	[102, 103]	1953	ATCC 11741	AF089108	AY835627	AM087721	NC007929	AM087815
sanfranciscensis	[104]	1984	ATCC 27651	X76327	AY700220	AM087754	-	AM087816
satsumensis	[105]	2005	DSM 16230	AB154519	-	AM087769	-	AM087859
secaliphilus	[106]	2007	DSM 17896	AM279150	-	-	-	-
senmaizukei	[107]	2008		AM297927	-	-	-	AM395074
sharpeae	[5]	1982	ATCC 49974	M58831	-	AM087765	AJ621675	AM263518
siliginis	[108]	2006	NBRC 101315	DQ168027	-	-	-	-
spicheri	[109]	2004	DSM 15429	AJ534844	-	AM087752	-	AM087818
suebicus	[110]	1989	ATCC 49375	AJ306403	-	AM087772	AJ621676	AM087865
thailandensis	[20]	2007	BCC 21235	AB257863	-	-	-	-
vaccinostercus	[111, 112]	1983	ATCC 33310	AJ315640	AJ621724	AM087750	AJ621678	AM087846
vaginalis	[113]	1989	ATCC 49540	X61136	AY123651	AM087751	AJ621679	AM087868
versmoldensis	[114]	2003	ATCC BAA478	AJ496791	-	AM087755	AJ621680	AM087821
vini	[115]	2006	DSM 20605	AJ576009	-	-	-	-
vitulinus	[100]	1973	ATCC 27783	AB219312	-	AM087759	AJ621681	-
zeae	[22, 23]	1996	ATCC 15820	D86516	AF429696	AM087761	-	AM087829
zymae	[2]	2005	LMG 22198	AJ632157	-	AM087753	-	AM087817
Paralactobacillus								
selangorans	[116]	2000	LMG 17710					
Pediococcus								
acidilactici	[4]	1887	ATCC 33314	AJ305320	AY123702	AM899818	-	AM899868
argentinicus	[117]	2008	LMG 23999	AM709786				
cellicola	[118]	2005	LMG 22956	AY956788	-	AM899811	-	AM899857

claussenii	[119]	2002	ATCC BAA344	AJ621555	AF405395	AM899832	AJ621696	AM899864
damnosus	[4]	1903 1980	ATCC 29358	D87678	AF405400	AM899820	AJ621694	AM899851
ethanolidurans	[120]	2006	LMG 23354	DQ400914	-	AM899848	-	AM899888
inopinatus	[121]	1988	ATCC 49902	AJ271383	AF405420	AM899821		AM899861
parvulus	[4, 122]	1961	ATCC 19371	D88528	AY123707	AM899829		AM899856
pentosaceus	[4]	1934	ATCC 33316	M58834	AF405404	AM899822		AM899891
siamensis	[20]	2007	NRIC 0675	AB358357	-	AM899944		AM899945
stilesii	[123]	2006	LMG 23082	AJ973157	-	AM899824		AM899896

References

1. Entani E, Masai H, Suzuki KI: ***Lactobacillus acetotolerans,*** **a new species from fermented vinegar broth.** *Int J Syst Bacteriol* 1986, **36**:544-549.

2. Vancanneyt M, Neysens P, De Wachter M, Engelbeen K, Snauwaert C, Cleenwerk I, van der Meulen M, Hoste B, Tsakalidou E, de Vuyst L, Swings J: ***Lactobacillus acidifarinae*** **sp. nov. and** ***Lactobacillus zymae*** **sp. nov., from wheat sourdoughs.** *Int J Syst Evol Microbiol* 2005, **55**:615-620.

3. Tanasupawat S, Shida O, Okada S, Komogata K: ***Lactobacillus acidipiscis*** **sp. nov. and** ***Weissella thailandensis*** **sp. nov., isolated from fermented fish in Thailand.** *Int J Syst Evol Microbiol* 2000, **50**:1479-1485.

4. Skerman VBD, McGowan V, Sneath PHA (editors): **Approved lists of bacterial names.** *Int J Syst Bacteriol* 1980, **30**:225-420.

5. Weiss N, Schillinger U, Laternser M, Kandler O: ***Lactobacillus sharpeae*** **sp. nov. and** ***Lactobacillus agilis*** **sp. nov., two new species of homofermentative,** ***meso*****-diaminopimelic acid-containing lactobacilli.** *Zentralbl Mikrobiol Parasitenkd Infektionskr Hyg Abt 1 Orig ,* 1981, **C2**:242-253.

6. Kato Y, Sakala RM, Hayashidani H, Kiuchi A, Kaneuchi C, Ogawa M: ***Lactobacillus algidus*** **sp. nov., a psychrophilic lactic acid bacterium isolated from vacuum-packaged refrigerated beef.** *Int J Syst Evol Microbiol* 2000, **50**:1143-1149.

7. Reuter G: ***Lactobacillus alimentarius*** **sp. nov., nom. rev. and** ***Lactobacillus farciminis*** **sp. nov., nom. rev.** *Syst Appl Microbiol* 1983, **4**:277-279.

8. Bohak I, Back W, Richter L, Ehrmann M, Ludwig W, Schleifer KH: ***Lactobacillus amylolyticus*** **sp. nov., isolated from beer malt and beer wort.** *Syst Appl Microbiol* 1998, **21**:360-364.

9. Nakamura LK, Crowell CD: ***Lactobacillus amylophilus,*** **a new starch-hydrolyzing species from swine waste-corn fermentation.** *Dev Ind Microbiol* 1979, **20**:532-540.

10. Naser SM, Vancanneyt M, Snauwaert C, Vrancken G, Hoste B, de Vuyst L, Swings J: **Reclassification of *Lactobacillus amylophilus* LMG 11400 and NRRL B-4435 as *Lactobacillus amylotrophicus* sp. nov.** *Int J Syst Evol Microbiol* 2006, **56**:2523-2527.

11. Nakamura LK: ***Lactobacillus amylovorus*, a new starch-hydrolyzing species from cattle waste-corn fermentations.** *Int J Syst Bacteriol* 1981, **31**:56-63.

12. Fujisawa T, Benno Y, Yaeshima T, Mitsuoka T: **Taxonomic study of the *Lactobacillus acidophilus* group, with recognition of *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and synonymy of *Lactobacillus acidophilus* group A3 (Johnson *et al*. 1980) with the type strain of *Lactobacillus amylovorus* (Nakamura 1981).** *Int J Syst Bacteriol* 1992, **42**:487-491.

13. Dent VE, Williams RAD: ***Lactobacillus animalis* sp. nov., a new species of *Lactobacillus* from the alimentary canal of animals.** *Zentralbl Bakteriol Parasitenkd Infektionskr Hyg Abt 1 Orig* 1982, **C3**:377-386.

14. Roos S, Engstrand L, Jonsson H: ***Lactobacillus gastricus* sp. nov., *Lactobacillus antri* sp. nov., *Lactobacillus kalixensis* sp. nov. and *Lactobacillus ultunensis* sp. nov., isolated from human stomach mucosa.** *Int J Syst Evol Microbiol* 2005, **55**:77-82.

15. Osawa R, Fujisawa T, Pukall R: ***Lactobacillus apodemi* sp. nov., a tannase-producing species isolated from wild mouse faeces.** *Int J Syst Evol Microbiol* 2006, **56**:1693-1696.

16. Fujisawa T, Shirasaka S, Watabe J, Mitsuoka T: ***Lactobacillus aviarius* sp. nov.: a new species isolated from the intestines of chickens.** *Syst Appl Microbiol* 1984, **5**:414-420.

17. Kandler O, Schillinger U, Weiss N: ***Lactobacillus bifermentans* sp. nov., nom. rev., an organism forming CO2 and H2 from lactic acid.** *Syst Appl Microbiol* 1983, **4**:408-412.

18. Manes-Lazaro R, Ferrer S, Rodas AM, Urdiain M, Pardo I: ***Lactobacillus bobalius* sp. nov., a lactic acid bacterium isolated from Spanish Bobal grape must.** *Int J Syst Evol Microbiol* 2008, **58**:2699-2703.

19. De Bruyne K, Camu N, de Vuyst L, Vandamme P: ***Lactobacillus fabifermentans* sp. nov. and *Lactobacillus cacaonum* sp. nov., isolated from Ghanaian cocoa fermentations.** *Int J Syst Evol Microbiol* 2009, **59**:7-12.

20. Tanasupawat S, Pakdeeto A, Thawai C, Yukphan P, Okada S: **Identification of a lactic acid bacteria from fermented tea leaves (miang) in Thailand and proposals of *Lactobacillus thailandensis* sp. nov., *Lactobacillus camelliae* sp. nov., and *Pediococcus siamensis* sp. nov.** *J Gen Appl Microbiol* 2007, **53**:7-15.

21. Chao SH, Tomii Y, Sasamoto M, Fujimoto J, Tsai YC, Watanabe K: ***Lactobacillus capillatus* sp. nov., a motile bacterium isolated from stinky tofu brine.** *Int J Syst Evol Microbiol* 2008, **58**:2555-2559.

22. Judicial commission of the international committee on systematics of prokaryoes: **The type strain of *Lactobacillus casei* is ATCC 393, ATCC 334 cannot serve as the type because it represents a different taxon, the name *Lactobacillus paracasei* and its subspecies names are not rejected and the revival of the name '*Lactobacillus zeae*' contravenes Rules 51b (1) and (2) of the International Code of Nomenclature of Bacteria. Opinion 82.** *Int J Syst Evol Microbiol* 2008, **58**:1764-1765.

23. Dicks LMT, Du Plessis EM, Dellaglio F, Lauer E: **Reclassification of *Lactobacillus casei* subsp. *casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 15820 as *Lactobacillus zeae* nom. rev., designation of ATCC 334 as the neotype of *L. casei* subsp. *casei*, and rejection of the name *Lactobacillus paracasei*.** *Int J Syst Bacteriol* 1996, **46**:337-340.

24. Vela AI, Fernandez A, Espinosa de los Monteros,A., Goyache J, Herraez P, Tames B, Cruz F, Dominguez L, Fernandez-Garayzabal JF: ***Lactobacillus ceti* sp. nov., isolated from beaked whales (*Ziphius cavirostris*).** *Int J Syst Evol Microbiol* 2008, **58**:891-894.

25. Nikolaitchouk N, Wacher C, Falsen E, Andersch B, Collins MD, Lawson PA: ***Lactobacillus coleohominis* sp. nov., isolated from human sources.** *Int J Syst Evol Microbiol* 2001, **51**:2081-2085.

26. Carr JG, Davies PA: **The ecology and classification of strains of *Lactobacillus collinoides* nov. spec.: A bacterium commonly found in fermenting apple juice.** *J Appl Bacteriol* 1972, **35**:463-471.

27. Endo A, Okada S: ***Lactobacillus composti* sp. nov., a lactic acid bacterium isolated from a compost of distilled shochu residue.** *Int J Syst Evol Microbiol* 2007, **57**:870-872.

28. Tong H, Dong X: ***Lactobacillus concavus* sp. nov., isolated from the walls of a distilled spirit fermenting cellar in China.** *Int J Syst Evol Microbiol* 2005, **55**:2199-2202.

29. Cato EP, Moore WEC, Johnson JL: **Synonymy of strains of "*Lactobacillus acidophilus*" group A2 (Johnson *et al*. 1980) with the type strain of *Lactobacillus crispatus* (Brygoo and Aladame 1953) Moore and Holdeman 1970.** *Int J Syst Bacteriol* 1983, **33**:426-428.

30. Scheirlink I, van der Meulen M, Van Schoor A, Huys G, Vandamme P, de Vuyst L, Vancanneyt M: ***Lactobacillus crustorum* sp. nov., isolated from two traditional Belgian wheat sourdoughs.** *Int J Syst Evol Microbiol* 2007, **57**:1461-1467.

31. Torriani S, van Reenen CA, Klein G, Reuter G, Dellaglio F, Dicks LMT: ***Lactobacillus curvatus* subsp. *curvatus* subsp. nov. and *Lactobacillus curvatus* subsp. *melibiosus* subsp. nov. and *Lactobacillus sake* subsp. *sake* subsp. nov. and *Lactobacillus sake* subsp. *carnosus* subsp. nov., new subspecies of *Lactobacillus curvatus* Abo-Elnaga and Kandler 1965 and *Lactobacillus sake* Katagiri, Kitahara, and Fukami 1934 (Klein *et al*. 1996, emended descriptions), respectively.** *Int J Syst Bacteriol* 1996, **46**:1158-1163.

32. Klein G, Dicks LMT, Pack A, Hack B, Zimmerman K, Dellaglio F, Reuter G: **Emended descriptions of *Lactobacillus sake* (Katagiri, Kitahara, and Fukami) and *Lactobacillus curvatus* (Abo-Elnaga and Kandler): numerical classification revealed by protein fingerprinting and identification based on biochemical patterns and DNA-DNA hybridizations.** *Int J Syst Bacteriol* 1996, **46**:367-376.

33. Weiss N, Schillinger U, Kandler O: ***Lactobacillus lactis, Lactobacillus leichmannii* and *Lactobacillus bulgaricus* subjective synonyms of *Lactobacillus delbrueckii* subsp. *lactis* comb. nov. and *Lactobacillus delbrueckii* subsp. *bulgaricus* comb. nov.** *Syst Appl Microbiol* 1983, **4**:552-557.

34. Dellaglio F, Felis GE, Castioni A, Torriani S, Germond JE: ***Lactobacillus delbrueckii* subsp. *indicus* subsp. nov., isolated from Indian dairy products.** *Int J Syst Evol Microbiol* 2005, **55**:401-404.

35. Haakensen M, Dobson CM, Hill JE, Ziola B: **Reclassification of *Pediococcus dextrinicus* (Coster and White 1964) Back 1978 (Approved Lists 1980) as *Lactobacillus dextrinicus* comb. nov., and emended description of the genus *Lactobacillus*.** *Int J Syst Evol Microbiol* 2009, **59**:615-621.

36. Krooneman J, Faber F, Alderkamp AC, Oude Elferink SJHW, Driehuis F, Cleenwerck I, Swings J, Gottschal JC, Vancanneyt M: ***Lactobacillus diolivorans* sp. nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage.** *Int J Syst Evol Microbiol* 2002, **52**:639-646.

37. Morotomi M, Yuki N, Kado Y, Kushiro A, Shimazaki T, Watanabe K, Yuyama T: ***Lactobacillus equi*** **sp. nov., a predominant intestinal *Lactobacillus* species of the horse isolated from faeces of healthy horses.** *Int J Syst Evol Microbiol* 2002, **52**:211-214.

38. Endo A, Roos S, Satoh E, Morita H, Okada S: ***Lactobacillus equigenerosi*** **sp. nov., a coccoid species isolated from faeces of thoroughbred racehorses.** *Int J Syst Evol Microbiol* 2008, **58**:914-918.

39. Endo A, Okada S: ***Lactobacillus farraginis*** **sp. nov. and *Lactobacillus parafarraginis* sp. nov., heterofermentative lactobacilli isolated from a compost of distilled shochu residue.** *Int J Syst Evol Microbiol* 2007, **57**:708-712.

40. Dellaglio F, Torriani S, Felis GE: **Reclassification of *Lactobacillus cellobiosus* Rogosa *et al*. 1953 as a later synonym of *Lactobacillus fermentum* Beijerinck 1901.** *Int J Syst Evol Microbiol* 2004, **54**:809-812.

41. Dicks LMT, Silvester M, Lawson PA, Collins MD: ***Lactobacillus fornicalis*** **sp. nov., isolated from the posterior fornix of the human vagina.** *Int J Syst Evol Microbiol* 2000, **50**:1253-1258.

42. Charlton DB, Nelson ME, Werkman CH: **Physiology of *Lactobacillus fructivorans* sp. nov. isolated from spoiled salad dressing.** *Iowa State of Science* 1934, **9**:1-11.

43. Weiss N, Schillinger U, Kandler O: ***Lactobacillus trichodes,*** **and *Lactobacillus heterohiochii,* subjective synonyms of *Lactobacillus fructivorans*.** *Syst Appl Microbiol* 1983, **4**:507-511.

44. Muller MRA, Ehrmann MA, Vogel RF: ***Lactobacillus frumenti*** **sp. nov., a new lactic acid bacterium isolated from rye-bran fermentations with a long fermentation period.** *Int J Syst Evol Microbiol* 2000, **50**:2127-2133.

45. Sakala RM, Kato Y, Hayashidani H, Murakami M, Kaneuchi C, Ogawa M: ***Lactobacillus fuchuensis*** **sp. nov., isolated from vacuum-packaged refrigerated beef.** *Int J Syst Evol Microbiol* 2002, **52**:1151-1154.

46. Berger B, Pridmore RD, Barretto C, Delmas-Julien F, Schreiber K, Arigoni F, Brussow H: **Similarity and Differences in the *Lactobacillus acidophilus* Group Identified by Polyphasic Analysis and Comparative Genomics.** *J Bacteriol* 2007, **189**(4):1311-1321.

47. Roos S, Engstrand L, Jonsson H: ***Lactobacillus gastricus*** **sp. nov.,** ***Lactobacillus antri*** **sp. nov.,** ***Lactobacillus kalixensis*** **sp. nov. and** ***Lactobacillus ultunensis*** **sp. nov., isolated from human stomach mucosa.** *Int J Syst Evol Microbiol* 2005, **55**:77-82.

48. Nielsen DS, Schillinger U, Franz CMAP, Bresciani J, Amoa-Awua W, Holzapfel WH, Jakobsen M: ***Lactobacillus ghanensis*** **sp. nov., a motile lactic acid bacterium isolated from Ghanaian cocoa fermentations.** *Int J Syst Evol Microbiol* 2007, **57**:1468-1472.

49. Beck R, Weiss N, Winter J: ***Lactobacillus graminis*** **sp. nov., a new species of facultatively heterofermentative lactobacilli surviving at low pH in grass silage.** *Syst Appl Microbiol* 1988, **10**:279-283.

50. Valcheva R, Korakli M, Onno B, Prevost H, Ivanova I, Ehrmann MA, Dousset X, Ganzle MG, Vogel RF: ***Lactobacillus hammesii*** **sp. nov., isolated from French sourdough.** *Int J Syst Evol Microbiol* 2005, **55**:763-767.

51. Mitsuoka T, Fujisawa T: ***Lactobacillus hamsteri*****, a new species from the intestine of hamsters.** *Proc Jpn Acad Ser B Phys Biol Sci* 1987, **63**:269-272.

52. Miyamoto M, Seto Y, Hao DH, Teshima T, Sun YB, Kabuki T, Yao LB, Nakajima H: ***Lactobacillus harbinensis*** **sp. nov., consisted of strains isolated from traditional fermented vegetables 'Suan cai' in Harbin, Northeastern China and** ***Lactobacillus perolens*** **DSM 12745.** *Syst Appl Microbiol* 2005, **28**:688-694.

53. Morita H, Shiratori C, Murakami M, Takami H, Kato Y, Endo A, Nakajima F, Takagi M, Akita H, Okada S, Masaoka T: ***Lactobacillus hayakitensis*** **sp. nov., isolated from intestines of healthy thoroughbreds.** *Int J Syst Evol Microbiol* 2007, **57**:2836-2839.

54. Naser SM, Hagen KE, Vancanneyt M, Cleenwerck I, Swings J, Tompkins TA: ***Lactobacillus suntoryeus*** **Cachat and Priest 2005 is a later synonym of** ***Lactobacillus helveticus*** **(Orla-Jensen 1919) Bergey** ***et al*****. 1925 (Approved Lists 1980).** *Int J Syst Evol Microbiol* 2006, **56**:355-360.

55. Douglas HC, Cruess WV: **A** ***Lactobacillus*** **from California wine:** ***Lactobacillus hilgardii*****.** *Food Research* 1936, **1**:113-119.

56. Kitahara K, Kaneko T, Goto O: **Taxonomic studies on the hiochi-bacteria, specific saprophytes of sake. II. Identification and classification of hiochi-bacteria.** *J Gen Appl Microbiol* 1957, **3**:111-120.

57. Rouse S, Canchaya C, van Sinderen D: ***Lactobacillus hordei*** **sp. nov., a bacteriocinogenic strain isolated from malted barley.** *Int J Syst Evol Microbiol* 2008, **58**:2013-2017.

58. Falsen E, Pascual C, Sjoden B, Ohlen M, Collins MD: **Phenotypic and phylogenetic characterization of a novel *Lactobacillus* species from human sources: description of *Lactobacillus iners* sp. nov.** *Int J Syst Bacteriol* 1999, **49**:217-221.

59. Baele M, Vancanneyt M, Devriese LA, Lefebvre K, Swings J, Haesebrouck F: ***Lactobacillus ingluviei*** **sp. nov., isolated from the intestinal tract of pigeons.** *Int J Syst Evol Microbiol* 2003, **53**:133-136.

60. Felis GE, Vancanneyt M, Snauwaert C, Swings J, Torriani S, Castioni A, Dellaglio F: **Reclassification of *Lactobacillus thermotolerans* Niamsup *et al*. 2003 as a later synonym of *Lactobacillus ingluviei* Baele *et al*. 2003.** *Int J Syst Evol Microbiol* 2006, **56**:793-795.

61. Fujisawa T, Itoh K, Benno Y, Mitsuoka T: ***Lactobacillus intestinalis*** **(*ex* Hemme 1974) sp. nov., nom. rev., isolated from the intestines of mice and rats.** *Int J Syst Bacteriol* 1990, **40**:302-304.

62. Gasser F, Mandel M, Rogosa M: ***Lactobacillus jensenii*** **sp. nov., a new representative of the subgenus *Thermobacterium*.** J *Gen Appl Microbiol* 1970, **62**:219-222.

63. Fujisawa T, Adachi S, Toba T, Arihara K, Mitsuoka T: ***Lactobacillus kefiranofaciens*** **sp. nov. isolated from kefir grains.** *Int J Syst Bacteriol* 1988, **38**:12-14.

64. Vancanneyt M, Mengaud J, Cleenwerck I, Vanhonacker K, Hoste B, Dawyndt P, Degivry MC, Ringuet D, Janssens D, Swings J: **Reclassification of *Lactobacillus kefirgranum* Takizawa *et al*. 1994 as *Lactobacillus kefiranofaciens* subsp. *kefirgranum* subsp. nov. and emended description of *L. kefiranofaciens* Fujisawa *et al*. 1988.** *Int J Syst Evol Microbiol* 2004, **54**:551-556.

65. Kandler O, Kunath P: ***Lactobacillus kefir*** **sp. nov., a component of the microflora of kefir.** *Syst Appl Microbiol* 1983, **4**:286-294.

66. Yoon JH, Kang SS, Mheen TI, Ahn JS, Lee HJ, Kim TK, Park CS, Kho YH, Kang KH, Park YH: ***Lactobacillus kimchii*** **sp. nov., a new species from kimchi.** *Int J Syst Evol Microbiol* 2000, **50**:1789-1795.

67. Mukai T, Arihara K, Ikeda A, Nomura K, Suzuki F, Ohori H: ***Lactobacillus kitasatonis*** **sp. nov., from chicken intestine.** *Int J Syst Evol Microbiol* 2003, **53**:2055-2059.

68. Edwards CG, Haag KM, Collins MD, Hutson RA, Huang YC: ***Lactobacillus kunkeei*** **sp. nov.: a spoilage organism associated with grape juice fermentations.** *J Appl Bacteriol* 1998, **84**:698-702.

69. Back W, Bohak I, Ehrmann M, Ludwig W, Schleifer KH: **Revival of the species *Lactobacillus lindneri* and the design of a species specific oligonucleotide probe.** *Syst Appl Microbiol* 1996, **19**:322-325.

70. Farrow JAE, Phillips BA, Collins MD: **Nucleic acid studies on some heterofermentative lactobacilli: description of *Lactobacillus malefermentans* sp. nov. and *Lactobacillus parabuchneri* sp. nov.** *FEMS Microbiol Lett* 1988, **55**:163-168.

71. Kaneuchi C, Seki M, Komagata K: **Taxonomic study of *Lactobacillus mali* Carr and Davis 1970 and related strains: validation of *Lactobacillus mali* Carr and Davis 1970 over *Lactobacillus yamanashiensis* Nonomura 1983.** *Int J Syst Bacteriol* 1988, **38**:269-272.

72. Morlon-Guyot J, Guyot JP, Pot B, Jacobe de Haut I, Raimbault M: ***Lactobacillus manihotivorans*** **sp. nov., a new starch-hydrolysing lactic acid bacterium isolated during cassava sour starch fermentation.** *Int J Syst Bacteriol* 1998, **48**:1101-1109.

73. Ehrmann MA, Müller MRA, Vogel RF: **Molecular analysis of sourdough reveals *Lactobacillus mindensis* sp. nov.** *Int J Syst Evol Microbiol* 2003, **53**:7-13.

74. Roos S, Karner F, Axelsseon L, Jonsson H: ***Lactobacillus mucosae*** **sp. nov., a new species with *in vitro* mucus-binding activity isolated from pig intestine.** *Int J Syst Evol Microbiol* 2000, **50**:251-258.

75. Hemme D, Raibaud P, Ducluzeau R, Galpin JV, Sicard P, van Heijenoort J: ***Lactobacillus murinus*** **n. sp., une nouvelle espèce de la flore dominante autochtone du tube digestif du rat et de la souris.** *Ann Microbiol* 1980, **131A**:297-308.

76. Edwards CG, Collins MD, Lawson PA, Rodriguez AV: ***Lactobacillus nagelii*** **sp. nov., an organism isolated from a partially fermented wine.** *Int J Syst Evol Microbiol* 2000, **50**:699-702.

77. Scheirlink I, van der Meulen R, van Schoor A, Cleenwerck I, Huys G, Vandamme P, de Vuyst L, Vancanneyt M: ***Lactobacillus namurensis*** **sp. nov., isolated from a traditional Belgian sourdough.** *Int J Syst Evol Microbiol* 2007, **57**:223-227.

78. Valcheva R, Ferchichi MF, Korakli M, Ivanova I, Gänzle MG, Vogel RF, Prévost H, Onno B, Dousset X: ***Lactobacillus nantensis*** **sp. nov., isolated from French wheat sourdough.** *Int J Syst Evol Microbiol* 2006, **56**:587-591.

79. Kashiwagi T, Suzuki T, Kamakura T: ***Lactobacillus nodensis*** **sp. nov., isolated from rice bran.** *Int J Syst Evol Microbiol* 2009, **59**:83-86.

80. Koort J, Murros A, Coenye T, Eerola S, Vandamme P, Sukura A, Bjorkroth J: ***Lactobacillus oligofermentans*** **sp. nov., associated with spoilage of modified-atmosphere-packaged poultry product.** *Appl Environ Microbiol* 2005, **71**:4400-4406.

81. Farrow JAE, Collins MD: ***Lactobacillus oris*** **sp. nov. from the human oral cavity.** *Int J Syst Bacteriol* 1988, **38**:116-118.

82. Wiese BJ, Rainey FA, Diekmann H: ***Lactobacillus panis*** **sp. nov., from sourdough with a long fermentation period.** *Int J Syst Bacteriol* 1996, **46**:449-453.

83. Liu B, Dong X: ***Lactobacillus pantheris*** **sp. nov., isolated from faeces of a jaguar.** *Int J Syst Evol Microbiol* 2002, **52**:1745-1748.

84. Vancanneyt M, Naser SM, Engelbeen K, De Wachter M, van der Meulen R, Cleenwerck I, Hoste B, de Vuyst L, Swings J: **Reclassification of *Lactobacillus brevis* strains LMG 11494 and LMG 11984 as *Lactobacillus parabrevis* sp. nov.** *Int J Syst Evol Microbiol* 2006, **56**:1553-1557.

85. Nigatu A: **Evaluation of numerical analyses of RAPD and API 50 CH patterns to differentiate *Lactobacillus plantarum, Lact. fermentum, Lact. rhamnosus, Lact. sake, Lact. parabuchneri, Lact. gallinarum, Lact. casei, Weissella minor* and related taxa isolated from *kocho* and *tef*.** *J Appl Microbiol* 2000, **89**:969-978.

86. Collins MD, Phillips BA, Zanoni P: **Deoxyribonucleic acid homology studies of *Lactobacillus casei, Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans,* and *Lactobacillus rhamnosus* sp. nov., comb. nov.** *Int J Syst Bacteriol* 1989, **39**:105-108.

87. Suzuki K, Funahashi W, Koyanagi M, Yamashita H: ***Lactobacillus paracollinoides*** **sp. nov., isolated from brewery environments.** *Int J Syst Evol Microbiol* 2004, **54**:115-117.

88. Takizawa S, Kojima S, Tamura S, Fujinaga S, Benno Y, Nakase T: ***Lactobacillus kefirgranum*** **sp. nov. and** ***Lactobacillus parakefir*** **sp. nov., two new species from kefir grains.** *Int J Syst Bacteriol* 1994, **44**:435-439.

89. Cai Y, Okada H, Mori H, Benno Y, Nakase T: ***Lactobacillus paralimentarius*** **sp. nov., isolated from sourdough.** *Int J Syst Bacteriol* 1999, **49**:1451-1455.

90. Curk MC, Hubert JC, Bringel F: ***Lactobacillus paraplantarum*** **sp. nov., a new species related to** ***Lactobacillus plantarum*****.** *Int J Syst Bacteriol* 1996, **46**:595-598.

91. Zanoni P, Farrow JAE, Phillips BZ, Collins MD: ***Lactobacillus pentosus*** **(Fred, Peterson, and Anderson) sp. nov., nom. rev.** *Int J Syst Bacteriol* 1987, **37**:339-341.

92. Back W, Bohak I, Ehrmann M, Ludwig W, Pot B, Kersters K, Schleifer KH: ***Lactobacillus perolens*** **sp. nov., a soft drink spoilage bacterium.** *Syst Appl Microbiol* 1999, **22**:354-359.

93. Bringel F, Castioni A, Olukoya DK, Felis GE, Torriani S, Dellaglio F: ***Lactobacillus plantarum*** **subsp.** ***argentoratensis*** **subsp. nov., isolated from vegetable matrices.** *Int J Syst Evol Microbiol* 2005, **55**:1629-1634.

94. Kostinek M, Pukall R, Rooney AP, Schillinger U, Hertel C, Holzapfel WH, Franz CMAP: ***Lactobacillus arizonensis*** **is a later heterotypic synonym of** ***Lactobacillus plantarum*****.** *Int J Syst Evol Microbiol* 2005, **55**:2485-2489.

95. Vogel RF, Bocker G, Stolz P, Ehrmann M, Fanta D, Ludwig W, Pot B, Kersters K, Schleifer KH, Hammes WP: **Identification of lactobacilli from sourdough and description of** ***Lactobacillus pontis*** **sp. nov.** *Int J Syst Bacteriol* 1994, **44**:223-229.

96. Lawson PA, Wacher C, Hansson I, Falsen E, Collins MD: ***Lactobacillus psittaci*** **sp. nov., isolated from a hyacinth macaw (*Anodorhynchus hyacinthinus*).** *Int J Syst Evol Microbiol* 2001, **51**:967-970.

97. Chenoll E, Macian MC, Aznar R: ***Lactobacillus rennini*** **sp. nov., isolated from rennin and associated with cheese spoilage.** *Int J Syst Evol Microbiol* 2006, **56**:449-452.

98. Kandler O, Stetter KO, Kohl R: ***Lactobacillus reuteri*** **sp. nov., a new species of heterofermentative lactobacilli.** *Zentralbl Mikrobiol Parasitenkd Infektionskr Hyg Abt 1 Orig* 1980, **C1**:264-269.

99. Corsetti A, Settanni L, van Sinderen D, Felis GE, Dellaglio F, Gobbetti M: ***Lactobacillus rossii*** **sp. nov., isolated from wheat sourdough.** *Int J Syst Evol Microbiol* 2005, **55**:35-40.

100. Sharpe ME, Latham MJ, Garvie EI, Zirngibl J, Kandler O: **Two new species of *Lactobacillus* isolated from the bovine rumen, *Lactobacillus ruminis* sp. nov. and *Lactobacillus vitulinus* sp. nov.** *J Gen Microbiol* 1973, **77**:37-49.

101. Pedersen C, Roos S: ***Lactobacillus saerimneri*** **sp. nov., isolated from pig faeces.** *Int J Syst Evol Microbiol* 2004, **54**:1365-1368.

102. Rogosa M, Wiseman RF, Mitchell JA, Disraely MN, Beaman AJ: **Species differentiation of oral lactobacilli from man including descriptions of *Lactobacillus salivarius* nov. spec. and *Lactobacillus cellobiosus* nov. spec.** *J Bacteriol* 1953, **65**:681-699.

103. Li Y, Raftis E, Canchaya C, Fitzgerald GF, van Sinderen D, O'Toole PW: **Polyphasic analysis indicates that *Lactobacillus salivarius* subsp. *salivarius* and *Lactobacillus salivarius* subsp. *salicinius* do not merit separate subspecies status.** *Int J Syst Evol Microbiol* 2006, **56**:2397-2403.

104. Weiss N, Schillinger U: ***Lactobacillus sanfrancisco*** **sp. nov., nom. rev.** *Syst Appl Microbiol* 1984, **5**:230-232.

105. Endo A, Okada S: ***Lactobacillus satsumensis*** **sp. nov., isolated from mashes of shochu, a traditional Japanese distilled spirit made from fermented rice and other starchy materials.** *Int J Syst Evol Microbiol* 2005, **55**:83-85.

106. Ehrmann MA, Brandt M, Stolz P, Vogel RF, Korakli M: ***Lactobacillus secaliphilus*** **sp. nov., isolated from type II sourdough fermentation.** *Int J Syst Evol Microbiol* 2007, **57**:745-750.

107. Hiraga K, Ueno Y, Sukontasing S, Tanasupawat S, Oda K: ***Lactobacillus senmaizukei*** **sp. nov., isolated from Japanese pickle.** *Int J Syst Evol Microbiol* 2008, **58**:1625-1629.

108. Aslam Z, Im WT, Ten LN, Lee MJ, Kim KH, Lee ST: ***Lactobacillus siliginis*** **sp. nov., isolated from wheat sourdough in South Korea.** *Int J Syst Evol Microbiol* 2006, **56**:2209-2213.

109. Meroth CB, Hammes WP, Hertel C: **Characterisation of the microbiota of rice sourdoughs and description of *Lactobacillus spicheri* sp. nov.** *Syst Appl Microbiol* 2004, **27**:151-159.

110. Kleynmans U, Heinzl H, Hammes WP: ***Lactobacillus suebicus* sp. nov., an obligately heterofermentative *Lactobacillus* species isolated from fruit mashes.** *Syst Appl Microbiol* 1989, **11**:267-271.

111. Dellaglio F, Vancanneyt M, Endo A, Vandamme P, Felis GE, Castioni A, Fujimoto J, Watanabe K, Okada S: ***Lactobacillus durianis* Leisner *et al*. 2002 is a later heterotypic synonym of *Lactobacillus vaccinostercus* Kozaki and Okada 1983.** *Int J Syst Evol Microbiol* 2006, **56**:1721-1724.

112. Okada S, Suzuki Y, Kozaki M: **A new heterofermentative *Lactobacillus* species with *meso*-diaminopimelic acid in peptidoglycan, *Lactobacillus vaccinostercus* Kozaki and Okada sp. nov.** *J Gen Appl Microbiol* 1979, **25**:215-221.

113. Embley TM, Faquir N, Bossart W, Collins MD: ***Lactobacillus vaginalis* sp. nov. from the human vagina.** *Int J Syst Bacteriol* 1989, **39**:368-370.

114. Krockel L, Schillinger U, Franz CMAP, Bantleon A, Ludwig W: ***Lactobacillus versmoldensis* sp. nov., isolated from raw fermented sausage.** *Int J Syst Evol Microbiol* 2003, **53**:513-517.

115. Rodas AM, Chenoll E, Macian MC, Ferrer S, Pardo I, Aznar R: ***Lactobacillus vini* sp. nov., a wine lactic acid bacterium homofermentative for pentoses.** *Int J Syst Evol Microbiol* 2006, **56**:513-517.

116. Leisner JJ, Vancanneyt M, Goris J, Christensen H, Rusul G: **Description of *Paralactobacillus selangorensis* gen. nov., sp. nov., a new lactic acid bacterium isolated from chili bo, a Malaysian food ingredient.** *Int J Syst Evol Microbiol* 2000, **50**:19-24.

117. De Bruyne K, Franz CMAP, Vancanneyt M, Schillinger U, Mozzi F,de Valdez GF, de Vuyst L, Vandamme P: ***Pediococcus argentinicus* sp. nov. from Argentinean fermented wheat flour and identification of *Pediococcus* species by *pheS*, *rpoA*, and *atpA* sequence analysis.** *Int J Syst Evol Microbiol* 2008, **58**:2909-2916.

118. Zhang B, Tong H, Dong X: ***Pediococcus cellicola* sp. nov., a novel lactic acid coccus isolated from a distilled-spirit-fermenting cellar.** *Int J Syst Evol Microbiol* 2005, **55**:2167-2170.

119. Dobson CM, Deneer H, Lee S, Hemmingsen S, Glaze S, Ziola B: **Phylogenetic analysis of the genus *Pediococcus*, including *Pediococcus claussenii* sp. nov., a novel lactic acid bacterium isolated from beer.** *Int J Syst Evol Microbiol* 2002, **52**:2003-2010.

120. Liu L, Zhang B, Tong H, Dong X: ***Pediococcus ethanolidurans* sp. nov., isolated from the walls of a distilled-spirit-fermenting cellar.** *Int J Syst Evol Microbiol* 2006, **56**:2405-2408.

121. **Validation List No 25.** *Int J Syst Bacteriol* 1988, **38**:220-222.

122. Gunther HL, White HR: **The cultural and physiological characters of the pediococci.** *J Gen Microbiol* 1961, **26**:185-197.

123. Franz CMAP, Vancanneyt M, Vandemeulebroecke K, De Wachter M, Cleenwerck I, Hoste B, Schillinger U, Holzapfel WH, Swings J: ***Pediococcus stilesii* sp. nov., isolated from maize grains.** *Int J Syst Evol Microbiol* 2006, **56**:329-333.