**Molecular Mechanisms of Hemolysis by *Brachyspira hampsonii***

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By

Brandon Alexander Keith

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**Abstract**

The re-emergence of swine dysentery in Western Canadian swine herds has resulted in dramatic production losses for pork producers. The observation that pathogenic strains of the causative agents *Brachyspira hyodysenteriae* and *Brachyspira hampsonii* are strongly β-hemolytic in contrast to weakly β-hemolytic avirulent strains suggests that the production of one or more hemolysins by these bacteria may play a critical role in the pathogenesis of swine dysentery. Our research aimed to characterize the *in vitro* functionality of two hemolysins produced by *Brachyspira hampsonii*, termed TlyA and native hemolysin. TlyA protein from *Brachyspira hampsonii* was purified via heterologous expression from an *E. coli* host and found to possess both hemolytic and rRNA methyltransferase activities. Two amino acid residues, S9 and C80, were found to inhibit the oligomerization and hemolytic activity of TlyA partially or completely when mutated. These residues are conserved in TlyA proteins from both pathogenic *Brachyspira* species and unrelated pathogenic bacteria, suggesting a common mechanism of oligomerization for these proteins. Next, we purified a potent hemolysin from *Brachyspira hampsonii* broth cultures which we have termed native hemolysin. Although this toxin had previously been linked to a gene termed *hlyA*, the recent discovery of an operon encoding for a Streptolysin S homologue within the *Brachyspira hampsonii* strain 30446 genome has raised doubts on the identity of this toxin. The *sagABCD* genes from *Brachyspira hampsonii* strain 30446 were cloned and heterologously expressed in *E. coli* using a co-expression strategy, and the purified SagA peptide was found to possess hemolytic activity against pig erythrocytes, albeit with a much lower specific activity than the native hemolysin purified from *Brachyspira hampsonii* strain 30446 broth cultures. To assess the role native hemolysin plays in the pathogenesis of swine dysentery, we first exposed a colonic cell line to varying doses of native hemolysin to assess the cytotoxicity of this toxin. Surprisingly, no cytotoxic activity was observed in cells

treated with 8 HU of native hemolysin for 24 hours. Rather, the observation that DIDS, a potent inhibitor of cellular anion-exchange proteins, could dramatically inhibit the hemolytic activity of native hemolysin suggested that the toxic effects of this peptide are not a result of direct pore-formation, but rather are mediated by the overactivation of $Cl^-/HCO_3^-$ exchangers such as band 3 or DRA. To test this hypothesis, Caco-2 monolayers were exposed to native hemolysin for 1 or 24 hours, and changes in electrogenic ion transport were assessed utilizing Ussing chamber studies. Remarkably, a significant decrease in electrogenic $Na^+$ absorption through ENaC was observed within an hour of native hemolysin treatment, providing evidence that *Brachyspira* induced diarrhea initially occurs due to $Na^+$ malabsorption within the colon of infected animals. Addition of DIDS to monolayers treated with native hemolysin ameliorated the decrease in electrogenic $Na^+$ absorption, indicating that these effects are directly linked to the overactivation of the $Cl^-/HCO_3^-$ exchanger DRA by native hemolysin. Altogether, these results not only confirm that both TlyA and a Streptolysin S homologue produced by *Brachyspira hampsonii* strain 30446 are hemolytically active proteins, but also provide the first direct evidence of a link between the primary sequence of a TlyA protein and bacterial pathogenicity, along with providing a mechanistic explanation for the development of diarrhea during the acute stages of *Brachyspira* infection.

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## Dedication

I dedicate this thesis to the memory of my grandfather Nick Yaschuk (September 2, 1929 – September 17, 2021). His hard work and dedication paved the way for future generations to pursue their dreams in Canada.

## Table of Contents

## List of Tables

## List of Figures

## List of Abbreviations

| | | |
|---|---|---|
| µCi | – | microcurie ($10^{-6}$ Ci) |
| µg | - | microgram ($10^{-6}$ g) |
| µL | – | microlitre ($10^{-6}$ L) |
| µM | – | micromolar ($10^{-6}$ moles/L) |
| Å | – | Angstrom unit ($10^{-1}$ nm) |
| βME | – | Beta mercaptoethanol |
| AA | – | amino acid |
| ACP | – | Acyl carrier protein |
| ADAM- | – | A disintegrin and metalloprotease |
| AIS | – | Avian intestinal spirochetosis |
| AKT | – | Protein kinase B |
| ANOVA | – | Analysis of variance |
| BCA | – | Bicinchonic acid assay |
| BCG | – | *Mycobacterium bovis* strain *Bacillus* Calmette-Guerin |
| BHI | – | Brain heart infusion |
| Caco-2 | – | Human colon adenocarcinoma cell line |
| CD | – | Circular dichroism |
| CDC | – | Cholesterol dependent cytolysin |
| CFTR | – | Cystic fibrosis transmembrane conductance regulator |
| CLS | – | Clostridiolysin S |
| DIDS | – | 4,4'-diisothiocyanatostilbene-2,2'-disulphonate |
| DMEM | – | Dulbecco's modified Eagle Medium |
| DOC | – | Deoxycholic acid/deoxycholate |
| DRA | – | Downregulated in adenoma |
| DTT | – | Dithiothreitol |
| *E. coli* | – | *Escherichia coli* |
| EDTA | – | Ethylene diamine tetra-acetic acid |
| ENaC | – | Epithelial sodium channel |
| ERK | – | Extracellular signal-regulated kinase |

| | | |
|---|---|---|
| FBS | – | Fetal bovine serum |
| FMN | – | Flavin mononucleotide |
| GAS | – | Group A *Streptococcus* |
| Gnd-HCL | – | Guanidine hydrochloride |
| GSK-3β | – | Glycogen synthase kinase 3 beta |
| HEPES | – | (4-(2-hydroxyethyl)-1-pierazineethanesulfonic acid) |
| HIS | – | human intestinal spirochetosis |
| HU | – | Hemolytic units |
| IBMX | – | 3-isobutyl-1-methylxanthine |
| IL- | – | Interleukin |
| $I_{sc}$ | – | Short circuit current |
| ITC | – | Isothermal titration calorimetry |
| $K_D$ | – | Dissociation constant |
| kDa | – | Kilodalton ($10^3$ g/mol) |
| LDH | – | Lactate dehydrogenase |
| LLO | – | Listerolysin O |
| LLS | – | Listerolysin S |
| LPS | – | Lipopolysaccharide |
| MAPK | – | Mitogen activated protein kinase |
| MBP | – | Maltose binding protein |
| MccB17 | – | Microcin B17 |
| MCP- | – | Monocyte chemoattractant protein |
| MUC- | – | Mucin |
| NF-κB | – | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NHE- | – | Sodium hydrogen exchanger |
| nm | – | Nanometer ($10^{-9}$ m) |
| OMV | – | Outer-membrane vesicle |
| PBS | – | Phosphate buffered saline |
| PD | – | Potential difference |
| PDB | – | Protein data bank |

| | | |
|---|---|---|
| PEG | – | Polyethylene glycol |
| PFT | – | Pore-forming toxin |
| PIS | – | Porcine intestinal spirochetosis |
| PL | – | Phospholipase |
| PMSF | – | Phenylmethylsulfonyl fluoride |
| PTC | – | Peptidyl transferase center |
| RL | – | Rhamnolipid |
| ROS | – | Reactive oxygen species |
| rRNA | – | ribosomal RNA |
| sag- | – | Streptolysin associated gene |
| SAMe- | – | S-adenosyl-l-methionine |
| SD | – | Swine dysentery |
| SDS-PAGE | – | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SLO | – | Streptolysin O |
| SLS | – | Streptolysin S |
| SM | – | Sphingomyelinase |
| TCEP | – | tris(2-carboxyethyl)phosphine hydrochloride |
| TDH | – | Thermostable direct hemolysin |
| TEER | – | Transepithelial electrical resistance ($\Omega/cm^2$) |
| TEV | – | Tobacco etch virus |
| TNF- | – | Tumor necrosis factor |
| TOMM | – | Thiazole/oxazole modified microcin |
| TrpLE | – | Tryptophan operon leader sequence |
| UPR | – | Unfolded protein response |

# Chapter 1 – Introduction

## 1.1 Rationale

First identified in the 1920s, swine dysentery is an infectious disease of pigs caused by *Brachyspira hyodysenteriae* [1-3]. While swine dysentery had been largely eliminated from North American swine herds by the late 1980s, the re-emergence of this disease over the past 20 years has led to massive production losses in infected herds, resulting in estimated yearly losses of $200 million within the United States [4]. Confounding these concerns are the recent emergence of *Brachyspira hampsonii* and *Brachyspira suanatina*, two related species capable of causing disease indistinguishable from *Brachyspira hyodysenteriae* induced swine dysentery [5-8]. Recent studies into the mechanism of swine dysentery have concluded that the diarrhea characteristic of this disease is malabsorptive in nature, with the downregulation of the $Na^+/H^+$ exchanger NHE3 [9] and the $Cl^-/HCO_3^-$ exchanger DRA [10] leading to decreased absorption of $Na^+$ and $Cl^-$ ions and subsequent osmotic movement of water into the colon of infected pigs. Additionally, the downregulation of the $Cl^-$ channel CFTR during *Brachyspira* infection leads to a decrease in apical $Cl^-$ secretion [11], altering the thickness of colonic mucous and promoting colonization of the colonic epithelium by *Brachyspira* species [12]. Despite these recent advances in our understanding of swine dysentery pathology, the molecular mechanisms by which *Brachyspira* infection induces these physiological changes within the colon is unknown. However, the positive correlation between a strong β-hemolytic phenotype and virulence in *Brachyspira* strains indicates that one or more hemolysins produced by these bacteria are critical virulence factors required for disease pathology.

While four hemolysin genes have been identified in *Brachyspira hyodysenteriae* [13, 14], minimal characterization of the encoded proteins has been carried out. Furthermore, although all

these genes are present in *Brachyspira hampsonii*, the recent discovery of this bacterium means that none of these hemolysins has received further study in *Brachyspira hampsonii*. Of the four identified hemolysin genes (termed *tlyA, tlyB, tlyC,* and *hlyA*), the *tlyA* gene has received the most study due to the identification of *tlyA* homologues within other pathogenic bacteria such as *Mycobacterium tuberculosis* [15], *Helicobacter pylori* [16], and *Campylobacter jejuni* [17]. While research on TlyA homologues from these bacteria has determined that TlyA is both a pore-forming hemolysin and a rRNA methyltransferase [18], this has yet to be confirmed for the TlyA proteins from *Brachyspira hyodysenteriae* or *Brachyspira hampsonii*. Furthermore, while a highly potent hemolysin produced by *Brachyspira hyodysenteriae* broth cultures in the presence of yeast RNA core (referred to as native hemolysin throughout this thesis) has been linked to a gene termed *hlyA* [14], this toxin bears striking similarities to streptolysin S, a hemolysin produced by *Streptococcus pyogenes* [19]. While the *hlyA* gene is present in the genomes of both the pathogenic *Brachyspira hampsonii* strain 30446 and the nonpathogenic *Brachyspira hampsonii* KL180, the recent discovery of an operon encoding for a streptolysin S homologue within the genome in strain 30446 but not strain KL180 [20] has raised doubts about the true identity of this hemolysin. Given the previously mentioned link between a β-hemolytic phenotype and pathogenicity in *Brachyspira* spp. it stands to reason that the hemolysins produced by these bacteria play an indispensable role in swine dysentery pathology, but these specific contributions are not currently known.

This thesis aims to characterize the major hemolysins of *Brachyspira hampsonii* and determine their role in the overall pathogenesis of swine dysentery. Not only is this research vital to the overall understanding of *Brachyspira* infections in pigs, these studies will also improve our understanding of important human diseases such as *tuberculosis* and soft tissue infections, due to

the fact that these hemolysins are also expressed by pathogens such as *Mycobacterium tuberculosis* and *Streptococcus pyogenes*.

## 1.1 Objectives

1. Clone, overexpress, purify, and characterize the hemolytic activity of TlyA from *Brachyspira hampsonii*
2. Purify and identify the native hemolysin produced by *Brachyspira hampsonii* broth cultures in the presence of yeast RNA core
3. Purify and characterize functional recombinant Streptolysin S-like peptide from *Brachyspira hampsonii* utilizing an *Escherichia coli* based expression system
4. Examine the effects of the *Brachyspira hampsonii* native hemolysin on colonic epithelial function.

## 1.2 Hypotheses

1. Alterations to the primary sequence of TlyA affect both the hemolytic phenotype and pathogenicity of bacteria expressing these proteins.
2. The native hemolysins produced by *Brachyspira hampsonii* and *Brachyspira hyodysenteriae* are modified peptides homologous to Streptolysin S.
3. The pathogenic effects of swine dysentery can be recapitulated *in vitro* by treating colonic epithelial cells with the native hemolysin of *Brachyspira hampsonii*.
4. The pathogenicity of *Brachyspira hampsonii* and *Brachyspira hyodysenteriae* strains are correlated with expression of a Streptolysin S-like peptide.

# Chapter 2 - Literature Review

## 2.1 The genus *Brachyspira*

*Brachyspira* (FKA *Treponema/Serpula/Serpulina*) is a genus comprised of aerotolerant anaerobic spirochetes, consisting of 9 named species at the time of writing (Table 2.1). *Brachyspira* species range from 2.0 to 11.0 µm in length and are highly motile due to the presence of flagella [21]. While species such as *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* are known to survive in soil or standing water for extended periods of time [22, 23], *Brachyspira* naturally inhabit the large intestines of a variety of mammalian and avian hosts [21, 24]. Some species such as *Brachyspira innocens* are harmless commensals [25], however, other species are important pathogens in both human and animal health.

Table 2.1 Host tropism and β-hemolytic phenotypes of recognized *Brachyspira* species

| **Name** | **Host(s)** | **β-hemolysis** | **Diseases caused** |
|---|---|---|---|
| *Brachyspira hyodysenteriae* | Pigs, rheas, chickens, mallards | **Strong** | Swine dysentery, necrotizing typhlo*coli*tis (rheas), |
| *Brachyspira hampsonii* | Pigs, geese, mallards | **Strong** | Swine dysentery |
| *Brachyspira suanatina* | Pigs, mallards | **Strong** | Swine dysentery |
| *Brachyspira pilosicoli* | Pigs, humans, rheas, chickens, mallards, dogs, horses | Weak | Porcine intestinal spirochetosis (PIS), human intestinal spirochetosis (HIS), avian intestinal spirochetosis (AIS) |
| *Brachyspira aalborgi* | Humans | Weak | HIS |
| *Brachyspira intermedia* | Chickens, pigs | Weak | AIS |
| *Brachyspira alvinipulli* | Chickens | Weak | AIS |
| *Brachyspira murdochii* | Pigs | Weak | Mild *coli*tis [26] |
| *Brachyspira innocens* | Pigs, dogs | Weak | None |

### 2.1.1 Human/Avian/Porcine Intestinal Spirochetosis

While spirochetes have been known to colonize the human gastrointestinal tract since the development of light microscopy, it was not until the early 20th century that researchers began to suspect that these bacteria may cause disease [27-29]. This disease, termed human intestinal spirochetosis (HIS), is characterized by moderate to severe watery diarrhea and abdominal pain [30-34]. While HIS is rare, prevalence is higher in men who have sex with men and $HIV^+$ patients [30, 31, 34-36], leading to speculation that HIS is a sexually transmitted infection. Higher HIS rates are also observed in developing regions such as the aboriginal communities of Australia [37, 38] and rural areas of India [39] due to poor sanitation practices in these regions leading to increased transmission through the fecal-oral route. HIS is caused by *Brachyspira aalborgi* and *Brachyspira pilosicoli*, both of which exhibit a weak β-hemolytic phenotype when cultured on blood agar [34, 40-42]. In addition to humans, *Brachyspira* species are also known to cause disease in both pigs and poultry, leading to diseases termed porcine intestinal spirochetosis (PIS) [43-45] and avian intestinal spirochetosis (AIS) [21, 46, 47], respectively. PIS is caused by *Brachyspira pilosicoli* [43] and is characterized by the development of watery or mucoid diarrhea, resulting in production losses due to poor feed conversion [42-45]. Conversely, *Brachyspira pilosicoli*, *Brachyspira intermedia*, and *Brachyspira alvinipulli* are all capable of causing AIS [46-49]. Similar to HIS and PIS, symptoms of AIS include watery feces or diarrhea, in addition to impaired egg production and quality [46, 47].

Regardless of the bacterial or host species, intestinal spirochetosis is typified by the end-on attachment of bacteria to the surface of the colonic epithelium, forming a "false brush border" in histological sections [31, 32, 34, 40, 48-53]. This attachment results in inflammation and a loss of absorptive capacity within the colon, with this inhibition of absorption resulting in watery diarrhea characteristic of intestinal spirochetosis [31, 32, 51, 54]. While invasion of *Brachyspira*

*pilosicoli* into epithelial cells and macrophages has been observed in HIS [50, 55, 56], this has not been seen in PIS or AIS. Additionally, *in vitro* experiments with *Brachyspira pilosicoli* have shown that this bacterium can induce apoptosis, destroy tight junctions, and upregulate expression of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and IL-8 in cultured colonic epithelial cells [53]. The authors observed that cell-free culture supernatants were unable to upregulate any of these cytokines under identical experimental conditions [53], suggesting that the pathogenic effects of *Brachyspira pilosicoli* are not due to the action of one or more secreted toxins.

While several promising vaccine candidates towards HIS, PIS, and AIS have been identified in recent years [57, 58], no vaccines against these diseases are commercially available at the time of writing. As a result, treatment of HIS/PIS/AIS is dependent on antibiotics. While *Brachyspira pilosicoli* strains implicated in human disease exhibit susceptibility to tetracycline, chloramphenicol, meropenem, moxifloxacin, metronidazole, and ceftriaxone [59], metronidazole is most commonly used for the treatment of HIS caused by *Brachyspira pilosicoli* or *Brachyspira aalborgi* [60-63]. Conversely, susceptibility to tiamulin, lincomycin, valnemulin, tylosin, carbadox, doxycycline, and tylvalosin has been reported for pig strains of *Brachyspira pilosicoli*, with tiamulin being the most common antimicrobial used for treatment [64-66]. Similar susceptibility profiles are observed for strains of *Brachyspira pilosicoli*, *Brachyspira intermedia* and *Brachyspira alvinipulli* implicated in AIS [67, 68]. While the prevalence of antimicrobial resistance in *Brachyspira pilosicoli* has remained low in recent years [66], mutations in the 23s rRNA gene have been linked to resistance to macrolides, lincosamides, and tiamulin [69, 70], underscoring the need for diligent biosecurity practices and responsible antibiotic use in order to prevent wider resistance in these bacteria.

### 2.1.2 Swine Dysentery

Swine dysentery (SD) is an infectious disease characterized by severe bloody mucoid diarrhea in grower and finisher pigs resulting in massive production losses and up to 30% mortality in infected herds [71]. SD was first identified in the 1920s [1] and was attributed to infection with *Campylobacter coli* by researchers in the 1960s [72, 73]. However, later studies definitively proved *Brachyspira hyodysenteriae* to be the causative agent [2, 3]. While antimicrobial usage and improved animal husbandry practices had largely succeeded in eliminating swine dysentery from American swine herds by the 1990s, re-emergence of the disease was observed in the early 2000s, with some authors partially attributing this to the decreased use of antibiotics in livestock production [8, 74].

#### 2.1.2.1 Epidemiology and Treatment

Swine dysentery is presently found in pig herds worldwide, with the strongly β-hemolytic spirochete *Brachyspira hyodysenteriae* first being linked to SD in 1972 [3]. Since this time, *Brachyspira hyodysenteriae* has remained an important pathogen in swine production, being detected in pig herds in North America [75, 76], Europe [77-80], Australia [81, 82], and Asia [83-85] within the past decade. In addition to infecting pigs, *Brachyspira hyodysenteriae* is also known to cause disease in rheas [86, 87], chickens [88], and mallards [89, 90]. While *Brachyspira hyodysenteriae* remains the primary causative agent of swine dysentery, recent outbreaks have been attributed to the newly described species *Brachyspira hampsonii* and *Brachyspira suanatina* [6, 7]. Several outbreaks of swine dysentery in the late 2000s produced symptoms indistinguishable from *Brachyspira hyodysenteriae* induced disease; however, *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* were not detected [5]. The causative agent was a strongly hemolytic *Brachyspira* species, termed *Brachyspira hampsonii* by the authors [5]. Two clades of *Brachyspira hampsonii* were identified [5], both of which are virulent

in pigs [6, 91]. An outbreak in Western Canada in 2009 was linked to *Brachyspira hampsonii* clade II (strain 30446) [6], and subsequent retrospective studies have indicated that *Brachyspira hampsonii* has been circulating in Western Canadian pig herds since at least 2002 [92]. *Brachyspira hampsonii* has since been detected in several European swine herds [93, 94]. Of note is the detection of *Brachyspira hampsonii* in migratory birds in both Spain [95, 96] and the Canadian arctic [97], indicating that these birds may serve as vectors for the spread of *Brachyspira hampsonii* from farm to farm. Similarly, a novel species termed *Brachyspira suanatina* was observed in both farmed and wild birds in Sweden in the early 2000s [89, 98]. Like *Brachyspira hyodysenteriae* and *Brachyspira hampsonii*, *Brachyspira suanatina* infects pigs, causing identical clinical signs to *Brachyspira hyodysenteriae* induced swine dysentery [7, 99, 100]. While *Brachyspira suanatina* has yet to be detected outside of Scandinavia and Germany [7, 99, 100], this bacterium is also known to colonize migratory birds [89, 90, 98], representing a potential mechanism by which *Brachyspira suanatina* could spread outside this geographical area in the future. Interestingly, while mallard ducklings experimentally challenged with *Brachyspira suanatina* did not develop clinical symptoms, epithelial damage and goblet cell hyperplasia were observed, suggesting that *Brachyspira suanatina* may possess the ability to cause disease in birds [90].

Currently, no vaccines against swine dysentery are available. While several groups attempted to produce bacterin vaccines in the 1980s and 1990s, these candidates provided only marginal protection against swine dysentery [101-105], with the authors of one study reporting increased disease severity in vaccinated animals [106]. Further complicating this approach is the fact that bacterin vaccines generally only provide protection against the same serotype used to prepare the vaccine [107]. Given that at least 17 serotypes of *Brachyspira hyodysenteriae* are known to exist

[108], research into swine dysentery vaccines in recent years has focused on the use of subunit vaccines. Vaccines utilizing outer membrane lipoproteins (BmpB/Bpmp72/SmpB) [57, 109, 110], oligopeptide-binding proteins [58], ferritin proteins [111], or flagellar proteins [112] have been evaluated and in some cases provided protection against *Brachyspira hyodysenteriae* or *Brachyspira pilosicoli* infection in pre-clinical trials. However, none of these vaccines have been approved for commercial use. In recent years, two avirulent, weakly β-hemolytic *Brachyspira hyodysenteriae* strains (D28/MU1) have been shown to provide protection against virulent *Brachyspira hyodysenteriae* strains [113, 114]. However, protection against other pathogenic species such as *Brachyspira hampsonii* and *Brachyspira suanatina* was not evaluated in these studies.

Treatment of swine dysentery is reliant on antibiotics at the present time, with macrolides (tylosin/tylvalosin/erythromycin), lincosamides (lincomycin/clindamycin), and pleuromutilins (tiamulin/valnemulin) being most commonly used [115]. An area of concern for veterinary medicine is the increased prevalence of antibiotic-resistant *Brachyspira* strains, with increased resistance to tylvalosin, lincomycin, tiamulin, and valnemulin reported in *Brachyspira hyodysenteriae* isolates during the early-mid 2000s [116-118]. These drugs all exhibit their antimicrobial function through binding to the ribosome, thereby inhibiting protein synthesis and leading to cell death [119-123]. As a result, mutations in the 23s rRNA have been linked to antibiotic resistance in *Brachyspira* species [69, 70, 118, 123, 124]. Specifically, an A2058T/G mutation in the 23s rRNA gene of *Brachyspira hyodysenteriae* leads to decreased tylosin, erythromycin, clindamycin, tiamulin, and valnemulin susceptibility [118, 124, 125], G2032A, C2055A, G2447T, C2499A, T2504G, and A2572T mutations in the 23s rRNA gene were associated with tiamulin resistance [69], and an N148S mutation in the ribosomal protein L3 is

associated with tiamulin and valnemulin resistance [69, 125]. In addition to ribosomal RNA/protein mutations, the acquisition of specific genes has also been linked to antibiotic resistance in *Brachyspira hyodysenteriae*. Increased resistance to tiamulin, valnemulin, and streptrogramins A and B has been linked to expression of a gene termed *tva*(A) [126, 127]. The chromosomally encoded *tva*(A) gene exhibits homology to *vgaA*, an ATP-binding cassette F protein produced by lincosamide resistant *Staphylococcus aureus* strains, and as such it is hypothesized that the *tva*(A) gene product functions by displacing pleuromutilins from the peptidyl transferase center of the ribosome [128]. Additionally, a gene termed *lnu*(C) has been shown to confer lincosamide resistance to *Brachyspira hyodysenteriae* by adenylating lincomycin molecules [129, 130]. Of note is the fact that the *Brachyspira hyodysenteriae lnu*(C) gene is found on a transposon [129], suggesting that lincosamide resistance in *Brachyspira* may arise due to horizontal gene transfer.

#### 2.1.2.2 Pathophysiology

Pigs suffering from swine dysentery typically present with soft yellow or grey feces accompanied with elevated temperature during the initial stages of the disease, after which point severe watery diarrhea containing blood and mucous is observed [3, 5, 6, 71, 91]. Postmortem examination typically reveals severe inflammation, lesion formation, hemorrhage, and fibrinous exudate within the colon of infected animals [131, 132]. While the end-on attachment of spirochetes and subsequent "false brush border" formation of *Brachyspira pilosicoli* infections is not observed, *Brachyspira hyodysenteriae*/*hampsonii*/*suanatina* are commonly found within colonic crypts and have been reported to invade goblet cells [131-133]. This association of *Brachyspira* spp. with the colonic epithelium appears to be a result of the strong chemotaxis these bacteria show towards mucins [134-136], as virulent *Brachyspira hyodysenteriae* strains exhibit stronger chemotaxis towards porcine colonic mucins in comparison to avirulent strains of

*Brachyspira hyodysenteriae*, *Brachyspira intermedia*, and *Brachyspira innocens* [134]. *Brachyspira hyodysenteriae* infection results in increased production of mucins in the colon, with the mucin gene *MUC5AC* being the most up-regulated [12, 133] despite the fact that this gene is not expressed in the colon under normal conditions [137-140]. This increase in mucus production corresponded to an increase in guanidine hydrochloride insoluble mucin levels [12, 133], indicating that increased mucus thickness during *Brachyspira* infection may reduce the absorptive capacity in the colon and contribute to the development of diarrhea. Additionally, *Brachyspira hyodysenteriae* more readily binds to mucus derived from pigs experimentally infected with swine dysentery than to that derived from control animals [12] and substantially modifies the diversity, chain length, and chemistry of O-glycan carbohydrates attached to colonic mucins [133]. In particular, sialic acid-containing mucins were shown to be more prevalent in infected animals [133], with the authors of this study later observing increased attachment of *Brachyspira hyodysenteriae* to these mucins in addition to increased growth when *Brachyspira hyodysenteriae* was grown in media containing free sialic acid [141]. While *Brachyspira hyodysenteriae* does not appear to be able to cleave sialic acid residues from colonic mucins *in vitro*, the authors hypothesized that sialidase activity by other commensal bacteria may increase the concentration of free sialic acid during swine dysentery [142, 143], thus providing an energy source for *Brachyspira hyodysenteriae* within the colonic mucosa [141]. Infection with *Brachyspira hyodysenteriae* and *Brachyspira hampsonii* also decreases expression levels of *CFTR* (cystic fibrosis transmembrane conductance regulator) [11], a $Cl^-$ channel whose impaired function in diseases such as cystic fibrosis or *Citrobacter rodentium* infection leads to increased mucous thickness [144-146]. Taken together, these observations suggest a positive feedback mechanism in which *Brachyspira hyodysenteriae* alters the mucin expression profile of the

colonic epithelium during infection, thereby improving both adhesion to the epithelial surface and growth.

Mechanistic studies into swine dysentery in the 1980s concluded that the diarrhea characteristic of the disease was malabsorptive in nature, as $Na^+$, $Cl^-$, and $HCO_3^-$ absorption in colonic loops infected with *Brachyspira hyodysenteriae* was essentially abolished [147]. Additional studies found no effect of the phosphodiesterase inhibitor theophylline on ion transport in *Brachyspira hyodysenteriae* infected colonic loops, leading the authors of this study to conclude that *Brachyspira hyodysenteriae* induced diarrhea did not possess a secretory component, unlike diarrhea induced by other bacteria such as *Salmonella* or *Escherichia coli* [148]. Later studies examining ion transport in the colon of pigs infected with *Brachyspira hyodysenteriae* or *Brachyspira hampsonii* concluded that $Cl^-$ secretion in infected pigs was inhibited due to a decrease in *CFTR* expression [11], further confirming that *Brachyspira* diarrhea is not secretory in nature. The same authors later found a significant decrease in $Na^+$ absorption in the colon of infected animals and linked this to decreased expression of an apical $Na^+/H^+$ exchanger termed *NHE3* [9]. *NHE3* has been previously shown to play an important role in colonic $Na^+$ absorption and maintenance of cellular pH, and *NHE3* knockout mice exhibit chronic diarrhea [149-151]. The downregulation of both *CFTR* and *NHE3* was shown to be a direct effect of *Brachyspira* infection rather than the host inflammatory response, as sonicated *Brachyspira hampsonii* could recapitulate these effects *in vitro* while direct application of the pro-inflammatory cytokine IL-1α had no effect [9, 11].

In addition to the aforementioned effects on mucus production/composition and ion transport, *Brachyspira* infection also induces a strong inflammatory response in infected animals. *In vitro* studies showed upregulation of IL-1β and IL-8 when macrophages were exposed to endotoxin

derived from *Brachyspira hyodysenteriae* [152, 153]. Notably, the levels of IL-1β and IL-8 expression were lower in cells treated with *Brachyspira hyodysenteriae* endotoxin than those treated with 50-fold lower concentrations of *E. coli* derived endotoxin [153], suggesting that endotoxins of *Brachyspira* spp. are less potent than those derived from other bacteria. In contrast, contradictory reports on the production of TNF-α during *Brachyspira* infection have emerged. While increases in TNF-α expression have been reported in macrophages treated with *Brachyspira hyodysenteriae* endotoxin [152], colonic tissue explants exposed to *Brachyspira hampsonii* [154] and the blood of pigs experimentally infected with *Brachyspira hyodysenteriae* [155], other groups did not observe increases in TNF-α expression during *Brachyspira hyodysenteriae* or *Brachyspira hampsonii* infection [10, 156]. Of note is the observation by Kruse *et al.*, that two of the pigs infected with *Brachyspira hyodysenteriae* did not exhibit clinical signs of swine dysentery yet had increases in TNF-α levels of similar magnitude to those that developed disease [155]. These results suggest that the observed increase in TNF-α levels may thus be due to asymptomatic colonization by *Brachyspira hyodysenteriae* or distress associated with the inoculation procedure, as no sham-inoculated controls were used in this study [155]. Besides contributing to the colonic inflammation characteristic of swine dysentery, induction of inflammatory cytokine expression has been shown to directly contribute to the pathogenesis of this disease through modulation of mucin and ion channel gene expression. *In vitro* infection of a mucus-producing cell line with *Brachyspira hyodysenteriae* resulted in increased production and apical transport of mucins only when the cells were treated with neutrophil elastase or IL-17A [156]. Inhibitor studies showed that this effect was mediated by the MAPK3/ERK1 signaling pathway [156]. Conversely, treatment of a colonic cell line with IL-1α resulted in decreased expression of the $Cl^-/HCO_3^-$ exchanger *DRA*, an ion channel downregulated

in swine dysentery [10]. The same study also showed that this downregulation of *DRA* lead to a decrease in $Cl^-$ absorption within the colon of infected pigs [10], indicating that the host inflammatory response is partially responsible for the development of diarrhea during *Brachyspira* infection. Overall, it appears that a complex interplay between the host inflammatory response and direct alteration of host cell physiology by *Brachyspira* spp. is involved in the pathogenesis of swine dysentery. As such, the well documented link between a strong β-hemolytic phenotype and pathogenicity in *Brachyspira* spp. suggests that one or more hemolysins produced by these bacteria may be responsible for the altered colonic epithelial function observed during swine dysentery pathogenesis. However, to examine this hypothesis it is critical to understand the varied functions of bacterial hemolysins and their known contributions to a variety of diseases.

### 2.2 Bacterial hemolysins – structure, function, and contribution to virulence

Microbial culture on blood agar is an important phenotypic test in diagnostic microbiology. Microorganisms cultured in this manner will typically exhibit one of three hemolysis patterns, termed α-hemolysis (partial breakdown of red blood cells in the media), β-hemolysis (complete clearing of red blood cells), or γ-hemolysis (no hemolysis). Hemolysis occurs through the action of hemolysins, a diverse family of toxins produced by bacteria capable of lysing red blood cells *in vitro*. While hemolytic phenotype is important for identification of pathogenic bacteria, it is important to note that lysis of red blood cells is rarely the primary effect of these toxins *in vitro* [157]. Hemolysins are known to have deleterious effects on a variety of cell types, even at concentrations orders of magnitudes lower than those required for hemolysis [157-160]. This section will focus on the structures and functions of the three known families of bacterial hemolysins and the contribution of these toxins to bacterial disease.

### 2.2.1 Surfactants

While most known hemolysins are proteins, several bacteria are known to produce lipids with hemolytic and cytolytic properties. Rhamnolipids (RLs) are a family of surfactants, consisting of one or two rhamnose sugars bonded to fatty acids of varying length [161-164]. Initially identified in the respiratory pathogen *Pseudomonas aeruginosa* [163, 165, 166], RLs have since been found in other bacteria such as *Burkholderia* spp. [167-170], *Renibacterium salmoninarum* [171], *Cellulomonas cellulans* [172], and several *Nocardioides* spp. [173]. Production of these surfactants by bacteria is believed to facilitate uptake of insoluble molecules such as alkanes, allowing RL producing bacteria to metabolize these substrates [174-176]. As a result, these molecules have seen extensive use in fields such as oil extraction [177, 178] and bioremediation of polluted areas [174, 175]. In addition to these applications, RLs produced by *Pseudomonas aeruginosa* also function as critical virulence factors. RL production has been shown to induce swarming motility (coordinated movement of a bacteria population) [179, 180] and biofilm formation [181, 182] in *Pseudomonas aeruginosa*, with these surfactants also exhibiting potent antimicrobial activity against gram positive bacteria [183, 184]. RLs are potent cytotoxic agents against erythrocytes [185-188], keratinocytes [189], and various cell lines [167, 190], where they function by solubilizing the host cell plasma membrane, leading to leakage of intracellular contents and subsequent cell death. RLs have been directly linked to virulence in *Pseudomonas aeruginosa* as a result, with higher RL concentrations in sputum from cystic fibrosis patients infected with *Pseudomonas aeruginosa* correlating with increased disease severity [191], and knockout strains of *Pseudomonas aeruginosa* deficient in RL production genes exhibiting decreased virulence *in vivo* [192]. RLs are also known to inhibit $Na^+$ absorption in bronchial and tracheal epithelia and inhibit ciliary beating [193, 194], both of which lead to increased *Pseudomonas aeruginosa* virulence in cystic fibrosis patients.

### 2.2.2 Lipases

In addition to solubilization of the membrane by surfactants such as rhamnolipids, bacteria have evolved enzymes capable of hydrolyzing specific lipids within the plasma cell membrane. Sphingomyelinases (SMs) are a family of hemolytic enzymes that hydrolyze sphingomyelin a sphingolipid highly abundant in the membranes of erythrocytes, nervous tissue, and the lenses of the eye [195-197]. SMs are classified by the site of cleavage, cleaving sphingomyelin either at the lipid backbone connecting to the phosphate headgroup (Sphingomyelinase C) or at the connection between the phosphate headgroup and phosphocholine chain (Sphingomyelinase D) [198, 199] (Figure 2.1). Bacterial SMs are $Mg^{2+}$ dependent enzymes possessing high levels of structural similarity to eukaryotic SMs and DNase I, despite little similarity at the primary sequence level [198, 200-202]. Two conserved histidine residues are responsible for the catalytic activity of SMs, while a conserved glutamate is responsible for coordination of the $Mg^{2+}$ cofactor and several conserved aspartate residues are required for recognition of the sphingomyelin substrate [200, 201]. Bacterial SMs are hemolytic to erythrocytes from a variety of species, exhibiting a phenomenon known as hot-cold hemolysis, where erythrocytes treated with an SM will exhibit little hemolysis when incubated at 37°C but will rapidly lyse when the cells are cooled to 4°C [203-205]. While the precise mechanism by which this phenomenon occurs is not fully understood, it is hypothesized that the binding of $Mg^{2+}$ ions to the plasma membrane is inhibited at lower temperatures, resulting in decreased membrane stability and subsequent lysis following sphingomyelin cleavage [203]. SMs are known to function as crucial virulence factors in a variety of pathogenic bacteria. The skin pathogen *Staphylococcus aureus* produces an SM known as β-toxin [205]. In addition to lysing erythrocytes *in vitro* [203, 205], β-toxin is known to possess cytotoxic activity against monocytes [206], T lymphocytes [205], and polymorphonuclear leukocytes [207], contributing to virulence of *Staphylococcus aureus*

through inhibition of the host immune response. As a result, β-toxin expressing strains of *Staphylococcus aureus* exhibit increased virulence in models of cutaneous [208, 209] and respiratory infections [210, 211]. Similarly, the food pathogen *Bacillus cereus* produces an SM known to increase virulence of this bacterium in mice models [212-214], while expression of SMs in pathogenic *Leptospira* isolates is hypothesized to contribute to the pathogenesis of leptospirosis [202, 215]. Finally, expression of an outer-membrane bound SM in *Mycobacterium tuberculosis* has been linked to improved bacterial growth within macrophages [216], providing another example of the role SMs play in bacterial infection.

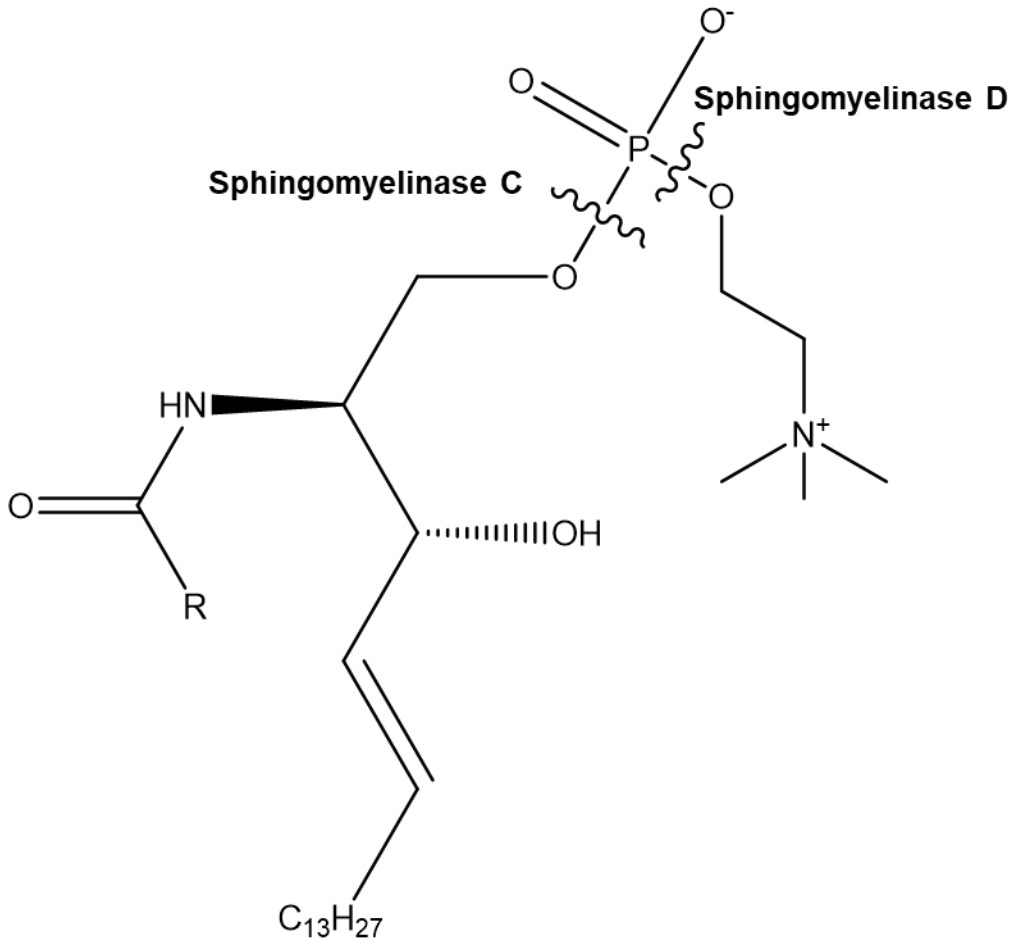


Figure 2.1 Structure of sphingomyelin and sites of bacterial sphingomyelinase cleavage. Figure is adapted from Flores-Diaz *et al.*, [199].

In addition to sphingomyelin, phospholipids are commonly targeted by bacterial enzymes known as phospholipases (PLs). In contrast to SMs, PLs produced by bacteria are highly diverse in both their structures and catalytic mechanisms [199]. PLs can be categorized by their cleavage site on the target phospholipid (Figure 2.2): $PLA_1$ enzymes cleave the fatty acid furthest from the phosphate moiety, $PLA_2$ enzymes cleave the fatty acid adjacent to the phosphate, PLB enzymes cleave both of these fatty acids, PLC enzymes cleave the phosphodiester bond immediately adjacent to the glycerol moiety, while PLD enzymes cleave the phosphodiester bond adjacent to the head group attached to the phosphate moiety [199, 217]. Expression of *pldA1*, an enzyme produced by virulent strains of *Helicobacter pylori*, has been shown to confer hemolytic activity to *Helicobacter pylori* and to improve colonization of the gastric mucosa by altering the lipid composition of gastric epithelial cells [218-220]. The crystal structure of OMPLA, a homologous PLA found in *Escherichia coli*, reveals a dimeric membrane-bound antiparallel β-barrel structure containing a catalytic serine-histidine-asparagine triad along with a coordinated $Ca^{2+}$ ion required for activity [221]. A similar structure is found in PldA from the human gastrointestinal pathogen *Yersinia pseudotuberculosis*, the expression of which has been linked to increased virulence *in vivo* [222]. Conversely, while PlaB from *Legionella pneumophilia* possesses a similar serine-asparagine-histidine catalytic triad, this protein possesses a mixed α/β secondary structure and is inactivated upon oligomerization, a phenomenon hypothesized to serve as a regulatory mechanism of phospholipase activity [223-225]. While *Legionella pneumophilia* is known to possess at least 15 different PL enzymes [217], only PlaB has been definitively shown to contribute to *Legionella* virulence [226-228], as guinea pigs infected with *plaB* knockout

strains of *Legionella pneumophilia* exhibit less lung inflammation and tissue destruction in comparison to wild-type strains [228].

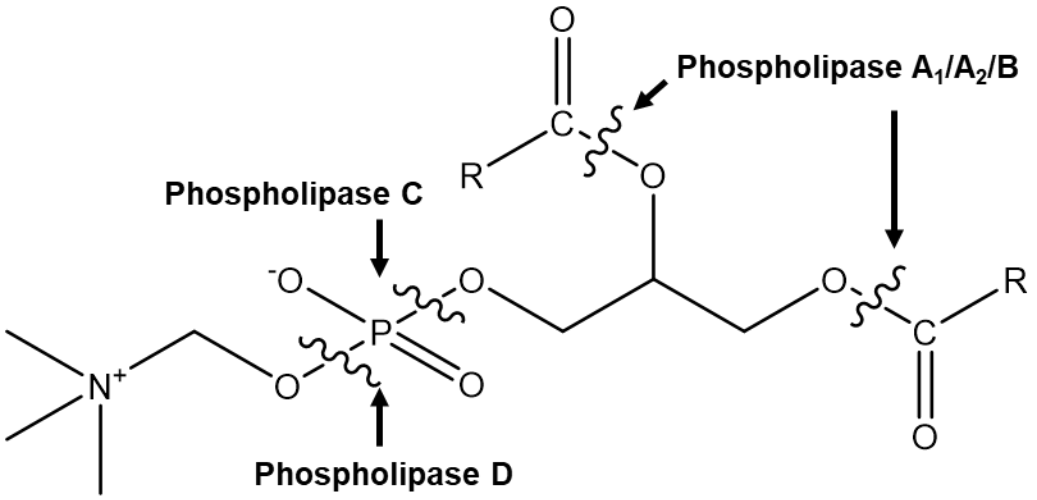


Figure 2.2 Structure of a phospholipid and sites of cleavage by bacterial phospholipases. Figure is adapted from Flores-Diaz *et al.*, [199].

### 2.2.3 Pore-forming toxins

In addition to lysing erythrocytes through the action of surfactants and lipases, certain bacteria produce toxins capable of forming pores within the host cell membrane. These proteins, termed pore-forming toxins (PFTs), represent an ancient family of bacterial toxins with a striking diversity in both structure and function. In this section the structure of bacterial pore-forming toxins will be reviewed, along with the effects of these toxins on host cells.

#### 2.2.3.1 Structure of PFTs

Pore-forming toxins are produced by bacteria as soluble monomers which are then secreted from the cell, bind a receptor on the host-cell membrane, oligomerize, and then insert into the host-cell membrane to form a solvent-accessible pore [229-233]. PFT receptors can be carbohydrates [234], proteins [235-237], or lipids [238, 239], and the PFT-receptor interaction is hypothesized to facilitate PFT oligomerization and pore-formation by increasing the local concentration of PFT monomers on the host-cell surface [157-159, 233]. The number of monomers required to form a pore is highly variable and dependent on the specific PFT in question; *Staphylococcus aureus* α-toxin forms a pore 2.4 nm in diameter comprised of 7 monomers [229], ClyA from *Escherichia coli*, *Salmonella enterica*, and *Shigella flexneri* utilizes 12 monomers to form a pore of 4.0 nm diameter [240], while pneumolysin from *Streptococcus pneumoniae* utilizes 44 monomers to form pores 26 nm in diameter [241]. This structural diversity has led to several classification systems for PFTs, the simplest of which is based on the secondary structure of the transmembrane segment [157-159, 233]. The α-PFTs utilize α-helices to span the membrane, while β-PFTs utilize β-sheets [157-159, 233] (Figure 2.3).

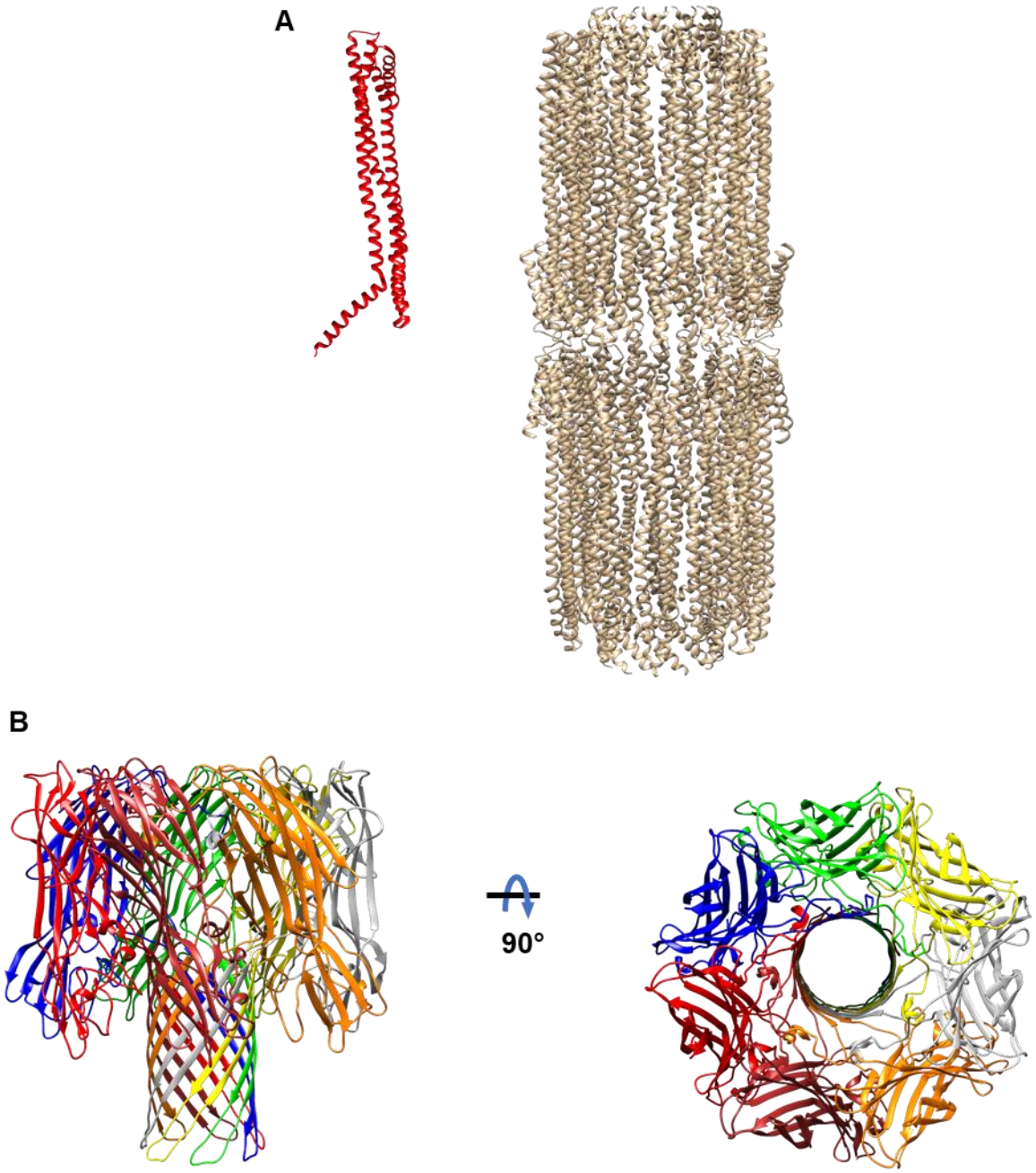


Figure 2.3 Structures of representative α and β bacterial pore-forming toxins *A*, monomeric (red) and oligomeric (tan) forms of ClyA (α-PFT) from *Escherichia coli* (PDB 2WCD) [240]. The mature pore is comprised of two 12-mers arranged end to end. *B*, oligomeric form of alpha toxin (β-PFT) from *Staphylococcus aureus* (PDB 7AHL) [229]. Each subunit of the heptameric pore is coloured independently, and the assembly has been rotated 90° towards the viewer in the second image.

*Coli*cins are a family of α-PFTs produced by *Escherichia coli* that target prokaryotic cells exclusively, functioning to kill competing bacteria [242-244]. *Coli*cin genes are found within plasmid encoded operons containing both the functionally active *coli*cin gene and a lysin gene involved in export of *coli*cin proteins [242, 245]. Exposure to DNA damaging agents triggers expression of both *coli*cin and lysin proteins, with lysin functioning to poke holes within the bacterial cell membrane leading to export of *coli*cin into the extracellular medium [242]. *Coli*cin proteins then bind to specific receptors such as the vitamin B12 transporter *btuB* on the outer membrane of competing bacteria, after which point the toxins are translocated to the inner membrane by either the Tol or TonB protein complexes depending on the *coli*cin in question [246-248]. *Coli*cins then oligomerize and form a voltage dependent nonspecific ion channel within the inner membrane, leading to a loss of membrane potential and subsequent cell death [242, 244, 249]. In addition to *coli*cins, the ClyA/HlyE toxins from *Escherichia coli*, *Salmonella enterica*, and *Shigella flexneri* are known to function as α-PFTs [240, 250, 251]. While ClyA lacks a known signal sequence for secretion, it has been demonstrated that outer-membrane vesicles (OMVs) from *Escherichia coli* and *Salmonella enterica* contain hemolytically active ClyA oligomers, representing an unconventional mechanism for PFT secretion [252-254]. Additionally, incubation of soluble monomeric ClyA with *n*-dodecyl-β-D-maltopyranoside triggers the spontaneous oligomerization of ClyA into 12-mers visible by electron microscopy [255]. Oligomerization and pore-formation by ClyA involves an initial membrane binding step to an unknown receptor [240, 251-253]. Membrane binding is facilitated by a so-called β-tongue region at the end of the ClyA monomer, and following this initial binding step a β-sheet to α-helix transition occurs in this β-tongue region, switching the ClyA monomer from a membrane parallel position to one perpendicular to the host cell membrane [240, 255]. This transition is

triggered by the movement of a phenylalanine residue (F190) away from a cluster of aromatic residues (F50, Y54, F159, and Y165) responsible for stabilizing the β-tongue region [240]. The rearrangement of proline 36 then results in the breakage of one α-helix (αA) into two α-helices (αA1/A2), the former of which swings out 90° to associate with the host cell membrane and binds to the same α-helix on another ClyA monomer, triggering the insertion of the ClyA pore into the membrane [240]. Remarkably, over half of the amino acid residues of ClyA are affected by these structural transitions during the processes of oligomerization and pore-formation [240], illustrating the intricacies inherent in the action of PFTs.

The *Staphylococcus aureus* α-toxin is a prototypical β-PFT with highly potent activity towards rabbit erythrocytes [229, 231, 235, 256-258]. While α-toxin possesses a canonical signal sequence for secretion [259], recent evidence has indicated that delivery of α-toxin to the host cell can also be accomplished through the use of membrane vesicles in a manner similar to that of ClyA [260]. High concentrations of α-toxin are capable of forming pores in artificial lipid membranes [261-263], however, the fact that rabbit erythrocytes are ~1000 times more susceptible to α-toxin than human erythrocytes lead researchers to suspect that a high-affinity receptor for α-toxin exists [257, 264]. This receptor was later determined to be disintegrin and metalloprotease 10 (ADAM10), a zinc metalloprotease highly expressed on the surface of rabbit erythrocytes but absent from human erythrocytes [236, 265]. In contrast to ClyA, oligomerization and pore-formation by α-toxin does not involve dramatic structural alterations. Binding of the α-toxin monomer to the host cell surface enables oligomerization into a heptameric pre-pore complex through hydrogen bonding and salt-bridge formation by specific residues within the N-terminus of the protein (D24, H35, K37, H48, K58, and D100) [229, 266-268]. A stem-domain comprised of two anti-parallel β-sheets from each α-toxin protomer then

inserts into the host-cell membrane to form a solvent-accessible pore 2.4 nm in diameter [229].

Similarly, cholesterol dependent cytolysins (CDCs) are a family of β-PFTs primarily produced by Gram positive bacteria [230, 269, 270], although examples from Gram negative bacteria have been identified [271]. As with α-toxin, CDCs possess typical signal sequences and are secreted from the host bacterium by conventional secretion pathways [270, 272]. As suggested by the name, CDCs target cholesterol rich regions of host cell membranes for binding and pore-formation, leading early researchers to conclude that cholesterol serves as the cellular receptor for these toxins [230, 269, 270, 273]. Strengthening this hypothesis is the fact that pre-treating CDCs with cholesterol inhibits the hemolytic activity of these toxins [273-275], however, LLO from *Listeria monocytogenes* maintains membrane binding capability and is able to activate inflammatory cytokine expression in infected cells even after cholesterol treatment [275, 276], suggesting that other cellular receptors may also play a role. Recent studies have since determined that all known CDCs can bind various glycan moieties with similar affinity to cholesterol, providing an explanation for the host cell tropism exhibited by many of these toxins [277, 278]. CDCs are comprised of four distinct protein domains, termed D1-D4 [279, 280]. The C-terminal D4 domain recognizes and binds cholesterol through a universally conserved threonine-leucine sequence, and mutation of either amino acid dramatically inhibits cholesterol binding and hemolytic activity of CDCs [281, 282]. Cholesterol binding by this TL motif induces the insertion of two loops (L2 and L3) and a highly conserved undecapeptide sequence (ECTGLAWEWWR) into the host-cell membrane, leading to structural alterations within the CDC monomer which serve to dissociate D3 from D2 [241, 283-285]. After these binding and initial insertion steps, oligomerization of CDC monomers into a pre-pore complex comprised of 30-50 monomers occurs [241, 280, 286]. Structural studies involving pneumolysin from

*Streptococcus pneumoniae* have revealed that the monomer-monomer interface is comprised of 85 amino acids; while hydrogen bonding and salt-bridge formation plays a crucial role in the stabilization of this interface, it is believed that the exclusion of water from the interface due to the complementarity of the intermolecular surfaces is the primary contributor to the stability of the monomer-monomer interaction [286]. Following pre-pore assembly D1 and D2 completely dissociate from D3, this dissociation then triggers the conformational change of two α-helices found within D3 (termed TMH1 and TMH2) to β-sheets which then insert into the host-cell membrane to form the CDC pore [241, 286]. Remarkably, this complex mechanism is conserved across CDCs from a wide variety of bacterial species and shares similarities to the membrane attack complex perforin utilized within the immune system [287], suggesting an ancient evolutionary origin for these toxins.

#### 2.2.3.2 Host cell responses to pore-forming toxins

Given the widespread diversity present in PFT structures, it is not surprising to learn that these toxins contribute to bacterial virulence through a multitude of effects on host cells. While the hemolytic activities possessed by many PFTs provide an easy mechanism by which the biological functions of these toxins can be evaluated, it is important to recognize that the lysis of erythrocytes is rarely the primary *in vivo* function of PFTs [288]. Listeriolysin O (LLO), a CDC produced by *Listeria monocytogenes*, provides what is perhaps the most direct example of PFT contribution to bacterial virulence by forming pores large enough to enable this bacterium to translocate across host cell membranes [289]. In contrast to other CDCs, LLO is inactive at pH 7.0 and maximally active under acidic conditions [290]. This pH dependency, combined with the ability of LLO to form pores within phagosomal membranes [291] enables *Listeria monocytogenes* to escape from macrophage phagosomes and replicate cytosolically [292]. *Listeria monocytogenes* mutants deficient in LLO production are consequently incapable of

phagosomal escape and are less virulent in mice as a result [292-295]. In contrast to these dramatic effects of LLO, the uncontrolled movement of ions through channels formed by PFTs often plays a crucial role in bacterial pathogenesis [159, 288]. *Vibrio parahaemolyticus*, a causative agent of gastroenteritis often found in improperly prepared seafood, produces a hemolysin termed thermostable direct hemolysin (TDH) capable of forming pores in both epithelial cells and artificial lipid bilayers [296-299]. TDH pores function as cation selective ion channels and directly contribute to the development of diarrhea during *Vibrio parahaemolyticus* infection by increasing intracellular $Ca^{2+}$ fluxes, leading to an increase in apical $Cl^-$ secretion through the stimulation of calcium activated chloride channels [300-302]. Along with this direct effect of TDH on $Cl^-$ secretion, a wealth of evidence has emerged indicating that pore formation has dramatic effects on the activation and inhibition of various host cell signaling pathways. The first evidence for the direct modulation of host cell signalling by PFTs was the observation that the treatment of respiratory epithelial cells with sublytic concentrations of pneumolysin, streptolysin O, anthrolysin O, or *Staphylococcus aureus* α-toxin results in high levels of p38 MAPK phosphorylation within 30 minutes [303]. Later studies revealed that this activation is a result of intracellular $K^+$ leakage through PFT pores, as evidenced by the fact that replicating these experiments in high extracellular $K^+$ concentrations inhibited p38 phosphorylation by eliminating the chemical gradient for $K^+$ leakage from intoxicated cells [304]. PFT mediated activation of p38 MAPK has since been demonstrated with VCC from *Vibrio cholerae* [304], HlyA from *Escherichia coli* [304], Cry1AB, Cry11Aa, and Cry5B from *Bacillus thuringiensis* [305, 306], βh/c from *Streptococcus agalactiae* [307], α-toxin from *Clostridium septicum* [308], and β-toxin from *Clostridium perfringens* [309], suggesting that p38 MAPK phosphorylation is a universal response to pore-formation. Activation of p38 MAPK has been shown to play a crucial

role in the host immune response to bacterial infection by inducing the transcription of inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α, and the chemical inhibition of p38 MAPK signaling increases the severity of *Streptococcus pneumoniae* infection [310-312]. Furthermore, PFT mediated p38 MAPK activation activates the unfolded protein response (UPR) [313], a cellular stress response pathway capable of promoting cell survival or apoptosis in response to alterations in endoplasmic reticulum function [314]. *Caenorhabditis elegans* worms deficient in the UPR activators *xbp-1* or *ire-1* were between 6 and 18 fold more sensitive to the *Bacillus thuringiensis* toxin Cry5B, indicating that activation of the UPR by p38 MAPK is a critical protective mechanism for eukaryotic cells against PFT intoxication [313]. However, deleterious effects of PFT mediated p38 MAPK activation have also been observed, with neuronal cells undergoing p38 MAPK mediated apoptosis in response to pneumolysin intoxication [315].

### 2.3 Hemolysis in *Brachyspira* species

Given that pathogenic isolates of *Brachyspira hyodysenteriae/hampsonii/suanatina* are strongly β-hemolytic in contrast to weakly β-hemolytic avirulent isolates [3, 5, 7, 8, 79, 81, 316] it is hypothesized that *Brachyspira* virulence is dependent on the production of one or more hemolysins. At the time of writing 4 genes coding for hemolysins have been identified in *Brachyspira hyodysenteriae*, while an additional 4 putative hemolysin genes and 4 putative phospholipase genes have been predicted by genomic analyses [317]. This section will discuss our current understanding of these toxins both in the context of swine dysentery and in diseases caused by unrelated bacteria.

### 2.3.1 The native hemolysin of *Brachyspira hyodysenteriae/hampsonii*

Efforts to understand the mechanisms of *Brachyspira* hemolysis began soon after the development of methods to culture this bacterium *in vitro*. The first attempt at characterizing a hemolysin from *Brachyspira hyodysenteriae* occurred in 1980, with the authors of this study reporting the purification of a hemolytic molecule from broth cultures of *Brachyspira hyodysenteriae* supplemented with yeast RNA [318]. This hemolysin, referred to as native hemolysin in this thesis to differentiate it from other named hemolysins in *Brachyspira* spp., was oxygen stable and was inactivated by pronase, lipases, and heat treatment, leading the authors to conclude it was a protein associated with lipids [318]. Attempts to determine the molecular weight of this protein were complicated by the fact that it migrated along with the tracking dye during SDS-PAGE analysis, however size exclusion chromatography indicated a molecular weight of approximately 78 kDa [318]. A similar experiment by a different group the next year also identified a hemolytic molecule sensitive to pronase treatment, however the molecular weight of this hemolysin was reported to be ~68 kDa based on SDS-PAGE [319]. Interestingly, expression of this hemolysin in *Brachyspira hyodysenteriae* broth cultures increased dramatically upon addition of yeast RNA core, a fraction of yeast RNA resistant to treatment with ribonucleases [320], to the culture media. Addition of yeast RNA core has been shown to improve the production of streptolysin S, an oxygen stable hemolysin produced by *Streptococcus pyogenes* [320, 321], suggesting that this hemolysin from *Brachyspira hyodysenteriae* may be related to streptolysin S. A later study found a hemolysin with similar properties to the two described earlier, with the authors of this study reporting a molecular weight of 19 kDa based on native PAGE analysis [322]. Notably, a band of ~66 kDa was observed during SDS-PAGE analysis of impure hemolysin preparations in this study [322], indicating that the band of ~68 kDa identified by the authors of the previous study may have in fact been an impurity [319].

While the common purification methods and the fact that all toxins identified were susceptible to pronase treatment suggests that these groups all identified the same toxin, the wide discrepancies in reported molecular weights of the toxin highlighted the need to definitively link a gene sequence in *Brachyspira hyodysenteriae* to hemolytic activity.

### 2.3.2 TlyA

#### 2.3.2.1 TlyA proteins in *Brachyspira*

The first attempts to link the β-hemolytic phenotype of *Brachyspira hyodysenteriae* to a specific gene were carried out in the early 1990s. A gene library of a pathogenic, strongly β-hemolytic *Brachyspira hyodysenteriae* strain was transformed into *E. coli* and these transformants were then plated on blood agar, leading to the identification of one putative hemolysin gene in an initial experiment [323]. This gene, termed *tly* and later renamed to *tlyA*, codes for a 26.9 kDa protein and was reported to be absent in the weakly β-hemolytic, avirulent *Brachyspira innocens* (genomic sequencing later revealed that *tlyA* is in fact present in *Brachyspira innocens*) [323]. Experiments involving osmotic shock supernatants from *E. coli* carrying the *tlyA* gene indicated that this protein was cytotoxic to a variety of cell lines and maintained its activity after treatment with EDTA or a variety of protease inhibitors [323, 324]. A *tlyA* knockout strain of *Brachyspira hyodysenteriae* was less hemolytic on blood agar than the wild-type strain and exhibited reduced virulence in both mice and pigs [325, 326], suggesting that the TlyA protein is a crucial virulence factor in the pathogenesis of swine dysentery. However, the observation that after several passages the *tlyA* knockout *Brachyspira hyodysenteriae* strain reverted to a strong β-hemolytic phenotype raised doubts about the knockout efficiency of this strain and the overall significance of the *tlyA* gene in swine dysentery [323]. Further confounding these results was the later observation that heterologous expression of foreign genes in *E. coli* via plasmid can induce

expression of a so-called “silent hemolysin” [327], raising doubt on the true functionality of the *tlyA* gene.

#### 2.3.2.2 TlyA proteins in other bacteria

Despite first being identified in *Brachyspira hyodysenteriae*, the majority of research into the functionality of TlyA proteins has been carried out on TlyA homologues found in unrelated bacteria. The first such homologue to be identified was in *Mycobacterium tuberculosis*, the causative agent of *tuberculosis*, with the same study also finding *tlyA* genes in *Mycobacterium leprae*, *Mycobacterium avium,* and *Mycobacterium bovis* strain BCG [15]. Similar to *Brachyspira hyodysenteriae* TlyA, the authors of this study demonstrated contact dependent hemolytic activity in both *Mycobacterium smegmatis* transformed with the *tlyA* gene and in cell lysates from *E. coli* expressing an N-terminal His-tagged variant of the *tlyA* gene [15]. Later studies showed that purified TlyA from *Mycobacterium tuberculosis* possessed hemolytic activity against rabbit erythrocytes *in vitro* [18], confirming that this hemolytic activity was intrinsic to TlyA and not a result of silent hemolysin expression in *E. coli*. The authors of this study reported that the hemolytic activity of TlyA was very slow (~18-24 hour incubation period required for hemolysis), dose dependent, and could by inhibited by the addition of reducing agents [18]. Additionally, higher order oligomers of TlyA formed on both erythrocyte and phagosome membranes and were resistant to both heat and SDS treatment but degraded in the presence of reducing agents [18]. A TlyA homologue was subsequently identified in the gastric pathogen *Helicobacter pylori* and was demonstrated to improve adhesion of this bacterium to the gastric epithelial surface, with TlyA knockouts being unable to colonize mice during experimental infection [16].

Similar to TlyA from *Mycobacterium tuberculosis*, *Helicobacter pylori* TlyA possessed slow dose dependent hemolytic activity against human erythrocytes when heterologously expressed in *E. coli* and purified as a His-tagged variant [328]. Experiments with osmoprotectants suggested that *Helicobacter pylori* TlyA exhibited its hemolytic effect through the formation of a pore <1.0 nm in diameter [328]. In addition to its activity against erythrocytes, *Helicobacter pylori* TlyA was shown to be cytotoxic to a human gastric adenocarcinoma cell line, providing a potential mechanism by which TlyA expression increases *Helicobacter pylori* virulence [328]. *Helicobacter pylori* TlyA is also capable of agglutinating liposomes comprised of asolectin and cholesterol, suggesting that TlyA may possess broad host cell selectivity [329].

Along with their toxic effects against a variety of cell types *in vitro*, TlyA proteins have been demonstrated to directly contribute to bacterial virulence *in vivo*. Expression of *Mycobacterium tuberculosis* TlyA in the nonpathogenic *Mycobacterium smegmatis* improved the ability of this bacterium to colonize both the lungs and spleen of mice [330]. Immunization of mice with TlyA and Freund's incomplete adjuvant significantly reduced colonization of these organs by bacteria expressing TlyA, further confirming that TlyA is a crucial virulence factor in Mycobacterial pathogenesis [330]. The authors of this study attributed this increase in virulence to the ability of TlyA to inhibit phagolysosome acidification, a crucial step in the pathogenesis of *Mycobacterium tuberculosis* that enables this bacterium to survive in infected macrophages [330]. Similarly, a TlyA knockout strain of *Mycobacterium tuberculosis* was unable to efficiently colonize the lungs and spleen of infected mice, resulting in fewer lung granulomas in animals infected with the knockout strain versus wild-type *Mycobacterium tuberculosis* [331]. In contrast to the impairment of phagolysosome acidification implicated in the previous study, the authors of this study attributed this loss of virulence to a modulation of the host immune response by TlyA.

Animals challenged with the TlyA knockout strain had increased Th1 and Th17 cell counts in comparison to those challenged with the wild-type strain, along with decreased Th2 and Treg cell counts [331]. The modulation of the host immune response by *Mycobacterium tuberculosis* is a crucial aspect of *tuberculosis* pathology and it has been previously demonstrated that upregulation of Th2 and Treg cells by wild-type *Mycobacterium tuberculosis* inhibits the Th1 response, inhibiting the adaptive immune response against *Mycobacterium tuberculosis* infection [332, 333]. Additionally, macrophage autophagy, a critical mechanism for antigen presentation during *Mycobacterium tuberculosis* infection [334, 335], was impaired when macrophages were infected with wild-type *Mycobacterium tuberculosis* in comparison to a TlyA deficient strain, representing another mechanism by which TlyA modulates the host immune response during *tuberculosis* [331].

In addition to their roles as hemolysins, TlyA proteins also possess a secondary function as rRNA methyltransferases. Early studies showed that mutation of the *tlyA* gene in *Mycobacterium tuberculosis* caused resistance to capreomycin, a cyclic peptide antibiotic used for the treatment of drug resistant *tuberculosis* infections [336], with this phenomenon being linked to a previously unknown rRNA methyltransferase function of TlyA [337]. Specifically, TlyA from *Mycobacterium tuberculosis* is capable of methylating nucleotides C1409 of 16s rRNA and C1920 of 23s rRNA at the 2'O position utilizing S-adenosyl-l-methionine (SAMe) as a cofactor, and both of these methylations are required for capreomycin to tightly bind the ribosome and inhibit protein translation [337, 338]. As a result, mutations to the 16s rRNA gene [339-341] or the *tlyA* gene [336-338, 342-344] have been linked to capreomycin resistance in *Mycobacterium tuberculosis*. Later comparative studies involving TlyA proteins from a variety of bacteria identified two distinct families of TlyA homologues, classified by the authors based on their

rRNA methylation patterns [338]. TlyA$^{I}$ proteins are only capable of methylating C1920 in 23s rRNA, while TlyA$^{II}$ proteins methylate both positions and possess extra amino acids at their N and C termini in relation to the shorter TlyA$^{I}$ proteins [338]. Despite the well documented role TlyA mediated rRNA methylation plays in antibiotic resistance, the biological implications of these methylations are not well understood. Recent evidence has suggested that rRNA methylation by a TlyA$^{I}$ family protein in *Campylobacter jejuni* has important effects on bacterial physiology and virulence, as mutation of residues required for rRNA methylation by TlyA resulted in decreased motility and biofilm formation in affected bacteria [345]. Interestingly, expression of *Mycobacterium smegmatis* TlyA (a TlyA$^{II}$ protein) in *Campylobacter jejuni* improved adhesion and invasion of Caco-2 cells in comparison to wild-type bacteria expressing a TlyA$^{I}$ family protein, in addition to dramatically increasing IL-8 expression in infected cells [346]. These observations indicate that in addition to contributing to bacterial virulence through their function as pore-forming hemolysins, the rRNA methyltransferase functionality of TlyA proteins also plays an important role in bacterial infection, likely contributing to virulence by increasing the efficiency of protein synthesis in bacteria expressing these proteins.

While our understanding of the functionality of TlyA proteins has improved immensely in the past decade, the difficulty inherent in crystallization of these proteins has meant that less is known about the structure of TlyA proteins. At the time of writing the only solved full length TlyA structure is that of *Streptococcus thermophilus*, annotated by the authors as a putative hemolysin [347]. This structure revealed two distinct protein domains in TlyA, comprised of an N-terminal S4 domain and a C-terminal Rossmann methyltransferase fold connected by a flexible linker region susceptible to proteolytic attack [18, 347, 348]. The S4 domain is homologous to ribosomal protein S4, a protein responsible for binding to 16s rRNA during

assembly of the ribosome [349-351]. Previous studies have reported the S4 domain to be highly flexible in solution [349-351], with this flexibility rendering S4 domain containing proteins difficult or impossible to crystallize. One research group reported that they were unable to crystallize full length TlyA protein from *Mycobacterium tuberculosis* due to this flexibility, only obtaining crystals suitable for x-ray diffraction after proteolytically removing the N-terminal region of this protein [352]. While the function of the S4 domain in TlyA has not yet been determined, it is hypothesized that this domain is responsible for binding the rRNA substrate methylated by TlyA proteins and that the flexibility inherent in the S4 domain may enable TlyA$^{II}$ proteins to discriminate between their 16s and 23s rRNA substrates [352]. In contrast to the S4 domain, the Rossmann methyltransferase fold of TlyA is a well-defined domain found in a wide variety of proteins utilizing dinucleotide cofactors [353-355]. While a putative SAMe binding motif is present within the Rossman methyltransferase fold of *Mycobacterium tuberculosis* TlyA, the isolated C-terminal region of TlyA containing this motif is incapable of binding SAMe alone [352]. Instead, a tetrapeptide motif (RAWV) present in the flexible linker region of *Mycobacterium tuberculosis* is required, but not sufficient for SAMe binding, and the structural plasticity within this region appears to play a crucial role in the recognition of rRNA substrates [352]. Paradoxically, the dual rRNA methyltransferase and hemolysin functionalities of TlyA imply that this protein must be present both within the bacterial cytoplasm as well as secreted into the extracellular space in order to make contact with the host cell membrane. Complicating this matter is the fact that TlyA proteins do not contain any putative signal sequences for extracellular secretion [15, 16, 323, 348, 356]. Despite this, expression of *Mycobacterium tuberculosis* TlyA in both *Mycobacterium smegmatis* and *Escherichia coli* results in the localization of this protein to the cellular membrane, with this localization still occurring in

bacteria deficient in three major secretion pathways [356]. Additionally, hemolytically active TlyA was present in outer membrane vesicles derived from *Escherichia coli* expressing TlyA and membrane vesicles derived from *Mycobacterium smegmatis* expressing TlyA [356]. These data suggest that TlyA secretion may thus be achieved using outer membrane vesicles in a similar manner to ClyA from *Escherichia coli* [252, 253]. Given the wealth of data on the contribution of TlyA proteins to the pathogenesis of bacteria such as *Mycobacterium tuberculosis* and *Campylobacter jejuni*, it therefore stands to reason that expression of TlyA in *Brachyspira hampsonii* and *Brachyspira hyodysenteriae* may play a crucial role in the pathogenesis of swine dysentery, and in-depth studies on the functionality of this protein are required to fully understand these contributions.

### 2.3.3 TlyB/TlyC

Several years after initially identifying the *tlyA* gene in *Brachyspira hyodysenteriae* the group responsible for this discovery repeated the same experiment. In contrast to the initial experiment, the authors reported the discovery of two more genes capable of conferring a hemolytic phenotype to *E. coli* which they termed *tlyB* and *tlyC* [13]. Southern blotting revealed that both the *tlyB* and *tlyC* genes were present in all seven *Brachyspira hyodysenteriae* strains tested by the authors, with the presence of weakly reacting bands in *Brachyspira innocens* genomic DNA indicating that this bacterium possessed a homologous gene [13]. The *tlyB* gene encodes a 93.3 kDa protein homologous to Clp proteases, a diverse group of proteolytic enzymes found in both prokaryotic and eukaryotic cells [357, 358], while the *tlyC* gene encodes a 30.8 kDa protein with no homology to any other known proteins [13]. However, in contrast to these initial results obtained with *E. coli* expressing the *tlyB* and *tlyC* genes on a plasmid, a later study involving *tlyB* and *tlyC* cloned from the pathogenic spirochete *Leptospira interrogans* concluded that neither of these proteins possessed a hemolytic function when expressed and purified from an *E.*

*coli* host [359]. While the authors of this study did not ascribe a function to TlyB, they concluded that TlyC functioned in extracellular matrix binding, as the purified protein was capable of binding to laminin, collagen IV, and plasma fibronectin at nanomolar concentrations [359]. While these results were limited by the fact that a truncated version of the TlyC protein lacking a predicted transmembrane region was used [359], we have observed that full length TlyC protein from *Brachyspira hampsonii* does not possess hemolytic activity *in vitro* (Data not shown). Taken together, these observations indicate that TlyB and TlyC are not hemolytic in *Brachyspira*, and likely are not important virulence factors in the pathogenesis of swine dysentery.

### 2.3.4 HlyA

In addition to *tlyA*, *tlyB*, and *tlyC*, a fourth putative hemolysin gene termed *hlyA* has been identified in *Brachyspira hyodysenteriae* and *Brachyspira hampsonii*. In contrast to the gene library approach utilized for the identification of *tlyA*, *tlyB*, and *tlyC*, the authors of the study identifying *hlyA* attempted to directly link the amino acid sequence of the *Brachyspira hyodysenteriae* native hemolysin to a gene in *Brachyspira hyodysenteriae* utilizing N-terminal sequencing [14]. A degenerate oligonucleotide primer was synthesized based on a 13 amino acid sequence derived from purified *Brachyspira hyodysenteriae* native hemolysin via N-terminal sequencing. Hybridization of this primer to *Brachyspira hyodysenteriae* genomic DNA resulted in the identification of a gene coding for an 8.93 kDa peptide, termed *hlyA* by the authors [14]. Similarly to the *tlyA* gene, the authors reported that the *hlyA* gene was not present in the nonpathogenic *Brachyspira murdochii* and *Brachyspira innocens* [14], however genomic sequencing later revealed that these bacteria do in fact possess the *hlyA* gene. Interestingly, capillary electrophoresis of this peptide revealed an estimated molecular weight of 19 to 21 kDa in comparison to the calculated 8.93 kDa, similar to the 19 kDa estimate of the mass of

*Brachyspira hyodysenteriae* native hemolysin obtained by native PAGE [322]. Analysis of the *hlyA* coding sequence revealed the presence of 7 amino acids on the N-terminus of the HlyA protein that were not detected by N-terminal sequencing, leading the authors to hypothesize that this sequence represented a non-canonical signal peptide that was proteolytically cleaved during export of this peptide to the extracellular space [14]. While no homology between HlyA and the TlyA/B/C proteins was observed, a phosphopantetheine binding motif homologous to acyl carrier proteins was present [360], suggesting that post-translational modification of this peptide may be required for activity [14]. While the authors of this study reported that *E. coli* carrying the *hlyA* gene on a plasmid exhibited a β-hemolytic phenotype and that a hemolytic molecule could be obtained after sonication and lysozyme treatment of the bacteria [14], studies in our laboratory with HlyA protein expressed and purified from an *E. coli* host found that HlyA alone was not hemolytic (Data not shown). One possible explanation for this discrepancy is the fact that the cloned insert containing the *hlyA* gene also carried two additional genes flanking the hlyA gene annotated as an ACP reductase and an ACP synthase [14]. Post-translational acylation is required for activation of *Escherichia coli* alpha-hemolysin [361, 362], indicating that similar activation by these flanking enzymes may be a perquisite for activation of HlyA proteins from *Brachyspira* species. Regardless, no further studies on the structure or function of HlyA from *Brachyspira* species have been carried out since the initial identification of this peptide 20 years and its overall contribution to the pathogenesis swine dysentery remains unknown.

### 2.3.5 Streptolysin S (SLS)

Despite the identification of the hlyA gene in *Brachyspira hyodysenteriae*, questions on the exact nature of the native hemolysin of *Brachyspira hyodysenteriae* and the contributions of the *tlyA*, *tlyB*, *tlyC*, and *hlyA* genes to the virulence of *Brachyspira hyodysenteriae* and *Brachyspira hampsonii* remain. Further confounding these questions is the recent discovery of a gene cluster

coding for a peptide homologous to streptolysin S (SLS) in *Brachyspira hampsonii* and an apparent link between the presence of this gene cluster and virulence [20]. This section will discuss the structures and functions of SLS and related peptides from a variety of pathogenic bacteria, in addition to the recent discovery of an SLS like peptide in *Brachyspira hampsonii* and the broader implications of this discovery on our understanding of the pathogenesis of swine dysentery.

#### 2.3.5.1 Identification and purification of SLS

*Streptococcus pyogenes*, AKA group A *Streptococcus* (GAS), is a strongly β-hemolytic bacterium capable of causing life-threatening infections in humans [363]. Two distinct hemolysins, termed streptolysin O (SLO) and streptolysin S (SLS), are responsible for this β-hemolytic phenotype in *Streptococcus pyogenes* [19]. These toxins were initially differentiated based on their oxygen stability and antigenicity – SLO is quickly inactivated by atmospheric oxygen and is highly antigenic, while SLS is stable in oxygen and non-antigenic [19]. While SLO has been well-characterized as a member of the cholesterol-dependent cytolysin family [158, 230], biochemical characterization of SLS was hampered by the idiosyncrasies inherent in the purification of this toxin [364]. While SLS was initially purified from *Streptococcus pyogenes* cultures grown in the presence of horse serum [19], later researchers found that addition of sodium ribonucleate to cultures resulted in improved yields of SLS [320, 365]. The degree of SLS induction by sodium ribonucleate is dependent on the species from which the RNA is derived, with one study finding beef liver RNA to be the most efficient followed by wheat germ and yeast, while tobacco mosaic virus RNA was incapable of inducing SLS formation [320]. Interestingly, yeast RNA digested with bovine pancreatic ribonuclease was approximately ten times more effective at stimulating SLS formation than intact RNA from any species studied, while ethanol precipitation of this digested RNA and resuspension of the

precipitate yielded a so-called "active fraction" capable of increasing SLS formation over 100 fold in comparison to untreated RNA [320]. While serum albumin and detergents such as Tween or Triton are also capable of inducing SLS activity in *Streptococcus pyogenes* cultures [366, 367], the aforementioned nuclease resistant RNA fraction, termed RNA core by later researchers, is the most effective inducer of SLS production and has subsequently become the standardized reagent for production and purification of SLS and related toxins [320, 321, 365, 368, 369]. While removal of yeast RNA core from SLS preparations by isoelectric focusing does not affect the hemolytic activity of SLS, the free peptide degrades rapidly, suggesting that the primary function of yeast RNA core and other inducers is stabilization of the SLS peptide in solution [370]. Strengthening this hypothesis is the fact that lipoteichoic acid, a major constituent of Gram positive bacterial cell membranes, is also a potent inducer of SLS activity [371]. As such, the majority of SLS produced by *Streptococcus pyogenes* is likely bound to lipoteichoic acid on the bacterial membrane until the bacterium reaches the host cell membrane where SLS can exert its effect [364, 371], and the addition of yeast RNA core to broth cultures of *Streptococcus pyogenes* serves to liberate SLS from the bacterial membrane, enabling purification of the toxin from cell-free supernatants [320, 321, 365, 368, 370].

#### 2.3.5.2 Lytic activity of SLS

Despite over 8 decades of study, the precise mechanism by which SLS lyses erythrocytes remains elusive. In addition to its potent hemolytic activity against erythrocytes [19, 320, 365, 366, 368], SLS also exhibits lytic activity against platelets [372], lysosomes [373, 374], and bacterial spheroplasts [375], suggesting that the toxin may be active against any lipid membrane. While the observation that treatment of phospholipid liposomes with SLS resulted in the leakage of $Na^+$ and $K^+$ ions lead early researchers to hypothesize that SLS was a phospholipase [376], later studies found SLS to be incapable of cleaving either phosphatidylcholine or

phosphatidylethanolamine *in vitro* [377]. While pores large enough to be visible by electron microscopy are not observed on the membranes of cells intoxicated with SLS [378], experiments with osmoprotectant molecules have suggested that SLS may form small pores in the host cell membrane, causing leakage of intracellular contents and subsequent cell lysis through colloid osmotic processes [379-381]. The size of SLS pores in membranes appears to be heterogenous, with one group reporting pores between 8.8 and 54.2 Å in diameter in erythrocyte ghosts [380], while another group reported sizes between 2.8 and 7.2 Å in diameter in intact erythrocytes [381]. Additionally, incubation of SLS with erythrocytes for > 5 minutes renders SLS partially resistant to inactivation by papain, suggesting that SLS forms pores in the erythrocyte membrane that are inaccessible to proteases [381]. However, analysis of the SLS amino acid sequence indicates that the mature SLS peptide does not possess a papain cleavage site [382], indicating that papain treatment may in fact cleave a host-cell receptor for SLS rather than SLS itself. Furthermore, while SLS is capable of binding erythrocyte membranes at temperatures above 10°C the majority of SLS in these reaction mixtures remained in solution even at 37°C, indicating that the interaction between SLS and erythrocyte membranes is relatively weak and reversible [383]. Later experiments revealed that intoxication of erythrocytes with SLS results in a dramatic influx of $Cl^-$ ions into treated cells, leading the authors to hypothesize that SLS can alter cellar anion channel function [384]. The same study also found that the hemolytic activity of SLS could by inhibited by both the addition of the anion-exchange inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulphonate (DIDS) to SLS reaction mixtures or blockage of the $Cl^-/HCO_3^-$ exchanger band 3 with a polyclonal antibody [384]. Furthermore, clinical symptoms of *Streptococcus pyogenes* infection were dramatically reduced in mice treated with DIDS, indicating that the alteration of anion exchanger function is a critical part of *Streptococcus*

*pyogenes* pathology [384]. These results indicate that rather than forming a pore in host-cell membranes, SLS may in fact function by activating the band 3 anion exchanger, leading to a massive influx of $Cl^-$ secretion into the cell and resulting in hemolysis through the osmotic movement of water. However, these results cannot explain the fact that SLS possesses activity against phospholipid liposomes lacking transmembrane ion channels such as band 3 [376, 377, 380], and as such it remains an open question as to whether SLS does in fact possess a pore-forming functionality.

#### 2.3.5.3 Genetics of SLS and homologous toxins

In contrast to these investigations of the biochemical properties of SLS carried out from the 1930s to the 1980s, the gene or genes responsible for SLS production remained unknown until the widespread adoption of genomic sequencing technology in the 1990s. A genomic region responsible for SLS production was first identified by comparative genomic studies involving nonhemolytic *Streptococcus pyogenes* mutants in 1998 [385]. While these studies identified a single gene responsible for SLS production termed *sagA* [385], later studies found that this gene was part of an operon consisting of 9 genes, termed *sagA-sagI* [386]. The *sagA* gene encodes for a 53 amino acid peptide similar to microcin B17 (MccB17) from *E. coli*, the first described member of a family of bacterial peptides now known as thiazole/oxazole-modified microcins (TOMMs) [386-388]. MccB17 is a potent antimicrobial agent, capable of inducing double stranded DNA breaks in infected cells at low micromolar concentrations by modifying the activity of DNA gyrase [389, 390]. MccB17 requires extensive post-translational modification for activity, with serine and cysteine residues in the C-terminal region of the MccB17 precursor being chemically modified to oxazole and thiazole rings [391, 392] (Figure 2.4). After these modifications occur the peptide is cleaved at a glycine-glycine repeat yielding a 26 amino acid N-terminal leader sequence and the 43 amino acid core MccB17 peptide [387, 393]. Like

MccB17, *sagA* requires post-translational modification of serine, cysteine, and threonine residues to form functional SLS [364]. Modification of the serine, cysteine, and threonine residues of SagA is performed by a complex comprised of the SagBCD proteins, with SagC functioning as a cyclodehydratase, SagB functioning as a flavin mononucleotide (FMN) dependent dehydrogenase, and SagD functioning as scaffolding protein to promote assembly of the SagABCD complex [394] (Figure 2.4).

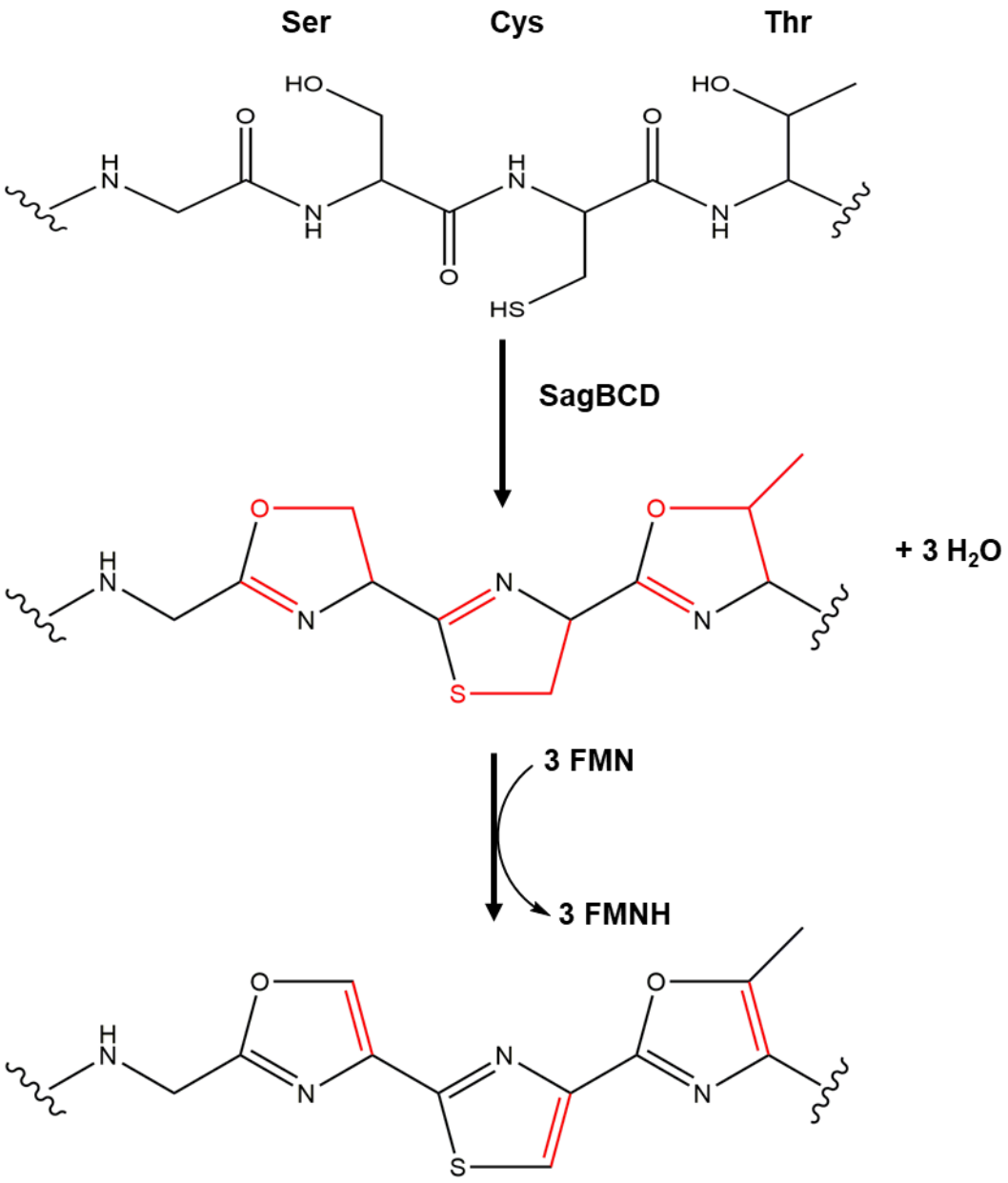


Figure 2.4 Formation of oxazole/thiazole rings in SagA residues by the SagBCD complex. Serine, cysteine, and threonine residues within the SagA peptide are first cyclized by the SagB catalyzed nucleophilic attack of the peptide carbonyl group by an alcohol or thiol found within the ser/thr/cys R group (Step 1+2). Following cyclization, the modified residues are then aromatized to oxazoles/thiazoles by the SagC catalyzed reduction of FMN to FMNH. (Step 3). Sites of modification at each step are indicated in red.

In contrast to earlier studies involving sag gene knockouts in *Streptococcus pyogenes* which concluded that the *sagA-sagI* genes were all required for the production of functional SLS [386], heterologous expression of the *sagABCD* genes in *E. coli* revealed that these were the only genes required for the production of functional SLS *in vitro* [394, 395]. Interestingly, sag operons have since been found in a wide variety of prokaryotes including firmicutes, bacterioidetes, actinobacteria, cyanobacteria, proteobacteria, and archaea [394]. Despite possessing low levels of sequence identity to one another, SagBCD proteins have been shown to be highly promiscuous in their recognition and modification of SagA substrates, as the SagBCD homologues from *Pyrococcus furiosus*, a hyperthermophilic archaeon [396], were capable of converting SagA peptides from both *Streptococcus pyogenes* and *Clostridium botulinum* into hemolytically active molecules [394]. Binding and recognition of SagA by the SagBCD enzymes involves both FxxxB (B = L, I, or V) and FQV motifs present in the N-terminal leader sequence of the SagA precursor [397]. In addition to SagA, the FxxxB motif is conserved in both MccB17 and ClosA, a precursor for a hemolytic toxin produced by *Clostridium botulinum* [398], providing an explanation for the substrate promiscuity demonstrated by SagBCD enzymes. Proteolytic cleavage of the SagA precursor is performed by SagE, a transmembrane protease homologous to eukaryotic CAAX proteases [399-401]. Similar to MccB17, proteolytic cleavage of the SagA precursor by SagE occurs at a glycine-glycine repeat, resulting in the removal of a 23 amino acid leader sequence from the 31 amino acid mature SLS peptide [364, 394, 399]. The transmembrane nature of the SagE protease, combined with the fact that only the SagBCD mediated modifications of the SagA peptide are required for hemolytic activity [394, 395], suggests that SagE mediated cleavage of the SagA precursor is a requirement for extracellular export of the mature peptide through an ATP-transporter formed by the SagGHI proteins [399].

#### 2.3.5.4 Contribution of SLS to disease

Since its identification in the 1930s, researchers have considered SLS to be a critical virulence factor in the pathogenesis of *Streptococcus pyogenes* infection [19]. While SLS$^-$ strains of *Streptococcus pyogenes* are capable of causing disease in humans [402], the vast majority of pathogenic GAS isolates produce SLS [403]. While recent research has shown that SLS contributes to disease through a variety of mechanisms, the first of these to be identified was impairment of the host immune response through the direct cytotoxicity of SLS towards macrophages, neutrophils, B cells, and T cells [274, 379, 404]. Studies with macrophages have observed that lysis of macrophages by SLS involves the production of mitochondrial reactive oxygen species (ROS) and subsequent alteration of mitochondrial transmembrane potential, leading to programmed cell death [405, 406]. Interestingly, SLS can inhibit the degradation of the host-cell kinase GSK-3β through an unknown mechanism, with this increase in cellular GSK-3β concentrations resulting in increased mitochondrial ROS production and subsequent cell death [406]. In addition to these cytotoxic effects, sublytic concentrations of SLS are capable of significantly inhibiting phagocytosis of *Streptococcus pyogenes* by macrophages, providing an additional mechanism by which SLS can inhibit the host immune response during *Streptococcus pyogenes* infection [274]. Activation of mast cells has been shown to be solely dependent on the expression of SLS during *Streptococcus equi* infection, as SLS knockout strains were incapable of inducing expression of the pro-inflammatory cytokines IL-6, TNF-α, or MCP-1 in cultured mast cells [407]. This SLS induced mast cell activation occurred several hours prior to detectable lysis within the infected cells and was dependent on activation of p38 and ERK1/2 MAPKs, indicating that the pro-inflammatory effects of SLS were not due to direct lysis of mast cells [407]. SLS is also cytotoxic towards keratinocytes and is considered a crucial virulence factor in the pathogenesis of soft tissue infections caused by *Streptococcus pyogenes* as a result [364, 385,

386]. Intoxication of epithelial keratinocytes with SLS results in downregulation of AKT [408], a serine/threonine kinase responsible for the activation of anti-apoptotic and pro-cell survival pathways [409, 410]. This inhibition of AKT signaling results in the activation of p38 MAPK which subsequently activates NF-κB, thus leading to increased expression of the pro-inflammatory cytokine IL-1β [408, 411]. This inflammatory response results in keratinocyte cell death through the induction of programmed necrosis [408], a caspase-independent form of programmed cell death often observed during inflammatory diseases [412]. Additionally, increased IL-1β leads to a dramatic increase in the severity of *Streptococcus pyogenes* infection, as evidenced by the fact that tissue damage associated with subcutaneous *Streptococcus pyogenes* infection is substantially decreased in mice treated with either the NF-κB inhibitor curcumin or antibodies against IL-1β [411]. Aside from these effects on keratinocytes, SLS also improves the ability of *Streptococcus pyogenes* to translocate across epithelia through the degradation of tight-junctions [413]. The observations that the host-cell protease calpain is translocated to the plasma membrane in cells infected with wild-type *Streptococcus pyogenes* and that the degradation of tight-junctions can be stopped by treatment with calpain inhibitors suggests that SLS is capable of inducing translocation of calpain to tight-junctions, thus enabling calpain mediated cleavage of the tight-junction proteins occludin and E-cadherin [413]. These data, along with the previously mentioned observations that SLS can alter the activity of the $Cl^-/HCO_3^-$ exchanger band 3 [384], suggest that SLS is an incredibly dynamic toxin capable of altering the activity of a wide variety of host cell proteins through as-yet unexplored mechanisms.

In addition to *Streptococcus pyogenes*, hemolytic peptides homologous to SLS have been identified in the human pathogens *Listeria monocytogenes* [414] and *Clostridium botulinum*

[398]. While listeriolysin S (LLS), the SLS homologue produced by *Listeria monocytogenes*, was initially reported to be toxic against both erythrocytes and a colonic cell line *in vitro* [414], later studies found that LLS possessed only marginal hemolytic activity and did not exhibit cytotoxic activity against macrophages or epithelial cells [415]. Rather, LLS functions exclusively as an antimicrobial toxin, functioning to kill competing members of the gut microbiota during *Listeria monocytogenes* infection [415-417]. While several studies have found that LLS expression increases the virulence of *Listeria monocytogenes* through this antimicrobial effect [414-417], a surprising observation was that expression of *llsB* (a modifying enzyme homologous to *sagB*), but not *llsA* (the precursor to the mature LLS peptide) improved the ability of *Listeria monocytogenes* to colonize the spleen and liver of infected animals [415]. This finding lead the authors to hypothesize that LlsB may in fact be a critical virulence factor in *Listeria monocytogenes* infection, however at the time of writing no further studies examining this hypothesis have been published. In contrast to LLS, the function of clostridiolysin S (CLS) remains relatively unknown. Unlike LLS, CLS appears to function effectively as a hemolysin, as deletion of either the *closA* or *closC* genes from *Clostridium botulinum* results in a loss of the β-hemolytic phenotype characteristic of this bacterium [398]. Likewise, incubation of the ClosA peptide (homologous to SagA) with the SagBCD enzymes from *Streptococcus pyogenes* resulted in the formation of a hemolytically active peptide [397-399]. Despite this, no in-depth studies on the *in vitro* toxicity of CLS towards other eukaryotic cell types or its contribution to the pathogenicity of *Clostridium botulinum in vitro* have been undertaken. The recent discovery of a gene cluster homologous to the sag operon in several *Brachyspira* species suggests that production of a toxin homologous to SLS may also be crucial in the pathogenesis of swine dysentery [20]. This operon is present in *Brachyspira hampsonii* clade I and clade II strains, as

well as the type strains of *Brachyspira hyodysenteriae*, *Brachyspira innocens*, and *Brachyspira murdochii*, but is notably absent in the nonpathogenic *Brachyspira hampsonii* strain KL180 [20, 97]. While the absence of this operon in *Brachyspira hampsonii* strain KL180 is strong evidence that a toxin homologous to SLS is critical for *Brachyspira* virulence, the observation that *Brachyspira hampsonii* KL180 is strongly β-hemolytic on blood agar obfuscates this idea [97]. Furthermore, these operons are also present in the weakly β-hemolytic, non-pathogenic *Brachyspira innocens* and *Brachyspira murdochii*, while the *sagA* homologue encoding for the toxin precursor is absent in the pathogenic *Brachyspira pilosicoli* [20]. As such, it appears likely that *Brachyspira* hemolysis and disease involves the contributions of multiple toxins such as TlyA, TlyB, TlyC, HlyA, and an SLS homologue, and in-depth analyses of the biological functions of each toxin are necessary to fully understand the pathology of swine dysentery.

# Chapter 3 – Mutational Analysis of TlyA from *Brachyspira hampsonii* Reveals Two Key Residues Conserved in Pathogenic Bacteria Responsible for Oligomerization and Hemolytic Activity

**Brandon A. Keith[1], John C.S. Harding[2], Matthew E. Loewen[1*]**

[1]Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

[2]Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

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TlyA proteins are expressed in a variety of pathogenic bacteria and possess dual hemolytic and ribosomal RNA methyltransferase functions. While the mechanism of TlyA mediated rRNA methylation is well understood, relatively little is known about the mechanism of TlyA induced hemolysis. TlyA protein from the pig pathogen *Brachyspira hampsonii* was heterologously expressed and purified from an *E. coli* host. Hemolytic activity and rRNA methylation by TlyA were assessed *in vitro*. Site-directed mutagenesis was used to mutate amino acid residues believed to be involved in TlyA mediated hemolysis. Purified TlyA-His protein exhibited both hemolytic and rRNA methyltransferase activities *in vitro*, with partial inhibition of hemolysis observed under reducing conditions. Mutation of a cysteine 80 to alanine impaired hemolytic activity. A C27A/C93A mutant was capable of dimerizing under non-reducing conditions, indicating that a C80-C80 disulfide bond is involved in TlyA oligomerization. A mutation conserved in several avirulent *Brachyspira* species (S9K) completely abolished hemolytic activity of TlyA. This loss of activity was attributed to impaired oligomerization in the S9K mutant, as assessed by ITC and size-exclusion chromatography experiments. Oligomeric assembly and hemolytic activity of TlyA from *Brachyspira hampsonii* is dependent on the formation of an intermolecular C80-C80 disulfide bond and noncovalent interactions involving serine 9. The conservation of these amino acids in TlyA proteins from pathogenic bacteria suggests a correlation between mutations in the tlyA gene and bacterial virulence. Our results further elucidate the mechanisms underlying TlyA mediated hemolysis and provide evidence of a conserved mechanism of oligomerization for TlyA family proteins.

## 3.1 Introduction

TlyA is a bifunctional hemolysin/rRNA methyltransferase found in important pathogens such as *Mycobacterium tuberculosis* [15], *Helicobacter pylori* [418], *Brachyspira hyodysenteriae* [323], and *Campylobacter jejuni* [17]. While the precise mechanism by which TlyA proteins exhibit their hemolytic function has not yet been elucidated, the hemolytic activity of TlyA proteins from *Mycobacterium tuberculosis* and *Helicobacter pylori* has been linked to disulfide bond formation, as two separate groups reported inhibition of hemolytic activity under reducing conditions [18, 419]. Site-directed mutagenesis of the *tlyA* gene from *Helicobacter pylori* showed the involvement of two specific cysteines (C124 and C128) in TlyA oligomerization and hemolytic activity [419]. Interestingly, cysteines are only found at these positions in *Helicobacter pylori* TlyA, suggesting that disulfide bond formation and subsequent mechanism of oligomerization differs in other TlyA proteins.

TlyA proteins also function as rRNA methyltransferases capable of 2'O methylating nucleotide C1920 of 23s rRNA, while some homologues are capable of also methylating nucleotide C1409 of 16s rRNA [338]. Selectivity of rRNA methylation is related to the primary sequence of TlyA, as TlyA proteins with truncated N and C termini referred to as $TlyA^{I}$, only methylate 23s rRNA, while full length TlyA proteins referred to a $TlyA^{II}$, methylate 16s and 23s rRNA [338]. Methylation of both C1409 and C1920 by *Mycobacterium tuberculosis* TlyA is required for binding of the cyclic peptide antibiotic capreomycin to the ribosome [336, 338, 420-422], with mutation of the tlyA and/or rRNA genes being linked to capreomycin resistance in *Mycobacterium tuberculosis* [336, 344, 420, 423]. In addition to this role in capreomycin resistance/susceptibility, recent evidence has suggested that TlyA encoded rRNA methylations are crucial for both fitness and virulence of host bacteria as TlyA methyltransferase dead strains

of *Campylobacter jejuni* exhibit decreased motility, biofilm formation, and host cell adhesion [345, 346]. These observations indicate that TlyA functions as an important virulence factor for many pathogenic bacteria through two distinct mechanisms, underscoring the need for further understanding of the functions of these proteins.

Similar to *Mycobacterium tuberculosis*, *Helicobacter pylori*, and *Campylobacter* jejuni, TlyA proteins are hypothesized to be important virulence factors produced by *Brachyspira hyodysenteriae* and *Brachyspira hampsonii*, the causative agents of swine dysentery. Swine dysentery is an infectious disease of pigs characterized by severe mucohaemmorhagic diarrhea, resulting in massive production losses due to decreased weight gain and increased mortality in infected animals [424, 425]. Initially attributed to infection with the spirochete *Brachyspira hyodysenteriae* [424-427], recent outbreaks of swine dysentery in North America have been attributed to the recently identified species *Brachyspira hampsonii* [6, 91] while sporadic outbreaks in Northern Germany and Scandinavia have been associated with *Brachyspira suanatina* [7, 99, 100]. While these species are not known to infect humans, the weakly β-hemolytic species *Brachyspira pilosicoli* and *Brachyspira aalborgi* can cause mild diarrhea and *coli*tis in immunocompromised patients [31, 40, 428]. Regardless of the specific species, a common observation with strongly pathogenic *Brachyspira* isolates is a strong β-hemolytic phenotype when these bacteria are cultured on blood agar in contrast to the weak β-hemolytic phenotype exhibited by less virulent *Brachyspira* species [6, 7, 79, 91, 323, 326, 429]. To date four putative hemolysin genes have been identified in *Brachyspira hampsonii* and *Brachyspira hyodysenteriae*, termed *tlyA/B/C* and *hlyA* [323, 325, 326, 430, 431]. While the functionality of these gene products in either *Brachyspira hyodysenteriae* or *Brachyspira hampsonii* has not been studied, *tlyA* knockout strains of *Brachyspira hyodysenteriae* exhibit a weak β-hemolytic

phenotype and reduced virulence in swine [325, 326] suggesting that TlyA may be a crucial virulence factor in the pathogenesis of swine dysentery.

In this study we aimed to assess the hemolytic activity of TlyA from *Brachyspira hampsonii*, a causative agent of swine dysentery, and to further understand the mechanisms by which TlyA proteins exhibit their hemolytic function. Here we find *Brachyspira hampsonii* TlyA expressed with a C-terminal His-tag possessed both hemolytic and rRNA methyltransferase functionalities and like *Mycobacterium tuberculosis* [18] and *Helicobacter pylori* [419], reducing agents were capable of significantly inhibiting the hemolytic activity. Site-directed mutagenesis of the three cysteine residues present in TlyA (C27, C80, and C93) revealed that only C80 was necessary for oligomerization and hemolysis. Notably, C80 is highly conserved across all bacterial TlyA protein sequences, with 71.9% of TlyA homologues possessing a cysteine at this position. *In silico* analysis of TlyA protein sequences from members of the *Brachyspira* genus showed two notable mutations in the N-terminal S4 domain of TlyA from the weakly hemolytic species *Brachyspira innocens*, *Brachyspira murdochii*, and *Brachyspira aalborgi*. One of these mutations (S9K) completely abolishing the hemolytic activity of *Brachyspira hampsonii* TlyA. This is in keeping with Serine 9 being conserved in the sequences from TlyA$^{I}$ family members previously shown to exhibit hemolytic activity *in vitro*. This inhibition of hemolytic activity in the S9K mutant was attributed to impaired oligomerization of this mutant compared to wild-type TlyA, as dilution isothermal calorimetry experiments revealed a 2.7 fold increase in the dimer dissociation constant of the S9K TlyA mutant relative to wild-type TlyA. Additionally, size-exclusion chromatography showed clear differences in the oligomeric states of S9K TlyA in relation to the wild-type protein.

## 3.2 Materials & Methods

### 3.2.1 Molecular modelling

Modelling of the *Brachyspira hampsonii* TlyA protein structure was carried out utilizing the phyre2 server utilizing a crystal structure annotated as a putative hemolysin from *Streptococcus thermophilus* as a template (PDB ID 3HP7) [432]. UCSF chimera was used for visualization of the structure [433].

### 3.2.3 Cloning & site-directed mutagenesis

For the TlyA-His construct, a codon-optimized variant of the *Brachyspira hampsonii* strain 30446 gene was chemically synthesized (Genscript), digested with NdeI and XhoI, and ligated into a pET-24-a-d (+) vector. The ligated plasmid was then transformed into DH5α competent *E. coli* cells (Invitrogen). Plasmid DNA was isolated from overnight cultures utilizing a miniprep kit (Qiagen) and success of cloning was confirmed by PCR analysis and sequencing. Site directed mutagenesis was undertaken by chemical synthesis (Genscript) using the codon-optimized TlyA-His construct as a template. Synthesized mutant *tlyA* sequences were digested with NdeI and XhoI and ligated into a pET-24-a-d (+) vector.

### 3.2.3 Expression and purification of TlyA constructs

BL21 (DE3) *E. coli* cells were used for overexpression and purification of all TlyA constructs used in this study. Overnight cultures transformed with the appropriate plasmid were grown in 4 x 500 mL 2YT broth cultures (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) containing 1% (v/v) glucose, 50 µg/mL kanamycin (Fisher Bioreagents) at 37 °C until mid-late log phase ($OD_{600} \approx 0.6$), after which point protein expression was induced with 0.2 mM IPTG (Fisher Scientific) overnight at 18 °C. Cultures were harvested by centrifugation, suspended in buffer A (250 mM KCl, 10 mM HEPES pH 7.4) containing 10% glycerol (Fisher Bioreagents), 50 µg/mL DNAse I (Sigma Aldrich), 50 µg/mL lysozyme (Sigma Aldrich), and 1 mM

phenylmethylsulfonyl fluoride (PMSF, ThermoFisher Scientific), and lysed by sonication. Insoluble cell debris was pelleted by centrifugation, and the soluble fraction was loaded onto an equilibrated XK 26/20 column (GE Healthcare) packed with $Ni^{2+}$ charged Profinity $Ni^{2+}$ IMAC resin (Bio-Rad). The column was subsequently washed with 10 column volumes of buffer A, followed by 10 column volumes of buffer A + 5 mM imidazole and 10 column volumes of buffer A + 10 mM imidazole, after which point TlyA protein was eluted with buffer A + 100 mM imidazole. Purity of eluted fractions was assessed by 12% SDS-PAGE. Fractions containing pure TlyA were pooled and dialyzed against storage buffer (1 M NaCl, 10 mM HEPES pH 7.4). Concentration of purified TlyA protein was estimated by the BCA assay (ThermoFisher Scientific) using bovine serum albumin as a standard [434].

### 3.2.4 Semi-native SDS-PAGE

Semi-native SDS-PAGE was performed according to the protocols previously used by Samainukul *et al.,* for the analysis of *Helicobacter pylori* TlyA [419]. Protein samples were diluted 1:1 with 2x Laemelli sample buffer (65.8 mM Tris-HCl pH 6.8, 26.3% (w/v) glycerol, 0.01 bromophenol blue) lacking SDS and incubated at room temperature, 37 °C, or 95 °C for 5 minutes as indicated. 10 mM β-mercaptoethanol was added to samples ran under reducing conditions. To induce oligomerization of TlyA samples without boiling the proteins the samples were dialyzed against PBS containing 0.1% sodium deoxycholate overnight at 4 °C.

### 3.2.5 Hemolysis and osmoprotection assays

Whole heparinized pig's blood was obtained from healthy animals, washed with three volumes of 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$) three times, and the washed erythrocytes were resuspended in PBS to a final concentration of 2% (v/v). TlyA proteins were dialyzed against PBS overnight, after which point 50 µL of protein was added to 50 µL of the erythrocyte suspension and incubated at room temperature for 18-24 hours.

Reaction mixtures were centrifuged at 2000 × g to pellet the erythrocytes, and hemoglobin release into the supernatant was quantified by the spectrophotometric detection of hemoglobin in the supernatant at 545 nm. Percent hemolysis was calculated by comparison of values obtained from TlyA treated samples to that of a PBS treated negative control (0% hemolysis) and a positive control lysed in distilled water (100% hemolysis). For Osmoprotection experiments 50 µL of purified TlyA-His protein (125 µg/mL final concentration) was added to 50 µL of a 2% pig erythrocyte suspension in PBS containing an osmoprotectant (30 mM mannitol/PEG 300/400/600/1000, or 15 mM PEG 3000/6000) and incubated at room temperature for 18-24 hours. Percent hemolysis was calculated as described previously, then compared to a reaction mixture containing 125 µg/mL TlyA in a 2% pig erythrocyte suspension with no added osmoprotectants (100% hemolysis relative to control).

### 3.2.6 Quantification of rRNA methylation by TlyA

A 1 L culture of DH5α *E. coli* cells was pelleted and resuspended in 10 mM Tris-HCl (pH 7.4) containing 10 mM $MgCl_2$, 60 mM $NH_4Cl$, and 6 mM β-mercaptoethanol. The cells were lysed by sonication and the insoluble fraction was removed by centrifugation at 39,410 × g for 45 minutes. Intact ribosomes were then pelleted by ultracentrifugation of the soluble fraction at 150,000 × g for 1 hour. The pelleted ribosomes were resuspended in 50 mM Tris-HCl (pH 7.4) containing 3 mM Mg(O)Ac, 200 mM $NH_4Cl$, and 5 mM dithiothreitol. RNA content of the purified ribosomes was estimated by measurement of absorbance at 260 nm. Methylation assay reaction mixtures containing 18 µg ribosome, 8 µg TlyA protein, and 0.5 µCi (120 µM) $^3$H-S-adenosylmethionine were incubated at 37 °C for 2 hours, after which point the reaction was terminated by addition of Trizol reagent (Invitrogen). Ribosomal rRNA was then purified using Trizol-chloroform according to the manufacturer's protocol, precipitated with ethanol,

resuspended in RNAse free water and added to 4 mL liquid scintillation cocktail. Radioactivity of the tlyA treated and control rRNAs was measured by liquid scintillation counting.

### 3.2.7 Circular dichroism spectroscopy

Proteins (100 µg/mL) were dialyzed into 50 mM sodium phosphate buffer (pH 7.4) containing 250 mM NaF and CD spectra were collected at 20 °C utilizing a PiStar-180 circular dichroism spectrometer with a 0.05 cm path-length cuvette. Four spectra from 260-185 nm were recorded with 0.5 nm resolution for each protein, averaged, and buffer subtracted. Estimation of secondary structure was performed utilizing the CDNN software package, using the "simple" database [435]

### 3.2.8 Isothermal titration calorimetry

WT and S9K TlyA-His were dialyzed against 10 mM HEPES (pH 7.4) containing 500 mM NaCl and 1 mM tris(2-carboxyethyl)phosphine HCl (Thermo Fisher Scientific) overnight and diluted to 100 µM. Isothermal titration calorimetry experiments were carried out on an ITC4200 microcalorimeter (Calorimetry Sciences Corporation) at 25 °C. The reaction cell contained 1400 µL of dialysis buffer (10 mM HEPES pH 7.4, 500 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine HCl) obtained directly from the beaker used for dialysis of WT and S9K TlyA-His to minimize dilution artifacts. Experiments comprised 22 injections of 10 µL WT or S9K TlyA-His into the reaction cell containing only buffer. Heats of dilution were integrated, and the heat of the 22$^{nd}$ dilution for each protein was considered to be the enthalpy of dilution and subtracted from all other injection enthalpies. Integrated enthalpies were then fit to a dimer dissociation model using NanoAnalyze version 3.11.0 (TA Instruments).

### 3.2.9 Analytical size exclusion chromatography

WT and S9K TlyA-His proteins were dialyzed against PBS overnight and diluted to a final concentration of 1 mg/mL. Two mL of protein was loaded onto a pre-equilibrated Superdex 200

pg 16/600 column (Cytiva Life Sciences) and eluted with 1 column volume of PBS at a flow rate of 0.75 mL/min. Elution profiles of WT and S9K TlyA-His were compared to a set of gel-filtration standards comprised of thyroglobulin (MW = 670 kDa), γ-globulin (MW = 158 kDa), ovalbumin (MW = 44 kDa), myoglobin (MW = 17 kDa), and vitamin B12 (MW = 1.35 kDa).

### 3.2.10 Statistical analysis

Data are expressed as mean + standard deviation or mean ± standard deviation as indicated. All data were normally distributed (Shapiro-Wilk test; $P > 0.05$) All data were analyzed by One-Way ANOVA followed by Holm-Sidak post-hoc with $\alpha = 0.05$. Post-hoc tests were compared to either control or WT TlyA-His as indicated. All statistical analyses were performed in Sigmaplot version 11.0 (Systat Software Inc). Significance is denoted by * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$).

## 3.3 Results

### 3.3.1 TlyA from *Brachyspira hampsonii* is a bifunctional hemolysin/rRNA methyltransferase

The *tlyA* gene from *Brachyspira hampsonii* codes for a 240 amino acid protein with a calculated molecular weight of 27.0 kDa. Bioinformatics and structural analyses have indicated that the TlyA protein consists of two separate domains – an S4 like domain at the N-terminus and a Rossman like methyltransferase fold comprising the C-terminus, with both regions linked by a flexible linker region susceptible to proteolytic attack [18, 422] (Figure 3.1A). For our initial studies the *tlyA* gene was amplified by PCR from *Brachyspira hampsonii* strain 30446 genomic DNA and ligated into a modified pET-28 vector encoding an N-terminal His-tag and a TEV protease recognition site, producing the His-TlyA construct . The resulting His-TlyA protein was easily expressed in *Escherichia coli* and purified from the soluble fraction by a one-step $Ni^{2+}$ affinity chromatography procedure (Figure 3.1B), however His-TlyA caused negligible hemolysis of pig erythrocytes at concentrations up to 250 µg/mL (Figure 3.1C). We hypothesized that steric hindrance due to the His-tag on the N-terminus of our construct was behind this lack of activity and attempted to cleave this tag utilizing TEV protease. While our TEV cleavage was inefficient and did not completely remove the His-tag from the His-TlyA construct, the isolated TlyA protein also did not exhibit hemolytic activity (Data not shown). In light of these results, we next cloned a codon optimized version of the *tlyA* gene into a pET-24-a-d(+) vector containing a C-terminal His-tag, producing the TlyA-His construct Following overexpression and purification of TlyA-His by $Ni^{2+}$ affinity chromatography (Figure 3.1B) the hemolytic activity of TlyA-His against pig erythrocytes was examined. In contrast to the His-TlyA construct, TlyA-His possessed dose dependent hemolytic activity against pig erythrocytes

(Figure 3.1C). Hemolytic activity was only observed after long (18-24 hour) periods at room temperature, a result consistent with those obtained for other TlyA proteins [18, 436]. TlyA-His lysed erythrocytes in a dose dependent manner), exhibiting ~43% hemolysis at the highest dose of 250 µg/mL and ~2% hemolysis at the lowest dose of 1.95 µg/mL. Addition of the reducing agent β-mercaptoethanol to reaction mixtures reduced the hemolytic activity of TlyA-His by ~4 fold at a dose of 250 µg/mL (Figure 3.1E), indicating that the hemolytic activity of TlyA from *Brachyspira hampsonii* is dependent on disulfide bond formation The size of the pores formed by TlyA in the pig erythrocyte membranes was estimated by addition of osmoprotectants (mannitol and PEG 300/400/600/1000/3000/6000) to reaction mixtures containing TlyA-His and pig erythrocytes. Statistically significant inhibition of hemolysis was observed upon addition of PEG 1000 and PEG 3000, while complete inhibition was observed with addition of PEG 6000 (Figure 3.1F), indicating that TlyA-His forms pores between 1.88 and 2.88 nm in diameter in pig erythrocytes. To further confirm that both the His-TlyA and TlyA-His constructs were properly folded both proteins were examined by far-UV circular dichroism spectroscopy (Figure 3.1D). While the CD spectra of both proteins were consistent with those of folded proteins possessing mixed α/β secondary structure (Table 3.1), a dramatic shift towards β-sheet rich secondary structure was observed for His-TlyA.

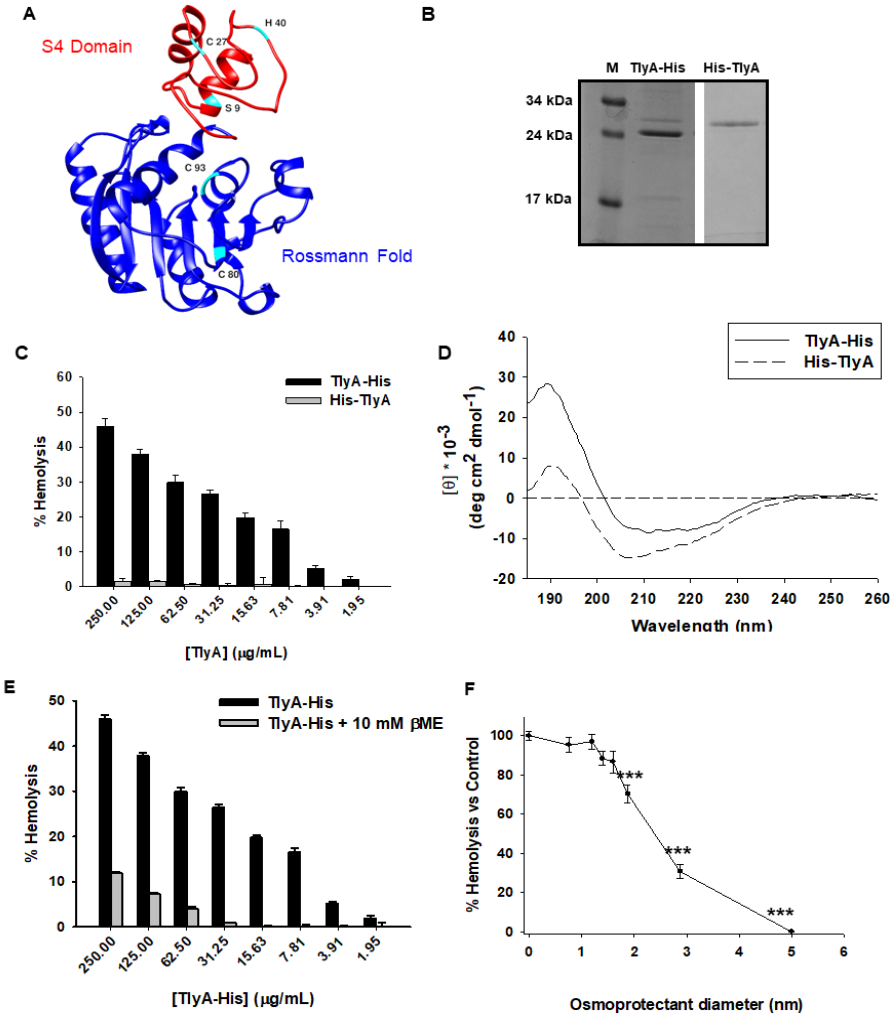


Figure 3.1 Hemolytic activity of TlyA from *Brachyspira hampsonii*. *A*, homology model of TlyA from *Brachyspira hampsonii*. Homology modelling was carried out using the phyre2 server [432] utilizing a putative hemolysin from *Streptococcus thermophilus* (PDB ID 3HP7) as a model. Amino acid residues comprising the N-terminal S4 domain are coloured red, amino acid residues comprising the Rossman-like methyltransferase fold are coloured blue, and amino acids mutated in this study (S9, C27, H40, C80, and C93) are labelled and coloured cyan. *B*, purification of TlyA-His and His-TlyA. TlyA protein constructs were expressed in *E. coli* and purified from the soluble fraction by $Ni^{2+}$ IMAC chromatography. Purity of proteins was assessed by 12% SDS-PAGE and Coomassie staining. Each lane contains ~500 ng of purified TlyA protein. *C*, hemolytic activity of TlyA-His (black bars) and His-TlyA (grey bars). TlyA-His lysed erythrocytes in a dose-dependent manner, with the highest dose tested (250 µg/mL) causing 46% hemolysis. Data are presented as mean + SEM (n = 10 per group). *D*, far-UV circular dichroism spectrum of TlyA-His protein. *E*, inhibition of hemolytic activity of TlyA-His by reducing agents. Addition of 10 mM β-mercaptoethanol (grey bars) to hemolysis reaction mixtures resulted in a ~4 fold inhibition of the hemolytic activity of TlyA-His. *F*, estimation of TlyA-His pore size. TlyA-His was incubated with a 2% pig erythrocyte suspension and various osmoprotectants. Osmoprotectants between 1.88 and 2.88 nm in diameter blocked hemolysis to a significant degree, while a 5.00 nm diameter osmoprotectant completely blocked hemolysis (One Way-Anova, Holm-Sidak Post-hoc, *** indicates significant difference from control at $p < 0.001$, n = 10 per group, data are presented as mean ± SEM).

Table 3.1 Estimated Secondary Structure Composition of TlyA-His and His-TlyA.

| | **TlyA-His** | **His-TlyA** |
|---|---|---|
| **α-Helix** | 33.40% | 28.40% |
| **Antiparallel β-sheet** | 8.00% | 21.60% |
| **Parallel β-sheet** | 8.40% | 7.90% |
| **β-Turn** | 16.70% | 19.70% |
| **Random Coil** | 32.30% | 24.80% |

In addition to functioning as a pore-forming hemolysin, previous studies have shown that TlyA is also a SAMe-dependent rRNA methyltransferase [336, 338, 345, 420]. To determine if TlyA from *Brachyspira hampsonii* also possesses this functionality we incubated TlyA protein with *E. coli* ribosomes and $^{3}H$ labelled SAMe. Both His-TlyA and TlyA-His could incorporate a radiolabeled methyl group into rRNA (Figure 3.2A). While TlyA encoded rRNA methylations are known to decrease capreomycin susceptibility in *Mycobacterium* species and *E. coli* carrying tlyA genes on a plasmid [18, 336], transformation of *E. coli* with *Brachyspira hampsonii tlyA* did not affect or growth rate in the presence of capreomycin, as the growth rate of *E. coli* containing the *tlyA* gene was slightly higher in the presence of capreomycin (Figure 3.2B). These results not only indicate that *Brachyspira hampsonii* TlyA possesses rRNA methyltransferase activity but suggests that the addition of additional N-terminal residues in our His-TlyA construct inhibits hemolytic activity without affecting the secondary rRNA methyltransferase functionality of this protein.

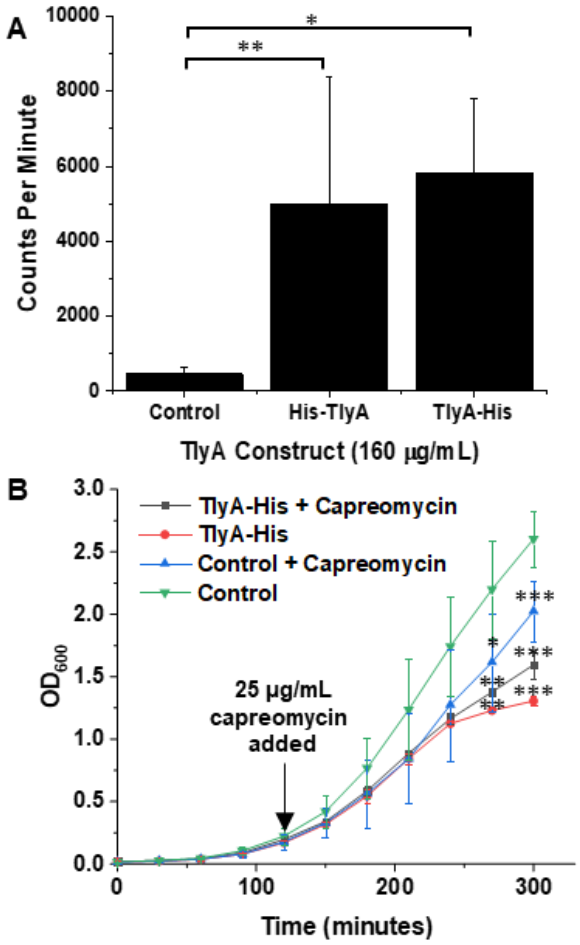


Figure 3.2 Ribosomal RNA methyltransferase activity of TlyA from *Brachyspira hampsonii*. *A*, rRNA methyltransferase activity of His-TlyA and TlyA-His. His-TlyA and TlyA-His were both capable of incorporating a $^{3}H$ labelled methyl group into rRNA (One Way-Anova, Holm-Sidak Post-Hoc, * indicates significant difference from no TlyA control at $p < 0.05$, ** indicates significance at $p < 0.01$, n = 4 per group, data are presented as mean + S.D.). *B*, growth rate of *E. coli* transformed with either TlyA-His or an empty pET-24-a-d (+) vector (Control). *E. coli* broth cultures were grown for 120 minutes, after which point 25 µg/mL capreomycin was added to randomly selected cultures and growth was monitored for an additional 180 minutes. While impaired growth was observed for both TlyA-His and control transformed cultures dosed with capreomycin, growth of the TlyA-His construct was slower in the absence of capreomycin, suggesting no increase in capreomycin susceptibility upon expression of *Brachyspira hampsonii* TlyA (One Way-Anova, Holm-Sidak Post-Hoc, * indicates significant difference from capreomycin free control vector at $p < 0.05$, ** indicates significance at $p < 0.01$, *** indicates significance at $p < 0.001$, n = 3 per group, data are presented as mean ± S.D.).

### 3.3.2 Heat and detergents promote the *in vitro* oligomerization of TlyA

Previous studies have demonstrated that intermolecular disulfide bond formation is a prerequisite for oligomerization and hemolytic activity in TlyA proteins from both *Mycobacterium tuberculosis* [18] *and Helicobacter pylori* [419]. These studies, combined with our previous observation that the reducing agent β-mercaptoethanol dramatically inhibits the hemolytic activity of *Brachyspira hampsonii* TlyA (Figure 3.1E) led us to hypothesize that intermolecular disulfide bond formation is also necessary for the oligomerization and hemolytic activity of *Brachyspira hampsonii* TlyA. While we first attempted to assess disulfide bond formation of TlyA-His under non-reducing conditions utilizing conventional SDS-PAGE, only minute amounts of dimeric TlyA-His protein were observed under these conditions (Data not shown). As a result, we next utilized a modified semi-native SDS-PAGE protocol previously used by Samainukul *et al.,* for analysis of the oligomeric state of *Helicobacter pylori* TlyA [419, 437] to examine disulfide bond formation between *Brachyspira hampsonii* TlyA-His monomers. Semi-native SDS-PAGE clearly demonstrated the presence of ~56 kDa, ~130 kDa, and >170 kDa oligomeric forms of TlyA-His following boiling of the protein for five minutes (Figure 3.3A). Interestingly, the molecular weights of these oligomers roughly correspond to dimeric (56 kDa), pentameric (130 kDa), and aggregated (>170 kDa) forms of TlyA-His. Notably, these higher order oligomers were only present after boiling TlyA-His and not upon incubating this protein at room temperature or 37 °C for five minutes. To assess the ability of TlyA-His to form oligomers under physiologically relevant temperatures we first attempted to demonstrate the presence of TlyA oligomers on erythrocyte membranes by Western blotting but were unable to reliably detect higher order oligomers (data not shown). As studies have previously demonstrated that detergents are capable of inducing oligomerization of pore-forming toxins such as ClyA from *E. coli* [255], we next examined the oligomerization of TlyA-His in the presence of the bile salt

sodium deoxycholate (Na-DOC). Semi-native SDS-PAGE revealed that dialysis of TlyA-His against PBS containing Na-DOC was capable of inducing oligomerization of TlyA-His, even in samples that were not boiled prior to semi-native SDS-PAGE analysis (Figure 3.3B). While treatment of these samples with β-mercaptoethanol resulted in the loss of the ~56 kDa band representing dimeric TlyA-His, another band of ~72 kDa was resistant to β-mercaptoethanol, suggesting that both intermolecular disulfide bonding and non-covalent interactions may play critical roles in the oligomerization of TlyA.

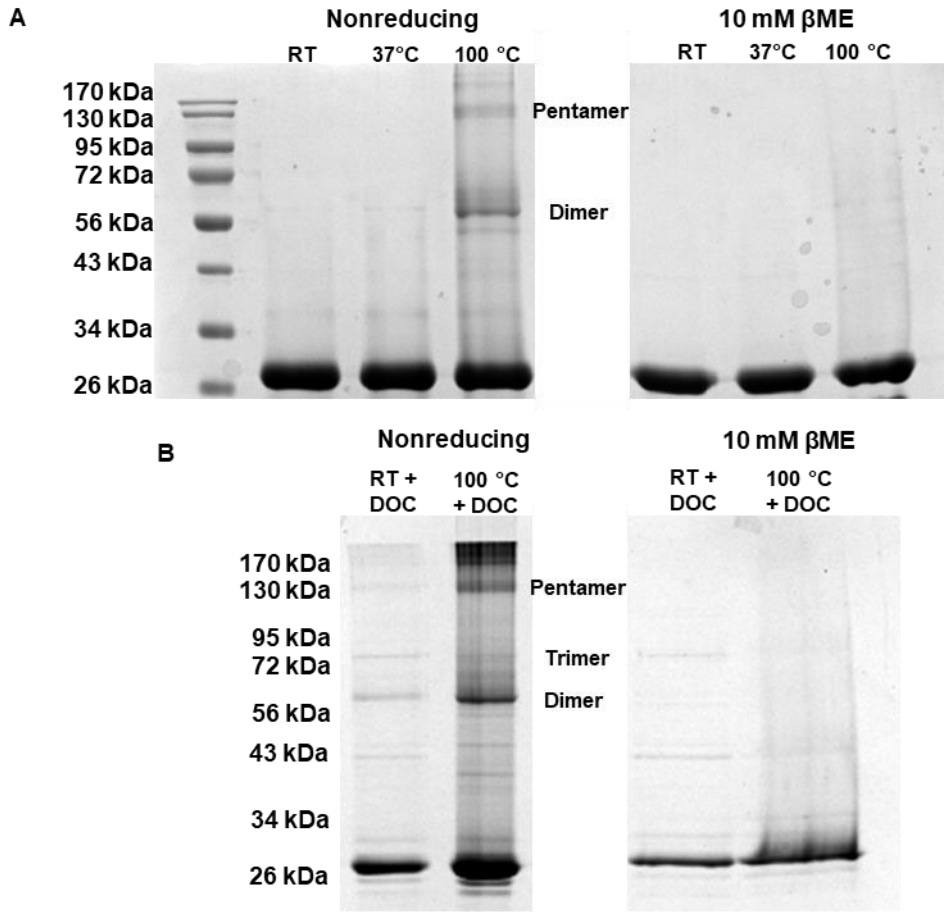


Figure 3.3 Heat and DOC micelles induce oligomerization of TlyA-His. *A*, oligomerization of TlyA-His in response to heat treatment. TlyA-His (~1000 ng/lane) was diluted 1:1 with 2x Laemelli sample buffer lacking SDS and incubated at 25°C (RT), 37°C, or 100°C for 5 minutes in the presence and absence of 10 mM β-mercaptoethanol (βME) as indicated. Dimeric (~56 kDa), pentameric (~130 kDa) and higher order (>170 kDa) species were observed in samples heated to 100°C under non-reducing conditions, however these species were not observed in βME treated samples. *B*, oligomerization of TlyA-His in the presence of DOC micelles. TlyA-His was dialyzed overnight against PBS containing 0.1% (w/v) sodium deoxycholate (DOC). The resulting protein (~500 ng/lane) was diluted 1:1 with 2x Laemelli sample buffer lacking SDS and incubated at 25°C (RT) or 100°C in the presence or absence of 10 mM βME as indicated. Dimeric (~56 kDa), trimeric (~72 kDa), pentameric (~130 kDa) and higher order (> 170 kDa) species were observed under nonreducing conditions regardless of heat treatment. Treatment with βME resulted in the degradation of all but the trimeric species, suggesting that the ~72 kDa trimer arose from noncovalent interactions between TlyA monomers.

### 3.3.2 Mutation of cysteine 80 in TlyA reduces, but does not abolish hemolytic activity

To understand the specific mechanisms of intermolecular disulfide bond formation in *Brachyspira hampsonii* TlyA and the contribution of these disulfide bonds to the activity of the protein, we sequentially mutated the three cysteine residues present in TlyA (C27, C80, and C93) were to alanine. The resulting C27A, C80A, and C93A TlyA-His constructs were expressed and purified in the same manner as wild-type TlyA-His, and the hemolytic activity was assessed as described previously. A statistically significant increase in hemolytic activity compared to wild-type (WT) TlyA-His was observed for the C27A and C93A mutants (Figure 3.4A), while a statistically significant decrease in hemolytic activity compared to WT TlyA-His was observed for the C80A mutant. The magnitude of this decrease was similar to that observed upon addition of β-mercaptoethanol (4.3 fold for C80A TlyA-His vs 3.9 fold for β-mercaptoethanol), suggesting that while a C80-C80 disulfide bond is involved in TlyA oligomerization, the loss of this bond does not completely abolish hemolytic activity. Next, we aimed to determine what role, if any, this disulfide bond played in TlyA's rRNA methyltransferase functionality. Once again, all the TlyA constructs possessed rRNA methyltransferase activity (Figure 3.4B). Remarkably, all the cysteine mutants were more efficient at methylating rRNA than WT TlyA-His, showing that disulfide bond formation is not a requirement for rRNA methylation by TlyA and may in fact play an inhibitory role. To definitively demonstrate that a C80-C80 intermolecular disulfide bond is necessary for oligomerization of TlyA-His, we next created a double mutant (C27A/C93A TlyA-His) containing only a single cysteine at position 80. Semi-native SDS-PAGE analysis revealed that the C27A/C93A TlyA-His mutant was in fact capable of oligomerization upon heating, existing primarily as a ~56 kDa dimer under these conditions with minimal evidence of higher order oligomer formation (Figure 3.5A). The observation that that the hemolytically impaired C80A TlyA-His mutant does not form a ~56 kDa dimer suggests that

this dimeric form of TlyA may thus be a critical intermediate in the overall assembly of the mature TlyA pore. Dialysis of C27A/C93A TlyA-His against PBS containing Na-DOC lead to the formation of both dimeric (~56 kDa) and trimeric (~72 kDa) species on semi-native SDS-PAGE (Figure 3.5B), but not the formation of higher order oligomers as observed with the wild-type protein. As with WT TlyA-His, the ~72 kDa C27A/C93A TlyA-His trimer was resistant to β-mercaptoethanol treatment, suggesting once again that this trimeric species arises from non-covalent interactions, rather than intermolecular disulfide bonding. Sequence analyses of the tlyA gene revealed that cysteine 80 is universally conserved in all *Brachyspira* TlyA protein sequences available on GenBank (Figure 3A). Additionally, 71.9% (359/500) of the top 500 non-*Brachyspira* homologues, including *Mycobacterium tuberculosis* and *Campylobacter jejuni* but not *Helicobacter pylori*, possess a cysteine at this position (Figure 3B/C), suggesting a conserved mechanism of oligomerization for these proteins.

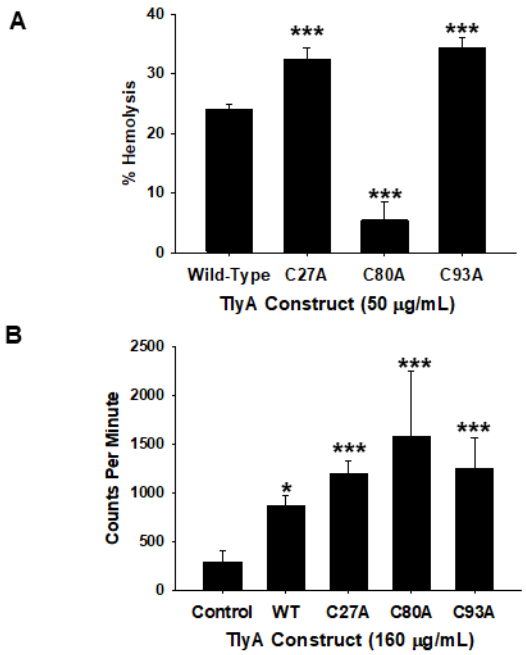


Figure 3.4 Disulfide bond formation is required for hemolytic activity of TlyA-His, but not methyltransferase activity. A, effect of cysteine mutations on hemolytic activity of TlyA-His. Two cysteine mutants (C27A and C93A) possessed significantly greater hemolytic activity than WT TlyA-His, while hemolytic activity of C80A was significantly inhibited in comparison to WT TlyA-His (One Way-Anova, Holm-Sidak Post-Hoc, *** indicates significant difference from WT TlyA-His at $p < 0.001$, $n = 5$ per group, data are presented as mean + SEM). B, effect of cysteine mutations on methyltransferase activity of TlyA-His. All cysteine mutants (C27A, C80A, and C93A) were more efficient at methylating rRNA than WT TlyA-His (One Way-Anova, Holm-Sidak Post-Hoc, * indicates significant difference from no TlyA control at $p < 0.05$, *** indicates significance at $p < 0.001$, $n = 4$ per group, data are presented as mean + SEM).

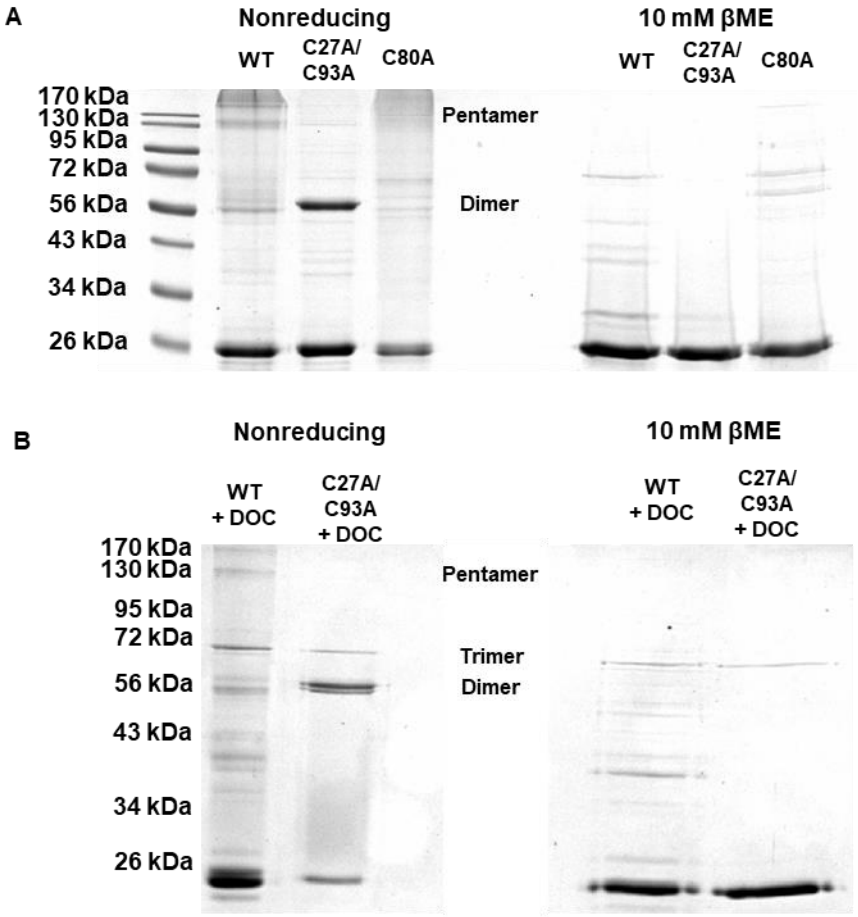


Figure 3.5. Dimerization of TlyA-His is dependent on a C80-C80 disulfide bond. *A*, oligomerization of wild-type TlyA and TlyA cysteine mutants in response to heat treatment. WT TlyA-His, C27A/C93A TlyA-His, and C80A TlyA-His (~500 ng/lane) were diluted 1:1 with 2x Laemelli sample buffer lacking SDS and incubated at 100°C for 5 minutes in the presence or absence of 10 mM βME as indicated. In the absence of βME C27A/C93A TlyA-His readily dimerized, while small amounts of dimeric WT TlyA-His were detected. Redox sensitive pentamers and higher order oligomers (≥ 130 kDa) were readily formed by WT and C80A TlyA-His, but not by C27A/C93A TlyA-His. *B*, oligomerization of WT TlyA-His and C27A/C93A TlyA-His in the presence of DOC micelles. Proteins were dialyzed overnight against PBS containing 0.1% (w/v) DOC and the resulting protein (~500 ng/lane) was diluted 1:1 with 2x Laemelli sample buffer lacking SDS and incubated at 25°C for five minutes in the presence or absence of 10 mM βME as indicated. C27A/C93A TlyA-His readily formed both dimeric and trimeric species in the absence of βME, however treatment with βME resulted in the degradation of dimeric species in both WT and C27A/C93A TlyA-His and the pentameric and higher order species (≥130 kDa) formed by WT TlyA-His.

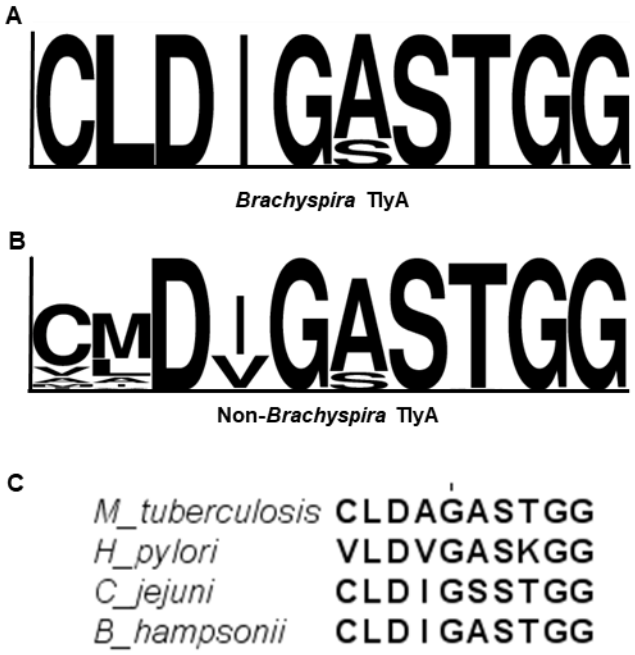


Figure 3.6 Conservation of cysteine 80 and the corresponding SAMe binding motif in TlyA proteins from *Brachyspira* and other bacteria. *A*, consensus logo of conservation of amino acids 80-89 in *Brachyspira* TlyA sequences [438]. *B*, consensus logo of conservation of amino acids 80-89 in non-*Brachyspira* TlyA sequences. The top 500 non *Brachyspira* TlyA homologues of *Brachyspira hampsonii* TlyA were obtained via BLAST search. *C*, multiple sequence alignment of amino acids 80-89 of TlyA from clinically relevant bacteria species. TlyA proteins from *Mycobacterium tuberculosis* (WP_055352317.1), *Helicobacter pylori* (WP_052936759.1), *Campylobacter jejuni* (WP_044305887.1), and *Brachyspira hampsonii* (WP_069726089.1) were aligned using the MUSCLE algorithm [439].

### 3.3.4 A serine to lysine mutation found in nonpathogenic *Brachyspira* spp. abolishes hemolytic activity of TlyA

The results of our site-directed mutagenesis and semi-native SDS-PAGE experiments suggested that both intermolecular disulfide bonding and non-covalent interactions are necessary for the proper oligomerization and pore-formation by *Brachyspira hampsonii* TlyA. As such, we hypothesized that additional intermolecular interactions were involved in TlyA oligomerization. We hypothesized that the weak hemolytic phenotype exhibited by less virulent members of the *Brachyspira* genus was a result of mutations in the *tlyA* gene, and that these mutations may provide further information on the mechanism of TlyA oligomerization. Bioinformatics analyses revealed that the *tlyA* gene was present in all nine *Brachyspira* species (Figure 3.7A), with the TlyA protein possessing 76.25% (183/240) amino acid identity between all species. Two notable mutations were present within the N-terminal S4 domain of TlyA (AAs 1-50). The virulent swine dysentery species *Brachyspira hampsonii* and *Brachyspira hyodysenteriae* possessed a serine at position 9 which was mutated to a glycine in *Brachyspira suanatina*, *Brachyspira intermedia*, and *Brachyspira aalborgi*, a histidine in *Brachyspira pilosicoli*, an isoleucine in *Brachyspira alvinipulli*, and a lysine in *Brachyspira innocens*, *Brachyspira murdochii*, and *Brachyspira aalborgi* (Figure 3.7A). Additionally, *Brachyspira hampsonii* and *Brachyspira hyodysenteriae* possessed a histidine at position 40 which was mutated to a tyrosine in *Brachyspira suanatina* and *Brachyspira intermedia* and a glutamine in *Brachyspira innocens/murdochii/alvinipulli/pilosicoli*. Site directed mutagenesis was used to mutate serine 9 to lysine (S9K TlyA-His) and histidine 40 to glutamine (H40Q TlyA-His). In both cases the mutations from *Brachyspira innocens/murdochii/aalborgi* were chosen due to the fact that these bacteria are weakly hemolytic and are only capable of causing mild disease [440, 441] in comparison to the strongly β-hemolytic, highly virulent *Brachyspira hampsonii/hyodysenteriae*

[6, 91, 424, 426, 427, 442]. The S9K and H40Q TlyA mutants were expressed and purified with a C-terminal His-tag as described previously, and the hemolytic activity of the mutants against pig erythrocytes was quantified (Figure 3.7B). While the hemolytic activity of H40Q TlyA-His did not significantly differ from WT TlyA-His, a complete abolition of hemolysis was observed with the S9K TlyA-His mutant. To assess the effect of these mutations on the rRNA methyltransferase activity we once again performed the methylation assay with the S9K and H40Q TlyA-His mutants. Both S9K TlyA-His and H40Q TlyA-His retained rRNA methyltransferase activity at similar levels to that of WT TlyA-His protein (Figure 3.7C), suggesting that these amino acids do not play an important role in rRNA methylation by TlyA.

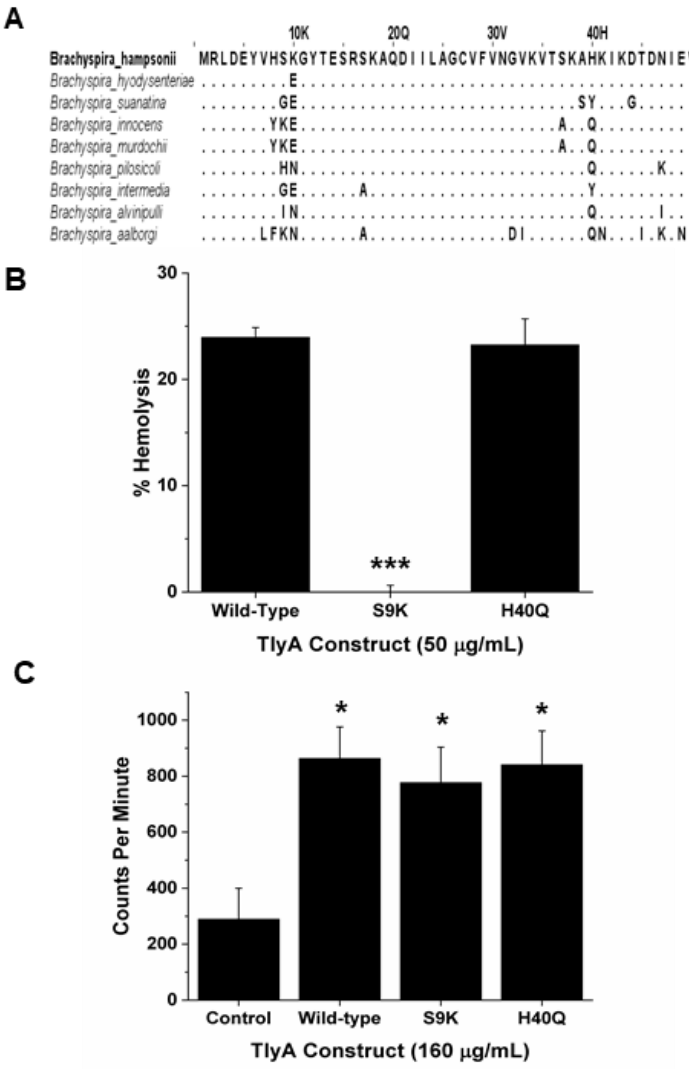


Figure 3.7 The S9K mutation present in TlyA of *Brachyspira innocens* and *Brachyspira murdochii* completely abolishes hemolytic activity of TlyA-His without affecting methyltransferase activity. *A*, multiple sequence alignment of the N-terminal S4 domain (AAs 1-50) of TlyA proteins from *Brachyspira* species. Two notable mutations (S9K and H40Q) are found when comparing the pathogenic, strongly β-hemolytic species *Brachyspira hampsonii* and *Brachyspira hyodysenteriae* and the nonpathogenic, weakly β-hemolytic species *Brachyspira innocens* and *Brachyspira murdochii*. *B*, hemolytic activity of S9K TlyA-His and H40Q TlyA-His. Hemolytic activity was completely abolished in the S9K TlyA-His mutant (One Way-Anova, Holm-Sidak Post-Hoc, *** indicates significant difference from WT TlyA-His at $p < 0.001$, $n = 5$ per group, data are presented as mean + S.D.). *C*, rRNA methyltransferase activity of S9K TlyA-His and H40Q TlyA-His. Both the S9K and H40Q TlyA-His were capable of methylating rRNA (One Way-Anova, Holm-Sidad Post-Hoc, * indicates significant difference from no TlyA control at $p < 0.05$, $n = 4$ per group, data are presented as mean + S.D.).

### 3.3.5 The S9K mutation impairs oligomerization of TlyA

In order to exert their cytotoxic effect, pore-forming toxins must first oligomerize before inserting into the host cell membrane [443, 444]. As such, we hypothesized that TlyA oligomerization and subsequent pore-formation were dependent on both serine 9 and cysteine 80. To assess the effect of the S9K mutation on TlyA oligomerization we utilized dimer dilution isothermal titration calorimetry (ITC), a method wherein highly concentrated (primarily oligomeric) protein is diluted into protein free buffer, enabling quantification of the thermodynamics of protein dimerization [445, 446]. WT or S9K TlyA-His at an initial concentration of 50 µM in a reducing buffer containing 1 mM TCEP was diluted into a reaction cell containing only buffer (Figure 3.8A/B) and the resulting enthalpies of dilution were integrated (Figure 3.8C/D). In both cases data analysis was complicated by a significant increase in dilution enthalpy at injection 12. Given that these abnormal injections were consistently observed, we hypothesized that these aberrations represented aggregation of monomeric TlyA-His back into dimers or higher order oligomers. Despite this, the enthalpies of dilution for WT TlyA-His were significantly more exothermic than those observed for S9K TlyA-His and fitting our data to a dimer dissociation model provided dimer dissociation constants ($K_D$) of 5.47 µM for WT TlyA-His versus 14.8 µM for S9K TlyA-His, suggesting that oligomerization is impaired in the S9K mutant. To further confirm oligomerization, we then analyzed the oligomerization of TlyA utilizing size-exclusion chromatography. At an initial concentration of 1 mg/mL (equivalent to 35.7 µM) WT TlyA-His eluted in two peaks at 68 mL and 76 mL, corresponding to trimeric and monomeric protein respectively, while S9K TlyA-His eluted in a broad single peak at 76 mL, indicating that this mutant was primarily monomeric at the concentration tested (Figure 3.9). Taken together, these data indicate that the S9K mutant is unable to properly

oligomerize *in vitro*, providing an explanation for the lack of hemolytic activity observed with this mutant in weakly pathogenic bacteria. Comparing the sequence of *Brachyspira hampsonii* TlyA to those of other TlyA$^{I}$ family proteins reveals that serine 9 is also conserved in both *Campylobacter jejuni* and *Campylobacter lari* (Figure 3.10), suggesting a conserved mechanism of oligomerization in these proteins.

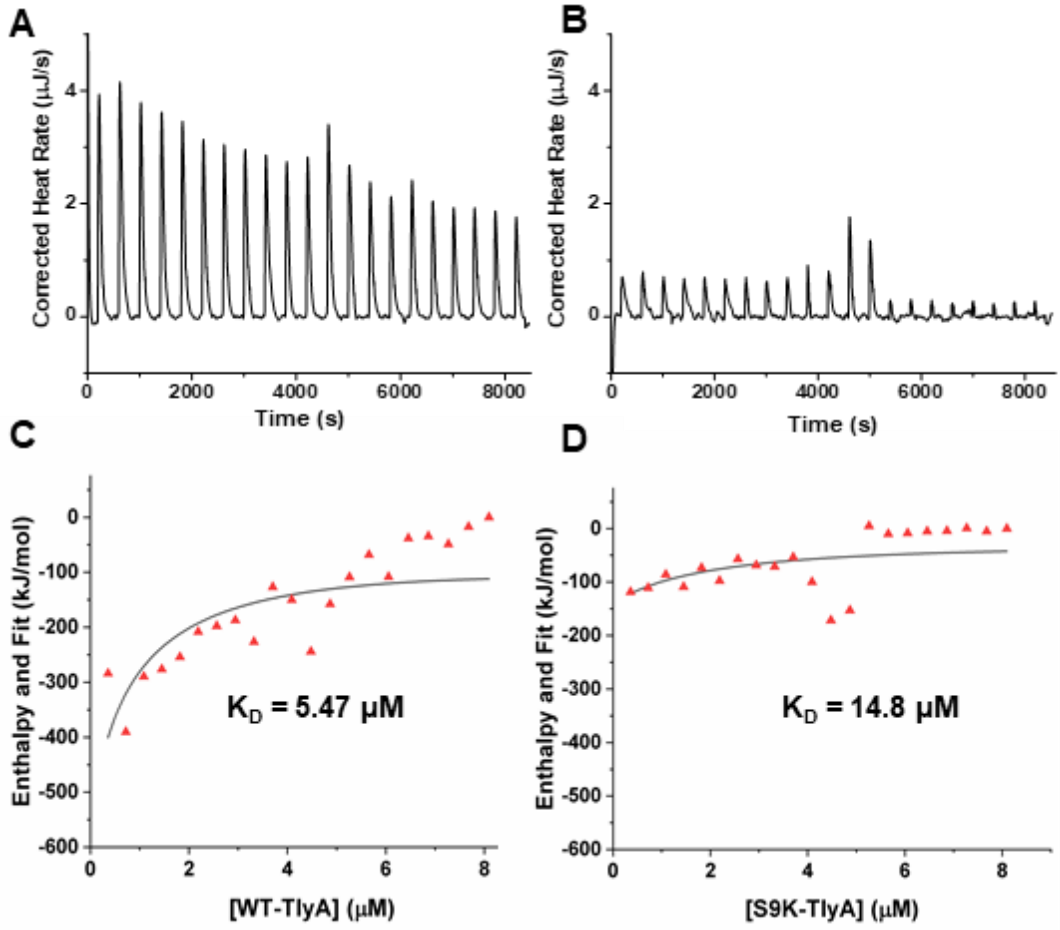


Figure 3.8 Oligomerization of TlyA-His is negatively affected by the S9K mutation. A, ITC thermogram of dilution of WT TlyA-His (50 μM) into buffer. B, ITC thermogram of dilution of S9K TlyA-His (50 μM) into buffer. C, fitting of integrated enthalpies of dilution of WT TlyA-His to a dimer dissociation model. D, fitting of integrated enthalpies of dilution of S9K TlyA-His to a dimer dissociation model.

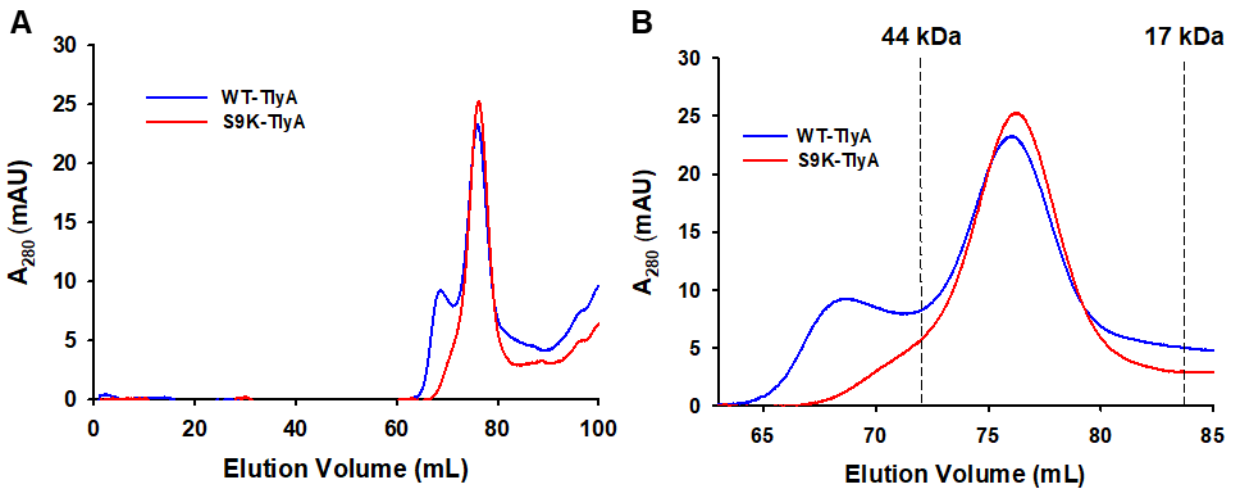


Figure 3.9 Size-exclusion chromatography elution profiles of WT and S9K TlyA-His. A, 2 mL of a 1 mg/mL protein solution was separated on a Superdex 200 pg size-exclusion column, and the elution profiles of WT (Blue) and S9K TlyA-His (Red) were compared to that of a set of gel-filtration standards as indicated in the inset from 65 to 85 mL elution volume (B).

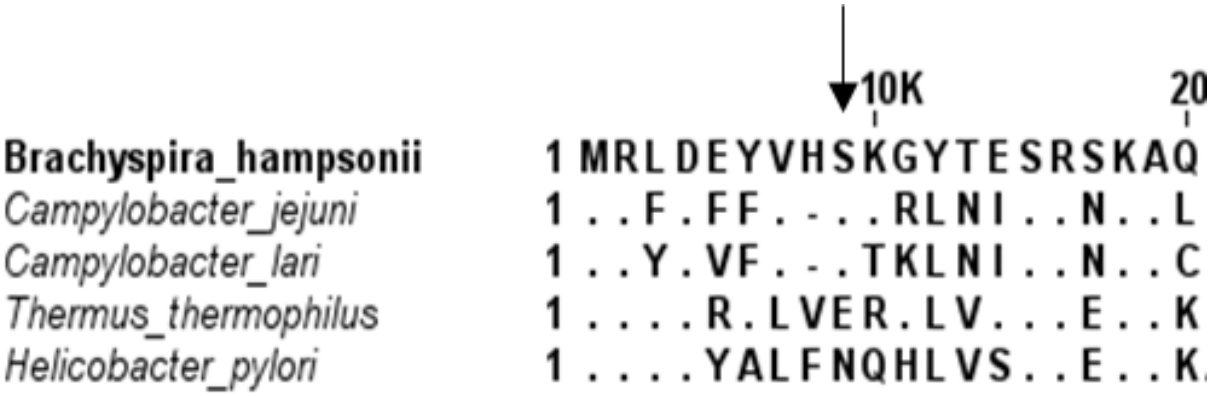


Figure 3.10 Conservation of serine 9 in previously described TlyA$^{I}$ family members. TlyA proteins from *Brachyspira hampsonii (*WP_069726089.1), *Campylobacter jejuni* (WP_044305887.1), *Campylobacter lari* (ECK2066597.1), *Thermus thermophilus* (BAW01290.1), and *Helicobacter pylori* (WP_052936759.1) were aligned using the MUSCLE algorithm [50]. The position of serine 9 is indicated by an arrow.

## 3.4 Discussion

### 3.4.1 A cysteine residue conserved in TlyA proteins from pathogenic bacteria is required for oligomerization and hemolytic activity

In this study we aimed to assess the *in vitro* functionality of TlyA from *Brachyspira hampsonii* to further understand the contribution of this toxin to virulence *in vivo*. While previous studies have confirmed the hemolytic functionality of TlyA proteins from bacterial pathogens such as *Mycobacterium tuberculosis* [18], *Helicobacter pylori* [436], and *Campylobacter jejuni* [17], no conserved mechanism for oligomerization and hemolysis of TlyA proteins has been identified to date. While two cysteine residues (C124 and C128) have been identified in intermolecular disulfide bonding in *Helicobacter pylori* TlyA [419], these cysteines are not conserved across other TlyA sequences. In addition to identifying a cysteine (C80) involved in disulfide bond formation in *Brachyspira hampsonii* TlyA that is highly conserved across bacterial TlyA sequences, we also identified a conserved serine residue (S9) required for oligomeric assembly of the TlyA pore in several $TlyA^{I}$ family members. These results identify a potentially conserved mechanism of oligomerization for $TlyA^{I}$ family proteins, in addition to correlating changes in the TlyA primary sequence to virulence in *Brachyspira* spp.

To exert their physiological effects, pore-forming toxins must assemble into an oligomeric state prior to inserting into the host cell membrane [157, 159]. While previous studies have provided evidence that *Mycobacterium tuberculosis* TlyA oligomerizes into a heptameric pore on the membranes of both erythrocytes and phagosomes [18], the mechanisms of oligomeric assembly of the *Brachyspira hampsonii* TlyA pore are not known. While we were unable to detect oligomeric forms of TlyA-His utilizing both Western blotting and conventional denaturing SDS-PAGE (data not shown), the use of a semi-native SDS-PAGE system enabled detection of multiple TlyA-His oligomers in, ranging from dimers to pentamers and larger aggregates (Figure

2A). Treatment of these samples with the reducing agent β-mercaptoethanol resulted in a near complete destruction of these higher order oligomers (Figure 3.3A), indicating that these species are the result of intermolecular disulfide bonding. Interestingly, this oligomerization was only observed upon boiling the protein, suggesting that while this oligomerization may occur during the assembly of the TlyA pore on the host cell membrane, it does not readily occur in solution at physiologically relevant temperatures. As such, we next dialyzed TlyA-His protein against PBS containing 0.1% Na-DOC, a bile salt capable of forming micelles in solution at concentrations ≥2 mM [447]. Semi-native SDS-PAGE analysis of TlyA-His protein treated in this manner revealed not only the presence of higher order oligomers in samples that had not been heated above room temperature (Figure 3.3B), but also the existence of a trimeric TlyA species resistant to the effects of reducing agents. While these data do not provide an unambiguous picture of the stoichiometry of the oligomeric TlyA pore, we hypothesize that the disulfide bonded dimer and non-covalently associated trimer may represent intermediates in the oligomerization of the TlyA pore, leading to the final assembly of a mature hexameric or nonameric pore in the membrane of the host cell. Taken together, these results suggested that the *in vivo* oligomerization of *Brachyspira hampsonii* TlyA was dependent on both intermolecular disulfide bonding and non-covalent interactions, leading us to further examine the mechanics of this oligomerization through site-directed mutagenesis studies.

In previous studies reducing agents have been shown to inhibit the hemolytic activity of TlyA proteins from *Mycobacterium tuberculosis* [18] and *Helicobacter pylori* [419]. Addition of β-mercaptoethanol to reaction mixtures containing WT TlyA-His and pig erythrocytes resulted in a decrease in hemolytic activity (Figure 3.1E) similar in magnitude to those observed in similar experiments involving *Mycobacterium tuberculosis* [18] and *Helicobacter pylori* [419]. While

the specific cysteine residue(s) involved in disulfide bond formation have not yet been determined for *Mycobacterium tuberculosis*, mutagenesis studies in *Helicobacter pylori* TlyA have shown that two cysteine residues, Cys124 and Cys128, are required for intermolecular disulfide bond formation [419]. Mutation of either of these cysteines to serine resulted in a complete loss of hemolytic activity [419]. Surprisingly, cysteines were not present at these positions in *Brachyspira hampsonii* TlyA, with this protein instead possessing three cysteines at positions 27, 80, and 93. Of the three *Brachyspira hampsonii* TlyA-His cysteine mutants created in this study (C27A, C80A, and C93A), only C80A TlyA-His exhibited lower levels of hemolytic activity than WT TlyA-His (Figure 3.4A). The hemolytic activity of C80A TlyA-His was 4.3 fold lower than WT TlyA-His, a decrease similar in magnitude to the 3.9 fold observed upon addition of β-mercaptoethanol (Figure 3.1E/3.4A). Surprisingly, increases in hemolytic activity were observed for C27A TlyA-His and C93A TlyA-His, and we hypothesize that this is due to a decrease in aggregation and subsequent inactivation of TlyA due to improper disulfide bond formation involving cysteines 27 and 93. Semi-native SDS-PAGE analyses provided further evidence to support this hypothesis, as evidenced by the fact that the C27A/C93A TlyA-His mutant was unable to form the high molecular weight (≥170 kDa) aggregates observed for both WT TlyA-His and C80A TlyA-His (Figure 3.5A). Furthermore, the finding that the C27A/C93A TlyA-His mutant was able to efficiently form a dimer under nonreducing conditions (Figure 3.5B) provides additional evidence that a C80-C80 intermolecular disulfide bond is formed during oligomerization and pore formation of *Brachyspira hampsonii* TlyA. Cysteine 80 is located immediately upstream of a highly conserved region of TlyA annotated as a SAMe binding motif [422]. Analysis of TlyA primary sequences reveals that cysteine 80 is not only universally conserved in TlyA proteins from the genus *Brachyspira*, but also in 71.9% of the top

500 non *Brachyspira* homologues available on GenBank, including *Mycobacterium tuberculosis* and *Campylobacter jejuni* (Figure 6). While this cysteine is substituted with a valine in *Helicobacter pylori*, mutational studies involving *Helicobacter pylori* TlyA have suggested that the oligomerization of this protein involves disulfide bond formation between cysteines at positions 124 and 128 [419], residues not found in other TlyA proteins. These data provide the first link between a highly conserved residue of TlyA and the hemolytic function of this protein. These observations also suggest that two distinct mechanisms of intermolecular disulfide bond formation by TlyA exist, involving a C80-C80 disulfide bond in *Brachyspira hampsonii*, *Mycobacterium tuberculosis*, and *Campylobacter jejuni*, and a C124-C128 disulfide bond in *Helicobacter pylori* [419].

### 3.4.2 Serine 9 is correlated with in vitro hemolytic activity in TlyA$^{I}$ family proteins

Pore-forming hemolysins are secreted from bacteria as soluble monomers which then diffuse to the host cell membrane, bind to a receptor on the host cell surface, oligomerize, and insert into the host cell membrane to form a solvent accessible pore [443, 444]. As previously mentioned, reducing agents and the C80A mutation both inhibited hemolytic activity to a similar degree (Figure 3.1E/3.4A) but were unable to abolish hemolytic activity completely. These observations, along with the fact that our mutagenesis studies indicated that only a single C80-C80 disulfide bond was necessary for hemolytic activity (Figure 3.4A/3.5A), suggested that additional amino acid residues were required for formation of higher order oligomers and subsequent pore formation by *Brachyspira hampsonii* TlyA. This in addition to the complete abolishment of hemolytic activity in N-terminal his-tagged TlyA (Figure 3.1C) focused our attention on the N-terminal region. Comparing the strongly β-hemolytic, virulent swine dysentery species *Brachyspira hampsonii, Brachyspira hyodysenteriae*, and *Brachyspira suanatina* [6, 7, 91, 99, 442] to the weakly β-hemolytic species *Brachyspira innocens*,

*Brachyspira murdochii,* and *Brachyspira aalborgi* [440, 441] revealed two notable amino acid substitutions in the N-terminal S4 domain of TlyA: serine to lysine at position 9 and histidine to glutamine at position 40 (Figure 3.7A). Site directed mutagenesis was used to create two TlyA-His mutants (S9K TlyA-His and H40Q TlyA-His) based on these results. Expression and purification of these constructs revealed that while the H40Q mutation had no effect on the hemolytic activity of TlyA-His, the S9K mutation resulted in a complete loss of hemolytic activity (Figure 3.7B). Given that neither of these mutations inhibited rRNA methyltransferase activity (Figure 3.7C) we hypothesized that serine 9 of *Brachyspira hampsonii* TlyA plays a critical role in the assembly of the oligomeric TlyA pore To test this hypothesis, we first attempted to quantify differences in solution dimerization between WT and S9K TlyA-His by dimer dilution ITC. While these experiments were complicated by the likely aggregation of both WT and S9K TlyA-His within the reaction cell (Figure 3.8A/B), dilution of WT TlyA-His was considerably more exothermic than S9K TlyA-His, suggesting a higher portion of the wild-type protein existed in an oligomeric state before dilution. Furthermore, analytical size-exclusion chromatography indicated that WT TlyA-His was capable of forming both monomeric (calculated MW of ~29 kDa) and trimeric species in solution (calculated MW of ~68 kDa) in contrast to the S9K TlyA-His mutant which was only present as a monomer under the given conditions (Figure 3.9). These results are in agreement with those obtained by semi-native SDS-PAGE of TlyA protein which indicated that TlyA can form redox-stable trimers in the presence of Na-DOC micelles (Figure 3.3B/3.5B). Looking specifically at $TlyA^{I}$ family proteins (TlyA proteins with shortened N and C termini capable of methylating only C1920 of 23s rRNA [338]), this serine is conserved in *Campylobacter jejuni* and *Campylobacter lari* and substituted with a glutamate in *Thermus thermophilus* (Figure 3.10). While serine was not present at this position in

*Helicobacter pylori*, a conservative substitution of asparagine was observed. Interestingly, TlyA proteins from both *Campylobacter jejuni* and *Camplyobacter lari* are hemolytic when heterologously expressed in *E. coli* [448] while TlyA from *Thermus thermophilus* is not [449], providing further evidence that serine 9 is indispensable for oligomerization and hemolytic activity. The conservative serine to asparagine substitution found in the hemolytic TlyA protein from *Helicobacter pylori* suggests that this residue may be involved in intermolecular hydrogen bonding, although further study is required to confirm this.

### 3.4.3 Loss of TlyA's hemolytic function does not impair rRNA methyltransferase activity

In addition to functioning as pore-forming hemolysins, TlyA proteins also function as rRNA methyltransferases, Specifically, TlyA proteins methylate nucleotides C1409 of 16s rRNA and C1920 of 23s rRNA [336, 338, 420-422, 450]. TlyA proteins capable of methylating both nucleotides are considered to be members of the $TlyA^{II}$ family while $TlyA^{I}$ family members only methylate the C1920 position [338]. While the biological relevance of TlyA encoded rRNA methylations is not yet fully understood, recent evidence has suggests that these rRNA modifications confer a variety of physiological benefits to the host bacterium with tlyA knockouts of *Campylobacter jejuni* exhibiting decreased biofilm formation, motility, and virulence [345, 346]. We thus hypothesize that the S9K mutation found in the weakly hemolytic *Brachyspira innocens*, *Brachyspira murdochii*, and *Brachyspira aalborgi* may thus represent a mechanism by which these bacteria can maintain the advantageous rRNA methyltransferase activity of TlyA without damaging their host.

In summary, this study has shown that TlyA from *Brachyspira hampsonii* functions as both a rRNA methyltransferase and a pore-forming hemolysin dependent on both cystine and intermolecular bonds. The oligomerization need for the hemolytic activity of TlyA is dependent

on both an intermolecular C80-C80 disulfide bond and noncovalent interactions involving serine 9, as mutation of both cysteine 80 to alanine or serine 9 to lysine inhibited or completely abolished the hemolytic activity of TlyA. Isothermal titration calorimetry and size exclusion chromatography experiments revealed that the S9K mutation inhibited the ability of TlyA to spontaneously dimerize in solution, providing a partial explanation for the complete abolition of hemolytic activity observed with this mutant. Taken together these results provide important information on the dynamics of oligomeric assembly and hemolytic activity of TlyA from *Brachyspira hampsonii* in addition to homologues from other bacteria such as *Mycobacterium tuberculosis* and *Campylobacter jejuni*. Additionally, the finding that non-pathogenic *Brachyspira* species possess a mutation known to completely abolish TlyA's hemolytic activity without affecting rRNA methyltransferase activity (S9K) suggests an evolutionary pressure on these commensal bacteria to maintain the rRNA methyltransferase activity without causing damage to the host organism.

### 3.5 Conclusion

Despite first being identified in *Brachyspira hyodysenteriae* nearly 30 years ago, the vast majority of research into TlyA family proteins has been carried out in human pathogens such as *Mycobacterium tuberculosis* and *Helicobacter pylori*. As such, our objective was to clone, overexpress, purify, and characterize the hemolytic activity of TlyA from *Brachyspira hampsonii*, in order to better understand the contributions of this protein to the overall pathology of swine dysentery. Alongside this specific objective, we hypothesized that mutations within the primary amino acid sequence of TlyA could be predictive of bacterial pathogenicity not only in

*Brachyspira* species, but TlyA expressing bacteria as a whole. In addition to confirming that *Brachyspira hampsonii* TlyA does in fact possess the hemolytic and rRNA methyltransferase functionalities described for other TlyA family proteins, the hemolytic activity of TlyA was shown to be dependent on both serine 9 and cysteine 80. Remarkably, both of these residues are conserved in TlyA proteins from a wide variety of pathogenic bacteria, lending support to our previously mentioned hypothesis. However, our study was limited by the fact that the activity of the TlyA mutants was only assessed *in vitro*, and experimental infection of pigs with a *Brachyspira hampsonii* mutant possessing the C80 or S9K mutations within the TlyA gene is required to confirm that the loss of hemolytic activity observed *in vitro* translates to a decrease in pathogenicity *in vivo*. Although these results confirm that *Brachyspira hampsonii* TlyA is a pore forming hemolysin, the high concentrations and long incubation times required for hemolysis with this protein suggests that *Brachyspira hampsonii* also possesses a more potent hemolysin. As such, the remainder of my doctoral research aimed to identify the hemolysin or hemolysins responsible for the strong β-hemolytic phenotype possessed by *Brachyspira hampsonii* and identify the gene or genes responsible.

# Chapter 4 – Functional Comparison of the Native Hemolysin of *Brachyspira hampsonii* to an SLS-like Peptide Produced by Pathogenic *Brachyspira hampsonii* Strains

**Brandon A. Keith[1], Lisa A. Johnson[2], Janet E. Hill[2], John C.S. Harding[3], Matthew E. Loewen[1*]**

[1]Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

[2]Deparatment of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

[3]Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

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Infection of pigs by *Brachyspira hyodysenteriae* or *Brachyspira hampsonii* can lead to swine dysentery, a serious disease responsible for massive production losses in infected herds. A positive correlation between a strong β-hemolytic phenotype and pathogenicity has been observed in *Brachyspira* isolates, suggesting that production of one or more hemolysins by these bacteria contributes to the development of the mucohaemmorhagic diarrhea characteristic of swine dysentery. While several putative hemolysin genes have been identified within the genomes of *Brachyspira hyodysenteriae* and *Brachyspira hampsonii*, the most potent hemolysin produced by these bacteria appears to be native hemolysin, a toxin produced upon addition of yeast RNA core to *Brachyspira hyodysenteriae* broth cultures. While the native hemolysin has been previously linked to a gene termed *hlyA*, the discovery of an operon encoding for a streptolysin S homologue within the genome of a pathogenic *Brachyspira hampsonii* strain has raised doubts on the identity of this toxin. To determine if the native hemolysin produced by *Brachyspira hampsonii* is in fact a streptolysin S homologue, the *sagABCD* genes from *Brachyspira hampsonii* were cloned, expressed, and purified from an *E. coli* host. While both the *Brachyspira hampsonii* native hemolysin and the purified SagA peptide were hemolytic against pig erythrocytes, the specific activity of the SagA peptide was several orders of magnitude lower than that of the native hemolysin. Additionally, both size exclusion chromatography and tris-tricine SDS-PAGE showed the native hemolysin and SagA peptide to be approximately the same size. While these data provide strong evidence that the *Brachyspira hampsonii* native hemolysin is a streptolysin S homologue, further analyses are required to definitively prove the identity of this toxin.

## 4.1 Introduction

Infection of pigs by *Brachyspira hyodysenteriae* or *Brachyspira hampsonii* causes a production limiting mucohaemmorhagic diarrhea known as swine dysentery [1-3, 73]. Although the precise mechanism by which these bacteria cause disease is not fully understood, the observation that pathogenic strains of *Brachyspira hyodysenteriae* or *Brachyspira hampsonii* exhibit strong β-hemolysis on blood agar has long lead to speculation that production of one or more hemolysins are necessary for swine dysentery pathogenesis [2, 6, 79, 114, 319]. At the time of writing four hemolysin genes have been identified in *Brachyspira hyodysenteriae* and *Brachyspira hampsonii*, termed TlyA/B/C and HlyA [13, 14, 323]. While TlyA homologues have been shown to play an important role in bacterial diseases such as *tuberculosis* [15, 330, 331] and *Helicobacter pylori* induced gastric ulceration [16, 218], *in vitro* studies involving TlyB and TlyC homologues from *Leptospira interrogans* have suggested that these proteins have been misannotated as hemolysins [359]. The *hlyA* gene was originally linked to a hemolytic peptide produced by *Brachyspira hyodysenteriae* broth cultures using N-terminal sequencing, however, the *hlyA* gene product does not possess appreciable homology to any known hemolysins [14]. Rather, the fact that the HlyA peptide is structurally related to acyl carrier proteins involved in fatty acid biosynthesis [360], combined with the observation that HlyA is highly abundant in broth cultures of both *Brachyspira hyodysenteriae* and the moderately virulent *Brachyspira pilosicoli* [451] suggest that the *hlyA* gene may also have been misannotated as a hemolysin. As such, the true identity of the hemolysin or hemolysins contributing to the β-hemolytic phenotype of *Brachyspira hyodysenteriae* and *Brachyspira hampsonii* remains unknown.

Several independent research groups purified a hemolytic molecule, referred to as native hemolysin in this manuscript, from *Brachyspira hyodysenteriae* broth cultures in the 1980s [318, 319, 322]. Although these reports disagreed on the molecular weight of the isolated hemolysin, similarities in the potency and susceptibility of the native hemolysin to enzymes such as pronase indicates that these groups had likely purified the same toxin [318, 319, 322]. Although the native hemolysin was later linked to a gene termed hlyA as described previously [14], both the high specific activity of the native hemolysin and the fact that high levels of native hemolysin expression can be induced by the addition of yeast RNA core are similar to previous reports on streptolysin S, a hemolysin produced by *Streptococcus pyogenes* [19, 320, 321, 369]. Furthermore, the recent discovery of a sag operon within the *Brachyspira hampsonii* 30446 genome encoding for a putative SLS homologue and several accessory genes required for modification of the SLS precursor into an active peptide [20] lead us to hypothesize that the native hemolysins produced by *Brachyspira hyodysenteriae* and *Brachyspira hampsonii* may in fact be an SLS homologue. In this study we report that like *Brachyspira hyodysenteriae*, *Brachyspira hampsonii* strain 30446 produces a highly potent hemolysin upon the supplementation of broth cultures with yeast RNA core, along with the finding that co-expression of the SagABCD genes from *Brachyspira hampsonii* 30446 produces a hemolytic peptide. While both toxins possessed hemolytic activity against pig erythrocytes, the specific activity of the heterologously expressed SagA peptide was several orders of magnitude lower than that observed for the *Brachyspira hampsonii* native hemolysin (222 HU/mg SagA protein vs $3.2*10^5$ HU/mg native hemolysin protein). While we were unable to definitively determine that the *Brachyspira hampsonii* native hemolysin to the SagA gene product, the similar molecular weights observed for both hemolysins via size exclusion chromatography and SDS-PAGE

analyses provide compelling evidence that these toxins are one and the same. Furthermore, efforts to optimize the SagA expression and purification protocols succeeded in increasing yields of the SagA peptide from ~50 µg/L of *E. coli* culture in our initial experiments to ~1.4 mg/L of *E. coli* culture in later experiments, enabling both nuclear magnetic resonance studies of the structure of the SagA peptide and the assessment of this toxin as a vaccine candidate in future studies.

## 4.2 Materials & Methods

### 4.2.1 Bacterial culture

Glycerol stocks of *Brachyspira hampsonii* strain 30446 [6] were inoculated into 10 mL of brain heart infusion broth (BHI) (BD – DF0418-15) containing 10% heparinized sheep's blood. Cultures were incubated anaerobically at 42 °C using a GasPak system (BD – B260001) for 3 days. Growth was monitored daily through visual observation of gas produced by the growing culture and microscopic examination. Cultures in log phase were then subcultured into BHI + 10% fetal bovine serum (BHIS) (Gibco – 26140087) and grown for an additional 3 days. Native hemolysin production was induced by the addition of yeast RNA core (Sigma-Aldrich – R6875) to a final concentration of 0.5 mg/mL and cultures were incubated overnight at 42 °C prior to purification.

### 4.2.2 Purification of *Brachyspira hampsonii* native hemolysin

*Brachyspira hampsonii* 30446 cultures induced with yeast RNA core were centrifuged for 20 minutes at 10,000 x g, after which point the supernatant was filtered through a 0.2 µm filter. Filtered cell-free supernatant was acidified to a pH of ~2.0 by the addition of 6.0 N HCl until a cloudy precipitate formed and the cell-free supernatant was centrifuged for 20 minutes at 10,000 x g. The resulting pellet was dissolved in 100 mM $NaPO_4$ buffer (pH 7.4) and loaded onto an equilibrated Resource Q anion exchange column (Cytiva Life Sciences – 17117901). The column was washed with 5 column volumes of running buffer (10 mM HEPES pH 7.4, 10 mM KCl), after which point proteins were eluted with a linear gradient from 0-60% elution buffer over 10 column volumes (10 mM HEPES pH 7.4, 1000 mM KCl), followed by 5 column volumes of 60% elution buffer. A sharp peak eluted during the 60% elution buffer wash step was then loaded onto a pre-equilibrated Superdex 200 pg 16/600 column (Cytiva Life Sciences - 28989335) and eluted with 1 column volume of PBS at a flow rate of 0.75 mL/min.

### 4.2.3 Hemolysis assays

Whole heparinized pig's blood was obtained from healthy animals and washed with 3 volumes of PBS. Washed blood was centrifuged for 5 minutes at 2,000 x g, the plasma and buffy coat were removed by aspiration, and the wash and centrifugation steps were repeated twice more. Washed erythrocytes were then suspended in PBS to a final concentration of 2% (v/v). Twofold serial dilutions of the native hemolysin purified by size exclusion chromatography were added to an equal amount of the pig erythrocyte suspension and incubated at 37 °C for 1 hour, after which point the cells were pelleted by centrifugation for 5 minutes at 2,000 x g and hemoglobin release into the supernatant was quantified by spectrophotometric detection at 545 nm. Percent hemolysis was calculated by comparison of test samples to a negative control (PBS only, 0% hemolysis) and a positive control (erythrocytes lysed in distilled water, 100% hemolysis). Each dose in the dilution series was assayed in triplicate. One hemolytic unit (HU) was defined as the reciprocal of the highest dilution capable of causing 50% hemolysis in this assay, and was used as a standardized measure of hemolysin activity between preparations.

### 4.2.4 Cloning of *sag* operon genes from *Brachyspira hampsonii* and *Brachyspira hyodysenteriae*

All genes were chemically synthesized by Genscript. The His-MBP-*sagA* gene was digested with NdeI and XhoI and ligated into a pET-DUET1 expression vector, while the *sagD* gene was digested with BamHI and NotI and ligated into the same pET-DUET1 expression vector at a differing MCS (Figure 4.2). The *sagB* gene was digested with NcoI and NotI and ligated into a pRSF-DUET1 vector, while the *sagB* gene was digested with NdeI and XhoI and ligated into the same vector at a differing MCS (Figure 4.2). The His-*sagA* construct was created by amplification of the *sagA* gene from the pET-DUET1 vector utilizing specific primers containing an NdeI restriction site on the forward primer and an XhoI restriction site on the reverse primer (Table 4.1). The resulting PCR product was then digested with NdeI and XhoI and ligated into a

pET-28a(+) expression vector. The TrpLE-*sagA* construct was chemically synthesized by Genscript, digested with NdeI and XhoI, and ligated into a pET-28a(+) expression vector containing an N-terminal His-tag. Success of all cloning reactions was verified by both vector sequencing and restriction digests.

### 4.2.5 Expression and purification of SagA constructs

The pET-DUET1 vector containing the His-MBP-*sagA* and *sagD* genes was transformed into Rosetta (DE3) cells alongside the pRSF-DUET1 vector containing the *sagB* and *sagC* genes and transformants were selected by culture on LB agar containing 50 µg/mL kanamycin, 100 µg/mL ampicillin, and 35 µg/mL chloramphenicol. A single, well-isolated colony was picked and incubated overnight at 37°C in LB media containing 50 µg/mL kanamycin, 100 µg/mL ampicillin, and 35 µg/mL chloramphenicol. 2YT media (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) was inoculated the next morning with the overnight culture and grown to an $OD_{600}$ of ~0.6, after which point protein expression was induced by the addition of 0.2 mM IPTG (Fisher BioReagents – BP1755) and allowed to proceed overnight at 18 °C. Cultures were harvested by centrifugation at 6,000 x g for 20 minutes, and the pellet was resuspended in Buffer A (10 mM HEPES, pH 7.4, 250 mM KCl) containing 10% glycerol, (Fisher Bioreagents – BP229), 50 µg/mL DNAse I (Sigma Aldrich - 11284932001), 50 µg/mL lysozyme (Sigma Aldrich - 1052810010), and 1 mM phenylmethylsulfonyl fluoride (PMSF, ThermoFisher Scientific - PI36978). The resuspended bacteria were disrupted by sonication, centrifuged at 39,410 x g for 30 minutes, and the resulting soluble fraction was loaded onto an equilibrated XK 26/20 column (Cytiva Life Sciences - 28988948) packed with $Ni^{2+}$ charged Profinity $Ni^{2+}$ IMAC resin (Bio-Rad - 156-0123). After washing with 10 column volumes of buffer A, followed by 5 column volumes of 5% buffer B (10 mM HEPES pH 7.4, 250 mM KCl, 500 mM imidazole) and 5 column volumes of 10% buffer B, protein was eluted with a linear gradient from 10% to 30%

buffer B over 10 column volumes. The resulting protein was loaded onto an equilibrated XK 26/20 column (Cytiva Life Sciences – 28988948) packed with amylose resin (New England Biolabs – E8021L). After washing with 10 column volumes buffer A, protein was eluted with 5 column volumes buffer C (10 mM HEPES pH 7.4, 250 mM KCl, 10 mM maltose). Eluted His-MBP-SagA protein was then treated with ~1 mg TEV protease and 0.5 mg/mL yeast RNA core overnight at 4°C, then purified by anion-exchange and size-exclusion chromatography as described previously for the *Brachyspira hampsonii* native hemolysin. Both the His-*sagA* and His-TrpLE-*sagA* constructs were transformed into Rosetta (DE3) cells and selected on LB agar containing 50 µg/mL kanamycin and 35 µg/mL chloramphenicol. Large scale cultures were grown in 2YT media as described previously, however cultures were incubated at 18°C for 3 hours after addition of IPTG before centrifugation. The resulting pellets were resuspended in Buffer A (10 mM HEPES, pH 7.4, 250 mM KCl) containing 10% glycerol, (Fisher Bioreagents – BP229), 50 µg/mL DNAse I (Sigma Aldrich - 11284932001), 50 µg/mL lysozyme (Sigma Aldrich - 1052810010), and 1 mM phenylmethylsulfonyl fluoride (PMSF, ThermoFisher Scientific - PI36978), lysed by sonication, and centrifuged at 39,410 x g for 30 minutes. The resulting pellet was then resuspended in wash buffer (100 mM Tris pH 7.0, 5 mM EDTA, 5 mM DTT, 2 M urea, 2% (w/v) Triton X-100) and centrifuged at 39,410 x g for 30 minutes. The washing step was repeated once more with wash buffer, then once again with wash buffer without Triton X-100. The pellets were then treated with ~40 mL extraction buffer (10 mM HEPES pH 7.4, 250 mM KCl, 6 M guanidine HCl) and incubated at 37°C overnight. The solubilized mixture was briefly sonicated and centrifuged at 39,410 x g to remove insoluble material, and loaded onto an equilibrated XK 26/20 column (Cytiva Life Sciences - 28988948) packed with $Ni^{2+}$ charged Profinity $Ni^{2+}$ IMAC resin (Bio-Rad - 156-0123). The column was

washed with 10 column volumes extraction buffer, and protein was eluted with a linear gradient from 0% to 30% Gnd-HCl elution buffer (10 mM HEPES pH 7.4, 250 mM KCl, 6 M guanidine HCl, 500 mM imidazole). Purified His-SagA was dialyzed against PBS overnight and used in downstream experiments, while His-TrpLE-SagA was dialyzed against distilled water. The resulting pellet was resuspended in 0.1 M HCl, and the His-TrpLE tags were removed by overnight treatment with ~50 mg of cyanogen bromide (Sigma-Aldrich - C91492). The CNBr treated mixture was dialyzed extensively against distilled water, and loaded onto a pre-equilibrated Superdex 200 pg 16/600 column (Cytiva Life Sciences - 28989335) and eluted with 1 column volume of PBS + 3 M guanidine HCl. The eluted SagA peptide was then dialyzed against PBS and used in downstream experiments.

### 4.2.6 Tris-tricine SDS-PAGE analysis of peptides

Purified native hemolysin and SagA peptides were diluted in 2x Lamelli buffer (4% SDS, 20% glycerol, 0.1 M Tris pH 6.8, and 0.005% bromophenol blue) + 10% β-mercaptoethanol and boiled for 5 minutes. Samples were then loaded onto a 12% polyacrylamide gel. Cathode buffer (0.1 M Tris, 0.1 M tricine, 0.1% SDS) was added to the gel chamber, while anode buffer (0.2 M Tris pH 8.9) was added to the buffer reservoir. Samples were electrophoresed at 130 volts for ~50 minutes until the dye front reached the end of the gel, and separated proteins were visualized by silver staining (Thermo Scientific – 24612).

## 4.3 Results

### 4.3.1 A potent hemolysin is produced by *Brachyspira hampsonii* broth cultures supplemented with yeast RNA core

Addition of yeast RNA core to broth cultures of *Brachyspira hyodysenteriae* induces the formation of a highly potent hemolysin, referred to as native hemolysin in this thesis [318, 319, 322]. To determine if a similar hemolysin is produced by *Brachyspira hampsonii* strain 30446, broth cultures of this bacteria were first grown in brain heart infusion media + 10% fetal bovine serum (BHIS), after which point the cultures were centrifuged and filtered through a 0.22 µm filter to yield the cell free supernatant. No hemolytic activity was observed in this cell free supernatant. As such, we next grew *Brachyspira hampsonii* strain 30446 in BHIS until late-log phase, then supplemented these cultures with 0.5 mg/mL yeast RNA core. Centrifugation and filtration of the culture supernatant yielded the cell-free supernatant fraction (Figure 4.1/Table 4.1), a fraction with a total hemolytic titer of 19200 hemolytic units (HU). Acid precipitation was then used to concentrate the hemolysin(s) within the cell free supernatant [318], after which point the acid precipitate was resuspended in $NaPO_4$ buffer and separated on an anion exchange column. Each of the peaks obtained from this anion exchange chromatography step was tested for hemolytic activity, revealing hemolytic activity was exclusively found in a sharp peak obtained after washing the column with 600 mM NaCl, termed the anion exchange fraction (Figure 4.1/4.2 A). While less than half of total hemolytic titer was recovered in the anion exchange fraction (7680 HU vs 19200 HU), this purification step served to concentrate the hemolysin 8-fold based on specific activity (16000 HU/mg of protein vs 2024 HU/mg) (Table 4.1). This fraction was then pooled and further separated on a size exclusion column. Out of the 4 peaks observed in the size-exclusion chromatogram eluting at ~185 mL, ~225 mL, ~245 mL, and ~250 mL, only 225 mL peak, termed the size exclusion fraction, contained hemolytic

activity (Figure 4.1/Figure 4.2B). While the total hemolytic titer recovered in the size exclusion fraction was again lower than that obtained in previous purification steps (2560 HU), this fraction was both highly pure (Figure 4.2D) and possessed a specific activity 20 times greater than that of the anion exchange fraction (320 000 HU/mg vs 16 000 HU/mg) (Table 4.1). All fractions possessed dose dependent hemolytic activity against pig erythrocytes, with the size exclusion fraction exhibiting measurable activity at concentrations as low as 1.35 ng/mL (Figure 4.2C) Although the amount of protein recovered was very low (8 µg of total protein as assessed by the BCA assay), concentrating the protein with a 3 kDa filter and separating this concentrated sample by SDS-PAGE revealed a band of < 10 kDa running with the tracking dye, suggesting that the purified hemolysin is a small peptide. Addition of Trypan Blue to reaction mixtures containing purified native hemolysin and pig erythrocytes caused a near complete abolition of hemolysis similar to that previously reported both for the *Brachyspira hyodysenteriae* native hemolysin and Streptolysin S from *Streptococcus pyogenes* (Figure 4.2E) [274, 318].

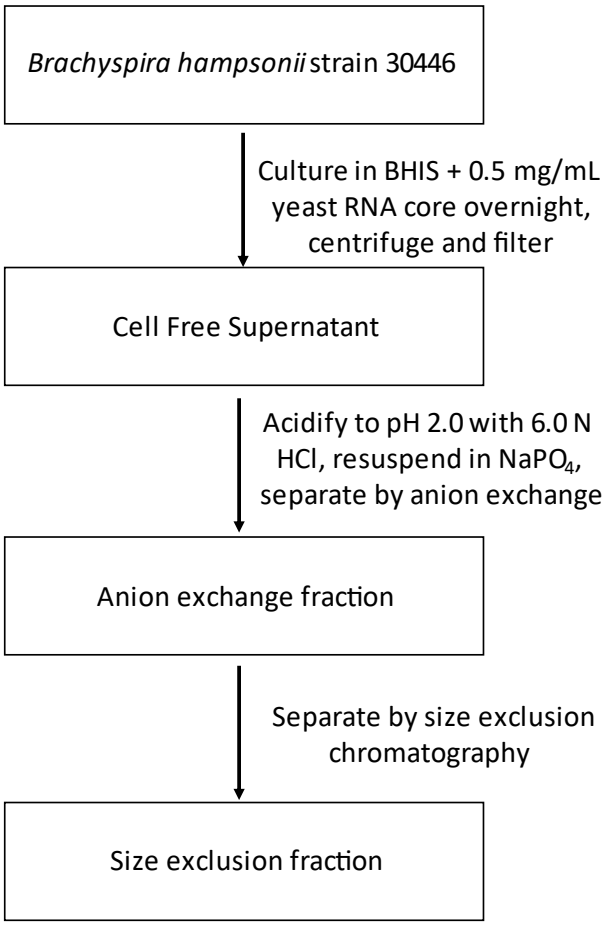


Figure 4.1 – Diagrammatic representation of native hemolysin purification.

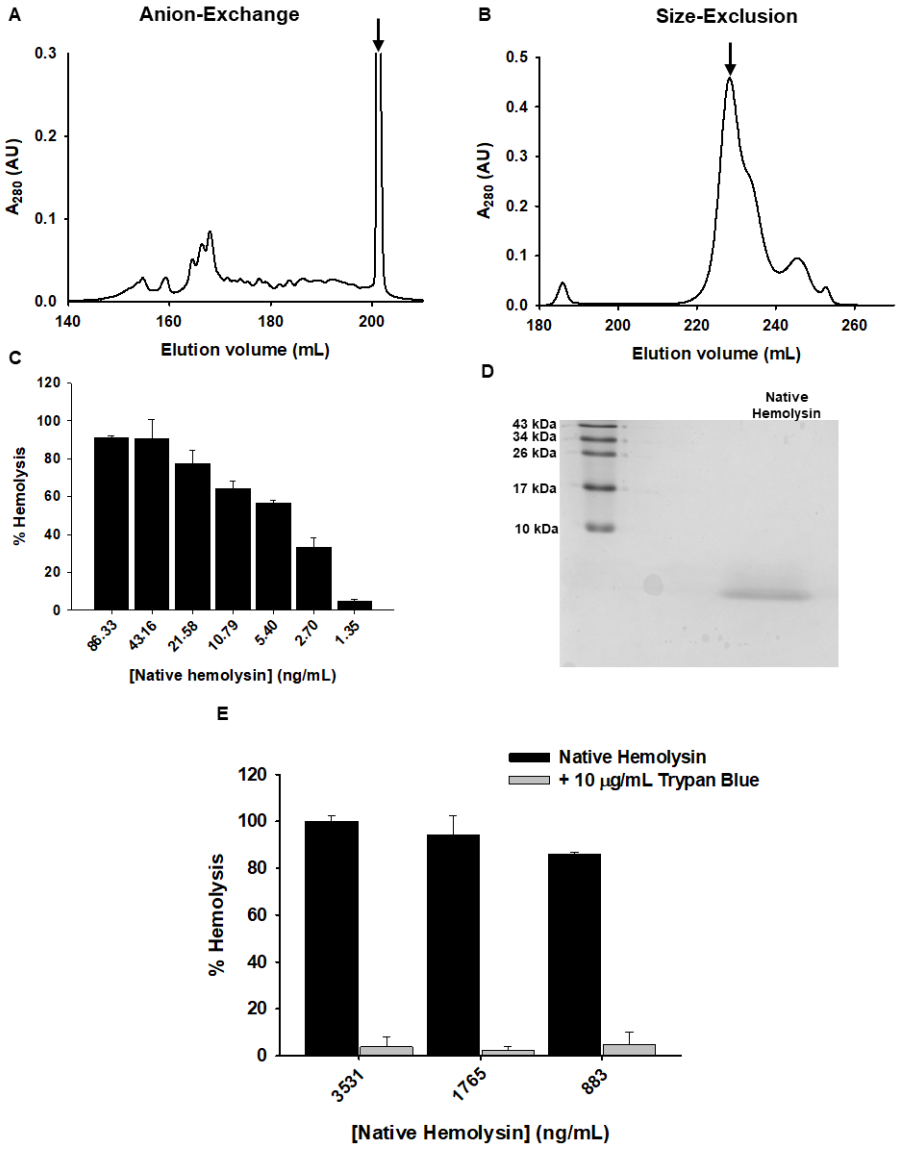


Figure 4.2 – Purification and activity of *Brachyspira hampsonii* native hemolysin. Yeast RNA core was added to *Brachyspira hampsonii* broth cultures to a final concentration of 0.5 mg/mL and incubated at 42 °C overnight. The cell free culture media was acidified to ~ pH 1.5 and the resulting pellet was resuspended in $NaPO_4$ and separated by anion exchange (A). Eluted fractions were assayed for hemolytic activity against pig erythrocytes, revealing that all hemolytic activity was contained within the indicated peak ($A_{280} \approx 3.0$, graph has been truncated for clarity). This peak was pooled and further separated by size-exclusion chromatography (B). Once again, all peaks were assayed for hemolytic activity and the peak possessing activity is indicated with an arrow. The protein concentration of the purified native hemolysin was quantified by the BCA assay, serial dilutions of the native hemolysin were then mixed with an equal volume of a pig erythrocyte suspension and incubated at 37°C for one hour (C). Hemolytic activity was readily detected at toxin concentrations as low as 1.35 ng/mL. Data are presented as mean + SD (n = 3 per dose). The purified native hemolysin was concentrated with a 3 kDa centrifugal filter and separated by 15% SDS-PAGE. Coomassie staining revealed a band <10 kDa running with the tracking dye (D). Addition of 10 μg/mL Trypan Blue to reaction mixtures containing pig erythrocytes and native hemolysin resulted in an almost complete abolition of hemolysis (E).

Table 4.1 Purification of *Brachyspira hampsonii* native hemolysin.

| **Fraction** | **Volume (mL)** | **Total activity (HU)** | **Total protein (mg)** | **Specific activity (HU/mg)** | **Purification Factor** |
|---|---|---|---|---|---|
| **Cell Free Supernatant** | 10 | 19200 | 9.487 | 2024 | 1 |
| **Anion Exchange** | 6 | 7680 | 0.480 | 16000 | 8 |
| **Size Exclusion** | 4 | 2560 | 0.008 | 320000 | 158 |

### 4.3.2 Co-expression of the SagABCD genes from *Brachyspira hampsonii* yields a hemolytic peptide

While previous studies have indicated that the *Brachyspira hyodysenteriae* native hemolysin is an acylated protein termed HlyA [14], expression and purification of the HlyA protein from *Brachyspira hampsonii* utilizing an *E. coli* expression system did not yield a hemolytic protein (Figure 4.3). These results, combined with the observation that production of both the *Brachyspira hampsonii* native hemolysin and Streptolysin S is induced by the addition of yeast RNA core [320, 365, 368, 369], lead us to hypothesize that the *Brachyspira hampsonii* native hemolysin is in fact a peptide homologous to SLS. Supporting this hypothesis is the recent discovery of a sag operon encoding for an SLS precursor within the genomes of both *Brachyspira hampsonii* strain 30446 and the type strain of *Brachyspira hyodysenteriae* [20]. To test this hypothesis, we chemically synthesized the *sagA/B/C/D* genes from *Brachyspira hampsonii* strain 30446 and cloned into pET-DUET and pRSF-DUET vectors [395, 452] (Figure 4.4).

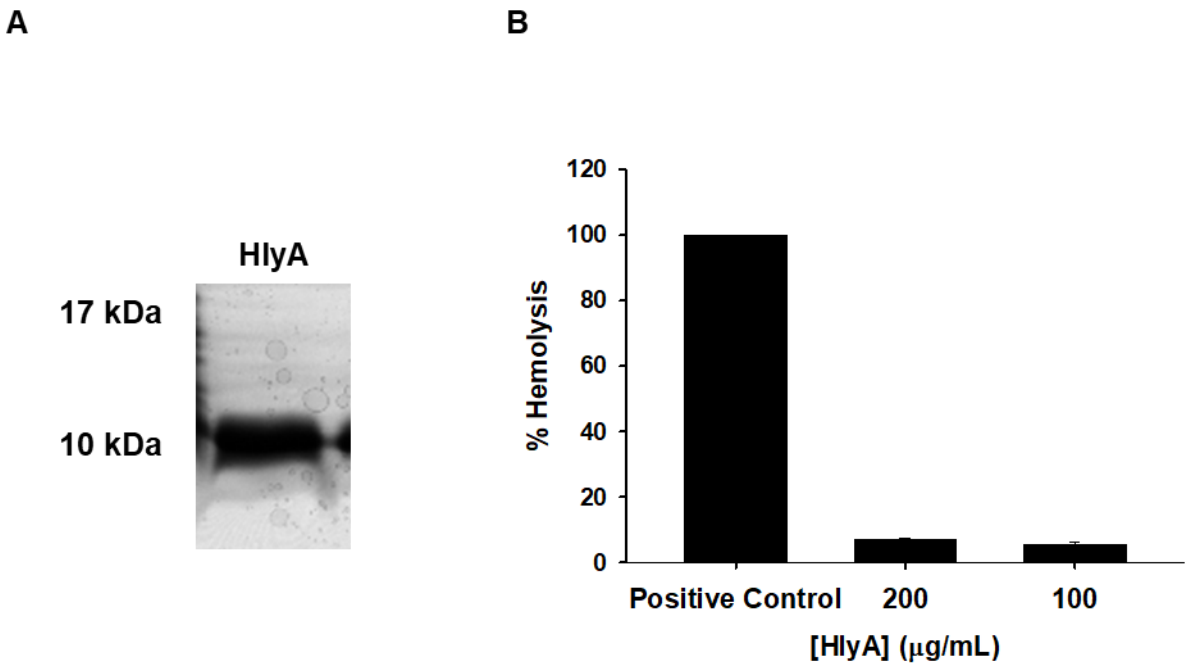


Figure 4.3 – Purification and hemolytic activity of HlyA protein from *Brachyspira hampsonii* strain 30446. The *hlyA* gene from *Brachyspira hampsonii* strain 30446 was cloned into a pET-28a expression vector, transformed into E. *coli*, and overexpressed. Pure His-HlyA protein was obtained from the soluble fraction by Ni2+ affinity chromatography and purity was assessed by 15% SDS-PAGE (A). Negligible hemolytic activity (≤6% hemolysis) was observed upon incubation of the purified His-HlyA protein with pig erythrocytes for up to 24 hours at room temperature (B). Data are presented as mean + S.D. (n = 3 per dose).

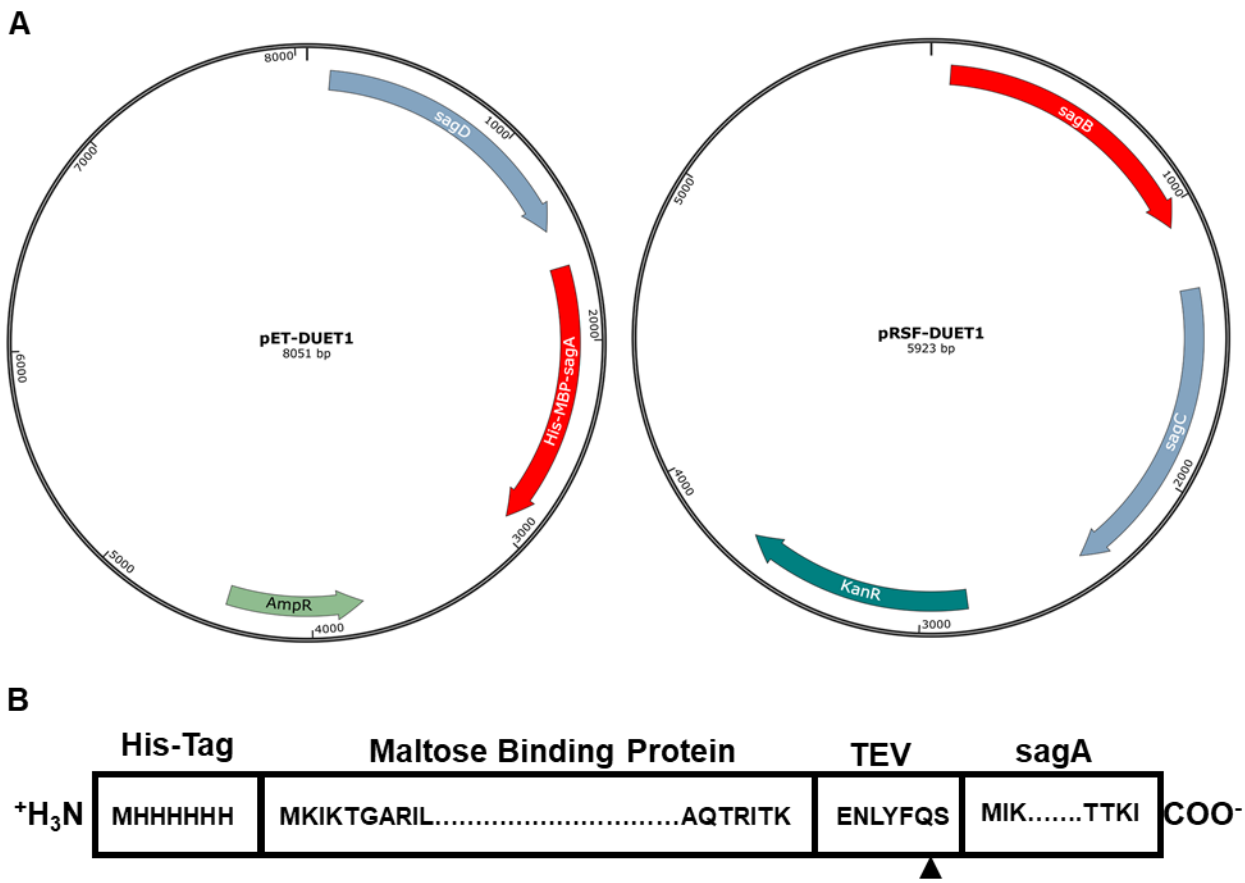


Figure 4.4 – Organization of *sagABCD* plasmids and His-MBP-sagA protein construct. The *sagA* (Streptolysin S preprotoxin), *sagB* (dehydrogenase), *sagC* (cyclodehydratase), and *sagD* (docking protein) [364] genes from *Brachyspira hampsonii* strain 30446 were chemically synthesized and ligated into the pET-DUET1 and pRSF-DUET1 plasmids (A). A diagrammatic representation of the His-MBP-SagA protein is shown in (B).

Both vectors were transformed into *E. coli*, and the addition of IPTG to late-log phase cultures lead to robust expression of all 4 genes (Figure 4.5). After harvesting and lysis of cell cultures, the His-MBP-SagA protein was purified from the soluble fraction by $Ni^{2+}$ and amylose affinity chromatography. Removal of the His-MBP tags from the SagA peptide was relatively inefficient (Figure 4.5), however, the addition of yeast RNA core to the TEV cleavage mixture enabled the purification of pure SagA following anion exchange and size exclusion chromatography (Figure 4.7).

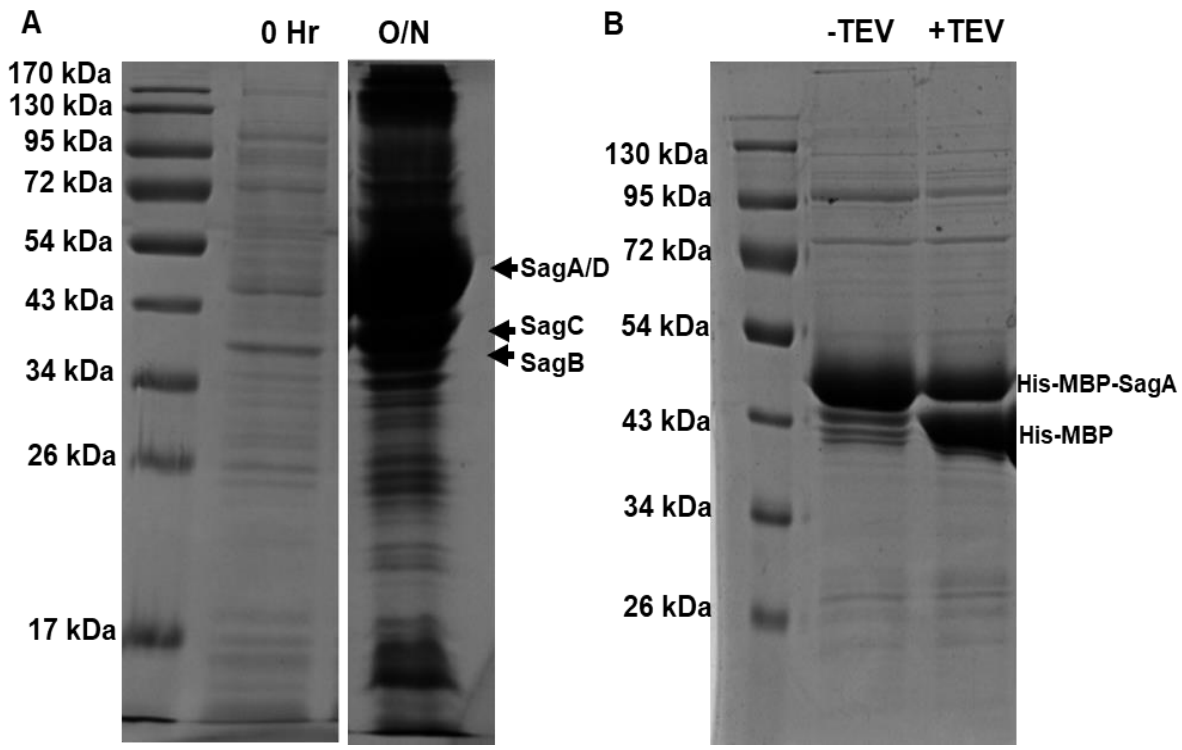


Figure 4.5 – Expression of Sag proteins and purification of His-MBP-SagA. The pET-DUET 1 vector containing the His-MBP-*sagA* and *sagD* genes and the pRSF-DUET1 vector containing the *sagB* and *sagC* genes were transformed into Rosetta (DE3) competent *E. coli* cells. Transformants were grown at 37 °C until the $OD_{600}$ of the cultures reached approximately 0.6, after which point protein expression was induced by the addition of 0.2 mM IPTG. Samples taken immediately prior to the addition of IPTG (0 Hr) and approximately 18 hours later (O/N) were isolated and separated on 12% SDS-PAGE in order to assess the expression levels of the SagABCD genes (A). Robust expression of His-MBP-SagA (~47 kDa), SagD (~51 kDa), SagC (~42 kDa), and SagB (~37 kDa) was observed following the 18 hour incubation. Following the 18 hour expression period cells were lysed and His-MBP-SagA protein was purified from the soluble fraction by $Ni^{2+}$ and amylose affinity chromatography. Purified His-MBP-SagA was then treated with TEV protease (~1 µg TEV protease to 50 µg His-MBP-SagA) for ~18 hours in order to remove the His-MBP affinity tags from the SagA peptide (B). SDS-PAGE analysis of the His-MBP-SagA protein before (-TEV) and after (+TEV) TEV cleavage indicates that only partial cleavage of the His-MBP affinity tags from the SagA peptide occurred.

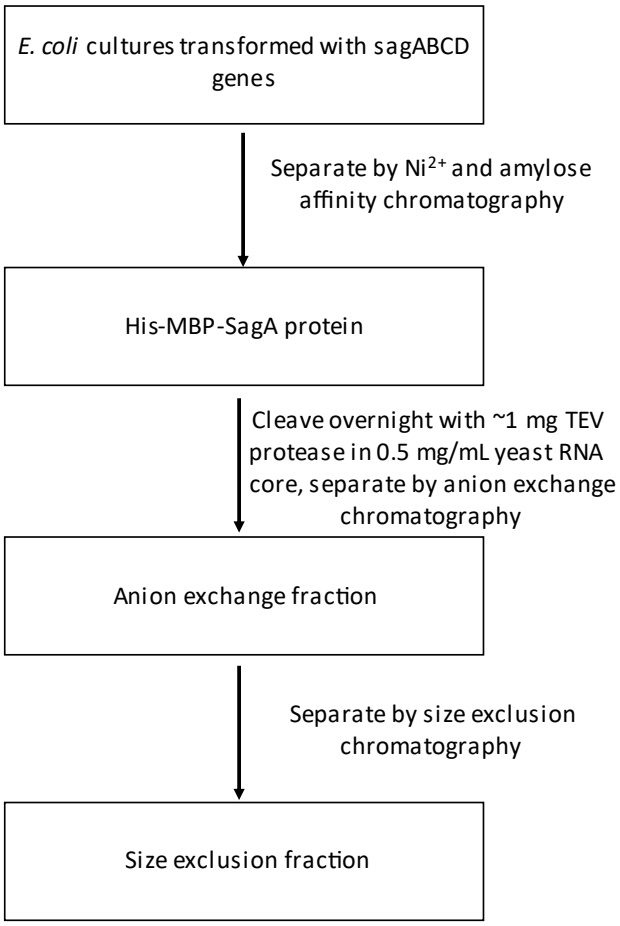


Figure 4.6 – Diagrammatic representation of sagA peptide purification

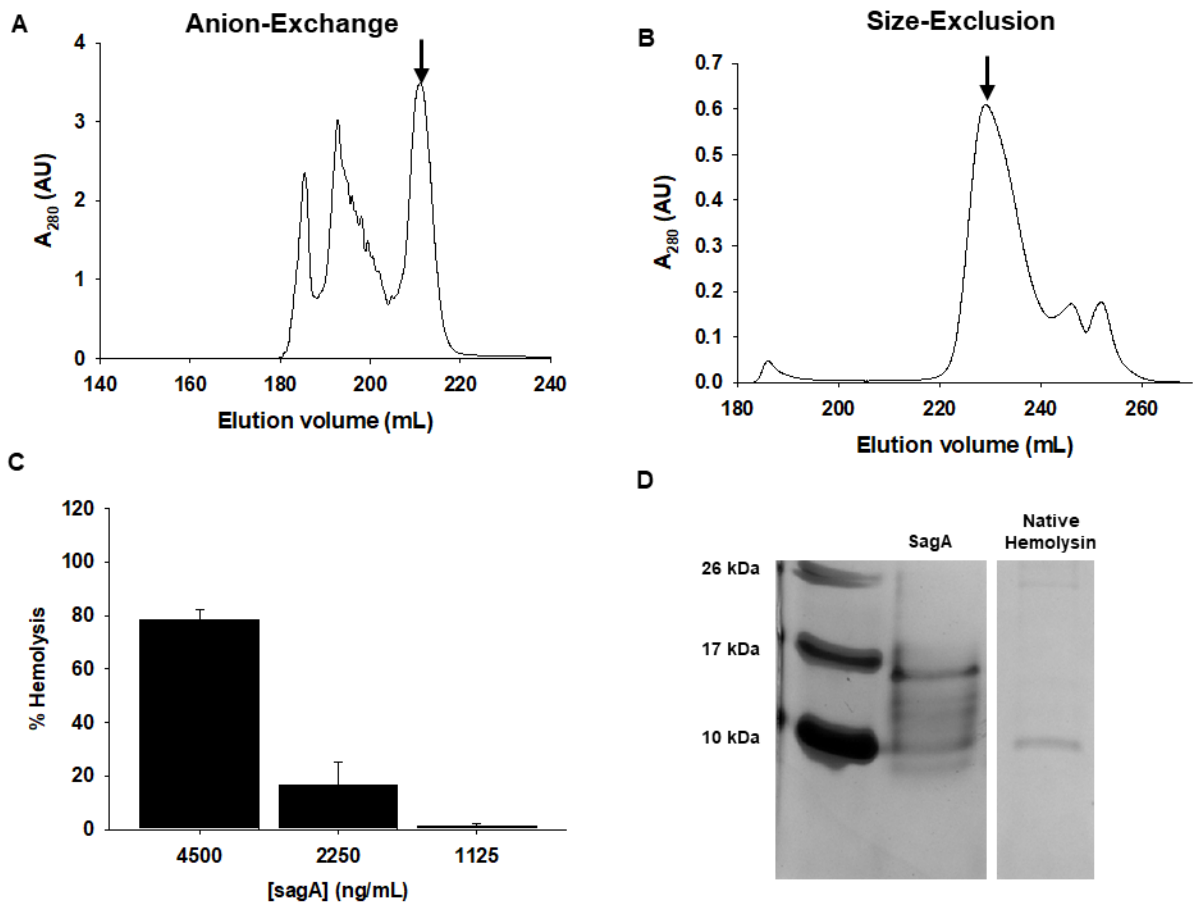


Figure 4.7 – Purification of TEV cleaved SagA peptide. Yeast RNA core (final concentration 0.5 mg/mL) was added following TEV cleavage of the His-MBP-SagA fusion protein and the resulting solution was separated utilizing anion-exchange (A) and size-exclusion chromatography (B). Hemolytic activity was only observed in the peaks indicated with arrows. Following the final size-exclusion chromatography step the hemolytic activity of the purified SagA peptide against pig erythrocytes was assessed (C). Like the native hemolysin, the SagA peptide was hemolytically active against pig erythrocytes, however, the hemolytic activity of this peptide required much higher concentrations (4500 ng/mL vs 5.40 ng/mL) and longer incubation times (24 hours vs 1 hour) than that observed for the *Brachyspira hampsonii* native hemolysin. Both the purified SagA peptide and the *Brachyspira hampsonii* native hemolysin were separated by 12% Tris-Tricine SDS-PAGE and visualized by silver staining (D). A single band of ~10 kDa was observed in the native hemolysin preparation. While this band was also apparent in the SagA preparation, additional bands ranging in size from ~15 kDa to ~8 kDa were also observed.

Despite this, addition of yeast RNA core to the TEV cleavage mixture enabled purification of small amounts of the SagA peptide utilizing anion exchange and size-exclusion chromatography (Figure 4.7A/B). Interestingly, comparison of the size exclusion chromatograms obtained following the purification of both the *Brachyspira hampsonii* native hemolysin (Figure 4.2B) and the heterologously expressed SagA peptide (Figure 4.7B) show that both peptides eluted at roughly the same volume (~225 mL), indicating that these toxins are roughly the same mass. Incubation of the purified SagA peptide with pig erythrocytes revealed that this peptide is hemolytically active (Figure 4.7C), albeit to a much lesser degree than that observed with the *Brachyspira hampsonii* native hemolysin (Figure 4.2C).

### 4.3.3 Optimized expression and purification of *Brachyspira hampsonii* and *Brachyspira hyodysenteriae* SagA peptides

While these attempts to purify and characterize the SagA peptide from *Brachyspira hampsonii* were successful, the yields obtained were disappointing (≤ 50 µg SagA peptide per liter of *E. coli* culture). Due to the inefficient cleavage of the His-MBP affinity tags by TEV protease, we cloned the *sagA* gene into an expression vector containing only an N-terminal His-tag. While we were unable to recover soluble His-SagA following expression of this construct in *E. coli*, moderate amounts of protein (~400 µg per L of culture) were recovered from the insoluble fraction following solubilization of inclusion bodies in guanidine HCl and separation on a size exclusion column. Given the tendency of the SagA peptide to accumulate in insoluble inclusion bodies when expressed without the MBP tag, we next aimed to maximize the efficiency of expression by focusing on inclusion body based purification strategies. For these studies, the sagA gene was cloned into an expression vector encoding for both a His-tag and the TrpΔLE 1413 sequence on the N-terminus of the SagA peptide. The *sagA* gene from *Brachyspira hyodysenteriae* was used for these studies, due to the increased prevalence of *Brachyspira*

*hyodysenteriae* in North American swine herds in recent years, while the TrpΔLE 1413 sequence is an artificial 17 amino acid sequence derived from portions of the *E. coli trp* operon that promotes high levels of insoluble peptide expression in *E. coli* [453-456]. The His-TrpLE-SagA fusion peptide was expressed in *E. coli* and inclusion bodies were solubilized in guanidine HCl, after which point the His-TrpLE-sagA peptide was purified by $Ni^{2+}$ affinity chromatography. Robust levels of His-TrpLE-sagA expression were observed, translating to a high recovery of the purified fusion peptide (~10 mg per L of culture). Cyanogen bromide was then utilized to chemically cleave the His-TrpLE fusion tags from the SagA peptide. While CNBr treatment did not completely cleave the His-TrpLE fusion tags (Figure 4.8B), much higher levels of cleavage were observed with this system in comparison to cleavage of the His-MBP tags by TEV protease, and at the time of writing efforts are ongoing to isolate the cleaved SagA peptide from the His-TrpLE fusion tags. The dramatic improvement in SagA yields utilizing the His-TrpLE-SagA expression strategy provides a vastly improved protocol for the production and characterization of related streptolysin S homologues in other bacteria such as *Streptococcus pyogenes*.

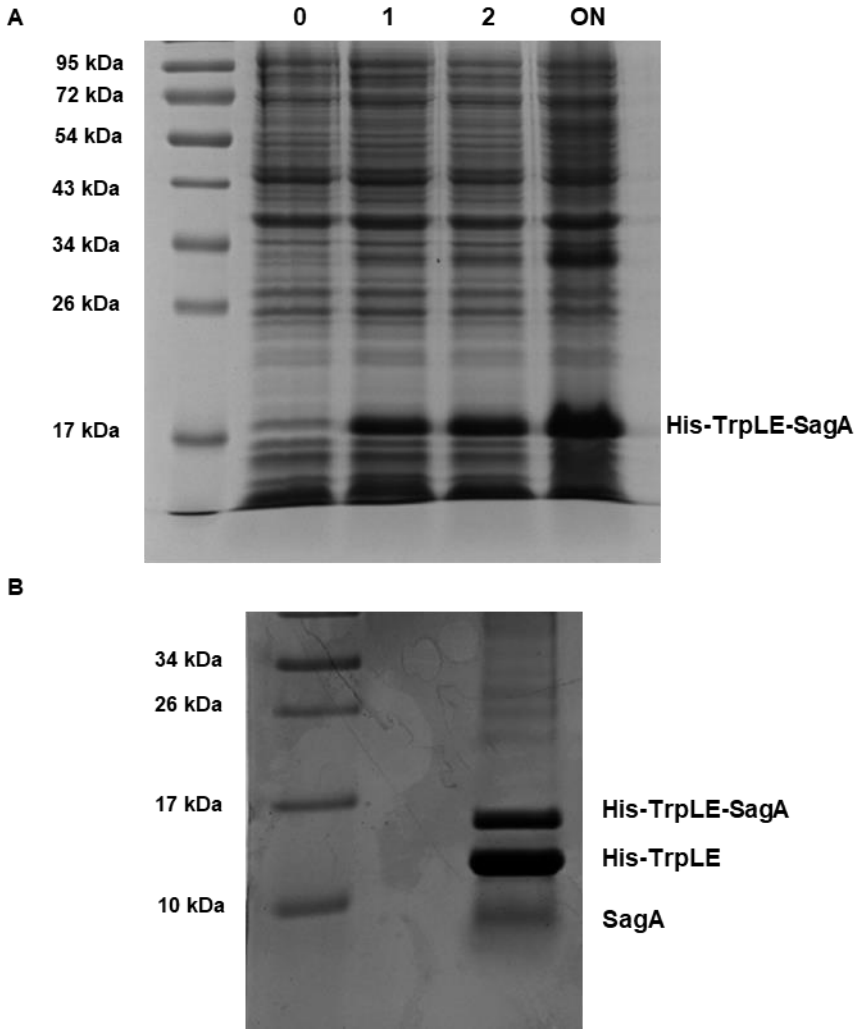


Figure 4.8 Expression and CNBr cleavage of His-TrpLE-SagA. *A, E. coli* BL21 (DE3) cells were transformed with the His-TrpLE-SagA construct and grown to an $OD_{600}$ of ~0.6. Protein expression was induced with 0.2 mM IPTG, and samples were taken immediately prior to IPTG addition (0), and 1, 2, or 18 (ON) hours after. Cells were lysed in Laemelli buffer and separated on 12% SDS-PAGE. Robust translation of the His-MBP-SagA peptide was observed within an hour of IPTG treatment. *B*, the His-TrpLE-SagA peptide was purified by $Ni^{2+}$ affinity chromatography under reducing conditions and cleaved overnight with CNBr, after which point the cleavage mixture was analyzed by 12% SDS-PAGE utilizing a tris-tricine buffer system. Approximately 50% cleavage of the His-TrpLE-SagA peptide was observed, and a diffuse ~10 kDa band roughly corresponding to that seen with the *Brachyspira hampsonii* native hemolysin was observed.

## 4.4 Discussion

### 4.4.1 Characterization of the *Brachyspira hampsonii* native hemolysin

Following the identification of *Brachyspira hyodysenteriae* as the causative agent of swine dysentery in 1972 [3], three research groups independently purified a hemolytic molecule from *Brachyspira hyodysenteriae* broth cultures [318, 319, 322]. Subsequently, while *Brachyspira hampsonii* is both highly pathogenic to pigs and strongly β-hemolytic on blood agar [5, 6], the recent identification of *Brachyspira hampsonii* within North American swine herds has meant that no investigation into hemolysins produced by this bacterium has been undertaken. To determine if *Brachyspira hampsonii* also produces a hemolysin similar to the native hemolysin of *Brachyspira hyodysenteriae*, we supplemented *Brachyspira hampsonii* broth cultures with yeast RNA core, a compound derived from RNAse digested yeast RNA that is known to induce the formation of a potent hemolysin in both *Streptococcus pyogenes* [320, 365, 368, 369] and *Brachyspira hyodysenteriae* [318, 319, 322]. Addition of yeast RNA core to *Brachyspira hampsonii* broth cultures induced the formation of a hemolytic molecule within the cell-free supernatant (Table 4.1) which was then further purified by anion-exchange and size-exclusion chromatography (Table 4.1, Figure 4.1). Losses in total activity were apparent after each purification step, as only 2560 HU of total hemolytic activity were recovered after the final size-exclusion purification step in contrast to the 19200 HU contained within the crude cell free supernatant (Table 4.1). While the acid precipitation step utilized in our purification protocol may be responsible for this loss of activity, this step served to quickly concentrate and purify the native hemolysin for downstream purification steps and we considered these losses to be an acceptable tradeoff as a result. Additionally, a previous study involving the *Brachyspira hyodysenteriae* native hemolysin reported a near complete loss of hemolytic activity within days unless the toxin was lyophilized and stored at -20°C or -80°C [319]. While the native hemolysin

obtained following size-exclusion chromatography retained its activity for months when stored in solution at -80°C, the dialysis and chromatography steps present in our purification procedure necessitate keeping this protein at 4°C for several days, potentially resulting in partial degradation of this toxin prior to storage.

In addition to the difficulties inherent in purifying the native hemolysins of *Brachyspira hyodysenteriae* and *Brachyspira hampsonii*, contradictory reports on the molecular weight of the purified native hemolysin have complicated efforts to characterize this toxin [318, 319, 322]. Initial studies on the *Brachyspira hyodysenteriae* native hemolysin reported a molecular weight of ~78 kDa based on size-exclusion chromatography [318], while another study the following year reported a molecular weight of ~68 kDa by SDS-PAGE [319]. Further confounding these results was a later study reporting a molecular weight of ~18 kDa based on size-exclusion chromatography, with the authors of this study also concluding that the ~68 kDa band observed on SDS-PAGE by Knoop [319] was an impurity rather than the active toxin [322]. Subsequently, we have also observed a ~68 kDa band in SDS-PAGE analyses of impure *Brachyspira hampsonii* native hemolysin preparations and believe that it is bovine serum albumin derived from the *Brachyspira* culture media. Correspondingly, comparison of the size exclusion elution profile of the *Brachyspira hampsonii* native hemolysin to size exclusion standards previously used for calibration of the specific column used in these experiments [457] suggests a molecular weight of ~31 kDa for this protein (Figure 4.2B). While we cannot discount the possibility that this represents the true molecular mass of the mature native hemolysin, we hypothesized that this estimated molecular weight may represent an oligomeric form of the native hemolysin or may be the overall mass of the native hemolysin – RNA complex formed upon the addition of yeast RNA core to *Brachyspira hampsonii* broth cultures. Furthermore, when the purified native

hemolysin was separated by SDS-PAGE and stained with Coomassie blue only a single band running alongside the tracking dye was observed (Figure 4.2D), suggesting that the native hemolysin is in fact a small peptide. In light of these complications we next utilized Tris-tricine SDS-PAGE to analyze the native hemolysin, a technique capable of enhancing the separation of small peptides and highly hydrophobic proteins in comparison to the standard Tris-glycine buffer used in SDS-PAGE [458, 459]. Separation of the native hemolysin by Tris-tricine SDS-PAGE followed by silver staining of the gel revealed a band of ~10 kDa in size (Figure 4.7D). While we attempted to excise this band for mass spectrometry peptide fingerprinting, the peptides identified following this analysis did not correlate to any annotated genes within the *Brachyspira hampsonii* genome, likely due to both the highly modified nature of this peptide, along with the fact that *sagA* is not annotated within any publicly available *Brachyspira hampsonii* genome.

### 4.4.2 Pathogenic strains of *Brachyspira hyodysenteriae* and *Brachyspira hampsonii* possess a sag operon encoding for a Streptolysin S like peptide

#### 4.4.2.1 Evidence that the HlyA gene product is not analogous to the *Brachyspira hampsonii* native hemolysin

Twenty years after the initial identification of the *Brachyspira hyodysenteriae* native hemolysin N-terminal sequencing was carried out on this peptide in order to definitively link this toxin to a gene sequence [14]. The peptide sequence obtained from this experiment was then used to create a degenerate oligonucleotide probe, enabling identification of a gene termed *hlyA* [14]. While the authors of this study reported *E. coli* expressing the *hlyA* gene possessed a β-hemolytic phenotype on blood agar [14], we did not observe significant hemolytic activity when the *hlyA* gene from *Brachyspira hampsonii* was cloned, expressed, and purified from an *E. coli* host (Figure 4.3). Furthermore, a later proteomics study identified the hlyA gene product among the top 10 most abundant proteins secreted by both *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* grown in broth culture [451], suggesting that the HlyA peptide identified by Hsu *et al.*,

may once again be a contaminant rather than the native hemolysin [14]. The high abundance of this peptide in *Brachyspira* broth cultures is also at odds with the fact that several purification steps are required to isolate microgram quantities of native hemolysin, even after supplementation of cultures with yeast RNA core (Table 4.1).

#### 4.4.2.2 Evidence that the SagA peptide is the *Brachyspira hampsonii* native hemolysin

These observations, combined with the similarity of the *Brachyspira* native hemolysin to Streptolysin S, lead us to hypothesize that this toxin is in fact a Streptolysin S homologue. While the *Brachyspira hyodysenteriae* WA1 genome contains genes annotated as *sagB* (WP_012671837.1), *sagC* (WP_012671838.1), and *sagD* (WP_012671839.1), the *sagA* gene encoding for the Streptolysin S precursor is not annotated on any publicly available *Brachyspira* genome, likely because conventional genome annotation methods are often inefficient at identifying small genes within prokaryotic genomes [460, 461]. Subsequently, manual curation of the *Brachyspira hampsonii* strain 30446 genome by a member of our research group found an operon containing the *sagABCDEGI* genes, while the genome of the nonpathogenic *Brachyspira hampsonii* KL180 did not possess any of these genes [20]. To test our hypothesis that the *Brachyspira hampsonii* native hemolysin is an SLS homologue, we cloned the *sagABCD* genes from *Brachyspira hampsonii* 30446 into a dual expression vector system allowing for co-expression of all four genes, a methodology previously used to express functional SLS in *E. coli* [395] (Figure 4.4) Due to the small size of the SagA peptide and the previously reported fragility of both the *Brachyspira hyodysenteriae* native hemolysin [322] and SLS [370] we expressed the SagA peptide with an N-terminal maltose binding protein fusion partner. Maltose binding protein has been extensively used for heterologous protein expression in *E. coli* due to its ability to

improve solubility of fusion proteins and its high affinity for commercially available amylose resins, allowing for easy purification of MBP tagged proteins [462, 463]. While the MBP tag was successful in enabling high expression of soluble His-MBP-SagA protein (Figure 4.6), we were unable to efficiently remove the His-MBP tags from the SagA peptide using TEV protease (Figure 4.5B), likely due to steric hinderance caused by the bulky MBP tag. Despite these difficulties, addition of yeast RNA core to the TEV cleavage reaction enabled us to purify small amounts of the tag free SagA peptide utilizing anion exchange and size exclusion chromatography (Figure 4.7A/B). Of note is the fact that both the *Brachyspira hampsonii* native hemolysin and the heterologously expressed SagA peptide eluted at roughly the same volume during the final size exclusion chromatography step (Figure 4.2B/4.7B), indicating that both peptides possess similar hydrodynamic radii equivalent to a globular protein ~31 kDa in size. Like the *Brachyspira hampsonii* native hemolysin and TlyA, the purified SagA peptide possessed hemolytic activity against pig erythrocytes, causing ~80% hemolysis at a concentration of 4500 ng/mL. While the specific activity of purified SagA is greater than that observed with TlyA (Figure 4.7C), this value is several orders of magnitude lower than the *Brachyspira hampsonii* native hemolysin (Figure 4.1C). Additionally, SagA mediated hemolysis was only observed after incubating the reaction mixtures overnight at 37 °C, in contrast to the 1 hour incubation required for hemolysis with the *Brachyspira hampsonii* native hemolysin. We hypothesize that this altered activity is due to impaired functionality of the SagBCD complex when expressed in *E. coli* in comparison to the native *Brachyspira hampsonii* host. While mass spectrometry has been used to qualitatively detect SagBCD induced modification of purified SagA peptides [394], these methods have not been refined to the point of quantifying *in vitro* SagA modifications and comparing these modifications to native SLS. Furthermore, no effort

was made in our experiments to remove a putative leader sequence contained within the *Brachyspira hampsonii* SagA protein sequence. While studies with *Streptococcus pyogenes* SagA have indicated that cleavage of this leader sequence is necessary for export of the mature peptide into the extracellular space [394, 395, 399], we cannot discount the possibility that the removal of this region is necessary for optimal hemolytic activity. Although the diminished activity observed with the SagA peptide in comparison to the native hemolysin is disappointing, these results are consistent with previous studies involving heterologously expressed SagA from *Streptococcus pyogenes* [394, 395, 399], lending strength to our hypothesis that the *Brachyspira* native hemolysins are SLS homologues. Furthermore, when both the SagA peptide and the *Brachyspira hampsonii* native hemolysin were separated by Tris-tricine SDS-PAGE a band of ~10 kDa was present in both preparations (Figure 4.7D). While this band was the only band present in native hemolysin preparations, additional bands were ranging from ~8 kDa to ~15 kDa were also observed in the SagA preparation. Given that the specific activity of the SagA peptide was much lower than the purified native hemolysin and that purified SLS is known to be highly fragile in solution when yeast RNA core is not present, it is reasonable to assume that some of these bands may represent degradation products. While yeast RNA core was added to the SagA preparation at the TEV cleavage step, this compound was not present during the expression and initial purification steps, potentially allowing for degradation to occur. Additionally, the ~15 kDa band observed may in fact represent a multimeric form of the SagA peptide. Of the 42 amino acids in the SagA peptide, 9 are cysteine. Although the sagBCD enzymes would theoretically function to modify these cysteines into thiazole residues *in vivo*, it is possible that these modifications do not efficiently occur within our *E. coli* expression host. If one or more of these cysteine residues were to remain unmodified within the mature SagA peptide intermolecular

disulfide bonding could occur, leading to the appearance of multimeric SagA species detected by Tris-tricine SDS-PAGE. While further approaches such as mass spectrometry or nuclear magnetic resonance must be undertaken in the future to definitively prove the identity of the *Brachyspira hampsonii* native hemolysin, these results provide compelling evidence that this toxin may in fact be an SLS homologue produced by pathogenic *Brachyspira* species.

### 4.4.3 Strategies for improving expression levels of SagA peptides utilizing an E. *coli* expression system

In addition to improving our understanding of swine dysentery pathogenesis, a goal of our research has been to evaluate the hemolysins identified in this research as components of a vaccine against swine dysentery. Given that the nonpathogenic *Brachyspira hampsonii* KL180 does not possess the *sag* operon present within the pathogenic *Brachyspira hampsonii* 30446 [20], we hypothesized that the SagA peptide studied in the previous sections could also be an effective candidate for this research. However, the lengthy culture procedures and expensive reagents required for purification of native hemolysin from *Brachyspira hampsonii* 30446 broth cultures necessitated the use of an *E. coli* heterologous expression system to produce significant quantities of this peptide for immunological studies. While we were able to produce small quantities of tag-free SagA peptide utilizing the His-MBP-SagA expression system, the yields obtained by this method were on the order of ~50 µg/L of *E. coli* culture. In contrast, previous studies in our lab have determined that a dose of 100-1000 µg of antigen is required to produce a robust immune response in pigs. As such, we determined that the His-MBP-SagA expression system was woefully inadequate for the large scale production of SagA peptide for these studies. To address these shortcomings, we first cloned the *sagA* gene into an expression vector containing only an N-terminal His-tag, because removal of the His-MBP affinity tags utilizing TEV protease was the primary yield limiting step in the previous protocol (Figure 4.3). While the

His-SagA expression system benefited from the fact that only a single purification step was required to purify this peptide and that the affinity tags did not need to be removed for downstream experiments, the fact that this peptide was not soluble when expressed in *E. coli* was a notable downside. This finding was not surprising, as SLS is known to tightly bind lipoteichoic acid found within the cell walls of Gram positive bacteria [371] and is only observed within the culture media following the addition of an inducer such as yeast RNA core [273, 320, 365, 368, 369]. Although the His-SagA expression system provided improved the yield of SagA from ~50 µg/mL to ~400 µg/mL, higher yields still are necessary for large scale studies. In order to improve the expression level of the SagA peptide we next utilized the Trp ΔLE 1413 sequence, referred to as TrpLE for brevity. TrpLE is an artificial 17 amino acid sequence derived from the *E. coli trp* operon leader sequence fused to a short portion of the TrpE protein sequence that efficiently promotes inclusion body formation when fused to short peptides and expressed in *E. coli* [453, 455, 456]. The His-TrpLE-SagA peptide was highly expressed in *E. coli*, as evidenced by the fact that extraction of inclusion bodies and purification utilizing $Ni^{2+}$ affinity chromatography under denaturing conditions yielded nearly 10 mg of His-TrpLE-SagA fusion peptide per liter of *E. coli* culture. Given the poor performance of TEV protease in removing the His-MBP affinity tags from the SagA peptide, we utilized cyanogen bromide to remove the His-TrpLE fusion partners from the purified His-TrpLE-SagA peptide. Cyanogen bromide (CNBr) cleaves peptides at the C-terminal end of methionine residues (only one methionine corresponding to the N-terminus of the peptide is present in SagA); this reaction is highly specific when cleavage is carried out under acidic conditions, providing a convenient mechanism for the removal of fusion tags from peptides that has been extensively used since the 1960's [453, 464-466]. While CNBr cleavage of the His-TrpLE-SagA peptide was more efficient than

TEV protease (Figure 4.7/4.8), large amounts of uncleaved His-TrpLE-SagA were still present within the reaction mixture. Future experiments will aim to both improve the efficiency of the CNBr cleavage reaction through optimization of the CNBr concentration and buffer system used, along with developing reliable methods for isolation of the cleaved SagA peptide following CNBr treatment.

## 4.5 Conclusion

A strong β-hemolytic phenotype is correlated with pathogenicity in *Brachyspira hyodysenteriae* and *Brachyspira hampsonii*. While a hemolysin produced by *Brachyspira hyodysenteriae* broth cultures in the presence of yeast RNA core has been previously linked to a gene termed *hlyA* [14], we believe this to be a mis-annotation, as an operon encoding for an SLS homologue has recently been discovered within the *Brachyspira hampsonii* genome [20]. As such, our specific objectives for this chapter were to purify the native hemolysin produced by *Brachyspira hampsonii* broth cultures and link this protein to a specific gene product, and to clone, express, and purify the *sagA* homologue found within the *Brachyspira hampsonii* genome. These experiments were carried out to test the hypothesis that the *Brachyspira* native hemolysin is in fact an SLS homologue. Both the native hemolysin and the heterologously expressed SagA peptide were hemolytically active against pig erythrocytes, however the specific activity of the SagA peptide was much lower than that of the native hemolysin. While amino acid sequencing of the native hemolysin will be required in order to prove the identity of this toxin, the similar sizes of the native hemolysin and SagA peptide, along with the previous observations that the specific activity of heterologously expressed SagA is low compared to SLS from *Streptococcus pyogenes* [394, 395, 397] lend strength to our hypothesis that the *Brachyspira hampsonii* native hemolysin and the SagA peptide are one and the same. In light of these observations, we hypothesized that the *Brachyspira hampsonii* native hemolysin/SagA is a critical virulence factor required for the pathogenesis of swine dysentery and set out to determine if the treatment of cultured cells with this toxin could recapitulate aspects of this disease *in vitro*.

# Chapter 5 – Acute Exposure of Caco-2 Monolayers to the Native Hemolysin of *Brachyspira hampsonii* Recapitulates a Diarrheic Phenotype Through the Inhibition of the ENaC $Na^+$ Channel

**Brandon A. Keith[1], Nitin Challa[1], John C.S. Harding[2], Matthew E. Loewen[1*]**

[1]Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

[2]Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

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*Brachyspira hyodysenteriae* and *Brachyspira hampsonii* are two causative agents of swine dysentery, a production limiting disease typified by severe mucohaemmorhagic diarrhea in infected pigs. While recent studies have determined that *Brachyspira* induces a malabsorptive diarrhea in infected animals through downregulation of electroneutral $Na^+$ and $Cl^-$ absorption, the molecular mechanisms by which these physiological changes occur is not completely understood. The recent discovery of a sag operon coding for a streptolysin S homologue in the pathogenic *Brachyspira hampsonii* strain 30446, but not the nonpathogenic KL180 strain, lead us to hypothesize that the action of this toxin may play a critical role in the development of diarrhea during *Brachyspira* infection. To test this hypothesis, we exposed Caco-2 monolayers to the native hemolysin of *Brachyspira hampsonii*, a potent hemolysin believed to be a streptolysin S homologue. While the *Brachyspira hampsonii* native hemolysin did not cause appreciable cytotoxicity in treated Caco-2 monolayers, the addition of the anion exchange inhibitor DIDS to reaction mixtures containing native hemolysin and pig erythrocytes resulted in a complete abolition of hemolysis. In response to this finding, we utilized pharmacological inhibitors to assess alterations in ion transport upon treatment of Caco-2 monolayers with native hemolysin. Significant changes in the cellular response to amiloride were observed in native hemolysin treated monolayers, suggesting that this toxin is capable of inhibiting electrogenic $Na^+$ absorption through ENaC.

## 5.1 Introduction

Infection of pigs by *Brachyspira hyodysenteriae* or *Brachyspira hampsonii* results in the development of swine dysentery, a disease characterized by severe mucohaemmorhagic diarrhea resulting in severe production losses in infected herds [1, 74, 425, 467]. Recent studies have described *Brachyspira* induced diarrhea as being malabsorptive in nature, with the downregulation of the $Na^+/H^+$ transporter NHE3 and the $Cl^-/HCO_3^-$ exchanger DRA resulting in decreased $Na^+$ and $Cl^-$ absorption within the colon of infected animals [9, 10]. Additionally, a decrease in apical $Cl^-$ secretion due to the downregulation of CFTR during *Brachyspira* infection, a phenomenon believed to facilitate colonization of the colonic mucosa by *Brachyspira* through modification of the chemical properties of colonic mucins [11, 12, 141, 156]. While the downregulation of DRA during *Brachyspira* infection is mediated by host IL-1α production [10], addition of IL-1α to a colonic cell line could not recapitulate the downregulation of NHE3 and CFTR observed during swine dysentery [9, 11], suggesting that a toxin or toxins produced by *Brachyspira hyodysenteriae* and *Brachyspira hampsonii* may be directly responsible for these effects.

Pathogenic *Brachyspira hyodysenteriae* and *Brachyspira hampsonii* isolates are strongly β-hemolytic on blood agar, leading to speculation that the production of hemolysins by these bacteria is a critical aspect of their pathology [71, 74, 114, 425, 468]. While four putative hemolysin genes termed *tlyA/B/C* and *hlyA* have been identified in the genomes of *Brachyspira hyodysenteriae* and *Brachyspira hampsonii* [13, 14], a small peptide termed native hemolysin appears to be the largest contributor to the β-hemolytic phenotype of these bacteria [318, 319, 322]. Despite being linked to the HlyA gene product by one study [14], the native hemolysin appears to be a streptolysin S homologue, as evidenced by both the functional similarity of the

two toxins and the recent discovery of an operon encoding for an SLS homologue within the *Brachyspira hampsonii* genome [19, 20, 318-320, 322, 368]. In addition to its well documented hemolytic activity, SLS is also known to dramatically alter host cell physiology during *Streptococcus pyogenes* infection by activating pro-inflammatory signaling pathways and directly modifying the activity of the band 3 anion exchanger [384, 411]. These findings, along with the observation that a nonpathogenic *Brachyspira hampsonii* strain lacks the sag operon responsible for SLS production [20], lead us to hypothesize that the native hemolysin of *Brachyspira hampsonii* directly contributes to the development of diarrhea through either direct cytotoxicity, modulation of the activity of the $Cl^-/HCO_3^-$ exchanger DRA, or a combination of these effects. Direct addition of the *Brachyspira hampsonii* native hemolysin to a colonic cell line did not lead to measurable cytotoxicity within 24 hours, suggesting that this toxin does not possess cytotoxic effects against epithelial cells. Addition of the anion exchange inhibitor DIDS to reaction mixtures containing native hemolysin and pig erythrocytes completely abolished the hemolytic activity of the *Brachyspira hampsonii* native hemolysin, indicating that this toxin directly activates the erythrocyte anion exchanger band 3. The effects of native hemolysin on epithelial ion transport were assessed by Ussing chamber studies of Caco-2 monolayers treated with native hemolysin for 1 or 24 hours. Pharmacological inhibitors revealed that treatment with native hemolysin results in a drop in electrogenic $Na^+$ absorption through ENaC. Addition of the anion exchange inhibitor DIDS to the Caco-2 monolayers before the addition of these pharmacological inhibitors prevented native hemolysin induced alterations to ion transport, suggesting that these effects are due to modulation of DRA by native hemolysin. While these results are at odds with those observed with tissue obtained from pigs experimentally infected with *Brachyspira hyodysenteriae* or *Brachyspira hampsonii*, this tissue was obtained 3-7 days

post inoculation [9-11] in contrast to the 1 and 24 hour timepoints used in our study, suggesting that the mechanisms of *Brachyspira* induced diarrhea may change during the progression of swine dysentery.

## 5.2 Materials & Methods

### 5.2.1 Cell Culture

Caco-2 cells derived from a human colorectal adenocarcinoma (ATCC # HTB-37) were cultured in Dulbecco's modified Eagle Medium (DMEM) (Corning) containing 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin (Life Technologies), and 1% MEM non-essential amino acids (Gibco) in a humidified 37°C incubator under a 5% $CO_2$ atmosphere. For cytotoxicity experiments cells were seeded in 96 well plates (Corning - 3343) at an initial density of $1.0*10^4$ cells/well and incubated overnight before being used in downstream experiments. For Ussing chamber experiments cells were seeded on polyester Snapwell inserts containing 0.4 µm pores (Corning - CLS380) and grown to confluency, after which point cells were cultured for one week in DMEM without FBS on the apical surface and DMEM + 10% FBS on the basolateral surface. Transepithelial electrical resistance (TEER) of the Caco-2 monolayers was regularly assessed using a Millicell ERS-1 voltohmmeter (MilliporeSigma), only monolayers with TEER $\geq 200$ $\Omega/cm^2$ were utilized for Ussing chamber experiments.

### 5.2.2 Cytotoxicity assays

Caco-2 cells were grown overnight in 96 well plates as described previously, after which point the media was removed by aspiration and replaced with 100 µL FBS free DMEM containing *Brachyspira hampsonii* native hemolysin or an equivalent amount of PBS as a negative control. After 24 hours of incubation 50 µL of media was removed from each well and cytotoxicity was assessed utilizing the Pierce LDH cytotoxicity assay kit (Thermo Scientific - 88953). Briefly, 50 µL of culture media was mixed with 50 µL LDH reaction mixture and incubated at room temperature for 30 minutes protected from light, after which point the reaction was stopped by adding 50 µL of stop solution. LDH release into the culture media was quantified by spectrophotometric measurement at 490 and 680 nm, and % cytotoxicity was calculated by

comparison of $A_{490}$-$A_{680}$ values for dosed wells to that of the negative control (DMEM + PBS, 0% cytotoxicity) and a positive control (1X LDH assay lysis buffer, 100% cytotoxicity).

### 5.2.3 Inhibition of Native Hemolysin Activity

Heparinized pig's blood was obtained from healthy animals, washed with 3 parts PBS to 1 part blood, and centrifuged for 5 minutes at 2000 x g. The supernatant was removed, and the PBS wash and centrifugation steps were repeated twice more. Washed erythrocytes were then suspended in PBS or PBS + 50 µM DIDS (Sigma-Aldrich – D3514) to a final concentration of 2% (v/v), mixed with an equal amount of native hemolysin (3.5 µg/mL ≈ 1120 HU), and incubated at 37 °C for 1 hour. Cells were then pelleted by centrifugation, and hemoglobin release into the supernatant was quantified by spectrophotometric measurement at 545 nm. % hemolysis was calculated by comparison of $A_{545}$ values to that obtained for the negative control (PBS only, 0% hemolysis) and the positive control (distilled water, 100% hemolysis). No hemolysis was observed with PBS + 50 µM DIDS alone.

### 5.2.4 Electrogenic Ussing Chamber studies

Caco-2 monolayer were grown to confluency on Snapwell inserts (Corning – CLS380) and cultured for 1 week in FBS free DMEM as described previously. Monolayers were then mounted in the Ussing chamber utilizing 1.12 $cm^2$ cell culture inserts (Physiologic Instruments – P2302). The apical and basolateral chambers were filled with 5 mL of Kreb's buffer (113 mM NaCl, 5 mM KCl, 1.6 mM $Na_2HPO_4$, 0.3 mM $NaH_2PO_4*H_2O$, 25 mM $NaHCO_3$, 1.1 mM $MgCl_2*6H_2O$, 2.2 mM $CaCl_2*2H_2O$, and 10 mM glucose, pH 7.4) and continuously gassed with a 95% $O_2$ – 5% $CO_2$ mixture. Chambers were maintained at 37°C by a heated circulating water bath. Ag-AgCl electrodes and salt bridges containing 3M KCl in agar were used to clamp the monolayers to 0 mV transepithelial potential difference utilizing 1 mV pulses every 30 seconds [469]. Tissues were allowed to equilibrate for 20 minutes before the addition of 100 µM amiloride to

the apical chamber (Sigma-Aldrich – A7410). After the traces reached steady state 10 µM isoproterenol (Sigma-Aldrich – I6504) was added to the apical and basolateral chambers. Once the traces again reached steady state 10 µM forskolin (Sigma-Aldrich – F6886) and 1000 µM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich – I5879) were added to the apical and basolateral chambers. For experiments involving the inhibition of DRA 500 µM DIDS (Sigma-Aldrich – D3514) was added to the apical and basolateral chambers prior to the addition of any drugs.

### 5.2.5 Statistical Analysis

All data were normally distributed (Sharpiro-Wilk test, $p > 0.05$). Differences in $I_{sc}$ between native hemolysin treated and control Caco-2 monolayers were analyzed by One-Way ANOVA followed by Holm-Sidak Post-hoc ($\alpha = 0.05$), while differences in $I_{sc}$ between native hemolysin treated and control Caco-2 monolayers following DIDS treatment were assessed using Student's T-test ($\alpha = 0.05$). All statistical analyses were performed in Sigmaplot version 11.0 (Systat Software Inc). Significance is denoted by * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$).

## 5.3 Results

### 5.3.1 The *Brachyspira hampsonii* native hemolysin does not possess *in vitro* cytotoxic activity against an epithelial cell line

The potent hemolytic activity of the native hemolysin reported in the previous chapter lead us to hypothesize that this toxin also possessed cytotoxic activity against colonic epithelial cells, and that this cytotoxicity may play a critical role in the pathogenesis of swine dysentery. To test this hypothesis, we exposed Caco-2 cells to varying concentrations of *Brachyspira hampsonii* native hemolysin and assessed cytotoxicity through the release of lactate dehydrogenase into the culture supernatant. Caco-2 cells were chosen due to the fact that previous studies have demonstrated that treatment of this cell line with sonicated *Brachyspira hampsonii* recapitulates aspects of swine dysentery pathology [9-11]. Surprisingly, no cytotoxicity was observed in Caco-2 cells treated with 8 hemolytic units (HU) of native hemolysin for 24 hours (Figure 5.1), indicating the *Brachyspira hampsonii* native hemolysin does not contribute to swine dysentery by destroying the colonic epithelium. In response to these findings, we hypothesized that the *Brachyspira hampsonii* native hemolysin may instead directly modify ion transport within the colonic epithelium.

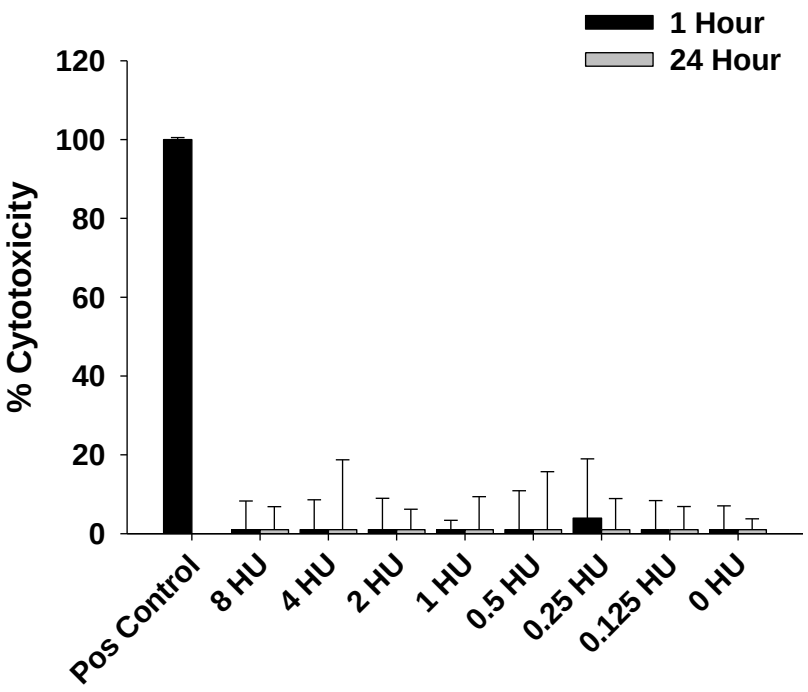


Figure 5.1 Cytotoxicity of *Brachyspira hampsonii* native hemolysin. Caco-2 cells were plated in a 96 well plate ($1.0 * 10^4$ cells per well) and incubated overnight at 37°C, after which point the cells were dosed with native hemolysin for 1 or 24 hours as indicated. Native hemolysin induced cytotoxicity was assessed by spectrophotometric measurement of lactate dehydrogenase activity in the culture media. No appreciable cytotoxicity was observed in cells treated with ≤ 8 HU of native hemolysin for 1 or 24 hours. Cells lysed with LDH assay lysis buffer were used as a positive control (100% cytotoxicity) while cells dosed with only media were used as a negative control (0% cytotoxicity. Data are presented as mean + SEM (n = 3 per group).

### 5.3.2 DIDS is a potent inhibitor of the *Brachyspira hampsonii* native hemolysin

While SLS was initially hypothesized to function as a pore-forming toxin [380, 381], recent evidence has suggested that SLS may in fact lyse erythrocytes by modifying the activity of the band 3 anion exchanger [384]. To better understand the hemolytic mechanism of the *Brachyspira hampsonii* native hemolysin, we assessed the ability of DIDS to inhibit hemolysis by this toxin. Addition of DIDS to reaction mixtures containing *Brachyspira hampsonii* native hemolysin and pig erythrocytes resulted in inhibition of hemolysis (Figure 5.2). DIDS is a potent inhibitor of the band 3 anion exchanger [470] and its inhibition of native hemolysin activity suggests that like SLS, the hemolytic activity of the *Brachyspira hampsonii* native hemolysin is a consequence of its overactivation of band 3 [384].

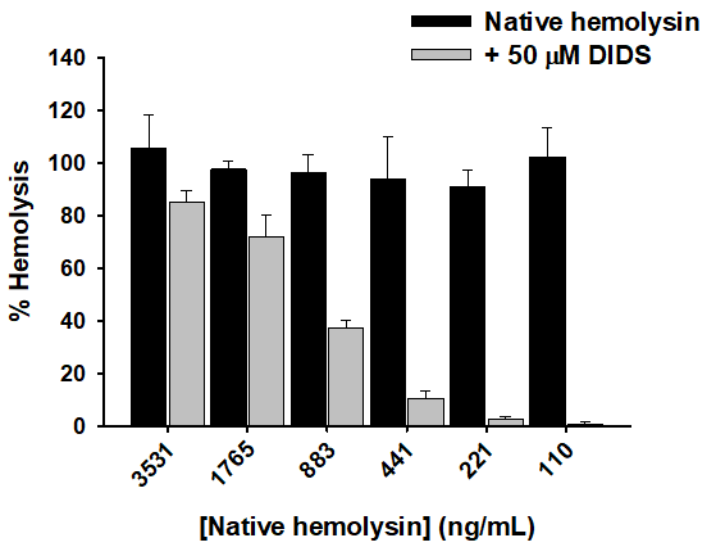


Figure 5.2 Effect of DIDS on *Brachyspira hampsonii* native hemolysin activity**.** A pig erythrocyte suspension was exposed to twofold serial dilutions of the *Brachyspira hampsonii* native hemolysin in the presence (grey bars) or absence (black bars) of 50 µM DIDS. While a small inhibitory effect of DIDS was observed at the highest native hemolysin dose of 3531 ng/mL (128 HU), at lower doses a near complete inhibition of hemolysis was observed. Data are presented as mean + SD (n = 3 per group).

### 5.3.3 A physiologically relevant dose of the *Brachyspira hampsonii* native hemolysin alters electrogenic $Na^+$ absorption and $Cl^-$ secretion in Caco-2 monolayers

Previous studies in our lab have shown that the inhibition of apical $Cl^-$ absorption is an important aspect of the pathogenesis of swine dysentery, and that this inhibition is a result of the downregulation of the apical $Cl^-/HCO_3^-$ exchanger DRA [10]. Our findings in the previous section led us to hypothesize that in addition to band 3, the *Brachyspira hampsonii* native hemolysin also interacts with DRA and that this interaction contributes to the development of diarrhea during *Brachyspira* infection. To test this, we exposed Caco-2 monolayers to 3.6 hemolytic units of *Brachyspira hampsonii* native hemolysin for either 1 or 24 hours, after which point the monolayers were mounted on the Ussing Chamber and the effects of native hemolysin treatment on electrogenic ion transport were assessed using pharmacological inhibitors. No significant differences in trans-epithelial electrical resistance were observed in monolayers treated with native hemolysin in relation to controls (Figure 5.3A), providing further evidence that the *Brachyspira hampsonii* native hemolysin is not cytotoxic towards colonic epithelial cells. Additionally, a slight decrease in initial membrane potential differences was observed in native hemolysin treated monolayers (Figure 5.3B), however this decrease was not statistically significant. Treatment with the ENaC inhibitor amiloride decreased $I_{sc}$ in both native hemolysin treated and control monolayers, with this drop being significantly greater in control monolayers (Figure 5.3C). These data indicate that native hemolysin is capable of inhibiting $Na^+$ absorption through ENaC, and that these effects occur within an hour of exposure. Stimulation of apical $Cl^-$ secretion by the β-adrenergic agonist isoproterenol or the adenyl cyclase activator forskolin and the phosphodiesterase inhibitor IBMX resulted in increased $I_{sc}$ in all monolayers tested (Figure 5.3D/E). While isoproterenol induced increases in $I_{sc}$ compared to control monolayers were

observed in those treated with native hemolysin for 1 or 24 hours, this increase was only significant after 24 hours of treatment. Similarly, increases in $I_{sc}$ relative to control were once again observed in monolayers treated with native hemolysin for 1 or 24 hours after the addition of forskolin/IBMX, however, this increase was only significant in the 1 hour treatment group.

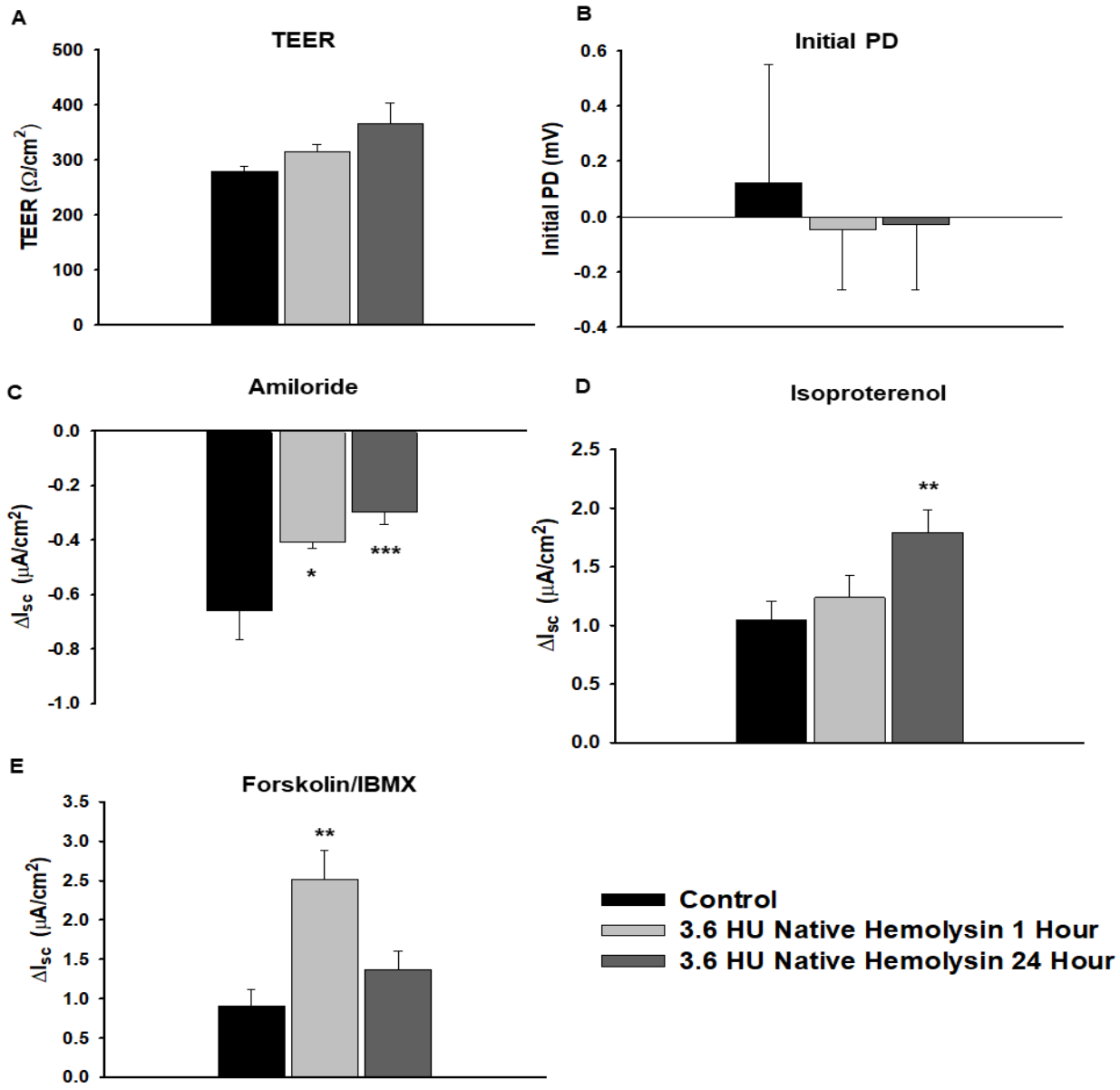


Figure 5.3 – Modulation of electrogenic $Na^+$ and $Cl^-$ transport by the *Brachyspira hampsonii* native hemolysin. Caco-2 monolayers grown on Snapwell supports were exposed to 3.6 HU of *Brachyspira hampsonii* native hemolysin for 1 or 24 hours, then mounted on the Ussing chamber. No differences in transepithelial electrical resistance (A) or initial membrane potential (B) were observed between native hemolysin treated and control monolayers. A muted amiloride response was observed in native hemolysin treated monolayers (B), indicating a decrease in electrogenic $Na^+$ absorption through ENaC. An increase in isoproterenol mediated $Cl^-$ secretion was observed after 24 hours of native hemolysin treatment (D), while an increase in forskolin/IBMX mediated $Cl^-$ secretion was observed after 1 hour of treatment (E). Data are presented as mean ± SEM (n = 16 per group). * indicates significant difference from control at $p < 0.05$, ** indicates significant difference from control at $p < 0.01$, *** indicates significant difference from control at $p < 0.001$ (One-Way ANOVA, Holm-Sidak Post-hoc)

### 5.3.4 The effects of *Brachyspira hampsonii* native hemolysin treatment on epithelial ion transport are blocked by the addition of DIDS

To determine if the effects seen in the previous section were a result of the modulation of DRA activity by the *Brachyspira hampsonii* native hemolysin we exposed Caco-2 monolayers to 3.6 HU of native hemolysin for 1 hour then mounted the cells in the Ussing Chamber. DIDS was added to the apical side of the chamber at a concentration of 500 µM to inhibit DRA activity [471], after which point pharmacological inhibitors were added as in the previous section. Both control and native hemolysin treated monolayers exhibited a decrease in $I_{sc}$ following amiloride treatment (Figure 5.4A), however this response was blunted in comparison to monolayers that were not previously treated with DIDS. No significant differences in $\Delta I_{sc}$ were observed between control and native hemolysin treated monolayers, indicating that the effects of the *Brachyspira hampsonii* native hemolysin on apical $Na^+$ absorption are mediated through DRA. Additionally, no significant differences in $\Delta I_{sc}$ between control and native hemolysin treated monolayers upon the addition of either isoproterenol or forskolin/IBMX (Figure 5.4B/C), indicating that the increased responses observed following the treatment of Caco-2 monolayers with native hemolysin are also a direct result of the stimulation of DRA activity by this toxin.

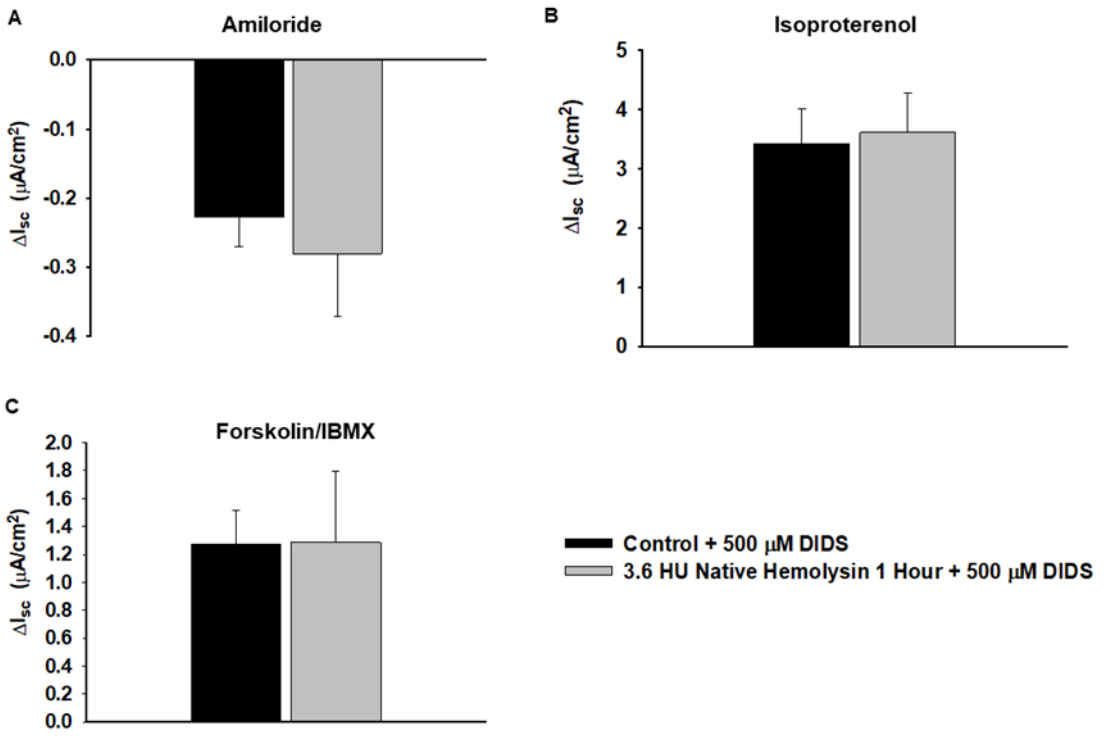


Figure 5.4 Effect of DIDS on native hemolysin induced ion transport alterations. Caco-2 monolayers were treated with 3.6 HU native hemolysin for 1 hour then mounted on the Ussing chamber. Following addition of 500 μM DIDS to the apical chamber electrogenic $Na^+$ absorption was inhibited by the addition of amiloride (A). Electrogenic $Cl^-$ secretion was stimulated by the addition of the β-adrenergic agonist isoproterenol (B) and the adenyl cyclase activator forskolin/IBMX (C). No significant differences between control and native hemolysin treated hemolysin monolayers were observed upon addition of any drugs (Student's T-test, $\alpha = 0.05$), indicating that the effects of native hemolysin on ion transport are due to a stimulation of DRA activity. Data are presented as mean + SEM (n = 11 for control monolayers, n = 9 for native hemolysin treated monolayers).

## 5.4 Discussion

### 5.4.1 The lack of in vitro cytotoxicity observed with the *Brachyspira hampsonii* native hemolysin is consistent with the pathology of swine dysentery

SLS is considered to be a crucial virulence factor in soft tissue infections caused by *Streptococcus pyogenes*, with SLS$^-$ negative *Streptococcus pyogenes* strains failing to induce the large-scale tissue destruction observed during severe infection [472]. As a result, we hypothesized that the native hemolysin of *Brachyspira hampsonii*, a putative homologue of Streptolysin S, could directly contribute to the development of swine dysentery through the direct destruction of the colonic epithelium, as severe cases of swine dysentery are often accompanied by lesion formation, inflammation and hemorrhage within the colon of infected animals [131]. To assess the cytotoxic effects of the *Brachyspira hampsonii* native hemolysin on colonic epithelial cells we utilized the Caco-2 cell line, a colonic adenocarcinoma cell line that has been previously used for studies of swine dysentery and porcine intestinal spirochetosis [9-11, 53, 136]. In contrast to the potent hemolytic activity the *Brachyspira hampsonii* native hemolysin displayed against pig erythrocytes (Figure 4.1C), this toxin did not possess measurable cytotoxic activity against Caco-2 cells at concentrations up to 8 HU (Figure 5.1). While we cannot discount the possibility that higher concentrations of native hemolysin would possess a cytotoxic effect towards Caco-2 cells, 8 HU was deliberately chosen as the highest dose in our experiment due to the fact that this dose is roughly equivalent to the dose of *Brachyspira hampsonii* lysate determined to be a physiological concentration in previous studies (approximately a 50 fold dilution of a dense broth culture) [9, 11]. Furthermore, while epithelial necrosis has been observed in both animals and tissue explants experimentally infected with *Brachyspira hyodysenteriae* or *Brachyspira hampsonii* [131, 132, 154, 473], this damage does not translate to a loss of trans-epithelial electrical resistance or increased tight junction

permeability, indicating that the barrier function of the colon is unaffected [9-11]. This epithelial necrosis appears to be a consequence of the host inflammatory response, as both live and sonicated *Brachyspira hyodysenteriae* cells are incapable of inducing necrosis in swine testicle or HeLa cells [474], while treatment of colon explants with *Escherichia coli* derived LPS induced necrosis to a similar degree as that observed with *Brachyspira hampsonii* co-culture [473]. Interestingly, co-culturing of colonic tissue explants with the non-pathogenic *Brachyspira hampsonii* KL180 did not induce epithelial necrosis to the same degree as that observed with the pathogenic *Brachyspira hampsonii* 30446 [473]. The *sag* operon is absent in *Brachyspira hampsonii* KL180 [20], suggesting that production of an SLS homologue may play a role in epithelial necrosis through stimulation of the host inflammatory response, rather than through direct cytotoxicity. Similarly, treatment of keratinocytes with SLS from *Streptococcus pyogenes* results in increased IL-1β production and subsequent cell death [408, 411], providing a potential mechanism by which *Brachyspira hampsonii* could induce epithelial necrosis during severe infection.

### 5.4.2 Proposed model of erythrocyte hemolysis by the native hemolysins of *Brachyspira hyodysenteriae* and *Brachyspira hampsonii*

While SLS was initially believed to function as a pore-forming toxin, contradictory findings on the size of this pore have raised doubt on the true functionality of this toxin [380, 381]. Recent evidence has indicated that SLS is capable of inducing cell death through a variety of mechanisms, including increasing production of mitochondrial ROS and inhibiting AKT signaling [405, 406, 408]. These observations, combined with the recent finding that SLS induces hemolysis through the overactivation of the band 3 anion exchanger [384], indicate that SLS has profound effects on cellular signaling and physiology aside from simple cytotoxicity due to pore formation. To determine if the *Brachyspira hampsonii* native hemolysin also lyses

erythrocytes through activation of band 3, and to further confirm the identity of this toxin as an SLS homologue, we assessed the ability of the anion exchange inhibitor DIDS to prevent native hemolysin mediated hemolysis. A complete abolition of hemolysis was observed upon treatment with 50 µM DIDS, confirming the involvement of band 3 in *Brachyspira hampsonii* mediated hemolysis. These data led to a putative model of SLS/native hemolysin mediated hemolysis in which the overactivation of the band 3 anion exchanger leads to a rapid influx of $Cl^-$ into the cell, leading to an influx of water and subsequent lysis of the cell through osmotic stresses. While this mechanism provides an explanation for the hemolytic activity of SLS/native hemolysin, the fact that band 3 is only expressed in erythrocytes and the kidney raises new questions about the biological role of the native hemolysin during *Brachyspira* infection [475, 476]. While it has been hypothesized that *Brachyspira* species may utilize one or more hemolysins to acquire cholesterol during infection [477, 478], it is unlikely that this is the primary function of these toxins *in vivo*. Indeed, while both *Brachyspira hampsonii* strain 30446 and strain KL180 are strongly β-hemolytic [97], the absence of the sag operon in the non-pathogenic KL180 strain suggests that this gene product has additional contributions to disease beyond nutrient acquisition. As such, we hypothesized that the primary *in vivo* function of the *Brachyspira hampsonii* native hemolysin is not the destruction of erythrocytes, but rather the direct modulation of colonic ion transport through the modulation of the $Cl^-/HCO_3^-$ exchanger DRA.

### 5.4.3 Acute treatment of Caco-2 monolayers with the *Brachyspira hampsonii* native hemolysin induces physiological changes consistent with malabsorptive diarrhea

While the *Brachyspira hampsonii* native hemolysin did not possess a cytotoxic effect towards Caco-2 cells (Figure 5.1), studies involving pharmacological inhibitors revealed not only a decrease in amiloride mediated inhibition of $Na^+$ absorption (Figure 5.3C), but also an increase in isoproterenol and forskolin/IBMX mediated $Cl^-$ secretion (Figure 5.3D/E) in Caco-2

monolayers treated with 3.6 HU of *Brachyspira hampsonii* native hemolysin. These findings are at odds with previous studies on the pathophysiology of swine dysentery, which concluded that *Brachyspira* induced diarrhea is a result of electroneutral $Na^+$ and $Cl^-$ absorption due to downregulation of the $Na^+/H^+$ exchanger NHE3 [9] and the $Cl^-/HCO_3^-$ exchanger DRA [10], while a decrease in $Cl^-$ secretion was observed due to downregulation of the $Cl^-$ channel CFTR [11]. However, the tissue utilized in these studies was obtained from pigs approximately 4-8 days after infection with *Brachyspira hampsonii* or *Brachyspira hyodysenteriae* [9-11], in contrast to the 1 and 24-hour timepoints used in our study. As a result, these data suggest that the early stages of *Brachyspira* induced diarrhea may in fact arise from the downregulation of electrogenic $Na^+$ absorption through ENaC. Overactivation of DRA by the *Brachyspira hampsonii* native hemolysin would lead to a large efflux of $HCO_3^-$ into the lumen of the colon, accompanied by a corresponding influx of $Cl^-$ into the cell. This $HCO_3^-$ efflux would result in a decreased intracellular pH, a condition previously shown to dramatically inhibit ENaC function [479]. Additionally, the loss of luminal $Cl^-$ through overactivation of DRA would also affect the folding and functionality of secreted colonic mucins [480], a phenomenon previously demonstrated to improve colonization and epithelial attachment of *Brachyspira* species within the colons of infected pigs [11, 133, 141, 156]. While the increased $\Delta I_{sc}$ observed upon treatment with isoproterenol and forskolin/IBMX (Figure 5.3D/E) is indicative of a $Cl^-$ secretory response, the fact that the initial membrane potential differences were negative in native hemolysin treated monolayers (Figure 5.3B) indicates that this phenomenon may be an artifact arising from the intense activation of DRA by native hemolysin. While the exact stoichiometry of $Cl^-/HCO_3^-$ exchange mediated by DRA remains a subject of debate, there is evidence to suggest that this transporter functions to bring 2 $Cl^-$ ions into the cell while expelling a single $HCO_3^-$ ion out of

the cell [481]. If this is indeed the case, overactivation of DRA by native hemolysin would result in a net movement of negative charge into the cell, accounting for the minor drop in PD observed in native hemolysin treated monolayers (Figure 5.3B). Furthermore, this increased absorption of $Cl^-$ through DRA would further serve to alter the rheological properties of colonic mucins, potentially facilitating colonization of the colonic epithelium by *Brachyspira hampsonii* [11, 12, 133, 141, 156]. Perplexingly, previous studies have shown that DRA is downregulated at both the mRNA and protein level during severe *Brachyspira* infection [10], meaning that this response would be muted during the later stages of disease. *In vitro* studies have shown that the downregulation of DRA expression during swine dysentery is a result of the host inflammatory response, as the addition of the pro-inflammatory cytokine IL-1α to Caco-2 monolayers resulted in a drop in DRA mRNA levels after 24 hours of exposure [10]. Respectively, increased expression of the related cytokine IL-1β is observed ≥6 hours after infection of keratinocytes with $SLS^+$ *Streptococcus pyogenes*, and this increased IL-1β production leads to keratinocyte cytotoxicity and the development of tissue lesions in mice infected with *Streptococcus pyogenes* [408, 411]. While we have not assessed the pro-inflammatory effects of native hemolysin alone on colonic tissue, previous results from our lab have shown that sonicated *Brachyspira hampsonii* 30446 induces an inflammatory response in Caco-2 cells after 48 hours of exposure [9, 10], providing a potential positive feedback mechanism by which DRA expression is downregulated during the progression of swine dysentery. Additionally, activation of the p38 MAPK pathway is observed not only during the treatment of Caco-2 cells with sonicated *Brachyspira hampsonii* [9], but also in keratinocytes treated with SLS [408, 411], suggesting that a conserved mechanism is responsible for the inflammatory response observed during these two events. Altogether, these data provide a potential mechanism for the acute development of

diarrhea during the early stages of *Brachyspira* infection (1-24 hour exposure), along with a potential mechanism for the downregulation of DRA observed in pigs suffering from severe diarrhea several days after infection.

## 5.5 Conclusion

Previous studies have shown that the diarrhea induced by *Brachyspira hyodysenteriae* or *Brachyspira hampsonii* infection is malabsorptive, occurring due to the downregulation of the electroneutral $Na^+/H^+$ exchanger NHE3 and the electroneutral $Cl^-/HCO_3^-$ exchanger DRA [9, 10]. The potent hemolytic activity observed with the *Brachyspira hampsonii* native hemolysin, alongside the fact that an operon believed to contain the genes responsible for native hemolysin production is absent in the nonpathogenic *Brachyspira hampsonii* KL180 [20] lead us to hypothesize that the native hemolysin is a crucial virulence factor in the pathogenesis of swine dysentery. As such, we set out to examine the effects of the *Brachyspira hampsonii* native hemolysin on a colonic cell line to determine if aspects of swine dysentery could be recapitulated *in vitro*. Ussing chamber studies involving Caco-2 cells treated with native hemolysin revealed a loss of electrogenic $Na^+$ absorptive capacity through ENaC. Although these results are at odds with what has previously been reported in the literature, the fact that these responses were seen within 1 hour of treatment, rather than several days following experimental infection with *Brachyspira*, suggest that swine dysentery may follow a biphasic progression, however, further experiments tracking the effects of longer-term exposure to native hemolysin must be carried out in the future to confirm this. Additionally, our experiments were limited by the fact that only electrogenic ion transport was assessed – flux studies involving radiolabelled tracers are required to fully understand the alterations to ion transport induced by native hemolysin. We also hypothesized that the pathogenicity of *Brachyspira* isolates is correlated with the expression of the SLS homologue/native hemolysin. Although these results suggest that native hemolysin plays a critical role in swine dysentery pathogenesis, experimental infection with $SLS^-$ strains of

*Brachyspira hyodysenteriae* or *Brachyspira hampsonii* must be carried out to definitively prove this.

# Chapter 6 – General Discussion

## 6.1 Implications

The re-emergence of swine dysentery within the previous 20 years has had profound effects on the pork industry due to the large economic costs associated with outbreaks of this disease. Although our understanding of the pathophysiology of swine dysentery has dramatically improved in recent years, the detailed molecular mechanisms by which *Brachyspira* species induce diarrhea in their porcine hosts remains unsolved. My doctoral research aimed to identify and characterize the major hemolysins produced by the recently discovered *Brachyspira hampsonii*, and to determine the role these hemolysins play in the overall pathogenesis of swine dysentery. This research was focused primarily on TlyA and native hemolysin, as previous reports have shown these toxins to be major contributors to disease in both *Brachyspira* species and unrelated pathogenic bacteria.

Although the *tlyA* gene was first discovered in *Brachyspira hyodysenteriae* several decades ago [323], the vast majority of research into this toxin has been carried out in homologues from *Mycobacterium tuberculosis* [15], *Helicobacter pylori* [16], and *Campylobacter jejuni* [17]. Our results confirm that like the TlyA homologues from these species, the *Brachyspira hampsonii* TlyA protein is both a pore-forming hemolysin and a rRNA methyltransferase. Additionally, our mutational studies have identified two specific amino acid residues necessary for the hemolytic activity of TlyA (S9 and C80). While the role of intermolecular disulfide bonding in TlyA oligomerization and pore-formation has been previously examined [18, 419], one study did not determine the specific cysteine residues involved in disulfide bond formation in *Mycobacterium tuberculosis* TlyA [18], while another study involving *Helicobacter pylori* TlyA determined that the cysteines involved in this disulfide bonding were not present in TlyA proteins from different

species [419]. In contrast, we have found that intermolecular disulfide bond formation in *Brachyspira hampsonii* TlyA involves cysteine 80, a residue highly conserved in a variety of TlyA proteins from various species, including *Mycobacterium tuberculosis* and *Campylobacter jejuni*. Additionally, the fact that a serine to lysine mutation found in nonpathogenic *Brachyspira* species completely abolished the hemolytic activity of TlyA by impairing oligomerization of this monomer suggests certain commensal bacteria have developed evolutionary strategies to maintain the beneficial rRNA methyltransferase functionality of TlyA without causing damage to their host. The observation that this serine residue is conserved in the hemolytic TlyA protein from *Campylobacter jejuni*, but not the nonhemolytic TlyA homologue from *Thermus thermophilus*, suggests that this may be a widely conserved mechanism of TlyA inactivation across multiple bacterial families.

Although the results in chapter 3 suggested that TlyA may play a role in the progression of swine dysentery, the high concentrations and long incubation times required for TlyA induced hemolysis suggested that TlyA is not the primary contributor to the β-hemolytic phenotype observed in pathogenic *Brachyspira* strains. As such, we turned our focus to native hemolysin, a highly potent hemolysin produced by *Brachyspira hyodysenteriae* broth cultures supplemented with partially digested yeast RNA (yeast RNA core) [318, 319, 322]. Although a previous study has linked the *Brachyspira hyodysenteriae* native hemolysin to a gene termed *hlyA* [14], the similarities of native hemolysin to streptolysin S of *Streptococcus pyogenes* [19], along with the fact that an operon encoding for a streptolysin S homologue has been found in the genomes of several pathogenic *Brachyspira* strains [20] suggested that this toxin may have been erroneously linked to the *hlyA* gene. We have determined that like *Brachyspira hyodysenteriae*, *Brachyspira hampsonii* strain 30446 produces a highly potent hemolysin in the presence of yeast RNA core.

Interestingly, the activity of this toxin was strongly inhibited upon the addition of Trypan blue, a known inhibitor of streptolysin S [274]. Subsequently, co-expression of the *sagA* gene from *Brachyspira hampsonii* strain 30446 with the *sagBCD* genes yielded a hemolytic peptide. Although the specific activity of the purified SagA peptide was much lower than that of the *Brachyspira hampsonii* native hemolysin, the similar molecular weights obtained for each toxin by size exclusion chromatography and SDS-PAGE strongly suggest that these toxins are one and the same. Additionally, our efforts to improve the yield of the heterologously expressed SagA peptide led us to develop an expression system capable of yielding ~5 mg of pure SagA peptide per liter of *E. coli* culture, greatly improving the feasibility of future structural and functional studies on streptolysin S homologues within both *Brachyspira* species and unrelated bacteria.

The potent hemolytic activity of the *Brachyspira hampsonii* native hemolysin, combined with the observation that the sag operon is absent in the nonpathogenic *Brachyspira hampsonii* strain KL180 [20], led us to hypothesize that this hemolysin plays a critical role in swine dysentery pathology. While we initially hypothesized that this was due to direct cytotoxicity of native hemolysin against the colonic epithelium, no cytotoxicity was observed when Caco-2 cells were treated with this toxin. This result, combined with the recent observation that streptolysin S can activate the erythrocyte anion exchanger band 3 [384], suggested that native hemolysin may in fact contribute to the development of diarrhea through direct modulation of colonic ion transport. To test this, we first assessed the inhibitory effect of DIDS on the *Brachyspira hampsonii* native hemolysin. Treatment with DIDS substantially inhibited the hemolytic activity of native hemolysin, suggesting that like streptolysin S, this toxin exerts its hemolytic effect through the activation of the band 3 anion exchanger. Given these results, we next aimed to assess the direct effects of native hemolysin on ion transport utilizing a cell culture model of the colonic

epithelium. Treatment of Caco-2 monolayers with native hemolysin resulted in a significant decrease in electrogenic $Na^+$ absorption through ENaC, confirming that this toxin has profound effects on epithelial ion transport. Furthermore, treatment of the dosed Caco-2 monolayers with DIDS completely reversed these effects, suggesting that these effects are a result of direct modulation of the $Cl^-/HCO_3^-$ exchanger DRA by the native hemolysin. Although our results are contradicted by *in vivo* studies involving tissue obtained from pigs experimentally infected with *Brachyspira hyodysenteriae* or *Brachyspira hampsonii* [9-11], the effects of native hemolysin treatment were apparent within 1 hour of exposure in contrast to the several days required for the development of clinical signs following *Brachyspira* infection, suggesting that the progression of swine dysentery may follow a biphasic progression.

## 6.2 Future Research

Although the effects of TlyA on the colonic epithelium were not examined in this thesis, research in our laboratory to understand the role TlyA plays in swine dysentery pathogenesis is ongoing. Furthermore, recent evidence has suggested the rRNA methyltransferase function of TlyA plays an important role in *Campylobacter jejuni* pathogenesis [345, 346]. As such, it stands to reason that the rRNA methyltransferase functionality of *Brachyspira hampsonii* TlyA may also play a crucial role in swine dysentery pathogenesis, and our research group is actively studying the effects of TlyA methyltransferase mutants on cultured colonic epithelial cells. Although our research has provided a wealth of evidence suggesting that the native hemolysin of *Brachyspira hampsonii* is in fact a streptolysin S homologue, we were unable to perform mass spectrometry fingerprinting of this peptide to definitively confirm this. Additionally, although the non-pathogenic *Brachyspira hampsonii* strain KL180 does not possess a sag operon encoding for the streptolysin S homologue, this bacterium is also strongly β-hemolytic on blood agar [97]. This

suggests that while the native hemolysin may in fact be the primary contributor to *Brachyspira* hemolysis, the β-hemolytic phenotype exhibited by these bacteria is likely due to the expression of multiple hemolysins. As such, transcriptomics analyses of all known *Brachyspira* hemolysins are necessary to completely understand the contributors to the hallmark β-hemolytic phenotype of these bacteria. Finally, although treatment of Caco-2 monolayers with the *Brachyspira hampsonii* native hemolysin induced significant alterations in ion transport, these experiments were limited in both the time points examined and the techniques used to assess these alterations. The time from initial infection with *Brachyspira hyodysenteriae* or *Brachyspira hampsonii* to the development of mucohaemmorhagic diarrhea is approximately 3-7 days [9-11], and as such, our experiments only recapitulated the first 24 hours of *Brachyspira* infection. Future experiments involving extended native hemolysin exposure times (48-72 hours) are necessary to test our hypothesis that a biphasic progression of *Brachyspira* induced disease exists. Additionally, our Ussing chamber experiments only assessed electrogenic ion transport through pharmacological inhibition of ion channels. Given that *Brachyspira* induced diarrhea is known to involve the downregulation of electroneutral transporters such as NHE3 [9] and DRA [10], flux experiments involving radiolabelled $Na^+$ and $Cl^-$ isotopes are necessary to fully understand the net movement of ions across the colonic epithelium in response to native hemolysin intoxication.

## References


[1] R.A. Whiting, L.P. Doyle, R.S. Spray, **Swine dysentery**, Purdue University Agricultural Experiment Station, 1921.

[2] D.J. Taylor, T.J.L. Alexander, **The production of dysentery in swine by feeding cultures containing a spirochaete**, British Veterinary Journal, 127 (1971) lviii-lxi.

[3] D.L. Harris, R.D. Glock, C.R. Christensen, J.M. Kinyon, **Inoculation of pigs with *Treponema hyodysenteriae* (new species) and reproduction of the disease**, Veterinary medicine, small animal clinician : VM, SAC, 67 (1972) 61-64.

[4] E.R. Burrough, McKean J., K.J. Schwartz, **Bloody scours (swine dysentery): A costly re-emerging disease that is preventable.,** in: D. Weiss, R. Baker (Eds.) Pork Information Gateway, vol. 2021, U.S. Pork Center of Excellence, Clive Iowa, 2013.

[5] Y. Chander, A. Primus, S. Oliveira, C.J. Gebhart, **Phenotypic and molecular characterization of a novel strongly hemolytic *Brachyspira* species, provisionally designated "*Brachyspira hampsonii*"**, Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 24 (2012) 903-910.

[6] J.E. Rubin, M.O. Costa, J.E. Hill, H.E. Kittrell, C. Fernando, Y. Huang, B. O'Connor, J.C. Harding, **Reproduction of mucohaemorrhagic diarrhea and *coli*tis indistinguishable from swine dysentery following experimental innoculation with *"Brachyspira hampsonii"* strain 30446**, PloS one, 2 (2013) e57146.

[7] R. Thérése, J.D. S., J. Karl-Erik, F. Claes, **A novel enteropathogenic, strongly haemolytic spirochaete isolated from pig and mallard, provisionally designated '*Brachyspira suanatina*' sp. Nov**, Environ. Microbiol., 9 (2007) 983-991.

[8] D.J. Hampson, T. La, N.D. Phillips, **Emergence of *Brachyspira* species and strains: Reinforcing the need for surveillance**, Porcine Health Management, 1 (2015) 8.

[9] C.B. Enns, B.A. Keith, N. Challa, J.C.S. Harding, M.E. Loewen, **Impairment of electroneutral na(+) transport and associated downregulation of NHE3 contributes to the development of diarrhea following in vivo challenge with *Brachyspira* spp**, American journal of physiology. Gastrointestinal and liver physiology, 318 (2020) G288-g297.

[10] C.B. Enns, **Pathophysiological effects of *Brachyspira* species on ion transport within the porcine colon resulting in diarrheal disease,** Veterinary Biomedical Sciences, Doctor of Philosophy, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, 2018.

[11] C.B. Enns, J.C.S. Harding, M.E. Loewen, **Decreased electrogenic anionic secretory response in the porcine colon following in vivo challenge with *Brachyspira* spp. Supports an altered mucin environment**, American journal of physiology. Gastrointestinal and liver physiology, 316 (2019) G495-g508.

[12] M.P. Quintana-Hayashi, M. Mahu, N. De Pauw, F. Boyen, F. Pasmans, A. Martel, P. Premaratne, H.R. Fernandez, O. Teymournejad, L. Vande Maele, F. Haesebrouck, S.K. Lindén, **The levels of *Brachyspira hyodysenteriae* binding to porcine colonic mucins differ between individuals, and binding is increased to mucins from infected pigs with de novo MUC5AC synthesis**, Infection and Immunity, 83 (2015) 1610-1619.

[13] A.A.H.M. ter Huurne, S. Muir, M. van Houten, B.A.M. van der Zeijst, W. Gaastra, J.G. Kusters, **Characterization of three putative *Serpulina hyodysenteriae* hemolysins**, Microb. Pathog., 16 (1994) 269-282.

[14] T. Hsu, D.L. Hutto, F.C. Minion, R.L. Zuerner, M.J. Wannemuehler, **Cloning of a beta-hemolysin gene of *Brachyspira* (*Serpulina*) *hyodysenteriae* and its expression in *Escherichia coli***, Infect Immun, 69 (2001) 706-711.

[15] B.W. Wren, R.A. Stabler, S.S. Das, P.D. Butcher, J.A. Mangan, J.D. Clarke, N. Casali, T. Parish, N.G. Stoker, **Characterization of a haemolysin from *Mycobacterium tuberculosis* with homology to a virulence factor of *Serpulina hyodysenteriae***, Microbiology, 144 (1998) 1205-1211.
[16] M.C. Martino, R.A. Stabler, Z.W. Zhang, M.J. Farthing, B.W. Wren, N. Dorrell, ***Helicobacter pylori* pore-forming cytolysin orthologue TlyA possesses *in vitro* hemolytic activity and has a role in colonization of the gastric mucosa**, Infect Immun, 69 (2001) 1697-1703.
[17] A. Sałamaszyńska-Guz, D. Klimuszko, **Functional analysis of the *Campylobacter jejuni cj0183* and *cj0588* genes**, Curr. Microbiol., 56 (2008) 592-596.
[18] A. Rahman, S.S. Srivastava, A. Sneh, N. Ahmed, M.V. Krishnasastry, **Molecular characterization of *tlyA* gene product, *rv1694* of *Mycobacterium tuberculosis*: A non-conventional hemolysin and a ribosomal RNA methyl transferase.**, BMC Biochem., 11 (2010) 35.
[19] E. Todd, **The differentiation of two distinct serological varieties of streptolysin, streptolysin o and streptolysin s**, Journal of Pathology and Bacteriology, 47 (1938) 423-445.
[20] L.A. Johnson, **Detection and characterization of pathogenic and non-pathogenic *Brachyspira*,** Veterinary Microbiology, Master of Science, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, 2017.
[21] L.J. Mappley, R.M. La Ragione, M.J. Woodward, ***Brachyspira* and its role in avian intestinal spirochaetosis**, Veterinary Microbiology, 168 (2014) 245-260.
[22] S.L. Oxberry, D.J. Trott, D.J. Hampson, ***Serpulina pilosicoli*, waterbirds and water: Potential sources of infection for humans and other animals**, Epidemiology and Infection, 121 (1998) 219-225.
[23] M. Boye, S.B. Baloda, T.D. Leser, K. Møller, **Survival of *Brachyspira hyodysenteriae* and *B. pilosicoli* in terrestrial microcosms**, Veterinary Microbiology, 81 (2001) 33-40.
[24] D.J. Hampson, **The spirochete *Brachyspira pilosicoli*, enteric pathogen of animals and humans**, Clinical Microbiology Reviews, 31 (2018) e00087-00017.
[25] J.M. Kinyon, D.L. Harris, ***Treponema innocens*, a new species of intestinal bacteria, and emended description of the type strain of *Treponema hyodysenteriae*,** International Journal of Systematic and Evolutionary Microbiology, 29 (1979) 102-109.
[26] T.K. Jensen, A.S. Christensen, M. Boye, ***Brachyspira murdochii coli*tis in pigs**, Vet Pathol, 47 (2010) 334-338.
[27] W.A. Harland, F.D. Lee, **Intestinal spirochaetosis**, British Medical Journal, 3 (1967) 718-719.
[28] H.B. Fantham, **Remarks on the nature and distribution of the parasites observed in the stools of 1305 dysenteric patients**, The Lancet, 187 (1916) 1165-1166.
[29] L.W. Parr, **Intestinal spirochetes**, The Journal of Infectious Diseases, 33 (1923) 369-383.
[30] C. Cooper, D.W. Cotton, M.J. Hudson, N. Kirkham, F.E. Wilmott, **Rectal spirochaetosis in homosexual men: Characterisation of the organism and pathophysiology**, Genitourinary medicine, 62 (1986) 47-52.
[31] C.M. Surawicz, P.L. Roberts, A. Rompalo, T.C. Quinn, K.K. Holmes, W.E. Stamm, **Intestinal spirochetosis in homosexual men**, The American Journal of Medicine, 82 (1987) 587-592.
[32] M. Esteve, A. Salas, F. Fernández-Bañares, J. Lloreta, M. Mariné, C.I. Gonzalez, M. Forné, J. Casalots, R. Santaolalla, J.C. Espinós, M.A. Munshi, D.J. Hampson, J.M. Viver, **Intestinal**

**spirochetosis and chronic watery diarrhea: Clinical and histological response to treatment and long-term follow up**, Journal of Gastroenterology and Hepatology, 21 (2006) 1326-1333.
[33] R.M. da Cunha Ferreira, A.D. Phillips, C.R. Stevens, M.J. Hudson, H.C. Rees, J.A. Walker-Smith, **Intestinal spirochaetosis in children**, Journal of pediatric gastroenterology and nutrition, 17 (1993) 333-336.
[34] A. Calderaro, S. Bommezzadri, C. Gorrini, G. Piccolo, S. Peruzzi, V. Villanacci, C. Zambelli, G. Dettori, C. Chezzi, **Infective *coli*tis associated with human intestinal spirochetosis**, Journal of Gastroenterology and Hepatology, 22 (2007) 1772-1779.
[35] J.R. Kostman, M. Patel, E. Catalano, J. Camacho, J. Hoffpauir, M.J. DiNubile, **Invasive *coli*tis and hepatitis due to previously uncharacterized spirochetes in patients with advanced human immunodeficiency virus infection**, Clinical Infectious Diseases, 21 (1995) 1159-1165.
[36] M. Körner, J.O. Gebbers, **Clinical significance of human intestinal spirochetosis—a morphologic approach**, Infection, 31 (2003) 341-349.
[37] R.G. Heine, P.B. Ward, A.S. Mikosza, V. Bennett-Wood, R.M. Robins-Browne, D.J. Hampson, ***Brachyspira aalborgi* infection in four australian children**, Journal of Gastroenterology and Hepatology, 16 (2001) 872-875.
[38] J.I. Lee, D.J. Hampson, **Intestinal spirochaetes colonizing aborigines from communities in the remote north of western australia**, Epidemiol Infect, 109 (1992) 133-141.
[39] P.S. Teglbjaerg, **Intestinal spirochaetosis**, Current topics in pathology. Ergebnisse der Pathologie, 81 (1990) 247-256.
[40] K. Hovind-Hougen, A. Birch-Andersen, R. Henrik-Nielsen, M. Orholm, J.O. Pedersen, P.S. Teglbjaerg, E.H. Thaysen, **Intestinal spirochetosis: Morphological characterization and cultivation of the spirochete *Brachyspira aalborgi* gen. Nov., sp. Nov**, Journal of clinical microbiology, 16 (1982) 1127-1136.
[41] J.I. Lee, D.J. Hampson, **Genetic characterisation of intestinal spirochaetes and their association with disease**, Journal of medical microbiology, 40 (1994) 365-371.
[42] D.J. Trott, T.B. Stanton, N.S. Jensen, D.J. Hampson, **Phenotypic characteristics of *Serpulina pilosicoli* the agent of intestinal spirochaetosis**, FEMS microbiology letters, 142 (1996) 209-214.
[43] D.J. Taylor, J.R. Simmons, H.M. Laird, **Production of diarrhoea and dysentery in pigs by feeding pure cultures of a spirochaete differing from *Treponema hyodysenteriae***, The Veterinary record, 106 (1980) 326-332.
[44] N. Muniappa, M.R. Mathiesen, G.E. Duhamel, **Laboratory identification and enteropathogenicity testing of *Serpulina pilosicoli* associated with porcine colonic spirochetosis**, Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 9 (1997) 165-171.
[45] J.R. Thomson, W.J. Smith, B.P. Murray, **Investigations into field cases of porcine *coli*tis with particular reference to infection with *Serpulina pilosicoli***, The Veterinary record, 142 (1998) 235-239.
[46] C.P. Stephens, D.J. Hampson, **Prevalence and disease association of intestinal spirochaetes in chickens in eastern australia**, Avian pathology : journal of the W.V.P.A, 28 (1999) 447-454.
[47] C.P. Stephens, D.J. Hampson, **Experimental infection of broiler breeder hens with the intestinal spirochaete *Brachyspira* ( *Serpulina* ) *pilosicoli* causes reduced egg production**, Avian Pathology, 31 (2002) 169-175.

[48] E. Ivanics, M. Dobos-Kovács, R. Glávits, E. Kaszanyitzky, C. Nemes, L. Szeredi, A. Beregszászi, L. Dencso, **Experimental study on the role of *Brachyspira alvinipulli* in intestinal spirochaetosis of geese**, Acta veterinaria Hungarica, 55 (2007) 315-326.
[49] A. Thuma, A. Dán, E. Kaszanyitzky, B. Fazekas, A. Tóth, R. Glávits, **Experimental inoculation of day-old ducks with *Brachyspira pilosicoli* and *B. alvinipulli***, Acta veterinaria Hungarica, 59 (2011) 165-174.
[50] F.G. Rodgers, C. Rodgers, A.P. Shelton, C.J. Hawkey, **Proposed pathogenic mechanism for the diarrhea associated with human intestinal spirochetes**, American journal of clinical pathology, 86 (1986) 679-682.
[51] N. Muniappa, G.E. Duhamel, M.R. Mathiesen, T.W. Bargar, **Light microscopic and ultrastructural changes in the ceca of chicks inoculated with human and canine *Serpulina pilosicoli***, Veterinary Pathology, 33 (1996) 542-550.
[52] N. Prapasarakul, K. Lugsomya, S. Disatian, T. Lekdumrongsak, W. Banlunara, P. Chetanachan, D.J. Hampson, **Faecal excretion of intestinal spirochaetes by urban dogs, and their pathogenicity in a chick model of intestinal spirochaetosis**, Research in Veterinary Science, 91 (2011) e38-e43.
[53] R. Naresh, Y. Song, D.J. Hampson, **The intestinal spirochete *Brachyspira pilosicoli* attaches to cultured caco-2 cells and induces pathological changes**, PloS one, 4 (2009) e8352.
[54] D.J. Trott, A.J. McLaren, D.J. Hampson, **Pathogenicity of human and porcine intestinal spirochetes in one-day-old specific-pathogen-free chicks: An animal model of intestinal spirochetosis**, Infect Immun, 63 (1995) 3705-3710.
[55] J.O. Gebbers, D.J. Ferguson, C. Mason, P. Kelly, D.P. Jewell, **Spirochaetosis of the human rectum associated with an intraepithelial mast cell and IgE plasma cell response**, Gut, 28 (1987) 588-593.
[56] J.O. Gebbers, D.J. Ferguson, C. Mason, V. Crucioli, D.P. Jewell, L**ocal immune reaction in human intestinal spirochetosis**, Schweizerische medizinische Wochenschrift, 117 (1987) 1087-1091.
[57] T. La, N.D. Phillips, D.J. Hampson, **Vaccination of chickens with the 34 kDa carboxy-terminus of Bpmp72 reduces colonization with *Brachyspira pilosicoli* following experimental infection**, Avian pathology : journal of the W.V.P.A, 48 (2019) 80-85.
[58] A. Movahedi, D.J. Hampson, **Evaluation of recombinant *Brachyspira pilosicoli* oligopeptide-binding proteins as vaccine candidates in a mouse model of intestinal spirochaetosis**, Journal of medical microbiology, 59 (2010) 353-359.
[59] C.J. Brooke, D.J. Hampson, T.V. Riley, ***In vitro* antimicrobial susceptibility of *Brachyspira pilosicoli* isolates from humans**, Antimicrobial agents and chemotherapy, 47 (2003) 2354-2357.
[60] J.M. Suarez-Peñaranda, F. Macías-García, J. Llovo, J. Forteza, **Histopathological diagnosis of intestinal spirochetosis in a nonimmunocompromised patient**, International journal of surgical pathology, 18 (2010) 73-74.
[61] J. Iwamoto, S. Ogata, A. Honda, Y. Saito, M. Murakami, T. Ikegami, Y. Adachi, Y. Matsuzaki, **Human intestinal spirochaetosis in two ulcerative *coli*tis patients**, Internal medicine (Tokyo, Japan), 53 (2014) 2067-2071.
[62] P.L. Peghini, J.G. Guccion, A. Sharma, **Improvement of chronic diarrhea after treatment for intestinal spirochetosis**, Digestive diseases and sciences, 45 (2000) 1006-1010.

[63] S. Warren, A. Freedman, N. Lomax, A. Christian, **Intestinal spirochaetosis causing chronic diarrhoea in a patient with HIV infection**, International journal of STD & AIDS, 28 (2017) 616-618.
[64] M. Pringle, A. Landén, A. Franklin, **Tiamulin resistance in porcine *Brachyspira pilosicoli* isolates**, Res Vet Sci, 80 (2006) 1-4.
[65] G.E. Duhamel, J.M. Kinyon, M.R. Mathiesen, D.P. Murphy, D. Walter, ***In vitro* activity of four antimicrobial agents against north american isolates of porcine *Serpulina pilosicoli***, Journal of Veterinary Diagnostic Investigation, 10 (1998) 350-356.
[66] M. Pringle, A. Landén, H.E. Unnerstad, B. Molander, B. Bengtsson, **Antimicrobial susceptibility of porcine *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* isolated in sweden between 1990 and 2010**, Acta veterinaria Scandinavica, 54 (2012) 54.
[67] D.W. Trampel, J.M. Kinyon, N.S. Jensen, **Minimum inhibitory concentration of selected antimicrobial agents for *Serpulina* isolated from chickens and rheas**, Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 11 (1999) 379-382.
[68] D.J. Hampson, C.P. Stephens, S.L. Oxberry, **Antimicrobial susceptibility testing of *Brachyspira intermedia* and *Brachyspira pilosicoli* isolates from australian chickens**, Avian pathology : journal of the W.V.P.A, 35 (2006) 12-16.
[69] M. Pringle, J. Poehlsgaard, B. Vester, K.S. Long, **Mutations in ribosomal protein l3 and 23s ribosomal RNA at the peptidyl transferase centre are associated with reduced susceptibility to tiamulin in *Brachyspira* spp. Isolates**, Molecular microbiology, 54 (2004) 1295-1306.
[70] M. Karlsson, C. Fellström, K.E. Johansson, A. Franklin, **Antimicrobial resistance in *Brachyspira pilosicoli* with special reference to point mutations in the 23s rRNA gene associated with macrolide and lincosamide resistance**, Microbial drug resistance (Larchmont, N.Y.), 10 (2004) 204-208.
[71] J.Z. Jeffrey, A.K. Locke, R. Alejandro, J.S. Kent, W.S. Gregory, Z. Jianqiang, **Diseases of swine**, Wiley-Blackwell, Hoboken, NJ, 2019.
[72] G. Lussier, **Vibrionic dysentery in swine in ontario-part ii: Morphological, biochemical and serological characteristics of *Vibrio coli***, Can Vet J, 3 (1962) 267-278.
[73] R.C. Meyer, J. Simon, C.S. Byerly, **The etiology of swine dysentery: I. Oral inoculation of germ-free swine with *Treponema hyodysenteriae* and *Vibrio coli***, Veterinary Pathology, 11 (1974) 515-526.
[74] E.R. Burrough, **Swine dysentery: Etiopathogenesis and diagnosis of a reemerging disease**, Veterinary Pathology, 54 (2016) 22-31.
[75] N.S. Mirajkar, P.R. Davies, C.J. Gebhart, **Antimicrobial susceptibility patterns of *Brachyspira* species isolated from swine herds in the united states**, Journal of clinical microbiology, 54 (2016) 2109-2119.
[76] L.A. Johnson, C. Fernando, J.C.S. Harding, J.E. Hill, **Characterization of *Brachyspira* communities from clinical cases of swine mucohaemorrhagic diarrhea through deep sequencing of the NADH oxidase (nox) gene**, Vet Microbiol, 214 (2018) 81-88.
[77] S. Löbert, W. Zimmermann, S. Bürki, J. Frey, H. Nathues, P. Scheer, M.G. Doherr, U. Stalder, F. Zeeh, **Occurrence of *Brachyspira hyodysenteriae* in multiplier pig herds in Switzerland**, Tierarztliche Praxis. Ausgabe G, Grosstiere/Nutztiere, 44 (2016) 13-18.
[78] F. Zeeh, S. De Luca, P. Nicholson, N. Grützner, C. Nathues, V. Perreten, H. Nathues, ***Brachyspira hyodysenteriae* detection in the large intestine of slaughtered pigs**, Journal of

veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 30 (2018) 56-63.
[79] R.M. Card, T. La, E.R. Burrough, R.J. Ellis, J. Nunez-Garcia, J.R. Thomson, M. Mahu, N.D. Phillips, D.J. Hampson, J. Rohde, A.W. Tucker, **Weakly haemolytic variants of *Brachyspira hyodysenteriae* newly emerged in europe belong to a distinct subclade with unique genetic properties**, Veterinary research, 50 (2019) 21.
[80] G. Rugna, P. Bonilauri, E. Carra, F. Bergamini, A. Luppi, Y. Gherpelli, C.F. Magistrali, A. Nigrelli, G.L. Alborali, P. Martelli, T. La, D.J. Hampson, G. Merialdi, **Sequence types and pleuromutilin susceptibility of *Brachyspira hyodysenteriae* isolates from Italian pigs with swine dysentery: 2003-2012**, Veterinary journal (London, England : 1997), 203 (2015) 115-119.
[81] T. La, J. Rohde, N.D. Phillips, D.J. Hampson, **Comparison of *Brachyspira hyodysenteriae* isolates recovered from pigs in apparently healthy multiplier herds with isolates from herds with swine dysentery**, PloS one, 11 (2016) e0160362.
[82] T. La, N.D. Phillips, D.J. Hampson, **An investigation into the etiological agents of swine dysentery in australian pig herds**, PloS one, 11 (2016) e0167424.
[83] K. Lugsomya, F. Zeeh, T. La, N. Phillips, D.J. Hampson, **First identification and characterisation of *Brachyspira hyodysenteriae* in pigs in hong kong**, Porcine Health Manag, 5 (2019) 27.
[84] X. Qi, F. Xu, W. Zhang, F. Ge, L. Shen, J. Wang, X. Wang, K. Ning, P. Liu, **Isolation and identification of *Brachyspira hyodysenteriae* and pathological examination of swine dysentery**, Zhongguo Yufang Shouyi Xuebao / Chinese Journal of Preventive Veterinary Medicine, 39 (2017) 42-44.
[85] J.C. Yeh, D.Y. Lo, S.K. Chang, H.C. Kuo, **Antimicrobial susceptibility patterns of *Brachyspira* species isolated in taiwan**, Microbial drug resistance (Larchmont, N.Y.), 24 (2018) 685-692.
[86] J.E. Sagartz, D.E. Swayne, K.A. Eaton, J.R. Hayes, K.D. Amass, R. Wack, L. Kramer, **Necrotizing typhlocolitis associated with a spirochete in rheas (*Rhea americana*)**, Avian diseases, 36 (1992) 282-289.
[87] N.S. Jensen, T.B. Stanton, D.E. Swayne, **Identification of the swine pathogen *Serpulina hyodysenteriae* in rheas (*Rhea americana*)**, Vet Microbiol, 52 (1996) 259-269.
[88] A. Feberwee, D.J. Hampson, N.D. Phillips, T. La, H.M. van der Heijden, G.J. Wellenberg, R.M. Dwars, W.J. Landman, **Identification of *Brachyspira hyodysenteriae* and other pathogenic *Brachyspira* species in chickens from laying flocks with diarrhea or reduced production or both**, Journal of clinical microbiology, 46 (2008) 593-600.
[89] D.S. Jansson, K.E. Johansson, T. Olofsson, T. Råsbäck, I. Vågsholm, B. Pettersson, A. Gunnarsson, C. Fellström, ***Brachyspira hyodysenteriae* and other strongly beta-haemolytic and indole-positive spirochaetes isolated from mallards (anas platyrhynchos)**, Journal of medical microbiology, 53 (2004) 293-300.
[90] D.S. Jansson, T. Råsbäck, C. Fellström, R. Feinstein, **Experimental challenge of mallards (anas platyrhynchos) with *Brachyspira hyodysenteriae* and "*Brachyspira suanatina*" isolated from pigs and mallards**, Journal of comparative pathology, 141 (2009) 211-222.
[91] M.O. Costa, J.E. Hill, C. Fernando, H.D. Lemieux, S.E. Detmer, J.E. Rubin, J.C. Harding, **Confirmation that "*Brachyspira hampsonii*" clade i (canadian strain 30599) causes mucohemorrhagic diarrhea and *coli*tis in experimentally infected pigs**, BMC veterinary research, 10 (2014) 129.

[92] J.E. Hill, S.E. Detmer, C. Fernando, S.A. Derbawka, R. Nosach, J.C.S. Harding, **Retrospective detection of *Brachyspira hampsonii* in archived *coli*tis cases from western canadian swine**, Transboundary and emerging diseases, 66 (2019) 381-388.
[93] J. Rohde, K. Habighorst-Blome, F. Seehusen, **"*Brachyspira hampsonii*" clade i isolated from belgian pigs imported to germany**, Vet Microbiol, 168 (2014) 432-435.
[94] M. Mahu, E. de Jong, N. De Pauw, L. Vande Maele, V. Vandenbroucke, T. Vandersmissen, C. Miry, F. Pasmans, F. Haesebrouck, A. Martel, F. Boyen, **First isolation of "*Brachyspira hampsonii*" from pigs in europe**, The Veterinary record, 174 (2014) 47.
[95] F.J. Martínez-Lobo, Á. Hidalgo, M. García, H. Argüello, G. Naharro, A. Carvajal, P. Rubio, **First identification of "*Brachyspira hampsonii*" in wild european waterfowl**, PloS one, 8 (2013) e82626.
[96] L.M. Aller-Morán, F.J. Martínez-Lobo, P. Rubio, A. Carvajal, **Experimental infection of conventional pigs with a '*Brachyspira hampsonii*' isolate recovered from a migrating waterfowl in spain**, Veterinary journal (London, England : 1997), 214 (2016) 10-13.
[97] J.E. Rubin, N.J. Harms, C. Fernando, C. Soos, S.E. Detmer, J.C. Harding, J.E. Hill, **Isolation and characterization of *Brachyspira* spp. Including "*Brachyspira hampsonii*" from lesser snow geese (chen caerulescens caerulescens) in the canadian arctic**, Microbial ecology, 66 (2013) 813-822.
[98] D.S. Jansson, C. Bröjer, D. Gavier-Widén, A. Gunnarsson, C. Fellström, ***Brachyspira* spp. (*Serpulina* spp.) in birds: A review and results from a study of swedish game birds**, Animal Health Research Reviews, 2 (2001) 93-100.
[99] M. Mushtaq, S. Zubair, T. Rasback, E. Bongcam-Rudloff, D.S. Jansson, ***Brachyspira suanatina* sp. Nov., an enteropathogenic intestinal spirochaete isolated from pigs and mallards: Genomic and phenotypic characteristics**, BMC microbiology, 15 (2015) 208.
[100] J. Rohde, M. Majzoub-Altweck, A. Falkenau, W. Hermanns, E.R. Burrough, M. Ritzmann, J. Stadler, **Occurrence of dysentery-like diarrhoea associated with *Brachyspira suanatina* infection on a german fattening pig farm**, The Veterinary record, 182 (2018) 195.
[101] D.J. Hampson, I.D. Robertson, J.R.L. Mhoma, **Experiences with a vaccine being developed for the control of swine dysentery**, Australian Veterinary Journal, 70 (1993) 18-20.
[102] R. Diego, I. Lanza, A. Carvajal, P. Rubio, P. Cármenes, ***Serpulina hyodysenteriae* challenge of fattening pigs vaccinated with anadjuvanted bivalent bacterin against swine dysentery**, Vaccine, 13 (1995) 663-667.
[103] W.R. Waters, B.A. Pesch, R. Hontecillas, R.E. Sacco, F.A. Zuckermann, M.J. Wannemuehler, **Cellular immune responses of pigs induced by vaccination with either a whole cell sonicate or pepsin-digested *Brachyspira* (*Serpulina*) *hyodysenteriae* bacterin**, Vaccine, 18 (1999) 711-719.
[104] W.R. Waters, R.E. Sacco, A.D. Dorn, R. Hontecillas, F.A. Zuckermann, M.J. Wannemuehler, **Systemic and mucosal immune responses of pigs to parenteral immunization with a pepsin-digested *Serpulina hyodysenteriae* bacterin**, Veterinary immunology and immunopathology, 69 (1999) 75-87.
[105] D.S. Fernie, P.H. Ripley, P.D. Walker, **Swine dysentery: Protection against experimental challenge following single dose parenteral immunisation with inactivated *Treponema hyodysenteriae***, Res Vet Sci, 35 (1983) 217-221.
[106] L.D. Olson, K.I. Dayalu, G.T. Schlink, **Exacerbated onset of dysentery in swine vaccinated with inactivated adjuvanted *Serpulina hyodysenteriae***, American journal of veterinary research, 55 (1994) 67-71.

[107] D.E. Briles, J.C. Paton, R. Mukerji, E. Swiatlo, M.J. Crain, **Pneumococcal vaccines**, Microbiology Spectrum, 7 (2019).
[108] W. Herbst, S. Schneider, G. Baljer, S.A. Barth, **An update of *Brachyspira hyodysenteriae* serotyping**, Research in Veterinary Science, 111 (2017) 135-139.
[109] T. La, N.D. Phillips, M.P. Reichel, D.J. Hampson, **Protection of pigs from swine dysentery by vaccination with recombinant bmpb, a 29.7kda outer-membrane lipoprotein of *Brachyspira hyodysenteriae***, Veterinary Microbiology, 102 (2004) 97-109.
[110] J. Holden, P.J. Coloe, P.M. Smooker, **An evaluation of the immunogenicity and protective responses to *Brachyspira hyodysenteriae* recombinant smpb vaccination**, Vet Microbiol, 128 (2008) 354-363.
[111] A.J. Davis, S.C. Smith, R.J. Moore, **The *Brachyspira hyodysenteriae* ftna gene: DNA vaccination and real-time pcr quantification of bacteria in a mouse model of disease**, Current microbiology, 50 (2005) 285-291.
[112] J.D. Gabe, R.J. Chang, R. Slomiany, W.H. Andrews, M.T. McCaman, **Isolation of extracytoplasmic proteins from *Serpulina hyodysenteriae* b204 and molecular cloning of the flab1 gene encoding a 38-kilodalton flagellar protein**, Infect Immun, 63 (1995) 142-148.
[113] M. Mahu, F. Boyen, S. Canessa, J. Zavala Marchan, F. Haesebrouck, A. Martel, F. Pasmans, **An avirulent *Brachyspira hyodysenteriae* strain elicits intestinal iga and slows down spread of swine dysentery**, Veterinary research, 48 (2017) 59.
[114] T. La, N.D. Phillips, F. Coiacetto, D.J. Hampson, **An atypical weakly haemolytic strain of *Brachyspira hyodysenteriae* is avirulent and can be used to protect pigs from developing swine dysentery**, Veterinary research, 50 (2019) 47.
[115] M. Archambault, J.E. Rubin, **Antimicrobial resistance in *Clostridium* and *Brachyspira* spp. And other anaerobes**, Microbiology Spectrum, 8 (2020).
[116] J. Prášek, D. Sperling, D. Lobova, J. Smola, A. Cizek, **Antibiotic susceptibility of *Brachyspira hyodysenteriae* isolates from czech swine farms: A 10-year follow-up study**, Acta Veterinaria Brno, 83 (2014) 3-7.
[117] A. Hidalgo, A. Carvajal, C. García-Feliz, J. Osorio, P. Rubio, **Antimicrobial susceptibility testing of spanish field isolates of *Brachyspira hyodysenteriae***, Res Vet Sci, 87 (2009) 7-12.
[118] Á. Hidalgo, A. Carvajal, B. Vester, M. Pringle, G. Naharro, P. Rubio, **Trends towards lower antimicrobial susceptibility and characterization of acquired resistance among clinical isolates of *Brachyspira hyodysenteriae* in spain**, Antimicrobial agents and chemotherapy, 55 (2011) 3330-3337.
[119] P. Vannuffel, C. Cocito, **Mechanism of action of streptogramins and macrolides**, Drugs, 51 Suppl 1 (1996) 20-30.
[120] S. Schwarz, J. Shen, K. Kadlec, Y. Wang, G. Brenner Michael, A.T. Feßler, B. Vester, **Lincosamides, streptogramins, phenicols, and pleuromutilins: Mode of action and mechanisms of resistance**, Cold Spring Harbor perspectives in medicine, 6 (2016).
[121] J. Spížek, T. Řezanka, **Lincosamides: Chemical structure, biosynthesis, mechanism of action, resistance, and applications**, Biochemical pharmacology, 133 (2017) 20-28.
[122] C. Davidovich, A. Bashan, T. Auerbach-Nevo, R.D. Yaggie, R.R. Gontarek, A. Yonath, **Induced-fit tightens pleuromutilins binding to ribosomes and remote interactions enable their selectivity**, Proceedings of the National Academy of Sciences of the United States of America, 104 (2007) 4291-4296.

[123] S. Paukner, R. Riedl, **Pleuromutilins: Potent drugs for resistant bugs-mode of action and resistance**, Cold Spring Harbor perspectives in medicine, 7 (2017) a027110.
[124] M. Karlsson, C. Fellström, M.U. Heldtander, K.E. Johansson, A. Franklin, **Genetic basis of macrolide and lincosamide resistance in *Brachyspira* (*Serpulina*) *hyodysenteriae***, FEMS microbiology letters, 172 (1999) 255-260.
[125] S. Hillen, H. Willems, W. Herbst, J. Rohde, G. Reiner, **Mutations in the 50s ribosomal subunit of *Brachyspira hyodysenteriae* associated with altered minimum inhibitory concentrations of pleuromutilins**, Vet Microbiol, 172 (2014) 223-229.
[126] A.B. García-Martín, S. Schwendener, V. Perreten, **The tva(a) gene from *Brachyspira hyodysenteriae* confers decreased susceptibility to pleuromutilins and streptogramin a in *Escherichia coli***, Antimicrobial agents and chemotherapy, 63 (2019) e00930-00919.
[127] R.M. Card, E. Stubberfield, J. Rogers, J. Nunez-Garcia, R.J. Ellis, M. AbuOun, B. Strugnell, C. Teale, S. Williamson, M.F. Anjum, **Identification of a new antimicrobial resistance gene provides fresh insights into pleuromutilin resistance in *Brachyspira hyodysenteriae*, aetiological agent of swine dysentery**, Frontiers in microbiology, 9 (2018) 1183.
[128] R. Ero, V. Kumar, W. Su, Y.G. Gao, **Ribosome protection by abc-f proteins-molecular mechanism and potential drug design**, Protein science : a publication of the Protein Society, 28 (2019) 684-693.
[129] S. De Luca, P. Nicholson, C.F. Magistrali, A.B. García-Martín, L. Rychener, F. Zeeh, J. Frey, V. Perreten, **Transposon-associated lincosamide resistance lnu(c) gene identified in *Brachyspira hyodysenteriae* st83**, Veterinary Microbiology, 214 (2018) 51-55.
[130] A. Achard, C. Villers, V. Pichereau, R. Leclercq, **New lnu (c) gene conferring resistance to lincomycin by nucleotidylation in *Streptococcus agalactiae* ucn36**, Antimicrobial agents and chemotherapy, 49 (2005) 2716-2719.
[131] B.L. Wilberts, P.H. Arruda, J.M. Kinyon, D.M. Madson, T.S. Frana, E.R. Burrough, **Comparison of sesion severity, distribution, and colonic mucin expression in pigs with acute swine dysentery following oral inoculation with "*Brachyspira hampsonii*" or *Brachyspira hyodysenteriae***, Vet Pathol, 51 (2014) 1096-1108.
[132] B.P. Wilcock, H.J. Olander, **Studies on the pathogenesis of swine dysentery:I. Characterization of the lesions in colons and colonic segments inoculated with pure cultures or colonic content containing *Treponema hyodysenteriae***, Veterinary Pathology, 16 (1979) 450-465.
[133] V. Venkatakrishnan, M.P. Quintana-Hayashi, M. Mahu, F. Haesebrouck, F. Pasmans, S.K. Lindén, ***Brachyspira hyodysenteriae* infection regulates mucin glycosylation synthesis inducing an increased expression of core-2 o-glycans in porcine colon**, Journal of Proteome Research, 16 (2017) 1728-1742.
[134] J.A. Milner, R. Sellwood, **Chemotactic response to mucin by *Serpulina hyodysenteriae* and other porcine spirochetes: Potential role in intestinal colonization**, Infect Immun, 62 (1994) 4095-4099.
[135] M.J. Kennedy, R.J. Yancey, **Motility and chemotaxis in *Serpulina hyodysenteriae***, Veterinary Microbiology, 49 (1996) 21-30.
[136] R. Naresh, D.J. Hampson, **Attraction of *Brachyspira pilosicoli* to mucin**, Microbiology (Reading, England), 156 (2010) 191-197.

[137] H.W. Hovenberg, J.R. Davies, A. Herrmann, C.J. Lindén, I. Carlstedt, **MUC5AC, but not MUC2, is a prominent mucin in respiratory secretions**, Glycoconjugate journal, 13 (1996) 839-847.
[138] T. Inatomi, S. Spurr-Michaud, A.S. Tisdale, Q. Zhan, S.T. Feldman, I.K. Gipson, **Expression of secretory mucin genes by human conjunctival epithelia**, Investigative ophthalmology & visual science, 37 (1996) 1684-1692.
[139] H. Nordman, J.R. Davies, G. Lindell, C. de Bolós, F. Real, I. Carlstedt, **Gastric MUC5AC and MUC6 are large oligomeric mucins that differ in size, glycosylation and tissue distribution**, The Biochemical journal, 364 (2002) 191-200.
[140] S.K. Linden, P. Sutton, N.G. Karlsson, V. Korolik, M.A. McGuckin, **Mucins in the mucosal barrier to infection**, Mucosal Immunology, 1 (2008) 183-197.
[141] M.P. Quintana-Hayashi, V. Venkatakrishnan, F. Haesebrouck, S. Lindén, **Role of sialic acid in *Brachyspira hyodysenteriae* adhesion to pig colonic mucins**, Infection and Immunity, 87 (2019) e00889-00818.
[142] B.J. Moncla, P. Braham, S.L. Hillier, **Sialidase (neuraminidase) activity among gram-negative anaerobic and capnophilic bacteria**, Journal of clinical microbiology, 28 (1990) 422-425.
[143] Fraser, **Neuraminidase production by clostridia**, Journal of medical microbiology, 11 (1978) 269-280.
[144] J.K. Gustafsson, N. Navabi, A.M. Rodriguez-Piñeiro, A.H. Alomran, P. Premaratne, H.R. Fernandez, D. Banerjee, H. Sjövall, G.C. Hansson, S.K. Lindén, **Dynamic changes in mucus thickness and ion secretion during *Citrobacter rodentium* infection and clearance**, PloS one, 8 (2013) e84430.
[145] J.K. Gustafsson, A. Ermund, D. Ambort, M.E. Johansson, H.E. Nilsson, K. Thorell, H. Hebert, H. Sjövall, G.C. Hansson, **Bicarbonate and functional CFTR channel are required for proper mucin secretion and link cystic fibrosis with its mucus phenotype**, Journal of Experimental Medicine, 209 (2012) 1263-1272.
[146] P.M. Quinton, **Role of epithelial $HCO_3^-$ transport in mucin secretion: Lessons from cystic fibrosis**, American Journal of Physiology-Cell Physiology, 299 (2010) C1222-C1233.
[147] R.A. Argenzio, S.C. Whipp, R.D. Glock, **Pathophysiology of swine dysentery: Colonic transport and permeability studies**, J Infect Dis, 142 (1980) 676-684.
[148] L.M. Schmall, R.A. Argenzio, S.C. Whipp, **Pathophysiologic features of swine dysentery: Cyclic nucleotide-independent production of diarrhea**, American journal of veterinary research, 44 (1983) 1309-1316.
[149] C. Bookstein, A.M. DePaoli, Y. Xie, P. Niu, M.W. Musch, M.C. Rao, E.B. Chang, **$Na^+/H^+$ exchangers, NHE-1 and NHE-3, of rat intestine. Expression and localization**, The Journal of clinical investigation, 93 (1994) 106-113.
[150] S. Akhter, M.E. Cavet, C.M. Tse, M. Donowitz, **C-terminal domains of $Na^+/H^+$ exchanger isoform 3 are involved in the basal and serum-stimulated membrane trafficking of the exchanger**, Biochemistry, 39 (2000) 1990-2000.
[151] P.J. Schultheis, L.L. Clarke, P. Meneton, M.L. Miller, M. Soleimani, L.R. Gawenis, T.M. Riddle, J.J. Duffy, T. Doetschman, T. Wang, G. Giebisch, P.S. Aronson, J.N. Lorenz, G.E. Shull, **Renal and intestinal absorptive defects in mice lacking the NHE3 $Na^+/H^+$ exchanger**, Nature genetics, 19 (1998) 282-285.

[152] J.M. Greer, M.J. Wannemuehler, **Pathogenesis of *Treponema hyodysenteriae*: Induction of interleukin-1 and tumor necrosis factor by a *Treponema*l butanol/water extract (endotoxin)**, Microb Pathog, 7 (1989) 279-288.
[153] R.E. Sacco, S.K. Nibbelink, M.J. Baarsch, M.P. Murtaugh, M.J. Wannemuehler, **Induction of interleukin (IL)-1Beta and IL-8 mRNA expression in porcine macrophages by lipopolysaccharide from *Serpulina hyodysenteriae***, Infect Immun, 64 (1996) 4369-4372.
[154] M.O. Costa, C. Fernando, R. Nosach, J.C.S. Harding, J.E. Hill, **Infection of porcine colon explants with "*Brachyspira hampsonii*" leads to increased epithelial necrosis and catarrhal exudate**, Pathogens and disease, 75 (2017).
[155] R. Kruse, B. Essén-Gustavsson, C. Fossum, M. Jensen-Waern, **Blood concentrations of the cytokines IL-1beta, IL-6, IL-10, TNF-alpha and IFN-gamma during experimentally induced swine dysentery**, Acta veterinaria Scandinavica, 50 (2008) 32.
[156] M.P. Quintana-Hayashi, N. Navabi, M. Mahu, V. Venkatakrishnan, H.R. Fernandez, F. Haesebrouck, F. Pasmans, S.K. Lindén, **Neutrophil elastase and interleukin 17 expressed in the pig colon during *Brachyspira hyodysenteriae* infection synergistically with the pathogen induce increased mucus transport speed and production via mitogen-activated protein kinase 3**, Infection and Immunity, 85 (2017) e00262-00217.
[157] M.R. Gonzalez, M. Bischofberger, L. Pernot, F.G. van der Goot, B. Frêche, **Bacterial pore-forming toxins: The (w)hole story?**, Cellular and Molecular Life Sciences, 65 (2008) 493-507.
[158] R.J. Gilbert, **Pore-forming toxins**, Cellular and molecular life sciences : CMLS, 59 (2002) 832-844.
[159] M. Dal Peraro, F.G. van der Goot, **Pore-forming toxins: Ancient, but never really out of fashion**, Nature reviews. Microbiology, 14 (2016) 77-92.
[160] A. Etxaniz, D. González-Bullón, C. Martín, H. Ostolaza, **Membrane repair mechanisms against permeabilization by pore-forming toxins**, Toxins, 10 (2018).
[161] R. Rahim, U.A. Ochsner, C. Olvera, M. Graninger, P. Messner, J.S. Lam, G. Soberón-Chávez, **Cloning and functional characterization of the *Pseudomonas aeruginosa* rhlc gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis**, Molecular microbiology, 40 (2001) 708-718.
[162] G. Soberón-Chávez, M. Aguirre-Ramírez, R. Sánchez, **The *Pseudomonas aeruginosa* rhla enzyme is involved in rhamnolipid and polyhydroxyalkanoate production**, Journal of industrial microbiology & biotechnology, 32 (2005) 675-677.
[163] G. Soberón-Chávez, F. Lépine, E. Déziel, **Production of rhamnolipids by *Pseudomonas aeruginosa***, Applied microbiology and biotechnology, 68 (2005) 718-725.
[164] A.M. Abdel-Mawgoud, F. Lépine, E. Déziel, **Rhamnolipids: Diversity of structures, microbial origins and roles**, Applied microbiology and biotechnology, 86 (2010) 1323-1336.
[165] F. Jarvis, M. Johnson, **A glyco-lipide produced by *Pseudomonas aeruginosa***, Journal of the American Chemical Society, 71 (1949) 4124-4126.
[166] G. Hauser, M.L. Karnovsky, **Studies on the production of gly*coli*pide by *Pseudomonas aeruginosa***, Journal of bacteriology, 68 (1954) 645-654.
[167] S. Häussler, M. Nimtz, T. Domke, V. Wray, I. Steinmetz, **Purification and characterization of a cytotoxic exolipid of *Burkholderia pseudomallei***, Infect Immun, 66 (1998) 1588-1593.
[168] J. Andrä, J. Rademann, J. Howe, M.H. Koch, H. Heine, U. Zähringer, K. Brandenburg, **Endotoxin-like properties of a rhamnolipid exotoxin from *Burkholderia* (*Pseudomonas*)**

***plantarii*: Immune cell stimulation and biophysical characterization**, Biological chemistry, 387 (2006) 301-310.
[169] D. Dubeau, E. Déziel, D.E. Woods, F. Lépine, ***Burkholderia thailandensis* harbors two identical Rh1 gene clusters responsible for the biosynthesis of rhamnolipids**, BMC microbiology, 9 (2009) 263.
[170] A. Manso Pajarron, C.G. De Koster, W. Heerma, M. Schmidt, J. Haverkamp, **Structure identification of natural rhamnolipid mixtures by fast atom bombardment tandem mass spectrometry**, Glycoconjugate journal, 10 (1993) 219-226.
[171] N. Christova, B. Tuleva, Z. Lalchev, A. Jordanova, B. Jordanov, **Rhamnolipid biosurfactants produced by *Renibacterium salmoninarum* 27bn during growth on n-hexadecane**, Zeitschrift fur Naturforschung. C, Journal of biosciences, 59 (2004) 70-74.
[172] S. Arino, R. Marchal, J.-P. Vandecasteele, **Production of new extracellular *glycolipids* by a strain of *Cellulomonas cellulans* (*Oerskovia xanthineolytica*) and their structural characterization**, Canadian journal of microbiology, 44 (1998) 238-243.
[173] E. Vasileva-Tonkova, V. Gesheva, **Glycolipids produced by antarctic *Nocardioides* sp. During growth on n-paraffin**, Process Biochemistry, 40 (2005) 2387-2391.
[174] Y. Zhang, R.M. Miller, **Effect of a *Pseudomonas* rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane**, Applied and environmental microbiology, 60 (1994) 2101-2106.
[175] G.S. Shreve, S. Inguva, S. Gunnam, **Rhamnolipid biosurfactant enhancement of hexadecane biodegradation by *Pseudomonas aeruginosa***, Molecular marine biology and biotechnology, 4 (1995) 331-337.
[176] R. Hommel, **Biochemistry of microbial degradation**, (1994).
[177] H. Amani, M.M. Müller, C. Syldatk, R. Hausmann, **Production of microbial rhamnolipid by *Pseudomonas aeruginosa* mm1011 for ex situ enhanced oil recovery**, Applied biochemistry and biotechnology, 170 (2013) 1080-1093.
[178] E.J. Gudiña, A.I. Rodrigues, E. Alves, M.R. Domingues, J.A. Teixeira, L.R. Rodrigues, **Bioconversion of agro-industrial by-products in rhamnolipids toward applications in enhanced oil recovery and bioremediation**, Bioresource technology, 177 (2015) 87-93.
[179] E. Déziel, F. Lépine, S. Milot, R. Villemur, **Rhla is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (haas), the precursors of rhamnolipids**, Microbiology (Reading, England), 149 (2003) 2005-2013.
[180] N.C. Caiazza, R.M. Shanks, G.A. O'Toole, **Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa***, Journal of bacteriology, 187 (2005) 7351-7361.
[181] S.J. Pamp, T. Tolker-Nielsen, **Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa***, Journal of bacteriology, 189 (2007) 2531-2539.
[182] R.A. Al-Tahhan, T.R. Sandrin, A.A. Bodour, R.M. Maier, **Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: Effect on cell surface properties and interaction with hydrophobic substrates**, Applied and environmental microbiology, 66 (2000) 3262-3268.
[183] S. Lang, E. Katsiwela, F. Wagner, **Antimicrobial effects of biosurfactants**, Lipid/Fett, 91 (1989) 363-366.
[184] S. Itoh, H. HONDA, F. TOMITA, T. SUZUKI, **Rhamnolipids produced by *Pseudomonas aeruginosa* grown on n-paraffin (mixture of c12, c13 and c14 fractions)**, The Journal of antibiotics, 24 (1971) 855-859.

[185] M.K. Johnson, D. Boese-Marrazzo, **Production and properties of heat-stable extracellular hemolysin from *Pseudomonas aeruginosa***, Infection and Immunity, 29 (1980) 1028-1033.
[186] G. Sierra, **Hemolytic effect of a glycolipid produced by *Pseudomonas aeruginosa***, Antonie van Leeuwenhoek, 26 (1960) 189-192.
[187] S. Kurioka, P.V. Liu, **Effect of the hemolysin of *Pseudomonas aeruginosa* on phosphatides and on phospholipase c activity**, Journal of bacteriology, 93 (1967) 670-674.
[188] K. Fujita, T. Akino, H. Yoshioka, **Characteristics of heat-stable extracellular hemolysin from *Pseudomonas aeruginosa***, Infect Immun, 56 (1988) 1385-1387.
[189] T. Stipcevic, T. Piljac, R.R. Isseroff, **Di-rhamnolipid from *Pseudomonas aeruginosa* displays differential effects on human keratinocyte and fibroblast cultures**, J Dermatol Sci, 40 (2005) 141-143.
[190] K. Rahimi, T.B. Lotfabad, F. Jabeen, S. Mohammad Ganji, **Cytotoxic effects of mono- and di-rhamnolipids from *Pseudomonas aeruginosa* Mr01 on MCF-7 human breast cancer cells**, Colloids and surfaces. B, Biointerfaces, 181 (2019) 943-952.
[191] R. Kownatzki, B. Tümmler, G. Döring, **Rhamnolipid of *Pseudomonas aeruginosa* in sputum of cystic fibrosis patients**, Lancet (London, England), 1 (1987) 1026-1027.
[192] M. Van Gennip, L.D. Christensen, M. Alhede, R. Phipps, P. Jensen, L. Christophersen, S.J. Pamp, C. Moser, P.J. Mikkelsen, A.Y. Koh, T. Tolker-Nielsen, G.B. Pier, N. Høiby, M. Givskov, T. Bjarnsholt, **Inactivation of the rhla gene in *Pseudomonas aeruginosa* prevents rhamnolipid production, disabling the protection against polymorphonuclear leukocytes**, APMIS : acta pathologica, microbiologica, et immunologica Scandinavica, 117 (2009) 537-546.
[193] R.C. Read, P. Roberts, N. Munro, A. Rutman, A. Hastie, T. Shryock, R. Hall, W. McDonald-Gibson, V. Lund, G. Taylor, et al., **Effect of *Pseudomonas aeruginosa* rhamnolipids on mucociliary transport and ciliary beating**, Journal of applied physiology (Bethesda, Md. : 1985), 72 (1992) 2271-2277.
[194] A.T. Hastie, S.T. Hingley, M.L. Higgins, F. Kueppers, **The effect of rhamnolipid hemolysin of *Pseudomonas aeruginosa* on respiratory epithelium**, The American review of respiratory disease, 135 (1987) 513-514.
[195] M. Koval, R.E. Pagano, **Intracellular transport and metabolism of sphingomyelin**, Biochimica et biophysica acta, 1082 (1991) 113-125.
[196] C.M. Talbott, I. Vorobyov, D. Borchman, K.G. Taylor, D.B. DuPré, M.C. Yappert, **Conformational studies of sphingolipids by nmr spectroscopy. II. Sphingomyelin**, Biochimica et biophysica acta, 1467 (2000) 326-337.
[197] B. Ramstedt, J.P. Slotte, **Membrane properties of sphingomyelins**, FEBS Letters, 531 (2002) 33-37.
[198] C.J. Clarke, C.F. Snook, M. Tani, N. Matmati, N. Marchesini, Y.A. Hannun, **The extended family of neutral sphingomyelinases**, Biochemistry, 45 (2006) 11247-11256.
[199] M. Flores-Díaz, L. Monturiol-Gross, C. Naylor, A. Alape-Girón, A. Flieger, **Bacterial sphingomyelinases and phospholipases as virulence factors**, Microbiology and molecular biology reviews : MMBR, 80 (2016) 597-628.
[200] S. Fujii, B. Inoue, H. Yamamoto, K. Ogata, T. Shinki, S. Inoue, M. Tomita, H. Tamura, K. Tsukamoto, H. Ikezawa, K. Ikeda, **$Mg^{2+}$ binding and catalytic function of sphingomyelinase from *Bacillus cereus***, Journal of biochemistry, 124 (1998) 1178-1187.

[201] T. Obama, Y. Kan, H. Ikezawa, M. Imagawa, K. Tsukamoto, **Glu-53 of *Bacillus cereus* sphingomyelinase acts as an indispensable ligand of $Mg^{2+}$ essential for catalytic activity**, Journal of biochemistry, 133 (2003) 279-286.
[202] S.A. Narayanavari, N.M. Kishore, M. Sritharan, **Structural analysis of the *Leptospiral* sphingomyelinases: In silico and experimental evaluation of Sph2 as an mg-dependent sphingomyelinase**, Journal of molecular microbiology and biotechnology, 22 (2012) 24-34.
[203] C.J. Smyth, R. Möllby, T. Wadström, **Phenomenon of hot-cold hemolysis: Chelator-induced lysis of sphingomyelinase-treated erythrocytes**, Infect Immun, 12 (1975) 1104-1111.
[204] L.R. Montes, D.J. López, J. Sot, L.A. Bagatolli, M.J. Stonehouse, M.L. Vasil, B.X. Wu, Y.A. Hannun, F.M. Goñi, A. Alonso, **Ceramide-enriched membrane domains in red blood cells and the mechanism of sphingomyelinase-induced hot-cold hemolysis**, Biochemistry, 47 (2008) 11222-11230.
[205] M. Huseby, K. Shi, C.K. Brown, J. Digre, F. Mengistu, K.S. Seo, G.A. Bohach, P.M. Schlievert, D.H. Ohlendorf, C.A. Earhart, **Structure and biological activities of beta toxin from *Staphylococcus aureus***, Journal of bacteriology, 189 (2007) 8719-8726.
[206] I. Walev, U. Weller, S. Strauch, T. Foster, S. Bhakdi, **Selective killing of human monocytes and cytokine release provoked by sphingomyelinase (beta-toxin) of *Staphylococcus aureus***, Infect Immun, 64 (1996) 2974-2979.
[207] M.J. Marshall, G.A. Bohach, D.F. Boehm, **Characterization of *Staphylococcus aureus* beta-toxin induced leukotoxicity**, Journal of natural toxins, 9 (2000) 125-138.
[208] S. Martínez-Pulgarín, G. Domínguez-Bernal, J.A. Orden, R. de la Fuente, **Simultaneous lack of catalase and beta-toxin in *Staphylococcus aureus* leads to increased intracellular survival in macrophages and epithelial cells and to attenuated virulence in murine and ovine models**, Microbiology (Reading, England), 155 (2009) 1505-1515.
[209] Y. Katayama, T. Baba, M. Sekine, M. Fukuda, K. Hiramatsu, **Beta-hemolysin promotes skin colonization by *Staphylococcus aureus***, Journal of bacteriology, 195 (2013) 1194-1203.
[210] A. Hayashida, A.H. Bartlett, T.J. Foster, P.W. Park, ***Staphylococcus aureus* beta-toxin induces lung injury through syndecan-1**, The American journal of pathology, 174 (2009) 509-518.
[211] W. Salgado-Pabón, A. Herrera, B.G. Vu, C.S. Stach, J.A. Merriman, A.R. Spaulding, P.M. Schlievert, ***Staphylococcus aureus* β-toxin production is common in strains with the β-toxin gene inactivated by bacteriophage**, J Infect Dis, 210 (2014) 784-792.
[212] M. Oda, M. Takahashi, T. Matsuno, K. Uoo, M. Nagahama, J. Sakurai, **Hemolysis induced by *Bacillus* cereus sphingomyelinase**, Biochimica et biophysica acta, 1798 (2010) 1073-1080.
[213] M. Oda, M. Hashimoto, M. Takahashi, Y. Ohmae, S. Seike, R. Kato, A. Fujita, H. Tsuge, M. Nagahama, S. Ochi, T. Sasahara, S. Hayashi, Y. Hirai, J. Sakurai, **Role of sphingomyelinase in infectious diseases caused by *Bacillus cereus***, PloS one, 7 (2012) e38054.
[214] M. Oda, H. Imagawa, R. Kato, K. Yabiku, T. Yoshikawa, T. Takemoto, H. Takahashi, H. Yamamoto, M. Nishizawa, J. Sakurai, M. Nagahama, **Novel inhibitor of bacterial sphingomyelinase, SMY-540, developed based on three-dimensional structure analysis**, Journal of enzyme inhibition and medicinal chemistry, 29 (2014) 303-310.
[215] S.A. Narayanavari, K. Lourdault, M. Sritharan, D.A. Haake, J. Matsunaga, **Role of sph2 gene regulation in hemolytic and sphingomyelinase activities produced by *Leptospira interrogans***, PLoS neglected tropical diseases, 9 (2015) e0003952.

[216] A. Speer, J. Sun, O. Danilchanka, V. Meikle, J.L. Rowland, K. Walter, B.R. Buck, M. Pavlenok, C. Hölscher, S. Ehrt, M. Niederweis, **Surface hydrolysis of sphingomyelin by the outer membrane protein RV0888 supports replication of *Mycobacterium tuberculosis* in macrophages**, Molecular microbiology, 97 (2015) 881-897.
[217] C. Lang, A. Flieger, **Characterisation of *Legionella pneumophila* phospholipases and their impact on host cells**, European Journal of Cell Biology, 90 (2011) 903-912.
[218] N. Dorrell, M.C. Martino, R.A. Stabler, S.J. Ward, Z.W. Zhang, A.A. McColm, M.J. Farthing, B.W. Wren, **Characterization of *Helicobacter pylori* PldA, a phospholipase with a role in colonization of the gastric mucosa**, Gastroenterology, 117 (1999) 1098-1104.
[219] T. Tannaes, I.K. Bukholm, G. Bukholm, **High relative content of lysophospholipids of *Helicobacter pylori* mediates increased risk for ulcer disease**, FEMS immunology and medical microbiology, 44 (2005) 17-23.
[220] R. Sitaraman, D.A. Israel, J. Romero-Gallo, R.M. Peek, Jr., **Cell-associated hemolysis induced by *Helicobacter pylori* is mediated by phospholipases with mitogen-activated protein kinase-activating properties**, Journal of clinical microbiology, 50 (2012) 1014-1018.
[221] H.J. Snijder, B.W. Dijkstra, **Bacterial phospholipase a: Structure and function of an integral membrane phospholipase**, Biochimica et biophysica acta, 1488 (2000) 91-101.
[222] A.V. Karlyshev, P.C. Oyston, K. Williams, G.C. Clark, R.W. Titball, E.A. Winzeler, B.W. Wren, **Application of high-density array-based signature-tagged mutagenesis to discover novel *Yersinia* virulence-associated genes**, Infect Immun, 69 (2001) 7810-7819.
[223] J. Bender, K. Rydzewski, M. Broich, E. Schunder, K. Heuner, A. Flieger, **Phospholipase plab of *Legionella pneumophila* represents a novel lipase family: Protein residues essential for lipolytic activity, substrate specificity, and hemolysis**, The Journal of biological chemistry, 284 (2009) 27185-27194.
[224] M. Diwo, W. Michel, P. Aurass, K. Kuhle-Keindorf, J. Pippel, J. Krausze, C. Lang, W. Blankenfeldt, A. Flieger, **NAD(H)-mediated tetramerization controls the activity of *Legionella pneumophila* phospholipase PlaB**, bioRxiv, (2020) 2020.2009.2001.246603.
[225] K. Kuhle, J. Krausze, U. Curth, M. Rössle, K. Heuner, C. Lang, A. Flieger, **Oligomerization inhibits *Legionella pneumophila* PlaB phospholipase a activity**, The Journal of biological chemistry, 289 (2014) 18657-18666.
[226] A. Flieger, B. Neumeister, N.P. Cianciotto, **Characterization of the gene encoding the major secreted lysophospholipase a of *Legionella pneumophila* and its role in detoxification of lysophosphatidylcholine**, Infection and immunity, 70 (2002) 6094-6106.
[227] S. Banerji, M. Bewersdorff, B. Hermes, N.P. Cianciotto, A. Flieger, **Characterization of the major secreted zinc metalloprotease-dependent glycerophospholipid: Cholesterol acyltransferase, plac, of *Legionella pneumophila***, Infection and immunity, 73 (2005) 2899-2909.
[228] E. Schunder, P. Adam, F. Higa, K.A. Remer, U. Lorenz, J. Bender, T. Schulz, A. Flieger, M. Steinert, K. Heuner, **Phospholipase plab is a new virulence factor of *Legionella pneumophila***, International journal of medical microbiology : IJMM, 300 (2010) 313-323.
[229] L. Song, M.R. Hobaugh, C. Shustak, S. Cheley, H. Bayley, J.E. Gouaux, **Structure of *Staphylococcal* alpha-hemolysin, a heptameric transmembrane pore**, Science (New York, N.Y.), 274 (1996) 1859-1866.
[230] R.K. Tweten, M.W. Parker, A.E. Johnson, **The cholesterol-dependent cytolysins**, Current topics in microbiology and immunology, 257 (2001) 15-33.

[231] G. Menestrina, M.D. Serra, G. Prévost, **Mode of action of beta-barrel pore-forming toxins of the *Staphylococcal* alpha-hemolysin family**, Toxicon : official journal of the International Society on Toxinology, 39 (2001) 1661-1672.
[232] G. Menestrina, M. Dalla Serra, M. Comai, M. Coraiola, G. Viero, S. Werner, D.A. *Coli*n, H. Monteil, G. Prévost, **Ion channels and bacterial infection: The case of beta-barrel pore-forming protein toxins of *Staphylococcus aureus***, FEBS Lett, 552 (2003) 54-60.
[233] M.W. Parker, S.C. Feil, **Pore-forming protein toxins: From structure to function**, Progress in biophysics and molecular biology, 88 (2005) 91-142.
[234] J.M. Mancheño, H. Tateno, I.J. Goldstein, M. Martínez-Ripoll, J.A. Hermoso, **Structural analysis of the *Laetiporus sulphureus* hemolytic pore-forming lectin in complex with sugars**, The Journal of biological chemistry, 280 (2005) 17251-17259.
[235] B.J. Berube, J. Bubeck Wardenburg, ***Staphylococcus aureus* α-toxin: Nearly a century of intrigue**, Toxins, 5 (2013) 1140-1166.
[236] G. von Hoven, A.J. Rivas, C. Neukirch, S. Klein, C. Hamm, Q. Qin, M. Meyenburg, S. Füser, P. Saftig, N. Hellmann, R. Postina, M. Husmann, **Dissecting the role of ADAM10 as a mediator of *Staphylococcus aureus* α-toxin action**, The Biochemical journal, 473 (2016) 1929-1940.
[237] C.R. MacKenzie, T. Hirama, J.T. Buckley, **Analysis of receptor binding by the channel-forming toxin aerolysin using surface plasmon resonance**, The Journal of biological chemistry, 274 (1999) 22604-22609.
[238] S. Seveau, **Multifaceted activity of listeriolysin o, the cholesterol-dependent cytolysin of *Listeria monocytogenes***, Sub-cellular biochemistry, 80 (2014) 161-195.
[239] M. Madegowda, S. Eswaramoorthy, S.K. Burley, S. Swaminathan, **X-ray crystal structure of the b component of hemolysin bl from *Bacillus cereus***, Proteins, 71 (2008) 534-540.
[240] M. Mueller, U. Grauschopf, T. Maier, R. Glockshuber, N. Ban, **The structure of a cytolytic alpha-helical toxin pore reveals its assembly mechanism**, Nature, 459 (2009) 726-730.
[241] S.J. Tilley, E.V. Orlova, R.J.C. Gilbert, P.W. Andrew, H.R. Saibil, **Structural basis of pore formation by the bacterial toxin pneumolysin**, Cell, 121 (2005) 247-256.
[242] E. Cascales, S.K. Buchanan, D. Duché, C. Kleanthous, R. Lloubès, K. Postle, M. Riley, S. Slatin, D. Cavard, **Colicin biology**, Microbiology and molecular biology reviews : MMBR, 71 (2007) 158-229.
[243] H.R. Herschman, D.R. Helinski, **Purification and characterization of colicin e2 and colicin e3**, The Journal of biological chemistry, 242 (1967) 5360-5368.
[244] M.W. Parker, F. Pattus, A.D. Tucker, D. Tsernoglou, **Structure of the membrane-pore-forming fragment of colicin a**, Nature, 337 (1989) 93-96.
[245] A.P. Pugsley, **Genetic analysis of coln plasmid determinants for colicin production, release, and immunity**, Journal of bacteriology, 158 (1984) 523-529.
[246] C.J. Lazdunski, E. Bouveret, A. Rigal, L. Journet, R. Lloubès, H. Bénédetti, ***Coli*cin import into *Escherichia coli* cells**, Journal of bacteriology, 180 (1998) 4993-5002.
[247] V. Braun, S.I. Patzer, K. Hantke, **Ton-dependent colicins and microcins: Modular design and evolution**, Biochimie, 84 (2002) 365-380.
[248] Z. Cao, P.E. Klebba, **Mechanisms of colicin binding and transport through outer membrane porins**, Biochimie, 84 (2002) 399-412.

[249] H. Ridley, C.L. Johnson, J.H. Lakey, **Interfacial interactions of pore-forming colicins**, Advances in experimental medicine and biology, 677 (2010) 81-90.
[250] S. Hunt, J. Green, P.J. Artymiuk, **Hemolysin E (HlyE, ClyA, SheA) and related toxins**, Advances in experimental medicine and biology, 677 (2010) 116-126.
[251] M. Vaidyanathan, P. Sathyanarayana, P.K. Maiti, S.S. Visweswariah, K. Ayappa, **Lysis dynamics and membrane oligomerization pathways for cytolysin a (ClyA) pore-forming toxin**, RSC Advances, 4 (2014) 4930-4942.
[252] S.N. Wai, B. Lindmark, T. Söderblom, A. Takade, M. Westermark, J. Oscarsson, J. Jass, A. Richter-Dahlfors, Y. Mizunoe, B.E. Uhlin, **Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin**, Cell, 115 (2003) 25-35.
[253] A. Ludwig, S. Bauer, R. Benz, B. Bergmann, W. Goebel, **Analysis of the SlyA-controlled expression, subcellular localization and pore-forming activity of a 34 kda haemolysin (ClyA) from *Escherichia coli* k-12**, Molecular microbiology, 31 (1999) 557-567.
[254] F.J. del Castillo, F. Moreno, I. del Castillo, **Secretion of the *Escherichia coli* k-12 SheA hemolysin is independent of its cytolytic activity**, FEMS microbiology letters, 204 (2001) 281-285.
[255] N. Eifler, M. Vetsch, M. Gregorini, P. Ringler, M. Chami, A. Philippsen, A. Fritz, S.A. Müller, R. Glockshuber, A. Engel, U. Grauschopf, **Cytotoxin ClyA from *Escherichia coli* assembles to a 13-meric pore independent of its redox-state**, The EMBO journal, 25 (2006) 2652-2661.
[256] L.Z. Cooper, M.A. Madoff, L. Weinstein, **Hemolysis of rabbit erythrocytes by purified *Staphylococcal* alpha-toxin. I. Kinetics of the lytic reaction**, Journal of bacteriology, 87 (1964) 127-135.
[257] L.Z. Cooper, M.A. Madoff, L. Weinstein, **Heat stability and species range of purified *Staphylococcal* alpha-toxin**, Journal of bacteriology, 91 (1966) 1686-1692.
[258] A. Glenny, M.F. Stevens, **Staphy-lococcus toxins and antitoxins**, Journal of Pathology and Bacteriology, 40 (1935) 201-210.
[259] G.S. Gray, M. Kehoe, **Primary sequence of the alpha-toxin gene from *Staphylococcus aureus* wood 46**, Infect Immun, 46 (1984) 615-618.
[260] B. Thay, S.N. Wai, J. Oscarsson, ***Staphylococcus aureus* α-toxin-dependent induction of host cell death by membrane-derived vesicles**, PloS one, 8 (2013) e54661.
[261] G. Weissmann, G. Sessa, A.W. Bernheimer, **Staphylococcal alpha-toxin: Effects on artificial lipid spherules**, Science (New York, N.Y.), 154 (1966) 772-774.
[262] J.P. Arbuthnott, J.H. Freer, B. Billcliffe, **Lipid-induced polymerization of staphylococcal -toxin**, Journal of general microbiology, 75 (1973) 309-319.
[263] A. Valeva, N. Hellmann, I. Walev, D. Strand, M. Plate, F. Boukhallouk, A. Brack, K. Hanada, H. Decker, S. Bhakdi, **Evidence that clustered phosphocholine head groups serve as sites for binding and assembly of an oligomeric protein pore**, The Journal of biological chemistry, 281 (2006) 26014-26021.
[264] A. Hildebrand, M. Pohl, S. Bhakdi, ***Staphylococcus aureus* alpha-toxin. Dual mechanism of binding to target cells**, The Journal of biological chemistry, 266 (1991) 17195-17200.
[265] G.A. Wilke, J. Bubeck Wardenburg, **Role of a disintegrin and metalloprotease 10 in *Staphylococcus aureus* alpha-hemolysin-mediated cellular injury**, Proceedings of the National Academy of Sciences of the United States of America, 107 (2010) 13473-13478.

[266] B. Walker, M. Krishnasastry, L. Zorn, H. Bayley, **Assembly of the oligomeric membrane pore formed by S*taphylococcal* alpha-hemolysin examined by truncation mutagenesis**, Journal of Biological Chemistry, 267 (1992) 21782-21786.
[267] A. Valeva, M. Palmer, S. Bhakdi, ***Staphylococcal* alpha-toxin: Formation of the heptameric pore is partially cooperative and proceeds through multiple intermediate stages**, Biochemistry, 36 (1997) 13298-13304.
[268] A. Valeva, J. Pongs, S. Bhakdi, M. Palmer, ***Staphylococcal* alpha-toxin: The role of the n-terminus in formation of the heptameric pore -- a fluorescence study**, Biochimica et biophysica acta, 1325 (1997) 281-286.
[269] R.J. Gilbert, **Cholesterol-dependent cytolysins**, Advances in experimental medicine and biology, 677 (2010) 56-66.
[270] A.P. Heuck, P.C. Moe, B.B. Johnson, **The cholesterol-dependent cytolysin family of gram-positive bacterial toxins**, Sub-cellular biochemistry, 51 (2010) 551-577.
[271] E.M. Hotze, H.M. Le, J.R. Sieber, C. Bruxvoort, M.J. McInerney, R.K. Tweten, **Identification and characterization of the first cholesterol-dependent cytolysins from gram-negative bacteria**, Infect Immun, 81 (2013) 216-225.
[272] C.R. Harwood, R. Cranenburgh, ***Bacillus* protein secretion: An unfolding story**, Trends in microbiology, 16 (2008) 73-79.
[273] L. Hewitt, E. Todd, **The effect of cholesterol and of sera contaminated with bacteria on the haemolysins produced by haemolytic streptococci**, Journal of Pathology and Bacteriology, 49 (1939) 45-51.
[274] I. Ofek, S. Bergner-Rabinowitz, I. Ginsburg, **Oxygen-stable hemolysins of group a streptococci. 8. Leukotoxic and antiphagocytic effects of streptolysins s and o**, Infect Immun, 6 (1972) 459-464.
[275] T. Jacobs, A. Darji, N. Frahm, M. Rohde, J. Wehland, T. Chakraborty, S. Weiss, **Listeriolysin o: Cholesterol inhibits cytolysis but not binding to cellular membranes**, Molecular microbiology, 28 (1998) 1081-1089.
[276] T. Nishibori, H. Xiong, I. Kawamura, M. Arakawa, M. Mitsuyama, **Induction of cytokine gene expression by listeriolysin o and roles of macrophages and nk cells**, Infect Immun, 64 (1996) 3188-3195.
[277] K.S. Giddings, J. Zhao, P.J. Sims, R.K. Tweten, **Human CD59 is a receptor for the cholesterol-dependent cytolysin intermedilysin**, Nature structural & molecular biology, 11 (2004) 1173-1178.
[278] L.K. Shewell, C.J. Day, F.E. Jen, T. Haselhorst, J.M. Atack, J.F. Reijneveld, A. Everest-Dass, D.B.A. James, K.M. Boguslawski, S. Brouwer, C.M. Gillen, Z. Luo, B. Kobe, V. Nizet, M. von Itzstein, M.J. Walker, A.W. Paton, J.C. Paton, V.J. Torres, M.P. Jennings, **All major cholesterol-dependent cytolysins use glycans as cellular receptors**, Science advances, 6 (2020) eaaz4926.
[279] J. Rossjohn, S.C. Feil, W.J. McKinstry, R.K. Tweten, M.W. Parker, **Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form**, Cell, 89 (1997) 685-692.
[280] M.P. Christie, B.A. Johnstone, R.K. Tweten, M.W. Parker, C.J. Morton, **Cholesterol-dependent cytolysins: From water-soluble state to membrane pore**, Biophysical reviews, 10 (2018) 1337-1348.
[281] A.J. Farrand, S. LaChapelle, E.M. Hotze, A.E. Johnson, R.K. Tweten, **Only two amino acids are essential for cytolytic toxin recognition of cholesterol at the membrane surface**,

Proceedings of the National Academy of Sciences of the United States of America, 107 (2010) 4341-4346.
[282] S.A. Park, Y.S. Park, S.M. Bong, K.S. Lee, **Structure-based functional studies for the cellular recognition and cytolytic mechanism of pneumolysin from *Streptococcus pneumoniae***, Journal of structural biology, 193 (2016) 132-140.
[283] R. Ramachandran, R.K. Tweten, A.E. Johnson, **Membrane-dependent conformational changes initiate cholesterol-dependent cytolysin oligomerization and intersubunit beta-strand alignment**, Nature structural & molecular biology, 11 (2004) 697-705.
[284] J. Rossjohn, G. Polekhina, S.C. Feil, C.J. Morton, R.K. Tweten, M.W. Parker, **Structures of perfringolysin o suggest a pathway for activation of cholesterol-dependent cytolysins**, Journal of molecular biology, 367 (2007) 1227-1236.
[285] K.J. Dowd, A.J. Farrand, R.K. Tweten, **The cholesterol-dependent cytolysin signature motif: A critical element in the allosteric pathway that couples membrane binding to pore assembly**, PLoS pathogens, 8 (2012) e1002787.
[286] J.E. Marshall, B.H. Faraj, A.R. Gingras, R. Lonnen, M.A. Sheikh, M. El-Mezgueldi, P.C. Moody, P.W. Andrew, R. Wallis, **The crystal structure of pneumolysin at 2.0 å resolution reveals the molecular packing of the pre-pore complex**, Scientific reports, 5 (2015) 13293.
[287] C.F. Reboul, J.C. Whisstock, M.A. Dunstone, **Giant MACPF/CDC pore forming toxins: A class of their own**, Biochimica et Biophysica Acta (BBA) - Biomembranes, 1858 (2016) 475-486.
[288] M. Bischofberger, I. Iacovache, F. Gisou van der Goot, **Pathogenic pore-forming proteins: Function and host response**, Cell Host & Microbe, 12 (2012) 266-275.
[289] J.A. Vázquez-Boland, M. Kuhn, P. Berche, T. Chakraborty, G. Domínguez-Bernal, W. Goebel, B. González-Zorn, J. Wehland, J. Kreft, ***Listeria* pathogenesis and molecular virulence determinants**, Clinical microbiology reviews, 14 (2001) 584-640.
[290] C. Geoffroy, J.L. Gaillard, J.E. Alouf, P. Berche, **Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin o from *Listeria monocytogenes***, Infect Immun, 55 (1987) 1641-1646.
[291] G.C. Kingdon, C.P. Sword, **Effects of *Listeria monocytogenes* hemolysin on phagocytic cells and lysosomes**, Infect Immun, 1 (1970) 356-362.
[292] D.A. Portnoy, P.S. Jacks, D.J. Hinrichs, **Role of hemolysin for the intracellular growth of *Listeria monocytogenes***, The Journal of experimental medicine, 167 (1988) 1459-1471.
[293] S. Kathariou, P. Metz, H. Hof, W. Goebel, **Tn916-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes***, Journal of bacteriology, 169 (1987) 1291-1297.
[294] J.L. Gaillard, P. Berche, J. Mounier, S. Richard, P. Sansonetti, **In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line caco-2**, Infect Immun, 55 (1987) 2822-2829.
[295] P. Cossart, M.F. Vicente, J. Mengaud, F. Baquero, J.C. Perez-Diaz, P. Berche, **Listeriolysin o is essential for virulence of *Listeria monocytogenes*: Direct evidence obtained by gene complementation**, Infect Immun, 57 (1989) 3629-3636.
[296] F. Raimondi, J.P.Y. Kao, C. Fiorentini, A. Fabbri, G. Donelli, N. Gasparini, A. Rubino, A. Fasano, **Enterotoxicity and cytotoxicity of *Vibrio parahaemolyticus* thermostable direct hemolysin in in vitro systems**, Infection and Immunity, 68 (2000) 3180.

[297] S.P. Hardy, M. Nakano, T. Iida, **Single channel evidence for innate pore-formation by *Vibrio parahaemolyticus* thermostable direct haemolysin (TDH) in phospholipid bilayers**, FEMS microbiology letters, 240 (2004) 81-85.
[298] T. Honda, Y. Ni, T. Miwatani, T. Adachi, J. Kim, **The thermostable direct hemolysin of *Vibrio parahaemolyticus* is a pore-forming toxin**, Canadian Journal of Microbiology, 38 (1992) 1175-1180.
[299] L. Seyama, H. Irisawa, T. Honda, T. Takeda, T. Miwatani, **Increase in membrane conductance and positive inotropic action of hemolysin produced by vibrio parahaemolyticus on rabbit myocardium**, Recent advances in studies on cardiac structure and metabolism, 11 (1976) 621-625.
[300] A. TAKAHASHI, Y. SATO, Y. SHIOMI, V.V. CANTARELLI, T. IIDA, M. LEE, T. HONDA, **Mechanisms of chloride secretion induced by thermostable direct haemolysin of *Vibrio parahaemolyticus* in human colonic tissue and a human intestinal epithelial cell line**, Journal of medical microbiology, 49 (2000) 801-810.
[301] F. Raimondi, J.P.Y. Kao, J.B. Kaper, S. Guandalini, A. Fasano, **Calcium-dependent intestinal chloride secretion by *Vibrio parahaemolyticus* thermostable direct hemolysin in a rabbit model**, Gastroenterology, 109 (1995) 381-386.
[302] W. Baffone, A. Casaroli, R. Campana, B. Citterio, E. Vittoria, L. Pierfelici, G. Donelli, **'In vivo' studies on the pathophysiological mechanism of *Vibrio parahaemolyticus* TDH(+)-induced secretion**, Microb Pathog, 38 (2005) 133-137.
[303] A.J. Ratner, K.R. Hippe, J.L. Aguilar, M.H. Bender, A.L. Nelson, J.N. Weiser, **Epithelial cells are sensitive detectors of bacterial pore-forming toxins**, The Journal of biological chemistry, 281 (2006) 12994-12998.
[304] N. Kloft, T. Busch, C. Neukirch, S. Weis, F. Boukhallouk, W. Bobkiewicz, I. Cibis, S. Bhakdi, M. Husmann, **Pore-forming toxins activate MAPK p38 by causing loss of cellular potassium**, Biochemical and biophysical research communications, 385 (2009) 503-506.
[305] D.L. Huffman, L. Abrami, R. Sasik, J. Corbeil, F.G. van der Goot, R.V. Aroian, **Mitogen-activated protein kinase pathways defend against bacterial pore-forming toxins**, Proceedings of the National Academy of Sciences of the United States of America, 101 (2004) 10995-11000.
[306] A. Cancino-Rodezno, C. Alexander, R. Villaseñor, S. Pacheco, H. Porta, Y. Pauchet, M. Soberón, S.S. Gill, A. Bravo, **The mitogen-activated protein kinase p38 is involved in insect defense against cry toxins from *Bacillus thuringiensis***, Insect biochemistry and molecular biology, 40 (2010) 58-63.
[307] M. Bebien, M.E. Hensler, S. Davanture, L.-C. Hsu, M. Karin, J.M. Park, L. Alexopoulou, G.Y. Liu, V. Nizet, T. Lawrence, **The pore-forming toxin β hemolysin/cytolysin triggers p38 MAPK-dependent IL-10 production in macrophages and inhibits innate immunity**, PLoS pathogens, 8 (2012) e1002812.
[308] A. Chakravorty, M.M. Awad, J.K. Cheung, T.J. Hiscox, D. Lyras, J.I. Rood, **The pore-forming α-toxin from *Clostridium septicum* activates the MAPK pathway in a RAS-C-RAF-dependent and independent manner**, Toxins, 7 (2015) 516-534.
[309] M. Nagahama, M. Shibutani, S. Seike, M. Yonezaki, T. Takagishi, M. Oda, K. Kobayashi, J. Sakurai, **The p38 MAPK and JNK pathways protect host cells against *Clostridium perfringens* beta-toxin**, Infect Immun, 81 (2013) 3703-3708.
[310] J.C. Lee, J.T. Laydon, P.C. McDonnell, T.F. Gallagher, S. Kumar, D. Green, D. McNulty, M.J. Blumenthal, J.R. Heys, S.W. Landvatter, J.E. Strickler, M.M. McLaughlin, I.R. Siemens,

S.M. Fisher, G.P. Livi, J.R. White, J.L. Adams, P.R. Young, **A protein kinase involved in the regulation of inflammatory cytokine biosynthesis**, Nature, 372 (1994) 739-746.
[311] R. Beyaert, A. Cuenda, W. Vanden Berghe, S. Plaisance, J.C. Lee, G. Haegeman, P. Cohen, W. Fiers, **The p38/rk mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor**, The EMBO journal, 15 (1996) 1914-1923.
[312] B. van den Blink, N.P. Juffermans, T. ten Hove, M.J. Schultz, S.J. van Deventer, T. van der Poll, M.P. Peppelenbosch, **P38 mitogen-activated protein kinase inhibition increases cytokine release by macrophages *in vitro* and during infection *in vivo***, Journal of immunology (Baltimore, Md. : 1950), 166 (2001) 582-587.
[313] L.J. Bischof, C.Y. Kao, F.C. Los, M.R. Gonzalez, Z. Shen, S.P. Briggs, F.G. van der Goot, R.V. Aroian, **Activation of the unfolded protein response is required for defenses against bacterial pore-forming toxin *in vivo***, PLoS pathogens, 4 (2008) e1000176.
[314] C. Hetz, **The unfolded protein response: Controlling cell fate decisions under er stress and beyond**, Nature Reviews Molecular Cell Biology, 13 (2012) 89-102.
[315] A.K. Stringaris, J. Geisenhainer, F. Bergmann, C. Balshüsemann, U. Lee, G. Zysk, T.J. Mitchell, B.U. Keller, U. Kuhnt, J. Gerber, A. Spreer, M. Bähr, U. Michel, R. Nau, **Neurotoxicity of pneumolysin, a major pneumococcal virulence factor, involves calcium influx and depends on activation of p38 mitogen-activated protein kinase**, Neurobiology of disease, 11 (2002) 355-368.
[316] S. Barth, M. Gömmel, G. Baljer, W. Herbst, **Demonstration of genes encoding virulence and virulence life-style factors in *Brachyspira* spp. Isolates from pigs**, Veterinary Microbiology, 155 (2012) 438-443.
[317] M.I. Bellgard, P. Wanchanthuek, T. La, K. Ryan, P. Moolhuijzen, Z. Albertyn, B. Shaban, Y. Motro, D.S. Dunn, D. Schibeci, A. Hunter, R. Barrero, N.D. Phillips, D.J. Hampson, **Genome sequence of the pathogenic intestinal spirochete *Brachyspira hyodysenteriae* reveals adaptations to its lifestyle in the porcine large intestine**, PloS one, 4 (2009) e4641.
[318] S.A. Saheb, L. Massicotte, B. Picard, **Purification and characterization of *Treponema hyodysenteriae* hemolysin**, Biochimie, 62 (1980) 779-785.
[319] F.C. Knoop, **Investigation of a hemolysin produced by enteropathogenic *Treponema hyodysenteriae***, Infect Immun, 31 (1981) 193-198.
[320] A.W. Bernheimer , M. Rodbart **The effect of nucleic acids and of carbohydrates on the formation of streptolysin**, Journal of Experimental Medicine, 88 (1948) 149-168.
[321] C.Y. Lai, M.T. Wang, J.B. de Faria, T. Akao, **Streptolysin s: Improved purification and characterization**, Archives of biochemistry and biophysics, 191 (1978) 804-812.
[322] K.A. Kent, R.M. Lemcke, R.J. Lysons, **Production, purification and molecular weight determination of the haemolysin of *Treponema hyodysenteriae***, Journal of medical microbiology, 27 (1988) 215-224.
[323] S. Muir, M.B. Koopman, S.J. Libby, L.A. Joens, F. Heffron, J.G. Kusters, **Cloning and expression of a *Serpula (Treponema) hyodysenteriae* hemolysin gene**, Infect. Immun., 60 (1992) 529-535.
[324] D.R. Hyatt, L.A. Joens, **Analysis of the lytic activity of the *Serpulina hyodysenteriae* hemolysin**, Infection and Immunity, 65 (1997) 4877.
[325] A.A.H.M. ter Huurne, M. van Houten, S. Muir, J.G. Kusters, B.A.M. van der Zeijst, W. Gaastra, **Inactivation of a *Serpula (Treponema) hyodysenteriae* hemolysin gene by**

**homologous recombination: Importance of this hemolysin in pathogenesis of *S. Hyodysenteriae* in mice**, FEMS Microbiol. Lett., 92 (1992) 109-113.
[326] D.R. Hyatt, A.A. ter Huurne, B.A. van der Zeijst, L.A. Joens, **Reduced virulence of *Serpulina hyodysenteriae* hemolysin-negative mutants in pigs and their potential to protect pigs against challenge with a virulent strain.**, Infect. Immun., 62 (1994) 2244-2248.
[327] A. Ludwig, C. Tengel, S. Bauer, A. Bubert, R. Benz, H.J. Mollenkopf, W. Goebel, **SlyA, a regulatory protein from *Salmonella typhimurium*, induces a haemolytic and pore-forming protein in *Escherichia coli***, Molecular & general genetics : MGG, 249 (1995) 474-486.
[328] K. Lata, K. Paul, K. Chattopadhyay, **Functional characterization of *Helicobacter pylori* TlyA: Pore-forming hemolytic activity and cytotoxic property of the protein**, Biochemical and biophysical research communications, 444 (2014) 153-157.
[329] K. Lata, K. Chattopadhyay, ***Helicobacter pylori* TlyA agglutinates liposomes and induces fusion and permeabilization of the liposome membranes**, Biochemistry, 53 (2014) 3553-3563.
[330] E. Mittal, S. Kumar, A. Rahman, M.V. Krishnasastry, **Modulation of phagolysosome maturation by bacterial *tlyA* gene product**, Journal of biosciences, 39 (2014) 821-834.
[331] M.A. Rahman, P. Sobia, V.P. Dwivedi, A. Bhawsar, D.K. Singh, P. Sharma, P. Moodley, L. Van Kaer, W.R. Bishai, G. Das, ***Mycobacterium tuberculosis* TlyA protein negatively regulates T Helper (TH) 1 and TH17 differentiation and promotes *tuberculosis* pathogenesis**, The Journal of biological chemistry, 290 (2015) 14407-14417.
[332] V.P. Dwivedi, D. Bhattacharya, S. Chatterjee, D.V. Prasad, D. Chattopadhyay, L. Van Kaer, W.R. Bishai, G. Das, ***Mycobacterium tuberculosis* directs T helper 2 cell differentiation by inducing interleukin-1β production in dendritic cells**, The Journal of biological chemistry, 287 (2012) 33656-33663.
[333] D. Bhattacharya, V.P. Dwivedi, M. Maiga, M. Maiga, L. Van Kaer, W.R. Bishai, G. Das, **Small molecule-directed immunotherapy against recurrent infection by *Mycobacterium tuberculosis***, The Journal of biological chemistry, 289 (2014) 16508-16515.
[334] V. Deretic, M. Delgado, I. Vergne, S. Master, S. De Haro, M. Ponpuak, S. Singh, **Autophagy in immunity against *Mycobacterium tuberculosis*: A model system to dissect immunological roles of autophagy**, Current topics in microbiology and immunology, 335 (2009) 169-188.
[335] E.K. Jo, **Autophagy as an innate defense against mycobacteria**, Pathogens and disease, 67 (2013) 108-118.
[336] C.E. Maus, B.B. Plikaytis, T.M. Shinnick, **Mutation of TlyA confers capreomycin resistance in *Mycobacterium tuberculosis*.**, Antimicrob. Agents Ch., 49 (2005) 571-577.
[337] S.K. Johansen, C.E. Maus, B.B. Plikaytis, S. Douthwaite, **Capreomycin binds across the ribosomal subunit interface using tlya-encoded 2'-o-methylations in 16s and 23s rrnas**, Molecular cell, 23 (2006) 173-182.
[338] T. Monshupanee, S.K. Johansen, A.E. Dahlberg, S. Douthwaite, **Capreomycin susceptibility is increased by TlyA-directed 2′-O-methylation on both ribosomal subunits**, Mol. Microbiol., 85 (2012) 1194-1203.
[339] C.E. Maus, B.B. Plikaytis, T.M. Shinnick, **Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis***, Antimicrobial agents and chemotherapy, 49 (2005) 3192-3197.
[340] A. Engström, N. Perskvist, J. Werngren, S.E. Hoffner, P. Juréen, **Comparison of clinical isolates and in vitro selected mutants reveals that *tlya* is not a sensitive genetic marker for**

**capreomycin resistance in *Mycobacterium tuberculosis***, The Journal of antimicrobial chemotherapy, 66 (2011) 1247-1254.
[341] Q. Du, G. Dai, Q. Long, X. Yu, L. Dong, H. Huang, J. Xie, ***Mycobacterium tuberculosis* rrs a1401g mutation correlates with high-level resistance to kanamycin, amikacin, and capreomycin in clinical isolates from mainland china**, Diagnostic microbiology and infectious disease, 77 (2013) 138-142.
[342] Q. Du, Q. Long, J. Mao, T. Fu, X. Duan, J. Xie, **Characterization of a novel mutation in the overlap of *tlyA* and *ppnK* involved in capreomycin resistance in *Mycobacterium***, IUBMB life, 66 (2014) 405-414.
[343] A. Sowajassatakul, T. Prammananan, A. Chaiprasert, S. Phunpruch, **Molecular characterization of amikacin, kanamycin and capreomycin resistance in m/xdr-tb strains isolated in thailand**, BMC microbiology, 14 (2014) 165.
[344] J. Zhao, W. Wei, H. Yan, Y. Zhou, Z. Li, Y. Chen, C. Zhang, J. Zeng, T. Chen, L. Zhou, **Assessing capreomycin resistance on *tlyA* deficient and point mutation (g695a) *Mycobacterium tuberculosis* strains using multi-omics analysis**, International journal of medical microbiology : IJMM, 309 (2019) 151323.
[345] A. Sałamaszyńska-Guz, S. Rose, C.A. Lykkebo, B. Taciak, P. Bącal, T. Uśpieński, S. Douthwaite, **Biofilm formation and motility are promoted by *cj0588*-directed methylation of rRNA in *Campylobacter jejuni***, Front Cell Infect Microbiol, 7 (2017) 533-533.
[346] A. Salamaszynska-Guz, I. Serafinska, P. Bacal, S. Douthwaite, **Virulence properties of *Campylobacter jejuni* are enhanced by displaying a mycobacterial TlyA methylation pattern in its rRNA**, Cell. Microbiol., (2020) e13199.
[347] J. Osipiuk, Hatzos, C., Cobb, G., Joachimiak, A., X-ray crystal structure of putative hemolysin from *Streptococcus* thermophilus, in, 2009.
[348] N.E. Arenas, L.M. Salazar, C.Y. Soto, C. Vizcaíno, M.E. Patarroyo, M.A. Patarroyo, A. Gómez, **Molecular modeling and in silico characterization of *Mycobacterium tuberculosis* tlya: Possible misannotation of this tubercle bacilli-hemolysin**, BMC structural biology, 11 (2011) 16.
[349] C. Davies, R.B. Gerstner, D.E. Draper, V. Ramakrishnan, S.W. White, **The crystal structure of ribosomal protein S4 reveals a two-domain molecule with an extensive rna-binding surface: One domain shows structural homology to theETSs DNA-binding motif**, The EMBO journal, 17 (1998) 4545-4558.
[350] M.A. Markus, R.B. Gerstner, D.E. Draper, D.A. Torchia, **Refining the overall structure and subdomain orientation of ribosomal protein S4 delta41 with dipolar couplings measured by NMR in uniaxial liquid crystalline phases**, Journal of molecular biology, 292 (1999) 375-387.
[351] L. Aravind, E.V. Koonin, **Novel predicted rna-binding domains associated with the translation machinery**, Journal of molecular evolution, 48 (1999) 291-302.
[352] M.A. Witek, E.G. Kuiper, E. Minten, E.K. Crispell, G.L. Conn, **A novel motif for S-Adenosyl-L-Methionine binding by the ribosomal RNA methyltransferase TlyA from *Mycobacterium tuberculosis***, The Journal of biological chemistry, 292 (2017) 1977-1987.
[353] I. Hanukoglu, **Proteopedia: Rossmann fold: A beta-alpha-beta fold at dinucleotide binding sites**, Biochemistry and molecular biology education : a bimonthly publication of the International Union of Biochemistry and Molecular Biology, 43 (2015) 206-209.

[354] B.P.S. Chouhan, S. Maimaiti, M. Gade, P. Laurino, **Rossmann-fold methyltransferases: Taking a "β-turn" around their cofactor, S-Adenosylmethionine**, Biochemistry, 58 (2019) 166-170.
[355] W.H. Shin, D. Kihara, **55 years of the Rossmann fold**, Methods in molecular biology (Clifton, N.J.), 1958 (2019) 1-13.
[356] S. Kumar, E. Mittal, S. Deore, A. Kumar, A. Rahman, M.V. Krishnasastry, **Mycobacterial tlya gene product is localized to the cell-wall without signal sequence**, Front Cell Infect Microbiol, 5 (2015) 60.
[357] P.D.B. Olinares, J. Kim, K.J. van Wijk, **The Clp protease system; a central component of the chloroplast protease network**, Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1807 (2011) 999-1011.
[358] C. Moreno-Cinos, K. Goossens, I.G. Salado, P. Van Der Veken, H. De Winter, K. Augustyns, **ClpP protease, a promising antimicrobial target**, International journal of molecular sciences, 20 (2019).
[359] E. Carvalho, A.S. Barbosa, R.M. Gómez, A.M. Cianciarullo, P. Hauk, P.A. Abreu, L.C. Fiorini, M.L.S. Oliveira, E.C. Romero, A.P. Gonçales, Z.M. Morais, S.A. Vasconcellos, P.L. Ho, ***Leptospiral* TlyC is an extracellular matrix-binding protein and does not present hemolysin activity**, FEBS Letters, 583 (2009) 1381-1385.
[360] D.M.B.M. Byers, H. Gong, **Acyl carrier protein: Structure–function relationships in a conserved multifunctional protein family**, Biochemistry and Cell Biology, 85 (2007) 649-662.
[361] P. Stanley, V. Koronakis, C. Hughes, **Acylation of *Escherichia coli* hemolysin: A unique protein lipidation mechanism underlying toxin function**, Microbiology and molecular biology reviews : MMBR, 62 (1998) 309-333.
[362] K.B. Lim, C.R. Walker, L. Guo, S. Pellett, J. Shabanowitz, D.F. Hunt, E.L. Hewlett, A. Ludwig, W. Goebel, R.A. Welch, M. Hackett, ***Escherichia coli* alpha-hemolysin (HlyA) is heterogeneously acylated in vivo with 14-, 15-, and 17-carbon fatty acids**, The Journal of biological chemistry, 275 (2000) 36698-36702.
[363] K. Ryan, C.G. Ray, N. Ahmad, W.L. Drew, J. Plorde, **Sherris medical microbiology, fifth edition**, Mcgraw-hill, 2010.
[364] E.M. Molloy, P.D. Cotter, C. Hill, D.A. Mitchell, R.P. Ross, **Streptolysin s-like virulence factors: The continuing saga**, Nature reviews. Microbiology, 9 (2011) 670-681.
[365] It, Ocirc, Ryo, T. Okami, M. Yoshimura, **Studies on the effect of nucleic acid upon the synthesis of oxygen stable streptolysin by certain strains of *Streptococcus pyogenes*:Part XII. On the production of streptolysin by resting hemolytic *Streptococci* suspended in nucleic acid solution**, The Japanese Medical Journal, 1 (1948) 253-259.
[366] I. Ginsburg, T.N. Harris, N. Grossowicz, **Oxygen-stable hemolysins of group a *Streptococci*. I. The role of various agents in the production of the hemolysins**, The Journal of experimental medicine, 118 (1963) 905-917.
[367] I. Ginsburg, N. Grossowicz, **A cell-bound hemolysin of group a S*treptococci***, Bulletin of the Research Council of Israel. Section E: Experimental medicine, 7e (1958) 237-246.
[368] B. Cinader, L. Pillemer, **The purification and properties of streptolysin s**, The Journal of experimental medicine, 92 (1950) 219-237.
[369] H. Ishikura, **Fractionation of RNAase I core by gel filtration and its relation to streptolysin s-forming activity**, Biochimica et biophysica acta, 51 (1961) 189-190.

[370] C. Loridan, J.E. Alouf, **Purification of RNA-core induced streptolysin s, and isolation and haemolytic characteristics of the carrier-free toxin**, Journal of general microbiology, 132 (1986) 307-315.
[371] T.S. Theodore, G.B. Calandra, **Streptolysin s activation by lipoteichoic acid**, Infect Immun, 33 (1981) 326-328.
[372] A.W. Bernheimer, L.L. Schwartz, **Effect of staphylococcal and other bacterial toxins on platelets in vitro**, The Journal of pathology and bacteriology, 89 (1965) 209-223.
[373] A.W. Bernheimer, L.L. Schwartz, **Lysosomal disruption by bacterial toxins**, Journal of bacteriology, 87 (1964) 1100-1104.
[374] G. Weissmann, H. Keiser, A.W. Bernheimer, **Studies on lysosomes. III. The effects of streptolysins o and s on the release of acid hydrolases from a granular fraction of rabbit liver**, The Journal of experimental medicine, 118 (1963) 205-222.
[375] A.W. Bernheimer, **Disruption of wall-less bacteria by streptococcal and staphylococcal toxins**, Journal of bacteriology, 91 (1966) 1677-1680.
[376] A.D. Bangham, M.M. Standish, G. Weissmann, **The action of steroids and streptolysin s on the permeability of phospholipid structures to cations**, Journal of molecular biology, 13 (1965) 253-IN228.
[377] H. Okazaki, **Streptolysin s of *Streptococcus pyogenes*: Studies on phospholipase activity**, Journal of biochemistry, 70 (1971) 867-868.
[378] I. Ginsburg, **Is streptolysin s of group a streptococci a virulence factor?**, APMIS : acta pathologica, microbiologica, et immunologica Scandinavica, 107 (1999) 1051-1059.
[379] W. Hryniewicz, J. Pryjma, **Effect of streptolysin s on human and mouse t and b lymphocytes**, Infect Immun, 16 (1977) 730-733.
[380] L. Buckingham, J.L. Duncan, **Approximate dimensions of membrane lesions produced by streptolysin s and streptolysin o**, Biochimica et Biophysica Acta (BBA) - Biomembranes, 729 (1983) 115-122.
[381] A. Carr, D.D. Sledjeski, A. Podbielski, M.D.P. Boyle, B. Kreikemeyer, **Similarities between complement-mediated and streptolysin s-mediated hemolysis***, Journal of Biological Chemistry, 276 (2001) 41790-41796.
[382] A. Cstorer, R. Ménard, **[33] Catalytic mechanism in papain family of cysteine peptidases**, in: Methods in enzymology, vol. 244, Academic Press, 1994, pp. 486-500.
[383] J.L. Duncan, L. Mason, **Characteristics of streptolysin s hemolysis**, Infection and immunity, 14 (1976) 77-82.
[384] D.L. Higashi, N. Biais, D.L. Donahue, J.A. Mayfield, C.R. Tessier, K. Rodriguez, B.L. Ashfeld, J. Luchetti, V.A. Ploplis, F.J. Castellino, S.W. Lee, **Activation of Band 3 mediates group a *Streptococcus* streptolysin s-based beta-haemolysis**, Nature microbiology, 1 (2016) 15004.
[385] S.D. Betschel, S.M. Borgia, N.L. Barg, D.E. Low, J.C. De Azavedo, **Reduced virulence of group a streptococcal tn916 mutants that do not produce streptolysin s**, Infect Immun, 66 (1998) 1671-1679.
[386] V. Nizet, B. Beall, D.J. Bast, V. Datta, L. Kilburn, D.E. Low, J.C. De Azavedo, **Genetic locus for streptolysin s production by group a *Streptococcus***, Infect Immun, 68 (2000) 4245-4254.
[387] P. Yorgey, J. Davagnino, R. Kolter, **The maturation pathway of Microcin B17, a peptide inhibitor of DNA gyrase**, Molecular microbiology, 9 (1993) 897-905.

[388] J.O. Melby, N.J. Nard, D.A. Mitchell, **Thiazole/oxazole-modified microcins: Complex natural products from ribosomal templates**, Current opinion in chemical biology, 15 (2011) 369-378.
[389] J.G. Heddle, S.J. Blance, D.B. Zamble, F. Hollfelder, D.A. Miller, L.M. Wentzell, C.T. Walsh, A. Maxwell, **The antibiotic Microcin B17 is a DNA gyrase poison: Characterisation of the mode of inhibition**, Journal of molecular biology, 307 (2001) 1223-1234.
[390] J.L. Vizán, C. Hernández-Chico, I. del Castillo, F. Moreno, **The peptide antibiotic Microcin B17 induces double-strand cleavage of DNA mediated by *E. coli* DNA gyrase**, The EMBO journal, 10 (1991) 467-476.
[391] A. Bayer, S. Freund, G. Nicholson, G. Jung, **Posttranslational backbone modifications in the ribosomal biosynthesis of the glycine-rich antibiotic Microcin B17**, Angewandte Chemie International Edition in English, 32 (1993) 1336-1339.
[392] P. Yorgey, J. Lee, J. Kördel, E. Vivas, P. Warner, D. Jebaratnam, R. Kolter, **Posttranslational modifications in Microcin B17 define an additional class of DNA gyrase inhibitor**, Proceedings of the National Academy of Sciences of the United States of America, 91 (1994) 4519-4523.
[393] J. Davagnino, M. Herrero, D. Furlong, F. Moreno, R. Kolter, **The DNA replication inhibitor Microcin B17 is a forty-three-amino-acid protein containing sixty percent glycine**, Proteins, 1 (1986) 230-238.
[394] S.W. Lee, D.A. Mitchell, A.L. Markley, M.E. Hensler, D. Gonzalez, A. Wohlrab, P.C. Dorrestein, V. Nizet, J.E. Dixon, **Discovery of a widely distributed toxin biosynthetic gene cluster**, Proceedings of the National Academy of Sciences of the United States of America, 105 (2008) 5879-5884.
[395] A.L. Markley, E.R. Jensen, S.W. Lee, **An *Escherichia coli*-based bioengineering strategy to study streptolysin s biosynthesis**, Analytical biochemistry, 420 (2012) 191-193.
[396] G. Fiala, K.O. Stetter, ***Pyrococcus furiosus* sp. Nov. Represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°c**, Archives of Microbiology, 145 (1986) 56-61.
[397] D.A. Mitchell, S.W. Lee, M.A. Pence, A.L. Markley, J.D. Limm, V. Nizet, J.E. Dixon, **Structural and functional dissection of the heterocyclic peptide cytotoxin streptolysin s**, The Journal of biological chemistry, 284 (2009) 13004-13012.
[398] D.J. Gonzalez, S.W. Lee, M.E. Hensler, A.L. Markley, S. Dahesh, D.A. Mitchell, N. Bandeira, V. Nizet, J.E. Dixon, P.C. Dorrestein, **Clostridiolysin s, a post-translationally modified biotoxin from *Clostridium botulinum***, The Journal of biological chemistry, 285 (2010) 28220-28228.
[399] T. Maxson, C.D. Deane, E.M. Molloy, C.L. Cox, A.L. Markley, S.W. Lee, D.A. Mitchell, **Hiv protease inhibitors block streptolysin s production**, ACS chemical biology, 10 (2015) 1217-1226.
[400] J. Pei, N.V. Grishin, **Type II CAAX prenyl endopeptidases belong to a novel superfamily of putative membrane-bound metalloproteases**, Trends in biochemical sciences, 26 (2001) 275-277.
[401] M.O. Bergo, P. Ambroziak, C. Gregory, A. George, J.C. Otto, E. Kim, H. Nagase, P.J. Casey, A. Balmain, S.G. Young, **Absence of the CAAX endoprotease RCE1: Effects on cell growth and transformation**, Molecular and cellular biology, 22 (2002) 171-181.

[402] L. James, R.B. McFarland, **An epidemic of pharyngitis due to a nonhemolytic group a *Streptococcus* at lowry air force base**, The New England journal of medicine, 284 (1971) 750-752.
[403] M.R. Wessels, **Streptolysin s**, The Journal of Infectious Diseases, 192 (2005) 13-15.
[404] T. Miyoshi-Akiyama, D. Takamatsu, M. Koyanagi, J. Zhao, K. Imanishi, T. Uchiyama, **Cytocidal effect of *Streptococcus pyogenes* on mouse neutrophils *in vivo* and the critical role of streptolysin s**, J Infect Dis, 192 (2005) 107-116.
[405] O. Goldmann, I. Sastalla, M. Wos-Oxley, M. Rohde, E. Medina, ***Streptococcus pyogenes* induces oncosis in macrophages through the activation of an inflammatory programmed cell death pathway**, Cellular microbiology, 11 (2009) 138-155.
[406] N. Tsao, C.F. Kuo, M.H. Cheng, W.C. Lin, C.F. Lin, Y.S. Lin, **Streptolysin s induces mitochondrial damage and macrophage death through inhibiting degradation of glycogen synthase kinase-3β in *Streptococcus pyogenes* infection**, Scientific reports, 9 (2019) 5371.
[407] C. von Beek, I. Waern, J. Eriksson, F.R. Melo, C. Robinson, A.S. Waller, M.E. Sellin, B. Guss, G. Pejler, **Streptococcal sagA activates a proinflammatory response in mast cells by a sublytic mechanism**, Cellular microbiology, 21 (2019) e13064.
[408] R.A. Flaherty, J.M. Puricelli, D.L. Higashi, C.J. Park, S.W. Lee, **Streptolysin s promotes programmed cell death and enhances inflammatory signaling in epithelial keratinocytes during group a *Streptococcus* infection**, Infect Immun, 83 (2015) 4118-4133.
[409] G. Song, G. Ouyang, S. Bao, **The activation of AKT/PKB signaling pathway and cell survival**, Journal of cellular and molecular medicine, 9 (2005) 59-71.
[410] T.J. Wiles, B.K. Dhakal, D.S. Eto, M.A. Mulvey, **Inactivation of host AKT/protein kinase B signaling by bacterial pore-forming toxins**, Molecular biology of the cell, 19 (2008) 1427-1438.
[411] R.A. Flaherty, D.L. Donahue, K.E. Carothers, J.N. Ross, V.A. Ploplis, F.J. Castellino, S.W. Lee, **Neutralization of streptolysin s-dependent and independent inflammatory cytokine IL-1β activity reduces pathology during early group a *Streptococcal* skin infection**, Front Cell Infect Microbiol, 8 (2018) 211.
[412] E.H. Kim, S.-W. Wong, J. Martinez, **Programmed necrosis and disease: We interrupt your regular programming to bring you necroinflammation**, Cell Death & Differentiation, 26 (2019) 25-40.
[413] T. Sumitomo, M. Nakata, M. Higashino, Y. Jin, Y. Terao, Y. Fujinaga, S. Kawabata, **Streptolysin s contributes to group a *Streptococcal* translocation across an epithelial barrier**, The Journal of biological chemistry, 286 (2011) 2750-2761.
[414] P.D. Cotter, L.A. Draper, E.M. Lawton, K.M. Daly, D.S. Groeger, P.G. Casey, R.P. Ross, C. Hill, **Listeriolysin s, a novel peptide haemolysin associated with a subset of lineage I *Listeria monocytogenes***, PLoS pathogens, 4 (2008) e1000144.
[415] J.J. Quereda, M.A. Nahori, J. Meza-Torres, M. Sachse, P. Titos-Jiménez, J. Gomez-Laguna, O. Dussurget, P. Cossart, J. Pizarro-Cerdá, **Listeriolysin s is a streptolysin s-like virulence factor that targets exclusively prokaryotic cells *in vivo***, mBio, 8 (2017).
[416] J.J. Quereda, J. Meza-Torres, P. Cossart, J. Pizarro-Cerdá, **Listeriolysin s: A bacteriocin from epidemic *Listeria monocytogenes* strains that targets the gut microbiota**, Gut microbes, 8 (2017) 384-391.
[417] R. Mohammadzadeh, A. Azadegan, B.S. Kalani, **Listeriolysin s may inhibit the anti-*Listeria*l properties of *Lactobacillus plantarum***, Microb Pathog, 137 (2019) 103744.

[418] M.C. Martino, R.A. Stabler, Z.W. Zhang, M.J.G. Farthing, B.W. Wren, N. Dorrell, ***Helicobacter pylori* pore-forming cytolysin orthologue Tlya possesses *in vitro* hemolytic activity and has a role in colonization of the gastric mucosa**, Infect. Immun., 69 (2001) 1697-1703.
[419] N. Samainukul, A.K. Linn, M.B. Javadi, S. Sakdee, C. Angsuthanasombat, G. Katzenmeier, **Importance of the Cys124−Cys128 intermolecular disulfide bonding for oligomeric assembly and hemolytic activity of the *Helicobacter pylori* Tlya hemolysin**, Biochem. Bioph. Res. Co., 514 (2019) 365-371.
[420] S.K. Johansen, C.E. Maus, B.B. Plikaytis, S. Douthwaite, **Capreomycin binds across the ribosomal subunit interface using Tlya-encoded 2'-O-methylations in 16s and 23s rRNAs**, Mol. Cell., 23 (2006) 173-182.
[421] T. Monshupanee, **Increased bacterial hemolytic activity is conferred by expression of Tlya methyltransferase but not by its 2′-O-methylation of the ribosome**, Current Microbiology, 67 (2013) 61-68.
[422] M.A. Witek, E.G. Kuiper, E. Minten, E.K. Crispell, G.L. Conn, **A novel motif for S-Adenosyl-L-Methionine binding by the ribosomal RNA methyltransferase Tlya from *Mycobacterium tuberculosis***, J. Biol. Chem., 292 (2017) 1977-1987.
[423] R. Akbergenov, D. Shcherbakov, T. Matt, S. Duscha, M. Meyer, D.N. Wilson, E.C. Böttger, **Molecular basis for the selectivity of antituberculosis compounds capreomycin and viomycin**, Antimicrob. Agents Ch., 55 (2011) 4712-4717.
[424] A. Alvarez-Ordóñez, F.J. Martínez-Lobo, H. Arguello, A. Carvajal, P. Rubio, **Swine dysentery: Aetiology, pathogenicity, determinants of transmission and the fight against the disease.**, Int. J. Env. Res. Pub. He., 10 (2013) 1927-1947.
[425] D.J. Hampson, E.R. Burrough, **Swine dysentery and *Brachyspiral* colitis**, in: J.J. Zimmerman, L.A. Karriker, A. Ramirez, K.J. Schwartz, G.W. Stevenson, J. Zhang (Eds.) Diseases of swine, John Wiley & Sons, Hoboken, NJ, 2019, pp. 951-970.
[426] I.T. Egan, D. L. Harris, and H. T. Hill. , **Prevalence of swine dysentery, transmissible gastroenteritis, and pseudorabies in Iowa, Illinois and Missouri swine.,** in: 86th U.S. Annual Health Assoc. Meeting, 1982, pp. 497-502.
[427] D.J. Taylor, T.J. Alexander, **The production of dysentery in swine by feeding cultures containing a spirochaete.**, Brit. Vet. J., 127 (1971) 58-61.
[428] E. Tsinganou, J.O. Gebbers, **Human intestinal spirochetosis--a review**, German medical science : GMS e-journal, 8 (2010) Doc01.
[429] J. Osorio, A. Carvajal, G. Naharro, P. Rubio, T. La, N.D. Phillips, D.J. Hampson, **Identification of weakly haemolytic *Brachyspira* isolates recovered from pigs with diarrhoea in spain and portugal and comparison with results from other countries**, Res. Vet. Sci., 95 (2013) 861-869.
[430] A.A.H.M. ter Huurne, S. Muir, M. van Houten, B.A.M. van der Zeijst, W. Gaastra, J.G. Kusters, **Characterization of three putative *Serpulina hyodysenteriae* hemolysins**, Microbial Pathogenesis, 16 (1994) 269-282.
[431] T. Hsu, D. Hutto, F. Minion, R.L. Zuerner, M.J. Wannemuehler, **Cloning of a beta-hemolysin gene of *Brachyspira (Serpulina) hyodysenteriae* and its expression in *Escherichia coli***, Infect. Immun., 69 (2001) 706-711.
[432] L.A. Kelley, S. Mezulis, C.M. Yates, M.N. Wass, M.J.E. Sternberg, **The PHYRE2 web portal for protein modeling, prediction and analysis**, Nat. Protoc., 10 (2015) 845.

[433] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, T.E. Ferrin, **UCSF Chimera--a visualization system for exploratory research and analysis**, Journal of computational chemistry, 25 (2004) 1605-1612.
[434] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, **Measurement of protein using bicinchoninic acid**, Anal. Biochem., 150 (1985) 76-85.
[435] G. Böhm, R. Muhr, R. Jaenicke, **Quantitative analysis of protein far UV circular dichroism spectra by neural networks**, Protein Eng. Des. Sel., 5 (1992) 191-195.
[436] K. Lata, K. Paul, K. Chattopadhyay, **Functional characterization of *Helicobacter pylori* tlya: Pore-forming hemolytic activity and cytotoxic property of the protein.**, Biochem. Bioph. Res. Co., 444 (2014) 153-157.
[437] W. Sriwimol, A. Aroonkesorn, S. Sakdee, C. Kanchanawarin, T. Uchihashi, T. Ando, C. Angsuthanasombat, **Potential prepore trimer formation by the *Bacillus thuringiensis* mosquito-specific toxin: Molecular insights into a critical prerequisite of membrane-bound monomers**, Journal of Biological Chemistry, 290 (2015) 20793-20803.
[438] G.E. Crooks, G. Hon, J.M. Chandonia, S.E. Brenner, **Weblogo: A sequence logo generator**, Genome research, 14 (2004) 1188-1190.
[439] R.C. Edgar, **Muscle: Multiple sequence alignment with high accuracy and high throughput**, Nucleic Acids Res., 32 (2004) 1792-1797.
[440] T.B. Stanton, E. Fournie-Amazouz, D. Postic, D.J. Trott, P.A.D. Grimont, G. Baranton, D.J. Hampson, L. Saint Girons, **Recognition of two new species of intestinal spirochetes: *Serpulina intermedia* sp. Nov. And *Serpulina murdochii* sp. Nov**, Int. J. Syst. Evol. Micr., (1997).
[441] D.J. Hampson, T. La, **Reclassification of *Serpulina intermedia* and *Serpulina murdochii* in the genus *Brachyspira* as *Brachyspira intermedia* comb. Nov. And *Brachyspira murdochii* comb. Nov**, Int. J. Syst. Evol. Micr., 56 (2006) 1009-1012.
[442] N.S. Mirajkar, T.J. Johnson, C.J. Gebhart, **Complete genome sequence of *Brachyspira hyodysenteriae* type strain b-78 (atcc 27164)**, Genome announcements, 4 (2016).
[443] M.R. Gonzalez, M. Bischofberger, L. Pernot, F.G. van der Goot, B. Frêche, **Bacterial pore-forming toxins: The (w)hole story?**, Cell. Mol. Life Sci., 65 (2008) 493-507.
[444] M. Dal Peraro, F.G. van der Goot, **Pore-forming toxins: Ancient, but never really out of fashion**, Nat. Rev. Microbiol., 14 (2015) 77-92.
[445] M. Lovatt, P. Cooper A Fau - Camilleri, P. Camilleri, **Energetics of cyclodextrin-induced dissociation of insulin**, Eur. Biophys. J., 24 (1996) 354-357.
[446] D. McPhail, A. Cooper, **Thermodynamics and kinetics of dissociation of ligand-induced dimers of vancomycin antibiotics**, J. Chem. Soc. Faraday Trans, 93 (1997) 2283-2289.
[447] K. Matsuoka, Y. Moroi, **Micelle formation of sodium deoxycholate and sodium ursodeoxycholate (part 1)**, Biochimica et biophysica acta, 1580 (2002) 189-199.
[448] K. Matsubara, T. Nakajima, J.E. Moore, B.C. Millar, T. Murayama, M. Matsuda, **Molecular analysis of the *tlya* gene in *Campylobacter lari***, Folia Microbiologica, 60 (2015) 505-514.
[449] T. Monshupanee, **Increased bacterial hemolytic activity is conferred by expression of Tlya methyltransferase but not by its 2′-o-methylation of the ribosome**, Curr. Microbiol., 67 (2013) 61-68.
[450] A. Sałamaszyńska-Guz, B. Taciak, A. Kwiatek, D. Klimuszko, **The cj0588 protein is a *Campylobacter jejuni* RNAmethyltransferase**, Biochem. Bioph. Res. Co., 448 (2014) 298-302.

[451] V. Casas, S. Vadillo, C. San Juan, M. Carrascal, J. Abian, **The exposed proteomes of *Brachyspira hyodysenteriae* and *B. pilosicoli***, Frontiers in microbiology, 7 (2016) 1103.
[452] R. Novy, K. Yaeger, D. Held, R. Mierendorf, **Coexpression of multiple target proteins in e. *Coli***, Innovations, 15 (2002) 2.
[453] P.M. Hwang, J.S. Pan, B.D. Sykes, **Targeted expression, purification, and cleavage of fusion proteins from inclusion bodies in *Escherichia coli***, FEBS Letters, 588 (2014) 247-252.
[454] R. Derynck, A.B. Roberts, M.E. Winkler, E.Y. Chen, D.V. Goeddel, **Human transforming growth factor-α: Precursor structure and expression in *E. Coli***, Cell, 38 (1984) 287-297.
[455] G. Miozzari, C. Yanofsky, **Translation of the leader region of the *Escherichia coli* tryptophan operon**, Journal of bacteriology, 133 (1978) 1457-1466.
[456] G.A. Cook, S. Stefer, S.J. Opella, **Expression and purification of the membrane protein p7 from hepatitis c virus**, Peptide Science, 96 (2011) 32-40.
[457] B.A. Keith, J.C.H. Ching, M.E. Loewen, **Von willebrand factor type a domain of HCLCA1 is sufficient for u-937 macrophage activation**, Biochemistry and Biophysics Reports, 18 (2019) 100630.
[458] H. Schägger, **Tricine–sds-page**, Nature Protocols, 1 (2006) 16-22.
[459] H. Schägger, G. Von Jagow, **Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kda**, Analytical biochemistry, 166 (1987) 368-379.
[460] M.R. Hemm, B.J. Paul, T.D. Schneider, G. Storz, K.E. Rudd, **Small membrane proteins found by comparative genomics and ribosome binding site models**, Molecular microbiology, 70 (2008) 1487-1501.
[461] A.S. Warren, J. Archuleta, W.-c. Feng, J.C. Setubal, **Missing genes in the annotation of prokaryotic genomes**, BMC Bioinformatics, 11 (2010) 131.
[462] P. Sun, J.E. Tropea, D.S. Waugh, **Enhancing the solubility of recombinant proteins in *Escherichia coli* by using hexahistidine-tagged maltose-binding protein as a fusion partner**, Methods in molecular biology (Clifton, N.J.), 705 (2011) 259-274.
[463] M. Lebendiker, T. Danieli, **Purification of proteins fused to maltose-binding protein**, Methods in molecular biology (Clifton, N.J.), 681 (2011) 281-293.
[464] W. Schroeder, J.B. Shelton, J.R. Shelton, **An examination of conditions for the cleavage of polypeptide chains with cyanogen bromide: Application to catalase**, Archives of biochemistry and biophysics, 130 (1969) 551-555.
[465] R. Joppich-Kuhn, J.A. Corkill, R.W. Giese, **Oxidation of methionine to methionine sulfoxide as a side reaction of cyanogen bromide cleavage**, Analytical biochemistry, 119 (1982) 73-77.
[466] R. Kaiser, L. Metzka, **Enhancement of cyanogen bromide cleavage yields for methionyl-serine and methionyl-threonine peptide bonds**, Analytical biochemistry, 266 (1999) 1-8.
[467] N.S. Mirajkar, N.D. Phillips, T. La, D.J. Hampson, C.J. Gebhart, **Characterization and recognition of *Brachyspira hampsonii* sp. Nov., a novel intestinal spirochete that is pathogenic to pigs**, Journal of clinical microbiology, 54 (2016) 2942-2949.
[468] M. Mahu, N. De Pauw, L. Vande Maele, M. Verlinden, F. Boyen, R. Ducatelle, F. Haesebrouck, A. Martel, F. Pasmans, **Variation in hemolytic activity of *Brachyspira hyodysenteriae* strains from pigs**, Veterinary research, 47 (2016) 66.

[469] L.L. Clarke, **A guide to ussing chamber studies of mouse intestine**, American journal of physiology. Gastrointestinal and liver physiology, 296 (2009) G1151-1166.
[470] T. Janas, T. Janas, **Reversible DIDS binding to Band 3 protein in human erythrocyte membranes**, Molecular membrane biology, 17 (2000) 109-115.
[471] J.M. Whittamore, M. Hatch, **Loss of the anion exchanger DRA (SLC26A3), or PAT1 (SLC26A6), alters sulfate transport by the distal ileum and overall sulfate homeostasis**, American journal of physiology. Gastrointestinal and liver physiology, 313 (2017) G166-G179.
[472] D. Humar, V. Datta, D.J. Bast, B. Beall, J.C. De Azavedo, V. Nizet, **Streptolysin s and necrotising infections produced by group g *Streptococcus***, Lancet (London, England), 359 (2002) 124-129.
[473] M.O. Costa, J.C.S. Harding, **Swine dysentery disease mechanism: *Brachyspira hampsonii* impairs the colonic immune and epithelial repair responses to induce lesions**, Microb Pathog, 148 (2020) 104470.
[474] B.P. Wilcock, H.J. Olander, **Studies on the pathogenesis of swine dysentery. II. Search for a cytotoxin in spirochetal broth cultures and colon content**, Vet Pathol, 16 (1979) 567-573.
[475] R.A.F. Reithmeier, J.R. Casey, A.C. Kalli, M.S.P. Sansom, Y. Alguel, S. Iwata, **Band 3, the human red cell chloride/bicarbonate anion exchanger (AE1, SLC4A1), in a structural context**, Biochimica et Biophysica Acta (BBA) - Biomembranes, 1858 (2016) 1507-1532.
[476] K. Hsu, **Exploring the potential roles of Band 3 and Aquaporin-1 in blood $CO_2$ transport–inspired by comparative studies of glycophorin b-a-b hybrid protein gp.** Frontiers in Physiology, 9 (2018).
[477] R.A. Kunkle, D.L. Harris, J.M. Kinyon, **Autoclaved liquid medium for propagation of *Treponema hyodysenteriae***, Journal of clinical microbiology, 24 (1986) 669.
[478] H. Plaza, T.R. Whelchel, S.F. Garczynski, E.W. Howerth, F.C. Gherardini, **Purified outer membranes of *Serpulina hyodysenteriae* contain cholesterol**, Journal of bacteriology, 179 (1997) 5414-5421.
[479] M.M. Reddy, X.F. Wang, P.M. Quinton, **Effect of cytosolic pH on epithelial $Na^+$ channel in normal and cystic fibrosis sweat ducts**, The Journal of membrane biology, 225 (2008) 1-11.
[480] M.A. Garcia, N. Yang, P.M. Quinton, **Normal mouse intestinal mucus release requires cystic fibrosis transmembrane regulator-dependent bicarbonate secretion**, The Journal of clinical investigation, 119 (2009) 2613-2622.
[481] N. Shcheynikov, Y. Wang, M. Park, S.B. Ko, M. Dorwart, S. Naruse, P.J. Thomas, S. Muallem, **Coupling modes and stoichiometry of $Cl^-/HCO_3^-$ exchange by SLC26A3 and SLC26A6**, The Journal of general physiology, 127 (2006) 511-524.