MOLECULAR AND PHYSIOLOGICAL RESPONSES OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS ATCC 4931 TO TRISODIUM PHOSPHATE

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By

BALAMURUGAN SAMPATHKUMAR

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University of Saskatchewan

51 Campus drive, Saskatoon, Saskatchewan

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ABSTRACT

Salmonella species continue to be commonly associated with cases of food-borne disease in developed countries. In the United States in 2001, the incidence per 100,000 people was highest for salmonellosis (15.1), followed by campylobacteriosis (13.8) and shigellosis (6.4). Enteric pathogens usually contaminate the surface of raw animal products during slaughter and primary processing (scalding, defeathering or dehiding, rinsing, cutting, mixing, and grinding, etc.) and can attach and/or reside in the regular and irregular surfaces of the skin, multiply and, thereafter, contaminate food preparation surfaces, hands and utensils. Trisodium phosphate (TSP) has been approved by the USDA as a sanitizer to reduce surface loads of *Salmonella* on chicken carcasses. A number of studies had demonstrated that TSP effectively removes surface contamination of carcasses by food-borne pathogens. However, very little scientific evidence is available which identifies the actual mechanisms of TSP antimicrobial activity and the response of food-borne pathogens exposed to TSP.

This study examined both the physiological and molecular response of *Salmonella enterica* serovar Enteritidis to TSP treatment. The role of high pH during TSP treatment on its antimicrobial activity was examined. Adaptation of *S. enterica* serovar Enteritidis to TSP treatment was also examined by analyzing the proteome of serovar Enteritidis cells using two-dimensional gel electrophoresis and mass spectrometry.

The role of high pH on the antimicrobial activity of TSP was examined using comparative studies involving treatment solutions containing different concentrations of TSP, treatment solutions adjusted to the equivalent pH as in each of the TSP treatments and TSP solutions pH adjusted to 7.0. Direct and indirect indices of cell survival, membrane damage, and cellular leakage were also employed to examine specific antimicrobial effects. Cell viability, loss of membrane integrity, cellular leakage, release of lipopolysaccharides and cell morphology were accordingly examined and quantified under the above treatment conditions. Exposure of serovar Enteritidis cells to TSP or equivalent alkaline pH made with NaOH resulted in the loss of cell viability and membrane integrity in a TSP concentration- or NaOH-alkaline pH-dependent manner. In contrast, cells treated with different concentrations of TSP whose pH was adjusted to 7.0 did not show any loss of cell viability or membrane integrity. These results indicate that TSP is a potent membrane-acting agent, and provide compelling evidence that high pH during TSP treatment was responsible for its antimicrobial activity.

Adaptation of *S. enterica* serovar Enteritidis with a sublethal concentration of TSP resulted in the induction of the alkaline stress response. Alkaline stress response involves induced thermotolerance, resistance to higher concentrations of TSP, high pH and sensitivity to acid. Examination of the proteome of TSP-adapted cells revealed differential expression of a number of proteins but did not include the common heat shock proteins involved in thermotolerance. However, TSP adaptation caused a shift in the membrane fatty acid composition from unsaturated to a higher saturated and cyclic fatty acid. This shift in fatty acid composition increases the melting point of the cytoplasmic membrane so that it remains functional at high temperatures.

Biofilm bacteria are more resistant to sanitizers, heat and antimicrobial agents than their planktonic counterparts. Examination of the proteome of TSP-adapted biofilm cell of *S. enterica* serovar Enteritidis revealed little overlap in the protein profile compared to TSP-adapted planktonic cells. Proteomic examination of planktonic and biofilm cells of *S. enterica* serovar Enteritidis revealed differential expression of a number of proteins involved in DNA replication, stress survival and transport of newly synthesized proteins. These results clearly indicate that changes in the expression of specific genes are involved in the biofilm mode of growth, which could play a significant role in resistance to antimicrobial agents.

The results of the current study provide a better understanding of the mechanisms of antimicrobial action of TSP and also elucidate the response of *S. enterica* serovar Enteritidis to TSP and high pH adaptation. The study also raises new questions regarding stress tolerance of *S.* Enteritidis following TSP or alkaline pH adaptation with relevance to food safety.

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Truly,

Bala Sampathkumar

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1.0 GENERAL INTRODUCTION

The confidence that consumers have in the safety and quality of meat products is closely linked to the consumer's buying habits and can only be built and maintained through a long-term commitment to quality at each stage of the supply chain. However, highly publicized reports of food-borne illness resulting from ingestion of contaminated meat products, particularly by verotoxigenic *Escherichia coli* O157:H7 and *Salmonella typhimurium*, and more recently Bovine spongiform encephalopathy (BSE) and foot and mouth disease have impacted consumer buying preferences in both Canada and the United States. When consumers perceive a food as unsafe, demand for the food can drop dramatically. For example, ever since the 1993 Jack-in-the-Box restaurant chain outbreak of *E. coli* O157:H7 was linked to undercooked hamburgers, the restaurant chain lost an estimated $160 million in sales in the first 18 months after the outbreak occurred and did not recover to pre-outbreak revenue levels for another 5 years. The same demand drop can be seen in other countries as well. In 1996, Britain announced a possible link between BSE, also known as "mad cow disease" in cattle and a new variant of Creutzfeldt Jakob Disease (vCJD) in humans. Immediately after the announcement, domestic sales and consumption of beef products in the U.K. fell by 40 percent. Trade was also heavily affected as the European Union, United States and Canada banned imports of live cattle and bovine products from the U.K. More recently in May 2003, the discovery of a single BSE-infected cow in Canada has resulted in a Canadian beef

import ban by a number of countries, most notably the United States and Japan. The result is that any agricultural and food production practice is now under the microscope of public scrutiny.

Bacteria such as *E. coli* come from the intestines of animals and contaminate the surface of the meat as the carcasses are processed. It is virtually impossible to eliminate all bacteria from meat products. With every new knife cut into the meat, more bacteria are spread, and when meat is ground, more new surface is created inviting more contamination. Increased plant output over recent years to meet demands from local and international markets has placed additional strain on the capacity of many processors to ensure good sanitation, quality control and product safety. This has led to a number of recalls in recent years where meat products have been found contaminated with pathogenic bacteria. For example, in 1997, Hudson Foods (Rogers, Ark.) recalled 25 million pounds of hamburger due to an *E. coli* O157:H7 contamination (Loader and Hobbs, 1999; Martin, 1999). Hudson Foods was eventually driven out of business and the plant, one of the most modern in the country, was sold to IBP (Tyson Foods, Inc., Dakota Dunes, SD). In another case in December 2000 involving Supreme Beef (Dallas, TX), two million pounds of beef, to be used in the school lunch program, were recalled due to repeated tests showing high levels of *Salmonella*. In the summer of 2002 ConAgra (Omaha, NE) recalled 19 million pounds of hamburger suspected of contamination by *E. coli* (FSIS, 2002a). This outbreak has been linked to 25 illnesses. In October 2002 Pilgrim's Pride (Pittsburg, TX) recalled 27.4 million pounds of fresh and frozen ready-to-eat turkey and chicken because of possible *Listeria* contamination (FSIS, 2002b). The outbreak was ultimately linked to 23 deaths and 120 illnesses (Fabi, 2002).

Canada is the worlds 10th largest beef producer, representing 2.5 % of total world output. In 2001, the United States accounted for 80 % of Canada's beef exports (Statistics Canada, 2002). With the increase in trade across the border the food safety regulations of Canada and the United States are almost identical except in some cases. For instance, the United States has a "zero tolerance" policy for *Listeria monocytogenes* in ready-to-eat (RTE) food. This means that the detection of any *L. monocytogenes* in either of two 25 g samples of a food renders the food adulterated as defined by the Federal Food, Drug, and Cosmetic Act, 21 U.S.C. 342(a)(1) (Shank et al., 1996). On the other hand Canada has a "non-zero tolerance" for *L. monocytogenes* for some classes of foods (ICMSF, 1994). For example, in Canada, RTE foods that have not been associated with an outbreak and do not allow any growth of *L. monocytogenes* during a 10-day period of refrigerated storage, may contain up to 100 *L. monocytogenes* organisms per gram of food (Health Canada, 1994). Since the United States is the main trading partner to Canada, food safety regulations in Canada are being aligned to those currently required in the United States. The introduction of Hazard Analysis Critical Control Point (HACCP) system to monitor and maintain plant sanitation and product quality in both the countries is one example. Another is the intervention treatment of animal carcasses with antimicrobial agents such as polyphosphates to reduce populations of bacteria on poultry carcasses.

The use of trisodium phosphate (TSP) as a potential sanitizer has been demonstrated by a number of investigators, and was approved by the United States Department of Agriculture (USDA) for the treatment of chicken carcasses in 1993, and currently has been extended for the treatment of other animal carcasses. The use of TSP has been included in the HACCP generic model for chilled ready to cook whole chicken

(http://www.inspection.gc.ca/english/fssa/polstrat/haccp/ckslaabpou/ckslaabpoue.shtml) in the food safety enhancement program as a bactericidal agent by the Canadian Food Inspection Agency (CFIA). Trisodium phosphate treatment of carcasses has been shown to reduce surface loads of a number of pathogens. However, very little scientific understanding is available which identifies the actual mechanisms of TSP antimicrobial activity. Possible modes of action of TSP include exposure of microorganisms to high pH, enhancing detachment of bacteria from food surfaces by sequestration of metal ions (Lee et al., 1994b) and/or by removal of fat from the skin surface, thereby allowing bacteria to be washed from the food surfaces more effectively (Bender and Brotsky, 1992; Giesse, 1992; Kim and Slavik, 1994a). To date, work has mainly focused on evaluating the efficiency of *Salmonella* removal by the process (Coppen et al., 1998; Delmore et al., 2000; Xiong et al., 1998a, b; Yang et al., 1998), and no published research exists that attempts to elucidate the actual mechanism of TSP antimicrobial action or analyze the genomic/proteomic responses of *Salmonella* to TSP treatment.

This thesis work reported in three chapters; 1) High pH during trisodium phosphate treatment causes membrane damage and destruction of *Salmonella enterica* serovar Enteritidis, 2) Differential expression of proteins by *S. enterica* serovar Enteritidis exposed to trisodium phosphate, and 3) Proteomic analysis of planktonic and biofilm cells of *S. enterica* serovar Enteritidis exposed to trisodium phosphate, describes the results of my findings on the antimicrobial action of TSP and the responses of *S.* Enteritidis to TSP.

The thesis research was based on the following hypotheses and technical objectives:

1.1 Hypotheses

1. The antimicrobial activity of trisodium phosphate is due to its alkaline pH in solution,

2. Pretreatment of *S. enterica* serovar Enteritidis with a sub-lethal concentration of TSP induces a response similar to alkaline stress response,

3. Pretreatment of *S. enterica* serovar Enteritidis with a sub-lethal concentration of TSP induces the synthesis of heat shock proteins, and

4. *Salmonella enterica* serovar Enteritidis biofilm bacteria respond differently to TSP treatment compared to planktonic serovar Enteritidis cells.

1.2 Technical objectives

1. Examining the effect of high pH of TSP on *S. enterica* serovar Enteritidis cells using a series of comparative studies involving treatment solutions containing different concentrations of TSP, treatment solutions adjusted to the equivalent pH as in each of the TSP treatments and TSP solution pH adjusted to 7.0,

2. Performing a comparative proteomic analysis of *S. enterica* serovar Enteritidis cells exposed to TSP and its equivalent alkaline pH made with NaOH by 2D-PAGE and MS analysis, and

3. Performing a comparative proteomic analysis of biofilm and planktonic cells of *S. enterica* serovar Enteritidis exposed to TSP.

2.0 REVIEW OF LITERATURE

2.1 *Salmonella*

Salmonellae are important zoonotic pathogens (Bynoe and Yurack, 1964; Edwards et al., 1948; Fox, 1974; Galton et al., 1964; McCoy, 1975; Payne and Scudamore, 1977; Sickenga, 1964). Salmonellae often cause an inapparent infection, but the infection may also, although less frequently, result in enteritis, septicemia, and other serious illness in animals and humans, especially in the young (Sadler et al., 1969; Turnbull, 1979). Excretion of the organism causes widespread contamination of the environment and of foods and feeds (Edel et al., 1977; McCoy, 1975; Oosterom, 1991; Wray and Sojka, 1977). Salmonellae are readily and frequently transferred from animals to animals and from animals to humans, mainly by indirect but also by direct means (Atkinson, 1964; Edwards, 1956; Galton et al., 1964; Peluffo, 1964; Sickenga, 1964). Transfer from humans to humans is somewhat less common (Turnbull, 1979), and indirect spread from humans to animals has been reported occasionally (Johnston et al., 1981; Kinde et al., 1996).

2.2 Different species of *Salmonella*

2.2.1 *Salmonella enterica* serovar Enteritidis

Salmonella serovars can be divided into those that are host adapted and those that are not (Buxton, 1957; Clarke and Gyles, 1993; McCoy, 1975). The serovars adapted to

humans include *S. typhi*, the causative agent of typhoid fever, and the paratyphoid Salmonellae (*S. paratyphi* A, *S. paratyphi* B, and *S. paratyphi* C) that cause paratyphoid fever (Mandal, 1979; Seeliger and Maya, 1964). Animal host-adapted Salmonellae include *Salmonella* Choleraesuis causing septicemia, pneumonia, and enterocolitis in pigs (Schofield, 1944; Schwartz, 1990); *Salmonella* Dublin causing mucohemorrhagic enteritis, septicemia, pneumonia, and abortion in cattle, and septicemia, necrotic enteritis, pneumonia, and mortality in calves (Wray and Sojka, 1977); *Salmonella abortusequi* causing abortion in horses (Schofield, 1945); *Salmonella abortusovis* causing abortion in sheep (Buxton, 1957); and *Salmonella* Gallinarum and *Salmonella* Pullorum, which causes fowl typhoid (Hewitt, 1928) and pullorum disease (Rettger, 1900, 1909), respectively, in chicken and turkeys.

Based on the taxonomic description of *Salmonella* by Le Minor and Popof (1987), all members of the nontyphoidal Salmonellae are designated as serovars of Salmonella subspecies enterica and may be written as *Salmonella* (italicized) followed by the serovar (nonitalicized). Thus *Salmonella enterica* subspecies *enterica* serovar Enteritidis is written as *Salmonella* Enteritidis (*S*. Enteritidis).

Salmonella Enteritidis is a Gram-negative, motile, facultative anaerobic rod-shaped bacterium. *Salmonella* Enteritidis, like other Gram-negative bacteria, is covered by a thick extra membrane layer- the Outer Membrane (OM), which is located outside a very thin peptidoglycan layer followed by the cytoplasmic membrane (Fig. 2.1). The OM is made of lipids and polysaccharides. This layer is effectively a second lipid bilayer, but is not constructed solely of phospholipids, as in the cytoplasmic membrane; instead it contains polysaccharide and protein. The lipid and polysaccharide are intimately linked in the outer layer to form specific lipopolysaccharide structures.

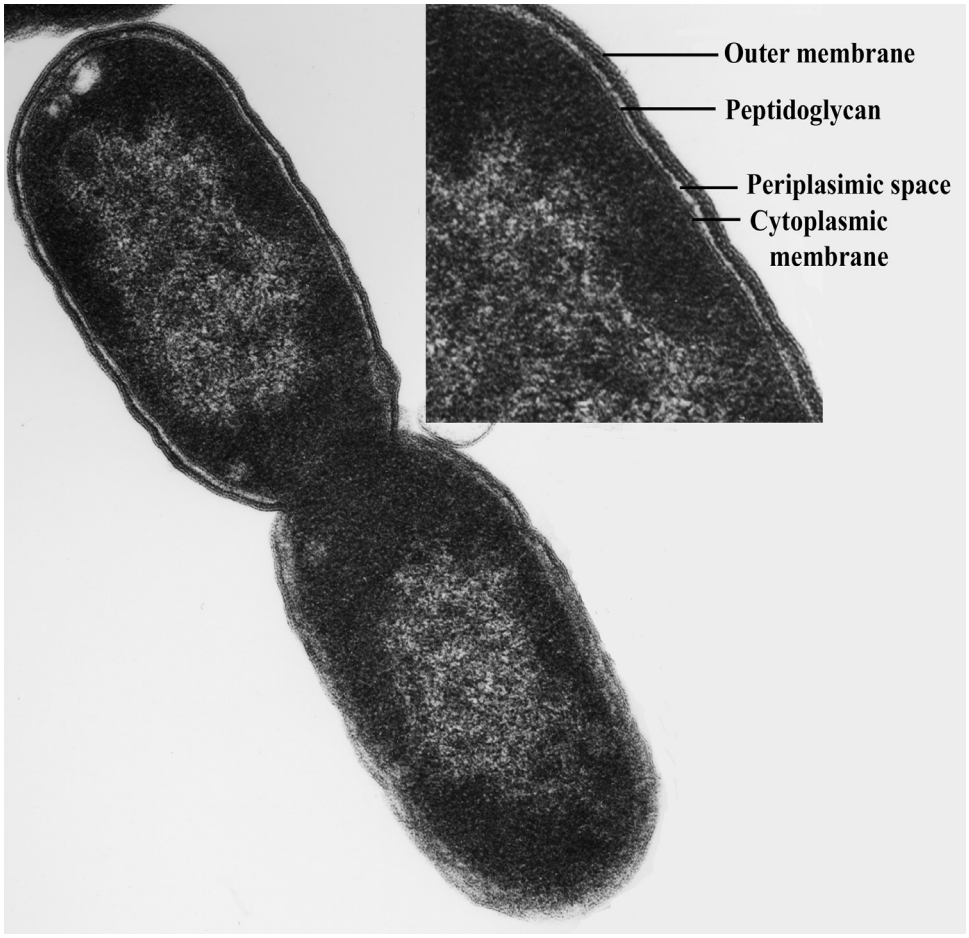


Figure 2.1 Electron micrographic thin section of *S. enterica* serovar Enteritidis ATCC 4931. Inset is a magnified view of a section of the electron micrograph showing various cellular membranes.

Because of the presence of lipopolysaccharide, the OM is frequently called the lipopolysaccharide layer, or simply LPS. The inner side of the OM contains lipoproteins complexes which serve as anchors between the OM and peptidoglycan. In Gram-negative bacteria a region called the periplasm between the outer surface of the cytoplasmic membrane and the inner surface of the LPS-containing OM occupies a distance of about 12-15 nm and is gellike in consistency, presumably because of the abundance of periplasmic proteins found there. The periplasm of Gram-negative bacteria generally contains three types of proteins: hydrolytic enzymes, binding proteins and chemoreceptors. The cytoplasmic membrane is a phospholipids bilayer which contains both highly hydrophobic (fatty acid) and relatively hydrophilic (glycerol) moieties and can exist in many different chemical forms as a result of variation in the manner of the fatty acids or phosphate-containing groups attached to the glycerol backbone. As phospholipids aggregate in an aqueous solution, they tend to form bilayer structures spontaneously- the fatty acids point inward towards each other in a hydrophobic environment, and the hydrophilic portions remain exposed to the aqueous external environment. The cytoplasmic membrane acts as an envelope that encloses the cytoplasm the genomic material.

2.2.2 Prevalence in humans

Salmonella Enteritidis belongs to the large number of more than 2,300 non-host-adapted *Salmonella* serovars (Le Minor and Popoff, 1992). The non-host specific *Salmonella* serovars cause salmonellosis in humans and in a wide variety of animal hosts

(Seeliger and Maya, 1964). During the first 5-6 decades of the 1900s, the main issues with respect to salmonellosis were the occurrence of typhoid fever in humans, which declined dramatically during that period in Europe and North America (Bynoe and Yurack, 1964; Edwards, 1958; Ranta and Dolman, 1947; Seeliger and Maya, 1964; Sommers, 1980), and the widespread prevalence of pullorum disease and fowl typhoid in chickens and turkeys (Chase, 1947; Hinshaw and McNeil, 1940; Moore, 1946), which caused high mortalities in flocks worldwide and prevented the establishment and growth of the poultry industry until the development and widespread application of testing and control measures (Bullis, 1977a; Chase, 1947; Hinshaw and McNeil, 1940; McDemott, 1947; Pomeroy, 1984; Schaffer et al., 1931; Snoeyenbos, 1984). Since the 1940s, there has been a rapid increase in the isolation of the non-host-specific *Salmonella* serovars from humans and animals (Galton et al., 1964; Guthrie, 1992). This was particularly the case with *S.* Typhimurium, which, until more recently, has been the most prevalent serovar isolated from humans and animals in many countries (Bullis, 1977b; Ferris and Miller, 1990; Hargrett-Bean and Potter, 1995; Kelterborn, 1967; Lior, 1989; McCoy, 1975; Rodrigue et al., 1990; Wray, 1985). However, during the last 10-15 years, there has been a dramatic rise in the prevalence of *S.* Enteritidis infections in humans, commencing initially in the European countries and later reported worldwide (Binkin et al., 1992; Laszlo et al., 1985; Rodrigue et al., 1990; Threlfall et al., 1993; Wong et al., 1994). During the 1985-95 period, 582 *S.* Enteritidis outbreaks were reported in the United States, and accounted for 24,058 cases of illness, 2290 hospitalizations and 70 deaths (CDC, 1996). In the process *S.* Enteritidis replaced *S.* Typhimurium as the most common serovar isolated from humans in many countries (CDC, 1992; Hargrett-Bean and Potter, 1995; Rodrigue et al., 1990). In Canada, the annual isolation rates of *S.*

Enteritidis varied between 3.7 % and 8.7 % of all *Salmonella* isolated from humans during the period 1976-82 (Khakhria et al., 1991). During the 12-year period from 1983-94, *S*. Enteritidis ranked as the third or fourth most commonly isolated serovar, except in 1991 and 1994, when it ranked second, and in 1993, when it ranked first. The percentage of *S*. Enteritidis of all *Salmonella* isolates from human sources during the 12-year period covering 1983-94 increased from a low of 2.7 % in 1984 to 16.9 % in 1994 (Khakhria et al., 1994; Khakhria et al., 1995; Khakhria et al., 1997).

Salmonella Enteritidis infection in humans were often thought to be associated with the consumption of contaminated eggs or food products containing eggs (Edwards, 1956; Watt, 1945) while recent studies have shown that *S*. Enteritidis infections are also associated with the consumption of meat (Hennessy et al., 1996; Humphrey et al., 1988; Reilly et al., 1988) or raw milk contaminated with this serovar (Wood et al., 1991). Shell eggs, scrambled eggs, food products containing raw or partly cooked eggs (including home-made mayonnaise, eggnog, milk shakes, mousse, and egg sandwiches), soft-boiled eggs, lightly cooked eggs, lightly cooked omelets, dishes containing raw egg white, ice cream containing uncooked eggs, and poultry meat have all been implicated in outbreaks (Cowden et al., 1989; Coyle et al., 1988; Hennessy et al., 1996; Humphrey et al., 1988; Mawer et al., 1989; Paul and Batchelor, 1988; Perales and Audicana, 1989; Stevens et al., 1989). Contamination of the egg contents may occur by direct transmission from an infected ovary and/or oviduct, or by contamination of the eggshell with feces in a laying bird excreting Salmonellae (Borland, 1975; Timoney et al., 1989; Williams et al., 1968). Contamination of egg contents may also occur in a bird that had only an enteric infection. The Salmonellae present in the feces may penetrate the eggshell pores as the egg cools (Stokes et al., 1956; Williams et al., 1968). During the

last decade, many of the outbreaks of *S.* Enteritidis affecting humans involved grade-A table eggs, suggesting that these outbreaks were not the result of dirty, soiled, or cracked eggs but due to internal contamination of eggs because of oophoritis of the laying hens. Infection of the ovaries of laying hens with *S.* Enteritidis has been reported on many occasions (Cooper et al., 1989; Faddoul and Fellows, 1966; Gordon and Tucker, 1965; Hopper and Mawer, 1988; Snoeyenbos et al., 1969; Timoney et al., 1989).

2.2.3 Symptoms of Salmonellosis in humans

The incubation period in infected humans varies from a few hours to 72 h and the duration of illness varies from 4 to 10 days. The carrier stage can last for weeks to months. Symptoms commonly observed are diarrhea, headache, abdominal pain, nausea, chills, fever, and vomiting (CDC, 1992; Steinert et al., 1990). In patients with underlying disease, septicemia is not uncommon and, in healthy subjects, there may be a wide range of sequelae, including pericarditis, neurological and neuromuscular diseases, reactive arthritis, ankylosing spondylitis, and osteomyelitis. Damage to the mucous membrane of the small intestine and colon may occur that may lead to malabsorption and nutrient loss (Baird-Parker, 1990). Severe dehydration, bloody diarrhea, and hematogenous spread of *S.* Enteritidis to bone, the meninges, and soft tissues have occurred in infants (Cross et al., 1989).

2.3 Stress responses of food-borne pathogens

Food-borne pathogens are commonly stressed during food processing. In cheese manufacture, for example, the pathogens present in the raw milk undergo a series of stresses, which include heat during milk thermal treatment, hydrogen peroxide that may

be added to raw milk, acid produced by the starter cultures during fermentation, and salt added to the curd. During sausage fermentation, food-borne pathogens are stressed by added salt, acid developed during fermentation, and heat during cooking and smoking. Food preservation and safety assurance are associated with these stresses, which are considered hurdles to food-borne pathogens (Leistner, 1995). For effective food preservation and safety assurance, the food industry relies on a combination of intrinsic, extrinsic and implicit inhibitory and lethal factors (hurdles) which when applied simultaneously or sequentially, are intended to curtail or inactivate the development of contaminating pathogenic bacteria in food. However, a phenomenon called stress hardening, which refers to increased resistance to lethal factors after adaptation to environmental stresses, may counteract the effectiveness of food preservation hurdles and compromise food safety. Compelling evidence has been accumulated by a number of workers which shows that this is the case, where for many food-borne bacterial pathogens, exposure to sublethal environmental stresses hardens these bacteria, and as a consequence, stress-adaptive strains have increased resistance to normally lethal levels of the same (homologous) or different (heterologous) inimical stresses (Gahan et al., 1996; Leyer and Johnson, 1993; Lou and Yousef, 1997; Rowe and Kirk, 1999). In effect, stress adaptation to the first encountered hurdle, hardens or "arms" the pathogens and increases the resistance to subsequent stresses, which may counteract the effectiveness of food preservation hurdles and compromise food safety.

2.3.1 Heat shock response

Food preservation by elevation of temperature (i.e. blanching, pasteurization, sterilization) is a common process of food preservation in use today. Reduction or

inactivation of microbial populations can be achieved by thermal processes using different means such as water, steam, hot air, electrical, light, ultrasound or microwave energy (Heldman and Lund, 1992). However, in the majority of cases, the process variables and controls have been derived by empirical investigations of the effect of temperature and time of exposure on microbial survival kinetics. Cells contain several targets for the action of heat and so it can be proposed that the basal heat resistance of microorganisms may be due to the intrinsic stability of their macromolecules, i.e. ribosomes, nucleic acids, enzymes and proteins inside the cell and the membrane. Ribosomal subunits may lose their specific secondary and tertiary structure whereas RNA and proteins may coagulate upon heating. Single stranded RNA, as well as single stranded DNA are susceptible to heat damage, however the latter is ~30 times less sensitive (Earnshaw et al., 1995). Membrane damage is mentioned (Russell and Fukanaga, 1990) as another target for the heat inactivation of bacterial cells. However, mild heat treatments can lead to adaptation of the cell membrane by increasing the saturation and the length of the fatty acids in order to maintain optimal fluidity of the membrane and activity of intrinsic proteins (Russell and Fukanaga, 1990).

Bacterial thermotolerance was shown to increase upon exposure to sublethal heating temperatures, viral infections and chemical compounds such as ethanol, methylating agents, antibiotics (such as kanamycin, streptomycin) and amino acid restrictors (Neidhardt et al., 1984). Protection against heat may be achieved by the accumulation of osmolytes that may enhance protein stability and protect enzymes against heat activation (Earnshaw et al., 1995; Taneja and Ahmed, 1994). Another successful adaptation of certain microorganisms, like the members of the genera *Bacillus* and *Clostridium*, to resist exposure to heat is their ability to produce spores

(Gould et al., 1995). However, a significant amount of evidence suggests a connection between the synthesis of heat shock proteins (HSPs) and induction of thermotolerance (Georgopoulos and Welch, 1993; Hecker et al., 1996). When bacterial cells are exposed to temperatures above their optimum for growth they synthesize increased amounts of a specific set of proteins known as heat-shock proteins (Lindquist, 1986; Neidhardt and VanBogelen, 1987). Studies on the heat-shock response *per se* began in 1962 with the report of Ritossa (1962) describing the induction by heat of a new set of puffs on the salivary gland chromosomes of a fruit fly, *Drosophila bucskii*. Since then, this phenomenon had been observed to be universal and induction of HSPs has been reported in bacteria, fungi, plants, animals, and humans (Lindquist, 1986; Neidhardt et al., 1984). Heat-shock response in *Salmonella* was reported as early as 1969 by Ng and coworkers who reported that the D_{60} value of *S. seftenberg* 775W was increased as the growth temperature was increased from 15 to 44°C (Ng et al., 1969). Similarly, Dega et al. (1972a, b) showed that *Salmonella* cells grown in concentrated milk at 43°C tolerated heating better than those grown at 22°C. Lee and coworkers (1983) reported that the synthesis of heat shock proteins in *S. typhimurium* LT2 was analogous to those found previously in *E. coli*. These proteins were found to be the only proteins synthesized after the temperature shift from 28 to 50°C (Lee et al., 1983). Mackey and Derrick reported that the survival at 52°C of *S. typhimurium* cells grown at 37°C was enhanced by several orders of magnitude if cells were preincubated at 42, 45, and 48°C (Mackey and Derrick, 1986) and coincided with the induction of 4 major heat-shock proteins of molecular weight about 83 K, 72K, 64K, and 25 K (Mackey and Derrick, 1990).

Neidhardt and Van Bogelen (1987) characterized the HSPs induced in *E. coli*. They reported as many as 17 heat-shock proteins which were diverse with respect to size, net charge, cellular abundance, and extent of inducibility by heat. These HSPs include the chaperonins GroEL, GroES, DnaK, DnaJ, and GrpE as well as the housekeeping RNA polymerase sigma factor σ^{70} (RpoD), comprise a regulon whose temperature-dependent induction requires an alternate sigma factor, σ^{32} (RpoH). σ^{32} confer upon RNA polymerase (E) promoter specificity for heat-shock promoters (Yura et al., 1993). Some of the resulting HSPs bind to proteins denatured as a result of elevated temperature, protecting them from further degradation or facilitating their refolding (Georgopoulos and Welch, 1993; Parsell and Lindquist, 1993). Other HSPs are involved in proteolysis (Lon and Clp proteases of *E. coli*). Most HSPs are synthesized at low levels under non-stress conditions but are induced rapidly and transiently upon exposure to high temperature. In *E. coli* and *B. subtilis*, the induction of HSPs is obtained through alternative sigma factors (σ^{32}, σ^{E}, σ^{N} or σ^{B}) which modify the promoter recognition specificity of the RNA polymerase to enable the expression of heat shock genes. The increase in σ^{32} in *E. coli* results from both increased synthesis and stabilization of σ^{32} which is ordinarily very unstable. Transcription of some 30 genes, constituting the σ^{32} regulon, is transiently increased as a consequence of elevated cellular levels of σ^{32}. This response is feedback controlled by the DnaK machinery that sequesters σ^{32} under physiological conditions and may also deliver it to FtsH, a protease that degrades σ^{32}. In *E. coli*, a second set of heat-inducible genes is controlled by σ^{E}. This sigma factor belongs to a class of sigma factors that respond to extracytoplasmic stimuli, such as unfolded proteins in the periplasm. A third heat-shock regulon in *E. coli*

is controlled by σ^N, an alternative sigma factor that requires an activator (PspF) for transcriptional activation (Model et al., 1997).

In addition to the transient induction in the synthesis of HSPs to a sudden upshift of temperature, modifications in membrane fluidity have also been shown to be involved in the adaptation to thermal stress. Bacterial cytoplasmic membrane, which consists mainly of lipids, has been shown to be a site for thermal injury (Dennis and Yatvin, 1981; Hansen, 1971; Hansen and Skadhauge, 1971; Yatvin et al., 1986). This cytoplasmic membrane, the boundary between the cytoplasm and the external environment, regulates the flow of nutrients and metabolic products in and out of the cell, thereby permitting homeostasis of the cytoplasmic environment (Brown et al., 1997; Kadner, 1996). Cytoplasmic membranes are not simply impermeable structural entities, but are both specific and selective, and actively modified in response to changes in the external environment. Growth conditions such as the composition of growth medium (Annous et al., 1997; Annous and Kozempel, 1998; Julak and Mara, 1973; Kadner, 1996; Lechevalier and Moss, 1977; Schweizer, 1989), the growth phase (age) of the cells (Kadner, 1996; Lechevalier and Moss, 1977; McGarrity and Armstrong, 1981; Rees et al., 1995; Yatvin et al., 1986), the incubation temperature at which the bacteria were cultured (Annous et al., 1997; Dennis and Yatvin, 1981; Hansen, 1971; Kadner, 1996; Lechevalier and Moss, 1977; McGarrity and Armstrong, 1981; Russell, 1984; Russell et al., 1995; Schweizer, 1989; Sinensky, 1974; Suutari and Laakso, 1994), and the pH (Brown et al., 1997; Lechevalier and Moss, 1977; Russell et al., 1995; Schweizer, 1989) markedly affect the composition of the membrane lipid. These changes in the composition of the membrane lipid are mainly important in maintaining

both membrane integrity and functionality in the face of external perturbation, since it is the membrane that first comes in contact with the stress (Russell et al., 1995; Suutari and Laakso, 1994). The major way in which bacteria maintain this ideal membrane fluidity is by changing their fatty acid composition (Annous et al., 1997; Russell, 1984; Suutari and Laakso, 1994). Thermal inactivation of bacteria was shown to be dependent on environmental parameters such as the growth medium (Annous and Kozempel, 1998; Casadei et al., 1998; Hansen and Skadhauge, 1971; Hansen and Riemann, 1963; Smith et al., 1986), the growth temperature (Dennis and Yatvin, 1981; Hansen, 1971; Hansen and Skadhauge, 1971; Hansen and Riemann, 1963; Rees et al., 1995; Rowan and Anderson, 1998), the growth phase (Hansen and Riemann, 1963; Rees et al., 1995), and the pH (Annous and Kozempel, 1998; Hansen and Riemann, 1963). These changes in thermal resistance of bacteria due to changes in growth conditions were positively correlated with alterations in the membrane fluidity (Dennis and Yatvin, 1981; Hansen, 1971; Hansen and Skadhauge, 1971; Yatvin et al., 1986; Yatvin and Schmitz, 1980). These researchers reported that an increase in the fluidity of the bacterial membrane due to the changes in the growth conditions corresponded to a decrease in thermal resistance. *Escherichia coli* subjected to an abrupt temperature shift from 30° to 45°C and held at the high temperature for various periods of time revealed a gradual decrease in the total unsaturated to total saturated fatty acid ratio in the cytoplasmic membrane, resulting in reduced membrane fluidity (Mejía et al., 1999). Mejía et al. (1999) observed that the main changes corresponded with a decrease in the long-chain unsaturated *cis*-vaccenate (18:1*c*11) and an increase in the saturated palmitate (16:0) proportions as the heat-shock time increases. They also found that the fluidity reduction occurred during the cellular

heat-shock response, as detected by the change in the induction levels of GroEL and DnaK (Lee-Rivera and Gomez-Eichelmann, 1994; Mejía et al., 1995). In *Pediococcus* sp. a decrease in the unsaturated to saturated/cyclic fatty acid ratio during the transformation from log to stationary phase of growth was accompanied by an increase in the D-values, suggesting a relationship between thermotolerance and reduced membrane fluidity (Annous et al., 1999).

Thus, it is important to note that microorganisms develop a complicated, tightly regulated response upon an upshift in temperature. Different stressors can activate this stress regulon by which they can increase heat-tolerance. The process of adaptation and initiation of defense against elevated temperatures is clearly an important target when considering food preservation and the use of hurdle technology.

2.3.2 Cold stress

The extended use of frozen and chilled foods and the increased popularity of fresh or minimally processed food, often preservative-free, greatly increased the interest in cold adaptation of microorganisms and in particular food-borne pathogens (Berry and Foefeding, 1997; Graumann and Marahiel, 1998; Yamanaka et al., 1998). Due to longer time intervals between production and consumption of food products and the extended use of refrigerators, the importance of food-borne psychrotrophic pathogens, such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Bacillus cereus* and *Clostridium botulinum*, increased. Microorganisms that are a major concern to food poisoning show a wide variety in minimum growth temperatures (Gould, 1998).

Mechanisms that permit low-temperature growth involve membrane modifications maintaining membrane fluidity (involving nutrient uptake; Russell, 1990)

and the maintenance of structural integrity of macromolecules and macromolecule assemblies such as proteins and ribosomes (Berry and Foefeding, 1997; Jaenicke, 1991). Microorganisms have developed a number of strategies to maintain their membrane lipids fluid and functional at low growth temperatures. In general, as the growth temperature is decreased, an increase in the proportion of shorter and/or unsaturated fatty acids in the lipids is observed. One of the most important consequences of membrane lipid changes in microorganisms is to modulate the activity of intrinsic proteins that perform functions such as ion pumping and nutrient uptake (Russell and Fukanaga, 1990). The membrane adaptation response has been most extensively studied for *E. coli* and it has been shown that the proportion of *cis*-vaccenic acid (C18:1) increases at low temperature at the expense of palmitic acid (C16:0). The increase in average chain length has the opposite effect on membrane fluidity, but is outweighed by the greater fluidity effect of increased unsaturation. The increase in C18:1 is catalyzed by the constitutive enzyme β-keto-acyl-acyl- carrier protein synthase I, which is active only at low temperatures (De Mendoza and Cronan, 1983; De Mendoza et al., 1983; Garwin and Cronan, 1980). Compatible solutes such as betaine, proline and carnitine may also play a role in osmoprotection and in cold adaptation. For *L. monocytogenes*, growth at 7°C was stimulated in the presence of betaine and cells transported betaine 15-fold faster at 7°C that at 30°C (Ko et al., 1994). For different compatible solutes, such as betaine, ectoine and mannitol, a protective effect during freeze drying has been reported. The mechanisms behind this effect remain to be elucidated but increased levels of compatible solutes have positive effects on cell survival and activity of enzymes (Louis et al., 1994).

One of the best studied reactions to low temperature is the synthesis of cold-shock proteins, which are reported to be involved in protein synthesis and mRNA folding (Graumann and Marahiel, 1998). It has been observed that many bacteria synthesize increased amounts of small (7 kDa) proteins upon a sudden decrease in temperature, the so-called cold-shock proteins (CSPs). These proteins share a high degree of similarity (>45 %) in a variety of Gram-positive and Gram-negative bacteria, including food-related microorganisms like *E. coli* (Goldstein et al., 1990), *B. subtilis* (Willimsky et al., 1992), *B. cereus* (Mayr et al., 1996), *S. enteritidis* (Jeffereys et al., 1998), *S. typhimurium* (Craig et al., 1998), *Lactobacillus lactis* (Wouters et al., 1998) and *L. plantarum* (Mayo et al., 1997). However, CSPs are not observed in all bacteria, for example, *Helicobacter pylori* (Tomb et al., 1997) and *Campylobacter jejuni* (Hazeleger et al., 1998). The most extensively studied CSPs are CspA of *E. coli* ($CspA^E$) and CspB of *B. subtilis* ($CspB^B$). Both $CspA^E$ and $CspB^B$ proteins have been characterized as single strand DNA binding proteins (Graumann and Marahiel, 1994; Jones et al., 1992). $CspA^E$ acts as a transcriptional activator of at least two other genes encoding cold-induced proteins, GyrA (Jones et al., 1992) and H-NS (LaTeana et al., 1991), both involved in DNA supercoiling. Both $CspA^E$ and $CspB^B$ are RNA binding proteins because they possess a highly conserved RNA-binding motif (Jones and Inouye, 1994; Schindelin et al., 1993) and act as RNA chaperones (Jiang et al., 1997). The functioning of the ribosomes seems to play a central role in the cold adaptation process. A downshift in temperature causes a cold-sensitive block in initiation of translation, resulting in a decrease in polysomes and an increase in 70S monosomes and ribosomal subunits. The mRNAs of cold-shock genes are highly unstable at 37°C and stabilize

after cold-shock resulting in the induction of CSPs, which lead to restoration of the ribosomal structure to form an intact translation initiation complex (Golderberg et al., 1997; Jones and Inouye, 1996; Mitta et al., 1997).

The significance of psychrotrophic organisms is well understood, however, the significance of the cold-shock response in these organisms is still uncertain. Different cold-shock treatments prior to freezing result in clear differences in microbial survival after freezing (Goldstein et al., 1990; Willimsky et al., 1992). This might result in a high survival rate of bacteria in frozen food products. Furthermore, low temperature-adapted bacteria show shorter lag times at cold temperature and their higher growth rate at low temperature may be relevant to food quality and safety.

2.3.3 Osmotic stress

Among the many ways used to preserve food products, increased osmotic pressure, i.e. lowering of water activity (a_w), is one of most widely used. Desiccation or addition of high amounts of osmotically active compounds such as salts or sugars lowers the water activity of the food. Therefore, understanding the processes underlying osmotic adaptation of pathogenic microorganisms is of crucial importance in trying to design new ways for controlling growth of food spoilage and pathogenic bacteria in low and medium water activity foods. The internal osmotic pressure in bacterial cells is higher than that of the surrounding medium. This results in a pressure exerted outwards on the cell wall, called the turgor pressure, which is thought to provide the mechanical force necessary for the cell to maintain its shape (Csonka, 1989). The universal response to the temporary loss of turgor following a hyperosmotic shock is the cytoplasmic accumulation of a certain class of solutes that do not interfere too seriously

with the functioning of cytoplasmic enzymes, the so-called "compatible solutes" (Booth et al., 1994; Csonka, 1989; Csonka and Epstein, 1996) which include K+, glycine-betaine, glutamate, or trehalose. These solutes counterbalance the increase in external pressure.

Glycine-betaine and proline serve as osmoprotectants when added extracellularly, but glutamate and trehalose must be synthesized internally (Christman et al., 1985). Osmotically stressed *S. typhimurium* may synthesize glutamate by a route other than by glutamate synthase or glutamate dehydrogenase, the normal routes of glutamate synthesis (Botsford et al., 1994). The uptake of betaine-glycine and proline can be subdivided into two groups: (i) the binding protein-dependant, ATP-driven system (Ames et al., 1990) that includes ProU of *E. coli* and *S. typhimurium*, and OpuA and OpuD of *B. subtilis*; (ii) the ion-motive force-driven transporters exemplified by ProP of *E. coli*, OpuD and OpuE of *B. subtilis*, BetL of *L. monocytogenes*, and the *S. aureus* betaine and proline transporters. Beumer and coworkers (Beumer et al., 1994) showed that exogenously supplied carnitine can contribute significantly to growth of *L. monocytogenes* at high osmolarity. L-Carnitine occurs ubiquitously in biological material because of its crucial function in the oxidation of fatty acids in mitochondria (Bieber, 1988). An ATP-dependent high-affinity L-carnitine transport system in *L. monocytogenes* enables the bacterium to scavenge L-carnitine when it is available at trace levels in foods (Verheul et al., 1995). Carnitine has also been reported to function as an osmoprotectant in *E. coli*, including the enterohaemorrhagic *E. coli* O157:H7, and it is taken up via the ProP and ProU transporters both under aerobic and anaerobic conditions (Verheul et al., 1998).

Changes in the expression of outer membrane porin proteins in response to osmotic stress have also been reported. The genes encoding the major outer membrane porins OmpC and OmpF are inversely regulated in response to changes in osmolarity (Nikaido and Vaara, 1987). OmpC levels increase and OmpF levels decrease when cells are grown in high osmolarity. Regulation of this system is mediated in part by a two-component regulatory system involving a membrane sensor, EnvZ, and a signal-transducing regulatory protein, OmpR (Csonka and Hanson, 1991). EnvZ senses some consequence of high osmolarity and phosphorylates OmpR. Osmotic control of *ompCF* genes appears to be based on the concentration of OmpR-P and the presence of multiple binding sites for this protein.

Apparently, food-borne pathogens can accumulate a wide spectrum of compatible solutes, most of which are present in significant amounts in foods, thereby allowing growth at reduced water activities. Knowledge about the osmoregulation, including information about the regulation of the synthesis and activity of the various transport systems for compatible solutes, and their role in cryoprotection, may provide clues to impose osmotic stress and low temperature optimally as hurdles in the preservation of foods.

2.3.4 pH stress

2.3.4.1 Acid stress response

Sudden, as well as gradual, exposures to acid stress occur in a variety of ecological niches occupied by food-borne pathogens. The acidification of foods is an age-old means of food preservation, and is still used as a method to control the growth of food-borne pathogens and spoilage bacteria. Acid mine drainage, acid rain, and weak

acids produced by microorganisms themselves all contribute to acid stress. The ability to survive and flourish during these encounters is crucial to perpetuation of the species. Some microorganisms (acidophiles) have evolved to the point that an extreme acid environment is a preferred ecological niche. *Salmonella typhimurium*, *E. coli* O157:H7, *Shigella flexneri* and *L. monocytogenes* are neutrophiles; they grow best at neutral pH. These microorganisms may be exposed to dramatic pH fluctuations in nature, for example in foods, and during pathogenesis. However, these organisms nevertheless must cope with frequent although often transient, encounters with potentially lethal levels of acid. *Salmonella* appears to have developed several systems for surviving exposures to low pH. These include inducible systems for acid growth as well as log-phase and stationary phase acid tolerance responses (ATRs). Huttanen (1975) reported a minimal growth pH of 4.3 for *S. typhimurium* in minimal growth medium. This value compares to pH 4.6 for *E. coli*. Huttanen (1975) found that *S. typhimurium* could adapt to grow at lower pH on solid media. He found that cells struck across a pH gradient grew up to a specific distance towards the most acid part of the medium. Cells taken from the acidic-growth edge were more acid resistant and could grow farther into the acidic end of a fresh gradient plate. Huttanen also found that this induced acid resistance was not a result of selecting more acid resistant mutants because these cells lost their acid resistance after passage on a neutral pH medium. In addition to its ability to grow at pH 4.3, *S. typhimurium* can survive severe acid conditions (down to pH 3.0) if it is allowed to induce one of two ATRs (Foster and Hall, 1990; Lee et al., 1994a). One system involves log-phase cells, the other can be demonstrated in stationary phase cells. Interruption of log-phase growth at neutral pH by a shift to pH 4.0-4.5 (called acid shock) induces 50 acid shock proteins (ASPs), of which ~20 are induced only by acid,

which leads to the development of profound acid tolerance to low pH (Foster, 1991, 1993). In contrast, stationary-phase cells shifted to pH 4.3 only induce 15 ASPs, four of which are also log-phase ASPs that can still provide resistance to extreme acid. Both these ATR systems require protein synthesis during the acid shock adaptive period but not during acid challenge (Foster, 1993; Lee et al., 1994a). Transition to acid pH environments also leads to dramatic changes in outer membrane porin synthesis. During acid adaptation at pH 5.8, OmpF synthesis decreases while OmpC expression increases (Foster and Hall, 1990; Foster et al., 1994). Even more dramatic is the complete repression of OmpC and OmpF during acid shock (Foster, 1991). Acid shocked cells also develop significant cross protection to heat, oxidative stress, and osmotic stress. Conversely, neither heat shock nor osmotic shock induces acid tolerance (Leyer and Johnson, 1993). Cross protection of acid-shocked and acid-adapted *S. typhimurium* and *E. coli* O157:H7 against thermal and osmotic stresses has been reported (Garren et al., 1998; Leyer and Johnson, 1993; Ryu and Beuchat, 1998). Shifts in pH have been shown to induce heat-shock proteins. As many as 16 heat-shock proteins were reported to be induced in *E. coli* shifted to lower pH (Hyde and Portalier, 1990). Farber and Pagotto (1992) showed that exposure of *L. monocytogenes* to low pH (4.0) resulted in significantly enhanced heat resistance of the acid-shocked cells as compared to control cells. Similar induction of thermotolerance was reported by Foster and Hall (1990) in *S. typhimurium*.

Acid-adapted cells are those that have been exposed to a gradual decrease in environmental pH, whereas acid-shocked cells are those which have been exposed to an abrupt shift to an acid pH. These processes may result in cells exhibiting different physiological states and thus, potentially different tolerances to secondary stresses

imposed by food environments (Ryu and Beuchat, 1998). Acid-adapted, but not acid-shocked *E. coli* O157:H7 cells in acidified tryptic soy broth (TSB) and low pH fruit juices were shown to have enhanced heat tolerance in TSB at 52°C and 54°C, and in apple cider and in orange juice at 52°C (Ryu and Beuchat, 1998). Acid-adapted *S. typhimurium* were also found to have increased tolerance to osmotic stress, an activated lactoperoxidase system, and the surface-active agents crystal violet and polymyxin B in addition to heat (Leyer and Johnson, 1993). Acid-adaptation in *Salmonella* involves induction and repression of a number of cellular proteins which include a few HSPs while no apparent induction of HSPs was observed in *E. coli* (Foster, 1995; Foster and Hall, 1990, 1991; Hickey and Hirshfield, 1990; O'Hara and Glenn, 1994; Raja et al., 1991; Rowbury and Goodson, 1993). In addition to the changes in expression of a number of cellular proteins, changes in the cell surface properties such as increase in cell surface hydrophobicity and induction of specific outer membrane proteins like OmpC and OmpF were also observed in *Salmonella* and *E. coli* during acid-adaptation (Leyer and Johnson, 1993; Sato et al., 2000). The fatty acid composition of an acid-adapted log-phase cell of *E. coli* is very different from that of non-adapted cells. During acid-adaptation, monounsaturated fatty acids (16:1ω7c and 18:1ω7c) present in the phospholipids of *E. coli* are either converted to their cyclopropane derivatives (cy17:0 and cy19:0), or replaced by saturated fatty acids. The acid tolerance of *E. coli* strains appeared to be correlated with membrane cyclopropane fatty acid content and, thus, it is postulated that an increased level of cyclopropane fatty acids may enhance the survival of microbial cells exposed to acid pH (Brown et al., 1997). Acid-adaptation can be induced very rapidly, within the period of a cell doubling and thus the ability of a

pathogen to retain an enhanced degree of acid tolerance following exposure to mild acidity may be an important factor which permits food-borne pathogens to survive gastric acidity, and subsequently precipitate an illness. However, this adaptation phenomenon is at least partially reversed by neutralization of the growth medium (Billie and Marquis, 1991; Brown et al., 1997; O'Hara and Glenn, 1994), which suggests that the ability of the pathogen to both adapt rapidly and to reverse this phenomenon will undoubtedly influence its survival.

Thus, acid-induced stress resistance may reduce the efficiency of hurdle technologies which rely upon the imposition of multiple stresses, i.e. "combination preservation" techniques (Gould, 1998).

2.3.4.2 Alkaline stress response

Enterobacteria like *E. coli* and *Salmonella* species are commonly exposed to alkaline pH. Environmental alkalinization can follow exposure to polluted waters, extreme alkalinity resulting from sewage, chemical industry and agricultural effluents (Rowbury et al., 1989). Enterobacteria can also be subjected to alkalinity in some foods (e.g., egg whites; Humphrey et al., 1991) and some food processing treatments (Humphrey et al., 1981). Mild alkaline pH values can also affect organisms in the phagolysosomes (Segal et al., 1981) and intestine. Studies have shown that mild alkalinity induces alkali tolerance, after which tolerant (alkali-habituated) organisms survive pH values lethal to non-habituated ones (Goodson and Rowbury, 1990; Rowbury et al., 1989). Adaptation of *Salmonella* or *E. coli* to alkaline conditions is accompanied by induced thermotolerance, increased resistance to bile salts, and increased resistance to external alkalinization (Flahaut et al., 1997; Goodson and

Rowbury, 1990; Humphrey et al., 1991; Taglicht et al., 1987). On the contrary, exposure of *S.* Enteritidis or *E. coli* to alkaline adaptation conditions sensitize them to acid stress (Rowbury et al., 1993) and vice versa (Rowbury and Hussain, 1996). Cross-protection against heat as a result of alkaline stress has been documented for both Gram-positive and Gram-negative bacteria. Heat resistance (55°C) of *S.* Enteritidis PT 4 in Lemco broth at pH 7.0 ± 0.2 was significantly increased by previous exposure to pH 9.2 ± 0.2 for 5 min or longer (Humphrey et al., 1991). Similarly, tolerance to heating at 62°C was induced by treating *E. faecalis* cells for 30 min at pH 10.5 (Flahaut et al., 1997). Increased resistance of *Listeria monocytogenes* to heating at 56°C has been demonstrated following exposure of cells to starvation conditions, ethanol, acid and H_2O_2 (Lou and Yousef, 1997). Rowbury and coworkers (1996) examined the regulatory aspects of alkali tolerance induction in *E. coli*. *Escherichia coli* shifted from external pH 7.0 to pH 8.5-9.5 rapidly became tolerant to pH 10.0-11.5. They reported that this induction in alkali tolerance needs protein synthesis which makes organisms resistant to DNA damage by alkali and better able to repair any damage that occurs. The ability of bacteria to grow at or withstand alkaline pH could also require the acquisition of enzymes capable of remaining active at high pH values (Kakinuma and Igarashi, 1990) and could be accomplished by modifications in gene expression. Studies have shown the induction of heat shock proteins (HSP) by a mild pH upshift (from pH 7.0 to 8.8) (Taglicht et al., 1987), induction of the SOS system by alkalinization of intracellular pH (Schuldiner et al., 1986), and RecA-independent DNA repair in *E. coli* (Goodson and Rowbury, 1990). In other specific studies, an unknown alkaline shock protein (23 kDa) in *Staphylococcus aureus* (Kuroda et al., 1995) and some genes whose expression

changes as a function of alkaline pH (Bingham et al., 1990; Foster and Aliabadi, 1989; Rowbury et al., 1996) have been described.

2.4 Stress-adaptation and enhanced virulence

Whilst the induction of a stress-resistant phenotype in sublethally stressed bacterial enteropathogens has profound implications for their survival in foods, there is strong evidence that sublethal environmental stressors can also modulate the synthesis of virulence factors in these microorganisms. Successful pathogens have evolved highly sophisticated signal transduction systems which, in response to environmental signals, such as inimical stresses, control the coordinated expression of various virulence determinants (Archer, 1996; Lee et al., 1995; Mekalanos, 1992). The goals of the pathogen are simple: survive and multiply. As stated by Mekalanos (1992), "disease is simply a manifestation of the complex interactions required to accomplish these goals (survive and multiply) within the milieu of host tissue". Hopkins (1994) pointed out that, "although life on a Petriplate with a few simple sugars and a handful of amino acids might not seem like much, to a bacterium it's a night in Trump Tower with breakfast in bed". In contrast, to the Petriplate with minimal nutrients, we consider human food as a friendly environment for bacteria, but more often it is probably an unfriendly environment, containing complex substrates for growth, competition in the form of bacteria, lack of moisture perhaps, and the presence of acids and other byproducts that may have been produced by competitors. Many of the stresses encountered in food, such as lack of iron, heat and cold stress, oxidative stresses, osmolarity extremes, and starvation, are the same stresses imposed on a bacterium by host defenses. For instance, environmental cues that are currently recognized as having a modulating influence on

the expression of coordinately regulated virulence determinants in bacteria include CO_2 (*Bacillus anthracis* and *Vibrio cholerae*), temperature (*Bordetealla pertussis*, *E. coli*, *S. typhimurium*, *L. monocytogenes*, *V. cholerae*, *Shigella* and *Yersinia* species), iron (*Corynebacterium diphtheriae*, *E. coli*, *L. monocytogenes*, *Pseudomonas aeruginosa*, *S. typhimurium* and *V. cholerae*), pH (*S. typhimurium*, *E. coli*, *L. monocytogenes*, *V. cholerae* and *Agrobacterium tumefaciens*), starvation (*L. monocytogenes*, *E. coli* and *S. typhimurium*), carbon source (*L. monocytogenes* and *E. coli*), osmolarity (*P. aeruginosa* and *L. monocytogenes*), growth phase (*S. typhimurium* and *Staphylococcus aureus*), Ca^{2+} (*Yersinia* species), oxidative stress (*S. typhimurium*), phenolic compounds, monosaccharides, and phosphates (*A. tumefaciens*), SO_4 and nicotinic acid (*B. pertussis*) and amino acids (*V. cholerae*) (Foster and Spector, 1995; Hill et al., 1995; Mekalanos, 1992; Rowan and Anderson, 1998). The ability of bacterial pathogens like *L. monocytogenes*, *E. coli* and *S. typhimurium* to adapt to low-pH environments is of particular interest because the organisms encounter such environments in foods and during passage through the stomach (~pH 3.5) and during their transient residence in the macrophage phagosome (~pH 4.4-5.7) (Foster and Spector, 1995; Hill et al., 1995). The isolation of acid-tolerant *L. monocytogenes* and *S. typhimurium* suggests that these low-pH environments have the potential to select for strains with increased acid resistance. However, acid-tolerant variants of *L. monocytogenes* were reported to have increased lethality for mice relative to their non-stressed wild type counterparts when inoculated by the intraperitoneal route (Marron et al., 1997). This increased capacity of acid-tolerant mutants of *L. monocytogenes* to grow under conditions of low pH may account for their increased virulence in the mouse model. The induction of virulence determinants in *L. monocytogenes* is also modulated by a variety of other environmental

factors. Almost all the known virulence genes in *L. monocytogenes* are coordinately regulated by the pleiotropic transcriptional activator PrfA (Bubert et al., 1999; Chakraborty et al., 1992). PrfA switches between transcriptionally inactive and active forms depending upon interaction with a protein factor termed Paf (Prf activating factor/s) that is responsible for transducing signals from the environment to the PrfA system. Increased expression of the *prfA* gene occurs in response to osmotic stress and heat shock, and during the stationary phase of growth (Freitag and Jacobs, 1999). Other environmental stimuli that influence the expression of PrfA include the preservatives nitrite and sorbate, carbon source (e.g. down-regulation in the presence of glucose), low levels of iron, charcoal and starvation (Park et al., 1992; Sokolovic et al., 1993).

The survival of *S. typhimurium* within macrophages is essential for its virulence (Fields et al., 1986; Riikonen et al., 1992). Many genes associated with survival within macrophages have been identified (Baumler et al., 1994; Belden and Miller, 1994; Fang et al., 1992; Fields et al., 1986). These include genes such as *htrA*, *purD*, *phoPQ*, *nagA*, and *fliD*. The best characterized system to date is the *phoPQ* system. One reason why phoPQ mutants survive macrophage invasion poorly is a diminished resistance to defensins. Several genes regulated by PhoPQ have been identified using *lacZ* or *phoA* fusion studies (Behlau and Miller, 1993; Belden and Miller, 1994; Miller et al., 1989; Miller et al., 1992). PhoP activated genes (*pag*) and PhoP repressed genes (*prg*) include *pagC*, *pagD*, *pagK*, *pagM*, and *prgH*, all of which are required for virulence in mice (Behlau and Miller, 1993; Miller et al., 1989; Miller et al., 1992). Other genes regulated by PhoPQ but not implicated in macrophage survival include *pagA* and *pagB*, which along with *pagC* are induced by intramacrophage acid pH (Alpuche-Aranda et al., 1992).

Expression of the general stress response RpoS sigma factors is necessary for both environmental-stress adaptation and virulence in enteric bacteria e.g. *E. coli* and *S. typhimurium* (Foster and Spector, 1995). In *E. coli* O157:H7 there appears to be a link with normal expression of the verocytotoxin gene and the ability of this pathogen to tolerate inimical stresses. Under certain osmotic stress and heat-shock conditions, the attenuated strain of *E. coli* O157:H7 (which does not produce verocytotoxin) is less resistant and adapts poorly to these stresses compared to wild-type *E. coli* O157:H7 strains that synthesize this toxin (Daboob, 1999). This indicates the dual-function of this particular virulence determinant in both environmental stress-adaptation and pathogenecity. Though the culture characteristics of the *E. coli* O157:H7 verocytotoxin-negative strain are similar, food safety studies using these attenuated strains as a measurement of the survival capabilities of normal wild-type *E. coli* O157:H7 are likely to be underestimating the true resistance of this important pathogen, and are likely to be flawed.

2.5 Trisodium phosphate

Trisodium phosphate (TSP) is an orthophosphate salt of phosphoric acid. It has a minimum of 41.5% P_2O_5 and a pH of approximately 11.8 in a 1% solution (Brotsky and Bender, 1991). Trisodium phosphate is also available as the dodecahydrate of the formula:

$Na_3PO_4.12H_2O$.

The dodecahydrate is also available as a technical grade with a formula of:

$5(Na_3PO_4.12H_2O)NaOH$

or as a food grade chemical with a formula of:

$4(Na_3PO_4.12H_2O)NaOH$.

Uses of TSP include: food additives, dietary supplements, paint removers, and detergent or industrial cleaners. The beneficial effects of phosphates on food products include: (i) water binding, (ii) retardation of oxidative rancidity, (iii) emulsification, and (iv) color development and stabilization (Wagner, 1986). The ability of phosphates to enhance microbial safety and stability of certain food has been discussed by Wagner (Wagner, 1986).

2.5.1 Av-Gard ™ process

The Av-Gard™ process is an USDA-approved procedure where TSP solution is applied to reduce the incidence of *Salmonella* contamination during poultry processing (Giesse, 1992). The Av-Gard™ process was the first commercial process for which a claim of successfully reducing Salmonellae (2 to 5 log decrease) on processed carcasses could be made (Brotsky and Bender, 1991). The process has been patented for poultry (Brotsky and Bender, 1991), specifically covering solutions of trialkali metal phosphate in concentration of 4% (wt./vol.) or greater with pH values of 11.5. TSP treatment has been reported to successfully reduce numbers of *E. coli*, *Campylobacter jejuni* and *L. monocytogenes* (Dickson et al., 1994; Kim and Slavik, 1994b; Slavik et al., 1994; Somers et al., 1994). The treatment with TSP is as effective as alkali treatment without the adverse effects on the meat or skin associated with alkali chemical reaction (Dickson et al., 1994). The process also was found to have little or no effect on the taste, texture, and appearance of poultry (Hollender et al., 1993). The phosphate residue can therefore be left on the poultry surfaces to provide reduced bacterial activity and improved shelf life (Brotsky and Bender, 1991). Trisodium phosphate is generally recognized as safe

by the Food and Drug Administration and has been approved by the United States Department of Agriculture for use as a food ingredient (Federal, 1982) and for the reduction of *Salmonella* contamination during poultry processing (Federal, 1994). The process involves immersing post-chill whole birds for 15 sec in a 10 % solution of Av-Gard™ TSP, allowing the excess TSP solution to drip from the bird, presumably leaving minimal amounts of phosphate on the carcass (Rhône-Poulenc, 1992). At 8 to 15 % w/v, TSP has been demonstrated to reduce the number of artificially inoculated Gram-negative pathogens surviving on surfaces of foodstuffs (Dickson et al., 1994; Dorsa et al., 1997; Hwang and Beuchat, 1995; Salvant et al., 1997; Taormina and Beuchat, 1999; Xiong et al., 1998b; Zhuang and Beuchat, 1996). Log reductions in viable counts of 1.4 and 0.9 were obtained for *Escherichia coli* O157:H7 and *S. typhimurium*, respectively, on beef adipose tissue, though significantly lower reductions (0.9 and 0.5, respectively) were observed on fascia (Kim and Slavik, 1994b). On chicken carcasses, Slavik et al. (1994), Kim and coworkers (1994) showed that TSP treatment at 10° or 50ºC reduced *Salmonella* counts by 1.6 to 1.8 log cycles per carcass;, and in a scanning electron microscopic study, Kim and Slavik (1994a) showed TSP effectively removed attached *Salmonella* from chicken skin. Trisodium phosphate in solution results in a very alkaline pH and it is thought that this alkaline pH is could be responsible for its antimicrobial activity. Several studies have examined the effectiveness of high pH treatments on the destruction of gram-negative food-borne pathogens. Kinner and Moats (1981) showed that when *S. typhimurium* broth cultures were exposed to pH 11.0 at 50°C, viable cells were eliminated in less than 1 min. Humphrey et al. (1981) found that *S. typhimurium* on chicken skin could be killed more rapidly in water at pH > 9.0

than at neutral pH values. Sodium hydroxide and KOH treatments (10 %) have been found to be effective in lowering *S. typhimurium* by as much as 4 log units on lean and fat beef tissure (Dickson, 1988). Catalano and Knabel (1994b) demonstrated that *Salmonella* spp. were rapidly destroyed in egg washwater maintained at a pH of 11.0 or higher, with D-values determined to be less than 0.14 min when the temperature was increased to 43.3°C. Another study by Catalano and Knabel (1994a) determined that no cross-contamination or penetration of *S. enteritidis* occurred when eggs were washed in pH 11.0 washwater at 37.7°C. Possible modes of action of high pH could involve solubilizing the membrane proteins (Duncan et al., 1972; Labbe et al., 1978) and also saponification of membrane lipids (Yatvin, 1977), leading to the weaking and disruption of the membranes of food-borne pathogens. Mendonca et al. (1994) had shown that destruction of gram-negative food-borne pathogens like *E. coli* O157:H7, *S. enteritidis* and *L. monocytogenes* by high pH involved disruption of the cytoplasmic membrane. Trisodium phosphate dip solutions (8 to 12 %) have been used to reduce *Salmonella* spp., *E. coli* O157:H7 and *L. monocytogenes* on the surface of beef tissue (Dickson et al., 1994; Kim et al., 1993; Kim and Slavik, 1994b). In light of these findings, modes of action of TSP likely include: i) disruption of the cell's membranes by exposure to high pH, ii) detachment of bacteria from food surfaces by sequestration of metal ions (Lee et al., 1994b) and iii) removal of fat from the skin surface, thereby allowing bacteria to be washed from the food surfaces more effectively (Bender and Brotsky, 1992; Giesse, 1992; Kim and Slavik, 1994a). To date, work has mainly focused on evaluating the efficiency of *Salmonella* removal by the process (Coppen et al., 1998; Delmore et al., 2000; Xiong et al., 1998a, b; Yang et al., 1998), and no published research exists that attempts to elucidate the actual mechanism of TSP antimicrobial action.

2.6 Proteomics

Proteomics is the study of the proteome, the protein complement of the genome. It studies protein characteristics and functions to obtain a global, integrated view of normal and abnormal cellular processes, protein-protein interactions, and regulatory networks at the protein level (Blackstock and Weir, 1999). The terms "proteomics" and "proteome" were coined by Marc R. Wilkins, vice president and head of bioinformatics at Proteome Systems in Sydney, Australia, in 1994 and mirror the terms "genomics" and "genome", which describe the entire collection of genes in an organism. Proteomics collectively analyzes the proteins that are regulated, expressed, or modified in the cell under different conditions (Liebler, 2002). The exact definition of proteomics varies depending on whom you ask, but most scientists agree that it can be broken down into three main activities: identifying all the proteins made in a given cell, tissue or organism; determining how those proteins join force to form networks; and outlining the precise three-dimensional structures of the proteins in an effort to find drugs that might turn their activities off or on. Thus, proteomics can be divided into two main subcategories: expression proteomics, the study to identify specific targets and markers, and functional proteomics, the study to define structure, interaction, and function. Expression proteomics begins with comparison of gels, which is an excellent way to observe up/down regulation, on/off expression, and modification of proteins. While expression proteomics can be viewed as the method of discovery- it allows identification of a protein of interest- functional proteomics allows more detailed analysis of this protein's structure, role, cellular location, and interaction with other proteins.

Proteins have diverse structural, enzymatic, and regulatory roles in cells, and methods that offer opportunities to study such a broad range of processes are in demand.

When scientists want to find out which proteins are present in selected cells or tissues, they usually rely on two techniques: two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry. Two-dimensional-PAGE is a very powerful and widely used technique to provide a visual map for comparison of protein expression at given times and under known conditions. With 2D-PAGE, scientists add a mixture of proteins to the edge of a thin gel that separates proteins in one direction according to their electrochemical charge and in a perpendicular direction according to their size. Since any given protein has a characteristic size and charge, each one shows up as a discrete dot on the gel. Researchers can cut individual dots from the gels to identify the proteins they contain using other techniques like mass spectrometry. Mass spectrometry employs magnets or electrical fields to resolve distinct proteins according to the masses of their constituent atoms. The results are displayed as peaks on a graph.

2.6.1 Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional-PAGE has become the standard proteomic separation technique, because it has the ability to resolve complex mixtures of thousands of proteins in a single gel. Two-dimensional-PAGE sorts proteins according to two independent properties in two discrete steps: in the first-dimension step, isoelectric focusing (IEF) separates proteins according to their isoelectric point (pI); in the second-dimension step, sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins according to their molecular weights (MW). Two-dimensional-PAGE was first introduced by O'Farrell (1975) and Klose (1975). In the original technique, the first-dimension separation was performed in carrier ampholyte-containing polyacrylamide gels cast in narrow tubes. Under the influence of an electric

current, carrier ampholytes form a pH gradient, a critical component of IEF. Sample was applied to one end of each tube gel and separated at high voltage. After IEF the gel rods were removed from their tubes, equilibrated in SDS sample buffer, and placed on vertical SDS-polyacrylamide gels for the second-dimension separation. Although 2D-PAGE is the most effective means of resolving complex protein mixtures, it was not widely used for many years after it was first introduced in the early 1970s. This was due to: 1) the relative technical difficulty of performing the IEF step, and 2) getting the focused proteins into the SDS-PAGE gel. In the original technique, the IEF step relied on "tube gels", which were tricky to set up and run. Moreover, the pH gradients in the tube gels were difficult to reproduce. Finally, getting the delicate tube gel containing the focused proteins set up to efficiently transfer the proteins in the SDS-PAGE slab gel was a technical challenge. Thus, 2D-PAGE was technically challenging to perform and even more difficult to perform reproducibly. This situation has changed much for the better with the introduction of new, dedicated 2D-PAGE systems that use immobilized pH gradient (IPG) strips and relatively foolproof hardware to facilitate the transfer of proteins from the IPG strip into the SDS-PAGE slab gel. The IPG strip is based on the use of immobilized pH gradients, in which polycarboxylic acid ampholytes are immobilized on supports to reproducibly create stable pH gradients. One can now purchase IPG strips from major suppliers that afford reproducible protein separations over a variety of wide and narrow pH ranges. The strip is hydrated with a buffer and the protein is loaded onto the strip under voltage. Then the voltage is increased to achieve focusing. After the focusing step, the strip is treated with a buffer that contains a thiol reductant and SDS and then is joined to the SDS-PAGE slab gel. Here the IPG strip containing the focused proteins acts as a "stacking gel" in one-dimensional PAGE (1D-

PAGE). The proteins then are resolved on SDS-PAGE slab gel in the same manner as for 1D-PAGE. Proteins separated by 2D-PAGE are visualized by conventional staining techniques, including silver, Coomassie, and amido black stains. Although there are many different protocols for all of these staining techniques, not all of them are compatible with subsequent analysis of the proteins. For example, silver-staining with formalin fixation of the proteins tends to fix proteins in the gel, preventing both their digestion and the recovery of any peptides formed. Similar problems result from prolonged exposure of gels to acetic acid. Thus, it is important to use staining protocols that are compatible with subsequent digestion and elution steps.

2.6.2 Mass spectrometry for identification and characterization of proteins

Mass spectrometry (MS) is now regarded as an indispensable tool for peptide and protein primary structure analysis. Mass spectrometry instrumentation had undergone tremendous change over the past decade, culminating in the development of highly sensitive, robust instruments that can reliably analyze biomolecules, particularly proteins and peptides. Mass spectrometry instrumentation can offer three types of analyses, all of which are highly useful in proteomics. First, MS can provide accurate molecular mass measurements of proteins as large as 100 kDa or more. Thus, MS analysis, rather than migration of SDS-PAGE is preferable for the estimation of protein masses. Highly accurate protein mass measurements generally are of limited utility, however, because they often are not sufficiently sensitive and because net mass often is insufficient for unambiguous protein identification. Mass spectrometry also can provide accurate mass measurements of peptides from proteolytic digests. In contrast to whole protein mass measurements, peptide mass measurements can be done with higher

sensitivity and mass accuracy. The data from these peptide mass measurements can be searched directly against databases, frequently to obtain definitive identification of the target proteins. Finally, MS analyses can provide sequence analysis of peptides obtained from proteolytic digests. Mass spectrometry is now considered the state-of-the-art method for peptide-sequence analysis. Mass spectrometry sequence data provide the most powerful and unambiguous approach to protein identification.

Mass spectrometers have three essential parts. The first part is the source, which produces ions from the sample. The second part is the mass analyzer, which resolves the ions based on their mass/charge (*m/z*) ratio. The third part is the detector, which detects the ions resolved by the mass analyzer. In short, the mass spectrometer converts components of a mixture of ions and then analyzes them on the basis of their *m/z*. The data are automatically recorded by the data system and can then be retrieved for manual or computer-assisted interpretation. Modern MS instruments are controlled by sophisticated computers and software and the data the instruments generate are handled by similarly sophisticated computer data systems. For the purpose of proteomics, good data on peptide mass or good data that describe peptide fragmentation are needed. The quality of the data is determined by three things. The first is sensitivity. In much of proteomic work, the amounts of proteins are limited. Thus, we need instruments that are routinely capable of obtaining data on femtomole (10^{-15} mole) quantities of peptides or less. Second, we need resolution, which is the measure of how well we can distinguish ions of very similar *m/z* values. The MS instruments that deliver the highest resolution reliably distinguish between ions that differ in *m/z* by as little as 0.001 amu. However, these instruments are extremely expensive and are not routinely used for proteomics work. Instruments commonly used in MS need to be able to distinguish ions that differ

in *m/z* values of at least one Da (i.e. the mass of a single hydrogen atom). Finally, we need mass accuracy. This means that the measured values for peptide ions or their fragment ions must be as close as possible to their real values. This is particularly useful when we use the data to identify peptides based on comparisons with database values.

2.7 Peptide mass fingerprinting

Peptide mass fingerprinting is a protein identification technique which uses MS to measure the masses of proteolytic peptide fragments. The protein is then identified by matching the measured peptide masses to corresponding peptide masses from protein or nucleotide sequence databases.

Imagine for a moment that we could take the entire proteome of an organism and cleave it into a collection of tryptic peptides. Trypsin cleaves proteins very selectively by cutting at lysine and arginine residues (except those next to prolines) yielding a specific number of peptides of specific length, sequence, and most importantly, of specific mass. As long as each peptide in the collection is associated with its protein of origin and amino acid sequence position, all of the information in the proteome would be maintained. Of course, this does not have to be done experimentally. We can use a computer to generate this list of peptides by performing a virtual digestion of all the proteins in a database. We can also do this with nucleotide sequence information by converting it to protein sequence information and then digesting. This list of peptides is a very valuable reference tool. One can rank these peptides from lowest mass to highest. An inspection of this list would reveal that some of the peptides over about six amino acids in length (about 700 Da) would have unique masses.

Now, if we have an unknown protein from that organism and we wish to identify it, we would start by digesting the protein with trypsin to generate tryptic peptides. Each peptide we would get from this digestion has a mass. Let's assume that we have the exact mass of each of our tryptic peptide digestion products by MS. If we were to take one of the tryptic peptide masses and compare it to the entries on the list, we would find a peptide on the list with exactly the same mass. If the measured mass was unique in the list of all peptide masses, we would be certain that the peptide came from that protein. Because we know the sequence location and origin of the peptide match in the list, we can be fairly sure that our tryptic peptide came from that same protein. We could then take a second tryptic peptide from our unknown protein and match it to the list in the same way. Again, a match would indicate which peptide and parent protein correspond to our unknown. Several matches between our tryptic peptides and tryptic peptide masses all from the same protein in the list would confirm the identity of our unknown. Even if multiple entries in the peptide mass list matched one of our unknown tryptic peptides, a consistent set of "hits" on peptides all derived from the same protein in the list would confirm its identity. Thus, as long as we can match peptide masses to a good list, we can identify unknowns simply by measuring the masses of their tryptic digest.

Of course, identifying a protein would be this simple if we are blessed with perfect mass measurements of our unknown tryptic peptides and a perfect list of all possible tryptic peptides from the proteins in our target organism. Thus, successful protein detection by peptide mass fingerprinting requires two things. First, one must be able to make accurate measurements of peptide masses. Second, one must have accurate databases of protein sequences to work with. Thus, successful application of peptide mass fingerprinting depends on how close we can come to perfection in the real world.

However, several factors complicate peptide mass fingerprinting. First, MS data are not perfect. Although most modern MALDI-TOF (Matrix-Assisted Laser Desorption Ionization-Time Of Flight) instruments are capable of measuring the *m/z* values of peptide ions to within 0.005 amu or better, errors nevertheless are inevitable. Second, there are frequently a lot of signals in MALDI-TOF spectra of real samples and many are from more than one protein. Consider that most spots on 2D gels contain 2-3 proteins, that a typical 50 kDa protein may give rise to 25-40 tryptic peptides, and that other contaminants may be present in the sample (e.g., human keratin from careless sample handling). These factors combine to produce spectra that are complex and represent peptides from multiple proteins. Finally, there is always a possibility that some database matches are owing to chance alone, rather than actual identity. The possibility of false-positive matches is greater for larger proteins, mainly because they yield more tryptic peptides than do smaller proteins.

2.8 Applications of proteomics

Proteomics technology is indeed impressive, but what does characterizing the proteome amount to in practical terms? In current practice, proteomics encompasses four principal applications. These are: 1) data mining, 2) protein-expression profiling, 3) protein-network mapping, and 4) mapping of protein modifications.

2.8.1 Mining proteomes

Proteome mining is simply the exercise of identifying all (or as many as possible) of the proteins in a sample. The point of mining is to catalog the proteome directly, rather than infer the composition of the proteome from expression data from

genes (e.g., by DNA microarrays). Mining is the ultimate brute-force exercise in proteomics as one simply resolves proteins to the greatest extent possible and then uses MS and associated database and software tools to identify what is found. Two-dimensional-PAGE along with MS analysis has been used effectively to create the proteome map of a number of organisms like *E. coli* and *S. typhimurium* (VanBogelen et al., 1996), *Arabidopsis thaliana* (Chivasa et al., 2002), *Helicobacter pylori* (Bumann et al., 2002), and various other tissues (e.g., human cerebrospinal fluid, Sickmann et al., 2002; human pituitary, Beranova et al., 2002; mouse oocyte, Coonrod et al., 2002). The European pathogenic microorganism proteome database consists of proteomic data from *Mycobacterium tuberculosis*, *H. pylori*, *Borrelia garinii*, *Francisella tularensis*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, Jurkat T-cells and mouse mammary gland projects (http://www.mpiib-berlin.mpg.de/2D-PAGE/). These proteome maps serve as databases for further comparative proteomic analysis.

2.8.2 Protein-expression profiling

Protein-expression profiling is the identification of proteins in a particular sample as a function of a particular state of the organism or cell (e.g., differentiation, developmental state, or disease state) or as a function of exposure to a drug, chemical, or physical stimulus. Expression profiling is actually a specialized form of mining. It is most commonly practiced as a differential analysis, in which two states of a particular system are compared. For example, normal and diseased cells or tissues can be compared to determine which proteins are expressed differentially in one state compared to the other. This information has tremendous appeal as a means of detecting potential targets for drug therapy in diseases. The most widely used approach to protein-

expression profiling is to subject samples to 2D-PAGE and compare spot patterns. Two-dimensional-PAGE is particularly well-suited to comparative proteome analysis because it effectively resolves many proteins reproducibly. Developments in MS analysis now make it possible to identify essentially any protein one can detect by staining the 2D-PAGE gel.

Protein-expression profiling requires that we compare the 2D gels from two different samples for differences in the occurrence or intensity of protein spots. The problem with this is that it is very hard to run multiple 2D gels with exact reproducibility. There usually are slight variations in the location of spots for specific proteins. This makes it hard to compare spots on two gels if their locations are slightly different. A great deal of work has been done to develop software tools to analyze the patterns of protein spots on 2D gels. Among the most widely used programs for 2D gel-image analysis are Melanie™ (developed by the Swiss Institute for Bioinformatics), PDQuest (Bio-Rad Laboratories) and ImageMaster 2D Elite (Amersham Biosciences). These software tools accommodate slight differences in the location of the spots on the 2D gel by allowing users to identify "landmarks", which are proteins that occur in both (or all) of the gels to be compared. These spots are then "paired" by the software to create a series of pairs by which the gels can be aligned or "matched". In other words, the gel images are lined up pixel-wise so that all the spots match. This process can entail some transformations or spatial "warping" of images to compensate for local geometric distortion in the gel. Statistical comparison of the spots may be done once the gels are matched. This allows the user to identify spots that differ between two or more samples. The proteins in these spots of interest can then be identified by MS analysis. Examples include studies of characteristics and effects of disease (Barcelo et al., 2002;

Brunagel et al., 2002; Choi et al., 2002; Jones et al., 2002; Leman et al., 2002; Pang et al., 2002; Shibahara et al., 2002; Zabel et al., 2002; Zhou et al., 2002) responses to experimental treatments such as drugs (Dahmen et al., 2002; Michishita et al., 2002; Reardon and Kim, 2002; Thongboonkerd et al., 2002), nutrients (Maeder et al., 2002), or environmental conditions/stress (Duche et al., 2002; Periago et al., 2002; Wilkins et al., 2001, 2002), developmental processes such as cell and tissue differentiation, morphogenesis (Brejning and Jespersen, 2002; Finnie et al., 2002; Nishioka et al., 2002; Oosthuizen et al., 2002; Thiel and Bruchhaus, 2001), and aging (Bulteau et al., 2002). Expression profiling have been very useful in understanding the evolutionary relationship of closely related organisms (Enard et al., 2002; Fullaondo et al., 2001; Tokita et al., 2002) and understanding mechanisms of pathogenesis (Monahan et al., 2001; Sherman et al., 2001; Shimizu et al., 2002).

2.8.3 Protein-network mapping

Protein-network mapping is the proteomics approach to determining how proteins interact with each other in living cells. Most proteins carry out their functions in close association with other proteins. It is these interactions that determine the functions of protein functional networks, such as signal-transduction cascades and complex biosynthetic or degradation pathways. Currently, much of our knowledge of protein-protein interactions has been gained through *in vitro* studies with individual purified proteins and with the yeast two-hybrid system. However, proteomics approaches offer the opportunity to characterize more complex networks coupled with analytical proteomics methods. Mass spectrometry-based proteomic analysis offers a new way to identify the components of multiprotein complexes. One approach is to

resolve the protein complex on 1D SDS-PAGE gel, stain and select the protein bands, digest them, and analyze by MALDI-TOF or LC-MS-MS (Liquid chromatography- MS-MS). The proteins can then be identified from the MS spectrum with the aid of peptide mass fingerprinting algorithms.

A potential problem with the use of SDS-PAGE is that it often results in loss of proteins, owing both to incomplete in-gel digestion and to incomplete recovery of the peptides from the gel. This can complicate detection of low-abundance proteins in the mixture. However, this can be overcome by immunoprecipitating the protein complex, which is then digested directly (without first separating proteins from each other) and then analyzing the peptide-digest mixture by MALDI-TOF or by LC-MS-MS. Potential problems with immunoprecipitation include specificity of the antibody against the target protein when in a complex, and contamination of the MS spectrum of the captured protein complex with peptides from the antibody. An alternative to the use of antibodies for capturing protein complexes is to use the "bait" approach, in which the target protein is immobilized on a solid support, such as epoxyalkyl sepharose, which reacts covalently with nucleophilic amine or thiol groups on the protein. Alternatively, recombinant proteins containing His-tag or FLAG-tag sequences associate tightly with immobilized nickel resins or immobilized anti-FLAG antibodies can be generated to capture putative target proteins. The immobilized complex is then harvested by centrifugation or filtration and the associated proteins are dissociated from the complex, digested and analyzed by MS. Other approaches that can be used to study protein-protein interactions include using multiprotein-nucleic acid complexes which uses specific nucleic acid sequences that interact with proteins associated with critical cellular functions.

2.8.4 Mapping of protein modifications

This is the task of identifying how and where proteins are modified. Many common posttranslational modifications govern the targeting, structure, function, and turnover of proteins. In addition, many environmental chemicals, drugs and endogenous chemicals give rise to reactive electrophiles that modify proteins. The two most widely used approaches are antibodies and site-directed mutagenesis. The mapping of phosphorylation sites in proteins provides a useful example of how these two techniques are used. Monoclonal antibodies directed against phophoserine/phosphothreonine or phophotyrosine can be used to map these residues to intact proteins or cleaved peptides. Site-directed mutagenesis allows investigators to systematically "knock out" serine, threonine or tyrosine residues thought to be phosphorylated in the system under study. Western blot analysis of these proteins or their peptide fragments with the antibodies can confirm whether phosphorylation was knocked out by specific amino acid substitution. This allows the investigator to infer which amino acids are the sites of modifications. However, there are two problems with this approach. First, one can never be sure whether the amino acid substitutions used in site-directed mutagenesis have changed some other aspect of the system, such as the association of a kinase, for example. Even subtle structural changes affect phosphorylation at adjacent acceptor sites. This is particularly troublesome in peptides where multiple potential phosphorylation targets occur together in close proximity. The second problem is a practical one. It takes tremendous effort to generate the antibodies and mutant proteins to do these types of studies. The issue of antibody specificity must be addressed every time a new protein system is studied.

The introduction of MS methods to analyze peptides now offers the best means to characterize protein modifications. If we use MALDI-TOF to obtain the MS-MS spectrum of a mixture of peptides, the data provide accurate mass measurements of the peptide ions. The measured masses reflect the amino acid composition of the peptides, plus the masses of any modifications. Thus, MALDI-TOF MS analysis can tell us which peptides may be present in the modified form. For example, MALDI-TOF analysis of a mixture of phosphorylated peptide and its nonphosphorylated counterpart will yield two signals. The one at lower *m/z* is for the unphosphorylated peptide, whereas the one at an *m/z* value of 80 units higher corresponds to the phosphorylated peptide.

To successfully utilize a MS spectrum to map protein modification, two factors are critical. One is the sequence coverage of the MS spectrum and second is the availability of MS spectra for modified peptides. The extent to which the entire protein sequence is represented by MS data is often referred to as "sequence coverage". For example, if we analyze a tryptic digest of a 100 amino acid protein and we obtain MS data on tryptic peptides corresponding to 60 residues, we say that we have 60 % sequence coverage. For purposes of simply identifying a protein, this is usually more than enough. However, the situation is very different when one tries to map protein modifications by MS. We can only detect peptide modifications if we have MS data for the modified peptide. To check a protein for modifications on all possible amino acids, we must have MS data for all the peptides. Recent improvements in MS technology like the Q-TOF MS (Quadruple-Time of Flight) have enabled generation of very good quality MS data with very high sequence coverage. Even with the availability of very high quality MS data, the biggest problem with mapping modifications is simply

obtaining MS spectra of modified peptides. Of all copies of any particular protein in a cell, only a small fraction may bear any specific modification. For example, many protein kinase substrates are rapidly phosphorylated and dephosphorylated, such that only a few phosphorylated copies of a protein may be present at a particular time. Thus, during MS analysis the peptide ions corresponding to the unmodified peptides are more intense and are more likely to be selected for MS-MS fragmentation than the ions from the modified peptides. One obvious solution to this problem is to employ an enrichment strategy to increase the fraction of modified proteins or peptides in the sample to be studied. This can be done at the protein or peptide level, depending on the nature and the abundance of the modification. Most enrichment strategies are directed at some chemical, physical, or immunological property of the modifying moiety itself. The immobilized metal affinity chromatography method has been used for isolating phosphopeptides from protein digests as the anionic phosphate group in the phosphorylated peptide has a strong affinity to polyvalent metal cations. Other methods like immobilized antibodies directed against the modifying moiety have been successfully capturing modified protein for subsequent MS analysis.

Protein modification could be responsible for a wide variety of biological processes (Boylan et al., 2001; Iwafune et al., 2002; Kim et al., 2002) and disease states like cancer. Thus, a better understanding of these modifications would help investigators design better treatment strategies.

2.9 Biofilms

Biofilms have been defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces. This definition includes microbial aggregates

and floccules and also adherent populations within the pore spaces of porous media (Costerton et al., 1995). Surface associated growth of microbial cells has been observed in a number of ecosystems (Costerton, 2000; Costerton et al., 1987, 1994, 1995), leading to biofilm development. During the complex process of adhesion, bacterial cells alter their phenotypes in response to the proximity of a surface (Fletcher, 1991; Lappin-Scott and Costerton, 1995.). During the earliest stages of biofilm formation, sessile bacteria find themselves in a stable juxtaposition with cells of the same species and with those of other species, as single-species and mixed-species microcolonies are formed (Lawrence et al., 1995; Marshall, 1992). These cellular juxtapositions, and the exopolysaccharide matrix production within the developing biofilm, condition the microenvironment of each biofilm bacterium (Van Loosdrecht et al., 1990) within the matrix, resulting in the establishment of a mature biofilm consisting of very efficient microbial communities adherent to surfaces (Fletcher, 1987).

2.10 Ubiquity of biofilms

The existence of microbial biofilms has been known for some time. The first to speculate on the existence of attached bacteria in oligotrophic systems was Zobell in 1943 (Zobell, 1943). Since this initial observation, virtually any surface, biotic or abiotic, has been shown to be colonized with biofilm communities of microorganisms, including contact lenses, ship hulls, dairy and petroleum pipelines, dental caries, water distribution systems, rocks in streams, heat exchangers and all varieties of biomedical implants and transcutaneous devices (Carpentier and Cerf, 1993; Costerton et al., 1987, 1995, 1999; Dankert et al., 1986; Elder et al., 1995; Gibbons and van Houte, 1980;

LeChevallier et al., 1987; Lehmann et al., 1992; Lewin, 1984; Meadows and Anderson, 1979; Ridgeway and Olson, 1981).

2.10.1 Biofilms on food contact surfaces

Interest in microbial biofilms in the food processing industry has intensified over the past several years. In food systems, microbial attachment and biofilm formation may be beneficial in their use to produce fermented foods. In vinegar production, for example, acetic acid-producing bacteria (*Gluconobacter* and/or *Acetobacter*) are grown in biofilms on wood chips to efficiently convert substrate to product. Microbial biofilms are also used in food processing waste water treatment. In trickling bed filters, microorganisms in biofilms adhered to filtering material remove organic and inorganic compounds from the waste water to facilitate its reuse. However, microbial attachment and biofilm formation on food processing equipment are more commonly detrimental and undesirable in food processing premises. Biofilms by pathogenic bacteria such as *Salmonella* (Dhir and Dodd, 1995; Humphrey et al., 1995; Jones and Bradshaw, 1996; Somers et al., 1994), *Klebsiella* (Jones and Bradshaw, 1996; Morin et al., 1996), *Pseudomonas* (Brown et al., 1995), *Campylobacter*, enterohaemorrhagic *E. coli* (Somers et al., 1994) and *Listeria* (Mafu et al., 1990; Ren and Frank, 1993) have been reported. Heat resistant *Streptococcus thermophilus* biofilms have been observed in the pasteurized milk section of a pasteurizer, inoculating milk at a rate of 10^6 cells/ml (Bouman et al., 1981). Czechowski (1990) reported on biofilms found in dairies and breweries, on bends in pipes, rubber seals, conveyor belts, waste water pipes, floors, etc. Teflon® and Buna-n® seals are excellent sites for biofilm formation and when cracked provide reservoirs for microbes which are difficult to inactivate or kill during cleaning.

Listeria monocytogenes persists on the rubber fingers of poultry pluckers and the trolleys that carry the carcasses after cleaning (Toquin et al., 1991), as does *S. aureus* (Le Gros et al., 1986). Notermans and co-workers (1991) have observed biofilms in poultry slaughterhouses by scanning electron microscopy. They found that after several weeks of operation the whole surface of the rubber plucker finger was pitted, trapping microorganisms in the same way as the factory floor and metal welds so that microorganisms become protected from routine cleaning and disinfection. Such biofilms represent a continuous source of contamination to foods coming in contact with them, potentially resulting in a public health concern. Increased resistance of biofilm cells to antibacterial agents and sanitizers (Carpentier and Cerf, 1993; Costerton et al., 1987; Costerton and Lappin-Scott, 1989; Ren and Frank, 1993) make the threat of food-borne illness an even more serious one.

2.10.2 Biofilms on medical devices

Evidence of the occurrence of biofilms on bio-medical devices has come from studies in which the devices either were examined upon removal from the patients or were tested in animal or laboratory systems. Costerton et al. (1999) provided a partial listing (Table 2.1) of bio-medical devices on or in which biofilms have been shown to develop. These devices include central venous catheters, central venous catheter needle-less connectors, contact lenses, endotracheal tubes, intrauterine devices, mechanical heart valves, pacemakers, peritoneal dialysis catheters, prosthetic dialysis catheters, prosthetic joints, tympanostomy tubes, urinary catheters and voice prostheses. This listing also provides documentation of various infections or diseases involving biofilms and the common biofilm bacterial species associated with these infections.

Table 2.1 Partial list of bio-medical devices in human infection involving biofilms.

Infection or disease	Common biofilm bacterial species
Arteriovenous shunts	*S. epidermidis* and *S. aureus*
Bacterial prostatitis	*E. coli* and other Gram-negative bacteria
Biliary stent blockage	A variety of enteric bacteria and fungi
Biliary tract infection	Enteric bacteria (e.g., *Escherichia coli*)
Central venous catheters	*S. epidermidis* and others
Contact lens	*P. aeruginosa* and Gram-positive cocci
Cystic fibrosis pneumonia	*Pseudomonas aeruginosa* and *Burkholderia cepacia*
Dental caries	Acidogenic Gram-negative cocci (e.g., *Streptococci*)
Exit sites	*S. epidermidis* and *S. aureus*
ICU pneumonia	Gram-negative rods
IUDs	*Actinomyces israelii* and many others
Mechanical heart valves	*S. aureus* and *S. epidermidis*
Musculoskeletal infections	Gram-positive cocci (e.g., *Staphylococci*)
Native valve endocarditis	Viridans group streptococci
Necrotizing fascilitis	Group A streptococci
Orthopedic devices	*S. aureus* and *S. epidermidis*
Osteomyelitis	Various bacterial and fungal species-often mixed
Otitis media	Nontypable strains of *Haemophilus influenzae*
Penile prostheses	*S. aureus* and *S. epidermidis*
Periodontitis	Gram-negative anaerobic oral bacteria
Peritoneal dialysis (CAPD) peritonitis	A variety of bacteria and fungi
Schleral buckles	Gram-positive cocci
Sutures	*Staphylococcus epidermidis* and *S. aureus*
Urinary catheter cystitis	*E. coli* and other Gram-negative cocci
Vascular grafts	Gram-positive cocci

Adapted from Costerton et al. (1999).

Maki et al. (1997) noted that central venous catheters (CVCs) pose a greater risk of device-related infection than does any other indwelling medical device, with infection rates of 3 to 5 %. Catheters may be inserted for administration of fluids, blood products, medication, nutritional solutions, and hemodynamic monitoring (Flowers et al., 1989). Biofilms have been shown by scanning electron microscopy and transmission electron microscopy to be universally present on catheters and may be associated with either the outside of the catheter or the inner lumen (Raad et al., 1993). Organisms that colonize the CVCs originate either from the skin insertion site, migrating along the external surface of the device, or from the hub, due to manipulation by the health care workers, and migrating along the inner lumen. Organisms colonizing CVCs include coagulase-negative staphylococci, *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *Klebsiella pneumoniae*, *E. faecalis*, and *Candida albicans* (Elliott et al., 1997; Raad, 1998). Urinary catheters are tubular, latex, or silicon devices that are inserted through the urethra into the bladder to measure urine output, collect urine during surgery, prevent urinary retention, or control incontinence (Kaye and Hessen, 1994). One study (Zimakoff et al., 1993) found that the percentage of patients undergoing indwelling urinary catheterization was 13.2 % for hospital patients, 4.9 % for nursing homes, and 3.9 % for patients receiving home care. Stickler noted that 10 to 50 % of patients undergoing short-term catheterization (up to 7 days) develop urinary tract infection, whereas essentially all patients undergoing long-term catheterization (greater than 28 days) will develop urinary tract infection (Stickler, 1996). Scanning and transmission electron microscopy on urinary catheters removed from patients revealed biofilms (Nickel et al., 1989; Stickler, 1996; Stickler et al., 1993b). Initially, catheters are colonized by single species, such as *S. epidermidis*, *E. faecalis*, *E. coli*, or *Proteus mirabilis*. As the catheters remain in place, the number

and diversity of organisms increase. Mixed communities develop, containing such organisms as *Providencia stuartii*, *P. aeruginosa*, *P. mirabilis* and *Klebsiella pneumoniae* (Stickler, 1996). Other organisms isolated from urinary catheter biofilms include *Morganella morganii*, *Acinetobacter calcoaceticus* (Stickler et al., 1993b) and *Enterobacter aerogenes* (Stickler et al., 1993a).

2.11 Biofilm ultrastructure

Biofilms were perceived as homogenous unstructured accretions of bacterial cells, surrounded by the cells' exopolysaccharide matrices for the first decade (1978-1990) following the discovery of the importance and ubiquity of biofilms. These perceptions were based on techniques for direct observation which did not give an accurate representation of the biofilm ultrstructure, in that electron microscopy required complete dehydration of highly hydrated biofilm matrices and in that light microscopy was badly distorted by out-of-focus effects. The advent of inexpensive high-speed computers and increasingly sophisticated software has transformed traditional microscopic observation into the discipline of analytical imaging. Confocal scanning laser microscopy (CLSM) has proven particularly well suited for the study of microbial biofilms and its use has produced a whole series of revelations that are the basis of current biofilm models (Shotton, 1989). Foremost has been the observation that biofilms are not structurally homogenous monolayers of microbial cells on a surface, but are heterogeneous in both space and time (Lewandowski, 2000). The basic building block or structural unit of the biofilm is the microcolony, which may be composed of single-species populations or multimember communities of bacteria, depending on the environmental parameters under which they are formed. Numerous conditions, such as

surface and interface properties, nutrient availability, hydrodynamics, and composition of the microbial community, can affect biofilm structure (Stoodley et al., 1997). For example, under high shear stresses, such as on the surface of teeth during chewing, the biofilm (dental plaque) is typically stratified and compacted (Bowden and Li, 1997; Wimpenny and Colasanti, 1997). Biofilms have also been examined under various hydrodynamic conditions such as laminar and turbulent flows, and it was shown that biofilm structures are altered in response to flow conditions (Stoodley et al., 1998). Biofilms grown under laminar flow were found to be patchy and consisted of rough round cell aggregates separated by interstitial voids. Biofilms grown in the turbulent flow cells were also patchy, but elongated "streamers" that oscillated in the bulk fluid were observed. Moreover, by observing biofilm development under continuous flow, Stoodley and coworkers (1998) were able to evaluate the effect of perturbations on established biofilms. They showed that the biofilm was polymorphic and structurally adapted to changes in nutrient availability.

Lawrence and coworkers (1991) using CSLM and biofilms of *P. aeruginosa*, *P. fluorescens*, and *Vibrio parahaemolyticus* were able to create an enhanced conceptual image of bacterial biofilm architecture, as it exists in nature. They generated biofilms with each of these organisms using continuous-flow slide culture chambers and examined them at various time intervals. The results showed that *V. parahaemolyticus* and *P. aeruginosa* produced biofilms that were approximately two times the thickness of *P. fluorescens* biofilms. Each biofilm demonstrated variation in depth and in the ratio of cellular to non-cellular material. All biofilms were highly hydrated, open structures composed of 73 to 98 % non-cellular material, including water channels and exopolysaccharide. The cells within *Pseudomonas* biofilms were more tightly packed at

the surface and less dense near the periphery of the biofilm, i.e. pyramidal in shape. Conversely, biofilms produced by *V. parahaemolyticus* showed the opposite arrangement; cell density was greatest near the periphery. In addition, *V. parahaemolyticus* biofilms had extensive void spaces within the inner regions of the biofilm. These voids or channels are an integral part of the biofilm structure. Using particle-tracking techniques, researchers have been able to demonstrate water flow through these channels (Stoodley et al., 1994). Therefore, the channels are, in essence, a distribution system for the biofilm, since they provide a means of circulating nutrients as well as exchanging metabolic products with the bulk fluid layer (Costerton, 1995) (Fig. 2.2). For instance, *in situ* measurements of dissolved oxygen using microelectrodes revealed that oxygen is available in the biofilm as far down as the substrata, indicating that the channels are transporting the oxygenated bulk fluid throughout the biofilm to the surface. The concentration of this dissolved oxygen decreases very rapidly after encountering the biofilm-liquid interface (Lewandowski et al., 1993). Thus it is presumed that these channels are a vital part of the biofilm structure and function, and therefore there are likely to be mechanisms for the formation as well as the maintenance of these structures.

2.12 Resistance to antimicrobial agents

The structure and physiological attributes of biofilms and their organisms have been demonstrated to confer an inherent resistance to antimicrobial agents, whether these antimicrobial agents are antibiotics, disinfectants, or germicides. A number of studies have reported the increased resistance of biofilm bacteria to selected antibiotics

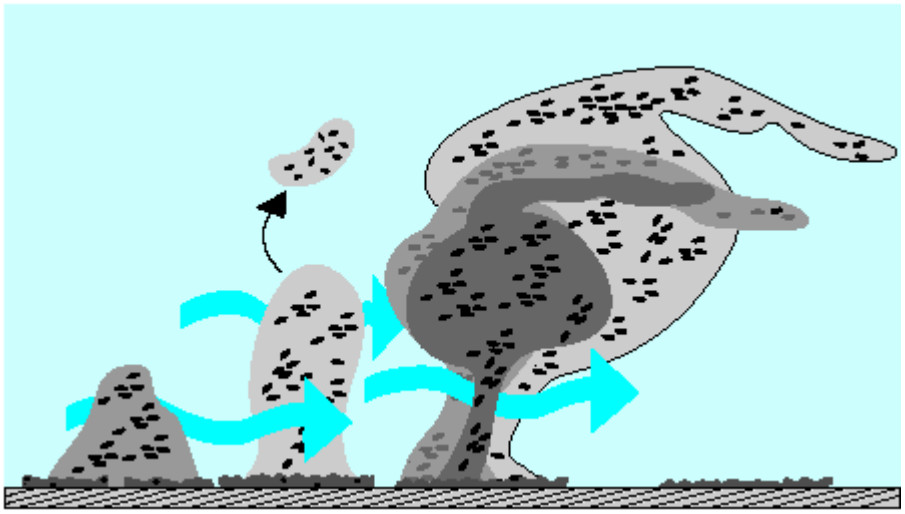


Figure 2.2 Conceptual model of the architecture of a single-species biofilm based on data collected by CSLM of living biofilms (adapted from Costerton et al., 1995).

when compared with their planktonic counterparts. Williams et al. (1997) reported that cells of *S. aureus* biofilms on silicon surfaces were 10 times more resistant to vancomycin than were their planktonic counterparts. Ceri and coworkers (1999) reported that *P. aeruginosa* required as much as 1,024 μg of imipenem and *E. coli* required a much as 512 μg of ampicillin as the minimal biofilm eradication concentrations compared to just 1 and 2 μg, respectively, for their planktonic counterparts. A similar increase in resistance was reported against ceftazidime and doxycycline by as much as 100 and 50 fold, respectively, in both *P. pseudomallei* and *Streptococcus sanguis* biofilms, respectively, compared to their planktonic counterparts (Larsen and Fiehn, 1996; Vorchit et al., 1993). Investigators have observed similar increases in resistance of biofilms and attached microorganism on food contact surfaces to various sanitizers (Caldwell et al., 1992; Cargill et al., 1991; Frank and Koffi, 1990; Lee and Frank, 1991; Mosteller and Bishop, 1993; Yu et al., 1993). Frank and Kofi (1990) found that attached microcolonies of *L. monocytogenes* were resistant to 12-20 min treatment with benzalkonium chloride, an anionic acid sanitizer, whereas unattached cells were killed by a 30 sec exposure to the sanitizer. Results of work conducted by Mustapha and Liewen (1989) and Wirtanen and Mattila-Sandholm (1992) suggest that the age of a biofilm affects the resistance of microorganisms to sanitizers. Sodium hypochlorite and quaternary ammonium compounds were effective against a 24 h biofilm of *L. monocytogenes* on food contact surfaces. Resistance of 24-144 h biofilms of *P. fluorescens*, *L. monocytogenes*, and *Bacillus subtilis* was evaluated against 0.1 % and 1.0 % chlorine at 25°C. Resistance to chlorine increased with biofilm age.

At least three mechanisms have been proposed to account for the increased resistance of biofilm bacteria to antimicrobial agents and include: (i) delayed penetration of the antimicrobial agent; (ii) altered growth rate of biofilm organisms; and (iii) physiological changes due to biofilm mode of growth.

2.12.1 Delayed penetration of the antimicrobial agent

Antimicrobial molecules must diffuse through the biofilm matrix in order to reach and inactivate the encased cells. The EPS constituting this matrix presents a diffusion barrier for these molecules by influencing either the rate of transport of the molecule to the biofilm interior or the reaction of the antimicrobial material with the matrix material. Suci and coworkers (1994) demonstrated a delayed penetration of ciprofloxacin into *P. aeruginosa* biofilms; what normally required 40 s for a sterile surface required 21 min for a biofilm-containing surface. Hoyle and coworkers (1992) found that dispersed bacterial cells were 15 times more susceptible to tobramycin than were cells in intact biofilms. DuGuid and coworkers (1992) examined *Staphylococcus epidermidis* susceptibility to tobramycin and concluded that the organization of cells within biofilms could in part explain the resistance of this organism to this antimicrobial agent. Studies examining antimicrobial agent penetration and interaction with the EPS of biofilms have revealed reduced efficacy due to poor diffusion of the antimicrobial agent through the biofilm matrix. Hatch and Schiller showed that a 2.0 % suspension of alginate from *P. aeruginosa* inhibited diffusion of gentamicin and tobramycin, and this effect was reversed by using alginate lyase (Hatch and Schiller, 1998). Souli and Giamarellou demonstrated the ability of *S. epidermidis* slime to hinder the antimicrobial

susceptibility of *Bacillus subtilis* to a large number of agents (Souli and Giamarellou, 1998).

2.12.2 Altered growth rate of biofilm organisms

Another proposed mechanism for biofilm resistance to antimicrobial agents is that biofilm-associated cells grow significantly more slowly than planktonic cells and, as a result, take up antimicrobial agents more slowly. Using a method of cell culture designed to determine the effect of growth rate apart from other biofilm processes, Evans et al. (1990) found that the slowest growing cells of *E. coli* were more resistant to cetrimide. Eng et al. (1991) determined that by controlling the growth rate of bacteria through nutrient limitation only fluoroquinolone antibiotics produced bactericidal effects against stationary-phase Gram-negative organisms. Furthermore, no class of antimicrobial agent was bactericidal against growth-limited *S. aureus*. An increase in the nutrient concentration and subsequently growth rate was followed by an increase in the efficacy of multiple classes of antimicrobial agents. Another study showed that *S. epidermidis* biofilm growth rates strongly influenced their susceptibility; the faster the rate of cell growth, the more rapid the rate of inactivation by ciprofloxacin (DuGuid et al., 1990). Anwar and coworkers (1992) were able to demonstrate age-related differences in the response of *S. aureus* biofilms to antimicrobial therapy. Exposure of 4-day-old biofilms to tobramycin and/or cephalexin produced a rapid reduction in viable-cell counts, whereas biofilms developed over a 13-day period demonstrated marked resistance to either drug or a combination of both. The authors suggest that these findings are due, at least in part, to the reduced metabolic activity of cells embedded in the aged biofilm.

2.12.3 Physiological changes due to biofilm mode of growth

This mechanism is more speculative than the previous two mechanisms. Gram-negative bacteria respond to nutrient limitations and other stresses by synthesizing sigma factors. In *E. coli*, those sigma factors that are under the control of the *rpoS* regulon regulate the transcription of genes whose products mitigate the effects of stress. By studying *E. coli* biofilms formed by strains with or without *rpoS* genes, Adams and McLean (1999) found that the *rpoS*$^{+}$ *E. coli* biofilms had higher densities and a higher number of viable cells. Since *rpoS* is activated during slow growth of this organism, it appears that conditions that elicit the slowing of bacterial growth, such as nutrient limitation or build-up of toxic metabolites, favor the formation of biofilms. Nutrient limitation and increase in toxic metabolite concentrations might be particularly acute within the depths of established biofilms. Tresse et al. (1995) found that agar-entrapped *E. coli* cells were more resistant to an aminoglycoside as oxygen tensions were decreased. They suggested that the effect was due to lowered uptake of the antibiotic by the oxygen-starved cells. It is also thought that at least some of the cells in a biofilm are thought to adopt a distinct and protected biofilm phenotype. This phenotype is not a response to nutrient limitation, but is thought to be a biologically programmed response to growth on a surface. Dagostino et al. (1991) proposed that initial bacterial association with a surface may result in the repression or induction of genes, which in turn results in a number of physiological responses.

2.13 Events in the development of biofilms

The development of a biofilm has been proposed to occur as a two-step (Marshall et al., 1971), three-step (Busscher and Weerkamp, 1987; Notermans et al.,

1991), and five-step process (Characklis and Cooksey, 1983; Lawrence et al., 1987). According to Marshall et al. (1971), cell attachment and biofilm formation are thought to occur in two stages, the reversible stage and the irreversible stage. The first stage involves the association of cells near, but not in actual contact with, the substratum. If allowed to remain associated with the substratum, the cells eventually synthesize exopolysaccharide (EPS) that exudes from the cell surface and directly binds the cell to the substratum. This bridge that is formed between the cell surface and solid substratum serves as the 'glue' that binds the cell irreversibly to the surface. Busscher and Weerkamp (1987) in their three-step model describe the interactions occurring between the substratum and the bacterium (Fig. 2.3). Initially at distances >50 nm, only attractive Van der Waal's forces operate. At this distance, the microbial and solid surfaces are too far apart for any recognition of surface features, including surface charge, hydrophobicity, and/or appendages. However, at 10 to 20 nm from a solid surface, interplay between attractive Van der Waal's and repulsive electrostatic forces occur. At this stage, adhesion is reversible because no true bonds are attaching the bacterium to the surface and a sheer force will remove the cells. For the organism to then be able to reach the surface and attach irreversibly, it has to overcome an interaction barrier- a barrier that the Derjaguin-Landau-Verwey-Overbeck (DLVO) theory describes as a high energy repulsion barrier which is affected by the surface area of a particle, or cell for that matter (Van Loosdrecht et al., 1989), fluid environment (Busscher and Weerkamp, 1987; Rijnaarts et al., 1995; Van Loosdrecht et al., 1989, 1990), surface charge (Van Loosdrecht et al., 1987a), hydrophobicity (Van Loosdrecht et al., 1987b), and microtopography of the surface. The high energy interaction barrier

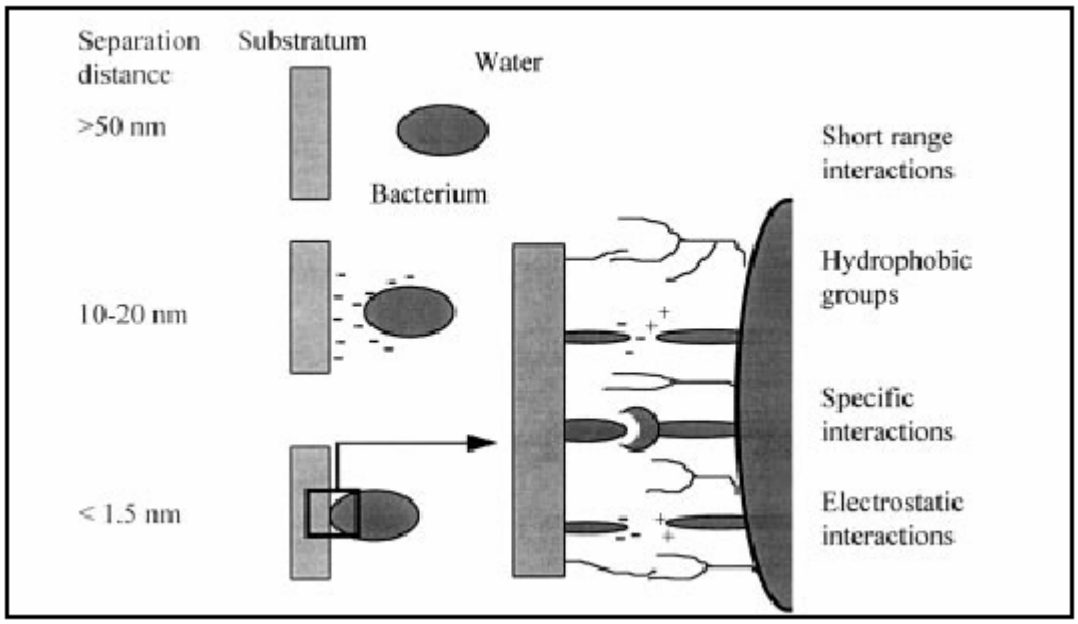


Figure 2.3 The adhesion process suggested by Busscher and Weerkamp for biofilm development (adapted from Busscher and Weerkamp, 1987). At separation distances of >50 nm, only attractive Van der Waal's forces occur. At 10 to 20 nm, Van der Waal's and repulsive electrostatic interactions influence adhesion. At <1.5 nm, short-range interactions can occur, irreversibly binding a bacterium to a surface.

described by the DLVO theory also acts a physical barrier preventing the bacterium from coming in direct contact with the surface. However, a bacterium with surface protrusions such as pili and/or flagella could conceivably overcome this barrier and these could assist the cell in coming to a stable region where microcolony and biofilm growth begins. This suggests that these thread-like structures may have a significant role in the cells adhering to the surface and may be important in the eventual formation of a biofilm. Similarly, the effect of the ionic strength of the solution also greatly affects the ability of the bacteria to approach closely to a surface, as described by DLVO. At low ionic strength, the amount of energy required by the bacterium to overcome electrostatic repulsion is so high that the electrostatic repulsion acts as a barrier for adhesion. However, with an increase in the ionic strength of the solution, the electrostatic repulsion may be completely balanced, allowing bacteria to freely approach a surface and irreversibly attach to it. Thus the properties of the fluid environment can have a profound effect on the bacteria's ability to adhere to a surface (Busscher and Weerkamp, 1987; Rijnaarts et al., 1995; Van Loosdrecht et al., 1989, 1990). Characklis and Cooksey (1983) expanded on the two-step model to include: (1) transport to a wetted surface; (2) adsorption of a 'conditioning' film; (3) adhesion of microbial cells to a wetted surface; (4) reactions within the biofilm; (5) detachment of biofilm. Briefly summarized, the organic molecules deposit on the substratum first and develop a layer of small, organic molecules. Subsequently, microbial adhesion to the conditioned surface occurs and is affected by free energy, charge, EPS, and cations. It is still unclear whether microorganisms attach preferentially to this layer of organic molecules. Physiological processes of the attached cells determine growth rate and metabolism.

These processes may be interrupted by the detachment of biofilm cells due to oxygen concentrations, fluid flow rate and chemical treatment.

Thus the formation of a mature biofilm is believed to occur in a sequential process of (1) transport of a microorganism to a surface, (2) initial microbial attachment, (iii) formation of microcolonies, and (iv) formation of mature biofilms (Characklis and Cooksey, 1983; Marshall, 1985; Van Loosdrecht et al., 1990).

2.14 Events in the transition from planktonic to biofilm mode of growth

Research in this decade has begun to reveal information about the molecular and genetic basis of biofilm formation. Biofilms involving several different bacterial species have been studied (Costerton et al., 1995). Four organisms, *P. aeruginosa*, *P. fluorescens*, *E. coli*, and *V. cholerae*, have become prominent model organisms for biofilm research. Biofilm bacteria are markedly different from their planktonic counterparts, as judged by gene expression, cellular physiology and the biofilm cell's increased resistance to antibiotics (Costerton et al., 1995; Hoyle and Costerton, 1991). Previous studies exploring biofilm formation have generally focused on identifying the organisms that comprise biofilms, their physical and chemical properties and the architecture of the biofilm (Costerton et al., 1995). In contrast, little is known about the cellular factors and molecular mechanisms required for the transition from planktonic to sessile mode of growth and subsequent development of the biofilm.

The initial attachment of bacterial cells to surfaces is believed to begin when bacteria sense certain environmental parameters that trigger the transition from planktonic growth to life on a surface (Fletcher and Pringle, 1986; Nyvad and Kilian, 1990; O'Toole et al., 2000; O'Toole and Kolter, 1998b; Poulsen et al., 1993; Stanley,

1983; Wang et al., 1996). The environmental cues that control this transition vary greatly among organisms. *Pseudomonas aeruginosa* will form biofilms under most conditions that allow growth (O'Toole and Kolter, 1998a), but some strains of *E. coli* K-12 will not form biofilms in minimal medium unless supplemented with amino acids (Pratt and Kolter, 1998), and *E. coli* O157:H7 had been reported to make biofilms only under low-nutrient conditions (Dewonti and Wong, 1995). O'Toole and Kolter (1998b) reported biofilm formation by *P. fluorescens* under every condition tested. Interestingly, a genetic analysis of biofilm formation by this organism revealed that it utilizes multiple genetic pathways to initiate biofilm development. For example, mutants unable to form a biofilm when grown on glucose were rescued for this defect by growth on citrate, suggesting an alternative citrate-dependant pathway for biofilm formation (O'Toole and Kolter, 1998b). Similarly in *V. cholerae* Herrigton et al. (1988) reported different pathways for initial attachment depending on the surface to which the organism attaches. For example, *V. cholerae in vivo* required the Tcp pilus for colonization of the intestine but it played no role in attachment to abiotic surfaces which are probably one environment exploited by this organism when not in humans. On abiotic surfaces, it is the pilus encoded by the *msh* locus (having no role in pathogenesis (Thenlin and Taylor, 1996) that is required for attachment. Abiotic surfaces can be subdivided into nonnutritive (plastic, glass, metal, etc.) and nutritive (e.g., chitin). While *mshA* is required for colonization of nonnutritive abiotic surfaces, it did not seem to play a role in the colonization of nutritive abiotic surfaces (e.g., cellulose; Watnick et al., 1999), suggesting the presence of a third set of functions required for the colonization of nutritive surfaces. Other environmental signals that can also influence initial attachment are osmolarity, pH, iron availability, oxygen tension, and temperature (Fletcher, 1996;

Nyvad and Kilian, 1990; O'Toole and Kolter, 1998a, b; Pratt and Kolter, 1998). Although the details of the environmental signals triggering biofilm development may vary from organism to organism, it is clear that environmental parameters have a profound impact on the transition between planktonic and biofilm growth.

Extra-cellular components such as flagella, pili and adhesins have been shown to be required for the sequence of events leading to biofilm formation, and have been best characterized primarily through mutation analysis. The primary function of flagella in biofilm formation is assumed to be in transport and in initial cell-to-surface interactions (De Weger et al., 1987; DeFlaun et al., 1994). The absence of flagella impaired *P. fluorescens* and *P. putida* in colonization of potato and wheat surfaces and reduced cellular adhesion of *P. aeruginosa* to polystyrene surfaces (O'Toole and Kolter, 1998a). Pili and pili-associated adhesins have been shown to be important for the adherence to and colonization of surfaces (Dorel et al., 1999; O'Toole and Kolter, 1998a; Pratt and Kolter, 1998; Vidal et al., 1998). In *E. coli*, attachment is reduced by mutations in the *csgA* gene, a biosynthetic curlin gene (Dorel et al., 1999; Vidal et al., 1998), and in the type I pili biosynthetic gene *fimH*, which encodes the mannose-specific adhesin in *E. coli.* There is also evidence of adhesive properties of type IV pili of *P. aeruginosa*, since mutants were reduced in the ability to form microcolonies when pili were absent (O'Toole and Kolter, 1998a). Mutations in other genes like *ica*, the gene for the polysaccharide intercellular adhesin of *S. epidermidis*, in *atlE*, the gene for autolysin of *Staphylococcus aureus* (Heilmann et al., 1997; Mack et al., 1994; Rupp et al., 1999), and in the genes for the mannose-sensitive hemagglutinin pilus of *V. cholerae* El Tor (Watnick et al., 1999) all reduced adhesion to surfaces. Membrane proteins may also influence the bacterial attachment process. Mutations in surface and membrane proteins,

including a calcium-binding protein, a hemolysin, a peptide transporter, and a potential glutathione-regulated K^+ efflux pump caused defects in attachment of *P. putida* to corn (Espinosa-Urgel et al., 2000). Matthysse et al. (1996) demonstrated the requirement for ABC transport systems in attachment and virulence of *A. tumefaciens*. Deletion of genes encoding components of the polyamine ABC transporter *potB*, *potH*, *potC*, and *potI* abolished attachment of *A. tumefaciens* to carrot suspension culture cells, and the resulting deletion mutants were avirulent (Matthysse et al., 1996). Bacterial extracellular polysaccharides have been shown to influence attachment and initial biofilm development, since these factors contribute to cell surface charge, which affects electrostatic interactions between bacteria and substratum (Van Loosdrecht et al., 1989). Adhesiveness of *Pseudomonas* species is related to the presence and composition of lipopolysaccharides (Williams and Fletcher, 1996). Substantially reduced attachment to biotic and abiotic surfaces was observed in O-polysaccharide-deficient *Pseudomonas* spp. (DeFlaun et al., 1999; Dekkers et al., 1998) and in *E. coli* strains with mutations in the lipopolysaccharides core biosynthesis genes *rfaG, rfaP, and galU* (Dekkers et al., 1998; Genevaux et al., 1999; Rodriguez-Herva et al., 1999).

Attachment to surfaces is thought to initiate a cascade of changes in the bacterial cells. The use of well-characterized mutant strains studied with phase contrast microscopy has proven instrumental in determining the mechanisms by which bacteria can initiate biofilm formation. A simple genetic screen has been implemented utilizing plastic 96-well microtiter dishes as a substrate for biofilm development, allowing the large-scale isolation of mutants defective in biofilm formation among a wide variety of organisms (Genevaux et al., 1999; Heilmann et al., 1996; Nyvad and Kilian, 1990; O'Toole and Kolter, 1998a, b; Pratt and Kolter, 1998). These biofilm-defective mutants

have been used both to identify functions required for biofilm development and to define various stages in the process. A number of surface attachment defective mutants of *P. aeruginosa*, designated as *sad*, have been described (O'Toole and Kolter, 1998a). One group of strains is defective in flagellum-mediated motility- these strains appeared to be blocked in the initial interaction with a surface. A second class of *sad* mutants is defective in the biogenesis of type IV pili, which are known to be involved in surface-associated movement referred to as twitching motility. Strains unable to make functional type IV pili attach to the surface and form a monolayer much as the wild type does, yet they are unable to form the microcolonies that are a hallmark of early biofilm development in *P. aeruginosa*. Therefore, twitching motility is required for the assembly of a monolayer of *P. aeruginosa* cells into microcolonies (O'Toole and Kolter, 1998a). With time, microcolonies develop into a mature biofilm that is often associated with the production of EPS. In *P. aeruginosa*, changes in gene expression following bacterial adhesion include surface-induced gene activation of *algC*, a gene involved in lipopolysaccharide core biosynthesis and in the biosynthesis of the exopolysaccharide alginate (Davies et al., 1993; Davies and Geesey, 1995). Wozniak and colleagues linked down-regulation of flagellum synthesis with the up-regulation of alginate synthesis (Garrett et al., 1999). As cells adjust to an immobile life on a surface, they lose their flagella and increase the production of EPS. It is not clear if there is a causal link between down-regulation of flagella synthesis and up-regulation of the genes required for alginate synthesis. In *V. parahaemolyticus*, it has been shown that interfering with flagellar rotation (i.e. by the cell's being in close proximity to the surface) directly leads to the induction of a signal transduction cascade that up-regulates the expression of a second flagellar machinery required for surface swarming (McCarter et al., 1988;

McCarter and Silverman, 1990). In *E. coli*, up-regulation after attachment was observed for OmpC, the *proU* operon, colanic acid exopolysaccharide production, tripeptidase T, and the nickel high-affinity transport system (*nikA*) (Prigent-Combaret et al., 1999). Another important step in the development and maturation of biofilms is the formation of the characteristic biofilm architecture. Although numerous techniques have been utilized to document the biofilm architecture of bacteria, until recently it was not clear if this structural complexity was regulated or the consequence of stochastic processes. The observation that a mutant of *P. aeruginosa* unable to synthesize the major quorum-sensing molecules, acylhomoserine lactones (acyl-HSLs), was radically altered in biofilm architecture clearly demonstrated that these molecules regulate the formation of biofilm structures in this organism. As visualized in a continuous-flow system, the *lasI* mutant (which is defective in acyl-HSLs) formed a biofilm without the usually well-spaced microcolonies (attaining heights of over 100 μm) and resistance to 0.2 % SDS treatment typically seen in the wild-type strain (Davies et al., 1998). Instead, the biofilm formed by the *lasI* mutant was a homogenous layer of cells approximately 20 μm thick that had completely lost the ability to resist SDS treatment. An exposure to SDS for as little as 5 min stripped the biofilm from the glass slide on which it formed. The typical biofilm architecture and resistance to SDS was restored by the addition of exogenous acyl-HSLs (Davies et al., 1998). These data strongly suggest that cell-cell communication is essential for this bacterium to establish a well-ordered surface community.

Recent investigations have been directed at determining the degree to which gene regulation during biofilm development controls the switch from planktonic to attached

growth. Brözel and coworkers monitored the global gene expression patterns in attached *P. aeruginosa* cells and observed as many as 11 proteins whose levels were altered during various stages of attachment (Brözel et al., 1995). More recently, Sauer et al. (2001; 2002) reported as many as 30 genes and 40 gene products were altered in *P. putida* within 6 h following attachment, and were also able to characterize five stages of biofilm development in *P. aeruginosa* using direct microscopic observation and whole-cell protein analysis. They grouped proteins showing differential regulation during the course of biofilm development into four general classes. Class I included proteins that encoded factors for metabolic processes, such as amino acid metabolism, carbon catabolism, and cofactor biosynthesis. The majority of these proteins were found to be up-regulated following adhesion. Class II included β-hydroxydecanoyl-acyl carrier protein (ACP) dehydrogenase, which is involved in various lipid biosynthesis reactions such as fatty acid, phospholipid, lipopolysaccharides, and acyl homoserine lactone biosynthesis. Class III contained membrane proteins primarily involved in molecular transport, such as the bacterial extracellular solute binding proteins (Tam and Saier, 1993) and porin (OprE), which forms a small channel in the outer membrane (Obara and Nakae, 1992). These membrane proteins were found to be up-regulated following adhesion. Class IV included proteins involved in adaptation and protection. With the exception of the probable ribosomal protein L25, the proteins falling into this class, such as alkyl hydroxyperoxide reductase subunit C and superoxide dismutase, were elevated in expression following adhesion. Similar differential expression of proteins between planktonic, microcolony and mature biofilm cells of *B. cereus* was observed by Oosthuizen and coworkers (2002). These studies have demonstrated that biofilm

bacteria are very different from their planktonic counterparts and have also shown that planktonic cells undergo a complex change in their global protein/gene expression pattern during their transition to a biofilm mode of growth. An understanding of various protein/gene expression changes during transition from planktonic to the biofilm mode of growth could help us design methods and agents necessary for the control of biofilms on food contact surfaces and prevent cross contamination of food during processing.

The current research approaches TSP, both as an antimicrobial agent as well as a stress at sublethal concentrations to *S.* Enteritidis. It is hypothesized that the antimicrobial activity of TSP is due to its alkaline pH and exposure to a sublethal concentration of TSP induces the alkaline stress response in *S.* Enteritidis. Thus the effect of TSP on *S.* Enteritidis was evaluated by comparative studies involving treatment solutions containing different concentrations of TSP, treatment solutions adjusted to the equivalent pH as in each of the TSP treatments and TSP solutions pH adjusted to 7.0. The study will then evaluate and compare the response of *S.* Enteritidis cells to a sublethal concentration of TSP or its equivalent alkaline pH made with NaOH when grown in suspension or attached to a surface.

3.0 HIGH pH DURING TRISODIUM PHOSPHATE TREATMENT CAUSES MEMBRANE DAMAGE AND DESTRUCTION OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS

3.1 Abstract

Trisodium phosphate (TSP) is now widely used during the processing of poultry and red meats, but the mechanism whereby it inactivates Gram-negative bacteria such as *Salmonella* spp. remains unclear. Thus, *Salmonella enterica* serovar Enteritidis (ATCC 4931) cells were treated with different concentrations of TSP (1.5, 2.0 and 2.5 % w/v) and compared with: i) cells treated with the same pH as the TSP treatments (pH 10.0, 10.5 and 11.0, respectively), and ii) cells treated with different concentrations of TSP (1.5, 2.0 and 2.5 % w/v) adjusted to a pH of 7.0 ± 0.2. Cell viability, loss of membrane integrity, cellular leakage, release of lipopolysaccharides and cell morphology were accordingly examined and quantified under the above treatment conditions. Exposure of serovar Enteritidis cells to TSP or equivalent alkaline pH made with NaOH resulted in the loss of cell viability and membrane integrity in a TSP concentration- or NaOH-alkaline pH-dependent manner. In contrast, cells treated with different concentrations of TSP whose pH was adjusted to 7.0 did not show any loss of cell viability or membrane integrity. A 30 min pretreatment with 1.0 mM EDTA significantly enhanced the loss of membrane integrity only when followed by TSP or NaOH-alkaline pH treatments.

Measuring the absorbance at 260 nm, agarose gel electrophoresis, Bradford assay and Tricine-SDS gel electrophoresis of filtrates of treated cell suspensions revealed considerable release of DNA, proteins and lipopolysaccharides compared to controls and pH 7.0 TSP treatments. Electron microscopic examination of TSP- or NaOH-alkaline pH-treated cells showed disfigured cell surface topology, and cells appeared wrinkled and showed evidence of a TSP concentration/pH-dependent disruption of the cytoplasmic and outer membranes. These results demonstrate that TSP treatment permeabilizes and disrupts the cytoplasmic and outer membranes of serovar Enteritidis because of the alkaline pH, which in turn leads to release of intracellular contents and eventual cell death.

3.2 Introduction

Salmonella species continue to be commonly associated with cases of food-borne disease in developed countries. In the United States in 2001, incidence per 100,000 people was highest for salmonellosis (15.1), followed by campylobacteriosis (13.8) and shigellosis (6.4) (CDC, 2002). Enteric pathogens usually contaminate the surface of raw animal products during slaughter and primary processing (scalding, defeathering or dehiding, rinsing, cutting, mixing, and grinding, etc.) and can attach and/or reside in the regular and irregular surfaces of the skin, multiply and, thereafter, contaminate food preparation surfaces, hands and utensils. Food spoilage and illness can result from this carryover of bacteria if these contaminated products are undercooked (CDC, 1993) or handled improperly. Due to the wide range of potential handling abuses, it is highly desirable to significantly reduce the numbers of pathogenic bacteria attached to the surfaces of these products.

An array of methods for reducing the load of potential pathogens on the surfaces of meat products has been developed. These methods use ionizing radiation (Farkas, 1998), organic acid sprays (Delmore et al., 2000; Dickson and Anderson, 1992; Podolak et al., 1996; Smulders and Greer, 1998), and phosphates (e.g., trisodium phosphate, polyphosphates) dips/sprays (Delmore et al., 2000) to reduce the numbers of bacterial pathogens present on raw animal products following processing. Trisodium phosphate is generally recognized as safe by the Food and Drug Administration and has been approved by the United States Department of Agriculture for use as a food ingredient (Federal, 1982) and for the reduction of *Salmonella* contamination during poultry processing (Federal, 1994). The process involves immersing post-chill whole birds for 15 sec in a 10 % solution of Av-Gard™ TSP, allowing the excess TSP solution to drip from the bird, presumably leaving minimal amounts of phosphate on the carcass (Rhône-Poulenc, 1992). Taste tests showed that TSP treatment at these relatively high concentrations had no effect on flavor, texture or appearance of treated poultry (Capita et al., 2000; Hollender et al., 1993; Kim and Marshall, 1999). At 8 to 15 % w/v, TSP has been demonstrated to reduce the number of artificially inoculated Gram-negative pathogens surviving on surfaces of foodstuffs (Dickson et al., 1994; Dorsa et al., 1997; Hwang and Beuchat, 1995; Salvant et al., 1997; Taormina and Beuchat, 1999; Xiong et al., 1998a; Zhuang and Beuchat, 1996). Log reductions in viable counts of 1.4 and 0.9 were obtained for *Escherichia coli* O157:H7 and *Salmonella typhimurium*, respectively, on beef adipose tissue, though significantly lower reductions (0.9 and 0.5, respectively) were observed on fascia (Kim and Slavik, 1994b). On chicken carcasses, Slavik et al. (1994) and Kim et al. (1994) showed that TSP treatment at 10° or 50ºC reduced

Salmonella counts by 1.6 to 1.8 log cycles per carcass, and in a scanning electron microscopic study, Kim and Slavik (1994a) showed that TSP effectively removed attached *Salmonella* from chicken skin.

Very little scientific evidence is available which identifies the actual mechanisms of TSP antimicrobial activity. Possible modes of action of TSP include: i) exposing of microorganisms to high pH, which might particularly affect cell membrane components (Mendonca et al., 1994), ii) enhancing detachment of bacteria from food surfaces by sequestration of metal ions (Lee et al., 1994b), and iii) removing of fat from the skin surface, thereby allowing bacteria to be washed from the food surfaces more effectively (Bender and Brotsky, 1992; Giesse, 1992; Kim and Slavik, 1994a). To date, work has mainly focused on evaluating the efficiency of *Salmonella* removal by the process (Coppen et al., 1998; Delmore et al., 2000; Xiong et al., 1998a, b; Yang et al., 1998), whereas there is no published information that documents possible mechanisms of TSP antimicrobial action. This study was interested in the role of high pH generated by dissolved TSP, and examines the hypothesis that the high pH during TSP treatment of *Salmonella enterica* serovar Enteritidis could be responsible for its antimicrobial action. This hypothesis was examined using a series of comparative studies involving treatment solutions containing different concentrations of TSP, treatment solutions adjusted to the equivalent alkaline pH as in each of the TSP treatments with NaOH and TSP solutions pH adjusted to 7.0. Direct and indirect indices of cell survival, membrane damage, and cellular leakage were also employed to examine specific antimicrobial effects.

3.3 Materials and Methods

3.3.1 Media and Chemicals

Tryptic soy agar (TSA), tryptic soy broth (TSB), propidium iodide (PI), magnesium chloride ($MgCl_2$) and proteinase K were purchased from Sigma Chemical Co. (St. Louis, MO.). Peptone water was obtained from Difco Laboratories (Detroit, MI.); trisodium phosphate dodecahydrate (TSP) from Rhodia (Cranbury, N.J.); sodium hydroxide (NaOH) from Fisher Scientific (Nepean, ON.); sodium chloride (NaCl) from MERCK (Darmstadt, Germany); ethylenediaminetetraacetic acid (EDTA) from J. T. Baker Chemical (Phillipsburg, N.J.); tris base from Life Technologies (Grand Island, N.Y.); and 6 N HCl from BDH Chemicals (Toronto, ON.). All chemicals used in the preparation of specimens for electron microscopy were purchased from Electron Microscopy Supply (Fort Washington, PA.).

3.3.2 Bacteria and culture conditions

Salmonella enterica serovar Enteritidis (ATCC 4931) was obtained from the American Type Culture Collection (Rockville, MD.). The stock culture was grown on TSA at 37°C for 18 h and maintained for a maximum period of one month at 4°C, after which a new stock culture was prepared from the frozen stock.

3.3.3 Preparation of cell suspensions for antimicrobial studies

A loopful of colony material from TSA was transferred to 3.0 ml of TSB and incubated on a gyratory shaker (100 rpm) at 37°C for 24 h. Cells in the stationary phase

of growth were prepared by transferring 1.0 ml of this batch culture into 100 ml of fresh TSB, and incubating at 37°C on a gyratory shaker (100 rpm) for 16 h.

3.3.4 Exposure of cells to TSP and high pH

Stationary phase cells were harvested by centrifugation at 12,000 x g for 10 min at 4°C (Sorvall RC-2B SuperSpeed Refrigerated Centrifuge, Kendro Laboratory Products, Asheville, NC.). The pellet was then washed once with phosphate buffered saline (PBS) (pH 7.0). Washed cells were harvested again by centrifugation and resuspended in 100 ml of treatment solution at room temperature (RT; 23 ± 2.0°C) containing either: i) TSB (control), ii) TSB with 1.5, 2.0 or 2.5 % (w/v) TSP, iii) 1.5, 2.0 or 2.5 % (w/v) TSP (in a TSB solution) that was pH adjusted to 7.0 ± 0.2 with 6 N HCl, and iii) TSB that was pH adjusted to 10.0 ± 0.2, 10.5 ± 0.2 or 11.0 ± 0.2 with NaOH (these solutions correspond to the pH values of 1.5, 2.0 and 2.5 % TSP treatment solutions above, respectively).

3.3.5 Cell viability

One-milliliter aliquots of each treated cell suspensions were removed at predetermined time intervals (0, 5, 10, 20, 30, 40, 50, and 60 min) and serially diluted in 0.1 % peptone water. Appropriate dilutions were then plated on TSA. The plates were counted for colony forming units (CFU) following incubation at 37°C for 24 h and plotted against time (survival curves). Decimal reduction time (D-value), defined as the time required to destroy 90% of the organisms, was calculated from these survival curves. All cell viability experiments were repeated three separate times.

3.3.6 Propidium iodide uptake

Washed stationary phase cells resuspended in different treatment solutions (as described above) were incubated in 250 ml Erlenmeyer flasks on a gyratory shaker (100 rpm) at RT for 30 min. Propidium iodide was then added to each flask (final concentration of 5.0 μM) and incubated for an additional 30 min under the same conditions. Following incubation with PI, cells were centrifuged and washed twice with PBS. Washed cells were diluted to an optical density at 680 nm (OD_{680}) of 0.5 ± 0.2 with PBS. Fluorescence of the resultant cell suspensions was then measured with a fluorometer (G. K. turner Associates, Palo Alto, CA.); the excitation wavelength was set at 495 nm, and the emission wavelength was set at >595 nm. Fluorescence values obtained from untreated control cells were subtracted from all treatment values.

Experiments were also performed to delineate the effects of TSP on the outer membrane; cells were pretreated with EDTA, which removes stabilizing divalent cations from the outer membrane by chelation, before exposure to TSP. Washed stationary phase cells were resuspended in 100 ml of 0.1 M Tris-HCl (pH 7.5) containing 1.0 mM EDTA and incubated on a gyratory shaker (100 rpm) at RT for 30 min. After incubation, cells were centrifuged and washed once with PBS. Washed cells were then resuspended in treatment solutions containing either: i) TSB (control), ii) TSB with 1.5, 2.0 or 2.5 % TSP, iii) 1.5, 2.0 or 2.5 % (w/v) TSP (in a TSB solution) that was pH adjusted to 7.0 ± 0.2 with 6 N HCl, and iv) TSB that was pH adjusted to 10.0 ± 0.2, 10.5 ± 0.2 or 11.0 ± 0.2 with NaOH, and measured for PI uptake as described above.

To isolate the effect of Mg^{2+} on the outer membrane of cells pretreated with EDTA, washed stationary phase cells were resuspended in 100 ml of 0.1 M Tris-HCl (pH 7.5) containing different concentrations of $MgCl_2$ (0.5, 2.5, 5.0. 7.5 and 10.0 mM) along with 1.0 mM EDTA. The resuspended cells were incubated on a gyratory shaker (100 rpm) at RT for 30 min before exposure to 2.0 % TSP for 1 h, and then measured for PI uptake, as above. Simultaneously, washed stationary phase cells were incubated on a gyratory shaker (100 rpm) at RT for 1 h in 100 ml of TSB containing 2.0 % TSP along with different concentrations of $MgCl_2$ (5.0, 10.0, 15.0, 20.0 and 25.0 mM) and measured for PI uptake.

3.3.7 Measurement of osmotic response

Approximately 1.0 ml of cells was exposed for 1 h to either: i) 1.5, 2.0 or 2.5 % TSP (in a TSB solution), ii) 1.5, 2.0 or 2.5 % TSP (in a TSB solution) that was pH adjusted to 7.0 ± 0.2 with 6 N HCl, or iii) TSB that was pH adjusted to 10.0 ± 0.2, 10.5 ± 0.2 or 11.0 ± 0.2 with NaOH, were centrifuged and washed once with PBS. Washed cells were resuspended in 1.0 ml of PBS, after which 100 µl of this suspension was added in triplicate to: i) 1.0 ml of PBS and ii) 1.0 ml of PBS containing 0.75 M NaCl. The OD_{680} of these suspensions was measured 4 min after mixing using a Spectronic 601 spectrophotometer (Milton Roy, N.Y.). The increase in OD_{680} was calculated by subtracting the mean value of the three measurements in PBS from the mean value of the three measurements in PBS containing 0.75 M NaCl. These OD increases were expressed as a percentage of the mean value obtained with PBS alone.

3.3.8 Measurement of cellular leakage

Leakage of cytoplasmic contents was determined by measuring the absorbance at 260 nm (Spectronic 601) of the cell filtrates following various treatments. Washed stationary phase cells were resuspended in 100 ml of sterile distilled water (dH_2O) containing different concentrations of TSP (0 [control], 0.5, 1.0, 1.5, 2.0 and 2.5 %) and dH_2O containing different concentrations of TSP (0.5, 1.0, 1.5, 2.0 and 2.5 %) pH adjusted to 7.0 and incubated on a gyratory shaker at RT for 1 h. The pH of the treatment solutions containing 0, 0.5, 1.0, 1.5, 2.0 or 2.5 % TSP were 7.2, 12.10, 12.28, 12.35, 12.40, and 12.44, respectively. The treatment solutions were not prepared in TSB as in previous studies, as the absorbance of TSB at 260 nm was unreadable by the spectrophotometer at this wavelength. After incubation, 3.0 ml of each treated cell suspension were filtered through a 25 mm diameter, 0.2 μm pore size Nalgene syringe filter (VWR CanLab, Mississauga, ON.). The filtrates were then examined for the presence of nucleic acids by measuring the absorbance at 260 nm (A_{260}). The Bradford assay (Bradford, 1976) was also performed on the filtrates to quantify release of proteins by the various treatments.

3.3.9 Agarose gel electrophoresis

Washed stationary phase cells were resuspended in 10.0 ml of sterile dH_2O containing 0.0, 0.5, 1.0, 1.5, 2.0 or 2.5 % TSP and dH_2O containing different concentrations of TSP (0.5, 1.0, 1.5, 2.0 and 2.5 %) pH adjusted to 7.0 and held at RT for 10 min. Cells were not resuspended in 100 ml of treatment solution to prevent

dilution of released nucleic acids in the filtrates. After 10 min incubation, 1.0 ml of each treated cell suspension was centrifuged at 14,000 rpm using a bench-top Eppendorf centrifuge (model 5415C, Brinkmann Instruments Inc., Westbury, N.Y.) for 10 min. The supernatant was then filtered through a 25 mm diameter 0.2 μm Nalgene syringe filter. Three hundred microliters of the cell filtrate were concentrated by 2 separate extractions with phenol: chloroform: isoamyl alcohol (25:24:1 v/v) (Life Technologies, Grand Island, N.Y.), followed by ethanol precipitation. A 95 μl aliquot of each concentrated supernatant was mixed separately with 5 μl of gel loading buffer. Fifteen microliters of each preparation were loaded onto 0.75 % agarose gel containing 0.5 μg of ethidium bromide per ml. The gels were run at 7 V/cm for 1.0 h at RT.

3.3.10 Release of lipopolysaccharides (LPS)

The possible release of outer membrane components like lipopolysaccharides (LPS) following various treatments were examined as described by Vaara (1992). One milliliter of bacterial suspension was removed after 1 h treatment using either: i) TSB (control), ii) 1.5, 2.0 or 2.5 % TSP (in a TSB solution), iii) 1.5, 2.0 or 2.5 % TSP (in a TSB solution) that was pH adjusted to 7.0 ± 0.2 with 6 N HCl, and iv) TSB that was pH adjusted to 10.0 ± 0.2, 10.5 ± 0.2 or 11.0 ± 0.2 with NaOH. After centrifugation at RT, 0.9 ml of the cell-free supernatant was freeze-dried. The freeze-dried supernatants were dissolved in 100 μl of SDS-PAGE buffer and heated at 100°C for 10 min. Each sample was then divided in two equal portions, to one of which was added a 10 μl aliquot of proteinase K solution (2.5 mg/ml). The samples were then held at 60°C for 1 h before analysis using Tricine SDS-PAGE in 14 % acrylamide gels (Schagger and Jagow, 1987).

A 10 µl aliquot of sample was applied to each lane. The gels with proteinase K-treated samples were silver stained (Amersham Biosciences Corp., Uppsala, Sweden) in accordance with the manufacturer's instructions.

3.3.11 Electron microscopy

Cells exposed to TSP or NaOH-alkaline pH were harvested by centrifugation and washed twice in 1X PBS, and pelleted by centrifugation at 12,000 x g for 10 min. Pelleted cells were resuspended in fresh fixative containing 3.0 % glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB). The cells were pelleted by centrifugation, washed three times with 0.1 M SCB, and fixed with 1.0 % osmium tetroxide (OsO_4) in 0.1 M SCB for 1 h at RT. The cells were then stained *en bloc* in saturated uranyl acetate and dehydrated in a gradient series of ethanol (50, 70, 95 and 100 % [v/v]) for 5.0 min per immersion. The cells were then washed three times for 5 min each with propylene oxide and perfused with epon-ardite. Preparations were thin sectioned and viewed using a Phillips 410 LS electron microscope.

3.4 Results and Discussion

3.4.1 Effect of TSP on cell viability

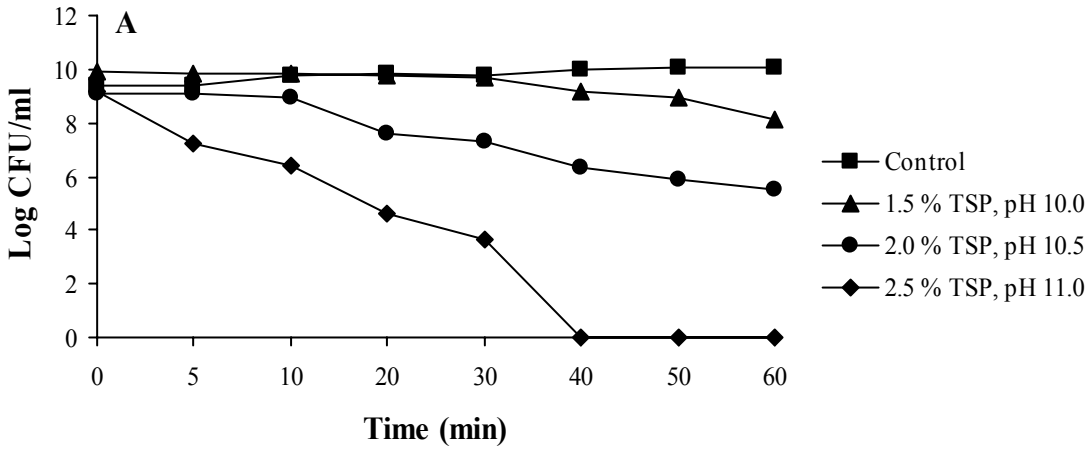
The results of this study showed that TSP reduced the viability of serovar Enteritidis cells in a concentration dependent manner (Fig. 3.1A). This effect was lost, however, if cells were exposed to TSB solutions containing 1.5, 2.0 or 2.5 % w/v TSP adjusted to pH 7.0 (Fig. 3.1B). Serovar Enteritidis cells were only slightly inhibited when treated with 1.5 % TSP (D-value = 36.90 min) or equivalent NaOH-alkaline pH

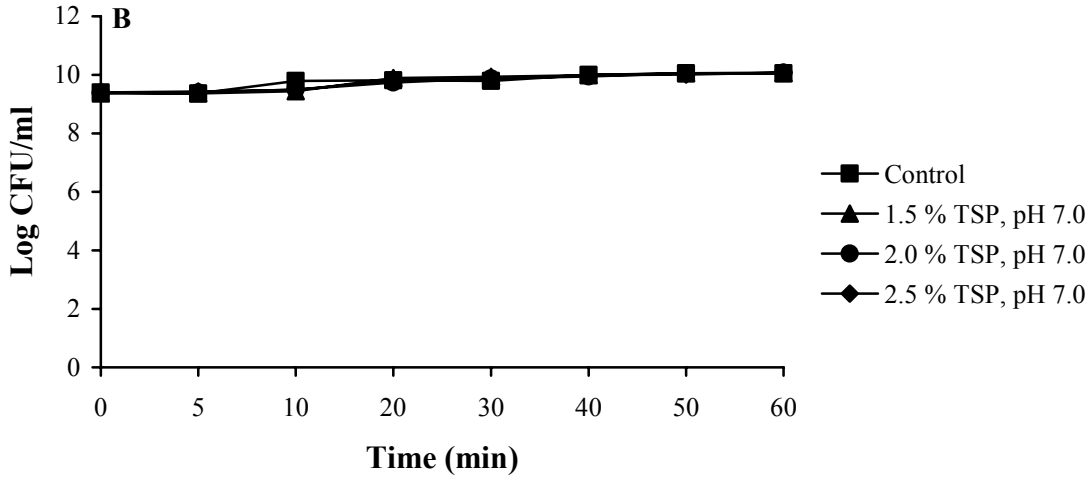
(10.0) TSB (D-value = 25.12 min) solutions for 1 h (Fig. 3.1 A and C). However, increasing the concentration of TSP in the treatment solution from 1.5 to 2.5 % resulted in a rapid loss of viability (D-values for 1.5, 2.0 and 2.5 % TSP were 36.90, 15.17 and 6.27 min, respectively), with no detectable survivors after 1 h. These decreases in survival were similar to those observed when cells were exposed to high pH TSB solutions (Fig. 3.1C). Cells of serovar Enteritidis better survived treatment with 1.5 % TSP, or equivalent NaOH-alkaline pH (pH 10.0) TSB solutions, and showed only a 2- and 3-log reduction in CFU, respectively, after a 1 h exposure (Fig. 3.1 A and C) compared to 5- and 6-log reduction in CFU within 20 min exposure to 2.5 % TSP or equivalent NaOH-alkaline pH (pH 11.0), respectively. It is noteworthy that when the pH of the various TSP treatment solutions was adjusted to 7.0, no loss in bacterial survival was observed (Fig. 3.1B), demonstrating that TSP had no effect on cell viability at pH 7.0. Thus, the effectiveness of TSP appeared to directly correlate with the alkaline pH of TSP solutions.

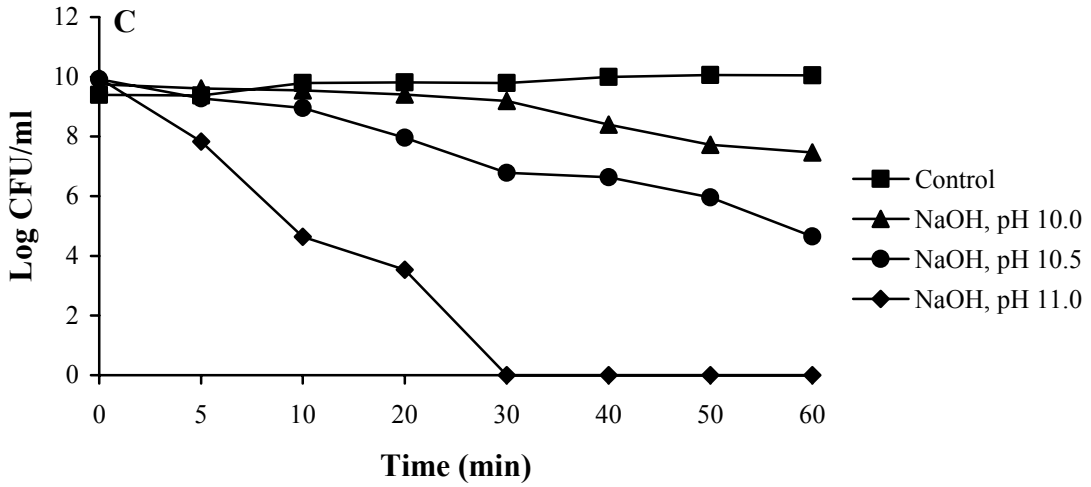
3.4.2 Propidium iodide uptake

When serovar Enteritidis cells were exposed to increasing concentrations of TSP, an increase in the uptake of the membrane-impermeant fluorescent dye, PI, was seen, suggesting that TSP treatment permeabilized both the outer and cytoplasmic membrane (Fig. 3.2). However, when cells were exposed to treatment solutions containing different concentrations of TSP where the pH had been adjusted to 7.0, the PI uptake by cells was similar to that of the control (Fig. 3.2).

Figure 3.1 Effect of different concentrations of TSP (A), TSP pH adjusted to 7.0 (B), or NaOH-alkaline pH (C) on the viability of *S. enterica* serovar Enteritidis ATCC 4931 cells over time. Following treatment, cell suspensions were diluted and plated on TSA, and CFUs enumerated after incubation at 37°C for 24 h. Log CFUs were plotted against time. Data from each treatment are the mean of three separate replications. Standard deviations were in the range of 10 to 15 % of the given values.


A
Log CFU/ml
Time (min)
Control
1.5 % TSP, pH 10.0
2.0 % TSP, pH 10.5
2.5 % TSP, pH 11.0


B
Log CFU/ml
Time (min)
Control
1.5 % TSP, pH 7.0
2.0 % TSP, pH 7.0
2.5 % TSP, pH 7.0


C
Log CFU/ml
Time (min)
Control
NaOH, pH 10.0
NaOH, pH 10.5
NaOH, pH 11.0

Gram-negative bacteria have previously been shown to be more susceptible to high pH because of their very thin (2 to 3 nm) peptidoglycan layer (Murray et al., 1965). The peptidoglycan layer stabilizes the cytoplasmic membrane of intact bacterial cells against turgor pressure (Csonka, 1989). The membranes of Gram-negative bacteria once weakened by high pH may be less capable of preventing the cytoplasmic membrane from bursting as shown by Mendonca et al. (1994) for *S. enteritidis* and *E. coli* suspended in $NaHCO_3$-NaOH buffer solution at pH 10.0.

To understand the effect of TSP or equivalent NaOH-alkaline pH on the outer membrane of serovar Enteritidis cells, stationary phase cells were pretreated with EDTA to sequester stabilizing divalent cations from the outer membrane. EDTA pretreatment of serovar Enteritidis cells significantly ($P < 0.05$) enhanced PI uptake following TSP or equivalent NaOH-alkaline pH exposure, compared to the PI uptake of cells treated similarly without EDTA pretreatment (Fig. 3.2). EDTA is known to have a significant effect on the outer membrane permeability of Gram-negative bacteria (Leive, 1974) and removes, by chelation, stabilizing divalent cations like Mg^{2+} and to a lesser extent Ca^{2+} from their binding sites in LPS. As a consequence of EDTA treatment, the outer membrane becomes more permeable to agents that otherwise would not cross the cell membrane (Helander and Vaara, 1987; Leive, 1974; Lugtenberg and van Alpen, 1983; Nikaido and Vaara, 1985). Short-term EDTA treatments of bacteria have been used to introduce macromolecules and hydrophobic compounds through the outer membrane without affecting cell viability (Helander et al., 1996; Leive, 1974; Scudamore et al., 1979; Stevens et al., 1992). In this study, short-term (30 min) treatment with EDTA alone did not affect cell viability or induce PI uptake. The addition of $MgCl_2$ during pretreatment of cells with EDTA before exposure to 2.0 % TSP inhibited PI uptake-

enhancing activity seen in EDTA pretreated cells, resulting in fluorescence levels similar to those seen in cells exposed to 2.0 % TSP without any pretreatment (Fig. 3.3). This effect is likely the consequence of the chelation of excess Mg^{2+} with EDTA, thus making EDTA unavailable for reaction with Mg^{2+} in the outer membrane and causing increased outer membrane permeability. Pretreatment of serovar Enteritidis cells with $MgCl_2$ at 0.5 to 10.0 mM (in 0.1 M Tris-HCl, pH 7.5) before exposure to TSP did not have any effect on the PI uptake following 2.0 % TSP treatment (Fig. 3.3). However, the addition of $MgCl_2$ along with 2.0 % TSP in the treatment solution resulted in a reduction of PI uptake compared to cells exposed to 2.0 % TSP alone (Fig. 3.4). This reduction in PI uptake was observed at 10 mM $MgCl_2$ concentration, and at concentrations of 25 mM $MgCl_2$ PI uptake was completely inhibited, possibly due to direct complex formation between TSP and $MgCl_2$, thus making TSP unavailable to act on the outer membrane. Alternatively, $MgCl_2$ stabilizes the outer membrane of the cells, making it resistant to the effects of TSP. The former explanation seems most likely as pretreatment with $MgCl_2$, unlike EDTA, had no effect on the uptake of PI (Fig. 3.3) following TSP exposure (i.e., TSP resulted in an increase in PI uptake with or without $MgCl_2$ pretreatment). These observations suggest that TSP, forms a complex with divalent cations in the outer membrane leading to increased permeability of the outer membrane.

Cells exposed to either 2.5 % TSP or equivalent NaOH-alkaline pH (11.0) TSB solution rapidly lost viability (Fig. 3.1) and accordingly exhibited the greatest PI uptake (Fig. 3.2). This suggested that the cells had sustained membrane damage and become permeable to the membrane impermeant dye, PI. In contrast, cells exposed to either

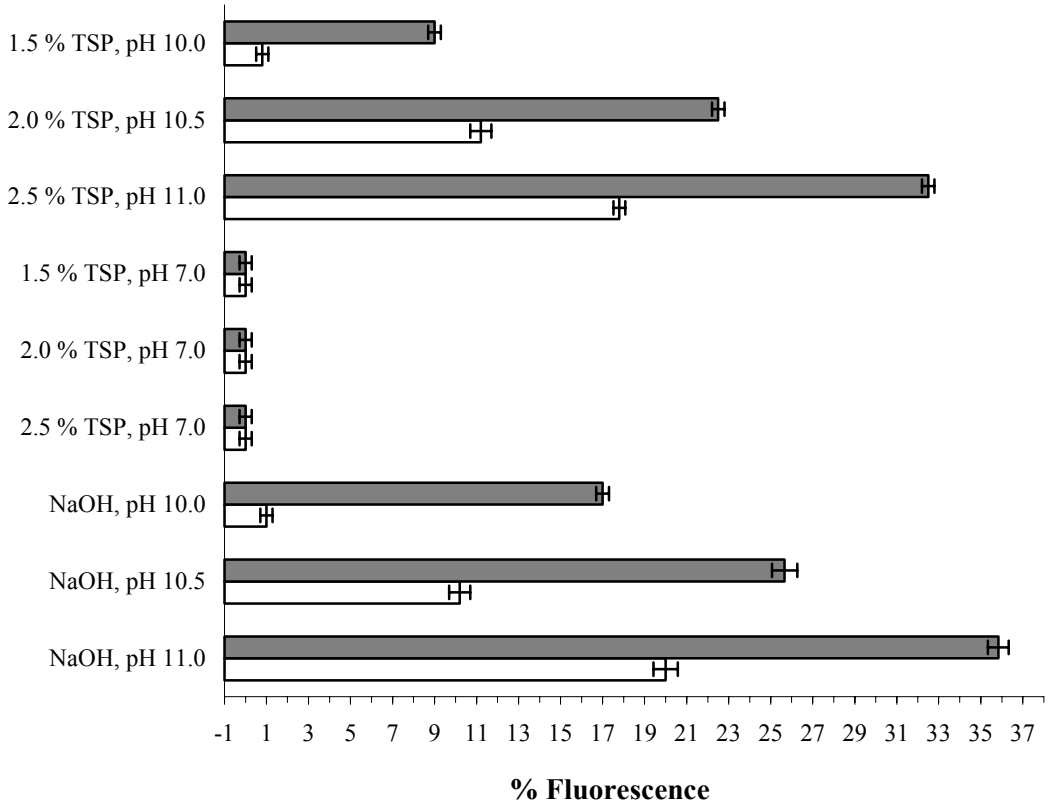


Figure 3.2 Propidium iodide uptake of *S. enterica* serovar Enteritidis ATCC 4931 cells exposed to different concentrations of TSP, TSP pH adjusted to 7.0, or NaOH-alkaline pH for 1 h following pretreatment with (solid bars), or without (open bars), 1.0 mM EDTA in 0.1 M Tris-HCl for 30 min. Percentage fluorescence value for each treatment is the mean of three separate replications. Fluorescence value obtained for untreated control cells was subtracted from all experimental values. Error bars represent ± one standard deviation.

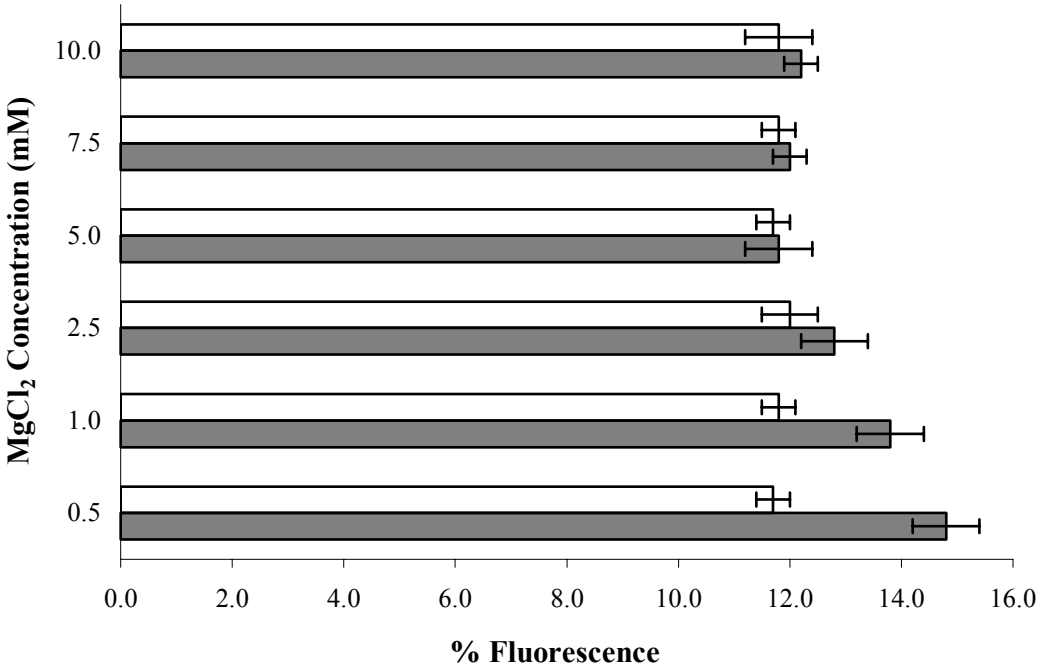


Figure 3.3 Propidium iodide uptake by *S. enterica* serovar Enteritidis ATCC 4931 cells pretreated with different concentrations of $MgCl_2$ (open bars) or $MgCl_2$ in combination with 1.0 mM EDTA (solid bars) for 30 min prior to 1 h treatment with 2.0 % TSP. Percentage fluorescence value for each treatment is the mean of three separate replications. Fluorescence value obtained for untreated control cells was subtracted from all experimental values. Error bars represent ± one standard deviation.

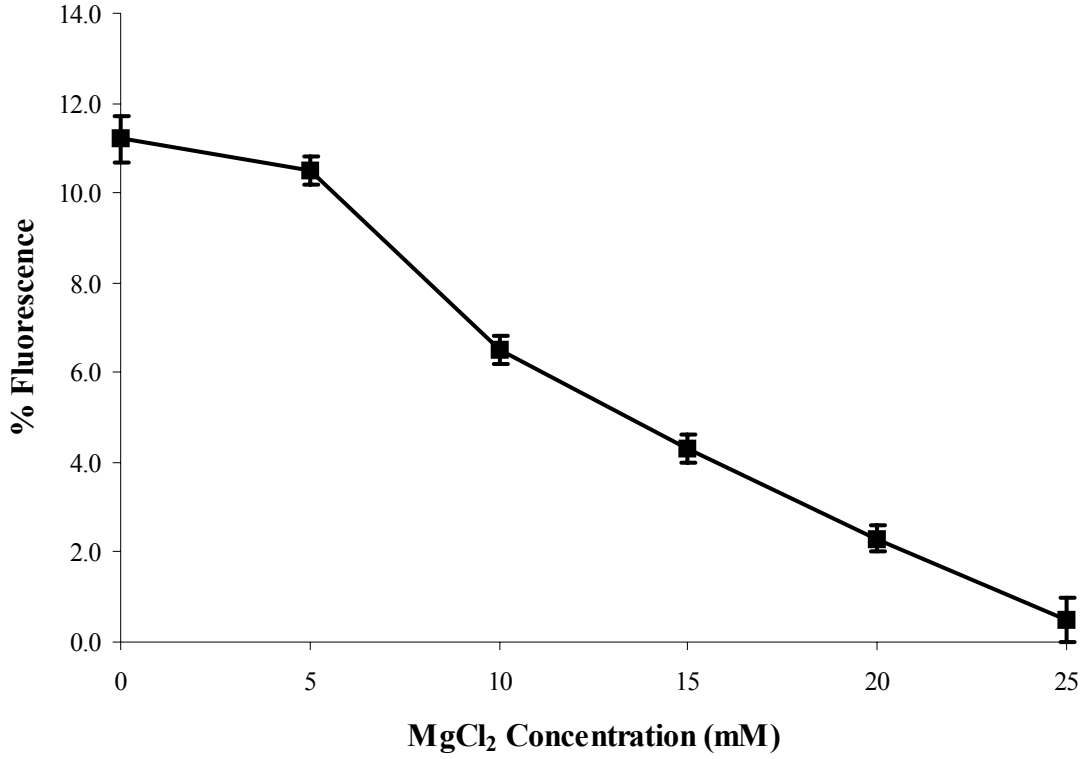


Figure 3.4 Propidium iodide uptake by *S. enterica* serovar Enteritidis ATCC 4931 cells exposed for 1 h at RT to a mixture of 2.0 % TSP and different concentrations of $MgCl_2$. Percentage fluorescence value for each treatment is the mean of three separate replications. Fluorescence value obtained for untreated control cells was subtracted from all experimental values. Error bars represent ± one standard deviation.

1.5% TSP or equivalent NaOH-alkaline pH (10.0) TSB solution, showed very little loss of viability and the least uptake of PI.

3.4.3 Osmotic response

When cell suspensions are placed in hypertonic salt solutions, an increase in OD results as a consequence of light scattering, and has been shown to be related to the extent of plasmolysis (Mager et al., 1956). This ability to plasmolyse in hypertonic salt solutions is referred to as osmotic response. In cold-shocked *Klebsiella aerogenes* cells, a good correlation between this loss of osmotic response and cell death was found to exist (Strange, 1964). Korber et al. (1996) developed a microscopic method for the direct measurement of plasmolysis in single cells, demonstrating that loss of osmotic response (i.e., loss of membrane integrity) correlated with the death of individual cells. During the present study, serovar Enteritidis cells exposed to increasing TSP concentrations lost their ability to plasmolyse in 0.75 M NaCl in a TSP concentration-dependent manner (Fig. 3.5), suggesting that the cells had sustained membrane damage. A similar response was observed when cells were exposed to high pH. Results from PI uptake and osmotic response studies confirmed that a negative relationship existed between cell viability and membrane damage when cells were exposed to TSP or equivalent NaOH-alkaline pH TSB solutions.

3.4.4 Leakage of cellular contents

While the A_{260} of filtrates of cells exposed to TSP was significantly ($P < 0.05$) higher than for untreated control values, no significant ($P < 0.05$) difference in A_{260} was

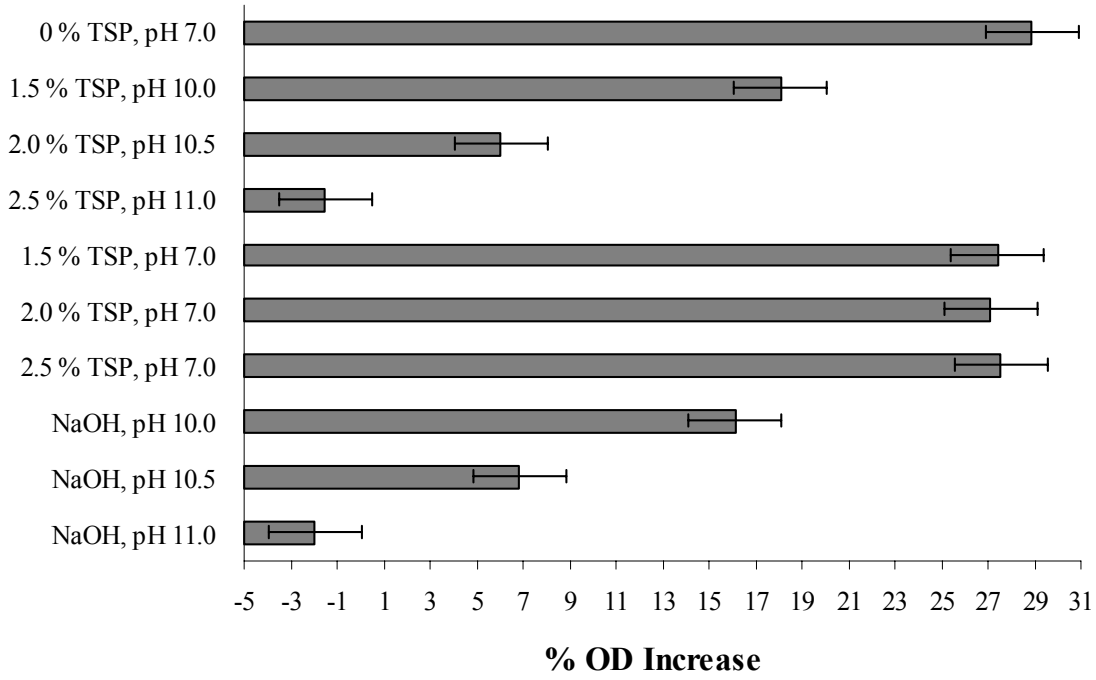


Figure 3.5 Osmotic response of *S. enterica* serovar Enteritidis ATCC 4931 cells following 1 h treatment with different concentrations of TSP, TSP pH adjusted to 7.0, or NaOH-alkaline pH. The OD_{680} of cell suspensions was measured in both PBS, and PBS containing 0.75 M NaCl. The osmotic response of treated cells was measured as the increase in OD_{680} of the cell suspension placed in 0.75 M NaCl divided by the OD_{680} in PBS. The OD_{680} increase was then expressed as a percentage of the value obtained with PBS alone. Percentage OD increase for each treatment is the mean of three separate experiments. Error bars represent ± one standard deviation.

Table 3.1 Absorbance at 260 nm and concentration of proteins in cell-free filtrates of *S. enterica* serovar Enteritidis ATCC 4931 exposed to different concentrations of TSP.

TSP Concentration (% w/v)	A_{260} nm[a]	Protein Concentration (μg/μl)[b]
0.0	0.12	0.00
0.5	1.96	1.57
1.0	1.98	1.99
1.5	1.98	2.82
2.0	1.98	3.11
2.5	1.98	3.12

Data are means of three separate replications. [a] Absorbance values from the control treatment (0 % TSP) were subtracted from the treatment values. [b] Protein content estimated by Bradford assay.

observed in filtrates of cells exposed to different concentrations of TSP (Table 3.1). Thus, increasing the time of exposure to different concentrations of TSP did not have any significant effect on A_{260} values. The presence of nucleic acids in the cell-free filtrates of TSP- or NaOH-alkaline pH-treated cells was confirmed by agarose gel electrophoresis, whereas none could be detected in untreated control (Fig. 3.6) and pH 7.0 adjusted TSP treatments (data not shown). Bradford assays performed on filtrates of cells exposed to TSP also revealed the presence of a very high concentration of protein; no protein was detected in filtrates of untreated control (Table 3.1) and pH 7.0 TSP-treated cell suspensions. Tricine SDS-PAGE analysis of cell-free supernatants of TSP-, or NaOH-alkaline pH-treated serovar Enteritidis cells revealed a prominent ladder pattern characteristic of smooth type LPS, indicating these treatments facilitated the release of LPS from the outer membrane (Fig. 3.7). Very little LPS was present in the supernatant of control or pH 7.0 TSP-treated cell suspensions.

Thus membrane damage due to TSP treatment was in fact found to be consistent with the increased release of A_{260} nm absorbing material to the medium (Table 3.1) and the presence of DNA, proteins (Table 3.1) and LPS in the filtrates of TSP-treated cell suspensions.

3.4.5 Electron microscopy

The cells used for this study were stationary phase washed cells. As a result, the electron micrographs of the untreated control (cells suspended in fresh TSB for 1h) consisted mostly of single or dividing ‘doublet’ cells (Fig. 3.8A). The dimensions and size of these cells were 0.75 to 1.0 by 0.5 micron in length and diameter. Internal ultrastructures of these cells show a centrally situated genome surrounded by the

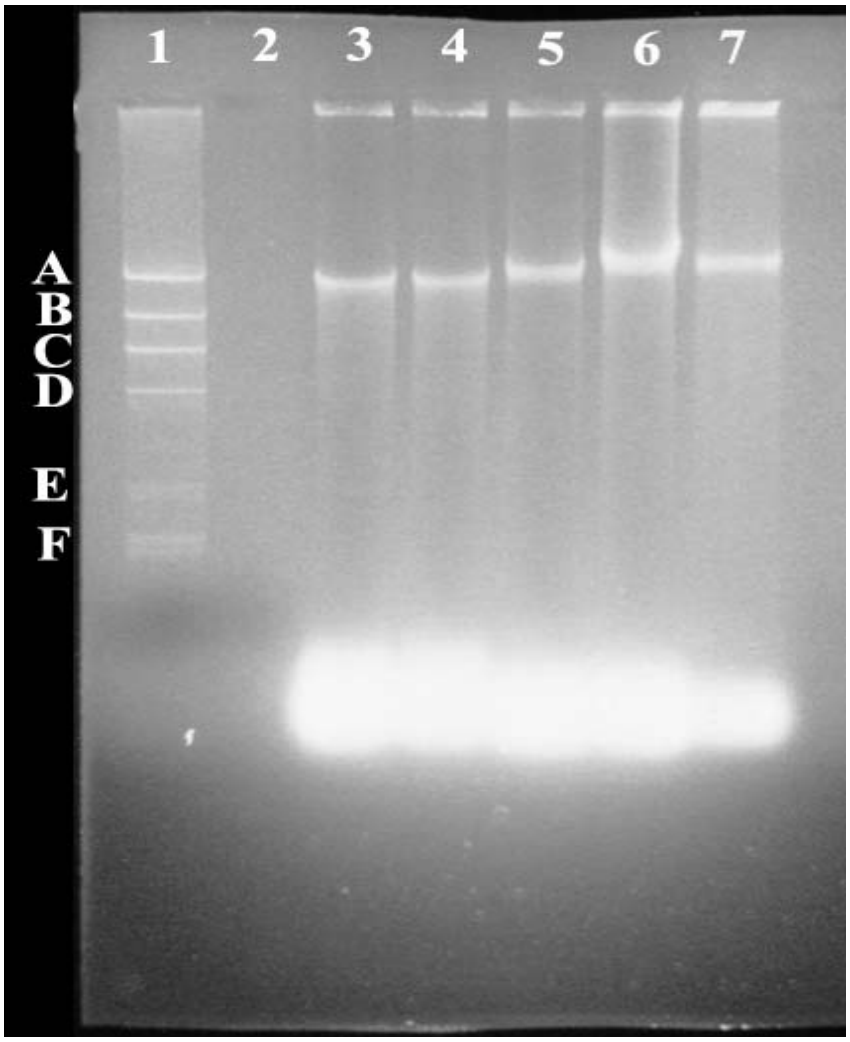


Figure 3.6 Ethidium bromide stained 0.75 % agarose gel of filtrates of *S. enterica* serovar Enteritidis ATCC 4931 cells treated with different concentrations of TSP. Lane 1- λ marker; 2- Control; 3- 0.5 % TSP; 4- 1.0 % TSP; 5- 1.5 % TSP; 6- 2.0% TSP; 7- 2.5 % TSP. λ marker: A- 23,130 bp; B- 9,416 bp; C- 6,557 bp; D- 4,361 bp; E- 2,322 bp; F- 2,027 bp.

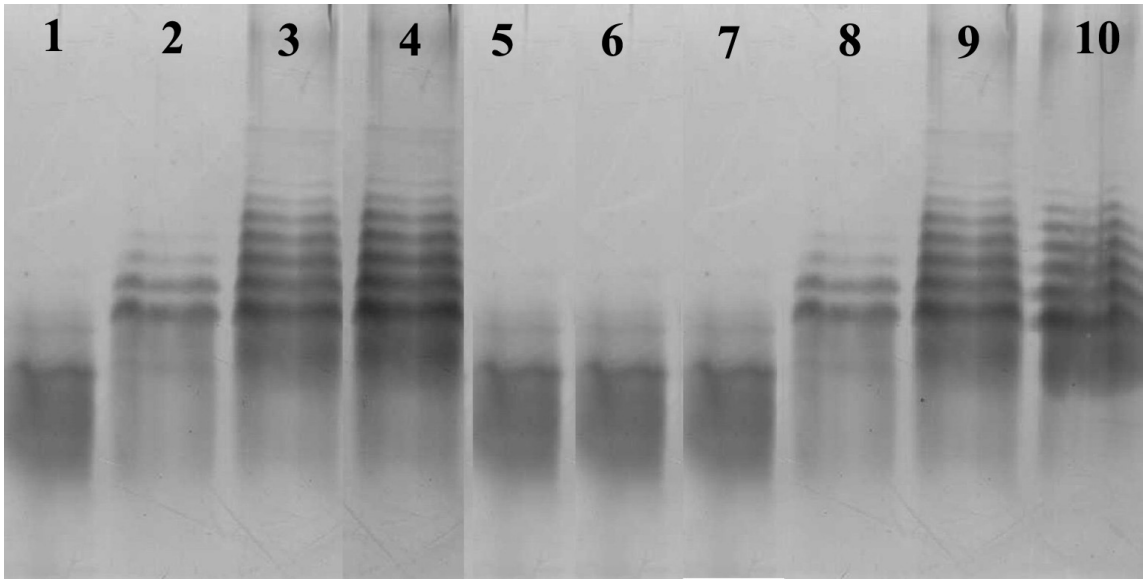


Figure 3.7 Silver-stained SDS-polyacrylamide gel (14.0 %) of proteinase K-treated filtrates of *S. enterica* serovar Enteritidis ATCC 4931 cells treated with either different concentrations of TSP, TSP pH adjusted to 7.0, or NaOH-alkaline pH. Lanes: 1- untreated filtrate; 2- 1.5 % TSP filtrate; 3- 2.0 % TSP filtrate; 4- 2.5 % TSP filtrate; 5- 1.5 % TSP pH adjusted to 7.0 filtrate; 6- 2.0 % TSP pH adjusted to 7.0 filtrate; 7- 2.5 % TSP pH to 7.0 filtrate; 8- pH 10.0 with NaOH filtrate; 9- pH 10.5 with NaOH filtrate; 10- pH 11.0 with NaOH filtrate.

cytoplasmic area with tightly packed ribosomes. The cell membranes and walls and the periplasmic spaces of these cells can be clearly distinguished. Cells treated with TSP showed that cells had lost their general shape (Fig. 3.8B). The genome area was disorganized and appeared as loosely packed DNA and polyribosomes. These effects are expected, if DNA degradation and release (Fig. 3.6) are associated with TSP treatment. TSP-treated cells also showed distortions and collapse in the membrane-wall continuity. In many instances, and especially with 2.5% TSP treatments (Fig. 3.8C), breaks in the outer membranes were evident. Finally, distinct condensation and 'beading" of cytoplasmic material (arrow heads without shafts in Fig. 3.8B) preferentially towards the inner membrane or the polar regions of the cells and some vacuolization were seen. Cells exposed to NaOH-alkaline pH (Fig. 3.9) showed a number of similarities to TSP-treated cells. Disappearance of the outer membrane and breaks between the cell wall and outer membranes observed in TSP-treated cells were also evident during NaOH-alkaline pH treatment (Fig. 3.10 A and B). Vacuolization (arrows in Fig. 3.9A), exaggerated condensation and 'beading' in the cytoplasmic area were observed similar to TSP treatments. In most cases, disrupted cytoplasmic membranes could be observed in close association with the condensed cytoplasm (wide arrow with shaft in Fig. 3.9B). The condensation of the cytoplasm in cells exposed to TSP or NaOH-alkaline pH could, in part, be explained by the fact that proteins and other cytoplasmic constituents would denature and precipitate at high pH. DNA, being highly hydrophilic (Sambrook, 1989), would remain water soluble and separate in the liquid phase from the rest of the cytoplasm and would be released out of the cell once the outer and cytoplasmic membranes were disrupted (long arrows in Fig. 3.8C and 3.10 C, and D). This was confirmed by the increased A_{260} values of filtrates of cells exposed to TSP

Figure 3.8 Electron micrographic thin sections of untreated control *S. enterica* serovar Enteritidis ATCC 4931 cells (A), cells exposed to 1.5 % TSP (B), and cells exposed to 2.5 % TSP (C). Arrows in panel B identify clear vacuolar regions. In panel C, long arrows show disruption and holes in the outer and cytoplasmic membranes, whereas the arrowhead (without shaft) shows what appeared to be compacted regions of residual ribosomes and amorphous bodies situated close to disrupted membrane. Bars, 0.5 μm.

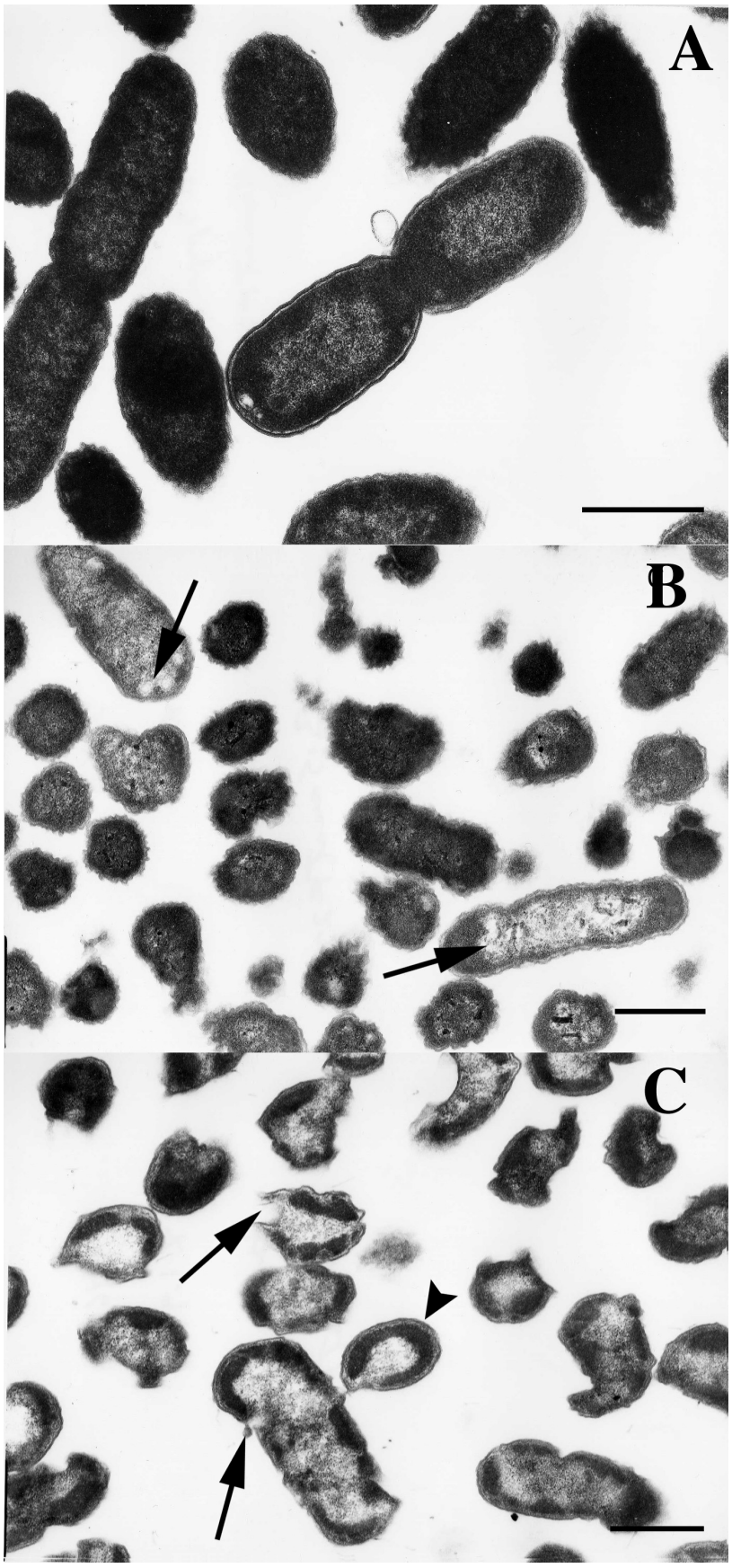
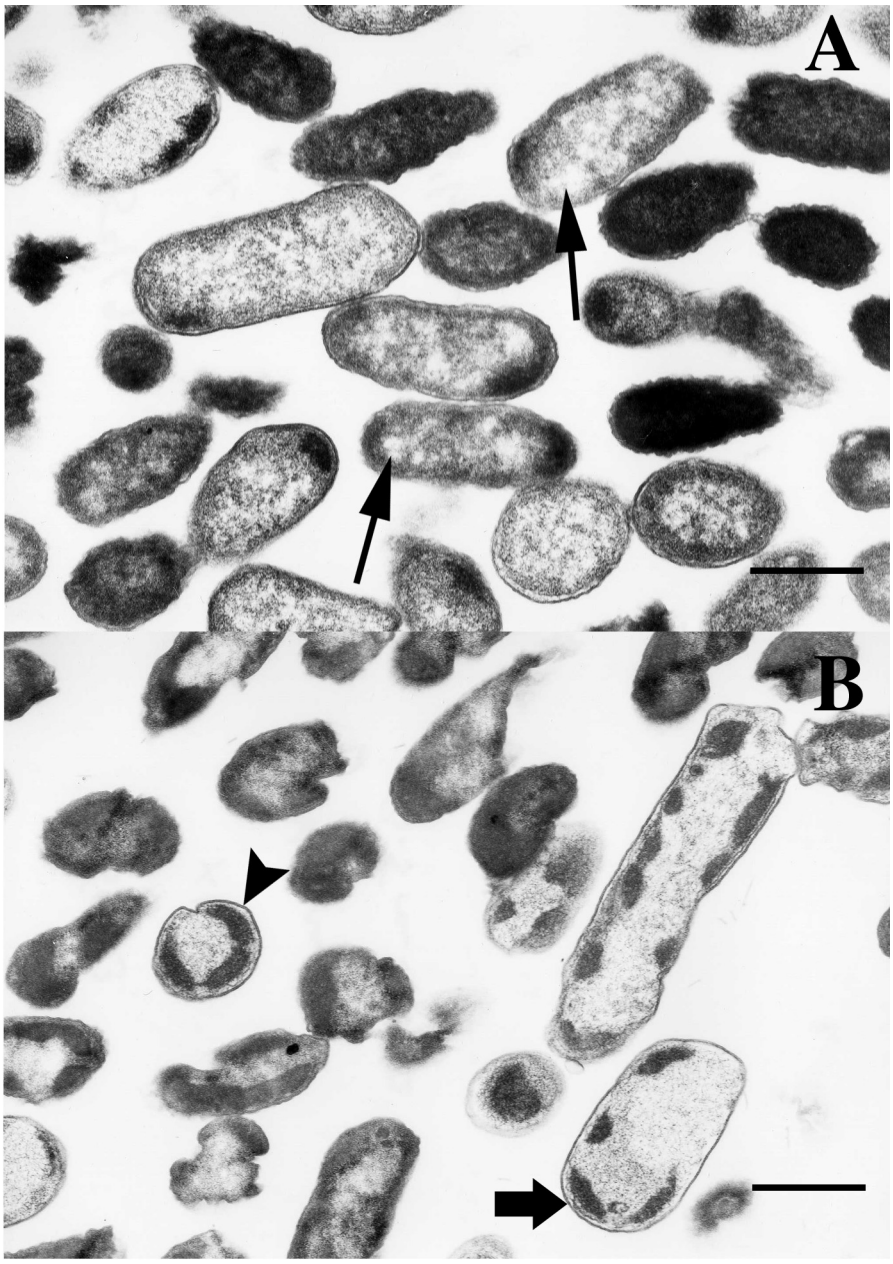

A
B
C

Figure 3.9 Electron micrographic thin sections of *S. enterica* serovar Enteritidis ATCC 4931 cells exposed to pH 10.0 (A) and 11.0 (B) TSB solutions. Arrows in panel A show clear vacuolar regions. In panel B, the arrowhead (without shaft) shows what appeared to be compacted regions of residual ribosomes and amorphous bodies situated close to the disrupted membrane, whereas the wide arrow with shaft shows an example of the disrupted cytoplasmic membrane closely associated with the condensed cytoplasm. Bars, 0.5 μm.


A
B

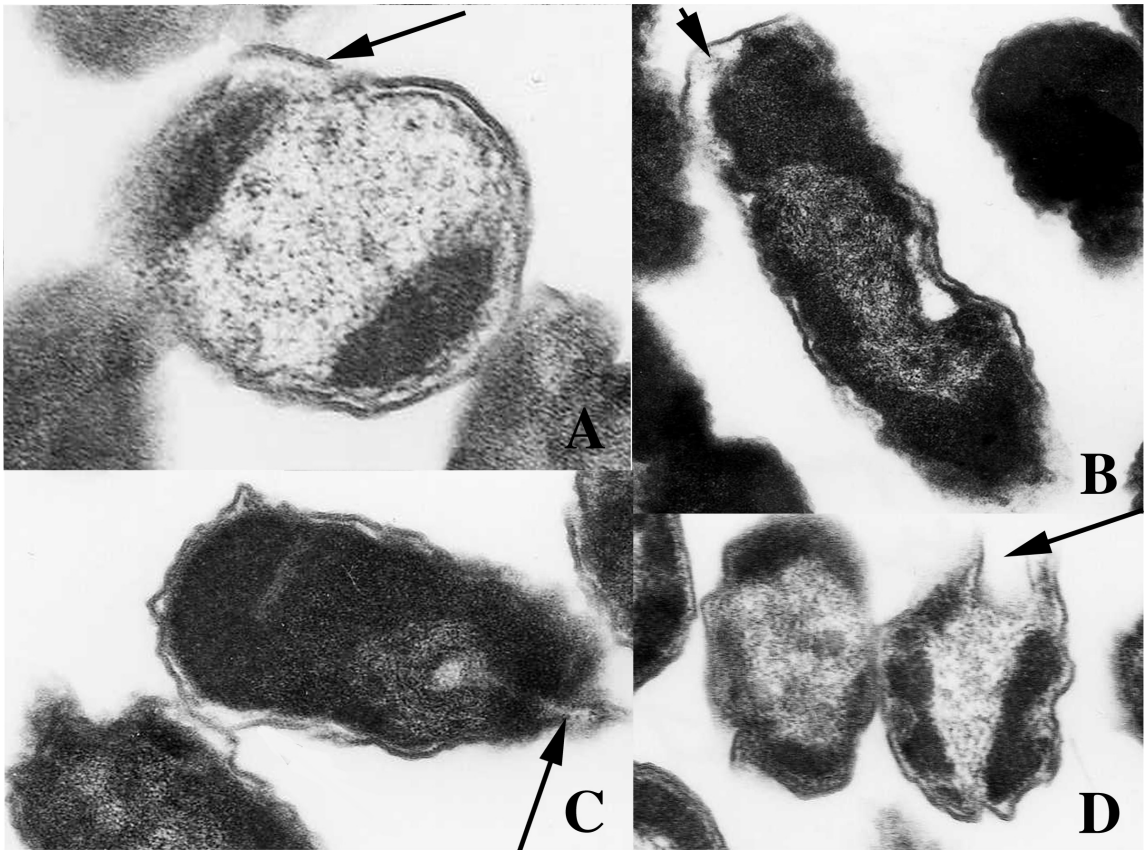


Figure 3.10 Electron micrographic thin sections of *S. enterica* serovar Enteritidis ATCC 4931 cells exposed to either pH 11.0 TSB (A, B) or 2.5 % TSP (C, D) solutions. In these magnified presentations (from Figs. 3.8 and 3.9), arrows in panels A and B indicate disrupted outer and cytoplasmic membranes and arrows in panels C and D indicate sites of discharge of intracellular contents.

or high pH and also detection of DNA on agarose gels in these filtrates (Table 3.1). Mendonca et al. (1994) reported similar membrane damage and discharge of intracellular material in *S. enteritidis* and *E. coli* exposed to pH 12 at 45°C. Membrane damage is not restricted to exposure to high pH alone. Electron microscopic studies have shown similar effects in *Salmonella* sp. and *E. coli* following starvation, freezing and exposure to lysozyme (El-Kest and Marth, 1992; Mackey et al., 1994) or high pressure (250 MPa), and in *Listeria monocytogenes*, after freezing and prolonged frozen storage or exposure to high pressure (500 MPa). A similar response was observed when *L. monocytogenes* cells were exposed to pH 12.0 (Mendonca et al., 1994).

The results presented in this study indicate that TSP is a potent membrane acting agent, as indirectly evidenced by its ability to induce the uptake of the membrane impermeant dye PI and release of LPS, DNA/RNA, and intracellular proteins from treated cells. The TSP action on membranes occurs at both functional and structural levels as directly evidenced by electron microscopic examination. The inhibition of PI uptake by $MgCl_2$ added in excess during TSP treatment suggests that the mode of action of TSP could involve chelation of divalent cations in the outer membrane. However, when the pH of the treatment solutions containing TSP was adjusted to 7.0, TSP had no effect on cell viability, and cells showed no evidence of membrane damage. Overall, these results provide compelling evidence that high pH during TSP treatment, and to a lesser extent sequestration of metal ions in the outer membrane by TSP, led to membrane damage, release of intracellular contents and cell death.

4.0 DIFFERENTIAL EXPRESSION OF PROTEINS BY *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS EXPOSED TO TRISODIUM PHOSPHATE

4.1 Abstract

Bacteria have developed adaptive mechanisms to survive stress. In this study, the responses of *Salmonella enterica* serovar Enteritidis to a sublethal dose of trisodium phosphate (TSP) or its equivalent alkaline pH made with NaOH were examined. Pretreatment of *S.* Enteritidis cells with 1.5 % TSP or pH 10.0 solutions resulted in a significant increase in thermotolerance, resistance to 2.5 % TSP, resistance to high pH and sensitivity to acid and H_2O_2. Thermotolerance, unlike resistance to high pH, was dependent on *de novo* protein synthesis. However, no heat shock proteins were up-regulated following TSP or NaOH-alkaline pH pretreatments. Total cellular and detergent-insoluble outer membrane proteins from pretreated and untreated control cells were examined by two-dimensional gel electrophoresis and SDS-PAGE, respectively. Two-dimensional-PAGE of total cellular proteins from untreated control cells resolved as many as 232 proteins, of which 22 and 15 % were absent in TSP- or NaOH-alkaline pH-pretreated cells, respectively. More than 50 % of the proteins that were either up- or down-regulated by TSP pretreatment were also up- or down-regulated by NaOH-alkaline pH pretreatment. SDS-PAGE analysis of detergent-insoluble outer membrane

proteins revealed the up-regulation of at least four proteins. Mass spectrometric analysis identified the up-regulated proteins to include those involved in the transport of small hydrophilic molecules across the cytoplasmic membrane and those that act as chaperones and aid in the export of newly synthesized proteins by keeping them in an open conformation. Other up-regulated proteins included common housekeeping proteins like those involved in amino acid biosynthesis, nucleotide metabolism and aminoacyl-tRNA biosynthesis. In addition to the differential expression of proteins following TSP or NaOH-alkaline pH treatments, change in the membrane fatty acid composition was also observed. Trisodium phosphate- or NaOH-alkaline pH-pretreated cells showed a higher saturated and cyclic to unsaturated fatty acid ratio than did the untreated control cells. These results suggest that the cytoplasmic membrane could play a significant role in thermotolerance or resistance to other stressors following TSP or NaOH-alkaline pH treatments.

4.2 Introduction

Bacteria have evolved adaptive mechanisms to face challenges of changing environments and to survive under conditions of stress. The causes of stress are numerous and include extremes of temperature and pH, salts in high concentration (osmotic pressure), oxidants, detergents, alcohol, antimicrobial agents, antibodies in sublethal doses, nutritional deprivations, etc. These stresses reflect the range of conditions that bacteria face in food and agricultural products, in animal systems, or in the environment. One of the best characterized stresses is the heat-shock response (Georgopoulos and Welch, 1993; Hecker et al., 1996). Treatment with elevated but non-lethal high temperatures can condition organisms to be able to tolerate subsequent

exposure to otherwise lethal high temperatures (Humphrey et al., 1993; Mackey and Derrick, 1986). Increased thermotolerance is conferred by the induction of the heat-shock proteins (HSPs), which enables cells to survive subsequent treatments with higher temperature (Gross, 1996). However, it has been shown that thermotolerance of *Escherichia coli* and *Salmonella* can also be increased by prior exposure to a number of sublethal stresses such as low-nutrient environments (Jenkins et al., 1990), alkaline pH (Humphrey et al., 1991), acidic pH (Hickey and Hirshfield, 1990), starvation (Jouper-Jaan et al., 1992), and a wide number of chemical pollutants found in nature (Blom et al., 1992). It is now becoming increasingly clear that exposure to one stress factor can afford protection against another, often unrelated, stress factor. The heat-shock response has been shown to involve the induction of a number of HSPs, some of which bind proteins denatured as a result of elevated temperature, protecting them from further degradation or facilitating their refolding (Georgopoulos and Welch, 1993; Parsell and Lindquist, 1993). Other HSPs are involved in proteolysis (Lon and Clp proteases) of denatured proteins. Most HSPs are synthesized at low levels under non-stress conditions but are induced rapidly and transiently upon exposure to high temperature.

In addition to the role of HSPs in the induction of thermotolerance, modifications in membrane fluidity have also been shown to be involved in the adaptation to thermal stress. Bacterial cytoplasmic membranes consist mainly of lipids and have been shown to be a site for thermal injury (Dennis and Yatvin, 1981; Hansen, 1971; Hansen and Skadhauge, 1971; Yatvin et al., 1986). The cytoplasmic membrane represents the boundary between the cytoplasm and the external environment and regulates the flow of nutrients and metabolic products in and out of the cell, thereby permitting homeostasis of the cytoplasmic environment (Brown et al., 1997; Kadner, 1996). Growth conditions

such as the composition of growth medium (Annous et al., 1997; Annous and Kozempel, 1998; Julak and Mara, 1973; Kadner, 1996; Lechevalier and Moss, 1977; Schweizer, 1989), the growth phase of the cells (Kadner, 1996; Lechevalier and Moss, 1977; McGarrity and Armstrong, 1981; Rees et al., 1995; Yatvin et al., 1986), the incubation temperature at which the bacteria were cultured (Annous et al., 1997; Dennis and Yatvin, 1981; Hansen, 1971; Kadner, 1996; McGarrity and Armstrong, 1981; Russell, 1984; Russell et al., 1995; Schweizer, 1989; Sinensky, 1974; Suutari and Laakso, 1994), and the pH (Brown et al., 1997; Lechevalier and Moss, 1977; Russell et al., 1995; Schweizer, 1989) markedly affect the composition of the membrane lipid. One of the most important consequences of membrane lipid changes in microorganisms is to modulate the activity of intrinsic proteins that perform functions such as ion pumping and nutrient uptake (Russell and Fukanaga, 1990). Russell and Fukanaga (1990) reported that an increase in the fluidity of the bacterial membrane due to the changes in the growth conditions corresponded to a decrease in thermal resistance. *Escherichia coli* cells subjected to an abrupt temperature shift from 30° to 45°C and held at the high temperature for various periods of time revealed a gradual decrease in the total unsaturated to total saturated fatty acid ratio in the cytoplasmic membrane, resulting in reduced membrane fluidity and a higher melting point (Mejía et al., 1999). Mejía et al. (1999) observed that the main changes corresponded to a decrease in the long-chain unsaturated *cis*-vaccenate (18:1*c*11) and an increase in the saturated palmitate (16:0) proportions as the heat-shock time increases. They also found that the fluidity reduction occurred during the cellular heat-shock response, as detected by the change in the induction levels of GroEL and DnaK (Lee-Rivera and Gomez-Eichelmann, 1994; Mejía

et al., 1995). In *Pediococcus* sp. a decrease in the unsaturated to saturated/cyclic fatty acid ratio during the transformation from log to stationary phase of growth was accompanied by an increase in the D-values, suggesting a relationship between thermotolerance and reduced membrane fluidity (Annous et al., 1999). An exact opposite effect was observed during cold stress. As the growth temperature was decreased, an increase in the proportion of shorter and/or unsaturated fatty acids in the lipids was observed. The membrane adaptation response has been most extensively studied for *E. coli* and it has been shown that the proportion of *cis*-vaccenic acid (C18:1) increases at low temperature at the expense of palmitic acid (C16:0). The increase in average chain length has the opposite effect on membrane fluidity, but is outweighed by the greater fluidity effect of increased unsaturation.

Similar changes in membrane fatty acid composition were also observed during acid adaptation. Acid-adapted, but not acid-shocked, *E. coli* O157:H7 cells were shown to have enhanced heat tolerance at 52°C and 54°C (Ryu and Beuchat, 1998). Acid-adaptation in *Salmonella* involves induction and repression of a number of cellular proteins which include a few HSPs, while no apparent induction of HSPs was observed in *E. coli* (Foster, 1995; Foster and Hall, 1990, 1991; Hickey and Hirshfield, 1990; O'Hara and Glenn, 1994; Raja et al., 1991; Rowbury and Goodson, 1993). In addition to the changes in expression of a number of cellular proteins, changes in the cell surface properties such as increase in cell surface hydrophobicity and induction of specific outer membrane proteins (OmpC and OmpF) were also observed in *Salmonella* and *E. coli* during acid-adaptation (Leyer and Johnson, 1993; Sato et al., 2000). The fatty acid composition of acid-adapted log-phase cells of *E. coli* is very different from that of non-

adapted cells. During acid-adaptation, monounsaturated fatty acids (16:1ω7c and 18:1ω7c) present in the phospholipids of *E. coli* are either converted to their cyclopropane derivatives (cy17:0 and cy19:0), or replaced by saturated fatty acids. The acid tolerance of *E. coli* strains appeared to be correlated with membrane cyclopropane fatty acid content and, thus, it is postulated that an increased level of cyclopropane fatty acids may enhance the survival of microbial cells exposed to acid pH (Brown et al., 1997). Brown et al. (1997) also suggested that the changes in the fatty acid composition from saturated to unsaturated and cyclopropane derivatives could also be responsible for the induced thermotolerance of acid-adapted cells.

Enterobacteria like *E. coli* and *Salmonella* species are commonly exposed to alkaline pH. Environmental alkalinization can follow exposure to polluted waters, for example from extreme alkaline sewage, chemical industry and agricultural effluents (Rowbury et al., 1989). Enterobacteria can also be subjected to alkalinity in some foods (e.g., egg whites; Humphrey et al., 1991) and some food processing treatments (Humphrey et al., 1981). The response to alkaline pH can be characterized as alkaline shock (exposure to alkaline pH for up to 30 min) and alkaline adaptation (exposure to alkaline pH for periods ranging from more than 60 min to several generations). Adaptation of *Salmonella* or *E. coli* to alkaline conditions is accompanied by induced thermotolerance, increased resistance to bile salts, and increased resistance against external alkalinization (Flahaut et al., 1997; Goodson and Rowbury, 1990; Humphrey et al., 1991; Taglicht et al., 1987). In contrast, alkaline adaptation of *S. enteritidis* or *E. coli* sensitizes the cells to acid stress (Rowbury et al., 1993) and vice versa (Rowbury and Hussain, 1996). The ability to grow at, or withstand, alkaline pHs could require the acquisition of enzymes capable of remaining active at high pH values (Kakinuma and

Igarashi, 1990) and could also be accomplished by modifications in gene expression. Although alkaline adaptation increases thermotolerance in *E. coli* and *Salmonella*, it has not been correlated with the expression of HSPs (Hickey and Hirshfield, 1990). In contrast, studies have shown the induction of heat shock proteins (HSP) by a mild pH upshift (from pH 7.0 to 8.8; 5-10 min; alkaline stress) (Taglicht et al., 1987), induction of the SOS system by alkalinization of intracellular pH (Schuldiner et al., 1986), and RecA-independent DNA repair in *E. coli* (Goodson and Rowbury, 1990). In other specific studies, an unknown alkaline shock protein (23 kDa) in *Staphylococcus aureus* (Kuroda et al., 1995) and some genes whose expression changes as a function of alkaline stress (Bingham et al., 1990; Foster and Aliabadi, 1989; Rowbury et al., 1996) have been described. However, the relationship between alkaline stress and heat shock response is not very clear. In *Enterococcus faecalis*, an alkaline shift in the external medium induced the synthesis of heat shock proteins (HSPs) but did not induce maximal thermotolerance (Flahaut et al., 1997). Thus, the mechanism underlying thermotolerance due to alkaline adaptation is not clear and is hypothesized to involve changes in the physiology of alkaline adapted cells. However, to our knowledge there exists no published report suggesting that this could be the case.

The United States Department of Agriculture (USDA) has approved the use of TSP as a post-chill processing aid to reduce bacteria on raw poultry carcasses (Bender and Brotsky, 1992; Giesse, 1992). Trisodium phosphate treatment has been shown to significantly reduce the population of *E. coli* O157:H7 (Dorsa et al., 1998; Somers et al., 1994; Taormina and Beuchat, 1999), *S. enteritidis* (Goto et al., 1997), *Salmonella typhimurium* (Dorsa et al., 1998; Salvant et al., 1997; Somers et al., 1994; Xiong et al., 1998a), *Clostridium sporogenes* (Dorsa et al., 1997, 1998), *Campylobacter jejuni*

(Salvant et al., 1997; Somers et al., 1994), and *Listeria monocytogenes* (De-Rodriguez et al., 1996; Salvant et al., 1997; Somers et al., 1994) on animal carcasses. Trisodium phosphate, when dissolved, results in an alkaline solution with a pH of 12.3 (in water) and 10.0 (in tryptic soy broth) for a 1.5 % w/v solution. Studies to understand the antimicrobial mode of action of TSP have shown that the alkaline pH of TSP and sequestration of metal ions by TSP is responsible for its antimicrobial activity and *S. enterica* serovar Enteritidis (ATCC 4931) can survive exposure to 1.5 % TSP, or its equivalent alkaline pH of 10.0 made with NaOH, for an hour (Sampathkumar et al., 2003). Therefore, it is hypothesized that TSP induces a response similar to that observed when bacteria are exposed to an alkaline pH. In this study the effect of pretreatment of *S. enterica* serovar Enteritidis with a sublethal concentration of TSP, or NaOH-alkaline pH, on tolerance to lethal stress and protein synthesis was examined.

4.3 Materials and Methods

4.3.1 Media and chemicals

Tryptic soy agar (TSA), tryptic soy broth (TSB), magnesium chloride ($MgCl_2$), phenylmethylsulfonyl fluoride (PMSF), 3-[(3-cholamidopropyl)-dimethylammonio] propane sulfonate (CHAPS), DNase, RNase A, chloramphenicol and bromophenol blue were purchased from Sigma Chemical Co. (St. Louis, MO.). Peptone water was obtained from Difco Laboratories (Detroit, MI.); trisodium phosphate dodecahydrate (TSP) from Rhodia (Cranbury, N.J.); sodium hydroxide (NaOH) from Fisher Scientific (Nepean, ON.); dithiothreitol (DTT), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), glycerol, lysozyme, mercaptoethanol, sodium dodecyl sulfate (SDS), sodium *N*-lauroyl sarcosinate (Sarkosyl), tris base and urea from Life Technologies

(Grand Island, N.Y.); 6 N HCl from BDH Chemicals (Toronto, ON.); and immobilized pH gradient (IPG) buffer (pH 4.0 to 7.0), and Immobiline™ DryStrip gels were from Amersham Bioscience Corp. (Uppsala, Sweden).

4.3.2 Bacteria and culture conditions

Salmonella enterica serovar Enteritidis (ATCC 4931) was obtained from the American Type Culture Collection (Rockville, MD.). The stock culture was grown on TSA at 37°C for 18 h and maintained for a maximum period of one month at 4°C, after which a new stock culture was prepared from the frozen stock.

4.3.3 Preparation of cell suspension

A loopful of colony material from TSA was transferred to 3.0 ml of TSB and incubated on a gyratory shaker (100 rpm) at 37°C for 24 h. Cells in the stationary phase of growth were prepared by transferring 1.0 ml of this batch culture into 100 ml of fresh TSB, and incubating at 37°C on a gyratory shaker (100 rpm) for 16 h.

4.3.4 Treatment with sublethal dose of trisodium phosphate or NaOH-alkaline pH and antibiotic treatment

Stationary phase cells were harvested by centrifuging at 12,000 x g for 10 min at 4°C (Sorvall RC-2B SuperSpeed Refrigerated Centrifuge, Kendro Laboratory Products, Asheville, NC.), washed twice with phosphate buffered saline (PBS; pH 7.0) and resuspended in 100 ml of TSB containing 1.5 % (w/v) TSP or TSB adjusted to pH 10.0 (pH equivalent of 1.5 % TSP in TSB) with NaOH prior to use. Fresh TSB (pH 7.2) was

used for control experiments. Treatment with TSP or NaOH-alkaline pH was carried out for 1 h at room temperature (RT; 23 ± 2.0ºC) in a rotary shaker (200 rpm). The sublethal dose of TSP was determined from preliminary experiments. Protein synthesis inhibition experiments were conducted with chloramphenicol (50 μg/ml) during TSP or NaOH-alkaline pH treatment.

4.3.5 Challenge conditions

Cellular pellets of control, TSP- or NaOH-alkaline pH-treated serovar Enteritidis were resuspended in TSB and exposed to: (i) pH 11.0 (adjusted with NaOH) at 37°C, (ii) pH 2.6 (adjusted with 6 N HCl) at 37°C, (iii) 30 mM H_2O_2 at 37°C, (iv) 2.5% (w/v) TSP at RT or (v) heat (55°C). The concentration of each lethal factor was determined from preliminary experimentation where several concentrations of each lethal factor were tested. At pre-determined time intervals, 1.0 ml aliquots of sample were removed, diluted in 0.1% peptone water, and appropriate dilutions plated on TSA plates and incubated for 24 h at 37°C. Colony forming units (CFU) were then enumerated.

In the case of heating at 55°C, the pellets were resuspended in 100 ml of TSB in a standard 160 ml screw-cap milk dilution bottle preheated to 55°C (Haake model F-3, Germany). Each bottle contained a sterile magnetic stir bar and a water-driven magnetic stirrer was operated under each heating vessel to ensure that a uniform temperature was maintained throughout the vessel. An uninoculated control bottle containing a thermometer was used to monitor the heating vessel temperature.

4.3.6 Data analysis

Survivor curves (Log CFU/ml versus time) were constructed using Microsoft Excel (Redmond, WA), and the negative inverse of the slope of a linear best-fit line for each survivor curve used to calculate the D-value (time at a give temperature or treatment that results in a 90% reduction in the number of surviving cells). The student *t* -test for samples of unequal size (Snedcor and Cochran, 1980) was used to test for significant ($P<0.05$) differences between average D-values.

4.3.7 Analysis of total cellular proteins

4.3.7.1 One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS-PAGE) and immunoblotting of total cellular proteins

Total cellular proteins extracted by sonicating (10 bursts, 10 s each at 60 W; Sonifier cell disruptor, Branson Ultrasonic Corp., Danbury, CT.) the cells in 50 mM HEPES (pH 7.4) were separated by 1D-SDS-PAGE (Laemmli, 1970). Two µg of the protein preparation were loaded per lane on a SDS-polyacrylamide gel (4.0 % stacking gel and 12.0 % resolving gel) and run at 100 V constant voltage for 2 h at RT using a Bio-Rad Mini-Protean® II system. The gels were silver stained as per the manufacturer's instructions (Amersham Biosciences Corp., Uppsala, Sweden). Another SDS-polyacrylamide gel was loaded with 25 µg of protein per lane and used for western blotting. The proteins were transferred to a nitrocellulose membrane using a Bio-Rad Semidry system at 20 V for 18 min. Mouse monoclonal antibodies against DnaK and GroEL HSPs were purchased from StressGen Biotechnologies Corp. (Victoria, Canada) and used as per the manufacturer's instructions for immunoblotting.

4.3.7.2 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of total cellular proteins

4.3.7.2.1 Sample preparation

Total cellular protein was extracted by resuspending washed cell pellets in 1.0 ml of lysis buffer (40 mM Tris [pH 7.0], 0.1 mg/ml lysozyme, 1.5 mM PMSF, 9 M urea, 4.0 % [w/v] CHAPS, and 100 mM DTT) for 1 h at RT. The extracts were centrifuged at 16,000 x g for 10 min (Eppendorf bench top centrifuge, model 5415C, Brinkmann Instruments Inc., Westbury, N.Y.) to remove cell debris. The resulting supernatant was treated with 0.1 volume of buffer containing 50 mM $MgCl_2$, 1 mg/ml DNase I and 0.25 mg/ml RNase A. The reaction was stopped after 15 min at 4°C with 3 volumes of ice-cold acetone. Proteins were then precipitated for 2 h at -20°C. The precipitate was collected by centrifugation at 4,500 x g for 15 min (Eppendorf bench top centrifuge, model 5804R, Brinkmann Instruments Inc., Westbury, N.Y.) and resuspended in 2.0 ml of protein solubilizing solution (9.0 M urea, 4 % [w/v] CHAPS, 100 mM DTT, 2 % [v/v] pH 4.0 to 7.0 IPG buffer) and held at RT for 1 h. The dye-binding assay of Bradford was then performed to quantify the protein concentration (Bradford, 1976).

4.3.7.2.2 Isoelectric focusing

Protein extracts were subjected to high-resolution 2D-PAGE according to the method described by O'Farrell (1975) and modified by Görg et al. (2000). Equal amounts of proteins (8 µg) were loaded onto a first-dimension gel strip. Isoelectric focusing was performed using a Multiphor™ II electrophoresis unit with Immobiline™

DryStrip Kit (Amersham Biosciences Corp., Uppsala, Sweden). The Immobiline DryStrip gels (pH 4.0 to 7.0) were rehydrated in 125 µl of rehydration solution (9.0 M urea, 2 % [w/v] CHAPS, 100 mM DTT, 2 % [v/v] pH 4.0 to 7.0 IPG buffer and 0.01 % [w/v] bromophenol blue) containing 8 µg of protein for 16 h at RT. Isoelectric focusing was achieved when the total running time yielded 60 KVh at 20°C. Following isoelectric focusing, the gels were equilibrated twice for 10 min each in fresh isoelectric focusing gel equilibration buffer (50 mM Tris-HCl [pH 8.8], 6 M urea, 30 % [v/v] glycerol, 2 % [w/v] SDS, 65 mM DTT, and 0.01 % [w/v] bromophenol blue).

4.3.7.2.3 SDS-PAGE

Equilibrated isoelectric focused strips were placed on top of a uniform SDS-14 % polyacrylamide gel for second-dimension electrophoresis with the Mini-Protean® II electrophoresis system (Bio-Rad, CA.). Second-dimension separation was carried out at 100 V constant voltage for 2 h at RT. The gels were silver stained as per the manufacture's instructions (Amersham Biosciences Corp., Uppsala, Sweden).

4.3.8 Analysis of protein spots on 2D gels

Individual gels were scanned on an Epson 1200C scanner with a transparency adapter as 8-bit grayscale 300 dpi images and then stored. Differentially expressed proteins were then detected and quantified from the stored images with PDQuest™ 2-D Analysis Software (Bio-Rad, CA.).

4.3.9 One-dimensional SDS-PAGE of detergent-insoluble outer membrane (OM) proteins

4.3.9.1 Extraction of detergent-insoluble OM proteins

Detergent-insoluble OM proteins were obtained from the cell pellets by a modification of the procedure of Filip et al. (1973) and Portnoy et al. (1984). Cell pellets were resuspended in 0.5 ml of 50 mM HEPES buffer (pH 7.4) and, while kept on ice, sonicated at the same settings as mentioned in section 4.3.7.1. The sonicated cells were then centrifuged at 16,000 x g for 2 min at 4°C (Eppendorf bench top centrifuge, model 5804R, Brinkmann Instruments Inc., Westbury, N.Y.) to remove cell debris. The supernatant was transferred to a 1.5 ml centrifuge tube, and the cell membranes were sedimented from the supernatant by centrifugation at 16,000 x g for 30 min at 4ºC. The supernatant was decanted and the cell membrane pellet was thoroughly suspended in 0.2 ml of 50 mM HEPES (pH 7.4) by repeated pipetting. The cytoplasmic membranes were solubilized by addition of an equal volume of detergent (2 % Sarkosyl in 50 mM HEPES, pH 7.4) and incubated at RT for 30 min with intermittent mixing. The detergent-insoluble OM proteins were then pelleted by centrifugation (setting mentioned above), and the protein pellet was washed once (without resuspending the pellet) with 0.5 ml of 50 mM HEPES buffer. The washed protein pellets were then suspended in 50 µl of 50 mM HEPES buffer and protein concentration quantified using the dye-binding assay of Bradford (Bradford, 1976).

4.3.9.2 SDS-PAGE of detergent-insoluble OM proteins

The detergent-insoluble OM protein preparation was separated by 1D-SDS-PAGE (Laemmli, 1970). Three micrograms of the protein preparation were loaded per lane on a SDS-polyacrylamide gel (4.0 % stacking gel and 12.0 % resolving gel) and run at 100 V constant voltage for 2 h at RT using a Bio-Rad Mini-Protean® II system. The gels were silver stained as per the manufacturer's instructions (Amersham Biosciences Corp., Uppsala, Sweden). The gels were scanned as mentioned in section 4.3.8 and densitometric analysis of differentially expressed proteins was performed using IS-1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, CA.). Different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µg) of bovine serum albumin (BSA; Pierce Biotechnology, Rockford, IL.) run on the same gel were used as concentration standards for densitometric analysis of differentially expressed proteins.

4.3.10 Mass spectrometry of proteins

Proteins spots of interest were excised from the gel, destained and in-gel digested with trypsin according to the established protocols for the MassPrep robotic workstation (Water/Micromass, Manchester, UK.). The samples were then dried in a speed-vac and reconstituted in 0.1 % trifluoroacetate (TFA). The samples were then desalted and concentrated with ZipTips C18 (Millipore Corp., Bedford, MA.) before analysis by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)-mass spectrometry (MS). Briefly, the ZipTips were wetted with 75 % acetonitrile (10 µl) twice and then equilibrated with 0.1 % TFA (10 µl) 4 times. The samples were then aspirated and dispensed through the ZipTips 5 times to bind the peptides to the ZipTips.

Trifluoroacetate (0.1 %) was then aspirated through the ZipTip 5 times to remove salts, and the sample was then eluted directly onto the MALDI plate with 75 % acetonitrile containing 5 mg/ml α-cyano-4-hydroxy cinnamic acid.

The MALDI-TOF-MS (Voyager DE-STR; Applied Biosystems, Foster City, CA.) was operated in the positive ion reflectron mode. Four hundred laser shots were averaged and processed with Data Explorer software (Applied Biosystems). The samples were internally calibrated using trypsin autolytic fragments, and database searches carried out with Protein Prospector (UCSF Mass Spectrometry Facility, San Francisco, CA.). Sample preparation and MS analysis were performed by the Mass Spectrometry facility at the National Research Council, Plant Biotechnology Institute (Saskatoon, Canada).

4.3.11 Fatty acid analysis

The total fatty acids were extracted and methyl esterified from 40 to 50 mg (wet weight) of cell pellet as described by Annous et al. (1997). A Hewlett-Packard 5890 series 2 gas-liquid chromatograph (Hewlett-Packard, Avondale, Pa.) equipped with a flame ionization detector and a capillary column Ultra 2-Hewlett Packard No. 19091B-102 (cross-linked 5% phenyl-methyl silicone; 25 m x 0.22 mm i.d.; film thickness, 0.33 mm; phase ratio, 150) with hydrogen as the carrier gas was used for the separation and detection of the fatty acid methyl esters (FAME). The FAME peaks were automatically integrated by Hewlett-Packard 3365 ChemStation software and FAMEs identified using the MIDI Microbial Identification Software (Sherlock TSBA Library version 3.80; Microbial ID, Inc., Newark, DE).

4.4 Results and Discussion

4.4.1 Influence of TSP or NaOH-alkaline pH pretreatment on thermotolerance and sensitivity against other stresses

The loss of cell viability of *S. enterica* serovar Enteritidis ATCC 4931 following exposure to TSP or its equivalent alkaline pH made with NaOH increased in a TSP concentration- or pH-dependent manner, respectively (Chapter 3.0). However, when the pH of the treatment solutions containing different concentrations of TSP was adjusted to 7.0 no loss in cell viability was observed. These results suggest that the antimicrobial activity of TSP was due to its alkaline pH. Studies on the survival of *S.* Enteritidis at different concentrations of TSP or its equivalent NaOH-alkaline pH reveal that serovar Enteritidis cells can survive exposure to 1.5 % TSP or pH 10.0 (NaOH) for 1 h without any significant loss of cell numbers (Chapter 3.0). These results suggest that serovar Enteritidis would respond in a similar fashion when exposed to a sublethal concentration TSP or its equivalent NaOH-alkaline pH. Table 4.1 shows the D-values of 1.5 % TSP- or pH 10.0-pretreated cells of *S.* Enteritidis challenged with either 55°C, 2.5 % TSP, pH 11.0 (pH equivalent to a treatment solution containing 2.5 % TSP), pH 2.6 or 30 mM H_2O_2. Pretreatment with 1.5 % TSP or pH 10.0 significantly ($P<0.05$) induced thermotolerance, resistance to TSP and high pH while sensitizing serovar Enteritidis cells to acid and H_2O_2 (Table 4.1). Chloramphenicol inhibition of protein synthesis during TSP or NaOH-alkaline pH pretreatment significantly ($P<0.05$) reduced the acquisition of induced thermotolerance or resistance to increased TSP concentration (Table 4.1), suggesting that a mechanism involving *de novo* protein synthesis is

necessary for increased thermotolerance and resistance to TSP. However, the resistance to pH 11.0 was independent of *de novo* protein synthesis as no significant ($P<0.05$) difference in resistance was observed (Table 4.1) even when protein synthesis was inhibited using chloramphenicol. Similar reports of induced thermotolerance and resistance to high pH in *S. enteritidis*, *E. coli*, and *E. faecalis* following alkaline adaptation (durations ranging from 30 to 60 min) were reported by a number of workers (Flahaut et al., 1997; Goodson and Rowbury, 1989; Humphrey et al., 1991; Humphrey et al., 1993; Rowbury et al., 1996). In *E. coli* (Rowbury et al., 1993; Rowbury and Hussain, 1996) and *E. faecalis* (Flahaut et al., 1997) pH adaptation results in the induction of a cross-response between alkaline and acid adaptation (i.e., alkaline pH-adapted cells became sensitive to acid, and vice versa). A similar response of acid sensitivity was observed in 1.5 % TSP- or pH 10.0-pretreated cells of serovar Enteritidis (Table 4.1). Alkaline adaptation involves exposure to alkaline pH for durations of 60 min and more, or even growth for up to a few generations in an alkaline growth medium. On the contrary, alkaline stress/shock involves exposure to alkaline pH for very short periods of time (5 to 10 min). The induction of thermotolerance following alkaline adaptation has previously been shown to be dependent on protein synthesis (Humphrey et al., 1993), whereas resistance to high pH appeared to be due to a phenotypic change (Goodson and Rowbury, 1989; Humphrey et al., 1991) during alkaline adaptation. The results from this study (Table 4.1) also show that the induction of thermotolerance, unlike the resistance to high pH, was dependent on protein synthesis. Therefore, it would be expected that the proteins induced during alkaline adaptation would belong to the family of HSPs responsible for induction of thermotolerance. However, to my knowledge no reports exist where increased thermotolerance following alkaline

Table 4.1 D-values of pretreated *S. enterica* serovar Enteritidis ATCC 4931 challenged with different lethal stresses.

Treatment	D-value (min)[a]				
	55°C	2.5 % TSP (w/v)	pH 11.0	pH 2.6	30 mM H_2O_2
Control	2.53A	1.37A	0.85A	1.65A	4.12A
1.5 % TSP	6.46B	2.86B	1.75B	1.29B	3.70B
1.5 % TSP + 50 μg/ml Chloramphenicol*	4.12C	2.44C	1.66B	0.57C	2.95C
pH 10.0 (NaOH)	4.04B	4.65B	2.39B	1.08B	3.61B
pH 10.0 (NaOH) + 50 μg/ml Chloramphenicol*	2.39C	1.52C	2.12B	0.43C	2.4C

[a] Values in the same column followed by different letters are significantly different ($P<0.05$).

***** D-values for protein inhibition treatments using chloramphenicol were compared with their corresponding 1.5 % TSP or pH 10.0 (NaOH) treatments for significance. Data from each treatment are the mean from three separate replications. Standard deviations were in the range of 10 to 15 % of the given values.

adaptation correlated with the induction of HSPs. Alkaline stress (5 to 10 min) in *E. coli* induced the heat shock response which involved a six-fold induction in the expression of HSP DnaK and GroEL (Taglicht et al., 1987). A similar response was also observed in *E. faecalis*, where alkaline stress (30 min) caused a 2.9- and 8.5-fold increase in DnaK and GroEL expression, respectively, but did not induce maximum thermotolerance (Flahaut et al., 1997). Therefore, the mechanism of induction of thermotolerance during alkaline adaptation is not clear and does not seem to involve the induction of HSPs.

4.4.2 Effect of TSP or NaOH-alkaline pH pretreatment on protein synthesis

Total cellular and detergent-insoluble OM proteins extracted from untreated, 1.5 % TSP- or pH 10.0-pretreated serovar Enteritidis cells were analyzed by 2D-PAGE and SDS-PAGE, respectively. Total cellular proteins separated by 1D-SDS-PAGE (Fig. 4.1A) were transferred to nitrocellulose membranes and probed with anti-DnaK (Fig. 4.1B) and anti-GroEL (Fig. 4.1C) monoclonal antibodies. No significant difference in the expression of either of the HSPs was observed in TSP- or NaOH-alkaline pH-pretreated cells compared to the untreated control, suggesting that TSP or NaOH-alkaline pH adaptation does not induce the universal stress response observed during heat-shock. This led to the hypothesis that thermotolerance induced by TSP or NaOH-alkaline pH pretreatment could involve proteins other than the common HSPs and/or could be due to some physiological changes that could occur to the cell during TSP or NaOH-alkaline pH pretreatment.

To examine differential expression of proteins due to TSP or NaOH-alkaline pH pretreatment, total cellular proteins extracted from untreated control, 1.5 % TSP- or pH 10.0-pretreated cells were separated by 2D-PAGE. Two-dimensional gels for each

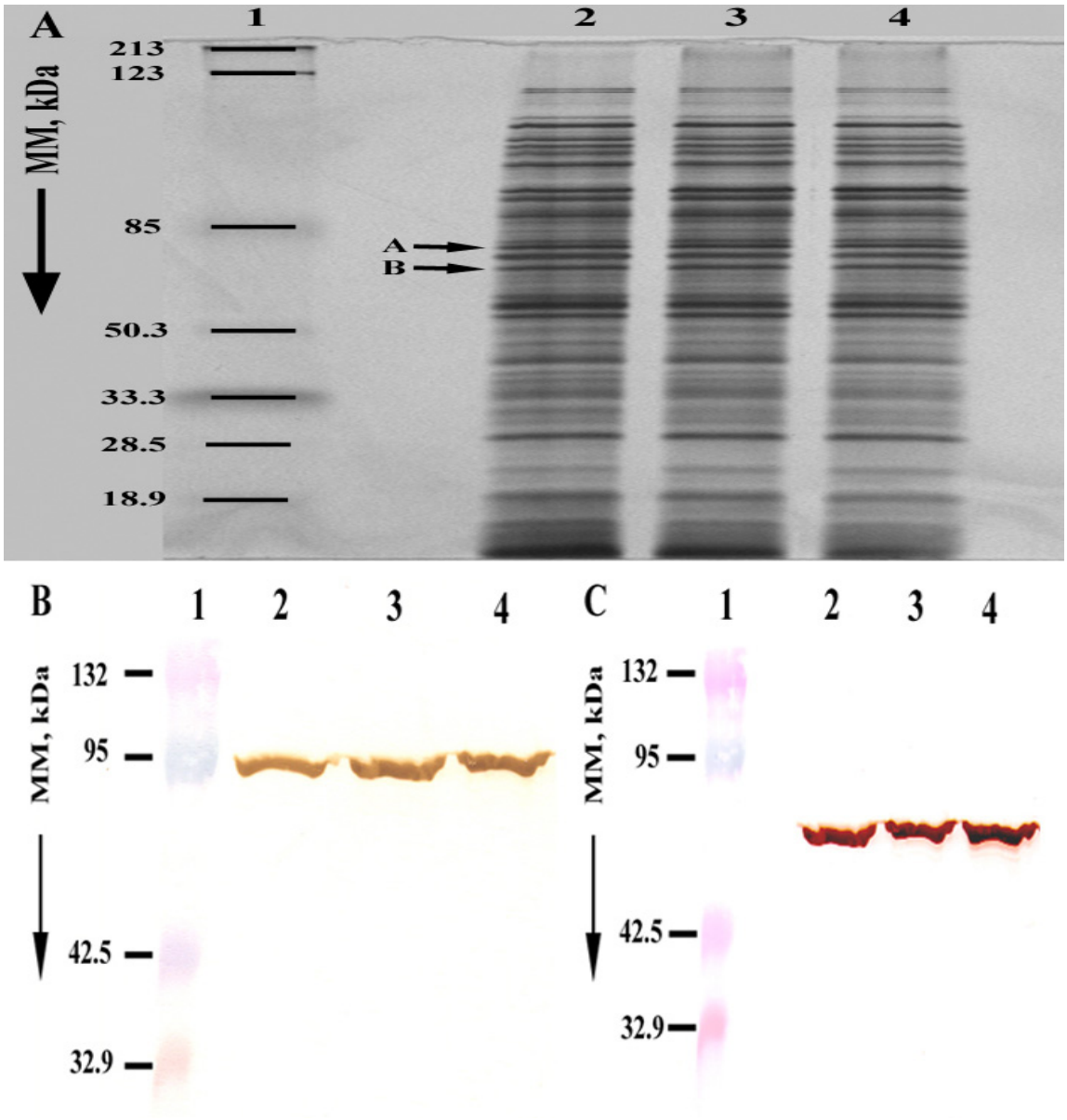


Figure 4.1 One-dimensional-SDS-PAGE profile of total cellular proteins (A) from untreated control (lane 2), 1.5 % TSP- (lane 3), and pH 10.0- (lane 4) pretreated cells of *S. enterica* serovar Enteritidis ATCC 4931 and their corresponding immunoblots probed for DnaK (B) and GroEL (C) HSPs. Lane 1 in all panels is prestained protein molecular mass ladder. Arrows labeled A and B in panel A point to DnaK and GroEL.

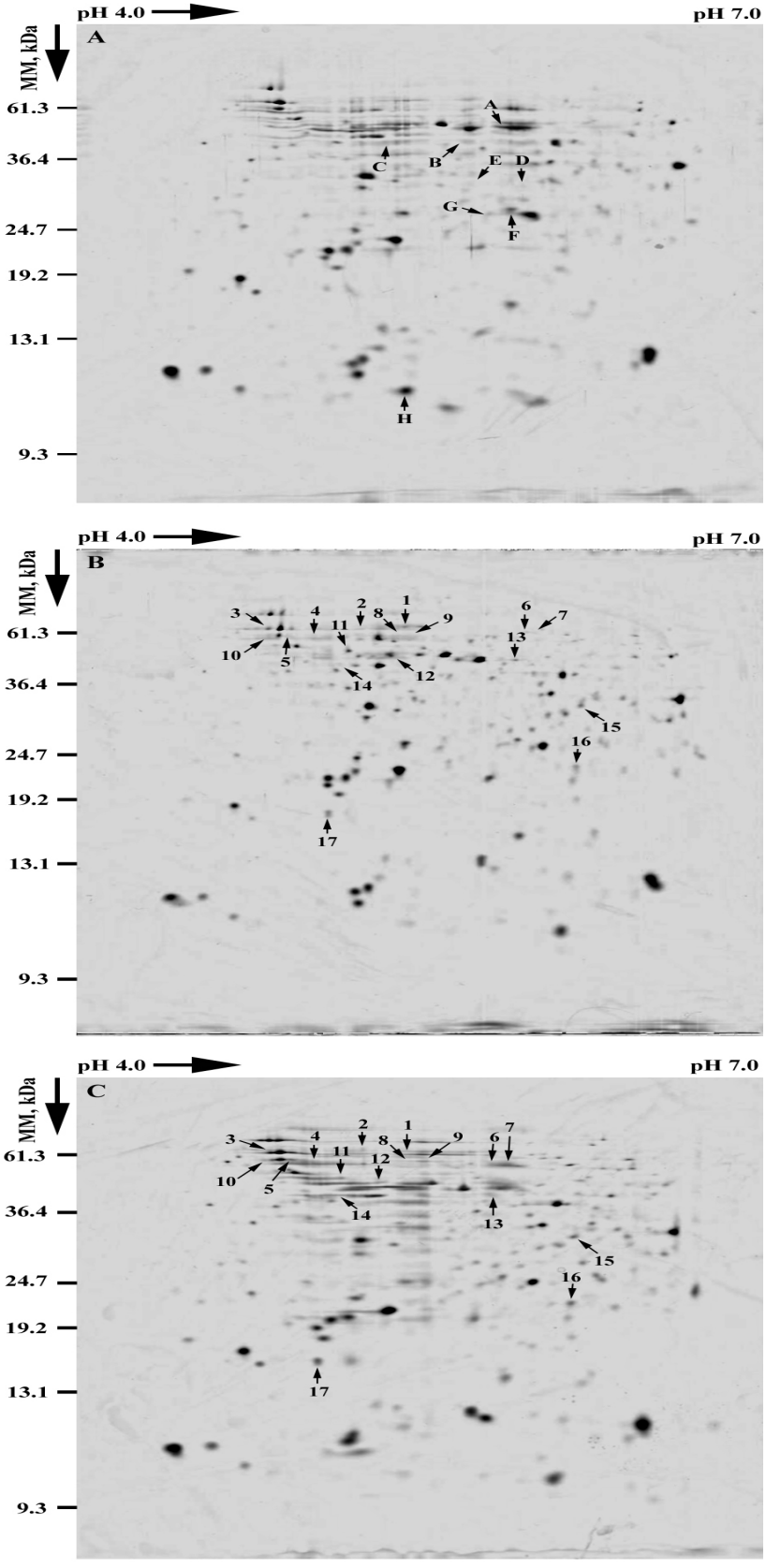
treatment were run in duplicate three times to confirm reproducibility of the protein pattern. Only protein spots that appeared in 4 out of 6 gels were selected for analysis. Of the approximately 232 proteins expressed in control cells (Fig. 4.2A), 22 % were absent in 1.5 % TSP-treated cells (Fig. 4.2B) compared to 15 % absent in pH 10.0-treated cells (Fig. 4.2C). Thirty-one proteins were up-regulated more than 2-fold by TSP pretreatment. Out of these, 4 proteins were up-regulated by more than 4-fold. An alkaline up-shift of pH of 7.0 to 10.0 up-regulated 54 proteins by more than twofold, out of which 11 and 5 proteins were up-regulated more than 4-fold and 8-fold, respectively. However, what is significant is that more than 50 % of the proteins that were either up- (Fig. 4.2B) or down-regulated (Fig. 4.2A) by 1.5 % TSP pretreatment were also up- (Fig. 4.2C) or down-regulated (Fig. 4.2A), respectively, by pH 10.0 pretreatment (Table 4.2). Differential expression of proteins by *E. coli* and *Salmonella* due to changes in their growth environment or exposure to various stresses has been reported by a number of workers. Spector et al. (1986) examined differential expression of proteins in *S. typhimurium* during phosphate limitation, ammonium limitation, carbon limitation, nicotinic acid limitation, anaerobiosis, and heat-shock. They reported that as many as 29 proteins were phosphate limitation-inducible, 24 were ammonium limitation-inducible, 11 were glucose limitation-inducible, 30 were anaerobically induced and 28 were HSPs. They also compared overlaps between the various differentially expressed proteins and reported that a few proteins induced during heat-shock or anaerobiosis was also identified as substrate limitation-inducible. Studies on the exposure of *E. coli* to a number of pollutants revealed that as many as 13 to 39 proteins were induced. Some of these proteins overlapped with HSPs and carbon starvation proteins, but at least 50 % were unique to a given pollutant (Blom et al., 1992). Hickey and Hirshfield (1990)

studied acid adaptation effects on patterns of protein synthesis in *E. coli* and *S. typhimurium* by 2D-PAGE. They reported that in *E. coli*, 13 polypeptides were elevated 1.5- to 4-fold, whereas in *S. typhimurium*, 19 polypeptides were increased 2- to 14-fold over the pH 7.0 control. However, they did not observe any apparent induction of HSPs upon acid-adaptation. Similarly, none of the proteins up-regulated by TSP or NaOH-alkaline pH pretreatment in the present study were DnaK or GroEL (Figure 4.1).

The cytoplasmic membrane, being the boundary between the cytoplasm and the external environment, is the first site in the bacterial cell that is exposed to various stresses. Accordingly, it could be expected that a response to stress would start from the cytoplasmic membrane. Thus, detergent-insoluble OM proteins from untreated control, 1.5 % TSP-, and pH 10.0-pretreated cells were examined by 1D-SDS-PAGE. The SDS-PAGE profile of these proteins showed very prominent differences in the expression of as many as four different proteins compared to their untreated controls (Fig. 4.3). Densitometric analysis of these differentially expressed proteins revealed at least a 1.5-fold increase in expression compared to the untreated control (Table 4.3).

Mass spectrometric analysis of selected differentially expressed detergent-insoluble OM proteins identified them as those involved in the transport of small hydrophilic molecules across the cytoplasmic membrane and proteins that act as chaperones and aid in the export of newly synthesized proteins by keeping them in open conformation (Table 4.4). The survival of *S.* Enteritidis to TSP or NaOH-alkaline pH exposure decreases in a concentration/pH dependent manner (Sampathkumar et al., 2003). However, *S.* Enteritidis can survive 1.5 % TSP or pH 10.0 treatments for 1 h without any significant loss in viability. This suggests that *S.* Enteritidis is capable of

Figure 4.2 Two-dimensional-PAGE profile of total cellular proteins from untreated control (A), 1.5 % TSP- (B), and pH 10.0- (C) pretreated cells of *S. enterica* serovar Enteritidis ATCC 4931. Proteins labeled in panel A are proteins that are down-regulated by both 1.5 % TSP and pH 10.0 pretreatments. Proteins labeled in panel B and C are proteins that up-regulated during both 1.5 % TSP and pH 10.0 pretreatments.


pH 4.0
pH 7.0
A
MM, kDa
61.3
36.4
24.7
19.2
13.1
9.3
A
B
C
D
E
F
G
H
pH 4.0
pH 7.0
B
MM, kDa
61.3
36.4
24.7
19.2
13.1
9.3
1
2
3
4
5
6
7
8
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10
11
12
13
14
15
16
17
pH 4.0
pH 7.0
C
MM, kDa
61.3
36.4
24.7
19.2
13.1
9.3
1
2
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17

Table 4.2 Estimated pI and molecular mass of proteins differentially expressed in *S. enterica* serovar Enteritidis ATCC 4931 following 1.5 % TSP or pH 10.0 pretreatments.

Spot I.D.[a]	Estimated pI (pH)	Estimated Mol. Mass (kDa)	Fold up-regulation[b]
A	5.93	47.56	4
B	5.72	42.52	2
C	5.35	41.80	2
D	5.95	31.99	2
E	5.72	30.97	2
F	5.89	27.07	2
G	5.76	26.73	2
H	5.44	11.37	4
1	5.41	65.69	4
2	5.24	65.69	4
3	4.83	64.96	2
4	5.03	60.53	2
5	4.94	60.53	2
6	5.90	60.53	2
7	5.93	60.53	2
8	5.39	59.76	2
9	5.45	59.76	4
10	4.86	57.45	2
11	5.10	48.28	2
12	5.29	46.48	2
13	5.90	45.02	4
14	5.16	44.32	2
15	6.19	34.02	2
16	6.12	23.60	2
17	5.09	17.76	2

[a] Spot I.D.s correlate with the protein spot I.D.s in Fig. 4.2. [b] Fold up-regulation is the relative intensity of protein spots of the TSP- or NaOH-alkaline pH-pretreated cells divided by the relative intensity of protein spots of the control.

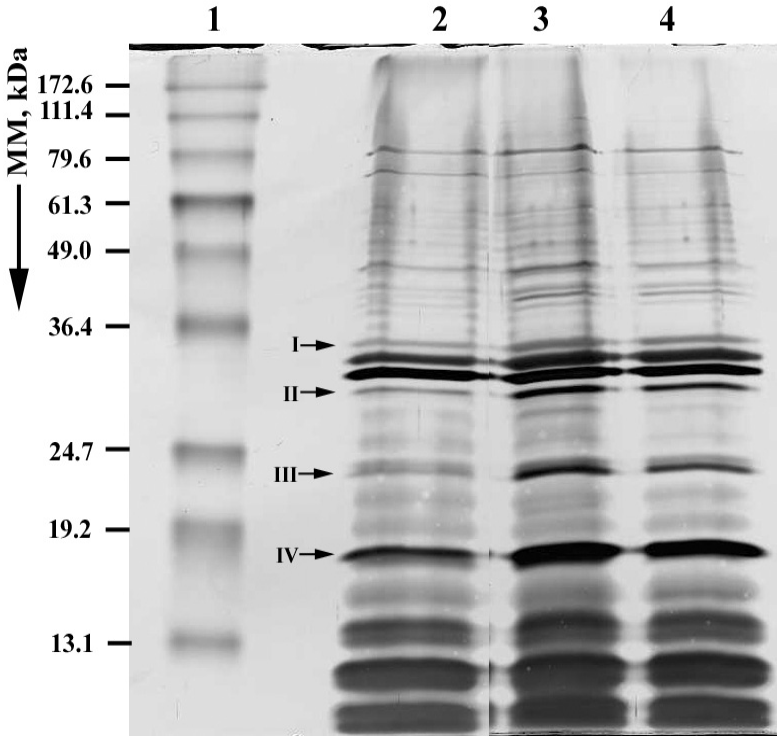

Figure 4.3 One-dimensional-SDS-PAGE profile of detergent-insoluble outer membrane proteins from untreated control (lane 2), 1.5 % TSP- (lane 3), and pH 10.0- (lane 4) pretreated cells of *S. enterica* serovar Enteritidis. Lane 1 is pre-stained protein molecular mass ladder. Arrows point to proteins that were differentially expressed following the different treatments.

Table 4.3 Estimated molecular mass and induction rate of detergent-insoluble outer membrane proteins from *S. enterica* serovar Enteritidis pretreated with TSP or NaOH-alkaline pH.

Protein I.D.[a]	Estimated Mol. Mass (kDa)	Fold-Induction[b]	
		1.5 % TSP	pH 10.0
I	39.9	2.0	1.5
II	32.7	3.0	2.2
III	23.3	2.9	1.8
IV	16.6	1.7	1.5

[a] Protein I.D.s correlates with the protein band I.D.s in Fig. 4.3. [b] Fold-induction is the integrated density value of protein bands of the TSP- or NaOH-alkaline pH-pretreated cells divided by the integrated density value of protein bands of the control. Integrated density values are determined using known concentration of BSA as a standard.

maintaining essential cellular activities at these conditions. Mass spectrometric analysis of selected differentially expressed proteins in TSP- or NaOH-alkaline pH-pretreated cells revealed up-regulation of proteins involved in amino acid biosynthesis, nucleotide metabolism and aminoacyl-tRNA biosynthesis (Table 4.4). These proteins play an important role in regulating the housekeeping functions of the cells. This may in part explain *S.* Enteritidis survival in 1.5 % TSP or pH 10.0 treatment solutions. However, pretreatment with TSP or NaOH-alkaline pH resulted in the down-regulation of a translational elongation factor (Table 4.4) involved in the binding of aminoacyl-tRNA to the A-site of ribosomes during protein synthesis. This would mean that protein synthesis in TSP or NaOH-alkaline pH pretreatment cells would be inhibited compared to untreated control cells. This factor could explain the reduction in the number of proteins expressed during TSP or NaOH-alkaline pH pretreatments compared to the untreated control.

4.4.3 Fatty acid analysis

A number of studies have suggested a relationship between membrane fluidity and stress tolerance. The composition of membrane fatty acids is responsible for the maintenance of membrane fluidity. One of the most important consequences of membrane fatty acid changes in microorganisms is to modulate the activity of intrinsic proteins that perform functions such as ion pumping and nutrient uptake (Russell and Fukanaga, 1990). Thermotolerance in *E. coli* and *Salmonella* induced by prior heat shock is accompanied by decreased membrane fluidity (Lee-Rivera and Gomez-Eichelmann, 1994; Mejía et al., 1995), in addition to the transient induction of the HSPs.

Table 4.4 Identification of selected proteins that were differentially expressed by *S. enterica* serovar Enteritidis ATCC 4931 cells following TSP or NaOH-alkaline pH pretreatment.[a]

Protein I.D.[b]	Protein	Function/Family	pI (pH)	Mol. Mass (kDa)
A	Translational elongation factor	Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis	5.3	43.3
1	Arginyl-tRNA synthetase	ATP + L-Arginine + tRNA(ARG) = AMP + Diphosphate + L-arginyl-tRNA(ARG); Belongs to class-I aminoacyl-tRNA synthetase family	6.3	72.2
3	Arginosuccinate lyase	N-(L-Argino)Succinate = Fumarate + L- Arginine; Arginine biosynthesis	5.1	50.8
11	Trigger factor	Involved in protein export. Acts as a chaperone by maintaining the newly synthesized protein in an open conformation	4.5	48.9
I	Aspartokinase 2	First step in the common biosynthetic pathway leading from ASP to diaminopimelate and LYS	4.8	44.3
II	Outer membrane protein C	Forms passive diffusion pores which allow small molecular weight hydrophilic materials across the outer membrane	4.6	41.2
III	Outer membrane protein W	Belongs to the outer membrane OMPW/ALKL family	5.6	22.9
IV	Xanthine phosphoribosyl transferase	Nucleotide metabolism; purine metabolism	5.2	17.4

[a] The protein spots were identified by MS analysis. Function/Family analyses were based on the search results from the NCBI database. [b] Protein I.D.s correlate with spot and band I.D.s in Figs. 4.2 and 4.3.

This decrease in membrane fluidity is bought about by a reduction in the ratio of unsaturated to saturated fatty acids in the membranes. In *Pediococcus* sp., a decrease in unsaturated to saturated/cyclic fatty acid ratio during transformation from log to stationary phase was accompanied by an increase in thermotolerance (Annous et al., 1999). In *E. coli*, thermotolerance induced by acid adaptation did not induce the heat shock response but was accompanied by conversion of unsaturated fatty acids present in the phospholipids to either their cyclopropane derivatives or were replaced by saturated fatty acids (Brown et al., 1997). In the present study, pretreatment of serovar Enteritidis cells with 1.5 % TSP or pH 10.0 resulted in a reduction in the concentration of unsaturated fatty acids (16:1 ω7c, 18:1 ω7c) and was accompanied by a increase in the concentration of saturated (16:0) and cyclic fatty acids (17:0, 19:0) (Table 4.5). Such a change in membrane fatty acid composition has been associated with decreased membrane fluidity and increased thermotolerance (Annous et al., 1999). Although these previous studies have correlated similar changes in the composition of the membrane fatty acids during heat shock or acid adaptation to the induction of thermotolerance, this is the first report, linking thermotolerance induced during alkaline adaptation to the changes in the membrane fatty acid composition.

The results presented in this study indicate that *S.* Enteritidis responds to both TSP and alkaline adaptation in a similar manner. Exposure of *S.* Enteritidis to a sublethal dose of TSP, or its equivalent alkaline pH made with NaOH, induces thermotolerance, resistance to high pH, resistance to increased concentration of TSP, as well as sensitizes serovar Enteritidis cells to acid and H_2O_2. While protein inhibition

Table 4.5 Fatty acid composition of *S. enterica* serovar Enteritidis ATCC 4931 exposed to different treatments.

Fatty Acid	Fatty Acid Composition (% ± SD)[a]		
	Control	1.5 % TSP (w/v)	pH 10.0
SFA			
12:0	5.17 ± 0.51	5.52 ± 0.66	5.84 ± 0.70
14:0	10.80 ± 1.40	13.11 ± 1.44	12.38 ± 1.48
16:0	25.07 ± 3.25	32.10 ± 3.53	31.59 ± 3.16
USFA			
16:1 ω7c	12.66 ± 1.39	2.40 ± 0.31	2.27 ± 0.27
18:1 ω7c	11.50 ± 1.61	4.19 ± 0.50	5.11 ± 0.61
CFA			
17:0	10.69 ± 1.38	16.85 ± 2.02	17.92 ± 2.32
19:0	6.11 ± 0.67	8.85 ± 1.06	8.95 ± 1.07

[a] Data from each treatment are the mean from three separate replications. USFA- Unsaturated fatty acid; SFA- Saturated fatty acid; CFA- Cyclic fatty acid.

studies suggested that thermotolerance induced by TSP or alkaline adaptation was dependent on protein synthesis, none of the common HSPs attributed to thermotolerance was detected. However, analysis of total cellular and detergent-insoluble OM proteins from TSP- or NaOH-alkaline pH-pretreated cells revealed the differential expression of a number of proteins involved in transport and housekeeping functions, findings that fail to explain induced thermotolerance. The relationship between membrane fatty acid composition and thermotolerance has been shown in *E. coli* following heat shock or acid adaptation. Thermotolerance due to acid adaptation does not involve the induction of HSPs, but seems to involve changes in membrane fluidity. Alkaline adaptation or TSP pretreatment results in a significant reduction in the ratio of unsaturated to saturated/cyclic fatty acid composition, similar to the changes observed during heat shock or acid adaptation. Therefore, the results from this study suggest that while TSP or NaOH-alkaline pH adaptation does not induce the universal stress response, the induction of thermotolerance and resistance to other lethal stresses in *S.* Enteritidis may be the consequence of changes in the cytoplasmic membrane such as reduction in membrane fluidity and expression of outer membrane transport proteins.

5.0 PROTEOMIC ANALYSIS OF PLANKTONIC AND BIOFILM CELLS OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS EXPOSED TO TRISODIUM PHOSPHATE

5.1 Abstract

The proteomes of *Salmonella enterica* serovar Enteritidis grown in suspension or attached to glass beads were analyzed to study the differential protein pattern expressed by planktonic and biofilm bacteria. Two-dimensional polyacrylamide gel electrophoresis, matrix-assisted laser desorption ionization-time of flight and database searches were used to identify differentially expressed proteins. In biofilm bacteria, a general reduction in the number of proteins expressed was seen compared to their planktonic counterparts (180 vs. 201 proteins for biofilm vs. planktonic bacteria), but the expression levels of 33 and 18 proteins were up- and down-regulated respectively. SDS-PAGE analysis revealed a down-regulation of most of the detergent-insoluble outer membrane proteins in biofilm bacteria. Exposure of the biofilm bacteria to TSP revealed a protein expression pattern with little similarity to the protein expression pattern of planktonic bacteria exposed to TSP. The variations in the protein patterns indicated that biofilm development is probably controlled by regulation of specific proteins involved in cell multiplication, substrate utilization and transport of newly synthesized proteins across the membrane.

5.2 Introduction

Microbial biofilms consist of microorganisms immobilized at a substratum surface, usually embedded in an organic polymer matrix of bacterial origin (Marshall, 1992). Surface-associated growth of microbial cells leading to the development of biofilms has been observed in most ecosystems (Costerton, 2000; Costerton et al., 1987; 1995). Biofilm bacteria are markedly different from their planktonic counterparts, as judged by gene expression, cellular physiology and the biofilm cell's increased resistance to antibiotics (Costerton et al., 1995; Hoyle and Costerton, 1991). Previous studies exploring biofilm formation have generally focused on identifying the organisms that comprise biofilms, their physical and chemical properties and the architecture of the biofilm (Costerton et al., 1995). In contrast, little is known about the cellular factors and molecular mechanisms required for the transition from planktonic to sessile mode of growth and subsequent development of biofilm.

The formation of a mature biofilm has been shown to occur in a sequential process that involves: (i) transport of microorganisms to a surface, (ii) microbial attachment, (iii) formation of microcolonies, and (iv) formation of mature biofilms (Marshall, 1985; Van Loosdrecht et al., 1990). Biofilm development initiates when bacteria switch from a planktonic to an attached mode of growth. Cellular components such as flagella, pili and adhesins have been shown to be involved in the formation of biofilms by various bacteria, and have been best characterized primarily through mutation analysis. The primary function of flagella in biofilm formation is assumed to be in transport and initial cell-surface interactions (De Weger et al., 1987; DeFlaun et al., 1994). Pili and pili-associated adhesins have been shown to be important for the

adherence to and colonization of surfaces (Dorel et al., 1999; O'Toole and Kolter, 1998a; Pratt and Kolter, 1998; Vidal et al., 1998). Recent investigations have been directed at determining the degree to which gene regulation during biofilm development controls the switch from planktonic to attached growth. Brözel and coworkers (1995) monitored the global gene expression patterns in attached *Pseudomonas aeruginosa* cells and observed as many as 11 proteins whose levels were altered during various stages of attachment. More recently, Sauer et al. (2001; 2002) reported as many as 30 genes and 40 gene products were altered in *Pseudomonas putida* within 6 h following attachment, and were also able to characterize five stages of biofilm development in *P. aeruginosa* using direct microscopic observation and whole-cell protein analysis. These studies have demonstrated that biofilm bacteria are very different from their planktonic counterparts and that planktonic cells undergo a complex change in their global protein/gene expression pattern during transition to the biofilm mode of growth. An understanding of various protein/gene expression changes that occur during transition from planktonic to biofilm mode of growth could help in the design of methods and agents that better control the development of biofilms on food contact surfaces and prevent cross contamination of food during processing.

Microorganisms respond to their constantly changing environments in order to compete successfully. As a response to harmful environmental conditions, the cells may produce additional proteins, often referred to as stress proteins. Well-characterized prokaryotic examples include heat-shock response (Neidhardt et al., 1984), the SOS response (Walker, 1984), oxidative stress (Christman et al., 1985; Morgan et al., 1986), starvation response (Groat et al., 1986; Matin, 1990, 1991; Matin et al., 1989), and anaerobiosis (Spector et al., 1986). Chemicals such as cadmium chloride, chlorpyrivos,

benzene, 2,4-dichloroaniline, dicotylphtalate, hexachlorobenzene, pentachlorophenol, trichloroethylene, and tetrapropylbenzosulfonate induce the synthesis of stress proteins in *Escherichia coli*, some of which are unique (Blom et al., 1992). Similar responses have been observed against antibiotics (Nakajima et al., 1998), detergents (Adamowicz et al., 1991; Flahaut et al., 1996), bile salts (Flahaut et al., 1996) and trisodium phosphate (Chapter 4.0). Exposure of *Salmonella enterica* serovar Enteritidis to sublethal concentrations of TSP results in the up-regulation of a number of proteins involved in transport of small hydrophilic molecules across the cytoplasmic membrane and proteins that act as chaperones and aid in the export of newly synthesized proteins by keeping them in an open conformation. Trisodium phosphate treatment also up-regulated common housekeeping proteins like those involved in amino acid biosynthesis, nucleotide metabolism and aminoacyl-tRNA biosynthesis (Chapter 4.0). However, all these stress response studies have been done on bacteria grown in suspension and no information regarding the response of attached bacteria to stress is available.

A number of studies have reported the increased resistance of biofilm bacteria to selected antibiotics when compared with their planktonic counterparts. Williams et al. (1997) reported that cells of *Staphylococcus aureus* biofilms on silicon surfaces were 10 times more resistant to vancomycin than were their planktonic counterparts. Ceri and coworkers (1999) reported that *P. aeruginosa* required as much as 1,024 μg of imipenem and *E. coli* required a much as 512 μg of ampicillin as the minimal biofilm eradication concentrations compared to just 1 and 2 μg, respectively, for their planktonic counterparts. A similar increase in resistance was reported against ceftazidime and

doxycycline by as much as 100 and 50 fold, respectively, in both *Pseudomonas pseudomallei* and *Streptococcus sanguis* biofilms, respectively, compared to their planktonic counterparts (Larsen and Fiehn, 1996; Vorchit et al., 1993). Investigators have observed similar increases in resistance of biofilms and attached microorganisms on food contact surfaces to various sanitizers (Cargill et al., 1991; Frank and Koffi, 1990; Lee and Frank, 1991; Mosteller and Bishop, 1993; Yu et al., 1993). Frank and Koffi (1990) demonstrated the increased resistance of *Listeria monocytogenes* attached to glass. After an initial reduction of 2-3 log cycles, the remaining attached cells survived 20 min of exposure to benzalkonium chloride (400 and 800 ppm) and acid anionic (200 and 400 ppm) sanitizers, while planktonic cells could no longer be detected after 30 sec exposure to the lowest concentration of sanitizer. Lee and Frank (1991) discovered that the time of attachment on surfaces also has a great effect on increased resistance to antimicrobial agents. Eight-day adherent cells were 100 times more resistant than the 4 h adherent population following 30 sec exposure to 200 ppm hypochlorite. Mosteller and Bishop (1993) reported that iodophor, hypochlorite, acid anionic, peroxyacetic acid, fatty acid, and quaternary ammonium sanitizers failed to provide an adequate reduction of 2-3 log cycle in numbers when 10^4 to 10^5 cells/mm^2 of *Pseudomonas fluorescens, Yersinia enterocolitica*, and *L. monocytogenes* were attached on rubber or Teflon® surfaces. However, the efficacy of these sanitizers versus a bacterial suspension of the same test bacteria resulted in a ≥5 log-cycle reduction under the same concentrations and time. Somers et al. (1994) examined the effectiveness of trisodium phosphate (TSP) against planktonic and biofilm cells of *Campylobacter jejuni*, *E. coli* O157:H7, *L. monocytogenes* and *Salmonella typhimurium*. Of the four bacteria tested, *E. coli* O157:H7 and *C. jejuni* were most sensitive to TSP treatment; 10^6 CFU/ml

of planktonic or 10^5 cfu/cm^2 of biofilm cells were eliminated by a 30 sec treatment with 1.0 % TSP. *Listeria monocytogenes* was the most resistant to the effect of TSP, requiring exposure to 8.0 % TSP for 10 min at room temperature to reduce biofilm bacteria by at least one log. Biofilm cells of *S. typhimurium* and *L. monocytogenes* were more resistant than planktonic cells. Treatment with 1.0 % TSP for 30 sec did not significantly reduce the cell population, however, increasing contact time and TSP concentrations significantly decreased survival. Slightly more than a 2.5 log decrease was observed with a 30 sec contact time at 8.0 % TSP. In contrast, planktonic cells were reduced by more than 6 logs. Possible explanations for the increased resistance of biofilm bacteria to antimicrobial agents include (i) limitations to the free diffusion of antimicrobial agents through the biofilm matrix, (ii) interaction of the antimicrobial agents with the biofilm matrix (cells and polymer), (iii) variability in the physical and chemical environments associated with individual biofilm bacteria or regions of the biofilm (e.g., varied conditions of pH, osmotic strength, or nutrients), and (iv) varied levels of metabolic activity within the biofilm milieu (Brown et al., 1988; Gilbert et al., 1990; Korber et al., 1994).

The present study examines the changes in the global protein expression patterns between *S. enterica* serovar Enteritidis cells grown in suspension (planktonic) or attached to glass beads (biofilm). The study also examines the effect of TSP exposure on the changes in the global protein expression pattern of serovar Enteritidis cells grown attached to glass beads.

5.3 Materials and Methods

5.3.1 Media and chemicals

Tryptic soy agar (TSA), tryptic soy broth (TSB), magnesium chloride ($MgCl_2$), phenylmethylsulfonyl fluoride (PMSF), 3-[(3-cholamidopropyl)-dimethylammonio] propane sulfonate (CHAPS), DNase, RNase A and bromophenol blue were purchased from Sigma Chemical Co. (St. Louis, MO.). Dithiothreitol (DTT), *N*-2-hydroxyethypiperazine-*N'*-2-ethanesulfonic acid (HEPES), glycerol, lysozyme, mercaptoethanol, sodium dodecyl sulfate (SDS), sodium *N*-lauroyl sarcosinate (Sarkosyl), tris base and urea from Life Technologies (Grand Island, N.Y.); trisodium phosphate dodecahydrate (TSP) from Rhodia (Cranbury, N.J.); immobilized pH gradient (IPG) buffer (pH 4.0 to 7.0), and Immobiline™ DryStrip gels were from Amersham Bioscience Corp. (Uppsala, Sweden).

5.3.2 Bacteria and culture conditions

Salmonella enterica serovar Enteritidis (ATCC 4931) was obtained from the American Type Culture Collection (Rockville, MD.). The stock culture was grown on TSA at 37°C for 18 h and maintained for a maximum period of one month at 4°C, after which a new stock culture was prepared from the frozen stock.

5.3.3 Preparation of cell suspension

A loopful of colony material from TSA was transferred to 3.0 ml of TSB and incubated on a gyratory shaker (100 rpm) at 37°C for 24 h. Cells in the stationary phase

of growth were prepared by transferring 1.0 ml of this batch culture into 100 ml of fresh TSB, and incubating at 37°C on a gyratory shaker (100 rpm) for 16 h.

5.3.4 Culturing of *S. enterica* serovar Enteritidis cells in suspension (planktonic cells)

One milliliter of the above stationary phase cells were transferred to 100 ml of TSB and incubated at room temperature (RT; 23 ± 2.0°C) for 12 h in a rotary shaker (200 rpm). After 12 h incubation, planktonic cells were harvested by centrifuging at 12,000 x g for 10 min at 4°C and washed twice with phosphate buffered saline (PBS; pH 7.0) and the pellet stored at -70°C until protein extraction.

5.3.5 Culturing of *S. enterica* serovar Enteritidis cells attached to glass beads (biofilm cells)

A 60 ml sterile syringe filled with acid washed sterile 3.0 mm glass beads was used to cultivate biofilms. The glass bead filled syringe was held in an upright position and attached to sterile silicon tubing (1.5 mm diameter) at the bottom and top, so that TSB could be pumped (Watson Marlow 5017) in from the bottom and effluent TSB collected from the top in a reservoir. To inoculate the bead column 30 ml of the above stationary phase serovar Enteritidis cells were injected into the syringe and held for 30 min at RT to enable attachment before the flow of TSB was initiated. A TSB flow velocity of 0.008 cm/sec was maintained for culturing serovar Enteritidis cells attached to glass beads. The pore space flow velocity for the 60 ml syringe filled with 3.0 mm glass beads was calculated using the following formula:

$$\text{Flow velocity} = \frac{Q}{(\text{Cross sectional area x porosity})}$$

Q is the bulk flow rate in ml (cm^3)/sec which was calculated by recording the volume of liquid pumped per unit time. The cross sectional area of the syringe is equal to πr^2 (cm^2). Porosity is calculated by subtracting the volume of liquid retained in the syringe filled with glass beads (25.8 ml) from the total volume of liquid in the syringe without the glass beads (60 ml). Porosity was expressed as a ratio and hence is unitless. The volume of liquid in the syringe without glass beads was taken as 1. The porosity of the bead filled column was determined to be 0.57 (1- 0.43).

Serovar Enteritidis biofilms were allowed to grow on the surface of the glass beads under the above flow regime for 12 h at RT. After 12 h, TSB flow was stopped and the residual TSB and floating serovar Enteritidis cell in the syringe were drained thoroughly before transferring the glass beads into a sterile test tube (20 mm diameter). Firmly attached serovar Enteritidis biofilm cells were then dislodged in 10 ml of PBS (pH 7.0) by repeated vigorous vortexing (5 times at 30 sec each). The suspended cells were harvested by centrifuging at 12,000 x g for 10 min at 4°C (Sorvall RC-2B SuperSpeed Refrigerated Centrifuge, Kendro Laboratory Products, Asheville, NC.) and washed twice with PBS (pH 7.0) and the pellet stored at -70°C until protein extraction.

5.3.6 Exposure of *S. enterica* serovar Enteritidis biofilm cells to trisodium phosphate

Serovar Enteritidis biofilms were grown on glass beads for 12 h as detailed in the previous section. After 12 h, TSB flow was stopped and the residual TSB and floating serovar Enteritidis cells in the syringe were drained before refilling the syringe with TSB

containing 1.5 % TSP. The attached cells were then held at these conditions for 1 h at RT. After 1 h, the entire contents of the syringe was transferred to a 100 ml centrifuge tube and cells bought into suspension by repeated vortexing (5 times at 30 sec each) with 10.0 ml PBS (pH 7.0). The suspended cells were harvested by centrifuging at 12,000 x g for 10 min at 4°C and washed twice with PBS (pH 7.0) and the pellet stored at -70°C until protein extraction.

5.3.7 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of total cellular proteins

5.3.7.1 Sample preparation

Total cellular protein was extracted by resuspending washed cell pellets in 1.0 ml of lysis buffer (40 mM Tris [pH 7.0], 0.1 mg/ml lysozyme, 1.5 mM PMSF, 9 M urea, 4.0 % [w/v] CHAPS, and 100 mM DTT) for 1 h at RT. The extracts were centrifuged at 16,000 x g for 10 min (Eppendorf bench top centrifuge, model 5415C, Brinkmann Instruments Inc., Westbury, N.Y.) to remove cell debris. The resulting supernatant was treated with 0.1 volume of buffer containing 50 mM $MgCl_2$, 1 mg/ml DNase I and 0.25 mg/ml RNase A. The reaction was stopped after 15 min at 4°C with 3 volumes of ice-cold acetone. Proteins were then precipitated for 2 h at -20°C. The precipitate was collected by centrifugation at 4,500 x g for 15 min (Eppendorf bench top centrifuge, model 5804R, Brinkmann Instruments Inc., Westbury, N.Y.) and resuspended in 2.0 ml of protein solubilizing solution (9.0 M urea, 4 % [w/v] CHAPS, 100 mM DTT, 2 % [v/v] pH 4.0 to 7.0 IPG buffer) and held at RT for 1 h. The dye-binding assay of Bradford (Bradford, 1976) was then performed to quantify the protein concentration.

5.3.7.2 Isoelectric focusing

Protein extracts were subjected to high-resolution 2D-PAGE according to the method described by O'Farrell (1975) and modified by Görg et al. (2000). Equal amounts of proteins (8 µg) were loaded onto a first-dimension gel strip. Isoelectric focusing was performed using a Multiphor™ II electrophoresis unit with Immobiline™ DryStrip Kit (Amersham Biosciences Corp., Uppsala, Sweden). The Immobiline DryStrip gels (pH 4.0 to 7.0) were rehydrated in 125 µl of rehydration solution (9.0 M urea, 2 % [w/v] CHAPS, 100 mM DTT, 2 % [v/v] pH 4.0 to 7.0 IPG buffer and 0.01 % [w/v] bromophenol blue) containing 8 µg of protein for 16 h at RT. Isoelectric focusing was achieved when the total running time yielded 60 KVh at 20°C. Following isoelectric focusing, the gels were equilibrated twice for 10 min each in fresh isoelectric focusing gel equilibration buffer (50 mM Tris-HCl [pH 8.8], 6 M urea, 30 % [v/v] glycerol, 2 % [w/v] SDS, 65 mM DTT, and 0.01 % [w/v] bromophenol blue).

5.3.7.3 SDS-PAGE

Equilibrated isoelectric focused strips were placed on top of a uniform SDS-14 % polyacrylamide gel for second-dimension electrophoresis with the Mini-Protean® II electrophoresis system (Bio-Rad, CA.). Second-dimension separation was carried out at 100 V constant voltage for 2 h at RT. The gels were silver stained as per the manufacture's instructions (Amersham Biosciences Corp., Uppsala, Sweden).

5.3.8 Analysis of protein spots on 2D gels

Individual gels were scanned on an Epson 1200C scanner with a transparency adapter as 8-bit grayscale 300 dpi images and then stored. Differentially expressed proteins were then detected and quantified from the stored images with PDQuest™ 2-D Analysis Software (Bio-Rad, CA.).

5.3.9 One-dimensional SDS-PAGE of detergent-insoluble outer membrane (OM) proteins

5.3.9.1 Extraction of detergent-insoluble OM proteins

Detergent-insoluble OM proteins were obtained from the cell pellets by a modification of the procedure of Filip et al. (1973) and Portnoy et al. (1984). Cell pellets were resuspended in 0.5 ml of 50 mM HEPES buffer (pH 7.4) and sonicated (10 bursts, 10 s each at 60 W; Sonifier cell disruptor, Branson Ultrasonic Corp., Danbury, CT.) on ice. The sonicated cells were then centrifuged at 16,000 x g for 2 min at 4°C (Eppendorf bench top centrifuge, model 5804R, Brinkmann Instruments Inc., Westbury, N.Y.) to remove cell debris. The supernatant was transferred to a 1.5 ml centrifuge tube, and the cell membranes were sedimented from the supernatant by centrifugation at 16,000 x g for 30 min at 4ºC. The supernatant was decanted and the cell membrane pellet was thoroughly suspended in 0.2 ml of 50 mM HEPES (pH 7.4) by repeated pipetting. The cytoplasmic membranes were solubilized by addition of an equal volume of detergent (2 % Sarkosyl in 50 mM HEPES, pH 7.4) and incubated at RT for 30 min with intermittent mixing. The detergent-insoluble OM proteins were then pelleted by centrifugation (setting mentioned above), and the protein pellet was washed once

(without resuspending the pellet) with 0.5 ml of 50 mM HEPES buffer. The washed protein pellets were then suspended in 50 µl of 50 mM HEPES buffer and protein concentration quantified using the dye-binding assay of Bradford (1976).

5.3.9.2 SDS-PAGE of detergent-insoluble OM proteins

The detergent-insoluble OM protein preparation was separated by 1D-SDS-PAGE (Laemmli, 1970). Three micrograms of the protein preparation were loaded per lane on a SDS-polyacrylamide gel (4.0 % stacking gel and 12.0 % resolving gel) and run at 100 V constant voltage for 2 h at RT using a Bio-Rad Mini-Protean® II system. The gels were silver stained as per the manufacturer's instructions (Amersham Biosciences Corp., Uppsala, Sweden). The gels were scanned as mentioned in section 5.3.8 and densitometric analysis of differentially expressed proteins was performed using IS-1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, CA.). Different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µg) of bovine serum albumin (BSA; Pierce Biotechnology, Rockford, IL.) run on the same gel were used as concentration standard for densitometric analysis of differentially expressed proteins.

5.3.10 Mass spectrometry of proteins

Proteins spots of interest were excised from the gel, destained and in-gel digested with trypsin according to the established protocols for the MassPrep robotic workstation (Water/Micromass, Manchester, UK.). The samples were then dried in a speed-vac and reconstituted in 0.1 % trifluoroacetate (TFA). The samples were then desalted and concentrated with ZipTips C18 (Millipore Corp., Bedford, MA.) before analysis by

matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)-mass spectrometry (MS). Briefly, the ZipTips were wetted with 75 % acetonitrile (10 µl) twice and then equilibrated with 0.1 % TFA (10 µl) 4 times. The samples were then aspirated and dispensed through the ZipTips 5 times to bind the peptides to the ZipTips. Trifluoroacetate (0.1 %) was then aspirated through the ZipTip 5 times to remove salts, and the sample was then eluted directly onto the MALDI plate with 75 % acetonitrile containing 5 mg/ml α-cyano-4-hydroxy cinnamic acid.

The MALDI-TOF-MS (Voyager DE-STR; Applied Biosystems, Foster City, CA.) was operated in the positive ion reflectron mode. Four hundred laser shots were averaged and processed with Data Explorer software (Applied Biosystems). The samples were internally calibrated using trypsin autolytic fragments, and database searches carried out with Protein Prospector (UCSF Mass Spectrometry Facility, San Francisco, CA.). Sample preparation and MS analysis were performed by the Mass Spectrometry facility at the National Research Council, Plant Biotechnology Institute (Saskatoon, Canada).

5.4 Results and Discussion

Total cellular and detergent-insoluble OM proteins extracted from serovar Enteritidis planktonic and biofilm cells were analyzed by 2D PAGE and SDS-PAGE. Two-dimensional PAGE of total cellular proteins from both planktonic and biofilm cells were performed in duplicate three times to confirm reproducibility of the protein pattern. Only spots that appeared in 4 out of 6 gels were selected for analysis. Differences in protein profiles between planktonic and biofilm cells could be observed over a wide range of molecular mass and pH. In biofilm bacteria, a general reduction in the number

of proteins expressed was seen compared to their planktonic counterparts (180 vs. 201 proteins for biofilm vs. planktonic cells), but the expression levels of a number of proteins in biofilm bacteria were up-regulated compared to the planktonic cells. Of the approximately 201 proteins expressed by serovar Enteritidis planktonic cells, 10 % were not expressed in biofilm bacteria (Fig. 5.1). Planktonic serovar Enteritidis cells had as many as 18 proteins up-regulated by more than twofold, out of which 5 proteins were up-regulated fourfold (Fig. 5.1A, Table 5.1). In contrast, biofilm bacteria had as many as 33 proteins up-regulated more than twofold, of which 8 proteins were up-regulated more than fourfold and 3 were up-regulated eightfold (Fig. 5.1B, Table 5.2). Altogether, 21 unique proteins were seen in planktonic cells (Fig. 5.1A, Table 5.3), while biofilm bacteria expressed 8 unique proteins (Fig. 5.1B, Table 5.4). Steyn et al. (2001) and Oosthuizen et al. (2001) have shown in *P. aeruginosa* and *Bacillus cereus*, respectively, differential expression of proteins following growth attached to surfaces. Steyn et al. (2001) used glass wool as a substratum for *P. aeruginosa* biofilms and observed that the levels of as many as 18 proteins were higher in biofilm cells and for five proteins the levels were lower in biofilm cells when compared to planktonic cells. They also observed six unique proteins in planktonic cells, while the biofilm cells contained five unique proteins. Similarly, Oosthuizen et al. (2001) observed 10 proteins differentially expressed as a result of surface attached growth of *B. cereus* on glass wool, of which 4 were unique to the biofilm profile. In another study Oosthuizen et al. (2002) identified some of these differentially expressed proteins and reported them to include the catabolic ornithine carbamoyltransferase and L-lactate dehydrogenase. In a more detailed study, Sauer and co-workers (2001, 2002) showed that during the transition from a planktonic to a biofilm mode of growth *P. putida* and *P. aeruginosa* displayed

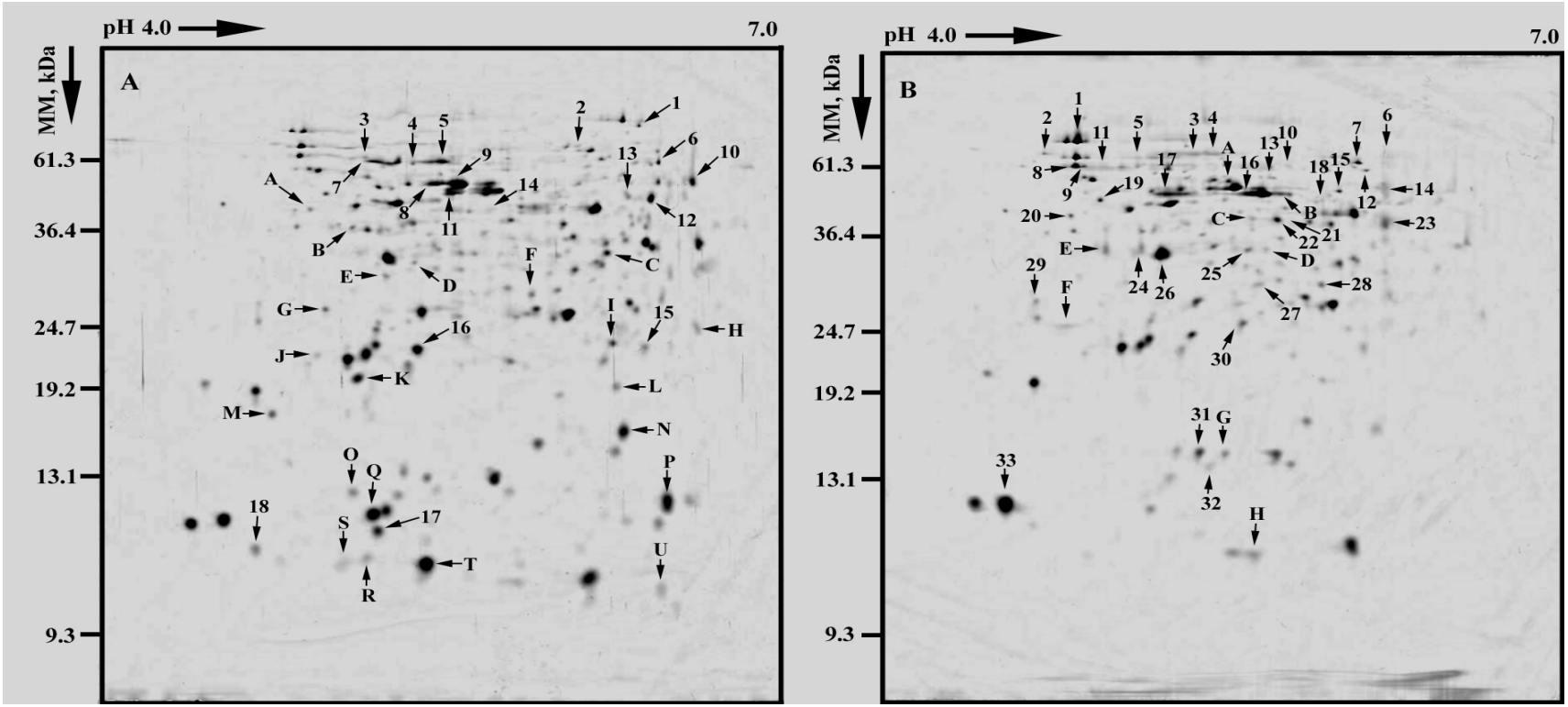

Figure 5.1 Two-dimensional-PAGE profile of total cellular proteins from cells of *S. enterica* serovar Enteritidis ATCC 4931 grown in suspension (A) or as biofilms (B). Proteins labeled with numbers in either panel indicate up-regulated proteins. Proteins labeled alphabetically indicate unique proteins.

Table 5.1 Estimated pI and molecular mass of proteins up-regulated in *S. enterica* serovar Enteritidis ATCC 4931 cells grown in suspension.

Spot I.D.[a]	Estimated pI (pH)	Estimated Mol. Mass (kDa)	Fold-Induction[b]
1	6.3	77.4	2
2	6.0	64.2	2
3	5.1	59.9	2
4	5.4	59.9	4
5	5.5	59.9	4
6	6.4	59.1	2
7	5.1	55.5	4
8	5.5	48.6	2
9	5.5	48.6	2
10	6.5	48.3	2
11	5.5	45.3	4
12	6.3	44.9	4
13	6.2	44.2	2
14	5.7	43.1	2
15	6.3	25.2	2
16	5.4	24.1	2
17	5.2	12.3	2
18	4.7	11.9	2

[a] Spot I.D.s correlate with protein spot I.D.s in Fig. 5.1A. [b] Fold-induction is the relative intensity of protein spots from cells grown in suspension divided by the relative intensity of protein spots from cell grown attached to glass beads.

Table 5.2 Estimated pI and molecular mass of proteins up-regulated in *S. enterica* serovar Enteritidis ATCC 4931 cells grown as biofilms.

Spot I.D.[a]	Estimated pI (pH)	Estimated Mol. Mass (kDa)	Fold-Induction[b]
1	4.9	74.5	2
2	4.7	66.4	4
3	5.3	66.4	2
4	5.5	66.4	4
5	5.1	64.2	2
6	6.2	64.2	2
7	6.1	61.3	2
8	4.9	59.9	2
9	4.9	59.9	2
10	5.8	59.9	2
11	5.0	59.1	2
12	6.1	57.7	4
13	5.7	53.3	2
14	6.2	48.6	10
15	6.0	47.5	2
16	5.6	47.1	8
17	5.2	46.8	2
18	5.9	44.9	10
19	5.0	44.9	2
20	4.8	40.8	2
21	5.8	40.1	4
22	5.7	39.7	2
23	6.2	38.6	4
24	5.2	34.4	2
25	5.6	34.2	2
26	5.2	34.1	2
27	5.7	30.0	2
28	5.9	30.0	2
29	4.7	28.0	2
30	5.6	25.2	2
31	5.4	14.9	2
32	5.4	14.0	2
33	4.5	12.5	2

[a] Spot I.D.s correlate with protein spot I.D.s in Fig. 5.1B. [b] Fold-induction is the relative intensity of protein spots from cells grown as biofilms divided by the relative intensity of protein spots from cell grown in suspension.

Table 5.3 Estimated pI and molecular mass of proteins that are unique to *S. enterica* serovar Enteritidis ATCC 4931 cells grown in suspension.

Spot I.D.[a]	Estimated pI (pH)	Estimated Mol. Mass (kDa)
A	4.9	41.2
B	5.1	37.1
C	6.2	33.7
D	5.4	33.2
E	5.2	31.7
F	5.8	30.2
G	5.0	27.9
H	6.5	25.2
I	6.2	24.6
J	5.0	23.1
K	5.1	21.2
L	6.2	21.1
M	4.7	18.0
N	6.2	17.7
O	5.1	13.7
P	6.4	13.1
Q	5.2	12.8
R	5.1	11.7
S	5.0	11.5
T	5.4	11.5
U	6.4	10.9

[a] Spot I.D.s correlate with protein spot I.D.s in Fig. 5.1A.

Table 5.4 Estimated pI and molecular mass of proteins that are unique to *S. enterica* serovar Enteritidis ATCC 4931 cells grown as biofilms.

Spot I.D.[a]	Estimated pI (pH)	Estimated Mol. Mass (kDa)
A	5.5	51.9
B	5.7	48.3
C	5.6	40.1
D	5.7	34.2
E	5.0	34.2
F	4.8	24.9
G	5.5	14.8
H	5.6	11.1

[a] Spot I.D.s correlate with protein spot I.D.s in Fig. 5.1B.

multiple phenotypes as well as a complex change in their protein induction profiles. The authors were able to correlate the expression of proteins with various stages of biofilm development and have also shown significant differential expression of proteins that could have been regulated by interaction of *Pseudomonas* with surfaces.

In this study a comparison of 51 proteins that were differentially expressed in the 2D PAGE profiles of biofilm and planktonic cells of serovar Enteritidis indicated that the majority of the proteins (33) appeared to be up-regulated in biofilm bacteria although the overall number of proteins expressed by biofilm bacteria was reduced by 10 %. While biofilm bacteria showed up-regulation of 33 proteins, planktonic bacteria showed up-regulation of only 18 proteins. In addition, eight proteins present in biofilm bacteria (Fig. 5.1B, Table 5.4) were absent in planktonic cells. These results indicate that the proteome of biofilm bacteria differed from the proteome of planktonic bacteria. Mass spectrometric analysis of selected differentially expressed proteins identified them as those involved in metabolism of sugars, DNA replication and chaperone proteins that aid in the export of newly synthesized proteins by keeping them in an open conformation (Table 5.5). The up-regulation of glucose-6-phosphate 1 dehydrogenase (GPD) and isocitrate dehydrogenase, enzymes involved in sugar metabolism and ATP generation, indicates that the central metabolism of *S.* Enteritidis is affected during biofilm development. Similar up-regulation of enzymes involved in carbon metabolism and ATP generation have been reported in *L. monocytogenes* and *E. coli* during a biofilm mode of growth (Tremoulet et al., 2002a, 2002b). Glucose-6-phosphate 1 dehydrogenase and isocitrate dehydrogenase are key enzymes in the pentose phosphate pathway (PPP) and citric acid cycle, respectively. Glucose-6-phosphate 1

dehydrogenase catabolizes the breakdown of 6-carbon sugars (glucose) to 5-carbon sugars and generates reducing equivalents (NADPH) to power reductive biochemical reactions within cells. These 5-carbon sugars are utilized for nucleotide synthesis or are driven back into glycolysis for ATP generation. Aerobic organisms utilize glycolysis and the citric acid cycle to generate ATP; however, under carbon limiting conditions by-products of glycolysis are converted back to glucose by the PPP and used for ATP generation. Thus, the up-regulation of proteins involved in sugar metabolism and chaperone proteins in serovar Enteritidis biofilm bacteria suggests that the biofilm bacteria could be facing some kind of a stress (e.g., nutrient starvation). Brown and Gilbert (1993) noted that cells respond to nutrient limitation by: 1) reducing the requirement for the deficient nutrient by limiting cell components containing the element by using alternate substrates or by reorganizing cellular metabolic pathways, 2) altering the cell surface and inducing higher-affinity transport systems, and 3) reducing cellular growth rate. The up-regulation of GPD and chaperone proteins in biofilm bacteria supports the observations of Brown and Gilbert. Glucose-6-phosphate 1 dehydrogenase could play a key role in reorganizing the cellular metabolic pathway and ATP generation, while the chaperone protein might be involved in transport across the membrane.

SDS-PAGE analysis of detergent-insoluble OM proteins from planktonic and biofilm cells revealed a down-regulation of two of the OM proteins by at least 2-fold, while a 30 kDa OM protein was down-regulated more that sevenfold (band II, Fig. 5.2). Mass spectrometric analysis identified these detergent-insoluble OM proteins as those involved in amino acid biosynthesis (band I, Fig. 5.2), passive transport of small molecular weight hydrophilic materials across the membrane (band II, Fig. 5.2) and

Table 5.5 Identification of selected proteins differentially expressed by *S. enterica* serovar Enteritidis ATCC 4931 grown as biofilms.[a]

Protein I.D.[b]	Protein	Function/Family	pI (pH)	Mol. Mass (kDa)
1	DNA gyrase subunit B, novobiocin-resistant	Negatively supercoils closed circular double-stranded DNA in an ATP-dependent manner and also catalyzes the interconversion of other topological isomers of double-stranded DNA rings	5.8	74.6
8	Trigger factor	Involved in protein export. Acts as a chaperone by maintaining the newly synthesized protein in an open conformation	4.8	48.9
15	Glucose-6-phosphate 1 dehydrogenase	D-glucose 6-phosphate + $NADP^+$ = D-glucose-1,5-lactone 6-phosphate + NADPH	6.1	55.3
19	Isocitrate dehydrogenase	Isocitrate + $NADP^+$ = 2-oxogultarate + CO_2 +NADPH	5.4	52.2
I	Aspartokinase 2	First step in the common biosynthetic pathway leading from ASP to diaminopimelate and LYS	4.8	44.3
II	Outer membrane protein C	Forms passive diffusion pores which allow small molecular weight hydrophilic materials across the outer membrane	4.6	41.2
III	Xanthine phosphoribosyl transferase	Nucleotide metabolism; purine metabolism	5.2	17.4

[a] The protein spots were identified by MS analysis. Function/Family analysis was based on search results from the NCBI database.

[b] Protein I.D.s correlate with the protein spot or band I.D.s in Figs. 5.1B and 5.2, respectively.

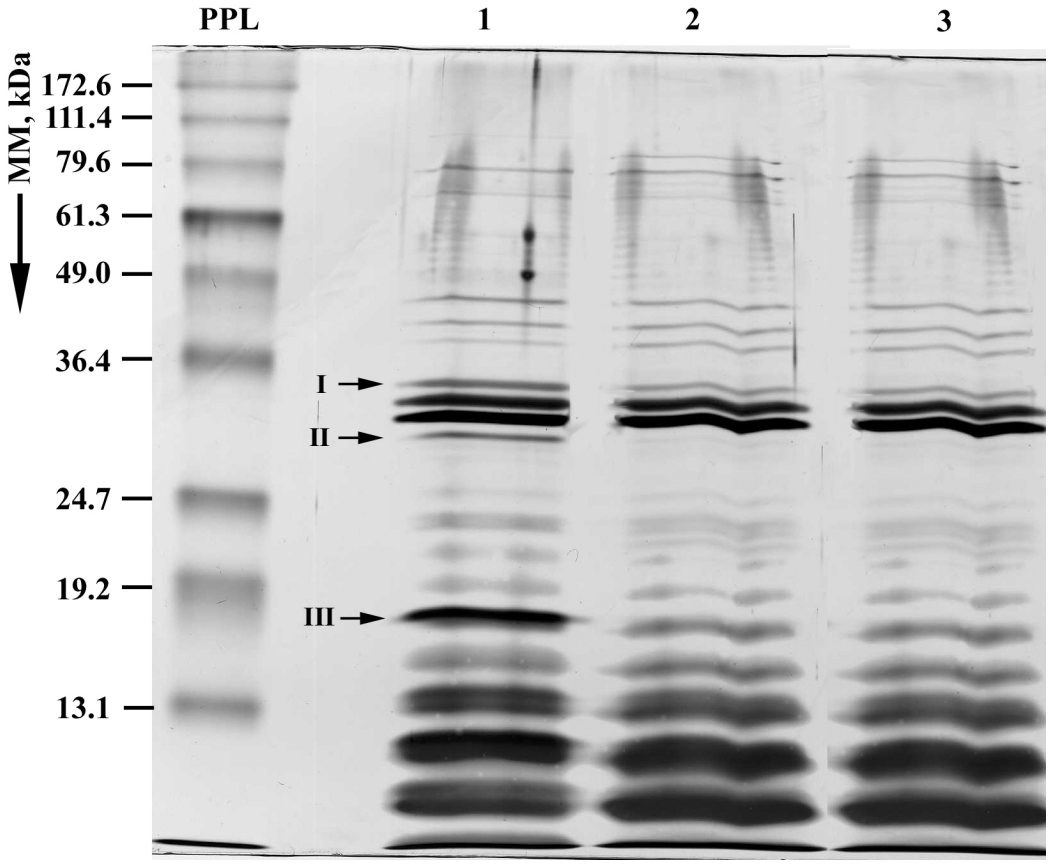

Figure 5.2 One-dimensional-SDS-PAGE profile of detergent-insoluble outer membrane proteins from *S. enterica* serovar Enteritidis ATCC 4931 grown in suspension (lane 1), biofilms attached to glass beads (lane 2), and biofilms attached to glass beads exposed to 1.5 % TSP (lane 3). PPL is a pre-stained protein molecular mass ladder. Arrows point to proteins that were differentially expressed.

nucleotide metabolism (band III, Fig. 5.2). These proteins are involved in cellular housekeeping functions and are expressed at very low concentrations in stationary phase cells. Thus, a down-regulation of these housekeeping proteins could suggest that the bacteria within biofilms could be in a physiological state with decreased growth rate similar to stationary phase cells. Bacteria within biofilms have been shown to have varying growth rates and express a wide range of metabolic activity. This phenomenon is attributed as a possible mechanism for resistance of biofilm bacteria to antimicrobial agents (Brown et al., 1988; Brown and Gilbert, 1993; Eng et al., 1991; Evans et al., 1990; Gilbert et al., 1990). Korber et al. (1994) found that when established *P. fluorescens* biofilms were exposed to the fluoroquinolone fleroxacin, a gradient of cell elongation was observed from the biofilm-liquid (where maximum cell elongation occurred) to the base of the biofilm (where the least cell elongation occurred). Quinolone compounds, which inhibit DNA-gyrase activity, have previously been used to estimate growth rates of cells by measuring the amount of cell elongation that occurs following treatment with antimicrobial agents (Bottomley and Maggard, 1990). This suggests that cells at different depths in the biofilm were growing at different rates at the time of fleroxacin exposure.

It has been previously shown that exposure of serovar Enteritidis cells grown in suspension to a sub-lethal concentration of TSP resulted in the differential expression of proteins involved in transport of small hydrophilic molecules across the cytoplasmic membrane and proteins that act as chaperones (chapter 4.0). Trisodium phosphate treatment also up-regulated common housekeeping proteins like those involved in

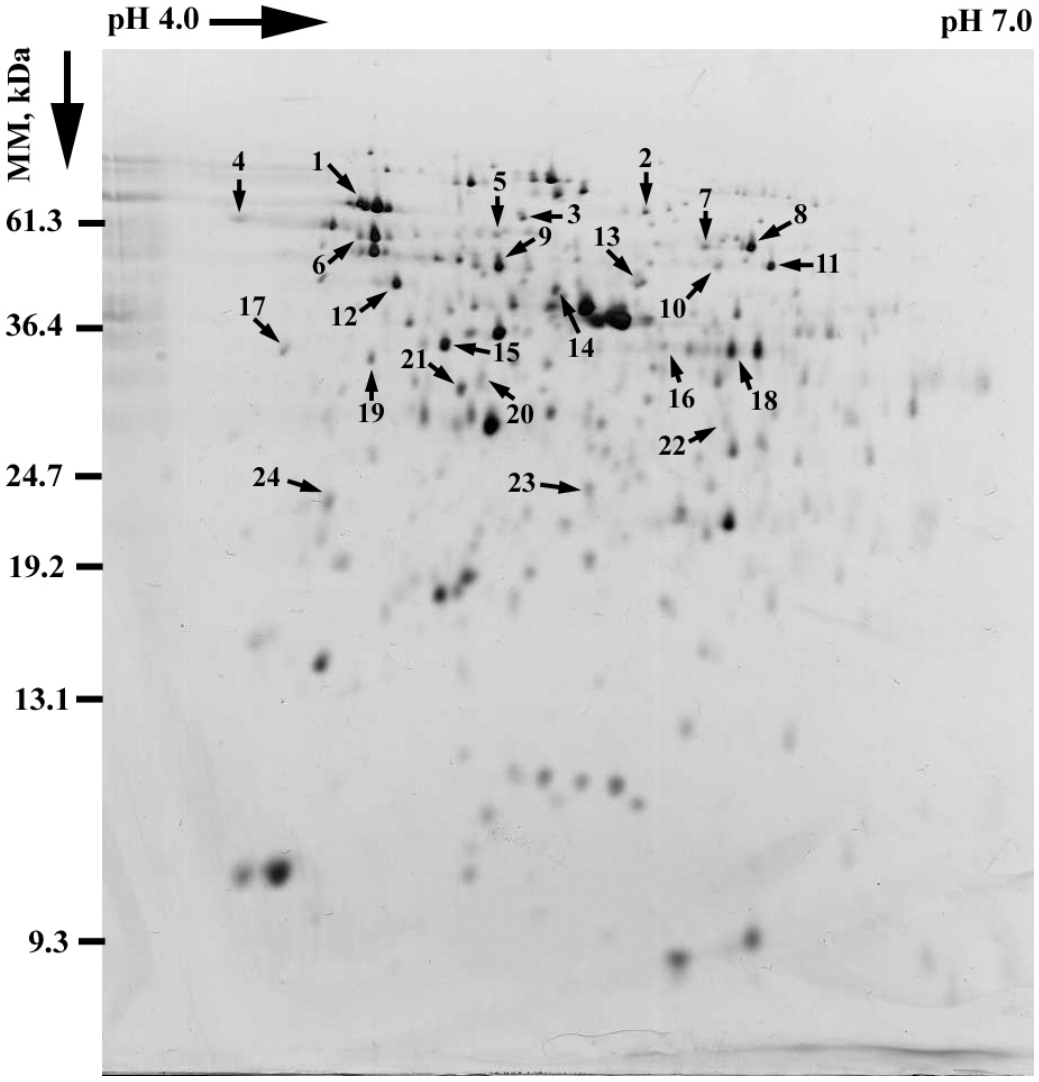

Figure 5.3 Two-dimensional-PAGE profile of total cellular proteins from cells of *S. enterica* serovar Enteritidis ATCC 4931 grown as biofilms and exposed to 1.5 % TSP. Proteins labeled with numbers indicate proteins that were up-regulated.

Table 5.6 Estimated pI and molecular mass of proteins up-regulated in *S. enterica* serovar Enteritidis ATCC 4931 grown as biofilms and exposed to 1.5 % TSP.

Spot I.D.[a]	Estimated pI (pH)	Estimated Mol. Mass (kDa)	Fold-Induction[b]
1	4.83	70.55	2
2	5.76	68.76	2
3	5.35	67.51	2
4	4.43	67.26	4
5	5.26	63.65	2
6	4.83	64.96	2
7	5.95	61.36	2
8	6.08	61.36	2
9	5.29	57.64	2
10	5.99	57.64	2
11	6.15	57.64	2
12	4.94	54.55	2
13	5.74	54.55	2
14	5.46	53.37	2
15	5.09	44.58	2
16	5.80	44.25	2
17	4.58	43.60	2
18	6.02	43.60	2
15	5.09	44.58	2
16	5.80	44.25	2
17	4.58	43.60	2
18	6.02	43.60	2
19	4.86	42.50	2
20	5.22	39.63	2
21	5.16	38.63	4
22	6.02	34.09	2
23	5.56	27.65	2
24	4.73	26.66	2

[a] Spot I.D.s correlate with protein spot I.D.s in Fig. 5.3. [b] Fold-induction is the relative intensity of protein spots from biofilm cells exposed to 1.5 % TSP divided by the relative intensity of protein spots from cell grown in suspension and exposed to 1.5 % TSP.

amino acid biosynthesis, nucleotide metabolism and aminoacyl-tRNA biosynthesis (Chapter 4; Figs. 4.2B and 4.3; Table 4.2) Exposure of serovar Enteritidis biofilm bacteria to TSP resulted in the up-regulation of as many as 24 proteins by 2-fold (Fig. 5.3, Table 5.6) compared to biofilm cells not exposed to TSP. However, a comparison of the up-regulated proteins between planktonic (Fig. 4.2B, Table 4.2) and biofilm bacteria (Fig. 5.3, Table 5.6) that have been exposed to TSP revealed little similarity. This suggests that the biofilm bacteria respond to TSP very differently than planktonic cells.

This study clearly shows the variation in the proteome of *S. enterica* serovar Enteritidis biofilm cells compared to the planktonic cells. Biofilm bacteria compared to their planktonic counterparts expressed fewer proteins, while up- and down-regulating the amounts of a number of proteins. Comparative proteomic analysis of biofilm and planktonic serovar Enteritidis cells exposed to TSP revealed little similarity suggesting that biofilm cells respond differently to TSP treatment. Differentially expressed proteins identified by MS analysis implicated them at various levels of cellular physiology, indicating that biofilm phenotype results in complex patterns of gene regulation. Understanding the role of these specific proteins during biofilm development should permit a better understanding of the mechanisms of resistance of biofilm bacteria to various antimicrobial agents.

6.0 SUMMARY

Trisodium phosphate (TSP) has been approved by the USDA for use as a carcass sanitizer in the poultry industry. A number of studies have demonstrated that TSP effectively removes surface contamination of carcasses by food-borne pathogens. However, very little scientific evidence is available that identifies the actual mechanisms of TSP antimicrobial activity. Possible modes of action of TSP include: i) exposing of microorganisms to high pH, which might particularly affect cell membrane components, ii) enhancing detachment of bacteria from food surfaces by sequestration of metal ions, and iii) removing of fat from the skin surface, thereby allowing bacteria to be washed from the food surfaces more effectively. To date, work has mainly focused on evaluating the efficiency of *Salmonella* removal from the surface of carcasses, whereas there is no published information that documents possible mechanisms of TSP antimicrobial action.

Trisodium phosphate in unbuffered solution results in very high pH. We hypothesized that it is this alkaline pH experienced by *Salmonella enterica* serovar Enteritidis cells during TSP treatment that was responsible for its lethal action. This hypothesis was examined using a series of comparative studies involving treatment solutions containing different concentrations of TSP, treatment solutions adjusted to the equivalent pH as in each of the TSP treatments and TSP solutions pH adjusted to 7.0. Direct and indirect indices of cell survival, membrane damage, and cellular leakage were

also employed to examine specific antimicrobial effects. Cell viability, loss of membrane integrity, cellular leakage, release of lipopolysaccharides and cell morphology were accordingly examined and quantified under the above treatment conditions. Exposure of serovar Enteritidis cells to TSP, or equivalent alkaline pH solutions made with NaOH, resulted in the loss of cell viability and membrane integrity in a TSP concentration- or alkaline pH-dependent manner. In contrast, cells treated with different concentrations of TSP whose pH was adjusted to 7.0 did not show any loss of cell viability or membrane integrity. A 30 min pretreatment with 1.0 mM EDTA significantly enhanced the loss of membrane integrity only when followed by TSP or NaOH-alkaline pH treatments. Measuring the absorbance at 260 nm, and performing agarose gel electrophoresis, Bradford assay and Tricine-SDS gel electrophoresis of filtrates of treated cell suspensions revealed considerable release of DNA, proteins and lipopolysaccharides compared to controls and pH 7.0 TSP treatments. Electron microscopic examination of TSP- or NaOH-alkaline pH-treated cells showed disfigured cell surface topology, wrinkling of cell membranes and evidence of a TSP concentration/pH-dependent disruption of the cytoplasmic and outer membranes. These results demonstrate that TSP treatment permeabilizes and disrupts the cytoplasmic and outer membranes of serovar Enteritidis because of the alkaline pH, which in turn leads to release of intracellular contents and eventual cell death.

A number of studies have shown that bacteria have developed adaptive mechanisms to survive stress. Treatment with elevated but non-lethal high temperatures can condition organisms to tolerate subsequent exposure to otherwise lethal high temperatures. Thermotolerance of *E. coli* and *Salmonella* can also be increased by prior

exposure to a number of sublethal stresses such as low-nutrient environments, acidic pH, starvation, alkaline pH, and to a wide number of chemical pollutants found in nature. Adaptation of *Salmonella* or *E. coli* to alkaline conditions has been shown to be accompanied by induced thermotolerance, increased resistance to bile salts, and increased resistance against external alkalinization.

Studies examining the physiological responses of *S. enterica* serovar Enteritidis ATCC 4931 to TSP or equivalent alkaline pH made with NaOH revealed that they can survive a 1 h exposure to 1.5 % TSP or pH 10.0 without any significant loss of cell viability. This led to the hypothesis that TSP treatment of *S.* Enteritidis induces a response similar to that observed when bacteria are exposed to alkaline pH. To examine this hypothesis, cells of *S.* Enteritidis were pretreated with 1.5 % TSP, or it equivalent alkaline pH of 10.0 made with NaOH, for 1h at room temperature followed by exposure to lethal stresses. Pretreatment of serovar Enteritidis with 1.5 % TSP or pH 10.0 solutions resulted in a significant increase in thermotolerance, resistance to increased concentration of TSP, resistance to external alkalization and sensitivity to acid and H_2O_2. Thermotolerance, unlike resistance to external alkalization, was dependent on *de novo* protein synthesis. However, western blot analysis showed that no heat shock proteins (HSPs) were up-regulated during TSP or NaOH-alkaline pH pretreatments. Total cellular and detergent-insoluble OM proteins from pretreated and untreated control cells were examined by two-dimensional gel electrophoresis and SDS-PAGE, respectively. Two-dimensional-PAGE of total cellular proteins from untreated control cells resolved as many as 232 proteins, of which 22 and 15 % of the proteins were absent in TSP- or NaOH-alkaline pH-pretreated cells, respectively. More than 50 % of the proteins that were either up- or down-regulated by TSP pretreatment were also up- or

down-regulated, respectively, by NaOH-alkaline pH pretreatment. SDS-PAGE analysis of detergent-insoluble OM proteins revealed up-regulation of at least four proteins. Mass spectrometric analysis identified the up-regulated proteins as transport proteins and chaperones. Other up-regulated proteins include common housekeeping proteins like those involved in amino acid biosynthesis, nucleotide metabolism and aminoacyl-tRNA biosynthesis. Another response to TSP or NaOH-alkaline pH treatment was the change in membrane fluidity, as elucidated by the membrane fatty acid composition. Alkaline pH- or TSP-pretreated cells showed a higher saturated and cyclic to unsaturated fatty acid ratio compared to the untreated control. These results suggest that the cytoplasmic membrane could play a significant role in thermotolerance and resistance to other stressors following TSP or NaOH-alkaline pH treatments.

Bacteria growing attached to surfaces (biofilms) have been shown to be more resistant to sanitizers, heat and antimicrobial agents than their planktonic counterparts. *Salmonella enterica* serovar Enteritidis were grown attached to glass beads and the total cellular protein profile (proteome) was examined using 2D-PAGE and compared with the protein profile from cells grown in suspension. The proteome of biofilm bacteria showed a 10 % reduction in the number of proteins expressed compared to the proteome of planktonic cells, while up-regulating as many as 33 proteins. Some of the differentially-expressed proteins were identified by MS and included those involved in sugar metabolism and energy generation, as well as chaperone proteins. Proteins involved in amino acid biosynthesis, nucleotide metabolism and proteins involved in passive transport of small molecules across the cytoplasmic membrane were down-regulated in biofilm bacteria, suggesting that the cell within the biofilm might be stressed and possibly nutrient limited. The protein profile of biofilm bacteria exposed to

TSP had no correlation to the protein profile of planktonic bacteria exposed to TSP, suggesting that biofilm bacteria might respond differently to TSP.

The results from this study indicate that TSP is a potent membrane acting agent and its antimicrobial action is due to its alkaline pH. *Salmonella enterica* serovar Enteritidis ATCC 4931 is resistant to 1.5 % TSP or its equivalent alkaline pH made with NaOH and responds to TSP or high pH adaptation by acquiring resistance to lethal stresses, via, altered protein synthesis and membrane fatty acid composition. Altered protein synthesis during TSP or high pH adaptation of planktonic cells of *S*. Enteritidis did not include the HSPs observed during universal stress response, and did not correlate with the protein synthesis pattern observed in biofilm cells of *S*. Enteritidis exposed to TSP suggesting that biofilm bacteria respond differently to TSP.

7.0 CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions

1. TSP treatment permeabilizes and disrupts the cytoplasmic and outer membranes of *S. enterica* serovar Enteritidis because of the alkaline pH, which in turn leads to release of intracellular contents and eventual cell death.

2. Pretreatment of *S. enterica* serovar Enteritidis with a sub-lethal concentration of TSP induces the alkaline stress response.

3. Pretreatment of *S. enterica* serovar Enteritidis with TSP resulted in the up-regulation of proteins involved in the transport of small hydrophilic molecules across the cytoplasmic membrane and proteins that act as chaperones. Other up-regulated proteins include common housekeeping proteins such as those involved in amino acid biosynthesis, nucleotide metabolism and aminoacyl-tRNA biosynthesis.

4. Trisodium phosphate treatment does not induce the universal heat shock response in *S. enterica* serovar Enteritidis.

5. Trisodium phosphate treatment causes a shift in the membrane fatty acid composition from unsaturated to saturated and cyclic fatty acids.

6. Proteomic analysis of *S. enterica* serovar Enteritidis cells grown attached to glass beads showed differential expression of a number of proteins involved in nucleotide metabolism, sugar metabolism, DNA replication and a significant difference in protein expression profiles compared to cells in suspension.

7. The protein expression profile of *S. enterica* serovar Enteritidis biofilm cells exposed to TSP did not correlate with the protein expression profile of *S. enterica* serovar Enteritidis planktonic cells exposed to TSP.

7.2 Future Directions

The results of this study raise three main questions. First, does the interaction of TSP with divalent cations on the surface of Gram-negative bacteria contribute to its antimicrobial action? Second, what is the effect of changes in membrane fatty acid composition following TSP treatment on thermotolerance? Third, what is specific nature of differences in the proteomic response of *S.* Enteritidis biofilm cells to TSP treatment compared to planktonic cells? Answers to these questions may be instrumental in understanding antimicrobial resistance mechanisms and in engineering effective methods to control food-borne pathogens.

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