THE EFFECT OF ENVIRONMENTAL CONDITIONS ON THE ANTIGENICITY OF THE OUTER MEMBRANE 35K PROTEIN PRESENT IN *SALMONELLA*

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Natasha Sabrina Tamara Taiarol

In partial Fulfilment of the
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of

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of Master of Science

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To
Scott
my Parents
and Family

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ABSTRACT

A monoclonal antibody (MAb) was developed by Jaradat and Zawistowski (1998) against an outer membrane 35K protein using whole, heat attenuated cells of *Salmonella typhimurium*. This MAb was designated as MAb 1D6 and identified as being of the immunoglobulin A (IgA) class. Outer membrane 35K protein, if antigenetically stable, could be a very useful serological and epidemiological marker of Salmonellae spp. To assess the stability of the 35K protein, outer membrane protein (OMP) extracts were prepared from several species of *Salmonella* grown under various environmental conditions. These conditions included factors such as pH, osmolarity and heat treatment. The antigenicity of the outer membrane 35K protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. Electrophoretic results showed that bacteria grown under stressful conditions produced several additional minor outer membrane proteins. This could have been due to the structural alteration of the 35K protein, since existing minor polypeptides had common antigenic properties. Furthermore, it was observed that production and antigenicity of the 35K protein was always conserved despite environmental treatment of the bacteria.

I. INTRODUCTION

Salmonellae species are Gram negative enteric organisms implicated as causative agents of food borne diseases such as salmonellosis, typhoid fever, and paratyphoid fever. These bacteria are known to cause gastrointestinal illness in humans by direct transfer from either human or animal sources. Once ingested, these organisms can invade the intestinal mucosa, epithelial cells and reticuloendothelial system and functions as an intracellular parasite in animals or humans (Foster and Spector, 1995). Upon invasion of the host, by either proliferating in the tissue or colonizing the intestinal tract (Foster and Spector, 1995) they produce various exotoxins, such as enterotoxin and cytotoxin (Stainer *et al.*, 1986). This causes symptoms such as vomiting, headache, fever and nausea.

Depending on the average incubation time of two to six days, the severity of the infection varies from diarrhea to death (Frazier and Westhoff, 1988). According to the report submitted by the Canadian Disease Control Center (CDC), the pathogen-borne disease, salmonellosis ranked second to campylobacterosis during 1994 with 7441 cases. However, it ranked first the same year in the number of actual documented outbreaks with a total of 297. The latter data did not discriminate between gastroenteritis and food borne illness.

Among all salmonellae species, *S. typhimurium* has been ranked as the number one serotype of human enteric pathogen before 1990 (Madden, 1994). In 1985, this organism was implicated as the causative agent associated with the contamination of 2% pasteurized milk at a dairy plant in Illinois. According to Ryan *et al.*, (1987), this was the largest recorded outbreak of salmonellosis in the United States. An extensive follow-up was done by surveying the local counties consumption of milk and whether the organism was still present in

the plant. The survey determined that the extent of the outbreaks occurred over a two-month period. The authors also concluded that the *S. typhimurium* had continually recontaminated the fluid milk of two different brands after pasteurization (Ryan *et al.*, 1987).

This organism is adaptable to a wide range of temperatures but has an optimum growth temperature of 37°C (Frazier and Westhoff, 1988). It can survive in a pH environment as low as 5.5 depending on the type of acidic environment but optimally grows between 6.5 to 7.5 (Frazier and Westhoff, 1988).

The main vehicles implicated in food borne infections are humans or animals who are either asymptomatic carriers or clinical carriers infected by the organism (Lim, 1986). The major food sources implicated in outbreaks of salmonellosis are foods of animal origin such as meat, poultry and eggs.

The human immune system acquires immunity toward a specific pathogen by passive immunization or by building up induced resistance. Immunization with antigens or antibodies is a technique to facilitate and induce immune resistance. Antibodies can be developed against pathogens by introducing an antigen, specifically, bacterial cell components within the outer membrane such as flagellin, lipopolysaccharide or protein into the host's body. The outer membrane is usually responsible or associated with the virulence of the organism and therefore antibodies against these cellular components are most efficient. Antibodies have been extensively developed against the lipopolysaccharide (LPS) portion of the outer membrane due to the immunogenic nature of the somatic O antigen found there, which is discriminatory between species of Gram negative bacteria (Tortora *et al.*, 1986). According to Lindberg and Minor (1994), similarities exist between serogroups and the structure of the repeating unit.

Flagellar H antigens also have antigenic determinants but the amino acid sequence has been proven similar in species such as *Bacillus subtilis* and *Salmonella adalaide* (Smith *et al.*, 1979).

The outer membrane proteins (OMPs) are important for serological identification, as epidemiological markers and for immunization purposes. Yamaura *et al.* (1992) produced a monoclonal antibody toward *Salmonella* O5-antigen ($O5^+$carrier type). It enabled them to isolate *S. typhimurium* as the causative agent in mass outbreaks of gastroenteritis and used the antibody as an epidemiological marker.

Outer membrane proteins have been used to produce antibodies against salmonellae (Kuusi, 1981) and *S. typhimurium* (Svensor *et al.*, 1979). Both studies have reported to produce vaccines against infectious Gram Negative bacteria (Kuusi *et al.*, 1981).

Stull *et al.* (1985) found that immunizing children with OMP provided better immunity than using other cellular components, such as the capsule of *Haemophilus influenzae*, which shows poor immunity in children. According to Sciortino *et al.* (1985), OMPs are good candidates for immunization against bacteria. Since some OMPs are found on the surface of the membrane, they are easily reached by antibodies produced against them by the immune system. OMPs are important for both identification and immunization purposes. Therefore, stability of the antigen is an important assessment. The antibody response could be compromised by denatured or stressed proteins (Kuusi *et al.*, 1981). Stressful conditions include temperature, low pH, high osmolarity and nutrient depletion.

The purpose of this study was to decide whether the antigenicity of the 35K protein could be compromised by subjecting the bacterial cells to certain environmental growth

conditions before harvesting and extraction the outer membrane proteins. SDS-electrophoresis and immunoblotting provided evidence to show that the antigenetic stability of the 35K protein was not compromised despite the treatment. Subsequently, the 35K protein was still reactive with the 1D6 MAb. The monoclonal antibody, (MAb) 1D6 directed against the 35K protein was produced in a previous study by Jaradat and Zawistowski (1995). This antibody was produced against the 35 kilodalton (K) outer membrane protein of *Salmonella typhimurium* to attain a common molecular marker across all salmonellae species. This would enable the use of a monoclonal antibody-based immunoassay for rapid detection of salmonellae spp.

This method would be faster than conventional methods where confirmatory tests take approximately five to seven days to complete. The MAb developed by Jaradat and Zawistowski (1995) reacted specifically with the 35K outer membrane protein present in all tested salmonellae species. As well, it cross-reacted with the 34K protein in non-salmonellae species. The 1D6 monoclonal antibody is an immunoglobulin A (IgA) and has eight paratopes that can bind low molecular weight antigens. Therefore this antibody cannot be used for an enzyme linked immuno sandwich assay (ELISA) detection system in a mixed microflora. Future studies may find a common OMP antigen that can be used to produce an antibody that does not cross-react with non-salmonellae species. The MAb 1D6 did not react with the *S. arizona* antigen in the previous study conducted by Jaradat and Zawistowski (1998). This could be due to cross-contamination with non-*Salmonella* species. In this study, the monoclonal reacted with 35K protein of S. arizona, detected by immunoblotting. *S. arizona* was confirmed to be a pure culture as tested by the Food Development Center, Portage La

Prairie, for this study.

Jaradat and Zawistowski (1998) used ELISA to screen different genera of bacteria. They showed that the MAb 1D6 cross-reacted with non-salmonellae species. Immunoblotting studies revealed that MAb 1D6 reacted with OMP 35K of salmonellae spp. and with OMP 34K of non-salmonella spp. Their work suggested that native (non-denatured) immunoblotting, as opposed to ELISA, could be a suitable assay to discriminate salmonellae from non-salmonellae spp. using MAb 1D6 antibodies.

The epitope configuration of Omp D (porin) of *Salmonella typhimurium* was changed due to high temperature treatment and/or SDS exposure (Pai *et al.*, 1992). This study indicated that OMPs may not be antigenically stable and may be influenced by various environmental factors and nutritional conditions during bacterial growth and protein synthesis.

The objective of the first study was to determine whether certain essential elements are required to maintain the antigenicity, specifically the epitope structure of the major OMPs. Under certain conditions, protein synthesis is regulated by the availability of nutrients in the cell and the ability of the cell to compensate by synthesizing or increasing the production of other proteins.

The purpose of the second study was to determine the effect of osmolarity, heat or cold and pH on the expression of OMP and the 35K protein. The objective of this study was to determine whether extreme or harsh environmental factors such as high osmolarity, heat and slow freezing affect the antigenicity (epitope structure) of the 35K major OMPs. Cells can undergo injury or stress because of suboptimal growth conditions. Protein synthesis may be

altered or slowed when injured or stressed cells are unable metabolically to function normally, Under conditions inducing cellular injury, protein denaturation may occur to some extent. This could cause the epitope structure to change, rendering it inaccessible for attachment by the antibody.

This thesis focuses on the outer membrane, in particular, the major outer membrane proteins present in Gram negative bacteria, specifically *Salmonella*.

II. LITERATURE REVIEW

Eubacteria share a common cytoplasmic membrane that is responsible for active transport, oxidative phosphorylation and biosynthesis of specific macromolecules (Dirienzo *et al.*, 1978). The latter are unable to enter the cell but their constituents can enter the cytoplasm following degradation by hydrolytic enzymes (Stainer *et al.*, 1996).

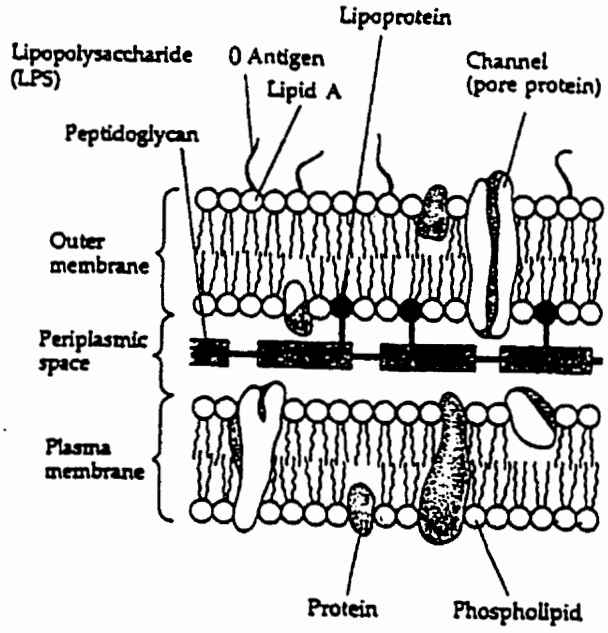
Bacteria can be divided into two major groups, Gram negative and positive bacteria, based upon the chemical and structural characteristics of the cell wall.

A. Cell wall

The primary function of the cell wall is to provide an osmotic barrier between the interior and exterior of the cell, and to provide structure and rigidity to the cell as a whole (Tortora *et al.*, 1986).

The cell wall of Gram negative bacteria is made up of a plasma membrane, a periplasmic space (peptidoglycan layer) and an outer membrane (Figure 1). Gram positive bacteria contain a cell wall consisting of peptidoglycan layers and a plasma membrane which surrounds the cytoplasm. They have no outer membrane.

Figure 1. - The Gram-negative cell wall. It is divided into three uniquely structures based on structure and chemical components. Situated from the interior outward: the plasma membrane (protein and phospholipid), periplasmic space (peptidoglycan) and outer membrane (lipid A, O antigen, lipoprotein and pore protein). (Adapted from Tortora *et al.*, 1986)


Lipoprotein
Lipopolysaccharide (LPS)
O Antigen
Lipid A
Channel (pore protein)
Peptidoglycan
Outer membrane
Periplasmic space
Plasma membrane
Protein
Phospholipid

The physiological characteristics of Gram negative bacteria evidently differ from Gram positive bacteria with respect to the cellular structure, function, permeability to different solutes and the effect of environmental conditions, such as temperature, pH and osmolarity. Because of differences in the cell walls of the two groups of bacteria, researchers have characterized the cellular components. These components are also responsible for the maintenance and integrity of the outer membrane of Gram negative bacteria (Dirienzo *et al.*, 1978).

B. Plasma membrane

The plasma or cytoplasmic membrane is the inner envelope that surrounds the cell. The cytoplasmic membrane is composed of 60% protein and 40% lipids. It appears as a thin layer around the cytoplast. The cytoplasmic membrane (CM) is involved in several functions such as production of lipopolysaccharides (LPS), phospholipids, peptidoglycan, cell division, DNA replication and oxidative phosphorylation (Lugtenberg and Van Alphen, 1983). The CM acts as a permeability barrier for the cytoplasm. This is done by facilitating transport of nutrients across the cell membrane. Directly, it gives the cell the energy it requires for maintenance and growth via enzymes in the membrane that convert the nutrients to utilizable energy.

C. Periplasmic space

Costerton and coworkers (1974) describe the periplasmic space as a zone sandwiched between the outer membrane - exterior of the cell, and the plasma membrane - interior of the cell. The building block of this region is peptidoglycan (murein). Its main purpose is to provide structural rigidity to the cell wall. This carbohydrate moiety attached by a β-1,4 linkage is composed of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM)

(Costerton *et al.*, 1974). Along with amino acids that form tetrapeptide side chains and peptide cross bridging, this forms a highly cross linked structural support (Inouye *et al.*, 1976). The periplasmic space in Gram negative bacteria is composed of a single layer of peptidoglycan in comparison to the numerous layers that comprises the cell wall of Gram positive bacteria. Within this space reside hydrolytic enzymes and binding proteins protected by the outer membrane (Dirienzo *et al.*, 1978).

According to Korhonen *et al.* (1985) the periplasmic space compromises 4% of the total cell protein. The three different types of proteins existing within this space are classified according to their function (Korhonen *et al.*, 1995). The first include proteins that have a high affinity for solutes such as sugars, amino acids and ions. These proteins bind to the nutrients to provide transportation other parts of the cell. Secondly, enzymes that destroy or transform antibiotics and heavy metals. Thirdly, proteins provide a catabolic function to breakdown large solutes that then enable them to pass through the cytoplasmic membrane.

D. Outer membrane

The outer membrane is primarily composed of lipopolysaccharides, phospholipids and proteins and contains, 30%, 20-25% and 40-45% of these components, respectively (Lugtenberg and Van Alphen, 1983). The outer membrane, as a whole, provides several functions for the cell. This includes, a differential barrier against hydrophobic compounds; passive diffusion to allow the passage of small molecular weight compounds; an uptake system for certain nutrients; involvement in cell division, conjugation and septum formation (Dirienzo *et al.*, 1978).

E. Lipopolysaccharide

Lipopolysaccharide (LPS) is found solely in the exterior region of the outer membrane (Nikaido and Vaara, 1987 and Brass, 1986) and is not present in the cytoplasmic membrane. LPS constitute 20% of the outer membrane (Nakae, 1986). Chemically, LPS is an amphiphatic molecule made up of a hydrophobic lipid portion - lipid A, a core polysaccharide and a hydrophilic polysaccharide region - O antigen. LPS is primarily responsible for stabilization and protection of the outer membrane (Lugtenberg, 1981).

(i) Lipid A

The hydrophobic portion of LPS is found toward the interior of the outer membrane (Lugtenberg and Van Alphen, 1983). This glycolipid is made up of a D-glucosaminyl β (1→D)- D glucosamine backbone (Nikaido and Nakae, 1979). Attached to this by amide and ester linkages are saturated fatty acids that contribute to its rigidity and hydrophobicity (Nikaido and Nakae, 1979).

Lipid A is responsible for producing the endotoxin that causes fever and intravascular hemolysis upon entry into the host's bloodstream (Tortora *et al.*, 1986).

(ii) O antigen

The polysaccharide portion of LPS is a sugar moiety with repeating units of oligosaccharides (Nikaido and Vaara, 1987). It is located on the exterior of the outer membrane and is negatively charged (Nikaido and Nakae, 1979). The hydrophilicity of the cell surface (highly negatively charged divalent cations) is primarily due to the carbohydrate moiety of the O antigen that contributes to the permeability of the cell (Nikaido, 1979). The hydrophobic lipid portion is attached to the hydrophilic portion of the LPS by an 'R' core.

The R core is composed of either an eight-carbon sugar acid 3-deoxyoctulosonic acid or a 7-carbon sugar, L-glycero-D-mannoheptose (Nikaido and Nakae, 1979). Since the core is the point of attachment between the O antigen and Lipid A, it shares highly charged groups associated with Lipid A.

The O antigen is responsible for the virulence of *Escherichia coli* and *Salmonella typhimurium* because it can elicit an immunogenic response in the host cell (Nikaido and Vaara, 1987). Rough strains caused by mutations, lack the O antigen and are more susceptible to hydrophobic antibiotics and dyes (Nikaido and Nakae, 1979).

F. Phospholipid

The outer membrane shares some similarities with the inner membrane. Both contain phospholipids composed of phosphatidylethanolamine, phosphatidyglycerol and diphosphatidyglycerol (cardiolipin) (Nikaido and Nakae, 1979). Phospholipid can be transposed between either membrane. Numerous authors have stated that phosphatidylethanolamine varies between the inner and outer membrane and the species of Gram negative organism (Lugtenberg and Van Alphen, 1983). One assumed function of the phospholipid bilayer is to provide stability for the LPS (Lugtenberg and Van Alphen, 1983).

G. Protein

The outer membrane of the cell wall is composed of a few major and minor proteins and these are present in appreciable amounts. These proteins are selectively positioned throughout the outer membrane, with some being involved in the structure of the cell wall and thus are usually exposed on the surface of the cell (Dirienzo *et al.*, 1978). Other proteins form oligomers with themselves to act as transportation channels between the environment and

the inner membrane of the cell (Lugtenberg and Van Alphen, 1983). The protein structure is primarily composed of β-sheets.

(i) Non-porins

Outer membrane proteins can be categorized into two groups based on their function. The first group, so-called non-porins, provide structure and rigidity to the cell wall. Non-porins can contribute to virulence of Gram negative organisms, by uptake of required iron via transferrin or lactoferrrin and contribute to the ability of a pathogen to attach to epithelial tissue. These characteristics are attributed to the presence of a virulence plasmid (Owen *et al.*, 1991). For example, *E. coli* (a pathogenic organism) can enter epithelial cells and cause invasive diarrhea due to gene products and plasmid-encoded proteins (Owen *et al.*, 1991). This causes evasion of the immune system by blocking antibodies (Owen *et al.*, 1991).

(ii) Porins

The second group, porins, act as transportation channels and provide a permeable barrier across the cell wall. These channel proteins extend into the interior of the cell and thus provide a conduit through which nutrients and minerals can pass into the cell. Differential permeability is regulated by molecular sieving (size) or charged groups present inside the channel that are selective toward hydrophilic substances. The charged groups prevent hydrophobic compounds such as antibiotics from entering the cell.

Large molecular weight compounds present in the environment surrounding the cell must be broken down by enzymes on the exterior of the cell surface, such as alkaline phosphatase, to gain entrance into the cell. These 'channels' or porin proteins exist as either tripolysaccharides or as monomeric forms.

H. Extraction of OMPs

(i) History

To study OMPs the outer membrane must be selectively separated from the cytoplasmic membrane layer, since both contain proteins. The classical work for fractionation of cell envelopes by isopycnic sucrose gradient centrifugation is attributed to researchers such as Miura and Mizushima (1969) and Osborne *et al.* (1972). The first step consisted of applying lysozyme or ethylenediamine tetra acetic Acid (EDTA) to the cells to induce spheroplast formation. In Gram negative organisms, spheroplast formation directly influences the effectiveness of separation of the inner and outer membrane in organisms such as *Salmonella typhimurium* and *Serratia marcescens*. These organisms require a higher concentration of lysozyme as opposed to *E. coli*, which form spheroplasts more readily (Larsen and Biedermann, 1993). Since lysozyme does not diffuse through the outer membrane channels of *Enterobacteriaceae*, EDTA is used to make the outer membrane permeable to lysozyme (Korhonen, 1995).

(ii) Effects of EDTA

EDTA contains monovalent cations that replace multivalent cations and causes destabilization of the OM by removing the cross-bridging divalent cations (Brass, 1986). It can effectively remove up to 50% of the cellular LPS (Brass, 1986) that supports the cell wall via its lipopolysaccharide-protein units. Since the protein is associated with the LPS, this may affect the total yield of recoverable protein using isopycnic centrifugation (Larsen and Biedermann, 1993). In the presence of Tris (hydroxymethyl aminomethane Tris, EDTA acts as a chelating agent. The extent of EDTA action is not the same for every organism. It

disintegrates the cell wall of *Pseudomonas* (Gram negative) but increases permeability of the cell wall of *E. coli* (Gram negative)(Sarvas, 1985a).

In 1974, Osborn and Munson developed a separation protocol based on sucrose-gradient density that became the foundation for further studies in this area. An outline of this method was reviewed by Sarvas (1985a). The principle behind this method involves the selective isolation of the inner membrane based on the differences between density of both membranes. The inner membrane has a density of 1.16 g/ cm^3 while the outer membrane density is 1.22g/ cm^3 (Osborn *et al.*, 1972). This method deals with subjecting the cell to plasmolysis in the presence of sucrose and Tris, and the formation of spheroplasts using EDTA. The spheroplasts are ruptured and subjected to a density gradient using a 30-55% (w/v) sucrose solution. The mixture is then centrifuged and OM is collected in the lower portion of the tube. Preliminary work conducted on OMPs provided information categorizing characteristic proteins as major proteins, including Protein I and Protein II, and minor proteins such as Ton C. Based on the results obtained by Schnaitman (1970), this method is the most reproducible and is the most commonly used.

(iii) French Pressure Method

The technique of using the French press to disrupt cells was also established. Under a pressure of 1000 atm, cells subjected to EDTA would shatter once the pressure was rapidly released. This step was followed by density gradient centrifugation. Kamio and Nikaido (1977) used lysozyme after this step to denature the β-1-4-glycosidic bond of the peptidoglycan layer that can entrap and may be attached to LPS.

(iv) Selective solubilization

More recently, several non-ionic detergents have been developed to dissolve or solubilize the cytoplasmic membrane. These include, Triton-X-100, sodium sarkosylate and lithium chloride. Triton-X-100 in the presence of Mg^{2+} was used by Lugtenberg (1985) selectively to separate the insoluble portion of the OM from the cytoplasmic membrane (soluble portion). According to Schnaitman (1970) Mg^{2+} acts as a protectant against solubilization of the outer membrane by Triton X-100. This is followed by subjecting the OM to 2% SDS to solubilize peptidoglycan-associated protein that can be subsequently isolated for further studies.

Larsen and Biedermann (1993) compared two methods of extraction; isopycnic centrifugation and solubilization using sarkosyl, to isolate the OMPs of *Serratia marcescens*. Using enzyme markers, such as succinate dehydrogenase and L-lactate dehydrogenase that are bound to the inner membrane, they found the extracted protein contained less enzyme activity; therefore the method yielded less contamination with inner membrane proteins than was obtained by the traditional method. This was attributed to the effectiveness of spheroplast formation, and was influenced by several factors such as the concentration of lysozyme and the rate at which the lysozyme-EDTA solution was added. These procedures influenced the rate of spheroplast formation. Using the solubilization method, Larsen and Biedermann (1993), however, obtained a lower yield with sarkosyl. This was believed due to rinsing the outer membrane with sarkosyl to eliminate any contamination by inner membrane proteins. Stull and coworkers (1985), conducted a study using rapid techniques to isolate the outer membrane proteins from *Haemophilus influenzae* Type b^1. The three rapid techniques

included: selective solubilization using non-ionic detergents, salt extraction of whole organisms using heat and chaotropic agents, and the collection of outer membrane fractions released into media during growth. The outer membrane was also extracted using: lithium; lithium plus EDTA; Triton-X-100; sodium lauroyl sarcosinate; sodium dodecyl-B-D-maltoside; and octyl B-D-glycopyranoside. By examining the electrophoretic profiles of the polypeptides, Stull and coworkers (1985) concluded that the Triton-X-100 and lithium extracts produced the closest pattern to the isopycnic technique. According to Stull *et al.* (1985) the polypeptide pattern identified by isopycnic sucrose gradient centrifugation could not be reproduced using any of the techniques they examined.

Filip *et al.* (1973) proved that the cytoplasmic membrane was solubilized by sodium-lauryl sarcosinate and protein was released from the outer membrane fractions. In their work, the outer membrane of *E. coli* B was labeled with ^{14}C- leucine and the inner membrane was labeled with ^{3}H from the growth media. Following electrophoresis, the extract that contained the outer membrane fraction contained an electrophoretic band at the correct density of 1.22 g/cm^3.

Ion exchange chromatography is another technique used to fractionate solubilized membrane polypeptides. Moldow *et al.* (1972) used guanidinium thiocyanate to solubilize the outer membrane of *E. coli*. Dialysis in the presence of 6M urea, a chaotropic agent, was used to remove the guanidinium thiocyanate. The authors concluded that the combination of the two chaotropic agents, guanidinium and thiocyanate was more effective in solubilizing protein than other agents. Moldow and coworkers, (1972) also concluded that guanidinium

thiocyanate was comparable to solubilization in SDS. This was shown by determining the protein concentration of the supernatant and by [^{14}C] leucine-labeled membranes.

(v) Porins vs non-porins

Pai *et al.* (1992) made the distinction between porins and non-porin proteins. After applying the French pressure method, the cells were subjected to 2% SDS in 10 mm Tris-HCl, pH 7.7, to extract the non-porin proteins. Roy *et al.* (1994) proved that the insoluble portion removed from the extraction step using 10mM Tris-HCl buffer (pH 7.7) containing 2% SDS is, in fact, the porin. Roy and coworkers (1994) confirmed this by the introduction of trypsin. The major immunogenic protein isolated from *Shigella* spp. was not degraded by this enzyme. The porin proteins were extracted using NaCl buffer that contained: 1% SDS; 50 mm Tris-HCl; 0.4 M NaCl; 5 mm EDTA; 0.05% 2-mercaptoethanol; and 3 mm sodium azide at pH 7.7 (Pai *et al.*, 1992). The porins were eluted from a Sephacryl S-200 column using NaCl buffer. Pai and co-workers (1992) also found that these porins were still closely associated with the cell wall peptidoglycan layer.

Several problems associated with this methodology have been identified by Lugtenberg and Van Alphen (1983). To solubilize proteins completely and remove the bonds present between the outer membrane and OMPs, temperatures of 70°C or greater must be used with *E. coli* and *S. typhimurium*. Therefore, the standard protocol requires boiling in 2% SDS for several minutes. Problems stemming from incomplete solubilization result in poor electrophoretic mobility, insufficient binding of SDS and prevention of the protein from entering the gel.

I. Heat modifiable proteins

Heat modification is a change in mobility of protein bands in SDS-gel electrophoregrams because of subjecting membrane proteins to SDS in the presence of heat (Inouye *et al.*, 1973). The different migration pattern resulting is due to induced conformational changes (Nakamura and Mizushima, 1976). The proteins denature which result in an unfolding of the B sheet complex into an alpha helix form. This conformational change occurs at two different transition temperatures based on the type of gel system used. In an experiment to determine the effect of solubilization temperature on the electrophoretic mobility of OMPs from *E. coli* K-12, Nakamura and Mizushima (1976) used two different systems involving urea-SDS and SDS gel. They credited the change in the electrophoregram to the solubilization temperature. The transition temperature using SDS fell between 70 and 100°C. Using urea-SDS, the transition temperature was between 50 and 70°C. According to Nakamura and Mizushima (1976) the conformational change of outer membrane proteins does not occur in SDS unless heat is applied. Using infrared and circular dicroism (CD) spectroscopy on dried films of specific outer membrane proteins, O-8, O-9, and O-10, they found that the electrophoretic mobilities change because of the conformational change from a β-sheet to an α-helix. The authors also attributed different or abnormal migration patterns to unusual charge or conformation and the presence of glycoproteins. A change in mobility was also attributed to the presence of covalently attached carbohydrates, or was due to repression or induction of a protein, or to a missense or a nonsense mutation. Based on the results obtained by Nakamura and Mizushima (1976) several conclusions were drawn. Upon removal of SDS, the β conformation can be reformed. With respect to electrophoretic mobility (m_o)

the dissociation of a dimer to a monomer occurs because of a decrease in surface charge density. However, in contrast, the binding of SDS molecules to the proteins causes an increase in surface charge density.

Ames (1974) investigated the identification of membrane proteins isolated from *S. typhimurium* using polyacrylamide gel electrophoresis (PAGE). The conditions used involved a solubilization treatment (37°C, 30 min) and a heat treatment (100°C, 2 min) in SDS. This study identified a characteristic set of major protein components in the 33K and 36K region. The treatment at 37°C for 30 minutes solubilized protein bands above and below the 33K and 36K region, but did not affect an electrophoretic band at 44K.

J. Peptidoglycan-associated proteins

Outer membrane protein can be categorized as either peptidoglycan-associated or non-associated protein. The peptidoglycan-associated proteins have a molecular weight between 33K to 44K (Lugtenberg *et al.*, 1977). Three major outer membrane proteins, 39K, 38.5K and 36K have been identified. Only the two higher molecular weight proteins are considered peptidoglycan-associated proteins. Nelson and Robinson (1983) compiled tables of well-characterized membrane proteins that have known structural models. Three of the identified outer membrane proteins belong to *Salmonella typhimurium.* These include protein C, D, and F with MW of 39.8, 38.0, and 39.3, respectively. Dirienzo and co-workers (1978) termed the peptidoglycan-associated proteins as 'matrix proteins' and denoted specific proteins in *E. coli* as Matrix proteins Ia and Ib. To release the peptidoglycan from the protein, the complex must be incubated in 2% SDS at 70°C or at 37°C in SDS using 0.5 M NaCl (Lugtenberg, 1983). Dirienzo and co-workers (1978) listed several factors that could cause discrepancy or variation

in the different MW using SDS-electrophoresis. These include: acrylamide concentration; extent of cross-linking; pH of the buffer; ionic strength; voltage; current; and method of outer membrane solubilization. It has been suggested that the type of bacterial strain, growth and environmental conditions could also influence the results. The conformational structure or charge on the protein, and the fact that glycoproteins could be present can contribute to different migration of identical proteins (Dirienzo *et al.*, 1978).

Lugtenberg and co-workers (1988) and Anwar (1991) showed the presence of the 40K electrophoretic band on SDS-PAGE. Armstrong *et al.* (1986) identified this polypeptide and further characterized it as a porin that is responsible for the formation of anionic-selective channels using lipid bilayer membrane systems. Browne and Hormache (1989) identified three polypeptides in the region of 34-36K using extracts boiled and treated with SDS. These proteins were characterized as porins by Kuusi *et al.* (1981) and Nikaido *et al.*, (1987).

Peptidoglycans can be associated with the outer membrane of the cell wall in two ways according to Lugtenberg *et al.* (1988). This may be by either peptidoglycan-associated proteins held by ionic bonds or by lipoprotein linked covalently to the peptidoglycan in *Enterobacteriaceae*. The main function of these peptidoglycan-associated proteins is to stabilize the hydrophilic channels that are found in the outer membrane. These channels allow nutrients that are hydrophilic to pass through the membrane and restrict hydrophobic compounds such as antibiotics and detergents from entering the cell. Solutes diffuse through these pores according to size, charge, hydrophobicity and permeability coefficients (Lugtenberg and Van Alphen, 1983). To transport nutrients to the cytoplast, a channel exists from the outer membrane through the periplasmic space and cytoplasmic membrane. A

difference in potential exists between the concentration at the end of each channel. This is termed 'a diffusion rate' (Lugtenberg and Van Alphen, 1983). For example, *Salmonella typhimurium* contains pores that allow oligosaccharides between 600-800 daltons to enter (Lugtenberg *et al.*, 1988).

Witkowska *et al.* (1988) used the extraction procedure outlined by Schnaitman (1970) to isolate the OMP *Hafnia alvei* by Triton X-100 in the presence of EDTA. Electrophoresis results showed that major proteins not related to peptidoglycan were susceptible to both the proteolytic enzymes trypsin and pronase. They were also heat modifiable. Peptidoglycan-associated proteins are those proteins that are not susceptible to proteolytic enzymes. They could be released from the cell wall using heat treatment at 100°C in the presence of SDS. Outer membrane proteins of *Hafnia* strains were characterized and identified as the 34, 35, 36 and 37K major proteins (peptidoglycan associated). The 33K (non-associated) proteins were similar to the outer membrane composition of Enterobacteriaceae including, *Salmonella, Escherichia* and *Shigella.*

Table 1- Characteristics and functions of outer membrane protein of Gram-negative bacteria

Protein	Mol wt	Number of molecules per cell	Condition for induction or derepression	Function
Lipoprotein	7200	Bound form $2.5x10^5$ free form $5x10^5$	Constitutive	Anchoring outer membrane to peptidoglycan; influence on cell shape; stabilization of outer membrane structure
OmpA protein	35159 (boiled)	10^5	Constitutive	Role in acceptor cell in stabilization of mating aggregates in F-pilus mediated conjugation; influence on cell shape; requires LPS for biological activity
OmpC porin	36000	Up to 10^5	Preferentially expressed in media of high osmolarity	General pore for hydrophilic solutes with a mol wt up to 700; requires LPS for biological activity
OmpF porin	37205	Up to 10^5	Preferentially expressed in media of high osmolarity	General pore for hydrophilic solutes with a mol wt up to about 700; requires LPS for biological activity
PhoE porin	40000	$1\text{-}2x10^5$	Phosphate limitation	General pore for hydrophilic solutes; preference for anionic solutes; recognized by polyphosphate; probably required for uptake of polyphosphate
Maltoporin	50000	$1\text{-}2x10^5$	Presence of maltose	Pore for small hydrophilic solutes; recognized by maltose and maltodextrins, required for uptake of higher maltodextrins
Tsx protein	28000	$\sim 10^4$		Pore for nucleosides
BtuB protein	60000		Vitamin B_{12} limitation	Uptake of vitamin B_{12}
Cir protein	74000		Fe^{3+}	Uptake of complexed Fe^{3+}
TonA protein	78000		Fe^{3+}	Uptake of ferrichrome
Fec protein	80500		Fe^{3+}	Uptake of Fe^{3+}citrate
Fep A protein	81000		Fe^{3+}	Uptake of Fe^{3+} enterocholin
Protein a	40000	Up to $\sim 4x10^4$	Constitutive	Protease

(Adapted from Lugtenberg, 1981)

K. Major outer membrane proteins

Four predominant proteins exist in the outer membrane of *S. typhimurium* and *E. coli* and these include lipoprotein, OmpA protein, OmpC porin and OmpF porin (Table 1).

(i) Lipoprotein

Lipoprotein has the smallest molecular weight, (MW= 7K) of macromolecules found in this region but is the most abundant form (Dirienzo *et al.*, 1978). It resides on the surface of the cell wall and therefore is highly immunogenic (Browne and Hormache, 1989). According to Lugtenberg and Van Alphen, (1983), the lipoprotein is covalently bound to the peptidoglycan. A solubilization temperature of 100°C in SDS is not able to remove the covalent bond between the lipoprotein and the peptidoglycan layer (Hofstra and Dankert, 1979a). Lipoprotein is made up of 58 amino acid residues excluding histidine, tryptophan, glycine, proline and phenylalanine (Lugtenberg and Van Alphen, 1983). It is primarily composed of α helical polypeptides (Nakamura and Mizushima, 1976). The bound form, therefore functions to connect and stabilize the outer membrane and peptidoglycan and contributing to the cell shape (Lugtenberg, 1981). By extracting the membrane fraction from *E. coli* followed by SDS-gel electrophoresis, Inouye proved that lipoprotein can exist in a free form (Dirienzo *et al.*, 1978).

(ii) Surface antigens

These antigens are proteins on the surface of the cell wall. They include porins that act as both filtered channels and provide structure and rigidity to the cell wall. These antigens, *in vitro*, act as phage receptors and react with antibodies to produce vaccines against pathogens providing antibacterial immunity (Kuusi *et al.*, 1981). Mice can be protected from

salmonellosis by immunity using major outer membrane protein preparations from *S. typhimurium* as a vaccine (Kuusi *et al.*, 1981). These are the only components available for attachment with the effector arm of the host immune system (Sciortino *et al.*, 1985). Therefore, antibodies produced against them (Anwar, 1991) can elicit a higher immunogenic response (Browne and Hormaeche, 1989).

(iii) *TolG* protein or OmpA

TolG has five other names given by various authors but the common name adapted by Lugtenberg and Van Alphen, is the OmpA protein (1985). This protein is characteristically different from the matrix protein as outlined by Dirienzo *et al.* (1978). Exposed to heat, the MW of the *tolG* protein increases. The matrix protein, in contrast, decreases in molecular weight upon heating in SDS. This shows that the *tolG* protein is heat modifiable. Due to variation in electrophoretic techniques, Dirienzo *et al.*(1978) cited several different molecular weights for the native form of the protein including, 27K, 38K, and 28.5K. The native proteins have a high Beta structure content that unfolds above 50°C to yield 36K, 48K, 33K forms respectively. Using the OmpA nomenclature, Lugtenberg and Van Alphen (1983) determined that this heat modifiable protein has a M_w of 35K and a MW of 28K in the native form. The protein requires LPS for its biological activity and is involved in determining cell shape (Lugtenberg, 1981).

OmpA is considered a major outer membrane protein present in the cell wall of Gram negative bacteria, specifically *Enterobacteriaceae* such as, *E. coli* and *Salmonella* (Lugtenberg and van Alphen, 1983). Several functions of this major protein include, F-pilus-mediated conjugation and uptake of colicin and ferrichrome iron (Overbeeke *et al.*, 1980). In a study

conducted by Overbeeke *et al.* (1980) to determine the antigenic relationship between pore proteins of *Escherichia coli* K12, a monoclonal antibody raised specifically against OmpA was homologous only toward this protein. Whereas, when antibodies were raised against OmpC, another protein, OmpF cross-reacted with these proteins. This showed that the OmpA protein is homologous with respect to its amino acid composition (Overbeeke *et al.*, 1980). According to Puohiniemi *et al.* (1990) the OmpA porin has unique characteristics and these are as follows: 10^5 copies exist per cell; the protein is made of a 325 amino acid residue that covers eight times the length of the outer membrane of *E. coli*; four loops bind bacteriophage to the outer membrane, are present on the surface of the bacteria. Although OmpA resides on the surface of the outer membrane, it can also be associated with the peptidoglycan-protein bound layer in minute amounts (Lugtenberg *et al.*, 1977).

(iv) OmpC

According to Lugtenberg (1981) OmpC has an electrophoretic band corresponding to a MW of 36K. In environments where the osmolarity of the medium is high, this protein is distinctively expressed. This protein is identified as a channel protein that allows only hydrophilic solutes with a molecular weight less than 7K to enter.

(v) OmpF

In comparison to OmpC, OmpF is distinctively expressed in environments with low osmolarity. It has the same number of molecules per cell as OmpC, but it has a higher MW of 37.2K (Lugtenberg, 1981).

Overbeeke *et al.* (1980) discussed the homology of OmpC, OmpF and protein E of *E. coli*, stating that the OMPs are not only cross-reactive but also share similar amino acid composition and amino acid termini.

Both OmpC and OmpF are exclusively bound to the peptidoglycan layer and cannot be released at temperatures of 60°C or lower using SDS solubilization (Lugtenberg, 1976).

L. Permeability

As previously mentioned, small molecular weight solutes are allowed to enter the cell via hydrophilic channels termed 'porins' using passive diffusion at concentrations higher than 10^{-6}M (Nakae, 1975). Larger solutes that cannot pass through these permeability barriers can enter at low concentrations via a substrate or substrate-chelator complex (Brass, 1986) using facilitated diffusion (Decad and Nikaido, 1976).

M. Environmental factors

Environmental affect toward the bacterial cell and the antigenicity of the 35K protein.

(i) Solutes

One requirement of the cell is to maintain osmotic pressure. The type of solute an organism requires is dependent upon the nature of the surrounding medium and the ability of the organism to adapt. Salmonellae, overall, are considered non-halophilic. However, this species still requires specific ions to maintain proper turgor pressure within the cell. Stainer *et al.* (1986) state that a direct relationship exists between the concentration of potassium ions and osmotic pressure, as well, K^+ is accumulated in the cell in greater amounts over Na^+. Though the K^+ ion is favored over Na^+ it does not affect the expression of outer membrane

proteins (Van Alphen and Lugtenberg, 1977). To prove this, *E. coli* was grown on supplemented growth media with a high concentration of NaCl, KCl, or sucrose. Cell envelope proteins were extracted and SDS-PAGE was conducted. It was concluded that any difference in the expression of the bands was due to the osmolarity of the media rather than the effect of a specific type of ion.

(ii) Iron

Although most enteric bacteria utilize organic compounds such as carbohydrates as an energy source, auxotrophic species within the enteric group may use certain inorganic compounds for specific growth requirements.

The need for bacteria, more specifically *Enterobacteriaceae*, to sequester iron stems from the cell's requirement to use iron. Iron is required for more than twenty functions. These include the following: acting as a cofactor for H_2O_2 and O_2 metabolism; the tricarboxylic acid cycle; and as iron enzymes such as, cytochromes, heme and non-heme proteins (Neiland, 1981a, Stainer *et al.*, 1986). The virulence of most enterobacteria can be attributed to their assimilation of iron regulated at the membrane level (Neiland, 1981a).

The availability of iron in the host is restricted from access by the invading organism. It is bound by proteins such as hemoglobin, myoglobin and cytochromes within the cell. It is bound extracellularly by transferrin in the blood and in the small intestine and tissues by transferrin, and intracellularly by ferritin (Stainer, 1986).

Since *Salmonella* is a pathogenic intracellular organism, it can acquire the iron that is protein bound in the vertebrate host by a ligand type siderophore, which releases the iron from its binding compound (Stainer, 1986). A 'siderophore' is a low molecular weight compound

that is chemically composed of hydroxamates that forms 'ferrichromes' or phenolates-catecholates and produces ferric 'enterobactin' (Neilands, 1981b).

Enterobactin is not stable under pH conditions lower than neutrality. Therefore it is susceptible to oxidation and hydrolysis (Neilands, 1981a). Siderophores have a strong affinity for iron in the ferric form (Neilands, 1981b) formed in the outer membrane (Neilands, 1982). Two forms of iron exist, ferric and ferrous. The cell transforms the former into the latter once it passes into the membrane.

Iron can be assimilated into the cell by either low or high affinity systems. Using the former system, Fe (III) enters the cell without the presence of any kind of solubilizing or transporting compound or without membrane receptors (Neilands, 1981a). By the formation of insoluble complexes between iron and hydroxyl ions, the cell can use these polymeric forms of iron (Neilands, 1981a).

The high affinity pathway uses siderophores (iron bearers) and receptors to incorporate iron into the cell. The iron assimilation in aerobic and facultative anaerobic microorganisms was schematically diagramed by four mechanisms of siderophore-mediated iron uptake by Neilands (1982). These include the high affinity pathways listed below: the ferric iron complex; enterobactin; the complex plus the receptor; and the ferric ion without the insertion of the siderophore into the cell.

The low affinity pathway involves the transfer of ferric ion to ferrous ion across the membrane. Neilands (1982) cited several authors who were responsible for devising tests to detect iron deficiency in an organism. These include the ferric perchlorate reaction, catechol or hydroxamate-type siderophores or by over-production of certain membrane proteins, such as

revealed by SDS-PAGE. The latter of the three methods will be used in this thesis to detect differences in protein patterns. According to Klebba *et al.* (1982) growth in iron-deprived media induces the synthesis of certain membrane proteins, including 83K, 81K, 78K, 74K and 25K. *S. typhimurium* binds to citrate and uses it as a carbon source whereas *E. coli* does not. Therefore, citrate would affect iron metabolism due to the ferrous citrate complex that is usually formed (Neilands, 1981a).

(iii) Phosphate

Phosphate represents 3% of the dry weight composition of the microbial cell. It is important in the physiological function of the cell because it forms part of the nucleic acids, phospholipids, and coenzymes (Stainer, 1996). Phosphate enters the cell via OMP porins, specifically protein P, 48000 M_w, from *Pseudomonas aeruginosa* that forms small anion specific channels responsible for high affinity phosphate transport (Hancock and Benz, 1986).

Poole and Hancock (1986) noted two outer membrane proteins from the families *Enterobacteriaceae* and *Pseudomonadaceae*, PhoE and protein P, respectively that are both phosphate regulated porin proteins. However, both have different entrance permeability characteristics. Distinctively therefore, PhoE has larger permeability channels and as a result, shows poor anion selectivity and allows compounds to enter by general diffusion pathways (Benz, 1984).

In comparison, protein P is specifically selective toward anions and phosphate via their positively charged lysine residues (Poole and Hancock, 1986). To prove its selective affinity toward phosphate, Hancock and Benz used conductance procedures to determine that protein P had higher affinity for phosphate than the chloride anion (1986).

Poole and Hancock (1986) also showed that heat modifiable protein, considered being major outer membrane proteins, include, OmpC, OmpD and OmpF of *S. typhimurium*. They are involved in the formation of porin channels and they are induced by phosphate starvation (Poole and Hancock, 1986). Poole and Hancock (1986) tested 12 species of *Pseudomonadaceae*, three strains of *E. coli K-12* and one strain of S. typhimurium. Temperatures of 30 or 37°C under phosphate-sufficient (1.0 mm) and phosphate-deficient conditions (0.2 mm) were examined. Solubilization using SDS, SDS-PAGE and Western Immunoblotting using a protein P trimer-specific polyclonal antiserum was studied to validate the oligomeric forms of the proteins.

Environmental factors play a role in determining the viability of the cell. According to Allwood and Russell (1970), the cell will not produce certain proteins, including OMPs under nutrient deficiency or cofactor limitation. For example, if the growth medium is deficient in iron, certain proteins normally synthesized by the cell will not be produced. This was evident by studying the electrophoretic profile of the outer membrane proteins. Alternately, if synthesis of the proteins is repressed by starvation, other proteins may be synthesized to compensate for the absence of this protein (Allwood and Russell, 1970).

Most cells produce different proteins to adapt to changing environmental conditions, unless faced with extreme conditions where anabolic activity is halted. To adapt to temperature change, the cell responds by producing specific proteins known as either 'cold shock' or 'heat shock' proteins, in these respective environments (Kaufmann, 1991).

(iv) Cellular injury

Bacteria have minimum and maximum thresholds of response to different environments. The cell usually undergoes some form of changes due to the environmental stress that results in either lethal or sublethal injury. Either way, the cell undergoes loss of cellular components, their metabolic activity is altered and they become more susceptible to sanitizers and antibiotics (Busta, 1976). Two situations develop in response to this stress. If stress occurs over an extended period, the cell is not able to repair itself, causing death. The second situation is where the cell is injured, the stress is removed and the cell is allowed to resuscitate. During the latter stage, the cell can restore or resynthesize components destroyed during injury. These include structural elements in the cell wall, cell membrane and functional damage to RNA or DNA, chromosomes and enzymes as reviewed by Hurst *et al.* (1976). More specifically they include, ribosomes denatured by heat, phospholipid lost due to rupture of the cell wall, and proteins induced by cold or heat (Busta, 1976). Though the cell can repair itself, it enters a lag phase and is not able to replicate. Busta (1976) distinguished injured cells by their inability to form colonies on media, lack of turbidity in broth or by the inability to use added substrates.

Cellular injury occurs when the cell membrane permeability has been changed due to extrinsic factors that caused the environment outside the cell to alter. These factors include, temperature extremes, altered pH and osmolarity, water activity (a_w), ionic strength, turgor pressure, and humidity. Cellular injury can also occur due to cell starvation during growth period. Lack of iron, maltose, phosphate and NaCl can induce injury. Starvation injury

involves disruption of cellular activity including protein synthesis, cell maintenance, regulation and reproduction.

According to Ray (1973) injured cells can be divided into two nonlethal injured groups, metabolically injured and structurally injured. Metabolically injured cells are those that are not able to form colonies on minimal agar that contains inorganic salts. Therefore they must be grown on a nonselective complete agar medium. Structurally injured cells are those cells that can grow on selective complete agar medium. An assessment of injury can be established by evaluating whether the treated cells can grow on the same growth media and under the same conditions as uninjured cells (Martin and Katz, 1991).

(v) Growth conditions

Growth of an organism is dependent upon several environmental factors. These include type of substrate, pH, temperature, osmolarity and oxidation-reduction potential. Under extreme conditions, these factors may have an inhibitory or negative effect on the cell by preventing or altering protein composition, cell regulation or fatty acid composition.

Deprivation of nutrients including carbon, nitrogen, phosphate, iron, oxygen or even a change of environment such as pH, temperature and osmolarity could come about due to several circumstances. These include competing organisms, destruction of nutrients during processing, or heat treatment causing lethality to the organism.

Organisms respond to environmental stress (present outside the cell) by synthesizing proteins regulated by genes. The proteins are regulated relevant to the type of stress involved.

(vi) pH

The pH of an environment can be changed by competing organisms that are present among the existing natural microflora. For example, salmonellae can survive at a minimum pH of four. However, competitors such as, lactic acid bacteria produce waste products that acidify the media to the extent that salmonellae are eliminated. Processing can also alter the pH of the environment.

Foster and Spector (1995) described the route salmonellae take upon entering a host. They must first encounter the low pH of the stomach. Salmonellae are an enteric group of bacteria that colonize the gut and intestinal tract. They can adapt to bile salts, weak acids, competing microflora and a higher osmolarity. They must also contend with epithelial and M cells in the intestinal mucosa and macrophages. They must survive the antibacterial effects of the blood. These organisms can adapt to these changes by the activation of regulatory genes and synthesis of adaptive proteins. The effect of heat, starvation, iron, osmolarity, oxidative stress, cationic peptides and low pH on the stress-response system was studied by Foster and Spector (1995). The induction of regulatory genes usually occurs over three intervals: immediately after a stress is invoked, 1-2 hours after the onset of stationary phase and 4-5 hours after a stationary phase has started (Foster and Spector, 1995).

The effect of low pH on a bacterial cell is dependent on the type of acid present. Chung and Goepfert (1970) provided a table of minimum pH values at which salmonellae can grow using different types of acid. Chung and Goepfert (1970) also categorized these acids into three groups, based on their permissivity: most permissive; intermediate; and restrictive. The last category includes short chain fatty acids, as well as acetic and propionic acid.

Therefore, for this class, the minimum pH that salmonellae could grow would be pH 5.0. These values are also affected by factors such as: temperature; type of medium; and strain of bacteria. In a food matrix, pH alone does not inhibit growth of organisms. Chung and Goepfert (1970) also suggested that factors such as temperature, oxygen reduction potential and the water activity of the medium play a synergistic role. They determined through their work that salmonellae cannot become tolerant to a lower pH by transfer from an environment of near optimum conditions. This is in contradiction to a more recent study conducted by Foster and Hall (1990) using *S. typhimurium*. Two types of pH shifts were conducted. A pH shift from 7.6 to 5.8 and 3.3 and a pH shift from 7.6 directly to 3.3. The cells were subjected to a gradual decline in pH were better able to survive at a final pH of 3.3 than cells directly subjected to pH 3.3. Foster and Hall (1990) attribute this to acid tolerance response (ATR) by which the cells can adapt to the lethal effect of an acid environment through three available mechanisms. These include reduced membrane conductivity, higher proton extrusion, or a higher buffering capacity of excess H^+ by cellular components. The survival of *S. typhimurium* to a pH of 3.0 was based on two ATR systems (Foster and Spector, 1995). The first occurs during the logarithmic stage and is termed an 'acid shock' which produces 50 acid-shock proteins (ASPs). The second system consists of the production of 15 proteins during the stationary phase. Cells subjected to mild acidic conditions or 'preshock' can react to an increasing H^+potential or by changing the ATR balance to reduce the stress. This causes an increased production of proteins and is called a 'buffering effect' (Foster and Hall, 1990). Leyer and Johnson (1993) conducted a study to determine whether the effect of acid adaptation cross-protects against other environmental stresses in *Salmonella typhimurium*. They

concluded that acid-adapted *S. typhimurium* developed better tolerance toward thermal and osmotic stress, the antimicrobial lactoperoxidase system present in milk as well as to hydrophobic and surface active compounds. This was attributed to an increase in the production of outer membrane proteins that changed the membrane structure, for example, heat shock proteins (Leyer and Johnson, 1993). Hickey and Hirshfield (1990) compared *Salmonella typhimurium* and *Escherichia coli* on minimal type medium at a low pH, (5.0). Several factors that affect growth rates were studied including the effect of external pH, internal pH, cell density and production of polypeptides. Hickey and Hirshfield concluded that *S. typhimurium* can adapt better to a pH shift from 7.0 to 5.0 than *E. coli*. This was attributed to the fact that *S. typhimurium* produces H_2S gas from sulfur-containing substrates, such as tetrathionate and thiosulfate, which depresses the external pH of the medium (Hickey and Hirshfield, 1990). Therefore, *Salmonella* can adapt to survive in the reduced pH based on its natural ecology and the increased production of polypeptides under stressed conditions (Hickey and Hirshfield, 1990). To show ATR response either minimal nutrient media or chemically complex media can be used (Foster and Spector, 1995).

(vii) Osmolarity

Microbial growth is dependent on the available water or water activity (a_w) of the medium. Each genus of bacteria has different a_w requirements. The minimum theoretical value for salmonellae to grow is 0.93 (Macrae *et al.*, 1993). High salt or sugar concentrations affect the osmolarity of the medium. The higher concentration of solutes outside the cell causes pressure on the cell wall. In effect this exerts an inward pressure on the cell membrane (Foster and Spector, 1995). Van Alphen and Lugtenberg (1977) conducted an experiment to determine the

influence of osmolarity of the growth medium on the outer membrane protein pattern of *E. coli*. The supplementation of yeast broth with either 300 mm NaCl, 300 mm KCl or 600 mm sucrose generated a reduced level of protein B and an increased level of protein C. In a separate study, the expression of OmpC of *Salmonella typhi* and *E. coli* was determined to be influenced either by low or high osmolarity. Nutrient broth (no sucrose added) was used as the control and represented a low level of osmolarity. A 20% sucrose solution was added to nutrient broth to develop conditions of high osmolarity.

Puente *et al.* (1991) showed that although the OmpC pore protein was expressed in *E. coli* under conditions of high osmolarity but repressed under conditions of low osmolarity, the OmpC in *S. typhi* was expressed under both conditions. Puente and coworkers (1991) also studied the effect of temperature (4, 20, 37, and 42°C) with respect to expression of Omps and concluded that alteration occurs because of the effects of both temperature and osmolarity.

To compensate for the increase in external pressure due to high osmolarity, the cell produces glutamate and trehalose or externally transports compatible solutes such as K^+, glycine-betaine and proline into the cell via a 'homeostatic mechanism' (Foster and Spector, 1995). Several genes are regulated by a single operon including proV, proW and proX in salmonellae to provide uptake of these components into the cell (Foster and Spector, 1995). Osmotic pressure differentials are constantly present between the environment and the inside the cell. The periplasmic space expands to allow entry of carbohydrate into the cell (Kawaji *et al.*, 1979). The outer membrane and the peptidoglycan layer must compensate for the osmotic pressure produced by the periplasm (Kawaji *et al.*, 1979). In contrast, the osmotic pressure

between the periplasm and the cytoplasm are negligible since both share an iso-osmotic relationship (Kawaji *et al.*, 1979). Therefore, results showing the OMPs O-8 and O-9 of *E. coli* synthesized and suppressed due to a high osmotic strength, is questionable (Kawaji *et al.*, 1979). It is more likely that the outer membrane and peptidoglycan layer recognize the discrepancy in osmotic pressure and regulate the production of these proteins to compensate (Kawaji *et al.*, 1979).

(viii) Oxygen-reduction potential

The oxygen-reduction potential (OR potential) of the environment can restrict microbial growth in the surrounding medium (Frazier and Westhoff, 1980). There are also oxidizing and reducing compounds that can have a bacteriostatic effect or encourage growth depending on concentration and the type of materials present. For example, cysteine can encourage biological activity by reducing the oxygen potential of the medium and this provides a suitable environment for multiplication (Allyn and Baldwin, 1930). Aerobic bacteria normally grow under a high oxygen potential. Facultative organisms such as *Salmonella typhimurium* can grow under a low oxygen-reduction potential that produces an anaerobic environment. To overcome the shift from aerobic to anaerobic conditions, *S. typhimurium* produces over 30 stress proteins or stimulons (Spector *et al.*, 1986). Under anaerobic conditions, the repression of the 24.6K major outer membrane protein of *S. typhimurium* was demonstrated by electrophoresis using a 16% polyacrylamide gel (Schiemann and Shope, 1991). Ames (1974) found that *S. typhimurium* cells grown under anaerobic conditions did not contain two high molecular weight proteins, 80K and 81K, and two low molecular weight proteins, 43K and 36K that existed under aerobic conditions.

N. Environmental effects of temperature

(i) Freezing

Freezing is used to preserve or extend the shelf life of food. Processors depend on this added value step usually after the food is cooked to provide more convenience to the consumer. The main purpose of freezing is to slow the deterioration of the food. This process may not, however, significantly reduce the initial microbial population present (Raccach and Juven, 1976). Loss in bacterial viability is primarily dependent on the rate of freezing.

Exposed to freezing temperatures, water freezes within the cell at a slow or fast rate. Slow freezing yields larger ice crystals causing a greater extent of cellular damage compared with fast freezing. This is due to the rupture of the cell wall causing the release of cellular fluids and DNA material (Ray and Ordal, 1973). Fast freezing limits osmotic damage by forming a protective layer of ice from the external water that surrounds the cell and limits the size of ice crystals, thus reducing damage from their formation (Ray and Ordal, 1973). During slow freezing, the cell is frozen from the interior outwards and during fast freezing, the cell is frozen from the exterior toward the interior. Raccach and Juven (1976) proved that a recovery better than 60% was possible with *Salmonella gallinarum* at a freezing rate of 18°C per minute as compared with freezing at a rate of 0.5°C per minute.

The overall effect of freezing causes an increased concentration of solutes both inside and outside the cell, precipitation of low and high molecular weight solutes and the deceleration of chemical reaction rates in the cell (Ray, 1973). Cellular components damaged by freezing include the permeability barrier, LPS, RNA and proteins. This causes leakage of cellular fluid and the cellular matter such as proteins. Free radical reactions occur due to a

loss in the oxidation-reduction potential of the cell. Hydrophobic bonds weaken due to an increase in ionic concentration (Ray, 1973). A loss of periplasmic enzymes causes formation of spheroplasts due to cellular damage. Thus, the cell becomes more susceptible toward detergents and antibiotics because of this (Hurst, 1977).

Cryoprotectants aid in protecting organisms from death due to freezing based on their ability to bond with water. They are primarily composed of organic compounds and inorganic solutes, most of which contain -OH or $-NH_2$ groups such as amino acids, proteins, glucose, acetamide and yeast extract. Ray (1973) has defined these as cryoprotective agents but was not able to provide an explanation of the exact mechanism of protection.

Several factors may interact to affect the lethal action of freezing and thawing. This includes, nature of the organisms, species, strain, stage of growth, and surrounding environmental conditions, such as pH, time and temperature (Ray, 1973, Raccach and Juven, 1973, and Arpai, 1962). The ability of cells to recover from injury is dependent on injury and the ability of the organism to use the available organic nutrients for energy and repair (Ray, 1973). According to Raccach and Juven (1976), organisms that are non-lethally affected but metabolically injured cannot be revived on minimal agar; the agar must be supplemented with casein or catalase. Raccach and Juven (1976) used tryptic soy agar (non-selective) and a minimal agar (selective) to calculate the percentage injury of *Salmonella gallinarum.* The cells were exposed cold at a rate of 22°C per minute. The final temperature of the medium reached -25°C. The cells were thawed at a rate of 15-20°C per minute. The final temperature was 4°C. Based on the colony forming units enumerated on both types of media, 35% of the viable cells had been injured.

(ii) Heat

Salmonella is a mesophilic organism. Its maximum growth temperature is 45°C but the organism achieves optimum growth at 37°C. To destroy salmonellae thermally, a heat treatment of 66°C for 12 minutes is required in food processing but this is dependent on the food product type (Frazier and Westhoff, 1988). According to Mackey and Derrick (1982a), the pasteurization process used for food products such as whole eggs or albumin is more detrimental to contaminating organisms than conditions used in a laboratory setting.

Allwood and Russell (1970) extensively reviewed the mechanisms by which bacterial cells are inactivated by heat. The review concerned the collective literature compiled on the destruction of the bacterial cell due to heat and relevant data are outlined below.

Specific sites affected by heat include the cell wall, cytoplasmic membrane, functional and structural proteins and enzymes, RNA and DNA. The effect that thermal inactivation has on the cell is dependent on several conditions. These include, the duration of the heat treatment, composition and pH of the surrounding environment, the initial number of cells present and resistance of the organisms.

Inactivation of the bacterial cell by heat is due to leakage of cellular material through the cell wall and cytoplasmic membrane. Products lost by the cellular damage include Ca^{2+}, Mg^{2+}, K^{+}, phosphate ions, amino acids and substances spectrophotometrically absorbing between 260 nm (Lee and Goepfert, 1975). The denaturation of protein also occurs. In order for the heat treatment to cause the protein moiety to unfold, water must be introduced with the heat treatment. This disrupts the hydrogen bonds between the polypeptide chain and causes coagulation of the protein. Therefore, moist heat is more detrimental to the cell than dry heat.

Consequently, lower moist heat temperatures can be used to injure or reduce the microbial load during processing. Dry heat will still cause injury to the cell but its effect is primarily due to oxidation. The cell can resynthesize protein in a suitable recovery medium.

Bacterial enzymes are also inactivated by heat although they are less sensitive to heat than other proteins. Specifically, heat inactivates enzymes such as dehydrogenase in *S. typhimurium* and *S. aureus* (Hurst, 1977). In a study conducted by Mackey and Derrick (1982a), the catalase enzyme was inactivated in *S. typhimurium* cells exposed to a heat treatment at 48°C for 45 minutes. Hydrogen peroxide is then formed by autoxidation and interacts with the injured cells. That H_2O_2 is toxic to the cells is more evident on solid recovery media than in liquid (Mackey and Derrick, 1982b).

Loss of viability is also due to RNA degradation from the membrane. Of the three different types of RNA (transfer, messenger, and ribosomal), ribosomal RNA is the functional unit of protein synthesis. After the cell is injured, a delay in the recovery of the cell occurs because the cell is not able to reproduce until the ribosomes destroyed are resynthesized (Allwood and Russell, 1970). The 30S and 50S ribosomal subunits are essential for protein synthesis. Witter and Ordal (1977) experimented with ribosomes and found that they were degraded by heat although RNA is stable at temperatures between 50-90°C (Allwood and Russell, 1970). Heat as well contributes to chromosomal damage by forming single strand breaks in bacterial DNA (Hurst, 1977). The ability of bacteria to survive heat treatment is regulated more by the ability to restore the damaged components injured by the heat than the ability of the cell to tolerate heat (Allwood and Russell, 1970).

According to Tomlins *et al.* (1982) the type of fatty acid composition of the organism affects its tolerance toward heat injury due to the ratio of saturated/unsaturated fatty acids which affects lipid fluidity. The alteration of the fatty acid composition is changed by the temperature and composition of the menstrum. Modification of fatty acids can be done by adding deoxycholate or benzoate to the medium (Tomlins *et al.*, 1982).

A study was conducted to determine the effect of heat treatment at varying pH on the survival of *S. typhimurium* (Clark and Ordal, 1969). The results showed that the ability of the organism to withstand heat treatment at 48°C was improved at a pH less than 8. Lee and Goepfert (1975) conducted a study to determine the effect of certain solutes toward thermal injury of *Salmonella typhimurium*. The extent of injury was dependent on the type of solute and its concentration. Based on the results, solutes such as sucrose up to a concentration of 30% increased the resistance of *S. typhimurium* by reducing the internal a_w of the cell. Goepfert (1975) assessed the number of injured cells by enumerating the total number of colonies on Tryptic Soy Agar supplemented with yeast extract (TSA-YE) and eosin methylene blue agar supplemented with 2% NaCl (EMB-NaCl). The viable cells enumerated on TSA-YE minus the uninjured cells enumerated on EMB-NaCl yielded the number of injured cells. Those organisms that can recover usually require a three-hour resuscitation period.

O. Detection of salmonellae

The standard protocol to evaluate a food product for the presence of salmonellae, requires an enrichment period on complex medium. This allows a recovery period for injured salmonellae to repair themselves and these would not be detected using selective medium. Salmonellae are isolated from the other existing microflora in the food product. The cells are

transferred and allowed to incubate in selective media such as Selenite Cystine Broth and/or Tetrathionate Broths. The cells are transferred onto plates of Brilliant Green Lactose Bile or Xylose Lysine Deoxycholate (XLD) and incubated to retrieve isolated colonies of salmonellae. Specific biochemical and serological tests can be done on typical colonies that form on the plates (AOAC, 1978). The conventional method takes 5 days. The fourth day allows confirmation of negative Andrews, 1995.

(i) Rapid methods vs conventional methods

Rapid methods are valuable for both the food industry and regulatory agencies. Advantages rapid methods include safe finished product assurance, fast detection of poultry and meat contaminated with salmonellae and rapid response to any contaminated products (Swaminathan *et al.*, 1985).

Several rapid method detection methods have been developed to decrease the time required to confirm a positive. Andrews (1995), compared rapid methods with approved conventional methods. A method must be accepted by the Association of Official Analytical Chemists (AOAC) and the International Commission for Microbiology Specifications for Food (ICMSF) in order for it to be acknowledged as an approved standard protocol. Methods must have a sensitivity level of 1 cell/ 25g and be applicable to a variety of food products based on collaborative studies.

(ii) Direct fluorescent-antibody (FA) technique

This method employs immunological staining using fluorescein-labeled antibodies directed toward specific antigens isolated from *Salmonella* (Flowers, *et al.*, 1992). The approved AOAC method consists of a 24-hr preenrichment, a 24-hr selective enrichment and a

4-hr post-enrichment stage; cells isolated from the post-enrichment stage are stained to determine the fluorescent cells (Flowers *et al.*, 1992). Based on morphological assessment of the fluorescent cells, a presumptive-positive is determined that must be confirmed by conventional methods (Flowers, *et al.*, 1992).

A fluorescent-antibody (FA) method for *Salmonella* was used as a pre-enrichment to restrict the growth of non-*Salmonella* organisms, and it subsequently increased the growth of *Salmonella* while decreasing background fluorescence (Insalta *et al.*, 1975). Over 100 samples including raw materials, animal feed and contaminated food were analyzed during this study using FA as a preenrichment broth followed by four different methods of selective enrichment: a microcolony technique; selenite-F FA technique; tetrathionate Brilliant Green FA microcolony technique; and FA technique from broth enrichment. Isolates from all four methods were confirmed by AOAC cultural confirmation. In comparison with the other three methods, Insalta (1975) concluded that the FA technique produced only 4.9% false negatives and no false positives on tetrathionate broth. FA can also be used as a slide staining technique by observing FA smears made on agar-prepared slides.

(iii) **Oxoid *Salmonella* rapid test** (OSRT)

Holbrook *et al.* (1989) conducted a study to determine the specificity of the Oxoid *Salmonella* Rapid Test which is based on Selective Motility Enrichment Techniques (SMET). This rapid 2-day test works by allowing salmonellae to migrate from enrichment media in the bottom compartment of a vessel to the top compartment containing selective media and novobiocin (sodium salt). A positive result is indicated by a colour change in the media of the top compartment from the motile *Salmonella*. The change in colour represents a biochemical

reaction between the protein (H antigen) and the enzyme-conjugated-antibody (polyvalent H) system (AOAC, 1995). In these tests, they found a specificity of 96.8% positives among 296 strains of *Salmonella*. No positives were obtained from the non-salmonellae species tested.

(iv) ELISA

Mattingly and Gehle (1984) used an indirect enzyme-linked immunoassay (ELISA) as a rapid method to examine 100 pure strains of *Salmonella* cultures and found that 94% of the strains tested positive as *Salmonella*. The monoclonal antibody Mattingly and Gehle (1984) produced was directed toward a structural antigen or amino acid sequence on the flagella of *Salmonella*. The heat-extracted strains were diluted to a known concentration of 10^4-10^6 colony forming units (CFU)/ ml. The sensitivity level of this rapid test method was determined to be 10^5 CFU/ml. Using a mixed culture of *E. coli* and *Salmonella* in a ratio of 1000:1, the ELISA still detected the *Salmonella*. In comparison with the FA method, which offers subjective results by allowing determination of agglutination or fluorescent organisms, ELISA is objective since it produces a colour reaction measured using a spectrophotometer (Mattingly and Gehle, 1984). Minnich *et al.* (1982) also achieved a sensitivity level of 10^6CFU/ ml and the method gave a strong positive visual result using an indirect ELISA. A total of 98 naturally contaminated food products were tested. They contained less than one cell per gram of *Salmonella*. They were evaluated using this technique and were compared with the pure culture method, immunofluorescence (IF) and enrichment serology (ES). Out of all the samples, only 13 subsamples were positive by all four techniques. Results were obtained, however, after a 4-6 hour pre-enrichment period, using the ELISA method. Disadvantages of other techniques are presented below. The FA test yields too many false positives and requires

a concentration of 10^7 salmonellae/ ml. The IF method develops problems when high numbers of organisms are encountered and results are subjective (Minnich *et al.*, 1982). Mattingly *et al.* (1985) also noted that cross-reactivity between antibodies in the antisera yielded between 5-9% false positives when the FA method was used. The same problem with false positives can occur due to cross-reactivity of polyclonals with the ES method (Mattingly *et al.*, 1985).

(v) DNA method

DNA hybridization (DNAH) has also been developed to detect salmonellae in foods. This method is based on developing probes that identify unique nucleotide sequences in target organisms (Flowers *et al.*, 1985). According to Flowers *et al.* (1985) there are two types of DNAH; isotopic and non-isotopic. The isotopic DNAH method involves 42 hr of culture steps and a 4 hr assay. The non-isotopic colorimetric DHAH method involves 44 hr of culture steps and a 2.5 hr assay. False negatives are reported as low as 1.8% and 0.8% for isotopic and non-isotopic DNAH method, respectively (Flowers *et al.*, 1985). Both DNAH techniques are only used as screening methods and conventional culture methods must be performed. Fitts (1985) attributes the reliability of this method to the presence of DNA that is never lost due to environmental conditions. He also noted that the DNA sequences are highly conserved within a specific genus.

Based on a review of rapid methods to detect salmonellae in comparison to the conventional culture method, both ELISA and DNA are not able to detect low numbers of salmonellae found in pre-enrichment and non-selective post-enrichments (Flowers, 1985).

(vi) Cross-reactivity of rapid methods

According to Kerr *et al.* (1992) there are different modes of capture that can be used with the ELISA system. This includes either polyclonals raised against flagella or monoclonals that can be raised against either heat extracted protein or flagellar antigen. Kerr *et al.* (1992) conducted a study to determine whether monoclonal antibodies could be raised against outer membrane protein and be specifically used to detect salmonellae. Kerr and coworkers (1992) suggested that adopting OMPs as antigens would be more advantageous than flagellins. Several disadvantages exist for using the flagellar antigen, but most importantly this includes the occurrence of non-flagellar serovars within a variety of salmonellae species that produce false negatives and cross-react with other *Enterobacteriaceae* causing false positives. Kerr and coworkers (1992) produced MAbs that did not react with four non-*Salmonella* organisms which share similarities among them, including: *E. coli*; *Klebsiella pneumoniae*; *Serratia marcescens* and *Citrobacter freundii*. However, they did react with 57 of the *Salmonella* species examined. Immunoblotting was performed using two of the seven MAbs produced. The MAbs were reactive with a 36K polypeptide using the nitrocellulose immuno-blot. According to Brown and Hormache (1989), the 36K polypeptide band represented OmpA and porins which share characteristics of being highly immunogenic and have similar migration patterns in SDS-PAGE. Using the ELISA capture method, 3.1% false positives were obtained from samples of small intestine gathered from clinical trials. The cross-reactivity was eliminated by boiling the cultures for 30 minutes before testing. Biswas and Chakrabarti (1994) extracted OMPs using a sarkosyl-insoluble fraction and studied the antigenicity and antigenic cross-reactivity of OMPs of *Vibrio parahaemolyticus*. The

insoluble-fraction which contained the OMPs also contained LPS. According to Kerr *et al.* (1992), the LPS can be eliminated by extraction with a chaotropic agent such as, guanidine thiocyanate to solubilize the protein fraction. This can be followed by ultracentrifugation to remove the insoluble LPS fraction.

P. Heat modifiable proteins

A heat modifiable protein is characterized by having two different migration patterns on SDS-PAGE based on the solubilization temperature in SDS used before loading. Zhang *et al.* (1989) reported that the OmpA protein to have a native molecular weight at a temperature of 37°C, compared with solubilization at 100°C in SDS. Analysis of SDS-PAGE of protein extracted from *Yersinia pseudotuberculosis* strain 78, revealed a major band at 35K using 100°C solubilization and a band at 28K after 37°C solubilization (Zhang *et al.*, 1989). To confirm the heat modifiable behavior of the protein, the 28K band was extracted from the gel and reheated at 37°C and 100°C. The 28K band migrated to the 35K position due to heat treatment at 100°C. Using immunoblotting, both the 28K and 35K protein bands reacted with the Ye-2 MAb. Zhang and coworkers (1989) used amino acid sequencing to reveal that the first 10 residues of the NH_2-terminus on *Yersinia pseudotuberculosis* OmpA were identical to that of OmpA sequences of *S. typhimurium* and *E. coli* researched by other authors. Although Zhang and coworkers (1989) were not able to distinguish whether the reactivity of the 35K protein was due to the carboxyl-terminus or NH2-terminus. Zhang and coworkers (1989) also used gene coding to determine that mutants lacking the OmpA protein did not produce the 35K band but did express the 35K band upon insertion of a plasmid coded for the OmpA gene.

Q. Antigenicity

The antigenic cross-reactivity of major OMPs in *Enterobacteriaceae* species was examined by Hofstra and Dankert (1979, 1980b). The antigenicity of the proteins studied could not be assessed because the method of separating these proteins into single molecules by boiling and denaturing was highly detrimental. This is contradictory when compared with later findings reported by Sarwar *et al.* (1992). They studied the effect of growth phase on the conservation of certain epitopes of the major OMPs of *Branhamella catarrhalis*. The cells were harvested at various stages of the growth phase. Sarwar and coworkers (1992) found boiling irrelevant with respect to conserving the antigenic properties of the epitopes.

III. MATERIALS AND METHODS

A. Materials

Bacto M Broth, used as the basic growth medium, was prepared using a formula from Difco (Detroit, MI, USA). The ingredients included the following; 5 g of yeast extract (Merck, West Germany), 12.5 g Tryptone (BBL, Darmstadt, West Germany), 2 g D-mannose (Mallickrodt Chemical, Chesterfield, Missouri) , 5 g sodium citrate (Mallickrodt), 5 g sodium chloride (Mallickrodt), 5 g dipotassium phosphate (Mallickrodt), 0.14 g manganese chloride (Fisher Scientific, Ottawa, Ontario), 0.08 g magnesium sulfate (Fisher), 0.04 g ferrous sulfate (Fisher) and 0.75 g Tween 80 (BDH, Darmstadt, West Germany) per litre. Media and reagents were rehydrated and solutions were made using distilled deionized water (Barnstead NANOpure, series 550, ULTRApure water system D4754 115 VAC; Barnstead/Thermolyne Corp., Dubuque, IA). The media used for selective isolation and enumeration of bacteria were as follows: selenite cystine (Difco), Tetrathionate Broth (Difco), Brilliant Green Lactose Agar (BBL), Salmonella Shigella agar (Difco), Nutrient Broth (BBL), Brain Heart Infusion Agar (Difco), and Standard Plate Count Agar (SPC, BBL). All other chemicals used were of analytical reagent grade and were purchased from Sigma Chemical Co. (St. Louis, MO) or Mallinckrodt Specialty Chemicals Co. (Paris, KY).

(i) Isolation of the outer membrane proteins

2-Amino-2-hydroxymethyl-1,3-propanediol (Tris) from Bio-Rad (Richmond, CA), sodium dodecyl sulphate (SDS) Bio-Rad, 37% HCl (Mallinckrodt), and 0.85% w/v saline were used. DNAase D-4527 (20,000 units) from Type II bovine pancreas, RNAase type II-A from bovine pancreas (85 k units/mg) and sodium-N-lauroyl sacrosine were purchased from Sigma

Diagnostics (Mississauga, Ontario). Ethylenediamine tetra acetic acid (EDTA) was from Fisher, and sodium azide was from J.T. Baker.

(ii) Protein determination of the extracted outer membrane proteins

The Pierce BCA Protein Assay Reagent Kit with bicinchonic acid (Rockford, Illinois) was used to determine protein concentration.

(iii) Antibody preparation -propagating MAb 1D6

RPMI Medium 1640 with L-glutamine and fetal calf serum were purchased from Gibco BBL Life Technologies (Burlington, Ontario).

(iv) Ammonium sulfate precipitation of MAb 1D6

Ammonium sulfate was purchased from Bio-Rad (CA, USA). Sodium chloride, dipotassium phosphate and disodium phosphate were purchased from Mallinckrodt (UK). Ammonium sulfate (Mallinckrodt), phosphate buffered saline (PBS) pH 7.2, and dialysis membrane (MW cutoff 12-14000, Mallinckrodt) were also used.

(v) ELISA for antibody titre

Alkaline phosphatase goat antimouse IgG (AP-GAM-IgG) and P-nitrophenyl phosphate (PNPP) 5 mg tablets were both purchased from Bio-Rad (Richmond, CA, USA). Diethanolamine, magnesium chloride, sodium carbonate anhydrous, sodium bicarbonate, potassium phosphate monobasic, sodium phosphate dibasic anhydrous and sodium chloride were purchased from Mallinckrodt (USA).

(vi) Electrophoretic chemicals

Acrylamide, bis-acrylamide, Tris, SDS, ammonium persulfate (AP), N.N.N'N'-tetramethylethylenediamine (TEMED), Bromophenol Blue, glycine, Coomassie blue R-250 (CBB), were from Bio-Rad. Glycerol, butanol, methanol, acetic acid (glacial) were from Mallinckrodt. 2-Mercaptoethanol (ME) was from Eastman Kodak Company (Rochester, NY). SigmaMarker molecular weight standards were from Sigma Diagnostics (Mississauga, Ontario).

(vii) Immunoblotting chemicals

Tris, glycine, affinity purified goat-anti-mouse IgG alkaline phosphatase conjugate were from Bio-Rad. NaCl, Tween-20, magnesium chloride, N,N-dimethylformamide, sodium hydroxide were from Mallinckrodt. Colour development reagents, alkaline phosphatase substrate tablets (5mg) were from Sigma. 5-bromo-4-chloro-3-indoyl phosphate ρ-toluidine salt (BCIP) and ρ-nitro blue tetrazolium chloride (NBT) and nitrocellulose membranes (0.45 μm) were from BIO-RAD. All reagents were of analytical grade.

B. Methods

Study 1 : Deficiency of specific nutrients or minerals in growth medium at different temperatures.

M broth media (growth media) were made without salt, iron or phosphate. The omission of each of these elements represents individual treatments. Furthermore, different growth temperatures were studied 37, 23, 42, 15°C with each individual treatment.

(i) Bacteria and growth conditions

The species of *Salmonella* used in this study included, *S. typhimurium* ATCC 13311 (serotype B), *S. thompson* (serotype C), *S. gallinarum* (serotype D_1), *S. enteritidis* PT4 (serotype D_1) and *S. arizona* (A typical). *S. typhimurium* was obtained from American Type Culture Collection (Rockville, MD, USA). *S. thompson*, *S. gallinarum* and *S. arizona* were obtained from University of Manitoba, Microbiology Department. *S. enteritidis* PT4 was obtained from Laboratory Center for Disease Control, Ottawa, Canada. Stock cultures of various *Salmonella* spp. were kept in 10% skim milk powder at -20°C until needed. Before use, the cells were thawed at room temperature. To maintain a pure culture of the working serotype, the conventional plating method was performed as follows. The entire content of the vial (1 ml aliquot) was transferred into 6 ml of nutrient broth (NB) and incubated at 37°C for 18 hr. Next, 1 ml was transferred to nutrient broth and incubated at 37°C for 18 hr. Biochemical plating followed. A loopful of the bacteria was transferred to selenite cystine and incubated (37°C, 24 hr), transferred to tetrathionate broth and incubated (42°C, 24 hr). A loopful of the organism from either tube was transferred onto Brilliant Green Lactose Broth

agar (BGA) or Salmonella Shigella agar (SS) and incubated at 37°C for 24 hr. The cultures were then transferred onto standard plate count agar (SPC) slants and stored at 4°C until used.

(ii) Cultivation of bacterial cells

To cultivate cells for extraction, cells were transferred from the agar slant into 5 ml of NB and incubated overnight at 37°C. One millilitre of this suspension was then inoculated into 250 ml of M-broth. These cells were incubated for 18 hr at 37°C in a controlled environment incubator shaker (Lab-Line Instruments, Inc., Melrose Park, Illinois) at 250 rpm. Six-500 ml flasks of M broth were inoculated with 1 ml of cells (late-logarithmic stage) grown and harvested. Cells harvested from six-250 ml flasks represented one treatment. The OMPs were extracted from these cells. The effect of environmental conditions was studied separately.

(iii) Selective omission of nutrients and effect of growth temperature

This study examined the effect of selective omission of specific nutrients and minerals on the OMPs' electrophoretic pattern from several salmonellae species. The treatments included the following; four different growth temperatures, 37°C (optimum), 23°C (room temperature), 42°C (upper maximum) and 15°C (lower minimum), three modified versions of M Broth; M Broth with no NaCl, M Broth with no ferrous sulphate and M Broth with no dipotassium phosphate, and unaltered M Broth (control). Four species of *Salmonella* including, *typhimurium*, *gallinarum*, *arizona* and *thompson* used in this study were subjected to 16 different treatments (Table 2). Based on the type of treatment, the bacteria were monitored and grown to their individual late logarithmic stages that took from 18 to 24 hours under the different incubation conditions. Depending on the species of *Salmonella*, the bacteria grown

at 15°C had a delayed logarithmic stage which required incubation to be extended to 3-5 days.

Before extraction of OMPs, salmonellae species were grown in M broth (control) or without 1.4% (w/v) NaCl, 1.4% (w/v) K_2HPO_4 or 0.01% (w/v) $FeSO_4$, for 18 hours at 37, 23, 42 and 15°C. The study objective was to determine the effect of the absence of sodium chloride, ferrous sulfate or dipotassium phosphate on the antigenicity of the 35K protein. All four species of *Salmonella* were cultured, extracted and subjected to SDS-PAGE electrophoresis and immunoblotting.

Study 2 : Effect of environmental conditions

Cell preparation was conducted as described under Study 1 sections (i) and (ii).

S. typhimurium was subjected to different environmental conditions (Table 3) to examine the effect on the protein electrophoretic pattern of extracted OMP's. Minimal medium was used in this section to study the effect of altering certain factors. The study consisted of a timed period of injury and resuscitation. To transfer injured cells into the resuscitation medium, cells were centrifuged at 4,500 x g and the pellet was submerged into a solution of buffered saline solution to prevent osmotic shock. The treatments used in this study either involved injury or injury followed by resuscitation to assess the stability of the 35K OMP. Refer to Study 2 sections i, ii, iii and iv for the specific protocol for the four injury conditions and applications; osmolarity, pH, heat and cold. A cell viability study was conducted to determine whether the cells were in fact injured. The numerical difference between colonies grown on Brilliant Green Agar +2% NaCl (selective medium) and Brain Heart Infusion Agar (non-selective) were noted but not reported.

The following formula was used to estimate the injured proportion of the population (Ray, 1979):

$$\%injury = 1 - \frac{colony\ count\ selective\ medium}{colony\ count\ non\text{-}selective\ medium} X100$$

Environmental conditions that were varied included osmolarity, pH, heat and cold. These are all a range of factors that bacteria could be subjected to in foods during harvesting, processing or storage.

(i) Effect of osmolarity

Nutrient broth (NB) medium was used to subject the bacteria to different levels of osmolarity (0.0, 0.6, 0.8, 1.0M) at 37 °C for 18 hours. Preliminary work was conducted before the treatments were begun as described below. The molarity (0.0-2.0) of the NB was adjusted by adding different percentages of NaCl to the rehydrated medium. The water activity (a_w) of the adjusted osmolarity medium was measured using a Deagon a_w meter CX-1, (Deagon Devices, Inc., Pullman, Washington). The machine was calibrated with NaCl using predicted a_w values of NaCl solutions between 15 and 50 °C provided by Resnik and Chirife (1988). The a_w of the adjusted medium was measured before and after autoclaving and allowing the temperature to reach 37 °C. A growth curve was constructed at 37 °C (250 rpm on the shaker using 500 ml flasks containing 250 ml of nutrient broth at each of the different molarity levels). Growth was assessed over a 24 hr period at 480 and 580 nm using a Spectronic 20 spectrometer (Bausch and Lomb, USA). Based on these results, it was concluded that a molarity over 1000 mm could not support the growth of *S. typhimurium.*

Therefore, from the information obtained during the preliminary work, it was determined that 0.0 mM NaCl (control- a_w 1.0), 600 mM NaCl (a_w 0.978), 800 mM NaCl (a_w 0.972) and 1000 mM NaCl (a_w 0.965) would be used to study the effect of osmolarity.

(ii) Effect of pH

Initially the pH of the medium was adjusted by adding 10 μl of 37% HCl or 1.0 N NaOH incrementally to 250 ml of nutrient broth. The pH was measured at two different temperatures, 25 °C and 37 °C. Minor differences in pH resulted at these two temperatures. From this data, tables were derived to display numeric amounts to adjust pH values from 3.0-9.0. This data represented amounts required to adjust the pH of the medium. After a 3 hr incubation, *S. typhimurium* was plated from the different pH media onto BGA and BHI before resuscitation to determine injury. Cells were injured for either 3 or 18 hr and then transferred to the resuscitation medium for the same period as the injury treatment (3 hr or 18 hr). This study involved five salmonellae species: *S. enteritidis*, *S. typhimurium*, *S. gallinarum*, *S. thompson*, and *S. arizona*. All five species were subjected to 3 hr injury followed by a 3 hr resuscitation period as well (Table 3). Cells grown at pH 3, 4, and 5 were assessed as injured but recovered during the resuscitation period.

(iii) Effect of heat

Cells were grown to stationary phase (absorbance of 0.2 at 480 nm) in a 500 ml flask containing 250 ml of nutrient broth for 19 hr at 37 °C. The cells were also plated on BHI to determine the initial concentration before transferring the cells into the heat-treated medium. One mililitre of 10^6cells/ml was dispensed into four individual flasks of Tryptic Soy Broth (TSB). Each flask was incubated 48°C for 30, 60 or 90 minutes. A HAAKE K Digital

Water Bath with HAAKE F3 digital readout and Water Circulator (Diesel Strauss 4, West Germany) was used as the heating chamber. Immediately after the heat treatment, the flasks were removed from the water bath and placed on ice. The medium was allowed to cool down to 35°C before the flask was removed and the cells plated on BGA and BHI to determine extent of injury. After the cells had been placed in the resuscitation medium, they were plated each hour for a total of three hr to determine the difference of injured cells to recovered cells. The cells were resuscitated for 18 hr.

(iv) Effect of freezing

The effect of slow freezing was also studied by dropping the temperature over 21 hr to a final temperature of -21°C. This was monitored using an Omega Data Logger (Omega Engineering, Inc., Stanford, CT). An Omega Data Logger was also used to monitor the temperature during thawing of cells. The freezing chamber consisted of a -20°C Kenmore upright freezer. Before injury, cells were incubated overnight at 37 °C for 18 hours in NB. The cells were centrifuged at 9,500 x g for 15 min at 4°C.

The pellet was resuspended in a Nalgene polysulfone Oak Ridge (28.5 x 104 mm) 50 ml capacity centrifuge tube (Baxter Diagnostics Corp., Mississauga Ontario) containing 10 ml 0.85% saline solution. The tube was emersed into a 11.5 x 13.5 cm polypropylene container containing 500 ml of a 40.23% (w/v) ethylene glycol (MW_r 62.07) solution placed in the -20°C freezer. Three probes were used to monitor the individual temperatures of the freezer, the cells in 0.1% saline solution and the ethylene glycol solution. The temperature of the ethylene glycol solution dropped approximately 0.5°C/ minute. After 21 hr, the tube containing the cells was removed from the freezer and immersed into the HAAKE water bath.

The HAAKE PG-20 control box was programmed to heat the water bath. The following conditions were programmed: T_0 = 5.0°C, ΔT = 25.0 °C/min (α) 1.00 for 25 min. The cells were thawed at a rate of 2°C/min to a final temperature of 8°C. One mililitre of the freeze-treated cells was transferred into TSB and the cells were allowed to resuscitate in TSB for 3 hr at 37°C. The cells were plated every hour on BHI and BGA to determine viability and injury but not reported.

Table 2. The deficiency of specific nutrients/ minerals in growth media incubated at different temperatures during study of the antigenicity of the OMP 35K protein in *Salmonella*.

Temperature (°C)	Control	without NaCl	no $FeSO_4*8H_2O$	no K_2HPO_4
37	A1	B1	C1	D1
23	A2	B2	C2	D2
42	A3	B3	C3	D3
15	A4	B4	C4	D4

Each block represents a different treatment applied to the bacterial cells of four different salmonellae *spp*.
Cells were incubated between 18-24 hr at 37 °C.

Table 3. Environmental stress conditions used to study the antigenicity of the OMP 35K protein in *Salmonella*.

	Stage of Harvest	
Treatment	Injure (NB)	Resuscitate (TSB)
Osmolarity (0, 0.6, 0.8, 1.0 M)	18 hr	18 hr
pH (3, 4, 5, 6, 7, 9)	3 hr, 18 hr	3 hr, 18 hr
Freezing	21 hr	18 hr
Heating (48°C)	30, 60, 90 min	18 hr

1. Extraction of OMPs

The protocol followed for extracting, electrophoresis and immunoblotting in these studies was outlined by Jaradat (1995). Briefly, the extraction procedure used in this investigation was adapted from the sarkosyl method as outlined by Filip *et al.* (1973).

The cells from 1.5L of M broth medium were harvested after reaching the late logarithmic stage. The cells were centrifuged at 9,000 xg in a GSA rotor using a Sorval RC2-B centrifuge (Dupont, Wilmington, Delaware) for 15 min at 4°C to collect the pellet. The cells were resuspended in 50 ml 4°C 50mM Tris-HCl (pH 7.2) and centrifuged at 9,000 x g using a Sorval SS-34 rotor for 15 minutes at 4°C. The pellet was resuspended in 50 mm Tris-HCl containing 10 ml of DNAase (0.1 μg/ml) and 10 ml of RNAase (0.1 μg/ml). The cells were then surrounded by a large beaker of ice to maintain a cool environment while they were sonicated. The cells were sonicated at 300 Watts using a Braun-Sonic 1510 (B. Braun, Melsungen, AG, Germany) for 45 sec X 5 replications with 1 min off intervals to prevent overheating. The disrupted cells were then centrifuged at 1,000 x g using a SS-34 rotor for 10 min at 4°C. The supernatant was collected and centrifuged at a speed of 20,000 x g using the SS-34 rotor for 1 hour at 4°C. The pellet was collected and incubated in 20 ml 2% sodium-N-lauroyl sarcosine in 50mM Tris-HCl buffer (pH 7.7) for 30 min at room temperature. The suspension was then centrifuged for 1 hr at 20,000 x g using the SS-34 rotor at 4°C. The supernatant was discarded and the pellet was incubated in 5 ml 2% sodium dodecylsulphate (SDS) in 10 mm tris-HCl (pH 7.7) for 30 minutes at 37°C. The suspension was again centrifuged for 30 minutes at 4°C at 20,000 x g using the SS-34 rotor. The supernatant contained the OMP non-porin portion and the pellet contained porins. To collect the porins,

the pellet was resuspended in a buffer which contained 1% w/v SDS, 5 mm EDTA, 0.05% w/v 2-mercaptoethanol and 3 mm sodium azide, all of which were solubilized in a 0.4 M NaCl buffer. The suspension was then centrifuged at 20,000 x g using the SS-34 rotor for 30 min at 4°C.

Both the outer membrane and porins were stored separately in 1.5 ml polypropylene Eppendorf conical micro centrifuge tubes, 1.5 ml capacity (Canlab, Baxter Diagnostics Corp., Mississauga, Ontario) at -20°C until further use.

2. Protein determination of extracted outer membrane proteins

Prior to electrophoresis the protein concentration of the OMPs was determined using a Pierce BCA Protein Assay Reagent Kit with bicinchoninic acid (Pierce Biochemicals, Rockford, Illinois, USA). Protein extracts were stored at -20°C. Prior to use, the vials were removed from the freezer and temperated. Aliquots of 10 μl of the protein extracts were dispensed into a 96-well Falcon 3912 Microtest III flexible assay plate (Becton Dickinson, Franklin Lakes, NJ). A set of standards was made from concentrate (2 mg/ml) bovine albumin fraction IV albumin standard (Pierce Biochemicals, Rockford, Illinois) and stored at 4°C prior to assay. A set of standards (200-1200 μg/ml) was used for each 96-well plate assay. Reagent A and Reagent B from the kit were combined at a ratio of 50:1 portions. Volumes of 200 μl were dispensed into each well containing either the protein extract or the standard. The plate was incubated at 37 °C for 1 hr. The absorbance value was measured at 570 nm using an ELISA plate reader system (Titertec Multiskan ELISA, Flow Laboratories, Mclean, VA, USA). The standards were plotted using a Scientific Figure Processing 6.0 computer program (Fig. P Corp, Cambridge, UK). The program calculated the mean and

standard deviations of the protein extracts performed in triplicate and a linear regression was calculated from the data. The unknown values of the protein extracts were calculated using the formula $y=mx+b$, where 'y' represented the wavelength and 'x' represented the concentration. To obtain a good separation and prevent overloading during electrophoresis the protein concentration was adjusted to 2μg protein/μl of extract (Schiemann and Shope, 1991). Prior to electrophoresis, the bacterial extracts that contained a lower protein concentration (<20 μg/ml) were passed through a Centricon 10 Concentrator according to the manual provided by Amicon (Beverley, MA, USA). The Centricon 10 Concentrator is based on membrane filtration chromatography (10,000 M_w cutoff). The Pierce BCA protein assay is not discriminatory toward a particular protein. Therefore, values obtained by this method represent total protein content of the extract.

3. Electrophoresis of outer membrane proteins

Electrophoresis was carried out using a discontinuous SDS-PAGE procedure under reducing conditions. A Bio-Rad dual vertical slab gel electrophoresis cell and Bio Rad 500/200 power supply were used. A monomer stock solution containing 30% w/v acrylamide and 2.7% w/v of bis-acrylamide was used to make up 5% stacking gels and 12% separating gels for the electrophoretic runs (Laemmli, 1970). Electrophoresis was carried out for 4 hr under a constant current of 30 mA for each gel. Before loading, extracts were boiled for 5 min in a treatment buffer (0.125M Tris-HCl, 4% (w/v) SDS, 20% (w/v) glycerol, 10% (w/v) mercaptoethanol). Initially each extract contained 25-40 μg/ ml of protein. Appropriate dilutions were made to obtain a final concentration of 2 μg/ ml. They were diluted in treatment buffer with the final concentration/ well being 1 μg protein/ μl. Two identical gels

were loaded for each run. One gel was stained overnight at room temperature in a staining solution (50% w/v methanol and 10% w/v acetic acid) containing 0.125% (w/v) Commassie blue R-250 (Kodak). This was followed by a 1 hr treatment in destaining solution I (50% w/v methanol and 10% w/v acetic acid). The gel was then transferred into destaining solution II (7% w/v acetic acid and 5% w/v methanol) for 1 to 2 days. The other gel was used for immunoblotting and stored in 2L transfer buffer containing of 25 mm Tris and 192 mm glycine for 30 min before electrophoretic transfer onto a nitrocellulose membrane.

4. Antibody preparation from MAb 1D6

Hybridoma cells which produced the monoclonal antibody ($1D6H_6A_3$) were developed (Jaradat, 1995) from parental mouse cells (2^{nd} clone), and stored at -80°C in liquid nitrogen. These cells were quickly thawed and transferred into a 250 ml polystyrene cell culture flask (Corning Glass Ware, Corning, NY, USA) which contained RPMI-1640 medium with 10% fetal calf serum. The cells were grown in culture flasks placed in a sterile atmosphere of 5% CO_2 in a temperature controlled water- jacketed incubator (Forma Scientific) at 36°C. The cells were monitored and allowed to propagate. Their growth was monitored periodically by observation under a phase contrast microscope (Nikcn). The cells were transferred aseptically and consecutively into 500 and 1000 ml flasks as the cell density increased. They were harvested by centrifugation when a dense population of cells was observed after approximately two weeks. The cells were centrifuged using a bench top centrifuge (Damno/ IEC Division, Mass., USA) at 1000 x g for 10 min. The supernatant containing antibodies was collected and used for further studies.

5. Ammonium sulfate precipitation of MAb 1D6

The procedure for preparation of the ammonium sulfate solution (761g/L) and phosphate buffered saline (PBS) pH 7.2, is outlined by Harlow and Lane (1988). A large 4L flask containing 2L supernatant was placed on ice. An equal volume of ammonium sulfate solution was added to the starting volume of antibody-containing supernatant (usually 2L). The flask was stored overnight at 4°C. The precipitate was centrifuged at 9,000 x g for 30 minutes at 4 °C. The pellet was resuspended to its original volume in PBS . The antibody solution was then transferred into Spectra/Por molecular porous dialysis tubing with a 12-14000 MW cutoff (Spectrum, USA). The dialysis tubing was placed in a flask containing PBS at 4 °C. The PBS solution was changed three times and the impurities precipitated out over the next two days. The antibody solution was then collected from the tubing and stored at -20°C.

6. ELISA for antibody titre

An ELISA was performed to determine the antibody titre as outlined by Brennand *et al.* (1986). A 96-well microtitre plate, except for the first column which was left as a blank, was coated using the OMP extract from *S. typhimurium.* Each well contained 2 μg/100 μl of OMP antigen diluted in 50 mm carbonate buffer. The plate was incubated overnight at 4 °C. The plate was washed with PBST (PBS with 0.05% Tween) 6 times using a manual plate washer. The plate was covered with blocking buffer (5% skim milk powder in PBS, pH 7.2) and incubated for 1 hr at 37°C. The plate was washed again 6 times with PBST. Dilute 0.1% blocking buffer (100 μl) was added to each well except for the initial well. The first well contained 200 μl of MAb 1D6 antibody. A serial dilution of the antibody was performed by

removing 100 μl from the first well and dispensing it into the next well. The plate was incubated for 1 hr at 37°C and washed 6 times in PBST. The commercial conjugate antibody alkaline phosphatase goat-antimouse IgG (AP-GAM-IgG, Bio-Rad) was diluted (1:3000) in blocking buffer and 100 μl was added to each well. The plate was incubated for 1 hr at 37 °C and washed 6 times with PBST. Diethanolamine buffer was prepared according to the procedure described by the manufacture (Sigma Chemical Company, St. Louis, MO, USA). The substrate p-nitrophenyl phosphate (100 μl) was added to each well. The ELISA plate was incubated overnight in a dark enclosure at room temperature. The plate was read at 405 nm using the ELISA microplate reader.

7. Immunoblotting of the outer membrane protein and MAb 1D6

Immunoblotting was performed according to the modified procedure of Davis *et al.* (1994). Electrophoretic transfer from the unstained gel to nitrocellulose membrane (0.45 μm retention, Bio-Rad) was conducted using a Bio-Rad Trans-Blot Cell with a Model 250/2.5 Power Supply (Bio-Rad). The transfer was carried out for 4 hr under a constant current of 200 mA at 4°C. Following transfer the membrane was washed twice in Tris buffered saline (TBS) and once in 2L of TBS, pH 7.5 for 5 min that contained 20 mm Tris and 500 mm NaCl. The membrane was blocked with 3% gelatin TBS for an hour. The membrane was washed three times in a Tween-20 wash solution containing TBS (TTBS). The membrane was incubated in a MAb 1D6 (1:5) TTBS 1% gelatin solution at room temperature on a shaker overnight. The following day, the membrane was washed three times in TTBS. The membrane was conjugated with goat anti-mouse IgG using a dilution of 1:3000 in TTBS for 1 hour at room temperature. The membrane was washed three times in TTBS and once in TBS.

The substrate was added using a stock solution that consisted of 0.1M Tris and 0.5 mm $MgCl_2$ at pH 9.5. Both 5-bromo-4-chloro-3-indoyl phosphate ρ-toluidine salt (BCIP) and ρ-nitro blue tetrazolium chloride (NBT) were added to this solution at a concentration of 1 mg/ ml. The time of colour development varied between 10-15 minutes and was stopped once the bands were observed. To avoid overexposure, the developer was suctioned from the membrane. The membrane was rinsed with distilled water several times to stop the reaction.

IV. RESULTS AND DISCUSSION

The expression of OMPs and antigenicity of the 35K protein.

A. Study 1 : Deficiency of specific nutrients or minerals in growth medium at specific temperatures.

S. typhimurium, *S. gallinarum*, *S. arizona* and *S. thompson* were exposed to four growth temperatures and four growth conditions. The setup and combination of conditions are outlined in Table 2 (Methods section). The electrophoretic gels and immunoblot for study 1 are not shown. A total of 12 gels and immunoblots were developed to conduct the study. The results of the treatments are compiled in Tables 4-7 for *S. typhimurium* (Table 4), *S. gallinarum* (Table 5), *S. arizona* (Table 6) and *S. thompson* (Table 7). The temperatures appear on the tables according to resistance toward temperature -optimum (37°C), room temperature (23°C), maximal high (42°C) and maximal low temperature (15°C).

The effect of the absence of specific nutrients and alteration of growth temperature on the expression of proteins and antigenicity are discussed below.

i) Effect of temperature

The only growth temperature that did not display a change in the expression of the outer membrane proteins was 37°C. This of course, is the optimal growth temperature of *Salmonella* spp. *S. typhimurium* (Table 4) showed an increase in the expression of the 60K protein at both 23°C and 42°C. Additional bands were recognized by MAb 1D6 at suboptimal growth temperatures including 28K (23°C) and 34K (15°C), respectively. *S. gallinarum* (Table 5) on the other hand, grew poorly and had an extended lag phase at 23°C, 42°C and 15°C yielding faint electrophoretic bands. The MAb still recognized the 35K at the four different growth temperatures. At 37°C, the Mab also reacted with the 28K and 34K proteins. For *S. arizona* (Table 6), a growth temperature of 23°C caused an increase in the expression of 12K. At 42°C, the 36K was missing from the profile. A lower growth temperature of 15°C caused an increase in the expression of the 35K and a decrease of the 34K. A low cell density was produced at this suboptimal growth temperature. The MAb detected both the 35K and 28K proteins.

The only temperature that affected the expression of the outer membrane proteins of *S. thompson* (Table 7) was 15°C. However, the MAb also recognized the 28K at 15°C, 23°C and 42°C. An additional band was recognized at 42°C and it was 34K.

ii) Effect of salt

The effect of eliminating salt from the medium while the growth temperature remained at 37°C varied significantly among species (Table 4-7). *S. typhimurium*(Table 4) showed an increase in the expression of 35K and 37K. *S. gallinarum* (Table 5) results were affected by low cell density. *S. arizona* without salt in the medium, showed a wider 34K band compared

with the control medium with salt at 37°C. The expression of the outer membrane protein bands for *S. thompson* (Table 7) was unaffected by the absence of salt in the medium.

iii) Effect of temperature and salt

There was a decrease in the expression of the 35 and 37K at 23°C and 42°C by *S. typhimurium* (Table 4) when salt was absent from the medium. At 23°C there was also an increase in the expression of the 55K. At 15°C the cells were affected by the low temperature and the absence of salt that resulted in a low cell density. The only temperature which caused an effect on the omps of *S. gallinarum* (Table 5) was at 42°C, without salt in the medium. Production of the 15, 34 and 35K proteins increased at 42°C. *S. arizona* (Table 6) was affected by the absence of salt in the medium at both temperature extremes. The 34 and 35K decreased in expression due to a low cell density at 42°C. Because of the low cell density, the reaction of the 35K protein with the MAb was faint. At 15°C, there was an increase in the intensity of the 35K. The expression of both the 35 and 37K proteins decreased at 42°C for *S. thompson* (Table 7). However, at this temperature, an additional band, at 28K, was recognized by the MAb.

iv) The effect of iron

The bands from *S. typhimurium* (Table 4) at 37°C showed no growth difference of cells in the presence or absence of iron in the medium. A low cell density was obtained during growth of *S. gallinarum.* The desired protein concentration could not be achieved for the extraction. As a result, the fixed amount of protein applied to each lane could not be loaded onto the gel. Therefore, there is a faint appearance to the bands (Table 5). The 32K and 34K proteins from *S. arizona* (Table 6) both increased in intensity compared with the controls.

Without the presence of iron in the medium *S. thompson* (Table 7) expressed a greater amount of the 35K and 36K proteins.

v) The effect of iron and temperature

S. typhimurium (Table 4) expressed an increase in the 60K at 23°C and 15°C. A notable decrease was evident, however, for the 35K and 36K without iron in the medium at 23°C. In the absence of iron at 42°C, *S. typhimurium* showed a visible decrease in the 36K protein without iron at 42°C. A different protein, the 15K was expressed at 15°C without iron with a much greater intensity than in the other treatments. The only temperature that affected the OMPs of *S. gallinarum* (Table 5) without iron was 23°C. At this temperature, both the 34K and 35K protein showed an increase in expression. The MAb at this temperature reacted with the 28K band as well. The use of three suboptimal temperatures void of iron, resulted in the absence of several proteins from *S. arizona* (Table 6). There were bands missing in the region of 36K at 42°C, and at 34K and 37K (15°C). Several OMPs increased in intensity, 32K at 23°C and 37K at 42°C. The MAb also recognized the 28K protein at 15°C. The combination of a low growth temperature of 23°C and the absence of iron caused *S. thompson* (Table 7) to grow poorly, yielding low cell density. Both temperatures, 42°C and 15°C, produced an increase in the expression of the 37K protein. At 42°C, the MAb recognized the 32K and 35K protein, but at 15°C, only the 34K and 35K proteins were present.

vi) Effect of phosphate

The absence of phosphate in the medium did not affect the OMP bands normally produced by *S. typhimurium*(Table 4). However, the lack of phosphate caused the appearance of an extra band on the immunoblot at 28K. With *S. gallinarum* (Table 5) it increased the

intensity of the 35K. It decreased the expression of both the 35K and 36K from *S. arizona* (Table 6). *S. thompson* (Table 7) showed a low cell density and faint bands were produced without phosphate.

vii) Effect of phosphate and temperature

The absence of phosphate in the medium and a higher growth temperature (42°C) both contributed to an increase in the 60K protein from *S. typhimurium* compared with the control (Table 4). The intensity of the 60K protein was also greater at 15°C compared with the control. The 35K protein, however, at the lower growth temperature (15°C), was reduced in intensity. An extra band, 34K, was recognized by the MAb after this exposure. *S.gallinarum* without phosphate (Table 5) displayed an increased intensity of the following bands; 35K and 36K at 23°C, 16K, 17K and 18K at 42°C and 6K, 18K, 22K and 35K at 15°C compared with the control at the same temperatures. At 42°C and 15°C, the MAb can recognize the 28K and 34K as well. *S. arizona* (Table 6) showed a reduced expression of the 34K at 23°C without phosphate compared with 37°C (control). The 18K band appeared very large and was greater than in the control at the two temperature extremes, 42°C and 15°C. *S. thompson* (Table 7) showed a difference in the electrophoretic profile only at the highest and lowest growth temperature. At 42°C there was an increase in the expression of the 18K and 60K. At 15°C the 35K, 36K and 37K all were increased in intensity. These two temperatures extremes also showed the 28K and 34K were recognized by the MAb.

Overall, these results indicated the expression of the 60K for *S. typhimurium*(Table 4) was influenced by the effect of suboptimal growth temperature (23°C and 42°C). The absence of iron and phosphate in the medium at a low growth temperature (15°C) affected the

60K. Overall, the absence of salt and iron in the medium affected the production of the 35K and 37K (salt) and 35K and 36K proteins (iron).

The effect of temperature was more evident with *S. gallinarum* (Table 5), as a low cell density was obtained at 23°C and at the two extremes, 42°C and 15°C. The absence of phosphate at the two extreme temperatures caused an increased expression in the lower molecular weight outer membrane proteins, 6K, 16K, 17K, 18K and 22K, more than of those seen in the mid-region of the gel. The repression of several OMPs for *S. arizona* (Table 6) was caused by the absence of both salt and iron at different temperatures. The expression of the 35K and 36K by *S.thompson* (Table 7) was influenced primarily by salt and phosphate at different temperatures.

Table 4. Influence of temperature and lack of NaCl, $FeSO_4$ or PO_4 on stress induced outer membrane proteins of *S. typhimurium*

Exposure	Protein band detection method	
Stress- 18 hr	SDS-PAGE (M_w) [a]	Immunoblot bands (M_w) [b]
37°C (optimum)- control	regular[c]	35K
23°C (room temperature)	60K intense	28 & 35K
42°C (maximal high)	60K intense	35K
15°C (minimum low)	normal appearance of bands[d]	34 & 35K
37°C, no NaCl	35 & 37 > control	35K
23°C, no NaCl	55 intense 35 &36 < control	35K
42°C, no NaCl	35 & 37 < control	35K
15°C, no NaCl	LCD[e]	35K
37°C, no Fe	normal appearance of bands[d]	35K
23°C, no Fe	35 & 36 < control 60K intense	35K
42°C, no Fe	36K < control	35K
15°C, no Fe	60 & 55K 60K > control	35K
37°C, no PO_4	normal appearance of bands[d]	28 & 35K
23°C, no PO_4	35, 36 & 55=control[b] other bands faint	28 & 35K
42°C, no PO_4	60K > control	35K
15°C, no PO_4	60K huge 35K very thin	28, 34 & 35K

[a] changes of electrophoregram patterns from omps based on cell growth conditions prior to extraction

[b] molecular weight (M_w) of antigen bands reactive with MAb 1D6

[c] regular electrophoretic profile, no stress induced

[d] same intensity of bands as under non-stressed growth conditions

[e] low cell density (LCD) of bacterial cells under stressed conditions

Table 5. Influence of temperature and lack of NaCl, $FeSO_4$ or PO_4 on stress induced outer membrane proteins of *S. gallinarum*

Exposure	Protein band detection method	
Stress- 18 hr	SDS-PAGE (M_w) [a]	Immunoblot bands (M_w) [b]
37°C (optimum)- control	regular[c]	28, 34 & 35K
23°C (room temperature)	LCD[e]	35K
42°C (maximal high)	LCD[e]	35K
15°C (minimum low)	LCD[e]	35K
37°C, no NaCl	LCD[e]	35K
23°C, no NaCl	LCD[e]	35K
42°C, no NaCl	bands below 15 & 35 K sharper than control	35K
15°C, no NaCl	LCD[e]	35K
37°C, no Fe	LCD[e]	35K
23°C, no Fe	34 & 35 > control	35K
42°C, no Fe	LCD[e]	28, 34 & 35K
15°C, no Fe	normal appearance of bands[d]	35K
37°C, no PO_4	35K > control	35K
23°C, no PO_4	35 & 36 > control	35K
42°C, no PO_4	16, 17 & 18K large bands	28, 34 & 35K
15°C, no PO_4	6,18,22 & 35K >control	28, 34 & 35K

[a] changes of electrophoregram patterns from omps based on cell growth conditions prior to extraction

[b] molecular weight (M_w) of antigen bands reactive with MAb 1D6

[c] regular electrophoretic profile, no stress induced

[d] same intensity of bands as under non-stressed growth conditions

[e] low cell density (LCD) of bacterial cells under stressed conditions

Table 6. Influence of temperature and lack of NaCl, $FeSO_4$ or PO_4 on stress induced outer membrane proteins of *S. arizona*

Exposure	Protein band detection method	
Stress- 18 hr	SDS-PAGE (M_w) [a]	Immunoblot bands (M_w) [b]
37°C (optimum)- control	34K very thin regular[c] 12K intense	35K
23°C (room temperature)		35K
42°C (maximal high)	36K missing	35K
15°C (minimum low)	34K very thin, LCD[d] 35K intense	28 & 35K
37°C, no NaCl	34K wide thicker than control	35K
23°C, no NaCl	12K intense	35K
42°C, no NaCl	34 & 35K very thin LCD[d]	35K faint
15°C, no NaCl	34K very thin 35K very intense	35K
37°C, no Fe	32 & 34 very prominent	35K
23°C, no Fe	32K very prominent LCD[d]	35K
42°C, no Fe	36K missing 37K > control	35K
15°C, no Fe	34 & 37K absent vs. control	28 & 35K
37°C, no PO_4	34, 35 & 36K very thin	35K
23°C, no PO_4	34K < control	35K
42°C, no PO_4	18K very large	35K
15°C, no PO_4	18K > control	35K

[a] changes of electrophoregram patterns from omps based on cell growth conditions prior to extraction

[b] molecular weight (M_w) of antigen bands reactive with MAb 1D6

[c] regular electrophoretic profile, no stress induced

[d] low cell density (LCD) of bacterial cells under stressed conditions

Table 7. Influence of temperature and lack of NaCl, $FeSO_4$ or PO_4 on stress induced outer membrane proteins of *S. thompson*

Exposure	Protein band detection method	
Stress- 18 hr	SDS-PAGE (M_w) [a]	Immunoblot bands (M_w) [b]
37°C (optimum)- control	regular[c]	35K
23°C (room temperature)	normal appearance of bands[d]	28 & 35K
42°C (maximal high)	normal appearance of bands[d]	28, 34 & 35K
15°C (minimum low)	60K band missing	28 & 35K
37°C, no NaCl	normal appearance of bands[d]	35K
23°C, no NaCl	normal appearance of bands[d]	35K
42°C, no NaCl	35 & 37 < control	28 & 35K
15°C, no NaCl	LCD[e]	35K
37°C, no Fe	35 & 36K > control	35K
23°C, no Fe	LCD[e]	35K
42°C, no Fe	37K > control	32 & 35K
15°C, no Fe	37K > control	32, 34 & 35K
37°C, no PO_4	35 & 36K only bands that are clear	35K
23°C, no PO_4	35 & 36K only bands that are clear	35K
42°C, no PO_4	large 18 & 60K	28, 34 & 35K
15°C, no PO_4	35, 36 & 37 share same intensity	28, 34 & 35K

[a] changes of electrophoregram patterns from omps based on cell growth conditions prior to extraction

[b] molecular weight (M_w) of antigen bands reactive with MAb 1D6

[c] regular electrophoretic profile, no stress induced

[d] same intensity of bands as under non-stressed growth conditions

[e] low cell density (LCD) of bacterial cells under stressed conditions

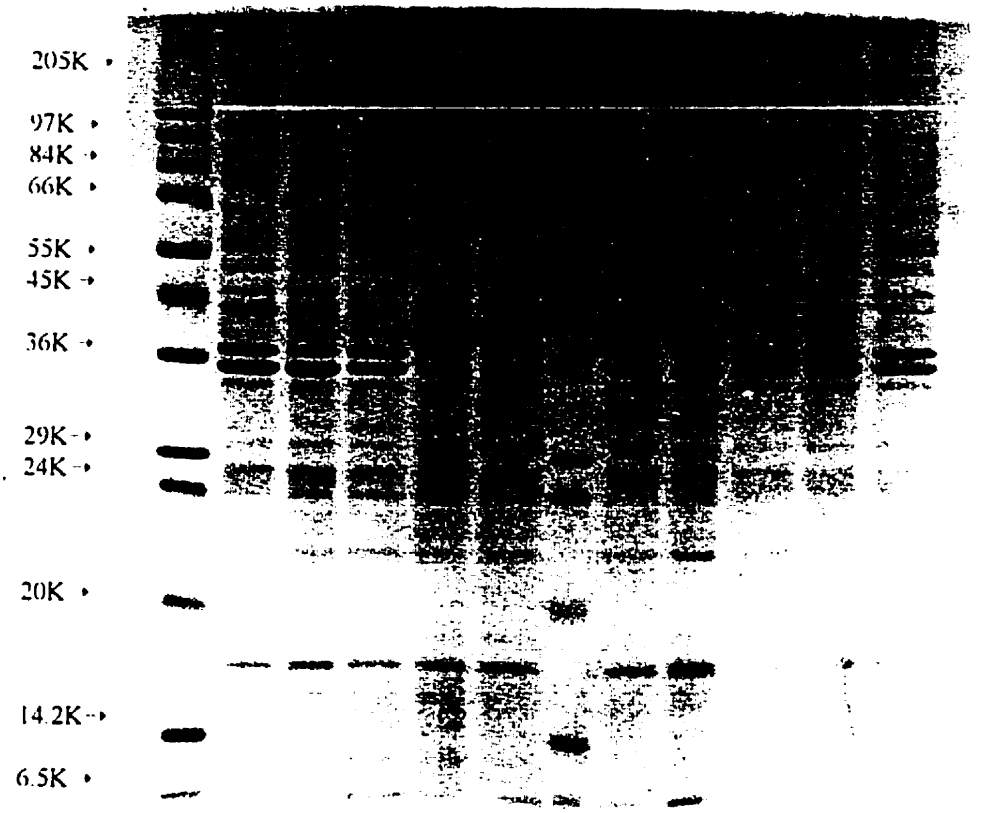
B. Study 2 : The effect of environmental conditions

The external growth medium was altered by osmolarity, heat, freezing and pH to determine the effect of environmental conditions on the expression of OMPs and antigenicity of the 35K protein.

(i) Effect of osmolarity

Outer membrane proteins were extracted from *S. typhimurium* (Fig 1a, 1b) grown in nutrient broth at varying levels of added salt (0.0M-control, 0.6M, 0.8M, and 1.0M). The treatments consisted of a 18 hr injury period (lowered a_w) followed by a 18 hr resuscitation period in trypticase soy broth. To determine whether the resuscitation period allowed recovery of altered or depleted proteins, one set of extracts was prepared from cells given only a 18-hour injury exposure with no resuscitation. The results of the effect of osmolarity on OMPs of *S. typhimurium* are shown in Table 8. The electrophoretic profile of the OMPs extracted from *S. typhimurium* cells grown with no added NaCl (control-injury plus resuscitation) and 0.6M NaCl (injury plus resuscitation) showed a normal profile of bands (Fig. 2a). Following growth with 0.6M NaCl (injury only), the 37K appeared thinner than in the control. The profile from extracts of 0.8M NaCl and 1.0M NaCl -exposed cells showed a decrease in the intensity of the 35K as opposed to the control. As the molarity increased, the 35K band became thinner. The MAb detected only one band, 35K, in the control sample without added NaCl (Fig 2b, lane 1). The lane containing OMPs extracted from cells grown at 0.6 M NaCl (Fig 2b, lane 2 and lane 3) had five bands recognized by the MAb, 28K, 32K, 34K and 35K. At 0.8M NaCl (Fig 2b, lane 4) and 1.0M NaCl (Fig 2b, lane 5), three proteins reacted with the MAb, 28K, 34K and 35K.

Fig. 2a. SDS-PAGE of outer membrane proteins extracted from *Salmonella typhimurium* grown at various levels of added NaCl (0.0, 0.6M, 0.8M, 1.0M). Two sets of time treatments were involved in this experiment: #1; cells were exposed to adjusted NaCl levels in Nutrient broth for 18 hr at 37°C that represents an injury treatment (I); #2 cells were exposed to a resuscitation treatment (R) in Trypticase Soy broth for 18 hr at 37°C. Lane M_r: molecular weight standards ('000s). Lanes: 1-control represented by 'C' (grown in normal nutrient broth). Lanes 2-5 (bold face) treated lanes represented by 'T'. Lane 2- 0.6M NaCl (injury and resuscitation), Lane 3- 0.6M NaCl (injury only), Lane 4- 0.8M NaCl (injury and resuscitation), Lane 5- 1.0M NaCl (injury and resuscitation).



M_r 1 2 3 4 5 M_r

C T

Fig. 2b. Immunoblot of outer membrane proteins extracted from *S.typhimurium*. Proteins in the polyacrylamide gel shown in Fig. 2a were electrophoretically transferred to a nitrocellulose membrane and reacted with MAb 1D6. Lane 1; control 'C', lanes **2-5**; treated '**T**'. Results are shown in duplicate.

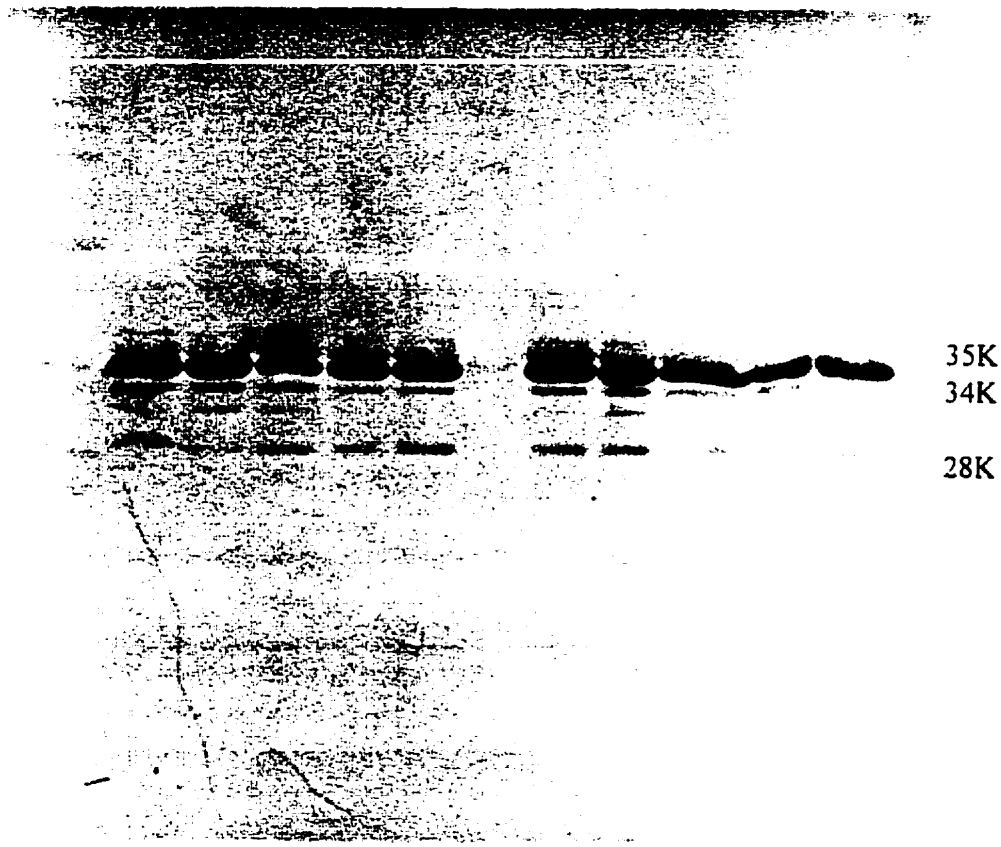

35K
34K
28K
1 2 3 4 5
C T

Table 8. Effect of osmolarity on stress induced outer membrane proteins of *S. typhimurium*

Stress	Protein band detection method	
18 hr nutrient broth (injury- I), 18 hr Tryptic Soy broth (recovery- R)	SDS-PAGE (M_w) [a]	Immunoblot bands (M_w) [b]
1) 0.0 M NaCl 18 hr NB, 18 hr TSB	regular[c]	35K
2) 0.6 M NaCl, injury and resuscitation	normal appearance of bands[d]	28, 32, 34 & 35K
3) 0.6 M NaCl, injury only	37K > control	28, 32, 34 & 35K
4) 0.8 M NaCl, injury and resuscitation	thin 35K < control	28, 34 & 35K
5) 1.0 M NaCl, injury and resuscitation	thinner 35K <control	28, 34 & 35K

[a] changes of electrophoregram patterns from omps based on cell growth conditions prior to extraction

[b] molecular weight (M_w) of antigen bands reactive with MAb 1D6

[c] regular electrophoretic profile, no stress induced

[d] same intensity of bands as under non-stressed growth conditions

ii) Effect of shock treatment: heat and cold

Heat

Outer membrane proteins were extracted from *S. typhimurium* cells grown in nutrient broth for 18 hr at 37°C . The cells were injured by subsequent exposure to 48°C for various periods. The separate times used for heat challenge were 0, 30, 60 and 90 minutes (Table 9).

Cold

Following growth in nutrient broth (37°C, 18 hr), cells were subjected to freezing (21 hr, -21°C). This injury represented slow freezing. The rate of cooling was recorded as 0.5°C/min. After thawing the cells (2°C/min). They were resuscitated in TSB for 18 hr at 37°C. The unchallenged control was also grown in nutrient broth for 18 hr at 37°C. Injuries by rapid or quick freezing was not conducted in these experiments.

The results of the effect of shock treatment on OMPs of *S. typhimurium* are shown in Table 9. The electrophoretic profile (Fig. 3a) represents OMPs extracted from *S. typhimurium* cells grown in nutrient broth (18 hr, 37°C), exposure to heat or cold, and resuscitation in tryptic soy broth (18 hr, 37°C). The thickness of the 35K and 36K protein bands decreased as the length of the heat treatment increased. This relationship was also evident toward the proteins detected by the antibody in the immunoblot (Fig. 3b). Heat exposure affected the epitopes at 28K, 32K and 34K that were not recognized by the MAb in the control sample. The extra bands decreased in intensity as the heat treatment was extended. The cells exposed to slow freezing produced a normal electrophoretic profile (Fig 3a, lane 5). The MAb still reacted with 28K, 32K, 34K and 35K proteins (Fig 3b, lane 5).

Fig. 3a. SDS-PAGE of outer membrane proteins extracted from *Salmonella typhimurium* grown in Nutrient broth (NB) (37°C, 18 hr). The cells were subjected to heat or cold. The heat treatment varied from 0, 30, 60 or 90 min at 48°C. The cold treatment was exposure at 21°C for 21 hr (rate of cooling 0.5°C/min). The cells were thawed (2°C/min) and resuscitated in Trypticase Soy broth (37°C, 18 hr). Lane M_r: molecular weight standards ('000s). Lanes: 1-control represented by 'C'; grown in NB (37°C, 18 hr) and TSB (37°C, 18 hr). Lanes **2-5** (bold face) treated lanes represented by 'T'. Lane 2- heated 30 min, Lane 3- heated 60 min, Lane 4- heated 90 min, Lane 5-cold (-21°C, 21 hr).

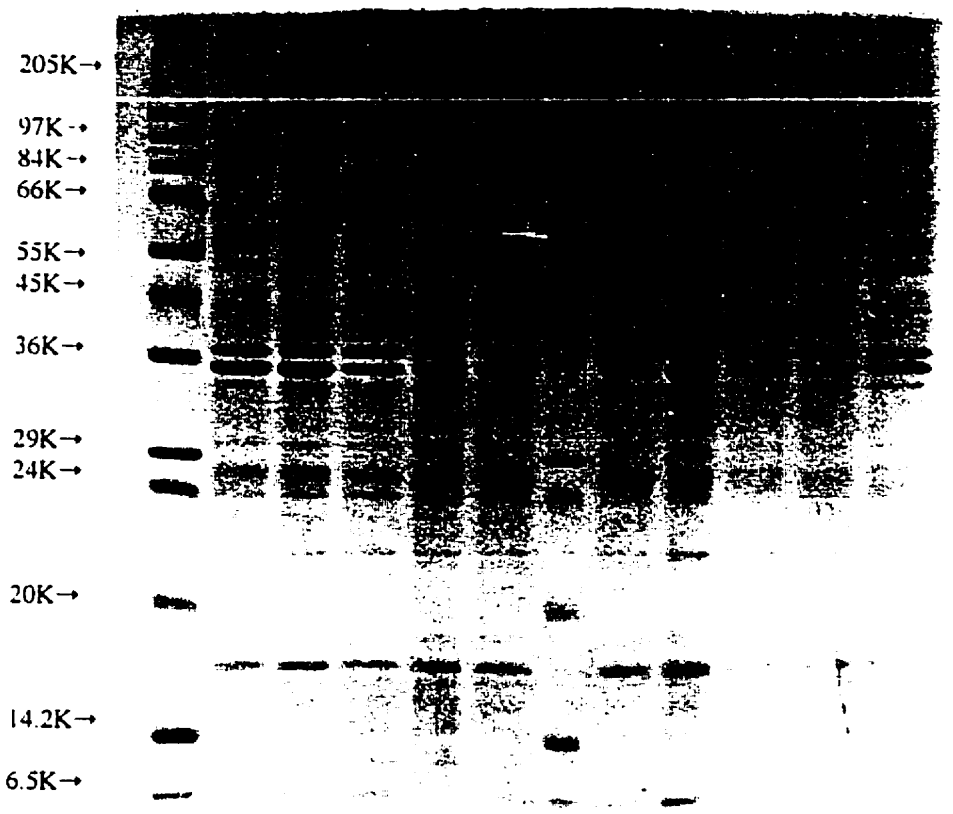

205K→
97K→
84K→
66K→
55K→
45K→
36K→
29K→
24K→
20K→
14.2K→
6.5K→
M_r 1 2 3 4 5
C T

Fig. 3b. Immunoblot of outer membrane proteins extracted from *S. typhimurium*. Proteins in the polyacrylamide gel shown in Fig. 3a were electrophoretically transferred to a nitrocellulose membrane and reacted with MAb 1D6.

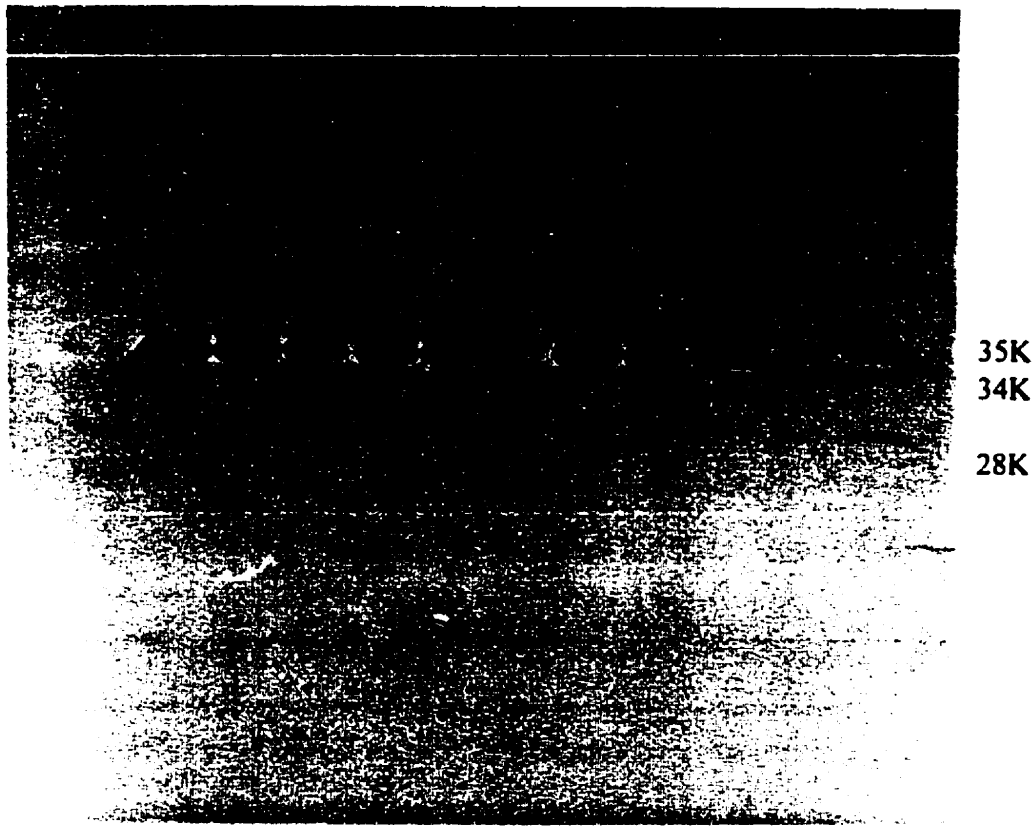

35K
34K
28K
1 2 3 4 5
C T

Table 9. Effect of thermal shock on stress induced outer membrane proteins of *S. typhimurium*

Stress	Protein band detection method	
Step 1) 18 hr in nutrient broth Step 2) shock treatment (injury - I) Step 3) 18 hr Tryptic Soy broth (recovery-R)	SDS-PAGE (M_w) [a]	Immunoblot bands (M_w) [b]
0 min, control (no heat or cold exposure)	35K wide	35K
30 min, 48°C (heat shock)	thickness of 35K & 36K affected by length heat exposure	28, 32, 34 & 35K
60 min, 48°C (heat shock)		28, 32, 34 & 35K[c]
90 min, 48°C (heat shock)		28, 32, 34 & 35K[c]
21 hr, -21°C (cold shock)	normal appearance of bands[d]	28, 32, 34 & 35K

[a] changes of electrophoregram patterns from omps based on cell growth conditions prior to extraction

[b] molecular weight (M_w) of antigen bands reactive with MAb 1D6

[c] Intensity of the 28, 32 & 34K decreased as the length of the heat treatment increased

[d] same intensity of bands as under non-stressed growth conditions

(iii) Effect of pH

According to Ray (1979) most of the injury caused to the cell by exposure to environmental extremes, occurs during the early logarithmic stage of growth.

As noted in the literature, salmonellae will grow in many environments between a pH range from 4-9 (Frazier and Westhoff, 1988). The minimum pH at which this organism can grow, however, is dependent upon the type of acid present in the medium. According to Chung and Goepfert (1970), the minimum pH value for growth in trypticase yeast extract medium adjusted using hydrochloric acid was about 4.0.

Two sets of experiments were conducted to determine whether the stage of growth affected the expression of the 35K epitope. The first experiment studied *S. typhimurium* under an 18 hr injury exposure followed by an 18-hr period of resuscitation. The second experiment included 5 species of Salmonella studied under a 3 hr injury exposure and 3 hr resuscitation period.

(iiia) Effect of pH- 18 hr injury

Before exposure to the adjusted pH(s), *S. typhimurium* cells were grown in nutrient broth for 18 hr to develop adequate cell mass for extraction. Cells were then harvested and resuspended to original volume in pH adjusted nutrient broth. Cells were grown at different pH's in nutrient broth (ranging from pH 4-9). The cells were collected after harvesting and resuspended in TSB (pH 7.0) to allow cells to resuscitate. The cells recovered during the resuscitation period. A text version of the electrophoretic profile for the 18 hr injury and 18 hr resuscitation experiment is shown in Table 10. The lanes that contain OMPs extracted from cells grown at pH 4 and 5 (injury-18 hr) and given a resuscitation period of 18 hr showed a

decrease in the expression of the 35K and 36K proteins (Fig. 4a). The cells grown at pH 5 (Fig 4a, lane 4) which did not receive a resuscitation period were not allowed to recover. Two immunoblot bands were detected 34K and 35K (Fig. 4b). At pH 6, 7 and 9, the electrophoretic profile appeared normal. Lanes 1, 6, 8 and 10 contained bands 28K, 34K, and 35K detected by the MAb. The extra bands, excluding 35K, were detected quite possibly due to a conformational change in epitope structure. There was an exception at pH 9 where the MAb detected only the 34 and 35K proteins.

Fig. 4a. SDS-PAGE of outer membrane proteins extracted from *Salmonella typhimurium*. The cells were initially grown in nutrient broth (18 hr, 37°C). They were harvested and transferred into various pH adjusted (pH 4-9) medium of nutrient broth (18 hr, 37°C) to sustain injury. The cells were resuscitated in Trypticase Soy broth (18 hr at 37°C). Lane M_r: molecular weight standards ('000s). Lanes: 1- control (C); pH 6.8 grown in NB (18 hr, 37°C) and TSB (18 hr, 37°C). Treated lanes **2-11** (**T**) are identified by **bold face**. Lane 2- pH 4.0 (injury and resuscitation), lane 3- pH 4.0 (injury only), lane 4- pH 5.0 (injury and resuscitation), lane 5- pH 5.0 (injury only), lane 6- pH 6.0 (injury and resuscitation), lane 7- pH 6.0 (injury only), lane 8- pH 7.0 (injury and resuscitation), lane 9- pH 7.0 (injury only), lane 10- pH 9 (injury and resuscitation), lane 11- pH 9 (injury only).

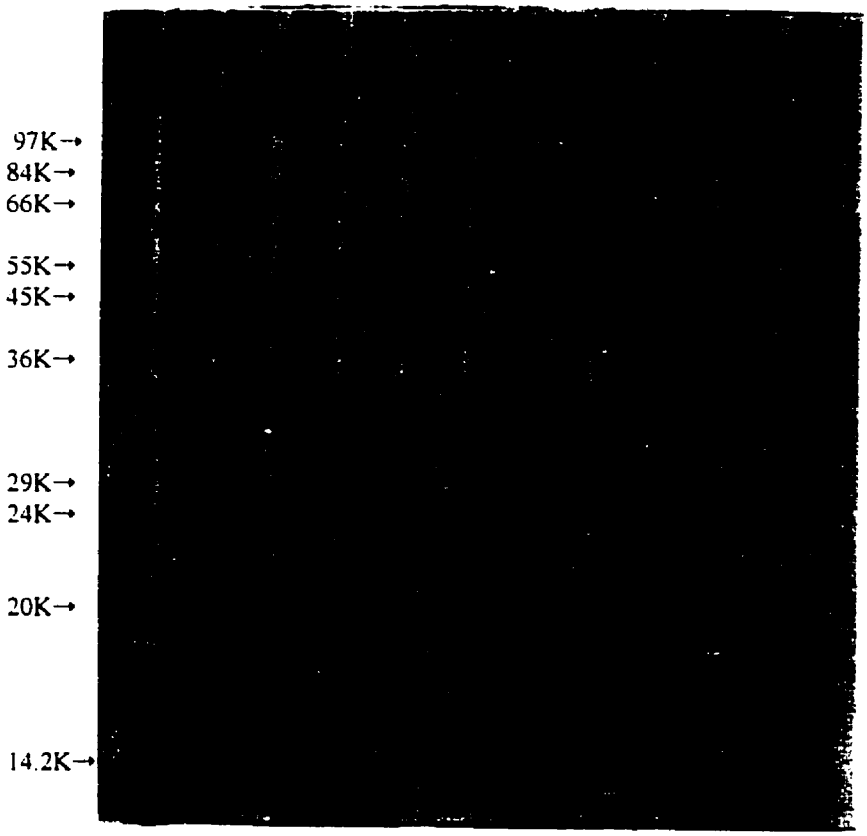

97K→
84K→
66K→
55K→
45K→
36K→
29K→
24K→
20K→
14.2K→
M_r 1 2 3 4 5 6 7 8 9 10 11
C
T

Fig. 4b. Immunoblot of outer membrane proteins extracted from *S. typhimurium*. Proteins in the polyacrylamide gel shown in Fig. 4a were electrophoretically transferred to a nitrocellulose membrane and reacted with MAb 1D6. Lane 1- control (C). Treated lanes **2-11** (**T**) are identified by **bold face**.

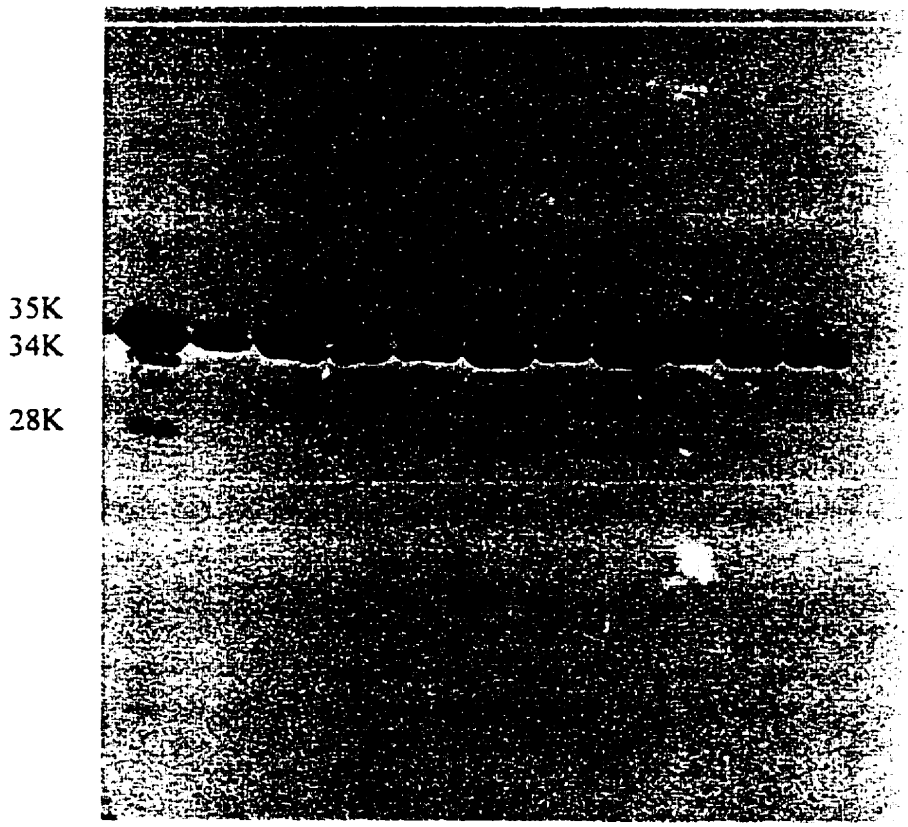

35K
34K
28K
1 2 3 4 5 6 7 8 9 10 11
C
T

Table 10. Effect of pH change on stress induced outer membrane proteins of *S. typhimurium*

Stress	Protein band detection method	
18 hr nutrient broth (injury- I), 18 hr Tryptic Soy broth pH 7.0 (recovery- R)	SDS-PAGE (M_w) [a]	Immunoblot bands (M_w) [b]
pH 4, injury & resuscitation	35 & 36K thinner than control	34 & 35K
pH 4, injury only	35 & 36K thinner than control	34 & 35K
pH 5, injury & resuscitation	35 & 36K thinner than control	34 & 35K
pH 5, injury only	bands <35K appear faint	34 & 35K
pH 6, injury & resuscitation	N[c]	28, 34 & 35K
pH 6, injury only	N[c]	35K
pH 7, injury & resuscitation	N[c]	28, 34 & 35K
pH 7, injury only	N[c]	35K
pH 9, injury & resuscitation	N[c]	34 & 35K
pH 9, injury only	N[c]	35K
pH 6.8, injury & resuscitation (pH control)	regular[d]	28, 34 & 35K
pH 6.8; injury only (pH control)	regular[d]	35K

[a] changes of electrophoregram patterns from omps based on cell growth conditions prior to extraction

[b] molecular weight (M_w) of antigen bands reactive with MAb 1D6

[c] normal appearance of bands (N), same intensity of bands as under non-stressed growth conditions

[d] regular electrophoretic profile, no stress induced

(iiib) Effect of pH- 3 hour injury

The second experiment involved five species of *Salmonella*, *S. enteritidis*, *S. typhimurium*, *S. gallinarum*, *S. thompson* and *S. arizona*. Cells were grown in nutrient broth (37°C, 18 hr) to develop an adequate cell mass for extraction. The cells were harvested and resuspended to original volume in the pH adjusted nutrient broth. The cells were grown between pH 3 and 9 in nutrient broth (37°C, 3 hr). Cells were then harvested and resuspended to original volume in trypticase soy broth (neutral pH). They were resuscitated in TSB for 3 hr at 37°C. The outer membrane proteins were extracted from cells. The purpose was to characterize the effect of pH on the electrophoretic profile (Fig. 5a; pH 3, 4 and 7) (Fig 6a; pH 5, 6 and 9) and antigenicity (Fig. 5b; pH 3, 4 and 7)(Fig 6b; pH 5, 6 and 9) of the OMPs are presented in Tables 11-15. The protocol for this experiment was adopted from Chart *et al.* (1994).

The results for protein band differences for Fig 5a, 6a (electrophoregram) and Fig 5b, 6b (immunoblot) for *S. enteritidis* was noted in Table 11. *S. enteritidis* had a typical profile of electrophoretic bands after pH 3-9 exposure (Fig. 5a, 6a). The immunoblot of *S. enteritidis* revealed faint band of 28K, 32K, and 34K by the MAb at pH 7 and 4 (Fig 5b, lane 1 and 11) and pH 5 and 6 (Fig 6b, lane 1 and 6). The appearance of these bands at pH 3 (Fig 5b, lane 6) and 9 (Fig 6b, lane 11) was not affected by pH. The appearance of the 35K band was not compromised by the effects of pH (Fig 5b, 6b).

The results for protein band differences for Fig 5a, 6a (electrophoregram) and Fig 5b, 6b (immunoblot) for *S. typhimurium* was noted in Table 12. *S. typhimurium* showed a normal electrophoretic profile for the six pH levels studied (Fig. 5a, 6a). At pH 3 (Fig 5a, lane 7)

the 54K band appears thick and more distinctive than at the other pH(s). The 100K band is absent at pH 4 (Fig 5a, lane 13). This species also has two very distinctive bands, 19 and 20K at pH 4 (Fig 5a, lane 13) that are not as apparent at the other pH. The only difference in the electrophoretic pattern between pH 7, control (Fig 5a, lane 2) and pH 6 (Fig 6a, lane 7) is the very thin band at 36K. The immunoblot (Fig 5b, 6b) displayed the 28K, 32K, 34K and 35K at pH 3 and 4 (Fig 5b, lane 7 and 13), also at pH 5 and 9 (Fig 6b, lane 2 and 13). At pH 6 (Fig 6b, lane 7) and pH 7 (Fig 5b, lane 2) only the 32, 34 and 35K were detected by the MAb.

The results for protein band differences for Fig 5a, 6a (electrophoregram) and Fig 5b, 6b (immunoblot) for *S. gallinarum* was noted in Table 13. *S. gallinarum*, at pH 3 (Fig 5a, lane 8) the 36K appeared faded compared with the thick 36K band at pH 7 (Fig 5, lane 4), pH 4 (Fig 5, lane 12) and pH 5 (Fig 6, lane 4). The bands that appear in the lower region of the gel (below 35K) at pH 3 and 4 (Fig 5, lane 8 and 12) are more defined compared with the control, pH 7 (Fig 5, lane 4). The intensity of the 35K band at pH 5, 6 and 9 (Fig 6, lane 4, 8 and 12) remained the same as the control, pH 7 (Fig 5, lane 4). Although the rest of the bands in these lanes are not as defined as the control, pH 7 (Fig 5, lane 4).

The immunoblot (Fig. 5b, 6b) displayed protein bands of 28K, 32K, 34K and 35K at pH 3 and 4 (Fig 5b, lane 8, 12). These bands were also displayed at pH 5, 6 and 9 (Fig 6b, lane 4, 8 and 12). The extract from pH 7 (Fig 5b, lane 4) treatment is missing the 28K band detected by the MAb at the other pHs. Additionally, a 33K band was detected by the Mab at pH 7 (Fig 5b, lane 4).

The results for protein band differences for Fig 5a, 6a (electrophoregram) and Fig 5b, 6b (immunoblot) for *S. thompson* was noted in Table 14. The only distinct difference among pH treatments with *S. thompson* was the intensity of the 36K band (Table 14). The 36K band at pH 7, control (Fig 5, lane 3), is faded and thin compared with the thick 36K band at pH 4 (Fig 5, lane 14). The clarity of the 36K band at pH 5 (Fig 6, lane 3) and pH 9 (Fig 6, lane 14) is blurred. The appearance of the bands in Fig. 6a, lane 9 (pH 6) and lane 14 (pH 9) above and below the mid-region of the gel is less distinguishable. Otherwise, the electrophoretic pattern over the pH range of 3-9 (Fig 5a, 6a) remained the same.

The immunoblot showed bands at 28K, 32K, 34K and 35K at pH 3, 4 and 7 (Fig 5b, lane 9, 14 and 3). These bands were also detected at pH 5, 6 and 9 (Fig 6b, lane 3, 9 and 14) by the MAb.

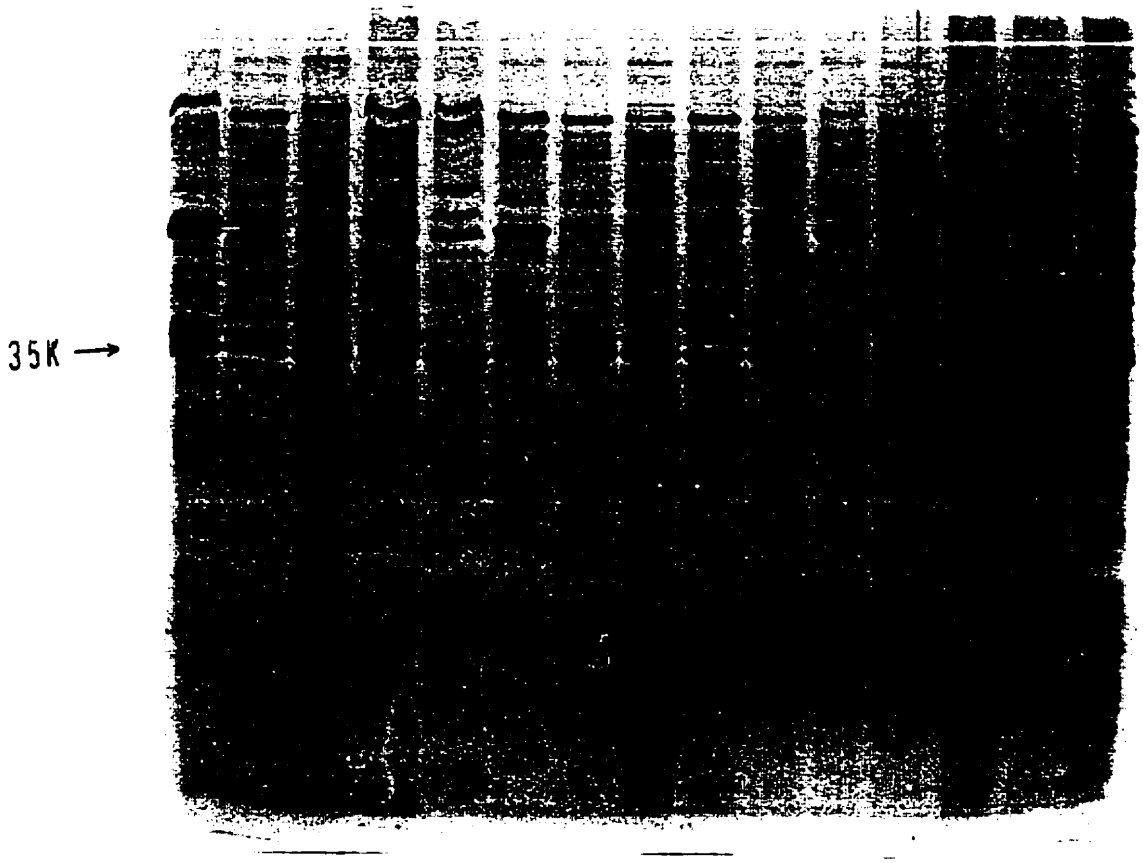
The results for protein band differences for Fig 5a, 6a (electrophoregram) and Fig 5b, 6b (immunoblot) for *S. arizona* was noted in Table 15. *S. arizona* displayed a normal electrophoretic pattern over the pH range of 3-9 (Fig 5a, 6a), with the exception that the 36K band was present only at pH 4 (Fig. 5a, lane 15). The immunoblot showed proteins at 28K, 32K, 34K and 35K detected by the MAb at pH 3 (Fig. 5b, lane 10). At pH 4 (Fig 5b, lane 15) 32K, 34K and 35K proteins reacted with the MAb. At pH values of 5, 6, 9 (Fig. 6b, lane 5, 10 and 15) and pH 7 (Fig. 5b, lane 5) only the 35K protein was detected.

Compared with the other 4 species, *S. enteritidis* displayed an intense band in the 62K region despite exposure to different pH. *S. typhimurium* and *S. thompson* uniquely displayed a 100K band. A 37K band was present in *S. enteritidis*, over the pH range of 4-9 (Fig 5a, 6a). This band was present in *S. arizona* but only over the pH range from 6-9 (Fig 6a, lane 10, pH

6, Fig 6a lane 15, pH 9 and Fig 5a, lane 5, pH 7.0). *S. arizona* and *S. gallinarum* expressed the 36K band, but the bands were thin or faded in some lanes. *S. arizona* expressed the 32 and 34K proteins at pH 3 and 4 (Fig. 5a, lane 10, pH 3 and lane 15, pH 4). This region appeared more intensely stained than in the other 4 species, but these proteins were not detected over the pH range of 5 to 9 (Fig. 5a, lane 5; pH 7, Fig 6a, lane 5; pH 5, lane 10; pH 6 and lane 15, pH 9).

The antigenicity of the 35K protein was conserved for *S. enteritidis*, *S. typhimurium*, *S. gallinarum*, *S. thompson* and *S. arizona*, despite exposure to pH. The 35, 34, 32 and 28K proteins were detected by the MAb, except for *S. arizona*. At pH 3 (Fig 5b, lane 10), pH 4 (Fig 5b, lane 15), pH 5 (Fig 6b, lane 5) and pH 9 (Fig 6b, lane 15) only the 35K was detected. The 28K protein was not detected by the Mab for the following organisms: *S. typhimurium*, pH 6 and 7 (Fig 6b, lane 7 and Fig 5b, lane 2), *S. gallinarum* pH 7 (Fig 5b, lane 4) and *S. arizona*, pH 7 and 4 (Fig 5b, lane 5 and 15) pH 5, 6 and 9 (Fig 6b, lane 5, 10 and 15).

Fig. 5a. SDS-PAGE of outer membrane proteins extracted from *Salmonella* spp. The cells were initially grown in nutrient broth (18 hr, 37°C). They were harvested and transferred into various pH adjusted (pH 3, 4 and 7) medium of nutrient broth (3 hr, 37°C), injury. The cells were resuscitated in Trypticase Soy broth (3 hr, 37°C). Lanes: 1-5: pH 7.0, lanes 6-10: pH 3.0, lanes 11-15: pH 4.0. Lanes 1, 6, 11- *S. enteritidis*, lanes 2, 7, 13- *S. typhimurium*, lanes 4, 8, 12- *S. gallinarum*, lanes 3, 9, 14- *S. thompson*, lanes 5, 10, 15- *S. arizona*.

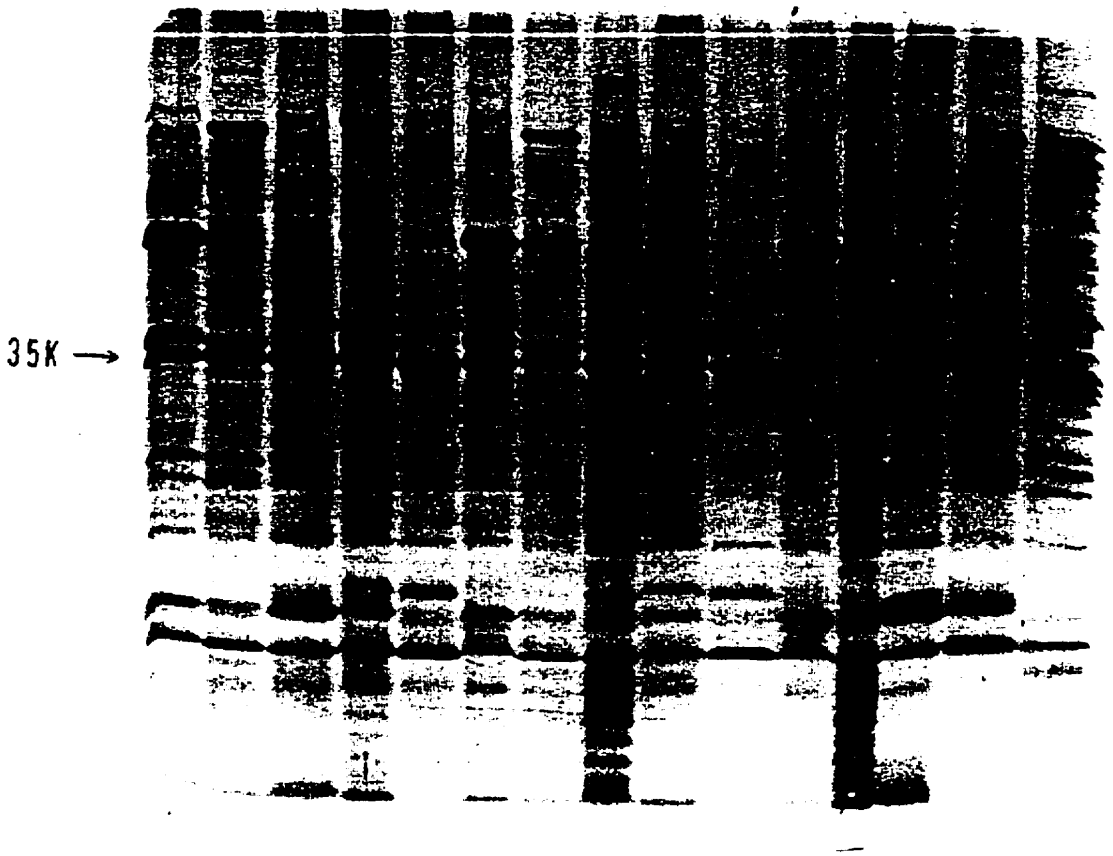


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Fig. 5b. Immunoblot of outer membrane proteins extracted from *Salmonella* spp. Proteins in the polyacrylamide gel shown in Fig. 5a were electrophoretically transferred to a nitrocellulose membrane and reacted with MAb 1D6.


35K
28K
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Fig. 6a. SDS-PAGE of outer membrane proteins extracted from *Salmonella* spp. The cells were initially grown in nutrient broth (18 hr, 37°C). They were harvested and transferred into various pH adjusted (pH 5, 6 and 9) medium of nutrient broth (3 hr, 37°C), injury. The cells were resuscitated in Trypticase Soy broth (3 hr, 37°C). Lanes: 1-5: pH 5.0, lanes 6-10: pH 6.0, lanes 11-15: pH 9.0. Lanes 1, 6, 11- *S. enteritidis*, lanes 2, 7, 13- *S. typhimurium*, lanes 4, 8, 12- *S. gallinarum*, lanes 3, 9, 14- *S. thompson*, lanes 5, 10, 15- *S. arizona.*



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Fig. 6b. Immunoblot of outer membrane proteins extracted from *Salmonella* spp. Proteins in the polyacrylamide gel shown in Fig. 6a were electrophoretically transferred to a nitrocellulose membrane and reacted with MAb 1D6.

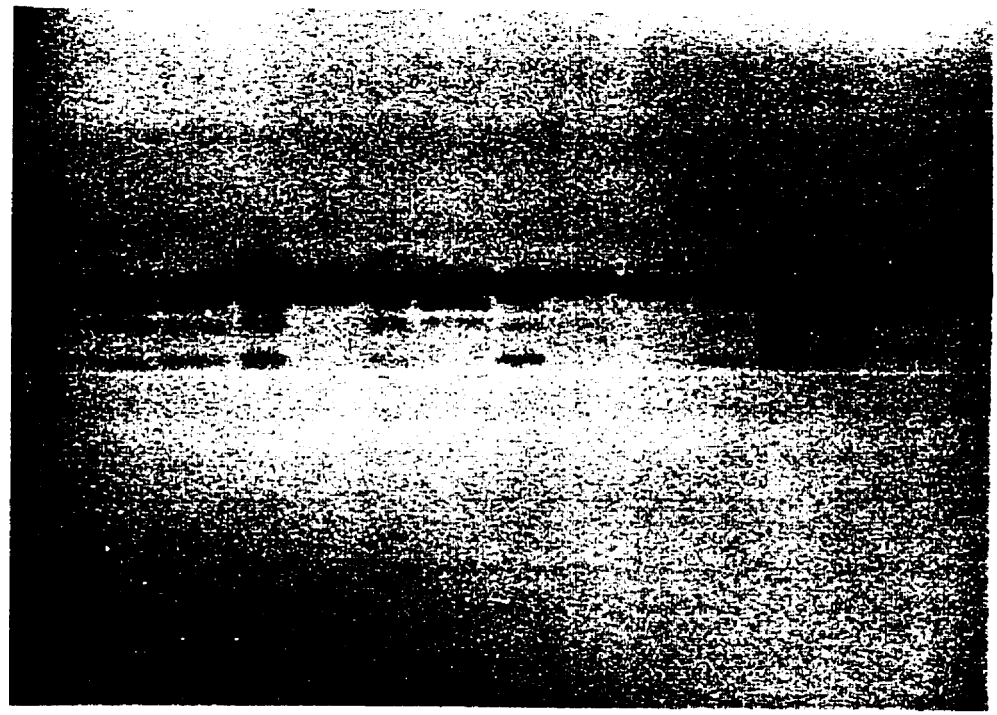

35K
28K
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Table 11. Stress-induced outer membrane proteins from *S. enteritidis*[e]: response to pH challenge

Stress	Protein band detection method	
3 hr nutrient broth (injury- I), 3 hr Tryptic Soy broth pH 7.0	SDS-PAGE (M_w) [a]	Immunoblot bands (M_w) [b]
pH 3, injury & resuscitation	normal appearance of bands[c]	28, 32, 34 & 35K
pH 4, injury & resuscitation	normal appearance of bands[c]	28, 32, 34 & 35K[f]
pH 5, injury & resuscitation	normal appearance of bands[c]	28, 32, 34 & 35K[f]
pH 6, injury & resuscitation	normal appearance of bands[c]	28, 32, 34 & 35K[f]
pH 7, injury & resuscitation	regular[d]	28, 32, 34 & 35K[g]
pH 9, injury & resuscitation	normal appearance of bands[c]	28, 32, 34 & 35K

[a] changes of electrophoregram patterns from omps based on cell growth conditions prior to extraction

[b] molecular weight (M_w) of antigen bands reactive with MAb 1D6

[c] same intensity of bands as under non-stressed growth conditions

[d] regular electrophoretic profile, no stress induced

[e] Prominent bands from *S. enteritidis* (pH 7) were: 17, 19, 28, 35, 36 and 60K

[f] Intensity of the 28, 32 and 34K was very faint (pH 4-6)

[g] Intensity of the 32 and 34K was very faint (pH 7)

Table 12. Stress induced outer membrane proteins from *S. typhimurium* [e]; response to pH challenge

Stress	Protein band detection method	
3 hr nutrient broth (injury- I), 3 hr Tryptic Soy broth; pH 7.0	SDS-PAGE (M_w) [a]	Immunoblot bands (M_w) [b]
pH 3, injury & resuscitation	54K thick	28, 32, 34 & 35K
pH 4, injury & resuscitation	100K absent 19 & 20K intense	28, 32, 34 & 35K
pH 5, injury & resuscitation	N[c]	28, 32, 34 & 35K
pH 6, injury & resuscitation	36K very thin	32 (faint), 34 & 35K
pH 7, injury & resuscitation used as pH control	regular[d]	32, 34 & 35K
pH 9, injury & resuscitation	N[c]	28, 32, 34 & 35K

[a] changes of electrophoregram patterns from omps based on cell growth conditions prior to extraction

[b] molecular weight (M_w) of antigen bands reactive with MAb 1D6

[c] normal appearance of bands (N), same intensity of bands as under non-stressed growth conditions

[d] regular electrophoretic profile, no stress induced

[e] Prominent bands present for *S. typhimurium* (control pH 7) were : 17, 28, 34, 35, 36, 92 and 100K

Table 13. Stress induced outer membrane proteins from *S. gallinarum*[e]; response to pH challenge

Stress	Protein band detection method	
3 hr nutrient broth (injury- I), 3 hr Tryptic Soy broth; pH 7.0 (recovery R)	SDS-PAGE (M_w) [a]	Immunoblot bands (M_w) [b]
pH 3, injury & resuscitation	faint 36K 23, 25 & 27K intense	28, 32, 34 & 35K
pH 4, injury & resuscitation	normal appearance of bands[c]	28 and 32K faint 34 & 35K
pH 5, injury & resuscitation	normal appearance of bands[c]	28 and 32K faint 34 & 35K
pH 6, injury & resuscitation	36K thin of bands[c]	28 and 32K faint 34 & 35K
pH 7, injury & resuscitation used as normal pH control	regular[d]	32, 33, 34 & 35K
pH 9, injury & resuscitation	36K thin	28, 32, 34 & 35K

[a] changes of electrophoregram patterns from omps based on cell growth conditions prior to extraction

[b] molecular weight (M_w) of antigen bands reactive with MAb 1D6

[c] same intensity of bands as under non-stressed growth conditions

[d] regular electrophoretic profile, no stress induced

[e] Prominent bands present for *S. gallinarum* (control pH) were : 17, 19, 28, 35 & 36K

Table 14. Stress-induced outer membrane proteins from *S. thompson*[e]: response to pH challenge

Stress	Protein band detection method	
3 hr nutrient broth (injury- I), 3 hr Tryptic Soy broth; pH 7.0 (recovery R)	SDS-PAGE (M_w) [a]	Immunoblot bands (M_w) [b]
pH 3, injury & resuscitation	36K intense	28, 32, 34 & 35K
pH 4, injury & resuscitation	36K very intense	28(faint), 32, 34 & 35K
pH 5, injury & resuscitation	36K faint	28, 32 & 34K (all 3 faint) 35K
pH 6, injury & resuscitation	normal appearance of bands[c]	28, 32 & 34K (all 3 faint)
pH 7[b], injury & resuscitation	regular[d]	28, 32, 34 & 35K
pH 9, injury & resuscitation	36K faint	28, 32, 34 & 35K

[a] changes of electrophoregram patterns from omps based on cell growth conditions prior to extraction

[b] molecular weight (M_w) of antigen bands reactive with MAb 1D6

[c] same intensity of bands as under non-stressed growth conditions

[d] regular electrophoretic profile, no stress induced

[e]Prominent bands present for *S. thompson* (control pH 7) were: 17, 19, 28, 34, 35, 60, 100 & 110K

Table 15. Stress-induced outer membrane proteins from *S. arizona* [e]; response to pH challenge

Stress	Protein band detection method	
3 hr nutrient broth (injury- I) 3 hr Tryptic Soy broth; pH 7.0 (recovery R)	SDS-PAGE (M_w) [a]	Immunoblot bands (M_w) [b]
pH 3, injury & resuscitation	normal appearance of bands[c]	28, 32, 34 & 35K
pH 4, injury & resuscitation	36K expressed at this pH only	32 (very faint), 34 & 35K
pH 5, injury & resuscitation	normal appearance of bands[c]	only 35K
pH 6, injury & resuscitation	normal appearance of bands[c]	only 35K
pH 7[b], injury & resuscitation	regular[d]	34 (very faint) & 35K
pH 9, injury & resuscitation	normal appearance of bands[c]	only 35K

[a] changes of electrophoregram patterns from omps based on cell growth conditions prior to extraction

[b] molecular weight (M_w) of antigen bands reactive with MAb 1D6

[c] same intensity of bands as under non-stressed growth conditions

[d] regular electrophoretic profile, no stress induced

[e] Prominent bands present from *S. arizona* (control pH 7) were : 17, 20, 33, 34 and 35K

iv) Excessive heat

Previous experiments conducted by Jaradat and Zawistowski (1995) identified the 35K OMP as a heat modifiable protein. This was done by solubilizing the extract in SDS at 100°C before electrophoresis. The 35K band was the only band recognized by immunoblotting using the MAb 1D6. The 35K protein is considered a polypeptide with several forms displayed by bands at 28K and minor protein bands between 34-40K under native conditions (native-PAGE electrophoresis, Jaradat and Zawistowski, 1995).

The extracts from *S. typhimurium* and *S. arizona* cells grown at 37°C, 42°C, 23°C and 15°C were subjected to moist heat at 121°C for 15 minutes to determine whether high temperature affected antigenicity.

Although the clarity of the electrophoresis bands was compromised, no change was evident in the electrophoretic pattern (data not shown). The clarity of the bands was lost due to the excessive heat treatment. The heat treatment may have denatured the protein causing a reduction in protein concentration. Though protein content was altered due to the applied heat, the antigenicity of the 35K band was not lost.

C. Conclusion

The antigenicity of the 35K outer membrane protein was not compromised by any of the treatments used in this study as assessed by SDS-PAGE and immunoblotting. The only deterent was low cell density caused by harsh treatments. This affected the appearance of OMP profiles and allowed expression of only very faint electrophoretic bands.

The monoclonal antibody produced against the 35K OMP recognized this band in all five species; *S. typhimurium*, *S. gallinarum*, *S. arizona*, *S. thompson*, and *S. enteritidis* by

immunoblotting. Even under suboptimal growth conditions, the antigenic structure of the 35K protein was conserved. The MAb 1D6 also reacted with major OMPs including those having a M_w of 34K, 32K and a minor 28K OMP. An epitope structure similar to the antigenic 35K OMP may have been induced in these other proteins by the stress applied to the growing cells under suboptimal conditions.

V. GENERAL DISCUSSION

Salmonella typhimurium is an enteric human pathogen and a major causative agent of salmonellosis, along with other *Salmonella* species. *Salmonella* is regarded as the most prevalent organism responsible for food infection. A trade off has existed with other organisms. Over the years, *E. coli* has become the most evasive bacterium to cause food borne illness (Frazier, 1985). It is regarded as a versatile organism: able to adapt to environmental diversity; nature and climate, environmental stress; the gastrointestinal tract of humans and nutrient limitation (Spector *et al.*, 1986).

In the previous study conducted by Jaradat and Zawistowski (1995) a monoclonal antibody against the 35K OMP from *Salmonella typhimurium* was produced. The MAb 1D6 could also recognize this protein in other *Salmonella* spp. and in non-*Salmonella* organisms (34K band) as detected by electrophoresis followed by immunoblotting.

In this study, SDS-PAGE was conducted on the outer membrane protein extracts of five *Salmonella* species including; *S. typhimurium, S. gallinarum, S. enteritidis, S. arizona* and *S. thompson.* Different treatments were applied to whole cells of *Salmonella* such as nutrient starvation and environmental stress.

The purpose of this study was to examine the electrophoretic profile pattern and establish whether the type of treatment used caused a change in expression or repression of proteins. Organisms can respond to environmental stresses, inducing genes or 'stimulons' to produce or increase the production of specific proteins (Spector *et al.*, 1986).

The changes that occurred in the electrophoretic pattern, due to stress-related response, of the outer membrane protein usually occurred within the 33-38K region, involving in

particular 34, 35 and 36K proteins. This region is considered to contain the major proteins of the outer membrane (Lee and Schnaitman, 1980).

Lee and Schnaitman (1980) compared the outer membrane porin proteins present in *E. coli* and *S. typhimurium*. They concluded that the OmpC, OmpF and OmpD found in both organisms do not produce the same electrophoretic profile, but they share similarities in regulation of expression and in function. As well, gene loci OmpC and OmpF are comparable. The authors identified the three outer membrane porins of *S. typhimurium*, OmpC, Omp F and Omp D, with molecular weights of 36, 35 and 34K, respectively.

Effect of temperature

The outer membrane proteins are affected by temperature. The electrophoretic pattern showed that at 42°C and 23°C, the protein pattern was more prominent than during the other temperature trials. For example, with *S. gallinarum* (Table 5) the 36K band was more apparent at 42°C than at the band 37°C. Growing the cells at 23°C and 42°C, showed an increased expression of the 34 and 37K bands and two minor bands appearing below this region than at 37°C. Thus, in the current study the effect of growing *S. typhimurium*, *S. gallinarum*, *S. arizona* and *S. thompson* at different temperatures showed minor changes in the protein pattern.

The effect of two different growth temperatures, 25°C and 37°C on *Yersinia enterocolitica* was assessed by Ogasawara *et al.* (1985). Ogasawara and coworkers (1985) found that neither growth temperature resulted in different reactivity when tested using an ELISA with the monoclonal antibody 4G1 produced against protein III. Ogasawara and coworkers (1985) concluded that the antigenicity of the heat modifiable protein found in the

Triton X-100-insoluble membrane fraction was conserved and not affected by growth temperature.

In a study conducted by Puente *et al.* (1991), the outer membrane protein from *S. typhi* and *E. coli* HB 101 were extracted from cells grown in nutrient broth at 4, 20, 37, and 42°C for 18 hr. Results of electrophoresis were conducted in a discontinuous system using 8M urea as the reducing agent. The growth temperature of the medium did not affect the protein electrophoretic patterns of the organism. The temperature effect on major OMPs, such as OmpC, were also studied by Roy *et al.* (1994). Results obtained using four different *Shigella* spp. grown at 30, 37 and 42°C for 18 hr proved this. Roy and coworkers (1994) also concluded that the effect of temperature alone did not alter the protein profile. Murine polyclonal antibodies were raised against each of the four different species. SDS-PAGE followed by immunoblotting revealed that the region between 34 and 38 K contained the major immunogenic protein. Except *S. dysenteriae* that displayed two separate bands at 37°C using immunoblotting, no change was evident in the electrophoretic profile.

Effect of salt

Temperature alone does not alter the expression of OMP; change usually occurs through the influence of a combination of both temperature and osmolarity. This view is shared by several authors including, Van Alphen and Lugtenberg (1977), Verhoef *et al.* (1979), Kawaji *et al.* (1979), and Lugtenberg *et al.* (1981),

Results obtained by Ames *et al.* (1974), based on studies conducted to assess the protein composition of the outer membrane of *S. typhimurium,* revealed that four major outer membrane proteins exist within the region of 33-36K. These proteins were not altered by

growth conditions. Three media; minimal salt medium E with 0.4% glucose as a carbon source, nutrient broth (0.8%) with 0.5% salt, or proteose-peptone (1%) with 0.5% NaCl, was used. Three different incubation temperatures before protein solubilization in SDS were also studied with no substantial difference resulting in the electrophoretic profile. The temperature of solubilization at 37°C compared with 100°C affected the ability of the proteins to undergo solubilization. The major protein bands, and the 44K protein were not present in the lower temperature extract. Ames and coworkers (1974) also concluded that the outer membrane protein content was affected by changes in the LPS content of the cell, specifically mutants lacking the O-antigen (*gal*E mutants). Slight decreases in the level of outer membrane proteins, excluding the 35K protein, was noted (Ames *et al.*, 1974). This experiment studied the effect of lipopolysaccharide mutations on the protein composition of the outer membrane of *S. typhimurium.* This work showed that the 35K protein interacts with the outer core of the LPS (Ames *et al.*, 1974). Four major protein bands in the M_w region of 33-36 K extracted from an *S. typhimurium* strain, were not affected by growth conditions.

The antigenicity of the 35K omp in this study was not compromised by the absence of salt in the media. Overall, the four spp. of *Salmonella* showed minor changes outer membrane protein bands from the electrophoretic profile. The combination of temperature and absence of salt changed the expression of the 34K omp from *S. arizona.*

Effect of iron

The effect of the absence of iron in growth media was similar to the results obtained without salt. Both minerals are essential for cell growth and necessary for normal cellular functions.

Notably, certain treatments appeared to affect the organisms causing development of a faint overall appearance of the protein bands specifically, *S. gallinarum* (Table 5; 37°C, without iron; 23°C, with iron; 42°C without iron) and *S. arizona* (Table 6; 15°C without iron). Despite the effect of temperature or iron in the growth medium, the 36K band of *S. arizona* was absent. *S. typhimurium*, *S. gallinarum*, *S. arizona* and *S. thompson* grown at 23°C without iron only showed the 35, 36 and 37K bands to be prevalent.

The epitope of the 35K protein was conserved for *S. typhimurium*, *S. gallinarum*, *S. arizona* and *S. thompson*. The ability of the MAb 1D6 to recognize the epitope was not compromised by any treatment. This includes the individual growth temperatures and temperature treatments in combination with the exclusion of iron.

This study was not in agreement with results obtained by Chart *et al.* (1993). The latter authors studied the effect of iron restriction on the growth of *S. enteritidis* and concluded that additional bands were expressed without iron.

Effect of phosphate

In three species, not including *S. arizona*, the absence of phosphate in the medium caused an increase in the appearance of major OMPs, 35 and 36K. Phosphate limitation usually depressed synthesis of several OMPs whereas Pho E (37K) was induced (Korhonen *et al.*, 1985).

The 60K polypeptide was affected by growth without phosphate at 15°C and 42°C. As a result, the electrophoretic band was absent or very faint in *S. gallinarum* and *S. arizona* but present in *S. typhimurium* and *S. thompson*. This electrophoretic band was identified as a major heat shock protein according to Sarvas (1985b). The cells grown at 42°C without

phosphate increased the amount of the 60K protein in *S. typhimurium* and *S. thompson*. This response is a result of heat shock response and absence of phosphate.

Effect of osmolarity

A study conducted by Van Alphen and Lugtenberg (1977) showed the effects of salt to the growth medium. OMPs extracted from *E. coli* K12 cells grown using 0.3M NaCl in yeast broth, displayed an increase in protein B (37K band). However, the addition of 0.8 and 1.0 M NaCl to the nutrient broth caused a thinner 37K protein band. Van Alphen and Lugtenberg (1977) stated that when salt is added to the growth medium, a depletion of certain proteins occurs that are normally present in high amounts. Noticeable differences were not evident in the 36K band in response to changes in NaCl concentration.

Puente *et al.* (1991) proved that the expression of the 36K band in *S. typhi* remains the same whether the cells were grown in a medium of low or high osmolarity. In contrast, high salt concentration ($>0.5\%$) was reported to suppress the expression of the OmpF protein (Lee and Schnaitman, 1980).

The major 35K electrophoretic omp and immunoblot bands decreased in thickness as the molarity of the medium increased in this study. Nakamura and Mizushima (1976) reported that the osmolarity of the growth medium can regulate the expression of the 35K porin of *S. typhimurium*.

Heat or cold shock

The antigenicity of the 35K protein was not compromised by suscepting the cells to heat (48° C) or a slow freezing rate. Immunoblot bands detected included 28, 32, 34 and 35K proteins (Fig 3b, lanes 1, 2, 3, 4 and 5). The heat treatment for 60 and 90 minutes at 48°C (Fig 3b, lane 3 and lane 4), showed that as the length of heat treatment increased, the 34, 32, and 28K immunoblot bands became more faint. The immunoblot bands were also less intense for the OMPs extracted from cells exposed to slow freezing.

With respect to antigenicity, the MAb 1D6 can recognize and react with the 35K outer membrane protein. Under restrictive conditions, certain treatments altered the epitope structure of other OMPs, 28K, 32K, and 34K, but the MAb also recognized these OMPs. Therefore, the expression of the epitope structure of these proteins must be similar to that of the 35K protein. Several factors may explain MAb 1D6's ability to react with other protein bands. These proteins may be composed of common antigenic subunits (Ogasawara *et al.*, 1985). Chemical or conformational changes to the protein may be induced by environmental stresses or the absence of certain nutrients or minerals (Ogasawara *et al.*, 1985). In addition common epitope structure may be exposed only during specific cultural conditions (Ogasawara *et al.*, 1985).

A monoclonal antibody A59/05F01/C09 was raised against the 25 K protein of *Brucella abortus* by Cloeckaert *et al.* (1996). The immunoblot revealed the 50 and 60 K bands that may have been multimers of the Omp 25. Specifically, it was thought that the 50K could have been a dimer of the Omp 25 (Cloeckaert *et al.*, 1996). The occurrence of molecular aggregation can contribute to increased numbers of band patterns by OMPs during

electrophoresis. In the previous study, Jaradat and Zawistowski (1995), assumed the 35K was a denatured polypeptide fraction of the native 28K protein.

pH

According to Chart *et al.* (1994), the OMPs from *S. enteritidis* exhibited varying amounts of the 3 major OMPs, 33, 35 and 36K, depending on the pH. In contrast with these results, the 33K OMP was not expressed by *S. enteritidis* (Fig. 5a, lane 1, 6 and 11, Fig 6a, lane 1, 6 and 11). Instead three bands in the 35, 36 and 37K region were found to vary depending on pH.

The protein band at the region of 36K resembled the 35.5K protein reported by Chart and coworkers (1994). These authors observed that certain strains of *S. enteritidis* also can produce higher M_w protein that can overlap with the 35K band.

VI. CONCLUSION & RECOMMENDATIONS

The expression of major and minor outer membrane protein bands was more evident without iron and phosphate in the growth medium compared with the absence of salt. Also, an increase in expression of certain proteins was induced when the organisms were grown under stressed conditions. Induction could be invoked by temperature change or nutrient limitation or both. However, the various salmonellae spp. responded differently at the two extreme temperatures used, 42°C and 15°C.

The outer membrane protein extracts from the cells subjected to a medium without salt at 42°C and 15°C showed reduced expression of the 35 and 36K proteins, assessed by electrophoresis. This could have been due to both treatments being beyond the optimum growth temperature of 37°C for *Salmonella*. As well, the temperature extremes affected cell density and caused a decrease in total protein content.

Regarding extreme conditions including high and low pH, osmolarity and growth temperature, the salmonellae spp. tested can recover from these suboptimal conditions by increasing the production of specific proteins. This was evident because the cells recovered after 3 or 18 hr resuscitation period, independent of the growth stage of the cells. A decrease in the total protein content of the outer membrane was observed when bacteria were exposed to suboptimal environments. This was shown by low cell density (LCD, Tables 4-15).

In summary, electrophoretic results showed that bacteria grown under stressful conditions synthesized new minor outer membrane protein bands usually prevalent in the upper region and lower regions of the electrophoregram (60 and 18K). The major OMPs detected by the MAb 1D6 included 34, 32 and 28K proteins. The MAb 1D6 can cross-react with these

minor proteins due to: structural alteration during synthesis; injury response; or the presence of common antigenic properties in existing minor polypeptides. The antigenicity of the 35K protein was always conserved despite environmental stress. The results suggest the expression of the 35K epitope structure is not dependent on the type of medium or growth stage of the organism. These results are consistent with those of Sarwar *et al.* (1992).

Conservation of the 35K OMP may have a beneficial use toward the development of a vaccine against salmonellosis, if the antibodies against this molecular marker could recognize the protein independent of the state (injured/uninjured) of the organism invading the host. Conservation of the antigenic properties of the 35K protein could also be used to develop a rapid method to detect *Salmonella* in food provided cross-reactivity of non-salmonella species was eliminated via selective enrichment or isolation procedures. We now know that despite whether the organism is injured or stressed due to heating, freezing or processing, the epitope on the 35K will still allow for recognition by the MAb.

To further the research in this area, better control over restrictive growth media is required to ensure that media is totally without the nutrient under study, particularly iron. Kadurugamuwa *et al.* (1987) suggested experimenting with spent media with chelating agents, biological agents such as iron scavengers or synthetic iron chelators (desferoxamine).

Other research could involve exposure to animal cells (epithelial cells) and environmental conditions to study cell invasions and the effect of adherence of *S. typhimurium* to animal cells (Schiemann and Shope, 1991).

In the previous study, Jaradat and Zawistowski (1995) demonstrated using immuno-gold staining (immuno-staining with colloidal gold) the 35K protein was localized on the

surface of the outer membrane. Proteins buried in the hydrophobic core of the outer membrane normally have strong association with phospholipids or LPS in the native state (Smyth, 1985). Therefore, purification of hydrophobic proteins may change their immunogenicity upon separation from the polysaccharide moiety (Smyth, 1985). Thus, the antigenicity of an OMP would be more accurately determined by targeting in situ surface localized proteins used as antigenic determinants.

To conclude, the 35K outer membrane protein represents an antigenetically stable determinant that would be ideally suited for the development of a serological and epidemiological marker of salmonellae spp.

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