STUDIES ON THE ANTIGENICITY OF THE OUTER MEMBRANE OF B-SEROGROUP SALMONELLA

A thesis
Submitted to the Faculty

of

Graduate Studies
The University of Manitoba

by

Ziad Waheed Jaradat

In partial Fulfilment of the
Requirements for the Degree

of

Master of Science

Food Science Department

May 1995

ISBN 0-612-13211-0

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Ziad Jaradat

To
My Parents
My brother Ahmed
My supervisor Dr. Zawistowski

ACKNOWLEDGEMENTS

Words can not describe my appreciation and gratitude for my dearest friend and supervisor Dr. J. Zawistowski for giving me the opportunity to work with his Agri-Food biotechnology group and for his guidance through out my studies.

Special thanks are also extended to Dr. G. Blank for his friendship, continuous support and his invaluable advices through the course of my study. Thanks are aslo extended to Dr. R. Holley for his continues support and encouragement and Dr. R. Lyric for his participation on the thesis examining committee.

Appreciation is also extended to Andria Masi and Mark Yoshimasu for their support, friendship and technical contributions; Jim Rogers for technical assistance.

My tremendous appreciation is extended to my Family. My father who spent all his fortune to educate us and who always emphasize the supreme value of education until he imprint that in our minds. My mother who always support me with her blessings and encouragement. My brothers and sisters, specially Ahmed who advised me in taking the decision to come and pursue my studies in Canada.

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LIST OF ABBREVIATIONS

A_w	Water activity
ATCC	American Type Culture Collection
BCIP	5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt
BSA	Bovine serum albumin
CDC	Centres for Disease Control
CM	Cytoplasmic membrane
DNA	Deoxyribonucleic acid
EDTA	Ethylene diaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
kDa	Kilodalton
KDO	2-Keto-3-Deoxy-D-manno-octulosonic acid
L-broth	Lactose broth
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
M_r	Molecular Weight
NBT	p-nitro blue tetrazolium chloride
OM	Outer membrane
OMP	Outer membrane protein
PBS	Phosphate buffered saline

PBST	Phosphate buffered saline containing Tween 20
PG	Peptidoglycan
pNPP	p-nitophenyl phosphate
R LPS	Rough type lipopolysaccharide
S LPS	Wild type lipopolysaccharide
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SPC	Standard Plate Count
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing Tween 20

ABSTRACT

Studies of *Salmonella* outer membrane components are important in order to characterize surface antigens which could be used as a diagnostic tool for *Salmonella*. In order to pursue such a study, four monoclonal antibodies were produced by a fusion of P3X63-Ag. 653 myeloma cells and spleenocytes of mouse immunized with *S. typhimurium* cells. MAbs 5A5 and 5B2 were of IgM class, MAb 4A8 was of IgG_{2a} class while MAb 1D6 was of IgA class; all with κ light chains. Results indicated that MAbs 5A5, 5B2 and 4A8 were reactive only to *S. typhimurium* and other B-serogroups;*S. heidelberg, S. stanley* and *S. paratyphi* B without any crossreactivity to other 33 tested bacteria, while MAb 1D6 recognized only two of 23 *Salmonella* and non-salmonellae species tested as assessed by ELISA. In contrast, when MAb 1D6 was tested against OMP extracts of the above 23 species, it reacted to all of them. Further analysis revealed that MAbs 5A5, 5B2 and 4A8 recognized the LPS O-5 antigen, unique for *S. typhimurium* and other members of *Salmonella* B-serogroup, while, MAb 1D6 was found to be reactive with outer membrane protein (OMP) of *Salmonella*. Titration curves of MAbs 5A5, 5B2 and 4A8 revealed that the IgM MAbs 5A5 and 5B2 exhibited higher avidity to their epitopes than the IgG_{2a}. Additive ELISA revealed that the three MAbs recognized the same epitope. All MAbs were found equally reactive with both live and heat treated *S. typhimurium*. The ability of MAbs to detect live cells was visualized by immunoelectron microscopy. Treatment of *S. typhimurium* cells with cholic acid had no

effect on antibody recognition of LPS;nor the extreme acid conditions had a destructive effect on the reactivity of the O-5 antigen with MAbs. However, alkaline conditions rendered the O-acetyl group unique for factor O-5 undetectable by MAbs. Furthermore, anti-OMP MAb 1D6 along with mouse antisera, were used to study the antigenicity of *Salmonella* outer membrane protein components. OMP extracts prepared from 19 *Salmonella*, representing seven serogroups and four non-salmonellae species of *Enterobacteriaceae* were studied using electrophoresis and immunoblotting. Immunoblotting with antisera revealed two major proteins with apparent molecular weights(M_r) of 35 and 24 kDa. The 35 kDa protein was present only in *Salmonella* species. MAb 1D6 bound to the 35 kDa OMP for all tested *Salmonella* species with the exception of *S. arizona*. These results demonstrated that the 35 kDa protein is common antigen for all tested *Salmonella*, however, an epitope recognized by MAb 1D6 is absent in the protein obtained from atypical *S. arizona*. The type of growth media had no effect on the antigenicity of both 35 and 24 kDa in crude OMP extracts. Treatment with reducing agents prior to separation did not affect the antigenicity of these OMPs. However, the electrophoretic appearance of the 35 kDa was influenced by temperature. Analysis of OMP extracts by SDS-PAGE and immunoblotting revealed that MAb 1D6 bound several isoforms of this protein; one major band at 28 kDa and about eight minor forms in the range of 34 to 40 kDa. Glycoprotein blot revealed that none of these forms contained carbohydrate moieties that may be responsible for the polymorphic appearance of the protein. However, these forms were converted to a single form with M_r 35 kDa upon heat treatment (100°C, 5min) which indicated that the 35 kDa protein is most likely

heat-modifiable. In addition, extended heat-treatment (121°C, 15 min) did not affect the antigenicity of the 35 kDa OMP. Furthermore, the 35 kDa protein was found to be exposed on the surface of the cells as revealed by gold labelling and immunoelectron microscopic studies. However, the accessibility of this OMP to MAb 1D6 was some-what detracted by the long LPS chains surrounding the cell.

FOREWORD

This thesis has been written in the manuscript style. Although these manuscripts are interrelated for the most part, each is independent of the other, and has been prepared according to the instructions given by the specific journal. Manuscript I, describes the production and characterization of monoclonal antibodies specific to factor O-5 of B-serogroup *Salmonella*. This paper was submitted to the Applied and Environmental Microbiology. Manuscript II, describes studies on the antigenicity of the outer membrane protein *of Salmonella*, and will be submitted to Food and Agricultural Immunology Journal.

The authors and titles of manuscripts are:

I. Jaradat, Z. W. and Zawistowski, J. 1995. Production and characterization of monoclonal antibodies against O-antigen 5 of *Salmonella typhimurium* lipopolysaccharide (Submitted).

II. Jaradat, Z. W. and Zawistowski, J. 1995. Antigenically stable 35 kDa outer membrane protein of *Salmonella* (In preparation).

1. INTRODUCTION

Salmonella genera contains over 2200 serovars. They are classified according to somatic O-antigen along with the flagellar antigen into several groups. Serotypes A, B, C, D and E are found to be responsible for more than 95% of human *Salmonella* isolates (Kerr *et al.*, 1992).

Upon ingestion of contaminated food or water with *Salmonella*, these pathogens can invade and colonize the small intestine in humans causing a variety of disease conditions known as salmonellosis. It reflects the outcome of a confrontation between the host defence system and the bacterial virulence determinants. Human salmonellosis has been subclassed into four clinical symptoms; asymptomatic intestinal carrier, acute gastroenteritis, bacteraemia and enteric fever (Butler and Kaye, 1992).

Salmonella typhimurium, belongs to the B-serogroup, has been recognized as the most common *Salmonella* serovar incriminated in food poisoning cases for the last few decades. *S. typhimurium* was first recognized in 1888 as a foodborne pathogen when 50 people consumed raw beef contaminated with this pathogen became infected and one person died in outbreak of salmonellosis in Germany (Tauxe, 1991).

In 1965, *S. typhimurium* was responsible for one of the largest waterborne outbreaks in the United States. Over 16,000 people were infected after drinking water became contaminated with this pathogen (Gouthrie, 1992). Twenty years later, over 16,000 people were infected after consuming pasteurized milk contaminated with *S.*

typhimurium. The cost of latter outbreak was estimated to be more than 60 million dollars.

In order to control the prevalence of *Salmonella* outbreaks, a fast and reliable detection method must be developed. Traditional culture methods are time consuming, labour intensive and expensive (Van-Poucke, 1990, Brooks *et al.*, 1992). Rapid methods such as enzyme linked immunosorbent assays (ELISAs) provide good alternative tests for detection of *Salmonella*. However, the accuracy of detection depends on the type of molecular marker to be recognized by the employed antibody. Enzyme immunoassays utilizing flagellin, fimbrial and lipopolysaccharide cell markers have been developed (Lee *et al.*, 1990, Thorns *et al.*, 1994). Nevertheless, these markers have certain drawbacks. Flagellin, for instance, is found only in motile bacteria, and the genes encoding for it possess a high degree of homology which lead to similarities in the structure. Consequently, this leads to high crossreactivity (Kawagishi, *et al.*, 1992).

In contrast, the LPS O-antigen of *Salmonella* has been subjected to tremendous diversification during the pathogen evolution (Nikaido and Nakae, 1979, Nghiem *et al.*, 1992). As a result, each *Salmonella* group is characterized by a set of factors present on the distal part of the LPS molecule. The sugar groups and their sequence in the repeating units are distinctive for each strain and encompasses its serological specificity (Nghiem *et al.*, 1992). This property renders the LPS O-antigen a very specific marker.

Outer membrane proteins are reported to be conserved among different *Salmonella* serogroups. Therefore, studies of outer membrane proteins are important to identify and characterize surface antigens which can be used as an immunodiagnostic tool or to be

used as a possible vaccine candidate against the whole *Salmonella* genera. Furthermore, such studies can be useful to identify and characterize the virulence factors or/and elucidate the structure-function relationships.

The objective of this thesis was to study the antigenicity of *Salmonella* outer membrane components in order to identify a surface marker(s) specific to *Salmonella* which would be expressed consistently under various growth and environmental conditions, yet conserved only among *Salmonella* species.

2. Literature Review

2.1 Salmonella

Salmonella is a gram-negative mesophilic, facultative intracellular parasite capable of penetrating, surviving and multiplying within diverse eukaryotic cell types such as epithelial and phagocytic cells (Finlay and Falkow, 1989a). They are natural inhabitants of the intestinal tract of domestic animals including cattle, swine, sheep, chicken, turkey and ducks. Once they become a part of the food chain these animals were found to be a vehicle of *Salmonella* transmission to humans. Pets such as dogs, cats and turtles have also been implicated in human salmonellosis (Gouthrie, 1992).

Human salmonellosis, which is an intestinal infection caused by *Salmonella,* is considered a world wide serious problem (Ootserom, 1991). The primary symptoms of this disease are nausea, diarrhoea, abdominal pain and fever. These symptoms usually occur after 12-18 hours upon ingesting the organism and persist for 2-3 days, several weeks or even months (Foster, 1978). Since 1943 salmonellosis has been considered to be the most significant and notifiable foodborne illness in the United States and other countries of the world (Tauxe, 1991).

The history of *Salmonella* outbreaks goes back to more than a hundred years, to 1888 when 50 people became ill and one person died because of consumption of infected raw ground beef. A causative factor of this outbreak was later identified as *Salmonella typhimurium* (Tauxe, 1991).

Salmonella outbreaks are divided into three time periods (Tauxe, 1991). First period dated between the end of nineteenth century and 1949 was dominated by outbreaks of *S. typhi*. This *Salmonella* species were known to cause typhoid fever in humans, while other *Salmonella* serovars were endemic among animals and poultry species. For instance, *S. choleraesuis* was predominant among swine while *S.pullorum* and *S.gallinarum* among poultry. In the second period which extended from 1950 to 1969 outbreaks of *S.typhi* were reduced and cases of typhoid fever were nearly eliminated in most of the developed countries. However, during this time non-typhoid *Salmonella* species started to rise steadily shifting the nature of human salmonellosis from typhoid fever to gastroenteritis.

The number of outbreaks of gastroenteritis caused by non-typhoid *Salmonella* continues to increase in the most recent, third period (1970-present). Foods of animal origin, particularly poultry and beef are the major causes of most of human salmonellosis.

2.2 Epidemiology of salmonellosis

Gouthrie (1992) in his new book "*Salmonella*" stated that *Salmonella* infection is the single most common foodborne disease in the U.S and Canada, with an increase in reported non-typhoid cases from zero in 1945 to more than 70,000 in 1985.

Bryan (1981), showed that in the U.S, between 1973-1978, *Salmonella* alone accounted for 23% of the reported outbreaks of foodborne diseases and 40% of the reported cases. The most incriminated foods included beef, turkey, ice-cream, eggs, pork

and chicken. Canadian statistics for 1973-1975 are almost the same. *Salmonella* accounted for 25% of the reported outbreaks and 39% of reported cases; turkey meat being mostly incriminated.

More recent epidemiological data show that *Salmonella* continues to be the most common foodborne pathogen. In the United States alone in the period of 1973-1987, *Salmonella* accounted for 42% of reported foodborne outbreaks (total of 7,458) and 51% of reported cases (total of 273,545) (Bean and Griffin, 1990). Estimated costs of salmonellosis outbreaks was $3.9 to $4.2 billion annually (Bean and Griffin, 1990).

In Canada, in the period between 1978 and 1982, Todd (1989) estimated the number of foodborne disease cases caused annually by *Salmonella* as a total of 627,200 with an economic loss of $ 846 million.

2.3 Salmonella typhimurium

Salmonella typhimurium has been recognized as the most common *Salmonella* serovar implicated in food poisoning cases for the last few decades. One of the biggest waterborne outbreaks of salmonellosis caused by *S.typhimurium* was in 1965 in California. About 16,000 people were infected after drinking contaminated water (Gouthrie, 1992). In 1985, twenty years later, another significant outbreak of *S.typhimurium* occurred in Chicago area, during which, over 16,000 people were infected after consuming milk contaminated with these bacteria. Of those, over 2,700 persons were hospitalized and 14 died (Bean and Griffin, 1990).

In 1969-1977 *S. typhimurium* was the most common serovar among foodborne

salmonellosis in the U.S. The number of cases have increased every year and almost doubling from 5514 in 1969 to 9380 cases in 1977 (Bryan, 1981).

This trend continued over the years and during the 1984-1986 period, *S.typhimurium* was the most common human isolate in the U.S yielding 40-60% of the total *Salmonella* outbreaks (Hargreat-Bean *et al.*, 1989). Between 1985 and 1991, *S.typhimurium* was a main pathogen accounted for 21% of total (8665 isolates) *Salmonella* isolates reported to the Centres for Disease Control (CDC) (Mishu *et al.*, 1994).

The endemic status of *S. typhimurium* was also similar in Canada. This pathogen accounted for the highest number of Salmonella isolates implicated in foodborne disease in 1992 (1596 isolates) as well as in 1993 (1341 isolates) (Khakhria *et al.*, 1994).

The presence of *Salmonella* in food is of growing concern in many countries. Traditional cultural methods required between 4 to 7 days. The methods for presumptive detection of this pathogen are labour intensive and expensive (Brooks *et al.*, 1992). In addition, sublethally injured bacteria might be missed, consequently giving a false-negative results. In contrast, enzyme-linked immunosorbent assays (ELISAs) are good alternative methods for the detection of these pathogens. They are inexpensive, and require less labour and time to perform. However, the accuracy of the ELISAs depends on the type of a molecular marker utilized as an antigen. Enzyme immunoassays utilizing flagellin, fimbrial, and lipopolysaccharide cell markers have been developed. Nevertheless, these markers have certain drawbacks. For example, some of these markers such as flagellin are found only in motile bacteria. In addition, it has been

documented that genes which encode for flagellin have a high degree of homology within the family *Enterobacteriaceae*, which leads to a great similarity in the structure of the encoded flagellin and consequently high cross reactivity among *Salmonella* and non *Salmonella* species (Kawagishi *et al.*, 1992). In contrast, LPS O-antigen of *Salmonella* has been subjected to tremendous diversification during evolution of pathogens (Nikaido and Nakae, 1979, Nghiem *et al.*, 1992). As a result, each *Salmonella* group is characterized by a set of factors present on the cell surface. The sugar residues, their sequence in the O-chain repeating units and the nature of glycosidic linkages are distinctive for each strain which accounts for its serological specificities (Nghiem *et al.*, 1992). This property, however, renders the LPS O-antigen marker too specific (Tsang *et al.*, 1991, Yamaura *et al.*, 1992). Above problems necessitates the need to search for a *Salmonella* cell marker which would be unique in the *Salmonella* genus, yet consistently expressed under various growth conditions. This aspect is important for a good marker, since some of the markers of *Salmonella* are either lost during the process of preparation prior to testing or being altered due to diverse physical and chemical composition of food and consequently rendered non-immunogenic. In turn, this could result in a higher number of false negatives, which would be a threat to consumer as well as it could lead to an economic loss due to the product recall.

In order to identify these markers, a thorough study of the structure of the *Salmonella* cell wall is mandatory. Based on such a study, a potential marker or markers present in the genus *Salmonella* could be identified and utilized in an immunoassay to improve the process of detection and identification of *Salmonella* in foods.

2.4 Structure of Salmonella cell wall

The cell envelope of *Salmonella* as well as other gram-negative bacteria consists of three morphologically distinct layers, the cytoplasmic membrane, peptidoglycan or murein layer and outer membrane (Figure 2.1) (Osborne *et al.*, 1972, Smyth, 1985, Benz, 1988). The outer membrane is not always an outmost layer of the cell envelope. It might be covered by an amorphous capsule layer in some bacteria as *S. typhimurium*. Furthermore, appendages like flagella, fimbria and pili might be attached to the cell envelope (Lugtenberg and van Alphen, 1983). Each of these constituents will be reviewed separately.

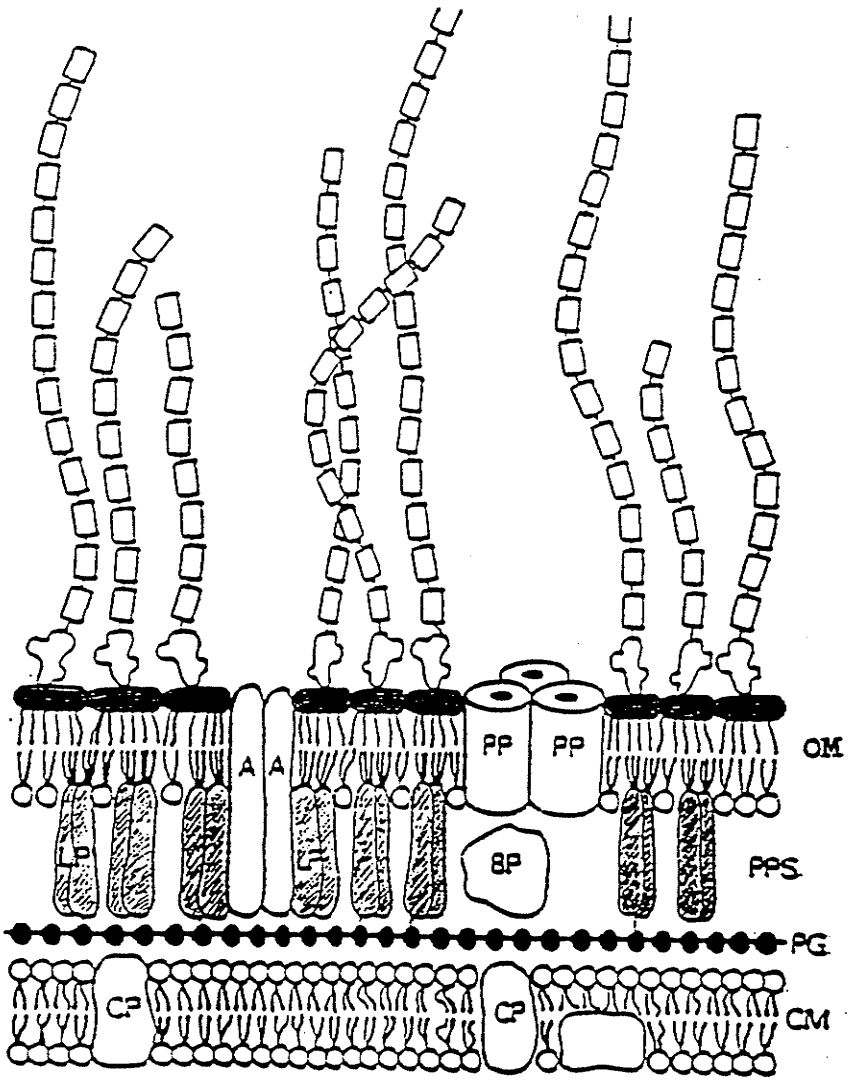
2.4.1 Cytoplasmic membrane

The cytoplasmic membrane shows the typical feature of a unit membrane having a thickness of 75 Å. It consists mainly of phospholipids and proteins in about equal amounts (Inouye, 1980, Lugtenberg and van Alphen, 1983). This membrane displays many important functions such as energy metabolism, active transport of nutrients, as well as synthesis of lipids, peptidoglycan and lipopolysaccharides (Inouye, 1980). In addition, it is involved in the cell division and serves as an anchor for DNA during replication (Lugtenberg and van Alphen, 1983).

2.4.2 Peptidoglycan layer

The peptidoglycan layer gives the bacteria its characteristic form and protects it from osmotic lysis (Benz, 1988). It has a 3 nm thickness (Beveridge, 1981) and it

Figure 2.1 Molecular structure of the gram-negative bacterial outer membrane. The membrane composed of three layers: outer membrane (OM); peptidoglycan (PG); cytoplasmic membrane (CM); other aspects of the cell envelope like the lipoprotein (LP), pore proteins (PP), OmpA protein (A), periplasmic space (PPS), binding protein (BP) and carrier protein (CP) have also been drown. (Adapted from Lugtenberg and van Alphen, 1983).


A
A
PP
PP
OM
LP
BP
PPS
PG
CP
CP
CM

represents 2-10% of the cell wall dry weight. The layer consists of N-acetylmuramic acid, N-acetylglucosamine, L-alanine, D-glutamic acid, m-diamino palmitic acid and D-alanine in the molar ratio of 1:1:1:1:1:1, respectively (Drews *et al.*, 1978). The N-acetylglucosamine and N-acetylmuramic acid are linked together by β1-4 glycosidic bonds (Drews *et al.*, 1978). These constituents, present in form of long strands, are covalently linked to each other and form the peptidoglycan net-work (Benz, 1988, Lugtenberg and van Alphen, 1983). Two matrix compounds, 36.5 kDa protein and the 7.0 kDa lipoprotein are strongly bound to the peptidoglycan layer of *Salmonella* (Beveridge, 1981). Both compounds extend to the outer membrane and maintain the outer membrane-peptidoglycan complex by providing a chemical link between the layers (Beveridge, 1981).

2.4.3 Outer membrane

The outer membrane (OM) accounts for 9-12% of cellular protein (Smyth, 1985). It contains approximately 20-25% phospholipids, 30% lipopolysaccharide and 40-45% protein (Beveridge, 1981). The outer membrane is covalently attached to the peptidoglycan layer *via* lipoprotein (Lugtenberg and van Alphen, 1983) and possesses certain functions. The functions of the outer membrane are diversified and have been reviewed by Dirienzo *et al.* (1978) and Inouye (1980). Briefly, the OM acts as a diffusion barrier against various compounds, contains receptors for bacteriophages and colicins, involves in the process of the conjugation and cell division, contains various specific uptake systems for nutrients such as iron, vitamins and carbohydrates,

participates in maintaining the integrity of the cell, and contains non-specific passive diffusion pores that allow for diffusion of low molecular weight substances.

The outer membrane harbours a number of components which are reviewed below.

2.4.3.1 Lipids

The lipid components of the outer membrane is found in the form of phospholipids composing around 20-25% of the outer membrane. The major lipid component of enteric bacteria is zwitterionic phosphatidylethanolamine which represents about 90% of the outer membrane phospholipids (Lugtenberg and van Alphen, 1983, Benz, 1988). In addition, the outer membrane contains small amount of negatively charged phospholipids called phosphatidylglycerol and cardiolipin (Dirienzo *et al.*, 1978, Benz, 1988). These phospholipids are exclusively located in the inner monolayer of the outer membrane.

2.4.3.2 Lipopolysaccharide (LPS)

Lipopolysaccarides (LPS) are amphipathic macromolecules present as common components in the cell wall of gram-negative bacteria constituting about 40% of the outer membrane surface area. It has been reported that one *S.typhimurium* cell may contain approximately 2.5 X 10^6 LPS molecules (Dirienzo *et al.*, 1978). It is believed that the main function of LPS is related to the maintenance of the structural integrity and selective permeability of the outer layer of cells (McCartney and Wardlaw, 1985).

Figure 2.2 shows a schematic structure of LPS as exemplified by the *S.typhimurium* LPS. All LPS molecules regardless of their origin, share a general architecture consisting of three structural regions: a lipid A region, a core oligosaccharide part and, in the case of members of the family *Enterobacteriaceae* a serotype-specific O-polysaccharide chain which formes O-chain or O-antigen (Dirienzo *et al.*, 1978, Nikaido and Nakae, 1979, McCartney and Wardlaw, 1985, Benz, 1988).

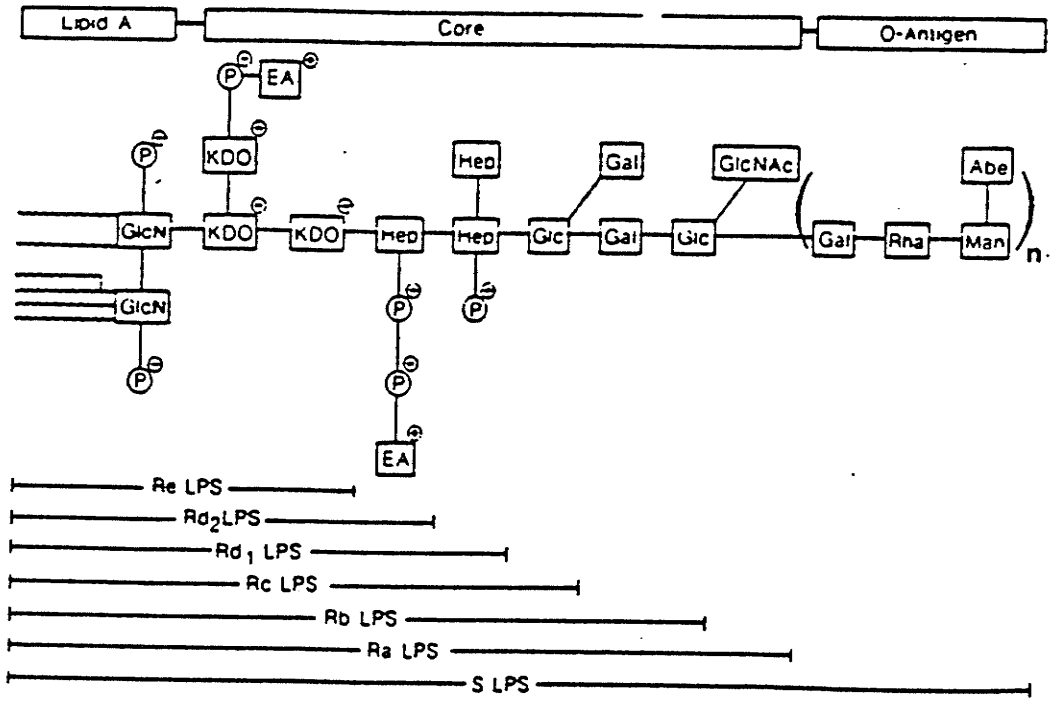
2.4.3.2.1. Lipid A

Lipid A, which anchors the molecule in the bacterial outer membrane is the hydrophobic part of the LPS molecule (Lugtenberg and van Alphen, 1983, Benz, 1988). It consists of D-glucosaminyl-β-D-glucosamine backbone on which phosphorus and long chain fatty acids are bound. These fatty acids form amide or ester bonds to the amino sugars (Drews *et al.*, 1978, Nikaido and Nakae, 1979, Benz, 1988). All fatty acids in lipid A are saturated, thus the absence of unsaturated fatty acids probably contributes to the low fluidity exhibited by this hydrophobic portion (Nikaido and Nakae, 1979).

2.4.3.2.2 Core region

An intermediate core region called also the R-core, is the hydrophillic part of the molecule (Lugtenberg and van Alphen, 1983). The characteristic constituents of the R-core oligosaccharide are 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) and L-glycero-D-mannoheptose (Drews *et al.*, 1978, McCartnay and Wardlaw, 1985). In addition, it contains a number of more common sugars like glucose, galactose and N-acetyl-D-

Figure 2.2 General structure of *S.typhimurium* lipopolysaccharide.
Gal, D-galactose; Rha, Rhamnose; Man, Mannose; Abe, Abequose; Glc, D-glucose; GlcNAc, N-acetyl-D-glucosamine; Hep, L-glycero-D-mannoheptose; P, phosphate; EA, ethanolamine; KDO, 2-keto-3-deoxy-D-manno-octulosonic acid; GlcN, D-glucosamine; ,hydroxy and non-hydroxy fatty acids; Ra to Re, incomplete R-form lipopolysaccharides; S LPS, wild smooth type LPS. (Adapted from Lugtenberg and van Alphen, 1983).


Lipid A
Core
O-Antigen
EA
KDO
KDO
KDO
Hep
Hep
Hep
Hep
Gal
Gal
GlcNAc
Glc
Glc
Abe
Gal
Rha
Man
n
GlcN
GlcN
EA
Re LPS
Rd2LPS
Rd1 LPS
Rc LPS
Rb LPS
Ra LPS
S LPS

glucosamine (Lugtenberg and van Alphen, 1983). Although seven types of LPS core structure, namely R_a, R_1 to R_4, K_{12} and B, have been found in members of the family *Enterobacteriaceae*, in general, chemical composition and structure of the core moiety are highly conserved (Jansson *et al.*, 1981)

2.4.3.2.3 O-antigen

A distal part of LPS is a specific O-antigen which is composed of repeating units (up to 70) of polysaccharides (Peterson and McRoarty, 1985). The nature of O-antigen is unique for a given LPS. The repeating units contains 3 to 6 sugar residues (McCartnay and Wardlaw, 1985) and their number may vary even among the same organism. The O-antigen chains are distinguished by a high diversity of the strain specific sugar compositions which occur due to rapid changes of the cell surface during evolution to allow bacteria to escape a host defence system by continually developing a new cell surface (Nikaido and Nakae, 1979, Ngiem *et al.*, 1992). This property is extremely useful in the O-serotyping and in the production of serogroup-specific antibodies (Drews *et al.*, 1978).

Lipopolysaccharides form an effective barrier against hydrophobic molecules which otherwise may diffuse through the outer membrane. This barrier is partly composed of the long oligosaccharide side chains attached to lipid A, and to the ionic bridges between charged groups in the polysaccharide moiety. It also shows a considerable affinity to the pore forming complex and some other proteins (Benz, 1988). Furthermore, the O-antigen contains carbohydrates which increase the hydrophobicity of

the cell surface enabling the cell to escape phagocytosis (Nikaido and Nakae, 1979).

2.4.3.3 Proteins

Proteins in the outer membrane layer are named after their structural genes (Lugtenberg and van Alphen, 1983, Smyth, 1985). These proteins are classified into major and minor proteins with the aid of polyacrylamide gel electrophoresis. The major proteins constitute about 80% of the total proteins in the OM layer, while the minor proteins constitute from 10 to 20% (Smyth, 1985).

A protein is considered major in terms of the number of molecules per cell and when the amount of major outer membrane protein per unit of outer membrane surface is constant under various growth conditions (Lutgtenberg and van Alphen, 1983). An apparent molecular weight of proteins has no role in this classification, where there is not any molecular weight distinctive to either group (Smyth, 1985).

2.4.3.3.1 Heat modifiable proteins

Among a number of major proteins studied in *E.coli* and *S.typhimurium*, heat modifiable proteins are the most mysterious due to their abnormal electrophoretic behaviour. One of these proteins has been given different names, 3a (Shnaitman, 1973); O-10, (Nakamura and Mizushima, 1976) ; tolG, (Dirienzo *et al.*, 1978); d, (Lugtenberg, 1983). There was, however, a consensus to name it as in case of other major proteins, after names of their structural genes. Thus, this protein which is present in about 10^5 molecules per cell was designated ompA (Lugtenberg and van Alphen, 1983). The OmpA

protein exhibits heat modifiability showing a higher apparent molecular weight for a heat treated form (35 kDa) than for a native form (28 kDa) as determined by SDS-PAGE (Shnaitman, 1973, Nakamoura and Mizushima, 1976, Lugtenberg and van Alphen, 1983). The high electrophoretic mobility of the native protein is ascribed to the high content of β-structure, excessive binding of SDS and to changes in viscosity. The viscosity of the native protein is low and increases upon heating above 70°C, which slows down proteins migration (Shnaitman, 1973). This protein together with lipoproteins is involved in maintaining both, the structural integrity of the outer membrane as well as the rod shape of the bacteria (Lugtenberg and van Alphen, 1983).

2.4.3.3.2 Peptidoglycan associated proteins

In the outer membrane, porins are bound tightly to the peptidoglycan layer by ionic linkages (Beveridge, 1981). They have molecular weight in the range of 30-50 kDa, and most of them exist as trimers of identical subunits. Three porins were identified and characterized in *S. typhimurium*; ompD (34 kDa), ompF (35 kDa), and ompC (36 kDa), and each was found in 10^5 molecules per cell (Nikaido and Nakae, 1979, Lugtenberg and van Alphen, 1983, Benz, 1988). These proteins share a common antigenic structure which suggests that they are derived from a common ancestral gene and the structure of the gene has been conserved very well during evolution (Lugtenberg and van Alphen, 1983). Porins play a very important role in the functionality of gram-negative bacteria. Mainly they are responsible for forming aqueous trans-membrane channels for the passive diffusion of waste and nutrients in the range of 500-600 daltons (Beveridge, 1981, and

Benz,1988). The expression of some of the porins is influenced by physiological conditions like osmolarity of culture media, and diverse growth conditions. For instance, some porins were induced when bacteria are grown on medium depleted of phosphate such as PhoE, and LamB of *E.coli*, and *S. typhimurium*, respectively (Benz, 1988).

2.4.3.3.3 Minor outer membrane proteins

Approximately 20 minor proteins which are not antigenically related to peptidoglycan-associated pore proteins have been identified in the outer membrane of gram-negative bacteria (Lugtenberg and van Alphen, 1983). Most of these proteins have certain functions. Some of these proteins act as a receptor of bacteriophage T6 and colicin K. Phage lambda receptor, a 55 kDa outer membrane protein, plays an important roll in the uptake of maltose and maltodextrins. Another protein, *butB* having an apparent M_r of 60 kDa is believed to be a glycoprotein involved in vitamin B_{12} uptake. Furthermore, there is a group of minor proteins (Cir, FhuA, Fec and 83 K) that are involved in the uptake and regulation of iron (Dirienzo *et al.*, 1978, Beveridge, 1981, Lugtenberg and van Alphen, 1983).

2.4.3.3.4 Murein lipoprotein

In 1969, Broun and Rehn reported the existence of a lipoprotein of M_r 7 kDa linked covalently to the peptidoglycan layer (Dirienzo *et al.*, 1978). This protein has been extensively characterized in *E.coli* and *S.typhimurium*. Amino acid sequence revealed that it contains 58 amino acids and it was found in approximately 7×10^5 molecules per cell.

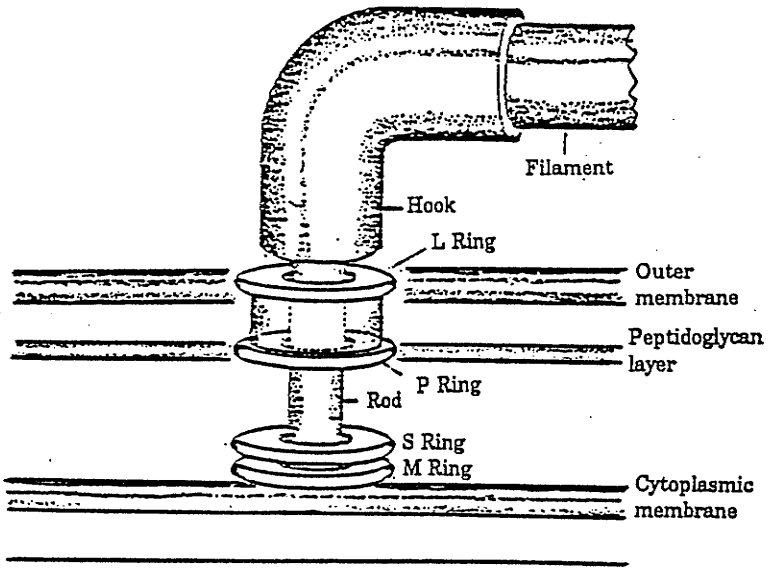
This high number of molecules makes it the most abundant protein in the cell (Nikaido and Nakae, 1979, Lugtenberg and van Alphen, 1983). This protein is mainly composed of α-helix, and it is extremely well conserved in *Enterobacteriaceae*. The function of this protein is not elucidated but physiological and morphological defects were observed in mutants lacking this protein (Dirienzo *et al.*, 1978).

2.4.3.4 Flagella, fimbriae and pili

Flagella, fimbria and pili are appendages consisting of protein subunits. The bacterial flagellum is composed of three structurally defined parts; the filament, the hook and the basal body (Figure 2.3) (Depamphilis and Adler, 1971). Two general structures, sheathed and unsheathed have been identified (Joys, 1968). Flagella are responsible for the cell motility and they are connected with all three layers of the cell envelope (Lugtenberg and van Alphen, 1983). Fimbria are filamentous appendages or whisker like organelles expressed on the surface of the bacteria, particularly those in the family *Enterobacteriaceae* (Thorns *et al.*, 1994). They are smaller, more rigid and numerous than flagella.

Pili or type I fimbriae were initially thought to be composed of identical repeating subunits of M_r about 17-21 kDa. Now it is recognized that they contain a minor protein on their surface. This protein, contains a D-mannose binding site which is believed to play a role in the attachment to eukaryotic cells. The attachment enables cells to colonize the intestinal or the urinary tract and invade the host (Duguid, 1959, Finlay and Falkow, 1989a). There is another type of appendage called N-methylphenylalanine pili, which

Figure 2.3 Diagrammatic model of the basal end of the flagellum of *E.coli*. (Adapted from Depamphilis and Adler (1971).


Filament
Hook
L Ring
Outer membrane
Peptidoglycan layer
P Ring
Rod
S Ring
M Ring
Cytoplasmic membrane

is also considered a virulence determinant (Finlay and Falkow, 1989b).

2.4.3.5 Capsular and slime layers

Capsular and slime layers are the outer-most components present on the cell periphery expressed in some of gram-negative bacteria. These compounds possess negatively charged polysaccharides (Lugtenberg and van Alphen, 1983). It has been demonstrated that some members of *Enterobacteriaceae* also produce extracellular carbohydrate slime layers consisting mainly of colanic acid. Slime layers are a loose network of unordered gel which extend from the cell surface. The capsules are more compact than the slime layers. Both layers are stabilized by combined electrostatic and hydrophobic-hydrophillic forces (Costerton *et al.*, 1974, Beveridge, 1981).

2.5 Antigenicity of Salmonella cell envelope

Cell envelope of *Salmonella* as well as other gram-negative bacteria harbours many compounds which due to their chemical complexity and high molecular weight are immunogenic. Compounds such as flagellin (H antigen), capsular (K-antigen), and lipopolysaccharide (O-antigen) are specific antigenic markers which are used for the serological classification of *Salmonella* (Kauffman-White, 1972). Furthermore, there are other compounds such as outer membrane proteins including porins, and fimbrial proteins which exhibit antigenic properties and were used as target markers in the detection of *Salmonella* by immunochemical tests.

Not all bacterial antigens exert an equal quality as cell markers. Some of them

like Broun lipoprotein or lipid A are buried inside the cell membrane and are not accessible for detecting antibodies. Others such as flagellin and capsular antigens need to be heat-treated to be accessible by antibodies. Two groups of cell markers, LPS and OMP are relatively easy accessible to adequate antibodies. They are present in all gram-negative bacteria and possess a degree of heterogeneity which makes them good candidates for the strain specific (LPS-O-antigen) or interspecies (OMP) identification.

2.5.1 Antigenicity of Salmonella LPS

Endotoxin was discovered by Pfeiffer in 1892 who isolated it from the late log phase culture of *Vibrio cholera*. The endotoxin was later found to be a causative factor of septic shock and death in experimental animals. Since that time endotoxin has been the most widely studied bacterial component. Recently, endotoxin was named lipopolysaccharide (LPS), although the name endotoxin still is in use (Luk *et al.*, 1991).

Salmonella LPS is both a virulence factor and a protective antigen (Watson *et al.*, 1992). Its virulence is due, in a part, to its ability to resist the complement activation of the alternative pathway in a host (Watson *et al.*, 1992), while protective properties are due to the physiological role it plays in the maintaining of the outer membrane integrity by forming an impermeable barrier against entrance of antibiotics, bile salts and detergents into the cell (Hancock, 1987, Rozalski *et al.*, 1989a). It is also required for the assembly of porins (Nghiem *et al.*, 1992).

Bacterial LPSs are structurally heterogenous molecules because of different degrees of polymerization of the repeating units. In addition, their heterogeneity is

affected by glycosylation and/or acetylation (Galanos *et al.*, 1977), non-stoichiometric modification of lipid A and core polysaccharides, and variations in the number of repeating units of the O-antigen (Peterson and McGroarty, 1985). All three principal domains which form the LPS molecule (Lipid A, Core LPS, and O-antigen chain) possess distinct antigenicity and physiopathological properties (Luk *et al.*, 1991).

2.5.1.1 Antigenicity of Lipid A

Lipid A is the inner part of the LPS molecule, and it is responsible for the insertion of LPS into the bacterial membrane by virtue of its hydrophobic properties (Luk *et al.*, 1990). The structure of lipid A is highly conserved within members of family *Enterobacteriaceae* (Elkins *et al.*, 1985). Its function includes many physiopathological activities associated with its endotoxin properties such as lethal toxicity, pyrogenicity, and complement activation. The latter leads to fever, hypotension, neutropenia and intravascular coagulation (Galanose *et al.*, 1977, Elkins *et al.*, 1985, Luk *et al.*, 1991).

It was shown that acid treatment of bacteria would expose lipid A on the wall surface. The acid treated bacteria was successfully used to produce anti-lipid A antibodies. It has been also reported that the immunodominant part of lipid A involves the linkage between amide fatty acids and the glucosamine backbone (Galanos *et al.*, 1977, Elkins *et al.*, 1985). Since the structure of lipid A is conserved among *Enterobacteriaceae* family, produced anti-lipid A antibodies exhibited cross-reactivity within this family and in turn their importance in the detection of *Salmonella* was limited (Kuhn *et al.*, 1992, Wood *et al.*, 1992).

Few studies pertaining to monoclonal antibodies against lipid A have been performed. Most of these studies were conducted to evaluate their usefulness for neutralization of a septic shock in animals caused by endotoxicity of lipid A (Elkins *et al.*, 1985, Parent *et al.*, 1992, Kuhn *et al.*, 1992, Warrn *et al.*, 1993).

2.5.1.2 Antigenicity of LPS Core

The chemical structure of the oligosaccharide core has been elucidated recently and found to be conserved among *Salmonella* and other related *Enterobacteriaceae* such as *E. coli* and *Shigella* (Luk *et al.*, 1991, Tsang *et al.*, 1991b, Gibb *et al.*, 1992).

The core structure can be subdivided into an inner part which contains L-glycero-D- mannoheptose (Hep),2-keto- 3-deoxy-D-manno-octulosonic acid (KDO), and the outer hexose (Gal, Glc, Glc, NAc) region. The latter region is the most variable part of the oligosaccharide chain (Gibb *et al.*, 1991, Luk *et al.*, 1991).

Antigenic determinants present on the glycolipid core in smooth bacteria are often not recognized by the immune system in animal hosts because of their subjacent location in the LPS molecule (Luk *et al.*, 1991). Once the O-antigen part is removed from LPS as in rough mutants, the core would gain immunogenicity. Due to such a deadlock, only few antibodies were produced against the LPS core, more specifically against the inner core region (KDO), using rough mutants (Rozalski *et al.*, 1989b, Lind *et al.*, 1991). These antibodies are of no significance for detection of *Salmonella* for a number of reasons. They could bind to most of bacteria within *Enterobacteriaceae* family because of the similar antigenicity of the LPS core. Furthermore, binding of antibodies is limited

due to epitope inaccessibility in intact bacteria. In contrast, these antibodies could have a certain application in clinical medicine, since anti-KDO antibodies could protect against gram-negative septicemia by inhibiting the effect of lipid A endotoxic activity (Rolzalski *et al.*, 1989b, Lind *et al.*, 1991).

2.5.1.3 Antigenicity of O-side Chain of LPS Molecule

The O-antigen is the outmost region of the LPS molecule. It is composed of a polymer of oligosaccharide molecules containing up to 70 repeating units with 3 to 6 sugar residues. Each unit can serve as an antibody recognition site (Lugtenberg and van Alphen, 1983, Peterson and McRoarty, 1985). The structure of the repeating units is unique for a given LPS. This gives the organisms a distinctive immunoreactive property (Rozalski *et al.*, 1989a).

The O-antigen is the most variable portion of bacterial LPS and it is essential for the serospecificity of gram-negative bacteria (Elkins *et al.*, 1985). The variability of the O-antigen is largely due to a phenomenon called an antigenic drift or due to slow accumulation of mutations in the structural genes which encodes surface components of the bacterial cell (Seifert and So, 1988).

The side chains of O-antigen, particularly dideoxyhexosyl residues such as abequose in the factor O-4 of B-serogroup and tyvelose in the factor O-9 of D-serogroup are considered important virulence factors. They protect bacteria in host animals by resisting their defence mechanisms (Luk *et al.*, 1990, 1991). Also, it has been reported that a slight alteration in the distribution of O-antigen could drastically alter the resistance

of bacterium to the host immunodefence system (Goldman and Hunt, 1990).

The high diversity of O-antigen side chains has been exploited for the production of highly specific monoclonal antibodies used for the serological identification of etiologic agents as well as mapping and recognition of potentially important antigenic epitopes (Luk *et al.*, 1988, 1990).

The *Salmonella* serogroup B has O-antigenic factors that distinguish it from other serogroups. This feature was utilized for the production of B-serogroup specific monoclonal antibodies. The chemical structure of *Salmonella* serogroup B O-antigen factors along with the production of monoclonal antibodies against these factors and some of their applications will be reviewed.

The O-antigen of B-serogroup *Salmonella* including *S. typhimurium* is composed of the tetrasaccharide repeating unit consisting of galactose, rhamnose, mannose and abequose (Goldman and Hunt, 1990). The position of certain glycosyl and acetyl substituent groups and side monosaccharides, contributes to the different O-antigenic factors of *Salmonella* B serogroup (Helander *et al.*, 1992). These factors are divided into major and minor components.

2.5.1.3.1 Major Factors of O-Antigen

There are two major factors in the O-antigen of B-serogroup *Salmonella*. Factor O-4 is present in all serovars belonging to B-serogroup. It is associated with the abequose residue which is linked to D-mannose through an α 1,3 glycosidic linkage. While factor O-5 is present only in certain serovars known as O-5 antigen carriers (Yamaura *et al.*,

1992). It is associated with the presence of the O-acetyl group linked to the abequose residue through an α 1,2 linkage. This O-acetyl group is found to be sensitive to alkaline treatment and can be removed by treatment with sodium methoxide in methanol (Hellerqvist *et al.*, 1969).

2.5.1.3.2 Minor Factors of O-Antigen

In addition to the major factors there are two minor; factor O-12 and O-1. These factors are associated with the position of D-galactose substituent. Factor O-1 contains the 2-D-glucopyronose residue at the position 6 as a substitute of D-galactose, while factor O-12 contains this residue at the position 4. These two factors are considered to be minor in terms of their immunogenicity (Hellerqvist *et al.*, 1969)

Lind and Lindberg (1992) produced four monoclonal antibodies which were reactive with factor O-4 of Salmonella B-group. These MAbs were not tested for detection of Salmonella B-group in foods or clinical samples but studies on the epitope size, specificity and their equilibrium constants were performed.

Moreover, Tsang *et al.* (1991a) produced six monoclonal antibodies against the same factor of *Salmonella* B-serogroup. Four out of six MAbs were found to be specific to *Salmonella* B serogroup, while two antibodies exhibited cross-reactivity with *S. paratyphi* of serogroup A. Using the slide agglutination method it was demonstrated that some of these antibodies could be useful for *Salmonella* serotyping.

Monoclonal antibodies have been also produced against factor O-5 of the B-serogroup. Yamaura and coworkers (1992) developed an antibody that was specific to

factor O-5. This antibody could be used to discriminate *S. typhimurium* into two classes: the O-5 positive and O-5 negative type (variety copenhagen).

Kosimar and Cebra (1983) produced a panel of MAbs against *S. typhimurium* O-antigen factors O-5, O-4, and O-1. These MAbs were found to react with cell surface-exposed components as determined by agglutination assays.

Metcalf *et al.* (1983), produced three MAbs against O-antigen factor O-1 of *S. typhimurium*. These antibodies showed extensive cross-reactivity with other serogroups containing the same factor. This cross-reactivity limited the usefulness of the antibodies for detection and identification of *S. typhimurium* in foods.

2.5.2 Antigenicity of the Outer Membrane Proteins

Studies of outer membrane proteins (OMP) from several gram-negative pathogens led to the identification of proteins that have been useful as epidemiologic and virulence markers. Analysis of OMPs has involved electrophoresis, immunoblotting and immunoelectron microscopy characterization as well as studies on the effect of growth media on their stability.

2.5.2.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Immunoblotting

Analysis of bacterial OMP extracts by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been exploited by many researchers in attempts to use this method for bacterial serotyping. However, a high degree of similarities between

electrophoretic band patterns of tested bacterial proteins makes electrophoresis difficult to apply for this purpose.

Newton *et al.* (1990) analysed OMP extracts from 33 *Edwardsiella ictaluri* isolates by SDS-PAGE. Twenty eight tested isolates showed similar electrophoretic profiles. Ten protein bands with M_r ranging from 19.5 to 71 kDa were identified among these isolates, with a major polypeptide band corresponding to 55 kDa. Only slight differences were observed in the minor OMP bands. However, some success was achieved by Hansman and Lawrence (1993) who analysed OMP extracts from 65 strains of *Haemophilus influenzae*. Electrophoretic analysis revealed seven distinguishable patterns among tested strains.

SDS-PAGE followed by immunoblotting has been used extensively in order to identify antigenic determinants in bacterial OMPs. Singupta *et al.* (1989) used immunoblot analysis to identify antigenic determinants of OMP extracts from *Vibrio cholera* serovars. Immunoblotting of OMP extracts with antisera raised against outer membrane proteins of *Vibrio cholera* strains revealed two immunogenic bands corresponding to 36 and 25-26 kDa proteins.

Bacterial cells have many antigenic markers exposed at the surface. Identification of these markers is an important asset, since these markers can be utilized for the production of monoclonal antibodies specific for the bacterium of interest. Consequently, such antibodies can be then used for the identification of microorganisms in food or clinical samples. Furthermore, these markers can be utilized as possible vaccine candidates.

Sinha and Chakraborti (1992) identified surface exposed antigenic markers in *Shigella dysenteriae* using SDS-PAGE followed by immunoblotting with antisera obtained from convalescent patients. Immunoblotting of OMP extracts revealed one major antigenic protein (57 kDa) along with some other minor proteins. In a similar study, Anwar (1991) used antisera from a convalescent patient recovering from whooping cough in order to identify surface exposed antibody-accessible outer membrane proteins of *Bordetella pertusis*. The antisera recognized a 69 kDa OMP and a 40 kDa porin.

Monoclonal antibodies produced against whole cells have also been used to identify surface exposed antigenic determinants. Spinola *et al.* (1993) carried out SDS-PAGE on OMP extracts from 35 strains of *Haemophilus ducreyi* followed by immunoblotting with monoclonal antibodies raised against whole cells. It was reported that only a 18 kDa protein was detected by antibodies used. Since whole bacterial cells were used to raise these antibodies, it is most likely that the 18 kDa protein was exposed on the surface of the bacterial cell.

Pai and coworkers (1992) produced monoclonal antibodies against *S. typhimurium* porin OmpD (34 kDa) in order to study the antigenic cross-reactivity of cell surface markers by means of immunoblotting and enzyme-linked immunosorbent assay. They demonstrated that OmpD shares more epitopes and had greater structural similarity with OmpC (36 kDa) than with OmpF porin (35 kDa).

2.5.2.2 Immunoelectron Microscopy

Identification of exposed antigens on bacterial cell surfaces is an important step

toward the production of MAbs which can be later used for its identification or the development of a vaccine which protect against it. Immunoblotting utilizing MAbs raised against whole cells is used widely for such a purpose. However, some surface exposed antigens might be denatured through the process of electrophoresis and blotting, and consequently they may lose their ability to be recognized by MAbs. Therefore, immunoelectron microscopy with its minimal requirements for sample treatment is an effective tool for ascertaining the exposure of an antigen expressed on the surface of intact cell. Furthermore, immunoelectron microscopy has been useful in elucidating the avidity of MAbs and the frequency of the epitopes expressed on the surface of the cell.

Tagawa *et al.* (1993) identified a single protein in *Haemophilus somnus* using a monoclonal antibody produced against its OMP extracts. Immunoelectron microscopy was then used to verify the exposure of this protein on the surface of the bacterial cells.

Electron microscopy and immunostaining with colloidal gold was also used to verify surface exposure of a protein marker identified in the outer membrane of *H. ducreyi* (Spinola *et al.*, 1992). Four strains of *H. ducreyi* and *H. influenza* were probed with either MAbs produced against *H. influenza* or with tissue culture supernatant from myeloma cells as a negative control. The number of gold spheres attached to the target cells were counted and compared statistically to the number of spheres attached to the control. A significant difference was observed between the sample and control suggesting that the marker protein was indeed a surface exposed epitope.

Cloeckaert and coworkers (1990) studied the surface exposure of seven *Brucella* OMPs by immunoelectron microscopy. They found that the accessibility of MAbs to their

epitopes was hindered by the presence of long LPS O-side chains.

2.5.2.3 Effect of Growth Media on Expression of OMP and Their Antigenicity

The effect of growth media on expression of outer membrane proteins has been studied using several bacterial species. No significant effect on the omp profiles of 16 serotypes of *Pasturella multocida* was determined by SDS-PAGE (Choi *et al.*, 1989). The same trend was reported in other studies on the expression of OMP from *Bacteroides distasonis* (Wexler *et al.*, 1992).

Sarwar and coworkers (1992) studied the effect of growth phase and growth media on the expression of epitopes of a heat modifiable protein of *Branhamella catarrhalis*. These proteins have M_r of 51 kDa at room temperature and 60 kDa when boiled under reducing conditions. These studies also concluded the that phase of growth had no effect on the expression of epitopes.

However, no investigation has yet been conducted on the identification of antigenic determinants of *Salmonella* outer membrane proteins. Therefore, this thesis reports results of studies undertaken in order to identify common antigenic determinants in *Salmonella* genera, and investigate the effect of growth media on the expression of these antigenic markers. In this report, production and characterization of MAbs to *Salmonella* are also described.

3. MANUSCRIPT I

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST O-ANTIGEN 5 OF *SALMONELLA TYPHIMURIUM* LIPOPOLYSACCHARIDE

3.1 Abstract

Three murine monoclonal antibodies (MAbs) were produced by a fusion of P3X63-Ag8.653 myeloma cells and spleenocytes of a mouse immunized with attenuated *Salmonella typhimurium* cells. MAbs 5A5 and 5B2 were of IgM class while MAb 4A8 was of IgG_{2a}; all with κ light chains. All MAbs were reactive only to *S. typhimurium* and other B serotypes;including *S. heidelberg, stanley* and *paratyphi* B and did not crossreact with other 33 tested bacteria. Further analysis revealed that all three MAbs recognized LPS O-5 antigen unique for *S.typhimurium* as determined by SDS-PAGE followed by immunoblot. Both IgM MAbs exhibited higher avidity to their epitopes than the IgG_{2a}. Furthermore all MAbs recognized the same epitope as determined by additive ELISA. The MAbs were found to be equally reactive to both, live and heat attenuated cells. The ability of the MAbs to detect live cells without further treatment to expose epitopes was confirmed by transmission electron microscopy. Treatment of *S. typhimurium* cells with cholic acid had no significant effect on the antibody binding to LPS. Low pH had no adverse effect on the reactivity of MAbs with the O-5 antigen, while alkaline conditions rendered LPS O-5 not antigenic. The MAbs ability to recognize live cells demonstrate their usefulness in an ELISA capture system for *S. typhimurium* detection and the serological differentiation between both *S. typhimurium* and *S. typhimurium* var. *copenhagen.*

3.2 Introduction

There are two variants of *S. typhimurium*; *S. typhimurium* O-antigen 5 carrier, and *S. typhimurium* var. *copenhagen* none O-5 carrier. The latter variant is believed to be involved in salmonellosis of domestic fowl. The outbreaks caused by this pathogenic strain were reported in broiler chickens and pigeons (Seuna, 1979, Grund and Stople, 1992).

Salmonella typhimurium has been recognized as the most common foodborne *Salmonella* serovar world wide. In 1985-1991, *S. typhimurium* was the most prevalent pathogen implicated in foodborne salmonellosis in the United States accounting for 21.6% of the total isolates (Mishu *et al.*, 1994). Also in Europe, the increase in prevalence of *S. typhimurium* in foodborne diseases continued to be significant (Tsang *et al.*, 1991). In Canada, this serovar accounted for the highest number of *Salmonella* isolates implicated in foodborne disease in 1992 as well as in 1993 (Khakhria *et al.*, 1994).

Salmonella B-serogroup including *S. typhimurium* has been reported to be more virulent than other *Salmonella* serogroups. It has been suggested that the structure of the O-antigen may account for this property. The presence of abequose in the O-polysaccharide chain makes LPS less potent in activating the complement system, and in turn enables the organism to escape the host defense mechanisms (Lind and Lindberg, 1992). Epidemiological investigations on the prevalence of *S. typhimurium* conducted worldwide appears to be consistent with these findings.

Further investigations revealed that beef and turkey meat are the most

incriminated foods as vehicles of infection by *S. typhimurium* (Bryan, 1981).

The detection of *Salmonella* in foods is often difficult. Traditional cultural methods require from 4 to 7 days. In addition, the methods for presumptive detection of this pathogen are labour intensive, involving isolation of the organism using pre-enrichment procedures and serological confirmation (Andrews, 1985, D'Aoust and Sewell, 1988, Brooks *et al.*, 1992, Van der Zee, 1994). In contrast, immunoassays are good alternative methods for the fast detection of these pathogens.

Recently, several rapid detection methods for *Salmonella* including enzyme-linked immunosorbent assays (ELISA) employing polyclonal and monoclonal antibodies have been developed. However, the specificity of ELISA depends on the type and quality of antibodies used in the assay format (Eckner *et al.*, 1992). In contrast to polyclonal antisera, use of monoclonal antibodies makes immunoassays more specific and provides unlimited supply of antibodies.

A few attempts have been made to produce MAbs against *S. typhimurium*. Tsang and coworkers (1991) reported the production of six MAbs to *Salmonella* B-serogroup. Two Mabs cross-reacted with *S. paratyphi* A, while four were found to be reactive with LPS O-4 and were used to serotype heat attenuated *Salmonella* in a slide agglutination assay. Moreover, Lee and coworkers (1990) developed an ELISA for detection of *S. typhimurium* in food. This assay utilized a polyclonal antiserum as a source for capture antibodies and a monoclonal detector antibody. Although a reported MAb (unknown epitope specificity) was more specific than polyclonal antibodies, it exhibited some cross-reaction with other *Salmonella* serotypes. Monoclonal antibody against *S. typhimurium*

which was specific to LPS O-5 has also been developed (Yamaura *et al.*, 1992). The latter antibody was used in epidemiological studies for identification of the source of infection by *S. typhimurium* and other B-serovars. The reactivity of this antibody with heat-killed bacteria was evaluated only by an agglutination assay, thus adaptability of this antibody to an ELISA format remained to be determined.

In this paper we describe the production and characterization of three monoclonal antibodies that are specific to LPS O-5 of B-serogroup *Salmonella*. These antibodies bound with equal avidity to live and heat attenuated *Salmonella* and can be used to develop an ELISA for serological discrimination between both types of *S. typhimurium,* and *S. typhimurium* var. *copenhagen*.

3.3 Materials and Methods

3.3.1 Materials.

Standard plate count agar (SPC), nutrient broth and M-broth were purchased from Difco Laboratories (Detroit, MI, USA). Brain heart infusion and RPMI 1640 media were from Gibco (Grand Island, NY, USA). Lipopolysaccharides from *S. typhimurium, S. enteritidis* and *E. coli*, hypoxanthine, aminopterin, thymidine, cholic acid, ρ-nitrophenyl phosphate were purchased from Sigma Chemical Co. (St Louis, MO, USA). Acrylamide, sodium dodecyl sulphate, nitrocellulose membrane (0.45 μm), alkaline phosphatase-conjugated goat anti-mouse immunoglobulins, gold (20 nm)-conjugated anti-mouse immunoglobulins, mouse-type subisotyping kit, 5-bromo-4-chloro-3-indolyl phosphate ρ-toluidine salt (BCIP) and ρ-nitro-blue tetrazolium chloride (NBT) were

purchased from BioRad (Richmond, CA, USA). Diethanolamine buffer was from Mallinckrodt (Paris, KY, USA). Pristane (2,6,10,14-tetramethylpentadecane) and phenol were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Myeloma cells P3X63.Ag8.653 were obtained from the American Type Culture Collection (Rockville, MD, USA). Polyethylene glycol, M_r 4000 (cat. no. 9727) was from Merck (Germany). Dimethelysulfoxide was purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA) while tissue culture flasks, 24 and 96 well polystyrene tissue culture plates were purchased from Corning Glassware (Corning, NY, USA). Falcon Microtest III polyvinyl chloride flexible 96-well plates were purchased from Canlab (Mississauga, ON, Canada). All of other chemicals and reagents were of analytical grade.

3.3.2 Bacteria and Growth Conditions

Stock bacterial cultures were maintained on SPC slants at 4°C until use. To prepare sufficient amount of *Salmonella*, a loopful of stock culture from SPC slants was inoculated into 5 ml of nutrient broth and incubated overnight at 37°C. Next, about 1 ml of broth was transferred into 250 ml M-broth and incubated with gentle shaking for additional 16 h at 37°C. Then, cells were harvested by centrifugation (9,000 x g, 10 min), washed and resuspended in 50 ml saline. Non-salmonellae species were grown in brain heart infusion broth at 37°C, harvested and resuspended in saline. Cells were enumerated as colony forming units, diluted to demanded concentration with saline, heat treated (20 min, 80°C) and used for ELISA as described below. Live bacteria were prepared by washing SPC slants with saline. All bacterial species used in this study are

shown in Table 1.

3.3.3 Lipopolysaccharide Preparation

Salmonella lipopolysaccharide (LPS) extracts were prepared according to the modified method of Johnson and Perry (1976). Briefly, three grams of freeze dried bacteria were resuspended with 50 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM ethylene diaminetetraacetic acid (EDTA) and 0.05% sodium azide (buffer L) and sonicated five times for 45 sec at 4°C using Braun Sonic 1510 (B.Braun, Melsungen, AG) set at 300 W. The resulting suspension was incubated with pancreas ribonuclease and deoxyribonuclease (0.1 μg/ml) in 20 mM $MgCl_2$ for 10 min at 37°C followed by another 10 min at 60°C. After incubation, the suspension was mixed with an equal volume of 90% preheated phenol to 70°C, incubated for 15 min at 70°C, and centrifuged at 18,000 x g for 1 h yielding aqueous and phenol phases. The aqueous phase was collected and dialysed against distilled water until no detectable phenol odour remained. The LPS extracts were then lyophilized and stored at -20°C until use.

3.3.4 Production of Monoclonal Antibodies

3.3.4.1 Immunization

A group of six female BALB/c mice (6-8 weeks old) were immunized with heat attenuated (80°C, 20 min) *S. typhimurium* cells at one week intervals. Initially, 100 μl of cell suspension (10^8/ml) was injected subcutaneously. The second and following injections were performed intraperitonally. The mice were killed 3 days after the final

injection. Tailbleedings were taken before the first immunization (pre-immune serum), ten days after each subsequent injection, and shortly before the mice were sacrificed. Serially diluted sera were tested for the presence of antibodies against *S. typhimurium* using an indirect non-competitive enzyme-linked immunosorbent assay (ELISA) as described below.

3.3.4.2 Hybridoma Production

The fusion was performed essentially as described by Goding (1983). Spleen cells were fused with P3X63-Ag8.653 myeloma cells at a ratio of 3:1 in RPMI 1640 using 50% (w/v) polyethylene glycol M_r 4000 as a fusing agent. The fused cells were resuspended in HAT selective medium (RPMI-1640 medium containing 10% fetal calf serum, 10^{-4} M hypoxanthine, 4 X 10^{-7} M aminopterin and 1.6 X 10^{-5} M thymidine) to a concentration of 10^6 cells/ml, and they were plated out onto five 96-well tissue culture plates containing feeder cell suspension of 10^4 cells/well in standard tissue culture medium. Two weeks after fusion, hybridomas were screened for antibody production by ELISA using attenuated cells of *S. typhimurium* ATCC 13311, *S. enteritidis* PT4 and *E. coli* ATCC 25995 as antigens. The positive hybridomas that were specific to *S. typhimurium* were cloned at least twice by limiting dilution.

3.3.4.3 MAbs Propagation and Isotyping

Once established, the hybridoma lines were expanded in tissue culture flasks and frozen in liquid N_2 for future use. Monoclonal antibody secreting clones 5A5, 4A8, and 5B2 were propagated either in tissue culture or as ascites fluid according to the procedure

of Harlow and Lane (1988). Pristane-primed adult male BALB/c mice were injected intraperitonally with 10^6 cells per mouse. Ascites fluid was collected, clarified by centrifugation and stored at -80°C until use, while spent medium were purified using 50% ammonium sulphate precipitation, dialysed against several changes of distilled water and stored at -80°C until use.

The isotyping of monoclonal antibodies was performed on MAbs from culture supernatant using a mouse monoclonal isotyping kit according to the manufacturer's instructions.

3.3.5 ELISA Protocol

An indirect ELISA was used to screen hybridoma spent media or to determine dilution titer of antisera. Heat-attenuated or live bacterial cells were diluted with carbonate buffer (pH 9.6) to 10^8 cells/ml and 100 μl/well suspension was used to coat 96-well plates for overnight at 4°C. Then, plates were washed six times with PBST (PBS with 0.5% Tween 20) and blocked by incubating for 1 h at room temperature with 200 μl PBST containing 1% (w/v) skim milk. After washing plates six times, 100 μl of monoclonal antibody diluted 1:3 (or appropriate dilution of antiserum) in PBS-Tween containing 0.1% (w/v) skim milk was added to the plates followed by 1 h incubation at 37°C. The plates were then washed six times and 100 μl of alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (diluted 1:3,000 in PBS-Tween containing 0.1% skim milk) was added, and incubated for 1 h at 37°C. The plates were rinsed six times with PBST and developed by adding 100 μl/well of substrate solution (pNPP)

followed by incubation for 1 h at 37°C or overnight at room temperature. The plates were read at 405 nm using a Titertec Multiskan ELISA (Flow Laboratories, McLean, VA, USA). Preimmune sera were used instead of MAb solutions as a negative controls.

3.3.6 Electrophoresis and Immunoblotting

3.3.6.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a discontinuous buffer system as described by Laemmli (1970) in a Protein II Cell vertical electrophoresis apparatus (BioRad, Richmond, CA, USA). Separating gel contained 15% acrylamide, while stacking gel comprised 4% acrylamide. LPS samples were solubilized in treatment buffer containing β-mercaptoethanol and boiled for 5 minutes at 100°C. About 20 μl (0.5 μg/μl) of samples were used per lane. Electrophoresis was carried out at constant current (30 mA per slab gel) for about 4 h. After the run was completed, the gels were either silver stained according to the method of Kittelberger and Hilbink (1993) or used for electroblotting.

3.3.6.2 Immunoblotting

Immediately after completion of the electrophoretic run, LPSs were transferred electrophoretically from the SDS-PAGE gel to nitrocellulose membrane according to the method of Weintraub et al. (1992). The electroblotting was carried out for 16 h at a constant current (180 mA) and temperature (4°C) in a Transblot Cell (BioRad, Richmond, USA) using a transfer buffer composed of 25 mM Tris, 152 mM glycine and

20% (v/v) methanol. After the transfer, the membrane was incubated with gentle shaking in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) containing 3% gelatin for 1 h at room temperature to block non specific binding sites. Then, membranes were washed three times with TBST (TBS with 0.05% Tween 20) and incubated with the appropriate MAbs (5A5, 5B2 or 4A8) diluted 1:10 in TBST containing 1% gelatin overnight at room temperature. After three washings with TBST, membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulins diluted 1:3,000 in TBST for 1 h at room temperature. The membranes were then washed three times with TBST, once with TBS and developed by incubating in BCIP/NBT substrate solution (1 mg/ml) and applying gentle shaking at room temperature until bands were visible. Colour development was stopped by rinsing the membranes with distilled water.

3.3.7 Dot Blot Assay

Dot blotting was performed in a Bio-Dot SF microfiltration apparatus (BioRad, Richmond, CA, USA) using nitrocellulose membrane (0.45 μm). About 100 μl of *S. typhimurium* whole cell suspension (10^7 cells/ml) was spotted on membrane and pulled through by passive filtration by applying gentle vacuum. After washing three times with TBS, membranes were incubated in 5% NaOH or in 38% HCl for 10 sec or left untreated. Immunoblotting was performed as described above.

3.3.8 Immunoelectron Microscopy

Immunolabelling was essentially performed as described by Cloeckaert *et al.*

(1990). Briefly, 5μl of native bacterial cell suspension in distilled water (5 X 10^8 cells /ml) was placed on formvar coated nickel grids. After being air dried for 2 h at room temperature, grids were first blocked in PBS containing 3% BSA for 30 min at room temperature, and then incubated with ascites fluid diluted to 1:200 in PBS-Tween 20 for 2 h at 37°C. Next, grids were incubated with colloidal gold (20 nm)-conjugated goat anti-mouse immunoglobulins diluted 1:25 in dilution buffer (20mM Tris, 150mM NaCl, 0.1% BSA, 0.005% Tween 20, and 0.4% gelatin, pH 9) for 20 hours at room temperature. After each incubation step, grids were washed four times with Tris-NaCl buffer containing Tween 20. Final wash was followed by rinsing grids with distilled water. Grids were viewed with the Hitachi H-7000 transmission electron microscope.

3.4 Results and Discussion

Three fusions resulted in about 400 hybridomas were performed in order to produce monoclonal antibodies (MAbs) to *S. typhimurium*. Approximately 10% of the hybridomas secreted antibodies were reactive with *S. typhimurium*. Of this only three hybridomas secreted antibodies that exhibited high specificity to *S. typhimurium*. These clones MAbs 5A5, 5B2 and 4A8 were propagated either in tissue culture flasks or as ascites fluid, purified and used for further studies. MAbs 5A5 and 5B2 were of IgM class while MAb 4A8 was of IgG_{2a} type, all possess κ light chains as assessed by a mouse-type subisotyping assay.

The specificity of MAbs was determined by non-competitive ELISA using various heat-attenuated (80°C, 20 min) bacteria (Table 3.1). Antibodies prominently reacted with

TABLE 3.1 Specificity of monoclonal antibodies to *S.typhimurium* as assessed by ELISA[a].

Group	Bacteria	O-antigen formula	Absorbance		
			MAb 4A8	MAb 5B2	MAb 5A5
B	*S.typhimurium* ATCC 13311[c]	1,4,[5],12	1.839	1.776	1.905
	S.heidelberg[d]	1,4,[5],12	1.822	1.840	1.887
	S.stanley[d]	1,4,[5],12,27	1.856	1.823	1.887
	S.paratyphi B[d]	1,4,[5],12	1.863	1.860	1.855
	S.albert[e]	4,12	0.061	0.095	0.046
	S.typhimurium var. *copenhagen*[b]	1,4,12	0.075	0.066	0.136
	S.kingston[e]	1,4,12,27	0.038	0.058	0.063
	S.agona[e]	1,4,12	0.031	0.077	0.025
	S.brandenburg[e]	1,4,12	0.018	0.084	0.032
C_1	*S.mbandaka*[e]	6,7	0.068	0.089	0.081
	S.thompson[d]	6,7	0.038	0.116	0.050
	S.choleraesuis[e]	6,7	0.028	0.087	0.048
C_2	*S.muenchen*[e]	6,8	0.036	0.069	0.040
	S.hadar[e]	6,8	0.024	0.062	0.028
D_1	*S.pullorum* ATCC 19945[c]	6,8	0.051	0.178	0.049
	S.gallinarum[d]	1,9,12	0.044	0.111	0.027
	S.berta ATCC 8392[c]	1,9,12	0.036	0.089	0.040
	S.enteritidis PT4[b]	1,9,12	0.036	0.062	0.033
	S.enteritidis PT8[b]	1,9,12	0.031	0.071	0.056
	S.enteritidis PT13[b]	1,9,12	0.029	0.058	0.057
	S.enteritidis PT1[b]	1,9,12	0.026	0.054	0.045
	S.enteritidis PT13a[b]	1,9,12	0.033	0.086	0.044
D_2	*S.maarssen* ATCC 15793[c]	9,46	0.029	0.082	0.039
E_1	*S.anatum*[e]	3,10	0.026	0.092	0.065
E_3	*S.thomasville*[e]	3,15,34	0.020	0.099	0.033
E_4	*S.senftenberg*[e]	1,3,19	0.032	0.062	0.024
F	*S.rubislaw*[e]	11	0.020	0.065	0.033
G_2	*S.havana*[e]	1,13,23	0.050	0.085	0.019
atypical	*S.arizona*[d]		0.025	0.148	0.056
	Y.enterocolitica[e]		0.023	0.159	0.024
	Shigella flexneri[e]		0.015	0.099	0.013
	E.coli ATCC 25992[c]		0.008	0.084	0.018
	E.coli ATCC 11775[c]		0.019	0.129	0.039
	C.freundii ATCC 8090[c]		0.028	0.072	0.021
	Enterobacter cloacae[e]		0.013	0.076	0.015
	Psudomonas fluorescence[e]		0.048	0.061	0.021
	Mycobacterium fortuitum[e]		0.008	0.073	0.012

[a] Bacterial cells heat treated for 20 min at 80°C were used as coating antigen in ELISA.
[b] Laboratory Center for Disease Control, Ottawa, Canada.
[c] American Type Culture Collection, Rockville, MD, USA.
[d] University of Manitoba, Winnipeg, Canada.
[e] Economic Innovation and Technology Council, Winnipeg, Canada.

four serovars belonging to serogroup B (*S. typhimurium* ATCC 13311, *heidelberg*, *stanley* and *paratyphi* B). None of these antibodies reacted with *S. typhimurium* var. *copenhagen* as well as other nine *Salmonella* serogroups (C1, C2, D1, D2, E1, E3, E4, F, G, and an atypical *S. arizona*). In addition, all three antibodies showed no reactivity to a number of *Enterobacteriaceae* (*Y. enterocolitica, C. freundii, E. coli, E. cloacae, shigella flexneri*) and other bacteria (*P. fluorescence*, and *M. fortuitum*).

It appeared that the MAbs were specific only towards *Salmonella* B-serovars containing the LPS O-5 antigen which is an unique factor for some of B-serovars such as *S. typhimurium* ATCC 13311, *S. stanley, S. heidelberg* and *S. paratyphi B.* This factor is a result of chemical modification of factor 4 and contains an acetyl group which is linked to the abequose residue (Figure 3.1).

Furthermore, the antigenic specificity of all MAbs were analysed by SDS-PAGE (Figure 3.2) followed by immunoblotting (Figure 3.3). Two LPSs extracted from *S. typhimurium* ATCC 13311 (Figure 3.2, lane 1) and *S. typhimurium* var. *copenhagen* (Figure 3.2, lane 2) were used for electrophoretic studies. Both LPSs share the same tetrasaccharide repeating unit, but the latter LPS lacks the factor 5. In addition, LPS from *S.enteritidis* (Figure 3.2, lane 3) which shows the same trisaccharide backbone with B-srogroup LPSs and LPS from *E. coli* (Figure 3.2, lane 4) which lacks the structural similarities were used. Silver staining of SDS-PAGE gels (Figure 3.2) revealed that all LPS were heterogenous consisting of three predominant subpopulations in a form of ladder-like patterns typical for smooth gram-negative bacteria (Yeh and Jacobs, 1992). These bands represent the LPS molecules containing increasing lengths of O-antigen

Figure 3.1 Chemical structure of the O-specific side chain of lipopolysaccharide from *Salmonella* B-serogroup (Adapted from Hellerqvist *et al.*, 1969).

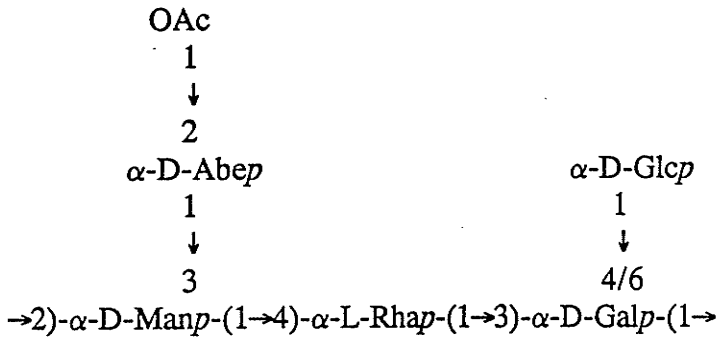

OAc
1
2
α-D-Abe*p*
1
3
α-D-Glc*p*
1
4/6
→2)-α-D-Man*p*-(1→4)-α-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→

Figure 3.2 SDS-polyacrylamide gel electrophoresis of LPSs extracted from different bacteria and developed by silver staining. Lanes; (1) *S. typhimurium* (Sigma), (2) *S. typhimurium* var. *copenhagen*, (3) *S. enteritidis* (Sigma), (4) *E. coli* (Sigma).

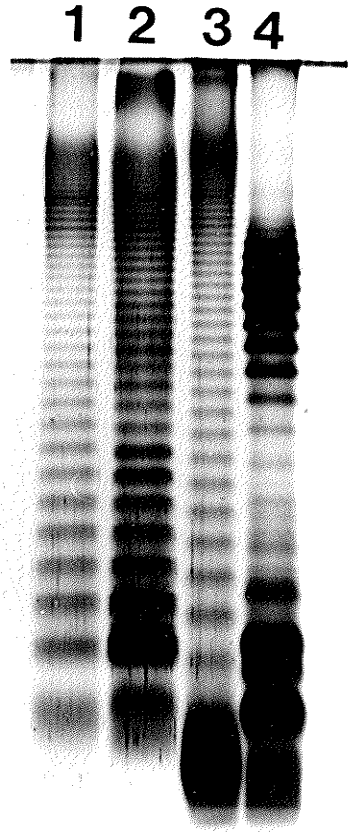
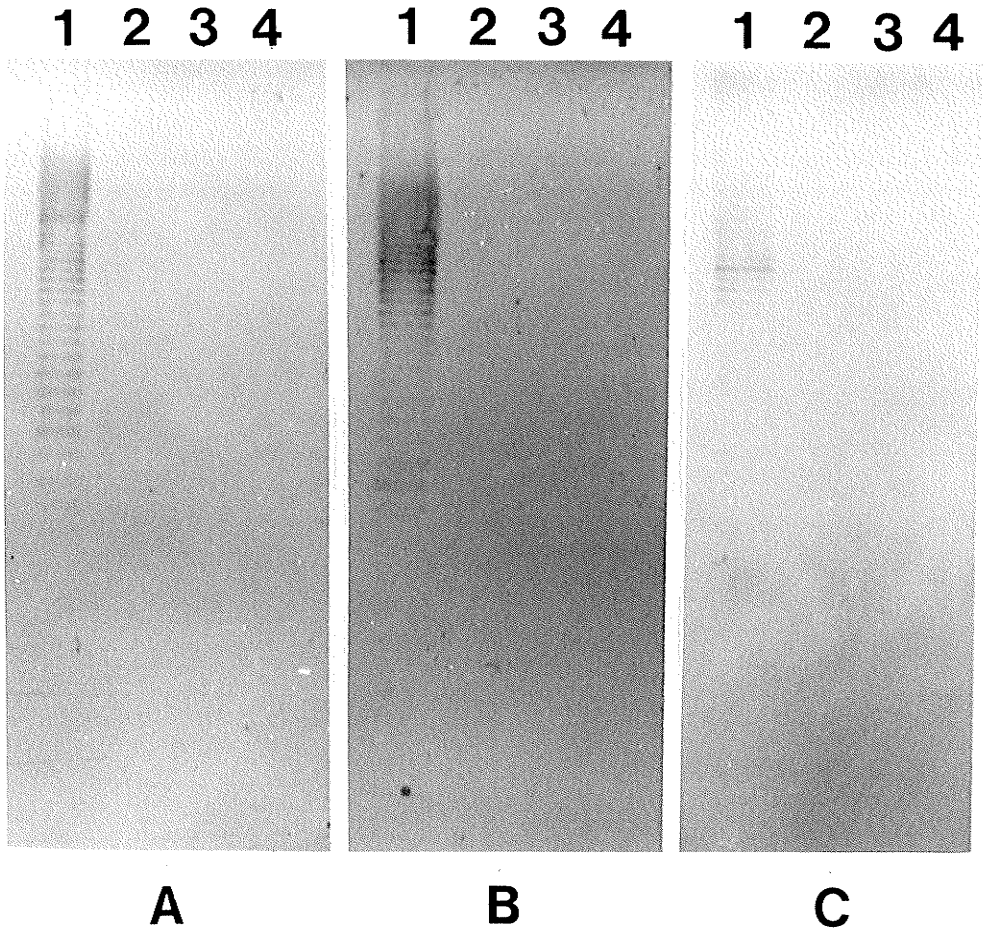

1 2 3 4
Long-Chain LPS
Short-Chain LPS

Figure 3.3 Immunoblot of SDS-PAGE of LPSs extracted from different bacteria (corresponding to gel of Figure 3.2) and probed with MAb 5A5 (A), MAb 5B2 (B), and MAb 4A8 (C). Lanes: (1) *S. typhimurium* (Sigma),(2) *S. typhimurium* var. *copenhagen*, (3) *S. enteritidis* (Sigma), (4) *E. coli* (Sigma).


1 2 3 4
1 2 3 4
1 2 3 4
A
B
C

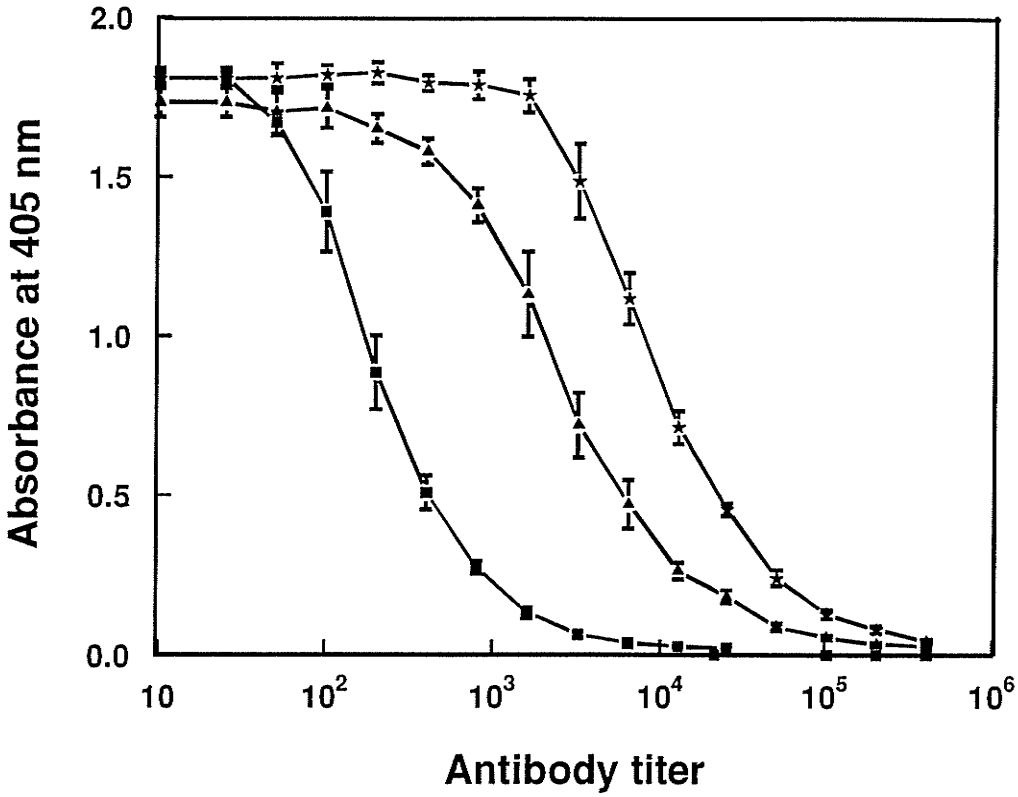
chains. It has been reported that the fastest migrating molecules are short chain LPS which contain a complete core oligosaccharide but lack O-antigen units, while slower migrating molecules represent LPSs containing long chain O-antigen (Peterson and McRoarty, 1985).

The specificity of MAbs for LPS O-antigen was confirmed by immunoblotting (Figure 3.3). The antibodies reacted only with *S. typhimurium* which contain the O:1,4,5,12 antigen (Figure 3.3 A, B, C, lane 1), and did not recognize LPS from *S. typhimurium* var. *copenhagen* as well as *S. enteritidis* and *E. coli* (Figure 3.3 A, B, C, lanes 2,3 and 4). The MAbs selectively bound to LPS present in the upper region of gel indicating that the recognized sites were in the long chain O-antigen region bearing the O-acetyl group. The presence of such long chain populations in *S. typhimurium* has been reported by Munford and coworkers (1980).

The avidity of MAbs to their epitopes was analysed using ELISA. Titration curves obtained for all three MAbs are shown in Figure 3.4. It appeared that IgM MAbs exhibited higher avidity than the IgG. This difference may be due to the fact that the IgM is a pentameric molecule with 10 antigen binding sites as opposed to IgG that has only two. It is also worthwhile to note that at dilution of 1:100,000 the IgM MAbs still reacted with the cells giving an absorbance value of 0.2 while the IgG gave the same absorbance at dilution of only 1:1,000.

To determine whether MAbs recognize the same epitope, all MAbs were tested by the additivity index ELISA (Friguet *et al.*, 1983). In this assay, an additivity index value close to zero indicates that two tested antibodies recognize the same or two closely

Figure 3.4 Reactivity of MAbs 5A5 (★), 5B2 (▲), and 4A8 (■) with heat attenuated (80°C, 20 min) whole cells of *S. typhimurium* ATCC 13311. Each point represents the mean of three replicates and the bars represents standard deviation of the mean.


2.0
1.5
1.0
0.5
0.0
Absorbance at 405 nm
10
10^2
10^3
10^4
10^5
10^6
Antibody titer

associated epitopes, while a high value indicates the simultaneous binding of both antibodies to distinct epitopes. Table 3.2 shows results of the additivity ELISA for all three monoclonal antibodies. The additivity index values for all MAbs were low; 7.99 for MAbs 5A5 and 5B2, 5.56 for MAbs 5A5 and 4A8, and the lowest value was 1.24 for MAbs 5B2 and 4A8. These low values suggested that these MAbs most likely recognized the same epitopes or epitopes that have similar structure.

Table 3.3 shows that MAbs also recognized live *Salmonella* B-serovars containing O-antigen 5. All antibodies were highly reactive with *S. typhimurium* ATCC 13311 and *S. heidelberg* exhibiting similar absorbance values that resulted from reactions with heat-attenuated cells. These findings suggest that LPS O-5 antigenic sites are easily accessible by MAbs without a need of heat treatment in order to expose the epitopes. It is possible, that the LPS O-antigen structure of *Salmonella* serogroup B accounted for this phenomenon as a result of the protrusion of the O-acetyl group which forms factor 5 (Figure 3.1) (Lind and Lindberg, 1992). Moreover, none of the antibodies was reactive with bacteria lacking the LPS O-5 factor.

The ability of MAbs to detect live *S. typhimurium* was further confirmed by immunoelectron transmission microscopy. Figure 5 shows labelling of a *S. typhimurium* live cell with MAb 4A8 conjugated with colloidal gold. Similar results were obtained with the other two MAbs. A noteworthy density of gold spheres covered the whole bacterial cell suggesting that factor O-5 is a significant part of its external leaflet. This appeared to be consistent with other reports indicated that bacterial LPS is expressed in a high frequency accounting for 50 to 70% of surface of outer monolayer (Lugtenberg

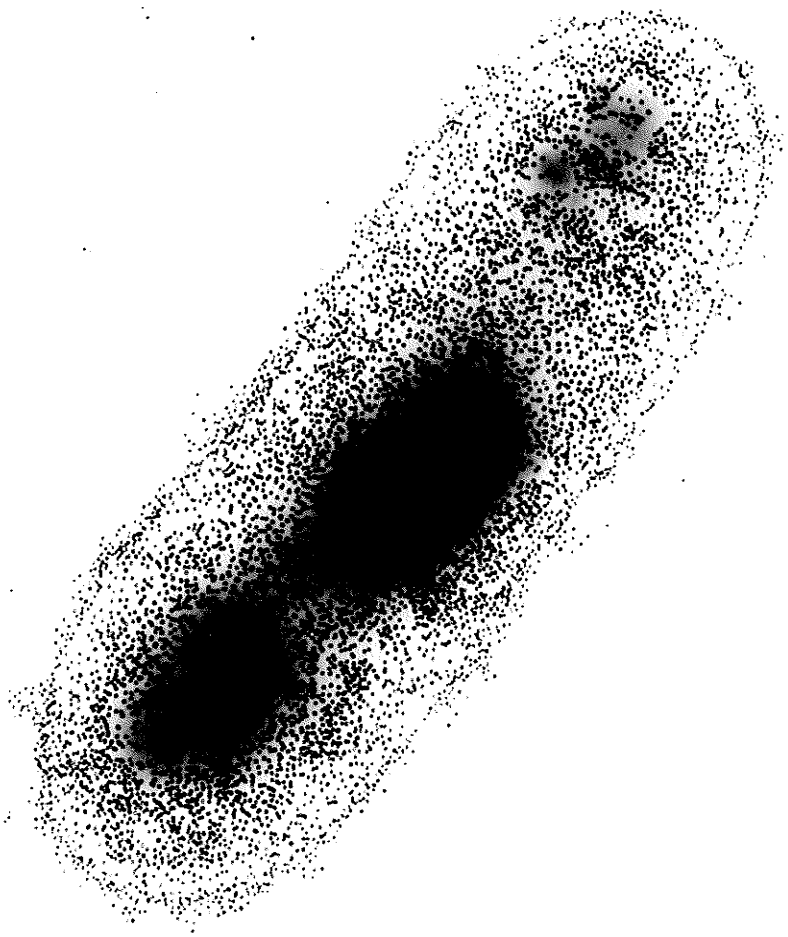
TABLE 3.2 Additivity indices of MAbs 5A5, 5B2, and 4A8 as assessed by ELISA.

MAb	5A5	5B2	4A8
5A5	_	7.99	5.56
5B2	7.99	_	1.24
4A8	5.56	1.24	_

TABLE 3.3 The reactivity of monoclonal antibodies against live *S. typhimurium* and other serogroups as assessed by ELISA.

Group	Bacteria	MAb		
		4A8	5B2	5A5
B	*S. typhimurium* ATCC 13311	1.805	1.807	1.777
	S. typhimurium var. *copenhagen*	0.107	0.030	0.064
	S. heidelberg	1.847	1.840	1.769
C	*S. mbandaka*	0.085	0.018	0.047
D	*S. enteritidis* PT13	0.119	0.115	0.080
E	*S. thomasville*	0.194	0.182	0.173
F	*S. rubislaw*	0.102	0.026	0.043
G	*S. havana*	0.138	0.095	0.076
	C. freundii	0.145	0.080	0.124
	E. coli ATCC 25995	0.108	0.038	0.069

Figure 3.5 Electron micrograph of *S. typhimurium* ATCC 13311 probed with MAb 4A8 ascites and goat anti-mouse immunoglobulins coupled to 20 nm gold spheres: Magnification: x 17,000.



and van Alphen, 1983). Moreover, the LPS O-antigen in *S. typhimurium* consists of more than 70 repeating units (Peterson and McRoarty, 1985).

The ability of MAbs to bind live *S. typhimurium* makes them suitable to be used for the isolation of this pathogen from food, environmental and clinical samples in an ELISA capture system. MAbs immobilized on magnetic beads or dipstics could be utilized directly for capturing live *S. typhimurium* cells from any sample prior to enrichment, thus a selective enrichment step could be eliminated (Wayatt, 1992).

Since all three MAbs were shown to be specific to bacterial LPS, the effect of various treatment such as detergent and extreme pH on reactivity of antibodies were further investigated.

The effect of cholic acid is shown in Table 3.4. Cholic acid and its derivatives are frequently used detergents to treat gram-negative bacteria in order to expose LPS to binding antibodies (Wang *et al.*, 1995). In our studies, cholic acid appeared to have mostly an adverse effect on the binding of all three MAbs to *S. typhimurium*, although a slight increase in absorbance values was observed for binding MAb 5B2 and 5A5 to *S. typhimurium* treated with 1% cholic acid (Table 3.4). It seemed that the decrease in the absorbance values caused by higher concentrations of cholic acid was related to an adverse effect on bacterial cells rather than LPS molecules, since free LPS extracted from *S. typhimurium* was not affected. Treatment of *S. heidelberg* with cholic acid also also had no effect on binding of this pathogen to monoclonal antibodies.

Furthermore, the acid treatment had a negligible effect on an enhancement of detection of *S. typhimurium* by monoclonal antibodies as assessed by dot blotting (Figure

TABLE 3.4 Effect of cholic acid on the reactivity of monoclonal antibodies to whole cells of *S.typhimurium* ATCC 13311 and LPS from *S.typhimurium* and *S.heidelberg*.

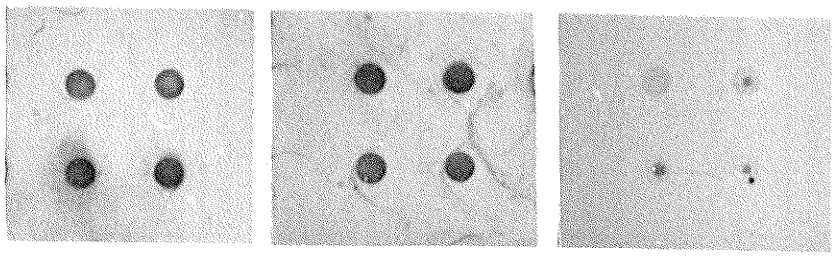
	Absorbance								
	S.typhimurium			*S.heidelberg*			LPS		
Cholic acid	4A8	5B2	5A5	4A8	5B2	5A5	4A8	5B2	5A5
0%	1.536	1.562	1.398	1.867	1.882	1.872	1.887	1.923	1.937
1%	1.285	1.750	1.763	1.829	1.858	1.916	1.822	1.859	1.916
5%	0.939	1.501	1.170	1.834	1.862	1.902	1.799	1.863	1.870
10%	0.805	1.239	1.012	1.838	1.850	1.889	1.674	1.828	1.794
13.5%	1.049	1.268	0.767	1.806	1.850	1.916	1.811	1.849	1.864

3.6). However, the antigenicity of LPS O-5 was conserved which is in disagreement with findings by Yamaura and coworkers (1992) that prolonged acid treatment destroys the acetyl group. In contrast, when *S. typhimurium* whole cell suspension was spotted on nitrocellulose membrane and exposed to alkaline conditions, no detection by all three MAbs was observed (Figure 3.3 C). Komisar and Cebra (1983) suggested that alkaline pH could structurally alter the O-acetyl group linked to the abequose residue. Since this group forms the factor 5, it is most likely that alkaline pH also rendered changes in the antigenicity of this epitope. It is worthwhile to indicate, however, that the effect of pH on the antibody binding to bacterial cells is dependant on the nature of antibodies. Todd and coworkers (1991) have reported that acid and alkaline treatment of bacterial cells was useful to expose epitopes and enhance an antigen-antibody interaction for certain anti-LPS antibodies, while it proved ineffective for other types of antibodies.

3.5 Conclusions

In this study we have produced and characterized monoclonal antibodies specific to the O-5 antigen of B-serogroup *Salmonella*. These antibodies did not exhibit any cross-reactivity to other tested serogroups. The ability of the MAbs to recognize equally live and heat treated bacteria was demonstrated by ELISA and immunoelectron microscopy. These MAbs can be utilized in an ELISA system for serological discrimination between *S. typhimurium* and *S. typhimurium* var. *copenhagen*.

Figure 3.6 Dot blot assay of whole cells of *S. typhimurium* ATCC 13311 (10^8 cells/ml) and probed with MAb 5A5. A- no treatment, B- cells were treated with 38% HCl for 10 sec prior incubating with MAb, C- cells were treated with 5% NaOH for 10 sec prior incubating with MAb. *S. typhimurium* cells were spotted on nitrocellulose membranes in quadruplicates.



A B C

4. MANUSCRIPT II

ANTIGENICALLY STABLE 35 kDa OUTER MEMBRANE PROTEIN OF *SALMONELLA*

4.1 Abstract

Identification of antigenic surface components of *Salmonella* genera may facilitate the development of reagents to diagnose and prevent salmonellosis. Outer membrane protein (OMP) extracts were prepared from 19 *Salmonella* representing 7 serogroups and four non-salmonellae species of *Enterobacteriaceae*. Extracts were examined by SDS-PAGE followed by immunoblotting. Immunoblotting with polyclonal antibodies revealed two major proteins with apparent molecular weight (M_r) of 35 and 24 kDa. The latter protein was present only in *Salmonella* species. A monoclonal antibody, designated MAb 1D6 (IgA) bound to a 35 kDa OMP for all tested *Salmonella* species, with the exception of *S. arizona*. The results demonstrated the 35 kDa protein was a common antigen for all tested *Salmonella*, however, an epitope recognized by MAb 1D6 was absent in this protein obtained from atypical species of *Salmonella* (*S. arizona*). Types of growth media did not affect the antigenicity of both, 35 and 24 kDa proteins in crude OMP extracts examined by SDS-PAGE and immunoblotting. Antigenicity of these OMPs was not affected by heat treatment and reducing agents prior SDS-PAGE. However, the appearance of the 35 kDa protein was influenced by treating OMP extracts with excessive temperature.

Analysis of OMP extracts by of SDS-PAGE and immunoblotting without heat-treatment revealed that MAb 1D6 bound several isoforms of this protein; one major at 28 kDa and about eight minor forms in the range between 34 and 40 kDa. None of these forms contained carbohydrate moieties that may be responsible for the polymorphic appearance of the protein. These forms were converted to a single form by heat-

treatment at 100°C indicating that the 35 kDa OMP is most likely a heat-modifiable protein. Furthermore, extended heat-treatment (121°C, 15 min) did not affect the antigenicity of the 35 kDa OMP. The 35 kDa OMP protein was found to be exposed on surface of the cells as assessed by immunoelectron microscopy.

4.2 Introduction

Salmonella cell envelope contains an outer membrane which is mainly composed of lipopolysaccharide (LPS) and outer membrane proteins (OMP) (Lugtenberg and van Alphen, 1983, Bentley and Klebba, 1988, Ogawa *et al.*, 1992). The outer membrane proteins contains pore forming proteins called porins (Pai *et al.*, 1992). Porins are present in the membrane as oligomers, usually trimers, and have molecular weights (M_r) in the range of 28 to 48 kDa. These porins are often non-covalently bound to other membrane compounds such as peptidoglycans and lipopolysaccharides (Hancock, 1987). Porins act as non-specific molecular sieving channels for hydrophillic molecules that diffuse into the cell. They also bear important immunogenic determinants (Hancock, 1987, Ogawa *et al.*, Spinola *et al.*, 1993).

It has been reported that under normal growth conditions *S. typhimurium* as well as *S. enteritidis* express three porins named OmpD (34 kDa), OmpF (35 kDa), and OmpC (36 kDa) (Pai *et al.*, 1992, Singh *et al.*, 1992, Chart *et al.*, 1993) while *E. coli* K_{12} produces two porins OmpC (36 kDa) and OmpF (35 kDa) (Lee and Shnaitman, 1980). In addition, *Enterobacteriaceae* contain at least one heat-modifiable non-peptidoglycan bound major outer membrane protein. The electrophoretic mobility of this protein is greatly influenced by heat-treatment. Hofestra and Dankert (1980) have reported that this protein in *Salmonella* species has an apparent M_r of 28 kDa when solubilized at 37°C prior to loading on SDS-PAGE gels, but it has M_r of 35 kDa when boiled for 5 min. This OMP was only partially characterized for its antigenicity with antisera and results were not conclusive whether it was a membrane surface exposed

protein.

The aim of this paper was to study the antigenicity of the *Salmonella* outer membrane proteins in an attempt to identify a surface exposed protein common to *Salmonella* species.

4.3 Materials and Methods

4.3.1 Materials

Standard plate count agar (SPC), lactose broth (L-broth), nutrient broth and M-broth were purchased from Difco Laboratories (Detroit, MI, USA). Brain heart infusion and RPMI 1640 media were from Gibco (Grand Island, NY, USA). Universal pre-enrichment broth and peptone water broth were made according to the Difco manual instructions. Protein assay reagents were obtained from Pierce (Rockford, IL, USA), while hypoxanthine, aminopterin, thymidine, N-lauroylsarcosine (Sarkosyl) ρ-nitrophenyl phosphate were purchased from Sigma Chemical Co. (St Louis, MO, USA). Diethanolamine buffer was from Mallinckrodt (Paris, KY, USA). Acrylamide, sodium dodecyl sulphate, nitrocellulose membrane (0.45 μm), alkaline phosphatase-conjugated goat anti-mouse immunoglobulins, gold (20 nm)-conjugated anti-mouse immunoglobulins, 5-bromo-4-chloro-3-indolyl phosphate ρ-toluidine salt (BCIP) and ρ-nitro blue tetrazolium chloride (NBT) were purchased from BioRad (Richmond, CA, USA). Myeloma cells P3X63.Ag8.653 were obtained from the American Type Culture Collection (Rockville, MD, USA). Polyethylene glycol, M_r 4000 (cat. no. 9727) was from Merck (Germany). Dimethylsulfoxide was purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA).

Tissue culture flasks, 24 and 96 well polystyrene tissue culture plates were purchased from Corning Glass Ware (Corning, NY, USA), while Falcon Microtest III polyvinyl chloride flexible 96-well plates were purchased from Canlab (Mississauga, ON, Canada). All of other chemicals and reagents were of analytical grade.

4.3.2 Bacteria and Growth Conditions

All bacterial cultures were maintained on SPC slants until use. Prior to extracting outer membrane proteins all *Salmonella* species were grown on nutrient broth for 16 h at 37°C. The following day, six flasks containing about 1.5 L of media (M-broth, L-broth, Peptone water broth, and Universal preenrichment broth) were inoculated each with 1 ml of turbid bacterial inoculum and incubated for additional 16 h. Then, cells were either attenuated by heating at 121°C for 15 min or left without attenuation, harvested by centrifugation, washed with Tris-HCl pH 7.2, and used immediately for OMP extraction. For ELISA, *Salmonella* was grown only in M-broth at 37°C for 16 h, harvested, washed and resuspended in saline. The bacterial strains used in this study are listed in Table 1.

4.3.3 Outer Membrane Protein Preparation

Outer membrane proteins were prepared using the modified sarkosyl method described by Philip *et al.* (1973). Harvested *Salmonella* cells were suspended in 50 mM Tris-HCl, pH 7.7 and treated with 0.1 μg of both bovine ribonuclease and deoxyribonuclease. The cells were then sonicated five times for 45 sec at 300 watts (0°C)

using a Braun-Sonic 1510 (B. Braun, Melsungen, AG). Unbroken cells were removed by centrifugation at 1000 x g for 15 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 45,000 x g for 1 h. The pellet was treated with 2% sarkosyl in 50 mM Tris-HCl, and the mixture was incubated for 30 min at room temperature followed by centrifugation at 45,000 x g for 1 h at 4°C. The pellet containing outer membranes and the peptidoglycan layer was resuspended in 10 mM Tris-HCl containing 2% SDS, incubated for 30 min at 32°C (Pai *et al.*, 1992) and centrifuged for 30 min at 45,000 x g at 4°C yielding supernatant which contained OMP. The OMP supernatant was collected and assessed for protein content using the bicinchoninic acid method according to the manufacturer's instructions. The OMP extracts were then aliquoted and stored at -20°C until use.

4.3.4 Production of Polyclonal Antibodies

Female BALB/c mice (4-8 weeks) were injected twice subcutaneously with 40 μg of *S. typhimurium* OMP extracts in complete or incomplete Freund's adjuvants with two weeks intervals. Then mice were injected twice intraperitonally with 40 μg OMP/mouse at a one-week interval. Next, tailbleedings were taken and serum was analysed for titer, aliquoted and stored at -20°C until use.

4.3.5 Production of Monoclonal Antibodies

Production of MAbs was performed essentially as described earlier by Jaradat and Zawistowski (1995, submitted).

4.3.6 Monoclonal Antibody Propagation and Isotyping

A stabilized hybridoma secreting MAb 1D6 was expanded in tissue culture flasks. The supernatant was purified by precipitation with 50% ammonium sulphate, dialysed against three changes of PBS and stored at -80°C until use. The isotyping of the MAb was performed on tissue culture spent medium using a mouse monoclonal type-subisotyping kit (BioRad, Richmond, CA, USA) according to the manufacturer's instructions.

4.3.7 ELISA Protocol

An indirect ELISA was used to screen hybridoma spent media or to determine dilution titer of antisera using either OMP extracts or bacterial cell suspension as antigens. When OMP was used as an antigen, plates were coated with 100 μl/well of OMP extract in carbonate buffer, pH 9.6, (1 μg/well) and incubated overnight at 4°C. Bacterial cell coated plates were prepared using either heat-attenuated (80°C for 20 min) or live bacteria. Cells were diluted with carbonate buffer (pH 9.6) to 10^8 cells/ml and resulting suspension (100 μl/well) was used to coat 96-well plates overnight at 4°C. Then, plates were washed six times with PBST (PBS with 0.5% Tween 20) and blocked by incubating with 200 μl PBST containing 1% (w/v) skim milk for 1 h at room temperature. After washing plates six times, 100 μl/well of monoclonal antibody solution diluted 1:3 (or appropriate dilution of antiserum) in PBS-Tween containing 0.1% (w/v) skim milk was added to the plates followed by 1 h incubation at 37°C. The plates were washed again six times and 100 μl/well of alkaline phosphatase-conjugated goat anti-

mouse immunoglobulins (diluted 1:3,000 in PBS-Tween containing 0.1% skim milk) was added, and incubated for 1 h at 37°C. The plates were rinsed six times with PBST and developed by adding 100 μl/well of substrate solution (pNPP) followed by incubation for 1 h at 37°C or overnight at room temperature. The plates were read at 405 nm on a Titertec Multiskan ELISA (Flow Laboratories, McLean, VA, USA). Preimmune sera were used instead of MAb solutions as a negative controls.

4.3.8 Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed on 12% separating and 5% stacking gels using a discontinuous buffer system in BioRad Protein II vertical unit (BioRad, Richmond, CA, USA) as described by Laemmli (1970). Outer membrane protein extracts were solubilized in treatment buffer containing β-mercaptoethanol. Samples (25-30 μg/ml) were either boiled for 5 min and loaded in the gel or loaded without boiling. The separation was carried out at a constant current 30 mA per gel for about 4 h. Gels were stained with Coommassie Brilliant Blue R-250 or used for immunoblotting.

4.3.9 Immunoblotting

Immediately after electrophoresis, gels were equilibrated in the transfer buffer (25 mM Tris and 192 mM glycine) for 30 min according to the method of Davis *et al.* (1994). Proteins were transferred electrophoretically from the SDS-PAGE gel to nitrocellulose membranes for 3 h at constant current (200 mA) and temperature (4°C).

Then the nitrocellulose membranes were blocked with 3% gelatin in Tris-buffered saline (TBS) for 1 hour at room temperature. After three washings with TBST, the membranes were incubated overnight with either mouse antisera raised against OMP extracts and diluted 1:25,000 in TBST buffer containing 1% gelatin or MAb 1D6 diluted 1:5 in the same buffer. After several washings with TBST, the blots were incubated with alkaline phosphatase-conjugated anti-mouse immunoglobulins diluted 1:3,000 in TBST for 1 h at room temperature. Membranes were then washed three times with TBST and once with TBS and incubated with 1 mg/ml BCIP/NBT substrate solution in Tris buffer (0.1 M Tris, 0.5 mM $MgCl_2$ pH 9.5) at room temperature with continuous shaking until bands were visible. Colour development was stopped by rinsing the membranes with distilled water.

4.3.10 Carbohydrate Staining

Proteins were stained for the carbohydrate content *in situ* according to the method of Kondo *et al.* (1991). Briefly, SDS-PAGE and immunoblotting were performed as described above, and membranes were then washed in Tris-saline buffer (10 mM Tris-HCl, pH 7.5, 0.5% (v/v) Tween 20, and 150 mM NaCl) for 40 min at room temperature. Blots were oxidized with 0.2% sodium periodate for 10 min at room temperature. The reaction was stopped by washing membranes three times with PBS, followed by incubation for 30 min with 0.1 mM biotin hydrazide dissolved in 0.1 N NaOH at room temperature. The membranes were washed twice with 0.1 N NaOH, twice with Tris-Tween saline and then incubated in alkaline phosphatase-conjugated

streptavidin diluted 1:10,000 in Tris-Tween-20 saline for 10 min. Bands containing sugars were visualized by incubating the membranes in BCIP/NBT substrate solution.

4.3.11 Immunoelectron Microscopy

Immunolabelling of cells using MAb 1D6 and transmission electron microscopy were performed as described previously by Jaradat and Zawistowski (1995, submitted).

4.4 Results and Discussion

4.4.1 Epitope Specificity of MAb 1D6

To study the antigenicity of *Salmonella* outer membrane proteins, polyclonal and monoclonal antibodies against *S. typhimurium* were produced. Polyclonal antibodies were developed by immunizing mice with the OMP extract of *S. typhimurium*, while monoclonal antibodies were produced by immunizing mice with heat attenuated *S. typhimurium* whole cells. Initial screening identified one hybridoma designated MAb 1D6 that was reactive with *S. typhimurium* whole cells. This antibody was found to be of IgA class with κ light chain as assessed by mouse-subisotyping assay. To reveal its specificity, MAb 1D6 was tested against heat attenuated (80°C, 20 min) whole cells and OMP extracts obtained from a number of *Salmonella* and non-salmonellae species using an indirect non-competitive ELISA (Table 4.1). Monoclonal antibody 1D6 reacted only with two B-serovars, *S. typhimurium* and *S. stanley* when the whole heat-treated cell suspensions were used to coat ELISA plates. In contrast, MAb 1D6 exhibited reactivities against all tested bacterial OMP extracts yielding the highest reaction with *S.*

TABLE 4.1 Reactivity of MAb 1D6 with heat attenuated cells and outer membrane proteins isolated from different bacteria as assessed by ELISA.

Group	Bacteria	Absorbance	
		Cells[e]	OMP[f]
B	*S.typhimurium* ATCC 13311[a]	0.176[g]	0.364
	S.heidelberg[d]	0.128	0.237
	S.stanley[d]	0.263	0.297
	S.paratyphi B[d]	0.110	0.261
	S.agona[b]	0.122	0.261
	S.brandenburg[c]	0.107	0.262
C_1	*S.mbandaka*[b]	0.109	0.261
	S.thompson[d]	0.132	0.221
	S.choleraesuis[d]	0.117	0.166
C_2	*S.hadar*[b]	0.106	0.273
D	*S.gallinarum*[d]	0.114	0.307
	S.berta ATCC 8392[a]	0.104	0.234
	S.enteritidis PT13[c]	0.090	0.217
E_1	*S.anatum*[b]	0.092	0.256
E_3	*S.thomasville*[b]	0.096	0.224
E_4	*S.senftenberg*[b]	0.092	0.183
F	*S.rubislaw*[b]	0.093	0.222
G	S.havana[b]	0.083	0.233
Atypical	*S.arizona*[d]	0.092	0.191
	Y.enterocolitica[b]	0.110	0.252
	Shigella flexneri[b]	0.119	0.252
	E.coli ATCC 25992[a]	0.111	0.258
	C.freundii ATCC 8090[a]	0.123	0.276
	Control[h]	0.125	0.150

[a] American Type Culture Collection, Rockville, MD, USA.
[b] Economic Innovation and Technology Council, Winnipeg, Canada.
[c] Laboratory Center for Disease Control, Ottawa, Canada.
[d] University of Manitoba, Microbiology Department, Winnipeg, Canada.
[e] Bacterial cells heat treated (10^7/well) for 20 min at 80°C were used as a coating antigen.
[f] Extracted OMPs (1 μg protein/well) were used as a coating antigen
[g] The results are expressed as the means of triplicate determinations.
[h] Controls were made using preimmune antisera.

typhimurium (Table 4.1). In both cases, however, the absorbance values were relatively low. Furthermore, MAb 1D6 exhibited only weak binding to live *S. typhimurium* and other tested bacteria (Table 4.2). It is possible that the presence of LPS on bacterial cells adversely affected the binding of MAb to a proteinaceous epitope. It has been suggested that the MAb accessibility of epitopes on the OMP of gram-negative bacteria is hindered by a LPS leaflet that surrounds the bacterial cell. Such a steric hindrance has been described for *Coxiella burnetii*, some members of family *Enterobacteriaceae, Nesseria gonorrhoea* and *N. meningitides* (Cloeckaert, *et al.*, 1990, Singh *et al.*, 1992). Moreover, OMP epitopes may be transiently exposed at the cell surface as a consequence of a natural turn-over of cell wall materials during certain periods in the life cycle of bacterial cells (Singh *et al.*, 1992).

4.4.2 Cross Reactivity in Salmonella Species

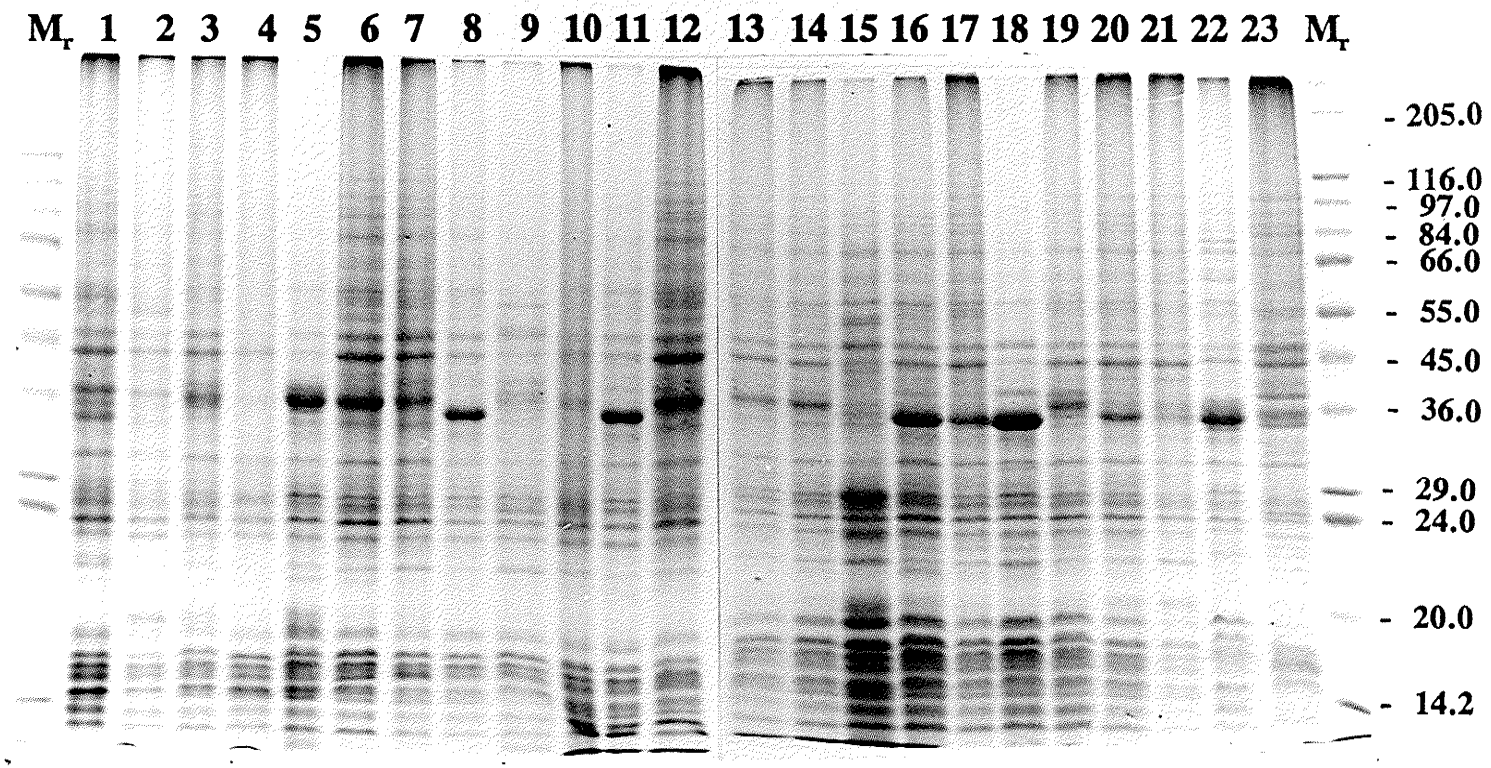
Outer membrane proteins extracted from 19 *Salmonella* and four other *Enterobacteriaceae* species were studied by SDS-PAGE. In general, protein electrophoretic profiles of all tested OMPs were remarkably similar within *Salmonella* serogroups or even among *Salmonella* and non-salmonellae species. However, some differences were detected among protein bands in the M_r range of 34 to 38 kDa. It appeared, that some bacteria such as *Y. enterocolitica* (Figure 4.1, lane 1), *S. arizona* Figure 4.1, lane 3), *S. rubislaw* (Figure 4.1, lane 5), *S. senftenberg* (Figure 4.1, lane 6), *S. thomasville* (Figure 4.1, lane 7), *S. hadar* (Figure 4.1, lane 12) and *S. heidelberg*

TABLE 4.2 The reactivity of MAb 1D6 with live bacteria.

Group	Bacteria	Absorbance
B	*S. typhimurium* ATCC 13311	0.164[a]
	S. typhimurium var. *copenhagen*	0.091
	S. heidelberg	0.087
C	*S. mbandaka*	0.074
D	*S. enteritidis* PT13	0.082
E	*S. thomasville*	0.131
F	*S. rubislaw*	0.075
G	*S. havana*	0.103
	C. freundii	0.147
	E. coli ATCC 25995	0.095

[a] The results are expressed as the means of triplicate deteminations.

Figure 4.1 Sodium dodecyl polyacrylamide gel electrophoresis of outer membrane proteins extracted from different bacteria and stained with Coommassie Brilliant Blue R-250. Lanes: 1- *Y. enterocolitica*, 2- *C. freundii*, 3- *S. arizona*, 4- *S. havana*, 5- *S. rubislaw*, 6- *S. senftenberg*, 7- *S. thomasville*, 8- *S. anatum*, 9- *S. gallinarum*, 10- *S. berta*, 11- *S. enteritidis* PT (13), 12- *S. hadar*, 13- *S.mbandaka*, 14- *S. thompson*, 15- *S. choleraesuis*, 16- *S. agona*, 17- *S. stanley*, 18- *S. brandenburg*, 19- *S. heidelberg*, 20- *S. typhimurium*, 21- *S. paratyphi* B, 22- *Sh. flexneri*, 23- *E. coli*. Lane M_r: Molecular weight standards ('000s).


M_r 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 M_r
- 205.0
- 116.0
- 97.0
- 84.0
- 66.0
- 55.0
- 45.0
- 36.0
- 29.0
- 24.0
- 20.0
- 14.2

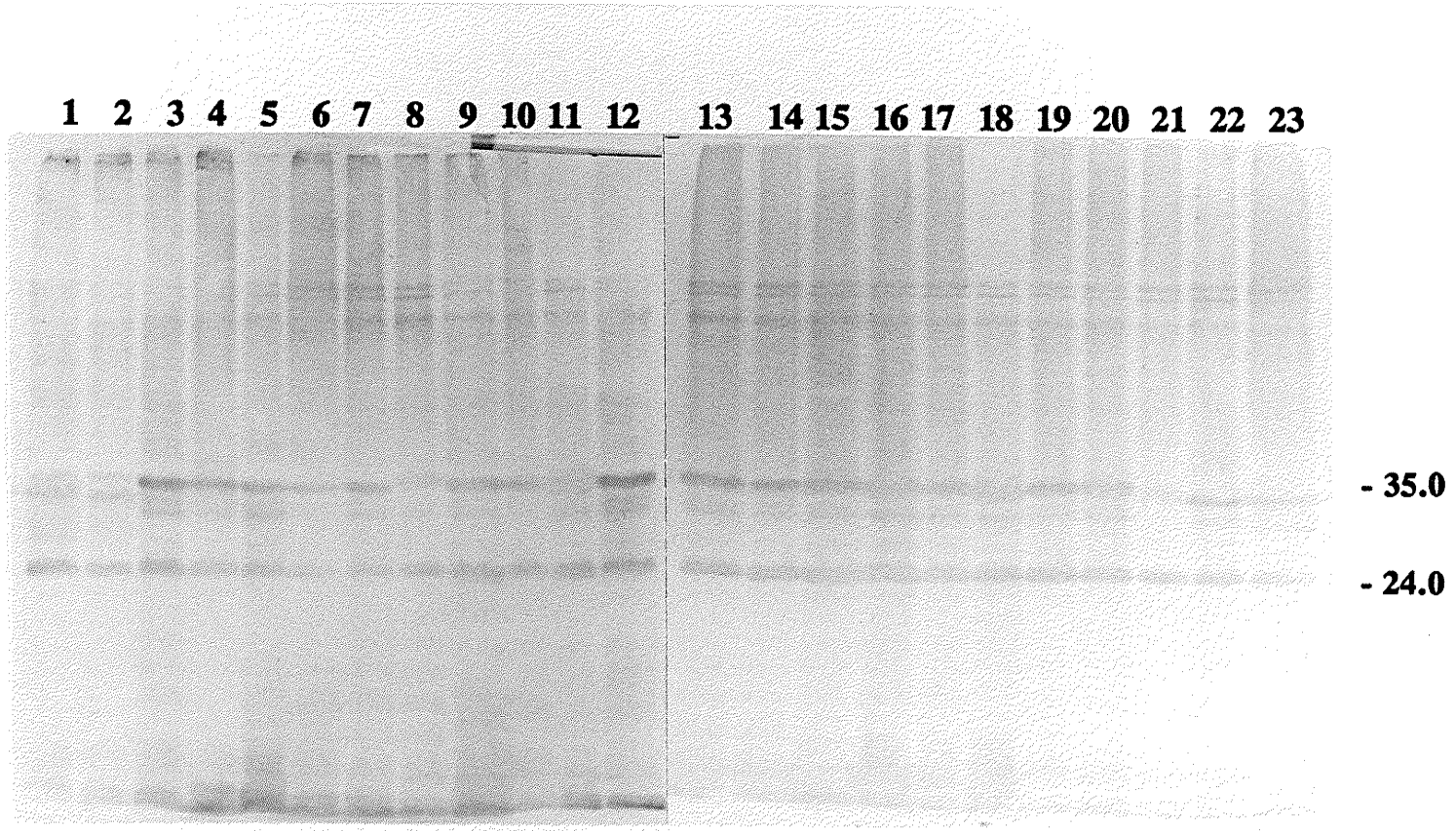
(Figure 4.1, lane 18) exhibited a major OMP band at 36 kDa, while *S. anatum* (Figure 4.1, lane 8), *S. enteritidis* (Figure 4.1, lane 11), S. agona (Figure 4.1, lane 16), *S. stanley* (Figure 4.1, lane 17), *S. brandenburg* (Figure 4.1, lane 18), *S. typhimurium* (Figure 4.1, lane 20) and *Sh. flexneri* (Figure 4.1, lane 22) exhibited a major band at M_r 35 kDa. These OMPs appear to be expressed in a higher number of molecules per bacterial cell in the aforementioned species. Furthermore, they may be more heat-resistant in some species than in other tested organisms such as *C. freundii, S. havana, S. gallinarum, S. berta, S. mbandaka, S. thompson, S. choleraesuis* and *S. paratyphi* B (Figure 1, lanes 2, 3, 9, 10, 13, 14, 15, and 21 respectively) which were devoid or exhibited only a faint electrophoretic band at the 35 to 36 kDa region.

Antigenic cross-reactivity between OMPs was investigated by immunoblotting using antisera produced against OMP extract of *S. typhimurium*. Incubation with antisera revealed two major proteins (35 and 24 kDa) and some minor polypeptides (Figure 4.2). These two proteins may be composed of a higher number of epitopes than other polypeptides. Such a conclusion is inferred from the intensity of the band recognized by antisera. Furthermore, the 35 kDa protein was present only in *Salmonella* serovars tested (Figure 4.2, lanes 3-21) while the 24 kDa protein was present in all organisms studied (Figure 4.2).

It is worthwhile to notice that yet another polypeptide with slightly lower M_r of 34 kDa was recognized by antisera in non-salmonellae species (Figure 4.2, lanes 1-2 and 22-23).

Crossreactivity between OMPs were further studied using MAb 1D6 in

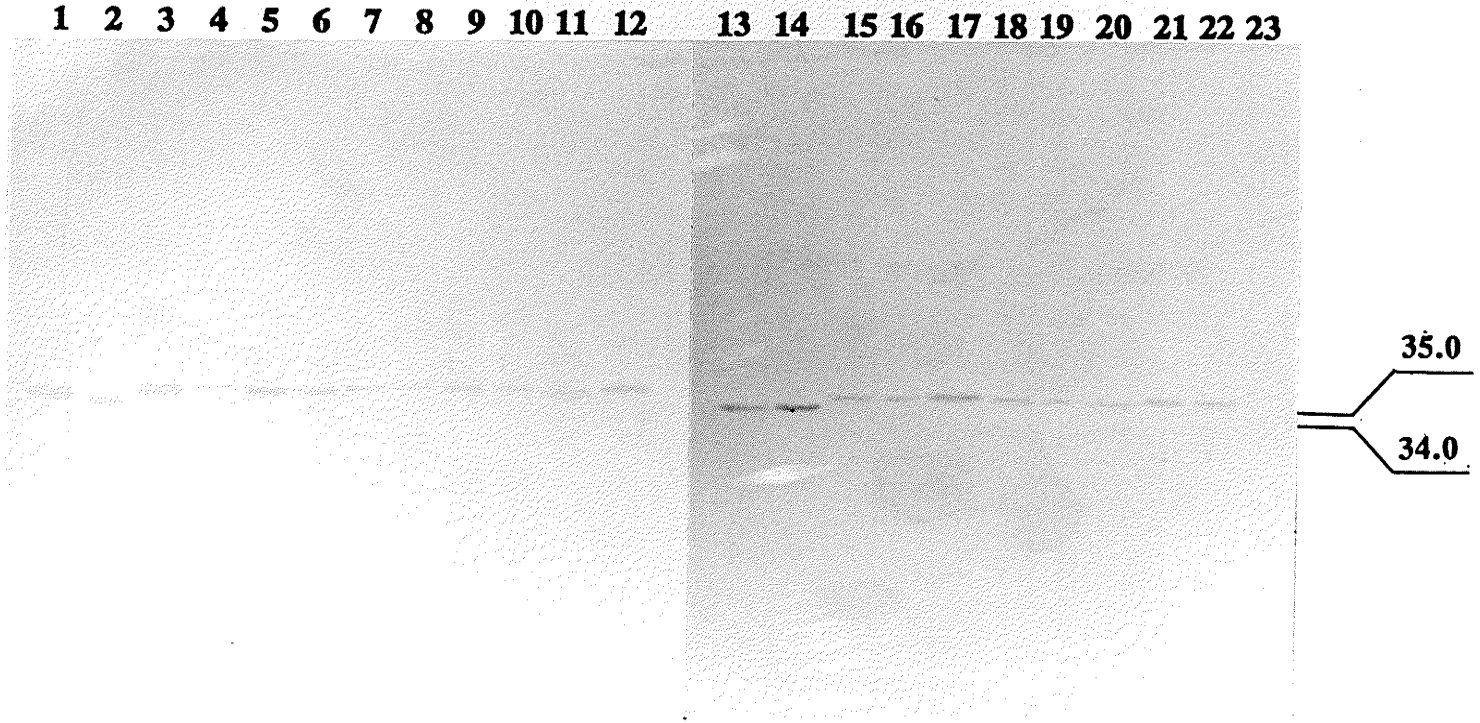
Figure 4.2 Immunoblot of SDS-PAGE of outer membrane proteins extracted from different bacteria (corresponding to gel Figure 4.1) and probed with polyclonal antisera. Lanes: 1- *Y. enterocolitica*, 2- *C. freundii*, 3- *S. arizona*, 4- *S. havana*, 5- *S. rubislaw*, 6- *S. senftenberg*, 7- *S. thomasville*, 8- *S. anatum*, 9- *S. gallinarum*, 10- *S. berta*, 11- *S. enteritidis* PT (13), 12- *S. hadar*, 13- *S. mbandaka*, 14- *S. thompson*, 15- *S. choleraesuis*, 16- *S. agona*, 17- *S. stanley*, 18- *S. brandenburg*, 19- *S. heidelberg*, 20- *S. typhimurium*, 21- *S. paratyphi* B, 22- *Sh. flexneri*, 23- *E. coli*.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
- 35.0
- 24.0

conjunction with immunoblotting. MAb 1D6 bound a single polypeptide at M_r 35 kDa present in all tested *Salmonella* species (Figure 4.3, lanes 3-12; 15-22) except an atypical S. *arizona* (Figure 4.3, lane 23). A different single polypeptide at M_r 34 kDa in non-salmonellae species was also recognized by MAb 1D6. Both polypeptides may be different allelic forms of the same protein, since both exhibited the same antigenic properties. Although applied monoclonal antibodies were produced against *S. typhimurium*, they reacted more strongly with *S. paratyphi* B (Figure 4.3, lane 3) and *S. heidelberg* (Figure 4.3, lane 5), both of B-serogroup. It is possible that the epitope on the OMP in these species is more repeated than in *S. typhimurium*.

The results clearly demonstrated that the 35 kDa polypeptide is a common antigen for all tested *Salmonella*, with the exception of *S. arizona*, containing the common epitope recognized by MAb 1D6. In addition, the 35 kDa polypeptide can be a useful molecular marker for the detection of *Salmonella* using immunoblotting. However, the application of this marker in an ELISA detection system is impeded by crossreactivity of MAb 1D6 with the non-salmonellae. It is noteworthy the absence of binding of monoclonal antibodies to *S. arizona* (Figure 4.3, lane 23). The 35 kDa polypeptide is indeed present in OMP extract obtained from this species as it has been revealed by the reaction with polyclonal antibodies (Figure 4.2, lane 3), however, it was not recognized by MAb 1D6. It is possible that the common epitope is missing, expressed in a low number on a protein molecule or buried in protein structure and rendered unaccessible to MAb 1D6.

Figure 4.3 Immunoblot of SDS-PAGE of outer membrane proteins extracted from different bacteria (corresponding to gels of figure 4.1) and probed with MAb 1D6. Lanes: 1- *E. coli*, 2- *Sh. flexneri*, 3- *S. paratyphi B*, 4- *S. typhimurium*, 5- *S. heidelberg*, 6- *S. brandenburg*, 7- *S. stanley*, 8- *S. agona*, 9- *S. choleraesuis*, 10- *S. thompson*, 11- *S. mbandaka*, 12- *S. hader*, 13- *Y. eterocolitica*, 14- *C. freundii*, 15- *S. enteritidis* PT (13), 16- *S. berta*, 17- *S. gallinarum*, 18- *S. natum*, 19- *S.thomasville*, 20- *S. senftenberg*, 21- *S.rubislaw*, 22- *S. havana*, 23. *S.arizona*. The M_r :('000) of OMPs (35.0 and 34.0) recognized by MAb 1D6 on the right-hand side.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
35.0
34.0

4.4.3 Effect of Heat Treatment on the Antigenicity of 35 and 24 kDa Proteins

Prior to extraction of OMPs, heat treatment (121°C for 15 min) was used to inactivate the bacteria. In order to determine what kind of effect the heat treatment had on the antigenicity of the 35 and 24 kDa proteins, a panel of *Salmonella* species was attenuated by sonication and subsequently OMPs were extracted as previously described. Extracted OMPs were subjected to SDS-PAGE in reducing and non-reducing conditions (Figure 4.4). The protein pattern of OMPs appeared to be different from the pattern of protein distribution as is apparent in Figure 4.1. Heat treatment of bacteria prior to extraction of OMPs yielded a higher number of electrophoretic bands (Figure 4.1) than in the case of sonication of organisms. However, the major protein band with the apparent M_r of 35 kDa was much more predominant when extracted from sonicated bacteria (Figure 4.4) than from heat treated organisms. In addition, all tested species exhibited another major protein at M_r about 38 kDa while *S. enteritidis* PT13 exhibited two other major proteins corresponding to M_r 37 and 38 kDa (Figure 4.4,lane 3). Reducing (Figure 4.4 B) and non-reducing (Figure 4.4 A) conditions had no effect on the distribution of the extracted proteins in SDS-PAGE. The excessive heat treatment of organisms may contribute to the high number of electrophoretic bands resulting from heat-modifiable proteins (Figure 4.1). Regardless of these differences, when the separated proteins were transferred to nitrocellulose and probed with antisera, the 35 and 24 kDa proteins were recognized predominantly in both cases (Figure 4.5).

Figure 4.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis of outer membrane proteins extracted from different bacteria, stained with Coommassie Brilliant Blue R-250 and solubilized in treatment buffer without reducing agent (A) or with reducing agent (B). Lane M_r: Molecular weight standards ('000s). Lanes: 1- *S. typhimurium*, 2- *S. mbandaka*, 3- *S. enteritidis* PT (13), 4- *S. thomasville*, 5- *S. rubislaw* , 6- *S. havana.*

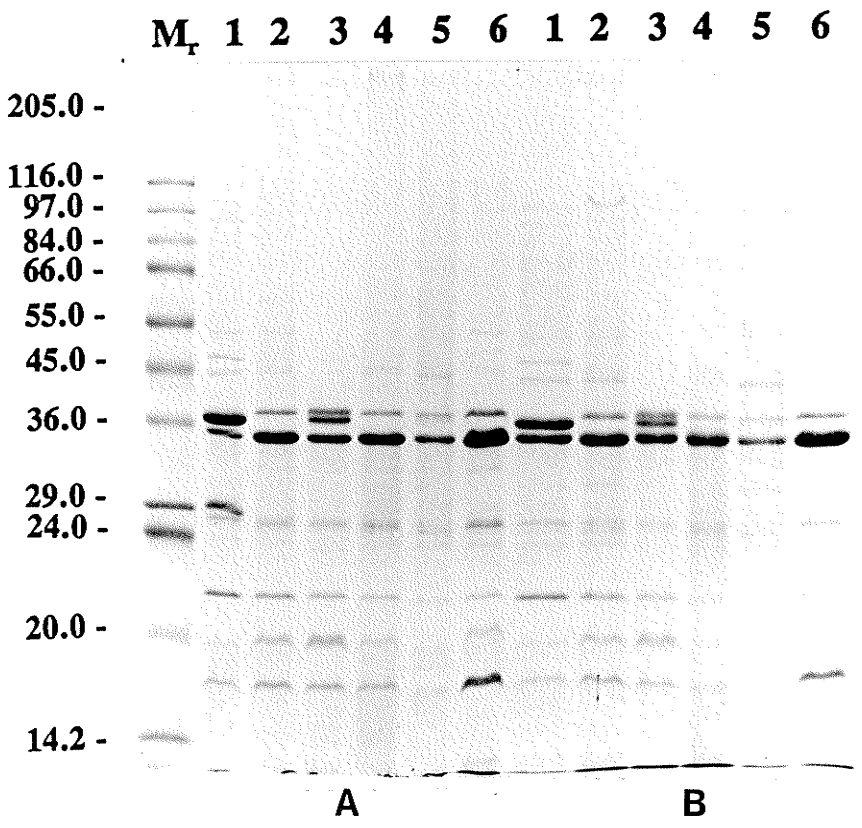
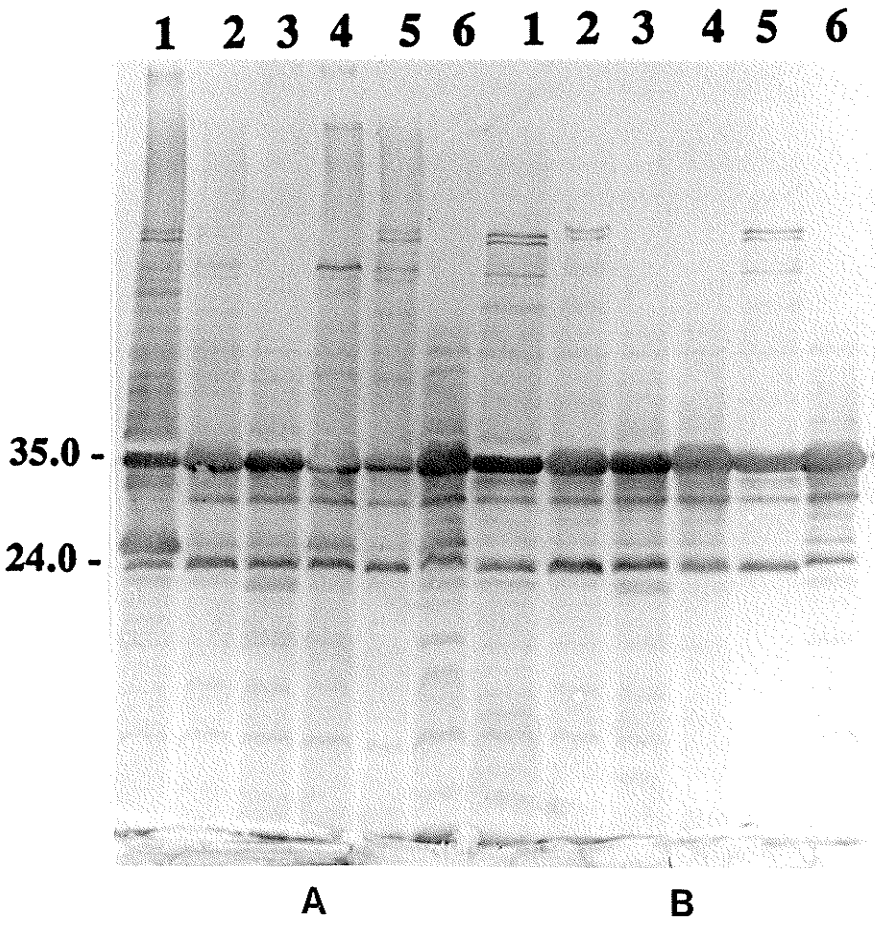

M r 1 2 3 4 5 6 1 2 3 4 5 6
205.0 -
116.0 -
97.0 -
84.0 -
66.0 -
55.0 -
45.0 -
36.0 -
29.0 -
24.0 -
20.0 -
14.2 -
A
B

Figure 4.5 Immunoblot of SDS-PAGE of outer membrane proteins extracted from different bacteria (corresponding to gel of Figure 4.4) and probed with polyclonal antisera. The M_rs ('000) of OMPs (35.0 and 24.0) recognized by the antisera are given on the left-hand side. Lanes: 1- *S. typhimurium,* 2- *S. mbandaka,* 3- *S. enteritidis* PT (13), 4- *S. thomasville*, 5- *S. rubislaw*, 6- *S. havana.*


1 2 3 4 5 6 1 2 3 4 5 6
35.0 -
24.0 -
A
B

4.4.4 Effect of Temperature on the OMP Profiles

The OMP extracts obtained from sonicated bacteria were analysed either as native or denatured proteins on SDS-PAGE and immunoblotting. Native SDS-PAGE revealed the presence of only one major protein band with an apparent M_r 28 kDa for all tested *Salmonella* species (Figure 4.6 A). After OMPs were heat treated at 100°C for 5 min in the presence of β-mercaptoethanol, the resulting SDS-PAGE revealed the presence of two predominant protein bands with apparent molecular weight of 35 and 38 kDa, but the absence of 28 kDa band for all tested bacteria (Figure 4.6 B).

Figure 4.7 shows immunoblot of SDS-PAGE on to nitrocellulose membrane probed with MAb 1D6. The antibody bound one major OMP polypeptide present at 28 kDa and about eight minor OMP polypeptides in the range between 34 and 40 kDa present in unheated extracts (Figure 4.7 A). In contrast, when heated OMPs were probed with MAb 1D6, only one major band at 35 kDa was recognized. This phenomenon could be attributed to the presence of heat modified proteins and has been ascribed to i) the high content of β-structure, ii) excessive binding of the protein to SDS and iii) changes in the viscosity which tend to increase upon heat treatment, which in turn detracts its mobility (Shnaitman, 1973, Nakamaura and Mizushima, 1976, Lugtenberg and van Alphen, 1983).

It is noteworthy the intensity and somewhat different band width of a M_r 35 kDa OMP polypeptide in heat-treated extracts (Figure 4.6 B) which may indicate that this protein is composed of more than one polypeptide of similar molecular weight. To confirm this hypothesis, all OMP extracts were subjected to more excessive heat

Figure 4.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis of outer membrane proteins extracted from different bacteria, solubilized in non-reducing buffer and loaded without boiling (A) or with reducing buffer and boiled for 5 min at 100°C (B) and stained with Coommassie Brilliant Blue R-250. Lanes: 1- *S. havana*, 2- *S. rubislaw*, 3- *S. thomasville*, 4- *S. enteritidis* PT (13), 5- *S. mbandaka*, 6- *S. typhimurium*. Lane M_r:Molecular weight standards ('000s).

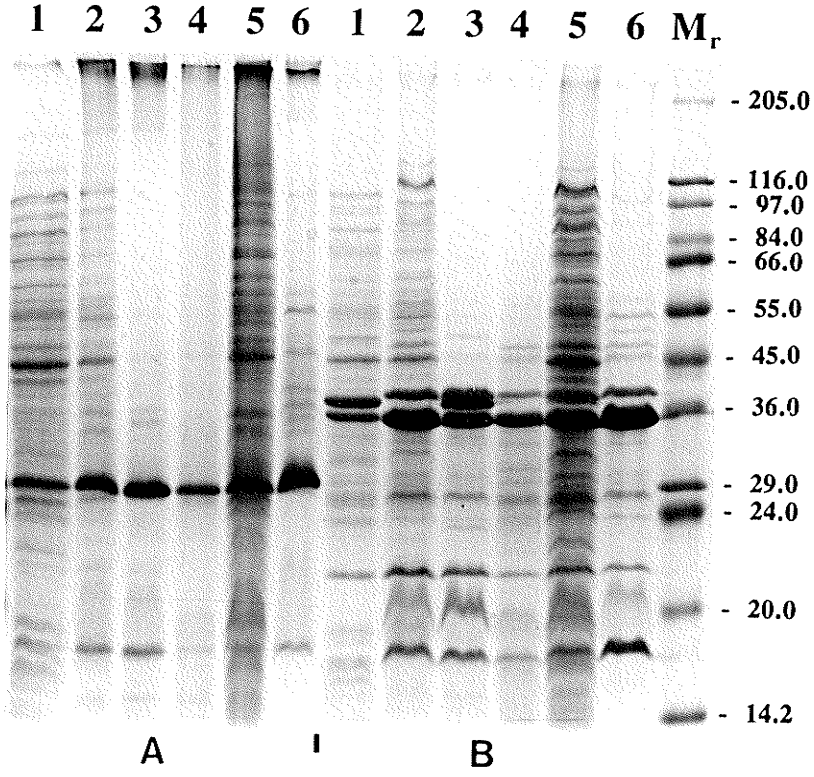
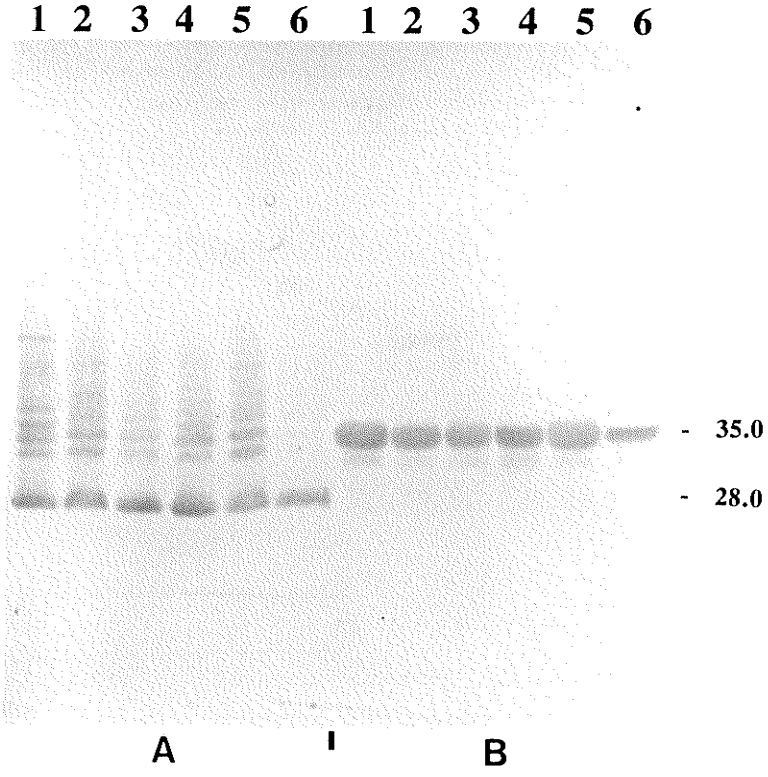

1 2 3 4 5 6 1 2 3 4 5 6 Mr
- 205.0
- 116.0
- 97.0
- 84.0
- 66.0
- 55.0
- 45.0
- 36.0
- 29.0
- 24.0
- 20.0
- 14.2
A
B

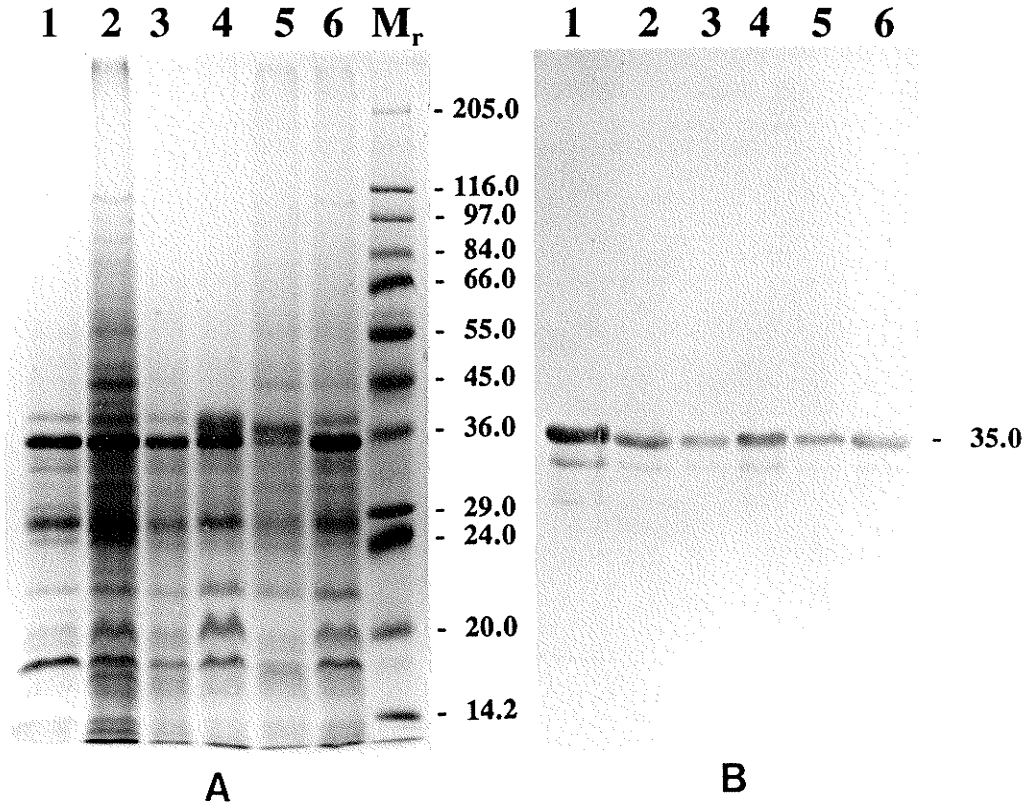
Figure 4.7 Immunoblot of SDS-PAGE of outer membrane proteins extracted from different bacteria (corresponding to gel of Figure 4.6) and probed with MAb 1D6. Lanes: 1- *S. havana*, 2- *S. rubislaw*, 3- *S. thomasville*, 4- *S. enteritidis* PT13, 5- *S. mbandaka*, 6- *S. typhimurium*.


1 2 3 4 5 6 1 2 3 4 5 6
35.0
28.0
A
B

treatment (121°C, 15 min) prior SDS-PAGE (Figure 4.8 A) and immunoblotting (Figure 4.8 B). The results revealed that increasing the exposure to high temperature did not affect the overall protein electrophoretic pattern (Figure 4.8 A), however, the appearance of the 35 kDa protein was somewhat different. Heating of OMP extracts resulted in the narrower protein band and an apparent increase in the reactivity with MAb 1D6 (Figure 4.8 B). This suggested that the 35 kDa protein was heat modifiable, displaying an apparent molecular weight in the range between 28 to 40 kDa when OMP extracts were only solubilized with SDS at room temperature. Upon heating, however, this protein was antigenically stable and highly reactive with MAb 1D6. It was also observed that MAb 1D6 recognized its epitope much stronger in blots than in ELISA. Two reasons could stand behind this observation; the hidden epitopes were exposed after the protein was subjected to denaturing conditions during SDS-PAGE and electrotransfer, and nitrocellulose has much higher capacity for protein binding than the polystyrene solid phase leading to a significant increase in the sensitivity of immunoblotting over ELISA (Sarasombath *et al.*, 1988).

To further scrutinize the heterogeneity of OMPs, electrophoretically separated OMP forms (native and denatured) (Figure 4.7 A) were analysed for carbohydrates using the method of Kondo and coworkers (1991). None of these forms, however, contained sugar moieties that may be responsible for their heterogeneity. In addition, no sugar was detected in the 35 kDa protein as apparent in Figure 4.7 B (data not shown).

Figure 4.8 SDS-PAGE of heat treated (121°C, 15 min) outer membrane proteins extracted from different bacteria and stained with Coommassie Brilliant Blue R-250 (A) or immunobloted and probed with MAb 1D6 (B). Lanes: 1- S. *havana*, 2- *S. rubislaw*, 3- *S.thomasville*, 4- *S.enteritidis* PT 13, 5- *S. mbandaka*, 6- *S. typhimurium*. Lane M_r: Molecular weight standards ('000).


1 2 3 4 5 6 Mr
1 2 3 4 5 6
- 205.0
- 116.0
- 97.0
- 84.0
- 66.0
- 55.0
- 45.0
- 36.0
- 29.0
- 24.0
- 20.0
- 14.2
- 35.0
A
B

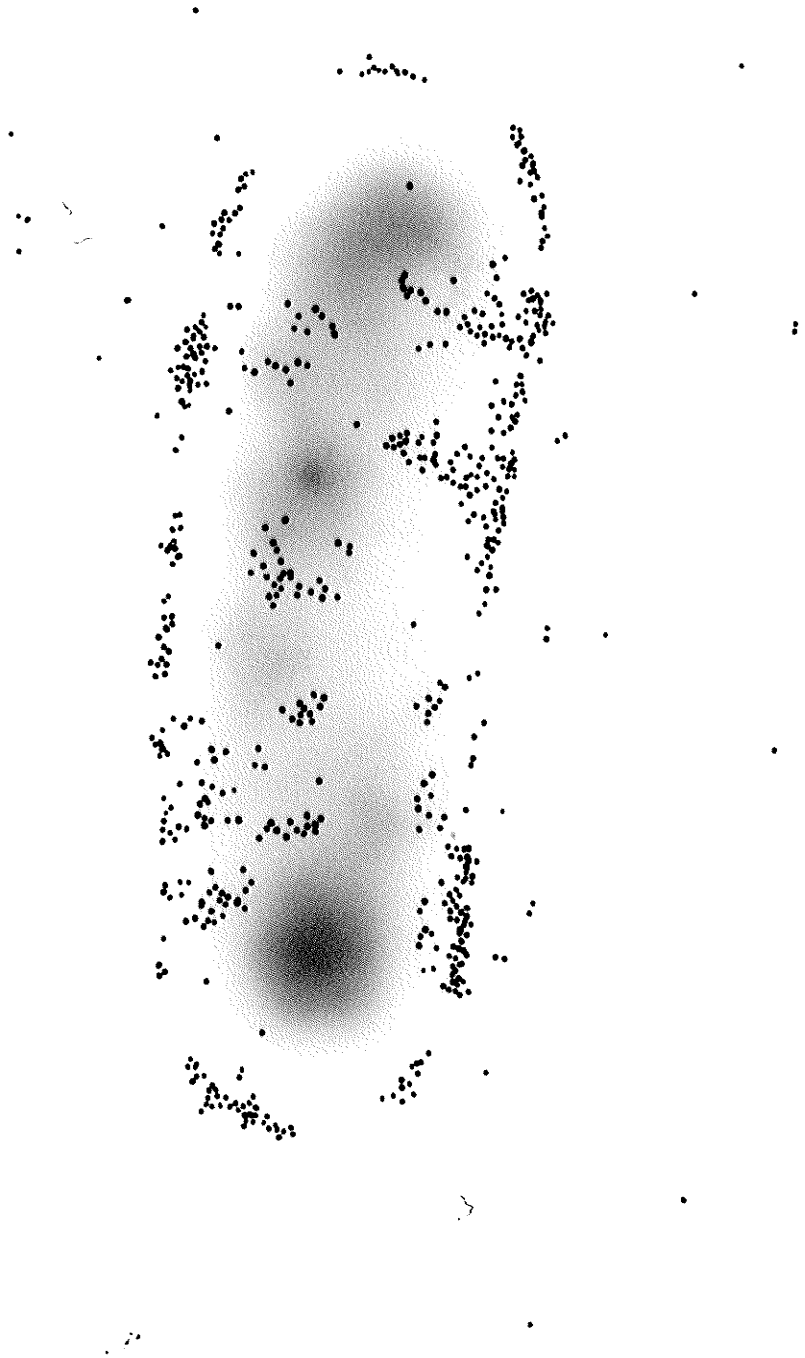
4.4.5 Epitope Accessibility to MAb 1D6

Although the 35 kDa protein was recognized by MAb 1D6 which was produced against *S. typhimurium* cells, there was no conclusive evidence that this protein is a surface exposed compound on a bacterial cell. It is because *Salmonella* cells were heat treated before immunization which could lead to rupture of some cells and consequently exposing hidden epitopes to the host defence system and in turn, triggered the synthesis of antibodies. To confirm location of the 35 kDa OMP within a *Salmonella* cell envelope, transmission immunoelectron microscopy on the intact whole cells of *S. typhimurium* was performed. Incubation of the cells with MAb 1D6 led to visualization of electrondense patches of gold particles outside the cell wall (Figure 4.9) indicating the location of the 35 kDa protein. This protein, however, was significantly less abundant on the surface of *S. typhimurium* cells than the LPS-O antigen as shown in previous studies (Jaradat and Zawistowski, 1995). In addition, the steric hindrance imposed by the long chains of LPS-O antigen may impede the accessibility of MAb 1D6 to its epitope.

4.4.6 Effect of Media on the OMP Profiles

In order to study the effect of the growth media on the antigenicity of OMPs (24 and 35 kDa), *Salmonella* representing six serogroups were grown on different media such as lactose broth, peptone water broth, universal pre-enrichment broth and M-broth. The media were chosen according to their nutrient content. Universal and M-broth are very rich in nutrients and minerals while lactose broth and peptone water are relatively poor. OMP extracts from *S. typhimurium, S. mbandaka, S. enteritidis, S. thomasville, S.*

Figure 4.9 Electron micrograph of *S. typhimurium* probed with MAb 1D6 and goat anti-mouse immunoglobulins coupled to 20 nm gold spheres. Magnification: x 20,000.



rubislaw and *S. havana* representing groups B, C, D, E, F and G, respectively, were subjected to SDS-PAGE electrophoresis (Figure 4.10). The electrophoretic profiles of separated OMPs obtained from *Salmonella* grown on all four media were generally similar, these results appeared to be consistent with results obtained by Choi and coworkers (1989) and Wexler and coworkers (1992). Both reported that changes in culture media did not appreciably affect the appearance of the OMP profiles extracted from *Pasteurella multocida* and *Bacteroides distasonis*. However, some minor differences were observed. These differences pertained to some proteins in the region of 35-36 kDa. For example, the 35 kDa OMP electrophoretic band was stained more intense in all *Salmonella*, except *S. typhimurium* obtained from universal broth (Figure 4.10, lanes 2, 6, 10, 14, 18) and M-broth (Figure 4.10, lanes 4, 8, 12, 16, 20). For *S. typhimurium*, the 35 kDa OMP was only observed in extracts from universal broth (Figure 4.10, lane 22). It is possible that the expression of this protein was dependant on composition of growth media. It has been reported that when bacteria are grown under shortage or excess of nutrients or minerals, some proteins may be suppressed or expressed in a low number of molecules (Pettersson *et al.*, 1993).

When OMP extracts were transferred electrophoretically to nitrocellulose membranes and probed with mouse antisera, two proteins turned to be the most antigenically stable, 35 and 24 kDa (Figure 4.11). These proteins were predominantly recognized with equal avidity by polyclonal antibodies, regardless of the type of media used to support bacterial growth. These findings are in agreement with our earlier results obtained during the course of these studies that both proteins represent major outer

Figure 4.10 Sodium dodecyl polyacrylamide gel electrophoresis of outer membrane proteins extracted from bacteria grown in different media and stained with Coommassie Brilliant Blue R-250. Lanes:1 to 4, *S. havana* grown in peptone water broth, universal preenrichment broth, lactose broth and M-broth respectively (the arrangement of the media is used for all the bacteria appear in this gel and the corresponding blot); 5 to 8, *S. rubislaw*; 9 to 12, *S. thomasville*; 13 to 16, *S.enteritidis* PT (13); 17 to 20, *S. mbandaka*; 21 to 24, *S. typhimurium*. Lane M_r: Molecular weight standards ('000).

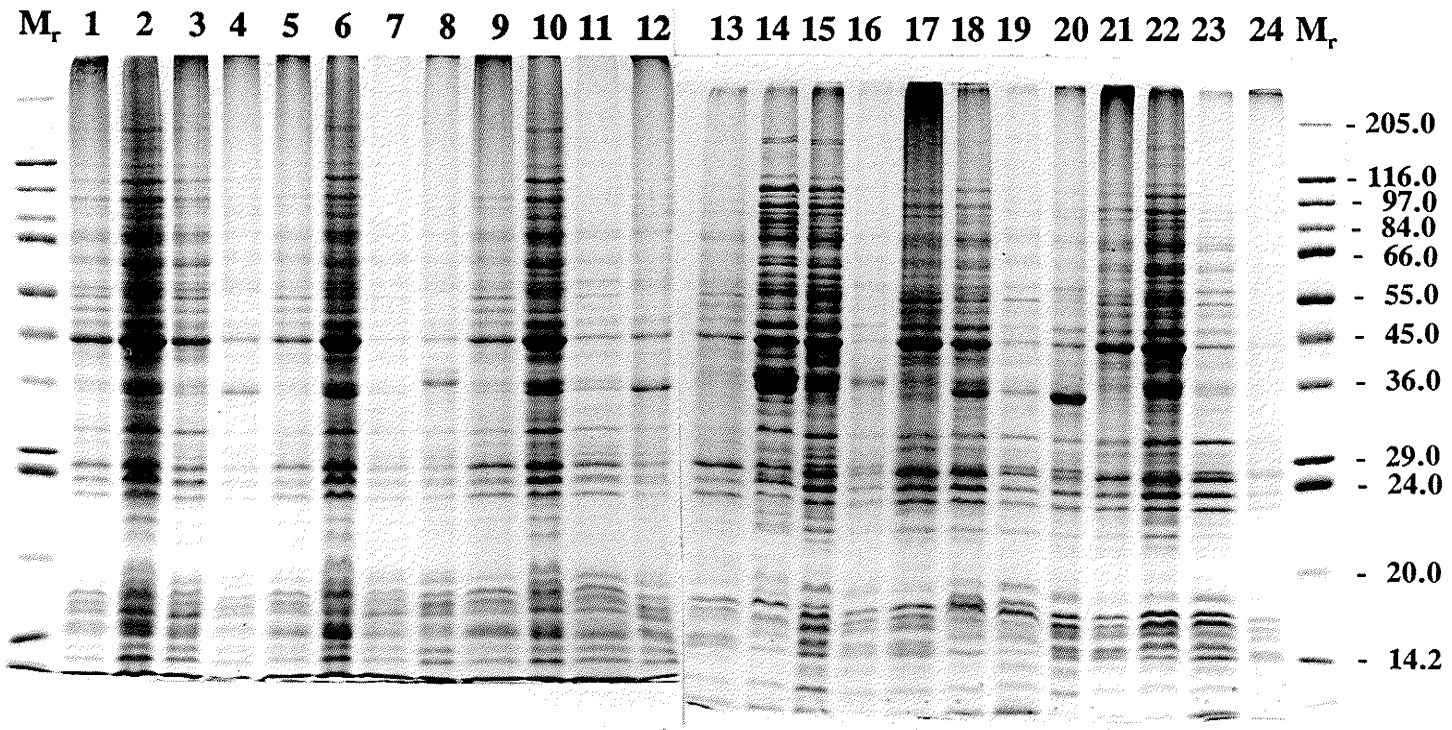
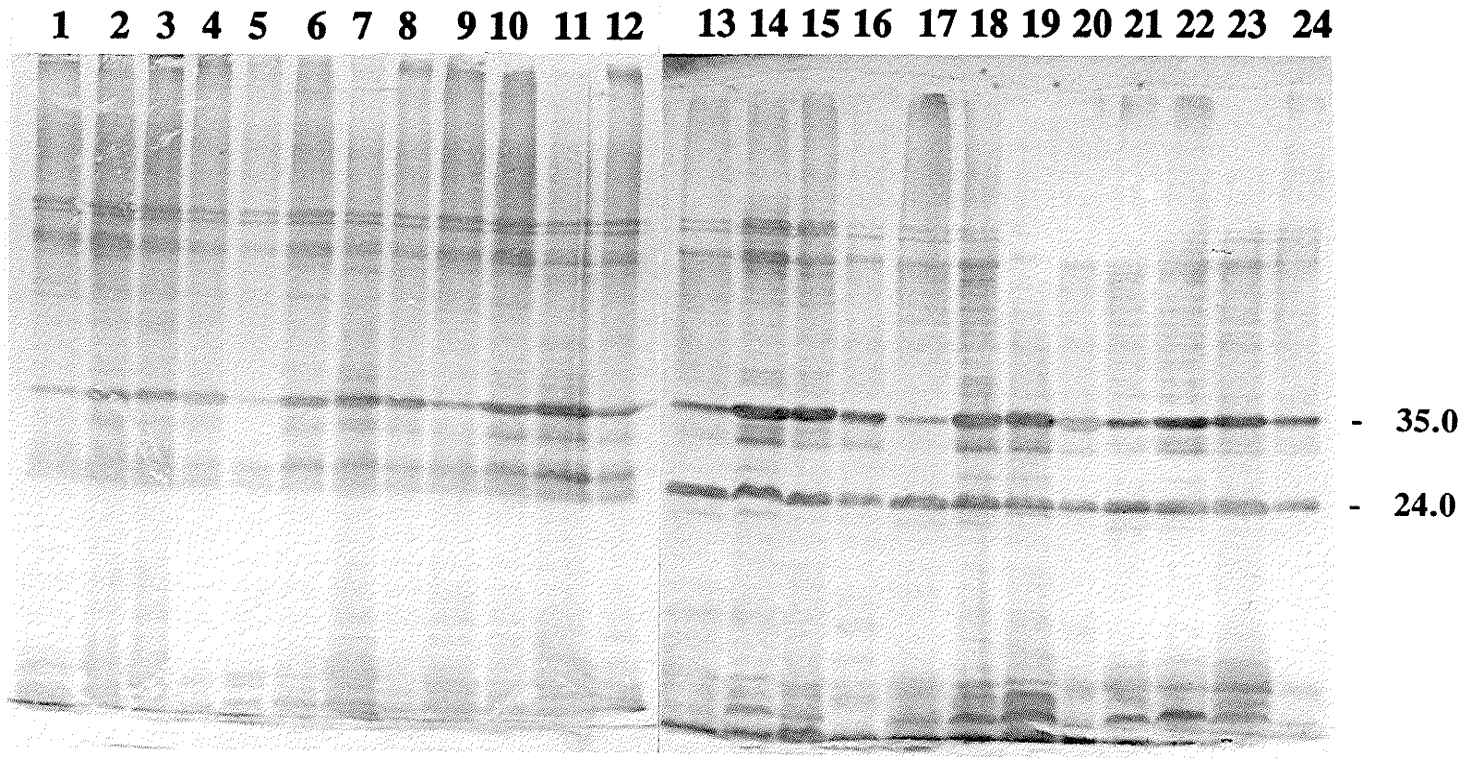

M_r 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 M_r
- 205.0
- 116.0
- 97.0
- 84.0
- 66.0
- 55.0
- 45.0
- 36.0
- 29.0
- 24.0
- 20.0
- 14.2

Figure 4.11 Immunoblot of SDS-PAGE of outer membrane proteins extracted from different bacteria (corresponding to gel of Figure 4.10) and probed with polyclonal antisera. Lanes:1 to 4, *S. havana* grown in peptone water broth, universal preenrichment broth, lactose broth and M-broth respectively (the arrangement of the media is used for all the bacteria); 5 to 8, *S. rubislaw*; 9 to 12, *S. thomasville*; 13 to 16, *S. enteritidis* PT (13); 17 to 20, *S. mbandaka*; 21 to 24, *S.typhimurium*. The M_r: ('000) of OMPs (35.0 and 24.0) recognized by the antisera on the right hand side.


1 2 3 4 5 6 7 8 9 10 11 12
13 14 15 16 17 18 19 20 21 22 23 24
- 35.0
- 24.0

membrane proteins of *Salmonella* and both featured relatively high antigenic stability.

4.5 Conclusions

In this study, we have identified two major antigenic outer membrane proteins in *Salmonella* species with an apparent M_r 35 and 24 kDa by the use of SDS-PAGE and immunoblotting. Monoclonal antibody designated MAb 1D6 reacted only to the 35 kDa protein in all tested *Salmonella* strains with the exception of an atypical *S. arizona*. The type of growth media and reducing conditions had no effect on the antigenicity of the 35 kDa protein. However, the electrophoretic appearance of the 35 kDa protein was influenced by heat-treatment which indicates that it is a heat modifiable protein. Electron microscopy studies confirmed that the 35 kDa protein appeared to have epitopes on the surface of the bacterial cell. These epitopes are either, expressed in low number or shielded by the long LPS chains as can be inferred from the low number of gold particles attached to the bacterial cells.

5. GENERAL DISCUSSION

Salmonella is considered to be the most common foodborne pathogen worldwide (Gouthrie, 1992). In order to control the spread of infection and eliminate the prevalence of this pathogen, a fast and reliable detection method must be developed. Enzyme-linked immunosorbent assays utilizing monoclonal antibodies can fulfil this requirement. Four monoclonal antibodies (MAbs) 5A5, 5B2, 4A8 and 1D6 were produced against B-serogroup *Salmonella*. Although antibodies of similar specificities have been produced previously (Kosimar and Cebra, 1983, Lee *et al.*, 1990, Yamaura *et al.*, 1992) they were not thoroughly characterized.

MAbs 5A5, 5B2 and 4A8 were found to be reactive with B-serogroup *Salmonella* bearing LPS O 5 antigen as revealed by ELISA, while MAb 1D6 reacted with the 35 kDa protein present in *Salmonella* species. Furthermore the specificity of MAbs to LPS O-antigen was confirmed by immunoblotting. All three MAbs selectively bound O-antigen bearing the acetyl group.

All MAbs reacted with both live and heat-attenuated (80°C, 20 min) *Salmonella* as assessed by ELISA and confirmed by immunoelectron microscopy. Anti-LPS MAbs bound to the whole S. typhimurium cell with high frequency indicating LPS-O5 antigen is a significant part of the external leaflet of the bacterial outer membrane. This appeared to bo consistent with other reports that have indicated that bacterial LPS is expressed in high frequency accounting for 50-70% of surface of the outer monolayer (Lugtenberg and

van Alphen, 1983). In contrast, MAb 1D6 exhibited low reactivity with live cells, evidenced by the low density of gold spheres bound to the cell. The shielding effect of LPS leaflet may be accounted for the limited accessibility of MAb 1D6 to its epitope (Cloeckaert *et al.*, 1990).

Since MAbs (5A5, 5B2 and 4A8) were shown to be specific to LPS, the effect of various treatments such as detergent and extreme pH on the reactivity of antibodies with *Salmonella* were investigated. Cholic acid used to extract LPS, had an adverse effect on binding of all MAbs to *S. typhimurium*. Acid treatment had a negligible effect on enhancement of detection of *S. typhimurium* by anti-LPS MAbs although the antigenicity of LPS O-5 was conserved. This is in disagreement with findings by Yamoura and coworkers (1992) that prolonged acid treatment could destroy the acetyl group. In contrast, alkaline conditions found to structurally alter the O-acetyl group thus rendered this antigen undetectable.

MAb 1D6 and mouse antisera raised against *S. typhimurium* were used to study the antigenicity of *Salmonella* outer membrane proteins by SDS-PAGE and immunoblotting. Two major antigenic proteins 35 and 24 kDa were identified. The 35 kDa protein was present only in *Salmonella* species while 24 kDa OMP was detected in all tested *Salmonella* and non-salmonellae.

Crossreactivity between OMPs was further studied using MAb 1D6 in conjunction with immunoblotting. MAb 1D6 reacted with a single polypeptide with M_r of 35 kDa present in all tested *Salmonella* species except an atypical *S. arizona*. Our results demonstrated that the 35 kDa polypeptide is a common antigen for all tested *Salmonella*

with the exception of *S. arizona*.

Antigenicity of the 35 kDa protein appeared to be stable and not affected by different growth media, heat treatment as well as reducing conditions. However, analysis of OMP extracts in their native form by immunoblotting, revealed that MAb 1D6 bound several isoforms of the 35 kDa protein; one major band at M_r 28 kDa and about eight minor forms in the range between 34 and 40 kDa. These forms were converted to a single form with M_r 35 kDa upon heating at 100°C indicating that this protein is most likely a heat modifiable protein. Further heat treatment (121°C, 15 min) did not affect the antigenicity of this 35 kDa protein.

6. CONCLUSIONS AND RECOMMENDATIONS

Four monoclonal antibodies (MAbs) 5A5, 5B2, 4A8 and 1D6 were produced against *S.typhimurium* outer membrane components. MAbs 5A5, 5B2 and 4A8 recognized the LPS O 5 antigen of *Salmonella* B-serogroup as revealed by ELISA, SDS-PAGE and immunoblotting, while MAb 1D6 reacted with a 35 kDa outer membrane protein present in all tested Salmonella species except *S. arizona*. All monoclonal antibodies were found to bind both live and heat-treated *Salmonella* cells.

The ability of MAbs to detect live cells of *S. typhimurium* was further confirmed by immunoelectron transmission microscopy. The high frequency of binding suggested that the LPS-O5 factor is a significant component of a LPS leaflet surrounding *S. typhimurium* cells. The MAb 1D6 also bound intact cells of *S. typhimurium* but to the lesser extent than other MAbs. This is because, the 35 kDa OMP may be present in a low number on the bacterial cell surface or it may be less accessible by MAb 1D6 due to the steric hindrance of LPS chains surrounding the bacterial cells. Further studies should be performed on treatment *S. typhimurium* cells with a detergent such as EDTA which is known to extract some of the LPS, or using rough *S. typhimurium* mutant in order to ascertain whether the low binding of MAb 1D6 to the cells is due to the steric hindrance or to the low amount of 35 kDa OMP expressed on the surface.

MAb 1D6 and mouse antisera were used to study the antigenicity of the *Salmonella* outer membrane proteins. Two OMPs with apparent M_r 35 and 24 kDa were

identified by using antisera. MAb 1D6 bound a single protein band at M_r 35 kDa in all tested *Salmonella* species except *S. arizona* and a 34 kDa protein in non-salmonella species as assessed by SDS-PAGE and immunoblotting. Above findings indicated that both 35 and 34 kDa proteins may be different allelic forms of the same protein. Further studies should be conducted to investigate the reasons behind the inability of MAb 1D6 to recognize *S. arizona*.

Reducing and non-reducing conditions had no effect on the antigenicity of the 35 kDa protein. However, the electrophoretic appearance of the 35 kDa OMP was influenced by heat-treatment. It is most likely, the 35 kDa OMP is a heat modifiable protein. It exhibited an apparent M_r of 28 to 40 kDa when OMP extracts were solubilized at room temperature and separated by SDS-PAGE, while it appeared as an electrophoretic band at M_r 35 kDa when OMP extracts were boiled for 5 min prior separation. Furthermore, the type of growth media had no effect on the antigenicity of the 35 and 24 kDa proteins. However, further studies should be conducted to examine the effect of environmental conditions such as pH, A_w, growth temperature and effect of excesses or shortages of certain minerals and nutrients such as iron and maltose on the antigenicity of the 35 kDa protein.

The 35 kDa protein can be useful as a marker for detection of *Salmonella* using immunoblotting, however, the application of this marker in an ELISA detection system is impeded by the crossreactivity of MAb 1D6 with *Salmonella* and non-salmonella. In contrast, MAbs recognized LPS can be much useful in the ELISA due to the high frequency of LPS antigen and the ability of MAbs to equally recognize live and heat

treated cells without any further treatment in addition to the absence of minimal crossreactivity with other serogroups. The aforementioned properties of MAbs 5A5, 5B2 and 4A8 make them good candidates to be utilized in a capture ELISA to detect *Salmonella* in food and clinical samples, thus eliminating a selective enrichment step. However, studies should be conducted to develop an ELISA using these MAbs to explore their ability to detect *S. typhimurium* in foods.

The 35 kDa protein appears to have epitopes exposed at the surface of the bacterial cells which may be utilized to develop vaccine against salmonellosis and enteric fever to replace the traditional toxic whole cell vaccines, yet further studies should be conducted to explore this area.

7. REFERENCES

Andrews, W. H. 1985. A review of culture methods and their relation to rapid methods for the detection of *Salmonella* in foods. Food Technol. 39:77-82.

Anwar, H. 1991. Surface-exposed antibody-accessible outer membrane proteins of *Bordetella pertussis*. Can. J. Microbiol. 37:590-593.

Bean, N. H. and Griffin, P. M. 1990. Foodborne disease outbreaks in the United States, 1973-1987: Pathogens, vehicles, and trends. J. Food Prot. 53:804-817.

Bentley, A. T. and Klebba, P. E. 1988. Effect of lipopolysaccharide structure on reactivity of anti-porin monoclonal antibodies with the bacterial cell surface. J. Bacteriol. 170:1063-1068.

Benz, R. 1988. Structure and function of porins from gram-negative bacteria. Ann. Rev. Microbiol. 42:359-393.

Beveridge, T. J. 1981. Ultrastructure, Chemistry and function of the bacterial wall. Int. Rev. Cytol. 72:229-317.

Brooks, J.L., Mirhabiballahi, B. and Kroll, R. G. 1992. Experimental enzyme-linked amperometric immunosensors for the detection of *Salmonella* in foods. J. Appl. Bacteriol. 37:189-196.

Bryan, F. L. 1981. Current trends in foodborne salmonellosis in the United States and Canada. J. Food Prot. 44:394-402.

Butler, J. and Kaye, D. 1992. Enteric infection. In: Cecil Textbook of Medicine. Ed. J. B. Wyngaarden, L. H. Smith and J. C. Bennet. W. B. Saunders Company, Philadelphia.

Chart, H., Frost, J. A. and Rowe, B. 1993. Expression of a new porin 'OmpE' by strains of Salmonella enteritidis. FEMS. Microbiol. Lett. 109:185-188.

Choi, K. H., Maheswaran, S. K. and Felice, L. J. 1989. Characterization of outer membrane protein-enriched extracts from *pasteurella multocida* isolated from turkeys. Am. J. Vet. Res. 50:676-683.

Cleoeckaert, A., Wergifosse, P., Dubray, G. and Limet, J. N. 1990. Identification of seven surface-exposed *Brucella* outer membrane proteins by use of monoclonal antibodies:Immunogold labelling for electron microscopy and enzyme-linked immunosorbent assay. Infect. Immun. 58:3980-3987.

Costerton, J. W., Ingram, J. M. and Cheng, K. J. 1974. Structure and function of the cell envelope of gram-negative bacteria. Biol. Rev. 38:87-110.

D'Aoust, J. Y. and Sewell, A. M. 1988. Detection of *Salmonella* with the BioEnzabead immunoassay technique. J. Food Prot. 51:538-541.

Davis, R. L., Parton, R., Coote, J. G., Gibbs, H. A. and Freer, J. H. 1994. Evaluation of different methods for the detection of outer membrane proteins and lipopolysaccarides of *Pasteurella haemolytica* by immunoblotting. J. Immunol. Meth. 167:35-45.

Depamphilis, M. L. and Adler, J. 1971. Purification of intact flagella from *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. 105:376-383.

Dirienzo, M. J., Nakamura, K. and Inouye, M. 1978. The outer membrane proteins of gram-negative bacteria: Biosynthesis, assembly and functions. Ann. Rev. Biochem. 47:481-532.

Drews, G., Weckesser, J. and Mayer, H. 1978. Cell envelopes. In the photosynthetic bacteria (Eds. R.Y. Clyton, W.R. Sistrom), pp. 61-78. Plenum Prss, New York, London.

Duguid, J. P. 1959. Fimbriae and adhesive properties in *Klebsiella* Strains. J. Gen. Microbiol. 21:271-286.

Eckner, K. F., Mciver, D., Lepper, W. A., Fanning, L., Curiale, M. S., Flowers, R. S. and Robison, B. 1992. Use of an elevated temperature and novobiocin in a modified enzyme-linked immunosorbent assay for the improved recovery of *Salmonella* from foods. J. Food Prot. 55:758-762.

Elkins, K. and Metcalf, E. S. 1985. Binding activity of a murine anti-lipid A monoclonal antibody. Infect. Immun. 48:597-600.

Finlay, B. B. and Falkow, S. 1989a. *Salmonella* as an intracellular parasite. Mol. Microbiol. 3:1833-1844.

Finlay, B. B. and Falkow, S. 1989b. Common themes in microbial pathogenicity. Microbiol. Rev. 53:210-230.

Foster, E. M. 1978. Foodborne hazards of microbial origin. Fed. Proc. 37: 2577 2581.

Friguet, B., Djavadi-ohaniance, L. and Goldberg, M. 1983. In "Immunoenzymatic Techniques". S. Avrameas (Ed.) pp. 171-174.

Galanos, C., Lüderitz, O., Rietschel, E. T., and Westphal, O. 1977. New aspects of the chemistry and biology of bacterial lipopolysaccharide with special reference to their lipid A component. In International Review of Biochemistry. Biochemistry of lipids II. volume 14. (Ed. Goodwin T.W.), pp 239-335. University Park Press. Baltimore.

Gibb, A. P., Barclay, G. R., Poxton, I. R. and Padova, F. D. 1992. Frequencies of lipopolysaccharide core types among clinical isolates of *Escherichia coli* defined with monoclonal antibodies. J. Infect. Dis. 166:1051-1057.

Goding, J. W. 1983. In monoclonal antibodies: principles and practice. Academic Press, London.

Goldman, R. C. and Hunt, F. 1990. Mechanism of O-antigen distribution in lipopolysaccharide. J. Bacteriol. 172:5352-5359.

Grund, S. and Stople, H. 1992. Adhesion of *Salmonella typhimurium* var.*copenhagen* in the intestines of pigeons. Int. J. Food. Microbiol. **15**:299-306.

Guling, P. A. and Curtiss, R. 1987. Plasmid-associated virulence of *Salmonella typhimurium*. Infect. Immun. 55:2891-2901.

Guthrie, R. K. 1992. *Salmonella*. CRC. Press. Inc., Boca Raton. FL.

Hancock, R. E. W. 1987. Role of porins in outer membrane permeability. J. Bacteriol. 169:929-933.

Harlow, E. and Lane, D. 1988. Growing hybridoma. In antibodies: A laboratory manual. Cold Spring Harobor Laboratory. New York, pp.

Hansman, D. and Lawrence, A. 1993. Outer membrane protein and immunoblot analysis of Australian isolated of *Haemophilus influenzae*. J. Med. Microbiol. 38:378-383.

Hargrett-Bean, N. T., Pavia, A. T. and Tauxe, R. V. 1988. *Salmonella* isolates from humans in the United States, 1984-1986. Morbid. Mortal. Weekly Rep. 37(ss-2):25031.

Helander, L. M., Moran, A. P. and Mäkelä, P. H. 1992. Separation of two lipopolysaccharide populations with different contents of O-antigen factor 12_2 in *Salmonella enterica* serovar *typhimurium*. Mol. Microbiol. 6:2857-2862.

Hellerqvist, C. J., Larm, O. and Lindberg, B. 1969. Structural studies on the O-specific side chains of the cell wall lipopolysaccharide from *Salmonella bredeney*. Acta Chem. Scand. 23:2217-2222.

Hofestra, H. and Dankert, J. 1980. Major outer membrane proteins: Common antigens in *Enterobacteriaceae* species. J. Gen. Microbiol. 119:123-131.

Inouye, M. 1979. Lipoprotein of outer membrane of *Escherichia coli*. In Biomembranes, Vol. 10 (Ed. Manso, L. A.),pp.141-208. Plenum Press, New York, London.

Jansson, P. J., Lindberg, A. A., Lindberg, B. and Wolkin, R. 1981. Structural studies on the hexose region of the core in lipopolysaccharides from *Enterobacteriaceae*. Eur. J. Biochem. 115:571-577.

Jaradat, Z. W. and Zawistowski, J. 1995. Production and characterization of monoclonal antibodies produced against O-antigen factor 5 of Salmonella typhimurium lipopolysaccharide. J. Appl. Environ. Microbiol. Submitted.

Johnson, K. G. and Perry, M. B. 1976. Improved techniques for the preparation of bacterial lipopolysacchaides. Can. J. Micorbiol. 22:29-34.

Joys, T. M. 1968. The structure of flagella and the genetic control of flagellation in *Eubacteriales*. A review. Antonie van Leevwenhoek. 34:205-225.

Kauffman, F. 1972. Serological diagnosis of *Salmonella* species. Kaufmann-White scheme. Munkes gaard. Copenhagen.

Kawagishi, I., Müller, V., Williams, A. W., Irikura, V. M. and Macnab, R. M. 1992. Subdivision of flagellar region III of the *Escherichia coli* and *Salmonella typhimurium* chromosome and identification of two additional flagellar genes. J. Gen. Microbiol. 138:1051-1065.

Kerr, S., Ball, H. J., Mackie, D. P., Pollock, D. A. and Finlay, D. A. 1992. Diagnostic applications of monoclonal antibodies to outer membrane protein for rapid detection of *Salmonella*. J. Appl. Bacteriol. 72:302-308.

Khakhria, R., Johnson, W. and Lior, H. 1994. Canada's most common *Salmonella* serotypes and *Salmonella enteritidis* phage types (1992-1993). Safety watch. 3 (summer):4.

Kittelberger, R. and Hilbink, F. 1993. Sensitive silver-staining detection of bacterial lipopolysaccharides in polyacrylamide gels. J. Biochem. Biophys. Meth. 26:81-86.

Kondo, M., Harada, H., Sunada, S. and Yamaguchi, T. 1991. Increased selectivity in the detection of glycoproteins on nitrocellulose membranes by washing with sodium hydroxide solution. Electrophoresis. 12:685-686.

Komisar, J. L., and Cebra, J. J. 1983. Monoclonal antibodies to *Salmonella typhimurium* and *Escherichia coli* lipopolysaccharides. Adv. Exp. Med. and Biol. 162:303-311.

Kuhn, H. M., Brade, L., Appelmelk, B. J., kusumoto, S., Rietschel, E. T. and Brade, H. 1992. Characterization of the epitope specificity of murine monoclonal antibodies directed against lipid A. Infect. Immun. 60:2201-2210.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

Lee, D. R. and Schnaitman, C. A. 1980. Comparison of outer membrane porin proteins produced by *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. 142:1019-1022.

Lee, H. A., Wyatt, G. M., Bramham, S. and Morgan, M. R. A. 1990. Enzyme-linked immunosorbent assay for *S. typhimurium* in food: Feasibility of 1-day Salmonella detection. Appl. Environ. Microbiol. **56**:1541-1546.

Lind, S. M., Kenne, L. and Lindberg, A. A. 1991. Mapping of the binding specificity for five monoclonal antibodies recognizing 3-deoxy-D-manno-octulosonic acid in bacterial lipopolysaccharides. J. Immun. 146:3864-3870.

Lind, S. and Lindberg. A. A. 1992. Epitope size, specificity and equilibrium constant for four monoclonal antibodies binding to the O:4 polysaccharide antigen of *Salmonella* serogroup B bacteria. Mol. Immun. 29:1013-1023.

Lugtenberg, B. and van Alphen, L. 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim. Biophys. Acta. 737:51-115.

Luk, J. M. C., Chan, K. H., Tsang, R. S. W. and Ng, M. H. 1988. Characterization and application of a murine monoclonal antibody specific fro the serogroup C2 *Salmonella*. J. Med. Microbiol. 26:115-119.

Luk, J. M. C., Lind, S. M., Tsang, R. S. W. and Lindberg, A. A. 1991. Epitope

mapping of four monoclonal antibodies recognizing the hexose core domain of *Salmonella* lipopolysaccharide. J. Biol. Chem. 34:23215-23225.

Luk, J. M. C., Nnalue, N. A. and Lindberg, A. A. 1990. Efficient production of mouse and rat monoclonal antibodies against the O antigens of *Salmonella* serogroup C_1, using LPS-coated bacteria as immunogen. J. Immun. Meth. 129:243-250.

McCartney, A. C. and Wardlaw, A. C. 1985. Endotoxic activities of lipopolysaccharides In Immunology of bacterial cell envelope. (Eds. Stewart-Tull, D. E. S. and Davis, M.), pp. 203-238. John Willy and Sons Ltd.

Metcalf, E. S., O'Brian, A. D., Laveck, M. A. and Biddison, W. E. 1983. Characterization of monoclonal antibodies which recognize specific cell surface determinants on *Salmonella typhimurium*. Adv. Exp. Med. 162:313-317.

Mishu, B., Koehler, J., Lee, L. A., Rodrigue, D., Brenner, F. H., Blake, P. and Tauxe, R. V. 1994. Outbreaks of *Salmonella enteritidis* infections in the United States, 1985-1991. J. Infect. Dis. 169:547-552.

Munford, R. S., Hal, C. L. and Rick, P. D. 1980. Size heterogeneity of *Salmonella typhimurium* lipopolysaccharides in outer membranes and culture supernatant membrane fragments. J. Bacteriol. 144:630-640.

Nakamura, K. and Mizushima. S. 1976. Effects of heating in dodecyl sulfate solution on the conformation and electrophoretic mobility of isolated major outer membrane proteins from *Escherichia coli* K-12. J. Biochem. 80:1411-1422.

Newton, J. C., Blevins, W. T., Wilt, G. and Wolfe, L. G. 1990. Outer membrane protein profiles of *Edwardsiella ictaluri* from fish. Am. J. Vet. Res. 51: 211-215.

Nghiem, H. O., Himmelspach, K. and Mayer. H. 1992. Immunochemical and structural analysis of the O polysaccharides of *Salmonella zuerich* [1,9,27,(46)]. J. Bacteriol. 174:1904-1910.

Nikaido, H. and Nakae, T. 1979. The outer membrane of gram-negative bacteria. Adv. Microb. Physiol. 20:163-250.

Ogawa, T. Kuribayashi, S. Shimauchi, H. Toda, T. and Hamada, S. 1992. Immunological and biological characterization of outer membrane proteins of *porphyromonas endodontalis*. Infect. Immun. 60:4528-4533.

Oosterom, J. 1991. Epidemiological studies and proposed preventive measures in the fight against human salmonellosis. Int. J. Food Microbiol. 12:41-52.

Osborn, M. J., Gander, J. E. Parisi, E. and Carson, J. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. J. Biol. Chem. 247:3692-3972.

Pai, S. R., Upshaw, Y. and Singh, S. P. 1992. Characterization of monoclonal antibodies to the outer membrane protein (OmpD) of *Salmonella typhimurium*. Can. J. Microbiol. 38:1102-1107.

Parent, J. B., Gazzano-Santoro, H., Wood, D. M. Lim, E., Pruyne, P. T., Trown, P. W. and Conlon, P. J. 1992. Reactivity of monoclonal antibody E5 with endotoxin. II. Binding to short-and long-chain smooth lipopolysaccharides. Cir. Shock. 38:63-73.

Peterson, A. A. and McGroarty, E. J. 1985. High-molecular-weight components in lipopolysaccharides of *Salmonella typhimurium, Salmonella minnesota*, and *Escherichia coli*. J. Bacteriol. 162. 738-745.

Pettersson, A., Ley, P. V. D., Poolman, J. T. and Tossmssen, J. 1993. Molecular characterization of the 98-Kilodalton iron-regulated outer membrane protein of *Neisseria meningitidis*. Infection and Immunity. 61:4724-4733.

Philip, C., Fletcher, G., Wulff, J. L. and Earhart, C. F. 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. J. Bacteriol. 115: 717-722.

Rozalski, A., Brade, L., Kosma, P., Appelmelk, B. J., Krogmann, C. and Brade, H. 1989a. Epitope specificities of murine monoclonal and rabbit polyclonal antibodies against enterobacterial lipopolysaccharides of the Re chemotype. Infect. Immun. 57:2645-2652.

Rozalski, A., Brade, L., Kuhn, H. M., Brade, H., Kosma, P., Appelmelk, B. J., Kusumoto, S. and Paulsen, H. 1989b. Determination of the epitope specificity of monoclonal antibodies against the inner core region of bacterial lipopolysaccharides by use of 3-Deoxy-D-manno-octulosonate-containing synthetic antigens. Carbohydr. Res. 193:257-270.

Rosenbusch, J. P. 1974. Characterization of the major envelope protein from *Escherichia* coli. J. Biol. Chem. 249: 8019-8029.

Sarasombath, S., Lertmemongkolchai, G. and Banchuin, N. 1988. Characterization of monoclonal antibodies to protein antigen of *Salmonella typhi*. J. Clin. Microbiol. 26:508-512.

Sarwar, J., Campagnari, A. A., Kirkham , C. and Murphy, T. F. 1992. Characterization of an antigenically conserved heat-modifiable major outer membrane protein of *Branhamella catarrhalis*. Infect. Immun. 60:804-809.

Schnaitman , C. A. 1973. Outer membrane proteins of *Escherichia coli* 1. Effect of preparative conditions on migration of protein in polyacrylamide gels. Arch. Biochem. Biophys. 157:541-552.

Seifert, H. S. and So. M. 1988. Genetic mechanisms of bacterial antigenic variation. Microbiol. Rev. 52:327-336.

Sengupta, D., Datta-Roy, K., Banerjee, K. and Ghose, A. C. 1989. Identification of some antigenically related outer-membrane proteins of strains of *Vibrio cholera* O1 and non O1 serovars involved in intestinal adhesion and the protective role of antibodies to them. J. Med. Microbiol. 29:33-39.

Seuna, E. 1979. Sensitivity of young chickens to *Salmonella typhimurium* var. copenhagen and *S. infantis* infection and the preventive effect of cultured intestinal microflora. Avian. Dis. **23**:392-400.

Singh, S. P., Upshawa, Y., Abdullah, T., Singh, S. R. and Klebba, P. E. 1992. Structural relatedness of enteric bacterial porins assessed with monoclonal antibodies to Salmonella typhimurium OmpD and OmpC. J. Bacteriol. 174: 1965-1973.

Sinha, A. K. and Chakraborti, S. 1992. Immunoblot analysis of antibody responses to *Shigella dysenteriae* type I infection. Ind. J. Med. Res. 95:112-114.

Smyth, C. J. 1985. Immunology of outer membrane proteins of gram-negative bacteria. In Immunology of bacterial cell envelope. (Ed. Stewart-Tull, D. E. S. and Davis, M.), pp. 177-201. John Wiley and Sons Ltd.

Spinola, S. M., Griffiths, G. E., Bogolan, J. and Menegus, M. A. 1992. Characterization of an 18,000-Molecular-weight outer membrane protein of *Haemophilus ducreyi* that contains a conserved surface-exposed epitope. Infect. Immun. 60:385-391.

Spinola, S. M., Griffiths, G. E., Shanks, K. L. and Blake, M. S. 1993. The major outer membrane protein of *Haemophilus ducreyi* is a member of the OmpA family of proteins. Infect. Immun. 61:1346-1351.

Tagawa, Y. Haritani, M. and Yuasa, N. 1993. Characterization of an immunoreactive 17.5-kilodalton outer membrane protein of *Haemophilus somnus* by using a

monoclonal antibody. Infect. Immun. 61:4153-4257.

Tauxe, R. V. 1991. *Salmonella*: A postmodern pathogen. J. Food Prot. 54:356-368.

Thorns, C. J., McLaren, I. M. and Sojka, M. G. 1994. The use of latex particle agglutination to specifically detect *Salmonella enteritidis*. Int. J. Food. Microbiol. 21:47-53.

Todd, E. C. D. 1978. Foodborne disease in six countries-A comparison. J. Food Prot. 41:559-565.

Todd, E. C. D. 1989. Preliminary estimates of costs of foodborne disease in Canada and costs to reduce Salmonellosis. J. Food Prot. 52:586-594.

Todd, E. C. D., Mackenzie, J. M., Parrington, L. J., Sharpe, A. N., Paterkin, P. I., Diotte, M. P., Gidney, M. A. J., Nielson, K., Frazer, A., Rahn, K., Tiffin, A. I., Peterson, G. and Gehle, W. 1991. Evaluation of *Salmonella* antisera for an optimum enzyme-linked antibody detection of *Salmonella* using hydrophobic grid membranes filters. Food Microbiol. 8:311324.

Tsang, R. S. W., Chan, K. H., Lau, N. W. H., Choi, D. K. W., Law, D. K. S. and Ng, M. H. 1991a. Characterization of murine monoclonal antibodies against serogroup B *Salmonella* and application as serotyping reagent. J. Clin. Micorobiol. 29:1899-1903.

Tsang, R. S. W., Schlecht, S., Aleksic, S., Chan, K. H. and Chau, P. Y. 1991b. Lack of the α-1, 2-linked N-acetyl-D-glucosamine epitope in the outer core structures of lipopolysaccharides from certain O serogroups and subspecies of *Salmonella enterica*. Res. Microbiol. 142:521-533.

Van der Zee, H. 1994. Conventional methods for the detection and isolation of *Salmonella enteritidis*. Int. J. Food Micorobiol. 21:41-46.

Van Poucke, L. S. G. 1990. Salmonella-Tek, a rapid screening method for Salmonella species in food. Appl. Environ. Microbiol. 56:924-927.

Wang, H., Blais, B. and Yamazaki, H. 1995. Rapid and economical detection of *S. enteritidis* in eggs by the polymyxin-cloth enzyme immunoassay. Int. J. Food Microbiol. 24:397-406.

Warren, H. S., Amato, S. F., Fitting, C., Black, K. M., Loiselle, P. M., Pasternack, M.S. and Cavaillon, J.M. 1993. Assessment of ability of murine and human anti-lipid A monoclonal antibodies to bind and neutralize lipopolysaccharide. J.

Exp. Med. 177:89-97.

Watson, D. C., Robbins, J. B. and Szu, S. C. 1992. Protection of mice against *Salmonella typhimurium* with an O-specific polysaccharide-protein conjugate vaccine. Infect. Immun. 60:4679-4686.

Wayatt, G. M. 1992. Immunoassays for food poisoning bacteria and bacterial toxins. Chapman and Hall, London.

Weintraub, A., Johnson, B. N., Stocker, B. A. D. and Lindberg, A. A. 1992. Structural and immunochemical studies of the lipopolysaccharides of *Salmonella* strains with both antigen O 4 and O 9. J. Bacteriol. 174:1916-1922.

Wexler, H. M., Getty, C. and Fisher, G. 1992. The isolation and characterization of major outer membrane protein from *Bacteroides distasonis*. J. Med. Microbiol. 73:165-175.

Wood, D. M., Parent, J. B., Gazzano-Santoro, H., Lim, E., Pruyne, P. T., Watkins, J. M., Spoor, E. S., Reardan, D. T., Trown, P. W. and Conlon, P. J. 1992. Reactivity of monoclonal antibody E5 with endotoxin. I. Binding to Lipid A and rough lipopolysaccharides. Cir. Shock. 38:55-62.

Yamaura, N., Uchiyama, T. and Terkado, N. 1992. Production and epidemiological application of a monoclonal antibody specific for *Salmonella* O-5 antigen.Kitasato. Arch. Exp. Med. 65: 13-22.

Yeh, H. Y. and Jacobs, D. M. 1992. Characterization of lipopolysaccharide fractions and their interactions with cells and model membranes. J. Bacteriol. 174:336-341.