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PORIN PROTEINS OF SALMONELLA TYPHIMURIUM MEDIATE ADHERENCE TO MACROPHAGES

BY

Robert Solomon Negm

B.S., University of New Hampshire, 1992

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
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Doctor of Philosophy
in
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ABSTRACT

PORIN PROTEINS OF SALMONELLA TYPHIMURIUM MEDIATE ADHERENCE TO MACROPHAGES

by

Robert Solomon Negm

University of New Hampshire, September,1997

Salmonellosis continues to be a major infectious disease in both the United States and elsewhere. The outer membrane porin proteins (Omp) from *Salmonella typhimurium* play a key role in the initial adherence of this microorganism to murine macrophages and this mechanism is a critical event in the pathogenesis of infections. In this study macrophages were found to make contact with an OmpC-like protein of *S. typhimurium* and inhibition assays using the purified protein significantly reduced bacterial binding to macrophage. The *ompC* gene in a strain of *S. typhimurium* that is resistant to killing by macrophage was inactivated by transposon mutagenesis. Bacterial binding assays with these *ompC*-deficient bacteria and isogenic wild-type strains confirmed the role of the OmpC protein in mediating bacterial adherence to macrophages. The *ompC* gene of *S. typhimurium* was amplified using a polymerase chain reaction and the corresponding nucleotide sequence indicated that this gene was unique to our test strain. These results support the role of porin proteins as ligands in the initial adherence of this pathogen to host defense cells and may be helpful in developing strategies for disease prevention.

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INTRODUCTION

Salmonellosis continues to be one of the major infectious diseases affecting people worldwide (46). About 1.2×10^6 cases of human salmonellosis are reported annually, excluding most of the self-limiting forms of enterocolitis that go unreported (22). Forty thousand cases per year are reported in the United States alone, resulting in 500 fatalities and more than 50 billion dollars in health care expenses (12). About 25-30% of these cases in the United States are due to *S. typhimurium* (20), which is also a prominent cause of disease in animals. In general, people in poor health, neonates, the elderly, and individuals with compromised immune systems, *e.g.*, AIDS (2,27) are particularly prone to infection with *Salmonella* spp. Animals are the main reservoir of *Salmonella* spp. , with the exception of *S. typhi*—the causative agent of typhoid fever, for which humans are the only reservoir (20). Clinically salmonellosis may present as enteric fever, gastroenteritis, empyema, bone and joint infections, or a combination of these.

Most *Salmonella* infections arise from oral ingestion of contaminated water or food products of animal origin (20). Ingested organisms move to the distal small bowel where they invade epithelial cells through their apical membrane (22). Specialized intestinal cells, known as mouse ileal membranous or M cells appear to be the major epithelial cell associated with uptake of these pathogens (10). In systemic disease the organisms penetrate past the basolateral membrane of these epithelial cells and travel to their principal target of infection, the Macrophage (Mø) underlying the lamina propria (22). These cells are professional phagocytic cells that form an important line of defense against invading microorganisms. These bacteria may be subsequently transported

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within the Mø to regional lymph nodes, the spleen and liver, where *Salmonella* may multiply further and become disseminated hematogenously (15).

To survive, even thrive, under these varied and, at times, inimicable conditions, *Salmonella* spp. must be remarkably versatile organisms. Mounting evidence strongly suggests that a major contributor to this adaptability is a complex set of genetically regulated responses leading to expression of new or altered proteins. For example, to survive the low pH found in the stomach as well as in phagolysosomes of Mø (31), *Salmonella* may utilize one of several acid-tolerance response genes (18). The products of this inducible system are called acid-shock proteins (18). It has been proposed that *Salmonella* spp. adhere to animal cells in a two-stage process: an early reversible step and a subsequent irreversible one. The latter but apparently not the former requires *de novo* RNA and protein synthesis by the microorganisms (22). *Salmonella* have been shown to synthesize new proteins during binding to epithelial cells (24) as well as to Mø (8). The stress conditions associated with encounters with host cells such as Mø can be mimicked *in vitro* during growth under anaerobic conditions (26). This feature has been used to identify a protein (p44) that is expressed in significantly higher amounts under anaerobic growth and that appears to be a major site of attachment to peritoneal Mø (36).

At the same time, some of our basic models for infection are being challenged. Thus, type 1 pili of *S. typhimurium* appear to offer no advantage for bacterial colonization of the intestine and subsequent infection compared to non-piliated controls (28), although changes in expression of type I pilin, a major protein of bacteria pili, is associated with

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delayed clearance of *S. typhimurium* from animal tissues (29). A virulence plasmid is associated with *S. typhimurium*, but recent evidence suggests that it is not necessary for adherence or invasion of eukaryotic cells nor for resistance to phagocytosis or killing by Mø (42). Of course, microbial virulence factors and corresponding host defenses may be demonstrable only under certain conditions. Indeed, how the potential pathogen and host cell initially interact may greatly influence the clinical result. For example, bacteria entering Mø through opsonin-mediated processes are likely to trigger the production of reactive oxygen molecules associated with intracellular killing, whereas the same organisms entering via opsonin-independent mechanisms may fail to trigger production of these oxygen products (32).

Adherence to host cells is considered to be a major contributing factor for infections by many pathogens, including *Salmonella* (3). Salmonellae typically encounter several different types of host cells during the course of infection and the binding requirements may be quite distinct in each case. Unlike pathogens such as *Yersinia pseudotuberculosis*, *S. typhimurium* and *Y. enterocolitica* have genetically separate loci for adherence and invasion (39), although in epithelial cells adherence and internalization of *Salmonella* are closely linked events (16). These two events can be separately studied in *S. typhimurium* models. Adherence and uptake of *Salmonella* appear to be regulated by environmental conditions (17). Such binding may, in turn, induce global changes in the host cells, thereby facilitating bacterial uptake by specific receptors not usually involved in phagocytosis (39). The nature of the bacterial ligands of *S. typhimurium* involved in attachment to host cells is not known (39). Studies from out laboratory suggest that porins may be one such ligand.

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Porins are protein trimers that produce transmembrane channels in the outer membrane of *S. typhimurium* and other bacteria (35). Although recognized initially for their role in the transport of low molecular weight compounds in and out of the bacterial cell, porins have also been associated with immune defenses against infection. Porins, with variable amounts of copurified lipopolysaccharide, have been shown to induce protective immunity in mouse models (30,34). The contribution of contaminating lipopolysaccharide (LPS) in such studies is unclear, but at a minimum LPS appears to enhance both the humoral and the cellular immune response to porins (34). Indeed, it has been proposed that porins must be associated with LPS to be biologically active (11). Porins from which LPS has been removed lose their ability to form pores but retain their ability to bind C1q (25). To our knowledge no studies have been published on the possible role of porins in the interaction of salmonellae with host phagocytic cells. However, porins from *Neisseria* species are capable of inserting into the membranes of human neutrophils (5). These highly purified neisserial porins (with no detectable contamination with other outer membrane proteins and with >0.01% LPS contamination) reduced expression of Fc receptors, impaired upregulation of complement receptor (CR) 1 and CR3, and inhibited phagocytic capacity of neutrophils for serum-opsonized meningococci (5).

One member of the porin family of proteins from *Salmonella* and related bacteria, OmpC, is synthesized and incorporated into the bacterial envelope under conditions of both low- and high-osmolarity, suggesting that it might be expressed under both free-living conditions (low-osmolarity) and during infection (high-osmolarity) (40). Studies suggest that the *ompR* system regulates the relative expression of OmpC and OmpF,

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with OmpC being preferentially expressed under the high-osmolarity conditions associated with infection (38). An *ompC*-like gene was found in all 17 clinical isolates of *S. typhi* examined with no restriction fragment length polymorphism, supporting the idea that this gene is associated with the infectious process. Although the *ompC* gene sequence from *S. typhimurium* has not been published, hybridization studies with the *ompC* genes from *E. coli* and from *S. typhi* with DNA from *S. typhimurium* suggest a similar, but not identical match in each case (9,13) . The *S. typhi ompC* gene has been cloned into a porin-less mutant of *E. coli* and the protein product identified on the outer membrane of the transformed *E. coli* host (48). Preliminary studies from our laboratory suggest that OmpC from *S. typhimurium* is involved in recognition of this microorganism by murine peritoneal Mø.

The role of opsonophagocytosis in protection against infection of pyogenic bacteria is firmly established; however, there is good evidence that phagocytosis can occur in the absence of opsonins, suggesting that animals may rely on alternative mechanisms for early recognition of invading microorganisms (reviewed in (37)). This evidence derives from studies of both gram-positive and gram-negative bacteria, animal and human systems, and various phagocytic cell populations, including peritoneal Mø, alveolar Mø, and granulocytes. Receptors on these phagocytic cells implicated in direct recognition of bacteria include CR 1 (23,24,43), CR3 (19,23,24,43), CR4 (19,23,43) . Recent studies in our laboratory with group B streptococci (45) and *S. typhimurium* (1), using blocking studies with monoclonal antibodies to CD11b and CD18, provide evidence for the involvement of CR3 in the recognition of these bacteria by murine peritoneal Mø.

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When blocking experiments are used to indicate involvement of corresponding receptors, complete inhibition of bacterial binding is seldom achieved, suggesting that multiple receptors may be responsible for microbial recognition by Mø. This has been shown for *Leishmania* promastigotes, in which both CR3 and the mannose/fucose receptor on Mø were required for optimal adherence (6) and *Mycobacterium avium*, which required the mannose/fucose receptor, CR3, and the fibronectin receptor for effective recognition by human Mø (4). A model is emerging that includes both receptor redundancy and cooperativity in this recognition (33). An intriguing possibility that remains to be confirmed experimentally is that microbial interaction with different receptors on phagocytic cells determines the fate of these potential pathogens. For instance, some receptors, such as the mannose and Fc receptors, seem particularly well-suited to convey the bound *Leishmania* promastigotes to phagolysosomes and to trigger a lethal respiratory burst, whereas recognition of the same microorganism by another receptor, *e.g.*, CR1, leads to parasite survival, possibly due to a lack of effective transduction of specific cellular signals intracellularly following receptor ligation (33). Conversely, intracellular events may affect the binding of externally expressed receptors (41).

Not unexpectedly multiple pathways have been described by which LPS interacts with Mø, and this initial binding may result in cell activation (47). It has been proposed that CR3 and CR4 are normally immobilized in the plane of the cytoplasmic membrane and thus unable to directly promote phagocytosis, although particles may avidly bind to them (21). High doses of LPS can activate these receptors, leading to their mobilization and, presumably, internalization of bound components. Microbial recognition by Mø

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may involve multiple receptors, *e.g.*, (43), and they may be sequentially activated (14). Moreover, the same receptors, *e.g.*, CR3 and CR4, may exist in various states of activity (44), so detection and quantification of these surface molecules in the absence of functional binding assays may not provide accurate information regarding their role in microbial recognition. Clearly, the information currently available suggests that the initial recognition of microorganisms by Mø in the absence of exogenous opsonins is a complex process.

The resurging interest in innate immunity in recent years reflects a growing appreciation of its importance in early defenses. As recently presented in an editorial introducing a series of papers on this subject: "Because of its essential role in immunity, and its importance in pathology and pathogenesis of various diseases, and because of the increasing number of severely immunocompromised hosts encountered in clinical medicine, the study of antigen-independent host defense has become increasingly important" (7). The subtitle of this editorial—Innate immunity: 50 ways to kill a microbe—provides a fitting closing statement. Understanding the initial events in host–microbe interactions can provide valuable information on both the pathogenesis of infectious disease and strategies for intervention. This is the overall rationale for the present study.

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CHAPTER ONE

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CHAPTER ONE

Macrophage Recognize and Make Contact with an OmpC- like Protein of *Salmonella typhimurium.*

ABSTRACT

Murine peritoneal Mø bind to *S. typhimurium in vitro* in the absence of exogenous opsonins. An outer membrane protein of *S. typhimurium* mediates this interaction. Biotin-labeled Mø were used to probe electroblotted envelope proteins of *S. typhimurium* that had been previously resolved by polyacrylamide electrophoresis under denaturing and reducing conditions. Mø bound to an outer membrane protein with an apparent molecular mass of 44-kDa. The protein was purified to homogeneity and free of detectable lipopolysaccharide. Limited microsequencing of this protein resulted in a 15 amino-acid query sequence of A-E-V-Y-N-K-D-G-N-K-L-D-L-Y-G, which shares complete identity with the OmpC polypeptide (residues 22-36) of *Escherichia coli* K-12. Picomolar concentrations of this purified protein significantly inhibited the subsequent adherence of ^{35}S-labeled *S. typhimurium* to Mø in monolayers. It is proposed that this 44-kDa protein is involved in the recognition of *S. typhimurium* by Mø during the initial stages of infection.

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INTRODUCTION

The global incidence of human *Salmonella* infections has markedly increased in recent years (49). A more complete understanding of the interactions of *Salmonella* spp. with the host immune system may contribute to approaches for reducing the incidence of salmonellosis. The course of infection by mouse-virulent *S. typhimurium* in the mouse has been described (17) and serves as a model of the human disease typhoid fever, caused by *S. typhi*. Although non-typhoid strains of *Salmonella* are most commonly associated with a self-limiting gastroenteritis in humans, individuals who are immunocompromised may develop disseminated disease with these strains (43). Salmonellae normally enter the body via the oral route and establish an invasive infection in the lining of the bowel. In systemic infections these organisms spread from the bowel into underlying tissues where they encounter tissue Mø (16). Studies have shown that *Salmonella* spp. require genetic loci both to invade host cells and for intracellular survival (11,15) within many types of mammalian cells. Furthermore, attachment to and subsequent invasion of mammalian cells by *S. typhimurium* appear to be independent phenomena (15,24). Our investigations have focused on early events in the interaction between the host defense cell, the Mø, and *S. typhimurium* in the absence of exogenous opsonins.

Surface structures on the outer membranes of Gram-negative bacteria include a class of proteins called porins, which produce relatively non-specific channels that allow the passage of small hydrophilic molecules across the outer membrane (38). Porins exist as tightly associated trimers and in this form are highly associated with lipopolysaccharide (LPS). Porins have also been reported to act as receptors for the attachment of various bacteriophage, colicins, and complement components (9,21,45). Although recognized initially for their role in the transport of low molecular weight compounds in and out of

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the bacterial cell, porins have also been associated with immune defenses against infection (25,34).

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MATERIALS AND METHODS

Chemicals. Unless otherwise stated all chemicals used were obtained from Sigma Chemical Co, St. Louis, Mo.

Bacterial Strains and Growth Conditions. *S. typhimurium* strain 14028 (Difco Laboratories, Detroit, MI) and strain 1826 (Max Planck Institut für Immunbiologie, Freiburg, Germany), were used. Both strains have an LD_{50} in mice of ~10 microorganisms when administered intraperitoneally. Cultures were grown to post-exponential phase in Luria-Bertani broth (Difco) at 37°C under anaerobic, non-agitated conditions, (5% CO_2, 10% H_2, 85% N_2) using a bi-phasic batch culture method (47).

Envelope protein preparations from *S. typhimurium*. Bacterial cultures were harvested, concentrated, washed 2X, and resuspended in ice-cold sonication buffer consisting of 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1.0 mM dithiothreitol (DTT), 50 µg milliliter^{-1} (ml) each of ribonuclease A and deoxyribonuclease I (Worthington Biochemical Corp., Freehold, NJ), 10 µg ml^{-1} leupeptin, 10 µg ml^{-1} pepstatin, 1.0 mM phenylmethylsulfonylfluoride, and 1.0% aprotinin (Calbiochem, San Diego, CA) Sonic extracts were made using four 30-s exposures to sonic oscillation (Ultrasonics, Inc., Heat-Instruments Co, Plainview, NY). Whole cells were removed by centrifugation at 10,000 × *g*; bacterial envelopes contained in the supernatant fluid were sedimented by centrifugation at 44,000 × *g* for 1 h. The supernatant fluid was decanted and the cell envelopes washed 2X with 5 ml of sonication buffer and collected by centrifugation. Following the third centrifugation the envelope proteins were solubilized by the addition of 500 µl of solubilization buffer containing 2.3% sodium dodecylsulfate (SDS), 5% 2-mercaptoethanol, 10 % glycerol, and 60 mM Tris-HCl, pH 6.8. This mixture was

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incubated at 60°C for 30 min and the insoluble material was removed by centrifugation at 44,000 $\times g$ at 37°C for 1 h. The supernatant fluid containing solubilized envelope proteins was transferred to a clean tube and stored at –20°C until used.

Mø. Peritoneal exudate Mø from 8-12 week-old Balb/c mice, obtained from our breeding facility, were elicited by intraperitoneal injection of 3.0 ml of aged Brewer thioglycollate (Difco). After 5 d the Mø were harvested by lavage using 10 ml of Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} (DPBS). The Mø were washed twice by centrifugation at 200 $\times g$, using cold DPBS and resuspended at a final concentration of 10^8 cells ml^{-1} in DPBS. Approximately 95% of these cells were Mø, as judged by cell morphology.

Labeling Mø. The biotin labeling reagent, sulfosuccinimydal-6-[biotinamido] hexanoate, 100 µg, (Pierce Chemical Co., Rockford, IL) was solubilized in DPBS. This was added to a viable Mø suspension (10^8 cells ml^{-1}) and incubated for 1 h at 4°C with end-over-end rocking (18). Triple washed, labelled Mø were resuspended in opsonin-free Morgan's 199 medium (M199) at a final concentration of 10^8 cells ml^{-1} and used immediately for probing. Mø viability after labeling was assessed by trypan blue exclusion.

***Salmonella* envelope proteins probed with labeled Mø.** Envelope proteins from *S. typhimurium* were separated by discontinuous electrophoresis (11.5% acrylamide gel) in the presence of SDS, electrophoretically transferred to nitrocellulose, and probed using modifications of other previously reported protocols (20,29). Fifty micrograms each of envelope proteins of *S. typhimurium* strains 1826 and 14028 were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 11.5% acrylamide) (26) and transferred

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to nitrocellulose membranes (46). These nitrocellulose replicas were blocked with 2.0% gelatin, washed with DPBS for 30 min, and transferred to a polyester heat-sealable pouch (Kapak, Inc., Minneapolis, MN). Labelled Mø (10^8 cells ml^{-1}) in M199 medium were allowed to attach to electroblotted envelope proteins through incubation without agitation for 30 min at 37°C, then the membranes were transferred to a platform rocker for an additional 30 min. The membranes were removed and washed 4X with DPBS for 1 min each, then fixed for 5 min in DPBS, pH 7.2, containing glutaraldehyde (1%), to enhance visualization. Washed membranes were incubated with avidin-conjugated horseradish peroxidase (avidin-HRP, Pierce) at a concentration of 2 μg ml^{-1} in 10 ml of DPBS. Unbound avidin-HRP was removed by washing the membrane with PBS for 30 min. Biotin-labelled Mø that had adhered to electroblotted envelope proteins were detected by the addition of the peroxidase substrate 4-chloro-1-naphthol (Bio-Rad, Richmond, CA).

Separation Of Envelope Proteins. Solubilized envelope protein samples were buffer-exchanged and concentrated against three volumes of elution buffer (20 mM Hepes, pH 8.2, containing 4.0 M urea and 1% n-octyl-β-D-glucopyranoside) by centrifugal dialysis (Centricon-30, Amicon, Inc., Beverly, MA). The protein samples were then applied to an anion-exchange chromatography column (MonoQ HR 5/5) and resolved by Fast Protein Liquid Chromatography (Pharmacia LKB, Upsala Sweden,) (27). Fractions enriched in protein, identified by A_{280} analysis, were pooled, concentrated, and further separated by SDS-PAGE. Coomassie blue-stained proteins excised from the electrophoretic gels were electrodialyzed against a buffer containing 0.01% SDS, 15 mM ammonium bicarbonate, pH 8.2, in a Micro-electroelutor (Amicon) and dialyzed 3X against DPBS through centrifugal dialysis. Dilutions of this protein were used to treat Mø monolayers in M199.

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Protein, Endotoxin and Phosphatase Assays. Subsamples were assayed for protein concentration, and endotoxin, and phosphatase contamination. LPS was assayed using *Limulus* amebocyte lysate (E-toxate®, Sigma) and purified endotoxin from *E. coli* 055:B5 as a reference. Protein concentration assays were performed by the bicinchoninic acid (Pierce, CA) method or by ultraviolet spectrophotometry at 280 nm using bovine serum albumin as the reference. Phosphatase activity was measured by colorimetric spectrophotometry (405 nm) using p-nitro-phenyl-phosphate as a substrate (36).

***In vitro* Adherence Assays.** All cell culture manipulations were done using media free of exogenous opsonins and serum components.

Biosynthetic radiolabeling of *Salmonella*. Bacterial cultures were grown as described above to a final culture density of ~10^9 cfu ml^{-1}. Bacteria from 0.4 ml of culture were washed with 1.0 ml of DPBS at 4°C and resuspended in 0.1 ml of methionine-free medium (Methionine Assay Medium, Difco) containing 250 µCi of [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-label, ICN, Irvine, CA). Samples were incubated at 37°C for 10 min and the reaction was quenched by the addition of 10 µl of 0.10 M L-methionine. The labeled bacteria were collected by centrifugation at 10,000 × *g* and washed repeatedly with PBS. Bacteria were resuspended in M199 medium and used for adherence assays.

Inhibition studies on Mø monolayers. Mø in M199 medium were allowed to attach to polystyrene tissue culture wells and held at 37°C for >6 h prior to washing and removal of unadherent cells with DPBS. Mø were then incubated in DPBS containing

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1.0% bovine serum albumin for 30 min at which time the medium was replaced with M199 medium. The effect of the 44-kDa protein on adherence of *S. typhimurium* was investigated by preincubating the Mø with the electrodialyzed and DPBS buffer-exchanged protein for 30 min at 37°C at varying concentrations before the addition of bacteria. Radiolabeled bacteria were added to each plastic well containing Mø (the ratio of Mø to bacteria was 1:100). Labeled *S. typhimurium* attached to Mø were detected by solubilization of the cell culture well contents with heated (70°C) SDS (10% w/v). The contents of the well were transferred to liquid scintillation vials containing fluor (Ecolite, ICN). Counts min^{-1} were obtained using an scintillation counter (LS7000, Beckman Instruments, Co.).

N-terminal sequencing. Greater than 1 μg (>22 picomoles) of protein samples were purified by SDS-PAGE separation, as described above, and excised from the gel after staining with Coomassie blue. The protein samples contained in gel fragments were either reduced with dithiothreitol, alkylated with iodoacetamide, and digested with cyanogen bromide, or left untreated. Proteins were electroeluted, concentrated, and applied directly to a polypeptide support disk for sequencing. The resultant *in situ* digested fragments were electrophoresed in gels containing SDS (15% acrylamide) and electroblotted to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA) (33). Protein bands on these membranes were located by staining with 0.1% Coomassie blue, excised and applied to a protein sequenator (Model 475A, ABI, Foster City, CA). Automated Edman degradation was performed for 15 cycles for each protein analyzed. Computer-assisted, basic local alignment search tools (2) were used to identify the sequence of the obtained query sequences through the network service of the National Center for Biotechnology Information using the Genpept (Release 85.0), SWISS-PROT (release 30.0) and PIR (release 41.10) databanks.

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RESULTS

Probing envelope proteins with labeled Mø. Nitrocellulose membranes containing transferred envelope proteins of these two serovars, were used to detect an *in vitro* interaction between a 44-kDa outer membrane protein of *S. typhimurium* strains 14028 and 1826 and viable Mø that had been surface-labeled with biotin (Fig. 1, lanes a and b). Mø bound to no other electroblotted envelope proteins. The binding of Mø to the 44-kDa protein occurred in the absence of serum opsonins. Viability of the Mø used for probing was greater than 90 percent.

Purification of the 44-kDa protein by anion-exchange chromatography and SDS-PAGE. The recovery of a 44-kDa protein free of LPS was achieved after sequential purifications (Fig. 2) beginning with sonic bacterial lysates (lane d), enrichments for envelope proteins (lane c), then NaCl-elution of proteins from the anion-exchange chromatography column (lane b), and finally gel electrophoresis and excision of the stained 44-kDa protein band from acrylamide gels (lanes a and f). Endotoxin assays indicated that the protein sample recovered from chromatography fractionation contained significant amounts of LPS and no detectable periplasmic phosphatase (Table 1). Protein samples obtained after electroelution from polyacrylamide gel contained no detectable LPS.

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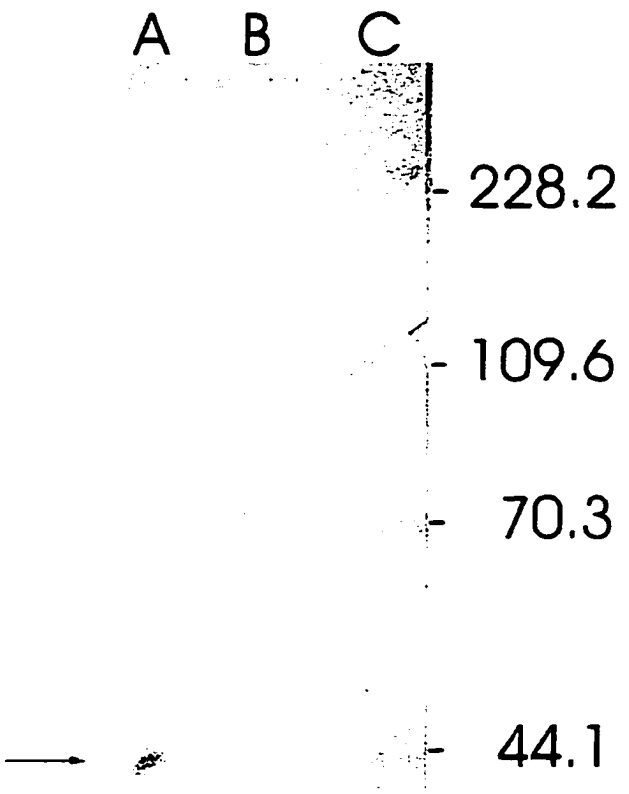

Fig. 1. Nitrocellulose membranes containing 50 µg of envelope proteins from *S. typhimurium* 1826 (lane A) and *S. typhimurium* 14028 (lane B) and probed with labeled Mø. The arrow on the left indicates envelope proteins to which viable biotinylated Mø have attached. The binding of Mø was revealed by the avidin-peroxidase activity. Molecular weight standards are shown on the right (lane C).

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Fig. 2. Protein composition of various preparations of the 44-kDa protein. Proteins were heated in SDS-containing sample buffer (26) at 100°C for 2 min and then separated by SDS-PAGE (11.5% acrylamide) and stained with Coomassie blue. Lane D contains bacterial sonic lysates (150 µg); lane C contains envelope proteins enriched by differential centrifugation and solubilization with SDS (100 µg); lane B contains pooled fractions recovered after NaCl elution from the anion-exchange chromatography column (100µg); and lanes A and F contains electrodialyzed 44-kDa protein excised from SDS-PAGE (10µg). Lane E contains molecular weight standards and their weights are shown on the right.

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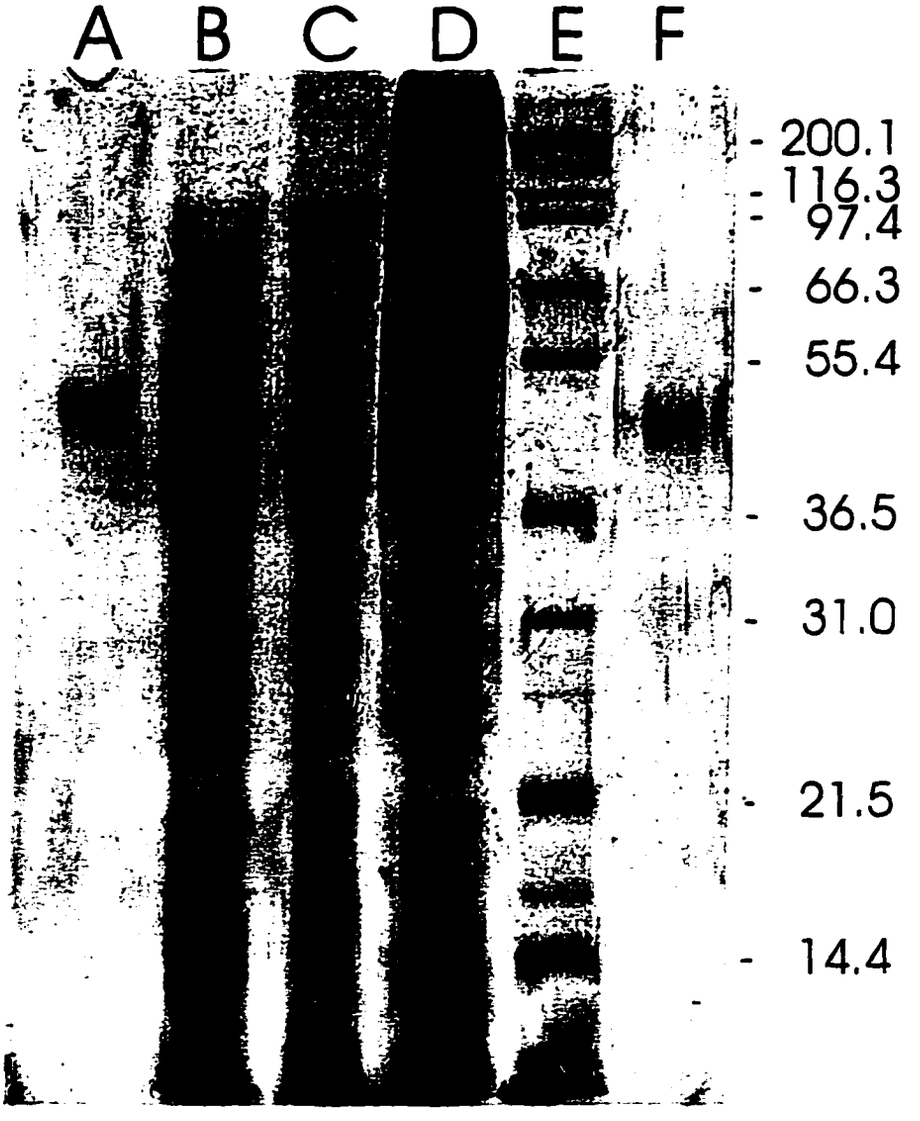

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TABLE 1. Levels of contaminating lipopolysaccharide (LPS) and phosphatase in preparations of the 44-kD protein from *S. typhimurium* at sequential stages of purification

Sample	LPS (EU/mg)[a]	Phosphatase (mU/mg)
Bacterial lysate[b]	3300	4.2
Envelope, solubilized[c]	6.5	0
Anion-exchange column[d]	3.0	0
Electroelution[e]	0	0

[a] Endotoxin units, using *E. coli* O55:B5 as reference

[b] Prepared by sonic disruption

[c] Derived from lysate by differential centrifugation and solubilization with SDS

[d] Pooled fractions recovered from anion-exchange chromatography column

[e] Derived by electrodialysis of Coomassie blue-stained bands from SDS-PAGE of anion-exchange column pooled fractions

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Bacterial adherence to Mø treated with the enriched 44-kDa protein. Adherence of ^{35}S-labeled *S. typhimurium* to Mø monolayers, pretreated with the 44-kDa protein and sham-treated, were measured. The 44-kDa protein inhibited bacterial adherence to tissue culture monolayers of Mø in a dose-dependent manner (Fig. 3). When Mø were treated with 100 or 1000 picomolar concentrations of the LPS-free 44-kDa protein, there was a 40 and 60% reduction in the bacterial attachment to Mø, respectively, compared to untreated controls.

Microsequencing the 44-kDa protein. Partial digestion of the 44-kDa protein yielded several lower molecular weight products; however, only the undigested 44-kDa band provided a readable sequence. Partial N-terminal sequencing of the 44-kDa protein of both serovars 14028 and 1826 provided a 15-mer of A-E-V-Y-N-K-D-G-N-K-L-D-L-Y-G. Comparison of this partial N-terminal sequence with known sequences in world-wide nucleotide and protein sequence databases generated complete sequence identity with amino acid residues 22 through 36 of the OmpC precursor protein of *E. coli* K-12. The partial sequence obtained in this study differed by one or two residues in this highly conserved porin protein region from other previously sequenced porins (Table 2). N-terminal sequencing the 36-kDa envelope protein of *S.typhimurium* 14028 confirmed that the 44-kDa protein is similar to, but not identical with the OmpC porin protein of *S. typhimurium* 14028. The 36-kDa protein yielded a 15-mer sequence of A-E-I-Y-N-K-D-G-N-K-L-D-L-F-G. This amino acid sequence is identical to the amino acid sequence for the OmpC of *S. typhimurium* LT2 SH7457 (42) (Table 2).

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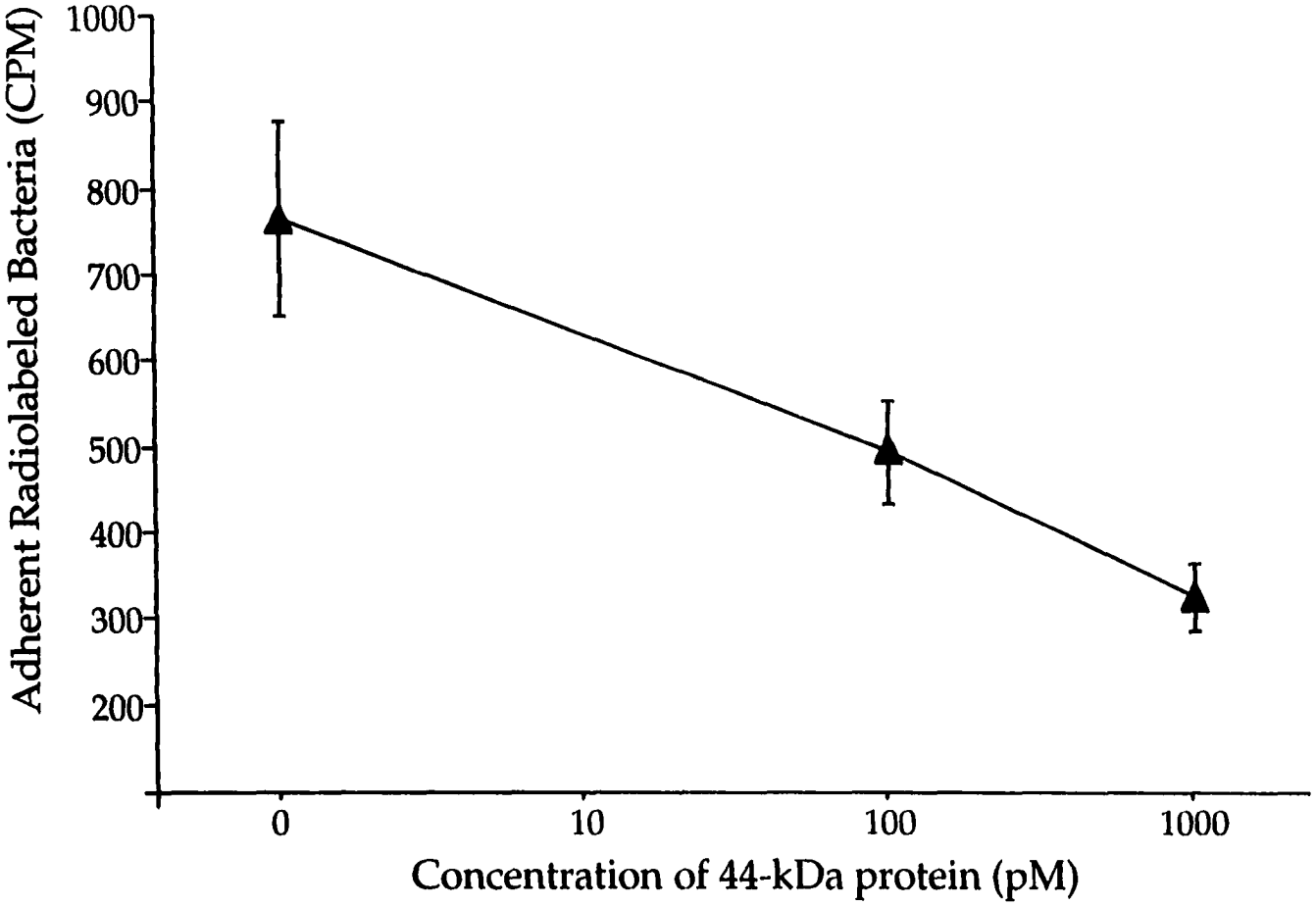

Fig. 3. Effect of pre-incubating Mø with purified 44-kDa protein on the adherence of *S. typhimurium*. Mø were treated for 30 min with the appropriate concentration of the 44-kDa protein, followed by the addition of ^{35}S-labeled *S. typhimurium*. Bacterial adherence measurements were determined in triplicate after 1 h incubation, and the means ± standard deviations of the results are shown. It was found that the effect of the 44-kDa protein inhibited adherence in a dose-dependent fashion, r^2= 0.997.

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TABLE 2. Alignment of N-terminal amino acid sequences of related porin proteins

Protein	Organism/Strain	Database	ID[a]	N-C No.	Partial Sequence	Ref.
44-kDa	*Stm* 14028	PIR[b]	A57558	1-15	-A-E-V-Y-N-K-D-G-N-K-L-D-L-Y-G-	[c]
OmpC	*E. coli* K-12 W620	S-P[d]	P06996	22-36	A-E-V-Y-N-K-D-G-N-K-L-D-L-Y-G-	(37)
OmpC	*Stm* LT2 SH 7457	[e]	n.d.	22-36	-A-E-I-Y-N-K-D-G-N-K-L-D-L-F-G-	(42)
OmpC	*Stm* 14028				A-E-I-Y-N-K-D-G-N-K-L-D-L-F-G-	[c]
OmpF	*Stm* LT2	NCBI[f]	468741	23-37	-A-E-I-Y-N-K-D-G-N-K-L-D-L-Y-G-	[g]
PhoE	*Stm* LT2 SJ2353	S-P	P30705	21-35	-A-E-V-Y-N-K-N-G-N-K-L-D-V-Y-G-	(44)
OmpC	*S. typhi* Ty2	S-P	P09878	22-36	-A-E-I-Y-N-K-D-G-N-K-L-D-L-F-G-	(48)
OmpS1	*S. typhi* IMSS-1	NCBI	311957	22-36	-A-E-I-Y-N-K-N-G-N-K-L-D-L-Y-G-	(10)
OmpF	*E. coli* K-12 KSJ02	S-P	P02931	23-37	-A-E-I-Y-N-K-D-G-N-K-V-D-L-Y-G-	(19)
PhoE	*E. coli* K-12 CS520	S-P	P02932	22-36	-A-E-I-Y-N-K-D-G-N-K-L-D-V-Y-G-	(6)
nmpC	*E. coli* K-12 CS384	NCBI	145856	24-38	-A-E-I-Y-N-K-D-S-N-K-L-D-L-Y-G-	(6)
lc	phage PA-2	NCBI	215369	24-38	-A-E-I-Y-N-K-D-S-N-K-L-D-L-Y-G-	(6)

[a] Accession or sequence identity number
[b] Protein Information Resource
[c] This study
[d] Swiss-Prot
[e] Unlisted; published as a partial amino acid sequence
[f] National Center for Biotechnology Information
[g] Unpublished

DISCUSSION

Adherence to host cells is considered to be necessary for the initiation of infection by many pathogens, including *Salmonella* (15). Salmonellae encounter different host cell types during the infection process, although the molecular participants in these interactions are poorly understood (40). Surface components of salmonellae, including fimbriae (7,8), flagella (22), and LPS (23), have been implicated in host-cell attachment, but these findings have not been universally accepted (30,31).

A key host defense cell in *Salmonella* infections is the Mø. Previous studies in our laboratory have shown that *S. typhimurium* adhere to Mø in the absence of opsonins (39) and that multiple receptors on Mø are involved in recognition of these bacteria (1). To identify the molecular participants of *S. typhimurium* involved in the early adherence to Mø, this study focused on the envelope proteins of *S. typhimurium*. Bacteria were grown under anaerobic conditions since this has been associated with enhanced attachment and entry into mammalian cells (12,28) . Probing immobilized bacterial envelope proteins with labeled Mø allowed us to identify the 44-kDa protein. Standard biochemical techniques were used to purify the protein free of detectable LPS. The final purified material was also devoid of phosphatase activity, whose presence is associated with periplasmic contamination (35). Picomolar to nanomolar concentrations of this 44-kDa protein were able to block attachment of *S. typhimurium* to Mø.

Partial amino acid sequence data obtained from the N-terminus of the 44-kDa protein completely matched a 15-mer from OmpC of *E. coli* K-12 (37). A similar but not identical sequence is associated with 36-kDa OmpC from *S. typhimurium* LT2 (42) and 14028. Based on this sequence data, the bacterial protein recovered in this study is

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OmpC-like; its physiological relationship with OmpC and other recognized porin proteins is, at present, unknown.

In situ porins are tightly associated with LPS. Indeed, it has been proposed that porins must be associated with LPS to be biologically active *in vitro* (9). Our studies suggest that bacterial adherence mediated through the 44-kDa outer membrane protein does not require LPS.

Salmonella porins are immunogenic in mice (25) and have been shown to confer significant protection against challenge with live *S. typhimurium* (25,34). The binding of *S. typhimurium* porins to human polymorphonuclear leukocytes induces a decreased oxidative burst in these cells as well as inhibits their migration in the presence of chemotactic agents (14). *Salmonella* porins also induce the release of tumor necrosis factor-α, interleukin-1 α and interleukin-6 by human monocytes (13). Subcellular events in Mø following attachment of the 44-kDa protein have not yet been characterized. Studies with two other Gram-negative species provide possible models.

Porins of *Neisseria* species are capable of inserting into the membranes of human neutrophils (4), resulting in the inhibition of actin polymerization, degranulation, and opsonin receptor expression. These porins translocate from live meningococci and gonococci and integrate directionally into the lipid-bilayer membrane of mammalian cells (5,32,41). Bernardini *et al.* (3) have shown that the OmpC of *Shigella flexneri* is involved in the invasion of HeLa cells, perhaps by directly interacting with cytoskeletal components.

This study suggests that Mø recognition of porin-like molecules may be an key event

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in early defense against *S. typhimurium* infections. Understanding how porin proteins interact with host defense cells may help in developing preventive strategies for reducing the morbidity and mortality of salmonellosis world-wide.

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CHAPTER TWO

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CHAPTER TWO

OmpC of *Salmonella typhimurium* Mediates Attachment to Mø and Resistance to Intracellular Killing.

ABSTRACT

Murine peritoneal Mø recognize, adhere to, and phagocytose *S. typhimurium* in the absence of serum opsonins. *OmpC* mutants were developed in our test strain of *S. typhimurium* and measurements of both bacterial adherence and phagocytic internalization were compared to those of the parent wildtype strain. These studies were performed in an opsonin-free environment that models *in vitro* the mammalian innate host defenses against *S. typhimurium*. The *ompC* mutation resulted in a five-fold decrease in the adherence of bacteria to the Mø. Moreover, internalized *ompC* mutants were susceptible to Mø killing in contrast to the wildtype strain. Evidence from this study supports a role for the OmpC protein in both initial recognition by Mø and in subsequent intracellular survival.

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INTRODUCTION

Non-typhoidal salmonellosis remains a major foodborne disease. Intervention strategies hold promise for reducing the incidence of self-limiting gastroenteritis caused by *Salmonella* spp. Major efforts have been developed to prevent the spread of this organism within the population (25). This study focused on innate defenses against *Salmonella* infections and is based on the premise that such defenses are an important means for preventing development of clinical salmonellosis and that a good understanding of the interaction between *Salmonella* spp. and the key cell of the innate defense system—the Mø—can lead to the development of intervention approaches at the individual level. Research has shown that *Salmonella* bacteria adhere to specialized small intestinal epithelial cells called M cells (29). After attachment, these bacteria mediate cytoskeletal and cell surface rearrangements causing internalization within a membrane-bound vesicle (22). Once internalized *Salmonella* destroy these cells (29) and enter Mø in the mesenteric lymph follicles. Following entry into Mø, these bacteria reside in membrane-bound vacuoles, resist killing, and replicate (47). Survival within the Mø is critical for *Salmonella* virulence because it enables these bacteria to evade key defenses of the immune system and to disseminate to other tissues (11,34).

The role of innate defenses in thwarting the initial microbial aggression has become increasingly recognized as an important component of our ability to prevent disease development following exposure to potential pathogens (8). A key defense cell in *Salmonella* infections is the Mø. Usually understood as a major cell involved in phagocytic uptake and internal destruction of microbial pathogens, for some intracellular bacterial pathogens such as *Salmonella* spp. the Mø becomes a haven, a protected environment in which the microorganism may thrive (28). Involvement of the

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porin protein OmpC in recognition of *S. typhimurium* by Mø supports a role for this protein in linking salmonellae with key cells of the innate defense system.

Constituting up to 2% of the total cellular protein in enteric bacteria (43), porins are strong candidates for recognition by host defense cells. They are highly antigenic, particularly in their native association with lipopolysaccharide (32), and may confer protection to challenge with the live microorganism (32,39,57). Cell-mediated immune responses to porins from *S. typhi* (4) and from *S. typhimurium* (40) support a role for these outer membrane proteins in inducing a specific immune response.

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MATERIALS AND METHODS

Chemicals. Unless otherwise stated all chemicals used were obtained from Sigma Chemical Co, St. Louis, Mo.

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. All strains were cultivated in Luria-Bertani broth (LB) or on LB agar plates unless otherwise stated. Wild-type *S. typhimurium* 14028 is a virulent strain isolated from a cow that had died from septicemia. This strain has been shown to be resistant to killing by Mø, to survive intracellularly and to resist killing by 1% Na deoxycholate (21). Unlike more commonly used *S. typhimurium* LT2 strains *S. typhimurium* 14028 retains its virulence *in vitro* (21). This strain has a lethal dose (LD_{50}) ≈ 30 organisms when injected intraperitoneally in BALB/c mice. *S. typhimurium* strain 103 is an *ompC* mutant derived from the parent 14028 developed in this study. Tetracycline concentrations used in LB agar were at 20 μg ml^{-1}. The *Escherichia coli* strain HB101 was used as a control for a internalization experiments, because it is readily phagocytosed and killed (20,31).

Bacteriophages. The bacteriophage used in this study are listed in Table 2. Unless otherwise stated bacteriophage lysates were propagated in exponential cultures of the host strain *S. typhimurium* 14028 which was grown in Luria Bertani (LB) broth to an early exponential phase of growth to a concentration of 1×10^9 CFU ml^{-1}. Bacteria were removed from lysates by centrifugation at 10,000 x *g* for 10 min and the supernatant fluids transferred aseptically to sterile tubes by filtration through a sterile 0.2 μm filter syringe. One ml of chloroform was added to each lysate, which was then incubated for 18 h at 4°C. Viable bacteria were monitored in these lysates by inoculating LB agar with 0.1 ml of the phage lysate and incubating at 37°C for 18 h.

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TABLE 1. Bacterial strains used.

Strain	Genotype	Source
S. typhimurium 14028	smooth wild-type	Difco[a]
S. typhimurium 14028	*ompC*::Tn*10*	this study[b]
S. typhimurium 103	*ompC* mutant	this study[c]
S. typhimurium LT2 23564	smooth wild-type	ATCC
S. typhimurium LT2 SH7241	*ompC*::Tn*10*	SGSC[d]
S. typhimurium LT2 SH5014	*rfa, fla*	SGSC[e]
S. typhimurium LT2 SH6017	*ompC*::Tn*10*	SGSC[e,f]
E. coli HB101	smooth wild-type	CGSC[g]
E. coli HB101	harbors plasmid pBR328	CGSC[h]

[a] American Type Culture Collection strain 14028, CDC 6516-60.
[b] Derived from *S. typhimurium* 14028 *ompC*::Tn*10*.
[c] *S. typhimurium* strain 103 is an *ompC* mutant derived from the parent 14028 *ompC*::Tn*10* through positive selection for *tet*^{r}.
[d] Kenneth E. Sanderson , *Salmonella* Genetic Stock Center, University of Calgary, CANADA, In this strain, the *ompC* gene has been inactivated by transposon mutagenesis through the transposition of Tn*10* after nucleotide residue 396 of the *ompC* open reading frame (18).
[e] Derived from *S. typhimurium* LT2 strain SL1027 and is an *rfa* mutant (44,53). This rough mutant contains less than a complete LPS (27) and is *fla*$^-$, therefore lacks flagellar-driven motility (53).
[f] Derived from *S. typhimurium* LT2 SH5014 (44).
[g] Barbara Bachmann and Mary Berlyn, Coli Genetic Stock Center, Yale, New Haven, CT.
[h] Plasmid pBR328, genbank accession number L08858, contains genes *tet* which encodes for the tetracycline resistance protein at nucleotide residues 86-1276, *bla* which encodes for β-lactamase at residues 2209-2997, and *chl*, which encodes for chloramphenicol-acetyl transferase at residues 3477-4136 (16,45,51).

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TABLE 2. Bacteriophages used.

Phage	Genotype	Source
P22 HT105/1	*int-201*	SGSC[a]
H5	*c2*	SGSC[b]
PH105	wild-type	P. Helena Mäkelä[c]

[a] Kenneth E. Sanderson , *Salmonella* Genetic Stock Center, University of Calgary, Canada, Derived from P22. P22 was isolated by Zinder and Lederberg from *S. typhimurium* LT22. It adsorbs to *Salmonella* with O-antigen 12 (serogroups A,B,D1) and has been shown to be a generalized transducing phage (59). Schmiëger isolated the lysogen deficient (*int*-) high-transducing phage HT105/1 (49) because of lytics problems in genetics using P22 (55,59).

[b] Derived from P22. The *c2* mutation of P22 is a clear-plaque mutant which does not lysogenize and the *c2* gene encodes a repressor equivalent to *cI* of bacteriophage lambda and this mutant bacteriophage is called H5 (35).

[c] P. Helena Mäkelä, National Public Health Institute, Helsinki, Finlandand this phage can only infect OmpC+ *S. typhimurium* strains without discrimination for smooth or rough LPS (27).

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Preparation of transducing particles. *S. typhimurium* LT2 strain SH7241 was used as the donor strain for preparing transducing lysates of *ompC*::Tn*10*. P22-transducing lysates were obtained by mixing 4 ml of P22 lysate with 1 ml of post-exponential *S. typhimurium* SH7241 culture. This mixture was incubated for 8-16 h at 37°C and the phage (transducing particles) were obtained as described above. P22 titers were determined by a soft agar overlay method with *S. typhimurium* 14028 as the host (35). This transducing lysate was used to infect wild type *S. typhimurium* 14028.

Transduction. This experiment was carried out by mixing LB-grown post-exponential cultures of the lysogenic recipient strain *S. typhimurium* 14028 (~2 x 10^9 ml^{-1}) with phage lysate at a ratio of 0.01 to 0.1 bacteriophage per bacterial cell (49). The infected cultures were incubated at 37°C for 1 h without agitation and serial dilutions of this were plated onto LB agar containing tetracycline (25 μg ml^{-1}). The plates were incubated for 2 d at 37°C. Tetracycline-resistant (*tet*^{r}) transductants were isolated. Transductants were screened to eliminate pseudolysogens and stable lysogens. Selected strains were used for curing *S. typhimurium* 14028 of Tn*10*.

Screening transductants for pseudolysogeny. Pseudolysogens were differentiated from nonlysogens by the growth of *tet*^{r} transductants overlaid with nutrient agar (0.8%) containing the host *S. typhimurium* 14028 and incubated at 37°C for 18 h (6). The presence of pseudolysogens was detected by the appearance of a plaque formation around the transductant colony (pers. comm., R. Zsigray).

Screening transductants for stable lysogeny. Stable lysogens are immune to P22

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superinfection and these transductants were not useful for further genetic studies in the event of lysis of the bacterial host. The transductants were tested for susceptibility to bacteriophage H5 infection by the soft-agar overlay technique. Those sensitive to this phage were considered nonlysogens as previously described (6,17) and were retained for further study.

Preparation of O-Antigen bacterial vaccine. Heat-killed *Salmonella* were used to elicit antibodies in rabbits to the O antigen (12,19). *S. typhimurium* 14028 was grown in LB broth from frozen glycerol stocks for 18 h at 37°C with shaking. The bacteria (≈2 x 10^9 CFU ml^{-1}) were concentrated by centrifugation (10,000 x *g*) for 10 min and resuspended in PBS, pH 7.3. The thrice-washed bacterial mass was resuspended in absolute ethanol and heated at 60°C for 1 h (12). The pelleted bacteria (10,000 x *g*) were resuspended in formal-saline (0.6% formaldehyde in 0.85% NaCl) and washed three times. The bacteria were resuspended in 0.85% NaCl at a final concentration of ≈1 x 10^9 bacteria ml^{-1} .

OmpC-specific antibody preparation. Four New Zealand rabbits were immunized over a 4-week period to obtain predominantly IgG-specific antiporin antibodies (12,19,50). Each rabbit was injected intravenously in the lateral ear vein with 0.1 ml of bacterial vaccine on d 1, 3, 5, 7, 8, 10, 12, 15, 17, and 21. On d 28 the rabbits were starved for 24 h and sacrificed. Approximately 100 ml of blood from each rabbit was collected by cardiac puncture. Rabbit serum was obtained by centrifugation to remove cellular material (800 x *g*). An IgG-rich fraction was obtained by precipitation with 50% saturated ammonium sulfate, centrifugation at (10,000 x *g*), and exhaustive dialysis against PBS pH 7.3 containing 0.1% thimerosal. This material was further purified by affinity chromatography on a Protein G Surperose column (Pharmacia LKB,

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Biotechnology, Piscataway , NJ) equilibrated with PBS, pH 7.3, containing 0.1 % thimerosal. The IgG was eluted with 0.10 M glycine, pH 2.7, and collected as 1.5 ml fractions in tubes containing 45 µl 1.0 M Tris-HCl, pH 9.0 to preserve the activity of the acid-labile IgG. Column size and flow rates were determined by the rat IgG_a and IgG_b binding properties quoted in the data sheet accompanying the affinity chromatography product. Anti-*Salmonella* activity was measured using a bacterial agglutination assay (12). OmpC-specific IgG was obtained through polyclonal sera absorption with an *ompC*-deficient strain of *S. typhimurium*. Approximately 10^{10} PBS-washed *S. typhimurium* SH7241 *ompC*::Tn*10* were added to rabbit anti-O antigen-specific *S. typhimurium* 14028 IgG and the mixture incubated for 30 min at 37°C. Bacteria were removed by centrifugation (10,000 x *g*) and the supernatant fluids containing OmpC-specific IgG were kept frozen at -20°C until used.

Screening transductants for phenotypic mutations. Western profiles of envelope proteins from the mutant strains were compared to that of the wild-type parent *S. typhimurium* 14028. Envelope proteins from *ompC* mutants and wild type strains were prepared as previously described in Chapter One. Approximately100 µg of envelope proteins was separated by electrophoresis in acrylamide gels (11.5%) containing SDS (33), and transferred to nitrocellulose membranes (58). These transferred proteins were probed with rabbit anti-*S. typhimurium* strain 14028 OmpC-specific IgG, and the nitrocellulose membranes washed with PBS pH 7.3. The bound antibody was detected by goat anti-rabbit IgG conjugated to horseradish peroxidase followed by the addition of the substrate 4-chloro-1-naphthol (58).

Transposon curing. Tn*10* was cured from *S. typhimurium* 14028 *ompC*::Tn*10* mutants by inoculating these strains on minimal medium agar containing fusaric acid (12

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μg ml^{-1}) and chlortetracycline (50 μg ml^{-1}) according to standard procedures for positive selection for tetracycline sensitivity (*tet*^{S}) of *Salmonella* (7,36). Loss of Tn*10* is accompanied by the loss of tetracycline resistance; therefore; fusaric acid-treated isolates were further screened for sensitivity to this antibiotic and for susceptibility to the OmpC-specific phage PH105.

Screening transductants for reversion. Selected *tet*^{S} cured strains were screened to determine if reversion to wild-type *ompC* had occurred using PH105 (27,44). Isolates that were *tet*^{S} and phage^{r} were considered phenotypically deficient of the OmpC protein.

Isolation of a *tet*^{r} gene fragment probe. The gene coding for the tetracycline resistance protein at nucleotide residues 86-1276 (*tet*^{r}) of pBR328 was isolated and used as a probe. *E. coli* HB101 containing plasmid pBR328 (16,45,51) was inoculated into a 10-ml shake flask of LB broth containing tetracycline (50 μg ml^{-1}) and incubated for 8 h at 37°C. This culture was used to inoculate 500 ml of LB broth containing tetracycline (50 μg ml^{-1}) and grown for 12 h. Plasmid DNA was isolated by the alkaline lysis method of Birnboim and Doly (3) and double digested with restriction endonucleases *Eco*RV and *Sal* I following the supplier's recommendations (New England Biolabs, Beverly, MA). These unique restriction sites at residues 187 and 651, respectively contain a portion of the *tet*^{r} gene. This mixture was separated by agarose electrophoresis (1% agarose, (37)). A 464-bp fragment was excised from the gel and digested with 5 U of β-agarase overnight at 37°C (FMC, Rockport, ME, (15)). The recovered fragment was precipitated by the addition of 1/10 volume of 5 M NaCl and two volumes of absolute ethanol, sedimented by centrifugation (10,000 x *g*), dried, and resuspended in milliQ-H_2O. Approximately 5 μg of isolated DNA was randomly labeled by the method of nick-translation using commercially supplied enzymes, deoxynucleosidetriphosphates (Gibco

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BRL, Gaithersburg, MD), and 65 μCi of ^{32}P-αlabeled deoxycytidinetriphosphate (3,000 Ci mMol $^{-1}$, ICN Pharmaceuticals, Irvine, CA).

Tn*10*::*tet* Southern hybridization. Selected *tet*^{s} strains of *S. typhimurium* were tested for the presence Tn*10* insertions in their chromosomal DNA by Southern hybridization analysis (52,54). Genomic DNA of *ompC* mutants and of wildtype *S. typhimurium* was purified by the method of Marmur (38). Restriction fragment analysis of *ompC* from *S. typhimurium* was performed by digesting 5 μg of chromosomal material with *Eco*RV (46). These digests were separated by electrophoresis in 1 % agarose gels, transferred to nylon membranes, probed with radiolabeled *tet* (= 10^{9} dpm μg^{-1}), and exposed to Hyper-film MP® (Amersham) for 24 h by the method of Southern (52,54).

Mø. Peritoneal exudate Mø from 8-12 week-old Balb/c mice, obtained from our breeding facility, were elicited by intraperitoneal injection of 3.0 ml of aged Brewer thioglycollate (Difco). After 5 d the Mø were harvested by lavage using 10 ml of Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} (DPBS). The Mø were washed three times by centrifugation (200 × *g*), using cold DPBS and the final Mø resuspensions were made in cell culture medium M199 deficient in serum components and without supplements at a final concentration of either 5×10^{5} or 5×10^{6} Mø ml^{-1}. Approximately 95% of these cells were Mø, as judged by cell morphology.

Growth and preparation of bacteria for adherence and internalization assays. Stationary-phase cultures of bacteria (=2×10^{9} CFU ml^{-1}) were prepared by inoculating LB with bacteria (=10^{3} CFU ml^{-1}) from frozen glycerol stocks and incubating them with vigorous shaking for 18 h at 37°C. Bacterial cultures were collected by centrifugation (5 min at 3,000 x *g* at 37°C) and suspended in prewarmed (37°C) M199 without

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supplements. From this suspension either 5×10^9 or 5×10^{10} bacteria ml^{-1} were prepared.

Internalization assay. Internalization of *ompC*$^+$ and *ompC*$^-$ *S. typhimurium* strains was measured at two different Mø concentrations in the absence of serum opsonins. Bacterial inocula (10 µL per tube) were added to siliconized microcentrifuge tubes and the Mø suspensions were added to the bacteria as 1.0 ml suspensions (time zero [t = 0]). The ratio of *Salmonella* to Mø was 100:1. At each time point measurements were made in triplicate. The infected cells were incubated at 37°C for 15 to 120 min (t = 15 to 120), end-over-end on a Rotamix (Applied Technical Resources, MD) at 12 revolutions min^{-1}. At either 15- or 30-min intervals the appropriate tubes were placed on ice and the Mø were washed with DPBS by differential centrifugation (400 x g) for 5 min at 4°C to remove nonadherent bacteria. This wash step was repeated three times. The supernatant fluids containing nonadherent bacteria were transferred to a clean tube and the bacterial concentration determined by direct colony counts on Plate Count Agar (PCA, Difco, MI). The number of colony-forming units (CFU) was counted following 18 h of incubation at 37°C . The Mø were overlaid with 1.0 ml of PBS containing 1% Na deoxycholate to release the bacteria from the Mø and incubated for 10 min at room temperature with occasional mixing. One-half of Mø lysate was plated on PCA agar directly and the other half volume was serially diluted and plated. The number of CFU was determined following 18 h of incubation at 37°C . Bacterial sedimentation in the centrifugation step was controlled by monitoring bacteria without Mø in the same test conditions. A positive control was the nonpathogenic *E. coli* strain HB101, which is recognized as susceptible to Mø killing.

Biosynthetic radiolabeling of *Salmonella*. Bacterial cultures were grown as

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described above to a final culture density of ~10^9 CFU ml^{-1}. Bacteria from 1.0 ml of culture were washed with 1.0 ml of DPBS prewarmed to 37°C and resuspended in 0.5 ml of sterile methionine-free medium (Methionine Assay Medium, Difco) containing 250 µCi of [^{35}S]-methionine and [^{35}S]-cysteine (Tran^{35}S-label, ICN, Irvine, CA). Samples were incubated at 37°C for 10 min and the reaction was quenched by the addition of 10 µl of 0.10 M L-methionine. The labeled bacteria were collected by centrifugation at 10,000 $\times g$ and washed three times with PBS, pH 7.3. Bacteria were resuspended in M199 medium and used for *in vitro* binding assays.

Adherence assays. Mø were infected with ^{35}S-labeled *S. typhimurium* as in the internalization assays. After 1 h the infected cells were fixed by the addition of an equal volume of 2% glutaraldehyde in PBS and incubated for 10 min. Mø were collected by centrifugation (200 x *g*) for 5 min. The supernatant fluids were collected and transferred to a scintillation vial for the determination of the radioactivity (dpm μl^{-1}). The sedimented Mø were washed three times with PBS to remove the non-adherent bacteria from the interstitial cellular space between cells. The sediment was resuspended in 1 ml (10% SDS, 70°C), transferred to a scintillation vial and counts min^{-1} of solubilized Mø pellet were obtained. The percentage of radiolabeled bacteria bound to Mø was calculated from the division of the radioactivity in the Mø sediment (Mø with adherent bacteria) by the total activity in the reaction vessel which included the sum of the radioactivity obtained from the sedimented Mø and supernatant fluids (adherent plus non-adherent bacteria).

Statistics. Each system was measured in triplicate and the assays repeated at least twice. In each experiment the data were expressed as mean± standard deviation of the cpm for adherence assays or viable bacteria recovered from the internalization assays.

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RESULTS

Transduction. The phage lysate prepared from *S. typhimurium* SH7241 had a titer of 4.93 x 10^9 PFU ml^{-1}. The phage lysate was free of viable *S. typhimurium* as determined by the failure of growth on LB agar inoculated with 0.1 ml of the phage lysate. *S. typhimurium* 14028 transduction with this lysate developed 633 tet^r transductants of which 267 were discarded as pseudolysogens. There were 13 isolates that were resistant to PH105 infection ($PH105^r$). Six of these 13 isolates were susceptible to P22 *c2* infection and confirmed as non-lysogenic strains.

OmpC-specific Western blot analysis. Serum prepared in this study had a titer of 1280 for *S. typhimurium* strain 14028, based on bacterial agglutination assays. Western blot profiles of envelope proteins of the six selected tet^r *S. typhimurium* 14028 transductants were probed with OmpC-specific antiserum (fig. 1). Antibody bound to the 36-kDa protein from envelope preparations of the wild type strain *S. typhimurium* 14028. No antibody binding to the envelope proteins of the six tet^r strains was seen and these were considered to be phenotypically OmpC-deficient.

Adherence assay. Binding of the *S. typhimurium ompC* mutants and the wild-type parent strain of *S. typhimurium* 14028 to murine peritoneal Mø was quantitatively measured *in vitro* using a ^{35}S-labeled bacterial binding assay. The percentage of radiolabeled wildtype *S. typhimurium* 14028 bound to Mø was five-fold higher than that of the six derived *ompC*::Tn*10* mutants derived from *S. typhimurium* 14028 (fig. 2). Furthermore, the binding activity of the wildtype *S. typhimurium* LT2 strain 23564 was comparable to that of the mutant strains.

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Figure 1. Western blot of electroblotted envelope proteins (100 µg per lane) probed with rabbit anti-*S. typhimurium* 14028 polyclonal sera previously absorbed with *S. typhimurium* SH7241 *ompC*::Tn*10*. Goat anti-rabbit antibody conjugated to peroxidase was used to visualize OmpC protein. Lane **A** contains molecular weight standards. Lane **B** contains envelope proteins from *ompC* wildtype *S. typhimurium* 14028, lane **C -G** contains envelope proteins from *S. typhimurium ompC*::Tn*10* trandsductants. Lane **H** contains envelope proteins from *S. typhimurium* LT2 strain SH7241 *ompC*::Tn*10*. Lane **I** contains 10 µg of purifed bovine serum albumin.

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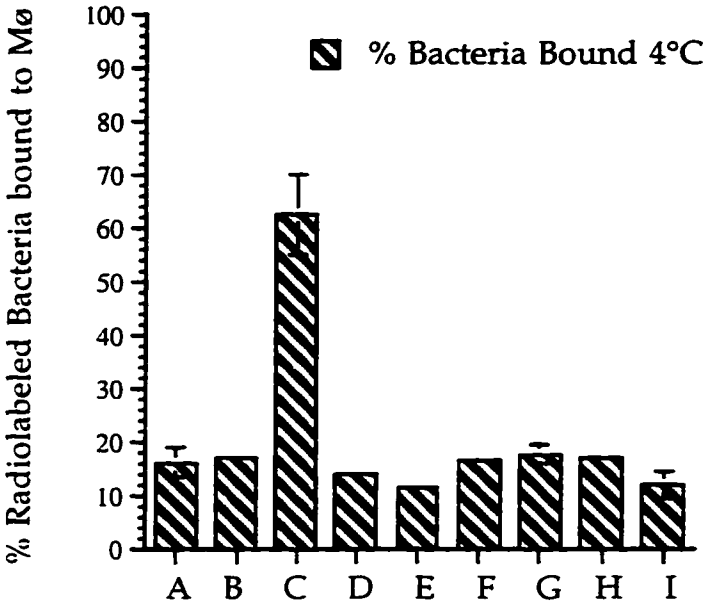

Figure 2. The percentage of radiolabeled bacteria that bind to Mø in suspension for one hour was determined in triplicate by measuring the amount of [35S]-methionine labeled bacteria bound to Mø divided by the total amount of radiolabeled bacteria present in each assay tube multiplied by 100 percent. The above histogram indicates that on average *S. typhimurium* 14028 *ompC*::Tn*10* mutants adhere to Mø in suspension 14.81% as compared to the wild-type *ompC S. typhimurium* 14028 which occurs at 62.54%. Binding assays were done with the following strains: wild-type *S. typhimurium* LT2 ATCC 23564 *ompC+*, **A**, *S. typhimurium* strain SH7241 *ompC*::Tn*10*, **B**, wild-type *S. typhimurium* 14028 *ompC+*, C, *S. typhimurium* 14028 *ompC*::Tn*10*, **D-I**.

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Curing *Salmonella* of Tn*10*. Drawbacks to these studies include the potential for wildtype revertants from unstable transpositions, which occur at a frequency of 1 in 10,000 and the requirement for the presence of the antibiotic, tetracycline (R. Zsigray, pers. comm.). Out of 166 fusaric acid-treated isolates tested 35 were *tet*^{s} and of these 18 were PH105^{r}. One of these isolates, *S. typhimurium* 103 ,was selected for the internalization studies. All 18 strains were considered prototrophic by their ability to grow as well as wild type strain in 18 h at 37°C on Davis minimal medium agar in the absence of supplements. Genomic DNA preparations from all of these selected strains failed to hybridize with a ^{32}P labelled *tet*^{r} fragment and were confirmed cured of Tn*10*. The cured strain *S. typhimurium* strain 103 was used in the internalization assays.

Internalization assay. Wild-type *S. typhimurium* 14028 attached to, was internalized by, and continued to survive and grow within Mø; however, the *ompC* mutant *S. typhimurium* 103 could no longer be recovered from Mø. OmpC mutants appeared to be completely killed within 90 min after phagocytosis by Mø (fig. 3 and fig. 4.). *S. typhimurium* LT2 strains 5014 and 6017 were both killed within the first 60 min subsequent to phagocytosis. The uptake assays did not determine whether *ompC* conferred a selective advantage of the *S. typhimurium* LT2 strains 6017 or 5014. *Escherichia coli* strain HB101 was internalized by Mø after 30 min of infection and no viable bacteria were recovered after 60 min. The recovery of Mø less unbound bacteria was confirmed with sham controls of bacteria without Mø as these did not recover bacteria by relative centrifugal forces used (400 x *g* for 5 min) in this assay.

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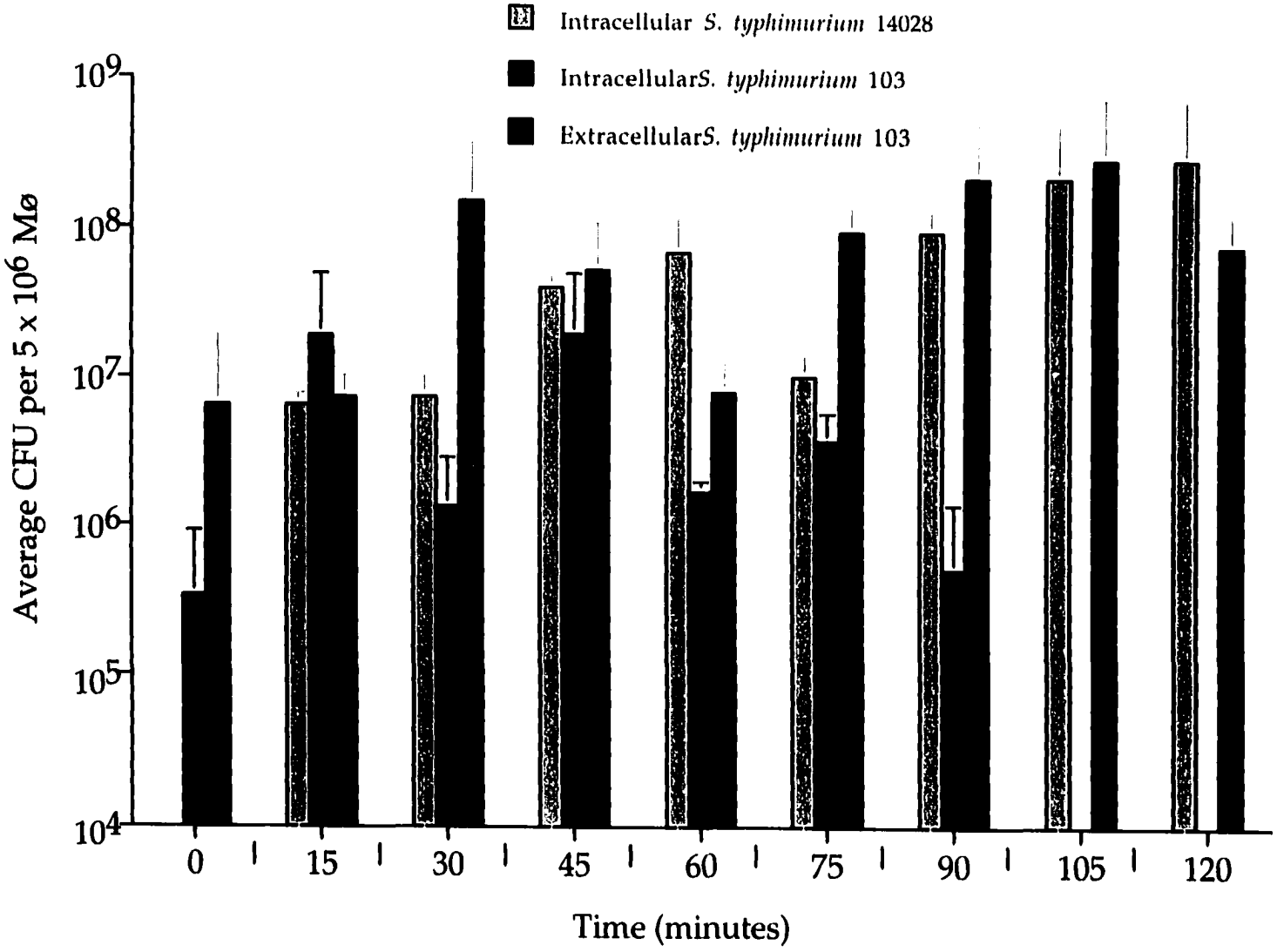

Fig. 3. Mø internalization assay at a ratio of 100 bacteria to one Mø.

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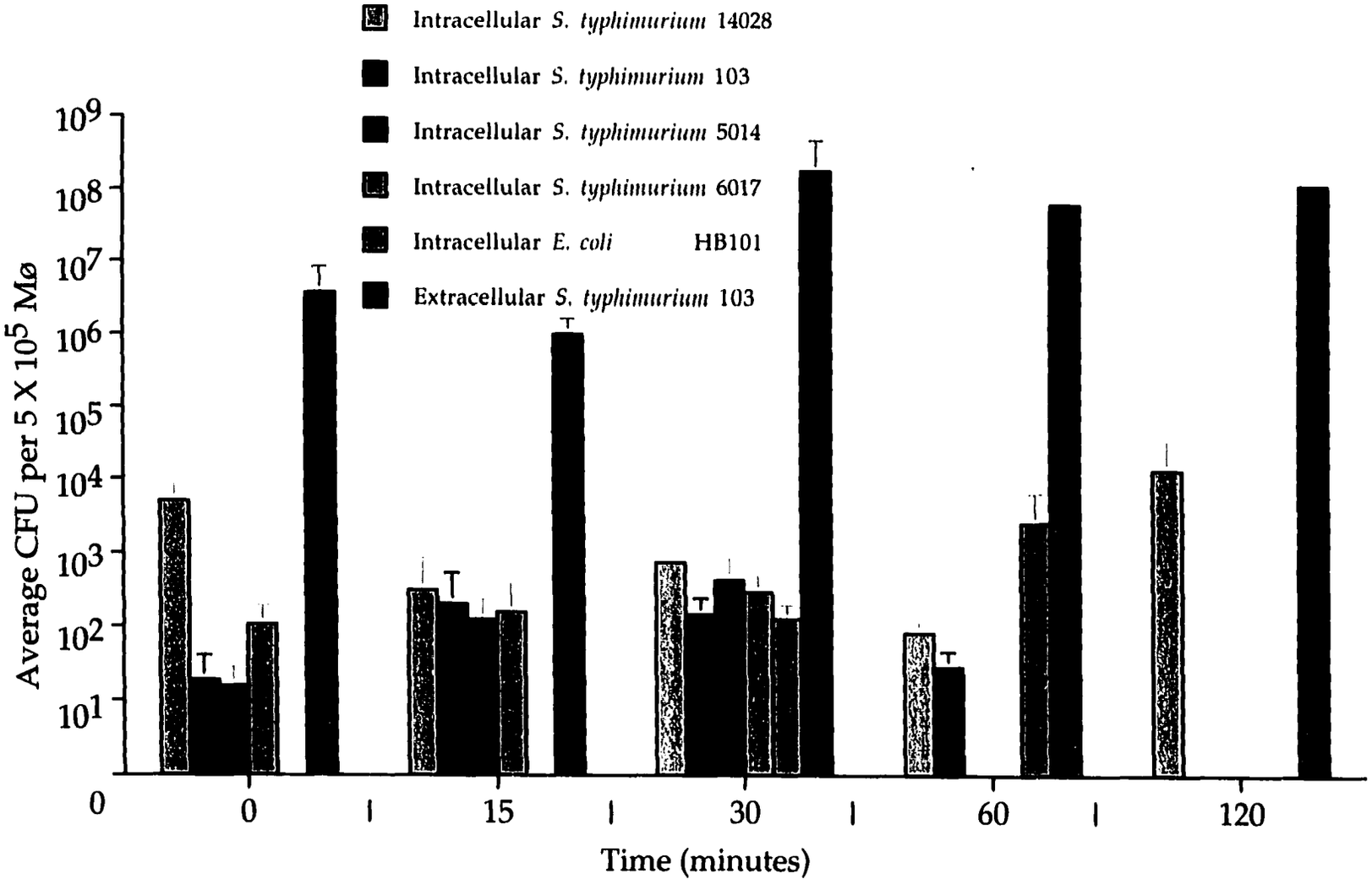

Fig. 4. Mø internalization assay at a ratio of 100 bacteria to one Mø.

DISCUSSION

Early host recognition and effective control by components of the innate defense system are a likely means for intervention strategies at the individual host level. Beyond binding to key host cells, porin proteins from *Salmonella* spp. have been shown to activate the complement system (24), to induce the secretion of several cytokines (23), and to stimulate free radical production by Mø (56). Binding and subsequent internalization of mammalian cells by *S. typhimurium* are independent phenomena (31). This study indicates that adherence of the bacteria to Mø involves the OmpC protein and that this process is independent of serum factors. Support for this finding is seen in studies with *Shigella flexneri,* in which the OmpC protein has been shown to be involved in the invasion of epithelial cells (1,2,48).

To determine whether OmpC expression in *S. typhimurium* is linked to adherence and internalization, *ompC*::Tn*10* was transduced into the Mø-resistant strain *S. typhimurium* 14028 (9,21). These mutant strains were confirmed as OmpC protein-deficient (42). Findings from the adherence assay confirm that the OmpC protein of *S. typhimurium* is involved in early recognition and attachment by host phagocytic cells. Mutants cured of Tn*10* and deficient in OmpC protein did not reveal selective advantages or disadvantages in the number of bacteria internalized by Mø at different times along the course of phagocytic uptake by Mø as compared to the wild type strains. The data do show that at select times viable *ompC*-deficient *S. typhimurium* is no longer recovered from the lysed Mø. Explanations are 1) that the OmpC protein enhances *S. typhimurium* resistance to Mø killing, 2) that bacterial intoxication renders Mø unable to internalize bacteria (47), or 3) that bacteria can no longer enter Mø due to host cell lysis. Arguments for the last possibility are weak, however, since the wild type strain

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continues to be recovered from Mø after 2 h in these infection assays. OmpC-mediated survival of *S. typhimurium* is further supported by the observation that non-pathogenic *E. coli* are killed by Mø (20,31).

Bacterial entry into Mø is a complex process because the constituents of both cells contribute to adherence and phagocytosis. Bacteria can invade Mø while Mø phagocytose bacteria. Not only is the identity of the subcellular participants involved in these internalization mechanisms unknown, but how proteins contribute to the trafficking and the final location of these bacteria within Mø is also unclear. Electron microscopic studies of Mø infected with microbes have revealed that intracellular pathogens employ diverse survival strategies (10). *S. typhimurium* LT2 strains has been observed within fused phagolysosomes (5,13) , and the final destiny and death of these bacteria was accepted (30). However, new evidence has shown that *S. typhimurium* 14028 escapes phagosome-lysosome fusion (26) and that this process is independent of the O chains of the lipopolysaccharide (10).

Bernardini *et al.* observed microscopically that the OmpC of *Shigella flexneri* mediates the extracellular spread of this pathogen from infected to uninfected epithelial cells (1), and that these bacteria lyse the phagosomal membrane and escape into the cytoplasm (14). This model supports the findings observed here with *S. typhimurium*. First, virulent *S. typhimurium* strains adhere better to Mø as than do avirulent *S. typhimurium* strains in the absence of opsonins (41). Second, *ompC*$^-$ bacteria adhere significantly less to Mø than do the virulent isogenic wildtype strains. The internalization findings show that the OmpC protein offers protection to the bacteria from intracellular killing by Mø. Confocal microscopy of infected Mø would be helpful to confirm the subcellular location of these *ompC*$^-$ bacteria. In view of the possibilities, this study provides provides insight into the

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mechanism involved in the virulence *S. typhimurium* and the promise of new strategies directed at the prevention and protection of enteric disease caused by *Salmonella* spp.

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ACKNOWLEDGEMENTS

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CHAPTER THREE

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CHAPTER THREE

Complete Nucleotide Sequence and Comparative Analysis of the *ompC* Gene of *Salmonella typhimurium*

ABSTRACT

Recent evidence suggests that the OmpC protein of *S. typhimurium* is involved in the adherence to host phagocytic cells and offers protection to the bacteria from intracellular killing by Mø. The nucleotide sequence of genes that encode for the classiscal porin proteins OmpC, OmpF, OmpD and PhoE of *Escherichia coli, S. typhi,* and *S. typhimurium* have each been determined except for the *ompC* gene of *S. typhimurium.* This study reveals that there are several distinct differences between the nucleotide sequence of this porin gene and its homologous gene in related enteric bacteria. The deduced OmpC amino acid sequence of *S. typhimurium* shares 77 and 98 percent identity with OmpC amino acid sequences of *E. coli* and *S. typhi,* respectively. This study offers strategies that target specific regions of the nine extracellular loops of this porin protein that either participate in host cell recognition of bacteria by phagocytic cells or are involved in bacterial resistance to phagocytic killing.

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INTRODUCTION

The surface of Gram-negative bacteria include a class of proteins called porins that form diffusion channels for the transfer of small hydrophilic molecules across the outer membrane (1,17,22) . The classical porin proteins of *S. typhimurium* are encoded by the structural genes *ompC, ompF, ompD,* and *phoE* (24,30-32,34). Support for the role of the outer membrane porin protein C in the pathogenesis of *Salmonella* species has been reported (15,36) . These molecules are likely candidates to participate in the virulence of *Salmonella* since they are major outer membrane proteins ($\approx 10^5$ per cell(16)). *S. typhimurium ompC* and *ompF* mutants are attenuated as shown by the increase of the lethal dose 50 by a 1,000-fold in orally challenged Balb/c mice (5). In addition, the homologous OmpC protein of *Shigella flexneri* mediates extracellular spreading of the bacteria from one epithelial cell to another and resistance to host cell killing (2). There is no known nucleotide (nt) sequence for the *ompC* gene of *S. typhimurium.* The literature cites nt sequences upstream of the *ompC* gene of *S. typhimurium* (10), limited amino acid (aa) sequence of the OmpC protein of *S. typhimurium* (32), or reports that the *ompC* of *S. typhi* and *S. typhimurium* are not heterogeneous (26). This study determined the complete nt sequence of *ompC* and showed it is unique to *S. typhimurium.* This information may provide insight as to how this protein is involved in the pathogenesis of *Salmonella* species.

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MATERIALS AND METHODS

Media and culture conditions. *S. typhimurium* ATCC strain 14028 was cultured in 1 l of LB broth for 18 h at 37°C. Cells were concentrated by centrifugation (10,000 x *g*) at 4°C. Genomic DNA was isolated from the cells by the method of Marmur (19).

PCR methodology. The *GenAmp*® PCR core kit (Perkin Elmer, CA) was used to amplify regions of the *ompC* DNA. All deoxyoligonucleotide primers were developed by a commercial supplier (Integrated DNA Technologies, IA). Synthesized oligonucleotide non-overlapping primers complementary to regions near or within the 1134 bp *ompC* reading frame of *S. typhi* (26) were designed to anneal to conserved regions of the related gene of *S. typhimurium* by use of the Lazer Gene Navigator Software Package (DNA Star®, Inc., Madison, WI). The oligonucleotides used for PCR reactions were also used for nt sequencing reactions. PCR reactions were performed with 0.1 µmol of each primer in a 100 µl of reaction mixture. *S. typhimurium* genomic DNA (100 ng) was mixed with 2 units of *Taq* DNA polymerase in 100 µl of reaction buffer and subjected to polymerase chain reaction (9). The *ompC* gene fragments were selectively amplified by several PCR reactions using oligonucleotide primers (fig 1A and B). The reaction conditions were as follows: 5.00 min at 95° C, 1 min at 55° C and 1 min at 72° C for 25 cycles ending with a 7-min extension step. The PCR products were separated from PCR reagents by DNA gel electrophoresis.

Analysis of plasmid and PCR products. The presence of double-stranded PCR products were confirmed by agarose gel electrophoresis. The size of the PCR products were determined by comparison with known ds-DNA molecular weight markers (18). Electrophoresis was carried out at 5 V/cm for 3 hr in a horizontal electrophoresis unit

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(Mini-cell, Bio-rad, CA) in the same Tris-acetate-EDTA buffer. The gel slabs were stained with 0.5% ethidium bromide and viewed using an ultraviolet transilluminator (C-63, Ultra violet products). Selected fragments were excised from gels and digested with 5 U of β-agarase overnight at 37°C (FMC, Rockport, ME, (6)). The recovered fragments were precipitated by the addition of 1/10 volume of 5 M NaCl and two volumes of absolute ethanol, sedimented by centrifugation (10,000 x *g*), dried, and resuspended in milliQ-H_2O.

Southern hybridization. PCR products were confirmed as *ompC* DNA by hybridization against the chromosomal *ompC* gene (33,35). PCR fragments were prepared and radiolabelled with ^{32}P-deoxycytidine triphosphate and resuspended in water as described in Chapter Two. Genomic DNA from wildtype *S. typhimurium* 14028 (5 µg) was digested with the restriction endonuclease *Eco* RV (26). These mixtures were separated by electrophoresis in 1 % agarose gels, transferred to nitrocellulose membranes, and probed with radiolabeled PCR products (≈ 10^9 dpm μg^{-1}). Membranes were exposed to Hyper-film MP®(Amersham) for 24 h. Hybridization of the radiolabelled PCR products with a 2.1-kb *Eco* RV fragment containing the *ompC* gene of *S. typhimurium*(26) was detected autoradiography.

DNA sequencing using PCR products. The ds-DNA obtained above was sequenced with fluorescently labeled dideoxy chain termination reactions (29) and an automated DNA sequencer (ABI Model 373, (Perkin-Elmer Corporation, Foster City, CA) (25). PCR -amplified DNA was primed for Sanger dideoxy sequencing as shown in Fig 1 C & D with 10 pmols of one of the following oligonucleotides: 74, 75, 63, 80, 81, 83, 66, 76, 77, 78, or 79. Sequence data were edited with the computer-assisted application (SeqEd version 1.0.3, Perkin-Elmer Corporation, Foster City, CA). Codon

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usage tables for *S. typhimurium* were provided from Genbank Release 99 (Feb 15, 1997) and used for direct DNA to protein sequence translations. Nt and deduced aa sequence identity comparisons were made with the *ompC* sequences from *E. coli* and *S. typhi* using Lazer Gene Navigator Software Package (DNA Star®, Inc.) and MacDnasis version 3.0 (Hitachi Software Engineering Co., Ltd.) computer-assisted application programs.

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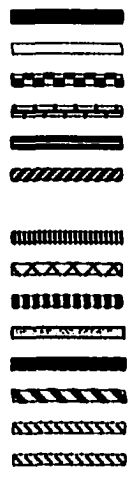

forward	#82 (24 mer)	GCTTTGAAATAGGGGTAAACAGAC*	-	strand nt 5-25
forward	#65 (24 mer)	ATTTCCGTATATTGTCTCC	+	strand nt 376-394
forward	#74 (24 mer)	AAAGACCGCAACAAATTAGACCTG	-	strand nt 482-505
forward	#62 (24 mer)	TTACGGTTTTGCCAACAAAG	-	strand nt 1234-1253
forward	#77 (20 mer)	GCATCAACACCGACGACATC	-	strand nt 1491-1510
forward	#79 (22 mer)	CGTAGTTGTGGCTGCTGTAG	+	strand nt 1491-1510
reverse	#80 (20 mer)	ACCAGTCGGCAAGTCCATTC	+	strand nt 335-316
reverse	#76 (24 mer)	CAGGTCTAATTTGTTGCCGTCTTT	+	strand nt 505-482
reverse	#78 (24 mer)	GTCCAGATTAAACAACGGCAGAAA	-	strand nt 505-482
reverse	#63 (22 mer)	CTTTGTTGGCAAAACCGTAA	+	strand nt 1253-1234
reverse	#75 (22 mer)	GATGTCGTCGGTGTTGATGC	+	strand nt 1510-1491
reverse	#66 (22 mer)	CGTCCGGGAAATCGTTGTAGAAAA	-	strand nt 1569-1546
reverse	#81 (19 mer)	TTTGTACGCCGGAATAAGG	+	strand nt 1604-1586
reverse	#83 (22 mer)	ATCTTTGTACGCCGGAATAAGG*	+	strand nt 1606-1586

Table 1. Oligonucleotide legend.

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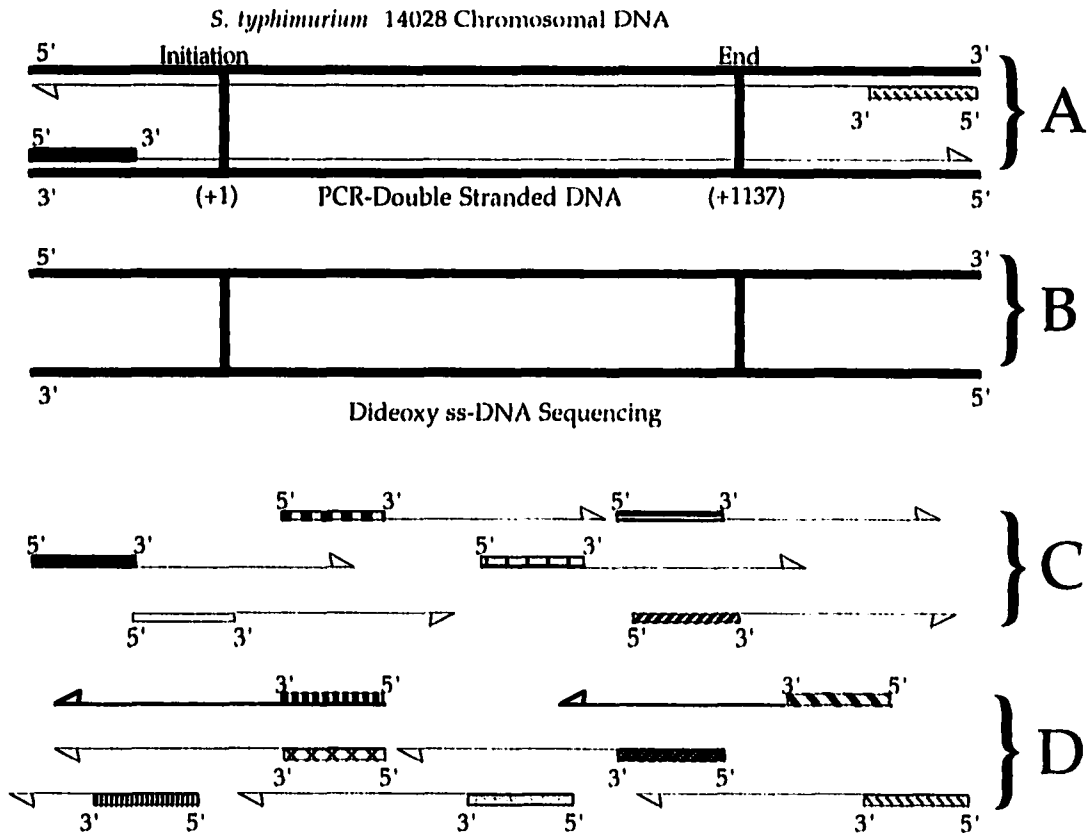

Fig. 1. PCR and nucleotide sequencing strategy of the *ompC* gene of *S.typhimurium* 14028.

RESULTS

PCR amplification. The *ompC* of *S. typhimurium* was amplified from the chromosome using the PCR strategy in Fig. 1. One of either the forward primers 74 or 82 were mixed with one of the reverse primers 63, 75, 80 and 81 as seen in Fig. 1 and 2 (26). PCR products were recovered from agarose gels as described in Chapter Two. PCR products were hybridized against the 2.1 kb *Eco* RV DNA fragment and confirmed as *ompC* DNA as shown in Fig. 3.

Sequencing OmpC. The *S. typhimurium ompC* gene was nt sequenced following the strategy shown in Fig. 1 C and D. The nt and deduced aa sequences of *ompC* are shown in Fig. 4. Numbering of nt and aa was based on the gene sequence of open reading frames (ORF) described from nt sequences of *ompC* from *S. typhi* (1134 nt) and *E. coli* (1101 nt (20,26)). The *ompC* sequence of *S. typhimurium* 14028 contains a single ORF of 1134 nt that corresponds to a protein (OmpC) of 378 aa. These results are consistent with the *ompC* reading frame of *S. typhi* (26).

Nt and aa sequence comparisons. At the nt level, the leader region (initiation codon at +1, Fig. 4) through nt 1134 has 98% and 77% nucleotide sequence identity with the *ompC gene* of *S. typhi* and *E. coli*. At the aa level, the OmpC of *S. typhimurium* shares 98 and 79% sequence identity with the related protein of *S. typhi* and *E. coli*. There are other differences at the nt level. There are 160 single nt substitutions between the alignments of *E. coli* and *S. typhimurium ompC*, which results in 38 different aa substitutions. There are also differences in the nt sequences that are the result of either deletions or insertions. In comparison with the *E. coli* gene, *S. typhimurium ompC* lacks six codons between nt 543 and 544, and has additional one, three, nine, and four

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codons, at 610, 685, 802, and 955, respectively, which are shown at aa positions 210, 233, 274, 325 in Fig. 5. Comparison with the *S. typhi ompC* gene reveals 12 nt substitutions at positions 124, 153, 315, 477, 621, 667, 774, 811, 828, 948, 1019, and 1066; however, aa changes only occur at positions 43, 229, 259, 264, 346, 362, and 363 (fig. 5.) There are no nt insertions or deletions between the *ompC* genes of *S. typhimurium* and *S. typhi.*

Protein secondary structure. The leader portion, which consists of 21 aa (starting before the arrow on Fig. 5), is identical in all three microorganisms. This shared identity was also confirmed by N terminal sequencing of the 36-kDa protein , as reported in Chapter One. There are 357 aa as deduced from the nt sequence for the *S. typhimurium* OmpC protein (calculated M_r of 39,215). The protein shares 77% and 98% similarity with the *E. coli* and *S. typhi* mature OmpC proteins, respectively.

The 5′ and 3′ end regions. The nt sequence of the 5′ untranslated region (UTR) upstream of the *ompC* of *S. typhimurium* shares 72.5% sequence identity with that of the UTR of *E. coli* and 99.6% sequence identity with that of the UTR of *S. typhi.* These non-identical nt residues are shown in Fig. 4 as either underlined or in bold type in Fig. 4 as compared to either *E. coli* or *S. typhi* UTR, respectively.

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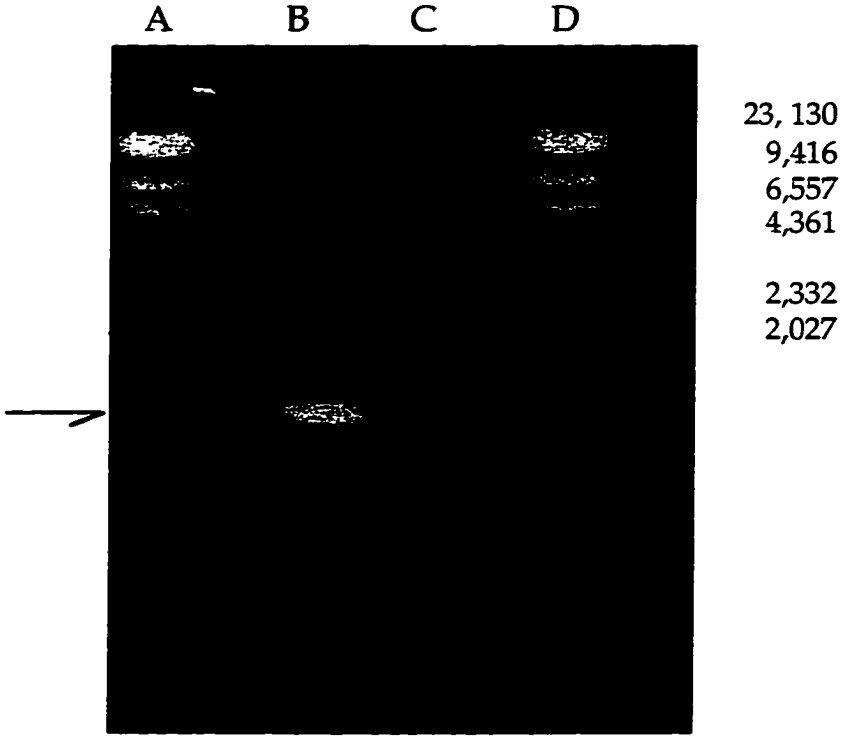

Fig. 2. Agarose gel (1.0%) used to separate a 1.03 kb *ompC* developed from a PCR reaction mixture using primers 74 and 75 and genomic DNA from *S. typhimurium* 14028 (lane **B**), negative control using no chromosomal template (lane **C**), λ*Hind* III molecular weight standards (lanes **A** and **D**) and their weights are shown on the right in base pairs.

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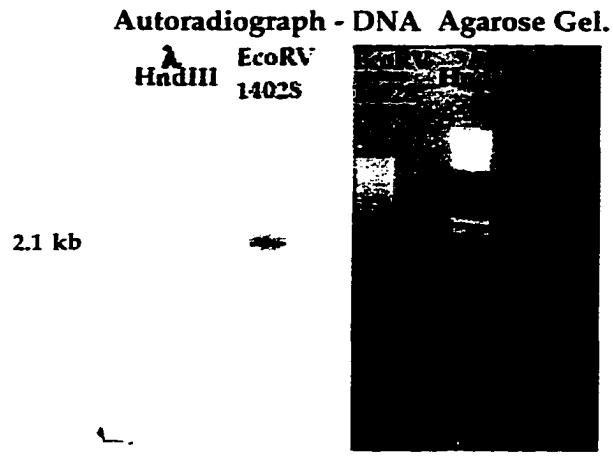

Fig. 3. Southern blot of *S. typhimurium* 14028 genomic DNA digested with *Eco*RV restriction endonuclease, separated using agarose gel electrophoresis, transferred to nitrocellulose and probed using the ^{32}P-labeled 1.03 kb PCR product which was developed with primers 74 and 75.

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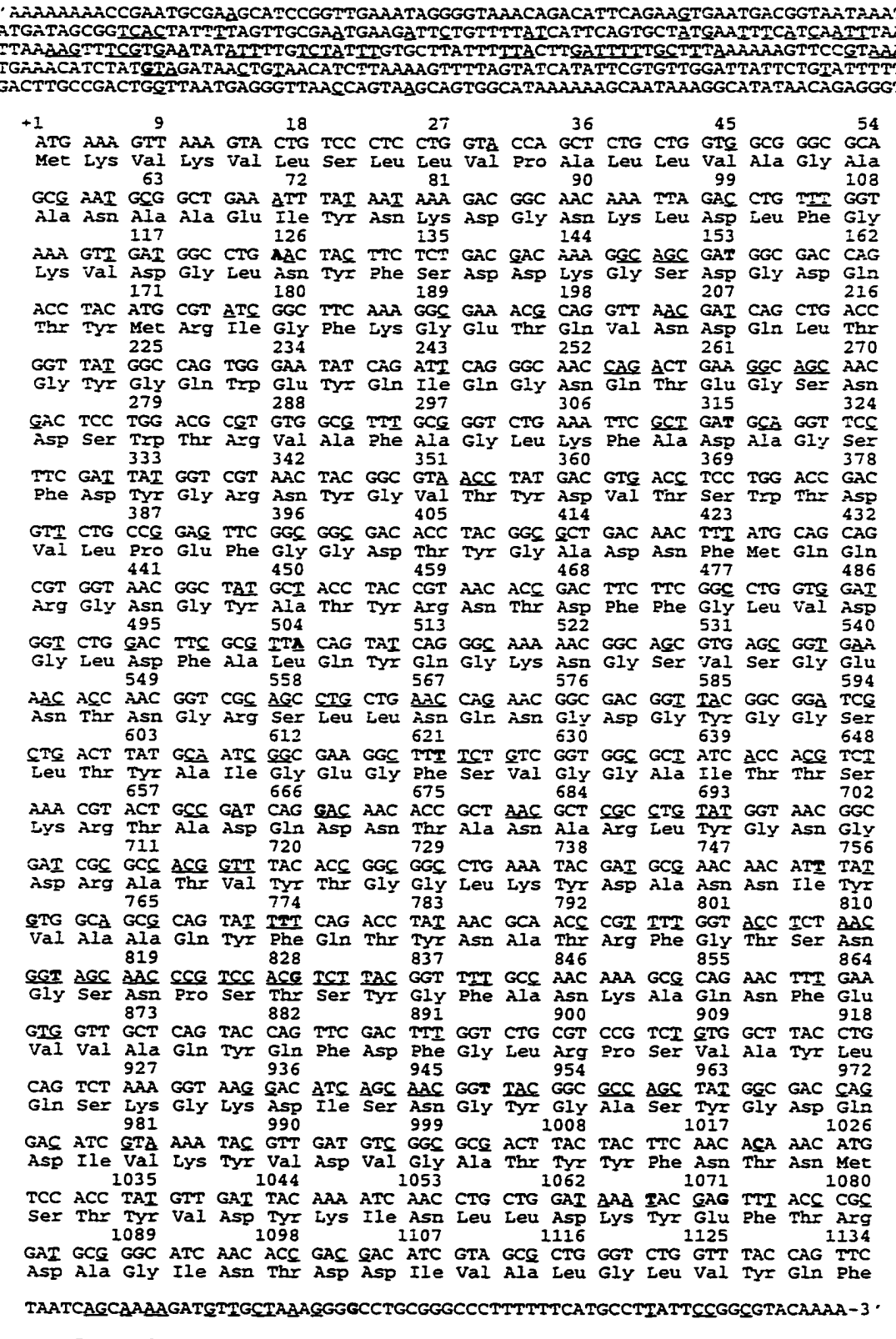

Fig. 4. Complete nt sequence of the *ompC* gene of *S. typhimurium* 14028. Nt residues that differ from *ompC* of *S. typhi* or *E. coli* are shown in bold or underlined, respectively.

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Fig. 5. Comparative alignment of deduced OmpC amino acid sequence from *S. typhimurium* 14028 (**A**) with OmpC sequences from *S. typhi* IMMS-1 (**B**), and *E. coli* K-12 (**C**). Shaded regions correspond to amino acids in boxes that differ from OmpC of *S. typhimurium*. The arrow indicates the site specific cleavage of the OmpC precursor protein. One letter amino acid abbreviations are shown.

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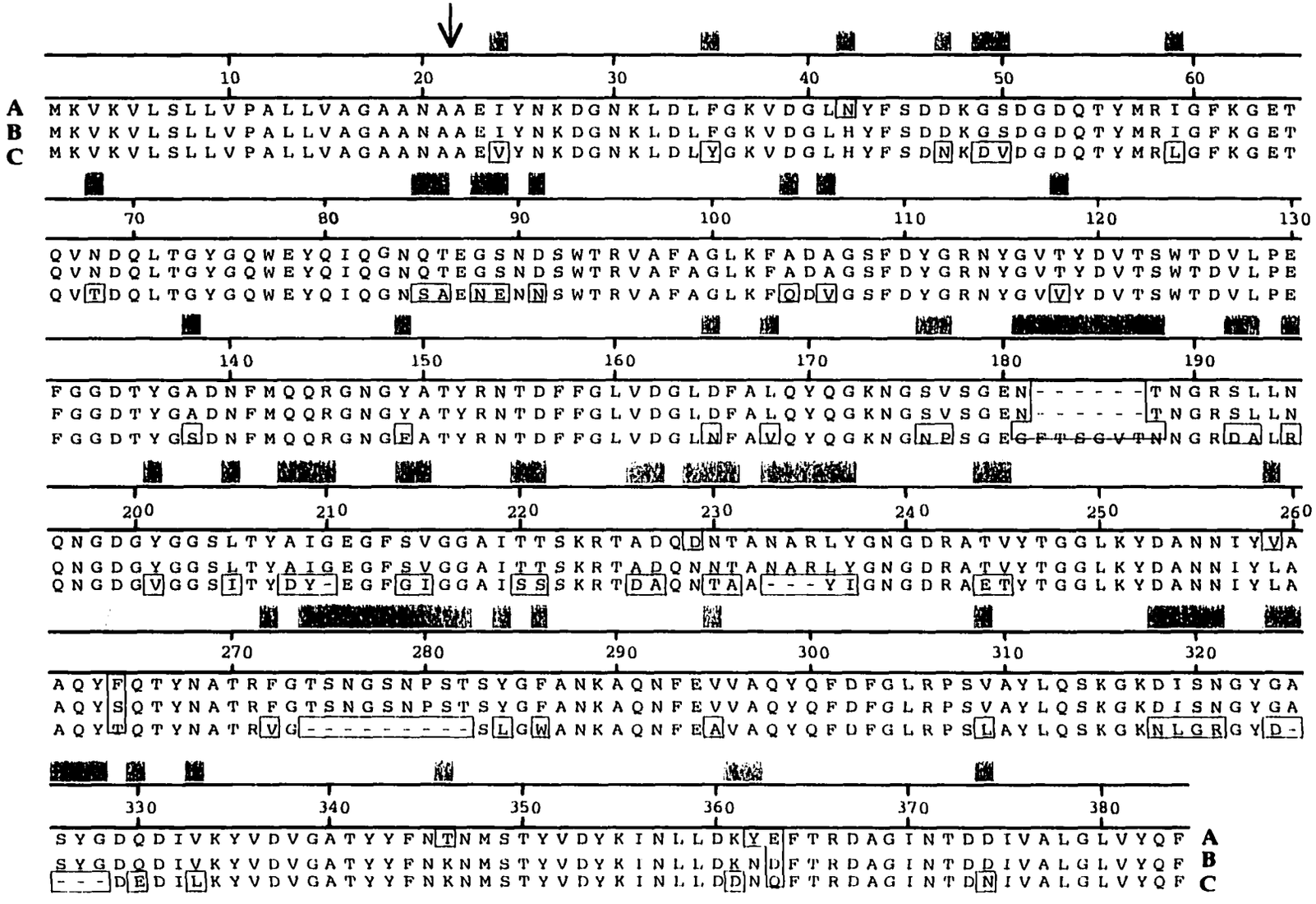

DISCUSSION

Many studies including this one use genetic approaches to study the mechanisms of disease (4,13,23). Nt sequences of the genomes from *Haemophilus influenzae, Methanococcus jannaschii, Mycobactererium genitalium* and *Saccharomyces cerevisiae* have already been completed (3,11,14). These findings provide an useful database of information for both known and unknown proteins (8). Genome sequencing of the human pathogens *Treponema pallidum, Borrelia burgdorferi, Mycobacterium tuberculosis, M. pneumoniae, E. coli* and *S. typhimurium* are currently underway (12,28). Many recombinant DNA experiments require knowledge of the DNA sequence as a prerequisite for creating detailed restriction enzyme maps, determining protein coding regions (ORF) and valuable DNA or protein motif domains.

The *ompC* gene of *S. typhimurium* is located at 49.3 min on the chromosome (28). It has been reported on the basis of Southern blot analysis that the *ompC* gene of *S. typhimurium* is identical to the *ompC* gene of *S. typhi* (26). This study has shown that there are seven substituted aa residues of the *S. typhimurium* OmpC protein when compared to the OmpC aa sequence of *S. typhi*. The high degree of homology in the UTR between *S. typhi and S. typhimurium* are consistent with those of nt sequence and identity comparisons obtained with the 5′ *ompC* UTR comparisons of *S. typhimurium* with other Gram negatives by Esterling *et al.* (10). Conserved regions amongst the enteric bacteria also include the N-terminus of the OmpC precursor protein which traffics the OmpC protein to the outer membrane following translation (21).

The ultrastructures of the OmpF and PhoE crystals of *E. coli* have been reported (7). A protein monomer folding pattern of 16 antiparallel β-barrel transmembrane (TM)

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regions that trimerize to form a pore has been proposed as the structural basis of porins (16). Comparison of the aa sequence of the members of the porin superfamily has led to the concept that the 16 TM β-strand sequences are highly conserved and are flanked by eight loops of variable aa sequences that promote turns of high hydrophilicity and low amphipathicity, oriented towards the bacterial cell surface (16). Based on these concepts, one monomer of *S. typhimurium* OmpC protein contains seven intracellular loops (from now on referred to as loops **1** through **7**) and eight extracellular loops (referred to as loops **a** through **i**),one of which does not span the membrane, and 16 TM spanning regions (referred to as **TM 1** through **16** (27)). The aa substitutions on the OmpC protein of *S. typhimurium* are as follows: an N to H substitution on extracellular loop **a**, N to D substitution at loop **f**, N to Y and D to E substitution at loop **i**, L to V, and S to F substitution at TM region number **11**, and a K to T substitution at intracellular loop number **7**.

The complete nt sequence of the *ompC* gene of *S. typhimurium* will be used for future projects related to the design of *S. typhimurium* partial OmpC chimeric molecules (*e.g.*, His-tagged, GST fusion proteins) that will identify subcellular components of Mø that are involved in binding interactions with this porin protein. Potential strategies for protection of the host from *Salmonella* at the individual level may arise from blocking the the surface exposed extracellular loops of this protein since they appear as likely candidates involved in the direct host defense cell recognition, bacterial adherence and bacterial resistance to phagocytic killing

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GENERAL DISCUSSION

Salmonella species infect both animal and human hosts and are the causative agents of diseases including enteric fever (typhoid), gastroenteritis, bacteremia, and a variety of localized systemic infections (9). Numerous virulence determinants that play key roles in the ability of *Salmonella* species to infect, colonize, invade, and replicate within the host have been identified (5-7,13). Much of this research has been conducted on *S. typhimurium*, a serotype of particular interest since it is one of the leading causes of food-borne disease in humans (3) It also induces a typhoid-like disease in susceptible mice (14). Murine typhoid pathogenesis has served as a model of human typhoid infection and has enabled a detailed analysis of the organism's spread beyond the bowel to deeper tissues and the systemic circulation. A key defense cell in *Salmonella* infections is the Mø. Usually understood as a major cell involved in phagocytic uptake and internal destruction of microbial pathogens, for some intracellular bacterial pathogens such as *Salmonella* spp, the Mø becomes a haven, a protected environment in which the microorganism may thrive (8). One trait of *S. typhimurium* thought to be essential for its disease potential is its ability to survive in Mø (4,10). To survive within Mø, pathogens have developed defense mechanisms to counter antibacterial assaults such as toxic oxygen derivatives, reactive nitrogen intermediates, and defensins.

The role of innate defenses in overcoming the initial microbial infection has become increasingly recognized as an important component of our ability to prevent disease development following infection by potential pathogens (2). Recognition is an important component of early defenses against salmonellosis and peritoneal Mø from mice

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associate with these organisms in the absence of exogenous opsonins such as specificantibody or complement (1). A global understanding of the events and subcellular participants involved in the initial interaction of *S. typhimurium* and host mononuclear phagocytes is essential for developing molecular strategies for disease prevention in individuals exposed to this microorganism.

Components of the innate defense system are an important first line of defense in controlling this disease early in the infectious process. A key bacterial ligand in this recognition process is a 44-kDa protein structurally related to the porin OmpC (11). Involvement of the porin protein OmpC in recognition of *S. typhimurium* by Mø and resistance to killing by Mø supports a role for porin proteins in linking salmonellae with key cells of the innate defense system (12).

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