

Abstract

Thomas, Gracie A. Expression of Virulence Genes in *Salmonella*. (Under the direction on Dr. Stephen J. Libby)

A role for *slyA* in *Salmonella* pathogenesis has been clearly demonstrated. Originally, *slyA* was identified as a gene that encoded a cryptic hemolysin. When cloned into *E. coli*, *slyA* was shown to be required for the cytolytic death of nucleated cells and the lysis of red blood cells. However, upon further investigation, it was discovered that expression of the hemolysin is directly regulated by *slyA* and is, therefore, not a function of the gene itself. It is previously reported that SlyA is required for the intracellular survival of the bacteria within host macrophages and that *slyA* mutant strains of *S. typhimurium* are severely attenuated for virulence in the murine model. In addition, Daniels *et al.* showed that SlyA is required for the destruction of murine M-cells but not for invasion. Further studies show that *slyA* may be required for resistance to oxidative stress and, more recently, that *slyA* may act as a regulator of virulence genes involved in the systemic phase of infection, but not the enteric phase.

Based on sequence homology with other transcriptional regulatory proteins of other Gram-negative bacteria, it was first suggested by Ludwig *et al.* that SlyA may act as a regulatory protein, affecting expression of both *E. coli* and *Salmonella* genes. In view of this, SlyA was initially grouped with MarR and EmrR (MprA) of *E. coli*, Hpr of *Bacillus subtilis*, and PecS of *Erwinia chrysanthemi*. However, it is now apparent that SlyA is more distantly related to these regulatory proteins than previously reported. SlyA is currently classified as a member of a unique family of low molecular weight transcriptional regulatory proteins that include RovA of *Yersinia pestis* and *Yersinia tuberculosis*, Rap of *Serratia marcescens*, and Hor of *Erwinia carotovora*.

SPI-4 is an approximate 25kb sequence insertion located at centisome 92 on the *Salmonella* chromosomal map. This pathogenicity island is flanked on either side by the *ssb* and *yciB* genes in both *S. typhimurium* and *S. typhi*. Originally, SPI-4 was identified via a transposon insertion as a chromosomal sequence required for survival inside of murine macrophages. Wong *et al.* originally claimed that SPI-4 contained 18 ORFs designated A-R. The recently completed annotation of both *S. typhimurium* LT2 and *S. typhi* genomes shows that only 6 open reading frames exist. Ahmer *et al.* demonstrated SPI-4 regulation by SirA, a transcriptional regulator of SPI-1 (invasion locus). Experiments in our laboratory have shown that the beta-galactosidase activity of a SPI4-K::MudJ insertion (SL3277) in a *slyA* mutant background is reduced 8-fold, suggesting that SPI-4 is regulated by *slyA*. We recently discovered that the predicted SPI4-K protein has significant homology to a class of autotransporter toxins found in pathogenic *E. coli*, *Bordetella*, *Neisseria*, and *Shigella* species, referred to as SPATE toxins. Members of this family are serine proteases possessing a characteristic GDSGS motif with a central catalytic Ser residue. We have constructed and purified a His-tagged SPI4-K protein fragment based on the earlier annotation, and used this protein to obtain a polyclonal antibody. In this study, we examined the protein expression characteristics of SPI4-K in *Salmonella typhimurium* and other Salmonellae. Here, we confirm through immunoblot analysis that SPI4-K is a large molecular weight protein that appears to be secreted from the intracellular compartment.

In an attempt to further clarify the role of both SlyA and SPI4-K in *Salmonella* pathogenesis, polyclonal antibodies were purified to these proteins. Here, we show that the anti-His-tagged SlyA polyclonal antibody reacted with several low molecular weight

proteins. In addition, we have found that SlyA may be expressed in early to mid logarithmic phase and *not* in stationary phase.

Expression of Virulence Genes in *Salmonella*

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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

MICROBIOLOGY

Raleigh

2002

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Acknowledgments

I would like to thank my parents for all of their support and positive encouragement they have given me over the years. I give a special thanks to my mentor, Dr. Stephen J. Libby, for showing me the ropes and always being patient. I offer my gratitude to Thomas A. Halsey, Michael J. Stewart, Elizabeth Boutt, and Kara Main for their helpful discussions and participation in the project. In addition, I would like to thank the other members of my committee, Dr. Amy M. Grunden and Dr. Geraldine Luginbuhl. Their input and guidance has led to the approval of the following thesis work.

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***Salmonella* Pathogenesis Review**

Disease Causing Mechanisms of *Salmonella*

Salmonella is a Gram-negative facultative intracellular pathogen that is able to infect and cause disease in humans and domestic animals. The World Health Organization reports that there are 2213 known serotypes of *Salmonella* to date, the majority of which are capable of causing disease in humans. The serotypes are categorized into seven taxonomic groups based on the variation in their biotypes. Groups I, II, IIIa, IIIb, IV, and VI are all members of *S. enterica*, while group V is composed of *S. bongori*. *Salmonella* infection in humans usually results from consumption of contaminated food and water. Symptoms following infection depend on the serotype and range from mild self-limiting enteritis where infection remains localized in the gut, to acute systemic salmonellosis where bacteria enter the bloodstream and disseminate to organs such as the spleen and liver.

For *Salmonella* to cause systemic disease, it must first survive the gastric acidity of the stomach, invade the intestinal epithelia, and resist host phagocytes. Because *Salmonella* is unable to survive at a pH lower than 4.5, a high oral concentration is required to cause infection. In the *ity*^s mouse model, the infectious dose is 10⁶ organisms. The site of infection is primarily initiated at M-cells of the Peyer's Patches in the small intestine (67). M-cells are specialized epithelial cells that constantly sample antigenic material in the lumen and present it to the immune system. The interaction between *Salmonella* and M cells facilitates host colonization. *Salmonella* gains entry into M-cells during macropinocytosis through the unique mechanism of cell membrane ruffling (71). *Salmonella* moves through the peripheral epithelial layer of the Peyer's patches by

transcytosis and enter the follicle dome of the lamina propria. The follicle dome houses host macrophages and lymphocytes that are constantly assessing the environment for bacteria. In some cases, the invading bacteria will be phagocytosed and killed by the professional phagocytes. Sometimes, however, virulent strains can invade and take up residence inside of host macrophages. The latter scenario leads to severe systemic infection by way of matriculating host macrophages that carry *Salmonella* to organs of the body such as the spleen and liver (41). In order to cause systemic infection, a sufficient number of *Salmonella* must transverse the M cells and survive within the patrolling phagocytes.

Surviving the Oxidative Burst within Phagocytes

The innate immune system of the host plays a vital role in resistance to *Salmonella* infection, as do a variety of other bacterial, viral, and parasitic pathogens. Professional phagocytes become activated when *Salmonella* is opsonized or tagged by complement factors and/or antibodies for recognition. Efforts have focused on understanding the ability of *Salmonella* to resist both oxygen-dependent and oxygen-independent antimicrobial activity of host phagocytic cells. Oxygen-independent killing mechanisms by host phagocytes include bioactive peptides, alteration of phagosomal ionic concentration or other nutrient limitation, and reduced pH (42). The oxidative mechanisms are the focus of this review and are discussed below.

The oxidative burst is the release of oxygen-dependent molecules with antimicrobial properties. The effects of the oxidative burst are most often detrimental to the bacterial cell, causing lipid, protein, and DNA damage. The inability to launch an oxidative burst

is extremely detrimental and is linked to a disorder in humans known as chronic granulomatous disease (GCD) (9). A deficiency in one of the protein components of the oxidase renders it non-functional and, as a result, humans are susceptible to repeated fungal and bacterial infections (90, 91, 104).

Intense effort has been directed at understanding the molecular mechanisms by which *Salmonella* is able to survive and replicate in host macrophages. *Salmonella* survival and replication in host phagocytic cells is required for the manifestation of virulence (39, 40, 42-44). Genes that have been shown to be required for *Salmonella* survival in host macrophages and virulence include *slyA*, *phoP*, *purE*, *aroA*, *rpoE*, SPI2, *sspJ*, *mig-14*, *pagC*, and *recA*, among many others (15, 19, 20, 29, 36, 61).

Until recently, the molecular mechanisms by which *Salmonella* is able to withstand reactive oxygen species produced by the respiratory burst of phagocytes were poorly understood. The respiratory burst of phagocytes following ingestion of bacteria results in the production of superoxide from molecular oxygen (4). The cytochrome b558 portion of the NADPH phagocyte oxidase (*phox*) associates with the phagosomal membrane following phagocytosis to form an active complex with the cytoplasmic subunits p47 and p67. This enzyme produces superoxide radical $\cdot\text{O}_2^-$ that can dismutate to form hydrogen peroxide (H_2O_2) near the ingested microbe. These two reactive oxygen species (ROS) can also react with nitric oxide (NO) to produce peroxynitrite (ONOO^-), a reactive nitrogen species (RNS). Hydrogen peroxide can react with Fe(II) (Fenton reaction) to form hydroxyl radical ($\cdot\text{OH}$). Thus, the production of superoxide radical by phagocytes can give rise to a variety of toxic and highly reactive antimicrobial compounds that must be resisted by pathogenic bacteria. Fang *et al.* has shown that

genetic and biochemical factors are used by *Salmonella* to resist or avoid phagocyte-derived oxidative antimicrobial products (15, 27-30, 37, 76). Several lines of evidence have demonstrated the importance of the phagocyte respiratory burst in resistance to *Salmonella*. *Salmonella* mutants defective in the DNA repair genes *recA* and *recBC* are able to survive and replicate in murine macrophages lacking the ability to generate a respiratory burst, but are killed in burst-competent macrophages (15). Mice that have a defined mutation in the gp91 subunit of the NADPH oxidase (*phox* knockouts) have increased susceptibility to *Salmonella* infection (29), and macrophages from these mice are unable to control *Salmonella* replication. An *S. typhimurium* *sodA* mutant lacking cytoplasmic Mn-superoxide dismutase remains virulent in mice and is able to replicate in macrophages (100). However, the importance of the periplasmic Cu,Zn-SOD for resistance to reactive oxygen and nitrogen compounds has recently been demonstrated (33). *S. typhimurium* *sodC* mutants are attenuated for virulence in mice and are unable to replicate in respiratory burst-competent macrophages. Restoration of virulence and macrophage growth can be observed in gp91*phox*^{-/-} mice or following inhibition of inducible nitric oxide synthase (iNOS). Recently, studies (21, 66) have demonstrated that the *Salmonella* Pathogenicity island 2 genes (SPI2) are required for survival in host phagocytes. Vazquez-Torres (101) subsequently showed that the SPI2 genes interfere with proper trafficking of NADPH oxidase-containing vesicles to phagosomes harboring *Salmonella*, even though the ability of these macrophages to generate a respiratory burst was not affected. There was an approximate 10-fold reduction of co-localized phagosome-associated NADPH oxidase in wild type *Salmonella*-infected macrophages as compared to SPI2 mutant *Salmonella*. These studies suggest that secreted protein

products from the SPI2 locus affect intracellular trafficking events to reduce delivery of the NADPH oxidase to the *Salmonella*-containing vacuole.

The ability of *E.coli* and *Salmonella typhimurium* to withstand oxidative stress has been shown to involve more than 60 genes (38). *S. typhimurium* has been utilized as a model pathogen for *in vivo* resistance to the oxidative environment of host phagocytes. It is interesting that many genetic loci required to resist oxidative stresses *in vitro* are not essential for *Salmonella* virulence. Only *slyA*, *sodC*, *rpoE*, *recA*, and *recBC* have clearly been demonstrated to mediate resistance to reactive oxygen species *in vitro* and play essential roles in survival within macrophages and virulence in mice *in vivo* (5, 14, 16, 98).

The Contribution of *Salmonella* Pathogenicity Islands to Virulence

Salmonella diverged from its most closely related counterpart, *E. coli*, approximately 100 million years ago (87). Evidence of this divergence is indicated by large, conserved clusters of virulence genes located in the chromosomal sequence of pathogenic *Salmonella* spp. that are not present in the analogous chromosomal regions of non-pathogenic spp. such as *E. coli* K12. These extended regions of unique DNA sequences are referred to as SPIs (*Salmonella* pathogenicity islands) (12). In general, the GC content of pathogenicity islands is much lower than that of the remaining chromosomal DNA sequence. Presumably, pathogenicity islands were attained through horizontal transfer from phage or plasmid of an undetermined source. Other bacterial pathogens such as *E. coli*, *Shigella*, *Yersinia*, *Helicobacter pylori*, *Vibrio cholera*, and *Pseudomonas syringae* possess pathogenicity islands as well (11, 17) (18) (34) (70, 72,

88), although none of these bacteria have more than two islands. Thus far, five pathogenicity islands have been identified in *S. typhimurium* and *S. typhi* (52, 104, 105), suggesting that a set of extremely complex systems for virulence exist. No matter what the species, however, each of these gene clusters uniquely contributes to the virulence of the pathogen they are associated with and its ability to cause disease.

The initial step leading to the divergence of *Salmonella* from *E. coli* is believed to be the acquisition of SPI-1 through phage or plasmid-mediated horizontal transfer. The first pathogenicity island was identified as a 40 kb chromosomal DNA sequence, absent from the *E. coli* K-12 genome, which codes for genes required for *Salmonella* invasion (47, 80). SPI-1 is found in both *S. enterica* and *S. bongori* isolates and is located at centisome 63 on the *Salmonella* chromosome map and contains the *inv/spa* gene cluster. In all, over 29 genes in SPI-1 are involved in the assembly of a type III secretion apparatus vital for entry into host epithelial cells (14-16)(24). The SPI-1 type III secretion system is composed of needle-like complexes that span the inner and outer membranes. The needle structures facilitate the transport of virulence factors from the bacterium into the cytosol of the host cell. Galan *et al.*, showed that if mutations are introduced in the *inv/spa* gene cluster, virulence is not attenuated when given intraperitoneally but is avirulent when administered orally (47). Clearly, this demonstrates a role for SPI-1 that is associated with the intestinal phase of disease and not systemic. In addition to host epithelial cell invasion, SPI-1 is also proposed to be involved in the recruitment of neutrophils (48) and intestinal fluid secretion at the site of infection (78).

The SPI-2 gene cluster is present at centisome 30.7 on the *Salmonella* chromosome and is an approximate 40 kb insertion absent from non-pathogenic bacteria (93). SPI-2 is only found in *S. enterica* isolates which suggests a more recent acquisition than that of SPI-1 (32, 85). Sequence analysis of the SPI-2 locus has identified 44 ORFs and functional analysis has revealed that only a 25 kb portion of the 40 kb insertion is required for virulence (59). The 25 kb region contains genes that encode the two-component regulatory system SsrAB and a type III secretion apparatus. SPI-1 and SPI-2 are the only *Salmonella* pathogenicity islands that code for type III secretion systems. However, SPI-2 encodes a type III secretion system functionally distinct from the SPI-1 system in that it is required for growth inside of host epithelial cells and macrophages (65) (23) (86). Shea *et al.* was the first to show that SPI-2 was required for virulence in a mouse model (93). Whether administered orally or intraperitoneally, SPI-2 mutants are severely attenuated for virulence, suggesting a role in systemic infection as they are unable to proliferate in the spleen or liver (92). In addition, the SPI-2 genes facilitate the evasion of *Salmonella* from intracellular killing. Vazquez-Torres *et al.* reveal that SPI-2 may interfere with the trafficking of the phagocyte NADPH oxidase to *Salmonella*-containing vacuoles, even though the ability to generate a respiratory burst was not affected (101). Because there is no physical contact between the two, the components of the respiratory burst are unable to effectively inhibit *Salmonella* infection. Presumably SPI-2 genes contribute to a reduction in oxidative stress and conceivably to increased oxidative damage to host tissues.

SPI-3 was first identified by Blanc-Potard *et al.* and described as a 17 kb region located at centisome 82 on the *Salmonella* chromosome that was required for survival

inside macrophages (10). The *mtgCB* operon lies within this virulence cluster and is required for both intramacrophage survival and growth in Mg^{2+} -limited conditions. Low magnesium conditions are found in the phagosomal compartment where nutrient availability is scarce (10, 51). Interestingly, the *mtgCB* operon is transcriptionally regulated by the two-component regulatory system PhoP/PhoQ (94). The PhoP/PhoQ system is a regulator of *Salmonella* pathogenesis and facilitates the metabolic adjustment at low Mg^{2+} conditions (50, 94).

The fourth pathogenicity island to be identified was SPI-4, an approximate 25 kb sequence located at centisome 92 on the *Salmonella* chromosomal map located between the *ssb* and *yciB* genes in both *S. typhimurium* and *S. typhi* (104). Originally, SPI-4 was identified via a transposon insertion as a chromosomal sequence required for survival inside of murine macrophages (6, 40). Wong *et al.* originally claimed that SPI-4 contained 18 ORFs designated A-R (104). The recently completed annotation of both *S. typhimurium* LT2 and *S. typhi* genomes shows that only 6 open reading frames with a single open reading frame (STM4261) proposed to encode a 660 kD protein (~16 kB gene) exist. Ahmer *et al.* (1) demonstrated SPI-4 regulation by SirA, a transcriptional regulator of SPI-1 (invasion locus). Allen *et al.* were the first to show that the genes on SPI-4 play a role in the invasion of cultured epithelial cells (3). This finding is significant because the ability to invade was predominantly attributed to SPI-1 in the past. However, Murray *et al.* showed that when the entire nucleotide sequence that codes for SPI-1 was deleted, the ability of *Salmonella* to invade was not completely eliminated (81). Perhaps the residual invasive properties of *Salmonella* are due to the genes encoded

on SPI-4. Clearly, further studies are needed to work out the contribution of SPI-4 to *Salmonella* pathogenesis.

SPI-5 was initially identified in *S. dublin* at centisome 25 on the chromosome (68, 105). Like SPI-4, the fifth pathogenicity island is found only in *Salmonella* spp. SPI-5 was absent from other enteropathogenic bacteria such as *Shigella sonnei*, EPEC, and *Yersinia pseudotuberculosis* when assayed through Southern blot analysis(105). Sequencing of SPI-5 revealed the presence of five novel genes: *pipA*, *pipB*, *pipC*, *pipD*, and *orfX*. *pipA*, *pipB*, and *pipD* mutants showed diminished fluid/chloride secretion and poor inflammatory responses when assayed in the ileal ligated loop model. Therefore, SPI-5 is most likely involved in the enteric phase of the disease. Interestingly, *sopB*, a gene present on SPI-5 that codes for a protein required for fluid secretion and neutrophil recruitment, is actually translocated by the SPI-1 *inv/spa* secretion system (46, 48, 60, 105). This would suggest an example of an exceedingly intricate network of cross-talk between genes located on different pathogenicity islands.

The contribution of the pathogenicity islands to *Salmonella* pathogenesis can be divided into three stages (55). The initiation of invasion of the small intestine comprises the first stage. Through the involvement of invasion genes located on SPI-1 and SPI-5, the bacteria attaches to the epithelial cell layer of the intestinal lumen. SPI-2 genes are incorporated in the second stage and abet in the penetration of the host epithelial layer. The final stage comprises replication inside of macrophages and infection of deep tissues. Genes located on SPI-2, SPI-3, SPI-4, and the *Salmonella* virulence plasmid (spv) are involved. The acquisition of unique virulence clusters has unquestionably led to the evolution of *Salmonella* into the champion pathogen that it is today.

The *Salmonella* Virulence Plasmid

The majority of *Salmonella* strains possess plasmids that carry virulence factors associated with the ability to cause systemic disease with the notable exception of *S. typhi* (56). Most isolates belonging to *Salmonella enterica* serovar Typhimurium carry a 90 kb virulence plasmid with a conserved 8 kb region harboring the *spv* genes (45, 53). The *spvRABCD* locus contains five genes that contribute to an increased rate of bacterial growth during the systemic phase of infection, primarily inside of macrophages, and influences the interaction of *Salmonella* with host cells (57). The exact molecular functions of *spvA*, *spvB*, *spvC*, and *spvD* remain somewhat elusive. Work by Spink *et al.* suggests that SpvA is a negative regulator of the *spv* operon (95), while SpvD is most likely secreted (35). Recent work by Matsui *et al.* suggests that SpvB and SpvC alone may directly mediate the virulent attributes of the *Salmonella* virulence plasmid (77). It has been shown that *spvB* encodes an ADP-ribosylating enzyme that is essential for virulence in the murine model (73). Although such enzymes have previously been described in extracellular bacterial pathogenesis (cholera and diphtheria toxin), the finding is novel for intracellular *Salmonella* pathogenesis. SpvR, the *spv* regulatory locus, is the best characterized of the five gene products produced by the operon. The expression of *spvABCD* relies on both the SpvR positive activator protein and RpoS (stationary phase sigma factor) (54). The virulence plasmid of *S. typhimurium* harbors other interesting loci required for virulence in addition to the *spv* operon. For instance, the plasmid-encoded fimbriae region (*pef*) is involved in the attachment of *Salmonella* to epithelial cells of the intestine (7). TlpA is a repressor protein that autoregulates its own transcription in accordance with the temperature inside the bacterial cell (69). Finally,

Rck is an outermembrane protein found only in *S. typhimurium* that is coded for on the virulence plasmid. This protein mediates resistance to host complement factors and is involved in intestinal epithelial cell invasion (22). Clearly, the *Salmonella* virulence plasmid is a principal element of *Salmonella* pathogenesis.

The Role of *slyA* in *Salmonella* Pathogenesis

slyA was originally identified as a gene that encoded a cryptic hemolysin (74). When cloned into *E. coli*, *slyA* was shown to be required for the cytolytic death of nucleated cells and the lysis of red blood cells (74). However, upon further investigation, it was discovered that expression of the hemolysin is directly regulated by *slyA* and is, therefore, not a function of the gene itself. Instead, the hemolytic activity is actually conferred by the gene *chyA* (also referred to as *sheA* or *hlyE*) in *E. coli* (76, 88). Based on sequence homology with other transcriptional regulatory proteins of other gram-negative bacteria at that time (31), it was suggested by Ludwig *et al.* that SlyA may act as a regulatory protein, affecting expression of both *E. coli* and *Salmonella* genes (75). Independent studies have demonstrated that *S. typhimurium slyA* mutants are profoundly attenuated for virulence in mice by intravenous, intraperitoneal, and oral routes of administration (26, 74). Analysis of *Salmonella* invasion in the murine model shows that while *slyA* is required for survival in Peyer's patches and the destruction of M-cells, it is not required for adherence to and invasion of M-cells (26). In addition, *slyA* mutants of *S. typhimurium* are unable to effectively replicate within the reticuloendothelial system of the host (74). Buchmeier *et al.* showed that *slyA* mutants of *S. typhimurium* were hypersusceptible to reactive oxygen species, namely hydrogen peroxide and paraquat,

found within the anti-microbial environment of macrophages suggesting a role for SlyA in oxidative stress resistance (14). While the murine model is used widely to examine typhoidal or systemic disease mechanisms, there is no convenient model for studying the enteric phase of disease. In view of this, Watson *et al.* performed infection studies in calves and showed that *slyA* mutants of *S. typhimurium* were not appreciably attenuated for virulence in cattle signifying a role for *slyA* in systemic, but not enteric infection (103). SlyA belongs to an eclectic family of transcriptional regulatory proteins (96). RovA of *Yersinia pestis* and *Yersinia tuberculosis*, Rap of *Serratia marcescens*, and Hor of *Erwinia carotovora* are some of the proteins that have recently been grouped with SlyA (82, 89, 99), while SlyA is more distantly related than previously reported to MarR and EmrR (MprA) of *E. coli*, Hpr of *Bacillus subtilis*, and PecS of *Erwinia chrysanthemi* (31). Future studies that examine the nature of *slyA* as a transcriptional regulator of virulence genes should reveal more about the nature of SlyA and its regulatory mechanisms.

Chapter 1: Purification of a Polyclonal Antibody for the Detection of SlyA Protein Expression

Introduction

Salmonella acquired infection remains a serious worldwide threat to public health, with illness ranging from minor gastroenteritis (*S. typhimurium*) to acute systemic disease (*S. typhi*). *Salmonella enterica* serovar Typhimurium is the most frequent origin of salmonellosis, with nearly all of the 2000 serotypes of the genus *Salmonella* able to cause disease in humans. Because the Center for Disease Control estimates that 1.4 million cases of the food-borne illness occur annually in the United States with 1,000 deaths resulting from severe salmonellosis infections, further investigation into the mechanisms of disease caused by this pathogen are warranted.

A role for *slyA* in *Salmonella* pathogenesis has been clearly demonstrated. Originally, *slyA* was identified as a gene that encoded a cryptic hemolysin (74). When cloned into *E. coli*, *slyA* was shown to be required for the cytolytic death of nucleated cells and the lysis of red blood cells (74). However, upon further investigation, it was discovered that expression of the hemolysin is directly regulated by *slyA* and is, therefore, not a function of the gene itself. It is previously reported that SlyA is required for the intracellular survival of the bacteria within host macrophages and that *slyA* mutant strains of *S. typhimurium* are severely attenuated for virulence in the murine model (74). In addition, Daniels *et al.* showed that SlyA is required for the destruction of murine M-cells but not for invasion (26). Further studies show that *slyA* may be required for resistance to

oxidative stress (13) and, more recently, that *slyA* may act as a regulator of virulence genes involved in the systemic phase of infection, but not the enteric phase (103).

Based on sequence homology with other transcriptional regulatory proteins of other gram-negative bacteria (31), it was first suggested by Ludwig *et al.* that SlyA may act as a regulatory protein, affecting expression of both *E. coli* and *Salmonella* genes (75). In view of this, SlyA was initially grouped with MarR and EmrR (MprA) of *E. coli*, Hpr of *Bacillus subtilis*, and PecS of *Erwinia chrysanthemi* (31). However, it is now apparent that SlyA is more distantly related to these regulatory proteins than previously reported. SlyA is currently classified as a member of an unique family of low molecular weight transcriptional regulatory proteins that include RovA of *Yersinia pestis* and *Yersinia tuberculosis*, Rap of *Serratia marcescens*, and Hor of *Erwinia carotovora* (82, 89, 96, 99).

In an attempt to further clarify the role of SlyA in *Salmonella* pathogenesis, a polyclonal antibody was raised to His-tagged SlyA. We used this antibody investigate the intracellular expression levels of SlyA within the Salmonellae and in other pathogenic bacteria. Here, we show that the anti-His-tagged SlyA polyclonal antibody reacted with several low molecular weight proteins. In addition, we have found that SlyA may be expressed in early to mid logarithmic phase and *not* in stationary phase.

Materials and Methods

Bacterial strains, plasmids and media. Bacterial strains used in this study are described in Table 1. All strains were grown in Luria-Bertani (LB) and were

supplemented with kanamycin (50 µg/ml), penicillin (200 µg/ml), and chloramphenicol (25 µg/ml) when required.

Construction of Expression Vector. A DNA fragment from *Salmonella typhimurium* ATCC 14028s was derived by PCR amplification and cloned into the expression vector pET-16b (Novagen) in the same reading frame as the 10xHis affinity tag with the tag placed at the N-terminal end. An oligonucleotide primer was constructed that spanned the ATG start codon to create a *NdeI* site. The reverse primer was made past the stop codon of the *slyA* fragment. These primers were used to amplify a 469 bp fragment using *S. typhimurium* ATCC 14028s as a template under the following conditions: denature at 95°C for 5 min, 94°C—30 sec—50°C—30 sec—72°C—1 min with vent polymerase (NEB, MA). The PCR fragment was gel purified and cloned into the *EcoRV* site of pSK (Stratagene, CA). Colonies containing inserted DNA were digested with *NdeI* and *BamHI* and the fragments were purified. The vector, pET-16b was restricted with *NdeI* and *BamHI* and ligated with the *NdeI/BamHI slyA* fragment. Following transformation into DH5α *E. coli*, DNA was purified from random colonies and restricted with *NdeI/BamHI* to check for the insert.

Expression of Recombinant Proteins. pET-16b:*slyA* was transformed in the *E. coli* strain BL21(DE3) pLysS for expression analysis studies. This strain harbors a lambda lysogen with an inducible bacterial T-7 RNA polymerase. The plasmid pLysS encodes T-7 Lysozyme and is used to repress transcription from the T-7 promoter.

To purify His-tagged SlyA, a 1 ml freezer stock of pET-16b:*slyA* was added to 1 L of LB media supplemented with 100 µg/ml ampicillin (SIGMA, MO) in a 2 L flask. Cells were grown vigorously with agitation (250rpm) at 37°C until log phase was reached (a cell density of $A_{600} = 0.6-0.8$). Protein expression was induced with the addition of up to 5mM IPTG (RPI Corp., IL). Cells were harvested by centrifugation for 20 min at 4°C at 10,000g after additional agitation (250rpm) at 37°C for 4 hours to ensure ample expression. The cell pellet was frozen at -80°C until protein preparation. The cell pellet was resuspended in lysis buffer (50 mM NaH_2PO_4 , pH 8.0; 300 mM NaCl; 10 mM imidazole) followed by disruption on ice with a microtip sonicator (Heat Systems-Ultrasonics, Inc. Cell Disrupter model W-370) with 15 second bursts followed by 1 minute rests on ice until low viscosity was achieved. The lysate was centrifuged at 10,000g at 4°C for 30 min to spin down the cellular debris.

Purification of recombinant Protein Using Nickel Nitrilotriacetic acid Agarose

under Native Conditions. Column chromatography was performed at room temperature using Ni-NTA Agarose coupled to Sepharose CL-6B (Qiagen, CA). This system facilitates a generous binding potential of 5-10 mg of 10xHis-tagged protein per ml of resin. The column was assembled according to the manufacturer's instructions (Bio-Rad, CA). The column was packed with Ni-NTA slurry until the desired bed size was achieved and equilibrated with 5 column volumes of lysis buffer. The lysate was added to the column and washed with 10 column volumes of wash buffer (50 mM NaH_2PO_4 , pH 8.0; 300 mM NaCl; 20 mM imidazole). His-tagged protein was eluted off the column in 1 ml fractions with elution buffer (50 mM NaH_2PO_4 , pH 8.0; 300 mM

NaCl; 500 mM imidazole). All fractions were analyzed by SDS-PAGE analysis on a 12% denaturing polyacrylamide gel. Eluted fractions containing a significant amount of SlyA protein (determined by SDS-PAGE) were pooled in dialysis tubing (10,000 M.W. cut-off) and concentrated using PEG (polyethylene glycol 7,000-9,000 MW). Proteins were dialyzed overnight in phosphate buffered saline to remove any remaining imidazole and prepare the antigen for immunization.

Production of goat anti-SlyA antibody. Prior to the initial challenge, goat serum was collected from the test animal (provided by Dr. Glenn Songer at the University of Arizona, Department of Veterinary Science and Microbiology) and analyzed for a lack of cross-reaction with SlyA through immunoblot analysis. A booster injection was given 2 weeks and 6 weeks after the first injection. Antiserum was collected 4 weeks and 8 weeks after the initial injection. The primary and secondary bleeds were both analyzed for SlyA specificity via recognition with a horseradish peroxidase (HRP)-conjugated anti-goat whole molecule antibody (SIGMA, MO) through Western blot analysis. Production of anti-His tagged SlyA antibody is described in Figure 1.

Western blot analysis. Bacteria from 10ml of an overnight culture were grown for 16 to 17 hours in LB medium and supplemented with appropriate antibiotics. A 1:1000 dilution of overnight culture in 25 ml LB was grown to fresh stationary phase (O.D. 2.5-3.0), harvested by centrifugation at 6,000 rpm for 10 minutes, washed several times with phosphate buffered saline, and lysed by the addition of 125 μ l of B-Per (Pierce, IL) and 75 μ l of Dnase with vortexing at 5 minute intervals for a total of 15 minutes on ice. The

lysate was centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatant was collected. Protein concentration was determined using the Bio-Rad protein assay, using bovine serum albumin as the standard. Two volumes of sample buffer (2.9 ml deionized water, 1.0 ml 0.5 M Tris-Hcl, pH 6.8, 2.0 ml glycerol, 1.6 ml 10% w/v SDS, 0.1 ml 1.0% bromophenol blue, 0.4 ml beta-mercaptoethanol) were added to one volume of the protein fraction. Protein fractions were boiled for 5 minutes and clarified by centrifugation at 14,000 rpm for 3 minutes. Equal amounts of total cell protein were loaded onto a 12% denaturing polyacrylamide gel. In some instances the polyacrylamide gel was cut so that only proteins in the low molecular weight range were transferred. Immunoblotting was performed and the separated proteins were transferred onto a nitrocellulose membrane. The proteins were probed using either polyclonal goat or rabbit anti-SlyA serum and detection of bound antibodies was achieved by the addition of horseradish peroxidase (HRP)-conjugated monoclonal anti-goat IgG (SIGMA, MO).

Growth Phase Analysis of wild-type *S. typhimurium*.

Bacteria from 10 ml of an overnight culture of wild-type *S. typhimurium* were grown for 16 to 17 hours in LB medium. A 1:1000 dilution of overnight culture in 25 ml LB was grown to fresh stationary phase (O.D. 3.0), with samples taken throughout the growth cycle (O.D. 0.308, 0.508, 0.702, 0.940, 1.53, 1.88, 2.29, 2.97) along with an aliquot from the original overnight culture. Cells were harvested by centrifugation at 6,000 rpm for 10 minutes, washed several times with phosphate buffered saline, and lysed by the addition of 75 µl of B-Per (Pierce, IL) and 25µl of Dnase with vortexing at 5 minute intervals for a total of 15 minutes on ice. The lysate was centrifuged at 14,000

rpm for 10 minutes at 4°C and the supernatant was collected. Protein concentration was determined using the Bio-Rad protein assay, using bovine serum albumin as the standard. Two volumes of sample buffer (2.9 ml deionized water, 1.0 ml 0.5 M Tris-HCl, pH 6.8, 2.0 ml glycerol, 1.6 ml 10% w/v SDS, 0.1 ml 1.0% bromophenol blue, 0.4 ml BME) were added to one volume of the protein fraction. Protein fractions were boiled for 5 minutes and clarified by centrifugation at 14,000 rpm for 3 minutes. Equal amounts of total cell protein were loaded onto a 12% denaturing polyacrylamide gel. Immunoblotting was performed and the separated proteins were transferred onto a nitrocellulose membrane. The proteins were probed using polyclonal goat anti-SlyA serum and detection of bound antibodies was achieved by the addition of horseradish peroxidase (HRP)-conjugated monoclonal anti-goat IgG (SIGMA, MO).

Results

Expression and Purification of His-tagged SlyA protein fragment. Figure 1 shows the results of the His-tag SlyA purification system used in this study. The protein was expressed well, as it was the dominant band in the total protein fraction (Fig. 1, lane 2). A comparison of the total protein with the flow through showed that the Ni-NTA chromatography column worked effectively to bind the His-tag on SlyA (Fig. 1, lanes 2 and 3), since the protein was absent in the flow through fraction. Proteins were eluted off the column with a high molar concentration of imidazole (500 mM) to increase the yield of protein per 1 ml fraction. Most of the protein became unbound within elution fractions 2-8 (Fig. 1, Lanes 5-11). The predicted molecular weight of the SlyA fragment with the

6x His-tag is 21 kD and this coincides with the size of the protein visualized on the gel. This purification procedure provided SlyA sufficiently pure enough to be used for the preparation of a polyclonal antibody.

The SlyA polyclonal antibody reacts with a small molecular weight protein in the pSX34::P_{BAD}-slyA strain.

Equal amounts of total protein derived from fresh stationary phase were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, reacted with the SlyA polyclonal antibody, and detected using chemiluminescence. The anti-his-tagged SlyA antiserum reacts with the 21 kD 6x His-tagged SlyA protein (Fig. 2, Lane 9) An immunoreaction is detected in only one strain. A small molecular weight protein running at approximately 17 kD (Fig. 2, Lane 6) is observed in SL2571 (pSX34::P_{BAD}-slyA). No immunogenic reaction was observed in the other strains: SL2757 (wild-type *S. typhimurium* ATCC 14028s) (Fig. 2, Lane 1), SL3129 (pCR2.1:slyA DH5 α) (Fig. 2, Lane 2), SL2317 (pSL101:slyA *S. typhimurium*) (Fig. 2, Lane 3), SL2774 (*E. coli* 0157:H7) (Fig. 2, Lane 4), SL2570 (pSX34::P_{BAD}) (Fig. 2, Lane 5), SL2818 (MG1655) (Fig. 2, Lane 7), and SL3343 (*slyA::km S. typhimurium*) (Fig. 2, Lane 8).

The SlyA polyclonal antibody reacts with two small molecular weight proteins.

Fig. 3 reveals that the anti-His-tagged SlyA polyclonal antibody reacts with two small molecular weight proteins. Equal amounts of total protein derived from fresh stationary phase were separated by 12% SDS-PAGE, the gel was cut, proteins in the low molecular weight range were transferred to a nitrocellulose membrane, reacted with the SlyA polyclonal antibody, and detected using chemiluminescence. The polyclonal

antibody reacted with the 6xHis-tagged SlyA protein (Fig. 3, Lane 1). Two small molecular weight proteins were also observed along with an interesting doublet banding pattern. A single protein in the 17 kD SlyA molecular weight range was detected in SL2570 (pSX34::P_{BAD}) (Fig. 3, Lane 3), SL3343 (*slyA::km S. typhimurium*) (Fig. 3, Lane 4), and SL3129 (pCR2.1:*slyA* DH5 α) (Fig. 3, Lane 6). Two distinct proteins in the SlyA molecular weight range were visualized SL2571 (pSX34::P_{BAD}-*slyA*) (Fig. 3, Lane 2), SL3203 (*S. dublin*) (Fig. 3, Lane 5), SL2818 (MG1655) (Fig. 3, Lane 7), and SL2757 (wild-type *S. typhimurium* ATCC 14028s) (Fig. 3, Lane 8).

The aforementioned experiment described in Fig. 4 was repeated with the addition of two MarR expressing strains, SL3298 (pWSK:*marR* (MG1655) in pEP185.2::*slyA*) and SL3291 (pWSK129:*marR* *E. coli* DH5 α). These strains were added to check for cross-reactivity of the antibody. Data in Fig. 4 reveals a doublet banding pattern that is identical to the one described previously. Two distinct proteins in the SlyA molecular weight range were visualized in SL2757 (wild-type *S. typhimurium* ATCC14028s) (Fig. 4, Lane 1), SL2818 (MG1655) (Fig. 4, Lane 2), SL3203 (*S. dublin*) (Fig. 4, Lane 4), and SL2571 (pSX34:P_{BAD}-*slyA*) (Fig. 4, Lane 7). Again, a single protein in the 17 kD SlyA molecular weight range was detected in SL3129 (pCR2.1:*slyA* DH5 α) (Fig. 4, Lane 3), SL3203 (*S. dublin*) (Fig. 4, Lane 4), and SL2570 (pSX34:P_{BAD}) (Fig. 4, Lane 6). The MarR expressing strains pWSK:*marR* (MG1655) in pEP185.2::*slyA* (Fig. 4, Lane 8) and pWSK129:*marR* *E. coli* DH5 α (Fig. 4, Lane 9) revealed the presence of only one protein band.

Analysis of Growth Phase Proteins.

Bacterial cells were collected at various times throughout the growth cycle of SL2757 (wild-type *S. typhimurium* ATCC 14028s). The optical densities at which cells were collected along with the results are depicted in Fig. 6, Lanes 1-9. A strong immunogenic reaction was detected in early log phase at O.D. 0.308 (Fig. 6, Lane 2). A weaker reaction was detected at mid log phase at O.D. 0.508 (Fig. 6, Lane 3). SlyA was not detected in any other phase of the growth cycle. The reaction appeared as a doublet pattern, possibly denoting two small molecular weight proteins that ran in the low molecular weight range. The approximate 21 kD His-tagged SlyA protein (Fig. 6, Lane 10) was used as a reference point.

Discussion

This report describes the expression system and purification method for the production of a polyclonal antibody for the detection of SlyA protein. SlyA had previously been identified only by Daniels *et al.* through immunoblot analysis when induced on a high copy plasmid and in stationary phase wild-type *S. typhimurium.*, albeit this immunoreaction was very poor (26). Therefore, we thought it would be interesting to develop a polyclonal antibody of our own to investigate SlyA expression in the Salmonellae.

Expression of SlyA

The pET system is the prevailing and most powerful system developed for the expression of recombinant proteins in *E. coli*. We chose to use the pET-16b expression vector

because a 10xHis affinity tag could be engineered in the same open reading frame as SlyA. In doing so, we could readily purify the protein through His-tagged column chromatography. In this case, the 6x His-tag was engineered onto the N-terminal end of the protein. The expression vector contained an ampicillin antibiotic marker for selection and a thrombin cleavage site that followed the N-terminal his-tag sequence. The system was under the control of an inducible T7-*lac* promoter. The gene plasmid construct used to prepare the His-tagged SlyA protein is shown in Fig. 5.

Up to 5 mM IPTG was added when bacteria reached log phase (O.D. 0.6-0.8) and cells were grown with agitation for another 4 hours to ensure ample protein expression. The outcome of the SlyA purification steps is shown in Fig. 1. After a 4 hour induction, the cell lysate reveals that SlyA was expressed well, as it was the prevailing band in the total protein fraction (Fig. 1, Lane 2).

Purification of SlyA

Column chromatography was performed at room temperature using Ni-NTA Agarose coupled to Sepharose CL-6B (Qiagen, CA). The high affinity of Ni-NTA matrix for biomolecules containing a 6x histidine tag supports a generous binding potential of 5-10 mg of 6xHis-tagged protein per ml of resin and minimal non-specific binding.

After 4 hours of induction, cells were harvested and the cell pellet was lysed by the addition of lysis buffer, sonication, and the intrinsic pLysS activity of the plasmid. The cellular debris was separated from the supernatant by centrifugation, and the supernatant was added to the Ni-NTA column. Following 10 column volumes of wash buffer, the

protein was eluted off the column by the addition of a high molar concentration of imidazole (500 mM).

A comparison of the cell lysate after the induction (Fig. 1, Lane 2) with the flow through fraction (Fig. 1, Lane 3) demonstrated that the matrix worked effectively to bind the His-tag on the protein, since there was no His-tagged protein visible in the flow through fraction. The maximal binding capacity of the affinity column, therefore, was not exceeded.

The molecular weight of His-tagged SlyA is estimated to be approximately 21 kD. This estimation corresponds with the size of the eluted protein fractions visualized by SDS-PAGE post-induction (Fig. 1, Lanes 4-13). Eluted fractions containing a significant amount of SlyA protein were pooled together in dialysis tubing and concentrated to the mg/ml range. Dialysis in phosphate buffered saline overnight was performed to remove any remaining imidazole and prepare the antigen for immunization. The Ni-NTA purification procedure provided SlyA sufficiently pure enough to be used for the preparation of a polyclonal antibody.

The polyclonal antibody reacts with more than one protein in 17 kD molecular weight range.

Western blot analysis was performed with cells grown to fresh stationary phase. The proteins were transferred to nitrocellulose and the entire membrane was probed with the anti-His-tagged SlyA polyclonal antibody. The detection of SlyA was visualized only in SL2571 (pSX34:P_{BAD}-*slyA*) and is shown in Fig. 2. In this strain, the *slyA* gene is cloned in front of an inducible promoter, whereby SlyA expression is induced by the addition of arabinose. So, the fact that SlyA is detectable in SL2571 (pSX34:P_{BAD}-*slyA*)

and not in SL2757 (wild-type *S. typhimurium* ATCC 14028s), SL2317 (pSL101:*slyA* *S. typhimurium*), SL2774 (*E. coli* 0157:H7), and SL2818 (MG1655) could be attributed to the levels of SlyA produced within the cell. Studies to determine how many copies per cell of SlyA are produced have yet to be performed. The *slyA* mutant strain SL3343 does not detect the presence of SlyA, nor does SL2570 (pSX34:P_{BAD}). Although, it should be noted that the expression levels for SL2757 (wild-type *S. typhimurium* ATCC 14028s) and SL2570 (pSX34:P_{BAD}) should be similar since SL2570 is in a *S. typhimurium* background. We can not explain why an immunoreaction was not detected in the SlyA expressing strains pCR2.1:*slyA* or pSL101:*slyA* *S. typhimurium*.

Cross-reaction is commonly seen with polyclonal antibodies. Fig. 2 shows that an immunogenic reaction with several other proteins was detected, specifically a protein at approximately 35 kD. The possibility that this band represents a SlyA dimer is quickly discounted by the fact that the reaction is detected in the *slyA* mutant strain, SL3343 . Furthermore, the denaturing conditions used in the protein preparation and the SDS-PAGE gel itself would most likely reduce non-specific interaction.

If SlyA is made at very low levels then the amount of protein transferred to the membrane for detection by chemiluminescence would be minute. Moreover, because of the strong affinity of the polyclonal antibody for non-SlyA proteins, we hypothesized that the amount of antibody needed to detect the low levels of SlyA protein may not be accessible. Therefore, only proteins in the low molecular weight range were transferred to the membrane. In this manner, there would be additional antibody available to bind SlyA specific protein.

The results are shown in Fig. 3. Indeed, the antibody detected two proteins in the low molecular weight range. We observed an interesting banding pattern. Two distinct bands were observed in SL2571 (pSX34:P_{BAD}-*slyA*), SL 3203 (*S. dublin*), SL2818 (MG1655), and SL2757 (wild-type *S. typhimurium* ATCC 14028s). Only one upper band was detected in SL2570 (pSX34:P_{BAD}), SL3343 (*slyA::km S. typhimurium*), SL3129 (pCR2.1:*slyA*), SL3298 (pWSK:*marR* (MG1655) in pEP185.2::*slyA*) (Fig. 4, Lane 8), and SL3291 (pWSK129:*marR E. coli* DH5 α). We proposed that the bottom band represented SlyA, since it was detected in strains that contained *slyA*—with the unexplainable exception again, SL3129 (pCR2.1:*slyA* DH5 α). We proposed that the top band might be MarR or some other low molecular weight protein that shares homologous regions with SlyA (31, 82, 89, 96, 99). Construction of a *marR* mutant might provide some insight as to whether or not the top band is indeed MarR.

SlyA may be expressed in early to mid log phase.

Previous work by Buchmeier *et al.* suggested that SlyA expression is induced during stationary phase (14). This finding was based on the observation that *slyA* mutant cells exhibited changes in protein expression patterns when compared to *S. typhimurium* 14028s during stationary phase, but not log phase. Daniels *et al.* also showed that the production of SlyA in *S. typhimurium* 14028s was barely detectable through immunoblot analysis in stationary phase, but not detectable at all in log phase (26). For these reasons, we conducted all of our SlyA expression studies with cells that were grown to stationary phase. However, because of the extreme conditions used to visualize detectable levels of SlyA protein in *S. typhimurium* 14028s (i.e. transferring specific molecular weight

regions of the gel to the membrane, long exposure times) we thought it would be interesting to examine the expression patterns of SlyA through the growth cycle to verify the previous findings.

In fact, recent experiments in our laboratory suggest that perhaps SlyA is expressed in early to mid log phase and *not* in stationary phase (Fig. 6). This novel finding would explain the difficulty in detecting SlyA expression natively within the *Salmonellae*. The evidence presented here is quite compelling. Obviously, further experiments need to be performed to confirm this finding.

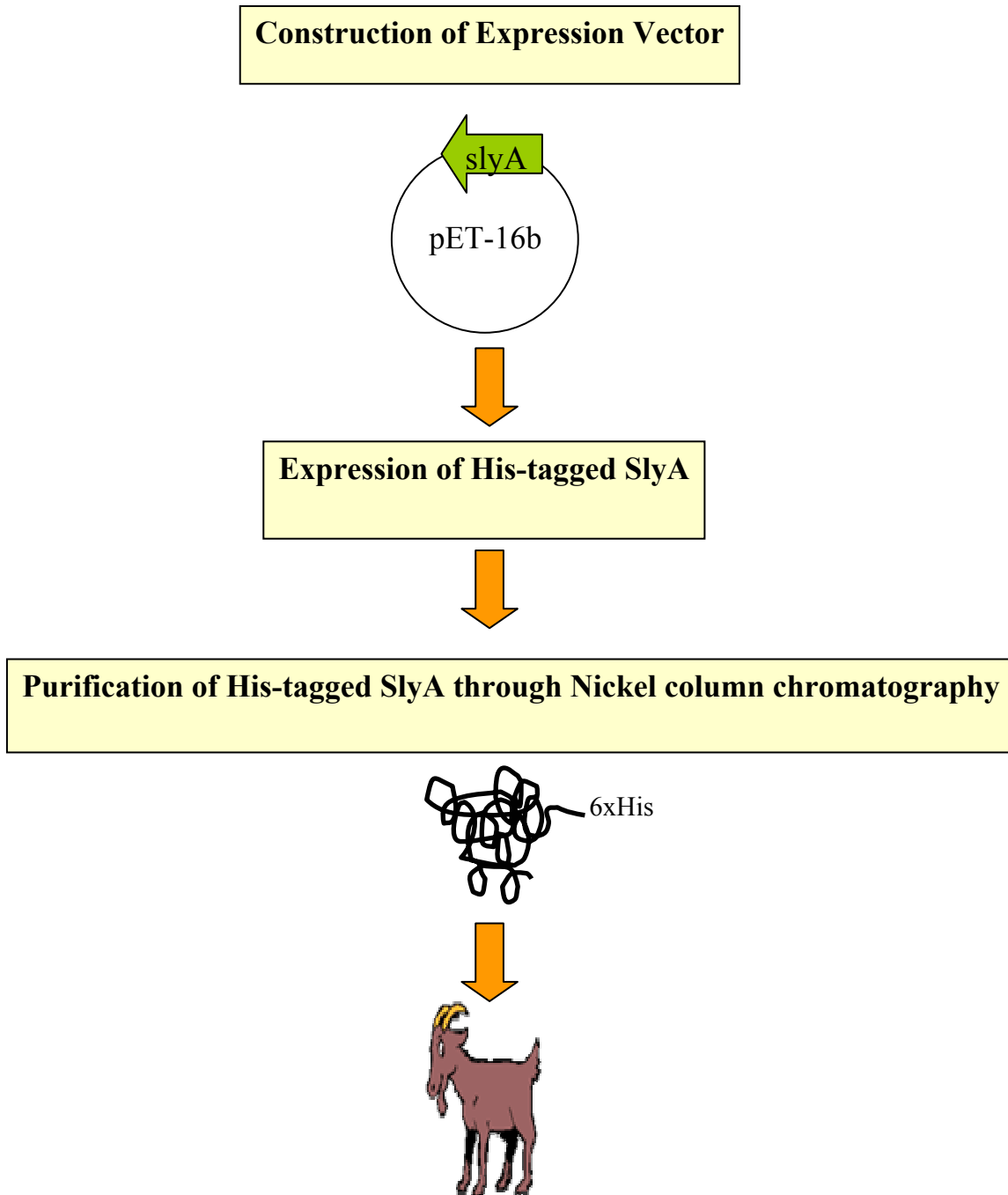


FIG. 1. Schematic of SlyA His-tag antibody production..

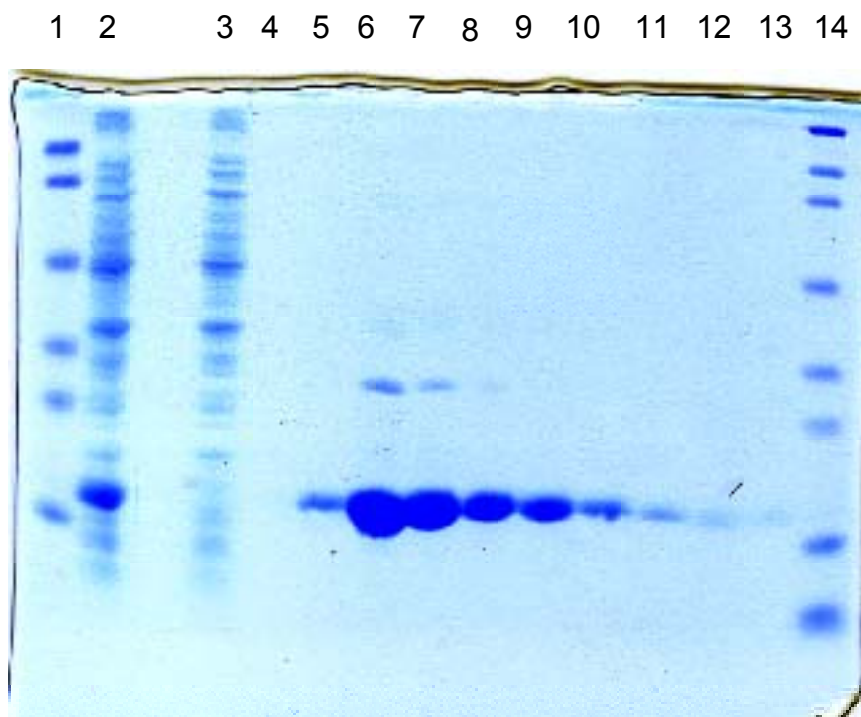


FIG 2. SDS-PAGE gel of His-tag protein purification steps. Lane 1, molecular weight markers, 103, 77, 50, 34.3, 28.8, and 20.7 kD; Lane 2, cell lysate after a 4-h induction; Lane 3, flow through; Lanes 4-13, 500 mM imidazole elutions; Lane 14, molecular weight markers, 209, 124, 80, 49.1, 34.8, 28.9, 20.6, and 7.1 kD.

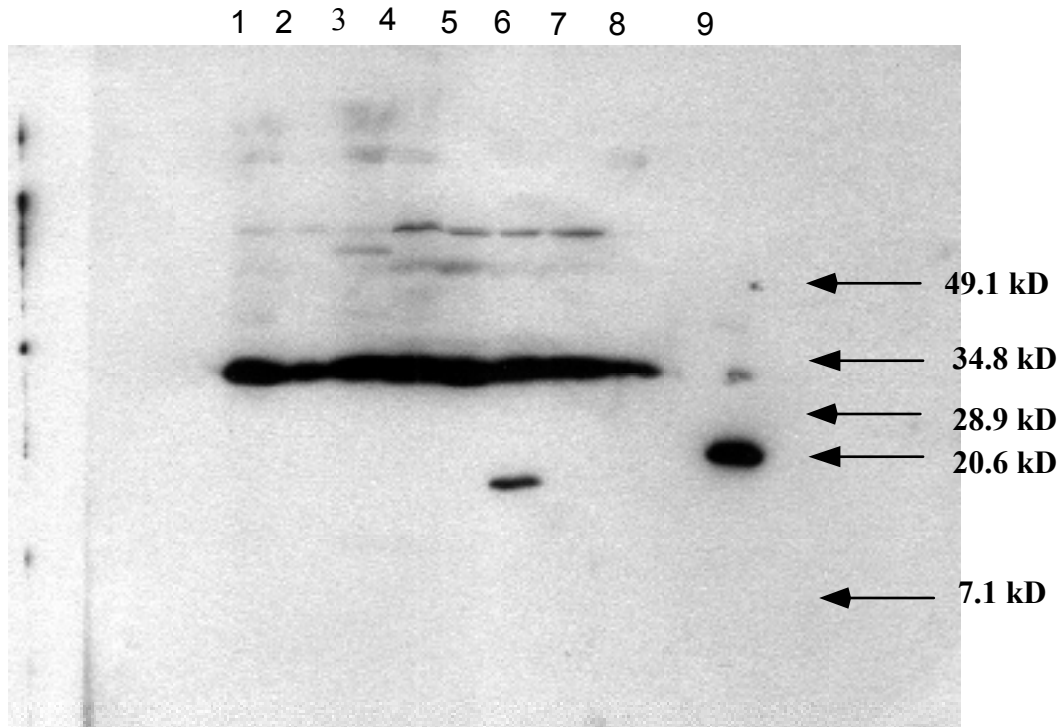


FIG. 3. Western blot analysis of SlyA stationary phase protein expression. Lane 1, SL2757; Lane 2, SL3129; Lane 3, SL2317; Lane 4, SL2774; Lane 5, SL2570; Lane 6, SL2571; Lane 7, SL2818; Lane 8, SL3343; Lane 9, His-tagged SlyA protein.

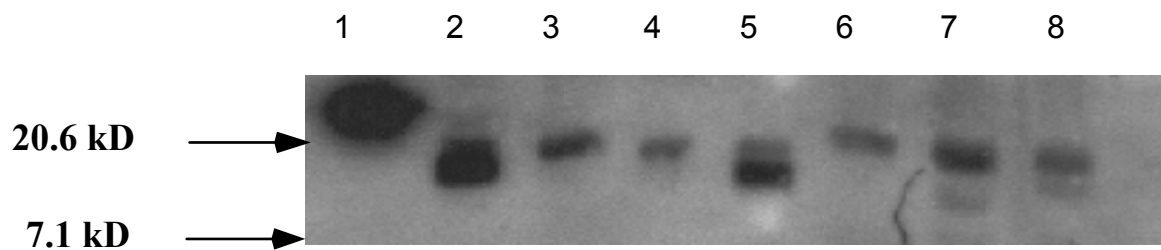


FIG. 4. Western blot analysis of SlyA stationary phase protein expression. Lane 1, His-tagged SlyA protein; Lane 2, SL2571; Lane 3, SL2570; Lane 4, SL3343; Lane 5, SL3203; Lane 6, SL3129; Lane 7, SL2818; Lane 8, SL2757.

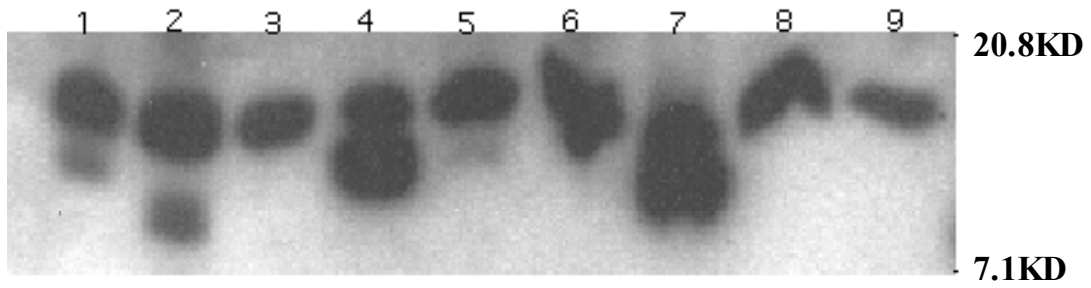


FIG. 5. Western blot analysis of SlyA stationary phase protein expression. Lane 1, SL2757; Lane 2, SL2818; Lane 3, SL3129; Lane 4, SL3203; Lane 5, SL3343; Lane 6, SL2570; Lane 7, SL2571; Lane 8, SL3298; Lane 9, SL3291 .

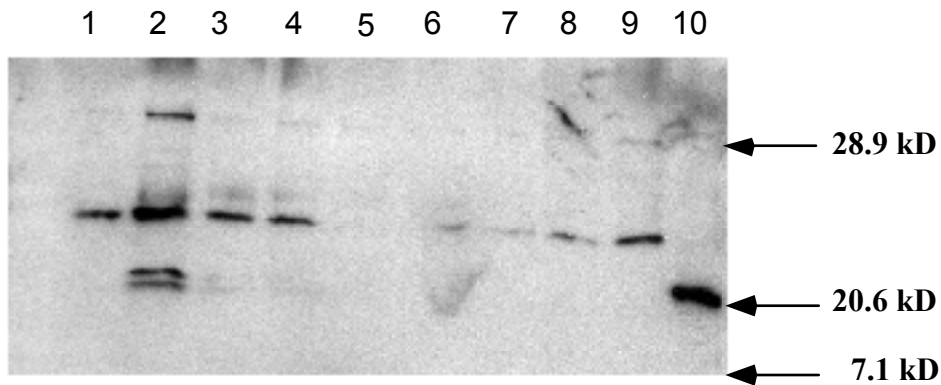


FIG. 6. Growth curve expression of SlyA. Lane 1, overnight stationary phase cells; Lane 2, O.D. 0.308; Lane 3, O.D. 0.508; Lane 4, O.D. 0.702; Lane 5, O.D. 0.940; Lane 6, O.D. 1.52; Lane 7, O.D. 1.88; Lane 8, O.D. 2.27; Lane 9, O.D. 2.97, Lane 10, His-tagged SlyA protein.

TABLE 1. Strains and plasmids used

Strain	Relevant features	Source
<i>S. typhimurium</i> 14028s (SL2757)	Wild type	ATCC
SL3129	pCR2.1: <i>slyA</i> DH5 α	Lab stock
SL2317	PSL101: <i>slyA</i> <i>S. typhimurium</i>	Lab stock
SL2774	<i>E. coli</i> 0157:H7	Lab stock
SL3203	<i>S. dublin</i>	Lab stock
SL2570	pSX34:P _{BAD}	Lab stock
SL2571	PSX34: P _{BAD} - <i>slyA</i>	Lab stock
SL2818	MG1655	Lab stock
SL3343	<i>slyA::km</i> <i>S. typhimurium</i>	Lab stock
SL3298	PWSK129: <i>marR</i> (MG1655) in pEP185.2:: <i>slyA</i> <i>S. typhimurium</i>	Lab stock
SL3291	PWSK129:: <i>marR</i> DH5 α	Lab stock
<i>E. coli</i> DH5 α	<i>endA1 hsdR17(r_K⁻m_K⁺)supE44 thi-1 recA1 pyrA(Nal^r) relA1Δ(lacIAA-argF)U169 deoR[Δ80dlacΔ (lacZ)M15]</i>	Invitrogen
BL21(DE3)pLysS	F ⁻ <i>ompT hsdS_B (r_B⁻m_B⁻) gal dcm</i> (DE3) pLysS (Cm ^R)	Novagen
Plasmids		
pET16b	His-tag vector	Novagen
pSK Bluescript	Cloning vector	Stratagene

Chapter 2: Purification of a Polyclonal Antibody for the Detection of SPI4-K Protein Expression

Introduction

Salmonella acquired infection remains a serious worldwide threat to public health, with illness ranging from minor gastroenteritis (*S. typhimurium*) to acute systemic disease (*S. typhi*). *Salmonella enterica* serovar Typhimurium is the most frequent origin of salmonellosis, with nearly all of the 2000 serotypes of the genus *Salmonella* able to cause disease in humans. Because the Center for Disease Control estimates that 1.4 million cases of the food-borne illness occur annually in the United States with 1,000 deaths resulting from severe salmonellosis infections, further investigation into the mechanisms of disease caused by this pathogen are warranted.

Salmonella diverged from its most closely related counterpart, *E. coli*, approximately 100 million years ago (87). Evidence of this divergence is indicated by large, conserved clusters of virulence genes located in the chromosomal sequence of pathogenic *Salmonella* spp. that are not present in the analogous chromosomal regions of non-pathogenic spp. such as *E. coli* K12. These extended regions of unique DNA sequences are referred to as *Salmonella* pathogenicity islands (12). Other bacterial pathogens such as *E. coli*, *Shigella*, *Yersinia*, *Helicobacter pylori*, *Vibrio cholera*, and *Pseudomonas syringae* possess pathogenicity islands as well (11, 17) (18) (34) (70, 72, 88), although none of these bacteria have more than two islands. Thus far, five pathogenicity islands have been identified in *Salmonella typhimurium* (52, 104, 105), suggesting that a set of extremely complex systems for virulence exist. SPIs are required for virulence and play roles in both enteric and systemic infection. They have been

implicated in invasion (47, 80) as well as intracellular survival within macrophage (6, 40).

SPI-4 is an approximate 25 kb sequence insertion located at centisome 92 on the *Salmonella* chromosomal map. This pathogenicity island is flanked on either side by the *ssb* and *yciB* genes in both *S. typhimurium* and *S. typhi* (104). Originally, SPI-4 was identified via a transposon insertion as a chromosomal sequence required for survival inside of murine macrophages (6, 40). Unfortunately, the amount of information available about SPI-4 is modest. Wong *et al.* originally claimed that SPI-4 contained 18 ORFs designated A-R (104). The recently completed annotation of both *S. typhimurium* LT2 and *S. typhi* genomes shows that only 6 open reading frames exist. Ahmer *et al.* (1) demonstrated SPI-4 regulation by SirA, a transcriptional regulator of SPI-1 (invasion locus). Experiments in our laboratory have shown that the beta-galactosidase activity of a SPI4-K::MudJ insertion (SL3277) in a *slyA* mutant background is reduced 8-fold, suggesting that SPI-4 is regulated by *slyA*. Allen *et al.* were the first to show that the genes on SPI-4 play a role in the invasion of cultured epithelial cells (3). This finding is significant because the ability to invade was predominantly attributed to SPI-1 in the past. However, Murray *et al.* showed that when the entire nucleotide sequence that codes for SPI-1 was deleted, the ability of *Salmonella* to invade was not completely eliminated (81). Perhaps the residual invasive properties of *Salmonella* are due to the genes encoded on SPI-4.

In collaboration with Dr. Brian Ahmer, our laboratory has isolated MudJ transposon insertions localized to an open reading frame designated SPI4-K. This ORF was initially predicted to encode a 90 kD protein, but the recently completed annotation suggests it is a

part of the larger STM4261 ORF proposed to encode a 660 kD protein (~16 kB gene). We recently discovered that the predicted SPI4-K protein has significant homology to a class of autotransporter toxins found in pathogenic *E.coli*, *Bordetella*, *Neisseria*, and *Shigella* species, referred to as SPATE toxins (2, 8, 49, 58, 61-64, 79, 83, 84, 97, 102). Members of this family are serine proteases possessing a characteristic GDSGS motif with a central catalytic Ser residue. We have constructed and purified a His-tagged SPI4-K protein fragment based on the earlier annotation, and used this protein to obtain a polyclonal antibody. In this study, we examined the protein expression characteristics of SPI4-K in *Salmonella typhimurium* and other Salmonellae. We present preliminary evidence through Western blotting that SPI4-K is a large molecular weight protein that appears to be secreted.

Materials and Methods

Bacterial strains and plasmids. Bacterial strains used in this study are described in Table 1. All strains were grown in Luria-Bertani (LB) and were supplemented with kanamycin (50 µg/ml) or chloramphenicol (25 µg/ml) when required.

Construction of Expression Vector. A DNA fragment from *Salmonella typhimurium* ATCC 14028s was derived by PCR amplification and cloned into the expression vector pET-16b (Novagen) in the same reading frame as the 6x His affinity tag with the tag placed at the N-terminal end. An oligonucleotide primer was constructed that spanned the ATG start codon to create a *NdeI* site. The reverse primer was made past the stop codon

of the SPI4-k fragment. These primers were used to amplify a 900 bp fragment using *S. typhimurium* ATCC 14028s as a template under the following conditions: denature at 95°C for 5 min, 94°C—30 sec—50°C—30 sec—72°C—1 min with pfu for 40 cycles. The PCR fragment was gel purified and cloned into the *EcoRV* site of pSK (Stragene, CA). Colonies containing inserted DNA were digested with *NdeI* and *BamHI* and the fragments were purified. The vector, pET-16b was restricted with *NdeI* and *BamHI* and ligated with the *NdeI/BamHI* SPI4-k fragment. Following transformation into DH5 α *E. coli*, DNA was purified from random colonies and restricted with *NdeI/BamHI* to check for the 900 bp insert.

Expression of Recombinant Proteins. pET-16b:SPI4-k was transformed in the *E. coli* strain BL21(DE3) pLysS for expression analysis studies. This strain harbors a lambda lysogen with an inducible bacterial T-7 RNA polymerase. The plasmid pLysS encodes T-7 Lysozyme and is used to repress transcription from the T-7 promoter.

To purify His-tagged SPI4-K, 1 ml freezer stocks were added to 1 L of LB media supplemented with 100 μ g/ml ampicillin (SIGMA, MO) in a 2 L flask. Cells were grown vigorously with agitation (250 rpm) at 37°C until log phase was reached (a cell density of $A_{600} = 0.6-0.8$). Protein expression was induced with the addition of up to 5mM IPTG (RPI Corp., IL). Cells were harvested by centrifugation for 20 min at 4°C at 10,000g after additional agitation (250 rpm) at 37°C for 4 hours to ensure ample expression. The cell pellet was frozen at -80°C until protein preparation. The cell pellet was resuspended in lysis buffer (50 mM NaH_2PO_4 , pH 8.0; 300 mM NaCl; 10 mM imidazole) followed by disruption on ice with a microtip sonicator (Heat Systems-Ultrasonics, Inc. Cell Disrupter

model W-370) with 15 second bursts followed by 1 minute rests on ice until low viscosity was achieved. The lysate was centrifuged at 10,000g at 4°C for 30 min to remove the cellular debris. Construction of His-tagged SPI4-K is described in Figure 1.

Purification of Recombinant Protein Using Nickel Nitrilotriacetic acid Agarose under Native Conditions.

Column chromatography was performed at room temperature using Ni-NTA Agarose coupled to Sepharose CL-6B (Qiagen, CA). This system facilitates a generous binding potential of 5-10 mg of 6x His-tagged protein per ml of resin. The column was assembled according to the manufacturer's instructions (Bio-Rad, CA). The column was packed with Ni-NTA slurry until the desired bed size was achieved and equilibrated with 5 column volumes of lysis buffer. The lysate was added to the column and washed with 10 column volumes of wash buffer (50 mM NaH₂PO₄, pH 8.0; 300 mM NaCl; 20 mM imidazole). His-tagged protein was eluted off the column in 1 ml fractions with elution buffer (50 mM NaH₂PO₄, pH 8.0; 300 mM NaCl; 500 mM imidazole). All fractions were collected for SDS-PAGE analysis and electrophoresed on a pre-cast 12% denaturing polyacrylamide gel (Bio-Rad). Eluted fractions that contained a significant amount of protein (determined by SDS-PAGE) were pooled in dialysis tubing (10,000 M.W. cut-off) and concentrated using PEG (polyethylene glycol 7,000-9,000 MW). Proteins were dialyzed overnight in phosphate buffered saline (PBS) to remove any remaining imidazole and prepare the antigen for immunization.

SDS-PAGE Analysis of His-tag Purification. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed so that protein fractions could be

analyzed. 100 μ l of 2X laemmli buffer (3.0 ml deionized water, 1.0 ml 1.0 M Tris-HCl, pH 6.8, 1.6 ml glycerol, 1.6 ml 20% w/v SDS, 0.4 ml 0.5% bromophenol blue) was added to each 100 μ l protein fraction collected at each step of the column chromatography process. Protein fractions were boiled for 5 minutes and clarified by centrifugation at 14,000 rpm for 3 minutes. Protein fractions were loaded in 10 μ l increments on a pre-cast 12% SDS-PAGE denaturing gel (Bio-Rad, CA) and visualized by Coomassie brilliant Blue staining.

Production of Rabbit Anti-SPI-4 Antibody. Antibody production was performed in a rabbit by Scantibodies, Ramon, CA. Prior to the initial challenge, serum was collected from the test animal and analyzed for a lack of cross-reaction with SPI4-K through immunoblot analysis. A booster injection was given 4 weeks and 8 weeks after the first injection. Antiserum was collected 1 week, 5 weeks, and 9 weeks after the initial injection. The primary and secondary bleeds were both analyzed for SPI4-K specificity via recognition with HRP-conjugated monoclonal anti-rabbit IgG (SIGMA, MO) through Western blot analysis. Production of anti-His tagged SPI4-K antibody is described in figure 2.

Western blot analysis of total cell protein. Bacteria from 10ml of an overnight culture were grown for 16 to 17 hours in LB medium and supplemented with appropriate antibiotics. A 1:1000 dilution of overnight culture in 25 ml LB was grown to fresh stationary phase (O.D. 2.5-3.0), harvested by centrifugation at 6,000 rpm for 10 minutes, washed several times with phosphate buffered saline, and resuspended in 125 μ l of B-Per

(Pierce) and 75 μ l of DNase with vortexing at 5 minute intervals for a total of 15 minutes on ice. The lysate was centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatant was collected. Protein concentration was determined using the Pierce BCA protein assay, using bovine serum albumin as the standard. Two volumes of sample buffer (2.9 ml deionized water, 1.0 ml 0.5 M Tris-HCl, pH 6.8, 2.0 ml glycerol, 1.6 ml 10% w/v SDS, 0.1 ml 1.0% bromophenol blue, 0.4 ml BME) were added to one volume of the protein fraction. Protein fractions were boiled for 5 minutes and clarified by centrifugation at 14,000 rpm for 3 minutes. Equal amounts of total cell protein were loaded onto a 12% denaturing polyacrylamide gel and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred onto a nitrocellulose membrane. The proteins were probed using a polyclonal rabbit anti-SPI4-K serum and detection of bound antibodies was achieved by the addition of horseradish peroxidase (HRP)-conjugated monoclonal anti-rabbit IgG (SIGMA, MO). Immunoblotting was performed with a 1:500 dilution of the primary antibody and 1:10,000 of the secondary antibody.

Western blot analysis of secreted protein. Bacteria from 10 ml of an overnight culture were grown for 16 to 17 hours in LB medium and supplemented with appropriate antibiotics. A 1:1000 dilution of overnight culture in 50 ml LB was grown to fresh stationary phase (O.D. 2.5-3.0) and the cell pellet harvested by centrifugation at 6,000 rpm for 10 minutes. The supernatant was centrifuged at 12,000 rpm for 30 minutes at 4°C several times, filter sterilized, supplemented with a 10% volume of 100% TCA overnight on ice, and centrifuged at 8,000 rpm for 15 minutes at 4°C. The pellet was

washed with 10 ml acetone and allowed to air-dry. The amber-colored pellet was resuspended in 50 μ l 2x laemmli buffer (3.0 ml deionized water, 1.0 ml 1.0 M Tris-HCl, pH 6.8, 1.6 ml glycerol, 1.6 ml 20% w/v SDS, 0.4 ml 0.5% bromophenol blue) and 50 μ l 0.1M Tris-HCl, pH 6.8 and stored at -20°C until further use. BME was added immediately before use to the 100 μ l fraction and boiled for 10 minutes. Protein was loaded onto a pre-cast 12% denaturing polyacrylamide gel (BIO-RAD, CA) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was performed and the separated proteins were transferred onto a nitrocellulose membrane. The proteins were probed using polyclonal rabbit anti-SPI4-K serum and detection of bound antibodies was achieved by the addition of horseradish peroxidase (HRP)-conjugated monoclonal anti-rabbit IgG (SIGMA, CA).

Results

Expression and Purification of His-tagged SPI4-K protein fragment. The pET system is the prevailing and most powerful system developed for the expression of recombinant proteins in *E. coli*. We chose to use the pET-16b expression vector because a 6x His affinity tag could be engineered in the same open reading frame as SPI4-k. In doing so, we could readily purify the protein through His-tagged column chromatography. In this case, the 6x His-tag was engineered onto the N-terminal end of the protein. The expression vector contained an ampicillin antibiotic marker for selection and a thrombin cleavage site that followed the N-terminal his-tag sequence. The system was under the control of an inducible T7-*lac* promoter. Column chromatography was performed at room temperature using Ni-NTA Agarose coupled to Sepharose CL-6B (Qiagen, CA).

The high affinity of Ni-NTA matrix for biomolecules containing a 6x histidine tag supports a generous binding potential of 5-10 mg of 6x His-tagged protein per ml of resin and minimal non-specific binding.

Fig. 3 shows the results of the His-tag SPI4-K purification system used in this study. The protein was expressed well, as it was the dominant band in the total protein fraction (Fig. 3, lane 1). A comparison of the total protein (Fig. 3, Lane 1) with the flow through fraction (Fig. 3, Lane 2) showed that a significant concentration of the over expressed protein went through the column without binding. This is probably the result of overloading the matrix with His-tagged protein. The Ni-NTA chromatography column worked effectively to bind the His-tag on SPI4-K (Fig. 3, lanes 4-13). Proteins were eluted off the column with a high molar concentration of imidazole (500 mM) to increase the yield of protein per 1 ml fraction. Most of the protein eluted from the column in fractions 2-13 (Fig. 3, Lanes 6-13). The predicted molecular weight of the SPI4-K fragment with the his-tag is 33 kD (Fig. 1) and this corresponds with the size of the protein visualized on the gel. This purification procedure provided SPI4-K sufficiently pure enough to be used for the commercial preparation of a polyclonal antibody.

The SPI4-K polyclonal antibody reacts with a large molecular weight protein. Equal amounts of total protein were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, reacted with the SPI4-K polyclonal antibody, and detected using chemiluminescence. The anti-his-tagged SPI4-K antiserum reacts with the 33 kD 6x His-tagged SPI4-K protein (Fig. 4, Lane 5). In addition, the polyclonal antibody detected a large molecular weight protein running above the 208 kD marker on the gel in

SL2757 (wild-type *S. typhimurium* ATCC 14028s) and SL3421 (SPI4-K Ser-to-Ala) (Fig. 4, Lanes 1 and 4), but not in the *slyA* or *sirA* mutant strains (Lanes 2 and 3).

The results of a second Western blot are shown in Fig. 5. We examined the expression of SPI4-K in *S. typhi*, *S. dublin*, *S. cholerasuis*, and *S. typhimurium*. An immunogenic reaction was visualized in the following strains: SL3203 (*S. dublin*) (Lane 1), SL2476 (*S. cholerasuis*) (Lane 2), SL2154 (*S. enteritidis*) (Lane 3), SL2757 (wild-type *S. typhimurium*) (Lane 5), and SL3421 (Spi4-K Ser-to-Ala) (Lane 8). No reaction was detected in SL2754 (*S. typhi*) (Lane 4), SL3343 (*slyA::km S. typhimurium*) (lane 6), SL3272 (*sirA::cm S. typhimurium*) (Lane 7), SL2818 (MG1655) (Lane 9), and SL3277 (SPI4-K MudJ *S. typhimurim*) (Lane 10). With the exception of SL2754 (*S. typhi*), a large molecular weight protein was detected in the strains that harbor SPI4-K. In every case, strains that lacked SPI4-K were not observed to have this protein.

The total protein immunoblot (Fig. 5) also revealed the presence of another conserved band at approximately 34 kD. The immunoreacton is seen in SL3203 (*S. dublin*) (Lane 1), SL2154 (*S. enteritidis*) (Lane 3), and SL3272 (*sirA::cm S. typhimurium*) (Lane 7). In addition, the point mutant SL3421 (SPI4-K Ser-to-Ala) revealed a conserved protein that lies between the 209 kD and 115 kD molecular weight markers. These peptides may be the result of autoprocessing of SPI4-K.

Analysis of secreted proteins. Equal amounts of secreted protein were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, reacted with the SPI4-K polyclonal antibody, and detected using chemiluminescence (Fig. 6). The SPI4-K polyclonal antibody reacted specifically with a large molecular weight protein that runs

higher than the 208 kD marker on the gel. A strong immunogenic reaction was seen in the following strains: SL2757 (wild-type *S. typhimurium* ATCC 14028s) (Lane 1) and SL2476 (*S. choleraesuis*) (Lane 3). A weak immunogenic reaction was seen in SL3203 (*S. dublin*) (Lane 2), although it is poorly visible in the figure. No reaction was detected in SL2614 (*S. typhi* TY2) (Lane 4), SL2818 (MG1655) (Lane 5), SL3421 (SPI4-K Ser-to-Ala) (Lane 6), SL3277 (SPI4-K MudJ *S. typhimurium*) (Lane 7), SL3272 (*sirA::cm S. typhimurium*) (Lane 8) and SL3343 (*slyA::km S. typhimurim*) (Lane 9). Again, with the exception of *S. typhi*, strains that harbored SPI4-K possessed a large molecular weight protein. Strains that lacked SPI4-K did not detect the polyclonal antibody.

Discussion

This report describes the expression system and purification method for the production of commercially produced polyclonal antibody to recombinant SPI4-K protein, and the molecular weight characterization of SPI4-K through immunoblot analysis. While genetic data predict that SPI4-K is approximately 200 kD, the current annotation for the size of the protein in Genbank is approximately 90 kD. The molecular weight of SPI4-K has not previously been observed by SDS-PAGE. The former annotation for SPI-4 alleged that this island contained 18 ORFs designated A-R (104). The recently completed annotation of both *S. typhimurium* LT2 and *S.typhi* genomes suggests otherwise. Only 6 open reading frames are now assigned to SPI-4, with a single open reading frame (STM4261) proposed to encode a 660 kD protein (~16 kB gene). Within STM4261 lies the previously annotated ORF, SPI4-k (Figure 1). Although SPI4-K only represents a portion of the open reading frame (STM4261), the function of this region seems to be

extremely important. The predicted SPI4-K protein has significant homology to a class of autotransporter toxins found in pathogenic *E.coli*, *Bordetella*, *Neisseria*, and *Shigella* species, and are referred to as SPATE toxins (2, 8, 49, 58, 61-64, 79, 83, 84, 97, 102). Members of this family are serine proteases that contain a central catalytic Ser residue with a characteristic GDSGS motif.

Purification of SPI4-K

The SPI4-K polyclonal antibody reacts with a large molecular weight protein

Western blot analysis confirms that the His-tag polyclonal antibody made in the rabbit is capable of detecting SPI4-K. Data from the initial immunoblot showed that a strong immunogenic reaction occurred with the 33 kD His-tagged SPI4-K protein (Fig. 4, Lane 5). This observation corresponded to the proposed molecular weight of His-tagged SPI4-K (Fig. 1). The anti-His-tag SPI4-K polyclonal antibody also recognized a protein that ran above the 209 kD molecular weight marker in SL3202 (wild-type *S. typhimurium* 14028s) and SL3421 (SPI4-K Ser-to-Ala) (Fig. 4, Lanes 1 and 4).

Depicted in Fig. 5, a large molecular weight protein was detected in SL3203 (*S. dublin*) (Lane 1), SL2476 (*S. choleraesuis*) (Lane 2), SL2154 (*S. enteritidis*) (Lane 3), and SL3202 (wild-type *S. typhimurium* ATCC 14028s) (Lane 5). Through blast sequence analysis, we confirmed that the aforementioned serotypes in fact contain SPI-4. This same large molecular weight protein was observed in SL3421 (SPI4-K Ser-to-Ala) as well (Lane 8). This mutant was constructed by a site-specific mutation that changes the putative active site Ser of SPI4-K to an Ala residue. Although the serine protease activity

of SPI4-K is abolished, there are no downstream effects associated with this type of point mutant. So, one would expect to visualize the >209 kD protein. Interestingly, no immunogenic reaction was detected for SPI4-K in *S. typhi* (Fig. 5, Lane 4). The recently sequenced genome provides definitive proof that *S. typhi* contains this pathogenicity island and the SPI4-K ORF in particular. We can not currently explain this anomaly. Perhaps SPI4-K is present but is not expressed. It is also possible that the SPI4-K of *S. typhi* is antigenically distinct from the SPI4-K of other Salmonellae.

Work by Ahmer *et al.* (1) showed that SirA, a transcriptional regulator of the SPI-1 virulence locus, regulates the expression of SPI-4. Genetic and DNA microarray approaches to identify SlyA-dependent genes in our laboratory have indicated that SPI4 is regulated by SlyA. Therefore, we rationalized that the *slyA* and *sirA* *S. typhimurium* mutants would show decreased expression or no expression at all. In fact, the antibody showed no reaction to proteins of either SL3343 (*slyA::km S. typhimurium*) or SL3272 (*sirA::cm S. typhimurium*). These observations led us to believe that we were indeed detecting SPI4-K. Although this immunoblot cannot definitively resolve current uncertainty regarding the size of the SPI4-K protein, these data indicate that SPI4-K in fact corresponds to a large molecular weight protein.

SPI4-K may be a secreted protein

Blast sequence analysis of (STM4261) designates this ORF as a possible putative inner membrane or exported protein, and shows significant homology to the RTX (repeats in toxin) family of exoproteins. This family of cytolytic toxins is widely distributed among pathogenic Gram-negative bacteria and possesses a hallmark C-

terminus distinguished by a series of glycine-rich repeats (25). RTX members are secreted from the cell through recognition of the C-terminal end of the protein via membrane-associated proteins that facilitate the export of the toxin. ORFs around STM4261 show high homology to ABC-transported proteins. It is possible that SPI4-K is transported by these systems.

To investigate whether or not SPI4-K was secreted, proteins were made from the cell culture supernatants. We utilized TCA to precipitate secreted protein from the supernatant. Fig. 6 shows that a single band representative of a > 209 kD protein was detected in SL3202 (wild-type *S. typhimurium* ATCC 14028s) (Lane 1), SL3203 (*S. dublin*) (Lane 2), and SL2476 (*S. cholerasuis*) (Lane 3). A weak immunogenic reaction is seen in *S. dublin* (Lane 2), although it is poorly visible in the scan, signifying that the expression of secreted SPI4-K in *S. dublin* was significantly lower than *S. typhimurium* or *S. cholerasuis*. The high molecular weight band has been detected in *S. dublin*, *S. cholerasuis*, and *S. typhimurium* as well. Therefore, the results from total and secreted immunoblots are consistent with each other. No immunogenic reaction was identified in SL2818 (MG1655) (Lane 5), SL3421 (SPI4-K Ser-to-Ala) (Lane 6), SL3277 (SPI4-K MudJ *S. typhimurium*) (Lane 7), SL3272 (*sirA::cm S. typhimurium*) (Lane 8), and SL3343 (*slyA::km S. typhimurium*) (Lane 9). Once again SPI4-K was not detected in *S. typhi* (Lane 4) even though we made proteins from a different isolate this time. Immunoblot analysis shows convincing evidence that SPI4-K may be secreted from the intracellular compartment.

SPI4-K may be processed

Often, secreted proteins must be processed before they are exported outside of the cell to remove leader sequences, etc. We have made several interesting observations that may provide some insight as to whether SPI4-K is processed before being exported. Fig. 5 reveals the presence of an approximate 34 kD protein observed in several strains. An immunoreacton was detected in SL2757 (wild-type *S. typhimurium* ATCC 14028s) (Lane 1), SL2154 (*S. enteriditis*) (Lane 3), and SL3272 (*sirA::cm S. typhimurium*) (Lane 7). While it would seem likely that SPI4-K processing would occur in *S. enteriditis* and *S. dublin*, one can only speculate as to why this protein, if indeed involved in processing, would show up in the *sirA S. typhimurium* mutant. In addition, SL3421 (SPI4-K Ser-to-Ala) reveals a conserved protein that lies between the 208 kD and 115 kD molecular weight markers. This protein is only seen in the point mutant. Perhaps the point mutation that abolishes the serine protease activity of this protein also affects the method by which this protein is prepared for export.

At present, the nature of these bands is unknown. The smaller peptides detected by the antisera to SPI4-K could be processing intermediates of the larger peptide. At present, we are not sure how the large peptide is secreted from the cell. However, genes that flank the SPI4-K open reading frame are highly homologous to ABC transporters that are involved in the transport of large proteins from bacteria. Alternatively, the smaller peptides could result from the autoprocessing of the SPI4-K protein itself. We have identified a putative catalytic serine motif in SPI4-K. It is possible that SPI4-K is synthesized as a pre-protein and is processed during secretion. Clearly, further

experimentation is required to answer these questions. Regardless, the Western blot analysis clearly demonstrates that SPI4-K is indeed a larger, secreted protein.

We have demonstrated a parallel expression pattern that is consistent for both total and secreted SPI4-K protein. In either case, a large molecular weight protein that runs above the 209 kD molecular weight marker is observed. The polyclonal antibody appears to have a specificity for SPI4-K, since the protein is not visualized in SPI4-K mutant strains or in *E. coli*. Therefore, we propose that SPI4-K is a large molecular weight protein that is secreted from *Salmonella*.

Future studies should focus on the characterization of the role of (STM4261) in *Salmonella* pathogenesis. Specifically, the serine protease activity of SPI4-K should be investigated functionally and biochemically. In addition, the effects of SPI4-K mutants in the murine model would be interesting to investigate.

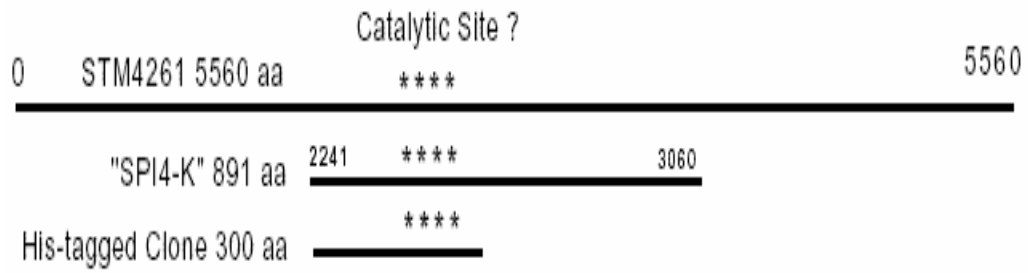


Figure 1. Map of STM4261 and location of His-tagged protein construction.

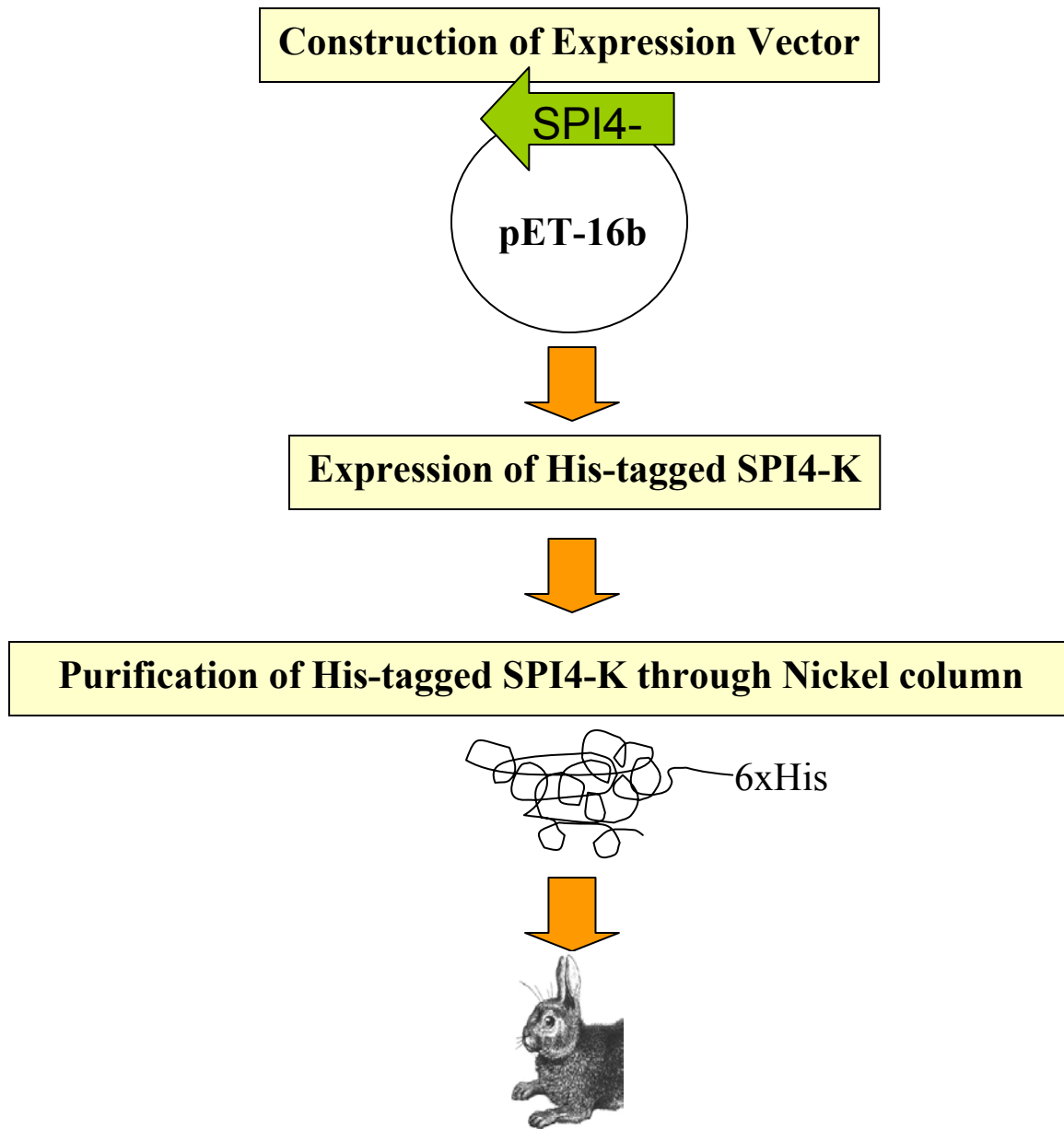


FIG. 2. Schematic of SPI4-K His-tag antibody production.

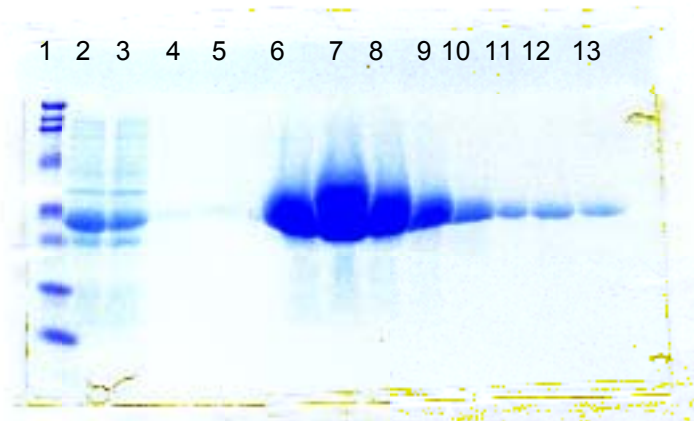


FIG. 3. SDS-PAGE gel of His-tag protein purification steps. Lane 1, molecular weight markers, 209, 124, 80, 49.1, 34.8, 28.9, 20.6, and 7.1 kD; Lane 2, cell lysate after a 4-h induction; Lane 3, flow through; Lanes 4-13, 500 mM imidazole elutions.

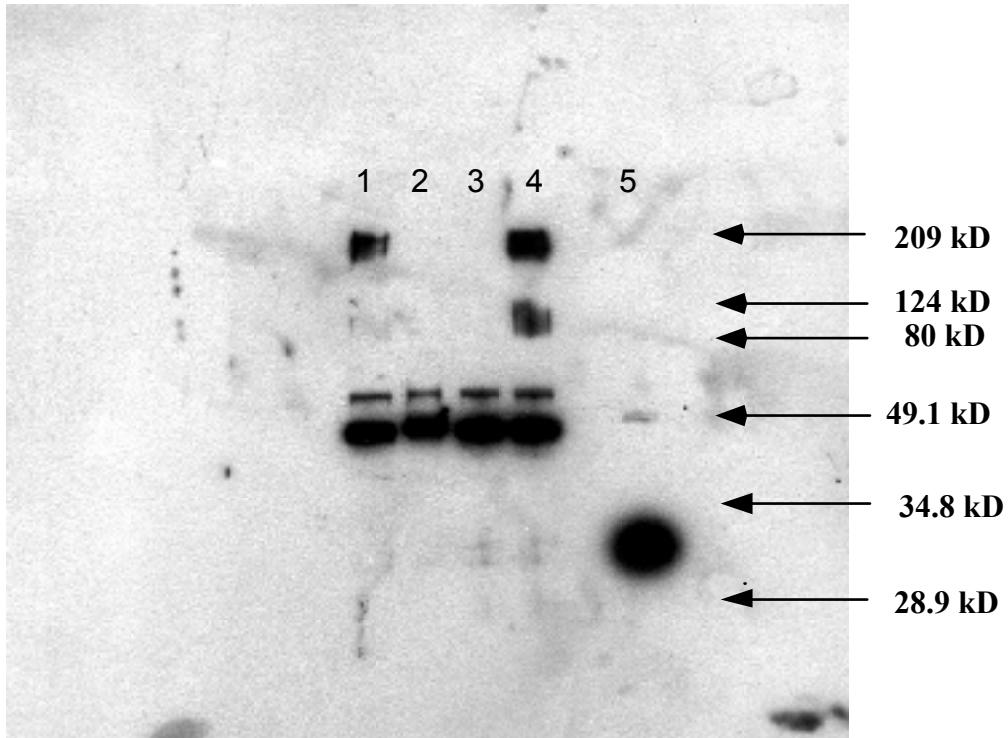


FIG. 4. Western blot analysis of SPI4-K total protein expression. Lane 1, SL2757; Lane 2, SL3343; Lane 3, SL3277; Lane 4, SL3421; Lane 5, His-tagged SPI4-K.

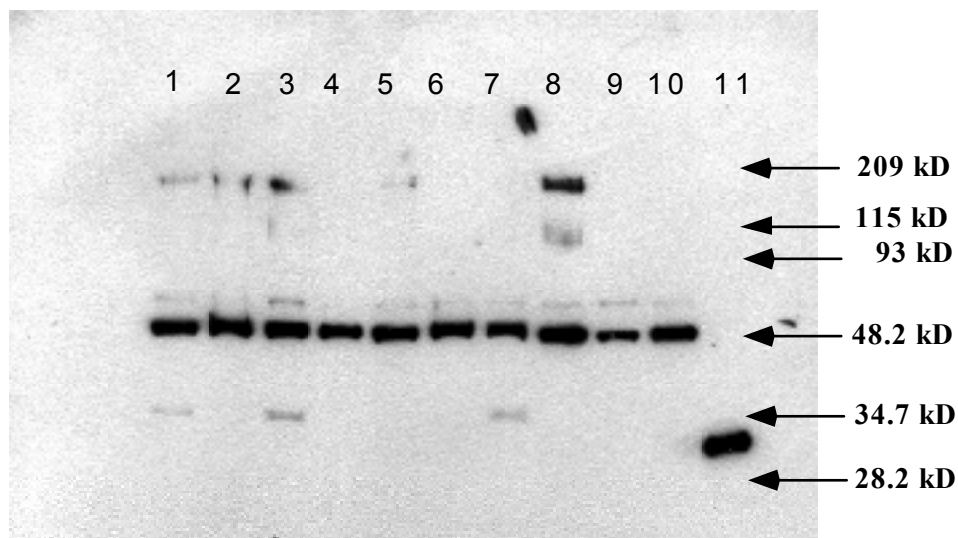


FIG. 5. Western blot analysis of SPI4-K total cell protein expression. Lane 1, SL3203; Lane 2, SL2476; Lane 3, SL2154; Lane 4, SL2754; Lane 5, SL2757; Lane 6, SL3343; Lane 7, SL3272; Lane 8, 3421; Lane 9, SL2818; Lane 10, SL3277; Lane 11, His-tagged SPI4-K protein.

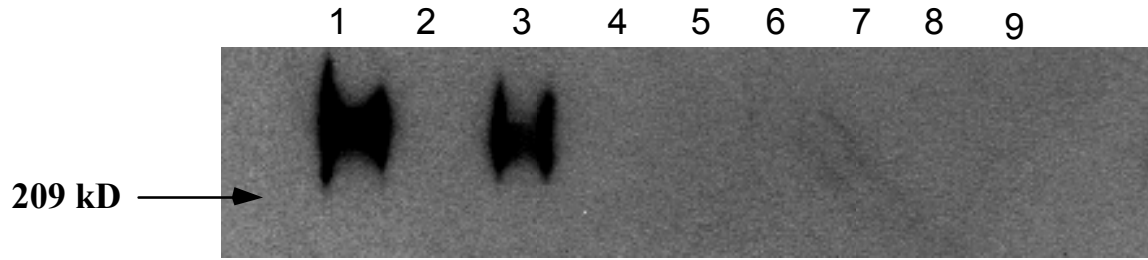


FIG. 6. Western blot analysis of secreted SPI4-K protein. Lane 1, SL2757; Lane 2, SL3203; Lane 3, SL2476; Lane 4, SL2614; Lane 5, SL2818; Lane 6, SL3421; Lane 7, SL3277; Lane 8, SL3272; Lane 9, SL3343.

TABLE 1. Strains and plasmids used

Strain	Relevant features	Source
<i>S. typhimurium</i> ATCC 14028s (SL2757)	Wild type	Lab stock
SL3343	<i>slyA::km S. typhimurium</i>	Lab stock
SL3277	SPI4-K::MudJ <i>S. typhimurium</i>	Lab stock
SL3421	SPI4-K Ser-to-Ala <i>S. typhimurium</i>	Lab stock
SL3203	<i>S. dublin</i>	Lab stock
SL2476	<i>S. cholerasuis</i>	Lab stock
SL2154	<i>S. enteritidis</i>	Lab stock
SL2754	<i>S. typhi</i>	Lab stock
SL2818	MG1655	Lab stock
SL3343	<i>slyA::km S. typhimurium</i>	Lab stock
SL3272	<i>sirA::cm S. typhimurium</i>	Lab stock
SL2614	<i>S. typhi</i> TY2	Lab stock
<i>E. coli</i> DH5 α	<i>endA1 hsdR17(r_K⁻m_K⁺)supE44 thi-1 recA1 pyrA(Nal^r) relA1Δ(lacIAA- argF)U169 deoR[Δ80dlacΔ (lacZ)M15]</i>	Lab stock
BL21(DE3)pLysS	F ⁻ <i>ompT hsdS_B (r_B⁻m_B⁻) gal dcm</i> (DE3) pLysS (Cm ^R)	Novagen
Plasmids		
pET16b	His-tag vector	Novagen
pSK Bluescript	Cloning vector	Stratagene

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