Genetic Analyses of *Salmonella* Virulence

A DISSERTATION

by

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CERTIFICATE OF APPROVAL

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Acknowledgements

I dedicate this thesis to my mother for sending me a lot of Email while I was in graduate school and to Fred for the opportunity to work in his laboratory and for his support.

Abstract

Salmonella is a gram negative bacterial pathogen that can cause human disease. *Salmonella* pathogenesis is complicated and only beginning to be understood on a molecular level. However, many of the virulence properties of *Salmonella* as well as other pathogens can be attributed to two distinct classes of proteins: those that are surface exposed and those that are injected directly into host cells.

Surface exposed proteins are often sought in pathogenesis research because they are so frequently implicated in virulence. These proteins can mediate adhesion and entry into host cells, and also are useful in the development of diagnostic tests and vaccines. In the first section of this thesis, the development of a novel genetic method for the identification of such proteins is described.

In addition to surface exposed proteins, proteins that are secreted directly into host cells are also important in bacterial pathogenesis. *Salmonella* secretes multiple proteins into host cell cytosol via a type III secretion system harbored by *Salmonella* pathogenicity island two (SPI-2). The injected effectors subvert cytoplasmic components of host cells to promote virulence. SsrAB is a two component regulator encoded within SPI-2 that activates the SPI-2 effectors, which are adjacent to the export apparatus on the chromosome. SsrAB has been thought to exclusively act within SPI-2. I

have found that *ssrB* in fact controls a global regulon of previously undescribed genes.

CHAPTER 1: Introduction

Salmonella overview

Salmonella is a gram negative bacterial pathogen that can infect diverse hosts including birds, reptiles and mammals. *Salmonella typhimurium* causes a self limiting gastroenteritis in humans whereas *Salmonella typhi* causes frequently fatal typhoid fever. *Salmonella* infection is a major public health problem with three million cases of infection per year in the U.S. alone (Neidhardt, 1996).

In addition to immediate public health concerns, *Salmonella* is also studied because it is a convenient model pathogen for dissecting basic pathogenic processes. *Salmonella* is able to invade eukaryotic cells and can evade detection and destruction by the immune system – traits that are essential to many human pathogens. Further, a murine model for studying *Salmonella* exists, allowing for the dissection of the complex interactions that occur between a pathogen and a eukaryotic immune system. In addition, *Salmonella* is easily cultivated, is genetically tractable and amenable to molecular biology manipulations.

Salmonella generally enters the body with the ingestion of contaminated foodstuffs. After passing through the stomach, *Salmonella* preferentially invades the M cells of the intestinal epithelium which is usually

accompanied by a large inflammatory response, characterized by the infiltration of professional phagocytes into the area and water flux. This first level of the disease is referred to as the intestinal phase (Baumler et al., 1997a; Baumler et al. 1997b; Baumler et al., 1996). After penetrating the intestinal epithelium, the systemic phase ensues, characterized by *Salmonella's* ability to survive within normally bactericidal macrophages (Rous and Jones, 1916). The ability of *Salmonella* to survive within macrophages, professional phagocytes of the immune system, is a critical component of systemic virulence (Fields et al., 1986). *Salmonella* not only survives within, but actually replicates (Fields et al., 1986) within macrophages and eventually kills them (Chen et al., 1996; Lindgren et al., 1996; Hersh et al., 1999); presumably after using them as vehicles for dissemination into the spleen and liver (Lindgren et al., 1996; Lindgren and Heffron, 1997). At these systemic sites of infection, little is known about the disease except that *Salmonella* replicates extensively (Richter-Dahlfors,et al., 1997; Shea et al., 1999). There are three possible outcomes to the systemic phase of disease: first, a large infiltration of leukocytes into the spleen and liver can clear the infection; second, the disease can be fatal; or third, a person can be converted to an asymptomatic carrier and shed the pathogen continuously for life, with no overt symptoms of disease (Baumler et al., 1998).

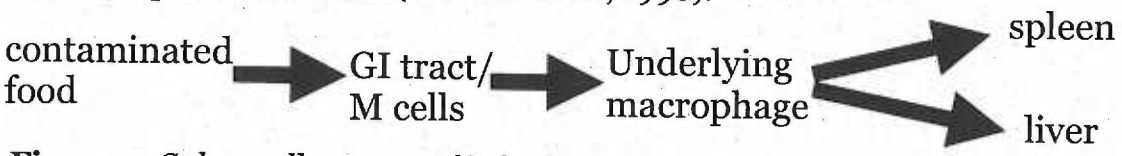


Figure 1. *Salmonella* course of infection. Worley, M.J., unpublished.

Salmonella disease is obviously very complicated and multi-factorial. However, exported proteins are implicated in nearly all of its pathogenic processes. In fact, virtually all virulence factors are exported to the cell envelope or secreted into host cells. Because these proteins are so frequently implicated in pathogenic processes, it is desirable to be able to readily identify them. In the first section of this thesis, a new genetic system for identifying surface exposed proteins is described.

Salmonella not only promotes virulence with surface exposed proteins, but also with ones that it secretes directly into eukaryotic cells through a 'type III' export pathway. Type III protein secretion systems are sophisticated export apparatuses that deliver virulence effectors directly from bacterial to host cell cytosol. The secreted proteins intimately engage components of eukaryotic cells, subverting them to promote virulence. In the second half of this thesis, a genetic study that analyzes the regulation of a *Salmonella* type III secretion system is described.

Protein Export

All bacteria need to secrete proteins to the cell envelope and to the external milieu. For this reason, bacteria have developed complex molecular machines to translocate proteins across membranes. *Salmonella*, like most gram negative bacterial pathogens, has three primary export pathways for secreting proteins to its surface or into host cells (Finlay and Falkow, 1997).

Type I secretion

Type I (ABC transporters) exporters are universally conserved among gram negative bacteria. These exporters are generally specific for a single substrate that is targeted to its cognate transporter through a poorly defined carboxyl terminal signal. With the hydrolysis of ATP, the substrate is extruded directly to the outside of the cell in a one-step process, without a periplasmic intermediate. While ABC transporters are often used to promote virulence, they can also be used for benign purposes. The type I secretion system is exemplified by the extrusion of haemolysin to the external environment from the cytoplasm of pathogenic strains of *Escherichia coli*, proteases by *Erwinia chrysanthemi*, and alkaline protease by *Pseudomonas aeurginosa* (Fath and Kolter, 1993; Salmond and Reeves, 1993; Binet et al., 1997).

The general secretory pathway

The invasin system described in chapter two, is a tool for analyzing type II export. The type II export system is frequently referred to as the general secretory pathway (GSP). It is the primary protein secretion system for gram negative bacteria. Many proteins that travel through the GSP fulfill housekeeping roles. However, in pathogens, many critical virulence factors also travel through the GSP.

Substrates of this system contain a characteristic signal sequence at their amino terminus that promotes interaction with components of the GSP machinery. Signal sequences have little primary sequence homology, but their overall features are conserved, permitting functional complementation. Signal sequences are generally rich in alanine and leucine, favoring the formation of an alpha helix. They are generally hydrophobic and often contain a glycine in the middle (Pugsley, 1993; Danese and Silhavy, 1998). Secretory proteins carrying a signal sequence that includes a conserved peptidase cleavage site are proteolytically processed at the inner membrane by one of two signal peptidase enzymes, and released into the periplasmic space. Some will remain in the periplasm, while some will insert into the outer membrane and a few will be secreted across it. The majority of secretory proteins contain signal sequences without cleavage sites, resulting in their permanent association with the inner membrane (Figure 2) (Pugsley, 1985; Pugsley, 1988; Pugsley,

1989; Pugsley, 1993; Danese and Silhavy, 1998; Worley et al., 1998). The only characterized components of the GSP are SecA, which recognizes the secondary structure of signal sequences (which allows for signal sequence complementation), binds them, and pilots them to the membrane; SecB, which binds along secretory proteins to act as a chaperone to prevent premature folding; and SecY, SecE, SecG, SecD and SecF which are inner membrane proteins that form a 'translocase' complex that transports proteins across the inner membrane. The terminal branch of the GSP that can secrete proteins across the outer membrane to the external milieu is not well understood (Pugsley, 1993; Danese and Silhavy, 1998).

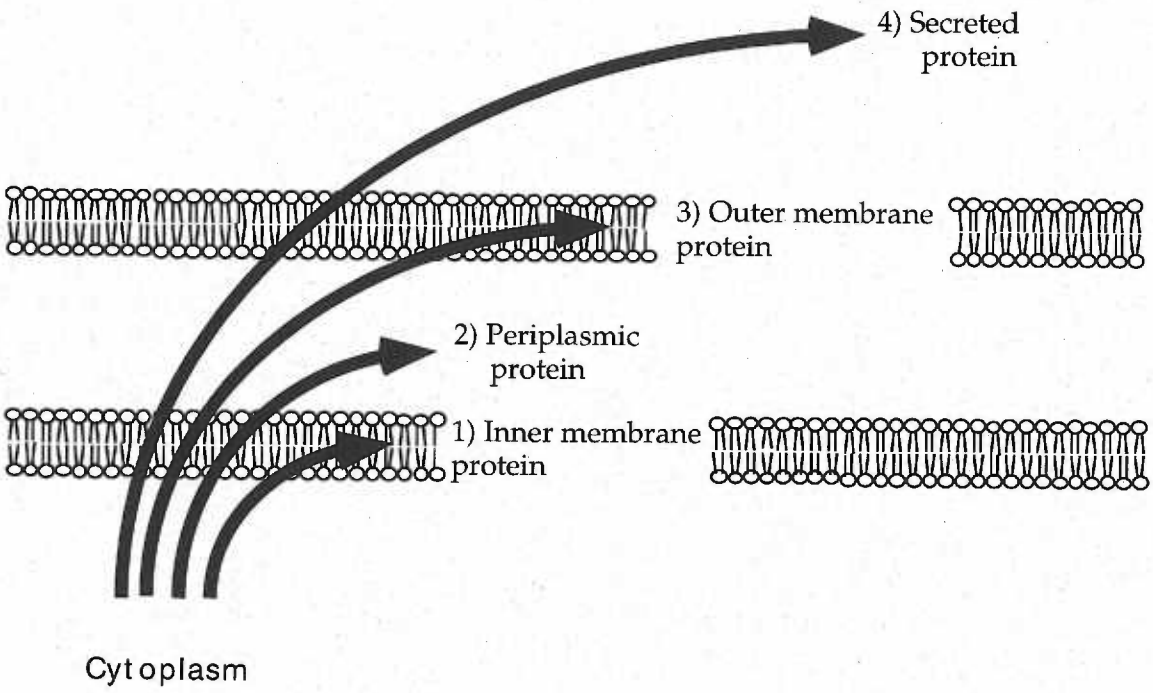


Figure 2. The general secretory pathway. Proteins that enter the GSP can localize to four distinct areas. The majority of these proteins insert into the inner membrane; those with cleavable signal sequences are released from the inner membrane into the periplasm, some of these will remain in the periplasm, some will insert into the outer membrane, and a few will be secreted across it to the external milieu. Worley, M.J., unpublished.

Type III secretion

The third chapter of this thesis deals with research centered around regulatory circuits that control type III export. Type III secretion systems function exclusively to promote virulence. These elaborate devices are composed of 20-25 membrane proteins that can facilitate a pseudo-fusion event between the two bacterial membranes and the plasma membrane, or vacuolar membrane of a eukaryotic cell. This sophisticated secretion system acts as a 'molecular syringe', injecting proteins directly from the bacterial cytoplasm into the eukaryotic cytoplasm. The injected effectors have a variety of effects on host cells that promote bacterial survival and proliferation (Cirillo et al., 1998; Hensel et al., 1998), often by interfering with the host cell's normal signaling pathways (Kaniga et al., 1996; Mecsas and Strauss, 1996). These effectors extensively and elegantly manipulate fundamental host cell processes, largely governing how bacterial pathogens interact with their hosts (Stephens and Shapiro, 1996; Cornelis, 1997; Cornelis and Wolf-Watz, 1997). Thus, understanding the relationships dictated by these effectors enhances our knowledge of both the host and the pathogen, making them of considerable interest in bacterial pathogenesis. Further, because homologous type III protein secretion systems are present in almost all gram negative bacterial pathogens, they are an attractive target for new anti-microbial therapies.

Type III secretion systems share several core structural components. These include several predicted outer and inner membrane proteins. The type III export apparatus of *S. typhimurium* was recently isolated and visualized with electron microscopy (Kubori et al., 1998). The supramolecular structure appeared as a 'needle complex', spanning the inner and outer membranes of the bacterial cell envelope. Two distinct regions were clearly identifiable: a cylindrical base that presumably anchors the complex in the cell envelope, and a needle-like extension that reaches away from the envelope. The 'needle' appeared to be a hollow structure about 120nM in length. It may serve as a conduit through which the effectors can traverse the three membranes. Recent evidence suggests that the type III apparatus is evolutionarily related to the flagellar basal body (Young et al., 1999).

The nature of the secretion signal that directs effectors into the type III machinery is controversial. For some time it has been known that the amino termini of type III effectors can be fused to a variety of other proteins, and these hybrids will be secreted. However, sequence alignments reveal no common motifs at the amino termini of type III effectors, and the amino termini are not processed as with GSP substrates (Sory et al., 1995). It was reported that a type III export signal resided within the mRNA that encodes the effectors. This study by Schneewind et. al. elegantly demonstrated that frameshift mutations that completely altered the peptide sequence for the first 15 amino acids did not abolish secretion. But point mutations in this region of

the mRNA that were predicted to interfere with a predicted RNA hairpin (but did not alter the peptide sequence) did inhibit secretion (Anderson and Schneewind, 1997). This result indicated that there was a type III secretion signal within the 5' mRNA of the effectors.

The significance of the mRNA signal is complicated by the observation that an alternative pathway appears to be present. The signal for this second pathway lies within the peptide sequence, in the middle of the protein rather than at the amino terminus. Because chaperone binding sites were mapped to these regions, it was proposed that in this second pathway, chaperones might pilot their cognate effectors to the export apparatus. Interestingly, inactivating either signal does not significantly diminish secretion (Cheng et al., 1997). Thus it seems that the mRNA signal pathway and the chaperone pilot pathway coexist and are at least partially redundant. The salient feature of both may be their ability to prevent intrabacterial protein aggregation. For reasons that are not clear, effectors tend to aggregate with each other as well as with the translocation machinery (Wattiau et al., 1996; Woestyn et al., 1996; Cheng et al., 1997). A specialized chaperone that recognizes a cognate effector and delivers it to the export machinery would obviously prevent aggregation. Recently, translation and secretion of YopQ by *Yersinia* were shown to be coupled (Anderson and Schneewind, 1999). Thus, an mRNA signal coupled with co-translational extrusion could also prevent premature association of the effectors. It is not clear why both pathways are maintained.

Pathogenicity islands

Type III exporters, which are important in the third chapter of this thesis, are intimately associated with pathogenicity islands. Pathogenicity islands are species specific genome regions that harbor virulence genes. These blocks of DNA are referred to as pathogenicity islands, because they are missing from a pathogen's nearest non-pathogenic relative. Some of these islands harbor type III exporters. The islands that encode type III exporters are essentially 'virulence cassettes'; encoding the structural components of a type III secretion system, as well as an array of cognate effectors and chaperones. Acquisition of such an island can confer super powers upon a previously innocuous organism allowing it to wreak havoc upon eukaryotic cells. Interestingly, pathogenicity islands are often associated with mobile genetic elements such as plasmids, transposons and bacteriophages (So et al., 1979; Mel and Mekalanos, 1996; Waldor and Mekalanos, 1996; Boyd et al., 1997). Frequent lateral transfer of pathogenicity islands has been predicted to occur, and to greatly affect the evolution of bacterial virulence (Mel and Mekalanos, 1996).

Escherichia coli is the nearest non-pathogenic relative of *Salmonella*; the two diverged approximately 100 million years ago, and retain 90% homology at the gene level (Neidhardt, 1996; Lawrence and Ochman, 1997; Lawrence and Ochman, 1998). Many of the virulence properties that

distinguish *Salmonella* from *E. coli* are attributable to two 40Kb *Salmonella* islands termed *Salmonella* pathogenicity island 1 (SPI-1) and *Salmonella* pathogenicity island 2 (SPI-2) (Galan et al., 1992; Galan and Ginocchio, 1994; Groisman and Ochman, 1996; Hensel et al., 1998; Uchiya et al., 1999). SPI-1 and SPI-2 encode distinct type III secretion systems. SPI-1 confers upon *Salmonella* the ability to invade epithelial cells and invoke the inflammatory response (Galyov et al., 1997; Galan et al., 1992) – important components of the early, intestinal phase of disease. SPI-2 allows *Salmonella* to proliferate intracellularly (Ochman et al., 1996; Cirillo et al., 1998; Hensel et al., 1998) and is involved in the late, systemic stages of infection (Baumler et al., 1998).

Salmonella type III exported effectors

Type III effector functions are key to understanding how bacterial pathogens manipulate their hosts. SopB, SopE and SipA are three of the best characterized *Salmonella* type III secreted effectors. All three are secreted into epithelial cells via SPI-1 during the intestinal phase of disease. SopB mediates virulence by interdicting inositol phosphate signaling pathways in a manner that mediates water flux and induces an inflammatory response. Specifically, SopB was found to hydrolyze phosphatidylinositol 3,4,5-trisphosphate, which normally acts to inhibit Ca^{2+} dependent chloride secretion. SopB also hydrolyzes inositol 1,3,4,5,6 pentabisphosphate,

producing inositol 1,4,5, 6-tetrabisphosphate. This is a signaling molecule that increases chloride secretion by antagonizing the chloride secretion inhibition mediated by phosphatidylinositol 3,4,5-trisphosphate (Galyov et al., 1997; Jones et al., 1998; Norris et al., 1998; Wood et al., 1998).

The function of the effectors SopE and SipA were both recently described. SopE was elegantly demonstrated to trigger a eukaryotic phenomenon termed ruffling. Ruffling is a large reorganization of the cytoskeleton that is accompanied by membrane protrusions (ruffles). A concomitant macropinocytic event promotes bacterial internalization. Microinjecting purified SopE or expressing it inside eukaryotic cells from a viral vector is sufficient to promote ruffling. SopE was shown to stimulate GDP/GTP nucleotide exchange in several Rho GTPases, including Rac-1 and CDC42, which provokes the cytoskeletal rearrangements associated with ruffling. Interestingly, mutating *sopE* does not diminish the ability of *Salmonella* to induce ruffling. Thus, SopE is sufficient but not necessary for the ruffling phenotype, indicating complete functional redundancy (Hardt et al., 1998).

SopE induced ruffling is global, instead of localized to the point of bacterial contact as it is normally. SipA was recently found to act in concert with SopE, focussing the ruffles to the site of bacterial contact. SipA accomplishes this by binding directly to actin, decreasing its critical concentration, and inhibiting the depolymerization of actin filaments. This

results in a more pronounced outward extension of the membrane ruffles, facilitating more efficient bacterial uptake (Kaniga et al., 1995; Zhou et al., 1999).

Interestingly, SopB and SopE are both injected into host cell cytosol by the SPI-1 encoded type III export apparatus, despite not being genetically linked to it. Until this recent finding, type III exported effectors were thought to be exclusively located on the same pathogenicity island as their cognate export apparatus. Considering these findings, one can easily envision that individual effectors could be 'shuffled' between different bacteria, allowing for new combinations of effectors to be rapidly generated. Thus, once a bacterial pathogen gained a type III 'gun', it could easily amass 'ammunition' for it that would allow it to explore new host niches and quickly evolve as a pathogen.

Pathogenicity island regulation

Bacterial pathogens are confronted with radically different microenvironments within a host in rapid succession. *Salmonella* must survive passage through the stomach, navigate and survive within the intestine, cross the intestinal epithelium and then survive within phagocytic cells of the immune system. Adapting to these new microenvironments requires elaborate environmental regulation of distinct arrays of genes. For example, extracellular expression of LPS modifications important for survival

within macrophages precipitates annihilation by the immune system (Guo et al., 1997). For a bacterial pathogen, precise gene regulation is literally a matter of life or death.

This elaborate gene regulation is largely achieved via two component regulators. Two component regulators, typified by ToxRS and OmpR/EnvZ are composed of an inner membrane protein (the sensor kinase) and a cognate DNA binding protein (the response regulator/transcription factor) that is free in the cytoplasm (Figure 3). In response to external cues, the sensor kinase autophosphorylates and then phosphorylates the response regulator, modifying its activity (Stock et al., 1989; Groisman and Heffron, 1995). Upon activation, the response regulator binds promoters to activate or repress gene transcription. Two component regulators are thus simple phosphorelay systems that allow bacteria to coordinate gene expression with the external environment.

Pathogenicity islands extensively 'listen' to endogenous two component housekeeping regulators to sense where they are within a host, and regulate gene expression appropriately. For example, in both *E. coli* and *Salmonella*, the two component regulator PhoPQ activates magnesium uptake systems in response to Mg^{2+} starvation. However, because divalent cation levels are low in the endosome, *Salmonella* also uses PhoPQ to activate intracellular virulence genes (Garcia Vescovi et al., 1996; Blanc-Potard and Groisman, 1997; Vescovi et al., 1997; Groisman, 1998; Blanc-Potard et al., 1999).

Many pathogenicity islands achieve even greater control over their expression by encoding their own transcriptional regulators. These regulators are sometimes referred to as adapter regulators because they are often regulated by endogenous two component regulators. Until recently, adapter regulators were thought to exclusively activate genes within the island in which they reside (Ahmer et al., 1999).

HilA is the best characterized adapter regulator. It is encoded within SPI-1 and can activate SPI-1 gene expression (Lee et al., 1992; Bajaj et al., 1995). Multiple distinct environmental signals activate *hilA* transcription, including osmolarity, pH, oxygen and divalent cation concentration (Bajaj et al., 1996). *ssrAB* is the adapter regulator for SPI-2 (Figure 4) and is required for expression of all of the genes within SPI-2 (Cirillo et al., 1998; Hensel et al., 1998). The environmental cue sensed by SsrA that results in SPI-2 gene expression is not known (Deiwick et al., 1999).

Figure 3. Model of a two component regulator. Two component regulators allow bacteria to coordinate gene expression with environmental conditions. Worley, M.J., unpublished.

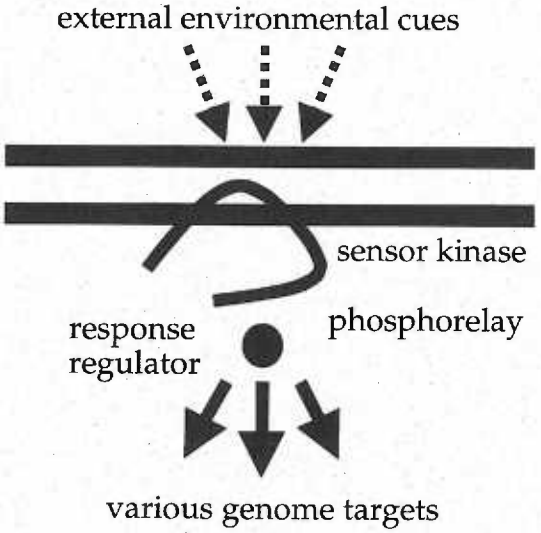
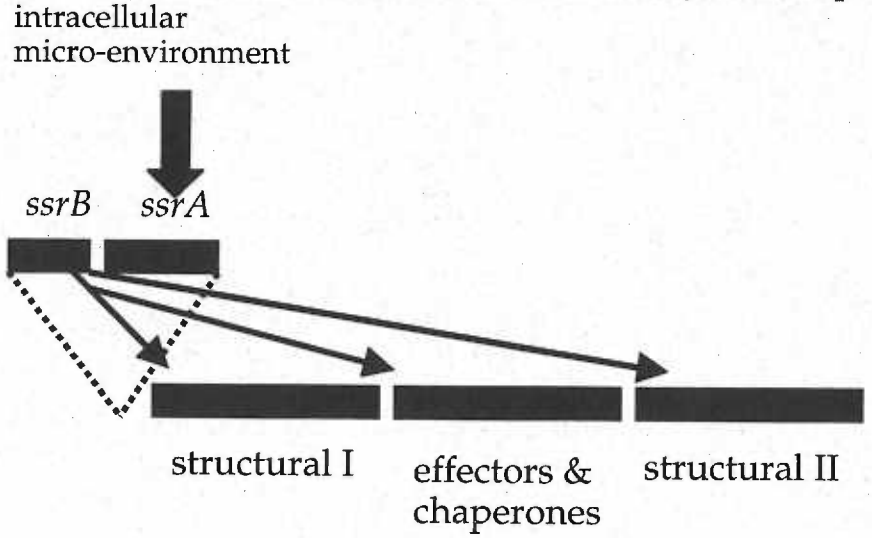


Figure 4. Map of the SPI-2 area of the *Salmonella* genome. Structural operons I & II encode >20 membrane proteins. The intervening region encodes 7 effectors and two specialized chaperones. *ssrAB* is the adapter regulator for SPI-2; in response to an unknown cue in the eukaryotic intracellular micro-environment, SsrA activates SsrB which in turn activates the expression of all of the genes in SPI-2. Until recently, SsrAB was thought to only act locally. Figure is not drawn to scale. Worley, M.J., unpublished.



Intracellular pathogenesis

The ability of *Salmonella* to survive within phagocytes was first noticed 83 years ago (Rous and Jones, 1916). The third chapter of this thesis revolves around the molecular basis for this observation: SPI-2.

When thinking about intracellular pathogenesis, it is useful to first consider the typical fate of bacteria that become internalized within eukaryotic cells. Bacteria that enter animal cells are generally enveloped in a membranous vacuole referred to as an endosome (for normally non-phagocytic cells), or a phagosome (for professional phagocytes). Normally these vesicles will fuse with lysosomes where the bacteria are subjected to an acidic micro-environment, degradative enzymes, and in the case of professional phagocytes, a barrage of bactericidal compounds. Many pathogens, including *Salmonella*, posses a variety of mechanisms to either survive within the lysosomes of professional phagocytes, prevent being delivered to them, or break out of them and run amuck in the cytoplasm (Goren et al., 1976; Sansonetti et al., 1986; Fields et al., 1989; Hacker et al., 1991; Falkow et al., 1992; Martinez de Tejada et al., 1995; Alvarez-Dominguez et al., 1997; Gunn et al., 1998; Guo et al., 1998; Uchiya et al., 1999).

Salmonella first becomes internalized within eukaryotic cells as it breaches the intestinal epithelium by invading intestinal M cells apically and rapidly passing through them. M cells are specialized epithelial cells that play a role in immunological surveillance (Clark et al., 1994; Jones et al., 1995).

In vitro, when *Salmonella* has resided within cultured epithelial cells for approximately six hours, structures termed *Salmonella* induced filaments (sifs) become noticeable. Sifs are filamentous tubular structures containing lysosomal glycoprotein. Sifs are not known to be induced by any other pathogen and thus are assumed not to be a host response to infection, but rather a *Salmonella* directed process. The function of sifs are unknown, however their formation is associated with intracellular bacterial replication (Garcia-del Portillo et al., 1993b; Garcia-del Portillo et al., 1993a).

Sif production requires a gene termed *sifA*. *sif*A displays no homology to any entries in the database and is associated with mobile element remnants (Stein et al., 1996); both features are hallmarks of horizontally acquired virulence factors. Because SPI-2 is induced inside epithelial cells (Cirillo et al., 1998), it is possible that *sif*A is a SPI-2 effector.

In addition to *sif*A, the two component regulator OmpR/EnvZ is also required for sif formation (Mills et al., 1998). The porins encoded by *ompC*, *ompF* and *tppB*, which are the only genes known to be regulated by *ompR* in *S. typhimurium*, are not required for sif formation (Mills et al., 1998). Sif formation dependence on *ompR* may be attributable to the *ompR* regulation of the *ssrB* regulon demonstrated in Chapter 3.

When *Salmonella* exits the M cells of the intestinal epithelium, it encounters underlying professional phagocytes of the immune system, predominantly macrophages. Macrophages are specialized immune system

cells that posses a variety of mechanisms for destroying microorganisms. *Salmonella* can enter macrophages via two distinct mechanisms. First, as previously discussed, *Salmonella* can cause many eukaryotic cells, including macrophages, to ruffle, resulting in *Salmonella* internalization. Second, macrophages are naturally phagocytic, so *Salmonella* can enter passively via receptor-mediated endocytosis.

Once internalized within macrophages, two distinct populations of *Salmonella* can be observed (Buchmeier and Heffron, 1991) (Figure 5). One population is present is 'spacious phagosomes' (Alpuche-Aranda et al., 1994). These endocytic vesicles differ from macropinosomes (which completely shrink within 15 minutes) by persisting in the cytoplasm, often fusing with macropinosomes as well as other spacious phagosomes. Over time, this vesicle can grow to the point that it occupies the majority of the cell (Figure 5). The second distinct population of *Salmonella* persists in a tight phagosome. Quite interestingly, these bacteria are viable, but are not growing, whereas the bacteria present in the spacious phagosome are proliferating rapidly (Abshire and Neidhardt, 1993). It appears that the 'remodeling' of the phagosome occurs immediately after *Salmonell*a entry (Rathman et al., 1997) and is dependent upon PhoPQ (Alpuche-Aranda et al., 1994). The molecular mechanism underlying the existence of the two different populations of *Salmonella* in the macrophage is not understood.

Salmonella has been reported independently by three different groups to be cytotoxic to cultured macrophages. However, there has been disagreement over the timing and mechanism of cytotoxicity. Two groups reported that cytotoxicity was dependent on the type III export apparatus of SPI-1 and happened early in infection (Chen et al., 1996; Monack et al., 1996). One of these groups reported that internalization was not required and that killing could be observed in as little as five minutes (Chen et al., 1996). However, a seemingly contradictory report suggested that macrophage killing did not require SPI-1 and could be observed very late in infection, and was dependent upon *ompR* (Lindgren et al., 1996). This discrepancy has been recently resolved through the observation that there are in fact two distinct killing pathways. One pathway acts early in infection and involves SPI-1. The SPI-1 type III export system injects the effector SipB into macrophages that activates the proapoptotic protease caspase-1 to trigger cell death (Hersh et al., 1999). A second pathway is also present however, and is independent of SPI-1. This second pathway involves SPI-2 (A. van der Velden and F. Heffron, unpub. data), is dependent on *ompR,* and acts late in infection (Lindgren et al., 1996). The observation that *ompR* regulates the *ssrB* regulon (Chapter 3), coupled with the fact that *ompR* affects SPI-1 gene expression in response to osmolarity (B. Ahmer, personal communication), indicates that OmpR is a shared component of both killing pathways. This may explain why *ompR* was

originally isolated as the least cytotoxic mutant possible (Lindgren et al., 1996; Lindgren and Heffron, 1997).

It is interesting to consider why *Salmonella* might posses two independent mechanisms for killing macrophages. Perhaps before the acquisition of the *ssrB* regulon, *Salmonella* needed to kill macrophages upon contact to avoid being killed itself. In this stage of *Salmonella* evolution, the pathogen may have simply persisted as long as possible in the mucosal epithelium in the face of an immunological onslaught, which ultimately cleared the infection. Upon acquisition of the *ssrB* regulon, the endgame changed, because *Salmonella* could now survive within macrophages and use them as a vehicle for dissemination to systemic sites of infection. At these systemic sites, *Salmonella* has the ability to replicate extensively, and to potentially become asymptomatic and be shed continuously for life. For these reasons, a delayed macrophage killing mechanism would be desirable. It is possible that the SPI-1 mediated mechanism is employed in vivo when *Salmonella* encounters macrophages on the luminal side of the intestinal epithelium.

The remainder of this thesis describes research that revolves around the two classes of virulence proteins discussed in the introduction: those that are surface exposed and those that are secreted directly into host cells. One chapter describes a powerful new system for the identification of surface

exposed proteins, which is particularly useful with genetically intractable bacteria. The following chapter delves more deeply into *Salmonella* pathogenesis, describing the identification and characterization of the *ssrB* regulon. The results presented in this chapter have interesting implications for understanding the dynamics of *Salmonella* infection as well as the general logic underlying virulence regulation.

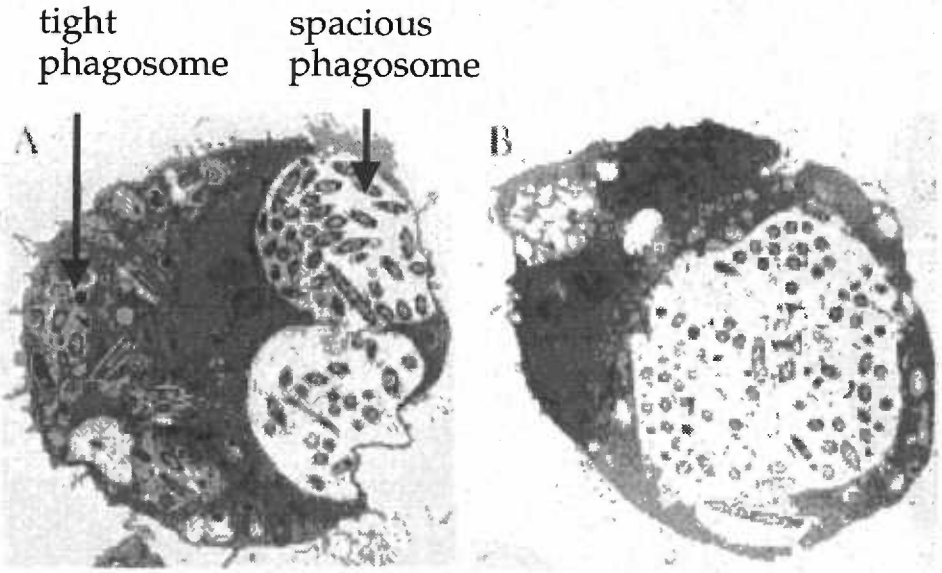


Figure 5. *Salmonella* within a macrophage. In the left panel, the two distinct population of *Salmonella* present inside macrophages are obvious. On the right, the vacuole that *Salmonella* replicates within can be seen to nearly occupy the entire cytoplasm of the macrophage. Modified from (Lindgren et al., 1996).

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CHAPTER II: Manuscript #1

Title: The Identification of Exported Proteins with Gene Fusions to Invasin

Running title: Gene Fusions to Invasin

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ABSTRACT

Exported proteins are integral to understanding the biology of bacterial organisms. They have special significance in pathogenesis research because they can mediate critical interactions between pathogens and eukaryotic cell surfaces. Further, they frequently serve as targets for vaccines and diagnostic tests. The commonly used genetic assays for identifying exported proteins utilize fusions to alkaline phosphatase or beta-lactamase. These systems are not ideal for identifying outer membrane proteins because they identify a large number of inner membrane proteins as well. We addressed this problem by developing a gene fusion system that specifically identifies proteins that contain cleavable signal sequences and are released from the inner membrane. This system selects fusions that restore outer membrane localization to an amino terminal truncated *Yersinia pseudotuberculosis* invasin derivative. In the present study, a variety of *Salmonella typhimurium* proteins that localize beyond the inner membrane were identified with gene fusions to this invasin derivative. Previously undescribed proteins identified include ones that share homology with components of fimbrial operons, multiple drug resistance efflux pumps, and a hemolysin. All of the positive clones analyzed contain cleavable signal sequences. Moreover, over 40% of the genes identified encode putative outer membrane proteins. This system has several features that may make it especially useful in the study of genetically intractable organisms.

INTRODUCTION

Almost all bacterial virulence factors are localized to the cell envelope or secreted (Finlay and Falkow, 1997). This group includes proteins involved in adhesion to and invasion of eukaryotic cells, components of type I and type III exporters, toxins, proteases, and almost all components of all fimbrial operons. Exported proteins contain a signal sequence at their amino terminus that targets them for export through the general secretory pathway (GSP). Signal sequences share little primary sequence homology, but their overall characteristics are conserved, permitting functional complementation. Signal sequences that include a peptidase cleavage site are proteolytically removed at the inner membrane and the mature proteins are released into the periplasmic space. These proteins can remain in the periplasm, insert into the outer membrane, or be secreted to the extracellular milieu. In contrast, proteins that contain non-cleavable signal sequences permanently associate with the inner membrane (Pugsley, 1993).

The primary established methods for the identification of exported proteins, fusions to alkaline phosphatase (PhoA) (Hoffman and Wright, 1985; Manoil and Beckwith, 1985; Taylor et al., 1989; Blanco et al., 1991; Giladi et al., 1993) or beta-lactamase (Bla) (Broome-Smith and Spratt, 1986; Smith et al., 1987; Broome-Smith et al., 1990, screen and select respectively for signal sequences. These systems do not require that signal sequences be cleavable.

Thus, non-cleavable signal sequences as well as generally hydrophobic stretches of amino acids are identified with these approaches (Friedlander and Blobel, 1985; Manoil and Beckwith, 1986; Smith et al., 1987; Calamia and Manoil, 1992; Lee and Manoil, 1994). In fact, the majority of the clones obtained with these methods do not contain proteins that are released from the inner membrane (Smith et al., 1987; Blanco et al., 1991; Giladi et al., 1993; Cleavinger et al., 1995).

Here, we describe a new fusion system devised to specifically identify the subset of secreted proteins that are released from the inner membrane. This fusion system uses the *Y. pseudotuberculosis* outer membrane protein invasin (Isberg and Falkow, 1985; Isberg et al., 1987). When invasin inserts into the outer membrane, its carboxyl terminus becomes surface exposed, and can contact eukaryotic integrins (Isberg and Leong, 1990; Leong et al., 1990), promoting bacterial internalization by the eukaryotic cells (Rankin et al., 1992). We found that only proteins that contain cleavable signal sequences and are normally released from the inner membrane can complement the export defect of an amino terminal truncated invasin and restore the invasive phenotype.

In this study, random *S. typhimurium* chromosomal fragments were fused to an invasin derivative (*Δinv*) and HeLa cells were infected with *E. coli* containing the resulting library. The survivors of gentamicin protection assays were found to contain a variety of periplasmic and outer membrane proteins

fused to the invasin derivative. Components of fimbrial operons, a putative drug efflux pump and a putative hemolysin were identified. Only translational fusions composed of cleavable signal sequences and ΔInv were observed. No cytoplasmic or inner membrane proteins were identified. This approach thus facilitates the identification of proteins released from the inner membrane - a group enriched for outer membrane proteins and virulence factors.

RESULTS

Constructions and Library Enrichment. The vector pICOM II (invasin complementation) was generated by cloning a deletion derivative of invasin into pWKS30 (Wang and Kushner, 1991) (Fig. 1). This derivative lacks the amino terminal 148 amino acids of the full length invasin and therefore cannot be translocated past the inner membrane. Because it is not exported past the inner membrane, ΔInv cannot promote invasion (Fig. 2). However, ΔInv retains the ability to insert into the outer membrane and promote invasion when it is fused to a cleavable signal sequence, and released into the periplasmic space. These isolates are easy to identify with simple gentamicin protection assays (Fig. 1).

S. typhimurium chromosomal fragments were cloned into pICOM II upstream of Δ*inv* and HeLa cells were infected with *E. coli* containing the resulting library. The survivors of one invasion assay were pooled and used to infect HeLa cells again. Three sequential invasion assays were used in this fashion to enrich the library for invasive clones. The dramatic rise in the fraction of the inoculum recovered after each round of selection (Fig. 3) demonstrates that the library was progressively enriched for invasive clones.

After the third round of selection, four clones from each of the three pools were arbitrarily chosen for analysis in individual invasion assays (Fig 4). Each of these twelve clones was found to be at least 1,000-fold more invasive

than pICOM II alone, suggesting that ΔInv was being exported past the inner membrane in these isolates.

Sequence Analysis. Plasmids from the twelve clones were isolated and sequenced. Sequence analysis revealed that all twelve isolates contain gene fragments fused in-frame to Δ*inv* that encode cleavable signal sequences. Three classes of clones were recovered: 1) secreted proteins, 2) outer membrane associated proteins and 3) periplasmic proteins. As expected, no cytoplasmic or inner membrane associated proteins were identified. Some properties of the clones identified are described below and are summarized in table I. The deduced amino acid sequences of the twelve clones that promoted ΔInv export are reported in table I.

Analysis of Clones. One gene fragment fused to Δ*inv* encodes a putative secreted protein. This clone, MJW41, shares homology with an *Actinobacillus pleuropneumoniae* protein that confers hemolytic activity upon *E. coli* (Ito et al., 1993). It also shares homology with uncharacterized regions of the *E. coli* (Blattner et al., 1997) and *Haemophilus influenzae* genomes (Fleischmann et al., 995).

Four fragments fused to ΔInv share homology with proteins that localize to the outer membrane. The first isolate in the outer membrane associated group, MJW37, contains a *S. typhimurium* homolog of *E. coli* MltB

(membrane bound lytic transglycosylase B). This protein resides in the *E. coli* outer membrane and functions as a murein hydrolase (Ehlert et al., 1995). Murein hydrolases are involved in metabolizing the periplasmic murein saccullus, thus affecting bacterial shape and mechanical stability (Holtje and Tuomanen, 1991). They have been implicated in pathogenesis in other organisms (Melly et al., 1984; Dillard and Seifert, 1997), may play a role in inducing septic shock (Takada et al., 1979), and can serve as targets for antibiotics (Dijkstra and Thunnissen, 1994). Another clone in this group, MJW38, encodes a *S. typhimurium* homolog of *E. coli* Slp (starvation lipoprotein). *E. coli* Slp is an outer membrane lipoprotein. Expression of Slp in *E. coli* is induced upon bacterial entry into stationary phase and under starvation conditions. The function of Slp is unknown (Alexander and St John, 1994). The third clone in this group, MJW39, shares homology to the *Pseudomonas aerugionosa* protein OprM. This protein is the outer membrane component of a proton motive force dependent efflux pump termed MexA-MexB-OprM that confers multiple drug resistance (Poole et al., 1993a; Poole et al., 1993b). The last isolate in this group, MJW40, contains two unrelated gene fragments. A homolog of TraI, an F plasmid helicase is fused in frame to ΔInv. However, a homolog of a *Helicobacter pylori* outer membrane protein (Tomb et al., 1997) is fused in frame to TraI at an HpaII site with an open reading frame extending throughout the fusion. Apparently

the outer membrane protein homolog was capable of effecting the translocation of the entire fusion through the inner membrane.

Several proteins that reside in the periplasmic space were identified. Polypeptides of two previously identified periplasmic proteins were fused to ΔInv in MJW46 and MJW47. MJW46 contains a fragment of the *S. typhimurium* lysine-arginine-ornithine-binding protein (Lao). Lao functions as the periplasmic binding component of an ABC transporter and contains a standard cleavable signal sequence (Higgins and Ames, 1981). *cpdB* is fused to Δ*inv* in MJW47. It encodes the periplasmic 2',3' cyclic phosphodiesterase signaling molecule that is regulated by carbon source availability (Anraku, 1966; Neu, 1968; Kier et al., 1977; Higgins and Ames, 1981; Liu et al., 1986; Liu and Beacham, 1990) and as such may be involved in adaptive mutation (Taddei et al., 1995). It also contains a standard cleavable signal sequence and is located in the periplasm (Liu and Beacham, 1990). Another clone in this group, MJW48, shares homology with a region of the *E. coli* genome predicted to be involved in carnitine metabolism (Blattner et al., 1997. However, MJW48 does not show homology to a previously described *E. coli* operon necessary for carnitine metabolism (Eichler et al., 1994). In any event, the *E. coli* and *S. typhimurium* genes appear to encode cleavable signal sequences (table II) and are presumably located in the periplasm.

Four polypeptides in the periplasmic group share homology with proteins involved in pilus biogenesis. MJW42 contains *orf5*, a component of

the previously described *pef* (plasmid encoded fimbriae) fimbrial operon Friedrich et al., 1993). The *pef* operon is located on the 90kb resident *S. typhimurium* virulence plasmid. It contributes to *S. typhimurium* virulence in the murine model of infection by mediating adhesion to the small intestine (Baumler et al., 1996). *orf5* is the fifth of thirteen open reading frames in *pef*. The protein encoded by *orf5* is required for pilus assembly but its exact function is unknown (Friedrich et al., 1993). MJW45 contains the previously identified *orf8* of the pef operon (Friedrich et al., 1993). This gene was recently shown to be regulated by the *S. typhimurium* quorum sensor SdiA, and was renamed SrgA (Ahmer et al., 1998). SrgA is a homolog of *E. coli* DsbA, a periplasmic disulfide oxidoreductase required for many proteins to fold properly, including several virulence determinants from multiple organisms (Yamanaka et al., 1994; Foreman et al., 1995; Okamoto et al., 1995; Watarai et al., 1995; Zhang and Donnenberg, 1996). Because this DsbA homolog is encoded within a fimbrial operon, it presumably plays some role is Pef assembly, although it is not required for it (Rodriguez-Pena et al., 1997). Perhaps it is required for optimal assembly. It is interesting that SrgA is an outer membrane associated lipoprotein (table II), whereas *E. coli* DsbA is free within the periplasm (Bardwell et al., 1991). Homologs of two additional periplasmic proteins involved in pilus assembly, TrbC and TraW (Maneewannakul et al., 1991; Maneewannakul et al., 1992; Neidhardt, 1996) are fused to ΔInv in MJW43 and MJW44 respectively. These genes may

represent one or two undescribed *S. typhimurium* fimbrial operons depending upon whether they participate in the synthesis of the same or different pili.

DISCUSSION

This report describes a simple assay for the identification of proteins that contain cleavable signal sequences. We find that when the *Y. pseudotuberculosis* protein invasin is deleted for its amino terminus, only a cleavable signal sequence will complement its export defect and allow it to insert into the outer membrane. Such fusions are easy to select from a genomic library with simple gentamicin protection assays that are routinely performed in pathogenesis research. Twelve arbitrarily chosen fusions, selected from a library of *S. typhimurium* clones expressed in *E. coli* were all found to contain the amino termini of open reading frames and cleavable signal sequences fused in-frame to ΔInv. No cytoplasmic or inner membrane associated proteins were identified. Because of its high specificity for proteins that are released from the inner membrane, invasin gene fusions should facilitate the identification of outer membrane proteins and putative virulence factors.

Inner membrane associated proteins are probably seldom identified with invasin complementation for several reasons. Any fusion tethered to the inner membrane, even a protein with a cleavable signal sequence that was retained in the inner membrane by a lipid anchor, would have to stretch across the entire periplasmic space, breaching the peptidoglycan layer, and contact the outer membrane itself, for ΔInv to be able to insert into the outer

membrane. Intuitively, it seems unlikely that such a fusion would occur. Further, if ΔInv was even transiently tethered to the inner membrane, it would probably be subject to proteolytic degradation, since it does not normally reside in the periplasm. Finally, even if inner membrane associated proteins did promote outer membrane localization of ΔInv, they probably would not do so efficiently and would be out-competed in the enrichments. In any event, no cytoplasmic or inner membrane associated proteins were identified in this study.

Several proteins exported past the inner membrane of *S. typhimurium* that were identified in this study deserve further consideration. One of the more interesting isolates contains a protein that shares homology ($p=10^{-46}$) with a hemolysin from the veterinary pathogen *A. pleuropneumoniae* (Ito et al., 1993). This isolate is of interest because hemolysins are secreted virulence factors that are often required for bacterial survival within a host. They are usually secreted through dedicated transporters independent of the GSP. However, it is certainly possible that a protein with hemolytic activity could be extruded through the GSP. Enigmatically, this fusion contains both putative standard (signal peptidase I) and lipoprotein (signal peptidase II) cleavage sites (table II). The presence of both types of cleavage sites raises the interesting possibility that this protein is differentially sorted upon entering the periplasm. Further study will be required to determine the routing and function of this protein.

In addition to the hemolysin, the components of fimbrial operons identified are also of interest. Fimbriae initiate the disease process by mediating adherence to host tissue and are necessary for full virulence of *S. typhimurium* in mice. *S. typhimurium* expresses four characterized fimbriae and is known to express others which remain to be identified (van der Velden, 1998). Fimbriae are complex structures that require numerous gene products to be exported past the inner membrane for assembly (Neidhardt, 1996). Thus, this system may significantly facilitate the identification of undescribed fimbrial operons.

Finally, the putative drug efflux pump component is also of interest. Based on homology, the full length *S. typhimurium* operon that includes the gene identified in this study belongs to the resistance-nodulation-division (RND) family of multicomponent, multidrug efflux systems (Nikaido, 1996; Paulsen et al., 1996), exemplified by *E. coli* AcrAB (Ma et al., 1994). More specifically, the outer membrane component shares an extremely high level of homology with the *P. aerugionosa* and *Neisseria gonorrhea* energy dependent efflux systems MexA-MexB-OprM (Poole et al., 1993a; Poole et al., 1993b; Li et al., 1995) and Mtr (Hagman et al., 1997) ($p=10^{-107}$ and $p=10^{-73}$ respectively). These efflux systems confer multiple drug resistance, resistance to toxic hydrophobic agents such as bile salts (Maier et al., 1975; Pan and Spratt, 1994; Hagman et al., 1995; Delahay et al., 1997; Hagman et al., 1997; Thanassi et al., 1997) and Mtr was recently reported to mitigate gonococcal

susceptibility to antibacterial peptides (Shafer et al., 1998). It will be interesting to determine if this *S. typhimurium* efflux pump confers drug resistance, is involved in gastrointestinal survival, and if it can mitigate the toxic effects of antibacterial peptides, particularly those found in the macrophage. Further, with the availability of an excellent animal model for *Salmonella* pathogenesis, it will be interesting to assess the importance of an RND efflux pump *in vivo*.

The invasin complementation system was designed to identify substrates of the GSP, which is the primary bacterial export pathway, and the only known route to the outer membrane. However, bacteria utilize two other export systems as well, the type I (ABC transporters) (Fath and Kolter, 1993; Binet et al., 1997) and type III pathways (contact dependent secretion) (Stephens and Shapiro, 1996; Cornelis and Wolf-Watz, 1997). Because proteins exported through these alternative pathways do not involve periplasmic intermediates (Fath and Kolter, 1993; Stephens and Shapiro, 1996; Binet et al., 1997; Cornelis and Wolf-Watz, 1997), they are not likely to be identified with this approach. However, all of the components of type I and type III exporters travel through the GSP (Fath and Kolter, 1993; Stephens and Shapiro, 1996; Binet et al., 1997; Cornelis and Wolf-Watz, 1997). In fact, the type III export machinery consists of over 20 different gene products, many of which rely on the GSP for export past the inner membrane (Stephens and Shapiro, 1996; Cornelis and Wolf-Watz, 1997). Therefore, in large scale

selections, which we are currently conducting, all of the type I and type III exporters are identifiable with the invasin system. Furthermore, type III exported effector molecules are often genetically linked to their export apparatus, rendering their identification straight forward once an export apparatus is identified. Quite recently, a novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins was described (Berks, 1996; Santini et al., 1998; Weiner et al., 1998). This system is Sec independent and utilizes a "twin arginine" leader motif (Berks, 1996; Santini et al., 1998; Weiner et al., 1998). None of the proteins identified here are likely to be exported *via* this newly discovered pathway. However, substrates of this system that are localized to the periplasm use cleavable amino terminal signals, and thus would probably score positively in this assay.

This approach will complement ongoing sequencing projects for several reasons. Leader peptides share little primary sequence homology (Pohlschroder et al., 1997) and are difficult to reliably identify using automated procedures. The invasin selection identifies functional signals not merely homology, and thus can be used to definitively assign subcellular locations to the proteins encoded by uncharacterized open reading frames. For example, we recently identified several *Chlamydia trachomatis* envelope proteins that do not resemble known exported proteins but do contain signal sequences (unpub. results). These proteins would not have been easily found,

if at all, with genome sequence analysis alone. Furthermore, only a small subset of bacterial genomes will ever be sequenced.

The assay described in this report may be especially useful in the study of genetically intractable organisms. For example, *Treponema pallidum*, *Mycobacterium leprea* and *C. trachomatis* are all ideal candidates for study with this system. Evolutionarily distant organisms are amenable to this type of analysis because the general properties of leader peptides and the GSP are highly conserved (Pugsley, 1993), and because the selection is so dramatic, that a low level of invasin export is probably sufficient for detection in this system. This system will additionally be useful in the study of fastidious organisms because it is not limited by transposon host range or availability of a delivery system. Finally, the $_{p}$*lac* promoter allows the isolation of fusions from organisms whose promoters are not active in *E. coli*. For these reasons, we anticipate that invasin gene fusions will be a useful new tool for the identification of surface exposed proteins and potential virulence factors from diverse organisms.

Experimental Procedures

Reagents and Strains. Cell culture plates were purchased from Falcon, cell culture reagents and T4 DNA ligase from Life Technologies, formerly Gibco-BRL. Restriction enzymes were obtained from Boehringer Mannheim, shrimp alkaline phosphatase from United States Biochemical, and *Epicurian Coli*® XL-2 Blue ultracompetent cells from Stratagene®.

Culture Methods. HeLa cells were maintained in tissue culture flasks (Falcon) at 37°C with 5% CO_2 and Dulbecco's modified Eagles medium (DMEM), supplemented with 10% fetal calf serum and 1mM sodium pyruvate. HeLa cells for the library enrichments were grown into nearly confluent monolayers in six well tissue culture plates. HeLa cells for individual invasion assays were similarly maintained, except they were grown in 24 well plates.

Construction of pICOM II. A 2.7kb *inv* derivative, missing the DNA encoding the first 148 amino acids of the invasin protein, was released from the plasmid pRI203 (Isberg et al., 1987) with *Cla*I and *Nru*I. This *inv* derivative was ligated into the *Cla*I and *Eco*RV sites of pWKS30 (Meerman and Georgiou, 1994), yielding pICOM II (invasin complementation) (Fig. 1).

Construction of a Recombinant Library in *E. coli*. *S. typhimurium* 14028S chromosomal DNA was isolated and partially digested with *Taq*I and *Hpa*II, and size-selected for fragments greater than 100 bp with a QIAquick™ PCR purification column (Qiagen®). The partially digested chromosomal

DNA was ligated into *Cla*I digested, dephosphorylated pICOM II. The ligation mixture was transformed into Epicurian Coli® XL-2 blue ultracompetent cells and the resulting transformants were selected on three groups of Luria-Bertani (LB) agar plates, containing 200 μg/ml ampicillin (amp). The library was selected on three groups of plates and the groups enriched separately to minimize subsequent sibling isolation. The library was determined by serial dilution to contain 9,400 in-frame fusions.

Library Enrichments/Invasion Assays. The library was removed from the selective plates with dacron swabs (Falcon), and suspended in Hanks Balanced Salt Solution (HBSS). Approximately 10^7 colony forming units from each of the three suspensions were used to infect HeLa cell monolayers that had been washed twice with 37°C HBSS, and overlaid with 37°C DMEM without serum. The cell culture plates were gently spun at 1000 rpm in a Sorvall table top centrifuge at ambient temperature for ten minutes, to bring the bacteria in close proximity to the eukaryotic cells. The cell culture was then incubated at 37°C with 5% CO_2 for two hours, allowing the bacteria a chance to invade. After this incubation, the media was aspirated, and the monolayers were washed five times with 37°C HBSS to remove extracellular bacteria. The monolayers were then incubated for another two hours with DMEM containing 75 μg/ml gentamicin, an antibiotic that only kills extracellular bacteria. After two hours of additional incubation, the medium was aspirated, and the monolayers were washed three times with HBSS to

remove any remaining extracellular bacteria. The HeLa cells were then lysed with 0.1% Triton X-100 to release intracellular bacteria. The intracellular bacteria were recovered on LB-amp-200 plates. The survivors of one invasion assay were pooled and used to infect HeLa cell culture again. The library was sequentially enriched in this manner with three rounds of invasion assays.

Sequence Analysis. The oligonucleotide 5'-AGGAGCCAGCCAATCAAGAGAG-3', was synthesized by the OHSU Microbiology Core Facility on an Applied Biosystems 394 synthesizer for use in determining the sequences of inserts fused to the *inv* derivative in pICOM II. This oligonucleotide recognizes the minus strand (bases 998 to 977) of the full length *Y. pseudotuberculosis inv* sequence. Plasmid DNA for sequencing was isolated with Qiagen® columns. DNA sequence determination was performed by the OHSU Microbiology Core facility with an Applied Biosystems 377 fluorescent DNA sequencer with chain terminator chemistry. Sequence analysis was performed with the computer programs MacVector® 6.0 from Oxford Molecular Group and BlastX (Altschul et al., 1990) from the National Centers for Biotechnology Information.

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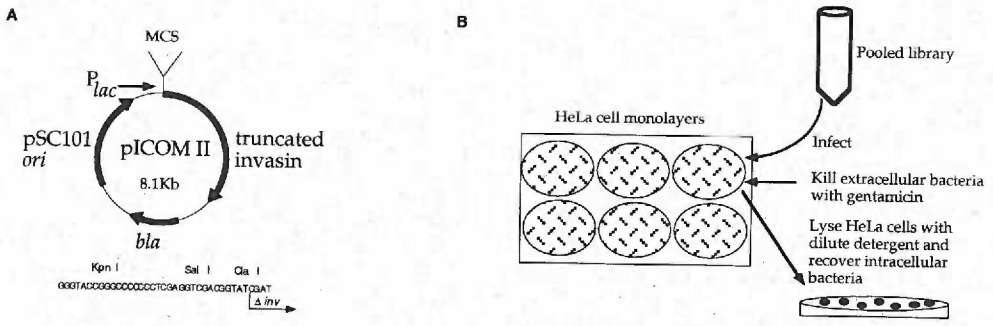

Figure 1A. Selection of clones encoding cleavable signal sequences with invasin and 1B. Design of pICOM II (invasin complementation).

Figure 1a. Selection of clones encoding cleavable signal sequences with invasin. Chromosomal DNA is cloned into pICOM II (Fig. 1b) upstream of an invasin derivative lacking its signal sequence. A library of transformants are pooled and HeLa cell culture is infected with them. Only clones containing cleavable signal sequences allow this invasin derivative to insert into the outer membrane. These are the only clones that enter the HeLa cells and become protected from gentamicin.

Figure. 1b. Design of pICOM II (invasin complementation). A deletion of *Y. pseudotuberculosis inv* that lacks the first 148 N-terminal amino acids it encodes was cloned into the ClaI and EcoRV sites of pWKS30 (Wang and Kushner, 1991). This deletion mutant contains the sequences necessary to direct outer membrane insertion and has the potential to facilitate invasion of eukaryotic cells. However, it does not have access to the periplasm because it is not exported past the inner membrane. pICOM II contains the pBluescript®KS polylinker. The confirmed unique sites are shown. Fusions can be expressed from the promoter of the gene cloned into the vector, or from the $_{P}$lac promoter.

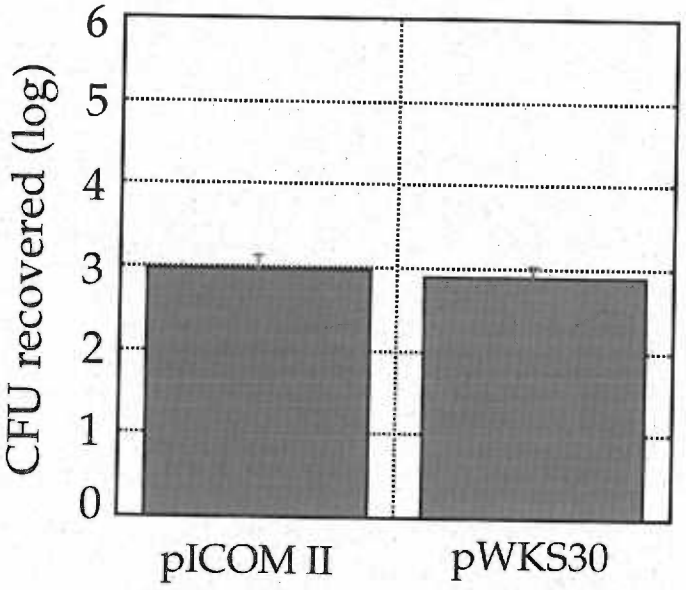
CFU recovered (log)
6
5
4
3
2
1
0
pICOM II
pWKS30

Figure 2. pICOM II does not promote bacterial invasion of HeLa cells more than the parent vector.

Figure 2. pICOM II does not promote bacterial invasion of HeLa cells more than the parent vector. An invasion assay was performed with the background strain containing pICOM II and the background strain containing the parent vector (pWKS30). Data points represent the averages of two independent experiments. Error bars represent the standard deviation.

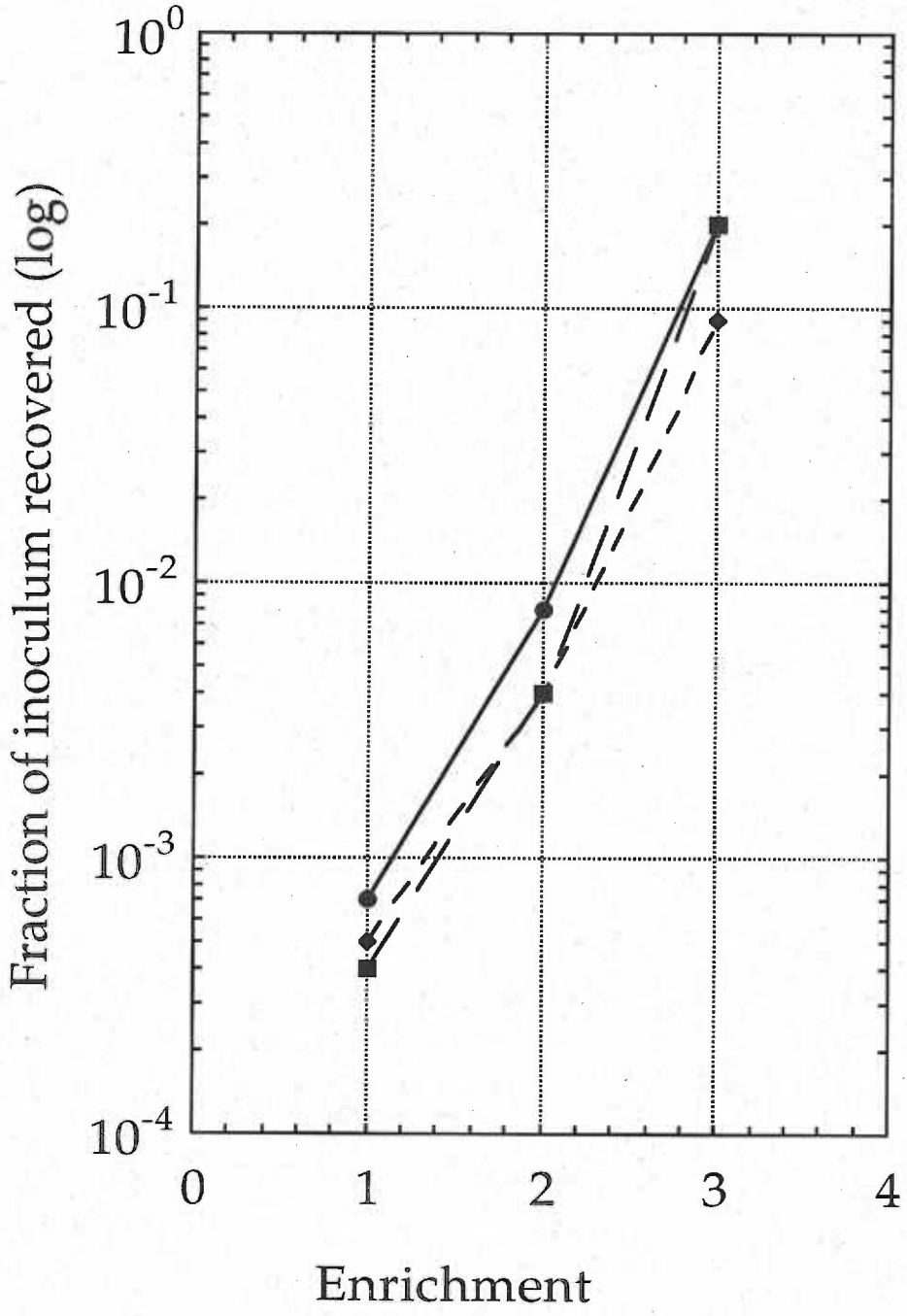

Figure. 3. Fraction of inocula recovered for each of three independent pools after each passage.

Figure. 3. Fraction of inocula recovered for each of three independent pools after each passage. For each enrichment, approximately 10^7 bacteria were added to one well of a 6 well cell culture plate containing HeLa cells. The number of invasive bacteria recovered after each passage was determined by plating serial dilutions. A substantially higher fraction of the inoculum was recovered with each progressive enrichment. The library was enriched in three independent pools to minimize sibling isolation. The power of the selection is so strong that fewer rounds of enrichment might be adequate.

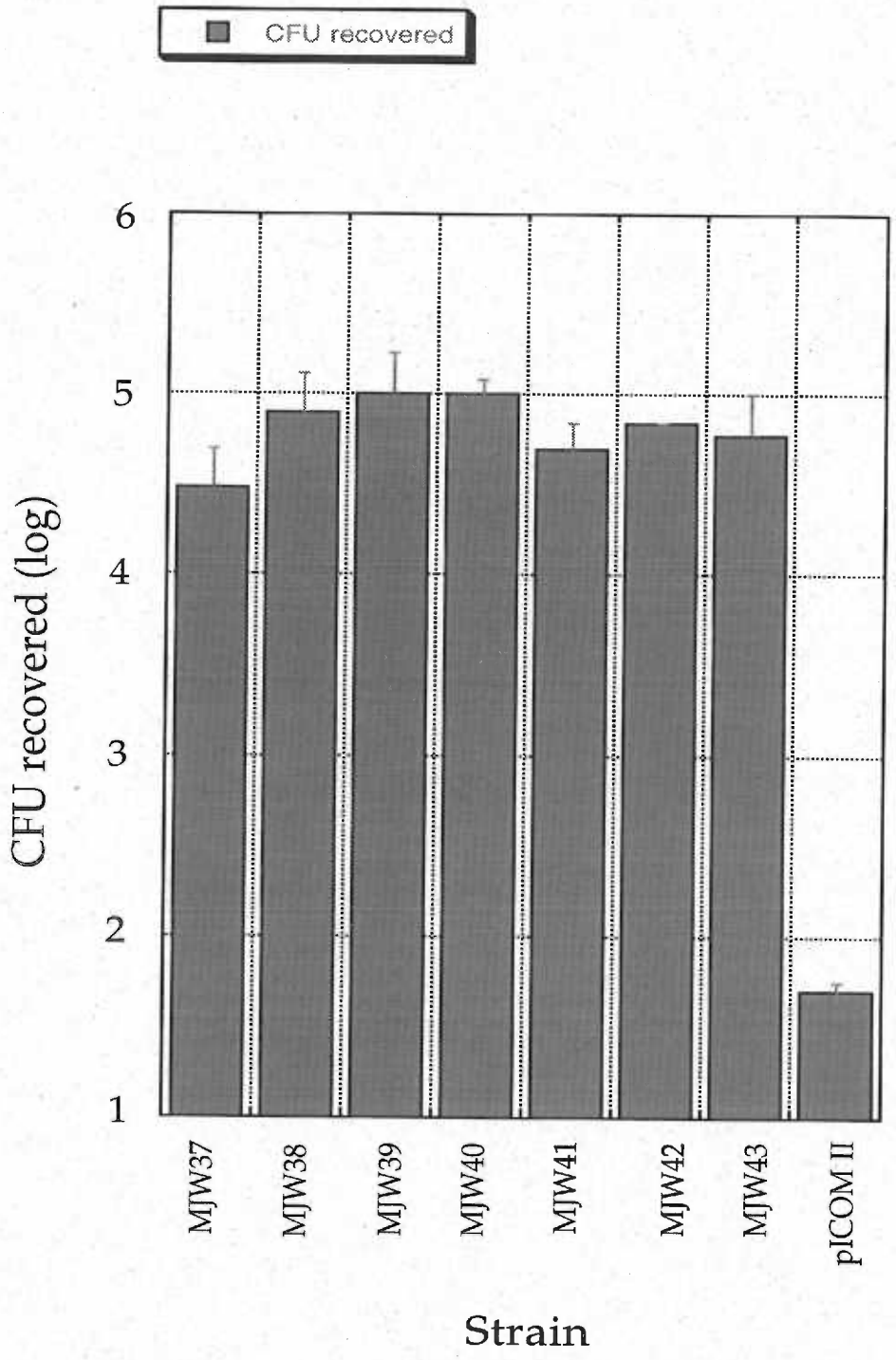

Fig 4. Invasion assays with individual clones.

Fig 4. Invasion assays with individual clones. Invasion assays were performed on arbitrarily selected isolates that survived three enrichments. pICOM II is included to provide a base line. The values shown are the averages of two separate invasion assays. Error bars represent the standard deviation. After three cycles of selection, each isolate analyzed was three logs more invasive that the background strain containing the pICOM II without an insert. The selection is so powerful that fewer rounds of selection would probably be adequate.

Plasmid	Gene fused to Δ*inv*	% identity	Location
MJW37	*E. coli mlt*B (murein lytic transglycosylase B) homologue	81	outer membrane
MJW38	*E. coli slp* (starvation lipoprotein) homologue	82	outer membrane
MJW39	*P. aerugionosa opr*K drug efflux pump homologue	44	outer membrane
MJW40	*H. pylori* outer membrane protein homologue	51	outer membrane
MJW41	*A. pleuropneumoniae hly* (hemolysin) homologue	57	secreted?/OM?
MJW42	*S. typhimurium orf*5 from the *pef* fimbrial operon	100	periplasmic
MJW43	F plasmid *trb*C homologue (pilus biogenesis)	56	periplasmic
MJW44	F plasmid *tra*W homologue (pilus biogenesis)	81	periplasmic
MJW45	*E. coli dsb*A homologue (disulfide oxidoreductase)	100	periplasmic
MJW46	*S. typhimurium lao* (amino acid transporter)	100	periplasmic
MJW47	*S. typhimurium cpd*B (cyclic phosphodiesterase)	100	periplasmic
MJW48	*E. coli cai*A carnitine oxidoreductase homologue	85	periplasmic

Table I. Genes identified and predicted subcellular locations of the proteins they encode.

Table I. Genes identified and predicted subcellular locations of the proteins they encode. All twelve fusions analyzed after the third enrichment contain the 5' ends of open reading frames fused in-frame to Δ*inv*. All twelve encode proteins that are predicted to localize to the periplasm, outer membrane or extracellular environment. No cytoplasmic or inner membrane proteins were identified.

MJW37
MLYHTSPWLNLFNGCLMFKR *RYVALLPLCVL* **LAAC** SSTPKSSETQATTGTP
606 bases fused

MJW38
VSRKRSRIMAVQKRLI *KGALACAFALM* **LSGC** VTIPDAIKGSSPTPQQDLVR
243 bases fused

MJW39
MISKHNDGNGIMKITFTGY *RQTATLATLAFVTT* **LAGC** TMAPKHERPASPTA
321 bases fused

MJW40
MGLRQSL *RIAASTLLLACGLQF* **AHA** DSSPQTIVFGVAPGPYGDMVKQAIAP
241 bases fused

MJW41
M*KAFSPLAVLISALL***LQGC**VAAAVVGTA **AVG**TKAATDPRSVGTQVDDGTLE
531 bases fused

MJW42
MMRRSEIRWMK *RHVCAGALVTGLLFPLATE* **AAS** KENITIPFTLTNPQQTCK
216 bases fused

MJW43
MHRKI*KYLAGLIMVISGTV* **SAG** GVANTPENRQFLKQQEELSRQLRDRPDAE
210 bases fused

MJW44
VSAYEGRAVKW*RGLTALLIWGQSV***AAA**DLGTWGDLWPVQEPDMLAVIMHR
189 bases fused

MJW45
MNYARDLFSL *KGILFSFL* **LAGC** VCPVVAQEWESITPPVVDAPAVVEFFSFY
210 bases fused

MJW 46
MK *KTVLALSLLIGLGAT* **AAS** YAALPQTVRIGTDTTYAPFSSKDAKGEFIGF
>500 bases fused

MJW47
MI *KFSATLLATLI* **AAS** VNAATVDLRIMETTDLHSNMMDFDYYKDTATEKFG
>600 bases fused

MJW48
MK *RXSFITLTIIGAYSALQA* **AWA** VDYPLPPEGSRLIGQNQTYTVQEGDKN
171 bases fused

Table II. Sequences that promote ΔInv export.

Table II. Sequences that promote ΔInv export. All twelve isolates analyzed contain putative cleavable signal sequences fused to ΔInv. These sequence conform to the consensus characteristics of signal sequences previously summarized (Pugsley, 1993). The proposed signal sequences are shown in italics with the proposed cleavage sites in boldface. The lack of a +2 D residue for the lipoproteins indicates outer membrane association (Yamaguchi et al., 1988; Gennity and Inouye, 1991; Gennity et al., 1992. Two different types of possible cleavage sites (SPase I and Spase II) (Pugsley, 1993) are indicated for the hemolysin homolog. MJW37 and MJW42 both contain "twin arginines", the hallmark characteristic of substrates of the *mtt*ABC operon (Berks, 1996; Santini et al., 1998; Weiner et al., 1998); however they are most likely not cofactor-containing redox proteins and thus are not likely exported *via* this pathway. Interestingly, ΔInv was functional even when fused to fairly large polypeptides; over one-half of the gene fragments fused to Δ*inv* encode greater than 165 amino acids.

CHAPTER #3: Manuscript #2

Salmonella SsrB Activates a Global Regulon of Horizontal Acquisitions

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Abstract

Salmonella enterica is a bacterial pathogen of humans that can proliferate within epithelial cells as well as professional phagocytes of the immune system. This ability requires a *S. enterica* specific locus termed *Salmonella* pathogenicity island 2 (SPI-2). SPI-2 encodes a type III secretion system that injects effectors encoded within the island into host cell cytosol to promote virulence. SsrAB is a two component regulator encoded within SPI-2 that was thought to exclusively activate SPI-2 genes. Here it is shown that SsrB in fact activates a global regulon. At least ten genes outside of SPI-2 are SsrB regulated within epithelial and macrophage cells. Nine of these ten SsrB regulated genes outside of SPI-2 reside within previously undescribed regions of the *Salmonella* genome. Most share no sequence homology with current database entries. However, one is remarkably homologous to human glucosyl ceramidase, an enzyme involved in the ceramide signaling pathway. The SsrB regulon is modulated by the two component regulatory systems PhoP/PhoQ and OmpR/EnvZ, and its expression is strictly limited to the intracellular micro-environment.

Introduction

Salmonella enterica is a gram negative bacterial pathogen that can infect a range of hosts including reptiles, birds and mammals. *S. enterica* infection can result in diseases ranging from self limiting gastroenteritis to frequently fatal typhoid fever. Many of the virulence traits of *S. enterica* as well as other bacterial pathogens are attributable to pathogenicity islands. Pathogenicity islands are discrete chromosomal regions that harbor virulence genes that are absent from related non-pathogenic species. Some of these islands harbor type III secretion systems. These elaborate devices are composed of 20-25 membrane proteins that can act as a 'molecular syringe', injecting proteins directly from the bacterial cytoplasm into host cell cytoplasm. The injected effectors subvert cytoplasmic components of host cells to promote virulence. Pathogenicity islands that encode type III secretion systems are essentially virulence cassettes; encoding the structural components of a type III secretion system, as well as an array of cognate effectors and chaperones (Groisman and Ochman, 1996; Galyov et al., 1997; Hacker et al., 1997; Hueck, 1998). These systems were thought to be encoded entirely within contiguous blocks of DNA. However, recently two *S. enterica* effectors were discovered that are not physically linked to their export apparatus (Hardt et al., 1998; Hong and Miller, 1998).

Escherichia coli is the nearest non-pathogenic relative of *S. enterica*; the two diverged approximately 100 million years ago, and retain 90% homology at the gene level (Groisman and Ochman, 1996). Many of the virulence properties that distinguish *S. enterica* from *E. coli* are attributable to two 40Kb *Salmonella* islands termed *Salmonella* pathogenecity island 1 (SPI-1) and *Salmonella* pathogenecity island 2 (SPI-2) (Galan et al., 1992; Galan and Ginocchio, 1994; Groisman and Ochman, 1996; Hensel et al., 1998; Uchiya et al., 1999). Several smaller species specific clusters of virulence genes have been identified and termed pathogenicity islets (Groisman and Ochman, 1997). SPI-1 and SPI-2 encode distinct type III secretion systems. SPI-1 is an older genetic acquisition and confers upon *S. enterica* the ability to invade epithelial cells and invoke the inflammatory response (Galyov, et al., 1997; Galan et al., 1992; Groisman and Ochman, 1993) – important components of the early, intestinal phase of disease. SPI-2 allows *S. enterica* to proliferate intracellularly (Ochman et al., 1996; Cirillo et al., 1998; Hensel et al., 1998) and is involved in the late, systemic stages of infection (Baumler et al., 1998).

Pathogenicity island gene expression is generally limited to specific host compartments. Pathogenicity islands often 'plug' in to endogenous two component housekeeping regulators to 'sense' where they are within a host, and regulate gene expression appropriately. Many pathogenicity islands achieve even greater control over their expression by encoding their own transcriptional regulators. Until recently, these regulators were thought to

exclusively activate genes within the islands in which they reside (Morschhauser et al., 1994; Gomez-Duarte and Kaper, 1995; Deiwick et al., 1998; Ahmer and Heffron, 1999; Ahmer et al., 1999a).

SsrAB is a two component regulator encoded within SPI-2 that is required for SPI-2 gene expression (Cirillo et al., 1998; Hensel et al., 1998). SsrA is a sensor kinase and SsrB is a transcriptional activator. The environmental cue sensed by SsrA that results in SPI-2 gene expression is not known (Deiwick et al., 1999). Until now, SsrB was assumed to exclusively activate genes within SPI-2.

Because the adapter regulator of SPI-1 regulates a SPI-1 effector which is not physically linked to SPI-1 (Ahmer et al., 1999b), we hypothesized that SsrB may play a broader role in *S. typhimurium* pathogenesis than merely activating genes within SPI-2. To address this hypothesis, we performed a global screen for genes outside of SPI-2 that are activated by SsrB. We report here that within host cells, SsrB activates an extensive global regulon of genes within previously undescribed horizontal acquisitions that are unlinked to SPI-2.

Results

Identification of SsrB-regulated genes outside of SPI-2

To identify SsrB regulated genes outside of SPI-2, the strategy depicted in Figure 1 was employed. The chromosomal copy of *S. enterica* serovar *typhimurium* 14028s (*S. typhimurium*) *ssrB* was disrupted with a chloramphenicol cassette through allelic exchange. A plasmid that contains *ssrB* under the control of a tightly regulated arabinose promoter was then introduced into this strain, yielding strain MJW141. Growth of MJW141 in the presence of arabinose results in expression of SsrB whereas growth in its absence does not. This strain was mutagenized with MudJ (see Experimental procedures), generating *lacZY* promoter fusions throughout the genome.

20,000 MJW141::MudJ mutants were patched in grids onto M9 glucose plates versus M9 arabinose plates. These plates were supplemented with the colorimetric ß-galactosidase indicator X-gal, allowing for the observation of changes in ß-galactosidase activity. 288/20,000 mutants screened in this manner were determined to be differentially regulated on arabinose plates versus glucose plates. One half of these mutants (144) were characterized further. Fusions whose phenotype was attributable to secondary mutations, vector sequence, arabinose regulation, and fusions within SPI-2

were eliminated from this collection (see Experimental procedures), leaving approximately 100 fusions for further study.

Molecular characterization

Chromosomal DNA flanking ten arbitrarily chosen SsrB regulated fusions was obtained and characterized. Sequence analysis indicates that all but one of these fusions reside within previously undescribed regions of the *S. typhimurium* genome. The sequence characteristics of the chromosomal area surrounding each of these insertions is diagrammed in Figure 2 and described below.

Three horizontal acquisitions that contain multiple genes were explored (Fig. 2). Horizontal acquisition of these genes is suggested through the presence of mobile element remnants which are frequently associated with horizontally acquired DNA (Groisman and Ochman, 1996); and also by a GC content that does not reflect the genome average, suggesting that the loci evolved in a different organism (Lawrence and Ochman, 1997). Horizontal acquisition is further suggested by association with tRNA genes, which are a preferred integration site for some bacteriophages (Cheetham and Katz, 1995) and finally by the lack of an *Escherichia coli* homolog, suggesting that the encoded proteins do not fulfill housekeeping roles (Groisman et al., 1993).

In the first of three multi-gene horizontal acquisitions described here (Fig. 2), an SsrB regulated gene was found within a *S. enterica* specific chromosomal area 7Kb in length, flanked by a housekeeping gene on each side (Fig. 2). The complete sequence of this area was determined (Genbank accession # TBA) which revealed the presence of three genes that appear to form an operon based on gene spacing. The GC content of this area is 56%, significantly higher than the genome average of 52% (Neidhardt, 1996; Lawrence and Ochman, 1997). The first gene (5' to 3') in this horizontal acquisition, *srlA* (SsrB regulated loci), shares modest homology with nuclear antigens ($P=10^{-7}$), and may be secreted to effect nuclear transcription. *srlB*, the second gene, shares no homology with current database entries and does not posses any conserved functional motifs. The third gene, *srlC* also shares no homology to current database entries. SrlC is predicted to form a coiled coil domain (>80% likelihood), which suggests that it is a type III secretion system substrate (Pallen et al., 1997).

Two distinct SsrB regulated genes were found within the second multi-gene horizontal acquisition described in this report (Fig 2). One of these two genes shares homology with DNA inversion systems such as the *pinB* system of *Shigella boydii* ($P=\approx10^{-15}$) (Tominaga et al., 1991). Interestingly, the MudJ insertion in *srlK* is only a few hundred base pairs away from the insertion site of a transposon (MS5076) in a previously described mutant with a macrophage survival defect (Fields et al., 1986; Baumler et al., 1994). This

phenotype coupled with activation by SsrB prompted downstream sequence analysis. Downstream, a horizontal acquisition over 7Kb in length was discovered. Intriguingly, the *Salmonella* O antigen acetylase (*oafA*) (Slauch et al., 1996) is embedded within this island and appears to be flanked by the putative inversion system (unpub. results).

Downstream of *oafA*, but within the same horizontal acquisition is the site of a second SsrB regulated fusion (*srlE*), within an *msgA* (macrophage survival gene) homolog ($P=10^{-9}$) (Fig 2). MsgA mediates both macrophage survival and mouse virulence (Gunn et al., 1995). *srlE* is followed by a tRNA gene. This 7kb horizontal acquisition has a GC content of 42%, significantly less than the genome average of 52% (Neidhardt, 1996; Lawrence and Ochman, 1997). The involvement of *srlK* in macrophage survival, and *oafA* in mediating host-pathogen interactions (Fields et al., 1986; Baumler et al., 1994; Slauch et al., 1995; Slauch et al., 1996) establish this area of the genome as a pathogenicity islet.

In the third multi-gene horizontal acquisition discovered (Fig. 2) an SsrB regulated fusion (*srlH*) was found within a homolog of *S. typhimurium sspH1* ($P=10^{-36}$) and *slrP* ($P=10^{-19}$) (Fig. 3). *sspH1* and *slrP* are homologs of *ipaH* and *yopM* which are type III exported effectors from *Shigella flexneri* and *Yersinia pseudotuberculosis* respectively (Fig. 3) (Miao et. al, 1999; Tsolis et. al., 1999). Downstream of *srlH* are mobile elements similar to those present in the enterohemorrhagic *E. coli* (EHEC) locus of enterocyte

effacement (Lee) pathogenicity island ($p=10^{-46}$ to orf L0015), suggesting similar ancestry, followed by another *msgA* (macrophage survival gene) homolog ($P=10^{-15}$). Interestingly, the area around this *msgA* homolog contains the attachment site for the Gifsy-2 prophage. Sequence analysis revealed that the entire horizontal acquisition described above is completely contained within a previously undescribed region of Gifsy-2 (Fig. 2). This fully functional prophage was recently determined to carry the *sodC* gene and to be important for *S. typhimurium* virulence (Figueroa-Bossi and Bossi, 1999).

The DNA sequence surrounding another MudJ insertion analyzed is homologous to a human enzyme involved in intracellular signaling. *srlJ* has no DNA homology to any entries in the database but shares remarkable amino acid homology with human lysosomal glucosyl ceramidase ($P=10^{-52}$). This enzyme is involved in the eukaryotic ceramide signal transduction pathway. Ceramide generation, which is presumably being promoted by SrlJ, is linked to diverse cellular responses such as cell cycle arrest, apoptosis and the induction of inflammatory responses (Hannun and Obeid, 1995; Hannun, 1996; Perry and Hannun, 1998). A clone containing the full length *S. typhimurium* gene was obtained and the sequence determined (Genbank accession # TBA). A Clustal alignment of the human and *S. typhimurium* proteins is displayed in Figure 4, which highlights their striking similarity.

Five additional distinct SsrB regulated genes outside of SPI-2 were identified: *srlI*, *srlD*, *srIG srlL*, and *srlM*. All of these genes reside within

previously undescribed regions of the genome, sharing no homology with database entries. The sequence surrounding these insertions is depicted in Figure 2.

Intracellular activation by SsrB

All of the fusions reported here were identified by expressing SsrB from a plasmid with a strong inducible promoter. This was necessary because SsrB is not normally active outside of eukaryotic cells. It was not clear if expressing a non phosphorylated form of SsrB would faithfully mimic the affects of its normal phosphorylation within cells. Therefore, to ascertain if the regulation observed was physiologically relevant, the ability of a chromosomal copy of *ssrB* to affect the expression of the identified genes, within eukaryotic cells, needed to be assessed.

Each *lacZ* fusion described above was moved into a wild type strain and into an *ssrB* mutant strain. These strains were used in parallel to infect cell culture and the ß-galactosidase activity of the intracellular bacteria measured (see Experimental procedures). A chromosomal copy of *ssrB* activated every fusion within either epithelial or macrophage cells (Table 1). The level of *ssrB* mediated activation ranged from 2 to 80 fold. Interestingly, SsrB preferentially activated some genes within macrophages versus epithelial cells – most notably, *srlK*, shown in Figure 5.

Environmental specificity of regulation

To determine the specificity of SsrB regulation to the intracellular micro-environment, the ratio between the level of expression of genes in a wild type background versus in an *ssrB::cm* background outside of host cells was determined, and compared to the same ratio determined when the bacteria were within host cells (Table 1.). The extracellular ratio for the three most highly regulated fusions was ≤1.32. By comparing these extracellular ratios with the ones determined within cells (Table 1), it was concluded ≥99.96% of the regulatory activity of SsrB upon its regulon is confined to the intracellular microenvironment (Figure 6).

SsrB mediated activation within murine spleen

In addition to the cell culture induction experiments, the ability of SsrB to activate genes *in vivo*, at systemic sites of infection was also tested. Mice were intravenously injected with wild type *S. typhimurium* carrying the *srlH* fusion (the fusion that produced the most ß-galactosidase activity in cell culture) and the *ssrB* mutant strain carrying the same fusion. One day after infection, ß–galactosidase activity was measured from spleen homogenates. As can be seen in Figure 7, a chromosomal copy of *ssrB* activates *srlH* within murine spleen 166 fold.

OmpR and PhoP regulation

ompR and *phoP* both encode transcription factors that are part of the two component regulatory systems OmpR/EnvZ and PhoP/PhoQ respectively. *Salmonella phoP* is known to regulate virulence genes and is required for survival within macrophages (Fields et al., 1989; Miller et al., 1989). *Salmonella ompR* mutants are unable to kill macrophages, however the basis for this phenotype is not clear (Lindgren et al., 1996; van der Velden et al. 1999). The three most highly transcribed SsrB regulated genes were tested for *ompR* and *phoP* regulation (see Experimental procedures). *srlH*, *srlJ* and *srlK* expression was significantly affected inside of macrophages by chromosomal alleles of both *ompR* and *phoP* (Figure 8).

Discussion

This study describes the discovery of a global regulon in *S. typhimurium* that is activated exclusively within eukaryotic cells. All members of this regulon are known (*srlK* and SPI-2) or putative virulence factors. Surprisingly, this global regulon is activated by SsrB, a regulator encoded within SPI-2. Until recently, regulators within pathogenicity islands were only thought to activate genes within the locus in which they are encoded. The complexity of genetic cross-talk between horizontal acquisitions revealed here is unprecedented. Several areas of the genome identified have interesting characteristics that may have implications for understanding *S. typhimurium* pathogenesis.

Within one previously undescribed pathogenicity islet, a MudJ inserted into what appears to be a site-specific recombination system (*srlK*). Such systems are sometimes used by bacteria to control gene expression (Neidhardt, 1996). Thus, it was interesting to find *oafA*, flanked by this putative *S. typhimurium* inversion system. OafA is an LPS modification enzyme that acetlyates the O-antigen. This acetylation affects a large conformational change, creating and destroying a series of epitopes (Slauch et al., 1996). Intriguingly *srlK* was preferentially activated by SsrB in macrophages versus epithelial cells. It can be inferred from this data that SsrB is activated by a generic intracellular cue and that at least one additional cell-

type-specific signal is integrated to distinguish macrophage cells from epithelial cells.

While the implications for gene regulation are interesting, the biological implications of SsrB activating the inversion system also deserve consideration. The cell envelope protects *S. typhimurium* within macrophages from a variety of bactericidal mechanisms. SsrB may activate *oafA* within macrophages, via the inversion system, to trigger a protective LPS conformational change. However, outside of cells, this protective conformation might expose immunogenic regions of LPS to the immune system. This scenario would explain why *ssrB* is required but not sufficient for *oafA* activation. The inversion system may also provide an explanation for the enigmatic observation that two distinct populations of *S. typhimurium* exist within macrophages (Buchmeier and Heffron, 1991; Abshire and Neidhardt, 1993; Alpuche-Aranda et al., 1994). This inversion system is currently being characterized at a molecular level, and the biological implications of its activation assessed.

The second horizontal acquisition described in this report contains the most highly regulated locus obtained, *srlH*. *srlH* is activated nearly 100 fold by SsrB in both cultured epithelial and macrophage cells. *In vivo*, *srlH* was found to be 166 fold activated by SsrB in murine spleen. This high level of activation within multiple cell types makes the biological function of the encoded protein of interest. Sequence analysis indicates that the MudJ in this

strain inserted roughly in the middle of a sequence that shares homology with *sspH1* and *slrP* (Fig. 3). *sspH1* was recently discovered to be an effector for SPI-2 (Miao et. al.) and *slrP* was recently identified as a virulence gene in a signature tagged transposon screen (Tsolis, R.M. et. al., 1999). Both *slrP* and *sspH1* are homologous to *ipaH* and *yopM*. These two proteins are type III exported effectors from *S. flexeneri* and *Y. pestis* respectively. YopM has been described as binding human alpha-thrombin and inhibiting platelet aggregation (Leung et al., 1990). However, a recent report suggests that YopM is targeted to the nucleus (Skrzypek et al., 1998). A function has not yet been ascribed to IpaH. It is tempting to speculate SlrP, SspH1 and SrlH form a family of *S. typhimurium* type III secreted effectors.

It was interesting that *srlH* and the surrounding area described in this report reside within an uncharacterized region of the prophage Gifsy-2 (Figure 2). Gifsy-2 is one of at least two fully functional prophages present on the *S. typhimurium* chromosome (Figueroa-Bossi and Bossi, 1999). Bacteria that have been cured for Gifsy-2 are significantly attenuated in their ability to promote disease in the murine model of Salmonellosis (Figueroa-Bossi and Bossi, 1999). This virulence defect is partly attributable to the *sodC* gene that is harbored by Gifsy-2 (Figueroa-Bossi and Bossi, 1999). SodC is a Cu^{2+}, Zn^{2+} superoxide dismutase implicated in bacterial defences against the microbicidal activities of macrophages (De Groote et al., 1997; Farrant et al., 1997). However, the Gifsy-2 borne *sodC* can not completely account for the

virulence defect of curing *S. typhimurium* for Gifsy-2 (Figueroa-Bossi and Bossi, 1999). The alleles described here, *srlH* and the *msgA* homolog may thus contribute to the virulence defect imparted through the absence of Gifsy-2.

It is significant that members of the SsrB regulon are regulated by both *ompR* and *phoP*. *phoP* is global housekeeping regulator present in both *E. coli* and *S. typhimurium*. In *S. typhimurium*, in addition to regulating housekeeping genes, *phoP* also activates numerous virulence genes that are necessary for intramacrophage survival (Fields et al., 1989.; Miller et al., 1989). Like *phoP*, *ompR* is present in both *E. coli* and *S. typhimurium*. In both organisms, in response to osmolarity changes, OmpR activates porin gene expression. *S. typhimurium ompR* mutants are unable to kill macrophages and are also defective in *Salmonella* induced filament (SIF) formation (Mills et al., 1998). This study reveals that PhoP, OmpR and SsrB control overlapping regulons that include *Salmonella* specific genes. OmpR was not previously appreciated to play a role in coordinating gene expression in response to the intracellular state. Members of the SsrB regulon are currently being tested for their ability to induce macrophage death and SIF formation.

One of the most interesting genes regulated by SsrB outside of SPI-2 is *srlJ*. *srlJ* shares high homology with human glucosyl ceramidase. This enzyme can increase intracellular levels of free ceramide as part of a lipid

signaling pathway implicated in several fundamental cell biology processes. The most noteworthy are the activation of JNK kinases and phosphatases, leading to apoptosis and the induction of an inflammatory response (Hannun and Obeid, 1995; Hannun, 1996; Perry and Hannun, 1998). SrlJ does not posses a classical amino terminal signal sequence, raising the possibility that it is a substrate of the SPI-2 type III export system. However, SrlJ could also be exported by an ABC transporter or alternatively SrlJ may affect host cell ceramide levels from within the bacterial cytoplasm. The location, induction of ceramide production, and the biological implications thereof, are currently being assessed and will be presented elsewhere.

In addition to understanding the genetic coordination of virulence and *S. typhimurium* pathogenesis, this study may also have implications for vaccine design. In recent years, bacterial pathogens have been systematically attenuated and used to express heterologous antigens to engender protective immune responses against multiple pathogens simultaneously. *Salmonella* is being extensively manipulated for this purpose because it can replicate and persist within professional antigen presenting cells, and can stimulate CD8, Th1 and Th2 responses. One of the challenges in using attenuated pathogens as vaccine carriers is confining heterologous gene expression to the interior of host cells. *srlH* is almost 100 fold induced upon entering eukaryotic cells, and can drive the production of nearly 500 Miller units of ß-galactosidase activity within host cells. This fusion is so strongly and specifically expressed, it will

almost certainly be an improvement over the promoters currently being used to drive heterologous protein expression in vaccine strains. Of additional interest, *srlK* is specifically induced within macrophages. The ability to limit heterologous antigen expression to the interior of professional antigen presenting cells may allow modulation of the nature of the response elicited.

The SsrB regulon extends significantly beyond the SPI-2 locus and the ten genes reported here. Ten arbitrarily chosen fusions were characterized here, however, nearly 100 independent, re-transduced fusions that are unlinked to SPI-2 were identified as being regulated by plasmid encoded SsrB. We are currently delineating the remainder of the SsrB regulon and determining the roles of these genes in intracellular virulence.

Experimental procedures

Bacterial strains and media

Bacteria were grown in Luria-Bertani (LB) broth (Difco) unless indicated otherwise. M9 media contained per liter: 11g of Na_2HPO_4 X 7 H_2O, 3g of KH_2PO_4, 0.5 g of NaCl and 1g of NH_4CL, 2mM $MgSO_4$, 0.1mM $CaCl_2$, 40μg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal), 0.2% glucose or arabinose, and carbenicillin. Antibiotics were used at the following concentrations: kanamycin, 60μg/ml; carbenicillin, 200μg/ml; streptomycin, 100μg/ml and chloramphenicol, 30μg/ml. Cell culture was maintained in Dulbecco's modified Eagles medium (DMEM), supplemented with 10% fetal calf serum, sodium pyruvate and non essential amino acids in a standard 37°C water jacketed tissue culture incubator with 100% humidity. All cell culture reagents were purchased from Gibco-BRL.

The strains used in this study are described in Table II. All transductions were performed with P22 HT *int* as previously described (Maloy et al., 1996). All transductants were streaked to isolation two times in the presence of 10mM EGTA. Smooth LPS and the lack of pseudolysogeny were confirmed by cross-streaking transductants against P22 on Evan's Uranine plates (Maloy et al., 1996).

Molecular biology techniques

All enzymatic manipulations of DNA were performed using established techniques (Ausubel et al.; Sambrook et al., 1989) with reagents purchased from Boehringer Mannheim (restriction endonucleases and alkaline phosphatase), New England Biolabs (T4 DNA ligase) and Stratagene (pfu DNA polymerase). Southern hybridization analysis was performed with the NEN Renaissance® kit manufactured by DuPont, according to the manufacturer's directions. Plasmid DNA for sequencing was isolated with ion exchange columns from Qiagen®. Oligonucleotide synthesis was performed by Applied Biosystems automated solid-phase synthesis at the OHSU Microbiology Core facility. Our departmental core facility also performed DNA sequence determination, using an Applied Biosystems 377 fluorescent DNA sequencer with chain terminator chemistry.

Inactivating *ssrB*

ssrB was PCR amplified with native Pfu DNA polymerase from the *S. typhimurium* 14028s genome with primers 5'CTTAATTTTCGCGAGGGCAGC 3' and 5'TAGAATACGACATGGTAAAGCCCG3', and ligated into the cloning vector pCR-Blunt (Invitrogen), yielding pMJW92. The chloramphenicol acetyl transferase (cat) cassette was released from pCMXX with SmaI digestion and

cloned into a unique SspI site in pMJW92, disrupting the cloned *ssrB* allele. *ssrB::cat* was released from the vector with EcoRI digestion and ligated into the EcoRI site of pKAS32 in SM10λpir (Skorupski and Taylor, 1996), yielding pMJW114. MJW114 was mated with MJW104 (14028s *rpsL*, nal) and a double cross over at the *ssrB* allele was selected with strep and cm – the dominant strep sensitivity allele carried on pKAS32 ensured that only double cross overs were obtained (Skorupski and Taylor, 1996). The disrupted chromosomal *ssrB* allele was transduced into wild type *S. typhimurium* 14028s with P22 HT *int* (Maloy et al., 1996) producing MJW129. The properties of this strain were confirmed by both PCR and Southern blot analysis (data not shown).

Creating an inducible *ssrB* allele

A cloned *ssrB* allele was released from pMJW92 (described above) with EcoRI digestion and ligated into the EcoRI site of pBAD30 generating pMJW120. The pBAD series of vectors allows for the tight regulation of cloned genes via the arabinose inducible pBAD promoter (Guzman et al., 1995). The proper orientation of *ssrB,* (under the control of the pBAD promoter) was determined by both restriction endonuclease and sequence analysis using primer 5' TACCCGTTTTTTTGGGCTAGCG 3' which was designed to match the pBAD30 vector. pMJW120 was electroporated into

MJW129 (described above) creating MJW141. All SsrB regulated fusions were originally derivatives of this strain.

Generation of transcriptional fusions

lacZ transcriptional fusions were generated throughout the MJW141 chromosome with a previously described genetic system (Hughes and Roth, 1988). Briefly, P22 HT *int* was grown on TT10288 and a high titer lysate obtained. TT10288 contains two prophages on its chromosome, MudI and MudJ, which cannot simultaneously be packaged by P22. MudJ is defective for transposition, however it can be complemented in cis by the proximal end of MudI which can be packaged along with MudJ by P22. When such a fragment is introduced into a new cell, the MudJ can hop once into the chromosome. The Km marker and the promoterless *lacZ* allele of MudJ thus allow the easy generation of *lacZ* transcriptional fusions throughout the *S. typhimurium* chromosome.

Screening for SsrB regulated genes

MJW141 was infected with a TT10288 P22 lysate at a low MOI and plated onto M9 plates supplemented with kanamycin and carbenicillin. 20,000 colonies containing independent *lacZ* transcriptional fusions were

obtained and patched with toothpicks onto M9-glucose plates supplemented with 4mg/ml X-gal versus M9-arabinose, also supplemented with 4mg/ml X-gal. Plates were incubated for three days at 37°C and differential color development was noted on each day. All MJW141::MudJ mutants that showed altered ß-galactosidase activity on M9-arabinose plates versus M9-glucose plates were streaked to isolation, transduced back into MJW141, and their phenotype confirmed.

Excluding fusions that respond to vector sequence or arabinose

To exclude fusions from this collection that were regulated by vector sequences or arabinose, in lieu of SsrB, 144 of the original 288 regulated fusions were transduced into MJW136 (the 800 series). This strain is isogenic to MJW141 except pBAD30 in this strain does not contain the *ssrB* allele. Because this strain does not carry the *ssrB* allele, fusions that continued to be expressed differentially on M9-arabinose versus M9-glucose could not be SsrB regulated. These strains, roughly one-third of the total, were discarded.

Excluding SPI-2 genes from the bank

Co-transductions were used to genetically eliminate fusions within SPI-2 from the mutant collection. P22 HT *int* was grown on each strain in the 400

series and used to infect *S. typhimurium* carrying no markers. If a P22 lysate of a strain could be used to transduce 14028s to both chloramphenicol (from *ssrB*) and kanamycin (from MudJ) resistance, it was assumed that the MudJ in this strain was within SPI-2, and the strain was not studied further.

Fusion junction sequence determination

The fusion junctions of 10 SsrB regulated fusions unlinked to SPI-2 were determined. Chromosomal DNA was isolated as previously described (Ausubel et al.,). The DNA was digested with the restriction endonuclease SalI, and ligated with T4 DNA ligase to SalI digested and dephosphorylated pWKS29 (Wang and Kushner, 1991). The ligations were transformed into *E. coli* and MudJ-containing transformants were selected on LB plates supplemented with kanamycin. The DNA sequence of the fusion junctions as well as the flanking DNA was obtained by sequencing with the primer 5' CTACAGGCTTGCAAGCCCCAC 3' which was designed to the end of the MudJ left end.

Sequence analysis

Sequence analysis was performed with the computer programs MacVector® 6.0 and Assemblylign from Oxford Molecular Group, the suite of

molecular biology algorithms available from the National Centers for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov/, the bacterial blast server from the Genome Sequencing Center at Washington University in St. Louis at http://genome.wustl.edu/gsc/bacterial/bacterial_blast_server.html and ftp://genome.wustl.edu/pub/gsc1/sequence/st.louis/bacterial/salmonella/ as well as the Sanger Centre *Salmonella typhi* blast server at http://www.sanger.ac.uk/Projects/S_typhi/blast_server.shtml. Several of the inserts were primer walked with numerous custom oligonucleotides, the sequences of which are available from the authors upon request. Protein sequence analysis (deduced from DNA sequences) was analyzed for conserved motifs with the prosite and coils algorithms at http://www.expasy.ch/tools/scnpsite.html and http://www.ch.embnet.org/software/COILS_form.html.

Physiological relevance/intracellular induction

Two new strains were created for each fusion to determine if a chromosomal copy of *ssrB* results in the activation of these fusions within eukaryotic cells. Each fusion was transduced into *S. typhimurium* 14028s and MJW129 (14028s *ssrB::cm*). Because SsrB is not active outside of cells, the ß-

galactosidase activity of each pair of strains was determined inside both HeLa (epithelial) cells and J774A (macrophage) cells.

Cell culture was grown to near confluency in 6 well plates. Monolayers were infected with standing cultures grown overnight in Luria Bertani (LB) broth, at an MOI of approximately 50:1. Bacteria were centrifuged onto the monolayers for 10 minutes at 1,000 rpm in a Sorvall table top centrifuge at ambient temperature. The plates were then incubated for 1 hour in a tissue culture incubator. After one hour, the cell culture was washed two times with 37°C DMEM without growth supplements. The cell culture was then overlaid with 37°C tissue culture media supplemented with 100μg/ml gentamicin, an antibiotic that selectively kills extracellular bacteria, and incubated for two hours. The cell culture was then washed two times with 37°C DMEM, overlaid with 37°C tissue culture media containing 10μg/ml gentamicin, and incubated for an additional four hours. The cell culture was then washed two times with 37°C DMEM and lysed with 1% triton X-100 in PBS to release intracellular bacteria. The released bacteria were pelleted and resuspended in 1ml of PBS. A ß-galactosidase activity was performed on 500μl of the resuspended bacteria, as previously described (Miller, 1992). The number of intracellular bacteria was determined by plating serial dilutions. ß-galactosidase units were determined per intracellular bacteria in lieu of the OD_{600} value normally used. Although *ssrB* mutants have an intracellular survival defect, *S. typhimurium* does not grow significantly inside cells in the first 6 hours, thus bacteria were

recovered in similar numbers. The level of induction dependent upon an intact chromosomal copy of *ssrB* was determined by simply dividing the intracellular ß-galactosidase activity for each fusion in the wild type background by the activity in the *ssrB::cm* background.

SsrB mediated activation within murine spleen

Female Balb/C mice (Jackson laboratories) that were approximately 8 weeks old were intravenously injected with wild type *S. typhimurium* carrying the *srlH* fusion and the *ssrB::cm* strain carrying the same fusion. The inoculum for both strains was 10^5. One day after infection, spleen homogenates from mice infected with each strain were assayed for ß–galactosidase activity, and the levels compared. The ß-galactosidase assay was performed as previously described (Miller, 1992), except the units were expressed per cfu recovered.

Determination of the specificity of SsrB regulation

The specificity of SsrB activity to the intracellular environment was determined. Each fusion in the wild type background, and in the *ssrB::cm* background were grown in parallel in tissue culture media with growth supplements, for six hours, and concentrated 10-fold. ß-galactosidase activity

and dependence on *ssrB* for induction was determined as described above. The ability of SsrB to activate genes in tissue culture media was then compared with the ability of SsrB to activate genes in macrophages (Table I).

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Figure 1. Strategy for identifying *ssrB* regulated genes.

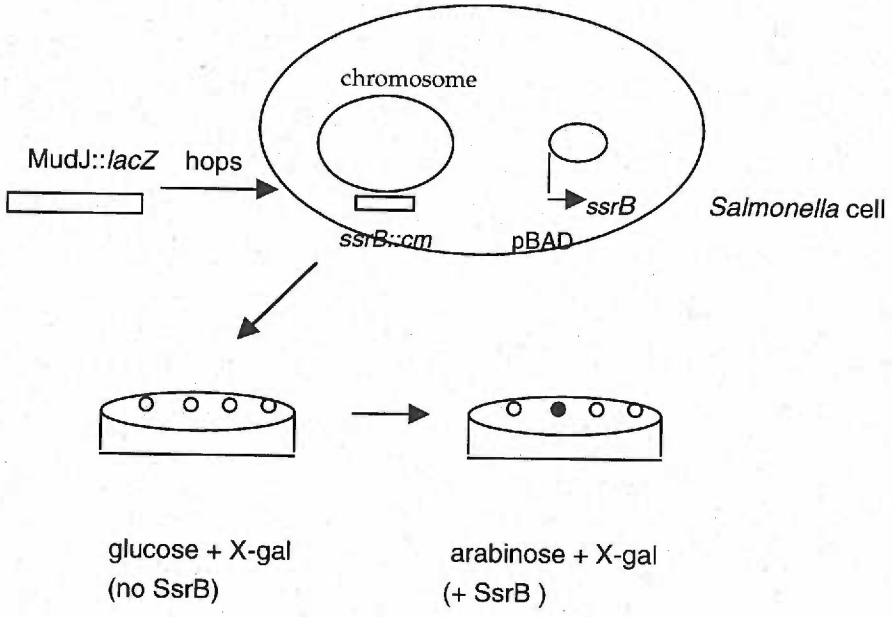

Figure 1. Strategy for identifying SsrB regulated genes. A plasmid that contains a copy of *ssrB* under the control of an arabinose inducible promoter was introduced into a strain deleted for chromosomal *ssrB*. Growth of this strain with glucose as the sole carbon source results in no SsrB being produced; whereas, growing this strain with arabinose as the sole carbon source results in SsrB expression. *lacZY* transcriptional fusions were generated throughout the genome of this strain as previously described (Hughes and Roth 1988). 20,000 independently isolated clones bearing *lacZY* promoter fusions were patched in grids onto plates containing M9-glucose versus M9-arabinose. The plates were supplemented with the colorimetric ß-galactosidase indicator X-gal, allowing for the observation of differential regulation. Fusions whose phenotype was attributable to secondary mutations, vector sequence, arabinose regulation, and fusions within SPI-2 were eliminated from this collection (see Experimental procedures).

Figure 2. Maps of horizontal acquisitions in the SsrB regulon.

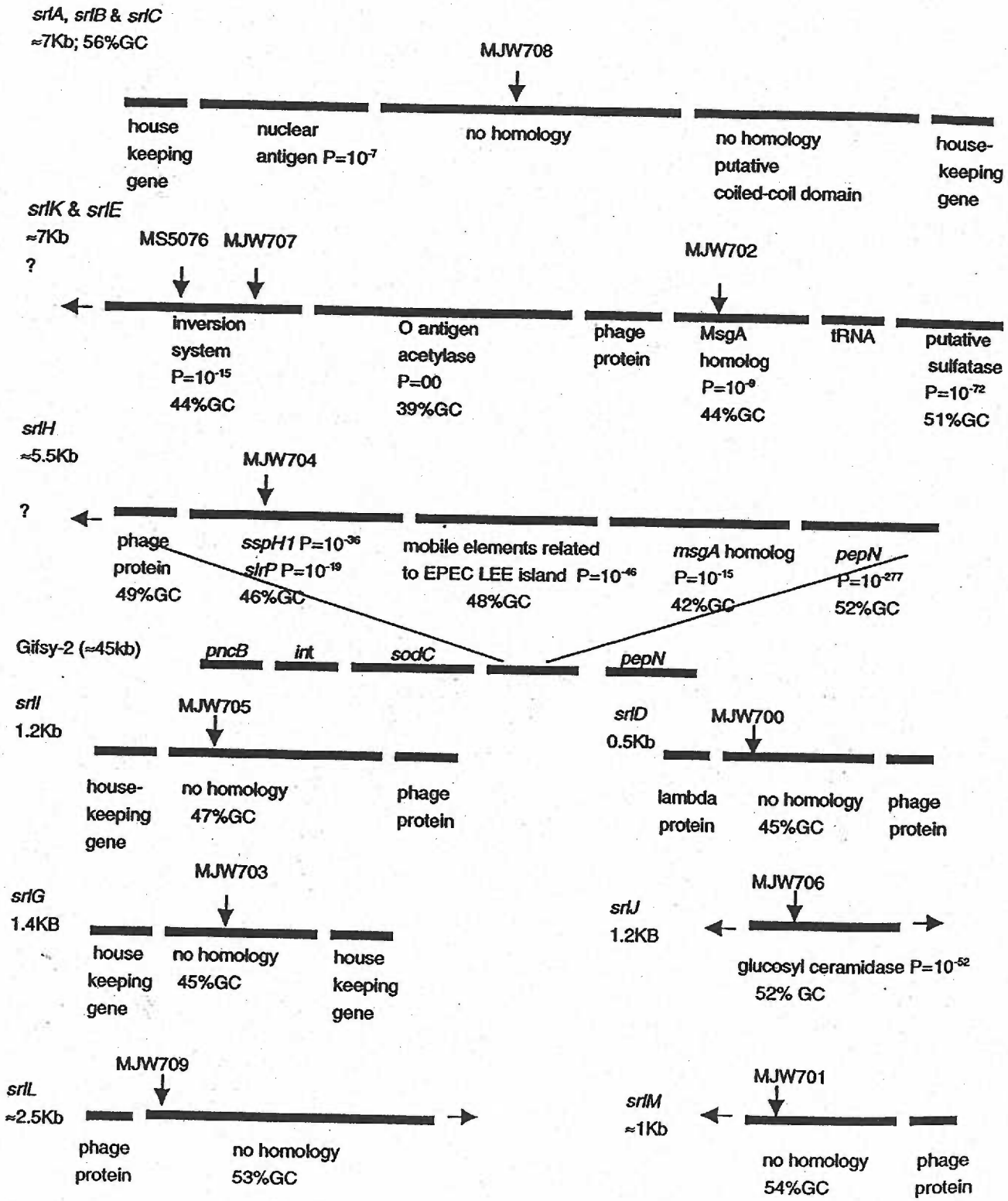

Figure 2. Maps of horizontal acquisitions in the SsrB regulon. Maps show as blocks the regions of the various horizontal acquisitions identified. Transposon insertion points are designated with arrows. Gene identities, homologs, and GC contents are shown under the blocks. The borders are designated if they are known. The maps are approximately drawn to scale, except for Gifsy-2. Additional details can be found within the text. MS5076 designated in *srlK* is the site of a previously isolated transposon insertion that resulted in a macrophage sensitive strain (Fields et al., 1986; Baumler et al., 1994). The Gifsy-2 map was adapted from (Figueroa-Bossi and Bossi, 1999).

Figure 3. Clustal alignment of SrlH, SlrP and SspH1.

```
SlrP      1  MFNITN--IQSTARHQSISNEASTEVPLKEEIWNKISAFFSSEHQVEAQNCIAYLCHPPETASPEEIKSKFECL  72
SrlH      1 MPFHIGSGCLPAIISNRRIYRIAWSDTPPEMSSWEKMKEFFCSTHQAEALECIWTICHPPAGTTREDVVSRFELL  75
SspH1     1  MFNIRN--TQPSVSMQAIAGAAAPEASPEEIVWEKIQVFFPQENYEEAQQCLAELCHPARGMLPDHISSQFARL  72
                *.*          . *   *  .       * *. **   . ** *.  .***      . . *.*  *

SlrP     73 RMLAFPAYADNIQYSRGGADQYCILSENSQEILSIVFNTEG-YTVEGGGKSVTYTRVTESEQ-------ASSASG 139
SrlH     76 RTLAYDGWEENIHSGLHGENYFCILDEGSQEILSVTLDDVGNYTVNCQGYSETHHLTMATEP------------ 137
SspH1    73 KALTFPAWEENIQCNRDGINQFCILDAGSKEILSITLDDAGNYTVNCQGYSEAHDFIMDTEPGEECTEFAEGASG 147
            . *..    .**.    *   .***  .*.****.     * ***   * * .      .*

slrP    140 ---------SKDAVNYELIWSEWVKEAPAKEAANREEPVQRMRDCLKNNKTELRLKILGLTTIPAYIPEQITTLI 205
SrlH    138 ------------------------------------------------------------------------- 137
SspH1   148 TSLRPATTVSQKAAEYDAVWSKWERDAPAGESPGRAAVVQEMRDCLNNGNPVLNVGASGLTTLPDRLPPHITTLV 222

SlrP    206 LDNNELKSLPENLQGNIKTLYANSNQLTSIPATLPDTIQEMELSINRITELPERLPSALQSLDLFHNKISCLPEN 280
SrlH    138 ------------------------------------------------------------------------- 137
SspH1   223 IPDNNLTSLPELPEGLRELEVSGNLQLTSLPS----------------------LPQGLQKLWAYNNWLASLPT- 274

SlrP    281 LPEELRYLSVYDNSIRTLPAHLPSEITHLNVQSNSLTALPETLPPGLKTLEAGENALTSLPASLPPELQVLDVSK 355
SrlH    138 ------------------------------------------------------------------------- 137
SspH1   275 ---------------------LPPGLGDLAVSNNQLTSLPE-MPPALRELRVSGNNLTSLPA-LPSGLQKLWAYN 326

SlrP    356 NQITVLPETLPPTITTLDVSRNALTNLPENLPAALQIMQASRNNLVRLPESLPHFRGEGPQPTRIIVEYNPFSER 430
SrlH    138 ---------------------------------GVERTDITYN------------------------------ 147
SspH1   327 NRLTSLPEMSP----------------------GLQELDVSHNQLTRLPQSLTGLS----SAARVYLDGNPLSVR 375
                                              ..    . *

SlrP    431 TIQNMQRLMSSVDYQGPRVLVAMGDFSIVRVTRPLHQAVQGWLTSLEE---EDVNQWRAFEAEANAAAFSGFLDY 502
SrlH    148 ----------------------------------------LTSDID-------------------------- 153
SspH1   376 TLQALRDIIG---HSGIRIHFDMAGPSVPREARALHLAVADWLTSAREGEAAQADRWQAFGLEDNAAAFSLVLDR 447
                                                    *** .

SlrP    503 LGDTQNTRHPD-FKEQVSAWLMRLAEDSALRETVFIIAMNATISCEDRVTLAYHQMQEATLVHDAERGAFDSHLA 576
SrlH    154 ---------AA-------AYLEELKQNPIINN----KIMNPVGQCESLMTPVSNFMN--------EKG-FDN--- 196
SspH1   448 LRETENFKKDAGFKAQISSWLTQLAEDAALRAKTFAMATEATSTCEDRVTHALHQMNNVQLVHNAEKGEYDNNLQ 522
                          . *  * .   .               **  .*   . *.         *.* .*

SlrP    577 ELIMAGREIFRLEQIESLAREKVKRLFFIDEVEVFLGFQNQLRESLSLTTMTRDMRFYNVSGITESDLDEAEIRI 651
SrlH    197 ------------------------------------------------------------IRYRGIFIWDKPTEEIPN 214
SspH1   523 GLVSTGREMFRLATLEQIAREKAGTLALVDDVEVYLAFQNKLKESLELTSVTSEMRFFDVSGVTVSDLQAAELQV 597
                                                                          *.   *    *.

SlrP    652 KMAENRDFHKWFALWGPWHKVLERIAPEEWREMMAKRDECIETDEYQSRVNAELEDLRIADDSDAERTTEVQMDA 726
SrlH    215 KSFCSG--------WK                                                          222
SspH1   598 KTAENSGFSKWILQWGPLHSVLERKVPERFNALREKQ-ISDYEDTYRKLYDEVLKSSGLVDDT----------DA 661
            *            *

SlrP    727 ERAIGIKIMEEINQTLFTEIMENILLKKEVSSLMSAYWR   765
SrlH    223                                       222
SspH1   662 ERTIGVSAMDSAKKEFLDGLRA--LVDEVLGSYLTARWRLN 700
```

Figure 3. Clustal alignment of *S. typhimurium* SspH1, SlrP and SrlH proteins. The entire sequence of *srlH* was determined (Genbank accession number TBA) and the deduced amino acid sequence was aligned with its two homologs with the software program MacVector® 6.51. Asterisks indicate identical residues and dots denote chemically similar residues. SspH1 and SlrP are homologs of the *Y. pseudotuberculosis* and *Shigella flexneri* type III exported effectors *yopM* and *ipaH*. SspH1, SlrP and SrlH are fairly similar at their amino terminus, however SrlH is significantly shorter than SspH1 and SlrP.

Figure 4. Clustal alignment of Salmonella (SrlJ) and human glucosyl ceramidase proteins.

```
Salmonella    1                    MKGRLISSDPYRQQFLVERAVSFSHRQRDCS----------------  31
Human         1 MEFSSPSREECPKPLSRVSIMAGSLTGLLLLQAVSWASGARPCIPKSFGYSSVVCVCNAT  60
                              *.          *. .***  .   * *

Salmonella   32 ----------------------------------------ELISVLPRHALQQIDGF  48
Human        61 YCDSFDPPTFPALGTFSRYESTRSGRRMELSMGPIQANHTGTGLLLTLQPEQKFQKVKGF 120
                                                       *... *  .  *.. **

Salmonella   49 GGSFTEGAGVVFNSMSEKTKAQFLSLYFSAQEHNYTLARMPIQSCDFSLGNYAYVDSSAD 108
Human       121 GGAMTDAAALNILALSPPAQNLLLKSYFSEEGIGYNIIRVPMASCDFSIRTYTYADTPDD 180
                 **. *. * .   ..*  ..   *  *** .  .*.. *.*. *****. .*.* *.  *

Salmonella  109 LQQGRLSFSR-DEAHLIPLISGALRLNP-HMKLMASPWSPPAFMKTNNDMNGGGKLR--- 163
Human       181 FQLHNFSLPEEDTKLKIPLIHRALQLAQRPVSLLASPWTSPTWLKTNGAVNGKGSLKGQP 240
                 *    *    *    **** **.*    . *.****. *. .***. .** * *.

Salmonella  164 -RECYADWADIIINYLLEYRRHGINVQALSVQNEP----VAVKTWDSCLYSVEEETAFAV 218
Human       241 GDIYHQTWARYFVKFLDAYAEHKLQFWAVTAENEPSAGLLSGYPFQCLGFTPEHQRDFIA 300
                       **   . .*  *  * ..  *.. .***    ..      .  .. * .  *

Salmonella  219 QYLRPRLARQGMDEMEIYIWDHDKDGLVDWAELAFADEANYKGINGLAFHWYTGDHFSQI 278
Human       301 RDLGPTLANSTHHNVRLLMLDDQRLLLPHWAKVVLTDPEAAKYVHGIAVHWYLDFLAPAK 360
                 . * * **     . . . *  .  *  ** .  .*    * ..*.* ***

Salmonella  279 QYLAQ---CLPDKKLLFSEGCVPMES-DAGSQIRHWHT---YLHDMIGNFKSGCSGFIDW 331
Human       361 ATLGETHRLFPNTMLFASEACVGSKFWEQSVRLGSWDRGMQYSHSIITNLLYHVVGWTDW 420
                   * .    *   *  ** **     .  .. *    * * .* *      *  **

Salmonella  332 NLLLNSEGGPNHQGNLCEAPIQYDAQNDVLRRNHSWYGIGHFCRYVRPG-ARVMLSSSYD 390
Human       421 NLALNPEGGPNWVRNFVDSPIIVDITKDTFYKQPMFYHLGHFSKFIPEGSQRVGLVASQK 480
                 ** ** *****   *  ..**  *   *   ..  * .***.... *  ** * .*

Salmonella  391 NLLEEVGFVNPDGERVLVVYNRDVQERRCRVLDGDKEIALTLPP-SGASTLLWRQESI 447
Human       481 NDLDAVALMHPDGSAVVVVLNRSSKDVPPTIKDPAVGFLETISPGYSIHTYLWHRQ  536
                 * *. *  ..***  *.** **  ..    . *      *. *    * **...
```

Figure 4. Clustal alignment of human and *S. typhimurium* glucosyl ceramidase proteins. The entire sequence of *srlJ* was determined (Genbank accession number TBA). The deduced amino acid sequence was aligned with its human homolog with the software program MacVector® 6.51. Asterisks indicate identical residues and dots denote chemically similar residues.

Table I. Fold induction by chromosomal *ssrB* within cultured epithelial and macrophage cells.

fusion	intraepithelial induction by *ssrB*	intramacrophage induction by *ssrB*
srlK	2	21
srlE	2	5
srlH	74	78
srlB	1	2
srlI	1	3
srlD	2	2
srlG	3	12
srlL	2	2
srlM	1	2
srlJ	24	35

Table I. Fold induction by chromosomal *ssrB* within cultured epithelial and macrophage cells. Cell culture was infected with each fusion in the wild type background and in the *ssrB::cm* background. ß-galactosidase assays were performed on intracellular bacteria to determine how much a chromosomal *ssrB* allele affects the various fusions identified within eukaryotic cells (see Experimental procedures for details). All fusions tested were activated within eukaryotic cells by a chromosomal a *ssrB* allele, indicating that SsrB expression from a plasmid is not required for activation of these genes, establishing physiological relevance. These experiments were performed on three independent occasions and the values averaged. Although *ssrB* mutants have an intracellular survival defect, *S. typhimurium* does not grow significantly inside cells in the first 6 hours, thus bacteria were recovered in similar numbers. It is important to consider that this assay is fairly insensitive; the ß-galactosidase is subject to proteolytic degradation and the number of bacteria that can be recovered from a host cell is significantly less the number normally used in a ß-galactosidase assay. Thus, the numbers reported here are probably underestimates of the actual affects of *ssrB* upon these genes.

Figure 5. *srlK* is preferentially activated by SsrB within macrophages.

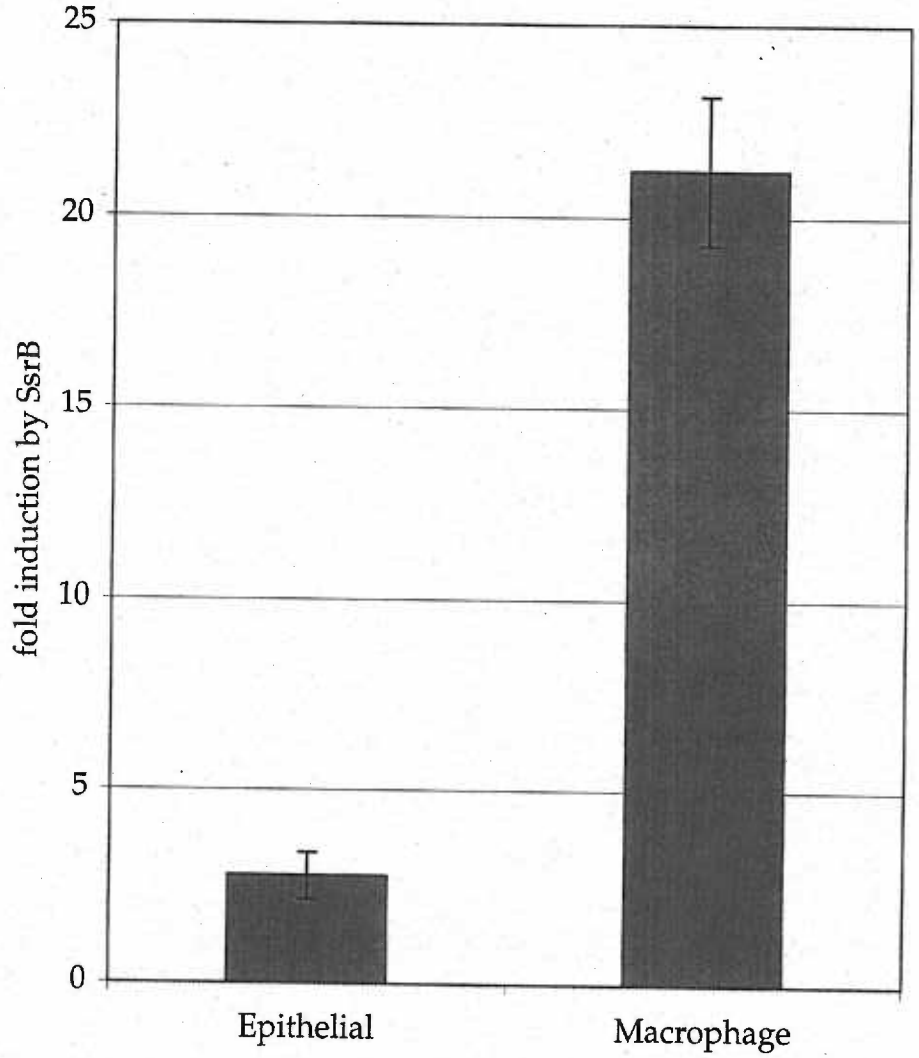

Figure 5. Macrophage specific SsrB mediated activation of *srlK*. SsrB preferentially activated some of the fusions inside J774A cells (macrophages) versus HeLa (epithelial) cells, most notably *srlK*. This experiment was performed on three independent occasions and the values averaged. The error bars represent the standard deviation.

Figure 6. Specificity of SsrB regulation to the intracellular micro-environment.

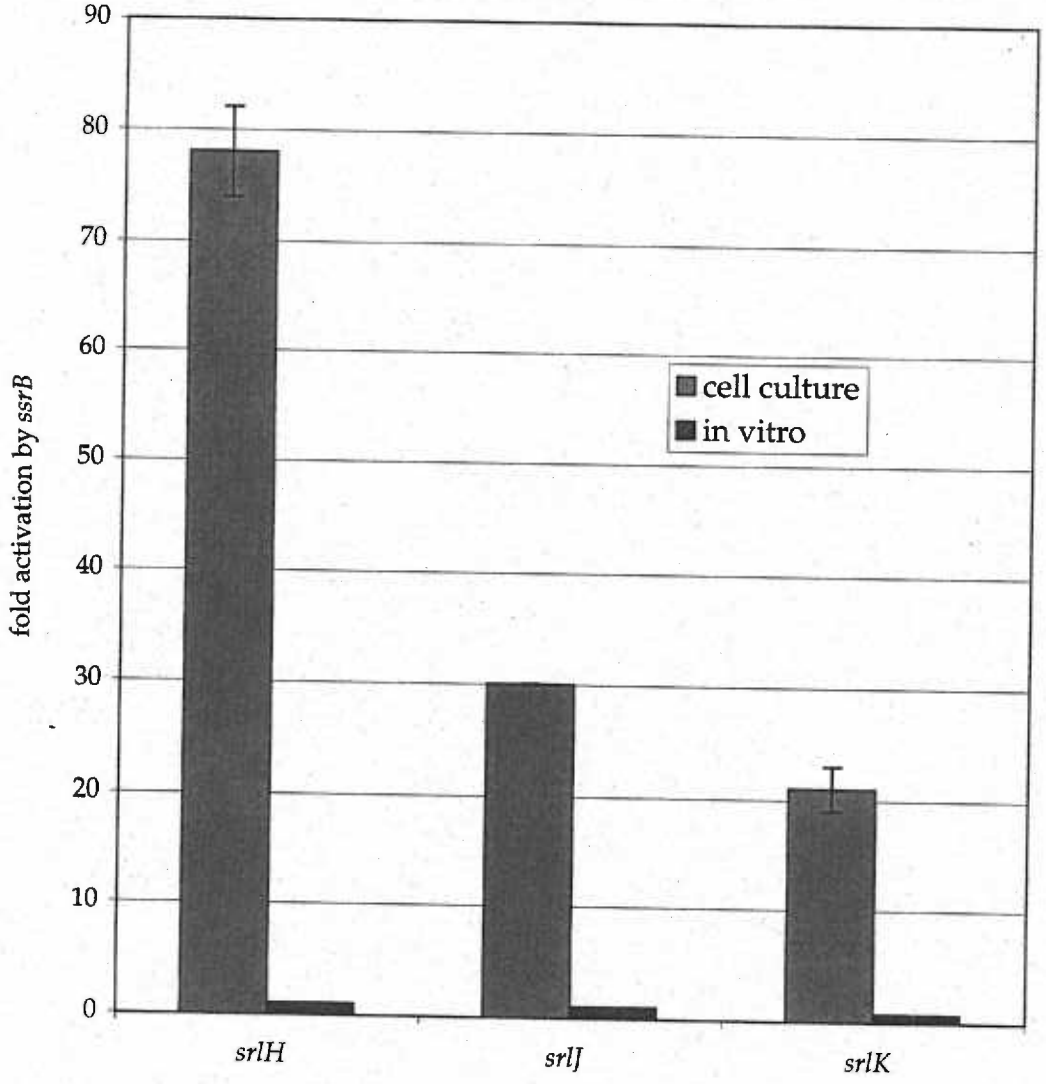

Figure 6. Specificity of SsrB regulation to the intracellular microenvironment. The specificity of SsrB regulation was determined by comparing the ratio of ß-galactosidase activity in a wild type background to the activity in an *ssrB::cm* background, in tissue culture media versus within cells (Table I). For the extracellular induction, strains were grown in a tissue culture incubator without agitation, in tissue culture media supplemented with 10% fetal bovine serum for six hours, concentrated 10-fold and the ß-galactosidase activity measured. Each pair of columns represents one strain. The column on the left for each pair is the level of SsrB activation within J774A (macrophage) cell culture. The column on the right for each pair is the level of SsrB activation in tissue culture media. The ratio between the wild type and the *ssrB::cm* backgrounds in tissue culture media was ≤1.32 for all three fusions tested, thus 99.96% of the regulatory activity of SsrB is confined to the intracellular microenvironment. This experiment was performed three times independently and the values averaged. Error bars represent the standard deviation. Several error bars are too small to be visible.

Figure 7. *srlH* induction by SsrB in murine spleen.

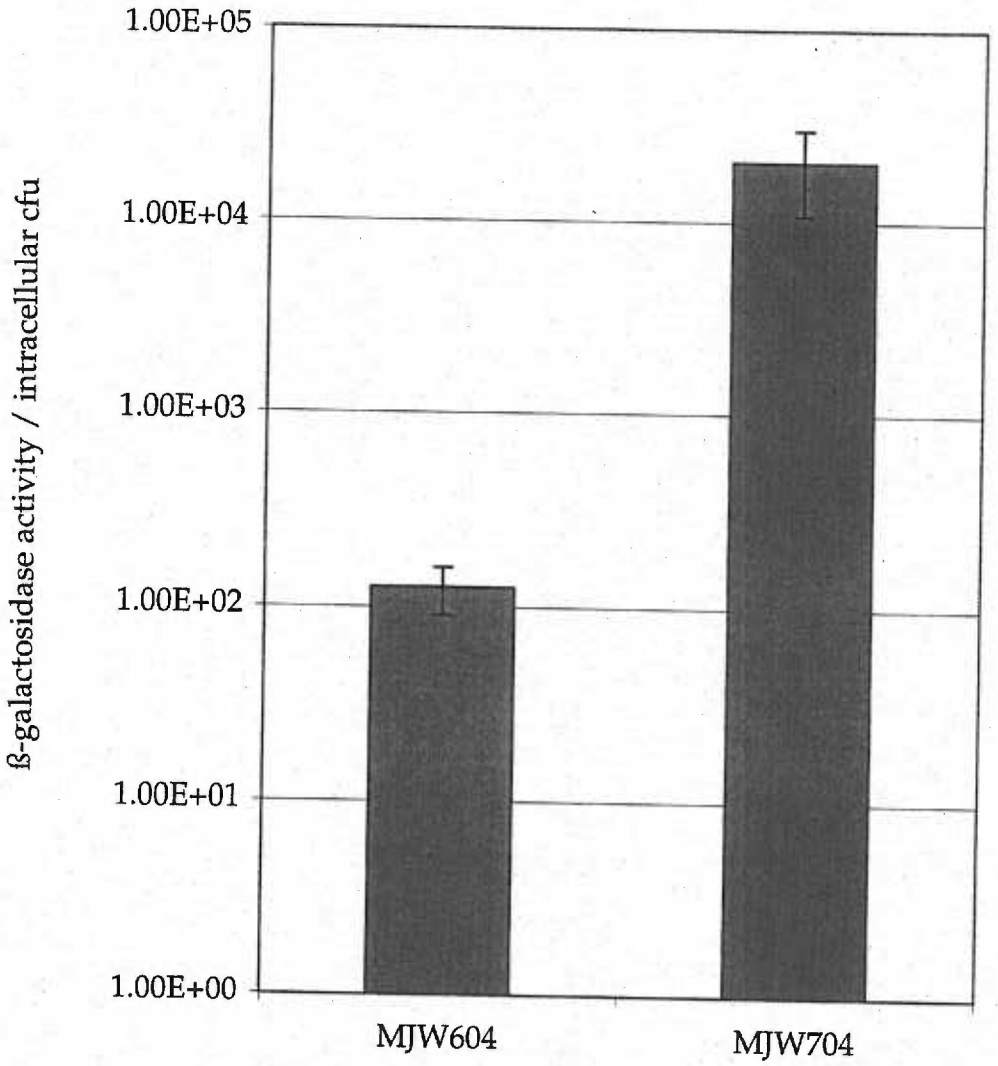

Figure 7. *srlH* induction by SsrB in murine spleen. Mice were intravenously infected with *srlH* in the wild type background (604) and in the *ssrB::cm* background (704). The inoculum was 10^5 bacteria for each strain. 24 hours after infection, ß-galactosidase activity was measure from spleen homogenates as previously described (Miller, 1992); except ß-galactosidase activity was expressed per cfu recovered. As can be seen, a chromosomal *ssrB* allele activates *srlH* 166 fold in murine spleen. This experiment was performed twice independently and the values averaged. The error bars represent the standard deviation.

Figure 8. The SsrB regulon is modulated by both OmpR and PhoP.

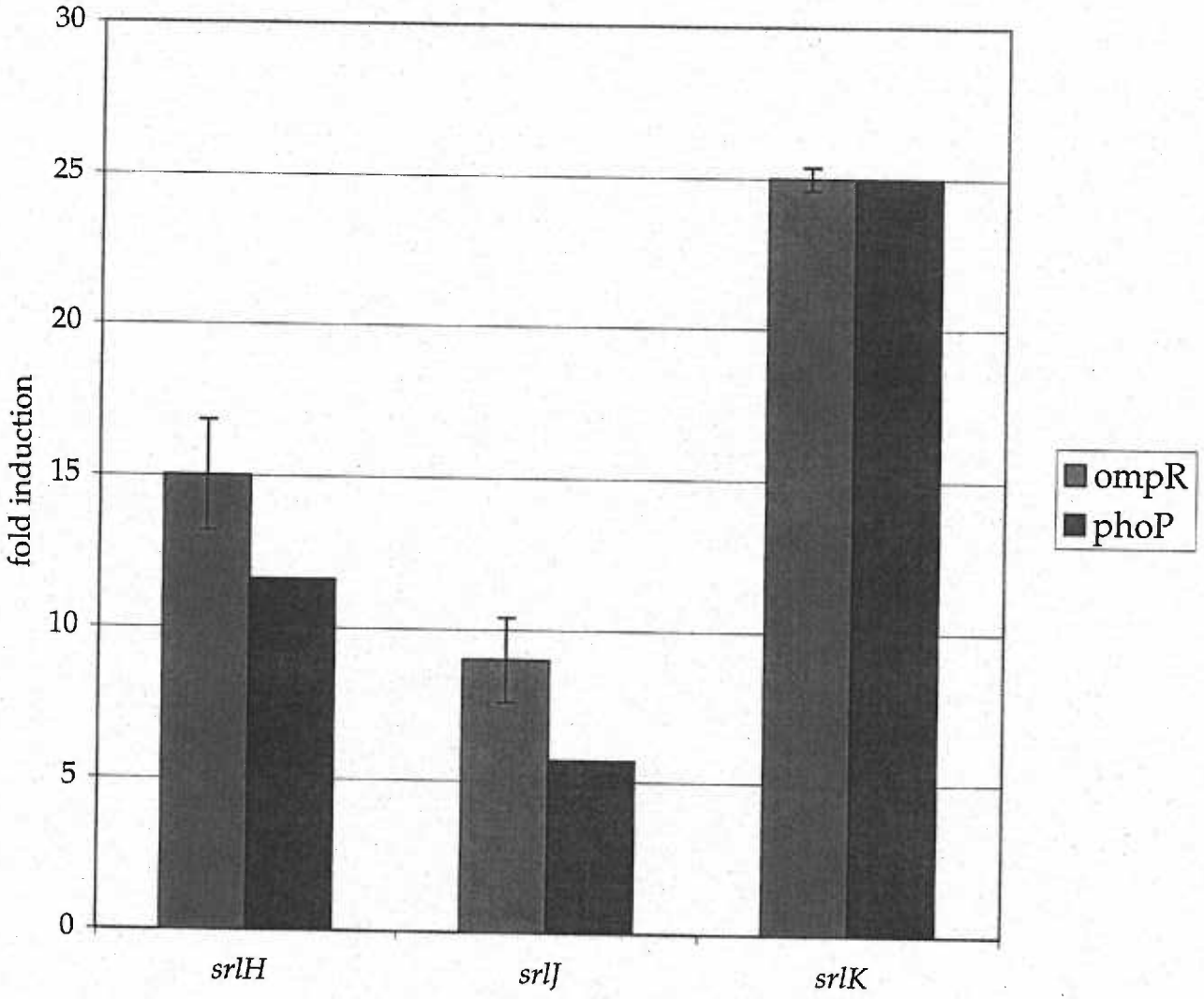

Figure 8. OmpR and PhoP regulation of the SsrB regulon. SsrB regulated fusions were transduced into mutant *ompR* and *phoP* backgrounds, and the affects of these chromosomal alleles determined. This induction experiment was identical to the one in Table 1. The units reported are the fold increase in expression with a wild type copy of the designated allele. All three fusions were significantly regulated by both OmpR and PhoP within macrophages. This experiment was performed three times independently and the values averaged. The error bars represent the standard deviation. Several error bars are too small to be visible.

Table II. Strains and plasmids used in this study.

Strain or plasmid	Characteristics	Reference
strains		
14028s	*Salmonella typhimurium*	ATCC
MJW129	14028s *ssrB::cm*	This study
MJW141	MJW129 + pMJW120	This study
MJW136	MJW129 + pBAD30	This study
MJW400 series	MJW141::MudJ	This study
MJW500 series	*E. coli* with pWKS129 + fusion junctions	This study
MJW600 series	MJW129::MudJ	This study
MJW700 series	14028s::MudJ	This study
MJW800 series	MJW136::MudJ	This study
MJW104	14028s *rpsL*, nal^r	This study
SM10λpir	*E. coli thi*-1, *thr*, *leu*, *tonA*, *lacY*, *supE*, *recA*::RP4-2-Tc::Mu, kan^r, λpir	Miller & Mekalanos, 1988
TT10288	*S. typhimurium* LT2 *hisD*9953::MudJ *hisA*9944::MudI	Hughes & Roth, 1988
plasmids used		
pBAD30	plasmid for arabinose induction	Guzman, 1995
pKAS32	R6K suicide vector ($carb^r$ $rpsL^+$)	Skorupski & Taylor, 1996
plasmids constructed		
pMJW120	pBAD30 + *ssrB* for arabinose conditional expression	This study
pMJW92	pCR-Blunt + *ssrB*	This study
pMJW98	pCR-Blunt + *ssrB::cm*	This study
pMJW114	pKAS32 + *ssrB::cm*	This study
pMJW178	pCR-Blunt + *srlJ* PCR product for sequencing	This study
pKHC2	pCR-Blunt + *srlH* PCR product for sequencing	This study

CHAPTER 4: Discussion

The work presented in this thesis makes several contributions to the field of bacterial pathogenesis. The invasin system allows for the rapid identification of surface exposed proteins which are frequently implicated in virulence. This system is applicable to the study of diverse organisms and may be especially useful in the study of genetically intractable bacteria. It will work well with most bacterial organisms because of the high level of conservation of components of the GSP, and of protein motifs that promote export (Pugsley, 1993). In addition, this system is not limited by transposon host range or the availability of a gene delivery system. Also, the vector promoter can facilitate the transcription of fusions that might not otherwise be expressed. To date, the invasin system has not identified any false positives and thus provides strong functional evidence as to the sub-cellular location of a protein. Several groups studying *Mycobacterium tuberculosis* and one group studying *Treponema pallidum*, both serious human pathogens, are currently using the invasin system.

While originally intended for use with gram negative bacteria, the invasin system appears to exclusively select secreted proteins when used to study gram positive bacteria (M. Worley and F. Heffron, unpublished results). The competitive nature of the selection employed by this system is so powerful that it may be possible to adapt it for the identification of eukaryotic membrane proteins as well.

Both projects described in this thesis will be useful in vaccine development. The invasin system is the most effectual method available to identify surface exposed proteins. Because these proteins are accessible to the immune system, they are frequently efficacious as components of protective vaccines. The invasin system is probably most useful with genetically intractable bacteria, several of which, such as *Chlamydia trachomatis*, *Mycobacterium leprea* and *Treponema pallidum*, are serious threats to public health.

The *ssrB* project also has implications for vaccine design. In recent years, bacterial pathogens have been systematically attenuated for virulence so that they can be safely used to generate protective immune responses against heterologous antigens. These live carrier vaccines have many advantages over traditional vaccines. Attenuated bacteria expressing heterologous antigens can be inexpensively propagated and can be desiccated for storage and transport, removing the need for refrigeration. Further, several heterologous antigens can be carried by the same strain, Thus, potentially, one oral dose of a carrier vaccine could provide an individual with life-long protection from a dozen or more different pathogens.

Salmonella is being extensively manipulated for use as a vaccine carrier for several reasons. *Salmonella* is known to replicate and persist within professional antigen presenting cells. Further, *Salmonella* possesses proteins (that exogenous ones can be fused to) that can stimulate both Th1 and Th2 responses. More is known about *Salmonella* pathogenesis than most

pathogens, but none-the-less serious challenges for exploiting *Salmonell*a as a vaccine carrier remain. One of the biggest problems is confining heterologous gene expression to proper host compartments. Ideally, expression of heterologous antigens should be driven by a strong promoter whose activation is strictly limited to the intracellular environment. Several of the promoters identified in Chapter 3 are excellent candidates for use in such vaccine strains. *srlH* is almost 100 fold induced upon entry into eukaryotic cells, and produces nearly 500 Miller units within eukaryotic cells. This fusion is so strongly and specifically expressed within host cells that it will almost certainly be an improvement over the promoters currently being used to drive heterologous protein expression in vaccine strains. Of additional interest, *srlK* is specifically induced within macrophages. Being able to limit heterologous antigen expression to the interior of professional antigen presenting cells may allow modulation of the nature of the response elicited.