# *SALMONELLA TYPHIMURIUM* GENES INDUCED UPON BACTERIAL INVASION INTO MAMMALIAN CELLS

by

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## Abstract

In causing gastroenteritis, *Salmonella typhimurium* are able to invade and grow within non-phagocytic intestinal cells. They are also able to survive within phagocytic cells, and have the potential to cause systemic disease. The ability of these bacteria to be intracellular is important in the disease process.

*Salmonella* express an unique set of genes inside macrophages (not expressed with other stresses, *e.g.* heat, low pH, starvation), however, only a few have been identified. Therefore, the goal of this study was to search for *Salmonella* genes only expressed inside cells, thereby identifying genes essential for intracellular survival and perhaps virulence.

A light-based reporter system was developed to specifically detect gene expression from intra-cellular bacteria. *Vibrio harveyi* luciferase genes, *luxAB*, were fused to *Salmonella* plasmid virulence genes, *spvRAB*, and the construct was shown to be upregulated after *Salmonella* invasion into epithelial cells. Upregulation was also demonstrated using a similar ß-galactosidase (*lacZ*) reporter gene fusion. The results indicated luciferase was a sensitive reporter, able to monitor bacterial gene expression from within host cells, and able to differentiate live from dead bacteria.

Subsequently, a library of *S. typhimurium* mutants was made by randomly inserting promoterless *luxAB* genes into the bacterial chromosome. Individual mutants were screened for luciferase expression (*i.e.* light production) within phagocytic cells. Extracellular bacterial mutants expressing light during growth in rich media or in the presence of cell-secreted factors were eliminated.

From this screen, four *S. typhimurium* genes were identified as upregulated within both phagocytic and non-phagocytic cells. Three genes were found located within "*Salmonella* pathogenicity islands" (SPI): *sigD/sopB* and *pipB*within SPI-5 and *ssaR* within SPI-2. The *sigD/sopB* gene encodes an inositol phosphate phosphatase affecting host cell signalling pathways and chloride secretion. The *ssaR* gene potentially encodes a type III secretion apparatus component. Bacterial type III secretion systems are highly regulated and specialized

for secretion of bacterial proteins directly into host cells. The fourth gene (*iicA* for induced intracellularly) was completely novel. The four bacterial mutants retained their ability to invade and grow within cultured cells, and all but *iicA* were required for virulence in a mouse model.

This work confirms *Salmonella* pathogenicity includes genes expressed in the intracellular environment. The ability to identify such genes, and their further characterization has led to an enhanced understanding about how *Salmonella* functions as an intracellular pathogen, in addition to identifying specific virulence factors.

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| | |
|---|---|
| amp | ampicillin |
| BALB.BM1 | cultured bone-marrow-derived macrophages from BALB/c mice |
| BALB/c | inbred Bagg albino mice; used for models of various bacterial infections |
| cAMP | cyclic-adenosine monophosphate |
| CDC42 | cell-division-cycle; small GTP-binding protein |
| cfu | colony-forming unit |
| cm | chloramphenicol |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | dimethyl sulfoxide |
| dNTP | deoxy-nucleotide tri-phosphate |
| EDTA | ethylene-diamine tetra-acetic acid |
| EEA1 | early endosomal antigen 1 |
| FBS | fetal bovine serum |
| FDG | fluorescein-di-galactopyranoside |
| HeLa | human cervix epitheliod cell line |
| $ID_{50}$ | bacterial dose at which 50% of cells or animals become infected |
| IgA | immunoglobulin A |
| *in vitro* | (Latin: in glass) here, to mean within labware or within cultured cells |
| *in vivo* | (Latin: in the living organism), here, to mean within the host animal |
| IP | intraperitoneal |
| IV | intravenous |
| J774A.1 | cultured macrophage-like cells from BALB/c mice |
| JNK | c-Jun N-terminal kinase |
| LAMP | lysosomal-associated membrane protein |
| LB | Luria-Bertani; rich bacterial media |

| | |
|---|---|
| $LD_{50}$ | bacterial dose at which 50% of animals die |
| LPS | bacterial lipopolysaccharide |
| MDCK | cultured Madin-Darby canine kidney cells |
| MEM | minimal Eagle's medium |
| MHC | major histocompatibility complex |
| MOI | multiplicity of infection |
| NAPS Unit | Nucleic Acid and Protein Services Unit at University of British Columbia |
| NP-1 | neutrophil peptide defensin 1 |
| Nramp | natural resistance-associated macrophage protein |
| ORF | open reading frame |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PETG | phenyl-ethyl-thio-galactoside |
| PMN | polymorphonuclear cell or neutrophil |
| polymyxin-CAP | cyclic-antibacterial peptides |
| PVC | polyvinylchloride |
| SCV | *Salmonella*-containing vacuole |
| SDS | sodium dodecyl sulfate |
| SOC | rich bacterial media containing tryptone, yeast extract and glucose |
| str | streptomycin |
| TE | buffered solution containing Tris-HCl and EDTA |
| tet | tetracycline |
| UBC | University of British Columbia |

## List of Bacterial Genetic Abbreviations

| | |
|---|---|
| A1A1 | *S. typhimurium* mutant with insertion in *sopB/sigD* gene |
| *agf* | thin aggregative fimbrial gene |
| *ahp* | alkyl hydroperoxide gene |
| AIDA-1 | adhesin involved with diffuse adherence in EPEC |
| *atr* | acid tolerance response gene |
| *brk* | gene encoding *Bordetella* resistance to complement |
| *cys* | gene encoding a protein component in the cysteine pathway |
| D11H5 | *S. typhimurium* SL1344 with insertion in *ssaR* gene |
| E12A2 | *S. typhimurium* SL1344 with insertion in *pipB* gene |
| *emrR* | E-multidrug resistance gene; involved with low-energy shock adaptation |
| *envZ* | histidine kinase; sensor component of two component regulatory system OmpR/EnvZ |
| EPEC | enteropathogenic *E. coli* |
| *fhlA* | formate hydrogenlyase |
| *fim* | type 1 fimbrial gene |
| *fliA* | flagellar gene; specifically an alternative sigma factor |
| *flgM* | flagellar gene; specifically an anti-sigma factor |
| *fur* | ferric uptake regulator gene |
| G+C | total guanosine plus cytosine content of the DNA |
| G5D5 | see: G7H1 |
| G7H1 | *S. typhimurium* SL1344 with insertion in *iicA* gene |
| G8B1 | see: G7H1 |
| *iic* | gene induced intra-cellularly |
| *inv* | invasion gene |
| *ipa* | invasion plasmid antigen gene |

| | |
|---|---|
| *kat* | gene encoding catalase |
| *lacZ* | gene encoding ß-galactosidase, from lactose operon |
| *lpf* | long polar fimbrial gene |
| *luxAB* | genes encoding bacterial luciferase |
| *marR* | multiple antibiotic resistance gene |
| *mgt* | magnesium transport gene |
| *mutS* | mutator gene involved in methyl-directed mismatch repair |
| *ompR* | regulator component of two component regulatory system OmpR/EnvZ |
| *orf* | open reading frame |
| P22 | *Salmonella*-specific bacteriophage |
| *pag* | PhoP-activated gene |
| *pef* | plasmid-encoded fimbrial gene |
| *phoP* | regulator component of two component regulatory system PhoP/Q |
| *phoQ* | histidine kinase; sensor component of two component regulatory system PhoP/Q |
| *pip* | pathogenicity island-encoded protein |
| *pmr* | polymyxin resistance gene |
| *prg* | PhoP-repressed gene |
| *rho* | gene encoding transcription terminator factor Rho |
| *rpoS* | gene encoding RNA polymerase alternative sigma factor 38 |
| *sap* | genes corresponding to sensitivity to antimicrobial peptides |
| *sec* | amino-terminal secretion signal of a protein |
| *selC* | selenocysteine tRNA gene; selenium metabolism |
| *sif* | *Salmonella*-induced filament gene |
| *sig* | *Salmonella* invasion gene |
| *sip* | *Salmonella* invasion protein gene |
| *sod* | superoxide dismutase gene |

| | |
|---|---|
| *sop* | *Salmonella* outer protein gene |
| *spa* | surface presentation of antigen gene |
| SPI | *Salmonella* pathogenicity island |
| *spi* | *Salmonella* pathogenicity island encoded gene |
| *spv* | *Salmonella* plasmid virulence gene |
| *ssa* | *Salmonella* secretion apparatus gene |
| *sse* | *Salmonella* secretion effector gene |
| *ssr* | *Salmonella* secretion regulator gene |
| *yop* | *Yersinia* outer protein gene |

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# Chapter 1: Introduction

## 1.1. *Salmonella* and salmonellosis

*Salmonella* are Gram-negative bacteria within the family Enterobacteriaceae, tribe Salmonellae, genus *Salmonella*, and (according to the Ewing classification scheme) are divided into three species: *typhi, choleraesuis, or enteritidis* (168). *S. typhi* is solely a human pathogen, and is the causative agent of typhoid fever (169, 252). Humans are the reservoir for this bacterium, and spread is most often through the consumption of water contaminated with human feces. Symptoms of typhoid fever may take from one week to one month after ingestion of the bacteria to manifest and are characterized by a sustained high fever, bacteremia followed by infection of the bilary system and other tissues, and an initial constipation period followed by diarrhea, possibly bloody (169). The disease is often severe, with a death rate from 2-10% and a 20% rate of relapse. Even after the person has apparently recovered, bacteria may survive in the gall bladder and be shed for up to a year (even with antibiotics and surgery) or longer (168, 169, 274).

*S. choleraesuis* is able to cause disease in both animals and humans, with the reservoir being farm animals, including swine. In humans it causes a severe disease in the form of a prolonged bacteremia characterized by fever, chills, and anorexia. Gastroenteritis is not common, however due to its systemic nature, focal lesions may develop in any tissue to cause osteomyelitis, pneumonia, pulmonary abscesses, meningitis or endocarditis. In patients with depressed immune systems (*e.g.* due to AIDS, organ transplantation, or cancer), *S. choleraesuis* is able to cause a severe typhoid-like disease (168, 274).

*S. enteritidis* is the most common of the three species, consisting of over 2000 serotypes according to the Kauffmann-White antigen classification scheme, and are almost ubiquitous in the environment (14, 20). They are found associated with many different types of animals and food products ranging from radish sprouts and eggs (227) to chicken and beef (14, 168, 274). Most often the bacteria are referred to by their common serotype

nomenclature, *i.e. S. typhimurium* rather than *S. enteritidis* serotype *typhimurium* (20). Of the more than 2000 serotypes existing, only 40% of them account for over 95% of all the clinical isolates (168) and some clinically relevant serovars include: *S. typhimurium, S. dublin,* and *S. paratyphi A*.

In humans, the ingestion of food or water contaminated with *S. enteritidis* results in gastroenteritis (274). Symptoms appear within 6 to 24 hr after ingestion and last up to a week, with severity varying from person to person, depending on serotype and dose ingested. The disease is characterized by initial nausea and vomiting followed by abdominal pain and diarrhea, with or without fever. After recovery, a person may continue to shed the bacteria in their feces for up to three months. Interestingly, antibiotic treatment of uncomplicated gastroenteritis has been reported to prolong the carrier state (168). Most often the disease is self-limiting and localized to the intestinal tract, but in a small number of people, especially young children or immunocompromised patients, the infection may become systemic and thereby more severe (227). In rare cases, *S. enteritidis* serotypes have been implicated in systemic disorders, including arthritis (313), pancreatitis (8), endocarditis (168), pericarditis (168), and mediastinitis (234). *S. enteritidis* serotypes are also able to cause disease in a number of animals, resulting in symptoms which range from barely detectable to severe typhoid-like resulting in death. This has allowed for the development of animal models. For example, infection with either *S. typhimurium* or *S. dublin* causes gastroenteritis in humans, yet result in a severe typhoid-like disease in BALB/c mice (10, 156). The remainder of this introduction describes research concerning *Salmonella enteritidis* species, specifically *S. typhimurium* or *S. dublin*. Many of the aspects described below also apply to *S. choleraesuis* and *S. typhi* (218), although the differences will not be expounded on.

## 1.2. Model Systems for the Study of Bacterial Pathogens

The study of bacteria and their interaction with the environment and hosts is done through the use of different model systems, all of which have their advantages and their limitations.

### 1.2.1. Bacterial culture

The growth of a pure bacterial culture in the lab can yield a wealth of information regarding the physiology of the organism. This is considered one of the most basic and essential aspects of bacteriology, and in the case of pathogenic bacteria, it is required to fulfill Koch's postulates to determine the cause of a disease (274). Specific biochemical and genetic information regarding the bacteria can only be obtained through the use of a pure culture. Furthermore, environmental cues can be controlled either individually or in combination, and thereby the specific bacterial responses to them determined. Some examples of parameters which can be tested include bacterial responses to changes in temperature, pH, osmolarity, nutritional requirements, resistance to complement, oxidative radicals, heavy metals, antibiotics, the production of toxins, and growth phase (115, 119, 295).

However, the growth of a single culture in the lab does not reveal all aspects of how bacteria are able to survive within an environment or cause disease. Often, the synergism and competition with other living organisms in the environment cannot be duplicated in a test tube (*in vitro*). Furthermore, species or tissue tropisms associated with a particular bacterial disease cannot be deduced. It is the rule rather than the exception that clinical isolates of bacteria display very different phenotypes than those passaged many times over in the lab. It is clear that while bacteria are free-living entities, they are greatly influenced by their surroundings and spend a great deal of their time reacting to and altering their environment (115). *Salmonella* are able to survive in a wide range of temperatures and pHs. They are not only able to react to environmental cues (*e.g.* pH, temperature, organic and inorganic substrates, oxidative radicals, etc.) (295), but are able to interact with and actually communicate at the cellular level with animate entities such as other bacteria, and even plants and animals (97, 250). It is this interaction and communication (or 'argument') between bacteria and host that is the basis of disease in salmonellosis, and most other bacterial diseases. The chemical signaling between bacteria and other organisms is very complex and is extremely difficult to reproduce in the lab with a pure bacterial culture. For example, the bacteria are able to survive in contaminated

water and food products. Once ingested, they are subjected to the salivary enzymes of the mouth and then the bile salts and low pH of the stomach. After the shock of the stomach, the bacteria pass through into the small intestine where they encounter the peristaltic movement of the intestines and the mucus continually moving along the ciliated brush border lining the intestinal surface. Furthermore, the ingested bacteria encounter many other microorganisms that are already resident in the intestine. The *Salmonella* are able to bypass these barriers, to interact with the intestinal cell surfaces and ultimately engineer their own uptake into these cells. Within the host cells, *Salmonella* remain inside a vacuolar space, where they must adapt in order to survive.

### 1.2.2. Cell culture

To avoid confusion throughout this discourse, bacterial cells will not be referred to as "cells" but as simply "bacteria"; while host cells will be referred to as "cells". The interactions of bacteria with host cells can be studied more closely with the use of cultured mammalian cells. There are a number of host cell types available for study, from various tissues, and from various types of animals. Examples of epithelial cells include HeLa cells which originated from a human cervical tumor and MDCK cells which originated from immortalized canine kidney cells. Examples of phagocytic cells include J774A.1 and BALB.BM1 cells, which are both mouse macrophage-like cells originating from BALB/c mice. The discovery of numerous *Salmonella* virulence factors has been made through the controlled infection of cultured host cells (22), specifically many factors required for bacterial invasion of cells and for survival within cells. The discovery of a new bacterial protein secretion system, the type III secretion system (TTSS), is especially noteworthy since bacterial effectors are secreted into host cells predominantly upon contact of the cells with the bacteria (188, 208).

*Salmonella* were long thought to produce disease from extracellular locations within the body for a number of reasons. The bacteria could be isolated from fecal and blood samples, and in EM studies, the destruction of *S. typhimurium* within host polymorphs and macrophages was seen (160). Moreover, the bacteria were able to replicate in extracellular

spaces, such as the peritoneal cavity (160). Over the past decade, there has been a shift, and many researchers now believe that the ability of *Salmonella* to persist intracellularly greatly influences the extent and severity of the disease (51, 76, 81, 85, 195, 247, 263).

The intracellular stage appears to be especially important as mutants which either cannot invade cells or survive within cells are attenuated (76, 195, 247). However, the bacteria are able to move via flagella (171) and adhere to cells via various fimbrial adhesins and pili (183, 324). They ultimately engineer their own uptake into the intestinal cells, targeting the M cells of Peyer's patches (165, 288). During invasion *Salmonella* induces selective aggregation and internalization of host cell surface proteins, such as MHC class I heavy chain, fibronectin-receptor, CD-44 and $ß_2$-microglobulin (109). The bacteria also trigger host cell signaling pathways in a CDC42-dependent manner (42, 172), (although uptake is Fc receptor independent (205)), that lead to cytoskeletal and nuclear responses resulting in the uptake of the bacteria (5, 90, 91, 267, 272). The mechanisms required for the invasion of cells by *Salmonella* and for their subsequent survival within cells are complex and require the coordination of a number of bacterial factors (see virulence factors section below) (79).

In culture, *Salmonella* are able to enter every mammalian cell type tested (81, 328). However, in order to separate the bacteria-mediated invasion from phagocytic uptake, invasion studies have used non-phagocytic epithelial cells (79). There is evidence that similar events take place in phagocytes and that the bacteria mediate their own uptake into these cells as well rather than relying on Fc-receptor or complement-receptor mediated uptake (110). *Salmonella* do not bind intimately to the cell surface in order to enter into cells, rather the bacteria initiate a splash or membrane-ruffling event which causes the cell to macropinocytose everything within the immediate vicinity (90, 105, 169). The bacteria are thus taken up into a large spacious vacuole, where they can be seen to actively move around (4, 5, 84). Once the bacteria have been engulfed, the cell surface returns to normal (84).

Inside the cell, the *Salmonella*-containing vacuole (SCV) does not traffic in the same manner as would a regular vacuole and the intracellular environment of the SCV is actively

modified by the bacteria (31, 108). While the pH of the vacuole is lowered, it only falls to around pH 5.0, rather than to pH 4.0 (3, 108). There is evidence that while the SCVs do accumulate some of the markers of the regular trafficking pathway (EEA1 indicating fusion with early endosomes; lysosomal glycoproteins and LAMPs indicating fusion with late endosomes (219, 240, 241) and Nramp1 (220, 284)), they prevent the accumulation of other markers in any great amount such as cathepsin D or the mannose-6-phosphate receptor. It was earlier reported that the mannose-6-phosphate receptor was not associated with the SCV at all (106, 256), but recently more sensitive evidence indicates that as many as 10% of SCVs may contain this marker; however this is still significantly different from the normal trafficking pattern seen with a cell (219). About 4-6 hr after invasion, the cell is seen to produce filamentous structures which extend out from the SCV, called Sifs (*Salmonella*-induced filaments), which correspond to bacterial replication within the cells (195, 221, 299). The Sifs can be labeled with lysosomal glycoprotein, and are connected to the SCV. They are not actually large enough to contain a bacterium although bacterial membrane blebs are found within these structures (299).

Other stimulated pathways include inositol phosphate signaling pathways (270) and those stimulated by rapid calcium fluxes within cells (65, 243), although cyclic adenosine 3':5'-monophosphate (cAMP) levels do not increase (37). The result is the secretion of fluid into the intestine and the influx of neutrophils to the site of infection, resulting in diarrhea (78, 206). Furthermore, *Salmonella* are toxic to cells. Invasion of the bacteria into macrophages induces apoptosis, which can be seen both *in vitro* (224) and *in vivo* (263).

Although ultimately more complex than the study of single bacterial cultures, the limitations of using cell culture are very similar, and this model is still considered an *in vitro* model. Cultured cells exist in an artificial environment where they cannot interact with other cell types for stimulation, cross-feeding, differentiation, or to get rid of toxic wastes. Often they are transformed such that they will not have a limited life-span but will be able to be passaged many times, similar to the passage of bacteria. However, as occurs with bacteria, the

more a cell is passaged in a lab setting, the more it is removed from specific contacts, hormones and other chemical gradients, the less likely it is to resemble the tissue from which it was taken. The use of primary cell cultures for study helps to lessen this 'de-differentiation effect', although they are more difficult to work with in the lab and often their availability is limited. Similarly, combined cell and bacterial cultures are difficult to work with and the results difficult to interpret because there are many variables, known and unknown.

### 1.2.3. Animal models

Ultimately, the best way to determine bacterial virulence and tissue specificity is to infect the host. In place of experimenting on humans, animal models are used to reproduce a given disease and determine whether a specific bacterial factor, or conversely a host factor, may increase or decrease the extent of bacterial pathogenesis. A good animal model requires that the bacterial infection can be established reproducibly. However this trait may actually make them less sensitive when assaying for bacterial virulence, as the animals are often deficient in some way or immunologically immature in order to give the bacteria an edge. Furthermore they are fairly crude measures of virulence as they reflect the cumulative effects of many steps including bacterial colonization and the production of symptoms. This makes it possible to overlook important virulence determinants.

There are a number of ways to determine virulence using an animal model. Traditionally researchers have used a whole animal model, looking at either the infectious dose ($ID_{50}$) or the lethal dose ($LD_{50}$) (274). The infectious dose ($ID_{50}$) is the number of bacteria necessary to infect 50% of the exposed animals. This parameter only reflects the infectivity of the bacteria and not the morbidity of the disease resulting from infection. The lethal dose ($LD_{50}$) is the number of bacteria necessary to kill 50% of the infected animals. A variation of this involves the determination of the day on which 50% of the animals died. Other parameters also used as indicators of virulence include the measurement of signs of disease such as weight loss, diarrhea, presence of bacteria in animal fluids (*e.g.* blood, urine, cerebrospinal fluid) or organs (*e.g.* spleen, liver), or the extent of the immunological response (*e.g.* PMN influx,

number and size of infection foci). Another model is the ileal loop model, where the intestine within an anesthetized animal is tied off and the bacteria injected directly into it. Only more localized effects of the bacterial infection can be determined with this model (*i.e.* fluid accumulation or PMN influx), however it has the advantage in that more than one bacterial strain at a time may be analyzed within the same animal.

For salmonellosis, a number of animal models have been used. The most widely used model involves the infection of BALB/c mice with *S. typhimurium* resulting in a disease which resembles typhoid fever in humans (233, 306). The BALB/c mice are particularly susceptible to intracellular bacterial infections, which has been attributed to a defective host resistance locus *nramp*1 (formerly known as the *ity/bcg/lsh* locus) (64, 116, 327). Other animals used include guinea pigs (160), rabbits (330), chickens (53, 142, 318), pigs (123, 314), sheep (27), and cows (94, 330).

In the mouse, *Salmonella* are able to cause disease when given by various routes. The bacteria may be induced orally, which is the route thought to most mimic the natural course of infection (112, 156). A typical $LD_{50}$ is around 1 x $10^6$ bacteria per mouse (195, 299, 306). The bacteria are forced to contend with not only the acid and gastric juices within the stomach, but also with various cellular barriers. Although *Salmonella* invade many cell types *in vitro*, the bacteria appear to be targeted to the membranous cells (M-cells) *in vivo* (94, 156). M-cells are specialized cells, found in Peyer's patches within the intestine, which are thought to internalize luminal materials for presentation to underlying antigen-presenting cells (*e.g.* monocytes, macrophages, and neutrophils). *Salmonella* are able to pass through the intestinal cell layer to the sub-layers (83, 85, 170), where they are able to colonize (204) and/or are picked up by waiting macrophages. The bacteria are able to survive within phagocytes (263), and possibly travel to other organs via this intracellular route (57). Using this route of infection, factors required for invasion are often detected as their loss results in a loss in virulence. When bacteria are injected intraperitoneally (IP) into mice (10), they no longer have to deal with these particular barriers, nor do they have to compete with the normal intestinal

flora. Consequently, the number of *Salmonella* required to cause disease is reduced using IP inoculation, with an $LD_{50}$ around 100 bacteria per mouse (299). As well, bacteria with mutations in invasion genes are not attenuated for infection resulting from IP inoculation, but those with mutations in genes needed for subsequent bacterial growth within cells are attenuated. Intravenous (IV) inoculation of *Salmonella* into mice bypasses the most barriers, putting the bacteria into direct contact with circulating macrophage cells and providing the most direct route to the organ of choice in which to establish an infection (*e.g.* liver and spleen). Again, the number of *Salmonella* required to cause disease is drastically reduced, with an $LD_{50}$ around 10 bacteria per mouse (131, 263).

## 1.3. Bacterial physiology

### 1.3.1. Secretion systems (Gram-negative bacteria only)

The type I *sec*-independent secretion system involves the three components: an inner membrane transport ATPase (coined ABC protein for ATP-binding cassette), a periplasmic-spanning protein which is anchored in the inner membrane, and an outer membrane protein which is secreted via a *sec*-dependent pathway (80, 161, 208). The genes encoding the secretion apparatus and the secreted protein are usually clustered. Proteins secreted via the type I pathway contain the signal for secretion in their carboxy terminus, however this signal appears to vary slightly between subfamilies of the secretion system. In contrast to the sec-dependent secretion pathways, this carboxy signal sequence is not cleaved off and there is no periplasmic intermediate of the secreted protein. Examples of proteins secreted via this pathway include the subfamily of hemolysins *Escherichia coli* alpha-hemolysin, *Bordetella pertussis* adenylate cyclase, and *Pasteurella haemolytica* leukotoxin, as well as the subfamily of proteases from *Pseudomonas aeruginosa* and *Erwinia chrysanthemi* (80, 161). There are no reported *Salmonella* proteins secreted by this pathway so far, however the outer membrane component of the secretion apparatus has been described and is functionally interchangeable with the *E. coli* homolog (TolC) (305).

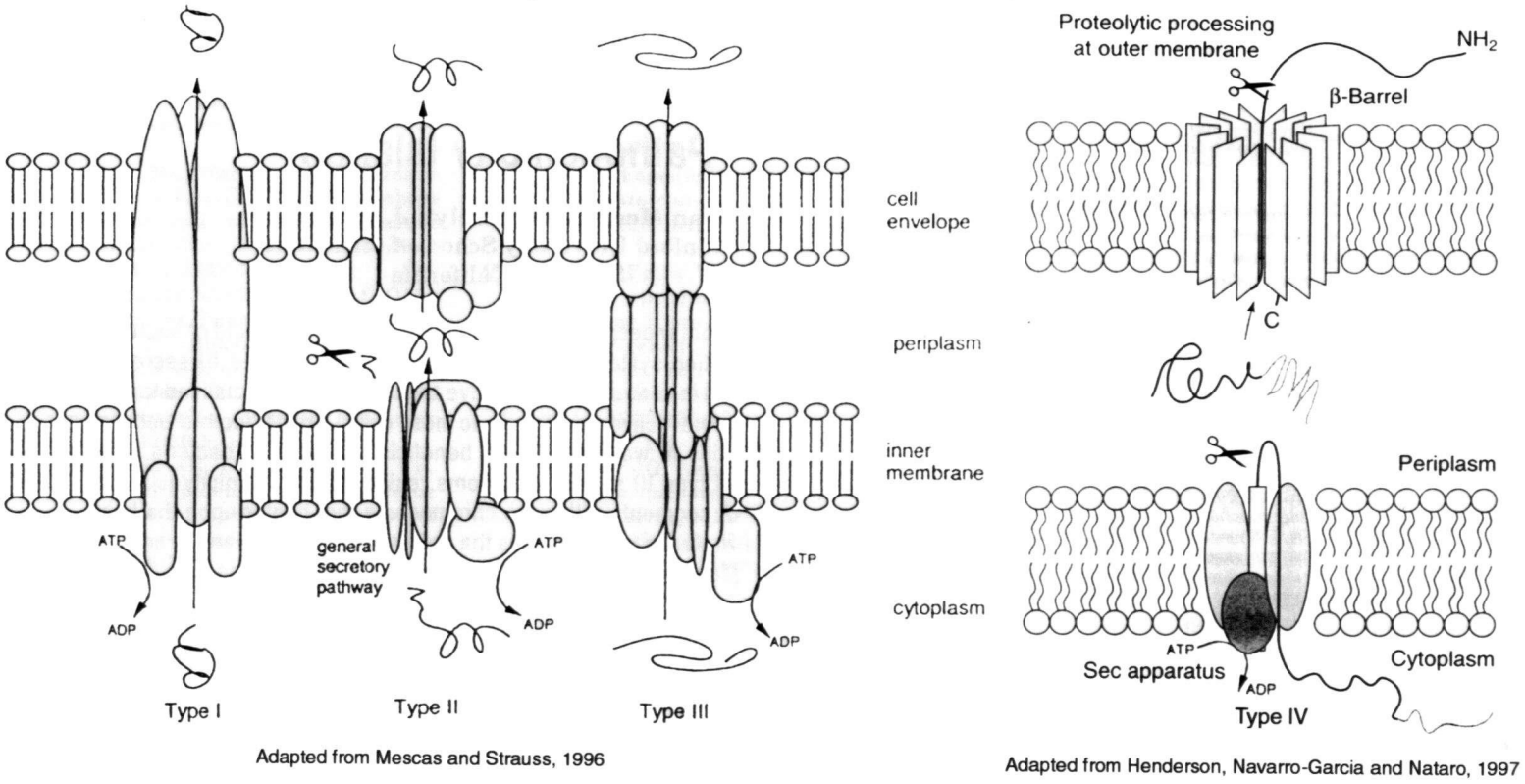
Both the type II and type IV secretion pathways are *sec*-dependent and involve two separate steps for transport across the inner and outer membranes. The first step (the *sec*-dependent step) is energized by ATP hydrolysis and requires the protein to have an amino-terminal signal sequence which is cleaved off during export to the periplasm. Components of this first step include a number of inner membrane proteins, an inner membrane associated ATPase, a chaperone to bind presecretory target proteins, a periplasmic signal peptidase, as well as a number of accessory proteins (161, 208).

Type II secretion is thought to be the main export pathway for gram-negative bacteria and is known as the generalized secretory pathway (80, 208, 273). The second step for translocation through the outer membrane includes a periplasmic chaperone and a number of outer membrane associated proteins which multimerize to form a channel (273). It is thought that the secretion signal for this step may be encoded by multiple segments on the protein (273), and no signal sequence cleavage occurs. Secretion components usually are encoded by about 13-15 clustered genes. The type II secretion pathway is primarily used for the secretion of degradative enzymes. Some examples of proteins secreted via this pathway are *Klebsiella oxytoca* pullulanase, *P. aeruginosa* phospholipase C, exotoxin A, and elastase, and *Xanthomonas campestris* polygalacturonase (161).

Proteins which use the type IV secretion pathway are able to induce their own transport across the outer membrane, and are therefore also known as autotransporters (80, 149). Once through the inner membrane, the proteins are able to associate with the outer membrane to form a pore and mediate their own passage out of the cell, in the absence of any energy coupling or accessory factors. Once outside, the protein may remain associated with the outer membrane or be cleaved off. Examples of proteins secreted via this pathway include gonococcal IgA protease, *Haemophilus influenzae* IgA protease, *Helicobacter pylori* vacuolating cytotoxin, enteropathogenic *E. coli* EspC, and *Bordetella pertussis* pertactin and BrkA (80, 149, 161)

The type III secretion pathway is especially important for the specific secretion of a number of virulence factors. This secretion system has been found to be conserved amongst a

number of gram-negative pathogens including *Salmonella* (80, 98, 268), *Shigella* (98, 268), *Yersinia* (268), *E. coli* (80), *Erwinia* (325), and *Pseudomonas* (311, 325) species. Proteins of the type III secretion system, as well as many of the proteins to be exported, are contained within large regions of DNA dubbed pathogenicity islands (111, 208). These regions may be chromosomally encoded, as in *Salmonella*, or contained on the virulence plasmid, as in *Yersinia*. Type III secretion involves more than twenty components, many of which are homologous to those involved with flagellar assembly, including a multimeric ATPase and various proteins involved in the synthesis of a specialized surface organelle (113, 182). Amino terminal signal sequences target proteins to the secretion machinery, although the secretion sequences are not cleaved (188). There is some evidence to indicate that some secretion information is contained within the structural configuration of the mRNA (7), and that at least for some proteins (but not all (96)), secretion and translation may be coupled (188). Each protein to be secreted also associates with a corresponding specific cytosolic chaperone. The whole system is highly regulated and proteins are only secreted upon the bacteria receiving specific environmental cues. The method is *sec*-independent and proteins are secreted across both the inner and outer membranes in one step. Furthermore, proteins may be secreted from the bacteria directly into host cells, where they can influence host cell signaling. In contrast to other secretion systems, many of the structural components of the type III system are considered to be virulence genes rather than housekeeping genes because their only function appears to be the selective secretion of proteins required for pathogenicity in the host (188). Moreover, mutations within these structural genes often leads to reduced bacterial virulence without greatly affecting bacterial growth in broth culture.




Adapted from Mescas and Strauss, 1996

Adapted from Henderson, Navarro-Garcia and Nataro, 1997

**Figure 1: Schematic diagram of the type I, type II, type III, and type IV secretion systems of gram-negative bacteria. All systems use energy to drive secretion. Types I and III systems secrete proteins across both the inner and outer membranes, while types II and IV systems transport proteins in separate steps. Types I and III do not remove any part of the protein during transport, unlike types II and IV.**

### 1.3.2. Virulence genes

#### a. Pathogenicity islands

The definition of a 'virulence' gene is a gene that is required by the bacterium to specifically cause disease in the host or to subvert the host's immune system. This differs from a 'housekeeping' gene which is required for maintenance of regular bacterial functions for growth, DNA division, protein production, nutrient acquisition, *etc.* Generally, if a mutation is made within a virulence gene, the bacteria will show increased susceptibility within the host, but will not be greatly affected during growth in regular rich media (122).

While virulence genes can be found scattered throughout the *Salmonella* chromosome, the latest research has shown the existence of large regions of DNA encoding groups of genes which appear to coordinate various aspects of pathogenesis known as pathogenicity islands (122). These islands have also been described in other gram-negative pathogens and frequently include the genes needed for the type III secretion system, including the LEE (locus of enterocyte effacement) region of pathogenic *E. coli* (67, 249), the two PAI (pathogenicity island) regions of uropathogenic *E. coli* (264), the Mxi/Spa region of *Shigella* (161, 268), the Exs/Psc region of *P. aeruginosa* (161), the Hrc/Hrp region of *Ralstonia solanacearum* (161, 325), and the virulence plasmid-encoded Lcr/Vir region of *Yersinia* (161, 325). Pathogenicity islands are clusters of horizontally acquired virulence genes which are generally found inserted at tRNA loci (24) and have a G+C content that is lower than that of the bacterial chromosomal DNA (around 52%). Within *Salmonella* there have been five *Salmonella* pathogenicity islands described so far (SPI-1, -2, -3, -4, and -5), although only two encode the machinery for type III secretion. The genes within these islands are important for coordinating and secreting other virulence factors encoded outside of these specialized areas on the bacterial chromosome.

In *S. typhimurium*, SPI-1 is a 40 kb region located at 63 minutes on the chromosome between *mutS* and *fhlA* (49, 217). SPI-1 appears to be present in all *S. enterica* subspecific groups (238). The overall G+C content of the gene cluster was found to range from 42-47%. SPI-1 has been shown to be especially important for bacterial invasion of epithelial cells and

M-cells (6, 47, 49, 98, 99, 331). This active invasion of cells by the *Salmonella* is in contrast to the antiphagocytosis activity of *Yersinia* species (207). Many of the SPI-1-encoded genes are also required for cytotoxicity in macrophages (43) and for virulence *in vivo*. This region contains genes encoding components of a type III secretion system which encode structural proteins (*invA, invC, invG, invJ, spaO, spaP, spaQ, spaR, spaS, prgH, prgI, prgJ, prgK,* and *orgA*) (50, 99, 174, 277, 304), secreted proteins (*avrA, sptP, spaO, invJ, sipA, sip/sspB, sip/sspC,* and *sip/sspD*) (48, 50, 162, 175, 176, 277) and their corresponding chaperones (*sicA, sicP* , *invI* , and *prgI*)(95, 98), and proteins for regulation of secretion (*sip/sspD,* and *invE*) (162). Proteins required for regulation of gene expression are also encoded within the SPI-1 region (HilA and InvF)(11, 174). Additional proteins secreted by the SPI-1-encoded type III secretion system (*e.g.* SopB/SigD, SopE, SopD), as well as additional regulatory factors of this region (*e.g.* PhoP/PhoQ), are located in unlinked chromosomal locations (60, 101, 173).

Many of the SPI-1 secreted proteins are translocated directly into the host cell and elicit direct effects on the host cell signal transduction pathways. SptP has tyrosine phosphatase activity within host cells which disrupts the actin cytoskeleton, likely freeing up G-actin monomers necessary for the formation of host membrane ruffles during *Salmonella* invasion (96, 177). SptP also has sequence similarity to the cytotoxins YopE from *Yersinia* spp. (268) and ribosyltransferase exoenzyme S from *P. aeruginosa* (177). AvrA has significant sequence similarity to AvrX, an avirulence protein from the plant pathogen *Xanthamonas campestris* pv. vesicatoria, and to YopJ/P from *Yersinia* spp. (311, 325). SipB and SipC are also secreted and are thought to affect the host actin cytoskeleton, as deduced by their ability to block *Salmonella* invasion when they exist as membrane-associated receptor chimeras in the host cell (36). They are further required for secretion of other proteins into host cells via the SPI-1 type III system (96). Without SipB and SipC, bacteria are able to secrete proteins but are not able to translocate them into host cells (339). InvH also appears to be important for secretion of proteins via the SPI-1 type III system, however its mechanism is unclear. It may

be acting to post-translationally modify proteins to be secreted via the type III system, but it has not been shown to be secreted itself (55).

SPI-2 is located at 31 minutes and also encodes components of a type III secretion system (150, 286). SPI-2 is present in most subspecific groups of *S. enterica*, but not all (238). It is interesting to note that while cross complementation between type III systems of different species can occur, *e.g. Salmonella sipB* can complement a *Shigella ipaB* mutant (98, 154) and a *Shigella spa24* could complement a *Salmonella spaP* mutant (121), many of the type III components of SPI-2 do not compensate for mutations in the components of SPI-1 within the same bacterium. However, this may reflect other factors, such as regulation of each SPI, since certain mutations in SPI-2 affect the transcription of SPI-1 genes and the ability of *S. typhimurium* to secrete SPI-1 effector proteins and to invade cultured cells as well (60). The function of the genes within SPI-2 appear to be divergent from those of SPI-1, and are necessary for intra-macrophage survival and dissemination throughout the host rather than invasion. Mutations within various SPI-2 genes resulted in reduced systemic spread of the infection within the mouse, although colonization of the intestinal Peyer's patches was still seen (46). A recent report has further implicated the SPI-2 region as necessary for bacterial replication in the host rather than bacterial survival *per se* (285). The secretion apparatus (*ssa*) is encoded within two regions: a 10 kb operon containing the genes *ssaK, L, M, V, N, O, P, Q, R, S, T,* and *U* (152) and a second smaller operon containing the genes *spiC, A, B* (239) (*ssaED, C, B*) (153). These two regions are separated by a region containing the potential effector (*sse*)/chaperone genes *sseA, B, C, D, E, F,* and *G* (153). The regulatory genes, *ssrA* and *ssrB*, are transcribed in the opposite direction to all the other genes within SPI-2. While many of the potential effector proteins have not been shown conclusively to be translocated into host cells, their expression is upregulated by intra-macrophage *Salmonella* (46, 321, 322). Mutations within various structural genes have been observed to have pleiotropic effects resulting in the increased sensitivity of the bacteria to complement, gentamicin, and polymyxin (60).

SPI-3 (24) is a 17 kb region located at 82 minutes which is inserted within the *selC* tRNA locus. It contains at least two genes which contribute to bacterial survival within macrophages, *mgtCB* (24). Although the G+C content of *mgtCB* is similar to the overall G+C content of 52-54%, they are immediately surrounded by regions with lower G+C content, (39.8 and 49.3%). These genes are transcribed in an operon and enable *Salmonella* to transport magnesium in low $Mg^{2+}$ conditions (225). Although *Salmonella* contain other magnesium uptake systems, *mgtCB* are tightly regulated by the PhoP/PhoQ two-component regulatory system (see below) and required for full virulence in mice (312). It has been suggested that MgtB has another function besides magnesium transport that is required by the bacteria inside host cells (290, 291).

SPI-4 is a 27 kb region located at 92 minutes on the *S. typhimurium* chromosome which is inserted between *ssb* (encoding the single-stranded DNA binding protein) and *SoxSR* loci (encoding a superoxide regulatory gene) at sites encoding a tRNA-like gene (337). There are two 9 kb regions which have lowered G+C contents (37% and 44%), and one 7 kb region with a G+C content of 54%. SPI-4 encodes for 18 putative proteins, including seven with homology to type I secreted toxin proteins including *Pasturella haemolytica* leukotoxin and *Bordetella pertussis* adenylate cyclase-hemolysin. This region is also important for intramacrophage survival. An insertional mutation in this region renders the bacteria unable to grow within macrophages (13)

SPI-5 is an 8 kb region located at 25 minutes in *S. typhimurium* and in *S. dublin* (159, 338). It is also present in other *Salmonella* serovars but not in *Shigella sonnei, E. coli,* or *Yersinia pseudotuberculosis*. This island appears to consist of six genes: *pip D, orfX) sigD/sopB, sigE /pipC, pip B,* and *pip A,* which are flanked by the tRNA *serT* on one side and *copS* on the other side. Of these genes, *sigD/sopB* is secreted by the SPI-1 type III secretion apparatus and *sigE /pipC* is thought to be its specific chaperone (101, 159). In *S. dublin*, SopB has been shown to be an inositol phosphate phosphatase which acts to increase chloride secretion by the host cell. Activities of SopB include the hydrolysis of phosphatidylinositol

3,4,5-trisphosphate, an inhibitor of calcium-dependent chloride secretion and of inositol 1,3,4,5,6-pentakisphosphate, an indirect inhibitor of phosphatidylinositol 3,4,5-trisphosphate-dependent chloride secretion (235).

Mutations within the *S. typhimurium* homologs, *sigDE*, are reported to reduce bacterial invasion by ten-fold (159), however this is not observed with the *S. dublin* genes, *sopB* and *pipC*. Nonetheless, the enteropathogenicity of *S. dublin* is greatly reduced in calf ileal loop models with mutations the *sopB* and all the *pip* genes, although systemic disease appears unaffected in a mouse model (338).

**b. Other virulence genes**

SopE is secreted by the type III secretion system of the SPI-1 (140, 339), yet it is encoded outside of centisome 63 within a cluster of genes from a cryptic P2-like prophage (141). The *sopE* gene was not found to be present in all *S. enterica* serovars: *S. dublin,* a number of *S. typhimurium* isolates, as well as *S. typhi* contained the gene, whereas *S. choleraesuis* did not. The SopE protein is secreted into the host cell cytoplasm in a *sip*-dependent manner (339) where its actions include the activation of cytoskeletal rearrangements along a CDC42-dependent pathway (140) and the activation of JNK. Furthermore, SopE was able to bind to several (but not all) members of the Rho subfamily of GTPases to promote nucleotide exchange *in vitro* (140). Altogether, SopE is capable of stimulating signaling pathways that can lead to both nuclear and morphological responses by the host cell (140).

SopD (in *S. dublin*) is translocated into host cells and is secreted without amino-terminal processing, however direct secretion via the type III pathway has not been shown. It is located downstream of the *cysJIH* operon, outside of the known pathogenicity islands. SopD acts in concert with *S. dublin* SopB (SigD in *S. typhimurium*) to cause enteropathogenicity in the host, although the nature of its activity remains obscure (173).

A number of flagellar genes have also been implicated in *Salmonella* virulence (82). While flagellar mutants are still able to invade cells with high efficiency *in vitro*, the ability to

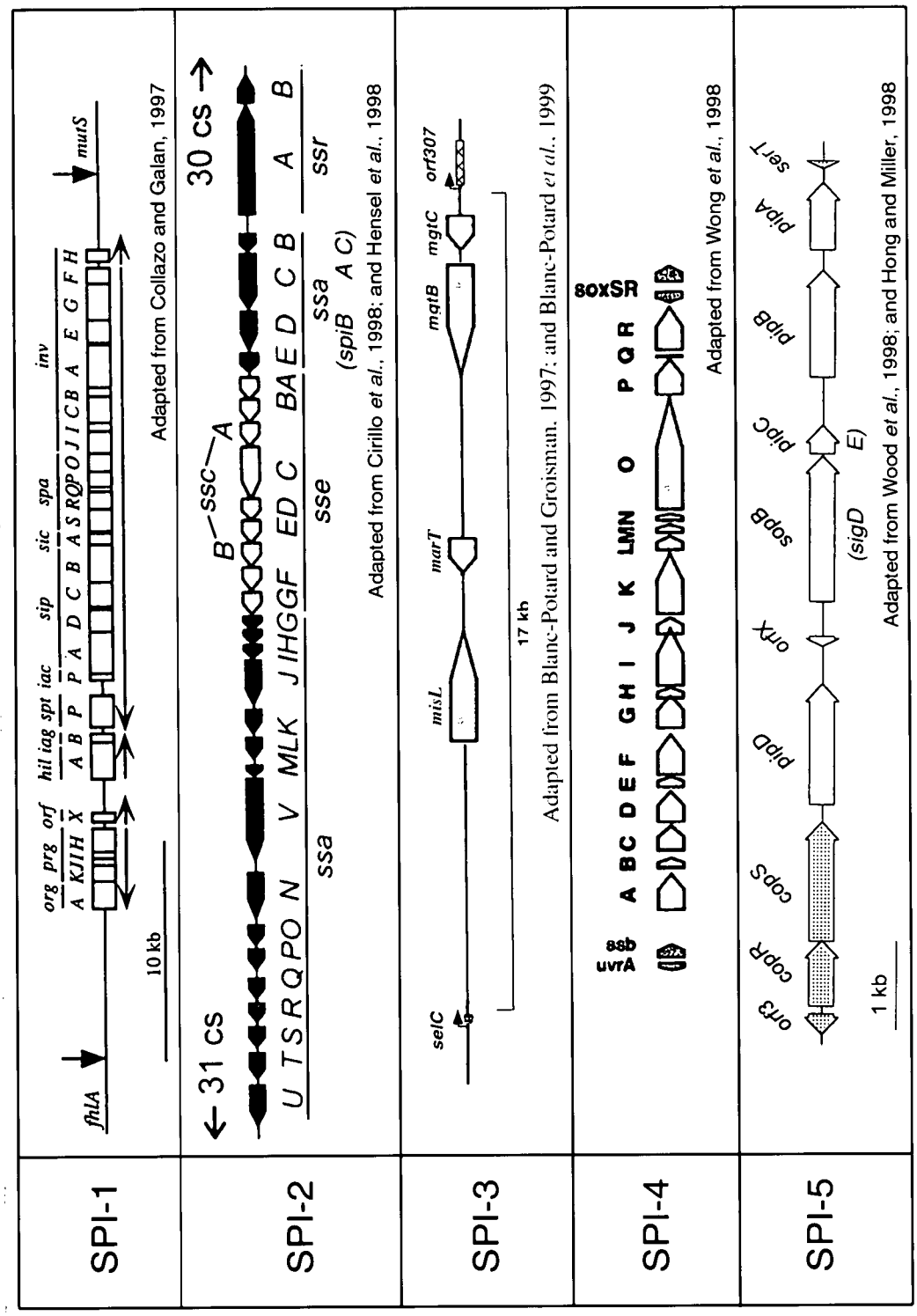



Figure 2: Schematic representation of *Salmonella* pathogenicity islands.

smooth swim has been shown to be advantageous during the initial interactions with the cells (171). Smooth swimming may cause the formation of propulsive bundles and free the majority of the bacterial surface from steric constraints, which may occur when the flagellar filaments are randomly distributed during tumbly swimming. Furthermore, the swimming motion may help the bacteria propel through mucus layers towards the cells. Other flagellar genes involved with regulation, such as the alternative sigma factor FliA and a corresponding anti-sigma factor FlgM, may act to coordinately regulate diverse virulence-associated genes (283).

Multiple fimbrial adhesins, involved with the initial adherence of *S. typhimurium* to the intestinal mucosa, are required for full virulence of the bacterium in mice (324). There are at least four fimbrial operons known in *S. typhimurium*: *lpf* (long polar finbriae) (324), *pef* (plasmid-encoded fimbriae) (324), *fim* (type 1 fimbriae) (70, 82, 248, 324), and *agf* (thin aggregative fimbriae) (265, 307, 324). Mutations in any one of these operons only moderately affect virulence, but a combination of mutations within all four operons results in more than 20-fold attenuation of the bacteria when given orally to mice. However, there is evidence of other fimbrial structures and/or adhesive organelles which may allow for colonization of the intestinal cells in the absence of the fimbriae encoded by the known operons (324). A cell-contact-stimulated formation of filamentous appendages has been implicated in the triggering of bacterial entry into host cells (258) and these appendages have been termed "invasomes" (113, 340). The formation of these contact-stimulated filaments is thought to be secreted via a type III secretion system, however they are formed in the absence of the SPI-1 type III secretion system, and their presence is not itself sufficient to trigger bacterial invasion (258).

Resistance to cationic antimicrobial peptides is mediated either directly or indirectly through PhoP/PhoQ regulation of a number of genes including *pagB-pmrAB, sapE, G, H, J, L, M,* and various LPS biosynthetic genes (117, 120, 245). The expression of these genes increases *Salmonella* resistance to the polymyxin-CAP family of peptides (216), and may have effects on other systems as well, *e.g.* expression of *pmrE* also results in modifications to the lipid A portion of LPS (134, 136, 148). A gene not regulated by PhoP, *sapM*, is involved

with resistance against the neutrophil peptide (NP-1) defensin (135). Resistance to antimicrobial peptides may be effected via an energized efflux pump system (117), biochemical alteration/inactivation of the peptide (117), or the prevention of peptide binding to the bacterial surface by LPS (319).

*Salmonella* resistance to complement includes a number of other factors such as many of the SPI-2 genes encoding type III secretion apparatus, the virulence plasmid-encoded Rck protein (45, 145), as well as LPS structure. LPS oligosaccaride side chains act as a physical barrier to complement such that the components are deposited away from the membrane and the pore-forming complex cannot penetrate the bacteria (82, 223).

Reactive oxygen intermediate-mediated damage, such as lipid peroxidation and amino acid carboxylation, is countered by *Salmonella* in a number of ways. A bacterial copper- and zinc-cofactored superoxide dismutase SodC (mapping between centisome 23.8 and 29.6) prolongs bacterial survival in the presence of superoxide *in vitro* and within a mouse model (75). SodA, a manganese-cofactored superoxide dismutase, may confer resistance to the early oxygen-dependent microbiocidal mechanisms of phagocytes, especially in the absence of iron (316). Resistance to alkyl peroxides is conveyed via an alkyl hydroperoxide (*ahp* ) (93), while resistance to hydrogen peroxide is conferred by catalase (*katG* and *katE*), as well as by a 59 kD protein (303). Reactive nitrogen intermediates are combated by homocysteine production by *Salmonella* which may directly interact with S-nitrosothiols such as S-nitrosoglutathione (59). S-nitrosothiols act as nitric oxide donors and have also been shown to have broad-spectrum microbiostatic activities themselves (59). Glucose 6-phosphate dehydrogenase has been shown to protect *S. typhimurium* against both oxygen and nitrogen intermediates *in vitro*, and is important during the early stages of salmonellosis (200).

LPS and various LPS-biosynthetic genes play an important role in the virulence of *Salmonella*. Not only do they act to provide resistance to cationic peptides and complement, but the lipid A portion of LPS is toxic to host cells and causes an endotoxic shock in the host.

LPS further is able to act as a chemokine and a macrophage activation factor (16), and may also be involved with the ability of *Salmonella* to transcytose cell layers (85).

Other factors known to be important for the survival of *S. enterica* species within the host are extra-chromosomal and found to be encoded within a large virulence plasmid (50-90 kb) (21, 128, 242). The *Salmonella*-plasmid virulence genes, encoded by the operon *spvRABCD* (129, 181), are upregulated within cultured cells (77) although they have not been found to be important for intracellular growth *in vitro* (128). These genes are important for extracellular growth under nutrient-limited conditions however (253). They are not required for bacterial invasion into cultured cells (44, 232, 330), but promote survival and growth of the bacteria within animals thereby increasing bacterial virulence (127, 130, 131, 327). The operon is regulated by a number of factors which include nutrient limitation (66, 72), short-chain fatty acids (66), RpoS (66), and SpvR (54, 296). SpvR is the operon regulator and is able to upregulate its own expression, while SpvA downregulates the operon (296). SpvB, C, D are thought to be membrane-associated proteins, although their activities remain obscure. The virulence plasmid may be involved with the lysis of resident and activated peritoneal macrophages *in vivo* which could influence the net growth of *Salmonella* during infection (124).

### 1.3.3. Regulation of virulence genes

It is known that bacterial pathogens, such as *Salmonella*, tightly regulate the expression of many of their genes required for virulence. Many of these genes are coordinated in response to very specific environmental conditions, *e.g.* in response to heat or osmolarity, and often there are multiple regulators for a particular virulence gene such that its expression, and subsequent translation, are in response to a variety of environmental parameters. The expression of *Salmonella* invasion genes are in response to specific environmental conditions resulting in the production of new proteins immediately prior to bacterial invasion (201).

### a. Environmental regulation

The environment provides many clues to pathogenic bacteria. Temperature, specifically the normal body temperature of the host, often serves to activate virulence genes, *e.g. Yersinia* only make Yops at 37°C, not at 26°C (211). Interestingly, the *Salmonella* virulence genes do not seem regulated to any great extent by temperature. pH serves to activate numerous *Salmonella* virulence genes (3, 211, 257), including Fur-regulated genes which are activated at low pH (317) and many of the type III secretion genes in SPI-1 which are expressed under slightly alkaline conditions (161). Starvation for different nutrients (*e.g.* carbon, nitrogen) results in the activation of RpoS regulated genes such as *spvRABCD* (66, 72). Environmental cues known to affect the expression level of a number of virulence genes, including those found in SPI-1, are osmolarity (12, 211), oxygen tension (12, 75, 91), growth phase (189), as well as the level of DNA superhelicity (100, 178). The effects of supercoiling on gene regulation is known to affect local gene expression, and can even affect the transcription of regions which are kilobases away (*e.g.* promoter relay) (74).

### b. Genetic regulation

*Salmonella* encodes two-component systems for the global regulation of genes, including both virulence and housekeeping genes. Examples of these two-component systems are encoded by the genes *phoP/phoQ* , *pmrA/pmrB,* and *ompR/envZ.* These systems consist of a sensor protein and a regulator protein. The sensor protein (PhoQ, PmrB or EnvZ) is a histidine kinase which spans the bacterial membrane with its extracellular domain acting to sense external signals and the intracellular domain acting as a kinase (329). Upon stimulation, the sensor protein undergoes autophosphorylation and is then able to phosphorylate the regulator protein (PhoP, PmrA or OmpR). This phosphorylation changes the ability of the regulator protein to bind to specific DNA sequences, allowing it to act as a transcriptional activator and/or repressor (12, 80, 102, 103). In addition, these genes are often positively autoregulated, and include regulation via two separate promoters, one of which is environmentally-sensitive and one which is not (135, 292). Moreover, the two-component

regulatory systems can interact to process multiple environmental signals in a complex hierarchical system (118, 135, 294).

The PhoP/PhoQ regulatory system (located at 27.4 centisomes (167) has been shown to modulate the expression of over 40 genes within *S. typhimurium* including the *phoPQ* operon itself, resulting in the activation or repression of these genes in response to external magnesium and calcium concentrations (103, 118, 211, 293, 312), pH (12, 15, 118, 211), osmolarity (12), oxygen tension (18), and starvation for both phosphate and carbon. There is evidence for the direct involvement of PhoP to all the conditions (329), however, PhoQ appears to respond specifically to magnesium and calcium (102, 118). The pathogenic properties which are regulated by the PhoP/PhoQ system include intra-macrophage survival (118, 215), resistance to antimicrobial peptides, such as polymyxin and NP-1 defensins (104, 135, 137, 212), the formation of spacious vacuoles (5), adaptive mutagenesis to growth-dependent mutations (139), and the down-regulation of the ability of phagocytic cells to present bacterial antigens to T-cells (332). Genes activated by this system are referred to as PhoP-activated genes (*pag*) and many virulence *pag*'s promote intracellular survival. Examples of virulence genes activated by PhoP include *mgtCB* (293), *pmrAB* (135), *pagP* (137) and possibly *pagC* (214, 304), *pagD, pagJ, pagK,* and *pagM* (19, 132-134, 136). Genes which are repressed are known as PhoP-repressed genes (*prg*), and many are involved with bacterial invasion and early survival within the phagolysosome (213, 332). Examples of virulence genes repressed by PhoP include SPI-1-encoded genes *hilA, prgHIJK, sip/sspA, sipC, invF* and *orgA* (246). A balance between activation and repression is needed for full virulence *in vivo*, since constitutive expression of PhoP attenuates *Salmonella* virulence and survival (215). Many genes responding to regulation by PhoP/PhoQ are co-regulated by other systems, such as activation by SirA (167), or may be responding to other regulators which also respond to PhoP, such as HilA (12, 18, 167) or PmrA (103, 120, 294).

The PmrA/PmrB regulatory system has been shown to modulate genes directly in response to magnesium concentrations and to pH (120, 294), as well as indirectly in response

to regulation by PhoP/PhoQ (135). Furthermore, many genes which are regulated by pH are coordinately regulated by both PhoP/PhoQ and PmrA/PmrB (15, 103, 294). The PmrA/PmrB system confers bacterial resistance to a number of cationic antimicrobial peptides, such as polymyxin (but not NP-1 defensin) through regulation of the *pmrE/pagA* and *pmrF* loci (120).

The OmpR/EnvZ regulatory system (located at 75 to 80 centisomes) (230) has been shown to regulate the formation of spacious vacuoles in macrophages, and affect the ability of the bacteria to kill these cells (199), resulting in bacterial attenuation (61). The OmpR/EnvZ system controls the expression of *Salmonella* outer membrane proteins, such as OmpF, OmpC, and the tripeptide permease TppB (40, 198, 199), and also affects the formation of *Salmonella*-induced filaments within host cells (221). The acid tolerance response also is dependent on this two component regulatory system during bacterial growth in minimal media (89).

SirA (*Salmonella* invasion regulator) is a transcriptional activator which similar to the two-component response-regulator FixJ/UvrY subfamily (167). FixJ of *Rhizobium meliloti*, has been shown to activate target gene expression after phosphorylation in response to changes in environmental oxygen (167). Although it is located outside of any identified pathogenicity island at 42.4 centisomes, SirA is known to activate a number of pathogenicity island-encoded genes, including SPI-1 encoded *hilA* and SPI-5 encoded *sigDE*. It is unclear whether its effects on these genes are direct or indirect (167).

HilA is encoded within SPI-1 (190) and is a transcriptional activator related to OmpR (11), although it contains neither a phosphoryl acceptor nor a membrane-spanning domain. It is required for the activation of many SPI-1 encoded invasion genes including the *inv--spa--prg* operon and *invF* (which further promotes *sipBCDA* expression). The *hilA* locus is coordinately regulated by both PhoP (12, 167) and SirA (167), although its expression does not necessarily affect the transcription of other PhoP or SirA regulated genes, *e.g. sigDE* transcription is unaffected by HilA but is dependent on SirA (159).

SlyA, originally reported to be a cytolysin (197), is actually a transcriptional regulator of *S. typhimurium* which is upregulated within macrophages and by entry into stationary

phase (29, 269). Other members of this family of proteins include MarR and EmrR which provide resistance to a wide range of toxic compounds by regulating the expression of membrane efflux systems. SlyA is required for bacterial resistance to oxidative products such as hydrogen peroxide, but not to nitric oxide products. When this gene is put into *E. coli*, it activates the cryptic hemolysin *clyA*, although the presence of a ClyA-like toxin in *Salmonella* has not been shown (29). Mutations within *Salmonella slyA* result in attenuation in mice by oral, IP, and IV routes of infection, and this gene has been implicated for intracellular survival rather than for the initial invasion or colonization steps (56).

Fur (ferric uptake regulator) is a gene repressor (located at 16.9 centisomes (230)) which is inactivated when iron availability is low allowing for the activation of genes involved in iron acquisition (88, 317). It is required for intra-macrophage survival (317). It is further required for a transient *Salmonella* acid-tolerance response (89, 191, 333), although this effect may be both direct and indirect as pH has a varied effect on different iron-regulated genes (88, 317). The Fur repressor may also act on genes required for resistance to reactive oxygen intermediates, such as *sodA* (316). The role of Fur regulation in bacterial virulence is unclear (107).

RpoS (sigma-38) is an alternative sigma factor which is required for *Salmonella* virulence. The avirulence of LT2 strains of *S. typhimurium* results (at least in part) from a defective *rpoS* gene (308, 334). RpoS is used to transcribe genes required during times of carbon and nitrogen starvation (73), during the accumulation of metabolic products (such as short-chain fatty acids) (66), and when the bacteria enter stationary phase (71, 335). While the expression of *rpoS* has been shown to increase after invasion into cells (41) and *rpoS* is thought to be exclusively required for systemic infection (125, 330), more recently it has been shown to play a role in the colonization of Peyer's patches (231). An example of virulence genes regulated by RpoS include the *spv* plasmid-encoded genes, which are required for long term survival of *Salmonella* in the host. However, not all genes upregulated in stationary phase use RpoS. For example, the increase in *slyA* expression during stationary phase is

independent of RpoS (29), and induction of SpvR by RpoS occurs during exponential growth but not during stationary phase (66). RpoS is also required for a sustained, low-pH-inducible acid tolerance response in *S. typhimurium* that occurs in stationary phase (88, 191, 192). It is not required for the acid tolerance response in logarithmic phase growth, although it may co-regulate the *atrB* locus which is required (191, 333).

SpvR is a virulence plasmid-encoded transcriptional activator which is upregulated during stationary phase (54, 296, 335), as well as during logarithmic phase in minimal salts media (but not rich media) (54, 335), and upon entry into host cells (77, 261, 262). SpvR positively regulates an operon immediately downstream encoding genes *spvABCD* (287, 296, 323). These genes are required for both gastroenteritis and systemic infection by the bacteria within animal models (196), and more recently they have been shown to be essential for replication of *Salmonella* within macrophages in the host (131). SpvR is acted upon by a number of different factors, including the upregulation by RpoS and autoregulation (335). It is downregulated by high levels of SpvA (296, 323), by iron (296), as well as cyclic-AMP (cAMP) and the cAMP receptor protein (CRP) (71, 236). SpvR has been shown to be regulated by a two-step model of transcription activation in a similar manner to LysR of the *Vibrio* luciferase operon (287).

## 1.4. Reporter systems for bacterial gene expression

Reporter enzymes make it possible to visualize the expression of bacterial genes whose products are not readily assayable. The expression of a specific gene can be monitored by first fusing that gene with a promoterless reporter gene and then measuring activity of the reporter gene product. However, the reporter enzyme must meet at least two criteria. First, it must have an activity that is distinct from endogenous cellular or bacterial enzymes in the system used. Second, (relative to the studies here), the reporter enzyme assay must be very sensitive, as numbers of invasive bacterial are often low and bacterial gene expression may be moderated.

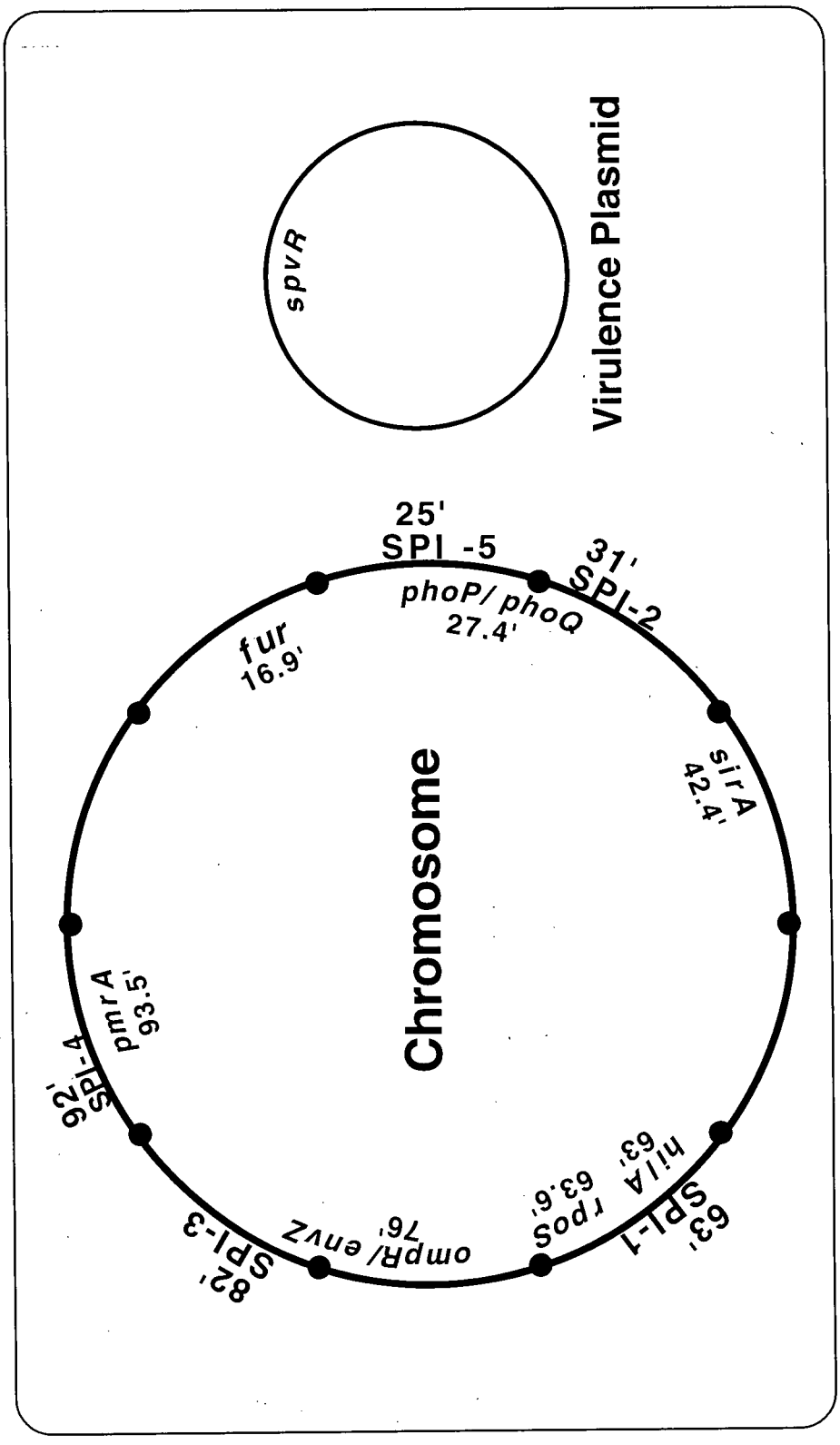



**Figure 3:** Schematic representation of the *Salmonella typhimurium* chromosome, which is divided into 100 centisomes. Known pathogenicity islands are shown on the outside of the chromosomal circle; while a number of virulence gene regulators are shown on the inside.

### 1.4.1. Reporter genes

#### a. ß-galactosidase

ß-Galactosidase has traditionally been the most widely used bacterial reporter enzyme and is a sensitive reporter of gene expression (38, 89, 163, 289, 293). It is encoded by a single gene, *lacZ*, which may be fused to a gene of interest as either a translational fusion, resulting in a hybrid protein, or a transcriptional fusion, in which both reporter and target genes are expressed under one promoter but the reporter protein is separate from the coding region (38). ß-galactosidase hydrolyzes a number of commercially-available substrates which may be assayed colorimetrically (28, 163), fluorescently (28, 77, 108, 251, 289) or chemiluminescently (28). The activity of the enzyme may be detected under a wide range of biochemical conditions (on agar plates, in solution, or within host cells), and does not require any co-factors for its activity (*e.g.* oxygen or bacterial energy). However, enzymatic activity persisting in a killed bacterium can not be differentiated from the activity in a viable bacterium and may possibly result in an apparent higher activity per bacterium than really exists. Furthermore, the bacterial and host cell membranes are not permeable to ß-galactosidase substrates and must be lysed in order to quantify the enzymatic activity in solution. Finally, while *Salmonella* do not produce an intrinsic ß-galactosidase, host cells do have a low level endogenous activity (28).

#### b. Bacterial luciferase

Bacterial luciferases may be found in many different bacteria and are generally encoded by two genes, *luxAB*, which result in a heterodimeric enzyme (210), and are transcribed as part of an operon (222), which also encodes gene products necessary for the production of an aldehyde substrate (210). In the absence of the substrate synthesis genes (*e.g. luxAB* only), the substrate must be added to the sample (138). In addition to an aldehyde substrate, the luciferase requires the presence of oxygen (143) and the bacterial energy source, $FMNH_2$ (17, 186), but does not require metals or cofactors (210). The product of enzymatic reaction is light in the visible range between 478-505 nm (17, 266). Most eukaryotic luciferases (*luc* firefly or

beetle luciferases (28)) are encoded by a single gene and require ATP as an energy source. They are more susceptible to changes in pH, ionic strength, and temperature than are the bacterial luciferases, and also require a metal cofactor (266).

Of the hundreds of different strains of luminescent bacteria, the most widely used reporter genes come from *Vibrio fischeri* (69, 92, 210), *V. harveyi* (187, 193, 210), *Photorhabdus (Xenorhabdus) luminescens* (52, 86, 210), *Photobacterium phosphorum* (210), or *P. leiognathi* (210). The most distinguishing feature between the different luciferases is their optimal temperature for activity. Luciferase from *V. fischeri* is stable and active between 20°C and 30°C but not at 37°C, whereas luciferases from *V. harveyi* and *P. luminescens* are stable at 37°C or higher (209, 251). The use of bacterial luciferases offers several advantages over other reporters. The luciferase aldehyde substrate, *n*-decanal, is volatile, amphipathic, and readily crosses membranes (138, 155, 210, 244, 276), unlike most ß-galactosidase substrates [Garcia-del Portillo, 1992 #22; (28, 77). Therefore, luciferase activity can be assayed without the need for bacterial or host cell lysis, and potentially, expression could be assayed within the same sample over time (186, 251). It is also possible to monitor bacterial gene expression from within a whole animal (35, 52). As well, unlike ß-galactosidase, most bacteria and tissue culture cells have no endogenous luciferase activity (52). Any light produced is the direct result of luciferase activity. Furthermore, light production is also an indicator of bacterial viability (180, 301, 302). In the absence of a sufficient supply of flavin mononucleotide ($FMNH_2$) within the bacteria, light is not produced even if luciferase is present, resulting in measurement of activity from viable organisms only (52).

### c. Other reporter genes

It has been possible to demonstrate the upregulation of various genes by *Salmonella* inside tissue culture cells using various other reporter genes. Examples include *papA* (type I fimbriae) (262), *cat* (chloramphenicol acetyl-transferase) (297), *phoA* (alkaline phosphatase) (28, 157), *gusA* (ß-glucuronidase) (28), and *galK* (galactokinase) (300).

### 1.4.2. Other detection methods

*In Vivo* Expression Technology (IVET) is a technique which enables researchers to positively select for *Salmonella* genes which are expressed upon infection of a host animal (147, 202, 203, 289). The bacteria are engineered with a promoter trap in which bacterial promoters drive the expression of a gene cassette. This cassette contains a gene which is required for growth in the host but can be supplemented for *in vitro* (*e.g.* either *purA* (auxotrophy) or *cat* (antibiotic resistance)) and the reporter *lacZ* gene (146, 202, 203). The final gene fusions are generated on chromosomal DNA, rather than on a plasmid, using gene duplication in a manner that avoids gene disruption unlike regular transposon insertional fusions. Since the genes are contained on the chromosome and not on a multi-copy plasmid, it is likely that the genes are regulated under the natural promoter environment. IVET identifies genes which are expressed upon infection of mice rather than upon invasion of a particular cell type. Therefore, bacterial genes that are necessary at any time during infection will be identified using this technique, providing the specific bacterial clones survive within the host until they can be recovered. IVET does not indicate at which point the bacterial genes are required, *i.e.* are they transiently induced or expressed *in vivo* all the time, and a large number of the identified bacterial genes are not specific for virulence (*i.e.* housekeeping). As well, genes which may be inhibitory or refractory to recombination events in the bacteria may be under-represented. Furthermore, complementation in the animal may demand high levels of expression relative to growth on laboratory medium, indicating that small increases in gene expression may be overlooked.

Signature-tagged mutagenesis (STM) is a negative selection technique that is also based on *in vivo* selection of bacterial mutants (147, 151). Transposons carrying gene tags, which consist of 40 bp of unique DNA flanked by 20 bp regions of DNA common to all the tags, are randomly integrated into the *Salmonella* chromosome. A pool of tagged insertion mutants is then used to infect a host animal and those bacterial mutants which cannot be recovered from the animal are deemed to carry insertions within genes necessary for infection. The presence or

absence of a particular bacterial mutant is assessed by dot blot hybridization, with the hybridization signals from the recovered pool compared with those from the input pool. The complexity or size of the pools must be restricted such that each mutant is present in sufficient numbers so that avirulent mutants are not falsely identified and that the hybridization signals do not become too weak to identify.

Differential display also utilizes an *in vivo* mouse model, however it looks for gene expression in the absence of bacterial mutagenesis, *i.e.* without the use of reporter genes. It requires the subtractive hybridization of two bacterial cDNA libraries, with the resulting cDNAs are used as probes to search for *in vivo*-expressed bacterial genes (147). One cDNA library is made from bacteria grown on laboratory medium and represents the pool of 'housekeeping' genes. The other cDNA library is made from bacteria recovered from infected tissue, and represents those genes required for bacterial virulence. The 'housekeeping' genes are subtracted or removed from the pool of genes expressed upon infection, and the remaining genes in the 'infected' pool are then used to screen the bacterial genome in search of genes required specifically for virulence. The crucial step of this technique lies in the ability to isolate and stabilize the bacterial mRNAs in order to make a representative cDNA library. Unlike most eukaryotic mRNAs, bacterial mRNAs do not have a poly-A tail.

Differential fluorescence induction (DFI) (320) is another technique utilizing a DNA promoter trap, where bacterial genes are selected on the basis of their expression within cultured macrophages rather than a whole animal (147, 321, 322). The gene encoding the green fluorescent protein (GFP) is transcriptionally fused with *Salmonella* genes and then, with the use of a fluorescence-activated cell sorter (FACS), host cells containing intracellular bacteria with genes expressing high levels of GFP are detected and sorted. An advantage of this technique is the ability to determine the bacterial gene expression within a single macrophage (321). A disadvantage of this technique is that gene fusions to GFP are contained on a multi-copy plasmid, rather than in the chromosome. This was done to increase the range of fluorescence obtained from the various *gfp* fusions, implying that low levels of *gfp*

expression may be missed with this technique. Furthermore, regulation of the genes may not have reflected the true nature of the expression within the chromosomal environment, *e.g.* repressors and/or activator proteins may have been titrated out by the multiple copies of the gene promoters, and there would be no gene regulation in *cis* or by promoter relay. A second disadvantage arises from the use of pooled clones, where competition between bacteria during growth (either intracellular or extracellular expansion of the pools) may result in the loss of some fusions.

Using two-dimensional protein gels, it has been shown that bacteria inside macrophage cells express different protein profiles than those grown outside cells (1, 30, 34, 254). With this technique, radiolabelled proteins in bacteria and/or cells can be separated into a discrete spots on a gel by separating them on both their isoelectric point and their molecular size. More recently, a technique involving the use of radiolabelled diaminopimelate (a lysine precursor specifically used by bacteria) has been used to identify the patterns of intracellular bacterial protein expression, without contamination by host proteins (33, 34, 147). These techniques are very powerful, however many of the proteins have yet to be identified.

## 1.5. Summary of thesis

In the past six years there has been a veritable explosion of data regarding the mechanisms of virulence for not only *Salmonella* but many other gram-negative bacteria as well. When this study was initiated, pathogenicity islands and type III secretion systems were unknown. At that time, it had been shown that *Salmonella* expressed an unique set of genes inside macrophages, that were not expressed in the presence of other stresses (heat, low pH, starvation, oxidative stress) either singly or in combination (1, 30). Moreover, this gene expression differed slightly depending on the isolate of *Salmonella* used as well as the type of cultured cells used. It was determined that over 100 bacterial genes were upregulated by intracellular *S. typhimurium*, with over 40 genes deemed unique to the intra-macrophage environment. However, only a handful of these genes could be attributed to those already known (30). It had furthermore been determined that the ability of *Salmonella* to invade cells

and to grow intracellularly were separate events and each were required for virulence in animal models (78). Therefore, the goal of this study was to develop a system to search for *Salmonella* genes which were only expressed inside cells, and thereby uncover genes which may be essential for intracellular survival and perhaps virulence.

The first section describes the development of a light-based reporter system by which to detect genes expressed from intracellular *Salmonella*. Initially, reporter gene fusions were made to the *Salmonella* plasmid virulence genes *spvRAB*, which had previously been shown to be required for *Salmonella* virulence in a mouse model. The *spv* genes were also known to be induced by carbon and nitrogen starvation and during stationary phase growth. Using the enzyme ß-galactosidase (*lacZ*), the *spv* genes were shown to be upregulated after *Salmonella* invasion into epithelial cells (77). Similar results of induction were confirmed by two other studies, where the genes were shown to be upregulated within phagocytic cells as well (262). Nevertheless, the use of ß-galactosidase as a reporter was not ideal, and therefore bacterial luciferase was tested as alternate reporter of intracellular bacterial gene expression. The luciferase genes were fused to the *spv* genes in order to be able to directly compare between the two reporter systems. The results indicated that while luciferase was also not a 'perfect' reporter, it was as sensitive as ß-galactosidase and offered a number of advantages as a reporter, namely the ability to monitor gene expression while the bacteria were inside the cells and the ability to differentiate between the activity from live and dead bacteria (251).

The second section describes the construction of a library of *Salmonella* mutants to be used to search for genes upregulated by intracellular bacteria. Also described in this section is the initial screening procedure used to look for genes which were only upregulated once *Salmonella* had become intracellular. The promoterless luciferase reporter genes were inserted as single random insertions throughout the chromosome, using a modified two-plasmid competition system (138). Bacteria were then tested for their ability to produce little to no light outside mammalian cells, while producing light from the intracellular environment. This

allowed for the identification of genes which were repressed during growth in rich media, but were activated or upregulated during *Salmonella* growth within cells.

The final section describes the characterization of the *Salmonella* mutants which were found to contain upregulated gene fusions. Two of the genes identified in this screen were found to be part of SPI-5, *sigD/sopB* and *pipB* one gene was found to be encoded within SPI-2, *ssaR*; and the fourth gene was completely novel, *iicA*. No obvious difference in phenotypes could be identified while the bacteria grew *in vitro* when comparing the insertional mutants to the wildtype bacteria. The bacterial mutants retained their ability to invade and grow within cultured cells (at least during short time periods). Interestingly, all four gene fusions were not only upregulated within phagocytic cells, but were upregulated within non-phagocytic cells as well. The insertions within all of these genes reduced the *in vivo* virulence of *S. typhimurium* to varying degrees, except for *iicA* which retained its virulence in the mouse model.

Within this study, a reporter system was described which was specifically able to detect the gene expression from bacteria residing inside mammalian cells. Moreover, the activity could be correlated to viable bacteria. By incorporating this system into the bacterial genome, several *Salmonella* virulence factors were identified and characterized. That not all the genes upregulated by intracellular bacteria were found to be virulence factors indicates the increasing complexity of animal models over cell culture models.

# Chapter 2: Materials and Methods

## 2.1. Media and chemicals

### 2.1.1. Chemicals and Assay Reagents

Detergents Triton X-100 and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich Canada, Ltd. (Oakville, ON) and made up as 10% stock solutions in sterile water, which were stored at room temperature. Trypsin (Gibco Life Technologies; Burlington, ON) was stored at -20°C and used undiluted during the passage of epithelial cells. Dimethyl sulfoxide (DMSO) (BDH Inc.; Toronto, ON) was used as a cryoprotectant when freezing cultured cells, at a final concentration of 10%. Miscellaneous chemicals such as ethidium bromide, agarose, ethanol, methanol, phenol, chloroform, sodium chloride, sodium hydroxide, hydrochloric acid, etc. were purchased from a number of different companies including: Sigma-Aldrich Canada, Ltd., BDH Inc., VWR Scientific Canada, Ltd. (London, ON), Amersham Canada, Ltd. (Oakville, ON), Baxter-Canlab (VWR-Canlab; Mississauga, ON), Difco Labs - Fisher Scientific (Ottawa, ON), and Gibco-BRL (Burlington, ON).

*n*-Decanal (99%; Sigma) was used for luciferase assays and kept stored at 4°C in an airtight container. A range of aldehyde concentrations were made by first adding 1 µl *n*-decanal into 1, 2, 4, or 10 ml of a solution of 70% (v:v) ethanol:8% (v:v) methanol. One milliliter of this solution was then added to 4.5 ml of MEM + 10% FBS, resulting in concentrations of 0.02%, 0.011%, 0.0055%, and 0.0022% (v:v) *n*-decanal, respectively. Two further concentrations of aldehyde were made by adding 1 µl *n*-decanal to 100 or 200 µl ethanol:methanol, and then adding 100 µl of this solution to 1 ml of tissue culture media, resulting in aldehyde concentrations of 0.099% and 0.0495% (v:v) respectively. Use of alcohol allowed the even dispersal of the aldehyde in the solutions. The concentration of alcohol was 14.2% (v:v) for the four lowest dilutions, and 7.8% (v:v) for the two higher dilutions. Due to their long-term instability in suspension, aldehyde solutions were kept in airtight containers, at room temperature, for no longer than 4 h. (Note: the final concentration

of the aldehyde in the luciferase assay well is another 10-fold diluted: see "luciferase assay" below.) (251)

For fluorescent ß-galactosidase assays, the fluorescent substrate fluorescein-di-galactopyranoside (FDG) (25) and the ß-galactosidase inhibitor phenyl-ethyl-thio-galactoside (PETG) (58) were used (Molecular Probes; Eugene, OR) (77, 108). FDG and PETG were initially dissolved in DMSO, and then diluted and stored at a concentration of 50 mM at -20°C for up to 1 month. The final concentration of DMSO in the stock solutions was 25% (v:v). For chemiluminescent ß-galactosidase assays, the Galacto-*STAR* ™ assay kit was used according to manufacturers instructions (Tropix - Perkin Elmer; Bedford, MA).

### 2.1.2. Antibiotics

All antibiotics were purchased from Sigma. Ampicillin (amp; 100 µg/ml (w:v)), tetracycline (tet; 15 µg/ml (w:v)), streptomycin (str; 25 µg/ml (w:v)), and chloramphenicol (cm; 30 µg/ml (w:v)) were used as selection agents for plasmid maintenance and bacterial growth. Gentamicin was used to inhibit extracellular bacterial growth during invasion assays (310) at a concentration of 100 µg/ml (total weight per volume) for the first four hours and at 10 µg/ml for incubations longer than four hours.

### 2.1.3. Molecular Biology Reagents

#### a. Reagents

Restriction enzymes *Hae*III, *Rsa*I, and *Bam*HI were purchased from both New England Biolabs (Mississauga, ON) and Boehringer Mannheim Canada (Laval, QC). T4 DNA ligase and deoxynucleotides (dNTPs) were purchased from Boehringer Mannheim Canada. The dNTPs were made as a 2.5 mM stock solution and stored at -20°C; all four deoxynucleotides were in equal proportions. The polymerase AmpliTaq (Perkin-Elmer Applied Biosystems; Norwalk, CT) was used for inverse polymerase chain reactions (inverse PCR). For sequencing reactions, ABI's AmpliTaq Dye Terminator Cycle Sequencing chemistry with FS Taq was used as recommended by the Nucleic Acid and Protein Services Unit (NAPS Unit) at the University of British Columbia. Centrisep spin columns (Princeton

Separations; Adelphia, NJ) were used to remove unused dyed dinucleotides from the sequencing reactions.

**b. Primers**

DNA primers were made by the NAPS Unit and are listed in Table 1. The primers LUX76 and LUX340 were made to regions within the *luxA* gene; all other primers were made to regions on the *S. typhimurium* chromosome. Primer set LUX76:LUX340 was used to amplify the regions upstream from the *luxA* gene in all six clones initially identified. Primer sets E-MINUS:E-PLUS and E3615:E3805 were used to amplify the region around the insertion site of E12A2; chromosomal DNA from both E12A2 and SL1344 was used. Primer set G-MINUS:G-Plus was used to amplify the region around the insertion site of G7H1; chromosomal DNA from both G7H1 and SL1344 was used. All primers except for LUX340 were used for sequencing.

**Table 1: Primers for Inverse PCR**

| Primers | Length | Sequence (5' to 3') |
|---|---|---|
| LUX76 | 18 nt | CAA GCG ACG TTC ATT CAC |
| LUX340 | 18 nt | TGC CGC ACA TCT ATT AGG |
| E-PLUS | 20 nt | CAG TTT TCC AAT TAC CTC CC |
| E-MINUS | 22 nt | TTC TGG AGG ATG TCA ACG GGT G |
| E3615 | 24 nt | ACA GCG TGT AGA TTT GCA CAA CAC |
| E3805 | 21 nt | GAC AGG TAG TCA ACA TAC CCC |
| G-MINUS | 20 nt | GGA GGA ATG CAC ACC TTT AG |
| G-PLUS | 19 nt | TAG TCC CTA ACC CCC ATT G |

### 2.1.4. Media

Tissue culture cells were grown in either minimal essential media (MEM #410-1500; Gibco Life Technologies) or Dulbecco's modified Eagle medium (D-MEM #430-2800; Gibco Life Technologies), both of which were supplemented with 10% fetal bovine serum (FBS) (Gibco Life Technologies). DMEM++ refers to basic D-MEM supplemented with both 10% serum and 20 mM HEPES (pH 7.4). Phosphate-buffered saline containing calcium and magnesium (PBS++) was used for washing and dilutions.

Bacteria were grown in a number of different media. Luria-Bertani (LB) broth and agar plates, MacConkey plates, and SOC broth, were used for routine bacterial culture, and recipes can be found in Sambrook *et al.* (275) and Ausubel *et al.* (9). Special "green plates" were used to isolate phage-free bacteria after transductions and made as previously described (68, 300). The green plates contain the dyes Alizarin Yellow and Aniline Blue, and infected colonies appear dark green in color while uninfected colonies remain pale. Bacteria were also grown in DMEM++ where indicated.

### 2.1.5. Buffers

A number of different buffers were used throughout this study. Phosphate-buffered saline (PBS-- or PBS++) was used as a diluent for bacteria and cells where indicated. PBS consisted of 0.2 g/L KCl, 0.2 g/L $KH_2PO_4$, 8 g/L NaCl, and 2.16 g/L $Na_2HPO_4.7H_2O$ and the final pH was adjusted to 7.4 with HCl. PBS-- contained no calcium or magnesium, whereas PBS++ contained 130 mM calcium and 200 mM magnesium. HEPES buffer (pH 7.4) was used as an extra buffering agent in DMEM++. TAE buffer was used for making and running agarose gels (185). TE buffer was used for resuspending preparations of DNA (275), and 50 mM TE (*i.e.* 50 mM Tris pH 8.0 + 50 mM EDTA) was used with chromosomal DNA preparations, while a 5 mM TE concentration was used with plasmid DNA samples (*i.e.* 5 mM Tris pH 8.0 + 5 mM EDTA). Tris-HCl buffers (made in a range from pH 7.0 to 8.0) were used for molecular biological manipulations and made according to Sambrook *et al.* (275). P22 buffer was used during the dilution and storage of the bacteriophage P22 (300).

## 2.2. Equipment

Horizon mini-gel apparatus (BRL; Burlington, ON) were used in conjunction with an FB105 power source (Fisher Scientific; Pittsburgh, PA) was used for resolving DNA on agarose gels as previously described (275). DNA gels were stained with ethidium bromide (275) and visualized using a long-wavelength transilluminator (Fisher Scientific). Pictures were taken using a Polaroid camera and 667 film (Polaroid, Ltd.; St. Albans, Hertfordshire, England). The Gene Pulser™ from BioRad (Richmond, CA) was used to electroporate DNA into competent bacterial cells. The Ericomp Twinblock System thermocycler (Ericomp Inc.; San Diego, CA) was used for PCR reactions (both inverse PCR and sequencing reactions). Sequencing gels were made and run by the NAPS Unit and the DNA sequences were determined using an automated 373 DNA Sequencer. Sequences were further analyzed using NCBI's BLAST program and the GenBank database.

Three different sets of apparatus were used for determining light production from bacterial samples. First, microtiter plate formats were exposed to X-ray film (X-OMAT by Eastman Kodak Company; Rochester, NY) and the resulting spots were quantitated with a computing densitometer (Molecular Dynamics; Sunnyvale, CA). Second, light from tubes of bacteria was measured using the 1250 Luminometer (LKB-Wallac; Finland). Third, the Luminograph LB980 (EG&G Berthold; Germany) (26) was used to measure light from a number of formats including agar plates and microtiter plates. Fluorescence was monitored in 96-well microtiter Fluoricon assay plates (IDEXX Corporation; Wesbrook, ME) which were read on a PANDEX Fluorescence Concentration Analyzer (IDEXX; Portland, OR).

Cultured cells were routinely expanded in sterile, flat-bottomed, tissue culture flasks (Becton Dickinson Canada; Mississauga, ON), which were 25 $cm^2$, 75 $cm^2$, or 125 $cm^2$ in size. During passage of the cultured macrophages, the cells were scraped with 25 cm disposable cell scrapers (Corning-Costar, Fisher Scientific; Pittsburgh, PA). For the invasion and reporter assays, cells were cultured overnight in 96-well plates, which were either completely clear (Falcon Microtest III tissue culture plates; Becton Dickinson Canada;

Mississauga, ON), or contained clear wells with white grids between each well (Immunoware 8-Well EIA strip plates; Pierce, Rockford, IL), or contained black wells with clear bottoms (Costar; Fisher Scientific). The EIA plates were sterilized with 70% ethanol prior to seeding with cells; all other tissue culture plates used were previously sterilized by gamma irradiation. For the ß-galactosidase assays, samples containing chloroform were transferred to Falcon Microtest III Flexible Assay Plates (Becton Dickinson Canada) for incubation, since the polyvinylchloride (PVC) plastic was more tolerant to chloroform than the polystyrene plastic of the other plates. Bacterial optical density was determined using an Ultraspec Plus Spectrophotometer (Pharmacia Biotech; Uppsala, Sweden).

Miscellaneous equipment such as Eppendorf tips, disposable pipettes, and sterilizing filters were purchased from VWR Scientific Canada, Ltd. (London, ON), Millipore Canada, Ltd. (Nepean, ON) and Amicon Division, W.R. Grace & Co.- Conn. (Beverly, MA).

## 2.3. Strains and Plasmids

### 2.3.1. Cells lines

Non-phagocytic cell lines used included Madin-Darby canine kidney cells MDCK (ATCC number: CCL-34) and the human epithelial cells HeLa (ATCC number: CCL-2). Phagocytic cells used included the BALB/c mouse macrophage-like cells J774A.1 (ATCC number: TIB-67) (255), and the mouse bone marrow-derived macrophage cell line BALB.BM1. For the initial studies, the MDCK and HeLa cells were grown in MEM at 37°C in an atmosphere of 95% air-5% $CO_2$, however for later studies, all cells were grown in DMEM++. For most studies, the cell lines were used between passages 5 and 20, although for the results obtained in Chapter 3 the cells may have been passaged up to 30 times.

### 2.3.2. Bacteria

The bacteria used in this study are listed in Table 2. The bacterium *S. typhimurium* SL1344 (158) was used throughout for all manipulations. All *Salmonella* strains were tested by agglutination to antiserum specific for *S. typhimurium* group B, O-antigen factors 1, 4, 5, 12 (Difco Labs).

**Table 2: Bacterial strains**

| Strain | Bacterial Type | Antibiotic Resistance | Other Features | References |
|---|---|---|---|---|
| **DH5α** | *Escherichia coli* | Naladixic acid | r-m+, *recA* | Sambrook *et al.*, 1989 |
| ***S. dublin* Lane** | *S. dublin* | Cm (≤6 μg/ml) | Wild type; virulence plasmid | Guiney *et al..*, 1990 |
| **LD842** | *S. dublin* Lane | Cm (≤6 μg/ml) | Cured of virulence plasmid | Fang *et al.* 1991 |
| **SL1344** | *S. typhimurium WRAY from S2337/65 parent* | Str | Used as wild type; *his*G46; virulence plasmid | Hoiseth&Stocker 1981 |
| **A1A1** | SL1344 | Tet & Str | *sigD::luxAB* | This work |
| **D11H5** | SL1344 | Tet & Str | *ssaR::luxAB* | This work |
| **E12A2** | SL1344 | Tet & Str | *sigF::luxAB* | This work |
| **G7H1** | SL1344 | Tet & Str | *iicA::luxAB* | This work |

### 2.3.3. Bacteriophage

The methods for isolating, storing, and using the bacteriophage P22 are described in (62, 63, 226, 278-282, 300, 336). The phage P22HT*int* was used as a vehicle to transfer both plasmids and chromosomal insertions from one *Salmonella* to the next, while P22H3 was used for cross-streaking experiments in order to determine whether bacteria were phage-sensitive (true transductants with no remaining phage lysogens) or resistant (lysogens) after transduction.

### a. Preparation of phage P22 stock

Bacteria were grown overnight in LB broth at 37°C shaking at 200 rpm. To 1 ml of this culture was added 4 ml of a P22 lysate containing $5x10^6$ plaque-forming units (pfu) per ml. This mixture was further incubated with aeration overnight at 37°C. The next day, a few drops of chloroform were added to the culture and incubation continued for another 10 min. The cells and debris were removed with centrifugation (*e.g.* 10 min at 10000 rpm). The clarified supernatant was then placed in a sterile tube and stored at 4°C. A couple drops of chloroform were added to this phage lysate stock to prevent bacterial growth during long-term storage.

Original phage P22 stocks of P22HT*int* and P22H3 were made using the host *Salmonella* strain SL1344. The phage P22HT*int* stock containing the plasmid pFUSLUX was then made in a similar way by making a lysate from the host bacteria SL1344 pFUSLUX, into which the pFUSLUX had originally been electroporated. The phage P22HT*int* was used because it cannot lysogenize within the host bacterium and also has the ability to mobilize plasmid DNA. The stocks typically contained $10^{10}$ pfu/ml, which was diluted one-hundred fold for use during transduction.

### b. Transduction of *Salmonella* with phage P22

Bacteria were grown overnight in LB broth at 37°C shaking at 200 rpm. From the overnight culture, a 1:100 dilution of the bacteria was made into fresh LB media and grown to approximately $1\text{-}5x10^8$ colony-forming units (cfu) per ml. One volume of bacteria was then mixed with a one-tenth volume of the appropriate P22HT*int* phage (at $1x10^8$ pfu/ml), and held at room temperature for 10 min. One hundred microliter aliquots were then plated onto LB plates, containing the appropriate antibiotics, and the plates incubated overnight at 37°C.

The resulting colonies were then purified on "green plates" to ensure they were not undergoing active infection, nor contained lysogenized phage. As stated earlier, on these specialized plates the infected bacterial colonies turned dark green, while uninfected colonies

remained a pale green color. The pale green colonies were further tested by cross-streaking them against a line of P22H3 phage lysate, which resulted in infected dark green colonies only if the original colony was lysogen-free.

### 2.3.4. Plasmid Preparation

**Table 3: Bacterial plasmids**

| Plasmid | Antibiotic Resistance | Other Features | References |
|---|---|---|---|
| **pACYC184** | Cm (>12 µg/ml) & Tet | Low copy number plasmid | Chang&Cohen 1978 |
| **pFF14** | Cm (>12 µg/ml) | *spvB::lacZ* translational fusion *(spvRAB::lacZ)* | Fang *et al.* 1991 |
| **pSPLUX** | Cm (>12 µg/ml) | *spvB::luxAB* transcriptional fusion *(spvRAB::luxAB::lacZ)* | Pfeifer&Finlay 1995 |
| **pTF421** | Amp | RNA1 overproduction | Guzzo&DuBow 1991 |
| **pFUSLUX** | Tet | *Tn5::luxAB::tet* gene cassette; ColEI origin of replication | Guzzo&DuBow 1991 |

The plasmids used in this study are listed in Table 3. The plasmid pFF14 containing an *spvB::lacZ* translational fusion was previously described by Fang *et al.* (72). The plasmid pSPLUX was made by inserting the *luxAB* gene cassette (251) into the *Bam*HI site of pFF14, as shown in Figure 11, to create a transcriptional fusion between *spvB* and *luxA*. Both pFF14 and pSPLUX are low copy number plasmids derived from pACYC184 (39). The plasmids pTF421 and pFUSLUX were used in concert (as described below) to randomly insert *luxAB* into the *Salmonella* chromosome (138).

## 2.4. Molecular Biology

### 2.4.1. DNA Isolation

#### a. Plasmid Preparation

Plasmid preparations were made using the alkaline lysis technique described in Sambrook *et al.* (275), with an extra phenol:chloroform step added. Bacterial plasmid DNA preparations were made from 2 ml overnight cultures. Isolated plasmid DNA was resuspended in either 5 mM TE or sterile water. Qiagen plasmid kits (Qiagen Inc.; Mississauga, ON) were also used to isolate plasmids from bacterial preparations, according to manufacturer's directions.

#### b. Chromosome Preparation

A cesium chloride preparation of chromosomal DNA from *S. typhimurium* SL1344 was made previously by Dr. Murry Stein. Chromosomal DNA preparations from *Salmonella* mutants were made as described below (9).

First, a stock solution of protease (*Streptomyces greisus* Type XXI) was made at 10 mg/ml in distilled $H_2O$ and incubated at 37°C for 1.5 h. Then 2 ml of overnight bacterial culture was pelleted at full speed in an Eppendorf microfuge (Fisher Scientific) for 5 min. The pellet was resuspended in 300 µl 50 mM TE using the flat end of a sterile toothpick, and the bacteria were then mixed with 100 µl of 20 mg/ml lysozyme for 15 min at room temperature. After this incubation, 20 µl of 10% SDS was added and the tube was mixed by gently inverting it. Then 100 µl of the predigested protease were added and the mixture was incubated at 37°C for 1 h with occasional mixing.

In the next step, 400 µl phenol was added and the tube was again mixed by inverting it. This was incubated at 37°C for 1 h with occasional mixing. After 1 h, 600 µl chloroform was added, gently mixed and the phases separated by centrifuging for 5 min at 8000 rpm. The upper aqueous phase containing the DNA was transferred into a new Eppendorf tube and respun. To transfer the upper phase, a modified 1 ml Eppendorf tip was used. This tip had about 1/4 cm of its end chopped off to enlarge the tip opening and thereby minimize shear

forces on the DNA. After the second centrifugation, the DNA was again transferred to a new tube and a one tenth volume of 3 M sodium acetate was added to the sample. The tube was then filled with -20°C 95% ethanol (about 1 ml or 2.2 volume of ethanol). The sample was not mixed, but instead the DNA globs were fished out with a hooked glass rod (melted Pasteur pipette). The 'glob' was briefly transferred to a new tube containing 1 ml 95% ethanol and then moved to an empty tube. The sample was dried at 37°C for about 1 h. The DNA was resuspended in 150 µl 50 mM TE and left to stand at 4°C overnight to achieve complete rehydration of the DNA. The next day, the purity of the DNA was checked by absorbance measurement with $OD_{260}$:$OD_{280}$ ratio.

### 2.4.2. Basic Method of DNA Precipitation with Ethanol

Ethanol-precipitation of DNA has been previously described (23). Briefly, a one-tenth volume of 3 M sodium acetate was added to the sample containing the DNA and then 2.5 volumes of 100% ethanol was added to the sample. This mixture was allowed to cool for a minimum of 15 min at -70°C, and then spun at 13000 rpm for 20 min. The pellet was washed once with 70% ethanol. The resulting pellet was then dried and the DNA was rehydrated in 5 mM TE.

### 2.4.3. Isolation of DNA from Agarose

Three separate methods were used to isolate bands of DNA from agarose gels. All three methods were equally effective and resulted in the recovery of more than 80% of the DNA in a band.

#### a. Sephaglas Bandprep Kit

The Sephaglas™ Bandprep Kit from Pharmacia Biotech was used to extract DNA from agarose gels, as directed by the manufacturer.

#### b. Freeze Squeeze Method for Isolating DNA Fragments

The DNA band of interest was cut out from the agarose gel, then wrapped in parafilm and set on dry ice or in -70°C to freeze (about 20 min). Once frozen, the liquid was squeezed

out from parafilm as band melted leaving a residue of agarose in the parafilm pocket. The DNA was then recovered using ethanol precipitation and resuspended in 5 mM TE or sterile distilled $H_2O$.

**c. Spin-Column Method for Isolating DNA Fragments**

A hot straight wire was used to poke a tiny hole in the bottom of a 500 µl Eppendorf tube. A small plug of loosely wound glass wool (about 0.5 0.75 cm deep) was then inserted into the end of the small Eppendorf tube (*i.e.* the column). This small tube was then placed into a larger 1.5 ml Eppendorf tube (*i.e.* the collection unit). The agarose gel plug containing the DNA was then placed on top of the glass wool in the small tube and the tubes were centrifuged for 45 sec at 6000 rpm in microfuge. This allowed maximum DNA recovery with little agarose contamination. The DNA was then recovered using ethanol precipitation and resuspended in 5 mM TE or sterile distilled $H_2O$ (144).

**2.4.4. Electroporation of bacteria**

**a. Preparation of electrocompetent bacteria**

Bacteria were grown overnight in LB broth at 37°C shaking at 200 rpm. From the overnight culture, a 1:100 dilution of the bacteria was made into fresh LB media and grown to an $OD_{600}$ of 0.3-0.5. Once bacteria reached proper OD, the culture flask was chilled on ice for 30 min and all subsequent steps were performed on ice or in refrigerated units. The bacteria were transferred to centrifuge bottles and spun at 10,000 rpm for 10 min in a Beckman Model J2-21 centrifuge (Beckman Instruments (Canada) Inc.; Mississauga, ON). The pellet was resuspended in 1 volume of chilled 10% glycerol for first wash. The sample was then recentrifuged as before. Subsequent washing of the bacteria was carried out in reduced volumes of 10% glycerol (1:2, 1:50, 1:100, and 1:500) in order to decrease the ionic strength of the sample and concentrate the bacteria to about $10^9$ to $10^{10}$ bacteria per 40 µl aliquot. Aliquots were placed into Eppendorf tubes and flash frozen using dry ice and ethanol. They were stored at -70°C for up to 6 months.

### b. Electroporation

Electrocompetent bacterial aliquots were thawed on ice only immediately before using. One to two microliters of DNA preparation was added to the thawed bacteria and the mix was incubated on ice for about 1 min. The sample was then transferred to a 0.2 cm electroporation cuvette and using the Gene Pulser™ from BioRad (Richmond, CA), the sample was pulsed at 2.5 kV with a 25 μF capacitance and 400Ω parallel resistance. The samples were immediately resuspended in 1 ml of SOC broth containing no antibiotics and incubated for 1 h at 37°C, shaking 200 rpm. After this recovery period, the sample was diluted and plated out onto selective medium to quantitate the transformants per μg of DNA.

## 2.4.5. Two plasmid competition system

A modified version of a two-plasmid competition system (138) was used to obtain random insertions of a promoterless luciferase gene cassette throughout the *Salmonella* chromosome. Competent *S. typhimurium* SL1344 bacteria were initially transformed using electroporation with either the plasmid pTF421 or pFUSLUX. Resulting transformants were selected on LB plates containing either ampicillin or tetracycline, respectively. A phage P22HT*int* lysate was then made of SL1344 pFUSLUX, and this lysate used to transfect SL1344 pTF421. The pSPLUX plasmid was transferred into SL1344 pTF421 about 100 fold more efficiently by the phage P22 compared with electroporation. SL1344 bacteria transfected with both plasmids were then grown for an extended period of time on LB plates containing both ampicillin and tetracycline. The plasmid pFUSLUX has a ColE1 origin of replication (184) and contains a gene cassette encoding a promoterless *luxAB* gene operon and a tetracycline resistance gene surrounded by insertional sequences from the transposon Tn5 (Figure 6) (138, 166). The plasmid pTF421 carries an ampicillin resistance gene and also encodes for the production of RNA1, which inhibits the replication of plasmids with ColE1 origins (131). This extended incubation allowed the bacteria to enter a hypermutability state that allowed for the random insertion of the *luxAB*-containing gene cassette into the

chromosome of the bacteria. Previous testing with *E. coli* showed that single chromosomal insertions resulted from this technique (138).

### 2.4.6. Inverse PCR

Inverse PCR was used for amplifying the regions either upstream or downstream from the site of reporter gene insertion (164, 237, 315). Chromosomal DNA was first cut with either the restriction enzyme *Hae*III or *Rsa*I at 37°C for a minimum of 1 h. After cutting, the restriction enzymes were removed using phenol:chloroform extraction and ethanol precipitation as described previously, and the DNA resuspended in 20 µl distilled $H_2O$. Five microliters of this cut DNA was placed in a 500 µl microfuge tube containing 1 µl ligase (5 U), 10 µl 5X ligase buffer and 34 µl distilled water, and incubated for 10 min in a sonicating waterbath set between 12°C and 16°C.

Inverse PCR reaction tubes contained: 5 µl ligated DNA, 5 µl of each of two primers (10 pmol stock), 5 µl 25 mM magnesium sulfate, 5 µl 10X AmpliTaq buffer, 4 µl dNTPs (2.5 mM stock), 20 µl distilled sterile water, and 1 µl AmpliTaq or AmpliTaq Gold (5 U). The PCR program consisted of an initial cycle of 94°C for 5 min and then 30 cycles of 94°C for 45 sec, 60°C for 45 sec, and 72°C for 2 min 30 sec. The reaction was then subjected to electrophoresis on a 1% agarose gel with 1X TAE buffer.

### 2.4.7. Sequencing

For sequencing, 90 ng purified template DNA was added to 3.2 pmol of primer and mixed with 8 µl of the FS Taq terminator premix reagent for a final volume of 20 µl. Each sequencing reaction consisted of 25 cycles of 96°C for 30 sec, 50°C for 15 sec, and 60°C for 4 min. Unused terminators (fluorescently pre-dyed dNTPs) were removed using Centrisep columns, as directed by the manufacturer. Sequencing gels were run by the NAPS Unit (UBC, Vancouver, BC). Sequences were further analyzed using NCBI's BLAST program and the GenBank database .

## 2.5. Invasion and Survival Assays

For the studies involving the development of the intracellular reporter system, and for the initial screening of bacteria with cultured cells, the *Salmonella* were grown overnight in LB broth at 37°C, with no shaking (159). For later studies involving the characterization of the mutants, *S. typhimurium* SL1344 and mutants A1A1, D11H5, E12A2, and G7H1 were first grown in 1 ml LB broth in culture tubes overnight, with shaking at 200 rpm. The next day the bacteria were subcultured at a 1:100 dilution in prewarmed, pre-equilibrated ($CO_2$ buffered) DMEM++ and grown for another 3 h, with shaking.

Invasion assays were done using a modified version of the gentamicin protection assay described by Tang *et al.* (310). Sterile 96-well plates were seeded with cultured cells 18 h in advance with 100 µl of 0.5-1.0x$10^5$ cells/ml (depending on cell type), in order to obtain 90% confluency by the next day. The cultured cells were then infected with 2 µl of bacterial cultures (multiplicity of infection (MOI) was 50-100 bacteria per cell). Bacteria were allowed to invade phagocytic cells for 30 minutes and non-phagocytic cells for 1 h. (Note that bacterial invasion of host cells took place in the presence of serum.) Following internalization of the bacteria, the cells were washed with PBS++ and incubated with 100 µl DMEM++ containing 100 µg/ml gentamicin. Where bacterial growth was studied for longer than 4 h, the gentamicin concentration was reduced to 10 µg/ml at 4 h in order to reduce any toxic effects of gentamicin on both the cells and intracellular bacteria. Cells were then lysed in 20 µl PBS containing 1% Triton X-100 + 0.1% SDS. Dilutions of bacteria were made in PBS-- and then plated onto LB agar. Bacterial colony-forming units (cfu) were enumerated by serial dilutions and plating. All counts were obtained from duplicates wells within triplicate experiments, and error bars represent standard error of the means, $P<(0.5)$ (228).

## 2.6. Reporter gene assays

The reporter assays were set up in the same manner as the invasion assays. Both bacterial and cell cultures were set-up the day prior to the assay and grown as indicated. Bacteria were allowed to invade the cells as described in section 2.5, and intracellular bacteria were differentiated from extracellular bacteria by their resistance to gentamicin while inside the host cells. Each experiment was performed in triplicate or quadruplicate, and each mutant was tested in duplicate in each experiment. Error bars represent standard error of the means, $P<(0.5)$ (228).

### 2.6.1. ß-galactosidase assays

For determination of bacterial ß-galactosidase activity, assays were done in 96-well microtiter plates. Activity from extracellular bacteria was determined from the bacteria remaining in the supernatant above the cells, prior to the addition of gentamicin. Activity from intracellular bacteria was determined after treatment of the cells with gentamicin. Note that the cells were washed once with PBS++ to remove gentamicin prior to assaying for enzyme activity. For ß-galactosidase assays, separate wells were used for the determination of enzyme activity and for viable bacterial counts. Over the course of this study, two different substrates were used to determine ß-galactosidase activity from bacteria: a fluorescent and a chemiluminescent substrate.

#### a. ß-galactosidase assay using fluorescent substrate

To each sample, 20 µl of 0.1% SDS was added and incubated for 5 10 min at 37°C. The wells were made up to approximately 100 µl with PBS++, mixed well, and the contents transferred to a 96-well PVC (polyvinylchloride) plate. One drop (~10 µl) chloroform was mixed with the 100 µl sample in flexible PVC plate and the plate was put on ice for at least 5 min. (Triton X-100 was not used for lysis since the two detergents (Triton X-100 and SDS) formed a precipitate formed when were combined with the substrate.) Two microliters of a 50 mM stock of FDG were then added to each well and the plate was incubated at 37°C for 1 h. To stop the reaction, the plate was again put on ice and 2 µl of a 50 mM stock of PETG

were added to each well. The reagents were light sensitive, therefore all sample manipulations were done with minimal exposure to light. To determine the enzyme activity, the samples were transferred to a Fluoricon assay plate and the fluorescence at 535 nm emission (485 nm excitation) was measured in a PANDEX Fluorescence Concentration Analyzer (IDEXX; Portland, OR). Duplicate wells for bacterial counts were run under the same conditions as wells for the ß-galactosidase assay, and viable bacteria were enumerated by serial dilutions and plating. Fluorescence was then correlated with viable counts to calculate ß-galactosidase activity as fluorescent units/cfu.

**b. ß-galactosidase assay using chemiluminescent substrate**

For the chemiluminescent assay, the bacteria and cultured cells were treated with SDS and chloroform as above, however the Galacto*Star* ™ Kit (Tropix Perkin Elmer) was used to detect ß-galactosidase activity. One hundred microliters of detection reagent (diluted 1:50 of stock solution as per instructions) was added to the 100 µl of sample, and then incubated at room temperature for 30 min. The chemiluminescence (or light output) of the sample was determined using the Luminograph LB980 photon imager. Duplicate wells were used for viable counts, and ß-galactosidase activity was correlated as photons/cfu.

### 2.6.2. Luciferase assay

For the luciferase assay, the aldehyde substrate was added directly to the sample of either intact cells or bacteria alone, and then viable bacterial counts were obtained from the sample well. Activity from extracellular bacteria was determined from the bacteria remaining in the supernatant above the cells, prior to the addition of gentamicin. Activity from intracellular bacteria was determined after treatment of the cells with gentamicin. In order to minimize any toxic effects during the assay, the media containing gentamicin was replaced with fresh media before adding aldehyde solution.

To determine light production (*i.e.* luciferase activity) from bacteria, 10 µl of the aldehyde solution was added to the 100 µl sample. Immediately the light production was read over the course of 1 min at maximum sensitivity on the Luminograph LB980. Light emissions

were obtained as photons/well. Light production was also assessed in microtiter plates with a 1 min exposure to X-ray film, and the results were quantitated using a computing densitometer. After the light production was determined, the host cells were lysed with 1% Triton X-100 and 0.1% SDS, and the bacteria plated out onto LB plates as indicated for the invasion assays. Luciferase activity was defined as photons/cfu (when using the LB980).

## 2.7. Screen for Transformed *Salmonella* Exhibiting Low Luciferase Activity Outside Host Cells

### 2.7.1. Extracellular Bacterial Screen

The *Salmonella* colonies obtained from the two-plasmid competition system (described above) were screened for low light production on LB plates. Approximately $1.5 \times 10^5$ bacterial colonies, resistant to both ampicillin and tetracycline, resulted from the transformations. These colonies were exposed to vapors of *n*-decanal (*e.g.* substrate was streaked onto the lid of the petri dish) and the resulting light output measured with a Luminograph LB980 low light video imaging system (Siemens) (251).

Colonies showing little to no light production were then restreaked onto fresh LB plates containing both antibiotics, and retested for both growth and low light production. Colonies passing through the second stage of the screen were then transferred to 96-well plates containing LB broth with both antibiotics; each well contained a separate colony. The bacterial mutants were also streaked onto green plates to ensure that the bacteria were not chronically infected with P22 phage (300).

### 2.7.2. Intracellular *versus* Extracellular Bacterial Screen

The selected bacterial mutants were further tested for low light production outside cells in broth, and tested for an induction of light production from within cells. Bacteria were initially grown in 100 µl of LB broth in 96-well plates sealed with parafilm, at 37°C and shaking at 150 rpm overnight. Sterile 96-well plates with black walls and clear bottoms (Costar) were used to grow tissue culture cells. Wells were seeded with 100 µl of

$1x10^5$ cells/ml in order to obtain 90% confluency by the next day. Both macrophage cell lines (J774A.1 and BALB.BM1) were used for the initial screen. The overnight bacterial plates were used to inoculate all other plates, and served as the extracellular stationary phase bacterial controls. For the intracellular test plates, 2 µl of each bacterial mutant from the stationary phase plates were used to inoculate the plates containing the cell lines. Bacteria were allowed to invade the cells for 1 h and then were washed off with PBS and the media replaced with DMEM++ containing 100 µg/ml gentamicin. Light from the intracellular bacteria was first determined at 2 h postinoculation and again at 4 h. The 2 h time point allowed the gentamicin to act on extracellular bacteria for at least 1 h, which has been found to be sufficient time to kill the bacteria and eliminate light production. Once the activity at the 2 h time point had been determined, the aldehyde-containing media was removed and replaced with fresh DMEM++ with gentamicin for another 2 h. At the 4 h time point, the intracellular activity was determined again. At 4 h, the viable bacteria were plated out on LB plates to determine bacterial numbers/well. During the screen for bacterial genes which were upregulated intracellularly, the cfu's were not determined for each sample. Instead, two to three samples per plate were used as an estimate of bacterial numbers and applied to the rest of the plate for convenience. For characterization of the mutants in later studies, actual cfu's were determined for each sample

For the extracellular logarithmic phase control plates, 2 µl from each of the wells in the stationary phase plates was first diluted in 200 µl PBS (1:100 dilution) and then 2 µl of this was added to 100 µl DMEM++ in a new plate. The plates were then incubated under similar conditions to the tissue culture cells, *i.e.* not shaking at 37°C in 5% $CO_2$. The light from the extracellular bacteria (both stationary and log phage bacteria) was detected at 4 h postinoculation of the log phase plate. Viable bacteria were plated out from the wells after luciferase activity was determined.

## 2.11. Mouse Studies

### 2.11.1. Typhoid Mouse Model

*Salmonella* suspensions were grown at 37°C in LB broth overnight, with shaking at 200 rpm. The next day, bacteria were diluted 1:100 into fresh LB broth and incubated with shaking. After 4 h, bacteria were washed once with PBS and resuspended in PBS containing 2% glucose. Control mice were given PBS with glucose only. BALB/c female mice, aged 6 to 10 weeks, were inoculated orally with bacterial suspensions (195, 299), after being deprived of water for 4 h. In the first experiment, the inoculation size was 25 µl. The dose of wild-type SL1344 was $2.5x10^6$ cfu/mouse, (approximately twice the reported $LD_{50}$ (195, 299)). The *Salmonella* mutants were given at 200 times this dose ($5x10^8$ cfu/mouse). Actual counts per mouse (per 25 µl) were as follows (± 10% error): for D11H5, $4.2x10^8$ cfu; for A1A1, $4.1x10^8$ cfu; for E12A2, $3.3x10^8$ cfu; and for G7H1, $5.0x10^8$ cfu. Four mice were used per group. In the second experiment, three doses were used of approximately $1x10^8$, $1x10^7$, and $1x10^6$ cfu/mouse, and the inoculation size was 10 µl.. Actual counts per mouse (per 10 µl) were 10-fold dilutions of the following (± 10% error): $7.6x10^7$ cfu of SL1344; $9.6x10^7$ cfu of A1A1; $1.3x10^8$ of E12A2; and $1.0x10^8$ cfu of G7H1. Five mice were used per group. Mice surviving after 28 days were sacrificed, and their livers and spleens were harvested, the organs homogenized and the resulting slurry spread onto MacConkey plates to obtain bacterial colony counts. The day at which 50% of the mice in each group had died was determined using the statistical calculations of Reed and Meunch (259,260) and the median survival time (228).

# Chapter 3: Development of an Intracellular Reporter System

Chapter 3 describes the development of an assay system for gene expression from intracellular bacteria. Specifically, the benefits and challenges of working with two different reporters, ß-galactosidase and luciferase, are discussed.

The ability of *Salmonella* to survive and grow within cells is crucial to its ability to cause disease within a host (27, 199). Many mutants which are defective in their ability to invade or replicate within cells are also avirulent in the host (4, 49, 76, 85). The development of cell-mediated immunity in addition to a humoral immune response against *Salmonella* infection, is also indicative of a host response to intracellular pathogens (271, 326). For *Salmonella* to propagate within the intracellular environment, the bacteria must adapt by regulating the expression of proteins necessary for growth within that environment. This global regulation has been observed by using two-dimensional protein gels, where bacteria have been shown to regulate the expression of proteins upon infection of cultured macrophages (1, 30, 33, 34). However, to study the expression of individual genes, fusion of the gene of interest to a reporter gene provides a simpler method to monitor expression.

The detection of reporter gene expression often uses bacteria grown as colonies on agar plates or grown to high density in broth cultures. The number of *Salmonella* within host cells after invasion will be 10-100 fold lower, therefore the suitability of using specific reporter genes to determine gene expression from bacteria within host cells was examined.

## 3.1. Results

### 3.1.1. Invasion assay to determine intracellular bacterial numbers

A gentamicin-protection assay (310) was used to define the bacteria which had invaded a host cell and thus become intracellular. *Salmonella* were added to medium containing cultured cells and allowed to invade the cells for a set period of time. The extracellular bacteria were then removed, the cells washed, and the new medium containing the antibiotic gentamicin was added. Bacteria residing inside the host cells were protected from the killing effects of gentamicin, while extracellular bacteria were killed (310). To enumerate the intracellular

bacteria, the infected host cells were then lysed with 1% Triton X-100, which destabilized the membrane of the host cell without impairing the bacterial membrane, allowing the bacteria to be released from the cell. The bacteria were then diluted and colony-forming units (cfu) were determined following growth on agar plates. Cellular 'ghosts' resulting from host cell lysis were observed under the microscope, and bacteria were seen swimming through the lysis buffer.

A possible concern with the method was that some of the bacteria might clump or associate with cellular debris, or that the intracellular vacuoles might not lyse with the same efficiency as the outer cell membrane. The result would be an underestimation of the number of intracellular bacteria. Therefore, the addition of the ionic detergent SDS was added to the lysis buffer in an effort to disrupt any remaining cell/bacterial interactions. Further experiments showed that 0.1% SDS was not damaging to *Salmonella* grown in media alone, *i.e.* cfu's were not reduced significantly by the presence of 0.1% SDS. A combination of 1% Triton X-100 and 0.1% SDS was tested and resulted in the recovery of 10-fold more bacteria from infected cells than with Triton X-100 alone (Figure 4). As the estimation of intracellular bacterial numbers is very important for the determination of specific activity of a reporter enzyme, the combination of 1% Triton X-100 and 0.1% SDS in the lysis buffer was used for the enumeration of intracellular bacteria in all further experiments.

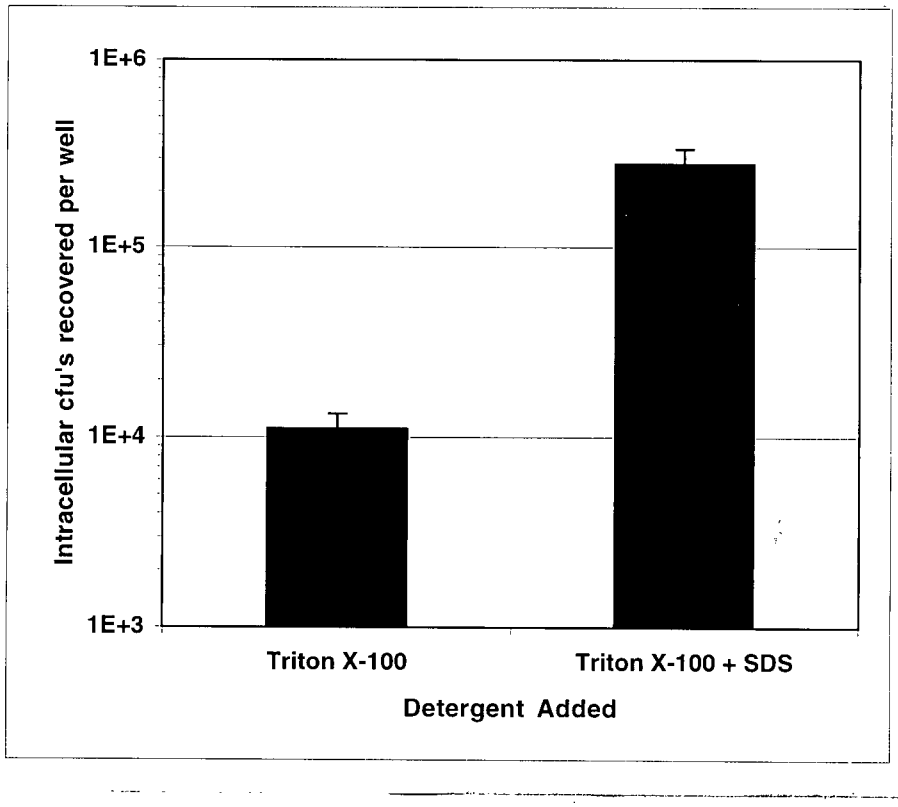



Figure 4: The effect of the detergents Triton X-100 (1.0%) and SDS (0.1%) on the recovery of intracellular *S. typhimurium* from HeLa cells. The experiments were done in triplicate and the error bars represent the standard deviation of the means.

### 3.1.2. ß-galactosidase as a reporter of intracellular bacterial expression

The plasmid pFF14 (11.2 kbp) is a pACYC184-based (39) low-copy number plasmid containing a translational fusion between the *S. dublin spvB* gene and the *lacZ* reporter gene (72, 77) (Table 2; Figure 5). It was previously demonstrated that the copy number of the plasmid pFF14 remained constant throughout the growth cycle of *S. dublin* (72), indicating that an increase in the expression of genes encoded on this plasmid was not due to growth cycle but to a change in regulation. The *spv* operon has previously been shown to be regulated by SpvR (72, 77, 129), and *spvB::lacZ* expression increased more than ten-fold upon bacterial entry into stationary phase or under conditions of carbon or nitrogen starvation (72, 125). Note that the regulation of the *spvB* gene remained under the control of SpvR with both the pFF14 and pSPLUX constructs.

The plasmid pFF14 was used to examine the production of ß-galactosidase resulting from expression of the *spvB::lacZ* fusion, from *Salmonella* within epithelial cells (MDCK and HeLa cells). After incubation of bacteria and host cells together as described above, the sample was treated with 0.1% SDS and chloroform to fully release the reporter enzyme. Activity of ß-galactosidase was assayed using a fluorescence assay based on the substrate FDG, which had previously been shown to be more sensitive than the colorimetric assay based on the substrate OMPG (28, 108). After a set period of incubation, the reaction was stopped and the fluorescence of the sample was measured using a Pandex fluorimeter. This reading was converted into specific activity (fluorescence/cfu) in combination with the number of colony forming units (cfu) or viable bacteria recovered from a parallel sample. Note that chloroform was not added to samples used for determining cfu's. The data shown in Figure 6A represents the expression from the *spvB::lacZ* gene fusion from *S. dublin* which were either extracellular (*i.e.* within the culture supernatant taken from above the cultured cells) or intracellular (*i.e.* inside cells and thus protected from gentamicin). The amount of ß-galactosidase activity increased per bacterium when the bacteria were intracellular as compared to those which were

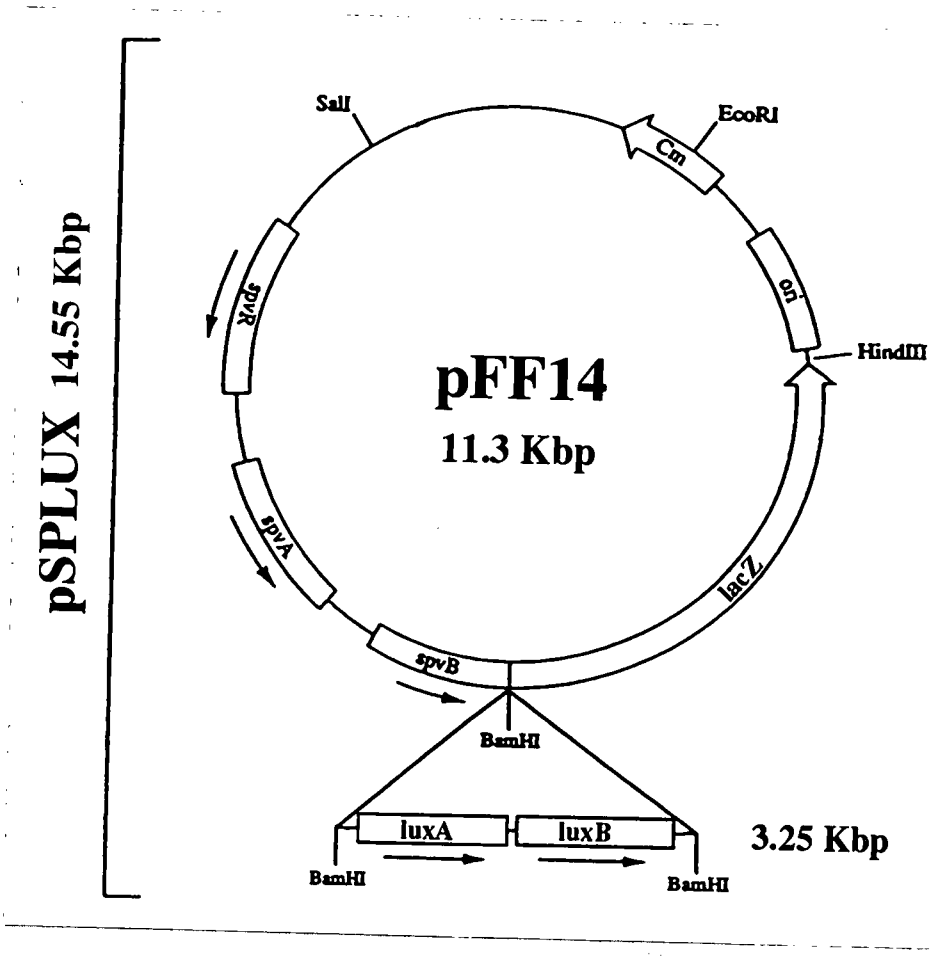



Figure 5: Plasmid maps of pFF14 and pSPLUX. The 11.3 kbp plasmid pFF14 (circular map) which contains *spvR*, *spvA*, and a translational *spvB::lacZ* fusion, was used for the ß-galactosidase studies. The 14.55 kbp plasmid pSPLUX was made by inserting a 3.25 kbp promoterless *luxAB* gene cassette into the *Bam*HI site between *spvB* and *lacZ*, thus placing *luxAB* under the transcriptional control of the *spvB* gene. The plasmid pFF14 was used for the ß-galactosidase studies, while pSPLUX was used for the luciferase studies.

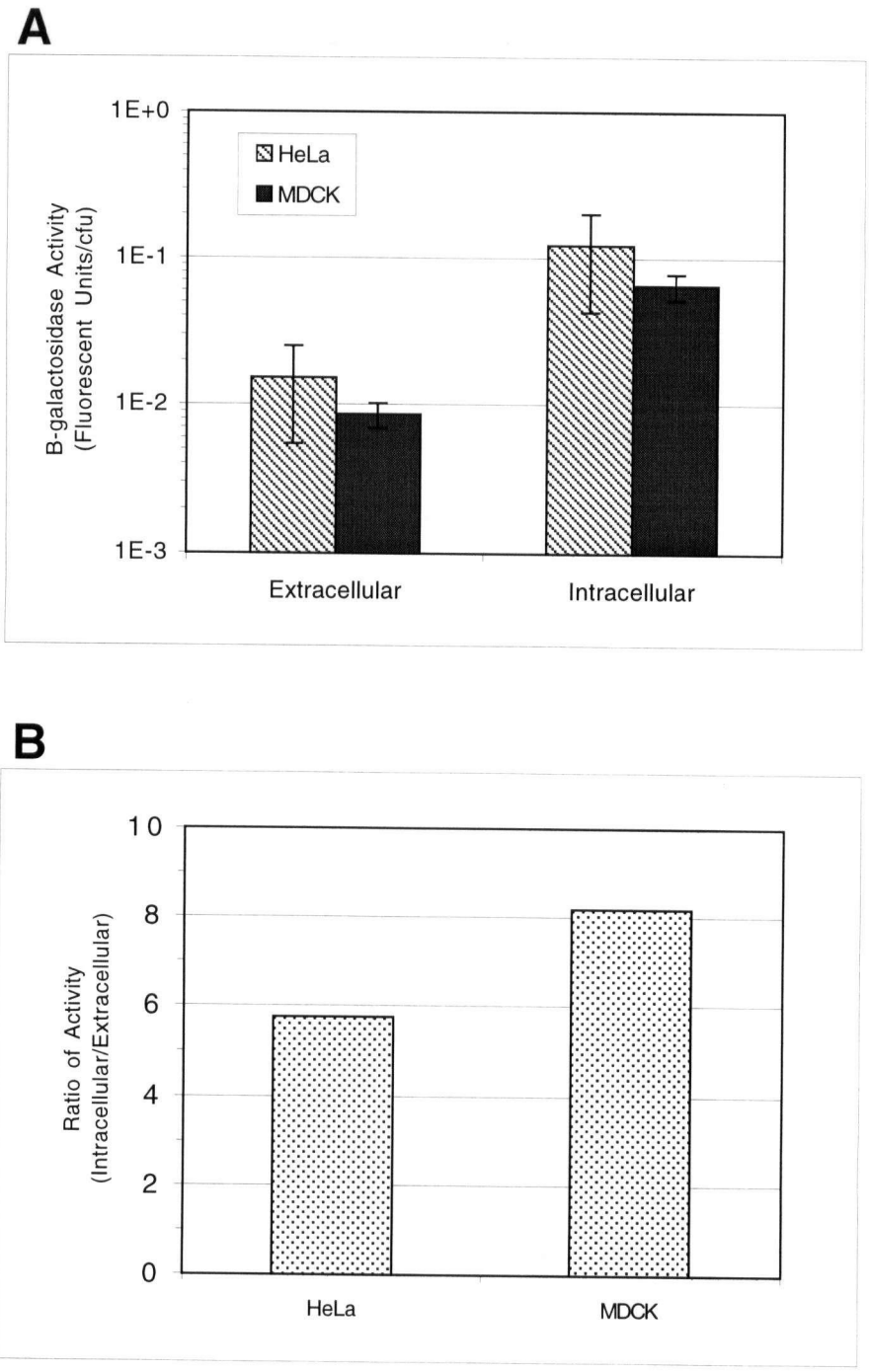



Figure 6: Expression of *spvB::lacZ* by bacteria inside non-phagocytic cells.

A) ß-galactosidase activity from bacteria which are either intracellular (inside HeLa or MDCK cells) or extracellular (remaining in the supernatant above the cells). B) Ratio of induction shown in (A) where intracellular activity is divided by extracellular activity.

extracellular. As shown in Figure 6B, this induction in activity was greater than five-fold within both epithelial cell types (MDCK and HeLa) tested.

Although ß-galactosidase was deemed to be a sensitive reporter of intracellular bacterial gene expression, there were problems associated with its use. First, the host cells had varying background levels of activity. This reduced the sensitivity of the assay when using these cells and made comparisons between cell types more difficult. Second, both the host cells and the bacteria needed to be completely lysed to accurately assay the enzymatic activity. Since chloroform was used in combination with low levels of detergent, the samples had to be transferred to special PVC plates as the chloroform reacted with the polystyrene components of regular multiwell plates. As well, higher amounts of detergent could not be used for lysis as it formed a precipitate in the substrate buffer, which interfered with the determination of fluourescence in the sample. Third, the FDG substrate was sensitive to light and therefore samples had to be kept in the dark as much as possible. Fourth, it was impossible to separate live bacteria (*i.e.* metabolically active bacteria that were able to be cultured) from those that had been killed by the host cell, therefore ß-galactosidase activity resulted from both live and dead bacteria within a sample. This was especially important for the determination of specific enzyme activity (defined as the number of fluorescent units per viable bacterium). The state of the *Salmonella* within cells has been reported to be in a constant flux which can be divided into two populations, one which is dying and the other which is growing (2, 32).

Another reporter, bacterial luciferase, was known to be detectable from only live bacteria due to its requirement for the bacterial energy $FMNH_2$ for activity. It was therefore tested to determine whether it could be used as a sensitive reporter of intracellular gene expression. A promoterless 3.25 kbp *luxAB* gene cassette from *Vibrio harveyi* (138) was ligated into the *Bam*HI of the plasmid pFF14 creating an *spvB::luxAB* transcriptional fusion, resulting in the isogenic plasmid pSPLUX (14.5 kbp) (Figure 5) (251).

### 3.1.3. Luciferase as a reporter of intracellular bacterial gene expression

#### a. Measurement of light production.

The product of luciferase is light, which is produced specifically at a wavelength of 490 nm from *V. harveyi* luciferase (210). Note that the bacterial substrate, *n*-decanal, was able to diffuse across both bacterial and host cell membranes, therefore lysis of bacterial and host cells was unnecessary for the determination of enzyme activity. Furthermore, the number of bacteria could be determined on the same sample from which the enzyme activity was detected (180). These details are discussed more fully in the following sections. Light production from bacteria was detected and quantitated with the use of different systems, including a tube luminometer, X-ray film combined with a densitometer, or a photon-imaging camera combined with a computer processor. The sensitivity and linearity of the different light-detection methods was analyzed.

A single tube luminometer was initially used for determining light production from bacteria free in culture, however, difficulties arose in determining light production from intracellular bacteria. First, the host cells could not be grown in the luminometer tubes, so the invasion portion of the assay had to be performed in a separate dish and then the host cells lysed in order to free the intracellular bacteria for transfer to the light assay tube. This introduced another variable into the system and raised the concern that detergents and other buffer components may alter the activity of the enzyme. It also resulted in the assaying of live bacteria which were no longer intracellular, and which could possibly adapt their gene expression. Only the host cells were completely lysed and the bacteria were exposed to not only low levels of detergents but to host cell contents containing lysosomal degradative proteins, etc. Since there was often a delay of 5-10 min during cell lysis, there was enough time for the bacteria to react to the 'new extracellular' environment, and perhaps exhibit an altered form of gene expression from that seen from truly intracellular bacteria. This differed from the assay for ß-galactosidase activity where both bacterial and host cells were completely

lysed at the same time. Finally, the luminometer could only measure one sample at a time and was not conducive to screening many samples.

X-ray film was tested and found to be capable of determining bacterial light production from both extracellular and intracellular bacteria. Both bacteria and host cells could be grown in 96-well microtiter plates, which were then exposed directly to the X-ray film for a set period of time, and the spots appearing on the X-ray film quantitated using a densitometer. This allowed for the testing of many samples at one time. However, a new set of problems was encountered. First, regular 96-well microtiter plates were made of clear plastic which allowed the light to spill over into neighbouring wells, altering the true reading of those wells. The cross-contamination of light between wells was eliminated with the use of 96-well white plastic grid plate with 8-well strips which could be snapped into place within the grid (Pierce Immunoware 8-Well EIA Strip Plates). The second problem was that while light could be detected from over a 1000-fold range, it was only linear over a 10-fold range as determined by densitometry scans of the X-ray film. Third, due to the small numbers of bacteria inside cells, the light produced from intracellular bacteria was often at the lower limit of detectable activity.

With the Luminograph LB980 photon-imaging camera/computer system it was possible to detect and quantitate light production over a 10000-fold range, which was linear within a 100-fold range (about $1.0x10^3$-$1.0x10^5$ photons/well). This system was also amenable to screening in a number of different formats, *e.g.* 96-well microtiter plates or colonies on agar plates. It was found to be more sensitive than X-ray film by at least five-fold (Figure 7). However, as shown in Figure 7, the white plastic from the microtiter plates produced a low level phosphorescence and was highly reflective. This problem was later reduced with the use of black microtiter plates with clear bottoms (Costar). The black plastic was not phosphorescent and less reflective, while the clear bottoms of the well allowed for the analysis of the host cell monolayer. The background level from the grid plates made of white polystyrene was about $1.0\text{-}4.0x10^3$ photons/well, while from the plates made of black polystyrene, it was reduced to about $2.0\text{-}5.0x10^2$ photons/well. This low background level of

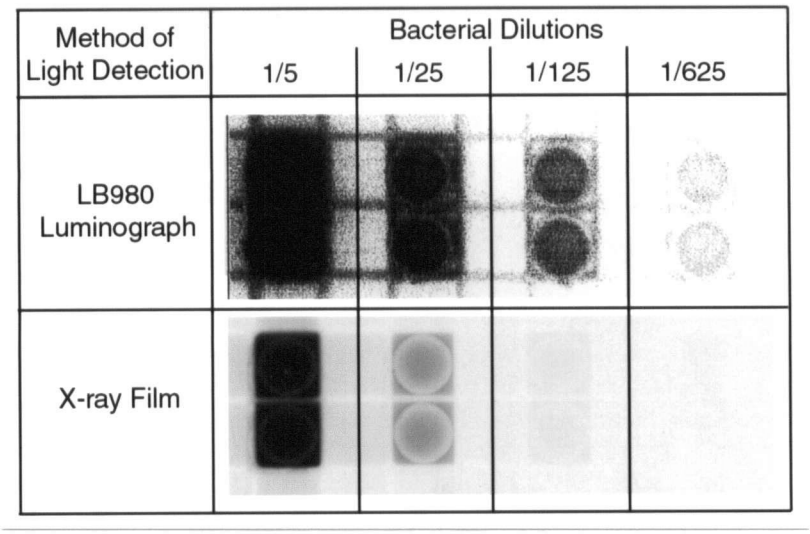



Figure 7: Comparison of two different methods to detect bacterial light production: Luminograph LB980 *versus* X-ray film. *S. dublin* pSPLUX were grown to stationary phase to induce *spvB::luxAB* expression and then diluted in PBS within 96-well gridded microtiter plates. The substrate *n*-decanal was added to a final concentration of 0.0022%. (Note that in order to directly compare the two methods, the picture obtained from the luminograph is shown as a negative image with the light output appearing as a dark image on a light background.)

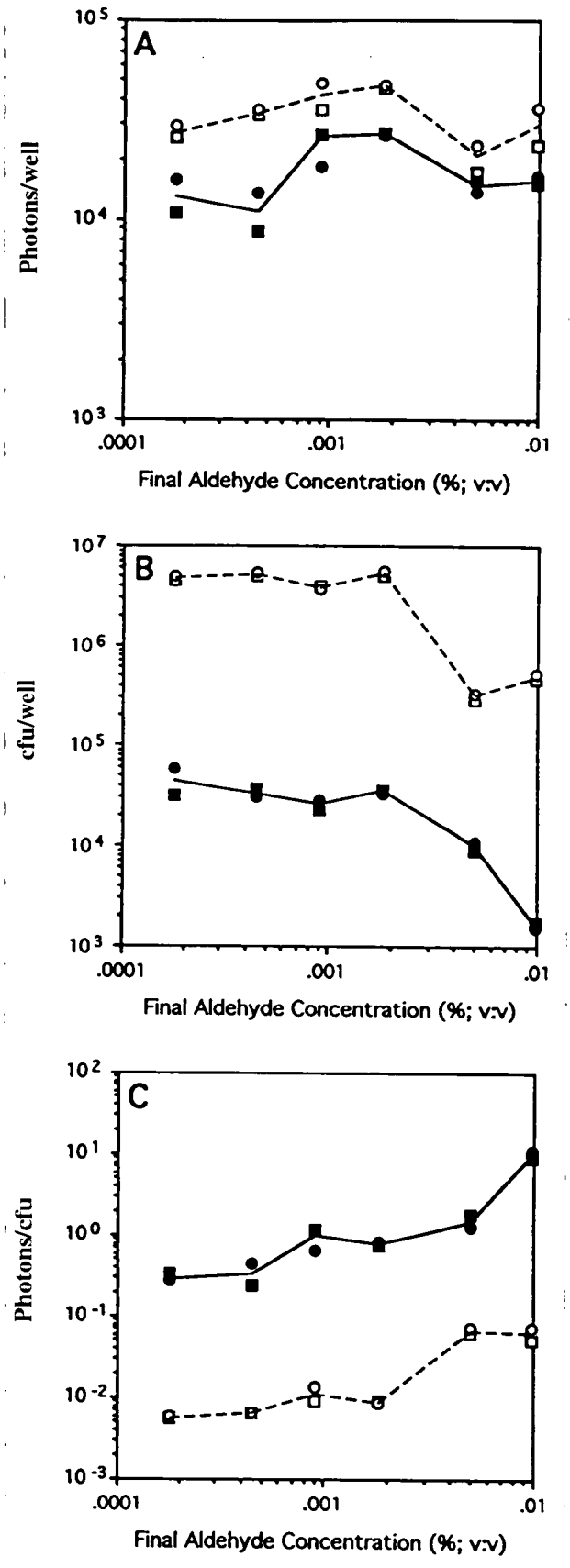
light (*i.e.* from static electricity, dark noise from the camera) was subtracted from the calculations.

**b. Effects of aldehyde concentration on bacteria and luciferase activity.**

As mentioned previously, to produce a blue-green light (490 nm), bacterial luciferase required an energy source (reduced flavin mononucleotide - $FMNH_2$), oxygen, and a long chain aldehyde (210). Therefore, when live bacteria were used, only the aldehyde substrate had to be added exogenously. Previous studies indicated that high aldehyde concentrations may be inhibitory to light production (194), therefore a range of concentrations was prepared to determine the optimal aldehyde concentration needed to measure light output from both intracellular and extracellular *Salmonella*. The final concentration of *n*-decanal in the assay wells ranged from 0.00022% to 0.0099%.

The effects of different aldehyde concentrations on both extracellular and intracellular *S. dublin* LD842 pSPLUX are shown in Figure 8. The results using *S. typhimurium* SL1344 pSPLUX were similar. In Figure 8A, a decrease in total light output from the wells was seen for the two highest concentrations of *n*-decanal (0.00495% and 0.0099%). Further investigation revealed that these concentrations of aldehyde were actually toxic to bacteria (Figure 8B). Viable counts (cfu's) were reduced by 5 to 10 fold in the presence of the two highest concentrations of aldehyde, whereas cfu's were unaffected for aldehyde concentrations of 0.0022% or less. The aldehyde concentrations which caused toxicity were similar for both intracellular and extracellular bacteria. Interestingly, the decrease in the number of viable bacteria was greater than the decline in light production per well. Therefore, the light output per viable bacterium appeared to rise as the aldehyde concentration increased (Figure 8C).

Figure 8: Effect of aldehyde concentration on bacterial viability and light production. MDCK cells were grown to confluency in 96-well microtiter plates and infected with *S. typhimurium* SL1344 pSPLUX as stated in the methods. Bacteria remaining with the cell monolayer after washing and gentamicin treatment were termed intracellular, whereas bacteria removed with the supernatant before washing were termed extracellular. This figure depicts the individual data points from one experiment, and the lines represent the means of the data points. (A) Light production as photons per 100 µl well; (B) Viable bacterial counts per 100 µl well; (C) Light production as photons/cfu: (closed symbols) intracellular bacteria; (open symbols) extracellular bacteria.


A
Photons/well
$10^5$
$10^4$
$10^3$
.0001
.001
.01
Final Aldehyde Concentration (%; v:v)
B
cfu/well
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
.0001
.001
.01
Final Aldehyde Concentration (%; v:v)
C
Photons/cfu
$10^2$
$10^1$
$10^0$
$10^{-1}$
$10^{-2}$
$10^{-3}$
.0001
.001
.01
Final Aldehyde Concentration (%; v:v)

Since the purpose of using the luciferase reporter was to correlate the expression of a gene to enzyme activity within a single live bacterium, it was crucial that the enzyme substrate not be toxic to that bacterium. Therefore, in subsequent experiments a final aldehyde concentration of 0.0022% was used, as this concentration produced the highest amount of light without a reduction in bacterial numbers.

Trypan blue exclusion studies were used to determine whether tissue culture cell death occurred (9). None of the aldehyde concentrations were toxic to the mammalian cells, even over extended periods of co-incubation of up to one hour (data not shown). It was further determined that gentamicin-killed bacteria did not produce light (Figure 9) and therefore, light produced from intracellular bacteria was not contaminated by killed bacteria remaining outside the cells. This provided further support that bacteria had to remain alive in order for luciferase activity to be detected; if they were killed by the cell, they would most likely cease to produce light.

The induction of the *spvB::luxAB* transcriptional fusions is shown in Figure 10, where equal numbers of bacteria were present in the extracellular and intracellular wells. Bacteria lacking the *luxAB* fusions (*S. dublin* or *S. typhimurium*) did not produce light. Wells containing extracellular *Salmonella* pSPLUX also did not produce much light since the bacteria were in logarithmic phase growth, and not entering into stationary phase. Figure 10 furthermore demonstrates that there is no light-producing activity detectable from the host cells. However, intracellular *Salmonella* pSPLUX expressing the *spvB::luxAB* transcriptional fusion showed increased light production.

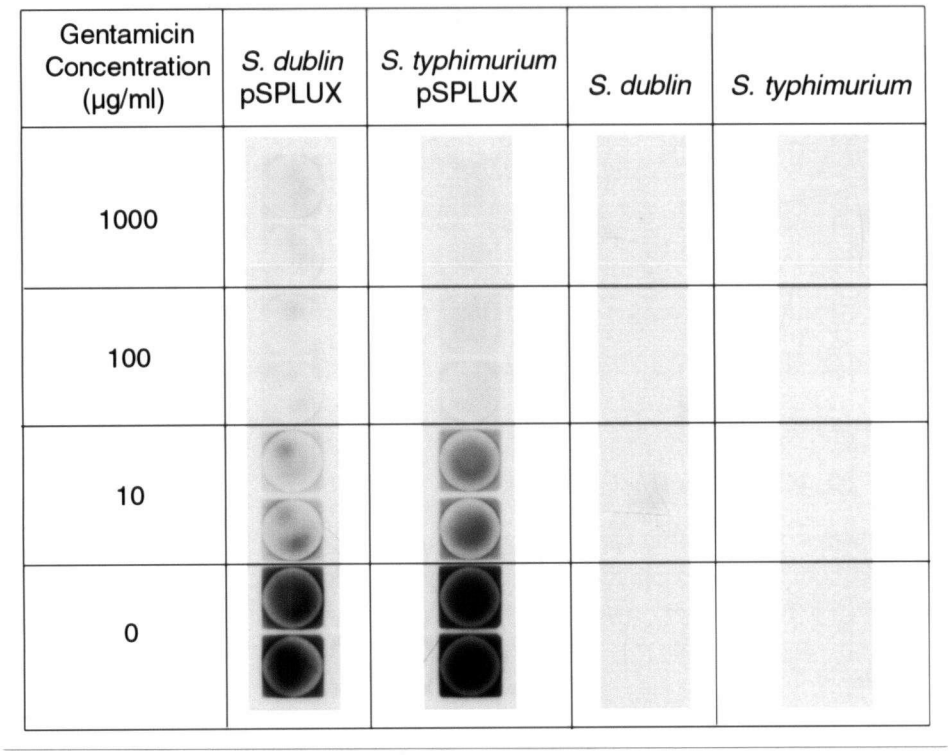



Figure 9: The action of different concentrations of gentamicin on the luciferase activity within bacteria. *Salmonella* were incubated for 45 min in the presence of DMEM++ plus gentamicin, at 37°C without shaking, prior to determining luciferase activity. Concentrations of gentamicin which killed more than 90% of the bacteria within the sample (>100 µg/ml) reduced the light output of the samples to near background levels. X-ray film was used to determine light output.

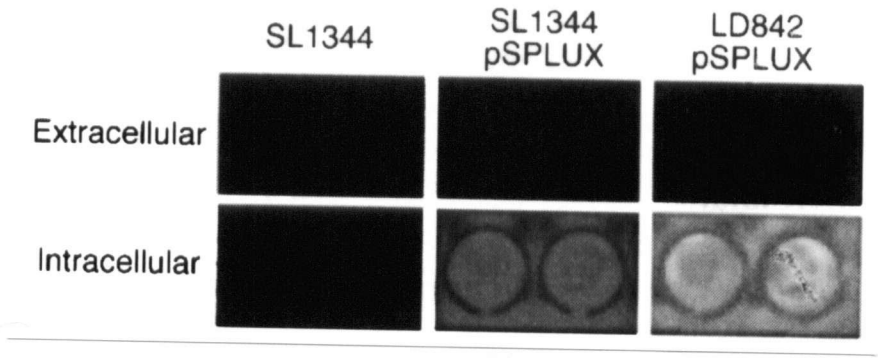



Figure 10: Detection of light production in *Salmonella* pSPLUX in an intracellular *versus* an extracellular environment. MDCK cells were seeded into 96 well microtiter plates with grids and infected with *Salmonella,* as described in the methods. After 2 h, extracellular bacteria were removed and placed into wells containing no MDCK cells. The samples were adjusted such that all wells contained equal numbers of bacteria (approximately $1x10^5$ cfu/well). No light was produced in the absence of the *luxAB* genes. A low level of light was detected from the extracellular bacteria containing the plasmid pSPLUX (*e.g.* under *spvB* repressing conditions). Increased light production was detected in wells containing intracellular bacteria with pSPLUX, indicating an increased expression of the *spvB::luxAB* transcriptional fusion. This figure was obtained using the Luminograph LB980.

### 3.1.4. Comparison of ß-galactosidase and luciferase as reporters of intracellular bacterial gene expression.

**Table 4: Induction of expression of reporter enzyme fusions by intracellular *S. dublin*.**

| Reporter Enzyme | Bacterial Location[a] | Specific Activity[b] | Relative Increase[c] |
|---|---|---|---|
| ß-Galactosidase | Extracellular | 0.0084 +/- 0.001 | |
| | Intracellular | 0.1872 +/- 0.174 | 22 |
| Luciferase | Extracellular | 0.0157 +/- 0.015 | |
| | Intracellular | 0.3820 +/- 0.130 | 24 |

[a] Extracellular location refers to the MDCK cells culture medium, 2 hr after initial infection by *S. dublin*. Intracellular location refers to the MDCK cells, 4 hr after initial infection.

[b] Units for specific enzyme activity. ß-galactosidase units are expressed as FDG fluorescence/cfu; luciferase units are expressed as photons/cfu. The error bars represent the standard deviation of the means.

[c] Relative increase for each enzyme is the specific activity of intracellular bacteria divided by specific activity of extracellular bacteria.

The sensitivity of luciferase as an intracellular reporter was compared to that of ß-galactosidase through the use of the isogenic plasmid fusions to *spvB* (126). Activities were first normalized to activity per bacterium (cfu) for each respective enzyme. Then the relative increase in activity after invasion was determined by dividing the intracellular activity by the extracellular activity. In side-by-side experiments, the increase in luciferase activity was similar to the increase in ß-galactosidase activity (24 *versus* 22 fold), as shown in Table 4. Therefore the luciferase was as sensitive as ß-galactosidase for the detection of intracellular gene expression.

For both assays, the minimum number of bacteria required in order to detect the *spvB* expression varied as the gene was induced or repressed. Under repressing conditions, $0.5\text{-}1.0\text{x}10^5$ bacteria were required, while only about $1.0\text{x}10^3$ bacteria were required when *spvB* was induced. It was previously shown (72) that the induction of *spv* gene expression was not the result of an increased plasmid copy number within the bacteria.

## 3.2. Discussion

Bacterial luciferases have been used in previous studies as reporters of intracellular bacterial gene expression, however, many of those studies were performed under conditions which were not physiological for mammalian cells (*e.g.* lower temperatures ranging from 22-30°C). Some examples include the monitoring of gene activation during plant-microbe interactions under conditions optimal for plant growth (187), and the induction of hydrogen peroxide-stimulated genes in *S. typhimurium* upon interaction with mouse macrophages at 30°C (92). This was of concern since temperature is the basis for induction of numerous virulence-associated genes, over a broad range of pathologenic bacteria (1, 30, 80, 211). Many virulence factors are optimally expressed at 37°C. The luciferase from *V. fischeri* is inadequate for temperature-dependent studies since it is inactivated above 30°C (69, 92, 155), however the *luxAB* gene cassette from *V. harveyi* encodes a heterodimeric luciferase which remains active at 37°C (69, 155, 210). In the work reported here, bacteria and tissue culture cells were grown and assayed at physiological temperatures (37°C), with minimal disruption to interactions occurring between the intracellular bacteria and the mammalian host cells. Although an alternative luciferase from *Xenorhabdis luminescens* remains thermostable up to 45°C (155, 210), it has a lower specific activity than *V. harveyi* luciferase (309). As well, *V. harveyi luxAB* genes were contained within a convenient 3.25 kbp *Bam*HI gene cassette which did not contain its own endogenous promoter. Not included within this cassette were the aldehyde biosynthetic genes *luxCDE*, encoded by an extra 4 kbp segment of DNA. While the presence of substrate synthesizing genes may appear to be an advantage, a fusion with high expression promoters would result in the high production of aldehyde, and potentially

increased *Salmonella* mortality. Even though the aldehyde substrate had to be exogenously supplied, previous studies have indicated that recombinant *lux* products exhibit high activity in the presence of externally-added decyl aldehyde (210, 309).

Langridge *et al.* (186) had previously found that vapors from high decanal concentrations (10% or more) resulted in increased levels of mortality among young plantlets. Therefore, the effects of aldehyde concentration on both bacteria and MDCK cells was addressed. None of the aldehyde concentrations tested appeared to harm the mammalian cells, but higher aldehyde concentrations (>0.0022%) were toxic to the bacteria (Figure 8B). It was not determined whether this toxicity was a direct or indirect effect of the aldehyde. The drop in numbers of colony-forming bacteria was greater than the drop in total light output per well (Figures 8A,B), indicating that higher aldehyde concentrations elicited more light production from the remaining viable bacteria (Figure 8C). It may be that the concentrations of aldehyde tested did not reach the substrate saturation range of the luciferase enzyme, or conversely, that the enzyme activity rendered them unculturable. The use of higher amounts of aldehyde therefore may have resulted in more efficient activity of the enzyme. However, since the mechanism of aldehyde toxicity was unknown, the concentration of aldehyde (0.0022%) which permitted the highest light production by luciferase without concomitant bacterial death was used.

The use of bacterial luciferase as a reporter of gene expression did pose some other problems. An article by Forsberg *et al.* (87) reported that an intrinsically curved segment of DNA in the 5' coding end of the *luxA* gene may influence promoter activity of the target gene. However, the luciferase activity correlated with the previously established ß-galactosidase data, indicating that the 5' end of the *luxA* gene did not interfere with *spv* regulation. Likewise, Gonzalez-Flecha and Demple (114) reported that luciferase activity (in the absence of *n*-decanal) was associated with an increase in oxidative radicals within the bacteria over time. However, since the *spv* operon is not influenced by the redox state of the bacteria (72), it seems unlikely that either the *luxAB* genes or the luciferase enzyme activity interfered with

*spvB* gene expression. Furthermore, it had been suggested by Meighen (210) that changes in intensity of luminescence *in vivo* may depend not only on the amount of functional luciferase available, but also on the availability of substrates ($FMNH_2$, aldehyde, and $O_2$). This implied that luciferase would be an inaccurate reporter in situations where either oxygen or energy were lacking. It appeared that the environment *Salmonella* encounters upon invasion of epithelial cells contained enough oxygen to support luciferase activity (Figures 8 and 10; and Table 4). As well, intracellular *Salmonella* remained viable, providing the aldehyde concentration was not too high. These results indicated that use of luciferase for *Salmonella* studies would not be limited by either the lack of oxygen or bacterial energy available to the intracellular bacteria.

Francis and Gallagher (92) showed that luciferase activity in response to oxidative stress was variably expressed within infected cells, suggesting that the intracellular environment may differ somewhat between individual cells or within a cellular subpopulation. Furthermore, there is a constant struggle between the bacteria and the host cells taking place during an infection (2, 32). As a result of the dynamic invasion process of the bacteria, there are two populations of *Salmonella* within cells, one static (and possibly decreasing in number) and the other rapidly dividing. These variables together may account for the variation in Table 4.

No reporter system is ideal for all situations and each system has its advantages and disadvantages. In this study, the use of luciferase as a reporter of intracellular bacterial gene expression was assessed using conditions optimal for *Salmonella* invasion of non-phagocytic mammalian cells. The bacterial luciferase, encoded by promoterless *luxAB* genes from *V. harveyi*, provided an alternative reporter system to ß-galactosidase, with several advantages. Luciferase was an accurate and sensitive reporter of intracellular *Salmonella spv* gene expression, as confirmed by data using ß-galactosidase assays (77). Moreover, the luciferase assay was faster and easier to perform than the ß-galactosidase assay. First, there was no need to lyse either the cells or the bacteria, and activity in the sample was measured immediately after substrate addition. For the ß-galactosidase assay, it was necessary to first

lyse the bacteria and then incubate the sample in the presence of the substrate for a set period of time. It was further necessary to protect the fluorescent ß-galactosidase substrate, FDG, from light. With luciferase, it was not only possible to determine bacterial gene expression from live extra-cellular bacteria but from intra-cellular bacteria as well. Another advantage of using luciferase was the absence of background activity. Neither the cells nor the bacteria had any endogenous luciferase activity and therefore any light detected resulted from the *luxAB* constructs, whereas endogenous ß-galactosidase activity could be detected from mammalian cells. The ability to monitor luciferase activity without physically disrupting either the bacteria or the cells would also allow for the monitoring of bacterial-cell interactions over time. Furthermore, the product (light) did not build up within the sample, and low dose applications of the aldehyde substrate were found to be non-toxic to both bacteria and tissue culture cells. Collectively, the results demonstrate that luciferase gene fusions are a sensitive way to monitor gene expression of bacterial pathogens found within mammalian host cells.

# Chapter 4: Development of a Screen for Bacterial Genes

Chapter 4 describes a screening system developed to search for *Salmonella* genes induced after bacterial invasion of host cells. Luciferase was used as the reporter of bacterial gene expression. This chapter first describes transfer of the *luxAB* reporter genes to the *S. typhimurium* chromosome, and the selection of the resulting bacterial mutants. Genes which were differentially expressed were then identified, specifically those genes induced by intracellular bacteria and repressed by extracellular bacteria.

## 4.1. Results

### 4.1.1. Transformation of *Salmonella* and Screen for Upregulated Bacterial Genes

A two plasmid competition system, described by Guzzo and DuBow (138), was used to obtain random insertions of the promoterless reporter gene cassette *luxAB* within the wild-type *S. typhimurium* SL1344 chromosome (Figure 11). The plasmid pFUSLUX contained a ColE1 origin of replication and a Tn5-*luxAB*-tetracycline resistance cassette, while the plasmid pTF421 encoded for ampicillin resistance and the production of RNA1 which acted to inhibit the replication of pFUSLUX. By growing the transformed bacteria in the presence of both tetracycline and ampicillin, the *luxAB* gene cassette was forced from the replication-inhibited pFUSLUX plasmid to integrate into the bacterial chromosome, such that the tetracycline resistance could be maintained by the bacteria. The tetracycline gene contained its own promoter; however, the *luxAB* genes were not expressed unless the cassette integrated such that it was under the control of an active bacterial promoter.

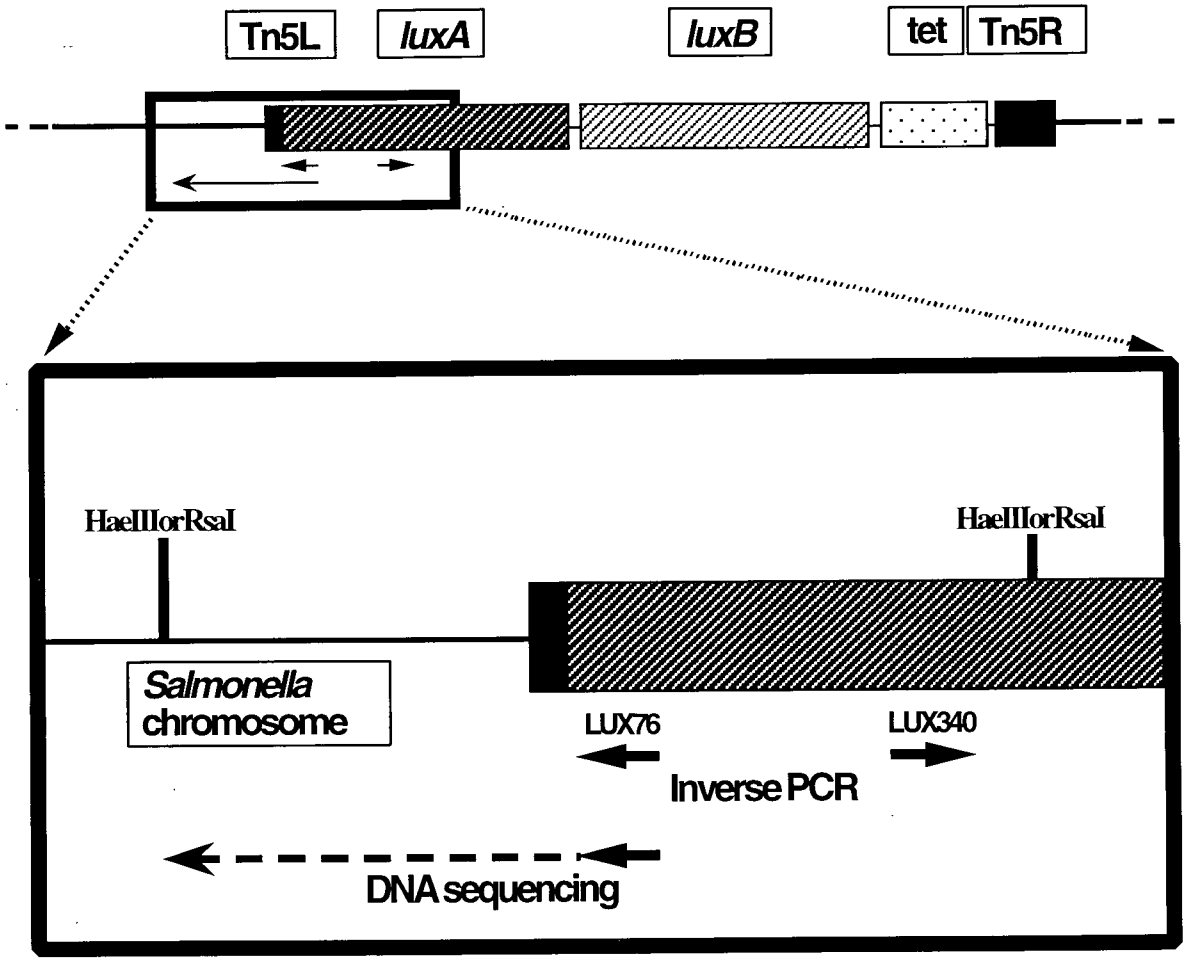
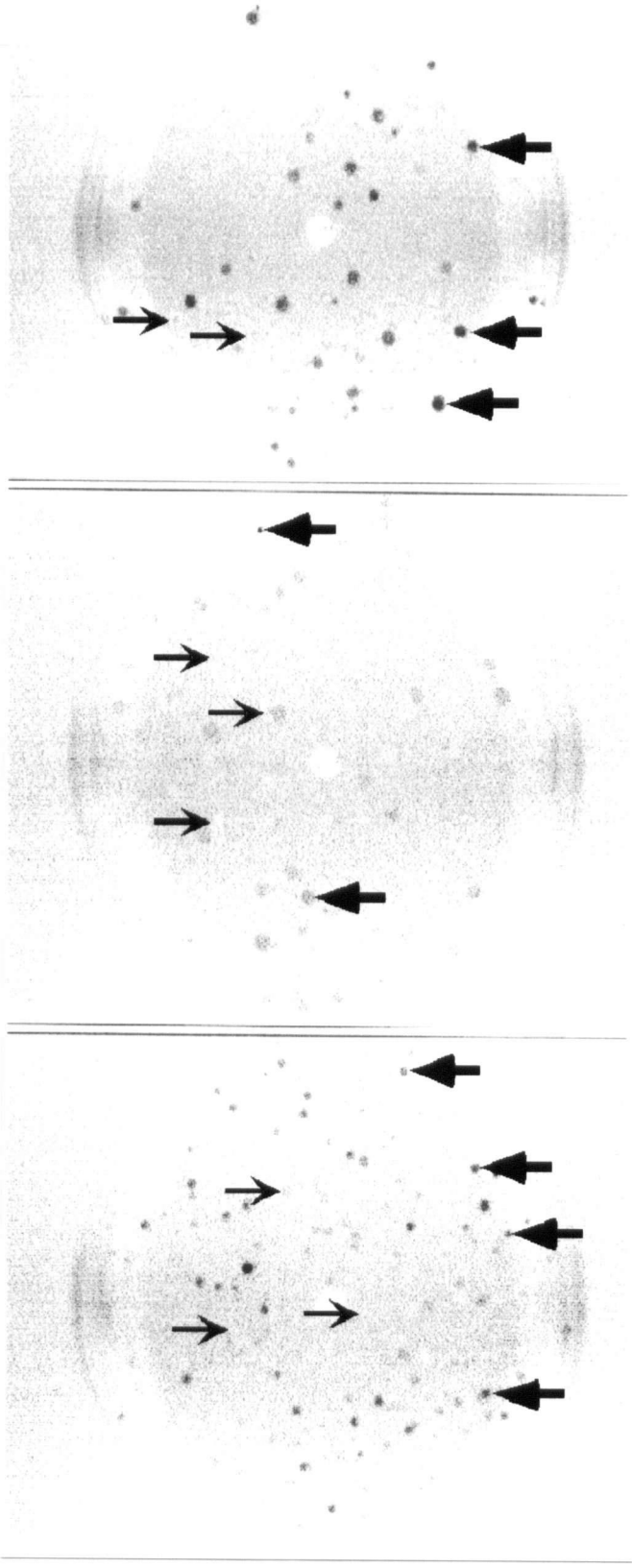



Figure 11: Schematic of the luciferase reporter gene cassette inserted within the bacterial chromosome, and the orientation of the primer pairs used for inverse PCR and subsequent DNA sequencing.

Initially, the two plasmids were sequentially transferred to *S. typhimurium* SL1344 using electroporation. First the pFUSLUX plasmid was transferred into a SL1344 bacterium. Purified pTF421 plasmid was then electroporated into SL1344 pFUSLUX. However transformation of the second plasmid by electroporation was inefficient and only a few hundred colonies with dual antibiotic resistance resulted from multiple attempts. Therefore, the phage P22HT *int* was used to transfer the plasmid pTF421 into SL1344 pFUSLUX. This procedure was much more efficient and approximately 15,000 *S. typhimurium* colonies resulted from the transductions.

To initially identify extracellular bacteria expressing the integrated reporter genes, the Luminograph LB980 photon imager was used to examine the transduced *Salmonella* colonies on agar plates for light production. The substrate was added to the colonies by streaking the aldehyde onto lids of the plates and allowing the vapors to penetrate the colonies. Those producing high relative amounts of light on the plates were discarded since they represented extracellular bacteria expressing high amounts of luciferase, while those producing little to no light on plates were retested (Figure 12). Each colony to be retested was streaked out onto a fresh plate to ensure the colony was truly resistant to both antibiotics and was also a single clone. Over 3500 colonies (about 2.4% of the original number of transformants) displaying little to no luciferase activity were detected in this manner. Each of these was further tested on green plates to ensure that they did not contain active P22 phage infections (*i.e.* lytic infections resulted in dark green colonies rather than light green colonies). Bacteria free of lytic infections were then transferred to broth cultures within individual wells of a 96-well plate.

Figure 12: Luminograph images of LB plates with *S. typhimurium* colonies transfected with both pTF421 and pFUSLUX plasmids. Bold, left-facing arrows point to examples of colonies producing light on LB agar alone, which were not further screened. Smaller, right-facing arrows point to examples of colonies producing little to no light on plates alone, which were picked for further screening.



At this point, the bacteria were stored at minus 70°C in 25% glycerol stocks within the 96-well plates and covered by a 96-well storage mat (Costar). Note that individual colonies were not pooled but placed into separate wells.

### 4.1.2. Screening for Bacterial Gene Induction Inside Cultured Macrophages

Each colony expressing low levels of light on agar plates was tested for luciferase activity during growth both outside and inside host cells. 96-well plates containing LB broth were inoculated with bacterial mutants and grown overnight, with shaking. The next day these plates, now considered the 'extracellular stationary phase' bacteria, were used to inoculate another plate of LB broth (the 'extracellular logarithmic phase' bacteria) and two plates of cultured macrophages, J774A.1 or BALB.BM1 (*i.e.* 'intracellular' bacteria). The time at which the bacteria was added to the cells was time zero. *S. typhimurium* typically has a lag period about 4 hr after it invades cells, during which no increase in cfu's is seen within the cells. This lag period is thought to allow the bacteria to adapt to the intracellular environment, and after this lag, the bacteria are able to grow exponentially, at least within epithelial cells.

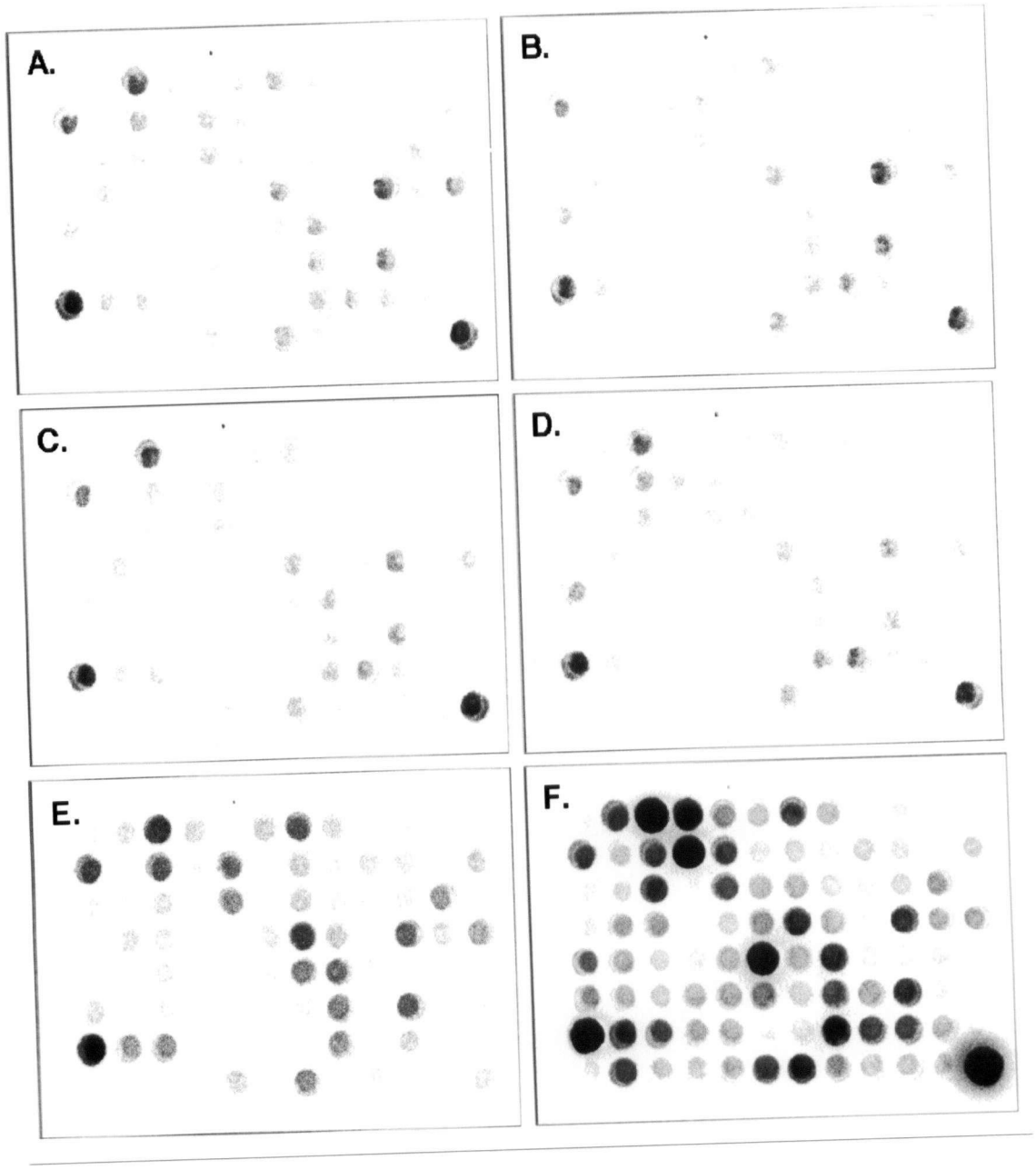
Therefore, both 2 and 4 hr after invasion, the bacterial samples were tested for the induction of light production, which was indicative of the promoter activity of genes expressed during intracellular growth. Bacteria were incubated with the cells for 1 hr, after which the medium was removed and the cells were washed. The infected cells were then treated with gentamicin for 1 to 3 hr in order to kill any bacteria remaining extracellular. Two and four hours after the initial infection of the cultured cells, the aldehyde substrate was added to the wells and the light production measured using the Luminograph LB980. After the 2 hr reading, the aldehyde-containing medium was replaced by fresh gentamicin-containing medium and the incubation continued for another 2 hr. The light production from the plates of extracellular bacteria (stationary and logarithmic phase) was only read 4 hr after time zero. An example of this screening procedure is shown in Figure 13, which demonstrates the light production from one 96-well plate of bacterial mutants under the six different conditions described: two extracellular (stationary and logarithmic phases of growth) and four

intracellular (2 and 4 hr inside either J774A.1 or BALB.BM1 cells). Light from each well was quantitated using the Luminograph LB980 and compared to the estimated bacterial count for that well. Bacterial numbers were estimated based on the averages of randomly picked wells within the various conditions, and generally, stationary phase bacteria averaged between $0.5\text{-}1.0\text{x}10^9$ cfu per well; logarithmic phase bacteria averaged between $0.5\text{-}1.0\text{x}10^8$ cfu per well; and intracellular bacteria averaged between $1.0\text{-}5.0\text{x}10^5$ cfu per well at both 2 and 4 hr. The thirty-three plates of frozen bacterial mutants were tested under each of the separate conditions at least twice. This was considered round one of the 96-well plate screen.

Mutants demonstrating the induction of light inside cells were then assembled into new 96-well plates and frozen as before. These mutants were again tested in all six conditions mentioned above, and this was considered round two. This was done to affirm that the reporter gene expression by these mutants was indeed induced and that the phenotypes were stably expressed. After screening, the mutants continuing to demonstrate the induction of light inside cells were once again assembled into new 96-well plates for round three of the screen.

From this final round of testing the *S. typhimurium* SL1344 mutants, 10 mutants (from the original 3500 mutants) consistently upregulated light production by more than an estimated five-fold at both 2 and 4 hr inside the mouse macrophage-like cell lines (both J774A.1 and BALB.BM1) when compared to the extracellular controls (both stationary and logarithmic phase).

Figure 13: Luminograph images of bacteria expressing light from a single sample 96-well plate of mutants. Bacterial growth conditions are as follows: A) inside BM1 cells 2 hr after infection; B) inside BM1 cells 4 hr after infection; C) inside J774A.1 cells 2 hr after infection; D) inside J774A.1 cells 4 hr after infection; E) extracellular bacteria in log phase growth; F) extracellular bacteria in stationary phase growth.


A.
B.
C.
D.
E.
F.

### 4.1.3. Transfer of Genes to Ensure Induction Phenotype is Linked to Gene Insertions

To be certain that the induction phenotype resulted from the chromosomal insertion of the gene cassette into a single gene, phage P22HT *int* lysates were made from each mutant. These lysates were used to transduce new *S. typhimurium* SL1344, and the resulting transduced bacteria were then tested for a pattern of light induction that matched the original parental mutant. Six mutants were identified and were named A1A1, E12A2, D11H5, G5D5, G7H1, and G8B1 (Figure 14). Green plates were once again used to ensure that the transductants no longer contained the P22 phage. This time however, the light green colonies of the transductants (*i.e.* no lytic P22 infection) were also cross-streaked against P22 H3. The transductants were considered free of lysogenic P22 only if they could be re-infected by the P22 H3 phage (300).

## 4.2. Discussion

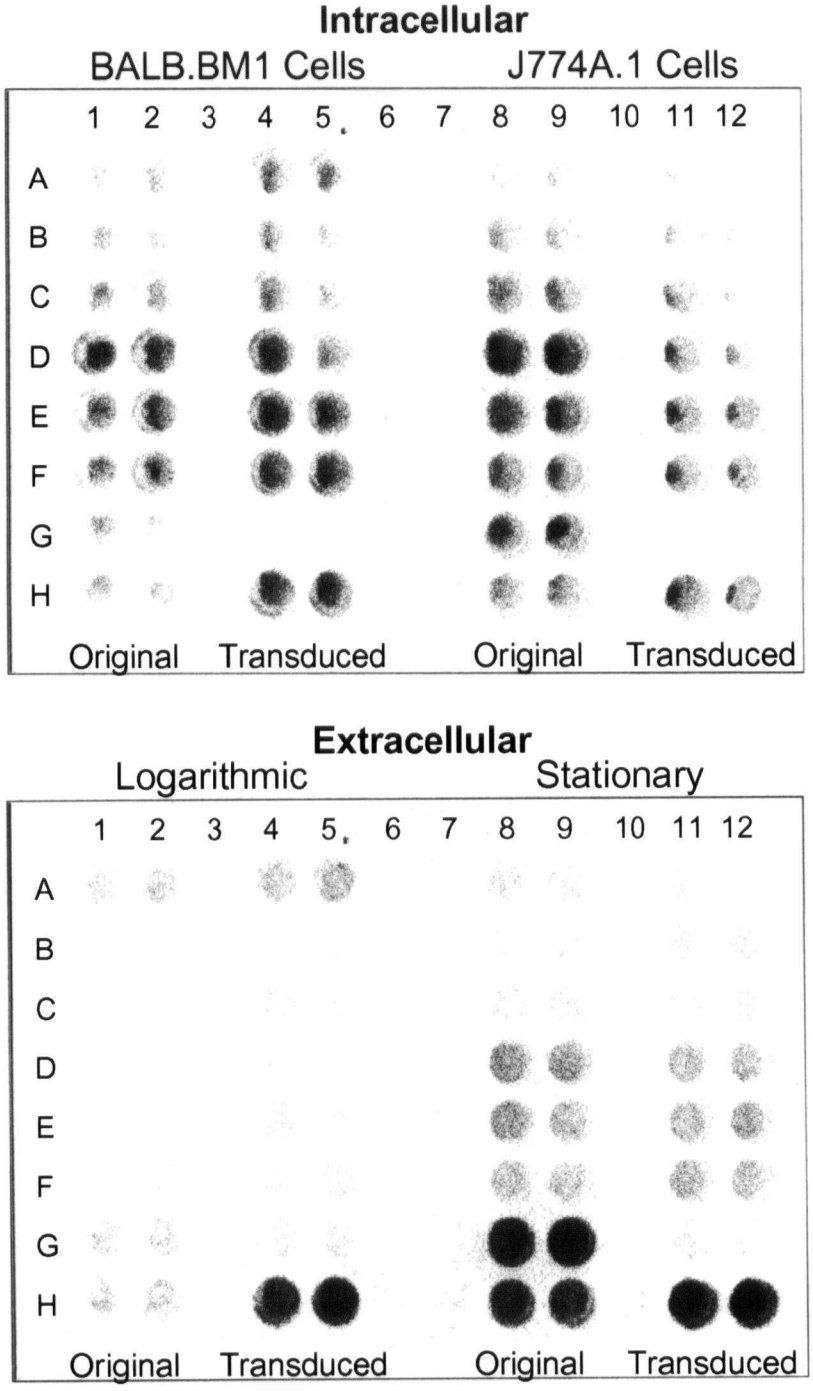
Intracellular survival and growth of *Salmonella* are important for the virulence of the bacteria, and recent evidence has suggested that many genes required for intracellular survival are not expressed by the bacteria when they are grown on regular media in the absence of cells. Therefore a screening procedure was designed to detect bacterial genes that were upregulated by intracellular environments of mammalian cells. A two-plasmid competition system wasused to obtain single random insertions of a promoterless *luxAB* reporter gene cassette within the *S. typhimurium* chromosome. This ensured the *luxAB* genes, (encoding luciferase), were under the transcriptional control of the natural *Salmonella* promoter environment, rather than from an alternate promoter or from a multi-copy number plasmid. The *Salmonella* genes were furthermore disrupted with this insertion and were not duplicated. While this reduced the chances of identifying genes crucial to bacterial viability (*i.e.* housekeeping genes), there was a concern that genes crucial to *Salmonella* survival within cells (*i.e.* virulence genes) may not have come out of the screen. However, despite this concern, genes required for

Figure 14: Comparison of the luciferase activity from the P22-transductional mutants with the original *S. typhimurium* insertional mutants. A) 96-well template showing placement of the various *S. typhimurium* clones. Clones with pSPLUX were included as positive controls. SL1344 (no *luxAB*) was included as a background level control. Uncharacterized *Salmonella* mutants which either constitutively expressed luciferase or not were further included as "on" or "off" controls, respectively. B) Corresponding luminograph images of the *S. typhimurium* clones expressing light while under different intracellular and extracellular conditions. The transduced mutants displayed luciferase activity in similar patterns to the corresponding original parent mutants.

Map of 96-Well Plate For Comparison of Original Mutants with Transductants

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | A1 | A1 | | A1A1 | A1A1 | | | A1 | A1 | | A1A1 | A1A1 |
| **B** | D11 | D11 | | D11H5 | D11H5 | | | D11 | D11 | | D11H5 | D11H5 |
| **C** | E12 | E12 | | E12A2 | E12A2 | | | E12 | E12 | | E12A2 | E12A2 |
| **D** | G5 | G5 | | G5D5 | G5D5 | | | G5 | G5 | | G5D5 | G5D5 |
| **E** | G7 | G7 | | G7H1 | G7H1 | | | G7 | G7 | | G7H1 | G7H1 |
| **F** | G8 | G8 | | G8B1 | G8B1 | | | G8 | G8 | | G8B1 | G8B1 |
| **G** | LD842 pSPLUX | LD842 pSPLUX | SL1344 | "Off" Mutant | "Off" Mutant | | | LD842 pSPLUX | LD842 pSPLUX | SL1344 | "Off" Mutant | "Off" Mutant |
| **H** | SL1344 pSPLUX | SL1344 pSPLUX | SL1344 | "On" Mutant | "On" Mutant | | | SL1344 pSPLUX | SL1344 pSPLUX | SL1344 | "On" Mutant | "On" Mutant |

"Original" Mutants | "Transduced" Mutants | "Original" Mutants | "Transduced" Mutants


Intracellular
BALB.BM1 Cells
J774A.1 Cells
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Original
Transduced
Original
Transduced
Extracellular
Logarithmic
Stationary
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Original
Transduced
Original
Transduced

intra-macrophage survival were identified (refer to Chapter 5 results and discussion), indicating that the screening parameters were not reliant on the ability of the bacteria to survive within cells.

*Salmonella* were initially grown on agar plates to test for colonies not producing significant amounts of light outside host cells. These low-light producers were then retested inside cells to see whether the amount of light could be induced by the intracellular environment. The screening effectively eliminated bacteria containing gene insertions that were constitutively expressed or induced by medium or serum alone. In retrospect, bacteria containing insertions within invasion genes were most likely eliminated by default as well since intracellular bacterial numbers would be too low to detect an increase in enzyme activity. For the initial screening phase, an estimate of bacterial numbers was used for the calculation of the specific activity of the luciferase, but later these numbers appeared to be an overestimation of the actual bacterial count. By lowering this threshold, it may be possible to observe more bacterial genes that were upregulated within host cells and not just the genes described in this study.

# Chapter 5: Characterization of Genes Upregulated by Intracellular *Salmonella*

In Chapter 5, the *S. typhimurium* mutants that were identified by the screen described in Chapter 4 are further characterized. The disrupted bacterial genes were sequenced and identified by comparison to known genes. The growth and pathogenicity of the mutants were also compared to that of the wild-type bacteria.

## 5.1. Results

### 5.1.1. Inverse PCR and Sequencing

Inverse PCR was used to amplify regions immediately upstream from the inserted *luxAB* genes using outfacing primers LUX76 and LUX340, which were specific for the *luxA* gene sequence, as indicated in Figure 11. Figure 15 demonstrates the single DNA fragment resulting from inverse PCR using chromosomal DNA from each of the mutants. No bands were seen using DNA from the wild-type bacteria SL1344. Based on the conditions of the inverse PCR used, it was highly unlikely that there was more than the one identified insertion of the *luxAB* cassette within an individual bacterial chromosome. For example, using a four-basepair cutting restriction enzyme results in frequent cutting of the chromosomal DNA, and using an extension times of 2 min or more in the PCR should be long enough to amplify segments of 2 to 3 kbp in length.

Amplified DNA fragments were isolated from the agarose gel and sequenced. Sequences of the upstream regions from each of the six mutants were compared with known sequences in GenBank. For the mutant E12A2, the primer set E PLUS/E MINUS was used to amplify and sequence further upstream and downstream from the insertion site. Similarly, the primer set G PLUS/G MINUS was used to identify the DNA region around the G7H1 insertion site. Figure 16 illustrates the positions of the insertions of the *luxAB* gene cassette within the *S. typhimurium* chromosome. D11H5 had the *luxAB* cassette inserted within the *ssaR* gene which is found within the *Salmonella* Pathogenicity Island 2 (SPI-2) ((152);

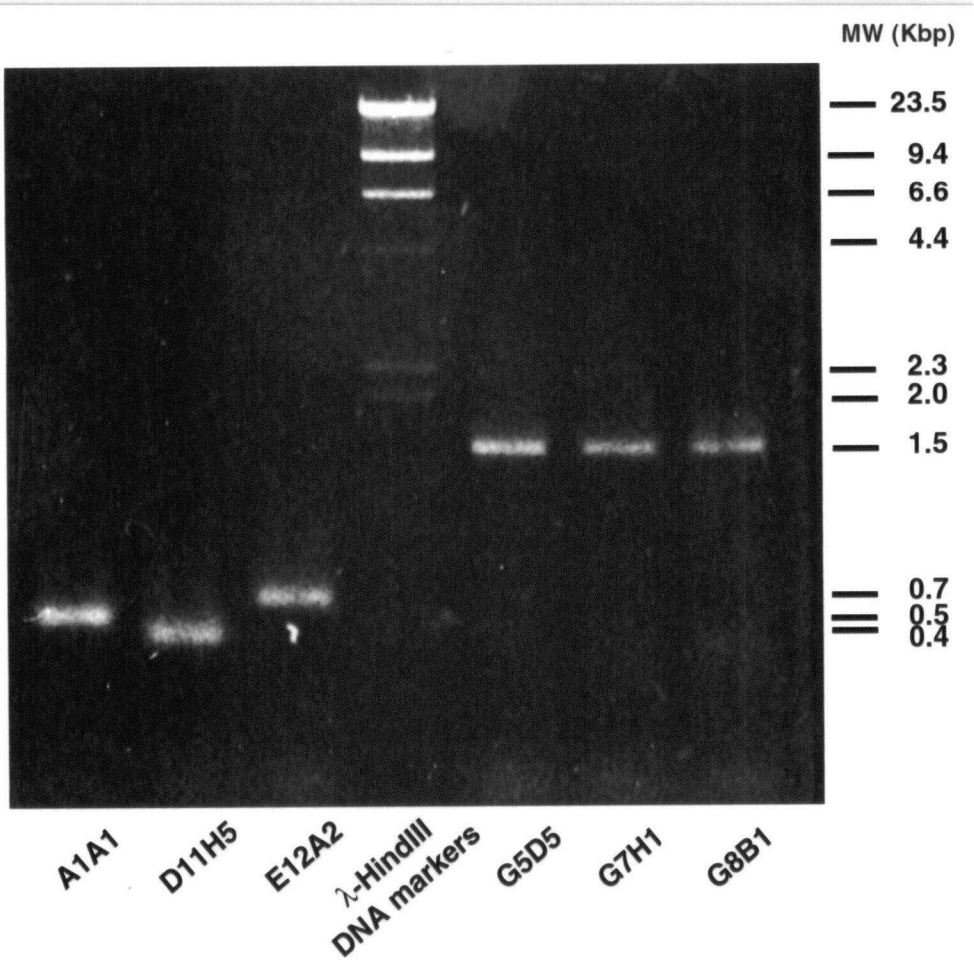
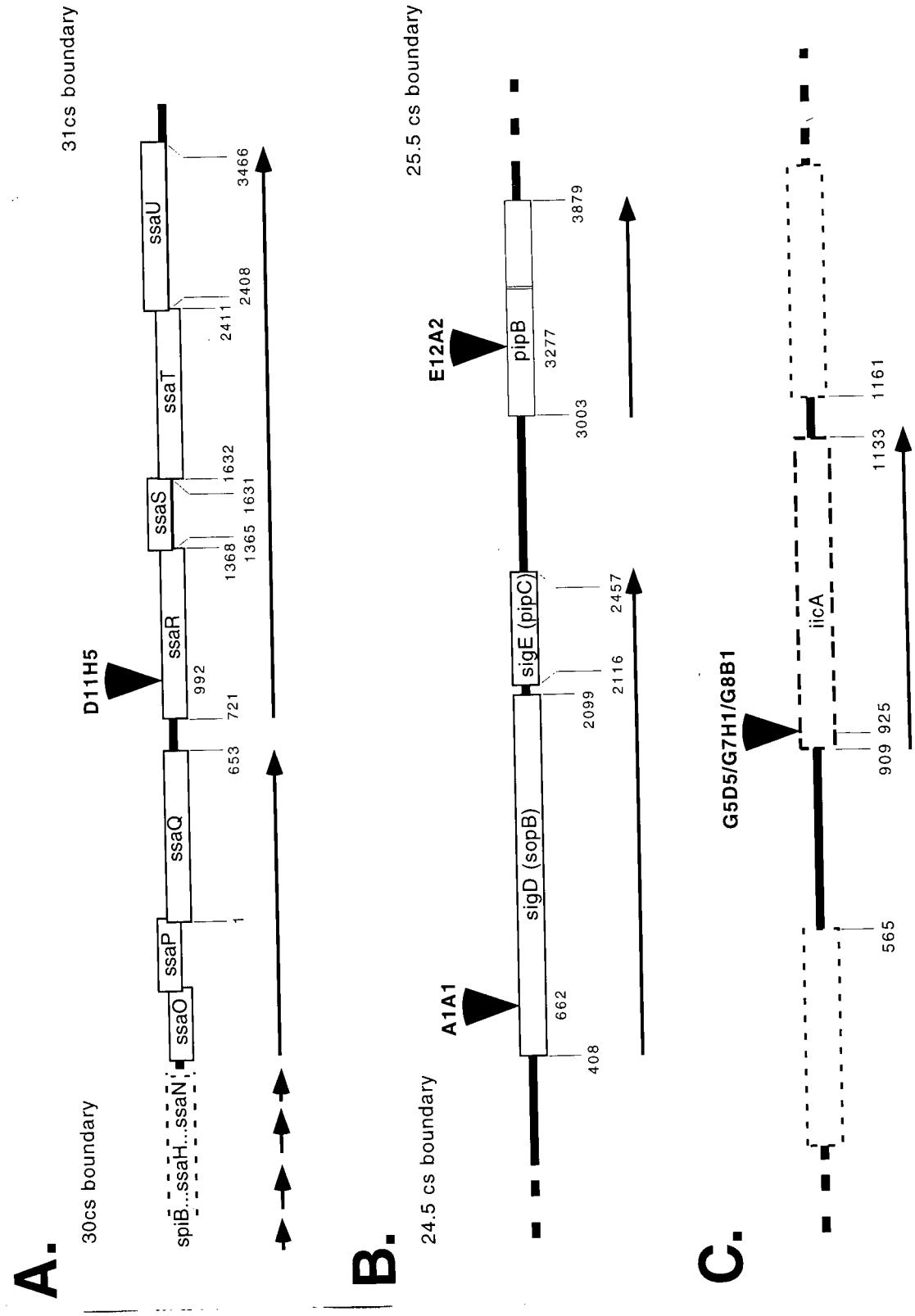



Figure 15: DNA bands resulting from inverse PCR are visualized on a 1% agarose gel. The chromosomal DNA from each mutant was cut with HaeIII, and then the fragments re-ligated prior to inverse PCR reactions using the LUX76:LUX340 primer set. The presence of a single band per mutant was indicative of a single insertion of the *luxAB* gene cassette within the chromosome.

Figure 16: Position of luciferase gene insertions within known *S. typhimurium* genes. Insertions are indicated by the large black arrowheads. Transcription direction is indicated by the arrows underneath the genes. Numbers underlying genes correspond to the sequence numbering within the Accession references for *S. typhimurium*. A) D11H5 had an insertion within the *ssaR* gene which is part of SPI-2 found between centisome 30-31 on the *S. typhimurium* chromosome (Accession #X99944). This region is part of an operon, where the open reading frames are found to overlap. B) Both A1A1 and E12A2 had insertions within SPI-5 located between near centisome 25 on the *S. typhimurium* chromosome (Accession #AF021817). A1A1 had an insertion in *sigD/sopB* while E12A2 had an insertion within a downstream ORF *pipB (S. dublin*: Accession #AF060858) . The corresponding genes in *S. dublin* are indicated in brackets. In *S. dublin* the corresponding region within SPI-5 was thought to be transcribed as one continuous mRNA, as indicated on the diagram. C) G7H1 had an insertion within a previously uncharacterized region of the *Salmonella* chromosome (Accession #AF164435). The insertion appeared to be within the 5′ end of a potential open reading frame.


A.
30cs boundary
31cs boundary
spiB...ssaH...ssaN
ssaO
ssaP
ssaQ
ssaR
ssaS
ssaT
ssaU
D11H5
1
653
721
992
1365
1368
1631
1632
2411
2408
3466
B.
24.5 cs boundary
25.5 cs boundary
A1A1
sigD (sopB)
sigE (pipC)
pipB
E12A2
408
662
2099
2116
2457
3003
3277
3879
C.
G5D5/G7H1/G8B1
iicA
565
909
925
1133
1161

Accession #X99944). A1A1 had the gene cassette inserted within the *sigD* gene found at centisome 25 on the chromosome ((159); Accession #AF021817). In *S. dublin* there is a homologous gene called *sopB*, which is found at the corresponding chromosomal position, within a region recently dubbed the fifth *Salmonella* pathogenicity island (SPI-5) ((338); Accession #AF060858). In the mutant E12A2, the *luxAB* gene cassette was inserted downstream of the *sigD/sopB* gene, in a region previously identified as a potential open reading frame. This gene was named *pipB,* as it was found to have high homology (>90% identity at the DNA level) with the *S. dublin pipB* gene, which is found downstream of *sopB*. The mutants G5D5, G7H1 and G8B1 were found to have the exact same insertion site, which was in a region that has not been identified yet. Therefore the potential open reading frame was named *iicA* for induced intracellularly *A* (GenBank Accession #AF164435). (For the sake of brevity, G5D5/G7H1/G8B1 will be referred to simply as G7H1.) Rapid mapping to locate the chromosomal position of the insert using locked-in P22 mapping sets was unsuccessful (179). However, this sequence appears to be present in both *S. typhimurium* and *S. typhi* genomes and matched a number of contigs found in the unfinished *Salmonella* sequencing projects at the Genome Sequencing Center at Washington University School of Medicine (http://genome.wustl.edu/gsc/bacterial/salmonella.shtml) (B_STM.CONTIG.1607; B_STMA2A.CONTIG.3097; B_STMA2A.CONTIG.3068) and at The Sanger Centre (http://www.sanger.as.uk/) (B_TYPHI2.hb56c04.s1).

See Appendix A for sequences of the four mutants: A1A1, D11H5, E12A2, and G5D5/G7H1/G8B1.

### 5.1.2. Extent of Gene Expression by the Mutants

The luciferase activity from both the intracellular and extracellular *Salmonella* mutants is shown in Figure 17. The activity from the intracellular bacteria can be seen to be induced when compared to the extracellular controls. This image is enhanced for low light and demonstrates the upregulation of bacterial gene expression which occurred within phagocytic cells, J774A.1, as well as within the non-phagocytic cells, HeLa and MDCK. The *spvB* gene was used as a positive control for intracellular gene expression by *S. typhimurium*. The *spvB* gene was actually induced by 10-fold inside cells, once light output and bacterial numbers had been normalized as photons/cfu. Figure 17 differs from Figure 10 in that the number of extracellular bacteria had not been adjusted to the same number found intracellularly. In Figure 17, the extracellular bacteria were 20-200 fold higher than the number found inside cells, as indicated by the relative bacteria numbers. Comparatively, the four *Salmonella* genes described in this study displayed lower expression outside of host cells and were more highly induced inside cells than was the *spvB* gene.

Figure 18 demonstrates the increase in luciferase activity by *Salmonella* within the intracellular environment over those remaining extracellular. The activity seen at the 1 hr time point represents only bacteria in the extracellular supernatant, while the other time points (3-7 hr ) represent only bacteria which were intracellular. The bacteria outside cells did not produce any more light than those grown in media alone, indicating that neither the proteins in the media and serum nor factors secreted by the cells were sufficient to induce the genes, as had been found with some *Shigella* genes (229). The *sigD/sopB* gene did not appear to be induced over time in media alone (Figure 18A), as had been reported by Hong and Miller, 1998 (159). Instead *sigD/sopB* was constantly expressed at a low level outside cells,

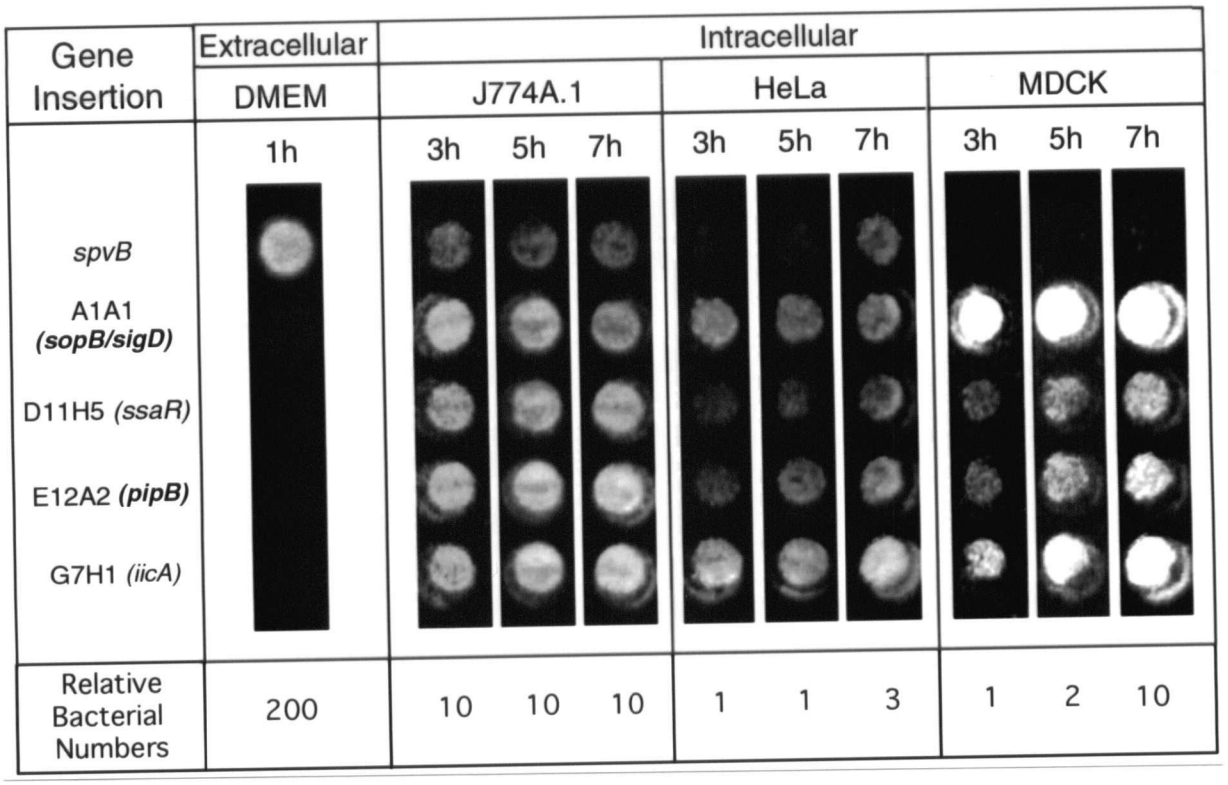
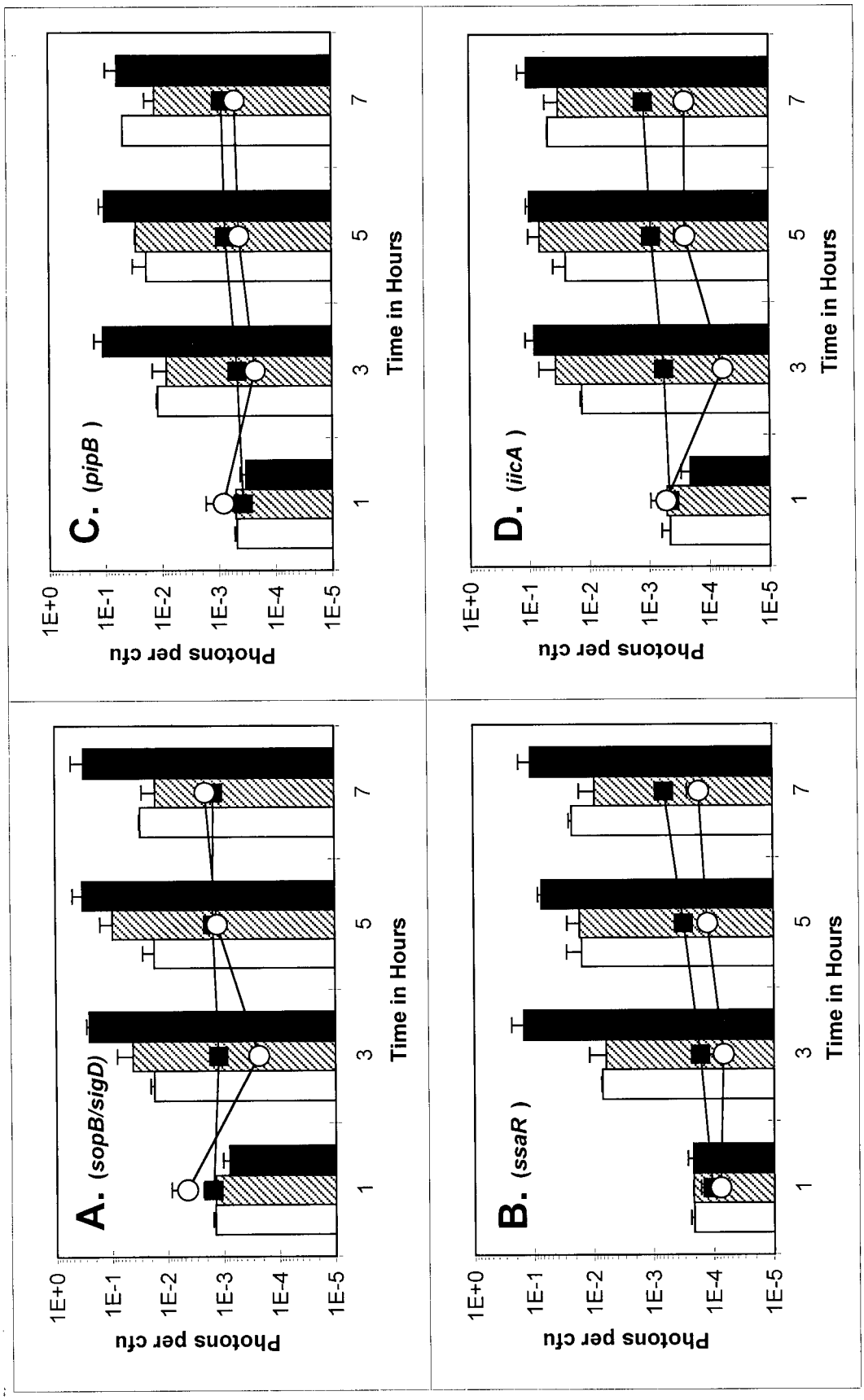



Figure 17: *S. typhimurium* mutants show increased light production inside mammalian cells. The image was taken using a Luminograph LB980 photon detector and provides a visual demonstration of the gene induction. Note that the number of extracellular bacteria are 20 to 200-fold higher than the number of intracellular bacteria. The *spvB* gene is included as a positive control and has previously been shown to be induced within cells by 5-20-fold compared to extracellular logarithmically-growing bacteria. The numbers shown at the bottom of the figure indicate the relative number of bacteria within the wells to further enable comparison of bacterial gene expression between the different conditions.

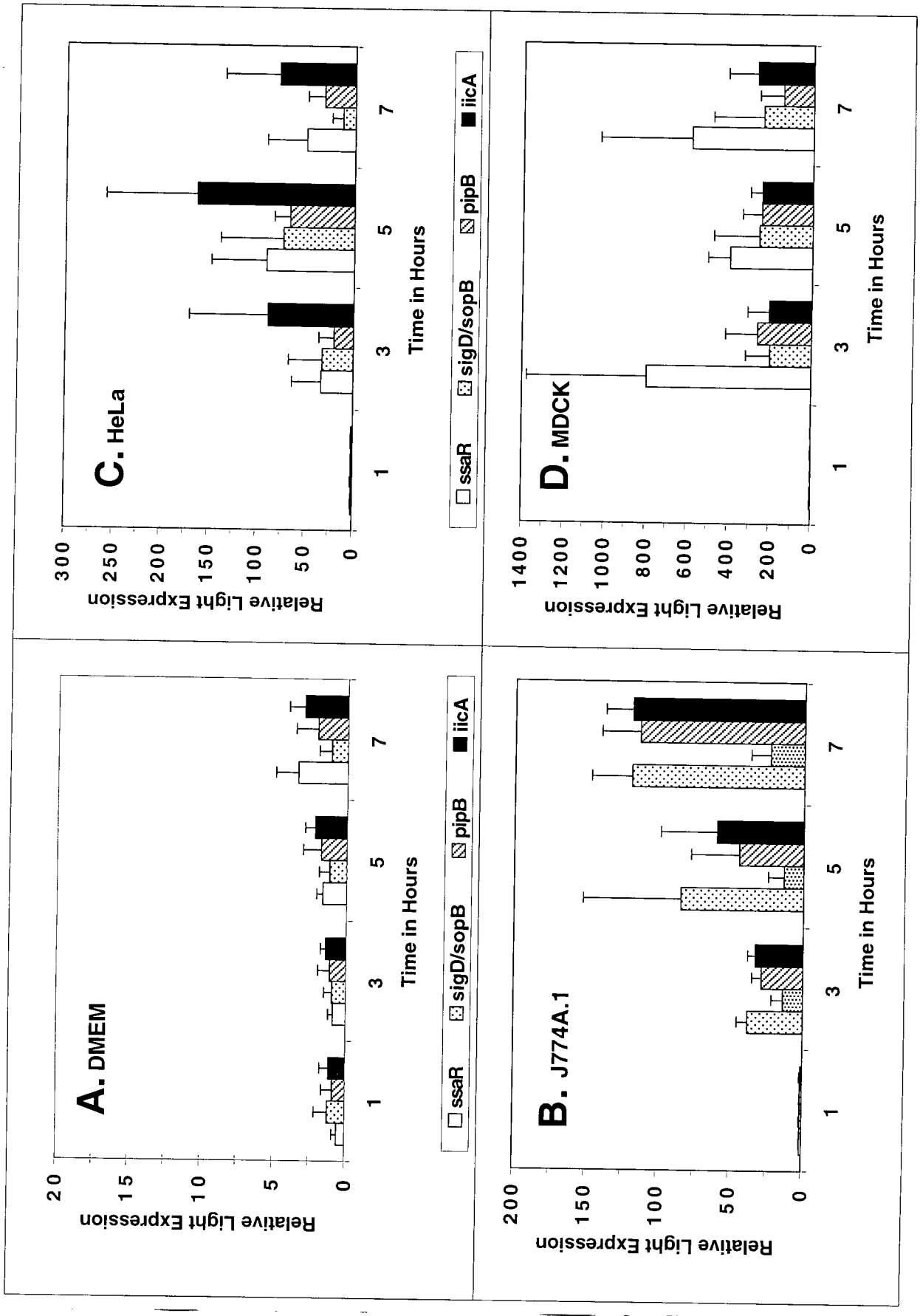
Figure 18: Comparison of light production of luciferase-expressing bacterial mutants exposed to different environmental conditions. Light was measured using a Luminograph LB980 photon detector. Individual bacterial mutants are represented in the panels: A) A1A1 (*sigD/sopB*); B) D11H1 (*ssaR*); C) E12A2 (*pipB* ); D) G7H1 (*iicA* ). The lines indicate bacteria grown in media alone, shaking while in LB broth and stationary while in DMEM++ media: DMEM++ (filled squares); LB broth (open circles). The bars for the 1 hr time points represent only the activity of the bacteria remaining in the supernatant outside the cells; the bars for the following time points (3, 5, and 7 hr) represent activity from only intracellular bacteria. J774A.1 (open bars); HeLa (hatched bars); MDCK (filled bars). Triplicate experiments were performed, and error bars represent standard error, $P<(0.5)$.


A. (sopB/sigD)
B. (ssaR )
C. (pipB )
D. (iicA )
Photons per cfu
1E+0
1E-1
1E-2
1E-3
1E-4
1E-5
1
3
5
7
Time in Hours

which was slightly higher than the other three genes described here (*pipB, ssaR, iicA*). As well, the other three genes were not found to be induced in media alone (Figure 18B-D). This was not unexpected, as the screening system was set up to eliminate mutants which were induced by growth phase.

Figure 19 summarizes the induction ratios of luciferase activity of the bacterial mutants in the various conditions. The ratios were determined by dividing the activity of the bacteria within each condition at the different time points (shown in Figure 18) by the average activity of each particular mutant in DMEM++ at 1 hr . Expression of the genes in LB broth was similar to that seen in DMEM++. The *ssaR* gene was upregulated within cultured macrophages by about 40-100 fold, and within cultured epithelial cells by 30-800 fold depending on the cell type (30-90 fold within HeLa cells and 400-800 fold within MDCK cells). The *sigD/sopB* gene was induced by 10-20 fold inside cultured J774A.1 macrophages, by 10-70 fold within HeLa epithelial cells, and by 200-250 fold inside MDCK kidney epithelial cells. The downstream *pipB* gene was induced more highly within J774A.1 cells (30-100 fold) than was the *sigD/sopB* gene, whereas induction patterns within epithelial cells were similar. The *pipB* gene was induced by 20-70 fold inside HeLa cells, and by 140-260 fold inside MDCK cells. The *iicA* gene was induced by 30-120 fold inside J774A.1 cells, by 80-160 fold inside HeLa cells, and by 200-270 fold inside MDCK cells. These data re-emphasize that none of the bacterial gene fusions were induced by the media alone, nor by cell-secreted factors; however, they do suggest that the conditions that *Salmonella* encounters within cells vary greatly between cell types. Interestingly, the gene fusions appear to be continuously induced over time, and expression only appears to decline once the cells begin to look sick (*i.e.* start to round up and lose contact with the monolayer).

Figure 19: Relative luciferase activity by extracellular and intracellular *Salmonella* mutants. The relative light expression is the ratio of the specific activity at each time point divided by the specific activity seen by bacteria grown in DMEM++ for 1 h. Bacteria were grown in A) DMEM++ media alone; B) macrophage-like J774A.1 cells; C) epithelial-like HeLa cells; and D) kidney-like MDCK cells. Symbols represent: D11H5 (*ssaR*) (clear bars); A1A1 (*sigD*) (speckled bars); E12A2 (*pipB*) (striped bars); G7H1 (*iicA*) (black bars). For panels B, C, and D, the 1 h time points represent the activity of extracellular bacteria remaining in the supernatant taken from outside the cells; the following time points (3, 5, and 7 h) represent activity from the intracellular bacteria. Triplicate experiments were performed, and error bars represent standard error of the ratio of two means.


A. DMEM
B. J774A.1
C. HeLa
D. MDCK
Relative Light Expression
Time in Hours
ssaR
sigD/sopB
pipB
iicA

HeLa cells were the most sensitive to *Salmonella* infections and would begin to "look sick" 5-6 hr after infection, while both MDCK and J774A.1 cells were more hardy and the cells would remain mostly intact up to 8-9 hr after infection.

### 5.1.3. Comparison of Growth Rate of the Mutants

The reporter gene insertions were targeted to genes which were upregulated upon bacterial invasion into cells; however, it was unknown whether the insertions affected bacterial functions or survival. Therefore, the relative growth of the bacteria was tested in order to determine whether the insertional mutations increased or decreased survival. Bacterial growth in media alone and within cells after invasion was examined, with the bacteria being grown in DMEM++ prior to invasion. Table 5 shows the growth of bacteria within the various conditions at 5 and 7 hr, as compared to the 3 hr time point. The growth rates of the four mutants did not appear to differ greatly from the growth rate of the wild type SL1344 when tested in extracellular media (LB broth or tissue culture medium plus serum, DMEM++). Within the epithelial cell lines (HeLa and MDCK), the growth patterns of all four mutants were also similar to the parent strain. Within the macrophage cells, the bacteria did not appear to increase in numbers; however, both the parental strain and all four mutants were able to survive within cultured macrophages. Longer time periods of growth within the cell lines were attempted, but the bacteria were visibly cytotoxic to the mammalian cells over longer time periods of incubation, *i.e.* longer than 9 hr (data not shown).

**Table 5: Growth of Bacteria Over Time in Various Conditions**

| Environment | *Salmonella* Mutant | Gene Insertion | Relative Growth[a] 5hr | 7hr |
|---|---|---|---|---|
| **J774A.1 Cells** | A1A1 | *sigD/sopB* | 1.2 | 0.9 |
| | D11H5 | *ssaR* | 1.0 | 1.2 |
| | E12A2 | *pipB* | 1.3 | 0.9 |
| | G7H1 | *iicA* | 1.1 | 0.9 |
| | SL1344 | - | 1.4 | 1.3 |
| **HeLa Cells** | A1A1 | *sigD/sopB* | 1.2 | 3.4 |
| | D11H5 | *ssaR* | 1.4 | 1.9 |
| | E12A2 | *pipB* | 1.5 | 2.5 |
| | G7H1 | *iicA* | 1.1 | 1.9 |
| | SL1344 | - | 1.9 | 3.6 |
| **MDCK Cells** | A1A1 | *sigD/sopB* | 2.0 | 7.0 |
| | D11H5 | *ssaR* | 2.6 | 10.0 |
| | E12A2 | *pipB* | 1.9 | 12.5 |
| | G7H1 | *iicA* | 1.0 | 4.2 |
| | SL1344 | - | 1.6 | 13.4 |
| **DMEM++ Broth** | A1A1 | *sigD/sopB* | 3.2 | 9.8 |
| | D11H5 | *ssaR* | 2.8 | 9.9 |
| | E12A2 | *pipB* | 3.3 | 10.3 |
| | G7H1 | *iicA* | 2.9 | 9.2 |
| | SL1344 | - | 2.6 | 10.6 |
| **LB Broth** | A1A1 | *sigD/sopB* | 3.9 | 6.5 |
| | D11H5 | *ssaR* | 3.5 | 10.1 |
| | E12A2 | *pipB* | 4.1 | 11.1 |
| | G7H1 | *iicA* | 3.2 | 6.6 |
| | SL1344 | - | 4.1 | 10.3 |

[a]Relative growth is the increase in bacterial numbers over time expressed as the number of cfu at *x*hr divided by the number of cfu at 3hr

### 5.1.4. Comparison of Invasiveness of the Mutants

All four mutants, when grown in DMEM++, remained as invasive as the wild type bacteria in the epithelial cells tested (Table 6). Since previous findings indicated that a small deletion in *sigD* resulted in a ten-fold reduction in invasion (159), the mutants were also tested by growing them in LB broth (both shaking overnight with subculture the next day, and standing overnight) prior to infection of the cells. Bacteria grown in LB broth were found to be about 10 fold more invasive than those grown in DMEM++ (data not shown); however, no significant difference between invasion of the mutants and the parent SL1344 was seen. Note that bacterial invasion took place in the presence of serum. The observation that the *ssaR* mutant D11H5 remained invasive correlates with previous findings where mutations within the *ssa* region of the SPI-2 did not affect invasion (60) Instead, the SPI-2 genes are thought to be necessary for the long-term survival of the bacteria within the mouse.

**Table 6: Relative Invasion of Bacterial Mutants Into Cultured Cells**

| *Salmonella* Mutant | Gene Insertion | % Invasion[a] in Different Cell Types J774 | HeLa | MDCK |
|---|---|---|---|---|
| A1A1 | *sigD/sopB* | 7.3 ±1.2 | 0.9 ±0.7 | 1.7 ±2.2 |
| D11H5 | *ssaR* | 8.6 ±1.1 | 1.2 ±0.7 | 1.4 ±2.2 |
| E12A2 | *pipB* | 6.7 ±1.4 | 0.9 ±0.1 | 2.1 ±3.3 |
| G7H1 | *iicA* | 9.1 ±3.0 | 1.0 ±0.3 | 3.0 ±2.5 |
| SL1344 | - | 7.4 ±0.3 | 0.9 ±0.7 | 1.5 ±2.4 |

[a] % invasion equals the number of intracellular bacteria at 3 hr divided by the total number of bacteria added to the well, then multiplied by 100. Bacteria were subcultured into DMEM++ prior to invasion.

### 5.1.5. Virulence of Mutants in Typhoid Mouse Model

Having identified genes upregulated by the intracellular environment, experiments were carried out to determine the requirement for these genes in the disease process. The insertional mutations did not affect the growth of the bacteria within cells or media, nor the invasion of the bacteria into cells. The virulence of the *S. typhimurium* mutants was therefore tested in a mouse infection model. The inbred mouse strain BALB/c was used for the infection model, as it has previously been shown to be susceptible to infection with a number of bacteria, including *Salmonella.* When infected with either *S. typhimurium* or *S. dublin,* BALB/c mice succumb to a systemic disease very similar to that of typhoid fever in humans, (which is caused by *S. typhi*). Signs of disease in the mouse were observed first as a lack of grooming and an overall scruffy appearance of the mice. As the disease progressed, the mice would appear more scruffy, their backs would become hunched, and they would begin to shiver uncontrollably. They would also become indifferent to the other mice in the cage. Eventually they would die. Although the disease is more systemic than the gastrointestinal disease caused by *S. typhimurium* in humans, this mouse model has proven very useful for identifying virulence factors.

The results of two experiments where mice were inoculated orally are presented in Figure 20. In the first experiment, the mice given D11H5 (*ssaR*) showed no detectable signs of disease even though they received 200 times the dose at which the wild type bacteria were 100% lethal (*i.e.* approximately 1000-fold higher than the reported $LD_{50}$ for SL1344). In the second experiment, insertion into the *pipB* gene (E12A2) attenuated the bacteria by reducing the mortality to approximately 60% that of wild-type, when given at a dose of $1.0 \times 10^6$ cfu/mouse. At this dose, the insertion within the *sopB/sigD* gene (A1A1) was also seen to attenuate virulence of the bacteria to approximately 80% mortality, although this is not statistically significant due to the small numbers of mice used for these experiments. The mutant G7H1 (*iicA*) remained as virulent as the wild type SL1344 bacteria. The possibility of polar effects caused by these insertions remains (see discussion).

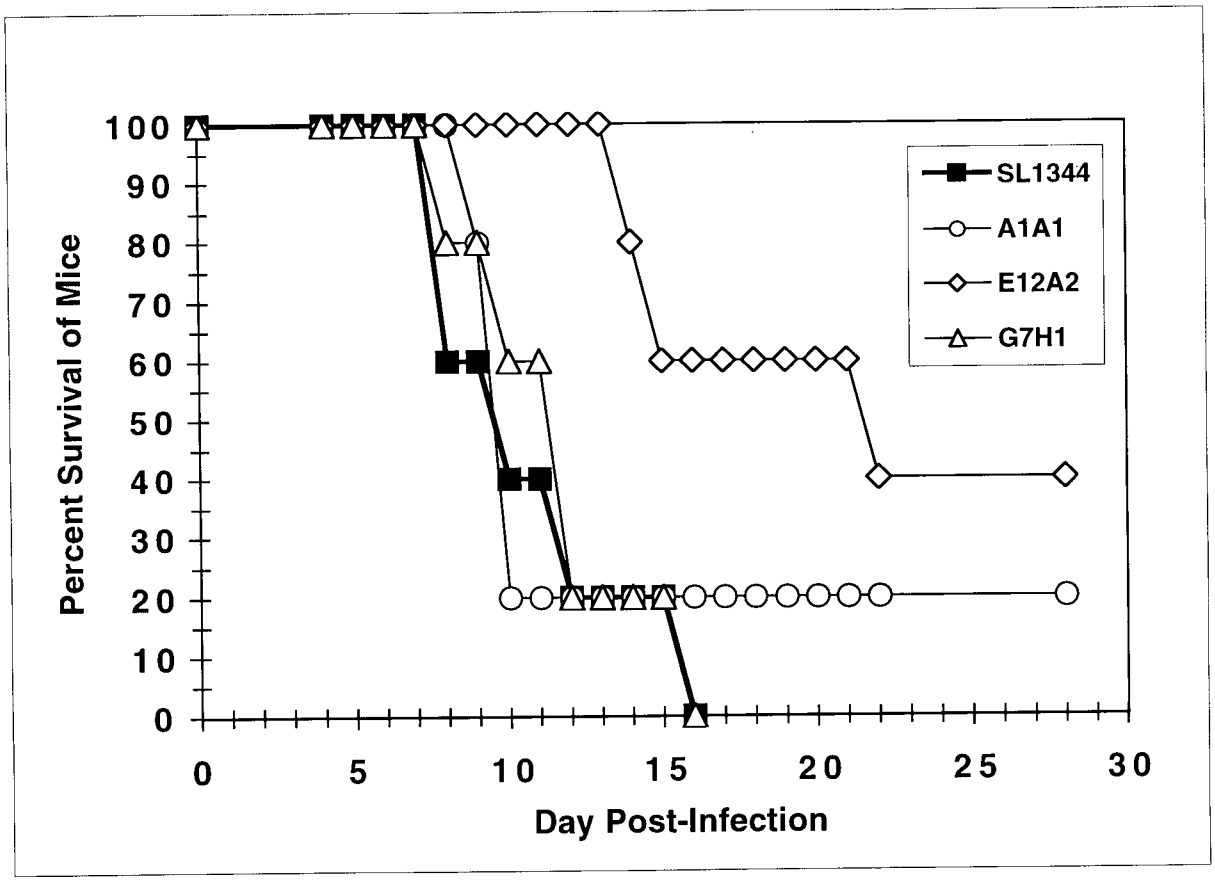



Figure 20: Virulence of *S. typhimurium* mutants in an orally infected typhoid mouse model. Mice were given $1x10^6$ cfu per mouse and disease progression was monitored for one month: SL1344 (wild type) (filled squares); A1A1 (*sigD*) (open circles); E12A2 (*pipB*) (open diamonds); G7H1 (*iicA*) (open triangles).

Although mutations in both the *sopB/sigD* (A1A1) and *pipB* (E12A2) regions appeared to cause attenuation, only a disruption of the *pipB* gene appeared to affect the rate at which disease developed in the mouse (Table 7). Generally, the higher the bacterial dose, the quicker the mice were to develop signs of disease and die. The delay in the time at which 50% of the mice in the group had died was largest when lower doses of bacteria were used (*i.e.* at a dose of $1x10^6$ cfu/mouse, 50% mouse mortality was seen on day 21 for E12A2 and on day 13 for wild-type SL1344) and was not as noticeable at higher doses (*i.e.* at a dose of $1.0x10^7$-$1.0x10^8$, 50% mouse mortality for E12A2 occurred only one to two days after that of the wild-type bacteria). A delay in mortality was also observed when the mice were inoculated intravenously with E12A2 (*pipB*). Mice inoculated with D11H5 (*ssaR*) showed no signs of disease (data not shown).

**Table 7: Bacterial dose affects kinetics of mortality of mice**

| *Salmonella* Mutant | Gene Insertion | Day of 50% Mouse Mortality[a] at various bacterial doses | | |
|---|---|---|---|---|
| | | $10^6$ | $10^7$ | $10^8$ |
| SL1344 | wild-type | 13 | 10 | 6 |
| A1A1 | *sigD/sopB* | 13 | 10 | 7 |
| E12A2 | *pipB* | 21 | 11 | 8 |
| G7H1 | *iicA* | 12 | 10 | 7 |

[a] Day post-infection at which over half the mice in the group died

After 28 days postinfection, the remaining mice were sacrificed and organ homogenates were prepared from spleens and livers. Only the mice inoculated with either the A1A1 (*sopB/sigD*) or the E12A2 (*pipB*) were tested in this manner as all mice inoculated with G7H1 (*iicA*) or wild type SL1344 had died. No *Salmonella* could be cultured from the homogenates, indicating the mice were able to clear the bacteria completely and were not chronically infected (47).

## 5.2. Discussion

The expression of four specific bacterial genes (*ssaR, sigD/sopB, pipB* , and *iicA*)was shown to be induced within the host cells, and this induction was not induced by rich media or by factors secreted by host cells (Figures 17, 18, and 19). These genes were also not only induced at early time points (2-4 hr), but remained upregulated after the bacteria had adapted and begun to grow within the cells (5-7 hr). Interestingly, reporter activity from all four genes indicated that expression was induced in both phagocytic cells and non-phagocytic cells. Expression from within cultured phagocytes continued to escalate over time, with the exception of the *sigD/sopB* gene, whereas intracellular gene expression leveled off or declined within epithelial cells.

### 5.2.1. D11H5 (*ssaR*)

The *ssaR* gene (D11H5), found within SPI-2, was homologous to the *Yersinia enterocolitica* gene *yscR*, which encodes a membrane-bound subunit of the type III secretion system (150, 152). The YscR forms a critical part of the secretion apparatus. The *ssaR* gene was upregulated after bacterial invasion of both cultured macrophages and epithelial cells, and continued to be expressed throughout the course of the infection. The insertion of the reporter gene cassette into the *ssaR* gene abolished virulence of *S. typhimurium* in the typhoid mouse model. These findings are novel for the *ssaR* gene but agree with previous reports of insertions within SPI-2 (46, 321). A *ssaH ::gfp* gene fusion (within another secretion apparatus-encoding gene) was induced by 400-fold within macrophages and rendered the bacteria avirulent in mice (321). Similarly, various other *gfp* insertions into regulatory, structural, and effector/chaperone genes of SPI-2 were also found to be induced within macrophage cells by 3 to 100 fold. Specifically the insertions within *ssrA* and *sscB* genes reduced the ability of the bacteria to spread to other organs within the mouse (46). One could argue that the *luxAB* insertion within the *ssaR* gene caused a polar effect on downstream genes, and therefore this gene is not directly related to virulence in mice. However, a previous report had indicated that an insertion within *ssaT,* a downstream gene within the same operon,

reduced bacterial invasion (152), whereas the mutant D11H5 was not impaired in its ability to invade non-phagocytic cells. The insertion is mostly likely having a direct effect on bacterial virulence, since type III secretion has been previously implicated as important to bacterial virulence (46), and this gene potentially encodes a structural subunit of the type III secretion system (152).

### 5.2.2. A1A1 (*sigD/sopB*) and E12A2 (*pipB* )

The mutant A1A1 had an insertion within the *sigD/sopB* gene (159), which is found within SPI-5. SPI-5 is located near centisome 25 in *S. typhimurium* (159). Previous studies have shown that SigD is a secreted protein, and have suggested that it is secreted into the host cell via the SPI-1 type III secretion system (159). Furthermore, the *S. typhimurium sigD/sopB* gene is more than 95% identical at the DNA level to the *S. dublin sopB* gene, which has recently been shown to act as an inositol phosphate phosphatase inside host cells (235). Norris *et al.* (235) showed that *Salmonella* affected the cellular signal transduction pathways and that SopB was able to hydrolyze phosphatidylinositol 3,4,5-trisphosphate, which is known to be a direct inhibitor of calcium-dependent chloride secretion. As well, SopB hydrolyzed 1,3,4,5,6-pentakisphosphate to 1,4,5,6-tetrakisphosphate, which is a signaling molecule that indirectly acts to increase chloride secretion. The result of these activities during infection was proposed to be increased chloride secretion by the gastrointestinal cells, ultimately resulting in diarrhea in the host (235).

The mutant E12A2 had an insertion within a previously unreported *S. typhimurium* gene, *pipB*. This gene was located downstream of *sigD/sopB* and was also contained within SPI-5. The hypothetical *pipB* gene product was homologous to that of the *S. dublin pipB* gene product, which has structural similarity to proteins involved with glycolipid biogenesis (338). As indicated in Appendix A, where the *S. dublin* gene is one ORF, the corresponding *S. typhimurium* region may actually be divided into two open reading frames (ORF). However, the size of the actual gene product has not been determined for either *S. typhimurium* or *S. dublin pipB*.

It has been suggested that within the SPI-5 of *S. dublin,* the *sopB* and genes downstream (*pipC, pipB,* and *pipA*) are contained within the same transcriptional unit, based on the analysis of the size of mRNA transcripts (338). Similar studies have not been performed for the *S. typhimurium* SPI-5 region, although there is evidence that the *sigDE* genes may be transcribed from a single promoter (159). In the study described here, the *sigD/sopB* (A1A1) and *pipB* (E12A2) genes had a similar expression pattern within epithelial cell types; however, the downstream *pipB* gene was more highly induced than was *sigD/sopB*. As well, the genes were not induced to the same extent within macrophages (Figures 18 and 19), with the expression of *pipB* continuing to escalate within the intramacrophage environment, while the expression of *sigD/sopB* leveled off over time. This suggests that *pipB* was transcribed from a separate promoter. Further support for a second promoter was that the insertion within the *pipB* gene greatly attenuated virulence of the *S. typhimurium*, while the insertion within the upstream *sigD/sopB* gene had only a marginal effect on virulence. The fact that the downstream gene (*pipB*) had a greater effect on virulence than the upstream gene (*sigD/sopB*) likewise argues that the insertion of the reporter gene cassette did not cause a downstream polar effect on gene expression, but that the attenuation resulted directly from inactivation of the genes containing the insertions.

Within cultured cell models, previous findings (159) indicated that insertions within either *sigD* or *sigE* resulted in a ten-fold reduction in invasion of epithelial cells when compared to wild type bacteria. In the assays described here, invasion was not significantly reduced by the insertions within either the *sigD/sopB* or *pipB* gene as compared to the parental strain. The cause of these differences is difficult to explain, although different *S. typhimurium* isolates and different cell lines were used here. It is interesting to note that others have reported variability in invasion efficiency of bacterial mutants depending on the point of mutation within a gene. Hensel *et al.* (152) reported that only one out of three insertions within the gene *ssaV* (SPI-2) resulted in a 10 fold reduction in bacterial invasion, while two other single insertions within this gene did not affect the level of invasiveness. Furthermore, it was found that

mutations within the *S. dublin* gene homologues did not affect invasion (101, 338, 339). However, when the *sigD/sopB* mutant described here was grown overnight and subcultured in LB, shaking at 37°C, and then allowed to invade cells in the absence of serum, there was a 30% reduction in invasion (298). (Note that there was no reduction in invasion by the other mutants (*ssaR, pipB, iicA*) in the absence of serum.) Future experiments with deletions of these genes may help determine their ultimate effect on bacterial invasion and virulence.

The insertions within the *sigD/sopB* and *pipB* genes resulted in attenuation of *S. typhimurium* in the typhoid mouse model, but did not totally abolish virulence of the bacteria as did the insertion within *ssaR*. The effect of the insertion within the *pipB* gene appeared to delay the onset of the disease allowing the infected mice to live longer, irrespective of dose (although the largest delay was seen with the $LD_{50}$ dose). In *S. dublin* studies by Galyov *et al.* (1997) and Wood *et al.* (1998), mutations within the SPI-5 region reduced intestinal secretion and inflammatory responses in a calf ileal loop model (101, 338). However, in a mouse model, the *S. dublin* mutants were recovered from organs in the same numbers as the wild-type strain (338). Their conclusion was that mutations in SPI-5-encoded genes affect the enteropathogenicity of *Salmonella*, but have no major effect on the development of systemic disease in mice. However the study here suggests there was a difference seen in the development of systemic disease, although the difference was only noticed when lower doses of bacteria were used for inoculation. In the previous *S. dublin* study, only one dose was reportedly tested on the mice and it could be that at that dose, the mouse immune response was overwhelmed such that no significant difference was seen when comparing the mutant to the wild-type bacteria.

### 5.2.3. G7H1 (*iicA*)

The mutant G7H1 contained an insertion within the previously undescribed region of the *S. typhimurium* chromosome which appeared to encode an open reading frame, and was thus named *iicA* for induced intracellularly. This gene fusion displayed upregulated reporter activity by intracellular *Salmonella,* within both macrophage and epithelial cells. However, the

insertion had no effect on invasion or survival of the bacteria within cells, nor did it appear to attenuate bacterial virulence in the mouse model. No significant similarity to any other known gene was found to this region, based on a search of the available DNA databases, and the function of the *iicA* gene and gene product remains to be determined.

## 5.3. Overall Conclusion

In conclusion, four individual genes of the *S. typhimurium* chromosome were identified that were upregulated in response to the intracellular environment of mammalian cells. The screen described in this report allowed for the identification of specific virulence factors, indicating that genes induced inside mammalian cells often play a key role in *Salmonella* pathogenesis. Three of the four genes (*ssaR, sigD/sopB,* and *pipB*) identified were virulence factors, inasmuch as insertional inactivation of these genes decreased mouse mortality in an animal model. The fourth gene, *iicA,* was not found to reduce mouse mortality, however its role in virulence has yet to be tested in other animal models, *e.g.* calf ileal loop model. The four genes identified were tightly regulated by the bacteria, with very little expression from extracellular bacteria. The three genes identified as virulence factors (*ssaR, sigD/sopB,* and *pipB*) were contained within chromosomal regions known as islands of pathogenicity (SPI), and at least two of the gene products had previously been shown to be involved with a type III secretion system (SsaR and SigD/SopB). Pathogenicity islands have been found in numerous bacterial pathogens, on their chromosomes and extrachromosomal elements. These regions often carry genes encoding type III secretions systems and proteins for secretion, as well as other products which allow the different bacteria to survive and cause disease within defined niches. The further characterization of these genes and their products will lead to an enhanced understanding about how *Salmonella* functions as an intracellular pathogen.

Future experiments will define the regulation of the various genes and the specific conditions required to induce their expression. For example, both *sigD/sopB* and *pipB* were contained within SPI-5 and both were induced within cells; however their expression levels varied over time indicating that they are expressed under separate and unique promoters.

Specific deletion mutations will help define which regions of the genes are important for bacterial virulence, *e.g.* the regions necessary for secretion of *sigD/sopB* have not been defined. Furthermore, the function of both PipB and SsaR have only been deduced by homology to other DNA sequences and the corresponding proteins have not been biochemically defined. Finally, other *Salmonella* genes may be discovered using this assay, and the techniques may be applied to other pathogenic bacteria to isolate virulence factors.

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# Appendix

## DNA and Predicted Protein Sequences Surrounding Insertional Mutations

The sites which are offset in the DNA sequences indicate the position of the insertion of the *luxAB* gene cassette. The names of the corresponding genes are given below the start of the predicted proteins. The sequences which are underlined were actually sequenced in this study; non-underlined sequences were obtained from previously published sequences.

**A: D11H5 (*ssaR*)**

ACCESSION X99944 (SPI-2 *S. typhimurium*)

```
        10         20         30         40         50         60
CCTGCAGTAA TCTACCACAT CAGCTAGCGT TGCATATTAA ATGGACAGTT GAAGAGCATG
GGACGTCATT AGATGGTGTA GTCGATCGCA ACGTATAATT TACCTGTCAA CTTCTCGTAC
..C..S..N. .L..P..H.. Q..L..A..L ..H..I..K. .W..T..V.. E..E..H..E
(ssaQ)

        70         80         90        100        110        120
AGTTCCATAG CATTATTTTT ACATGGCCAA CGGGTTTTTT GCGCAATATA GTCGGAGAGC
TCAAGGTATC GTAATAAAAA TGTACCGGTT GCCCAAAAAA CGCGTTATAT CAGCCTCTCG
..F..H..S. .I..I..F.. T..W..P..T ..G..F..L. .R..N..I.. V..G..E..L

       130        140        150        160        170        180
TTTCTGCTGA GCGACAACAG ATTTATCCTG CCCCTCCTGT GGTAGTCCCT GTATATTCAG
AAAGACGACT CGCTGTTGTC TAAATAGGAC GGGGAGGACA CCATCAGGGA CATATAAGTC
..S..A..E. .R..Q..Q.. I..Y..P..A ..P..P..V. .V..V..P.. V..Y..S..G

       190        200        210        220        230        240
GCTGGTGCCA GCTTACATTA ATCGAACTTG AGTCTATCGA AATCGGCATG GGCGTTCGGA
CGACCACGGT CGAATGTAAT TAGCTTGAAC TCAGATAGCT TTAGCCGTAC CCGCAAGCCT
..W..C..Q. .L..T..L.. I..E..L..E ..S..I..E. .I..G..M.. G..V..R..I

       250        260        270        280        290        300
TTCATTGCTT CGGCGACATC AGACTCGGTT TTTTTGCTAT TCAACTACCT GGGGGAATCT
AAGTAACGAA GCCGCTGTAG TCTGAGCCAA AAAAACGATA AGTTGATGGA CCCCCTTAGA
..H..C..F. .G..D..I.. R..L..G..F ..F..A..I. .Q..L..P.. G..G..I..Y

       310        320        330        340        350        360
ACGCAAGGGT GTTGCTGACA GAGGATAACA CGATGAAATT TGACGAATTA GTCCAGGATA
TGCGTTCCCA CAACGACTGT CTCCTATTGT GCTACTTTAA ACTGCTTAAT CAGGTCCTAT
..A..R..V. .L..L..T.. E..D..N..T ..M..K..F. .D..E..L.. V..Q..D..I
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        370         380         390         400         410         420
TCGAAACGCT ACTTGCGTCA GGGAGCCCAA TGTCAAAGAG TGACGGAACG TCTTCAGTCG
AGCTTTGCGA TGAACGCAGT CCCTCGGGTT ACAGTTTCTC ACTGCCTTGC AGAAGTCAGC
..E..T..L. .L..A..S.. G..S..P..M ..S..K..S. .D..G..T.. S..S..V..E

        430         440         450         460         470         480
AACTTGAGCA GATACCACAA CAGGTGCTCT TTGAGGTCGG ACGTGCGAGT CTGGAAATTG
TTGAACTCGT CTATGGTGTT GTCCACGAGA AACTCCAGCC TGCACGCTCA GACCTTTAAC
..L..E..Q. .I..P..Q.. Q..V..L..F ..E..V..G. .R..A..S.. L..E..I..G

        490         500         510         520         530         540
GACAATTACG ACAACTTAAA ACGGGGGACG TTTTGCCTGT AGGTGGATGT TTTGCGCCAG
CTGTTAATGC TGTTGAATTT TGCCCCCTGC AAAACGGACA TCCACCTACA AAACGCGGTC
..Q..L..R. .Q..L..K.. T..G..D..V ..L..P..V. .G..G..C.. F..A..P..E

        550         560         570         580         590         600
AGGTGACGAT AAGAGTAAAT GACCGTATTA TTGGGCAAGG TGAGTTGATT GCCTGTGGCA
TCCACTGCTA TTCTCATTTA CTGGCATAAT AACCCGTTCC ACTCAACTAA CGGACACCGT
..V..T..I. .R..V..N.. D..R..I..I ..G..Q..G. .E..L..I.. A..C..G..N

        610         620         630         640         650         660
ATGAATTTAT GGTGCGTATT ACACGTTGGT ATCTTTGCAA AAATACAGCG TAAACCTGAT
TACTTAAATA CCACGCATAA TGTGCAACCA TAGAAACGTT TTTATGTCGC ATTTGGACTA
..E..F..M. .V..R..I.. T..R..W..Y ..L..C..K. .N..T..A.. *

        670         680         690         700         710         720
AAGAAAAATA ATATGCGAAC AATATAATAG CGTTCCAGGT CGTGTCATGA GAGATACAGT
TTCTTTTTAT TATACGCTTG TTATATTATC GCAAGGTCCA GCACAGTACT CTCTATGTCA

        730         740         750         760         770         780
ATGTCTTTAC CCGATTCGCC TTTGCAACTG ATTGGTATAT TGTTTCTGCT TTCAATACTG
TACAGAAATG GGCTAAGCGG AAACGTTGAC TAACCATATA ACAAAGACGA AAGTTATGAC
M..S..L..P ..D..S..P. .L..Q..L.. I..G..I..L ..F..L..L. .S..I..L..
(ssaR)

CCTCTCATTA TCGTCATGGG AACTTCTTTC CTTAAACTGG CGGTGGTATT TTCGATTTTA
GGAGAGTAAT AGCAGTACCC TTGAAGAAAG GAATTTGACC GCCACCATAA AAGCTAAAAT
P..L..I..I ..V..M..G. .T..S..F.. L..K..L..A ..V..V..F. .S..I..L..

        850         860         870         880         890         900
CGAAATGCTC TGGGTATTCA ACAAGTCCCC CCAAATATCG CACTGTATGG CCTTGCGCTT
GCTTTACGAG ACCCATAAGT TGTTCAGGGG GGTTTATAGC GTGACATACC GGAACGCGAA
R..N..A..L ..G..I..Q. .Q..V..P.. P..N..I..A ..L..Y..G. .L..A..L..

        910         920         930         940         950         960
GTACTTTCCT TATTCATTAT GGGGCCGACG CTATTAGCTG TAAAAGAGCG CTGGCATCCG
CATGAAAGGA ATAAGTAATA CCCCGGCTGC GATAATCGAC ATTTTCTCGC GACCGTAGGC
V..L..S..L ..F..I..M. .G..P..T.. L..L..A..V ..K..E..R. .W..H..P..
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      970         980         990        1000        1010        1020
GTTCAGGTCG CTGGCGCTCC TTTCTGGACG T[CT]GAGTGGG ACAGTAAAGC ATTAGCGCCT
CAAGTCCAGC GACCGCGAGG AAAGACCTGC A[GA]CTCACCC TGTCATTTCG TAATCGCGGA
V..Q..V..A ..G..A..P. .F..W..T.. S..E..W..D ..S..K..A. .L..A..P..

      1030        1040        1050        1060        1070        1080
TATCGACAGT TTTTGCAAAA AAACTCTGAA GAGAAGGAAG CCAATTATTT TCGGAATTTG
ATAGCTGTCA AAAACGTTTT TTTGAGACTT CTCTTCCTTC GGTTAATAAA AGCCTTAAAC
Y..R..Q..F ..L..Q..K. .N..S..E.. E..K..E..A ..N..Y..F. .R..N..L..

      1090        1100        1110        1120        1130        1140
ATAAAACGAA CCTGGCCTGA AGACATAAAA AGAAAGATAA AACCTGATTC TTTGCTCATA
TATTTTGCTT GGACCGGACT TCTGTATTTT TCTTTCTATT TTGGACTAAG AAACGAGTAT
I..K..R..T ..W..P..E. .D..I..K.. R..K..I..K ..P..D..S. .L..L..I..

      1150        1160        1170        1180        1190        1200
TTAATTCCGG CATTTACGGT GAGTCAGTTA ACGCAGGCAT TTCGGATTGG ATTACTTATT
AATTAAGGCC GTAAATGCCA CTCAGTCAAT TGCGTCCGTA AAGCCTAACC TAATGAATAA
L..I..P..A ..F..T..V. .S..Q..L.. T..Q..A..F ..R..I..G. .L..L..I..

      1210        1220        1230        1240        1250        1260
TATCTTCCCT TTCTGGCTAT TGACCTGCTT ATTTCAAATA TACTGCTGGC TATGGGGATG
ATAGAAGGGA AAGACCGATA ACTGGACGAA TAAAGTTTAT ATGACGACCG ATACCCCTAC
Y..L..P..F ..L..A..I. .D..L..L.. I..S..N..I ..L..L..A. .M..G..M..

      1270        1280        1290        1300        1310        1320
ATGATGGTGT CGCCGATGAC CATTTCATTA CCGTTTAAGC TGCTAATATT TTTACTGGCA
TACTACCACA GCGGCTACTG GTAAAGTAAT GGCAAATTCG ACGATTATAA AAATGACCGT
M..M..V..S ..P..M..T. .I..S..L.. P..F..K..L ..L..I..F. .L..L..A..

      1330        1340        1350        1360        1370        1380
GGCGGTTGGG ATCTGACACT GGCGCAATTG GTACAGAGCT TTTCATGAAT GATTCTGAAT
CCGCCAACCC TAGACTGTGA CCGCGTTAAC CATGTCTCGA AAAGTACTTA CTAAGACTTA
G..G..W..D ..L..T..L. .A..Q..L.. V..Q..S..F ..S..*
                                            M..N..  D..S..E..L
                                            (ssaS)

      1390        1400        1410        1420        1430        1440
TGACGCAATT TGTAACGCAA CTTTTATGGA TCGTCCTTTT TACGTCTATG CCGGTAGTGT
ACTGCGTTAA ACATTGCGTT GAAAATACCT AGCAGGAAAA ATGCAGATAC GGCCATCACA

..T..Q..F. .V..T..Q.. L..L..W..I ..V..L..F. .T..S..M.. P..V..V..L

      1450        1460        1470        1480        1490        1500
TGGTGGCATC GGTAGTTGGT GTCATCGTAA GCCTTGTTCA GGCCTTGACT CAAATACAGG
ACCACCGTAG CCATCAACCA CAGTAGCATT CGGAACAAGT CCGGAACTGA GTTTATGTCC

..V..A..S. .V..V..G.. V..I..V..S ..L..V..Q. .A..L..T.. Q..I..Q..D
```

## B. A1A1 (*sigD/sopB*) and E12A2 (*pipB*)

ACCESSION AF021817 (*sigDE S. typhimurium*)

```
        10          20          30          40          50          60
TATCTGTTCA AGCATGGAAT AGGAAAAACG AATATTCTTC GTCACGGTCT TACTTGTCCG
ATAGACAAGT TCGTACCTTA TCCTTTTTGC TTATAAGAAG CAGTGCCAGA ATGAACAGGC

        70          80          90          100         110         120
GGGCTTTGCT GGCATACACA CACCTGTATA ACATTTGATG TAACGCCGTT ACTTTACGCA
CCCGAAACGA CCGTATGTGT GTGGACATAT TGTAAACTAC ATTGCGGCAA TGAAATGCGT

        130         140         150         160         170         180
GGAGTAAATC GGTGAATTTG ATCTGAGTCA AGAAGGTGGG TTTTCAATAA AAGTTGTGCC
CCTCATTTAG CCACTTAAAC TAGACTCAGT TCTTCCACCC AAAAGTTATT TTCAACACGG

        190         200         210         220         230         240
ATAAATTGTG AAGTTTGTAG ATTTTATGAA CATTTGATGT ACCGATCTCC CCCATGATCG
TATTTAACAC TTCAAACATC TAAAATACTT GTAAACTACA TGGCTAGAGG GGGTACTAGC

        250         260         270         280         290         300
CCACTACGCT ATGGACGTCA GGATGCCTCC CCGCCTGATC AGAAGCGTTT CCTCATTAAA
GGTGATGCGA TACCTGCAGT CCTACGGAGG GGCGGACTAG TCTTCGCAAA GGAGTAATTT

        310         320         330         340         350         360
AAGGACATTT TTTTAAAGTT CCTGGTGCAT AAAAGTCACA TCCTTTTAAA GGGTTGTTAA
TTCCTGTAAA AAAATTTCAA GGACCACGTA TTTTCAGTGT AGGAAAATTT CCCAACAATT

        370         380         390         400         410         420
CCCTGTTGAA TGTTCCCACT CCCCTATTCA GGAATATTAA AAACGCTATG CAAATACAGA
GGGACAACTT ACAAGGGTGA GGGGATAAGT CCTTATAATT TTTGCGATAC GTTTATGTCT
                                                    M..  Q..I..Q..S
                                                    (sigD/sopB)

        430         440         450         460         470         480
GCTTCTATCA CTCAGCTTCA CTAAAAACCC AGGAGGCTTT TAAAAGCCTA CAAAAAACCT
CGAAGATAGT GAGTCGAAGT GATTTTTGGG TCCTCCGAAA ATTTTCGGAT GTTTTTTGGA
..F..Y..H.  .S..A..S..  L..K..T..Q  ..E..A..F.  .K..S..L..  Q..K..T..L

        490         500         510         520         530         540
TATACAACGG AATGCAGATT CTCTCAGGCC AGGGCAAAGC GCCGGCTAAA GCGCCCGACG
ATATGTTGCC TTACGTCTAA GAGAGTCCGG TCCCGTTTCG CGGCCGATTT CGCGGGCTGC
..Y..N..G.  .M..Q..I..  L..S..G..Q  ..G..K..A.  .P..A..K..  A..P..D..A

        550         560         570         580         590         600
CTCGCCCGGA AATTATTGTC CTGCGAGAAC CCGGCGCGAC ATGGGGGAAT TATCTACAGC
GAGCGGGCCT TTAATAACAG GACGCTCTTG GGCCGCGCTG TACCCCCTTA ATAGATGTCG
..R..P..E.  .I..I..V..  L..R..E..P  ..G..A..T.  .W..G..N..  Y..L..Q..H
```

```
        610         620         630         640         650         660
ATCAGAAGGC GTCTAACCAC TCGCTGCATA ACCTCTATAA CTTACAGCGC GATCTTCTTA
TAGTCTTCCG CAGATTGGTG AGCGACGTAT TGGAGATATT GAATGTCGCG CTAGAAGAAT
..Q..K..A. .S..N..H.. S..L..H..N ..L..Y..N. .L..Q..R.. D..L..L..T

        670         680         690         700         710         720
CCGTCGCGGC AACCGTTCTG GGTAAACAAG ACCCGGTTCT AACGTCAATG GCAAACCAAA
GGCAGCGCCG TTGGCAAGAC CCATTTGTTC TGGGCCAAGA TTGCAGTTAC CGTTTGGTTT
..V..A..A...T..V..L...G..K..Q..D...P..V..L...T..S..M...A..N..Q..M

        730         740         750         760         770         780
TGGAGTTAGC CAAAGTTAAA GCGGACCGGC CAGCAACAAA ACAAGAAGAA GCCGCGGCAA
ACCTCAATCG GTTTCAATTT CGCCTGGCCG GTCGTTGTTT TGTTCTTCTT CGGCGCCGTT
..E..L..A. .K..V..K.. A..D..R..P ..A..T..K. .Q..E..E.. A..A..A..K

        790         800         810         820         830         840
AAGCATTGAA GAAAAATCTT ATCGAACTTA TTGCAGCACG CACTCAGCAG CAGGATGGCT
TTCGTAACTT CTTTTTAGAA TAGCTTGAAT AACGTCGTGC GTGAGTCGTC GTCCTACCGA
..A..L..K. .K..N..L.. I..E..L..I ..A..A..R. .T..Q..Q.. Q..D..G..L

        850         860         870         880         890         900
TACCTGCAAA AGAAGCTCAT CGCTTTGCGG CAGTAGCGTT TAGAGATGCT CAGGTCAAGC
ATGGACGTTT TCTTCGAGTA GCGAAACGCC GTCATCGCAA ATCTCTACGA GTCCAGTTCG
..P..A..K. .E..A..H.. R..F..A..A ..V..A..F. .R..D..A.. Q..V..K..Q

        910         920         930         940         950         960
AGCTTAATAA CCAGCCCTGG CAAACCATAA AAAATACACT CACGCATAAC GGGCATCACT
TCGAATTATT GGTCGGGACC GTTTGGTATT TTTTATGTGA GTGCGTATTG CCCGTAGTGA
..L..N..N. .Q..P..W.. Q..T..I..K ..N..T..L. .T..H..N.. G..H..H..Y

        970         980         990        1000        1010        1020
ATACCAACAC GCAGCTCCCT GCAGCAGAGA TGAAAATCGG CGCAAAAGAT ATCTTTCCCA
TATGGTTGTG CGTCGAGGGA CGTCGTCTCT ACTTTTAGCC GCGTTTTCTA TAGAAAGGGT
..T..N..T. .Q..L..P.. A..A..E..M ..K..I..G. .A..K..D.. I..F..P..S

        1030        1040        1050        1060        1070        1080
GTGCTTATGA GGGAAAGGGC GTATGCAGTT GGGATACCAA GAATATTCAT CACGCCAATA
CACGAATACT CCCTTTCCCG CATACGTCAA CCCTATGGTT CTTATAAGTA GTGCGGTTAT
..A..Y..E. .G..K..G.. V..C..S..W ..D..T..K. .N..I..H.. H..A..N..N

        1090        1100        1110        1120        1130        1140
ATTTGTGGAT GTCCACGGTG AGTGTGCATG AGGACGGTAA AGATAAAACG CTTTTTTTTG
TAAACACCTA CAGGTGCCAC TCACACGTAC TCCTGCCATT TCTATTTTGC GAAAAAAAAC
..L..W..M. .S..T..V.. S..V..H..E ..D..G..K. .D..K..T.. L..F..F..D

        1150        1160        1170        1180        1190        1200
ACGGGATACG TCATGGCGTG CTTTCCCCCT ATCATGAAAA AGATCCGCTT CTGCGTCACG
TGCCCTATGC AGTACCGCAC GAAAGGGGGA TAGTACTTTT TCTAGGCGAA GACGCAGTGC
..G..I..R. .H..G..V.. L..S..P..Y ..H..E..K. .D..P..L.. L..R..H..V
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      1210        1220        1230        1240        1250        1260
TCGGCGCTGA AAACAAAGCC AAAGAAGTAT TAACTGCGGC ACTTTTTAGT AAACCTGAGT
AGCCGCGACT TTTGTTTCGG TTTCTTCATA ATTGACGCCG TGAAAAATCA TTTGGACTCA
..G..A..E. .N..K..A.. K..E..V..L ..T..A..A. .L..F..S.. K..P..E..L

      1270        1280        1290        1300        1310        1320
TGCTTAACAA AGCCTTAGCG GGCGAGGCGG TAAGCCTGAA ACTGGTATCC GTCGGGTTAC
ACGAATTGTT TCGGAATCGC CCGCTCCGCC ATTCGGACTT TGACCATAGG CAGCCCAATG
..L..N..K. .A..L..A.. G..E..A..V ..S..L..K. .L..V..S.. V..G..L..L

      1330        1340        1350        1360        1370        1380
TCACCGCGTC GAATATTTTC GGCAAAGAGG GAACGATGGT CGAGGACCAA ATGCGCGCAT
AGTGGCGCAG CTTATAAAAG CCGTTTCTCC CTTGCTACCA GCTCCTGGTT TACGCGCGTA
..T..A..S. .N..I..F.. G..K..E..G ..T..M..V. .E..D..Q.. M..R..A..W

      1390        1400        1410        1420        1430        1440
GGCAATCGTT GACCCAGCCG GGAAAAATGA TTCATTTAAA AATCCGCAAT AAAGATGGCG
CCGTTAGCAA CTGGGTCGGC CCTTTTTACT AAGTAAATTT TTAGGCGTTA TTTCTACCGC
..Q..S..L. .T..Q..P.. G..K..M..I ..H..L..K. .I..R..N.. K..D..G..D

      1450        1460        1470        1480        1490        1500
ATCTACAGAC GGTAAAAATA AAACCGGACG TCGTCGCCGC ATTTAATGTG GGTGTTAATG
TAGATGTCTG CCATTTTTAT TTTGGCCTGC AGCAGCGGCG TAAATTACAC CCACAATTAC
..L..Q..T. .V..K..I.. K..P..D..V ..V..A..A. .F..N..V.. G..V..N..E

      1510        1520        1530        1540        1550        1560
AGCTGGCGCT CAAGCTCGGC TTTGGCCTTA AGGCATCGGA TAGCTATAAT GCCGAGGCGC
TCGACCGCGA GTTCGAGCCG AAACCGGAAT TCCGTAGCCT ATCGATATTA CGGCTCCGCG
..L..A..L. .K..L..G.. F..G..L..K ..A..S..D. .S..Y..N.. A..E..A..L

      1570        1580        1590        1600        1610        1620
TACATCAGTT ATTAGGCAAT GATTTACGCC CTGAAGCCAG ACCAGGTGGC TGGGTTGGCG
ATGTAGTCAA TAATCCGTTA CTAAATGCGG GACTTCGGTC TGGTCCACCG ACCCAACCGC
..H..Q..L. .L..G..N.. D..L..R..P ..E..A..R. .P..G..G.. W..V..G..E

      1630        1640        1650        1660        1670        1680
AATGGCTGGC GCAATACCCG GATAATTATG AGGTCGTCAA TACATTAGCG CGCCAGATTA
TTACCGACCG CGTTATGGGC CTATTAATAC TCCAGCAGTT ATGTAATCGC GCGGTCTAAT
..W..L..A. .Q..Y..P.. D..N..Y..E ..V..V..N. .T..L..A.. R..Q..I..K

      1690        1700        1710        1720        1730        1740
AGGATATATG GAAAAATAAC CAACATCATA AAGATGGCGG CGAACCCTAT AAACTCGCAC
TCCTATATAC CTTTTTTATTG GTTGTAGTAT TTCTACCGCC GCTTGGGATA TTTGAGCGTG
..D..I..W. .K..N..N.. Q..H..H..K ..D..G..G. .E..P..Y.. K..L..A..Q

      1750        1760        1770        1780        1790        1800
AACGCCTTGC CATGTTAGCC CATGAAATTG ACGCGGTACC CGCCTGGAAT TGTAAAAGCG
TTGCGGAACG GTACAATCGG GTACTTTAAC TGCGCCATGG GCGGACCTTA ACATTTTCGC
..R..L..A. .M..L..A.. H..E..I..D ..A..V..P. .A..W..N.. C..K..S..G
```

```
      1810        1820        1830        1840        1850        1860
GCAAAGATCG TACAGGGATG ATGGATTCAG AAATCAAGGG AGAGATCATT TCCTTACATC
CGTTTCTAGC ATGTCCCTAC TACCTAAGTC TTTAGTTCCC TCTCTAGTAA AGGAATGTAG
..K..D..R. .T..G..M.. M..D..S..E ..I..K..G. .E..I..I.. S..L..H..Q

      1870        1880        1890        1900        1910        1920
AGACCCATAT GTTAAGTGCC CCTGGTAGTC TTCCGGATAG CGGTGGACAG AAAATTTTCC
TCTGGGTATA CAATTCACGG GGACCATCAG AAGGCCTATC GCCACCTGTC TTTTAAAAGG
..T..H..M. .L..S..A.. P..G..S..L ..P..D..S. .G..G..Q.. K..I..F..Q

      1930        1940        1950        1960        1970        1980
AAAAAGTATT ACTGAATAGC GGTAACCTGG AGATTCAGAA ACAAAATACG GGCGGGGCGG
TTTTTCATAA TGACTTATCG CCATTGGACC TCTAAGTCTT TGTTTTATGC CCGCCCCGCC
..K..V..L. .L..N..S.. G..N..L..E ..I..Q..K. .Q..N..T.. G..G..A..G

      1990        2000        2010        2020        2030        2040
GAAACAAAGT AATGAAAAAT TTATCGCCAG AGGTGCTCAA TCTTTCCTAT CAAAAACGAG
CTTTGTTTCA TTACTTTTTA AATAGCGGTC TCCACGAGTT AGAAAGGATA GTTTTTGCTC
..N..K..V. .M..K..N.. L..S..P..E ..V..L..N. .L..S..Y.. Q..K..R..V

      2050        2060        2070        2080        2090        2100
TTGGGGATGA AAATATTTGG CAGTCAGTAA AAGGCATTTC TTCATTAATC ACATCTTGAG
AACCCCTACT TTTATAAACC GTCAGTCATT TTCCGTAAAG AAGTAATTAG TGTAGAACTC
..G..D..E. .N..I..W.. Q..S..V..K ..G..I..S. .S..L..I.. T..S..*

      2110        2120        2130        2140        2150        2160
TCTTGAGGTA ACTATATGGA AAGTCTATTA AATCGTTTAT ATGACGCGTT AGGCCTGGAT
AGAACTCCAT TGATATACCT TTCAGATAAT TTAGCAAATA TACTGCGCAA TCCGGACCTA
                M..E. .S..L..L.. N..R..L..Y ..D..A..L. .G..L..D..
              (sigE)

      2170        2180        2190        2200        2210        2220
GCGCCAGAAG ATGAGCCACT GCTTATCATT GATGATGGGA TACAGGTTTA TTTTAATGAA
CGCGGTCTTC TACTCGGTGA CGAATAGTAA CTACTACCCT ATGTCCAAAT AAAATTACTT
A..P..E..D ..E..P..L. .L..I..I.. D..D..G..I ..Q..V..Y. .F..N..E..

      2230        2240        2250        2260        2270        2280
TCCGATCATA CACTGGAAAT GTGCTGTCCC TTTATGCCAT TGCCTGACGA CATCCTGACT
AGGCTAGTAT GTGACCTTTA CACGACAGGG AAATACGGTA ACGGACTGCT GTAGGACTGA
S..D..H..T ..L..E..M. .C..C..P.. F..M..P..L ..P..D..D. .I..L..T..

      2290        2300        2310        2320        2330        2340
TTGCAGCATT TTTTACGTCT GAACTACACC AGCGCCGTCA CTATCGGCGC TGACGCAGAC
AACGTCGTAA AAAATGCAGA CTTGATGTGG TCGCGGCAGT GATAGCCGCG ACTGCGTCTG
L..Q..H..F ..L..R..L. .N..Y..T.. S..A..V..T ..I..G..A. .D..A..D..

      2350        2360        2370        2380        2390        2400
AATACTGCTT TAGTGGCGCT TTATCGCTTG CCGCAAACCA GTACCGAAGA AGAGGCGCTC
TTATGACGAA ATCACCGCGA AATAGCGAAC GGCGTTTGGT CATGGCTTCT TCTCCGCGAG
N..T..A..L ..V..A..L. .Y..R..L.. P..Q..T..S ..T..E..E. .E..A..L..
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      2410        2420        2430        2440        2450        2460
ACTGGTTTTG AATTATTCAT TTCAAACGTG AAGCAATTGA AAGAGCATTA TGCATAATTT
TGACCAAAAC TTAATAAGTA AAGTTTGCAC TTCGTTAACT TTCTCGTAAT ACGTATTAAA
T..G..F..E ..L..F..I. .S..N..V.. K..Q..L..K ..E..H..Y. .A..*

      2470        2480        2490        2500        2510        2520
AATACGTCAA CATACTTTCT TAATGAGATA AAACGCGATA CGTATGCCCT TTACAAGAGA
TTATGCAGTT GTATGAAAGA ATTACTCTAT TTTGCGCTAT GCATACGGGA AATGTTCTCT

      2530        2540        2550        2560        2570        2580
CAAGACCAGA ATCTTTGGTG GAAATGTAAG GGGCAAACGT TCATCTCTCT CATTTTGCTC
GTTCTGGTCT TAGAAACCAC CTTTACATTC CCCGTTTGCA AGTAGAGAGA GTAAAACGAG

      2590        2600        2610        2620        2630        2640
TGTTTGCGGG AGCATTTTTA GTGTGTAAGT ATTCCTGCTC ATCAGGTTTT TACGCCATCA
ACAAACGCCC TCGTAAAAAT CACACATTCA TAAGGACGAG TAGTCCAAAA ATGCGGTAGT

      2650        2660        2670        2680        2690        2700
CGCGCATTTA TTCTGGTATA AGTTGAAATA CTGCAAAAAA TATTGGTGCT TATTATTTTT
GCGCGTAAAT AAGACCATAT TCAACTTTAT GACGTTTTTT ATAACCACGA ATAATAAAAA

      2710        2720        2730        2740        2750        2760
TCTTTAAGTA AATTTTCGCT GAACAAACTT AATTGTTTAT TCAATGATGA TGAAGCGTAA
AGAAATTCAT TTAAAAGCGA CTTGTTTGAA TTAACAAATA AGTTACTACT ACTTCGCATT

      2770        2780        2790        2800        2810        2820
GCTATGCTGG AAATGAAGGA ATCAATAGCA AGGATAATCT TATTATTCAC GGGTGATATT
CGATACGACC TTTACTTCCT TAGTTATCGT TCCTATTAGA ATAATAAGTG CCCACTATAA

      2830        2840        2850        2860        2870        2880
ACTTCTGCTT CACCGTTATG GCAGATATCA TCGCCTCTTG TCAGATGCCA GACACCTACT
TGAAGACGAA GTGGCAATAC CGTCTATAGT AGCGGAGAAC AGTCTACGGT CTGTGGATGA

      2890        2900        2910        2920        2930        2940
CATACTCAAC CAAAGCTCTA AATACAAAAA TCACCTTATA TCTTTTTTTA TTATTCCTTG
GTATGAGTTG GTTTCGAGAT TTATGTTTTT AGTGGAATAT AGAAAAAAAT AATAAGGAAC

      2950        2960        2970        2980        2990        3000
TATAAATGTG ACTTGACTCA CACCTATAAG GAGTCGGCTC ACTTCCATAA GAAGGAATCA
ATATTTACAC TGAACTGAGT GTGGATATTC CTCAGCCGAG TGAAGGTATT CTTCCTTAGT

      3010        3020        3030        3040        3050        3060
AAATGCCAAT AACTAACGCG TCCCCAGAAA ATATATTAAG ATATTTGCAT GCGGCCGGTA
TTTACGGTTA TTGATTGCGC AGGGGTCTTT TATATAATTC TATAAACGTA CGCCGGCCAT
  M..P..I. .T..N..A.. S..P..E..N ..I..L..R. .Y..L..H.. A..A..G..T
  (pipB)

      3070        3080        3090        3100        3110        3120
CCGGTACGAA AGAAGCAATG AAAAGTGCAA CTTCACCACG CGGTATACTG GAATGGTTTG
GGCCATGCTT TCTTCGTTAC TTTTCACGTT GAAGTGGTGC GCCATATGAC CTTACCAAAC
..G..T..K. .E..A..M.. K..S..A..T ..S..P..R. .G..I..L.. E..W..F..V
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      3130        3140        3150        3160        3170        3180
TCAATTTTTT TACCTGTGGT GGAGTAAGAA GAAGCAATGA AAGATGGTTT CGGGAGGTAA
AGTTAAAAAA ATGGACACCA CCTCATTCTT CTTCGTTACT TTCTACCAAA GCCCTCCATT
..N..F..F. .T..C..G.. G..V..R..R ..S..N..E. .R..W..F.. R..E..V..I

      3190        3200        3210        3220        3230        3240
TTGGAAAACT GACCACATCA TTATTATATG TAAATAAAAA TGCTTTCTTC GATGGTAATA
AACCTTTTGA CTGGTGTAGT AATAATATAC ATTTATTTTT ACGAAAGAAG CTACCATTAT
..G..K..L. .T..T..S.. L..L..Y..V ..N..K..N...A..F..F.. D..G..N..K

      3250        3260        3270        3280        3290        3300
AAATATTTCT GGAGGATGTC AACGGGTGTT CTATAT[GT]CT GTCATGTGGA GCAGCATCCG
TTTATAAAGA CCTCCTACAG TTGCCCACAA GATATA[CA]GA CAGTACACCT CGTCGTAGGC
..I..F..L. .E..D..V.. N..G..C..S ..I..C..L. .S..C..G.. A..A..S..E

      3310        3320        3330        3340        3350        3360
AAAATACGGA TCCCATGGTC ATTATTGAAG TGAACAAAAA TGGAAAAACT GTAACGGATA
TTTTATGCCT AGGGTACCAG TAATAACTTC ACTTGTTTTT ACCTTTTTGA CATTGCCTAT
..N..T..D. .P..M..V.. I..I..E..V ..N..K..N. .G..K..T.. V..T..D..K

      3370        3380        3390        3400        3410        3420
AAGTTGATAG TGAGAGATTT TGGAATGTAT GTCGAATGTT AAAACTGATG AGTAAACATA
TTCAACTATC ACTCTCTAAA ACCTTACATA CAGCTTACAA TTTTGACTAC TCATTTGTAT
..V..D..S. .E..R..F.. W..N..V..C ..R..M..L. .K..L..M.. S..K..H..N

      3430        3440        3450        3460        3470        3480
ATATACAACA GCCTGATTCA CTTATAACCG GAGGATGGTT TTCTGAACCT GCGCGGAGTA
TATATGTTGT CGGACTAAGT GAATATTGGC CTCCTACCAA AAGACTTGGA CGCGCCTCAT
..I..Q..Q. .P..D..S.. L..I..T..G ..G..W..F. .S..E..P.. A..R..S..K

      3490        3500        3510        3520        3530        3540
AACCTGGCTC ATAAAGATTT CCAGGGGGAA GATTTGTCAA AAATAGATGC TTCTAATGCA
TTGGACCGAG TATTTCTAAA GGTCCCCCTT CTAAACAGTT TTTATCTACG AAGATTACGT
..P..G..S. .*                                                M..L.
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(corresponding *pipB* gene is continuous in *S. dublin*)

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      3550        3560        3570        3580        3590        3600
GATTTCCGTG AAACAACTTC TATCTAATGT AAATTTAGTC GGTGCAAATT TGTGTTGTGC
CTAAAGGCAC TTTGTTGAAG ATAGATTACA TTTAAATCAG CCACGTTTAA ACACAACACG
.L..M..Q.. I..S..V..K ..Q..L..L. .S..N..V.. N..L..V..G ..A..N..L.

      3610        3620        3630        3640        3650        3660
AAATCTACAC GCTGTAAATC TAATGGGTTC AAACATGACT AAAGCAAACC TGACTCACGC
TTTAGATGTG CGACATTTAG ATTACCCAAG TTTGTACTGA TTTCGTTTGG ACTGAGTGCG
.C..C..A.. N..L..H..A ..V..N..L. .M..G..S.. N..M..T..K ..A..N..L.
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      3670        3680        3690        3700        3710        3720
AGACCTGACT TGCGCTAACA TGTCCGGTGT AAACTTAACC GCTGCAATTC TATTCGGCTC
TCTGGACTGA ACGCGATTGT ACAGGCCACA TTTGAATTGG CGACGTTAAG ATAAGCCGAG
.T..H..A.. D..L..T..C ..A..N..M. .S..G..V.. N..L..T..A ..A..I..L.

      3730        3740        3750        3760        3770        3780
AGACTTAACT GACACCAAAC TAAATGGTGC GAAATTAGAT AAGATAGCTC TAACTTTAGC
TCTGAATTGA CTGTGGTTTG ATTTACCACG CTTTAATCTA TTCTATCGAG ATTGAAATCG
.F..G..S.. D..L..T..D ..T..K..L. .N..G..A.. K..L..D..K ..I..A..L.

      3790        3800        3810        3820        3830        3840
GAAAGCATTA ACAGGAGCCG ATCTGACAGG TAGTCAACAT ACCCCTACTC CACTCCCGGA
CTTTCGTAAT TGTCCTCGGC TAGACTGTCC ATCAGTTGTA TGGGGATGAG GTGAGGGCCT
.T..L..A.. K..A..L..T ..G..A..D. .L..T..G.. S..Q..H..T ..P..T..P.

      3850        3860        3870        3880        3890        3900
TTACAATGAT AGAACTCTTT TCCCCCATCC GATATTTTAG TCGAGATAAA GGGATTTTAT
AATGTTACTA TCTTGAGAAA AGGGGGTAGG CTATAAAATC AGCTCTATTT CCCTAAAATA
.L..P..D.. Y..N..D..R ..T..L..F. .P..H..P.. I..F..*

      3910        3920
AAACAAGAAG TATTCAAACA GA
TTTGTTCTTC ATAAGTTTGT CT
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## C. G7H1 (*iicA*)

ACCESSION #AF164435 (*iicA S. typhimurium*)

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        10         20         30         40         50         60
GAATGCAAAA GAGAAGTTAC TGGATTTTGT GGAGTTAGAA GAAAACGAAT CGCTGATTTT
CTTACGTTTT CTCTTCAATG ACCTAAAACA CCTCAATCTT CTTTTGCTTA GCGACTAAAA
E..C..K..R ..E..V..T. .G..F..C.. G..V..R..R ..K..R..I. .A..D..F..
(potential ORF)

        70         80         90        100        110        120
GGTAAAGATA TTCGCATCGT TCTGGCATCA GCGGATTTCA GTAAAGAATT AACGACAACC
CCATTTCTAT AAGCGTAGCA AGACCGTAGT CGCCTAAAGT CATTTCTTAA TTGCTGTTGG
G..K..D..I ..R..I..V. .L..A..S.. A..D..F..S ..K..E..L. .T..T..T..

       130        140        150        160        170        180
GCAATATGGC TAAGAGATAA AGGTGTCGAT ATTCGCTGTG TTCGCTTAAC GCCTTACAAC
CGTTATACCG ATTCTCTATT TCCACAGCTA TAAGCGACAC AAGCGAATTG CGGAATGTTG
A..I..W..L ..R..D..K. .G..V..D.. I..R..C..V ..R..L..T. .P..Y..N..

       190        200        210        220        230        240
TTTAAGGGTG AAGTGCTGAT TAATGCTGAA CAAATAATAC CAGTCCCTGA ACTGGAAGAA
AAATTCCCAC TTCACGACTA ATTACGACTT GTTTATTATG GTCAGGGACT TGACCTTCTT
F..K..G..E ..V..L..I. .N..A..E.. Q..I..I..P ..V..P..E. .L..E..E..

       250        260        270        280        290        300
TATCAGGTCA GATTCAGAGA GAAACGCACG GAACAAATTA TTAGCAGTCA AAAGTCGGAG
ATAGTCCAGT CTAAGTCTCT CTTTGCGTGC CTTGTTTAAT AATCGTCAGT TTTCAGCCTC
Y..Q..V..R ..F..R..E. .K..R..T.. E..Q..I..I ..S..S..Q. .K..S..E..

       310        320        330        340        350        360
AGGGATTATT CCTTATATAA ATATAAAGGA AAAACCTTCA ATAAACGGAA GCTTGCACTT
TCCCTAATAA GGAATATATT TATATTTCCT TTTTGGAAGT TATTTGCCTT CGAACGTGAA
R..D..Y..S ..L..Y..K. .Y..K..G.. K..T..F..N ..K..R..K. .L..A..L..

       370        380        390        400        410        420
GAACTTTTCA CTGACTGGAT TAATAAACAT AATCCTGCGA ATATAGATGA TCTCAAGAAT
CTTGAAAAGT GACTGACCTA ATTATTTGTA TTAGGACGCT TATATCTACT AGAGTTCTTA
E..L..F..T ..D..W..I. .N..K..H.. N..P..A..N ..I..D..D. .L..K..N..

       430        440        450        460        470        480
AAATTGAGTG AAGACTTACA GAAAAGAACA GTAGCACTGG TTGAGCAGAT CCCTGAAAAA
TTTAACTCAC TTCTGAATGT CTTTTCTTGT CATCGTGACC AACTCGTCTA GGGACTTTTT
K..L..S..E ..D..L..Q. .K..R..T.. V..A..L..V ..E..Q..I. .P..E..K..

       490        500        510        520        530        540
AGGAAAAACA GATATCATAT GCAGGAAGAT GCGTTGATTG AGTTGCCGTC CGGTGAGCGT
TCCTTTTTGT CTATAGTATA CGTCCTTCTA CGCAACTAAC TCAACGGCAG GCCACTCGCA
R..K..N..R ..Y..H..M. .Q..E..D.. A..L..I..E ..L..P..S. .G..E..R..
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        550         560         570         580         590         600
ATTGCTATAT CGATAAATGG GGGTTAGGGA CTATAGAACT GCTTATATGA TTTTGTTCGG
TAACGATATA GCTATTTACC CCCAATCCCT GATATCTTGA CGAATATACT AAAACAAGCC
I..A..I..S ..I..N..G. .G..*

        610         620         630         640         650         660
TCAGGATAAT TTTGTAGTTG AAAAAGTAGG TTGACAGGAA GTAATAATAA AATAGATCCC
AGTCCTATTA AAACATCAAC TTTTTCATCC AACTGTCCTT CATTATTATT TTATCTAGGG

        670         680         690         700         710         720
ATTCATTAAT GGGATCTCAC GTTTCATCCG ATACGAAGAC CATGGTCTCT TTGTCAGTAG
TAAGTAATTA CCCTAGAGTG CAAAGTAGGC TATGCTTCTG GTACCAGAGA AACAGTCATC

        730         740         750         760         770         780
CGTCATAATT ACGCAAGCCT CTTTACTTTG CTTATCATTT ATATTTAATG TAAATATTCA
GCAGTATTAA TGCGTTCGGA GAAATGAAAC GAATAGTAAA TATAAATTAC ATTTATAAGT

        790         800         810         820         830         840
CGCAACACCA TTAAAAAATA AGAAAAAATG GCTCACTGTT GAACTGATAT TAATACCTGA
GCGTTGTGGT AATTTTTTAT TCTTTTTTAC CGAGTGACAA CTTGACTATA ATTATGGACT

        850         860         870         880         890         900
ACCACTGAAT TAGAGTAATG TGGCGCTATT CATAGCGTAA TTTTTTCTGT TGCGGTTACA
TGGTGACTTA ATCTCATTAC ACCGCGATAA GTATCGCATT AAAAAAGACA ACGCCAATGT

        910         920         930         940         950         960
GGGGGAGGAA TGCACACCTT TAGACCATAC TCACTAAGGC ATAGCGATCT GTTATATGAA
CCCCCTCCTT ACGTGTGGAA ATCTGGTATG AGTGATTCCG TATCGCTAGA CAATATACTT
        M ..H..T..F. .R..P..Y.. S..L..R..H ..S..D..L. .L..Y..E..
   (iicA)

        970         980         990        1000        1010        1020
GATATTCCGT TAGAAATACG CGAGCAAATA ATCTTATTGA TTATCAATAC GCTAGGAAAC
CTATAAGGCA ATCTTTATGC GCTCGTTTAT TAGAATAACT AATAGTTATG CGATCCTTTG
D..I..P..L ..E..I..R. .E..Q..I.. I..L..L..I ..I..N..T. .L..G..N..

       1030        1040        1050        1060        1070        1080
TGCTCCTCTT TTTATGATAT GACATTATAC TGCTATCATA ATAGTCATTC TGACGAAGTT
ACGAGGAGAA AAATACTATA CTGTAATATG ACGATAGTAT TATCAGTAAG ACTGCTTCAA
C..S..S..F ..Y..D..M. .T..L..Y.. C..Y..H..N ..S..H..S. .D..E..V..

       1090        1100        1110        1120        1130        1140
TATCGAAGAA TATGTAAAAC GTTGCGCAAA GAGTATGGCT TATTCACCTT ATAGGCGCAT
ATAGCTTCTT ATACATTTTG CAACGCGTTT CTCATACCGA ATAAGTGGAA TATCCGCGTA
Y..R..R..I ..C..K..T. .L..R..K.. E..Y..G..L ..F..T..L. .*
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      1150        1160        1170        1180        1190        1200
TCAACGTCAT ATCTGGATGA AATGAGTAAT CTGTTATTAA AAACAGATGA TAAAAGAAAG
AGTTGCAGTA TAGACCTACT TTACTCATTA GACAATAATT TTTGTCTACT ATTTTCTTTC
                      M..S..N.. L..L..L..K ..T..D..D. .K..R..K..
                    (potential ORF2)

      1210        1220        1230        1240        1250        1260
CATATTGATA CCATTGAGCT TGCTTTTAAC TATATAGATA CCTACCTTCG GACCTATGAA
GTATAACTAT GGTAACTCGA ACGAAAATTG ATATATCTAT GGATGGAAGC CTGGATACTT
H..I..D..T ..I..E..L. .A..F..N.. Y..I..D..T ..Y..L..R. .T..Y..E..

      1270        1280        1290        1300        1310        1320
GTTACGCTTG GGTTAGAACC GGATAAGGCG ATTAGTGAAT TAAATAATAT ATTTCATGAG
CAATGCGAAC CCAATCTTGG CCTATTCCGC TAATCACTTA ATTTATTATA TAAAGTACTC
V..T..L..G ..L..E..P. .D..K..A.. I..S..E..L ..N..N..I. .F..H..E..

      1330        1340        1350        1360        1370
CATAGTTTAA AATATCGATA TGAAAAATGG TTAGGATTGT TAAGGTTGCG C
GTATCAAATT TTATAGCTAT ACTTTTTACC AATCCTAACA ATTCCAACGC G
H..S..L..K ..Y..R..Y. .E..K..W.. L..G..L..L ..R..L..R. .
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