HOST-PATHOGEN INTERACTIONS: THE IMPACT OF NRAMP1 ON *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM VIRULENCE GENE EXPRESSION

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ABSTRACT

Nramp1 (natural resistance-associated macrophage protein 1) is a host resistance gene that provides protection against several intracellular pathogens, including *Salmonella enterica* serovar Typhimurium. Little is known about the dynamic interplay that occurs between mammalian host resistance determinants such as Nramp1 and pathogens during infection. To explore these interactions we examined the effect of Nramp1 on selected *S.* Typhimurium (STM) genes.

Nramp1 is believed to be a divalent cation transport system responsible for transport of Fe^{2+} and Mn^{2+} from the phagolysosome to the cytoplasm of macrophages. We chose to investigate the role of the bacterial Mn^{2+} transporters MntH and SitABCD in STM pathogenesis. Nramp1-transfected cell lines infected with STM harboring *mntH* and *sitA* transcriptional fusions were used to investigate the intracellular patterns of expression of these STM genes. Both genes are expressed in the intracellular environment and are further upregulated in the presence of Nramp1. Analyses using axenic bacterial cultures demonstrated that expression of *mntH* and *sitA* responds to levels of Fe^{2+} and Mn^{2+} through the regulators Fur and MntR, while expression of *mntH* also responds to hydrogen peroxide through the regulator OxyR. Studies using strains deleted for *oxyR* or an expression plasmid deleted for the OxyR box indicated that hydrogen peroxide is important for initial expression of *mntH* inside macrophages both in the presence and absence of Nramp1. Similar studies investigating the role of Fur and MntR on *mntH* expression in cultured cells were inconclusive. Virulence studies using congenic Nramp1 knockout mice demonstrated that both *mntH* and *sitA* are essential for virulence of STM in *Nramp1*$^{+/+}$ animals. An *mntH sitA* double knockout strain was more attenuated than either single knockout strain, indicating that *mntH* and *sitA* contribute independently to the pathogenic nature of STM.

The effect of Nramp1 was also studied on selected STM virulence genes associated with the Type III Secretion System encoded within *Salmonella* pathogenicity island-2 (SPI2). *In vivo* studies demonstrated that SPI2 is essential for the ability of STM to replicate in the spleen of *Nramp1*$^{+/+}$ mice. To investigate a potential interaction between SPI2 and Nramp1, Nramp1-transfected cell lines were used to identify that the SPI2-associated genes *ssrA*, *sseA* and *sseJ*, but not *phoP* or the SPI1 regulator *hilA*, are upregulated in the presence of Nramp1. Studies using axenic cultures indicate that these SPI2-associated genes are responding to extracellular levels of Fe^{2+} via a novel iron-responsive regulatory system. Further, these genes are differentially regulated by the global regulatory systems PhoPQ and OmpR, suggesting a previously uncharacterized complexity of SPI2 gene regulation.

Overall STM is directly influenced on the genetic level by the presence of Nramp1. This impact on STM virulence gene expression correlates with the proposed function of Nramp1 as a divalent cation transport system. We propose that upregulation of STM virulence genes in response to limiting levels of divalent cations is a pathogenic strategy developed by the bacterium to maintain some level of replication in naturally resistant (i.e. *Nramp1*$^{+/+}$) hosts. We discuss how this may be involved in the development of chronic disease states by *Salmonellae*.

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AtNramp	*Arabidopsis thaliana* Nramp homologue
ATR	acid tolerance response
Ap	ampicillin
ASP	acid shock protein
BALB/c	inbred albino mouse lineage; *Nramp1*$^{-/-}$
C3H/HeN	inbred mouse lineage; *Nramp1*$^{+/+}$
C57/BL6	inbred black mouse lineage; *Nramp1*$^{-/-}$
cfu	colony-forming unit
Cm	chloramphenicol
cs	centisome
DiP	dipyridyl; "iron-specific" chelator
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPI	diphenylene iodium chloride; inhibitor of the macrophage oxidative burst
DTPA	diethylene triamine pentaacetic acid; "iron-specific" chelator
EDTA	ethylene diamine tetraacetic acid; general chelator
EGTA	ethylene bis (oxyethylenenitrilo) tetraacetic acid; general chelator, often considered to be Ca^{2+}-specific
EPEC	enteropathogenic *Escherichia coli*
FBS	fetal bovine serum
G418	geneticin
GALT	gut-associated lymphoid tissue

GEF	guanine exchange factor
GI	gastrointestinal
Gm	gentamicin
h	hour
HeLa	human cervix epitheliod cell line
IBD	inflammatory bowel disease
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
in vitro	(Latin: in glass); here to mean within cultured cells
in vivo	(Latin: in the living organism); here to mean within the host animal
Ity/Lsh/Bcg	various early gene designations for Nramp1
kbp	kilobase pairs
Km	kanamycin
LAMP	lysosomal associated membrane protein; lysosomal marker
LB	Luria-Bertani; rich bacterial growth media
LD_{50}	lethal dose at which 50% of infected animals succumb to infection
lgp	lysosomal membrane glycoprotein; lysosomal marker
LPS	lipopolysaccharide
LRR	leucine rich repeat
M6PR	mannose 6-phosphate receptor; late endosomal marker
MHC	major histocompatibility complex
min	minutes
MOI	multiplicity of infection
mvl	malvolio; Drosophila melanogaster Nramp1 homologue

NMM	N Minimal Medium; chemically defined minimal bacterial growth media
Nramp	Natural resistance-associated macrophage protein
ORF	open reading frame
OsNramp	*Oryza sativa* Nramp homologue
P22	*Salmonella*-specific bacteriophage
pag	PhoP-activated gene
PAI	pathogenicity island
PBS	phosphate buffered saline
PCR	polymerase chain reaction
phox	the genes encoding NADPH oxidase
PMA	phorbol myristyl acetate; inducer of the macrophage oxidative burst
PMN	polymorphonuclear cell
prg	PhoP-repressed gene
pSLT	*Salmonella* virulence plasmid
Rab5	small GTP-binding protein involved in regulating the traffic of intracellular transport vesicles
RAW	murine macrophage/monocyte cell line derived from BALB/c mice
Rac	small GTPase involved in cell signaling
Rho	small GTPase involved in cell signaling
RLU	relative light units
RNIs	reactive nitrogen intermediates
ROIs	reactive oxygen intermediates
SCV	*Salmonella*-containing vacuole

sec	seconds
SEM	standard error of the mean
Sif	*Salmonella*-induced filament
Slc11a1	solute carrier protein 11a1; a.k.a. Nramp1
Sm	streptomycin
SMF	family of metal transporters in *Saccharomyces cerevisiae* homologous to the Nramp family of proteins
SOD	superoxide dismutase
SPI	*Salmonella* pathogenicity island
STM	*Salmonella enterica* serovar Typhimurium
Tc	tetracycline
TCRS	two component regulatory system
TMD	transmembrane domain
TNF	tumour necrosis factor
tRNA	transfer ribonucleic acid
TTSS	type three secretion system
UTR	untranslated region
V-ATPase	vacuolar adenosine triphosphatase
WT	wild-type

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DEDICATION

This thesis is dedicated to the memory of my Grandfather, John Ernest Dehm.

He was the first member of our family to earn a graduate degree;

thanks to his love and support he ensured that he was not the last.

CHAPTER 1: INTRODUCTION

In the last twenty years there has been increasing interest in the field of host-pathogen interactions. Further, widely accepted model systems have come under intense scrutiny in the aim to improve model systems in order to obtain "the most relevant model" for a given infection. To this end we chose to investigate the impact of the host resistance protein Nramp1 on the murine typhoid model (i.e. infection of mice with *Salmonella enterica* serovar Typhimurium), which is currently the only accepted model for extrapolation to infection of humans with *Salmonella enterica* serovar Typhi. It was hoped that the introduction of this host resistance mechanism would be one step further in ameliorating the murine typhoid model by taking into account a naturally occurring host defense present in human infections. In order to understand the host-pathogen interaction in this manner, this introduction will discuss in some detail *S.* Typhimurium as a pathogen and bacterial factors involved in virulence of this microorganism. Significant time is devoted to the discussion of the regulation of virulence genes by various stimuli and how this may relate to niches encountered by the bacterium within the host. Finally, model systems for the study of *Salmonella* infections are discussed and a detailed explanation of Nramp1 as a host resistance mechanism and its role in human disease states is presented.

1.1 *Salmonella* and salmonellosis

The nomenclature for the genus *Salmonella* is continuously evolving. The standard classification scheme is the Kauffman classification, in which each serotype is identified on the basis of the serologic identification of O (somatic) and H (flagellar) antigens (141). Using this classification, each serotype is considered to be a separate species, resulting in the familiar *Salmonella typhimurium* designation. However if this scheme were to continue to be used, it would result in 2, 463 species of *Salmonella.* In

1986 it was recommended that the type species for *Salmonella* be changed to *Salmonella enterica*, which was adopted by Ewing in the 4th edition of *Edward's and Ewing's Identification of Enterobacteriaceae* (76), as well as by the Center for Disease Control (CDC). Currently according to the CDC there are two recognized species of the genus *Salmonella*, *S. enterica* and *S. bongori*, based on DNA-DNA hybridization analyses (40). *S. enterica* is further subdivided into six subspecies on the basis of biochemical differentiation and genomic relatedness: I, *enterica*; II, *salamae*; IIIa, *arizonae*; IIIb, *diarizonae*; IV, *houtenae*; and VI, *indica* (40). There remain over 2,000 serotypes within *Salmonella enterica* subspecies *enterica*, and the CDC names the serotypes in subspecies I, for example serotypes Enteritidis, Typhimurium, and Typhi. To emphasize that these named serotypes are not separate species, the serotype name is not italicized and the first letter is capitalized (40). This is the *Salmonella* nomenclature used throughout the course of this work.

Salmonellae of subspecies I cause three markedly different diseases in humans: typhoid fever, septicemic salmonellosis, and gastroenteritis. *S.* Typhi and *S.* Paratyphi cause the severe systemic disease typhoid fever. They are host restricted, highly adapted human pathogens that do not cause disease in animals. Humans are the reservoir for *S.* Typhi, which is spread through the fecal-oral route, most often by consumption of water contaminated with human feces. Symptoms of typhoid fever may appear from one week to one month after initial ingestion of the bacterium, and are characterized by a sustained high fever, bacteremia, infection of the bilary system, eventual ulceration of the intestine, and bloody diarrhea (138). The disease is often severe, with a mortality rate of 2-10% and a 20% rate of relapse. Even after apparent recovery, bacteria may survive in the gallbladder and viable bacteria are shed into the feces for up to a year or longer, forming an active carrier state that can manifest even after antibiotic treatment (137, 138, 219).

S. Cholerasuis and *S.* Dublin are etiological agents of disease in both animals and humans. Swine are the zoonotic reservoir of *S.* Cholerasuis and cause a severe systemic infection in these animals. In contrast, *S.* Dublin is primarily a bovine pathogen that causes severe gastroenteritis in cows. In humans both *S.* Cholerasuis and *S.* Dublin cause septicemic salmonellosis, a severe disease characterized by prolonged bacteremia with fever, chills and anorexia; gastroenteritis is not common. In immunosuppressed individuals *S.* Cholerasuis infection can lead to a severe typhoid-like disease (137, 219).

The most familiar human *Salmonella*-based disease is gastroenteritis, with *S.* Typhimurium (STM) being one of the most clinically relevant gastroenteritis-related serovars. In humans, ingestion of *S.* Typhimurium-contaminated food or water generally results in gastroenteritis (219). Symptoms appear within 6-24 h after ingestion and last up to a week with severity varying on an individual basis. Gastroenteritis is characterized by initial nausea and vomiting followed by abdominal pain and diarrhea. After recovery, a person may continue to shed viable bacteria in their feces for up to three months, and in 1-3% of cases shedding can persist for up to a year or longer (219). Most often the disease is self-limiting and localized to the gastrointestinal tract, but some individuals, especially children and the immunosuppressed, may develop a more severe systemic form of the disease (184).

1.2 Course of a systemic STM infection

As mentioned above, transmission of *S. enterica* generally occurs by ingestion of contaminated food or water. Sources of contamination are far-reaching, and have recently included contaminated water, vegetables, fruit, seeds, spices, and meat products. After passage through the stomach, STM displays a preference for the distal region of the intestine and the M cells of the Peyer's patches (136). M cells are naturally phagocytic cells of the intestinal epithelium that are involved in immunity by sampling antigens from

the intestinal lumen and passing them to the underlying macrophages. In the intestine, STM may cross the epithelial barrier in one or more of three ways: 1) uptake via the natural phagocytic ability of M cells; 2) invading M cells and/or surrounding epithelial cells; or 3) uptake by dendritic cells or other phagocytes. However the bacteria cross the epithelial barrier, they then encounter cells of the gut-associated lymphoid tissue (GALT). Current knowledge suggests that the macrophages of the GALT play the next major role in determining the outcome of a *Salmonella* infection. If the macrophages and other cells succeed in limiting the infection, in most cases the resulting salmonellosis is a self-limiting gastroenteritis. However, if the bacteria infect the macrophages to survive and replicate within them, a severe systemic salmonellosis can ensue.

The specific host cell type responsible for carrying *Salmonellae* from the GALT to the lymph nodes remains to be identified. However, as mobile cells, macrophages and dendritic cells are hypothesized to function in this capacity, delivering *Salmonellae* to the lymph nodes from which they disseminate into the bloodstream eliciting a bacteremic state. This permits bacterial seeding of major internal organs, including the liver and spleen where *Salmonellae* survive and replicate within hepatic and splenic macrophages. Eventually a second bacteremic state ensues. In humans the bacteria disseminate to the gallbladder where they are shed into the bile and into the intestine for the second time. This re-seeding of the intestine may be responsible for the eventual ulceration and perforation of the intestine observed in severe typhoidal infections. Once infection reaches this stage, in most cases the host animal dies, succumbing either to massive injury to internal organs or possibly to septic shock.

S. enterica spp. can also cause persistent infections, with the carrier state characterized by the inability to clear the infection from the liver and spleen. In this state the host organism can secrete bacteria in the feces for extended periods of time.

Approximately 2-5% of all new cases of typhoid fever develop into the carrier state. Individuals infected with STM normally shed the bacteria into the feces for up to three months after resolution of gastroenteritis, while 1-3% of cases continue to shed the bacteria for more than one year (219). Chronic carriage of *Salmonella* is recognized as a significant complication that facilitates spread of the disease and predisposes victims to additional medical problems. Manifestation of the pathogenic nature of STM in both self-limiting and persistent disease states relies on the expression of a large number of virulence genes, discussed in detail below.

1.3 Virulence Genes and Pathogenicity Islands

In the last two decades the field of bacterial pathogenesis has expanded rapidly due to intense investigation into how bacterial pathogens subvert or manipulate a host to cause disease. This has led to the identification of numerous bacterial virulence factors, defined as "any bacterial product or strategy that contributes to the ability of a bacterium to cause an infection" (219), and their respective virulence genes. Generally, if a mutation is made in a virulence gene, the bacteria are attenuated upon infection of a host (i.e. are impaired in their ability to cause a productive infection), but are not greatly affected during growth in regular laboratory media (103). Virulence genes are located scattered throughout the bacterial chromosome, either individually or in large groups of genes encompassing up to 200 kbp of DNA termed pathogenicity islands (PAIs).

PAIs can be generally defined as groups of genes that have been obtained from an (often) unrelated microorganism that enhance virulence of a bacterium during infection of a host. PAIs are most commonly characterized in Gram negative bacteria by having DNA G+C contents that are noticeably different than the rest of the bacterial genome, indicating horizontal transmission from other bacteria at some point in their evolutionary history (113). Identification of specific DNA sequences including direct repeats, insertion

sequence elements, bacteriophage attachment sites and tRNA loci flanking the PAIs provides further support for horizontal acquisition of these regions of DNA (113). Smaller clusters of virulence genes have been colloquially termed "pathogenicity islets". A number of bacterial pathogens have been identified to have at least one pathogenicity island, including STM, enteropathogenic *Escherichia coli* (EPEC), and *Yersinia* and *Shigella* species. Five pathogenicity islands have been identified to date in STM and are referred to as *Salmonella* pathogenicity islands (SPIs), illustrated in Figure 1.

The key to the pathogenic strategy of STM is linked to two Type III Secretion Systems (TTSS). Essentially, TTSSs are multiprotein molecular syringes which bacteria use to inject bacterial effector proteins directly into the host cell, and are often referred to as "needle complexes" due to their appearance by scanning electron microscopy. STM has two distinct TTSS with apparent separate functions, illustrated in Figure 2. The structural genes for the separate TTSS are encoded within SPI1 and SPI2 respectively, while the genes encoding their effector proteins are scattered throughout the chromosome, within the identified SPIs as well as in other pathogenicity islets. A description of the role of each TTSS is discussed below; the specific roles of certain effectors are discussed in the text where appropriate.

1.3.1 SPI1

SPI1 is a 40 kbp region located at centisome (cs) 63 on the *Salmonella* chromosome (58). SPI1 is present in all *S. enterica* subspecies (195), and has been shown to be essential for manifesting invasion of non-phagocytic cells, apoptosis of macrophages, cytotoxicity of M cells, and the production of gastroenteritis (55, 91, 183, 204, 264). It is believed that acquisition of SPI1 allowed the divergence of *Salmonellae* from other closely related microorganisms such as *E. coli*, and permitted *Salmonellae* to

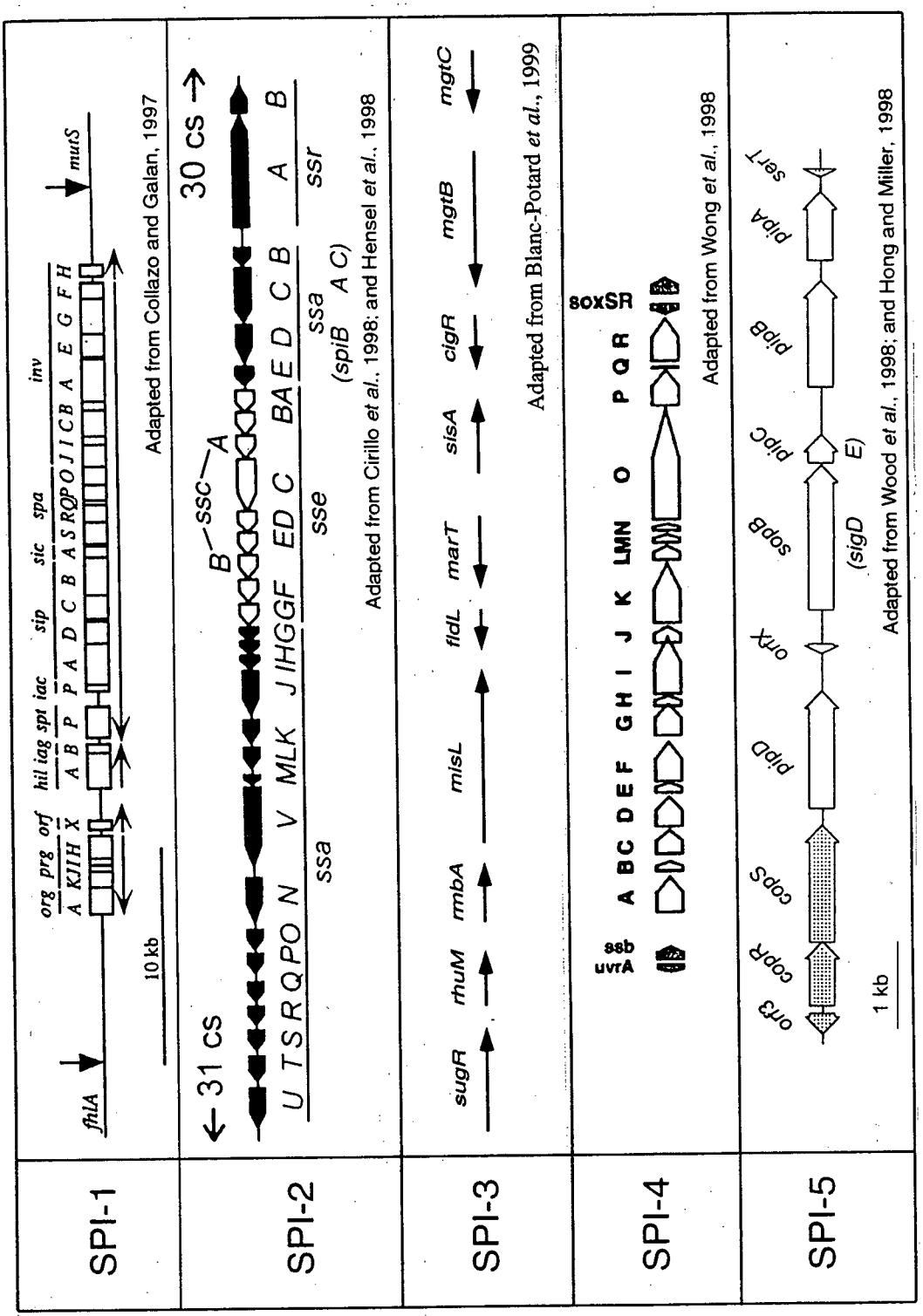


Figure 1. Schematic representation of the *Salmonella* pathogenicity islands.

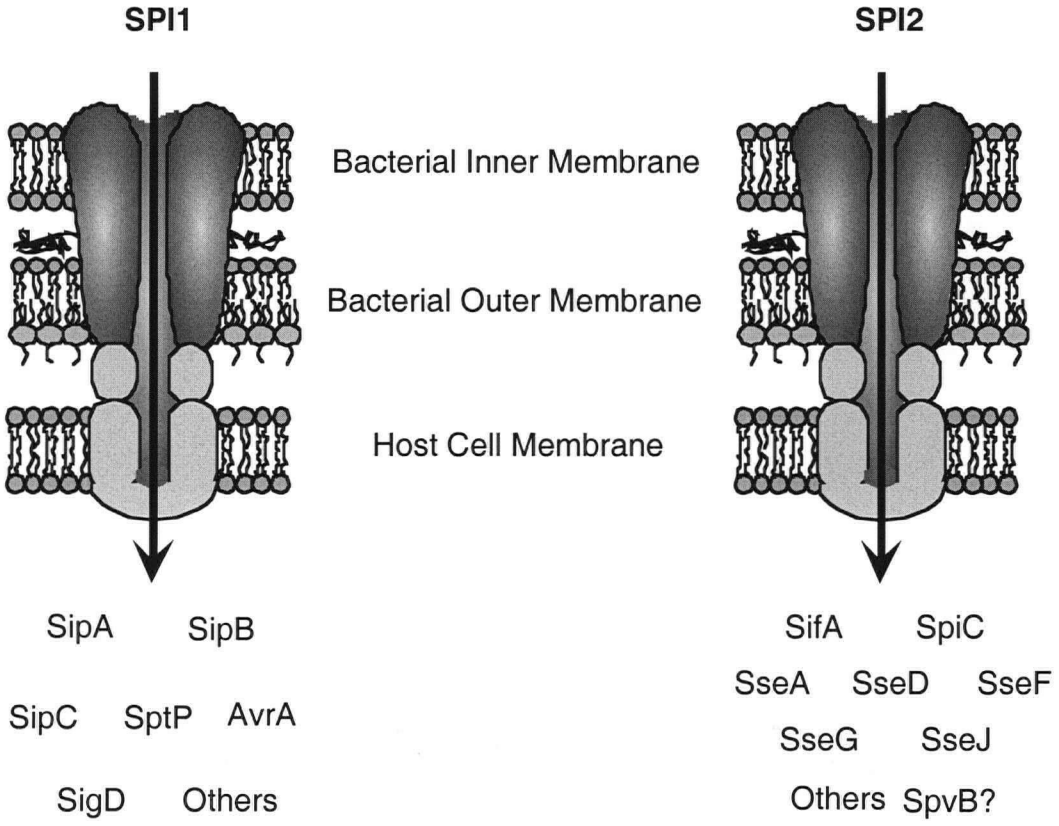


Figure 2. Illustration of the SPI1 and SPI2 Type III Secretion Systems. The SPI1 TTSS is involved in the invasion of non-phagocytic cells, while the SP2 TTSS is involved in intramacrophage survival. As a result, the SPI1 TTSS crosses the outer membrane of the host cell to inject secreted effectors into the cytosol of the host cell, while the SPI2 TTSS crosses the membrane of the SCV to pass secreted effectors into the cytosol.

become gastrointestinal diarrheagenic pathogens (21). SPI1 contains genes encoding structural components of the SPI1 TTSS (*invACGJ*, *spaOPQRS*, *prgHIJK* and *orgA*), SPI1 secreted proteins and potential effectors (*avrA, sptP, spaO, invJ, sipA, sip/sspBCD*), their corresponding chaperones (*sicA, sicP, invI* and *prgI*) and proteins governing the regulation of secretion (*sipD* and *invE*). Proteins required for regulation of gene expression are also encoded within SPI1 (*hilA* , *hilC, hilD*, and *invF*). Additional SPI1 secreted proteins as well as regulatory factors for this region are located elsewhere in the chromosome and are discussed below where relevant.

1.3.2 SPI2

SPI2 is located at 31 cs and encodes a TTSS that is distinct in components and function from the SPI1-encoded TTSS (228). The SPI2 TTSS does not play a role in invasion of non-phagocytic cells, although it has been recently suggested to be involved in mediating early trafficking of the *Salmonella*-containing vacuole (SCV; the specialized intracellular vacuole in which *Salmonellae* survive and replicate) in epithelial cells (239). Instead, the SPI2 TTSS is essential for intramacrophage survival and is absolutely required for systemic spread of the bacteria and generation of systemic phases of disease in the murine typhoid model (54). SPI2 is present in *S. enterica* but absent from *S. bongori*, and its acquisition is thought to be the key evolutionary step of *Salmonella* as an intracellular and systemic pathogen (21). It is interesting to note that although many of the SPI2 TTSS structural components are homologous to those in the SPI1 TTSS, mutations in these SPI1 TTSS structural genes cannot be complemented by their SPI2 homologues. This indicates that there are substantial differences between these two TTSS either at the level of function or of temporal expression and regulation.

The SPI2 secretion apparatus is encoded within two regions: a 10 kbp operon containing the genes *ssaJKLMVNOPQRSTU*, and a second smaller operon upstream

containing the genes *spiCAB* (a.k.a. *ssaBCD*). These two regions are separated by a region containing the potential effector (*sse*) or chaperone (*ssc*) genes *sseAB-sscA-sseCDE-sscB-sseFG*. The genes encoding a two component regulatory system, *ssrAB,* are located upstream of *ssaBCD* and are transcribed in the opposite direction to all the other genes within SPI2. Many potential effectors have yet to be shown to be translocated into host cells or to have a defined function, but as their expression is upregulated by the intramacrophage environment, a function directed towards intramacrophage survival has been hypothesized (155, 205).

1.3.3 SPI3

SPI3 is a 17 kbp region located at 82 cs inserted within the *selC* tRNA locus (37). It contains at least 10 identified ORFs (*sugR, rhuM, rmbA, misL, fldL, marT, sisA, cigR* and *mgtCB*) (38). *mgtCB* are the most extensively characterized ORFs, with *mgtB* identified as a magnesium transport system (136, 233). The function of *mgtC* remains unknown, although recent research indicates that it traffics to the Golgi apparatus (M.E. Maguire *et al.*, unpublished observations). The other ORFs of SPI3 are intriguing for their homology to other virulence factors, including ToxR of *Vibrio cholera (marT)*, the AIDA-1 adhesin of EPEC and members of the pathogen-restricted immunoglobulin A1 protease family (*misL*), and the PgaA antigen of the periodontal pathogen *Porphyromas gingivalis* (*sugR*) (38). *rhuM, rmbA, fidL, slsA* and *cigR* all have putative signal sequences and are presumably exported. Of the SPI3 genes, only *misL, marT* and *mgtCB* have been tested for invasion, survival within macrophages and virulence in the murine typhoid model (37, 38, 232); *mgtC* alone was found to be required for intramacrophage survival and to attenuate STM *in vivo* (37, 232). *mgtCB* is tightly regulated by the virulence-associated two component regulatory system PhoPQ; however PhoPQ does not regulate *sugR, rhuM, misL* or *marT* expression, nor is MarT involved in regulation of any

of the SPI3 loci in spite of its homology to ToxR (38). It is thought overall that the role of SPI3 lies in intramacrophage survival and development of a systemic infection (37, 38).

1.3.4 SPI4

SPI4 is a 27 kbp region located at 92 cs (270) currently believed to encode 18 putative proteins, including those with homology to type I secreted toxin proteins including *Pasteurella haemolytica* leukotoxin and *Bordetella pertussis* adenylate cyclase hemolysin (270). *ims8* is also located within SPI4, a gene previously identified to be important in intramacrophage survival (270). An insertional mutation in this region results in a lack of ability to grow within macrophages (20). Thus this region is important for intramacrophage survival, but its direct role and function remains unclear.

1.3.5 SPI5

SPI5 is an 8 kbp region located at 25 cs in STM, flanked by the *serT* and *copS* genes. SPI5 consists of six genes (nomenclature refers to *S.* Dublin/STM homologues): *pipD*, *orfX*, *sigD/sopB*, *sigE/pipC*, *pipB* and *pipA*. SPI5 has recently been shown to be mosaic in nature, with *sigD/sopB* being present in all *Salmonella* spp, but *pipB* is not found in *S. bongori* (147). Of the SPI5 genes, *sigD/sopB* is secreted by SPI1 TTSS and *sigE/pipC* is thought to be its specific chaperone. In *S.* Dublin SopB has been shown to be an inositol phosphate phosphatase which acts to increase chloride secretion by the host cell (191). Activities of SigD, the STM homologue of SopB, include hydrolysis of phosphatidylinositol 3,4,5-triphosphate, 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-dependant chloride secretion (168). SigD has also been shown to activate Akt in HeLa cells, and may be involved in delaying or preventing apoptosis of epithelial cells during infection ((240); O. Steele-Mortimer *et al.*, unpublished observations). Mutations in *sopB* and *pipCBA* greatly reduce

enteropathogencity of *S.* Dublin in the calf ileal loop model and therefore appear to play a role in gastroenteritis (271). In contrast, mutation of *sigDE* and *pipB* does not affect systemic disease in the murine typhoid model, but deletion of *pipA* results in attenuation of STM in naturally susceptible mice (147).

1.3.6 The *Salmonella* virulence plasmid (pSLT) and the *spv* locus

Virulence plasmids are found in only a small number of *Salmonella* serovars that can be divided into two groups: serovars that are host-adapted to domestic animals (e.g. *S.* Dublin, *S.* Choleraesuis, *S.* Gallinarum, *S.* Pullorum, *S.* Abortusovis) and those serovars with a broad host-range (*S.* Typhimurium and *S.* Enteritidis). Virulence plasmids range in size from 50 to 100 kbp, with STM harboring one of the largest virulence plasmids (pSLT; 100 kbp). Strains cured of their respective plasmids were found to be less virulent than their isogenic wildtype parent strains when inoculated by either oral or intraperitoneal routes of infection (106, 107). Several studies indicate that the virulence plasmid enhances bacterial growth within the intracellular environment of host cells and thus contributes to systemic disease (107, 120).

Of the 100 kbp of pSLT, an 8 kbp region (designated *spv* for *Salmonella* plasmid virulence) is present on all known *Salmonella* virulence plasmids and is responsible for the virulence phenotype in mice (216). The *spv* locus consists of the *spvR* regulatory locus and four structural genes, *spvABCD*. Recent work by Matsui *et al.* (171) indicates that *spvBC* are necessary and sufficient to replace the entire *spv* locus and pSLT itself, and can restore the ability of STM to cause systemic infection in BALB/c mice after subcutaneous infection. *spvR* is involved in regulating *spvABCD* expression (59, 190), while SpvA may function as a repressor of the *spv* operon (1). SpvR from *S.* Dublin was found to be required for the development of severe enteritis in cattle and is involved in enhancing intracellular proliferation of *S.* Dublin in blood-derived bovine monocytes

(161). This suggests that the *spv* genes promote enhanced intracellular proliferation in intestinal tissues and at extraintestinal sites in the natural host (160). *spv* genes have also been found to be required for an apoptosis-like cytopathology of host macrophages (161). SpvR itself has been determined to be involved in the formation of *Salmonella*-induced filaments (Sifs; intracellular filamentous structures formed within host cells thought to be required for intracellular replication of STM (241)) and is associated with intramacrophage survival (111). SpvB is a mono(ADP-ribosyl) transferase (158, 198) that uses actin as a substrate and depolymerizes actin filaments (158), preventing the conversion of G actin in to F actin (249), a function that appears to be essential for virulence in mice (158). Overall, the *spv* genes on pSLT are crucial for intramacrophage survival and systemic disease in the murine typhoid model.

1.3.7 Gifsys

In 1997 two large (approx. 50 kbp) cryptic lambda-like prophages were discovered in the *Salmonella* chromosome, located at 57 and 24 cs and named Gifsy-1 and Gifsy-2, respectively (81). Further work indicated that both prophages were fully functional and could be induced effectively by exposure of the bacteria to hydrogen peroxide (80). A third Gifsy prophage, Gifsy-3, was eventually identified (82). Interestingly, not all strains of *S.* Typhimurium have the same complement of Gifsys, as both 14028s and SL1344 carry Gifsy-1 and -2, but only 14028s carries Gifsy-3.

Gifsy-1 contains six genes, *gogA, gogB, pagJK, msgA* and *gipA*. *gogA* shows 72% identity with the PipA protein of *S.* Dublin (82). *gogB* has significant similarity to members of the leucine-rich repeat (LRR) family of proteins including YopM of *Yersinia pestis* and the TTSS effectors SlrP, SspH1 and SspH2 of STM, but has no effect on STM virulence in the murine typhoid model (82). The functions of *pagJK* and *msgA* are unknown, and recent studies into their role in pathogenesis were inconclusive (82).

However, initial studies on *pagJ* showed it to be essential for virulence in mice (24). *gipA* has been identified to be specifically induced in the small intestine of the mouse and is required for growth and survival of STM in the murine Peyer's patch (238). Gifsy-1 appears to be redundant in function to Gifsy-2, but does attenuate STM to a minor (although significant) extent in strains deleted for Gifsy-2 (80).

Gifsy-2 was found to be essential for establishment of a systemic infection in the murine typhoid model, as curing of Gifsy-2 results in over 100-fold attenuation of STM (80). Gifsy-2 contains six loci designated *gtgA* to *gtgF*, *sodCI* and *grvA* (82). *gtgA* is virtually identical (98%) to the *gogA* locus of Gifsy-1, the *pipA* homologue. Inactivation of *gogA* and/or *gtgA* had no effect on virulence in the mouse (82). *gtgB* was independently identified as a member of the SsrB regulon and encodes a TTSS protein also known as SrfH and SseI (175, 272). *gtgB* was not found to have an effect on virulence (82, 217). *gtgF* specifies a hypothetical protein with 76% identity to the putative *Salmonella* virulence factor with unknown function, MsgA (82).

STM has two discrete periplasmic Cu,Zn superoxide dismutase enzymes which protect the bacterium from exogenous oxidative damage, SodCI (encoded within Gifsy-2) and SodCII (encoded elsewhere in the chromosome) (77). All *Salmonella* serotypes appear to have a *sodCII* locus, but only the most highly pathogenic serotypes were found to harbor *sodCI*. Deletion of either *sodCI* or *sodCII* in isolation had little effect on STM virulence in a susceptible (*Nramp1*$^{-/-}$) mouse, which the authors considered to be "naturally deficient in production of reactive oxygen intermediates (ROIs) in response to infection", but the *sodCI-sodCII* double knockout strain was significantly attenuated in this background. However, all three deletion strains were significantly attenuated in naturally resistant (*Nramp1*$^{+/+}$) mice, considered in this specific work to be proficient at ROI production (77). *grvA* is an unusual locus in that its deletion results in enhanced

virulence in mice, and as such its function has been colloquially called "antivirulence" (125). Interestingly, this antivirulence phenotype required *sodCI* but not other genes of Gifsy-2, which has led to the hypothesis that in a wildtype situation GrvA decreases pathogenicity of STM in the host, most likely by affecting resistance to ROIs (125).

Gifsy-3 is located at 27-28 cs and carries a second *pagJ* gene as well as the gene for the secreted SPI1 effector SspH1 (82). As mentioned above, SspH1 is a member of the LLR family of proteins, and is the only *Salmonella* effector to date known to be secreted by both the SPI1 and SPI2 TTSS (176). A second LRR effector known as SspH2 is present in STM, and is only secreted by the SPI2 TTSS. Deletion of *sspH1* or *sspH2* in isolation, as well as curing of 14028s for Gifsy-3, has no effect on virulence in mice (82, 176), but a *sspH1-sspH2* double knockout was significantly attenuated (176). SspH1 alone was found to have no role in enteritis in the bovine model of infection (277). Thus it appears that Gifsy-3 is redundant to SspH2 for STM virulence.

1.3.8 SopEϕ

SopE is a substrate of the SPI1 TTSS and is involved in actin cytoskeleton rearrangements and invasion of non-phagocytic cells. The *sopE* gene is encoded within a cryptic P2-like prophage named SopEϕ located outside of SPI1 at cs 59-60 (118). Inactivation of *sopE* alone has no effect on virulence of STM during infection of BALB/c mice (118). *sopE* is only present in certain serovars of *S. enterica*, including *S.* Typhi, *S.* Heidelberg, *S.* Hadar, *S.* Newport, *S.* Dublin, *S.* Enteriditis and *S.* Pullorum. Further, *sopE* is only present in certain strains of STM including SL1344 but not 14028s, and in a small group of STM epidemic strains (180). SopE is 70% identical to another SPI1 effector known as SopE2 (73). SopE2 has been determined to be involved in production of gastroenteritis in the bovine enteritis model (277). Although SopE and SopE2 have been identified to activate different sets of Rho GTPases, SopE appears to be redundant in

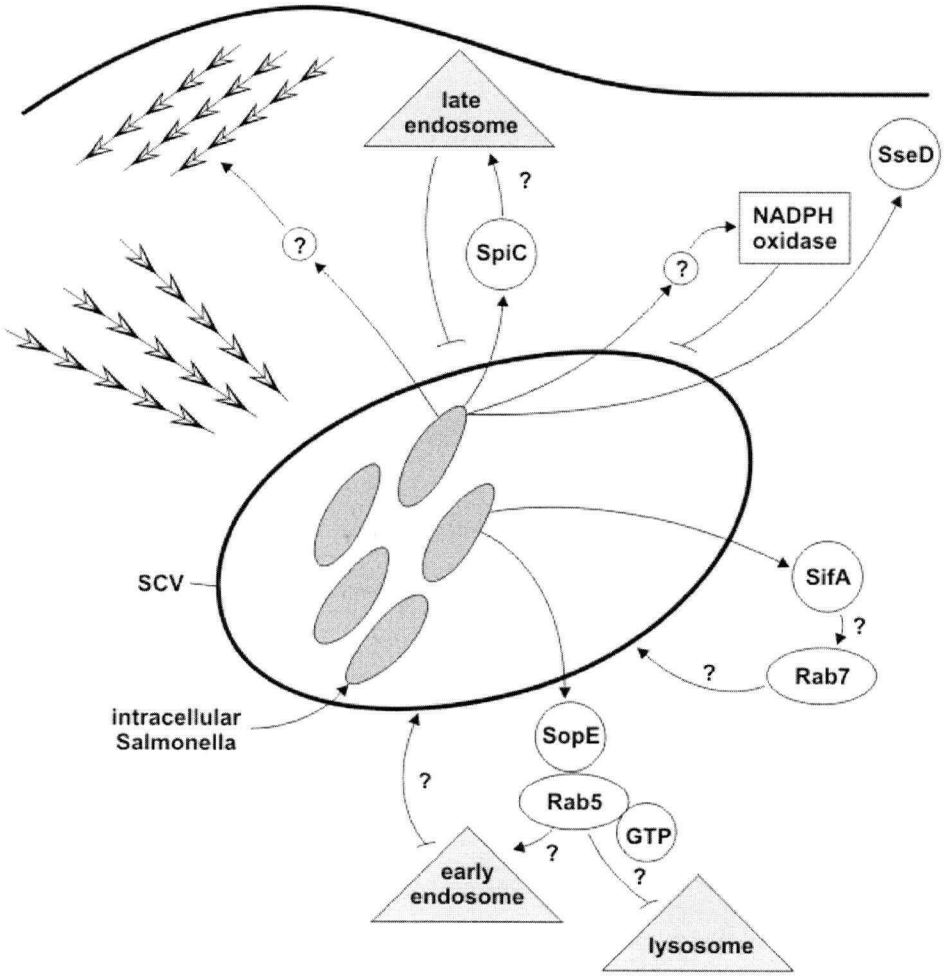
function to SopE2 (242). However, its presence in highly virulent STM strains indicates that although it may not have an overt role in virulence, it may be involved in fine-tuning the interaction of *Salmonella* spp. with their hosts (180).

1.4 Role of SPI2 in intramacrophage survival

Manifestation of systemic phases of *Salmonella*-based diseases relies upon the ability of *Salmonellae* to survive and replicate within macrophages. Due to this key role of intramacrophage survival and its relevance to the murine typhoid model, the role of SPI2 and its associated effectors in this phenomenon is detailed below and illustrated in Figure 3.

Once inside a macrophage, *Salmonellae* reside within a spacious vacuole known as the *Salmonella*-containing vacuole (SCV) from which stable lysosomal glycoprotein-containing filamentous structures known as Sifs (*Salmonella*-induced filaments) extend (L. Knodler *et al.*, unpublished observations). Sifs connect with the SCV, and in epithelial cells their formation coincides with replication of intracellular *Salmonellae* (241). It is hypothesized that Sifs are necessary for intracellular bacterial replication and may be involved in acquisition of nutrients and maintenance of a permissive niche for the bacterium (241). The SPI2 effector SifA is essential for maintenance of the SCV in macrophages and for intramacrophage survival and replication of STM (30, 41). SifA is hypothesized to act as an activator of the Rab7 membrane fusion machinery, possibly by promoting a continuous flow of membrane to the SCV to sustain the net increase in surface area resulting from intracellular *Salmonella* replication (30). SopE is also involved in mediating interactions between the SCV and the Rab network; the GTP-bound form of Rab5 forms a complex with SopE on the SCV membrane (185, 186) where it may promote the continuous fusion of the SCV with early endocytic compartments and prevent targeting to lysosomes. The SPI2 effectors SpiC and SseFG

Figure 3. Illustration of mechanisms for survival of STM within macrophages. Maintenance of the SCV as a permissive environment for the bacteria involves altering normal vesicular trafficking pathways. The effectors SpiC and SopE appear to prevent fusion of the SCV with late endosomes and lysosomes, respectively, SpiC by an unknown mechanism and SopE by binding the active, GTP-bound form of Rab5 that may promote fusion with early endosomes. SifA is involved in maintaining the SCV, possibly by interacting with Rab7 and directing other, novel fusion events with unidentified endosomal compartments. SseD may interfere with the assembly of a functional NADPH oxidase complex, while an unknown effector directly prevents trafficking of intact NADPH oxidase to the SCV, thereby diverting toxic ROIs generated by this enzyme from the SCV. Another unidentified effector is involved in promoting and maintaining a meshwork of actin filaments around the SCV, which has an as-yet-undefined role in vacuolar maintenance. Shaded ovals represent intracellular STM; barbed arrows represent actin filaments. Adapted from Zaharik *et al.* (276).


late
endosome
SseD
?
SpiC
NADPH
oxidase
?
?
SCV
SifA
?
?
Rab7
intracellular
Salmonella
SopE
?
Rab5
GTP
?
?
early
endosome
lysosome

were also identified as being required in some manner for Sif formation in epithelial cells and thus may have additional, as yet unidentified roles, in SCV formation, maturation, or maintenance (111).

An unidentified SPI2 effector has also been implicated in the formation of an F-actin meshwork around SCVs that is required for survival and replication of intracellular *Salmonella* as well as maintenance of the integrity of the SCV (173). It is tempting to speculate that SpvB may be involved in this actin accumulation due to its role as an ADP ribosylase (section 1.3.6). Based on homologies to other proteins, SseJ is hypothesized to act either as an acyltransferase or as a lipase. Further, SseJ has been implicated in regulating the dynamics of the SCV membrane although the mechanism by which it does so is currently unknown. It is hypothesized that SseJ may be modifying SCV membrane lipids to facilitate fusion of the SCV with selected vesicular compartments, or perhaps regulating budding or scission of the SCV (217).

While SpiC has been suggested to directly block fusion of the SCV with endosomes (254), other SPI2 effectors have been implicated in protecting the SCV from various non-specific cellular defense mechanisms. For example, SseD may block initial assembly of the NADPH oxidase complex (146), while an unidentified SPI2 effector is involved in preventing an intact NADPH oxidase complex from trafficking to the SCV (259). In addition, function of the SPI2 TTSS as a whole, and not any individual effector, has also been implicated in the diversion of iNOS (inducible nitric oxide synthase) from the SCV and exclusion of peroxynitrite from the SCV (49). This suggests that avoidance of reactive nitrogen intermediates (RNIs) as well as ROIs is important for intracellular adaptation and survival of STM.

1.5 Regulation of virulence genes

Bacterial pathogens including *Salmonellae* tightly regulate the expression of their virulence genes. These genes may be coordinated in response to very specific environmental conditions, and often are influenced by multiple regulators to fine-tune expression in response to a number of environmental parameters. *Salmonellae* have a number of two component regulatory systems (TCRS) for the global regulation of both virulence and "housekeeping" genes. These systems consist of a sensor protein and a regulator protein. The sensor protein is a histidine kinase that generally spans the bacterial inner membrane, with an extracellular domain acting to sense external signals and an intracellular domain acting as an autokinase. Upon stimulation the sensor protein undergoes autophosphorylation and then transphosphorylates the regulator protein. This phosphorylation relay changes the ability of the regulator protein to bind to specific DNA sequences, allowing it to act as a transcriptional activator and/or repressor. These TCRS can also interact to process multiple environmental signals in a complex hierarchical system. TCRS known to be involved in virulence gene regulation are discussed below.

1.5.1 EnvZ-OmpR

OmpR is the regulator protein of a TCRS with the sensory protein EnvZ. EnvZ senses the environmental stimulus of osmolarity via an unknown mechanism, and through OmpR, controls the expression of *Salmonella* outer membrane proteins, including OmpF and OmpC (51). This is the traditional interpretation of the role of EnvZ-OmpR in STM gene regulation. However *ompR* mutants are significantly attenuated during infection of BALB/c mice (70). Although *ompR* had no effect on invasion of epithelial cells, both *envZ* and *ompR* strains displayed reduced or completely inhibited Sif formation in epithelial cells (179). OmpR has been associated with

Salmonella-induced apoptosis of macrophages (162), and is involved in the acid tolerance response of STM (section 1.5.3). Recently, it was elucidated that expression of *ompR* is induced by acid shock as opposed to osmotic shock, which is essential for acid tolerance of STM (17). Thus it appears that the role of OmpR in STM virulence gene regulation may be related to its role in acid tolerance instead of to its function in response to osmolarity.

1.5.2 Ion-responsive regulatory systems

PhoPQ

PhoPQ is a TCRS that governs virulence and mediates the adaptation to Mg^{2+}-limiting environments in STM (102). Located at 27.4 cs on the STM chromosome, it modulates the expression of over 40 genes within STM (including *phoPQ* itself), in response to extracellular Mg^{2+} and Ca^{2+} concentrations (102). Phospho-PhoP is the active form of the PhoP regulatory protein. In conditions of high Mg^{2+} concentrations, PhoQ acts as a phosphatase and dephosphorylates PhoP, abolishing transcription of the subset of PhoP-regulated genes known as *pags* (PhoP-activated genes) while alleviating repression of another subset of genes called *prgs* (PhoP-repressed genes).

Many *pags* are associated with virulence and promote intracellular survival (101, 177, 178), including genes involved in the resistance to the antimicrobial peptide polymyxin (*pagD*, *pmrAB*, *ugd* and *pbgBE*). *prgs* are also associated with virulence, including resistance to bile salts (*prgC, prgH*; (256)) and invasion of nonphagocytic cells (*hilA*; (16)). *phoP* or *phoQ* null strains of STM are dramatically attenuated for virulence and have been associated with various phenotypes including the inability to survive within macrophages, and increased susceptibility to killing by antimicrobial peptides, bile salts and acid pH (102). Although a subset of PhoP-regulated genes is responsive to mild acid pH, this induction has been determined to be independent of both PhoP and PhoQ

(93). A balance between activation and repression of the PhoP regulon is essential for full virulence, since constitutive expression of *phoP* (which results in further repression of *prgs* and activation of *pags*) results in an attenuation for mouse virulence as dramatically as a *phoP* null mutant (178). These constitutive mutants ($PhoP^C$) are unable to invade nonphagocytic cells due to repression of transcription of *hilA* and therefore lack of expression of SPI1 genes. However, $PhoP^C$ strains are also attenuated when inoculated intraperitoneally and therefore are also defective for intramacrophage survival (8).

PmrA-PmrB

PmrAB is a TCRS that responds to extracytoplasmic ferric (Fe^{3+}) iron in order to promote transcription of genes essential for growth in high iron and for resistance to polymyxin and other antimicrobial proteins (273). It is essential for full virulence in mice (110). This appears to involve alteration of the lipid A moiety of LPS so that it no longer binds iron, or positively charged antimicrobial peptides like polymyxin, as efficiently (108, 273). Transcription of PmrA-activated genes is also induced in low Mg^{2+} conditions in a PhoPQ-dependent manner, and PmrAB is involved in the regulation of a subset of the PhoPQ regulon (109, 234). Expression of *pmrAB* is also induced by mild acid pH independently of PhoPQ and PmrAB itself (273). Thus the sensor for pH-regulated induction of virulence genes remains unknown. The genetic basis for the interaction between the PhoPQ and PmrAB regulons was determined to be PmrD, a *pag* that mediates the activity of the PmrAB system at the postranscriptional level (153). It is believed that PmrAB plays a role in surviving toxic iron levels in environments outside animal hosts. Such environments may include soil or groundwater where Fe^{3+} levels are high and the soil bacterium *Paenibacillus polymyxa* produces polymyxin (273). The co-regulation of the PmrAB regulon by low Mg^{2+} and high Fe^{3+} conditions reflects the ability of the bacterium to respond to a broader spectrum of environmental cues (153).

Fur

Fur (ferric uptake regulator) is a regulatory protein that governs expression of approximately 40 genes in response to iron availability (88). Fur binds Fe^{2+}, and to a lesser extent Mn^{2+}, as a cofactor (117). The cation alters affinity of Fur to bind a "Fur box" in the upstream regulatory region of genes within the Fur regulon (89). Originally thought to be functional only as a repressor, Fur is now believed to also act as a transcriptional activator (88). Interestingly, not all iron-inducible genes are regulated by Fur (88). Likewise, not all Fur-regulated genes are responsive to intracellular iron levels (88). The role of Fur in virulence is unclear, for although STM SL1344 deleted for *fur* is fully virulent (94), other STM *fur* strains display some level of attenuation (211). Fur may be involved in virulence as one of the regulators involved in the acid tolerance response of STM (86), discussed below.

1.5.3 The Acid Tolerance Response (ATR) and STM gene expression

The ATR of *Salmonellae* involves the synthesis of at least 51 acid shock proteins (ASPs) and allows the bacteria to survive exposures to potentially lethal acidic environments. The response and adaptation of bacteria to acid shock is a complex phenomenon, with different systems being engaged depending on growth phase and type of acid stress (organic vs inorganic) (87). The ATR also provides a significant degree of cross-protection to heat, osmotic and oxidative challenges (156, 159). Four global regulators have been implicated in the ATR: PhoPQ, RpoS, OmpR and Fur (18, 23, 85, 156). PhoPQ is involved in resistance to inorganic acid stress, while Fur and RpoS appear to be involved primarily in resistance to organic acid stress (23, 87). RpoS, but not Fur, has also been implicated in surviving acid stress imposed by volatile fatty acids (14). Notably Fur regulates ATR genes in an iron-independent manner (114). OmpR has not been classified as having a role in organic vs inorganic acid tolerance, but is required for

the stationary phase ATR (18). This is relevant as organic acids present in the gastrointestinal tract, even at neutral pH, can induce acid tolerance due to protonated short chain fatty acids crossing the bacterial membrane and releasing protons within in the cytoplasm (87). The ATR is involved in virulence, as deletion of the *atp* gene (encoding the Mg^{2+}-dependent proton translocating ATPase, a characterized ASP) resulted in attenuation of SL1344 when delivered by both oral and intraperitoneal routes of infection (94). Further studies showed a correlation between acid sensitivity and level of attenuation, the most sensitive being the most attenuated (211). Mutants were also deficient in intramacrophage survival. The association with PhoPQ, RpoS and OmpR, three key regulators of other virulence genes in STM, strongly indicate an important role of the ATR in numerous facets of *Salmonella* pathogenicity. This role could encompass survival of the low pH of the stomach, resistance to volatile fatty acids in the intestine, or adaptation to the low pH of the SCV, all key stresses that STM must overcome to maintain infection of a host.

1.5.4 Regulation of SPIs

Regulation of SPI1 is complex, and new regulators are seemingly discovered on a daily basis. The two apparently primary regulators of SPI1 and SPI1-associated genes are HilA and InvF, encoded within SPI1 itself (15). HilA is the primary regulator of SPI1, SPI4 and SPI5 (4, 15). HilA is thought to be a constitutively active regulator with no manner of post-transcriptional modulation of its activity; therefore, all regulation of HilA activity is thought to occur at the level of *hilA* transcription (15). *hilA* is maximally expressed in conditions of low oxygen, high osmolarity, the presence of endogenous fatty acids or fatty acid breakdown products, and slightly alkaline pH, conditions thought to exist in the proximal intestine (16, 166). However, the manner in which these environmental stimuli are sensed and relayed to control *hilA* expression are for the most

part unknown. Interestingly, although osmolarity is associated with regulation of *hilA* and SPI1, deletion of *ompR* had little or no effect on the response of *hilA* and SPI1 to osmolarity of the growth media (164). Other regulators that appear to influence *hilA* expression include HilD, HilC/SprA/SirC, the BarA-SirA two component regulatory system, SirB, CsrAB, and PhoRB (164). *hilA* is also a member of the PhoPQ regulon and is classified as a *prg*, or PhoP-repressed gene. It is thought that *hilA* is controlled by such a battery of regulators in order to most efficiently fine-tune expression of SPI1 genes to promote invasion only at the most opportune times during infection.

HilA itself regulates expression of the second major SPI1 regulator, InvF (140). HilA and InvF appear to regulate different subsets of SPI1-associated virulence genes. HilA appears to directly activate both TTSS apparatus genes and SPI1-secreted proteins, while InvF regulates expression of a subset of SPI1 secreted proteins that requires HilA acting indirectly through expression of *invF* (64, 74, 165). However, it appears that HilC, HilD and SirA can also directly activate *invF* expression without the need for *hilA* as an intermediary (165, 210). HilD can also directly bind the *invF* promoter in the absence of HilA (165), implying that under certain conditions a subset of invasion genes can be activated independently of HilA. However, this remains a theoretical possibility based solely on *in vitro* observations and has yet to be demonstrated to occur or have any relevance *in vivo*.

Regulation of SPI2 is much less well characterized than for SPI1 and SPI1-associated genes. To date all SPI2-associated genes have been identified as being positively regulated by SsrAB, a TCRS encoded within SPI2 whose sensory stimulus is currently unknown (68). Note that the STM SsrAB regulatory system is distinct and unrelated to the *E. coli ssrA* tmRNA involved in the targeting of polypeptides for degradation (97). Until recently SsrAB was assumed to activate SPI2 and SPI2-associated

genes exclusively. However, Worley *et al* . (272) have demonstrated that SsrB activates a global regulon, many of the loci residing in previously undescribed regions of the STM chromosome. Expression of *ssrAB* itself is regulated by OmpR but does not appear to be autoregulated (54, 155). As a result, OmpR indirectly influences the expression of genes downstream of SsrAB. The PhoPQ TCRS has also been implicated in SPI2 gene expression, although this remains a matter of controversy. Deiwick *et al.* (68) originally identified SPI2 loci as being expressed in minimal medium containing limiting amounts of Mg^{2+} or phosphate and suggested that *ssrAB* is positively regulated by PhoPQ. However recent work by Miao *et al.* (174) and Zaharik *et al.* (this work) do not observe an effect of PhoPQ on expression of *ssrAB* or other SPI2-encoded genes. In support of this, work by Beuzon *et al.* (31) determined that SsrAB and PhoPQ contribute independently to STM virulence in mice and therefore are most likely not cooperatively regulating all of the same genes. However, three of the SsrAB-regulated genes identified by Worley *et al.* (272), *srfH, srfJ* and *srfK,* were upregulated 4-12 fold by PhoPQ, and therefore it remains a possibility that a subset of SPI2-associated genes are influenced by the PhoPQ TCRS.

PhoPQ is the only global regulatory system known to be involved in the expression of any of the SPI3 genes, being a positive regulator of *mgtCB*. To date, HilA has been identified to positively regulate four SPI4 genes, *orfG, orfI, orfJ*, and *orfK* (4). Of the SPI5 genes, *pipD* appears to be independent of HilA and SsrAB (147), and regulation of *orfX* has not been studied. *sigD/sopB* is a member of the HilA regulon (4), but interestingly the adjacent *pipBA* operon is regulated by SsrAB ((147); L. Knodler, unpublished observations).

The *pipA* homologues *gogA* and *gtgA* located within Gifsy-1 and Gifsy-2, respectively, may be regulated in a similar manner to *pipA* and therefore may be

members of the SsrAB regulon, although this has not been directly investigated. The *pagJK* locus (Gifsy-1) were originally identified as PhoP-activated genes therefore forming part of the PhoPQ regulon. *gtgB* (Gifsy-2) is a member of the SsrAB regulon (82). Of the two loci of Gifsy-3, *pagJ* is a member of the PhoPQ regulon, while expression of *sspH1* is only known to be independent of SsrAB (176). Expression of *sopE* is dependant on the SPI1 regulator InvF as well on its chaperone SicA (65).

As mentioned in section 1.3.6, SpvR is the transcriptional activator of the *spvABCD* operon located on the virulence plasmid of STM. Expression of *spvR* and *spv* genes in general is upregulated during growth in minimal medium (268) and upon entry into host cells (79, 106). *spvR* is autoregulated, is positively regulated by the virulence-associated alternative sigma factor RpoS (190), and negatively regulated by SpvA (1) and iron concentration (237).

1.6 Model systems for studying *Salmonella* infections

Most work on bacterial physiology involves the use of axenic laboratory cultures (i.e. cultures that are not contaminated with any other kind of microorganism or living cell). This has the advantages of being able to easily examine large numbers of bacteria in synchronized cultures and the ability to control desired variables. However, axenic culture conditions do not reflect what occurs during infection of a host organism. Much remains unknown about the environments and host factors the pathogen encounters during infection, and therefore we are unable to accurately duplicate these conditions in a test tube.

To circumvent the limitations of axenic bacterial culture models, considerable research has been devoted to identifying molecular mechanisms of bacterial pathogenicity and associated virulence factors using *in vitro* cell culture models. This approach offers some improvement over axenic cultures, as there are a number of host

cell lineages available for study derived from various tissue types and species of animals. However, generally these cell lines are terminally differentiated, immortalized, or both, which does not reflect the true status of that cell type in the whole animal. In addition certain tissues, animals, or animal lineages have been exploited for derivation of cell lines and are commonly used for experiments that do not reflect an *in vivo* situation. Unfortunately, it is infrequent that pathogenic mechanisms identified in these less-than-ideal models are tested to determine if they function similarly, if at all, in a relevant infection model, and to what extent they contribute to disease. In addition even in cell culture models the contribution of host processes such as inflammation, host resistance and other factors to the development of an infection are missed. Since disease is usually a complex series of linked events, tissue culture cells are often poor models of the relevant primary cells that pathogens normally interact with during disease.

Ultimately, the best way to determine what goes on during an infection is to infect the host. Animal models are commonly used to reproduce a given disease and determine whether a specific bacterial or host factor is involved or implicated in bacterial pathogenesis. By utilizing animal models, one can follow the inflammatory and immune processes during disease, in addition to identifying new virulence factors that are not detected using tissue culture cells. However, it must be acknowledged that there are complications with animal model systems as well. Although you do see a representative infection, it is nearly impossible to work out a mechanism in such a complicated system. This is where the cell culture models do have a significant role to play. However, such studies must eventually be complemented with whole animal work to further test the hypothesis in the most relevant model system.

The choice of an animal model to study an infectious process is critical. Ideally, the animal model represents a naturally occurring infection in that animal that closely

mimics the human disease. Nonetheless, there are problems even with the most commonly used animal models. The most widely used model for salmonellosis is the BALB/c mouse. Infection of these inherently susceptible animals with STM results in a disease that resembles human typhoid fever. However until recently results obtained using this model were often extrapolated back to gastroenteritis in humans, in spite of the fact that STM infection of mice does not result in perceptible diarrhea or inflammation of the gut. Thus to accurately study gastroenteritis the standard accepted model system is *S.* Dublin infection of cows. To reinforce the importance of choosing an accurate model system, numerous STM genes have been identified to have no role in virulence in the murine typhoid model, but are required for gastroenteritis in cows (251). A further concern with the murine typhoid model is that, since no other model system has been found to represent *S.* Typhi infection of humans, much extrapolation from murine STM infection is necessary. However, as mentioned above, this uses a naturally susceptible mouse model to extrapolate back to a human population. This poses a problem, as except for inbred animal lineages, on the whole most animals have some natural resistance to STM. Therefore, if we are to continue to extrapolate data obtained with the murine typhoid model to human infections, the role of host resistance can not be ignored. The host resistance locus studied in most detail with respect to STM is Nramp1, and the inclusion of Nramp1 as a variable in the murine typhoid model may more accurately reflect naturally occurring *Salmonella* infections of humans, potentially yielding further strengths for this model system.

1.7 Nramp1

In 1974 it was found that a panel of inbred mouse strains infected with STM exhibited a dose-dependent susceptibility with a parenteral LD_{50} of either ≤ 10 bacteria or $\geq 10^4$ bacteria with no strains of intermediate resistance ((194) and references therein).

Similar phenomena were reported for the pathogens *Leishmania donovani* and *Mycobacterium bovis* BCG. Interestingly, the strain distribution of resistance and susceptibility was the same for all three pathogens, leading to the hypothesis that resistance to *M. bovis* BCG (*Bcg*), *S.* Typhimurium (*Ity*, for *I*mmunity to *ty*phimurium), and *Leishmania* (*Lsh*) was conferred by a single locus or group of tightly linked loci with a potentially broad role in resistance to antigenically unrelated intracellular pathogens. Mendelian segregation analyses and genetic mapping studies supported this theory, with resistance in all three cases segregating as a dominant gene on mouse chromosome 1.

Experiments *in vivo* determined the inherited resistance to *M. bovis BCG, S.* Typhimurium and *L. donovani* was the result of a single gene conferring enhanced bacteriostatic or bactericidal mechanisms in the mature macrophages of resistant mice. Positional cloning was used to isolate six candidate genes for *Bcg Ity/Lsh* (261), one of which was a novel gene expressed exclusively in the spleen and liver and was enriched in the macrophage populations derived from these organs. This candidate gene was named *Nramp1* (natural resistance-associated macrophage protein-1) and was predicted to be an integral membrane protein with 10-12 transmembrane domains (TMD) (263). Sequence analysis of *Nramp1* cDNA clones from 27 Bcg^R and Bcg^S inbred mouse strains determined that susceptibility to infection was associated with a single nucleotide change resulting in a non-conservative glycine to aspartic acid substitution at position 169 (G169D) within the predicted TMD4. Accordingly, introduction of a transgene containing the resistant *Nramp1* allele into a susceptible mouse strain conferred resistance to *Mycobacterium* and STM infections. In addition, creation of an $Nramp1^{-/-}$ mouse strain yielded animals that, although normal in appearance and longevity, were no longer naturally resistant to *Mycobacterium*, STM or *Leishmania*. Together, these

experiments proved that the various phenotypes described for the *Bcg*, *Ity* and *Lsh* loci resulted from a common genetic defect at *Nramp1*.

The effect of Nramp1 on infection is not universal, as it has been shown to play no role in resistance/susceptibility to other pathogens including *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* or *Chlamydia trachomatis* (199). It has also recently been identified that Nramp1 has the opposite effect on *Franciscella tularensis* infections, the G169 allele conferring susceptibility to this pathogen, while the A169 allele confers resistance (152). Recently, the role of Nramp1 in murine models of *Mycobacterium tuberculosis* infection has been questioned (192, 193), and it has been proposed that the Nramp1-mediated effects on *Mycobacteria* may be specific to the vaccine strain BCG. However, there is still evidence to suggest a role of NRAMP1 in controlling human *Mycobacterial* infections (see section 1.7.6). In contrast, Nramp1 plays a definitive and consistent role in controlling an STM murine infection. Differences in bacterial loads in the liver and spleen are seen early on in infection in resistant versus susceptible mice, within 24 h when bacteria are delivered intravenously and within 48 h if delivered intraperitoneally. ((98); this work). Susceptible mice die within one week of inoculation regardless of route of infection, whereas *Nramp1*$^{+/+}$ mice are eventually able to clear the infection successfully. It has not yet been determined whether NRAMP1 has an analogous effect on replication of *S.* Typhi in human infections.

1.7.1 Function of Nramp1

Nramp1 is an integral membrane phosphoglycoprotein (263) that is localized to late endocytic compartments of resting macrophages (105), and becomes associated with the membrane of the phagosome upon phagosomal biogenesis. This subcellular targeting pattern supports the hypothesis that Nramp1 controls the replication of intracellular

parasites by altering the intravacuolar environment of the bacteria-containing phagosome. All three of the microorganisms whose growth is classically regulated by Nramp1 are intracellular parasites that survive and replicate within mononuclear phagocytes. Macrophages from resistant and susceptible mice have been shown to take up equivalent numbers of bacteria (98); thus, phagocytic ability of these cells is not affected by Nramp1. Initially, it was not clear whether the role of Nramp1 was bacteriocidal or bacteriostatic in nature. A small (about threefold), but reproducibly greater degree of killing STM was observed in Ity^R than Ity^S mice during the first 4 h following intravenous infection postinfection (27). However, further experimentation concluded that the effect of Nramp1 on the growth rate of intracellular bacteria was greater than its effect on killing the bacteria (27, 98, 129). Thus the effect on Nramp1 on intracellular bacteria is primarily bacteriostatic in nature.

1.7.2 Nramp1 and divalent cation transport

The function of Nramp1 at the phagosomal membrane responsible for this bacteriostatic effect is a matter of controversy. Extensive homology of Nramp1 to other ion transport systems including Nramp2, MntH and the Smf family of proteins (discussed below), indicate that it may function as a divalent cation transport system. Atkinson and Barton (11) transfected RAW 264.7 cells ($Nramp1^{D169}$) with $Nramp1^{G169}$ and observed an increased iron flux into the cytoplasm from a calcein-inaccessible cellular location. *Xenopus* oocytes were used to demonstrate that Nramp1 is a divalent cation transporter with affinity for Fe^{2+}, Zn^{2+} and Mn^{2+} (96). However, Goswami *et al.* (96) also suggested that Nramp1 is a highly pH-dependant antiporter which fluxes metal ions in either direction against a proton gradient. As phagocytic vacuoles normally have a pH approximating 5.0, this suggests that Nramp1pumps divalent cations **into** the vacuole. Although it has become well-accepted that the transport affinity of Nramp1 is for divalent

cations and that its mechanism of transport is related to pH, the direction of proton transport (be it antiport or symport) remains an issue of controversy.

There are cases to be made for either direction of transport. Zwilling *et al.* have favored the iron-import function of Nramp1, suggesting that elevating Fe^{2+} in the vacuole would provide a catalyst for the formation of toxic oxygen compounds which would kill intracellular bacteria. In support of this hypothesis, they found that low doses of iron stimulated the ability of macrophages from *Nramp1*$^{+/+}$ mice to limit the growth of *M. avium* (279). This growth-inhibitory effect occurred only over a very narrow dose range and was abrogated by the addition of hydroxyl radical scavengers. Kuhn *et al.* subsequently found that phagosomes formed upon phagocytosis of latex beads isolated from resistant cells contained more Fe^{2+} and more hydroxyl radicals than latex-bead phagosomes isolated from susceptible cells (154).

Recently Jabado *et al.* developed a divalent cation-sensitive dye attached to zymosan (132). This conjugated dye was taken up by peritoneal macrophages isolated from *Nramp1*$^{+/+}$ and *Nramp*$^{-/-}$ mice. Quenching of the dye by Mn^{2+} was used to monitor the flux of divalent cations across the phagosomal membrane (132). It was found that phagosomes from *Nramp1*$^{+/+}$ mice **extruded** Mn^{2+} into the cytoplasm faster than their *Nramp1*$^{-/-}$ counterparts. This difference in rate of transport was eliminated when acidification of the phagosomal lumen was dissipated, reinforcing the idea that divalent metal ion transport through Nramp1 **is** H^+ dependent, but more likely to be via a symport rather than an antiport mechanism. More recently, Biggs *et al.* (32) demonstrated that there was 30-50% greater iron efflux from *Nramp1*^{G169}-transfected RAW264.7 macrophages than in the corresponding *Nramp1*^{D169} cells. The extent of this Nramp1-dependant iron release was also reduced by addition of bafilomycin A1 or endogenous nitric oxide synthesis (both inhibitors of V-ATPase), again suggesting transport of ions

out of the vacuole and a dependence on a proton gradient for transport. These data provide direct evidence that Nramp1 transports ions from the phagosome into the cytosol of the host cell.

1.7.3 Nramp1 and macrophage activation

Many studies using congenic mouse strains and *Nramp1*-transfected cell lines have indicated an effect of Nramp1 early in the macrophage activation pathway. Nramp1 appears to have many effects on macrophage function including regulation of IFN-γ, IL-1β, iNOS, MHC class II molecules, TNF-α, nitric oxide release, L-arginine flux, oxidative burst, and tumouricidal as well as antimicrobial activities (36). *Nramp1*$^{-/-}$ macrophages also have a defect in ability to process antigens, which is compounded by the influence of the gene on molecules regulating (TNFα, IL-1β) or directly involved in (MHC II) antigen presentation. This results in an *in vivo* bias towards development of a TH1 response in *Nramp1*$^{+/+}$ mice, but a TH2 response in *Nramp1*$^{-/-}$ mice ((36) and references therein). The role of Nramp1 in macrophage activation is still a source of controversy, and due to these pleiotropic effects is outside the scope of this work. Therefore discussion of this subject is limited to acknowledging the role of Nramp1 in the generation of the macrophage oxidative burst.

1.7.4 Nramp1 and trafficking

Recent studies indicate that Nramp1 has a role in altering trafficking within the macrophage. Many *Mycobacterial* species survive intracellularly by prematurely arresting the process of phagosomal maturation, allowing them to reside within vacuoles with attenuated acidity in order to promote their survival (67). However, this inhibition of phagosome-lysosome fusion appears to be reduced or abrogated in the presence of Nramp1, as lysosome fusion (measured by presence of acid phosphatase on the phagosome), was twice as frequent in Bcg^R than in Bcg^S macrophages (66). Further

studies demonstrated that phagosomes containing live *M. bovis* BCG displayed attenuated V-ATPase-dependant acidification in *Nramp1*$^{-/-}$ macrophages (pH 6.5) but acidified normally in wildtype macrophages (pH 5.5). This also correlated with increased fusion of the *Mycobacterial* phagosome with late endosomes/lysosomes as determined by LAMP2-acquisition (112). Nramp1, therefore, appears to bypass the arrest in phagosomal maturation normally observed in susceptible strains by enhancing fusion of the *Mycobacterial* phagosome with late endosomes/lysosomes, leading to increased V-ATPase activity at the phagosomal membrane, and thus enhanced phagosomal acidification.

STM is also known to alter vacuolar trafficking pathways during infection of *Nramp1*$^{-/-}$ macrophages, by diverting NADPH oxidase from the SCV, becoming isolated from extracellular fluid phase markers and not acquiring M6PR, a late endocytic marker. It was recently shown that in the presence of wildtype Nramp1, isolation of the SCV from normal trafficking pathways does not occur, demonstrated by acquisition of M6PR (60). Therefore in both *Mycobacterial* and *Salmonella* infection models, Nramp1 appears to alter the trafficking patterns of bacteria-containing vacuoles. As a result, these vacuoles are no longer secluded from normal lysosomal trafficking and are subject to the full battery of bactericidal agents present in the macrophage. It is possible that such exposure to the defenses of the macrophage would restrict replication of these pathogens in a manner sufficient to demonstrate the Nramp1-mediated bacteriostasis.

1.7.5 The *Nramp* gene family and divalent cation transport

NRAMP2

NRAMP2, a ubiquitously expressed gene located on human chromosome 12q13, encodes a 12 TMD protein that is highly homologous to *NRAMP1* (66% identity, 80% similarity) (260). The murine homologue, Nramp2, functions as a transporter of divalent

cations including Fe^{2+}, Mn^{2+}, Zn^{2+} and other divalent metals, but not Ca^{2+} or Mg^{2+} (148). Its transport capabilities are pH-dependent with maximum activity at pH 5.5. Although *Nramp2* is ubiquitously expressed, *Nramp2* is markedly upregulated in the proximal intestine following chronic iron depletion, and has recently been described as being upregulated by IFN-γ and LPS (265).

Mutation in *Nramp2* was identified as the cause of iron deficiency in the *mk /mk* mouse model of microcytic anemia (84), as well as the cause of hyperchromic anemia in the Belgrade (*b/b*) rat (83). Both animals demonstrate decreased or deficient uptake of iron from the gastrointestinal tract, and both carry the same mutation in *Nramp2*: a guanosine to adenosine transition causing a glycine to arginine substitution (G185R) in TMD4 of the predicted protein equivalent to the G169D mutation in the murine *Nramp1* allele (83, 245). Nramp2 has been localized to the brush border at the apical membrane of enterocytes in the proximal duodenum of mice (45), previously identified as being the site of iron uptake in the intestine. Thus, Nramp2 is believed to be responsible for the intestinal absorption of non-heme iron.

However, iron acquisition was also found to be decreased in peripheral cells and tissues in the *mk /mk* and *b/b* animals (78). The anemia can not be corrected by direct iron injections (19), suggesting that there is a second block of iron entry into peripheral tissues. Nramp2 was further identified as being an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes (104). Recent studies found that iron release from transferrin inside the endosome was normal in *mk/mk* reticulocytes (46), suggesting that these cells have a further defect in Fe^{2+} transport across the endosomal membrane. Nramp2 was subsequently found to colocalize with the transferrin receptor in red blood cells of these mice (46). This, along with the pH dependence of Nramp2 transport, strongly suggest that Nramp2 is responsible for transporting Fe^{2+} into the

cytoplasm after acidification of the transferrin-positive endosome. Lack of Nramp2 results in disrupted iron transport (83), due to alteration of function of Nramp2 and not to degradation of the protein (245). Thus, overall, Nramp2 is involved in dietary iron uptake from the duodenum and iron acquisition from transferrin in peripheral tissues.

Recently Nramp2 has been investigated for a possible role in inflammatory bowel disease (IBD), a syndrome which includes Crohn's disease and ulcerative colitis. *NRAMP2* is located within a 40 cM region on the long arm of chromosome 12 that was found to contain a susceptibility locus for IBD (244). As a result the frequency of four *NRAMP2* polymorphisms was assessed in a group of 155 Crohn's disease patients, 114 ulcerative colitis patients and 189 healthy controls (244). No evidence for linkage was found between *NRAMP2* and IBD, Crohn's disease or ulcerative colitis, and as a result *NRAMP2* does not appear to be involved in IBD.

Nramp *homologues*

Nramp homologues encoding conserved proteins have been identified in many phylogenetically distant organisms, including mammals, birds, fish, insects, nematodes, plants, fungi, yeast and bacteria ((206) and references therein). The degree of sequence conservation among these Nramp1 family members is suggestive of parallel conservation of function. The first homologues to be identified were the SMF genes in *Saccharomyces cerevisiae*. This yeast is now known to express three homologues of the Nramp family, SMF1, SMF2 and SMF3, which all encode metal transport proteins designated Smf1p, Smf2p and Smf3p which are responsible for transport of various divalent cations. Smf1p is a high-affinity manganese transporter with secondary affinity for cadmium, copper and iron (57, 163, 247). Smf2p can affect cobalt levels in yeast (163) and has also been implicated in manganese trafficking (267). Recently, both Smf1p and Smf2p have been shown to stimulate iron uptake into *Xenopus* oocytes (52). *SMF3* mutant yeasts show

symptoms of iron starvation, and, therefore, it is hypothesized that Smf3p may mobilize vacuolar stores of iron during iron starvation conditions (206). Accumulated evidence thus indicates that the *S. cerevisiae* Nramp homologues are involved in metal ion transport and homeostasis.

The *Drosophila melanogaster* homologue of *Nramp1*, known as *malvolio* (*mvl*) is involved in taste discrimination behavior of the fly. While wildtype flies will avoid media containing 100 mM sodium chloride and strongly favor media containing sugars, *mvl* flies show an increased acceptance of salt, and a greatly reduced preference for sugars. However, when the flies are fed with media supplemented with Fe^{2+} or Mn^{2+}, normal taste behavior is restored (213). *malvolio* flies were also raised from egg to adulthood on media containing elevated concentrations of metal ions. Mutant flies reared in the presence of 10 mM $MnCl_2$ or $FeCl_2$ developed into adults with recovered taste behavior; moreover, exposure of adult mutant flies also recovered normal taste behavior if exposed to these ions 2 h prior to testing taste behavior (197). Therefore, these data suggest that Mvl functions as a Mn^{2+}/Fe^{2+} transporter, and that one or both of these ions are involved in the signal transduction of taste perception in *Drosophila* adults (197). Further, expression of the human NRAMP1 protein in *D. melanogaster* in normal flies did not lead to any alterations in their behavior or physiology. However, in the *mvl* mutant flies, ubiquitous expression of *NRAMP1* totally rescued the typical taste defect. This result further supports a similar function of Nramp1 and Mvl (62).

Nramp1 homologues have also been identified in various bacteria, including *Mycobacterium tuberculosis*, *E. coli* and STM (69, 144). The M. *tuberculosis* homologue (previously Mramp; now MntH) was found to be pH-dependent and to transport zinc, iron, manganese and copper (3). Loss of function of the *M. tuberculosis* homologue did not affect virulence of this pathogen in a murine model of tuberculosis or intracellular

survival in bone marrow-derived macrophages (69). In contrast, loss of function of the STM MntH results in a modest attenuation in *Nramp1*$^{-/-}$ animals, but a marked attenuation in *Nramp1*$^{+/+}$ animals (this work). The STM and *E. coli* homologues have been shown to be proton-stimulated, highly selective manganese transporters with nM affinity for Mn^{2+} and Cd^{2+} and μM affinity for Fe^{2+} (144).

Plant homologues of Nramp1 have also been identified, including the rice (*Oryza sativa*) (OsNramp) and *Arabidopsis thaliana* homologues (AtNramp). *A. thaliana* has five homologues identified to date (61, 250), while three OsNramp homologues have been identified (26). Sequence comparison indicates that there are two classes of Nramp proteins in plants, class 1 consisting of AtNramp1, OsNramp1 and OsNramp2, and class 2 consisting of AtNramp2-5 and OsNramp3 (61). Class 1 homologues are capable of restoring growth to *fet3fet4 S. cerevisiae*, indicating that they complement the yeast iron –uptake function of *fet3* and *fet4*, whereas class 2 homologues do not complement (61). There is some evidence indicating that the AtNramp proteins may also be involved in transport of the toxic metal cadmium (250). Therefore it appears that at least some members of the plant Nramp family are involved in metal transport. Overall, Nramp proteins from phylogenically distant organisms from mammals to bacteria share a common function of divalent metal transport, presumably the major role of this family of conserved proteins.

1.7.6 *NRAMP1* and human disease

Association with bacterial infectious diseases

The *Nramp1* gene is evolutionarily conserved, with a homologue in humans designated *NRAMP1*. Although the G169D functional null mutation has not been found in human or other mammalian *NRAMP1* homologues, several promoter polymorphisms have been identified and linked with various disease states. Four promoter

polymorphisms within a possible enhancer element containing a Z-DNA forming dinucleotide repeat were initially identified (33, 224), with an order of descending frequency of allele 3>2>1≈4. In the absence of exogenous stimuli, alleles 1, 2 and 4 are poor promoters, while allele 3 drives high expression of *NRAMP1*. All four alleles were equally responsive to upregulation by IFN-γ, but LPS stimulation had no effect on alleles 1 and 4, caused a reduction in expression of driven by allele 2, and enhanced expression driven by allele 3 (224). Allele 2 has been associated with susceptibility to tuberculosis (25, 34), while allele 3 is associated with susceptibility to autoimmune diseases including the development of rheumatoid arthritis (221, 225). These potentially detrimental alleles may be retained in the human population because they carry an associated benefit: increased disease resistance with allele 3 and protection against autoimmune disease for allele 2 (224).

The association of human *NRAMP1* polymorphisms with disease susceptibility has been most extensively studied with the *Mycobacterial* diseases leprosy and tuberculosis. *NRAMP1* polymorphic variants were found to be associated with susceptibility to tuberculosis in a number of studies taken around the globe including a Gambia, West Africa (25), Guinea-Conakry (48), Canada (100), Korea (218), and Japan (92). In contrast to these positive studies, one study of a Brazilian population showed no linkage of any *NRAMP1* polymorphisms to susceptibility to tuberculosis (34), while another Brazilian-based study concluded that *NRAMP1* plays only a minor role in development of tuberculosis in this population (226).

Although *NRAMP1* alleles do not contribute to susceptibility to leprosy in certain populations analyzed (119, 214), positive results were obtained in a large study of susceptibility to leprosy in South Vietnam (5) and an unrelated study of 168 members of 20 leprosy families (2). Another interesting study of 273 patients with leprosy and 201

controls from Mali found that, although there was no evidence of association of *NRAMP1* polymorphism with leprosy per se, an *NRAMP1* 3′ UTR deletion allele was significantly associated with leprosy type (172). Therefore it appears that *NRAMP1* may have an influence on the clinical presentation of leprosy, if not completely on susceptibility itself, speculatively at the level of influencing cellular immune response type (172).

Effects of promoter polymorphism on human susceptibility to *Salmonella*-based diseases have been far less studied. Only one study has been conducted to date on correlation between polymorphisms and susceptibility to typhoid fever in populations in southern Vietnam (72). No allelic association was identified between 6 different *NRAMP1* alleles and susceptibility to typhoid fever. In addition, neither homozygotes nor heterozygotes for any *NRAMP1* variants were at increased risk of typhoid fever. However, the authors speculated that a polymorphism may play a role in the development of more serious sequelae of the disease. Studies investigating *NRAMP1* and susceptibility to leishmanial diseases in humans have been similarly negative (34, 167).

Association with other diseases

NRAMP1 has been studied for its association with a number of different human diseases. A number of studies support the hypothesis that Nramp1 is involved in some manner in the development of rheumatoid arthritis or juvenile rheumatoid arthritis (222, 230). Nramp1 has also been hypothesized to be involved in development of multiple sclerosis (151) and diabetes (75, 124), although further study is necessary before drawing further correlations between this host resistance factor and these unrelated disease states.

However, the most strenuously studied disease linked to *NRAMP1* is inflammatory bowel disease (IBD). IBD includes Crohn's disease and ulcerative colitis, and may be caused by a hyperactive immune response to normal intestinal flora. Originally one study found an association of a region spanning *NRAMP1* was present in

15% of patients with Crohn's disease but was only found in 5% of unrelated controls (126). However, when the analysis was done using alleles of *NRAMP1*, no significant association was observed, and a separate study found no association between *NRAMP1* alleles and any form of IBD (243). However, recently a Japanese group found that a novel promoter polymorphism of *NRAMP1* was significantly associated with IBD in a Japanese population (149). Therefore there may also be a link between *NRAMP1* and IBD.

1.8 Summary of thesis

The aim of this work was to study the potential interaction between the host resistance protein Nramp1 and STM virulence gene expression in the murine typhoid model. Two sets of STM virulence genes were studied, namely the genes encoding the STM Mn^{2+} transport systems, *mntH* and *sitA* (*mntH* being homologous to Nramp1), and SPI2 genes associated with intramacrophage survival. Monocopy transcriptional fusions to the genes of interest were created to study the expression of these genes in axenic cultures, in *in vitro* cell culture models, and upon recovery of viable bacteria from the spleens of infected mice.

mntH and *sitA* were expressed in the intracellular environment, but *mntH* appears to be most responsive to pH similar to that found in the SCV and may represent the primary Mn^{2+} transport system for STM residing within its intracellular niche. Although *sitA* alone was required for virulence of STM in *Nramp1*$^{-/-}$ mice, both transporters were essential for full virulence of STM in *Nramp1*$^{+/+}$ mice, demonstrating an interdependence between the genotypes of both host and pathogen. The SPI2-associated genes chosen for study were also upregulated in the presence of Nramp1 *in vitro* and *in vivo*. Further study correlated this upregulation with the deprivation of Fe^{2+} from the environment, consistent with the role of Nramp1 as a divalent cation transport system. It is possible that this

upregulation of genes required for intramacrophage survival in response to cation deprivation mediated by Nramp1 functions as a mechanism for STM to remain viable and capable of manifesting chronic infection of an *Nramp1*$^{+/+}$ host.

Therefore by introducing the variable of a host resistance protein into the currently accepted murine typhoid model we were able to gain significant insights into the STM-host interaction. This demonstrates that currently accepted model systems can be expanded or altered and still yield novel insights into host-pathogen interactions.

CHAPTER 2: MATERIALS AND METHODS

2.1 Strains and Plasmids

2.1.1 Bacterial strains and growth conditions

Bacterial strains used and/or constructed during this study are listed in Table 1. The bacterium *S.* Typhimurium (STM) 14028s and SL1344 were the wildtype bacteria used for experiments studying SPI2 or MntH and SitA, respectively. Luria-Bertani (LB) broth (29) and agar plates were used for growth and maintenance of bacterial strains. N minimal media (NMM), pH 7.4 was used for *in vitro* expression studies (68) and was supplemented with 0.3% glycerol, 0.1% casamino acids and 8 μM $MgCl_2$ (augmented to 200 μM $MgCl_2$.when working with *phoP*-deficient strains). When NMM was required at pH 4.5, MES (4-morpholine ethane sulfonic acid monohydrate) replaced Tris as the buffering system. When required, compounds were added at the final concentrations unless otherwise noted: ascorbate, 5 mM; DiP, 250 μM; DTPA, 250 μM; EDTA, 250 μM; EGTA, 250 μM; $MnSO_4$, 500 μM; $MgSO_4$, 500 μM; $FeSO_4$, 500 μM; $ZnSO_4$, 500 μM; ampicillin (Ap), 100 μg/mL; carbenicillin (Cb), 100 μg/mL; chloramphenicol (Cm, 30 μg/mL); gentamicin (Gm), 10 or 100 μg/mL; kanamycin (Km), 50 μg/mL; streptomycin (Sm), 25 μg/mL; tetracycline (Tc), 30 μg/mL; Xgal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside), 50 μg/mL.

2.1.2 Cell lines

The human cervical carcinoma cell line HeLa (ATCC CCL-2) and the *Nramp1*$^{-/-}$ murine macrophage-like cell line RAW 264.7 (ATCC TIB 71) were used as the non-phagocytic and phagocytic cell lines, respectively. Stably transfected RAW cell lines were obtained from Philippe Gros (98) and contained either an empty neomycin-resistant pCB6 vector (phenotypically Nramp1-) or pCB6 containing the wild-type murine *Nramp1* gene under the direction of a constitutive promoter (Nramp1+). The desired

plasmids were maintained by the addition of geneticin (G418; Gibco) at 200 μM to the growth media; expression of Nramp1 was periodically confirmed by immunofluorescence as previously described (98). These cultured cell lines were grown in MEM (minimal essential media (Gibco); HeLa cells) or DMEM (Dulbecco's minimal Eagle's medium (Gibco); RAW cells) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2, and were passaged by trypsinization (HeLa) or by scraping (RAW) as previously described (98, 240). 0.05% Trypsin (Gibco) was stored at –20°C and used undiluted during the passage of epithelial cells. Dimethyl sulfoxide (DMSO) (BDH Inc.) was used as a cryoprotectant at a final concentration of 10% when freezing cultured cells. When required DMSO (0.1%), DPI (diphenylene iodium chloride; 2 μM) or PMA (phorbol myristyl acetate; 5 nM) were added to activated RAW cells for 30 min prior to infection and maintained throughout the course of the experiment.

2.1.3 Plasmids

Plasmid vectors used as cloning and expression vehicles and their important characteristics are listed in Table 2. Plasmids used or constructed during the course of this work are listed in Table 3 and are described in the text where appropriate. The presence of DNA inserts in vector DNA was verified by agarose gel electrophoresis. Except where indicated, recombinant plasmids were isolated from *E. coli* DH10B and transferred to specific STM strains using KR1562 as an intermediate host.

Table 1. STM strains

Strain	Genotype; Remarks	Reference
LT2	wildtype lab strain; avirulent	Lab collection
KR1562	LT2 *metA22 metE55 galE496 rpsL120 xyl-404 (ilv) hsdL6 hsdSA29, pSLT-free*	Lab collection
14028s	wildtype	Lab collection
CS015	14028s *phoP*::Cm	(203)
CS022	14028s *pho*-24	(203)
CS382	14028s *ssrA*::Km	(174)
JSG421	14028s *pmrA*::Tn10	(110)
MZ201	14028s *fur*::Km; derived by P22 trandscuction from MM2646	This work
MZ202	14028s *ompR*::Tn10; derived by P22 transduction from SWL350	This work
MZ203	14028s *mntR*::Cm; derived by P22 transduction from MM2645	This work
SL1344	wildtype	(127)
ΔssaR	SL1344 *ssaR*	Lab collection
MM2165	SL1344 *mntH*::Km	(144)
MM2507	SL1344 x pMLZ104	(144)
MM2524	SL1344 *mntH*::Km *sitA*::Cm	This work
MM2529	SL1344 *oxyR*::Tn10 x pMLZ104	This work
MM2562	SL1344 *sitA*::Cm	(143)
MM2616	SL1344 x pDGK261	This work
MM2617	SL1344 x pDGK262	This work
MM2618	SL1344 x pDGK263	This work
MM2645	SL1344 *mntR*::Km x pMLZ104	This work
MM2646	SL1344 *fur*::Km x pMLZ104	This work
MZ204	SL1344 *hilA*::Km	This work
MZ205	SL1344 *phoP*::Cm; derived by P22 transduction from CS015	This work
MZ206	SL1344 *ssrA*::Km; derived by P22 transduction from CS382	This work
MZ207	SL1344 *pmrA*::Tc; derived by P22 transduction from JSG421	This work
SWL350	SL1344 *ompR*::Tc	Lab collection

Table 2. Plasmid vectors.

Plasmid vector	Relevant properties	Source/Ref.
pFZY1	Low-copy number transcriptional fusion vector containing a multiple cloning site facilitating in-frame *lacZY* fusions; Ap^R; 11.3 kbp	(150)
pBluescriptII SK	High copy number phagemid designed for sequencing, used here as a cloning vector; Ap^R; 2.9 kbp	Stratagene
pCR2.1	High-copy number cloning vector used for rapid ligation of PCR-amplified fragments by TA-cloning; Ap^R; 3.9 kbp	Invitrogen
pACYC184	Low-copy number cloning vector; Cm^R, Tc^R; 4.2 kbp	NEB

Table 3. Plasmid constructs.

Recombinant plasmid	Parental plasmid; comments
pDGK227	pBluescript; contains full-length *mntH* gene and promoter region
pDGK261	pMLZ104; deleted for the OxyR binding motif in the *mntH* promoter region
pDGK262	pMLZ104; deleted for the Fur binding motif in the *mntH* promoter region
pDGK263	pMLZ104; deleted for the MntR binding motif in the *mntH* promoter region
pHILA	pFZY1; contains 1.8 kbp fragment of the *hilA* promoter region extending into coding sequence for gene expression analysis
pMLZ104	pFZY1; contains 780 bp fragment of the *mntH* promoter region and coding region isolated as an *Eco*RI-*Hind*III fragment from pDGK227 generating an *mntH*::*lacZY* transcriptional fusion for gene expression analysis
pPHOP	pFZY1; contains 420 bp fragment of the *phoP* promoter region extending into coding sequence for gene expression analysis
pSSRA	pFZY1; contains 790 bp fragment of the *ssrA* promoter region extending into coding sequence for gene expression analysis
pSSEA	pFZY1; contains 500 bp fragment of the *sseA* promoter region extending into coding sequence for gene expression analysis
pSSEJ	pFZY1;contains 736 bp fragment of the *sseJ* promoter region extending into coding sequence for gene expression analysis
pSITA	pFZY1; contains 670 bp fragment of the *sitA* promoter region extending into the coding sequence for gene expression analysis

2.2 Genetic Techniques

2.2.1 Generalized transduction methods

Bacteriophage lysates were derived from P22 HTK. P22 transducing phage lysates were prepared from 1.5 mL mid-log phase cultures infected with phage at a multiplicity of infection (MOI) of 0.01. Cultures were grown overnight at 37°C, with aeration. In the a.m. cultures were chloroform-treated to lyse remaining cells and release internalized phage; cells and cell debris were removed by centrifugation at 14 krpm in a benchtop Eppendorf centrifuge, and supernatants sterilized by the addition of a saturating volume of chloroform. Transductions were achieved by mixing log-phase cells (approx. 10^9 cells/mL) grown in LB with phage lysate added at an MOI of approximately one. Mixtures were incubated at 37°C with aeration for 20 min before plating to selective media. EGTA was added to media at a final concentration of 10 mM to prevent lysogeny of transductants. When necessary, P22 transductants were screened for lysogeny by cross-streaking against phage H5.

2.2.2 DNA isolation

Plasmid preparation

Plasmid preparations were carried out using Qiagen plasmid isolation kits (Qiagen Inc.) as per the manufacturer's instructions. Bacterial plasmid isolations were normally carried out using 2 mL of overnight LB culture; isolation of pFZY1-based plasmids required plasmid isolation from a total volume of 5 mL of overnight culture.

Boiling method of isolation of chromosomal DNA for PCR

A 2 mL culture was grown in LB+desired antibiotic to saturation at 37°C with aeration. The entire culture was centrifuged at 14 krpm in a benchtop Eppendorf centrifuge. The bacterial pellet was washed once in 1 mL dH_2O and centrifuged again.

The pellet was ultimately resuspended in 200 μL dH_2O and boiled for 5 min in a 95°C heating block. RNaseA was added to a final concentration of 50 ng/μL and the preparation incubated on ice for 5 min prior to centrifugation as before. 150 μL of the resulting supernatant was transferred to a new tube, and 3 μL used per 50 μL PCR reaction.

Isolation of restriction digest fragments from agarose.

The isolation of restriction digest fragments was carried out using the Qiagen Qiaquick Gel Extraction Kit according to manufacturer's instructions.

2.2.3 Electroporation

Preparation of electrocompetent bacteria

Bacteria were grown overnight in LB broth at 37°C with aeration. 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 the desired density, the culture 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 centrifuged at 10 krpm for 10 min in a Beckman Model J2-21 centrifuge. The pellet was suspended in 1 volume of chilled 10% glycerol and centrifuged as before. Subsequent washing of the bacteria was carried out in reduced volumes of 10% glycerol (1:2, 1:50, 1:100, 1:500) in order to decrease the ionic strength of the sample and concentrate the bacteria to approx. 10^9 to 10^{10} bacteria per 40 μL aliquot. Aliquots were placed into Eppendorf tubes and frozen at –80°C.

Electroporation

Electrocompetent bacterial aliquots were thawed on ice immediately prior to use. 2 μL of DNA sample was added to the electrocompetant bacteria and incubated on ice for

5 min. The sample was then transferred to a pre-chilled 0.2 cm electroporation cuvette and using the Gene PulserTM from BioRad (Richmond, CA), the sample was pulsed at 2.5 kV with a 25 μF capacitance and either 200Ω or 400Ω parallel resistance for *E. coli* or *Salmonella*, respectively. Samples were immediately diluted in 1 mL of LB broth and incubated for 1 h at 37°C with aeration. Following recovery, the sample was plated onto selective medium. For pFZY1-based derivatives, the entire sample was centrifuged to concentrate the transformed bacteria and the entire aliquot plated to selective medium to maximize recovery of transformants.

2.2.4 Recombinant DNA methods

Recombinant DNA methodology was based on the laboratory manual of Sambrook *et al.* (220). Procedures drawn from this manual include: restriction endonuclease digestion and ligation of DNA; conversion of 5′ overhangs to blunt ends; and agarose gel electrophoresis. Restriction enzymes were purchased from NEB Biolabs and were used according to manufacturer's instructions. CIP (calf intestinal phosphatase; NEB) was used to remove 5′ phosphates from digested DNA to prevent ligation of digested vectors. T4 DNA ligase and deoxynucleotides (dNTPs) were purchased from Boehringer Mannheim Canada.

2.3 PCR methods

2.3.1 Reagents

dNTPs were made as a 2.5 mM stock solution and stored at –20°C with all four dNTPs in equal proportions. AmpliTaq Gold DNA polymerase (Perkin-Elmer Biosystems) was used for PCR except where noted.

2.3.2 Oligonucleotides

DNA oligonucleotides were made by Sigma or by the Nucleic Acid and Protein Services (NAPS) Unit at the University of British Columbia. Oligonucleotides for PCR amplification for cloning and sequencing are listed in Table 4. The oligonucleotides pFZY1FW and pFZYRV were made to regions within the transcriptional fusion plasmid pFZY1 for sequencing. All other oligonucleotides were made to regions of the STM chromosome.

2.3.3 Amplification of desired loci

Amplification of genes by PCR was achieved using a standard touch-down PCR method. Briefly, PCR reactions were prepared following the manufacturer's instructions for the DNA polymerase used (conditions described are specifically for AmpliTaq Gold). Cycling conditions involved an initial 10 min heat activation of the DNA polymerase at 94°C, followed by five cycles of denaturation at 94°C for 30 sec; annealing at 50°C for 30 sec and extension at 72°C for 2 min. Touch-down cycling involved 15 cycles of denaturation at 94°C for 30 sec, annealing beginning at a target temperature of 65°C for 30 sec and decreasing by 1°C increments per cycle, and ending with a 2 min extension at 72°C. Final amplification involved 25 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 2 min. A final 10 min extension at 72°C was added for ease of TA-cloning.

2.3.4 Sequencing

For sequencing reactions, ABI's AmpliTaq Dye Terminator Cycle Sequencing chemistry with FS Taq was used following instructions of the NAPS Unit at UBC. For sequencing, 500 ng of purified plasmid template DNA was added to 3.2 pmol of oligonucleotide and mixed with 4 μL version 3 Big Dye terminator premix for a final volume of 20 μL. Each sequencing reaction consisted of 25 cycles of 96°C for 30 sec,

Table 4. Oligonucleotides for PCR and sequencing.

Designation	Length (nt)	Sequence 5′ to 3′; engineered restriction sites
ssrAFW	21	CACAGGC**GAATTC**TATCATTC; *Eco*RI
ssrARV	22	GTATAAAGGA**AAGCTT**TTTCGC; *Hind*III
phoPQE1	27	TCGAC**GAATTC**TTAAATAATGCCTGCC;*Eco*RI
phoPRH3	28	ATGTGGAATG**AAGCTT**CGTCACGTAGTC; *Hind*III
sseAFW	23	GTAAA**GAATTC**TTAGAGCCTATC; *Eco*RI
sseARV	22	TCTTTA**AAGCTT**CTCGGCCTCC;*Hind*III
hilA4FW	25	GTCCAGATGACACTATCTCCTTCCG
hilART1	20	CAGAATGCTACCTCAGCATG
sifCreg5′	30	AGC**GAATTC**CTGAGTATCAAGCCAGCTCAT;*Eco*RI
Related2-6-3′	25	AGTCATATAGTCATTAGCCCCCAAT
sseJFW	20	AT**GTCGAC**TAAAACACTAGC; *Sal*I
sseJRV	22	**CTCGAG**TGGAATAATGATGAGC; *XhoI*
sitAFW	21	CACGCGCGATACGTTTACCAG
sitARV	22	CG**AAGCTT**CGGTAATGCCCATC; *Hind*III
pFZY1FW	20	TATCACGAGGCCCTTTCGTC
pFZRV	21	CATAAATTTACGTACGGTGGC
M13RV	17	CAGGAAACAGCTATGAC
M13FW-20	16	GTAAAACGACGGCCAG

50°C for 15 sec and 60°C for 4 min. Centrisep spin columns (Princeton Separations; Philadelphia, NJ) were used to remove unused dinucleotides from the completed sequencing reactions. Sequencing gels were run by the NAPS Unit. Sequences were analyzed using NCBI's Pairwise BLAST program and the GenBank database.

2.4 Invasion and Replication assay

Invasion assays were carried out using a modified version of the gentamicin protection assay as described by Tang *et al.* (248). Sterile 24-well plates were seeded with cultured cells either 48 h in advance with 1 mL of $2x10^5$ cells per mL (RAW cells) or 24 h in advance with 1 mL of $1x10^5$ cells/mL (HeLa cells). When using RAW cells, 24 h prior to infection the cells were activated by addition of 200 U/mL of IFN-γ. A 1 mL saturated culture of the desired bacteria in LB+desired antibiotic was grown at 37°C with aeration. Subsequently the bacteria were subcultured by diluting 300 µL of the saturated culture into 10 mL of fresh LB and grown for 3 h as before. 1 mL of this 3 h subculture was diluted 1:10 in PBS^{++} and OD_{600} values obtained for normalization. 50 µL of OD-normalized cultures were used to infect each well of the cultured cells for 30 min at 37°C in a CO_2 incubator. Following internalization of the bacteria, the mammalian cell monolayers were washed with PBS^{++} (i.e. PBS containing calcium and magnesium) and incubated with $Gm_{50\ \mu g/mL}$ (HeLa) or $Gm_{100\ \mu g/mL}$ (RAW) to kill any remaining extracellular bacteria. Where bacterial growth within HeLa cells was studied for longer than 2 h, the gentamicin concentration was reduced to 5 µg/mL. Where bacterial growth within RAW cells was studied for longer than 6 h, the gentamicin concentration was reduced to 10 µg/mL. At desired time points the monolayers were lysed with 500 µL of PBS^{++} containing 1% Triton X-100 and 0.1% SDS. Dilutions of bacteria were made in PBS^{++} and plated onto LB agar for enumeration of bacterial colony forming units (cfu). All

counts were obtained from duplicate wells within triplicate experiments, and error bars represent the standard error of the mean.

2.5 Reporter gene assays

2.5.1 Chemiluminescent β-galactosidase assays

50 μL of the bacterial suspension released from the host cell monolayers was lysed by the addition of 20 μL of chloroform. Tropix Inc. Galacton-Star chemiluminescent β-galactosidase substrate kits were used according to the manufacturer's instructions and incubated in Dynex Microlite-1 96-well plates for 30 min at room temperature. Plates were read using the luminescence detection function of the Spectrafluor Plus (TECAN, Austria) and data were evaluated using Magellan software, version 1.1 (TECAN, Austria). Light emissions were obtained as Relative Light Units (RLU) and are normalized to the number of bacteria present in each sample as determined by the bacterial enumeration counts, being expressed as RLU per viable bacterium. Graphs denote light emission as RLU per 10^6 bacteria in an effort to more clearly demonstrate the observations. Background β-galactosidase activity was eliminated by blanking readings to wells containing wild-type STM with no *lacZY*-expressing plasmid.

2.5.2 Gene expression assay, cultured cell lines

Intracellular gene expression assays were carried out with a modified version of the invasion assay described above. Briefly, the strains of interest were grown to saturation in 1 mL of LB broth plus desired antibiotic in culture tubes, with shaking at 200 rpm, subcultured at a 1:100 dilution in new, antibiotic-free LB broth and grown as before for a further 3 h. Naïve or stably transfected cultured RAW 264.7 cells were seeded at a density of 2x 10^5 cells/mL into 96 well plates 48 h prior to infection, allowed to adhere and activated with murine IFN-γ (Genzyme) as before. For the purposes of this study,

"activation" is defined as the exposure of macrophages to murine IFN-γ prior to infection. These activated cells reached approximately 90% confluency prior to infection and were infected with the bacterial strains of interest at an MOI of approximately 50-100:1. Bacteria were allowed to invade the cells for 30 min at which time the extracellular bacteria were removed and incubated in parallel in a separate 96 well plate. The monolayers were then washed with PBS^{++}, incubated with Gm and lysed as above. When free bacteria were being evaluated, the PBS+Triton+SDS was added directly to the growth media. The resulting lysate was split in half, 50 μL being transferred to serial 10^{-1} dilution blanks for bacterial enumeration on LB agar, and the other 50 μL used for determination of β-galactosidase activity as described above. Assays were performed on five independent occasions in duplicate and all results were averaged.

2.5.3 Gene expression assay, axenic cultures

Axenic bacterial cultures were grown to saturation in basic NMM plus the desired antibiotic at 37°C with shaking. These cultures were then subcultured at a 1:100 dilution to fresh, antibiotic-free NMM supplemented as described in the text. The subcultures were grown for three hours as before and assayed for bacterial enumeration and β-galactosidase activity as described above. Assays were performed in triplicate on three separate occasions and all results were averaged.

2.6 Mouse studies

2.6.1 Murine typhoid model, unrelated mice

Virulence assays were performed on 6-8 week old female BALB/c or C3H/HeN mice, housed in groups of five in cages equipped with individual isolation units. Aerobic bacterial cultures were grown for 3 h in LB, washed once in PBS^{++} and normalized by OD_{600} reading to contain the desired number of bacteria in 150 μL of PBS^{++}. Mice were

infected by oral gavage with 10 μL of the bacterial suspension for a final dose of $5x10^6$ cfu. Remaining mice were euthanized 28 days after infection. This work was approved by the committee on animal care at the University of British Columbia and Case Western Reserve University, Cleveland, OH.

2.6.2 Murine typhoid model, congenic mice

Virulence assays were performed on 6-8 week-old C57/BL6 or congenic $Nramp1^{Gly169}$ derivatives (gift of B. Zwilling, Ohio State University) by the laboratory of Dr. Ferric Fang, University of Washington. Wildtype STM SL1344 or isogenic *sitA*::Cm, *mntH*::Km, or *sitA*::Cm *mntH*::Km double knockout strains were diluted in 200 μL of PBS and administered intraperitoneally to groups of five female mice. Inocula were determined by dilutional plating to range from 400-800 cfu for the C57/BL6 mice and from 1800-3100 cfu for the more *Salmonella*-resistant $Nramp^{Gly169}$ mice. Infected animals were monitored daily for survival until the experiment was terminated 28 days after infection.

2.6.3 *In vivo* gene expression assay

Bacterial strains were grown to saturation in 1 mL of LB+desired antibiotic at 37°C with shaking. The saturated culture was diluted 1:100 in 10 mL of antibiotic-free LB and grown for a further 3 h. Strains were all normalized to an OD_{600} of 0.3, resuspended in PBS^{++} and diluted 1:6000 for inoculation. Age- and sex-matched 129sv *Nramp1*$^{-/-}$ and *Nramp1*$^{+/+}$ mice between the ages of 6-18 weeks were infected intraperitoneally with 300 μL of the desired strains for an infectious dose of approximately $2.5 x10^5$ bacteria per mouse as determined by bacterial plate counts. At 24 h post-infection the mice were sacrificed and their spleens harvested. Splenic tissue was homogenized in 2 mL PBS^{--} containing a complete EDTA-free protease inhibitor cocktail (Roche) and then centrifuged to concentrate the homogenate. Half of the homogenate

was used for bacterial enumeration and the other half for β-galactosidase assays as described above. Background splenic β-galactosidase activity was taken into account by infecting control mice with wild-type STM containing no *lacZY* fusion and blanking all further readings to this control. Assays were repeated on four separate occasions with groups of five mice.

2.6.4 Splenic bacterial enumeration assay

Bacterial strains were grown for 16 h in 10 mL LB with the desired antibiotic and diluted 100 μL into 11.9 mL of PBS^{++} and normalized to an OD_{600} of 0.04. Normalized cultures were further diluted 100 μL into 11.9 mL of PBS^{++} to obtain an approximate bacterial concentration of $3.33x10^5$ cfu/mL. Age- and sex-matched 129sv *Nramp1*$^{-/-}$ and *Nramp1*$^{+/+}$ mice between the ages of 6-18 weeks were infected intraperitoneally with 300 μL of the desired strains for an infectious dose of approximately 10^5 bacteria per mouse as determined by bacterial plate counts. At the specified time points of 1, 3 and 4 days post-infection, surviving mice were sacrificed and their spleens harvested. Splenic tissue was homogenized in 2 mL PBS^{++} and serially diluted for enumeration of bacterial cfu on selective media.

CHAPTER 3:ROLE OF *mntH* AND *sitA* IN VIRULENCE OF STM

3.1 Identification of *mntH* and *sitA*, and construction of transcriptional fusions

Upon completion of the sequencing of the STM genome, MntH was identified by Dr. D.G. Kehres, a post-doctoral fellow in the laboratory of Dr. Michael Maguire at Case Western Reserve University, on the basis of its similarity to the murine host resistance protein Nramp1. Since Nramp1 affects murine resistance to STM infection, the possibility that *mntH* may be a bacterial virulence factor in the murine typhoid model was of interest. Analysis of the sequence of the *mntH* promoter region identified two consensus sequences for the known global regulatory systems OxyR and Fur; a novel inverted repeat sequence was also found (eventually called the "MntR box") (144) (Figure 4A). A scan of the STM genome indicated that only one other STM gene appeared to contain this inverted repeat sequence in its promoter region (D.G. Kehres, personal communication): *sitA*, the first gene in an operon located within SPI1 thought to encode a virulence-associated iron-uptake system (135) (Figure 4A). This MntR box was of interest as it may represent a novel element which could be involved in the screening and identification of previously undescribed STM virulence factors.

Therefore, it was decided to investigate the expression patterns of *mntH* and *sitA* in axenic culture and upon infection of host cells. pDGK227, a plasmid containing most of the *mntH* ORF and its entire promoter region, was obtained from Dr. Kehres as part of a collaborative effort between the two laboratories. A monocopy *mntH::lacZY* transcriptional fusion plasmid was constructed by ligation of a 780 bp *Eco*RI-*Hind*III fragment including the *mntH* upstream regulatory region from pDGK227 into similarly digested pFZY1 and subsequent screening by agarose gel electrophoresis to yield pMLZ104 (Figure 4B).

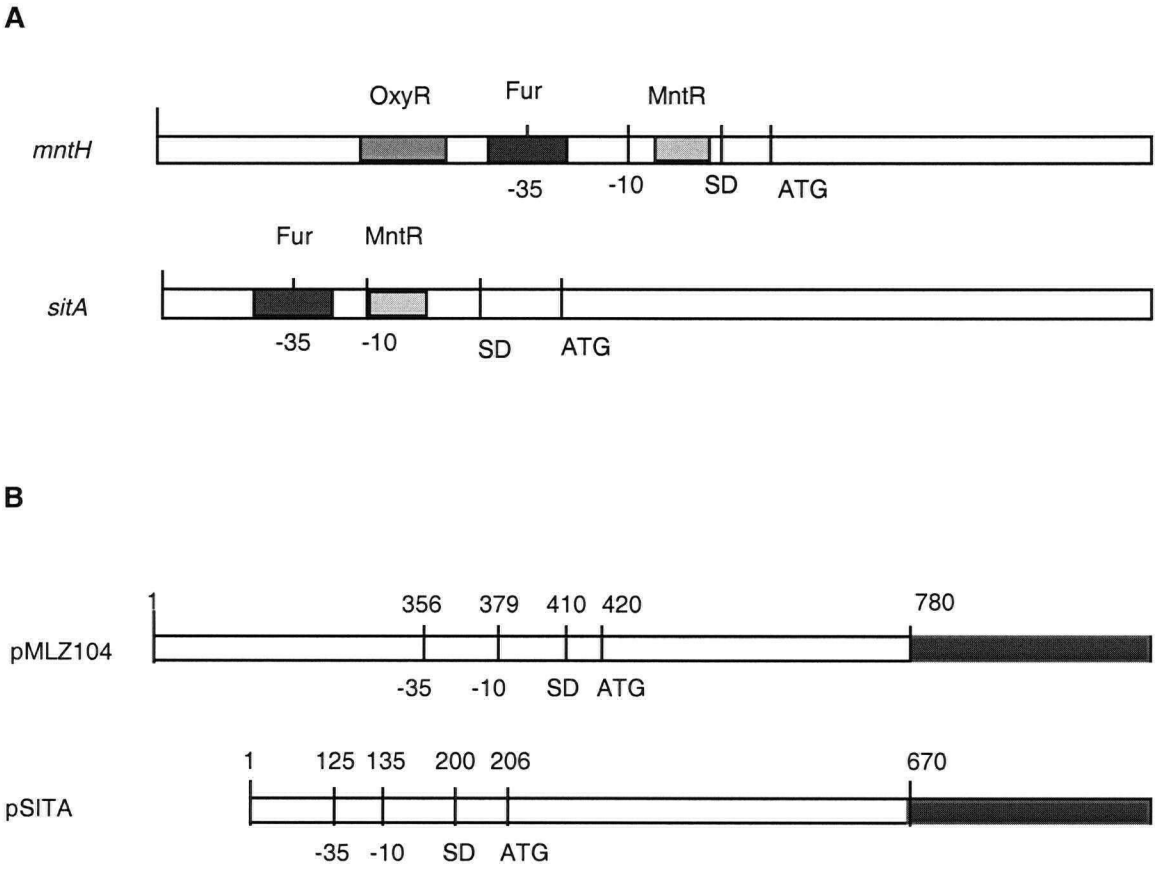


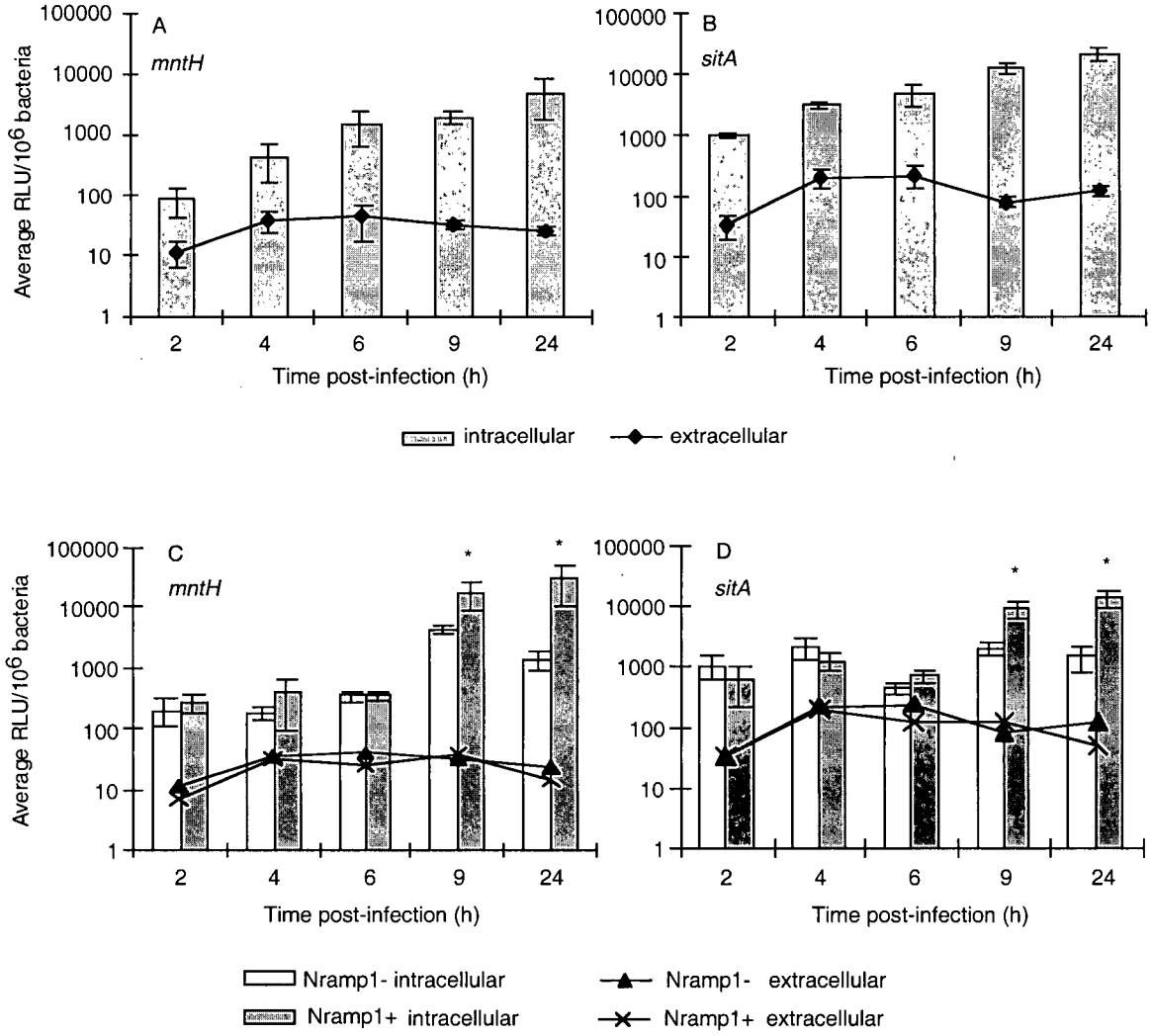
Figure 4. Illustration of the *mntH* and *sitA* promoter regions and transcriptional fusions. **Panel A**: Illustration of the placement of the OxyR, Fur and MntR boxes in the *mntH* and *sitA* promoter regions. The open box represents the cloned DNA sequence of interest. Elements of the promoter regions including –35, -10 and Shine-Dalgarno (SD) consensus sequences are shown. The translational start site of each gene is designated by an "ATG". **Panel B**: Illustration of the *mntH* and *sitA* transcriptional fusions. The open box represents the cloned DNA sequence of interest; the shaded box represents *lacZY* DNA from the transcriptional fusion plasmid pFZY1. Nucleotide positions from the beginning of the cloned insert depicting the placement of promoter elements are drawn above the bar; the corresponding promoter element is written below the bar. Diagram is not drawn to scale.

The transcriptional fusion plasmid, pSITA, was constructed by PCR amplification, direct ligation of the PCR product into pCR2.1, digestion with *Eco*RI and *Hind*III, and subsequent ligation into similarly digested pFZY1 (Figure 4B). This plasmid was used in parallel with pMLZ104 to investigate expression of *sitA* and the role of this novel inverted repeat in expression of *mntH* and *sitA*.

3.2 Expression of *mntH* and *sitA* in RAW 264.7 cells

IFN-γ-activated RAW 264.7 macrophage-like cells (which have a defective *Nramp1* allele and thus do not contain a functional Nramp1 protein) were infected with SL1344 harboring either pMLZ104 or pSITA and expression of *mntH* and *sitA* in internalized STM and extracellular STM was compared. As can be seen in Figure 5A, transcription of *mntH* is markedly induced upon reaching the intracellular environment, being induced 8-fold and 200-fold in comparison to its expression in extracellular bacteria at the 2 h and 24 h time points, respectively. Over time in the intracellular environment *mntH* expression increased a total of 58-fold between 2 and 24 h, while *mntH* expression in extracellular STM only increased a maximum of 2-fold over the same time course. In comparison, *sitA* is also induced following invasion of these macrophage-like cells (Figure 5B). Initial induction of *sitA* is greater than for *mntH*, being 30-fold higher in intracellular bacteria at 2 h compared to extracellular bacteria. However, *sitA* reaches a maximal induction of 170-fold by 24 h post-infection, similar to that of *mntH* at the same timepoint. In spite of its higher initial intracellular expression level, expression of *sitA* does not increase as dramatically as *mntH* over the 24 h time course, increasing only 20-fold between 2 and 24 h while inside cells. However, its extracellular expression levels are similar to *mntH*, reaching a 4-fold increase in expression in extracellular bacteria between 2 and 24 h.

Figure 5. Expression of *mntH* and *sitA* upon infection of activated RAW 264.7 cells and in the presence of Nramp1. **Panels A and B**: Bacteria were grown to mid-log phase and allowed to infect host cells for 30 minutes prior to either removal of remaining bacteria to a host-cell-free 96 well plate (extracellular bacteria), or to gentamicin treatment to kill remaining non-internalized bacteria (intracellular bacteria). Results shown represent the average number of Relative Light Units normalized to the number of bacteria present in each well as determined by parallel plate counts, and multiplied by 10^6 to obtain larger numbers for easier interpretation. Three experiments were performed with duplicate wells and all results were averaged. Error bars represent the SEM. **Panels C and D**: Bacteria cultured as described above were allowed to invade RAW 264.7 cells transfected with either empty pCB6 vector (Nramp1-) or pCB6 carrying a functional Nramp1 gene (Nramp1+) and gene expression monitored at selected time points over a 24 h time course. Three experiments were performed with duplicate wells and all results were averaged. Error bars represent the SEM. Asterisks indicate statistical significance to $P<0.05$ as determined by the Wilcoxan Rank Sum test for unpaired samples.


A
mntH
B
sitA
Average RLU/10^6 bacteria
100000
10000
1000
100
10
1
2
4
6
9
24
Time post-infection (h)
intracellular
extracellular
C
mntH
D
sitA
*
Nramp1- intracellular
Nramp1+ intracellular
Nramp1- extracellular
Nramp1+ extracellular

As MntH is a homologue of Nramp1, it was of interest to see if its expression pattern was altered in the presence of this host resistance protein. As shown in Figure 5C, no significant difference was observed in *mntH* expression for the first 6 h of infection between the Nramp1- RAW cells transfected with an empty vector and RAW cells transfected with a construct that constitutively expresses the murine *Nramp1* gene (Nramp1+). However *mntH* expression was approximately 5-fold and 22-fold higher in the Nramp1+ cell line at 9 and 24 h, respectively. Over the 24 h time course, *mntH* expression in the Nramp1- cell line increased approximately 7-fold; in contrast in the presence of Nramp1 *mntH* expression increased an average of 115-fold over the same time course. This again is markedly different from expression of *mntH* in extracellular bacteria, which remained 10^2- to 10^3-fold lower than in the intracellular bacteria and only increased an average of 2-fold over 24 h.

Expression of *sitA* was studied in the presence and absence of functional Nramp1, and a similar pattern was observed (Figure 5D): There was little difference in *sitA* levels within the first 6 h of infection, but *sitA* was upregulated 5- and 10-fold in the presence of Nramp1 at 9 and 24 h, respectively. Between 2 and 24 h post-infection in the absence of Nramp1 *sitA* expression increased a maximum of 2-fold, while in the presence of Nramp1 *sitA* expression increased 20-fold. As observed in the untransfected RAW cells, initial expression levels of *sitA* are nearly 10-fold higher than *mntH* in the intracellular environment. However, expression of *mntH* appeared to be more responsive to the presence of Nramp1, exhibiting a greater increase over time (115-fold vs 20-fold for *mntH* and *sitA,* respectively). For both genes, expression did not increase over time as dramatically in the RAW cell line transfected with empty vector as observed in untransfected RAW cells. In fact, in the presence of Nramp1 *sitA* transcription increased to the same extent over the 24 h time course as observed in initial experiments in the

untransfected RAW cells. However, *mntH* expression in the vector-only cells did increase 7-fold, compared to 58-fold in the RAW cells and 115-fold in Nramp1+ cells. It is possible that introduction of the plasmid used for stable transfection of the RAW cells (pCB6) sufficiently alters the cell and subsequently the intramacrophage environment such that the expression of these two genes is noticeably affected.

3.3 Effect of *mntH* and *sitA* on invasion, replication and survival within cultured cell lines

As *mntH* and *sitA* are significantly induced inside cells, we investigated if they demonstrated further aspects typical of virulence genes. STM is well-characterized to invade and replicate rapidly within epithelial cells and to replicate to a lesser extent in macrophages if allowed to be taken up by the phagocytic pathway (122, 239). In contrast if STM is allowed to actively invade macrophages we do not observe overt replication of the bacteria. Instead we observe a drop in bacterial numbers which does not reach complete clearance of the bacterial load over 24 h (Finlay laboratory, unpublished observations). For the purposes of this work we term such behavior in macrophages as "survival" of STM instead of "replication" or "clearance". Therefore we specifically investigated whether *mntH* and *sitA* affected invasion and/or replication of STM in epithelial cells (represented by the HeLa cell line) or invasion and/or survival in macrophages (RAW 264.7 cell line).

SL1344 bearing deletions of *mntH* (MM2165), *sitA* (MM2524), *mntH* and *sitA* (MM2562), and the gene encoding a novel Mn^{2+}-repressor, *mntR* (MM2645), were studied. MntR was included on the basis that it may control expression of both *mntH* and *sitA* (142), and therefore any effect *mntH* or *sitA* would have on invasion or survival, may also be observed in this knockout strain. As the *mntR* strain harbored pMLZ104, MM2507 (SL1344 x pMLZ104) was also studied in case carriage of the plasmid affected

intracellular replication and survival. None of these strains had any significant effect on invasion and replication of STM in HeLa cells (Figure 6A and B); in all cases the bacteria increased 100-fold in number over the 24 h time course. Similarly, none of these mutations had any effect on survival of STM in activated untransfected RAW cells (Figure 6C and D), with the bacterial load decreasing an average of 5-fold over 24 h.

As *mntH* and *sitA* are upregulated in the presence of Nramp1, it was possible that a replication defect would only be observed in an Nramp1+ background. Therefore, survival of these mutant strains was studied using the stably transfected cell lines previously described. As shown in Figure 7A, the number of wildtype SL1344 decreased in the absence of Nramp1 by approximately 10-fold over the 24 h time course. In the presence of Nramp1 survival of SL1344 was further affected, decreasing nearly 100-fold over the same 24 h. These results also indicate that Nramp1 is functional in the stably transfected cell lines. Figures 7B and C demonstrate that none of the mutations described above have any effect on survival in the stably transfected cell lines.

3.4 Effect of hydrogen peroxide and ion levels on expression of *mntH* and *sitA*

OxyR, Fur and MntR boxes were identified in the *mntH* promoter region (144). Similarly, Fur and MntR boxes are evident in the *sitA* promoter region (see Figure 4). OxyR is a global regulatory system that responds to hydrogen peroxide levels and may be involved in the defense against the oxidative burst (215), while Fur is a global regulatory system that responds to cellular Fe^{2+} levels (12), and MntR is a recently identified regulatory system that responds to cellular Mn^{2+} levels (200). As reactive oxygen intermediates (ROIs) are produced by phagocytic cells during infection and Nramp1 is hypothesized to function as a divalent cation transport system, alteration in peroxide and divalent cation levels are stimuli STM would encounter during infection of macrophages.

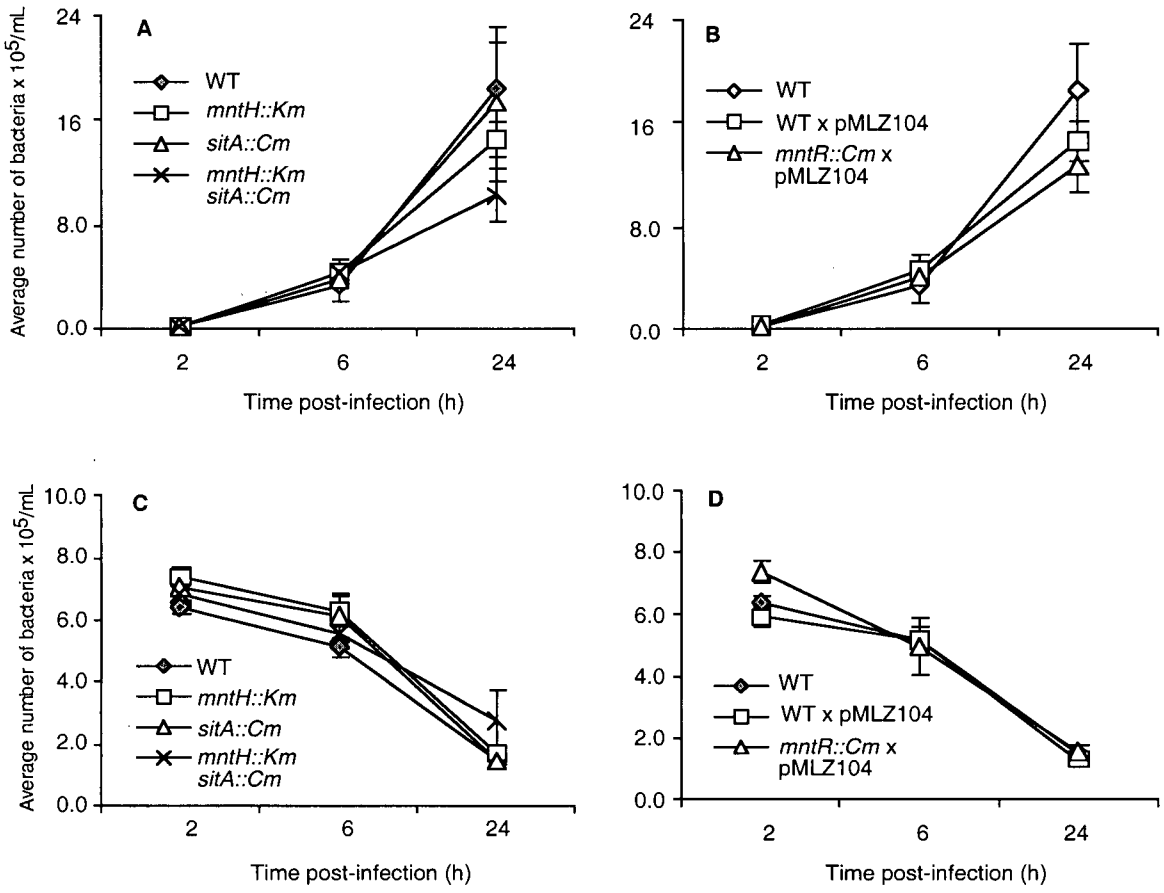


Figure 6. Effect of *mntH* and *sitA* on invasion or replication of STM in HeLa cells or survival of STM within activated RAW 264.7 cells. Bacteria were allowed to infect either HeLa cells (**panels A and B**) or activated RAW 264.7 cells (**panels C and D**) for 30 min prior to gentamicin treatment to kill non-internalized bacteria. Results shown depict the average number of cfu obtained at each time point after lysis of the host cell monolayer, serial dilution and plating. Three experiments were performed with duplicate wells and all results were averaged. Error bars represent the SEM. All strains were derived from the wildtype strain SL1344.

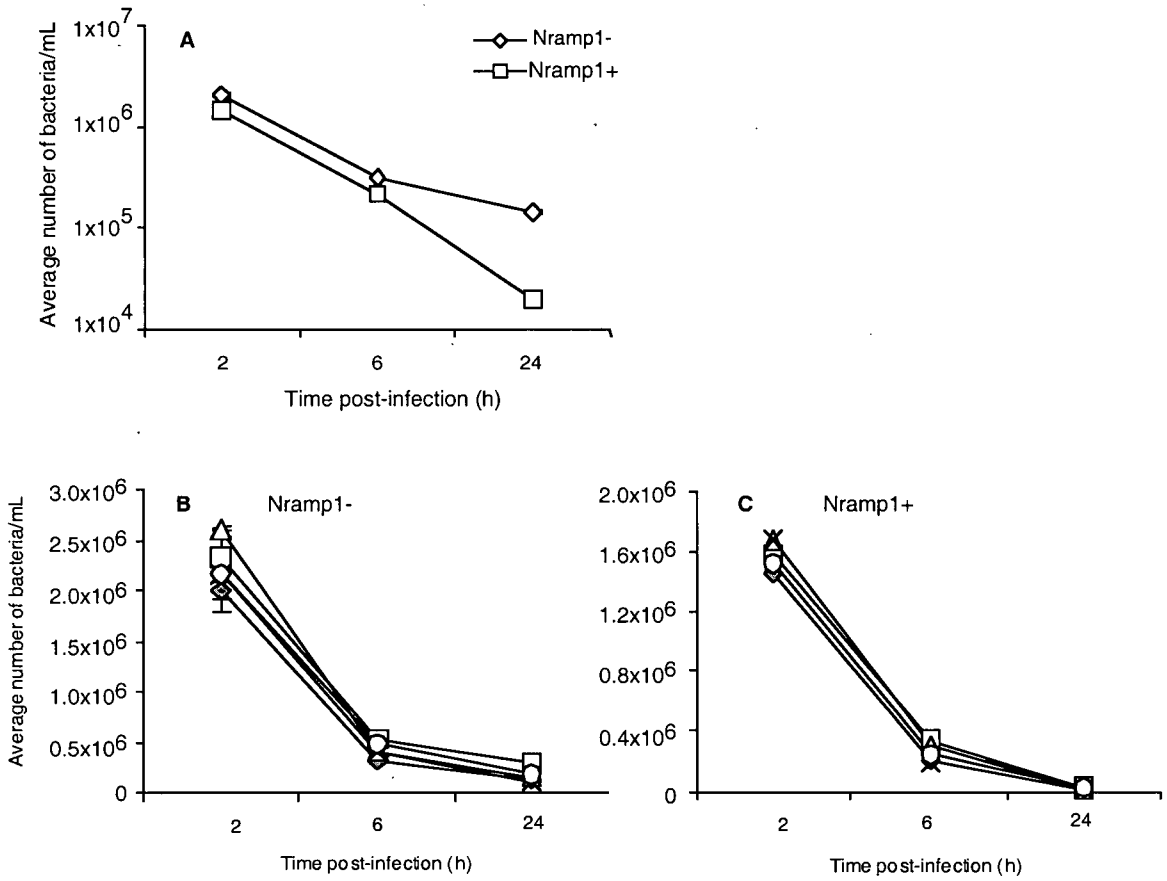


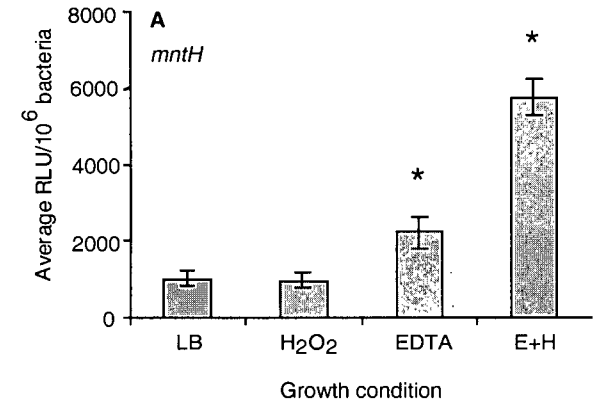
Figure 7. Effect of *mntH*, *sitA* and *mntR* on survival of STM in RAW 264.7 cells transfected with murine Nramp1. Results shown depict the average number of cfu obtained at each time point after lysis of the host cell monolayer, serial dilution and plating. Three experiments were performed with duplicate wells and all results were averaged. Error bars represent the SEM, and in many cases are occluded by the graph symbol. **Panel A** illustrates the effect of Nramp1 on SL1344 in the two transfected cell lines. **Panels B and C** illustrates the effect of *mntH*, *sitA* and *mntR* on survival of STM in the Nramp1- or Nramp1+ cell line, respectively. Closed diamonds, SL1344; open squares, MM2165 (*mntH::*Km); open triangles, MM2524 (*sitA::*Cm); ×, MM2562 (*mntH::*Km *sitA::*Cm); open circles, MM2645 (*mntR::*Cm).

Therefore the effects of both hydrogen peroxide and ion levels on expression of these loci was determined.

The general chelator EDTA and hydrogen peroxide were added to bacterial growth media to determine the effect of divalent cations and oxidative stress on *mntH* expression. Addition of hydrogen peroxide alone had no effect on *mntH* expression, while addition of EDTA induced *mntH* expression 2-fold (Figure 8A). The combination of hydrogen peroxide and EDTA resulted in an additive effect, increasing *mntH* expression 6-fold. As Nramp1 is thought to be transporting Fe^{2+} out of the SCV, the supposed Fe^{2+}-selective chelator DiP was also used to determine the effect of divalent cations on expression of *mntH*. Various divalent cations were added back to either EDTA- or DiP-chelated media to determine which cations were likely involved in regulation (Figure 8B). *mntH* was induced 2-fold by addition of EDTA to the growth media; this induction was reduced by 60% upon addition of excess $ZnSO_4$ or $MgSO_4$. However, addition of $MnSO_4$ or $FeSO_4$ reduced *mntH* expression below basal levels. A similar pattern was observed for *mntH* expression in the presence of DiP, with *mntH* being induced 6-fold in the presence of DiP, and $MnSO_4$ or $FeSO_4$ very effectively repressing the observed chelator-induced increase in transcription.

Due to the lack of an OxyR box in the *sitA* upstream regulatory region, *sitA* was not initially screened for the impact of hydrogen peroxide on expression levels. Chelation with EDTA resulted in a 3-fold increase in expression of *sitA*, while chelation with dipyridyl increased expression 7-fold (Figure 8C). Re-addition of cations to chelated media gave patterns of expression similar to those obtained with *mntH*. With both chelators addition of $FeSO_4$ repressed expression the most, with $MnSO_4$ the next most effective cation. This suggests that concentration of Fe^{2+} and/or Mn^{2+} may be regulating

Figure 8. Effect of oxidative stress and divalent cation concentration on *mntH* and *sitA* expression. Wild-type STM SL1344 bearing either the *mntH*::*lacZY* or *sitA*::*lacZY* transcriptional fusion plasmids were grown overnight in LB at pH 7.6 prior to subculturing for 3 h in LB (pH 7.6) supplemented with either 1) hydrogen peroxide (100 μM), EDTA (100 μM), or hydrogen peroxide and EDTA (100 μM each; **Panel A**), or 2) dipyridyl (DiP) or EDTA (250 μM) alone or supplemented with one of the following divalent cations to a final concentration of 500 μM: $ZnSO_4$, $MgSO_4$, $MnSO_4$ or $FeSO_4$ (**Panels B and C**). Results shown represent the average number of Relative Light Units representing β-galactosidase activity normalized to the number of bacteria present in each well as determined by parallel plate counts (described in Section 2.5.3), and multiplied by 10^6 for easier interpretation. Three experiments were performed in triplicate and all results were averaged. Error bars represent the SEM. Asterisks indicate statistical significance to $P<0.01$, as determined by the Student's t-test.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mntH
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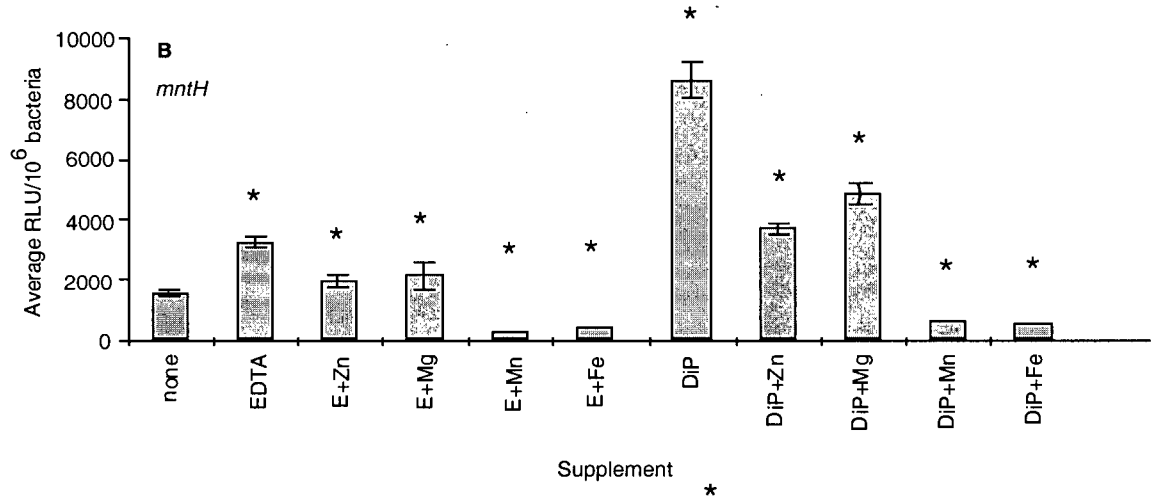
8000
6000
4000
2000
0
LB
H2O2
EDTA
E+H
Growth condition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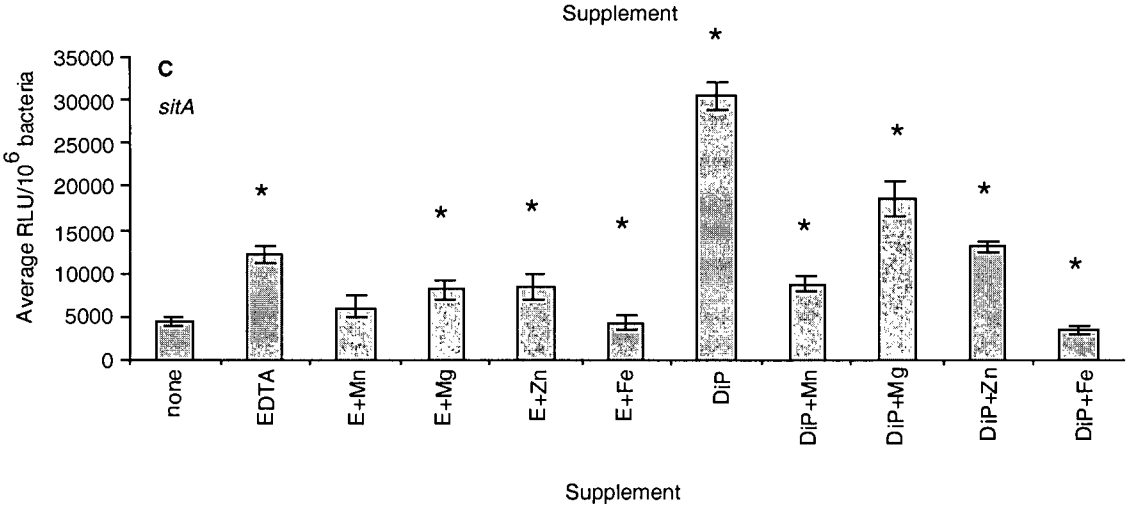
mntH
Average RLU/10^6 bacteria
10000
8000
6000
4000
2000
0
none
EDTA
E+Zn
E+Mg
E+Mn
E+Fe
DiP
DiP+Zn
DiP+Mg
DiP+Mn
DiP+Fe
Supplement


C
sitA
Average RLU/10^6 bacteria
35000
30000
25000
20000
15000
10000
5000
0
none
EDTA
E+Mn
E+Mg
E+Zn
E+Fe
DiP
DiP+Mn
DiP+Mg
DiP+Zn
DiP+Fe
Supplement

the expression of these two genes in the presence of Nramp1 during infection of murine macrophages.

3.5 Effect of pH on expression of *mntH* and *sitA*

The pH of the SCV has been shown to acidify during the course of an infection, regardless of the presence or absence of Nramp1 on the SCV membrane (60). As one of the conditions STM will encounter during infection of a macrophage is reduced pH, the effect of pH on *mntH* and *sitA* expression was determined in both N minimal medium (NMM) and LB. Culturing the bacteria overnight in NMM pH 4.5 did not support growth of the bacterial cultures. Therefore, overnight cultures were grown in NMM pH 7.4 and subcultured for 3 h in NMM pH 4.5. As seen in Figure 9A, using these growth conditions there is no significant effect of pH on expression of *mntH* or *sitA*. In contrast, satisfactory growth of the bacteria in LB pH 5.6, as suggested by Dr. Michael Maguire (personal communication) was sufficient for these experiments. Using these culture conditions, Figure 9B demonstrates that at pH 5.6 there is little effect of pH on EDTA or peroxide on *mntH* expression, but the presence of both EDTA and peroxide induced *mntH* 25-fold, compared to the approximately 5-fold induction observed at pH 7.6.

In contrast *sitA* was studied solely using conditions of ion depletion as it is unresponsive to conditions of oxidative stress (discussed below, and D.G. Kehres, personal communication). Bacterial subcultures at either pH 7.6 or 5.6 were supplemented with DiP and one of the following divalent cations: $ZnSO_4$, $MnSO_4$, $MgSO_4$ or $FeSO_4$. In contrast to *mntH* expression, *sitA* expression levels were reduced at least five-fold by growth at pH 5.6 when compared to growth at pH 7.6 (Figures 9C and D), although induction by DiP and repression by Zn^{2+}, Mn^{2+} and Fe^{2+} was somewhat apparent. Therefore it appears that of the two genes encoding the STM Mn^{2+} transport

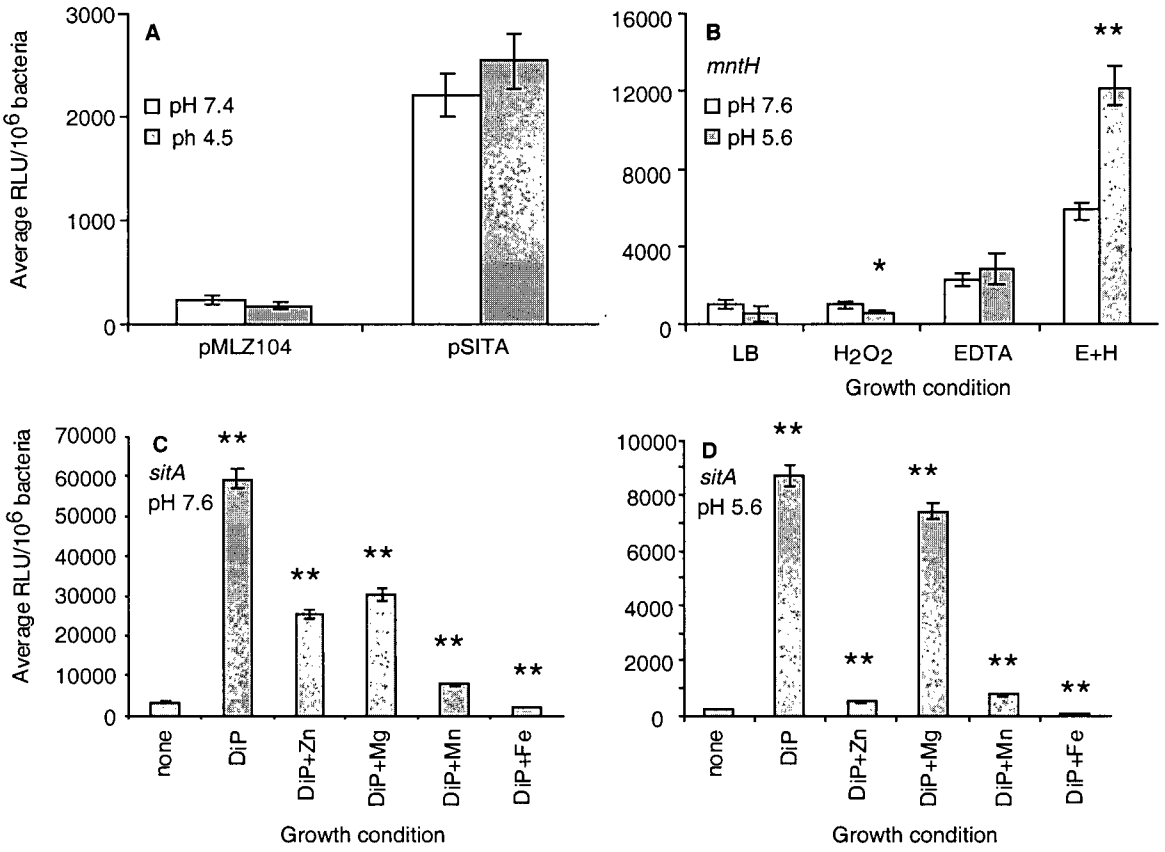


Figure 9. Effect of pH on expression of *mntH* and *sitA*. **Panel A**: Bacteria were grown overnight in NMM pH 7.4 prior to subculturing for 3 h in NMM at a pH of either 7.4 or 4.5. **Panels B and C**: Bacteria were grown overnight in LB pH either 7.6 or 5.6 prior to subculturing for 3 h in the same medium supplemented as indicated. β-galactosidase activity and bacterial cfu counts were performed as described in Section 2.5.3. Three experiments were performed in triplicate and all results were averaged. Error bars represent the SEM. * indicates statistical significance to $P<0.05$, as determined by the Student's t-test. ** indicates statistical significance to $P<0.01$ as determined by the Student's t-test. Significance was compared either between paired cultures (Panel B), or when compared to the untreated sample (Panels C and D).

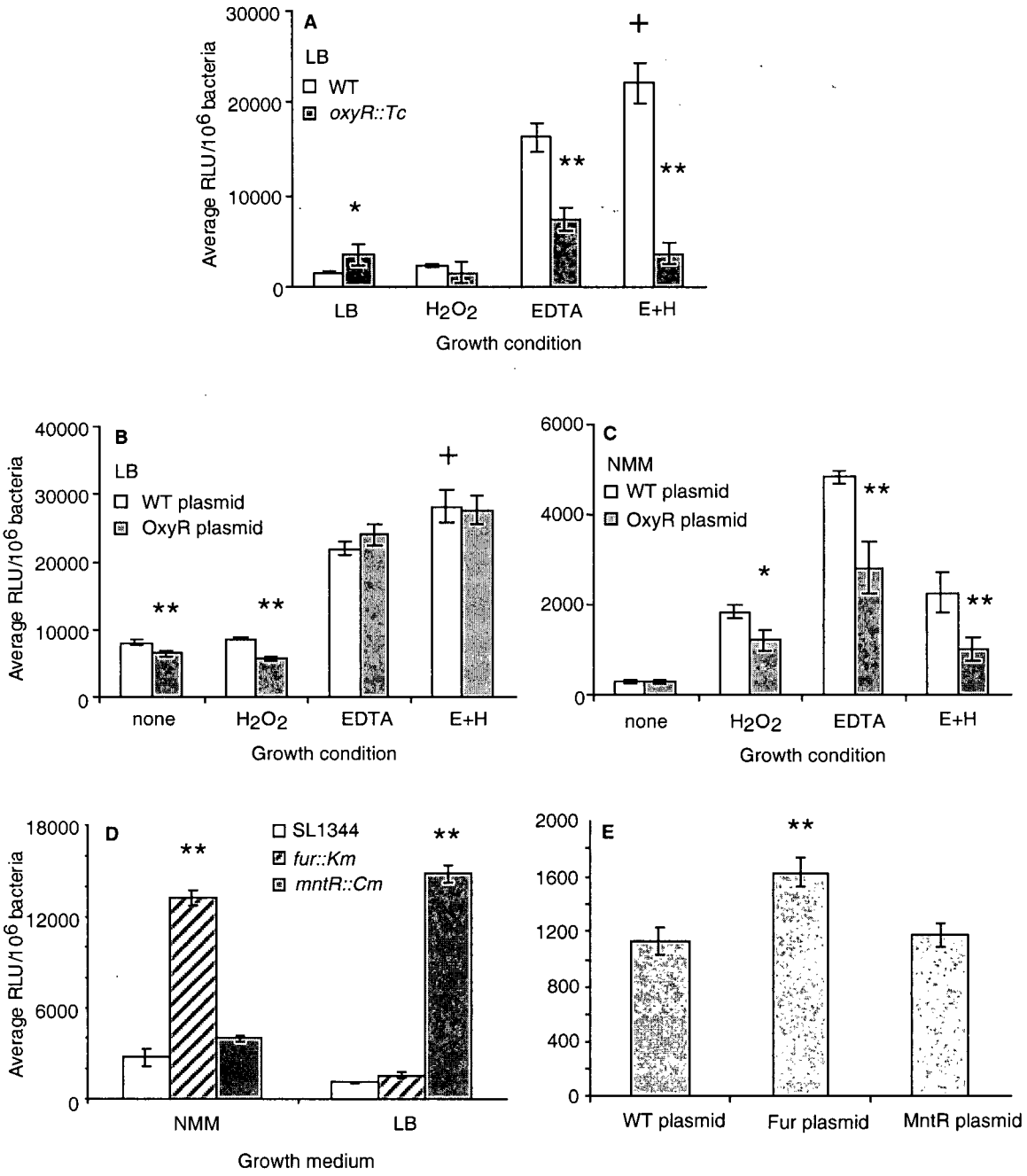
systems, *mntH* is expressed most efficiently at the pH encountered by STM in the intracellular environment of the macrophage.

3.6 Effect of Fur, OxyR and MntR on expression of *mntH* and *sitA* in bacterial culture

Ultimately we wanted to investigate which stimulus (hydrogen peroxide or divalent cation concentration) may be responsible for the induction of expression of *mntH* in the presence of Nramp1 *in vivo*. Therefore, we needed to characterize more completely the roles of OxyR, Fur and MntR on *mntH* expression. To confirm such an effect, SL1344 bearing chromosomal deletions in these three regulatory systems were investigated for their effect on *mntH* expression in either NMM or LB using induction conditions described in Chapter 2. *sitA* was also studied in parallel using similar strains. Further, a series of plasmids carrying the *mntH::lacZY* transcriptional fusion in which the OxyR, Fur or MntR boxes in the *mntH* upstream regulatory region had been deleted were obtained from Dr. Kehres (see Appendix) and tested for effects on *mntH* expression. Figure 10A shows that disruption of the chromosomal *oxyR* gene had no significant effect on expression of *mntH* in the presence of peroxide alone, but did result in a 50% decrease in expression in the presence of EDTA alone, and an 80% reduction of expression in combination with hydrogen peroxide. Interestingly, deletion of the OxyR box from the *mntH* promoter region on the transcriptional fusion plasmid had little effect on expression of *mntH* in strains cultured in LB (Figure 10B). However, when the bacteria were cultured in NMM, deletion of the OxyR box resulted in decreased expression of *mntH* similar to that observed by deletion of the chromosomal *oxyR* locus (Figure 10C).

Disruption of the chromosomal loci *fur* and *mntR* as well as deletion of the Fur and MntR boxes from the *mntH* promoter region resulted in an increase in expression of

Figure 10. Regulation of *mntH* by OxyR, Fur and MntR. Bacteria were grown as previously described in either LB or NMM alone or supplemented as indicated. β-galactosidase activity and bacterial cfu counts were assayed as described in Section 2.5.3. Three experiments were performed in triplicate and all results were averaged. Error bars represent the SEM. **Panel A**: Disruption of the chromosomal *oxyR* gene reduces expression of *mntH* in EDTA- or EDTA + hydrogen peroxide-supplemented cultures. **Panels B and C**: Deletion of the OxyR box from the *mntH* promoter region affects expression of *mntH* when bacteria are grown in NMM but not in LB. MM2507: SL1344 x pMLZ104 (*mntH::lacZY* with the complete promoter region); MM2616: SL1344 x pDGK261 (ΔOxyR//*mntH::lacZY*). **Panel D**: Disruption of the chromosomal *fur* and *mntR* genes differentially affects *mntH* expression dependant on growth medium. MM2645 (*fur*::Km); MM2646 (*mntR*::Cm). **Panel E**: Deletion of the Fur box but not the MntR box from the *mntH* promoter region affects expression of *mntH* during growth in LB culture. MM2617: SL1344 x pDGK262 (ΔFur//*mntH::lacZY*); MM2618: SL344 x pDGK263 (ΔMntR//*mntH::lacZY*). * indicates statistical significance to $P<0.05$ between paired cultures, as determined by the Student's t-test. ** indicates statistical significance to $P<0.01$ between paired cultures as determined by the Student's t-test. + indicates statistical significance to $P<0.01$ between the EDTA chelated sample and the EDTA+H_2O_2 sample, as determined by the Student's t-test.


A
LB
WT
oxyR::Tc
Average RLU/10^6 bacteria
30000
20000
10000
0
LB
H2O2
EDTA
E+H
Growth condition
B
LB
WT plasmid
OxyR plasmid
40000
30000
20000
10000
0
none
H2O2
EDTA
E+H
Growth condition
C
NMM
WT plasmid
OxyR plasmid
6000
4000
2000
0
none
H2O2
EDTA
E+H
Growth condition
D
SL1344
fur::Km
mntR::Cm
18000
12000
6000
0
NMM
LB
Growth medium
E
2000
1600
1200
800
400
0
WT plasmid
Fur plasmid
MntR plasmid

mntH during growth in both LB and NMM, although with different patterns of expression (Figure 10D). Disruption of the chromosomal *fur* gene resulted in a 5-fold increase in *mntH* expression when cultured in NMM, but only a 1.5-fold increase when strains were grown in LB. In contrast, disruption of *mntR* yielded a 1.5-fold increase in expression in NMM and a 13-fold increase when grown in LB. Deletion of the Fur box from the promoter region of the plasmid-borne *mntH* transcriptional fusion generated a 1.5-fold increase in expression when grown in LB, while the MntR box deletion had no appreciable effect on *mntH* expression in these growth conditions (Figure 10E). Therefore, OxyR positively regulates *mntH* expression, while Fur and MntR negatively regulate expression of this gene.

Similar studies were carried out to investigate the role of OxyR, MntR and Fur on *sitA* expression. Figure 11A illustrates that the loss of OxyR does not affect expression of *sitA*, nor is there a significant effect of peroxide on *sitA* expression, confirming that hydrogen peroxide does not induce *sitA* expression due to the lack of an OxyR box in its promoter region. In contrast, disruption of *fur* yielded an average 2-fold increase in *sitA* expression when strains were cultured in either NMM or LB, while disruption of *mntR* generated a 1.5-fold increase in *sitA* expression when grown in NMM and an 11-fold increase when grown in LB (Figure 11B). Therefore like *mntH, sitA* is negatively regulated by the ion-responsive regulators MntR and Fur, but unlike *mntH* is not affected by the OxyR regulatory system in response to oxidative stress.

It is interesting to note that the basal level of expression of *sitA* and *mntH* is lower when the bacteria were grown in LB compared to growth in NMM (see Figures 10D and 11B). This most likely reflects the concentrations of the cations found in these different growth media. As NMM is a chemically defined minimal medium its transition metal cation concentration is low (approximately 8 μM $MgCl_2$), while LB is a

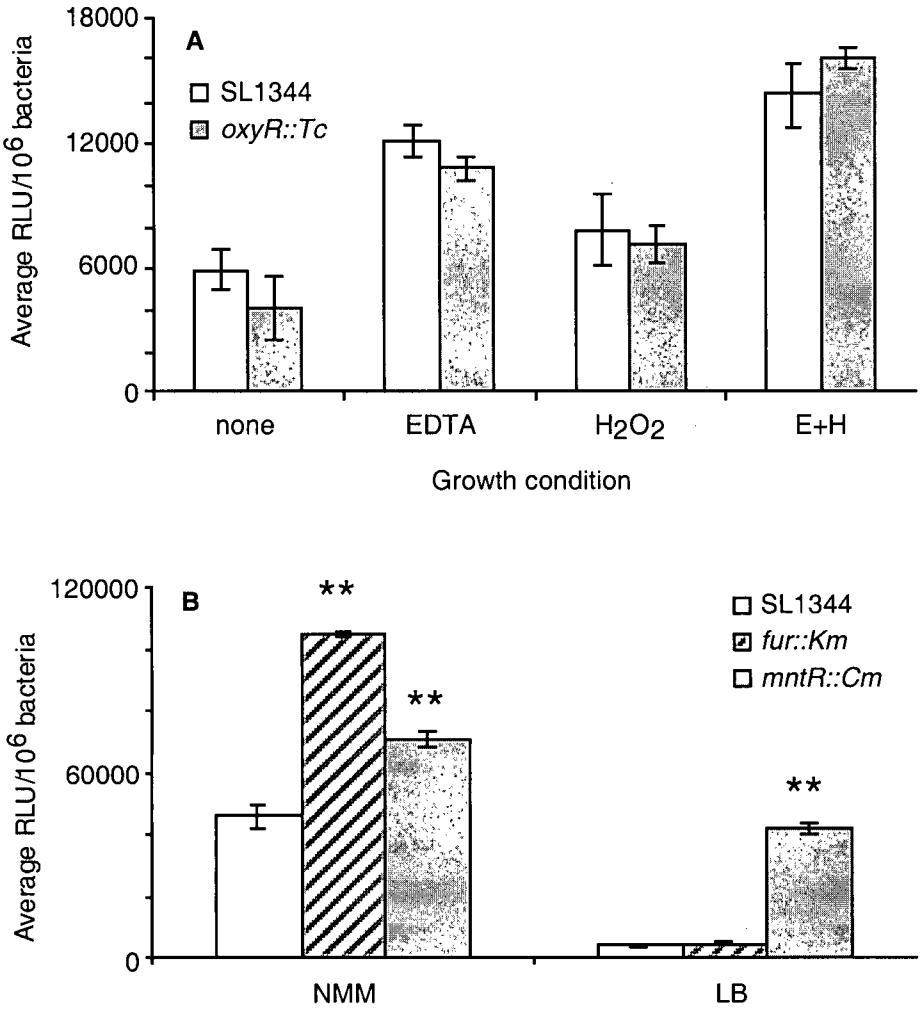


Figure 11. Effect of Fur, MntR and OxyR on expression of *sitA*. Bacteria were cultured in LB or NMM and supplemented as previously described. β-galactosidase activity and bacterial cfu counts were assayed as described in Section 2.5.3. Three experiments were performed in triplicate and all results were averaged. Error bars represent the SEM. **Panel A**: Disruption of the chromosomal *oxyR* gene does not affect expression of *sitA*. **Panel B**: Disruption of the *fur* and *mntR* genes results in an increase in expression of *sitA* dependent on growth medium. MM2645, *fur*::Km; MM2646, *mntR*::Cm. ** indicates statistical significance to $P<0.01$ as determined by the Student's t-test.

complex medium with many cations present at more elevated levels. As a result the expression of these genes is predicted to be partially derepressed in NMM and, therefore, their basal levels of expression would be higher than in LB. Interestingly deletion of Fur and MntR had a greater impact on expression of these genes during growth in NMM than in LB. As both Mn^{2+} and Fe^{2+} are present at similarly low concentrations in the defined medium, it would appear that Fur is not important for expression of *sitA* and *mntH* after growth in rich medium while MntR has a stronger regulatory role in these conditions, while the converse would be true in minimal media. As NMM has been used previously as a medium thought to mimic conditions found in the SCV (68), it is tempting to speculate that Fur may be more important than MntR in expression of these genes *in vivo*.

3.7 Effect of Fur, MntR and OxyR on *mntH* expression in cultured cell lines

It was of interest to determine if MntR, Fur or OxyR alone or in combination were involved in the expression of *mntH* expression *in vivo* and in the induction/derepression of this gene in the presence of Nramp1. This would further delineate the effect of Nramp1 on the bacterial pathogen *in vivo* and perhaps elucidate what intracellular condition Nramp1 is altering to mediate its bacteriostatic effect. Therefore, SL1344 harboring OxyR, Fur or MntR box deletion plasmids described above were used to infect the Nramp1-transfected RAW cell lines. Deletion of *oxyR* resulted in a 2-fold reduction in expression of *mntH* in the Nramp1- cells (Figure 12A). In the presence of Nramp1, deletion of the OxyR box marginally, yet significantly, reduced *mntH* expression within the first 6 h post-infection, while at later time points expression of *mntH* returned to wildtype levels (Figure 12B). This effect could not be studied in the *oxyR* knockout strain in the cell culture model, as this strain could not survive in the activated RAW cells, resulting in either no colonies or too few colonies to count upon plating throughout the

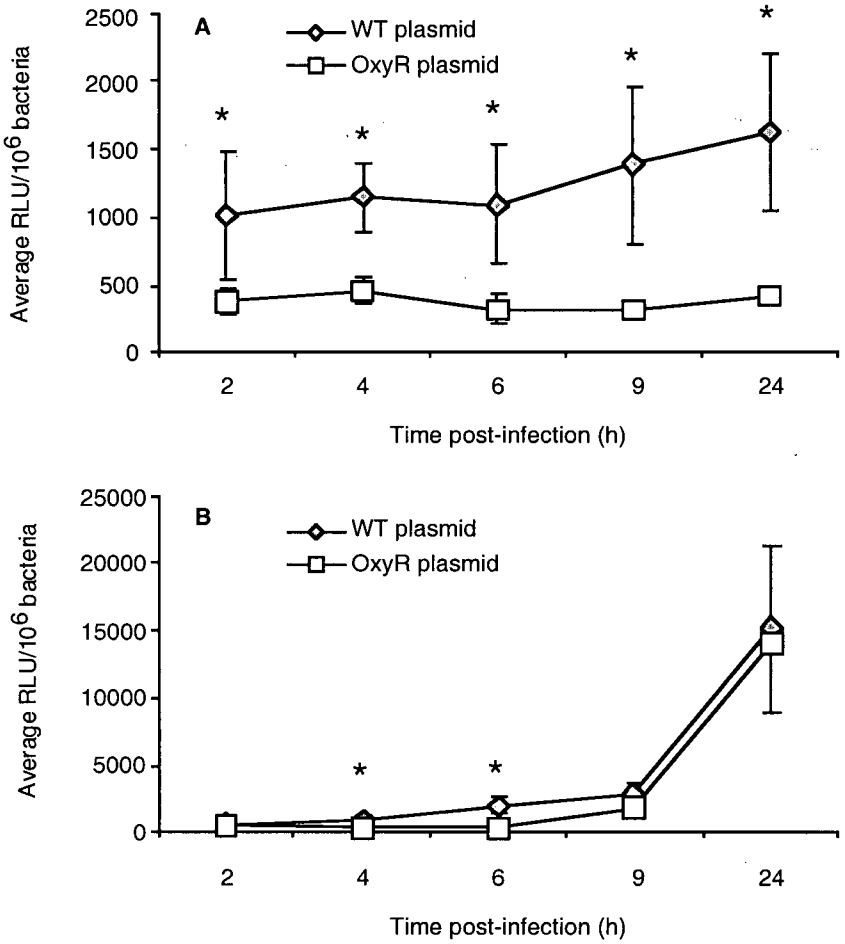


Figure 12. Role of OxyR in the expression of *mntH* in activated RAW 264.7 cells. Bacteria were allowed to invade RAW cells transfected with either the empty pCB6 vector alone (Nramp1-; **Panel A**) or pCB6 carrying a functional Nramp1 gene (Nramp1+; **Panel B**) and gene expression monitored at selected time points over a 24 h time course as described in Section 2.5.2. Three experiments were performed with duplicate wells and all results were averaged. Error bars represent the SEM. MM2507: SL1344 x *mntH::lacZY*; MM2616: SL1344 x pDGK261 (ΔOxyR/*mntH::lacZY*). Asterisks indicate statistical significance to $P<0.05$ as determined by the Wilcoxan Rank Sum test for unpaired samples.

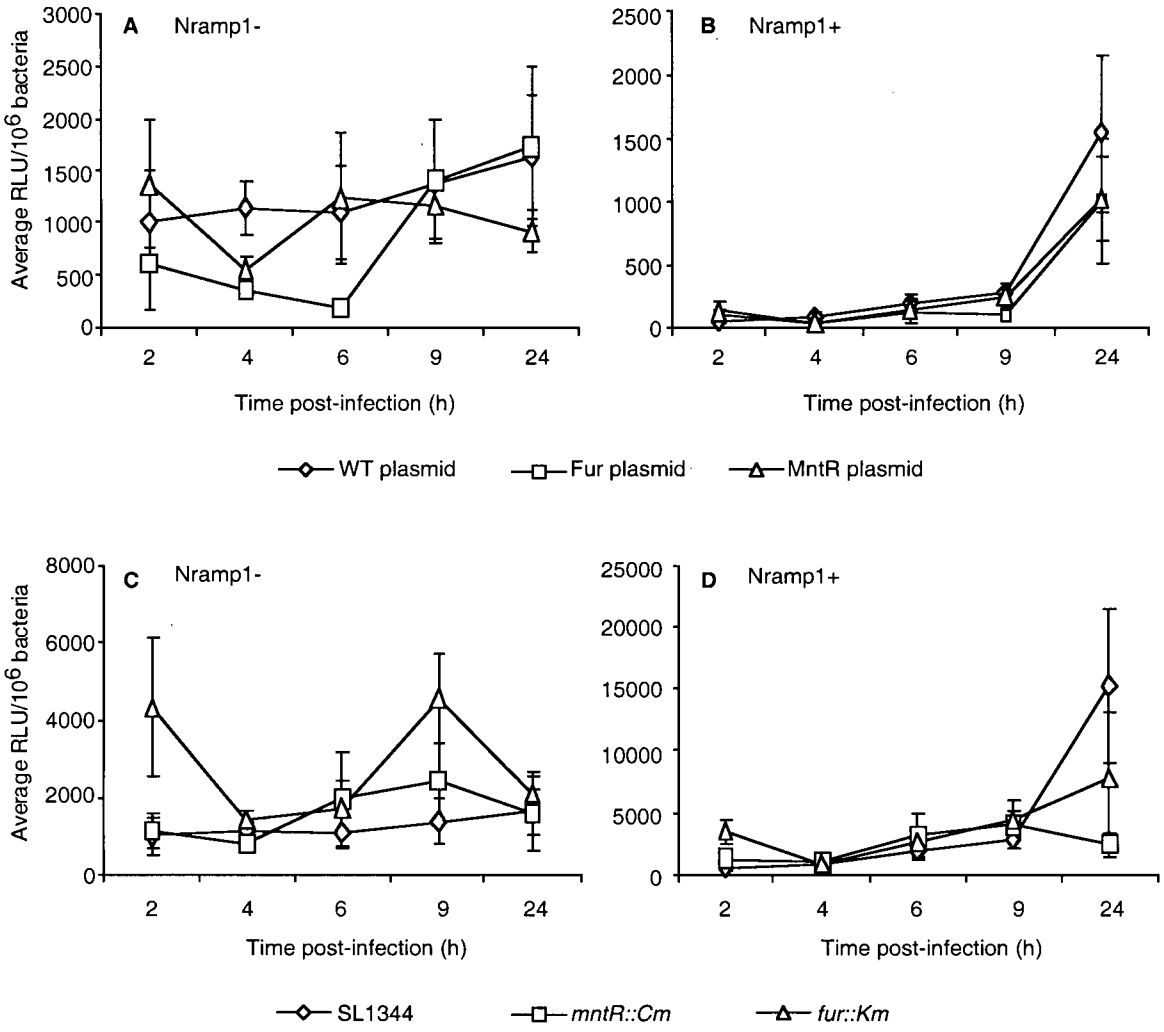


Figure 13. Effect of MntR and Fur on *mntH* expression in activated RAW 264.7 cells. Bacteria were allowed to invade RAW cells transfected with either empty pCB6 vector alone (Nramp1-; **Panels A and C**) or pCB6 carrying a functional Nramp1 gene (Nramp1+; **Panels B and D**) and gene expression monitored at selected time points over a 24 h time course as described in Section 2.5.2. Three experiments were performed with duplicate wells and all results were averaged. Error bars represent the SEM. Values were found not to be statistically significant by the Wilcoxan Rank Sum test for unpaired samples.

entire time course. Deletions of the Fur or MntR boxes had no appreciable effect on MntH expression in either cell line (Figure 13A and B). Fur or MntR chromosomal deletions similarly did not affect expression of *mntH* (Figure 13C and D).

3.8 Effect of the oxidative burst on *mntH* expression

We have confirmed that *mntH* is regulated by the global regulatory system OxyR, believed to respond to hydrogen peroxide generated during infection as part of the oxidative burst of phagocytic cells. Therefore, we tested whether we could alter the expression of *mntH* in cultured cells by either inducing or repressing the oxidative burst of IFN-γ-activated RAW 264.7 macrophages. Compounds known to induce (PMA; (128)) or repress (DPI; (95)) the oxidative burst of macrophages were added to transfected cells and the expression of *mntH* studied over a 24 h time course. DMSO was included as a control as the solvent for DPI. The results were counterintuitive as addition of DPI slightly but significantly increased *mntH* expression at later times post-infection, while PMA only affected *mntH* expression at 9 h post-infection, and then only modestly (Figure 14). As the oxidative burst is thought to occur rapidly upon infection of phagocytic cells, these results were perplexing.

As a control, cellular hydrogen peroxide levels following PMA or DPI treatment were assayed using a commercially available kit (Amplex Red, Molecular Probes). No hydrogen peroxide deriving from either the untreated, PMA-stimulated or DPI-repressed cell lines was detected. Dr. Ferric Fang (University of Washington) suggested that RAW 264.7 cells do not display an appreciable oxidative burst and, therefore, may not be sufficiently responsive to PMA and DPI or display measurable amounts of this ROI. Further, work by Carrie Rosenberger in this lab has determined that RAW 264.7 cells do exhibit a detectable oxidative burst upon stimulation with PMA; however even after PMA stimulation hydrogen peroxide levels could not be detected in this cell line.

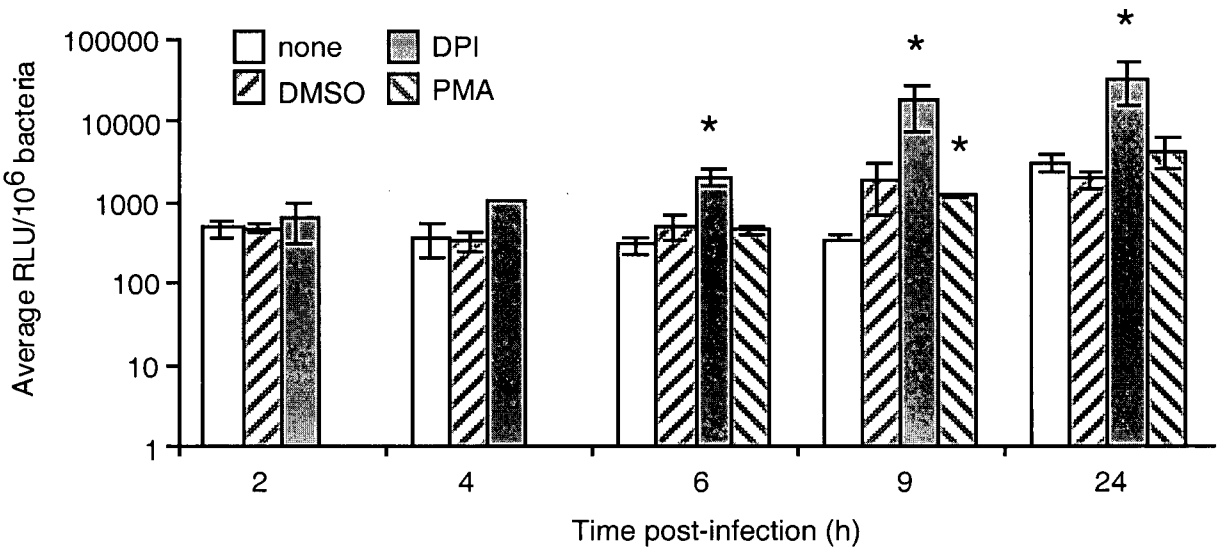


Figure 14. Effect of the alteration of the phagocyte oxidative burst on *mntH* expression in activated RAW 264.7 cells. RAW cells were either untreated, or treated with DMSO (0.1%), DPI (2 μM) or PMA (5 nM) for 30 min prior to infection with STM bearing pMLZ104. Gene expression was monitored at selected time points over a 24 h time course as described in Section 2.5.2. Three experiments were performed with duplicate wells and all results were averaged. Error bars represent the SEM. Asterisks indicate statistical significance to $P<0.01$ as determined by the Wilcoxan Rank Sum test for unpaired samples.

Therefore to study the effect of hydrogen peroxide on *mntH* expression in this system was futile.

To study further the effect of the oxidative burst on *mntH* expression in the presence and absence of Nramp1, elicited peritoneal macrophages were obtained from a BALB/c mouse as a test before attempting to obtain macrophages from congenic Nramp1 knockout mice. Sufficient numbers of macrophages were recovered; however, due to the high MOI required in order to detect expression of *mntH* in cultured cells, these elicited cells did not survive the infection for longer than 2 h, thus preventing analysis. Therefore, the effect of the oxidative burst on *mntH* expression remains poorly described.

3.9 Effect of other regulatory systems

To investigate the role of virulence-associated regulatory systems on *mntH* and *sitA* expression, strains of SL1344 harboring insertional activation mutations (*hilA*::Km; *phoP*::Cm; *ssrA*:Km; *pmrA*::Tn10) were constructed by P22 transduction and transformed with either pMLZ104 or pSITA . Expression of the genes of interest was investigated during growth in NMM (Figure 15). Lack of a functional PhoPQ and PmrAB TCRS resulted in a minor, yet significant, increase in the expression of *mntH*. In contrast, deletion of *hilA, ssrA* and *pmrA* resulted in a similar minor increase in *sitA* expression. However, this is contradictory to previous results indicating that neither HilA nor PhoP are involved in *sitA* expression (135). It is unknown whether this difference is biologically significant, and therefore the effect of other regulatory systems in *mntH* and *sitA* expression remains to be conclusively defined.

3.10 Virulence studies

Initial studies on the effect of *mntH* on STM virulence were carried out in the 14028s wildtype strain using the unrelated BALB/c (*Nramp1*$^{-/-}$) and C3H/HeN

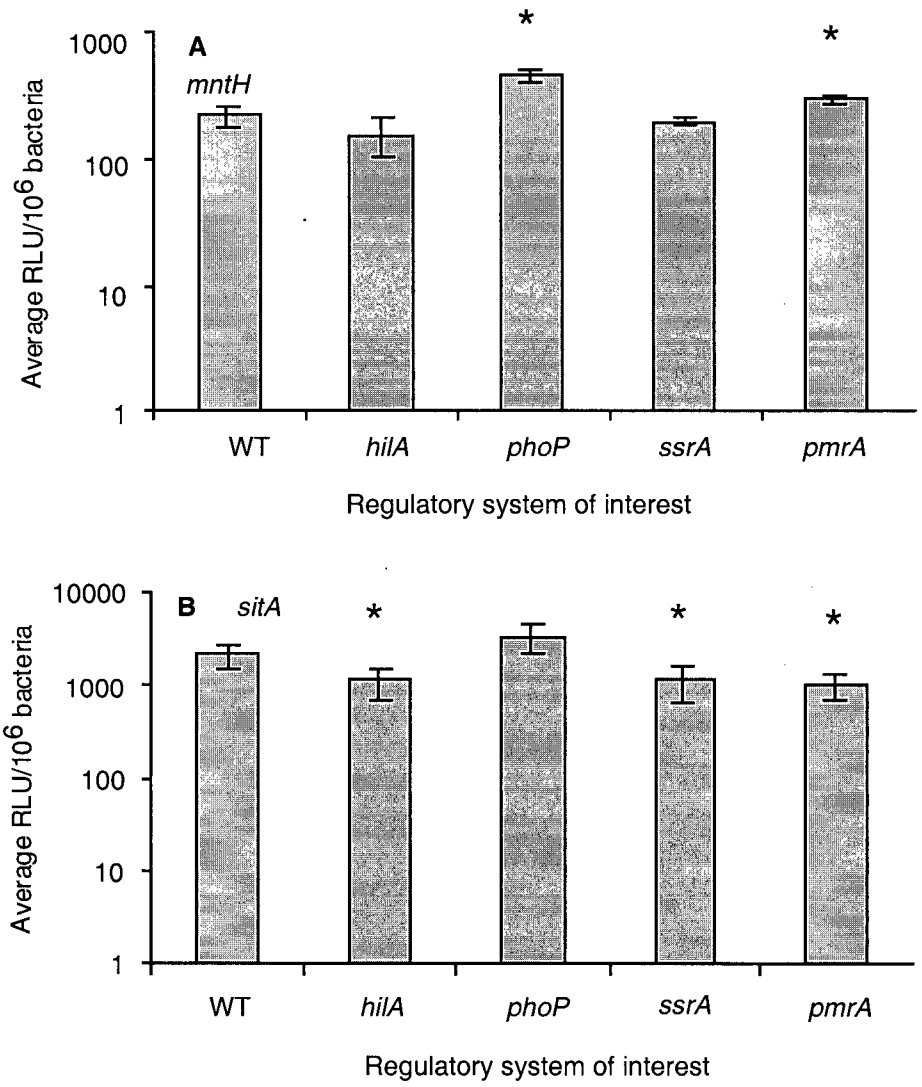


Figure 15. Effect of the virulence-associated regulatory systems HilA, PhoPQ, SsrA and PmrAB on *mntH* and *sitA* expression. Bacteria harboring insertions in these different regulatory genes were grown and subcultured in NMM. β-galactosidase activity and bacterial cfu counts were assayed as described in Section 2.5.3. Three experiments were performed in triplicate and all results were averaged. Error bars represent the SEM. Asterisks indicate statistical significance to $P<0.01$ as determined by the Student's t-test.

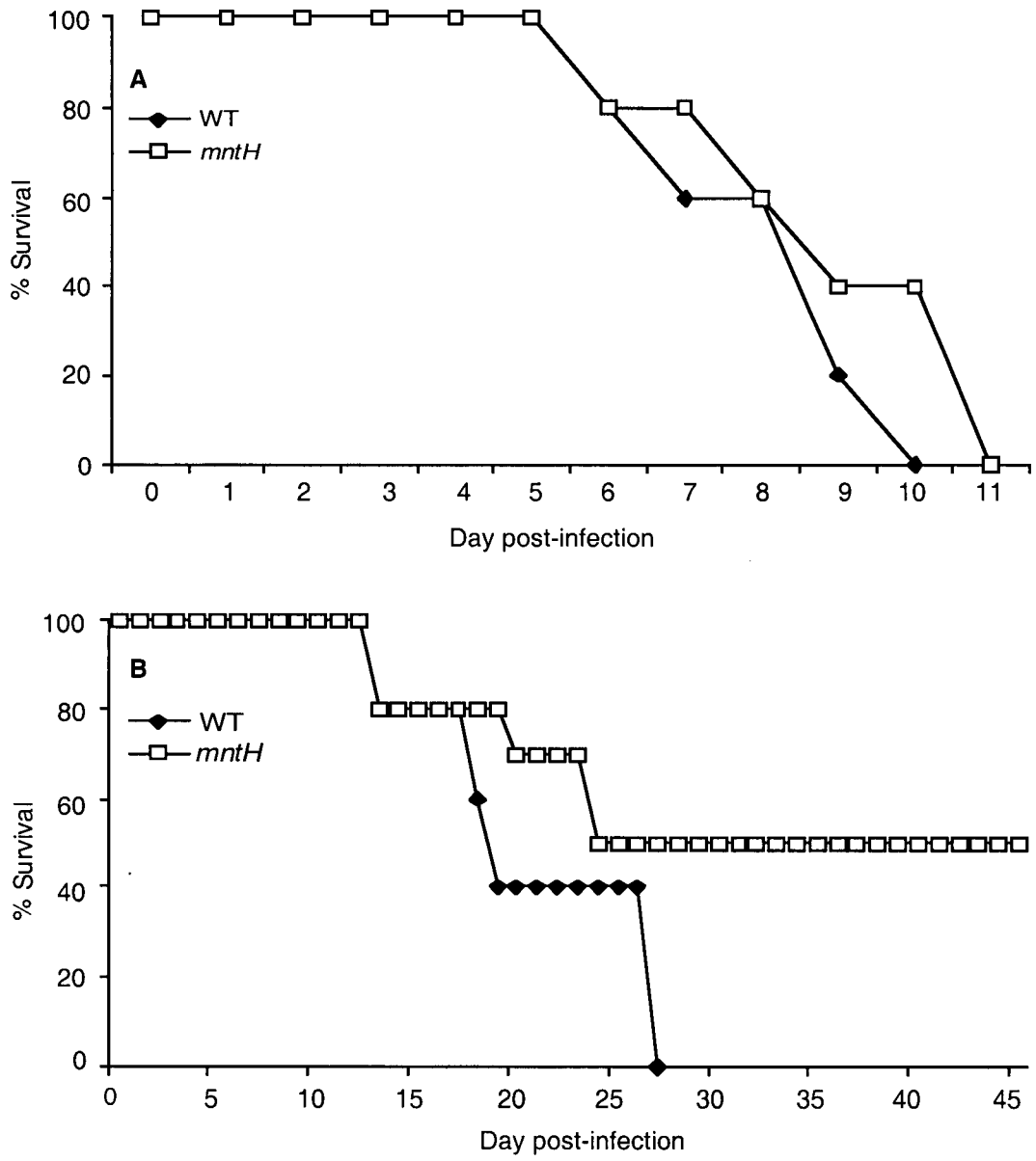
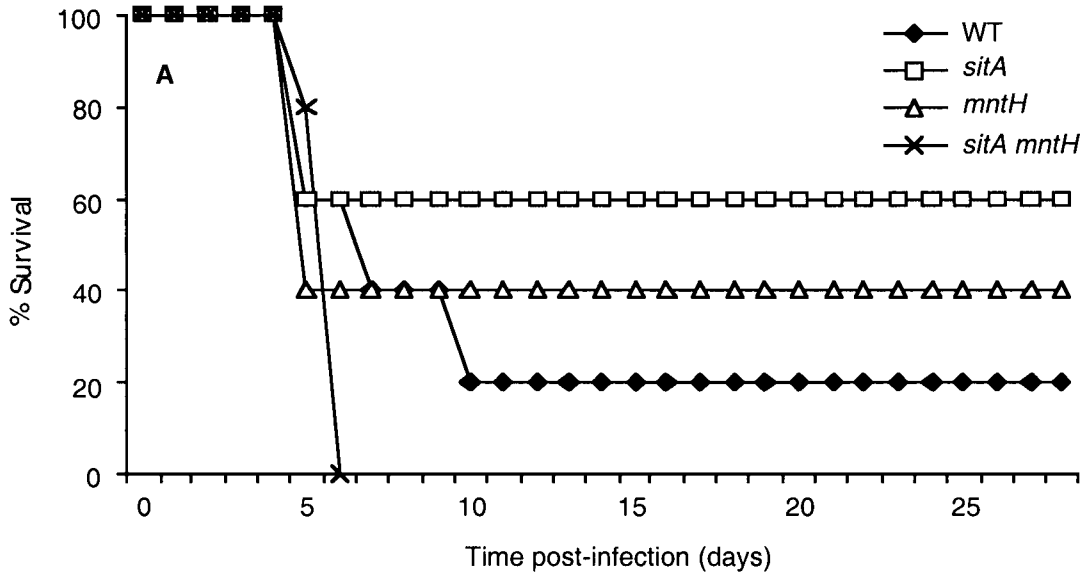


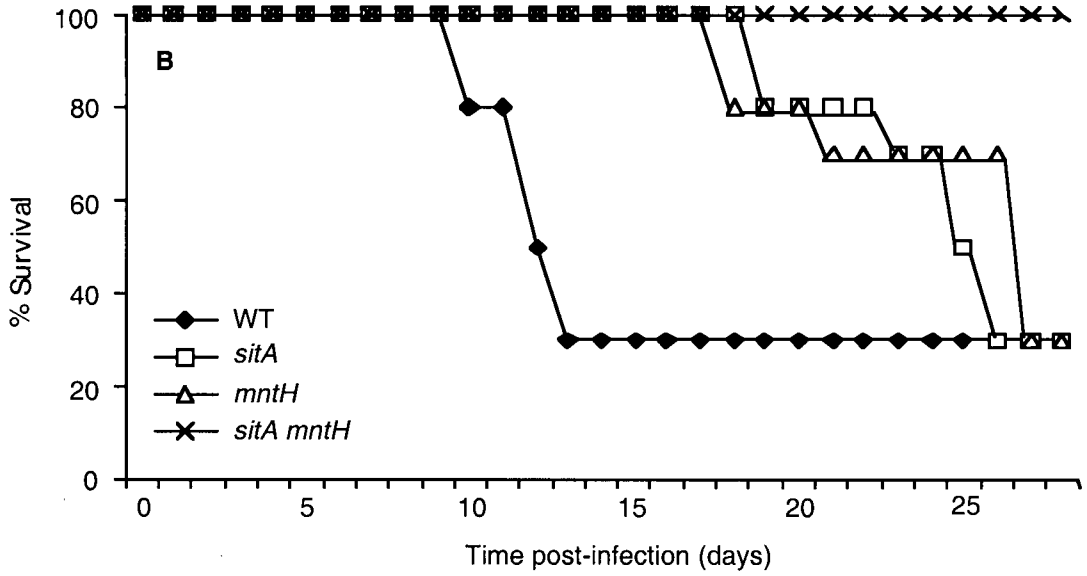
Figure 16. Effect of *mntH* on virulence of STM 14028s in BALB/c (Nramp1$^{-/-}$; **Panel A**) or C3H/HeN (Nramp1$^{+/+}$; **Panel B**) mice. Bacteria were grown to mid-log phase in LB prior to dilution in PBS. Groups of five (BALB/c) or ten (C3H/HeN) female mice were orally inoculated to a final dose of $5x10^6$ cfu/ mouse. Mice were monitored on a daily basis for signs of morbidity and mortality.

(*Nramp1*$^{+/+}$) mouse lineages; the C3H/HeN experiments were carried out at Case Western Reserve University by Dr. David Kehres during a temporary oversubscription of our animal facilities at UBC. In the *Nramp1*$^{-/-}$ BALB/c mice, deletion of *mntH* resulted in a mean delay in the time of death of the animal of about 2 days after peroral infection (Figure 16A). In the *Nramp1*$^{+/+}$ C3H/HeN mice, deletion of *mntH* resulted in significant attenuation of STM following peroral infection, with 100% of the infected control animals succumbing to oral infection by day 28 post-infection, while 50% of the *mntH*-infected animals succumbed by day 24; remaining animals survived until sacrifice on day 45 (Figure 16B). Although these experiments suggested that *mntH* may play a role in virulence in the presence of Nramp1, these experiments were not conclusive since the mouse lineages were not congenic. Therefore congenic Nramp1 mouse lineages were bred by Dr. Ferric Fang at the University of Washington for further studies.

SL1344 was used as the strain background and intraperitoneal infection was used for these experiments by agreement between the collaborating parties. Figure 17A demonstrates that deletion of *mntH* or *sitA* alone or in combination had little effect on virulence of STM in the C57/BL6 *Nramp1*$^{-/-}$ mice. However, in the C57/BL6 *Nramp1*^{G169} (*Nramp1*$^{+/+}$) mice both the *mntH* and *sitA* strains were attenuated (Figure 17B), as only 40% succumbed to infection by day 26 and 28, respectively, while the double knock-out was completely avirulent. This demonstrates that both of these Mn^{2+}-transport systems are required for virulence of STM in the murine typhoid model. Further as this effect is observed in the congenic *Nramp1*$^{+/+}$ lineage of mice, this suggests that there may be competition for a common substrate between *mntH, sitA* and Nramp1. If the bacteria are unable to acquire this divalent cation, be it Fe^{2+} or Mn^{2+}, they would be severely compromised for their ability to develop a productive infection.

Figure 17. Role of *mntH* and *sitA* in virulence in the Nramp1$^{+/+}$ mouse lineage C57/BL6 Nramp1^{G169}. Bacteria were grown to mid-log phase and diluted in PBS prior to intraperitoneal infection. Groups of ten female mice were infected with each strain to a final dose of 4-8 x 10^2 cfu/mouse for the C57/BL6 animals (Nramp1$^{-/-}$; **Panel A**), and 1.8-3.1 x 10^3 cfu/mouse for the C57/BL6 Nramp1^{G169} animals (Nramp1$^{+/+}$; **Panel B**). The doses were different due to the ability of the Nramp1$^{+/+}$ animals to withstand higher lethal doses of STM than the Nramp1$^{-/-}$ animals. Mice were monitored for signs of morbidity and mortality for 28 days. Closed diamonds, SL1344 wildtype; open boxes , *sitA*::Cm; open triangles, *mntH*::Km; crosses, *sitA*::Cm *mntH*::Km.


A
100
80
60
40
20
0
% Survival
0
5
10
15
20
25
Time post-infection (days)
WT
sitA
mntH
sitA mntH


B
100
80
60
40
20
0
% Survival
0
5
10
15
20
25
Time post-infection (days)
WT
sitA
mntH
sitA mntH

3.11 Discussion

Two genes were identified from the STM genome using different queries. *mntH* was identified based on its homology to the mammalian host resistance protein Nramp1. *sitA* was targeted due to the presence of a novel inverted repeat in its promoter region common only to the *mntH* promoter region. In view of the potential similarity in the regulation of expression of these two genes, combined with the important role of Nramp1 in resistance to infection and its homology to *mntH*, these genes were examined to see if they encode previously unidentified virulence factors. Work by Kehres *et al.* (144) identified MntH as a Mn^{2+}-transport system in STM with maximum activity at low pH, therefore being homologous in function to Nramp1. Initial studies on *sitABCD* suggested that it encoded an iron transport system that was not involved in STM virulence (278). Subsequent work demonstrated that disruption of *sitABCD* attenuated STM upon infection of naturally susceptible (BALB/c) mice (135). Further work by Kehres *et al.* (143) identified *sitA* as encoding an STM transport system responsible for Mn^{2+} but not Fe^{2+} transport in alkaline conditions. Since MntH and SitABCD have distinct pH optima, they may function at different points during the course of an infection. Identification of the function of these proteins suggested a number of interesting avenues of research, including: 1)the potential interplay of these two transport systems during infection; 2) a possible interaction between one or both of these divalent cation transport systems with the mammalian cation transporter Nramp1 at the level of substrate availability; and 3) a role of one or both of these transport systems in virulence of STM in the presence or absence of Nramp1.

Investigating these genes at the level of their expression demonstrated that they are induced in the intracellular environment, a characteristic identified for other STM virulence factors (54, 255). Moreover, both genes were upregulated in the presence of Nramp1. Neither *mntH* nor *sitA*, alone or in combination, had any effect on STM

invasion or replication in epithelial cells, or on survival within macrophages. Nevertheless, both loci were determined to be essential for full virulence of STM in mice containing a functional Nramp1 locus. Moreover, their impact on virulence was additive, suggesting that they play different roles in infection. Therefore these two Mn^{2+} transporters are STM virulence factors whose role in virulence is enhanced in the presence of the host-resistance protein Nramp1.

As expression of *mntH* and *sitA* was interdependent with host cell genotype (i.e. the presence or absence of Nramp1), we chose to use these genes as tools in an attempt to decipher the bacteriostatic effect of Nramp1 on the intracellular bacteria. In order to use these loci most effectively we had to characterize the regulatory networks involved in *mntH* and *sitA* expression more completely. Thus, the effect of Fur, MntR, OxyR and the virulence-associated regulatory systems HilA, SsrA, PhoPQ and PmrAB were investigated to determine their impact on *mntH* and *sitA* expression. The major SPI1 and SPI2 transcriptional regulators, HilA and SsrAB respectively, as well as the Fe^{3+}-responsive PmrAB had a minor effect on expression of *sitA*, while PhoPQ and PmrAB had a slight effect on expression of *mntH*. Although this represents the first study into the effect of two of these regulatory systems on *mntH* expression, these results contradict previous studies by Janakiraman and Slauch (135), which demonstrated that *sitA* was not a member of the HilA or PhoPQ regulons. Therefore the role of these regulatory systems remain undefined.

In contrast, OxyR positively regulates *mntH* in response to oxidative stress generated by growth in the presence of hydrogen peroxide. This effect was additive upon chelation of divalent cations. In contrast, *sitA* was not regulated by *oxyR*. However, both genes were negatively regulated by the Fe^{2+}-responsive regulatory protein, Fur, and the novel Mn^{2+}-repressor, MntR. These results corroborate recent work by Kehres *et al.* (142)

that confirmed that *mntH* is regulated by extracellular hydrogen peroxide, Fe^{2+} and Mn^{2+} levels. Their study further suggested that there is cross-talk between Fur and MntR, such that *mntH* is regulated by Fe^{2+} primarily through Fur and by Mn^{2+} primarily through MntR, although MntR can repress *mntH* expression in response to Fe^{2+} levels, especially if Fur is absent.

As discussed in section 1.7.2, Nramp1 is involved in transport of divalent cations within subcellular compartments. Nramp1 has also been implicated in altering the activation state of macrophages, which may influence the oxidative burst of these phagocytic cells and thus their production of reactive oxygen intermediates. Expression of *mntH* was found to be responsive to hydrogen peroxide levels as a measure of oxidative stress as well as divalent cation concentration. Thus, this presented us with the unique opportunity to use *mntH* expression as a tool to investigate the cellular conditions altered by the presence of functional Nramp1 in the macrophage. A series of plasmids deleted for the OxyR, MntR and Fur boxes of the *mntH* promoter region as obtained from Dr. Kehres along with insertional inactivation mutants of *oxyR*, *mntR* and *fur* to study expression of *mntH* inside cells. The deletion plasmids were crucial for these experiments, as deletion of chromosomal loci has been observed to affect intracellular behavior of the bacterium in a manner apparently unrelated to the predicted effect of the gene-specific deletion (discussed in ref (278)). Therefore, it was believed that the deletion plasmids would be preferable for studying *mntH* expression *in vivo* over knocking out entire global regulatory systems that would be predicted to have extensive effects on the bacterium unrelated to expression of *mntH*.

Of the three regulatory systems studied, only OxyR had a significant effect on *mntH* expression inside the murine macrophage-like RAW cells. Deletion of the OxyR box reduced expression of *mntH* in Nramp1- cell line over the entire time course, yet

diminished *mntH* expression only within the first 6 h post-infection in the Nramp1+ cell line. Thus, it appears that oxidative stress is a stimulus sensed by the bacterium upon infection, regardless of the host resistance background. This agrees with numerous other studies which demonstrate that reactive oxygen and nitrogen intermediates are important for host cell defense (ref (257) and references therein). In contrast, expression of *mntH* in the presence of Nramp1 at later time points is regulated by a stimulus other than oxidative stress and this later response must be due to a regulatory system other than OxyR. Further elucidation of the role of oxidative stress on *mntH* expression, including attempts to chemically induce or repress the oxidative burst of infected macrophages were inconclusive.

Experiments using the Fur box and MntR box deletion plasmids as well as the chromosomal knockout strains to test the roles of Fur and MntR on *mntH* expression in the cell culture model were inconclusive. Therefore no definitive conclusion can be drawn for the role of Fur and MntR on expression of *mntH* in the presence of Nramp1 during intracellular survival. However, a hypothesis combining the effects of oxidative stress and divalent cation limitation on the intracellular bacterium remains to be tested. It is tempting to speculate that the bacterium senses oxidative stress through OxyR as a signal of residence within a macrophage resulting in induction of certain loci required to combat this phase of the host defense. At a slightly later point in infection the impact of Nramp1 through depletion of cations from the SCV would provide a secondary stimulus for virulence-associated gene expression through the Fe^{2+}- and Mn^{2+} -responsive regulators, MntR and Fur. This hypothesis implies that Fe^{2+} and Mn^{2+} are important for the intracellular survival of STM and that the role of MntH and SitABCD in virulence is to supply STM with sufficient Fe^{2+} and/or Mn^{2+} to maintain cellular functions and intracellular survival. However, it also would explain the ability of Nramp1 to mediate

bacteriostasis on the basis of depriving the intracellular bacterium of essential divalent cations essential for intermediary metabolism and survival.

In conclusion we have identified that the STM Mn^{2+} transport systems MntH and SitABCD are virulence factors in the murine typhoid model and are interdependent on host genotype, specifically the presence of the host-resistance protein Nramp1. Expression of *mntH* and *sitA* was similarly regulated by the ion-responsive regulatory systems Fur and MntR, consistent with their role as divalent cation transport systems. In addition, *mntH* was responsive to oxidative stress, which was useful in attempting to identify the impact of Nramp1 on the intracellular environment *in vivo*. These studies indicate that the alteration of the oxidative burst via the production of hydrogen peroxide is not a significant function of Nramp1 during infection. However, a direct correlation between divalent cation concentration and Nramp1 function was not satisfactorily determined. Therefore, the impact of the function of Nramp1 on intracellular STM was not conclusively defined.

CHAPTER 4: INVESTIGATING INTERACTIONS BETWEEN SPI2 AND NRAMP1

4.1 Effect of SPI2 on replication in the presence of Nramp1

It has been established that the SPI2 TTSS and associated effectors are necessary for full virulence of STM in the murine typhoid model (228). However these studies have all been carried out in *Nramp1*$^{-/-}$ mice and cultured cell lines or in non-congenic *Nramp1*$^{+/+}$ and *Nramp1*$^{-/-}$ animal lineages (54, 122, 196, 227, 228). It is also well documented that expression of functional *Nramp1* controls replication of STM both *in vivo* and *in vitro* (99). Therefore, we compared the role of SPI2 in bacterial survival and replication in congenic *Nramp1*$^{+/+}$ and *Nramp1*$^{-/-}$ mice (Figure 18).

Previous studies in our laboratory using the same infection model determined that 100% of *Nramp1*$^{-/-}$ animals infected with wild-type STM were overwhelmed by the bacteria at the dose used and succumbed to infection by day 5 post-infection. In contrast, the bacterial load in the liver and spleen of *Nramp1*$^{+/+}$ animals peaked at day 4 post-infection and plateaued thereafter with the animals ultimately surviving the infection ((98); B.A. Vallance, personal communication). In agreement, we observed extensive replication of wild-type bacteria in the *Nramp1*$^{-/-}$ animals; by Day 4 30% of the animals in this group had succumbed to infection (Figure 18). Although *Nramp1*$^{+/+}$ mice do not usually succumb to the infection, we also observed that wild-type bacteria replicated significantly in these animals at the dose used. In contrast, bacteria in which SPI2 is not expressed and thus do not have a functional SPI2 TTSS due to an insertion in the SPI2 two component regulatory system *ssrAB* (CS382; *ssrA*::Km) behaved nearly identically in both strains of mice, replicating to a limited extent in both the presence and absence of functional Nramp1. This indicates that SPI2 is essential for maximal bacterial replication in both *Nramp1*$^{-/-}$ and *Nramp1*$^{+/+}$ mice.

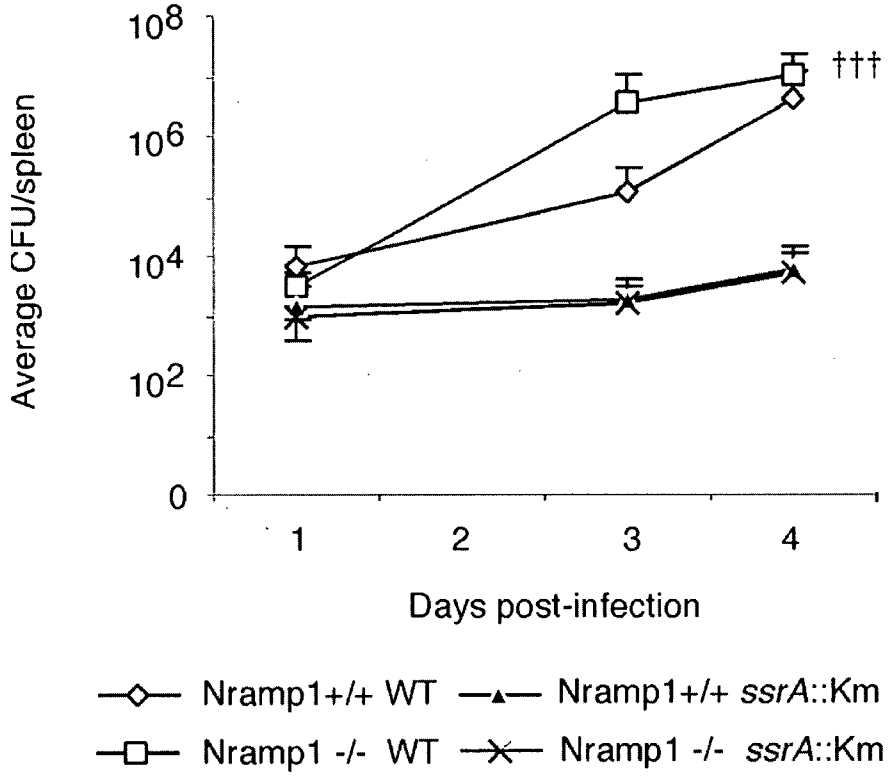


Figure 18. Role of SPI2 in replication of STM in murine spleen in the presence of Nramp1. Age and sex-matched congenic 129sv/J mice were infected intraperitoneally with 10^5 cfu of either wildtype or SPI2- (*ssrA*::Km) STM 14028s and spleens harvested for bacterial enumeration at designated time points as described in Section 2.6.4. Data represent the average ± the SEM of two experiments performed on experimental groups of five mice. † indicates death of an animal at the relevant time point.

4.2 Gene selection and creation of transcriptional fusions

The presence of Nramp1 in the membrane of the SCV during infection of macrophages may influence the composition of the intravacuolar environment due to Nramp1's putative role as a divalent cation transporter (228). As a number of virulence genes are regulated in response to divalent cation concentration (notably the PhoPQ regulon) it was possible that depletion of the SCV of divalent cations by Nramp1 could affect expression of STM virulence genes. Considering the importance of SPI2 for intramacrophage survival of STM, the characterized bacteriostatic effect of Nramp1 on STM could be the result of decreased transcription of SPI2 genes in response to altered divalent cation concentration in the SCV. Accordingly, three SPI2-associated genes were selected to investigate the effect of Nramp1 on STM virulence gene expression: *ssrA* , the sensor kinase of the SsrAB virulence-associated TCRS (155); *sseA* , the first gene of the proposed SPI2 effector operon; and *sseJ* (217), a recently characterized SPI2 effector encoded outside SPI2. In addition, the locus encoding the response regulator for the PhoPQ TCRS (*phoP)* and the SPI1-encoded regulatory gene *hilA* were also tested. PhoP is involved in intramacrophage survival and the control of the most extensive virulence-associated regulon known to date (102). *hilA* plays a central role in the expression of SPI1 loci (166), and was used as a negative control as SPI1-associated genes are believed to be poorly expressed in the intracellular environment.

Pioneering studies have identified the expression of certain bacterial genes during infection (63, 155, 205, 255), but with one recent exception (44) they are qualitative. They also do not give us much information on the time course of expression. To quantify virulence gene expression over time, transcriptional fusions to *phoP*, *ssrA* and *sseA* were generated by PCR amplification using oligonucleotides with engineered *Eco*RI and *Hind*III restriction sites in the 5′ and 3′ oligonucleotides, respectively. Fragments were digested with *Eco*RI and *Hind*III and ligated into the similarly digested monocopy

transcriptional fusion vector pFZY1 to yield pPHOP, pSSRA and pSSEA (Figure 19). Construction of pHILA involved PCR amplification as before and direct ligation of the fragment into pCR2.1 prior to *Eco*RI-*Hind*III digestion and ligation into pFZY1. Construction of pSSEJ took advantage of a naturally occurring *Hind*III site within the *sseJ* coding region. An *sseJ* fragment was PCR amplified and ligated directly into pCR2.1, digested with *Eco*RI-*Hind*III and ligated into pFZY1. Constructs were screened by agarose gel electrophoresis and sequenced to verify the integrity and accuracy of the insert. Correct plasmids were transformed into wildtype STM 14028s, were stably maintained by the bacterium during a 24 h infection of activated RAW 264.7 cells, and carriage of the transcriptional fusion plasmid had no appreciable effect on survival of the bacterium during infection (not shown).

4.3 Expression of SPI2-associated genes in cultured cell lines

STM 14028s bearing the transcriptional fusions described above was used to infect activated RAW 264.7 cells, and expression of these SPI2-associated genes in internalized versus extracellular STM was compared. As shown in Figure 20, expression of all five loci was low in DMEM alone, and for the most part decreased over the 24 h time course. In contrast, Figure 21 shows that expression of all five loci was induced in the intracellular environment compared to extracellular bacteria at the same time point. At 2 h post-infection *hilA* was induced 7-fold, *phoP* was induced 10-fold, *ssrA*, was induced 9-fold, *sseA* was induced 13-fold, and *sseJ* was induced 80-fold. Induction of *hilA* is a novel finding, as the major target of HilA regulation (SPI1) is not believed to be required for intramacrophage survival. Therefore, it has been hypothesized that *hilA* , and

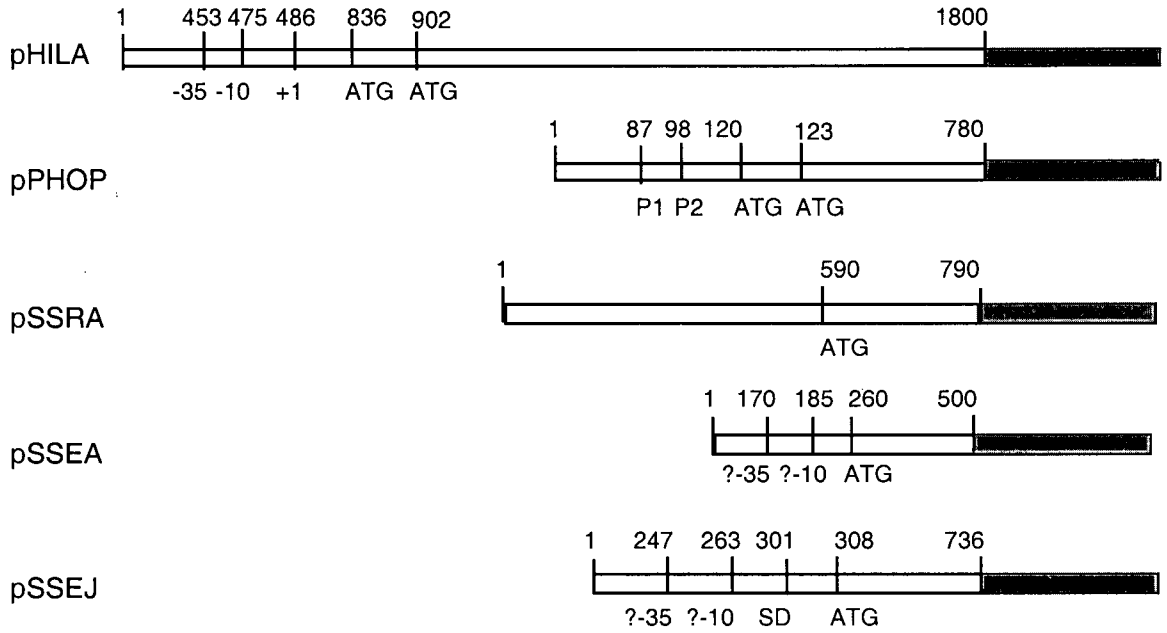


Figure 19. Illustration of the transcriptional fusion plasmids pHILA, pPHOP, pSSRA, pSSEA and pSSEJ. Open boxes represent cloned DNA of the gene of interest; closed boxes represent lacZY DNA from pFZY1. The numbers depicted above the boxes represent nucleotide positions determined from the beginning of the cloned sequence. The –35, -10 and transcriptional start sites (+1) for *hilA* are defined, and two translational start sites (ATG) have been postulated (223). The specific –35 and –10 regions for *phoP* have yet to be conclusively defined, but two transcriptional start sites have been characterized, shown here as P1 and P2 (235). The promoter region for *ssrA* remains undefined. Manual scanning of the upstream regions of *sseA* and *sseJ* identified potential –35 and –10 regions, marked with a "?". Diagram is not drawn to scale.

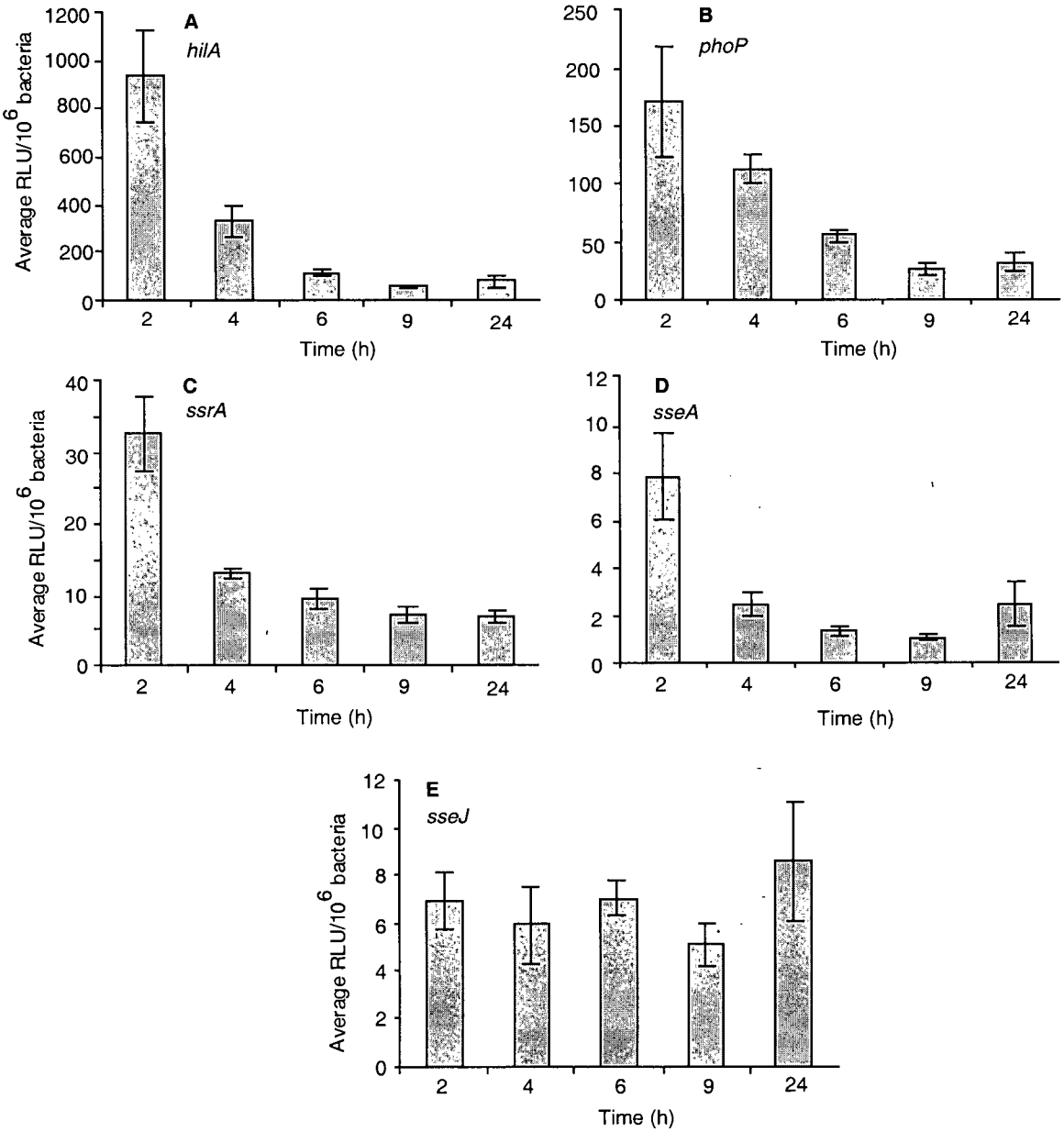
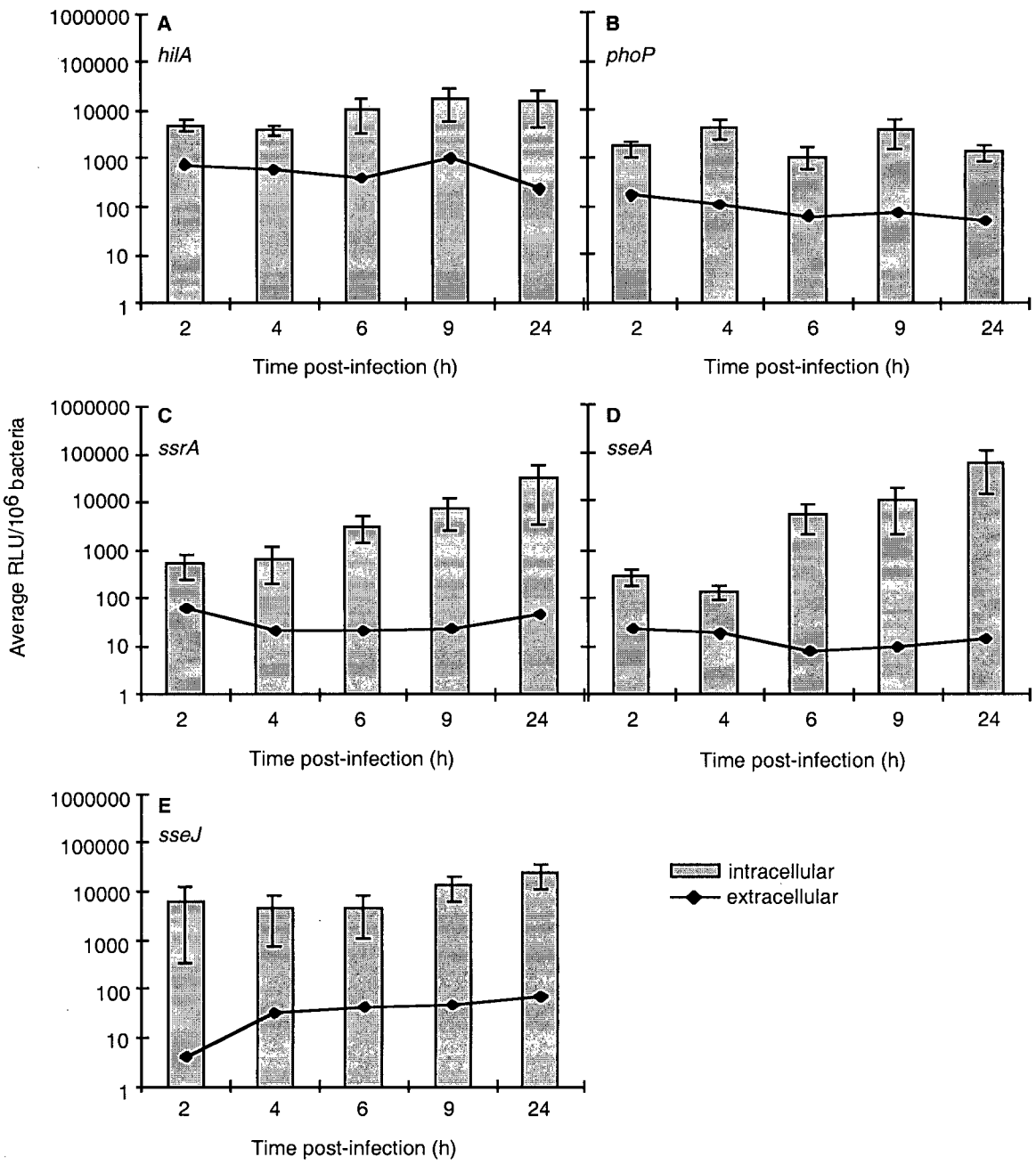


Figure 20. Expression of SPI2-associated genes in DMEM. Bacteria were subcultured into Dynex Microlite-1 96-well microtitre plates containing 100 µL of DMEM containing 200 U/mL of IFN-γ for up to 24 h, following time points identical to those described for the intracellular gene expression assay (Section 2.5.3). β-galactosidase activity and cfu counts were assayed as described in Section 2.5.3.

Figure 21. Regulation of STM virulence gene expression upon infection of activated RAW 264.7 cells. Bacteria were grown to mid-log phase and allowed to infect host cells for 30 minutes prior to either removal of remaining bacteria to a host-cell-free 96 well plate (extracellular bacteria), or to gentamicin treatment to kill remaining non-internalized bacteria (intracellular bacteria). Results shown represent β-galactosidase activity defined as the average number of Relative Light Units normalized to the number of bacteria present in each well as determined by parallel plate counts (see Section 2.5.2), and multiplied by 10^6 to obtain larger numbers for easier interpretation. Three experiments were performed with duplicate wells and all results were averaged. Error bars represent the SEM.

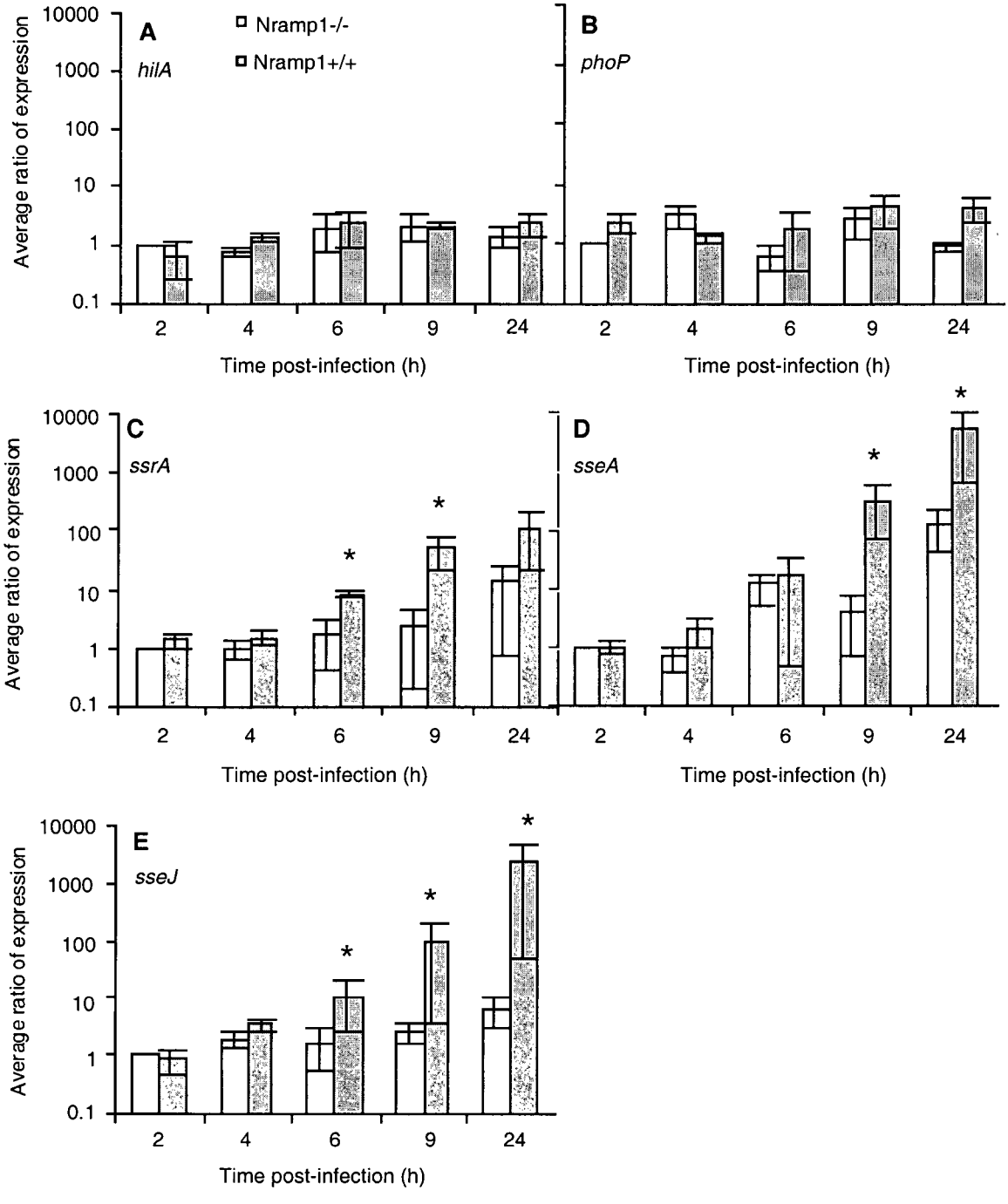

A
hilA
B
phoP
C
ssrA
D
sseA
E
sseJ
1000000
100000
10000
1000
100
10
1
2
4
6
9
24
Time post-infection (h)
Average RLU/10^6 bacteria
intracellular
extracellular

subsequently SPI1, would not be expressed in the intracellular environment. In contrast, *phoP* and other *pags*, and SPI2 genes including *ssrA*, are upregulated within the macrophage (7, 121, 155). The five genes differed in their patterns of intracellular expression over the 24 h time course. Expression of *hilA* and *phoP* did not change significantly over 24 h, while expression of the SPI2 genes increased dramatically (55-fold, 213-fold and 4-fold for *ssrA*, *sseA* and *sseJ*, respectively), a pattern previously described for SPI2 genes (155).

The impact of Nramp1 on the expression of the selected genes was determined by infecting the stably transfected Nramp1+ or Nramp1- cell lines (described in Chapter 3) with STM carrying the respective transcriptional fusion of interest. Gene expression is calculated as a ratio of the expression of each gene at the given time point compared to the value at the 2 h time point in the Nramp1- cell line. The expression of *hilA* and *phoP* was not affected by the presence of transfected Nramp1 (Figure 22, panels A and B). In contrast, the SPI2-associated genes demonstrated low expression levels for the first few hours of infection and then increased between 6 and 24 h post-infection (Figure 22, panels C-E). In the Nramp1- cell line expression of *ssrA*, *sseA* and *sseJ* increased approximately 15-, 125- and 7-fold respectively between 2 and 24 h. It is unknown why these induction levels are so much lower than those observed in the untransfected RAW cells. However, as this same phenomenon was also observed using the *mntH* and *sitA* transcriptional fusions as described in Section 3.2, this may be due to unknown effects of pCB6 on the host cell.

During infection of the Nramp1+ cell line induction of the SPI2-associated genes (again, normalized to the corresponding 2 h time point in the Nramp1- cells) rose to a total of 80-, 5,000- and 3,000- fold during the same time span (Figure 22, panels C-E).

Figure 22. Effect of Nramp1 on expression of SPI2-associated genes. Bacteria were allowed to invade RAW cells transfected with either empty pCB6 vector alone (Nramp1-) or pCB6 carrying a functional Nramp1 gene (Nramp1+) and gene expression monitored at selected time points over a 24 h time course. β-galactosidase activity and bacterial cfu were assayed as described in Section 2.5.2. The ratio of expression of each gene at each time point was set to the 2 h time point in the Nramp1- cell line. Six experiments were performed with duplicate wells and all results were averaged. Error bars represent the SEM. Asterisks indicate statistical significance to $P<0.05$ as determined by the Wilcoxan Rank Sum test for unpaired samples.


A
hilA
Nramp1-/-
Nramp1+/+
B
phoP
C
ssrA
D
sseA
E
sseJ
Average ratio of expression
10000
1000
100
10
1
0.1
2
4
6
9
24
Time post-infection (h)
*

Comparison of the expression of each gene at 24 h between the two cell lines demonstrated that *ssrA*, *sseA* and *sseJ* are upregulated a total of 8-, 40- and 360-fold, respectively, in the presence of Nramp1. This response is attributable to the alteration of intracellular conditions by Nramp1 as non-internalized (i.e. "extracellular") bacteria exposed to the Nramp1+ and Nramp1- macrophages displayed lower levels of gene expression than those observed in internalized bacteria and were not significantly different based on host cell genotype (Figure 23). Therefore the effect of Nramp1 on SPI2 gene expression is an intracellular phenomenon.

4.4 Expression of SPI2-associated genes in intact murine spleen

The observation that SPI2-associated genes were upregulated in the presence of Nramp1 was unexpected, as we had hypothesized that Nramp1 may be turning off expression of SPI2 in order to slow the growth of the bacterium so that the host had a better opportunity to eliminate the infection. To confirm that observations obtained with the transfected cell lines were representative of events that occur during *in vivo* infection, we studied the expression of these genes in congenic *Nramp1*$^{+/+}$ and *Nramp1*$^{-/-}$ mice. Both *hilA* and *phoP* were expressed in both strains of mice, but neither gene's expression was significantly affected by Nramp1 (Figure 24). In contrast, all three SPI2 loci displayed higher expression levels in the *Nramp1*$^{+/+}$ mice, on average 10-, 5- and 35-fold increases for *ssrA*, *sseA* and *sseJ*, respectively (*ssrA* and *sseJ* values are statistically significant) when normalized to expression levels observed in the *Nramp1*$^{-/-}$mice. Thus, in both a transfected cell line model and the murine typhoid infection model, the presence of functional Nramp1 correlated with significant upregulation of SPI2-associated genes.

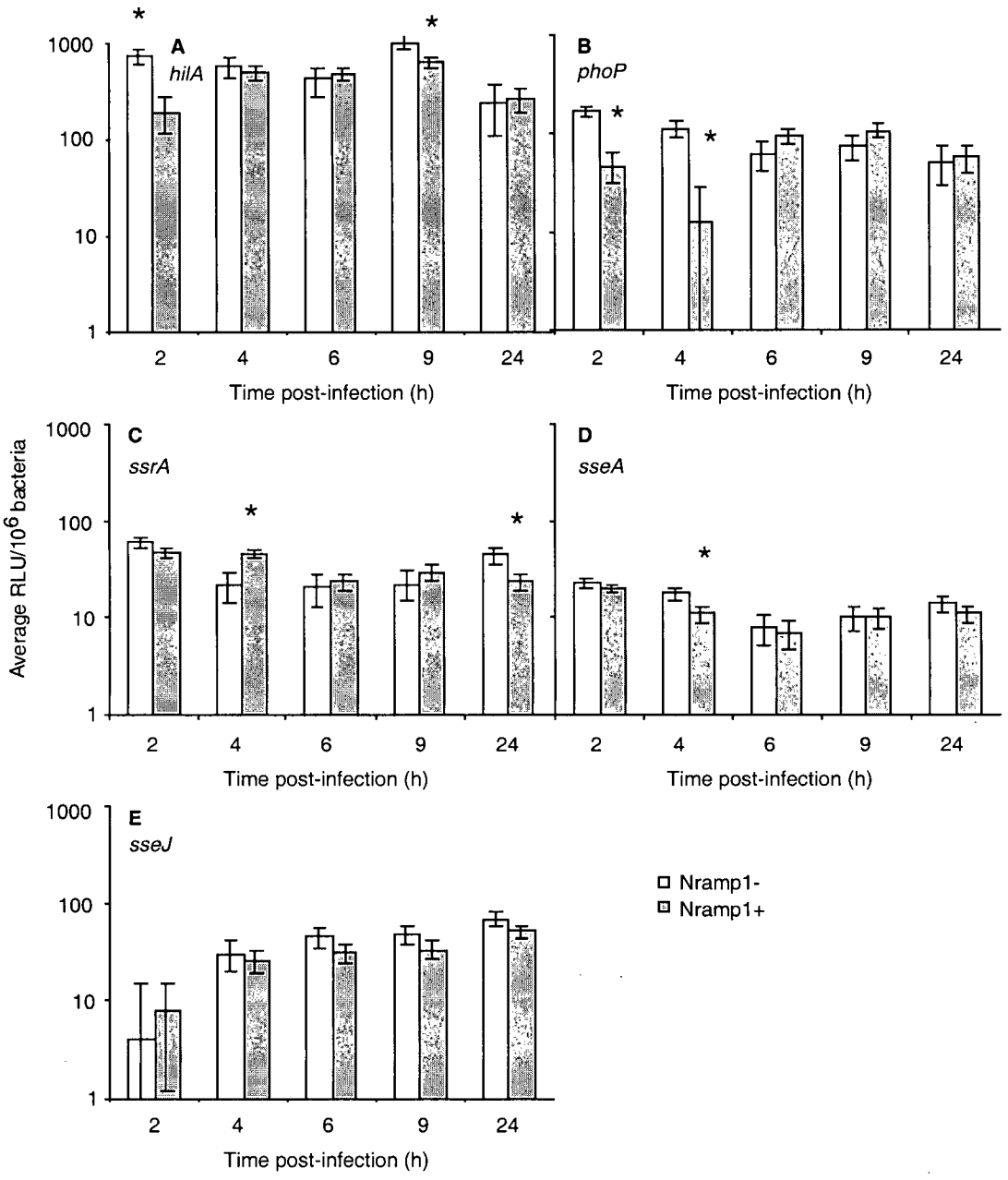


Figure 23. Expression of STM virulence genes in extracellular bacteria. Bacteria were allowed to infect the cell lines and non-internalized bacteria removed to a parallel host-cell-free plate for further incubation as described in Section 2.5.1. Three experiments were performed in duplicate and all results were averaged. Error bars represent the SEM. Asterisks indicate statistical significance to $P<0.01$ as determined by the Student's t-test.

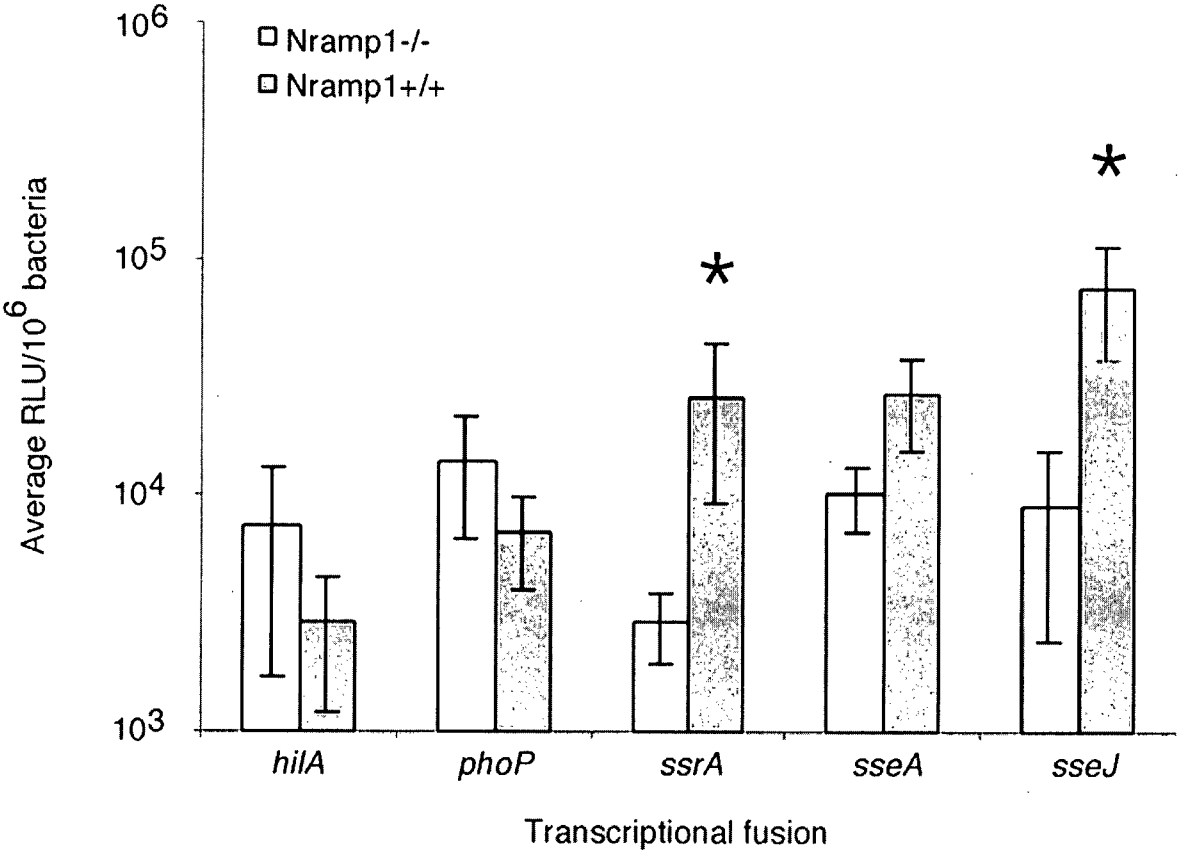


Figure 24. Expression of SPI2-associated genes is upregulated in spleens of STM infected *Nramp1*$^{+/+}$ 129sv mice. An average of 2.5x10^5 STM 14028s bearing the different transcriptional fusion plasmids were injected intraperitoneally into age- and sex-matched 129sv mice and spleens harvested at 24 h post-infection for evaluation of β-galactosidase activity as described in Section 2.5.3. These data represent the mean ± the SEM of five separate experiments performed on groups of five mice per fusion of interest. Asterisks indicate statistical significance to $P<0.05$, as determined by the Wilcoxan Rank Sum test for unpaired samples.

4.5 Effect of chelators of divalent cations on expression of SPI2-associated genes

The specific effects of Nramp1 on the intramacrophage environment have not been established, although some reports indicate that it affects divalent cation concentrations within the SCV. As Nramp1 appeared to be modulating the expression of SPI2-associated genes within the macrophage, we wanted to investigate if altering divalent cation concentration affected SPI2-associated gene expression. Therefore, we tested the effect of four different chelators on SPI2 gene expression in axenic cultures, including the general chelators EGTA and EDTA, and the putative "iron specific" chelators dipyridyl (DiP) and DTPA (135). The normal medium used in these studies was N minimal media pH 7.4, previously defined as SPI2 expression media (68) because it is thought to more closely mimic the intravacuolar environment.

Figure 25 demonstrates that addition of the general chelators EGTA and EDTA significantly induced the expression of all five genes to varying extents, while the "iron-specific" chelators DiP and DTPA had a significant impact on the expression of *phoP* and theSPI2-associated genes but had no effect of *hilA* expression. Between *phoP* and the SPI2-associated genes, EDTA, EGTA and DiP had essentially identical effects on gene expression levels (approximately 6-fold induction for *phoP, ssrA,* and *sseA* and 17-fold for *sseJ*) while DTPA was less effective (approximately 3-fold induction for *phoP, ssrA,* and *sseA* and 5-fold for *sseJ*). These results indicate that these virulence-associated loci respond to divalent cation levels and appear to either be repressed by high concentrations of divalent cations or activated by low concentrations of divalent cations.

4.6 Effect of specific divalent cations on virulence gene expression

Addition of chelators to growth media results in chelation of many different cations, and the only way to truly test which cation(s) are involved in this induction of expression of the genes of interest is to add excess cation back to chelated media. As the

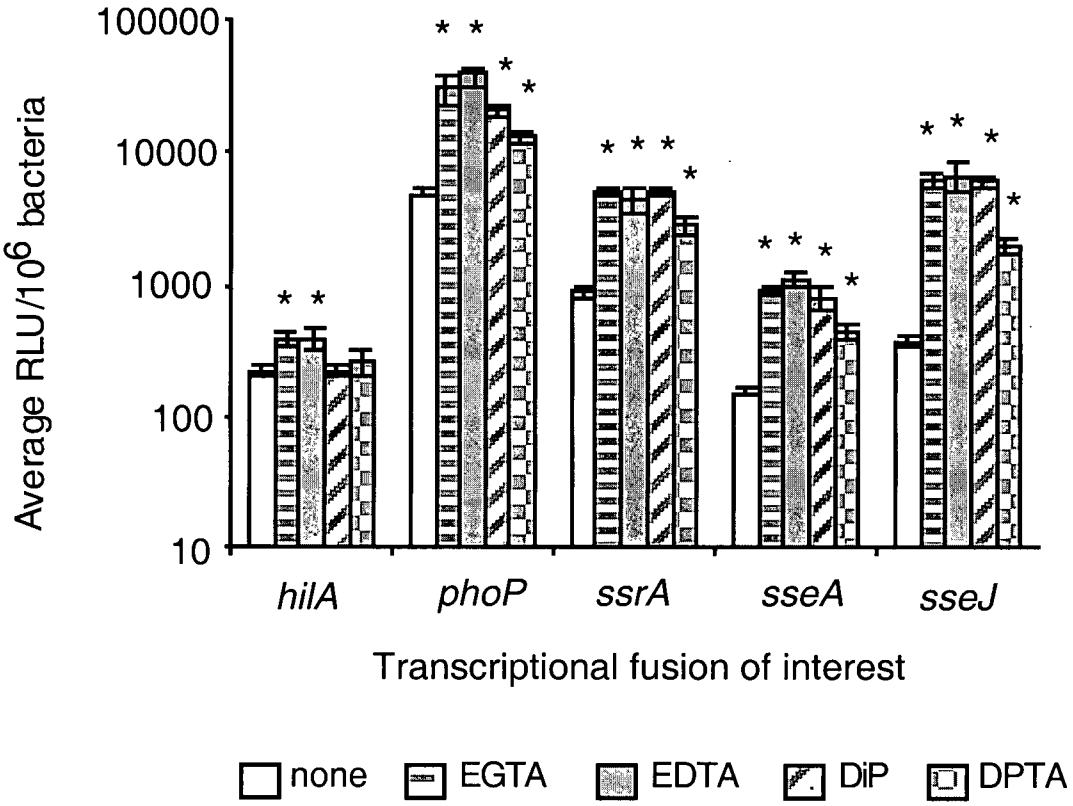


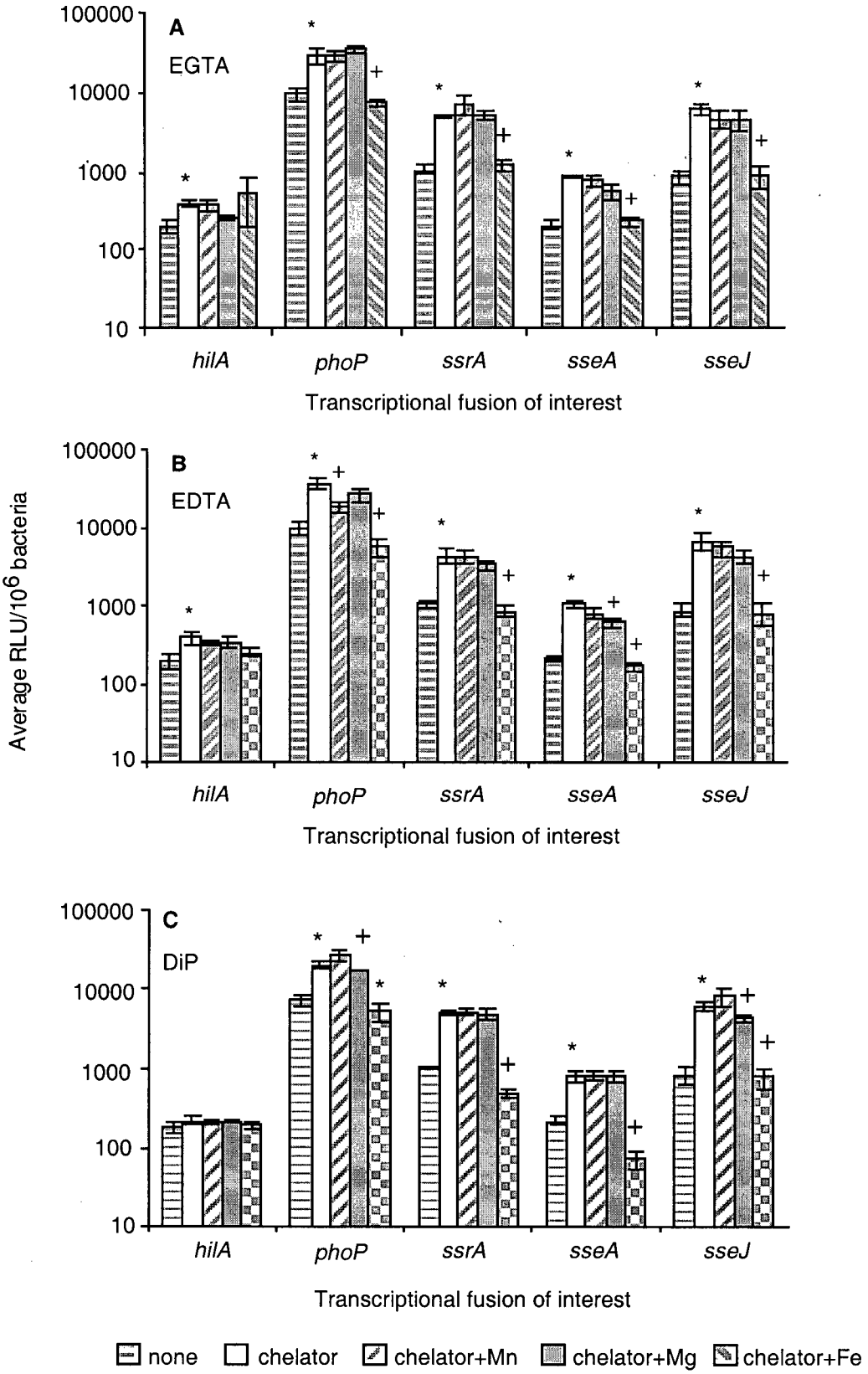
Figure 25. Effect of chelators of divalent cations on STM virulence gene expression. Bacteria were grown overnight in NMM (8 μM $MgCl_2$) supplemented with one of EGTA, EDTA, DiP or DTPA at a final concentration of 250 μM. β-galactosidase activity and bacterial cfu were assayed as described in Section 5.2.3. Experiments were performed three times in triplicate and results represent the average of all data points. Error bars are the SEM. Asterisks indicate statistical significance to $P<0.01$ as determined by the Student's t-test.

changes in expression patterns were most dramatic using EGTA, EDTA or DiP as chelators, further studies omitted DTPA. It was observed that upon re-addition of excess amounts (500 μM) of the various divalent cations to the chelated media, only excess $FeSO_4$ consistently repressed expression of these four genes to basal levels (Figure 26). Thus this upregulation of these four genes may be due to depletion of iron from the growth media.

These experiments were carried out in media at pH 7.4, a pH at which iron is normally in the insoluble Fe^{3+} form. As we were most interested in the effect of Fe^{2+} on gene expression, experiments using DiP as a chelator were repeated with the addition of ascorbate to keep iron in the soluble Fe^{2+} form. As shown in Figure 27, expression of *phoP* and the SPI2-associated genes was still induced by growth in DiP (approximately 3-fold for *phoP, ssrA* and *sseA*, and 4-fold for *sseJ*), and repressed upon re-addition of $FeSO_4$. Thus is appears that these genes can be regulated at the transcriptional level by either the ferrous or ferric forms of iron.

However, it was possible that adding such high concentrations of chelators and divalent cations may be affecting bacterial growth rate. To address this issue, doubling times for wild-type 14028s in NMM alone, NMM+DiP (250 μM), or NMM+DiP_{250}+$FeSO_4$ (500 μM) were calculated. It was determined that the doubling times for growth in NMM alone or NMM supplemented with both DiP and $FeSO_4$ were essentially the same, being 170 and 180 min, respectively. In contrast, the doubling time for growth in DiP-chelated media was nearly twice as long, measured at 335 min. As the cultures used in the above assays were all harvested at 180 min (3 h), they would be at very different stages of growth phase. As a result, the "inductive" effect observed with DiP-chelated media may be an artifact of growth phase and not a response to cation concentration.

Figure 26. Effect of addition of excess $FeSO_4$ to chelated NMM on STM SPI2-associated virulence gene expression. Bacteria were subcultured in NMM containing either: no supplement; the desired chelator at a final concentration of 250 μM; or the desired chelator (250 μM) and the divalent cation of interest at a final concentration of 500 μM. β-galactosidase activity and bacterial cfu were assayed as described in Section 2.5.3. Experiments were carried out three times in triplicate and the results depict the average of all data points. Error bars represent the SEM. * indicates statistical significance between the untreated control compared to the chelator-treated sample to $P<0.01$ as determined by the Student's t-test. + indicates statistical significance to $P<0.01$ between samples treated with the chelator alone and samples treated with excess divalent cations as determined by the Student's t-test.


A
EGTA
B
EDTA
C
DiP
100000
10000
1000
100
10
Average RLU/10^6 bacteria
hilA
phoP
ssrA
sseA
sseJ
Transcriptional fusion of interest
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chelator
chelator+Mn
chelator+Mg
chelator+Fe

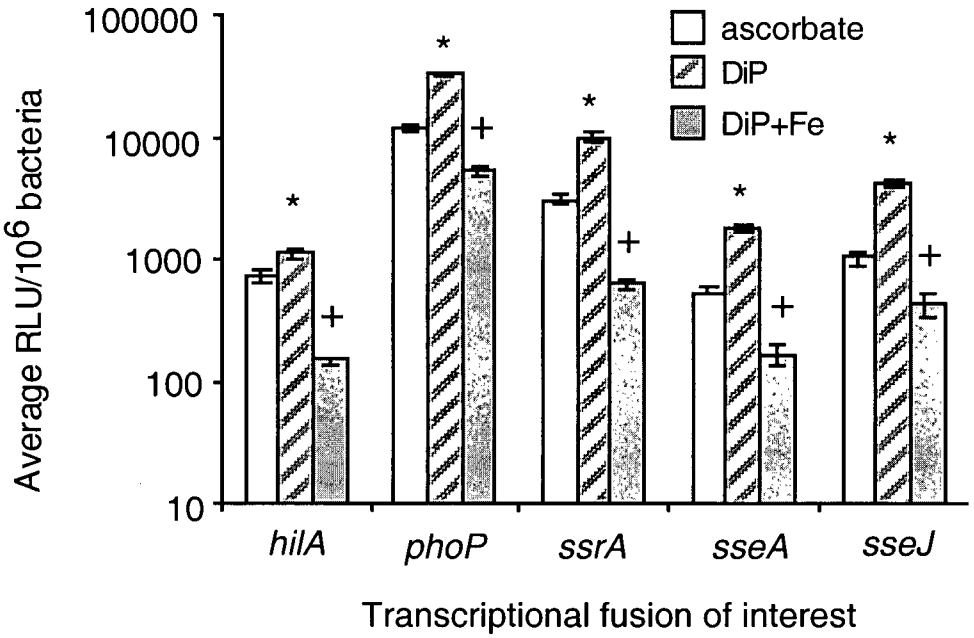


Figure 27. Effect of Fe^{2+} on expression of STM virulence genes. Bacteria were subcultured in NMM containing either: ascorbate (5 mM); ascorbate+DiP (250 μM); or ascorbate+DiP+$FeSO_4$ (500 μM). β-galactosidase activity and bacterial cfu were assayed as described in Section 2.5.3. Experiments were carried out three times in triplicate and the results depict the average of all data points. Error bars represent the SEM. * indicates statistical significance to $P<0.01$ between the ascorbate control and the DiP-treated sample, determined by the Student's t-test. + indicates statistical significance to $P<0.01$ between the DiP- and DiP+Fe-treated samples, determined by the Student's t-test.

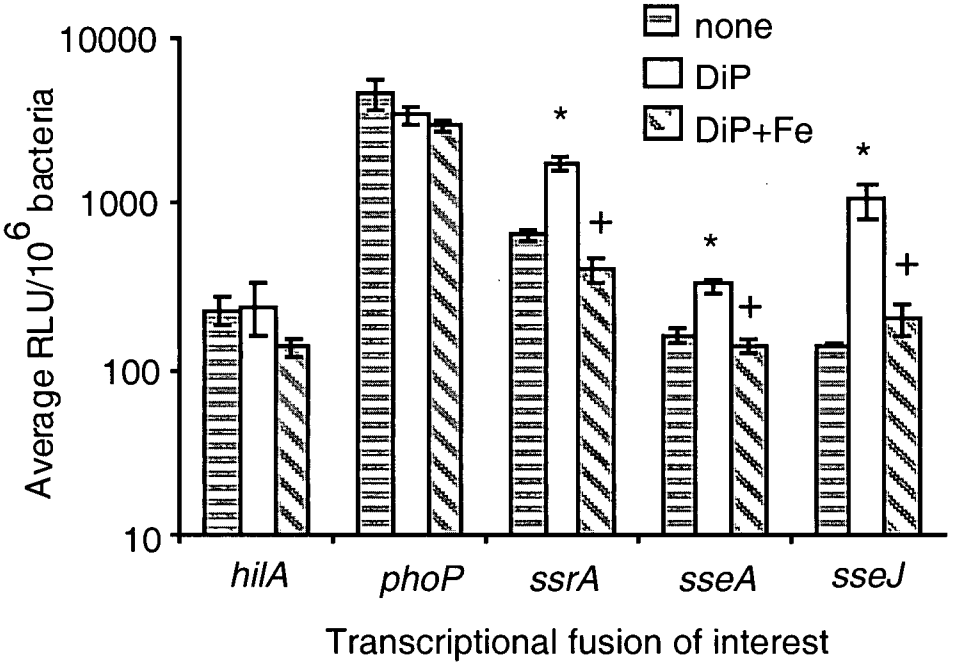


Figure 28. Expression of STM virulence gene expression upon addition of DiP after normalization for growth phase. Bacterial cultures were supplemented as in previous experiments and were harvested at either 180 min (NMM and DiP+ $FeSO_4$ supplemented cultures) or 350 min (DiP alone). β-galactosidase activity and bacterial cfu were assayed as described in Section 2.5.3. Results represent the average of three experiments performed in triplicate. Error bars represent the SEM. * indicates statistical significance to $P<0.01$ between the ascorbate control and the DiP-treated sample, determined by the Student's t-test. + indicates statistical significance to $P<0.01$ between the DiP- and DiP+Fe-treated samples, determined by the Student's t-test.

To address this issue, the DiP chelation and iron addition assay was performed as previously described with the NMM and $FeSO_4$-supplemented cultures harvested at 180 min, while the NMM+DiP cultures were harvested at 335 min. As shown in Figure 28, after this normalization for growth phase, *phoP* no longer demonstrated induction of expression in the presence of DiP and was not significantly affected by addition of excess iron. In contrast, the SPI2-associated genes were still significantly induced by DiP and repressed by excess iron. Therefore, the SPI2-associated genes appear to be responsive to iron concentration in axenic cultures.

As there has been controversy as to the direction of transport of ions by Nramp1, there remained a possibility that the effects on gene expression observed in the transfected cell lines and *in vivo* were the result of a response to an increase in divalent cation concentration. To address this issue, the same strains were grown in NMM alone or supplemented with one of $MgSO_4$, $MnSO_4$ or $FeSO_4$ at 50 μM, after ensuring that the growth rates were not changed upon addition of any of these ions alone. As shown in Figure 29, *hilA* was unresponsive to the addition of any of these cations. *phoP* alone was significantly affected by addition of $MgSO_4$; this is not unexpected for *phoP*, as *phoP* is positively regulated by PhoPQ and therefore repressed by high Mg^{2+} concentrations. In contrast, addition of any of the divalent cations had no significant effect on expression of *ssrA*, *sseA* or *sseJ*. Thus it appears that if STM is responding to an alteration in divalent cation concentration in the SCV mediated by activity of Nramp1, it is not the result of an increase in the concentration of divalent cations in the SCV.

4.7 Effect of regulatory systems on virulence gene expression

As iron levels may be a novel stimulus for SPI2-associated gene expression, we attempted to identify the regulatory network involved in this response. We used insertional inactivation mutants to test a number of different regulatory systems for their

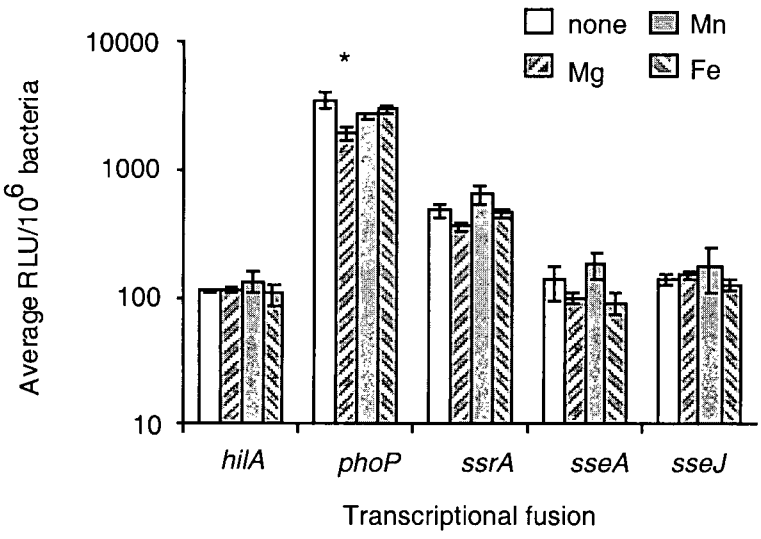


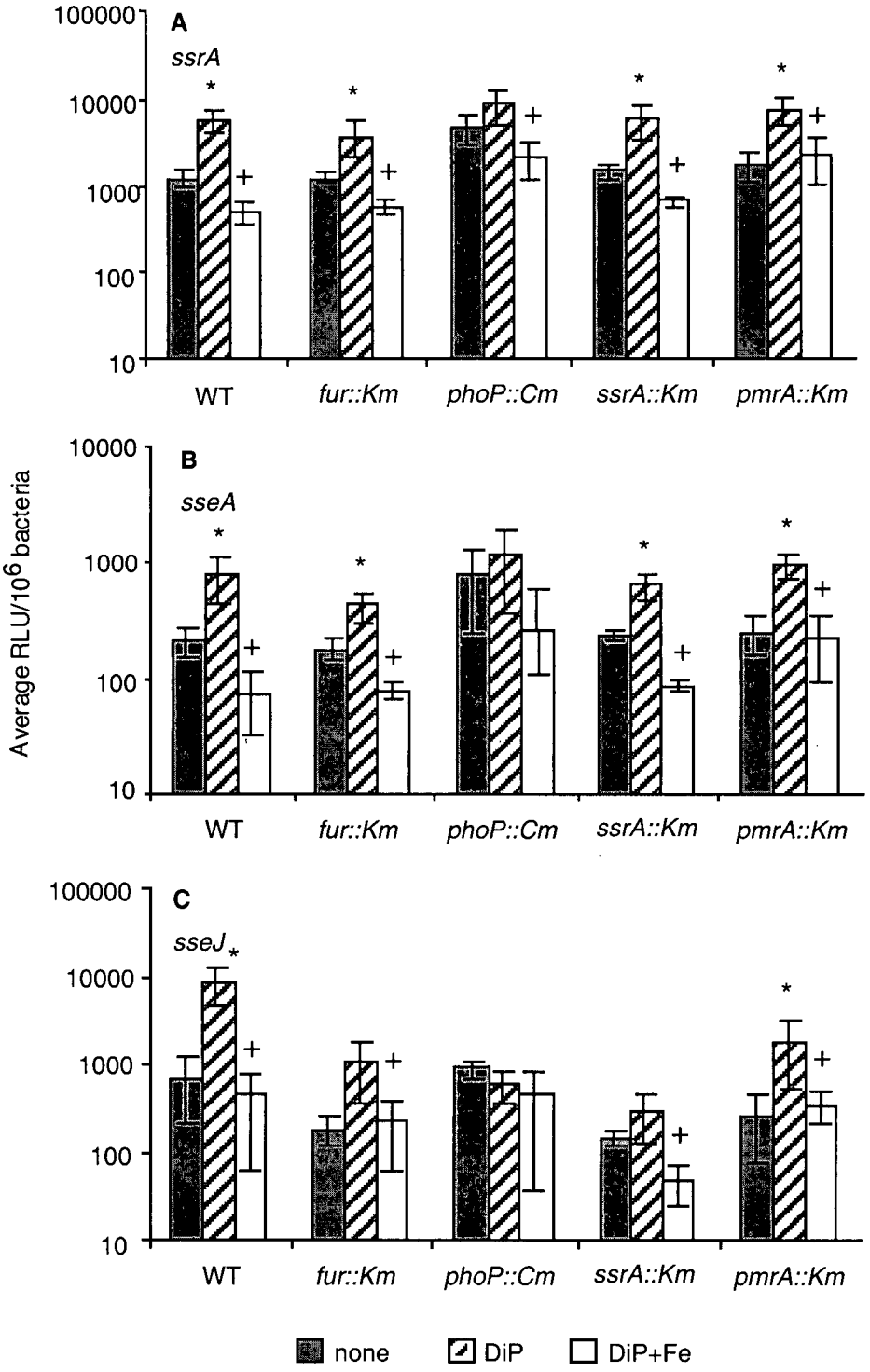
Figure 29. Effect of adding divalent cations to normal NMM on STM virulence gene expression. Bacterial cultures were supplemented with the divalent cation of interest at a final concentration of 50 μM. β-galactosidase activity and bacterial cfu were assayed as described in Section 2.5.1. Experiments were carried out three times in triplicate and all results averaged. Error bars represent the SEM. Asterisks indicate statistical significance to $P<0.01$ as determined by the Student's t-test.

potential involvement including the global regulator Fur (MZ201; *fur*::Km) and the Fe^{3+}-responsive TCRS PmrAB (JSG421; *pmrA*::Tn10). PhoPQ was investigated in case it may respond to physiological levels of Mn^{2+} *in vivo* even though it has not been documented to do so *in vitro* (102). SsrAB (CS382; *ssrA*::Km) was included in case we could identify Fe^{2+} concentration as the stimulus for this TCRS. In view of the data presented in Chapter 3, MntR was also investigated for a potential role in SPI2-associated gene expression.

Figure 30 demonstrates that neither Fur nor PmrAB is involved in the induction of the SPI2-associated genes with respect to Fe^{2+} concentration, as induction by DiP and repression by excess Fe^{2+} was still observed in these knockout strains. Fe^{2+} also does not appear to be a stimulus for SsrAB. The results with respect to the impact of PhoPQ on SPI2-associated gene expression can not be interpreted due to the extremely high variability. This is likely due to poor growth of the *phoP*::Cm strain in the growth medium used (NMM pH 7.4, 8 μM $MgCl_2$).

To address the role of PhoPQ in the response to iron concentrations more satisfactorily, NMM was supplemented with 200 μM $MgCl_2$ and the bacteria grown for 24 h prior to harvesting for the β-galactosidase assays. A constitutively active *phoP* mutant (CS022; $phoP^C$)was also used for these experiments (Figure 31). As expected, *hilA* (a known PhoP-repressed gene; (164)) was repressed in the $phoP^C$ strain and derepressed in the *phoP* null strain. *phoP* itself is a PhoP-activated gene (102) and accordingly we observed *phoP* to be upregulated in the $phoP^C$ strain and downregulated in null mutant. In contrast, expression of neither *ssrA* nor *sseA* was greatly affected by PhoPQ. However, *sseJ* was significantly downregulated in the *phoP*::Cm strain, indicating that it may in fact be a member of the PhoPQ regulon. Overall, we can eliminate PhoPQ as the TCRS involved in the response to Fe^{2+} as *ssrA* and *sseA* were not regulated by PhoPQ, while all three SPI2-associated loci appear to be regulated in

Figure 30. Effect of the regulatory systems Fur, PmrAB and SsrAB on regulation of SPI2-associated virulence genes in response to Fe^{2+} levels. Expression of *ssrA* (**Panel A**), *sseA* (**Panel B**) and *sseJ* (**Panel C**) was examined in wildtype bacteria versus bacteria harboring one of the following insertional mutations: *fur*::Km; *phoP*::Cm; *ssrA*::Km; or *pmrA*::Tn10. Bacteria were cultured in NMM with 8 μM $MgCl_2$ supplemented with DiP_{250} or DiP_{250}+$FeSO_{4(500)}$. Cultures were assayed for β-galactosidase activity and bacterial cfu as described in Section 2.5.3. Experiments were carried out four times in triplicate and all data points averaged. Error bars represent the SEM.* indicate statistical significance to $P<0.01$ between the untreated sample and the DiP-chelated sample, as determined by the Student's t-test. + indicate statistical significance to $P<0.01$ between the DiP-chelated sample and the DiP+Fe sample, as determined by the Student's t-test.


A
ssrA
B
sseA
C
sseJ
100000
10000
1000
100
10
Average RLU/10^6 bacteria
WT
fur::Km
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none
DiP
DiP+Fe

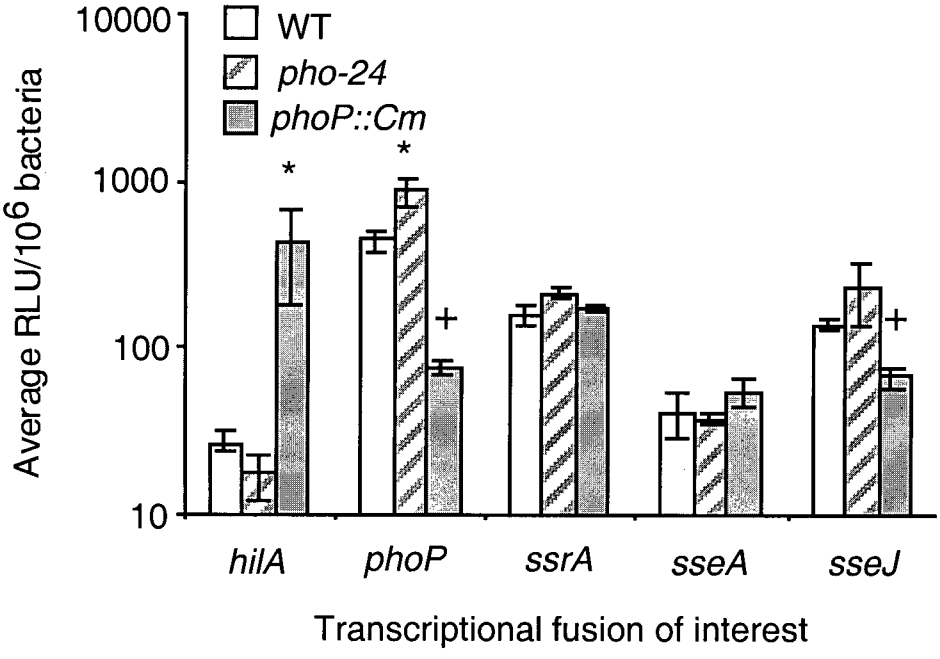

Figure 31. Effect of PhoPQ on regulation SPI2-associated virulence genes in response to Fe^{2+} levels. Expression of *hilA, phoP, ssrA, sseA* and *sseJ* was examined in wildtype bacteria, in a constitutively active *phoP*^{C} strain (CS022; *pho-24*), or in a *phoP*::Cm functional knockout strain, (CS015). Bacterial cultures were grown in NMM with 200 μM $MgCl_2$ for 24 h prior to harvesting for the assay. β-galactosidase activity and bacterial cfu were assayed as described in Section 2.5.3. Experiments were performed three times in triplicate and all results were averaged. Error bars represent the SEM. * indicates statistical significance to $P<0.05$, as determined by the Student's t-test. + indicates statistical significance to $P<0.01$ as determined by the Student's t-test.

response to Fe^{2+} levels. However the role of PhoPQ in regulation of SseJ, and therefore perhaps in a subset of SPI2-associated genes, remains undefined.

Upon examination of the data from Figure 30, it appeared that while the overall level of *sseJ* expression was reduced 5-fold in the *ssrA* mutant strain, expression of neither *ssrA* itself nor *sseA* was affected by disruption of *ssrA*. The lack of an autoregulatory effect on *ssrA* was not problematic, as it has been demonstrated previously that *ssrA* is not autoregulated (54, 155). However, both *sseA* and *sseJ* have been found to be regulated by SsrAB (122, 176). Therefore, the SPI2-associated plasmid constructs were re-tested for expression in wild type and *ssrA*::Km mutant strains in NMM. As shown in Figure 32, only *sseA* and *sseJ* were significantly affected by disruption of *ssrA*, demonstrating 43% and 90% decreases in expression levels, respectively . Overall, these results confirm previous data indicating that *sseA* and *sseJ*, but not *ssrA* itself, are members of the SsrAB regulon.

As an additional method to verify expression of the SPI2-associated genes and reconcile their expression patterns with previously published observations, we further examined the role of OmpR on SPI2-associated gene expression. As OmpR has been shown to function upstream of SsrAB and positively regulate *ssrAB*, it was hypothesized that disruption of *ompR* would decrease the expression of *ssrA, sseA* and *sseJ* . Figure 33 demonstrates that *hilA* and *phoP* are negatively regulated by OmpR, while *ssrA* and *sseJ* are positively regulated by OmpR. Surprisingly, expression of *sseA* was enhanced about 10-fold in the *ompR::Tc* strain unlike other SPI2-associated loci, indicating that *sseA* is negatively regulated by OmpR. The different regulatory patterns observed between the five loci of interest indicates that there is not enough commonality to suggest that OmpR is involved in responding to Fe^{2+} levels. Therefore, OmpR does not appear to be the

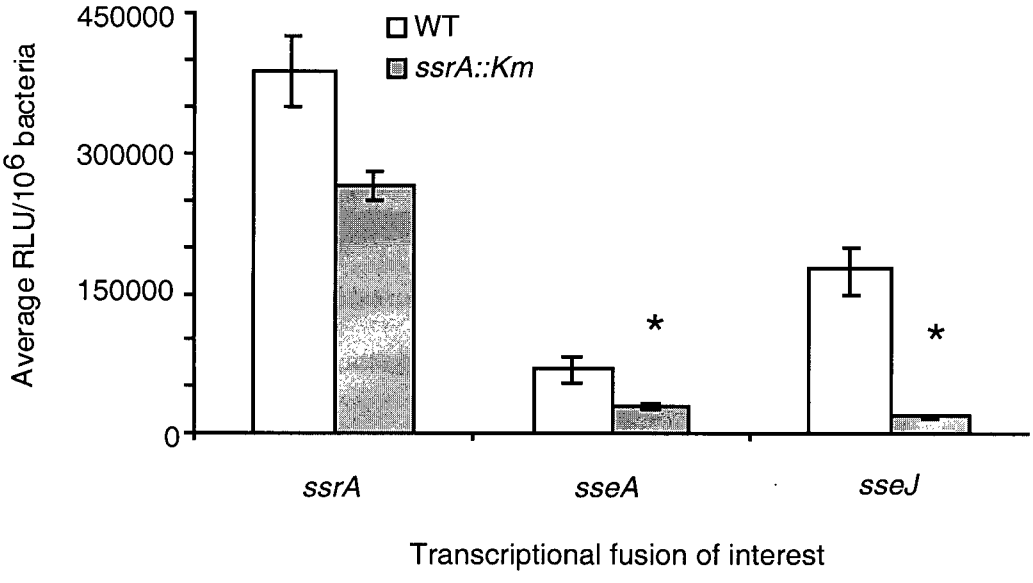

Figure 32. Effect of SsrAB on expression of *sseA* and *sseJ*. Expression of *ssrA*, *sseA* and *sseJ* was examined in 14028s (WT) bacteria versus CS382 (*ssrA*::Km) and were grown in normal NMM (8 μM $MgCl_2$) as previously described. β-galactosidase activity and bacterial cfu were assayed as described in Section 2.5.3. Experiments were carried out three times in triplicate and all results averaged. Error bars represent the SEM. Asterisks indicate statistical significance to $P<0.01$ as determined by the Student's t-test.

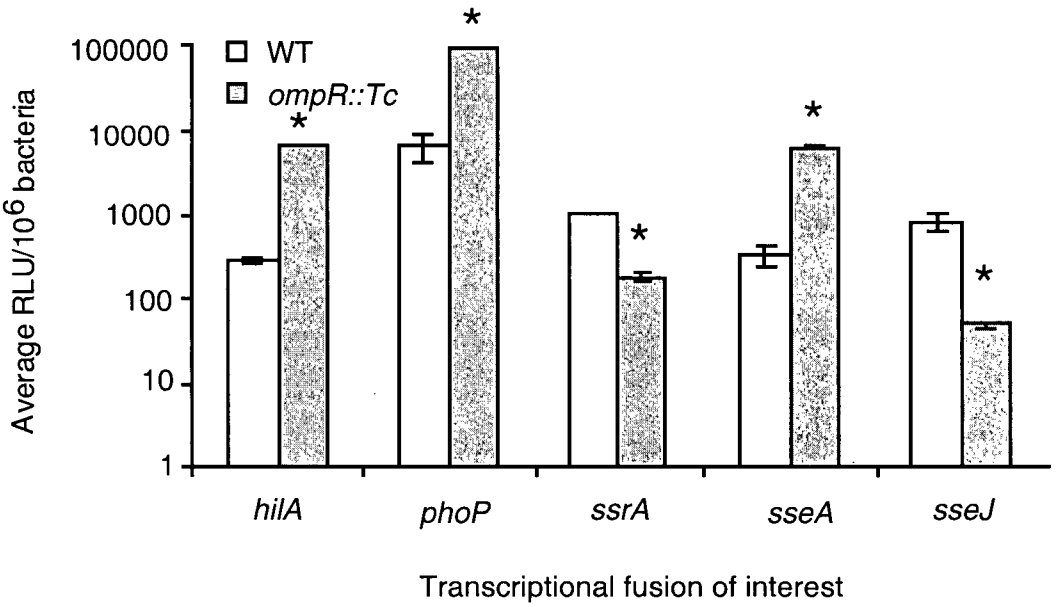

Figure 33. Effect of OmpR on expression of SPI2-associated genes. Expression of *hilA, phoP, ssrA, sseA* and *sseJ* was examined in wildtype bacteria versus bacteria carrying the *ompR*::Km functional knockout strain and were grown in normal NMM (8 μM $MgCl_2$). β-galactosidase activity and bacterial cfu were assayed as described in Section 2.5.3. Experiments were carried out three times in triplicate and all results averaged. Error bars represent the SEM. Asterisks indicate statistical significance to $P<0.01$ as determined by the Student's t-test.

regulatory system of interest in these studies. Further work on the role of OmpR in SPI2-associated gene expression was not pursued.

Chapter 3 of this work describes the role of MntR in regulation of two STM virulence factors, *mntH* and *sitA*. As MntR had been shown to be responsive to both Fe^{2+} and Mn^{2+} (142), we thought it prudent to examine the possible role of MntR in SPI2 regulation. As shown in Figure 34, deletion of *mntR* had no effect on expression of any of the genes of interest. This was not surprising, as MntR boxes have not been identified in SPI2 or in the promoter region of *sseJ*.

Therefore, the observed regulatory effects on the SPI2-associated genes in conditions of Fe^{2+} limitation are not mediated by any of the previously identified Fe^{2+}-sensing regulators or SPI2 regulatory systems, or by the novel virulence-associated regulator MntR. Thus, we may have identified the existence of a novel regulatory system in STM responsible for sensing divalent cation concentration involved in virulence gene regulation. Further we have identified that there is a similar complexity in the regulation of SPI2 akin to that of SPI1, in that PhoPQ and OmpR may be involved in the differential regulation of subsets of SPI2-associated genes.

4.8 Discussion

To our knowledge, a direct comparison of the effect of SPI2 in congenic *Nramp1*$^{+/+}$ and *Nramp1*$^{-/-}$ lineages of mice had not been undertaken. Similarly, the interplay between mammalian and pathogen resistance factors including potential interactions between Nramp1 and SPI2 have not yet been adequately examined. Using congenic knockout mice infected intraperitoneally with STM, we determined that minimal bacterial replication occurred in both *Nramp1*$^{+/+}$ and *Nramp*$^{-/-}$ mice in the absence of SPI2, reinforcing the importance of these virulence genes during infection in either model system. In *Nramp1*$^{-/-}$ mice infected with wild-type (SPI2+) STM, the

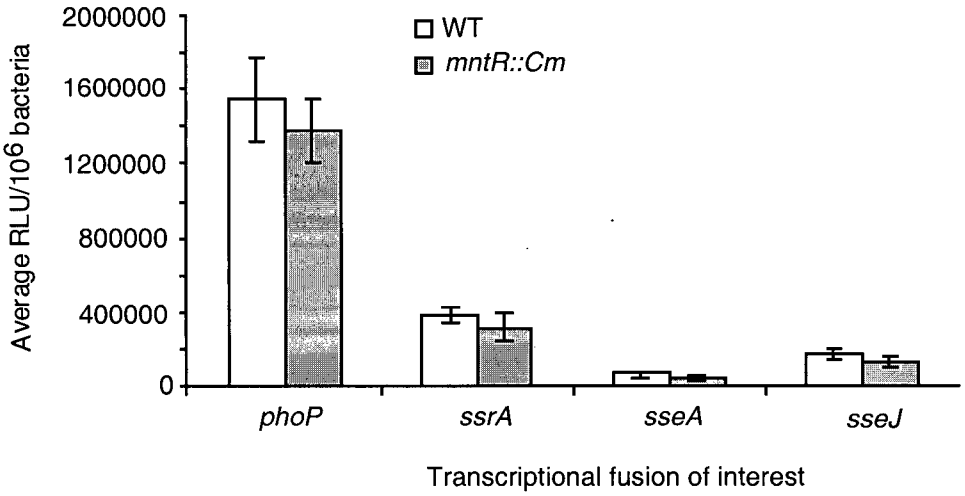

Figure 34. Effect of MntR on the expression of SPI2-associated genes. Expression of *phoP, ssrA, sseA*, and *sseJ* was examined in 14028s (WT) bacteria versus MZ203 (*mntR*::Cm) and were grown in normal NMM as previously described. β-galactosidase activity and bacterial cfu were assayed as described in Section 2.5.3. Experiments were carried out three times in triplicate and all results averaged. Error bars represent the SEM. Results were determined not to be statistically significant by the Student's t-test.

bacteria replicated rapidly and eventually the animals were overwhelmed, while during a similar infection of *Nramp1*$^{+/+}$ mice, significant bacterial replication in the spleen of the mice was observed. Therefore SPI2 is essential for maintaining a moderate level of bacterial replication in *Nramp1*$^{+/+}$ animals.

Interestingly, Nramp1 colocalizes with the phagosome and begins to extrude Mn^{2+} ions as early as 60 min post-infection in transfected cultured cells (132). Despite this apparent rapid activity of Nramp1, the most dramatic Nramp1-mediated effects in our experiments were not observed on loci that are expressed immediately upon reaching the intracellular environment (*hilA*, *phoP*), but with the SPI2-associated genes, loci that are expressed to higher levels at later stages of infection (i.e. after 6 h post-infection; (155)). In these experiments, the Nramp1-associated increase in SPI2-associated gene expression was maximal at 24 h post-infection. We observed an upregulation of SPI2-associated genes, not only in transfected cell lines, but also in viable bacteria from spleens of infected *Nramp1*$^{+/+}$ and *Nramp1*$^{-/-}$ animals 24 h after intraperitoneal infection. The *in vitro* and *in vivo* results correlate well, in that:1) *hilA* and *phoP* are not differentially expressed between the two mouse lineages ; and 2) the SPI2-associated genes *ssrA* and *sseJ* are significantly upregulated in the *Nramp1*$^{+/+}$ animals. To our knowledge, this represents the first study to demonstrate that the function of a particular host protein influences the expression pattern of STM genes required for intracellular survival.

Nramp1 has been suggested to be a H^+/divalent cation antiport system such that direction of transport of divalent cations can vary depending on the magnitude of a pH gradient (96). Jabado *et al.* demonstrated that the pH of a latex-bead phagosome reaches pH 6.5 by 1 h after phagocytosis, and that by this time Nramp1 is responsible for the active efflux of Mn^{2+} from the phagosome (132). Thus since we had identified a relationship between Nramp1 and expression of SPI2 genes within the SCV, it was of

interest to determine if this proposed function of Nramp1 was related to our gene expression data. The most strongly documented substrates for Nramp1 and homologous transporters are Fe^{2+} and Mn^{2+}, and as such these were the cations of focus in our *in vitro* experiments (99, 132, 144). An *in vitro* expression assay based on growth media known to induce expression of SPI2-associated genes (68) was developed and used to determine that the SPI2-associated genes were upregulated in chelated media and repressed by addition of excess Fe^{2+}. There was no significant impact of Mn^{2+} levels on expression of these genes. This is the first demonstration of *phoP* and SPI2-associated genes responding to iron levels. Therefore, we conclude that these virulence genes may be responding to iron limitation in the cell as a result of Nramp1 depleting the SCV of this essential divalent cation.

Studies to identify whether a known regulatory system was responsible for this Fe^{2+}-responsive regulation of SPI2-associated gene expression yielded some unexpected insights into SPI2 regulation. We determined that the Fur global regulatory system was not involved in this Fe^{2+}-responsive phenotype, which was not unexpected as no Fur boxes were identified within SPI2 (J.L. Puente, personal communication). In addition, the PmrAB TCRS (which responds to Fe^{3+} levels) or MntR (a novel virulence-associated regulatory protein which responds to Mn^{2+} and Fe^{2+} levels) were also not involved in the regulation of the SPI2-associated genes. We confirmed the role of SsrAB in expression of *sseA* and *sseJ*, but Fe^{2+} concentration does not appear to be the stimulus for this TCRS.

PhoPQ was similarly not involved in the response to Fe^{2+} levels in this system. However, these experiments generated an interesting twist in the controversy over the involvement of PhoPQ in SPI2 gene expression. While we observed no effect of PhoPQ on expression of genes encoded within SPI2 (*ssrA, sseA*), the SPI2 effector encoded outside of SPI2, *sseJ*, was regulated by PhoPQ, being moderately induced in the $PhoP^{C}$

strain and having a reduced basal level of expression in the *phoP*::Cm strain (characteristics of a PhoP-activated gene). Two studies have refuted the theory that PhoP is not involved in SPI2 expression (155, 174). However, our results suggest that PhoPQ may regulate a subset of SPI2-associated genes, and may in fact be restricted to those encoded outside of SPI2. This is not an unrealistic theory, as other effectors appear to have been acquired by horizontal transmission and have become members of a global regulon (e.g. *mgtCB* in SPI3 and *pagJ* of Gifsy-3).

The role of OmpR in SPI2 gene expression was also studied. Previously OmpR was shown to positively regulate *ssrAB* (155), which has been assumed to affect the expression of all members of the SsrAB regulon in an identical manner. However we observed a dichotomy in expression of SPI2-associated genes in response to OmpR regulation, in that *ssrA* and *sseJ* were positively regulated by OmpR while *sseA* was negatively regulated by OmpR. Therefore, in contrast to our observations with respect to regulation of SPI2-associated genes by PhoPQ, we observe differences in the regulation of genes encoded within SPI2 itself by OmpR. This is a novel observation. Although genes within SPI5 differ in their regulation by SsrAB such that *pipBA* is regulated by SsrAB yet the adjacent *sigD* is not (L. Knodler, unpublished observations), this is the first instance in which two genes within the same SPI are positively or negatively regulated by the same regulatory system.

After these experiments were completed, Bang *et al.* (17) identified a role for OmpR as an acid shock protein and showed that osmolarity was not the stimulus for OmpR-mediated regulation of SPI2. It is apparent that there is far more to be elucidated about the role of OmpR in SPI2 expression. It is tempting to speculate that there may be some hierarchy of expression of regulation of virulence genes by OmpR. It is possible that members of the SsrAB regulon are induced by OmpR as a result of its effects on

expression of *ssrAB*, but further inductive effects on *sseA* and other effector loci may be fine-tuned by the direct action of OmpR on these promoters.

Thus, it appears that SPI2-associated genes are controlled by an increasing number of regulatory systems, but are not always coordinately regulated. SPI2-associated genes all appear to be members of the SsrAB regulon yet are differentially regulated by PhoPQ and OmpR. In addition SPI2-associated genes may be further controlled by a distinct, uncharacterized, Fe^{2+}-responsive regulatory system. The increasingly complex mechanism of SPI2 regulation may reflect the various stimuli encountered by the bacterium during infection, including limiting concentrations of divalent cations. This would supposedly allow for the maximum benefit of expression of these genes required for intramacrophage survival and development of a productive infection.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

5.1 Role of Fe^{2+} and Mn^{2+} in infection

Divalent cations are absolutely required for the survival of all living things. They function in a variety of capacities, from acting as enzymatic co-factors and prosthetic groups to stabilizing macromolecular complexes such as DNA and cell membranes. Until recently iron has been thought to be the most important transition metal divalent cation in biological systems due to its numerous cellular functions (202). However, work presented in this study as well as a body of new literature supports the idea that other divalent cations such as Mn^{2+} may play just as important a role as Fe^{2+} during infection. Below is a brief discussion of the evidence for the importance of Mn^{2+} for maintenance of the pathogenic nature of STM.

The importance of iron in infection was inferred from observations that iron deprivation is often bacteriostatic, and that extreme iron deficiency for extended periods is often lethal for bacteria (43, 266). This link was further strengthened by evidence that incidence of fungal and bacterial infection increases under conditions of iron overload, while reduced iron levels are associated with enhanced resistance to infection (169, 201) and references therein). Limitation of free iron availability is one of the ways in which the host defends itself against infection (42). In vertebrates iron is present in body fluids at concentrations greater than 20 μM, but the quantity of free iron in these fluids is below 10^{-18} M (42), a concentration much lower than that required for bacterial growth (required free iron concentration of 0.05 to 0.5 μM; (169)). This low concentration of free iron reflects the poor solubility of Fe^{3+} in aerobic environmentsat physiological pH (13) and the presence of a number of iron-binding proteins including hemoglobin, iron-sulfur proteins, ferritin, transferrin and lactoferrin (201). In addition, Nramp1 has long been hypothesized to be involved in removing Fe^{2+} (and more recently, Mn^{2+}) from

intracellular compartments, contributing to the phagocyte's ability to reduce the amount of free iron available to intracellular pathogens.

However, bacteria have found a way around this host-mediated sequestration of iron. The most prevalent of these bacterial strategies include the manufacture and secretion of: 1) siderophores (specific binding agents with a high affinity for Fe^{3+}; e.g. enterobactin and aerobactin); 2) transferrin-binding proteins which bind transferrin and subsequently release Fe^{3+} for transport across the bacterial cell envelope; and 3) hemolytic cytotoxins and membrane proteins dedicated to the transport of heme ((201) and references therein). These bacterial strategies may enhance infection in two ways: by fostering more permissive conditions for bacterial multiplication and thus infection, or by enhancing the expression of other virulence factors. Further, iron is involved in the regulation of expression of a number of virulence-associated genes, primarily via the iron-sensing regulatory protein, Fur, as discussed in section 1.5.2. Therefore, it is apparent that iron plays an important role in the host-pathogen interaction and the subsequent determination of the outcome of an infection.

In spite of the dominant role of iron in infection, some bacterial pathogens have further subverted the host's strategy of iron limitation by becoming totally independent of an iron requirement. *Borrelia burgdorferi*, the etiological agent of Lyme's disease, has no requirement for iron in its metabolism, apparently having replaced iron as a cofactor with Mn^{2+} (207). In addition, there is evidence that several species of *Streptococci* as well as the bacterium *Lactobacillus plantarum* can grow in the total absence of iron (10, 134, 189, 236). Interestingly, while these bacteria no longer require iron, they appear to have acquired an absolute requirement for Mn^{2+} (133). Thus, Mn^{2+} is an essential divalent cation for microorganisms in this most extreme case of iron independence. It is possible

that even in iron-dependent pathogens, Mn^{2+} may play a greater role in virulence than previously anticipated.

There are a number of possible roles for Mn^{2+} in pathogenesis. First, non-enzymatic Mn^{2+} may be important for bacterial growth *in vivo* by acting as a protectant against ROIs in place of an enzymatic SOD (10). Non-enzymatic Mn^{2+} may also play a role in stabilizing bacterial cell walls (71), which would directly affect survival of a bacterium *in vivo* as it encounters various environmental niches with varying osmotic stresses. Secondly, Mn^{2+} may play a significant role in the regulation of expression of a number of virulence-associated genes. A number of Mn^{2+}-responsive regulatory systems have recently been identified in pathogenic microorganisms. The MntR regulatory system has been characterized in both STM and *E. coli* and defined to be involved in expression of the genes encoding the Mn^{2+}-transporters MntH and SitABCD (135, 142, 200). Mn^{2+}-responsive regulatory systems have also been identified in *B. subtilis* (MntR; (209)), *Staphylococcus aureus* (MntR; (131)), *Streptococcus gordonii* (ScaR; (134)), and *Treponema pallidum* (TroR; (208)). Unfortunately, the direct impact of MntR on virulence of STM has yet to be studied. However, members of the ScaR and MntR regulons in *S. gordonii* and *S. aureus* have been found to be virulence-related (131, 134), and the MntR-regulated STM loci *mntH* and *sitABCD* are involved in virulence in the murine typhoid model (this work; and (135)). Therefore Mn^{2+}-regulatory systems and their regulons appear to be important for the pathogenesis of a number of different microorganisms.

Finally, Mn^{2+} may impact virulence through its role as a cofactor or prosthetic group for various bacterial enzymes. Mn^{2+} metalloenzymes have many diverse functions within bacterial cells (53, 275). In addition, Mn^{2+} and other cations may be interchangeable in the metal biding sites of many proteins, and Mn^{2+} has been found to be

interchangeable with Mg^{2+} or Fe^{2+} (157). Mn-dependent enzymes that have been identified to date in STM are: PrpA and PrpB, SodA (superoxide dismutase), KatN (catalase) and a number of enzymes involved in intermediary metabolism.

Little is known about the role of any of these enzymes in pathogenesis of STM. PrpA and PrpB are Mn^{2+}-dependent serine/threonine phosphatases homologous to a family of type I eukaryotic phosphatases (229); (181). 2D gel analyses suggests that nearly 20 different phosphoproteins are substrates for dephosphorylation by PrpA or PrpB (181), potentially indicating a wide-reaching role in bacterial signal transduction pathways. Preliminary studies indicate that mutation of *prpA* or *prpB* markedly alters the peroxide and temperature sensitivity of STM (229). This suggests a possible role for these enzymes in virulence with respect to modulating the heat shock response and the response to oxidative stress *in vivo*. Similarly, SodA and KatN may be essential for the virulence-associated oxidative stress response. Although KatN has not been tested for a role in virulence, there is a correlation between expression of *katN* and an increase in peroxide resistance of STM (212). SodA was found to increase the resistance of STM to early killing by macrophages, but was not involved in virulence upon infection of *Nramp1*$^{-/-}$ mice (252). This issue must be re-investigated using *Nramp1*$^{+/+}$ animals before any firm conclusions as to the role of KatN or SodA in virulence can be assessed conclusively.

A number of enzymes appear either to require Mn^{2+} absolutely for function or to tolerate Mn^{2+} as their catalytic divalent cation. Such Mn^{2+}-dependent enzymes in STM include 3-phosphoglycerate mutase (involved in glycolysis), aminopeptidase P (peptide cleavage), and SpoT (AppppA synthase/hydrolase; involved in the stringent response) (133, 229). Enzymes which can function with Mn^{2+} as the catalytic ion include enzymes involved in nucleic acid degradation, aromatic acid metabolism, amino acid metabolism,

sugar metabolism, glycolysis and gluconeogenesis (133). A detailed description of the potential role of all of these enzymes in virulence is beyond the scope of this work. However, overall their potential role *in vivo* centres around their individual roles in intermediary metabolism. As STM resides within, at best, a microaerobic environment within the SCV, the bacterium will be dependent on glycolysis and/or the TCA cycle for generation of ATP. Therefore, if any one of the Mn^{2+}-dependent metabolic enzymes was rendered non-functional by the absence of their required divalent cation, metabolism of the bacterium would be dramatically impaired. In turn, this would be expected to significantly affect the bacterium's ability to cause or sustain an infection.

In summary, there are a number of ways in which Mn^{2+} could impact the pathogenicity of various microorganisms, from affecting virulence gene expression, playing a role in the oxidative stress response, to altering the function of enzymes essential for intermediary metabolism. As there is accumulating evidence that divalent cations other than Fe^{2+} are important *in vivo* for bacterial pathogens, this must be taken into account in further investigations on bacterial pathogenesis and the study of host-pathogen interactions.

5.2 Role of bacterial ion transport systems during infection

It is apparent that STM requires a number of different divalent cations to maintain normal metabolic processes as well as its pathogenic nature *in vivo*. We have discussed above the roles of Fe^{2+} and Mn^{2+} during infection. However these cations as well as others must be able to enter the bacterial cell in order to carry out one or all of their appointed tasks. To this end, STM encodes a number of ion transport systems responsible for the uptake of divalent cations across the bacterial inner membrane; their roles in virulence are discussed below.

Transport of iron unrelated to the siderophore-mediated uptake of Fe^{2+} involves a single characterized system, Feo (139, 253). Feo is responsible for the uptake of Fe^{2+} and is important for bacterial colonization of the intestine in the murine typhoid model but appears to have no role in systemic infection (253). Recently it has been suggested that the constitutively active Mg^{2+} influx/efflux system, CorA, may be responsible for transport of Fe^{2+} (50, 116). However, further investigation demonstrated that CorA either does not transport iron or only mediates transport of iron at concentrations too high to be physiological and, therefore, would play no role during *in vivo* infection (Krisztina Papp and ME Maguire, unpublished observations). However, in spite of this lack of involvement in iron transport, deletion of *corA* has been observed to result in a marked attenuation of STM in the murine typhoid model (Lin, Zaharik and Maguire, unpublished observations) which is hypothesized to be due to its role in Mg^{2+} transport.

In contrast to the single characterized Fe^{2+} transport system in STM, two Mn^{2+} transport systems have recently been identified in this bacterium. The first STM Mn^{2+} transport system was identified due to its homology to the human host resistance protein Nramp1 and was named MntH (144). The second STM Mn^{2+} transport system, SitABCD, was originally identified as a virulence-associated iron transport system encoded within SPI1 (135, 278). However, careful study revealed that SitABCD, like MntH, had a higher affinity for Mn^{2+} than Fe^{2+} (143). These two transport systems do not appear to be redundant as MntH is active at acidic pH while SitA is most active at a slightly basic pH (143).

Because of these different pH optima and the defined role of SitA in virulence, it was hypothesized that these transporters would function at different stages of infection or at different locations in the host. We subsequently found that deletion of *mntH* or *sitA* had no effect on STM virulence in *Nramp1*$^{-/-}$ mice. In *Nramp1*$^{+/+}$ animals, both *mntH* and

sitA deletion strains were markedly attenuated, and they had an additive effect on virulence in a double knockout strain. Therefore, both Mn^{2+} transport systems are essential for virulence of STM in the presence of Nramp1. Interestingly, Mn^{2+} transport systems homologous to SitABCD have been identified to play a role in the virulence of other pathogenic microorganisms including *Enterococcus faecalis, Streptococcus mutans* and *Y. pestis* (22, 145, 231). However, the MntH homologue of *Mycobacterium tuberculosis*, another intracellular pathogen affected by Nramp1, was found to play no role in virulence of this microorganism regardless of the presence or absence of a functional Nramp1 (39, 69).

Overall, it is obvious that divalent cation transport systems are essential for virulence of STM in the murine typhoid model. On the surface, the role of these transport systems in virulence appears to be intuitive: as divalent cations are necessary for the function of a number of prokaryotic enzymes either involved directly in virulence or involved in intermediary metabolism. Without these cations the bacterium can not survive within the host. However, further dissection of these observations leads us to a more complicated view of survival of STM in the host.

With respect to iron transport, transport of Fe^{2+} was only found to be important in colonization of the intestine and not in systemic phases of disease, as described above. However, although the enterobactin siderophore system is important for *S.* Typhi pathogenesis, no role for siderophores has been found for pathogenesis of STM in the murine typhoid model (28). Therefore, no role to date has been found for iron at later stages of systemic infection. This is contrary to the long-held belief that iron is the predominant divalent cation in bacterial growth and survival *in vivo*. If acquisition of iron were so important at systemic sites, it might be expected that Feo would play a much larger role in the infectious process, as iron at these anaerobic sites is expected primarily

to be in the Fe^{2+} form. It was speculated that there must be another iron transport system involved in STM pathogenesis, and briefly attention focussed on CorA (50). However, this hypothesis did not withstand further scrutiny (M.E. Maguire, unpublished observations). Thus it would appear that iron is not the pre-eminent divalent cation during infection.

The role of Mn^{2+} in infection is a rapidly expanding field and a novel topic of investigation. In the case of the defined STM Mn^{2+} transport systems, *sitA* is essential for virulence in both *Nramp1*$^{+/+}$ and *Nramp1*$^{-/-}$ animals ((135); this work), while a significant effect of *mntH* is observed only in the *Nramp1*$^{+/+}$ animals (this work). Further, *sitA* was found to be important in a number of sites of infection, including the small intestine and the liver and spleen within 4-7 days post-infection (135). However, our data indicate that both *sitA* and *mntH* have a role at later times in infection (day 18 and later). The lack of congruity between experiments may reflect the different methods used to determine attenuation: Janakiraman and Slauch (135) used competitive indices while we used time to death assays. However these potential differences must be addressed.

As SitABCD is most functional at basic pH, it suggests that this Mn^{2+} transport system would be responsible for uptake of Mn^{2+} at extracellular sites in infection. In contrast, MntH is active at acidic pH and its expression is induced by hydrogen peroxide. Thus, MntH would be expected to be the primary Mn^{2+} transport system in the SCV of phagocytic cells where the bacterium would encounter both of these stimuli. As Nramp1 is primarily effective at the level of the SCV and would not be predicted to have a role at extracellular sites of infection, this could explain why SitA attenuates virulence in both *Nramp1*$^{+/+}$ and *Nramp1*$^{-/-}$ strains of mice. Since deletion of *mntH* significantly affects virulence only in the presence of Nramp1, this suggests that the concentration of Mn^{2+} in

the *Nramp1*$^{-/-}$ vacuole may be low but Nramp1 must be functional and depleting the vacuole of Mn^{2+} to the point that it becomes critical for bacterial survival.

While attractive, this theory does not take into account our observations that *sitA* is not required for intraperitoneal infection of *Nramp1*$^{-/-}$ animals and that the effect of both Mn^{2+} transport systems in the *Nramp1*$^{+/+}$ animals is apparent only after day 18 post-infection. Due to the intraperitoneal route of infection used in these experiments, the bacteria should have reached the liver and spleen of the animals long before day 18. Therefore, any effect of Nramp1 as an efflux mechanism from the SCV would, in theory, have been apparent far earlier than this. Although we have an idea of what occurs during murine infection with STM at early times in infection, very little work has been done on the late stages of infection. This is primarily due to the limitation of the BALB/c mouse model, as these animals generally succumb to infection prior to day 10. Therefore it is possible that this delay in impact of the Mn^{2+} transport systems may reflect migration of the bacteria to a new intracellular niche where Mn^{2+} continues to be limiting. It is also possible that the effects of Mn^{2+} deprivation are cumulative. In this case perhaps deprivation of the initial SCV of Mn^{2+} by Nramp1 does affect STM within the first 5-7 days of infection by affecting intermediary metabolism or defenses against oxidative stress. It is possible that the bacterium could continue to survive in this stressful environment without proper functioning of these enzymes for a limited period of time. Then at later stages of infection the cumulative effects of constant Mn^{2+}-deprivation become critical and the bacterium is no longer capable of surviving in the host. However it is also possible that there is one critical function of Mn^{2+} or a critical Mn^{2+}-dependent enzyme required at this late stage of infection, without which the bacterium is no longer able to maintain a productive infection.

Alternatively, perhaps the role of Nramp1 at later time points in infection is not related to transport of divalent cations. As discussed in section 5.6, Nramp1 has been implicated in macrophage activation, MHC class II expression and T cell differentiation. These are all host responses to infection that have been implicated in clearance of STM in the murine typhoid model, and they appear to play a role in the later stages of disease. Therefore, it is possible that the role of Nramp1 at later stages of infection involves its poorly-characterized influence on these aspects of the immune response. Thus, although we have made significant advances in recent years with respect to understanding the role of divalent cations in general in pathogenesis, there is still much about the intracellular lifestyle and pathogenic requirements which remains unknown and requires further elucidation.

5.3 Role of divalent cations and virulence gene regulation

The importance of divalent cations on virulence gene expression has been discussed in previous sections of this work (see sections 1.5.2 and 5.2). One of the potential influences of the role of Nramp1 on pathogenic microorganisms relates to depletion of divalent cations from the vacuole that would affect virulence gene expression. We presented evidence in Chapter 4 that Nramp1 may function in such a capacity by depleting the vacuole of Fe^{2+} that in turn has an effect on SPI2-associated virulence gene expression. In a similar manner we determined that the presence of Nramp1 correlated with upregulation of *mntH* and *sitA in vivo*. However, we were unable to determine the role of MntR or Fur in expression of *mntH* in the presence of Nramp1 nor whether Fe^{2+} or Mn^{2+} is responsible for increased expression inside infected cells. However, in both cases it appears that the bacterium senses depletion of divalent cations from the vacuole and adjusts expression of virulence-associated genes accordingly.

In a recent paper, Frehel *et al.* (90) hypothesize that Nramp1 is depleting the vacuole of divalent cations essential for *Mycobacterial* gene expression, in the absence of

which numerous virulence-associated genes would not be expressed. As a result, the pathogenic strategy of the bacterium would be compromised. This was our original premise for the function of Nramp1 on SPI2 expression. However, as documented in Chapters 3 and 4 we observed exactly the opposite, such that virulence gene expression was increased in the presence of Nramp1. Therefore it is of interest to speculate why the obvious hypothesis of the impact of Nramp1 on virulence gene expression is not supported by this preliminary study. The key may lie in the manner in which ion-responsive regulatory systems function in bacteria.

Of the ion-responsive regulatory systems identified to date in STM, three (Fur, MntR and Zur) require direct binding of the cation of interest to the apotranscription factor to facilitate binding to the operator region of genes of interest (9, 115, 200). These three regulatory systems all function as negative regulators of gene expression. Although it has been proposed that Fur can act as a positive regulator for a certain subset of genes and that both Fe^{2+}-Fur and apo-Fur may activate transcription of these genes (88), none has been characterized in STM. In contrast, PhoPQ works in a different manner. In the absence of Mg^{2+}, PhoP is in its phosphorylated form and can bind DNA, thereby mediating activation of PhoP-activated genes (*pags*) and repression of PhoP-repressed genes (*prgs*). When excess Mg^{2+} is present it binds PhoQ, which stimulates the phosphatase activity of PhoQ to dephosphorylate phospho-PhoP. This results in elimination of expression of *pags* and increased expression of *prgs* because PhoP can no longer bind the promoter region of these genes.

Therefore, for all four of these regulatory systems if the concentration of the divalent cation of interest is low, the default position for these regulators is to turn on transcription of their target genes (with the exception of the *prgs*). This ensures that during growth in limiting concentrations of divalent cations, genes required for bacterial

survival will be expressed. As a result the evolution of gene expression systems in pathogens is already adapted for growth in low cation conditions. Therefore, the hypothesis that the action Nramp1 at the level of the vacuole would result in decreased expression of virulence genes does not correlate with current knowledge of ion-responsive regulatory proteins.

However, it must be remembered that we do not know what regulator or regulatory system is involved in the upregulation of SPI2-associated genes in the presence of Nramp1 described in Chapter 4. As a result, we do not know if it is acting as a positive regulator in the absence of iron or a negative regulator in the presence of iron. Nor do we know if this potential regulatory system is similar to Fur, MntR and Zur, and is determined by direct binding of divalent cations to the regulatory protein itself, or if it is a member of a phosphorelay system similar to PhoPQ. It is possible that this proposed system may function in a manner similar to PrpA/PrpB where a protein phosphatase is not functional in the absence of the required Mn^{2+} ion, thereby short-circuiting its role in a phosphorelay cascade and impacting virulence gene expression. In point of fact, we do not even know if this system acts at the transcriptional level. Clearly, much remains to be elucidated about this avenue of research with respect to STM virulence gene regulation and is important for our understanding of facet of bacterial pathogenesis.

5.4 Dissecting the host-pathogen interaction: phox, Nramp1 and iNOS

To the general public, studies into the host-pathogen interaction are of most relevance when they deal with human diseases. However, to date more information is available about the host-pathogen interactions occurring between plant pathogenic bacteria and their specific host plants. In such systems it has been shown that during infection of host plants these bacterial pathogens produce various symptoms of disease, whereas in non-host plants they trigger the hypersensitive response (HR), a rapid-defense

associated programmed death of plant cells at the site of invasion (6). Resistance to bacterial infection by plants appears to be determined by the presence of a resistance gene (*R*) in the plant and an avirulence gene (*avr*) in the bacterium. When both genetic determinants are present, host defense responses are triggered and pathogen colonization is limited. However, the absence of either genetic component results in disease (6). A discussion of this system is beyond the scope of this work. However it is of note that this system is dependent on a specific TTSS in the plant pathogenic bacteria and on secretion of effector proteins, encoded within large pathogenicity islands. Thus it appears that there are parallels to be drawn between plant-pathogen interactions and mammal-pathogen interactions. Therefore, there is likely to be a complex interplay between host and pathogen in both model systems that needs to be carefully dissected and investigated.

In contrast to the plant models, much less is known about the dissection of the host-pathogen interaction in animal model systems, or how innate, non-specific host defense mechanisms function to control infection. Overall, the only such defense mechanisms that have been studied in the murine typhoid model are Nramp1, NADPH phagocyte oxidase (phox) and inducible nitric oxide synthase (iNOS). Phox and iNOS were determined to play significantly different roles during infection, phox contributing to killing of STM within the first few hours post-infection (258) and iNOS contributing to a sustained bacteriostatic effect which is most pronounced 7 days post-infection (170). Temporally in between these documented effects of phox and iNOS, Nramp1 has been characterized to control bacterial replication within the first 5-7 days post-infection (262). But STM has devised ways of dealing with all three of these host defense mechanisms. First, unknown SPI2 effectors are involved in the diversion of phox from the SCV to prevent the localized action of ROIs on the intracellular bacterium (259). Second, our work demonstrates that the bacterium responds to the presence of Nramp1 by

upregulating virulence-associated gene expression that ostensibly would improve the ability of the pathogen to survive in the intracellular environment. Finally SPI2 is involved in the diversion of iNOS from the SCV to reduce the impact of RNIs on the pathogen (170).

However the theory presented above would indicate that the only effect of Nramp1 would have on the intracellular pathogen would occur within the early stages of infection (i.e. prior to day 7 post-infection). Yet our studies on *mntH* and *sitA* indicate that Nramp1 may play a role much later in infection, or may have a cumulative effect on the pathogen. As the bacteria are present in splenic and hepatic macrophages prior to day 18, Nramp1 may have some specific effect on later stages of infection. Nramp1 has been associated with regulation of macrophage activation as measured by production of nitric oxide, IL-1, and IFN-γ, MHC class II expression (269) and Th1/Th2 differentiation ((35)and references therein). Further, the MHC has been found to play an important role in late-phase susceptibility to STM infection (130). T lymphocyte subsets were also required for effective clearance of STM (182, 188). Therefore, it is possible that the observed effects we are seeing at the later stages of infection in the Nramp1 congenic mice is due to the effect of Nramp1 on these distinct host defense mechanisms. Due to the interconnectedness between Nramp1 and these other various defense mechanisms, it is obvious that the elucidation of the Nramp1-pathogen interaction is complicated and requires much further investigation.

5.5 Interplay between Nramp1 and STM during the development of a persistent infection

Using Nramp1-transfected cell lines and congenic knockout mice, we have been able to study the dynamic nature of infection in two relevant model systems. This has yielded some exciting insights into the STM– host interaction. Initially, finding that the

presence of functional Nramp1 resulted in the upregulation of genes required for intramacrophage survival was surprising. An effective strategy for halting replication of this pathogen inside host cells would have been to suppress SPI2 expression in some manner. However, we observed the opposite effect, such that in *Nramp1*$^{+/+}$ animals loci involved in intramacrophage survival were upregulated. Presumably this increase in SPI2 expression permits replication of STM at early time points in infection, but eventually *Nramp1*$^{+/+}$ mice control and ultimately survive an infection with wildtype STM.

This SPI2-mediated ability to replicate in the presence of Nramp1 may reflect the dynamic tug-of-war that occurs between host and pathogen during the infectious process. Thus, we propose that the challenge faced by *Salmonella enterica* during evolution was not only to become an intracellular pathogen but to become a pathogen that could withstand the effects of innate host defense mechanisms such as Nramp1. As described in Hentschel and Hacker (123), "the evolutionary success of a horizontal gene transfer event should be governed by the need to increase the fitness of the recipient bacterium". Therefore acquisition of SPI2 would not only allow the bacterium to survive within macrophages but would permit it to maintain a moderate replication rate in a normally non-permissive environment. Thus, this bacterium would effectively be increasing its fitness in a specific ecological niche.

It is commonly held that the most successful pathogens are those that infect and colonize a host but do not kill it, allowing for prolonged shedding and thus successful transmission to new hosts. Nramp1 is found in all mammals examined to date, and functional polymorphic variants are found in human populations. Therefore, it is expected that intracellular pathogens such as STM would continuously encounter this innate host defense system "in the wild". Thus SPI2-enhanced replication in *Nramp1*$^{+/+}$ hosts may be essential for the development of a "successful" pathogenic strategy – that of

the production of a self-limiting disease (STM and gastroenteritis) or the establishment of a carrier state (*S.* Typhi and typhoid fever).

Unfortunately, little is known about the persistence of STM in the murine typhoid model. In addition, the murine model is not appropriate for studying enteropathogenicity as mice infected with STM do not develop diarrhea. Although bacterial persistence is important in human infection by both *S.* Typhi and STM, animal models to study this interaction are poorly characterized. Recently, a model for the study of STM persistence in the murine typhoid model was developed. It involves the use of STM strain deficient in AgfA fibres required for efficient colonization of the intestine. *Nramp1*$^{-/-}$ animals infected orally with AgfA- STM develop a chronic carrier state, associated with persistence of the bacteria in the small intestine, spleen and liver (246). This model was used to identify PNPase (polynucleotide phosphorylase) as a global regulator of persistency in STM (56). PNPase was further found to regulate SPI2 genes, implicating this pathogenicity island and associated TTSS in persistence of infection. Therefore, there is evidence that SPI2 is involved in persistence in naturally susceptible mice. However, this model system has not been expanded to see if it can be used to model chronic infection in *Nramp1*$^{+/+}$ animals.

An unrelated study has recently identified a possible link between Nramp1 and persistence in a murine model of *Salmonella* infection (47). However, this work did not use STM but instead focussed on infection with *S.* Enteritidis. In this model, even the susceptible (*Nramp1*$^{-/-}$) animals survived infection with this microorganism. Surprisingly, these authors found that mice with the mutant allele of Nramp1 (i.e. *Nramp1*$^{-/-}$) had a greater capacity to clear the bacterial load from the reticuloendothelial system than did *Nramp1*$^{+/+}$ animals (47). Unfortunately clear conclusions can not be drawn from this model system and directly applied to STM infection due to the differences between STM and *S.* Enteritidis as pathogens. This study does suggest a role for Nramp1 in later stages

of infection. However, as its function appears to be the polar opposite of the currently accepted role for Nramp1 in controlling infections, it is obvious that the role of Nramp1 in infection remains controversial.

These novel models for the study of chronic infection could be applied to test our hypothesis of the role of SPI2 in chronic infection of naturally resistant hosts. It would be interesting to observe if either of these animal models could be exploited to expand our hypothesis. This would not only clarify the role of Nramp1 in later stages of infection, but would also address the importance of SPI2 in STM's pathogenic strategy and the development of a persistant infection. This would provide much-needed insight into a poorly understood area of the host-pathogen interaction.

5.6 The function of Nramp1: is "natural resistance" misleading?

Nramp1 was originally identified as a locus governing susceptibility to diseases caused by three intracellular pathogens. Study of this gene has thus focussed on host resistance on the assumption that this is Nramp1's only function relevant to pathogenesis. However recent work demonstrates that Nramp1 is involved in erythrophagocytosis and recycling iron from senescent red blood cells (32, 187, 274). Two points suggest that host defense was not the "original" role of Nramp1 in the host: 1) Nramp1 is primarily expressed in macrophages, which are scavenger cells involved in phagocytosis of senescent erythrocytes; and 2) Nramp1 is highly homologous to Nramp2, which is involved in transferrin-dependent iron uptake. Combining these two points, from an evolutionary standpoint it is possible that Nramp1 arose as a method to release iron from endosomes as part of the iron-recycling pathway.

As a result of Nramp1 depleting the vacuole of divalent cations including Fe^{2+} and Mn^{2+}, there would be an associated effect on the survival of intracellular pathogens. Nramp1 polymorphisms would still arise and be maintained in the host population for

their secondary effects on limiting infections by intracellular pathogens as well as effects on the development of autoimmune diseases. In addition, presence of NRAMP1 in the majority of the population would still provide the selective pressure to drive the evolution of STM and other intracellular pathogens in their adaptation to the cation-restricted vacuole. In this respect, there is support for the effort to rename Nramp1 as Slc11a1 (solute carrier 11a1). This is not intended to diminish the role of the protein in host resistance. However, it would acknowledge that the original function of Nramp1 is not likely rooted in host resistance. This would require us to shift our perspective from how Nramp1 evolved to mediate resistance to intracellular pathogens to the point of view of how STM evolved and adapted to deal with the ubiquitous presence of Nramp1 in mammalian hosts. Although this may appear to be a minor shift in viewpoint, it has significant implications for how we view the increasing complexity of the host-pathogen interaction. However, our ability to "think outside the box" may well be essential to unraveling the Gordian Knot of host-pathogen interactions.

5.7 Summary

The purpose of this work was to elucidate the interplay between the host resistance protein Nramp1 and the intracellular pathogen *S.* Typhimurium. During the course of this work we determined that the presence of Nramp1 results in an increase in expression of virulence-associated genes in STM, including SPI2-associated genes thought essential for intracellular survival and the genes encoding the two STM Mn^{2+} transport systems, *mntH* and *sitABCD*. We determined that divalent cation concentration was involved in this increase in expression of these loci and that depletion of Fe^{2+} was a stimulus for upregulation of SPI2-associated genes. We further identified a role for MntH, the STM Nramp1 homologue, in virulence of this pathogen. In so doing we began to focus on the role of Mn^{2+} in virulence and infection. This coincided with the

demonstration of the ability of Nramp1 to transport Mn^{2+}, which led other researchers to contemplate the role of divalent cations other than Fe^{2+} in host resistance to infection. Our data support the hypothesis that depletion of the vacuole of divalent cations affects *S.* Typhimurium virulence gene regulation. This may represent one facet of the pathogenic strategy of the bacterium as this increase in virulence gene expression does not appear to be detrimental to the bacterium. In contrast, it may be involved in maintaining some level of replication of *S.* Typhimurium in naturally resistant hosts. Therefore, we hypothesize that this pathogenic strategy contributes to the ability of *S.* Typhimurium to cause chronic infection in naturally resistant hosts.

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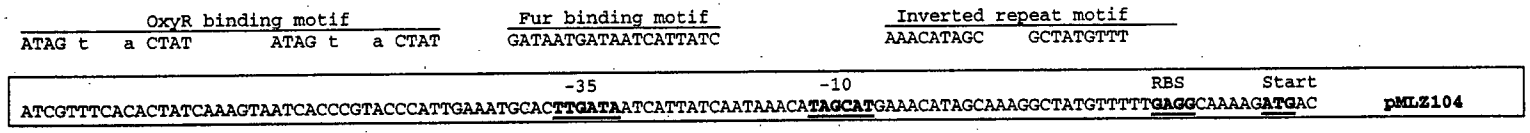
APPENDIX

Illustration of the construction of the *mntH* promoter deletion plasmids. The plasmids were constructed by Dr. D.G. Kehres, Case Western Reserve University, Cleveland, OH. These plasmids were derived from pMLZ104, the original *mntH::lacZY* transcriptional fusion plasmid. In order to avoid potential alterations in gene expression levels based solely on the deletion of plasmid DNA, the specific regions of interest were replaced with sequences of DNA corresponding to the P22 *ant* promoter. The P22 *ant* promoter was chosen as it is similar in its –35, -10, Shine-Dalgarno (SD) and ATG spacing to the *mntH* promoter, and as long as STM is P22- nothing is known to bind to these regions of the P22 *ant* promoter which would affect *mntH* expression. Replacements were made by first mutating pMLZ104 and the P_{ant} promoter region to contain one of the following restriction enzyme sites: *Xba*I, *Sac*I or *Kpn*I. Then the corresponding region of the Pant promoter was swapped for the native region of the pMLZ104 construct by restriction digest and ligation using a 5′ *Eco*RI site in the pMLZ104 construct. Plasmids were verified by DNA sequencing.

pMLZ104:



Wildtype P_{ant} promoter from which the replacement sequence was derived:

TAAAGTGCGGATCATCTCTAGCCATGCCATCACTCCAAGTTAGTGTATTGACATGATAGAAGCACTCTACTATATTCTCAATAGGTCCACGGTGGACCTGTATTGTGAGGTGAATATG P_{ant}

Sequences of the three mutant promoters derived by replacing the individual motif with the P_{ant} sequence and inserting a novel restriction site: