Genetic Analysis of the Effect of *Nramp1* on the Host and Pathogen Genomes in the Context of Chronic *Salmonella* Infection

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ABSTRACT

In humans, *Salmonella* infections cause two major clinical diseases: salmonellosis and typhoid fever. Silent carriage of the bacteria is frequent and contributes to disease dissemination. Using a genomic approach, we have reported the identification of ten loci (*Ses1*-*Ses10*) affecting *Salmonella* persistence in mice. A major locus, *Ses1*, was validated using a congenic approach. *Nramp1* remains a strong candidate gene for *Ses1* although we did not detect a significant interaction between *Ses1* and *Nramp1*$^{-/-}$. We also present the creation of new double congenic strains (*Ses1/Ses4* and *Ses1/Ses5*) that will be used to validate the inheritance model of *Salmonella* clearance in females. Furthermore, the influence of *Nramp1* on the transcriptome of *Salmonella* was investigated and diverse virulence mechanisms were shown to be involved. Notably, differential *phoP* expression, and the resulting differential expression of PhoP-regulated genes, was observed in the presence of *Nramp1*. Our results confirm the importance of host-pathogen interactions in determining the outcome of infection.

RÉSUMÉ

Les infections à salmonelles regroupent différentes maladies dont la salmonellose et la fièvre typhoïde. Le portage asymptomatique des salmonelles est fréquent et contribue à la dissémination de la maladie. En utilisant une approche de criblage génomique par locus, nous avons identifié dix loci (*Ses1*-*Ses10*) affectant la persistance de *Salmonella* chez la souris. Un locus majeur, *Ses1*, a été validé en utilisant des souris congéniques. *Nramp1* demeure un gène candidat de choix pour *Ses1* quoiqu'un test d'interaction *Ses1*/*Nramp1*$^{-/-}$ se soit avéré non significatif. Le modèle proposé de portage de *Salmonella* incluant des interactions entre les loci *Ses1*/*Ses4* et *Ses1*/*Ses5* sera exploré par la création de nouvelles lignées congéniques combinatoires qui ont été créées durant la préparation de cette thèse. L'influence de *Nramp1* sur le transcriptome de *Salmonella* a été étudiée au niveau des mécanismes bactériens de virulence. En particulier, nous avons observé une expression différentielle de *phoP*, et par conséquent l'expression différentielle de gène dont l'expression est contrôlée par PhoP, en présence de *Nramp1*. Nos résultats confirment l'importance des interactions hôte-pathogène dans l'issue de l'infection à salmonelles.

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LIST OF ABBREVIATIONS

129S6	129S6/SvEvTac
AMP	Antimicrobial peptides
ANOVA	Analysis of variance
BMDM	Bone marrow-derived macrophage
Btk	Bruton's tyrosine kinase
Ccl20	Chemokines (C-C motif) ligand 20
cDNA	Complementary DNA
CFU	Colony forming unit
CI	Competitive index
COG	Clusters of orthologous groups
DC	Dendritic cells
DEPC	Diethylpyrocarbonate
DIG-11-dUTP	Digoxigenin labeled deoxyuridine triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
gDNA	Genomic DNA
H-2	Mouse major histocompatibility complex
IFNγ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
Il8ra	Interleukin 8 receptor, alpha
Il8rb	Interleukin 8 receptor, beta
Inpp5d	Inositol polyphosphate-5-phosphatase D
Ity	*I*mmunity *t*o *T*yphimurium
kb	kilobase
KC	Keratinocyte-derived cytokine
LAMP	Lysosomal-associated membrane proteins

LBP	LPS-binding protein
LD_{50}	Lethal dose, 50%
LOD	Logarithm of odds
lpf	Long polar fimbrial
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
M cells	Microfold cells
M6PR	Mannose 6-phosphate receptor
Mb	Megabase
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MOI	Multiplicity of infection
NaAc	Sodium acetate
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor κB
NK	Natural killer
Nos2	Inducible nitric oxide synthase
Nramp1	Natural resistance-associated macrophage protein 1
NTS	Non-typhoidal salmonellosis
OD600	Optical density at a wavelength of 600 nm
OM	Outer membrane
Omp	Outer membrane proteins
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphonuclear leukocytes
PP	Peyer's patches
PRR	Pattern recognition receptors
QTL	Quantitative trait loci
Rb1-PCR	PCR using primers specific for the Rb1 sequence tag

rDNA	Ribosomal DNA
RES	Reticuloendothelial system
RIS	Recombinant inbred strain
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SCOTS	Selective capture of transcribed sequences
SCV	*Salmonella*-containing vacuoles
SDS	Sodium dodecyl sulfate
Ses	*Salmonella E*nteriditis *S*usceptibility
SGL	Significant gene list
SH3	Src homology 3
Ship	Src homology 2 domain-containing inositol 5-phosphatase
Sif	*Salmonella*-induced filaments
Slc11a1	Solute carrier family 11 member 1
SNP	Single nucleotide polymorphism
SPI	*Salmonella* pathogenicity islands
spv	*Salmonella* plasmid virulence
SSC	Saline-sodium citrate buffer
Th	T helper
Th2	T helper type 2
Tlr4	Toll-like receptor 4
TNFα	Tumor necrosis factor α
TSA	Trypticase soy agar
TSB	Trypticase soy broth
TTSS	Type III secretion system
xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

THESIS OBJECTIVES

A mouse model was developed to study the late phase of a *Salmonella* Enteritidis infection. Six weeks post-inoculation C57BL/6J mice clear the bacteria whereas 129S6/SvEvTac mice become chronic carriers despite being extremely resistant to acute infection. Ten loci affecting bacterial burden were identified by linkage analysis and designated *Ses (Salmonella* Enteritidis susceptibility) 1 to 10. The *Ses1* locus was validated using a congenic approach and *Nramp1* was identified as a strong candidate gene. Our hypothesis is that *Nramp1* is the gene underlying *Ses1*, and its interactions with other host loci (*Ses4* and *Ses5*) and with the bacterial transcriptome are responsible for bacterial clearance in C57BL/6J mice. To test this hypothesis, three objectives were established. First, we wanted to validate the candidacy of *Nramp1* as the gene underlying *Ses1* using a quantitative complementation approach. The second objective was to generate single and double congenic strains to investigate the interactions between *Ses1* and newly identified QTL (*Ses4* and *Ses5*) and to assess their influence on the clearance phenotype. In addition, we wanted to investigate the effect of *Nramp1* on bacterial gene expression using selective capture of transcribed sequences.

INTRODUCTION

Section 1: *Salmonella*

1.1 Introduction

Foodborne diseases are a public health burden in both developed and developing countries. These illnesses are most commonly caused by bacteria such as *Campylobacter jejuni*, *Escherichia coli* O157:H7, and *Salmonella* species (1, 2). *Salmonella* are rod-shaped Gram-negative bacteria found ubiquitously in nature. They are spread through contaminated water or food and can infect a broad range of hosts such as insects, birds, reptiles, and mammals (3). *Salmonella* infections can lead to a broad range of disease states ranging from the asymptomatic carrier state to sepsis.

Presently over 2500 *Salmonella* serovars have been identified (4). Serovars were initially divided according to their antigenic structures as defined by the Kauffmann-White serotyping scheme (5). With the advance of technology, *Salmonella* strains are now classified according to the allelic types of their housekeeping genes (6). The current classification scheme has 2 species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* (7). A majority of serovars (60%), including *Salmonella* Typhi, Paratyphi, Typhimurium, and Enteritidis, belong to *Salmonella enterica* subsp. *enterica* (5). Some strains are host specific, such as *Salmonella* Typhi and *Salmonella* Paratyphi in humans, *Salmonella* Dublin in cattle, *Salmonella* Gallinarum and *Salmonella* Pullorum in birds; whereas other strains such as *Salmonella* Typhimurium and *Salmonella* Enteritidis can infect a broad range of hosts.

1.2 Structure

Bacteria of the *Salmonella* genus have a single circular chromosome of approximately 5 megabases (Mb) that encodes 4600 genes (8-10). Comparative genomic analysis has revealed that 90% of the genome is conserved among *Salmonella* species (11). The remaining 10% of the genome consists of DNA sequences acquired through horizontal gene transfer which are unique to each serovar. These genomic regions usually encode for surface structure determinants and virulence genes that can cause variation in virulence and epidemiology between serovars. *Salmonella* species and *Escherichia coli* share 80% of their genomes (8). *Salmonella* pathogenicity islands (SPI) were initially

identified as gene blocks absent from *Escherichia coli* which confer unique virulence traits to *Salmonella* species. Currently, ten SPI have been identified. Some are present throughout the *S. enterica* species (SPI-1 and SPI-2), whereas others are serovar specific (SPI-7 in *Salmonella* Typhi, Paratyphi C, and Dublin) (12). Different serovars have approximately 98% homology between their common SPI (13, 14). SPI-1 and SPI-2 are the two most studied SPI and encode type III secretion systems (TTSS) involved in invasion and virulence, respectively. TTSS are complex syringe-like apparatuses composed of at least 20 proteins (15). These proteins form a needle complex which spans the bacterial cell wall and a translocon which associates with the host cell membrane. Secreted proteins are implicated in modulating cytoskeletal architecture, membrane trafficking, signal transduction, and cytokine gene expression (16). Other genetic elements, such as plasmids and bacteriophages, are present in *Salmonella* and contribute to the specificity of each serovar (11). Plasmids can be involved in virulence or provide drug-resistance genes; however, some of them are cryptic and have no known function (17). An 8 kilobase (kb) region encoding the *spv* (*Salmonella* plasmid virulence) operon is common to all virulence plasmids (18). This locus is required for progressive systemic infection of different serovars (19). For example, calves infected with mutant *spv Salmonella* Dublin do not develop systemic salmonellosis, unlike calves infected with wild-type *Salmonella* Dublin (20). The *spv* genes are required for host macrophage cytopathology. Most importantly, the *spvB* gene encodes an ADP-ribosylating enzyme that destabilizes the host cell's cytoskeleton (21, 22).

The bacterial cell envelope of Gram-negative bacteria is composed of the outer membrane (OM), the peptidoglycan layer, and the inner membrane. The OM plays an essential role in bacterial survival and virulence, as it is composed of a variety of proteins with immunogenic or virulent properties. OM proteins (Omp), also called porins, function as channels to provide the bacteria with nutrients and to eliminate toxic wastes. OmpC and OmpF are the most abundant porins on the cell surface (23). Early serological studies identified three main antigens present on the OM of *Salmonella*: the somatic, flagellar, and capsular antigens (24). The somatic antigen, also called O antigen or cell wall antigen, is composed of up to 40 repeat oligosaccharide units (25). The somatic antigen is the outermost component of lipopolysaccharide (LPS), an abundant glycolipid at the

bacterial OM (26). LPS is important for gut colonization, as shown by the decreased capacity of strains with a defective LPS structure to colonize the colon (27). LPS has three structural components: the somatic antigen, the core, and lipid A. Lipid A, or endotoxin, is the biologically active centre of LPS. Its recognition by the host immune system leads to the secretion of a variety of cytokines and inflammatory molecules. It is a key mediator of septic shock and can play a lethal role in *Salmonella* infections (28, 29). The flagellar antigen, or H antigen, represents the flagella, whip-like organelles anchored in the cell membrane which extend 15 to 20 μm outwards (30). Flagellin, the molecular subunit of flagella, is encoded by *fliC* and *fljB*. Expression of these two genes is mutually exclusive and results in the expression of flagellin with phase 1 (FliC) or phase 2 (FljB) antigenic specificities (31-33). Most *Salmonella* serovars are diphasic, as they can alternatively express *fliC* or *fljB* by going though phase variation (31). However, a few serovars (such as *Salmonella* Enteritidis and *Salmonella* Typhi) are monophasic and always express an H antigen with the same specificity (34). Currently, 114 H antigens have been recognized (35). The somatic and flagellar antigens are expressed on all *Salmonella* serovars. In contrast, the capsular antigen, also called K antigen or Vi antigen, can be expressed on three serovars only: *Salmonella* Typhi, *Salmonella* Paratyphi and *Salmonella* Dublin. The Vi antigen covers the bacterial wall to prevent recognition of *Salmonella* by the host, hence promoting bacterial survival in the blood (36). For example, the Vi antigen inhibits phagocytosis by preventing complement component C3 from attaching to the bacteria (37). In addition, expression of the Vi antigen reduces the production of the neutrophil chemoattractant interleukin 8 (IL-8), leading to a significant decrease in neutrophil influx in the intestinal mucosa (38). However, it is not essential for virulence as Vi antigen-negative mutants can establish an infection (39).

Section 2: Human infection

Salmonella infections lead to two major diseases in human: typhoid fever, a systemic disease, or non-typhoidal salmonellosis (NTS), a self-limiting gastroenteritis. Cases of typhoid fever usually occur in the developing world; whereas NTS cases are reported worldwide.

2.1 Typhoid Fever

Typhoid fever has an estimated incidence of 22 million cases worldwide with 200 000 associated deaths (40). The diseases' etiological agent is *Salmonella* Typhi, a human specific serovar. Typhoid fever is endemic in South America, Africa, and Asia (41). Paratyphoid fever is a clinically similar disease with less severe symptoms (42). It is caused by *Salmonella* Paratyphi A, B and C.

Clinical manifestation of the disease usually occurs 7 to 14 days following infection. Typical symptoms are fever, malaise, headache, anorexia, nausea, and abdominal pain (41). The severity of the disease depends on a number of factors such as age, immune state, and size and virulence of inoculum (43). Appropriate treatment alleviates symptoms within a few days. Untreated patients will show signs of sickness for 2 weeks before symptoms slowly attenuate over a period of up to 4 months, with a mortality rate of 12 to 30% (24).

Infected individuals can be effectively treated with antibiotics such as chloramphenicol, ampicillin, and amoxicillin to relieve symptoms within a week following the start of treatment. The development of multidrug resistant strains in areas of Africa and Asia has led to the use of fluoroquinones as the primary treatment for *Salmonella* Typhi infections (41). The emergence of new nalidixic acid resistant strains, which have a decreased sensitivity to fluoroquinones, poses a new challenge in the treatment of typhoid fever.

Asymptomatic chronic carriage can develop in approximately 1 to 5% of patients. In these individuals, which act as a unique reservoir for new *Salmonella* Typhi infection, the bacteria persist in the gallbladder and can be recovered from the stools at least one year following infection (42). However, approximately 25% of chronic carriers have no previous history of typhoid fever (24).

2.2 Non-typhoidal salmonellosis

Worldwide estimates of NTS vary from 200 million to 1.3 billion cases each year, with 3 million associated deaths (43). Symptoms appear 6 to 72 hours following the ingestion of at least 50 000 bacteria (43). NTS is characterized by an acute onset of fever, abdominal discomfort, diarrhea, nausea, and sometimes vomiting. Symptoms usually

resolve within a few days without antimicrobial treatment. The infection can also cause enterocolitis, an inflammation of the small and large intestine characterized by bloody diarrhea, fever, and severe abdominal pain lasting 2 to 3 weeks (44). Antibiotics may be used in the case of severe illness or at risk patients (45). NTS can be caused by many different serovars; however, 60% of cases are caused by four serovars: *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Newport, and *Salmonella* Heidelberg (46). A small number of individuals who get NTS may develop Reiter's syndrome, characterized by pains in the joints, irritation of the eyes, and painful urination which can lead to chronic arthritis (47).

2.3 Vaccines

Two vaccines against typhoid fever are commercially available. The first one, the Vi vaccine, stimulates a protective antibody response against *Salmonella*'s capsular antigen, which otherwise prevents the binding of antibodies to the somatic antigen (48). It is administered as a single intramuscular dose and is effective within 7 to 10 days. The second vaccine is a live vaccine from *Salmonella* Typhi strain Ty21a, made innocuous to humans by chemical mutagenesis (49). Different mutations, including the *GalE* mutation associated to defective O-antigen production, prevent its proliferation within the host (50). It is administered in 3 or 4 doses under the form of bacteria-containing capsules. Both vaccines provide 50 to 80% protection and require boosters every 2 to 5 years (48). Therefore, it is desirable to develop new vaccines which confer better protection for a longer period of time; and new live attenuated vaccines are currently being tested (48). Presently, no vaccine exists for NTS.

Section 3: *Salmonella* pathogenesis

3.1 Invasion

Salmonella pathogenesis was elucidated using mouse models of infection which will be discussed in section 4. *Salmonella* infections occur through the oral route, by ingestion of contaminated food or water. The strongly acidic environment of the stomach creates a bottleneck effect which kills approximately 99% of the infectious dose (51). The surviving bacteria reach the small intestine where they interact with the Peyer's patches

(PP) (52). The adhesion of *Salmonella* Typhimurium to the PP is mediated by genes of the long polar fimbrial (*lpf*) operon (53). *Salmonella* promotes its entry into microfold cells (M cells), which are located under the PP, via bacterial-mediated endocytosis (54). Upon contact with M cells the expression of the SPI-1 TTSS is induced (55). Transcriptional regulation of essential components of the SPI-1 TTSS such as InvA, InvB, InvG, OrgA, and SpaR is under the control of HilA and InvF (54, 56, 57). A mutation in any one of these essential genes renders the *Salmonella* non-invasive, and therefore non-virulent, but only if administered orally. If administered intraperitoneally into mice, these mutants remain virulent (54). Invasion proteins SipB, SipC, and SipD form a translocon which links the TTSS to the host cell to allow secretion of effector proteins into the host cytoplasm. SipA and SipC are also involved in actin polymerization and bundling. The other three secreted effectors required for invasion are SopE, SopB, and SptP. SopE is a guanine nucleotide exchange factor (GEF) which activates Cd42 and Rac1 GTPases (58). SopB is an inositol polyphosphatase which activates RhoG, as well as Cdc42, and Rac1. These effectors promote cytoskeletal rearrangements which lead to host cell membrane ruffles that enclose *Salmonella* in a vacuole. Once *Salmonella* is internalized, SptP can antagonize Cdc42 and Rac1 though its GTPase activating protein (GAP) and tyrosine phosphatase activities and mediate recovery of the host cell morphology (59). Furthermore, SipA, SopB, and SopE can disrupt the structure and function of tight junctions which usually maintain the integrity of the epithelium (60). Altogether, this leads to the destruction of M cells and depolarization of the intestinal epithelium and allows invasion of adjacent enterocytes by *Salmonella*.

Once *Salmonella* has crossed the intestinal epithelium, it can infect local submucosal phagocytes. Dissemination of the bacteria in the host occurs through phagocyte migration to organs of the reticuloendothelial system (RES) through the lymphatics and blood stream. Interestingly, *Salmonella* strains deficient for SPI-1 can also colonize the gut and cause systemic disease through an M cells independent mechanism (55). This invasion-genes independent mechanism relies on CD18-expressing leukocytes residing in the lamina propria of the ileum. These cells can uptake *Salmonella* in the gut lumen and transport it through the bloodstream to the liver and spleen (61, 62). *Salmonella* serovars which cause enteritis can invade enterocytes; however, they cannot

breach the intestinal epithelial barrier. Enteritis is associated with a massive recruitment of neutrophils into the submucosa of the intestine and transmigration of neutrophils into the intestinal lumen (52).

3.2 Intracellular survival

Once inside phagocytes, *Salmonella* survive and replicate within *Salmonella*-containing vacuoles (SCV). This intracellular niche protects *Salmonella* from antimicrobial agents produced by the host (63). Immediately after its formation the vacuole acquires early and recycling endocytic markers such as early endosomal antigen 1 (EEA1), transferrin receptor (TfnR), Rab5, and Rab11 (64). Within 30 minutes these markers are replaced by lysosomal-associated membrane proteins (LAMP1 and LAMP2). Despite the acquisition of these late endosomal markers, the SCV does not merge with lysosomes, thus preventing *Salmonella* degradation (65).

The biogenesis of the SCV requires the SPI-2 TTSS and, to a lesser extent, the SPI-1 TTSS (63). The importance of SPI-1 for intracellular survival was just recently established using a *Salmonella* Typhimurium *invA* mutant. This mutant cannot assemble a functional SPI-1 TTSS and therefore cannot invade non-phagocytic cells (56). Steele-Mortimer *et al* demonstrated that when the *invA* mutant was co-internalized with wild-type *Salmonella* in epithelial cells, the mutant was unable to replicate (66). More specifically, the formation of SCV requires SopB, a SPI-1 TTSS effector that promotes membrane fission through an unknown mechanism (67). Other SPI-1 effectors may be involved in the early stages of SCV formation to compensate for the lag-time necessary before SPI-2 proteins are expressed, approximately 3 to 4 hours post-invasion. SPI-2 effectors have been shown to control both SCV membrane dynamics and trafficking between the SCV and the endocytic pathway to protect *Salmonella*'s niche. The SPI-2 protein SpiC is required for translocation of effectors into the host cytosol (68). SpiC inhibits intracellular membrane fusion and is known to interact with host proteins with a possible role in phagosomal maturation (69). Currently, 19 effectors have been identified. Most effectors are thought to translocate to the host cell cytosol to promote intravacuolar replication. Only a few effectors such as SseI, SteC, SifA, SseJ, SseF, and SseG have known functions. SteC is involved in the formation of an actin meshwork that surrounds

the SCV and is thought to play a role in maintaining its membrane integrity (70). SseI (also known as SrfH) interacts with host proteins, thus promoting motility of infected phagocytes and accelerating systemic spread (71). The stability of the SCV requires the formation of long tubular extensions called *Salmonella*-induced filaments (Sif) (72). The SPI-2 secreted effector SifA directs the formation of Sif along microtubules (73). SseJ localizes to Sif and is involved in vacuolar membrane disruption of *sifA* mutant strains (74). SseF and SseG also contribute to Sif formation (75). In addition, SseF and SseG have been involved in the recruitment of exocytic vesicles to the SCV. This suggests that *Salmonella* hijacks both the endocytic and exocytic pathways of the host to promote its survival (76). The exact roles played by many SPI-2 effectors remain unknown and are currently being investigated. However, studies using *Salmonella* strains deficient for SPI-2 have confirmed its importance for virulence as no mutant was able to cause systemic disease (77, 78).

Furthermore, *Salmonella* has been shown to be cytotoxic to macrophages, both *in vitro* and *in vivo* (79, 80). SPI-1 dependent cell death in the early stage of infection relies on SipB (81). SipB binds and activates caspase-1, a key effector of pyroptosis. In turn, caspase-1 can cleave and activate the pro-inflammatory cytokines IL-1β and IL-18 (81). During the systemic phase of infection the SPI-2 TTSS is required to activate caspase-1 and cause cell death (82). The implications of *Salmonella*-mediated host cell death are not fully understood and it has been proposed to be a mechanism for *Salmonella* to avoid host defenses and invade new host cells. Alternatively, host cell death could benefit the host, preventing bacterial replication and exposing *Salmonella* to other antimicrobial mechanisms (82).

3.3 *Salmonella* transcription profile following macrophage infection

Bacterial survival within host cells depends on the ability of *Salmonella* to adapt to the intracellular environment. Eriksson *et al* have analyzed the gene expression profile of *Salmonella* Typhimurium recovered from infected J774-A.1 macrophages at 4, 8, and 12 hours post-infection (83). Bacterial RNA from each time point was applied to microarrays and compared to RNA obtained from opsonized *Salmonella* Typhimurium grown in medium. A total of 919 genes were found to be differentially regulated. As

expected, most SPI-1 genes were down-regulated while numerous SPI-2, SPI-3, and SPI-5 genes were induced 12 hours post-infection. Transcription of genes involved in LPS biosynthesis was decreased 4 hours post-infection. No change in gene expression was observed for *rpoS* and *phoP*, two important regulatory genes. RpoS and PhoP control the expression of over 200 genes involved in various aspects of virulence such as invasion, motility, acid tolerance, small molecules transport, antimicrobial peptide (AMP) resistance and bacterial membrane shuffling (84). For example, *Salmonella* Typhimurium up-regulated the expression of the *mgtCB* operon, which encodes the magnesium transporter gene *mgtB*, in response to magnesium limitation 4 hours post-infection. In contrast, PmrAB is a two-component regulator responsive to iron which was down-regulated 8 hours post-infection. These results may reflect the absence of a functional *Nramp1* (natural resistance-associated macrophage protein 1) allele in J774-A.1 cells. *Nramp1*, also known as *Slc11a1* (solute carrier family 11 member 1), is a divalent-cation transporter recruited to the membrane of phagosomes following *Salmonella* infection that affects maturation of the SCV and limits bacterial replication (85, 86). The role of *Nramp1* in *Salmonella* infection will be further discussed in section 7.4. Genes known to be expressed under acidic conditions such as *cadB*, *cysB* and *adiY* were up-regulated, thus confirming that the SCV is acidic. PgtE is a protease which cleaves AMP and was up-regulated by *Salmonella* Typhimurium inside cells. There was a decrease in the expression of genes encoding type 1 fimbriae or flagella, reflecting *Salmonella* Typhimurium's attempt to evade immune recognition. Furthermore, SodCI, an enzyme which catalyzes superoxide radicals to less toxic products, was induced inside macrophages. The expression of genes required to repair the damage caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) was also up-regulated. Finally, approximately 50% of the genes differentially regulated have unknown or putative functions. Overall these results provided great insight on the transcriptional profile of *Salmonella* Typhimurium during macrophage infection (83).

3.4 *Salmonella* persistence

An important aspect of *Salmonella* pathogenesis, as well as other pathogens such as *Mycobacterium tuberculosis* and *Helicobacter pylori*, is the establishment of a persistent infection. Long-term carriers act as a reservoir for future infections and allow

bacterial survival in restricted populations (87). In humans, *Salmonella* can persist in the gall bladder and bone marrow of persistently infected patients (88, 89). Carriers also have high titers of serum antibodies against the Vi and flagellar antigens. They do not show any signs of disease although they are at increased risk of developing hepatobiliary cancer (90).

3.4.1 Persistent *Salmonella* infection

Salmonella proteins involved in the establishment of a chronic carrier state have been identified (91-95). The *Salmonella* gene *aceA* encodes isocitrate lyase, an essential enzyme for the metabolism of fatty acids. Studies demonstrating the role of isocitrate lyase in *Mycobacterium tuberculosis* persistence triggered investigation of its role in *Salmonella* persistence (91, 96). Competitive index (CI) experiments were done using the *Salmonella* Typhimurium *aroA*$^-$ model of chronic infection. Mice were infected with equal numbers of *aroA* mutants and *aroA aceA* double mutants (91). Fourteen days post-inoculation a significant defect in the CI of the *aroA aceA* double mutant strain was observed. CI experiments were also done in which 129Sv mice were infected orally with 10^9 CFU. Two months post-inoculation the *aroA* mutant was detectable in the MLN but the *aroA aceA* double mutant had been cleared, thus demonstrating that *aceA* is required for the establishment of a persistent state of infection (91).

The *Salmonella* proteins Mig-14 and VirK are known to inhibit binding of cathelin-related AMP to *Salmonella*, and have recently been shown to play a role in *Salmonella* persistence (92, 93). 129X1/SvJ mice were infected with 10^7 CFU of *Salmonella* Typhimurium and bacterial load was determined 123 days post-infection. It was found that more mice infected with a *mig-14* mutant *Salmonella* Typhimurium were able to clear the bacteria compared to mice infected with wild-type *Salmonella*. However, among all mice that did not clear the infection, bacterial loads in the spleen were similar. This indicates a role for *mig-14* in the establishment, but not the maintenance, of chronic infection (92). Similarly, results from CI experiments showed that the *virK* mutant *Salmonella* Typhimurium was outcompeted by the wild-type bacteria in both the liver and the spleen of 129X1/SvJ mice 3 weeks post-infection, indicating a role for *virK* in bacterial persistence in the late phase of infection (93).

3.4.2 Genome-wide scan

A genome-wide approach using a negative selection screen identified 118 *Salmonella* genes required for long-term systemic infection (94). 129X1/SvJ mice were infected intraperitoneally with a *Salmonella* Typhimurium SL1344 Tn10 transposon-mutagenized library of 50 000 mutants. The intraperitoneal route of infection was used to bypass the 'bottleneck effect' created by the acidic environment of the stomach which could significantly influence the complexity of the mutant library. Bacteria from two independent infections were recovered from the spleen and liver at day 28 post-infection. Mutants which could not be recovered from either infection were identified by microarray analysis and a significant gene list (SGL) was compiled. The SGL included genes from SPI-1 to 6, as well as genes from the fimbrial and LPS operons. More specifically, thirteen SPI-2 secreted effectors were selected against, suggesting a novel role for SseK2 and SseI in persistence. The SPI-3 encoded gene *mgtC* was also identified as important for systemic disease. *mgtC* is known to be important for intracellular survival but its exact function remains unknown (97). The identification of SPI-4, -5 and -6 genes provided new insight on the role of these operons as they had not previously been implicated in systemic infection. Noticeably *mig-14* and *aceA* were not selected against, suggesting complementation for their function in the mutant library. Some virulence genes with no previously suggested role in long-term infection were further investigated, and CI experiments confirmed the contribution of SPI-1 effector genes *orgA*, *sipA*, *sipB*, *sipC*, and *sipD* to *Salmonella* persistence. These results provided further evidence that SPI-1 is not only required for the initial invasion phase of infection but also for persistence. The 118 genes in the SGL were functionally classified into clusters of orthologous groups of proteins (COG). Various aspects of *Salmonella* physiology such as RNA processing, energy production, signal transduction mechanisms, intracellular trafficking and secretion, and defense mechanisms were shown to be involved. Of interest are the 30% of genes which did not belong to any COG as they only have putative or unknown function. Different COG were found to be important at different stages of the infection, suggesting that specific virulence genes are required for virulence at different time points. Finally, the SGL was compared to that compiled from a previous study done using BALB/c mice

and only SPI-2 and LPS operons genes overlapped. This could be explained by the different genetic background of 129X1/SvJ and BALB/c mice which may interact differently with *Salmonella*. Particularly, the presence of a mutated *Nramp1* in BALB/c mice could have an impact on bacterial gene expression (94, 98).

Section 4: Mouse models

Salmonella can infect a broad range of hosts which can in turn be used as animal models for the study of *Salmonella* pathogenesis. The mouse is a small, easily managed animal with numerous well characterized inbred strains available in which to model human disease. Early studies on host resistance of mice to virulent *Salmonella* Typhimurium recognized differential susceptibility between inbred strains (Figure 1) (99). In this model the 129S6/SvEvTac (129S6) strain is extremely resistant with a 100% survival rate. In contrast, the C57BL/6J and C3H/HeJ strains are extremely susceptible and succumb to infection within 7 days. Some strains such as the A/J strain will always succumb to infection; however, decreasing infectious doses delay their time of death and they are considered to have intermediate susceptibility (3).

4.1 Typhoid model

In the mouse, *Salmonella* Typhi is avirulent and does not cause disease (100). However, infection of mice with *Salmonella* Typhimurium causes a systemic typhoid-like disease similar to that caused by *Salmonella* Typhi in humans.

Four distinct phases characterize the course of sublethal *Salmonella* Typhimurium infection in mice (Figure 2) (101). Within the first few hours following infection, approximately 90% of bacteria are cleared from the blood. Bacterial killing depends on pre-existing antibodies and on both classical and alternative complement pathways. The level at which the alternative pathway is activated has been linked to the structure of LPS, with rough-type LPS *Salmonella* being cleared more efficiently from the blood (102).

During phase 2, the surviving bacteria reach their intracellular location within macrophages of the RES where they undergo exponential growth for approximately 4 to 5 days. The importance of macrophages in controlling bacterial replication was initially shown using silica, a macrophage inactivating agent. Administration of silica in mice

Figure 1. Survival curve of classical inbred and wild-derived mouse strains to an intravenous challenge with 10^4 CFU of *Salmonella* Typhimurium strain Keller.
Adapted from Roy, M. F., and D. Malo. 2002. Genetic regulation of host responses to Salmonella infection in mice. *Genes and immunity* 3:381-393.

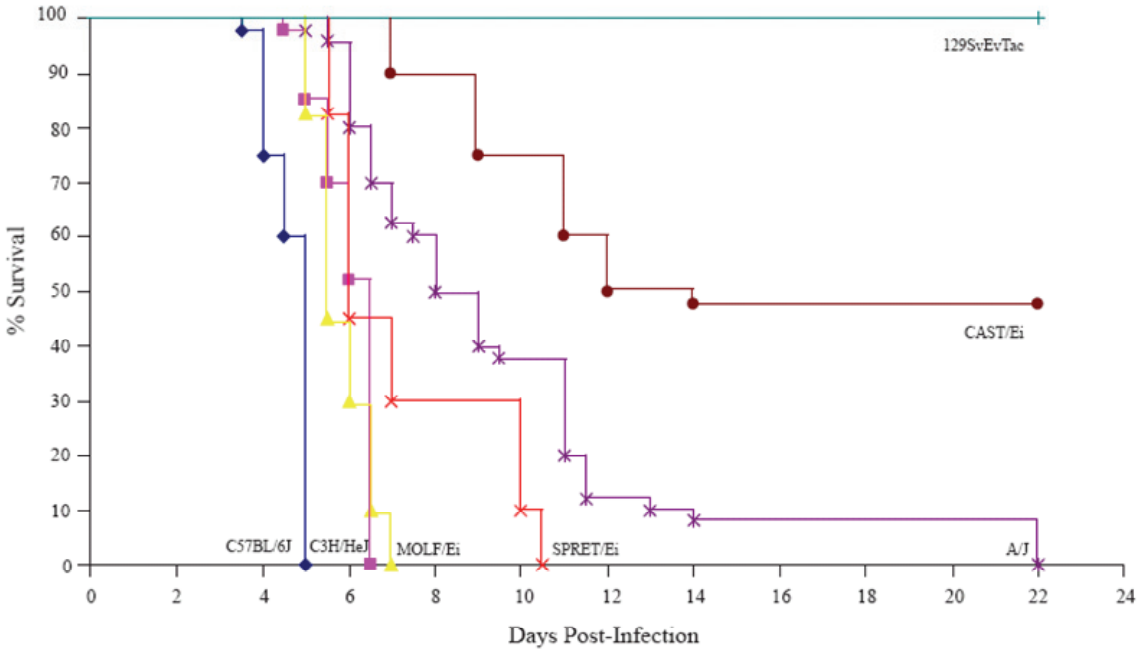
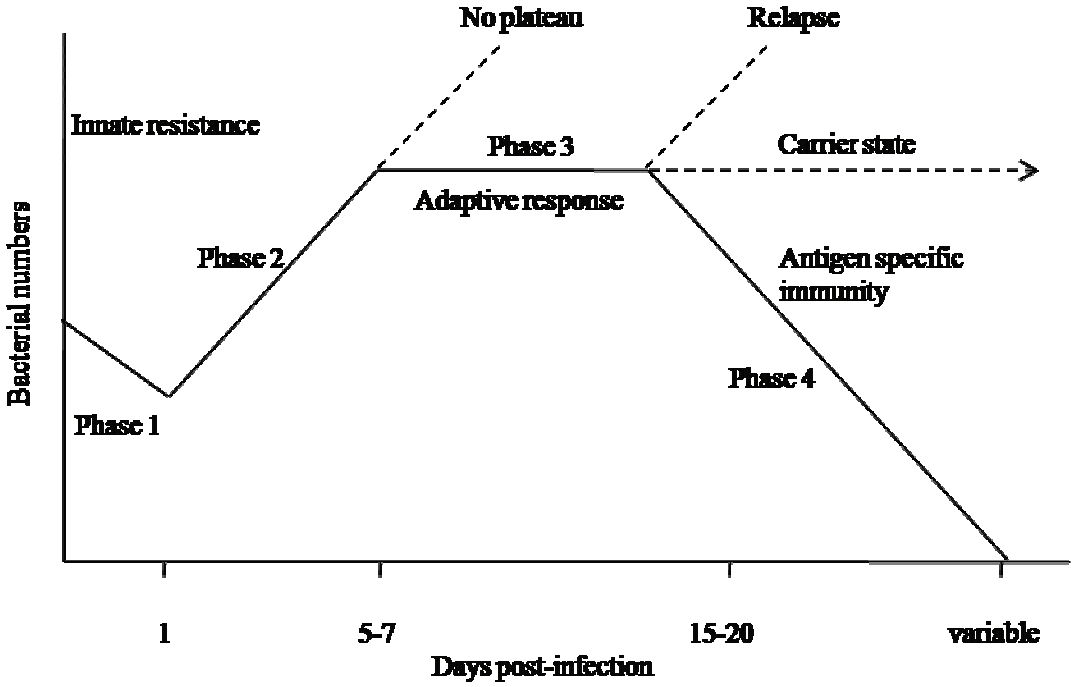

100
90
80
70
60
50
40
30
20
10
0
% Survival
0
2
4
6
8
10
12
14
16
18
20
22
24
Days Post-Infection
129SvEvTac
CAST/Ei
A/J
C57BL/6J
C3H/HeJ
MOLF/Ei
SPRET/Ei

Figure 2. The four phases of sublethal *Salmonella* infection in mice.

Within the first few hours a majority of bacteria are cleared from the blood. During phase 2 the surviving bacteria reach their intracellular location within the RES where they undergo exponential growth for approximately 4-5 days. *Nramp1* as well as ROS and RNS control the intracellular growth of *Salmonella* (1, 2). The plateau phase starts when exponential growth is stopped and the adaptive immune response is activated; it persists for about 1-2 weeks. The fourth phase of infection is clearance of the bacteria from the RES. The incapability to do so due to defects in the adaptive immune system can lead to a relapse of growth or to a chronic carrier state (3). From Mastroeni, P. 2002. Immunity to systemic Salmonella infections. *Current molecular medicine* 2:393-406.


No plateau
Relapse
Innate resistance
Phase 3
Carrier state
Adaptive response
Phase 2
Antigen specific immunity
Phase 4
Phase 1
Bacterial numbers
1
5-7
15-20
variable
Days post-infection

decreases the *Salmonella* Typhimurium 50% lethal dose (LD_{50}) by 100-fold (103). The importance of macrophages in controlling *Salmonella*'s exponential growth was further confirmed by the discovery of *Nramp1* (104). Another important antibacterial function of macrophage comes from the production of ROS, as *Salmonella* growth is more prominent in mice incapable of producing ROS (105).

The third phase of infection is characterized by the activation of the adaptive immune response leading to the suppression of exponential growth. This plateau phasepersists for about 1 to 2 weeks and is necessary for the survival of the animal. Several different host factors control this phase. Toll-like receptor 4 (Tlr4) recognizes the LPS component of *Salmonella*'s OM. Null alleles of *Tlr4* were identified in different inbred mice strains such as C57BL10/ScCr and C3H/HeJ making them more susceptible to acute infection. These mice can only partially control bacterial replication due to defects in macrophage activation and function (101). Furthermore, efficient suppression of growth requires the concerted action of several cytokines to form macrophage-rich granulomas (80). Interferon γ (IFNγ) is required for macrophage infiltration and activation within organs of the RES, and tumor necrosis factor α (TNFα) is involved in the formation of granulomas (106). The production of RNS, in conjunction with granuloma formation, is essential for the suppression of exponential growth (105).

The fourth phase of infection is clearance of the bacteria from the RES through the activation of T cells and B cells, which leads to antigen specific immunity. The incapability to completely clear the bacteria due to defects in the adaptive immune system can lead to a relapse of growth or to a chronic carrier state (101).

4.2 Enterocolitis model

Following oral infection of mice with *Salmonella* Typhimurium, only 1% of an infectious dose can breach the intestinal barrier and target the gut-associated lymphatic tissues to cause a systemic typhoid-like disease (51, 107). For reasons that remain unclear, the bacteria are unable to replicate in the intestine and cause inflammation. Poor colonization of the intestine results from the interplay between the pathogen and the gut flora which compete for nutrients and attachment sites (108). Moreover, the resident microflora produces inhibitory substances such as hydrogen peroxide and volatile fatty

acids which affect bacterial metabolism and survival (109). The intestinal flora also acts in an anti-inflammatory fashion by down-regulating nuclear factor κB (NF- κB) (110).

Previously, colitis was studied using a calf ileal loop model which required extensive logistics (111). Recently, a murine model has been developed in which C57BL/6J mice are pre-treated with 20 mg streptomycin before oral infection with 10^8 colony forming units (CFU) of *Salmonella* Typhimurium SL1344 (112). In this model, *Salmonella* Typhimurium SL1344 can effectively colonize the intestine and cause colitis, as well as a typhoid-like disease. Inflammation of the cecum is observed as early as 8 hours post-inoculation. Within 20 hours of infection, there is evidence of submucosal edema, epithelial erosion, and reduced goblet cells. Polymorphonuclear leukocytes (PMN) infiltration of the submucosa, the lamina propria, and the intestinal lumen is also observed. Significant increase in the liver and mesenteric lymph nodes (MLN) bacterial loads is only observed 48 hours post-inoculation, suggesting that intestinal inflammation occurs independently from systemic infection (112). Streptomycin pre-treated 129S6 mice develop a similar acute colitis following infection (113). Although this inbred strain did not show signs of systemic disease they did develop chronic crypt-destructive colitis within 6 weeks of infection (113).

4.3 Chronic infection model

Mouse models of chronic infection were developed to study the clearance phase of *Salmonella* infections and to gain insight into the mechanisms underlying the establishment of a chronic carrier state (114-118). Initially, models of chronic *Salmonella* infection used attenuated *Salmonella* Typhimurium strains and have provided the basis of our understanding of the acquired immune response to *Salmonella* infection. Currently, two models using wild-type bacteria exist to study the late phase of *Salmonella* infections (116, 117).

The two inbred mouse strains being used in the model of chronic *Salmonella* Enteritidis infection developed in our laboratory are C57BL/6J and 129S6. When infected with 10^3 CFU of *Salmonella* Enteritidis, these mice do not develop clinical disease. Previous work in our laboratory showed that C57BL/6J mice could completely clear the bacteria from their system 42 days post-inoculation whereas 129S6 mice could not

(Figure 3) (117). Loci affecting bacterial burden in the late phase of infection with *Salmonella* Enteritidis were identified by linkage analysis of the F2 progeny from an intercross between C57BL/6J and 129S6 (*Salmonella E*nteritidis *S*usceptibility 1, 2 and 3, *Ses1*-*Ses3*) (Figure 4). *Ses1* alone explained 14% of the phenotypic variance. *Nramp1* is the underlying candidate gene for *Ses1* as it maps 5kb from the maximum peak logarithm of odds (LOD) score and because of the presence of a known functional polymorphism in the *Nramp1* gene between the mice strains used in this study. Interestingly, in this model, a functional allele at *Nramp1* is associated with chronic infection whereas in the acute systemic model of infection with *Salmonella* Typhimurium, the wild-type *Nramp1* allele is associated with resistance and better survival. Seven additional quantitative trait loci (QTL) (*Ses4* to *Ses10*) linked to bacterial persistence were also identified through two-locus epistasis QTL linkage mapping in the segregating F2 population of C57BL/6J and 129S6 (Figure 4). The analysis was done separately in males and females as it was observed that bacterial clearance was influenced by gender. Sexual dimorphism can be explained by loci present on the sex chromosomes or by the influence of sex-specific hormones on the immune system. In females, the novel interaction model proposed explained 47% of the phenotypic variance (118). In this model, *Ses1* and *Ses*3 showed a significant effect alone; however, interactions of *Ses1* with *Ses4* (chromosome X) and/or *Ses5* (chromosome 7) accounted for 72% of the total variation explained. In males, only *Ses1.1* (which is proximal to *Ses1*) had an individual effect. Three interactions (*Ses1*-*Ses6*, *Ses7*-*Ses8*, and *Ses9*-*Ses10*) also contributed to bacterial clearance in the male F2 progeny. This model also explained 47% of the phenotypic variance. Furthermore the *Ses1* locus was validated using a congenic approach where the C57BL6/J *Ses1* interval was transferred to the 129S6 background by serial backcrosses (118). From these analyses, it was clear that *Ses1* was the locus having the greatest impact on *Salmonella* Enteritidis clearance both in females and males.

In a second model of chronic infection, 129X1/SvJ mice are inoculated orally with 10^8 *Salmonella* Typhimurium CFU (116). Initially mice show signs of acute *Salmonella* infection. These symptoms gradually decrease and 60 days post-infection mice do not display signs of illness anymore. However, systemic sites of infection are still colonized by *Salmonella*, and bacteria can be recovered from MLN cells positive for the monocyte

Figure 3. Kinetics of infection following an intravenous challenge with 10^3 CFU of *Salmonella* Enteritidis in C57BL/6J and 129S6 mice.

Forty-two days post-inoculation, C57BL/6J mice (dashed line) completely clear the bacteria from their system whereas 129S6 mice (solid line) become chronic carriers. From Caron, J., L. Lariviere, M. Nacache, M. Tam, M. M. Stevenson, C. McKerly, P. Gros, and D. Malo. 2006. Influence of Slc11a1 on the outcome of Salmonella enterica serovar Enteritidis infection in mice is associated with Th polarization. *Infect Immun* 74:2787-2802.

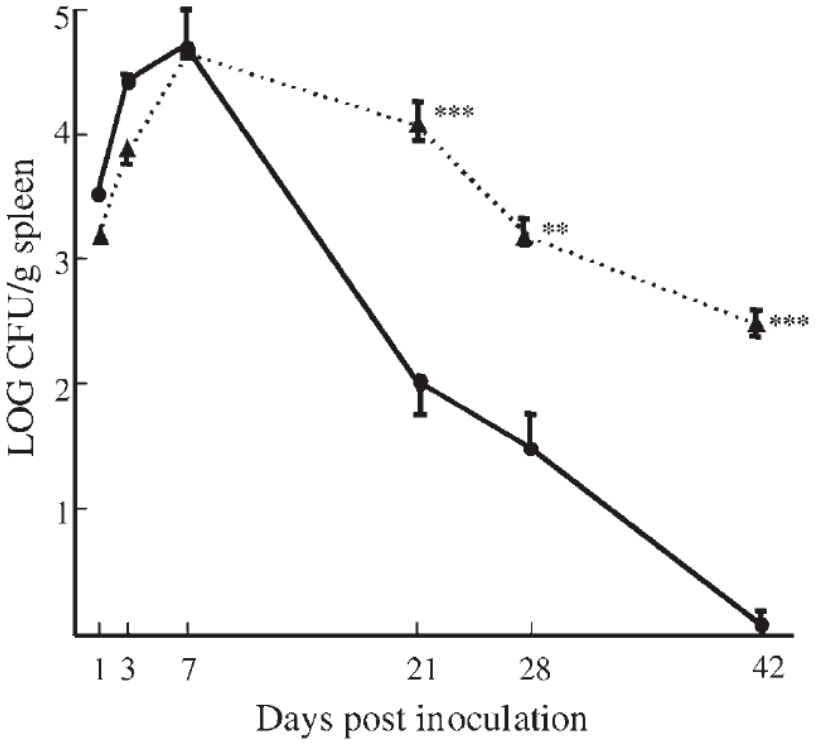
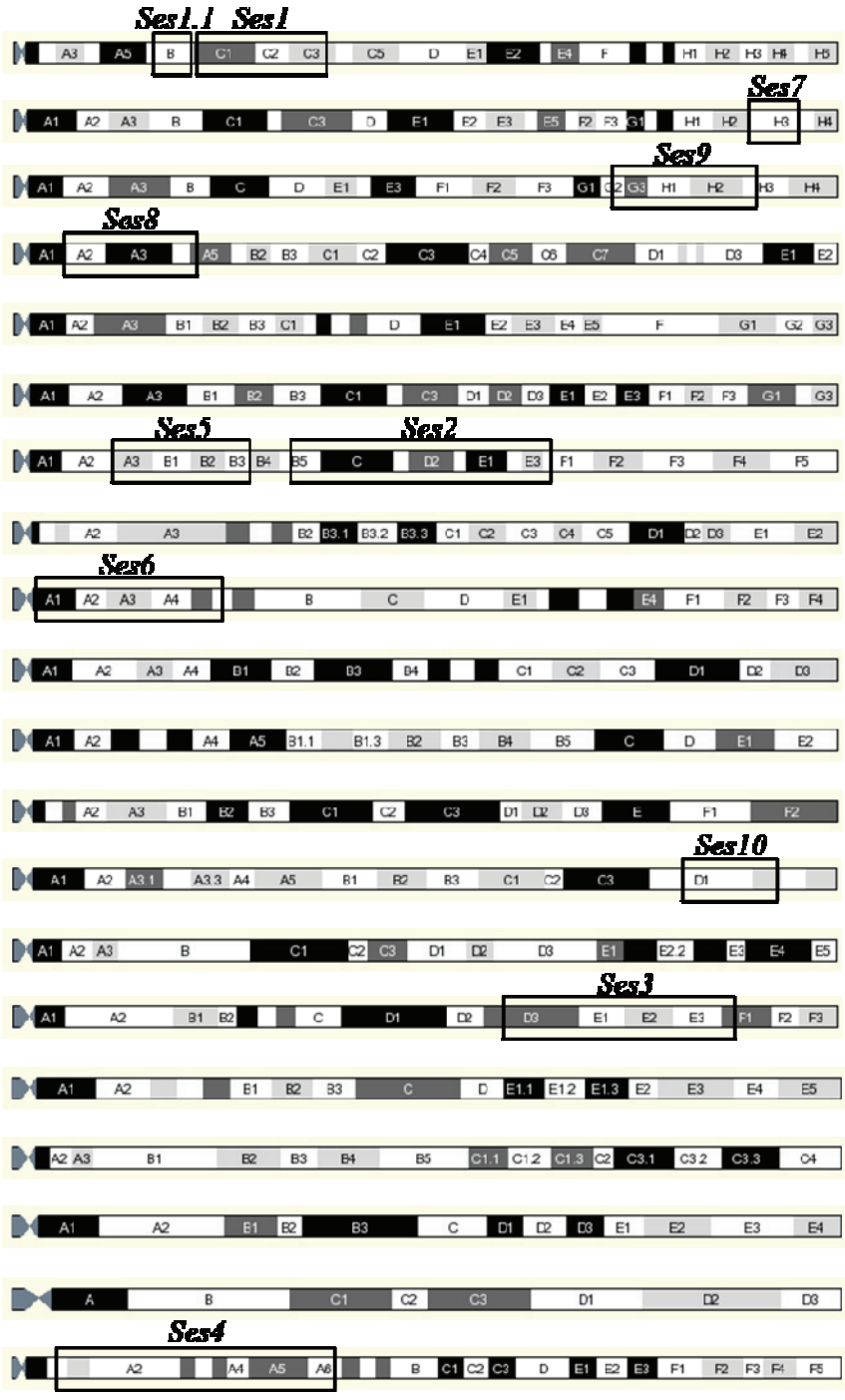

5
4
3
2
1
LOG CFU/g spleen
***
**
***
1 3 7 21 28 42
Days post inoculation

Figure 4. Chromosomal location of loci affecting bacterial burden in the late phase of infection with *Salmonella* Enteritidis.

Physical positions of the loci were determined using the Ensembl mouse genome browser (www.ensembl.org/Mus_musculus). Banding patterns are indicated and the chromosomes are not drawn to scale.


Ses1.1
Ses1
Ses7
Ses9
Ses8
Ses5
Ses2
Ses6
Ses10
Ses3
Ses4

and macrophage marker MOMA-2 up to a year following infection (116). In this model, chronically infected mice injected with IFNγ neutralizing antibody revert back to an acute disease state, underlying the importance of IFNγ in the maintenance of chronic infection. Moreover, it was noticed that some chronically infected mice shed the bacteria at higher rates than others. This "supershedder" phenotype was influenced by *Salmonella* virulence factors, such as SipB and SsaV. Mice infected with *Salmonella* strains mutant for SipB or SsaV, which are deficient for the translocation of all SPI-1 and SPI-2 TTSS effector proteins, respectively, shed low levels of bacteria (119). The phenotype is also influenced by the indigenous intestinal microflora as all streptomycin-pretreated mice infected with *Salmonella* became suppershedders (119).

Section 5: Innate immunity of the host

Phagocytes, and more specifically macrophages, are extremely important for the innate immune response to *Salmonella* infection (103, 120). Upon infection, macrophages can recognize the bacteria via pattern recognition receptors (PRR) such as Tlr4. Recognition of LPS-binding protein (LBP)/CD14-bound LPS by Tlr4 leads to up-regulation of NF-kB, which in turn induces the production of cytokines, chemokines, and co-stimulatory molecules essential for fighting infection (121). For example, TNFα, IL-1, IL-6, IL-12, and IL-18 are expressed and attract immune cells to the site of infection (122). In turn, IL-12 and IL-18 induce the expression of IFNγ (123, 124). *Salmonella* also induces IFNγ expression by natural killer (NK) cells in the early phase of infection (125). IFNγ and TNFα are both involved in macrophage activation, which leads to further secretion of IL-12 and TNFα (126). However, systemic expression of TNFα in excessive amounts leads to septic shock (127). Activated macrophages can destroy a pathogen through a combination of mechanisms. For example, NRAMP1 competes with *Salmonella* for essential ions and it prevents bacterial evasion from the endocytic pathway. As mentioned previously, the role of NRAMP1 in *Salmonella* infection will be discussed in section 7.4. Another important macrophage microbicidal system is the production of ROS and RNS (128).

5.1 Cytokines

Cytokine neutralization studies have provided crucial information for the understanding of the complex network of cytokines induced following *Salmonella* infection. Initially, it was found that administration of anti-IFNγ or anti-TNFα antibodies exacerbated the disease caused by *Salmonella* Typhimurium in CBA mice (129, 130). Similarly, treatment with anti-IL-12 antibodies leads to increased *Salmonella* Typhimurium growth in the RES of susceptible A/J mice and significantly impairs IFNγ production *in vivo* or *in vitro* (124, 131). A/J mice treated with anti-IL-18 antibodies have increased bacterial loads in their liver and spleen, and decreased levels of circulating IFNγ (123). These results demonstrate that both IL-12 and IL-18 are key mediators of the innate immune response to *Salmonella* and that they act through IFNγ. The results obtained from neutralization studies were confirmed using mice with a targeted mutation in the corresponding genes or their receptors. For example, mice deficient for the IFNγ receptor are extremely susceptible to the otherwise avirulent *Salmonella* Typhimurium *aroA*$^{-}$ strain (115). TNFα receptor p55-knockout mice are also highly susceptible to infection and fail to localize NADPH (nicotinamide adenine dinucleotide phosphate) oxidase-containing vesicles in proximity to SCV (132).

5.2 Tlr4

LPS hyporesponsive inbred strains such as C3H/HeJ, C57BL/10ScCr, and C57BL/6.KB2-mnd are extremely susceptible to Gram-negative bacteria, including *Salmonella* species. Genetic linkage analysis located the *Lps* locus responsible for this phenotype on mouse chromosome 4, and positional cloning identified *Tlr4* as a candidate gene (133). Sequence analysis of the *Tlr4* gene showed a non-conservative mutation from proline to histidine at position 712 in C3H/HeJ mice and a deletion of exon 2 in the C57BL/6.KB2-mnd sequence. In C57BL/10ScCr mice, no *Tlr4* transcript was detected because of a 75 kb deletion covering the genomic region of interest. The generation of *Tlr4* knockout 129/SvJ mice confirmed that *Tlr4* was the gene underlying the *Lps* locus as these mice were hyporesponsive to LPS stimulation and behaved in a similar fashion to C3H/HeJ mice (134).

Tlr4 encodes for an 841 amino acid protein expressed at the plasma membrane. It has an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic IL-1 receptor-like region, two characteristic Toll motifs (135). Tlr4 was the first member of the TLR family identified in mammals (135). The family now consists of 13 members, each of which recognizes a specific pathogen-associated molecular pattern (PAMP). TLRs are part of the PRR family of receptors. They are now known to play an essential role in the response to infection in plants, insects, and vertebrates (136).

5.3 NADPH oxidase and Nos2

The contribution of ROS and RNS to the antimicrobial properties of the cell is extremely important. NADPH oxidase and inducible nitric oxide synthase (Nos2) are the 2 enzymes which catalyze the reactions generating ROS and RNS, respectively (128). They are expressed in polymorphonuclear and mononuclear phagocytes. Generation of ROS is greater in neutrophils while RNS are more prevalent in macrophages (137). The variety of ROS and RNS produced is reflected in their antimicrobial actions. ROS injure the bacteria by DNA damage and protein modifications. RNS can inhibit DNA replication, and in conjunction with ROS, they can prevent bacterial respiration (128). The study of ROS and RNS in the context of infection is greatly aided by the availability of inbred mice strains with a targeted mutation in *Nos2* and in gp91*phox*, an essential subunit of NADPH oxidase. Following intravenous infection with 10^5 CFU of an intermediate virulent strain of *Salmonella* Typhimurium, gp91*phox* mutants have significantly higher bacterial loads in their spleen and liver compared to the wild-type C57BL/6J mice at day 1 post-inoculation. Within 5 days, all gp91*phox*$^{-/-}$ mice succumb to infection while C57BL/6J mice survive (105). In contrast, *Nos2* mutants show a significant increase in bacterial loads only as of day 7 post-infection. These results reveal a bactericidal role for ROS in the early phase of infection and a bacteriostatic role for RNS later in the infection. Similar results were obtained *in vitro* using peritoneal phagocytes (138).

In humans, a mutation in any one of the 4 essential subunits of NADPH oxidase can cause chronic granulomatous disease. This condition is characterized by increased susceptibility to bacterial and fungal infection (139). No NOS2 deficiency has ever been

reported in humans; however, polymorphisms in the promoter region of *NOS2* have been associated with resistance to malaria (140).

Section 6: Adaptive immunity of the host

Adaptive immunity to *Salmonella* Typhimurium was initially investigated using attenuated *Salmonella* Typhimurium strains such as *Salmonella* Typhimurium C5TS, a temperature-sensitive mutant, or *Salmonella* Typhimurium *aroA*$^{-}$, a mutant defective for aromatic amino acids biosynthesis (114, 115). Many components of the adaptive immune system that are essential for clearance of a *Salmonella* infection have been identified through these studies (126). The results obtained can be extrapolated to virulent infections based on the assumption that similar mechanisms regulate resistance to attenuated and virulent *Salmonella* strains. Recently, *Nramp1* has been shown to be involved in the late phase of a wild-type *Salmonella* Enteritidis infection and its role in the adaptive immune response will be discussed in section 7.6 (117).

6.1 T cells

Nude mice have no hair growth and are athymic due to a spontaneous mutation in the *Foxn1* (Forkhead box N1) gene. In consequence, these mice fail to develop mature T cells from bone marrow progenitors. Nude mice have been used to study the role of T cells during *Salmonella* infection (141). Kinetics of infection of athymic and euthymic mice are similar in the early phase of infection; however, during the late phase of infection athymic mice are unable to control bacterial growth while euthymic mice can. More specifically, depletion of CD4+ T cells prevents *Salmonella* clearance, whereas depletion of CD8+ T cells only slightly affects clearance (141). The role of different T-cells subsets was further investigated using different knockout mice. Mice deficient for the major histocompatibility complex (MHC) class II molecules, and thus devoid of mature CD4+ T cells, were found to be very susceptible to infection with an attenuated *Salmonella* Typhimurium. In contrast, $\beta_2m^{-/-}$ mice deficient for MHC class I molecules, and therefore lacking mature CD8+ T cells, were resistant to infection (115). Furthermore, higher protection against infection is conferred by the transfer of CD4+ T

cells from infected mice into naïve mice than the transfer of CD8+ T cells (141). However, both cell types are important for resistance to a secondary infection (142).

6.2 CD28

Efficient T cell activation requires not only recognition of a peptide:MHC complex by the T-cell receptor, but also a costimulatory signal between a B7 molecule, present at the surface of antigen presenting cells, and CD28, expressed at the surface of T cells and NK cells. The role of CD28 in *Salmonella* infections was investigated using CD28-deficient mice (143). These mice are more susceptible to high doses of *Salmonella* Typhimurium than mice expressing CD28. At lower doses, bacterial loads in the spleen and liver of $CD28^{-/-}$ mice are significantly higher compared to wild-type mice 10 days post-infection (143). When serum antibodies titers were analyzed, it was found that CD28-deficient mice had reduced immunoglobulin G3 (IgG3) levels and did not produce any IgG1 or IgG2. This deficiency could not be compensated for by the administration of immune serum. CD28-deficient mice also produce less IFN-γ upon infection. Finally, when infected with attenuated *Salmonella* Typhimurium *aroA*$^{-}$, these mice cannot clear the bacteria and develop chronic infection (143). These results show the critical role of CD28 in the late phase of *Salmonella* infection.

6.3 B cells

Antibody production is the main function of B cells. The first insight into the role of B cells during *Salmonella* infection was obtained by studying CBA/N mice. These mice have an X-linked immunodeficiency (*xid*), due to a mutation in Bruton's tyrosine kinase (*Btk*) that causes abnormal B cell development (144). CBA/N mice have improper B cell maturation, decreased IgM and IgG3 serum levels, and an impaired antibody response to thymus-independent type II antigens. The susceptibility of *xid* mice to *Salmonella* Typhimurium was first documented in 1979, when they were found to have a LD_{50} 1000-fold lower than that of immunologically normal, histocompatible CBA/CaHN mice (145). Furthermore, *xid* mice survive the infection for at least 10 days, while susceptible strains such as BALB/c die within a few days, suggesting a role in the late phase of infection (145). Serological analysis of a [CBA/N x DBA/2N] Fl progeny

provided further insight into the humoral response of these mice. Male mice hemizygous for the CBA/N X chromosome died between 2 to 4 weeks post-infection. This time period corresponds to the exponential phase of antibody production in immunologically normal offspring (146). Resistance to infection was restored when anti-*Salmonella* serum was transferred to *xid* mice. These results were validated using B cells deficient Igμ$^{-/-}$ mice created on a C57BL/6J background by targeted disruption of the Ig μ chain gene (147). Wild-type mice have a LD_{50} 10-fold greater than mutant mice infected with 10^3 CFU of *Salmonella* Typhimurium orally (148). When infected with *Salmonella* Typhimurium *aroA*$^-$, both Igμ$^{-/-}$ and Igμ$^{+/+}$ mice clear the bacteria from their spleen and liver within 42 days. However, when a secondary challenge with virulent *Salmonella* is administered, a significantly higher percentage of wild-type mice survive compared to mutant mice (126). Put together, these results demonstrate the importance of B lymphocytes in protective immunity against *Salmonella* infections in mice.

In humans, a mutation in *BTK* leads to X-linked agammaglobulinemia (XLA) (149). XLA patients fail to produce mature B lymphocyte cells and assemble non-functional antibodies. They are prone to bacterial infections and require antibiotic treatment to survive.

6.4 Mouse major histocompatibility complex

C57BL/10 mice congenic for different mouse major histocompatibility complex (H-2) regions were found to be differentially susceptible to *Salmonella* strains of low virulence (150). To become chronic carriers, mice with the H-2^b and H-2^d haplotypes required a smaller infectious dose than mice with the H-2^a, H-2^k, and H-2^f haplotypes. Furthermore, when infected with a virulent strain of *Salmonella* Typhimurium, mice with the H-2^b and H-2^d haplotype were less responsive to antibiotic treatment and could not survive without continued antibacterial therapy (151). A larger panel of C57BL/10 H-2 congenic mice was classified as susceptible (H-2^b), intermediate (H-2^d, H-2^f, H-2^k, H-2^p, H-2^r, H-2^s, H-2^v) or resistant (H-2^j, H-2^q, H-2^u) to infection (114). To investigate how the genetic background, and more specifically the *I*mmunity *t*o *T*yphimurium (*Ity*) resistance (R) or susceptible (S) allele, affects the phenotype associated with an H-2 haplotype, recombinant inbred strains (RIS) derived from C57BL/6J (*Ity*^{S}, H-2^b) and A/J (*Ity*^{R}, H-2^a)

mice were analyzed. Results suggested an epistatic effect between the *Ity* gene and the H-2^b haplotype (114). Further genotyping within the H-2 complex identified the D and K-Aα subregions to be of influence on bacterial clearance (152). A genetic association between MHC class II genes and typhoid fever has also been observed in humans (153).

Section 7: *Nramp1*

7.1 The *Bcg*/*Lsh*/*Ity* locus

In 1976 it was noticed that some inbred mouse strains such as CBA, C3H/He, and DBA/2 had a LD_{50} value 10,000-fold greater than other strains such as BALB/c, C57BL/6J, and DBA/1 when infected with *Salmonella* Typhimurium (154). Genetic linkage analysis identified a locus segregating as a simple Mendelian dominant trait involved in host resistance to *Salmonella* Typhimurium (154). The locus, called *Ity*, was located on chromosome 1 (155). Independently, two groups investigating host response to an attenuated form of *Mycobacterium bovis* (Bacille Calmette Guérin) and *Leishmania donovani* each identified a locus, called *Bcg* and *Lsh* respectively, which segregated with resistance or susceptibility in different inbred mice strains (156, 157). Interestingly, both loci mapped to the same genomic region as *Ity*. Furthermore, a similar pattern of susceptibility to *Salmonella* Typhimurium, *Mycobacterium bovis* (Bacille Calmette Guérin), and *Leishmania donovani* infection was observed in a survey of 14 inbred strains and 38 RIS, indicating that a single gene likely underlied all three loci (158, 159). Early studies showed that the *Bcg*/*Lsh*/*Ity* gene was expressed on macrophages of the spleen and liver and that expression of the resistant allele (*Bcg*/*Lsh*/Ity^R) promoted bacterial killing in the mouse (160).

Initial mapping located the 15 cM *Bcg*/*Lsh*/*Ity* locus to proximal chromosome 1 (155, 156, 159). Cosegregation analysis of 8 highly polymorphic markers in the vicinity of the locus in backcross mice and RIS mice allowed the identification of three markers tightly linked to the *Bcg* locus in a 0.3 cM interval (161). Physical mapping of this chromosomal region estimated it to be 1 Mb (162). A 400 kb bacteriophage and cosmid contig including the *Bcg* region were constructed and used to identify six novel transcriptional units within this interval (163). Tissue specific expression analysis identified one candidate gene exclusively enriched for in macrophages of the RES. This

gene was called natural resistance-associated macrophage protein 1 (*Nramp1*) and was found to encode an integral membrane protein with structural homology to some prokaryotic and eukaryotic proteins. Sequence comparison of 13 resistant and susceptible strains identified a glycine to aspartic acid mutation in predicted transmembrane domain 4 that correlated with susceptibility (163). A survey of 27 inbred strains confirmed the mutant *Nramp1* allele to be associated to the *Mycobacterium bovis* (Bacille Calmette Guérin) susceptibility phenotype (164). Furthermore, targeted disruption of the *Nramp1* gene on the resistant 129S6 background resulted in increased susceptibility to *Mycobacterium bovis*, *Leishmania donovani*, and *Salmonella* Typhimurium infection (165). Resistance to infection was restored in *Bcg*/*Lsh*/*Ity* susceptible C57BL/6J mice transgenic for the *Nramp1* Gly169 allele (166). In addition, the NRAMP1 protein was detected in macrophages isolated from these transgenic mice. Put together these results confirmed that *Nramp1* was the gene underlying the *Bcg*/*Lsh*/*Ity* locus. *Nramp1* has since been renamed *Slc11a1*.

7.2 *Nramp1* characterization

Since its identification in 1993, *Nramp1* has been the focus of various studies to better understand its bactericidal effect on *Salmonella* and other pathogens. The *Nramp1* gene is composed of 15 exons spanning 11.5 kb (167). *Nramp1* mRNA is detected in myeloid cells of the monocyte/macrophage or granulocyte lineages. It encodes for a 56 kDa integral membrane protein with 12 predicted transmembrane domains, a glycosylated extracytoplasmic loop, a putative Src homology 3 (SH3) binding domain, and three protein kinase C phosphorylation sites (104, 168). Posttranslational modifications result in a 90 to 100 kDa phosphoglycoprotein. In susceptible mice, the null *Nramp1* allele (G169D) causes the Nramp1 protein to be misfolded and retained at the endoplasmic reticulum (ER) membrane (169).

7.3 The Nramp family

Soon after the identification of *Nramp1*, homologs were identified in bacteria, yeast, plants, flies, and mammals (170). Notably, the chicken *NRAMP1* has been linked to *Salmonella* Typhimurium susceptibility (171). In humans, *NRAMP1* maps on

chromosome 2q35. Several human population studies in Africa and Asia have linked allelic variation at the *NRAMP1* locus to susceptibility to leprosy and tuberculosis (172, 173). The role of *NRAMP1* in *Salmonella* Typhi susceptibility was investigated in a population from southern Vietnam but no association was detected between different *NRAMP1* alleles and disease susceptibility (174). In addition, a second *Nramp* gene (*Nramp2*) was identified on mouse chromosome 15 (175). It is involved in iron transport but does not play a role in host response to infection (176).

The Nramp family of proteins share 33 to 75% sequence identity in their hydrophobic core that encodes for the highly conserved structural basis consisting of 12 transmembrane domains (177). Other structural characteristics, such as an amphipathic α-helix and conserved residues that are consistent with that of membrane transporters and channels, were identified. However, at that time, the molecular functions of these proteins remained unknown (177). The first breakthrough occurred when Smf1p, NRAMP1's homolog in yeast, was shown to mediate manganese uptake (178). Further insight was provided when DCT1, Nramp2's homolog in rats, was identified as a proton-coupled divalent metal ion transporter, with iron as its main substrate (179).

7.4 NRAMP1 function

Macrophage activation up-regulates *Nramp1* gene expression (104). *In vitro* activation of RAW264.7 macrophages with LPS or IFNγ and *in vivo* induction of peritoneal macrophages with LPS or thioglycollate lead to increased *Nramp1* mRNA expression (104).

Colocalization studies in resting macrophages detected anti-NRAMP1 antibodies on LAMP-1 positive compartments. Upon phagocytosis, NRAMP1 in parallel with LAMP-1, is recruited to the phagosomal membrane to which it remains associated during phagolysosomal maturation (85). These findings support the hypothesis that NRAMP1 controls the intravacuolar environment to limit bacterial replication. More specifically, NRAMP1 functions as a pH-dependent divalent cation transporter (180). Transport studies have shown that NRAMP1 is involved in iron transport from the endosomal compartment to the cytoplasm (181). Monitoring of manganese transport shows faster export of manganese from the endosome to the cytoplasm in *Nramp1*$^{+/+}$ macrophages

compared to *Nramp1*$^{-/-}$ macrophages. Decreased acidification of the phagosomal lumen eliminates the export rate difference (180). Divalent cations are essential cofactors to many enzymatic reactions and their depletion has a bacteriostatic effect on *Salmonella* (182). All together, these results demonstrate that NRAMP1 functions as a pH-dependent divalent cation transporter. An alternative model has been proposed in which NRAMP1 functions as an antiporter of protons and divalent cations. This hypothesis was formulated based on a study which showed that, in response to extracellular iron overload, iron accumulation in the phagosome was four times greater in NRAMP1 expressing macrophages compared to those lacking NRAMP1 (183). In this study, ligand binding was not differentiated from active transport of iron and only a narrow concentration range of extracellular iron overload was used. In addition, this model suggests NRAMP1's membrane orientation and function differs from that of NRAMP2, which is highly unlikely for closely related proteins (182). For all these reasons, this model will not be discussed further.

During *Salmonella* infection, SCV formed in the presence of NRAMP1 acquire the mannose 6-phosphate receptor (M6PR), an important protein for the delivery of hydrolases to the lysosome. In the absence of NRAMP1, SCV do not acquire the M6PR and are inaccessible to the endocytic pathway, promoting bacterial survival (184). The addition of iron chelators to NRAMP1 lacking RAW264.7 cells infected with *Salmonella* restores recruitment of the M6PR to the SCV (185). Iron is an essential cation for bacterial growth and survival. Therefore, *Salmonella* competes with the host for the iron available within phagosomes and encodes numerous iron transporters such as FepBCDG, SitABCD, FeoABC and MntH, the *Salmonella* homolog of NRAMP1 (186, 187).

Since its identification as a macrophage protein, NRAMP1 has also been found to be expressed in neutrophils and dendritic cells (DC) (188, 189). Expression of NRAMP1 occurs in the late stage of neutrophil maturation and persists in circulating PMN leukocytes in human blood (188). In resting neutrophils, NRAMP1 is expressed by gelatinase-containing tertiary granules. Following phagocytosis of a pathogen, NRAMP1 is recruited to the phagosomal membrane through the fusion of tertiary granules with the phagosome. In mice, NRAMP1 can be detected in late endosomes and lysosomes of

$CD11c^+$ DC (189). Finally, stimulated DC with a functional NRAMP1 are more efficient at antigen processing and presentation.

NRAMP1 also has multiple pleiotropic effects. For example, RAW264.7/*Nramp1* cells (RAW264.7 macrophages transfected with a functional *Nramp1* allele) stimulated with IFNγ express higher levels of MHC class II molecules than RAW264.7 macrophages. Moreover, NRAMP1 positive cells have an enhanced ability to process antigen (190). Consequently, a functional *Nramp1* allele has been associated with a predominant Th1 response in the early phase of infection (191). *Nramp1* also increases RNS production by macrophages (192).

7.5 *Nramp1*'s influence on *Salmonella* in the early phase of infection

The influence of *Nramp1* on *Salmonella* virulence gene expression in the early phase of infection has been previously investigated (95). This study focused on SPI-2 associated genes *ssrA*, *sseA*, and *sseJ*, SPI-1 regulatory gene *hilA*, and the response regulator gene *phoP*. *Salmonella* Typhimurium strains carrying LacZ fusion reporter plasmids for each gene of interest were created. It was observed that, 9 hours post-infection of RAW264.7 and RAW264.7/*Nramp1* macrophages, all SPI-2 associated genes were up-regulated in the presence of *Nramp1*. Results obtained in RAW264.7/*Nramp1* macrophages were reproduced by iron chelation of RAW264.7 cells, indicating that virulence gene expression is regulated in an iron-responsive manner. *In vivo* infection of 129S6 and 129S6-*Nramp1*$^{-/-}$ mice showed a significant increase in *ssrA* and *sseJ* expression in the presence of *Nramp1* 24 hours post-infection. The expression of *hilA* and *phoP* was not significantly different in either model (95). Interestingly, even though NRAMP1 is known to be recruited to the phagosomal membrane as early as 60 minutes post-infection, only genes whose expression is induced in the late phase of infection were affected by the the presence or absence of a functional *Nramp1* (95, 180). Furthermore, SPI-2 mutant *Salmonella* Typhimurium cannot survive in *Nramp1*$^{+/+}$ or *Nramp1*$^{-/-}$ mice. In contrast, wild-type bacteria are extremely virulent in *Nramp1*$^{-/-}$ mice and can efficiently replicate in *Nramp1*$^{+/+}$ mice, thus showing that the outcome of infection is influenced by the interaction between SPI-2 and *Nramp1* (95). These findings demonstrate the impact of *Nramp1* on bacterial gene expression and support the idea that

acquisition of SPI-2 by *Salmonella* was an efficient strategy to be able to infect a host and use it as a transmission agent without actually killing it (95).

The expression of *mntH* and *sitA* was also investigated using RAW264.7 macrophages (193). Both transporters were shown to be up-regulated 9 and 24 hours following bacterial internalization by RAW264.7/*Nramp1* macrophages. *Salmonella* strains mutant for either *sitA*, *mntH*, or both, were used to infect intraperitoneal macrophages isolated from C57BL/6J mice and congenic mice carrying a functional *Nramp1* allele. Compared to wild-type bacteria, the *sitA* mutant and the *sitA mntH* double mutant had reduced survival in the presence of a functional *Nramp1* allele 18 hours post-infection. The same bacterial mutants were used for *in vivo* infection of C57BL/6J mice and congenic mice carrying a functional *Nramp1* allele. All mutants were virulent in C57BL6/J mice. In contrast, the *mntH* and *sitA* mutants had decreased virulence in the presence of a functional *Nramp1* allele and the double mutant was completely avirulent (193). Similar results were obtained when 129S6 and 129S6-*Nramp1*$^{-/-}$ were infected with these mutants, with the exception that the *sitA mntH* double mutant was avirulent in 129S6-*Nramp1*$^{-/-}$ mice. These results clearly demonstrate the competition between host and pathogen for divalent cations available in the SCV.

The influence of *Nramp1* on *Salmonella* Typhimurium protein expression was also investigated (194). Proteomic analysis of *Salmonella* Typhimurium isolated from RAW264.7 and RAW264.7/*Nramp1* macrophages identified 315 bacterial proteins whose expression was induced upon infection. A large majority of these proteins were involved in housekeeping and their abundance level remained constant through the first 24 hours post-infection. Thirty-nine proteins were up-regulated at 2, 4, and/or 24 hours post-infection. Interestingly, *Nramp1* was found to differentially induce the expression of 6 proteins. SodCI, Upp, and STM3117 were detected in the absence of a functional *Nramp1*, whereas IHFα, IHFβ, and SerA were only observed in bacteria isolated from macrophages carrying a functional *Nramp1*. IHFα and IHFβ form a heterodimer called integration host factor involved in regulating the transcription of numerous virulence factors. SodCI is a superoxide dismutase which limits oxidative damage caused to *Salmonella* by host ROS and RNS. SerA, a D-3-phosphoglycerate dehydrogenase, and Upp, a uracil phosphoribosyltransferase, have no known role in virulence. STM3117 has

no known function; however, its role in virulence was confirmed as a mutant with a deletion in the gene encoding STM3117 had significantly reduced intracellular survival (194). These findings provide further evidence for the crucial role of *Nramp1* in controlling *Salmonella* infection.

7.6 *Nramp1* role in adaptive immunity

The impact of *Nramp1* on bacterial persistence was investigated using our mouse model of chronic *Salmonella* Enteritidis infection (195). 129S6 and 129S6-*Nramp1*$^{-/-}$ mice were infected with 10^3 CFU of *Salmonella* Enteritidis and followed over a 6-weeks period. As early as 7 days post-infection, the influence of *Nramp1* on bacterial clearance was observed. Forty-two days post-inoculation, the splenic bacterial load was 18-fold greater in 129S6 mice compared to 129S6-*Nramp1*$^{-/-}$ mice. The impact of *Nramp1* on bacterial clearance was further investigated and different factors involved in the immune response were analyzed. Microarrays analysis of spleen RNA obtained from female 129S6 and 129S6-*Nramp1*$^{-/-}$ mice provided a global view of differentially expressed genes following *Salmonella* Enteritidis infection. Ten days post-infection, genes involved in macrophage and/or DC activation were predominant in 129S6 mice while genes implicated in lymphocyte activation were up-regulated in 129S6-*Nramp1*$^{-/-}$ mice, suggesting T helper (Th) polarization. Forty-two days post-infection, the presence of a mutated *Nramp1* gene was associated with a marked increase in *Gata3*, *Cd28*, and *Il4*, hallmarks of Th type 2 (Th2) polarization. Serum analysis detected IL-4 only in *Nramp1* knockout mice at day 10 post-infection. Moreover, a significant increase in serum IgG2a titers in 129S6 mice was detected 42 days post-infection. These results show an impact of *Nramp1* on Th polarization in which bacterial persistence in the spleens of 129S6 mice is associated with a Th1 response, while bacterial clearance in 129S6-*Nramp1*$^{-/-}$ mice correlates with a Th2 response (195). The strong proinflammatory response developed by 129S6 mice delays the activation of the adaptive immune response through unknown mechanisms. For example, NRAMP1 is known to promote fusion of the SCV with the phagolysosome and thus may influence antigen presentation as the context in which *Salmonella*'s flagellar antigen is presented has also been associated with Th polarization

(184, 196). Furthermore, as described previously, the outcome of infection is also influenced by the effect of *Nramp1* on bacterial gene expression.

Based on the findings described in the introduction, we hypothesized that *Nramp1* is the gene underlying *Ses1*, and that its interactions with other host loci (*Ses4* and *Ses5*), as well as with the bacterial transcriptome, are responsible for bacterial clearance in C57BL/6J mice. The work presented in the following chapters of this thesis describes the approaches chosen to test our hypothesis and the results obtained.

MATERIALS AND METHODS

Section 1: Animals

The inbred mouse strains 129S6 and C57BL/6J were obtained from Taconic (Germantown, NY, USA) and The Jackson Laboratories (Bar Harbor, ME, USA), respectively. The 129S6-*Nramp1*$^{-/-}$ mice were provided by Dr. Philippe Gros (McGill University). The mice were created by gene targeting using the pMC.NR targeting vector and 129S6 embryonic stem cells. Chimeric offspring were crossed with 129S6 mice to generate the 129S6-*Nramp1*$^{-/-}$ strain (165). 129S6.B6-*Ses1* congenic mice were created by introgressing the C57BL/6J *Ses1* segment on the 129S6 background by serial backcrosses and marker-assisted selection. The *Ses1* locus transferred spans 16.73 Mb and is flanked by markers *D1Mit19* and *D1Mit48* (117). All mice were maintained in our animal facilities under conditions specified by the Canadian Council on Animal Care.

1.1 *Salmonella* Infection

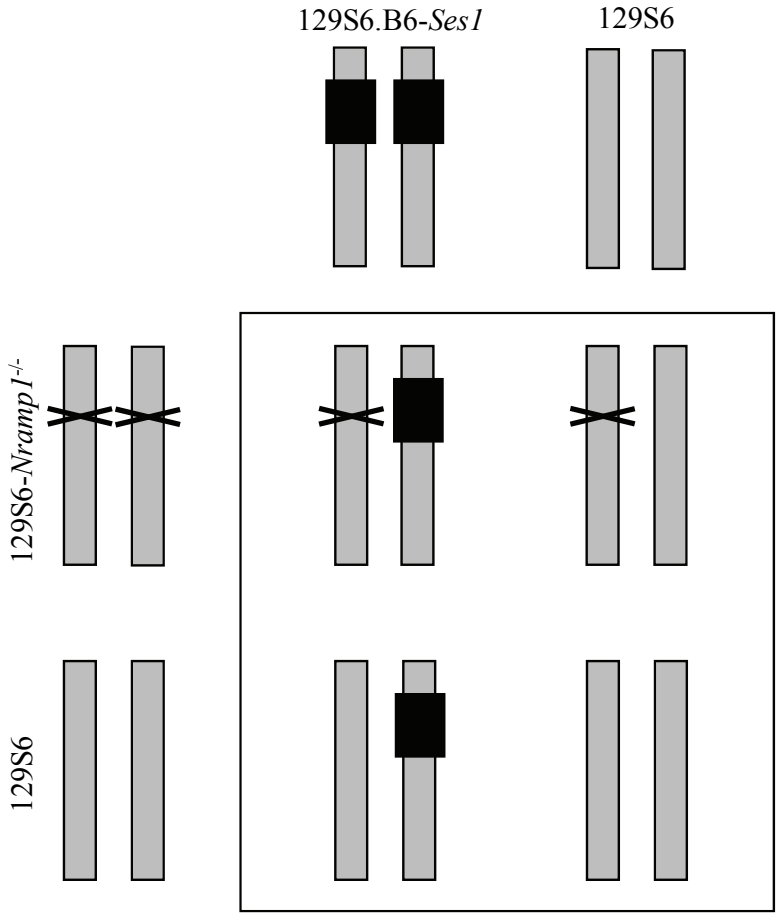
Mice between 6 and 12 weeks of age were challenged with *Salmonella* Enteritidis strain 3b, originally obtained from Dr. William Kay (University of Victoria). Mice were inoculated with 10^3 CFU in 0.2 mL of physiological saline through the caudal vein. At specific time points post-inoculation, spleens were harvested and used for CFU enumeration on trypticase soy agar (TSA) and for RNA extraction.

Section 2: Quantitative complementation test

To test whether the *Nramp1*$^{-/-}$ allele interacts with the *Ses1* locus, four crosses were set up: [129.B6-*Ses1* x 129S6-*Nramp1*$^{-/-}$], [129S6 x 129S6-*Nramp1*$^{-/-}$], [129S6 x 129S6.B6-*Ses1*], and [129S6 x 129S6] (Figure 5) (197). F1 hybrids were infected and the bacterial loads 42 days post-inoculation were determined. As appropriate C57BL/6J, 129S6-*Nramp1*$^{-/-}$, and 129S6.B6-*Ses1* mice were used as controls. Statistical analysis to test for differences in bacterial counts was carried out using one-way analysis of variance (ANOVA) with post-test comparison using the Tukey's test in GraphPad Prism 4. The interaction coefficient was calculated as ([129.B6-*Ses1* x 129S6-*Nramp1*$^{-/-}$] – [129S6 x 129S6-*Nramp1*$^{-/-}$]) - ([129S6.B6-*Ses1* x 129S6] – [129S6 x 129S6]). The interaction was analyzed using two-way ANOVA in GraphPad Prism 4. Complementation failure was indicated by an interaction coefficient with a significant associated P-value ($P < 0.05$).

Figure 5. Candidate gene validation by quantitative complementation

Phenotypes of offsprings from the 4 crosses shown above are compared. Quantitative complementation failure is detected if the effect of a C57BL/6J-*Ses1* segment on a wild-type or a knockout *Nramp1* allele is significantly different from the effect of a 129S6-*Ses1* segment on a wild-type or a knockout *Nramp1* allele. Adapted from Darvasi, A. *Trends Genet*, 2005. **21**(7): p. 373-6.


129S6.B6-*Ses1*
129S6
129S6-*Nramp1*$^{-/-}$
129S6

Section 3: Congenic Strains

Congenic mice strains for the *Ses1.2*, *Ses4*, and *Ses5* loci, as well as double congenics strains for *Ses1.2*/*Ses4* and *Ses1.2*/*Ses5* are being produced by transferring the C57BL/6J segments of interest on the 129S6 background. At first, two crosses were initiated between a C57BL/6J female and a 129S6 male and between a 129S6 female and a C57BL/6J male. Two females from each F1 generation were backcrossed to a 129S6 male to obtain the N2 generation. Individuals with the appropriate genotype were used to produce the N3 generation for the *Ses1.2*, *Ses4*, and *Ses5* congenic strains (Table 1). At the N5 generation, congenic mice were intercrossed to produce *Ses1.2*/*Ses4* and *Ses1.2*/*Ses5* double congenic N5F1 individuals. Brother-sister mating will be initiated at generation N10 or N5F1N5 to obtain congenic mice homozygous for the locus or loci of interest.

Section 4: Cell culture

4.1 *In vitro* cell culture

The RAW264.7 cell line (American Type Culture Collection Number TIB-71) was established by transforming a macrophage clone isolated from BALB/c mice (*Nramp1*$^{D169/D169}$) with Abelson leukemia virus (198). RAW264.7 cells were transfected with the *Nramp1*$^{G169/G169}$ allele using the pCB6 expression vector to obtain RAW264.7/*Nramp1* cells (provided by Dr. Philippe Gros, McGill University) (199). The RAW264.7 and RAW264.7/*Nramp1* cell lines were culture in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Life Technologies, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and grown at 37˚C in 5% CO_2. Cells were maintained in 75 cm^2 filter cap flasks with untreated hydrophobic surfaces (Sarstedt, Montreal, Quebec, Canada). RAW264.7/*Nramp1* cells were cultured in the presence of 200 μg/mL geneticin (Invitrogen Life Technologies, Burlington, Ontario, Canada).

Table 1. Description of new congenic strains

Name	Flanking markers
129.B6-*Ses1.2* [a]	*D1Mit48-D1Mit213*
129.B6-*Ses4*	*DXMit136-DXMit143*
129.B6-*Ses5*	*D7Mit76-D7Mit228*
129.B6-*Ses1.2*/*Ses4*	*D1Mit48-D1Mit213*; *DXMit36-DXMit143*
129.B6-*Ses1.2*/*Ses5*	*D1Mit48-D1Mit213*; *D7Mit76-D7Mit228*

[a] The previously constructed 129S6.B6-*Ses1* strain did not cover the proximal end of the QTL. The new 129S6.B6-*Ses1.2* strain will completely cover the 2-LOD confidence interval around the peak marker at *Ses1*.

4.2 *Ex vivo* cell culture

4.2.1 Production of L929 cells-conditioned medium

L929 cells were grown to confluence in Roswell Park Memorial Institute (RPMI) medium (Invitrogen Life Technologies, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated FBS, 2mM L-glutamine and 1% penicillin/streptomycin (Invitrogen Life Technologies, Burlington, Ontario, Canada) in 75 cm^2 filter cap flask with an adherent surface (Sarstedt, Montreal, Quebec, Canada). The supernatant was removed and cells were incubated in 5 mL of 0.05% Trypsin-EDTA (ethylene diamine tetraacetic acid) (Invitrogen Life Technologies, Burlington, Ontario, Canada) for 5 to 10 minutes at 37°C in 5% CO_2. Trypsin was inactivated in 25 mL of phosphate buffered saline (PBS) (Invitrogen Life Technologies, Burlington, Ontario, Canada). Cells were centrifuged for 10 minutes at 1200 rpm and 5 x 10^5 cells were seeded per 75 cm^2 flask and complemented with 40 mL complete media. Cells were allowed to grow for 14 days before the supernatant was removed, filtered using a 0.22 µm Express® Plus filter (Millipore, Etobicoke, Ontario, Canada), and aliquoted. The resulting L929 cells-conditioned medium was stored at -20°C.

4.2.2 Preparation of bone marrow-derived macrophages

129S6 and 129S6-*Nramp1*$^{-/-}$ female mice between 8 and 10 weeks of age were sacrificed by carbon dioxide asphyxiation. The skin and tissue surrounding the femoral bones were removed using scissors and femurs were cut out at the joints. Femurs were cleaned from any remaining adherent tissue before both ends were cut off and marrow cells flushed with 2 mL RPMI medium using a 26G needle. Cells from each mouse were pooled and incubated in 10 mL of red blood cells lysis buffer (Table 2) for 10 minutes, on ice. Cells were centrifuged for 10 minutes at 1200 rpm and resuspended in 15 mL of complete medium. Complete medium consisted of RPMI medium supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 1% penicillin/streptomycin and containing 30% L929 cells-conditioned medium. Bone marrow-derived cells were incubated at 37°C in 5% CO_2 in complete medium for 24 hours in petri dishes (Thermo Fisher Scientific, Kirkland, Quebec, Canada). Non-adherent cells were transferred to Primaria™ cell culture dishes (BD Bioscience, Mississauga, Ontario, Canada) and marrow cells were

Table 2. Red blood cells lysis buffer

Solution 1	Solution 2	Solution 3
0.65 M NH_4CL	20 mM $MgCl_2 \cdot 6H_2O$	0.26M $NaHCO_3$
25 mM KCl	11 mM $MgSO_4 \cdot 7H_2O$	
4 mM $Na_2HPO_4 \cdot 12H_2O$	30 mM $CaCl_2$	
8.75 mM KH_2PO_4		
27 mM Glucose		
0.1 mM Phenol red		

The three solutions were diluted in water to obtain a final concentration of 20% Solution 1, 5% solution 2, and 5% solution 3.

allowed to differentiate for 7 days. Prior to being used, bone marrow-derived macrophages (BMDM) were made quiescent by culturing them in complete medium lacking L929 cells-conditioned medium and penicillin/streptomycin for 18 hours.

Section 5: *In vitro* and *ex vivo Salmonella* infection

One day prior to infection, cells (RAW264.7 macrophages or BMDM) were centrifuged for 10 minutes at 1200 rpm, resuspended in medium supplemented with 10% heat-inactivated FBS without antibiotics, and counted to a concentration of 1 x 10^6 cells/mL. For CFU determination, 5 x 10^5 cells were seeded per well in a 24-wells plate. For RNA extraction, 2 x 10^7 cells were seeded in 100 mm-wide tissue culture dishes.

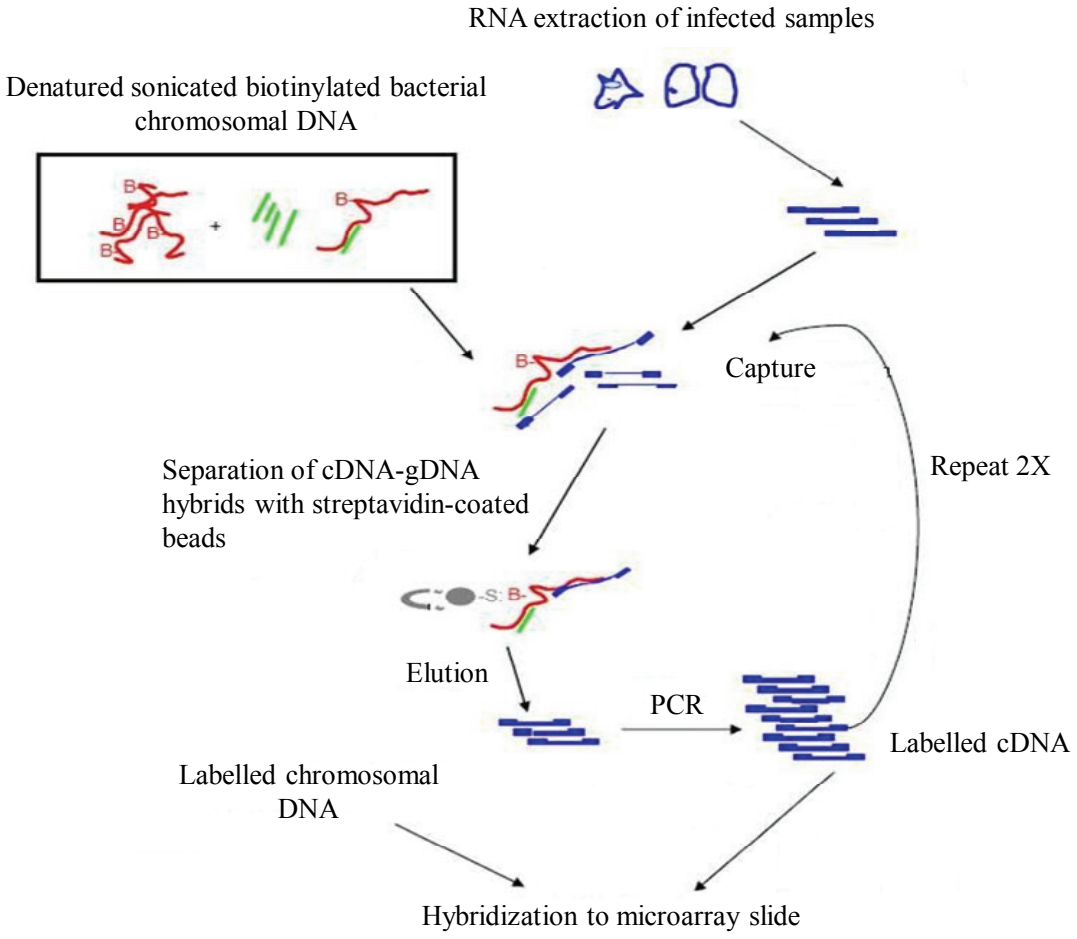
A single colony of *Salmonella* Enteritidis was grown in 5 mL trypticase soy broth (TSB) overnight at 37°C at 250 rpm. The overnight culture was diluted 1/25 and grown to mid-log phase (optical density at a wavelength of 600 nm (OD600) = 0.9). Bacteria were opsonized with 10% 129S6 serum in medium on ice for 30 minutes. Medium containing opsonized bacteria was added to the cells at a multiplicity of infection (MOI) of 10 bacteria per cell. Plates were centrifuged for 5 minutes at 2500 rpm to synchronize phagocytosis and incubated for 40 minutes at 37˚C in 5% CO_2; dishes were incubated for 45 minutes at 37˚C in 5% CO_2. To kill extracellular bacteria, infected cells were washed twice with PBS and incubated in medium containing 100 μg/mL gentamicin for 60 minutes before the concentration was lowered to 10 μg/mL for the remaining of the infection. This time point was defined as 1 hour post-invasion, and cells were washed twice in PBS and lysed. For CFU determination, cells were lysed in 500 μL of 1% Triton X-100 in PBS; lysates were diluted in PBS and enumerated on TSA. For RNA extraction, cells were lysed in 1 mL TRIzol reagent (Invitrogen Life Technologies, Burlington, Ontario, Canada) and stored at -80˚C.

Section 6: Selective Capture of Transcribed Sequences

Selective capture of transcribed sequences (SCOTS) enriched total complementary DNA (cDNA) samples for bacterial transcripts expressed within the host during infection as schematized in Figure 6. cDNA was prepared from infected samples and bacterial transcripts were selectively captured by hybridization to bacterial genomic

Figure 6. Selective capture of transcribed sequences (SCOTS)

Bacterial transcripts expressed within the host during infection are selectively captured from total cDNA prepared from infected cells or tissues by hybridization to rDNA-blocked biotinylated bacterial genomic DNA. Captured samples are eluted and amplified. After three rounds of SCOTS the products can be applied to microarrays. Adapted from Faucher, S. P., S. Porwollik, C. M. Dozois, M. McClelland, and F. Daigle. 2006. *Proc Natl Acad Sci U S A 103:1906*.


RNA extraction of infected samples
Denatured sonicated biotinylated bacterial chromosomal DNA
Capture
Repeat 2X
Separation of cDNA-gDNA hybrids with streptavidin-coated beads
Elution
PCR
Labelled cDNA
Labelled chromosomal DNA
Hybridization to microarray slide

DNA (gDNA) blocked with ribosomal DNA (rDNA). The captured sequences were amplified and used for 2 more rounds of selective capture. The resulting product consisted of a complex pool of bacterial cDNA sequences which were analyzed by slot blot and microarrays.

6.1 SCOTS sample preparation

6.1.1 Blocked, biotinylated genomic DNA

The pC6 plasmid, which contains the *Escherichia coli* ribosomal operon *rrnB*, was obtained from Dr. Squires (Tufts University). To obtain rDNA, the pC6 plasmid was amplified with a plasmid MAXI kit (Qiagen, Mississauga, Ontario, Canada).

A single colony of *Salmonella* Enteritidis was grown in 5 mL TSB overnight at 37°C at 250 rpm. Two mL of overnight culture were centrifuged at 8000 rpm for 2 minutes and resuspended in 50 µL of Tris-EDTA. The cells were lysed with proteinase K (Invitrogen Life Technologies, Burlington, Ontario, Canada) for 30 minutes at 65˚C. DNA was extracted by phenol/chloroform, precipitated in 100% ethanol, washed in 70% ethanol, and resuspended in sterile, DNase-free water (Invitrogen Life Technologies, Burlington, Ontario, Canada).

Each round of SCOTS required 0.3 µg of gDNA per sample, therefore the gDNA was quantified by A_{260}/A_{280} spectrophotometer readings and a sufficient quantity for all samples to go through three rounds of SCOTS was biotinylated. An equal quantity of photobiotin acetate (Sigma-Aldrich, Oakville, Ontario, Canada) was added to the gDNA and the mixture was irradiated for 20 minutes with a 250 watt incandescent light while being kept on ice. Fresh photobiotin acetate was added and the sample was irradiated for another 20 minutes to ensure even biotinylation of the gDNA. Excess photobiotin was extracted in isobutanol.

The addition of rDNA to the biotinylated gDNA was done at a 17:1 ratio and the mixture was sonicated to obtain DNA fragments of varying length. The resulting sonicated, biotinylated gDNA and rDNA mixture was aliquoted and precipitated with 2.5 volumes of 100% ethanol and 1/10 volume of 3M sodium acetate (NaAc).

Table 3. SCOTS buffers composition

Buffer	Composition
Hybridization buffer	10 mM EPPS (4-(2-Hydroxyethyl)piperazine-1-propanesulfonic acid) 1 mM EDTA
2X binding and washing buffer	10 mM Tris-Cl pH 7.5 1 mM EDTA 2.0 M NaCl
Washing buffer	20 mM NaCl 0.1% SDS

Before use, the precipitated biotinylated gDNA and rDNA mixture was washed in 70% ethanol and resuspended in hybridization buffer (4 µL per 0.3 µg of gDNA) (Table 3). Samples were incubated for 3 minutes at 99˚C under oil and NaCl was added at a final concentration of 0.2M. Samples were incubated for 30 minutes at 65˚C to allow hybridization of rDNA to gDNA to obtain biotinylated, rDNA-blocked gDNA.

6.1.2 Infected samples

6.1.2.1 RNA extraction

Samples from infected RAW264.7 and RAW264.7/*Nramp1* macrophages at 1 and 2 hours post-invasion were used for enrichment. RNA from infected cells from each time point was extracted with TRIzol reagent according to the manufacturer's instructions. Briefly, samples were lysed in 1 mL TRIzol and the RNA was recovered from the aqueous phase following the addition of 0.2 mL of chloroform. Samples were precipitated overnight in 0.7 volume isopropanol with 10 µg of glycogen. Samples were then resuspended in diethylpyrocarbonate (DEPC) treated water. RNA concentration and integrity were determined by A_{260}/A_{280} spectrophotometer readings and agarose gel electrophoresis, respectively. Samples were treated with TURBO DNase (Ambion, Austin, TX, USA) and the absence of DNA was verified by polymerase chain reaction (PCR) using primers for *invA*, a *Salmonella*-specific gene. All PCR amplifications required by SCOTS were done for 25 cycles with 1.5mM $MgCl_2$ in a total volume of 50 µL. Primer sequences and annealing temperatures are described in Table 4.

6.1.2.2 First and second strand cDNA synthesis

For each condition, 5 samples of 5 µg of total RNA were converted to cDNA using Superscript® II reverse transcriptase (Invitrogen Life Technologies, Burlington, Ontario, Canada) according to the manufacturer's instructions. First-strand synthesis was done by random priming using the conserved Rb1-RNA primer which had the defined Rb1 5' end and a random nonamer at the 3' end (Table 4). Second strand cDNA synthesis was done using Klenow fragment (New England Biolabs Ltd, Pickering, Ontario, Canada) according to the manufacturer's instructions. All samples were purified using the QIAquick PCR purification kit (QIAGEN, Mississauga, Ontario, Canada) prior to being

Table 4. SCOTS primers information

Primer name	Sequence (5' - 3')	Annealing temperature
RpoA-F [a]	ACGATCTGGAATTGACTGTCCGCT	55˚C
RpoA-R [a]	AGCCAGCACGTCTTTAATCTCGGT	
RpoB-F [a]	ATTCATCCAGCGTGCCTACGATCT	55˚C
RpoB-R [a]	GCTTCTTTCGCACCGTCAAACACT	
RpoD-F [b]	TCGTGAAGCGAAAGTGCTGCGTAT	55˚C
RpoD-R [b]	TTCGCGGGTAACATCGAACTGTTT	
InvA-F [b]	CTACAAGCATGAAATGGC	60˚C
InvA-R [b]	CGCCCAGATCCATACATCATCG	
Rb1-RNA [b]	CGGGATCCAGCTTCTCACGCANNNNNNNNN	N/A
Rb1 [b]	CGG GAT CCA GCT TCT CAC GCA	60˚C

[a] Primers were designed using IDT Oligo Design (http://www.idtdna.com/Scitools) using gene sequences obtained from *Salmonella* Enteritidis PT4 (http://www.sanger.ac.uk/Projects/Salmonella/).
[a] Primers were obtained from Dr France Daigle (Université de Montréal).

amplified by PCR using primers specific for the Rb1 sequence tag (Rb1-PCR) and quantified by gel electrophoresis. Equal quantities of PCR products (approximately 1/5 of the total volume) were precipitated overnight in 2.5 volumes of 100% ethanol, 1/10 volume of 3M NaAc, and 10 mg glycogen (Roche Diagnostics, Laval, Quebec, Canada).

6.2 Hybridization and capture

Precipitated Rb1-PCR products were washed in 70% ethanol and resuspended in 4 µL of hybridization buffer (Table 3). Samples were then incubated for 3 minutes at 99˚C under oil before NaCl was added at a final concentration of 0.2M. Following an initial incubation of 30 minutes at 65˚C to allow hybridization of abundant and non-specific sequences, biotinylated rDNA-blocked gDNA was added to each sample at a 1:1 volume ratio. Samples were hybridized at 65˚C for 18 hours.

Hybridized samples were diluted in 500 µL of sterile, DNAse-free water and captured with 60 µg of streptavidin-coated beads (Promega, Madison, WI, USA) resuspended in 500 µL of 2X binding and washing buffer (Table 3). Samples were washed three times in washing buffer (Table 3), eluted in 100 µL NaOH 0.4N and precipitated overnight in 2.5 volumes of 100% ethanol, 1/10 volume of 3M NaAc, and 10 mg glycogen.

Samples were resuspended in 40 µL of 10mM Tris-Cl, pH 8.5 and 1/10 volume was used to amplify the captured sequences by Rb1-PCR. Each PCR was done in duplicate. For each condition, the Rb1-PCR products were pooled, purified, quantified by gel electrophoresis, and precipitated overnight in 2.5 volumes 100% ethanol, 1/10 volume of 3M NaAc, and 10 mg of glycogen. These precipitated samples were used for the following round of SCOTS.

6.3 Southern Blot

The Rb1-PCR products from each round of capture were labeled and analyzed by Southern blot. Probes were labeled by PCR amplification using digoxigenin labeled deoxyuridine triphosphate (DIG-11-dUTP) (Roche Diagnostics, Laval, Quebec, Canada). Prior to hybridization, 5 µL of each probe was denatured by boiling for 5 minutes followed by rapid cooling on ice water.

Salmonella Enteritidis gDNA was extracted as previously described and 20 µg was used for digestion with DdeI (New England Biolabs Ltd, Pickering, Ontario, Canada) for 16 hours according to the manufacturer's instructions. Samples containing 2 µg of digested gDNA were migrated on a 1% agarose gel for 16 hours at 20 volts. After electrophoresis, the DNA was denatured by soaking the agarose gel for 45 minutes in a solution of 1.5M NaCl, 0.5N NaOH with gentle agitation. The DNA was then neutralized by soaking the gel for 30 minutes in a solution containing 1.5M NaCl, 1M Tris pH 7.4 with gentle agitation. Following neutralization, the DNA was transferred in 20X SSC (saline-sodium citrate buffer) to Hybond-N nitrocellulose membranes (Amersham, Oakville, Ontario, Canada) for 24 hours. Membranes were washed for 5 minutes in 6X SSC, dried for 30 minutes on a filter paper, and the DNA was fixed to the membrane by UV-crosslinking for 1.5 minutes.

Prehybridization, hybridization, and detection were done using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics, Laval, Quebec, Canada) according to the manufacturer's instructions. Briefly, pre-hybridization was performed at 42˚C for 3 hours in 10 mL of DIG Easy Hyb buffer per 100cm^2 of membrane. Hybridization was then done at 42˚C for 17.5 hours with 3.5 mL of DIG Easy Hyb buffer per 100cm^2 membrane, using 2 µL of DIG-labeled probe per mL of DIG Easy Hyb buffer. The membranes were then washed twice for 5 minutes in ample 2X SSC, 0.1% SDS (sodium dodecyl sulfate) at room temperature, followed by two 15 minute washes in 0.5X SSC, 0.1% SDS at room temperature. Hybrid detection was carried out using anti-digoxigenin alkaline phosphatase conjugate and the chemiluminescent substrate CSPD (disodium 3 - (4-methoxyspiro {1,2-dioxetane-3,2'- (5'- chloro) tricycle [3.3.1.1] decan}-4 yl) phenyl phosphate). The membranes were then exposed to X-ray films for 1 hour before the signals were visualized.

6.4 Slot Blot

Probes for selected *Salmonella* genes were amplified by PCR using the primers described in Table 5, and 10 µL of each probe was applied to a Hybond-N nitrocellulose membrane by use of a Minifold® II Slot-Blot System (GE Healthcare, Baie d'Urfé, Quebec, Canada). For each condition, the Rb1-PCR product from the third round of

Table 5. Primers for slot blot analysis

Name	Sequence (5' – 3')
16S-F	CGGGGAGGAAGGTGTTGTG
16S-R	GAGCCCGGGGATTTCACATC
aceA-F	ATCCACAGCATGTGGTTCAA
aceA-R	TGGGAAACAAAGGTGTAGCC
bcfD-F	GCCATATCCGGTCGTAGAAA
bcfD-R	GTTGACCCGCTTTCACATTT
ihfA-F	AAACGGTGAGCAGGTGAAAC
ihfA-R	CGCCGTGCTGTAATAGGAAT
mgtC-F	CGGCGACGATCATTATTCTT
mgtC-R	GGACCGAACCTAACCCTTGT
orgA-F	AGTTAATACTGGCGGCATGG
orgA-R	CTGATGCATTGCCAAAAATG
pgtE-F	CTCCTGATAGCGTCACGACA
pgtE-R	AGCGTCATGAACGAATAGGG
phoP-F	TGCGCGTACTGGTTGTAGAG
phoP-R	TCATCCGGCAGACCTAAATC
rfbA-F	GCATTAGTACTGGGTGACAA
rfbA-R	GCCACTTTGGTCAAACTCAA
rpoD-F	GCTGAGGTCAATGACCATCTGCC
rpoD-R	TTTCGCCTTCCCGGGTCAAC
serA-F	TCTGCACGTACCTGAAAACG
serA-R	GCACAAAGCCGGGATATCTA
sipA-F	CAGACGGCAGAGATTGTGAA
sipA-R	CGGTCGTACCGGCTTTATTA
sodCI-F	CGGTCTGCTTTTCACTCCTC
sodCI-R	ACCATCTGCATTGACAACCA
sseA-F	AAATCCGGGCTAAGGTGAGT
sseA-R	TAAATCCTTCTCGGCCTCCT
sseJ-F	ATGATGGACTTCCTGGGTTG
sseJ-R	CCCTTATACTCACCACGGCT
upp-F	ACACCCACTCGTCAAACACA
upp-R	GGTGACTTTTTCCGTTTCCA
virK-F	TCCTGTCCGAGGAACAAATC
virk-R	CTTCATGGCGGATAAAGGAA

selective capture was labeled as described in section 6.3. Pre-hybridization, hybridization, and detection were carried out as described in section 6.3, with the exception that membranes were exposed to X-ray films for 2 minutes only. Slot blot results were digitalized using the GeneSnap image acquisition software (Syngene, Vancouver, British Columbia, Canada). Raw slot brightness was determined using GeneTools (Syngene, Vancouver, British Columbia, Canada) and the data was normalized to the housekeeping gene *rpoD* using the formula: $N = (V / V_{rpoD})$; where N is the normalized slot brightness and V is the raw slot brightness.

6.5 *Salmonella* ORF microarrays

The STv7E_2 microarray slide is a non-redundant array consisting of PCR-amplified sequences from the annotated open reading frames (ORF) of several *Salmonella enterica* serovars. Initially, all probes were amplified from *Salmonella enterica* serovar Typhimurium LT2. The array was then supplemented with gene-specific probes from 6 different *Salmonella* strains, including *Salmonella* Enteritidis PT4 strain 125109, and now covers over 98% of each genome (200). More specifically, the STv7E_2 array has 5648 gene-specific probes covering 99% of the genome of *Salmonella* Enteritidis PT4 strain 125109. Furthermore, it has 31 sRNA elements, 6 human gene controls, 4 plasmid controls and 2 labeled controls. Three identical arrays are spotted on each slide (200).

6.5.1 Preparation of probes

Products from the third round of SCOTS for RAW264.7 and RAW264.7/*Nramp1* samples obtained 2 hours post-invasion were amplified by Rb1-PCR. The PCR products were purified using the QIAquick PCR purification kit, resuspended in sterile, DNase-free water and quantified with a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Kirkland, Quebec, Canada). Each labeling reaction used 1.5 µg of cDNA in a total volume of 21 µL. Following the addition of 20 µL of 2.5X random primer / reaction buffer mix, samples were incubated for 5 minutes at 95˚C then placed on ice, and 5 µL of 10X dNTP mix for labeled dCTP was added (Table 6). The reaction mixture was completed with 2 µl of Cy3-dCTP or Cy5-dCTP (GE Healthcare, Baie d'Urfé, Quebec, Canada) and 2µL of Klenow fragment (5U/µL). The samples were

Table 6. DNA labeling and microarrays hybridization solutions

Solution	Composition
2.5X random primer / reaction buffer mix	125 mM Tris-Cl pH 6.8 12.5 mM $MgCl_2$ 25 mM 2-mercaptoethanol 0.6 µg/µl random hexamers
10X dNTP mix for labeled dCTP	1.2 mM dATP 1.2 mM dGTP 1.2 mM dTTP 0.6 mM dCTP 10 mM Tris-Cl pH 8.0 1mM EDTA
Pre-hybridization solution	25% formamide 5X SSC 0.1%SDS 0.1 mg/mL BSA
2X Hybridization solution	50% formamide 10X SSC 0.2% SDS

incubated for 17.5 hours at 37˚C and the reaction was stopped by the addition of 5 µL 0.5M EDTA pH 8.0. In this preliminary experiment, the RAW264.7 sample was labeled with Cy3-dCTP while the RAW264.7/*Nramp1* sample was labeled with Cy5-dCTP. For each condition, 1.5 µg *Salmonella* Enteritidis gDNA was labeled with the opposite dye following the same procedure.

Labeled probes were purified using the QIAquick PCR purification kit and resuspended in 50 µL of sterile, DNase-free water. Dye incorporation was verified using a NanoDrop® ND-1000 Spectrophotometer. The volume of the probes was decreased to 20 µL using a Savant SpeedVac System (Thermo Fisher Scientific, Kirkland, Quebec, Canada) before each labeled cDNA sample was combined with a gDNA sample labeled with the opposite dye.

6.5.2 Pre-hybridization and hybridization

Slides were incubated in pre-hybridization solution (Table 6) at 42˚C for 1 hour with gentle agitation, followed by two 1 minute washes in sterile, DNase-free water. The slides were then dried by centrifugation for 2 minutes at 1000 rpm. Prior to use, the probes were mixed with 40 µL of 2X hybridization solution (Table 6) and incubated for 5 minutes at 95˚C. Probes were hybridized to the STv7E_2 microarray for 17.5 hours at 42˚C in a hybridization chamber (Corning, Lowell, MA, USA) submerged in water. Following hybridization, slides were washed in a solution of 2X SSC, 0.1% SDS for 5 minutes at 42 °C then in a solution of 0.1X SSC, 0.1% SDS for 10 minutes at room temperature. Traces of SDS were removed by four 1 minute washes in 0.1X SSC. Arrays were rinsed in sterile, DNase-free water for 10 seconds then in 96% ethanol for 5 seconds and dried by centrifugation.

6.5.3 Data acquisition and analysis

Slides were analyzed with a ScanArray Lite scanner (PerkinElmer Life Science, Woodbridge, Ontario, Canada) and quantitation was performed using the ScanArray Express 2.0 software (PerkinElmer Life Science, Woodbridge, Ontario, Canada). Preliminary data analysis was done using Microsoft Excel 2007.

Negative control spots were defined as having 100 base pairs sequences with less than 70% identity to any given sequence in the genomes of the *Salmonella* strains present on the array. The detection threshold was set as the median signal intensity of the negative controls plus three standard deviations. Signals below this threshold were considered to be undetected. The median signal intensity value of all detected genes was determined for both samples. All signal intensity values for the RAW264.7 sample were multiplied by the (RAW264.7/*Nramp1* / RAW264.7) median ratio. This normalization step allowed for comparison of the two data sets. To account for the differential labeling of the two samples, only genes detected with an intensity signal greater than 1000 were analyzed. Expression fold change for genes detected in both samples were calculated using the normalized intensity data and changes of two or greater were considered significant.

RESULTS

Section 1: Evaluation of *Nramp1* as a candidate gene for *Ses1*

For this aspect of the project we opted for a quantitative complementation approach, also called QTL-knockout interaction test. This method tests the interaction between a QTL (*Ses1*) and the null allele of a candidate gene located within the QTL region (*Nramp1*) by comparing the phenotypes of offspring from 4 different crosses (Figure 5) (197, 201). Three conditions are needed to accomplish this type of experiment: 1) the QTL must have a recessive mode of inheritance; 2) congenic strains of the QTL in question must be available (129S6.B6-*Ses1*); and 3) a null allele of the candidate gene on the appropriate background has to be available (129S6-*Nramp1*$^{-/-}$).

Mice with either the C57BL/6J or 129S6 *Ses1* locus were mated to animals with the knockout (KO) or wild-type (WT) allele at *Nramp1*. F1 animals with all possible combination of alleles (C57BL/6J-*Ses1*/KO, C57BL/6J-*Ses1*/WT, 129S6-*Ses1*/KO and 129S6-*Ses1*/WT) and control animals (C57BL/6J, 129S6.B6-*Ses1* and 129S6-*Nramp1*$^{-/-}$) were tested for susceptibility to *Salmonella* Enteritidis infection. If *Nramp1* underlies *Ses1*, complementation failure would be observed in F1 animals from the [129.B6-*Ses1* x 129S6-*Nramp1*$^{-/-}$] cross as they have no functional *Nramp1*, leading to a decrease in their mean bacterial phenotype. Mice issued from the other crosses would have at least one copy of the functional *Nramp1* allele leading to higher splenic bacterial loads. In numerical terms, interaction is observed if the difference in mean bacterial load between C57BL/6J-*Ses1*/KO and 129S6-*Ses1*/KO animals is significantly greater than the difference in mean bacterial load between C57BL/6J-*Ses1*/WT and 129S6-*Ses1*/WT animals.

Four infections have reached their experimental endpoint (day 42 post-infection). Based on the sex-specific models of bacterial clearance proposed previously, the data was analyzed separately in males and females. The mean bacterial loads 42 days post-inoculation were compared using one-way ANOVA. In addition, the interaction between the mutated *Nramp1* gene and the QTL alleles was analyzed using two-way ANOVA. Representative results for males and females are shown (Figure 7 and 8). In both males and females, 129S6 mice presented spleen CFU counts significantly higher than those observed in C57BL/6J mice ($P<0.001$). In males, the impact of *Ses1* was not detectable in this series of experiment (Table 7). In females, the congenic 129.B6-*Ses1* and 129S6-

Nramp1$^{-/-}$ mice presented intermediate values as previously observed and the difference between the two groups was not significant, as expected (Table 8) (117, 118). 129S6-*Nramp1*$^{-/-}$ animals had a mean bacterial load significantly lower than 129S6 animals ($P<0.05$). As expected, F1 animals issued from the 129.B6-*Ses1* x 129S6-*Nramp1*$^{-/-}$ cross had the lowest CFU counts compared to F1 animals issued from the other crosses. However, the differences were not significant.

The magnitude of the interaction was computed as an interaction coefficient and analyzed by two-way ANOVA. In males, the interaction coefficient was of 0.047 with an associated P value of 0.8814 (Figure 9 and Table 9). In females, the interaction coefficient was of -0.090, with an associated P value of 0.823 (Figure 10 and Table 10). Despite the observation that [129.B6-*Ses1* x 129S6-*Nramp1*$^{-/-}$] F1 animals present lower bacterial counts compared to 129S6, the interaction coefficient was not statistically significant in either males or females.

Figure 7. Male splenic bacterial load following *Salmonella* Enteritidis infection of the F1 progeny resulting from quantitative complementation crosses.

The [129.B6-*Ses1* x 129S6-*Nramp1*$^{-/-}$] F1 (n = 10), [129S6 x 129S6-*Nramp1*$^{-/-}$] F1 (n = 7), [129.B6-*Ses1* x 129S6] F1 (n = 9), 129S6 (n = 5), 129.B6-*Ses1* (n = 3), 129S6-*Nramp1*$^{-/-}$ (n = 4)**,** C57BL/6J (n = 3) were infected with 1000 CFU of *Salmonella* Enteritidis. Spleens were harvested 42 days post-infection, homogenized and plated without any dilutions on TSA. The data are the average LOG CFU per gram of spleen ± standard error of the mean. An asterisk represents a significant difference with respect to 129S6 mice using one-way ANOVA. The results are representative of 4 independent experiments.

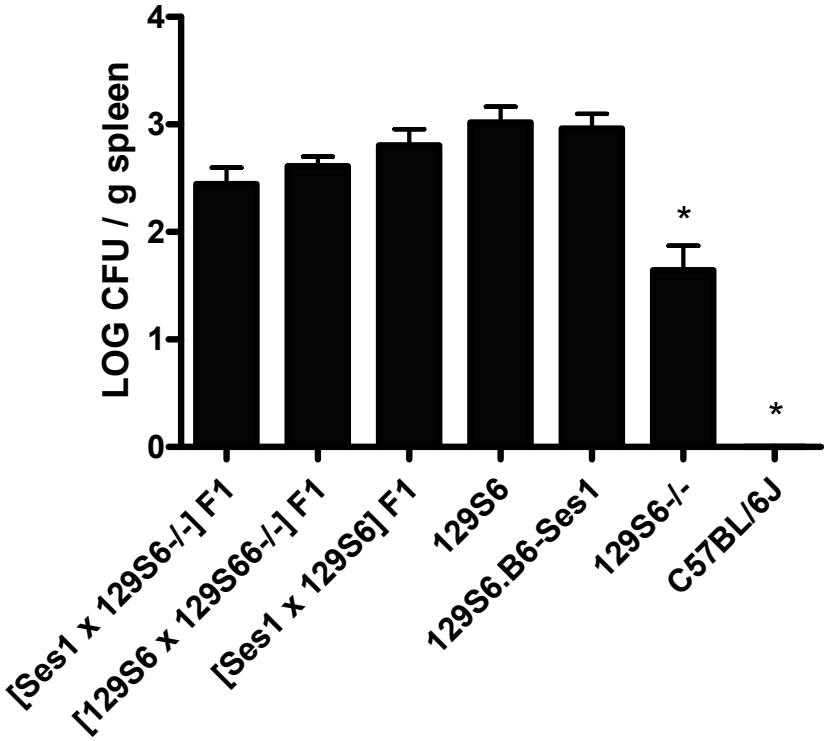

LOG CFU / g spleen
4
3
2
1
0
*
*
[Ses1 x 129S6-/-] F1
[129S6 x 129S66-/-] F1
[Ses1 x 129S6] F1
129S6
129S6.B6-Ses1
129S6-/-
C57BL/6J

Figure 8. Female splenic bacterial load following *Salmonella* Enteritidis infection of the F1 progeny resulting from quantitative complementation crosses.

The [129.B6-*Ses1* x 129S6-*Nramp1*$^{-/-}$] F1 (n =8), [129S6 x 129S6-*Nramp1*$^{-/-}$] F1 (n = 9), [129.B6-*Ses1* x 129S6] F1 (n = 9), 129S6 (n = 4), 129.B6-*Ses1* (n = 3), 129S6-*Nramp1*$^{-/-}$ (n = 4), C57BL/6J (n = 3) were infected with 1000 CFU of *Salmonella* Enteritidis. Spleens were harvested 42 days post-infection, homogenized and plated without any dilutions on TSA. The data are the average LOG CFU per gram of spleen ± standard error of the mean. An asterisk represents a significant difference with respect to 129S6 mice using one-way ANOVA. The results are representative of 4 independent experiments.

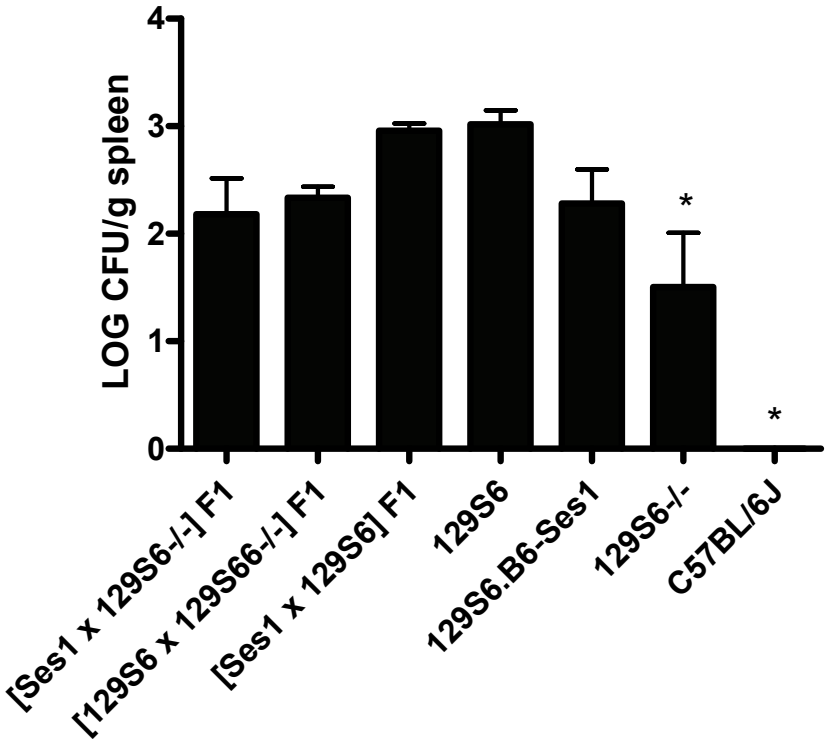

LOG CFU/g spleen
4
3
2
1
0
*
*
[Ses1 x 129S6-/-] F1
[129S6 x 129S6-/-] F1
[Ses1 x 129S6] F1
129S6
129S6.B6-Ses1
129S6-/-
C57BL/6J

Table 7. Statistical analysis of male splenic bacterial loads following *Salmonella* Enteritidis infection of the F1 progeny resulting from quantitative complementation crosses

Strains compared		Mean difference	95% confidence interval	P value
C57BL/6J	129S6	3.019	1.969 to 4.069	**P < 0.001**
129S6$^{-/-}$	129S6	1.373	0.5314 to 2.215	**P < 0.001**
129.B6-*Ses1*	129S6	0.05653	-0.8599 to 0.9730	P > 0.05
129.B6-*Ses1* x 129S6$^{-/-}$	129S6	-0.5756	-1.263 to 0.1117	P > 0.05
129.B6-*Ses1* x 129S6	129S6	-0.2149	-0.9148 to 0.4851	P > 0.05
129S6 x 129S6$^{-/-}$	129S6	-0.4068	-1.142 to 0.3280	P > 0.05
129.B6-*Ses1*	129S6$^{-/-}$	-1.317	-2.275 to -0.3582	**P < 0.01**
129S6$^{-/-}$	129.B6-*Ses1* x 129S6$^{-/-}$	0.7976	0.05519 to 1.540	**P < 0.05**
129.B6-*Ses1*	129.B6-*Ses1* x 129S6$^{-/-}$	-0.5191	-1.345 to 0.3070	P > 0.05
129.B6-*Ses1* x 129S6	129.B6-*Ses1* x 129S6$^{-/-}$	-0.3607	-0.9373 to 0.2159	P > 0.05
129S6 x 129S6$^{-/-}$	129.B6-*Ses1* x 129S6$^{-/-}$	-0.1688	-0.7872 to 0.4496	P > 0.05

Table 8. Statistical analysis of female splenic bacterial loads following *Salmonella* Enteritidis infection of the F1 progeny resulting from quantitative complementation crosses

Strains compared		Mean difference	95% confidence interval	P value
C57BL/6J	129S6	3.019	1.413 to 4.624	**P < 0.001**
129S6$^{-/-}$	129S6	1.514	0.2035 to 2.825	**P < 0.05**
129.B6-*Ses1*	129S6	0.7381	-0.6777 to 2.154	P > 0.05
129.B6-*Ses1* x 129S6$^{-/-}$	129S6	-0.8357	-1.971 to 0.2994	P > 0.05
129.B6-*Ses1* x 129S6	129S6	-0.06142	-1.175 to 1.053	P > 0.05
129S6 x 129S6$^{-/-}$	129S6	-0.6836	-1.798 to 0.4303	P > 0.05
129.B6-*Ses1*	129S6$^{-/-}$	-0.7762	-2.192 to 0.6396	P > 0.05
129S6$^{-/-}$	129.B6-*Ses1* x 129S6$^{-/-}$	0.6785	-0.4567 to 1.814	P > 0.05
129.B6-*Ses1*	129.B6-*Ses1* x 129S6$^{-/-}$	-0.09767	-1.353 to 1.157	P > 0.05
129.B6-*Ses1* x 129S6	129.B6-*Ses1* x 129S6$^{-/-}$	-0.7743	-1.675 to 0.1264	P > 0.05
129S6 x 129S6$^{-/-}$	129.B6-*Ses1* x 129S6$^{-/-}$	-0.1521	-1.053 to 0.7486	P > 0.05

Figure 9. Analysis of variance of the interaction between *Ses1* and *Nramp1*$^{-/-}$ in males.

Bacterial loads 42 days following *Salmonella* Enteritidis infection of the F1 progeny resulting from the QTL-knock-out interaction test crosses are shown. The data are the average LOG CFU per gram of spleen ± standard error of the mean.

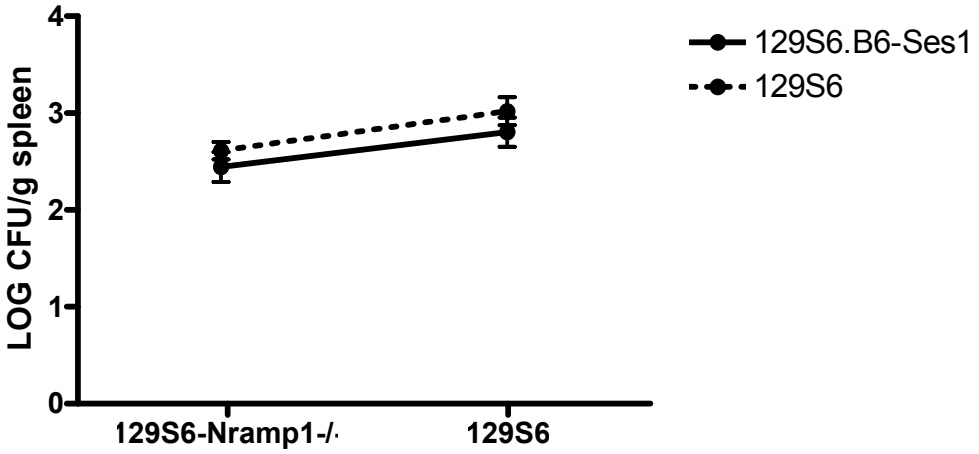

LOG CFU/g spleen
4
3
2
1
0
129S6-Nramp1-/-
129S6
129S6.B6-Ses1
129S6

Table 9. Analysis of variance of the interaction between *Ses1* and *Nramp1*$^{-/-}$ in males

	C57BL/6J *Ses1*	**129S6 *Ses1***	**Interaction coefficient**	**P-value**
Mutant	2.444	2.612	0.047	0.8814
Wild-type	2.804	3.019		

The mean bacterial load for the four combinations of QTL (129S6 *Ses1* or C57BL/6J *Ses1*) and genotype at *Nramp1*, and the QTL x gene interaction coefficient with its associated P value are shown.

Figure 10. Analysis of variance of the interaction between *Ses1* and *Nramp1*$^{-/-}$ in females

Bacterial loads 42 days following *Salmonella* Enteritidis infection of the F1 progeny resulting from the QTL-knock-out interaction test crosses are shown. The data are the average LOG CFU per gram of spleen ± standard error of the mean.

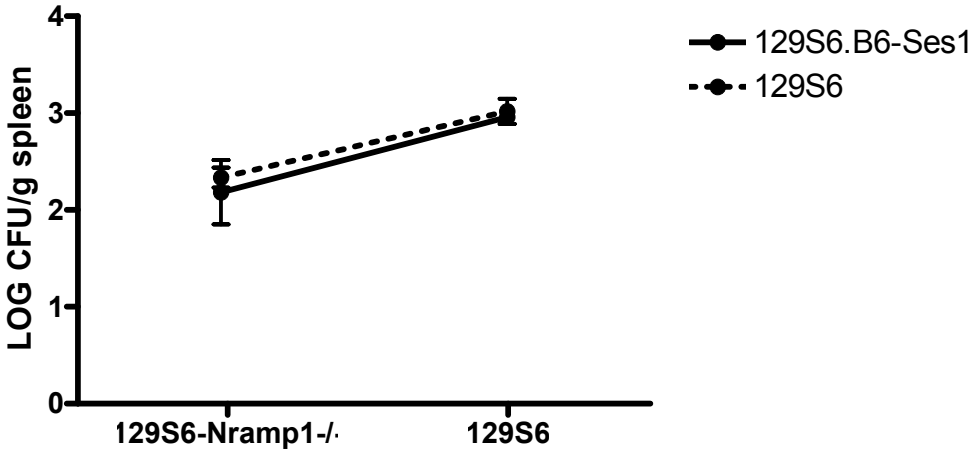

4
3
2
1
0
LOG CFU/g spleen
129S6-Nramp1-/-
129S6
129S6.B6-Ses1
129S6

Table 10. Analysis of variance of the interaction between *Ses1* and *Nramp1*$^{-/-}$ in females

	C57BL/6J *Ses1*	**129S6 *Ses1***	**Interaction coefficient**	**P-value**
Mutant	2.183	2.335	-0.090	0.823
Wild-type	2.957	3.019		

The mean bacterial load for the four combinations of QTL (129S6 *Ses1* or C57BL/6J *Ses1*) and genotype at *Nramp1*, and the QTL x gene interaction coefficient with its associated P value are shown.

Section 2: Host response to *Salmonella* Enteritidis infection is under complex genetic control

Testing the epistatic models of *Salmonella* Enteritidis clearance requires the creation of new congenic mouse strains based on the previous QTL interaction statistical analysis of the [C57BL/6J x 129S6] F2 panel. We concentrated our efforts on the female model as its associated loci create a more pronounced phenotype. In this model of bacterial clearance, *Ses1* and *Ses3* showed a significant effect alone; however, interactions between *Ses1* and *Ses4* (chromosome X) and/or between *Ses1* and *Ses5* (chromosome 7) accounted for 72% of the total phenotypic variance (118). To confirm these interactions *in vivo*, we are creating 5 congenic strains as described in Table 11. The choice of flanking markers was done based on the 2-LOD support interval around the peak marker for each locus. We are creating a new 129S6.B6-*Ses1.2* strain that will completely cover the 2-LOD confidence interval around the peak marker at *Ses1* as the previously constructed 129S6.B6-*Ses1* strain did not cover the proximal end of the QTL. First, we initiated 2 crosses between a C57BL/6J female and a 129S6 male and between a 129S6 female and a C57BL/6J male. Two females from each F1 generation were backcrossed to a 129S6 male to obtain the N2 generation. Offspring were genotyped for selected markers at each locus and individuals with the appropriate genotype were backcrossed to 129S6 animals. Selected N5 individuals from each single congenic strain were used in the new crosses (129.B6-*Ses1.2* x 129.B6-*Ses4* and 129.B6-*Ses1.2* x 129.B6-*Ses5*) to create double congenic mouse strains. Ten backcrosses are required to produce a congenic strain with a homogeneous background. We have reached generation N6 to N8 and N5F1N2 to N5F1N3 for the different single and double congenic strains, respectively. The specific breeding scheme for each new line is described in Table 11. Three to nine more months are required until completion of all congenic strains. We will then establish homozygous founders for all strains. Approximately 1/4 of single congenic F1 progeny will be homozygous at the locus of interest, and 1/16 of double congenic F1 progeny will be homozygous at both loci. F1 mice with the appropriate genotype will be crossed to produce the F2 generation that will be used to test our proposed model of bacterial clearance.

Table 11. Congenic strains breeding scheme

Strain name	Interval size (Mb) [a]	Generation	Estimated time to completion
129.B6-*Ses1.2*	46.6	N8	3 months
129.B6-*Ses4*	55.0	N7	6 months
129.B6-*Ses5*	27.7	N6	9 months
129.B6-*Ses1.2*/*Ses4*	46.6 and 55.0	N5F1N3	3 months
129.B6-*Ses1.2*/*Ses5*	46.6 and 27.7	N5F1N2	6 months

[a] Size based on the physical position of the markers at Ensembl mouse genome browser (www.ensembl.org/Mus_musculus).

Section 3: Investigation of the effect of *Nramp1* on the transcriptome of *Salmonella*

We decided to use SCOTS to study the global transcriptional profile of *Salmonella* during infection (Figure 6). Briefly, SCOTS enriches total cDNA samples for bacterial transcripts expressed within the host during infection. Low abundancy bacterial transcripts are captured by hybridization of total cDNA to rDNA-blocked biotinylated bacterial genomic DNA. Captured transcripts are eluted and amplified and the capture is repeated twice more (202, 203). SCOTS-derived cDNA samples are then analyzed by microarrays and results are confirmed by quantitative PCR on non-enriched samples.

Initially, we attempted to use spleens from infected mice for SCOTS. We decided to use RNA from samples obtained at early time points following infection, when *Salmonella* levels are similar and also at their peak in both 129S6 and 129S6-*Nramp1*$^{-/-}$ spleens. This would also allow us to determine the impact of *Nramp1* on bacterial gene expression before its effect on survival is observed. It has been previously reported that 129S6 and 129S6-*Nramp1*$^{-/-}$ mice have similar LOG CFU/g spleen values at days 3 and 5 post-infection, before 129S6 mice show signs of *Salmonella* Enteritidis carriage (118). However, upon replication of this experiment with increased sample size we detected a significant difference between bacterial loads in female spleens as early as day 5 (Figure 11). Several preliminary experiments were unsuccessful to amplify target bacterial housekeeping genes (*rpoA*, *rpoB*, or *rpoD*) from non-enriched cDNA obtained from infected tissues. Consequently, we opted for a different approach using *in vitro* and *ex vivo* models of *Salmonella* Enteritidis infection, in which sample size and bacterial levels are more easily controlled. First, we characterized *Salmonella* Enteritidis infection in an *in vitro* model using the RAW264.7 macrophage cell line expressing or not expressing a functional *Nramp1*. RAW264.7/*Nramp1* macrophages limited bacterial replication while RAW264.7 macrophages were unable to contain *Salmonella* Enteritidis (Figure 12). In the absence of a functional *Nramp1*, bacterial levels went up 122% (MOI of 1) and 112% (MOI of 10) than that of their initial levels 21 hours post-invasion. On the other hand, RAW264.7/*Nramp1* cells showed a slight increase in intracellular bacteria early in the infection (3 hours post-invasion) before the effect of *Nramp1* was observed. The MOI had a significant effect on the efficiency of clearance, with 72% of the bacteria surviving at a

MOI of 10 whereas 92% survived at a MOI of 1. Similar results were observed with BMDM from 129S6 and 129S6-*Nramp1*$^{-/-}$ mice infected at a MOI of 10 (Figure 13).

We repeated the infection using RAW264.7 and RAW264.7/*Nramp1* macrophages, and at 1 hour post-invasion we observed a LOG CFU/well of 5.9 ± 0.1 and 6.2 ± 0.2, respectively. These values were not significantly different and did not increase significantly 2 hours post-invasion (data not shown). Furthermore, we were able to detect housekeeping genes in non-enriched cDNA samples obtained from 2 x 10^7 cells infected at a MOI of 10. Therefore, infected cell samples from 1 and 2 hours post-invasion were used for SCOTS. For each condition, the complexity of the SCOTS-derived cDNA samples was assessed by Southern blot hybridization (Figure 14). Samples obtained 1 hour post-invasion were less complex than those obtained 2 hours post-invasion. Furthermore, samples obtained 2 hours post-invasion showed increasing complexity with consecutive capture rounds, as expected. To analyze the cDNA mixtures captured, we used both a candidate gene and a whole genome approach. Bacterial genes whose expression is known or suspected to be influenced by *Nramp1* were further investigated by slot blot hybridization (Figure 15). A list of genes tested and the rationale for their choice are presented in Table 12. After hybridization with the SCOTS products, the bands were quantified using an image analysis program. Using this approach, we did not detect any significant change in expression in the presence or absence of a functional *Nramp1* at 1 or 2 hours post-invasion, as all expression ratios were smaller than 2 (Table 13).

To investigate the global bacterial transcriptional profile we decided to apply our SCOTS-derived samples to *Salmonella* ORF array. This array covers the genomes of 5 different *Salmonella* serovars (Typhimurium LT2, Typhimurium SL1344, Typhi CT18, Typhi Ty2, Paratyphi SARB42, and Enteritidis PT4). Each array contains 5648 gene-specific probes and it is spotted in triplicate on a slide. Of importance to this experiment, the array covers 99% of the genome of *Salmonella* Enteritidis PT4. Initial experiments were done using SCOTS-derived samples from RAW264.7 and RAW264.7/*Nramp1* cells 2 hours post-invasion. Hybridization to the *Salmonella* ORF array showed patterns in which the cDNA hybridized to a limited number of spots. Nonetheless, a total of 866 genes were detected consistently in the triplicate arrays of the RAW264.7 and the RAW264.7/*Nramp1* samples. We further analyzed genes detected with an intensity signal

greater than 1000. Using this selection criterion for strong signals, we detected 131 and 104 genes in the RAW264.7 and RAW264.7/*Nramp1* samples, respectively (Tables 14 and 15). There were 34 genes in common to both groups. In the presence of a functional *Nramp1* allele, 4 genes (*mgtB*, *rpsD*, *manZ*, and *menG*) were up-regulated (expression ratio > 2) (Table 16). Furthermore, 7 genes (*osmE*, *rplP*, *yhdG*, *bglJ*, *treF*, *rplD*, and *msbB*) were down-regulated in the presence of a functional *Nramp1* allele (expression ratio < -2) (Table 17). The greatest fold change was observed for *mgtB*, a magnesium transporter, which had a 10-fold increase in expression in the presence of a functional *Nramp1*. Transcriptional regulation of *mgtB* is controlled by PhoP, which was detected only in the presence of a functional *Nramp1*. Consequently, PhoP-activated genes such as *yheR*, and *ybjY* were also detected in the presence of a functional *Nramp1* and PhoP-repressed genes such as *flgF*, *prgJ*, *sipB*, *sopB*, and *hilA* were detected in the absence of a functional *Nramp1*. Next, all genes strongly enriched for by SCOTS were classified according to their COG (8). Various aspects of *Salmonella* physiology were represented, as shown in Tables 18 to 20. In the absence of a functional *Nramp1*, more genes were detected in the intracellular trafficking and secretion COG, and in the pathogenicity COG. In the RAW264.7/*Nramp1* sample, a higher percentage of genes detected belonged to the replication, recombination and DNA repair COG, the posttranslational modification, protein turnover, and chaperones COG, and the energy production and conversion COG. Numerous genes belonged to the cell wall/membrane biogenesis COG, with 11.9% of the detected RAW264.7 genes and 7.8% of the detected RAW264.7/*Nramp1* genes being classified in this category. More specifically, genes involved in the biosynthesis of murein lipoprotein (*mipA*, *mepA*, and *mraY*) and genes involved in LPS biosynthesis (*glmU* and *htrB*) were detected in the absence of a functional *Nramp1* only. In addition, we detected *msbB*, an enzyme involved in increasing the potency of LPS, in both samples and observed a 2-fold decrease in its expression in the presence of *Nramp1* (Table 17). Known pathogenicity genes were detected in both samples; however, none were in common between the two samples. That is, in the presence of a functional *Nramp1*, *ssaN*, *typA*, *mgtC*, *sipB*, *sopB*, and *hilA* were detected, and in the absence a functional *Nramp1*, only *sipA* and *msgA* were detected. Interestingly, four of these genes (*sipB*, *sopB*, *hilA* and

sipA) are SPI-1 genes. Finally, it must be noted that the majority of genes (approximately 20%) detected in each of the samples had no known function.

Figure 11. Early response to *Salmonella* Enteritidis infection in 129S6 and 129S6-*Nramp1*$^{-/-}$ mice.

Spleens were harvested 3 and 5 days post-infection and one half was used for CFU enumeration. Each bar represents groups of 5 males (A) or females (B). The data are the average LOG CFU per gram of spleen ± the standard error of the mean. The asterisk represents the significance level of the difference in bacterial load between 129S6 and 129S6-*Nramp1*$^{-/-}$ males or female.

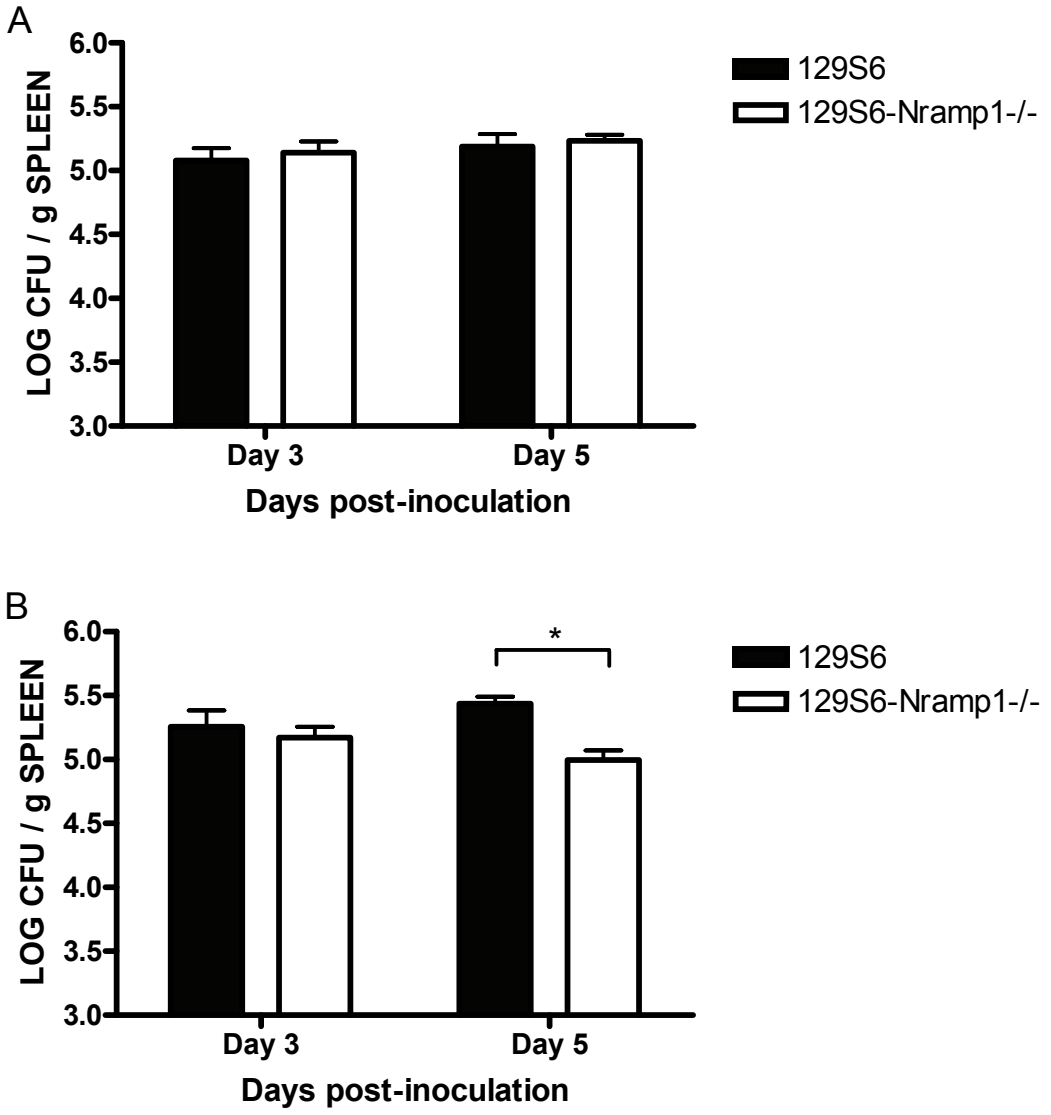

A
6.0
5.5
5.0
4.5
4.0
3.5
3.0
LOG CFU / g SPLEEN
129S6
129S6-Nramp1-/-
Day 3
Day 5
Days post-inoculation
B
*
6.0
5.5
5.0
4.5
4.0
3.5
3.0
LOG CFU / g SPLEEN
129S6
129S6-Nramp1-/-
Day 3
Day 5
Days post-inoculation

Figure 12. *Salmonella* Enteritidis replication in RAW264.7 and RAW264.7/*Nramp1* macrophages.

Bacteria were added at a MOI of 1 and 10 (open and closed points respectively) to RAW264.7 and RAW264.7/*Nramp1* macrophages (dashed and solid lines respectively) in triplicate wells. The results are the average LOG CFU per well (A) or average percent survival (B) ± standard error of the mean. The experiment was repeated three times with similar results.

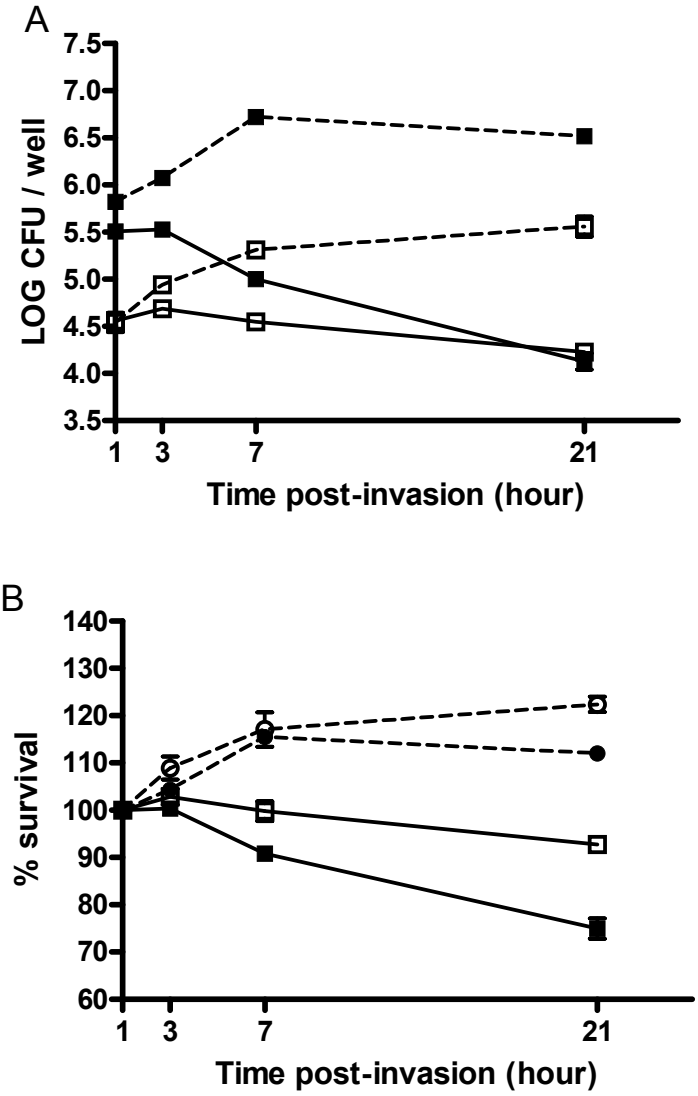

A
LOG CFU / well
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
1
3
7
21
Time post-invasion (hour)
B
% survival
140
130
120
110
100
90
80
70
60
1
3
7
21
Time post-invasion (hour)

Figure 13. *Salmonella* Enteritidis replication in 129S6 and 129S6-*Nramp1*$^{-/-}$ BMDM. Dashed and solid lines represent 129S6 and 129S6-*Nramp1*$^{-/-}$ BMDM respectively. The results are the average LOG CFU per well (A) or average percent survival (B) ± standard error of the mean. The experiment was repeated three times with similar results.

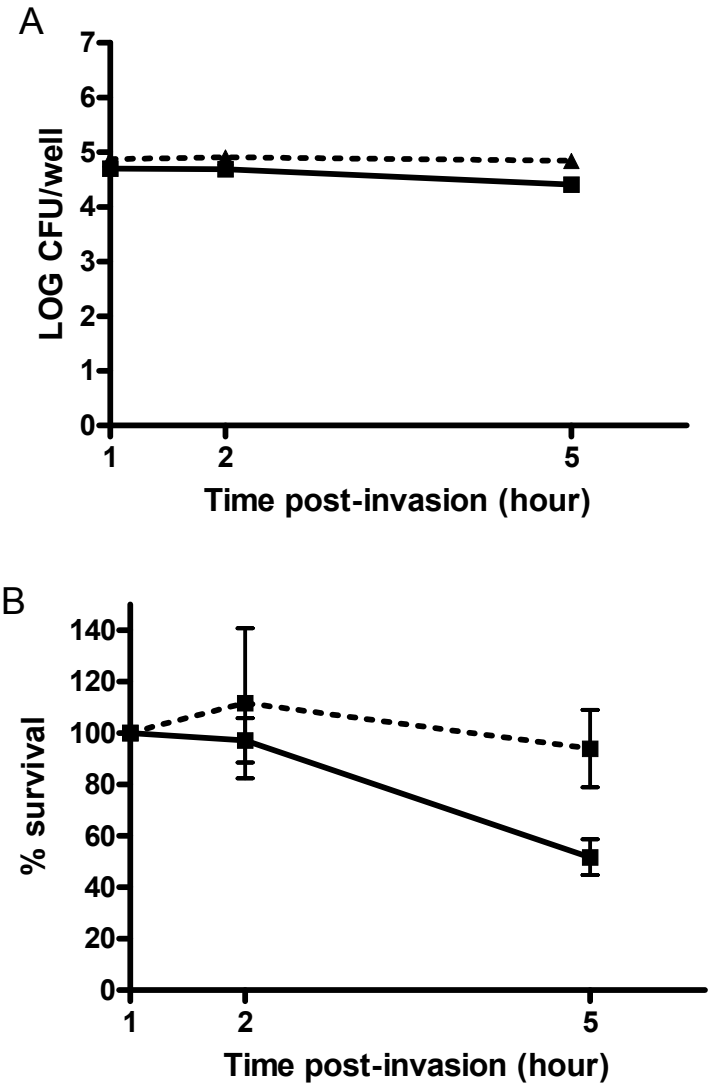
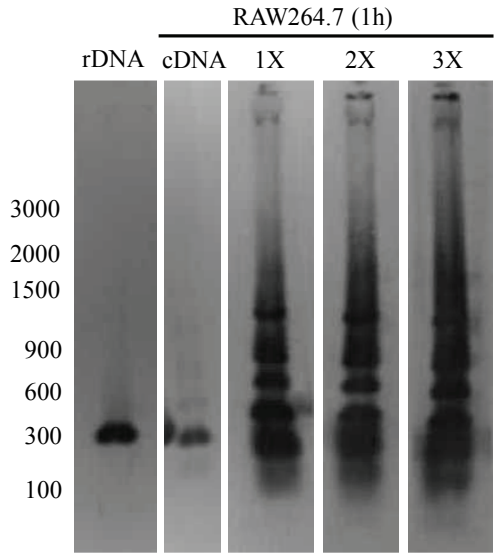
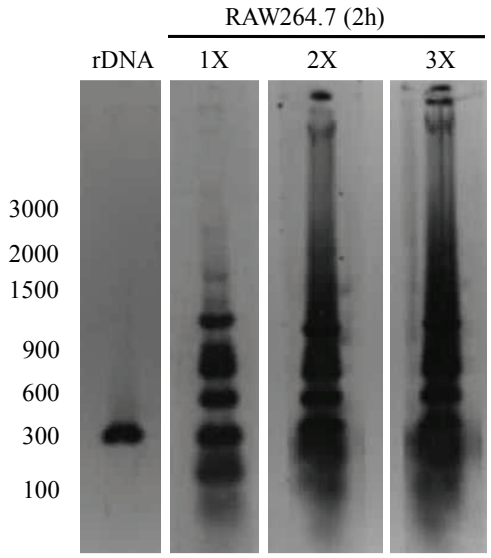
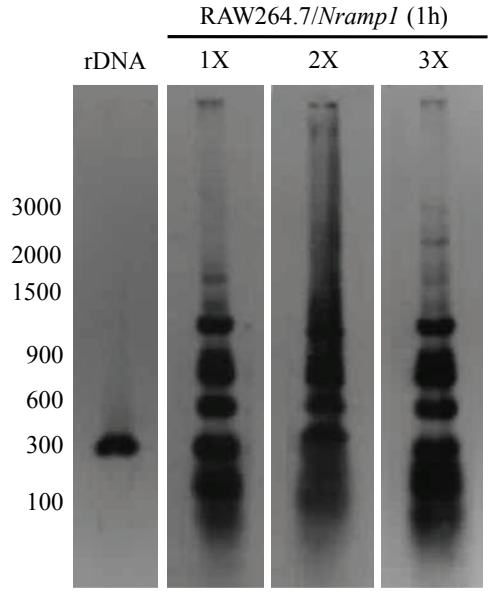

A
LOG CFU/well
0
1
2
3
4
5
6
7
1
2
5
Time post-invasion (hour)
B
% survival
0
20
40
60
80
100
120
140
1
2
5
Time post-invasion (hour)

Figure 14. Southern hybridization of SCOTS products to *Salmonella* Enteritidis genomic DNA.

Southern blot hybridization results for ribosomal DNA, cDNA (when visible), 1X SCOTS, 2X SCOTS, and 3X SCOTS for RAW264.7 and RAW264.7/*Nramp1* samples, at 1 and 2 hours post-invasion. Probes were prepared by PCR amplification using DIG-11-dUTP. Hybridization conditions are described in Materials and Methods. Molecular weight markers (in bp) are indicated to the left of each condition.


RAW264.7 (1h)
rDNA
cDNA
1X
2X
3X
3000
2000
1500
900
600
300
100


RAW264.7 (2h)
rDNA
1X
2X
3X
3000
2000
1500
900
600
300
100


RAW264.7/*Nramp1* (1h)
rDNA
1X
2X
3X
3000
2000
1500
900
600
300
100

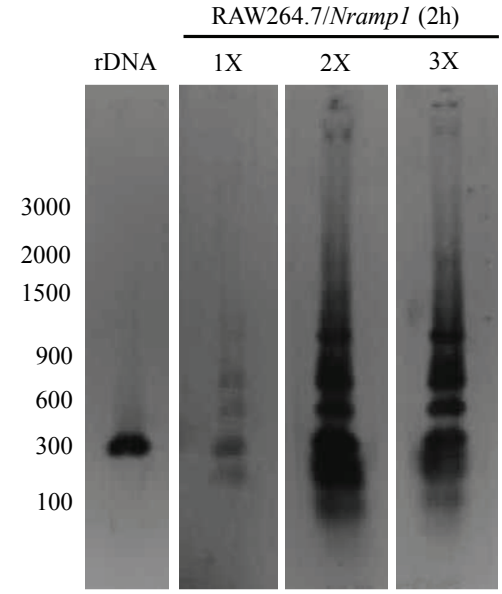

RAW264.7/*Nramp1* (2h)
rDNA
1X
2X
3X
3000
2000
1500
900
600
300
100

Figure 15. Slot blot hybridization of SCOTS-derived products to specific gene probes.

Gene probes were amplified by PCR using primers described in Table 5. RAW264.7 and RAW264.7/*Nramp1* SCOTS-derived samples obtained 1 and 2 hours post-invasion.were marked by PCR amplification using DIG-11dUTP. Hybridization conditions are described in Materials and Methods.

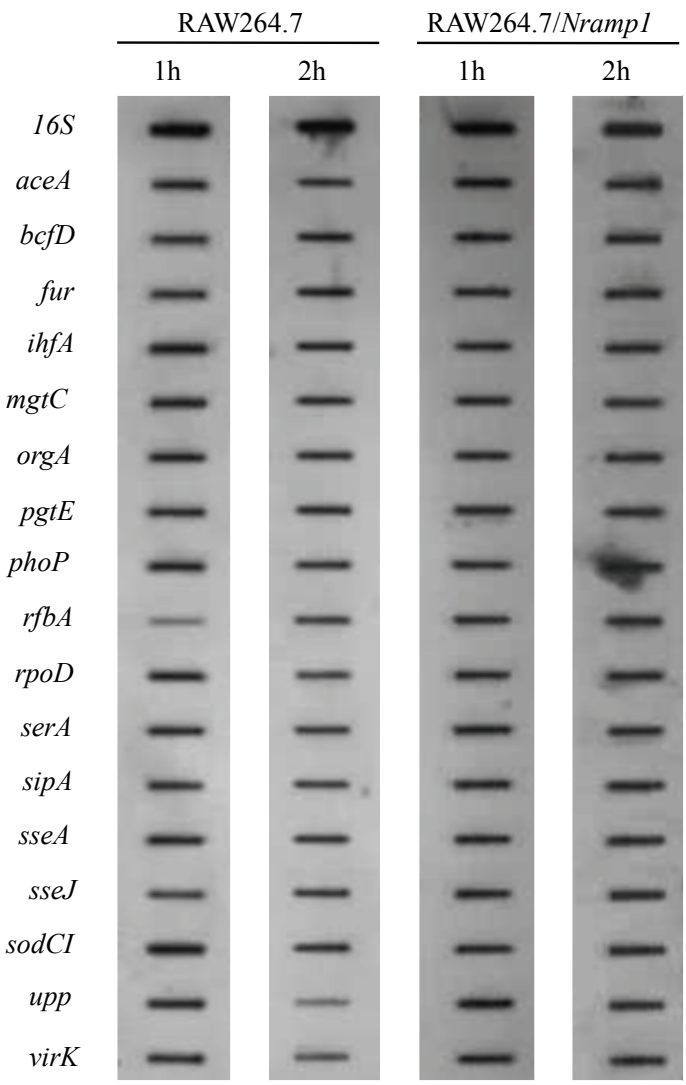

RAW264.7
RAW264.7/*Nramp1*
1h
2h
1h
2h
16S
aceA
bcfD
fur
ihfA
mgtC
orgA
pgtE
phoP
rfbA
rpoD
serA
sipA
sseA
sseJ
sodCI
upp
virK

Table 12. Rationale behind genes analyzed by slot blot hybridization

Gene	Name	Rationale
16S	Ribosomal RNA	Control
aceA	Isocitrate lyase	Defect in the CI of the *aroA aceA Salmonella* Typhimurium double mutant compared to the *aroA* mutant in chronic infection (91, 96).
bcfD	Fimbrial subunit	Contribution to systemic *Salmonella* infection identified through a negative selection screen (94).
fur	Ferric uptake regulator	Activated by manganese; negatively regulates operons encoding enzymes involved in iron transport.
ihfA	Integration host factor α	Protein detected in *Salmonella* isolated from RAW264.7 macrophages carrying a functional *Nramp1* only (194).
mgtC	Magnesium transport protein	Contribution to systemic *Salmonella* infection identified through a negative selection screen; expression up-regulated in J774-A.1 cells (83, 94).
orgA	Needle complex assembly protein	Contribution to systemic *Salmonella* infection identified through a negative selection screen and confirmed by CI experiments (94).
pgtE	Outer membrane protease	Activated by PhoP; cleaves α-helical antimicrobial peptides that bind *Salmonella*'s OM (204).
phoP	DNA-binding transcriptional regulator	PhoP control the expression of over 200 genes involved in various aspects of virulence (84).
rfbA	dTDP-glucose pyrophosphorylase	Contribution to systemic *Salmonella* infection identified through a negative selection screen (94).
rpoD	RNA polymerase σ factor D	Control
serA	D-3-phosphoglycerate dehydrogenase	Protein detected in *Salmonella* isolated from RAW264.7 macrophages carrying a functional *Nramp1* only (194).
sipA	Cell invasion protein	Control; contribution to systemic infection ruled out by CI experiment (94).
sseA	Secretion system chaperone	Expression up-regulated in RAW264.7/*Nramp1* cells compared to RAW264.7 cells 9 hours post-infection (95).
sseJ	Secreted effector J	Expression up-regulated in 129S6 mice compared to 129S6-*Nramp1*$^{-/-}$ mice 24 hours post-infection (95).
sodCI	Superoxide dismutase	Protein detected in *Salmonella* isolated from RAW264.7 macrophages carrying a null allele at *Nramp1* only (194).
upp	Uracil phosphoribosyltransferase	Protein detected in *Salmonella* isolated from RAW264.7 macrophages carrying a null allele at *Nramp1* only (194).
virK	Virulence protein	CI experiments show that *virK* mutant *Salmonella* Typhimurium is outcompeted by wild-type bacteria in both the liver and the spleen of 129X1/SvJ mice 3 weeks post-infection (93).

Table 13. Gene expression ratios comparing genotype at *Nramp1* and time post-invasion of SCOTS-derived products[a]

Gene	- *Nramp1*	+ *Nramp1*	1h	2h
	1h / 2h	1h / 2h	RAW264.7 / RAW264.7/*Nramp1*	RAW264.7 / RAW264.7/*Nramp1*
aceA	1.05	0.93	0.88	0.78
bcfD	0.99	0.92	0.91	0.84
fur	0.99	0.85	0.94	0.81
ihfA	1.22	0.84	1.10	0.77
mgtC	1.19	0.86	1.03	0.75
orgA	1.16	0.81	1.04	0.73
pgtE	1.10	0.84	0.94	0.73
phoP	1.17	N/A[b]	1.01	N/A[b]
rfbA	N/A[c]	1.06	N/A[c]	1.00
serA	1.11	0.86	0.90	0.70
sipA	1.17	0.84	0.92	0.66
sseA	1.21	0.84	0.99	0.69
sseJ	1.03	0.86	0.85	0.71
sodCI	1.25	0.83	1.10	0.7
upp	1.50	0.94	0.91	0.57
virK	1.25	0.89	0.90	0.64

[a] All expression ratios were normalized with *rpoD* as a housekeeping gene.
[b] Ratios could not be calculated correctly due to the background.
[c] Ratios could not be calculated due to technical problems during the blotting procedure.

Table 14. Genes detected with intensity >1000 in the RAW264.7 sample only

Gene symbol	Function	Intensity
nanA	N-acetylneuraminate lyase (aldolase)	19721
SEN4283	N/A	17974
ubiG	3-demethylubiquinone-9 3-methyltransferase and 2-octaprenyl-6-hydroxy phenol methylase	16117
envE	Putative envelope protein	14804
STM3835	N/A	14253
SEN4288	N/A	13302
secG	Preprotein translocase IISP family, auxillary membrane component	11904
ydiU	Putative cytoplasmic protein	10450
bcr	MFS family multidrug transport protein, bicyclomycin Resistance protein	10153
ibpB	Small heat shock protein	8272
purG	Phosphoribosylformylglycinamidine synthetase	7845
uxuR	Transcriptional repressor for uxu operon	7379
STY2054	N/A	7329
ssaN	Secretion system apparatus: homology with the YscN family of proteins	7208
N/A	Homologue of pipB, putative pentapeptide repeats (8 copies)	6791
bioA	7,8-diaminopelargonic acid synthetase	6698
gldA	Glycerol dehydrogenase, NAD	6474
STY1526	Putative Ni/Fe-hydrogenase 1 b-type cytochrome subunit	6242
yohL	Putative cytoplasmic protein	6205
sipB	Cell invasion protein	5968
ptsG	Sugar Specific PTS family, glucose-specific IIBCcomponent	5765
STM4529	Putative cytoplasmic protein	5737
yafV	Putative amidohydrolase	5300
ftsQ	Cell division protein; ingrowth of wall at septum	5206
STM1586	Putative periplasmic protein	5159
malP	Maltodextrin phosphorylase	5143
rplK	50 S ribosomal subunit protein L11	5134
yjeI	putative outer membrane lipoprotein	5038
mipA	Scaffolding protein for murein-synthesizing holoenzyme	4968
SEN1387	N/A	4965
sopB	*Salmonella* outer protein: homologous to ipgD of Shigella	4894
rcsB	Response regulator (positive) in two-component regulatory system with RcsC (LuxR/UhpA familiy)	4842
ybiR	Putative Di- and tricarboxylate transporters	4819

Table 14. Genes detected with intensity >1000 in the RAW264.7 sample only (continued)

Gene symbol	Function	Intensity
mgtC	Magnesium transport protein	4706
STM1624	Putative cytoplasmic protein	4489
gst	Glutathionine S-transferase	4480
rpsK	30S ribosomal subunit protein S11	4422
ynfD	Putative outer membrane protein	4416
yebS	Putative inner membrane protein	4377
mraY	Phospho-N-acetylmuramoyl-pentapeptide transferase	4366
holE	DNA polymerase III, theta subunit	4265
fldA	Flavodoxin 1	4117
yjeQ	Putative GTPase	4029
infC	Protein chain initiation factor IF-3	3994
yeaZ	Putative molecular chaperone	3992
yqjI	Putative transcriptional regulator	3908
yihZ	D-Tyr-tRNA(Tyr) deacylase	3868
pyrG	CTP synthetase	3831
yjgP	Putative permease	3667
ilvC	Ketol-acid reductoisomerase	3660
ccdB	Toxin addiction system: toxin	3471
secY	Preprotein translocase of IISP family, membrane subunit, Putative ATPase	3295
sbcB	Exonuclease I, 3' --> 5' specific; deoxyribophosphodiesterase	3237
ybaW	Putative esterase	3194
ybaP	Putative cytoplasmic protein	3181
ffs	Signal recognition particle, RNA component	3114
STM3133	Putative amidohydrolase	3096
yjbQ	Putative cytoplasmic protein	3081
ychK	Putative phosphoesterase	3076
smg	Putative cytoplasmic protein	3021
yohL	Putative cytoplasmic protein	3006
lrhA	NADH dehydrogenase transcriptional repressor (LysR family)	3005
yqjD	Putative inner membrane protein	2975
STY1293	N/A	2839
prfB	Peptide chain release factor 2	2711
aroP	APC family, aromatic amino acid transporter	2698
typA	GTP-binding elongation factor family protein	2698
ydcR	Putative regulatory protein, gntR family	2694

Table 14. Genes detected with intensity >1000 in the RAW264.7 sample only (continued)

Gene symbol	Function	Intensity
STM1251	Putative molecular chaperone (small heat shock protein)	2681
rplQ	50S ribosomal subunit protein L17	2674
mepA	Murein DD-endopeptidase, penicillin-insensitive	2628
ynfB	Putative periplasmic protein	2566
gsp	Bifunctional: glutathionylspermidine synthetase; Glutathionylspermidine amidase	2563
mopA	Chaperone Hsp60 with peptide-dependent ATPase activity, Affects cell division	2457
rtT	Regulatory RNA	2432
galK	Galactokinase	2430
STM3125	Putative cytoplasmic protein	2334
yrbC	Putative ABC superfamily, transport protein	2236
suhB	Inositol monophosphatase	2160
ydeV	Putative sugar kinase	2068
repA2	DNA replication	2067
nagB	Glucosamine-6-phosphate deaminase	2055
ycdW	Paral putative oxidoreductase	2004
PSLT026	Putative periplasmic protein	1975
STM1128	Putative sodium/glucose cotransporter	1939
glmU	N-acetyl glucosamine-1-phosphate uridyltransferase and glucosamine-1-phosphate acetyl transferase	1881
hpaR	4-hydroxyphenylacetate catabolism	1864
STM2938	Putative cytoplasmic protein	1856
htrB	Lauroyl/myristoyl acyltransferase involved in lipid A biosynthesis	1831
yejF	Putative ATPase component of ABC-type transport system, contain duplicated ATPase domain	1821
thrA	Aspartokinase I , bifunctional enxyme N-terminal is aspartokinaseI and C-terminal is homoserine dehydrogenase I	1813
yrbB	Putative STAS domain	1786
rpmH	50S ribosomal subunit protein L34	1778
rplC	50S ribosomal subunit protein L3	1765
rplB	50S ribosomal subunit protein L2	1735
rpsI	30S ribosomal subunit protein S9	1692
hilA	Invasion genes transcription activator	1688

Table 15. Genes detected with intensity >1000 in the RAW264.7/*Nramp1* sample only

Gene symbol	STM Function	Intensity
pgpB	Phosphatidylglycerophosphate phosphatase B	17029
STM4317	Putative helix-turn-helix protein, copG family	13694
icc	Cyclic 3',5'-adenosine monophosphate phosphodiesterase	13292
phoP	Response regulator in two-component regulatory system with PhoQ, transcribes genes expressed under low Mg+ Concentration (OmpR family)	10997
plsX	Putative fatty acid/phospholipid synthesis protein	10662
ycjI	Putative carboxypeptidase	10361
ygeD	Putative efflux protein, resistance protein	10148
creB	Response regulator in two-component regulatory system with CreC (OmpR family)	9024
yfaZ	Putative inner membrane protein	7269
fdnG	Putative molybdopterin oxidoreductases	7009
rpsJ	30S ribosomal subunit protein S10	6999
ygjO	Paral putative methyltransferase	6850
ygdL	Paral putative enzyme	6750
STM1791	Putative hydrogenase-1 protein	5752
yafA	Putative hydrolase of the alpha/beta superfamily	5599
yffH	Putative pyrophosphohydrolase	5289
STM1638	Putative SAM-dependent methyltransferases	5057
leuA	2-isopropylmalate synthase	4782
yjaB	Putative acetyltransferase	4695
mdoB	Phosphoglycerol transferase I	4577
STM2655	Putative cytoplasmic protein	4556
fdnG	Putative molybdopterin oxidoreductases	4516
lon	DNA-binding, ATP-dependent protease la; cleaves RcsA and SulA, heat shock k-protein (DNA binding activity)	4423
trkH	Trk family, potassium transport protein, requires TrkE	4307
STY4667	N/A	4229
rlgA	Putative resolvase	4082
bglA	6-phospho-beta-glucosidase A	4014
ycfR	Putative outer membrane protein	3697
PSLT007	Putative outer membrane protein	3515
hemA	Glutamyl tRNA reductase	3472
yaeC	Putative outer membrane lipoprotein	3377
ygdP	Putative invasion protein; NTP pyrophosphohydrolase	3018
sipA	Cell invasion protein	2920
msgA	Macrophage survival gene; reduced mouse virulence	2889

Table 15. Genes detected with intensity >1000 in the RAW264.7/*Nramp1* sample only (continued)

Gene symbol	STM Function	Intensity
lon	DNA-binding, ATP-dependent protease la; cleaves RcsA and SulA, heat shock k-protein (DNA binding activity)	2792
rplE	50S ribosomal subunit protein L5	2712
ygdE	Putative SAM-dependent methyltransferase	2668
dnaG	DNA biosynthesis; DNA primase	2652
STM4032	Putative acetyl esterase	2508
yceP	Putative cytoplasmic protein	2490
yeaA	Putative domain frequently associated with peptide methionine sulfoxide reductase	2479
rplN	50S ribosomal subunit protein L14	2465
nemA	N-ethylmaleimide reductase	2316
ymfC	Putative ribosomal large subunit pseudouridine synthase	2183
greA	Transcription elongation factor, cleaves 3' nucleotide of paused mRNA	2079
hemG	Protoporphyrin oxidase	1978
yieP	Putative regulatory protein, gntR family	1974
hflB	ATP-dependent zinc-metallo protease	1950
mobA	Putative molybdopterin-guanine dinucleotide biosynthesis protein	1940
yaeE	Putative ABC superfamily (membrane) transport protein	1813
murB	UDP-N-acetylenolpyruvoylglucosamine reductase	1682
recD	Exonuclease V, alpha chain	1548
purH	Bifunctional: phosphoribosylaminoimidazolecarboxamide formyltransferase; IMP cyclohydrolase	1394
envZ	Sensory histidine kinase in two-component regulatory system with OmpR	1368
arcA	Response regulator (OmpR family) in two-component regulatory system with ArcB (or CpxA), regulates genes in aerobic pathways	1326
glyS	Glycine tRNA synthetase, beta subunit	1286
SEN4243	N/A	1268
trxB	Thioredoxin reductase	1232
rimM	16S rRNA processing protein	1226
dedD	Paral putative lipoprotein	1204
STM2694	Fels-2 prophage: similar to late control gene B in phage	1186
yicH	Putative inner membrane protein	1164
STM0281	Putative cytoplasmic protein	1132
yheR	Putative NAD(P)H oxidoreductase	1125

Table 15. Genes detected with intensity >1000 in the RAW264.7/*Nramp1* sample only (continued)

Gene symbol	STM Function	Intensity
SPA2518	N/A	1106
ndh	Respiratory NADH dehydrogenase 2; cupric reductase	1089
ygfA	Putative ligase	1069
ybjY	Paral putative membrane protein	1058
STM1675	Putative short-chain alcohol dehydrogenase	1041
ydhD	Putative glutaredoxin protein	1035
ygcF	Putative Organic radical activating enzymes	1014
leuS	Leucine tRNA synthetase	1007

Table 16. Up-regulated genes in the presence of *Nramp1*[a]

Gene name	Gene function	Signal intensity		Fold change in the presence of *Nramp1*
		+Nramp1	*-Nramp1*	
mgtB	Magnesium transport protein	18966	1854	10.23
rpsD	30S ribosomal subunit protein S4	14792	3249	4.55
manZ	Sugar Specific PTS family, mannose-specific enzyme IID	6613	1877	3.52
menG	Putative methyltransferase in menaquinone biosynthesis protein	12300	4623	2.66
yidA	Putative hydrolase of the HAD superfamily	7406	3777	1.96
rplJ	50S ribosomal subunit protein L10	6414	3571	1.80
10Sa	Regulatory RNA	30518	17186	1.78
csrB	Regulatory RNA	56398	32918	1.71
tufA	Protein chain elongation factor EF-Tu (duplicate of tufB)	5603	3503	1.60
N/A	Putative carbonic anhydrase	3325	2204	1.51
rpoA	RNA polymerase, alpha subunit	4314	3315	1.30
N/A	Putative cytoplasmic protein	13828	10767	1.28
N/A	Putative integrase protein	5615	4437	1.27
rpoS	Sigma S (sigma 38) factor of RNA polymerase	8107	6497	1.25
SPA2929	6S RNA	63858	51953	1.23
dniR	Transcriptional regulator for nitrite reductase (cytochrome c552)	6981	5848	1.19
recA	DNA strand exchange and recombination protein with proteiase and nuclease activity	9427	8408	1.12
rnpB	Regulatory RNA	63024	59347	1.06
Pseudogene	Pseudogene; in-frame stop following codon 23	12255	11937	1.03
yaeL	Putative membrane-associated Zn-dependent protease	3752	3752	1.00

[a] Only genes expressed in both RAW264.7 and RAW264.7/*Nramp1* were analyzed.

Table 17. Down-regulated genes in the presence of *Nramp1* [a]

Gene name	Gene function	Signal intensity		Fold change in the presence of *Nramp1*
		+Nramp1	*-Nramp1*	
osmE	Transcriptional activator of ntrL gene	1031	5837	-5.66
rplP	50S ribosomal subunit protein L16	1699	8489	-5.00
yhdG	Putative TIM-barrel enzyme, possibly dehydrogenase	4588	20750	-4.52
bglJ	Transcriptional regulator (activator) of bgl operon (LuxR/UhpA family)	2330	9998	-4.29
treF	Cytoplasmic trehalase	1267	4651	-3.67
rplD	50S ribosomal subunit protein L4, regulates expression of S10 operon	1455	5121	-3.52
msbB	Myristoyl transferase in lipid A biosynthesis, suppressor of htrB (lpxL)	2863	5969	-2.09
N/A	Putative inner membrane protein	3563	6197	-1.74
pspC	Phage shock protein; regulatory gene, activates expression of psp operon with PspB	1223	2095	-1.71
nlpD	Lipoprotein	5579	8687	-1.56
serA	D-3-phosphoglycerate dehydrogenase	2026	2623	-1.29
N/A	Putative oxalacetate decarboxylase, subunit alpha	1665	2135	-1.28
gltI	ABC superfamily (bind_prot), glutamate/aspartate transporter	4069	4414	-1.08

[a] Only genes expressed in both RAW264.7 and RAW264.7/*Nramp1* were analyzed.

Table 18. COG classification of genes detected with intensity >1000 in the RAW264.7 sample

Category [a]	Genes
Translation	***csrB***[b], *ffs, infC, prfB,* ***rnpB****, rplB, rplC,* ***rplD****,* ***rplJ****, rplK,* ***rplP****, rplQ, rpmH,* ***rpsD****, rpsI, rpsK, rtT,* ***tufA****,* ***yhdG****, yhiZ*
Transcription	***bglJ****,* ***pspC****, rcsB,* ***rpoA****,* ***rpoS****, uxuR, ydcR*
Replication, recombination and repair	*holE,* ***recA****, sbcB*
Cell cycle control, mitosis, meiosis	*ftsQ*
Signal transduction mechanisms	***bglJ****,* ***gltI****,* ***pspC****, rcsB, suhB, typA, yrbB*
Cell wall, membrane biogenesis	***dniR****, envE, glmU, htrB, mepA, mipA, mopA, mraY,* ***msbB****, nanA,* ***nlpD****,* ***yaeL****, ybiR, yebS, yjeI, yjeQ, yrbC*
Cell motility	*ssaN*
Intracellular trafficking and secretion	*secG, secY, ssaN, yejF*
Posttranslational modification, protein turnover, chaperones	*gst, ibpB, yeaZ*
Energy production and conversion	*fldA, gldA*
Carbohydrate transport and metabolism	*galK, hpaR, malP,* ***manZ****, ptsG, suhB,* ***treF****, ydeV*
Amino acid transport and metabolism	*aroP,* ***gltI****, gsp, ilvC, nagB, nanA,* ***serA****, thrA, ycdW, ydcR*
Nucleotide transport and metabolism	*purG, pyrG*
Coenzyme transport and metabolism	*bioA, ilvC,* ***menG****,* ***serA****,* ***ubiE,*** *ubiG*
Inorganic ion transport and metabolism	***mgtB****, mgtC, nanA, ybiR*
Secondary metabolites biosynthesis, transport and catabolism	*yrbC*
Drug/analog sensitivity	*bcr*
Pathogenicity	*hilA, mgtC, sipB, sopB, ssaN, typA*
General function prediction only	*yafV, ybaW, ychK,* ***yidA****, yjgP, ynfB*
Function unknown	11 putative proteins, **6 putative proteins**, SEN1387, SEN4283, SEN4288, *smg*, SPA1121, **SPA2929**, *ybaP, ydiU, yjbQ, yohL*
Not in COG	***10Sa****, ccdB, irhA,****osmE****, repA2, ynfD, yqjD*

[a] Gene classification based on the COG database for *Salmonella* Typhimurium LT2 (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html) and on gene classification for *Salmonella* Typhi CT18 (http://www.sanger.ac.uk/Projects/S_typhi/St_gene_list_hierarchical.shtml).

[b] Genes in bold were detected in both samples.

Table 19. COG classification of genes detected with intensity >1000 in the RAW264.7/*Nramp1* sample

Category [a]	**Gene**
Translation	***csrB***[b], *glyS, leuS, rimM,* ***rnpB****,* ***rplD****, rplE,* ***rplJ****, rplN,* ***rplP****,* ***rpsD****, rpsJ,* ***tufA****,* ***yhdG****, yjgO, ymfC*
RNA processing and modification	*greA*
Transcription	***bglJ****, creB, phoP,* ***pspC****,* ***rpoA****,* ***rpoS****,* ***yieP****, yjaB*
Replication, recombination and repair	*dnaG,* ***recA****, recD, rlgA, yffH, ygdP*
Signal transduction mechanisms	*arcA,* ***bglJ****, creB, envZ,* ***gltI****, phoP,* ***pspC****,* ***yieP***
Cell wall, membrane biogenesis	***dniR****, mdoB,* ***msbB,*** *murB,* ***nlpD****, yaeC,* ***yaeL****, yicH, yjbY*
Posttranslational modification, protein turnover, chaperones	*hflB, lon, trxB, ydhD, yeaA, ygcF*
Energy production and conversion	*fdnG, hemG, ndh, nemA*
Carbohydrate transport and metabolism	*bglA,* ***manZ****,* ***treF****, ygeD*
Amino acid transport and metabolism	***gltI****, leuA,* ***serA****, ycjI, ygeD*
Nucleotide transport and metabolism	*purH*
Coenzyme transport and metabolism	*hemA, hemG, leuA,* ***menG****, mobA,* ***serA****,* ***ubiE****, ygdL, ygfA*
Lipid transport and metabolism	*pgpB, pslX*
Inorganic ion transport and metabolism	***mgtB****, trkH, yaeE, ygeD*
Pathogenicity	*msgA, sipA*
General function prediction only	*icc, ygdE, ygeD, yheR,* ***yidA***
Function unknown	**6 putative proteins**, 9 putative proteins, *dedD*, SEN4243, **SPA2929**, STY4667, *yafA*, *yceP*
Not in COG	***10Sa****,* ***osmE****, ycfR, yfaZ*

[a] Gene classification based on the COG database for *Salmonella* Typhimurium LT2 (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html) and on gene classification for *Salmonella* Typhi CT18 (http://www.sanger.ac.uk/Projects/S_typhi/St_gene_list_hierarchical.shtml).

[b] Genes in bold were detected in both samples.

Table 20. COG classification breakdown

	RAW264.7		RAW264.7/*Nramp1*		% in
Category [a]	# genes	%	# genes	%	genus
Translation	20	13.99	16	13.91	3.50
RNA processing and modification	0	0.00	1	0.87	0.02
Transcription	7	4.90	8	6.96	6.31
Replication, recombination and repair	3	2.10	6	5.22	4.05
Chromatin structure and dynamics	0	0.00	0	0.00	0.00
Cell cycle control, mitosis, meiosis	1	0.70	0	0.00	0.70
Nuclear structure	0	0.00	0	0.00	0.00
Defense mechanisms	0	0.00	0	0.00	0.93
Signal transduction mechanisms	7	4.90	8	6.96	3.31
Cell wall, membrane biogenesis	17	11.89	9	7.83	4.54
Cell motility	1	0.70	0	0.00	1.95
Cytoskeleton	0	0.00	0	0.00	0.00
Extracellular structures	0	0.00	0	0.00	0.02
Intracellular trafficking and secretion	4	2.80	0	0.00	2.23
Posttranslational modification, protein turnover, chaperones	3	2.10	6	5.22	3.11
Energy production and conversion	2	1.40	4	3.48	5.43
Carbohydrate transport and metabolism	8	5.59	4	3.48	7.06
Amino acid transport and metabolism	10	6.99	5	4.35	7.84
Nucleotide transport and metabolism	2	1.40	1	0.87	1.59
Coenzyme transport and metabolism	6	4.20	9	7.83	3.18
Lipid transport and metabolism	0	0.00	2	1.74	1.73
Inorganic ion transport and metabolism	4	2.80	4	3.48	4.81
Secondary metabolites biosynthesis, transport and catabolism	1	0.70	0	0.00	1.47
Drug/analog sensitivity	1	0.70	0	0.00	N/A
Pathogenicity	6	4.20	2	1.74	N/A
General function prediction only	6	4.20	5	4.35	9.84
Function unknown	28	19.58	21	18.26	6.43
Not in COGs	7	4.90	4	3.48	19.96
Total	143[b]	100	115[b]	100	100

[a] Gene classification based on the COG database for *Salmonella* Typhimurium LT2 (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html) and on gene classification for *Salmonella* Typhi CT18 (http://www.sanger.ac.uk/Projects/S_typhi/St_gene_list_hierarchical.shtml).

[b] Total different than that reported in text because some gene belong to 2 or more COGs.

DISCUSSION

The host response to bacterial infection is under complex regulation involving both genetic and environmental components. The host genetic architecture responsible for the development of a chronic carrier state following *Salmonella* Enteritidis infection was previously investigated in our laboratory. Several QTL were identified as discussed in section 4.3 of the introduction. In brief, *Ses1* and *Ses3* showed a significant effect alone and interactions of *Ses1* with *Ses4* and/or *Ses5* accounted for 72% of the total variation in females. In males, *Ses1.1* had an individual effect and three interactions (*Ses1*-*Ses6*, *Ses7*-*Ses8*, and *Ses9*-*Ses10*) also contributed to bacterial clearance.

The first aim of this project was to further investigate the role of *Ses1* and its candidate gene *Nramp1* in the context of chronic *Salmonella* infection. To validate a gene as underlying a QTL, different approaches can be taken to provide sufficient evidence in support of its candidacy (205). In our model of chronic *Salmonella* infection, *Nramp1* is an excellent candidate gene for *Ses1* because of its chromosomal location (5 kb from the maximum peak LOD score at *Ses1*) and because of the presence of a known functional polymorphism in the *Nramp1* gene between C57BL/6J and 129S6 mice. Furthermore, a wild-type allele at *Nramp1* is known to be associated with resistance to acute *Salmonella* infection in mice. Using 129S6-*Nramp1*$^{-/-}$ mice, the influence of *Nramp1* on the immune response to *Salmonella* Enteritidis infection was investigated and the presence of a null *Nramp1* allele was associated with more effective clearance of the bacteria (195). Quantitative complementation can provide further evidence that a gene underlies a QTL (197). This method tests the interaction between a QTL and the null allele of a candidate gene located within the QTL region. Interaction between the mutant gene and the homologous QTL allele would lead to complementation failure as seen by the phenotypic output (197). This method was developed in *Drosophila* and identified several QTL genes involved in bristle number (197). Recently, this method was successfully applied to mammals. Yalcin *et al* identified a 1 cM region of mouse chromosome 1 linked to susceptibility to depression and anxiety in mice which contained only 2 genes (*Rgs2* and *Rgs13*) (206). *Rgs2* was known to be involved in anxiety and aggression in mice and quantitative complementation confirmed it as the gene underlying the QTL of interest (206).

We repeated the quantitative complementation test 4 times and consistent results were obtained each time. The data was analyzed separately in males and females. Our data showed a trend towards lower CFU counts in the absence of a functional *Nramp1*, as expected. However, quantitative complementation could not be observed in support of the candidacy of *Nramp1* as the gene for *Ses1* since the interaction coefficients calculated were not significant. This may be due to a number of factors such as the magnitude of the impact of *Ses1* on the phenotype or the influence of other genetic variants located within the locus.

Ses1 alone explains 11% and 20% of the phenotypic variance in our male and female models of bacterial clearance, respectively. Combined with the size of the interval and the number of genes present within *Ses1*, this impact may not be sufficient to observe quantitative complementation. In our set of experiments, the impact of *Ses1* was detectable only in females and was less pronounced than in original experiments (118). This phenomenon can be observed during the breeding of congenic mice. The elimination through homologous recombination of small genomic regions bordering the interval may influence the phenotype. Better definition of the boundaries of the *Ses1* region transferred may explain and help prevent reduction in the expression of the phenotype.

Genetic variants located in the *Ses1* congenic interval may also influence the expression of the phenotype, individually or through their interaction with *Nramp1*. This hypothesis is supported by the observation that *Nramp1* knockout mice on a pure 129S6 background present lower bacterial counts compared to 129S6 mice. The *Ses1* interval, which was transferred from the C57BL/6J donor strain to the 129S6 recipient background, contains 187 genes of C57BL/6J origin. This list can be narrowed down to 50 immunological relevant genes using the knowledge-based ranking tool PosMed^{SM}. Genes of particular interest in this region are *Ccl20* (chemokine (C-C motif) ligand 20), *Inpp5d* (inositol polyphosphate-5-phosphatase D), *Il8ra* (interleukin 8 receptor, alpha), and *Il8rb* (interleukin 8 receptor, beta). In BALB/C mice, *Ccl20* expression is strongly induced following oral and intraperitoneal infection with *Salmonella* Enteritidis (207). Pretreatment with anti-Ccl20 antibodies induces bacterial dissemination to the spleen and increases the production of IL-4, leading to an IFNγ/IL-4 ratio skewed towards a Th2 response. Phosphatases are essential for signal transduction within and between cells and

have been shown to influence phagocyte function during *Leishmania* infection (208, 209). More specifically, the presence of a functional *Nramp1* gene inhibits protein tyrosine phosphatases activity, thus promoting proinflammatory functions of macrophages (210). *Inpp5d*, also called *Ship* (Src homology 2 domain-containing inositol 5-phosphatase), was shown to control *Salmonella* infection *in vivo* (211). Increased susceptibility of $Ship^{-/-}$ mice to *Salmonella* Typhimurium is proposed to be due to a bias towards macrophages displaying an alternatively activated (M2) phenotype, associated with inefficient clearance of Gram-negative bacteria (211). The IL-8 receptor ligand in mice is the keratinocyte-derived cytokine (KC). An 84–fold increase in KC mRNA levels is observed in the cecal mucosa of streptomycin-pretreated mice infected with *Salmonella* Typhimurium (212). Furthermore, *Nramp1* has been shown to regulate the expression of KC during mycobacterial infection (213). A role for any of these three genes in our model may be envisioned and will be further investigated. Single nucleotide polymorphisms (SNP) between C57BL/6J and 129S6 have been identified in the coding regions of *Inpp5d* and *Il8ra*. More specifically, a synonymous SNP in exon 19 and a SNP in the 3' untranslated region of *Inpp5d* have been identified. A point mutation leading to a threonine to isoleucine change is present in exon 2 of *Il8ra*. Some SNP are present in the intronic regions of *Il8rb*; and no known SNP are present in *Ccl20*. These polymorphisms could influence splicing, transcription, or translation of these genes and lead to a change in gene or protein expression. Expression levels in the spleens of infected animals would provide further insight into their role in the late phase of *Salmonella* Enteritidis infection. Additionally, the impact due to genetic variants could be eliminated by further fine mapping of the *Ses1* region to obtain an interval with a minimal number of genes.

Quantitative complementation failure would have strengthened our hypothesis that *Nramp1* underlies *Ses1*. However, negative results cannot rule out *Nramp1* as a candidate gene since *Nramp1* knockout mice on a pure 129S6 background have been shown to have significantly lower bacterial loads 42 days post-infection in comparison to their wild-type 129S6 counterparts.

The complexity of host response to *Salmonella* Enteritidis infection is highlighted by the fact that interactions account for 66% and 72% of the variation explained by the male and female models of bacterial clearance, respectively. In our female model of

bacterial clearance the interactions are between *Ses1.2* and *Ses4*, and between *Ses1.2* and *Ses5*. The second aim of this project was to functionally validate the inheritance model of *Salmonella* clearance in females by creating new congenic mouse strains. Single and double congenic strains are being developed by introgressing C57BL/6J chromosomal segments onto the 129S6 recipient background. Ten generations are necessary to obtain a congenic mouse with approximately 99.8% of its genome from the recipient strain. In the coming weeks, selected offspring from each new strain will be backcrossed to 129S6 mice. It will take approximately 9 months before all strains reach generation N10 or N5F1N5. Assessment of the model can then be done by infecting mice homozygous for the locus or loci of interest and determining the splenic bacterial loads 42 days post-inoculation.

Of the three single congenic strains being developed, only the 129.B6-*Ses1.2* strain is expected to influence bacterial clearance because linkage analysis detected its individual effect on the phenotype. Furthermore, the 129.B6-*Ses1* strain was previously shown to have significantly lower bacterial loads in the spleen 42 days following *Salmonella* Enteritidis infection compared to 129S6 mice (118). The 129.B6-*Ses1.2* strain is expected to behave in a similar fashion. The *Ses4* and *Ses5* loci were detected through two-locus epistasis QTL linkage mapping for their interaction with *Ses1* and therefore the single congenic strains carrying these loci individually are not expected to influence bacterial burden. However, the 129.B6-*Ses1.2/Ses4* and the 129.B6-*Ses1.2/Ses5* double congenic strains are expected to clear a *Salmonella* Enteritidis infection significantly more efficiently than 129S6 mice. Forty-two days post-inoculation, these strains are also expected to have lower splenic bacterial loads compared to 129S6-*Ses1.2* mice. This may be accounted for by the interactive effect between the 2 loci which will be observed in addition to the effect of *Ses1.2*. Moreover, assessment of subphenotypes such as cellular distribution in the spleen, cytokine expression, and antibody response in all new strains being created would help us elucidate the way the locus or loci of interest influence our model. Although beyond the scope of this thesis, confirming the interactions between *Ses1* and *Ses4* and/or *Ses1* and *Ses5* is a critical step towards the identification of new genes involved in bacterial clearance.

The *Ses4* and *Ses5* intervals cover regions of 55.0 and 27.7 Mb, respectively. If their statistical interaction with *Ses1* is confirmed through functional testing, these regions can be fine mapped to reduce the interval size. One of the most powerful tools to do so is the creation of subcongenic mice strains which carry a shorter chromosomal segment than their congenic parent (214). Ideally, the region is narrowed down to contain only a few candidate genes. However, strong candidate genes can already be identified based on a known or potential role in response to *Salmonella* infection such as *Elf4* (ets domain transcription factor 4), *Il13ra1* (interleukin 13 receptor alpha 1), and *Lamp2* (lysosomal membrane glycoprotein 2) within the *Ses4* interval. Candidate genes in *Ses5* are *Nfkbib* (nuclear factor of kappa light chain gene enhancer in B cells inhibitor beta), *Cd22*, *Rog* (repressor of Gata), and *Hamp* (hepcidin) (205).

The candidacy of hepcidin is supported by its key role in the regulation of iron homeostasis (215). Iron is an essential cation for both the host and the pathogen as it can modulate the innate and adaptive immune response of the host, and it acts as an essential cofactor for bacterial growth (216). Recent work in our laboratory has shown the critical role played by iron during *Salmonella* infection. AcB61 mice carrying a functional mutation within *Pklr* (pyruvate kinase) present a constitutive hemolytic anemia with reticulocytosis and tissue iron overload (217, 218). Consequently, they are extremely susceptible to *Salmonella* Typhimurium infection. Hepcidin influences iron homeostasis by binding to ferroportin, a macrophage iron efflux protein, leading to internalization and degradation of the hepcidin-ferroportin complex (219). Research efforts in our laboratory have concentrated on the development of C57BL/6J and 129S6 mice with a targeted disruption in the *Hamp* gene. Susceptibility to *Salmonella* infection was investigated and 7 days following intravenous infection with 10^3 CFU of *Salmonella* Enteritidis, C57BL/6J-*Hamp*$^{-/-}$ mice presented with significantly higher bacterial loads in their spleens and liver compared to wild-type mice; no difference was observed with *Salmonella* Typhimurium (data not published).

Both the quantitative complementation test and the development of congenic strains provided us with greater understanding of host response to chronic *Salmonella* Enteritidis infection. As the outcome of infection is dependent on host-pathogen interactions, the third aim of this project was to investigate the effect of *Nramp1* on the

bacterial transcriptome. The impact of *Nramp1* on the host transcriptome was previously examined *in vivo* and was associated with Th cell polarization (195). Mice expressing a non-functional *Nramp1* presented a lower bacterial burden, with an associated Th2 response (195). However, the exact mechanism of how *Nramp1* influences bacterial persistence is not known. We hypothesized that *Nramp1* promotes chronic carriage through reprogramming of the bacterial transcriptome that favors *Salmonella* persistence.

In vivo, the effect of *Nramp1* was detected as early as 5 days post-infection. To look at the transcriptional profile of *Salmonella* as the chronic carrier phase was being established, we decided to use samples obtained 3 days post-infection. The challenges faced with trying to detect bacterial housekeeping genes in infected mouse spleens could be due to the small bacteria:host mRNA ratio in infected samples. Furthermore, bacterial mRNA is short-lived, unstable, and only accounts for approximately 4% of total bacterial RNA (220). Using *in vitro* and *ex vivo* models, the effect of *Nramp1* on *Salmonella* Enteritidis replication could be observed 2 hours following infection. However, in RAW264.7/*Nramp1* macrophages and 129S6 BMDM, the presence of a functional *Nramp1* was associated with better clearance, similarly to what is observed during *Salmonella* Typhimurium infection (199). These results highlight the complex nature of the host response to infection. Macrophages alone may not be sufficient to exhibit a clearance phenotype in the absence of a functional *Nramp1*. Previous findings indicating that the influence of *Nramp1* on the outcome of infection is associated with Th polarization support this idea (195).

We then sought to analyze the effect of *Nramp1* on the bacterial transcriptome. Using SCOTS, we extracted and enriched total RNA for bacterial mRNA from infected RAW264.7 and RAW264.7/*Nramp1* cells. Southern blot hybridization confirmed the increasing complexity of our samples with consecutive rounds of capture. SCOTS-derived products were then analyzed by slot blot hybridization. We investigated the expression of genes with a known contribution to the establishment of chronic infection and genes whose expression is known to be influenced by *Nramp1*. Our results did not confirm previous findings as no significant expression changes were observed. This may reflect important differences between the models used. For example, the contribution to long-term *Salmonella* infection of several genes tested was identified through a negative

selection screen using *Salmonella* Typhimurium (94). Furthermore, the effect of *Nramp1* on *serA*, *ihfA*, *upp*, and *sodCI* was detected at the protein level and may be due to post-translational events, thus not reflecting gene expression levels.

To identify new genes required for persistence and gain new insight into the global transcriptional profile of *Salmonella* Enteritidis we decided to apply our SCOTS-derived samples to *Salmonella* ORF microarrays. Initially, samples obtained 2 hours post-invasion were analyzed. A total of 866 genes were detected in the RAW264.7 and the RAW264.7/*Nramp1* samples. A previous study, in which SCOTS samples were derived from THP-1 human macrophages infected with *Salmonella* Typhi, detected 3942 genes after three rounds of capture. This discrepancy in the number of genes detected suggests that, in our experiment, a limited pool of cDNA transcripts was captured during the first round and was further amplified with each round of SCOTS. Only 4 genes tested by slot blot hybridization were detected in our microarray analysis: *phoP* and *mgtC* were detected in the RAW264.7 sample, *sipA* was detected in the RAW264.7/*Nramp1* sample, and only *serA* was detected in both samples. There are 2 possible explanations for these conflicting results. First, although the same SCOTS products were used in both analyses, the hybridization probes were prepared differently. For the slot blot analysis, the probes were prepared by PCR amplification while the probes used for microarrays analysis were prepared by second strand cDNA synthesis. Including an amplification step during the labeling of the probes may have allowed low abundancy transcripts to reach the detection threshold in our slot blot analysis. A defect in the microarrays, such as degradation of spotted sequences, may also explain our results. More specifically, by microarray analysis, we only detected *phoP* in the presence of a functional *Nramp1*. However, by slot blot hybridization, we were able to detect *phoP* in both samples. This may also be due to the amplification step involved in the preparation of the slot blot hybridization probes. Furthermore, we did not detect a significant change in the expression of *phoP* between the two samples. The method used for preparation of the slot blot hybridization probes may have reduced the expression difference to levels that are below the sensitivity of our analysis. Nonetheless, preliminary data analysis has revealed that *Nramp1* does influence the bacterial transcriptome.

Expression fold change data indicated that *mgtB* expression was the most influenced by the genotype at *Nramp1*. The expression of *mgtB* was 10-fold greater in RAW264.7/*Nramp1* macrophages compared to RAW264.7 macrophages. As no significant fold change was detected for *mgtA* or other known PhoP-activated genes, our results suggest that *mgtB* may be under the control of a different transcriptional regulator responsive to divalent cations. Our results also illustrate the broad range of the activity of PhoP: numerous PhoP-activated genes were detected along with *phoP*, while PhoP-repressed genes were only detected in the absence of *phoP*. The PhoPQ two-component regulatory system, common to many Gram-negative bacteria, regulates bacterial gene expression in response to environmental signals. In *Salmonella*, the PhoPQ sensor system is composed of the PhoQ histidine kinase and the PhoP cytosolic response regulator protein (221). In response to environmental stimuli, PhoQ autophosphorylates, leading to phosphorylation of PhoP into its active state (222). PhoP can then regulate the expression of over 200 genes involved in various aspects of virulence such as invasion, motility, acid tolerance, small molecules transport, AMP resistance, and bacterial membrane shuffling (84). *In vitro*, low concentrations of divalent metal ions (especially magnesium) activate PhoQ. However, *in vivo*, phagosomal cations concentrations do not decrease sufficiently to activate PhoQ (223). Different *in vivo* studies show that PhoPQ responds to acidic pH and AMP in the phagosome (224, 225). Using a *phoP*::GFP expression system, expression of *phoP* was shown not to be influenced by *Nramp1*; in agreement with findings that the phagosomal pH is similar in both RAW264.7 and RAW264.7/*Nramp1* macrophages following *Salmonella* Typhimurium infection (184, 223). Experimental limitations prevent phagosomal pH determination later than 1 hour post-invasion. Currently, no information is available about the phagosomal pH 2 hours post-invasion, when our samples were collected. Finally, the response to a concentration change for a single cation is complex and pleiotrophic (226). Although magnesium is not a substrate of *Nramp1*, these results emphasize the importance of divalent cations in determining the outcome of host-pathogen interactions.

Previous studies that have investigated the bacterial transcriptome during macrophage infection, or the establishment of a persistent state, identified various aspects of *Salmonella* Typhimurium physiology as being important (83, 94, 194). We identified

similar COG, with 2 of special interest. Numerous genes were detected in the cell wall/membrane biogenesis category. Genes detected in the RAW264.7/*Nramp1* sample were involved in membrane biogenesis, which is important for cell growth. For example, *murB* (a UDP-N-acetylenolpyruvoylglucosamine reductase) was only detected in the presence of *Nramp1*. This enzyme has been shown to be essential for *Escherichia coli* growth (227). In the RAW264.7 sample, 12% of detected genes belonged to the cell wall/membrane biogenesis category. Most genes were involved in cell wall modulations, which can influence bacterial virulence. We identified 3 enzymes involved in murein lipoprotein biosynthesis: *mepA* (a murein endopeptidase), *mipA* (a scaffolding protein for murein-synthesizing holoenzyme), and *mraY* (an essential enzyme for the formation of the murein precursor lipid). Murein lipoprotein is a critical component of the cell wall and it is required for full virulence of *Salmonella* Typhimurium in mice (228). However, the most active component of *Salmonella*'s OM is the lipid A moiety of LPS (29). We detected 3 enzymes which belong to the LPS biosynthesis pathway in the absence of *Nramp1*. One of these genes, *glmU*, encodes an enzyme required for the biosynthesis of UDP-N-acetylglucosamine, a metabolite which leads to the production of LPS and peptidoglycan (229). Furthermore, we detected *htrB* (a lauroyl transferase of lipid A biosynthesis) uniquely in the RAW264.7 sample. Consequently, in the absence of *Nramp1* we observed a 2-fold increase in the expression of *msbB*, which encodes an enzyme required for the attachment of myristic acid to the lipid A component of LPS, thus increasing its toxicity (230). The enzymatic activity of *msbB* is optimal following laurate incorporation by *htrB*, leading to increased stimulation of host cells (230, 231). These results suggest that, in the absence of a functional *Nramp1*, there is an up-regulation in virulence factors present at the OM, while the presence of *Nramp1* prevents such cell wall modulations. Previously, a study identified several genes involved in LPS biosynthesis of *Salmonella* Typhimurium that were required for persistence, although none were in common with the genes we detected (94). Overall, these results highlight the importance of cell wall remodeling during macrophage infection. More specifically, the absence of a functional *Nramp1* allele is associated with increased modulations of the OM. Our data also showed an increased number of pathogenicity genes expressed in the absence of *Nramp1*, among which were 3 SPI-1 genes. In the absence of *Nramp1*, we

detected *sipB*, *sopB*, and *hilA*, which have all been shown to be required for long-term infection (94). On the other hand, *sipA* was the SPI-1 gene detected in the presence of *Nramp1*. A role for *sipA* in persistence was previously ruled out by CI experiments (94). These results demonstrate the importance of SPI-1 genes for bacterial survival during macrophage infection. In recent years, mounting evidence has demonstrated that SPI-1 is not exclusively involved in cell invasion, but is also required for the intracellular phase of infection (66, 232). Furthermore, SPI-1 may be required for the sustained invasion of new host cells necessary for persistence. Genes unrelated to SPI-1 were also identified. In the RAW264.7 sample, *ssaN*, *mgtC*, and *typA* were detected. Of interest, *typA* is a regulator known to up-regulate the expression of genes involved in virulence and in stress response (233). In the presence of a functional *Nramp1*, only *msgA*, a gene required for bacterial virulence and survival within macrophages, was detected in addition to *sipA* (234). Altogether, these results suggest that differential pathogenicity mechanisms are promoted in the presence or absence of a functional *Nramp1*. Finally, 20% of detected genes have no known function. Genes unique to *Salmonella* Enteritidis should be further investigated as they may represent important differences between *Salmonella* Enteritidis and Typhimurium. Although both serovars behave similarly *in vitro* with respect to the LOG CFU/well observed following infection, *Salmonella* Enteritidis-specific genes may be involved during *in vivo* infection.

To the best of our knowledge, no other studies have compared the effect of *Nramp1* alone on the transcriptome of *Salmonella*. Although our *in vivo* model is not reproduced *in vitro*, our results have confirmed those obtained from a genome-wide screen that identified bacterial genes essential for long-term infection. These results will be the foundation for future research endeavors. In particular, the influence of *Nramp1* on the transcriptome of *Salmonella* Enteritidis recovered from the spleens of infected mice will be investigated. Comparing these results to those obtained using *Salmonella* Typhimurium *in vivo* will provide great insight into *Salmonella* serovars-specific host-pathogen interactions.

The work presented in this thesis demonstrates the crucial role played by *Nramp1* during chronic *Salmonella* Enteritidis infection. Although we were unable to confirm the

candidacy of *Nramp1* as the gene for *Ses1*, our results emphasize the complexity of host response to infection. Furthermore, in our female model of bacterial clearance, interactions of *Ses1* with *Ses4* and *Ses5* account for 72% of the variance explained and we hope to confirm these interactions by creating new congenic mouse strains. Finally, we investigated the impact of *Nramp1* on the transcriptome of *Salmonella* using a combined approach of SCOTS and microarrays. Various aspects of *Salmonella* physiology were shown to be involved during macrophage infection. Notably, we observed the activation of differential virulence mechanisms under the regulation of PhoP in the presence or absence of a functional *Nramp1*. PhoP-activated genes were predominant in the presence of a functional *Nramp1* while PhoP-repressed genes were detected in the absence of a functional *Nramp1*, confirming the importance of host-pathogen interactions in determining the outcome of infection.

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