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Differential Screening in Immunodeficient Mice Reveals Bacterial Enzymes With Unexpected Roles in Host-Pathogen Interactions

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Differential screening in immunodeficient mice reveals bacterial enzymes with unexpected roles in host-pathogen interactions

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For my mother,
who could have been the first "Dr. Hisert",
but chose to be "Mom" instead.

and

For my father,
whose diligence provided me with opportunities
that he was not so fortunate to have had.

"To be a scientist – it is not just a different job, so that a man should choose between being a scientist and being an explorer or a bond-salesman or a physician or a king or a farmer. It is a tangle of very obscure emotions, like mysticism, or wanting to write poetry; it makes its victim all different from the good normal man. The normal man, he does not care much what he does except that he should eat and sleep and make love. But the scientist is intensely religious – he is so religious that he will not accept quarter-truths, because they are an insult to his faith."

- Max Gottlieb (in Arrowsmith, by Sinclair Lewis)

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Abbreviations

ABC transporter	ATP binding cassette transporter
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cells
ATP	Adenosine triphosphate
BCG	Bacillus Calmette Guerin
C'R	Complement receptor
Casp	Caspase
c-diGMP	Cyclic diguanylate
cfu	Colony forming units
CGD	Chronic granulomatous disease
C.I.	Competitive index
DC	Dendritic cell
DGC	Diguanylate cyclase
DNS	Data not shown
dsRNA	double stranded RNA
EEA1	Early endosomal antigen 1
FAD	Flavin adenine dinucleotide
FcR	Fc receptor
FFA	Free fatty acid
FMN	Flavin mononucleotide
GAP	GTPase-activating protein
GEF	Guanine-nucleotide exchange factor
GPI	glycosylphosphatidylinositol

GTP	Guanosine triphosphate
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ICE	Interleukin-1β converting enzyme
IDO	Indolamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase (see NOS2)
i.p.	Intraperitoneal
i.v.	Intravenous
KRPG	Krebs ringers with phosphate and glucose
LAM	Lipoarabinomannin
LAP	Lysosomal acid phosphatase
LB	Luria-Bertani
LBP	LPS binding protein
LDH	Lactate dehydrogenase
lgp	Lysosomal glycoproteins
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Man-LAM	Mannose-capped lipoarabinomannin
MAPK	Mitogen activator protein kinase
MBL	Mannose binding lectin
MDR	Multi-drug resistant
MHC	Major histocompatibility complex
MMR	Macrophage mannose receptor

MOI	Multiplicity of infection (bacteria:host cell)
MPO	Myeloperoxidase
MSD	Membrane spanning domain
Mtb	*Mycobacterium tuberculosis*
MyD88	Myeloid differentation antigen 88
NAD(P)H	Nicotinamide adenine dinucleotide (phospate), reduced form
NBD	Nucleotide binding domain
NK cells	Natural killer cells
NF-κB	Nuclear Factor-κB
NOS2	Nitric oxide synthase, isoform 2
Nramp	natural-resistance associated macrophage protein
ORF	Open reading frame
PAMP	Pathogen-associated molecular patterns
PDE	Phosphodiesterase
PDIM	Phthiocerol dimycocerosate
PGN	Peptidoglycan
Phox	Phagocyte oxidase
PLA_2	Phospholipase A2
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear cells
PPD	Purified protein derivative
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
SCV	*Salmonella*-containing vacuole
sifs	*Salmonella* induced filaments

SOD	Superoxide dismutase
SPI	*Salmonella* pathogenicity island
STM	Signature-tagged transposon mutagenesis
TCR	T cell receptor
TGF	Transforming growth factor
TGN	Trans Golgi network
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TTSS	Type three secretion system

Abstract

Over centuries of co-existence with their hosts, microbes that are exclusively vertebrate pathogens have evolved mechanisms for manipulation of host cells and evasion of the immune system. Some pathogens dodge the immune response by altering the functions of and hiding within the very cells that should be consuming and digesting them: macrophages. The aim of these studies was to explore the dynamic interactions of the host with two microbes that opt to live within the macrophage phagosome: *Mycobacterium tuberculosis* (Mtb) and *Salmonella typhimurium.* In order to identify bacterial genes that provide protection against specific host immune pathways, we have developed the strategy of "differential" signature-tagged transposon mutagenesis (STM). By evaluating bacterial mutants for differential virulence in strains of immuno-deficient mice, we identified five enzymes required for *in vivo* survival in the face of specific immune stresses: four Mtb enzymes essential for growth and rapid lethality in NOS2-deficient mice but not in IFN-γ-deficient mice, and one *S. typhimurium* enzyme that participates in resistance to phagocyte oxidase. The Mtb enzymes suggest novel pathways for countering IFN-γ-dependent immune mechanisms other than NOS2. Analysis of the sequence of the *S. typhimurium* enzyme suggests the existence in prokaryotes of an alternative signaling pathway that complements classical phospho-relay 2-component regulatory systems.

Preface

Although this doctoral thesis is intended for approval by a committee of prominent researchers in the fields of immunology and microbiology, I feel strongly that the information included herein should be accessible to non-scientist and scientist alike. Not only is it important for me share these accomplishments with my family and friends, but, additionally, I subscribe to the belief that science is too often discussed only by the few and not disseminated to the population at large. Especially as technology has advanced, science has become unapproachable to the layperson. This preface serves as my first attempt as a scientist to make one small but complex piece of science comprehensible, and hopefully interesting as well, to a wider audience.

The vertebrate immune system, an introduction

The cells of the vertebrate immune system, also known as white blood cells, can be divided into two subsets: innate and adaptive (Janeway and Medzhitov, 2002). The innate immune system is evolutionarily the original mechanism of host defense, and all organisms that can be said to possess immune systems contain variations of the innate immune system. The adaptive immune system is more recently evolved, and is unique to vertebrates. The two arms of the vertebrate immune system work together, complementing each other's function.

Some basic distinctions can be made between innate and adaptive immunity. The receptors of the innate immune system that recognize pathogens are fixed in the genome, and therefore they are present on all cells of one class. These receptors recognize molecular patterns that are present in many pathogens, or PAMPs (pathogen-associated

molecular patterns). Over evolutionary time, these PAMP receptors have been selected for recognition of foreign molecules, so that cells of the innate immune system are experts at distinguishing between self and non-self. PAMP receptor-bearing cells include macrophages, neutrophils, eosinophils, mast cells, dendritic cells (DCs), and natural killer (NK) cells. Triggering of these cells through PAMP receptors results in rapid anti-microbial responses, including production of toxic micromolecules and anti-microbial peptides, secretion of cytokines and chemokines, and upregulation of co-stimulatory molecules on their surfaces.

Cells of the adaptive immune system, which include B cells and T cells, rearrange segments of their genomic DNA to create sequences encoding unique receptors. Thus, no two naïve B cells or T cells share receptor specificity, and clonal expansion must occur to generate an army of cells with the same receptor. Because the process of constructing B and T cell receptors involves the generation of almost infinite diversity, receptors are inevitably produced that recognize self-molecules, and cells bearing these receptors are deleted. Detection is not perfect, however, and some cells B and T cells with auto-reactive receptors escape the deletion process.

B and T cells generally require activation by the innate immune system, and this interaction provides a second tier of protection against auto-reactive immune cells. Activation results through innate cell production of cytokines and chemokines, but also through the action of antigen presenting cells (APCs) that include macrophages and DCs. The T cell receptor (TCR) does not recognize intact proteins, but rather digested protein epitopes displayed as peptides in the context of major histocompatibility (MHC) molecules found on APCs. APCs constantly sample the extracellular milieu by

phagocytosing and digesting particles that are then presented in MHC class II molecules. Intracellular proteins are also broken down and presented to T cells, but in the context of class I MHC molecules. T cells come in two different flavors corresponding to the two classes on MHC molecules. TCRs on $CD4^+$ T cells recognize peptides presented in MHC class II molecules exclusively, whereas $CD8^+$ T cells respond only to peptides in the context of MHC class I molecules. Unlike the TCR, the B cell receptor, also known as immunoglobulin (Ig), recognizes unprocessed antigens in blood and tissues, but B cells generally also require co-stimulation by T cells to become activated. However, even this safeguard of requiring two cells to agree that a protein is foreign is not always sufficient, and thus one consequence of having an adaptive immune system is the possibility of developing auto-immune disease.

Once activated, B and T cells undergo clonal expansion, generating effector cells and long-lived memory cells. Memory, or the ability to mount a rapid and specific immune response to antigens previously encountered by the immune system, is one of the main hallmarks and benefits of adaptive immunity. The other substantial advantage of adaptive immunity is the ability to amplify anti-microbial responses without indiscriminately destroying host tissues. As the innate immune response gets more intense, non-specific tissue damage occurs. Because B and T cells focus their cytotoxic mechanisms against distinct epitopes, clonal expansion results in targeted killing. Activated B cells secrete Ig molecules that bind directly to foreign particles and target the particles for destruction by the innate immune system. Activation of $CD4^+$ T, or helper T cells, results in upregulation of costimulatory molecules and secretion of cytokines. $CD4^+$ T cells can mature into one of two classes of helper cells: Th1 cells that secrete the

cytokine interferon-γ (IFN-γ) and promote immune responses to intracellular pathogens (cellular immunity), or Th2 cells that secrete the cytokine interleukin-4 (IL-4) and promote B cell development and other immune responses to extra-cellular pathogens (humoral immunity). Th1 cells potentiate the cytotoxic mechanisms of innate cells as well those of $CD8^+$ T cells, also referred to as killer T cells or cytotoxic T cells (CTLs). Once activated, $CD8^+$ T cells lyse any cell bearing an MHC-peptide combination recognized by the T cell's TCR. Unlike MHC class II molecules, which are only found on APCs, class I molecules are present on virtually every cell in the body; thus, $CD8^+$ T cells are responsible for destruction of infected cells. Like $CD4^+$ T cells, $CD8^+$ T cells also produce cytokines that can feedback on the innate immune system.

Being the primordial, and in many species the only, immune system, the innate immune system not only triggers the adaptive response, but also possesses its own arsenal of anti-microbial mechanisms. Some phagocytic cells, including neutrophils and macrophages, are bona fide killing machines, which bombard phagocytosed microbes with an array of toxic substances. NK cells act as the innate version of CD8+ T cells: they kill aberrant host cells, although, unlike T cells, NK cells destroy cells that lack MHC molecules. Yet PAMP receptor-bearing cells are only part of the story. The sentinel activity as well as the microbicidal activity of the innate immune system extends beyond white blood cells. Other cells, including skin cells and the epithelial cells lining the lungs and gut, play key roles in defense against invading microbes, both by acting as barriers and also by secreting inflammatory mediators. In addition, the liver produces a number of acute-phase proteins during early infection that serve to opsonize microbes (bind microbes and label them for destruction). Acute phase proteins are effectively

soluble PAMP recognition molecules, and they include mannan-binding lectin (MBL), C-reactive protein (CRP) and serum amyloid protein (SAP). Another set of proteins, the complement system, consists of at least 20 serum proteins that interact in a highly regulated enzymatic cascade and participate in both recognition and destruction of many pathogens. Complement components can bind bacteria directly or indirectly via Ig molecules bound to bacterial surface antigens. Once complement components are bound, a proteolytic cascade is activated, resulting in the insertion of pores in the bacterial membrane followed by lysis of the cell. Complement also serves to facilitate ingestion of microbes by phagocytes. Phagocytes express complement receptors (C'R) and receptors (FcR) that bind the non-variable regions of Ig molecules (the Fc region). Thus, the more a microbe is decorated with complement and Ig molecules, the more likely that phagocytes will ingest it. The interplay between innate and adaptive immunity truly works in both directions: innate cells act to activate B and T cells, and Ig molecules and T cell cytokines function to improve the killing mechanisms of the innate immune system.

Classification of microbes

At the most basic level microbes, or microorganisms, can be categorized as either prokaryotes or eukaryotes. Prokaryotes are bacteria: single cells containing protein, DNA and RNA within one membrane. Bacteria sometimes contain a rigid cell wall outside the plasma membrane, but bacteria never contain membrane bound organelles. Bacteria can be subdivided into eubacteria and archea; however, because archea live in extreme environments and do not infect animals, this discussion will pertain to eubacteria only. All other cellular forms of life are eukaryotic, whether single-celled like protozoa,

or multi-cellular like plants and animals. (Viruses, which will not be discussed, are considered by some to be forms of life, but they are not cells; rather, they invade cells and hijack their replicative machinery. Thus, because they cannot survive independently of cells, many people do not consider them true "living" things.) Eukaryotic cells contain membrane bound organelles that segregate cellular components: the nucleus contains DNA; mitochondria contain the enzymes required for producing stockpiles of ATP, the molecule cells use to store energy; the endoplasmic reticulum and the Golgi apparatus prepare and modify proteins that are targeted for secretion or export to the plasma membrane. Organelles allow the cell to regulate events more rapidly and with finer precision, as well as to perform more complex functions. Additionally, eukaryotic cells possess a cytoskeleton composed of microtubules and actin filaments that permit the cell to change its shape.

Bacteria can be further subdivided based on their shape and on the structure of their outer cell layers. Spherical bacteria are referred to as "cocci", and rod-shaped bacteria are described as "bacilli", although some bacteria have complicated structures that defy either category. Additionally, most bacteria can be classified as either Gram positive or Gram negative depending on whether they retain or exclude Gram's Stain (the dye crystal violet). Gram positive cells have an inner membrane surrounded by a thick wall of peptidoglycan (PGN), which does not allow crystal violet to diffuse out once it has penetrated inside the cell. Gram negative cells have both an inner and an outer membrane, which are separated by the periplasmic space (Denyer and Maillard, 2002). The PGN layer that lies external to the inner membrane is much thinner than the PGN found in Gram positive bacteria, and thus Gram negative bacteria are unable to retain

crystal violet. Another key distinction between these two classes of bacteria is that the external leaflet of the outer membrane of Gram negative bacteria is composed of lipopolysaccharides (LPS). As discussed previously, LPS is highly immunostimulatory and can initiate endotoxic shock. Gram positive bacteria do not make LPS; however, they do make lipoteichoic acid and other immunostimulatory molecules.

Some bacteria have envelopes or capsules that make them resistant to the stains used to determine Gram designations. For example, the very waxy outer layer of mycobacterial species interferes with crystal violet staining, and thus a special dye, Carbol fuschin is used instead. Most bacteria will stain with Carbol fuschin; however, only mycobacteria retain this dye well enough to resist destaining with acidic alcohol. Thus, mycobacteria are often referred to as "acid-fast bacilli."

Chapter 1

Introduction

Microbial pathogenesis combines the fields of immunology, microbiology, and cell biology in order to understand the complex molecular interactions between host cells, microbes and microbial products (Cossart *et al.*, 1996; Finlay and Falkow, 1997). The bacteria *Mycobacterium tuberculosis* and *Salmonella typhimurium* preferentially reside *in vivo* within the macrophage, a host cell that normally destroys invading microbes. Comparison of these two bacteria reveals that evolution has generated multiple elegant solutions to the problems faced by microbes living inside one of the host's most effective killing machines.

I. Macrophages

Phagocytes and phagocytosis

Macrophages, neutrophils and DCs are the only mammalian cells that perform the specialized process of phagocytosis. This is in contrast to the process of endocytosis, which is used by most cells for the uptake of nutrients. Endocytosis is usually triggered by binding of receptors to their cognate ligands, and does not involve rearrangement of the actin cytoskeleton. Rather, small molecules including clathrin facilitate membrane invaginations and subsequent budding (Mousavi *et al.*, 2004). Phagocytosis involves the ingestion of large particles, such as whole cells, and requires actin polymerization at the plasma membrane and the activity of myosin, kinases, and GTPases (Amer and Swanson,

2002). Once a particle is enclosed inside the phagocyte, the phagosome matures through the endocytic pathway, a series of steps used by the cell to sequentially degrade ingested particles. Vesicles are recruited to the phagosome that contain degradative enzymes such as proteases and hydrolases as well as the proton ATPases, which acidify the phagosome. The order of fusion of these vesicles with the phagosome appears to be regulated by the Rab family of GTPases. Phagosomes start as early endosomes, evolve into late endosomes as vesicles fuse, and mature finally into lysosomes, in which the pH drops as low as 4.0 and degradation of ingested material is completed.

The three types of phagocytes serve different purposes in the host. DCs are professional APCs that ingest particles and migrate to lymph nodes where they initiate adaptive immune responses. Neutrophils, or polymorphonuclear cells (PMNs), are short-lived effector cells that ingest pathogens and bombard them with granules filled with anti-microbial peptides and toxic micromolecules. PMNs also release both cytokines and granular contents into the surrounding tissues, thus contributing to the inflammatory responses. Macrophages also ingest microbes and destroy them with toxic molecules; however, unlike PMNs, macrophages participate in physiologic as well as pathologic processes. In addition to phagocytosing invading pathogens, macrophages also scavenge dead and damaged host cells. If the macrophage determines that the ingested body represents danger, cytokines and chemokines are released to alert other immune cells.

Receptors for recognition of pathogens

Capture of microbes by macrophages occurs through an array of receptors including complement receptors and Fc receptors, as well as direct binding of microbes

by PAMP receptors, of which the macrophage expresses many types (Janeway and Medzhitov, 2002). The macrophage mannose receptor (MMR) binds bacteria and fungal pathogens and targets them for destruction in the lysosome. MARCO (macrophage receptor with collagenous structure), which is found only on macrophages, binds to bacterial cell walls and mediates bacterial phagocytosis. The macrophage scavenger receptor (MSR) binds a broad range of polyanionic ligands including double stranded RNA (dsRNA), which is found in some viruses, and the bacterial products lipopolysaccharide (LPS) and lipoteichoic acid (LTA). LPS, also known as endotoxin, causes endotoxic shock by over-stimulating the innate immune system, which results in a toxic systemic inflammatory response. In addition to binding pathogens for ingestion, MSR is thought to protect against endotoxic shock by scavenging free LPS.

Macrophages possess other PAMP receptors that serve not to capture microbes, but rather to recognize the presence of microbes and initiate appropriate immune activation. Toll-like receptors (TLRs) are a family of receptors that recognize different microbial products, but utilize common signaling pathways to alter cytokine expression and macrophage functions (Janeway and Medzhitov, 2002). Signals from these receptors are passed to an adaptor molecule, MyD88 or one of its homologs, which then triggers a signaling cascade culminating in the activation of the transcription factor NF-κB. NF-κB mediates the expression of numerous immune and inflammatory cytokines (May and Ghosh, 1998).

Currently, ten TLRs have been identified in mice and humans, although ligands have been established for only half of them. TLR2 recognizes the largest number of known ligands, in part because it forms heterodimers with either TLR1 or TLR6 to make

functional receptors with different specificities. TLR2 ligands include bacterial peptidoglycan and lipoproteins, LPS from some bacterial species, a surface antigen from the protozoan *Trypanosoma cruzi*, a component of yeast cell walls, as well as other bacterial factors. TLR4 recognizes LPS, LTA, the host heat shock protein hsp60, and the fusion protein of respiratory syncytial virus. The sole known ligand for TLR5 is flagellin, the protein subunit that makes up flagella, which are the tail-like structures bacteria use for swimming motility. TLR9 recognizes unmethylated CpG DNA. Although mammalian DNA also contains CpG sequences, they are almost always methylated. Thus unmethylated CpG DNA is a hallmark pathogenic microbes, many of which do not have methylating enzymes.

It should be noted that TLR4 is not the only receptor for LPS. Prior to the discovery of TLR4, the then yet-to-be-identified LPS receptor was one of the Holy Grails of innate immunity. Two proteins had been identified that bound to LPS, but neither appeared to have any capacity to convey a signal across the plasma membrane into the cell. LPS, which is very hydrophobic, aggregates in micelles in the serum. LPS-binding protein (LPB) extracts monomers of LPS from micelles and transports them to host cells where monomers are transferred to the surface protein CD14. CD14, however, is not a membrane-spanning protein, but rather is held in the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor, and by itself cannot transmit information to the inside of the cell. The discovery of TLR4 appeared to provide the answer to the riddle for how cells relay the LPS signal. Yet, there have been no definitive experiments demonstrating direct binding of LPS to any domain of TLR4. It is postulated that a

complex of TLR4, CD14, and a third protein, MD2, together bind LPS and induce the signaling cascade that results in inflammatory gene expression.

Several intracellular pathogen recognition systems exist as well. The cytosolic protein kinase PKR and the OAS/RNaseL pathway are activated by viral dsRNA. The recently identified NOD family of proteins appears to be involved in intracellular microbe detection as well. NOD proteins show similar domain architecture to plant resistance genes (R genes), which are triggered by microbial products to activate the hypersensitivity response, a major plant defense pathway. The range of ligands for the NOD proteins has yet to be identified; however, several distinct muropeptides from peptidoglycan, a component of both Gram positive and Gram negative bacterial cell walls, have been shown to bind NOD1 and NOD2 (Girardin *et al.*, 2003; Royet and Reichhart, 2003), and both NOD1 and NOD2 have been shown to activate NF-κB.

Macrophage anti-microbial mechanisms

The macrophage is equipped with a variety of anti-microbial mechanisms for destruction of ingested pathogens (Shiloh and Nathan, 1999). Generation of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) accounts for much of the microbicidal activity of macrophages (Nathan and Shiloh, 2000), but other mechanisms also contribute to pathogen destruction (Shiloh *et al.*, 1999).

1. ROI and RNI

Phagocyte oxidase (phox) is triggered to produce ROI in macrophages and PMN that have been activated by cytokine signaling or recognition of microbes. The subunits

of the inactive enzyme are located in both the cytosol and in vesicular membranes. When phagocytosis occurs, the vesicles fuse with the phagosome, and the cytoplasmic subuits are recruited to form a functional enzyme. This process can also occur at the plasma membrane at the site of pathogen recognition. Generation of ROI occurs within minutes of pathogen recognition. Activated phox transfers electrons from NADPH to molecular oxygen to form superoxide ($^{\cdot}O_2^-$). Superoxide is converted spontaneously or by superoxide dismutase (SOD) into hydrogen peroxide, which can be converted by transition metal chemistry into hydroxyl radical. Hydroxyl radical is highly reactive, and thus does not diffuse far. Hydrogen peroxide, which is toxic, diffuses readily through membranes and can reach the cytosol of microbes. In PMNs, the enzyme myeloperoxidase (MPO) is responsible for the generation of hypochlorite (HOCl, commonly known as bleach) from hydrogen peroxide. Activation of macrophages by IFN-γ enhances production of ROI by phox (Nathan *et al.*, 1983).

Macrophages and PMNs use inducible nitric oxide synthase (iNOS or NOS2) to generate RNI. Unlike phox, whose subunits are constitutively synthesized as inactive precursors, NOS2 is expressed only when macrophages are activated by cytokines such as IFN-γ. In the cytosol, two NOS2 monomers combine with the cofactors FAD, FMN, calmodulin, and tetrahydrobiopterin to form an enzyme that uses electrons from NADPH to convert L-arginine into L-citrulline and nitric oxide ($^{\cdot}NO$). Soluble nitric oxide diffuses into the phagosome where it reacts with microbial proteins as well as nucleic acids. Nitric oxide can also auto-oxidize into other toxic RNIs. When synthesized simultaneously, superoxide and nitric oxide can combine to make peroxynitrite, which gives rise to sodium nitrite and hydroxyl radical. Hydroxyl radical is perhaps the most

reactive of all the RNIs and ROIs. The toxicity of ROI and RNI results from modification of protein side chains, disruption of heme and Fe-S clusters, and damage to DNA.

2. Nutrient limitation

Macrophages can deprive ingested pathogens of nutrients, including divalent cations, amino acids, and possibly also carbohydrates. The exact mechanisms by which cation restriction occurs remain unclear, but studies of bacterial and protozoal mutants indicate that iron (Shiloh and Nathan, 1999) and magnesium (Buchmeier *et al.*, 2000) both appear to be limited in the phagosome. Efforts to identify a cation transporter have focused on the membrane proteins Nramp1 and Nramp2. Nramp1 (natural-resistance associated macrophage protein 1) was initially identified by cloning of the allele that make some inbred strains of mice more susceptible to *Salmonella typhimurium, Leishmania donovani,* and *Mycobacterium bovis* var. BCG (Govoni *et al.*, 1996). These organisms represent three widely differing groups of pathogens, but all three reside within the phagosome of the macrophage during infection. Mutations in a yeast Nramp1 homolog rendered the yeast *Saccharomyces cerevisiae* sensitive to low manganese. Nramp2 controls iron uptake in the intestine, and mutation of the Nramp2 gene leads to iron-deficiency anemia in mice and rats. Nramp2 has also been reported to be required for iron transport in endosomes. Thus, although there is no direct evidence, Nramp1, which is expressed predominantly in phagocytes, is considered a strong candidate for mediating iron and/or magnesium restriction in the phagosome. It has also been

suggested that Nramp1 exchanges metal cations for protons, thus participating in the acidification of the phagosome while restricting the nutrient content (Russell, 2001).

Activated macrophages, like some other mammalian cells, can express indoleamine 2,3-dioxygenase (IDO), which catalyzes oxidative cleavage of the indole ring of the amino acid L-tryptophan. IDO is upregulated by IFN-γ, and TNFα can act synergistically with IFN-γ to enhance IDO activity in some cell types. L-tryptophan is an essential amino acid for many pathogens, and thus IDO's depletion of intracellular L-tryptophan pools has crippling effects on some intracellular microbes, including *Toxoplasma* and *Chlamydia* species, as well as some extracellular bacteria, including Gram positive cocci (MacKenzie *et al.*, 1999). Bacterial DNA also triggers induction of IDO, and stimulation of macrophages with a synthetic DNA analog was found to inhibit *Mycobacterium avium* growth in an IDO-dependent manner (Hayashi *et al.*, 2001b). IDO-mediated depletion of L-tryptophan by APCs can also serve to restrict host cell activity, specifically T cell responses (Swanson *et al.*, 2003).

Recently, a number of pathogens that can survive in the macrophage phagosome have been shown to rely upon metabolism of fatty acids for survival *in vivo*. *Mycobacterium tuberculosis, Cryptococcus neoformans,* and *Candida albicans* all infect their hosts via inhalation, and thus one of the first cells encountered by these pathogens is the alveolar macrophage. Studies have demonstrated that full virulence of each microbe is dependent on expression of isocitrate lyase, an enzyme required to produce carbohydrates from fatty acids without using ATP when glucose is not available (Lorenz and Fink, 2001; McKinney *et al.*, 2000; Rude *et al.*, 2002). The importance of isocitrate

lyase for pathogenesis suggests that the macrophage may attempt to starve ingested microbes by restricting carbohydrate in the phagosome.

3. Acidification

The ability to acidify vesicles is not unique to phagocytes. Acidification is necessary for the activity of lysosomes, and lysosomes are responsible for two important functions in nucleated cells: the degradation of macromolecules retrieved from the external environment by endocytosis, and the process of autophagy, or digestion of organelles that have become dysfunctional or obsolete. Approximately 40 enzymes, referred to as acid hydrolases, mediate degradation that occurs in lysosomes, including lipases, phosphatases, glycosidases, nucleases, sulfatases, phospholipases and proteases. These acid hydrolases are optimally active at low pH, and thus acidification is essential for lysosomal functions. All cells that contain lysosomes also contain proton ATPases, which pump hydrogen ions into vacuoles targeted for fusion with lysosomes. The dependence of lysosomal hydrolases on acidity protects the host cell: if a lysosome were to rupture, the degradative enzymes would be non-functional in the neutral pH of the cytosol.

In phagocytes, lysosomes serve an additional function: digestion of pathogens for presentation of antigens on MHC class II molecules. When the ingested pathogen is still alive, phagosome-lysosome fusion, accompanied by acidification of the phagosome, acts as a potential killing mechanism. In the absence of acid hydrolases, it is not clear whether acidification alone kills microbes ingested by the macrophage. Many microbes that infect their host by the oral route have evolved acid-tolerance mechanisms for

surviving the low pH of the stomach, and are thus resistant to acid. Although some microbes are susceptible to killing by acid *in vitro*, acidification in the macrophage usually occurs simultaneously with phagosome-lysosome fusion. It is therefore difficult to determine if dependence on acid for cytotoxicity *in vivo* is due to acidity itself or due to activation of acid hydrolases. Moreover, acidic conditions render RNI toxic. Nitric oxide oxidizes to form nitrite, and, at acidic pH, nitrite is protonated to form nitrous acid (Stuehr and Nathan, 1989). Thus, the precise contributions of acidification to killing of pathogens by macrophages are difficult to dissect. At the least, acidification contributes to macrophage anti-microbial activity by activating other cytotoxic mechanisms.

Another complication to understanding how acidification participates in host defense arises from studies that use bafilomycin A1 to inhibit the V-ATPase. Inhibition of acidification by treatment of macrophages with bafilomycin A1 prevents killing of some pathogens (Philippe *et al.*, 2003; Schneider *et al.*, 2000). However, bafilomycin has been shown to affect macrophage functions in addition to inhibiting the V-ATPase, including maturation of the endocytic pathway (Xu *et al.*, 2003) and the production of ROI by phox (Bidani *et al.*, 2000). Thus, the results of experiments that use bafilomycin to inhibit macrophage killing are difficult to interpret, and may not actually reflect a role for acidification in the specific pathogen-host interactions examined.

4. Phospholipases

Phospholipases cleave and release fatty acids from membrane phospholipids. Phospholipase A_2 (PLA_2) contributes to inflammation by releasing arachadonic acid,

which can be modified by lipoxygenase to produce leukotrienes or by cyclooxygenase to generate prostaglandins, prostacyclins, and thromboxanes. These bioactive lipids have both pro- and anti-inflammatory effects (Yoshikai, 2001). PLA_2 also appears to participate directly in the anti-microbial immune response. Arachidonate and other free fatty acids (FFA) have bactericidal activity against various pathogens (Giamarellos-Bourboulis *et al.*, 2003; Saito and Tomioka, 1988). PLA_2 can also directly kill bacteria by hydrolysis of bacterial cell membranes (Beers *et al.*, 2002; Buckland and Wilton, 2000; Gronroos *et al.*, 2001; O'Callaghan *et al.*, 2003).

Multiple types of PLA_2 are found in macrophages, including both secretory forms, which might be targeted to the phagosome, as well as cytosolic forms (Dennis, 1994). Additional evidence that PLA_2 activity may be important for macrophage killing of intracellular pathogens comes from a recent report that compared expression profiles of bacteria undergoing different stresses. Bacteria experiencing membrane damage due to incubation in the detergent SDS exhibit transcriptional responses that are similar to those of bacteria ingested by macrophages (Schnappinger *et al.*, 2003). These data suggest that bacteria in the macrophage phagosome confront membrane injury, possibly as result of phospholipase activity.

5. Apoptosis

Apoptosis is a highly regulated, physiologic process by which mammalian cells that have been damaged beyond repair or that have become unnecessary can involute and die without generating inflammation (Hetts, 1998). Apoptosis can occur as a result of endogenous signals, such as mitochondrial damage, or as a result of signaling through

surface receptors that contain intracellular death domains (Bhardwaj and Aggarwal, 2003). The best described of these receptors are Fas and the TNF receptor, both of which have the capacity to trigger apoptosis, although they can relay signals for other responses as well. Apoptosis of infected cells can be either a physiologic or a pathologic response to infection. Some intracellular microbes promote apoptosis and thereby exit host cells without triggering an inflammatory response (Navarre and Zychlinsky, 2000). This kind of pathologic induction of apoptosis will be discussed later. Contributions of apoptosis to the immune system's ability to control and destroy intracellular pathogens will be highlighted here.

When an infected cell dies as a result of microbial proliferation and membrane lysis, pathogens are released to infect more cells, and an inflammatory response is provoked. However, when infected cells undergo apoptosis, intracellular microbes are sequestered in apoptotic bodies, preventing the spread of infection. These apoptotic bodies are cleared by phagocytic cells with little effect on the surrounding tissues (Fadok and Chimini, 2001). Despite these theoretical benefits, macrophage apoptosis is not frequently found as contributing factor in control of intracellular infection. In a mouse model of resolving pulmonary infection, one of only a few *in vivo* examples, apoptosis of alveolar macrophages is required for clearance of low dose *Streptococcus pneumoniae* without features of pneumonia (Dockrell *et al.*, 2003). A number of pathogens have been shown to block the apoptotic pathways of macrophages *in vitro*, suggesting that macrophages attempt to control infection by inducing programmed cell death. *T. gondii* has been shown to interfere with apoptotic pathways (Denkers, 2003), and attenuated strains of *Mycobacteria* induce macrophage apoptosis, whereas virulent strains inhibit

apoptosis (Fratazzi *et al.*, 1999). Apoptosis resulting from *Mycobacteria* infection appears to depend on macrophage production of TNFα and subsequent signaling through macropahge TNF receptors (Rojas *et al.*, 1997).

Although virulent *M. tuberculosis* blocks macrophage induction of apoptosis, both mouse and human antigen specific $CD4^+$ T cells can kill *M. tuberculosis*-infected macrophages by signaling through Fas ligand (FasL) (Lewinsohn *et al.*, 1998; Silva and Lowrie, 2000). Fas-mediated apoptosis results in decreased viability of intracellular *M. tuberculosis* within the dying macrophage (Oddo *et al.*, 1998). Fas-mediated apoptosis of macrophages also contributes to control of cutaneous *Leishmania* infections: mice deficient in either Fas or FasL are unable to heal skin lesions caused by *Leishmania major* (Conceicao-Silva *et al.*, 1998), and this appears to be due to persistence of infected macrophages in these mice.

II. Macrophages and Intracellular Pathogens

Despite the macrophage's efforts to convert the phagosomal milieu into a hostile environment, many pathogens colonize the macrophage and replicate within this cell. These pathogens represent a large phylogenetic range of single-celled organisms that include Gram positive and Gram negative bacteria, as well as protozoa and single-celled fungi. Although the species of pathogens that invade macrophages are diverse, the tactics for survival inside the cell fall within a limited set of known templates (Russell, 2000).

Strategies for avoiding degradation in the lysosome: localization within the cell

Macrophage phagosome maturation is a highly regulated process, and each step along the way represents a potential target for microbial disruption. Several families of GTPases are involved in the trafficking of vesicles within the endocytic pathway. The Rabs are small GTPases belonging to the Ras superfamily (Fratti *et al.*, 2001). (The Ras superfamily derives its name from the founding member, viral *ras*, an oncogene that was originally identified for its role in triggering a rat sarcoma). Each Rab is associated with trafficking of a specific compartment within the cell: Rab5 associates with maturing early endosomes, Rab 7 associates with maturing late endosomes, Rabs 4 and 11 mediate recycling of vesicles from early endosomes to the plasma membrane, and Rab 9 is associated with trafficking of vesicles from the trans Golgi network (TGN) to late endosomes. The ability of microbes to block phagosome-lysosome fusion has been correlated with retention and exclusion of specific Rab molecules, although the exact mechanisms by which this occurs are generally not well understood.

Rab GTPases function in both activated and unactivated macrophages. In IFN-γ activated macrophages, two other families of GTPases appear to participate in regulation of vesicular trafficking: a 47-kilodalton family and a 65-kilodalton family (Boehm *et al.*, 1998). Studies have shown that the 47-kilodalton family GTPases, which includes IGTP, LRG-47 and IRG-47, are important for control of phagosomal pathogens (Collazo *et al.*, 2001; MacMicking *et al.*, 2003; Taylor *et al.*, 2000), although the mechanism of regulation is not clear. Is has yet to be demonstrated whether pathogens dysregulate phagosome maturation by inhibiting either the 47-kilodalton family or the 65-kilodalton family of GTPases.

There are four basic strategies for avoiding degradation in the macrophage lysosome as a result of phagosome maturation: creation of a novel vacuole outside the endocytic network, inhibition of phagosome-lysosome fusion, expression of genes that allow survival within the fully mature phago-lysosome, and escape from the phagosome into the cytosol (Table I).

1. A unique vacuole

Microbes need first to get into the macrophage. A number of pathogens sneak in the back door, so to speak, by mediating their own uptake and thus avoiding recognition resulting from binding to macrophage phagocytic receptors. Members of the protist class Apicomplexa have evolved a set of organelles (micronemes, rhoptries, and dense granules) specially designed for initiating vacuolization in the macrophage (Hoff and Carruthers, 2002). Through the action of motors and secreted proteins and lipids, apicomplexan organisms force their way into host cells by causing invaginations of the host cell membrane. The resultant compartment, referred to as the parasitophorous vacuole, interacts with the host cell cytoskeleton, but does not fuse with endosomes or lysosomes. *Toxoplasma gondii* uses this mechanism to invade a variety host cell, including macrophages. In macrophages, residence in the parasitophorous vacuole enables *T. gondii* to manipulate the host immune response by interfering with macrophage cytokine expression while remaining protected from many of the macrophage's anti-microbial mechanisms (Denkers *et al.*, 2003).

Most pathogens enter macrophages through the front door: they are ingested by phagocytosis. This method of entry guarantees uptake with minimal work by the

Table I. Microbial strategies for avoiding degradation in the lysosome: localization inside the macrophage

Create a new vacuole	Block maturation of the phagosome	Resist killing within the phago-lysosome	Escape from the phagosome
Toxoplasma gondii	*Mycobacterium tuberculosis*	*Leishmania donovani*	*Listeria monocytogenes*
Legionella pneumophila	*Salmonella typhimurium**	*Coxiella burnetii*	*Shigella flexneri*
Brucella abortus	*Nocardia asteroides*	*Histoplasma capsulatum***	*Rickettsia prowazekii*
Chlamydia trachomatis	*Coccidioides immitis*		*Trypanosoma cruzi*

* The phagosome of *S. typhimurium* acidifies without full maturation into a phago-lysosome.
** The phagosome of *H. capsulatum* fuses with lysosomes but does not acidify.

microbe, but it places the microbe within the endocytic pathway and alerts the macrophage to the presence of danger. The Gram negative bacterium *Legionella pneumophila* has evolved mechanisms that delay fusion with lysosomes long enough to convert the phagosome into a compartment that no longer resemble any component of the endocytic network (Roy and Tilney, 2002). Not exactly a novel vacuole, the compartment created by *L. pneumophila* appears to be an extension of the endoplasmic reticulum (ER). *L. pneumophila* uses the Dot/Icm apparatus, a type IV secretion system, to inject effectors into the host cytosol, some of which act to recruit ER-derived vesicles to the phagosome, while others prevent normal endocytic maturation. Another Gram negative bacterium, *Brucella abortus*, also uses a type IV secretion system (the VirB system) to mediate recruitment of ER vesicles to the phagosome after ingestion by macrophage (Celli *et al.*, 2003).

2. Developmental arrest of the phagosome

Unlike *B. abortus* and *L. pneumophila*, which convert the early phagosome into a vacuole with no similarity to endocytic vesicles, *Salmonella typhimurium* and *Mycobacterium tuberculosis* arrest the maturation of the phagosome such that the modified vacuoles containing *S. typhimurium* and *M. tuberculosis* resemble stages of normal endosome development. *M. tuberculosis* arrests the phagosome almost immediately after ingestion, whereas *S. typhimurium* allows some maturation processes to occur but not others. Because several features of the *S. typhimurium* vacuole are reflective of early endosomes, while others are representative of lysosomes, there is some disagreement about whether or not phagosome-lysosome fusion occurs (Buchmeier and

Heffron, 1991; Oh *et al.*, 1996). *S. typhimurium*, a Gram negative bacterium like *B. abortus* and *L. pneumophila*, shares the strategy of injecting factors into the host cytosol to manipulate the fate of the phagosome; however, in the case of *S. typhimurium*, a type III secretion system is used to deliver the effector molecules. Discussion of disruption of trafficking in the macrophage by *S. typhimurium* and *M. tuberculosis* will be continued later in this document.

3. Survival despite full phagolysosome maturation

Most pathogens that replicate within the phagosome of the macrophage modify the vacuole to some extent. The distinction between microbes that survive by arresting endocytic development and microbes that survive within a fully mature phagolysosome is determined by the presence or absence of acid hydolases in the replicative niche. A few pathogens have evolved to resist killing in the fully mature, hydrolase-containing lysosome. The protozoa of the *Leishmania* genus and the Gram negative bacterium *Coxiella burnetii* both localize to and replicate within lysosomes that are fully acidified to a pH between 4.5 and 5.0. *Leishmania* vacuoles contain active cathepsins B, L, and D, and stain positively for the lysosomal membrane components LAMP-1 and LAMP-2 but not for the transferrin receptor, which associates only with early endosomes (Courret *et al.*, 2002). *Leishmania* resists damage from acid hydrolases by covering its surface with GIPLs, short lipidoglycans that are relatively impervious to attack. Additionally, endocytosis and exocytosis occur only at a small area of protected membrane called the flagellar pocket, thus protecting endocytic receptors from degradation by hydrolases. The mechanisms by which *C. burnetii* copes with acid hydrolases remain unknown (Baca

et al., 1994). Another mystery is how these two pathogens prevent destruction of their host cell by antigen-specific T cells, since antigen presentation machinery is functionally active within the pathogen-containing lysosomes. One possibility is that the pathogen actively degrades MHC molecules (Courret *et al.*, 2001). A second theory postulates that processing and presentation of microbial antigens occurs, but the resulting peptide-MHC complexes trigger an inappropriate Th2-type immune response that is unable to eradicate intracellular infection (Maekawa *et al.*, 1998).

4. Escape from the phagosome

Another strategy for avoiding the anti-microbial mechanisms associated with the endocytic network is to escape from the phagosome into the cytosol. Among the microbes that can lyse the phagosomal membrane after ingestion are the Gram positive bacterium *Listeria monocytogenes* (Cossart and Lecuit, 1998), and the Gram negative bacterium *Shigella flexneri* (Zychlinsky *et al.*, 1994). Macrophages are not the primary targets of these pathogens. Both bacteria invade epithelial cells, and in the process create vacuoles in these non-phagocytic cells that the bacteria lyse in order to access the cytosol. The factors used by *S. flexneri* to lyse the phagosome have yet to be identified, although a lowered pH is required for activation of the required mechanism. *L. monocytogenes'* escape is mediated by listeriolysion O (LLO), a thiol-activated cytolysin similar to exotoxins found in other Gram positive bacteria. Although the process of bacterial uptake is different with macrophages than with epithelial cells, phagosome lysis has been observed for *L. monocytogenes* and *S. flexneri* after ingestion by macrophages.

Escape to the cytosol prevents degradation by lysosomal acid hydrolases, but the cytosol is not a wholly safe environment either, and not all microbes can survive in this niche. *Brucella* engineered to express LLO are unable to replicate within macrophages (Kohler *et al.*, 2001), suggesting that cytosolic factors are toxic to *Brucella*. Recruitment of E.R. membrane by *B. abortus* may be important not only to deviate the phagosome from the endocytic network, but also to expand the size of the vacuole as replication proceeds and thus prevent phagosome rupture. A study in which intracellular pathogens were microinjected directly into the host cell cytoplasm found that only cyptoplasmic pathogens could survive in this niche, while microbes that normally reside within the vacuole were killed (Goetz *et al.*, 2001). As will be discussed later, *S. typhimurium* also recruits membrane to its vacuole inside the macrophage, and mutants that are not maintained in the phagosome are killed by yet-to-be defined factors in the cytosol (Holden, 2002).

Strategies for coping with host production of RNI and ROI

Regardless of where pathogens localize inside the macrophage, host production of toxic micromolecules is a danger that must be confronted by all intracellular pathogens. Virulence factors described as anti-oxidant or anti-RNI defenses encompass a range of different activities that interfere with ROI's and RNI's ability to cause damage. Some pathogens attempt to avoid toxic micromolecules by inhibiting phox or NOS2, thus preventing production of ROI and RNI. Examples of this strategy include secretion by *Bordetella pertussis* of an adenylyl cyclase that elevates macrophage cytosolic cAMP and stalls the activity of phox (Confer and Eaton, 1982), the tyrosine phosphatase YopH

from *Yersinia enterocolitica* that inhibits the respiratory burst (Green *et al.*, 1995), and an unknown mechanism of the yeast *Candida albicans* that inhibits NOS2 enzyme activity in macrophages (Schroppel *et al.*, 2001). A variant of this strategy is the diversion of phox or NOS2 away from the pathogen-containing phagosome. *S. typhimurium* injects unknown factors into its host macrophage that cause redistribution of phox from the phagosome to the plasma membrane (Gallois *et al.*, 2001). A similar effect on NOS2 localization has been reported during *S. typhimurium* infection of macrophages as well (Chakravortty *et al.*, 2002).

For microbes that cannot turn off the enzymatic sources of RNI and ROI, the next best defense is catabolism these molecules before they cause damage. Aerobic microbes express enzymes, which may include superoxide dismutases (SODs), catalases, peroxiredoxins, and glutathione reductases, for detoxification of endogenously generated superoxide and peroxides (Dukan and Nystrom, 1999; McCord *et al.*, 1971). These enzymes also protect against host-generated ROI (Faguy and Doolittle, 2000). Systems specifically expressed to detoxify exogenous ROI and RNI, but not endogenous ROI, are also common in the world of intracellular pathogens. In Gram negative bacteria, periplasmic SODs act as the first line of defense against exogenous ROI and play little to no role in detoxification of endogenous superoxide (Korshunov and Imlay, 2002). *S. typhimurium* mutants deficient in periplasmic SOD also demonstrate increased susceptibility to RNI *in vivo* (De Groote *et al.*, 1997), suggesting that SOD detoxification of superoxide prevents formation of peroxynitrite, thus greatly diminishing the effects of RNI as well as ROI. Several other defenses against ROI also double as protection against RNI. The peroxiredoxin alkyl hydroperoxide reductase, AhpC, which catalyzes the

reduction of organic hydroperoxides, can act in many organisms for defense against RNI as well as ROI (Shiloh and Nathan, 1999). AhpC's role in detoxification of RNI appears to be due to AhpC's ability to catalytically detoxify peroxynitrite (Bryk *et al.*, 2000). A role for homocysteine has been demonstrated in defense specifically against RNI, both as a sink to absorb RNI, and as a regulator of the *hmp* gene, which encodes flavohaemoglobin (Poole and Hughes, 2000). Flavohaemoglobin can convert nitric oxide to less toxic nitrogen species, and E. coli *hmp* mutants are hyper-susceptible to RNI.

Enzymes to repair oxidative and nitrosative damage are another important component of microbial defense against ROI and RNI. Some enzymes mend damaged DNA resulting from a range of molecular insults that include ROI and RNI. The recBC system repairs damage inflicted by RNI and ROI (Buchmeier *et al.*, 1995; Shiloh *et al.*, 1999), and the Uvr system is an important component of *Mycobacterium tuberculosis*'s defense against RNI (Darwin *et al.*, 2003). ROI and RNI (through the formation of peroxynitrite) can both cause oxidation of proteins, and the enzyme methionine sulfoxide reductase (Msr) has been shown to be important for defense against both stresses *in vitro* (St John *et al.*, 2001).

When all else fails, a final mechanism to defend against macrophage cytotoxicity is invoked by certain pathogens that cause chronic disease: reversion to a semi-dormant state until immune pressure subsides. Most infections with *M. tuberculosis*, *T. gondii*, *S. typhi*, and *L. donovani* include a prolonged and indefinite period of latency, during which the quiescent pathogens are harbored within host macrophages (Coppin *et al.*, 2003; Muñoz-Elías and McKinney, 2002). Because many macrophage anti-microbial mechanisms are induced or up-regulated by IFN-γ, a lapse in cellular immunity, such as

$CD4^+$ T cell depletion due to infection with HIV, results in decreased immune pressures on the intracellular pathogens. These organisms are then able to awake from dormancy and recommence intracellular replication.

III. *Mycobacterium tuberculosis*

Tuberculosis, which is caused by infection with the bacterium *Mycobacterium tuberculosis*, is one of the oldest recognized ailments in human society. Remains of mummies from Europe, South America, and Asia, dating as far back as 3000 B.C.E, contain traces of DNA from *Mycobacterium tuberculosis* (Fusegawa *et al.*, 2003; Nerlich *et al.*, 1997; Salo *et al.*, 1994). The first written accounts of tuberculosis can been traced to the Greek physician Hippocrates, who wrote in 460 B.C.E. about fatalities due to the widespread disease "phthisis" (Hippocrates, 400 BCE). The cause of the ubiquitous disease would not be revealed until centuries later. In the mid 1800's, Louis Pasteur popularized the germ theory of diseases: the concept that microorganisms could be the source of illness and pathology. Soon thereafter, Robert Koch identified *Mycobacterium tuberculosis* as the causative agent of tuberculosis. In addition, Koch further advanced the nascent field of microbiology by introducing criteria by which scientists could deduce whether a specific microorganism produces a specific disease. These criteria became known as Koch's postulates, and they are still in use today. To conclude that a certain microbe causes a disease, Koch's postulates state that 1) the microbe must be found in diseased tissues, but not in healthy ones, 2) the microbe must be able to be isolated from the diseased tissue and grown as a pure culture, 3) reintroduced of the isolated microbe into a healthy person or animal should induce the originally observed pathology, and 4)

the microbe must be able to be re-isolated following testing of postulate number 3. In the late 1800s, Koch demonstrated that all of these criteria were true for the disease tuberculosis and the bacterium we now know as *Mycobacterium tuberculosis.*

M. tuberculosis (Mtb) is sometimes categorized as a Gram positive bacterium, but Mtb truly cannot be described accurately by the Gram stain designation due to its unusual external envelope. Like Gram positive bacteria, Mtb has a single plasma membrane surrounded by a layer of PGN. However, external to the PGN, Mtb possesses a thick cell wall composed almost entirely of lipids and glycolipids. The main component of the cell wall is mycolic acid, a long chain branched β-hydoxyl fatty acid, which can be found either covalently or non-covalently attached to the PGN. Interspersed among the mycolic acid molecules are numerous complex lipid and glycolipids that remain associated to the cell wall through hydrophobic interactions. The lipid-rich cell wall inteferes with absorption of crystal violet, and thus a different chemical test is performed to identify Mtb and other mycobacterial species in the laboratory. Retention of Carbol fuschin dye in the presence of acidic alcohol is a unique characteristic of the lipid envelope, and thus the presence of "acid-fast bacilli" in microscopic preparations is diagnostic for mycobacteria.

Tuberculosis: the disease

There is no reservoir of Mtb outside of the human population, and thus Mtb is distinctly evolved to survive and replicate in the human body. Transmission of disease occurs exclusively by aerosol droplets produced when the host coughs. Adaptation of Mtb inside the human lung and transmission of Mtb are hugely effective, as evidenced by

recent estimates indicating that approximately 2 billion people, or one third of the world's population, are infected with Mtb (Dye *et al.*, 1999). Of these 2 billion individuals, 20 million manifest active disease, and approximately 2 million people die from the disease annually. Infection with Mtb results in more deaths every year than any other bacterial pathogen, rivaled in lethality only by the rapidly spreading epidemic of HIV. Moreover, HIV and Mtb infection exacerbate one another, and tuberculosis is the number one cause of death in HIV-positive individuals (Corbett *et al.*, 2003). The recent re-emergence of Mtb as a global threat has been attributed to the spread of HIV, continued increases in population growth and population density, and the development of multi-drug resistant (MDR) strains of Mtb (Corbett *et al.*, 2003; De Cock and Chaisson, 1999; Dye *et al.*, 1999; Snider and Castro, 1998).

Mtb's ability to exist latently causing subclinical disease in the human lung contributes to the continued prevalence of tuberculosis in the human population. The vast majority of people infected with Mtb manages to contain infection and control bacterial replication; however, Mtb is not sterilized in the lungs of these individuals. Rather, Mtb remains in a dormant state in which it survives despite a vigorous immune response. Latently infected individuals have no symptoms of clinical disease, and a positive tuberculin skin test (also known as the PPD test, referring to the purified protein derivative that is injected) is the only indication these individuals have encountered Mtb. Years later, infected individuals can experience reactivation of latent Mtb (Lillebaek *et al.*, 2002; Tufariello *et al.*, 2003). Approximately half of all active cases of tuberculosis are thought to result from endogenous reactivation of latent Mtb infection (Styblo, 1991). Reactivation appears to be the result of immunosuppression, although the cause of

decreased immunity is not always clear, and the underlying reasons for reactivation in some individuals remain a mystery. HIV infection has become the number one risk factor for reactivation of tuberculosis, increasing the risk of reactivation from 10%-20% per lifetime to 10% per year (Corbett *et al.*, 2003).

The natural history of disease

When bacteria are inhaled, they are rapidly ingested by alveolar macrophages, as well as resident lung DCs (van Crevel *et al.*, 2002). Many of these bacteria evade being killed and begin to replicate, eventually lysing the macrophage. Macrophage chemokines, as well as the presence of lysed host cells, attract inflammatory cells to the site of infection. As monocytes extravasate into the lung tissue, they mature into macrophages that ingest extracellular Mtb. As macrophages accumulate, Mtb continues to replicate, but tissue damage does not occur during this stage of infection. Two to three weeks after initial infection, T cell clones primed by infected lung DCs migrate to the lung where they proliferate and activate macrophages to kill intracellular Mtb. The arrival of T cells correlates with growth arrest of Mtb. The accumulation of cells at the site of infection is called a granuloma, and this structure is usually sufficient to contain infection. An equilibrium is achieved between the pathogen and the immune system, and Mtb remains in a latent state, so long as immune pressure does not taper off.

For unknown reasons, some people do not control initial infection and develop local disease, or primary tuberculosis. As bacterial replication persists, the inflammatory response amplifies, and clinical symptoms of disease begin to manifest: fever, weight loss, cachexia, and cough. If bacterial replication continues to exacerbate the immune

system, cavitation can occur, in which the center of the granuloma undergoes liquefactive necrosis due to the toxicity of an over-exuberant immune response. Cavitary tuberculosis is especially infectious because the liquefied cavities becomes linked to airway passages, and patients shed very high numbers of bacteria in their sputa (Muñoz-Elías and McKinney, 2002). In some extreme cases, dissemination of Mtb from the lungs to other organs occurs, resulting in systemic, or miliary, tuberculosis. Interestingly, although initial infection is always in the lung, and the lung is the most common site of reactivation, reactivation can occur in almost any organ ranging from nervous tissue to bones (Glickman and Jacobs, 2001). This suggests that initial infection includes a hematogenous dissemination to multiple tissues that is quickly controlled by the immune system, although not eradicated.

Treatment and prevention of tuberculosis

In addition to reactivation of latent disease, another factor that has led to Mtb's continued persistence within the human population is a lack of efficient medical interventions. Like most diseases caused by microbes, the strategy to eradicate tuberculosis consists of a two-pronged approach: chemotherapeutic agents for the treatment of infected individuals, and vaccination of uninfected individuals. Unfortunately, in the case of *M. tuberculosis*, neither approach has yielded an adequate solution.

The same antimicrobials that sterilize an *in vitro* culture of Mtb in a matter of days require months for effective treatment of a patient. Anti-mycobacterial drugs must be taken in combination for at least six months to provide a lasting cure (McKinney,

2000; Muñoz-Elías and McKinney, 2002). Even individuals with latent infection must adhere to a six-month treatment regimen to reduce the risk of reactivation later in life. Penetration of the drugs into the tissues does not account for the discrepancy between *in vitro* and *in vivo* killing (Barclay *et al.*, 1953). Moreover, killing of bacteria by anti-tubercular drugs is not accelerated if either the dose (McCune *et al.*, 1957) or the number of drugs administered (Jindani *et al.*, 1980) is increased. An altered metabolic state of the bacteria *in vivo* appears to be the source of Mtb's ability to persist despite high levels of multiple chemotherapeutic agents. Mitchison proposed that relative *in vivo* drug resistance results from the fact that latent Mtb are either slowly-dividing or non-replicating (Mitchison, 1980).

Because chemotherapy is so cumbersome, patient non-compliance is an over-whelming problem, frequently resulting in patient relapse and/or the emergence of multi-drug resistant strains of *M. tuberculosis*. Multi-drug resistant tuberculosis can be unresponsive to all available agents, thus leaving surgical removal of the tuberculous lesion as a final treatment option. Although a number of drugs have been added to the anti-mycobacterial arsenal over the years since the initial use of Streptomycin, no agent has been identified that substantially reduces the duration of medication necessary for effective treatment.

The development of a successful Mtb vaccine has been similarly challenging. There is currently only one vaccine in use, the bacillus Calmette Guerin (BCG), a live-attenuated strain of *Mycobacterium bovis*. Vaccination with BCG has yielded both highly variable and generally poor efficacy in prevention of pulmonary tuberculosis in adults, despite eliciting potent antigen-specific immune responses in the majority of

vaccinated individuals (Brewer, 2000). BCG does, however, appear to protect against the morbidity and mortality of Mtb meningitis and other disseminated forms of tuberculosis in infants, and thus it is consistently distributed to newborns throughout much of the world (Huebner, 1996).

Most of the current efforts to create new vaccines are focused on either augmentation of BCG with Mtb antigens, or else the development of live attenuated Mtb strains. It is possible, though, that a better vaccine for pulmonary tuberculosis may be elusive because acquired immunity to Mtb may be only partially effective at best. The fact that individuals who initially control infection can experience reactivation of disease years later demonstrates that immunity to live, virulent Mtb is not sterilizing (Lillebaek *et al.*, 2002; Tufariello *et al.*, 2003). In addition to the phenomenon of reactivation of latent Mtb, exogenous re-infection of asymptomatic individuals also appears to be common in TB-endemic areas, despite the presence of potent antigen-specific immune responses established by the previous infection (Caminero *et al.*, 2001; du Plessis *et al.*, 2001; van Rie *et al.*, 1999).

Pathogen-Host interactions

1. Interactions with the macrophage

Macrophages are the cells with which Mtb has the most interactions, and thus Mtb has evolved complex mechanisms for remodeling the macrophage into a niche where it can replicate in relative protection from the host. Even the first interactions between the mycobacterium and the macrophage are carefully orchestrated. Studies have shown that Mtb is promiscuous in its use of phagocytic receptors, thus ensuring its uptake by

macrophages. Mtb also promotes its survival by entering macrophages by routes that trigger minimal anti-microbial responses. Uptake of non-opsonized Mtb by complement receptors and macrophage mannose receptors (Aderem and Underhill, 1999) does not trigger phagocyte oxidase (phox) production of ROI (Astarie-Dequeker *et al.*, 1999; Wright and Silverstein, 1983). In the absence of these receptors, the macrophage scavenger receptor will also bind Mtb. Binding of lung surfactant protein (SpA) to Mtb has been shown to facilitate uptake by macrophages while at the same time suppressing macrophage production of RNI (Pasula *et al.*, 1999). Although the significance is not yet understood, phagocytosis of Mtb involves the recruitment of cholesterol to the phagosome, a phenomenon not usually associated with ingestion of pathogens (Gatfield and Pieters, 2000).

Once ingested, Mtb manipulates the macrophage to alter normal phagosome maturation (Deretic and Fratti, 1999; Russell, 2001). It is not clear exactly how Mtb disrupts trafficking, but it has been demonstrated that the Mtb phagosome in unactivated macrophages fails to advance beyond being the early endosomal stage, with retention of Rab5 and exclusion of Rab7 and lysosomal markers such as LAMP-1 and LAMP-2. This block in maturation may be due to the inability of Rab5 to bind EEA1 (early endosomal antigen 1), which appears to be inhibited after macrophage ingestion of Mtb or latex bead coated with Mtb envelope lipids, such as mannose-capped lipoarabinomannin (Man-LAM) (Fratti *et al.*, 2001). Moreover, like early endosomes, the Mtb phagosome maintains communication with the plasma membrane (Sturgill-Koszycki *et al.*, 1994). The proton ATPase is not recruited from the phagosome, and thus the pH remains relatively neutral. These events are dependent on live Mtb; after phagocytosis of dead

bacteria, the phagosome acidifies to a pH of 5.0, whereas phagosomes containing live Mtb acidify to a pH of approximately 6.5 (Sturgill-Koszycki *et al.*, 1994). TACO, a protein that normally transiently associates with endosomes in the process of maturation into lysosomes, is retained on the Mtb phagosome (Ferrari *et al.*, 1999b). The precise function of TACO in intracellular trafficking is not currently understood; it is not yet clear whether TACO acts to blocks phagolysosome fusion, or whether TACO is simply a marker indicating that phagosomal maturation has been arrested.

In addition to disrupting the endocytic machinery of the macrophage, Mtb also interferes with macrophage functions that promote activation of other immune cells. By avoiding the lysosome, Mtb prevents not only its own destruction, but also processing of Mtb antigens for MHC class II presentation to $CD4^+$ T cells (Pancholi *et al.*, 1993). In addition, Mtb has been shown to block antigen presentation by down-regulating macrophage expression of both MHC class II and CD1 (Stenger *et al.*, 1998). CD1 is a molecule similar to MHC molecules that presents lipid antigen to T cells, as opposed to protein antigens. The expression of the costimulatory molecule B7 is also decreased in Mtb infected cells (Samandari *et al.*, 2000).

During infections with most pathogens, macrophages and T cells communicate via an elaborate cytokine crosstalk: infected macrophages secrete IL-12, which activates naïve T cells to become Th1 cells and produce IFN-γ, which in turn feeds back and activates macrophages. Mtb dampens the immune response by interfering with these normal cytokine networks. Infection with Mtb inhibits IL-12 expression in macrophages (Nau *et al.*, 2002), thus decreasing generation of Th1 cells (Hickman *et al.*, 2002). Decreased IL-12 may be due to Mtb-induced macrophage production of IL-10 (Redpath

et al., 2001), which not only hinders IL-12 expression, but also blocks IFN-γ's ability to activate macrophages (Murray *et al.*, 1997). T cell activation and proliferation are suppressed by IL-6 produced by Mtb-infected macrophages (VanHeyningen *et al.*, 1997). Even if T cells manage to become completely functional, T cell cytokines are not fully effective because Mtb hinders the macrophage's ability to respond to IFN-γ (Kincaid and Ernst, 2003). Production of IL-6 contributes to this partial block in responsiveness to IFN-γ, which occurs in both infected and uninfected macrophages (Nagabhushanam *et al.*, 2003).

As described earlier, there is evidence that macrophages commit suicide in order to kill intracellular pathogens. Because the macrophage acts as a safe haven for Mtb, apoptosis would be detrimental to the bacterium, and Mtb has evolved mechanisms to evade this host defense. Ingestion of mycobacteria can result in macrophage apoptosis; however, the fate of the macrophage is dependent on the virulence of the infecting strain. Avirulent strains, such as the lab strain *M. tuberculosis* H37Ra, the attenuated vaccine strain *M. Bovis* BCG, and the non-virulent mycobacterial species *M. kansasii*, cause more apoptosis than virulent strains, including the lab strains *M. tuberculosis* H37Rv and *M. tuberculosis* Erdman, and various clinical isolates (Keane *et al.*, 2000). Several mechanisms for blocking apoptosis during infection with virulent strains have been described. Virulent *M. tuberculosis* H37Rv, but not avirulent H37Ra, induces the anti-apoptotic Bcl-2 family member Mcl-1 after infection of macrophages (Sly *et al.*, 2003). Other reports indicate that Mtb-induced IL-10 stimulates secretion of a soluble receptor for the pro-inflammatory and pro-apoptotic cytokine TNFα, thus preventing TNFα from

binding receptors on the macrophage and triggering apoptosis (Balcewicz-Sablinska *et al.*, 1998; Rojas *et al.*, 1999).

2. The immune response to *M. tuberculosis*

Perhaps because of Mtb's ability to derail so many aspects of cellular immunity, a stalemate is reached between the bacterium and the host immune system in most individuals, resulting in latent infection. Granulomas form around the invading mycobacteria, and replication of bacteria ceases. These granulomas are composed of a central core of necrotic tissue (dead macrophages) containing extracellular bacilli, surrounded by a mantle of macrophages harboring intracellular bacteria and a sheath of $CD4^+$ and $CD8^+$ T cells. The granuloma also includes B cells, DCs, endothelial cells, fibroblasts, and probably stromal cells (Gonzalez-Juarrero *et al.*, 2001).

The most powerful insights into which components of the immune response are key for maintaining granulomas have come from studies with mice made deficient in specific immune effectors, and from identification of human genetic or pathologic immune suppressive conditions. During acute infection, cellular immunity is critical for the control of Mtb, with little contribution from B cells or Ig. Studies in T cell-deficient mice indicate that T cell production of Th1 type cytokines is required for protection (Flynn and Chan, 2001), whereas B cell deficient mice show no overt insufficiency in controlling Mtb (Johnson *et al.*, 1997). These experiments are reinforced by the findings that AIDS patients with low $CD4^+$ T cell counts are predisposed to tuberculosis (Selwyn *et al.*, 1989), whereas multiple myeloma patients with defective B cell responses demonstrate no increased susceptibility to Mtb (Barnes *et al.*, 1994).

Of the two T cell subsets, $CD4^+$ T cells play the dominant role in control of Mtb, but $CD8^+$ T cells are also important (Stenger and Modlin, 1999). The role of $CD8^+$ T cells in control of Mtb is one area of research in which the immune system of the mouse appears to diverge from that of the human. $CD8^+$ T cells can mediate lysis of cells presenting Mtb antigens in the context of either MHC class I molecules or CD1 molecules. There is no mouse homolog for CD1b, the human CD1 molecule shown to present Mtb lipid antigens. Moreover, human CTLs appear to lyse macrophages and kill intracellular Mtb via the granule protein granulysin. Studies in mice made deficient in CTL granule toxins indicate that neither perforin nor granzyme is required for control of Mtb (Cooper *et al.*, 1997); however comparable studies cannot be performed to clarify the importance of granulysin, because mice do not encode a granulysin homolog.

Further confusion in the field of $CD8^+$ T cell responses to Mtb infection has arisen from the use of β-2-microglobulin (β2M)-deficient mice as a model for $CD8^+$ T cell deficiency. MHC class I molecules are made up of a polymorphic chain that comprises the antigen presenting cleft, and an invariant β2M molecule that stabilizes the MHC complex in the plasma membrane. In the absence of β2M, class I MHC molecules are unstable, and are not expressed on the cell surface. When there are no class I MHC molecules, developing $CD8^+$ T cells are deleted prior to maturation into functional cells. Thus, β2M-deficient mice have been used to model deficiency in $CD8^+$ T cells. In this model, $CD8^+$ T cells were found to play an important role in the control of Mtb (Flynn *et al.*, 1992). A later study demonstrated that MHC class I-deficient mice are much less susceptible to Mtb than β2M-deficient mice (Rolph *et al.*, 2001). These apparently inconsistent data can be explained by the fact that β2M molecules stabilize multiple

surface proteins in addition to MHC class I, including the protein HFE, which mediates iron uptake. Mutations in HFE result in iron overload. If β2M-deficient mice are treated with lactoferrin to chelate some of the extracellular iron, then Mtb infection proceeds identically to infection in MHC class I-deficient mice (Schaible *et al.*, 2002). These results are consistent with previous studies that found a correlation between iron-overload and susceptibility to Mtb (Boelaert *et al.*, 2003). Thus, although $CD8^+$ T cells contribute to control of acute infection with Mtb, their role is less important that initially presumed.

The importance of $CD4^+$ T cells in control of Mtb is well-established. Although $CD4^+$ T cells can lyse Mtb infected macrophages using the Fas-Fas Ligand (FasL) pathway (Stenger and Modlin, 1999), the $CD4^+$ T cell's main role during Mtb infection is to produce IFN-γ, which activates infected macrophages. IFN-γ is essential for control of Mtb, as evidenced by infection of IFN-γ-deficient mice (Cooper *et al.*, 1993; Flynn *et al.*, 1993) and IFN-γR1-deficient mice (MacMicking *et al.*, 2003), which are completely unable to curb bacterial replication. Naturally occurring mutations in IFN-γ-signaling pathways in humans result in highly increased susceptibility to mycobacterial infections as well (Casanova and Abel, 2002). Activation of macrophages by IFN-γ overrides the Mycobacteria-induced block in phagosome maturation (Schaible *et al.*, 1998; Via *et al.*, 1998), and activates mechanisms that result in killing of intracellular Mtb. IFN-γ activation alters expression of approximately 25% of the macrophage transcriptome (Ehrt *et al.*, 2001); however, only a few of these genes have been shown to help control replication of Mtb.

Expression of NOS2 and the production of RNI are induced by IFN-γ. Multiple studies have demonstrated that mice deficient in NOS2, or mice treated with NOS2-

inhibitors, are highly susceptible to infection by Mtb (Chan *et al.*, 1995; Flynn *et al.*, 1998; MacMicking *et al.*, 1997). Additional studies have shown direct toxic effects of RNI against Mtb (Chan *et al.*, 1992). The role of NOS2 in control of tuberculosis in humans has been more difficult to confirm, largely because there have been no reports of human genetic deficiencies in NOS2. However, evidence is accumulating that RNI contributes to the human anti-tubercular response (Nathan, 2002; Shiloh and Nathan, 2000), including multiple studies that have demonstrated that alveolar macrophages from the lungs of patients infected with Mtb express NOS2, whereas macrophages from uninfected controls do not (Choi *et al.*, 2002; Nicholson *et al.*, 1996).

Like NOS2, the IFN-γ-inducible GTPase LRG-47 is highly induced after stimulation of macrophages with IFN-γ. Mice disrupted in LRG-47 are also unable to control replication of Mtb, and succumb rapidly to infection (MacMicking *et al.*, 2003). In the absence of LRG-47, IFN-γ is unable to force the maturation of the Mtb-containing phagosome, suggesting that LRG-47 acts to regulate vesicular trafficking in activated macrophages. Inhibition of both NOS2 and LRG-47 has an additive effect and accounts for most of IFN-γ's anti-mycobacterial effects, although not all (MacMicking *et al.*, 2003).

Despite enhancement of ROI production by IFN-γ, induction of phox does not appear to be an important macrophage mechanism for the control of Mtb. The human condition known as chronic granulomatous disease (CGD) results from a deficiency in one or more of the components of phox and the consequent inability to mount a respiratory burst (Segal *et al.*, 2000). Patients who suffer from CGD are more susceptible to a number of bacterial pathogens (Holland, 2003), and yet there is no definitive

evidence that CGD predisposes individuals to mycobacterial infections, although there is still some controversy concerning this topic (Lamhamedi-Cherradi *et al.*, 1999). Studies using the mouse model of CGD lend further support to the conclusion that phox is not of primary importance for control of mycobacterium: phox-deficient mice show little (Adams *et al.*, 1997; Cooper *et al.*, 2000) or no (Jung *et al.*, 2002; Ng *et al.*, 2004) increase in susceptibility to Mtb.

Other cytokines with undisputed importance during Mtb infection include IL-12 and TNFα(Orme and Cooper, 1999). In addition to evidence from animal models, human cases have been identified to support the roles of these two cytokines in resistance to tuberculosis. Human genetic deficiencies in IL-12 signaling result in increased susceptibility to Mtb (Lammas *et al.*, 2002), and treatment of rheumatoid diseases with anti-TNF antibodies can result in reactivation of latent Mtb (Gomez-Reino *et al.*, 2003). IL-12 is required to promote T cell production of type 1 cytokines including IFN-γ, and thus IL-12 deficiency results in IFN-γ deficiency. The mechanism by which TNFα-deficiency increases the risk of Mtb appears to be more complex. Like IFN-γ, TNFα can overcome the block on phagosome-lysosome fusion in infected macrophages. However, mice deficient in either one of these two cytokines are extremely susceptible to Mtb, indicating that IFN-γ and TNFα do not play redundant roles during infection with Mtb (Flynn *et al.*, 1995; MacMicking *et al.*, 1997). Some IFN-γ effects are mediated indirectly through macrophage production of TNFα(Shi *et al.*, 2003); however, the difference in pathology in the two strains of immunodeficient mice during infection indicates that IFN-γ and TNFα play independent roles (Garcia *et al.*, 1997). Mice deficient in both IFN-γ and TNFα signaling are more susceptible to *M. bovis* var. BCG

than singly deficient mice (Garcia *et al.*, 1997); however, a similar comparison has not been performed with Mtb infected mice.

The correct balance of TNFα is a critical determinant of pathology during infection with Mtb. A lack of TNFα results in dysregulation of the immune response to Mtb, but excess production of TNFα results in increased lung pathology, and is thought to be responsible for many of the clinical symptoms of tuberculosis, including fever and cachexia (Tramontana *et al.*, 1995; Tufariello *et al.*, 2003). A recent study found that monocytes from patients with active tuberculosis secreted high levels of IL-10 and TNFα, but low levels of IL-12, whereas monocytes from asymptomatic infected individuals expressed the reverse cytokine profile (Pereira *et al.*, 2004). These data suggest that excess TNFα results in clinical symptoms, and that the inability to mount a Th1 type response during infection with Mtb leads to active disease.

Our understanding of the immune response to acute infection with Mtb is much better than our understanding of the immune factors that are important for maintaining latency. This discrepancy is due to a lack of an adequate animal model for latent infection as it occurs in humans (Tufariello *et al.*, 2003). Mice, rabbits, and guinea pigs are all susceptible to infection with Mtb and provide good models for active disease. In the mouse lung, Mtb replicates exponentially for the first 2-3 weeks after infection, at which time replication halts as a result of the development of adaptive immunity (Fig. 1A). The immune system maintains a constant titer of Mtb in the lungs for months, and thus the mouse is often used as a model of chronic infection in which a delicate equilibrium has been achieved between the pathogen and the host. However, this model is not a true representation of latency in the human lung, in which only small numbers of

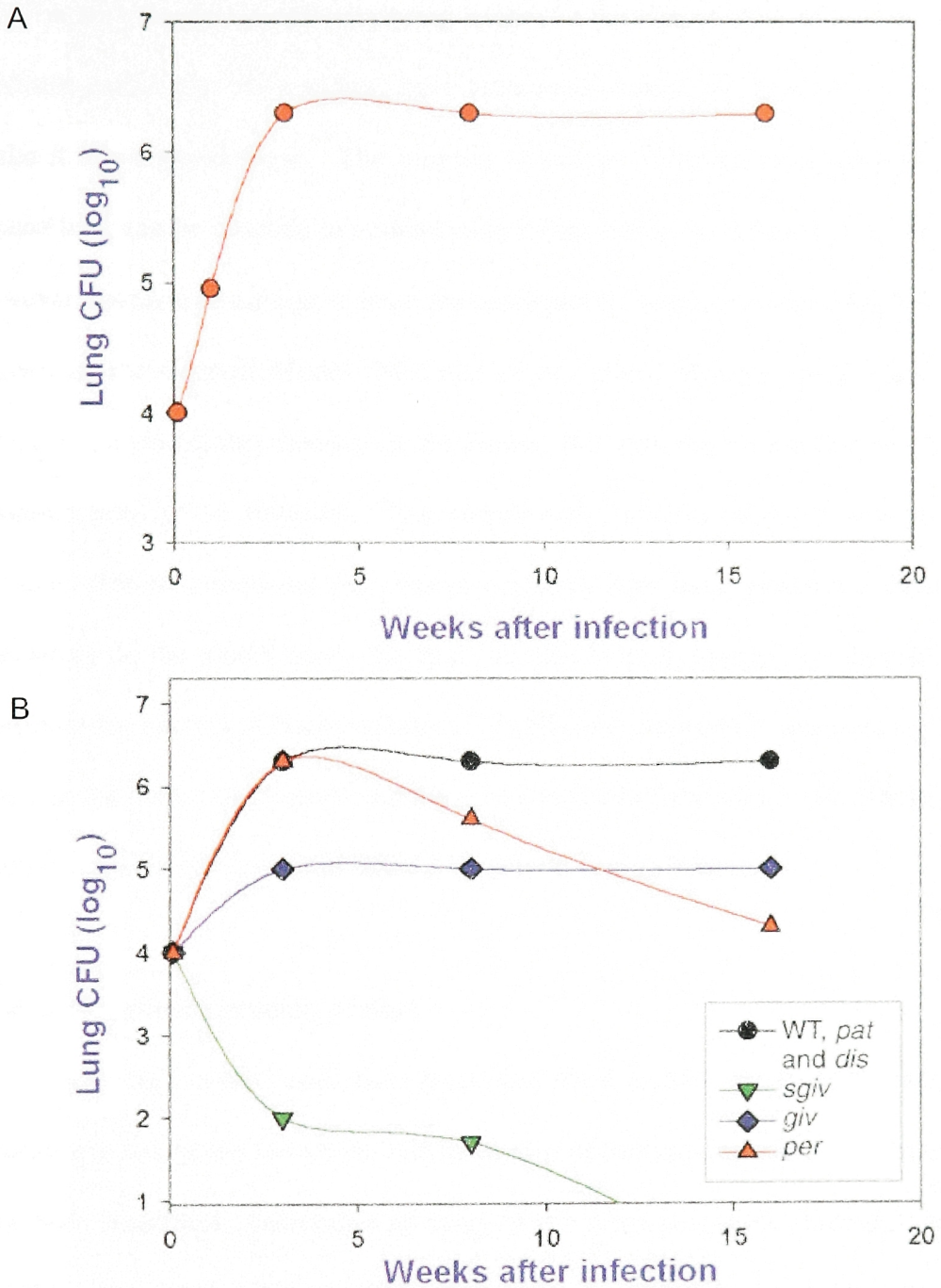


Figure 1. Growth of *M. tuberculosis* strains in mice after i.v. infection. C57BL/6 mice were infected intravenously with $1x10^6$ colony-forming units (CFU) of wild-type (WT) or mutant bacilli. (A) Growth of wild type *M. tuberculosis* in the lungs of immunocompetent mice. (B) Classification of mutants based on their growth in the lungs of immunocompetent mice.

Modified from Hingley-Wilson *et al.* (2003) *Nature Immunol* **4**: 949-955

bacteria are present. Moreover, during latency in the human lung, there is no progression in tissue pathology. Researchers have attempted to improve upon the latency model to make it more physiologic. The number of colony forming units (cfu) of Mtb in the mouse lung can be reduced to undetectable levels if mice are treated with chemotherapy; however, bacteria re-emerge if mice are subsequently immuno-suppressed. This strategy, known as the Cornell Model (McCune *et al.*, 1956; McCune and Tompsett, 1956), recreates paucibacillary latency in the mouse, but with the caveat that non-physiologic reagents need to be imposed. Not surprisingly, studies in the mouse to determine immune effectors required for control of latent Mtb have generated varying results depending on the model used. Several immune factors, though, are agreed upon to be important for control of latent infection. T cells are required for maintenance of latency, although the subset of primary importance is debated (Tufariello *et al.*, 2003), and NOS2 is also important for control of latent Mtb (Flynn *et al.*, 1998).

3. *M. tuberculosis* virulence factors

Although many genes have been described as Mtb virulence factors, there are surprisingly few genes for which both an *in vivo* phenotype and a mechanism of virulence have been identified. Disruption of many of the genes originally studied *in vitro* causes no deficit for growth of the resulting mutants *in vivo*. Conversely, the functions of many of the genes identified by screening of random mutants *in vivo* have not yet been determined. Mutants which do manifest *in vivo* phenotype have been grouped into categories based on replicative ability in the lungs of immuno-competent mice (Hingley-Wilson *et al.*, 2003). Those mutants that demonstrate suboptimal growth during acute

infection are referred to as *giv* mutants, indicating their deficit for "growth *in vivo*". Mutants, such as auxotrophs, that do not replicate at all and are rapidly eliminated from the host are called severe *giv* (*sgiv*) mutants. Mutants that demonstrate no deficit for rapid growth during the acute phase of infection but are unable to maintain high titers after adaptive immunity sets in are referred to as *per* mutants, because they do not persist (Fig. 1B).

Because of the important role played by NOS2 in control of Mtb, many studies have focused on understanding bacterial factors that have evolved to counter this host threat. A number of Mtb genes have been described that have *in vitro* activities for detoxification of RNI or repair of the lesions caused by these species, including *ahpC* (Bryk *et al.*, 2000), *msrA* (St John *et al.*, 2001), *noxR1* (Ehrt *et al.*, 1997), *noxR3* (Ruan *et al.*, 1999), and *glbN* (Ouellet *et al.*, 2002). However, Mtb mutants deficient in any one of these genes demonstrate no phenotype *in vivo*, suggesting that other RNI counter-immune mechanisms may be upregulated in reponse to the *in vivo* environment. Alternately, it may be that Mtb has evolved unconventional methods for coping with the toxicity of nitric oxide *in vivo*. A saturation screen for Mtb mutants that demonstrate increased susceptibility to killing by RNI *in vitro* revealed unexpected candidates: two proteasome-associated proteins (*mpa* and *paf*), a DNA repair enzyme that responds to a broad range of toxic insults (*uvrB*), and an enzyme required for the synthesis of a flavin cofactor (*fbiC*) (Darwin *et al.*, 2003). When tested in mice, the proteasome-associated protein mutants were attenuated for growth *in vivo*. When evaluated in $NOS2^{-/-}$ mice, the *mpa* mutant showed a partial restoration in virulence.

Thus, in the absence of one single enzyme to effectively detoxify nitric oxide, Mtb appears to have evolved a number of mechanisms, both common to other pathogens as well as unique to Mtb, which together act to counter RNI. Mtb's susceptibility to NOS2 is perhaps the result of Mtb's patchwork defense against RNI. It should be noted, however, that evolution might have favored Mtb's limited resistance to RNI. In the absence of NOS2, a mouse is unable to control bacterial replication and succumbs to disease relatively soon after infection. When RNI is present, Mtb achieves an unstable equilibrium with the host: bacterial replication halts, and Mtb persists in the host's lungs despite an ongoing immune response. In a human host, persistence of infection coupled with continued inflammation results in a chronic productive cough laden with Mtb bacilli. Because contagion is via aerosol droplets, Mtb's ability to cause chronic infection maximizes its opportunities to spread to other hosts. Evolution of effective defenses against all aspects of the host immune response would lead to earlier death of the host, and would decrease the distribution of the bacteria.

Several studies have revealed that phox's apparent lack of contribution to control of Mtb is due to efficient bacterial enzymes that have evolved specifically to detoxify host-produced ROI. Mtb possesses one catalase for the breakdown of hydrogen peroxide: KatG. A *katG* mutant is attenuated for growth in wild type mice, but grows identically to wild type Mtb in phox-deficient mice (Ng *et al.*, 2004). The fact that KatG is not required for virulence in the absence of phox suggests that the major, if not only, function of KatG *in vivo* is to defend Mtb against host ROI. Mtb also produces two superoxide dismutases (SODs) for the catalytic degradation of superoxide: SodA, which is iron-dependent, and SodC, which is copper/zinc-dependent. SodA is the predominant

SOD, and disruption of *sodC* does not alter growth *in vivo*; however, a *sodC* mutant is attenuated in IFN-γ-activated macrophage capable of generating ROI (Piddington *et al.*, 2001). When tested in phox-deficient macrophages, SodC mutants grow no differently than wild type Mtb. SodA appears to be required for defense against both endogenous and exogenous (host-generated) ROI. The inability to generate an Mtb mutant with a deleted *sodA* gene suggests that SodA is essential for *in vitro* growth; however, a mutant with reduced expression of SodA is severely attenuated *in vivo* (Edwards *et al.*, 2001), again demonstrating that Mtb has evolved efficient mechanisms for countering ROI. The extreme phenotype of the reduced expression SodA mutant suggests that seemingly ineffective immune pressures may become exceptionally potent when microbial counter-immune mechanisms are disrupted.

The onset of adaptive immunity halts the increase in bacterial titer in the lungs of Mtb infected mice, and results in a shift to the chronic phase of infection. The identities of genes for which disruption results in *per* phenotypes shed light on the nature of Mtb's environment within the granuloma during the chronic phase of infection. Under conditions of nutrient starvation, some bacteria initiate the stringent response, a coordinated shutdown of active metabolism that is mediated by production of hyperphosphorylated guanine nucleotides ((p)ppGpp). Disruption of the Mtb gene *relMtb,* which mediates the synthesis of (p)ppGpp, results in an inability of Mtb to persist *in vivo* (Dahl *et al.*, 2003), supporting the idea that IFN-γ-activated macrophages halt bacterial growth by depleting the phagosome of nutrients. Another stress-induced response that occurs in many bacteria is the "SOS" response, which leads to an increase in the generation of mutations during periods of adverse conditions. DnaE2, one of

Mtb's two major DNA polymerases, appears to be responsible for error-prone DNA synthesis during the SOS response. The Mtb *dnaE2* mutant, like the *relMtb* mutant, replicates like wild type Mtb during acute infection, but is defective in persistence during chronic infection (Boshoff *et al.*, 2003). Further evidence that Mtb is confronted with a state of starvation during chronic infection is provided by studies indicating that the Mtb *icl* mutant is also a *per* mutant (McKinney *et al.*, 2000). As mentioned previously, Icl, functioning as one of two enzymes in the glyoxylate shunt, allows bacteria to convert fatty acids into carbohydrates when glucose is unavailable. The harsh nature of the phagosomal environment has also been illustrated by microarray analysis of Mtb genes induced in the macrophage. Comparison of Mtb genes expressed inside IFN-γ activated macrophages with genes expressed when Mtb is subjected to specified *in vitro* stresses suggests that Mtb experiences iron depletion, decreased oxygen, carbohydrate restriction, and assault on the lipid membrane within the activated macrophage (Schnappinger *et al.*, 2003).

It is becoming increasingly apparent that, in addition to proteins, Mtb lipids and glycolipids act as effector molecules to subvert host cell functions (Beatty *et al.*, 2000). A large proportion of the Mtb genome is dedicated to lipid biosynthesis and lipid degradation genes (Glickman and Jacobs, 2001). Mtb encodes multiple homologs of the *E. coli* β–oxidation genes, enzymes used to break down fatty acids for fuel. However, some of the homologs found in Mtb do not participate in catabolism, but rather contribute to the synthesis of complex lipids. FadD28, one of 36 FadD genes, is required for the production of phthiocerol dimycocerosate (PDIM), a cell wall associated lipid found only in pathogenic mycobacteria that is required for replication of Mtb in the lungs (Cox *et al.*,

1999). Lipids may act as a barrier for Mtb, but their roles go far beyond that. The precise structure of these complex lipids is essential for their contributions to virulence. The cyclopropane synthetase PcaA adds a single propane rings to one chain of mycolic acid. Mutants disrupted in *pcaA* have a *per* phenotype *in vivo* (Glickman *et al.*, 2000). In addition, the *pcaA* mutant triggers an immune response in the lungs that is altered as compared with the response to wild type Mtb, suggesting that mycolic acid may be bioactive lipids and cause immune modulation.

In addition to *giv, sgiv*, and *per,* two newer terms were recently introduced to describe mutants with complex *in vivo* phenotypes. Mutants that produce altered pathology in the lungs without demonstrating altered bacterial growth *in vivo* make up the class of mutants referred to as *pat* mutants, short for "pathology". To date, two mutants have been identified that fit the *pat* designation, and both are related to sigma factor functions. The putative transcription factor WhiB3 is a binding partner of the sigma factor RpoV. WhiB3 is not required for growth *in vivo*, but a *whiB3* mutant causes less pathology in the lungs and thus requires significantly longer to kill its host that wild type Mtb (Steyn *et al.*, 2002). Disruption of the gene *sigH*, which encodes an extracytoplasmic (ECF) alternative sigma factor, also decreases virulence without affecting bacterial replication and persistence *in vivo* (Kaushal *et al.*, 2002).

The *sigH* mutant is another example of the lack of correlation between *in vitro* and *in vivo* data seen for many Mtb mutants. Mtb *sigH* was cloned by homology to the ECF sigma factor *sigE*, and was shown to be involved in resistance to heat shock and oxidative stress in *M. smegmatis* (Fernandes *et al.*, 1999). Soon thereafter, *sigH* was identified by SCOTS (selective capture of transcribed sequences) as being expressed in

Mtb after phagocytosis by human macrophages (Graham and Clark-Curtiss, 1999). Further research demonstrated increased susceptibility to heat shock and oxidative stress in an Mtb *sigH* mutant (Raman *et al.*, 2001). When the Mtb mutant was assayed for virulence in mice, it demonstrated no defect for growth and survival within the lungs, but still required significantly longer to kill the mice (Kaushal *et al.*, 2002). This attenuation was determined to be due to an altered immune response that was capable of controlling bacterial replication, but did not produce extensive immunopathology. Therefore, the increased susceptibly to oxidative stress seen *in vitro* did not appear to be the cause of the *sigH* mutant's attenuation in the mouse.

A fifth mutant classification was created for one mutant for which the phenotype was found to depend on route of infection. The *hbhA* mutant has difficulty disseminating from the lungs to other tissues, and therefore manifests a phenotype only when delivered by the aerosol route (Pethe *et al.*, 2001). This mutant has been dubbed a dissemination (*dis*) mutant. Like the other four categories of mutants, the term "*dis*" is descriptive of the phenotype, but in this case the phenotype provides a guideline for understanding the function of the gene. *hbhA* encodes heparin-binding hemagglutinin which has been shown to bind human complement component 3, as well as heparin and other sulfated sugars (Mueller-Ortiz *et al.*, 2002). The *hbhA* mutant demonstrates no inability to invade macrophages, but has altered interactions with epithelial cells (Mueller-Ortiz *et al.*, 2002; Pethe *et al.*, 2001). Although it has been thought that Mtb is disseminated from the lungs in phagocytes, these data suggest that the ability to bind and interact with epithelial cells contributes to Mtb pathogenesis.

The unique nature of the strategies used by Mtb for survival in the host complicate our ability to deduce the precise mechanisms by which particular Mtb genes lead to virulence. FadD28, PcaA, and Mpa were each identified during screens of random Mtb mutants for specific phenotypes (Cox *et al.*, 1999; Darwin *et al.*, 2003; Glickman *et al.*, 2000). Although mechanisms describing how these enzymes affect pathogenesis have yet to be revealed, the clues we do have about their functions steer us towards considering original ways in which bacteria may have evolved to interact with the host. Limiting the influence of our pre-conceived ideas about virulence may be the most productive way to identify new Mtb virulence factors.

IV. *Salmonella typhimurium*

Salmonellae are Gram negative facultative intracellular bacteria that infect a wide range of animal hosts. The genus *Salmonella* is further subdivided into serotypes that can be differentiated based on the structure of two surface antigens: LPS and flagella. Nomenclature for the different serotypes is commonly confused. There are only two *Salmonella* species, *S. bongori* and *S. enterica*, which encompass eight subspecies (Chan *et al.*, 2003; McClelland *et al.*, 2001). *S. bongori* is subspecies V, and the other seven subspecies –I, II, IIIa, IIIb, IV, VI, and VII- contain over 2200 serotypes that all fall within the species *S. enterica*. Technically, individual serotypes should be referred to as serovars of *S. enterica*; however, in practice, most serotypes are used in place of the name of the *Salmonella* species. For example, *S. enterica* serovar Typhimurium has become known as *S. typhimurium*.

S. typhimurium, one of a small number of *Salmonella* serotypes that infects humans, causes localized inflammation in the human intestine. Although *S. enteriditis*, along with *S. virchow*, is the main cause of Salmonella infection in chickens, it is not one of the main serotypes to infect humans. In addition to *S. typhimurium*, the serotypes that commonly cause enteritis in humans include Heidelberg, Derby and Infantis. *S. arizonae* rarely infects humans, and mostly colonizes reptiles. *S. dublin* is infective to cows, and *S. choleraesuis* infects pigs. *S. bongori* does not cause disease in mammals.

S. typhimurium is categorized in subspecies I of *S. enterica*, and exhibits 89% homology to the serotype Typhi, also in subspecies I (McClelland *et al.*, 2001). *S. typhi* is an exclusively human pathogen that causes the systemic disease typhoid fever. Typhoid fever, although not a pandemic like tuberculosis, causes substantial worldwide morbidity and mortality every year. Due to overcrowding and poor sanitation, typhoid fever affects 16 million people and results in 600,000 deaths each year (Kaufmann *et al.*, 2001). Infection of mice with *S. typhimurium* produces a systemic disease that resembles human infection with *S. typhi*, and thus is used as a model to understand the pathogenesis of typhoid fever.

Salmonella serotypes are highly homologous to several other Gram negative species, including *Escherichia coli, Yersinia* species, and *Shigella* species. With overall genome homology upwards of 70%, these 4 genera would arguably not receive separate designations if they were classified today. For this reason, *S. typhimurium* is frequently studied as a model for understanding bacterial genetics and bacterial cell biology. Moreover, *S. typhimurium* infection of mice can serve as a general model for studying microbial pathogenesis.

The immune response to Salmonella

The natural route of infection for *S. typhi* in humans and *S. typhimurium* in mice is oral. Some bacteria manage to survive gastric defenses, chiefly acid and RNI, and make their way to the small intestine, where they invade the epithelial lining of the terminal ileum to gain access to deeper tissues. Invasion is mediated by the bacteria and occurs through M cells (Jepson and Clark, 2001). M cells are specialized epithelial cells found in follicle-associated epithelium (FAE) that sample antigenic material from the gut lumen and transfer it to gut-associated lyphoid tissue (GALT) found below in the mucosa. In the intestine, the FAE overlies lympoid tissue known as Peyer's patches. *Salmonella* destroy the M cell through which they invade, providing a pathway for more bacteria to enter the mucosa and colonize Peyer's patches (Carter and Collins, 1974; Jones *et al.*, 1994; Kohbata *et al.*, 1986). Alternatively *S. typhimurium* has also been shown to be transported across gut epithelium by $CD18^+$ phagocytes (Vazquez-Torres *et al.*, 1999). This phase of infection is associated with recruitment of PMNs to the site of invasion, which attack bacteria in Peyer's patches and extravasate into the lumen of gut (Cheminay *et al.*, 2004; Gewirtz *et al.*, 2001a). Macrophages in Peyer's patches also participate in the initial immune response to *S. typhimurium*; however, *S. typhimurium* lyses these macrophages and migrates to the lymphatics and bloodstream for transport to lymph nodes, the liver and the spleen.

Within 3 days, most bacteria are found intracellularly within phagocytes. In the spleen, the majority of bacteria localize to macrophages of the red pulp and the marginal zone (Salcedo *et al.*, 2001). In the liver, *S. typhimurium* resides in infiltrating PMNs

during the first few days of infection, and in infiltrating macrophages at later times (Richter-Dahlfors *et al.*, 1997). Resident macrophages of the liver (Kupffer cells) and hepatocytes do not appear to be infected, and there is no appreciable infiltration of B or T cells (Richter-Dahlfors *et al.*, 1997). In susceptible mice, although replication of bacteria is not controlled, death ultimately results from sepsis due to bacterial products, including LPS and flagellin (Khan *et al.*, 1998).

As with Mtb, there are disagreements about the contributions of specific immune factors to the control of *S. typhimurium*. Many of the discrepancies between data can be traced to the use of different strains of mice as well as of different strains of bacteria. Many laboratories use C57BL/6 or BALB/c mice for experimental infections. These strains are homozygous for the susceptible allele of Nramp1 (*Nramp1*^{s}), a critical locus for control of *Leishmania*, *S. typhimurium*, and *M. bovis* var. BCG (Govoni *et al.*, 1996). Thus, even "wild type" C57BL/6 mice are immuno-compromised, as compared to strains such as 129Sv or CBA that are *Nramp1*^{r}.

Several laboratory strains of *S. typhimurium* are also commonly used as virulent "wild type" strains, although genetic differences between the strains have never been characterized. Additionally, some laboratories use clinical isolates of varying virulence. Virulence is determined by the ability of a bacterial strain to kill mice, generally an *Nramp1*^{s} strain such as C57BL/6 or BALB/c. Definitions of virulence vary. For non-oral routes of infection (i.p. or i.v.), some people define virulence as a lethal dose required to kill 50% of the mice (LD_{50}) that is less than 100 cfu (Swords *et al.*, 1997). A virulent LD_{50} for oral or gastric infection is usually considered to be between 10^5 and 10^6 cfu. When comparing mutant bacteria to the parental strain, individual laboratories

determine their own definitions of what attenuation means (Bowe *et al.*, 1998). The first complete genome to be published for *S. typhimurium* was sequenced from the LT2 strain, an attenuated strain which does not express the alternative sigma factor RpoS (Swords *et al.*, 1997). Despite acknowledgement of this genetic insufficiency, there are many laboratories that routinely use LT2 as a "wild type" strain. Some laboratories choose to use attenuated strains of *S. typhimurium* to prolong the duration of infection in susceptible mice; however, a recent study demonstrated that overlapping yet distinct sets of host immune factors are responsible for control of different attenuated *S. typhimurium* mutants with similar virulence phenotypes (Raupach *et al.*, 2003). Thus, infection with attenuated strains does not necessarily reflect on the immune response to virulent strains of *S. typhimurium.*

In addition to the *Nramp1* locus, the production of ROI by phox is an undisputed and critical component of host defense against *S. typhimurium*, as manifest by experiments in mice (Mastroeni *et al.*, 2000; Shiloh *et al.*, 1999), experiments in macrophages (Shiloh *et al.*, 1999; Vazquez-Torres *et al.*, 2000a), and by the increased susceptibility to *Salmonella* infections of individuals with chronic granulomatous disease (Segal *et al.*, 2000). Because *S. typhimurium* resides in the macrophage phagosome during most of the course of infection, it is not surprising that a macrophage cytotoxic mechanism is of primary importance for killing of *S. typhimurium.* Similarly, cytokines known to activate macrophages and promote phox activity are also important mediators of the immune response to *S. typhimurium* (Eckmann and Kagnoff, 2001). A deficiency in TNFα or the TNF receptor severely exacerbates disease and prevents localization of phox to *Salmonella*-containing phagosomes (Nauciel and Espinasse-Maes, 1992;

Vazquez-Torres *et al.*, 2001). This blockade in vesicle fusion in TNF-unresponsive mice cannot be overcome by activation of macrophages with IFN-γ. However, a deficiency in either IFN-γ or IL-12, which stimulates T cells and NK cells to produce IFN-γ, also increases host susceptibility to *S. typhimurium* in both mice (Kincy-Cain *et al.*, 1996; Nauciel and Espinasse-Maes, 1992) and humans (Jouanguy *et al.*, 1999). As with Mtb, mice rendered deficient in both TNFα and IFN-γ fare worse after infection with *S. typhimurium* than mice lacking either cytokine alone (Nauciel and Espinasse-Maes, 1992).

During *S. typhimurium* infection, which cells are making the important IFN-γ: T cells, NK cells, or other cells? Contributions of IFN-γ to control of *S. typhimurium* can be seen using resistant mice infected with virulent bacteria (Nauciel and Espinasse-Maes, 1992), susceptible mice infected with attenuated bacteria (Bao *et al.*, 2000; Kirby *et al.*, 2002), and susceptible mice infected with virulent bacteria (Gulig *et al.*, 1997), and the cells producing IFN-γ are not always the same. With attenuated bacteria in susceptible mice, macrophages and neutrophils were the chief sources of IFN-γ in one study (Kirby *et al.*, 2002), while NK cells produced IFN-γ in a separate study using the same strain of mice but a different attenuated strain of *S. typhimurium* (Schwacha *et al.*, 1998). Splenocytes from resistant mice produce much more IFN-γ in response to *S. typhimurium* than splenocytes from susceptible mice, and the big producers in the resistant mice are NK cells (Ramarathinam *et al.*, 1993a, b).

Although *Nramp1* and phox are both anti-microbial mechanisms found in phagocytes, and although the experiments with cytokine-deficient mice support the relevance of a Th1 response during infection, the roles of specific phagocyte populations

for control of *S. typhimurium* are also not entirely clear. When macrophages are depleted from susceptible mice before infection with virulent bacteria, the mice experience less morbidity and mortality than their counterparts with macrophages (Wijburg *et al.*, 2000). This result demonstrates how completely *S. typhimurium* has evolved to survive and replicate within the macrophage *in vivo*. Yet, macrophage production of TNFα is required for maximum production of IFN-γ by NK cells in the spleen during virulent infection of resistant mice (Ramarathinam *et al.*, 1993b). In the absence of *Nramp1* activity, macrophages appear to be relatively impotent to mount a protective immune response, and therefore become detrimental to the immune response because they function to conceal and protect *S. typhimurium*.

PMN also express phox and kill *S. typhimurium*. Not surprisingly, this difference in the role of macrophages in resistant and susceptible mice influences the relative importance of PMN during infection. In fact, Fierer has shown that a requirement for PMN during *S. typhimurium* infections depends on two specific factors: the *Nramp1* status of the host, and the presence or absence of the virulence plasmid (spv) in the bacterium (Fierer, 2001). *Nramp1*^{s} mice are so susceptible to spv^{+} bacteria that the presence or absence of PMN has no effect on disease progression; however, when susceptible mice are infected with spv^{-} *S. typhimurium*, PMN become an important component of the immune response. Likewise, PMN-depleted *Nramp1*^{r} mice have no problem controlling attenuated spv^{-} bacteria, but PMN do a play a role in resistant mice infected with fully virulent spv^{+} *S. typhimurium*.

Studies using attenuated strains of *Salmonella* to infect susceptible mice, as well as studies using *Nramp1*^{r} mice, demonstrate that both T cells (Lo *et al.*, 1999; Mittrucker

et al., 1999; Weintraub *et al.*, 1997) and B cells (Mittrucker *et al.*, 2000) play a role in immunity to *S. typhimurium*. Because infection of susceptible strains of mice with virulent *S. typhimurium* generally results in murine death before adaptive immunity can develop, the role of T and B cells in the control of *S. typhimurium* is often studied in the context of memory and secondary infection. That is to say: mice are immunized with an attenuated strain of *S. typhimurium*, and then later challenged with a virulent one. Development of resistance during immunization with attenuated strains is dependent on the interactions between B cells and T cells (Mastroeni and Menager, 2003). During secondary infection, Th1 memory is critical for immunity, and T cells become an important source of IFN-γ (Kirby *et al.*, 2002). $CD4^+$ T cells specific for an epitope found in FliC (flagellin), one of the monomers that comprises flagella, account for much of the protective memory response (McSorley *et al.*, 2000).

One of the biggest controversies regarding the immune response to *S. typhimurium* concerns the relevance of NOS2: some researchers find important contributions of NOS2 to control of *S. typhimurium* (Mastroeni *et al.*, 2000), while others do not (Shiloh *et al.*, 1999). A lack of susceptibility of NOS2-deficient mice infected with *S. typhimurium* may be in part due to an insufficiency in NK cell produced IFN-γ in *Nramp1*^{s} strains of mice (Ramarathinam *et al.*, 1993a), and in part due to death of mice before the development of an adaptive immune response and T cell production of IFN-γ. Even with inocula as low as 10 organisms, *S. typhimurium* kills immuno-competent C57BL/6 mice in approximately one week (Shiloh *et al.*, 1999). When experiments are performed during which *S. typhimurium*-infected mice are able to contain infection for at least 2 weeks, NOS2 appears to contribute to the immune response late in infection.

Several studies have demonstrated a role for NOS2 and RNI in control of attenuated strains of *S. typhimurium* (Alam *et al.*, 2002; Mastroeni *et al.*, 2000). Not only do C57BL/6 mice infected with attenuated strains live long enough for T cell responses to develop, but in addition, attenuated strains of *S. typhimurium* have been show to stimulate IFN-γ production by NK cells in *Nramp1*^{s} mice (Schwacha *et al.*, 1998). *Nramp1*^{r} mice, which survive a sufficient time to mount a T cell response, also trigger NK cells to produce early IFN-γ. If *Nramp1*^{r} 129Sv mice are infected with high numbers of virulent *S. typhimurium*, the bacteria do not kill the mice; however, NOS2-deficient 129Sv mice begin to die 10 days after infection, suggesting that late activation of NOS2 is required for continued suppression of infection (Mastroeni *et al.*, 2000).

Contributions of RNI to the macrophage's ability to kill *S. typhimurium* have also been evaluated by comparing macrophages deficient in NOS2 with immuno-competent macrophages. A small impact of NOS2 on killing of virulent *S. typhimurium* can be seen at 20 hours after infection in macrophages from *Nramp1*^{s} C57BL/6 mice, and this effect is enhanced by activation of macrophages with IFN-γ (Vazquez-Torres *et al.*, 2000a). When NOS2-deficient mice on a C57BL/6x129Sv background are evaluated, there is no detectable influence of NOS2 on the killing of virulent *S. typhimurium* by macrophages at 4 hours after infection (Shiloh *et al.*, 1999). The differences in the mouse strains used, the differences in the time points for evaluation, and/or the presence or absence of IFN-γ to activate macrophages may explain the dissimilar results. Considering how much variability in the immune response to *S. typhimurium* is generated depending on the strain of mice and strain of bacteria used, it is not ultimately surprising that researchers disagree about the roles of particular immune mediators in the control of *S. typhimurium* infection.

Defense against ROI and RNI

Many genes have been identified in *S. typhimurium* that contribute to protection against oxidative stress. *S. typhimurium* encodes both constitutive and inducible antioxidant mechanisms. Inducible antioxidant genes are triggered by several stresses including hydrogen peroxide, superoxide, and growth into stationary phase.

Sub-lethal doses of hydrogen peroxide induce about 30 proteins in *S. typhimurium* (Morgan *et al.*, 1986), at least 9 of which are dependent on the transcriptional activator OxyR that is active only when oxidized (Janssen *et al.*, 2003; Storz and Imlay, 1999). OxyR-dependent genes include the catalase *katG*, the peroxiredoxin *ahpCF*, the glutathione reductase *gorA*, the glutaredoxin *grxA*, the DNA binding protein *dps*, the regulatory DNA *oxyS*, and the iron regulatory locus *fur*. Catalases detoxify hydrogen peroxide by converting it to water and oxygen. Peroxiredoxins reduce hydrogen peroxide as well as organic hydroperxoides. Glutathione (GSH) acts as a hydrogen donor, thus maintaining a reducing environment in the cytosol. Glutaredoxins transfer hydrogen ions from GSH to disulfide-linked oxidized cysteine residues. This reaction generates disulfide-linked glutathione (GSSG), which is converted by glutathione reductase back to GSH (Dickinson and Forman, 2002). Fur represses uptake of iron. Because cytoplasmic iron can convert hydrogen peroxide by the Fenton reaction to the very reactive hydroxyl radical, regulation of cytoplasmic iron levels is an important method for minimizing damage resulting from exposure to ROI. The exact mechanism by which Dps prevents DNA mutagenesis specifically resulting from ROI and promotes virulence is not well

understood; however, it may involve sequestering iron and thus preventing localized Fenton reactions (Grant *et al.*, 1998; Halsey *et al.*, 2004).

The SoxR/S regulon, which contains at least 10 genes, is activated as a result of intracellular superoxide (Pomposiello and Demple, 2000; Storz and Imlay, 1999). One of the induced genes, *sodA*, encodes a cytoplasmic, Mn-dependent superoxide dismutase that converts superoxide to hydrogen peroxide. In addition to detoxifying endogenous superoxide, over-expression of SodA promotes resistance of *S. typhimurium* to killing by the J774.1 macrophage cell line (Tsolis *et al.*, 1995). *S. typhimurium* possesses two other SODS, SodCI and SodCII, which are both Cu, Zn-dependent and localize to the periplasm. However, neither *sodCI* nor *sodCII* is regulated by SoxR/S (Fang *et al.*, 1999). The *fur* locus is also part of the SoxR/S regulon, and is involved in a further level of regulation among these genes. When *E. coli* are grown in iron-rich media, Fur binds to the *sodA* promoter and prevents its expression (Niederhoffer *et al.*, 1990). In *S. typhimurium*, inactivation of the Fur repressor results in increased SodA activity and increased resistance to killing by macrophages (Tsolis *et al.*, 1995).

Bacteria grown to stationary phase are subject to a number of stresses associated with high-density cultures, including nutrient deprivation and accumulation of toxic waste products. Expression of genes to cope with these stresses is governed by several alternative sigma factors including σ^S, σ^H, and σ^E, referred to in *S. typhimurium* as RpoS, RpoH, and RpoE. The best studied of these is RpoS, which regulates at least 30 genes upon entry into stationary phase (O'Neal *et al.*, 1994; Talukder *et al.*, 1996), and is active when *S. typhimurium* resides inside host cells (Chen *et al.*, 1996a). RpoS regulates the genes encoding KatE and KatN, 2 of the 3 catalses used by *S. typhimurium* (Robbe-Saule

et al., 2001). RpoS exerts control over KatG expression in *E. coli*, but this relationship has not been evaluated in *S. typhimurium* (Loewen *et al.*, 1998). KatG and KatE both require a heme prosthetic group for their catalytic activity, whereas KatN appears to be a non-heme catalase, like AhpC. RpoE also contributes to regulation of *S. typhimurium*'s anti-oxidant defense, although the mechanism has not been described. An *S. typhimurium* strain deficient in RpoE is attenuated in mice, but is restored in virulence in mice lacking phox (Testerman *et al.*, 2002). RpoS mutants demonstrate a similar pattern of differential virulence, although *rpoE/rpoS* double mutants remain relatively attenuated in phox-deficient mice, highlighting the fact that these sigma factors regulate other genes in addition to anti-oxidant defenses (Testerman *et al.*, 2002).

The role of heme catalases in detoxification of ROI is underscored by the increased susceptibility to hydrogen peroxide of an *S. typhimurium* mutant defective in *hemA,* which catalyzes the first committed step in heme biosynthesis (Elgrably-Weiss *et al.*, 2002). Heme catalases appear to be important to *S. typhimurium* when bacterial density is high and concentrations of hydrogen peroxide are in the millimolar range. However, they may be redundant *in vivo*, as a katE/katG double mutant is fully virulent in both mice and macrophages (Buchmeier *et al.*, 1995). This resistance to ROI may be due to residual catalase activity provided by KatN or AhpC, or due to the fact that DNA repair enzymes (RecBC) appear to play a more important role in defenses againt ROI than catalases during exposure to lower concentrations of hydrogen peroxide (Buchmeier *et al.*, 1995).

A number of regulatory genes have been implicated in resistance to ROI, including RamA (van der Straaten *et al.*, 2004), SlyA (Buchmeier *et al.*, 1997), and ArcA

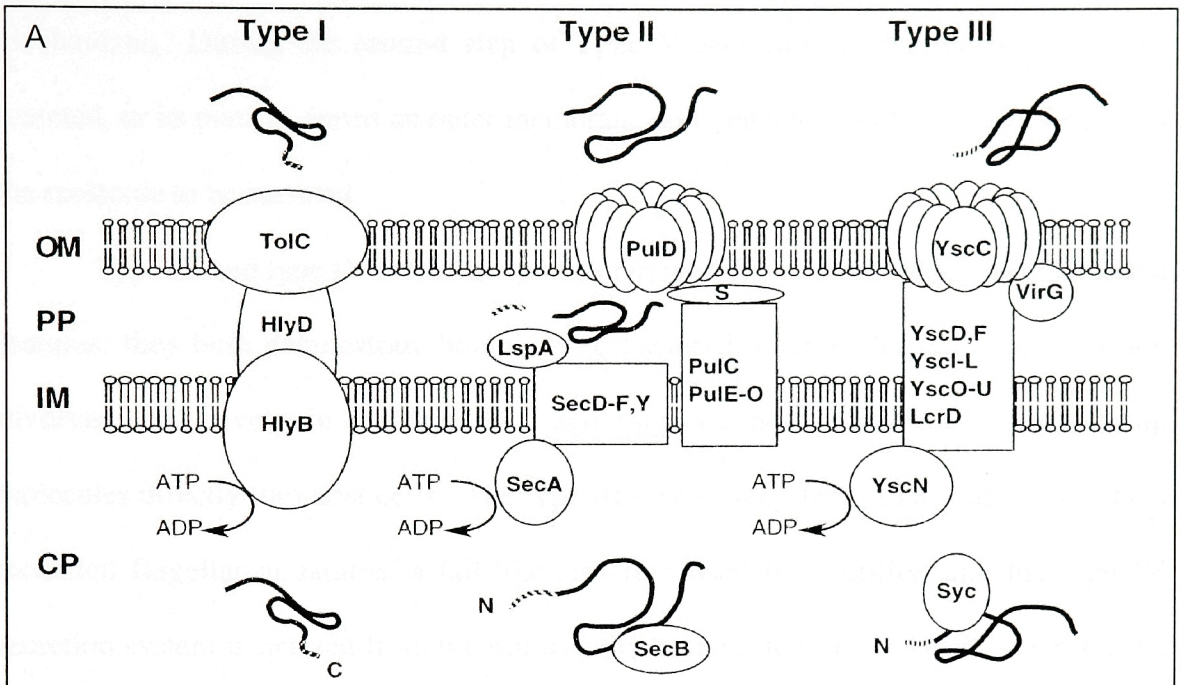
(Lu *et al.*, 2002). The ArcA locus is also associated with resistance to RNI (Lu *et al.*, 2002). Other *S. typhimurium* genes that protect against both RNI and ROI include *sodCI* (De Groote *et al.*, 1997), *msrA* (St John *et al.*, 2001), and *ahpCF* (Chen *et al.*, 1998). AhpCF can catalytically detoxify peroxynitrite (Bryk *et al.*, 2000). Resistance to RNI mediated by SodCI and MsrA also appears to be the result of interference with peroxynitrite's ability to cause damage. It is thought that elimination of superoxide by SodCI prevents peroxynitrite from forming, and MsrA, which reduces oxidized methionines, appears to repair oxidative lesions caused by peroxynitrite.

Type III secretion systems

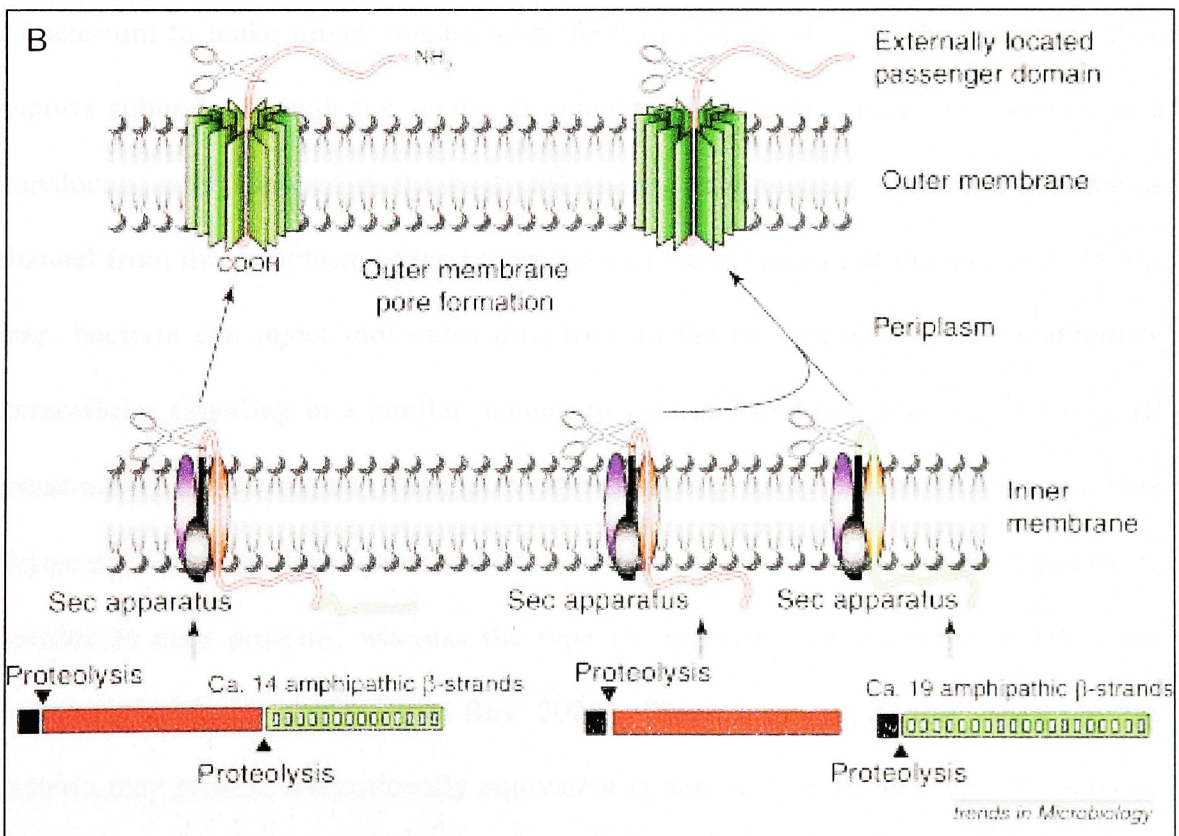
Virulence of virtually all pathogens relies upon secretion of protein or lipid mediators that manipulate host cells. For Gram negative bacteria, secretion is complicated by the challenge of two membranes, separated by the periplasmic space, across which molecules need to be transported. Evolution has provided Gram negative with at least five solutions to this problem (Fig. 2) (Henderson *et al.*, 2000; Hueck, 1998). Type I secretion and type III secretion involve direct transport of molecules through channels that span both membranes and the periplasmic space. Type II sec-dependent secretion is a two-step process. Proteins for export are first transported from the cytoplasm into the periplasm by the *sec* pathway, which is also used to transport proteins whose final destination is the periplasm. A second set of transporters then exports proteins targeted for secretion across the outer bacterial membrane. Type IV secretion can be *sec*-dependent or *sec*-independent, with some substrates initially brought into the periplasm, and others exported directly from the cytoplasm. Type V secretion, which

Figure 2. Secretion systems of Gram negative bacteria. (A) Schematic overview of the type I, II, and III secretion systems as exemplified by alpha-hemolysin secretion by *E. coli* (type I), pullulanase secretion by *Klebsiella oxytoca* (type II), and Yop secretion by *Yersinia* (type III). OM, outer membrane; PP, periplasm; IM, inner membrane; CP, cytoplasm. (B) The secretion pathway of the true autotransporters is depicted in the bottom left of the diagram and the 'unlinked' autotransporters in the bottom right. The three functional domains of the proteins are shown: the leader sequence (black), the passenger domain (red) and the β-domain (green). The autotransporter polyproteins are synthesized and generally exported through the cytoplasmic membrane via the Sec machinery (colored ovals). However, some autotransporters possess extended signal sequences that make it at least possible that alternative pathways are involved. Once through the inner membrane, the signal sequence is cleaved and the β-domain inserts into the outer membrane in a biophysically favored β-barrel structure that forms a pore in the outer membrane. After formation of the β -barrel, the passenger domain inserts into the pore and is translocated to the bacterial cell surface where it might undergo further processing.

Figure 2.



Modified from Hueck CJ (1998). *Microbiol Mol Biol Rev* **62**: 379-433.



Modified from Henderon C.J., Cappello R, and Nataro JP (200). *Trends Microbiol* **8**: 529-532

includes auto-tranporters and two-partner secretion systems, is two-step, *sec*-dependent mechanism. During the second step of Type V secretion, either the protein to be secreted, or its partner, forms an outer membrane pore intended solely for the transport of the molecule to be secreted.

Type III and type IV secretion systems required for virulence share two additional features: they both demonstrate homology to bacterial macromolecular complexes not involved exclusively in pathogenesis, and they are both capable of translocating molecules directly into host cells. The type III secretion system (TTSS) appears to be a modified flagellar apparatus, a tail-like structure used for motility, and the type IV secretion system is derived from the pili used by bacteria to transfer DNA between cells during conjugation (Fig. 3). The needle-like projections of these secretion systems allow a bacterium to make direct contact with the host cell membrane. The bacterium then exports subunits through the secretion apparatus that form a pore, also known as a translocon, which inserts in the host cell plasma membrane, providing a continuous channel from the cytoplasm of the bacterium into the cytoplasm of the host cell. In this way, bacteria can inject molecules directly into the host cells, and thus manipulate intracellular signaling in a similar manner to certain secreted exotoxins. The type III secretion apparatus, which is often to as referred as the "needle complex" because it both physically and functionally resembles a syringe (Kubori *et al.*, 1998), appears to translocate only proteins, whereas the type IV apparatus can export both DNA and proteins (Hueck, 1998; Nagai and Roy, 2003). There is one report that Gram positive bacteria may possess a functionally equivalent system to type III and type IV secretion that allows transfer of molecules from the bacterial cytosol directly into the host cytosol

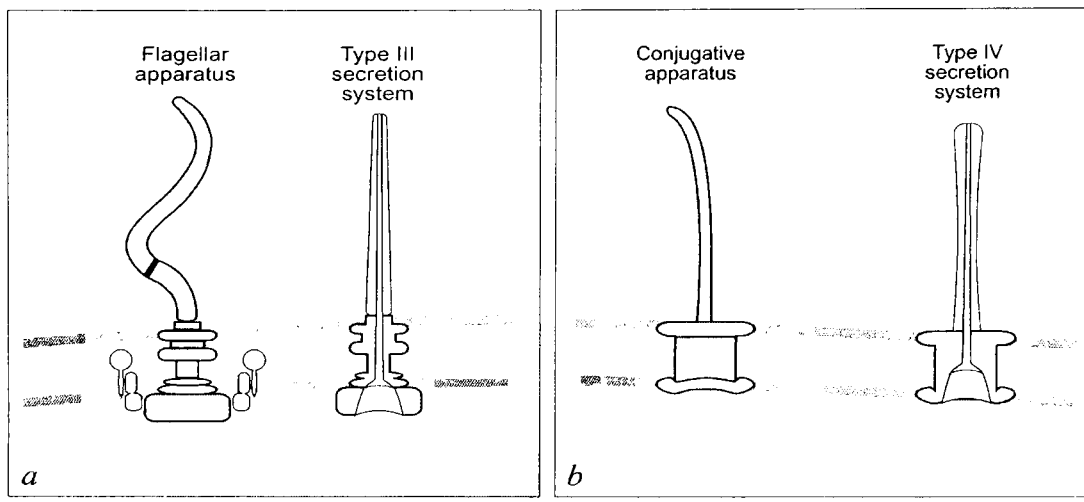


Figure 3. Schematic representation of a bacterial flagellum (*a*) and a conjugative pilus (*b*) showing their similarity to type III and type IV secretion systems, respectively.

Modified from Covacci, A. and Rappuoli, R. (2000). *J. Exp. Med.* **191**: 587-592.

(Madden *et al.*, 2001). Further studies will be required to determine whether this phenomenon is widespread, or unique to *Streptococcus pyogenes*.

Salmonella pathogenicity islands

"Virulence factors" can be defined as any gene required for growth of a pathogen within its host (Groisman and Ochman, 1997). However, many genes fitting this description, such as genes for nutrient synthesis, are also present in strains of commensal bacteria that live symbiotically with their hosts. A more rigorous definition of "virulence factors" are those genes required for growth *in vivo* that are not found in related nonpathogenic bacteria. Virulence factors clustered on the chromosome are called pathogenicity islands (PAIs) (Groisman and Ochman, 1997; Marcus *et al.*, 2000). The genes of a PAI often work together to enact a particular virulence function that is relevant at a specific time during infection, such as expression of a TTSS. PAIs appear to have been obtained by most bacteria through the process of horizontal gene transfer, based on their location in the genome (near sites common for insertions such a tRNA genes), their G+C content being dissimilar to the rest of the genome, and the fact that PAIs often encode all of the components required to enact complex phenotypes. Acquisition of a PAI virulence cassette can sometimes be sufficient to convert a nonpathogenic species into a virulent one (McDaniel and Kaper, 1997).

There are five *Salmonella* pathogenicity islands (SPIs) located on the chromosome, two of which encode virulence-related TTSSs. SPI-1 appears to have been the older of the two TTSS, acquired earlier in the evolution of *S. typhimurium* than SPI-2 (Amavisit *et al.*, 2003). The SPI-1 encoded TTSS confers upon *Salmonella* the ability to

invade epithelial cells, and is homologous to TTSSs in *Shigella, Yersinina, Erwinia*, and *Pseudomonas,* all with similar roles in pathogenesis (Groisman and Ochman, 1997). The SPI-2 TTSS is necessary for growth in macrophages, and appears to be unique to *Salmonella* species, although some of the genes encoding the needle apparatus demonstrate mild homology to *Yersinina* and *E. coli* genes. Perhaps because it is newer to the genus, or perhaps because of the nature of its function, SPI-2 is the most highly conserved of the SPIs among *Salmonella* serotypes (Amavisit *et al.*, 2003)

The TTSSs encoded by SPI-1 and SPI-2 require four classes of proteins for complete function: apparatus proteins, regulatory proteins, secreted effectors, and chaperones for the secreted effectors. Although the genes encoding the secretion apparati, as well as most of the chaperones and regulators, are located within the PAIs, genes encoding secreted effectors are found in the PAI and scattered throughout the chromosome. A number of TTSS effectors are clustered together in another PAI: SPI-5 (Hong and Miller, 1998; Wood *et al.*, 1998). SPI-5 is the most recently identified of the SPIs, and includes some genes with homology to *Shigella* invasion genes. SPI-5 encodes effectors secreted by both the SPI-1 and SPI-2 TTSSs (Knodler *et al.*, 2002). The SPI-1 TTSS effectors found at SPI-5 are required for enteropathogenesis in cattle, but have not been shown to play a role in disease in mice (Hapfelmeier *et al.*, 2004), despite promoting invasion of cell lines *in vitro* (Hong and Miller, 1998). The exact functions of the SPI-5 encoded SPI-2 effectors are not clear.

The nucleotide composition of SPI-3 and SPI-4 indicates that they were both horizontally acquired by the *Salmonella* genome; however, unlike the other SPIs, they exhibit mosaic structures, and there is no obvious functional relationship between the

gene products within each PAI. SPI-3 genes, including the *mgtBC* encoded magnesium transporter, are important for survival in macrophages (Blanc-Potard *et al.*, 1999). The function of SPI-4 is less clear, although a locus within the region contributes to intramacrophage survival (Wong *et al.*, 1998).

In addition to these chromosomal virulence cassettes, many *Salmonella* serotypes contain a virulence plasmid, which varies in size between different serotypes. In *S. typhimurium*, pSLT is 95 kb. The *spv* operon, *spvRABCD*, is present on all *Salmonella* virulence plasmids, and contributes to virulence in mice by promoting survival in macrophages (Gulig *et al.*, 1998). The actions of *spvB* and *spvC* are sufficient to confer the survival advantage (Matsui *et al.*, 2001). The exact mechanisms of these virulence factors are unknown, although SpvB acts can ADP-ribosylate actin in host cells, thus preventing actin polymerization (Lesnick *et al.*, 2001).

PhoP-PhoQ

Many prokaryotes use two-component regulatory systems to convert detection of environmental changes into transcriptional responses (Stock *et al.*, 2000). The archetypal sensor kinase is a transmembrane protein composed of two domains: an N-terminal sensor domain that detects changes in the periplasm, and a cytoplasmic kinase domain that autophosphorylates a specific C-terminal histidine residue. The activated sensor kinase transfers the phosphate to a specific aspartate residue on the cytoplasmic response regulator. Phosphorylation causes a conformational change in the response regulator,

allowing the protein to mediate a response to the extra-cytoplasmic change. Frequently this response consists of the response regulator binding to specific promoters, thus activating or repressing genes that will address the altered extra-cytoplasmic environment of the bacterium.

The PhoP-PhoQ system is the first example of a two-component regulatory system for which the primary function is to signal changes in extracellular magnesium levels (Groisman, 2001). Autophosphorylation of the PhoQ sensor kinase is inhibited by millimolar levels of extracellular Mg^{2+}, Ca^{2+}, or Mn^{2+}, whereas micromolar levels of these ions activate the system. PhoP and PhoQ are found in a number of bacterial species, including both pathogenic and non-pathogenic strains, and appear to regulate four main classes of genes: genes that regulate adaptation to limited environmental magnesium, genes that regulate virulence in pathogenic strains, genes that alter the bacterial outer envelope, and species-specific genes that confer unique properties to particular organisms.

In *S. typhimurium*, the PhoP/Q system regulates at least 40 genes, representing 1% of total ORFS, some of which appear to be ancestral while others appear to have been acquired by horizontal transfer (Lejona *et al.*, 2003). PhoP/Q is required for virulence in both mice and macrophages (Miller *et al.*, 1989). Two independent studies demonstrated that the majority of *S. typhimurium* genes identified as being induced within macrophages are PhoP-dependent (Heithoff *et al.*, 1999; Valdivia and Falkow, 1997). The contributions of PhoP/Q to virulence are presumably due to a combination of the multiple deficiencies of the *phoPQ* mutant demonstrated *in vitro*, including increased susceptibility to killing by antimicrobial peptides, bile salts, and low pH. The PhoP-

regulated genes involved in providing resistance to these host mechanisms can be divided into three categories: adaptations to changes in extracellular cations (specifically magnesium), resistance to antimicrobial peptides, and activation of virulence factors.

Adaptation to low extracellular magnesium takes many forms. The *mgt* genes (*mgtA*, *B*, and *C*) are directly activated by PhoP and encode magnesium transporters. *mgtC* is required for growth in limited magnesium as well as for growth in macrophages, suggesting that the intra-phagosomal environment of *S. typhimurium* is deficient in Mg^{2+} (Blanc-Potard and Groisman, 1997). In addition to the need to cope with nutritional deprivation, *S. typhimurium* must adjust for the change in ionic content of its environment. The bacterial surface chelates cations as a method of preventing repulsion of adjacent negatively charged LPS molecules. As extracellular magnesium decreases, there is less positive charge available to stabilize LPS molecules. PhoP induces the transcription of a second two-component regulatory system, PmrA/B, which activates the *ugd* and the *pbgPE* operon. These genes mediate the incorporation of 4-aminoarabinose into Lipid A of LPS, decreasing the overall net negative charge of LPS. This modification of Lipid A effectively balances the effects of reduced available magnesium, thus stabilizing surface interactions between adjacent LPS molecules to maintain the integrity of the bacterial envelope.

Addition of 4-aminoarabinose to Lipid A also serves to increase *S. typhimurium's* resistance to the antimicrobial peptide polymyxin B. Several other PhoP-activated genes have been identified that increase the bacterium's ability to defend against host-derived antimicrobial peptides. *pagP* encodes an enzyme that incorporates palmitate into Lipid A, a modification that heightens resistance to some antimicrobial peptides (although not

to polymyxin B). PgtE is an outer membrane protease that cleaves particular antimicrobial peptides. Studies comparing mutant strains of *S. typhimurium* suggest that other PhoP-activated genes involved in defense against antimicrobial peptides remain to be identified.

Beyond these specific PhoP-activated pathways, the PhoP/Q system contributes to the repression and activation of *S. typhimurium*'s major virulence mechanisms. Consistent with the role in adaptation to limited divalent cations and resistance to antimicrobial peptides, the genes influenced by PhoP are critical for survival once *S. typhimurium* has translocated from the intestine into host tissues. PhoP represses SPI-1 genes required for invasion of gut epithelial cells, but appears to positively regulate the *spv* genes, although this remains controversial.

Salmonella pathogenicity island –1 (SPI-1)

SPI-1 was originally identified as a critical locus for *S. typhimurium* invasion of epithelial cells in the intestine (Galán and Curtiss, 1989; Jones and Falkow, 1994; Mills *et al.*, 1995). *S. typhimurium* mutants defective in this locus have a higher LD_{50} (100-1000x) than wild type strains when infection occurs orally, although SPI-1 mutants demonstrate no deficit for survival and growth *in vivo* after i.p. infection (Galán and Curtiss, 1989; Penheiter *et al.*, 1997). Being the first TTSS and the first PAI identified in *S. typhimurium*, the locus was not originally designated "SPI-1", but rather "inv/spa" to indicate the contribution of the locus to invasion and the homology of some SPI-1 genes to the *Shigella spa* locus (Groisman and Ochman, 1993).

SPI-1 contains genes encoding a TTSS, several secreted effectors, chaperones, and regulators for expression of the locus, including *invF* and *hilA*. Expression of SPI-1 genes occurs during late log phase growth at 37°C *in vitro*. Not surprisingly, optimal expression occurs under conditions that approximate the environment in the lumen of the intestine, including neutral pH and oxygen-limitation (Bajaj *et al.*, 1996). Initially it was thought that secretion of SPI-1 effectors was dependent on bacterial contact with host cells; however, a shift in pH from acidic to alkaline, as would be expected to occur as bacteria move from the stomach to the intestine, is sufficient to induce SPI-1 secretion (Daefler, 1999).

HilA, the master regulator of SPI-1, activates expression of *invF*, which in turn acts in concert with the SPI-1 chaperone SicA to induce expression of SPI-1 effectors encoded both within SPI-1, as well at other loci on the chromosome (Darwin and Miller, 1999, 2001b). Many regulators have been shown to influence expression of *hilA* including RtsA and RtsB (Ellermeier and Slauch, 2003), HilC, HilD and HilE (Akbar *et al.*, 2003; Baxter *et al.*, 2003), Hha (Fahlen *et al.*, 2001), CsrA (Altier *et al.*, 2000), and the two component regulatory systems PhoP/PhoQ (Pegues *et al.*, 1995) and BarA/SirA (Johnston *et al.*, 1996; Lawhon *et al.*, 2002). The specific environmental signals that lead to activation of the individual regulators are not well understood; however, short chain fatty acids found in the intestine have been shown to trigger SPI-1 expression by signaling through BarA/SirA (Lawhon *et al.*, 2002).

There are at least 13 effectors known to be secreted by the SPI-1 TTSS (Zhou and Galán, 2001). Biochemical function has been established for more than half of the effectors, although almost all of the effectors have been characterized according to

contributions to virulence. Three of these secreted proteins act together to form the SPI-1 translocon: SipB, SipC, and SipD (also referred to as SspB, SspC, and SspD). SipA, SipC, SopB (also known as SigD), SopE, SopE2, and SptP are involved in actin rearrangements and mediate invasion of epithelial cells. SipA, SopA, SopB, SopD, and SopE2 are responsible for *S. typhimurium*-induced enteropathogenesis in cattle (Zhang *et al.*, 2002). SspH1, which can be secreted by both SPI-1 and SPI-2, is not found in all isolates of *S. typhimurium*; however, when present, it participates in lethal virulence in calves by an unknown mechanism (Miao *et al.*, 1999). SlrP and AvrA were identified as SPI-1 effectors based on homology to *Yersinia* TTSS secreted effectors, but their precise role has yet to be determined. Although AvrA is homologous to *Yersinia*'s YopJ, it was named based in its homology to the gene AvrRxx, a virulence factor found in the plant Xanthomonas (Hardt and Galan, 1997). Like SspH1, SlrP appears to have the unusual ability to be secreted by either SPI-1 or SPI-2.

The mechanism by which SPI-1 effectors mediate invasion of epithelial cells *in vitro* has been well characterized (Gruenheid and Finlay, 2003). The effectors SopE and SopE2, which act as guanine-nucleotide-exchange factors (GEFs), are first injected into the epithelial cell where they act to activate Cdc42 and Rac1. SopB/SigD, found in SPI-5 but secreted by the SPI-1 TTSS, can also activate these small GTPases indirectly. Activated Cdc42 and Rac1 mediate localized actin polymerization that results in plasma membrane ruffling. SipA binds to and stabilizes rearranged actin in the cytosol. SipC, which is present at the site of contact between the bacterium and the membrane because it functions as part of the translocon, nucleates and bundles actin at the membrane. SipC also appears to bind cytokeratins, suggesting that intermediate filaments may also

participate in the invasion process. SigD/SopB, which functions as an inositol phosphatase, breaks down phosphatidylinositol-4,5-bisphosphate (PIP_2), assisting in plasma membrane invaginations at the commencement of invasion and in vacuolar sealing at the end. Once the bacterium is enclosed in the newly formed SCV, the GAP (GTPase-activating protein) function of SptP reverses the actions of SopE and SopE2 by turning off Cdc42 and Rac1. SptP also demonstrates tyrosine phosphatase function, and may act to alter vimentin or the architecture of intermediate filaments. Microtubules do not appear to be involved.

The contributions of SPI-1 to penetration of the epithelial barrier in the intestine *in vivo* have recently been further dissected to reveal that, although SPI-1 is required for invasion of epithelial cells, SPI-1 is not required for dissemination of bacteria from the intestine after oral inoculation (Lostroh and Lee, 2001). *S. typhimurium* defective in both the SPI-1 gene *invA* and the fimbrial gene *lpfC*, which targets bacteria to Peyer's patches, gain access to the spleen and liver after being transported across the intestinal epithelium by $CD18^+$ phagocytes (Vazquez-Torres *et al.*, 1999). Moreover, although *hilA* mutants demonstrate a decreased ability to disseminate from the gut, mutants lacking the entire SPI-1 locus, which cannot invade tissue culture cells *in vitro,* colonize mice as effectively as wild type *S. typhimurium* after gastric inoculation (Murray and Lee, 2000). The ΔSPI-1 mutant, however, has a higher LD_{50} (>100x) than either the *hilA* mutant or the wild type strain. The difference in phenotypes of the two mutants can be explained if both *hilA*-dependent and *hilA*-independent SPI-1 encoded factors are required for full virulence. In a model proposed by Murray and Lee, a SPI-1-dependent, *hilA*-independent factor causes the recruitment of inflammatory cells to the site of infection. SipB, a *hilA*-dependent

SPI-1 TTSS effector, has been shown to exert cytotoxic effects on macrophages (Hersh *et al.*, 1999). Bacteria with intact *hilA* thus eliminate infiltrating macrophages, and proceed through the lamina propria to the bloodstream. Mutants lacking *hilA* are unable to induce macrophage death after they are ingested due to an inability to express *sipB*, and therefore *hilA* mutants are killed by macrophages. The results of Murray and Lee suggest that mutants lacking all of SPI-1 trigger decreased recruitment of inflammatory cells, and thus SPI-1 mutants transverse the epithelium without being deterred by immune cells. The attenuation of SPI-1 mutants despite being fully competent to colonize the mouse suggests either that SPI-1 mediates additional virulence functions, or that inflammatory responses to invasion are required for full virulence of *S. typhimurium.*

Salmonella pathogenicity island – 2 (SPI-2)

Unlike SPI-1, which appears to be dispensable during infection following i.p. or i.v. inoculation, the SPI-2 TTSS is required for growth of *S. typhimurium* in mice and in macrophages (Hensel *et al.*, 1995; Ochman *et al.*, 1996; Shea *et al.*, 1996). *In vivo* deficiencies of SPI-2 mutants can be observed starting at 8 hours after infection, at which time SPI-2 mutants fail to proliferate in the spleen and liver, in contrast to the more than 1000-fold increase in cfus of wild type bacteria in these organs during the first 3 days of infection (Shea *et al.*, 1999). The failure of SPI-2 mutants to grow in macrophages results from the mutants' inability to remodel the phagosome. Wild type *S. typhimurium* manipulates the macrophage's endocytic network to create a hospitable niche in which to replicate. Phox is diverted away from the phagosome, and phagosome maturation is

halted (Waterman and Holden, 2003). In the absence of these adjustments, SPI-2 mutants are unable to proliferate.

Expression of SPI-2 is induced after phagocytosis of *S. typhimurium* by the macrophage. Environmental signals trigger the membrane protein EnvZ to phosphorylate the response regulator OmpR, which in turn activates transcription of *ssrAB* (Lee *et al.*, 2000a). SsrA and SsrB act as the master regulators of SPI-2 TTSS expression. These two proteins, which function as a two-component regulatory system, are not related to the SsrA/tmRNA system that some bacteria use to mark proteins for degradation that have stalled during translation (Karzai *et al.*, 2000). Transcription of SPI-2 genes is triggered by low extracellular magnesium or calcium or by low extracellular phosphate levels (Deiwick *et al.*, 1999), suggesting that these conditions are representative of the environment within the macrophage phagosome. In contrast to SPI-1 effectors that are secreted when the pH shifts from acid to alkaline, SPI-2 secretion is stimulated by a shift to acid pH (Beuzon *et al.*, 1999). Although the ramifications are not known, SsrB exerts influence over SPI-1 genes, including *hilA*, in addition to SPI-2 genes (Deiwick *et al.*, 1998; Deiwick *et al.*, 1999).

SPI-2 effectors

When SPI-2 was identified, many of the genes were recognized to be homologous to genes from SPI-1, as well as from the TTSSs in *Yersinina* and *E. coli*. Accordingly, the SPI-2 genes were designated as *ssa* (secretion system apparatus), *ssr* (secretion system regulator), *sse* (secretion system effector) or *ssc* (secretion system chaperone) (Hensel *et al.*, 1997). The secreted effectors were later divided into two categories: true

effectors that are injected into host cells and translocon proteins that form a pore in eukaryotic cell membranes (Nikolaus *et al.*, 2001; Scherer *et al.*, 2000). The SPI-2 translocon is encoded by SseB, -C, and –D (Nikolaus *et al.*, 2001). In addition to the secretion system genes, the SPI-2 locus contains 12 genes that do not contribute to the TTSS, including a tetrathionate reductase complex and several ORFs of unknown function (Hensel *et al.*, 1999).

The precise functions of the at least 23 individual secreted effectors of SPI-2 are mostly unknown. The first gene identified to encode a secreted SPI-2 effector was *spiC*. *spiC* is found between the *ssr* genes and the *ssa* genes within SPI-2. Initial studies by Groisman's group indicated that a *spiC* mutant is greatly attenuated for growth in macrophages, and that SpiC is secreted into host cells where it disrupted trafficking of endosomes, thus potentially explaining how the *Salmonella*-containing vacuole (SCV) is diverted from normal endosome-lysosome fusion (Uchiya *et al.*, 1999). The role of SpiC as a secreted effector was later challenged by Freeman et al., who suggested that SpiC in fact acts as a chaperone for the translocon proteins SseB and SseC (Freeman *et al.*, 2002). In addition to demonstrating that the *spiC* mutant does not secrete SseB or SseC, the authors noted that the extreme attenuation of the *spiC* mutant resembles that of an apparatus mutant, not an effector mutant. Groisman and colleagues recently responded by demonstrating that SpiC interacts with the macrophage protein Hook3, and that a dominant negative Hook3 affects endosomal trafficking in the same manner as either expression of SpiC or infection by *S. typhimurium* (Shotland *et al.*, 2003). Additionally, another group identified the mammalian protein TassC as a SpiC binding partner, and depletion of TassC by anti-sense oligonucleotides resulted in enhanced survival of a *spiC*

mutant (Lee *et al.*, 2002). Thus, although it is clear that SPI-2 dysregulates vescicular trafficking in macrophages, it is uncertain which effectors are mediating this function.

Like SPI-1, SPI-2 encodes only a few secreted effectors. Most of the known SPI-2 effectors located outside SPI-2 were identified by genetic, not functional, strategies. Miao and Miller reasoned that, like secreted effectors of other TTSSs, SPI-2 effectors should contain a consensus amino acid sequence that targets them for secretion. Alignment of the sequences of effectors isolated from culture supernatants (SspH1, SspH2, and SlrP) revealed a conserved N-terminal domain that was used to identify five new effector genes: *sseI*, *sseJ*, *sifA*, *sifB*, and *sopD2* (Brumell *et al.*, 2003; Miao and Miller, 2000).

Expression of most of the TTSS structural genes within SPI-2 is governed by the SsrA/B two-component regulatory system. Considering the possibility that genes outside SPI-2 that function with the SPI-2 TTSS might be co-regulated, Worley, et al. investigated the scope of the SsrB regulon (Worley *et al.*, 2000). Transcriptional fusions revealed that more than 10 genes outside of SPI-2 are are regulated by SsrB. These genes, referred to as SsrB regulated factors (srfs), were mostly novel with the exception of *srfH* (*sseI*). The functions of SrfA-M still remain unknown.

SPI-2 was originally identified by Holden's group during an STM screen for *S. typhimurium* virulence genes (Hensel *et al.*, 1995). The technique of STM sets bacterial mutants to compete *in vivo* to reveal which mutants are less fit than their virulent counterparts. Holden extended this principle to identify secreted effectors of SPI-2: by competing single mutants with double mutants, Holden was able to determine whether virulence factors function in the same or different pathways. Using competitive

infections, Holden's group found that SifA acts in the same virulence mechanism as SPI-2 (Beuzon *et al.*, 2000). Furthermore, Holden's group demonstrated that SifA is secreted, and contributes to remodeling of the SCV membrane. SifA was named for its contribution to the formation of *Salmonella*-induced filaments (sifs), structures that result from complex actin and membrane rearrangements triggered by the bacteria. When SifA is not secreted, *S. typhimurium* is unable to maintain the integrity of the vacuolar membrane and individual bacteria end up in the cytoplasm of the macrophage where they are unable to replicate. SifA appears to be required for recruitment of membrane to the expanding SCV, and another SPI-2 effector, SseJ, participates in disassembling or remodeling the SCV membrane (Ruiz-Albert *et al.*, 2002). Thus, if both *sseJ* and *sifA* are mutated, the double mutant does not lose its vacuolar membrane in the macrophage, although it is attenuated *in vivo*.

The formation of Sifs can be observed microscopically after infection of epithelial cells with wild type but not SPI-2 mutant *S. typhimurium*. Using changes in host cell morphology as an indicator of SPI-2 effector activity, Guy et al. screened for mutants, which, like the *sifA* mutant, are unable to induce sifs (Guy *et al.*, 2000). *spiC* and several SPI-2 apparatus genes were identified, as well as a locus in the *smf-aroE* intragenic region and a locus on *S. typhimurium*'s virulence plasmid. The SPI-2 encoded genes *sseF* and *sseG* also influence sif formation. Although *sseBCD* encode the SPI-2 translocon, *sseFG* appears to encode true secreted effectors. Secretion of both proteins into culture supernatant as well as translocation into host cells and involvement in sif formation has been demonstrated (Hansen-Wester *et al.*, 2002; Kuhle and Hensel, 2002).

Finally, analysis of SPI-5 led to the discovery that, although some SPI-5 gene products are secreted by SPI-1, the gene *pipB* is part of the SPI-2 regulon. Furthermore, PipB is translocated by SPI-2 into the macrophage cytosol where it contributes to sif formation (Knodler *et al.*, 2002).

The Salmonella-containing vacuole and intracellular trafficking

Two separate *Salmonella*-containing vacuoles (SCVs) are generated during *S. typhimurium* infection of the mouse: the SCV formed after SPI-1 mediated invasion of epithelial cells, and the SCV created out of the phagosome resulting from the actions of SPI-2 within the macrophage. Both SCVs differ from the traditional phagosome due to manipulation of host cell enzymes and cytoskeletal structures by translocated TTSS effectors.

The process of SPI-1 forced entry into epithelial cells is complete within 90 minutes (Garcia-del Portillo and Finlay, 1995), at which time the SCV associates with the early endosomal markers EEA1 (early endosomal antigen 1) and the transferrin receptor (TF-R). Within 15 minutes of internalization, the SCV undergoes rab7-dependent fusion with compartments that contain lysosomal glycoproteins (lgps) and lysosomal acid phosphatase (LAP). Fusion appears to be controlled by the bacterium and results in segregation from the endocytic pathway. The SCV does not follow a conventional maturation resulting in the formation of a true lysosome because the SCV does not acquire the mannose-6-phosphate receptor or cathepsin D. However, the SCV is also isolated from the early endocytic/recycling network, resulting in only limited access to the SCV by fluid endocytic markers (Garcia-del Portillo and Finlay, 1995).

While SPI-1 enables *S. typhimurium* to invade normally non-phagocytic epithelial cells by inducing a "splash" in the membrane, *S. typhimurium* takes advantage of the phagocytic nature of macrophages to facilitate its uptake by these cells. SPI-2, therefore, mediates not the creation of a vacuole, but rather the modification of the phagosome into a niche suitable for *Salmonella* survival and replication (Holden, 2002). However, *Salmonella* does influence the macrophage during phagocytosis, instigating what resembles macropinocytosis, rather than traditional receptor-mediated endocytosis. Diffuse ruffling occurs at the surface of the macrophage. This is distinct from the ruffling mediated by SPI-1, which is localized on epithelial cells at the point of invasion. Morphologically, the SCV resembles a macropinosome with space between the bacterium and the membrane, rather than a close fitting phagosome (Alpuche-Aranda *et al.*, 1994). The spacious vacuole forms around *S. typhimurium* regardless of the method of opsonization, although vacuoles formed after IgG-opsonization are initially small and only later enlarge.

Also independent of the mode of bacterial entry, segregation of the SCV from the endocytic pathway can be detected starting at 15 minutes after internalization. The SCV prevents the accumulation of the late endosomal markers Rab 7 and Rab 9 and retains the early endosomal marker Rab5 (Amer and Swanson, 2002), although the SCV has minimal access to extracellular fluid markers. Trafficking of the SCV is controlled by the bacterium, as inhibition of bacterial protein synthesis prevents the divergence of the SCV from normal phagocytic maturation (Holden, 2002). As in the epithelial cells, the SCV in the macrophage contains some lgps including LAMP-1, but does not manifest all of the characteristics of a true lysosome, including the presence of degradative enzymes.

S. typhimurium's ability to evade destruction in late endosomes and lysosomes is the result of undefined actions of the PhoP/Q system. The SCVs of PhoP mutants gradually accumulate cathepsin D and other lysosomal markers that are not associated with the vacuoles of wild type bacteria (Garvis *et al.*, 2001).

PhoP/Q allows *S. typhimurium* to escape from destruction in the lysosome, but SPI-2 is required for creation of the niche in which bacteria can replicate. SPI-2 genes are up regulated 1-6 hours after entry into the macrophage by activation of the OmpR/EnvZ system, presumably due to alterations in the SCV environment. *In vitro*, translocon proteins are secreted only when the pH drops below 5.5, suggesting that acidification of the SCV may signal injection of SPI-2 effectors into the macrophage *in vivo* (Beuzon *et al.*, 1999). This usually occurs within the first hour, with the pH in the SCV dropping as low as 4.0. Three to six hours after uptake, bacterial replication begins and is accompanied by SPI-2 mediated changes in actin polymerization. An F-actin meshwork forms around the SCV, and the SCV begins to extend sifs along microtubules. Sifs, like the SCV, are enriched in lgps and the proton ATPase, and are formed through the coordinated action of multiple SPI-2 effectors, including SifA, SifB, SseJ, SseF, SseG, SopD2, and potentially PipB. It is not clear which effectors initiate the formation of actin meshwork around sifs; however, SspH2 and SseI associate with actin and actin-binding proteins. The virulence plasmid-encoded protein SpvB, which is not thought to be secreted by SPI-2, can ADP-ribosylate actin and disrupt actin meshworks, and thus may contribute to actin rearrangements formation (Gruenheid and Finlay, 2003).

As *S. typhimurium* duplicates within the macrophage, each daughter bacterium buds off into its own SCV, and membrane must constantly be recruited to maintain SCV

integrity (Beuzon *et al.*, 2000; Meresse *et al.*, 2001). The *S. typhimurium* effectors that act to form sifs mediate both the recruitment of endosomes to supply membrane to the enlarging SCV, as well as the removal of membrane. When certain SPI-2 effectors are disrupted, the resulting mutants are unable to maintain their SCVs, and these mutants end up in the cytosol where they are killed. The source of the cytosolic killing activity has yet to be identified, although it has been postulated to be ubiquicidin, a small cationic protein with anti-microbial activity (Hiemstra *et al.*, 1999). The bactericidal mechanism appears to be unique to macrophages, as evidenced by the fact that *S. typhimurium* manipulated to lose its vacuolar membrane in epithelial cells replicates better than enclosed bacteria (Brumell *et al.*, 2002).

In addition to sif formation, SPI-2 modifies the SCV in other unusual ways. Through the action of an unknown effector, SPI-2 prevents the fusion of vesicles containing phox with the SCV (Vazquez-Torres *et al.*, 2000b). In fact, *S. typhimurium*-infected macrophages redirect the trafficking of phox-containing vesicles to the plasma membrane instead of to the phagosome (Gallois *et al.*, 2001). SPI-2 secreted effectors may also exert a similar re-trafficking action on inducible nitric oxide synthase (NOS2) (Chakravortty *et al.*, 2002). Despite being originally identified as a cytosolic enzyme, studies have shown that NOS2 can associate with intracellular vesicles (Vodovotz *et al.*, 1995) and the actin cytoskeleton (Webb *et al.*, 2001); however, NOS2 does not appear to be targeted to phagosomes (Webb *et al.*, 2001). SPI-2 also appears to mediate recruitment of cholesterol and the GPI-anchored protein CD55 to the SCV, although it is not clear what role in intracellular survival this might have.

Flagellar genes and their overlapping regulation with SPI-1

Flagella are extracellular appendages used by microbes for motility. The number and localization of flagella vary between bacterial species. *Salmonella* species tend have to 6-8 peritrichous (distributed evenly over the surface) flagella, whereas other species, such as *Caulobacter crescentus*, have one polar flagellum. These long, tail-like structures are composed of the monomer flagellin, which is exported to the outside of the bacterium where it polymerizes to form the flagellum. The apparatus employed by the bacteria to secrete flagellin is known as the hook basal body (HBB), which consists of a double ringed pore that spans both bacterial membranes and the periplasmic space. This method of secretion is functionally and structurally related to secretion by virulence-related TTSSs. In fact, many of the proteins that make up the secretion apparati of the SPI-1 and SPI-2 TTSS have homologs in the flagellar HBB (Fig. 4).

Flagellin monomers are encoded by two independent genes that are situated at separate loci on the chromosome: *fliC*, which encodes phase-1 flagellin and *fljB*, which encodes phase-2 flagellin. The expression and secretion of these proteins requires more than 50 genes, including structural subunits, secretion chaperones, regulators, and proteins that provide motive force (Aldridge and Hughes, 2002). Expression of these genes follows a complex, three tier regulatory hierarchy. The master regulators in class I (FlhD and FllhC) are transcription factors that induce expression at class II promoters. Class II genes include structures required during early assembly of the HBB, as well as FliA (σ^{28}) and its anti-sigma factor FlgM. FlgM binds FliA until early assembly of the HBB is completed, at which point FlgM is secreted by the HBB. Free FliA then binds to

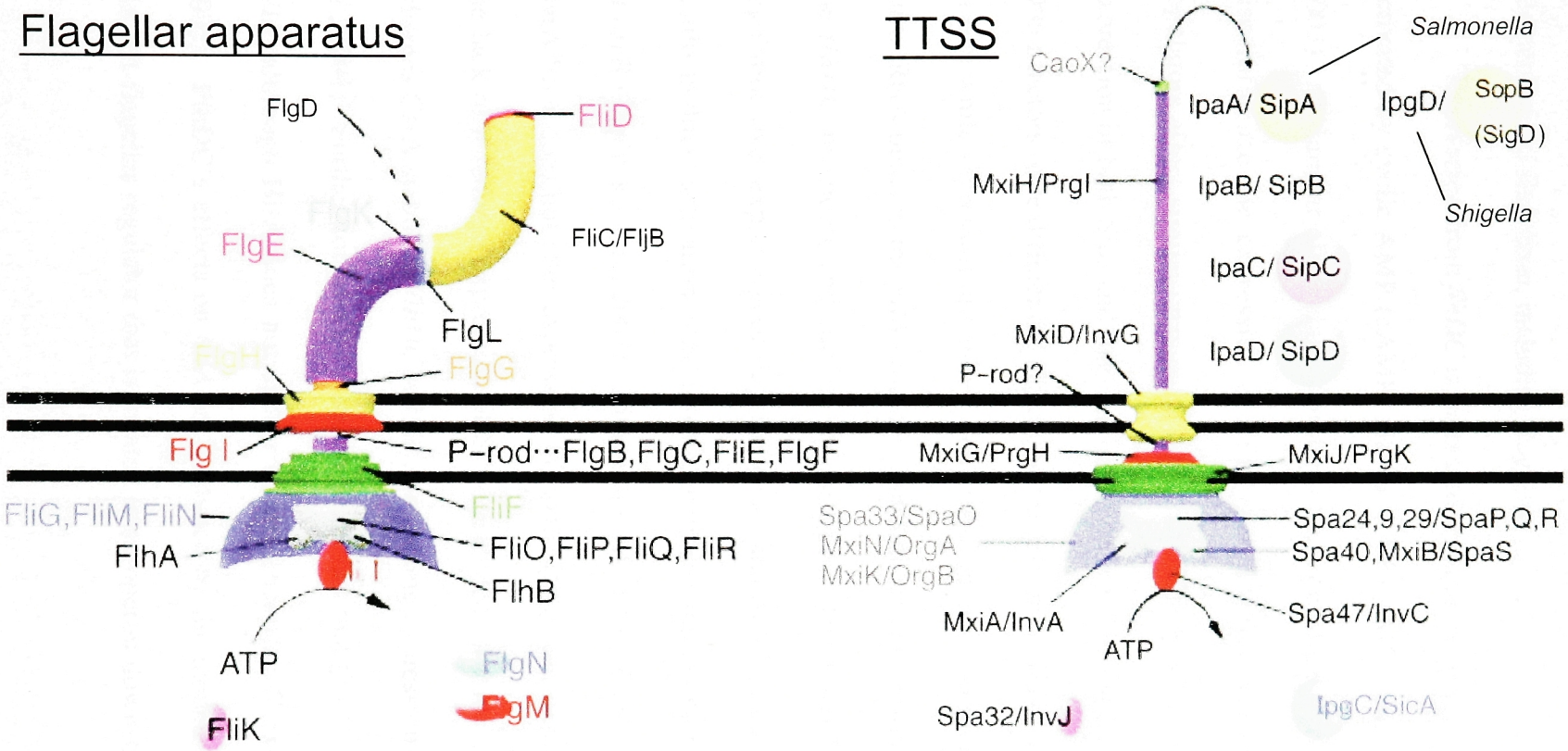


Figure 4. Diagrams of known positions of major flagellar components and established and hypothetical TTSS functional homologs. Functions of proteins conserved in both systems are marked by similar position, shading, and coloring whether they share sequence homologies or not.

Modified from Blocker A, Komoriya K, & Aizawa A. (2003) *PNAS* **100**: 3027-3030

class III promoters, thus activating expression of structures required for the external filament of the flagellum, including flagellin.

Expression from *flhDC* is repressed by high osmolarity via the OmpR protein, and activated by cyclic AMP (cAMP) via the CAP-cAMP complex (Francez-Charlot *et al.*, 2003). A number of regulatory proteins that contribute to control of *flhDC* expression also influence the expression of SPI-1 genes, often via regulation of *hilA*. Some regulators affect similar changes at both loci. The RcsB/C regulatory system represses expression of both Sips and flagellin in response to low osmolarity (Arricau *et al.*, 1998). Other factors have differential effects on expression of *flhDC* and *hilA*, causing activation of one while repressing the other. The *rtsAB* operon coordinately regulates *flhDC* and *hilA*. RtsA binds to the *hilA* promoter and induces expression of *hilA*, while RtsB binds the *flhDC* promoter and inhibits expression. The CsrA/B system exerts complex regulation over expression of flagellar genes and SPI-1 genes. Activation of BarA/SirA results in direct activation of *hilA*, while indirectly repressing *flhDC* through upregulation of *csrB* (Teplitski *et al.*, 2003). Although *csrA* and *csrB* are usually expressed together, BarA/SirA does not alter expression of *csrA*. Both over-expression of CsrA as well as the lack of it reduces expression of SPI-1 encoded invasion genes (Altier *et al.*, 2000), whereas CsrA stabilizes *flhDC* mRNA, promoting expression of flagellar genes (Wei *et al.*, 2001). Further complexity arises from the fact that FlhDC can activate expression of *hilA*, although HilA does not appear to influence *flhDC* expression (Teplitski *et al.*, 2003). FlhDC's effects on *hilA* are mediated by the induction of expression of *fliZ*, a class II flagellar regulator that is located in an operon downstream of *fliA* (Iyoda *et al.*,

2001; Lucas *et al.*, 2000). Thus, disruption of *fliA* also results in repression of SPI-1 by preventing expression of FliZ (Eichelberg and Galán, 2000; Lucas *et al.*, 2000).

Inflammatory role of flagellin

Flagellin is the key mediator of *S. typhimurium* induced inflammation in the intestine due to its stimulatory effects on epithelial cells (Zeng *et al.*, 2003). Invasion of gut epithelia by *S. typhimurium* is associated with recruitment of large numbers of PMN to the lamina propria, followed by translocation of PMN through the epithelia into the lumen of the gut (Fierer, 2001). These two processes are both mediated by the pathogen, but appear to be controlled by different bacterial mechanisms. PMN chemotaxis occurs as a result of epithelial cell production of IL-8. Epithelial cells are triggered to secrete basolateral IL-8 by signaling of bacterial flagellin through toll-like receptor 5 (TLR-5) (Gewirtz *et al.*, 2001a; Gewirtz *et al.*, 2001b; Hayashi *et al.*, 2001a). Signaling by TLR-5 is MyD88-dependent. TLR-5 is expressed on only the basolateral surfaces of the epithelial cells; however, *S. typhimurium* does not need to migrate across the epithelia to induce IL-8 production. Rather, *S. typhimurium* actively translocates flagellin across the epithelia. *Salmonella* flagellin alone cannot mediate its own translocation, as apical flagellin exerts no effect on intestinal epithelia. Moreover, pathogenic *S. typhimurium*, but not flagellated commensal *E. coli*, are able to induce IL-8 secretion, suggesting that translocation of flagellin is an active process that acts as a virulence determinant. The exact mechanism of flagellin translocation is still unknown, but it appears to be SPI-1-independent, as apically-situated *hilA*$^{-}$ mutants are still able to trigger basolateral IL-8 release from epithelial cells (Gewirtz *et al.*, 1999).

PMN cross the epithelial layer into the lumen of the gut in response to apical secretion of pathogen-elicited epithelial chemoattractant (PEEC) (Gewirtz *et al.*, 1999). PEEC secretion is mediated by bacterial SPI-1-dependent secretion of SipA (Lee *et al.*, 2000b) which results in targeting of epithelial cell ADP-ribosylation factor 6 (ARF6) and protein kinase C (PKC) to the apical membrane of the cell (Criss *et al.*, 2001). Transmigration of PMN does not require *Salmonella* uptake by or invasion of epithelial cells; however *Salmonella*'s interaction with the epithelia must occur on the apical side. Moreover, recombinant SipA appears to be sufficient to cause PMN transmigration. Infiltrating PMNs then halt the pro-inflammatory response by secreting proteases that degrade flagellin. Neutrophil elastase and cathepsin G dismantle both soluble and bacterial bound flagellin, destroying the bioactive portion and preventing further IL-8 secretion by epithelial cells (Lopez-Boado *et al.*, 2004).

Apoptosis and caspases

The process of eukaryotic cell death is not a single phenomenon. Initially the term "necrosis" encompassed all cell death processes, with "oncosis" referring to all non-organized or non-programmed cell death. Today the vocabulary of cell death is dominated by the two terms "apoptosis" and "necrosis", with modified definitions. The most simplistic models separate necrosis, or accidental and unorganized cell death, from apoptosis, also known as programmed cell death. Classic apoptosis is ATP-dependent and involves of chromatin condensation, laddering of chromosomal DNA, and blebbing of the plasma membrane with exposure of phosphatidylserine residues, resulting in disassembly of the cell into apoptotic bodies (Hetts, 1998). Necrotic death is

characterized by cellular swelling, random degradation of chromosomal DNA, and lysis of the plasma membrane. In this dualistic model, apoptosis is linked to innocuous clearing of dead cell via phagocytosis by neighboring cells, whereas necrosis tends to result in activation of inflammatory pathways and often further destruction of the tissue (Fadok and Chimini, 2001). In recent years cell death has been more commonly described as a continuum ranging from apoptosis to necrosis with specific examples demonstrating characteristics of both necrosis and apoptosis (Leist and Jaattela, 2001), thus resulting in categories such as apoptosis-like programmed cell death and necrosis-like programmed cell death. Many researchers acknowledge the limitations of assays to differentiate between apoptosis and necrosis (Aigner, 2002). Definitions are descriptive and often semantic, and thus the death category assigned to a phenomenon should be understood in the context of the assays used by the researchers to evaluate cell death.

Regulation of classical apoptosis by proteases was first observed in the nematode *Caenorhabditis elegans* (*C. elegans*) (reviewed in (Ellis *et al.*, 1991). The responsible enzymes had been identified based on their homology to the mammalian protease interleukin-1β converting enzyme (ICE). This family of enzymes was dubbed "caspases" to indicate their function as a cysteine proteases that cut at aspartic acid residues, a previously unidentified cleavage site for mammalian proteases (Howard *et al.*, 1991). Soon after the *C. elegans* caspases were recognized to mediate programmed cell death, mammalian caspases were identified with corresponding functions (Alnemri *et al.*, 1996).

There are about a dozen caspases in mammals that promote apoptosis (Los *et al.*, 1999; Thornberry and Lazebnik, 1998). The caspases are divided into 3 families based on gene homology: the ICE-like proteases (Casp1, 4, 5, 13, 14, and murine Casp11 and

12), the CED-3 family (Casp3, 6, 7, 8, 9, and 10), and Casp2. Each family exhibits a substrate preference. Although all of the caspases contain a similar catalytic core including of the amino acid sequence QACXG, the 4 or 5 amino acids N-terminal to the cleavage-site aspartate determine the particular substrate specificity of individual caspases.

Unactivated caspases exist as zymogens. Proteolytic cleavage results in activation of the protease domain, which then autocatalytically releases the enzymes' 3 domains: the prodomain, the large enzymatic subunit and the small enzymatic subunit. The two enzymatic subunits comes together to form a heterodimer with protease activity, although structural analysis suggests that active caspases are tetramers composed of 2 heterodimers (Mittl *et al.*, 1997). Some active caspases proceed to cleave other caspase family members, resulting in a proteolytic cascade. Based on their order in the cascade, caspases can be separated into initiator and effector caspases, with the initiators starting the cascade and effectors cleaving substrates that result in cell death.

Initiator caspases are activated by binding of the caspase's prodomain to adaptor molecules through homotypic association of interaction motifs such as the CARD (caspase recruitment domain) or DED (death effector domain). Two main pathways initiate adaptor molecule activity. Signaling through transmembrane death receptors, such as CD95 and TNF-R1, recruits FADD, which contains a DED, resulting in the activation of Casp8. Cytochrome c released from mitochondria as a result of toxins and other stresses activates the adaptor Apaf1 in the presence of ATP. Active Apaf1 then interacts with Casp9 via interaction of CARD domains. Additionally, these two pathways can activate each other. Casp9 cleavage of Casp6 can result in cleavage of

Casp8. Alternatively, Casp8 can activate Bid, which in turn can cause mitochondrial release of cytochrome c.

Release of mitochondrial cytochrome c can influence apoptosis in multiple ways. In addition to participating with Apaf1 and Casp9 to form the apoptosome, cytochrome c can increase intracellular calcium by binding to inositol (1,4,5) phosphate receptors on the endoplasmic reticulum and triggering calcium release. Intracellular calcium is usually tightly regulated, because sustained elevated cytosolic calcium is toxic and can result in cell death. Levels of calcium can reach the toxic threshold as a result of organelle membrane damage, hyperactivity of ligand-gated ion channels, or calcium dumping from the endoplasmic reticulum. Sustained elevated intracellular calcium is thought to promote apoptosis by activating multiple targets, including calcineurin, nitric oxide synthase isoforms 1 and 3, Ca^{++}-dependent endonucleases, phospholipase A2 (PLA_2), and Ca^{++}-dependent transglutaminases (Orrenius *et al.*, 2003). Additionally, calcium activates a family of proteases called calpains that can independently, and in collaboration with caspases, cause cell death (Kim *et al.*, 2002).

Casp3, 6, and 7 are considered the effector caspases. Effectors participate in three main strategies to cause disassembly and death of the cell. Inactivation of apoptosis inhibitors such as Bcl-2 proteins and I^{CAD} results in rapid initiation of several degradative pathways, including DNA fragmentation by the nuclease CAD (caspase-activated deoxyribonuclease). Dysregulation of enzyme activity by cleavage of regulatory domains from catalytic domains leads to disruption of cytoskeleton structure and DNA repair. Caspases target gelsolin, focal adhesion kinase (FAK), p21-activated kinase 2

(PAK2), and DNA-PK_{cs}. Finally, caspases help dismantle cells by destroying structures such as nuclear lamina.

Some researchers have described "caspase-independent apoptosis" (Leist and Jaattela, 2001) and "caspase-dependent necrosis" (Wang *et al.*, 2003). The definitions of apoptosis and necrosis in these articles are derived from morphologic changes in dying cells. Caspase-dependent necrosis, for example, is described for A549 cells undergoing hyperoxia-induced death with all the features of necrosis, yet death is dependent on Casp8 activation of Bid. Caspase-independent apoptosis involves chromatin condensation, but it is less complete than in classical apoptosis. Thus, caspase involvement in cell death does not define apoptosis, nor do classical apoptotic features necessarily imply the involvement of caspases.

A few of the caspases play important roles in inflammation in addition to regulation of cell death. Casp1, or ICE, was identified as the enzyme that converts the IL-1β precursor into an active cytokine. Casp1 also processes the precursors of IL-16 and IL-18. Unlike Casp3-deficient mice, which die either *in utero* or soon after birth due to aberrant neural development, Casp1-deficient mice show no developmental defects. Moreover, although over-expression of Casp1 promotes apoptosis by suppressing Bcl2, Casp1-deficient cells demonstrate no overt problems accomplishing programmed cell death. Thus, the role of Casp1 in inflammation and immunity is well defined, but the role of Casp1 in cell death is less clear.

Recently, Casp1 has been implicated in several specific cases of immune cell death. Death of microglial cells triggered by ATP signaling through P2Z-receptors appears to be partially dependent on Casp1, 3 and 8 (Ferrari *et al.*, 1999a). Apoptotic

features, such as chromatin condensation and DNA fragmentation, could be blocked with caspase inhibitors; however, inhibitors did not prevent cytoplasmic vacuolization and cell lysis. Casp1 may also play a role in neuronal cell death as a result of traumatic brain injury (TBI) (Sanchez Mejia *et al.*, 2001). This subject is currently open to debate with opponents presenting evidence that Casp3, and not Casp1, is required for TBI cytotoxicity (Allen *et al.*, 1999; Sullivan *et al.*, 2002). Finally, although a number of bacterial pathogens can cause host cell apoptosis, only a few have been demonstrated to activate Casp1. Two intracellular bacterial pathogens have been connected with Casp1-dependent cell death: *Shigella flexneri (*Edgeworth et al., 2002*)* and *S. typhimurium* (Knodler and Finlay, 2001; Monack *et al.*, 2001). Additionally, the oral Gram-positive species *Actinobacillus actinomycetemcomitans*, a cause of periodontal disease, produces a leukotoxin that lyses neutrophils and monocytes in a Casp1-dependent mechanism (Kelk *et al.*, 2003).

Salmonella-induced macrophage death

S. typhimurium is one of 3 enteric Gram negative bacteria that induce host cell death through the actions of a TTSS (Navarre and Zychlinsky, 2000). *Shigella*, *Yersinia*, and *Salmonella* are facultative intracellular pathogens that use pathogenicity island-encoded TTSSs to mediate invasion of host cells. Some TTSS-secreted effectors hijack actin filaments, enabling uptake of the pathogen, while other specific effectors are known to bind intracellular proteins, thus dysregulating host cell signaling and resulting in host cell death. While invasion effectors are most important for interactions with non-phagocytic cells such as epithelia, the proteins that mediate cytotoxicity are active

primarily in macrophages. *Shigella* and *Salmonella* effector proteins appear to cause death by directly activating macrophage caspases. *Yersinia* triggers cytotoxicity by triggering toll-like receptors (TLRs) to activate pro-apoptotic pathways, and simultaneously translocating YopP/J into the host cell cytosol, which prevents the activation of NF-κB that would normally prevent macrophage apoptosis. *Shigella* and *Salmonella*-induced cytotoxicity results in a large inflammatory response, whereas *Yersinia*-induced death resembles classical apoptosis in that it reduces the inflammatory component of the immune response.

There appear to be potentially four independent cytotoxic mechanisms by which *S. typhimurium* can kill macrophages (Table II). SPI-1-dependent killing accounts for 3 mechanisms of cytotoxicity, one of which occurs very rapidly (within 1-6 hours) and two of which occur late (8-12 hours) after infection of macrophages. SPI-2-dependent killing occurs only late after infection (6-14 hours) (van der Velden *et al.*, 2000). SPI-1 and SPI-2 appear to account for all of *S. typhimurium*'s cytotoxic activity: a double mutant that can express neither SPI-2 nor the SPI-1 encoded *sipB* demonstrates no ability to kill macrophages (van der Velden *et al.*, 2000).

If bacteria are grown to late log phase before infecting macrophages, thus inducing expression of SPI-1 and its effectors, macrophage cell death can be observed within a few hours. This phenomenon has been observed using bone marrow derived macrophages (Chen *et al.*, 1996b; Lundberg *et al.*, 1999; Monack *et al.*, 1996), as well as both the J774.1 (Chen *et al.*, 1996b; Lundberg *et al.*, 1999) and RAW264.7 (Hersh *et al.*, 1999; Monack *et al.*, 1996) macrophage-like cell lines. Death is dependent on macrophage Casp1 and *S. typhimurium* expression of the SP1-1 translocon protein SipB

Table II: Categories of *Salmonella*-induced macrophage death

	Type 1	Type 2	Type 3	Type 4
Rate	Fast	Slow	Slow	Slow
Growth phase of bacteria	Late log	Stationary	Late log	Late log
Bacterial factors required	SipB	SPI-2, SpvB? OmpR/EnvZ	SipB	SipB
Caspases involved	Casp1 (& Casp2?)	Casp2	Casp2, 3, 6, 8 and Cyt C	Caspase independent
Type of death	Necrosis	?	Apoptosis	Autophagy
Reference	(Brennan and Cookson, 2000; Hersh *et al.*, 1999; Lundberg *et al.*, 1999)	(Lindgren *et al.*, 1996)	(Jesenberger *et al.*, 2000)	(Hernandez *et al.*, 2003)
Notes	Casp1 Degradation of Raf1? (Jesenberger *et al.*, 2001)		Seen in $Casp1^{-/-}$ mice	Occurs when all caspases are inhibited

(Hersh *et al.*, 1999; Lundberg *et al.*, 1999). SipB is the homolog of the *S. flexneri* protein IpaB, which is necessary for *S. flexneri* –induced macrophage apoptosis (Zychlinsky *et al.*, 1992). IpaB binds to caspase-1 while inducing macrophage apoptosis (Guichon *et al.*, 2001), and SipB appears to function in the same way to promote macrophage cytotoxicity. In fact, microinjection of SipB is sufficient to induce macrophage death (Hersh *et al.*, 1999). However, despite the involvement of SipB and Casp1, early *Salmonella*-induced macrophage cell death does not appear to be true apoptosis. Two independent investigators have closely examined the phenomenon in both cell lines and primary macrophages and concluded that there are no true apoptotic features seen in SPI-1-dependent macrophage death, and that death more closely resembles necrosis (Brennan and Cookson, 2000; Watson *et al.*, 2000). Thus, SPI-1-dependent Salmonella-induced macrophage cell death is more properly referred to as caspase-1-dependent necrosis.

Because caspase-1 is not generally associated with apoptosis and because macrophage death appears to be necrotic, not apoptotic, it is unclear whether activation of caspase-1 by SipB results in rapid macrophage death. SPI-1-dependent caspase-1 activation, not unexpectedly, results in release of IL-1β and IL-18, which leads to inflammation at the site of infection (Hersh *et al.*, 1999; Monack *et al.*, 2001). However, cytokine release does not explain how caspase-1 and SipB induce macrophage death *in vitro*. One report indicates that infection of bone marrow macrophages with *Salmonella* induces degradation of Raf-1 that is both SipB- and caspase-1-dependent, and that *c-raf-1*- deficient macrophages are hyper-susceptible to *Salmonella*-induced death (Jesenberger *et al.*, 2001). Raf-1 is considered an entry point to the mitogen activated protein kinase (MAPK) cascade (Dhillon and Kolch, 2002); however, death of *c-raf-1*-deficient

macrophages occurred without disruption of MEK or ERK signaling and without increased mitochondrial fragility, suggesting a novel function of Raf-1. Raf-1 modification is also seen as a result of interactions with *S. typhimurium* porins and with the SPI-1 secreted effector SptP (Galdiero *et al.*, 2002; Lin *et al.*, 2003).

A second form of *Salmonella*-induced macrophage death has been described as a result of infection with over-night cultures of *S. typhimurium*. Stationary phase cultures of *S. typhimurium* do not express SPI-1, and J774.1 macrophages as well as bone marrow macrophages survive long enough to undergo a delayed, SPI-2 dependent death (Lindgren *et al.*, 1996). In addition to mutations in SPI-2, disruption of OmpR/EnvZ, the 2 component regulatory systems that modulate SPI-2, abrogates killing, suggesting that the intracellular environment modulates the bacterial mechanisms that result in cytotoxicity. The virulence plasmid *spv* genes, specifically *spvB*, are required for SPI-2-dependent cytotoxicity against human monocytes-derived macrophages (Browne *et al.*, 2002). The specific SPI-2-secreted effectors that trigger death pathways in the macrophage are unknown; however, SpvB, not traditionally considered a SPI-2 effector, may be responsible, in part, for the destruction of macrophages. SpvB acts as an ADP-ribosyl transferase that modifies actin, thus blocking polymerization to F-actin filaments (Lesnick *et al.*, 2001), and SPI-2-dependent death is associated with extensive disruption of macrophage actin.

SPI-2-dependent, SipB-independent macrophage death is mediated by Casp2 (Knodler and Finlay, 2001). The specific role of Casp2 in cell death is not fully understood, and there is some debate concerning whether Casp2 is an effector or an initiator caspase. Like the Casp1 null mouse, the Casp2-deficient mouse does not exhibit

a dramatic phenotype; however, Casp2 has been demonstrated to be required for apoptosis in several systems including beta-amyloid toxicity and trophic factor deprivation (Troy and Shelanski, 2003).

Although the involvements of SPI-1 and SPI-2 in early and late *Salmonella*-induced macrophage death appear to be exclusive, the role of caspases may not be so distinct. At least one study indicates that rapid SPI-1-dependent death occurs after simultaneous and independent activation of both Casp1 and Casp2 (Jesenberger *et al.*, 2000). Moreover, if macrophages deficient in Casp1 are studied, delayed SipB-dependent killing can be examined. Jesenberger, et al. found that Casp1-deficient bone marrow macrophages undergo apoptosis that is Casp2-dependent at 4-6 hour after infection, and that cytotoxicity is SipB (SPI-1)-dependent, suggesting a third mechanism of *Salmonella*-induced macrophage death. Unlike Casp1-dependent cytotoxicity, delayed SipB-dependent, Casp2-dependent death involves Casp3, 6 and 8, as well as cytochrome c translocation from mitochondria, thus resembling classical apoptosis.

A recent study has characterized a SipB-dependent cytotoxicity which appears to be caspase-independent (Hernandez *et al.*, 2003). Caspase-1-deficient bone marrow macrophages were killed by wild type *Salmonella* in a delayed, SipB-dependent manner that was not inhibited by the pan-caspase inhibitor Z-VAD. Microscopic examination revealed that SipB associates with macrophage mitochondria, resulting in mitochondrial remodeling and formation of autophagosomes. Recombinant SipB protein has been shown to insert into mammalian cell membranes without perturbing bilayer integrity, and to induce heterotypic membrane fusion (Hayward *et al.*, 2000). A SipB mutant that lacks the domain required to induce membrane fusions could still associate with mitochondria,

but was unable to cause formation of autophagosomes or macrophage death. Thus, caspase-independent induction of autophagy by SipB may represent a fourth mechanism of *Salmonella*-induced macrophage death.

It is difficult to study the relevance of *Salmonella*-induced macrophage death *in vivo*, but several studies have shed light on the possible role of cytotoxicity in systemic pathogenesis. Caspase-$1^{-/-}$ mice and wild type mice control *S. typhimurium* infection similarly after i.p. infection; however, caspase-$1^{-/-}$ mice are 1000x more resistant to infection after oral inoculation (Monack *et al.*, 2000; Monack *et al.*, 2001). Moreover, histologic analysis of ligated intestinal loops demonstrated that although membrane ruffling and invasion occur in caspase-$1^{-/-}$ mice during *S. typhimurium* infection, there is neither cell death in Peyer's patches nor PMN infiltration at the site of invasion. This suggests that *Salmonella*-induced macrophage death is of primary importance during invasion of the gastrointestinal tissue, particularly of cells in Peyer's patches. Because SPI-1 genes are upregulated by conditions found in the intestine, and SPI-2 genes are turned on within the phagosome, it is likely that rapid SipB-dependent killing occurs in the macrophages of the gut, whereas delayed SPI-2-dependent cytotoxicity may occur in tissue macrophages, allowing bacteria time to replicate within the phagosome.

Confocal microscopy of liver sections from *Salmonella*-infected mice revealed that bacteria live within phagocytes, not hepatocytes, in the liver, and are cytotoxic to phagocytes in which they reside (Richter-Dahlfors *et al.*, 1997); however, this study did not distinguish between dying macrophages and dying PMNs. Activated neutrophils will die rapidly independent of the presence of *Salmonella*, and therefore it is difficult to ascertain from this study whether *Salmonella* directly kill phagocytes during systemic

infection. It is possible, though, that phagocyte death seen in the liver results from SPI-2 dependent delayed cytotoxicity, thus enabling nascent intracellular bacteria to spread to new phagocytes.

Mice depleted of macrophages by injection of liposomes are more resistant to i.v. infection with *S. typhimurium* than control mice (Wijburg *et al.*, 2000), suggesting that macrophages function more as safe sites for bacterial survival and replication than they do as bactericidal cells. Thus, it would be detrimental to *S. typhimurium* to destroy all macrophages encountered *in vivo*, and cytotoxicity is presumably regulated to optimize both the spread and the proliferation of the bacteria.

Because gut associated macrophages are probably killed by SPI-1-expressing bacteria, some people have postulated that dendritic cells may be responsible for carrying *Salmonella* from the site of infection to the liver and spleen (Rescigno *et al.*, 2001). In the absence of SPI-1, intestinal *S. typhimurium* is transported across the gut epithelia by $CD18^+$ mononuclear phagocytes that resemble dendritic cells (Vazquez-Torres *et al.*, 1999). Yet *Salmonella* may be cytotoxic to dendritic cells, too. LDH release, phosphatidylserine redistribution, and caspase activation all occur rapidly after infection of dendritic cells with *S. typhimurium* (van der Velden *et al.*, 2003). Although cytotoxicity is both SipB- and caspase-1-dependent, death occurs with either late log phase or stationary phase cultures of *S. typhimurium*, suggesting that death may be occurring by a mechanism different from that seen in macrophages.

S. typhimurium causes systemic disease in mice that resembles typhoid fever caused by *S. typhi* in humans. SPI-1-dependent macrophage death appears to be largely responsible for dissemination of the bacteria from the gut tissue into the internal organs.

In humans and cattle, infection with *S. typhimurium* generally causes localized disease in the intestine resulting in diarrhea. Despite this difference in pathogenesis in the larger species, *S. typhimurium* induces SPI-1-dependent macrophage death of both bovine and human macrophages (Forsberg *et al.*, 2003; Santos *et al.*, 2001). However, SipB-dependent macrophage death appears either not to occur during or not to contribute to enteritis in calves (Zhang *et al.*, 2003). Thus, the systemic nature of *S. typhimurium* infection in mice may be due to specific conditions in the murine intestine that promote bacterial expression of genes required for host cell death, resulting in breach of the intestinal wall.

V. *S. typhimurium* and *M. tuberculosis*: some key distinctions

S. typhimurium and *M. tuberculosis* are very different in many ways: one is Gram negative, the other is acid-fast; infection with one causes a systemic fever in mice and gastroenteritis in humans, infection with the other can result in diffuse pathology, but most often presents as infection of the lungs. Yet, once inside the host, both pathogens take shelter within the macrophage phagosome and cause dysregulation of phagosome maturation. A comparison of some of the basic features of the microbial pathogenesis of each bacterium reveals both interesting parallels and differences.

Although *S. tyhphimurium* and Mtb both modify the macrophage phagosome as a survival strategy, the resulting niches are very different. Mtb's preferred phagosome resembles an early endosome. In addition to avoiding lysosomal enzymes, Mtb eschews the proton ATPase, and thus the pH in the phagosome does not drop below approximately 6.5 in unactivated macrophages. This is important because low pH is

required for optimal activity of macrophage acid hydrolases, and acidic pH amplifies and prolongs the effects of RNI. Nitrite, which can be formed from nitric oxide, is toxic in acidic pH, but loses its reactivity when the pH becomes neutral. The SCV in which *S. typhimurium* resides also resembles an early endosome, insomuch as it retains Rab5 and excludes Rab7 and many other lysosomal enzymes; however, unlike the Mtb phagosome, the SCV acidifies to a pH of 5.0. *S. typhimurium* appears to require low pH in order to activate its virulence mechanisms. SPI-2 effectors, which are required for *S. typhimurium* survival in the macrophage, are not secreted *in vitro* unless the pH drops below 5.5 (Holden, 2002). Thus, while Mtb has evolved to survive by blocking phagosome maturation and avoiding acidic environments, *S. typhimurium* has evolved to take advantage of aspects of phagosomal maturation, and may in fact require a low pH to survive *in vivo*.

Toxic micromolecules pose a threat to all phagosomal pathogens. Mtb and *S. typhimurium* have evolved similar strategies for confronting ROI produced by phox, and yet resist killing with different levels of success. Phox activity is triggered by phagocytosis, and does not require activation by the macrophage stimulatory cytokine IFN-γ. Thus, unlike NOS2, which must be transcribed and translated following macrophage activation, phox is an immediate threat to phagosomal pathogens. Mtb and *S. typhimurium* have both evolved mechanisms for hindering the efficacy of phox: Mtb directs its own uptake by binding to phagocytic receptors that tend not to stimulate phox, and *S. typhimurium* uses SPI-2 to divert phox away from the phagosomal membrane. These bacterial manipulations, however, are not sufficient to eliminate the effects of phox entirely, and thus both bacteria possess catalases and peroxidases for the detoxification of

ROI. Here is where we see a striking difference between Mtb and *S. typhimurium*: the effectiveness of KatG and SodA make Mtb virtually impervious to the activity of phox, whereas, despite an arsenal of anti-oxidant mechanisms, *S. typhimurium* is highly susceptible to killing by ROI. Comparison of disease in phox-deficient mice with immuno-competent mice clearly illustrates this point (Ng *et al.*, 2004; Shiloh *et al.*, 1999).

The opposite holds true for RNI. NOS2-deficient mice control infection with virulent strains of *S. typhimurium* as well as wild type mice; however, Mtb overwhelms NOS2-deficient mice, which die substantially earlier than their infected immuno-competent counterparts (MacMicking *et al.*, 1997; Shiloh *et al.*, 1999). The two bacteria share a number of enzymes that have been shown to be important defenses against RNI *in vitro*, including AhpC and MsrA (Shiloh and Nathan, 2000; St John *et al.*, 2001); however, as is the case with ROI, it is not clear why one pathogen appears to be so much more susceptible to RNI than the other. In fact, Mtb has been shown to possess RNI counter-immune mechanisms that are absent in *S. typhimurium*. The Mtb proteosome provides resistance to RNI *in vitro* and *in vivo* by an unknown mechanism; however, Gram negative bacteria do not encode proteasomes, and thus this is not counter immune strategy available to *S. typhimurium*.

As discussed earlier, *S. typhimurium*'s apparent resistance to RNI *in vivo* may reflect the use of *Nramp1*^{s} mice as a model for acute systemic typhoid fever. Unlike Mtb, which causes a chronic infection in mice during which adaptive immunity plays a critical role, *Nramp1*^{s} mice do not live long enough to benefit from IFN-γ generated by antigen-specific T cells. When *Nramp1*^{r} mice are infected, a role for NOS2 in control of *S.*

typhimurium can be demonstrated (Mastroeni *et al.*, 2000). Typhoid fever resulting from *S. typhi* infection of humans is generally an acute and self-limiting infection; however, 1-5% of infected individuals become asymptomatic chronic carriers (Muñoz-Elías and McKinney, 2002). A recent publication describes oral infection of *Nramp1*^{r} mice with low dose virulent *S. typhimurium* as a mouse model of chronic typhoid fever (Monack *et al.*, 2004). This model does not entirely mimic chronic disease in humans, during which bacteria evade immune recognition by colonizing the immune-privileged gall bladder. In chronically infected *S. typhimurium*-infected mice, bacterial populations persist in the lymph nodes; however, the model presents a novel opportunity to assess components of the adaptive immune response required for maintenance of chronic infection. When IFN-γ is neutralized in these chronically infected mice, there is a rebound in bacterial proliferation, and the mice demonstrate signs of illness. It will be interesting to learn more about which immune factors contribute to maintenance of chronic *Salmonella* infection, and if NOS2 is among them.

It is not surprising that Mtb and *S. typhimurium* encode homologous genes; most bacteria contain genes with homology to genes from other bacterial species. More interesting, though, is the evolution of analogous strategies by Mtb and *S. typhimurium* for interacting with the immune response. One of the most potent immuno-modulatory molecule synthesized by *S. typhimurium* is the Lipid A component of LPS. The outer lipids and glycolipids of Mtb are also immuno-modulatory. The use of lipids for both protection as well as manipulation of the immune response is beginning to be appreciated as a common strategy among pathogens (van der Kleij and Yazdanbakhsh, 2003). Bioactive lipids that have both pro- and anti-inflammatory effects are well characterized

in the host, including prostaglandins and leukotrienes (Yoshikai, 2001). The discovery of TLRs and the appreciation that TLR2 mediates signaling of many microbial lipids has revitalized the field of microbial bioactive lipids. Lipids from bacteria, single-celled parasites, as well as parasitic worms have all been shown to signal through TLR2, resulting in suppression or activation of the immune response.

The delivery of virulence factors into the macrophage is another strategy common to the microbial pathogenesis of both Salmonella and mycobacteria. *S. typhimurium* uses TTSSs to inject proteins across the phagosomal membrane and into the cytoplasm of the macrophage. Mtb releases both surface proteins and lipids that are transferred out of the phagosome and trafficked through the macrophage. Although the exact functions of these released proteins and lipids are not known, lipids released from intracellular BCG are able to stimulate macrophage responses (Beatty *et al.*, 2000; Rhoades *et al.*, 2003). It has been proposed that the transporters that export lipids, such as PDIM, to the surface of the bacterium are Mtb's equivalent of the TTSS (Glickman and Jacobs, 2001), although actual injection of lipids into the host has not been demonstrated. The ability to inject molecules into host cells where they can act to manipulate host functions at a distance may be a shared strategy of intracellular pathogens.

VI. Differential signature-tagged transposon mutagenesis (STM)

Although *in vitro* studies provide a simplified system in which to examine specific interaction between pathogens and immune cells, the results may not accurately reflect real scenarios that occur in the host. In recent years, a number of clever assays have been developed that take advantage of new technologies to reveal microbial genes

important for disease. DNA microarray technology permits the evaluation of expression of entire microbial genomes, reflecting both which genes are induced inside host cell, as well as the nature of the intracellular environment (Eriksson *et al.*, 2003; Schnappinger *et al.*, 2003). Differential fluorescence induction (DFI) uses expression of a green fluorescent protein (GFP) reporter in combination with fluorescence-activated cell sorting (FACS) to identify genes that are upregulated under specific conditions, including ingestion by macrophages (Valdivia and Falkow, 1997). IVET and RIVET use a similar reporter construct strategy, but rely on expression of antibiotic resistance for identifying genes, and thus IVET and RIVET can be used to analyze bacterial genes induced *in vivo*, rather than in cell culture. IVET (*in vivo* expression technology) creates a bacterial library of chromosomal fusions in which a promoter-less antibiotic resistance gene acts as the reporter. After infection with the library, animals are treated with antibiotics, and thus only clones in which the reporter fusion has inserted in a gene expressed *in vivo* will be recovered (Mahan *et al.*, 1995). RIVET (recombinase-based *in vivo* expression technology) uses reporter constructs that express the TnpR resolvase (Camilli *et al.*, 1994). When induced, TnpR recognizes res sites, and will excise any DNA contained within two res sites. A DNA sequence flanked by res sites is placed immediately downstream of the promoter of an antibiotic resistance cassette, thus blocking expression of antibiotic resistance until the TnpR resolvase is expressed. If a gene is expressed only briefly during infection, antibiotic resistance will continue to be expressed even after the gene has been turned off. Thus, RIVET can identify genes that are induced only at distinct times during infections.

Another strategy for understanding genes required for growth *in vivo* involves disrupting bacterial genes and assessing the bacterial mutants for virulence in an animal model. Making large numbers of random marked gene disruptions is easily achievable using transposon mutagenesis. However, in order to effectively screen libraries of mutants, one must have a method for identifying individual mutants from within a pool of mutants. More specifically, one must have a method for identifying the absence of a particular mutant that has been depleted from the pool because it was unable to survive *in vivo*. Two strategies have been developed with different to solutions to this problem. In signature-tagged transposon mutagenesis (STM), a set of transposons is generated such that each transposon bears an unique DNA tag, which acts like a bar code (Hensel *et al.*, 1995). If there are 48 individual tags, then 48 individual transposon mutants can be pooled and simultaneously screened for attenuation *in vivo*. Using Southern hybridization, DNA tags carried by bacteria recovered from animals can be analyzed to determine which tags are under-represented after passage *in vivo* as compared to the input pool. TraSH (transposon site hybridization) combines transposon mutagenesis with microbial genome microarrays to evaluate much larger pools of transposon mutants (Sassetti *et al.*, 2001). Pools of bacterial mutants are subjected to a specific stress, and then labeled RNA probes are generated from each surviving mutant by amplifying off the transposon into the disrupted gene. These probes are labeled, hybridized to a genome microarray, and compared to probes generated from non-stressed bacterial pools. Although TraSH permits rapid identification of many genes essential for growth under the conditions tests, it does not preserve the individual mutants that are attenuated for growth, and specific mutants must be reconstructed for further study. STM pools are

assembled from individual clones, and thus a stock of each mutant is available for follow up experiments.

Our interest was not general virulence factors, but rather bacterial mechanisms that counter specific immune stresses. *S. typhimurium* and *M. tuberculosis* persist within the macrophage *in vivo* despite bombardment with RNI, ROI, and other toxic substances. The bacterial genes responsible for the survival of *S. typhimurium* and *M. tuberculosis* despite these host defenses are only partially characterized. In order to identify bacterial genes that are required for growth in the face of distinct components of the host immune system, we developed a modified version of STM based on the hypothesis that a bacterial gene required for protection against a specific stress should be dispensable for bacterial growth in the absence of that stress. During Differential STM, pools of bacterial mutants are evaluated for differential virulence in at least two strains of mice with different degrees of immune-competency (Fig. 5). For example, mutants that are attenuated in wild type mice, but have no difficulty growing in NOS2-deficient mice, contain transposon insertions in genes that may provide specific resistance to RNI. This strategy enables dissection of bacterial counter immune mechanisms to distinct host stresses *in vivo*.

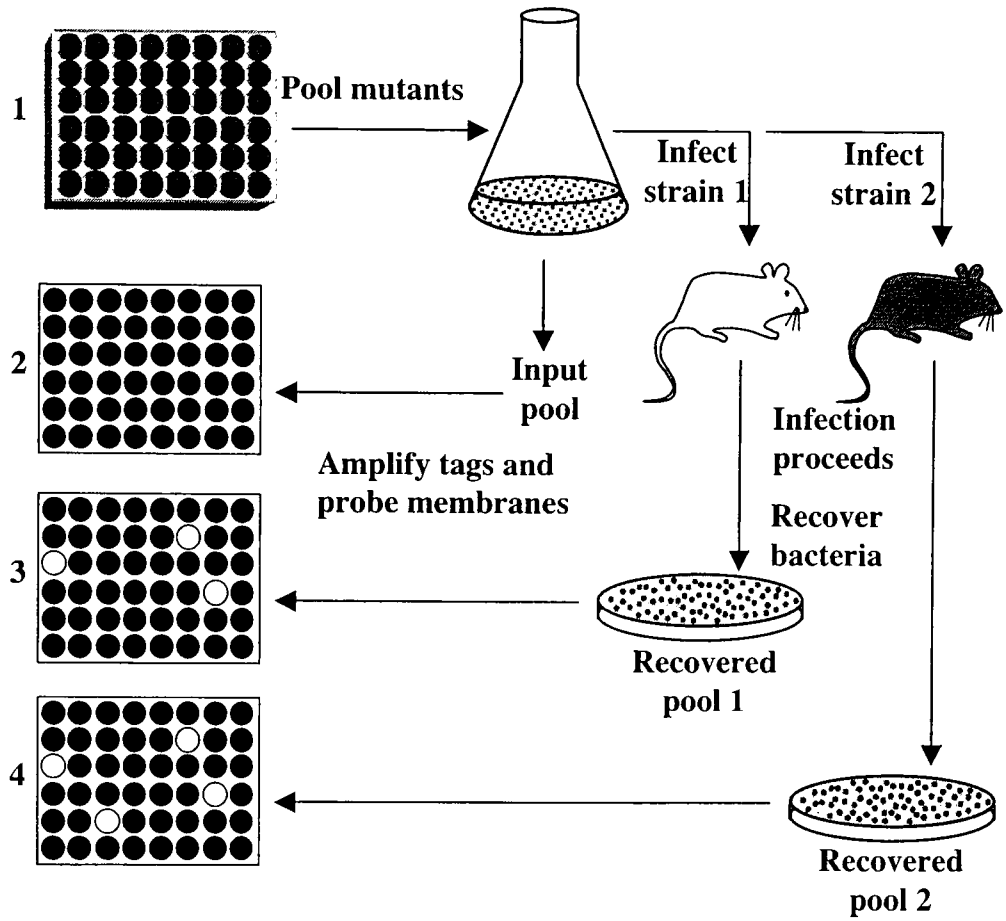


Figure 5. Differential signature-tagged transposon mutagenesis: a strategy to identify bacterial mutants deficient in the ability to counter specific immune mechanisms. Signature-tagged transposon mutants are arrayed in 48 or 96-well microtiter dishes (1). Pooled mutants are inoculated into 2 different strains of mice that differ in immune status. Strain 2 mice should either be immunocompetent, or deficient in a subset of the functions absent in strain 1 mice. Bacteria are recovered from the appropriate organ by plate culture at the indicated times post-infection. Signature tags in the recovered pools of bacteria are amplified and radiolabeled and used to probe membranes arrayed with the set of 48 or 96 signature tags. Tags that are detected in the input pool (2) but reduced or absent in the recovered pools (3,4) indicate mutants that are attenuated *in vivo*. The mutant spotted in column 3 row 5 is under-represented in pools recovered from strain 2 mice (4) but not in pools recovered from strain 1 mice (3), indicating that the corresponding *cim* gene is required for progressive bacterial growth in the face of a an immune mechanism present in mouse strain 2, but absent in mouse strain 1.

Chapter 2

Material and methods

M. tuberculosis experiments:

Bacterial strains and growth conditions

Wild-type Mtb (strains H37Rv and Erdman) and derivative strains were propagated at 37°C in Middlebrook 7H9 broth containing 10% OADC (DifCo), 0.5% glycerol, and 0.05% Tween-80, or on Middlebrook 7H10 agar containing 10% OADC and 0.5% glycerol. Antibiotics were hygromycin (50 μg/ml) and kanamycin (25 μg/ml). Frozen stocks were prepared by growing broth cultures to mid-log phase (OD_{600} ~ 0.5), adding glycerol to 15%, and storing in aliquots at -80°C.

Construction of STM mutant library

A panel of 48 transposons in the temperature-sensitive mycobacteriophage phAE87 was constructed as described (Cox *et al.*, 1999). Each phage contained a unique random 40mer signature tag within the *Tn*5370 transposon, which carries the hygromycin resistance marker (*hyg*). Phages were amplified individually in *Mycobacterium smegmatis* mc^2155 to final titers of ~5 x 10^{11} PFU/ml. Cultures of Mtb H37Rv were mutagenized with the phage stocks and transposon insertion mutants were selected on 7H10 agar containing 50 μg/ml hygromycin. Individual colonies were picked and inoculated into 7H9 broth containing 50 μg/ml hygromycin dispensed into 48-well microtiter plates, such that each of the 48 signature tags was represented by one mutant per plate. The microtiter plate cultures were grown to stationary phase at 37°C and

aliquots from each well were combined to generate pools of 48 signature-tagged mutants. The pools were diluted 1:100 in 7H9 broth containing 50 μg/ml hygromycin and grown at 37°C until the OD_{600} reached ~ 0.2. The amplified pools were then washed and resuspended in PBS containing 0.05% Tween-80 and 15% glycerol as a cryopreservant, aliquoted in cryovials, and stored at -80°C

Mouse infections

Male and female C57BL/6, IFN-$\gamma^{-/-}$, and $NOS2^{-/-}$ mice, 6-10 wk of age, were purchased from Jackson Laboratories. $phox^{gp91-/-}NOS2^{-/-}$ mice were generated and maintained as described (Shiloh *et al.*, 1999). Mtb frozen stocks were thawed, diluted in PBS, and sonicated briefly to disperse clumps. Mice were infected by injection of 0.1 ml (1×10^5 - 1×10^6 CFU) of the diluted bacterial stock into a lateral tail vein and euthanized by CO_2 exposure at specified time points; n = 4-5 mice per time-point unless otherwise noted. Organs were removed aseptically and homogenized in PBS containing 0.05% Tween-80 and 100 μg/ml ampicillin to prevent contamination. Bacteria in the lungs, spleen, and liver were enumerated by plating diluted organ homogenates on 7H10 agar, and counting colonies after 3-4 weeks at 37°C; error bars indicate standard deviations.

Isolation of Mtb genomic DNA and amplification of signature tags

The four STM mutants characterized in this study were identified from 2 pools as follows. Lung homogenates from mice infected with STM pools were plated at low density for enumeration of CFU and at high density for STM analysis. Genomic DNA was isolated from ~10,000 pooled colonies per lung homogenate (recovered pools) and

from colonies generated by plating the inocula that were used to infect the mice (input pools). STM tags were PCR amplified from input and recovered pools using primers specific for invariant regions on either side of the STM tags (Cox *et al.*, 1999), and PCR products were purified using the Qiaex II kit (Qiagen). Amplified tags were labeled with ^{32}P -dCTP using the Megaprime Labeling System (Amersham), and free ^{32}P -dCTP was removed using Probequant G-50 Micro columns (Amersham).

Southern hybridizations

The 48 individual signature tags were PCR amplified from purified phage DNA and cloned individually into pUC19 using *Hin*DIII. The 48 plasmid DNAs were arrayed onto 9 x 12 cm Zeta Probe nylon membranes (Bio-Rad) using a 48 well Bio-Dot SF Microfiltration Apparatus (Bio-Rad). Hybridizations using labeled signature tags from input and output STM pools were performed at 65°C in Rapid-Hyb solution (Amersham) and the hybridized membranes were processed per the manufacturer's instructions. Autoradiographic films (Kodak) were exposed to membranes for at least 2 hours and developed per the manufacturer's instructions.

Identification of transposon insertion sites

Genomic DNA was isolated from individual STM mutants grown in 7H9 broth. Transposon insertion sites in the genome were identified by inverse PCR as described (Cox *et al.*, 1999). Briefly, *Rsa*I-digested genomic DNA was purified using the Qiaex II kit (Qiagen), self-ligated, and PCR amplified with primers o84L-F (5'-GTCATCCGGCTCATCACCAG-3') and o84L-R (5'-AACTGGCGCAGTTCCTCTGG-

3') to amplify the genomic DNA fragments flanking the ends of the transposon. PCR products were separated on agarose gels and purified using the Qiaex II kit (Qiagen). The nucleotide sequences of the purified amplicons were determined by the Rockefeller University DNA Sequencing facility. In some cases, PCR products were cloned using the TopoTA Cloning Kit (Invitrogen) and the plasmid DNA was purified using the Qiagen miniprep kit prior to sequencing.

Statistical analysis

The Student's *t*-Test was used to evaluate differences in bacterial CFU in infected organs. One-tailed Student's *t*-Tests were applied to comparisons of CFU from H37Rv-infected wild type mice with CFU from H37Rv-infected IFN-$\gamma^{-/-}$ mice and NOS2$^{-/-}$ mice in Fig. 4A. Two-tailed Student's *t*-Tests were applied to the data in Fig. 1A-C, and the data in Fig. 4B-E. Kaplan-Meier curves were compared using the Mantel-Haenszel test as applied by the GraphPad Prism 4.0 program from GraphPad Software, Inc. (Motulsky, 2003). *P* values < 0.05 were considered statistically significant. Data are represented as means with error bars indicating the standard deviations from the means.

S. typhimurium experiments:

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Tables III and IV. STM pools were a generous gift from David Holden; STM mutants were derived from the parental strain 12023, also known as 14028 with spontaneous nalidixic acid resistance (14028^{R}) (Hensel *et al.*, 1995). Bacteria were grown in Luria-Bertani (LB) broth or on LB-agar

Table III. *S. typhimurium* strains used in this study

Strain	Relevant characteristics	Source or reference
12023	Nalidixic acid-resistant spontaneous mutant (14028^R)	Shea *et al.* 1999
SCN3	*ssrB*::mTn*5* in 12023 (Kan^R)	P08H06 in Hensel *et al.* 1995
SCN7	*ssaR*::mTN*5* in 12023 (Kan^R)	P02A02 in Hensel *s* 1995
SCN19	*ydiV*::mTn*5* in 12023 (Kan^R)	P01E12 in Hensel *et al.* 1995
SCN27	12023 + pwks30	*This study*
SCN31	SCN19 + pwks30	*This study*
SCN32	SCN19 + pKH7	*This study*
SCN72	*ydiU*::Cm^R in 12023	*This study*
SCN78	*ydiV*::Cm^R in 12023	*This study*
SCN79	*ydiV*::Cm^R *ssaR*::mTn*5* in 12023 (Kan^R)	*This study*
SCN84	*ydiV*::Cm^R-*lacZYA*/*ydiV*$^+$ in LT2	*This study*
SCN85	*ydiV*::Cm^R-*lacZYA*/*ydiV*$^+$ in SCN3	*This study*
SCN87	SCN78 + pKH7	*This study*
SL3201	Mouse virulent, fim$^-$	Hoiseth and Stocker 1981
SCN96	*fljB*::MudJ *fliC*::Tn*10* in SL3201 (Kan^R, Tet^R)	Schmitt *et al.* 1996
SCN97	*sipB::aphT* transduced into 12023 (Kan^R)	Kaniga *et al.* 1995
SCN98	*sipB::aphT* transduced into SCN78 (Kan^R)	*This study*
SCN99	*ydiV*::Cm^R in SL3201	*This study*
SCN100	*ydiV*::Cm^R *fljB*::MudJ *fliC*::Tn*10* in SL3201 (Kan^R, Tet^R)	*This study*
FW1	*ydiV*::Cm^R-*lacZYA*/*ydiV*$^+$ in 12023	*This study*
LT2	*rpoS* mutant	SGSC
SCN101	*flhDC*:: Cm^R-*lacZYA*/*flhDC*$^+$ in 12023	*This study*
SCN102	*flhDC*:: Cm^R-*lacZYA*/*flhDC*$^+$ in SCN19 (Kan^R)	*This study*
FW9A1	SL1344 *ydiV*::Cm^R *sipC*::Tn*5*-*lacZYA* (Kan^R)	*This study*
BJ68	SL1344 *sipC*::Tn*5*-*lacZYA* (Kan^R)	Penheiter *et al.* 1997
LB5000	*flaA66 metA22 trp-2 rpsL xyl-401 ilv-452 leu hsd mos*$^+$	Bullas and Ryu 1983
FW2A	*ahpC*:: Cm^R-*lacZYA*/*ahpC*$^+$ in12023	*This study*
FW3A	*ahpC*:: Cm^R-*lacZYA*/*ahpC*$^+$ in SCN19 (Kan^R)	*This study*

Table IV. *E. coli* strains and plasmids used in this study

Strain	Relevant characteristics	Source or reference
E. Coli		
S17-1λpir	Tp^R Str^R *RecA thi pro hsdR* RP4::2-Tc::Mu::Km Tn*7* λpir lysogen	Miller and Mekalanos 1988
ER2566	F-l-f*huA2*[*lon*] *ompT lacZ*::T*7 gene I gal sulA11* D(mcrC-mrr)*114::IS10* B(mcr-73::mTn*10*)2	Chong et al. 1997
ECN145	ER2566 + pETV10	*This Study*
Plasmids		
pwks30	Amp^R, low copy-number cloning vector	Wang and Kushner 1991
pFUSE	MobRP4 *ori*R6K, polylinker upstream of promoterless *lacZYA* (Cm^R)	Baumler *et al.* 1996
pKH7	fragment containing *ydiV* and putative promoter in pwks30 (Amp^R)	*This study*
PKH101	pFUSE containing a 1.2 kb fragment fusing 784 bp upstream of *ydiV* to *lacZYA* (Cm^R)	*This study*
pFlac	pFUSE containing a 728 bp fragment fusing 674 bp upstream of *flhD* to *lacZYA* (Cm^R)	*This study*
pkanπ-less	pEP185.2 containing a 1.7 kb BamHI fragment from pUTmtn5Kan2 (Kan^R, Cm^R)	*This study*
pKH201	pFUSE containing a SmaI fragment fusing ~800 bp of *ahpC* and it's 5' sequence to *lacZYA* (Cm^R)	*This study*
$pET24b^+$	Expression vector containing an IPTG-inducible T7 promoter, a T7-Tag, and a C-terminal His-Tag	Novagen
pETV10	pET24b+ with the coding sequence of ydiV replacing the T7-tag.	*This study*
pAJD107	Amp^R; medium copy-number cloning vector	Darwin and Miller 2001

plates. When necessary, growth medium was supplemented with carbenicillin (100 μg/ml), kanamicin (100 μg/ml), chloramphenicol (25 μg/ml for established strains, 12.5 μg/ml for selection after transduction or transformation), or tetracycline (15 μg/ml). Unless otherwise noted, "late log phase" refers to 20 μl of an overnight culture diluted 1:100 into 2 ml LB media +/- antibiotics in a 14 ml tube, and shaken at 250 rpm and 37°C for 5.5 hr, with a final culture density of A_{600} ~3.0. "Stationary phase" refers to an overnight culture.

Mice

Wild-type and NOS2$^{-/-}$ C57BL/6 mice were purchased from Charles River Laboratories, Taconic, or Jackson Laboratories. Additional NOS2$^{-/-}$ mice (MacMicking *et al.*, 1995), phox$^{gp91-/-}$ mice (Pollock *et al.*, 1995), and phox$^{gp91-/-}$NOS2$^{-/-}$ mice (Shiloh *et al.*, 1999) were bred in the Weill Cornell Medical College animal facility after backcrossing to C57BL/6 mice for at least 6 generations. MyD88$^{-/-}$ mice and corresponding wild-type C57BL/6 x 129/SvJ were also bred in the Weill Cornell Medical College animal facility.

***S. typhimurium* targeted gene disruptions**

Targeted mutations were made using the one-step inactivation protocol described by Datsenko and Wanner (Datsenko and Wanner, 2000). Briefly: 60-mer primers were constructed that contained 40 nucleotides of the gene to be disrupted and 20 nucleotides corresponding to regions on the plasmids pKD3 and pKD4. Amplification from pKD3 or pKD4 using these primers generated fragments containing either a kanamicin resistance

cassette or a chloramphenicol resistance cassette flanked by regions of homology to the gene to be disrupted. The plasmid pKD46, containing the λ Red recombinase under the control of the arabinose-inducible P_{araB} promoter, was electroporated into *S. typhimurium* LB5000 to create the strain SCN35. Purified PCR products were electroporated into SCN35 grown in 0.8% arabinose to express the λ Red recombinase, and plated on LB containing either kanamicin or chloramphenicol. Phage lysates were made from transformants containing the antibiotic resistance cassette in the correct location, and lysates were used to transduce the mutations into 12023 *S. typhimurium*.

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Methods bracketed by the asterisks were written by Michael Shiloh, and pertain to experiments performed by Michael Shiloh.

Mouse infection with STM pools and recovery of bacteria

Transposon-tagged *S. typhimurium* pools in sterile 96-well microtiter plates (provided by David Holden) were grown in LB (kanamycin 50 μg/ml, nalidixic acid 15 μg/ml) with gentle shaking at 37°C overnight. Following overnight growth, bacteria were reinoculated with a sterilized metal replicator into a fresh plate and grown with shaking at 37°C to early log phase (OD_{580}~0.2), pooled and collected by centrifugation at 1,000 x g for 10 min. Pooled cells were diluted to 5 x 10^5 CFU/ml in sterile saline, and mice were injected intraperitoneally with 200 μl (1 x 10^5 CFU/mouse). Two C57BL/6 mice were injected per pool in the first round. The remainder of the pooled cells was used for genomic DNA preparation (input pool). Three days after infection, mice were sacrificed

by CO_2 inhalation, their spleens were removed and homogenized, and serial dilutions were plated on LB-kanamicin-nalidixic acid plates (3 plates per dilution). Bacteria were harvested from plates containing approximately 1000 colonies such that approximately 4000 total colonies were collected. Pooled cells were pelleted and resuspended in 10 mM Tris-EDTA (TE).

When pools enriched for attenuated mutants were used as inocula, 4 mice per pool were injected. $phox^{gp91-/-}$ and $phox^{gp91-/-}NOS2^{-/}$ were injected with 1 x 10^4 CFU/mouse, and were sacrificed after two days, because a higher inoculum (10^5 CFU/mouse) caused rapid death in the mice, and because despite the lower inoculum, the phox-deficient mice did not survive for 3 days after infection (data not shown).

Salmonella genomic DNA preparation

Salmonella pools resuspended in TE were lysed by the addition of SDS (0.5% final conc.) and proteinase K (100 μg/ml final conc.) and incubated at 37°C for 1 h. DNA was separated from lipids and proteins by precipitation with cetyltrimethylammonium bromide (CTAB). Proteinase K was inactivated at 65°C for 10 min. DNA was extracted with choloroform:isoamyl alcohol (24:1), phenol:chloroform:isoamyl alcohol (25:24:1) and subsequently precipitated by isopropanol (0.7 volume). Genomic DNA was washed with 70% ethanol, resuspended in TE, and adjusted to a concentration of 1 mg/ml.

Colony blots

Bacteria were grown overnight in 96-well plates as described above. Nylon membranes (NEN) were placed on pre-dried 15 cm square (Greiner) LB plates (kanamycin, nalidixic acid) and the membranes were inoculated with a 96-prong replicator. Multiple membranes were inoculated for each pool depending on need. Bacteria were grown upside-down at 37°C for 8-10 h. Membranes were removed from the agar and dried on Whatman paper. Bacteria were lysed by placing the membranes on Whatman paper soaked with 0.4 M NaOH for 8 min. Membranes were neutralized in 0.5 N Tris, pH 7.0 followed by 4 washes in 2X SSC with vigorous shaking to remove bacterial debris. After washing, the membranes were dried and the DNA cross-linked to the membrane by UV irradiation. Membranes were stored at room temperature until needed.

Generation of probes and membrane hybridization

Probes were generated by first amplifying the tag region in a non-radioactive amplification. In a 100 μl volume, 5 μg of genomic template DNA was mixed with 770 ng of primer P2 5' TACCTACAACCTCAAgCT-3' and P4 5'-TACCCATTCTAACCAAgC-3' in a PCR mixture containing 20 mM Tris-Cl, pH 8.3; 50 mM KCl: 2 mM $MgCl_2$; 0.1% Tween 20; 200 μM each of dNTP; 2.5 U Taq polymerase (AmpliTaq). Amplification was by the following cycle: 95°C, 4'; 20 cycles [50°C, 45''; 72°C, 10''; 95°C, 30'']. The resulting 80 bp DNA fragments were purified by agarose gel electrophoresis, extracted (Qiaquick gel extraction kit), and used as templates for a second round of PCR. For the generation of radioactive probes, 20 ng template DNA was amplified in a 20 μl volume according to the protocol described above, except that each

reaction utilized 100 μCi ^{32}P-a-dCTP instead of non-radioactive dCTP. The invariable PCR arms were removed by HindIII (New England Biolabs) digestion for 1 h at 37°C. Probes were then denatured by boiling for 5 min, and placed immediately on ice. Membranes were hybridized with radioactive probes in a solution of Na_2HPO_4, pH 7.2, 0.25 M; NaCl, 0.25 M; EDTA, pH 8.0, 1 mM; SDS, 7%; Formamide, 50%; PEG 8,000, 10% w/v, in a rotating hybridization oven at 42°C for 16 h. Hybridization solution was removed, and the membranes washed twice with 2x SSC, followed by two washes in 2x SSC, 0.1% SDS at 65°C for 1 h. Colony hybridization was detected by autoradiography.

Identification of transposon insertion sites

Colonies selected for sequencing were grown overnight in liquid culture and genomic DNA prepared as described above. Genomic DNA was then digested with restriction enzymes having a known site within the mini-Tn5 transposon multiple cloning site (de Lorenzo *et al.*, 1990; Herrero *et al.*, 1990). Restriction fragments were precipitated and used for ligation into pBluescript SK(+) vector. The products of the ligation reaction were precipitated, resuspended in water, and used for electroporation of competent *E. coli* (XL-10 Gold). Transformed cells were grown on LB plates containing ampicillin and kanamycin, which allowed for selection of plasmids with inserts containing the kanamycin cassette and flanking DNA. Depending on the restriction enzyme chosen (either at the I or O end of the transposon), the adjacent DNA was sequenced on an ABI automated fluorescence sequencer (Applied Biosystems) using primers P6 (5'-CCTAGGCGGCCAGATCTGAT-3') when the DNA was digested at the

O end or P7 (5'-GCACTTGTGTATAAGAGTCAG-3') when the DNA was digested at the I end. Sequences were compared to Genbank sequences by the BLASTX algorithm.

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Alternatively, overnight liquid cultures of clones were mated with overnight liquid cultures of S17-1λpir *E. coli* (Nal^s) containing the plasmid pkanπ-less (Kan^rCm^r). Briefly, small volumes of both cultures were mixed and spotted on an LB plate, and mating was allowed to proceed for at least 2 hours. The bacteria were then recovered and plated on LB containing both chloramphenicol, kanamicin, and nalidixic acid to select for clones in which pkanπ-less from S17-iλpir had homologously recombined with the kanamicin cassette of the mini-Tn5 transposon in the *S. typhimurium* STM mutant. Genomic DNA was prepared from these clones and digested with SacII. Digests were self-ligated and transformed into electrocompetent s17-1λpir. Plasmid minipreps were performed using transformants that had been selected on LB containing chloramphenicol and kanamicin. DNA was sequenced using primer P6 as described above.

In vivo bacterial virulence assays

To compare *in vivo* survival, *S. typhimurium* 12023 and *S. typhimurium* STM mutants were inoculated from fresh overnights into LB at a dilution of 1:100 and grown for 1.5-2 hours to early log phase (A_{600} ~ 0.2-0.4). For monotypic infections, bacteria were pelleted, resuspended in 0.9% sterile saline, and diluted to 10^4 CFU/ml. Mice were injected intravenously with 100 μl (1 x 10^3 CFU/mouse). At various time points, mice were sacrificed, their spleens were harvested and homogenized in 0.9% NaCl, and serial dilutions were plated on LB to determine colony forming units (cfu) per spleen. Colonies

were counted the following day. For competition experiments (Beuzon and Holden, 2001), bacteria were pelleted, resuspended in 0.9% sterile saline and diluted to 5 x 10^3 CFU/ml. *S. typhimurium* 12023 and individual STM mutant strains were mixed in a 1:1 ration for a final concentration of 1 x 10^4 CFU/ml. Mice were injected intravenously with 100 μl (1 x 10^3 CFU/mouse). Spleen homogenates prepared at indicated timepoints were plated on LB containing nalidixic acid and kanamicin (to determine numbers of STM mutants) and LB containing nalidixic acid (to determine the total number of bacteria). Competitive index was determined by the following formula: $(mutant_{out}/12023_{out})/(mutant_{in}/12023_{in})$.

In vitro fitness

Overnight cultures of STM mutants and 12023 *S. typhimurium* were diluted 1:100 into fresh LB. 50 µl 12023 + 50 µl mutant strain were combined with 900 µl LB containing 20 µg/ml nalidixic acid in a 14 ml culture tube. Aliquots of the mixed cultures were diluted and plated on LB +/- kanamicin to determine the starting ratio of mutant:12023. Cultures were grown at 37°C, 250 rpm for 4-5 hr, then diluted and plated on LB +/- kanamicin. Competitive index was determined as before $[(mutant_{out}/12023_{out})/(mutant_{in}/12023_{in})]$.

Isolation and infection of macrophages

Mice were injected intraperitoneally with 1 ml of freshly prepared, filter-sterilized 5 mM sodium periodate ($NaIO_4$) in 0.9% saline. Four days later mice were sacrificed by CO_2 inhalation. When serum was needed, cardiac puncture was performed to acquire

blood, which was allowed to clot at room temperature, and then centrifuged to separate the serum. Serum was heated at 56°C for 30 min to inactivate complement MAC components. Peritoneal cells were harvested from the same mice by lavage with 10 ml cold DMEM. Red blood cells were lysed with hypotonic saline, and leukocytes were resuspended in DMEM containing 10% heat-inactivated fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin-G, and 100 μg/ml streptomycin. Cells were plated at a density of $5x10^5$ cells/well in 96 well microtiter plates unless otherwise specified. The following day, nonadherent cells and antibiotic-containing media were removed by washing cells twice with pre-warmed 1xPBS. Wells were replenished with DMEM containing 1% heat-inactivated FCS and 1 mM sodium pyruvate. Assays were performed on the second day after harvesting cells. Immediately prior to infection, cells were washed twice with pre-warmed 1xPBS, and wells were replenished with either 100 μl DMEM containing 1% heat-inactivated FCS and 1 mM sodium pyruvate for bactericidal assays, or with 200 μl Krebs Ringer phosphate plus glucose (KRPG) formulated as previously described (De la Harpe and Nathan, 1985) for cytotoxicity assays. Bacterial strains for infections were inoculated from fresh overnight cultures into LB at a dilution of 1:100 and grown for 5.5 hours to late log phase (A_{600} ~ 3-4.) Late log cultures were resuspended in 0.9% saline, diluted 1:50, and opsonized in 10% heat-inactivated murine serum for 30 min at 37°C with end-over-end rotation. Opsonized bacteria were resuspended in 0.9% saline, and 10 μl of opsonized bacteria was added per well of macrophages. Approximately 10^6 bacteria were added per well for an MOI of 2:1, unless otherwise specified. Plates were centrifuged at 250xg for 5 min at room temperature and then incubated at 37°C.

Salmonella-induced macrophage cytotoxicity assay

Immediately after centrifugation, samples for the -10 minute time point were harvested by removing 100 µl of supernatant from each well. All other plates were incubated 10 minutes at 37°C to allow phagocytosis of bacteria by macrophages, after which time the 0 minute time point was harvested, and gentamicin was added to wells in all other plates for a final concentration of 10 µg/ml. At indicated time points during infection, 100 µl of supernatant from each well was removed. Cytotoxicty was determined by amount of LDH released into supernatants using the Cytotoxicity Detection Kit (Roche Molecular Biochemicals) according to the equation: %cytotoxicity = (experimental LDH release uninfected LDH release) / (Maximum LDH release uninfected LDH release) x 100. Remaining medium was removed from the wells prior to microscopic examination and photography. Alternately, the Lowry protein assay was used to determine total protein per well as an estimate of macrophages remaining. At each time point, medium was removed, and wells were washed two times in 1xPBS to wash away excess serum protein. Macrophages were lysed in 100 mM NaOH for 30 min, and then 100 µl of Lowry reagent (0.5% $CuSO_4 \cdot 5H_2O$ + 1.0% Na_3-citrate + 4% $NaCO_3$) was added per well. Reactions were developed using 10 µl of Folin and Ciocalteu's Phenol reagent (Sigma), and plates were evaluated at A_{650}.

Bactericidal assays

After centrifugation, plates were incubated at 37°C, 5% CO_2 for 10 min to allow for phagocytosis. At time 0, wells were washed 3x with prewarmed 1x PBS. The

macrophages in one plate were then lysed with 0.1% Triton X-100, and 10 μl of lysates were transferred via multichannel pipette to wells containing 100 μl LB broth + 10% Alamar blue in a read-out plate. Wells in the unlysed plates were replenished with 100 μl DMEM containing 1% heat-inactivated FCS, 1 mM soduium pyruvate, and 10 μg/ml gentamicin, and plates were incubated at 37°C, 5% CO_2. At indicated time points, macrophages in one 96-well plate were lysed, and 10 μl of lysates was transferred to the read-out plate. Read-out plates were stored overnight at 4°C, and evaluated the following day (Shiloh *et al.*, 1997).

***In vitro* stress susceptibility assays**

Overnight cultures of *S. typhimurium* were diluted 1:100 in LB broth +/- carbenicillin 100 μg/ml in 14 ml tubes, and outgrown for 5.5 hour at 37°C, 250 rpm to an A_{600} of approximately 3.5. 5.5 hour cultures were diluted 1:100 into LB broth containing the specified stress. Samples were cultured at 37°C in 96-well microtiter plates with agitation. Unless indicated, starting pH of the cultures was approximately 7.0. Assays using sodium nitrite were performed at pH 5.5 in LB media +100 mM MES. Growth and/or killing of bacteria were evaluated by assessing A_{600} of the culture, plating for cfus, or the Alamar Blue assay (Shiloh *et al.*, 1997).

Production of recombinant YdiV and Antiserum

Primers were generated to PCR amplify *ydiV* from *S. typhimurium* chromosomal DNA such that the product would contain a 5' *NdeI* site around the start site and a 3' *XhoI* site: forward (5'-CATGGGACTGGCCATATGATTGCTTCACTTGATGAGCTT)

and reverse (5'-CGGCAAACAGAACTCGAGTCGCTGAACGAGTTTAATGAG). The product was cloned into pET24b$^+$, cut with *NdeI* and *XhoI*, and the resulting plasmid (pETV10) was used to transform *E. coli* ER2566. The hexahistidine fusion protein was over-expressed by induction with 1 mM IPTG for 3.5 hours. Sonication was used to disrupt *E. coli*, and His-tagged protein was purified on Ni-NTA agarose beads under native conditions. A portion of the purified protein was subjected to SDS-PAGE, and the Coomassie blue-stained band running at approximately 25 kDa was cut from the gel and sent to Covance (Denver, PA) for production of antisera. Rabbit were injected with a primary boost of purified protein in Freund's complete adjuvant, followed by 4 boosts in Freund's incomplete adjuvant. Rabbit sera was purified by adsorption with acetone powders made from *S. typhimurium* SCN19 and *E. coli* ER2566.

Protein analysis: Silver stained SDS-PAGE gels and Western Blots

Bacterial lysates were prepared by sonicating on ice in 50 mM Tris, pH 7.5. Cytosolic fractions were recovered by centrifugation to separate membrane fraction. Secreted and shed proteins were prepared by tricholoroacetic acid (TCA)-precipitation of bacterial supernatants. Briefly, supernatants were filtered through 0.45 μM syringe filters. 100% TCA (4°C) was added per sample for a final concentration of 10% TCA, and proteins were pelleted by spinning at 18K x g, 4°C for 10 min. Precipitated proteins were washed with cold acetone and re-pelleted. Remaining acetone was evaporated before resuspending supernatant proteins. Protein samples were separated by SDS-PAGE. 8% polyacrylamide was used when evaluating YdiV, and 15% polyacrylamide was used for all other assays. Silver staining was performed as described (Blum *et al.*,

1987). Briefly, polyacrylamide gels were fixed overnight in methanol (50%), acetic acid (12%) and 0.185% formaldehyde. Gels were pretreated in sodium thiosulfate, impregnated with silver nitrate, and developed with sodium carbonate. Developing reaction was stopped with methanol and acetic acid, and gels were equilibrated with glycerol. For immnoblots, proteins were transferred from polyacrylamide gels to nitrocellulose membranes and reacted with antiserum or anti-flagellin monoclonal antibody (Virostat) in (pH 7.5) Tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% nonfat milk. Anti-rMsrA and rMsrB antisera were prepared as described (St John *et al.*, 2001). Bound antibody was detected by enhanced chemiluminescence (Pierce).

Motility assays

Bacterial motility was assessed by spotting 2 μl of culture on the surface of agar plates containing 0.32% agar for swimming motility and 0.6% agar for swarming motility. Media in the plates contained 1% Tryptone and 0.5% yeast extract (with no added NaCl.) Plates were analyzed after either 5 hr incubation at 37°C or overnight incubation at room temperature.

Congo Red Agar

Bacterial production of exopolysaccharide was evaluated by plating liquid cultures of *S. typhimurium* on Congo Red agar plates. Congo red plates were made as follows: 5 g tryptone, 2.5 g yeast extract, and 7.5 g agar were dissolved in 500 ml water and autoclaved. When media was sufficiently cool, Congo Red was added to a final

concentration of 40 μg/ml, and Coomasie Brilliant Blue was added to a final concentration of 10 μg/ml. Plated bacterial cultures were incubated at 30°C or room temperature overnight (Romling *et al.*, 1998; Zogaj *et al.*, 2001).

Transcriptional fusion constructs and analysis of gene expression

Chromosomal *lacZYA* fusion reporters were constructed using the plasmid pFUSE (Baumler *et al.*, 1996). In order to include full promoter sequences, segments of chromosomal DNA selected for insertion into pFUSE included part of the upstream gene, the intervening region, and a portion of the 5' region of the gene to be evaluated. Restriction fragments of genomic DNA were subcloned into the multiple cloning site of pAJD107, and then recovered using XbaI and SmaI for ligation into pFUSE. Ligations were transformed into *E. coli* S17-1λpir. Clones generated by this method include pKH101 (784 bp of DNA upstream of *ydiV* fused to *lacZYA*), pFlac (728 bp of DNA upstream of *flhD* fused to *lacZYA*), and pKH201 (~800 bp of DNA upstream of *ahpC* fused to *lacZYA*). Fusion constructs were transferred by conjugation into SCN14 (Nal^R) or SCN19 (Nal^R,Kan^R), and integrants into the *S. typhimurium* chromosome were selected by plating on LB-agar containing chloramphenicol and nalidixic acid, +/- kanamicin. Integrants contain one intact copy of the gene (*ydiV*, *ahpC*, or *flhDC*) contiguous with the pFUSE construct containing the putative gene promoter fused to *lacZYA*. *sip* operon expression was evaluated using the strain BJ68, which includes a *lacZYA* fusion construct inserted within and disrupting *sipC* (Penheiter *et al.*, 1997). Transduction was used to transfer the fusion constructs into other *S. typhimurium* strains when needed.

Expression of *ydiV*, *flhDC*, *ahpC*, or the *sip* operon in strains containing fusion constructs was assayed by evaluation of β-galactosidase activity in whole cell lysates. Overnight cultures were diluted 1:100 into 30 ml fresh LB broth in 250 ml beveled flasks and grown with agitation at 37°C. At specified time points, 1 ml of culture was harvested and centrifuged at room temperature for 2 min at maximum speed in a microfuge. Supernatants were removed, and pellets were frozen at –20°C. After all time points had been harvested and frozen, pellets were thawed and resuspended in working buffer. Lysis of cells, measurement of β-galactosidase activity, and calculations of values were performed as described (Miller, 1972).

Transductions

To make P22 bacteriophage lysates, 0.5 ml of a stationary phase *S. typhimurium* culture was combined with 2 ml of LBEDϕ broth (LB + E salts + dextrose + P22 phage) (Maloy *et al.*, 1996). Phage infected cultures were incubated at 37°C with agitation for 6-16 hours to permit infection and lysis of *S. typhimurium*. Infected cultures were centrifuged for 2 min at maximum speed in a microfuge. Supernatants were transferred to clean tubes with two drops of chloroform and vortexed to kill any remaining bacteria. For transduction, 10-20 μl donor P22 lysate was mixed with 100-200 μl recipient bacteria. Samples were incubated at 37°C for 10-20 min, and then phage infection was arrested by addition of 1 ml LB + 10 mM EGTA. Cultures were returned to 37°C and incubation was continued for 20 min (for transduction of Kan^R or Amp^R) up to two hours (for transduction of Cm^R). Cultures were plated on LB-agar containing 10 mM EGTA

and the appropriate selective antibiotics. Absence of continued phage activity was confirmed by plating clones on LB-agar containing Evans-Blue-Uranine (EBU agar).

Quantitation of c-diGMP

Synthetic c-diGMP was kindly provided by Dr. Roger Jones of Rutgers University, New Jersey (Hsu *et al.*, 1985).

Samples analyzed for quantitation of endogenous c-diGMP levels were prepared as follows. Overnight cultures were diluted 1:100 into 50 ml LB broth + 100 μg/ml carbenicillin in 500 ml flasks. Cultures were grown at 37°C with agitation. After 5.5 hr, cultures were spun at 4000 rpm, 4°C for 30 min. Pellets were resuspended in 1 ml 50 mM Tris, pH 8.0, and sonicated on ice to disrupt cell membranes. Lysates were spun at 13K, 4°C for 30 min to isolate soluble fractions. Volumes of supernatant containing 1.8 mg of protein were combined with 1 volume chloroform, 4 volumes methanol, and 3 volumes of water to extract polar micromolecules. Samples were vortexed and spun at 13K for 5 min. Aqueous phases were recovered and frozen at –80°C.

For evaluation of phosphodiesterase activity in lysates, preparation of samples was as for quantitation of endogenous c-diGMP with the following modifications. Overnight cultures diluted 1:100 were grown for 2.5 hours, at which point 1 mM IPTG was added to ECN 145 cultures to induce expression of rYdiV. Cultures were harvested 2 hours later (total culture time = 4.5 hr.) After sonication, half of the lysate was spun to isolate supernatant (soluble fraction), and the other half was saved unseparated to include membrane bound proteins (total cell lysates). Volumes of soluble fraction and total cell lysate corresponding to 0.5 mg of protein in the soluble fraction were used for enzymatic

assays. Samples to be denatured were boiled for 10 min. 50 pmole of c-diGMP was added to each sample. Following incubation at 37°C for 10, 30, or 60 min, samples were extracted as above, frozen at –80°C, and shipped to Dr. Michael MacCoss at the University of Washington for evaluation of c-diGMP content. Dr. MacCoss fractionated the extracts using a home built 100 μm capillary column packed with 7 cm of HILIC material, producing a flow rate of ~500 nl/min. Fractions were subjected to selected reaction monitoring (SRM) mass spectrometry as described in the legend for Figure 22.

Chapter 3

M. tuberculosis counter-immune (*cim*) mutants identified by differential screening in immuno-deficient mice

IFN-γ-inducible NOS2 is an essential component of host defense against Mtb (MacMicking *et al.*, 1997). However, it is clear that IFN-γ-deficient mice are more susceptible to Mtb than NOS2-deficient mice, because time-to-death (MacMicking *et al.*, 2003; Mogues *et al.*, 2001) and bacterial growth in the tissues (MacMicking *et al.*, 2003) after infection are more rapid in the former. These differences indicate that NOS2 is not the only anti-mycobacterial mechanism controlled by IFN-γ. Recently, MacMicking, *et al.* demonstrated that the IFN-γ-inducible GTPase LRG-47 is also an essential component of murine defense against Mtb (MacMicking *et al.*, 2003), potentially accounting for the difference in susceptibility between $NOS2^{-/-}$ and IFN-$\gamma^{-/-}$ mice. Chemical inhibition of NOS2 further exacerbates infection in LRG-47-deficient mice, indicating that these mechanisms make independent contributions to host defense. However, the combined loss of LRG-47 and NOS2 does not increase susceptibility to the same level observed in IFN-γ-deficient mice (MacMicking *et al.*, 2003), suggesting further IFN-γ-mediated host mechanisms for the control of Mtb. Unidentified IFN-γ-dependent, NOS2-independent mechanisms have also been shown to be important for the control of other macrophage pathogens, including *Bordetella pertussis* (Mahon and Mills, 1999), *Mycobacterium avium* (Gomes *et al.*, 1999), *Toxoplasma gondii* (Suzuki *et al.*, 2000; Yap and Sher,

1999), *Leishmani donovani* (Murray and Delph-Etienne, 2000), *Francisella tularensis* (Polsinelli *et al.*, 1994), *Legionella pneumophila* (Heath *et al.*, 1996) and *Listeria monocytogenes* (Leenen *et al.*, 1994).

To better understand the role of IFN-γ during infection with Mtb, we sought to identify genes used by Mtb as counter-measures to IFN-γ mediated pathways. The interplay between the immune response and persistent pathogens like Mtb is a fine-tuned balance of host attacks and bacterial counter-measures (Muñoz-Elías and McKinney, 2002). Over thousands of years of co-existence with mammalian hosts, there has presumably been strong evolutionary pressure for pathogenic mycobacteria to evolve counter-measures against each of the potentially bactericidal elements of the host immune response. Host mechanisms against which the bacterium has evolved effective counter-measures appear to be of lesser significance during infection with wild type strains of Mtb, but are revealed as important immune pressures when the relevant bacterial genes are disrupted. One example of this phenomenon is detoxification by KatG catalase of the phox respiratory burst, which effectively neutralizes the impact of this bactericidal immune mechanism (Ng *et al.*, 2004).

Here we use a variant of signature-tagged transposon mutagenesis (STM) (Hensel *et al.*, 1995) as a screening method to identify Mtb genes that are involved in evading or countering IFN-γ-dependent, NOS2-independent immune responses. We report the identification of four Mtb transposon-induced mutants that are capable of progressive replication and rapid lethality in IFN-γ-deficient mice but not in NOS2-deficient mice, indicating increased susceptibility of the mutants to IFN-γ-dependent immune mechanisms other than NOS2. Identification of the genes disrupted in these mutants

suggests that such diverse processes as phosphate metabolism, polyketide synthesis, and cell wall biogenesis may be important for adaptation of Mtb to the *in vivo* environment as influenced by the host immune response.

Results

Growth and lethality of Mtb in IFN-γ$^{-/-}$, NOS2$^{-/-}$, and phox$^{gp91-/-}$ NOS2$^{-/-}$ mice

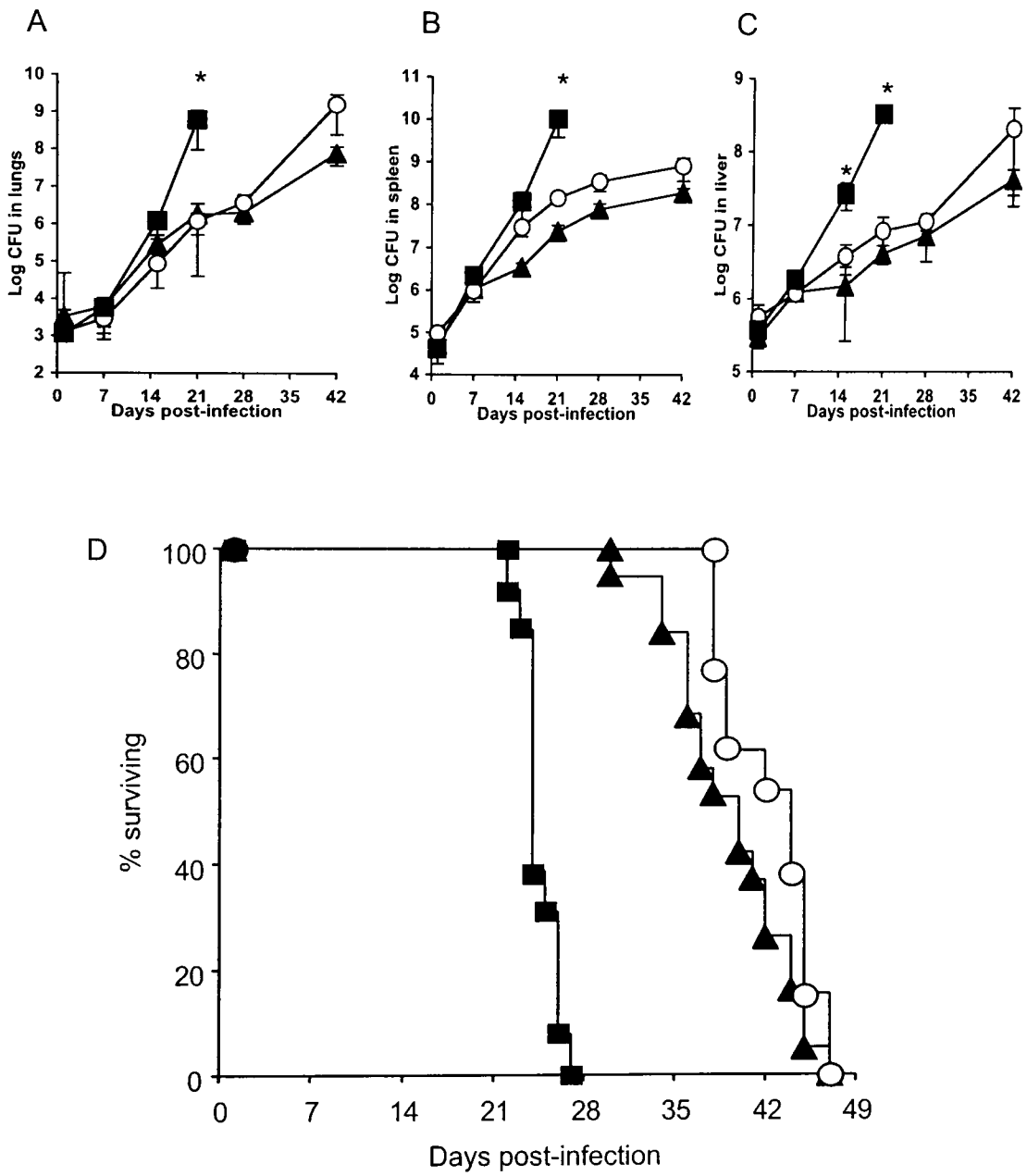
Previous infections of phox-deficient mice suggested that host production of ROI may play a detectable, albeit minor, role in control of Mtb (Adams *et al.*, 1997; Cooper *et al.*, 2000). We considered the possibility that the contributions of IFN-γ-activated phox to host defense against Mtb might be masked by the dominance of NOS2 in phox-deficient mice. To draw forward subtle anti-mycobacterial effects of phox, we compared susceptibility of NOS2$^{-/-}$ mice to Mtb with that of mice lacking both NOS2 and phox (Fig. 6). Phox^{gp91}-deficiency did not increase the susceptibility of NOS2-deficent mice in terms of survival (Fig. 6D) or bacterial growth kinetics in the tissues (Fig. 6A-C). These observations suggest the existence of an additional, as-yet unidentified IFN-γ-protective mechanism(s) that is independent of NOS2, LRG-47, and phox.

Identification of Mtb counter-immune (cim) mutants by differential screening in IFN-γ$^{-/-}$and NOS2$^{-/-}$ mice

The substantial difference in susceptibility of IFN-γ$^{-/-}$ and NOS2$^{-/-}$ mice to Mtb suggested the possibility of identifying mechanisms of bacterial defense against IFN-γ-dependent, NOS2-independent immune mechanisms by differential screening in mice. Our strategy was to passage pools of signature-tagged mutants through NOS2$^{-/-}$ mice and

Figure 6. Pathogenesis of Mtb in NOS2$^{-/-}$, phox$^{gp91-/-}$NOS2$^{-/-}$, and IFN-γ$^{-/-}$ mice. IFN-γ$^{-/-}$ mice (filled squares), NOS2$^{-/-}$ mice (open circles), and phox$^{gp91-/-}$NOS2$^{-/-}$ mice (filled triangles) were infected i.v. with ~2 x 10^5 CFU of wild-type Mtb Erdman. (A-C) Kinetics of bacterial growth in the lungs (A), spleens (B), and livers (C) of infected mice (n = 4-5 per group, except day 21 IFN-γ$^{-/-}$, n = 5; day 42 NOS2$^{-/-}$, n=3; day 42 phox$^{gp91-/-}$NOS2$^{-/-}$, n = 8). *$P < 0.05$ for comparison of IFN-γ$^{-/-}$ mice with NOS2$^{-/-}$ and phox$^{gp91-/-}$NOS2$^{-/-}$ mice. A representative graph depicting growth of Mtb in the lungs of wild type mice can be found in Figure 1A. (D) Survival of infected mice. Mean time-to-death was 24.5 days for IFN-γ$^{-/-}$ mice (n = 13), 42.4 days for NOS2$^{-/-}$ mice (n = 13), and 39.4 days for phox$^{gp91-/-}$NOS2$^{-/-}$ mice (n = 19). Survival times were significantly different for IFN-γ$^{-/-}$ mice vs. NOS2$^{-/-}$ mice ($P < 0.0001$) and IFN-γ$^{-/-}$ mice vs. phox$^{gp91-/-}$NOS2$^{-/-}$ mice ($P < 0.0001$) but not for NOS2$^{-/-}$ mice vs. phox$^{gp91-/-}$NOS2$^{-/-}$ mice ($P = 0.064$). Similar results were obtained in a second experiment using Mtb H37Rv. Wild type mice infected with a similar inoculum of Mtb live upwards of 250 days (data not shown).

Figure 6.


A
B
C
Log CFU in lungs
Log CFU in spleen
Log CFU in liver
Days post-infection
D
% surviving
Days post-infection

IFN-$\gamma^{-/-}$ mice in order to identify mutants that were under-represented in the former but not the latter. Mice were infected with pools representing 48 independently derived STM mutants and sacrificed at ~2 wk (IFN-$\gamma^{-/-}$ mice) or ~3 wk (NOS2$^{-/-}$ mice) post-infection for recovery of bacteria from the lungs. These time-points were selected to permit maximal expansion of the bacterial populations in the lungs; by sacrificing animals shortly before they would become moribund, the under-representation of attenuated mutants in the recovered pools can be maximized. Each pool of mutants was passaged through two NOS2$^{-/-}$ mice and two IFN-$\gamma^{-/-}$ mice, and mutants that showed a consistent phenotype in both mice of each genotype were selected for further analysis. Here we describe four *cim* mutants identified during initial screening of 2 pools that appear to be defective in counter-immune mechanisms specifically directed towards IFN-γ-dependent, NOS2-independent host pathways.

Identification of genes disrupted in the cim mutants

The transposon insertion sites (Table V) were identified in each of the four *cim* mutants using inverse PCR (Cox *et al.*, 1999). The first and second mutants were disrupted in Rv2958c and Rv0405 (a.k.a. *pks6*), respectively, neither of which are found in operons, and the third mutant was disrupted in Rv0930, which is the final gene in an apparent operon. The observed phenotypes of these mutants were therefore unlikely due to polar effects of the transposon insertion on neighboring genes. The fourth transposon insertion site was located in Rv0072, which appears to be co-transcribed with the downstream gene, Rv0073. Rv0072 and Rv0073 have been annotated as putative components of an ATP-binding cassette (ABC) transporter based on homology to genes

TABLE V. Time-to-death kinetics of mice infected with Mtb counter-immune (*cim*) mutants

MTB strain	Putative function	Median survival (d)		Ratio:	Mean survival (d)		Ratio:
		IFN-$\gamma^{-/-}$ mice	$NOS2^{-/-}$ mice	$\frac{NOS2^{-/-}}{IFN\text{-}\gamma^{-/-}}$	IFN-$\gamma^{-/-}$ mice	$NOS2^{-/-}$ mice	$\frac{NOS2^{-/-}}{IFN\text{-}\gamma^{-/-}}$
H37Rv	—	23	36*	1.57	24	39*	1.70
*Rv*0072	Glutamine ABC transporter MSD subunit	32	84*	2.63	32	94*	2.94
*Rv*0405	Polyketide synthase 6 (Pks6)	44	123*	2.80	45	126*	2.84
*Rv*0930	Phosphate ABC transporter MSD subunit	38	180*	4.74	39	182*	4.67
*Rv*2958c	Glycosyl transferase	43	133*	3.06	43	127*	2.95

Mice were infected i.v. with ~2 x 10^5 CFU of the indicated MTB strains (n = 4-6 mice per group).
*$P < 0.005$ in comparison with IFN-$\gamma^{-/-}$ mice.

of known function in other bacterial species (Braibant *et al.*, 2000). Rv0930 has been annotated as part of another putative ABC transporter. Expression of Rv2958c, which is homologous to glycosyl transferase genes in other species, was shown previously to increase survival of non-pathogenic *Mycobacterium smegmatis* in macrophages (Miller and Shinnick, 2000). *pks6*, encoding a putative polyketide synthase, was identified in a previous STM screen as being essential for full virulence of Mtb in wild-type BALB/c mice (Camacho *et al.*, 1999), however, the effect of *pks6* deficiency on Mtb replication in immuno-deficient mice has not been reported.

Confirmation of the cim mutants' in vivo phenotypes

None of the *cim* mutants displayed a diminished growth rate *in vitro* (Fig. 7), suggesting that their *in vivo* growth deficiencies were due to loss of functions required for Mtb's adaptation to *in vivo* environmental conditions. The *cim* mutants' *in vivo* phenotypes were confirmed by infection of wild-type (C57Bl/6), IFN-$\gamma^{-/-}$, and NOS2$^{-/-}$ mice with the individual strains (Fig. 8). As expected, wild-type Mtb (H37Rv strain) demonstrated a marked growth advantage in the lungs of IFN-$\gamma^{-/-}$ and NOS2$^{-/-}$ mice as compared to C57Bl/6 mice (Fig. 8A). All four of the *cim* mutants displayed a significant growth advantage in IFN-$\gamma^{-/-}$ mice as compared to C57Bl/6 mice; however, little or no growth advantage was observed in NOS2$^{-/-}$ mice as compared to C57Bl/6 mice, thus confirming the phenotype that had been selected in the screen (Fig. 8B-E). Additionally, all of the *cim* mutants demonstrated statistically significant deficits in their ability to grow in the lungs of C57BL/6 mice as compared with H37Rv (data not shown, $p < 0.05$).

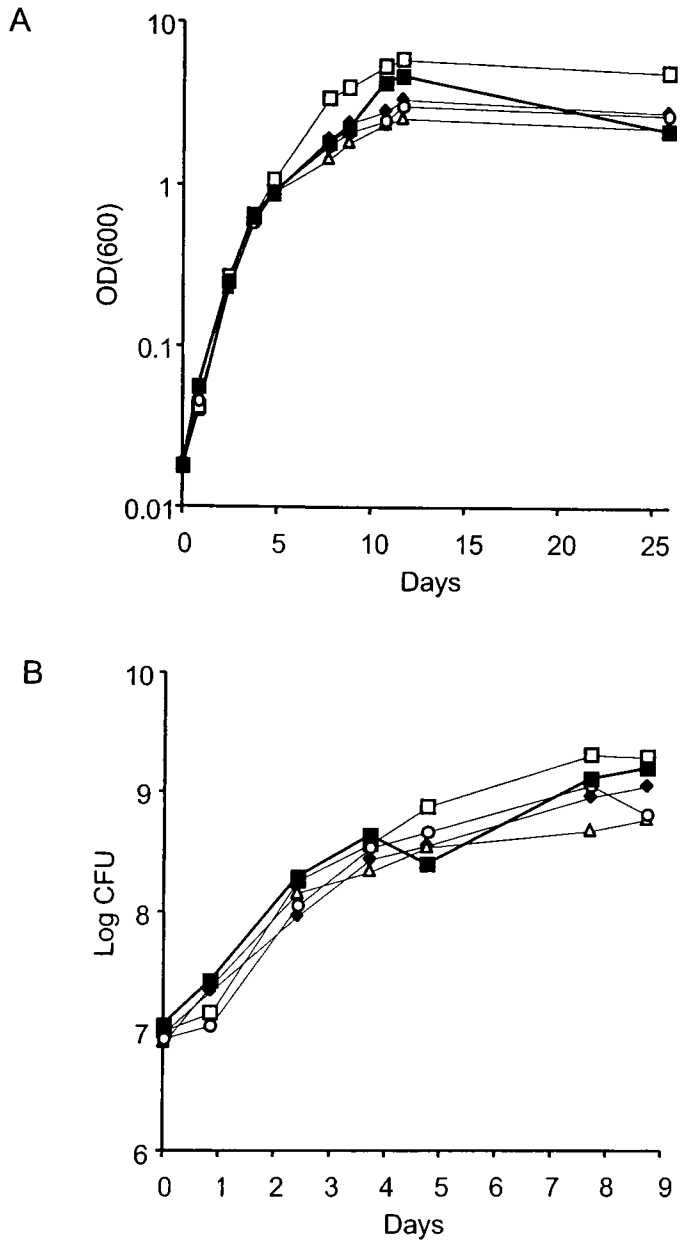
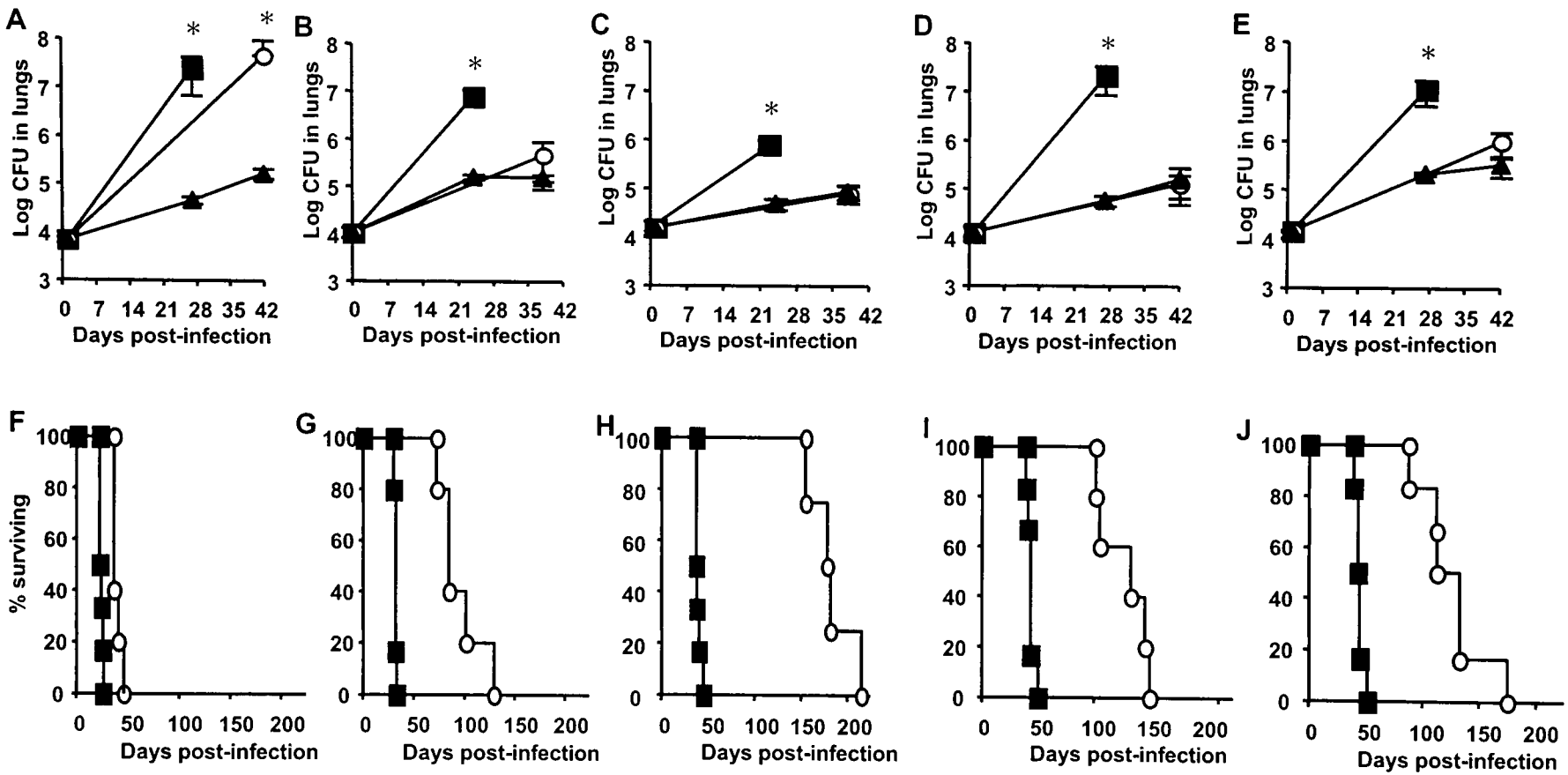


Figure 7. The Mtb *cim* mutants do not manifest any obvious growth deficiencies *in vitro*. Stationary phase cultures of the indicated Mtb strains were diluted 1:500 in fresh medium and incubated at 37°C. Aliquots were withdrawn at the indicated times for measurement of the optical density at 600 nm (A) or enumeration of CFU by plate culture (B). Symbols: wild-type Mtb H37Rv (filled diamonds); *Rv*0072 (open circles); *Rv*0405 (open triangles); *Rv*2958c (open squares), *Rv0930* (filled squares). At time-points later than 9 days, clumping of the cultures prevented accurate measurement of CFU (B). Results are representative of two experiments.

Figure 8. Pathogenesis of Mtb *cim* mutants in mice. Mice were infected i.v. with ~0.5-1.0 x 10^6 CFU of wild-type Mtb (A,F) or one of the following Mtb *cim* mutants: *Rv*0072 (B,G), *Rv0930* (C, H), *Rv*2958c (D,I), *Rv*0405 (E,J). Symbols: C57Bl/6 mice (filled triangles), NOS2$^{-/-}$ mice (open circles), and IFN-$\gamma^{-/-}$ mice (filled squares). (A-E) Bacterial growth kinetics in the lungs of mice (n = 4 per group). *$P < 0.05$ for comparisons of bacterial loads (CFU) in IFN-$\gamma^{-/-}$ mice vs. C57Bl/6 mice at ~4 wk post-infection or NOS2$^{-/-}$ mice vs. C57Bl/6 mice at ~6 wk post-infection. Results are representative of two experiments. (F-J) Survival of infected mice (n = 4-6 per group). In all cases, survival of infected NOS2$^{-/-}$ mice was significantly longer than survival of IFN-$\gamma^{-/-}$ mice infected with the same Mtb strain ($P < 0.005$ for all comparisons).

Figure 8.


A
B
C
D
E
Log CFU in lungs
Days post-infection
F
G
H
I
J
% surviving
Days post-infection

These observations suggest that the *cim*-encoded functions are essential for progressive bacterial growth in the presence, but not the absence, of IFN-γ-dependent immune mechanisms other than NOS2. This interpretation was further supported by the survival kinetics of mice infected with the *cim* mutants. Time-to-death was similar for IFN-γ-deficient animals infected with wild-type Mtb H37Rv or each of the *cim* mutants (Table V and Fig. 8F-J, solid squares). However, the time-to-death for NOS2-deficient animals was markedly delayed when animals were infected with each of the four *cim* mutants as compared to wild-type Mtb (Table V and Fig. 8F-J, open circles). These observations indicate that the role of the *cim* genes in Mtb pathogenesis is contingent on aspects of the *in vivo* environment that are modulated by the host immune response. The most straightforward interpretation of these results is that these *cim* genes encode functions that are required for protection against the onslaught of IFN-γ-dependent, NOS2-independent host immune mechanisms; confirmation of this hypothesis will require the elucidation of the precise mechanisms by which these *cim* genes contribute to virulence.

Discussion

Long-term persistence in immuno-competent individuals is a hallmark of Mtb infection, but little is known about the bacterial mechanisms involved in countering the onslaught of the host immune response. Here we describe a genetic strategy to identify Mtb genes that are involved in protecting the pathogen against immune mechanisms controlled by IFN-γ, a key cytokine required for macrophage activation and anti-mycobacterial defense. IFN-γ is known to activate a large number of pathways at the level of the macrophage (Ehrt *et al.*, 2001) , but, with few exceptions, the roles of these

pathways in anti-mycobacterial defense are unknown. The four *cim* genes described here appear to be involved in adaptation of Mtb to pressures exerted by IFN-γ-dependent mechanisms other than NOS2, because the corresponding mutants showed little or no defect for growth and lethality in IFN-γ-deficient mice yet were highly attenuated in NOS2-deficient mice. A similar strategy of characterizing virulence in immuno-deficient mouse strains was used to demonstrate the role of the Mtb KatG catalase (Ng *et al.*, 2004) and the *Salmonella* Pathogenicity Island-2 (Vazquez-Torres *et al.*, 2000b) in countering the host NADPH oxidase; in these cases, attenuation of the mutants was reversed in $gp91^{phox-/-}$ mice.

The *cim* mutants do not appear to be killed in NOS2-deficient mice, but they are incapable of progressive growth in these mice; indeed, the growth profiles of the *cim* mutants are very similar in C57Bl/6 and $NOS2^{-/-}$ mice, in sharp contrast to wild-type Mtb, which replicates much more extensively in $NOS2^{-/-}$ mice than in C57Bl/6 mice (Fig. 8). Of interest, none of the *cim* mutants appeared to be severely attenuated in wild-type C57Bl/6 mice despite being severely attenuated, compared to wild-type Mtb, in NOS2-deficient mice (Fig. 8). We anticipated this possibility, which is why we chose to screen mutant pools directly in immuno-deficient mice. Several explanations are possible. The impact of immune mechanisms that are engaged at later stages of infection, after NOS2 has already come into play, might be masked by the dominant effect of NOS2. It is also possible that alternative pathways that are up-regulated in the absence of NOS2 (Ehrt *et al.*, 2001) might exert an anti-bacterial effect only in that context. Direct screening in immuno-deficient mice should be particularly useful for identification of Mtb genes that permit continued bacterial growth in the face of otherwise bacteriostatic immune

mechanisms activated by IFN-γ; it is unlikely that such mutants would be identified by screening in C57Bl/6 mice, due to the masking effect of NOS2.

One of the *cim* genes, *Rv*0405, was previously identified as an Mtb virulence factor (Camacho *et al.*, 1999). *Rv*0405 is annotated as polyketide synthase 6 (*pks6*), one of 16 genes in the Mtb genome that are predicted to encode polyketide synthases (Cole *et al.*, 1998), although other polyketide synthases continue to be identified (Saxena *et al.*, 2003). To date, only two of the Mtb polyketide synthases have been assigned biochemical functions: Pks2 (Sirakova *et al.*, 2001) and Pks15/1 (Constant *et al.*, 2002) are involved in the biosynthesis of cell wall sulfolipids and phenolglycolipids, respectively. Although the polyketide synthesized by Pks6 has not been identified, a number of polyketide metabolites from other organisms—such as FK506, rapamycin, and cyclosporine—are potent immunosuppressants (Cardenas *et al.*, 1998). Thus, Pks6 might be involved in synthesis of a polyketide(s) product that interferes with the IFN-γ-dependent arm of the anti-mycobacterial immune response, which would explain why *pks6* is apparently dispensable for bacterial virulence in the absence of IFN-γ (Fig. 8).

*Rv*2958c and its *Mycobacterium leprae* homolog were identified previously in screens for genes that enhanced the survival of the non-pathogenic saprophyte *Mycobacterium smegmatis* following phagocytosis by macrophages (Miller and Shinnick, 2000; Mundayoor and Shinnick, 1994). *Rv*2958c is annotated as one of several putative glycosyl transferases in the Mtb genome (Cole *et al.*, 1998); these enzymes may be involved in the glycosylation of cell surface lipids or proteins that interact with host cells. Defects in glycosylation of secreted and surface proteins have been shown previously to affect the immune response to these Mtb antigens (Schmidt *et al.*, 2003). *Rv*2958c is

located within the genomic region that includes *pks15/1*. This gene cluster is conserved in order and sequence between Mtb and *M. leprae*, suggesting that these genes may function in a common pathway for the production of cell wall phenolglycolipids (Constant *et al.*, 2002).

Rv0072 and Rv0930 are annotated as components of a putative ATP binding cassette (ABC) transporters. ABC transporters are large multi-subunit permeases which function in eukaryotic cells as exporters and in prokaryotes as both importers and exporters (Braibant *et al.*, 2000). ABC transporters consist of two membrane-spanning domains (MSDs), and two nucleotide-binding domains (NBDs). Prokaryotic ABC importers often include an additional high affinity substrate binding protein (SBP) that specifies the cargo of the ABC transporter.

*Rv*0072 encodes one of two annotated MSD subunits for a putative glutamine transporter. The transposon insertion in *Rv*0072 may also exert polar effects on the downstream gene, *Rv*0073, which is annotated as encoding the glutamine transporter's NBD subunit. Mtb is a glutamine prototroph, and previous studies demonstrated that the Mtb glutamine synthetase, GlnA1, is important for survival in macrophages and in guinea pigs (Miller and Shinnick, 2000; Tullius *et al.*, 2003), indicating that the phagosomal concentration of glutamine is too low to support bacterial metabolism. Thus, it is unclear what role glutamine transport might play in Mtb pathogenesis. In other species of bacteria, ABC transporters have been implicated in the transduction of information from the extracellular milieu, leading to adaptive changes in bacterial gene expression (Decker *et al.*, 1999; Lucas *et al.*, 2000; Ruiz and Silhavy, 2003; Valdivia and Falkow, 1997). We speculate that the very low levels of glutamine in the macrophage phagosome (Miller and

Shinnick, 2000; Tullius *et al.*, 2003) might serve as an environmental cue that is transduced by the glutamine transporter to the appropriate cellular response pathways, resulting in adaptation of the organism to IFN-γ-dependent changes in the intracellular environment.

*Rv*0930, annotated as *pstA1*, is predicted to encode an MSD subunit for a putative high-affinity inorganic phosphate transporter (Braibant *et al.*, 1994; Braibant *et al.*, 1996a; Braibant *et al.*, 1996b; Lefevre *et al.*, 1997). *Rv*0930 is the last open reading frame in what appears to be an operon encoding another MSD subunit (*Rv*0929, a.k.a. *pstC2*) and a SBP subunit (*Rv*0928, a.k.a. *pstS3*) of the putative phosphate transporter. A homologous locus located ~4.2 kb downstream of the *pstA1* locus is thought to encode a second high-affinity phosphate transporter; the order of the genes in the two putative operons is conserved although the homology between the corresponding genes is limited (25-64%). It is unclear at present why loss of just one of the two phosphate transporters would result in such a marked phenotype *in vivo*. In *Escherichia coli*, the high-affinity Pst transport system is of primary importance when phosphate levels are very low, whereas the low-affinity Pit transport system is sufficient when extracellular phosphate is abundant (Harris *et al.*, 2001). The Mtb genome contains homologs of both of the *E. coli pit* genes (*pitA* and *pitB*), but their role in phosphate transport has not been elucidated in Mtb. The Pst system has been implicated in virulence in other pathogens, including *Streptococcus* (Orihuela *et al.*, 2001), *Salmonella* (Lucas *et al.*, 2000; Valdivia and Falkow, 1997), *Shigella* (Runyen-Janecky and Payne, 2002), and pathogenic *E. coli* (Daigle *et al.*, 1995). Attenuation of Pst system mutants in *Salmonella* is apparently due not only to disrupted phosphate metabolism *per se* but also to dysregulation of other

virulence pathways (Lucas *et al.*, 2000; Valdivia and Falkow, 1997), suggesting that Pst functions as a sensor of the intra-phagosomal milieu leading to adaptive changes in bacterial gene expression. Our results suggest that the Pst system in Mtb is particularly important for bacterial adaptation to environmental changes triggered by IFN-γ, but given the possibility that this system functions both as a transporter and as a regulator of gene expression, further studies will be necessary to elucidate the precise role of the Pst system in Mtb virulence.

The success of Mtb as a persistent pathogen can be attributed to the evolution of bacterial genes that act to counter elements of the host immune response. Although a number of Mtb virulence factors have been identified, few mutants with *in vivo* phenotypes have been described for which the mechanism of virulence has been clarified. Differential STM, the screening of bacterial mutants for differential attenuation in the face of varying *in vivo* stresses, allows for identification of mutants that are susceptible to specific pressures, and can be applied to a diverse range of scientific questions. Other studies from our laboratory are employing differential STM to pinpoint mycobacterial genes that are necessary for resisting other *in vivo* stresses, including RNI, ROI, and chemotherapeutic agents, as well as the set of pressures that occur during persistent infection. Although differential STM does not provide defined mechanisms of virulence, this method offers greater insight into which host pathways bacterial genes are countering. Understanding of the counter-immune mechanisms of Mtb might lead to potentially new and interesting anti-mycobacterial therapies. Inhibitors targeting these pathways would promote the clearance of infection not by killing the organism directly, but by increasing its vulnerability to the onslaught of the host immune response.

Technical Comments

Analysis of methods for preparation of bacterial inocula

Because Mtb's hydrophobic envelope promotes aggregation, bacterial preparations for injection into mice must be treated to eliminate clumps. Presence of large aggregates in inocula results in atypical distribution in mouse organs after i.v. infection. Because the capillary beds of the lungs are much narrower than the vessels comprising the reticulo-endothelial system in the spleen and liver, clumps of bacteria preferentially lodge in the lungs, resulting in upwards of 5% of the inocolum seeding this organ. Not surprisingly, pathogenesis of Mtb infection proceeds unusually, and mice die earlier than anticipated.

We tested three methods for dispersing aggregates of bacteria: vortexing, sonication in a water bath, and probe sonication. An aliquot of frozen H37Rv was thawed, separated into three tubes, diluted, and vortexed or sonicated accordingly. Mice were injected i.v. with 0.1 ml of bacteria. Organs were harvested at 24 hours after infection for determination of bacterial distribution and at 3 weeks after infection for evaluation of bacterial viability *in vivo* (Fig. 9). Probe sonication provided the best disruption of clumps, as assessed by the lowest percent of inocula lodging in the lungs (Fig. 9A) and the highest number of cfu in the inocula (Fig. 9B). The overall distribution of the inocula at 24 hours after infection was not appreciably different among the three methods of dispersion (Fig. 9A, B); however, by 3 weeks after infection it was clear that probe sonication had damaged the bacteria (Fig. 9C). This damage was insufficient to kill the bacteria, as indicated by cfus recovered from plating the inocula; rather, probe sonication rendered Mtb more vulnerable to the *in vivo* environment. We therefore chose

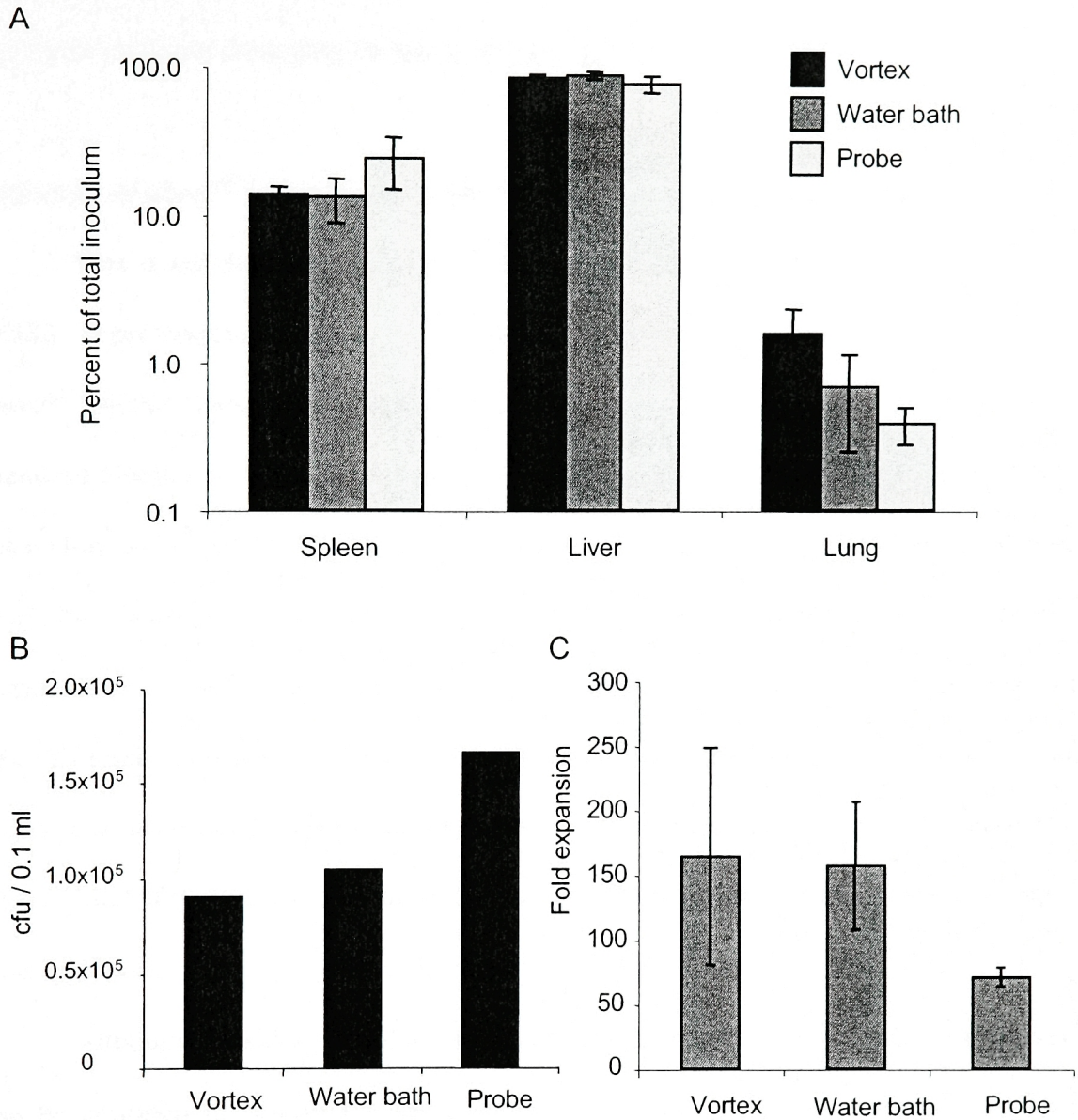


Figure 9. Evaluation of methods for preparing Mtb inocula prior to i.v. injection. Freezer stocks of Mtb were thawed, diluted and vortexed and/or sonicated to disperse clumps. Methods were analyzed by distribution of the inocula in the lung, liver, and spleen at 24 hours after infection (A), total cfu in the inocula prior to injection (B), and expansion of the inocula in the lungs at 3 weeks after injection, as compared with lung cfu at 24 hours (C). In panels A & C, values represent means of 5 mice +/- standard deviations.

water bath sonication for subsequent experiments, because it effectively dispersed aggregates without damaging the bacteria and with less variability than vortexing alone.

Infection of phox$^{gp91-/-}$NOS2$^{-/-}$ mice with Mtb

This is not the first report of Mtb infection of mice deficient in both phox and NOS2. A previous report by Jung *et al.* describes growth of Mtb H37Rv in the organs of phox$^{gp91-/-}$NOS2$^{-/-}$mice after aerosol infection, and indicates that Mtb replicates with identical kinetics in the lungs, livers, and spleens of phox$^{gp91-/-}$NOS2$^{-/-}$mice and NOS2$^{-/-}$ mice (Jung *et al.*, 2002). The data provided here are consistent with the findings of Jung *et al.*, but also provide further characterization of pathogenesis of Mtb in phox$^{gp91-/-}$NOS2$^{-/-}$ mice. Our studies include confirmation of Jung *et al.*'s results using a different strain of Mtb (Erdman), confirmation of Jung *et al.*'s results by a different route of infection (i.v), a direct head-to-head comparison of bacterial cfu in the organs of NOS2$^{-/-}$, phox$^{gp91-/-}$ NOS2$^{-/-}$ and IFN-$\gamma^{-/-}$ mice, and an examination of survival of the three strains of mice post-infection.

Although considered virulent strains of Mtb, no two "wild type" laboratory strains can be assumed to behave the same *in vivo* or *in vitro*. Within our laboratory, we observed *in vitro* phenotype differences between the strains Erdman and H37Rv. Although all strains of Mtb form aggregates if allowed to grow in liquid culture for extended periods, H37Rv demonstrates a particularly marked predisposition for developing clumps in liquid culture, presumably due to increased hydrophobicity of the H37Rv lipid envelope. When immunodeficient mice were infected i.v. with H37Rv or Erdman, IFN-γ-deficient mice succumbed sooner than NOS2-deficient mice regardless of

the Mtb strain; however, the difference in survival between IFN-γ-deficient mice and NOS2-deficient mice was less dramatic with H37Rv (Fig. 10), consistent with the findings of others (Mogues *et al.*, 2001). Thus, the more pronounced difference in survival seen during infection with Erdman provides a larger window in which to see potential subtle contributions of phox as evaluated by pathogenesis in phox$^{gp91-/-}$NOS2$^{-/-}$ mice and NOS2$^{-/-}$mice.

Although aerosol infection is more representative of the natural route of infection with Mtb, our interest in using modified STM required us to use the i.v. route for infection. Intravenous infection permits inoculation with larger numbers of bacteria, because immediate dissemination of bacteria through peripheral organs triggers a systemic immune response, which leads to more rapid containment of bacterial growth in the lungs, thus prolonging murine survival. With aerosol infection, bacteria do not disseminate from the lung to other organs for several weeks, delaying the onset of a systemic immune response and resulting in 10 to 100 times more bacterial replication in the lungs before immunity forces the bacteria to cease replicating. Thus, if more than 500-1000 cfu seed the lungs during an aerosol infection, death of the animal may occur before immunity can halt bacterial replication. STM requires that ample numbers of bacteria be delivered to the host species to ensure that all mutants in a pool are represented in the target organ. Our pools contained 48 mutants each, so we wanted at least 1000 cfu to seed in the lungs of the mice to ensure that all mutants were represented. Because we planned to use STM to identify counter-immune mutants susceptible to IFN-γ-dependent, NOS2-independent host mechanisms, we felt it was important to confirm the work of Jung, *et al.* in the i.v. model of disease.

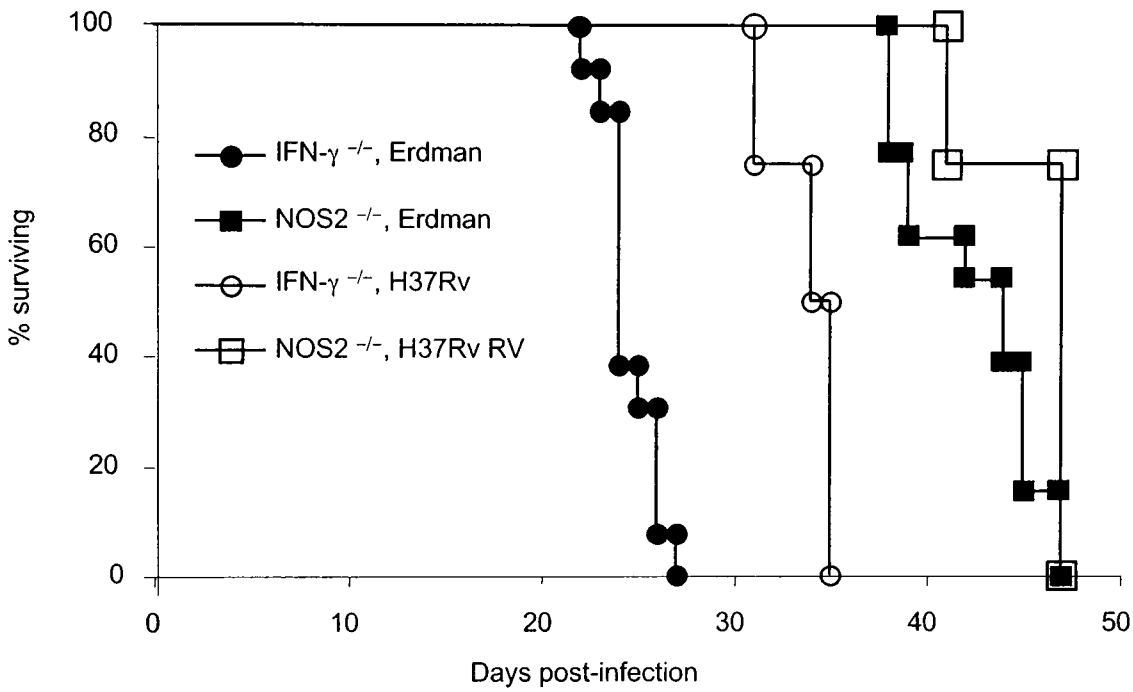


Figure 10. Pathogenesis of H37Rv and Erdman strains of Mtb in immunodeficient mice. Survival of IFN-$\gamma^{-/-}$ mice (circles) and NOS2$^{-/-}$ mice (squares) infected i.v. with $1x10^5$ cfu Mtb Erdman (closed symbols) or H37Rv (open symbols). For Erdman, infections n = 13, for H37Rv infections, n = 4.

Although the study by Jung *et al.* illustrates that phox does not affect the ability of Mtb to proliferate in host tissues, it does not directly examine the virulence of Mtb in phox$^{gp91-/-}$NOS2$^{-/-}$mice. Several studies have demonstrated that bacterial growth *in vivo* does not always correlate with virulence, as measured by mouse survival (Manca *et al.*, 1999; Manca *et al.*, 2001; North *et al.*, 1999; Steyn *et al.*, 2002). Two strains of bacteria can grow with the same kinetics *in vivo*, and yet cause death of mice at different rates, demonstrating the role of immune pathology in the disease process of tuberculosis. Studies have shown that phox and NOS2 act not only as antimicrobial effectors, but also as mediators of other macrophage functions (Ehrt *et al.*, 2001). It is possible that Mtb might grow similarly in NOS2$^{-/-}$ and phox$^{gp91-/-}$NOS2$^{-/-}$mice, but that phox$^{gp91-/-}$NOS2$^{-/-}$mice might generate more immune pathology in response to infection than their NOS2$^{-/-}$ counterparts. This scenario would not be detected by determination of evaluation of bacterial growth *in vivo*, and would be revealed only by analysis of murine survival after infection with Mtb or by examination of the host immune response during infection, including evaluation of tissue pathology. Our results indicate that NOS2$^{-/-}$ and phox$^{gp91-/-}$ NOS2$^{-/-}$mice die with roughly the same kinetics after infection with Mtb, suggesting that there is no detectable impact of phox on immune pathology during infection with wild type Mtb.

Chapter 4

Identification of a *Salmonella typhimurium* gene that modulates interactions with macrophages and contributes to virulence

Careful regulation of virulence factors and defense mechanisms allows *Salmonella typhimurium* to persist and replicate within the phagosome of the macrophage despite the macrophage's arsenal of immune defenses. *S. typhimurium* has specifically evolved to alter macrophage cell functions and take advantage of the phagosomal milieu; inside the macrophage, the bacterium is safe from attacks by other immune mechanisms. Many of the molecular mechanisms that shape the dynamics between *S. typhimurium* and the macrophage have been well characterized, largely due to the ease of genetic manipulation of *S. typhimurium.* Yet, despite genome sequencing, mutagenesis studies, and numerous mutant screens, many *S. typhimurium* genes with putative enzymatic functions remain unexamined, including many of the bacterial effectors translocated into host cells by the SPI-1 and SPI-2 TTSSs. Genes providing defense against ROI have been studied intensively, yet bacterial resistance to RNI is still poorly understood. Moreover, complex regulatory circuits used by bacteria to link the induction and repression of multiple pathways during the adaptation to new environments are just now beginning to be understood. Here we describe the *S. typhimurium* gene *ydiV*, discovered as contributing to anti-oxidant defense, but which in fact regulates multiple bacterial functions. Analysis of its domain architecture reveals that *ydiV* appears to belong to a

family of enzymes found throughout prokaryotes. Modulation of intracellular c-diGMP by YdiV, and perhaps by most of these enzymes, may act as a novel signaling alternative to the classical phospho-relay 2-component regulatory system.

Results

Identification of a novel contributor to S. typhimurium's antioxidant defense

A modified version of signature-tagged transposon mutagenesis was performed using the same library of 960 *S. typhimurium* STM mutants that was evaluated in the original description of STM (Hensel *et al.*, 1995). All 960 mutants were screened for attenuation in wild type C57BL/6 mice, as determined by under-representation of STM tags recovered from the spleen 3 days after infection. Attenuated mutants were reassembled alongside randomly chosen virulent mutants (ratio 1:2) into 3 new pools containing 96 mutants each. The new pools were used to infect wild type C57BL/6 mice, $NOS2^{-/-}$ mice, $phox^{gp91-/-}$ mice and $phox^{gp91-/-}NOS2^{-/-}$ mice (all on the C57BL/6 background), and virulence of individual mutants was evaluated in each strain of mouse. Of the original 960 mutants, 31 mutants were found to be under-represented in recovered bacteria from all 4 strains of mice (Table VI). Hensel *et al.* had previously identified thirteen of these mutants as attenuated for growth *in vivo* during a screen of the same STM library in BALB/c mice (Hensel *et al.*, 1995). An additional 7 mutants were found to be attenuated in wild type C57BL/6 mice, but restored in virulence in at least one strain of immunodeficient mice on the C57BL/6 background (Table VII).

SPI-2 mutants were among the mutants identified as being attenuated in all 4 strains of mice tested in our differential STM screen (Table VI); yet, these same SPI-2

Table VI. *S. typhimurium* STM clones negatively selected in all mouse strains

Mutant	Gene Insertion	Comments	*in vitro* C.I. *	*in vivo* C.I. *
SPI-2 genes				
P07A04	*ssaE*	SPI-2 apparatus		
P09B07	*ssaT*	SPI-2 apparatus		
P05H11	*ssaU*	SPI-2 apparatus		
P04H11	*ssaV*	SPI-2 apparatus		
P09B06	*ssaV*	SPI-2 apparatus		
P02D06	*ssaV*	SPI-2 apparatus		
P02D09	*ssaV*	SPI-2 apparatus		
P07G02	*ssaC*	SPI-2 apparatus		
P02A02	*ssaR*	SPI-2 apparatus	0.9 (0.1)	0.0005 (0.0008)
P04H10	*ssaR*	SPI-2 apparatus		
P10E11	*sscA*	SPI-2 chaperone		
P09G04	*sseB*	SPI-2 effector		
P04F08	*sseA*	SPI-2 effector		
P07B08	*ssrA*	SPI-2 regulator		
P03F04	*ssrA*	SPI-2 regulator		
P08H06	*ssrB*	SPI-2 regulator	0.9 (0.4)	0.0003 (0.0002)
P07F06	?	maps within SPI-2		
Other known virulence factors				
P09H12	*spvR*	plasmid virulence factor		
P05D10	*spvA*	plasmid virulence factor		
P07G06	*rfbK*	LPS biosynthesis		
P09G02	*rfbK*	LPS biosynthesis		
P06B11	*rfaK*	LPS biosynthesis		
Putative virulence factors				
P09H06	*pabB*	PABA synthesis	1.0 (0.2)	0.0057 (0.0035)
P03G07	*fimW*	fimbriae expression	0.8 (0.1)	0.52 (0.23)
P03C01	*exbB*	iron uptake	1.3 (0.3)	0.48 (0.23)
P09A02	SPI-4, gene "P"	pathogenicity island	0.9 (0.1)	0.60 (0.55)
P09A11	*ratC-shdA*	adaptation to exothermic hosts	0.8 (0.0)	0.37 (0.29)
P09G09	*sseI/srfH*	putative SPI-2 effector	1.9 (0.7)	0.18 (0.059)
P10D02	STM3116	putative acetyl-CoA hydrolase	0.6 (0.1)	0.25 (0.091)
P10H01	*purD*	purine biosynthesis		
P03H06	*potB*	polar effect on *sif* operon?		

* C.I.: competitive index when competed against wild type S. typhimurium 12023.

Table VII. Attenuation reversal (AR) mutants: *S. typhimurium* STM clones attenuated in wild type mice but restored to virulence in at least one strain of immunodeficient mice.

Mutant	Gene insertion	Comments	Differential STM phenotype**				*in vitro* C.I. ++	*in vivo* C.I. ++
			C57BL/6	$NOS2^{-/-}$	$phox^{gp91-/-}$	$phox^{gp91-/-}$ $NOS2^{-/-}$		
P09D06	*rafY**	outer membrane pore	-	-	+	+	1.0 (0.5)	0.70 (0.25)
P04D11	*rafY**	outer membrane pore	-	-	+	+	1.6 (0.4)	0.80 (0.21)
P09H08	*traG*	pilus formation	-	+	-	+	1.0 (0.4)	1.00 (0.20)
P08G03	*spvR*	plasmid virulence factor	-	-	+	+	1.2 (0.5)	0.68 (0.26)
P01E12	*ydiV*	putative EAL domain	-	-	+/-	+	0.8 (0.2)	0.090 (0.045)
P08E04	*ytfN*	unknown function	-	+	+	+	1.0 (0.1)	0.36 (0.22)
P09D03	ORF408/*rkh*	ribokinase-like protein	-	+	-	+	0.8 (0.05)	0.70 (0.21)

**rafY* = STM2816, not annotated by WUSTL, but demonstrates 87% identity to *E. coli rafY.*

** STM phenotype: "-" signs indicate under representation of mutant's tag after *in vivo* passage, "+" signs indicate equal representation of mutant's tag after *in vivo* passage as compared to input pool, "+/-" indicates inconsistent results for that mutant's tag when comparing tags recovered from individual mice.

++ C.I.: competitive index when competed against wild type S. typhimurium 12023.

mutants, which were unable to kill C57BL/6 mice, were found to be restored to virulence in phox-deficient mice by Vazquez-Torres *et al.* (Vazquez-Torres *et al.*, 2000b). This apparent discrepancy can be explained by the different methods used to evaluate virulence in the two studies. Vazquez-Torres *et al.* defined virulence as the ability of mutants to kill mice. STM determines virulence by the ability of mutants to compete with wild type bacteria in a mouse, and thus STM measures the rate of bacterial proliferation *in vivo*. When a 1:1 mixture of a SPI-2 mutant and wild type bacteria was used to infect C57BL/6 mice, the SPI-2 mutant was confirmed to be highly attenuated (competitive index (C.I.) = 0.0023 +/- 0.004), consistent with both our STM results and the survival studies of Vazquez-Torres *et al.* When the SPI-2 mutant was competed against wild type *S. typhimurium* in phox-deficient mice, the competitive index of the mutant increased, but remained less than 0.1 (CI = 0.05 +/- 0.05). SPI-2 mutants are therefore partially restored in their ability to proliferate in phox-deficient mice. This partial restoration is sufficient for the bacteria to overwhelm *Nramp1*^{S} mice, and thus SPI-2 mutants appear virulent when virulence is evaluated by murine survival after infection; however, mutants with a competitive index of less than 0.5 are identified as under-represented, and thus attenuated, by STM. The difference in the conclusions from the two original studies serves to illustrate the incredible sensitivity of STM to detect attenuated mutants.

Southern blot analysis of DNA from the 7 attenuation reversal (AR) mutants ruled out the possibility of multiple transposon insertions in the same mutant (data not shown). Additionally, transposon insertions in the AR mutants were transduced into fresh cultures of wild type 12023, decreasing the likelihood of additional unmarked mutations. The AR

mutants demonstrated no deficit in *in vitro* proliferation in LB broth under conditions of good aeration as compared with wild type *S. typhimurium* (Table VII). Using mixed infection, the 7 AR mutants were assayed to confirm attenuation in wild type mice. None of the AR mutants demonstrated dramatic deficits in ability to compete with wild type bacteria *in vivo*; however, two mutants consistently generated C.I.s of less than 0.5: AR5 and AR6 (table VII). The relative virulence of these two mutants was then evaluated in immunodeficient mice. AR6 demonstrated attenuation in immunodeficient that was equivalent to the deficit seen in wild type mice (Fig. 11A). AR5, however, exhibited no deficit in virulence in either phox$^{gp91-/-}$ mice or phox$^{gp91-/-}$NOS2$^{-/}$ mice despite being attenuated in both wild type and NOS2$^{-/-}$ mice (Fig. 11A), suggesting that the gene(s) disrupted in this mutant mediates resistance to a phox-dependent stress.

The transposon insertion in AR5 was located in the gene *ydiV*, which is located in the *S. typhimurium* genome downstream of *nlpC,* a putative membrane-bound peptidase, and 62 nucleotides upstream of *ydiU* (Fig. 11B). Other genes in the vicinity include the upstream *btu* operon, which is thought to be involved in Vitamin B12 transport, and aroH which participates in the synthesis of aromatic amino acids. The "y" designation of *ydiV*, *ydiU,* and *ydiE* indicates that these genes had not been described or assigned functions at the time of *S. typhimurium* genome annotation. The close proximity of *ydiV* and *ydiU* suggested that these two genes might be transcribed as an operon, and thus the transposon insertion in *ydiV* would disrupt expression of both *ydiV* and *ydiU*. To determine whether disruption of expression of *ydiU* contributed to the phenotype of AR5, a *S. typhimurium* strain was constructed in which most of *ydiU* was replaced with an antibiotic resistance cassette. Infection of C57BL/6 mice and phox$^{gp91-/-}$ mice with a mixture of wild type *S.*

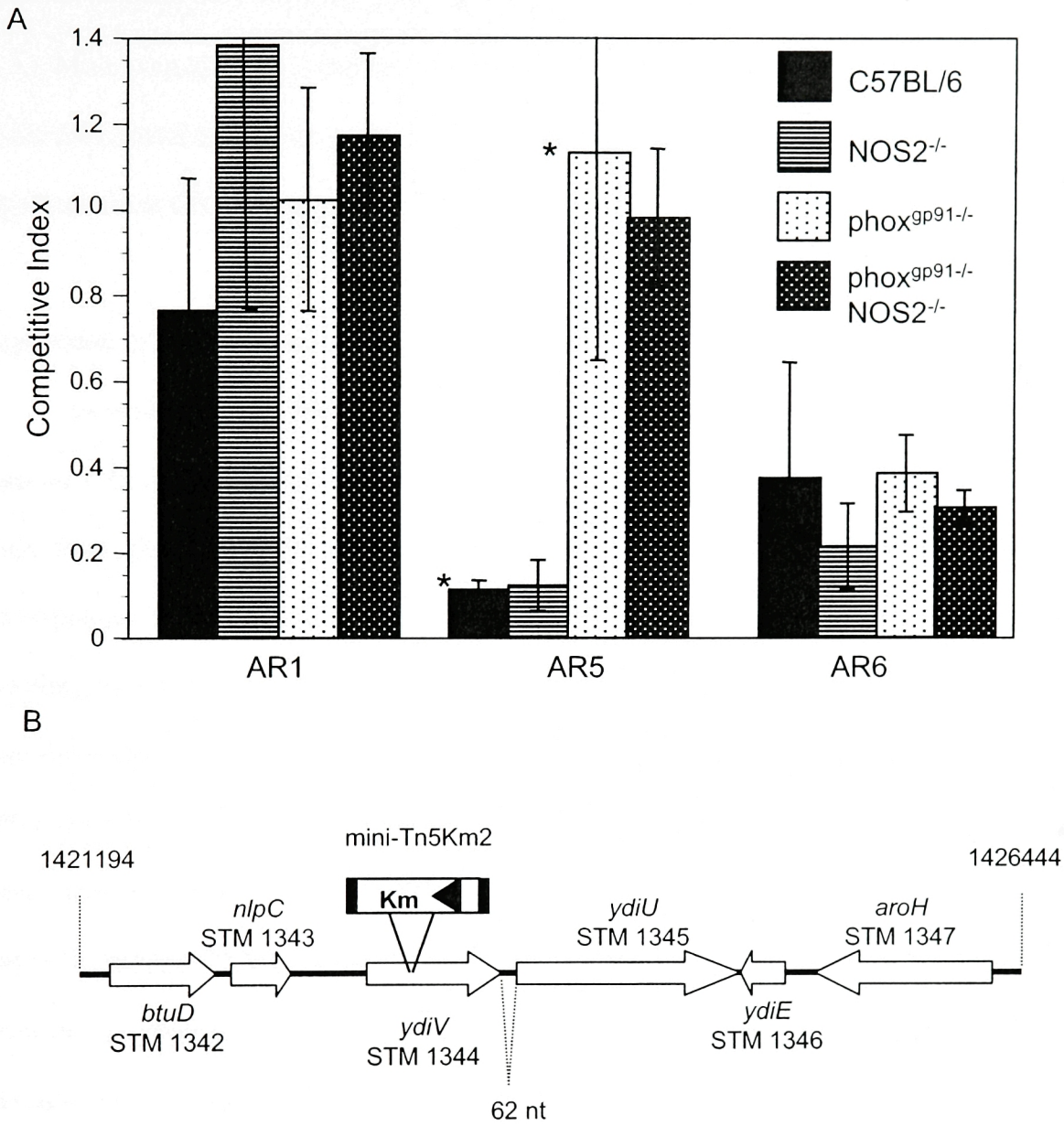


Figure 11. The *ydiV* locus in not required for growth of *S. typhimurium* in mice that lack phox, but contributes to virulence in wild type mice. (A) Mixed infection of mice with AR mutant. Values are means for 5 mice +/- standard deviation. *indicates average of 3 experiments. (B) Insertion site of the STM transposon in the mutant AR5.

typhimurium and the Δ*ydiU* strain revealed that *ydiU* was not required for virulence (Fig. 12A). Moreover, complementation of AR5 with *ydiV* expressed from a low copy plasmid under the control of its own promoter restored the mutant's ability to compete with wild type bacteria in C57BL/6 mice (Fig. 12B).

Expression of YdiV in late log phase mediates protection against ROI in vitro

Increased susceptibility to phox *in vivo* does not necessarily predict a decrease in resistance to oxidative stress or to ROI *in vitro*. SPI-2 mutants that are unable to divert phox from the phagosome in macrophages are rapidly killed by ROI in mice and macrophages, but they are not more vulnerable than wild type strains of *S. typhimurium* to killing by ROI *in vitro* (Vazquez-Torres *et al.*, 2000b). Moreover, many genes that are required for growth or survival *in vivo* are not constitutively expressed *in vitro*, including SPI-1 (Daefler, 1999), SPI-2 (Deiwick *et al.*, 1999), and many anti-oxidant defenses (Janssen *et al.*, 2003). A chromosomal *lacZYA* fusion was created at the *ydiV* locus such that both endogenous *ydiV* and β-galactosidase would be expressed off of the putative promoter region of *ydiV*. Analysis of the resulting *S. typhimurium* strain (FW1) indicated that *ydiV* is expressed in LB broth *in vitro*, with a spike in expression occurring at the transition between exponential growth and stationary phase (late log phase) (Fig. 13). Immunoblot of bacterial lysates using specific antibody generated against rYdiV confirmed levels of YdiV protein in the wild type strain during late log phase that were more robust than during stationary phase, as well as a lack of YdiV protein in the *ydiV* mutant (Fig. 14). Expression of YdiV was found to be not merely restored, but also greatly enhanced by the complementation vector. All subsequent *in vitro* assays were

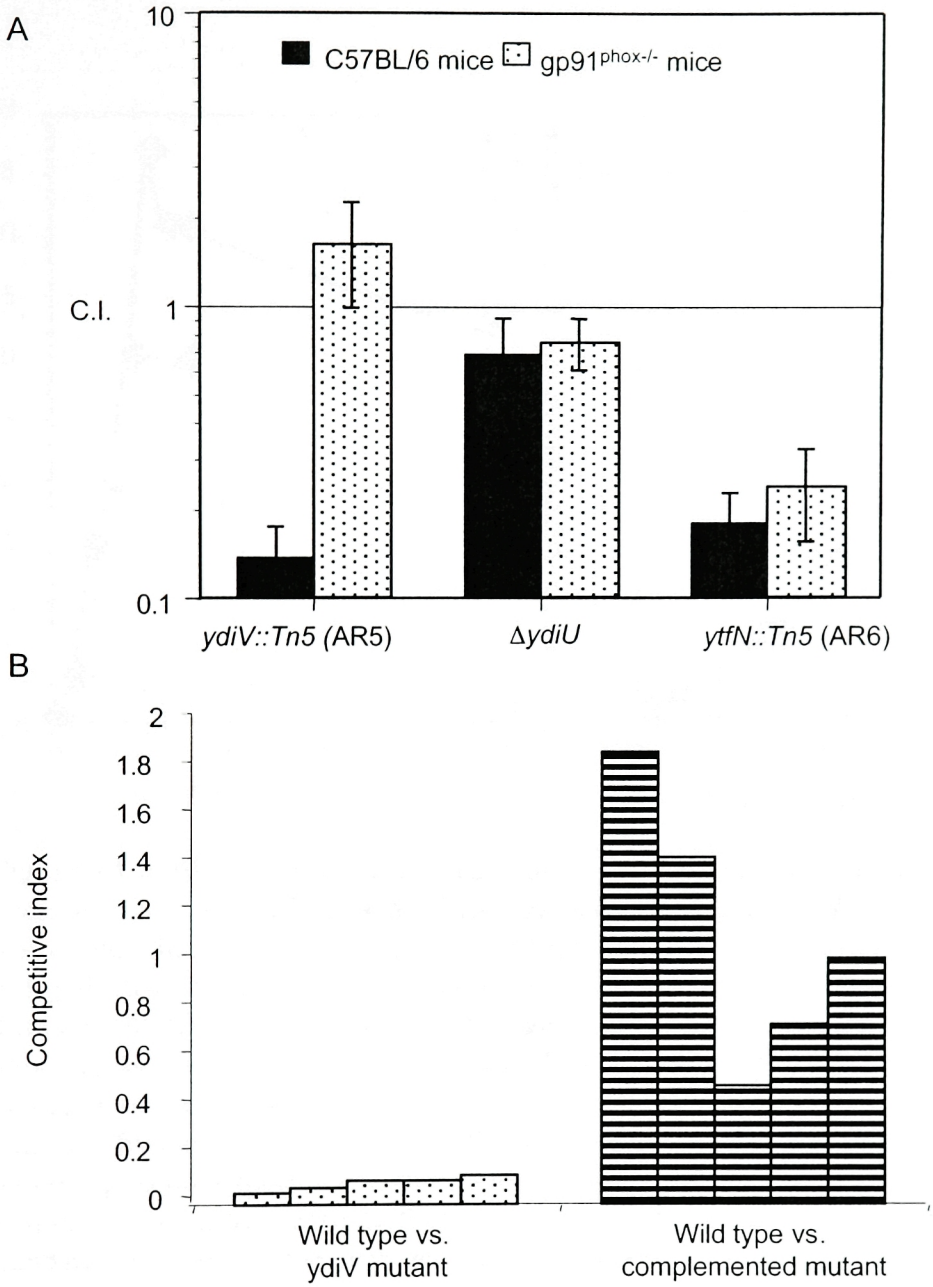


Figure 12. Deficiency in *ydiV* only accounts for the phenotype of the AR5 mutant. *(A) In vivo* phenotype of AR5, AR6, and Δ*ydiU S. typhimurium* in C57BL/6 and gp91$^{phox-/-}$ mice as evaluated by competitive mixed infection with wild type 12023 *S. typhimurium*. Values are means for 5 mice +/- standard deviation. (B) Mixed infections of C57BL/6 mice using wild type *S.typhimurium* vs. the *ydiV* mutant or wild type vs. the complemented mutant. Each bar represents the competitive index from mixed infection in an individual mouse.

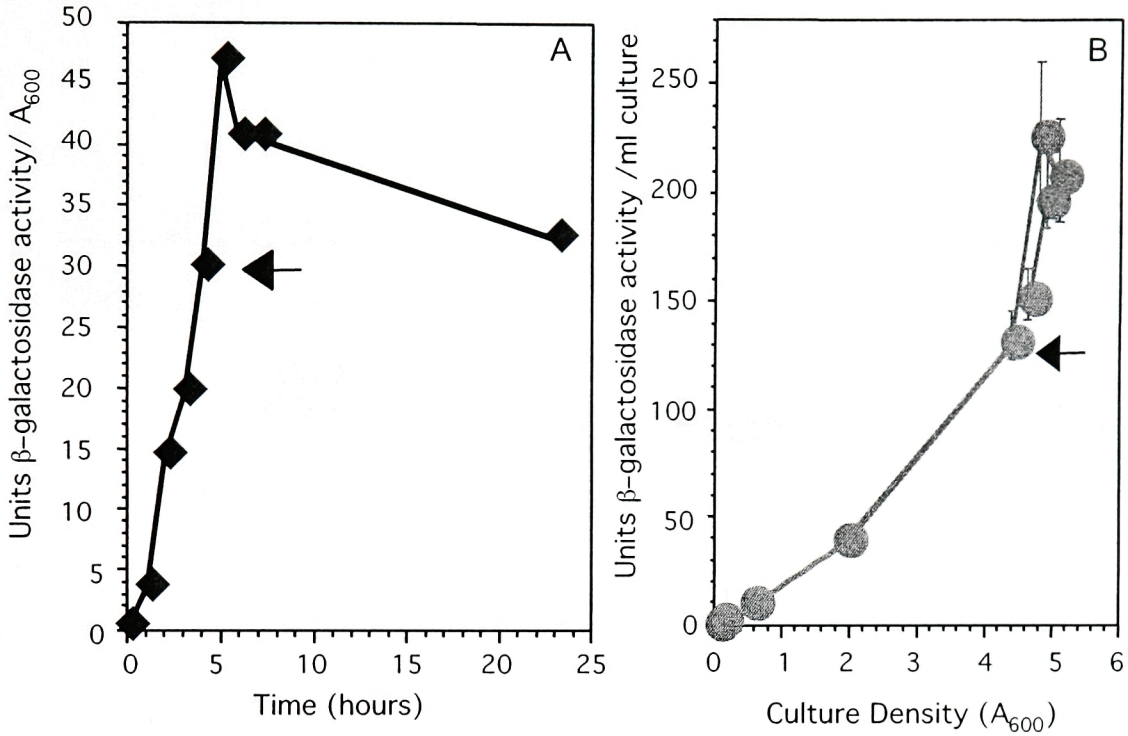


Figure 13. YdiV expression is induced during late log phase growth *in vitro*. An overnight culture of wild type *S. typhimurium* containing a *ydiV-lacZYA* fusion was diluted into fresh LB and grown with aeration at 37°C. (A) Graph of units of β-galactosidase activity/A_{600} versus time permits correlation of time after dilution with (B) spike in induction of β-galactosidase activity. The arrow in (B) indicates the culture density at which the slope of the line changes (signifying induction of the *lacZYA* fusion), and correlates with the time point (5 hr) indicated by the arrow in panel (A). Points represent the average of data from two independent experiments, and each experiment provided 2 independent evaluations of enzyme activity from each culture. Error bars represent the standard deviation for the average of the 4 data points.

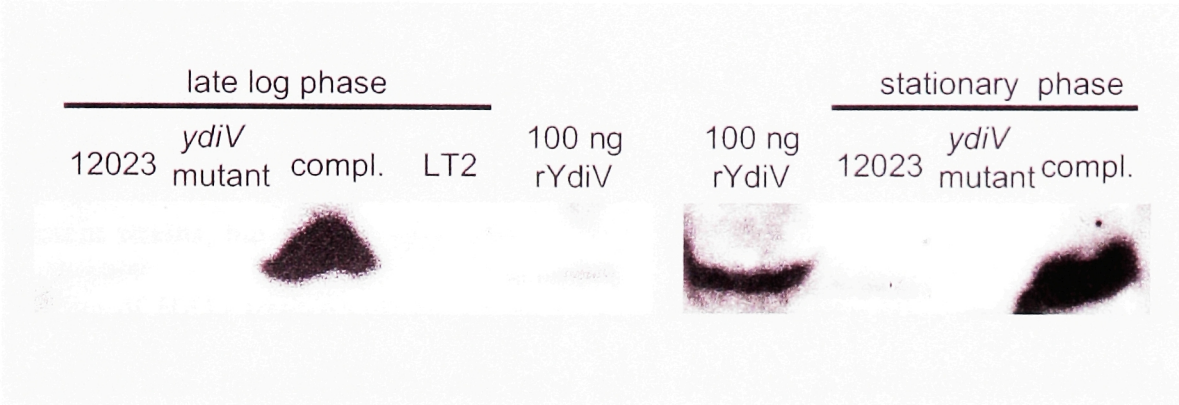


Figure 14. Expression of YdiV in different strains of *S. typhimurium* used in this study. Immunoblot of total cell lysates from late log phase cultures or stationary phase cultures. Polyclonals antibodies to rYdiV were used.

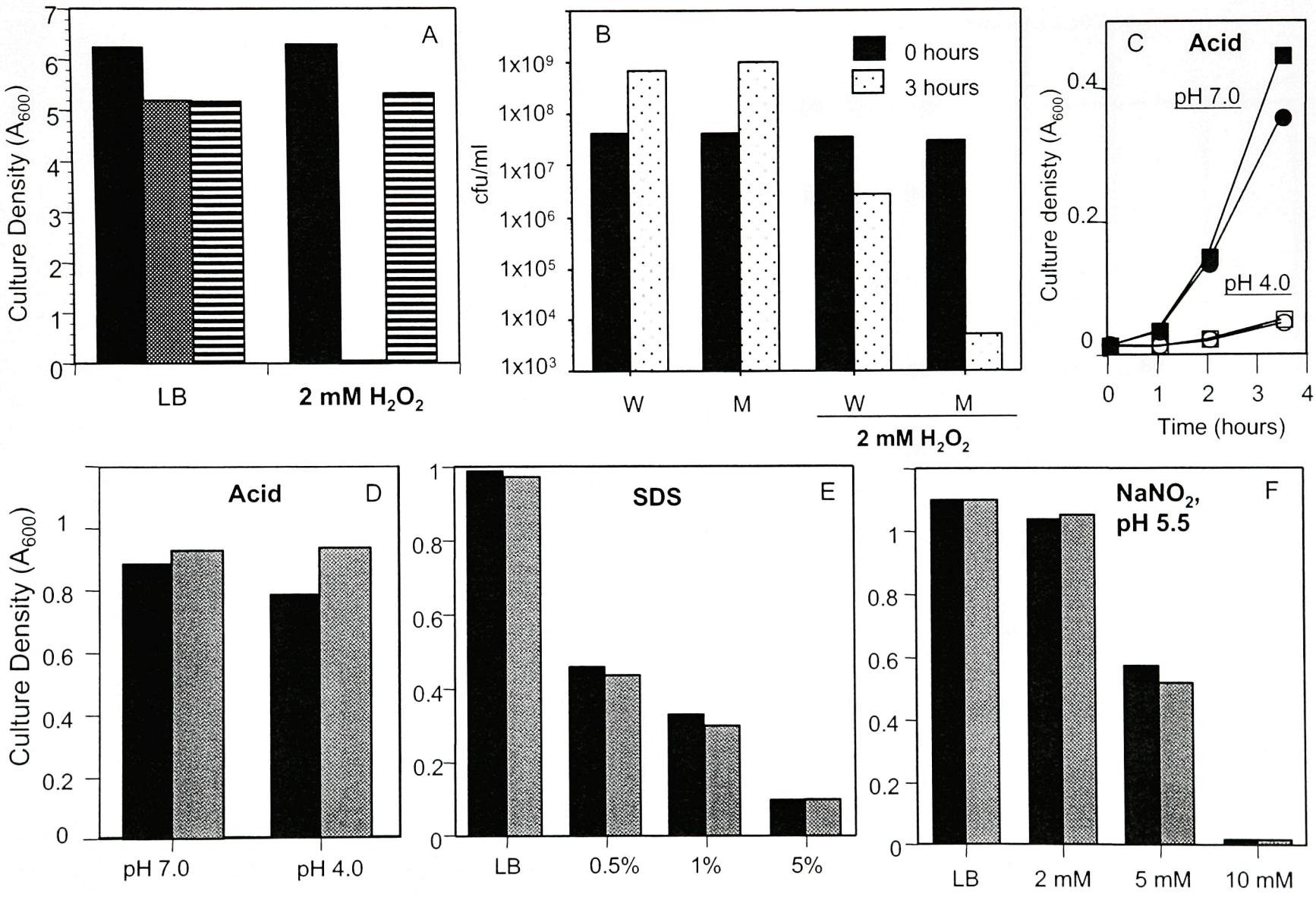
performed with bacterial cultures grown to late log phase (5.5 hour after 1:100 dilution of an overnight culture) to achieve optimal expression of YdiV in wild type *S. typhimurium*, and thus maximize the difference between wild type and *ydiV* mutant phenotypes.

Wild type *S. typhimurium*, the *ydiV* mutant and the complemented mutant were assayed for their ability to grow in medium containing various toxic stresses (Fig. 15). Although all three strains flourished in LB media, only the wild type and complemented mutant strains, but not the *ydiV mutant*, were able is grow in 2 mM H_2O_2 (Fig. 15A). Plating of H_2O_2- treated cultures to determine cfu revealed that the effects of H_2O_2 are cidal, not static (Fig. 15B). The protective role of *ydiV* appears to be specific for ROI, as the *ydiV* mutant did not show increased susceptibility to killing by SDS or $NaNO_2$, and the mutant was able to recover as well as wild type *S. typhimurium* from the static effects of an acidic environment (Fig. 15C-F)

Investigation of some of the known *S. typhimurium* mechanisms for resistance to H_2O_2 revealed no deficits in the *ydiV* mutant strain. The OxyR regulon was found to be fully induced in the *ydiV* mutant after exposure to sublethal H_2O_2 (Fig. 16), and sublethal H_2O_2 did not enhance expression of *ydiV* (data not shown). Levels of MsrA and MsrB protein were comparable in wild type and mutant lysates (data not shown). There are at least 2 OxyR-independent catalases in *S. typhimurium*: *katE* and *katN*, both of which are regulated by the alternative sigma factor RpoS (Robbe-Saule *et al.*, 2001). Transduction of the chromosomal *ydiV-lacZYA* fusion into several mutant and wild type strains of *S. typhimurium* revealed that *ydiV* was not expressed in the attenuated strain LT2 (Fig. 17), and a lack of YdiV protein in LT2 was demonstrated by immunoblot (Fig. 14). Although other mutations may also exist in LT2, LT2 is defective in expression of *rpoS*

Figure 15. Deficiency in *ydiV* results in increased susceptibility to killing by ROI. Late log phase cultures of *S. typhimurium* were diluted 1:100 into the LB containing specific stresses and incubated at 37°C, with agitation. (A) and (B), 2 mM H2O2; (C) and (D), pH 4.0; (E), SDS; (F), acidified sodium nitrite, pH 5.5. Susceptibility was determined by culture density after 24 hour exposure to bolus stress as evaluated by A_{600}, except in panel B, in which killing of bacteria was evaluated at specified time points after exposure to a bolus dose (2 mM) of H_2O_2 by diluting and plating cultures. In all panels except B & C: wild type, black bars; *ydiV* mutant, grey bars; complemented mutant, striped bars. Panel B: W = wild type, M = *ydiV* mutant; 0 hours in culture, black bars; 3 hours in culture, stippled bars. Panel C: wild type, squares; *ydiV* mutant, circles.

Figure 15.


A
Culture Density (A_{600})
LB
2 mM H_2O_2
B
cfu/ml
0 hours
3 hours
W
M
W
M
2 mM H_2O_2
C
Acid
pH 7.0
pH 4.0
Culture denisty (A_{600})
Time (hours)
D
Acid
pH 7.0
pH 4.0
E
SDS
LB
0.5%
1%
5%
F
$NaNO_2$, pH 5.5
LB
2 mM
5 mM
10 mM

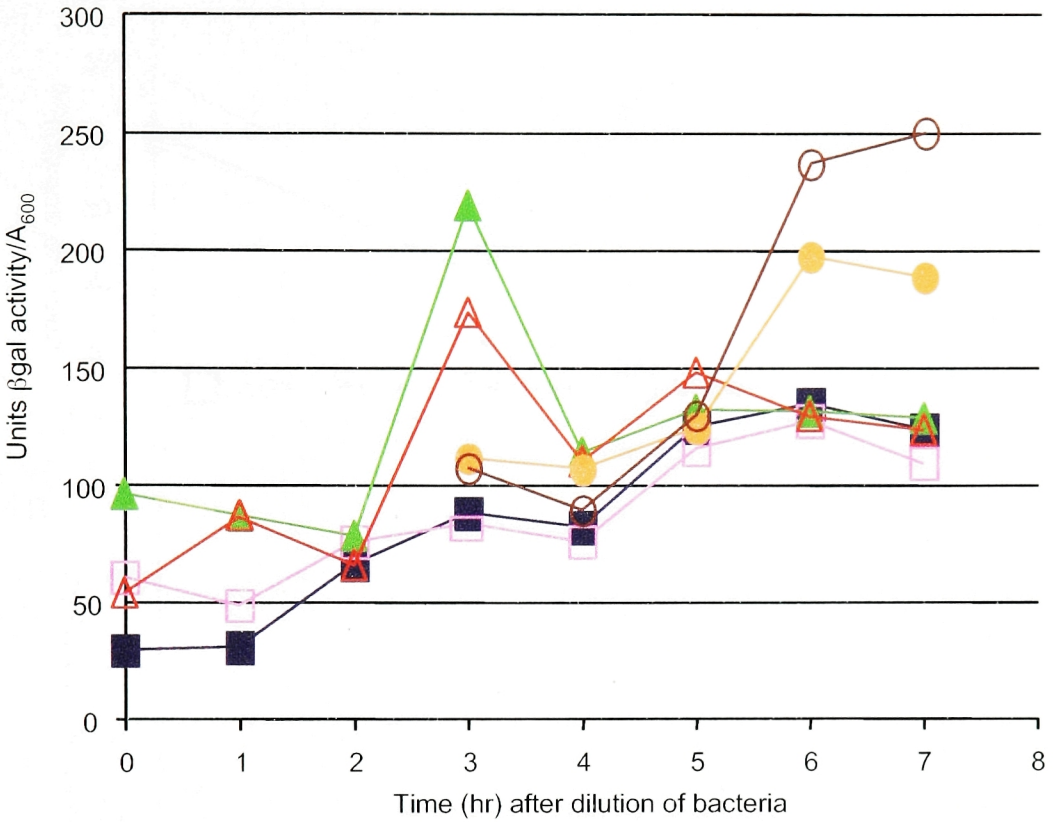


Figure 16. Disruption of *ydiV* does not affect induction of the OxyR regulon by sub-lethal hydrogen peroxide. Overnight cultures were diluted 1:100 at time 0 and grown with agitation at 37°C. A bolus of 60 μM hydrogen peroxide was added at specified times to specified cultures. Closed symbols, wild type bacteria containing the *ahpC-lacZYA* fusion; open symbols, ydiV mutant bacteria (SCN19) containing the *ahpC-lacZYA* fusion. Square, no peroxide; triangles, peroxide added at 2 hr after dilution; circles, peroxide added at 5 hr after dilution.

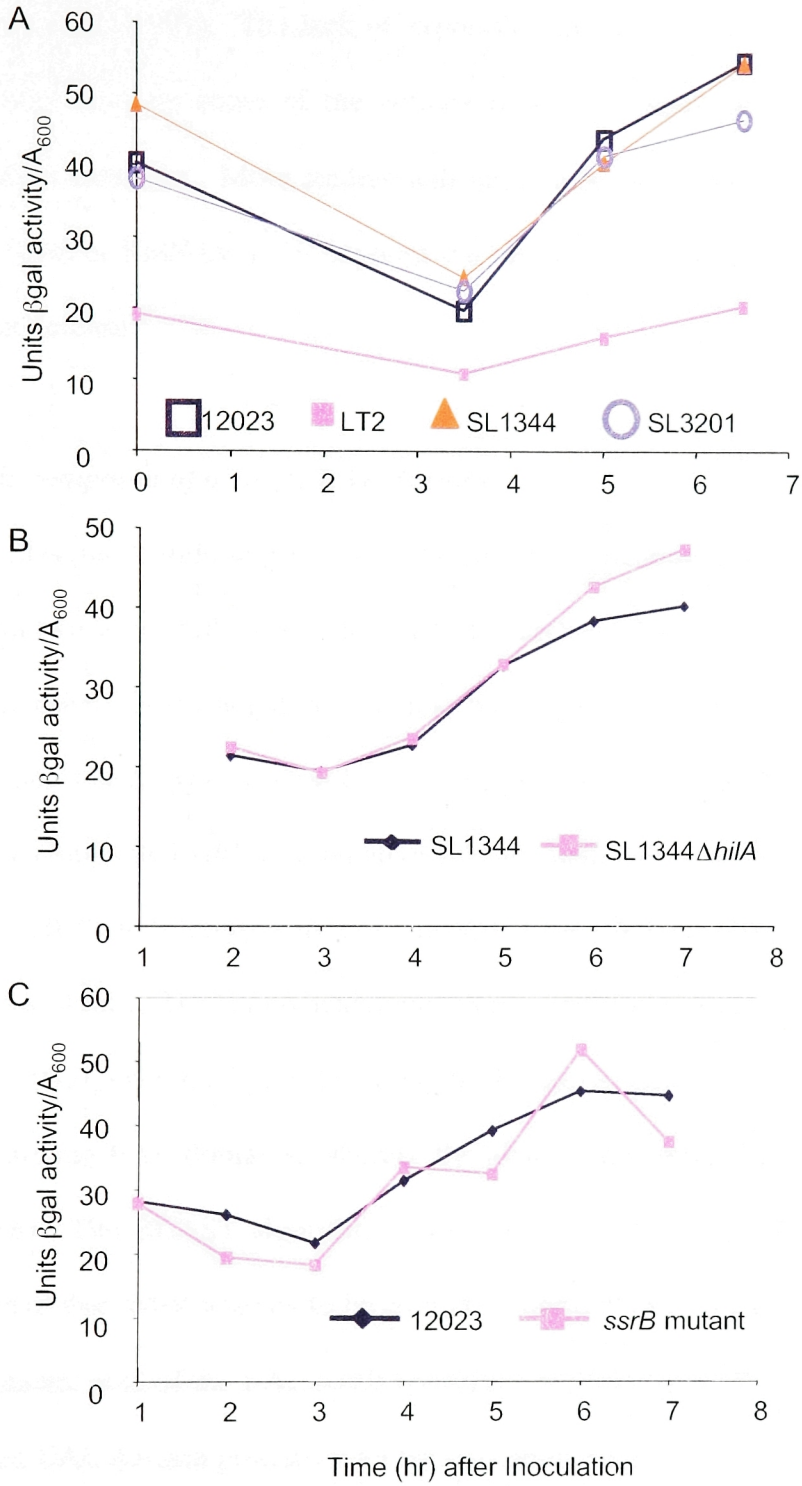


Figure 17. *ydiV* is regulated by an unknown mechanism that is absent in the *S. typhimurium* strain LT2. The *ydiV-lacZYA* fusion was transduced into different *S. typhimurium* strains. Overnight cultures of bacteria were diluted 1:100 into LB and grown at 37°C with aeration. YdiV expression in different "wild type" strains of *S. typhimurium* (A), a mutant of the SPI-1 master regulator *hilA* (B), and a mutant of the SPI-2 master regulator *ssrB* (C).

(Swords *et al.*, 1997). The lack of expression of *ydiV* in LT2 raises the possibility that *ydiV* may mediate some of the actions of RpoS, including activation of the RpoS-dependent catalases. More studies will need to be performed to clarify if induction of either KatE or KatN by YdiV explains the mechanism by which *ydiV* contributes to anti-oxidant defense.

YdiV is composed of a single EAL domain

The nucleotide sequence of *ydiV* gave no indication that *ydiV* encoded an enzyme with known anti-oxidant activity. Blast searches identified homologs in *E. coli* and several other Gram negative species, but none of these genes had been assigned functions. The *S. typhimurium* Genome Sequencing Center at Washington University in St. Louis annotated *ydiV* as a putative EAL domain based on low homology (E value = 10^{-04} – 10^{-20}) to a consensus domain sequence (Wilson, 2002). EAL domains are relatively short (~215 amino acids) with conserved acidic residues spread throughout the domain (Galperin *et al.*, 2001). Despite the name, only the glutamate is consistently found among EAL domains, whereas the alanine and leucine are much less conserved (Fig. 18). The BLAST algorithm cdart (conserved domain architecture retrieval tool) confirmed that YdiV appears to be composed entirely of a single EAL domain, with only the glutamic acid of the EAL motif conserved in YdiV (Fig. 19A). Alignments of other assigned EAL domain proteins with the consensus sequence demonstrated much stronger homology; however, because these other sequences had contributed to the derivation of the consensus sequence, they would be anticipated to demonstrate a higher degree of

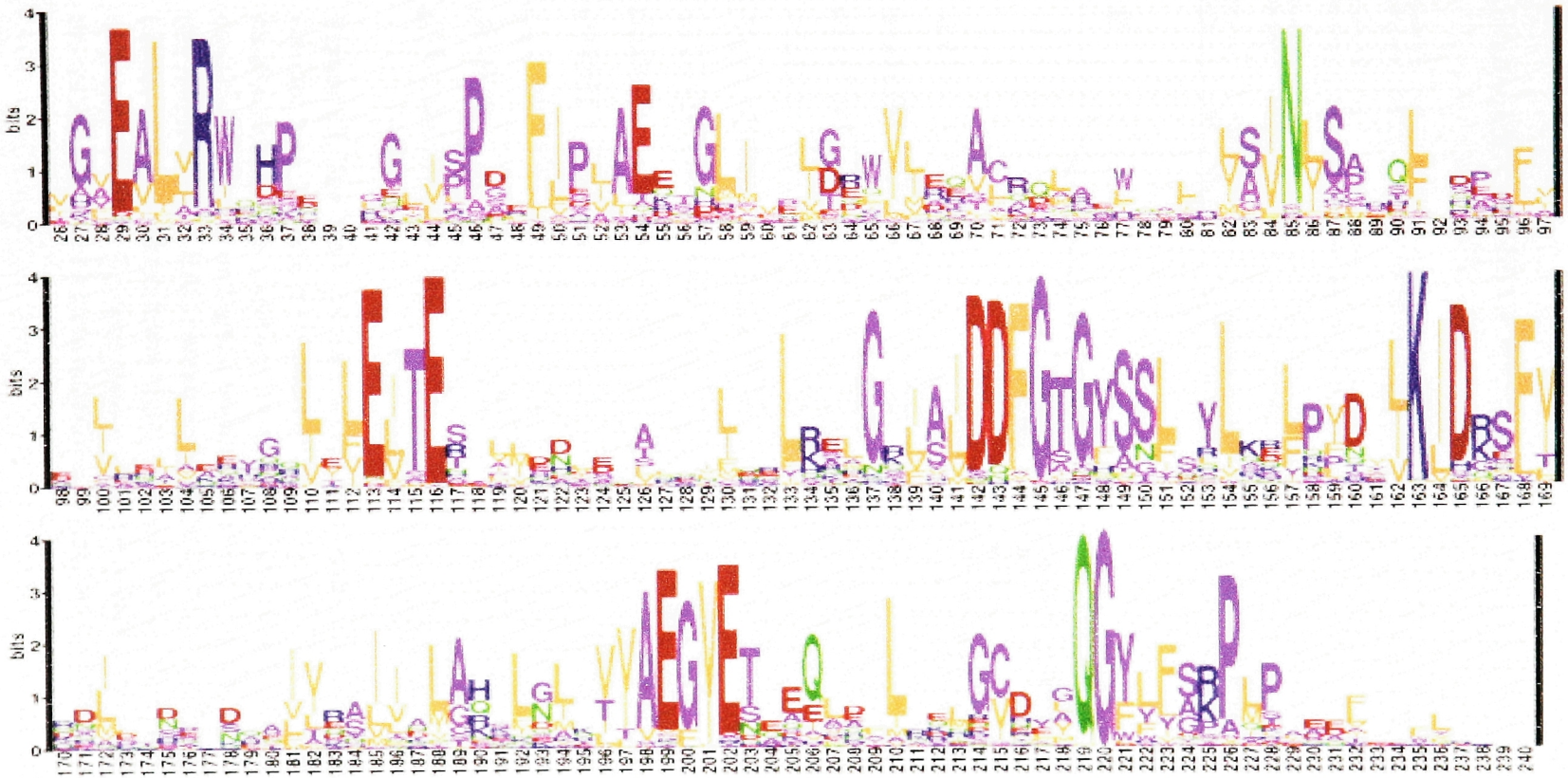
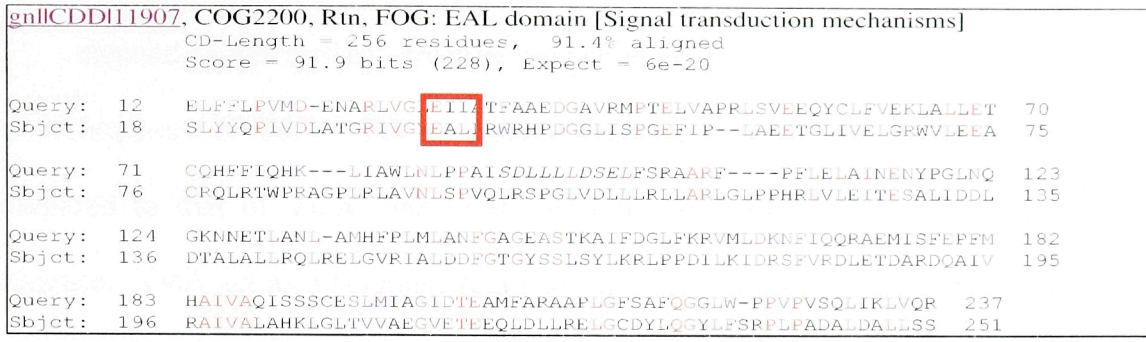


Figure 18. Consensus sequence of the EAL domain. The residue numbering is from *E. coli* YdiV. The EAL motif comprises residues 29–31. The consensus sequence represents 128 EAL domains encoded in complete microbial genomes. The letters in each position represent amino acid residues found in that position; the height of each letter reflects the fraction of sequences with the corresponding amino acid residue in that position (the degree of conservation). The total height of each column indicates statistical importance of the given position. The residues are colored as follows: N, Q – green; K, R, H – blue; D, E – red; F, L, I, M, V – yellow; the rest – purple.

Modified from Galperin MY, Nikolskaya AN, & Koonin EV. (2001) *FEMS Microbiol Lett* **203**: 11-21.

A



B

	Sequence source	% Identity to the consensus sequence	% Similarity to the consensus sequence	% of sequence aligned	E-value.
ydiV	*Salmonella typhimurium*	22 / 23	41 / 41	91.4 / 92.7	6e-20 / 2e-04
gil15641657	*Vibrio cholerae*	40 / 43	60 / 60	98.0 / 100.0	2e-64 / 2e-71
gil16127324	*Caulobacter crescentus*	52 / 51	68 / 68	94.5 / 93.9	9e-80 / 8e-79
gil2632230	*Bacillus subtilis*	24 / 27	41 / 45	94.5 / 91.9	3e-36 / 1e-42
gil17987736	*Brucella melitensis*	45 / 48	65 / 69	100.0 / 98.0	7e-77 / 9e-77
gil15594708	*Borrelia burgdorferi*	32 / 36	58 / 60	91.8 / 96.0	5e-51 / 5e-44
gil3659619	*Gluconacetobacter xylinus*	31 / 39	54 / 60	98.0 / 100.0	1e-49 / 2e-68
gil349482	*Klebsiella pneumoniae*	29 / 31	51 / 54	99.2 / 98.4	6e-34 / 2e-50
gil4467262	*Streptomyces coelicolor*	39 / 41	56 / 58	98.0 / 99.2	2e-43 / 1e-57
gil6458457	*Deinococcus radiodurans*	43 / 41	60 / 60	97.7 / 96.8	2e-53 / 2e-58
gil15641224	*Vibrio cholerae*	32 / 35	55 / 60	96.9 / 94.3	7e-53 / 1e-50

Figure 19. The YdiV primary amino acid sequence exhibits homology to the EAL domain. (A) Alignment by cdart of the YdiV amino acid sequence with the COGS database consensus sequence for EAL domains. Red = conserved, Blue = not conserved, Grey = unaligned residues not fully covered by all sequences.
(B) Alignments of EAL containing proteins to the consensus sequences of COGS (upper left) and Pfam (lower right). The sequences other than *ydiV* are expected to be more similar to the consensus sequences because they contributed to the derivation of the consensus sequences. Prepared by collaborator Shaneen Singh.

alignment than YdiV (Fig. 19B). Additionally, the short length of the YdiV amino acid sequence acts to decrease the significance of the E value produced by BLAST.

Secondary and tertiary structure prediction programs were used to analyze further the architecture of YdiV. For secondary structure analysis, the YdiV sequence was compared to that of VieA, one of the proteins used to derive the EAL consensus sequence. VieA act as a response regulator in *Vibrio cholerae*, and participates in the activation of cholera toxin expression (Tischler *et al.*, 2002). The YdiV and VieA amino acid sequences were each converted by 5 independent prediction algorithms into α-helices and β-sheets. The structure predictions were then aligned. Despite low homology at the primary sequence, the predicted secondary structures of YdiV and VieA mapped almost identically (Fig. 20). Moreover, protein threading programs predicted that both YdiV and EAL domain secondary structures would aggregate to form higher order structures known as a TIM, or α/β, barrel, a common protein fold found in as many as 10% of known enzymes (Nagano *et al.*, 2002) (Fig. 21A). Active site residues in TIM barrels are generally present at the carboxy termini of β-strands (Anantharaman *et al.*, 2003), and the residues in the postulated analogous positions in YdiV are highly conserved between YdiV and other EAL domains (Fig. 21B).

Cyclic di-GMP: a novel signaling molecule in pathogenic bacteria

In the plant colonizing bacterium *Acetobacter xylinum*, also known as *Gluconacetobacter xylinus*, proteins containing one EAL domain and a tandem GGDEF domain catalyze the synthesis and catabolism of the small molecule cyclic diguanylate (c-diGMP) (Tal *et al.*, 1998). (Fig. 22). Three different diguanylate cyclases (DCGs) genes,

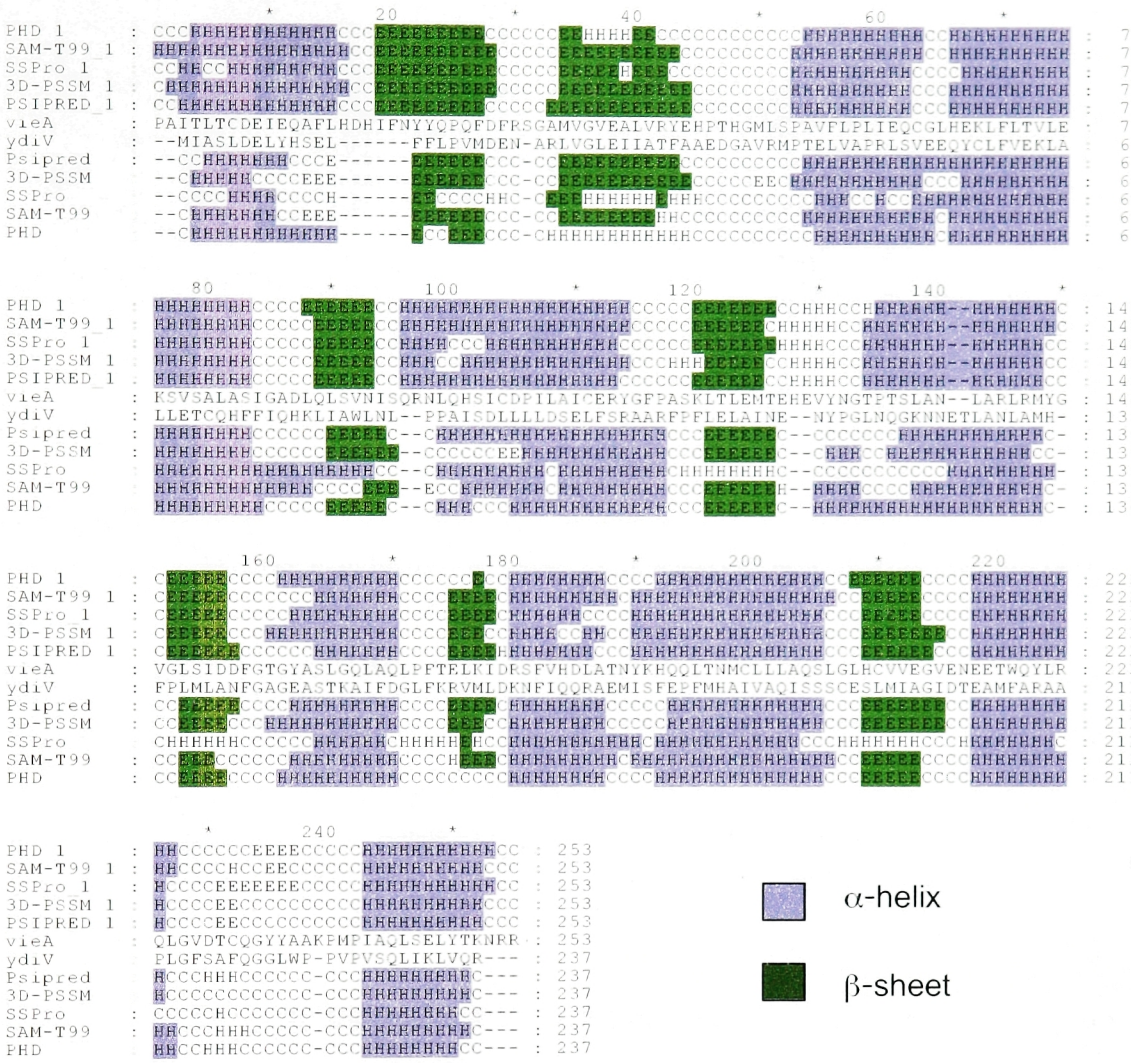


Figure 20. Secondary structure predications indicate that YdiV exhibits the same architecture as the EAL domain. Five independent secondary structure prediction algorithms were used to determine the putative arrangement of α-helices and β-sheets in YdiV and the EAL domain-containing protein VieA. The two uncolored rows in the middle of each alignment indicate the amino acid sequences of VieA and YdiV. The top 5 rows represent 5 predicted secondary structures for VieA produced by 5 different prediction algorithms (PHD, SAM-T99, SSPro, 3D-PSSM, and Psipred), and the bottom 5 rows represent predicted secondary structures for YdiV produced by the same 5 algorithms used to analyze VieA. Prepared by collaborator Shaneen Singh.

A

Modified from Branden & Tooze, Introduction to Protein Structure, 2nd ed. (1999)

B

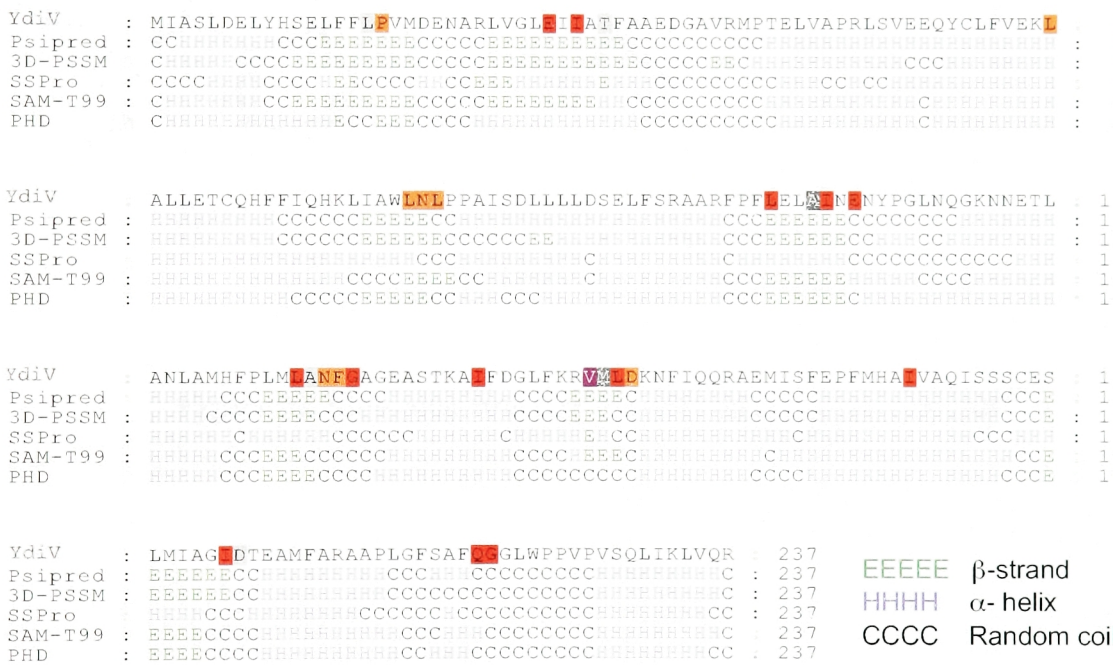


Figure 21. Residues conserved between EAL domains and YdiV map to postulated active site residues in the TIM barrel protein fold. (A) Structure of a TIM barrel, including active site residue positions. (B) Location of postulated active site residues in YdiV amino acid sequence (top line). Identity/similarity of residues between YdiV and 100% of EAL domain sequences (Purple), 95% of sequences (Red), and 90% of sequences (orange). Residues conserved in 90% (light grey) or 95% (dark grey) of other EAL domain sequences but not in YdiV. Secondary structure predictions for YdiV produced by 5 different prediction algorithms are aligned beneath the YdiV amino acid sequence. Prepared by collaborator Shaneen Singh.

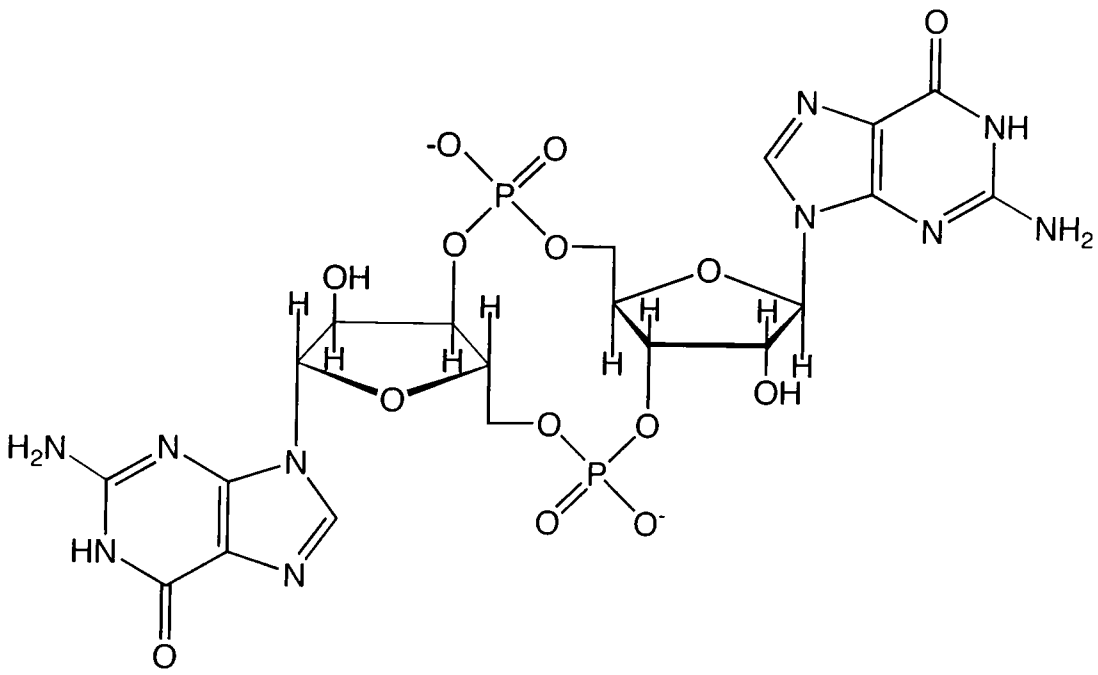


Figure 22. Molecular structure of cyclic diguanylate (c-diGMP)

each found in an operon with a phosphodiesterase (PDE) gene, are responsible for modulating an intracellular pool of c-diGMP, which in turns activates cellulose synthase (Ross *et al.*, 1985; Ross *et al.*, 1987). Although both the EAL and the GGDEF domains are present in DGCs and PDEs, studies suggest that the GGDEF domain is responsible for c-diGMP cyclase activity. Computational analysis has indicated that the GGDEF domain demonstrates homology to adenylyl cyclases, including similarities in secondary structure predictions, hydrophobicity patterns, and functionally and structurally important residues clustered in conserved motifs (Pei and Grishin, 2001). Moreover, different GGDEF-containing genes, including one from *E.coli* that includes no other associated domains, can complement a deficiency in cellulose synthesis in *Rhizobium leguminosarum*, a nitrogen fixing bacteria related to *A. xylinum* (Ausmees *et al.*, 2001). These data not only demonstrate functional complementation, but they also indicate that the GGDEF domain serves the same enzymatic function in *E. coli* as it does in *A. xylinum* and *R. leguminosarum.* Because GGDEF domains are postulated to act as c-diGMP cyclases, EAL domains are assumed to possess PDE activity, by default; however, there is currently no direct evidence, biochemical or computational, that supports this hypothesis.

YdiV represented an excellent candidate for evaluating whether EAL domains possess PDE activity, as it is composed of just the single domain. If YdiV catalytically degrades c-diGMP, we anticipated that the complemented mutant, which overexpresses YdiV, would contain lower levels of endogenous c-diGMP than either the wild type or the *ydiV* mutant strain. Soluble lysates of late log phase cultures of each of the three strains were extracted with methanol and chloroform to eliminate proteins, and then

extracts were fractionated by HPLC. Fractions were subjected to electrospray mass spectroscopy, and endogenous levels of c-diGMP were measured by comparison to known quantities of synthetic c-diGMP. At least ten times more c-diGMP was detected in extracts from *ydiV* mutant *S. typhimurium* than in extracts from the over-expressing strain (Fig. 23). Moreover, the amount of c-diGMP in extracts from the over-expressing strain was indistinguishable from background levels, suggesting that over-expression of YdiV leads to elimination of endogenous c-diGMP. Wild type extracts are in the process of being evaluated to determine if the *ydiV* mutant contains significantly more c-diGMP than wild type *S. typhimurium*. These data represent the first evidence that EAL domains possess PDE activity; moreover, this is the first evidence implicating c-diGMP in the regulation of virulence mechanisms of pathogenic bacteria.

EAL and GGDEF domains are abundant in the S. typhimurium genome

According to annotation by the Genome Sequencing Center at Washington University in St. Louis, the *S. typhimurium* genome contains 22 genes with either an EAL domain, a GGDEF domain, or both (Table VIII). Seven genes encode both domains, four genes include other domains in addition to EAL or GGDEF, but in more than half the genes, the EAL or GGDEF domain is the only recognized domain in the gene. However, all the genes that encode a sole GGDEF domain also encode a long N-terminal region that bears no homology to any domains in the database. In fact, *S. typhimurium* contains no genes that are composed of only a GGDEF domain, whereas 5 of the 18 genes with EAL domains are short genes composed entirely of an EAL domain, such as *ydiV*.

Figure 23. Over-expression of YdiV in *S. typhimurium* results in depletion of endogenous c-diGMP. Chloroform / methanol extracts of bacterial cytosolic fractions were analyzed for endogenous c-diGMP content. Methods are paraphrased from a description by Michael MacCoss who performed the analysis. (A) Positive ion electrospray ionization of synthetic c-diGMP, (M+H)+ ion = 691.1. (B) Spectrum after solation of the 691.1 ion in the first quadrupole (Q1) of a triple mass spectrometer, fragmentation of the 691.1 ion by low energy collisions with Ar gas in Q2, and detection of the product ions in Q3. The 540 Ion results from the loss of one guanine. (C) Selected reaction monitoring (SRM) mass spectrometry: chromatogram of 900 fmol of synthetic c-diGMP with mass spectrometer set to detect only molecules with a precursor ion of 691 and product ions of 540. (D) SRM mass spectrometry for *ydiV* mutant extract. The second peak results from c-diGMP chelation of cations. (E) SRM mass spectrometry for methanol (as a blank). Peak is carry-over from the previous sample due to c-diGMP's ability to bind to the metal syringe. (F) SRM mass spectrometry for complemented mutant extract (equal quantity to the amount of *ydiV* mutant extract used for (D)). Peak intensity is not distinguishable from that of the blank.

Figure 23.

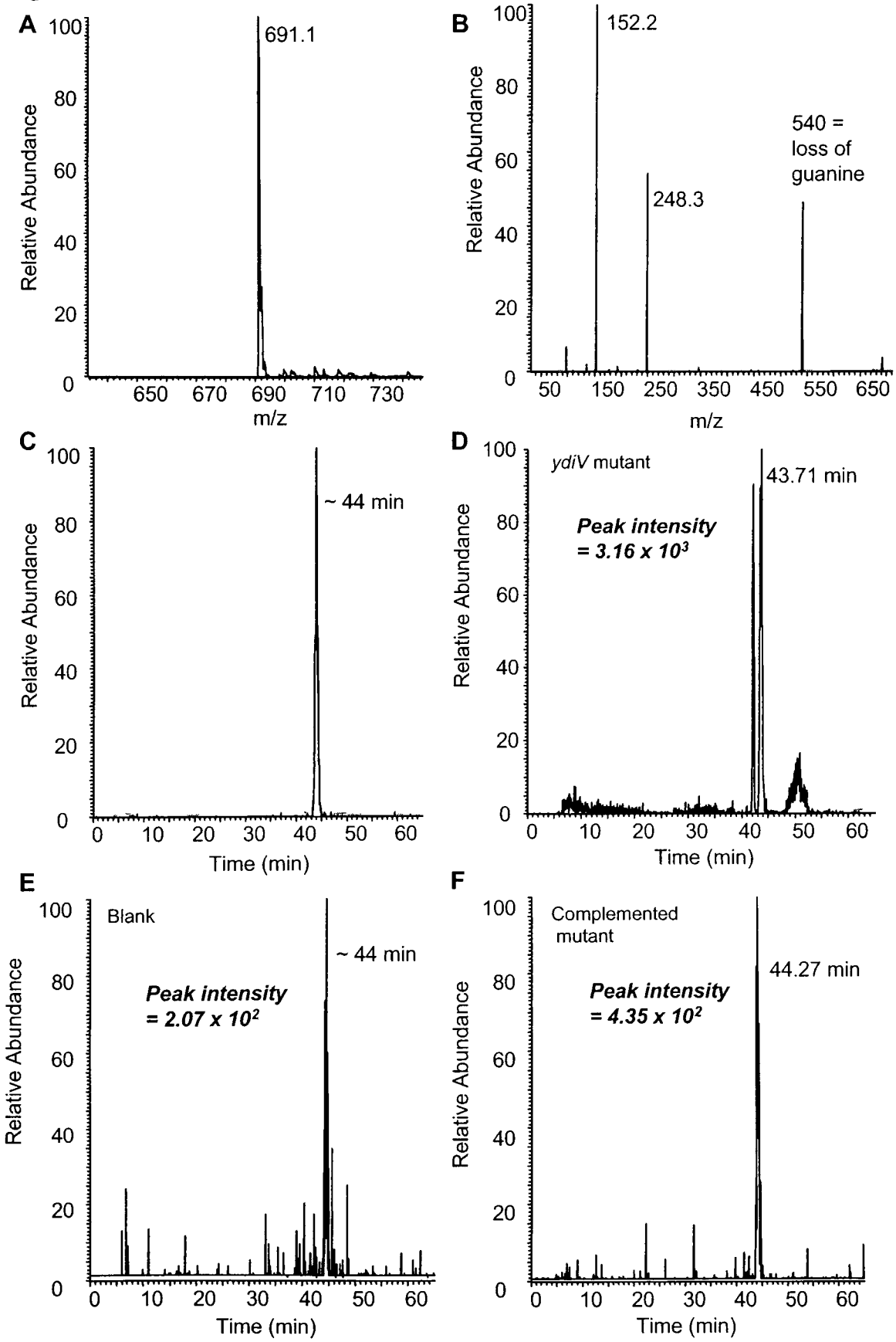

A
691.1
Relative Abundance
m/z
B
152.2
248.3
540 = loss of guanine
Relative Abundance
m/z
C
~ 44 min
Relative Abundance
Time (min)
D
ydiV mutant
43.71 min
Peak intensity = 3.16 x 10³
Relative Abundance
Time (min)
E
Blank
~ 44 min
Peak intensity = 2.07 x 10²
Relative Abundance
Time (min)
F
Complemented mutant
44.27 min
Peak intensity = 4.35 x 10²
Relative Abundance
Time (min)

Table VIII. *S. typhimurium* genes containing EAL and GGDEF domains

STM number[1]	name	alt. name	Pfam/COG[2] assigned by WUSTL[1]	Domains present			Is the EAL/ GGDEF domain part of a larger domain architecture?[3]
				GGDEF	EAL	other	
343			pf00563		*		
385	yaiC	adrA	pf0090	*		MASE2	
468	ylaB		pf00563		*		COG4943
551			COG2200		(see below)[4]		
1283	yeaJ		pf0090	*			
1344	**ydiV**		COG2200		*		
1697			COG2200		*		
1703	yciR		pf0090	*	*	PAS	COG5001?
1827			pf00563		*		COG4943
1987			pf0090	*			
2123	yegE		pf0090	*	*	MASE1	COG5001?
2215	**rtn**		pf00563		*		
2410	yfeA		pf00563	*	*		COG5001?
2503			pf00563	*	*		
2672	yfiN		pf0090	*			
3375	yhdA		pf0090	*	*		COG5001?
3388			pf0090	*	*	MHYT	
3611	yhjH		COG2200		*		
3615	yhjK		pf00672	*	*		
4264	yjcC		pf00563		*		COG4943
4551			pf0090	*	*		
pslt032[5]			COG2200		*		

[1]Assigned to each ORF in the *S. typhimurium*, in order of arrangement on the chromosome, by the Genome Sequencing Center, WUSTL (Washington University in St. Louis)

[2]Two of the three independent protein domain databases. EAL domains are designated rtn or COG2200 in COG (cluster of orthologous groups), pf00563 in Pfam, and DUF2 or Smart00052 in SMART (simple module architecture reseach tool). GGDEF domains are designated COG2199 in COG, pf0090 in Pfam, and DUF1 or smart00267 in SMART.

[3] COG4943 = signal sensor + EAL domain, COG5001 = N-terminal sequence + GGDEF + EAL

[4]The C-termini of STM551 (aa 69-100) aligns with low homology to residues 803-834 of a *B. japonicum* gene which is COG5001. The homologous portion compises the C-termini of the *B. japonicum* COG5001 EAL domain.

[5]pslt indicates that the gene is found on the virulence plasmid.

Only one of these genes had been previously studied: *adrA*, which appears to be the *S. typhimurium* homolog of *A. xylinum*'s cellulose regulating DGC (Romling *et al.*, 2000; Zogaj *et al.*, 2001). The only other member of this set described in the literature was the *E. coli* homolog of *rtn* (STM 2215), which had been identified in a screen for mutants with altered adaptation rates to exogenous stress (Hall, 1997). Blast searches indicated that GGDEF and EAL domains are widespread among prokaryotes, and are often found in tandem with signal sensor domains, such as the PAS or CheY domains (Galperin *et al.*, 2001). Most of the genes in the database have never been functionally analyzed; however, EAL and GGDEF domain-containing genes in other Gram negative bacteria, including *Bordertella pertussis* (Merkel *et al.*, 1998a), *Vibrio parahaemolyticus* (Boles and McCarter, 2002), and *Caulobacter crescentus* (Aldridge *et al.*, 2003; Hecht and Newton, 1995), appear to act as response regulators, influencing diverse bacterial functions, including synthesis of exopolysaccharide, expression of flagella, and regulation of virulence genes.

YdiV and regulation of cellulose synthesis in S. typhimurium

Endogenously produced c-diGMP has been characterized as a regulator of enzymatic activity in only one context: activation of cellulose synthesis in nitrogen-fixing, plant-colonizing bacteria. Recently, it has been appreciated that pathogeneic Gram negative bacteria also produce exopolysaccharides under specific conditions. In *Vibrio* species, synthesis of capsular polysaccharides is mediated by proteins containing both an EAL and a GGDEF domain (Boles and McCarter, 2002; Rashid *et al.*, 2003). Under certain environmental conditions that promote biofilm formation, *S. typhimurium*

produces an extracellular matrix composed of aggregative fimbriae and cellulose (Zogaj *et al.*, 2001). Cellulose synthesis is activated by expression of the gene *adrA*, which encodes a putative transmembrane domain, a MASE2 domain, and a GGDEF domain (Romling *et al.*, 2000). In light of the parallels to *A. xylinum*, it seems likely that AdrA activates cellulose synthesis by generating c-diGMP. However, unlike the DGCs and PDEs in *A. xylinum, adrA* does not encode an EAL domain, nor is it in an operon with a candidate PDE.

We considered the possibility that YdiV acts as the PDE that turns off cellulose synthesis in *S. typhimurium*. Zogaj *et al.* described four colony morphotypes resulting from growth on Congo Red agar that are produced depending on which elements of the extracellular matrix *S. typhiumurium* expresses: in the absence of matrix, colonies appear white; production of cellulose only leads to pink colonies; production of fimbriae only is associated with brown colonies; and secretion of both cellulose and fimbriae leads to red colonies (Zogaj *et al.*, 2001). If YdiV acts as the PDE complement to AdrA, overexpression of YdiV should inhibit cellulose synthesis, and thus result in white or brown colonies. Overnight cultures of wild type, *ydiV* mutant, and hypercomplemented *S. typhimurium* were diluted, plated on Congo Red Agar, and incubated at 30°C or 37°C. Colonies from each of the three strains grown at 30°C developed an identical morphotype with red colonies (Fig. 24). All strains produced white colonies when grown at 37°C (data not shown), consistent with inhibition of biofilm formation at this temperature. Thus, YdiV does not appear to act in conjunction with AdrA to regulate cellulose synthesis, although comparison with a cellulose-deficient strain of *S. typhimurium* on Congo Red agar and a more quantitative assay for cellulose production would be required

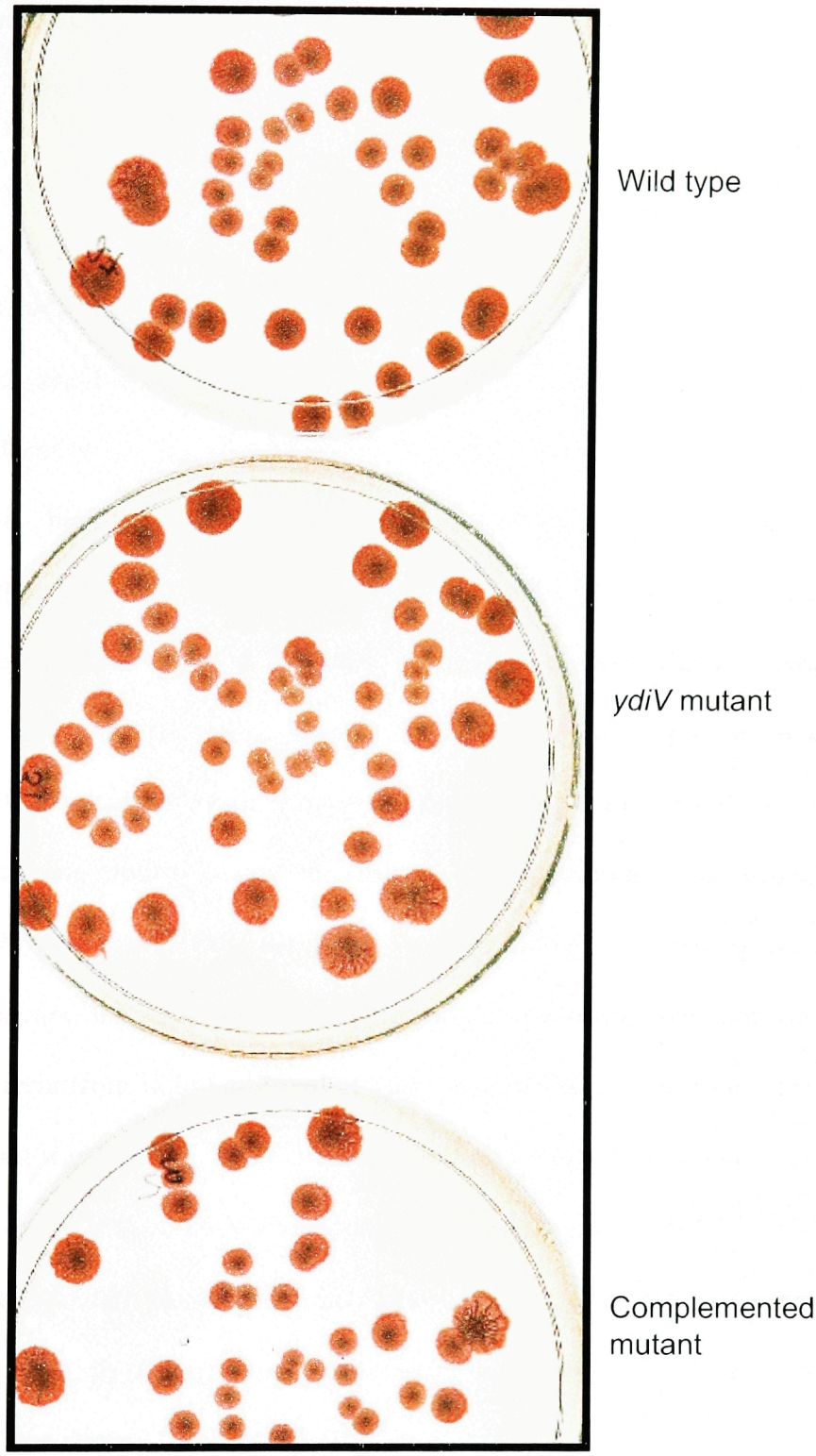


Figure 24. YdiV does not appear to contribute to the production of cellulose by *S. typhimurium*. Bacterial cultures were spread on LB-agar containing no NaCl augmented with 40 μg/ml Congo Red and 10 μg/ml Coomasie Brilliant Blue. Red color indicates the presence of polysaccharide.

to make a definitive conclusion. One of the other 17 EAL domain-containing proteins encoded by the *S. typhimurium* genome may act as the reciprocal phosphodiesterase for AdrA.

YdiV contributes to the regulation of flagellin

Several Gram negative bacteria use EAL or GGDEF domains to regulate motility either by effecting function of flagella (Aldridge *et al.*, 2003; Boles and McCarter, 2002) or pili (Huang *et al.*, 2003). We examined whether expression of *ydiV* influenced the motility of *S. typhimurium*. Swimming motility was assayed by growth through 0.3% LB-agar. Over a 4-6 hour period, the *ydiV* mutant swam slightly further than wild type bacteria (Fig. 25). Strikingly, the complemented mutant did not swim at all, even when agar plates were left to incubate overnight. When bacteria were inoculated on 0.65% agar to evaluate swarming motility (across the surface of the agar, rather than through it) (Young *et al.*, 1999b), the results were the same: the *ydiV* mutant swarmed further than wild type *S. typhimurium*, and the complemented mutant did not swarm (data not shown). Observation of bacteria from liquid cultures at 100x magnification confirmed that the complemented mutant was amotile. The extreme phenotype of the complemented mutant was consistent with expression of YdiV above wild type levels in this strain.

To determine whether the defect in motility was due to a functional defect in flagella or a lack of synthesis of flagella, protein content in bacterial culture supernatants was evaluated by silver stained SDS-PAGE (Fig. 26A). One protein was clearly over-abundant in supernatant from the *ydiV* mutant strain, but undetectable in the supernatant of the complemented strain. The apparent molecular weight of the over-expressed

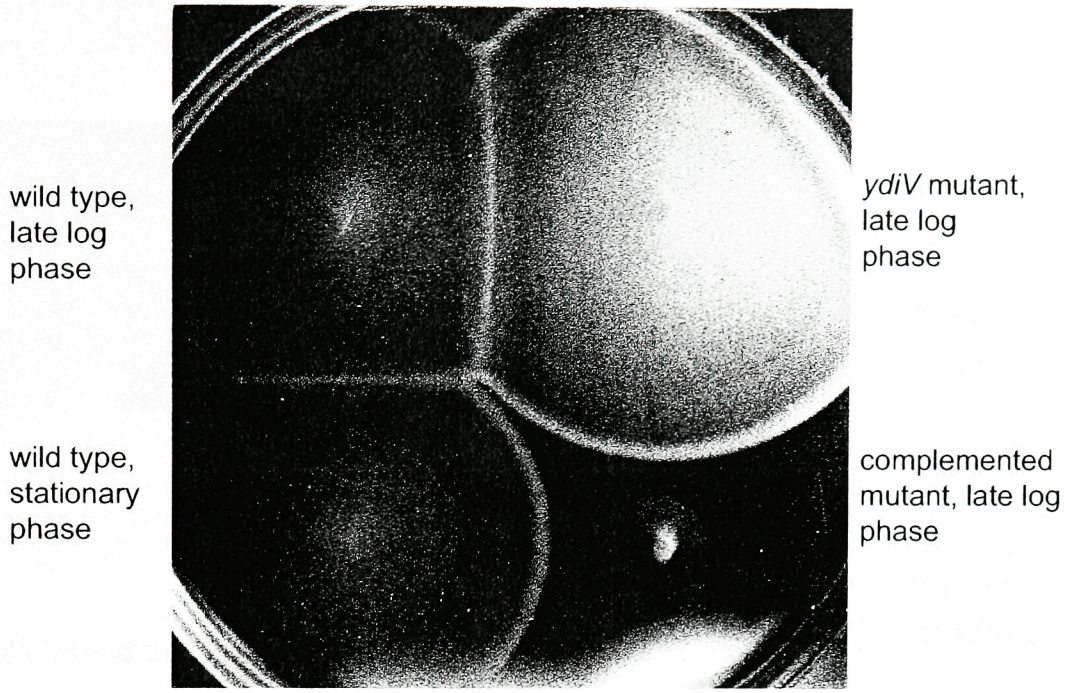


Figure 25. Over-expression of YdiV inhibits *S. typhimurium* swimming motility. 2 μl of bacterial culture was inoculated into LB media containing 0.35% agar, no NaCl. Plate was incubated at 37°C for 6 hours.

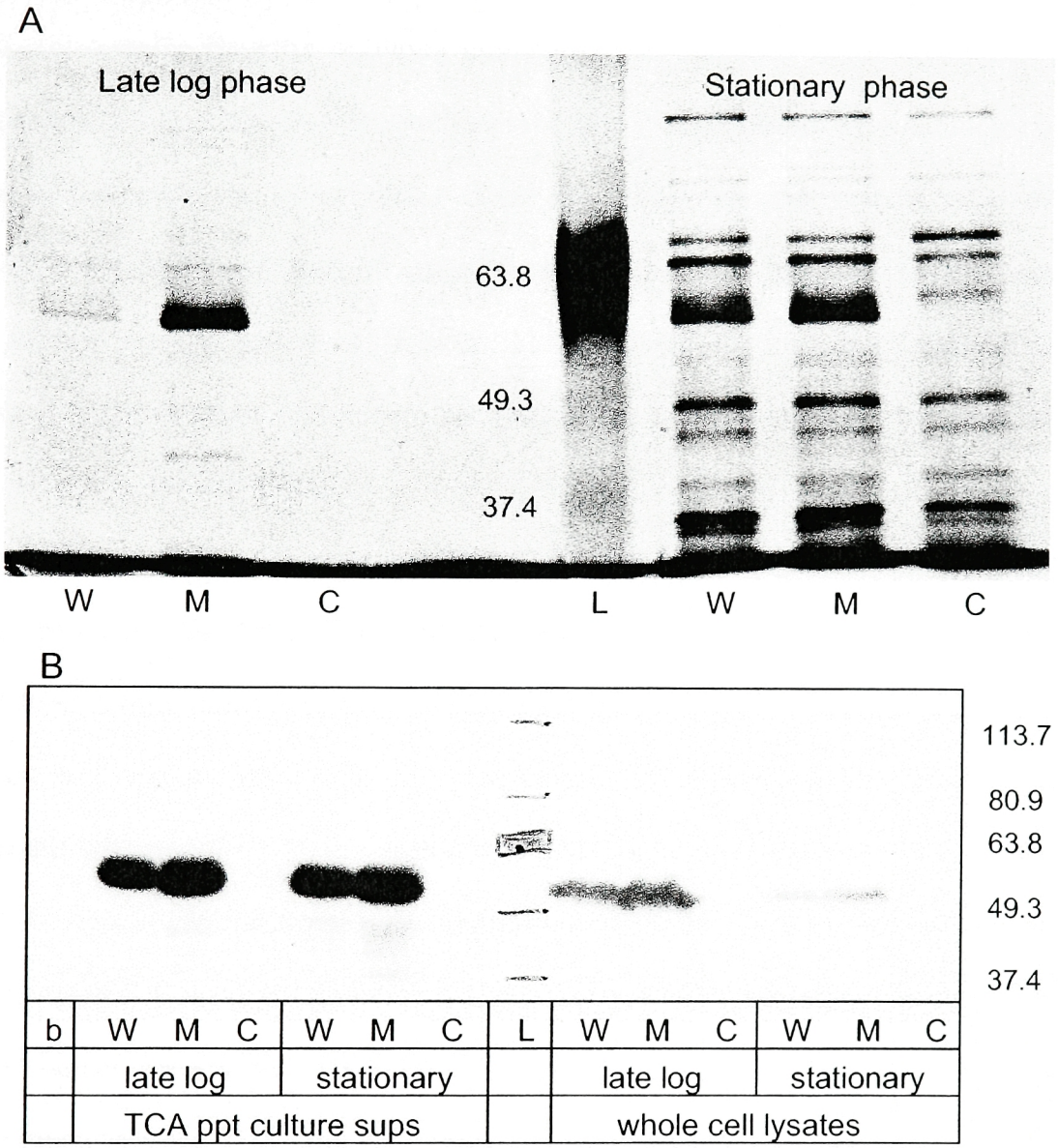


Figure 26. Expression of YdiV is inversely correlated with expression of flagellin. (A) Silver stained SDS-PAGE of TCA-precipitated bacterial culture supernatants. W, wild type. M, *ydiV* mutant. C, complemented mutant. L, ladder. (B) Immunoblot using anti-flagellin antibody. W, wild type. M, *ydiV* mutant. C, complemented mutant. L, ladder. b, LB broth.

protein was consistent with flagellin, the monomer encoded by *fliC* or *fljB* that polymerizes to form flagella. An immunoblot of bacterial supernatant proteins using anti-*Salmonella* flagellin antibody confirmed that flagellin levels were higher in supernatants from cultures of the *ydiV* mutant than in supernatants of wild type cultures, and undetectable in supernatants from the complemented mutant (Fig. 26B). Additionally, immunoblotting of whole bacterial lysates from the 3 strains demonstrated the same expression pattern of flagellin. This indicated that the lack of flagellin in the supernatant of the complemented mutant was not due to faulty secretion, but rather due to an inability to express flagellin.

Flagellin expression is controlled by a three-tiered regulatory hierarchy. The first tier, known as Class I genes, includes the master regulator genes, *flhD* and *flhC*. FlhD and FlhC form a transcriptional activator that is required for expression of the Class II genes, including the sigma factor *fliA*. Expression of FliA is required for transcription of flagellin (*fliC* and *fljB*). To investigate which tier might be affected by YdiV, an *flhDC-lacZYA* fusion was constructed and transduced into both wild type and *ydiV* mutant *S. typhimurium*. Counter-intuitively, the expression of *flhDC* was mildly repressed in the *ydiV* mutant, even though the mutant over-expresses flagellin (Fig. 27). This lack of correlation between *flhDC* and *fliC* expression was consistent with a previous report. The levels of FlhD and FlhC protein are regulated in *S. typhimurium* by Clp proteases (Tomoyasu *et al.*, 2002). When the genes encoding ClpXP are disrupted, levels of FlhD and FlhC protein increase, resulting in increased transcription of *fliA*, and concomitant increase in transcription of flagellin. In the absence of ClpXP, the transcription of *flhDC* decreases slightly, suggesting possible feedback inhibition. Over-abundance of YdiV

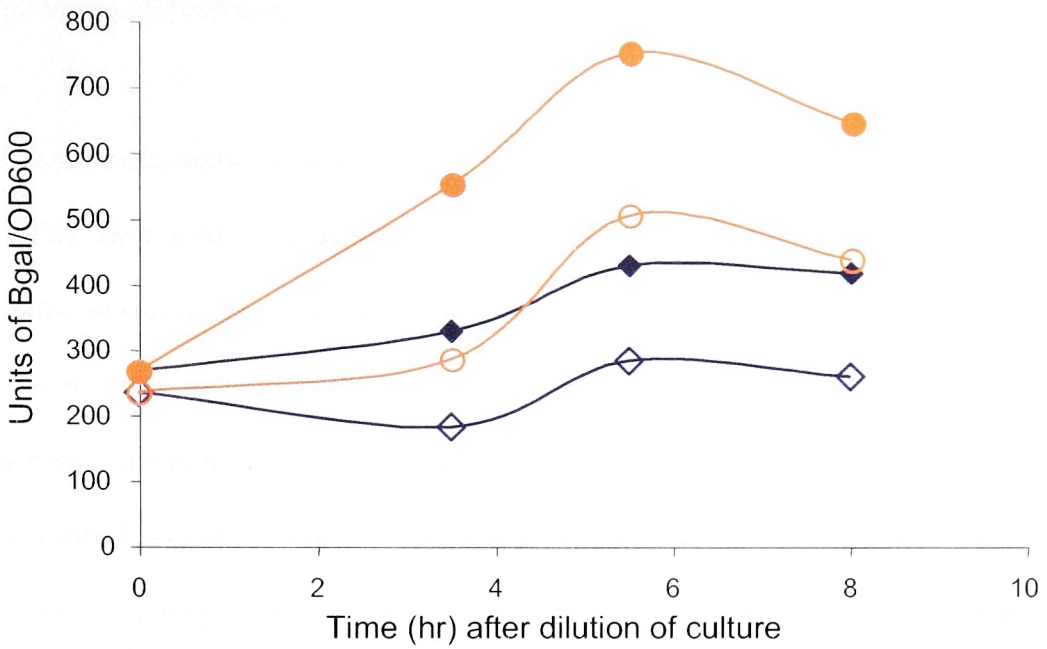


Figure 27. Enhanced production of flagellin by the *ydiV* mutant is not due to increased expression of *flhDC*. Wild type (closed symbols) and *ydiV* mutant (open symbols) *S. typhimurium* strains containing an *flhDC-lacZYA* fusion were grown in flasks (diamonds) or tubes (circles). Cultures grown in flasks received more aeration than cultures grown in tubes, which has been demonstrated to affect expression of flagellin.

may suppress flagellin expression by positively regulating either the activity or expression of Clp proteases.

Rapid Salmonella-induced macrophage death is suppressed by over-expression of YdiV

The first EAL domain-containing protein to be characterized, *bvgR* from *Bordetella pertussis*, was identified as a locus involved in the regulation of virulence genes. A *B. pertussis bvgR* mutant demonstrates decreased capacity to colonize the lungs after aerosol infection of mice, resulting in longer survival of mice before succumbing to infection (Merkel *et al.*, 1998b). In addition to contributing to *S. typhimurium*'s survival *in vivo* by mediating resistance to ROI, we found that YdiV regulates other functions associated with pathogen-host interactions. *S. typhimurium* grown to late log phase expresses the SPI-1 effector SipB, which induces rapid death of macrophages (Lundberg *et al.*, 1999; Monack *et al.*, 2001). Consistent with these studies, when we infected periodate-elicited macrophages with late log phase cultures of *S. typhimurium*, we observed rapid depletion of the macrophage monolayer (Fig. 28A).

Infection of macrophages with the *ydiV* mutant greatly enhanced killing of macrophages, with lysis of up to 100% of cells within 90 minutes after infection (Fig. 28A). Remarkably, over-expression of YdiV in the complemented mutant completely abolished *S. typhimurium*'s cytotoxic effect. Killing of macrophages by both the wild type strain and the *ydiV* mutant was dependent on growth phase and expression of *sipB*, but independent of SPI-2 (Fig. 29), confirming that we were witnessing the same phenomenon of rapid *Salmonella*-induced macrophage death that had been observed by others (Monack *et al.*, 2001). When macrophages were infected with a mixed culture of

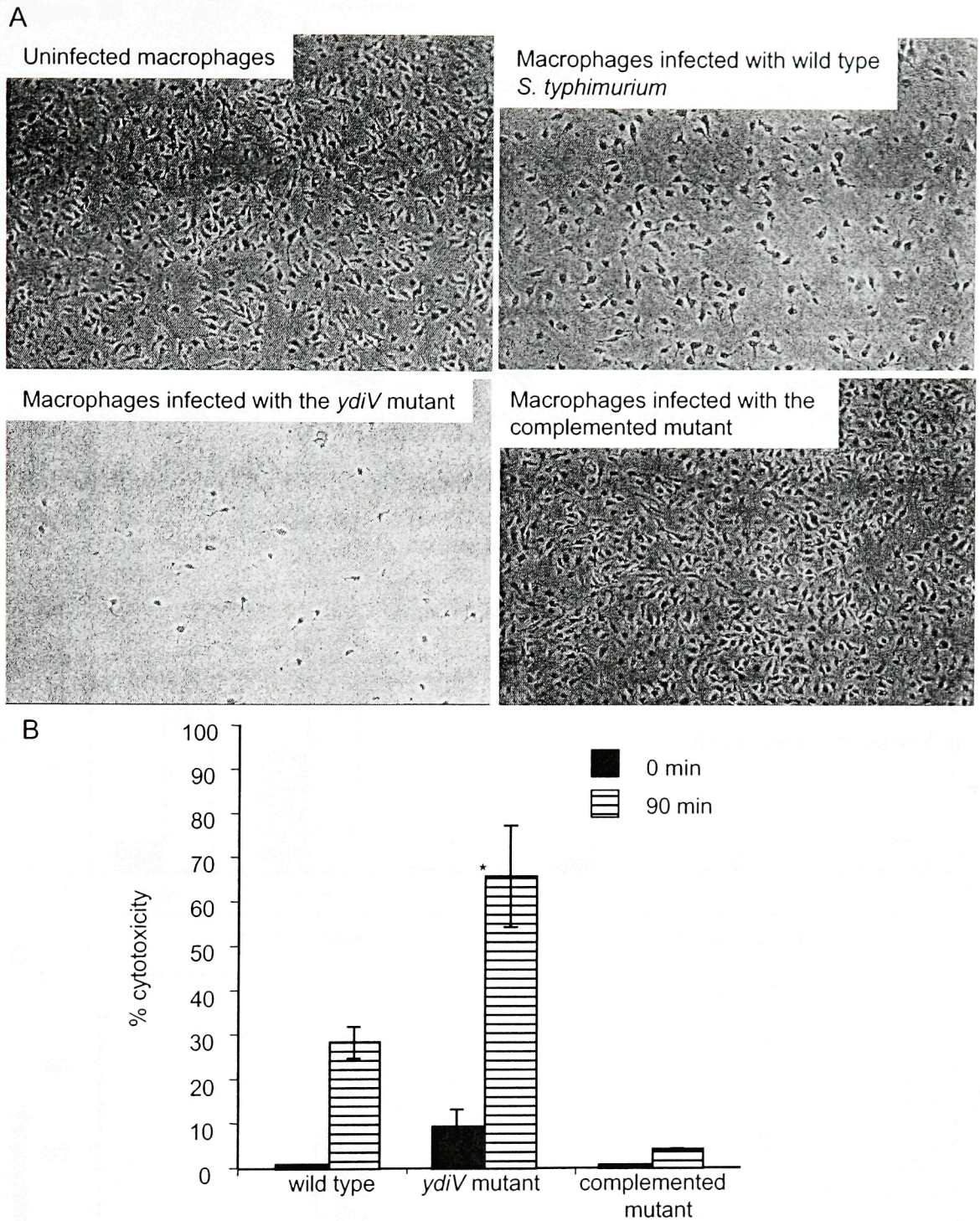
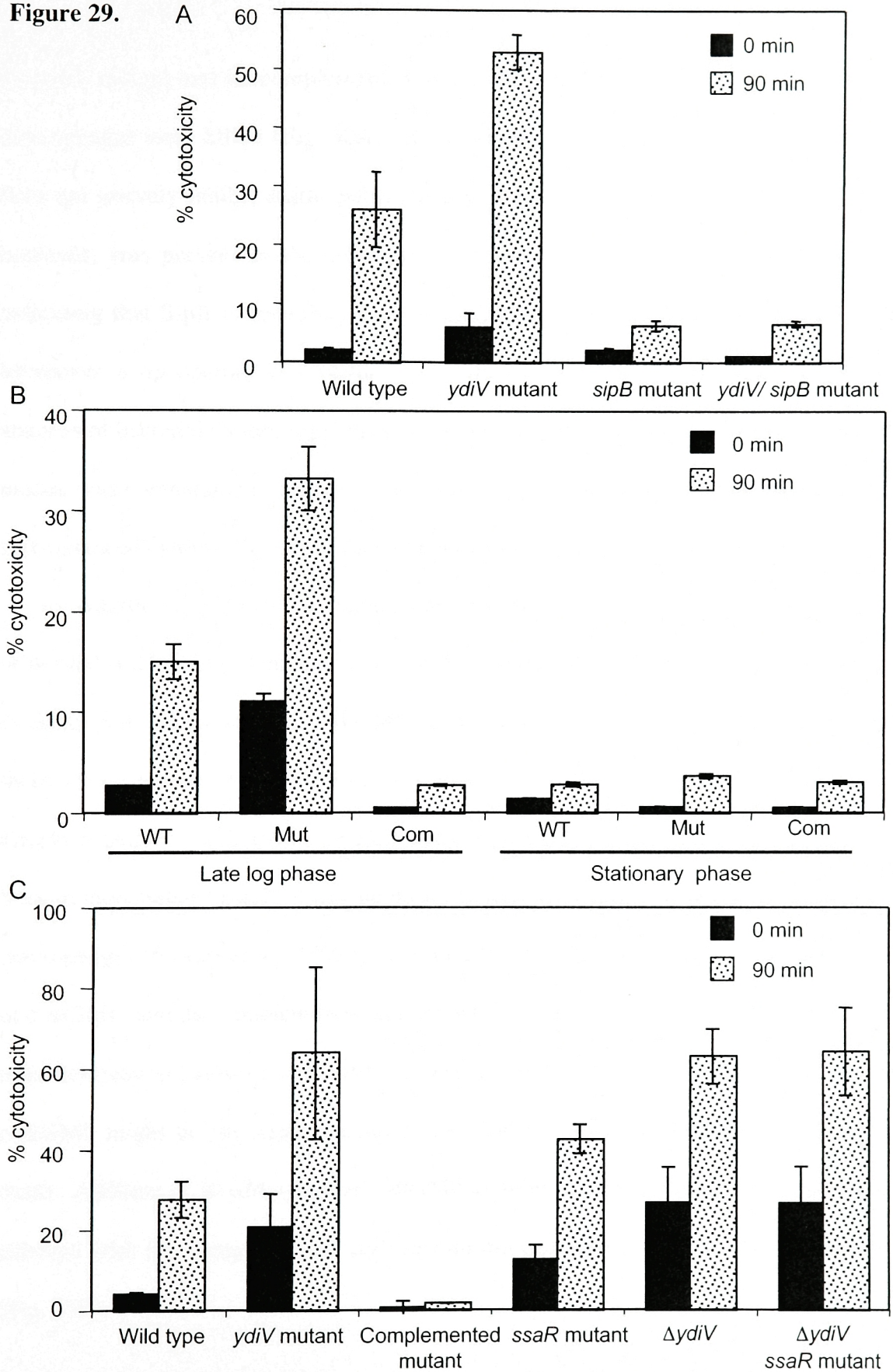


Figure 28. Expression of YdiV is inversely correlated with *S. typhimurium*'s ability to induce rapid macrophage death. (A) 20x magnification of periodate-elicited peritoneal macrophages 90 minutes after infection with opsonized, late log phase *S. typhimurium*. (B) Quantitation of macrophage death after infection with opsonized, late log phase *S. typhimurium* as measured by LDH release. Experiment shown is representative of 15 independent experiments. *Toxicity of the mutant is significantly greater than toxicity of wild type *S. typhimurium*. For this experiment, $p<0.01$; for compiled data, $p<10^{-6}$ by two-tailed Student's *t*-Test.

Figure 29. Rapid *Salmonella*-induced macrophage death is dependent on bacterial growth phase and expression of *sipB*, but not on secretion by SPI-2.

(A) Macrophages were infected with late log phase cultures of *S. typhimurium* disrupted in *ydiV* and/or *sipB*. (B) Macrophages were infected with late log phase or stationary phase cultures of wild type (WT), *ydiV* mutant (Mut) or complemented mutant (Com) *S. typhimurium*. (C) Macrophages were infected with late log phase cultures of *S. typhimurium* disrupted in *ydiV* and/or *ssaR*. *ssaR* encodes a component of the secretion apparatus of SPI-2. Δ*ydiV* contains a targeted disruption in *ydiV*, and demonstrates the same phenotypes as the *ydiV*::*mTn5* transposon mutant. Values are means for triplicates +/- standard deviations. Results are representative of two independent experiments.

Figure 29.


A
% cytotoxicity
0 min
90 min
Wild type
ydiV mutant
sipB mutant
ydiV/ sipB mutant
B
% cytotoxicity
0 min
90 min
WT
Mut
Com
WT
Mut
Com
Late log phase
Stationary phase
C
% cytotoxicity
0 min
90 min
Wild type
ydiV mutant
Complemented mutant
ssaR mutant
ΔydiV
ΔydiV ssaR mutant

the *ydiV* mutant and its complemented strain, the mutant phenotype prevailed and the macrophages were killed (Fig. 30A). This demonstrates that the complemented strain does not actively inhibit death; rather, it lacks a factor required for cytotoxicity. SipB, however, was present in the supernatant of the complemented mutant (Fig. 31A), indicating that SipB is necessary but not sufficient to induce rapid macrophage death. Moreover, a *sip* operon- *lacZYA* fusion construct, as well as silver-stained SDS-PAGE analysis of bacterial culture supernatant proteins, indicated that Sip expression in the *ydiV* mutant was comparable to expression in wild type *S. typhimurium* (Fig. 31B), suggesting that enhanced cytotoxicity of the *ydiV* mutant was not due to excessive secretion of SipB.

Addition of c-diGMP to mammalian cell lines has been shown to cause disruption of normal cell cycling (Amikam *et al.*, 1995; Steinberger *et al.*, 1999). Although there is evidence that plants generate c-diGMP as an allosteric regulator of cellulose synthase, there are no reports of c-diGMP being synthesized in eukaryotic cells from the animal kingdom (Amor *et al.*, 1991). Additionally, other nucleotides, specifically ATP, have been demonstrated to induce cell death by binding to P2Z receptors on the surface of macrophages (Ferrari *et al.*, 1999a). Because the *ydiV* mutant contains increased levels of c-diGMP, and the complemented mutant, which is unable to kill macrophages, appears to be deficient in cytosolic c-diGMP, we entertained the possibility that secreted bacterial c-diGMP might be the supplemental factor required for SipB-dependent macrophage death. Addition of 50 μM synthetic c-diGMP to uninfected macrophages or macrophages infected with the complemented *ydiV* mutant did not induce death of the macrophages (Fig. 30B).

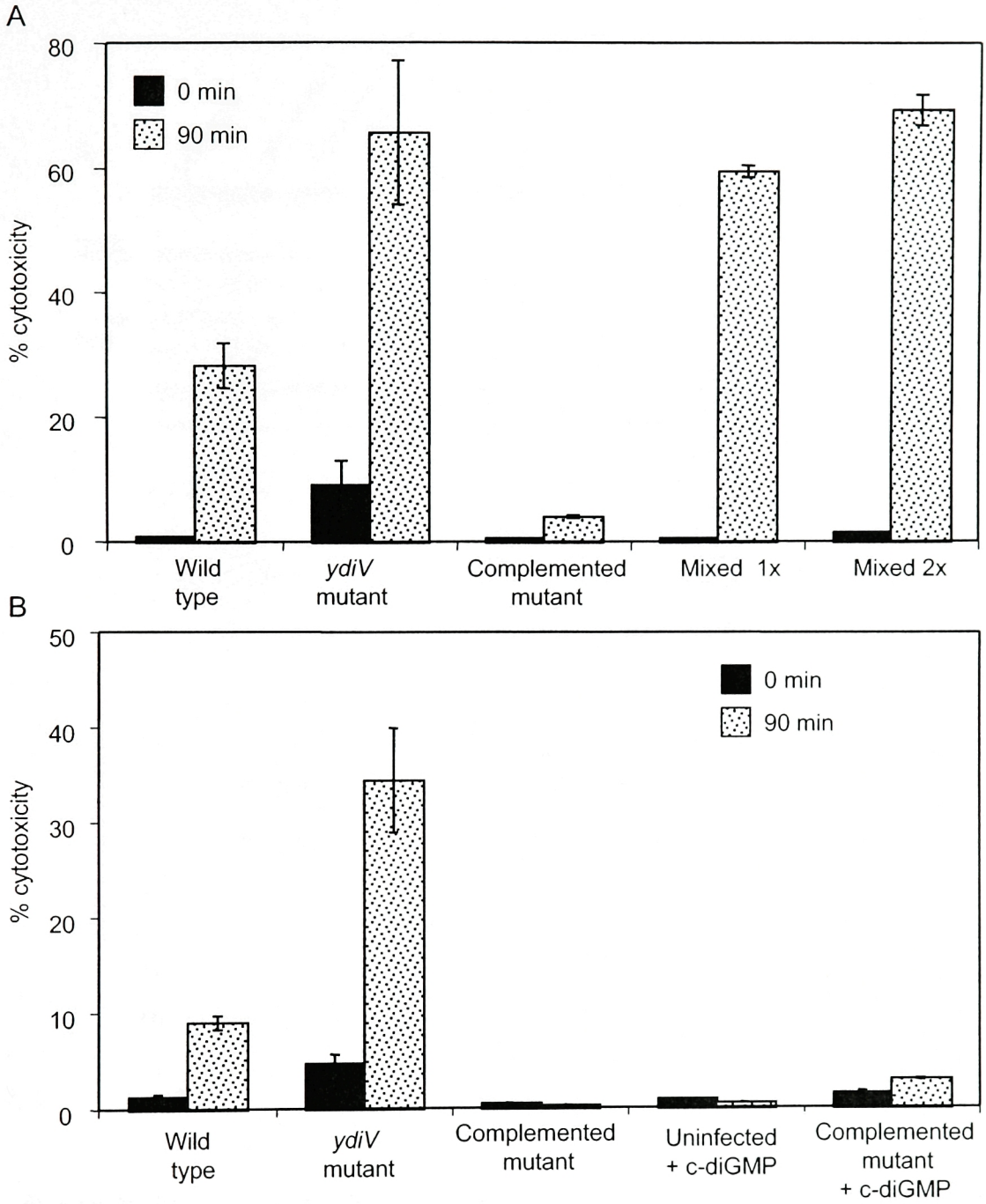


Figure 30. Production of a cytotoxic factor by the *ydiV* mutant. Late log phase cultures of *S. typhimurium* were used to infect macrophages. (A) Mixed infection with 1:1 ratio of *ydiV* mutant: complemented mutant. 1x represents the same total MOI as infection with one strain (2:1), but only half as much of each individual strain. Mix 2x represents twice the total MOI used for infection with one strain (4:1), but the same number of bacteria from each individual strain as used for infection with one strain. (B) 50 μM c-diGMP was added to uninfected macrophages or macrophages infected with the complemented mutant.

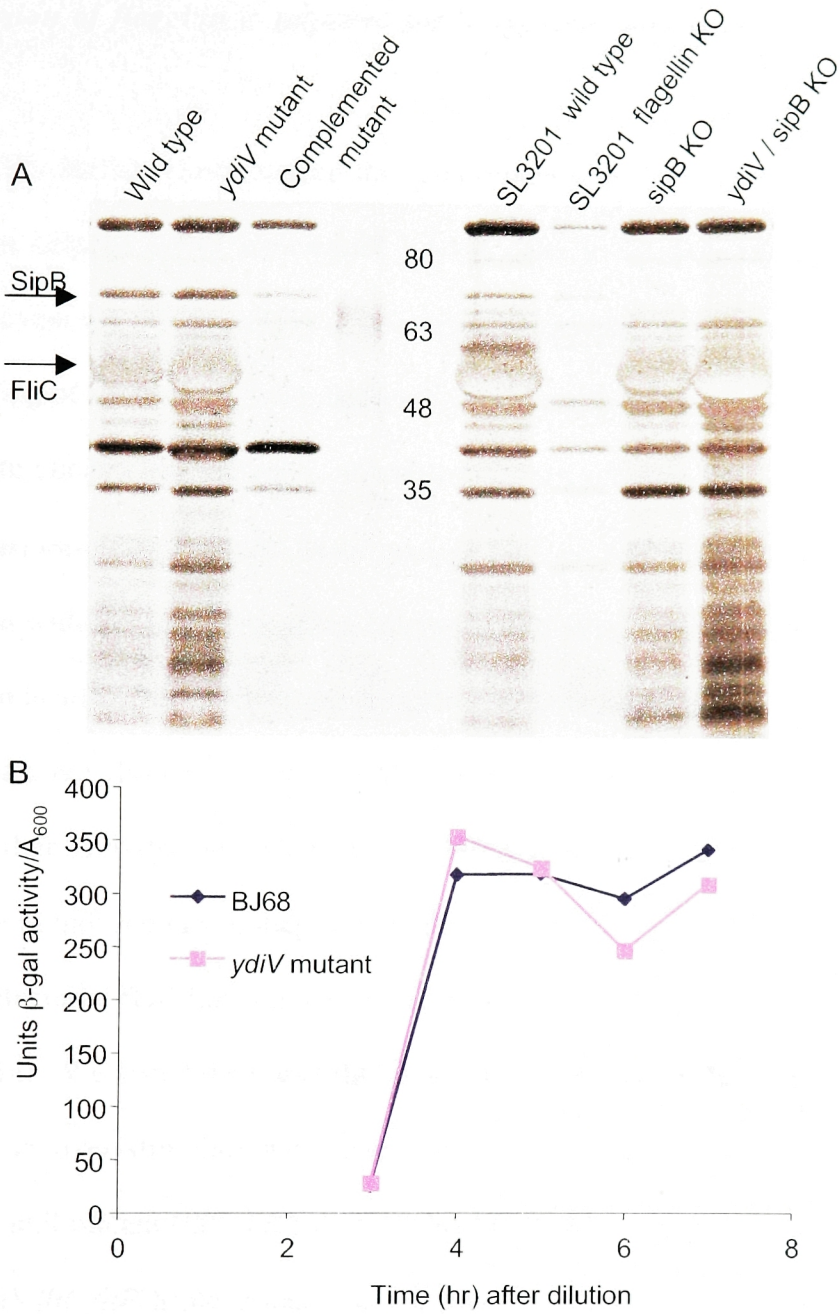


Figure 31. Expression of YdiV does not affect expression of SipB. (A) Silver stained SDS-PAGE of TCA-precipitated late log phase culture supernatants. Positions of molecular weight standards are indicated in kilodaltons. Flagellin is negatively stained due to over-abundance in relation to the other secreted proteins. Position of SipB was determined by absence of a protein band of the approximate correct molecular weight in *sipB* mutant strains. (B) Expression of the *sip* operon in *S. typhimurium* SL1344 with or without an intact *ydiV* gene. BJ68 contains *lacZYA* inserted in *sipC*. Graph depicts the average of 4 independent experiments. Comparison of enzyme activity in the two strains at each time point by 2-tailed Student's *t*-Test indicated no significant differences (P>0.5 for all time points).

Expression of flagellin is required for S. typhimurium to induce rapid macrophage death

We further characterized the requirements for *Salmonella*-induced macrophage death to help us better understand *ydiV*'s role in cytotoxicity. Opsonization of *S. typhimurium* was not required for macrophage death; however, it accelerated the process, and killing of bacteria by either heat or antibiotics abrogated cytotoxicity. Stimulation of periodate-elicited macrophages with IFN-γ did not promote macrophage resistance to *S. typhimurium*-induced death. Macrophages from phox$^{gp91-/-}$ mice were also lysed during infection with the *ydiV* mutant but left intact following infection with the complemented strain, indicating that macrophage death was not the result of host production of ROI by phox (data not shown). Inhibition of actin polmerization in macrophages by treatment with either dihydrocytochalasin B (DHCB) or cytochalasin D prevented rapid *Salmonella*-induced macrophage death (Fig. 32A).

Bacterial flagellin activates macrophages by signaling through TLR5 (Hayashi *et al.*, 2001a). We tested the possibility that hyper-secretion of flagellin by the *ydiV* mutant resulted in over-stimulation of macrophages, thus enhancing macrophage death. A flagellin null mutant (*fliC*$^{-}$*fljB*$^{-}$) was obtained from Alison O'Brien (Schmitt *et al.*, 1996), and a *ydiV fliC*$^{-}$*fljB*$^{-}$ triple mutant was constructed by transduction of the *ydiV* mutation in the flagellin null mutant. When added to murine macrophages, neither the flagellin mutant nor the *ydiV fliC*$^{-}$*fljB*$^{-}$ mutant caused any detectable macrophage death (Fig. 33). However, macrophages deficient in MyD88, the adaptor molecule used by TLRs to transduce signals, were still susceptible to killing by both wild type and *ydiV* mutant *S. typhimurium* (Fig. 32B).

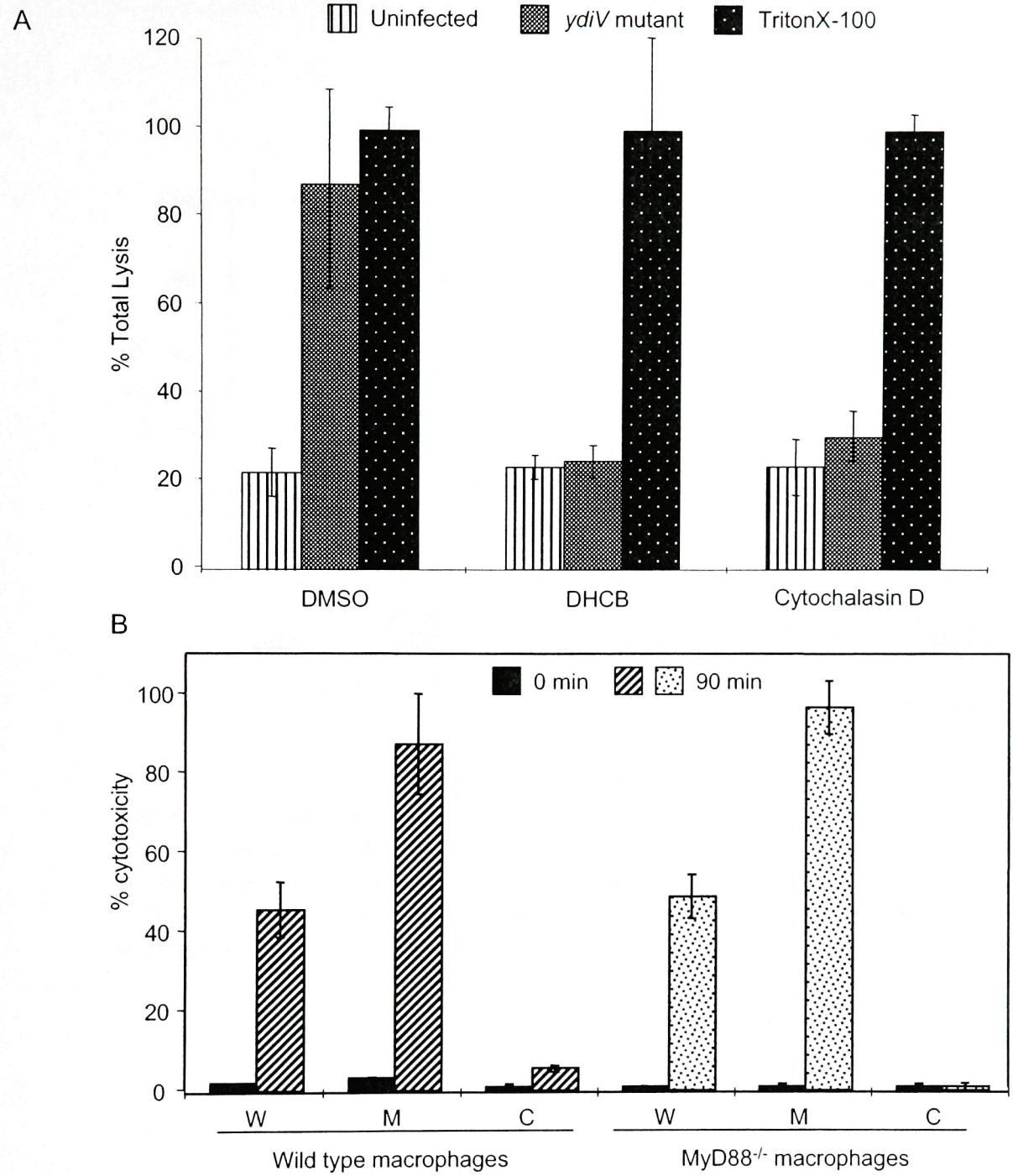


Figure 32. Characterization of macrophage functions required for rapid *Salmonella*-induced cytotoxicity. (A) Actin rearrangement is required for rapid macrophage death after infection with *S. typhimurium*. Macrophages were pre-treated with DMSO (vehicle control), dihydrocytochalasin B, or cytochalasin D for 30 minutes before infection. Toxicity is expessed as % lysis = (Exp LDH)/(maximum LDH release)x100. (B) MyD88-deficient macrophages are susceptible to rapid killing by *S. typhimurium*. Striped bars = wild type macrophages; speckled bars = MyD88-deficient macrophages. Values are means for triplicates +/- standard deviations.

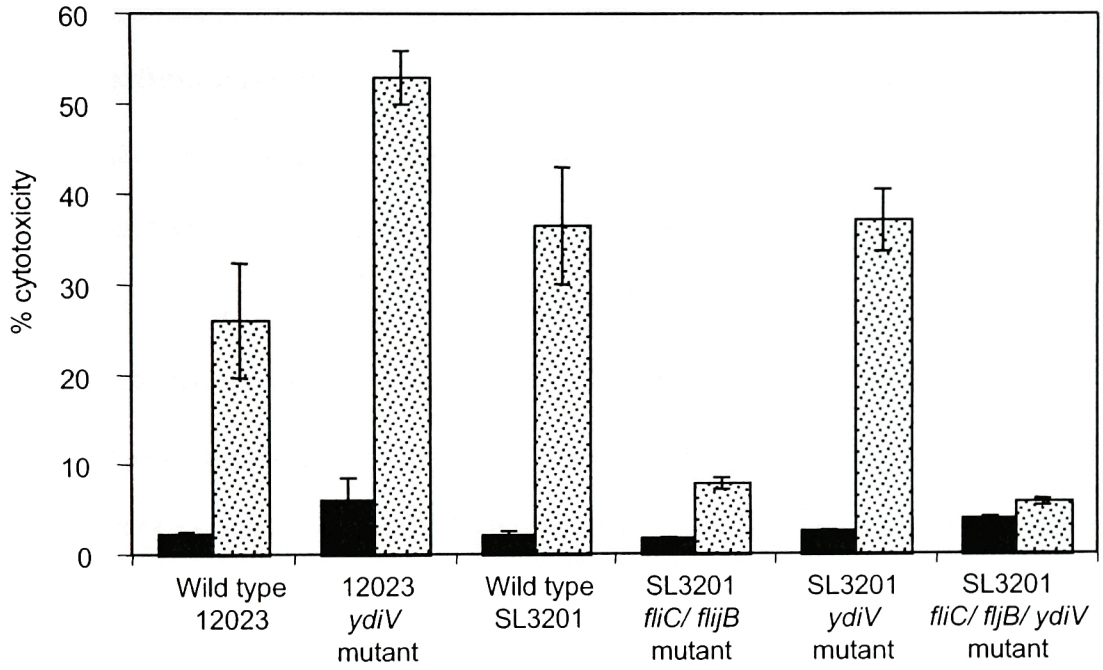


Figure 33. Rapid *Salmonella*-induced macrophage death requires expression of the genes encoding flagellin, *fliC* and *fljB*. Macrophages infected with late log phase cultures of *S. typhimurium* disrupted in *ydiV* and/or *fliC* & *fljB*. Values are means for triplicates +/- standard deviations. Results are representative of two independent experiments.

Although flagellin did not trigger macrophage death through the actions of MyD88 and thus probably not through TLR5, other macrophage receptors might recognize flagellin. To determine if the crucial toxic factor absent from cultures of the complemented strain was a secreted factor, such as secreted flagellin, gentamicin-treated supernatants from macrophages infected with the *ydiV* mutant and gentamicin-treated cultures of the *ydiV* mutant were added to macrophages with or without infection by the complemented mutant. Addition of sterile supernatants did not mediate killing of macrophages in the presence or absence of the co-infection (Fig. 34).

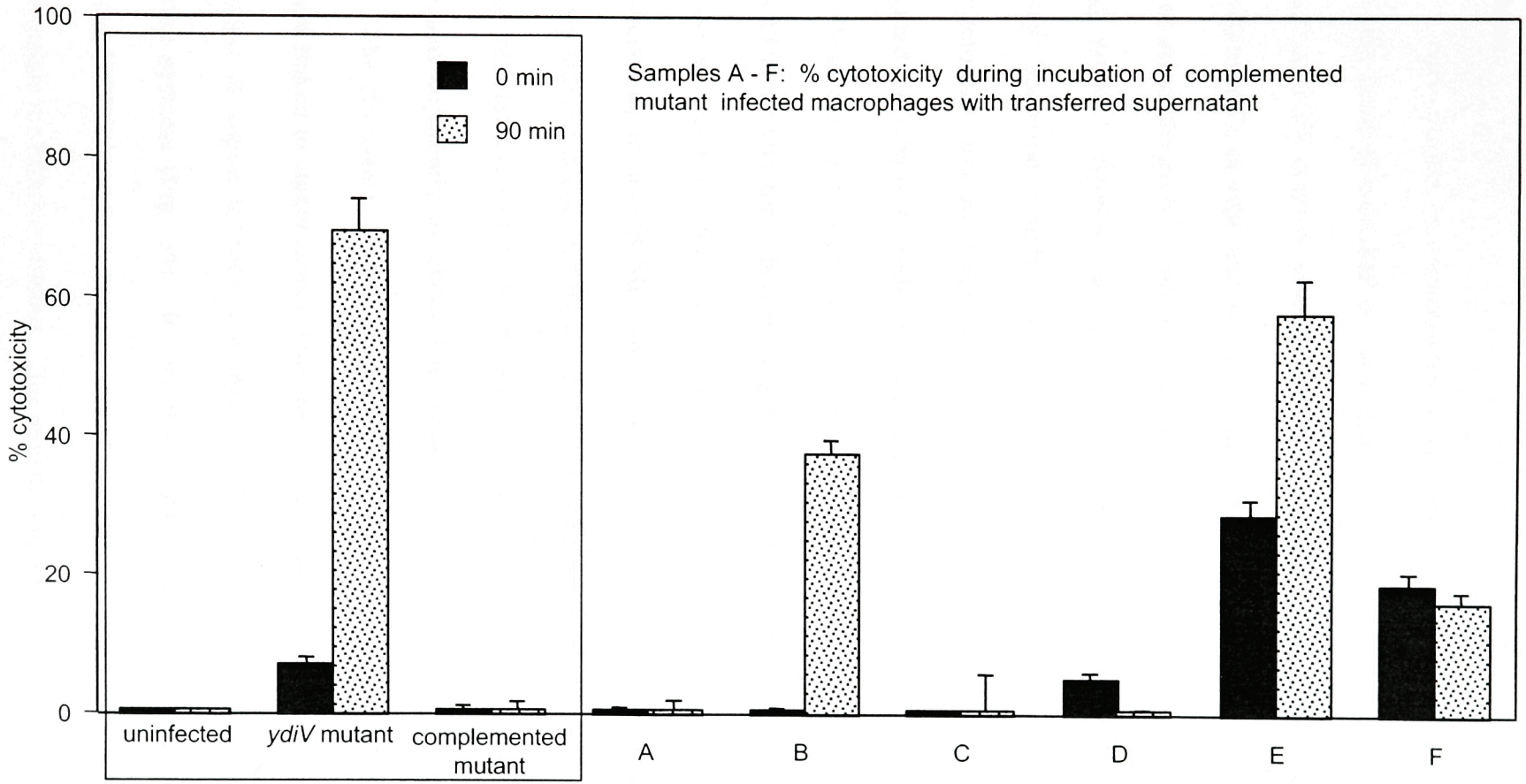
Several studies indicate that when secretion by either the flagellar basal body or SPI-1 TTSS is disrupted, secretion by the other system is also dysregulated (Eichelberg and Galán, 2000; Iyoda *et al.*, 2001; Komoriya *et al.*, 1999). Analysis of proteins secreted by *S. typhimurium* grown under aeraobic conditions in LB medium revealed that all proteins detected were either products of flagellar genes or secreted SPI-1 effector, including SipB (Komoriya *et al.*, 1999). Moreover, when individual genes encoding flagellar components were mutated, changes occured not only in secretion of the specific flagellar gene products, but also in secretion of the SPI-1 effector proteins (Komoriya *et al.*, 1999). Thus, mutations in the genes encoding flagellin were reported to dysregulate SipB secretion; however, silver-stained SDS-PAGE indicated that *fliC⁻fljB⁻ S. typhimurium* is competent to secrete SipB (Fig. 31), further verifying that SipB is necessary but not sufficient for rapid *Salmonella*-induced macrophage death.

Figure 34. Supernatants from *ydiV* mutant *S. typhimurium* or from *ydiV* mutant infected macrophages do not contain a soluble factor that induces rapid cell death. Wells donating supernatants contained either 200 µl of KRPG only (A, B, C) or $5x10^5$ macrophages in 200 µl of KRPG (D, E, F). Late log phase *ydiV* mutant *S. typhimurium* (10^6 cfu) was added to wells B, C, E, and F, and plates were centrifuged at 250xg for 5 min at room temperature. After incubation at 37°C for 30 min, gentamicin was added to wells A, C, D, and F at a final concentration of 250 µg/ml. Plates were returned to 37°C for another 30 min, and then 100 µl of supernatants were transferred to recipient wells. Prior to receiving supernatants, recipient wells containing $5x10^5$ macrophages in 100 µl of KRPG were infected with 10^6 cfu of complemented mutant *S. typhimurium*, centrifuged at 250xg for 5 min at room temperature, and incubated at 37°C for 10 min to permit ingestion of bacteria. After 10 minutes, 100 ml of donor supernatants were added wells, the time 0 time point was harvested, and gentamicin was added to the 90 min time point wells at a final concentration of 10 µg/ml. Plates were incubated at 37°C for 90 min, followed by harvest of supernatants.

Sample A = toxicity to macrophages of high dose gentamicin
Sample B = control for C
Sample C = tests whether live bacteria secrete a toxic molecule into the media
Sample D = toxicity to macrophages of high dose gentamicin
Sample E = LIVE bacteria; tests whether macrophages induce bacteria to secrete a toxic factor into the supernatant, or vice versa. Because supernatants are transferred from wells that had contained live bacteria and macrophages, LDH is transferred and is therefore present at 0 min.
Sample F = NO live bacteria; tests whether macrophages induce bacteria to secrete a toxic factor into the supernatant, or vice versa. The LDH present in these well represent LDH transferred from donor wells. However, because gentamicin was added before transfer of the supernatant, only dead bacteria were added, and no further LDH was released. Thus, the amount of LDH is these wells is the same at 0 and 90 min.

Values are means for triplicates +/- standard deviations.

Figure 34.



Supernatant from wells containing:	A	B	C	D	E	F
ydiV mutant		X	X		X	X
gentamicin	X		X	X		X
macrophages				X	X	X

Discussion

S. typhimurium, an enterobacterium pathogenic for humans and mice, contains c-diGMP; the level of c-diGMP is controlled by YdiV, a protein comprised of an EAL domain; and YdiV controls diverse aspects of the host-pathogen interaction, including resistance to H_2O_2 in vitro and to phox in mice; motility and synthesis of flagellin; and killing of macrophages by a process dependent on SipB, flagellin and additional factor(s). Lacking *ydiV, S. typhimurium* killed macrophages with extraordinary speed and efficiency. Secretion of SipB by the YdiV over-expressing complemented mutant, which lacks cytotoxicity towards macrophages, indicates that other factors in addition to SipB are required for *Salmonella*-induced macrophage death.

These are the first results providing evidence that the EAL domain alone may possess PDE activity for c-diGMP, and that modulation of c-diGMP contributes to the regulation of virulence mechanisms in addition to those that rely on cellulose synthesis. YdiV likely encodes a c-diGMP PDE or the catalytic subunit of a c-diGMP PDE whose regulatory subunit is encoded by another gene. The potential involvement of YdiV in depleting cellular levels of the regulatory molecule c-diGMP helps explain the seemingly diverse and apparently unrelated phenotypes observed for the *S. typhimurium ydiV* mutant. The abundance of EAL and GGDEF domains throughout eubacteria, many of which are linked to signal sensor domains, suggest that synthesis of c-diGMP acts as a mechanism of signal transduction that complements the classical two-component regulatory systems (Fig. 35). Rather than regulating phosphorylation of response regulators, GGDEF and EAL domains would modulate levels of c-diGMP, which would in turn regulate the effector proteins. This model, supported by the presence of multiple

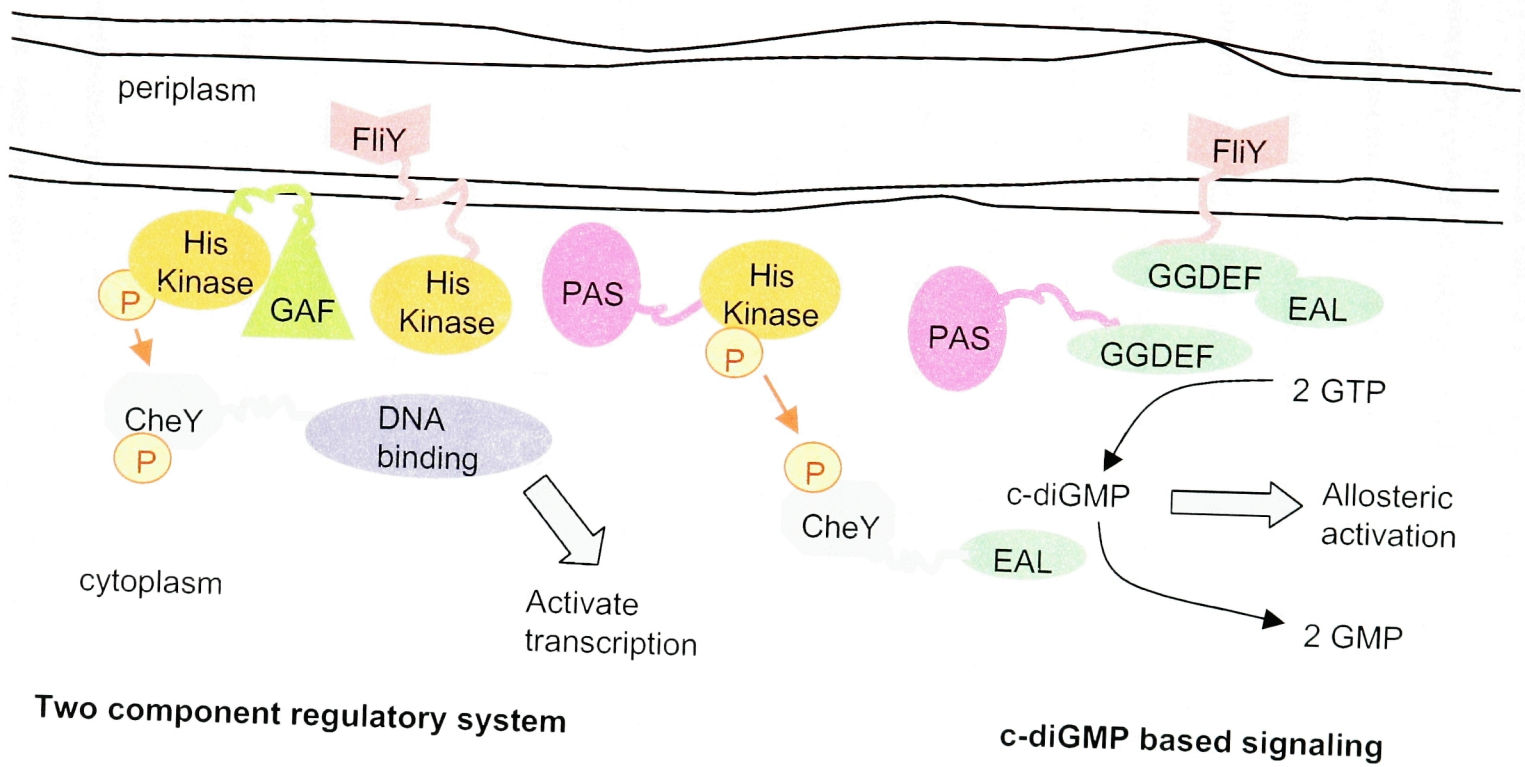


Figure 35. Diverse prokaryotic signaling domains: model for a c-diGMP-based signaling system that complements the role of the classical two component phospho-relay system. Signal sensor domains, including PAS (heme binding), GAF (cGMP binding, photopigment binding) and FliY (amino acid binding) are found associated with histidine kinases, but also with GGDEF and EAL domains, suggesting two independent mechanisms for signal transduction. In addition, CheY, a phosphoacceptor, is found associated with effector domains (like DNA binding domains) as well as GGDEF and EAL domains, suggesting overlap between the two pathways.

EAL and GGDEF domains within single genomes, suggests that there are microenvironments and/or regulons regulated by c-diGMP within bacteria, as are regulated by cAMP in eukaryotic cells (Rich *et al.*, 2001). The EAL domain encoded by *ydiV* represents one of 18 EAL domains present in the *S. typhimurium* genome; the phenotypes of the *ydiV* mutant illustrate that other EAL domains cannot compensate for the absence of YdiV, further supporting the idea that c-diGMP does not freely diffuse throughout the cell, but rather acts as a signaling molecule relaying information between specific proteins.

The crystal structure of c-diGMP indicates that 2 c-diGMP molecules stack together to form a cage-like dimer held together by a chelated divalent cation, with the 4 guanine backbones lying parallel to one another (Liaw *et al.*, 1990). Currently, allosteric activation of cellulose synthase is the only function described for c-diGMP (Ross *et al.*, 1990; Ross *et al.*, 1991), although the precise molecular interactions have not been determined. Considering that EAL and GGDEF domains are found in genes identified by their roles in virulence and motility, it is likely that c-diGMP can act as an activator for other proteins besides cellulose synthase. Alternatively, one study demonstrated that synthetic cyclic dinucleotides can act as competitive inhibitors of RNA polymerase (Hsu and Dennis, 1982), suggesting that direct disruption of transcription could be another mechanism by which c-diGMP mediates regulatory effects.

Salmonella-induced macrophage death

We were initially surprised to discover that macrophage monolayers were depleted after infection with *S. typhimurium* because previously published experiments

from our laboratory have demonstrated that macrophages both control and kill intracellular *S. typhimurium*. In these experiments, macrophages incubated with *S. typhimurium* over the course of 5 hours did not demonstrate any signs of cytotoxicity (Shiloh *et al.*, 1997; Shiloh *et al.*, 1999). The difference between these earlier experiments and our current experiments is the preparation of the inocula: previous experiments used early log phase or stationary phase cultures, while current studies were performed with late log phase cultures. We chose to grow the inocula to late log phase because expression studies had demonstrated peak *ydiV* expression occurring at this time.

Our observation that *S. typhimurium* kills murine macrophages is consistent with reports of *Salmonella*-induced cytotoxicity in the literature. The studies by Galan (Chen *et al.*, 1996b; Monack *et al.*, 1996), Falkow (Monack *et al.*, 1996), Baccarini(Lundberg *et al.*, 1999), and Zychlinsky (Hersh *et al.*, 1999) all use bacteria grown to late log phase, a condition which induces expression of SPI-1. The delayed killing of macrophages by *S. typhimurium* described by Heffron's group resulted from infection of macrophages with overnight cultures of bacteria grown to stationary phase (Lindgren *et al.*, 1996). Not surprisingly, the influence of bacterial growth phase on interactions between pathogens and macrophages has been observed with other species as well. If *L. pneumophila* is grown to post-exponential phase, bacteria-containing phagosomes isolate from the endocytic network during macrophage infection. However, if exponential-phase *L. pneumophila* is used to infect macrophages, bacteria end up in phagolysosmes that stain positively for LAMP-1 and cathepsin D (Joshi *et al.*, 2001).

Studies by Baccarini's group (Lundberg *et al.*, 1999) included the most rapid demonstration of *Salmonella*-induced macrophage cytotoxicity published to date: almost

complete death of macrophages by 90 minutes post infection with an MOI of 25:1. This rapid and efficient cytotoxicity is comparable to the killing we see using *ydiV* mutant *S. typhimurium*, and more extreme than what we observe with our wild type *S. typhimurium*. The "wild type" strain used in the Lundberg *et al.* study was LT2, a strain we have shown does not express *ydiV*. Therefore, LT2 is a functional *ydiV* mutant, providing a possible explanation for the rapid and efficient cytotoxicity observed in the study by Lundberg *et al.*

It is also possible that variability in efficiency of killing and elapsed time until maximum death observed in different laboratories can be explained by differences in the method of determination of "death". Monack, *et al.* (2001) demonstrate still frames from video microscopy indicating that macrophages show changes in morphology by 20 minutes after infection, and manifest alterations consistent with programmed death (including cell shrinkage, vacuolization and membrane blebbing) by 45 minutes after infection. The authors used inability to exclude a vital dye to measure death within the entire population of macrophages. This assay does not detect macrophages that have already died and completely disintegrated, and therefore results in an under-estimation of cell death. In fact, all assays that rely upon detection of dying cells (as opposed to a dead ones) such as DNA fragment staining, exclusion of vital dyes, and Annexin 5 staining will miss cells that have already died and broken apart completely. Baccarini's group evaluated cytotoxicity using the same assay used in our studies: LDH release. Evaluation of LDH release reflects cumulative death rather than a snapshot of dying cells, and is therefore a more reliable indicator of cytotoxicity that occurs very rapidly.

Although it appears that the groups of Falkow, Galan, Baccarini, and Zychlinsky are all observing the same pathogen-induced cytotoxic phenomenon that we have described, there are some details upon which there are still discrepancies. Studies by Galan's group indicated that cytochalasin D had no effect on cytotoxicity (Chen *et al.*, 1996b), whereas our studies and those of Falkow's group (Monack *et al.*, 1996) demonstrated that inhibition of actin by cytochalasin D halts *Salmonella*-induced macrophage death. There are several potential explanations for the requirement of actin cytoskeleton rearrangement during the process of Salmonella-induced macrophage death. Phagocytosis of *S. typhimurium* may be required; either to trigger signaling cascades in the macrophage, or simply to increase the area of contact between the bacterium and the macrophage. Likewise, phagocytosis may not be necessary so much as zippering, a phenomenon of close membrane communication between the bacterium and the host cell that creates a large surface for molecular interactions. In the case of rapid *Salmonella*-induced death, which appears to require the translocation of SipB into the cytosol of host cells, a large amount of membrane contact may be required for the mobilization of sufficient bacterial secretion systems, even if internalization of the bacteria is not required. Actin's involvement, however, may not occur during the delivery of the death signal; rather, actin may be required for the process of what appears to be catastrophic disintegration of the cell. The nature of macrophage death induced by *Salmonella* has been characterized extensively, and, although the process resembles necrosis (Brennan and Cookson, 2000; Watson *et al.*, 2000), macrophages are not passive participants: inhibition of macrophage caspase-1 prevents death (Hersh *et al.*, 1999). It is possible that actin rearrangement may also help mediate this necrosis-like process.

It is also unclear whether the activation state of the macrophage influences the extent of cytotoxicity. IFN-γ-activated RAW264.7 cells are highly susceptible to killing, but so are thioglycolate-elicited peritoneal macrophages (partially activated cells) and bone marrow macrophages (unactivated cells) (Chen *et al.*, 1996b; Monack *et al.*, 1996). As far as we can tell, our studies provide the only head to head comparison of IFN-γ–activated macrophages versus unactivated macrophages, and our results indicated that activation did not significantly impact *Salmonella*-induced macrophage death. However, periodate-elicited macrophages, used in our assays, are already relatively activated cells. Naive populations, such as resident peritoneal cells or bone marrow macrophages will need to be used to accurately evaluate the role of macrophage activation in resistance to *Salmonella*-induced macrophage death.

Finally, the data concerning the influence of bacterial opsonization on cytotoxicity are also inconsistent. Falkow's group found that serum-opsonized bacteria are less toxic (Monack *et al.*, 1996). A study using a human macrophage cell line also found that opsonized bacteria are less capable of killing macrophages (Forsberg *et al.*, 2003). We found the opposite to be true: opsonized and non-opsonized *S. typhimurium* both kill, but serum-opsonized bacteria do so more rapidly.

These inconsistencies between results may be the outcome of the slightly different experimental setups employed by the different laboratories. Unactivated bone marrow cells, macrophage cell lines, and elicited macrophages are physiologically different cells, and are likely to demonstrate a range of susceptibilities to pathogen-induced death. Moreover, "wild type" strains of *S. typhimurium* are not genetically identical. The

multiplicity of infection and the length of time allowed for infection also vary considerably between the different assays.

Flagellin and Salmonella-induced macrophage death

This is the first report demonstrating a role for the expression of flagellin in *Salmonella*-induced macrophage death. Multiple theories can be postulated that account for the dual requirements of *sipB* and *fliC/ fljB* expression for cytotoxicity. The role of motility as a factor in *S. typhimurium*'s ability to kill cannot be ruled out. Ideally, a mutant with paralyzed flagellin would be tested to address this point; however, mutations in the *mot* genes that control the motor of flagellin lead to dysregulation of expression of other flagellar genes, thus confounding interpretation of potential mutant phenotypes (Komoriya *et al.*, 1999). Centrifugation of culture plates containing both adherent macrophages and free-floating bacteria has been shown to effectively colocalize amotile bacteria with the macrophage monolayer (Tomita and Kanegasaki, 1982). From our own work, we have observed that, following centrifugation and 10 minutes of phagocytosis, the number of bacteria ingested by macrophages was equivalent for wild type bacteria, the *ydiV* mutant, and the complemented mutant (data not shown). Thus, the complemented mutant's defect in cytotoxicity is not due to an inability to penetrate the macrophage monolayer, despite a lack of flagellin.

Although MyD88 is not required for *Salmonella*-induced macrophage death, flagellin may convey death signals through another receptor, which couples with SipB activity to kill the macrophage. Alternatively, disruption of the genes encoding flagellin may alter expression of an unknown molecule that functions with SipB to induce

macrophage death. SipB has been shown to form a pore in eukaryotic cell membranes (Hayward *et al.*, 2000; Scherer *et al.*, 2000), and this unknown protein might be translocated through SipB into the macrophage cytosol where it could induce macrophage death.

Another possibility is that flagella secrete and/or translocate SipB. It has been shown for other gram negative enteric bacteria that some virulence factors are secreted by the flagellar TTSS apparatus (Young *et al.*, 1999a). Additionally, Lee and Galan recently demonstrated that the default pathway for *S. typhimurium* SPI-1 effectors that cannot bind their cognate SPI-1 chaperones is secretion through the flagellar apparatus (Lee and Galán, 2004). Wild type SipB may be differentially secreted by two different TTSSs, resulting in two different effector functions. When SipB is secreted through the SPI-1 needle apparatus, it acts as a translocon to facilitate delivery of SPI-1 effectors required for invasion; however, SipB may be secreted by the flagellar TTSS, thus inducing macrophage death.

Model for YdiV's contributions to virulence

We propose that YdiV acts as a PDE for c-diGMP, which in turn acts as an activator or repressor for diverse functions including anti-oxidant defense and the expression of flagellin. Disruption of *ydiV* results in accumulation of c-diGMP and dysregulation of the YdiV regulon. The *in vivo* phenotype of the *ydiV* mutant in this study is probably due mostly, if not entirely, to its deficit in anti-oxidant defense, as demonstrated by reversal of attenuation of the *ydiV* mutant in phox-deficient mice. Although YdiV plays a key role in mediating *Salmonella*-induced macrophage death, this

function probably does not contribute to the *in vivo* phenotype of the *ydiV* mutant. SPI-1 mutants are not attenuated when infected i.p. (Galán and Curtiss, 1989), suggesting that SPI-1 is not required after translocation from the gut into the blood; moreover, caspase-1-deficient mice demonstrate no difference in susceptibility to *S. typhimurium* when compared to wild type mice after i.p. infection (Monack *et al.*, 2000). Thus, SipB-dependent macrophage death probably occurs solely in macrophages recruited to Peyer's patches. Infection of mice by the oral route might reveal different phenotypes for the *ydiV* mutant and the complemented mutant. The complemented mutant, which cannot induce macrophage death, may be unable to cause systemic infection after oral inoculation.

YdiV's regulation of flagellin could affect virulence by a mechanism independent of *S. typhimurium*'s ability to kill macrophages. *S. typhimurium* mutants lacking flagella are equally effective at killing mice after oral infection as wild type strains (Lockman and Curtiss, 1990). In fact, *flhD* mutants are slightly more virulent than wild type strains when administered orally (Schmitt *et al.*, 2001). The increased virulence of the *flhD* mutant does not appear to be due to enhanced invasion of the gut, but rather due to the mutant's increased proliferation in macrophages in the spleen and liver. Yet, flagellin is repressed in wild type *S. typhimurium* after ingestion by macrophages *in vitro* (Eriksson *et al.*, 2003). When *S. typhimurium* is engineered to constitutively express flagellin, virulence is dependent on the specific flagellin monomer that is expressed: FliC-expressing strains retain virulence, but FljB-expressing strains fail to proliferate in the spleen (Ikeda *et al.*, 2001). The role of flagellin in pathogenesis during *S. typhimurium*

infection of mice will require further clarification before we can postulate that dysregulation of flagellin contributes to attenuation of the *ydiV* mutant.

Chapter 5

Some conclusions about pathogenesis of intracellular bacteria

The 5 genes identified in this study represent diverse categories of bacterial activities: modification of extracellular surface moieties (Rv*2958c*), production of bioactive compounds (*pks6*), recognition of changes in the environment and/or uptake of nutrients (Rv*0072*, *pstA1*), and regulation of the concentration of a small intracellular signaling molecule (*ydiV*). At the outset of this study, we had hoped that we might elucidate strategies for enhanced survival within the macrophage that were common to *M. tuberculosis* and *S. typhimurium*. Although no one gene or pathway was detected in both differential STM screens, some of the genes identified have homologs in both species.

Rv*0405* (*pks6*) appears to be the only *cim* gene with no counterpart in *S. typhimurium*. In fact there are no polyketide synthases encoded by *S. typhimurium*. Rv*2958c* has no close homologs in *S. typhimurium*, however *S. typhimurium* does encode glycosyl transferases, particularly *iroB*, which is part of an iron-regulatory operon (Hantke *et al.*, 2003), and *murG*, which involved in peptidoglycan synthesis (van den Brink-van der Laan *et al.*, 2003). These genes, however, probably do not represent counterparts of Rv*2958c* in *S. typhimurium*. In addition to not demonstrating strong similarity to these two genes, Rv*2958c* is found on the chromosome in the proximity of *pks1/15* and is thought to function with Pks1/15 in the synthesis of phenolglycolipids. Thus, the exact mechanism by which Rv2958c contributes to *M. tuberculosis*'s ability to

counter the effects of IFN-γ *in vivo* is probably not shared by *S. typhimurium*, which does not encode polyketide synthases.

The high-affinity phosphate and glutamine transporters to which Rv*0930* (*pstA1*) and Rv*0072* contribute respectively not only have homologs in *S. typhimurium*, but they have also been shown to contribute to pathogenesis of *S. typhimurium in vivo* (Klose and Mekalanos, 1997; Lucas *et al.*, 2000). Interestingly, the studies with *S. typhimurium* have implicated both the glutamine transporter and the Pst phosphate system as being important for signaling changes in the environment of the bacterium, rather than mediating uptake of nutrients. The *in vivo* requirement for the two transporters in both *S. typhimurium* and *M. tuberculosis* suggests that the macrophage phagosome likely contains low concentrations of glutamine and phosphate. It is worth noting that a third shared nutrient transporter, the MgtC magnesium transporter, was found to be required by both *S. typhimurium* and *M. tuberculosis* for growth in macrophages and in low concentrations of magnesium *in vitro* (Buchmeier *et al.*, 2000). These results suggest that magnesium is limited in the phagosome of the macrophage, and that uptake of magnesium by the MgtC transporter is required for growth of both bacteria when magnesium is limited. Further studies will be required to determine the true roles of the glutamine and phosphate transporters. Perhaps restriction of phosphate and glutamine *in vivo* can effectively starve *S. typhimurium* and *M. tuberculosis*, or perhaps the bacteria have merely evolved to recognize low concentration of these nutrients as signals to up-regulate particular virulence factors.

The *M. tuberculosis* genome contains EAL and GGDEF domain-containing proteins; however, unlike *S. typhimurium*, *M. tuberculosis* encodes only two. Rv*1354c*

includes an EAL domain, a GGDEF domain, and a GAF domain, while Rv*1357c* contains only an EAL domain. It is intriguing that the only 2 genes encoding EAL/GGDEF domain-containing proteins in the *M. tuberculosis* genome are found in such close proximity to each other (within 5000 nucleotides), suggesting that the gene products may function together to regulate c-diGMP. Most of the genes at this locus have not been assigned function, including Rv*1354c* and Rv*1357c*. Rv*1355* was annotated *moeY*, due to weak similarity to a molybdopterin biosynthesis protein. As our understanding of the role of c-diGMP in pathogenic bacteria expands, perhaps we will gain insight into the roles of these two *M. tuberculosis* genes.

M. tuberculosis and *S. typhimurium* both possess the ability to maintain chronic infections, residing for long periods of time within host macrophages. The interaction, therefore, between the bacterium and the host is most likely a constantly changing one, evolving as the immune response develops over time. The identification of genes that are involved with modulation of bacterial function and are required for countering specific components of the host immune response supports the idea that regulatory circuits are a critical component of the bacterium's ability to cause chronic infection. The putative and demonstrated functions of the 5 gene products detected in these differential STM screens point to novel, potentially widespread, bacterial pathways for gauging and responding to host immune pressures, whose roles in virulence are not yet fully appreciated.

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