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Isolation and characterization of two genetic loci from the intracellular pathogen *Francisella novicida*

by

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biochemistry and Microbiology

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ABSTRACT

Francisella novicida is a facultative intracellular pathogen capable of growing in macrophages. A spontaneous mutant of *F. novicida* defective for growth in macrophages was isolated on LB media containing the chromogenic phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (X-p) and designated GB2. Using an *in cis* complementation strategy, four strains were isolated which are restored for growth in macrophages. A locus isolated from one of these strains complements GB2 for the intracellular growth defect, colony morphology on LB (X-p) media, and virulence in mice. The locus consists of an apparent operon of two genes, designated *mglAB*, for *m*acrophage *g*rowth *l*ocus. Both *mglA* and *mglB* transposon insertion mutants are defective for intracellular growth and have a phenotype similar to GB2 on LB (X-p) media. Sequencing of *mglA* cloned from GB2 identified a missense mutation, providing evidence that both *mglA* and *mglB* are required for the intramacrophage growth of *F. novicida*. Preliminary studies have also identified a convergently transcribed gene, tentatively designated *mglC*, immediately downstream of *mglB*. *mglC* null mutants are defective for intracellular growth and show the same phenotype on LB (X-p) agar as GB2. *mglB* expression in GB2 was confirmed using antiserum against recombinant MglB. Western blot analysis revealed the

absence of MglA in an *mglB* null mutant, indicating MglB may influence MglA levels. Analysis of the regulation of *mglA* expression during growth in broth culture shows a decrease in expression upon entering late log-early stationary phase. *mglA* is also expressed during culture in macrophages. Cell fractionation studies revealed several differences in the protein profiles of *mgl* mutants compared with wild-type *F. novicida*, most notably the absence of a 70 kDa secreted protein. A candidate clone for the gene encoding this 70 kDa protein has been isolated. The deduced amino acid sequences of *mglA* and *mglB* show similarity to the SspA and SspB proteins of *Escherichia coli* and *Haemophilus* spp. In *E. coli*, SspA and/or SspB influence the levels of multiple proteins under conditions of nutritional stress, and SspA can associate with the RNA polymerase holoenzyme. Taken together, these observations suggest that in *Francisella* MglA and MglB may control the expression of genes whose products contribute to survival and growth within macrophages. Roles for the putative MglC and possibly the 70 kDa secreted protein in this activity are also indicated.

Acid phosphatases capable of inhibiting the respiratory burst of neutrophils have been identified in certain intracellular pathogens. The gene encoding AcpA, a respiratory burst-inhibiting acid phosphatase of *Francisella*, was cloned and sequenced. The deduced amino acid sequence of AcpA showed limited similarity to phospholipase C proteins present in *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*. An *F. novicida acpA* null mutant was

found to exhibit wild-type growth kinetics in both cell-line and inflammatory mouse macrophages as well as remaining virulent for mice. These data suggest that AcpA is not essential for the intracellular growth or virulence of *F. novicida*, and that any role it may play in virulence is subtle.

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ABC	ATP-binding cassette
ACP	acid phosphatase
Ap	ampicillin
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pairs
CAT	chloramphenicol acetyltransferase
Cb	carbenicillin
CFE	cell-free extract
cfu	colony forming units
CHA-B	cystine heart agar with horse blood
Cm	chloramphenicol
CoA	coenzyme A
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
dTTP	deoxythymidine triphosphate
ELISA	enzyme-linked immunosorbant assay
Em	erythromycin
FKBP	FK506-binding protein
fMLP	*N*-formyl-methionyl-leucyl-phenylalanine

GKO	gamma interferon knockout
GTP	guanosine triphosphate
h	hour(s)
id	intradermal
IFN	interferon
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
in	intranasal
ip	intraperitoneal
IPTG	isopropyl β-D-thiogalactopyranoside
IP_3	inositol 1,4,5-trisphosphate
iv	intravenous
kb	kilobases or kilobase pairs
kDa	kilodalton
Km	kanamycin
LAMP	lysosome-associated membrane glycoprotein
LAP	lysosomal acid phosphatase
LB	Luria-Bertani
LD_{50}	50% lethal dose
LVS	live vaccine strain
LPS	lipopolysaccharide
mCi	millicurie
MES	2-[N-morpholino]ethanesulfonic acid
min	minute(s)

μM	micromolar
mM	millimolar
mmol	millimole
M6PR	mannose 6-phosphate receptor
MUP	4-methylumbelliferylphosphate
NK	natural killer
NMMA	N^G-monomethyl-L-arginine
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
PM	polymyxin B
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocyte
PMSF	phenylmethylsulfonyl fluoride
pNPPC	p-nitrophenylphosphorylcholine
PPIase	peptidyl-prolyl *cis-trans* isomerase
PTPase	protein tyrosine phosphatase
rDNA	ribosomal deoxyribonucleic acid
s	seconds
sc	subcutaneous
scid	severe combined immunodeficient
SCV	*Salmonella*-containing vacuole

SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP	spacious phagosome
SPI	*Salmonella* pathogenicity island
TBS-T	Tris-buffered saline with Tween-20
TCR	T cell receptor
TDL	thoracic duct lymphocyte
TNF	tumor necrosis factor
Tris	Tris[hydroxymethyl]aminomethane
TSB-C	tryptic soy broth with cysteine
X-p	5-bromo-4-chloro-3-indolyl phosphate

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INTRACELLULAR PATHOGENS

Host organisms possess many defense mechanisms designed to resist infection. Primary barriers to infection are both physical and chemical in nature. Physical barriers such as skin and mucous membranes, which are generally impermeable to most infectious agents. Both are constantly sloughing cells from the surface, and in the mucous membrane, produce a mucous layer to trap foreign particles and block adherence to epithelial cells. Other inhibitory factors of the skin include low pH and dryness. Availability of essential nutrients, such as iron, is limited at certain locations by high affinity iron binding proteins. Also, a normal microbial flora exists at many locations which can block potential colonization sites and compete for nutrients. Secondary barriers are encountered on entry into the blood or underlying tissues and include complement and professional phagocytic cells (e.g. neutrophils, macrophages). At later time points, products are generated by effectors of a specific acquired immune response (activated macrophages, lymphocytes).

Successful pathogenic microbes have the capacity to infect a host organism, often by overcoming epithelial barriers to infection, becoming localized in an environment suitable to support replication, and proliferating in this environment prior to transmission of the amplified microorganisms to new hosts. One class of bacterial pathogens, intracellular pathogens, accomplish these tasks by entering into, and surviving within, eukaryotic cells. The

intracellular environment provides several advantages to the invading microbe. Invasion of epithelial cells can allow pathogens to cross epithelial barriers. The host cell provides a potential source of nutrients to support replication in the absence of competition with normal flora. Within the host cell, the bacterium gains some protection from the innate and acquired immune responses in the extracellular environment. Finally, infection of some cells may facilitate the spread of the organism to new host tissues.

An intracellular lifestyle does not come without complications. Both non-professional and professional phagocytic cells (macrophages) can serve as hosts to intracellular pathogens. However, growth within non-professional phagocytic cells requires the bacteria to have mechanisms to induce their own uptake by these host cells. In both host cell types, the invading microbe must possess strategies to deal with the potential consequences of phagosome-lysosome fusion, which could result in exposure to a number of toxic substances including hydrolytic enzymes (lysozyme, proteases, glycosidases, lipases), small cationic peptides (e.g. defensins), and lactoferrin (Moulder, 1985). Phagocytosis by macrophages is associated with the production of other antimicrobial molecules called reactive oxygen intermediates, and survival and replication within these cells requires additional specialized mechanisms.

The strategies that intracellular pathogens have evolved to permit survival and growth within eukaryotic cells can be broadly classified

into the following three categories: 1) escape from the phagosome prior to phagosome-lysosome fusion; 2) adaptation to survival within the phagolysosome; and 3) inhibition of phagosome-lysosome fusion. Bacteria in the first category replicate in the nutrient-rich environment of the cytoplasm and spread intercellularly to new host cells, thereby avoiding an extracellular phase. Pathogens in the latter two categories replicate within specialized vacuoles resulting from altered interactions with normal phagocytic pathways. This review will summarize the virulence factors contributing to the intracellular parasitism of one of the most well-studied model intracellular pathogens, *Salmonella enterica*.

Salmonella

INTRODUCTION

Salmonella enterica is recognized as a pathogen of a wide variety mammals from humans to mice. Infection can be manifested in the form of a self-limiting gastroenteritis, enteric fever, or a severe systemic infection known as typhoid fever. Many *Salmonella* serovars exhibit a high degree of host specificity. Hence, the type of disease resulting from infection is dependent on the species of the infected host and/or the serovar of the infecting bacteria. For example, *S. enterica* serovar *typhi* (*S. typhi*) specifically infects humans to cause typhoid fever. *S. enterica* serovar *typhimurium* (*S. typhimurium*) causes a typhoid-like disease in mice but self-limiting gastroenteritis in humans.

S. typhimurium infection of mice has been used extensively as a model to study *S. typhi* pathogenesis. *Salmonella* infection is initiated by ingestion of contaminated food or water. Bacteria breach the intestinal epithelial barrier by adhering to and invading the M cells of Peyer's patches (Jones *et al.*, 1994). M cell invasion leads to cell death within 60 minutes of addition of bacteria to ligated ileal loops. Two hours post-infection, bacteria are observed within adjacent enterocytes and penetrating the underlying follicle dome where they are phagocytosed by macrophages. Replication within these cells is followed by a bacteremia which results in the spread of

the bacteria to the spleen and liver. Salmonellae multiply to high numbers in these organs, apparently within macrophages (Richter-Dahlfors *et al.*, 1997), prior to being released into the bloodstream, resulting in septicemia and death in susceptible mice. Invasion of epithelial cells and intracellular replication are considered important factors in *Salmonella* pathogenesis, and the genes shown to contribute to these activities are the focus of the following discussion.

INVASION

Invasion of M cells in the ileal Peyer's patches is the earliest detectable interaction between *Salmonella* and host intestinal tissue (Jones *et al.*, 1994). Phenotypically, entry into M cells resembles that observed into cultured epithelial cells, and many of the host and bacterial factors involved have been identified using the tissue culture cell model. Bacterial contact with the host cell induces macropinocytosis, a process characterized by the formation of large membrane ruffles on the host cell membrane which enclose the bacteria in a membrane-bound compartment (Francis *et al.*, 1992; 1993). Membrane ruffling is associated with dramatic rearrangements of the actin cytoskeleton, which returns to normal shortly after bacterial entry.

The host cell signalling events which accompany *Salmonella* invasion include intracellular calcium and inositol phosphate fluxes, but the exact signalling pathway remains to be determined

(Ruschkowski *et al.*, 1992; Pace *et al.*, 1993). Cytoskeletal rearrangements may be mediated by affecting the activity of CDC42, one of three small guanosine triphosphate (GTP)-binding proteins involved in controlling the formation of actin-based structures, as *Salmonella* requires CDC42 for invasion (Chen *et al.*, 1996a).

Salmonella entry into macrophages by ruffling results in the induction of apoptosis in a high proportion of the host cells (Chen *et al.*, 1996b; Monack *et al.*, 1996). Other intracellular pathogens, including *Shigella* (Zychlinsky *et al.*, 1992) and *Legionella pneumophila* (Müller *et al.*, 1996), also induce apoptosis in macrophages. Mutants defective for invasion of epithelial cells invade a murine monocyte-macrophage cell line (RAW264.7) 7- to 10-fold less efficiently than wild-type *S. typhimurium* (Monack *et al.*, 1996). Invasive strains defective for intramacrophage growth retain the ability to induce apoptosis, indicating intracellular replication is not necessary for cytotoxicity. Reports are conflicting as to whether bacterial uptake is necessary to induce apoptosis. Although *S. typhimurium* has recently been shown to induce apoptosis in phagocytes of infected mice (Richter-Dahlfors *et al.*, 1997), the role of this activity in the interaction of *Salmonella* with macrophages is unclear as a non-invasive mutant (BJ66) is still capable of replicating in macrophages (Monack *et al.*, 1996) and resides in an intracellular compartment identical to that occupied by a wild-type strain (Rathman *et al.*, 1997). In addition to invasion-associated genes, genes regulated by the OmpR/EnvZ two-component regulatory

system are also involved in induction of cytotoxicity as *ompR* mutants fail to induce apoptosis in macrophages (Lindgren *et al.*, 1996).

Salmonella-induced modifications to host cell signalling pathways are predicted to be carried out by a set of effector proteins translocated to the surface of the bacterium or into the host cell by a type III or contact-dependent protein secretion system (Galan, 1996). The genetic loci encoding the secretory apparatus, secreted proteins, and regulatory proteins comprising this invasion-associated system are largely clustered in a 40 kb segment at 58 to 60 min on the *Salmonella* chromosome known as *Salmonella* pathogenicity island 1 (SPI-1) (Mills *et al.*, 1995). This region constitutes one of three pathogenicity islands in *Salmonella*, which are thought to be acquired by horizontal gene transfer from other microorganisms (Groisman and Ochman, 1996). The major genetic elements involved in *Salmonella* entry into host cells are discussed below.

The secretion apparatus

Of at least 28 genes present in SPI-1, 17 have been shown or are predicted to encode components of the secretion apparatus (Galan, 1996). These components are encoded in the *spa*, *inv*, and *prg* loci and have been identified through a variety of approaches. Putative functions have been assigned based mainly on amino acid sequence similarities to proteins in other systems such as *Shigella* and *Yersinia* spp. Homologs of the *spa*, *inv*, and *prg* genes in other bacteria are

similarly grouped (Groisman and Ochman, 1993; Pegues *et al.*, 1995) and, in some cases, functionally interchangeable (Groisman and Ochman, 1993; Rosqvist *et al.*, 1995). For example, a non-invasive *S. typhimurium spaP* mutant is complemented for invasion by *spa24*, the corresponding locus in *Shigella* (Groisman and Ochman, 1993).

The first SPI-1 genes identified were isolated by complementation of a spontaneous non-invasive mutant of *S. typhimurium* with a cosmid clone (Galan and Curtiss, 1989). Analysis of subclones of the cosmid led to the identification of a group of four genes, designated *invA*, *-B*, *-C*, *-D*, which complemented the mutant strain. *invA*, *-B*, and *-C* are organized within an operon while *invD* is located downstream in an independent transcriptional unit. Both *invA* and *invC* have been shown to be required for entry into epithelial cells (Galan *et al.*, 1992; Eichelberg *et al.*, 1994). *invA* mutants exhibit reduced virulence in mice following oral infection, but retain wild-type virulence when inoculated by the intraperitoneal route (Galan and Curtiss, 1989). Using a ligated ileal loop model, Jones and Falkow (1994) reported *S. typhimurium invA* mutants are defective for invasion and destruction of M cells in the follicle-associated epithelium of murine Peyer's patches. These data suggest that invasion of host cells by *Salmonella* plays an important role in establishing infection by the oral route. This has been supported by more recent studies using non-invasive strains with mutations in other loci (Penheiter *et al.*, 1997). Analysis of the predicted secondary structure of InvA suggests it is a membrane

protein with seven transmembrane domains localized in the amino-terminal half of the protein and a hydrophilic carboxyl terminus oriented into the cytoplasm (Galan *et al.*, 1992). Cell fractionation studies and analysis of translational fusions of *invA* to alkaline phosphatase (*phoA*) are consistent with InvA being a polytopic inner membrane protein (Galan, 1996). It has been suggested that InvA may form a channel to allow transport of secreted proteins across the inner membrane. Based on sequence similarity to the β subunit of the F_0F_1 proton-translocating ATPase, InvC is suggested to energize the secretion of proteins through this type III secretion pathway (Eichelberg *et al.*, 1994). Purified InvC has been shown to exhibit ATPase activity, and this activity is essential for invasion (Eichelberg *et al.*, 1994). Both InvA and InvC are required for the proper assembly and shedding/retraction of surface appendages termed invasomes, which are formed shortly after bacterial contact with epithelial cells but disappear immediately preceding entry (Ginocchio *et al.*, 1994).

Transposon mutagenesis of loci adjacent to *invABC* resulted in the isolation of two new genes required for invasion, *invF* (described below) and *invG* (Kaniga *et al.*, 1994). The deduced sequence of InvG contains a putative signal sequence and is similar to the PulD family of protein translocases. This family includes components of type III secretion systems in other Gram-negative bacteria, such as MxiD of *Shigella flexneri*, required for the surface presentation of the Ipa proteins, and YscC of *Yersinia enterocolitica*, involved in the secretion

of the Yop proteins (Kaniga *et al.*, 1994). By analogy with the role of PulD in pullulanase secretion in *Klebsiella*, InvG is postulated to be an outer membrane protein and assist in the transport of target proteins across this membrane. Evidence of a role for InvG in protein secretion comes from the observations that *invG* mutants are defective for the secretion of at least six proteins (Penheiter *et al.*, 1997) and in the assembly of surface appendages which are induced immediately after contact with epithelial cells (Ginocchio *et al.*, 1994).

The *S. typhimurium* PhoP/Q two-component regulatory system affects the expression of several phenotypes including virulence in mice and survival in macrophages (discussed in more detail below). PhoP/Q activate and repress the expression of various genes, termed *pag* (*phoP*-activated genes) and *prg* (*phoP*-repressed genes), respectively (Miller and Mekalanos, 1990). A strain expressing a mutant PhoQ (*phoPQ*^{c}) with increased net kinase activity (PhoP^{c} phenotype) has constitutive expression of *pag* and repression of *prg*, and is attenuated for virulence in mice, survival within macrophages, and invasion of epithelial cells, suggesting *prg* are virulence genes (Miller and Mekalanos, 1990; Gunn *et al.*, 1996). Using the transposon Tn*phoA*, Behlau and Miller (1993) identified a *prg* locus (*prgH*) which contributes to mouse virulence by the oral or intraperitoneal routes and to entry into epithelial cells. The data suggest this locus plays a role in both invasion of the mucosa and survival during interactions with phagocytic cells. However,

prgH::Tn*phoA* mutants exhibit wild-type survival in macrophages. Characterization of the region adjacent to *prgH* revealed that it is in an operon of four genes designated *prgHIJK* (Pegues *et al.*, 1995). The predicted sequences of PrgH, PrgI, PrgJ, and PrgK are similar to proteins required for secretion of Ipa (invasion protein antigens) and Yop (*Yersinia* outer proteins) virulence factors. PrgH and PrgK are both predicted to be lipoproteins on the basis of potential lipoprotein processing sites in the N-termini. The *Yersinia* (YscJ) and *Shigella* (MxiJ) homologs of PrgK are membrane-associated lipoproteins (Pegues *et al.*, 1995). Homologs for PrgH and PrgJ have only been found in *Shigella* (Lee, 1997). The localization and functions of PrgI and PrgJ are unknown. A *prgH*::Tn*phoA* mutant was shown to be defective for secretion of several proteins, suggesting at least one of the proteins in the *prgHIJK* operon has a role in protein secretion.

The ability of *Salmonella* to enter mammalian cells is regulated by a number of environmental conditions including oxygen tension (Lee and Falkow, 1990), growth state (Lee and Falkow, 1990), and osmolarity (Galan and Curtiss, 1990). Jones and Falkow (1994) identified a group of invasion genes by screening for oxygen-regulated *lacZY* transcriptional fusions. One gene, *orgA*, was sequenced and characterized in more detail. Similar to the *invA* mutant phenotype, an *orgA* mutant (BJ66) is attenuated for virulence in mice (>60-fold) by the oral route of infection but remains fully virulent by the intraperitoneal route. This correlates with the inability of BJ66 to invade and destroy M cells in a murine ligated

ileal loop model, and with the observation that it is defective in the secretion of several proteins (Penheiter *et al.*, 1997). The *orgA* gene is predicted to encode a 48 kDa hydrophilic protein which lacks a signal sequence. OrgA is similar to MxiK of *Shigella* spp., and is one of five putative secretion machinery components for which homologs have been found only in *Shigella* (Lee, 1997).

Secreted proteins

Several proteins secreted by the SPI-1 type III secretion system have been identified. These proteins may participate in a variety of activities including formation of invasomes, regulation of the secretion process, translocation of secreted proteins into host cells, and execution of putative effector functions within host cells. In addition, two secreted proteins, InvJ and SpaO, are necessary for the export of all proteins known to be secreted through this system to date, suggesting they may be components of the export apparatus (Collazo *et al.*, 1995; Collazo and Galan, 1996).

Among the first secreted proteins identified were the products of the *sip* (or *ssp*) operon (Kaniga *et al.*, 1995a; Kaniga *et al.*, 1995b). This operon encodes four proteins, SipB, SipC, SipD, and SipA, which show sequence similarity to the *Shigella* IpaB, IpaC, IpaD, and IpaA proteins, respectively. The *sip* operon is located just downstream of the *spa* operon. Of the four proteins, only SipA is not essential for invasion of epithelial cells and (with the exception of *sipB* which has not been tested) full virulence in mice by the oral route (Kaniga *et*

al., 1995a; Kaniga *et al.*, 1995b; Penheiter *et al.*, 1997). SipB and SipC have recently been shown to be translocated into cultured intestinal epithelial cells (Collazo and Galan, 1997). SipB, SipC, and SipD are required for the translocation of SipB and SipC into host cells. Also, it has been proposed SipD plays a role in modulating the export of some secreted proteins as culture supernatants from a *sipD* mutant contain increased levels of SipA, SipB, and SipC (Kaniga *et al.*, 1995a) as well as SopE (see below; Hardt *et al.*, 1998). Although SipA is detected on the surface of the bacteria after infection, it is not required for the translocation process (Collazo and Galan, 1997).

A second set of secreted proteins called Sops (*Salmonella* outer proteins) were identified in a *S. dublin* double *fliM*/polar *sipB* mutant (Wood *et al.*, 1996). These proteins, designated SopA to SopE, form large filamentous aggregates in the culture medium. One protein, SopE, has been independently characterized by two groups (Wood *et al.*, 1996; Hardt *et al.*, 1998). SopE is necessary for efficient invasion of HeLa cells and Henle-407 cells during short infection times (15 min). A *sopE* mutant induces less extensive cytoskeletal rearrangements and diffuse membrane ruffles as compared to those induced by a wild-type strain (Hardt *et al.*, 1998). SopE may function as an effector of host cell responses as it is translocated into host cells by a mechanism dependent on a product(s) of the *sip* operon (Wood *et al.*, 1996) but is not essential for secretion of other targets of the *inv/spa* type III secretion system or translocation of SipC into host cells (Hardt *et al.*, 1998). Interestingly, the *sopE* gene

is only present in a small subset of *Salmonella* serovars and, in *S. typhimurium*, maps within the genome of a cryptic bacteriophage located outside SPI-1 (Hardt *et al.*, 1998).

Kaniga *et al.* (1996) identified a secreted effector protein, denoted SptP, requiring the SPI-1 type III secretion apparatus for secretion. The encoding gene, *sptP*, is located downstream of the *sip* operon in SPI-1. The deduced amino acid sequence of SptP suggests the protein has a modular structural organization. The amino-terminal region shows similarity to the toxins ExoS of *Pseudomonas aeruginosa* and YopE of *Yersinia* spp., both of which are targets of type III secretion systems and are involved in host cell damage. The carboxy-terminal region is similar to the catalytic domain found in eukaryotic tyrosine phosphatases (PTPases) and another target of the *Yersinia* type III secretion system, the tyrosine phosphatase YopH. Purified SptP exhibits tyrosine phosphatase activity using phosphorylated peptide substrates. SptP is required for full virulence in mice, but is not required for invasion of epithelial or macrophage cell lines. Very recently, SptP was shown to be translocated into host cells in a *sip*-dependent fashion (Yu and Galan, 1998). Microinjection of purified SptP into cultured epithelial cells results in disruption of the actin cytoskeleton and disappearance of stress fibers. Either the amino-terminal or carboxy-terminal domain is sufficient to effect this activity, suggesting the two putative effector domains may function independently but affect similar cellular functions.

Regulatory proteins

The regulation of *S. typhimurium* invasion is complex and modulated *in vitro* by several environmental factors. Conditions such as oxygen tension, osmolarity, bacterial growth state, and pH have all been shown to influence *Salmonella* entry into host cells (Galan and Curtiss, 1990; Lee and Falkow, 1990; Behlau and Miller, 1993). The mechanism by which these conditions are sensed in order to modify the expression of invasion genes is poorly understood. Changes in the level of DNA supercoiling can affect *inv* gene expression (Galan and Curtiss, 1990). Current data suggest a model whereby the expression of invasion genes is coordinately regulated by activating the synthesis of a cascade of transcription factors in response to appropriate environmental cues. Sub-optimal conditions for any particular environmental or regulatory factor dramatically represses invasion gene transcription (Bajaj *et al.*, 1996). Invasion gene expression is under the control of at least four factors, InvF, HilA, SirA, and PhoP/Q, whose encoding genes are located both inside and outside SPI-1.

The *invF* gene is located immediately upstream of *invG* in SPI-1 and is required for entry into cultured epithelial cells (Kaniga *et al.*, 1994). The predicted sequence identifies InvF as a member of the AraC family of transcriptional activators, which includes the VirF invasion regulators of *Shigella* and *Yersinia*. InvF induces the

transcription of the secreted proteins encoded in the *sip* operon (Johnston *et al.*, 1996).

The *hilA* locus (hyperinvasion locus) was identified in a search for hyperinvasive *S. typhimurium* mutants able to enter cultured epithelial cells even after growth under invasion-repressing conditions (Lee *et al.*, 1992). As for InvF, HilA is necessary both for invasion of epithelial and M cells, and virulence in mice by the oral route of infection (Bajaj *et al.*, 1995; Penheiter *et al.*, 1997). The amino-terminal region of HilA is similar to the DNA binding and transcriptional activation domain of the OmpR/ToxR family of transcriptional activators. HilA coordinately regulates the expression of at least six genes including *sipA*, *sipC*, *prgH*, *prgK*, *orgA*, *invF*, and *orgA* (Bajaj *et al.*, 1995; Bajaj *et al.*, 1996). The activation of *invF* and *prgH* expression in *Escherichia coli* by HilA suggests HilA may activate transcription by directly binding to target promoters (Bajaj *et al.*, 1996). Since the expression of *hilA* is inhibited under conditions which repress invasion gene expression (acidic pH, aerobiosis, low osmolarity, presence of $phoPQ^c$ mutation), it has been suggested that regulation of *hilA* expression plays a key role in controlling the invasion phenotype.

Johnston *et al.* (1996) have shown that *hilA* expression is regulated by another protein called SirA (*Salmonella* invasion regulator). SirA is also required for the induction of *prgH* expression and secretion of SipA, SipB, SipC, and SipD. Not surprisingly, *sirA* mutants are defective for invasion of epithelial cells. Similar to the

phoPQ locus, the *sirA* gene is located outside SPI-1. The deduced amino acid sequence of SirA is similar to UvrY of *E. coli* and GacA of *P. fluorescens*, both of which are members of the FixJ subfamily of response regulators of two-component regulatory systems. The *sirA* mutant phenotype is suppressed by two unlinked loci called *sirB* and *sirC*. *sirC* is located in a previously uncharacterized region of SPI-1. These observations have led to the proposal of a model for the regulation of *Salmonella* invasion (Johnston *et al.*, 1996). Detection of "invasion signals" by an unidentified sensor kinase results in activation of SirA by phosphorylation. Phosphorylated SirA then activates *hilA* expression, either directly or indirectly through activation of *sirB* and/or *sirC* transcription, to continue the regulatory cascade leading to the synthesis of invasion genes. Since activation of PhoP/Q occurs under conditions thought to resemble the intracellular environment, PhoP/Q-mediated repression of invasion gene expression at an unknown point in the regulatory cascade inhibits the expression of these genes, which are presumably unnecessary for intracellular survival.

INTRACELLULAR REPLICATION

The ability of *S. typhimurium* to survive and replicate within epithelial cells and macrophages is essential for virulence (Fields *et al.*, 1986; Leung and Finlay, 1991). In both professional and non-professional phagocytes, *Salmonella* resides and replicates within a

membrane-bound compartment. Characterization of this compartment has been a subject of intense study, occasionally with conflicting results. Based on different criteria, some groups have reported that in murine macrophages, *S. typhimurium* is localized within phagosomes that fuse with lysosomes (Carrol *et al.*, 1979; Oh *et al.*, 1996), while others indicate that *S. typhimurium* inhibits phagosome-lysosome fusion (Ishibashi and Arai, 1990; Buchmeier and Heffron, 1991). However, it is now accepted that *Salmonella*-containing vacuoles (SCVs) are specialized vacuoles that differ from true phagolysosomes (Finlay and Falkow, 1997).

It is now understood that the formation of a phagolysosome is not the result of a single fusion event between a phagosome and a lysosome, but occurs through a continuum of fusion reactions with vesicles of the endosomal-lysosomal pathway (Desjardins *et al.*, 1994; Beron *et al.*, 1995). Unique compartments have been identified along the endocytic pathway, and markers specific for these compartments are used to follow the transformation of phagosomes into phagolysosomes. The three compartments of the endocytic pathway include early endosomes, late endosomes/prelysosomes, and lysosomes. Transformation of prelysosomes into lysosomes involves the accumulation and/or processing of high levels of lysosomal acid hydrolases, creating a degradative compartment.

The fate of SCVs after entry into host cells has been characterized using endocytic markers. Trafficking of SCVs in epithelial cells is similar to that observed in cultured and bone marrow-derived

macrophages (Garcia-del Portillo and Finlay, 1995; Rathman *et al.*, 1997). Within 15-30 min after bacterial internalization, SCVs fuse with vesicles containing lysosomal membrane glycoproteins (LAMP-1 and LAMP-2), which are markers for late endosomes and lysosomes (in higher levels). Lysosomal acid phosphatase (LAP) is also incorporated into SCVs with the same kinetics as LAMPs. In contrast, phagosomes containing latex beads or heat-killed bacteria progress along a degradative pathway, acquiring cathepsins and mannose 6-phosphate receptors (M6PRs), in addition to LAMPs and LAP. M6PRs function in the delivery of soluble lysosomal enzymes to prelysosomal compartments (Garcia-del Portillo and Finlay, 1995). Also, as opposed to latex bead-containing phagosomes, SCVs show limited interaction with fluid phase endocytic tracers, indicating SCVs do not readily fuse with incoming endocytic traffic. Collectively, the data show that *S. typhimurium* resides in an intracellular compartment which diverges from the normal degradative endocytic pathway.

It is important to note that the above observations represent the pathway followed by the majority of SCVs. A certain percentage of SCVs (approximately 25-30%) are trafficked along a degradative pathway (Buchmeier and Heffron 1991; Garcia-del Portillo and Finlay, 1995; Rathman *et al.*, 1997). Others have reported the presence of at least two bacterial populations within macrophages, one static and the other rapidly dividing (Abshire and Neidhardt, 1993b). This heterogeneity in the intracellular population of bacteria

may in part explain some of the earlier conflicting data regarding the fate of SCVs.

As described above, *S. typhimurium* can enter both epithelial cells and macrophages by macropinocytosis, although the membrane ruffling induced in epithelial cells is localized to the point of bacterial contact with the host cell (Alpuche-Aranda *et al.*, 1994; Garcia-del Portillo and Finlay, 1994). Roughly half of the bacteria initially reside in unusually large vacuoles called spacious phagosomes (SPs), which are morphologically similar to macropinosomes. However, while macropinosomes shrink completely within 15 min, SPs persist in the cytoplasm for as long as 45 min, sometimes enlarging by fusion with macropinosomes or other SPs.

At least two lines of evidence suggest formation and maintenance of SPs contributes to the intracellular survival of *Salmonella*. First, host-adapted *Salmonella* serotypes rarely isolated from mice and humans fail to form or maintain SPs in mouse macrophages, and do not survive in macrophages from mice or humans (Alpuche-Aranda *et al.*, 1995). Second, a *phoPQ*^{c} mutant presumably containing a constitutively active PhoP transcriptional regulator induces significantly fewer SPs in mouse macrophages and enters in close-fitting phagosomes (Alpuche-Aranda *et al.*, 1994). This mutant is avirulent and defective for survival in macrophages early after phagocytosis, suggesting a *phoP*-repressed gene(s) (*prg*) is involved in SP formation (Miller and Mekalanos, 1990). SPs are postulated to facilitate intracellular survival by diluting toxic compounds delivered

to SCVs or by reducing the rate of SCV acidification, allowing the bacteria time to adapt by expressing other genes necessary for intracellular survival (Alpuche-Aranda *et al.*, 1994).

SCVs in murine macrophages become acidified, although the rate and level of acidification is in dispute, perhaps due to technical differences in the protocols employed by different groups. Alpuche-Aranda *et al.* (1992) reported SCVs acidify slowly over a 4-5 h period compared to vacuoles containing heat-killed organisms. Others have found that the majority of SCVs acidify to a pH between 4.0 and 5.0 within 20 to 30 min after formation (Rathman *et al.*, 1996). Treatment with inhibitors of phagosome acidification reduced the number of bacteria recovered from infected macrophages, suggesting *Salmonella* may require an acidic environment for intracellular replication and survival (Rathman *et al.*, 1996). Other intracellular pathogens, including *Coxiella burnetii*, *Leishmania* spp., and *Francisella tularensis*, also replicate within acidified host cell compartments (Antoine *et al.*, 1990; Maurin *et al.*, 1992; Fortier *et al.*, 1995). However, unlike *Francisella*, acidification of *Salmonella* phagosomes is not linked to iron acquisition.

Several genes have been identified which affect the intracellular survival and replication of *Salmonella*. The following section summarizes the genes which have been characterized to date. Through a variety of techniques, a number of genes have been shown to be upregulated after entry into host cells or during infection of experimental animals, implying these genes contribute to

Salmonella fitness *in vivo* (reviewed in Heithoff *et al.*, 1997b and Valdivia and Falkow, 1997). However, in many cases the specific roles of these loci in virulence remains to be defined. The discussion here is limited to genes shown to make a measurable contribution to phenotypes associated with intracellular survival.

phoPQ

PhoP/PhoQ comprise a two-component regulatory system (Groisman *et al.*, 1989; Miller *et al.*, 1989) which regulates many virulence-associated phenotypes including spacious phagosome formation (Alpuche-Aranda *et al.*, 1994), invasion of epithelial cells (Behlau and Miller, 1993), inhibition of processing and presentation of antigens by macrophages (Wick *et al.*, 1995), resistance to antimicrobial peptides (Fields *et al.*, 1989), intramacrophage survival (Fields *et al.*, 1986), survival in infected mice (Fields *et al.*, 1986), adaptation to Mg^{2+}-limiting environments (Soncini *et al.*, 1996; Blanc-Potard and Groisman, 1997), and modification of lipid A (Guo *et al.*, 1997). PhoP/Q-mediated modulation of these phenotypes occurs through the activation and repression of the production of over 40 proteins (Miller and Mekalanos, 1990). Although many PhoP/Q-regulated loci have been isolated, in most cases, the genes contributing to each phenotype are unknown. In fact, several PhoP/Q-regulated genes are not required for virulence in mice, suggesting the regulatory role of PhoP/Q is not limited to *Salmonella* pathogenesis (Fields *et al.*, 1989; Gunn *et al.*, 1995). Genes shown to

affect some of the above PhoP/Q-regulated phenotypes are described below.

PhoQ is a membrane-bound sensor-kinase predicted to contain two transmembrane regions, a long cytoplasmic tail, and a large periplasmic domain (Miller *et al.*, 1989; Gunn *et al.*, 1996). PhoP is a 224 amino acid cytoplasmic response regulator belonging to the OmpR subgroup of two-component response regulators. By analogy with other two-component systems, detection of appropriate environmental signals by the periplasmic domain of PhoQ triggers the autophosphorylation of PhoQ on a conserved histidine residue. PhoQ then phosphorylates PhoP on a conserved aspartate residue in the amino-terminus. Phosphorylated PhoP then activates the transcription of *pag* (*phoP*-activated genes). Gunn *et al.* (1996) have shown evidence of phosphotransfer between PhoQ and PhoP.

Recent studies have shown that PhoQ is a sensor of extracellular divalent cations, specifically Mg^{2+} and Ca^{2+} (García Véscovi *et al.*, 1996; García Véscovi *et al.*, 1997). Micromolar concentrations of Mg^{2+} or Ca^{2+} activate the transcription of at least 25 PhoP-activated loci, while growth in millimolar levels of these cations represses transcription of PhoP-activated genes (García Véscovi *et al.*, 1996; García Véscovi *et al.*, 1997). The regulatory effect of the cations appears to be mediated by altering the conformation of the PhoQ periplasmic domain. Support for this hypothesis comes from several experiments. First, a protein chimera (ZhoQ) in which the PhoQ periplasmic domain is replaced by the periplasmic domain of EnvZ, a

related sensor protein which responds to osmolarity, allows the expression of a PhoP-activated gene in both high and low Mg^{2+} media in the absence of PhoQ (García Véscovi *et al.*, 1996). Second, Mg^{2+} alters the trypsin sensitivity of PhoQ but not ZhoQ *in vitro* at the same concentration required to repress expression of PhoP-activated genes (García Véscovi *et al.*, 1996). Since the modified trypsin sensitivity is detected in spheroplasts prepared from bacteria expressing PhoQ, soluble components from the periplasmic space are not necessary for this effect. More recently, García Véscovi *et al.* (1997) have shown that the tryptophan intrinsic fluorescence pattern of a purified polypeptide corresponding to the PhoQ periplasmic domain is significantly altered in the presence of Mg^{2+} or Ca^{2+} but not Ba^{2+}, which is unable to repress PhoP-activated genes. They also provided evidence for distinct, independent binding sites for Mg^{2+} and Ca^{2+}. Repression of PhoP-activated gene expression is achieved at lower concentrations of Mg^{2+} than Ca^{2+}, although both cations are necessary for maximal repression. Finally, a mutant PhoQ protein ($PhoQ^c$) which mediates the constitutive overexpression of several PhoP-activated genes displays a reduced affinity for Ca^{2+} but an unchanged affinity for Mg^{2+} (García Véscovi *et al.*, 1997). The mutation in $PhoQ^c$ is a Thr to Ile substitution at amino acid 48 which is located in the periplasmic domain immediately following a predicted membrane spanning segment (Gunn *et al.*, 1996). Amino acids involved in cation binding in the PhoQ periplasmic domain may include a region with several acidic residues (amino acids 135-154).

There are experimental data supporting this suggestion using the *E. coli* PhoQ protein (García Véscovi *et al.*, 1997).

Resistance to antimicrobial peptides. Fields *et al.* (1989) attempted to determine the microbicidal mechanism of macrophages responsible for decreased intracellular survival of *phoP* mutants. Crude granule extracts from human neutrophils and rabbit peritoneal macrophages were found to have a strong microbicidal effect on the mutants. Fractions of the extracts with the highest activity against the mutants were enriched in low molecular weight proteins that could correspond to defensins. Defensins are small molecular weight antimicrobial cationic peptides capable of adopting amphipathic alpha-helical structures and forming pores in membranes (Parra-Lopez *et al.*, 1993). Defensins are present in large amounts in the granules of neutrophils and macrophages of several mammals. Both *phoP* and *phoQ* mutants are hypersusceptible to purified defensins as well as to related antimicrobial peptides from frogs (magainins), insects (cecropin, mastoparan, mellitin), and pigs (cecropin) (Fields *et al.*, 1989; Groisman *et al.*, 1992).

The work of Fields *et al.* (1989) prompted a search for loci involved in resistance to antimicrobial peptides. This search identified several unlinked genes affecting virulence in mice and resistance to subsets of 6 different cationic peptides, but none of these genes have been shown to be regulated by PhoP (Groisman *et al.*, 1992). However, one of these loci, *sapABCDF*, is required for

survival in macrophages *in vitro* (Parra-Lopez *et al.*, 1993). The deduced amino acid sequences of *sapABCDF* are most similar to the components of two oligopeptide uptake systems in *S. typhimurium* and *Bacillus subtilis*. SapA shows sequence similarity to periplasmic solute binding proteins. The deduced sequences of SapD and SapF are similar to the sequences of several members of the ATP binding cassette (ABC) family of transporters. It was suggested the SapABCDF system may facilitate resistance to some antimicrobial peptides by transporting them into the cytoplasm where they could be degraded by proteases.

The only PhoP-regulated locus shown to control resistance to antimicrobial peptides is encoded within *pmrCAB* (Gunn and Miller, 1996; Soncini and Groisman, 1996). PmrC (also known as PagB) encodes a putative membrane protein of unknown function. PmrA-PmrB function as a two-component regulatory system controlling resistance to the cationic peptide antibiotic polymyxin B (PM). PM resistant mutants of *S. typhimurium* have mutations which map to the response regulator, *pmrA*, and are also resistant to other antimicrobial peptides, including protamine and the neutrophil peptides CAP37 and CAP57 but not defensins (Gunn and Miller, 1996). However, *pmrA* null mutants do exhibit wild-type virulence in mice. PmrA/B-dependent resistance to antimicrobial peptides is suggested to result from the induction of genes whose products covalently modify lipid A by substituting the 4' phosphate with 4-aminoarabinose (Helander *et al.*, 1994; Guo *et al.*, 1997). This

substitution decreases the negative charge of the LPS, which may reduce the accessibility of cationic peptides to lipid A. Since PhoP/Q also regulate a PmrA/B-independent lipid A modification, it has been proposed that other lipid A alterations, or a combination of lipid A alterations and expression of Pag proteins, contribute to a different cationic peptide resistance pathway effective against other peptides including defensins (Gunn and Miller, 1996).

Adaptation to Mg^{2+}-limiting environments. Both *phoP* and *phoQ* are necessary for growth in low Mg^{2+} media (García Véscovi *et al.*, 1996). Given that Mg^{2+} acts as a signal controlling the PhoP/Q regulatory system, this suggested the PhoP/Q regulon may allow adaptation to Mg^{2+}-limiting environments. Consistent with this hypothesis, several PhoP-activated genes have been shown to be essential for growth in low Mg^{2+} liquid or solid media (García Véscovi *et al.*, 1996; Soncini *et al.*, 1996; Blanc-Potard and Groisman, 1997). Two of these genes, *mgtCB*, form an operon located in a 17 kb pathogenicity island designated SPI-3 and encode a high affinity Mg^{2+} uptake system (Snavely *et al.*, 1991; Blanc-Potard and Groisman, 1997). The function of MgtC is not understood, but MgtB and MgtC may act independently in Mg^{2+} transport. *mgtC* is required for growth in macrophages (Blanc-Potard and Groisman, 1997). Addition of exogenous Mg^{2+} partially rescues *mgtCB* and *phoP* mutants for growth in macrophages. Since expression of PhoP-activated genes is induced in host cells, the data suggest the

Salmonella phagosome contains a limiting concentration of Mg^{2+} (Alpuche-Aranda *et al.*, 1992; Garcia-del Portillo *et al.*, 1992; Heithoff *et al.*, 1997a).

Survival within macrophages. As described above, *prg* loci appear to play a role in intracellular survival early after phagocytosis. A switch to expression of *pag* loci occurs at a later stage and is thought to permit continued survival and replication. Analysis of the induction of PhoP-activated genes after entry into epithelial cells or macrophages indicates transcription is minimal within 1 h after infection and reaches a maximal level at 4-6 h post-infection (Garcia-del Portillo *et al.*, 1992; Alpuche-Aranda *et al.*, 1992). This roughly coincides with the time at which bacterial numbers begin to increase inside both types of host cells (Leung and Finlay, 1991; Abshire and Neidhardt, 1993b), and the time when *phoP* mutants begin to show a defect in intramacrophage survival (Fields *et al.*, 1986; Miller and Mekalanos, 1990).

Of the *pag* loci identified to date, only *pagC* has been clearly shown to be required for virulence in mice and survival within macrophages (Pulkkinen and Miller, 1991). It remains possible that some of the PhoP-regulated phenotypes may be the result of a cumulative effect of multiple *pag*-encoded proteins, but strains with mutations in multiple *pag* loci have yet to be created to test this hypothesis. *pagC* encodes an 18 kDa outer membrane protein of unknown function (Pulkkinen and Miller, 1991). *pagC* mutants have

wild-type sensitivity to defensins, lysozyme, complement, and cationic peptides derived from mouse intestine. PagC shows similarity to other outer membrane proteins including Ail of *Yersinia enterocolitica*, Lom, a bacteriophage lambda-encoded protein of unknown function, Rck of *S. typhimurium*, and OmpX of *Enterobacter cloacae* (Pulkkinen and Miller, 1991). Both Ail and Rck have been shown to affect serum resistance (Bliska and Falkow, 1992; Heffernan *et al.*, 1992). Ail also mediates attachment to and invasion of epithelial cells by *Yersinia* (Miller and Falkow, 1988). There is no evidence supporting a role for PagC in invasion as *pagC* mutants display wild-type invasion of epithelial cells (Galan and Curtiss, 1989), and *pagC* is insufficient to confer an invasive phenotype on *E. coli* (Miller, 1991).

msgA

Analysis of the region adjacent to *pagC* led to the discovery of *msgA* (macrophage survival gene) (Gunn *et al.*, 1995). *msgA* mutants have more than 300-fold reduced virulence in mice and are defective for survival in macrophages. The intracellular survival defect is quantitatively equivalent to that of *phoP* mutants. MsgA is predicted to be a 79 amino acid, hydrophilic protein containing a large number of acidic amino acids at the C-terminus and lacking similarity to any known proteins. The deduced amino acid sequence lacks a putative signal sequence and any large stretches of hydrophobic residues. Expression of *msgA* is not regulated by PhoP.

Both *pagC* and *msgA* are part of a low G+C region of the *S. typhimurium* chromosome suggesting this region may have been acquired by horizontal transfer from another organism.

SPI-2

The second pathogenicity island identified in *Salmonella*, designated SPI-2, is a 40 kb virulence gene cluster located at minute 30.7 (Hensel *et al.*, 1995; Ochman *et al.*, 1996; Shea *et al.*,1996; Hensel *et al.*, 1997). Partial sequencing of SPI-2 has shown it contains several genes which encode a second type III secretion system (*ssa* genes) as well as a putative two-component regulatory system [*ssrAB*; *ssrA* is referred to as *spiR* by Ochman *et al.* (1996)] (Ochman *et al.*, 1996; Shea *et al.*, 1996). SPI-2 was initially proposed to be required for survival in macrophages. This was based on the observation that an *ssaC* (*spiA*) mutant is defective for survival in macrophages, but exhibits wild-type invasion of epithelial cells (Ochman *et al.*, 1996). Consistent with this proposition, expression of *ssaH*, a putative component of the secretion apparatus, is induced in macrophages and is regulated by SsrA/B (Valdivia and Falkow, 1997). However, others have been unable to demonstrate a role in intramacrophage survival for any of the SPI-2 genes identified to date (Hensel *et al.*, 1997). Given that all SPI-2 mutants are strongly attenuated for virulence in mice by the oral or intraperitoneal routes, SPI-2 gene products are likely to contribute to virulence after penetration of the intestinal epithelium (Hensel *et al.*, 1995; Ochman

et al., 1996; Shea *et al.*, 1996). It should be noted there is one report of mutations in some *ssa* genes affecting SPI-1-related functions (epithelial cell invasion, macrophage cytoxicity), suggesting the possibility of an interaction between the two type III secretion systems (Hensel *et al.*, 1997).

slyA

slyA was initially thought to encode a cytolysin as it conferred a hemolytic phenotype when expressed in multiple copies in *E. coli* (Libby *et al.*, 1994). Subsequent analysis of the deduced amino acid sequence showed SlyA is similar to a family of low molecular weight transcriptional regulators including MprA, EmrR, and MarR of *E. coli*, HprR of *B. subtilis*, and PecS of *Erwinia chrysanthemi* (Dehoux and Cossart, 1995). These proteins regulate diverse functions such as multiple antibiotic resistance (MarR, EmrR), sporulation and resistance to hydrogen peroxide (HprR), and synthesis of microcins (MprA) (Dehoux and Cossart, 1995). SlyA was later shown to induce the expression of a cryptic cytolysin in *E. coli* (Oscarsson *et al.*, 1996). *slyA*-hybridizing sequences are present in *Shigella* spp. and enteroinvasive *E. coli* and may be located on extrachromosomal plasmids (Libby *et al.*, 1994).

slyA mutants are defective for survival in macrophages and are avirulent in mice by oral, intraperitoneal, or intravenous routes of infection (Libby *et al.*, 1994). Studies of tissues dissected from orally infected mice indicated that relative to the wild-type strain, fewer

numbers of *slyA*⁻ bacteria reach the Peyer's patches, mesenteric lymph nodes, liver, and spleen, and that the *slyA*⁻ mutant is very rapidly cleared from the liver (Libby *et al.*, 1994). This is consistent with more recent studies showing that *slyA* is necessary both for the destruction of M cells and adjacent enterocytes, and survival in Peyer's patches (Daniels *et al.*, 1996). Proper expression of *slyA* is apparently important for *Salmonella* virulence as overexpression, mediated by a multicopy plasmid carrying *slyA*, fails to complement a *slyA* mutant for virulence in mice (Libby *et al.*, 1994) and impairs M cell destruction by wild-type *S. typhimurium* (Daniels *et al.*, 1996).

Buchmeier *et al.* (1997) have recently analyzed the expression of *slyA* and the function of SlyA. Expression of *slyA* is induced 15-fold in stationary phase and 12-fold in macrophages, reaching a maximal level 6 h post-infection. Induction of *slyA* transcription does not require the stationary phase sigma factor RpoS. SlyA was shown to positively and negatively regulate the expression of several proteins during stationary phase and within macrophages. The sensitivity of a *slyA* mutant to reactive oxygen intermediates, including hydrogen peroxide and products generated by the redox cycling compound paraquat, suggests SlyA may regulate genes whose products contribute to resistance to oxidative stress such as that encountered during the respiratory burst of phagocytic cells.

fkpA

Members of a family of proteins with peptidyl-prolyl *cis-trans* isomerase activity (PPIase) and the capacity to bind the immunosuppressant macrolide FK506 are called FK506-binding proteins (FKBPs) (Hacker and Fischer, 1993). The Mip outer membrane protein of the intracellular pathogen *L. pneumophila* has PPIase activity and its carboxyl terminus shows sequence similarity to FKBPs (Fischer *et al.*, 1992). A Mip-like protein has also been identified in the obligate intracellular pathogen *Chlamydia trachomatis* (Lundemose *et al.*, 1993). The PPIase activity of the Mip proteins of *Legionella* and *Chlamydia* contributes to the initiation of infection in host cells (Cianciotto *et al.*, 1989; Lundemose *et al.*, 1993).

The *fkpA* gene of *S. typhimurium* encodes a Mip-like protein contributing to survival in both epithelial cells and macrophages during the first 6 h after infection (Horne *et al.*, 1997). The intracellular survival defect is not related to efficiency of bacterial entry into host cells as a *fkpA* mutant displays wild-type levels of uptake by host cells. The function of the FkpA and Mip proteins are unknown. Since FKBPs may play a role in protein folding, it has been suggested that FkpA and Mips may facilitate proper folding of bacterial virulence factors or modify the activity of one or more host cell antimicrobial proteins to enhance intracellular survival (Horne *et al.*, 1997).

Francisella

INTRODUCTION

McCoy (1911) is credited with the first description of tularemia, the disease caused by the bacterium now known as *Francisella tularensis*. While studying an apparent outbreak of plague among ground squirrels in Tulare County, California, McCoy described an infection that caused lesions in squirrels and experimentally infected animals similar to those produced by the plague bacillus. The disease was differentiated from plague because the organism could not be easily isolated. A year later, McCoy and Chapin (1912) reported the isolation of the etiologic agent and named it *Bacterium tularense*. Around the same time, *F. tularensis* infections of humans were also described. In Brigham City, Utah, Pearse (1911) described six cases of an illness known as deer fly fever resulting from the bite of a fly. In their study of a diseased meat cutter in Cincinnati, Ohio, Wherry and Lamb (1914) identified *B. tularense* as the causative agent. Francis (1921) later isolated *B. tularense* from seven cases of deer fly fever and named the disease tularemia. The next eleven years saw descriptions of tularemia in other countries, including the former Soviet Union (Pollitzer, 1967), Canada (McNabb, 1930), Sweden (Carlberg, 1932), and Japan (Ohara, 1925; Francis and Moore, 1926), where the disease was known as *yato-byo* (wild rabbit disease). Further characterization of *B. tularense* led to its placement

in other genera including *Pasteurella* and *Brucella*, but it was eventually assigned its own genus and designated *Francisella tularensis* in recognition of the efforts of Edward Francis in studying tularemia.

Three other bacteria have been identified as belonging to the genus *Francisella*. A very close relative of *F. tularensis*, *F. novicida* was first isolated from a water sample taken from a bird refuge with large numbers of dead muskrats (Larson *et al.*, 1955). Since then it has only been isolated twice, from human patients, and is considered to be of low virulence for humans (Hollis, 1989). *F. philomiragia* has also been isolated from muskrats (Jensen *et al.*, 1969) and from 14 human patients (Wenger *et al.*, 1989). Common factors among these patients are that they experienced near-drowning incidents in salt water or had other underlying immunocompromised conditions (e.g. chronic granulomatous disease). This has led to the suggestion that *F. philomiragia* is an opportunistic pathogen of humans. Very recently, an endosymbiont of the wood tick *Dermacentor andersoni* called DAS (*D. andersoni* symbiont) was identified as belonging to the genus *Francisella* (Neibylski *et al.*, 1997). It was found to be most similar to *F. tularensis* (95.4%) on the basis of the 16S rDNA gene sequence. Its infectivity for humans is unknown. The organisms were localized to apparent phagosomes in ovarial tissue cells of the ticks, and experimental infection of guinea pigs could only be accomplished by direct injection of ovarial tissues or organisms propagated in tick or Vero cell cultures.

Exchanges of sera from tularemia patients between the United States and other countries showed the *F. tularensis* strains in North America, Europe, and Asia were all antigenically similar. These strains were later found to have identical lipopolysaccharide (LPS) O-antigen structures (Tokhtamysheva *et al.*, 1995). However, the North American isolates were clearly different as they caused a more severe form of tularemia in humans and were of higher virulence in experimental animals. Later studies indicated the European and Asian form of *F. tularensis* was also present in North America. This led to the subdivision of *F. tularensis* into two biotypes, initially labelled *F. tularensis* var *tularensis* (or *F. tularensis nearctica*) and *F. tularensis* var *palaearctica* (or *F. tularensis holarctica*), for the North American and European-Asian variants, respectively. Today, the most commonly used designations are type A (North American) and type B (European-Asian) as proposed by Jellison.

TULAREMIA - CLINICAL AND TRANSMISSION

There are six recognized forms of tularemia: oculoglandular, ulceroglandular, glandular, pneumonic or respiratory, oropharyngeal, and typhoidal. The clinical presentation is influenced by the biotype of the infecting bacterium and the route of infection. *F. tularensis* type A causes severe human illness which is fatal in a small percentage of untreated cases. Human infections with *F. tularensis* type B are milder and are rarely fatal. Persistent fever, chills,

headaches, myalgia, and general malaise are symptoms common to all forms of tularemia (Martin *et al.*, 1982).

F. tularensis is capable of infecting a wide variety of mammals, from various types of rodents to sheep (Martin *et al.*, 1982). Transmission of the type A strains is in most cases the result of bites from ticks or other insects or from direct contact with infected animals such as muskrats, rabbits, and beavers. Infections with *F. tularensis* type B can also result from bites of ticks or mosquitoes, although the important animal reservoirs are thought to include voles, hares, muskrats, and water rats. Ulceroglandular tularemia is associated with infection by insect bites or through skin lesions occurring during the dressing of wild game. Glandular tularemia results from infection by similar routes to the ulceroglandular form without the characteristic primary lesion of the latter. Direct inoculation of the eye by contaminated fingers or hands can cause oculoglandular tularemia. Both oropharyngeal and typhoidal tularemia can result from the ingestion of contaminated water or meat (Ohara *et al.*, 1996; Tärnvik *et al.*, 1996). Inhalation of *F. tularensis* can lead to either pneumonic or typhoidal tularemia, although pneumonia can occur secondary to infection by any route.

MORPHOLOGY

F. tularensis is a small, pleomorphic, non-motile, Gram-negative cocco-bacillus. It has a loosely associated capsule which can be removed by suspension in hypertonic sodium chloride solution (Hood, 1977). *F. novicida* is roughly three times larger, measuring 0.7 x 1.7 μm. Neither pili nor flagella have been detected on the surface of the bacterium (Nano, 1992). The DAS is also pleomorphic and on average measures 1.2 μm in diameter, as observed in electron micrographs of infected tick ovarial tissue cells (Niebylski *et al.*, 1997). Attempts to culture the DAS *in vitro* on agar plates were unsuccessful.

INTRACELLULAR GROWTH

Francisella is a facultative intracellular pathogen capable of growing within a variety of host cells, including both non-professional and professional phagocytes. Its interaction with macrophages has received by far the most attention and is an important component of its pathogenesis *in vivo*. In this section, I will discuss the current knowledge of the intracellular growth of *Francisella*, including preferred host cells, characteristics of the intracellular compartment, and virulence factors affecting intracellular growth.

Host cells

The first reports of intracellular replication of *F. tularensis* came from studies of hepatic cells and the endothelia of guinea pigs and the gut epithelium of the wood tick *D. andersoni* (Tärnvik, 1989). Buddingh and Womack (1941) noted the accumulation of large numbers of bacteria in ectodermal epithelial cells 24 hours after inoculation of *F. tularensis* onto the chorioallantois of chick embryos. Intracellular growth of *F. tularensis* has also been observed in HeLa cells (Shepard, 1959) and mouse fibroblasts (Merriott *et al.*, 1961). After aerosol infection of monkeys with a moderately virulent strain, Hall *et al.* (1973) detected *F. tularensis* in the epithelial cells of respiratory bronchioles and adjacent alveoli as well as in pulmonary macrophages. Quantitative cultures indicated that within 6 days the bacteria had spread to the regional lymph nodes, spleen, and liver and were replicating in these tissues. Similar dissemination to the lungs, spleen, and liver are observed after infection of rats (Downs *et al.*, 1949) and mice by the intravenous, intraperitoneal, or intranasal route (Fortier *et al.*, 1991). Early after intravenous infection of mice, *F. tularensis* was shown replicating inside both Kupffer cells and hepatocytes (Conlan and North, 1992). Neutrophils and macrophages accumulated at sites of infection and appeared to lyse infected hepatocytes, presumably to release intracellular bacteria and allow uptake by macrophages.

Early studies described the survival, or in one instance rapid killing, of *F. tularensis* in peritoneal or alveolar macrophages from

guinea pigs and rabbits (Stefanye *et al.*, 1961; Thorpe and Marcus, 1964b; Thorpe and Marcus, 1965). Interpretation of the results of the above experiments was complicated by the use of streptomycin in the culture medium, which may have affected the viability of intracellular bacteria. McElree and Downs (1961) showed rat peritoneal macrophages phagocytosed *F. tularensis* and that 72 hours after infection, substantially increased numbers of bacteria were recovered from the macrophages. Other investigators have demonstrated *F. tularensis* replication in alveolar macrophages from rabbits (Nutter and Myrvik, 1966) and mice (Polsinelli *et al.*, 1994), peritoneal macrophages from mice, guinea pigs, and rats (Anthony *et al.*, 1991a; Fortier *et al.*, 1992), and human monocytes (Fortier *et al.*, 1994). Phagocytosis and replication of *Francisella* within macrophages is not affected by immune serum (Nutter and Myrvik, 1966; Rhinehart-Jones *et al.*, 1994). Strains of increased virulence have a higher rate of intracellular growth (Nutter and Myrvik, 1966; Shepard, 1959). *In vivo* in infected animals, *Francisella* is rarely found extracellularly (Fortier *et al.*, 1994). In peritoneal cells of infected mice, it is exclusively localized in the macrophage subpopulation, in which it replicates (Fortier *et al.*, 1994; Fortier *et al.*, 1995).

There have been no data suggesting *F. tularensis* is capable of growth within polymorphonuclear leukocytes (PMNs). *In vivo*, *Francisella* has not been observed within PMNs. At late time points during the infection of chick embryos, bacteria are found in

fibroblasts and mononuclear phagocytes but not within PMNs (Buddingh and Womack, 1941). PMNs from monkeys, humans, and rats are unable to phagocytize *F. tularensis* in the absence of immune serum (Proctor *et al.*, 1975; Löfgren *et al.*, 1983). At high concentrations (20%), non-immune serum can facilitate uptake of *F. tularensis* by human PMNs (Löfgren *et al.*, 1983), but this fails to lead to appreciable induction of the respiratory burst (Löfgren *et al.*, 1984; Sandström *et al.*, 1988). However, regardless of the type of serum employed, a virulent, wild *F. tularensis* strain was more resistant to phagocytosis and killing by human PMNs than an attenuated, live vaccine strain (LVS) (Löfgren *et al.*, 1983). This was in part attributed to the higher resistance of the wild strain to hypochlorous acid (Löfgren *et al.*, 1984). Collectively, the data suggest that PMNs are unsuited for supporting the intracellular growth of *Francisella*.

Intracellular compartment

Little is known about the nature of the intracellular compartment in which *Francisella* replicates. Electron microscopy and immunofluorescence studies by several investigators have shown that after phagocytosis, *Francisella* is localized, and replicates within, membrane-bound vacuoles (Proctor *et al.*, 1975; Anthony *et al.*, 1991a; Conlan and North, 1992; Nano, 1992; Fortier *et al.*, 1994; Fortier *et al.*, 1995). At later stages of infection, large numbers of bacteria are commonly observed in the cytoplasm leading to swelling

of the host cell and displacement of the nucleus. It is not known whether additional replication occurs after entry into the cytoplasm, but maximal growth in a defined medium *in vitro* occurs under mildly acidic conditions and is inhibited above pH 7.2-7.4 (Traub *et al.*, 1955). *Francisella*-containing phagosomes apparently become acidified, an event which facilitates acquisition of iron essential for growth (Fortier *et al.*, 1995). Macrophages treated with endosome acidification inhibitors do not support replication of *Francisella* in the absence of an external, pH-independent iron source. Although lysosomes (as defined by thorium labelling and acid phosphatase staining) may become associated with the outer edges of vesicles containing *Francisella*, they do not seem to fuse (Anthony *et al.*, 1991a). Small vesicular inclusions possibly derived from the bacteria and staining positive for acid phosphatase activity were detected within the phagocytic vacuole. Thus, intracellularly *Francisella* is localized within an acidified compartment which does not undergo phagosome-lysosome fusion.

Virulence factors affecting intracellular growth

There are few reports available describing candidate factors of *Francisella* that affect intracellular growth. Candidate factors have been identified in both *F. tularensis* and *F. novicida*, but in most cases their exact role in promoting intracellular growth is not understood. The following is a brief account of the potential virulence factors related to intracellular growth identified thus far.

minD. Two loci have been identified in *F. novicida* which affect serum resistance and growth in macrophages (Anthony *et al.*, 1994; Mdluli *et al.*, 1994). One locus encodes a protein similar to MinD of *E. coli* (Anthony *et al.*, 1994). MinD is an inner membrane-associated ATPase whose function in *E. coli* involves regulation of septum formation during cell division (Anthony *et al.*, 1994). An *F. novicida* *minD* mutant (KEM7) grows approximately 10-fold less well than the wild-type strain in mouse macrophages and is avirulent in mice. The kinetics of the intracellular growth of KEM7 are similar to those of the wild-type strain, and the difference in absolute growth is attributed to increased sensitivity to killing mechanisms encountered within 1 h after interaction with macrophages. *In vitro*, KEM7 exhibits enhanced sensitivity to oxidative killing and serum. It is suggested that abnormal septum formation in *minD* mutants may disrupt cell wall integrity, permitting bactericidal agents access across the outer membrane.

valAB. The gene products of another locus, designated *valAB*, may also influence intramacrophage growth (Mdluli *et al.*, 1994). Two related mutants were isolated which are serum-sensitive, defective for growth in macrophages, and avirulent in mice. Partial complementation of the serum-sensitive phenotype of one mutant with *valAB* provided evidence for a genetic lesion in this locus. The deduced amino acid sequences of *valAB* are similar to those of two

essential proteins of *E. coli*, MsbA and LpxK. MsbA and ValA are members of the superfamily of ATP binding cassette (ABC) transporters and are believed to participate in the transport of LPS to the outer membrane (McDonald *et al.*, 1997). LpxK has recently been identified as a lipid A 4'-kinase (Garrett *et al.*, 1997). The exact nature of the mutations in the *F. novicida* mutants were not identified, and the inability to complement the intracellular growth defect suggested the possibility that an unlinked genetic lesion was responsible for this phenotype. Whether ValA/ValB-mediated alterations in outer membrane LPS content or structure affect *F. novicida* intramacrophage growth remains to be demonstrated.

LPS phase variation. A correlation between LPS O-antigen type and growth in rat macrophages has recently been described (Cowley *et al.*, 1996). *F. tularensis* strains segregate two colony variants at a low frequency, one virulent (known as "blue") and the other avirulent (known as "gray") (Eigelsbach, 1951). Gray variants express a new LPS O-antigen, which is also the predominant form found in *F. novicida* (Cowley *et al.*, 1996). Any strain producing the *F. novicida*-type LPS is unable to replicate in rat macrophages but retains the capacity to replicate in mouse macrophages (Anthony *et al.*, 1991a; Cowley *et al.*, 1996). Biochemical data using purified LPS and lipid A showed that the *F. novicida*-type molecules are sufficient to induce the production of nitric oxide by rat macrophages, thereby inhibiting *Francisella* intracellular growth (Cowley *et al.*, 1996). This

effect can also occur *in vivo* as co-infection of rats with an *F. tularensis* blue variant (LVS) and a 10-fold excess of *F. novicida* results in suppression of LVS growth in host tissues (Cowley *et al.*, 1997). This phase variation phenomenon has not been observed in *F. novicida*. The genes and mechanisms involved in this process are unknown, although Cowley *et al.* (1996) found reversion to the blue variant could occur under intramacrophage conditions.

Stress response. Studies of a rifampicin-resistant, temperature-sensitive, RNA polymerase mutant (Rif7) of *F. tularensis* have implicated a set of stress-induced proteins as contributors to intramacrophage growth and virulence (Bhatnagar *et al.*, 1994; Bhatnagar *et al.*, 1995). Rif7 is unable to replicate in mouse macrophages or host tissues, and has an intraperitoneal (ip) 50% lethal dose (LD_{50}) 10^5-fold greater than the wild-type strain. Exposure to various environmental stresses *in vitro* prior to infection, including high temperature (42 °C), low iron levels, or low pH, partially restores virulence in mice and fully restores growth in macrophages. Protein synthesis is required during the stress treatment to reverse the virulence phenotype. Envelope protein profiles of cells grown under different stress conditions shows increased levels of several proteins specific for each condition, suggesting that expression of these proteins may contribute to the survival of *F. tularensis* in macrophages and mice.

23 kDa protein. Very recently, Golovliov *et al.* (1997) analyzed the pattern of protein synthesis after infection of cell-line macrophages with LVS. After 24 h of intracellular growth, 24 major proteins are detected, only 4 of which are significantly induced. The most prominently induced protein is a 23 kDa protein upregulated at 6 and 24 h post-infection, which is also induced *in vitro* by exposure to hydrogen peroxide. The 23 kDa protein is localized to the cell-free extract fraction. The deduced amino acid sequence of the encoding gene shows no similarities to known proteins. The induced and repressed proteins identified, the 23 kDa protein in particular, may aid in the adaptation of *Francisella* to growth in an intracellular environment.

HOST RESPONSE AND IMMUNITY TO *F. TULARENSIS*

Much work has been devoted to characterizing the host immune response to *F. tularensis* infection and defining the mechanisms involved in clearance of primary infections and requirements for the development of protective immunity to tularemia. Survival of tularemia leads to long-lasting, specific protective immunity in humans (Burke, 1977) and animals (Fortier *et al.*, 1991). An attenuated, live vaccine strain (LVS) derived from an *F. tularensis* type B strain was developed in 1961 (Eigelsbach and Downs, 1961). LVS has been shown to induce good protection in humans against respiratory tularemia, and to reduce the severity but not the

incidence of ulceroglandular tularemia (Burke, 1977). Killed vaccines are not effective in inducing protective immunity. LVS has retained virulence for mice and causes a lethal infection similar to human tularemia (Fortier *et al.*, 1991). The outcome of murine infection with LVS is highly dependent on the route of inoculation (Fortier *et al.*, 1991). Challenge by the intranasal (in), intravenous (iv), or intraperitoneal (ip) routes results in LD_{50}s approaching a single bacterium. In contrast, the intradermal (id) LD_{50} is about 10^6 bacteria, and survival of id infection leads to strong specific protective immunity to lethal challenge by any route with up to 10^6 LD_{50}s (Elkins *et al.*, 1992a; Elkins *et al.*, 1992b). Thus, sublethal infections of mice with LVS are easily established and lead to a protective immune response which is easily measurable (Elkins *et al.*, 1996). Hence, LVS infection in mice has been employed as a model of immunity to intracellular pathogens. The following section contains a discussion of the relative roles of macrophages, and innate, humoral, and cellular factors in the immune response to *Francisella*, many of which have been examined using the murine model of infection.

Macrophages

Data from *in vitro* and *in vivo* studies suggests that macrophages are likely the main effector cells involved in eliminating *Francisella*. Early studies showed that passively transferred macrophages from immune animals increased resistance to challenge in normal

recipients, and suppressed the intracellular growth of *Francisella* (Allen, 1962; Thorpe and Marcus, 1964a; Thorpe and Marcus, 1964b; Thorpe and Marcus, 1965; Nutter and Myrvik, 1966). However, these passive transfer experiments did not exclude a contribution from activated lymphocytes in the cell preparations. More recent reports have demonstrated that murine alveolar and peritoneal macrophages, and human monocytes, inhibit the intracellular growth of *Francisella* when activated with gamma-interferon (IFN-γ) (Polsinelli *et al.*, 1994; Anthony *et al.*, 1992; Fortier *et al.*, 1992; Fortier *et al.*, 1994). Inhibitory activity of murine peritoneal macrophages also requires tumor necrosis factor alpha (TNF-α), a cofactor for the activation of certain cytotoxic effector functions including nitric oxide synthesis (Fortier *et al.*, 1992). Murine macrophages activated *in vivo* by infection with *Mycobacterium bovis* BCG also inhibit the growth of *Francisella*, and require IFN–γ and TNF-α for activation (Green *et al.*, 1993).

In murine peritoneal macrophages, anti-*Francisella* activity is mediated by reactive nitrogen intermediates, in particular nitric oxide, produced through an L-arginine-dependent metabolic pathway (Anthony *et al.*, 1992; Fortier *et al.*, 1992; Green *et al.*, 1993). Evidence supporting this conclusion comes from studies using the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (NMMA) and quantitative analysis of levels of nitrite and nitrate, which are stable oxidative intermediates derived from nitric oxide. Levels of nitrite elaborated by activated macrophages *in vitro* and

urinary nitrate excreted from *M. bovis* BCG-infected mice correlate with anti-*Francisella* activity. NMMA blocks nitrite synthesis and restores LVS growth in IFN-γ-activated macrophages *in vitro* (Anthony *et al.*, 1992; Fortier *et al.*, 1992). Treatment of *M. bovis* BCG-infected mice with NMMA reduces urinary nitrate excretion to normal levels, and macrophages harvested from these animals fail to inhibit LVS growth (Green *et al.*, 1993). Nitric oxide has been implicated as an effector molecule inhibiting the growth of other intracellular pathogens (Adams *et al.*, 1990; Green *et al.*, 1990; Adams *et al.*, 1991). Nitric oxide is thought to inhibit *Francisella* growth by inhibition of iron-dependent enzymes and by the formation of iron-nitrosyl complexes with free iron, thereby preventing acquistion of this essential nutrient (Fortier *et al.*, 1994). This is consistent with the observation that *Francisella* can replicate in IFN-γ-activated macrophages in the presence of an unlimited exogenous iron supply (Fortier *et al.*, 1994).

Innate factors

Studies using the murine model of tularemia have revealed that innate immune defences can provide significant resistance to *Francisella* infection. As mentioned earlier, neutrophils and macrophages may participate early after infection by lysing infected hepatocytes, thereby aborting *Francisella* replication in these permissive host cells and allowing uptake of released bacteria by macrophages (Conlan and North, 1992). This activity is also

important in limiting the growth of *S. typhimurium* and *Listeria monocytogenes* in the liver, and in the survival of sublethal infections with all three intracellular bacteria (Conlan and North, 1992). Neutrophils are essential for defence against primary infection as neutrophil-depleted mice rapidly succumb to otherwise sublethal infections (Sjöstedt *et al.*, 1994). Infection is exacerbated, but not always lethal, in neutrophil-depleted immune mice, implying a small contribution by neutrophils in resistance to reinfection. However, depletion of neutrophils has little effect on *F. tularensis* growth during the first 2 days. Therefore, rather than by ingestion and direct killing, neutrophils have been suggested to function by secreting cytokines which attract and help activate macrophages and T cells at the site of infection (Sjöstedt *et al.*, 1994).

Early lymphocyte-independent resistance to infection has been shown using LVS infection of lymphocyte-deficient *scid* mice (Elkins *et al.*, 1996). *scid* mice lack both mature B and T lymphocytes. Intradermally infected *scid* mice survive for about 20 days. This short term survival is dependent on IFN-γ, TNF-α, and neutrophils as mice depleted of any one of these die within 1 week of infection. Other studies using gamma interferon knockout (GKO) mice, and IFN-γ and TNF-α administration or depletion in normal mice have further emphasized the essential role of these cytokines early in infection (Anthony *et al.*, 1989; Leiby *et al.*, 1992; Green *et al.*, 1993; Elkins *et al.*, 1996). It should be noted that the requirement for TNF-α and IFN-γ in protective immunity to reinfection is apparently dependent

on the size of the challenge inoculum; as challenge inocula exceed 10 LD_{50}s, induction of a TNF-α- and IFN-γ-dependent immune response becomes necessary to clear the infection (Sjöstedt *et al.*, 1996). T cell-deficient mice infected by the id route survive for about 30 days, suggesting that B cells may augment this early inflammatory response (Elkins *et al.*, 1993; Conlan *et al.*, 1994; Elkins *et al.*, 1996). Recently, Culkin *et al.* (1997) identified a role for B cells in a novel, early protective immune response to LVS. Mice subjected to sublethal id infection (priming) rapidly develop protective immunity to reinfection by any route (Elkins *et al.*, 1992). Within 3 days after priming with LVS, mice are capable of surviving a lethal ip LVS challenge of 10^4 LD_{50}s. On the other hand, unprimed mice die within 4 to 8 days. Several lines of evidence indicate that this immunity requires B cells but not T cells: i) nude mice can generate this immune response (Elkins *et al.*, 1993); ii) early protective immunity in *scid* mice can be reconstituted with nude mouse or T-cell-depleted normal mouse spleen cells (Culkin *et al.*, 1997); iii) total T cell knockout mice survive id priming and ip challenge (Culkin *et al.*, 1997); iv) B cell knockout mice are defective for expression of early protective immunity (Culkin *et al.*, 1997). Primed mice are not protected against a subsequent challenge with *S. typhimurium* but are protected from a *L. monocytogenes* challenge (Culkin *et al.*, 1997), indicating protection is somewhat non-specific. This is consistent with the rapid development and T-cell-independent nature of this response (Elkins *et al.*, 1993). Anti-LVS

immunoglobulin M (IgM) (which is not protective) and very low levels of immunoglobulin G (IgG) antibodies are detected during the time course of early protective immunity. It is suggested that rather than by secreting specific antibodies secretion, B cells may participate by secreting soluble mediators which aid in macrophage activation directly or indirectly by enhancing cytokine production from other cells [e.g. IFN-γ from natural killer (NK) cells].

Humoral factors

F. tularensis infection induces a strong humoral immune response, but cell-mediated immunity plays a more important role in resistance to tularemia. In human vaccinees, antibodies to *F. tularensis* are first detected at 2 weeks after vaccination (Koskela and Herva, 1982). Diagnostically significant agglutination titers are not observed until 4 weeks post-vaccination, and reach maximal levels at 1.8 months, in parallel with maximal IgM titers. Immunoglobulin A (IgA) and IgG reach their respective maximal titers 1 week and 1 month later than IgM. These antibody titers decline but persist in significant amounts for up to 11 years, with IgG being the dominant class (Koskela and Herva, 1982). In LVS-infected mice, high levels of IgG2a and IgM are detected 4 weeks after sublethal id infection, and reach peak levels at 2 weeks post-infection (Rhinehart-Jones *et al.*, 1994). However, individuals vaccinated with a killed vaccine preparation are poorly protected in

spite of the presence of serum agglutinating antibodies (Burke, 1977).

The contribution of specific antibody to protection against tularemia has been evaluated by passive transfer of immune serum to non-immune animals. Thorpe and Marcus (1965) reported that immune serum does not alter the mortality rates of white mice, guinea pigs, or rabbits after subcutaneous (sc) challenge with a fully virulent type A strain. Transfer of serum from LVS-immunized rats to naive rats also does not protect against LVS replication in the spleen or liver (Kostiala *et al.*, 1975). Anthony and Kongshavn (1987) observed that treatment of mice with immune serum prior to iv LVS infection results in enhanced clearance from blood, preferential localization in the liver, and an overall two-fold reduction in bacteria recovered from the livers and spleens. A small protective effect of immune serum has been demonstrated in mice challenged with *F. tularensis* strains of low virulence but not against strains of high virulence (Pannell and Downs, 1953). Others have also reported transfer of immunity to lethal LVS infection in mice by immune serum and have attempted to analyze the mechanism of protection (Fortier *et al.*, 1991; Rhinehart-Jones *et al.*, 1994). Transfer of protection against an ip challenge of 3000 LD_{50}s was shown to be mediated by a high-titer IgG fraction of immune mouse serum (Rhinehart-Jones *et al.*, 1994). This is notably lower than the protection seen in intact immune animals, which can withstand ip challenges of 10^6 LD_{50}s. Although bacterial replication occurs in

reticuloendothelial organs (liver, spleen, and lungs) of mice treated with immune or normal serum, mice treated with immune serum have lower bacterial burdens and eventually clear the infection after 22 days. Clearance of the infection requires production of IFN-γ by recipient mice. It also requires T cells, as the protective effects of immune serum in athymic nude mice are only observed in animals reconstituted with T cells from normal mice. Thus, a specific antibody response may be a minor contributor in the immune response to *Francisella* infection, but as with other intracellular pathogens, clearance of the infection and survival of the host ultimately depends on the development of a specific T cell-mediated immune response. This is discussed in greater detail below.

Cellular factors

Many studies have emphasized the importance of cell-mediated immunity in resistance to *F. tularensis* infection. Several investigators made use of delayed-type hypersensitivity (DTH) as an indicator of cell-mediated immune responses to *F. tularensis*. DTH is demonstrable in humans early after either vaccination with LVS or natural infection (Buchanan *et al.*, 1971; Koskela and Herva, 1982). Claflin and Larson (1972) showed mice develop DTH to LVS after vaccination with live LVS and survive challenge with a virulent *F. tularensis* type A strain. Killed antigen preparations fail to elicit DTH and resistance to infection. Correlation between cellular

hypersensitivity and resistance to infection has also been reported in rats (Kostiala *et al.*, 1975) and humans (Burke, 1977).

Others have shown that both tularemia patients and LVS vaccinees develop immunospecific T cell responses to killed *F. tularensis* as measured by either *in vitro* lymphocyte blast transformation assays or isolation of T lymphocyte lines from peripheral blood mononuclear cells (PBMCs) (Tärnvik *et al.*, 1985; Surcel *et al.*, 1989; Surcel *et al.*, 1991). The activated T lymphocytes are $CD4^+$ and, after stimulation with *F. tularensis* antigen, respond with the production of IL-2 and IFN-γ (Surcel *et al.*, 1991). This pattern of cytokine expression resembles the phenotype of the type 1 subset of $CD4^+$ T helper cells (Th1) which support macrophage activation (Romagnani, 1996). This is consistent with the cytokine expression pattern observed in the liver during the early stages of murine tularemia, which includes IL-12 and IFN-γ, cytokines that promote the development of a Th1 response (Golovliov *et al.*, 1995; Romagnani, 1996).

More detailed analyses of the role of T cells in immunity to *F. tularensis* have been conducted using rodent models of infection. Thoracic duct lymphocytes (TDLs) from *Francisella* immune donor rats confer resistance to LVS replication in the spleens and livers of naive rats (Kostiala *et al.*, 1975). Transfer of immune serum had no effect on resistance to infection. Similar results have been reported in mice using non-adherent spleen cells (Anthony *et al.*, 1987; Fortier *et al.*, 1991). T cell-depleted mouse spleen cells from LVS

immunized mice fail to protect non-immune mice against lethal LVS ip infection (Fortier *et al.*, 1991). Suppression of T cell-mediated immunity in mice by treatment with cyclosporin A renders them significantly deficient in their ability to resolve primary LVS infection (Anthony *et al.*, 1987). Long-term survival and clearance of bacteria after primary LVS challenge of nude (*nu/nu*) mice requires *nu/+* spleen cells, which serve as a source of mature T cells (Elkins *et al.*, 1993). Reconstitution studies in *scid* mice have shown that B220$^-$ (B cell$^-$)/T$^+$ lymphocytes confer long-term survival of id LVS infection and clearance of bacteria from recipient mice (Elkins *et al.*, 1996).

More recently, Yee *et al.* (1996) have examined the relative contributions of T cell subsets to primary and secondary immunity to *F. tularensis*. Mice depleted of CD4$^+$ or CD8$^+$ T cells by either antibody treatment or gene disruption survive and clear bacteria after id infection with LVS. These immune mice are also resistant to subsequent lethal ip challenge of 10^4 LD_{50}s. In contrast, $\alpha\beta$ TCR$^-$ (T cell receptor) mice die within 35 days after sublethal id infection. Similar results have been obtained using mice depleted of CD4$^+$ and CD8$^+$ T cells by combined thymectomy and treatment with anti-CD4 and anti-CD8 monoclonal antibodies (Conlan *et al.*, 1994). Collectively, the results to date indicate that T cells are essential for control and elimination of primary *Francisella* infections as well as protective immunity to reinfection. However, either CD4$^+$ or CD8$^+$ T

cells are sufficient to mediate long-term survival and generate protective immunity.

RATIONALE OF RESEARCH

Intracellular pathogens cause infections of significant morbidity and mortality in humans worldwide. For example, the agents of dysentery (*Shigella* spp.), gastroenteritis and typhoid fever (*Salmonella*), tuberculosis (*Mycobacterium tuberculosis*), and Legionnaire's disease (*L. pneumophila*) are well known for the serious risks they pose to human health. Since replication within eukaryotic cells is critical for the virulence of these pathogens, an understanding of the bacterial determinants contributing to this process may lead to the development of therapeutic agents to combat this class of microbial pathogens.

The major objective of the research described here is to identify and characterize the factors necessary for bacteria to grow inside host cells. To accomplish this task, the Gram-negative, facultative intracellular pathogen *Francisella* was chosen as a model organism. The experiments in this study have employed *F. novicida* for several reasons. *F. novicida* grows quickly in laboratory media, allowing the rapid analysis of recombinant strains and the production of large quantities of cellular material for biochemical studies (e.g. protein purification). It shows low infectivity for humans, yet remains highly virulent in mice, a natural animal host for this organism. The pathology of *F. novicida* infection in mice resembles that of the very closely related human and animal pathogen *F. tularensis*. However, unlike *F. tularensis*, *F. novicida* is amenable to basic genetic

manipulation (transformation, complementation, mutagenesis), permitting the use of both biochemical and genetic approaches to identifying virulence factors. It is assumed that at least some of the molecules affecting the intracellular growth of *Francisella* will be conserved in other intracellular pathogens.

COLLABORATIONS

The following is a list of individuals who contributed to some of the experiments described in this study:

a) **Dr. M.S. Kuhlenschmidt, Dr. T.J. Reilly** (Department of Pathobiology, College of Veterinary Medicine, University of Illinois). Performed the purification, amino acid sequencing, mass spectrometry, and all enzymatic studies of AcpA.

b) **Sandy Kielland** (University of Victoria). Performed N-terminal amino acid sequencing of electroblotted 70 kDa protein.

c) **David Fenton** (University of Victoria). Performed construction of pGB3Em and helped in initial screening of the library of GB2 transformants.

d) **Steve Hendy** (University of Victoria). Provided advice and helpful discussions on performing CAT assays.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used in this study are listed in Table 1. Wild-type *F. novicida* U112 was obtained from the American Type Culture Collection (ATCC). *F. novicida* strains were cultured at 37 °C in tryptic soy broth (Difco) containing 0.1% cysteine hydrochloride (Sigma) (TSB-C), or on either cystine heart agar (Difco) supplemented with 5% defibrinated horse blood (PML Microbiologicals) (CHA-B) or LB agar (Sambrook *et al.*, 1989) containing 0.1% cysteine hydrochloride, 0.2% glucose, 40 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma) (X-p) and adjusted to pH 6.0 [LB (X-p)]. Erythromycin (Em, 25 μg/ml) and kanamycin sulfate (Km, 15 μg/ml) were added where necessary. *E. coli* strains were grown at 37 °C in LB broth supplemented with sodium ampicillin (Ap, 250 μg/ml), disodium carbenicillin (Cb, 250 μg/ml), erythromycin (Em, 250 μg/ml), or kanamycin sulfate (Km, 30 μg/ml) as required.

Isolation of a spontaneous mutant of *F. novicida*

When grown on LB (X-p) medium, colonies of wild-type *F. novicida* are pinpoint and intensely blue. On one plate, a large colony was observed which appeared light blue. This colony was restreaked on LB (X-p) medium and designated GB2.

Table 1. Bacterial strains, plasmids, and bacteriophages.

	Description	Reference or source
F. novicida		
U112	Wild-type	Larson *et al.*, 1955
JMB1	U112, *recA*::mTn*10*Km	Berg *et al.*, 1992
GB2	U112, *mglA*	this study
GB2-C3	GB2, 20-40 kb *Tsp*509I insert containing *mglA*::Em^R cassette	this study
GB2/pGB40Em	GB2, *mglAB*$^+$	this study
GB5	U112, *mglA*::mTn*10*Km	this study
GB6	U112, *mglB*::mTn*10*Km	this study
GB7	U112, *mglA'-cat*, *mglAB*$^+$	this study
GB8	U112, *mglC*::mTn*10*Km	this study
2L	U112, *acpA*::Em^R	this study
E. coli		
BL21	F$^-$ *ompT hsdS* ($r_B^-m_B^-$) *gal*	Grodberg and Dunn, 1988
DH11S	F$^-$*mcrA* Δ(*mrr hsdRMS mcrBC*) Δ (*lac-proAB*) Δ (*recA*1398) *deoR rpsL srl thi*/F' *proAB*$^+$ *lacI*^{q}*Z* Δ M15	Gibco BRL
DH5α	*supE*44 D *lac*U169 (ϕ80*lacZ* Δ M15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Sambrook *et al.*, 1989
E131	DH5α (λ-CH616)	Haas *et al.*, 1993
JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ(*lac-proAB*)	Sambrook *et al.*, 1989
XL-1 Blue	*supE44 hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi relA*1 *lac*- F'[*proAB*$^+$ *lacI*^{q} *lacZ* Δ M15Tn*10*(*tet*^{R})]	Bullock *et al.*, 1987

XL-1 Blue	*supE44 hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi relA*1 *lac*- F'[*proAB*+ *lacI*q *lacZ* Δ M15Tn*10*(*tet*R)]	Bullock *et al.*, 1987
Plasmids		
pTZ18U	ApR phagemid	Mead *et al.*, 1986
pUC18	ApR	Vieira and Messing, 1982
pRL498	KmR	Elhai and Wolk, 1988
pTn*Max2*	EmR, source of EmR cassette	Haas *et al.*, 1993
pQE30	ApR, expression vector	Qiagen
pREP4	KmR, multicopy plasmid containing *lacI*	Qiagen
pGB3	pUC18 with *acpA* *Hin*dIII insert	this study
pGB3Em	pGB3, *acpA*::EmR	this study
pC3-20	EmR, EmR cassette and *Sau*3AI insert containing *mglA*	this study
pGB40	pTZ18U with *mglAB*+ *Hin*dIII insert	this study
pGB40Em	pGB40 with EmR cassette in *Eco*RI site	this study
pGB48	pTZ18U with *mglA'* *Sac*I/*Bgl*II insert	this study
pGB48Em	pGB48 with EmR cassette in *Eco*RI site	this study
pGB51	pQE30 with *Bam*HI/*Hin*dIII *mglA* PCR product	this study
pGB56	pQE30 with *Bam*HI/*Hin*dIII *mglB* PCR product	this study
pGB60	pTZ18U with *mglABC* *Sac*I insert	this study

pGSB9	Ap^R Em^R, source of Em-Cm cassette	this study
pEmCm-1.6	Em^R, *mglA'-cat* fusion using Em-Cm cassette	this study
pGB63	pRL498 with 6 kb *Sac*I insert, hybridizes with 70kD-N_2 oligonucleotide	this study
pGB64	pUC18 with 6 kb *Sac*I insert from pGB63	this study
Bacteriophages		
λ1105	Replication-defective λ carrying mTn*10*Km	Way *et al.*, 1984
M13mp18	single-stranded filamentous phage	Yanisch-Perron *et al.*, 1985

Intracellular growth assay

The murine macrophage-like cell line J774A.1 [ATCC TIB-67 (Ralph and Nakoinz, 1975)] was cultured at 37 °C in a humidified atmosphere of 5% carbon dioxide in Dulbecco's Modified Eagle Medium (Gibco BRL Life Technologies Inc.) containing 5% (v/v) fetal bovine serum (Hyclone) (DMEM). Cell-line macrophages were harvested by gentle scraping and concentrated by centrifugation. Inflammatory macrophages were isolated by peritoneal lavage of female BALB/c mice (University of Victoria Animal Care facility) 3 days after intraperitoneal injection of 4% (w/v) Brewer's thioglycollate medium (Difco) and cultured in DMEM containing 10% fetal bovine serum. Intracellular growth of *F. novicida* strains was assayed as described previously (Anthony *et al.*, 1991a). Briefly, macrophages were plated in 96-well plates at a density of approximately 2 x 10^5 cells/well. *F. novicida* strains were grown in TSB-C and diluted in DMEM to approximately 2-5 x 10^6 colony forming units/ml (cfu/ml). Macrophage monolayers were inoculated with 150 µl of the bacterial suspension and centrifuged at 600 x *g* to enhance the association between bacteria and macrophages. Following incubation for 1 h to allow for phagocytosis, the wells were washed three times and incubated with 150 µl of DMEM. Bacterial growth was enumerated at various times by addition of 150 µl of 0.2% sodium deoxycholate (Sigma) in saline and plating serial dilutions of the macrophage lysates. Results shown are the average $\log_{10}$ cfu of triplicate wells ± 1 standard deviation (SD).

Mice and experimental infections

Female, specific-pathogen-free C57BL/6NCrlBR mice were purchased from Charles River Canada. *F. novicida* strains were cultured in TSB-C to a density of approximately 10^9 cfu/ml and stored in aliquots at -80 °C. Prior to infection, aliquots were thawed and diluted in saline to a concentration of about 0.5-1 x 10^5 cfu/ml. Mice were then injected intraperitoneally with 200 µl of diluted bacteria. Bacterial concentrations were verified retrospectively by plating serial dilutions on CHA-B. At specified timepoints, mice were euthanized by CO_2 asphyxiation and cervical dislocation. Spleens were removed using aseptic technique and homogenized in sterile 0.85% saline. Spleen homogenates were serially diluted and plated on CHA-B to determine bacterial numbers. Results represent the averages of four mice per timepoint ± 1 SD $\log_{10}$ cfu per spleen.

Deoxycholate and complement sensitivity assays

Assays for deoxycholate and complement sensitivity were performed essentially as described by others (Mdluli *et al.*, 1994). Briefly, overnight cultures were washed 3 times in phosphate-buffered saline (PBS) and diluted in PBS to a concentration of approximately 10^6 cfu/ml. One hundred microlitres of the bacterial suspension was added to the wells of a 96-well plate. To assess sensitivity to deoxycholate, 100 µl of either PBS or 0.2% sodium deoxycholate in saline was added to the bacteria and the mixture

was incubated at room temperature for 30 min. For complement sensitivity assays, 100 µl of either normal or heat-inactivated (56 °C, 30 min) guinea pig complement (Cedarlane Laboratories) was added to the bacteria and incubated at 37 °C for 4 h. After the incubation period, samples were diluted and plated on CHA-B to measure bacterial numbers. Results are based on the average percent survival ± 1 SD using triplicate wells.

Complementation of *F. novicida* GB2 for growth in macrophages

Chromosomal DNA from wild-type *F. novicida* was partially digested with the restriction endonuclease *Tsp*509I (New England Biolabs) which recognizes the tetranucleotide sequence AATT and cleaves very frequently in the AT-rich *Francisella* genome. The resulting DNA fragments were separated by pulsed field gel electrophoresis on a 1% agarose gel (24 h, 185 V, 5-80 s ramp; 15 °C), and 20-40 kb fragments were isolated by electroelution from excised gel slices. These fragments were ligated to an *Eco*RI-digested erythromycin resistance (Em^R) cassette derived from pTn*Max2* (Haas *et al.*, 1993). After ligation, this DNA was transformed (Mdluli *et al.*, 1994) into the spontaneous mutant GB2 where it integrated into the chromosome by homologous recombination. Em^R transformants were selected by plating on CHA-B containing Em. Stationary cultures of the Em^R transformants were subsequently grown overnight in 96-well plates in TSB-C supplemented with Em in 288 pools of 10

colonies/pool. These pooled cultures were used to infect monolayers of J774 macrophages as described above. Intracellular growth was monitored over a 48 h period by microscopic examination. Wells displaying evidence of intracellular growth were treated with 0.1% sodium deoxycholate in saline, and the resulting macrophage lysates were serially diluted and plated to recover the complemented bacteria.

Recombinant DNA techniques and DNA sequencing

Standard recombinant DNA procedures were used (Sambrook *et al.*, 1989). The *E. coli* strains JM109 and XL-1 Blue were used for routine cloning experiments. Restriction endonucleases and T4 DNA ligase were purchased from Gibco BRL Life Technologies Inc. or New England Biolabs. Chromosomal DNA was prepared by the method of Wilson (1987). Transposon shuttle mutagenesis of *F. novicida* using the mTn*10*Km transposon was performed as described previously (Anthony *et al.*, 1991b) with the exception that plasmid DNA prepared from λ1105-infected DH5α carrying the target recombinant plasmid was transformed into JM109 to isolate the mutagenized plasmids. The modification was necessary to overcome the apparent instability of recombinant plasmids carrying the mTn*10*Km element in DH5α. Transposon shuttle mutagenesis of *F. novicida* using the Tn*Max2* transposon was performed as described by Haas *et al.* (1993), followed by transformation of *F. novicida* with the mutagenized recombinant plasmids (Anthony *et al.*, 1991b).

DNA sequencing of both strands of the 1798 bp region spanning *acpA* and the 1309 bp region spanning *mglAB* was performed using the dideoxy chain-termination method (Sanger *et al.*, 1977) with a commercial T7 DNA polymerase ('Sequenase', US Biochemicals) or *Taq* DNA polymerase ('AmpliTaq DNA polymerase, CS', Perkin-Elmer). [α-^{35}S]dATP (1000 mCi/mmol) was purchased from NEN Life Science Products. Both universal and reverse as well as custom-designed primers were used in the sequencing reactions according to the manufacturer's instructions. Single-stranded templates were prepared from M13mp18 or phagemid pTZ18U clones in DH11S. Double-stranded templates were derived from pTZ18U clones in XL-1 Blue. DNA and deduced amino acid sequence information was analyzed using the family of programs included in Lasergene (DNASTAR). Multiple sequence alignments were conducted using the ClustalW program (version 1.7) available on-line from the Human Genome Center at the Baylor College of Medicine (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) and modified by inspection. Amino acid sequence similarities to sequences in protein databases available on-line through the National Center for Biotechnology Information were determined using the BLASTP or BLASTX programs (Altschul *et al.*, 1990). The *acpA* sequence was assigned the Genbank accession number L39831. The sequences of *mglA* and *mglB* have been submitted to Genbank and are available under the accession number AF045772.

Plasmid construction

A reverse genetics approach was used to clone the *acpA* gene encoding a respiratory-burst inhibiting acid phosphatase (AcpA) of *Francisella*. Purification and sequencing of portions of AcpA from *F. tularensis* B38 were performed by Dr. Thomas J. Reilly and Dr. Mark S. Kuhlenschmidt. A nondegenerate oligonucleotide (ACP#1: 5'-ACI GAT GTI AAT AAT III AAA CCI AAT GAT TAT GG-3') was prepared by reverse translation of the N-terminal peptide sequence. The codon usage in the *valAB* locus of *F. novicida* (Mdluli *et al.*, 1994) was used as a guide in designing the oligonucleotide. The oligonucleotide was 3'-end-labelled (ECL 3'-oligolabelling system, Amersham) as per the manufacturer's instructions and used to screen a *F. tularensis* LVS genomic library of partial *Sau*3AI fragments cloned into the *Bam*HI site of the vector pTZ18U. The oligonucleotide was found to hybridize specifically to *Francisella* DNA using a hybridization and stringency wash temperature of 50 °C. One of the hybridizing clones contained a 1.3 kb DNA insert (pACP1). Partial sequencing of this insert revealed one open reading frame (ORF) with a deduced amino acid sequence identical to the N-terminal amino acid sequence and the sequence of an internal peptide generated by cleavage of AcpA with cyanogen bromide (CNBr). This insert was used as a probe to identify a 3.1 kb *Hin*dIII fragment of *F. novicida* DNA that was cloned into pUC18 to create pGB3. The *F. novicida acpA* gene was sequenced to facilitate future experiments which can most easily be done in *F. novicida*.

To construct an *acpA* mutant strain of *F. novicida*, a plasmid containing a mutated gene was created. The Em^R cassette of pTn*Max2* was excised as a *Bam*HI fragment and ligated to pGB3 digested with *Bcl*I. This resulted in the replacement of an approximately 300 bp *Bcl*I fragment within *acpA* by the Em^R cassette, effectively truncating 486 bp from the 3' end of the gene. The new plasmid, designated pGB3Em, was transformed into wild-type *F. novicida* to generate an allelic replacement with the mutant *acpA* gene.

Southern hybridization analysis of *Hin*dIII-digested wild-type *F. novicida* chromosomal DNA using the locus cloned in pC3-20 as a probe identified a hybridizing fragment of approximately 2.9 kb. This fragment was cloned into the *Hin*dIII site of pRL498 using size-fractionated, *Hin*dIII-digested *F. novicida* chromosomal DNA and designated pGB39. Subcloning of this *Hin*dIII fragment into the *Hin*dIII site of pTZ18U generated pGB40. To create pGB40Em, the Em^R cassette of pTn*Max2* was excised as an *Eco*RI fragment and cloned into the *Eco*RI site in the multiple cloning site of pGB40.

To determine the ability of *mglA* to complement GB2 for intracellular growth, a 0.7 kb *Sac*I/*Bgl*II insert from pGB40 containing the first 522 bp of *mglA* was subcloned into *Sac*I/*Bam*HI-digested pTZ19U to form plasmid pGB48. To add a selectable marker to pGB48 which would function in *F. novicida*, the Em^R cassette of pTn*Max2* was subcloned as a *Eco*RI fragment into the *Eco*RI site of pGB48 to create pGB48Em.

All polymerase chain reactions (PCR's) described below were carried out using 50 µl reaction volumes and a mixture of *Taq* DNA polymerase (Pharmacia) and *Pfu* DNA polymerase (Stratagene) in a ratio of 12/1 (u/u) to minimize the number of *Taq* polymerase-induced errors (Cline *et al.*, 1996). PCR reactions also contained primer concentrations of 0.4 µM, 200 µM of each dNTP, and 2.5 mM $MgCl_2$ unless noted otherwise.

To clone the *mglA* allele from GB2, a 2 kb fragment containing *mglA* was amplified by PCR from GB2 chromosomal DNA. The primers used were mgl-EcoRI-2 (5'-CTTGAAAAAGAATTCCAAAACCAGCCA-3'), which corresponds to a region about 1.1 kb upstream of *mglA*, and mgl-PstI-1 (5'-AACCAAATTCTGCAGCATACATTCCT-3'), which corresponds to position 288-313 on the antisense strand of *mglB*. The sequences of both primers were modified (with respect to their complementary sequences) to incorporate internal restriction sites for *Eco*RI and *Pst*I. The reaction was carried out using 3.5 mM $MgCl_2$ and 5 cycles of denaturing (94 °C, 30 s), annealing (44 °C, 60 s), and extension (72 °C, 120 s), followed by 30 cycles of denaturing (94 °C, 30 s), annealing (50 °C, 30 s), and extension (72 °C, 120 s). The PCR product was digested with *Sac*I and *Bcl*I to yield an 873 bp fragment containing the entire *mglA* sequence which was cloned into *Sac*I/*Bam*HI-cut pTZ19U. This plasmid was designated pGB54.

Overexpression of *mglA* was achieved by amplifying the *mglA* gene by PCR and subcloning this fragment into the His_6-tag

expression vector pQE30 (Qiagen). The reaction used the primers mglA-F1 (5'-ACTAGGAGGGATCCATCTTGCTT-3') and mglA-R2 (5'-GCTCCTTTAAGCTTGATAGTTTT-3') with pGB40 serving as the template DNA. The reaction was performed using 25 cycles of denaturing (94 °C, 30 s), annealing (44.5 °C, 30 s), and extension (72 °C, 60 s). The (631 bp) PCR product was digested with *Bam*HI (GGATCC) and *Hin*dIII (AAGCTT) and cloned into pQE30 digested with *Bam*HI and *Hin*dIII to create plasmid pGB51. The vector/insert junction and the *mglA* insert were sequenced to ensure the absence of PCR-induced mutations.

For the purpose of overexpression, *mglB* was cloned into the expression vector pQE30. The *mglB* gene was amplified by PCR from pGB40 using the primers mglB-F1 (5'-CTTAATATGGGATCCCTTAGAGCA-3') and mglB-R1 (5'-AAGAGTAAAAAGCTTATCTAATGAAAG-3'). The reaction was performed using 25 cycles of denaturing (94 °C, 30 s), annealing (44.5 °C, 30 s), and extension (72 °C, 40 s). The 426 bp PCR product was digested with *Bam*HI and *Hin*dIII and cloned into *Bam*HI/*Hin*dIII-cut pQE30, generating the plasmid pGB56. DNA sequencing confirmed the integrity of the vector/insert junction and the absence of PCR-induced mutations in *mglB*.

To monitor gene expression in *F. novicida*, a cassette was constructed which permits transcriptional fusions to a promoterless *cat* gene. First, a plasmid consisting of only the Em^R cassette from pTn*Max2* (designated pEm^R) was created by isolating the Em^R

cassette from *Eco*RI-digested pTn*Max2*, self-ligating, and transforming into *E. coli* E131. Next, a promoterless *cat* cassette was excised from pCM7 (Pharmacia) as a *Hin*dIII fragment, treated with Klenow DNA polymerase to create flush ends, and ligated to *Sma*I-digested pRL498. The *cat* cassette was then isolated from the resulting plasmid (pRL498Cm) by digestion with *Bam*HI and treated with Klenow DNA polymerase in the presence of dATP and dGTP to partially fill-in the 5' overhang as described previously (Zabarovsky and Allikmets, 1986). This fragment was subsequently ligated at 4 °C overnight to similarly treated (in the presence of only dCTP and dTTP) *Sal*I-digested pEm^R and transformed into E131. Clones were screened by analysis of restriction digests for the presence of the *cat* cassette oriented in the opposite direction relative to the direction of transcription of *ermC'* in pEm^R. One clone confirmed to have this orientation was designated pEmCm. Finally, digestion of pEmCm with *Bam*HI released the Em-Cm cassette as a 2.1 kb fragment which was then cloned into the *Bam*HI site of pTZ18U generating plasmid pGSB9. The manipulations above resulted in the modification of the 5' region of the *cat* cassette by 15 bp: 5'-(G)GATCCGTCGATCCCCAGCTT-3' (*Bam*HI and former *Hin*dIII sites underlined).

A reverse genetics approach was used in attempts to clone the gene encoding a 70 kDa secreted protein of *F. novicida*. Based on the N-terminal amino acid sequence of the 70 kDa protein, two degenerate oligonucleotides were prepared (70kD-N_1: 5'-ATG CAT CAA CC(A/T) CAT CAA TAT-3'; 70kD-N_2: 5'-AAT AAA ATI GGI ATI

GAT AC(A/T) CAA TAT ATI GAT-3') which correspond to independent sections of the N-terminal sequence. Selections of codon usage were guided by a codon usage table (Table 2) compiled from the sequences of 13 *Francisella* genes. Southern blots were performed at various temperatures to determine the conditions under which each oligonucleotide probe bound specifically to *F. novicida* chromosomal DNA digests. When hybridized at 42 °C, the oligonucleotide 70kD-N_1 bound specifically to an approximately 2.8 kb *Xba*I DNA fragment. To clone this fragment, size-fractionated (2.5 - 3.0 kb), *Xba*I-digested *F. novicida* chromosomal DNA was cloned into pTZ18U. A clone hybridizing to 70kD-N_1 was identified and designated pGB62. Later analysis of pGB62 revealed it contains two *Xba*I inserts of 2.6 and 2.9 kb in size. Southern hybridization at 40 °C with the oligonucleotide probe 70kD-N_2 identified a 6 kb *Sac*I fragment which was cloned into pRL498 to create plasmid pGB63. Subcloning of the 6 kb *Sac*I insert in pGB63 into the *Sac*I site of pUC18 created the plasmid pGB64.

Table 2. Codon Usage Preferences of *Francisella*

Amino acid	Codon	Usage	% Usage	Amino acid	Codon	Usage	% Usage
Ala (388)	GCA	117	30.2	Leu	CUU	65	20.6
	GCC	8	2.06		UUA	135	42.7
	GCG	29	7.47		UUG	30	9.49
	GCU	234	60.3	Lys	AAA	274	78.1
Arg (133)	AGA	81	60.9		AAG	77	21.9
	AGG	11	8.27	Met	AUG	97	100
	CGA	5	3.76	Phe (155)	UUC	35	22.6
	CGC	5	3.76		UUU	120	77.4
	CGG	4	3.01	Pro (144)	CCA	71	49.3
	CGU	27	20.3		CCC	1	0.69
Asn (247)	AAC	70	28.3		CCG	8	5.56
	AAU	177	71.7		CCU	64	44.4
Asp (276)	GAC	31	11.2	Ser (268)	AGC	27	10.1
	GAU	245	88.8		AGU	75	28
Cys (37)	UGC	9	24.3		UCA	67	25
	UGU	28	75.7		UCC	4	1.49
Gln (163)	CAA	125	76.7		UCG	6	2.24
	CAG	38	23.3		UCU	89	33.2
Glu (278)	GAA	177	63.7	Ter (13)	UAA	12	92.3
	GAG	101	36.3		UAG	1	7.69
Gly (334)	GGA	50	15		UGA	0	0
	GGC	50	15	Thr (276)	ACA	100	36.2
	GGG	9	2.69		ACC	8	2.9
	GGU	225	67.4		ACG	16	5.8
His (54)	CAC	7	13		ACU	152	55.1
	CAU	47	87	Trp	UGG	24	100
Ile (321)	AUA	122	38	Tyr (130)	UAC	36	27.7
	AUC	64	19.9		UAU	94	72.3
	AUU	135	42.1	Val (296)	GUA	103	34.8
Leu (316)	CUA	67	21.2		GUC	22	7.43
	CUC	5	1.58		GUG	24	8.11
	CUG	14	4.43		GUU	147	49.7

Numbers in parentheses indicate total number of codons analyzed. Values are based on the codon usages as deduced from the sequences of the following 13 genes (accession numbers in parentheses): 23 kDa protein (Y08861), *valAB* (L17003), *acpA* (L39831), *mglAB*, *fopA* (M93695), *grpE-dnaK-dnaJ* (L43367), *cpn10* (X98853), *cpn60* (X98853), and 17 kDa lipoprotein/TUL4 (M32059).

Isolation of plasmids which complement GB2 for intracellular growth

Chromosomal DNA (0.5 μg) isolated from a GB2 strain complemented for growth in macrophages (designated GB2-C3) was partially digested with *Sau*3AI, self-ligated (at a DNA concentration of 40 μg/ml), and transformed into *E. coli* E131 by electroporation. Recombinant DNA molecules containing the Em^R cassette replicate as plasmids in E131 using the bacteriophage fd origin of replication encoded in the cassette. Plasmids isolated in this manner were then used to transform GB2 to screen for the ability to complement GB2 for growth in macrophages. One plasmid of approximately 5 kb, pC3-20, was chosen for further analysis.

SDS-PAGE and Western immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described previously (Laemmli, 1970) using 12% or 15% separating and 4% stacking gels. Proteins were visualized by staining with Coomassie brilliant blue R250 [0.1% (w/v) Coomassie blue in 40% (v/v) methanol/10% (v/v) acetic acid] followed by destaining in 40% methanol/10% acetic acid.

For Western blots, proteins were electrophoretically transferred to Immobilon-P membranes (Millipore) using a semi-dry transfer apparatus (Multiphor II Novablot, Pharmacia LKB) as per the manufacturer's recommendations. The membranes were blocked overnight with 5% skim milk (Difco) in Tris-buffered saline (50 mM

Tris-HCl, 137 mM NaCl, [pH 7.5]) containing 0.1% Tween-20 (TBS-T), followed by incubation in antiserum diluted in TBS-T. Chemiluminescent detection of bound primary antibody was achieved using horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG secondary antibody (Cedarlane Laboratories Ltd.) diluted in TBS-T and Renaissance chemiluminescence detection reagent (NEN Life Science Products).

Overexpression and purification of recombinant MglB and MglA

Overexpression of *mglB* was performed in *E. coli* BL21 carrying the *lacI* gene on a multicopy plasmid (pREP4) to increase control of transcription from the expression vector. Affinity purification of recombinant proteins was achieved essentially following the recommendations of the manufacturer of the metal affinity resin (CLONTECH Laboratories, Inc.). Fresh transformants of BL21/pREP4 with pGB56 were grown at 37 °C to mid-log phase in tryptone-phosphate medium (Wu *et al.*, 1995) containing 0.2% glucose, Km, and Cb. Expression of *mglB* was induced with the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and continued for 2 h. After chilling the culture on ice for 30 min, the cells were pelleted at 4000 x *g* for 15 min. Cell pellets were washed 2 times with sonication buffer (50 mM NaH_2PO_4, 10 mM Tris-HCl, 0.1 M NaCl, pH 8.0) and frozen at -80 °C. Frozen pellets were resuspended in 0.1 culture volumes of sonication buffer prior

to the addition of lysozyme (0.75 mg/ml) and phenylmethylsulfonyl fluoride (PMSF, 0.4 mM; Gibco BRL). After a 30 min incubation on ice, cells were lysed by sonication at a constant output of 60 W. Cell debris and unbroken cells were removed by centrifugation at 15000 x *g* for 20 min. The supernatant was transferred to a fresh tube containing 0.1 volumes of TALON Metal Affinity Resin (CLONTECH Laboratories, Inc.) pre-equilibrated with sonication buffer and rotated slowly for 1 h at 4 °C. Non-specifically bound proteins were removed by washing in a batch format with sonication buffer (4 x 10 bed volumes) followed by wash buffer (50 mM NaH_2PO_4, 0.3 M NaCl, 10% glycerol, pH 7.0; 3 x 10 bed volumes). Bound protein was eluted with elution buffer (50 mM NaH_2PO_4, 20 mM 2-[N-morpholino]ethanesulfonic acid (MES), 0.1 M NaCl, pH 5.3; 5 x 2.5 bed volumes).

Overexpression and purification of recombinant *mglA* was essentially as described for recombinant *mglB* with minor modifications. Fresh transformants of BL21/pREP4 with pGB51 were grown to mid-log phase at 37 °C. Expression of *mglA* was induced with the addition of IPTG and continued at either 37 °C for 3 h or 30 °C overnight. Bound protein was eluted from the resin with elution buffer adjusted to pH 5.0.

Preparation of antisera

Purified recombinant MglB was desalted in buffer A (20 mM NaH_2PO_4/10 mM $MgCl_2$, pH 7.4) by repeated centrifugal

ultrafiltration (Filtron). Approximately 570 μg of desalted protein was emulsified in complete Freund's adjuvant (Difco) and injected into a female New Zealand white rabbit at multiple sites. Two boost injections using 228 μg of protein emulsified in incomplete Freund's adjuvant (Difco) per injection were given 42 and 77 days later. Final collection of serum was conducted 28 days after the second boost injection. The serum was used in Western blots at a dilution of 1:1000.

Purified recombinant MglA was dialyzed overnight against buffer A and concentrated by lyophilization. Approximately 600 μg of protein was resuspended in sterile distilled water, emulsified in complete Freund's adjuvant, and injected into a female New Zealand white rabbit at multiple sites. Two boost injections using 300-350 μg of protein emulsified in incomplete Freund's adjuvant per injection were given 39 and 291 days later. Final collection of serum was conducted 10 days after the second boost injection. The final serum sample was filter sterilized and used in Western blots at a dilution of 1:1000.

Construction of an *mglA'-cat* fusion strain of *F. novicida*

A 1.6 kb *Bgl*II fragment containing the first 522 bp of *mglA* was isolated from pGB40. The *Bam*HI-EmCm cassette constructed above (pGSB9) was isolated, dephosphorylated by treatment with shrimp alkaline phosphatase (US Biochemicals), ligated to the 1.6 kb *Bgl*II fragment, and transformed into E131. A clone which contained the

1.6 kb *Bgl*II insert oriented as to create a transcriptional fusion of *mglA'* with the *cat* portion of the EmCm cassette was identified by analysis of restriction enzyme digests and designated pEmCm-1.6. After transformation of U112 with pEmCm-1.6, Em^R transformants were screened for growth in J774 macrophages. Of 24 transformants tested, all were positive for intracellular growth. Southern blot analysis of one transformant confirmed pEmCm-1.6 had integrated into the chromosome by a single cross-over homologous recombination event, thereby generating an *mglA'-cat* transcriptional fusion while preserving a wild-type copy of *mglAB*. This strain was designated GB7.

Regulation of *mglA* expression

i) preparation of cell extracts. To determine *mglA* expression levels *in vitro*, GB7 was cultured in Chamberlain's medium (Chamberlain, 1965). Overnight cultures of GB7 in Chamberlain's medium with 25 μg/ml Em were diluted 1/25 (v/v) in fresh medium and grown to early log phase (about 3 h). The cultures were then diluted again by 1/25 (v/v) in fresh pre-warmed medium and incubation was continued. Aliquots of the culture were taken at various timepoints and placed on ice. Cells were pelleted by centrifugation and washed 3 times with PBS. Prior to the final centrifugation step, the cells were split evenly to two tubes. One cell pellet was resuspended in PBS to determine bacterial numbers by

plating serial dilutions in triplicate. The second cell pellet was frozen in a dry ice/ethanol bath and stored at -80 °C.

To determine *mglA* expression levels in macrophages, J774 macrophages were plated at a cell density of $1x10^6$ cells/well in 6-well plates and cultured for 24 h prior to use. Infections were performed essentially as described above with a minor modification. Macrophages were infected with overnight stationary phase cultures of GB7 grown in Chamberlain's medium (with Em) and diluted in DMEM at a bacteria:macrophage ratio of about 50:1. At specified timepoints, infected macrophage monolayers were washed 3 times with PBS (room temperature) followed by a final vigorous wash with cold PBS to facilitate removal of the cells. An aliquot of the cell suspension was removed and lysed with 0.1% sodium deoxycholate to determine bacterial numbers (one agar plate per well with triplicate wells per timepoint). The remaining cells were pelleted by centrifugation, frozen in a dry ice/ethanol bath, and stored at -80 °C. Cells for the 0 h timepoint were harvested by resuspension in the DMEM supernatant immediately following centrifugation of the bacteria/macrophage mixture, ensuring recovery of both intracellular and extracellular bacteria. This cell suspension was transferred to a microfuge tube and the cells were pelleted and washed 3 times with PBS. Prior to the last centrifugation step, an aliquot was removed, lysed with 0.1% sodium deoxycholate, and the sample was processed as described above.

Immediately prior to assay for chloramphenicol acetyltransferase (CAT) activity, cell pellets were thawed at 37 °C for 5 min and resuspended in an appropriate volume (usually 0.1-0.4 ml) of 1X reporter lysis buffer (Promega). Cell extracts were prepared essentially following the manufacturer's recommendations.

ii) assay for CAT activity. Assays for CAT activity in cell extracts were performed using a modification of a procedure described previously (Sleigh, 1986). The final concentrations of [^{14}C]acetyl coenzyme A (specific activity of 60 mCi/mmol; Amersham) and unlabelled acetyl coenzyme A (lithium salt, Sigma) used in the assay were 0.2 μCi/ml and 18 μM, respectively. Each reaction contained a mixture of cell extract with 0.04 ml of a reagent cocktail consisting of 0.4 μl of [^{14}C]acetyl coenzyme A, 0.36 μl of 5 mM unlabelled acetyl coenzyme A, 19.24 μl of 0.25 M Tris-HCl (pH 7.8), and 20 μl of 8 mM chloramphenicol [diluted in 0.25 M Tris-HCl (pH 7.8) from a 34 mg/ml stock in ethanol]. The reactions were made up to a final volume of 0.1 ml with 1X reporter lysis buffer. Reactions were incubated at 37 °C for 1 h prior to extraction with 0.2 ml of cold ethyl acetate and quantitation of acetylated chloramphenicol by scintillation counting of 0.1 ml of the organic phase. Standard curves of CAT activity were prepared using appropriate dilutions of purified CAT (Promega) in 1X reporter lysis buffer and assayed in duplicate. For bacteria grown *in vitro*, activity is represented as the mean CAT activity of three replicates of a given

cell extract per mean cfu. For intracellular bacteria, activity is represented as the mean CAT activity/cfu ± 1 SD for triplicate wells. Results shown are representative of at least 2 (*in vitro*) or 3 (in macrophages) experiments.

Cell fractionation

Overnight stationary phase cultures of *F. novicida* strains in TSB-C (with antibiotic where necessary) were centrifuged at 4000 x *g* for 20 min at 4 °C. The supernatant was centrifuged again at 10 000 x *g* for 20 min at 4 °C, and the supernatant from this second centrifugation was saved as the culture supernatant fraction. Culture supernatant proteins were concentrated by precipitation with 1 volume of cold 10% trichloroacetic acid (TCA) on ice for 20 min. Precipitated proteins were pelleted by centrifugation. The pellets were washed once with cold ethanol, dried, and resuspended in Laemmli sample buffer.

The cell pellet was washed twice with PBS and sonicated at a constant output of 60 W for a total of 200 s. Cell debris and unbroken cells were removed by centrifugation at 4000 x *g* for 10 min at 4 °C. The supernatant was then centrifuged at 150 000 x *g* for 1 h at 4 °C to pellet the membranes. The pellet was resuspended in PBS (membrane fraction). The supernatant was saved as the cell-free extract (CFE) fraction. Lipopolysaccharide (LPS) was removed from the membrane fraction by hot-phenol extraction as described by Wong (1994). Briefly, the membrane fraction was treated with 1 volume of buffer-saturated phenol (Gibco BRL) at 70 °C for 10 min. The phenol layer was then back extracted with 1 volume of 20 mM Tris-HCl (pH 7.5) and heated again at 70 °C. Proteins in the phenol layer were precipitated by adding 2 volumes of cold acetone. The

pellet was washed once with acetone and once with ether. The sample was then dried and resuspended in Laemmli sample buffer.

Ammonium sulfate fractionation of *F. novicida* culture supernatants

Overnight cultures of *F. novicida* were centrifuged at 12 000 x *g* for 30 min at 4 °C. The supernatant was removed and ammonium sulfate was added slowly to 55% of saturation. After stirring for 1 h at 4 °C, the mixture was centrifuged at 12 000 x *g* for 20 min and the pellet was saved as the 0-55% fraction. The supernatant was removed and ammonium sulfate was slowly added to 65% of saturation. This solution was stirred and centrifuged as described above. The pellet was saved as the 55-65% fraction, which is enriched for a 70 kDa protein. Both pellets were resuspended in 20 mM Tris-HCl (pH 7.5) and dialyzed extensively against 10 mM Tris-HCl (pH 7.5). Dialyzed samples were concentrated by lyophilization.

Southern blotting

DNA digests were separated on 0.8% agarose gels in TBE buffer (Sambrook *et al.*, 1989). DNA was depurinated and denatured *in situ* prior to transfer to Hybond-N$^+$ nylon membrane (Amersham) using a vacuum transfer apparatus (Bio-Rad) as recommended by the manufacturer. For non-radioactive Southern hybridizations, fluorescein-labelled probes were prepared using the ECL random prime labelling kit (Amersham). High stringency hybridization and

chemiluminescent detection of bound probe was achieved as suggested by the manufacturer.

For radioactive blots, oligonucleotides were 5'-end-labelled with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) or [γ-^{33}P]ATP (1000-3000 Ci/mmol; Amersham) and T4 polynucleotide kinase (New England Biolabs). Membranes were prehybridized in prehybridization solution [5X SSC, 0.01 M sodium phosphate (pH 6.8), 5X Denhardt's reagent (Sambrook *et al.*, 1989), 0.5% SDS, 1 mM EDTA] for 1 h. Hybridization was carried out in hybridization solution (prehybridization solution with 1X Denhardt's reagent) containing radiolabelled probe. After hybridization, the membranes were washed twice for 5 min in 2X SSC, 0.1% SDS at room temperature followed by two 15 min stringency washes in 1X SSC, 0.1% SDS at the prehybridization/hybridization temperature.

Protein microsequencing

N-terminal amino acid sequencing of an abundant *F. novicida* 70 kDa secreted protein was performed essentially as described by Matsudaira (1987). Protein samples consisted of either 0-65% or 55-65% ammonium sulfate fractions of *F. novicida* U112 culture supernatants. Protein samples were mixed with Laemmli sample buffer (Laemmli, 1970) and heated at 70 °C for 5 min prior to separation on 6% polyacrylamide gels. Before electrophoresis, gels were precleared at 100 V for 1 h with 0.05 mM glutathione (Sigma) added to the upper buffer reservoir. Samples were electrophoresed

at 100 V with 0.1 mM sodium thioglycolate (Sigma) added to the upper buffer reservoir. Proteins were electrophoretically transferred to Immobilon-P membrane (Millipore) by wet transfer (Towbin *et al.*, 1979) using a Bio-Rad Trans-Blot cell. After transfer, the membrane was rinsed briefly in water. Electroblotted proteins were visualized by staining for 5 min in 0.1% Coomassie brilliant blue R250 in 50% methanol followed by destaining in 50% methanol. The band of interest was excised and sequenced by automated Edman degradation on an Applied Biosystems model 470A gas phase sequencer equipped with an on-line phenylthiohydantoin-amino acid analyzer.

CHAPTER 1. Identification of a locus of *Francisella novicida* encoding two putative global regulators of intramacrophage growth

INTRODUCTION

To initiate an infection, pathogenic microbes must enter into a host organism and find a location which provides an environment suitable for replication. For a number of important microbial pathogens, the preferred site of replication is within host cells. Of particular interest are those invaders which can elude or resist the battery of killing mechanisms of macrophages and successfully proliferate in these cells. To accomplish this task, intracellular pathogens have evolved one of three general strategies. One strategy involves escaping from the phagosome prior to phagosome-lysosome fusion. This mechanism is used by *Shigella* and *Listeria* (Finlay and Falkow, 1997). Some microorganisms, for example *Salmonella*, *Leishmania*, and *Coxiella,* have adapted to survive in phagolysosomes or acidified vacuoles which contain some lysosomal markers (Finlay and Falkow, 1997; Rathman *et al.*, 1997; Rabinovitch and Veras, 1996). Other pathogens, including *Mycobacteria*, *Chlamydia*, and *Legionella,* are apparently able to block phagosome-lysosome fusion (Clemens, 1996; Hackstadt *et al.*, 1997; Horwitz, 1983; Horwitz and Maxfield, 1984). With the development of improved cell biology techniques, it has become evident that the two latter strategies are

oversimplified descriptions of the complex interactions between pathogen-containing vacuoles and host cell vesicular pathways, which do not follow normal phagocytic pathways.

Francisella tularensis is a Gram-negative, facultative intracellular pathogen (Tärnvik, 1989). It is the causative agent of tularemia, an acute febrile illness of humans and a wide range of animals. The two recognized biovars of *F. tularensis* are designated type A and type B. The highly virulent *F. tularensis* type A is found only in North America and is responsible for the majority of tularemia cases in the United States. *F. tularensis* type B is found in Europe, Asia, and North America and causes a less severe form of tularemia in humans. Disease manifestation may take many forms including oropharyngeal, respiratory, glandular, ulceroglandular, or oculoglandular depending on the route of infection. Infection by any route is sometimes accompanied by pneumonia. A typhoidal tularemia occasionally occurs by inhalation or ingestion of *F. tularensis*. The type A strains are most commonly transmitted by tick bites, while infection with the type B strains is often associated with a variety of insect bites, dressing of wild hares, or ingestion of contaminated water (Nano, 1992). Infection with the type A strains can be fatal in untreated individuals.

F. tularensis is capable of growing in macrophages isolated from rodents including mice, rats, and guinea pigs (Anthony *et al.*, 1991a), as well as in human monocytes (Fortier *et al.*, 1994). Similar to the third class of pathogens above, *Francisella* appears to be able to

inhibit phagosome-lysosome fusion (Anthony *et al.*, 1991a). However, the intracellular compartment occupied by *Francisella* is acidified, an event thought to facilitate acquistion of iron which is essential for growth (Fortier *et al.*, 1995). There is also evidence that *Francisella* can replicate in non-professional phagocytic cells such as fibroblasts and hepatocytes (Conlan and North, 1992; Tärnvik, 1989). Data from experimental animal infections suggests that *in vivo F. tularensis* is rarely found extracellularly and that it replicates predominantly within macrophages (Fortier *et al.*, 1994; Fortier *et al.*, 1995).

F. novicida is closely related to *F. tularensis*, being virtually identical by 16S rDNA gene sequencing and DNA hybridization criteria, but it has low virulence for humans (Hollis *et al.*, 1989; Forsman *et al.*, 1994). *F. novicida* grows aggressively in murine macrophages, and its pathogenesis in mice is similar to *F. tularensis*. However, *F. tularensis* has more fastidious growth requirements and is less amenable to genetic manipulation than *F. novicida*. Consequently, we have chosen the latter as a model intracellular bacterium to identify factors of *Francisella* required for growth in macrophages. Presumably, the study of these factors will further the understanding of the mechanisms by which bacteria enter and replicate within eukaryotic cells.

During a study of a respiratory burst-inhibiting acid phosphatase (AcpA) (Reilly *et al.*, 1996), we isolated a spontaneous mutant of *F. novicida* which appears to have reduced phosphatase activity and is

defective for growth in macrophages. This work describes the isolation and characterization of the genetic locus mutated in this strain, which was designated *mglAB* for *m*acrophage *g*rowth *l*ocus.

RESULTS

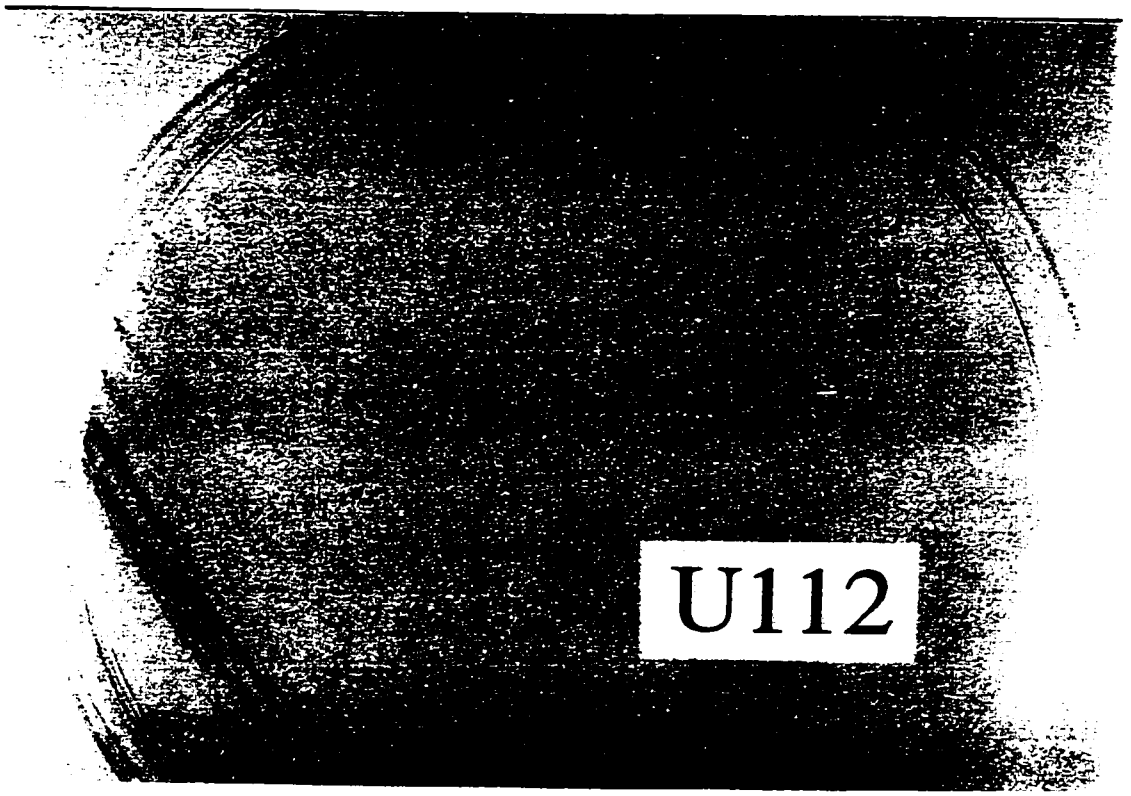
Isolation of a spontaneous mutant of *F. novicida*

Studies of a respiratory burst-inhibiting acid phosphatase (AcpA) of *Francisella* led to the development of a media on which to assay phosphatase activity in *F. novicida*. This media consisted of a modified LB agar containing the chromogenic phosphatase substrate, X-p [LB (X-p)]. When cultured on this media, isolated colonies of wild-type *F. novicida* are pinpoint and dark blue (Fig. 1A). In the absence of X-p, growth of wild-type *F. novicida* on the modified LB media is not inhibited. Apparently, the products of X-p hydrolysis (presumably the 5-bromo-4-chloro-indigo dye) inhibit the growth of *F. novicida*. In support of this, a similar effect was observed during attempts to construct *lacZ* fusions in *F. novicida*. On one of several plates, a large isolated colony was observed (colony diameter about 1 mm) which had a light blue colour. This strain was streak purified and designated GB2 (Fig. 1B). The phenotype of GB2 on LB (X-p) media is stable for at least two passages in broth or solid media in the absence of X-p, suggesting any reversion to wild-type must occur at a very low frequency.

GB2 is defective for growth in macrophages

The light blue phenotype of GB2 on LB (X-p) suggested this strain may be a spontaneous phosphatase mutant or altered in the expression of an exported phosphatase. This was interesting in light

A.



B.

C.

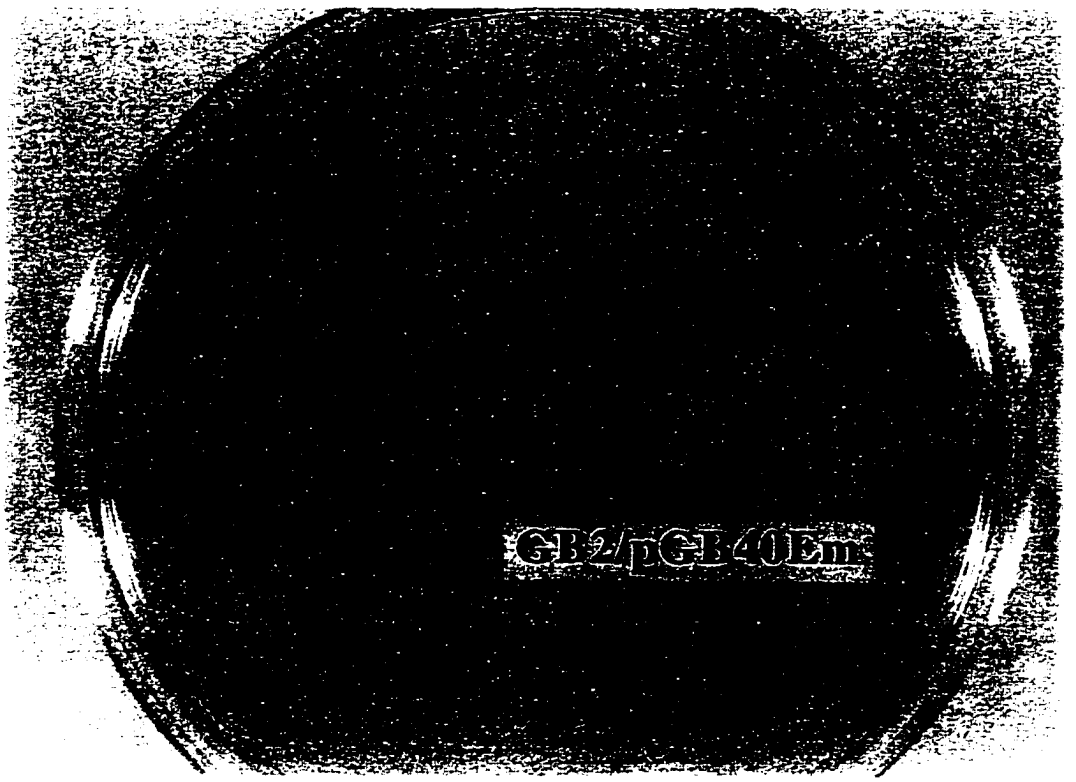
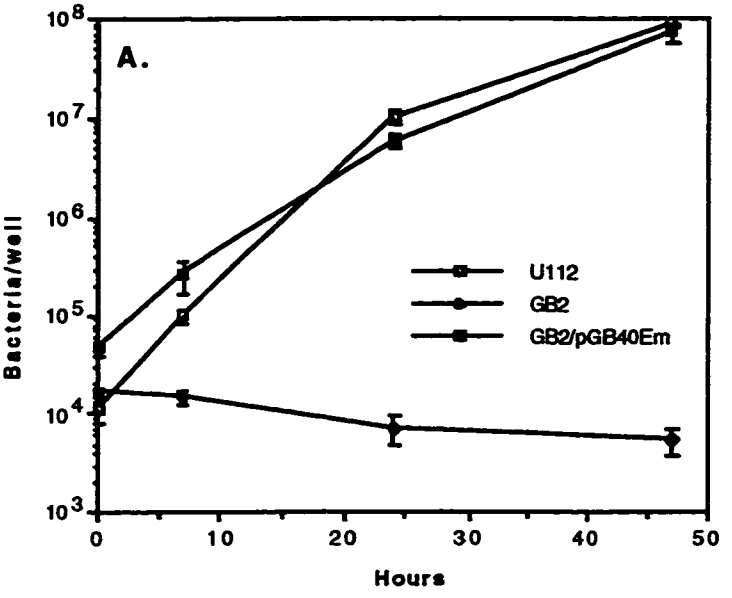
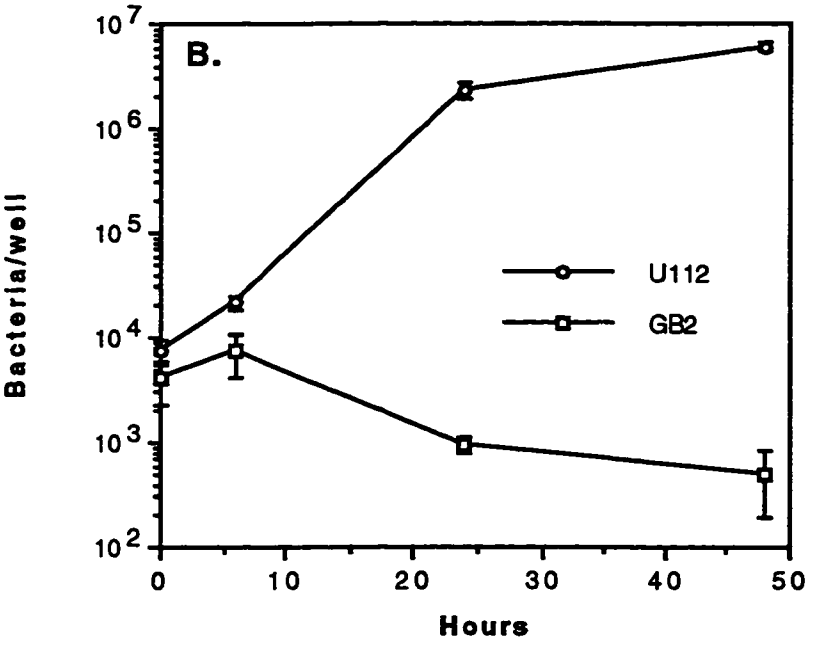


Figure 1. Growth of *F. novicida* on LB (X-p) agar. Plates were incubated for 36 h at 37 °C. A. U112 (wild-type). B. GB2 (*mglA*). C. GB2/pGB40Em (GB2, *mglAB*$^{+}$).

of the fact that other investigators had recently isolated an acid phosphatase (AcpA) from *Francisella* which displays enzymatic activity which could contribute to *Francisella* virulence (Reilly *et al.*, 1996). Although later data showed the mutation in GB2 to be located elsewhere (i.e. not in an exported phosphatase-encoding gene), it was on this basis that GB2 was examined for the ability to grow in the murine macrophage-like cell line J774. The GB2 strain is defective for growth and survival in J774 macrophages by 4-5 orders of magnitude as compared to wild-type *F. novicida* (Fig. 2A). Similar results are obtained using inflammatory murine peritoneal macrophages (Fig. 2B). The intracellular growth assay makes use of sodium deoxycholate to release intracellular bacteria from the macrophages. Control experiments confirmed that GB2 has wild-type resistance to 0.1% sodium deoxycholate, showing that the decreased cell numbers are not an artifact of the lysis procedure. However, GB2 exhibits the same growth rate as wild-type *F. novicida* in bacteriological medium, suggesting that the mutant has no obvious defects in *in vitro* growth (Fig. 2C). GB2 was also examined for sensitivity to serum since others have described serum-sensitive mutants of *F. novicida* which are unable to grow in macrophages (Anthony *et al.*, 1994; Mdluli *et al.*, 1994). GB2 exhibited wild-type resistance to serum killing (108% ± 30 survival vs. 62% ± 31 survival for wild-type) while less than 0.4% (i.e. below the detection limit of the assay) of a control rough mutant strain of *F. tularensis* (LVSR;


A.
Bacteria/well
Hours
U112
GB2
GB2/pGB40Em


B.
Bacteria/well
Hours
U112
GB2

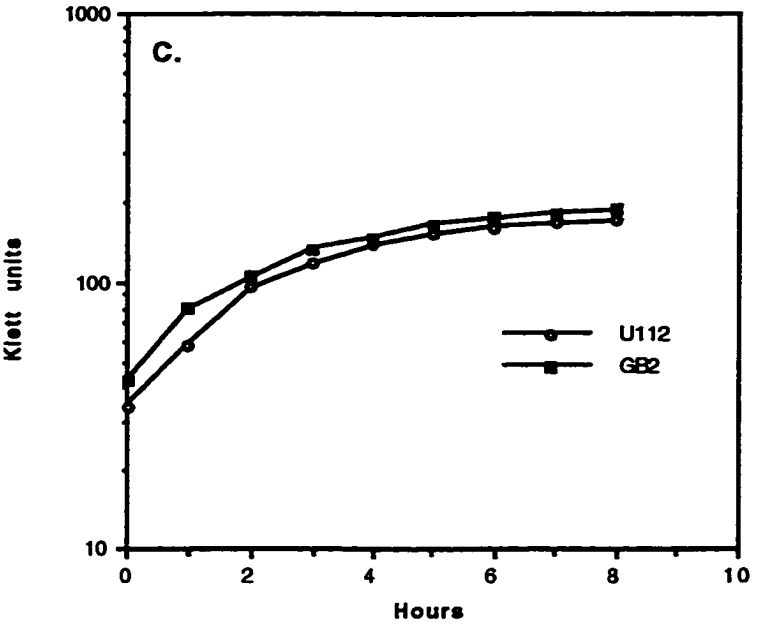


Figure 2. Growth of *mgl* mutants in macrophages and in TSB-C. U112 (wild-type), GB2 (*mglA*), GB2/pGB40Em (GB2, *mglAB*$^+$). A. Growth in J774A.1 cultured macrophages. B. Growth in thioglycollate-elicited murine peritoneal macrophages. C. Growth in TSB-C.

Sandström *et al.*, 1988) survived the treatment. This suggested that GB2 differed from the *F. novicida* mutants of other investigators and that the phenotype of GB2 may not be the result of dramatic alterations to outer membrane integrity.

Complementation of GB2 for growth in macrophages

To identify the genetic locus required to complement GB2 for growth in macrophages, an *in cis* complementation strategy was devised (Fig. 3) by modifying a technique previously used to construct mutants of *F. novicida* (Mdluli *et al.*, 1994). Large fragments of a partial, random digest of wild-type chromosomal DNA were ligated to an Em^R cassette derived from Tn*Max2* (Haas *et al.*, 1993). This ligation mixture was used to transform GB2 where the transformed ligation products integrated into the chromosome by homologous recombination. The resulting bank of Em^R GB2 transformants should therefore contain large fragments of wild-type DNA linked to the Em^R marker, in effect creating a wild-type genomic library in GB2. It should be noted that all complementation experiments described in this study involve complementation *in cis*. Consequently, in GB2 transformants complemented for growth in macrophages, the Em^R cassette will presumably mark the region of the *F. novicida* chromosome containing the complementing locus. From a bank of 2880 Em^R GB2 transformants, four strains were isolated which showed both the same growth kinetics in

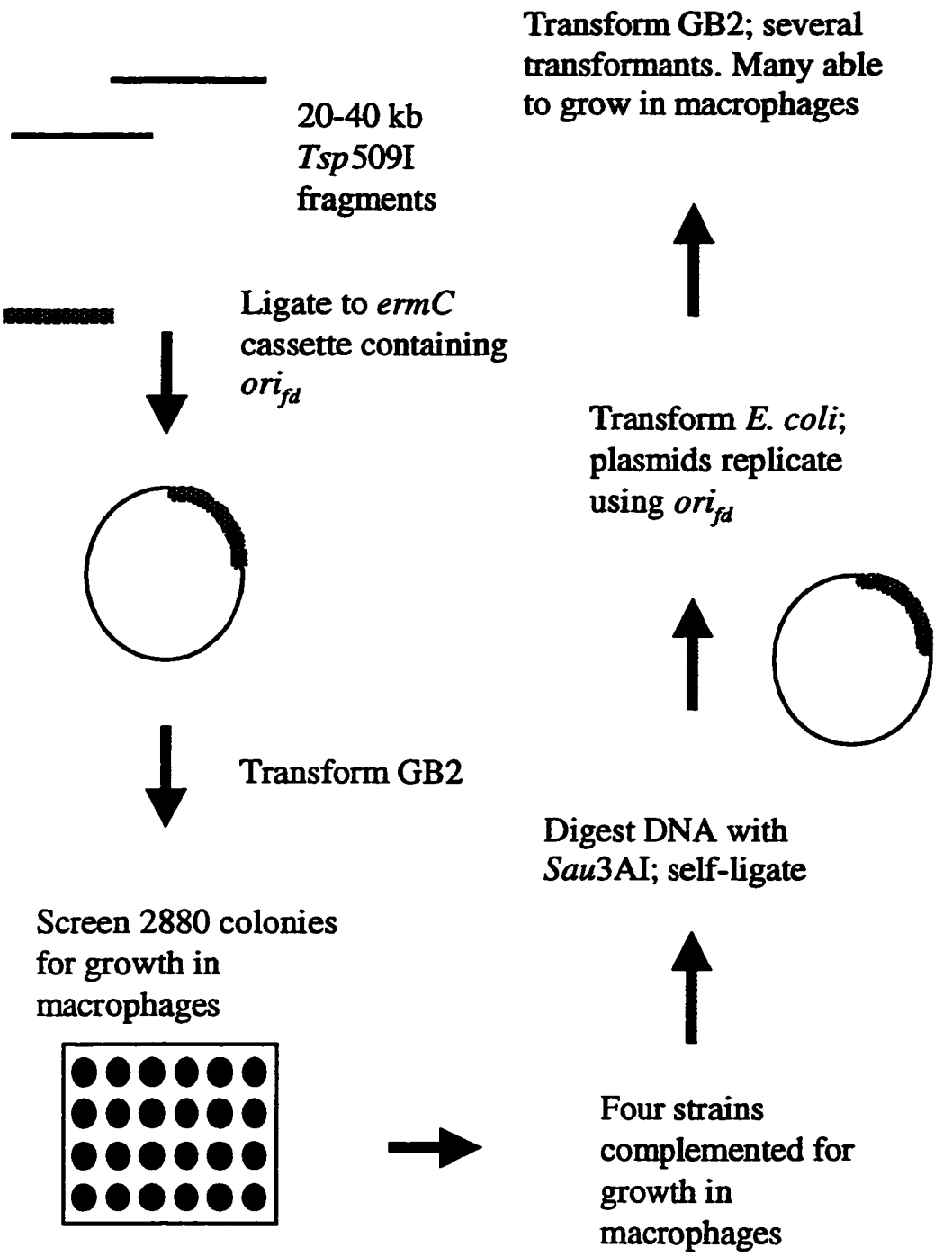

20-40 kb
*Tsp*509I
fragments
Ligate to *ermC*
cassette containing
ori_{fd}
Transform GB2
Screen 2880 colonies
for growth in
macrophages
Four strains
complemented for
growth in
macrophages
Digest DNA with
*Sau*3AI; self-ligate
Transform *E. coli*;
plasmids replicate
using ori_{fd}
Transform GB2; several
transformants. Many able
to grow in macrophages

Figure 3. Complementation of GB2 for growth in macrophages. Large fragments of a random partial digest of wild-type chromosomal DNA were ligated to an Em^R (*ermC'*) cassette and transformed into GB2. Transformants were pooled and screened for growth in J774A.1 macrophages. The Em^R marker and flanking DNA from complemented strains were rescued in the form of plasmids in *E. coli*. These plasmids were then screened for the ability to complement GB2.

macrophages and phenotype on LB (X-p) medium as wild-type *F. novicida* (intramacrophage growth of a representative strain is shown in Fig. 4). One of these strains, designated GB2-C3, was chosen for further analysis.

Isolation of plasmids which complement GB2 for growth in macrophages

An important characteristic of the Em^R cassette used in this study is the incorporation of the bacteriophage fd origin of replication into the cassette. This origin of replication is functional only in strains expressing the bacteriophage fd gene *2* protein, such as *E. coli* E131, where it is supplied by a recombinant λ prophage (Haas *et al.*, 1993). This feature allows the rescue of the Em^R cassette and flanking DNA from GB2 strains complemented for growth in macrophages (GB2-C strains) by digesting chromosomal DNA from such strains with restriction endonucleases, self-ligating this DNA to circularize the digestion products, and transforming an appropriate *E. coli* strain (E131). Recombinant molecules containing the Em^R cassette will then replicate as plasmids in *E. coli*. Several plasmids were isolated using self-ligated, partial *Sau*3AI digests of chromosomal DNA from the GB2-C strain GB2-C3. Of four plasmids transformed into GB2, three complemented this strain for intracellular growth. Data for one of the complementing plasmids is shown in Fig. 5. The smallest plasmid (5 kb), designated pC3-20, was chosen for further analysis.

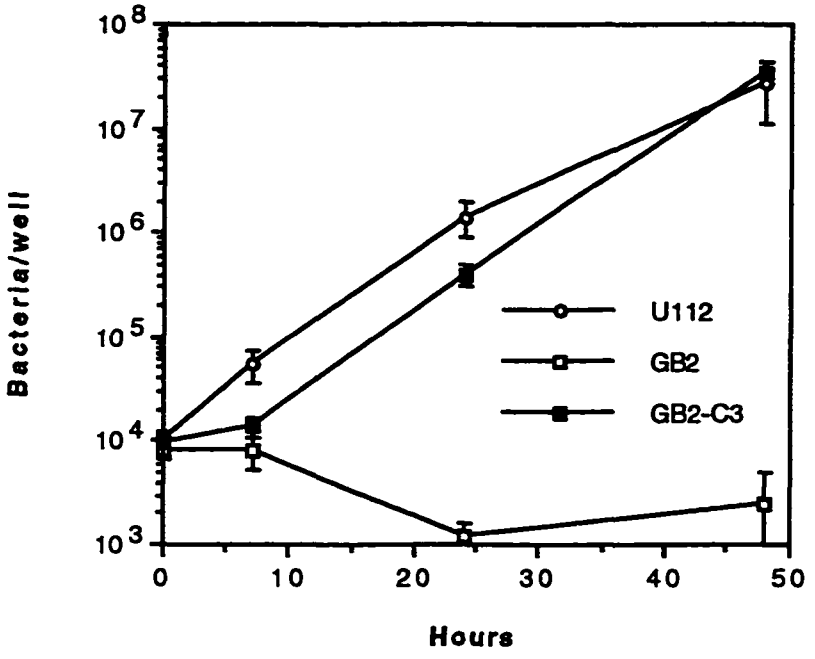


Figure 4. Growth of complemented GB2 strain in J774A.1 macrophages. U112 (wild-type), GB2 (*mglA*), GB2-C3 (complemented GB2 strain from bank of 2880 Em^R GB2 transformants).

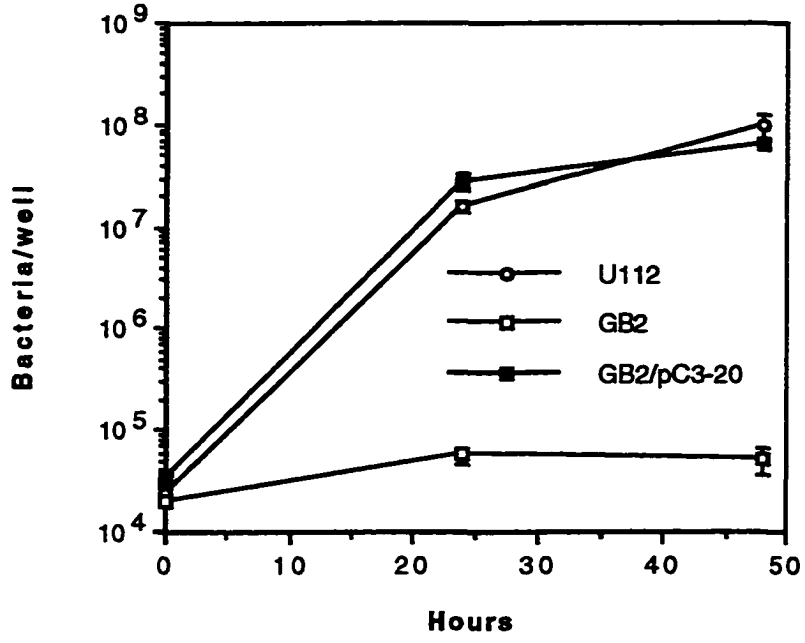


Figure 5. Complementation of GB2 for growth in J774A.1 macrophages with pC3-20. U112 (wild-type), GB2 (*mglA*), GB2/pC3-20 (GB2 transformed with pC3-20).

Cloning of the intracellular growth locus defective in GB2

The complementation strategy that I used initially creates a duplication of the transformed DNA (Fig. 3). Thus, in GB2-C strains there may be two copies of the locus of interest, a mutant and a wild-type allele. Evidence indicating the presence of a duplication in GB2-C3 was obtained when DNA from this strain was analyzed by Southern blot using the locus cloned in pC3-20 as a probe (Fig. 6). To ensure that I had a wild-type copy of the complementing locus, the *F. novicida* insert cloned in pC3-20 was used as a probe to clone a homologous fragment from wild-type *F. novicida*. A Southern blot of wild-type *F. novicida* DNA cut with *Hin*dIII and probed with the locus cloned in pC3-20 identified a hybridizing fragment of approximately 2.9 kb (Fig. 6). However, a *Hin*dIII digest of GB2 DNA produced two hybridizing fragments of approximately 1.4 and 1.5 kb, showing that a mutation in GB2 created a new *Hin*dIII restriction site close to the center of the wild type 2.9 kb *Hin*dIII fragment (Fig. 6). The 2.9 kb *Hin*dIII fragment was subsequently cloned into pTZ18U, and this plasmid was designated pGB40.

To determine if pGB40 was capable of complementing GB2 for intracellular growth, an additional antibiotic resistance marker was cloned into this plasmid as *F. novicida* is Ap^R (Hollis *et al.*, 1989). The Em^R cassette (used above) was cloned into the *Eco*RI site in the multiple cloning site of pGB40 to create plasmid pGB40Em. GB2 was

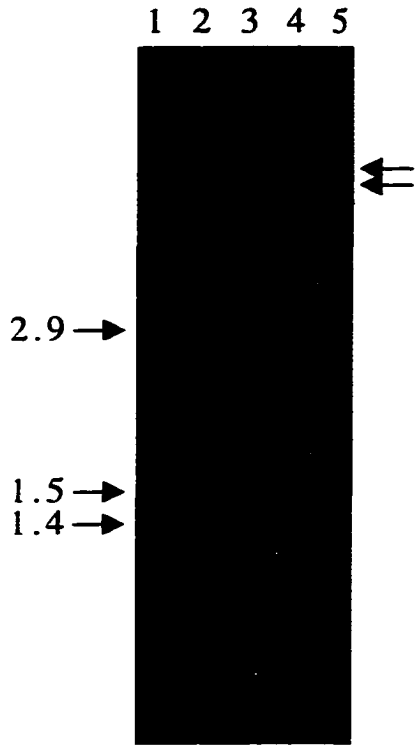


Figure 6. Southern blot analysis of GB2 mutant and complemented strain from library of GB2 transformants. Band sizes (in kb) were estimated using 1 kb ladder DNA standards. Lanes: 1, U112 (*Hin*dIII); 2, U112 (*Pst*I); 3, GB2 (*Hin*dIII); 4, GB2 (*Pst*I); 5, GB2-C3 (*Pst*I). Chromosomal DNA from each strain was digested with the indicated restriction endonuclease and probed with the insert in pC3-20. Arrows on the left indicate the *Hin*dIII fragment which is altered in GB2. Arrow on the right indicates a duplicated fragment in GB2-C3.

then transformed with pGB40Em, and the resulting transformants were screened for growth in macrophages. Several transformants (28/47) were restored for growth in macrophages. One representative transformant (GB2/pGB40Em) was shown to be fully complemented both for colony morphology on LB (X-p) media (Fig. 1C) and growth in macrophages (Fig. 2A). Southern blot analysis of both complemented and non-complemented transformants probed with the locus cloned in pGB40 (Fig. 7A) showed that only the complemented strains carry a copy of the 2.9 kb *Hin*dIII fragment (in addition to the GB2 allele). Together, these data indicated the locus required to complement the genetic lesion in GB2 and the site of the genetic lesion itself mapped within the 2.9 kb *Hin*dIII fragment.

DNA sequencing and analysis of *mglAB*

A 1309 bp portion of the 2.9 kb *Hin*dIII fragment cloned in pGB40 was sequenced. Three putative open reading frames (ORFs) were found (Fig. 8). The first ORF (*rpsI*), beginning at bp 3 and ending at bp 137 (Fig. 9), has a deduced amino acid sequence with significant identity (78%) to the C-terminal 45 amino acids of the ribosomal S9 protein of *Haemophilus somnus* (Theisen and Potter, 1992) and other bacteria. Two putative ORFs are organized in an apparent operon which begins near the centre of the *Hin*dIII fragment. The genes corresponding to these ORFs have been named *mglAB* for *m*acrophage *g*rowth *l*ocus. Incomplete sequence analysis

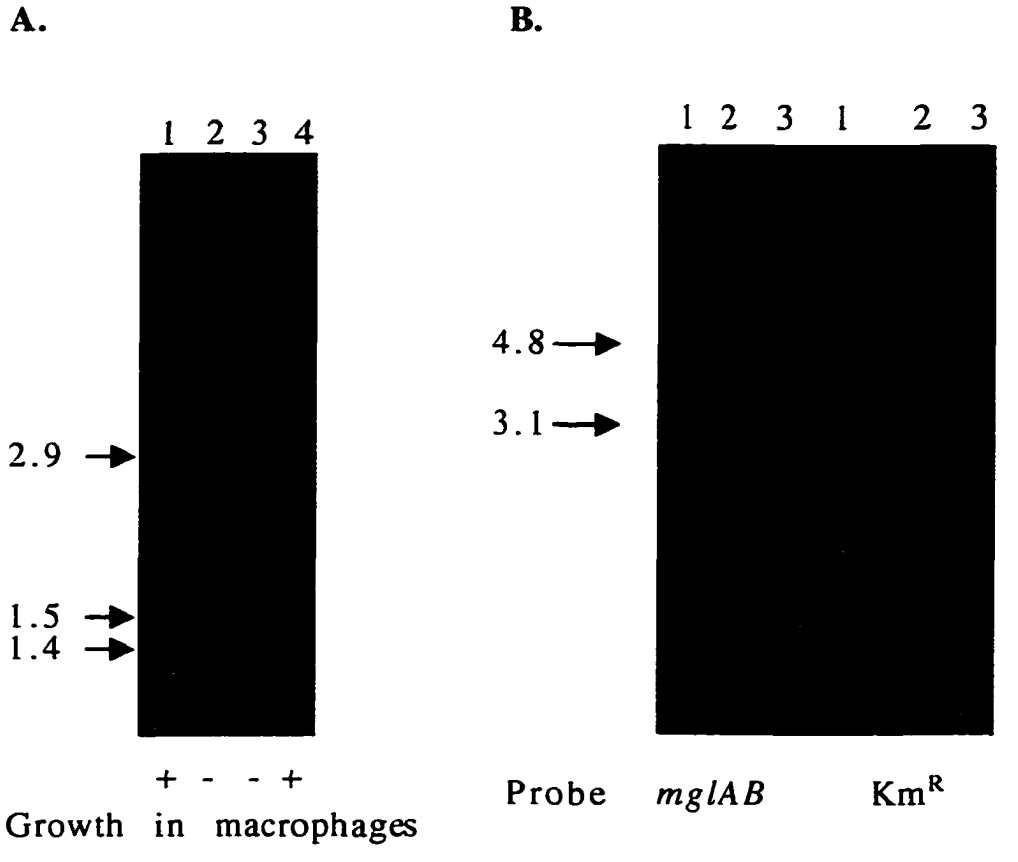

A.
1 2 3 4
2.9
1.5
1.4
+ - - +
Growth in macrophages
B.
1 2 3 1 2 3
4.8
3.1
Probe
mglAB
Km^R

Figure 7. Southern blot analysis of *F. novicida mgl* mutants and complemented GB2 strain. Band sizes (in kb) were estimated using 1 kb ladder DNA standards.

A. Lanes: 1, U112 (wt); 2, GB2 (*mglA*); 3, GB2/pGB40Em* (representative transformant not complemented for intracellular growth); 4, GB2/pGB40Em (representative transformant complemented for intracellular growth). Chromosomal DNA from each strain was digested with *Hin*dIII and probed with the insert in pGB40 (*mglAB*).

B. Lanes: 1, U112; 2, GB5 (*mglA*::mTn*10*Km); 3, GB6 (*mglB*::mTn*10*Km). Chromosomal DNA from each strain was digested with *Xba*I and probed with either the insert in pGB40 (*mglAB*) or the kanamycin resistance cassette (Km^R). The smaller hybridizing fragment (about 1.4 kb) detected by the *mglAB* probe corresponds to the *Xba*I fragment shown in Fig. 8B.

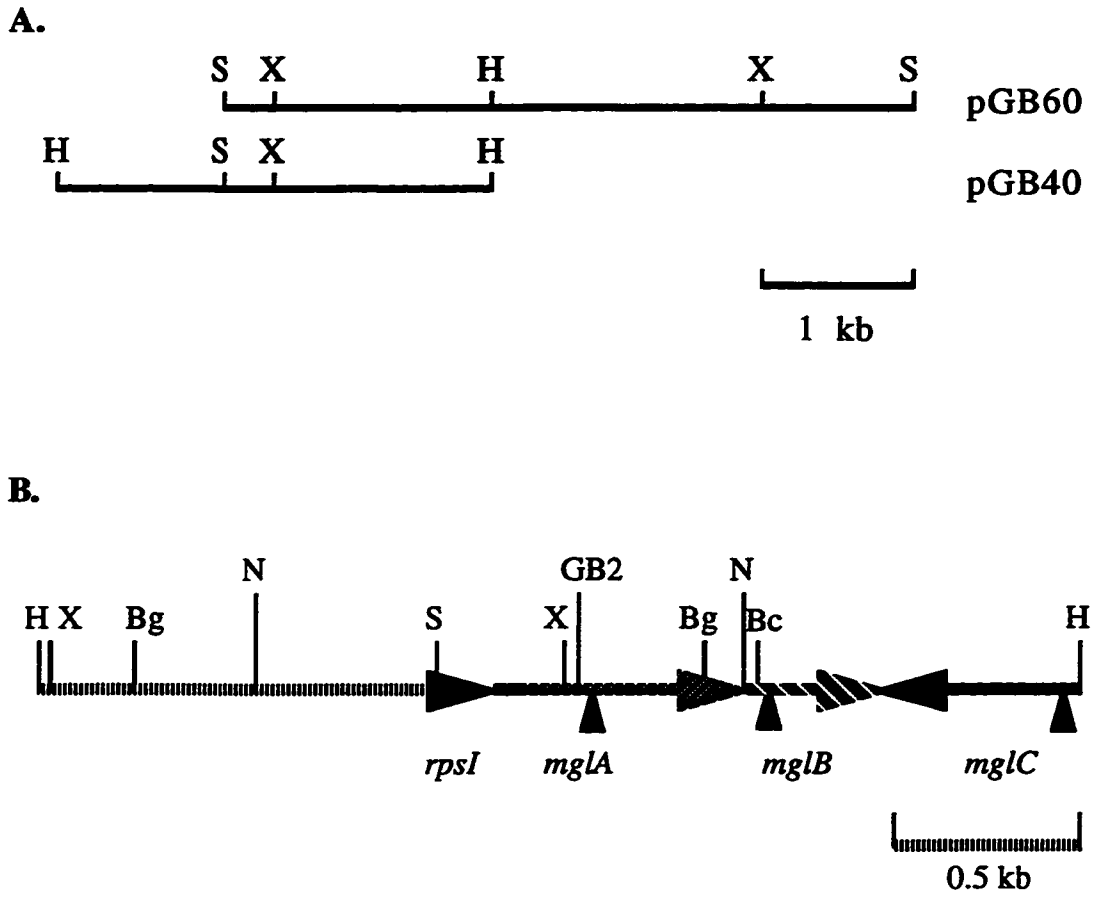


Figure 8. Restriction map and ORF organization of the *mglAB* region. Restriction sites are abbreviated as follows: Bg, *Bgl*II; Bc, *Bcl*I; H, *Hin*dIII; N, *Nde*I; S, *Sac*I; X, *Xba*I. A. The inserts in pGB40 (2.9 kb *Hin*dIII) and pGB60 (4.6 kb *Sac*I) were cloned from digests of *F. novicida* chromosomal DNA. B. Detailed restriction and ORF map of the 2.9 kb insert in pGB40. Arrows indicate putative ORFs. The 1309 bp segment starting from the *Sac*I site and proceeding right was sequenced on both strands. Two short segments within *mglC* (about 100 bp and 160 bp) and approximately 440 bp (starting from the left hand *Hin*dIII site) were sequenced on one strand only. A small

section to the left of the *Sac*I site was not sequenced on either strand. Triangles indicate the location of mTn*10*Km insertions which were recombined into the *F. novicida* chromosome. The location of the point mutation in *mglA* from GB2 is also indicated.

```
GAGCTCTAATCGAGTATGATGAAGAACTTAAACCAGCTCTTCGTGAAGCAGGTTTTGTTA  60
  A  L  I  E  Y  D  E  E  L  K  P  A  L  R  E  A  G  F  V  T

CTCGTGACCCACGTAAAGTTGAGCGTAAGAAATTTGGTCTTAGAAAAGCTCGTAGAAGAA 120
  R  D  P  R  K  V  E  R  K  K  F  G  L  R  K  A  R  R  R  R

GACAATTCTCTAAGCGTTAATCTATTTTATATTACCTTTCTATTATTCTTTTTTATTTTA 180
  Q  F  S  K  R  *
                -35                      -10

TTACTAGGAGGATACAATCTTGCTTTTATACACAAAAAAAGATGATATCTATAGCGATAT 240
       SD          M  L  L  Y  T  K  K  D  D  I  Y  S  D  I

AGTCCGCATGATCCTTCTTATTAAAGGGGCTAATGCGAAAATTGTAGATGTTTCTAAAGA 300
  V  R  M  I  L  L  I  K  G  A  N  A  K  I  V  D  V  S  K  E
                                                            ↓
AGAAAACTCAAAACATCTAGAAGAGCTAAATATCATTACACCTAATGGTAATATACCTAC 360
  E  N  S  K  H  L  E  E  L  N  I  I  T  P  N  G  N  I  P  T

GCTTAGCACAGATGATTTTGCAGTGTATAGGCTTAGTGTGATTATAGAAGCTATAGAGGA 420
  L  S  T  D  D  F  A  V  Y  R  L  S  V  I  I  E  A  I  E  D

TCTATATCCCTTTCCTCCGATGTTTCCAGTATTTCCAAAACAGCGAGCTAATGCAAGAAT 480
  L  Y  P  F  P  P  M  F  P  V  F  P  K  Q  R  A  N  A  R  I

ATTGTTAGAATATGTTAATAAGACGTTTCTACAAAATATTATTAAATTACAAAGTCCTGA 540
  L  L  E  Y  V  N  K  T  F  L  Q  N  I  I  K  L  Q  S  P  D

TTTGGATGAAAAACAAGCTAACGAAATAAAAATGCTAATGCAAAGGGATATAATAAGCAC 600
  L  D  E  K  Q  A  N  E  I  K  M  L  M  Q  R  D  I  I  S  T

TTATAAGACAATAGTTAGTGAAAGAGAAGTAAATGCAGAAAGTAATCCAGATGCTCAAAA 660
  Y  K  T  I  V  S  E  R  E  V  N  A  E  S  N  P  D  A  Q  N

TATCAACGTATTGACTCTGATAATAACTTTCGTTTTTTATTATTTCATTAAGTTAAAGAT 720
  I  N  V  L  T  L  I  I  T  F  V  F  Y  Y  F  I  K  L  K  I

CTCAATACCTACCAAAGATAAAAACATTATCAAAGAGATCAAAGAATTACTTAGCGAACC 780
  S  I  P  T  K  D  K  N  I  I  K  E  I  K  E  L  L  S  E  P

TAACTTTATAAAAACTATCAAAGCAAAAGGAGCTTAATATGGCTATGCTTAGAGCATATG 840
  N  F  I  K  T  I  K  A  K  G  A  *
                              SD       M  A  M  L  R  A  Y  V

TAGTTAAAGCTACATACAACTGGCTAGTTGATCATGGATTTACACCTTATGTTTTAGTTG 900
  V  K  A  T  Y  N  W  L  V  D  H  G  F  T  P  Y  V  L  V  D

ATACTGAGTATGAAGGTGTTATAGTACCAGCAAACTATATTGATGAAGATAAAAAAATAC 960
  T  E  Y  E  G  V  I  V  P  A  N  Y  I  D  E  D  K  K  I  L

TTTTAGATTTATCTCCTCAAGCAATACAAGATTTAGTTATAGATGATAATCATATTAGCT 1020
  L  D  L  S  P  Q  A  I  Q  D  L  V  I  D  D  N  H  I  S  F

TTGCTGCAACGTTTGATAGTGAGCCAATGTCTATAAATATTCCTATCGAGGCTGTCTTAG 1080
  A  A  T  F  D  S  E  P  M  S  I  N  I  P  I  E  A  V  L  E
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```
AAGTATTTTCTAAAGAAACAGAGCAAGGAATGTATGCTCGCGAATTTGGTTATGGAATTA  1140
  V  F  S  K  E  T  E  Q  G  M  Y  A  R  E  F  G  Y  G  I  N

ATATCAATGAAGGCGAAGATGATGAAACTGCTAATCCTAAGAAATTAGGAGAAACTAATT  1200
  I  N  E  G  E  D  D  E  T  A  N  P  K  K  L  G  E  T  N  S

CAGATAACGTTCTTTCATTAGATTAATTCTTTACTCTTAATAACCTTCTGTATTTATCTG  1260
  D  N  V  L  S  L  D  *
                        *  N  K  V  R  L  L  R  R  Y  K  D

TAATTATAGGCAAATATCCTACTATAAAACTCAATGGTATGCATATAAA             1309
T  I  I  P  L  Y  G  V  I  F  S  L  P  I  C  I  F ←mglC
```

Figure 9. Nucleotide and deduced amino acid sequence of the 1309 bp *mglAB* region of pGB40. The sense strand (with respect to *mglAB*) is shown with amino acids represented by single letter abbreviations below the nucleotide sequence. Putative promoter sequences and potential ribosome-binding sites are indicated by underlining and in bold, respectively. Stop codons are represented by asterisks. The sequence corresponding to *rpsI* spans bp 1 to bp 137. *mglA* is predicted to span from bp 200 to bp 814. The *mglB* ORF begins at bp 819 and ends at bp 1223. The site of the C to A transversion mutation in GB2 is shown by the arrow. The 3' region of the putative *mglC* from bp 1309 to bp 1126 is also shown.

of the DNA adjacent to *mglB* suggests that the only possible ORF is oriented in the opposite direction relative to *mglB*.

The potential UUG initiation codon of *mglA* at bp 200 is preceded by a consensus *E. coli* Shine-Dalgarno sequence 8 nucleotides upstream. A consensus ribosome binding site for *mglB* is located 6 nucleotides upstream of the potential AUG initiation codon at bp 819, overlapping the 3'-end of the coding region of *mglA*. Notably, the intergenic region between *rpsI* and *mglA* (60 bp) is much shorter than that observed between *rpsI* and *sspA* (the gene immediately downstream of *rpsI*) in both *E. coli* (393 bp) (Blattner *et al.*, 1997) and *H. somnus* (143 bp) (Theisen and Potter, 1992), although the significance of this is unknown.

The deduced amino acid sequence of *mglA* is 20% identical to SspA (stringent starvation protein) from *E. coli* (Serizawa and Fukuda, 1987), 21% identical to SspA from *Haemophilus influenzae* (Fleischmann *et al.*, 1995), and 23% identical to SspA from *H. somnus* (Theisen and Potter, 1992) (Fig. 10A). MglA is predicted to be a protein of approximately 24 kDa with an isoelectric point (pI) of 7.1. The deduced amino acid sequence of *mglB* is 30% identical to *E. coli* SspB (Williams *et al.*, 1991) and 36% identical to *H. influenzae* SspB (Fleischmann *et al.*, 1995) (Fig. 10B). MglB is predicted to be a protein of approximately 15 kDa with a pI of 3.9. Hydropathy plots of the sequences of MglA and MglB are similar to those of SspA and SspB from other bacteria in being largely hydrophilic with no obvious membrane spanning regions. The deduced N-terminal

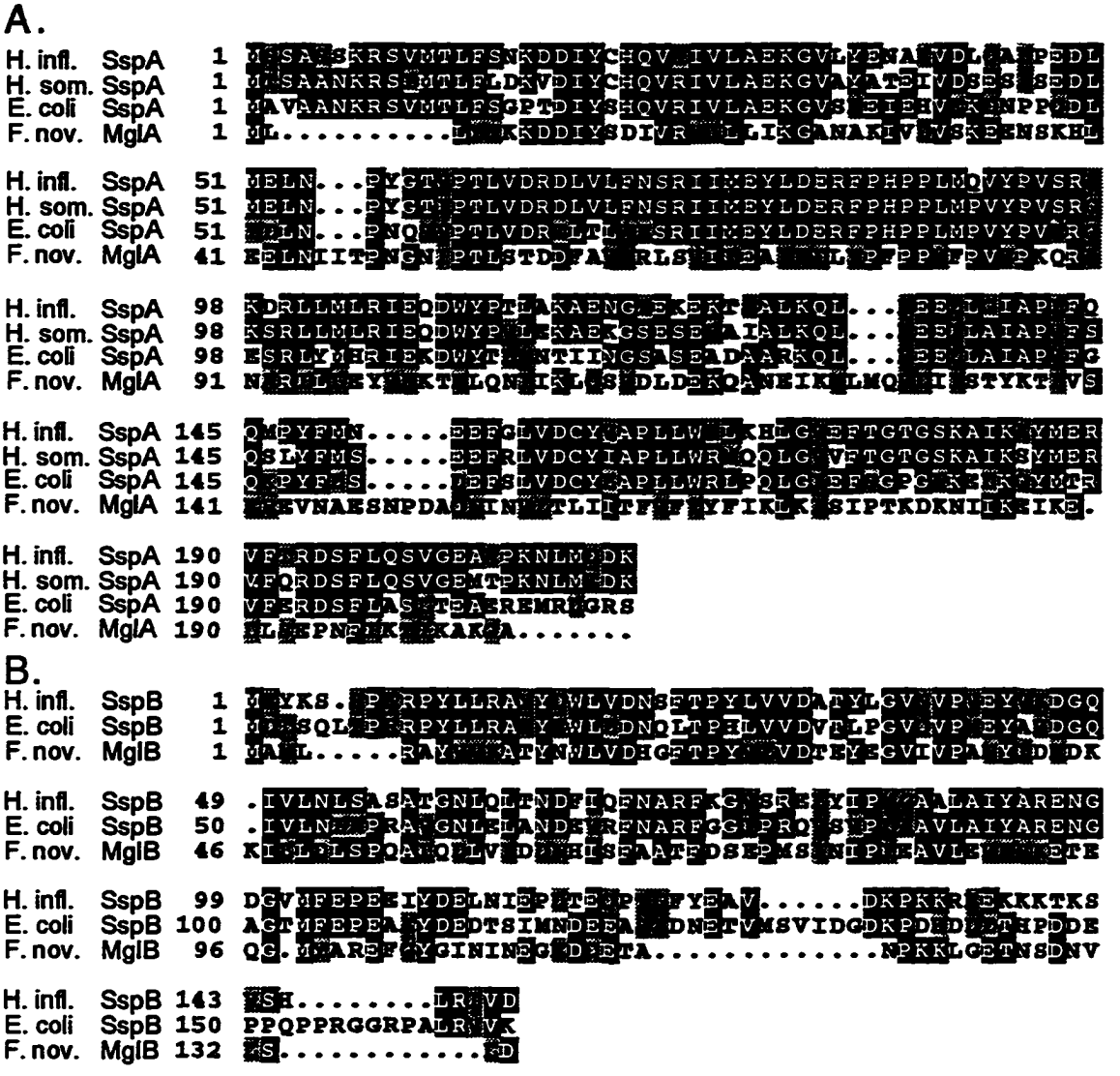


Figure 10. Amino acid alignment between MglA and SspA (A) and between MglB and SspB (B). Identical residues are shaded black. Conserved residues are shaded gray. Abbreviations: *H. influenzae*, (H. infl.); *H. somnus*, (H. som.); *F. novicida* (F. nov.).

sequences of MglA and MglB also lack the features of a signal peptide (Izard and Kendall, 1994).

mglAB insertion mutants are defective for growth in macrophages

The ability of pGB40 to complement GB2 for intracellular growth suggested the insert in pGB40 may contain a gene(s) required by *F. novicida* for growth in macrophages. In an attempt to regenerate mutants defective for intracellular growth, pGB40 was subjected to random mutagenesis using a mTn*10*Km transposon as described in the experimental procedures. Plasmids with insertions in *mglA* (198 bp downstream of the putative start codon) or *mglB* (91 bp into the coding region) were transformed into wild-type *F. novicida*. Transformants were first screened for growth in macrophages. Representative examples of strains positive and negative for intracellular growth were then analyzed by Southern hybridization using *Xba*I-digested chromosomal DNA. Southern blots were probed with the insert in pGB40 and with the mTn*10*Km transposon. Recombinant strains in which allelic replacement occurred had a shift in the wild type 3.1 kb *Xba*I fragment to a 4.8 kb fragment which hybridized with both probes, consistent with the insertion of the 1.7 kb mTn*10*Km transposon (Fig. 7B). Allelic replacement was confirmed by the absence of hybridization to vector DNA in these strains. The recombinant strains with insertions in *mglA* and *mglB* were designated GB5 and GB6, respectively. Both GB5 and GB6 are

defective for growth in macrophages, having growth kinetics similar to those of GB2 (Fig. 11). Furthermore, both strains exhibit a phenotype similar to GB2 when grown on LB (X-p) media (Fig. 12). Transformants lacking allelic replacements in *mglA* or *mglB* retained the wild-type phenotype on LB (X-p) media and in macrophages (data not shown). These data demonstrate that *mglB* is required for the intracellular growth of *F. novicida*. Given the possible polar effect of the insertion mutation, a role for *mglA* in intracellular growth could not be confirmed from these experiments.

mglA is required for intracellular growth

Data from the Southern blot of *Hin*dIII-digested GB2 DNA probed with the insert in pGB40 indicated the site of the mutation in GB2 (as identified by the newly created *Hin*dIII restriction site) was approximately 1.4-1.5 kb from the end of the wild-type 2.9 kb *Hin*dIII fragment (Fig. 7A). This localized the site of the mutation to being within *mglA*. To identify the mutation in GB2, a 2 kb fragment containing *mglA* from GB2 was amplified by PCR. An 873 bp *Sac*I/*Bcl*I fragment (Fig. 8) derived from the PCR product and containing *mglA* was cloned and sequenced. A C to A transversion mutation was discovered at bp 160 in the *mglA* coding sequence (shown by arrow in Fig. 9). This results in the creation of a *Hin*dIII restriction site (AAGCTT), consistent with the Southern blot data.

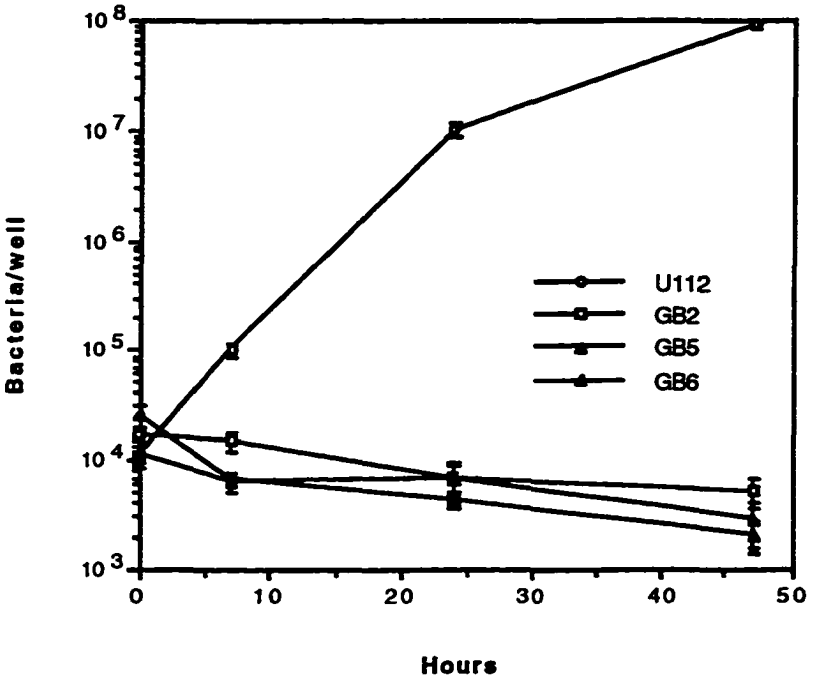
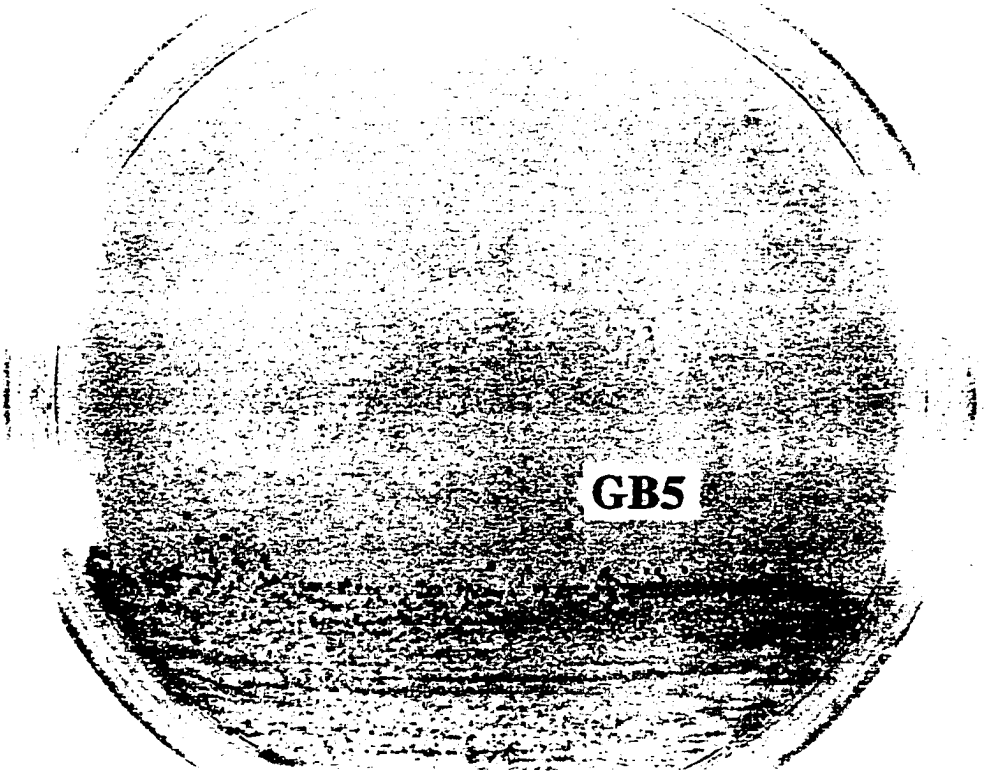


Figure 11. Growth of *F. novicida mgl* mutants in J774A.1 macrophages. U112 (wild-type), GB2 (*mglA*), GB5 (*mglA*::mTn*10*Km), GB6 (*mglB*::mTn*10*Km).

A.



B.

C.

Figure 12. Growth of *F. novicida* mutants on LB (X-p) agar. Plates were incubated for 36 h at 37 °C. A. GB5 (*mglA*::mTn*10*Km). B. GB6 (*mglB*::mTn*10*Km). C. GB8 (*mglC*::mTn*10*Km).

The predicted consequence of this mutation at the translational level is a non-conservative substitution of a lysine (Lys) residue (AAG) for a threonine (Thr) residue (ACG) at amino acid 54 of MglA. Analysis of the predicted secondary structure of MglA by both the Garnier-Robson and Chou-Fasman algorithms shows no significant alterations as a result of the substitution (Garnier *et al.*, 1978; Chou and Fasman, 1974). The three known SspA sequences all have a threonine residue at the position corresponding to Thr54 of MglA as well as conserved residues flanking this position (Fig. 10A). Given this information, a clone was generated (pGB48Em) which contains only wild-type *mglA*. When transformed into GB2, this clone is able to complement GB2 for intracellular growth (Fig. 13). The data demonstrate that *mglA* is required for the intramacrophage growth of *F. novicida*, and suggest that Thr54 plays an important role in the function of MglA.

mglA is required for virulence in mice

Data from experimental infections of mice suggest *mglA* may also be required for virulence. The virulence of GB2 and GB2/pGB40Em in mice was examined using bacterial replication in the spleen as a measure of virulence. The kinetics of the growth of GB2/pGB40Em in mouse spleens was identical to wild-type *F. novicida* over a 52 h time period (Fig. 14), while by 36 h bacteria were cleared (i.e. below

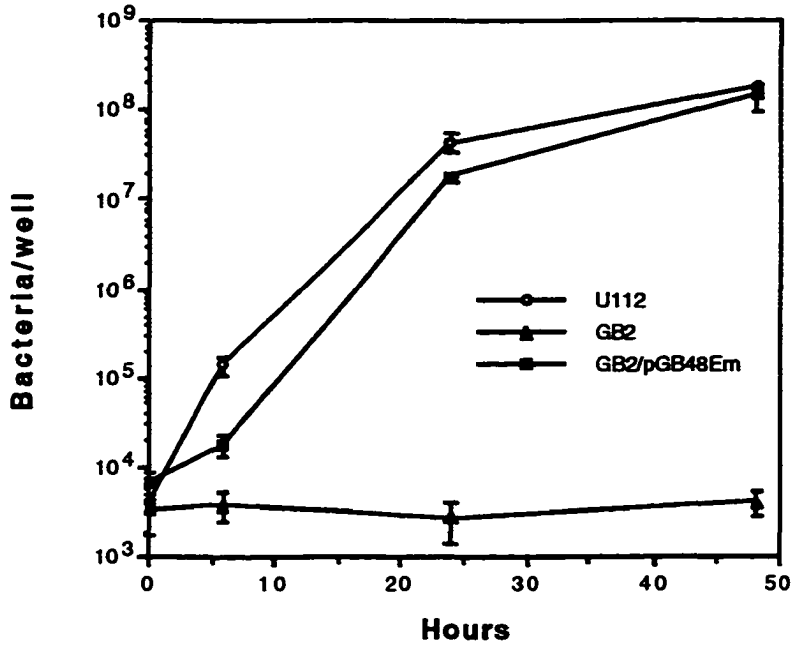


Figure 13. Complementation of GB2 for growth in J774A.1 macrophages with pGB48Em. U112 (wild-type), GB2 (*mglA*), GB2/pGB48Em (GB2, *mglA'*).

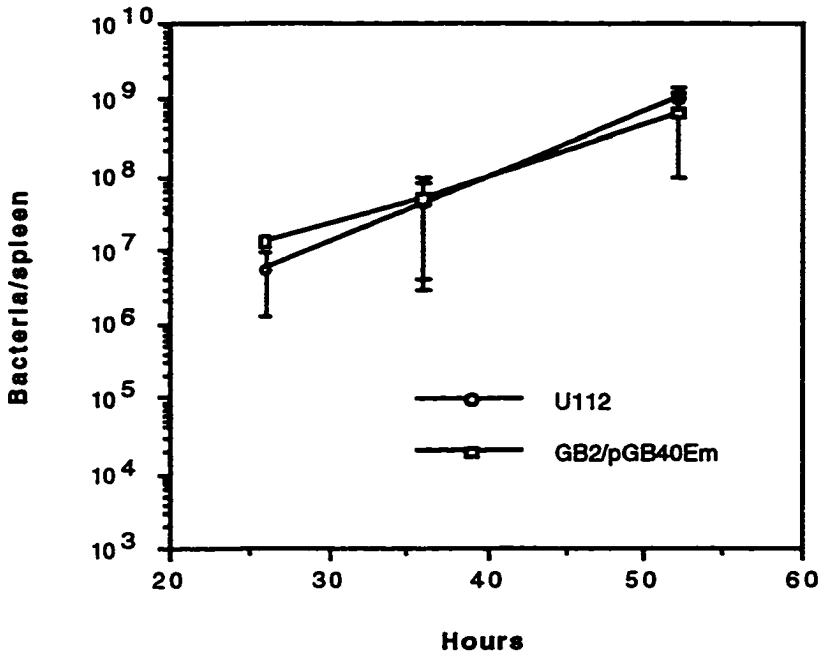


Figure 14. Growth of complemented GB2 strain in spleens of infected mice. Mice were inoculated ip with 1-2 x 10^4 bacteria. Bacterial replication in the spleen was monitored at selected timepoints. U112 (wild-type), GB2/pGB40Em (GB2, *mglAB*$^+$).

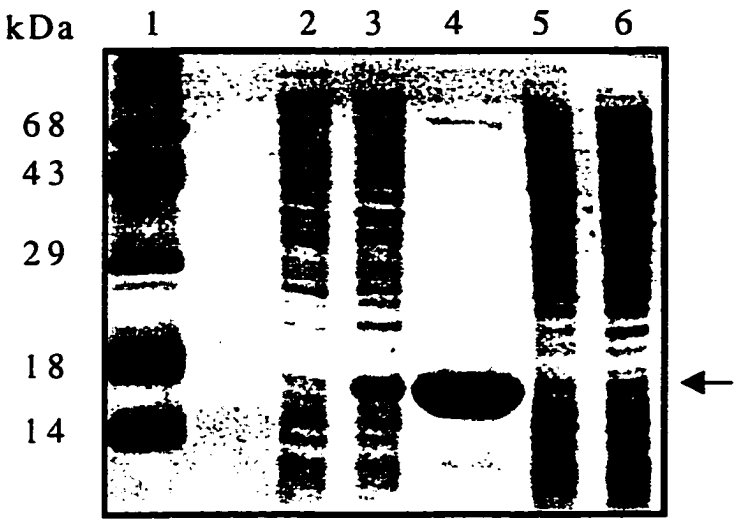
the detection limit of the assay; not shown in Fig. 14) from the spleens of mice infected with GB2. Mice infected with GB2/pGB40Em also exhibited symptoms of murine tularemia including hackling of the fur, splenomegaly, conjunctival discharge, and general lethargy, whereas GB2-infected mice showed none of these symptoms and remained active in their cages throughout the course of the experiment.

Western blot analysis of *mglB* expression

DNA corresponding to the *mglB* ORF was cloned into the His-tag expression vector pQE30 and expressed in *E. coli* as a recombinant protein containing an N-terminal fusion of 6 histidine residues. Overexpression of *mglB* and purification of the recombinant protein is shown in Fig. 15A. The apparent molecular mass of the recombinant protein is in agreement with that predicted for the recombinant protein (16.6 kDa).

Expression of *mglB* in the various *mgl* mutants was analyzed by Western blotting using antiserum against recombinant MglB. The production of MglB detected in GB2 shows that *mglB* expression is not affected by the point mutation in *mglA*, and confirms that *mglA* is required for the intracellular growth of *F. novicida* (Fig. 15B, lane 2). The absence of MglB in the *mglB* insertion mutant strain GB6 supports the Southern blot evidence indicating disruption of *mglB* in this strain (Fig. 15B, lane 4). The absence of MglB in the *mglA*

A.



B.

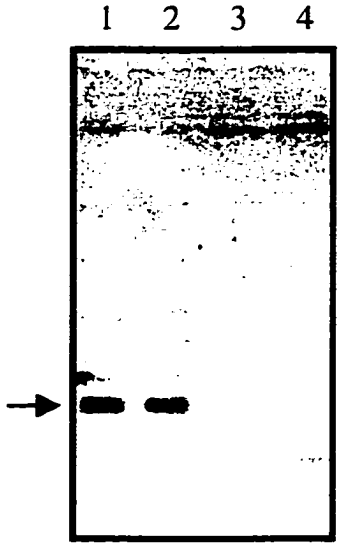


Figure 15. Analysis of MglB expression in *F. novicida*. Arrows indicate location of recombinant MglB (A) or MglB (B).

A. Purification of recombinant His-tagged MglB. SDS-PAGE was performed and the proteins were visualized by staining with Coomassie brilliant blue. Apparent molecular masses are indicated on the left in kDa. Lanes: 1, molecular mass standards; 2, BL21/pREP4/pGB56 (no IPTG); 3, BL21/pREP4/pGB56 (2 h after addition of IPTG); 4, purified His-MglB; 5, BL21/pREP4/pQE30 (no IPTG); 6, BL21/pREP4/pQE30 (2 h after addition of IPTG).

B. Western blot analysis of MglB expression in *F. novicida*. Lanes: 1, U112 (wt); 2, GB2 (*mglA*); 3, GB5 (*mglA*::mTn*10*Km); 4, GB6 (*mglB*::mTn*10*Km).

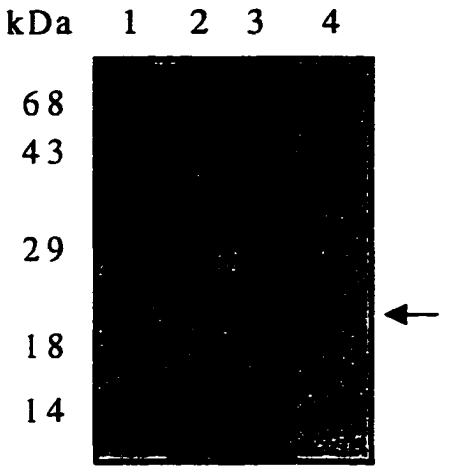
insertion mutant strain GB5 provides further evidence that *mglA* and *mglB* are organized in an operon.

Western blot analysis of *mglA* expression

To prepare MglA antiserum, *mglA* was first expressed as an N-terminal His_6-tag fusion protein in *E. coli*. Overexpression of *mglA* and purification of the recombinant protein is shown in Fig. 16A. The predicted molecular mass of the recombinant protein (24 852 Da) is in agreement with the apparent molecular mass as measured by SDS-PAGE. Rabbit antiserum against the recombinant protein was then generated using purified His-MglA. As shown in Fig. 16B, the polyclonal rabbit antiserum specifically reacts with a protein of similar electrophoretic mobility to His-MglA in lysates of *E. coli* expressing recombinant MglA which is absent from *E. coli* cells containing the vector DNA (pQE30).

Western blot analysis of *mglA* expression in the *mgl* mutant strains indicates that the mutant MglA is produced in GB2 (Fig. 16C, lower arrow). Consistent with the Southern blot data, MglA is absent from the *mglA* insertion mutant strain GB5. A *mglB* mutant (GB6) also lacks detectable levels of MglA, suggesting that MglB may be required for expression of *mglA* or that MglA is unstable in the absence of MglB. A cross-reacting protein of approximately 55 000 Da was detected in GB5 and GB6 (Fig. 16C, upper arrow). Longer

A.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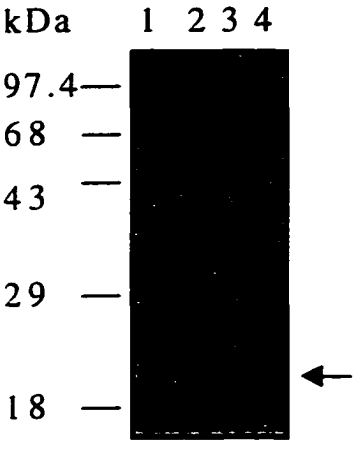
68
43
29
18
14

B.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68
43
29
18

C.

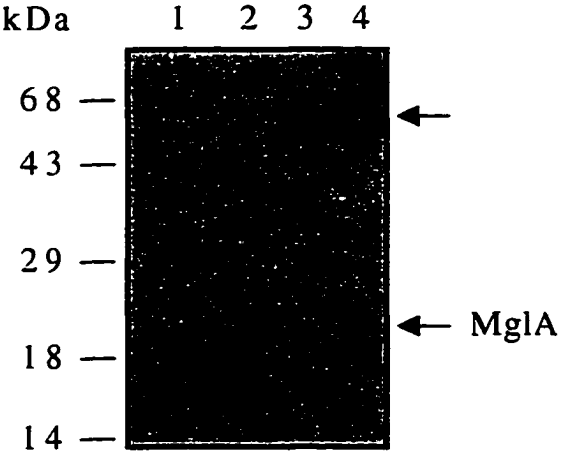

kDa 1 2 3 4
68
43
29
18
14
MglA

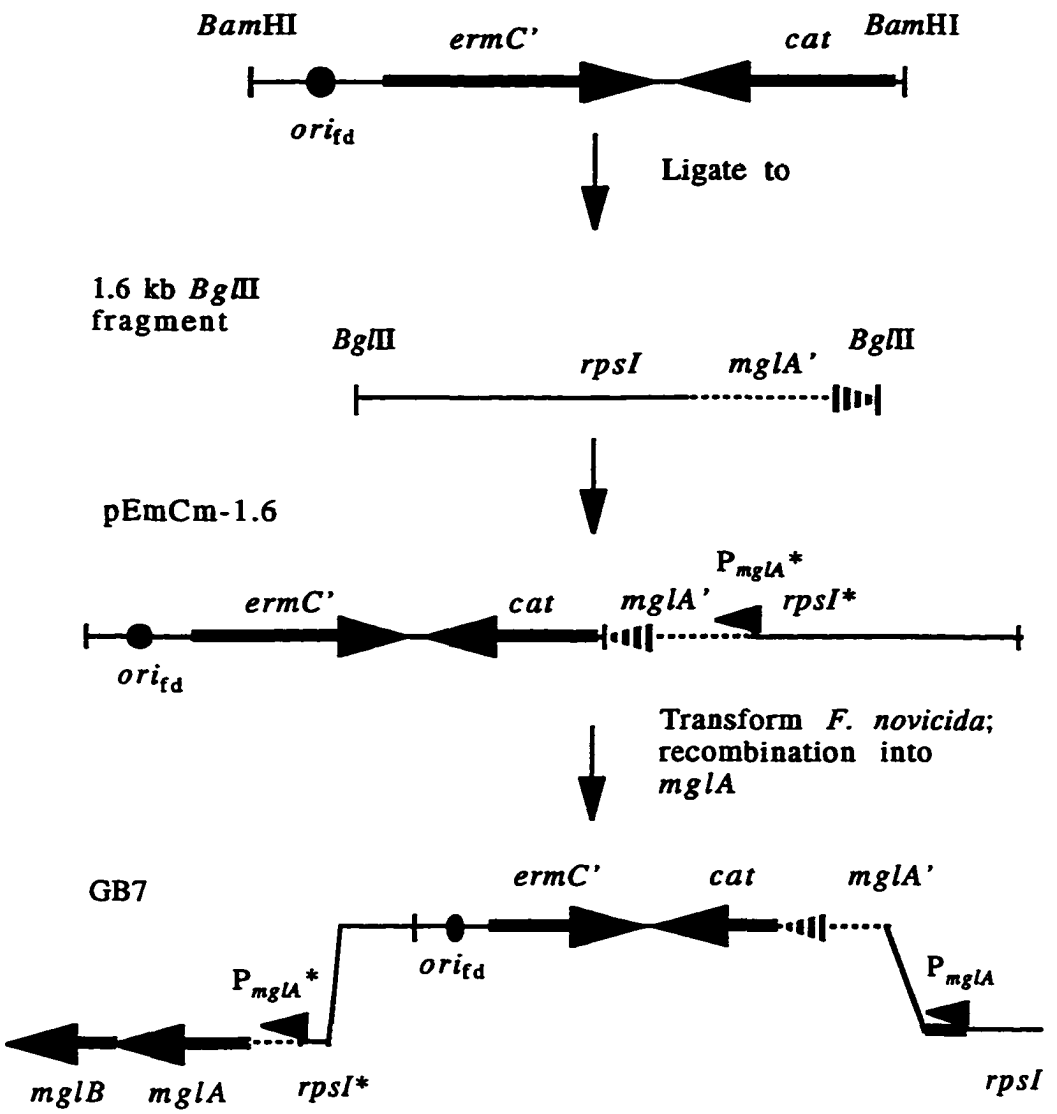
Figure 16. Analysis of MglA expression in *F. novicida*. Apparent molecular mass markers are indicated on the left in kDa. A. Purification of recombinant His-tagged MglA. SDS-PAGE was performed and the proteins were visualized by staining with Coomassie brilliant blue. Lanes: 1, molecular mass standards; 2, BL21/pREP4/pGB51 (no IPTG); 3, BL21/pREP4/pGB51 (3 h after addition of IPTG); 4, purified His-MglA. B. Western blot analysis of MglA expression in *E. coli*. Lanes: 1, BL21/pREP4/pQE30 (no IPTG); 2, BL21/pREP4/pQE30 (3 h after addition of IPTG); 3, BL21/pREP4/pGB51 (no IPTG); 4, BL21/pREP4/pGB51 (3 h after addition of IPTG). C. Western blot analysis of MglA expression in *F. novicida*. Lanes: 1, U112 (wild-type); 2, GB2 (*mglA*); 3, GB5 (*mglA*::mTn*10*Km); 4, GB6 (*mglB*::mTn*10*Km). Upper arrow indicates protein cross-reactive with MglA antiserum.

exposures revealed the presence of a corresponding cross-reactive protein in U112 and GB2 (data not shown). The reason for the higher cross-reactivity with the GB5 and GB6 lysates is unknown. One explanation is that this protein may be up-regulated in MglB$^-$ strains.

Regulation of *mglA* expression

As a preliminary examination of the conditions affecting *mglA* expression at the transcriptional level, the level of *mglA* expression in *F. novicida* grown in broth culture and in macrophages was measured. To accomplish this task, a reporter cassette suitable for use in *Francisella* was constructed. The cassette is a modification of the Em^R cassette (used in the experiments described above) containing a promoterless *cat* gene to which transcriptional fusions can be made (Fig. 17). Using this cassette, an *F. novicida* strain (GB7) was constructed which contains an *mglA'-cat* transcriptional fusion *in cis* with a wild-type copy of *mglAB* so the strain will retain the ability to grow intracellularly. Southern blot analysis of chromosomal DNA from GB7 probed with *mglA* and the EmCm reporter cassette confirmed the expected genotype (Fig. 18). When grown in Chamberlain's medium, peak expression occurs during exponential growth, apparently begins to decline during late log phase, and reaches a minimum of about one half the maximal level during stationary phase (Fig. 19A). Similar results are observed during growth in TSB-C. In infected macrophages, an increase in

A.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BamHI
ermC'
cat
BamHI
ori fd
Ligate to
1.6 kb BglII fragment
BglII
rpsI
mglA'
BglII
pEmCm-1.6
P mglA*
ermC'
cat
mglA'
rpsI*
ori fd
Transform F. novicida; recombination into mglA
GB7
ermC'
cat
mglA'
ori fd
P mglA*
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rpsI

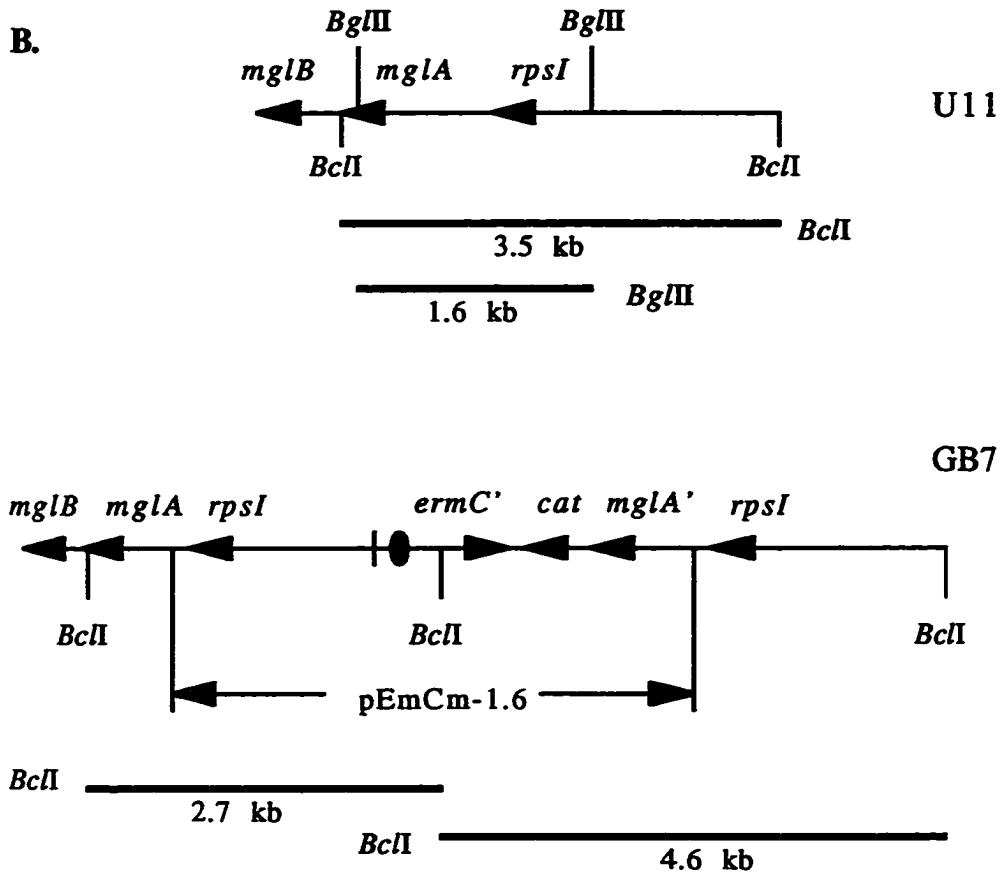


Figure 17. Construction of *F. novicida mglA'-cat* transcriptional fusion strain (GB7). A. The *Bam*HI-cut Em^R-Cm cassette containing the ori_{fd}, Em^R (*ermC'*), and promoterless *cat* fragments was isolated as described in the experimental procedures and ligated to a 1.6 kb *Bgl*II insert from pGB40 containing a portion of *mglA*. The resulting plasmid (pEmCm-1.6) containing an *mglA'-cat* transcriptional fusion was isolated from *E. coli* and recombined into the wild-type *F. novicida* (U112) chromosome. The integration event is arbitrarily shown as having occurred in *mglA*. The *mglA'-cat* fusion strain so created was designated GB7. The linear form of pEmCm-1.6 is shown for illustrative purposes. Dashed lines and asterisks indicate

recombinant *F. novicida* DNA. B. Predicted fragments produced by digestion of U112 and GB7 DNA with *Bcl*I. Not drawn to scale. A *Bcl*I site is located 3.5 kb upstream of the *Bcl*I site in *mglB*. Integration of the 3.8 kb pEmCm-1.6 into the chromosome by single cross-over homologous recombination, as predicted to have occurred in GB7, increases the distance between the above *Bcl*I sites to 7.3 kb (3.8 kb + 3.5 kb). Due to a *Bcl*I site within *ermC'*, the fragments produced by *Bcl*I digestion of GB7 DNA would be 2.7 kb and 4.6 kb. This is consistent with the Southern blot data shown in Fig. 18.

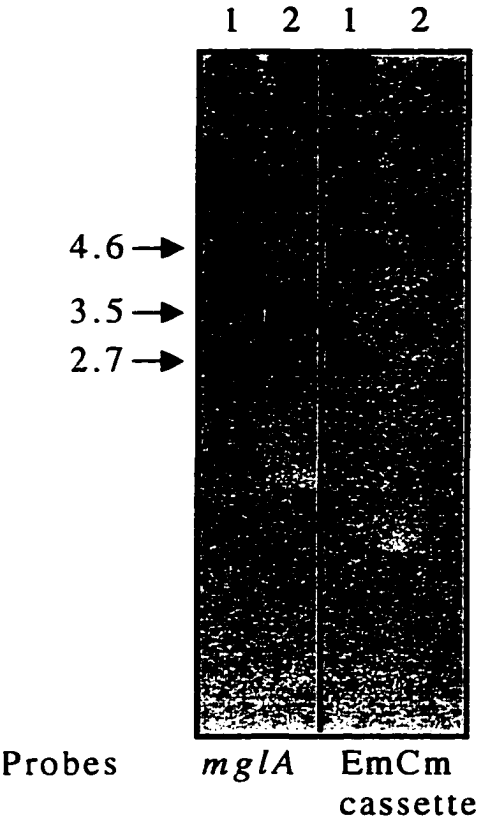
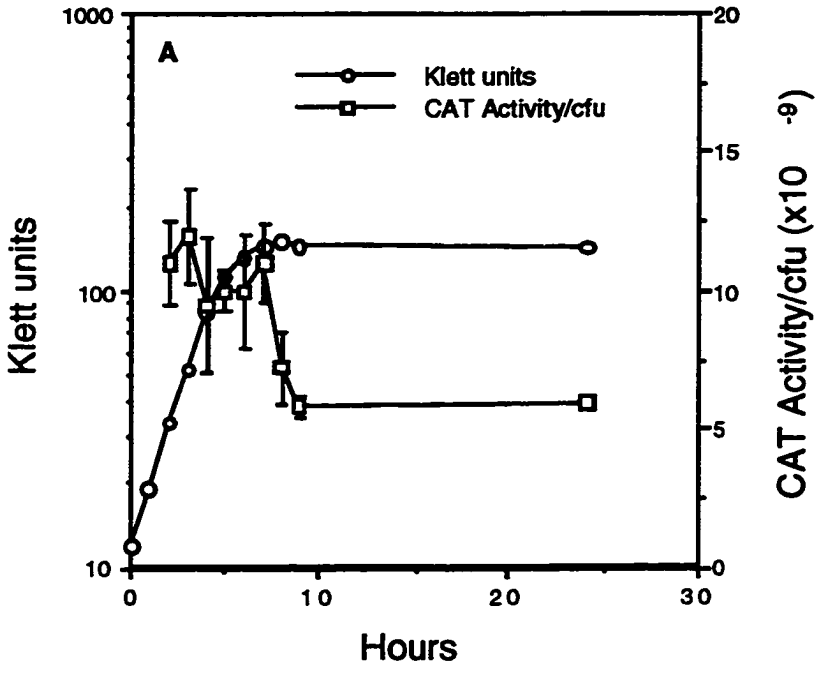


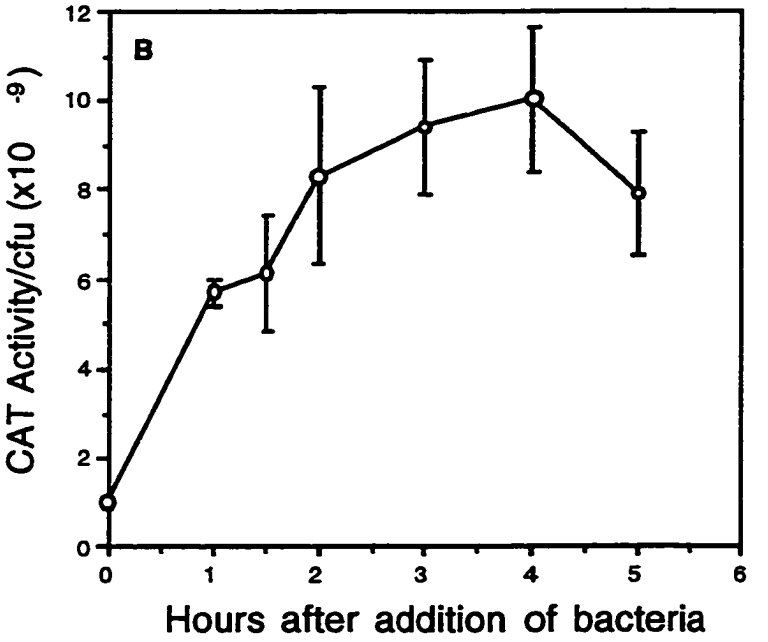
Figure 18. Southern blot analysis of *F. novicida mglA'-cat* fusion strain. Band sizes (in kb) were estimated using 1 kb ladder DNA standards. Lanes: 1, GB7 (U112, *mglA'-cat*); 2, U112 (wild-type). Chromosomal DNA from each strain was digested with *Bcl*I and probed with *mglA* or the EmCm cassette.

mglA expression can be detected within 1.5 h after the addition of bacteria (Fig. 19B). Maximal levels of CAT activity occur 3 to 4 h after infection. The magnitude of the increase in *mglA* expression ranged from 4-10 fold in three independent experiments (one representative is shown in Fig. 19B). When incubated in tissue culture medium in the absence of macrophages, no significant increase in *mglA* expression is detected (Fig. 19C). The data indicate that *mglA* expression increases early after infection of macrophages and during exponential growth *in vitro*.

Cell fractionation of *mgl* mutants

Previous studies of SspA in *E. coli* revealed that expression of several proteins during both exponential growth and stationary phase is altered in an *sspA* insertion mutant (Williams *et al.*, 1994a). To determine if MglA or MglB could alter the expression of other proteins in *Francisella*, crude cell fractions of various *mgl* mutants were compared by SDS-PAGE. The cells were divided into supernatant, envelope, and cell-free extract (CFE) fractions. When compared with wild-type *F. novicida*, the *mgl* mutants all exhibit altered levels of proteins in each of the fractions examined (Fig. 20). The most notable differences included 4 proteins of 70 kDa, 38 kDa, 33 kDa, and 17 kDa apparent molecular mass in the CFE fraction, an approximately 20 kDa envelope protein, and a 70 kDa secreted protein. The 17 kDa CFE protein is present in GB5 and absent from the other strains, suggesting its expression may be repressed by


A
Klett units
CAT Activity/cfu
1000
100
10
20
15
10
5
0
0
10
20
30
Hours
Klett units
CAT Activity/cfu (x10 -9)


B
12
10
8
6
4
2
0
0
1
2
3
4
5
6
Hours after addition of bacteria
CAT Activity/cfu (x10 -9)

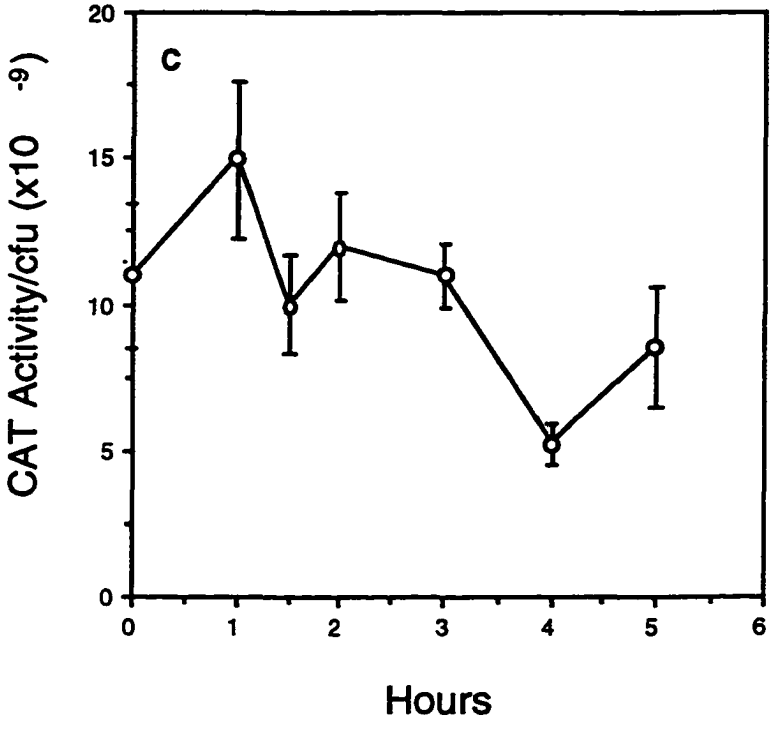


Figure 19. Analysis of the regulation of *mglA* expression. CAT activity and bacterial counts of the *mglA'-cat* fusion strain GB7 were measured during incubation under the following conditions: (A) growth in Chamberlain's medium; (B) infection of J774A.1 macrophages; (C) incubation in DMEM.

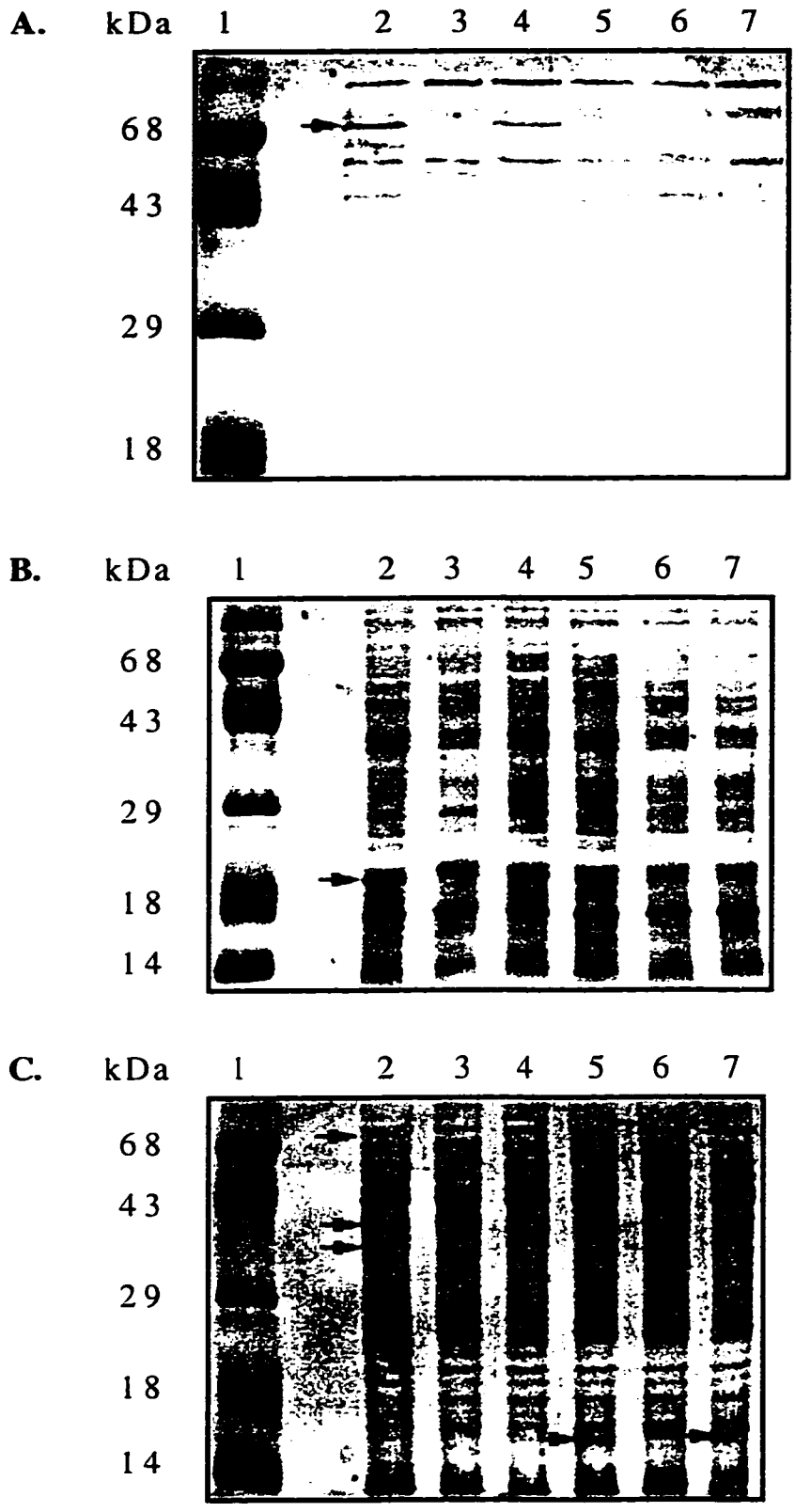

A.
kDa
1
2
3
4
5
6
7
68
43
29
18
B.
kDa
1
2
3
4
5
6
7
68
43
29
18
14
C.
kDa
1
2
3
4
5
6
7
68
43
29
18
14

Figure 20. Cell fractionation of *mgl* mutants. Crude cell fractions of stationary phase cultures of *F. novicida* strains were analyzed by SDS-PAGE and staining with Coomassie brilliant blue. Apparent molecular masses are indicated on the left in kDa. Arrows indicate proteins with altered levels in the *mgl* mutants. Cell fractions: (A) supernatant; (B) envelope; (C) cell-free extract. Lanes: 1, molecular mass markers; 2, U112 (wild-type); 3, GB2 (*mglA*); 4, GB2/pGB40Em (GB2, *mglAB*$^+$); 5, GB5 (*mglA*::mTn*10*Km); 6, GB6 (*mglB*::mTn*10*Km); 7, GB8 (*mglC*::mTn*10*Km).

MglA. The other proteins are present in wild-type *F. novicida* and the complemented GB2 strain (GB2/pGB40Em) but are absent from all *mgl* mutant strains. These data demonstrate that MglA and MglB can influence the levels of other *Francisella* proteins and suggest that they may act as global regulators, although the mechanism of the regulation is unknown.

Evidence for a third *mgl* gene - *mglC*

Random transposon mutagenesis of pGB40 also generated a plasmid with a mTn*10*Km insertion which, by analysis of restriction digests, maps downstream of *mglB* very near (within 200 bp) the end of the insert DNA (Fig. 8). Later, the site of the insertion was found to be immediately adjacent to the *Hin*dIII restriction site at the end of the insert DNA by sequencing using primers which hybridize to the transposon. This insertion mutation was recombined into the chromosome of wild-type *F. novicida*, and the transformants were screened for growth in macrophages. Out of 20 transformants screened, half were defective for intracellular growth (one example is shown in Fig. 21). As shown in Fig. 22, Southern blot analysis of *Xba*I-digested chromosomal DNA from a representative transformant defective for intramacrophage growth showed a band of approximately 4.8 kb in size which hybridizes to both the mTn*10*Km transposon and the 1 kb *Nde*I/*Hin*dIII fragment which spans *mglB* and the downstream region (Fig. 8). The 1.7 kb increase in the size

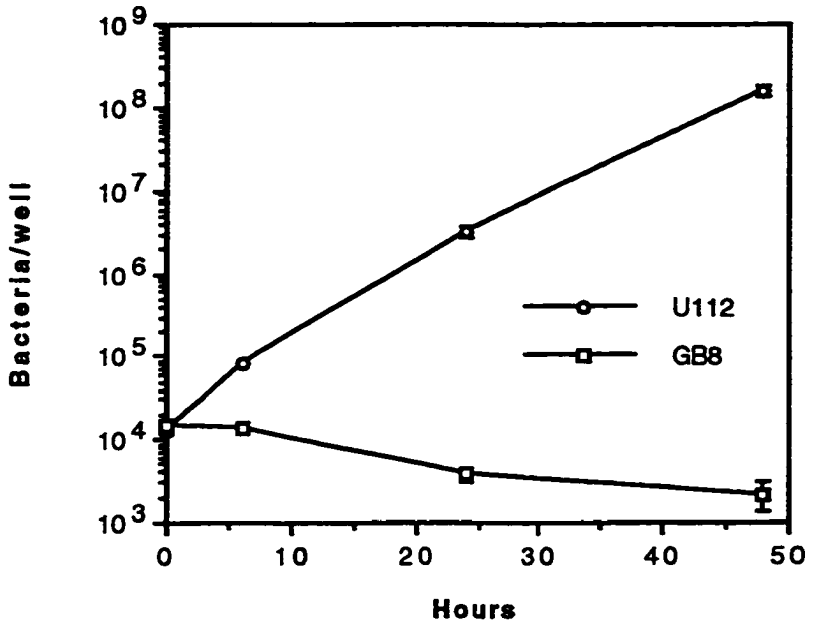


Figure 21. Growth of *F. novicida mglC* mutant in J774A.1 macrophages. U112 (wild-type), GB8 (*mglC*::mTn*10*Km).

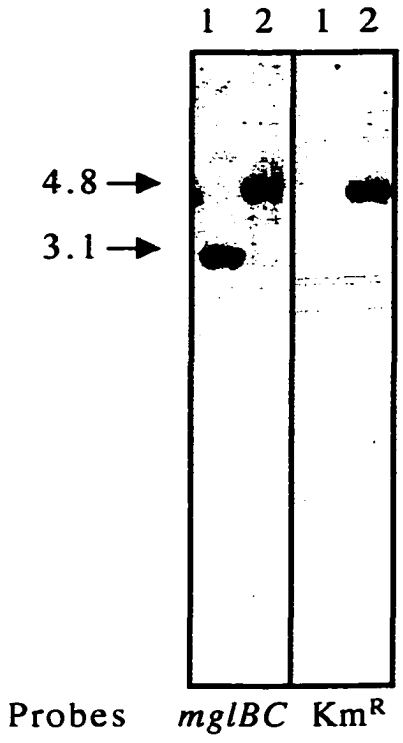


Figure 22. Southern blot analysis of *F. novicida mglC* mutant. Band sizes (in kb) were estimated using 1 kb ladder DNA standards. Lanes: 1, U112 (wild-type); 2, GB8 (*mglC*::mTn*10*Km). Chromosomal DNA from each strain was digested with *Xba*I and probed with either the 1 kb *Nde*I/ *Hin*dIII fragment of pGB40 (*mglBC*) or the kanamycin resistance cassette (Km^R).

of this hybridizing fragment as compared to the wild-type fragment is consistent with allelic replacement, and the strain was designated GB8. When grown on LB (X-p) agar, GB8 exhibits a phenotype similar to *mglA* and *mglB* mutants, although the GB8 colonies are slightly smaller (Fig. 12C). Analysis of cell fractions of GB8 by SDS-PAGE showed a protein profile similar to GB5 (Fig. 20). These data indicate that the mutation in GB8 may have inactivated a gene which is adjacent, and functionally related to *mglAB*.

Incomplete DNA sequence analysis of the region downstream of *mglB* in pGB40 and in an overlapping clone (pGB60) identified a putative 867 bp ORF (Fig. 8) transcribed convergently relative to *mglAB*. Due to the incomplete nature of this sequence (about 500 bp of the ORF is sequenced on only one strand), the analysis of the data here is restricted to a few comments. The corresponding gene has been tentatively designated *mglC*. The deduced amino acid sequence of *mglC* predicts a highly hydrophobic protein of approximately 33 kDa molecular mass with no significant similarity to sequences or motifs in the Genbank and PROSITE databases. The orientation of the *mglC* ORF confirms that the mTn*10*Km insertion in *mglB* does not exert a polar effect on a downstream gene in the *mglAB* operon and that *mglB* is required for intracellular growth.

N-terminal sequencing of a 70 kDa secreted protein

The cell fractionation studies demonstrated that the localization or production of a prominent 70 kDa secreted protein is dependent upon functional MglA and MglB (and the putative MglC). A number of important virulence factors in bacterial pathogens are secreted proteins. Thus, this is a good candidate for an *mgl*-regulated gene whose product contributes to the intracellular growth of *F. novicida.* To clone the gene encoding this 70 kDa protein, a reverse genetics approach was adopted. The 70 kDa protein is one of five abundant secreted proteins (Fig. 20A). Ammonium sulfate fractionation of *F. novicida* culture supernatant proteins identified a fraction (55-65%) which is enriched for the 70 kDa protein (Fig. 23). The 70 kDa protein-enriched fraction was separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and the 70 kDa band was excised. The first twenty amino acids of the N-terminal sequence (MHQPHQYSSNKIGIDTQYID) were determined by automated Edman degradation.

Isolation of a candidate clone for the gene encoding the 70 kDa protein

To clone the gene encoding the 70 kDa protein, two oligonucleotides were constructed by reverse translation of residues 1-7 (oligonucleotide 70kD-N_1) and residues 10-20 (oligonucleotide

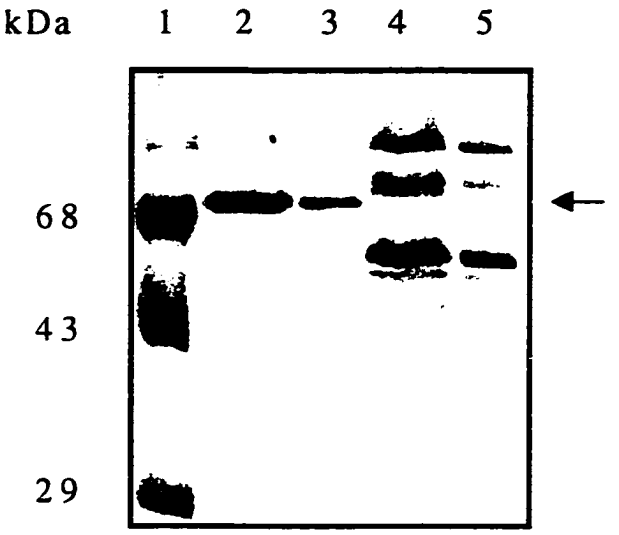


Figure 23. Ammonium sulfate fractionation of *F. novicida* culture supernatant proteins. Apparent molecular masses are indicated on the left in kDa. Arrow indicates the 70 kDa protein. Lanes: 1, molecular mass markers; 2 and 3, 55-65% fraction; 4 and 5, 0-55% fraction. Lanes 2 and 4 contain three times the volume of sample loaded in lanes 3 and 5.

70kD-N_2) of the N-terminal sequence. Both oligonucleotides contained a single (A/T) degeneracy corresponding to the third positions of the codons for proline-4 [CC(A/T), 70kD-N_1] and threonine-16 [AC(A/T), 70kD-N_2], but were otherwise non-degenerate. Nucleotide selections were made using the average codon preferences of 13 out of the 14 published *Francisella* gene sequences (Table 2).

When hybridized to *F. novicida* chromosomal DNA under conditions of reduced stringency (at 30 °C), 70kD-N_1 bound to multiple DNA fragments (Fig. 24A). Hybridization under higher stringency conditions at 42 °C showed 70kD-N_1 binds specifically to an approximately 2.8 kb *Xba*I fragment (Fig. 24B). In attempting to clone this fragment, a hybridizing clone (pGB62) was isolated which contained two *Francisella Xba*I fragments of about 2.6 kb and 2.9 kb in size, most likely resulting from a tri-molecular ligation event. A 263 bp portion of pGB62 was sequenced using 70kD-N_1 as a primer in the sequencing reaction. A putative ORF from bp 193 to bp 11 was found whose deduced amino acid sequence shows very high similarity to the C-terminus of prolyl-tRNA synthetases from several bacteria. This suggested pGB62 does not contain the 70 kDa protein gene as sequencing with the 70kD-N_1 primer is expected to produce DNA sequence corresponding to the ORF for the 70 kDa protein gene in a positive reading frame, while the ORF observed in pGB62 is oriented in a negative direction. The present data do not rule out the possibility that the central *Xba*I site joining the 2.6 kb and 2.8 kb

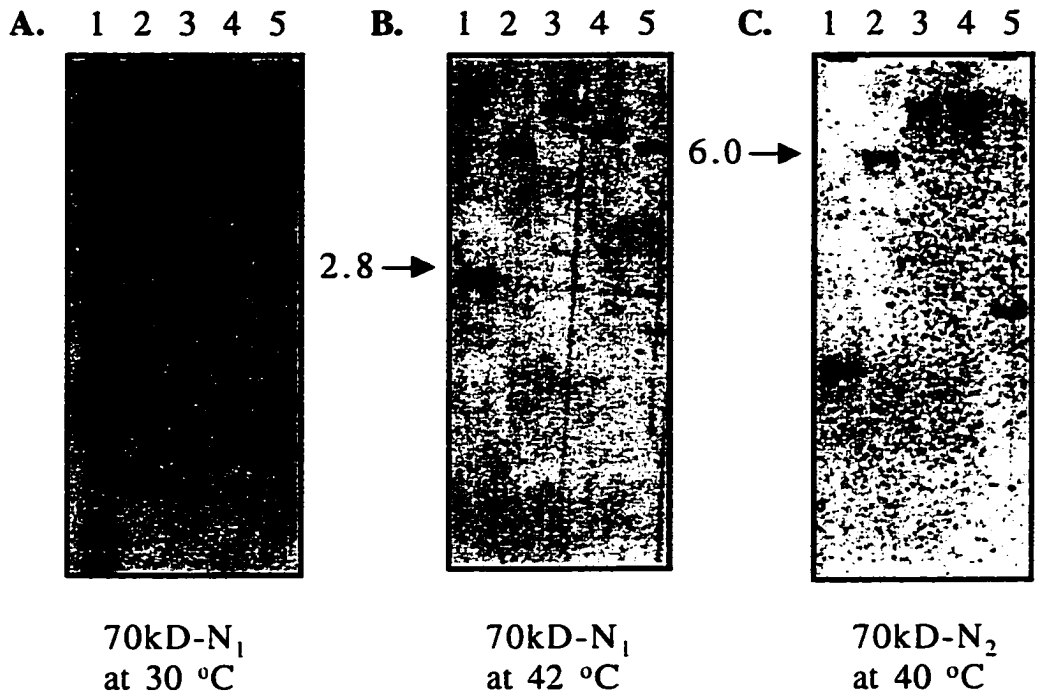


Figure 24. Southern blot analysis of *F. novicida* with oligonucleotides corresponding to the N-terminus of the 70 kDa protein. Chromosomal DNA from wild-type *F. novicida* (U112) was digested with the indicated restriction endonucleases and probed with oligonucleotides derived from the N-terminal sequence of the 70 kDa protein. The probes and hybridization/stringency wash temperature used are shown under each blot. Arrows indicate fragments which were cloned. Lanes: 1, *Xba*I; 2, *Sac*I; 3, *Pst*I; 4, *Eco*RI; 5, *Bgl*II.

fragments in pGB62 is located between the binding site of 70kD-N_1 and the start of the interpretable sequence data, which may be 50-100 bp downstream of the primer binding site. Consequently, the 263 bp sequence obtained would correspond to a presumably unrelated region located at the end of the adjacent *Xba*I fragment in pGB62.

Given the possibility that 70kD-N_1 was not hybridizing to the gene of interest (under stringent conditions), a Southern blot of *F. novicida* DNA was performed using the 70kD-N_2 oligonucleotide as a probe. At 40 °C, a 6 kb *Sac*I fragment hybridized with the probe (Fig. 24C) and was subsequently cloned into pRL498 to form pGB63. It is interesting to note that under hybridization conditions that are stringent for each oligonucleotide, 70kD-N_1 and 70kD-N_2 hybridize to different fragments (compare Fig. 24B and Fig. 24C). Since the oligonucleotides were designed using independent sections of the N-terminal sequence, hybridizing bands common to both probes would also serve as good candidates for the DNA fragment of interest. Under conditions of reduced stringency, 70kD-N_1 hybridized to some fragments (*Eco*RI, *Pst*I, and *Xba*I) which may correspond to fragments hybridizing to 70kD-N_2, but since there were no matches for the *Bgl*II and *Sac*I digests these hybridizing fragments are not likely to be identical. The data to this point indicated pGB63 is a candidate clone for the gene encoding the 70 kDa secreted protein.

Analysis of mutants defective for secretion of the 70 kDa protein

To determine if the 6 kb *Sac*I fragment contained the gene encoding the 70 kDa protein, I attempted to generate *F. novicida* null mutants with insertions in the corresponding region of the chromosome. I subjected pGB64 to random transposon mutagenesis using the Tn*Max2* transposon and transformed pools of mutagenized plasmids into wild-type *F. novicida*. The supernatants from cultures of several random colonies were then analyzed by SDS-PAGE. Out of 26 strains screened, 13 appear defective for secretion of the 70 kDa protein. Profiles of a random selection of 13 of the 26 strains are shown in Fig. 25. A 44 kDa protein is also missing from the supernatants of strains lacking the 70 kDa protein. It is not known whether the 44 kDa protein is a breakdown product derived from the 70 kDa protein. The sites of the transposon insertions in pGB64 have not yet been mapped. Although not definitive, the data support the possibility that the 6 kb *Sac*I fragment may contain at least a portion of the 70 kDa protein gene.

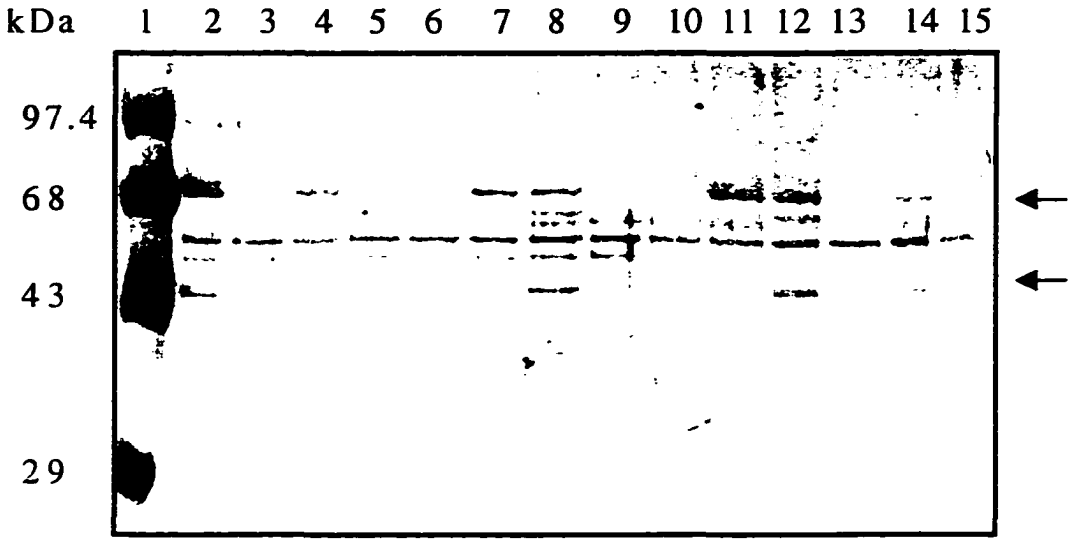


Figure 25. Analysis of culture supernatants of putative *F. novicida* 70 kDa protein mutants. Concentrated culture supernatants of *F. novicida* strains transformed with pGB64 carrying random Tn*Max2* insertions were analyzed by SDS-PAGE and staining with Coomassie brilliant blue. Apparent molecular masses are indicated on the left in kDa. Arrows indicate the 70 kDa protein and the 44 kDa protein. Lanes: 1, molecular mass markers; 2, U112; 3-15, U112/pGB64::Tn*Max2* transformants.

DISCUSSION

Francisella is one of many bacterial pathogens capable of intracellular growth. Although the nature of the host immune response and development of protective immunity to *Francisella* infection have received much attention, few virulence factors have been identified (Hood, 1977; Sandström *et al.*, 1988), especially those affecting intracellular growth (Anthony *et al.*, 1994; Bhatnagar *et al.*, 1994; Mdluli *et al.*, 1994; Cowley *et al.*, 1996). This may in part be a result of a paucity of genetic techniques for the study of *Francisella*. In a search to identify genes of *Francisella* required for intracellular growth, I isolated a spontaneous mutant, GB2, which is defective for growth in macrophages. The data have shown that the gene mutated in GB2, *mglA*, as well as an adjacent gene, *mglB*, are both required for the intracellular growth of *F. novicida*. To my knowledge, this is the first report of full complementation of a defined genetic lesion in *Francisella*. Mdluli *et al.* (1994) reported partial complementation of the serum-sensitive phenotype of a mutant defective for intramacrophage growth, but the essential nature of the target locus and lack of genetic tools limited their analysis of this mutant.

The phenotype of serum resistance distinguishes GB2 from two previously described *F. novicida* intracellular growth mutants, KEM7 and KM14 (Anthony *et al.*, 1994; Mdluli *et al.*, 1994). KEM7 is a *minD* mutant which shows increased sensitivity to oxidative killing and macrophage killing mechanisms occurring early after phagocytosis

(Anthony *et al.*, 1994). In *E. coli*, MinD is one of three proteins involved in controlling septum formation during cell division (de Boer *et al.*, 1989). KM14 apparently has a mutation in a locus (*valAB*) whose products are believed to transport LPS to the outer membrane (ValA) and phosphorylate lipid A (ValB) (Mdluli *et al.*, 1994; McDonald *et al.*, 1997). Thus, the mutations in KEM7 and KM14 occur in genes whose products contribute to maintaining the structural integrity of the cell. This correlates with their serum sensitive phenotype. By contrast, the serum resistant phenotype of GB2 suggests that its inability to replicate in macrophages is not a consequence of compromised cell structure.

It has been shown that intramacrophage replication contributes significantly to the virulence of *Francisella* in the murine model of tularemia (Bhatnagar *et al.*, 1994). The data presented here using mice infected with GB2 and a complemented GB2 strain (GB2/pGB40Em) supports this observation. In addition, this experiment also suggests *mglA* is required for *F. novicida* virulence in mice. Bhatnagar *et al.* (1994) described a temperature-sensitive RNA polymerase mutant (Rif7) defective for growth in macrophages. The phenotype of Rif7 was temporarily reversed by exposure to various *in vitro* stress conditions prior to macrophage infection, suggesting it was unable to mount an adequate stress response *in vivo*, though none of the proteins induced by the stress conditions were identified (Bhatnagar *et al.*, 1995). Although the stress response was not examined, GB2 is capable of growth at 42 °C.

The deduced amino acid sequences of *mglA* and *mglB* show similarity to the SspA and SspB proteins of *E. coli* and *Haemophilus* spp. Similar to their analogues in *E. coli* and *Haemophilus* spp., *mglAB* are organized in an apparent operon located immediately downstream of *rpsI*, which encodes the ribosomal protein S9 (Theisen and Potter, 1992; Fleischmann *et al.*, 1995; Blattner *et al.*, 1997). In addition, just as *sspA* null mutants fail to express *sspB* (Williams *et al.*, 1994a), an *mglA* null mutant lacks detectable MglB, supporting the notion that *mglA* and *mglB* are in an operon. The predicted initiation codon of *mglA* (UUG) is rare (Sprengart and Porter, 1997), but not unprecedented as the predicted start codon for *E. coli lacA* is also UUG (Büechel *et al.*, 1980). However, it seems the most likely choice considering the next possible start codon (AUG) of the *mglA* ORF, located 45 nucleotides downstream, is not preceded by a Shine-Dalgarno sequence and initiation at this point would remove a region of the deduced MglA sequence which shows significant similarity to SspA (Fig. 10A).

Relatively little is known about the functions of SspA and SspB in other bacteria. Δ*sspA* mutants (which are also *sspB*$^-$) exhibit reduced survival during extended amino acid starvation (Williams *et al.*, 1994a). Williams *et al.* (1994a) showed that *E. coli sspA* insertion mutants have altered expression of several proteins during both exponential and stationary phase. Combined with reports that SspA can stably associate with RNA polymerase holoenzyme (Ishihama and Saitoh, 1979; Williams *et al.*, 1994b) and *E. coli sspA* insertion

mutants fail to support bacteriophage P1 replication as a result of defective expression of P1 late genes (Williams *et al.*, 1991), it has been suggested that SspA (and perhaps SspB) may function as a transcriptional regulator by altering RNA polymerase promoter specificity (Williams *et al.*, 1994a). Given the similarity of MglA and MglB to SspA and SspB, I examined the various *mgl* mutants for altered expression of other proteins. The data show that the protein profiles of *mglA* and *mglB* mutants are different from wild-type *F. novicida* for several proteins. Additional differences may well have been observed with more sensitive analytical techniques. The light blue phenotype of the *mgl* mutants on LB (X-p) plates also suggests that MglA and MglB may control the expression of an exported phosphatase. It is not known whether MglA and MglB affect the expression of the periplasmic acid phosphatase *acpA* or perhaps other unidentified phosphatases. Based on these observations, we hypothesize that MglA and MglB may function as global regulators of the expression of a set of genes, at least some of which encode proteins that contribute to the intramacrophage growth of *Francisella*. Since the protein profiles of MglA and MglB mutants are quite similar, it is possible that MglA and MglB may act together in regulating gene expression.

One likely candidate for an *mgl*-regulated protein involved in mediating intracellular growth is an abundant 70 kDa secreted protein identified in this study. Many bacterial virulence factors are surface-localized or secreted, as might be expected to permit

interaction with the host. This protein is absent from the supernatants of all *mgl* mutant strains. I have described the preliminary work in the characterization of this protein. Using the N-terminal amino acid sequence obtained from partially purified 70 kDa protein, oligonucleotides were synthesized and used to isolate a candidate clone for the corresponding gene. Analysis of secreted protein profiles of transposon mutants of *F. novicida* constructed using this candidate clone suggest that the insert contains, at the very least, a gene(s) affecting the expression or secretion of the 70 kDa protein. Further analysis is necessary to determine if the clone (pGB63) contains the gene of interest and to entirely rule out the first clone isolated (pGB62) as a candidate.

There is also evidence for a third *mgl* gene, putatively designated *mglC*. A *mglC* null mutant (GB8) is defective for growth in macrophages, forms large, light blue colonies on LB (X-p) media, and on cell fractionation has a protein profile similar to an *mglA* null mutant. A convergently transcribed gene (relative to *sspB*) has been identified immediately downstream of *sspB* in both *E. coli* (Blattner *et al.*, 1997) and *H. influenzae* (Fleischmann *et al.*, 1995). The *E. coli* gene, *yhcL*, is predicted to encode a 48.8 kDa hydrophobic protein showing strong sequence similarity to a dicarboxylate transport protein. In *H. influenzae*, the gene downstream of *sspB* is predicted to encode a 625 amino acid protein which, by sequence similarity, has been identified as 1-deoxyxylulose-5-phosphate synthase. These sequences show no similarity to each other or MglC with the

exception of the high hydrophobicity of YhcL. Thus, it is possible that any functional interaction between MglC and MglA/MglB is unique to *Francisella*.

It is difficult to explain the mechanism by which substitution of Thr54 with Lys (T54K) disrupts MglA function in GB2. No significant changes in the secondary structure of the mutant MglA are predicted using two different algorithms for prediction of protein secondary structure. It is tempting to speculate that MglA activity may be regulated post-translationally by phosphorylation of Thr54 by a protein kinase as a terminal event in a signal transduction cascade. There are recent reports of protein threonine kinases and protein threonine phosphatases in prokaryotic systems though their functions remain to be elucidated (Kennelly and Potts, 1996; Zhang, 1996). However, interactions between two-component signal transduction pathways (the predominant type found in prokaryotes) and eukaryotic-type protein kinases have been demonstrated in eukaryotic systems (Zhang, 1996). Alternatively, the T54K mutation could alter the tertiary structure of an MglA domain involved in dimerization or interaction with another protein, perhaps RNA polymerase or MglB. Gel filtration chromatography of purified SspA suggests it exists as a dimer in native form (Ishihama and Saitoh, 1979). However, we have yet to investigate MglA protein-protein interactions. Alignment of the sequence of MglA with the sequences of SspA from *E. coli* and *Haemophilus* spp. revealed all four proteins are predicted to have a Thr residue corresponding to Thr54 of MglA.

Thus, elucidation of the role of Thr54 in MglA function may provide insight into the function of SspA in other bacteria.

In *E. coli*, *sspA* expression is induced during starvation for carbon, nitrogen, phosphate, or required amino acids and increases with decreasing growth rate (Williams *et al.*, 1994a). This was found to correlate with the similarity of the *sspAB* promoter to several gearbox promoters, which are starvation-inducible, growth-rate dependent, and contain the gearbox element (CGGCNAGTA) (Williams *et al.*, 1994a). Our initial studies of *mglA* expression have shown that *mglA* is maximally expressed during exponential growth in broth culture. The absence of MglA in an *mglB* null mutant suggests *mglAB* expression could be positively regulated by MglB. Alternatively, MglA may be degraded in the absence of MglB. In *E. coli*, *sspAB* expression is apparently not autoregulated (Williams *et al.*, 1994a). Thus, our results suggest that regulation of *mglA* expression may differ from that of *sspA* in *E. coli*. This is in agreement with the absence of the gearbox element in the putative *mglA* promoter region. However, our data do not rule out the possibility that *mglA* expression is responsive to exhaustion of some nutrient in the growth medium. We have also shown that *mglA* expression increases early after infection of macrophages, consistent with a role for MglA in intracellular growth. This pattern of *mglA* expression is not a consequence of the incubation conditions as bacteria incubated under identical conditions in the absence of macrophages show no significant increase in *mglA* expression.

It should be noted that the CAT assay employed in this study prohibits the direct comparison of CAT levels between bacteria from infected macrophages and bacteria grown in broth culture. This is the result of the detection of residual acetyl-CoA-consuming or CAT inhibitory activity in the cell extracts of J774 macrophages, in spite of the heat treatment which inactivates such activity in other cell lines (Sleigh, 1986). Admittedly, this is a limitation inherent in the assay which may have been resolved by the use of an alternative reporter gene, or by the use of methods to directly measure CAT protein levels such as enzyme-linked immunosorbant assays (ELISAs) or Western immunoblotting.

We are presently attempting to identify environmental conditions which might modulate *mglA* expression. Indeed, it would not be surprising to find nutritional starvation among these conditions as it appears to be an obstacle encountered by many bacteria in the intracellular environment. Auxotrophic mutants of *S. typhimurium*, *Mycobacterium bovis* BCG, *Brucella mellitensis*, and *L. pneumophila* defective for amino acid or nucleotide biosynthesis fail to grow intracellularly (Leung and Finlay, 1991; Bange *et al.*, 1996; Drazek *et al.*, 1995; Mintz *et al.*, 1988). Starvation for specific nutrients *in vitro* induces the synthesis of several *S. typhimurium* proteins which are also induced during infection of macrophages (Abshire and Neidhardt, 1993a). Furthermore, the alternative sigma factor RpoS, a global regulator of gene expression in response to nutrient deprivation, is required for the survival of *Salmonella* under

nutrient-limiting conditions which may be encountered in the intracellular environment, and controls the expression of important virulence genes (Fang *et al.*, 1992; Guiney *et al.*, 1995). Finally, the *Salmonella* PhoP/PhoQ two-component regulatory system essential for survival in macrophages (reviewed in Groisman and Heffron, 1995) senses (García Véscovi *et al.*, 1996) and, among other functions contributing to intramacrophage survival, mediates adaptation to the apparently low Mg^{2+} environment of the *Salmonella* phagosome by inducing the expression of the *mgtCB* operon located on a third *Salmonella* pathogenicity island (SPI-3) (Blanc-Potard and Groisman, 1997).

The identification and characterization of virulence factors of *Francisella* has been hampered by a lack of tools for genetic manipulation of the bacterium. Transformation, allelic replacement, and *in cis* partial complementation of a mutation in *Francisella* have been described previously (Anthony *et al.*, 1991b; Berg *et al.*, 1992; Mdluli *et al.*, 1994). Recently, crude cloning vectors have been developed based on a cryptic plasmid isolated from an *F. novicida*-like strain of *Francisella* (Norqvist *et al.*, 1996; Pavlov *et al.*, 1996). Building upon the efforts of Mdluli *et al.* (1994), this work further expands the repertoire of genetic techniques now available for the study of *Francisella*, which in theory could be applied to the study of other bacteria. I have identified a useful antibiotic resistance marker (Em^R encoded by *ermC'*) and created a *cat* reporter cassette for the analysis of gene expression in *F. novicida*. This Em^R marker

has the advantage of consistently producing an increased transformation frequency (at least 5-10 fold) as compared to the commonly used kanamycin resistance marker derived from Tn*903* (Way *et al.*, 1984) when transforming identical loci into *F. novicida* (data not shown). Additionally, the method used to construct the *mglA'-cat* fusion strain in this study (GB7) provides a valuable strategy for the analysis of the regulation of expression of chromosomal genes. This method preserves the location (i.e. chromosome as opposed to a plasmid), copy number, and genotype (*mglAB*$^+$) of the target locus and thus should provide a more reliable representation of the expression level.

Further characterization of MglA and MglB may lead to a better understanding of the functions of SspA and SspB and the general mechanisms by which bacteria control transcription. Identification of loci regulated by MglA and/or MglB should reveal genes required for the intramacrophage growth of *Francisella*, some of which may be conserved in other intracellular pathogens.

CHAPTER 2. Analysis of AcpA, a respiratory burst-inhibiting acid phosphatase of *Francisella*

INTRODUCTION

The production of toxic oxygen species constitutes an important component of the antimicrobial arsenal of phagocytic cells. In response to phagocytosis or stimulation by various agents, a large amount of superoxide anion is produced, which can subsequently dismutate into hydrogen peroxide (H_2O_2) (Chanock *et al.*, 1994). These molecules may give rise to other microbicidal species including singlet oxygen, hydroxyl radical, and hypochlorous acid (Robinson and Badwey, 1994). The production of superoxide anion is catalyzed by the enzyme NADPH oxidase, which reduces oxygen to superoxide anion at the expense of NADPH (Chanock *et al.*, 1994). This antimicrobial response has been termed the respiratory (or oxidative) burst, due to the rapid consumption of oxygen associated with superoxide anion synthesis.

NADPH oxidase is a multimeric enzyme consisting of at least five different polypeptides localized in either the plasma membrane or cytosol in the inactive state (McPhail, 1994). The membrane component is cytochrome b_{558}, a heterodimeric protein composed of gp91^{phox} and p22^{phox} subunits (Chanock *et al.*, 1994). The three essential cytosolic proteins are p47^{phox}, p67^{phox}, and a small GTP-binding protein called Rac (McPhail, 1994). Another cytosolic

protein, $p40^{phox}$, may play a regulatory role in NADPH oxidase function (Tsunawaki *et al.*, 1996; Fuchs *et al.*, 1997; Sathyamoorthy *et al.*, 1997) but is not required in a cell-free model of oxidase activation/assembly (Abo *et al.*, 1992).

During the activation of NADPH oxidase, the cytosolic components are translocated to the plasma membrane where they associate with cytochrome b_{558} to form the active enzyme (Heyworth *et al.*, 1994; De Leo *et al.*, 1996; Fuchs *et al.*, 1997). NADPH oxidase activation also results in the phosphorylation of $p47^{phox}$ (El Benna *et al.*, 1994; Waite *et al.*, 1997), $p67^{phox}$ (El Benna *et al.*, 1997), and $p40^{phox}$ (Fuchs *et al.*, 1997). It has been suggested these phosphorylation events alter the intermolecular interactions of $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ to allow assembly of the functional oxidase (De Leo *et al.*, 1996; Fuchs *et al.*, 1997).

Over the past 14 years, evidence has accumulated that certain intracellular pathogens may enhance their survival in phagocytic cells by inhibiting the respiratory burst using tartrate-resistant, non-specific acid phosphatases (ACPs). The first burst-inhibiting ACP described was a cell-surface ACP ($ACP\text{-}P_1$) purified from *Leishmania donovani* (Remaley, *et al.*, 1984; Remaley *et al.*, 1985). Shortly thereafter, a burst-inhibiting ACP (ACP_2) was isolated from *Legionella micdadei* (Saha *et al.*, 1985). Both the *Leishmania* and *Legionella* ACPs hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) and inositol 1,4,5-trisphosphate (IP_3), and it has been suggested this activity may disrupt the signalling pathways that lead

to the activation of the respiratory burst (Das *et al.*, 1986; Saha *et al.*, 1988). More recently, Baca *et al.* (1993) detected tartrate-resistant, burst-inhibiting ACP activity in the obligate intracellular pathogen *Coxiella burnetii*. Unfortunately, conclusive evidence of a role for burst-inhibiting ACPs as virulence factors has yet to be obtained. Evaluation of their possible contribution to virulence has been limited by the absence of any sequence information or ACP mutants.

Recently, a tartrate-resistant ACP (AcpA) was isolated from *Francisella* (Reilly *et al.*, 1996). Maximal hydrolysis of monophosphate ester substrates by AcpA occurs at pH 6.0. It has a broad substrate specificity, with phosphotyrosine and a phosphotyrosyl peptide being among the best substrates examined to date. AcpA was found to inhibit the oxidative burst of porcine neutrophils stimulated with either the chemoattractant peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol 12-myristate 13-acetate (PMA) (Reilly *et al.*, 1996). This study describes the cloning and sequencing of the gene encoding AcpA (*acpA*), and the preliminary examination of its role in *Francisella* virulence, especially its contribution to growth in macrophages.

RESULTS

Cloning and sequencing of *acpA*

To permit further characterization of AcpA, particularly with regard to assessing its potential role in *Francisella* virulence, the structural gene for AcpA (designated *acpA*) was cloned and sequenced. An oligonucleotide probe was used to isolate a clone (pACP1) containing a portion of *acpA* from a *F. tularensis* LVS genomic library. The insert in pACP1 was then used as a probe to clone a 3.1 kb *Hin*dIII fragment from *F. novicida* containing the full length *acpA* gene (Fig. 26). The first 640 bp of the *F. tularensis* LVS *acpA* coding region was sequenced and is identical to the corresponding sequence in *F. novicida acpA*, consistent with the close relatedness of these bacteria indicated by other studies (Hollis *et al.*, 1989; Forsman *et al.*, 1994). The complete sequence of *F. novicida acpA* is shown in Fig. 27. A putative ribosome binding site is located 7 nucleotides upstream of the predicted start codon. The first 21 amino acids of the deduced sequence, preceding the N-terminal Thr residue of AcpA, contains many of the functional elements of a standard Gram-negative signal peptide (Izard and Kendall, 1994). The following 20 amino acid sequence is identical to the N-terminal sequence (TDVNNSKPNDYGTLVKIEQK) determined by Edman degradation of purified AcpA. Furthermore, the deduced sequence (MYPNAKNPEGE) at position 422-454 is identical to the

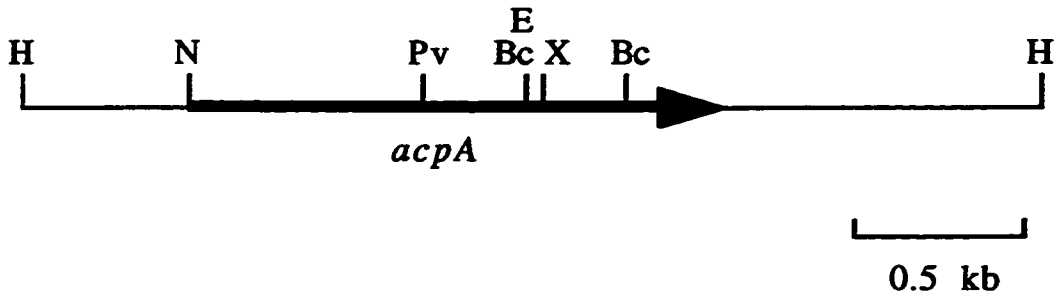


Figure 26. Restriction map and ORF organization of the *acpA* region. The 3.1 kb insert in pGB3 is shown. Restriction sites are abbreviated as follows: Bc, *Bcl*I; E, *Eco*RI; H, *Hin*dIII; N, *Nde*I; Pv, *Pvu*II; X, *Xba*I. The region from approximately 200 bp upstream to 50 bp downstream of the *acpA* ORF was sequenced. Replacement of the 300 bp *Bcl*I fragment with an Em^R cassette created a recombinant plasmid (pGB3Em) which was used to generate an *F. novicida acpA* null mutant (2L).

```
AAAAATATTGCTTTTACACCGAAGTCTAAAATATAATTATTCCCCTGTTATTTTAAATAT   60

TGATGCTTTATATAAAAAAGTAAATATTCTAGAGCAAAAATATAAATATTTAAATCTTTT  120

TCTTAAAAAAATTTTTTTTAGCAATAATTTATTTAATTTTGTTATTAATATTTCCTTTGAG  180
                    -35                          -10

TCTAACAAACTAAAGGTATCATATGAAGCTCAATAAAATTACTTTAGGAATTTTAAGTCT  240
              SD        M  K  L  N  K  I  T  L  G  I  L  S  L

AAGTATCGCAACAACGACTTTTGCCACAGATGTGAATAATAGCAAACCAAATGATTATGG  300
 S  I  A  T  T  T  F  A  T  D  V  N  N  S  K  P  N  D  Y  G

AACTCTTGTAAAAATAGAACAAAAATTATTTAATAATGCGAATACTCTAAAAACTACAAC  360
 T  L  V  K  I  E  Q  K  L  F  N  N  A  N  T  L  K  T  T  T

TCCAATAAAGCACGTAGTAATAATATTCCAAGAGAATAACTCTTTTGATAGATACTTTGG  420
 P  I  K  H  V  V  I  I  F  Q  E  N  N  S  F  D  R  Y  F  G

AATGTACCCTAATGCCAAAAACCCAGAGGGTGAGCCAAAATTTGTAGCCAAAGAAAATAC  480
 M  Y  P  N  A  K  N  P  E  G  E  P  K  F  V  A  K  E  N  T

TCCAAATGTTAATGGTCTGACAAAACAATTATTAGAGAATAATCCAAATACAAAAAATCC  540
 P  N  V  N  G  L  T  K  Q  L  L  E  N  N  P  N  T  K  N  P

TTATCGTTTAGATAGAAATTTCCAACCTTGCTCACAAAATCATGAGTACCATCAAGAAAT  600
 Y  R  L  D  R  N  F  Q  P  C  S  Q  N  H  E  Y  H  Q  E  I

TTCTTCTTTTAATGGTGGATTAATGAACAAATTTGTTGAACATGGTGGTCATGATAATGA  660
 S  S  F  N  G  G  L  M  N  K  F  V  E  H  G  G  H  D  N  D

CACCTATAAACAAAACTGTGATGGTCAAGTCATGGGATATTATGATGGTAATACTGTCAC  720
 T  Y  K  Q  N  C  D  G  Q  V  M  G  Y  Y  D  G  N  T  V  T

AGCATTATGGAATTACGCACAAAATTTCGCTCTAAATGATAATACGTTTGGTACAACTTT  780
 A  L  W  N  Y  A  Q  N  F  A  L  N  D  N  T  F  G  T  T  F

TGGTCCATCAACACCTGGTGCCCTTAACCTAGTGGCTGGTGCAAATGGTCCAGCAATGAG  840
 G  P  S  T  P  G  A  L  N  L  V  A  G  A  N  G  P  A  M  S

TCCAAGTGGTAATTTAGAAAATATTGAAAACAACTATATCATTGATGATCCTAACCCATA  900
 P  S  G  N  L  E  N  I  E  N  N  Y  I  I  D  D  P  N  P  Y

CTACGATGATTGCTCTTATGGTACAAGTAAATCTGGCGATACAAATACAGCTGTAGCAAA  960
 Y  D  D  C  S  Y  G  T  S  K  S  G  D  T  N  T  A  V  A  K

AATTACTGATGGTTATAATATTGGACACTATCTAACTCAAAAAGGTATTACTTGGGGTTG 1020
 I  T  D  G  Y  N  I  G  H  Y  L  T  Q  K  G  I  T  W  G  W

GTTCCAAGGAGGATTCAAACCAACAAGCTACTCTGGTAAAACAGCAATATGTGATGCTAT 1080
 F  Q  G  G  F  K  P  T  S  Y  S  G  K  T  A  I  C  D  A  M

GAGCACTAATAAGTTCGGTGTAAAATCAAGAGACTATATACCTCATCATGAGCCTTTTAA 1140
 S  T  N  K  F  G  V  K  S  R  D  Y  I  P  H  H  E  P  F  N
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CTACTGGAAAGAGACATCAAACCCTCATCATTTAGCACCAAGTGATGATAAGTATATAGG  1200
 Y  W  K  E  T  S  N  P  H  H  L  A  P  S  D  D  K  Y  I  G

TAGTAATGACCAAGCTAATCATCAGTACGACATAAGTGAATTCTGGAAGGCTCTTGATCA  1260
 S  N  D  Q  A  N  H  Q  Y  D  I  S  E  F  W  K  A  L  D  Q

AAACAACATGCCTGCGGTAAGTTACTTAAAAGCTCCTGGCTACCAAGATGGTCATGGAGG  1320
 N  N  M  P  A  V  S  Y  L  K  A  P  G  Y  Q  D  G  H  G  G

CTACTCAAACCCTCTAGATGAACAAGAATGGCTAGTCAATACCATTAATAGAATCCAGCA  1380
 Y  S  N  P  L  D  E  Q  E  W  L  V  N  T  I  N  R  I  Q  Q

ATCAAAAGCTGGGATAGCACATGCAATTATAATTATTTATGATGACTCTGATGGTGACTA  1440
 S  K  A  G  I  A  H  A  I  I  I  I  Y  D  D  S  D  G  D  Y

TGACCATGTCTACAGCCCAAAATCACAGTTTAGCGATATCAAAGGAAGACAAGGTTATGG  1500
 D  H  V  Y  S  P  K  S  Q  F  S  D  I  K  G  R  Q  G  Y  G

ACCAAGATTACCAATGCTTGTTATTTCTCCTTATGCTAAAGCAAACTATGTTGATCATTC  1560
 P  R  L  P  M  L  V  I  S  P  Y  A  K  A  N  Y  V  D  H  S

ATTACTTAATCAAGCATCTGTACTTAAGTTTATAGAGTATAACTGGGGCATCGGCTCAGT  1620
 L  L  N  Q  A  S  V  L  K  F  I  E  Y  N  W  G  I  G  S  V

TAGTAAGTATAGTAATGATAAATACTCAAACAATATCTTAAACATGTTTGATTTTAATAA  1680
 S  K  Y  S  N  D  K  Y  S  N  N  I  L  N  M  F  D  F  N  K

AGAACAAAAAACACTAAAACTGATTTTAGATCCTAAGACAGGATTAGTGATGGATAAATT  1740
 E  Q  K  T  L  K  L  I  L  D  P  K  T  G  L  V  M  D  K  L

AAACTAAAAATATTTACTCGGTAAGTTGCTTTAATCTATTGTTTTCGCTTATCGCCTC    1798
 N  *
```

Figure 27. Nucleotide and deduced amino acid sequence of the 1798 bp *acpA* region of pGB3. The sense strand is shown with amino acids represented by single letter abbreviations below the nucleotide sequence. Putative -35 and -10 promoter regions and a potential ribosome-binding site are indicated by underlining and in bold, respectively. The stop codon is represented by an asterisk. The *acpA* ORF spans bp 203 to bp 1744. The single underlined segment in the ORF is the start of the AcpA N-terminal peptide which is identical with that obtained by sequencing the purified protein. The

double underlined segment 3' to the AcpA N-terminal sequence is the deduced amino acid sequence identical with a cyanogen bromide peptide sequence prepared from AcpA.

peptide sequence of a cyanogen bromide fragment of AcpA determined by Edman degradation. The predicted molecular weight of signal peptide cleaved *F. novicida* AcpA based on the deduced sequence (55 413 Da) is in close agreement with the molecular weight of *F. tularensis* AcpA (55 759 Da) determined by mass spectrometry (Reilly *et al.*, 1996). These data indicate that the *acpA* nucleotide sequence in Fig. 27 encodes AcpA.

The deduced amino acid sequence of *acpA* shows no similarity to other known acid phosphatases, but it is similar to phospholipase C (PLC) proteins identified in *Pseudomonas aeruginosa* (Plc-N and Plc-H) (Pritchard and Vasil, 1986; Ostroff *et al.*, 1990) and *Mycobacterium tuberculosis* (MpcA and MpcB) (Leão *et al.*, 1995; Johansen *et al.*, 1996) (Fig. 28). The amino acid sequence of Plc-N is 40% identical overall to that of Plc-H with the majority of the homology occurring in the N-terminal two-thirds of the proteins (Ostroff *et al.*, 1990). MpcA shows overall identities of 72%, 36% and 38% to MpcB, Plc-H, and Plc-N, respectively (Johansen *et al.*, 1996). AcpA is 18% identical overall to MpcA, MpcB, and Plc-N and 17% identical overall to Plc-H, although there are several blocks of highly conserved regions (Fig. 29). Plc-H (730 amino acids) and Plc-N (692 amino acids) are considerably larger than MpcA (520 amino acids), MpcB (521 amino acids), and AcpA (514 amino acids). Comparison of the hydropathy plots of AcpA and the PLCs indicates these proteins are all largely hydrophilic, although AcpA contains fewer regions of

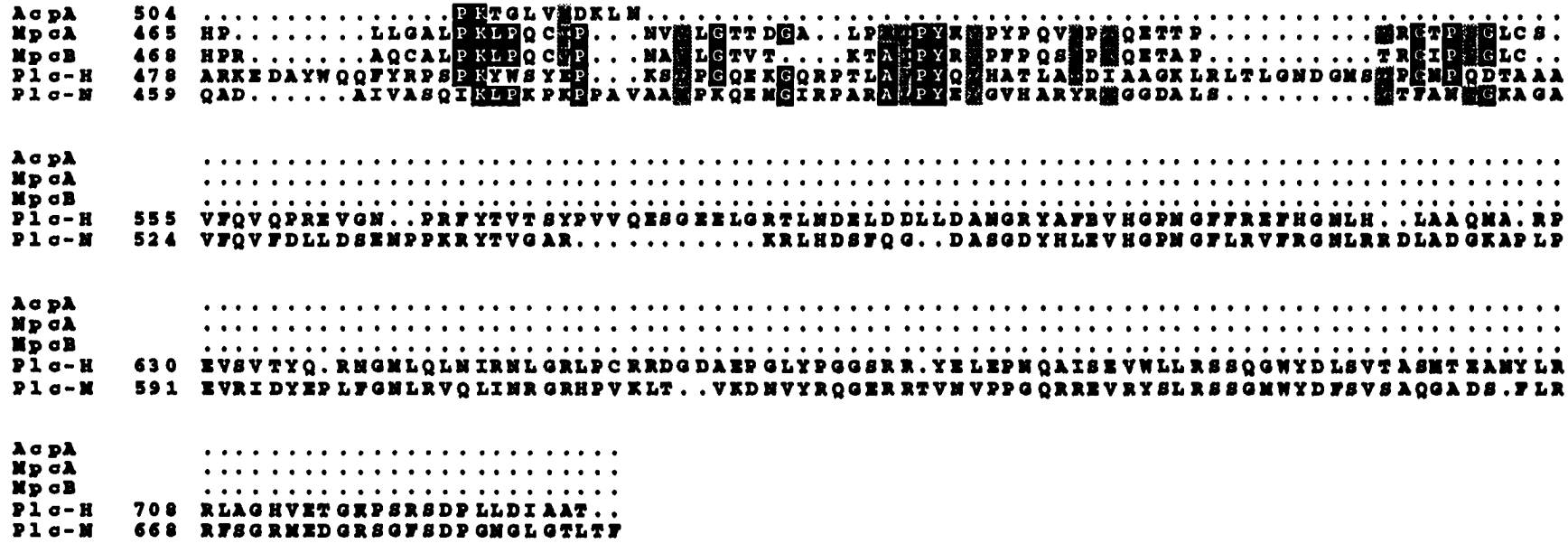


Figure 28. Amino acid alignment between AcpA and PLC proteins from *M. tuberculosis* (MpcA, MpcB) and *P. aeruginosa* (Plc-H, Plc-N). Identical residues are shaded black. Conserved residues are shaded gray.

hydrophobicity. Based on amino acid content AcpA, at 27.2%, is less hydrophobic than MpcA (35.6%), MpcB (35.5%), Plc-H (32.7% for residues 1-520; 34.5% overall), and Plc-N (34.2% for residues 1-520; 31.5% overall).

The *P. aeruginosa* and *M. tuberculosis* PLCs are all capable of using phosphatidylcholine as a substrate (Ostroff *et al.*, 1990; Johansen *et al.*, 1996). Preliminary experiments using the synthetic substrate p-nitrophenylphosphorylcholine (pNPPC) detected PLC activity in AcpA when assayed at pH 7.3 but not at pH 6.0, which is the optimum for the phosphomonoesterase activity of AcpA (performed by T.J. Reilly and M.S. Kuhlenschmidt). The PLC specific activity of AcpA (610 nmol of pNPPC hydrolyzed/h/mg) was comparable to that of a commercial *Clostridium* phospholipase [1040 nmol/h/mg (Sigma, Type XIV)], but was 3-4 orders of magnitude lower than its phosphomonoesterase specific activity assayed at pH 7.3 [1.5 x 10^6 nmol of 4-methylumbelliferylphosphate (MUP)/h/mg] and pH 6.0 (9.5 x 10^6 nmol/h/mg). Phosphomonoesterase activity using MUP as a substrate was undetectable in the *Clostridium* PLC sample.

Growth of an *F. novicida acpA* mutant in macrophages

To determine if AcpA contributes to the intracellular replication of *F. novicida*, an *acpA* mutant was constructed. A plasmid containing a copy of *acpA* in which a 300 bp *Bcl*I fragment of the gene (Fig. 26) is replaced by an Em^R cassette was created (pGB3Em)

and recombined into the chromosome of wild-type *F. novicida* by homologous recombination. Southern blot analysis of one strain, designated 2L, indicated gene replacement had occurred (Fig. 29). A 5.1 kb *Nde*I fragment in 2L hybridizes with both *acpA* and Em^R cassette probes. This represents an approximately 1 kb increase relative to the wild-type *acpA Nde*I fragment, corresponding to the addition of the Em^R cassette (1.35 kb) and the deletion of the 300 bp insert in the mutant allele. The mutation in 2L is predicted to truncate 486 bp from the 3'-end of *acpA*, removing the C-terminal 162 amino acids of AcpA. This represents 31.5% of the protein and includes 3 of the 5 most highly conserved regions between AcpA and the PLCs (Fig. 28).

The *acpA* mutant strain was then examined for growth in cell-line macrophages (J774A.1). The 2L strain exhibits growth kinetics identical to wild-type *F. novicida* (Fig. 30A). Others have isolated *F. novicida* mutants which grow in J774 but not in thioglycollate-elicited mouse macrophages, suggesting J774 macrophages may be deficient in some macrophage killing mechanisms (Mdluli, 1994). Thus, the 2L strain was also tested for growth in inflammatory mouse macrophages. 2L displays wild-type growth in inflammatory mouse macrophages (Fig. 30B), suggesting *acpA* is not required for the intramacrophage growth of *F. novicida*.

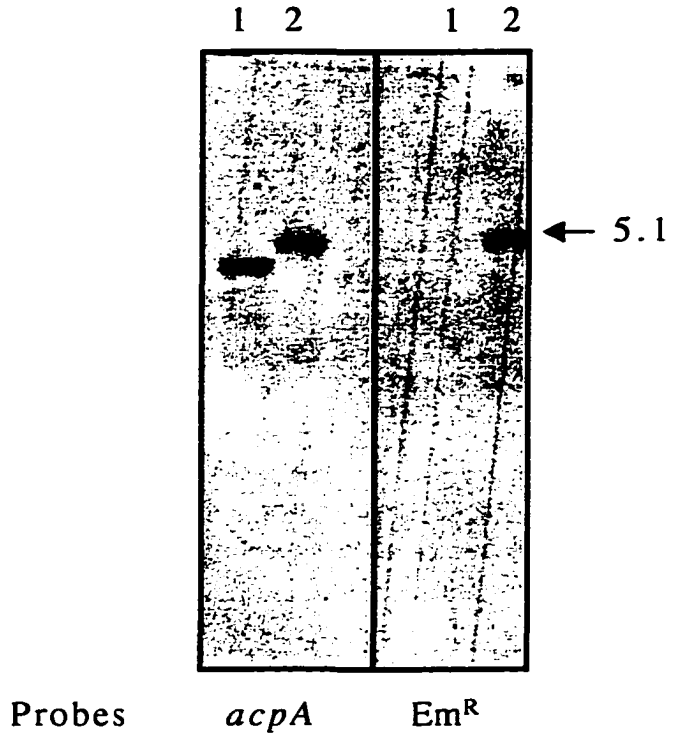
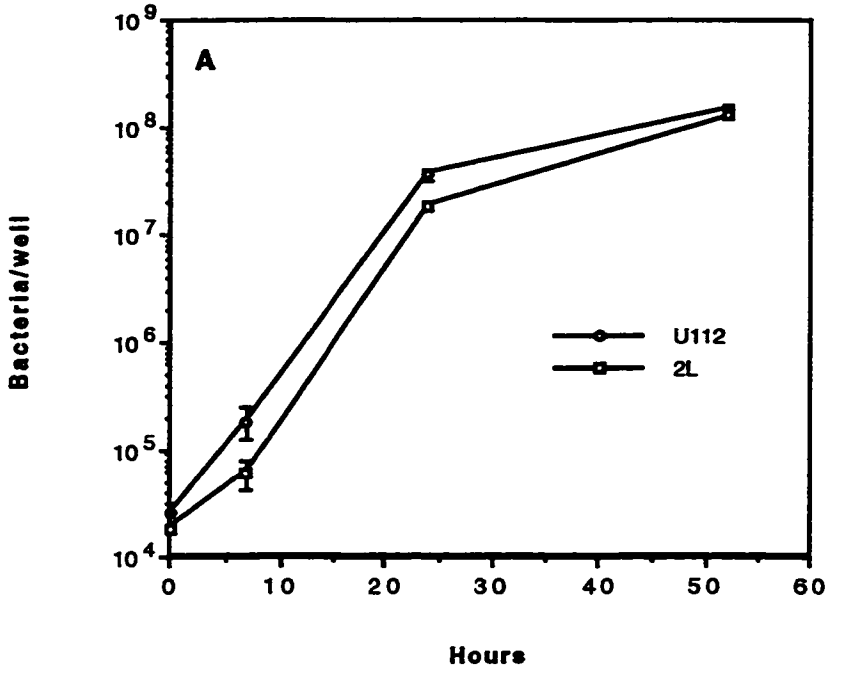


Figure 29. Southern blot analysis of *F. novicida acpA* mutant. Band size (in kb) was estimated using 1 kb ladder DNA standards. Lanes: 1, U112 (wild-type); 2, 2L (*acpA*::Em^R). Chromosomal DNA from each strain was digested with *Nde*I and probed with either *acpA* or the Em^R cassette.


A
Bacteria/well
10^9
10^8
10^7
10^6
10^5
10^4
0
10
20
30
40
50
60
Hours
U112
2L

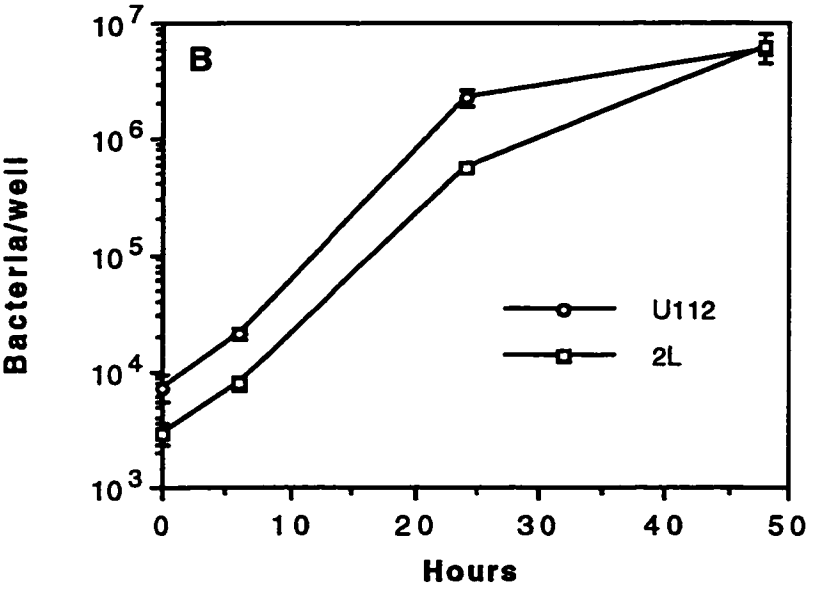

B
Bacteria/well
10^7
10^6
10^5
10^4
10^3
0
10
20
30
40
50
Hours
U112
2L

Figure 30. Growth of *F. novicida acpA* null mutant in macrophages. U112, (wild-type); 2L (*acpA*::Em^R). A. Growth in J774A.1 cultured macrophages. B. Growth in thioglycollate-elicited mouse peritoneal macrophages.

Virulence of *F. novicida acpA* mutant in mice

To examine the possibility that AcpA may contribute to the survival or growth of *F. novicida in vivo*, I analyzed the growth of 2L in the spleens of infected mice as a measure of virulence. Fifty-two hours after infection, roughly equivalent numbers of bacteria are detected in the spleens of mice infected with either wild-type *F. novicida* or 2L (Fig. 31). In addition, mice infected with 2L all show symptoms of murine tularemia. This suggests that AcpA is not essential for virulence of *F. novicida* in mice.

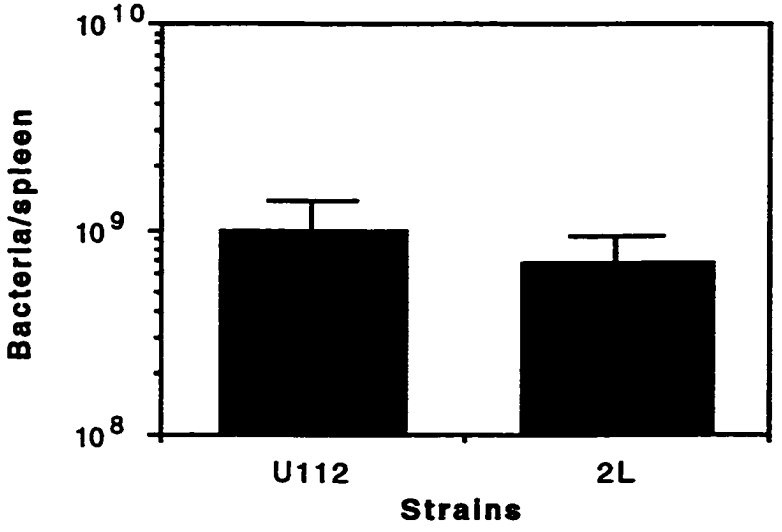


Figure 31. Growth of *F. novicida acpA* null mutant in spleens of infected mice. Mice were infected ip with 1-2 x 10^4 bacteria. Bacterial replication in the spleens was enumerated 52 h after injection. U112, (wild-type); 2L (*acpA*::Em^R).

DISCUSSION

Respiratory-burst inhibiting ACPs have been identified in three intracellular pathogens, *L. donovani*, *L. micdadei*, and *C. burnetii* (Remaley *et al.*, 1984; Saha *et al.*, 1985; Baca *et al.*, 1993). A study of ACP activity in *Francisella* led to the purification and characterization of a burst-inhibiting ACP designated AcpA (Reilly *et al.*, 1996). This work describes the cloning and sequencing of *acpA*, the gene encoding AcpA, and represents the first reported isolation of a gene encoding a burst-inhibiting ACP. Studies of an *acpA* mutant suggest that AcpA is not essential for growth in macrophages or for virulence in mice.

The deduced amino acid sequence of AcpA shows no similarity to any known phosphatases and lacks acid phosphatase (RHGXRXP) and protein tyrosine phosphatase (PTPase) [(I/V)HCXAGXXR(S/T)G] signature motifs (Van Etten *et al.*, 1991; Bliska, 1995). Interestingly, AcpA exhibits a low level of similarity to PLC enzymes from *P. aeruginosa* (Plc-N and Plc-H) and *M. tuberculosis* (MpcA and MpcB). The non-hemolytic (Plc-N) and hemolytic (Plc-H) *Pseudomonas* PLCs both hydrolyze phosphatidylcholine, but have different activities on other substrates. Plc-N can also hydrolyze phosphatidylserine whereas Plc-H is active on sphingomyelin (Ostroff *et al.*, 1990). The difference in substrate specificity has been proposed to be conferred by the C-terminal one-third of the proteins, which was found to have reduced homology compared to the N-terminal two-thirds (Ostroff *et*

al., 1990). MpcA and MpcB can both direct the hydrolysis of phosphatidylcholine and sphingomyelin when expressed in *M. smegmatis*, a mycobacterial strain devoid of PLC activity (Johansen *et al.*, 1996). It is interesting to note that AcpA, MpcA, and MpcB are substantially smaller (by about 200 amino acids) than Plc-H and Plc-N, although by comparison of their amino acid sequences with the *Pseudomonas* PLCs and AcpA, the *Mycobacterium* PLCs are more similar to Plc-H and Plc-N. To my knowledge, none of these PLCs have been assayed for phosphatase activity. The absence of phosphatase activity in a *Clostridium* PLC suggests phosphomonoesterase activity is not typical for PLCs. Using the synthetic substrate pNPPC, PLC activity was detected in the purified AcpA at pH 7.0, but the level of activity was over 3 orders of magnitude lower than the phosphomonoesterase activity at either pH 7.0 or pH 6.0 (Reilly *et al.*, 1996). The dramatically higher rate of hydrolysis of phosphomonoesters at acidic and neutral pH compared to phosphodiester substrates, including pNPPC, and the inhibition of the phosphomonoesterase activity by common ACP inhibitors, support the designation of AcpA as an acid phosphatase in spite of its partial sequence similarity to PLCs. However, a true assessment of PLC activity of AcpA must await studies using natural substrates.

The mechanism by which any ACP inhibits the respiratory burst is unknown. Both the *Leishmania* ACP-P_1 and *Legionella* ACP_2 hydrolyze PIP_2 and IP_3 *in vitro* (Das *et al.*, 1986; Saha *et al.*, 1988). In addition, when incubated with intact cells, ACP_2 reduces the levels

of PIP_2 in unstimulated neutrophils, and IP_3 and diacylglycerol in fMLP-stimulated neutrophils (Saha *et al.*, 1988). However, no ACP has been shown to gain access to the neutrophil or macrophage cytoplasm, which is a presumed requirement for the hydrolysis of the above substrates. Preliminary experiments using radiolabelled, catalytically active AcpA failed to demonstrate uptake of exogenously added AcpA by neutrophils (Reilly and Kuhlenschmidt, unpublished observations). This would suggest that AcpA may act by hydrolysis of surface-accessible substrates necessary for activation of the oxidative burst. Multiple, incompletely characterized signal transduction pathways appear to participate in NADPH oxidase activation in response to various stimuli, involving the generation of second messenger molecules from phospholipid precursors and the phosphorylation of several proteins on serine, threonine, and tyrosine residues (Baggiolini and Wymann, 1990; Dusi *et al.*, 1994; Robinson and Badwey, 1994; Ptasznik *et al.*, 1996; Waite *et al.*, 1997). Among the phosphorylated proteins identified in activated neutrophils are the $p47^{phox}$ (serine and tyrosine residues), $p67^{phox}$ (serine residues), and $p40^{phox}$ components of NADPH oxidase (El Benna *et al.*, 1994; El Benna *et al.*, 1997; Fuchs *et al.*, 1997; Waite *et al.*, 1997). Since phosphotyrosine and phosphoserine are both good substrates for AcpA (Reilly *et al.*, 1996), it is possible AcpA-mediated dephosphorylation of NADPH oxidase components could disrupt the assembly of the oxidase if AcpA does somehow enter the neutrophil cytoplasm. Determination of whether the

phosphomonesterase and/or low level PLC activities of AcpA are responsible for oxidative burst inhibition will require further investigation.

I attempted to determine the role of AcpA in the virulence of *Francisella* through the analysis of an *acpA* mutant strain, 2L. The wild-type intramacrophage growth of the *acpA* mutant strain indicates AcpA is not essential for *F. novicida* intracellular growth. Experimental infections of mice with the 2L strain also suggest AcpA is not required for virulence. However, these observations do not necessarily rule out a subtle role for AcpA in *Francisella* virulence. These experimental infections explored only a single route of infection with a relatively large inoculum. Natural infections with *Francisella* can occur through multiple routes (e.g. inhalation, ingestion) with relatively low doses of bacteria; 25 bacteria by the aerosol route are sufficient to induce tularemia in humans (Tärnvik, 1989). Thus, further studies with alternative infection protocols may be necessary to reveal any contribution by AcpA to *Francisella* survival *in vivo*. There is some evidence that the PLCs of *Pseudomonas* and *Mycobacterium* may contribute to the virulence of these organisms. The LD_{50} of a *plcS* mutant of *P. aeruginosa* (*plcS* encodes Plc-H) is increased 200-fold over the wild-type in a mouse model of infection (Ostroff *et al.*, 1989). Analysis of PLC activity in various mycobacterial species only detected PLC activity in extracts of the most virulent species (Johansen *et al.*, 1996).

The mutant AcpA is predicted to have a substantial truncation at its C-terminus, including three of the most similar regions between AcpA and the PLCs. However, in the absence of data regarding the activity of the purified mutant protein, the possibility that the truncated AcpA retains some residual enzymatic activity cannot be ruled out. Attempts to construct *acpA* mutants with transposon insertions closer to the N-terminus were unsuccessful, possibly indicating *acpA* may be an essential gene for *F. novicida*, although there is precedent for the influence of transposon location on the transformation efficiency of certain DNA fragments (Anthony *et al.*, 1991b).

The data to this point suggest that AcpA is not essential for the virulence of *Francisella*. Given the nature of the *acpA* mutant constructed in this study, a subtle role for AcpA in *Francisella* virulence cannot be excluded. Further investigation is warranted to test this potential role. However, having cloned the *acpA* gene, it is now possible to better evaluate the contribution of a respiratory burst-inhibiting ACP to the virulence of an intracellular pathogen.

CONCLUSIONS

Prior to this study, few virulence factors of *Francisella* had been identified, especially those contributing to the intramacrophage growth of this microorganism. This work describes the isolation and characterization of two genetic loci, *mglAB* and *acpA*, from *F. novicida*. One of these loci, *mglAB*, was clearly shown to be necessary for growth in macrophages and virulence in mice. Preliminary analysis of *mglA* expression suggests it is induced in macrophages but may be regulated differently when compared to genes encoding related proteins in other bacteria. Studies in *E. coli* suggest that the regulation of *sspA* expression is complex (Williams *et al.*, 1994a). Likewise, *mglA* expression may also be complex and evidence consistent with this comes from the apparent absence of MglA in an *mglB* mutant. Admittedly, the studies of the regulation of *mglA* expression are far from complete. However, this work describes the creation of genetic tools, strains, and protocols which may be used as the basis for the design of experiments to analyze this in more detail. There is also evidence that MglA and/or MglB may function in regulating the transcription of several genes in *F. novicida*.

The discovery of *mglAB* and the results presented here suggest two general lines of future investigation: i) determination of the mechanism by which *mglAB* expression is regulated and how MglA/MglB affect the levels of other *Francisella* proteins; and ii)

identification and characterization of MglA/MglB-regulated genes. The potential cloning of an MglA/MglB-regulated gene encoding a 70 kDa secreted protein not only provides a useful reagent for both sets of future experiments, but represents a possible virulence factor whose contribution to *Francisella* virulence may also be investigated. Presumably, characterization of *mglAB*-dependent genes will aid in understanding the function of MglA/MglB as well as revealing factors of *Francisella* essential for intramacrophage growth.

The assays employed in this work failed to show a role for AcpA in *Francisella* virulence in mice or growth in macrophages. Its phosphomonoesterase activity is interesting in light of its sequence similarity to phospholipase C proteins of other bacteria. However, these data do not eliminate the possibility that AcpA is a virulence factor. Perhaps AcpA enhances intramacrophage survival at a very early stage in the interaction with the host cell, which was not revealed in the experiments described here. Alternatively, there may be another protein(s) with similar functions to AcpA.

The genetic techniques and reagents for the analysis of *F. novicida* developed in this study serve to increase the value of *Francisella* as a model intracellular pathogen. The identification of additional virulence factors affecting the intracellular growth of this microorganism will likely contribute to our understanding of the basic biological requirements for replication within eukaryotic cells.

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