ISOLATION AND ANALYSIS OF THREE GENETIC LOCI FROM
THE INTRACELLULAR PATHOGEN Francisella novicida AND
gseA FROM Chlamydia trachomatis

by

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ABSTRACT

Francisella novicida, a close relative of F. tularensis, is a facultative intracellular pathogen that can survive and grow in macrophages by preventing phagolysosomal fusion. In this study we used in vitro cassette mutagenesis to generate a library of insertion mutants of F. novicida which was assayed for defective growth in murine macrophages. Two mutants, KM14 and KEM7, were isolated.

F. novicida KM14 was found to grow to less than 1/10 the final number of bacteria inside macrophages when compared to wild type or a random insertion mutant. It was also found to grow $10^3$ to $10^4$ fold less well in the livers and spleens of infected mice. KM14 had enhanced sensitivity to complement; related mutants were sensitive to complement and/or deoxycholate, and all were defective for growth in macrophages. The locus mutagenized in KM14 included genes that show high similarity in their deduced amino acid sequence to those of msbA and orfE of Escherichia coli; the former is a member of the superfamily of ABC-transporter proteins. We named the corresponding genes in F. novicida valAB (for virulence associated locus). We hypothesize that ValAB are responsible for the transport of lipids to the outer membrane, and are needed for maintaining the integrity of the outer membrane as well as for growth in macrophages. During the course of these studies we observed what appeared to be a high rate of induction of mutation accompanying integration of transforming DNA.
KEM7 was defective for growth or survival in macrophages in comparison with the wild type (WT) strain or another insertion mutant, KEM21. While all three strains exhibited intracellular growth, the number of viable KEM7 present after 24-48 h of infection was approximately 10-fold less than that of WT or KEM21. This observation was apparently due to a reduced number of viable KEM7 associated with the macrophages after the phagocytosis period of 1 h. Upon intravenous inoculation of C57BL/6 mice, the number of KEM7 in the livers and spleens 48 h post-infection was found to be 10³ to 10⁴ fold less than that of either KEM21 or WT.

A gene that is functionally analogous to the recA gene of *Escherichia coli* was cloned from *Francisella novicida*. The gene was found to suppress the sensitivity of an *E. coli* strain to DNA-damaging agents and to support genetic recombination in *E. coli*. After transposon mutagenesis, the recA-like gene was returned to *F. novicida* and a UV-sensitive *F. novicida* strain was isolated. In contrast to the wild-type strain, this UV-sensitive strain could not be transformed with chromosomal DNA.

DNA cloned from *Chlamydia trachomatis* is able to direct the formation of the genus-specific lipopolysaccharide epitope of chlamydiae in enteric Gram-negative bacteria. We demonstrated that a single *C. trachomatis* gene (*gseA*) is sufficient to impart this trait to *Escherichia coli*. The deduced amino acid sequence of *gseA* showed 23% identity (66% similarity) to *kdtA*, an *E. coli* gene that codes for a bifunctional enzyme catalyzing the addition of two 3-deoxy-D-manno-octulosonic acid (Kdo) residues to lipid A precursors.
Extracts of *E. coli* expressing *gseA* transferred at least one additional Kdo unit from cytidine monophosphate activated Kdo CMP-Kdo to precursors already bearing the two Kdo residues attached by the *kdtA* gene product. Introduction of *gseA* into an *E. coli* mutant with a thermolabile *kdtA* gene product endowed cell extracts with the ability to transfer not only the third but also the first two Kdos to lipid A precursors, demonstrating that the *C. trachomatis* enzyme is at least trifunctional. Given the similarities of these two Kdo transferases and how essential Kdo is in Gram-negative bacteria, lipopolysaccharide biosynthesis may be a target for development of novel drugs effective against chlamydiae.
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KIZZY
DEDICATION

To the memory of my mother. (Lalakahle Nhlane; Sikukhumbula njalo yeTOSHANA).
GENERAL INTRODUCTION

Intracellular Parasitism

Intracellular parasites spend most of their lives within host cells. They have adapted themselves to the intracellular habitat where the host cell environment provides many nutrients. Indeed, some obligate intracellular parasites even lack some essential metabolic processes that have become obsolete in light of this abundance. The intracellular existence also shields the parasite from adverse environmental conditions such as dehydration, freezing, and extreme pH. Intracellular existence though does not come without its own problems. The parasites have to have mechanisms for entering the host cell, avoiding being killed, multiplying intracellularly and spreading from cell to cell. These mechanisms require participation of virulence factors, which is the subject of this thesis.

The following is a summary of the various ways in which different intracellular bacteria have solved the problems of entry and survival inside host cells. This summary does not attempt to be comprehensive, but rather focuses on bacterial entry and survival as two stages in the life cycle of intracellular pathogens that involve virulence factors.

Some bacterial pathogens of mammals have evolved the ability to enter, persist and grow intracellularly within macrophages. Whereas macrophages are usually a component of the host's innate
defense system, ingesting and digesting foreign invaders, in these cases they provide the pathogen with a continuous supply of nutrients, and protection from the host's other innate defense mechanisms and acquired immune responses in the extracellular environment. A pathogen with such an ability is then capable of persisting inside the host tissues for extended periods of time, and spreading throughout the body.

A pathogen that invades, or is ingested by macrophages is usually exposed to the killing mechanisms of these professional phagocytes. These mechanisms include the production of hydrogen peroxide, superoxide radical, and other oxygen-derived radicals; activation of myeloperoxidase to produce microbe-damaging hypohalite; and phagolysosomal fusion that exposes the pathogen to the lysosomal acid hydrolases (glycosidases, proteases, and lipases), lysozyme, lactoferrin, and cationic proteins.

Clearly an organism that has to survive within macrophages must find a way of surviving or subverting these killing mechanisms. As should be expected, various organisms have evolved different strategies to evade the lethal consequences of life within a macrophage and focus here will be on the strategies used to defeat the effects of a fused phagolysosome. Survival mechanisms used by the pathogen include escape from the phagosome, preventing phagolysosomal fusion and resisting the consequences of phagolysosomal fusion. The following will be a limited survey of the mechanisms used in the survival of intracellular pathogens within
macrophages. This summary will highlight the various virulence factors involved in the three survival categories. The well-studied bacterial pathogens *Salmonella*, *Legionella*, and *Listeria* will be used as examples of pathogens that use resistance to lysosomal enzymes, inhibition of phagolysosomal fusion, and escape from the phagosome, respectively, as virulence mechanisms.

**Listeria monocytogenes**: An Organism that Escapes from the Phagosome

Some intracellular pathogens avoid the lethal consequences of phagolysosomal fusion by escaping from the phagosome soon after they enter the host cell. Four such organisms *Shigella flexneri*, *Rickettsia tsutsugamushi*, the protozoan *Trypanosoma cruzi*, and *Listeria monocytogenes*, also share the capability to elaborate hemolytic activity which seems to be associated with the ability to escape the phagosome and enter the host cytoplasm (Sansonetti et al., 1986; Nogueira and Cohn, 1976; Gaillard et al., 1987; Tilney and Portnoy, 1989). Rickettsiae enter host cells in membrane-bound vacuoles but are found free in the cytoplasm 30 min later (Andrese and Wisseman, 1971; Ewing et al., 1978; Rikihiisa and Ito, 1980; Rikihiisa and Ito, 1982). Phospholipase A has been implicated in the dissolution of the phagosome membrane to allow this rickettsial escape (Rikihiisa and Ito, 1982). A better characterized pathogen that
utilizes escape from the phagosome as a survival mechanism is *Listeria monocytogenes*.

*Listeria monocytogenes* is a gram-positive, non-spore forming, facultatively anaerobic coccobacillus that occurs ubiquitously in nature, having been isolated from soil, water, vegetation, and a variety of mammals, birds, fish, insects and other animals. *L. monocytogenes* is motile, with a characteristic tumbling motility in liquids when cultured at room temperature.

Although Listeriae are widely distributed in nature, human disease is uncommon, being restricted to several well-defined population groups including neonates, pregnant women, and immunocompromised patients, particularly those with a malignancy, or having had a recent kidney transplant. In neonates the early onset disease presents as disseminated abscesses and granulomas in multiple organs, while the late onset disease is meningitis. Adult patients present with endocarditis, meningitis or a remarkable history of chills and a high grade fever.

**Invasion.** Several lines of evidence indicate that *Listeria monocytogenes* starts its intracellular life by invading normally non-phagocytic host cells. In experimental animals infected either orally, or in the conjunctiva, *L. monocytogenes* first penetrates into those epithelial cells that are exposed to the bacteria, and then they are picked up by macrophages (Racz *et al.*, 1972; Racz...
et al., 1970). In vitro experiments demonstrate that \textit{L. monocytogenes} can enter and grow in various epithelial and fibroblast cell lines (Gaillard et al., 1987; Kuhn et al., 1990; Portnoy et al., 1988).

Invasion of non-phagocytic epithelial cells is a key virulence mechanism, particularly of pathogenic bacteria like \textit{Shigella}, \textit{Salmonella}, and \textit{Yersinia} that have to cross the intestinal barrier. The genetic determinants required for bacterial penetration of mammalian cells vary among the bacterial pathogens (Finlay and Falkow, 1989a). For \textit{Listeria} it was first observed that only those \textit{Listeria} species that were pathogenic were capable of penetrating an enterocyte-like cell line Caco-2 (Gaillard et al., 1987). This suggested that specific bacterial factors are required for cell invasion. Gaillard et al., (1991) analyzed a bank of transposon insertion mutants for their ability to invade mammalian cells. This work identified a protein, internalin, that enabled \textit{L. monocytogenes} to invade mammalian cells. Introducing DNA fragments that encode internalin into either non-invasive transposon mutants of \textit{L. monocytogenes}, or \textit{L. innocua} conferred the ability to invade (Gaillard et al., 1991). Immunogold labelling of \textit{L. innocua} expressing internalin produced a strong reaction around the bacteria while there was no reaction with a control strain, suggesting that internalin is on the surface of \textit{Listeria} and can presumably make contact with eukaryotic cells (Gaillard et al., 1991).
On further analysis it was found that internalin is a surface protein that contains features typical of certain cell-wall proteins, including the M protein of *Streptococcus pyogenes*, a gram-positive extracellular pathogen. The internalin gene encodes a protein of 744 amino acids, with a predicted molecular weight of about 80Kd and a calculated pI of 4.44. The protein is rich in threonine (13.3%) and serine (7.8%) residues. These amino acids are distributed evenly throughout the protein. Two-thirds of internalin is made up of two regions of repeats, designated region A and region B. Region A begins at residue 29 and extends to residue 357. It is composed of 15 successive repeats. Each repeat contains 22 amino acids, except the sixth which contains 21 amino acids. Thirteen of the 22 positions are highly conserved with either only one or two closely related amino acids found at the same position in at least 60% of the repeats. The repeats form the consensus sequence NQI-DI-PL-LTNL-L-L/I.

Region B is dissimilar to region A in its amino acid sequence. Region B begins at residue 462 and ends at residue 650. It is made of three successive repeats. The first two are made of 70 amino acids and the third is made of 49 amino acids. In the part common to the three repeats, the same amino acid is found in 27 of the 49 positions of the repeats. At the C-terminus, internalin has a 20 amino acid hydrophobic region followed by a 7 amino acid charged segment. Directly N-terminal to the hydrophobic sequence is a proline- and glycine-rich region ending with the hexapeptide
LPTTGD. These features are characteristic of the anchor region of surface proteins of gram-positive bacteria (Fischetti et al., 1990).

The mechanism by which internalin triggers listerial uptake by host cells is unknown. It was observed that internalin-deficient mutants of *L. monocytogenes* were unable to attach to host cells (Gaillard et al., 1991), as is the case for invasin-deficient mutants of *Y. pseudotuberculosis* (Isberg et al., 1987). It is therefore likely that internalin binds to a mammalian receptor, or is part of a structure that binds to a mammalian receptor; however a host cell receptor has not been identified. Internalin does not contain the tripeptide sequence RGD, which is present in fibronectin and other extracellular ligands recognized by integrins (Pierschbacher and Ruoslahti, 1984).

It should be noted that invasin of *Y. pseudotuberculosis* also does not have the RGD sequence but binds to β chain integrins, indicating that other sequences are important for recognition by these receptors (Isberg and Leong, 1990). Therefore, the receptor for internalin could still be a member of the integrin family of receptors.

Internalin shows no amino acid similarity to any known proteins, but it is structurally analogous to some cell-wall proteins with internal repeats that have been found in the genera *Staphylococcus* and *Streptococcus*. These include the protein A and fibronectin-binding protein from *Staphylococcus aureus* (Sjordahl, 1977; Uhlen et al., 1984; Signas et al., 1989), the M protein, Fc binding protein, and the IgA-binding protein from *Streptococcus pyogenes* (Hollingshead et al., 1986; Miller et al., 1988; Mouw et al.,
1988; Heath and Cleary, 1989; Frithz et al., 1989), the protein G from group G streptococcus (Fahnestock et al., 1986), and the wall-associated protein A from Streptococcus mutans (Ferretti et al., 1989). By analogy with the surface proteins from the Staphylococcus spp. and Streptococcus spp., internalin is presumably anchored in the cytoplasmic membrane by its C-terminus, with its N-terminal region distal to the cell. This way the N-terminal would be the functional domain of the molecule, and the repeats would play a central role in the interaction of internalin with the eukaryotic target host cell (Ferretti et al., 1989).

A variety of eukaryotic proteins contain leucine-rich repeats similar to that in the 22 repeat motif of region A of internalin. The function of these repeats is not clearly understood, but it has been proposed that regularly spaced leucine residues might promote coiled-coil structures (Ohkura and Yanagida, 1991). As well it is not known how internalin crosses the cytoplasmic membrane. Internalin does not have a typical signal peptide as seen on other surface proteins from gram-positive bacteria. This is not unusual as there are examples of secreted proteins that lack a leader peptide including the E. coli hemolysin family of proteins and the Yersinia outer membrane proteins (Felme et al., 1985; Michelis et al., 1990).

Internalin was the first cell-wall surface protein to be characterized in L. monocytogenes that appears to be involved in the interaction of the pathogen with the host cells. The tandemly repeated motifs in internalin correspond to intragenic repeats, which
are known to be hot spots for recombination in many organisms (Albertini et al., 1982). Therefore internalin may contribute to strain variability both functionally and antigenically. This might explain why certain listerial clones are more frequently associated with listeriosis than others (Piffaretti et al., 1989).

**Intracellular Growth.** The ability of *L. monocytogenes* to grow inside macrophages has been shown to be a virulence factor. The studies of Mackaness have established that *L. monocytogenes* can initially survive and multiply within macrophages, and that the virulence of this organism is dependent on this feature. In one of the most informative studies of the Listerial growth cycle in vivo, mice were injected with $5 \times 10^4$ living *L. monocytogenes* and in the successive days histological studies of liver and spleen tissue were performed, together with viable counts from these tissues. At 4 hours, no organisms could be found in these tissues. At 24 hours, discrete lesions were found in both tissues that consisted of bacterial cells with a mixture of neutrophils and mononuclear phagocytes. At 48 hours, the lesions were larger, with a central core of neutrophils surrounded by mononuclear phagocytes, with the bacteria located at the periphery in association with the mononuclear phagocytes. There were no bacteria at the center of the lesion. At 72 hours, the lesions were even larger. Healthy macrophages had continued to accumulate at the periphery and were proceeding to invade the center of the lesion. The bacteria were still associated with the
mononuclear phagocytes at the periphery. On the fourth day, there were even more macrophages, and neutrophils had completely disappeared. Lesions consisted primarily of healthy mononuclear cells. Bacteria, though even more numerous than on the third day as determined by Gram stain preparations, had fallen in viable counts. On the fifth and sixth days, the lesions were condensed and dispersing, and the viable counts were lower. The listerial in vivo growth cycle thus consisted of two phases, a logarithmic growth phase in the first four days, and a phase of bacterial inactivation.

The Hemolysin (Listeriolysin O). Tilney and Portnoy (Tilney and Portnoy, 1989) used electron microscopy and microbiological tools to describe the series of events that are involved in the cell to cell spread of \textit{L. monocytogenes} in cultured macrophages. They observed that after phagocytosis, \textit{L. monocytogenes} escapes the phagocytic vacuole by lysing the membrane to become free in the cytoplasm. In the cytoplasm the bacteria are surrounded by actin filaments, which become organized into an array that extends from only one end of the bacterial cell. The \textit{L. monocytogenes} moves to the cell surface with the actin tail extending towards the cell center. At the cell surface, finger-like projections form with \textit{Listeria monocytogenes} at their tips. The pseudopodal projection touches a neighboring cell, which engulfs the portion of it that contains the bacterial cell. The new phagosome
contains a vacuole within a vacuole. The vacuolar membrane is dissolved, followed by the lysis of the phagosome membrane.

The cell-cell spread mechanism presented above is interesting in that it suggests that the pathogen may never be extracellular through the whole infection process. *L. monocytogenes* would thus remain protected within the intracellular environment, away from serum antibodies and complement.

The listerial hemolysin had been shown to be capable of lysing organelles, a fact that lead to the speculation that the hemolysin is involved in the membrane lysing. This speculation was strengthened by the observations that listeriolyisin negative mutants remained in the phagolysosome and could not multiply (Gaillard *et al.* 1987).

Historically it was noted that all strains of *L. monocytogenes* isolated from infections produce a zone of hemolysis on blood agar (Groves and Welshimer, 1977). This spurred interests in isolating a soluble extracellular hemolysin. Harvey and Faber (1941) demonstrated the existence of such a molecule in culture supernatants and showed that it behaves as a typical sulfhydryl (SH)-activated toxin, of which streptolysin O is a prototype. Subsequent isolations have utilized sulfhydryl columns for better purifications, enabling the elucidation of some of its properties (Girard *et al.*, 1963; Jenkins *et al.*, 1964; Jenkins and Watson, 1971; Kingdon and Sword, 1970a; Njoku-obi *et al.*, 1963; Siddique *et al.*, 1974).
The hemolysin is a heat-labile protein with a molecular mass of 60 kDa. It is cardiotoxic, lethal to mice (Kingdon and Sword, 1970b) and cytolytic for many eukaryotic cells (Kingdon and Sword, 1970a; Watson and Lavizzo, 1973). It is also capable of lysing isolated organelles such as lysosomes (Kingdon and Sword, 1970a). Its lytic activity is enhanced by reducing agents, and is suppressed by oxidation (Girard et al., 1963; Njoku-obi et al., 1963) and by exposure to cholesterol, or anti-streptolysin O antiserum.

The pathogenic role of the hemolysin had remained unclear for years after it was discovered. The work of Gaillard et al., (1987) demonstrated a relationship between virulence and the ability of *L. monocytogenes* to produce the hemolysin, suggesting that it might be involved in intracellular growth of *L. monocytogenes* in macrophages. It was found that a non-hemolytic mutant obtained by transposon mutagenesis had lost the capacity to grow in host tissues of infected mice, and virulence was restored in its hemolytic revertant strain. Portnoy et al., (1988) showed a complete correlation between the production of a 58 kDa hemolysin, intracellular growth and virulence in mice. These researchers also showed that transposon insertion mutants lacking hemolysin activity were unable to multiply within murine cells, including macrophages and fibroblasts. They thus proposed that the hemolysin was required for intracellular growth in murine cells.

The exact mechanism by which listeriolyisin aids intracellular growth was not evident from these studies. Kingdon and Sword
Sword (1966) suggested a nutritional role for the hemolysin whereby it would liberate intracellular iron which would be utilized for intracellular growth. Geoffroy et al., (1987) have shown that the pH optimum of the hemolysin is 5.5, which is close to the pH of a phagolysosome, leading Portnoy et al., (1988) to speculate that the role of the hemolysin is to lyse the phagolysosomal membrane, liberating the bacteria to the macrophage cytoplasm.

Mutants of L. monocytogenes lacking a pore-forming listeriolysin O, do not escape from the phagosomal compartment (Gaillard et al., 1987; Tilney and Portnoy, 1989), and subsequently fail to become established in the cytoplasm (Gaillard et al., 1987; Portnoy et al., 1988; Kuhn et al., 1988). In an attempt to demonstrate the role of listeriolysin in phagosomal escape, Bielecki et al., (1990) cloned the structural gene for the L. monocytogenes haemolysin hlyA, into an asporogenic mutant of Bacillus subtilis under the control of an IPTG-inducible promoter. The resulting strain B. subtilis (hlyA) secreted a new polypeptide after induction which comigrated with native listeriolysin O as seen by SDS-PAGE. When B. subtilis (hlyA) was used to infect the phagocytic macrophage-like cell line J774 in the presence of IPTG, the bacteria multiplied inside the macrophages with an intracellular doubling time of 1 hour. This is the same doubling time as L. monocytogenes (Portnoy et al., 1988). In the absence of IPTG the B. subtilis (hlyA) was killed, and no growth was detectable. Light microscopy of
stained preparations showed extensive intracellular multiplication of the haemolytic *B. subtilis* strain. Unlike *L. monocytogenes*, which spreads to the periphery of infected cells and then from cell to cell (Tilney and Portnoy, 1989; Havell, 1986), haemolytic *B. subtilis* did not spread from cell to cell, but rather multiplied until the infected host cells lysed. Electron microscopy revealed that some of the haemolytic *B. subtilis* were growing freely in the host cytoplasm, and others were in intact vacuoles. By contrast, all of the non-haemolytic *B. subtilis* were in intact vacuoles. These data indicated that the haemolysin alone mediates lysis of the host vacuole. Electron microscopy also showed that the intracytoplasmic *B. subtilis* was not associated with host actin filaments as would be the case with intracytoplasmic *L. monocytogenes*. This observation indicated that the expression of the haemolysin was sufficient to mediate access to the host cytoplasm, but other *L. monocytogenes* gene product(s) are required to nucleate actin polymerization.

The *ActA* Gene Product. *L. monocytogenes* and the gram-negative *S. flexneri* share the ability to spread within tissues by direct cell to cell infection, without leaving the cytoplasm (Racz *et al.*, 1970; Oaks *et al.*, 1985; Havell, 1986). Shortly after having entered the host cell's cytoplasm, the bacteria become surrounded by filamentous actin that gets rearranged into "comet tails" protruding from the bacterial end opposite to the direction of movement (Bernadini *et al.*, 1989; Tilney and Portnoy, 1989; Dabiri *et al.*, 1990; Mounier *et al.*, 1990). The mechanism of the intra- and intercellular
bacterial movement is unknown. It is rapid, up to 1.45 μm/s, and independent of microtubules and myosin II. In the case of _L. monocytogenes_ actin and tropomyosin (Dabiri _et al._, 1990) and in the case of _S. flexneri_, vinculin (Kadurugamuwa _et al._, 1991) are involved in bacterially induced actin assembly. The polymerized actin consists of short, randomly oriented microfilaments that differ from the long actin filaments that are usually seen in cells (Tilney and Portnoy, 1989). Moving bacteria leave behind trails of F-actin several micrometers long, and some of it gets incorporated into finger-like cytoplasmic protrusions. The bacteria get internalized by adjacent host cells, ending up inside two host derived membranes (Tilney and Portnoy, 1989). The two membranes surrounding the bacteria are lysed releasing the bacteria into the new host cell cytoplasm where the bacteria can multiply and start another cycle of spreading. During this entire dissemination process, the _S. flexneri_ and the _L. monocytogenes_ cells are protected from the host immune system, making this cell to cell spreading mechanism a key virulence factor. _L. monocytogenes_ transposon-induced mutants impaired in their ability to spread from cell to cell have been isolated (Sun _et al._, 1990; Kuhn _et al._, 1990). Analysis of one such mutant (Kocks _et al._, 1992), has identified the first _L. monocytogenes_ protein involved in bacterial induction of actin assembly. This protein has an apparent molecular weight of 90 kDa and is expressed at the surface of wild-type bacteria. The gene encoding this protein, _actA_, is part of an operon (Mengaud _et al._, 1991), whose complete nucleotide sequence
has been determined (Vazquez-Boland et al., 1992). On the basis of
the nucleotide sequence, the mature actA product is a 610 amino
acid protein with a calculated pI of 4.74. The protein is rich in
glutamic acid (12%) and proline (9.2%). It contains two long proline-
rich repeats and a small motif, repeated seven times, containing
three residues.

The actA gene product has been shown to be involved in the
cell to cell spread of L. monocytogenes via the nucleation and control
of filament assembly in vivo, and rearrangement of F-actin into
comet tails (Kocks et al., 1992). It seems that there is more than one
bacterial product involved in these processes, because another
mutant with a lesion outside of actA has been described that was
able to polymerize actin but was unable to form actin tails (Kuhn et
al., 1990). Since the actA mutant could not associate with F-actin, the
actA product must act at an early step in the formation of the
microfilaments. The actA protein is expressed on the bacterial
surface and could be a nucleator, but there must be an intracellularly
induced bacterial or cellular cofactor that is required to initiate actin
polymerization because L. monocytogenes is not able to nucleate
actin assembly in vitro (Tilney et al., 1990). It is not yet clear
whether the actA product interacts directly with actin or other
cytoskeletal components. The predicted amino acid sequence of the
actA gene product did not reveal any clear consensus sequences for
globular (G-) or F-actin binding sites (Kocks et al., 1992). There was
also no sequence similarity between the \textit{actA} gene product and the \textit{S. flexneri virG} gene product which is also involved in actin assembly.

\textbf{Other listerial virulence determinants}

\textit{plcA}. Adjacent to the hemolysin gene, and transcribed in the opposite direction is a gene which encodes a phosphatidylinositol-specific phospholipase (PI-PLC). PI-PLC has been shown to hydrolyze both phosphatidylinositol (PI), and Phosphatidylinositol-glycan (PI-glycan). \textit{plcA} insertion mutants of \textit{L. monocytogenes} were found to have reduced virulence, but these mutations could be having a polar effect on the downstream regulatory gene \textit{prfA}. It is nevertheless, noteworthy that only pathogenic species in the genus \textit{Listeria} secrete PI-PLC. (Camilli \textit{et al.}, 1991; Mengaud \textit{et al.}, 1991; Notermans \textit{et al.}, 1991).

\textbf{Lecithinase operon}. Downstream from the hemolysin gene (\textit{hly}), lies an operon that encodes the \textit{L. monocytogenes} lecithinase. This operon is comprised of the genes \textit{mpl}, \textit{actA} (see above), and \textit{plcB}, and three open reading frames, ORFX, Y, and Z.

\textit{plcB}. This locus encodes a protein that exhibits lecithinase activity on egg yolk overlays of renatured SDS-PAGE gels. The nucleotide sequence predicts a protein of 289 amino acids with sequence similarity to the phosphatidylcholine-phospholipase C of \textit{Bacillus cereus} and the \textit{Clostridium perfringens} alpha toxin. \textit{plcB} mutants express no lecithinase activity and make small plaques on fibroblast monolayers, and it has been suggested that the lecithinase
might be involved in lysing the double-membrane vacuole which is formed around *L. monocytogenes* during cell-cell spread (Vazquez-Boland *et al.*, 1992).

**mpl.** The first gene of the lecithinase operon encodes a metalloprotease. Insertion mutants in *mpl* are of reduced virulence and are also reduced in lecithinase production (Mengaud *et al.*, 1991). The metalloprotease seems to be involved in the proteolytic processing of the lecithinase since *mpl* mutants produced only the unprocessed form of the lecithinase.

**prfA.** Downstream of *plcA* (see above), lies a gene, *prfA*, (Gormley *et al.*, 1989) that encodes a protein of 237 amino acids. Deletion mutants of this region express low levels of *hly* mRNA, suggesting that *prfA* is a positive regulatory factor for *hly*. Complementation of deletion mutants with a plasmid carrying *prfA* increased transcription levels of *hly, plcA, mpl*, and *plcB*, demonstrating that *prfA* is an activator of at least four genes, all of which seem to be involved in virulence. *prfA* is the second gene of an operon and can be expressed either from its own promoter located in the *plcA-prfA* intergenic region or from the *plcA* promoter, suggesting that *prfA* regulates its own synthesis (Mengaud *et al.*, 1991). It has been suggested that PrfA recognizes a 14 base pair palindromic sequence found in the -35 region of the promoters for *hly, plcA*, and *mpl*, where it would bind to mediate activation (Mengaud *et al.*, 1989).
**Legionella pneumophila**: An Organism that Inhibits Phagolysosomal Fusion

One way for an intracellular pathogen to avoid being killed after phago-lysosomal fusion is for it to prevent the fusion from taking place. Although *Legionella pneumophila* will be used as an example to illustrate inhibition of phagolysosomal fusion, many other organisms including *Mycobacterium tuberculosis*, *Chlamydia psittaci*, and *Toxoplasma gondii*, utilize this strategy.

*L. pneumophila* is the causative agent of Legionnaires' disease, a special form of pneumonia in humans (McDade *et al.*, 1977; Winn and Myerowitz, 1988; Horwitz, 1988). This pathogen is capable of infecting and multiplying intracellularly in human blood monocytes, human alveolar macrophages, and tissue culture cells (Fischer *et al.*, 1992). *Legionella* spp. produce an acute purulent pneumonia whereby the alveoli are filled with polymorphonuclear leukocytes (PMNs), macrophages, fibrin, and erythrocytes (Winn and Myerowitz, 1988). A common characteristic of the exudate in most cases is the lytic destruction of the inflammatory cells, a process that has been termed leucocytoclastic. The majority of bacteria are found within inflammatory phagocytic cells; only a few being extracellular (Glavin *et al.*, 1979; Winn and Myerowitz, 1988). Most of the intracellular bacteria are found within membrane-bounded cytoplasmic vacuoles (phagosomes) or, later on in the infection after extensive intracellular bacterial proliferation, within the disrupted cytosol of the host cells.
(Chandler et al., 1979). Similar histological observations were made in the lungs of guinea pigs inoculated with *Legionella* spp. intranasally (Katz and Hashemi, 1982), intratracheally (Myerowitz, 1982; Pasculle et al., 1985; Winn, 1988), by aerosol (Baskerville et al., 1983; Davis et al., 1983), and even intraperitoneally (Chandler et al., 1979).

*L. pneumophila* in Mononuclear Phagocytes

**Alveolar macrophages.** Electron micrographs of monkey alveolar macrophages infected with *L. pneumophila* showed that 3 hours after infection about 5% of the alveolar macrophages contained intracellular bacteria (Kishimoto et al., 1979). Twenty-four hours later, many macrophages contained distended vacuoles filled with *L. pneumophila*. Multiplication of the legionellae was so rapid that the cytoplasm of the macrophages became filled with vesicles containing bacteria, and the cells were ultimately destroyed. In the absence of antibody, pigtail monkey alveolar macrophages were able to phagocytose about 1% of the legionellae in the inoculum (Jacobs et al., 1984). The macrophages killed 60-97% of the ingested bacteria within 30 min. Phagocytosis was associated with a respiratory burst as evidenced by nitro blue tetrazolium reduction around ingested bacteria. Killing of the alveolar macrophage-associated legionellae was inhibited by mannitol and by the combination of superoxide dismutase and catalase. Addition of either enzyme alone had no
effect, suggesting that the killing was mediated by the hydroxyl radical (OH·), which is formed from O₂⁻ and H₂O₂ in the presence of Fe³⁺. The virulent legionellae which survived the killing multiplied more than 10² in 96 h following infection.

*L. pneumophila* also multiplied rapidly in human alveolar macrophages in vitro (Nash et al., 1984). Specific antibody combined with complement promoted phagocytosis, but alveolar macrophages were only able to kill less than 10% of an inoculum even in the presence of both opsonins. The legionellae multiplied 10².5-10⁵ over 3 days ultimately destroying the macrophage monolayer. Electron microscopy showed the bacteria located intracellularly within membrane bounded vacuoles which were studded with ribosomes. No extracellular multiplication of the bacteria took place (Elliott and Winn, 1986). Growth was inhibited when the alveolar macrophages were pretreated with cytochalasin D, which prevented phagocytosis of legionellae.

**Peripheral blood monocytes.** *L. pneumophila* cells multiplied several logs when incubated with human peripheral blood monocytes in vitro (Horwitz and Silverstein, 1980). The monocyte monolayer was destroyed at peak bacterial growth. Electron microscopy showed legionellae in membrane-bounded cytoplasmic vacuoles that were studded with host ribosomes. Peripheral blood monocytes bound more than three times as many virulent *L. pneumophila* in the presence of both specific antibody and
complement than when opsonized with complement alone (Horwitz and Silverstein, 1981). Monocytes required both antibody and complement to kill any legionellae cells, but even in the presence of both opsonins, only $10^{-0.25}$ of an inoculum of virulent legionellae were killed. The surviving bacteria multiplied several logs in the monocytes over 4 days after infection regardless of whether they were opsonized with both opsonins.

Potential Virulence Factors of Legionellae

The Peptide Toxin. The first *Legionella* factor observed to inhibit neutrophil activation was a cytotoxin from culture filtrates that was toxic for Chinese Hamster Ovary cells (CHO) (Friedman *et al.*, 1982). The cytotoxic activity was found to be heat stable, could pass through dialysis tubing with a molecular weight cutoff of 1,000, and was sensitive to pronase and papain but insensitive to trypsin. These features suggested that the factor was a small peptide. Quantities of partially purified toxin which had no effect on neutrophil viability or phagocytosis were able to depress the hexose monophosphate shunt (HMPS) activity and oxygen consumption during phagocytosis. Further characterization of the toxin has been hampered by the difficulty in purifying this peptide factor to homogeneity, but there is compelling evidence that the *Legionella* toxin is of importance in pathogenesis. The toxin is produced by all five *Legionella* spp. that have been examined (Hedlund and Larson,
1981). Legionellae secrete the toxin into the medium in which they are grown. Exogenously applied, partially purified toxin, and toxin produced by intraphagosomal bacteria had adverse affects on phagocytes (Hedlund and Larson, 1981). The toxin is stable at pH 3.5, making it stable at the low pH of an acidified phagosome. Treatment of neutrophils with the toxin causes a decrease in their bactericidal activity. Immunization of mice with toxin containing material protects against a lethal challenge of Legionella cells (Hedlund and Larson, 1981).

**Phosphatases.** All strains of L. pneumophila and at least one strain of L. micdade produce an acid phosphatase (Muller, 1981; Nolte et al., 1982; Thorpe and Miller, 1981). Acid phosphatase cytochemistry studies of phagosome-lysosome fusion in peripheral blood monocytes infected with L. pneumophila showed a thin layer of the lead phosphate reaction product between the inner and outer bacterial membranes of the intraphagosomal bacteria (Horwitz, 1983a). The active phosphatase seems to reduce the amount of the second messengers IP$_3$ and diacylglycerol produced following receptor-mediated stimulation during phagocytosis (Saha et al., 1989). The phosphatase catalyzes the dephosphorylation of PIP$_2$ both in vitro and in intact neutrophils. The phosphatase may directly dephosphorylate some of the IP$_3$ formed from the hydrolysis of PIP$_2$. The phosphatase cannot be solely responsible for the antiphagocyte effects of ingested legionellae because it does not
prevent $\text{O}_2^{-}$ production, that occurs following phagocytosis (Donowitz et al., 1990). Also, the legionellae phosphatase is heat sensitive and loses its phosphatase activity whereas ingested virulent legione!lae inhibit subsequent neutrophil and monocyte activation even if heat killed (Donowitz et al., 1989; Donowitz et al., 1990). Thus it is doubtful that the *Legionella* phosphatase can account fully for the capacity of the bacteria to block the respiratory burst of phagocytic cells. It is probable that multiple factors are involved in producing blocks in the activation pathway following phagocytosis (Dowling et al., 1992).

**Phospholipase C.** All of the various species of *Legionella* that have been analyzed except *L. micdadei* produce phospholipase activity. It has also been recognized that the histopathology of pneumonia caused by *L. pneumophila* is characterized by a necrosis of mononuclear and polymorphonuclear leukocytes, suggesting that the pathogenesis might involve an extracellular phospholipase (Baine, 1985). Also, it was shown that cultures of *L. pneumophila* could lyse erythrocytes from guinea pig, horse, sheep, rabbits, and humans (Baine et al., 1979). It was also demonstrated that washed *Legionella* cells catalyze the hydrolysis of the sphingomyelinase-phospholipase C substrate $p$-nitrophenylphosphorylcholine (Baine, 1985). Neutral detergents like Tween 20 and 80, and Triton X-100 also stimulated activity of the phospholipase. But purified phospholipase C was not hemolytic. The hemolytic activity that had
been observed was probably due to another molecular species, for example a metalloprotease, or the recently described non-proteolytic hemolysin termed legiolysin (Baine, 1985).

It has been suggested that the phospholipase C might be involved in the perturbation of the phagosome membranes by hydrolyzing phospholipid thus disrupting phagosome-lysosome fusion. However, since a number of strains which produce phospholipase C fail to inhibit phagolysosomal fusion, this activity is unlikely (Elliott and Winn, 1986; Oldham and Rodgers, 1985; Rechnitzer and Blom, 1989).

**Proteases.** Legionellae elaborate a number of proteolytic enzymes and aminopeptidases, some of which remain bound to the organism and some of which are secreted to the culture medium. Four strains of *L. pneumophila* were shown to be able to degrade a number of human serum proteins (Muller, 1980). Bacterial culture suspensions were incubated with serum and then immunoelectrophoresis was used to analyze proteolytic degradation of 23 different proteins. Five proteins were degraded: acid glycoprotein, antichymotrypsin, lipoprotein, globulin, and glycoprotein I. It was also demonstrated that cell-free culture filtrates of eight strains of *L. pneumophila* representing six serogroups contained proteolytic activity capable of digesting casein, and gelatin, but not elastin (Thompson *et al.*, 1981). It was also shown that *L. pneumophila* produces a variety of aminopeptidases
(Muller, 1981). All four strains of *L. pneumophila* tested were positive for 14 aminopeptidase activities. In addition a proline-specific endopeptidase, and a chymotrypsin-like activity have been demonstrated for *Legionella* species (Berdal *et al.*, 1982; Berdal *et al.*, 1983).

The first *Legionella* aminopeptidase to be isolated and purified was phenylalanine-aminopeptidase (Gul'nik *et al.*, 1986). It has a molecular weight of 35 kDa, a pI of 5.8 and a pH optimum of 8.0 to 9.5. It can be inactivated with incubation with EDTA, which suggests that it is a metalloprotein, but activity could not be reconstituted with Zn$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, or Cu$^{2+}$. It was concluded that the role of the aminopeptidase was to liberate hydrophilic free amino acids from peptides present in the growth medium, and that it might hydrolyze intracellular host cell peptides or the products of host cell proteolysis effected by *Legionella* spp. (Gul'nik *et al.*, 1986).

The *Legionella* zinc metalloprotease is a relatively acidic protein with a pI of 4.2 to 4.4 and a broad pH optimum (pH 5.5 to 7.5). Although it is a zinc metalloprotein, it is not inhibited by phenylmethylsulfonyl fluorid, chymostatin, or trypsin inhibitor. It is active as a 40 kDa monomer but is readily autolyzed to a 38 kDa species. It has been shown to be cytotoxic for a variety of cells, suggesting that production of this protease during infection may play an important role in causing cytolysis and the destruction of pulmonary tissue.
A genetic locus encoding the 38 kDa protease has been cloned from *L. pneumophila* serogroup 1 (Quinn *et al.*, 1989). It was shown through transposon inactivation analysis that a single polypeptide encoded on a 1.2 kb fragment is responsible for proteolytic, hemolytic, and cytotoxic properties. A probe consisting of the DNA sequence encoding the 38 kDa metalloprotease from *L. pneumophila* Philadelphia 1 strain hybridized to the chromosomal DNA of all serogroups of *L. pneumophila*, but not to DNA of any strains of *L. micdadei*, *L. dumoffii*, *L. feeleii*, or *L. jordanis* that were examined. Western blots performed with antisera to *L. pneumophila* protease demonstrated cross-reactions among 38 kDa proteins from strains of *L. pneumophila*, but no reactions were observed with proteins from other *Legionella* species. Also, the cloned protease from *L. pneumophila* reacted with convalescent-phase serum from patients infected with *L. pneumophila*, but not with antiserum from patients infected with other species of *Legionella* (Keen and Hoffman, 1989; Quinn *et al.*, 1989). The sequence of the structural gene was determined and found to contain a large open reading frame which is preceded by consensus promoter and ribosome binding sequences. The deduced polypeptide contained a putative signal sequence and a total of 543 amino acid residues with a calculated molecular mass of 60,775 Da, which is much larger than the observed 38 kDa of native and recombinant proteins (Black *et al.*, 1990). There was extensive amino acid identity with the elastase of *Pseudomonas aeruginosa*, which was most pronounced in the regions forming the enzymatic
active sites of the elastase. Competitive inhibitors for the elastase were also inhibitory for the \textit{L. pneumophila} protease, indicating that the protease and the \textit{P. aeruginosa} elastase share a similar molecular mechanism of proteolysis. An invasion-defective mutant of the fish pathogen \textit{Vibrio anguillarum} has been found and shown to produce lower levels of a zinc metalloprotease than wild type bacteria (Norqvist \textit{et al.}, 1990). There is considerable similarity between the \textit{Vibrio} enzyme and the \textit{P. aeruginosa} elastase, and small but significant sequence similarity with the \textit{L. pneumophila} protease.

A mutant of \textit{L. pneumophila} with a transposon insertion in the zinc metalloprotease gene could not synthesize the exoprotease, and could thus not produce hemolysis (Szeto and Shuman, 1990). However, this mutant strain was able to grow within and kill human macrophages as well as the wild type strain. It was also found that when guinea pigs were challenged with various doses of aerosolized mutant and wild type \textit{L. pneumophila}, the protease-negative and protease positive strains demonstrated equivalent 50\% and 100\% lethal doses. They had comparable growth rates in lungs of infected guinea pigs, and they produced comparable pathological lesions. These observations suggested that the protease is not required for intracellular infection, multiplication, nor for lethality in vivo. It also does not seem to be required for acute pneumonitis. The cytotoxic exoprotease has been implicated as a persistence factor that enhances virulence through the destruction of macrophages (Keen and Hoffman, 1989). It has further been suggested that self-limited
Legionella infections, such as Pontiac fever, may result from aproteolytic organisms colonizing lung macrophages, but failing to elicit the cytotoxic damage necessary for the development of more acute disease (Keen and Hoffman, 1989).

**Protein kinases.** The discovery of kinase activity, together with the finding of acid phosphatase activity on the outer surface of some intracellular prokaryotes (Remaley et al., 1985; Remaley et al., 1984) has led to the speculation that intracellular parasites may be capable of regulating the properties and function of host phagocytic cells by the phosphorylation and dephosphorylation of critical proteins. *Legionellae* have been shown to contain at least two protein kinases. One of these kinases has the capability of catalyzing, in the presence of ATP, the phosphorylation of not only tubulin, but also phosphatidyl inositol (PI). The function and significance of the kinases in *Legionella* are as yet unknown. Also, even though the enzyme has been shown to be able to phosphorylate PI and tubulin in vitro and PI in intact neutrophils, its physiologically relevant substrates are yet unknown. The presence of PI is rare in prokaryotes, having been found only in mycobacteria and myxobacteria (Wadee et al., 1983; Caillon et al., 1983), and not found in *Legionella* species (Finnerty et al., 1979). Since prokaryotes do not contain tubulin, it would seem likely that host phagocyte PI or tubulin or both are potential physiological substrates. The phosphorylation of either of these eukaryotic substrates might block
phagocyte activation or function. Phosphorylation of tubulin monomers inhibits assembly into microtubules (Wandosell et al., 1987; Wandosell et al., 1986), thus the kinase might affect various antibacterial activities mediated by tubulin, including motility, phagocytosis, and granule-membrane fusion.

Cell Surface Legionella Proteins

The Major Outer Membrane Protein (MOMP). All 10 serogroups of *L. pneumophila* examined possessed a 29 kDa MOMP. The MOMP of legionellae is closely associated with peptidoglycan and has been purified by a three-step procedure that involves extraction of the bacteria with calcium and detergent followed by ion-exchange and molecular-sieve chromatography. The protein seems to form aggregates through the formation of interchain disulfide bridges (Butler et al., 1985; Gabay et al., 1985). The MOMP of *L. pneumophila* has been shown to be a cation-selective porin (Gabay et al., 1985), which led to the speculation that one mechanism of *L. pneumophila* Philadelphia 1 strain to inhibit acidification of the monocyte phagosome might be to insert the cation-selective MOMP porin, into the monocyte membrane (Horwitz, 1988).

Immunoblot analysis of opsonized *L. pneumophila* indicated that C3 was fixed exclusively to MOMP (Bellinger-Kawahara and Horwitz, 1990). C3 is the major opsonin for the phagocytosis of the legionellae (Payne and Horwitz, 1987; Steffensen et al., 1985), so that
if the MOMP were modified it might lead to decreased internalization of the bacterium and subsequent failure to multiply. It has been suggested that reduced expression of MOMP could be the mechanism of attenuation for the strains which are not phagocytosed as effectively as the virulent strains (Rodgers and High, 1991). However since MOMP is present in both virulent and avirulent *Legionella* strains (Butler *et al.*, 1985), its role in pathogenesis has not been established.

**The Mip protein.** A 24 kDa outer membrane protein of *L. pneumophila* was found to be a dominant antigen when tested with antisera from rabbits immunized with killed bacterial cells (Pearlman *et al.*, 1985). This protein is distinct from the MOMP, and the structural gene has been cloned and deleted in mutants by site-specific mutagenesis involving allelic exchange with specific loss of 24 kDa antigen expression (Cianciotto *et al.*, 1989). The mutant was significantly impaired in its ability to infect transformed human U937 cells and human alveolar macrophages. An 80-fold greater inoculum of the mutant strain than of the parent strain was required to infect U937 cells. The mutant strain regained full infectivity following re-introduction of the cloned 24 kDa protein gene. Immediately following infection, the titer of the mutant strain was drastically reduced, but the growth rates of the mutant and parent strains in U937 cells were similar over the next 40 hours, indicating that the mutant was less able to initiate infection, but remained
capable of intracellular multiplication. Even when opsonized with specific antibody, the mutant strain demonstrated reduced infectivity despite equivalent cell association, indicating that the mutant did not lack a ligand required for macrophage attachment. The 24 kDa protein was suggested to be required either for optimal internalization of \( L.\ pneumophila \) by macrophages or for resistance to the bactericidal mechanisms that are operative immediately following phagocytosis. The protein was thus designated Mip (for \textit{macrophage infectivity potentiator}) and the gene encoding it \textit{mip}.

A \textit{mip} mutant strain killed fewer guinea pigs when inoculated intratracheally, and produced illness later after inoculation than did the isogenic parent strain (Cianciotto \textit{et al.}, 1990). Although the mutant strain could produce illness and death at a high dose, it was significantly less virulent than wild type, and reintroduction of the \textit{mip} gene restored virulence to wild type levels. There was no discernible difference between the \textit{mip} mutant and parent strains in proteolytic and phosphatase activities, complement fixation, serum resistance, or LPS structure. Mip is only one of many virulence factors since an \textit{mip}+ strain rendered avirulent by multiple passages on agar still expressed Mip.

The DNA sequence for the Mip protein has been determined and the deduced polypeptide is positively charged with an estimated \( \text{pI} \) of 9.8 (Engleberg \textit{et al.}, 1989). The amino acid sequence of the \textit{C}-terminal part of the Mip protein as deduced from the DNA sequence of the \textit{mip} gene from strain Wadsworth (Engleberg \textit{et al.}, 1989)
revealed a homology to the sequences of FK506-binding proteins (FKBps) found in various eukaryotic organisms (Standaert et al., 1990; Maki et al., 1990; Tropschug et al., 1990; Wiederrecht et al., 1991; Jin et al., 1991; Lane et al., 1991). The eukaryotic FKBPs are able to bind to the immunosuppressant macrolide, FK506. FKBPs and cyclophilins form the substance class of the immunophilins (Schreiber, 1991). Immunophilins possess peptidyl-prolyl cis/trans isomerase (PPIase) activity and catalyse the slow isomerization of prolyl bonds in oligopeptides and proteins in vitro (Lang et al., 1987; Fischer and Schmid, 1990; Tropschug et al., 1989). In addition immunophilins and their complexes with FK506 or cyclosporin A seem to play a key role in the signal transduction pathways of T cells (Schreiber, 1991).

The Mip protein cloned from L. pneumophila strain Philadelphia I was found to possess PPIase activity comparable in magnitude to other cyclophilin-type prokaryotic PPIases and to eukaryotic FKBPs. The inhibitory effect of FK506 on Mip was similar to that observed for human FKBP (Fischer et al., 1992). The role of Mip enzymatic activity in Legionella is still unclear but a few possibilities have been suggested. Mip may modify bacterial cell structures or surface proteins of the phagocytic cell, thereby enhancing their phagocytic capacity (Fischer et al., 1992). Mip activity in the phagocytic cell could modify proteins to inhibit phagosome-lysosome fusion and acidification of the vacuole (Horwitz,
1983b; Horwitz and Maxfield, 1984). Mip could also be deregulating signal-pathway proteins in phagocytes (Fischer et al., 1992).

The Mip protein is an example of a bacterial virulence factor possessing characteristics normally observed in eukaryotes. Another such example of a protein in pathogenic bacteria with enzymatic capacity normally ascribed to eukaryotes was recently detected in *Yersinia*. The YopH protein of *Yersinia pestis*, exhibits tyrosine phosphatase activity which is able to influence the phosphorylation pattern of the eukaryotic cell (Guan and Dixon, 1990; Bliska et al., 1991). This may suggest a new general class of bacterial virulence factors which are prokaryotic proteins with enzymatic properties related to eukaryotic cell signal factors, which contribute to survival and dissemination of intracellular bacterial pathogens (Fischer et al., 1992).

**Heat shock protein.** All *Legionella* species that have been examined express a 58-60 kDa protein which contains a genus-specific epitope recognized by monoclonal antibodies, as well as epitopes which are cross-reactive with many species of gram-negative bacteria (Hoffman et al., 1989; Sampson et al., 1990). The gene coding for this *L. pneumophila* common antigen has been cloned in *E. coli* and its nucleotide sequence determined (Hoffman et al., 1989; Hoffman et al., 1990; Sampson et al., 1990). The protein is preferentially synthesized upon heat shock. It is serologically cross-reactive, and has considerable similarity with other heat shock
proteins including GroEL protein of *E. coli*, and the *Mycobacterium tuberculosis* 65 kDa antigen. It is highly immunogenic and is the predominant *Legionella* protein reactive with human convalescent-phase serum from patients with confirmed cases of legionellosis. It is also antigenic for human T lymphocytes (Hoffman et al., 1990). This protein has also been shown to be located in the periplasmic space or expressed on the surface of intracellular bacteria (Hoffman et al., 1990). There is no direct evidence that this protein is a virulence factor, but it seems likely that it would be induced by the unfavorable conditions in the phagosome (Dowling et al., 1992).

The *dot* locus. *L. pneumophila* mutants specifically defective for intracellular replication were isolated using an intracellular thymineless death enrichment strategy (Berger and Isberg, 1993). Mutants belonging to two distinct phenotypic classes were isolated that could not grow in macrophage-like cultured cells. One class of mutants was defective for both inhibition of phagosome-lysosome fusion and association of host cell organelles with bacteria-containing phagosomes ('recruitment'). Another class of mutants was defective only for organelle recruitment, suggesting that recruitment may be necessary for intracellular growth. Recombinant clones were identified that complemented the intracellular growth defects of these mutants. A single genetic locus designated *dot* (for defect in organelle trafficking), restored wild-type phenotypes for intracellular growth, organelle recruitment, and inhibition of
phagosome-lysosome fusion to mutants belonging to both phenotypic classes. The *dot* locus may thus encode a positive regulatory factor controlling expression of gene(s) whose product(s) inhibit phagosome-lysosome fusion, cause recruitment of host cell organelles, and allow intracellular growth (Berger and Isberg, 1993). On the other hand, the *dot* locus may encode two products, perhaps in an operon, one product responsible for inhibition of phagosome-lysosome fusion, and a second required for organelle recruitment, and thus for intracellular growth (Berger and Isberg, 1993). It is also possible that the *dot* locus encodes a single multifunctional protein required for intracellular growth responsible for both inhibition of phagosome-lysosome fusion and organelle recruitment (Berger and Isberg, 1993). Further analysis of the *dot* locus is still required to establish its proper function.
Salmonella typhimurium: An Organism that Exhibits Resistance to Lysosomal Enzymes

Several intracellular pathogens that live inside macrophages resist killing by being resistant to the arsenal of lysosomal enzymes. This group includes *Yersinia pestis* (Straley and Harmon, 1984), *Salmonella typhimurium*, *Coxiella burnetti*, and the genus *Leishmania* (Moulder, 1985). The following is a summary of what is known about how *Salmonella typhimurium* interacts with the host cell.

Salmonellae are the etiologic agent of a variety of diseases collectively described as salmonellosis, which are characterized by gastroenteric disorders, enteric fever, or septicemia. *Salmonella typhimurium* is a facultative intracellular bacterium that, once ingested, invades intestinal epithelial cells and survives within them. The bacteria then proceed through the epithelial layer and enter deeper tissues, where they are engulfed by macrophages (Fields *et al.*, 1986a). Macrophages seem to provide transportation for the bacteria to organs of the reticuloendothelial system, such as the mesenteric lymph nodes, liver and spleen. Bacteria numbers can increase substantially within the macrophages (Fields *et al.*, 1986a).
Potential virulence factors of Salmonella

Invasion. Salmonellae invade enterocytes, and this process has been considered one of the earliest steps in the pathogenesis of these organisms. An in vitro system has been used to identify genes of S. typhimurium that confer on this organism the ability to penetrate eukaryotic cells (Galan and Curtiss, 1989). A cosmid clone was isolated that complemented a penetration-deficient mutant. This cosmid clone was further subcloned to produce an 18 kb HindIII insert that still conferred invasive properties to the invasion-deficient mutant. Further analysis of this DNA fragment revealed at least four genes involved in the invasive phenotype: invA, encoding a 54 kDa protein; invB, encoding a 64 kDa protein; invC, encoding a 47 kDa protein; and invD, encoding a 30 kDa protein. invA, -B, and -C were arranged in that order in the same transcriptional unit and invD was located downstream of this gene cluster, in an independent transcriptional unit. Insertions in invA and invB totally abolished the invasive phenotype, and insertions in invD yielded a reproducible 5 fold decrease in invasiveness. It was unclear how invD was related to invABC. Insertions in invC did not affect the invasive phenotype. A number of suggestions were made by the authors to explain these findings, one being that the defective mutant, DB4673, was not deficient in invC or that invC is not necessary for invasion. According to these studies, the processes of attachment to, and invasion into eukaryotic cells by Salmonella were distinct and independent phenomena. The penetration-deficient
strain DB4673 was able to adhere to cultured epithelial cells, and this phenotype was independent of the inv locus. Also, introduction of the mutated invA gene into virulent S. typhimurium did not significantly affect the ability of these mutants to attach to tissue culture cells. The significance of the inv locus in the pathogenesis of salmonellae remains unclear since inv mutants retain considerable virulence, indicating that these organisms must have alternative ways of invading host cells.

Another gene, invE, has been identified that is located immediately upstream of invA (Ginocchio et al., 1992). Mutations in this gene rendered S. typhimurium noninvasive, and this defect could be complemented in trans with a plasmid carrying only the invE gene. invE mutants remained fully capable of attaching to cultured cells, exhibiting adherence similar to that of wild type. It had been previously observed that Salmonella in close proximity to enterocytes, caused profound changes in the architecture of the enterocytes (Takeuchi, 1967; Finlay and Falkow, 1990). These changes were shown to be localized to the point of contact, and were transient. These changes were not observed with infections with invE mutants (Ginocchio et al., 1992). The authors reasoned that it is possible that upon contact with epithelial cells, wild-type Salmonella may trigger a sequence of events that lead to the reorganization of cytoskeleton components that normally maintain the structure of the microvilli. This would facilitate the endocytic event. invE mutants
would thus be unable to trigger intracellular events that lead to bacterial internalization.

A chromosomal region that conferred upon *E. coli* HB101 the ability to invade cultured human epithelial cells was cloned from *Salmonella typhi* Ty2 (Elsinghorst et al., 1989). Three invasion-positive recombinant cosmids were isolated and their analysis revealed a 33-kb region of identity. Electron microscopy of epithelial cells invaded by either *S. typhi* or *E. coli* carrying the invasion cosmid showed bacteria within endocytic vacuoles. Further transposon analysis of the recombinant cosmid identified at least four separate loci that were required for the invasion phenotype. The invasion cosmids were found to carry the *recA* and *srlC* genes indicating that the cloned DNA was located at about 58 minutes on *S. typhi* chromosome. When a segment of the *S. typhi* invasion region was used as a probe, homologous sequences were isolated from *S. typhimurium*. Two independent *S. typhimurium* recombinant cosmids containing the entire 33-kb common region identified in *S. typhi* were isolated, but these cosmids did not confer upon *E. coli* HB101 the ability to invade epithelial cells. It was noted by the authors that the large segment of chromosomal DNA required for the *S. typhi* invasion system was in sharp contrast to the relatively small DNA region required for invasion by *Yersinia* species. The *inv* locus of *Y. pseudothuberculosis* occupies 3.2-kb and encodes a single protein (Isberg *et al.*, 1987), while the *ail* locus of *Y. enterocolitica* occupies 650-bp (Miller and Falkow, 1988). On the other hand *Shigella*
*flexneri* requires a 37-kb segment of DNA from its virulence plasmid for its invasion-associated loci.

Yet another locus has been identified that was involved in invasion of aerobically grown *S. typhimurium* (Lee *et al.*, 1992). This locus was termed *hil* for hyperinvasion locus. If deleted, *hil* rendered *S. typhimurium* non-invasive. Both an increase and a decrease in *hil* expression resulted in changes in bacterial invasion, suggesting that the alteration in *hil* mutants was specific for invasion. It was also found that deletion of *hil* reduces invasiveness of bacteria grown under the normally inducing, low oxygen conditions, suggesting that *hil* mutants are affected in the oxygen-regulated invasion pathway. It was determined by P22-mediated transduction that *hil* is weakly linked to *srl* and *mutS* and likely lies between these two genes (Lee *et al.*, 1992). The two studies have identified two gene clusters required for bacterial invasion in this region of the chromosome: the *inv* locus (Elsinghorst *et al.*, 1989) which encompasses *recA* and *srl*; and the locus described by Galan and Curtiss (Galan and Curtiss, 1989), which lies near *mutS*. These results suggested that *hil* lies between these two *inv* loci. Further transduction data suggested that 20-30-kb of DNA lies between *hil* and the Elsinghorst *inv* genes, and a cosmid containing the Galan and Curtiss (Galan and Curtiss, 1989) *inv* locus did not complement the *hil* deletion mutation, suggesting that *hil* lies between the two previously identified *inv* loci.
Survival in Macrophages. The ability of *S. typhimurium* to survive and multiply within a host macrophage seems to be central to the bacterial pathogenesis and virulence (Carroll *et al.*, 1979; Fields *et al.*, 1986a). Within macrophages, *S. typhimurium* cells are generally found in phagolysosomes, in which the bacteria encounter both oxygen-dependent and oxygen-independent bactericidal processes (Elsbach and Weiss, 1988; Klebanoff, 1988). It has been shown that *S. typhimurium* survives the microbicidal mechanisms of macrophages by mounting adaptive responses that enable it to become an intracellular parasite (Carroll *et al.*, 1979). This adaptation is achieved by altering the pattern of *Salmonella* gene expression in response to conditions found within the macrophages. These changes have been demonstrated by the induction and repression of protein spots resolved on two-dimensional polyacrylamide gels (Abshire and Neidhardt, 1993a; Buchmeier and Heffron, 1990).

This ability to survive and persist within macrophages has been correlated with mouse typhoid fever pathogenesis (Fields *et al.*, 1986b), and is consistent with the pathophysiology of human typhoid fever. Inside the macrophage, Salmonellae are exposed to at least two widely different environments: the phagosome, which is at physiological pH, and the phagolysosome, which has low pH, oxygen radicals, and other toxic compounds. It has been shown that half the phagosomes in which *S. typhimurium* resides are fused with lysosomes and that the only phagosomes in which the bacteria are
seen in the process of cell division are those that are not fused with lysosomes (Buchmeier and Heffron, 1991). This suggests that *Salmonella typhimurium* survives in part by inhibiting phagosome-lysosome fusion and in part by adapting to conditions within fused phagolysosomes (Abshire and Neidhardt, 1993b).

Mutants of *Salmonella typhimurium* have been identified that are unable to survive within murine macrophages (Fields *et al.*, 1986a). The mutated genes were under the control of *phoP* and *phoQ*, which together compose a two-component regulatory system similar to those that control response to environmental stresses in other bacteria (Fields *et al.*, 1986a; Miller *et al.*, 1989; Ronson *et al.*, 1987).

**The PhoP and PhoQ proteins.** The PhoP/PhoQ proteins are environmental response regulators which regulate *Salmonella* mouse virulence (Miller *et al.*, 1989; Groisman *et al.*, 1989), survival within macrophages, resistance to low pH (Foster and Hall, 1990) and resistance to mammalian granule cationic proteins (Fields *et al.*, 1986a). Null mutations in the *phoP* locus lead to marked virulence defects as well as lack of expression of proteins that require the products of the *phoP* locus (PhoP/PhoQ) for transcriptional activation. These genes have been named *pags* (for *phoP* activated genes). One gene, *pagC*, encodes a virulence protein that is not involved in resistance to cationic proteins and survival at low pH (Pulkkinen and Miller, 1991). Therefore multiple virulence factors encoded by unlinked genes seem to comprise the PhoP regulon.
The phoP locus at 25 minutes on the Salmonella chromosome is composed of two genes, phoP and phoQ, located in an operon (Miller et al., 1989). phoP encodes a 224-amino-acid protein with extensive similarity to DNA-binding proteins of the OmpR subclass of the two component regulatory family (Ronson et al., 1987) that activate transcription and are phosphorylated at an aspartate residue by a sensor kinase protein (Stock et al., 1989). phoQ encodes a 487-amino-acid protein with similarity to sensor kinases that autophosphorylate at a histidine residue and then transfer phosphate to the amino-terminal domain of a transcriptional activator protein. When appropriate environmental signals within the macrophages are sensed by PhoQ, phosphate is presumably transferred from a PhoQ histidine residue at amino acid 276 to an aspartate in the amino-terminus of PhoP. Phosphorylated PhoP then promotes transcription of pag genes.

PhoQ has two predicted transmembrane domains (amino acids 17-44 and 191-218). Between these transmembrane domains is the periplasmic portion of PhoQ, which, because of its extracytoplasmic location, is probably involved in sensing environmental signals, such as might occur within the macrophage. One region of this periplasmic domain of PhoQ (amino acid 135-154) is extensively acidic and could thus function as a receptor for certain environmental signals which would result in conformational changes that may activate PhoQ autophosphorylation and subsequent phosphorylation of PhoP.
A number of genetic loci that require the PhoP and PhoQ proteins for transcriptional activation have been defined (Miller, 1991), but no pag loci have been demonstrated to be essential for acid or defensin resistance. Only mutations in the pagC locus have been demonstrated to attenuate S. typhimurium virulence for BALB/c mice. Most likely, more pag virulence loci will be defined since pagC mutants are not as attenuated for virulence as are phoP mutants. It is also possible that other pag loci collectively have important virulence properties that are not recognizable as defects in single gene mutations. Over 40 different proteins are presumed to be regulated in both a positive and negative manner by the PhoP and PhoQ proteins (Miller and Mekalanos, 1990).

The pagC locus has been characterized as being essential for S. typhimurium virulence and survival within macrophages (Pulkkinen and Miller, 1991), however, pagC mutants have wild type sensitivity to defensins, acid pH, lysozyme, guinea-pig complement, and cationic protein fractions derived from mouse gut. The pagC locus encodes a single 188-amino-acid envelope protein with extensive similarity to three other known bacteria outer membrane proteins, i.e. Ail (Yersinia enterocolitica), Lom (bacteriophage lambda), and OmpX (Enterobacter cloacae). Ail is an outer membrane protein that confers upon E. coli the ability to invade epithelial cells and it has been correlated with strain pathogenicity (Miller et al., 1990). Lom is expressed in bacteriophage lambda lysogens of E. coli, with a possible enhancement of virulence of the lysogens. OmpX
overproduction reduces the quantity of porins present in *E. cloacae* (Stoorvogel *et al*., 1991). Despite its similarity to Ail, PagC does not seem to be essential for epithelial cell invasion since *pagC* or *phoP* mutants are not decreased in invasion of epithelial cells both in vitro, and in gut invasion in mice (Galan and Curtiss, 1989). Also, the cloned *pagC* does not confer an attachment or invasion phenotype on *E. coli* (Miller, 1991). The role of PagC in promoting intracellular survival is unknown, but it could target *Salmonella* to a specific site through protein/protein interactions with intracellular components of macrophages (Miller, 1991). It has also been postulated that PagC might function as a molecular sink, protecting the cell from toxic compounds like halide ions or molecular oxygen by the covalent modification of its numerous tyrosine residues (Miller, 1991).

*phoP* locus mutants of *S. typhimurium* are highly sensitive to killing by cationic proteins termed defensins (Fields *et al*., 1986a; Miller *et al*., 1990). Defensins are small peptides of about 30 amino acids found in mammalian neutrophils and macrophages. They contain a characteristic consensus arrangement of cysteine, glycine, and arginine residues (Lehrer *et al*., 1991). The cysteine residues form three highly conserved disulphide bonds that contribute to the amphipathicity of native peptides. The antibacterial activity of defensins is not completely defined, but seems to be as a result of their detergent-like structure which would kill by permeabilizing the outer and inner bacterial membranes (Miller, 1991). The sensitivity of *phoP* mutants to defensins is not due to the absence of the PhoQ
membrane protein, but is due to the lack of regulation of a phoP-regulated gene or genes (Berger and Isberg, 1993). Since defensins have to cross the outer membrane barrier to permeabilize the inner membrane of \textit{E. coli}, resistance to defensins could involve the synthesis of proteases that would degrade defensins, or the suppression of a receptor that facilitates transport of defensins across the outer membrane (Berger and Isberg, 1993).

\textit{S. typhimurium} synthesizes a number of proteins at pH 5.5 which confer survival to cells subsequently exposed to pH 3.3 (Foster and Hall, 1990). However, this acid tolerance does not seem to be regulated by PhoP, and \textit{phoP} mutants are extremely sensitive to killing at pH 3.3, and they grow normally at pH 5.5. This acid sensitivity may explain, in part, the reduced virulence of PhoP mutants as the organisms encounter a low-pH environment in the phagolysosome. This low-pH sensitivity may be independent of defensin sensitivity as defensins are most active at low ionic strength and physiologic pH.

Recently, Libby \textit{et al.}(1994) have identified a hemolytic toxin (salmolysin) in \textit{Salmonella typhimurium} that they suggest might be a virulence factor. This protein had an observed and predicted molecular weight of 16 kDa, and an isoelectric point of pI 5.5. It was sensitive to heat (65°C for 15 min.), and insensitive to protease inhibitors (PMSF, leupeptin and pepstatin). It had no phospholipase or cholesterol oxidase activity. It was observed to be a pore-forming toxin with an ability to lyse a variety of cultured mammalian cells,
and was thus called a cytolysin. This toxin was required for survival in mouse peritoneal macrophages and for virulence in a mouse infection model. Expression of this toxin was found to be independent of the main intracellular regulation system of \textit{phoP/phoQ}. 
The Genus *Francisella*

**Historical**

*Francisella tularensis* was first described by McCoy in 1911, while studying an outbreak of a "plague-like disease" of ground squirrels in Tulare County, California. In 1912, this organism was isolated by McCoy and Chapin (1912), who named it *Bacterium tularense* after the county of its discovery. In 1914, Wherry and Lamb identified *Bacterium tularense* as the organism responsible for a diseased meat worker in Cincinnati, Ohio. Shortly after the extensive description of tularemia in the United States, a similar disease, Yato-byo (Hare disease) was described in Japan, and found to be caused by a similar organism (Ohara, 1925). In 1926, Soviet researchers isolated a similar organism from human cases in Siberia. The name of this disease agent has changed over the years from *Bacterium tularense*, to *Pasteurella tularensis*, to what is called today, *Francisella tularensis* in honor of the contributions of Edward Francis in understanding the disease. In 1950, another bacterium was discovered that was found to be so closely related to *F. tularensis* that it was placed in the genus *Francisella*, and designated *F. novicida*. Since its original isolation *F. novicida* has only been found twice. *F. novicida* seems to have a very low infectivity for humans.

In North America, tularemia is an acute, febrile, granulomatous, infectious, zoonotic disease. The severity of the disease varies according to the route of infection and the virulence of the infecting organism. Without antibiotic treatment, glandular, ulceroglandular,
and oculoglandular tularemia results in 5% mortality, and pulmonary, or typhoidal-type tularemia results in about 30% mortality rate. In Europe and Asia, mortality rates are less than 1% for all forms of the disease, indicating an inherent lower virulence of strains of the organism in those continents.

After exchanges of *F. tularensis* strains and convalescent sera, it was found that the bacterial strains from Asia, Europe, and North America were antigenically identical, but that the strains from North America were more virulent as demonstrated both by clinical signs of illness, and by virulence for laboratory animals. It was later found that there are two forms of *F. tularensis* in North America, the highly virulent form found in most clinical isolations, and the form that seemed to correspond to the European-Asian isolates. At least three nomenclature systems have been used to describe the different *F. tularensis* biovars. Olsufjev *et al.*, (1959) proposed the designation *F. tularensis* var *tularensis* (also referred to as *F. tularensis* var *nearctica*) for the highly virulent N. American strain and *F. tularensis* var *palaearctica* for the less virulent strain found throughout the Northern Hemisphere. Soviet investigators have used *F. tularensis* nearctica Olsufiev, and *F. tularensis* holarctica Olsufiev instead of the biovar designations. Jellison *et al.*, (1961), who were the first to report on the two kinds of tularemia in N. America, proposed the designations "A" and "B" for the highly virulent and the less virulent forms.
**Morphology**

*F. tularensis* is a gram-negative coccobacillus that measures 200 nm x 200-700 nm in size. The related organism, *F. novicida* is larger, measuring 700 nm x 1700 nm. These organisms seem to possess a loosely associated capsule which consists of carbohydrate, protein, and large amounts of lipid (Pavlova *et al.*, 1967; Hood, 1977). These organisms do not appear to have any flagella or pili associated with them.

**Transmission**

The natural reservoirs for the type A strains are a variety of small mammals from the orders *Rodentia* and *Lagomorpha*. The less virulent type B has been isolated from the external environment (streams, rivers, lakes or ponds), and its principal reservoirs in nature are voles and water rats in Eastern Europe, and beavers, muskrats, and voles in N. America. In all cases transmission to man or other vertebrates occurs through bites of a variety of parasites; handling or ingestion of contaminated meat or water; inhalation of airborne organisms in dusts contaminated by infected rodents; or bites and scratches of wild or domestic carnivores that have been contaminated by their infected prey (Eigelsbach and McGann, 1984).
Intracellular Growth

*F. tularensis* is thought of as a facultative intracellular parasite. Evidence for intracellular multiplication has come from studies with hepatic cells, and endothelia of guinea pigs (Councilman and Strong, 1921), epithelium of a tick's gut (Francis and Moore, 1926), HeLa cells (Shepard, 1959), and mouse fibroblasts (Merriott *et al*., 1961). Experiments with infected mammals have shown *F. tularensis* following a pattern of pathology similar to other intracellular pathogens. In monkeys, inhaled *F. tularensis* organisms were first found in the tracheobranchial lymph nodes, in which they seemed to multiply (Eigelsbach *et al*., 1962; White *et al*., 1964; Schricker *et al*., 1972; Hall *et al*., 1973). Fluorescent antibody staining showed *F. tularensis* in macrophages in the lumina of respiratory bronchioles (White *et al*., 1964). After a few days of infection, bacteria were found spreading into the spleen, liver, and bone marrow. With the highly virulent type A, animals developed fulminating disease and died before granulomas were formed, whereas with the less virulent type B, bacterial numbers eventually decreased and most of the animals survived (Schricker *et al*., 1972). Type B infections caused an initial influx of polymorphonuclear neutrophils (PMNs), that was shortly followed by an invasion of macrophages into the reticuloendothelial organs. The macrophages infiltrated diffusely and also formed granulomas (Hall *et al*., 1973). Intracutaneous inoculation of the Live Vaccine Strain (LVS) into monkeys produced a similar sequence of events and dissemination (McGavran *et al*.,
1962). The white rat, which shows a similar degree of natural resistance to *F. tularensis* as humans exhibited similar dissemination and histopathological changes as described above (Downs *et al.*, 1949; Olsufyev and Dunayeva, 1961; Moe *et al.*, 1975). Histopathological studies and results from in vitro studies suggest that *F. tularensis* is indeed a facultative intracellular parasite. Rat mononuclear phagocytes were found to ingest *F. tularensis*, with the rate of phagocytosis enhanced by serum (McElree and Downs, 1961). Other studies have shown survival and multiplication of *F. tularensis* in macrophages from guinea pigs (Stefanye *et al.*, 1961), and rabbits (Thorpe and Marcus, 1964a; Thorpe and Marcus, 1964b). Recently, Anthony *et al.*, (1991) have reported on in vitro observations of both *F. tularensis* and *F. novicida* proliferating in rodent macrophages. *F. tularensis* (LVS) grew in macrophage monolayers from mice, guinea pigs, or rats, whereas *F. novicida* grew in macrophages from mice and guinea pigs, but not in macrophages from rats. Transmission electron microscopy observations from these studies indicated both *Francisella* species surviving within macrophage phagosomes that were unfused with lysosomes. More recently, Conlan and North, (1992) showed that *F. tularensis*, like *Listeria monocytogenes* and *Salmonella typhimurium*, in addition to being able to parasitize macrophages, can also invade and multiply within hepatocytes. This indicated that for subsequent ingestion by macrophages, bacteria growing inside hepatocytes had to be released into the extracellular space. According to these investigators' results
this release is achieved by leukocytes (mostly PMNs) that accumulate at the sites of infection. Participation in the lysis of the infected hepatocytes of monocytes, NK cells, or T cells could not be ruled out.

**Immune Response to *F. tularensis***

**Humoral immunity.** A strong antibody response is usually observed during *F. tularensis* infections, but protective immunity seems to require cell-mediated immunity. In the third week of infection, agglutinating antibodies can be detected, and only a few days later, opsonizing antibodies can be found (Lofgren *et al.*, 1980). Maximal antibody titers are achieved during the second month of disease (Koskela and Herva, 1982), and thereafter, titers decline, but may still be detected for more than 10 years (Carlsson *et al.*, 1979). Whereas it was initially thought that humoral immunity might be protective against *F. tularensis* infections, it is now generally agreed that the humoral response has minimal value in host defense against virulent organisms. Passive transfer of immune serum to nonimmune animals was protective only when the animals were challenged with less virulent strains (Thorpe and Marcus, 1965). In the mouse, immune serum only affected the distribution of *F. tularensis* (LVS) in the various organs, so that bacteria were cleared from the blood sooner and accumulated in the liver (Anthony and Kongshavn, 1987). In rats, transferred immune serum had no effect on growth rates of *F. tularensis* (LVS) in the liver or spleen (Kostiala *et al.*, 1975). In humans, disease onset was common even after
demonstration of agglutinating antibodies (Overholt et al., 1961). *F. tularensis* is resistant to the bactericidal effects of both normal and immune sera, and this resistance seems to be due to a bacterial capsule since a capsule deficient mutant was rapidly killed by normal serum (Sandstrom et al., 1938).

**Cell-mediated immunity.** After infection with *F. tularensis*, delayed-type hypersensitivity (DTH), a common indicator of cell-mediated immunity, was demonstrable on average, one week before agglutinating antibodies (Buchanan et al., 1971). Passive transfer of mononuclear phagocytes from infected mice conferred protection on nonimmune mice when challenged with virulent strains (Eigelsbach et al., 1975). Mononuclear leukocytes from convalescent rats were protective to normal rats when challenged (Woodward et al., 1964). When lymphocytes from rats immunized with *F. tularensis* (LVS) were transferred to nonimmune rats, bacterial multiplication in the livers and spleens of the recipient rats was interrupted within 48 h of challenge (Kostiala et al., 1975). A similar protective effect was afforded by non-adherent spleen cells to mice and this effect was markedly reduced by treating the recipient mice with cyclosporin A, a T cell suppressive agent, suggesting the involvement of T lymphocytes in protection (Shevach, 1985). Cell-mediated immunity to *F. tularensis* infection as evidenced by DTH seems to correlate with subsequent resistance to infection. Mice that developed DTH to homologous antigen after infection survived subsequent challenge
with the highly virulent *F. tularensis* SCHU strain (Claflin and Larson, 1972). Mice that had been immunized with either heat killed bacteria or bacterial extracts developed no DTH, and were not protected. A similar correlation between DTH and protection against infection with *F. tularensis* was also observed in rats (Kostiala *et al.*, 1975), and in humans (Hornick and Eigelsbach, 1966). It has been noted, though, that in *F. tularensis* infections, just like in mycobacterial infections, the T cells involved in protection can be dissociated from those T cells involved in DTH (Ascher *et al.*, 1977; Miller, 1984; Mroczenski-Widely *et al.*, 1989).

Recently, studies with mice infected with *F. tularensis* (LVS) indicated major differences in the host response to infection by the intradermal route as opposed to any other route (intraperitoneal, intravenous, and intranasal) (Fortier *et al.*, 1991). It was found that a dose that was lethal by any other route was not lethal by the intradermal route. Immunity to lethal intraperitoneal LVS infections could also be transferred with both immune cells and immune serum. Further work by the same investigators (Elkins *et al.*, 1993) utilizing nude (*nu/nu*) mice indicated that the generation of immunity after intradermal inoculation was conventional α/β T cell independent, but specific and protective against a subsequent lethal intraperitoneal challenge. These investigators suggested that a specific protective mechanism was operational at the site of intradermal infection, and for this activity the γ/δ T cells were implicated due to their strategic location in the skin, and their
capacity to recognize the MHC-linked antigens, produce lymphokines, develop cytotoxic potential, and respond to bacterial heat shock proteins (Koning et al., 1987; Matis et al., 1987; Moingeon et al., 1987; O'Brien et al., 1989). The immunity afforded by this method was shown to be short-term, and conventional T lymphocytes were required for long-term survival of infected mice.
RATIONALE OF RESEARCH

Francisella tularensis is a Gram-negative etiologic agent of tularemia, an acute febrile illness of humans. F. tularensis is a facultative intracellular pathogen that seems to favor macrophages as its host cell, where it resides in an unfused phagosome. Most of the studies that have been conducted previously have been essentially on the immune response towards this organism. The purpose of this research was to attempt to learn more about the virulence properties of the bacterium since very little was known in this area. For this, F. novicida was chosen as a model since it shares a high DNA homology with F. tularensis, and like F. tularensis, it is virulent for mice, resides in macrophages and causes a similar disease pathology. But unlike F. tularensis, F. novicida is not infectious for humans, has no fastidious growth requirements, and is amenable to basic genetic manipulations. The objectives of this research were as follows:

1. To improve on the genetic manipulations that can be done on Francisella.
2. To identify virulence factors of F. novicida.
3. To make avirulent mutants defective in the identified virulence factors.
4. To reconstitute virulence by complementing the defective factors.
COLLABORATIONS

The following individuals were involved in some of the experiments described in this thesis:

a) Dr. C. R. H. Raetz, Dr. C. J. Belunis (Merck Research Laboratories, Department of Biochemistry, Rahway, New Jersey).
Performed the experiments on the catalytic activity of the $gseA$ gene product by assaying the extracts of $E. coli$ expressing the $gseA$ gene.

b) J. Berg (University of Victoria)
Cloned and mutagenized the $F. novicida$ recA gene

c) Dr. L. S. D. Anthony (University of Victoria)
Performed all the animal experiments and the preliminary DNA manipulations on mutant KEM7.

d) G. Baron (University of Victoria)
Performed some of the transposon mutagenesis of pKEM8, and created $F. novicida$ mutants of the DNA polymerase gene.

e) S. Cowley (University of Victoria)
Performed the subcloning and sequence of pKEM7.

f) M. McDonald (University of Victoria)
Performed the Temperature, Antibiotic, Detergent, and Complement sensitivities of the KM14 series of mutants.
MATERIAL AND METHODS

Bacterial strains and plasmids. Plasmid pFEN207 (Nano and Caldwell, 1985) is a pUC8 (Vieira and Messing, 1982) derived recombinant that contains a 6.1 Kb fragment of C. trachomatis LGV-434 (serovar L2) genomic DNA. Plasmids pFEN208, pFEN212 and pKEM1 are described in the text. Escherichia coli DH5α [F- φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rK-,mK+) supE44 l- thi-1 gyrA relA1] was used as host strain and was cultured in LB broth (Maniatis et al., 1982). Isopropyl-β-D-thiogalactopyranoside (1mM) (Sigma) and 5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside (40μg/ml) (Boehringer Mannheim) were added to media as needed to induce expression of cloned genes or detect clones expressing functional β-galactosidase.

Wild type F. novicida U112 (WT) was obtained from the American Type Culture Collection (Rockville, MD) and cultured at 37°C in tryptic soy broth (Difco, Detroit, MI) containing 0.1% cysteine hydrochloride (TSB-C), or on cysteine heart agar (Difco) containing 5% defibrinated horse blood (Prepared Media Laboratories, Richmond, B.C.) (CHA-B). The cap- mutant of F. tularensis strain LVS (Sandstrom et al., 1988), designated LVSR, was cultured in the same media as F. novicida. Escherichia coli DH5α and RC3 [met thy Δ(lac-pro) supD r- m- Tn10(TetR) ΔminCDE aph (KmR)] were grown in TSB-C containing appropriate antibiotics for selection.
E. coli strains were grown in LB broth (Sambrook et al., 1989) supplemented with 30 μg/ml kanamycin sulfate or 250 μg/ml sodium ampicillin as needed.

Genetic manipulation of F. novicida. Random insertional mutagenesis of F. novicida was accomplished essentially as described previously (Sharetsky et al., 1991). Briefly, chromosomal DNA from wild type F. novicida was digested to completion with the restriction endonuclease Pac I. The DNA was ligated at a concentration of 1μg/ml to favor intramolecular ligations thus generating circularized DNA. The circularized fragments were partially digested with Bam HI and then ligated with a Bam HI fragment containing the kanamycin resistance gene from plasmid pNK862 (Way et al., 1984). The ligation mixture was used to transform F. novicida according to methods previously described (Anthony et al., 1991), and kanamycin resistant clones were selected by plating on CHA-B containing 15 μg/ml kanamycin sulfate (see Figure 1). Plasmid clones were mutagenized with the transposase-less mTn10Km by delivering the element with λ1105.

Screening for mutants defective in intracellular growth and intracellular growth assay. Insertional mutants were screened for their ability to induce a cytopathic effect in macrophage monolayers using a modification of an intracellular growth assay, which has been described previously (Anthony et al., 1991). Briefly,
macrophages were harvested from mice by peritoneal lavage 3 days after intraperitoneal injection of 4% Brewers thioglycollate medium (Difco) and incubated in wells of to flat bottomed 96 well microtitre plates at a density of approximately $2 \times 10^5$ macrophages per well. *F. novicida* strains were grown in TSB-C and diluted in Dulbecco's Modified Eagle's Medium (ICN Flow) containing 10% fetal bovine serum (Hyclone) (DMEM) to a concentration of approximately $2-5 \times 10^6$ colony-forming units (CFU) per ml. The macrophage monolayers were seeded with 150 µl of this bacterial suspension and the plates centrifuged ($600 \times g$, 10 min) to accelerate the association between bacteria and macrophages. The cultures were incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO₂, after which the wells were washed three times and replenished with 150 µl of DMEM. The plates were incubated and the monolayers examined periodically for microscopic evidence of cytopathic effect, i.e. rounded or lysed cells. This screening method was repeated with clones which displayed delayed evidence of cytopathic effect.

To monitor intracellular growth, resident peritoneal, thioglycollate-elicited, or bone marrow-derived macrophages were infected with the *Francisella* strains as described above. Instead of observing microscopic evidence of cytopathic effect, the macrophage monolayers were lysed at various times following infection with 0.1% sodium deoxycholate dissolved in saline. Results were expressed as the mean ± 1 SD log₁₀ CFU *Francisella* per macrophage monolayer, based upon triplicate wells.
Mice and experimental infections. Female, specific pathogen free C57BL/6NCrlBR strain mice were purchased from Charles River Canada (Candiac, Quebec). They were housed in barrier-topped cages under conventional conditions and given food and water *ad libitum*. For infection studies, *Francisella* strains were cultured in TSB-C to a density of approximately $10^9$ CFU per ml and stored in aliquots at -80°C. Prior to infection, an aliquot was thawed at 37°C and diluted in sterile physiological saline to a concentration of $1-5 \times 10^3$ CFU per ml. The number of *Francisella* in the inoculum was verified retrospectively by plating on CHA-B. Mice were inoculated via the lateral tail vein with 0.2 ml of this suspension. At the indicated times following infection, the mice were exsanguinated by decapitation and the livers and spleens dissected and homogenized in sterile 0.85% saline. Serial dilutions of organ homogenates were plated on CHA-B and the number of CFU per organ determined after overnight incubation at 37°C. Results are expressed as the mean ± 1 SD log_{10} CFU *Francisella* per organ, based on 4 mice per group.

**SDS-PAGE and Western immunoblot analysis.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli, (1970). Proteins separated by SDS-PAGE were transferred to Immobilon-P membrane (Millipore) using a Tris (12.5 mM)/glycine (96 mM)/methanol (10%) buffer in a semi-dry immunoblotting apparatus (LKB) according to the
manufacturer's instructions. Blots were incubated sequentially with the primary antibody (1:1000), and then with ^{125}I-protein A (Dupont) and visualized by autoradiography.
DNA manipulation and sequence analysis. Standard recombinant DNA procedures were used (Maniatis et al., 1982). Restriction endonucleases and DNA ligase were obtained from Bethesda Research Laboratories, Burlington, Ont., Boehringer Mannheim Biochemicals, Dorval, Que, or New England Biolabs Inc., Beverly, MA. DNA sequence analysis was performed by the dideoxy-chain termination method of Sanger et al., (1977) with M13 and plasmid clones providing single and double stranded templates, respectively. A commercially available T7 DNA polymerase ("Sequenase", United States Biochemicals Corp., Cleveland, OH) was used for the sequencing reactions according to the manufacturer's instructions. Radiolabeled α-35S dATP (1,000 mCi/mmol) was obtained from Dupont, NEN Research Products, Boston, MA. Both strands of the pFEN212 insert were sequenced in their entirety using the universal sequencing primers to sequence restriction fragments or using custom designed primers to sequence from internal sites in the insert.

pKEM14-12 was isolated from a recombinant clone bank constructed by ligating partial Sau3A I digested DNA into the BamHI site of pRL498 (Elhai and Wolk, 1988). Both strands of the 3.2 kb fragment that encompasses the valAB operon were sequenced in their entirety using the universal sequencing primer to sequence deletion subclones generated with the "Cyclone" system (IBI), or using custom designed primers to sequence from internal sites in the fragment.
DNA and deduced amino acid sequence information was analyzed using the family of programs included in Geneworks (Intelligenetics). BLASTP (Altschul et al., 1990) was used to search for amino acid sequence similarities among six protein data bases available on-line through the National Library of Medicine (U.S.).

In vitro transcription/translation. Detection of polypeptides encoded by recombinant plasmid was accomplished by use of an in vitro coupled transcription/translation reaction. The method of Zubay, (1973) as modified by Collins, (1979) was incorporated in a commercial kit (Amersham Corp., Oakville, Ontario). Standardized counts of incorporated $^{35}$-S-labelled methionine (100,000 cpm) of each reaction mixture were solubilized in loading buffer and subjected to SDS-PAGE (Dreyfuss et al., 1984) and fluorography.

Antibody detection of recombinant products. Two monoclonal antibodies (L2I-6 and EVI-HI) that react with the genus-specific LPS epitope of Chlamydia (kindly provided by H. Caldwell) were used to detect this epitope in E. coli. L2I-6 (Caldwell and Hitchcock, 1984) is a murine immunoglobulin G3 monoclonal antibody and EVI-HI is a IgG2 monoclonal antibody that has the same reactivity as L2I-6. SDS-PAGE of LPS was performed on whole cell lysates by the method of Laemmli, (1970) with a 12.5% gel. For Western blot analysis, the gels were transferred electrophoretically to nitrocellulose and developed with monoclonal antibodies followed by incubation with
125I-labelled Protein A (10 µCi/µg). The nitrocellulose membranes were then exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) for 16–48 hours and the film developed according to the manufacturer’s instructions. Immunoblots of bacterial colonies were processed in an identical fashion as the Western blots.

Complement, detergent, temperature and antibiotic sensitivities.

Complement. Susceptibility of *F. novicida* WT, and the various mutants to complement killing was evaluated according to the following procedure. *Francisella* strains were cultured overnight, washed and resuspended three times in PBS. 100 µl of bacteria at a concentration of approximately 10^6 CFU per ml were inoculated into each well of a 96 well microtitre plate. 100 µl of guinea pig complement (Cedarlane Laboratories, Hornby, Ontario), either normal or heat-inactivated (56°C, 30 min), were added to the bacteria. The concentration of bacteria was measured in the inoculum (t=0 h) and in the wells after 2 and 4 h of incubation at 37°C. The results of this experiment are expressed as the mean ± 1 SD log10 CFU *Francisella* per ml based on triplicate wells.

Deoxycholate sensitivity was measured by adding 100 µl of bacteria at a concentration of approximately 10^6 CFU per ml to each well of a 96 well microtitre plate. To this was added 100 µl of 0.2% sodium deoxycholate (Sigma). The concentration of bacteria was
determined by plating and counting from control wells (PBS) and from the sodium deoxycholate wells after 30 minutes of incubation.

Temperature sensitivity was assessed by streaking colonies on CHA-B plates and incubation at different temperatures. Antibiotic sensitivities were assayed by measuring the zone of inhibition around antibiotic discs.

**In Vitro Analysis of Kdo Transerase Activity.** Performed by Dr. C. R. H. Raetz, and Dr. C. J. Belunis (Merck Research Laboratories, Department of Biochemistry. Rahway, New Jersey). [4'-32P]Kdo2-IVA and [1-14C]Kdo were prepared and stored as described previously (Brozek et al., 1989; Clementz and Raetz, 1991). CMP-Kdo synthase was purified from *E. coli* (Brozek et al., 1989).

Typical reaction mixtures (20μl) contained 50 mM Tris-HCl, pH7.5, 10 mM MgCl₂, 3.2 mM Triton X-100, 5 mM CTP, 2 mM Kdo, 25 μM [4'-32P]Kdo2-IVA (1-2 x 10⁴ cpm/nmol), 1.8 milliunits of CMP-Kdo synthase, and 2 mg/ml protein of crude *E. coli* extracts, unless otherwise indicated. The latter were prepared by French pressure cell disruption of late log phase cultures grown on LB broth (Maniatis et al., 1982) in the presence of 125 μg/ml ampicillin and induced for 3 h with 2.5 mM isopropyl-1-thio-β-D-galactopyranoside. After 60 min at 30°C, the reactions were terminated by spotting 5 μl onto a Silica Gel 60 thin layer plate (E. Merck, Darmstadt, Germany). The plates were air dried and developed in either Solvent A, chloroform/pyridine/88% formic acid/water (30:70:16:10, v/v) or
Solvent B, chloroform/methanol/water/acetic acid (25:15:4:2, v/v). In some experiments, 1 mM \([^{14}\text{C}]\text{Kdo} (4 \times 10^4 \text{ cpm/nmol})\) and nonradioactive 25 \(\mu\text{M} \text{Kdo}_2-\text{IVA}\) were employed as the substrates. The radiolabelled lipid products were detected by autoradiography of the thin layer plates and were quantitated by scraping into scintillation vials and counting in 10 ml of Biosafe (Research Products International Corp., Mount Prospect, IL). A unit of enzymatic activity is the amount required to generate 1 \(\mu\text{mol}\) of product/min.
CHAPTER 1. A *Francisella novicida* Virulence Associated Locus that Includes a Gene Encoding an ABC-Transporter Protein

INTRODUCTION

A number of bacterial and protozoan pathogens use intracellular growth in mononuclear phagocytes as a major virulence mechanism. Within macrophages, the organisms are supplied with nutrients while being protected from many of the natural and acquired host defense mechanisms, thus enabling them to spread from cell to cell and to persist in the tissues for long periods of time. One such facultative intracellular pathogen is *Francisella tularensis*, the Gram-negative bacterial etiologic agent of tularemia, an acute febrile illness of humans.

In experimentally infected animals *F. tularensis* is found both inside macrophages and extracellularly (White *et al.*, 1964). Unlike macrophages, polymorphonuclear leukocytes (PMN's) are unable to phagocytose *F. tularensis* in the absence of antiserum (Proctor *et al.*, 1975). In vitro *Francisella* can grow in rodent resident peritoneal, thioglycolate-elicited, or bone marrow-derived macrophages (Anthony *et al.*, 1991). There is some evidence that in situ *F. tularensis* can also enter non-professional phagocytic cells such as hepatocytes (Conlan and North, 1992). The growth of *Francisella* inside macrophages is similar to that of *Mycobacterium tuberculosis*
and *Legionella pneumophila* in that these bacteria prevent phagolysosomal fusion (Anthony *et al.*, 1991).


Although the immune response to *F. tularensis* infection has been studied considerably, the molecular characteristics of *Francisella* which allow it to grow intracellularly and cause disease have received little attention. A major reason underlying the lack of understanding of this aspect of the pathogenesis of tularemia has been poorly developed methods for the genetic manipulation of the bacteria. Natural isolates vary in their virulence for different animal hosts. The North American type A strain is highly virulent in primates, rabbits and rodents, whereas the type B strain, found throughout the world, is mildly virulent in primates and rabbits. A capsule has been found on both a virulent strain (Hood, 1977) and the live vaccine strain (Sandstrom *et al.*, 1988) that is apparently
needed for infectivity and virulence. The capsule acts to prevent phagocytosis by PMN's and to protect against complement (Sandstrom et al., 1988). Eigelsbach described opacity variants of a highly virulent strain that are avirulent in mice (Eigelsbach et al., 1951); these types of variants are resistant to complement and still grow in macrophages (Nicholson, Anthony, and Nano, unpublished).

We have chosen *F. novicida* as a model of infection since, like *F. tularensis*, it is virulent in mice (Owen et al., 1964) in which it causes an infection resembling that caused by *F. tularensis* with respect to pathology and mortality (Larson et al., 1955); it grows in macrophages (Anthony et al., 1991) yet it does not have fastidious growth requirements, is not infectious for humans, affecting mainly immunocompromised individuals (Hollis et al., 1989; Wenger et al., 1989). By DNA relatedness criteria *F. novicida* cannot be separated from *F. tularensis* (exhibiting greater than 90% DNA sequence identity) (Hollis et al., 1989) and therefore most of the information we learn from one species will be relevant to the other.

Thus, infection of mice with *F. novicida* is a useful paradigm of infection with virulent *F. tularensis*. Moreover, techniques for the genetic manipulation of *F. novicida* have been described (Anthony et al., 1992; Tyeryar and Lawton, 1969; Tyeryar and Lawton, 1970). Our laboratory has recently reported the generation of mutant *F. novicida* strains by allelic exclusion, which are deficient either in their expression of an outer membrane protein (Anthony et al., 1992) or in Rec A (Berg et al., 1992). Using these techniques, it is
now feasible to address the question of bacterial virulence in tularemia at the molecular level. In this study, we have employed insertional mutagenesis to construct mutant *F. novicida* strains. Two of these strains were found to exhibit an avirulent phenotype upon infection of mice, without an apparent impairment in their growth phenotype in vitro. The following is a detailed account of the characterization of one of these mutants, KM14, and a brief account on the other, KEM7.
RESULTS

Mutagenesis and isolation of mutants. A cassette mutagenesis protocol, designed originally for *Haemophilus influenzae* (Sharetzsky *et al.*, 1991), was tested for its usefulness in mutagenizing *F. novicida*. As described in the Materials and Methods this scheme uses ligation of an antibiotic cassette to DNA prior to transformation; integration of the ligated DNA should result in replacement of a chromosomal allele by the antibiotic cassette (Fig. 1). After carrying out this mutagenesis protocol using a kanamycin resistance (Km) gene cassette we examined 12 independent colonies for insertion of the Km cassette in the chromosome. Southern blot analysis showed that each clone contained one cassette insertion, and that the different insertions were located on separate restriction fragments (Fig. 2).

After we had determined that this procedure resulted in random insertion of the antibiotic cassette, we chose to carry out a pilot study to assess if the protocol could generate a variety of avirulent mutants. However, since we judged that the mutagenesis scheme as we were performing it was generating less than 500 random mutants, we screened only 1,000 mutants in the pilot study. One thousand of these mutant clones were inoculated into microtiter wells containing thioglycolate-elicited murine macrophages, and the wells were monitored microscopically for the destruction of the macrophage monolayers as a result of *F. novicida* intracellular growth. Approximately 100 isolates showing delayed
Figure 1. Mutagenesis scheme. Chromosomal DNA from *F. novicida* was digested with *Pac* I. The resulting *Pac* I fragments were circularized by self-ligation at a low DNA concentration in order to favor intramolecular ligation. The circularized DNA was then linearized by cutting partially with *BamH* I. The linearized DNA was ligated to a Km$^+$ cassette that had compatible cohesive ends at a high DNA concentration to favor intermolecular ligation.
Figure 2. Southern blot of 12 in vitro insertional mutants taken at random. Chromosomal DNA was isolated from each mutant and digested with Bcl I to completion and then the fragments separated on an agarose gel. The DNA was transferred onto a membrane and treated as described in Materials and Methods. The probe used was the Kanamycin cassette that was used for the insertional mutagenesis. Lane 1 is the 1 kilobase DNA ladder. Lane 2 is wild type F. novicida. Lane 3 is a positive control mutant #68-11 that harbours the kanamycin cassette in its genome. Lane 4 is the kanamycin cassette positive control. Lanes 5-15 are the different random insertional mutants.
cytopathic effects on the macrophage monolayers were found, and these mutants were subjected to the same analysis a second time in duplicate experiments. The growth of these F. novicida strains that appeared to destroy the macrophage monolayer more slowly were then measured by viable counts. This process revealed 12 mutant F. novicida that appeared to grow more slowly in macrophages than the parent strain. The growth rate of these 12 mutants was measured in bacteriological medium and only three were found to grow more slowly than the wild type (Fig. 3). We chose to study only mutants which had growth defects specifically for macrophages. On closer analysis, only two of the remaining nine showed a marked decrease in growth rates in macrophages compared to the wild type. One of these mutants, KM14, is described here.
Figure 3. Growth of mutants in bacteriological media (TSB-C). Those mutants that exhibited slow growth in macrophages were subjected to an in vitro growth assay in normal bacteriological media to ensure that their slow growth was not due to a generalized growth impairment that was irrelevant to intracellular growth. Bacterial growth was monitored every hour by taking Klettometer readings. Mutants #7 and #14 were chosen for further analysis.
Isolation of the mutagenized locus. Using the Km cassette as a probe of Southern blots of KM14 DNA, we identified and isolated the putative mutagenized locus in KM14 by ligating sized Bcl I cut KM14 DNA to BamH I digested pUC18. Sequence analysis of the DNA flanking the cassette revealed deduced amino acid sequences with similarity to the E. coli DNA polymerase I (Joyce et al., 1982) on one side and to the E. coli RNA polymerase β-subunit (Ovchinnikov et al., 1982) on the other side; we later concluded that an artifact of the mutagenesis procedure brought together the two polymerase genes (see Fig. 4). Using the DNA polymerase gene fragment as a probe we noted that two hybridizing bands appeared on the Southern blot of Bcl I digested KM14 DNA, one that co-migrated with a band in the wild type U112 DNA and one approximately 3 kb smaller (Fig. 5). The lower band co-migrated with a restriction fragment that hybridized with the Km cassette DNA, and was the fragment that was cloned to generate pKEM14. Although it was apparent from this Southern blot and others that a portion of the chromosome was duplicated, this data did not easily explain the mutagenic events that resulted in the observed phenotypes. In order to decipher the genetic event and obtain a recombinant clone of the wild type locus we screened a clone bank (see Materials and Methods) using the DNA polymerase fragment as a probe. Several clones of approximately 14 kb were isolated; one of these, pKEM14-12 and subclones of its insert DNA
Figure 4. Possible genetic events leading to mutants KM14 and KM14S. In vitro ligation of Km cassette between BamHI sites located in polA gene and rpoC gene. Transformation of *F. novicida* likely led to integration by a single crossover event of ligation products leading to duplication of a portion of the chromosome. The duplication apparently resolved leaving a mutation undetectable by Southern blot hybridization.
Figure 5. Southern blot hybridization of Bcl I digested chromosomal DNA. Lane 1, KM14S; lane 2, KM14; lane 3, U1112. The lower band in lane 2 is approximately 3 kb and co-migrates with a band in the KM14 that hybridizes with Km cassette. The upper band is estimated to be 6 kb.
were studied in detail (Fig. 6). Part of pKEM14-12 was sequenced, and presentation of the sequence data here will aid in understanding many of the experimental results that were obtained before we knew the nature of the genes in this locus.

**DNA sequence analysis of the cloned region.** We found four major open reading frames (ORF's, Fig. 7) between the left-most Xba I site and the BamHI site of pKEM14-8; the DNA sequence of the 3264 bp region from the Xba I site to just past the EcoRV site is shown in Figure 6. An ORF starting at bp 254 is apparently transcribed in a leftward fashion, and has a deduced amino acid sequence with 58% identity to the N-terminal 86 amino acids of the *E. coli* nucleotidyl transferase (Herdman, 1973). Starting at bp 485 and proceeding rightward to bp 3157 are two ORFs that are apparently arranged in an operon. We named the two genes containing these two ORFs, *valAB* (for virulence associated locus). Ninety-nine bp downstream of *valAB* an ORF starts that has approximately 50% identity to DNA polymerase I of *E. coli* (Joyce *et al.*, 1982).

The deduced amino acid sequence of *valAB* had significant similarity to the deduced amino acid sequence of two genes, *msbA* and *orfE*, in an operon found in *E. coli* (Karow and Georgopoulos, 1993). Both genes in this operon were shown to be essential for viability of *E. coli*. The putative protein ValA has 572 amino acids and a molecular mass of 62,555 Da as compared to MsbA with 582 amino acids and a molecular mass of 64,460 Da. ValA and MsbA
Figure 6 Restriction map and open reading frame organization of the valAB region. The insert DNA in pKEM14-12 was generated by ligating Sau3A I partially digested DNA into pRL498. All of the other plasmids, except pKEM14-1 are subclones of pKEM14-12. Plasmid pKEM14-1 is the left half of plasmid pKEM14 which was generated as a Bcl I-Bcl I clone from KM14 chromosomal DNA that contained the Km cassette (the lower band in lane 2 of Fig. 5). The insert DNA in pKEM14-1 is homologous (by DNA sequence) to the Bcl I-BamH I chromosomal fragment but there was an apparent deletion in the pKEM14-1 fragment as compared to the wild type fragment. Triangles indicate Km
cassette as they were located in the recombinant clones in *E. coli* prior to transformation of *F. novicida*. The inserts were associated with the recombinant plasmids as follows: KM18 insert in pKEM14-5; KM9 and 15 inserts in pKEM14-2; KM14S-C1 and GB1 inserts in pKEM14-8. Several *Hind* III sites in leftmost portion of upper restriction map not localized. Letters at the end of pKEM14-12 indicate restriction sites as follows: X=Xba I; S=Sal I; P=Pst I; Sp=Sph I; H=Hind III. The region from the leftmost Xba I site to approximately the *Kpn* I site was sequenced; the area left of the enlarged region shown in the lower restriction map was not completed and several small errors remain. However, there were several open reading frames of 200-400 bp that revealed deduced amino acid sequences that had approximately 50% identity with DNA polymerase I. These ORFs were arranged left to right to align amino-terminus to carboxy terminus with DNA polymerase I. The putative *polA* gene extended to approximately 300 bp to the right of the *BamH* I site in the middle of pKEM14-12. Restriction map of lower portion of figure derived from the DNA sequence.
1081 ATAAAGATTTTTGGTGCCGAACAAAAACACAAATAAAATAATTTCTTTAAAAATCTTGAATAT
IleArgIlePheGlyAlaGlnGlnGlnGlyGlnGlnAsnGlyPhePhePheAsnLeuAspTyr
1141 ACATACTCACACAAATAGAGATAGCACTAGAGCTGCTGAACTACTACACTACCTCAAT
ThrTyrSerGlnGlnIleArgThrIleAlaLeuAlaLeuThrSerProValIleGln
1201 ATATATGCTTCTTTTGGTTTTAGCTTCTATTATTACAAATTGCTATTATTGCTATTATAAT
IleIleAlaSerLeuValAlaPheSerLeuPheThrIleAlaIlePheGlyThrAsn
1261 GTATGGGCGGTTCATCCTGTTGACAGCAGGGTCTTTTGGCTTTTGGACAGCTGCA
AspGlyGlyGlySerSerTrpLeuThrAlaGlySerPheAlaSerPhePheAlaAlaAla
1321 GCCGCTATTCTAAACCAGATAAAGCTTACAAATTGTTATTTAATTCTCTGATATTCAAAAGCT
AlaAlaIleLeuLysProLeuThrAspLeuValAsnValValIleGlnGlyLys
1381 GTAGCAGCAACTGAAAGATATTAGTGTAAATGTTAATCAGCTAAAGCTGAGAGAGGT
ValAlaAlaThrGluAspIlePheTyrIleLeuAspSerProAlaGlyThrGluThrGly
1441 AGTAAAGAACTTAGCTTAAGGTTAGGTTAAGGTCATACATCAAAGATCTAGTCTTTCTTTT
SerLysGluLeuAlaLysValAspGlyAsnValThrIleAspLeuSerPheAla
1501 GTTGAACATTAAGTACTAGTGCTGTTAGTGACTATCAAACAGGGTACGCTTAGCA
GlyGluHisLysValThrValLeuGlnValSerAspAlaGlnGlyGlnThrValAla
1561 TTTGTTGTTAAGTGCAAGAGTGTGTTAAAGACTACTTTTGACGTTATAAGGAA ACTTTTGAG
PheValGlySerGlySerGlySerGlySerGlyThrThrLeuThrSerIleIleSerArgPhe
1621 ACTCAGCATAAAGGTGAGATTCTTCTTGATGGAGTTGATACAAGAGAATTATCTTTGAG
ThrGlnHisLysValThrValThrValAspArgGluLeuThrLeuGlu
1681 AATCTACGCTCAGACTTTTCATGACTAAAAGATGTTACATGCTATTGATAACAT
AsnLeuArgSerHisLeuSerIleValSerGlnAsnValHisLeuPheAspThrVal
1741 TATAATATGCTTTTGGCCCTCTGAAAGAGTTTCCGAGATGATAAATCTCAT
TyrAsnIleAspIleAlaPheValGlnGluSerSerGluArgGluValValIleAspAla
1801 CTAAAAAGAAGCTACTGCTAGATGGTTGCTTTGGGCTAAAGATGTTAATCTCTGATTTTATCAT
LeuLysArgAlaAsnAlaThrGluPheValGlnGluLeuSerGlyGlyGlnArgIleSerIleArg
1861 ATAGGCAATAATGGTTCAAGATCAGAGGCTCAGCAGCCACTACTGCTATTGATAGGAGGA
IleGlyAsnAsnGlySerPheValGluPheLeuIlePheAlaThrSerAlaLeuAspAsn
1921 GCTTTATTAAAAATGCTCTGCTGATATTGCTTAGGCAACTAGTGCTCTTGATAAT
AlaLeuLerLysAsnAlaProGluValLeuPheAspGluAlaThrSerAlaLeuAspAsn
1981 GAATCTGAGAGATGAGTAGCAGAGGTGCTCAGCTAAAAGATGTTACTACAGT
GluSerGluArgValValGlnGlnAlaLeuGluSerLeuThrGluSerCysThrThrIle
2041 GATATAGCCTAGGCTTACTGCTTTGAATATGGTAAAGATGTTACTACAGT
ValIleAlaHisArgLeuSerThrValGluAsnAlaAspLysIleValValMtrAspGly
2101 GTGAGGTGTTGAAAAAGCTGTTAGGCTAGAGTCTAGAGTTGCTGATGATGG
GlyLysValValGluSerGlySerHisGluLeuGluGlnGlyGlyGluLeuThr
GGCTCTATCAATCGGGACTTCAATAGTACATATGCTAGATAAGATTGTGTCACAGATCAA
GlySerIleAsnArgAspPheAsnSerThrTryAlaArgEnd

ACAAACTTGCTTAGCGGTGCTACAACCAATATATCTTTTTTATAGATATTGCAA
ProAsnLeuSerArgValLeuGlyProIleSerLeuValPheIleAspIleAlaAsn

AAAAGTAAAATATACACATCGCAACTGCTCATAAAATACATATAACATCTATTTTGATATTGTG
LysArgIleLeysIleYsIleGlnIleLeysIleIleGlnSerIleIleProIleVal

TGCGAATATTCCTGTGGCGGTACTGCAAACCTCAATGTTAGATTTGGGTCAGCAGC
GlyAsnIleSerValGlyGlyThrGlyGlyThrProValIleArgMetLeuAlaGlnGln

TTGGTGGTATCTGGATATAGCTTTGCAAGATTTGCTAGATAACATCTAGTTGCTAGC
GlAspProPheGluValThrSerArgGlyThrLeuAlaThrGlnCysGlyAspGluProGly

CTGCACACATTATAATACCTGCGAGATAAGAATGCTGTACATGCTATTAG
LeuGlnHisTryLysLeuIleArgAspGlyIleValValValAspIleAlaArgMet

GTTGCAACATAAACATATGTTTGGCCTGCGGCTGAGAGAGAGGAG
PheGlyAsnLeuCysLeuProAlaAlaGluProLeuArgGluArgLeuLys

AGAAGTAGCCAAATATATAGTTGACATTTGCTGAGATAAAAGATAAGTACTCAA
GluValAspGlnIleIleGlnAsnCysSerAspAspLysAlaAspGluLeuLys

AAACTAAAAATGGTCTTGATGCAAAGTCTGTACATCTGAATTGTTAATATATAC
AsnThrLysAsnValThrTryAlaLysValValThrLeuPheValAsnIleLeuThr

AGCTAAAAAGTACTGATTAGCAGACTAATATGTTAATAGCTAGCTAGAGGAT
AlaLysValValIleAlaLysThrGluPheAsnHisGlnAsnValValIleAlaGlyIle

TGGCAATCCACAAATATTATTTTAAAGACTTTAGAAGAGTGCTATAAACACATACAGCTAA
GlyAsnProThrPhePhePheLeuPheLeuGluSerAlaIleAsnIleThrAla

AAAAGTTTTAAAGATCCACAATAAAATTCTACAGAGGTATTTTGGGGTATAGTAT
LysValPhePheAspAspHisIleThrGluSerGlnSerAspPheGluGlyIleAspSerGlu

CAATACTGTAGTGACATATAAAGTACATTTGCTAATTAAGAATCCAAAATTTTGGCTAA
IleThrValValMetThrTryLysAspAlaIleLysCysLysAsnPheAlaLysAlaAsn

TTGGGTATCTGTTAGCTAGATCATTACATGCTATAACACATACAGCTAA
TryTrpTryLeuPheAspIleAlaLeuAspAsnValValEnd
Figure 7. DNA sequence and deduced amino acid sequence of 3264 bp region of pKEM14-12. \textit{valA} and \textit{valB} are arranged in an apparent operon that is transcribed left to right. A gene corresponding to \textit{cca} of \textit{E. coli}, which encodes tRNA nucleotidyl transferase, is transcribed right to left. Presumptive promoter regions are indicated by underlines and bases capable of serving as part of ribosome binding regions are indicated by asterisks. No apparent rho-independent transcriptional terminator regions are evident. This sequence has been assigned GenBank accession number L17003.
are 42% identical in their amino acid sequences (Fig. 8A) and both have Walker motifs A and B commonly found in the superfamily of ATP binding proteins (ABC proteins). Among the Group B ABC proteins defined by Pugsley (Pugsley, 1992) ValA and MsbA have 72% and 82% identity with the consensus sequence of the Walker motifs A and B, respectively (Fig. 8C). The hydrophobicity plots of both proteins are similar (Fig. 9); visual inspection reveals the same six transmembrane spanning regions in ValA as previously identified for MsbA (Karow and Georgopoulos, 1993).

The putative proteins ValB and OrfE have 39% identity in their amino acid sequences (Fig. 8B) but show no significant similarity to other proteins in any of the protein databanks as determined by the BLASTP program (Altschul et al., 1990). ValB has 322 amino acids and a molecular mass of 36,103 Da as compared to 328 amino acids and molecular mass of 35,588 Da for OrfE. The hydrophobicity plots of ValB and OrfE are similar (Fig. 9); although there are hydrophobic regions no clear membrane spanning regions can be found.

**Generation of mutants by transformation with recombinant clones.** Assuming that the region we had cloned corresponded to the mutagenized locus in KM14 we reasoned that mutagenizing the clones and performing gene replacement in *F. novicida* should result in mutants with a phenotype similar to KM14, i.e., defective growth in macrophages. Transposon mutagenesis of the *Sal I-Sal I* fragment in pKEM14-5
Figure 8. Amino acid alignment between ValA and MsbA (A) and between ValB and OrfE (B). Identities are indicated by single letter amino acid symbols between the sequences; aligned amino acids that have similar contribution to secondary structure are indicated with a "+". C. Alignment of ValA and MsbA to consensus Walker motifs A (top) and B (bottom). The ValA sequences are from residues 375-390 and 461-498.
Figure 9. Hydrophobicity plots comparisons between ValA and MsbA and between ValB and OrfE. Kyte-Doolittle algorithms were used to predict hydrophobic regions. The plots and visual inspection of ValA and MsbA predicts six membrane spanning regions. Although hydrophobic stretches exist in ValB and OrfE, all of them are interrupted by polar amino acids, suggesting that they are not transmembrane regions.
was performed, and allelic replacement with the transposon-mutagenized clones was attempted; some insertions were also made in pKEM14-2 (Fig. 6). Although we obtained kanamycin resistant transformants of *F. novicida* when we transformed with the mutagenized clones, we found that the same transforming DNA yielded colonies with different phenotypes: most transformants grew like the wild type in macrophages but a few transformants (KM18, KM9, and KM15) were defective for growth in macrophages. We examined the DNA from these new mutants that were defective for growth in macrophages and we found that the transposons did not integrate by a double cross-over event with a gene replacement. However, we did find that the restriction pattern of the *valAB* region in KM18 had changed (Fig. 10). We studied the clones that were defective for growth in macrophages for other phenotypes (see below).

In order to determine if mutations in the DNA polymerase gene were contributing to the phenotypes, we created a Km insertion in the DNA polymerase I gene approximately 300 bp to the left of the *BamH* I site in pKEM14-8. After transformation of *F. novicida* we isolated one transformant, GB1, that had a Southern blot profile consistent with gene replacement. This strain did not have any apparent defects for growth in macrophages nor was it sensitive to complement.
Figure 10. Southern blot analysis of *F. novicida* strains U112 (WT) (lanes 1, 2, 8 and 9) and KM18 (lanes 4, 5, 6, and 7). Chromosomal DNA from each strain was digested with *Bcl* I (lanes 1, 3, 7, and 9), or *Bgl* II (lanes 2, 5, 6, and 8). **Panel A.** The lower hybridizing *Bcl* I fragment in KM18 (Lane 3) migrates at a higher position compared to the same fragment in WT (Lane 1), indicating a Km insertion at this position. The smallest hybridizing *Bgl* II fragment in WT (Lane 2), disappears in KM18 (Lane 5), and is replaced by a new fragment at a much higher position.

**Panel B.** The wild type lanes (lanes 8 and 9) do not have any fragments that hybridize with the Km cassette probe. The KM18 lanes (lane 6 and 7) show rather faint bands corresponding to the unique bands seen on Panel A.
Phenotypic characterization of KM14 and related mutants. The original mutant, KM14, was first analyzed for the stability of its phenotype, particularly since we had evidence from the Southern blots that DNA had been duplicated (Fig. 5). We found that the kanamycin resistant phenotype was quickly lost, and we named a Km-sensitive variant KM14S. Its Southern blot profile showed one band hybridizing with pKEM14-1 indicating that the presumed duplication present in KM14 had been resolved (Figs. 4 and 5).

KM14, KM14S, and three strains, KM18, KM9 and KM15, isolated during attempts at gene replacement, were analyzed for a variety of phenotypes including virulence in mice, growth in macrophages, and detergent, complement, and antibiotic sensitivity. The wild type U112 strain and a strain with a random, stable Km cassette (KM21) were used as controls. Strains KM14, KM14S, KM9, and KM15 had varying degrees of sensitivity to complement, some being greater than 1,000 fold more sensitive than wild type (Table 1). All of the strains except U112 and KM21 were sensitive to vancomycin and polymixin B. Strain KM9 and KM15 were at least 1,000 fold more sensitive to 0.1% deoxycholate than the other strains.

When we measured growth in thioglycolate-elicited macrophages, all of the strains yielded viable counts after 48 hours that were approximately 25-fold lower than wild type or KM21 (Fig. 11A)
TABLE 1

Characteristics of *F. novicida* mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Temperature Sensitive</th>
<th>Deoxycholate sensitive&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Complement sensitive&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Growth in Macrophage&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Virulence in Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>U112/KM21</td>
<td>-</td>
<td>-</td>
<td>+(+)</td>
<td>+(+)</td>
<td>+</td>
</tr>
<tr>
<td>KM14</td>
<td>-</td>
<td>-</td>
<td>+(10&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>-(-)</td>
<td>-</td>
</tr>
<tr>
<td>KM14S</td>
<td>-</td>
<td>-</td>
<td>+(10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>-(-)</td>
<td>-</td>
</tr>
<tr>
<td>KM18</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-(-)</td>
<td>ND</td>
</tr>
<tr>
<td>KM9</td>
<td>+</td>
<td>+(10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>+(&gt;10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>-(-)</td>
<td>ND</td>
</tr>
<tr>
<td>KM15</td>
<td>+</td>
<td>+(&gt;10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>+(&gt;10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>-(-)</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number in parentheses indicate factor by which viable count was reduced.

<sup>b</sup> Symbol in parentheses indicates growth characteristics in the macrophage cell line J774.

"ND" indicates "not determined."
Figure 11. In vitro growth of *F. novicida* mutants in murine macrophages.
Panel A. Thioglycollate elicited macrophages. Panel B. J774 macrophage cell line. Bacteria were enumerated by plating serial dilutions. The results are expressed as the mean ±1 standard deviation log₁₀.
All of the mutant strains, except KM18, grew identically to wild type in the macrophage cell line J774, which we expected to be defective in some of the killing processes (Fig. 11B). The apparent rate of growth of KM18 during the first 10 hours was consistently lower than that of the other mutants in both thioglycolate and J774 macrophages whereas KM14S grew initially like wild type but then stopped growing. As a measure of virulence in the whole animal we tested the growth of KM14 in the liver and spleen of infected animals (Fig. 13). We found that KM14 grew 1,000 to 10,000-fold less well than wild type. In separate experiments we demonstrated that KM14S grew identically to KM14 and that KM21 grew slightly slower than wild type but had 3-4 logs more growth than KM14 (Fig. 12) in macrophages.

When we learned that ValAB were similar to MsbA and OrfE, which are essential for the viability of E. coli, we suspected that ValAB may be essential for viability for F. novicida. This led us to hypothesize that we were creating point mutations leading to missense changes in valAB. If this were true then we should expect to find an occasional mutant that would be temperature sensitive for growth, since ValA or ValB would be defective at high temperature and therefore growth would be interrupted. When individual colonies of KM18, KM9 or KM15 were streaked on petri plates and incubated at 38.5°C they were unable to grow, supporting the notion that a temperature-sensitive lesion in an essential gene had occurred.
Figure 12. In vitro growth of *F. novicida* mutants in murine thioglycollate elicited macrophages. Bacteria were enumerated by plating serial dilutions.
The results are expressed as the mean ±1 standard deviation log_{10}. Panel A comparing KM14 to KM18, KM21, and wild type. Panel B. Similar experiment as Panel A, with KM14S substituted for KM14. KM14 grows identically to KM14S. Both mutants grow slower than KM21, which grows slightly slower than wild type.
Figure 13. Growth of *F. novicida* mutants in spleens and livers of infected mice. At different times following intravenous inoculations livers and spleens were homogenized and liberated bacteria enumerated by plating serial dilutions. WT-L: U112 growth in livers. 14-L: KM14 growth in livers. WT-S: U112 in spleens. 14-S: KM14 in spleens. Bacteria were enumerated by plating serial dilutions. The results are expressed as the mean ±1 standard deviation log10.
Alternatively it may be that a genetic lesion results in a structural component of the cell that is temperature sensitive.

**Complementation of KM14S and *E. coli* mutants with *valAB***. Recombinant clones of *msbA* were originally identified by their ability to suppress a lesion in *htrB* (hence, multicity suppressor of *htrB*). Therefore we tested a clone containing *valA* for its ability to complement *E. coli htrB* mutants. The *E. coli htrB* mutant strain MLK53 is unable to grow at 42°C; however, when this strain harbours a recombinant plasmid encoding either the *E. coli msbA* locus or *valA* locus (pKEM14-2.3) the strain can grow at 42°C. Since *valA* can substitute for *msbA* in suppressing the *htrB* mutant it is likely that *valA* is functionally analogous to *msbA*.

The strains KM14, KM14S, KM9, KM15, and KM18 were generated in such a way that no specific genetic defect could be directly linked to their phenotype, although changes in restriction patterns were observed in the *valAB* region for KM14 and KM18. Several attempts were made to complement the defects in these strains by introducing wild type alleles in trans, however, this was not achieved due to the lack of genetic tools available for *F. novicida*. However, we were able to introduce a wild type allele of the *valAB* region in cis taking advantage of the kanamycin sensitivity of KM14S. A Km insert was placed by transposition into pKEM14-8 near the *BamH I* site, and the DNA was used to transform KM14S to kanamycin resistance. Our goal was to find strains that had integrated a functional *valAB* locus either by a single or double
cross-over event; the Km-tagged locus was to serve as a co-transforming marker. Of four rare transformants three were found to have increased resistance to complement relative to KM14S (Fig. 14). One strain, KM14S-C1, was found to be about 100 fold more resistant to complement than KM14S; however it failed to grow in macrophages. Southern blot analysis showed that KM14S-C1 and KM14S-C3 had incorporated the cloning plasmid and that these two strains and KM14S-C4 had duplications of at least part of the valAB region. In contrast, KM14S-C2 did not contain a copy of the plasmid in its chromosome nor did it contain an apparent duplication of the valAB region (Fig. 15). Thus it appears that a functional copy of valAB is able to partially reverse the defect in KM14S. Since functional ABC-transporter proteins are thought to be dimers it may be that the partial complementation seen in KM14S-C1, C3 and C4 is due to dimers being formed between functional and non-functional molecules of ValA.
Figure 14. Complement killing of KM14S-derived strains transformed with a recombinant clone of valAB. A Km cassette was inserted in the polA gene in pKEM14-8 and the resulting plasmid was used to transform KM14S to kanamycin resistance.
Figure 15. Southern blot analysis of transformants of KM14S. Xba I cut chromosomal DNA was separated by gel electrophoresis and blotted onto a membrane prior to hybridization. Arrow 1 points to a restriction fragment that is apparently the 8 kb Xba I fragment that contains valAB and polA and has acquired a Km cassette (1.6 kb). The Km cassette does not have an internal Xba I site. In KM14S-C2 this fragment is the only one that hybridizes with the pKEM14-8 insert, and presumably there has been a simple gene replacement event in the polA region in this strain. Arrow 2 points to restriction fragments that co-migrate with the one present in wild type DNA and present in KM14S. Presumably this fragment in KM14S-C1, C-2, C-3, and C-4 is identical to the one in their parent strain, KM14S. Arrow 3 points to the 2.8 kb pTZ18U in the pKEM14-8 probe. Arrow 4 points to an unidentified fragment in KM14S-C4 of approximately 2 kb that hybridizes with both pKEM14-8 insert DNA and with the Km cassette DNA. The hybridization pattern of KM14S-C3 DNA is consistent with a single cross-over integration of pKEM14-8 insert DNA and with Km cassette DNA. The hybridization pattern of KM14S-C3 DNA is
consistent with a single cross-over integration of pKEM 14-8::mTnJ0Km.

KM14S-C1 has a similar pattern with additional duplication of part or all of the 8 kb Xba I fragment and pTZ18U. KM14S-C4 has multiple rearrangements of portions of the 8 kb Xba I fragment which all have a portion or all of mTnJ0Km.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Characteristics</th>
<th>Source/Reference</th>
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<td>U112</td>
<td>Wild type (Ap&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Larson et al., 1955</td>
</tr>
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<td>U112::Km cassette, duplication of val&lt;sub&gt;AB&lt;/sub&gt; region</td>
<td>This Study</td>
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<td>KM14, val&lt;sub&gt;AB&lt;/sub&gt; duplication resolved, Km&lt;sup&gt;S&lt;/sup&gt;</td>
<td>This Study</td>
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<td>U112:: mTn&lt;sub&gt;10&lt;/sub&gt;Km, genome reorganization in val&lt;sub&gt;AB&lt;/sub&gt; region</td>
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<tr>
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<td>U112, polA::mTn&lt;sub&gt;10&lt;/sub&gt;Km</td>
<td>This Study</td>
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</table>

*E. coli* Strains
DH5α  F- Φ80 lacZΔM15 endA1 recA1 hsdR17 supE44 thiI gyrA96 relA1 Δ(lacZYA-argF)U169  Hanahan, 1983
MLK53  W3110 (wild type) htrB1::Tn10  Karow et al., 1991
MLK986  MLK53 msbB::Ωcam  Karow and Georgopoulos, 1992

Plasmids

pTZ18U  ApR, phagemid  Mead et al., 1986
pRL498  KmR, positive selection vector  Elhai and Wolk, 1988
pUC18  ApR  Vieira and Messing, 1982
pNK862  ApR, KmR, source of Km cassette  Way et al., 1984
pKEM14  pUC18:: Bcl I insert from KM14 containing the Km cassette and flanking DNA  This study
pKEM14-1  pUC18::EcoR I-BamH I fragment (lefthand) from pKEM14  This study
pKEM14-12  pRL498::Sau3A I (14 kb) F. novicida DNA  This study
pKEM14-2  pTZ18U::Bgl II fragment from pKEM14-12  This study
pKEM14-2.3  pTZ18U::Xba I-Xho I fragment from pKEM14-12  This study
pKEM14-5  pTZ18U::5 kb Sal I fragment from pKEM14-12
This study

pKEM14-8  pTZ18U:: 8 kb Xba I fragment from pKEM14-12
This study

**Bacteriophage**

λ1105  Replication defective λ carrying mTn10Km  Way et al., 1984
DISCUSSION

In this work we attempted to create mutants of *F. novicida* by allelic replacement but discovered mutants generated by point mutations or some unknown mechanism. Repeated attempts to generate allelic replacements in the *valAB* region have been unsuccessful. This result was unexpected since we have been able to generate gene replacement in other loci, with the resulting loss of a protein or a function (Anthony *et al.*, 1991; Berg *et al.*, 1992). Our failure to demonstrate gene replacement in *valAB* limits our ability to directly attribute a particular mutation with a phenotype. However, our success in partially reversing the complement sensitivity phenotype of KM14S by transformation with pKEM14-8::mTn10Km provides evidence that lesions in *valAB* contributed to the complement sensitive phenotype since the only functional *F. novicida* genes that were introduced into KM14S were *valAB*.

Despite these successes further work is needed to address the many unanswered questions relating to the mutagenic events that resulted in the phenotypes observed for the mutants.

If one makes the supposition that KM14, KM14S, KM18, KM9 and KM15 all have defects in *valAB* then how does one explain the various phenotypes? We hypothesize that ValAB are responsible for secretion of lipids to the outer membrane since overproduction of the *E. coli* homologues MsbA/OrfE results in the rescue of *E. coli htrB* mutants that accumulate intracellular phospholipid, and from the observation that the *F. novicida* mutants have phenotypes consistent
with compromised outer membranes. Such a hypothesis explains the sensitivity of the mutants to detergents, antibiotics and complement, but does not directly explain the lack of growth in macrophages.

One might explain the lack of growth in macrophages as simply the result of the sensitivity of the mutants to the killing action of the phagocytes. However, the growth pattern of various mutants does not support this theory. The pattern of growth of the mutants described in this paper shows a slower growth rate in macrophages than wild type. An unrelated *F. novicida* mutant that we have isolated that is sensitive to complement shows the same growth rate as wild type (KM7) but a reduction in the number of viable bacteria during its initial interaction with macrophages. Moreover, KM18, which is the most feeble strain in its growth in macrophages, is not sensitive to complement or deoxycholate. Therefore, there appears to be no direct link between complement or deoxycholate sensitivity and the ability to grow in macrophages.

We would like to offer an hypothesis that explains the role of ValAB as both essential gene products needed for growth of *F. novicida* and as direct or indirect virulence factors essential for growth in macrophages. We propose that ValAB (and analogues in all gram negative bacteria) are responsible for secretion of components to the outer membrane. Given the phenotype of phospholipid accumulation in *E. coli htrB* mutants --which the ValAB analogues, MsbA and OrfE suppress-- we propose that ValAB secrete phospholipids to the inner leaflet of the outer membrane. Any
dysfunction of ValAB would interfere with not only the integrity of the outer membrane but also its fluidity. Any change in the fluidity may affect the propensity of the outer membrane to form blebs. A pattern of unusual fatty acids -- hydroxy fatty acids (~20%), C20-26 fatty acids (~35%) (Jantzen et al., 1979; Hollis et al., 1989; Nichols et al., 1985) is diagnostic of bacteria in the genus Francisella. We therefore propose that these fatty acids incorporated into phospholipids may either contribute to the propensity to bleb or give important biological virulence properties to the blebs. Hence, we envisage that ValAB may play a role in virulence by being very active at secretion (relative to its analogues in non-pathogenic gram-negative bacteria) or we can envisage ValAB playing a totally passive role with the phospholipids determining the properties of the outer membrane. A defective ValAB, however, would not deliver sufficient phospholipids for the correct functioning of the outer membrane.

We see a number of possible roles for blebs in intracellular growth. First, the blebs could carry phospholipids to the phagosome membrane that disrupt its integrity; also, porins carried in the blebs may form channels in the phagosome membrane. A leaky phagosome membrane would allow hydronium ions to leak out thereby decoupling acidification of the phagosome; lack of acidification of the phagosome may prevent the fusion with lysosomes. The leaking phagosome would also allow nutrients to enter the phagosome to allow growth of the bacterium. Blebs could additionally carry bacterial products to the host cytoplasm.
We have observed blebs in *F. novicida*-laden phagosomes at the surface of the bacterial cell and adjacent to the phagosome membrane; cytochemical staining also revealed blebs containing acid phosphatase (Anthony *et al.*, 1991). Recently Reilly and co-workers reported large amounts of acid phosphatase associated with *F. tularensis* (Reilly *et al.*, 1992). By analogy with the action of the tyrosine phosphatase produced by *Yersinia pseudotuberculosis* (Bliska *et al.*, 1991) it is conceivable that *Francisella* elaborates a phosphatase that interferes with signal trafficking of host cells. The production of fusogenic blebs may be a vehicle for delivering the acid phosphatase to the cytoplasm of target cells.

The apparent operon organization of *valAB* is not unexpected given that of *msbA* and *orfE*. In contrast to the conserved operon organization of *valAB* with respect to *msbA/orfE* the genome organization is apparently quite different between *F. novicida* and *E. coli*. In *F. novicida valAB*, *polA* and *cca*, are all tightly linked on the chromosome whereas in *E. coli msbA* is located at 20 min, *polA* at 87 min and *cca* at 67 min on the chromosome.

Lastly, the high frequency generation of mutants by integration of transforming DNA is unusual but not unprecedented. The phenomenon of induction of mutations accompanying transformation and integration of homologous DNA was observed in the cyanobacterium *Anacystis nidulans* (Herdman, 1973). Transformation with linear chromosomal DNA resulted in mutations tightly linked to the selected genetic markers and occurred at a rate
comparable to what we have observed with *F. novicida*. Although in this work we have postulated that point mutations occur due to an error-prone recombinational system it is possible that in *F. novicida* and *A. nidulans* systems an unknown mechanism is creating pleomorphic mutations. In the case of transformation of *F. novicida* it is also possible that the mTn10Km in the transforming DNA can be induced to transpose or undergo another illegitimate recombination event by a *F. novicida* factor. With the DNA sequence of the *valAB* region and the strains developed in this study it should be possible to ascertain the basis for the mutagenic events and to determine if all of the observed phenotypes are due to a single genetic defect.
OTHER RESULTS (KEM7) Another mutant designated KEM7 was isolated by the same procedure as described above (see Materials and Methods). The rest of the work on this mutant was carried out by S. Cowley and L. S. D. Anthony, and is reported elsewhere. The following is a brief account of the preliminary work that I was involved in.

Intracellular growth of *F. novicida* KEM7 in macrophages in vitro. *F. novicida* KEM7, was also relatively slow to induce cytopathic effects in the macrophage monolayer and was thus selected for more detailed study. The time course of KEM7 growth was monitored in various macrophage populations in vitro, and compared to the growth of *F. novicida* WT and KEM21. The results of this experiment (Fig. 16), indicate that all three strains are capable of intracellular growth in macrophages. However, the absolute growth of KEM7 was consistently found to be approximately 10-fold less than that of WT or KEM21. Apparently, this difference is due mainly to the fact that the number of KEM7 present in the macrophages at the outset of infection (t=0 h) is markedly less than the numbers of the control strains. Since the number of bacteria inoculated into the macrophage monolayers (INOC) was essentially the same and all strains were capable of survival in DMEM to a similar extent, the difference observed between KEM7 and control bacteria at t=0 h must reflect
Figure 16. Time course of growth of *F. novicida* WT, KEM21 and KEM7 in (A) resident peritoneal, (B) thioglycollate-elicited (inflammatory) and (C) bone marrow-derived macrophages. Results are expressed as the mean ± 1 S.D. log_{10} *Francisella* per well, based on triplicate wells. INOC refers to the number of bacteria inoculated into the wells at the outset of infection.
differences in the early events of the interaction between the *Francisella* strains and the macrophages, for example phagocytosis or early intracellular killing. The results of this experiment also demonstrate that resident and thioglycollate-elicited peritoneal macrophages, as well as bone marrow-derived macrophages, all support the growth of the *Francisella* strains in a similar fashion.

**Virulence of* F. novicida* KEM7 in vivo.** In order to determine the virulence phenotype of *F. novicida* KEM7, C57BL/6 strain mice were infected intravenously with either *F. novicida* WT, KEM21 or KEM7. The time course of bacterial growth was monitored in the livers and spleens, and the results of this experiment are shown in Figure 17. The data from the hepatic bacterial enumeration indicate that approximately 10³ bacteria were present in the livers at 4 h following infection (Fig. 17), regardless of the strain. During the next 2 days, WT and KEM21 were observed to proliferate exponentially until approximately 10⁶-10⁷ bacteria were isolated per liver. In marked contrast, essentially no net growth of KEM7 was observed during the first 48 h of infection.

The splenic bacterial growth was found to exhibit a pattern different from the growth in the livers (Fig. 17). At 4 h post-infection, the number of KEM7 isolated from the spleens was 10-fold less than either KEM21 or WT. It is unlikely that this observation is due simply to the difference in the size of the initial
Figure 17. Time course of growth of *F. novicida* WT, KEM21 and KEM7 in the livers (A) and spleens (B) of C57BL/6 strain mice inoculated intravenously with approximately 700, 500 and 300 colony forming units, respectively. Results are expressed as the mean ± 1 S. D. log_{10} *Francisella* per mouse, based on groups of 4 mice.
inocula, because the difference in the number of bacteria in the inoculum between KEM7 and either KEM21 or WT was less than 3-fold whereas the difference between KEM7 and either WT or KEM21 4 h post-infection was greater than 10-fold. All three strains were observed to grow exponentially for the first 24 h following infection. During this time, the numbers of KEM21 and WT increased by approximately 1,000-fold, whereas the number of KEM7 increased only 100-fold. Between 24 and 48 h post-infection, WT continued to grow exponentially while the numbers of KEM7 and KEM21 began to stabilize such that by 48 h after infection, the number of KEM7 in the spleens was approximately 1,000-fold less than KEM21 and 10,000-fold less than WT.

The phenotypic differences in virulence between WT, KEM21 and KEM7 were also evident clinically. Mice infected with WT or KEM21 showed visible clinical signs of disease (e.g. ruffled fur, cachexia) by 2 or 3 days post-infection, respectively. Mice infected with these strains were euthanized at these times. In contrast, mice infected with KEM7 showed no overt symptoms of disease at any time during the observation period (19 days). When the livers and spleens of mice infected with KEM7 were assayed at the end of the observation period for the presence of bacteria, none of the bacteria could be detected. These results demonstrate clearly that KEM7 is an avirulent mutant of *F. novicida*.

We considered the possibility that the mutation in KEM7 caused a generalized growth impairment. To address this issue, we
inoculated cysteine-supplemented tryptic soy broth (TSB-C) with *F. novicida* WT, KEM21 or KEM7 and monitored the time course of bacterial growth. All three strains were found to grow at a similar rate in this cell free system. The doubling times for WT, KEM21 and KEM7 were determined from the exponential phase of the growth curves and found to be 0.8, 0.7 and 0.8 h, respectively when the cultures were incubated with vigorous aeration, and 1.1, 1.2 and 1.1 h when they were incubated under stationary conditions. Thus, the observed avirulence of KEM7 is not simply the result of a mutation which inhibits bacterial growth in general.

**DISCUSSION**

The mutant KEM7 was shown to be highly attenuated by virtue of its poor growth in the tissues of mice (Fig. 17), and its failure to induce disease symptoms when administered at a dose with which the wild type strain or the irrelevant mutant (KEM21) causes overt disease. KEM7 also exhibits an apparently enhanced susceptibility to the early killing events of macrophages in vitro (Fig. 16). The growth of the mutant in vivo is suppressed 10,000-fold in comparison with WT. It is likely that the combined effects of killing by different mechanisms, such as oxidative products and complement, result in a pronounced destruction of KEM7 in the tissues of infected animals.
CHAPTER 2. Cloning of the recA Gene and Construction of a recA Strain of Francisella novicida

INTRODUCTION

In an attempt to develop a genetic system in Francisella spp., we began to construct mutant strains, including the recA mutant that is described below.

The Escherichia coli recA gene is a multifunctional protein required for homologous recombination (Clark, 1973; Dressler and Potter, 1982; McEntee et al., 1979; Radding, 1981; Radding, 1982) and DNA repair (Hanawalt et al., 1979; Walker, 1984). The RecA protein can function as a protease which specifically cleaves the LexA repressor to induce the SOS response in E. coli (Horii et al., 1981; Little et al., 1980); the SOS network is a group of genes involved in DNA repair and mutagenesis (Walker, 1984; Walker et al., 1982). Recent evidence has suggested that the RecA protein plays a direct role in SOS mutagenesis by proteolytically cleaving and activating UmuD, one of the key proteins involved in the mutagenic pathway, and forming a part of the protein complex thought to be involved in the introduction of mutations into the DNA (Burckhardt et al., 1988; Dutreix et al., 1989; Kitagawa et al., 1985; Lu et al., 1986; Nohmi et al., 1988; Woodgate et al., 1989).

Considerable evidence suggests that the functional roles of the RecA protein may be conserved in a wide range of other bacterial species. Genes reported to have RecA-like activities have been
isolated from a variety of microorganisms, including *Neisseria gonorrhoeae*, *Vibrio anguillarum*, *Rhizobium meliloti*, *Proteus vulgaris*, and *Salmonella typhimurium* (Better and Helinski, 1983; Keener *et al.*, 1984; Koomey and Falkow, 1987; MacPhee, 1970; Singer, 1989). This work reports on the isolation of the *recA* gene from *Francisella novicida*. The gene was identified by its ability to suppress the RecA- phenotype of an *E. coli* mutant. The isolated gene was mutagenized with transposon Tn10-km and then reintroduced into the chromosome of *F. novicida* to create an *F. novicida recA* mutant strain deficient in DNA repair and related functions.

RESULTS

Cloning the *F. novicida recA* gene

A clone bank derived from wild-type *F. novicida* chromosomal DNA was constructed by using the positive selection vector pRL498 (Elhai and Wolk, 1988) and transformed into *E. coli* MM294R, a strain carrying a deletion in the *recA* gene. Chromosomal DNA was isolated from *F. novicida* and partially digested with *Sau3A* I and then size fractionated on a 0.8% agarose gel. DNA fragments ranging in size from 3 to 10 kb were ligated to *BamH* I-cut pRL498, and the ligation mixture was used to transform competent *E. coli* MM294R. Since *recA*-dependent DNA repair is required for growth in the presence of the DNA alkylating agent methyl methanesulfonate (MMS) (Shanabruch *et al.*, 1983; Todd *et al.*, 1981), clones that were able to
suppress the recA phenotype were identified by growth in LB medium containing concentrations of MMS that were lethal to or inhibited growth of the recA parent strain of *E. coli*. Approximately 2,500 kanamycin-resistant clones containing *F. novicida* inserts were pooled and inoculated into LB broth tubes containing a range of 1 to 32 mM MMS. Cultures were incubated standing at room temperature, and growth was assayed by turbidity after 30 h. Whereas the MM294R host strain was inhibited by 1 mM MMS, a pool of MM294R organisms harboring recombinant plasmids grew in 8 mM MMS. This MMS-resistant culture was diluted and plated. An isolated clone carrying a plasmid conferring MMS resistance was chosen for further study. This recombinant plasmid was designated pJB1.

**Analysis of Cloned DNA**

One of the features of a functional RecA protein is its proficiency at promoting genetic recombination between homologous DNA sequences (Clark, 1973; Dressler and Potter, 1982; McEntee *et al.*, 1979; Radding, 1981; Radding, 1982). The ability of the cloned recA gene of *F. novicida* to catalyze homologous recombination was assessed by scoring the number of Lac+ recombinants arising from the introduction of recA+ recombinant plasmids into *E. coli* JC14604 (Keener *et al.*, 1984). This recA strain of *E. coli* harbors two partially deleted non overlapping copies of the lac operon, and it is possible that double-crossover recombinational events give rise to Lac+.
recombinants (Zieg and Kushner, 1977). In each of three independent experiments, more than 1,000 colonies were examined on lactose MacConkey agar for the Lac\(^+\) phenotype. After 48 h of incubation at 37\(^\circ\)C, nearly 8% of the colonies of strain JC14604(pJB1) showed Lac\(^+\) papillae, whereas untransformed JC14604 showed no Lac\(^+\) papillae. By comparison, it was found that JC14604 harboring plasmid pVD308, which carries the \textit{N. gonorrhoeae} recA locus (Koomey and Falkow, 1987), generates approximately the same percentage of Lac\(^+\) papillae. By demonstrating the ability for pJB1 to impart MMS resistance and recombinational proficiency on \textit{E. coli} recA strains, it was concluded that pJB1 contained and expressed an \textit{F. novicida} gene that is analogous to the recA gene of \textit{E. coli}.

**Subcloning from pJB1**

In order to carry out transposon mutagenesis with \textit{Tn}10-Km, the recA region in pJB1 had to be subcloned into the Ampicillin resistance plasmid, pYUM1118. Also, a smaller subclone of pJB1 was made in the Kanamycin resistance plasmid pBGS19. Plasmid pJB1 was partially digested with \textit{Sau}3A I and ligated into the \textit{BamHI} I sites of pYUM1118 and pBGS19 to generate plasmids pJB3 and pJB10-4, respectively (Fig.18). These recombinant plasmids conferred MMS resistance on and restored recombinational
Figure 18. Restriction map of recombinant plasmid insert DNA containing the *F. novicida* recA region. Small triangles indicate the location of mini-Tn10-Km insertions which inactivate the recA gene; deletion of the *BamH I*-BstE II fragment also eliminates the RecA activity. The hatched bars indicate cloning vector sequences, and the thin lines indicate *F. novicida* insert sequences. Multiple cloning sites are expanded. Abbreviations are as follows: Ba, *BamH I*; BS, *BstE II*; E, *EcoR I*; H, *Hind III*; K, *Kpn I*; P, *Pst I*; Sa, *Sal I*; Sm, *Sma I*; Sp, *Sph I*; Ss, *Sst I*; X, *Xba I*. 
proficiency to recA E. coli strains.

**Transposon Mutagenesis and Construction of recA F. novicida**

For transposon mutagenesis, E. coli carrying plasmid pJB1 was grown to a density of $4.0 \times 10^9$ CFU/ml and mixed 1:1 with $\lambda$1105 phage ($10^8$ PFU/ml), which carries a mini Tn10-Km transposon (Way et al., 1984). After 30 min room temperature incubation, the cultures were incubated for 1 h with shaking (240 rpm) at 37°C, after which they were plated onto LB agar with kanamycin. After 24 h, the kanamycin-resistant survivors were pooled and a plasmid preparation was done by the alkaline lysis method (Maniatis et al., 1982). The mutagenized plasmid preparation was transformed into E. coli JC14604, and kanamycin resistant transformants were screened for the RecA phenotype. A number of transposon inserts in pJB3 which inactivated the recA gene were identified.

Plasmid pJB1 DNA containing transposon inserts that inactivated the RecA activity was used to transform F. novicida as described in Materials and Methods. Kanamycin-resistant F. novicida transformants were screened for the RecA phenotype by patching colonies onto agar plates which had 100 µl of MMS spread on their surfaces (Better and Helinski, 1983). One strain designated JB2, was unable to grow on plates containing MMS and was found to be more sensitive to killing by UV irradiation than the wild type F. novicida (Fig. 19). To assess recombinalional proficiency, JB2 and
Figure 19. Sensitivity of *F. novicida* U112 and JB2 to UV killing. Cell suspensions were irradiated for various lengths of time with a 254-nm germicidal lamp. After UV irradiation of cultures in the dark, the cell suspensions were diluted and the number of survivors was calculated by plate counts.
the wild-type strain U112, were transformed with DNA isolated from a spontaneous chloramphenicol (Cm\(^{r}\)) \(F.\) novicida strain. In duplicate experiments, strain JB2 yielded no Cm\(^{r}\) colonies, whereas the wild-type strain gave approximately 150 Cm\(^{r}\) colonies per ug of DNA. These data indicated that the \(\text{recA}\) gene in JB2 had been inactivated.

To verify that the mini-Tn10-Km transposon had inserted into the chromosome, a Southern blot analysis was performed on DNA isolated from \(F.\) novicida JB2. Blots probed with DNA carrying the \(F.\) novicida \(\text{recA}\) locus indicate that the hybridizing band in strain JB2 has a higher molecular weight than the band in the parent strain U112 (Fig. 20A). Also, the band in JB2 that hybridizes with the \(\text{recA}\) probe comigrates with the band that hybridizes with the kanamycin mini-transposon probe (Fig. 20B). These data indicated that the transposon had inserted into the chromosome at the \(\text{recA}\) locus. No bands that hybridized with the kanamycin transposon or with pUC DNA were found in wild-type \(F.\) novicida DNA.

In summary, this work reports on the cloning of a fragment of DNA from \(F.\) novicida that encodes activity which is functionally analogous to the RecA activity of \(E.\) coli. This conclusion was supported by the facts that (i) the cloned \(F.\) novicida DNA functionally complemented the DNA repair and recombination deficiencies of \(E.\) coli and (ii) an \(F.\) novicida mutant showed marked deficiencies in DNA repair and homologous recombination following
Figure 20. Southern blot analysis of Xba I-digested DNA isolated from *F. novicida* strains. Lanes 1, *recA* strain JB2; lanes 2, parent strain U112. Blots were probed with plasmid pJB10-4 (A) or isolated Km transposon DNA (B). Numbers to the left of panel A indicate sizes (in kilobases) of standard bands (not shown). Size of kanamycin transposon is 1.6 kb.
insertional mutation of the cloned DNA sequence and its reintroduction into the *F. novicida* chromosome.
CHAPTER 3. A Novel 3-Deoxy-D-manno-octulosonic Acid Transferase from Chlamydia trachomatis Required for Expression of the Genus-specific Epitope

INTRODUCTION

Chlamydia trachomatis is a procaryotic pathogen that replicates as an obligate intracellular parasite in animal cells and resembles Gram-negative bacteria in that it has two membranes (Schachter and Caldwell, 1980). Throughout the world C. trachomatis is one of the leading causes of sexually transmitted diseases. In many developing countries C. trachomatis also causes an eye infection, inflicting disease in approximately 350 million people, and causing blindness in 6 million of these (Schachter, 1978; Dawson et al., 1981). The related organism C. psittaci infects many animals species, including many domesticated animals.

The Chlamydiae have a complex life cycle, consisting of two distinct forms. Chlamydia elementary bodies (EBs) are rigid, metabolically inert cells that are the infectious form. After entry into a host cell the EBs differentiate into reticulate bodies (RBs) which are fragile and metabolically active. The RBs multiply in phagosomal compartments that are rendered incapable of fusing with lysosomes (Friis, 1972). The transition between the two Chlamydial forms correlates with the organization of the major outer membrane protein (MOMP) and two other cysteine rich outer
membrane proteins (Hatch et al., 1984; Newhall and Jones, 1983; Bavoil et al., 1984). In the elementary bodies the three cysteine rich proteins form a supramolecular structure that is held together by disulfide bonds, apparently substituting for peptidoglycan in giving rigidity to the cells. After being phagocytosed by host cells the disulfide bonds in the outer structure are reduced and the MOMP is thought to act as a porin (Bavoil et al., 1984).

The dominant immunogens on the chlamydial EB are the MOMP (Caldwell et al., 1981) and the lipopolysaccharide (Dhir et al., 1971; Nurminen et al., 1983). There is considerable antigenic variability present among the MOMPs of different serovars of C. trachomatis. However the dominant antigenic determinant on the LPS is found on all of the diverse Chlamydial strains and species, and has consequently been called a genus-specific epitope (Dhir et al., 1971; Nurminen et al., 1983; Perez-Martinez and Storz, 1985). Besides a possible role in attachment for the MOMP, the precise role in pathogenesis of the MOMP and the LPS has not been precisely defined. However, both of these structures are useful for diagnostic assays.

The LPS of C. trachomatis is similar to the Re LPS, the minimal LPS that consists of lipid A and two Kdo residues, essential for growth of E. coli and Salmonella (Brade et al., 1987a; Brade et al., 1987b). C. trachomatis LPS contains about 50% more Kdo than Re LPS does (Brade et al., 1987a; Brade et al., 1987b). Previously Nano and Caldwell, (1985) described a recombinant plasmid, pFEN207, that
directs the expression of the LPS epitope of chlamydiae in enteric bacteria. Strains of *E. coli* and *Salmonella* harbouring pFEN207 synthesize, in addition to their normal complement of LPS, a truncated LPS bearing the genus-specific epitope (Brade *et al.*, 1987b; Nano and Caldwell, 1985). Chemical analyses suggest that the epitope is generated by addition of a third Kdo residue to Re LPS molecules (Brade *et al.*, 1987b). The proposed Kdo trisaccharide that confers the genus-specific epitope is shown in Fig.21. The third Kdo somehow prevents addition of heptose (Brade *et al.*, 1987a; Brade *et al.*, 1987b) at position 5 of the innermost Kdo (Fig. 2).

This work describes the cloning and sequencing of the *gseA* gene of *C. trachomatis* and provide the first direct evidence for its catalytic activity by assaying extracts of *E. coli* expressing the recombinant *gseA* gene. The enzyme encoded by *gseA* consists of a single subunit with remarkable structural similarity to the bifunctional Kdo transferase of *E. coli* (Raetz, 1990), but it transfers at least one more Kdo residue to lipid A precursors than the *E. coli* enzyme does.
Figure 21. Proposed structure and biosynthesis of the genus-specific epitope of chlamydiae in *E. coli* strains bearing the recombinant *gseA* gene. Lipid IVA is a key biosynthetic precursor that is identical to lipid A except for the absence of laurate and myristate [673,345,343]. In a wild-type *E. coli* a Kdo disaccharide is incorporated by the *kdtA* gene product before the lipid A
moiety is completed and other core sugars or O-antigens of LPS are added \( (673,345,343) \). In strains bearing the recombinant \( gseA \) gene, a truncated LPS bearing only the Kdo trisaccharide of \( C. trachomatis \) on lipid A is generated \( (36,35) \). In extracts of \( E.coli \) cells expressing a recombinant \( gseA \) gene, at least three Kdos are added to lipid IVA, generating mainly metabolite a (the proposed structure at the bottom of the figure).
Figure 22. Space filling model of the proposed structure of the genus-specific epitope of chlamydiae. The model suggests that one of the carboxyl oxygens of the third outermost Kdo is capable of forming a hydrogen bond with the C-5 OH of the innermost Kdo, possibly preventing the normal addition of heptose \cite{673} at this position.
RESULTS

Subcloning and Sequencing of the DNA Conferring the Genus-specific Epitope

To determine the minimum amount of *C. trachomatis* DNA needed to generate the genus-specific epitope in *E. coli*, the original recombinant plasmid, pFEN207, was analyzed as shown in Fig. 23. An *SstI-*Sst I fragment, identified by transposon mutagenesis (Nano and Caldwell, 1986) to encode the relevant epitope conferring activity, was subcloned into M13mp18 to create pFEN208. This plasmid was opened at the *BamHI* site in the vector and digested with *Bal I*. An *EcoRI* linker was added, and a 2.3-kilobase *EcoRI* piece was cloned into pUC 13 to form pFEN212. The insert in pFEN212 was sequenced (Fig. 24) and two open reading frames were found. The smaller open reading frame encoded a putative protein of 17,068 Da of unknown function. The larger open reading frame (designated *gsea*) was then subcloned as a blunt-ended *SacI-*Sac II fragment into pUC18 to form pKEM1, and this plasmid was found to confer the production of the genus-specific epitope of chlamydiae on *E. coli*. Plasmid pKEM1 contained an open reading frame of 1203 bp, preceded by 80 bp on its 5' end and followed by 105 bp at its 3' end.

There is no genetic or biochemical evidence defining consensus DNA sequences of chlamydiae required for the initiation of transcription by RNA polymerase (Sardinia *et al.*, 1989). A
Figure 23. Restriction sites used to subclone the \textit{gseA} gene from pFEN207. The restriction sites in parentheses represent sites added to the \textit{C. trachomatis} DNA from the cloning plasmids or through linker addition. The \textit{ScaI}-\textit{SacII} fragment from pFEN212 was made blunt ended with the Klenow fragment of DNA polymerase before cloning into the \textit{SmaI} site of pUC18.
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<th>Interpretation</th>
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GlyAsnMetLysThrPheIleGluSerSerLeuAlaThrAsnArgArgAspPheTrpArg

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AlaLysLeuGlnIleSerSerGlnAspArgLeuIleValLeuGlySerMetHisProLys

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AspValGluValTrpAlaGluValSerHisProHisAsnSerSerThrLysIleLeu

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TrpValProArgHisLeuGluLysLeuLysGluHisAlaLysLeuGluLysAlaGly

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IleLeuPheGlyLeuTrpSerGlnAlaAlaSerPheArgGlnTyrAsnSerLeuIleMet

TGACGCTATGAGGTATTAAAAAGATTATTTTACTCTGCAAGCATATTGCCATTTGGAG
AspAlaMetGlyValLeuLysAspIleTyrSerAlaAspIleAspLeuValGlyGly

GAACCTCGATCCACTGAGGAGGCTATAATTTTATTAGAGCCTCTCTCAAAAGAGGCCG
ThrPheAspProSerValGlyGlyHisAsnLeuLeuGluProLeuGlnLysGluAlaPro

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GluAlaGlyLeuSerValAsnLysGluThrLeuLeuAspValValThrAspLeuLeuGln

AAAACGAAAAAACCGCAAGCTTATATGGAAAAGGGAAATCTTTTGTGAAGCAGGAA
AsnGluLysAsnProSerLeuTyrEnd
Figure 24. DNA sequence of pFEN212 and deduced amino acid sequence of two open reading frames. The gseA gene begins at nucleotide 147 and runs to nucleotide 1350. It is preceded by a putative ribosome binding site 12 bp upstream of the start codon (stars). Putative promoter regions are underlined.
A second open reading frame of unknown identity and function runs in the opposite orientation of \textit{gseA} from nucleotide 2284 to nucleotide 1834.
region upstream of the translational start of \textit{gseA} does show a pair of sequences resembling \textit{E. coli} promoter regions (Fig. 24). However, expression of the genus-specific epitope in \textit{E. coli} is greatly stimulated by the addition of isopropyl-\(\beta\)-\(D\)-thiogalactopyranoside (IPTG) to the medium, suggesting that the vector promoters play a dominant role in transcription. No rho-independent transcriptional stop sequences are evident at the 3' end of \textit{gseA}. A possible ribosome binding site is located upstream from the translational start of \textit{gseA} (Fig. 24).

\textbf{Similarity to the \textit{kdtA} Gene of \textit{E. coli}.}

The \textit{gseA} gene in pKEM1 encodes a protein of 401 amino acid residues with a molecular mass of 45,851 Da. The predicted size of the protein is in agreement with \textit{in vitro} transcription and translation of pFEN212, in which a translation product is observed at approximately 43 kDa (Fig. 25). FASTP analysis of the NBRF protein sequence data bank revealed significant similarity to only one other protein, the \textit{kdtA} gene of \textit{E. coli} described by Clementz and Raetz, (1991). The latter encodes a polypeptide of 425 amino acids that catalyzes the transfer of two Kdo residues from CMP-Kdo to the lipid A domain of LPS in \textit{E. coli} (Fig. 22). The deduced amino acid sequences of the \textit{gseA} gene and the \textit{kdtA} are 66\% similar and 23\% identical (Fig. 26). The most conserved region is between residues 313 and 346 in the \textit{C. trachomatis} sequence, but similarity is found throughout. The two polypeptides have similar
Figure 25. Fluorogram of peptides synthesized in an in vitro transcription/translation reaction. Lane A, pUC8 used as template; lane B, pFEN212 used as template. The arrow at the right indicates a putative gseA gene product. The numbers at the left indicate relative molecular weights (in kDa).
Figure 26. Relationship of \textit{gseA} to \textit{kdtA} at the amino acid level. Double stars indicate identity and single stars indicate similarity between amino acids aligned by the FASTP program.
hydropathy profiles, predicted secondary structures, and isoelectric points (9.5). Both lack typical leader sequences, suggesting that they are not exported to the periplasm or outer membrane.
The following enzymatic studies were performed by C Belunis and C. R. H. Raetz.

The *gseA* Gene Encodes a Novel Kdo Transferase

Efficient *in vitro* systems were developed for the enzymatic transfer of two Kdo units from CMP-Kdo to lipid IV$_A$ (Brozek *et al.*, 1989; Clementz and Raetz, 1991), a key precursor resembling mature lipid A, except for the absence of two fatty acyl chains (Fig. 22). The *kdtA* gene product has been overexpressed and purified (Brozek *et al.*, 1989; Clementz and Raetz, 1991), facilitating preparation of labelled Kdo$_2$-IVA (Fig. 22). When [4'-32P] Kdo$_2$-IVA and a CMP-Kdo generating system are incubated for 60 min in the presence of 2mg/ml crude extract of *E. coli* JM109 harboring a vector control (Fig. 27A, lane 2), very little further conversion of [4'-32P] Kdo$_2$-IVA to other more polar metabolites is observed, consistent with the fact that the *KdtA* gene product of the *E. coli* host is only capable of incorporating two Kdo units. In contrast, similar incubations with extracts made from JM109 derivatives harboring either pFEN207 (Fig. 27A, lane 3) or pKEM1 (Fig. 27A, lane 4) efficiently generate two more polar metabolites. The formation of the most prominent (Fig. 27A, metabolite a) is linear with time and protein concentration (Figs. 28 and 29). Metabolite b is not observed at protein concentrations below 0.5 mg/ml (Figs. 28 and 29) even though the conversion of [4'-32P]Kdo$_2$-IVA to metabolite a is very efficient under these conditions.
Figure 27. Conversion of [4'-32P]Kdo2-IVA to more polar metabolites in extracts of E. coli strains bearing the recombinant gseA gene. Panel A, all reactions contain 2 mg/ml crude extract protein of the indicated E. coli strain. Lane 1, no added enzyme; lane 2, pUC8 (vector control) in E. coli JM109(42); lane 3, pFEN207 in JM109; and lane 4, pKEM1 in JM109.

Panel B, in this experiment, 0.8 mg/ml extract from strain JM109/pFEN207 was used. Lane 1, no E.coli extract added; lane 2, complete system; lane 3, no Kdo added; lane 4, no CTP added; lane 5, no CMP-Kdo synthase added; lane 6, no MgCl2 added. SF, solvent front.
Figure 28. Time course of the conversion of $[4'\cdot^{32}\text{P}]Kdo_2\text{-IVA}$ to metabolite a. Reactions contained extracts of JM109/pFEN207 in the first 9 lanes. The reactions were terminated at the indicated times by spotting 5 µl samples onto a silica gel thin layer plate and analyzed as described in Materials and Methods. SF, solvent front.
Figure 29. Protein concentration dependence of the conversion of [4'-\(^{32}\text{P}\)]Kdo\(_2\)-IVA to metabolites a and b. Reactions were done with the addition of crude protein extract at the indicated concentrations in a final volume of 20 µl and analyzed as described in Materials and Methods.
Conversion of [4'-32P] Kdo2-IVA to metabolites a and b is dependent upon a CMP-Kdo generating system (Fig. 27B). The latter consists of E. coli CMP-Kdo synthase, CTP, Mg2+, and Kdo (Brozek et al., 1989). CMP-Kdo is generated by the reaction Kdo + CTP \rightarrow CMP-Kdo + PPi. CMP-Kdo is unstable in water at pH 7 (Raetz, 1990; Belunis and Raetz, 1992). Individual omission of any of the components of the CMP-Kdo generating system (Fig. 27B, lanes 3-6) abolishes synthesis of metabolites a and b. These results show that the addition of at least one Kdo residue from CMP-Kdo to [4'-32P] Kdo2-IVA occurs during the formation of metabolites a and b.

To determine the stoichiometry of Kdo addition to Kdo2-IVA, extracts of strains producing gseA gene product were incubated with 1mM Kdo and 25 μM Kdo2-IVA supplemented with either [1-14C] Kdo (4 x 104 cpm/nmol) or [4'-32P] Kdo2-IVA (1-2 x 104 cpm/nmol) in parallel tubes. Reactions were terminated after 2 h at 30°C by spotting 5μl onto a TLC plate followed by chromatography in Solvent B. Autoradiography confirmed that the incorporation of both [1-14C] Kdo and [4'-32P] Kdo2-IVA into metabolite a had occurred. The ratio of 14C to 32P in metabolite a was 0.8, consistent with the composition Kdo3-IVA. The yield of metabolite b was too low to permit estimation of the stoichiometry. Definitive conclusions about the structure of the Kdo units present in metabolites a and b await their analysis by spectroscopic methods, but so far, it has not been possible to recover them intact from thin layer plates.
The \textit{gseA} Gene Product Can Transfer at Least Three Kdo Residues to Precursor Lipid IV\textsubscript{A}

Previously, a mutant of \textit{E. coli} was described (Clementz and Raetz, 1991), and designated TC5, that possesses a thermolabile Kdo transferase. Extracts of TC5 convert [4'\textsuperscript{-32}P] lipid \textsubscript{IVA} to [4'\textsuperscript{-32}P] Kdo-IV\textsubscript{A} very slowly at 42°C compared with wild type (Fig. 30). Introduction of the \textit{gseA} gene into TC5 restores the ability of cell extracts to generate [4'\textsuperscript{-32}P] Kdo\textsubscript{2}-IV\textsubscript{A} from [4'\textsuperscript{-32}P] lipid IV\textsubscript{A} (Fig. 30). Metabolite a is also synthesized in this setting (Fig. 30), demonstrating that the Kdo transferase of \textit{C. trachomatis} minimally transfers three Kdo residues to [4'\textsuperscript{-32}P] lipid IV\textsubscript{A}. 
Figure 30. Introduction of the gseA gene into a mutant of E.coli with a thermolabile Kdo transferase restores the ability of extracts to generate $[4'-\text{32P}]\text{Kdo2-IVA}$ from $[4'-\text{32P}]\text{ipid IV}_A$. Lane 1, no enzyme added; lane 2, R477 (wild type); lane 3, TC5 ($kdtAI$); lane 4, TC5/pFEN207; lane 5, R477/pFEN207. SF, solvent front; a, metabolite a.
DISCUSSION

The work reported here shows that the genus-specific epitope of chlamydiae is conferred upon *E. coli* by expression of a single gene (*gseA*) encoding a novel Kdo transferase. This conclusion is based upon the sequence homology of *gseA* and *kdtA* (Fig. 26), the enzymatic assays of extracts of *E. coli* harboring *gseA* (Figs. 27-30), and the earlier studies of the Kdo content of *C. trachomatis* LPS (Brade *et al.*, 1987b).

The *kdtA* gene product consists of a single polypeptide (Clementz and Raetz, 1991) and is an unusual bifunctional enzyme that attaches two positionally distinct Kdo residues to lipid A or lipid A precursors (Fig. 22) (Clementz and Raetz, 1991). The enzyme encoded by *gseA* is capable of adding at least three Kdo moieties to such lipid A precursors (Figs. 22 and 27-30). Further spectroscopic analyses of the products generated by the *C. trachomatis* transferase will be necessary to determine the nature of the Kdo-Kdo linkages present in metabolites a and b. The high efficiency of the conversion of \([4'\text{-}^{32}\text{P}]\text{Kdo}_2\text{-IVA}\) to metabolite a may also prove useful for the preparation of novel lipopolysaccharide substructures and defined reagents for diagnostic assays.

The cloning of *gseA* was made relatively simple by the detection of the unique antigen that host cells harboring the *gseA* gene were able to synthesize (Nano and Caldwell, 1985). The *gseA* gene product is one of the few enzymes of chlamydiae for which the
structural gene has been cloned, expressed in *E. coli*, and studied by measuring its enzymatic activity in cell extracts. These findings demonstrate that it is possible to dissect metabolic processes in chlamydiae by using recombinant DNA techniques. The availability of authentic chlamydiae is limited by the difficulty of growing these organisms.

Analyses of 16 S RNA sequences show that chlamydiae are members of a distant phylum far removed from the one that includes *E. coli* (Woese, 1987). The differences between *gseA* and *kdtA* are likely to be much greater than between the Kdo transferase genes of most other Gram-negative bacteria. Consequently, the identification and cloning of other Kdo transferase genes from diverse sources should be possible. Moreover, the conservation of Kdo activation and metabolism strongly suggests that CMP-Kdo or other LPS biosynthesis inhibitors (Goldman *et al.*, 1987) have potential as novel antibiotics for infections caused by chlamydiae.
CONCLUSIONS AND FUTURE RESEARCH

Investigating the mechanisms underlying the ability of pathogenic microorganisms to cause disease requires that a number of conditions be met. Firstly, an appropriate system for the assessment of virulence must be established so that the differences between virulent and avirulent strains might be visualized. Ideally, this system must be sensitive enough to distinguish between forms that are merely attenuated and those that are totally incapable of causing disease. Animal models of infection are very useful in this respect, provided they mimic the parameters of natural infection. Secondly, it is useful if the assay system is adaptable to studies in vitro so that phenomena observed in vivo may be dissected simply and effectively without the problems inherent in studying the whole animal. Finally, mechanisms should be established for the genetic manipulation of the organism itself, thus enabling mutations to be engineered in specific and traceable genetic loci. We and others have shown that the experimental murine models of infection with *F. novicida* and *F. tularensis* have been shown to fulfill all these criteria, and so we have begun to address the question of virulence in this bacterial genus. We have developed an in vitro system where *Francisella* species are allowed to grow intracellularly within resident, inflammatory, bone marrow-derived, or cell line macrophages causing an easily detectable cytopathic effect on the macrophage monolayers.
The method of mutagenesis employed here was chosen for its inherent advantage that it labels the mutagenized region with a kanamycin resistance marker, thus simplifying the molecular cloning and subsequent genetic characterization of the mutated locus. There are no established plasmids or phages that work well in Francisella. Thus this technique also had the advantage that it required no Francisella-specific vectors for delivering the mutagenic element. It also became apparent in the subsequent procedures that although this mutagenesis scheme was well suited for our applications, it had several problems. Even though we had shown (Fig. 2) that in most cases there was only one kanamycin insertion per chromosome, it was possible to have multiple insertions which complicated further analysis of the mutation. It seemed that not all insertions were achieved through a double cross-over event which would result in a simple allelic replacement, some were single cross-overs, resulting in a high probability for complicated rearrangements that could not be easily explained except by inference from Southern blot hybridization patterns. Also, although we had found a way of mutagenizing Francisella without the need for a Francisella-specific vector, we still had the problem that we could not do complementations in trans which provides the best proof that a certain locus is responsible for a certain phenotype.

It is fortunate that both the Francisella mutated loci that were studied seem to have analogous loci and expressed gene products in Escherichia coli. This provides the opportunity to study these genes
in an *E. coli* background where the genetic tools are readily available. This way the phenotype of these genes can be easily established and then later, when the *Francisella* genetic manipulations have been improved, their exact participation in virulence can be determined. This work is presently being pursued in our laboratory.

The need to identify virulence factors is obvious because it leads directly to the understanding of the pathogenesis of, vaccine development against, and cure of microbial diseases. The discovery of a virulence factor in one pathogen usually suggests the presence of an analogous factor in a related pathogen. We certainly hope for such extrapolation between *Francisella* and other pathogens, most probably other Gram-negative intracellular pathogenic bacteria. The procedure for assigning virulence property status to a molecule is not trivial. First, the encoding gene must be eliminated exclusively, usually through mutagenesis resulting in a mutant that is not capable of causing disease, or has reduced virulence. Then expression of the gene product must be restored, usually by genetic complementation, thus bringing the mutant back to wild type and demonstrating that it can cause disease. This is what was attempted with the study reported in this thesis, except that the complementation was not precise due to lack of proper genetic tools. We hope that in the future *in trans* complementation will be possible in order to make this work more precise.

Most of the work reported here on *Francisella* was mainly preliminary and aimed at opening up possibilities for future work.
The purpose, as stated in the objectives, was to improve the repertoire of genetic manipulations that can be carried out on *Francisella*. Future work will most likely take advantage of the availability of the *recA* strain in which genetic elements may replicate extrachromosomally. The identification of virulence factors was mainly investigatory, done just to examine the possibility of effecting *in vitro* insertional mutagenesis technique in this organism. Future attempts will probably utilize modified protocols that will eliminate some of the problems encountered here.
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placement of the division site in *Escherchia coli*. *Journal of Bacteriology* **170**, 2106-2112
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair(s)</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CHA-B</td>
<td>Cysteine heart agar with horse blood</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hampster Ovary cells</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CMP-Kdo</td>
<td>Cytidine monophosphate-3 Deoxy-D-manno-octulosonic acid</td>
</tr>
<tr>
<td>CTP</td>
<td>Cytosine triphosphate</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxy-Adenosine triphosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed type hypersensitivity</td>
</tr>
<tr>
<td>EB</td>
<td>Elementary bodies</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK506-binding proteins</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HeLa</td>
<td>(Helen Lange) Human cervical carcinoma cells</td>
</tr>
<tr>
<td>HMPS</td>
<td>Hexose monophosphate shunt</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>125I</td>
<td>Radioactive iodine</td>
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</tbody>
</table>
IFN  Interferon
Ig   Immunoglobulin
IgG  Immunoglobulin G
iNOC Inoculum
IP3  Inositol triphosphate
IPTG Isopropyl-1-thio-β-D-galactopyranoside
kb(p) Kilobase or kilobasepairs
kDa  KiloDalton
kdo  3-deoxy-D-manno-octulosonic acid
kdo2-IVA 2(3-deoxy-D-manno-octulosonic acid)-lipid A analog
LB   Lauria-Bertani broth
LD50/100 Lethal dose that kills 50/100% of a tested animal population
LPS  Lipopolysaccharide
LVS  Live vaccine strain of Francisella tularensis
MgCl Magnesium chloride
MHC  Major histocompatibility complex
min  Minutes
mM  Milimolar
μM  Micromolar
MMS  methyl methanesulfonate
MOMP Major outer membrane protein
NK   Natural killer cells
OH   hydroxyl radical
ORF  Open reading frame
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>O2^-</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pfu</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidyl inositol</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidyl inositol diphosphate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PPIase</td>
<td>Peptidyl-prolyl cis/trans isomerase</td>
</tr>
<tr>
<td>RB</td>
<td>Reticulate bodies</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid sequence</td>
</tr>
<tr>
<td>35S</td>
<td>Radioactive sulphur</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris[hydroxymethyl]aminomethane</td>
</tr>
<tr>
<td>TSB-C</td>
<td>Tryptic soy broth with cysteine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>X</td>
<td>Xanthine</td>
</tr>
<tr>
<td>XD</td>
<td>Xanthine oxidase</td>
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</tbody>
</table>
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*Francisella novicida* virulence associated locus that includes a gene encoding an ABC-transporter protein. *Submitted*


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Title of Dissertation:

ISOLATION AND CHARACTERIZATION OF THREE GENETIC LOCI FROM THE INTRACELLULAR PATHOGEN *Francisella novicida* AND *gseA* FROM *Chlamydia trachomatis*

Author: __________________________

(Signature)

KHISIMUZI EDWARD MDLULI

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25th July, 1994

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