Isolation and identification of eight genetic loci from the intracellular pathogen *Francisella novicida*

by

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ABSTRACT

Shuttle transposon mutagenesis of the intracellular bacterial pathogen *F. novicida* resulted in eight mutants which showed decreased growth in intraperitoneal murine macrophages as compared to the wildtype strain. The genetic loci flanking the transposon were isolated and characterized by DNA sequencing. Five of the eight mutant clones showed similarity to database entries at the deduced amino acid level. One of the mutants, designated CG62, was interrupted in a presumed gene encoding a protein identical to a possible virulence factor found in *F. tularensis*. This protein is expressed only when the bacterium is growing inside macrophages, and when it is exposed to hydrogen peroxide. The protein has been termed Fip, for *Francisella* intramacrophage protein. Four of the *F. novicida* mutants had interrupted genetic regions similar to genes for a stress response protein, a purine biosynthesis protein, alanine racemase and a protein of unknown function found in *E. coli* and *V. cholerae*, respectively. The other three mutant strains appear to have been interrupted in unknown genetic regions, which may encode novel proteins involved in virulence.

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ABBREVIATIONS

Ap	ampicillin
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pairs
CFU	colony forming units
CHA-B	cystine heart agar with horse blood
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
Em	erythromycin
h	hour(s)
id	intradermal
IFN	interferon
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
in	intranasal
ip	intraperitoneal
IPTG	isopropyl-D-thiogalactopyranoside
iv	intravenous
kb	kilobases or kilobase pairs
kDa	kilodalton
Km	kanamycin
LB	Luria-Bertani
LD50	50% lethal dose
LVS	live vaccine strain
LPS	lipopolysaccharide
μM	micromolar
mM	millimolar
mmol	millimole
NK	natural killer
NMMA	NG-monomethyl-L-arginine
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocyter
DNA	ribosomal deoxyribonucleic acids
S	Second
sc	subcutaneous
scid	severe combined immunodeficient
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS-T	Tris-buffered saline with Tween-20

TCR	T cell receptor
TDL	thoracic duct lymphocyte
TNF	tumor necrosis factor
Tris	Tris[hydroxymethyl]aminomethane
tRNA	transfer ribonucleic acid
TSB-C	tryptic soy broth with cysteine
WHO	World Health Organization

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INTRODUCTION

Infectious disease

Infectious diseases are the leading causes of mortality worldwide, resulting in approximately one half of deaths of people under 45 each year (WHO, 2000). Since World War II the prevailing attitudes (especially in the developed world) on the control of infectious diseases reflected scientific confidence in the development of antibiotics and the widespread use of vaccines. This confidence has faltered in the last two decades as previously unknown pathogens such as human immunodeficency virus (HIV), Ebola virus, and the bacterium *Helicobacter pylori* have appeared. Additionally, diseases which were thought to be under control in the western world, such as tuberculosis, have reemerged as bacteria develop increased resistance to antibiotics. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is responsible for the most deaths annually due to an infectious disease, and there is great interest in preventing the spread of multiple drug resistant strains. The problem of resistance to commonly used antibiotics is compounded by the fact that few new agents are being discovered. These reasons alone have motivated scientists to renew their efforts to develop effective vaccines, and new antimicrobial drugs (WHO, 2000). Also of interest are the recent ideas, put forth by evolutionary biologists and epidemiologists, that many diseases with ill-defined causes may be infectious in origin. For example, there is recent epidemiological evidence suggesting a link between the Gram negative bacterium *Chlamydia pneumoniae* and atherosclerosis. Additionally, human papillomavirus is now accepted as the cause of at least 50 % of cervical cancers. Although there is little experimental support for these theories as yet, if these evolutionary biologists are correct and many major illnesses have microbial origins, the host-pathogen interaction will once again be pushed to the forefront of medical science (Ewald, 1993).

Bacterial pathogenesis

Bacteria can gain entry to a host through the gastrointestinal system, the respiratory tract, the genitourinary tract, and through openings in epithelial linings. Each of these routes require the bacteria to survive nonspecific host barriers such as skin, mucus, endocytosis and phagocytosis, and the inflammatory response. Generally the innate host response depends upon the route of entry. However, if these defenses cannot clear the infection, an acquired humoral or cell-mediated immune response may occur. A humoral response produces antibodies that bind to antigen on the bacterial surface, resulting in both activation of the complement system and increased phagocytosis by phagocytes. This type of immune response functions to control extracellular microbes and soluble toxins (Kuby, 1997).

Intracellular bacterial pathogens are able to enter the host cells to survive and replicate in an environment that offers protection from the immune response. They can avoid or subvert the defenses of the host to survive within phagocytic cells that are lethal to most bacteria. Bacteria that are able to infect phagocytic cells gain access to a specialized niche, which can provide nutrients, protect them from both acquired and innate immune responses, and enable them to avoid competition with pre-existing microbial populations. Once inside the cells these pathogens may trigger a cell-mediated immune (CMI) response, however. One characteristic CMI response to intracellular bacterial pathogens is termed delayed-type hypersensitivity (DTH), and is characterized by specific T-cells, T_{DTH}, that secrete cytokines such as the macrophage activator IFN-γ (Kuby, 1997). In addition, cytotoxic T-cells may be activated, which act to recognize and eliminate infected cells.

In order to live within a mammalian host, all bacteria must be able to evade or subvert the immune response to varying degrees. For example, pathogens such as *Helicobacter pylori* can avoid escalating an immune response for long periods of time. This bacterium, which colonizes the epithelial lining of the stomach, is able to survive for years within a host, often producing few symptoms. Other bacteria, such as *Yersinia pestis*, produce dramatic effects in a human host and induce a strong immune response, resulting in a relatively rapid (albeit uncomfortable) death.

Intracellular bacterial pathogens

Pathogens that survive within phagocytes need to avoid processing through the phagosome-lysosome fusion pathway. After initial phagocytosis, the phagosome fuses with lysosomes and ingested bacteria are exposed to a variety of toxic compounds. These include hydrolytic enzymes such as lysozyme, proteases, glycosidases, and lipases, small cationic peptides such as defensins (which form amphipathic alpha-helical structures and create pores in membranes), and lactoferrin (Parra-Lopez *et al.,* 1993). The phagocyte also produces reactive oxygen and nitrogen intermediates, which are toxic to bacteria (Kuby, 1997). Intracellular pathogens evade this pathway by three general strategies. Some pathogens (most notably *Shigella flexneri*) dissolve the initial vacuolar membrane and escape to the cytoplasm. In contrast, pathogens such as *Coxiella burnetti,* are able to survive inside the acidified phagolysosome. Finally, bacteria such as *Mycobacteria*, *Salmonella,* and *Legionella* prevent the vacuole from entering the phagolysosome fusion pathway. Most intracellular pathogens, including *Francisella*, appear to use the last strategy, and are almost always found inside vacuoles while living inside host cells. These vacuoles are manipulated to undergo various changes that are advantageous to the bacteria, and are not considered to be true phagolysosomes. They are characterized by the presence or absence of specific membrane molecules (or markers), acidification, and interactions with other organelles and membranes. For example, the vacuole surrounding *Legionella pneumophila* lacks any lysosomal markers, and initially the vacuole is surrounded by mitochondria, followed by ribosomes (Finlay and Falkow, 1997). *Salmonella* appear to require acidification of the vacuole for intracellular replication to occur (Rathman *et al.,* 1996). *Coxiella burnetti* and *Francisella tularensis* also replicate within acidified vacuoles (Antoine *et al.,* 1990; Fortier *et al.,* 1995). *Francisella* requires acidification in order to facilitate the acquisition of iron, but this is not the case for *Salmonella* or *Coxiella.* Instead, they appear to require an acidic pH as a signal for intracellular replication and survival.

Both *Mycobacteria* and *Legionella* reside in neutral compartments (Sturgill-Koszycki *et al*, 1994; Horwitz *et al*.,1984).

It is thought that initial interaction with the host cell determines the way in which the bacteria will be engulfed and the type of vacuole it will reside in. If *Salmonella* expresses the invasion protein invasin from *Yersinia* , it will enter by the invasin pathway. It is subsequently found to reside in a vacuole lacking the expected characteristics. Virulence factors such as capsules and LPS allow some bacteria to prevent phagocytosis. Bacteria can secrete proteases which degrade host proteins, and enzymes, such as catalase, which neutralize oxygen radicals. These armaments help the bacteria survive in the hostile environment of the host cell, and they are carefully regulated in order to maximize survival while minimizing effort. Presumably, factors additional to pH, such as the presence of lysosomal markers or the presence of oxygen products, trigger the expression of virulence factors and help regulate bacterial behavior (Finlay and Falkow, 1997).

Francisella

Francisella tularensis is a facultative intracellular pathogen, which is the etiologic agent of tularemia, a zoonotic bacterial disease. Tularemia is a febrile illness in humans, which can be lethal if not treated properly. Treatment with the antibiotics streptomycin or tetracycline clears the infection, although gentamicin and chloramphenicol are also effective (Nano, 1992). The disease can have different clinical presentations, dependent upon the route of infection. It is often transmitted via an insect bite, which produces an ulceroglandular form of the illness. The same symptoms present upon exposure via a cut or wound, most often after handling wild game meat. An ulcer develops at the site of infection, which is soon followed by a fever, general discomfort, and swelling of local lymph nodes. If the bacteria infect the eye, generally due to contact with contaminated hands, an oculargiandular form of tularemia develops. The oropharyngeal form occurs after ingestion of contaminated food and water. Inhalation of the bacteria results in a pneumonic or typhoidal form of the disease. Without treatment septicemia can occur regardless of the mode of entry, resulting in pneumonia when it spreads to the lungs (Tarnevik, 1989). Pneumonia may occur anywhere from two days to months after infection, and has a high mortality rate if not properly treated (Gill and Cunha, 1997).

F. tularensis is divided into two main biovars, both of which are antigenically similar, but which differ in their virulence properties. The type A biovar (previously called *F. tularensis* var *tularensis*) is the more virulent of the two, and exists in North America. It has been found most often in ticks and rabbits, and produces a severe form of tularemia in humans (Tarnevik, 1989). The type B biovar (previously called *F. tularensis* biovar *palaeartica*) is found in Europe and Asia, and North America. This biovar is less virulent in humans and rarely causes death. It is often found in rodents, and requires a 1000 to 10,000 fold higher dose than biovar A to cause the same illness in rabbits (Nano, 1992).

History

Tularemia was described in 1837 by a Japanese physician, Homma Soken, as an illness which occurred after the ingestion of hare meat. He described the prototypical symptoms of initial chills and fever, followed by the appearance of glandular tumors. In 1925, another Japanese physician, Hachiro Ohara, examined three patients who developed a similar illness after eating hare meat. He and his wife, Riki Ohara, hypothesized that the illness derived from the hare, and demonstrated this by exposing Mrs. Ohara's left hand to tissue and fluids derived from the animal's heart. This experiment worked, as she contracted the symptoms of tularemia, and an organism was subsequently isolated from her axillary lymph glands. The organism was designated the Ohara-Haga coccus. The disease was called Yato-Byo (wild hare disease), although the illness is sometimes called Ohara's disease in Japan. No further studies were done at this time, perhaps understandably (Evans *et al.*, 1985).

In 1911, a plague of ground squirrels in Tulare County, California attracted the attention of George McCoy, who was investigating possible plague outbreaks in

San Francisco after the fire of 1906 (McCoy, 1911). The organism causing this plague was previously unidentified, and he named it *Bacterium tularense* (from the location of its discovery) after isolating it on a coagulated egg medium (McCoy, 1912: Evans *et al.,* 1985). Within a few decades, *Francisella* was isolated from rodents in the former Soviet Union and Canada (Nano, 1992). The first case of infection in humans was described by Wherry and Lamb in a meat cutter from Cincinnati, Ohio in 1914 (Evans *et al.,* 1985; Bell, 1981). Dr. Edward Francis, U.S Public Health Service surgeon, went to Utah in 1919 to find the cause of a presumed novel disease in humans, called deer fly or rancher's fever. He inoculated animals, and then carefully observed and chronicled their symptoms. After extensive research he determined that the illness was caused by *Bacterium tularense*, and he proposed the name tularemia as a technical international name (Francis, 1921). He was very qualified to name the disease, not least for the fact that he infected himself with the bacterium on more then one occasion, and thus had ample opportunity to acquaint himself with the symptoms. All of his efforts were honored with a Nobel Prize nomination, the Gold Medal for Contributions to Medical Science from the American Medical Association, and the eventual renaming of the bacterium to *Francisella tularensis* (Rockwood, 1983).

Morphology

Francisella tularensis. is 0.2 x 0.2-0.7 um in diameter while *Francisella novicida* is 0.7 x 1.7 um in diameter. Both are Gram negative nonmotile coccobacilli when actively growing in adequate media and are pleomorphic otherwise. They are obligately aerobic and facultatively intracellular. Cysteine is often required for growth, or to increase growth rate (in the case of *F. novicida*). On cysteine-heart agar supplemented with blood, the bacterial colonies are smooth and gray (Eigelsbach and McGann, 1984). The lipid concentrations in the capsule and cell wall, 50 % and 70% respectively, are quite high for Gram-negative bacteria. The G + C content of the DNA is 33-36 mol %. It is unclear how *Francisella* reproduces, but budding has been observed, although there is speculation that binary fission may occur (Eigelsbach and McGann, 1984). *Francisella* can be differentiated from other bacteria which cause similar symptoms because they require special media for growth due to a requirement for sulfhydryl compounds. The thermal death point of *Francisella* is 56 °C for 10 minutes, as established by McCoy and Chapin (1912).

Francisella tularensis was initially successfully cultured on a coagulated egg yolk medium by McCoy and Chapin (1912). Colonies appear after 18 to 24 hours at 37 °C. Francis further improved culturing techniques, and was the first to add cysteine to the medium, which is still used (Bell, 1981). *In vitro* culturing of *Francisella* on blood plates results in a decrease in virulence, which can be restored by passage through animals.

Phylogeny

In addition to *F. tularensis*, the only other *Francisella* species recognized are *F. philomiragia* and *F. novicida*, but they rarely cause illness in humans. They are all situated in the gamma subdivision of the Proteobacteria. *F. novicida* is almost identical to *F. tularensis* on the basis of 16s ribosomal DNA sequence analysis, although it is lacking a bacterial capsule and is much larger than *F. tularensis* (Forsman *et al.*, 1994). It was first isolated from water, and it is thought that it originated from high numbers of dead muskrats in the area (Larson *et al.*, 1955). It has been isolated twice from humans since then, and is thought to only infect immunocompromised individuals (Hollis, 1989). *F. philomiragia* has been isolated from dead muskrats (Jensen *et al.*, 1969), as well as from sea mammals, and from over ten human patients. Many of the humans infected had experienced near-drowning incidents, and were immunocompromised after corticoid steroid treatment. Other patients were immunocompromised due to chronic granulomatous disease (Wenger *et al.*, 1989).

Recent evidence suggests that an endosymbiont of the wood tick *Dermacentor andersoni*, called DAS (*D. andersoni* symbiont), which was first isolated in western

Montana, is a member of the genus *Francisella*. Analysis of 16S rDNA sequences revealed 95.4% similarity with *F. tularensis*. Although it has yet to be shown whether it can infect humans, it seems unlikely as the bacteria had to be injected directly into the ovarial tissue of guinea pigs to initiate an infection. The tick endosymbiont was found in phagosomes in ovarial tissue cells of ticks, and can be cultured in Vero cells (Niebyliski *et al.*, 1997). Other endosymbionts closely related to *Francisella* have been found in different tick species (Noda *et al.*, 1997). These endosymbionts will probably be placed within the genus *Francisella*, along with the tick pathogen *Wolbachia persica*, which shares 97.85 % 16S rDNA sequence similarity with *F. tularensis* (Noda *et al.*, 1997; Forsman *et al.*, 1994). *Francisella* species are distantly related to *Coxiella burnetti*, the causative agent of Q-fever and an obligate intracellular pathogen found in over 40 species of ticks (Noda *et al.*, 1997).

Epidemiology

Tularemia primarily appears to be a disease of northern latitudes, although it is not understood why. It is endemic to many areas of North America, Europe and Asia (Nano, 1992). Tularemia occurrence is dependent upon the seasons. The vast majority of cases are due to direct contact with lagomorphs such as rabbits and hares, as first noted by the researchers Ohara and Francis. Many cases are reported during the hunting season in the fall and winter. Tick-associated (*Dermacentor* spp.) infection occurs primarily in the spring, while deer-fly mediated infection happens during the summer. A similar trend has been found in Europe and Asia. Tularemia can be transmitted by eating infected meat, by physical contact with tissue and fluids of infected animals, through cuts and sores, via vectors such as deer flies and ticks, and from animal bites (including domesticated animals such as cats and dogs). It appears to be able to survive in the salivary glands of animals such as coyotes for long periods of time after the predator has ingested an infected animal. The wood tick, *Dermacentor andersoni*, is one of the principal tick vectors in North America, and Parker *et al* first isolated *Francisella* from it in 1924 in Montana. It is believed that ticks ingest *Francisella* along with the blood meal, although this has not been shown (Bell, 1981).

In the United States, where the illness has been referred to as deer-fly fever, rabbit fever and tick fever, there has been a large decline in the number of reported cases since the 1950's. Approximately 200 cases of tularemia are reported in the United States each year, of which 10-20 % present in the pneumonic form, either as the primary form, or due to ulceroglandular or typhoidal tularemia (Gill and Gunha, 1997). Humans usually contract tularemia while hunting or hiking in the wilderness. Tularemia also often infects laboratory personnel investigating *Francisella tularensis*; it ranked third after brucellosis and tuberculosis among laboratory acquired infections in the U.S from 1950 to 1963 (Bell, 1981).

There are occasional outbreaks of the disease everywhere that it exists. An outbreak of the pneumonic form of the disease amongst vacationer's in Martha's Vineyard was well documented in 1979. Another epidemic occurred in Sweden during the late sixties when farmers were exposed to dust from hay contaminated during a vole infestation. Airborne infections have also been reported upon numerous occasions in laboratory workers (Bell, 1981). More recently, there was an outbreak in early 1998 in Castilla Y Leon, Spain, which affected hundreds of people. Serological testing was performed on over 350 people, and over half of them tested positive (Labayru *et al.*, 1999). Presently there is an epidemic in Kosovo, with 699 suspected cases as of spring 2000. The first case of tularemia in Kosovo was confirmed in August of 1999, and 56 other cases have been confirmed by laboratory tests since then. No deaths have been reported in this outbreak, and the mode of transmission has not yet been determined (WHO Outbreak News, 2000).

Ecology

F. tularensis has been isolated from a large variety of wild mammals including skunks, voles, mice, rats, ground squirrels, sheep, rabbits, hares, beavers, muskrats, horses, and various types of birds. Many different arthropod vectors have been identified. In North America, the biovar type A is most strongly associated with rabbits and the primary vectors are ticks, most notably *Dermacentor andersoni* (Jellison, 1974). *F. tularensis* is not believed to be hereditarily transmitted via transovarian passage; the mode of transmission between ticks appears to be primarily horizontal. The endosymbiont DAS was found to undergo transovarial transmission to female progeny, however. Another symbiont which forms a monophyletic group with *Francisella* and *Wolbachia* spp. has been found in the Malpighian tubules (organs of excretion) of the tick *Ornithodoros moubata* (the African soft tick) (Noda *et al.*, 1997). Infection of the ticks by classical *Francisella* species is primarily within the gut, but the hemolymph (blood) and various other organs are often also infected. Dr. Edward Francis believed that the gut epithilium was the site of replication. Anal excretions contain large amounts of the bacteria, while they also exist in the salivary glands, and are most likely passed on through the saliva when the ticks bite (Bell, 1981). Mites have been shown to be hosts, as have deer flies, and in one reported case, a brown spider. Transmission by many biting insects, such as mosquitoes and mites, is thought to be mechanical. Mosquitoes were implicated in an epidemic affecting humans in Scandinavia (Bell, 1981).

Waterborne infection has occurred often both in Europe and in North America, and has been attributed to free-living bacteria, the presence of dead infected animals such as muskrats in the water, and to bacteria within amoeba. Despite the fact that the bacteria can often be found in streams in North America, it is not often dessiminated via this route, probably because a large inoculum is required to initiate infection by an oral route (Bell, 1981). One research group has reported that *F. tularensis* lives within the amoebae *Achantamoeba castellanii*, and they postulate this may be one way in which it infects humans who ingest the protozoan. This idea has yet to be confirmed with controlled studies. *F. tularensis* is able to survive and replicate within *Acanthamoebae*, which are common soil and water protozoans and are bactivores. (Berdal *et al*, 1996).

Molecular biology

Genetic manipulations of *Francisella* are limited by a number of factors. *F. tularensis* is difficult to work with as it is a Level III pathogen, and is extremely infectious. An attenuated live vaccine strain (LVS) of *F. tularensis* has been developed, which is not infectious in humans. Consequently, most studies use *F. tularensis* live vaccine strain (LVS) or *F. novicida*. These strains are relatively easy to work with, as they grow quickly and genetic tools have been developed for them. *F. novicida* and the LVS can be chemically transformed or electroporated, provided the plasmids introduced contain *Francisella* DNA (Anthony *et al.*, 1991). The DNA can be interrupted by a transposon carrying a marker, and this DNA can undergo homologous recombination into the chromosome. Since both the LVS and *F. novicida* are sensitive to a number of antibiotics, antibiotic resistance markers can be used. The transposon mini-Tn*10*, which carries a kanamycin resistance cassette, and the Tn*1721* derived Tn*Max*2 (Haas *et al.*, 1993), which carries an erythromycin resistance cassette, have both been used successfully for random transposon mutagenesis in *F. novicida* (Mlduli *et al.*, 1994; Baron and Nano, 1998; Cowley *et al.*, 2000).

One recently overcome limitation for genetic study has been the lack of an autonomously replicating shuttle vector for both *E. coli* and *F. novicida*. A number of plasmids have been isolated from *Francisella* strains, and one of these, pFNL10 from a *F. novicida*-like strain, has been used to create a shuttle vector for *E. coli* using the origin of replication from plasmid pACYC184 (Pavlov *et al.*, 1996; Norqvist *et al.*, 1996). This plasmid had not been characterized thoroughly, however, and there have been no reliable published reports of its efficacy.

A major advance in studies of the pathogenesis of this organism will occur when the genome sequence of the highly virulent *F. tularensis* strain Schu 4 becomes publicly available. Currently the data is in raw form and is available at the *Francisella* genome web site: http//www.medicro.mds.qmw.ac.uk./ft/.

Intracellular survival

The presence of intracellular *F. tularensis* was first observed after inoculation of the chorioallantois (vascular membrane involved in gas exchange) of chick embryos (Buddingh and Womack, 1941). Intracellular growth was first described using hepatic cells and endothelium from guinea pigs , and gut epithelial cells from *Dermacentor andersoni* (Tarnevik, 1989). HeLa cells and mouse fibroblasts were also found to support bacterial replication (Shepard, 1959; Merriott *et al.*, 1961).

Histopathological studies have revealed patterns of dissemination common to many intracellular pathogens. A study in which monkeys were infected via aerosol demonstrated initial infection of the tracheobronchial lymph nodes. Bacteria were also found in epithelial cells of the respiratory bronchioles and in pulmonary macrophages. Within 6 days of initial infection, the infection spread to regional lymph nodes, the spleen, liver and bone marrow, and septicemia followed (Hall *et al.*, 1973). Similar patterns have emerged from studies in other animals such as rats and mice. Infection of mice with the LVS is believed to produce infections reflective of those found in humans infected with the wildtype organisms. Within three days of either an intravenous, intraperitoneal or intranasal route of infection, the bacteria have spread to the reticuloendothelial organs. By the fifth day, the mice have developed pneumonia, and death follows shortly thereafter. If the bacteria are introduced through the skin, the infection is not lethal and dissemination of the pathogen occurs more slowly (Fortier *et al.*, 1991; Fortier *et al.*, 1994).

While *in vivo*, the bacteria appear to be predominately intracellular, as they have been found primarily in macrophages, as well as some endothelial cells. After intraperitoneal infection, they are found almost exclusively in peritoneal macrophages (Fortier *et al.*, 1994), and are not found within polymorphonuclear lymphocytes (PMNs). *F. tularensis* have been found to replicate in the Kupffer cells and in hepatocytes of infected mice (Conlan and North, 1992). Multiple studies performed in cell culture have demonstrated bacteria growing inside cells. These cells include alveolar macrophages from rabbits and mice (Nutter and Myrvik, 1966), peritoneal macrophages from mice, guinea pigs and rats (Anthony *et al.*, 1991a; Fortier *et al.*, 1992), and human monocytes (Fortier *et al*, 1994). The greater the virulence of the strain, the higher the rate of intracellular growth.

As mentioned earlier, *Francisella* resides inside a vacuole while inside cells, however, unlike other pathogens such as *Salmonella*, almost nothing is known of its intracellular lifestyle (Anthony *et al.*, 1991a; Fortier *et al.*, 1994; Fortier *et al.*, 1995). The data available are largely electron microscopic and immunofluorescence studies. Uptake of the bacteria does not seem to require microfilament mobilization (based on studies with the microfilament inhibitor cytochalasin B), suggesting that they do not enter the cell by classic phagocytosis (Fortier *et al.*, 1994). This behavior is reminiscent of *Chlamydia* and *Rickettsia* uptake, but other similarities have not been studied. As bacterial growth progresses, they are released to the cytoplasm and the host cells round up.

The vacuole does not fuse with secondary lysosomes, although acidification is essential for bacterial growth (Anthony *et al.*, 1991a; Fortier *et al.*, 1995). This is thought to be necessary for obtaining iron by facilitating its release from an iron binding protein such as transferrin (Fortier *et al.*, 1995). If the macrophages are prevented from acidifying, *Francisella* cannot grow without exogenous iron. When murine macrophages are treated with interferon-γ, nitric oxide is produced and the bacteria stop growing. Bacterial growth can be revived by the addition of iron pyrophosphate, which suggests that NO combines with iron and makes it unavailable to the bacteria (Anthony *et al.*, 1992: Fortier *et al.*, 1992).

Francisella virulence factors

Although it is clear that *Francisella* can multiply within host cells, there is little information regarding how it is able to do this. A number of factors have been identified to be of importance, but their exact roles have yet to be elucidated. They range from classically accepted virulence factors (bacterial capsules and LPS), to unknown and unique proteins, such as the 23 kDa protein described in this thesis (Golovliov *et al.*, 1997).

Bacterial capsule

F. tularensis is surrounded by a carbohydrate rich capsule, which protects the bacteria from complement, and which may help prevent phagocytosis. This capsule can be removed with salt solution and bacteria lacking the capsule are avirulent (Hood, 1977). A capsule deficient mutant was shown to survive in PMNs, despite the fact that the wildtype strain cannot. Apparently this mutant cannot induce an antimicrobial response in these cells (Sandstrom *et al.*, 1988). *F. novicida* does not appear to have a capsule (Nano, 1992). No attempt to identify capsule biosynthetic genes has been made, most likely due to the lack of genetic systems for the investigation of *F. tularensis.*

LPS

LPS phase variation occurs in *F. tularensis* and has been found to affect intracellular growth, lipopolysaccharide antigenicity and nitric oxide production in macrophages (Cowley *et al.*, 1996). Eigelsbach first described the segregation of avirulent colony opacity variants at a low frequency per generation. The virulent variant was called blue, and the avirulent variant was called gray (Eigelsbach *et al.*, 1951). The gray variants express a different LPS O-antigen, which is the type found in *F. novicida.* All strains expressing this LPS O-antigen are unable to replicate within rat macrophages, but can still infect mouse macrophages (Cowley *et al.*, 1996). The LPS and lipid A of *F. tularensis* are unable to stimulate nitric oxide production in rat macrophages. These molecules in *F. novicida* and the gray variant induce NO production, inhibiting bacterial growth. If rats are coinfected with *F. tularensis* LVS blue variant and a 10-fold excess of *F. novicida*, suppression of bacterial growth occurs (Cowley *et al.*, 1997). A study to identify *F. novicida* mutants deficient in LPS biosynthesis was undertaken with the aim of identifying the genes and mechanisms involved in phase variation. This study identified three mutants with distinct LPS phenotypes. The deduced amino acid sequences from the interrupted genes of two of these mutants were similar to known LPS biosynthetic proteins (Cowley *et al.*, 2000).

23 kDa protein (Fip)

A 23 kDa protein has been identified which is upregulated when the LVS strain is growing inside macrophages (Golovliov *et al.*, 1997). The bacteria were grown in macrophages for 24 hours, and were pulse-labeled for 2 hours before analysis of protein synthesis. They found 24 major proteins were synthesized during this time, but only four were prominently induced. The 23 kDa protein was the most strongly induced inside macrophages, but it was also induced when the LVS was grown extracellularly and exposed to hydrogen peroxide. The protein was localized to the cytoplasmic fraction. The deduced amino acid sequence did not show any similarity to known proteins, and did not have a signal peptide that further supports cytoplasmic localization. The other three proteins were not characterized in this study. Unfortunately, they did not determine if the protein was present in the supernatant of the bacterial cultures exposed to hydrogen peroxide.

MglAB

A possible operon encoding regulatory proteins of virulence genes has been identified in *F. novicida*. An apparent operon of two genes, called *mglAB*, is required for intramacrophage growth and virulence in mice. Both mglA and mglB transposon insertion mutants were found to be almost completely defective for intracellular growth. The deduced amino acid sequences showed similarity to SspA and SspB of *E. coli* and *H. influenzae*. In *E. coli* these proteins are involved in regulating the levels of a number of proteins under nutritional stress conditions. SspA can associate with the RNA polymerase holoenzyme. Inactivation of *mglAB* results in the loss of expression of a number of proteins, most notably a secreted 70 kDa protein (Baron and Nano, 1998).

ValAB

A genetic locus, *valAB*, was found to be associated with sensitivity to complement and detergent in *F. novicida.* Transposon insertion mutants of *valAB* showed a 1000-fold decrease in growth of bacteria in the liver and spleens of mice, but the wildtype phenotype could not be complemented with this locus. These gene products were highly similar, at the deduced amino acid level, to MsbA and LpxK of *E. coli.* MsbA is a member of the superfamily of ATP binding cassette (ABC) transporters (Mdluli *et al.,* 1994). A temperature sensitive *valA* mutant was used to demonstrate that this gene is required for LPS assembly. ValA is thought to be a member of the ABC transporter family, and responsible for transport of lipid A through the inner membrane (McDonald *et al.,* 1997).

MinD

A mutant sensitive to serum and oxidative killing was created using transposon mutagenesis. The locus associated with these phenotypes was found to be an *E. coli minD* homologue. In *E. coli,* MinD is an inner membrane associated ATPase that is involved in the regulation of septum formation during cell division (Anthony *et al.,* 1994). This mutant also showed a 10-fold decrease in intramacrophage growth, as compared to wildtype and a random insertion strain. It was surmised that this decrease in intracellular growth was due to increased sensitivity to macrophage killing mechanisms during the first hour of infection, as the kinetics of growth was very similar to wildtype. Possibly this sensitivity is due to abnormal septum formation, which allows bactericidal agents across the outer membrane.

***Immunology of* Francisella**

In 1961, an attenuated live vaccine strain (LVS) of *F. tularensis* was developed in the U.S from a strain obtained from scientists in the USSR (Eigelsbach and Downs, 1961). Previous to this the phenol-killed Foshay vaccine was in use.

However killed *Francisella* vaccines are not effective in mounting a true long-term protective response. *F. tularensis* LVS was first used to immunize laboratory personnel working with the pathogen at Fort Detrick, Maryland. It protects well against respiratory tularemia, although the ulceroglandular form of the disease is just as prevalent despite exhibiting milder symptoms (Burke, 1977). Recovery from tularemia results in long-term specific protective immunity in both humans and animals (Burke, 1977; Fortier *et al.*, 1991).

The *F. tularensis* LVS provides a good model for immunity as it is not infectious for humans, but is lethal for mice. There is little or no cross-reactive immunity or tolerance, as *Francisella* is not related to the normal intestinal tract flora of the mouse (Elkins *et al.*, 1992). Both *F. tularensis* wildtype and LVS strain infections in mice are largely dependent upon the route of entry (as is also the case in other animals). When the bacteria are introduced intradermally, the LD_{50} is approximately 10^6 bacteria. Conversely, intranasal, intravenous and intraperitoneal routes of infection have an LD_{50} of 1-100 bacteria. This allows researchers to investigate the sublethal immune response in detail, as mice which have recovered from a sublethal intradermal infection are resistant to inoculations from other routes (Fortier *et al.*, 1994). This section provides a brief overview of the immune responses elicited by *Francisella*, and some of the ways in which these responses are overcome by the bacterium.

Innate defenses

Innate defense mechanisms are the first barrier a bacteria must overcome to infect a host. The skin is a natural physical barrier, as well as being an acidic and dry environment inhospitable to many microorganisms. *Francisella* is able to gain entry through tick bites and cuts. Some infections have been initiated merely through physical contact with an inoculum, but it is presumed they gain entry through microscopic breaks in the skin. *Francisella* can also gain entry by inhalation and by ingestion, as discussed earlier. After entering a previously exposed host, the bacterial lipopolysaccharide activates the classical complement cascade. However, *Francisella* is resistant to this defense mechanism (Fulop *et al.*, 1993; Anthony *et al*, 1994; Rhinehart-Jones *et al.*, 1994). It is possible that the bacterial capsule, or the LPS O-antigen provide protection.

Neutrophils are short-lived phagocytes that undergo an oxidative burst which releases several reactive oxygen intermediates (ROI), including hypochlorous acid. Neutrophils have been shown to be important in defense, as mice depleted of neutrophils and eosinophils using granulocyte specific antibodies succumb to sublethal primary infections (Sjostedt *et al.*, 1994; Elkins *et al.*, 1996). This effect is lessened in immune mice. Since neutrophil depletion only has an effect after 2 days, it is thought that neutrophils may secrete cytokines that attract and activate macrophages, rather than kill *Francisella* directly (Sjostedt *et al*, 1994). This idea is supported by evidence that phagocytosis and killing of *Francisella* only occurs efficiently after opsonization, and the bacterial capsule prevents phagocytosis by these cells (Lofgren *et al.*, 1983).

Macrophages

Another defense mechanism is phagocytosis by local and recruited macrophages and monocytes. Macrophages are active as a first line of defense against a pathogen both before and after an inflammatory response, and they can also behave as immune effector cells after the induction of pathogen-specific immunity (Kuby, 1997). It is thought that macrophages are the main line of defense against *Francisella* infection. When activated by IFN-γ, murine alveolar and peritoneal macrophages and human monocytes inhibit intracellular bacterial growth (Polsinelli *et al.*, 1994: Anthony *et al.*, 1992; Fortier *et al.*,1994). Stimulation by TNF-α is also important in murine peritoneal macrophages for the activation of nitric oxide synthesis (Fortier *et al.*, 1992).

Macrophages which have been activated by cytokines released from T and B cells undergo a number of changes which help destroy intracellular parasites. These include the downregulation of transferrin receptors in order to decrease intracellular iron availability, upregulation of effector molecules such as reactive oxygen and nitrogen intermediates and degradation of tryptophan (Fortier *et al.*, 1994).

Nitric oxide is the effector molecule which appears to kill intracellular *Francisella* (Anthony *et al.*, 1992; Fortier *et al.*, 1992). NO which is derived from L-arginine can kill intracellular organisms by reacting with free iron to form nitrosyl-iron complexes. This prevents any iron requiring enzymes in both the macrophage and the bacterium from functioning, and effects respiration, DNA synthesis, and the citric acid cycle. These effects can be reversed by continuous addition of exogenous iron (Fortier *et al.*, 1994).

Humoral immune factors

A strong antibody response is mounted during *Francisella* infection, although, it does not contribute significantly to clearance of virulent strains. The crucial defense against tularemia is a cell-mediated immune response (Arnevik, 1989). This is reflected in the inefficacy of killed vaccines in the generation of specific immunity. Studies in humans vaccinated with LVS showed that antibodies to LVS appeared after two weeks, but that agglutinating serum antibodies did not reach diagnostically significant levels until after 4 weeks, and peak almost 2 months later (Koskela and Herva, 1982).

Humoral factors appear to play a role in mice that are infected with LVS intradermally. Antibody produced can be used to passively transfer limited protection to naïve mice when challenged with LVS. IgM and IgG2a are the two main classes of induced antibody, and they offer some protection to mice when inoculated into naïve animals along with LVS (Rhinehart-Jones *et al.*, 1994). Antibody opsonizes LVS to killing by PMNs, however it doesn't affect ingestion by macrophages or intracellular replication.

Immune serum seems to facilitate clearance of the bacteria from blood, although how it accomplishes this is not clear. Mice that were treated with immune serum, and then inoculated with LVS and killed, had a proportionately higher number of bacteria in the liver and spleen. There was a two-fold reduction in the total number of bacteria, in the liver and spleen, as compared to non-immune serum treated controls (Anthony *et al.*, 1987). The antibody response appeared to be against lipopolysaccharide (LPS), and carbohydrate associated with the outer capsule. *F. tularensis* LVS strain without the capsule is quickly killed by normal human serum, suggesting that the capsule is involved in protection from both normal and homologous immune serum (Sandstrom *et al.*, 1988).

Immunoglobulin M (IgM) production in LVS-challenged mice is detected after 5 days and peaks at the second week. The IgG response (predominately of the IgG2A isotype) can be detected after 10 days. Both IgG and IgM are present for at least four months after initial infection (Rhinehart-Jones *et al.*, 1994). Humans which have been vaccinated with the LVS are found to have specific IgM, IgA and IgG antibodies two weeks after the vaccination, and these antibodies are present over a year later (Koskela *et al.*, 1982). Patients with tularemia have specific IgM, IgG and IgA serum antibodies present within 6-10 days of becoming ill (about 2 weeks after being infected). These antibodies are present in their highest amounts after 1-2 months, and are detectable a decade after the infection (Koskela *et al.*, 1985). Studies of humans which had been vaccinated with a killed virulent *Francisella* strain, and who subsequently contracted tularemia while working with a virulent strain of *Francisella*, demonstrated that they were still vulnerable to infection, despite having high titers of IgG and IgM antibodies (Burke *et al.*, 1977).

B-cells have recently been shown to contribute to the control of initial infections by *Francisella*, however this is probably not dependant upon antibody production. If a mouse is given a sublethal injection with LVS, it becomes resistant to a lethal injection 2-3 days later. Only severe-combined immunodeficient (SCID) mice and B cell knockout mice are not able to survive this treatment (Culkin *et al.*, 1997). B-cell knockout mice are slightly less able to clear a primary intradermal LVS infection, but are 100 fold less protected against a secondary lethal challenge (Elkins *et al.*, 1999). These results suggest that B-cells may contribute to secondary challenges, through unknown mechanisms that do not involve antibody production.

Cellular Immune Factors

T-lymphocytes have been found to be crucial to resolution of *Francisella* infections and to the generation of long-term specific immunity. T-lymphocytes produce cytokines that appear to activate macrophages to control intracellular bacterial growth, but their exact role beyond this remains unknown. It has been suggested that T-cells may destroy infected macrophages in the last stages of the immune response, an event which occurs in other systems, but there is no evidence for this as of yet for *Francisella* infections (Nano and Elkins, submitted; Kaufmann *et al.*, 1988).

Delayed-type hypersensitivity (DTH) response appears approximately one week after initial *F. tularensis* infection (Buchanan *et al.*, 1971). This response precedes the appearance of agglutinating serum antibodies by at least one week and has been used in the development of a skin test for diagnosis. DTH also occurs after vaccination, and has been shown to correlate with cell mediated immunity in humans, rats and mice (Kostiala *et al.*, 1975; Burke, 1977). LVS immunized mice survived challenge with virulent *F. tularensis*, and produced a DTH response upon exposure to homologous antigen (Claflin and Larson, 1972).

Sublethal infection with *F. tularensis* LVS in mice results in proliferation of both CD4+ and CD8+ T cells, both of which help to transfer protection and fight disease progression. If one of these T-lymphocyte subtypes is eliminated it has no effect on the degree of protection, but if both are removed, disease will progress unabated (Fortier, 1994). This dependence upon T-lymphocytes is apparent in studies showing that mice lacking T cells (either nude (*nu/nu*) or α/β T-cell knockout mice) are killed after one month of infection, despite a lack of pronounced symptoms. However, mice lacking only one subtype of T cell can survive an LVS intradermal infection (Elkins *et al.*, 1993: Conlan *et al.*, 1994; Yee *et al.*, 1996). Earlier studies demonstrated that resistance to *F. tularensis* could be passively transferred via lymphocytes from immune to nonimmune syngeneic rats and mice (Kostiala *et al.*, 1975; Anthony *et al.*, 1987). The importance of T-lymphocytes in this process was demonstrated by a decrease in resistance in passively immune mice due to treatment with cyclosporin A (a T-cell suppressive agent) (Anthony *et al.*, 1987).

Although the exact function of T-cells, beyond cytokine production, in clearing *Francisella* infection is not known, it is possible that they are responsible for the final elimination of the bacteria. T cells can kill macrophages infected with bacteria, and one study showed cytotoxic T-cells from peripheral blood lymphocytes of LVS vaccinated humans are specific for monocytes infected with *F. tularensis* (Kaufmann *et al.*, 1988; Surcel *et al.*, 1991).

METHODS AND MATERIALS

Bacterial strains

Bacterial strains used in this study are listed in Table 1. Wildtype Francisella novicida U112 was obtained from the American Type Culture Collection (ATCC). *F. novicida* was grown at 37 °C in tryptic soy broth (Difco) supplemented with 0.1 % cysteine hydrochloride (Sigma), or on cysteine heart agar (Difco) supplemented with 0.5 % defibrinated horse blood (PML Microbiological). Erythromycin (Sigma) was added at a concentration of 25 mg/ml when required. Escherichia coli strains were grown at 37°C in Luria Bertani (LB) broth or on LB agar (Sambrooke *et al.*, 1989), supplemented with 250 mg/ml erythromycin (Em), 250 mg/ml ampicillin (Ap) (Sigma), 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma) and 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Gibco BRL) as required.

Mice care

Female specific pathogen free Balb-C mice were purchased from Charles River, Canada. They were housed in the University of Victoria Animal Care Facility.

Recombinant DNA methods

Standard recombinant DNA techniques were used as per Sambrooke *et al* (1989). All restriction endonucleases used in this study were from New England Biolabs. Calf alkaline phosphatase and T4 DNA ligase were from Gibco BRL Life Technologies Inc. Chromosomal DNA was isolated using either a chemical miniprep extraction or a large-scale cesium chloride gradient preparation, as described by Ausubel *et al.* (1988). Plasmids were isolated using a modified version of the protocol designed by Birnboim and Doly (1979) or using the Qiaprep spin kits from Qiagen. Plasmids which were treated with alkaline phosphatase were isolated from a

Table 1 Bacterial strains and plasmids

Strains or plasmids	Description	Reference or source
F. novicida		
U112	Wildtype	Larson *et al.*, 1955
CG21	U112::Tn*Max2*	This study
CG45	U112::Tn*Max2*	This study
CG57	U112::Tn*Max2*	This study
CG62	U112::Tn*Max2*	This study
CG69	U112::Tn*Max2*	This study
CG93	U112::Tn*Max2*	This study
CG116	U112::Tn*Max2*	This study
CG124	U112::Tn*Max2*	This study
E. coli		
DH5α	*supE*44 D *lac*U169 (Φ80*lac*Z Δ M15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Sambrook *et al.*, 1989
E131	DH5α (λ-CH616)	Haas *et al.*, 1993
XL-1 Blue	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi relA*1 *lac*- F'[*proAB*$^+$ *lacI*^{q} *lacZ*Δ M15Tn10(*tet*R)]	Bullock *et al.*, 1987
Plasmids		
pTZ18U	Ap^R phagemid	Mead *et al.*, 1986
pUC18	Ap^R	Vieira and Messing, 1982

p*TnMax*2	Em^R, *TnMax*2	Haas *et al.*, 1993
pCG1	pTZ19u with the *BamH* I Tn*Max2* 1.5 kb fragment	This study
pCG21	Self-ligated CG21 *Nde* I Em^R fragment	This study
pCG21-1	pUC18 with *Bam*H I pCG21 insert, Ap^R	This study
pCG45	Self-ligated CG45 *Nde*I Em^R fragment	This study
pCG45-1	pTZ18u with *Bam*H I pCG45 insert, Ap^R	This study
pCG45-2	pTZ18u with *BamH* I pCG45 insert, Ap^R	This study
pCG57	pUC18 with *Bcl* I Em^R insert	This study
pCG62	pUC18 with *Bcl* I Em^R insert	This study
pCG69	pUC18 with *Bcl* I Em^R insert	This study
pCG93	pUC18 with *Bcl* I Em^R insert	This study
pCG116	pUC18 with *Bcl* I Em^R insert	This study
pCG124	Self-ligated CG124 *Nde* I Em^R fragment	This study
pCG124-1	pTZ19u with *Eco*R I pCG124 insert, Ap^R	This study
pCG124-2	PTZ19u with *Eco*R I pCG124 insert, Ap^R	This study
pFEN70-7	pTZ19u with 6.3 kb *Sac* I insert	Dr. Francis Nano

cesium chloride gradient using ultracentrifugation (Ausebel *et al.*, 1988). DNA separation was accomplished by electrophoresis through agarose gels (0.8%) using either TBE or TAE as the buffering solution. Electrocompetent cells were prepared as described by Sambrooke *et al.* and were stored at –76 °C. DNA sequences were analyzed using the BlastX family of programs available from the National Center for Biotechnology Information (***www.ncbi.nlm.nih.gov)***. The DNA sequences were used to search raw data downloaded from the Francisella genome project (***www.medmicro,mds.qmw.ac.uk/ft/***) with a local FASTA program available at http://fasta.bioch.virginia.edu/ (Pearson and Lipman, 1988). DNA and deduced amino acid sequence information was manipulated and analyzed using the Lasergene packages of programs (DNASTAR). Multiple sequence alignments were performed using the ClustalX program available from the Baylor College of Medicine Human Genome Center (http://dot.imgen.bcm.tnc.edu). Plasmids used in this study are listed in Table 1.

F. novicida library construction and shuttle mutagenesis

(performed with Siobhan Cowley)

A shuttle mutagenesis scheme, depicted in Figure 1, was used to create a library of over 10,000 *F. novicida* mutants, which were screened for deficiencies in intracellular growth. Chromosomal DNA from *F. novicida* U112 was partially digested with Tsp509 I (NEB), and was size fractionated on a 0.8 % TBE agarose gel. The DNA fragments 5 to 10 kb in size were purified by electroelution using an IBI apparatus and following manufacturer's instructions. Plasmid pUC18 was purified from a cesium chloride gradient, and was digested with EcoR I. The linearized plasmid was incubated with calf intestinal alkaline phosphatase (NEB) and was purified by agarose gel electrophoresis using the Qiaex II gel extraction kit (Qiagen). The size fractionated DNA was ligated into this vector. The resulting Francisella genomic DNA library was used for shuttle mutagenesis with the transposon TnMax2. Shuttle mutagenesis was performed by the procedure of Haas *et al.* (1993). The clone bank was electroporated into *E. coli* E131 carrying pTn*Max2*. Transformants were selected on LB agar plates containing Ap and Em. The transformants were collected into 120 pools of 100 colonies or more, and grown at 37 °C in selective LB broth. Transposition of the Tn*Max2* erythromycin resistance carrying transposon (Em^R) was induced by growing these cultures for 2 hours at 30 °C in selective broth containing 100 mM IPTG. Plasmids were isolated from E. coli transformants in pools of 100, and 120 pools of transposon-mutagenized plasmid DNA were transformed into *F. novicida* U112 (Anthony *et al.*, 1991; Mdluli *et al.*, 1994). Mutant *F. novicida* strains were created when the TnMax2 was rescued into the chromosome via homologous recombination. Ninety-six Em^R *F. novicida* mutant colonies were picked from each of these 120 transformation pools, and grown on selective CHA-B plates. These colonies formed a library of over 10,000 mutant strains.

Microscopic screening for intracellular growth

(performed with Siobhan Cowley)

The *F. novicida* mutant library was grown in selective TSB-C in microtitre plates and used to infect thioglycollate-elicited C57BL6 murine macrophages. The intracellular growth of each mutant was screened visually, using an inverted microscope (TMS Nikon), for a lack of growth in thioglycollate-elicited C57BL6 murine macrophages as compared to wildtype. Briefly, macrophages were cultured in 96-well microtitre plates in DMEM (Gibco), at a density of $2x10^5$ cells/well, after which nonadherent cells were washed off. These monolayers were infected with the *Francisella* mutants diluted to a multiplicity of infection of approximately 5 bacteria/macrophage. After an one hour incubation at 37 °C, nonadherent cells were washed off. The infected macrophages were incubated at 37 °C with 5% CO_2, and were observed over a period of two days. The macrophages were screened for cell lysis at 0, 24, 42 and 72 hour timepoints. The mutant *F. novicida* strains in microtitre wells containing healthy macrophages (not lysed or rounded up, and containing few bacteria) at 42 hours were chosen for further analysis. Thirty-nine different mutants were screened a second time, and nineteen of these were chosen for quantitative analysis (discussed in the following section). All initial screening was done using inflammatory murine intraperitonial macrophages.

In vitro intracellular growth assay

These intracellular growth assays used the macrophage-like murine cell line J774A.1 (ATCC TIB-67, Ralph and Nakoinz, 1975) or intraperitoneally harvested inflammatory murine macrophages, and have been described previously (Anthony *et al.*, 1991). Both types of cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5 % (v/v) fetal bovine serum and 2 mM L-glutamine in cell culture flasks (Sarstedt). They were incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide. The J774A.1 macrophages were harvested by scraping the monolayer off the cell culture flasks. Inflammatory macrophages were harvested by injecting mice intraperitoneally with 0.5 ml of a 4 % (w/v) Brewer thioglycollate medium (Difco) solution to stimulate macrophage proliferation (performed by University of Victoria Animal Care facility staff). Three days after the injection, the mice were killed by carbon dioxide gassing, and the inflammatory macrophages were harvested by peritoneal lavage with 8 ml of DMEM. After harvesting, the macrophages were washed and an aliquot was counted using a Bright-Line hemacytometer (Cambridge Instruments Inc) under the 40x objective lens of an Axioskop microscope (Zeiss). They were resuspended in an appropriate volume of DMEM and 100 µl was aliquoted into 96-well microtitre plates to a cell density of 2 x 10^5 cells/well. *F. novicida* strains were grown in TSB-C (supplemented with Em as required), and then diluted in DMEM. Macrophage monolayers were infected with 150 µl of this solution to give a multiplicity of infection of approximately 5-10 bacteria/macrophage. The plates were centrifuged in a Model TJ-6R centrifuge (Beckman) at 600 x *g*, and were incubated for 1 hour to allow phagocytosis to occur. The macrophages were washed three times, and incubated with 150 µl of DMEM. The potential growth mutants were analysed quantitatively by lysing the cells with 0.1 % deoxycholate at 0, 24, 48, and 72 hours, and inoculating the released *F. novicida* cells on agar medium. The number of viable bacteria in the macrophage cultures were calculated by counting colony forming units of serial dilutions on CHA-B (Difco) media.

Growth in bacteriological media

Mutants were grown in bacteriological media (TSB-C) to identify those with deficiencies not specifically needed for intracellular growth. Cultures of mutant strains grown overnight in TSB-C supplemented with 15 µg/ml Em were used to inoculate 10 ml cultures. Cell density was measured using a Klett-Summerson photoelectric colorimeter with a green filter, over an 8 hour period, at the indicated intervals.

SDS-PAGE and Western blotting of lipopolysaccharide (LPS)

Mutants were analyzed by Western blot for reactivity with anti-*F. novicida* LPS mAb. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli with a 7.5 % stacking gel and a 12 % separating gel (Laemmli, 1970). The gels were run on a Protean 2 mini-gel system (Biorad) at 200 V for 45 minutes. The *F. novicida* LPS was transferred to an Immobilon-P membrane (Millipore) by electrophoresis. The electrophoretic transfer was done with a semi-dry immunoblotting apparatus (LKB) using a Tris/glycine/methanol buffer as per the manufacturer's instructions. The blots were blocked with 10 % skim milk powder (Difco) in PBS with 0.05 % Tween overnight. The primary mouse monoclonal antibody was against *F. novicida* LPS (Cowley *et al.*, 1996), and the secondary antibody was horse-radish peroxidase (HRP)-conjugated sheep anti-mouse IgG mAb (Amersham-Pharmacia). The signal was generated using the ECL chemiluminescent system (Amersham Pharmacia).

Deoxycholate and hydrogen peroxide sensitivity assays

Francisella strains were exposed to varying concentrations of deoxycholate and hydrogen peroxide to determine their susceptibility, as described previously (Mdluli *et al*, 1994; McDonald *et al*, 1998). Bacteria were grown overnight with low aeration at 37 °C in TSB-C. The cells were washed three times with PBS, before resuspending in 1 ml of PBS. They were diluted approximately 5000 fold, and 100 ul was aliquoted into microtitre wells at a concentration of approximately 10^6 CFU/ml. These cells were incubated for 1 or 3 hours, with the indicated concentrations of deoxycholate or hydrogen peroxide, respectively. The cells were serially diluted and plated onto CHA-B plates. The results of this experiment are expressed as the log10 percentage survival CFU/ml based on triplicate wells.

Temperature sensitivity assay

Francisella strains carrying the transposon were tested for temperature sensitivity by growth at 42 °C overnight, on CHA-B plates supplemented with Em.

Southern blotting

DNA digestions were separated on 0.8 % agarose gels. The DNA was transferred to a Hybond N+ nylon membrane (Amersham-Pharmacia) using a Model 785 Vacuum Blotter (BioRad), after *in situ* denaturation. The DNA was heat fixed to the membrane using a Model 583 BioRad gel dryer at 80 °C for two hours. Alternatively it was UV fixed to the membrane with the UV Stratalinker set at 1200 μJoules x 100 (Stratagene). The membranes were hybridized under high stringency conditions with random-prime or 3'oligonucleotide fluorescein-labelled probes, prepared using ECL kits (Amersham-Pharmacia), and as instructed by the manufacturer. Positively hybridizing DNA was identified using ECL chemiluminescent detection.

Recovery of transposon containing plasmids

In order to retrieve the DNA flanking the transposon from mutants CG124, CG45 and CG21, *Nde* I was used to digest chromosomal DNA. The digests were diluted to a concentration of approximately 25 ng/μl, and self-ligated as shown in Figure 2. These self-ligated plasmids, carrying the ori^{fd} origin of replication within the transposon, were electroporated into *E. coli* E131. Transformants were plated on selective media, to identify those carrying the plasmids. As this approach was not effective with the remaining five mutants, possibly because of problems with the origin of replication or plasmid instability, an alternative approach was used. DNA libraries of the mutant DNA from CG57, CG62, CG69, CG93, and CG116 were created. Briefly, genomic DNA was incompletely digested with *Bcl* I, *Bgl* II or *Bam*H I, and ligated into alkaline phosphatase treated pUC18. Ligations were electroporated into *E. coli* XL1 Blue, and selected on LB media supplemented with ampicillin, X-gal and IPTG. Transformants were generally 80 % white, and selected white colonies were screened for insert size by running plasmids on a 0.8 % agarose gel. The *Bcl* I and *Bgl* II libraries (selected because *Bcl* I cuts once within the transposon), were replica plated onto LB media supplemented with 250 μg/mL erythromycin, and were grown at 37 °C for a minimum of 24 hours. Colonies over 1 mm in diameter were chosen for continued study. The plasmids were purified, and digested with *Bam*H I. Agarose gel analysis showed the presence of a 1.5 kb band, which corresponds with the 1.5 kb *Bam*H I cut Tn*Max2*. Southern blot analysis was used to confirm the presence of the Tn*Max2* transposon within these plasmids.

F. novicida wildtype library construction

A wild type library of *F. novicida* U112 DNA was created in the same manner as described above for the mutant strains. Genomic DNA was incompletely digested with three enzymes, *Bcl* I, *Bgl* II or *Bam*H I to create fragments approximately 7-12 kb in length. These fragments were cloned into alkaline phosphatase treated pUC18, and clones were screened with blue/white screening. Plasmids were purified from 10 of the white colonies, and the insert sizes were found to be approximately 5 kb on average (ranging from 3 to over 12 kb in size). Assuming the inserts are 4 kb on average and the genome is 3.0 Mb (both generous estimates), approximately 3400 clones are required for 99 % representation of every gene in the chromosome. Four thousand colonies were picked onto selective plates, and were transferred for culturing in selective media in 96-well microtitre plates. Aliquots of 10-20 uL of these cultures were pooled into pools of 50 or 100, and were stored at -76 °C until required.

F. novicida wildtype library screening

The wildtype library was plated onto LB plates containing Ap, which were incubated overnight at 37 °C. The plates were placed at 4 °C for at least one hour, and colonies were transferred onto 137 mm diameter Colony/Plaque Screen hybridization membranes (NEN Research Products). The DNA was denatured and neutralized *in situ*, as per the manufacturer's instructions. The DNA was heat fixed to the membrane using a Model 583 BioRad gel dryer at 80 °C for two hours. Alternatively, the blots were rinsed in 2X SSC and DNA was UV fixed to the membrane with the UV Stratalinker (Stratagene) set at 1200 μJoules x 100. Blots were hybridized with random-prime or 3' oligonucleotide fluorescein-labelled probes using ECL kits, and were processed following manufacturer's instructions (Amersham Pharmacia). Positively hybridizing colonies were selected for further analysis. Plasmids were isolated from these transformants, and were used for dot blot analysis. Between 2-5 μl of the crude plasmid preparations were dotted onto Hybond-N+ nucleic acid hybridization membranes, and processed as described above with 3'oligonucleotide probes.

DNA sequencing

Automated DNA sequencing was performed using the ABI PRISM Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase as per the manufacturer's instructions (Perkin Elmer). Template concentration used was dependant upon insert size and ranged from 200-500 ng/μl. Sequencing was accomplished using an ABI 373 sequencer (Perkin Elmer), and both universal and custom designed primers were used.

Ammonium sulfate fractionation of *F. novicida* culture supernatants (performed by Audrey Chong)

Overnight cultures of *F. novicida* mutants and wildtype strains were centrifuged at 12,000 x *g* for 30 minutes at 4 °C. Ammonium sulfate was dissolved slowly in the supernatant to 55% of saturation. The mixture was centrifuged at 12, 000 x *g* after stirring for one hour, and the pellet was saved as the 0-55% fraction. The supernatant was removed again, and ammonium sulfate was slowly added to 65% of saturation. This solution was stirred and centrifuged as before. The pellet was saved as the 55-65% fraction, which is enriched for the 70 kDa protein. Both pellets were resuspended in 20 mM Tris-HCl (pH 7.5) and dialyzed against 10 mM Tris-HCl (pH 7.5). These samples were concentrated by lyophilization.

SDS-PAGE and Western blotting (performed by Audrey Chong)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Laemmli (1970) using a Biorad Protean 2 minigel apparatus. Separating gels were 12 % and stacking gels were 4 %. Proteins were visualized by staining with Coomassie brilliant blue R250 followed by destaining in 40 % v/v methanol/10 % v/v acetic acid. Immunoblotting was performed by electrophoretically transferring the proteins to an Immobilon-P membrane using a semi-dry transfer apparatus (Multiphor II Novablot, Pharmacia LKB) as per the manufacturer's instructions. The membrane was blocked overnight with 5 % skim milk powder (Difco) in Tris-buffered saline solution (50 mM Tris-HCl, 137 mM NaCl, pH 7.5) containing 0.1 % Tween-2 (TBS-T). The blot was incubated in the same solution in the presence of antiserum to the 70 kDa protein. The proteins were detected using chemiluminescence. A horseradish peroxidase-conjugated sheep anti-rabbit secondary antibody (Cedarlane Laboratories Ltd) diluted in TBS-T was added to the bound primary antibody and was reacted with Renaissance chemiluminescence detection reagent (NEN Life Science Products).

Chapter 1: The identification and analysis of eight *F. novicida* intracellular growth mutants created using shuttle mutagenesis.

Introduction

Francisella is a facultative intracellular pathogen, which grows predominately in macrophages when living in mammalian hosts. Many studies have addressed the host immune response and the development of protective immunity, but little work has been done to identify the factors that allow *Francisella* to survive inside host cells. *In vitro*, they can grow in rodent resident peritoneal or bone marrow derived macrophages, as well as macrophage-like cell lines such as J774A.1 (Anthony *et al.*, 1991a). They also appear able to replicate in nonprofessional phagocytic cells such as fibroblasts and hepatocytes (Conlan and North, 1992). Microscopy studies suggest that they live inside an acidified phagosome which remains unfused with lysosomes (Anthony *et al.*, 1991b).

Genetic techniques have been developed for *F. novicida* that have aided in the study of virulence factors (Anthony *et al.*, 1991; Baron and Nano, 1998; Baron and Nano, 1999). Previously, three *F. novicida* mutant strains which showed decreased virulence in mice were identified in this laboratory: KEM7 (a *min*D mutant), KM14 (containing a presumed mutation in *val*AB) and GB2 (*mgl*AB mutant) (Anthony *et al.*, 1994; Mdluli *et al.*, 1994 and Baron and Nano, 1998).

F. novicida is closely related to *F. tularensis*, and has been shown to be almost identical on the basis of 16s rRNA sequencing, as well as DNA hybridization studies (Hollis *et al.*, 1989; Forsman *et al.*, 1994). It is an ideal model for studying *F. tularensis* pathogenesis as it is highly virulent in mice, but does not easily infect humans. It is more easily manipulated genetically, and has less fastidious growth requirements, unlike the *F. tularensis* LVS.

Recently, a 23 kDa protein has been identified in *F. tularensis* LVS, which is expressed while the bacteria are inside macrophages, but not when the bacteria are grown in broth culture. This protein is also induced in the presence of hydrogen peroxide. An inverse genetics approach identified the gene for this protein on an *Xba* I digested 1.3 kb DNA fragment. Sequence analysis showed no significant similarity to known proteins. Expression studies demonstrated the gene product was 23 kDa, and possesses the same immunoreactivity as the original 23 kDa protein (Golovliov, *et al*, 1997).

Studies of this nature have shown that a number of proteins are induced when a bacteria enters a host cell. These proteins are often stress response proteins (such as heat-shock proteins) which act as molecular chaperones to stabilize protein conformation (Morgan *et al.*, 1986; Young and Garbe, 1991). Other proteins, such as catalase (which converts H_2O_2 into O_2 and H_2O) and peroxidase (which reduces alkyl hydroperoxides), may be produced to protect bacteria against hydrogen peroxide produced during the respiratory burst (Manca *et al.*, 1999).

The respiratory burst is an important antimicrobial defense elicited after pathogens have been phagocytosed by host cells. Superoxide anion is produced in high amounts by the enzyme NADPH oxidase, and it can subsequently dismutate into hydrogen peroxide (Chanock *et al.*, 1994). Other compounds are produced from these molecules, such as singlet oxygen, hydroxyl radical, and hypochlorous acid. This respiratory burst coincides with a large consumption of oxygen. In both plants and animals, it has been associated with the initiation of programmed cell death (apoptosis). Hydrogen peroxide has been implicated as one molecule that signals cells to undergo apoptosis. Many viruses and bacteria have been shown to induce or repress apoptosis during infection (Weinrauch and Zychlinsky, 1999).

In this study, a shuttle mutagenesis scheme, depicted in Figure 1, was used to create a library of over 10,000 *F. novicida* mutants which could be screened for a lack of growth in murine peritoneal macrophages. Eight macrophage growth mutants were identified from over 10,000 mutants, and the genetic loci were characterized. In five of these mutant strains the transposon integrated into genes which show similarity to known genes. One of these genes is almost identical to the 23 kDa protein of *F. tularensis* at the deduced amino acid level. This protein has been termed Fip, for *Francisella* intramacrophage protein. This finding further supports the premise that *F. novicida* is a useful tool in the study of *Francisella* pathogenesis. Four of the *F. novicida* mutants had interrupted genetic regions similar to genes for a stress response protein, a purine biosynthesis protein, alanine racemase and a protein of unknown function found in *E. coli* and *V. cholerae*. The other three mutant strains appear to have been interrupted in unknown genetic regions, which may encode novel proteins involved in virulence.

Results

Construction of a *F. novicida* wildtype library and shuttle mutagenesis

The *F. novicida* U112 DNA library we made was screened for insert size by transforming into *E. coli* XL1 Blue and purifying plasmid DNA from selected colonies. After separation by agarose gel electrophoresis it was found that the majority of the inserts were over 5 kb in size, and thus fell within the targeted range of 5-10 kb. This library was used for transposon mutagenesis with the Em^R Tn*Max2* (as shown in Figure 1) in 120 pools of 100 colonies. Plasmid DNA was isolated from each of these colony pools, and the DNA was transformed into *F. novicida.*

The resulting erythromycin resistant *F. novicida* colonies were screened for random insertion of the transposon into the genome, and for the presence of pUC18 integration into the genome that would occur in the case of single crossover events. A Southern blot of 10 randomly chosen *F. novicida* transformants probed with Tn*Max2* demonstrated 5 out of 10 insertions were obviously unique. A similar Southern blot probed with pUC18 demonstrated that none of these transformants obtained the transposon via single crossover recombination (data not shown). This was done to insure the mutagenesis scheme could produce mutants with random transposon insertions in the *F. novicida* chromosome.

The *F. novicida* Em^R transformants were screened microscopically for a lack of growth in murine interperitoneal macrophages as compared to wildtype, and 39 mutants were chosen for further screening. These 39 mutants were again screened for intramacrophage growth, and one mutant was chosen from each of 19 pools found to contain intracellular growth mutants. In summary, 19 unique mutants defective for growth in macrophages were produced by shuttle transposon mutagenesis of *F. novicida*, and were chosen for further investigation.

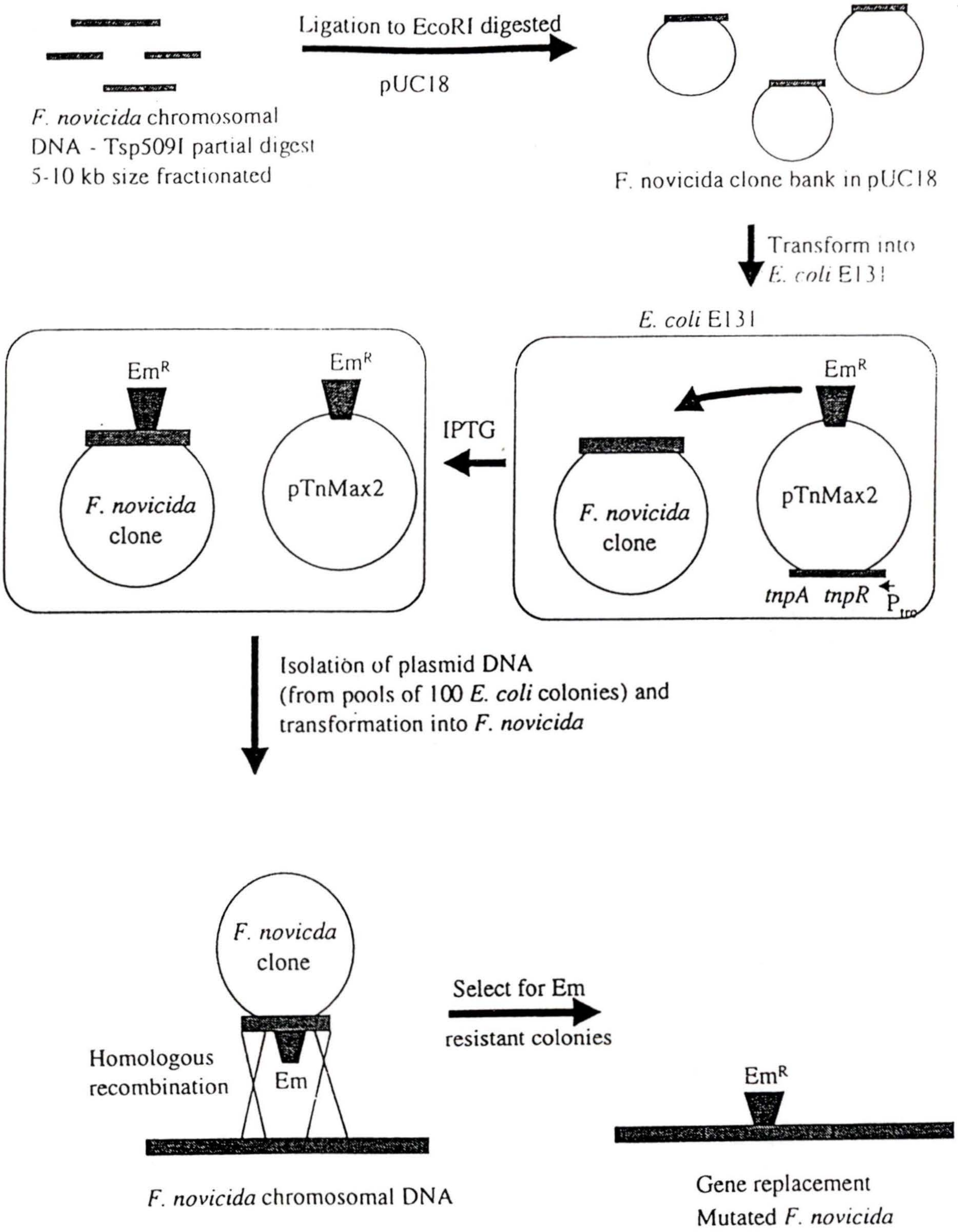


Figure 1. Schematic representation of the procedure using to perform shuttle mutagenesis of F. novicida. Details are found in Methods and Materials section.

Quantitative screening of intracellular growth and growth in bacteriological media

All of the 19 *F. novicida* intracellular growth mutants found during preliminary screening were analysed quantitatively for intracellular growth, as compared to the wildtype strain (only data from eight mutants is shown, as discussed below). All 19 mutants demonstrated decreased survival inside macrophages, although none of the mutants were completely unable to replicate inside host cells.

To help determine if these mutants were deficient in factors specifically required for intracellular growth, they were grown in bacteriological media, and analysed quantitatively. Eight of these 19 mutants did not have general growth deficiencies, as shown in Figure 2. Since the mutants were grown in selective media containing erythromycin, another mutant (CG85) which carries the Em^R cassette was used as a control. Only strains CG57 and CG124 show a slight decrease in their growth in selective TSB-C, as compared to CG85. This demonstrates that the transposon did not interrupt genetic loci required for growth outside of macrophages. These eight mutants were assumed to be deficient for components required for survival within macrophages, and were selected for further analysis.

The intracellular growth of the eight mutants in murine intraperitoneal macrophages, and the macrophage-like murine cell line J774A.1, can be seen in Figures 3 and 4, respectively. CG124 had a 1000-fold decrease in growth inside the murine macrophages, as compared to the wildtype strain U112, as seen in Figure 3. Strains CG21, CG93, CG116, and CG62 had almost a 100-fold decrease in intracellular growth in murine macrophages, while CG69, CG57 and CG45 had approximately a 10-fold decrease in growth. In general, all of the mutant strains grew less aggressively in the J774A.1 macrophage-like cell line than in murine intraperitoneal macrophages, with the exception of strain CG21. All eight *F. novicida* mutants exhibited at least a 10-fold decrease in intracellular growth, which indicated they may contain transposon insertions in genetic regions important for intracellular survival.

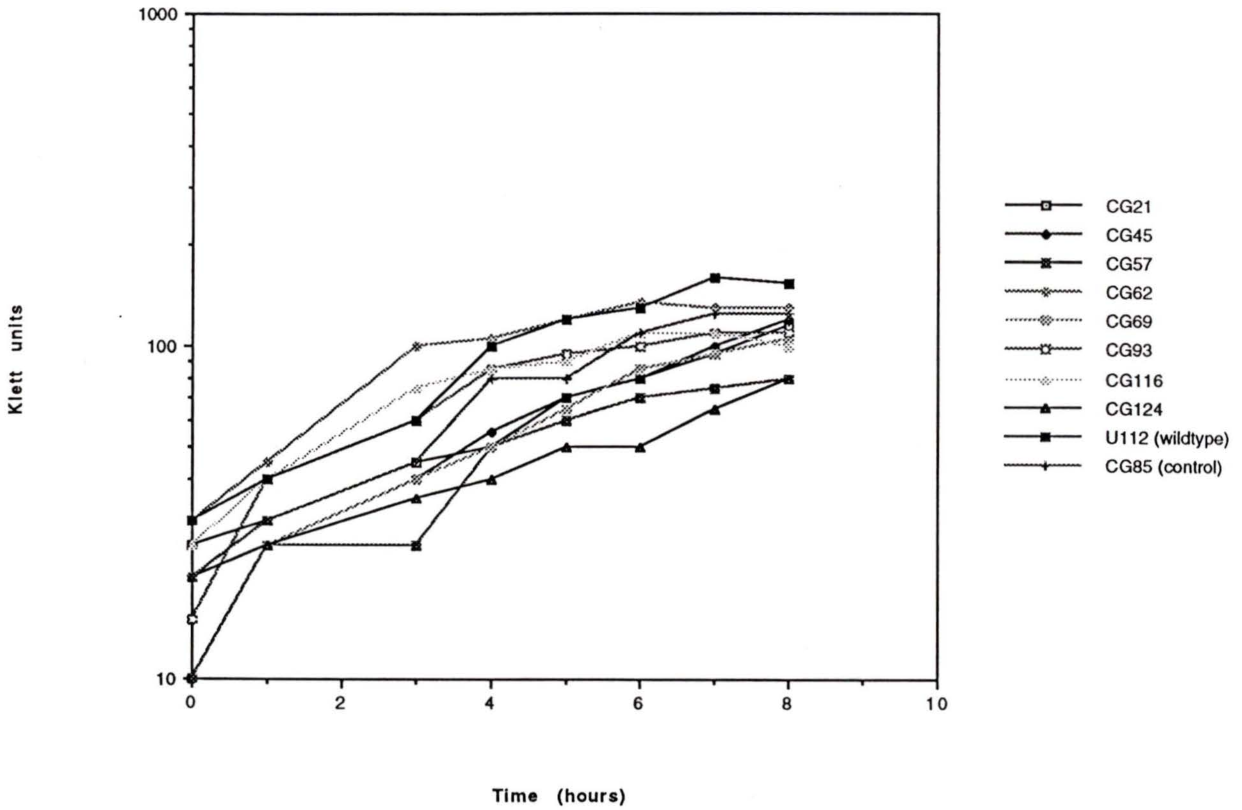


Figure 2. Growth of *F. novicida* mutants in bacteriological media (TSB-C). Mutants exhibited slow grow in macrophages underwent an in vitro growth assay to ensure they did not have general growth impairments. Bacterial growth was monitored every hours using a Klettometer. Only eight chosen mutants are shown. U112 (wildtype), CG21. CG45, CG57, CG62, CG69, CG93, CG116, CG124.

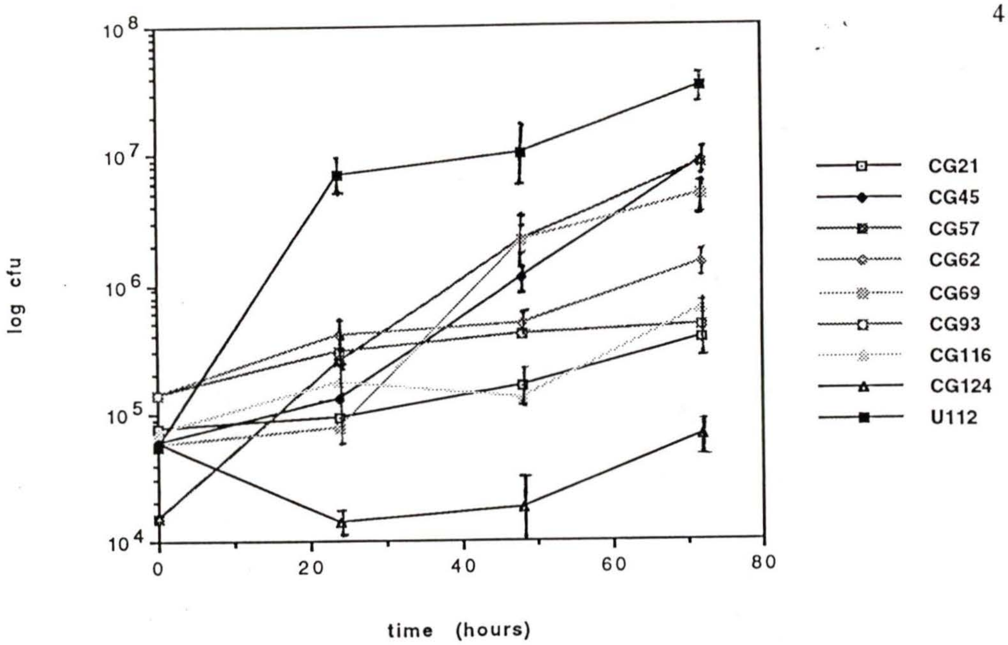


Figure 3. Growth of *F. novicida* Tn*Max*2 insertional mutants in intraperitoneal murine macrophages. Bacteria were enumerated by plating serial dilutions. U112 (wildtype), CG21, CG45, CG57, CG62, CG69, CG93, CG116, CG124.

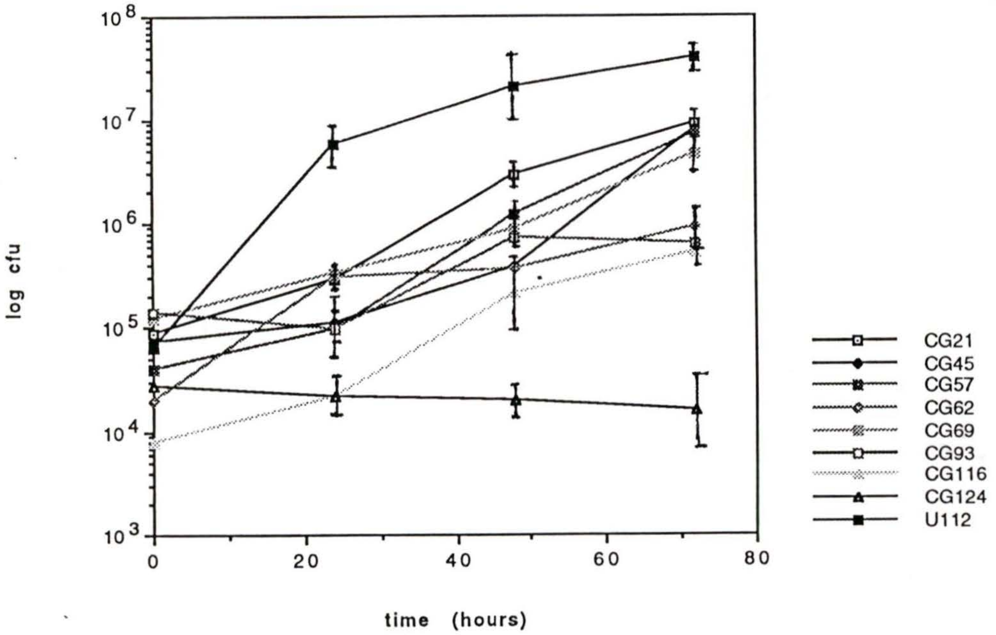


Figure 4. Growth of *F. novicida* Tn*Max*2 insertional mutants in J774A.1 macrophages. Bacteria were enumerated by plating serial dilutions. U112 (wildtype), CG21, CG45, CG57, CG62, CG69, CG93, CG116, CG124.

Phenotypic characterization of eight intracellular growth mutants

Deoxycholate sensitivity assay

Deoxycholate sensitivity assays were undertaken to insure that mutants were not affected by the use of the detergent deoxycholate when lysing macrophages during growth assays. It was possible that mutants could appear to be deficient in intracellular growth, merely due to sensitivity to the lysing agent used. This assay may also indicate that mutants had altered LPS, as Gram negative bacteria often depend upon interactions of LPS molecules to protect from hydrophobic antibiotics and detergents. Figure 5 shows that all of the eight strains have a similar sensitivity profile to wildtype, and none of the mutants were sensitive to deoxycholate at the concentration used to lyse the macrophages. Consequently, sensitivity to detergent is not responsible for deficiencies in intracellular growth.

Immunoblotting with anti-*F. novicida* LPS monoclonal antibody

The eight *F. novicida* strains were analysed by immunoblotting to determine if they had wildtype LPS structure. LPS extractions from the bacterial strains were separated by SDS-PAGE, and compared to *F. novicida* wildtype LPS in their reactivity with anti-*F. novicida* LPS monoclonal antibody. Figure 6 demonstrates that all of the mutant strains showed the characteristic ladder of the wildtype LPS, with the exception of CG124 and CG116. These mutants do show a pattern identical to wildtype, however the bands are very faint. This is most likely because less sample was loaded in these lanes, and thus it was determined that all the mutant strains possess wildtype LPS, and deficiencies in intracellular growth are attributable to other causes. This data supports the finding that none of the mutants were notably sensitive to deoxycholate.

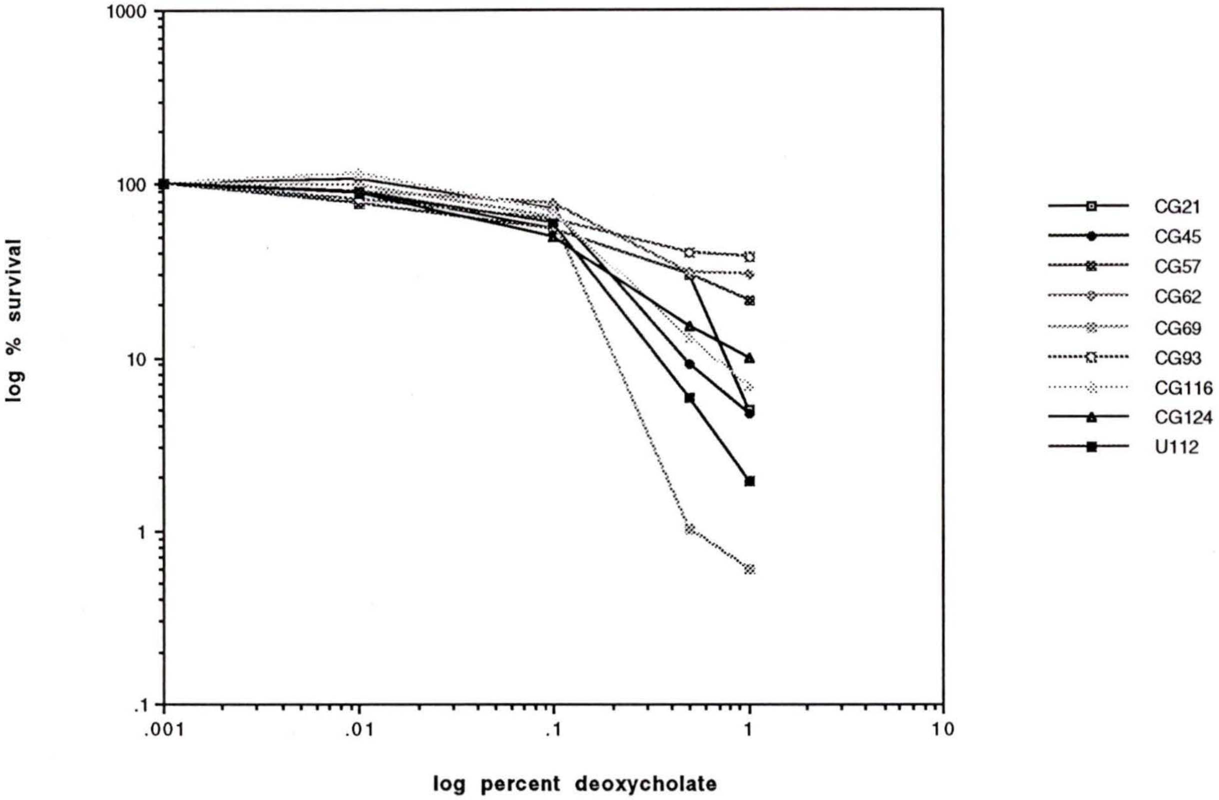


Figure 5. Sensitivity of *F. novicida* mutants to deoxycholate. The number of viable bacteria were quantitated after a 3 hr incubation period at the shown concentrations. Percent survival was found by comparing deoxycholate exposed wells with those exposed to PBS. U112 (wildtype), CG21, CG45, CG57, CG62, CG69, CG93, CG116, CG124.

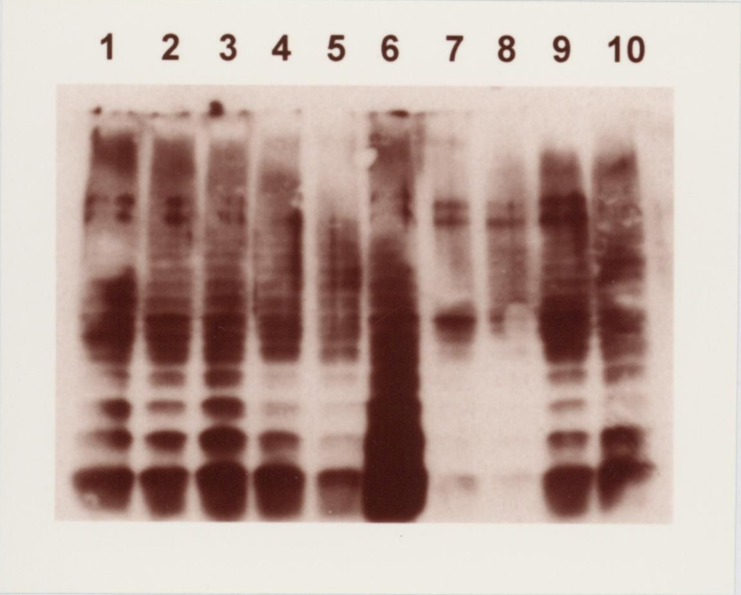


Figure 6. Western blot of whole cell lysates from *F. novicida* intracellular growth mutants. Samples were separated by electrophoresis on a 12 % (w/v) SDS-PAGE gel and transferred to a PVDF membrane. *F. novicida* LPS mAb was used to probe the membrane. Lane 1, CG21; lane 2, CG45; lane 3, CG57; lane 4, CG62; lane 5, CG69; lane 6, CG93; lane 7, CG116, lane 8, CG124; lanes 9 and 10, U112 (wildtype).

Southern blot showing single transposon insertion event in selected mutants

The eight mutant strains were found to contain single transposon insertions using Southern blot analysis. Figure 7 shows hybridization of a Tn*Max2* DNA probe with complete *Nde* I digests of chromosomal DNA from each of the eight mutants. The transposon inserted into *Nde* I fragments of the following approximated sizes: CG21, 10.5 kb; CG45, 5.3 kb; CG57, 7.5 kb; CG62, 14.5 kb; CG69, 14 kb; CG93, 6.5 kb, CG116, 13.0 kb; CG124, 3.8 kb. Three of these fragments containing the transposon are over 12 kb and the sizes reported here are an estimation, based on this blot and others not shown. A Southern blot of *Xba* I digested chromosomal DNA probed with the transposon indicated that the mutants were not siblings, with the possible exception of CG62 and CG116. These two mutants showed similar restriction digestion patterns (data not shown). In summary, Southern blot analysis was used to determine the size of the *Nde* I fragments containing the transposon in the chromosomal DNA of each mutant.

Recovery of Tn*Max2*-containing DNA by self-ligation

In order to retrieve the genetic loci affected by transposon insertion, the origin of replication in Tn*Max2* was exploited, as described by Haas *et al.*, (1993). This scheme is depicted in Figure 8. The restriction endonuclease *Nde* I was chosen, as it is one of the few enzymes which completely digests *F. novicida* DNA and does not cut within the transposon. The completely *Nde* I digested chromosomal DNA was diluted to concentrations of approximately 25 ng/μl, and self-ligated. The ligation mixtures were electroporated into *E. coli* strain E131, and plated onto selective agar. Colonies from the three mutants, CG21, CG124 and CG45, were visible between 24 to 48 hrs later. The plasmids were purified and cut with *Nde* I. Agarose gel electrophoresis revealed that the fragments were the expected size, and corresponded in size to the positively hybridizing *Nde* I fragments found in Figure

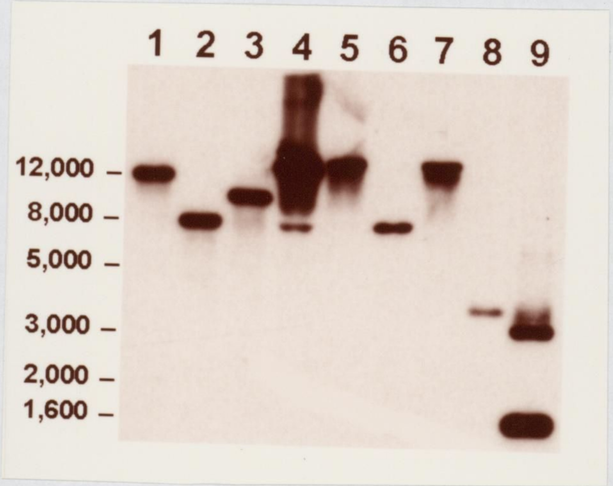


Figure 7. Southern blot analysis of *F. novicida* intracellular growth mutants. Chromosomal DNA was completely digested with *Nde* I, and probed with Tn*Max*2. The sizes were estimated from Gibco 1 kb DNA standards. Lane 1, CG21; lane 2, CG45; lane 3, CG57; lane 4, CG62; lane 5, CG69; lane 6, CG93; lane 7, CG116; lane 8, CG124; lane 9, pCG1 (containing Tn*Max*2 insert).

7. The plasmids were designated pCG21, pCG45 and pCG124, and were found to be 10.5, 5.3 and 3.8 kb, respectively.

Digestion of pCG124 with *Bam*H I produced two fragments of 2.4 and 1.2 kb, while digestion of pCG45 with *Bam*H I produced three fragments which were 2.8, 2.2, and 0.3 kb in length. As the transposon does not excise from these fragments neatly, it suggests that the DNA has undergone rearrangement, or the *Bam*H I and *Eco*R I restriction sites have been lost. Sequencing results revealed possible explanations for this, and are discussed later. *Bam*H I digestion of pCG21 produced the expected 1.5 kb fragment (corresponding with the excision of Tn*Max2*), and a 9 kb fragment. The largest of the fragments were cloned into *Bam*H I digested pUC18 or *Eco*R I digested pTZ18u, to create the plasmids pCG21-1, pCG124-1, pCG124-2, pCG45-1 and pCG45-2 (as described in Table 1). These plasmids were sequenced using universal primers, to identify the DNA flanking the transposon.

Recovery of Tn*Max2*-containing DNA from mutant DNA libraries

The strategy described above was not effective for cloning the transposon-interrupted loci from the five other mutant strains, perhaps due to problems with the origin of replication in the transposon, or plasmid instability. In order to recovery the DNA flanking the site of transposon insertion, DNA libraries were constructed, and transformants were screened for Em^R. Chromosomal DNA was isolated from *F. novicida* mutant strains by cesium chloride gradient ultracentrifugation. Three separate DNA samples were digested for 5 minutes with the enzymes *Bam*H I, *Bcl* I and *Bgl* II. This DNA was ligated into alkaline phosphatase-treated pUC18, and transformants were screened with blue/white screening for the presence of inserts. In general, the libraries contained at least 80 % inserts, and screening revealed that most inserts were 5 kb or larger. These clones were screened for the presence of the transposon by replica plating the libraries onto Em selective media. Positive Em^R colonies grew up after 48 hours. Plasmids were purified from selected Em^R colonies. Digestion of these plasmids with *Bam*H I demonstrated the excision of a

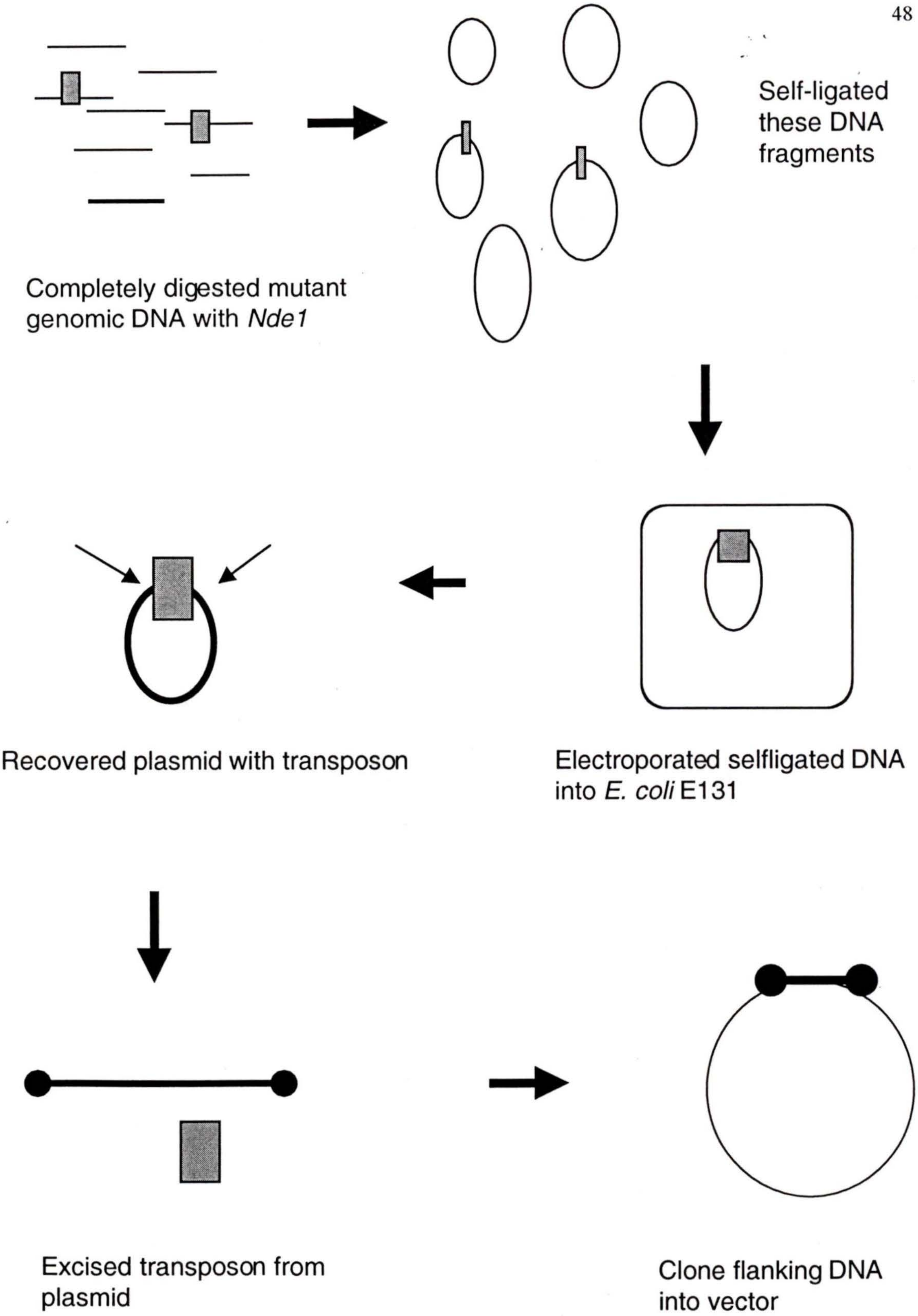


Figure 8. Schematic representation of the self-ligation procedure used to recover *F. novicida* mutant DNA fragments interrupted by the transposon. The transposon was excised from the recovered plasmids and the flanking DNA sequences were cloned.

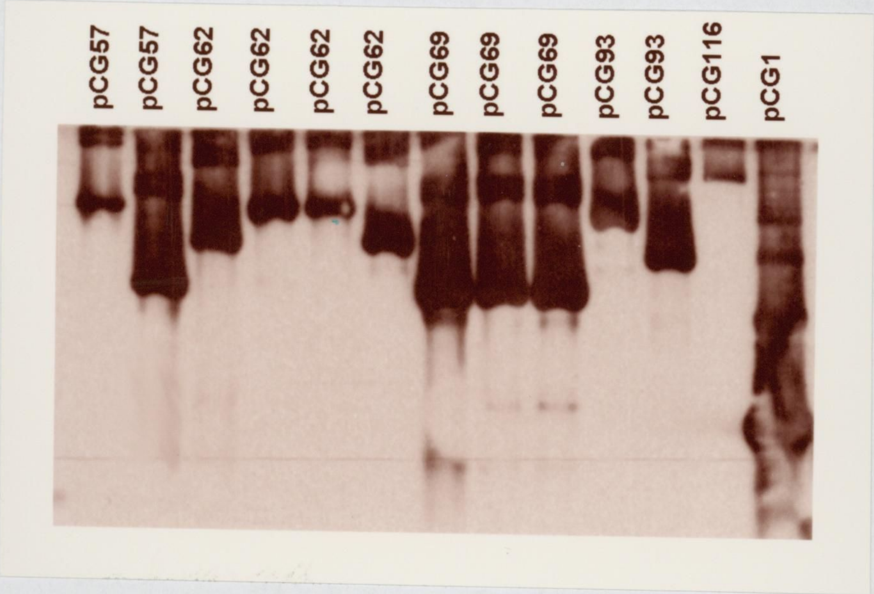


Figure 9. Southern blot analysis of Em^R colonies found during screening of *F. novicida* CG57, CG62, CG69, CG93 and CG116 mutant libraries on selective agar. Plasmids were isolated from randomly chosen colonies and probed with the ECL-labelled transposon Tn*Max2.* All of the chosen clones were found to contain the transposon.

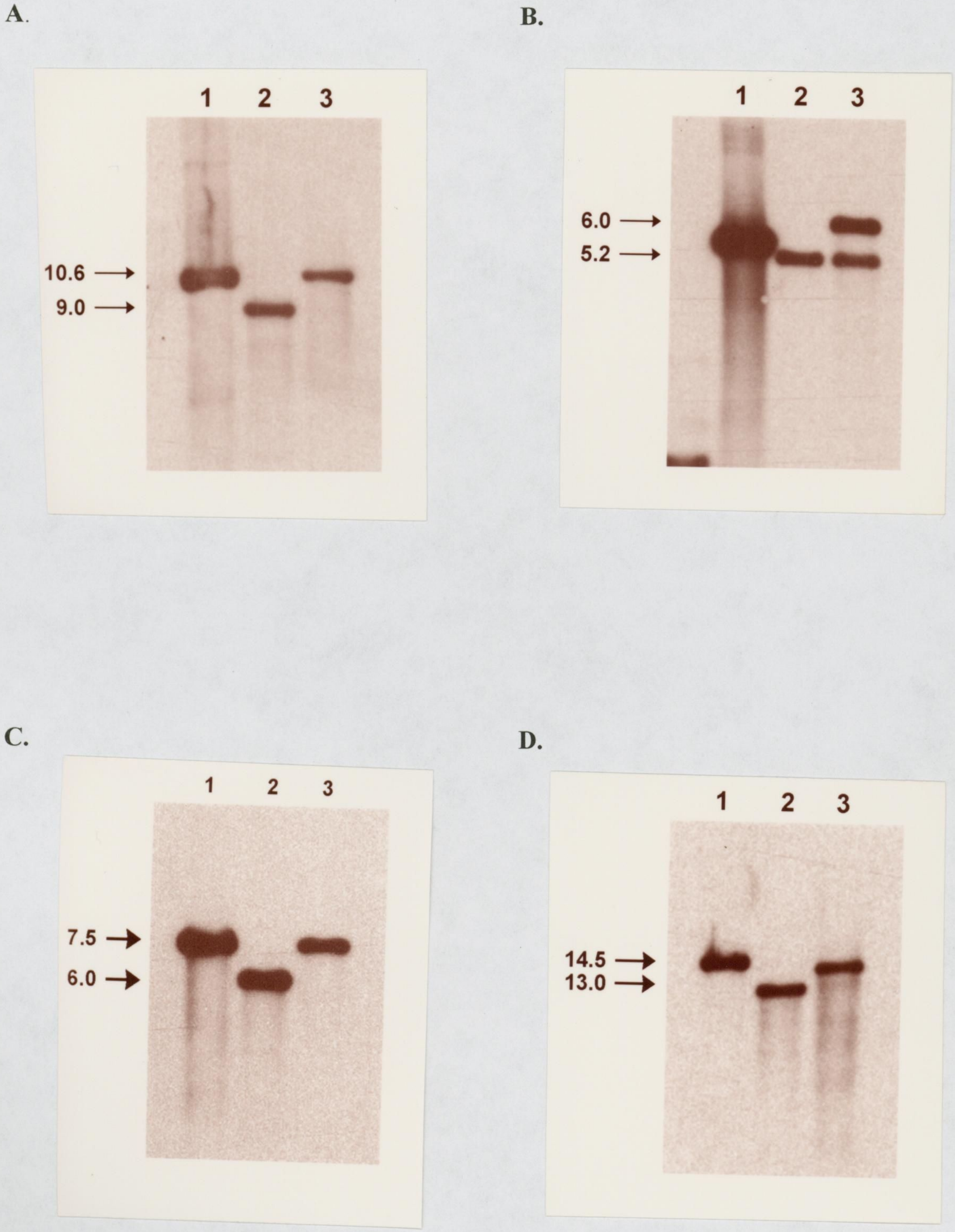
1.5 kb fragment which was assumed to be the transposon. Random colonies were chosen for screening by Southern blot, and probed with the transposon. All of the *F. novicida* mutant clones isolated from DNA libraries contained the transposon, as evidenced by the Southern blot shown in Figure 9.

Southern blot analysis of wildtype and mutant strains showing location of transposon insertion.

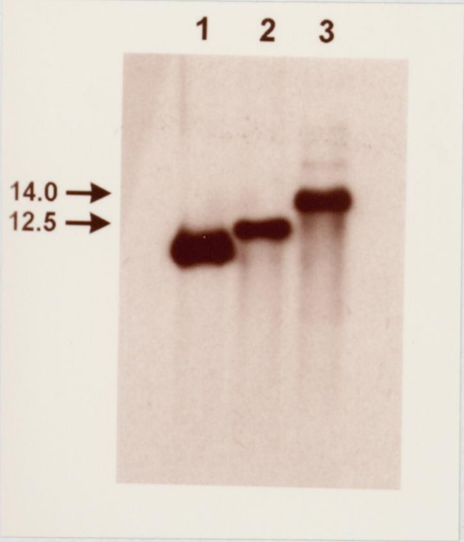
The genetic loci interrupted by the transposon were identified by hybridizing DNA adjacent to the transposon from each of the mutant strains with completely *Nde* I digested wildtype genomic DNA. This identified the DNA fragments that were disrupted by the transposon, and it also demonstrated that only one transposon was inserted into each loci. It was expected that there would be a 1.5 kb increase in the size of the *Nde* I fragments from the mutant strains, due to the integration of the transposon. This was found to be the case for mutant strains CG21, CG57, CG62, CG69, CG116 and CG124 and can be seen by comparing lanes 2 and 3 in the appropriate Southern blots (Figure 10).

It appears as though there was not a simple insertion of the transposon in strain CG45, (this is also supported by restriction endonuclease digestion results). The Southern blot in Figure 10B shows labelled plasmid pCG45 hybridized to a 5.2 kb *Nde* I fragment of wildtype DNA, shown in lane 2 of Figure 10B. However the wildtype DNA fragment was expected to be approximately 4.6 kb in size, as it lacks the 1.5 kb transposon. Additionally, the mutant CG45 completely *Nde* I digested DNA showed an unexpected positive hybridization to a DNA fragment approximately 6.0 kb in size, as well as to the expected 5.2 kb fragment (shown in lane 3 of Figure 10B). Sequencing analysis sheds some light on these results, as reported below.

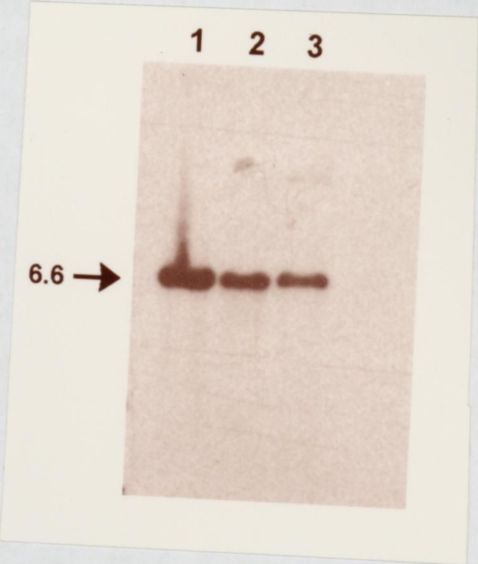
The expected size of the plasmid pCG124 is 3.2 kb, but the actual size was shown to be approximately 3.8 kb (this discrepancy can be seen in Figure 10H). In this case the wildtype fragment was the expected size, as it showed a decrease in


A.
1
2
3
10.6
9.0
B.
1
2
3
6.0
5.2
C.
1
2
3
7.5
6.0
D.
1
2
3
14.5
13.0

E.



F.



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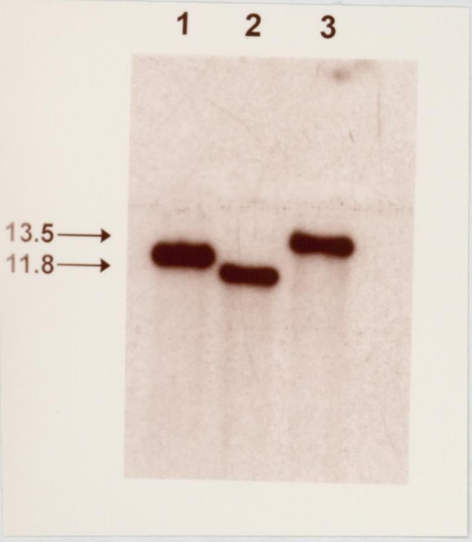


H.

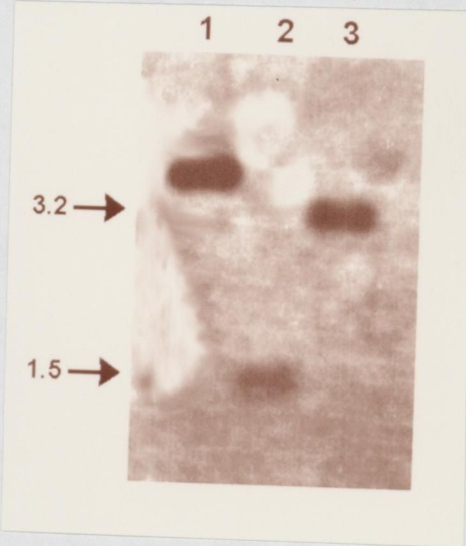


Figure 10.. Southern blot analysis of *F. novicida* intracellular growth mutants showing genetic loci containing the Tn*Max2* transposon and flanking DNA. Chromosomal DNA from the wildtype strain (in lane 2) and the mutant strains (in lane 3) were completely digested with *Nde* I and probed with plasmids containing the transposon. The left lane contains the DNA used to probe the blots, as a positive control. A. Strain CG21, lane 2, *Nde* I cut wildtype DNA; lane 3, *Nde* I cut CG21 DNA; probed with plasmid pCG21. B. Strain CG45; probed with plasmid pCG45 C. Strain CG57. D. Strain CG62. E Strain CG69. F. Strain CG93 G. Strain CG116. H. Strain CG124; probed with pCG124. Blots for CG57, CG62, CG69, CG93 and CG116 were probed with an *Nde* I fragment containing the transposon and flanking sequence from the indicated mutant.

size of 1.5 kb. This suggests that plasmid pCG124 was not due to a simple self-ligation of the mutant 3.2 kb DNA fragment, but also contained some contaminating DNA. Sequencing analysis confirmed this possibility, as reported below.

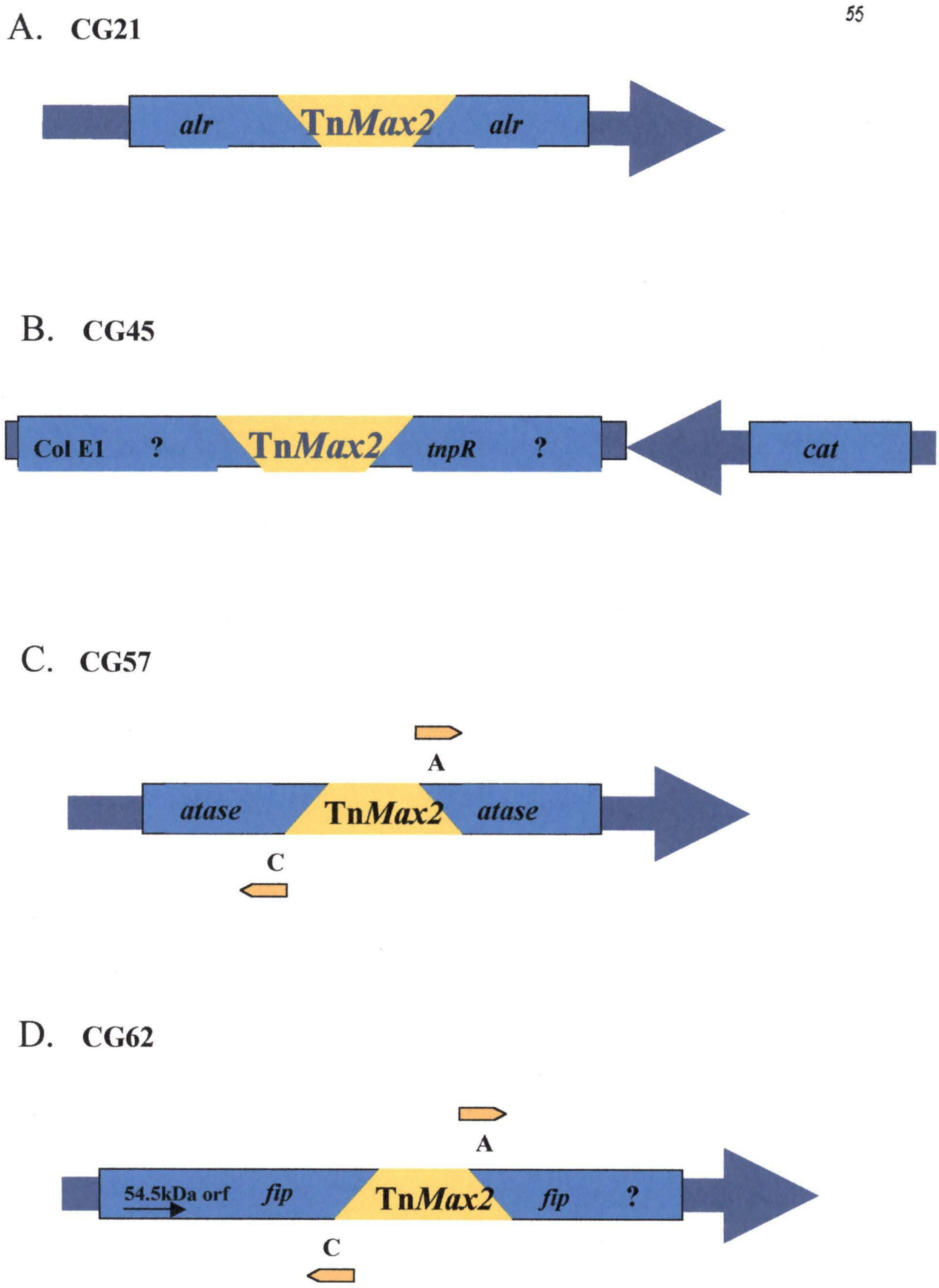
The DNA probe used for *F. novicida* strain CG93 did not appear to contain the loci interrupted by the transposon, as the blot in Figure 10F demonstrates. This result can be explained by the method used to generate the probe, and is discussed later.

In summary, these data showed single transposon insertion into *Nde* I fragments of DNA from mutants CG21, CG57, CG62, CG69, CG116 and CG124. The DNA used as a probe in Figure 10F did not contain DNA flanking the transposon, and therefore single transposon insertion was not shown for mutant CG93. According to the data shown here CG45 DNA appears to have undergone rearrangment upon transposon insertion. In addition, the plasmid pCG124 isolated from CG124 digested DNA is not of the expected size, and may contain contaminating DNA.

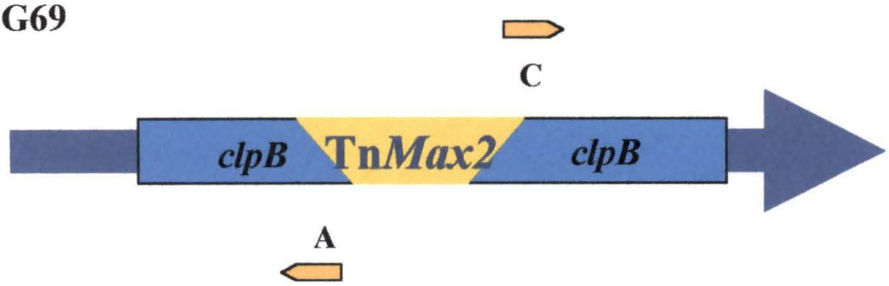
Sequencing of transposon-flanking DNA

The genetic loci interrupted by the transposon were characterized by DNA sequencing, using both universal and custom made primers. Universal primers were used to sequence the DNA flanking the transposon in plasmids pCG21-1, pCG124-1, pCG124-2, pCG45-1 and pCG45-2. Primers designed from sequence data from pCG1 (containing the *Bam*H I fragment of Tn*Max2*) were used to sequence the remaining five mutant plasmids pCG57-1, pCG62-1, pCG69-1, pCG93-1, and pCG116-1. These primers were 5'-CGTAAAGCACTAAATCCGAACCCTAA-3' (designated primer C) and 5'-ATCGCGCATGTCAATCTA-3' (designated primer A). Figure 11 shows an overview of the loci interrupted by the transposon as determined by sequencing data. Four of the mutant strains, CG21, CG57, CG62, and CG69, were interrupted in DNA sequences that showed similarity to known genes, and CG116 was interrupted in a DNA sequence similar to a proposed gene.

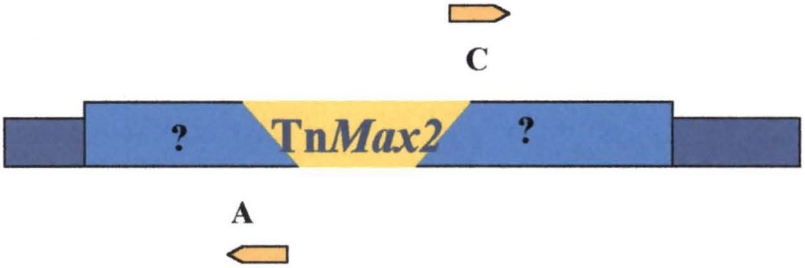
The deduced amino acid sequence corresponding to the genetic locus interrupted by the transposon in strain CG21 showed a 28 % identity to alanine


A. CG21
alr
TnMax2
alr
B. CG45
Col E1
?
TnMax2
tnpR
?
cat
C. CG57
A
atase
TnMax2
atase
C
D. CG62
A
54.5kDa orf
fip
TnMax2
fip
?
C

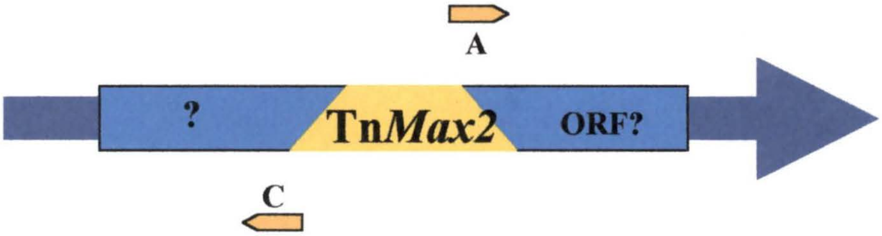
E. CG69



F. CG93



G. CG116



H. CG124

Figure 11. Summary of the DNA regions adjacent to the transposon. A: CG21, B: CG45, C:CG57, D:CG62, E:CG69, F: CG93, G: CG116, H: CG124. The arrow represents assumed ORFs in the same direction while the blocks represent sequences showing similarity to indicated proteins at the deduced amino acid level. Question marks represent *F. novicida* sequences showing no similarities to database entries. A and C are primers used to sequence from the ends of the transposon in CG57, CG62, CG69, CG93 and CG116. Details and abbreviations can be found in the results section.

racemase from *Staphylococcus aureus* over a 363 amino acid region, with an E-value of approximately 10^{-33}. The deduced amino acid alignment is shown in Figure 12.

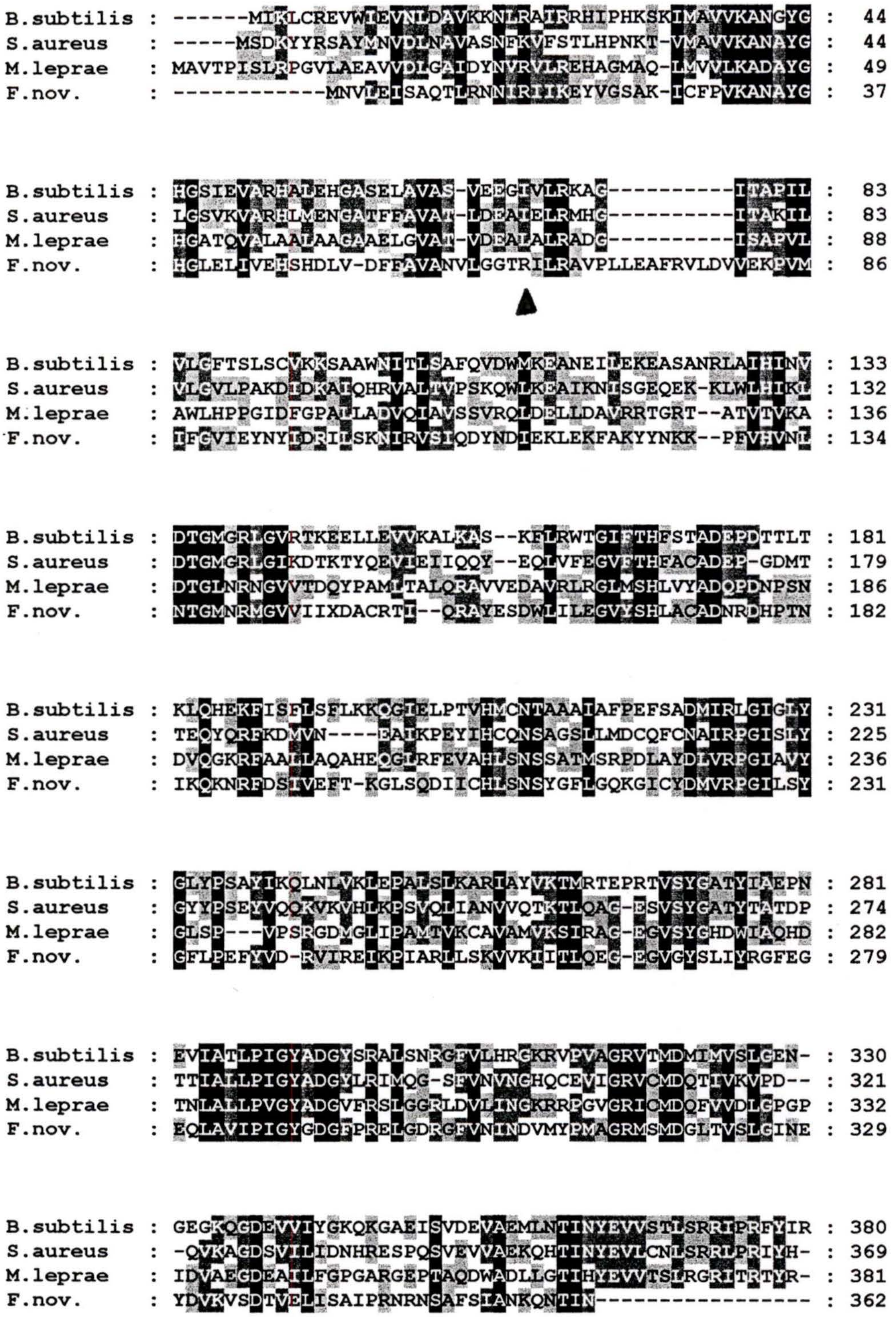
The DNA interrupted in strain CG57 showed a high degree of similarity to amidophosphoribosyltransferases (ATase) from a number of bacteria at the deduced amino acid level. It has over 50 % identity and an E-value of $10^{-100}$with the ATase from *E. coli* over a 358 amino acid sequence (as shown in Figure 13).

Transposon insertion into strain CG62 occurred in a gene that appears to encode a protein identical to a 23 kDa protein from *F. tularensis* (Golovliov *et al.*, 1997). This protein has been termed Fip, for *Francisella* intramacrophage protein, and the alignment is shown in Figure 14. The DNA sequence is shown in Figure 15.

In strain CG69 the transposon inserted into a putative gene encoding a homologous conserved stress response protein called ClpB, which shows higher than 60 % identity over a 415 amino acid region with the equivalent protein in *E. coli*. The E-value over this region was found to be 10^{-150}. The deduced amino acid alignments can be seen in Figure 16.

The transposon was found adjacent to a sequence similar to a gene encoding a hypothetical *E. coli* protein in strain CG116, as the alignment in Figure 17 demonstrates. A 31 % similarity over 112 amino acids with an E-value of 10^{-7} was found at the deduced amino acid level. The function of this protein remains unknown, and no conserved motifs or domains were found using Prosite and Prints analysis. Although it appears as though the transposon inserted just downstream of the beginning of this gene in strain CG116, the corresponding sequence was not found on the other side of the transposon (probably due to the quality of the sequence). Sequence from the opposite side of the transposon showed no similarity to known genes.

The *Francisella* DNA sequences found flanking the transposon in the other three proteins, CG45, CG93 and CG124 are shown in Figures 25, 26, and 27, respectively (in the appendix). The DNA sequence from CG93 was of poor quality, and this impeded analysis. The transposon appears to have integrated into genes which have not yet been characterized in both strains CG45 and CG124.



```
B.subtilis : ------MIKLCREVWIEVNLDAVKKNLRAIRRHIPHKSKIMAVVKANGYG :  44
S.aureus   : -----MSDKYYRSAYMNVDLNAVASNFKVFSTLHPNKT-VMAVVKANAYG :  44
M.leprae   : MAVTPISLRPGVLAEAVVDLGAIDYNVRVLREHAGMAQ-LMVVLKADAYG :  49
F.nov.     : ------------MNVLEISAQTLRNNIRIIKEYVGSAK-ICFPVKANAYG :  37

B.subtilis : HGSIEVARHALEHGASELAVAS-VEEGIVLRKAG----------ITAPIL :  83
S.aureus   : LGSVKVARHLMENGATFFAVAT-LDEAIELRMHG----------ITAKIL :  83
M.leprae   : HGATQVALAALAAGAAELGVAT-VDEALALRADG----------ISAPVL :  88
F.nov.     : HGLELIVEHSHDLV-DFFAVANVLGGTRILRAVPLLEAFRVLDVVEKPVM :  86
                                        ▲

B.subtilis : VLGFTSLSCVKKSAAWNITLSAFQVDWMKEANEILEKEASANRLAIHINV : 133
S.aureus   : VLGVLPAKDIDKAIQHRVALTVPSKQWLKEAIKNISGEQEK-KLWLHIKL : 132
M.leprae   : AWLHPPGIDFGPALLADVQIAVSSVRQLDELLDAVRRTGRT--ATVTVKA : 136
F.nov.     : IFGVIEYNYIDRILSKNIRVSIQDYNDIEKLEKFAKYYNKK--PFVHVNL : 134

B.subtilis : DTGMGRLGVRTKEELLEVVKALKAS--KFLRWTGIFTHFSTADEPDTTLT : 181
S.aureus   : DTGMGRLGIKDTKTYQEVIEIIQQY--EQLVFEGVFTHFACADEP-GDMT : 179
M.leprae   : DTGLNRNGVVTDQYPAMLTALQRAVVEDAVRLRGLMSHLVYADQPDNPSN : 186
F.nov.     : NTGMNRMGVVIIXDACRTI--QRAYESDWLILEGVYSHLACADNRDHPTN : 182

B.subtilis : KLQHEKFISFLSFLKKQGIELPTVHMCNTAAAIAFPEFSADMIRLGIGLY : 231
S.aureus   : TEQYQRFKDMVN----EAIKPEYIHCQNSAGSLLMDCQFCNAIRPGISLY : 225
M.leprae   : DVQGKRFAALLAQAHEQGLRFEVAHLSNSSATMSRPDLAYDLVRPGIAVY : 236
F.nov.     : IKQKNRFDSIVEFT-KGLSQDIICHLSNSYGFLGQKGICYDMVRPGILSY : 231

B.subtilis : GLYPSAYIKQLNLVKLEPALSLKARIAYVKTMRTEPRTVSYGATYIAEPN : 281
S.aureus   : GYYPSEYVQQKVKVHLKPSVQLIANVVQTKTLQAG-ESVSYGATYTATDP : 274
M.leprae   : GLSP---VPSRGDMGLIPAMTVKCAVAMVKSIRAG-EGVSYGHDWIAQHD : 282
F.nov.     : GFLPEFYVD-RVIREIKPIARLLSKVVKIITLQEG-EGVGYSLIYRGFEG : 279

B.subtilis : EVIATLPIGYADGYSRALSNRGFVLHRGKRVPVAGRVTMDMIMVSLGEN- : 330
S.aureus   : TTIALLPIGYADGYLRIMQG-SFVNVNGHQCEVIGRVCMDQTIVKVPD-- : 321
M.leprae   : TNLALLPVGYADGVFRSLGGRLDVLINGKRRPGVGRICMDQFVVDLGPGP : 332
F.nov.     : EQLAVIPIGYGDGFPRELGDRGFVNINDVMYPMAGRMSMDGLTVSLGINE : 329

B.subtilis : GEGKQGDEVVIYGKQKGAEISVDEVAEMLNTINYEVVSTLSRRIPRFYIR : 380
S.aureus   : -QVKAGDSVILIDNHRESPQSVEVVAEKQHTINYEVLCNLSRRLPRIYH- : 369
M.leprae   : IDVAEGDEAILFGPGARGEPTAQDWADLLGTIHYEVVTSLRGRITRTYR- : 381
F.nov.     : YDVKVSDTVELISAIPRNRNSAFSIANKQNTIN----------------- : 362
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```
B.subtilis : DGEIFKVSTPVLYV : 394
S.aureus   : DGDQRFVTNELLK- : 382
M.leprae   : --EAQTVDR----- : 388
F.nov.     : -------------- :   -
```

Figure 12. Deduced amino acid sequence alignment of DNA flanking the transposon in mutant CG21. The deduced amino acid sequence was aligned with alanine racemase from *B. subtilis*, *S.aureus*, and *M. leprae*. Arrow indicates approximate location of transposon insertion.

```
H.influ.    : MCGIVGIVSQSPVNESIYAALTLLQHRGQDAAGIVTVDDENRFRLRKANG :  50
P.multocid  : MCGIVGIVSQSPVNQSIYDALTLLQHRGQDAAGIVTVDDENRFRLRKANG :  50
E.coli      : -CGIVGIAGVMPVNQSIYDALTVLQHRGQDAAGIITIDANNCFRLRKANG :  49
N.menin.    : MCGVLGLVSHEPVNQLLYDGLQMLQHRGQDAAGIATAEG-GTFHMHKGKG :  49
F.nov.      : -------------------------------------------------- :   -


H.influ.    : LVSDVFHQEHMLRLQGNAGLGHVRYPTAGSS-SVSEAQPFYVNSPYGVTL :  99
P.multocid  : LVSDVFEQVHMLRLQGNAGIGHVRYPTAGSS-SVSEAQPFYVNSPYGLTL :  99
E.coli      : LVSDVFEARHMQRLQGNMGIGHVRYPTAGSS-SASEAQPFYVNSPYGITL :  98
N.menin.    : MVREVFRTRNMRDLTGNAGIAHVRYPTAGNAGSSAEAQPFYVSSPFGIVL :  99
F.nov.      : -------------------------------------------------- :   -


H.influ.    : VHNGNLTNSVELKEKVFKTARRHVNTNSDSELLLNILANHLD-----HIP : 144
P.multocid  : VHNGNLTNSSELKEKLFRLARRHVNTNSDSELLLNILANHLD-----HFE : 144
E.coli      : AHNGNLTNAHELRKKLFEEKRRHINTTSDSEILLNIFASELD-----NFR : 143
N.menin.    : AHNGNLTNTAELYENVCNKHLRHVNTSSDSEVLLNVFAHELRREVSKNAD : 149
F.nov.      : -----------------------------PELLLNFFACGMN------KS :  15


H.influ.    : QDHLDPQDIFYAVRKTHKDVRGAYACLAMIIGHGMVAFRDPFGIRPLVLG : 194
P.multocid  : KYQLDPQDVFSAVKQTHQDIRGAYACIAMIIGHGMVAFRDPNGIRPLVLG : 194
E.coli      : HYPLEADNIFAAIAATNRLIRGAYACVAMIIGHGMVAFRDPNGIRPLVLG : 193
N.menin.    : PHRLNADNIFNAVAQVHRLVRGAYGVIAMIAGYGMLAFRDPYGIRPLVLG : 199
F.nov.      : KXILRLLRLFIKHVNLFLHVKGGYACTAMIANFGLIAFRDPYGIRPLVLG :  65


H.influ.    : KR--EENGKTDYMFASETVALDIVGFEFVRDIAAGEAVYVTFDGELYSQQ : 242
P.multocid  : KR--EENGKTEYMFASESIALDTVGFEFVRDVQPGEAIYVTFEGEMYAQQ : 242
E.coli      : KRDIDEN-RTEYMVASESVALDTLGFDFLRDVAPGEAIYITEEGQLFTRQ : 242
N.menin.    : SQT-DSEGRKSYAVASESVAFNALTYDLERDIRPGEAVFVGFDGTMIARQ : 248
F.nov.      : FKEYDDG-EKAYMVASESVALDISGFKVLRDVEPGEVIIITEDRKVHSKI : 114


H.influ.    : CAESAVLNPCIFEYVYFARPDSTIDGVSVYAARVHMGEKLGQKIAKEWAD : 292
P.multocid  : CADKPTLTPCIFEYVYFARPDSCIDGVSVYAARVHMGQRLGEKIAREWAD : 292
E.coli      : CADNPVSNPCLFEYVYFARPDSFIDKISVYSARVNMGTKLGEKIAREWED : 292
N.menin.    : CSDRAKLSPCLFEYVYFARPDSVIDGVSVYQSRLDMGVSLAEKIKRELPV : 298
F.nov.      : CAKNPVLAPCLFEYVYFARPDSIMNGVSVYQARVDAGKILSKRSKEAWKD : 164


H.influ.    : EIDNIDVVIPVPETSTDIALQIARVLGKPYRQGFVKNRYVGRTFIMPGQA : 342
P.multocid  : -VDDIDVVIPVPETSNDIALRIARVLNKPYRQGFVKNRYVGRTFIMPGQA : 341
E.coli      : --LDIDVVIPIPETSCDIALEIARILGKPYRQGFVKNRYVGRTFIMPGQQ : 340
N.menin.    : --DGIDVVMPIPDTSRPSAMELAVHLDKPYREGLIKNRYIGRTFIMPGQA : 346
F.nov.      : --KDIDIVIPVPETGRASAQEIAAALGVEYREGFVKNRYVGRTFIMPENV : 212
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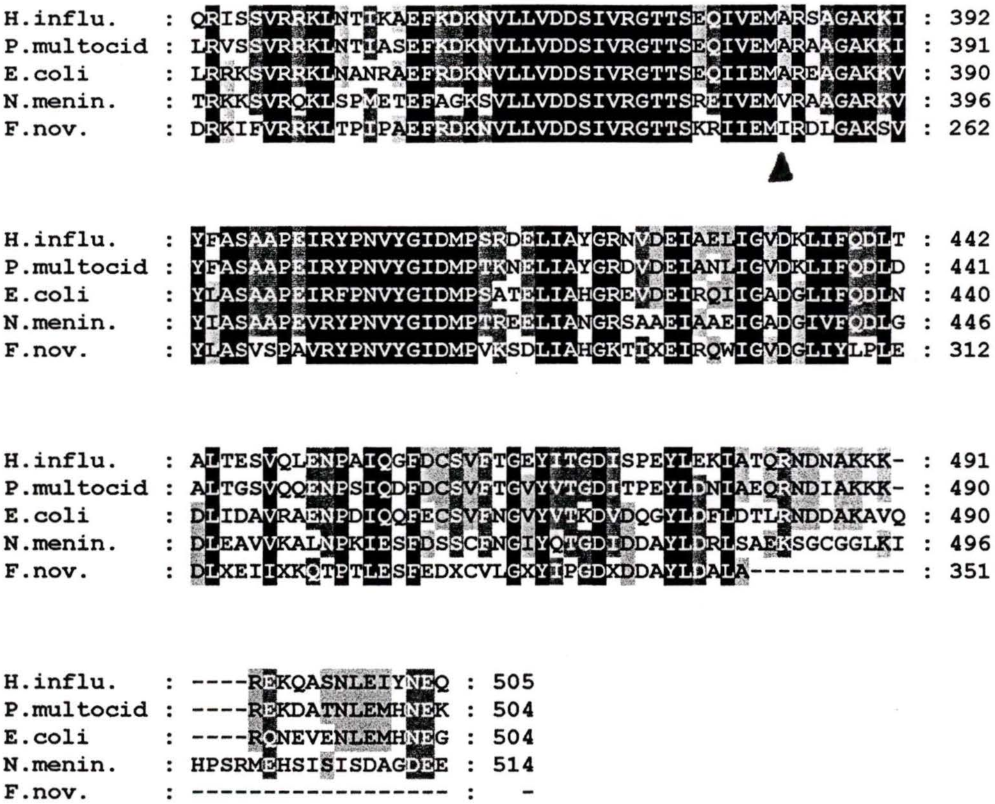

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H.influ.    : QRISSVRRKLNTIKAEFKDKNVLLVDDSIVRGTTSEQIVEMARSAGAKKI : 392
P.multocid  : LRVSSVRRKLNTIASEFKDKNVLLVDDSIVRGTTSEQIVEMARAAGAKKI : 391
E.coli      : LRRKSVRRKLNANRAEFRDKNVLLVDDSIVRGTTSEQIIEMAREAGAKKV : 390
N.menin.    : TRKKSVRQKLSPMETEFAGKSVLLVDDSIVRGTTSREIVEMVRAAGARKV : 396
F.nov.      : DRKIFVRRKLTPIPAEFRDKNVLLVDDSIVRGTTSKRIIEMIRDLGAKSV : 262

H.influ.    : YFASAAPEIRYPNVYGIDMPSRDELIAYGRNVDEIAELIGVDKLIFQDLT : 442
P.multocid  : YFASAAPEIRYPNVYGIDMPTKNELIAYGRDVDEIANLIGVDKLIFQDLD : 441
E.coli      : YLASAAPEIRFPNVYGIDMPSATELIAHGREVDEIRQIIGADGLIFQDLN : 440
N.menin.    : YIASAAPEVRYPNVYGIDMPTREELIANGRSAAEIAAEIGADGIVFQDLG : 446
F.nov.      : YLASVSPAVRYPNVYGIDMPVKSDLIAHGKTIXEIRQWIGVDGLIYLPLE : 312

H.influ.    : ALTESVQLENPAIQGFDCSVFTGEYITGDISPEYLEKIATQRNDNAKKK- : 491
P.multocid  : ALTGSVQQENPSIQDFDCSVFTGVYVTGDITPEYLDNIAEQRNDIAKKK- : 490
E.coli      : DLIDAVRAENPDIQQFECSVFNGVYVTKDVDQGYLDFLDTLRNDDAKAVQ : 490
N.menin.    : DLEAVVKALNPKIESFDSSCFNGIYQTGDIDDAYLDRLSAEKSGCGGLKI : 496
F.nov.      : DLXEIIXKQTPTLESFEDXCVLGXYIPGDXDDAYLDALA----------- : 351

H.influ.    : ----REKQASNLEIYNEQ : 505
P.multocid  : ----REKDATNLEMHNEK : 504
E.coli      : ----RQNEVENLEMHNEG : 504
N.menin.    : HPSRMEHSISISDAGDEE : 514
F.nov.      : ------------------ :   -
```

Figure 13. Deduced amino acid sequence alignment of DNA flanking the transposon in mutant CG57. The deduced amino acid sequence was aligned with amidophosphoribosyltransfase (Atase) proteins from *H. influenzae*, *P. multocida*, *E. coli*, and *N. meningitis*. Arrow indicates approximate location of transposon insertion.

```
F.tularens : MSEMITRQQVTSGETIHVRTDPTACIGSHPNCRLFIDSLTIAGEKLDKNIVAIEGGEDVT :  60
F.nov.Fip  : MSEMITRQQVTSGETIHVRTDPTACIGSHPNCRLFIDSLTIAGEKLDKNIVAIEGGEDVT :  60


F.tularens : KADSATAAASVIRLSITPGSINPTISITLGVLIKSNVRTKIEEKVSSILQASATDMKIKL : 120
F.nov.Fip  : KADSATAAASVIRLSITPGSINPTISITLGVLIKSNVRTKIEEKVSSILQASATDMKIKL : 120


F.tularens : GNSNKKQEYKTDEAWGIMIDLSNLELYPISAKAFSISIEPTELMGVSKDGMSYHIISIDG : 180
F.nov.Fip  : P-PNKKQEYKTDEAWGIMIDLSNLELYPISAKAFSISIEPTELMGVSKDGMSYHIISIDG : 179
              ▲

F.tularens : LTTSQGSLPVCCAASTDKGVAKIGYIAAA : 209
F.nov.Fip  : LTTSQGSLPVCCAASTDKGVAKIGYIAAA : 208
```

Figure 14. The deduced amino acid sequence alignment of DNA flanking the transposon in mutant CG62. The deduced amino acid sequence was almost completely identical with a 23 kDa protein from *F. tularensis*, here called Fip. Both of these proteins show no similarities to any other known proteins. Arrow indicates approximate location of transposon insertion.

AAAATAAATGTAATTCCCCACATTCAATTTGAAGGAATGAATACTACAATGACTATAGATACTAGGCTTGAACCAGAATT 80

K I N V I P H I Q F E G M N T T M T I D T R L E P E L

ATTCGGTACAAATAATAACTAAAAAA**AGGAG**AATGATTATGAGTGAGATGATAACAAGACAACAGGTAACAAGTGGCGAG 160

F G ? -10 SD *fip* M S E M I T R Q Q V T S G E

► ►

ACCATTCATGTGAGAACTGATCCTACTGCATGTATAGGATCTCATCCTAATTGTAGATTATTTATTGATTCTTTAACTAT 240

T I H V R T D P T A C I G S H P N C R L F I D S L T I

AGCTGGGGAGAAACTTGATAAAAATATCGTTGCTATAGAGGGTGGAGAGGATGTCACGAAAGCTGATTCGGCTACAGCTG 320

A G E K L D K N I V A I E G G E D V T K A D S A T A

CTGCTAGTGTAATACGTTTATCTATAACGCCAGGCTCTATAAATCCAACAATAAGTATTACTCTTGGTGTTCTAATTAAA 400

A A S V I R L S I T P G S I N P T I S I T L G V L I K

TCAAATGTCAGAACTAAAATTGAAGAGAAAGTTTCGAGCATATTACAAGCAAGTGCTACAGATATGAAAATTAAGTTAGG 480

S N V R T K I E E K V S S I L Q A S A T D M K I K L G

AGTTGCTAAAATAGGATATATTGCAGCTGCAGCTGCATAGTAAGATCGGAGTTGATTCTAATGTTTNTAGAAAGGATT 558

V A K I G Y I A A A A A ter ter

Figure 15. Nucleotide and deduced amino acid sequence of the 558 bp *fip* region of strain CG62. The sense strand is shown, with single letter abbreviations for deduced amino acids shown beneath. The 3' end of a deduced protein, showing similarity to uncharacterized proteins from *E. ictaluri*, and *V. cholerae,* precedes the *fip* gene. Possible –35 and –10 promoter regions are underlined and a potential ribosome binding site is in bold. Two possible stop codons are indicated by ter. The *fip* ORF spans bp 118 to bp 517.

```
E.coli     : MRLDRLTNKFQLALADAQSLALGHDNQFIEPLHLMSALLNQEGGSVSPLLTSAGINAGQL :  60
H.infl.    : MNIEKFTTKFQEALSERQSLAIGKDNQFIEPVHLLTALLNQQGGSIAPILTASGVNVALL :  60
F.nov.     : ----------------------------------------------------SGVNIQTF :   8
N.menin.   : MRYDKLTAKFQQALAEAQSLALAADGSYLEAGFVLKALLDDQNSGAAALLAHAGVNVPQV :  60

E.coli     : RTDINQALNRLPQVEGTGGDVQPSQDLVRVLNLCDKLAQKRGDNFISSELFVLAALESRG : 120
H.infl.    : RNELKTELNKLPQVIGNGGDVQLSRQLINLLNLCDKFAQQNQDKFISSELFLFAALEERG : 120
F.nov.     : IKAVNDMVDSLKKVSEEGILVPPSRDLIXXLHKMQELANKNGDEFISSEVFLLASLEDK- :  67
N.menin.   : KQRLQQHLNSLPKVSGQGGDILPSRELQAVLNLMDKAATKRSDAYIASELFLLALVQQND : 120

E.coli     : TLADILKAAGATTANITQAIEQMRGGESVNDQGAEDQRQALKKYTIDLTERAEQGKLDPV : 180
H.infl.    : TISDILKKCGAKKEQISQAIQHIRGGQNVNDQNAEESRQALEKYTIDLTARAESGKLDPV : 180
F.nov.     : SLXGLYNKFGITKEKLTKAVNDYRGGEKVSSQNQEDMKGALDKYTVDLTDLARKGKIDPI : 127
N.menin.   : ATGKILKEAGATEQNINAAIDAVRGGQNVNDANAEDQRDALKKYTLDLTQRARDGKLDPV : 180

E.coli     : IGRDEEIRRTIQVLQRRTKNNPVLIGEPGVGKTAIVEGLAQRIINGEVPEGLKGRRVLAL : 240
H.infl.    : IGRDEEIRRAIQVLQRRTKNNPVLIGEPGVGKTAIVEGLAQRIVNGEVPEGLKNKRVLSL : 240
F.nov.     : IGRDSEIRRTIQVLQRRTKNNPVLIGEPGVGKTAIVEGLAQRIVNDEVPGGTAEFGVLSL : 187
N.menin.   : IGRDDEIRRAIQVLQRRTKNNPVLIGEPGVGKTAIVEGLAQRIVNGEVPESLRNKRLLVL : 240
                                                                    ▲

E.coli     : DMGALVAGAKYRGEFEERLKGVLNDLAKQEGNVILFIDELHTMVGAGKADGAMDAGNMLK : 300
H.infl.    : DMGALIAGAKYRGEFEERLKAVLNELSKEEGRVILFIDEIHTMVGAGKTDGAMDAGNLLK : 300
F.nov.     : DMGALLAGAKFRGDFEERLKSVLKELSKQEGNVILFIDELHTMVGAGKAXGSMDAGNMLK : 247
N.menin.   : DLAALIAGAKYRGEFEERLKGVLNDLAKDDGNTLIFIDEIHTLVGAGKTDGAMDAGNMLK : 300

E.coli     : PALARGELHCVGATTLDEYRQYIEKDAALERRFQKVFVAEPSVEDTIAILRGLKERYELH : 360
H.infl.    : PSLARGELHCVGATTLDEYRQYIEKDAALERRFQKVFVDEPSVEDTIAILRGLKERYEIH : 360
F.nov.     : PALARGELKCVGATTLDEYREYVEKDPALERRFQKVLVDEPTVEDTIAILRGLKERYELH : 307
N.menin.   : PALARGELHCIGATTLDEYRQYIEKDAALERRFQKVLVGEPSVEDTIAILRGLQERYEIH : 360

E.coli     : HHVQITDPAIVAAATLSHRYIADRQLPDKAIDLIDEAASSIRMQIDSKPEELDRLDRRII : 420
H.infl.    : HHVDITDPAIVAAATLSHRYISDRQLPDKAIDLIDEAASSIRMEIDSKPEPLDRLERRII : 420
F.nov.     : HGVNITDSAIVSAATLSHRYITDRQLPDKAIDLVDEAASQIRMEIDSKPEKMESLYRRII : 367
N.menin.   : HGIDITDPAIVAAAELSDRYITDRFLPDKAIDLIDEAASRVKMEKETKPEAMDKIDRRLI : 420

E.coli     : QLKLEQQALMKESDEASKKRLDMLNEELSDKERQYSELEEEWKAEKASLSGTQTIKAELE : 480
H.infl.    : QLKLEQQALQKEEDEASRKRLEMLEKELAEKEREYAELEEVWKSEKATLSGSQHIKQELD : 480
F.nov.     : QLKMQREQLKKEKDDATKKRLEILEQEIKGLDSEYKGLKELWKSXKA------------- : 414
N.menin.   : QLRMEKAHVEKEKDDASKKRLELIDEEINGLQKEYADLDEIWKAEKAISDGAANIKKQID : 480
```

```
E.coli     : QAKIAIEQARRVGDLARMSELQYGKIPELEKQLEAATQLEGKTMR----LLRNKVTDAEI : 536
H.infl.    : TAKTELEQARRAGDLAKMSELQYGRIPDLEKQLEQAETSEGKEMT----LLRYRVTDEEI : 536
F.nov.     : ------------------------------------------------------------ :   -
N.menin.   : EVKIKIEQAKRQGDLALASKLMYEDLEHLEKQRAAAERADTDSTKPANKLLRNNVGAEEI : 540

E.coli     : AEVLARWTGIPVSRMMESEREKLLRMEQELHHRVIGQNEAVDAVSNAIRRSRAGLADP-- : 594
H.infl.    : AEVLSKATGIPVSKMMEGEKEKLLRMEDELHKRVIGQEEAVDAVANAIRRSRAGLSDPNR : 596
F.nov.     : ------------------------------------------------------------ :   -
N.menin.   : AEVVSRMTGIPVSKMMEGERDKLLKMEEVLHRRVVGQDEAVRAVSDAIRRSRSGLADPNK : 600

E.coli     : ------------------------------------------------------------ :   -
H.infl.    : PIGSFLFLGPTGVGKTELCKTLAKFLFDSEDAMVRIDMSEFMEKHSVSRLVGAPPGYVGY : 656
F.nov.     : ------------------------------------------------------------ :   -
N.menin.   : PYGSFLFLGPTGVGKTELCKALAGFLFDSEDHLIRIDMSEYMEKHAVARLIGAPPGYVGY : 660

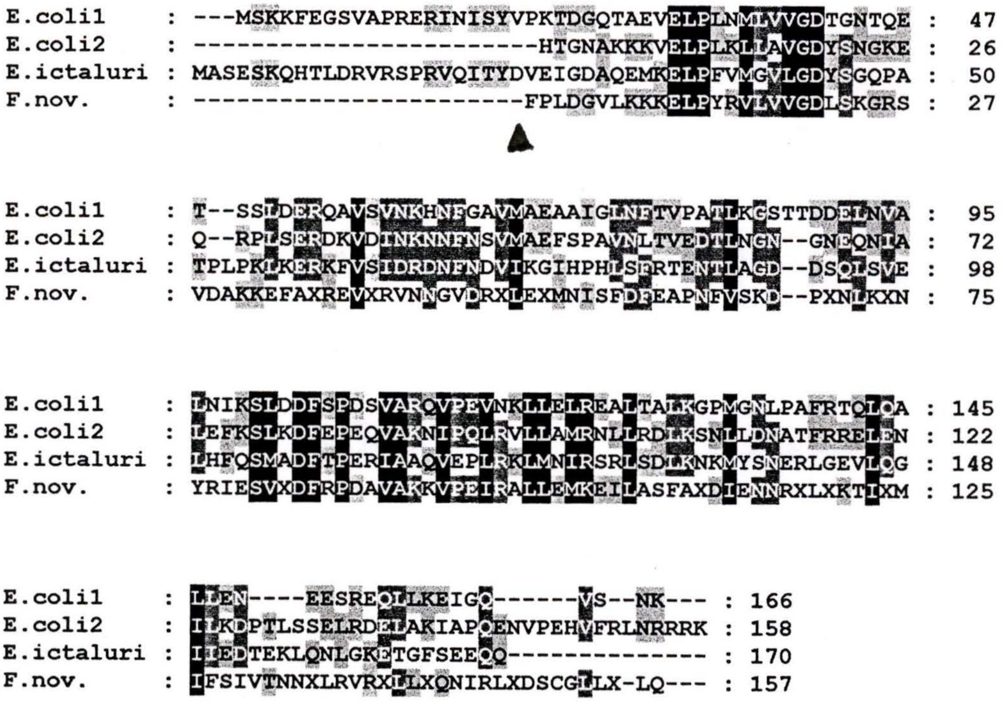
E.coli     : ------------------------------------------------------------ :   -
H.infl.    : EEGGYLTEAVRRRPYSVILLDEVEKAHADVFNILLQVLDDGRLTDGQGRTVDFRNTVVIM : 716
F.nov.     : ------------------------------------------------------------ :   -
N.menin.   : EEGGYLTEQVRRKPYSVILLDEVEKAHPDVFNILLQVLDDGRLTDGQGRTVDFKNTVIVM : 720

E.coli     : ------------------------------------------------------------ :   -
H.infl.    : TSNLGSDLIQGNKDESYSEMKALVMSVVSQHFRPEFINRIDETVVFHPLGKENIRAIASI : 776
F.nov.     : ------------------------------------------------------------ :   -
N.menin.   : TSNIGSQHIQQMGIQDYEAVKEVVMEDVKEHFRPEMINRIDEVVVFHGLDQDNIRNIAKI : 780

E.coli     : ------------------------------------------------------------ :   -
H.infl.    : QLERLAKRMETRGYELVFTDALLDFIGEVGYDPIYGARPLKRAIQQEIENSLAQQILSGA : 836
F.nov.     : ------------------------------------------------------------ :   -
N.menin.   : QLKGLEKRLEKQNLRLAVSDAALDIIAKAGFDPIYGARPLKRAIQSEIENPLAKALLAGN : 840

E.coli     : -------------------- :   -
H.infl.    : LLPGKVVTIDYANAEVQARQ : 856
F.nov.     : -------------------- :   -
N.menin.   : YAPESEIRVEADGDRLKFA- : 859
```

Figure 16. The deduced amino acid sequence alignment of DNA flanking the transposon in mutant CG69. This sequence aligns with a highly conserved group of stress response proteins, called ClpB, and was aligned with proteins from *E. coli, H. influenzae*, and *N. meningitis.* Arrow indicates approximate location of transposon insertion.



```
E.coli1     : ---MSKKFEGSVAPRERINISYVPKTDGQTAEVELPLNMLVVGDTGNTQE :  47
E.coli2     : ------------------------HTGNAKKKVELPLKLLAVGDYSNGKE :  26
E.ictaluri  : MASESKQHTLDRVRSPRVQITYDVEIGDAQEMKELPFVMGVLGDYSGQPA :  50
F.nov.      : -----------------------FPLDGVLKKKELPYRVLVVGDLSKGRS :  27

E.coli1     : T--SSLDERQAVSVNKHNFGAVMAEAAIGLNFTVPATLKGSTTDDELNVA :  95
E.coli2     : Q--RPLSERDKVDINKNNFNSVMAEFSPAVNLTVEDTLNGN--GNEQNLA :  72
E.ictaluri  : TPLPKLKERKFVSIDRDNFNDVIKGIHPHLSFRTENTLAGD--DSQLSVE :  98
F.nov.      : VDAKKEFAXREVXRVNNGVDRXLEXMNISFDFEAPNFVSKD--PXNLKXN :  75

E.coli1     : LNIKSLDDFSPDSVARQVPEVNKLLELREALTALKGPMGNLPAFRTQLQA : 145
E.coli2     : LEFKSLKDFEPEQVAKNIPQLRVLLAMRNLLRDLKSNLLDNATFRRELEN : 122
E.ictaluri  : LHFQSMADFTPERIAAQVEPLRKLMNIRSRLSDLKNKMYSNERLGEVLQG : 148
F.nov.      : YRIESVXDFRPDAVAKKVPEIRALLEMKEILASFAXDIENNRXLXKTIXM : 125

E.coli1     : LLEN----EESREQLLKEIGQ------VS--NK--- : 166
E.coli2     : ILKDPTLSSELRDELAKIAPQENVPEHVFRLNRRRK : 158
E.ictaluri  : ILEDTEKLQNLGKETGFSEEQQ-------------- : 170
F.nov.      : IFSIVTNNXLRVRXLLXQNIRLXDSCGLLX-LQ--- : 157
```

Figure 17. The deduced amino acid sequence alignment of DNA flanking the transposon in mutant CG116. The deduced amino acid sequence showed was aligned with two uncharacterized proteins from *E. coli*, and a protein of unknown function from *Edwardsiella ictaluri.* Arrow indicates approximate location of transposon insertion.

Sequencing results from CG45 demonstrate that the interrupted DNA region contains at least part of the plasmid pTn*Max2* DNA sequence. The plasmid appeared to have integrated into the genome, along with the transposon. The presence of the choramphenicol acetyltransferase (*cat*) gene, which is not present in pTn*Max2* but is found in pTn*Max1*, suggested that a mixed population of plasmids was used in the mutagenesis procedure. The sequence found for pCG124 contained some plasmid DNA, although the *cat* gene was not present. It is possible that a similar event to that found in strain CG45 occurred, in which part of the pTn*Max2* DNA was also transferred along with the transposon.

Hydrogen peroxide assay of CG62 mutant

Since the Fip protein in *F. tularensis* is induced in the presence of hydrogen peroxide (Golovliov *et al.*, 1997), the sensitivity of the putative *F. novicida fip* mutant strain to hydrogen peroxide was investigated. The viability of the CG62 mutant strain, and a control containing the transposon (CG88) are compared with the viability of the wildtype strain in Figure 18. It appeared as though the mutant strain was at least 4-fold more sensitive to hydrogen peroxide than the wildtype and CG88 strains.

Fasta search for fip gene in *F. tularensis* Schu 4 genome (raw data)

To obtain further data on the DNA region containing the *fip* gene, the raw data available at the website http://www.medmicro.mds.qmw.ac.uk/ft/. was searched using a local Fasta program. Portions of the sequence found upstream and downstream of the gene were used to search for the corresponding *F. tularensis* Schu 4 strain DNA clones. The process was repeated to find a number of genes immediately adjacent to this area. This data was used to help construct the general overview shown in Figure 11. A putative gene similar to a proposed gene from *Edwardsiella ictaluri* encoding a 54.5 kDa protein was found upstream of the *fip* gene. Searches downstream of the *fip* gene found no matches with database entries.

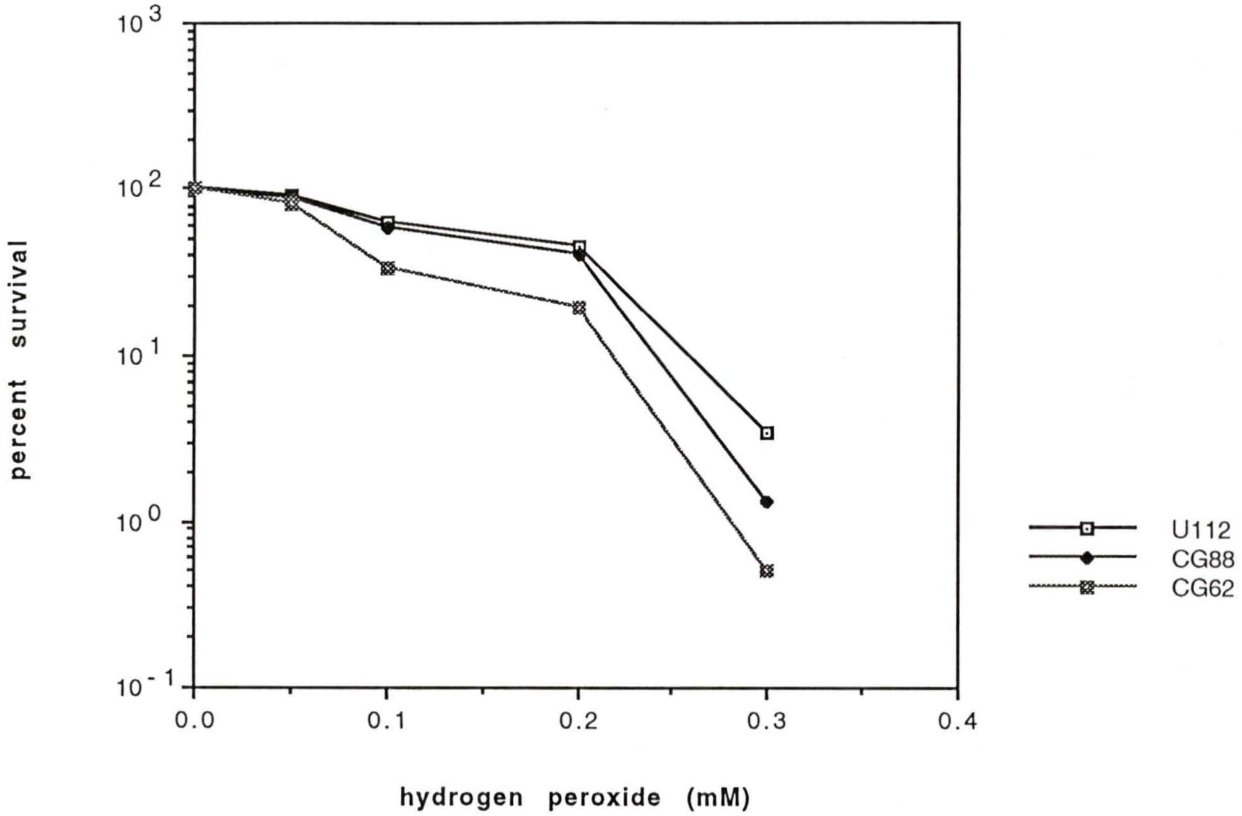


Figure 18. Hydrogen peroxide sensitivity assay of CG62 *fip* mutant. The number of viable bacteria were quantitated after a 3 hr incubation period with H_2O_2 at the amounts indicated.

Isolation of WT clones for CG124, CG21, CG45 and CG62.

In order to complement the mutations, the wildtype copies of the genes must be obtained. Two *Francisella* wildtype DNA libraries were used to obtain wildtype clones. The first library had been made previously by Dr. Larry Anthony. Plasmids were purified from 50 different pools. A dot blot using the plasmid pCG124 as the labelled probe identified a number of plasmid pools containing hybridizing DNA. Further investigation revealed two individual clones which hybridized with the pCG124 probe. One clone contained an insert of approximately 1.8 kb and the other contained an insert of approximately 2.9 kb (data not shown).

A second *F. novicida* wildtype library was created (as described previously). This library was used for colony blots and these blots were hybridized with the labelled plasmids pCG21, pCG45 and pCG62. An example of these results is shown in Figure 19, where labelled plasmid pCG21 was hybridized with a colony blot of the wildtype library. A number of positively hybridizing colonies were chosen for dot blot analysis. An oligonucleotide was designed from the alanine racemase gene sequence, 5'-TATAATAAAGAGTCTCAAACACGTG-3', and was used as a probe. Two wildtype clones, called A and B here, hybridized strongly with the oligonucleotide probe, as shown in Figure 20. Plasmid A is 8 kb in size while plasmid B is 3 kb in size. Both pCG45 and pCG62 colony blots contained positively hybridizing colonies, but these were not analyzed further.

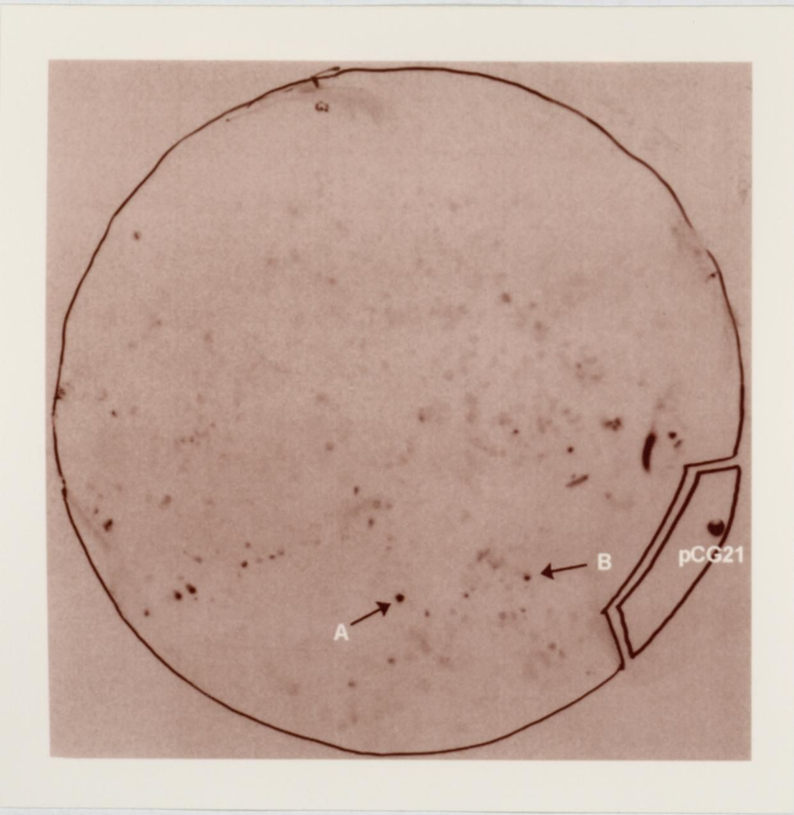


Figure 19. Colony blot of the *F. novicida* U112 (wildtype) library screened with ECL labelled pCG21 probe. The arrows indicate the positively hybridizing clones which correspond to clones found by dot blot analysis (Figure 20).

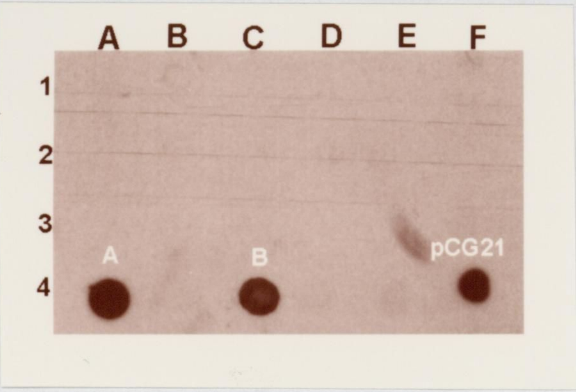


Figure 20. Dot blot analysis of the positively hybridizing *F. novicida* U112 clones found in the colony blot shown in Figure 19. The left clone is the control, while the remaining two clones, called A and B, hybridized with the CG21 oligonucleotide probe labelled with ECL.

Discussion

Francisella is an intracellular pathogen which resides in vacuoles that do not fuse with lysosomes, as do a number of well-studied bacterial pathogens such as *Legionella* and *Salmonella*. Unlike these organisms, *Francisella* intracellular growth has not been investigated in depth, and few virulence factors have been identified.

Transposon mutagenesis is a common technique used to create random bacterial mutants that can be screened for characteristics of interest to researchers. It has been used to identify genes involved in virulence in virtually all major bacterial pathogens studied, including *V. cholerae*, *L. pneumophila*, and *S. typhi*. Here we used a shuttle transposon mutagenesis scheme, which compensates for the lack of genetic systems available in *F. novicida* by allowing transposon mutagenesis to occur in a second organism, in this case *E. coli* (as shown in Figure 1). The genetic region containing the transposon can then be reintroduced to *F. novicida*, where it will integrate into the genome by a double recombination event. Over 10,000 *F. novicida* mutants were made in this way, and were screened for deficient intracellular growth. An initial visual screen identified 39 potential macrophage growth mutants, which were subsequently rescreened a second time. Nineteen mutants were identified and their growth in macrophages was determined quantitatively using macrophage growth assays. In order to insure they were not deficient in factors involved in general growth, assays of all of the 19 mutants were done in bacteriological media (TSB-C). Eight mutants were shown to grow as the wildtype strain, and these were chosen for further analysis.

F. novicida strains with defects in LPS have been shown to suffer decreased intracellular growth. KM14 appeared to have a mutation in the locus encoding ValA and ValB, which are thought to transport LPS to the outer membrane and phosphorylate lipid A, respectively (Mdluli *et al.*, 1994; McDonald *et al.*, 1997). SC66 which had altered LPS as shown by Western blot, also grew poorly in macrophages (Cowley *et al.*, 2000). All of the eight mutants described in this study possessed wildtype LPS as shown by Western blot. They are unlikely to have problems in the structural integrity of the cells as they were not sensitive to the detergent deoxycholate. Therefore their intramacrophage growth phenotype was thought to be due to factors other than those that affect cellular structure.

In past studies, *Francisella* mutants created with a transposon carrying a kanomycin (Km) resistance cassette were found to contain more than one transposon, and to have undergone genetic rearrangement (Mdluli *et al.*, 1994). Southern blot analysis of the interrupted loci in the eight mutants studied here indicated that one transposon insertion event had taken place. No single crossover events were detected, using pUC18 as the probe. DNA sequencing results and Southern blot analysis did reveal DNA rearrangement in at least one mutant strain, CG45, as discussed below.

As the tranposon Tn*Max2* contains an origin of replication, the interrupted DNA fragments can be retrieved by self-ligation followed by transformation into the appropriate *E. coli* strain. Three plasmids, pCG124, pCG45 and pCG21, were recovered in this manner. Both pCG124 and pCG45 *Francisella* DNA sequences did not show similarity to any known bacterial genes, suggesting that they may encode novel proteins. No open reading frames were distinguished, as there was not enough sequence for proper analysis. It is not uncommon for unknown genes to be found in studies of this type, and these genetic loci may play an important role in virulence. For example, a recent *in vivo* study using signature-tagged mutagenesis in *Legionella pneumophila* found 16 mutant clones that had attenuated virulence. Sequence analysis of the interrupted genetic loci revealed that three of the clones showed no similarity to known bacterial genes (Edelstein *et al.*, 1999).

Both CG124 and CG45 genetic loci affected by the transposon seemed to have undergone rearrangement of some type, however. The sequence obtained from CG45 revealed that at least portions of the plasmid pTn*Max2* integrated into the *F. novicida* genome. Presumably part of pTn*Max2* integrated into the *F. novicida* WT clone along with the transposon during shuttle mutagenesis (please see Figure 1). Only a small region of *Francisella* DNA was sequenced from this clone, and it had no significant similarity to known sequences. An overview of the CG45 genetic locus interrupted by the transposon can be seen in Figure 11. The Southern blot in Figure 10B demonstrates that there was not a simple insertion of the transposon into the genome, however further analysis is required to explain these results.

It appears as though the transposon integrated into CG124 via a simple insertion event, and the interrupted locus underwent a double recombination event into the *F. novicida* genome, as hoped for. The plasmid recovered from CG124 via self-ligation is 3.8 kb, instead of 3.2 kb as predicted, however. Digestion of this plasmid with *Nde* I results in two fragments of unexpected sizes. This suggests that there may be contaminating DNA present in this plasmid, although the source is unknown. The contamination probably occurred at the self-ligation step, and appears to be vector DNA. The *F. novicida* DNA sequenced from these CG124 clones showed no significant similarity with any database entries.

Partial DNA sequences obtained from CG21 DNA flanking the transposon showed similarity, at the deduced amino acid level, to alanine racemases from a number of bacteria. Alanine racemase catalyses the reversible conversion of the L-form of the amino acid to the D-form. Alanine racemase is unique to bacteria, and therefore is a target for drugs such as D-cycloserine. D-amino acids are found in bacterial cell walls and D-alanine is required for the synthesis of the mucopeptide component of cell walls of almost all bacteria. It is also found in lipoteichoic acids of Gram positive bacteria (Kullik *et al.*, 1998; Thompson *et al.*, 1998). If the intramacrophage growth phenotype is due to a defect in the *F. novicida* putative *alr* gene (which can only be shown by complementation studies), it may be due to alterations in the integrity of the cell wall, which make it more susceptible to microbicidal agents produced by macrophages. A loss of D-alanine in *Staphylococcus aureus* teichoic acids results in increased sensitivity to antimicrobial peptides, such as defensins. Teichoic acids are an integral component of the cell walls in Gram positive bacteria (Peschel *et al.*, 1999). Although *F. novicida* is Gram negative, it is conceivable that the loss of D-alanine in the cell wall renders the bacteria sensitive to positively charged antimicrobial peptides. Only biochemical studies can determine if this gene possesses alanine racemase activity, or if it serves some other function in *Francisella.*

Recently a *Listeria monocytogenes* strain has been created which requires D-alanine for growth. Two genes responsible for the synthesis of this compound, an alanine racemase gene (*alr*) and a D-amino acid aminotransferase gene (*dat*), have been inactivated. This is a highly attenuated strain which is being investigated as a possible vaccine vector. It can grow on media supplemented with D-alanine, but is unable to grow under natural conditions. This strain is completely attenuated in mice, but it can generate protective immunity against lethal challenge equal to that produced by the wildtype bacteria (Thompson *et al.*, 1998). It may be interesting to see if a similar *F. tularensis* mutant could be made.

The remaining five interrupted genetic loci were not recovered by self-ligation, but were found by screening DNA libraries of *F. novicida* strains CG57, CG62, CG69, CG93 and CG116 for Em^R colonies. Plasmids isolated from these colonies were shown to contain the transposon, and partial DNA sequences adjacent to the transposon were obtained.

Sequence obtained from CG57 showed a high degree of similarity at the deduced amino acid level to glutamine phosphoribosylpyrophosphate amidotransferases (ATases) from a number of bacteria. This enzyme catalyzes the first step in *de novo* purine biosynthesis, producing phosphoribosylamine. This reaction can be reversed to salvage purines. If this putative gene is responsible for the growth phenotype, than it may explain the slight decrease in growth shown in bacteriological media (see Figure 2). Genes of this type are often viewed as "housekeeping" genes which are not directly related to virulence, and may be expressed during other growth conditions. The putative ATase is probably especially required under stressful conditions, such as those found inside a macrophage. Recently, a probable ATase has been identified in *Pasteurella multocida,* using signature-tagged mutagenesis in a mouse model, as a protein which contributes to *in vivo* survival (Fuller *et al.*, 2000).

DNA sequences obtained from either side of the transposon in strain CG62 showed virtual identity at the deduced amino acid level to a 23 kDa protein found in *F. tularensis* LVS by Golovliov *et al.* (1997). Two-dimensional (2-D) polyacrylamide gel electrophoresis was used to look at differential protein expression between bacteria inside macrophages and those grown in culture media. Four proteins were found to be noticeably upregulated. One of these was the 23 kDa protein which was isolated from the 2-D gel, and partially sequenced. The corresponding DNA sequence was identified using a reverse genetics approach, and the gene encoding the protein was sequenced, and expressed in *E. coli.* The expressed protein was also 23 kDa, and possessed the same immunoreactivity as the original protein (Golovliov *et al.*, 1997).

The 23 kDa protein appears to be cytoplasmic as it was found predominately in the cytoplasmic fraction after ultrasonic disintegration, a finding which was supported by the lack of a putative signal peptide. They did not determine if the protein was secreted into the media, and there is a putative transmembrane region. A small amount of the protein was detected in the membrane fraction. The function of this protein is unknown but it may be involved in the stress response. However other stress proteins, such as DnaK and GroEL homologues, were not shown to be upregulated. In fact, since so few proteins were upregulated, these researchers suggested that there is a relatively modest *Francisella* stress response to macrophages, compared to other bacterial pathogens (Golovliov *et al.*, 1997).

The identification of an identical putative protein in *F. novicida* (designated Fip), which is connected with defective intracellular growth, strongly supports the idea that this protein is involved in virulence. The DNA sequences between the *F. tularensis* LVS protein and the *F. novicida* Fip protein discovered in this work, were found to be very similar, and the arrangement of the genes appeared to be identical. Both genes seemed to have a typical Shine-Delgarno sequence 6 base pairs upstream of the putative ATG initiation codon. Since the *F. tularensis* protein was induced with hydrogen peroxide, I tested CG62 for sensitivity to hydrogen peroxide. The results, shown in Figure 18, suggest that this mutant is slightly more sensitive to hydrogen peroxide than the wildtype. Fip may play a regulatory role in *Francisella* virulence, or it may be a virulence factor that directly affects intracellular growth. It is possible that it plays a role in protecting the bacteria from reactive oxygen intermediates produced during the respiratory burst. However, the fact that the protein is only expressed after the first two hours of infection, suggests it may have another role. Another possibility is that Fip interferes with host cell pathways involved in programmed cell death, as hydrogen peroxide can activate a signalling pathway resulting in apoptotis.

Sequence analysis of flanking regions of the transposon in CG69 found a putative gene encoding a protein which is a highly conserved stress response protein (Gottesman *et al.*, 1990). This genetic region appears to encode a member of a system of ATP-dependent proteases called ClpB (for caseinolytic protease). This system was first identified as a heat-shock inducible, multicomponent, ATP-dependent protease complex which hydrolyzed casein. It is composed of three gene families, *clp*ABCXY, *clp*P and *clp*Q, which are not homologous. ClpB is the only protein which does not interact with ClpP, and its function is not entirely understood (Celerin *et al.*, 1998). The other components of the Clp system form holoenzymes with ClpP which hydrolyze both aggregated and nonaggregated denatured proteins. The function of ClpB is not known, but it is upregulated in cyanobacterium under excess light stress conditions. Excess light results in the production of reactive oxygen byproducts in photosynthetic organisms, which can result in oxidative damage to proteins, lipids and DNA (Celerin *et al.*, 1998). It is possible that a ClpB protein in intracellular *Francisella* is expressed in response to the presence of reactive oxygen intermediates produced by the host cell.

DNA analysis of the sequence interrupted by the transposon in strain CG93 showed no significant similarity with known genes. Perhaps analysis was hampered by the poor quality of the sequence. Southern blot analysis of this locus proved inconclusive, as the DNA sequence used to probe the wildtype and CG93 genomic DNA did not contain the transposon, nor did it contain sequences flanking the transposon. The Southern blot should be repeated with a CG93 DNA fragment that has been shown to hybridize with the transposon.

Partial sequence analysis of the DNA adjacent to one side of the transposon in strain CG116 showed similarity to proteins of unknown function in *E. coli*, *V. cholerae* (data not shown), and *E. ictaluri*. The beginning of the protein was not found on the other side of the transposon, probably because of poor sequence, although it is possible that genetic rearrangement has occurred. One of these proteins is associated with a DNA fragment found in pathogenic *E. coli* strains causing neonatal meningitis, but lacking in nonpathogenic strains (Bonacorsi *et al.*, 2000). I am presently attempting to determine where in the *E. coli* and *V. cholerae* genomes these genes are found (the *V. cholerae* genome has recently become available for analysis).

This study demonstrates that it is possible to do relatively large mutagenesis studies in *Francisella* using the genetic techniques currently available. We have identified a number of putative genes which may help *Francisella* survive inside host cells. The identification of a putative gene, *fip*, previously associated with *Francisella* intracellular growth suggest that this approach can be used to identify virulence factors. Further characterization of these genetic loci may reveal other proteins involved in pathogenesis, and could lead to the identification of pathogenicity islands. The *Francisella* genome project should prove to be very useful in providing information on these genetic loci, and indicating future directions for investigation.

Chapter 2. Intramacrophage growth analysis of a possible *F. novicida* 70 kDa protein mutant.

Introduction

A spontaneous *F. novicida* mutant, called GB2, was found to be almost completely defective for intracellular growth. The growth phenotype was associated with an apparent operon, called *mgl*AB (for macrophage growth locus). Both *mgl*A and *mgl*B transposon insertion mutants were found to be unable to grow inside macrophages. The deduced amino acid sequences of these genes show similiarity to the SspA and SspB proteins of *E. coli* and *H. influenzae* (Baron and Nano, 1998).

In *E. coli,* these proteins have been shown to be induced under conditions of nutritional stress, and their presence influences the expression of a number of proteins. SspA can associate with the RNA holoenzyme, and the proteins are believed to be regulatory proteins (Williams *et al.*, 1994). For these reasons, MglAB was hypothesized to control the expression of genes whose products contribute to intracellular survival. Cell fractionation studies of *mgl* mutants showed several differences compared to the wildtype strain. The most notable difference was the lack of a 70 kDa protein in the mutant strains. This protein was localized to the culture supernatant, is believed to be secreted and was hypothesized to be involved in virulence. The N-terminal sequence of the protein was determined, and a degenerated oligonucleotide was designed from this sequence (Baron, 1998).

The oligonucleotide was labelled and found to hybridize to a clone with a 6.3 kb insert. Presumably, the gene encoding the 70 kDa protein gene is found within this insert. This 6.3 kb insert was subcloned into pUC18, and called pFEN70. The transposon Tn*Max2* was used to create insertional mutations, which were reintroduced to *F. novicida* to create a library of potential 70 kDa mutant strains. A number of these mutant strains lacked the 70 kDa protein, as determined by gel electrophoresis of cell fractions. Further studies, reported here, demonstrated a 10-fold decrease in macrophage growth in two of these mutant strains. This result supports the idea that the 70 kDa protein may be involved in virulence. Partial DNA sequences have been analyzed from the putative 70 kDa protein clone, and found to contain sequences similar to chitinase and endothelin-converting enzyme (ECE-1) homologue genes from other bacteria.

Results

Growth assay of possible *F. novicida* 70 kDa protein mutants in J774A.1 macrophages

Two putative *F. novicida* 70 kDa protein mutants were analyzed for growth in J774A.1 macrophages. Figure 21 demonstrates an approximately 10-fold decrease in growth as compared to a transposon-containing control strain. Later growth assays confirmed these results, using both the wildtype strain and another transposon-containing strain as controls (data not shown). These two strains were previously shown to be lacking the 70 kDa protein, as determined using cell fractionation studies and SDS-PAGE (Dr. Gerald Baron, personal communication). It is presumed that the transposon Tn*Max2* has interrupted the gene encoding the 70 kDa protein and if this is found to be correct, these data suggest that the protein may be involved in intracellular growth.

SDS-PAGE analysis of putative a 70 kDa mutant strain showing absence of a 70 kDa protein.

A putative *F. novicida* 70 kDa protein mutant was analyzed by SDS-PAGE for the absence of the protein as compared to the wildtype strain. In Figure 22A it can be seen that a band corresponding to approximately 70 kDa is missing from the mutant strain, as indicated by the black arrow. Lanes 1 and 4 contain supernatant extract from *F. novicida* wildtype strain, while lanes 2 and 3 contain supernatant extract from the 70 kDa mutant.

A Western blot was performed with antiserum against the 70 kDa protein, and is shown in Figure 22B. It is apparent that the anti-serum contains antibody to a number of other proteins, however, it is possible to see a difference between the wildtype strain (lane 2), and the putative 70 kDa mutant strain (lane 1). A band is missing in the mutant strain that corresponds in size to approximately 70 kDa.

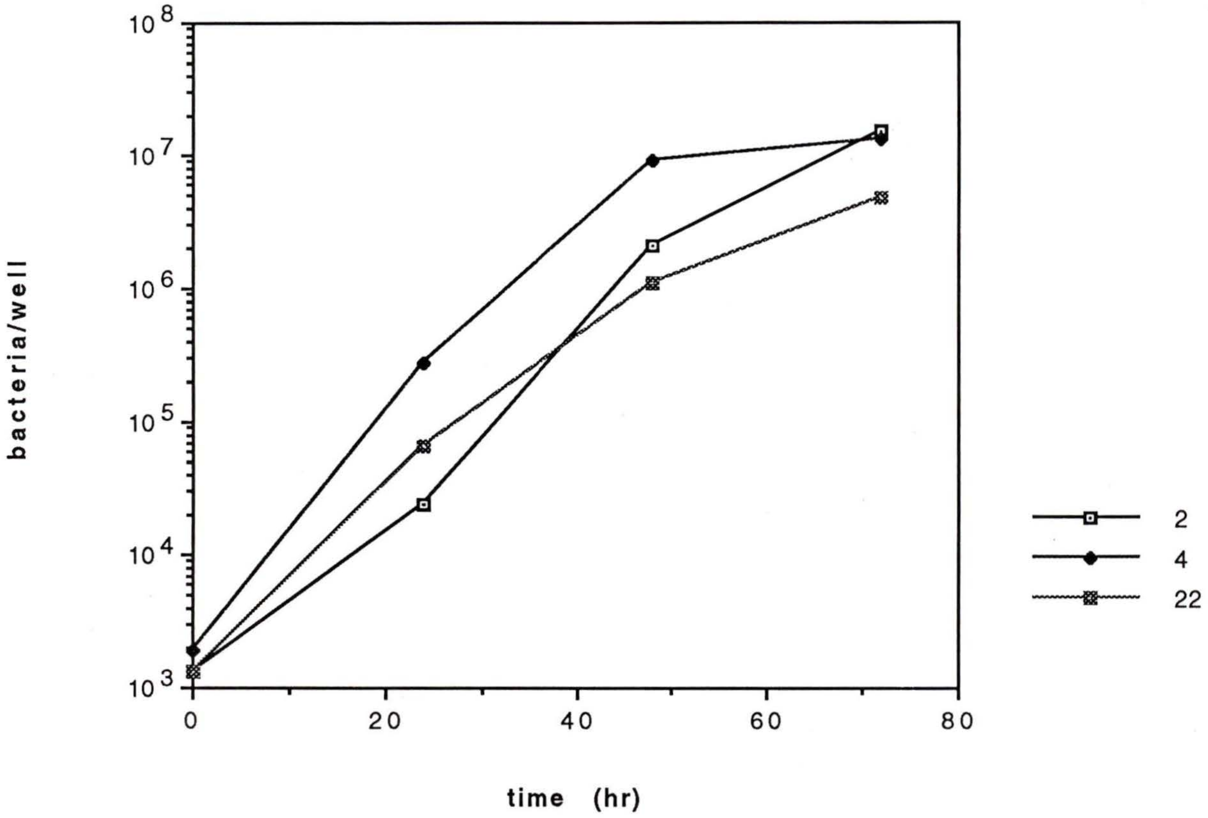


Figure 21. Growth of two *F. novicida* 70 kDa protein mutant strains in J774A.1 macrophages. Bacteria were enumerated by plating serial dilutions. *F. novicida* strain 22 and strain 2 lacked the 70 kDa protein, while strain 4 contains a transposon insertion which does not affect expression of the 70 kDa protein.

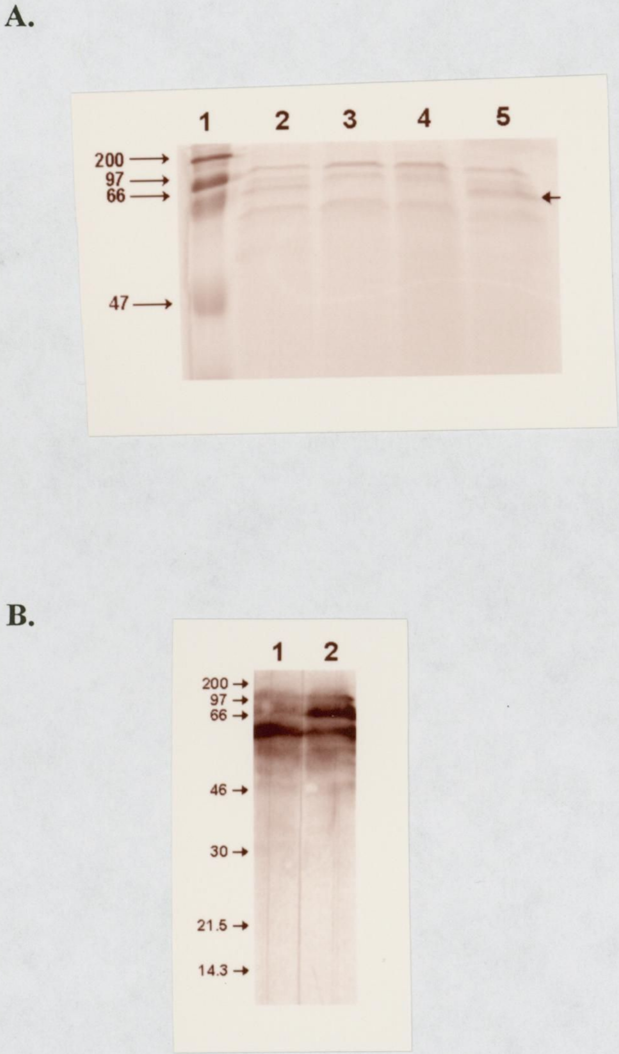


Figure 22. A. Coomassie brilliant blue stained SDS-PAGE gel of supernatant cell fractions from *F. novicida* 70 kDa protein mutant strains as compared to the wildtype strain. Lane 1: molecular mass markers, lanes 2 and 5: wildtype strain, lanes 3 and 4: 70 kDa protein mutant strain. Arrow indicates 70 kDa protein.
B. Western blot of *F. novicida* 70 kDa protein mutant strain as compared to the wildtype strain. The supernatant cell fractions of stationary phase cultures were analyzed by SDS-PAGE and proteins were electrophoretically transferred to a PVDF membrane. Anti-serum to the 70 kDa protein was used to probe the blot. Lane 1: *F. novicida* 70 kDa protein mutant. Lane 2: *F. novicida* wildtype strain.

Taken together these data may indicate that the 70 kDa protein is not being expressed in these mutant strains, although these results are preliminary.

Partial sequencing of the plasmid pFEN70.

A degenerate oligonucleotide probe was designed from the N-terminal sequence obtained from the 70 kDa protein. The plasmid pGB63, containing a 6.3 kb insert, was found to hybridize with this oligonucleotide (Baron, 1998). This insert was subcloned into pUC18 and was called pFEN70-7. Plasmid pFEN70-7 was used to create the putative *F. novicida* 70 kDa mutant.

Sequences from either end of this 6.3 insert, obtained with universal primers, were analyzed for similarity to database entries. Sequence alignments are shown in Figure 23 A and B, respectively.

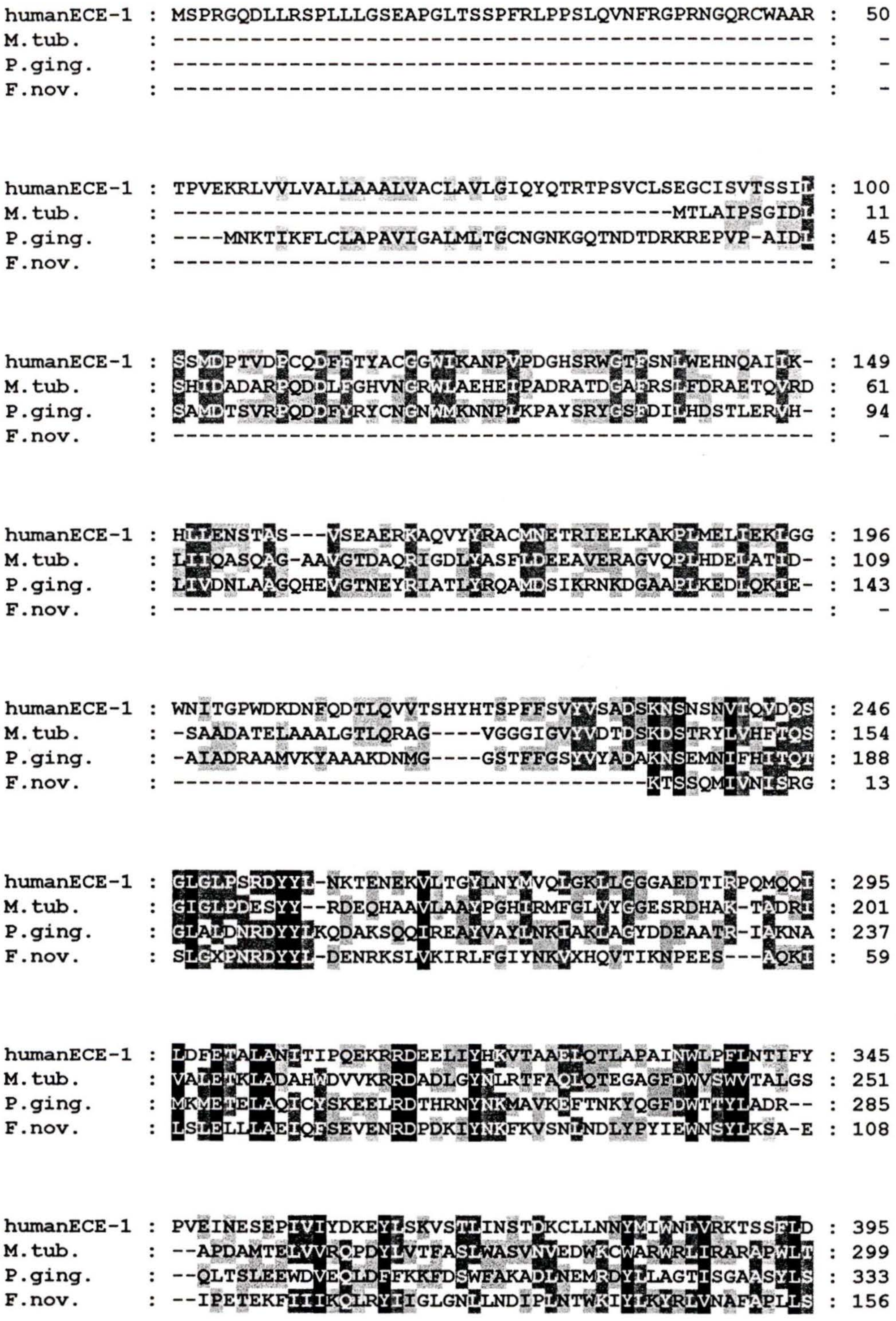
The forward sequence was shown to have similarity to chitinases from a variety of bacteria. The 5' end of the sequence overlaps with the beginning of a chitinase gene from *Serratia marcescens* in the reverse orientation over a 48 amino acid region. The identity between these two sequences is 47 %, with an E-value of approximately 10^{-4}.

The reverse sequence was found to contain a possible endothelin-converting enzyme homologue (ECE-1) at the deduced amino acid level. The *F. novicida* sequence shows 31 % identity, and has an E-value of 10^{-35} over 282 amino acids with a proposed ECE-1 homologue from *M. tuberculosis*.

Fasta search using pFEN70 sequences against *F. tularensis* Schu 4 data

The *F. tularensis* Schu 4 strain has recently been sequenced, and the raw data is available on a website. This allows researchers to search their sequences against this "database" of clone sequences. The two DNA sequences found above were used to search this database in order to identify other genes in the plasmid pFEN70 6.3 kb insert. The main aim of this search was to identify the 70 kDa protein gene

A.



```
humanECE-1 : MSPRGQDLLRSPLLLGSEAPGLTSSPFRLPPSLQVNFRGPRNGQRCWAAR :  50
M.tub.     : -------------------------------------------------- :   -
P.ging.    : -------------------------------------------------- :   -
F.nov.     : -------------------------------------------------- :   -

humanECE-1 : TPVEKRLVVLVALLAAALVACLAVLGIQYQTRTPSVCLSEGCISVTSSIL : 100
M.tub.     : ---------------------------------------MTLAIPSGIDL :  11
P.ging.    : ----MNKTIKFLCLAPAVIGALMLTGCNGNKGQTNDTDRKREPVP-AIDL :  45
F.nov.     : -------------------------------------------------- :   -

humanECE-1 : SSMDPTVDPCQDFFTYACGGWIKANPVPDGHSRWGTFSNLWEHNQAIIK- : 149
M.tub.     : SHIDADARPQDDLFGHVNGRWLAEHEIPADRATDGAFRSLFDRAETQVRD :  61
P.ging.    : SAMDTSVRPQDDFYRYCNGNWMKNNPLKPAYSRYGSFDILHDSTLERVH- :  94
F.nov.     : -------------------------------------------------- :   -

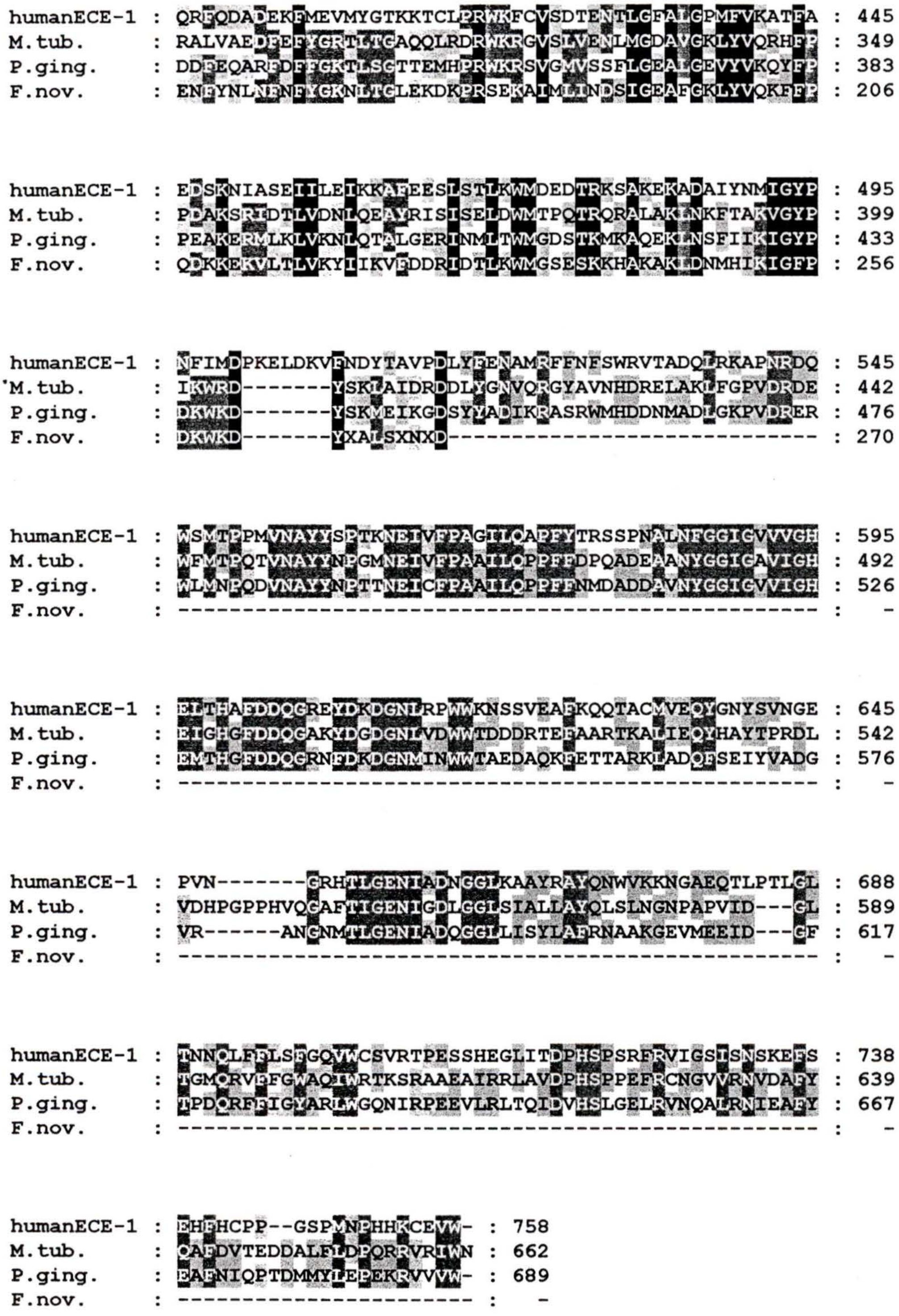
humanECE-1 : HLLENSTAS---VSEAERKAQVYYRACMNETRIEELKAKPLMELIEKLGG : 196
M.tub.     : LIIQASQAG-AAVGTDAQRIGDLYASFLDEEAVERAGVQPLHDELATID- : 109
P.ging.    : LIVDNLAAGQHEVGTNEYRIATLYRQAMDSIKRNKDGAAPLKEDLQKIE- : 143
F.nov.     : -------------------------------------------------- :   -

humanECE-1 : WNITGPWDKDNFQDTLQVVTSHYHTSPFFSVYVSADSKNSNSNVIQVDQS : 246
M.tub.     : -SAADATELAAALGTLQRAG----VGGGIGVYVDTDSKDSTRYLVHFTQS : 154
P.ging.    : -AIADRAAMVKYAAAKDNMG----GSTFFGSYVYADAKNSEMNIFHITQT : 188
F.nov.     : -------------------------------------KTSSQMIVNISRG :  13

humanECE-1 : GLGLPSRDYYL-NKTENEKVLTGYLNYMVQLGKLLGGGAEDTIRPQMQQI : 295
M.tub.     : GIGLPDESYY--RDEQHAAVLAAYPGHIRMFGLVYGGESRDHAK-TADRI : 201
P.ging.    : GLALDNRDYYLKQDAKSQQIREAYVAYLNKIAKLAGYDDEAATR-IAKNA : 237
F.nov.     : SLGXPNRDYYL-DENRKSLVKIRLFGIYNKVXHQVTIKNPEES---AQKI :  59

humanECE-1 : LDFETALANITIPQEKRRDEELIYHKVTAAELQTLAPAINWLPFLNTIFY : 345
M.tub.     : VALETKLADAHWDVVKRRDADLGYNLRTFAQLQTEGAGFDWVSWVTALGS : 251
P.ging.    : MKMETELAQICYSKEELRDTHRNYNKMAVKEFTNKYQGFDWTTYLADR-- : 285
F.nov.     : LSLELLLAEIQFSEVENRDPDKIYNKFKVSNLNDLYPYIEWNSYLKSA-E : 108

humanECE-1 : PVEINESEPIVIYDKEYLSKVSTLINSTDKCLLNNYMIWNLVRKTSSFLD : 395
M.tub.     : --APDAMTELVVRQPDYLVTFASLWASVNVEDWKCWARWRLIRARAPWLT : 299
P.ging.    : --QLTSLEEWDVEQLDFFKKFDSWFAKADLNEMRDYLLAGTISGAASYLS : 333
F.nov.     : --IPETEKFIIIKQLRYIIGLGNLLNDIPLNTWKIYLKYRLVNAFAPLLS : 156
```



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humanECE-1 : QRFQDADEKFMEVMYGTKKTCLPRWKFCVSDTENTLGFALGPMFVKATFA : 445
M.tub.     : RALVAEDFEFYGRTLTGAQQLRDRWKRGVSLVENLMGDAVGKLYVQRHFP : 349
P.ging.    : DDFEQARFDFFGKTLSGTTEMHPRWKRSVGMVSSFLGEALGEVYVKQYFP : 383
F.nov.     : ENFYNLNENFYGKNLTGLEKDKPRSEKAIMLINDSIGEAFGKLYVQKFFP : 206

humanECE-1 : EDSKNIASEIILEIKKAFEESLSTLKWMDEDTRKSAKEKADAIYNMIGYP : 495
M.tub.     : PDAKSRIDTLVDNLQEAYRISISELDWMTPQTRQRALAKLNKFTAKVGYP : 399
P.ging.    : PEAKERMLKLVKNLQTALGERINMLTWMGDSTKMKAQEKLNSFIIKIGYP : 433
F.nov.     : QDKKEKVLTLVKYIIKVFDDRIDTLKWMGSESKKHAKAKLDNMHIKIGFP : 256

humanECE-1 : NFIMDPKELDKVFNDYTAVPDLYFENAMRFFNFSWRVTADQLRKAPNRDQ : 545
M.tub.     : IKWRD-------YSKLAIDRDDLYGNVQRGYAVNHDRELAKLFGPVDRDE : 442
P.ging.    : DKWKD-------YSKMEIKGDSYYADIKRASRWMHDDNMADLGKPVDRER : 476
F.nov.     : DKWKD-------YXALSXNXD----------------------------- : 270

humanECE-1 : WSMTPPMVNAYYSPTKNEIVFPAGILQAPFYTRSSPNALNFGGIGVVVGH : 595
M.tub.     : WFMTPQTVNAYYNPGMNEIVFPAAILQPPFFDPQADEAANYGGIGAVIGH : 492
P.ging.    : WLMNPQDVNAYYNPTTNEICFPAAILQPPFFNMDADDAVNYGGIGVVIGH : 526
F.nov.     : -------------------------------------------------- :   -

humanECE-1 : ELTHAFDDQGREYDKDGNLRPWWKNSSVEAFKQQTACMVEQYGNYSVNGE : 645
M.tub.     : EIGHGFDDQGAKYDGDGNLVDWWTDDDRTEFAARTKALIEQYHAYTPRDL : 542
P.ging.    : EMTHGFDDQGRNFDKDGNMINWWTAEDAQKFETTARKLADQFSEIYVADG : 576
F.nov.     : -------------------------------------------------- :   -

humanECE-1 : PVN-------GRHTLGENIADNGGLKAAYRAYQNWVKKNGAEQTLPTLGL : 688
M.tub.     : VDHPGPPHVQGAFTIGENIGDLGGLSIALLAYQLSLNGNPAPVID---GL : 589
P.ging.    : VR------ANGNMTLGENIADQGGLLISYLAFRNAAKGEVMEEID---GF : 617
F.nov.     : -------------------------------------------------- :   -

humanECE-1 : TNNQLFFLSFGQVWCSVRTPESSHEGLITDPHSPSRFRVIGSISNSKEFS : 738
M.tub.     : TGMQRVEFGWAQIWRTKSRAAEAIRRLAVDPHSPPEFRCNGVVRNVDAFY : 639
P.ging.    : TPDQRFFIGYARLWGQNIRPEEVLRLTQIDVHSLGELRVNQALRNIEAFY : 667
F.nov.     : -------------------------------------------------- :   -

humanECE-1 : EHFHCPP--GSPMNPHHKCEVW- : 758
M.tub.     : QAFDVTEDDALFLDPQRRVRIWN : 662
P.ging.    : EAFNIQPTDMMYLEPEKRVVVW- : 689
F.nov.     : ----------------------- :   -
```

B.

```
S.mars.cbp  : MNKTSRTLLSLGLLSAAMFGVSQQANAHGYVESPASRAYQCKLQLNTQCG : 50
S.mars.chi  : MNKTSRTLLSLDLLSAAMFGVSQQANAHGYVESPASRAYQCKLQLNTQCG : 50
F.nov.chit  : -----MKLNKITLLTGLALLVSSEAYSHGYVESPASRALLCKEGKNKDCG : 45


S.mars.cbp  : -SVQYE------PQSVE : 60
S.mars.chi  : RCVQYE------PQSV- : 60
F.nov.chit  : ERARYPGILXSRPAGI- : 61
```

Figure 23. Partial sequence from ends of plasmid pFEN70 containing gene encoding 70 kDa protein.

A. Sequence obtained with forward universal primer showed similarity to ECE-1 proteins from humans, *M. tuberculosis* and *P. gingivalis* at the deduced amino acid level.

B. Sequence obtained with reverse universal primer showed similarity to a chitinase and a chitinase binding protein (cbp) from *S. marcescens* at the deduced amino acid level.

in pFEN70, however this proved unsuccessful. These searches were performed with a local Fasta program.

Additionally, an attempt was made to identify the 70 kDa protein using the N-terminal sequence MHQPHQYSSNKIGIDTQYID obtained previously, and a local Tfasta program. A clone was identified from the Schu 4 database which showed an exact match over the sequence SNKIGIDTQYI. A BlastX search determined that the Schu 4 clone containing this peptide showed no significant similarity with known proteins, however. A protein with a high degree of similarity to MutT proteins from *Streptococcus pneumoniae* and *Bacillus subtilis* was identified in a region adjacent to this area (data not shown). The E-value was approximately 10^{-17} over 142 aa. Neither of these sequences have been found in pFEN70, however.

A putative transposase gene was found adjacent to the ECE-1 homologue gene. It was most similar to a transposase from *S. pneumoniae*, with 31 % identity over 90 aa, and an E-value of 10^{-5}. A putative threonyl t-RNA synthetase was found adjacent to the chitinase gene, at the opposite end of the insert. It was highly similar to the equivalent protein in *N. meningitidis* with an identity of 55 % over 161 aa, and an E-value of approximately 10^{-53}. A possible genetic region was built up in this way, and is shown in Figure 24. Only sequencing of pFEN70 will confirm if this region in *F. novicida* is equivalent to the genetic region that was found by searching the *F. tularensis* Schu 4 clones.

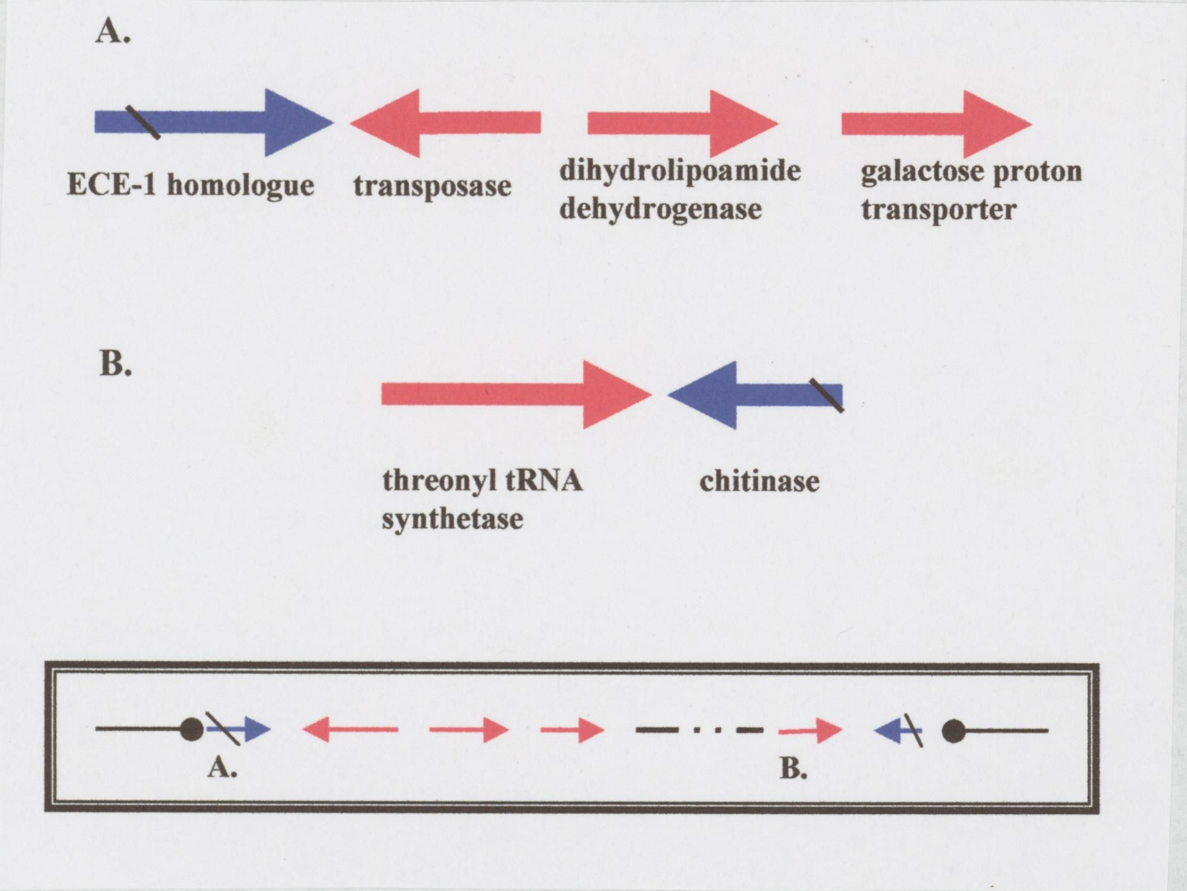

Figure 24. Overview of genetic regions analysed by Fasta search against *F. tularensis* Schu 4 raw genome data. DNA sequences from either end of the plasmid pFEN70 6.3 kb insert were used to search the *F. tularensis* library, and generate a possible arrangement of genes in the area.. The putative genes found in *F. novicida* are shown in blue, while the putative genes found in *F. tularensis* are shown in red. Sequencing analysis of this genetic region is required to confirm these data. The organization of the 6.3 kb insert is shown in the box. The dotted line represents indetermined sequence.

A. Possible genetic region, adjacent to the putative ECE-1 homologue ORF, encoding probable transposase, dihydrolipoamide dehydrogenase and galactose proton transporter proteins.

B. Possible genetic region, adjacent to the putative chitinase ORF, encoding a probable threonyl tRNA synthetase protein.

Discussion

The results reported here have begun to characterize a genetic locus that is thought to contain the gene for the 70 kDa protein. This protein is of interest as it's expression is believed to be under the control of a possible global regulatory operon, *mgl*AB, which is involved in intracellular growth. Figure 21 shows two possible 70 kDa mutant strains that have decreased growth in macrophages as compared to a control strain. A decrease in growth of this magnitude can be associated with effector molecules active in pathogenesis. It is therefore possible that the mutant strains are defective in a 70 kDa protein that is involved in survival and growth inside host cells.

Western blot analysis demonstrates that one of the mutant strains lacks the 70 kDa protein in the culture supernatant, while it is present in the wildtype strain. Although the anti-serum is not specific for the protein, the only difference between the wildtype and mutant strains is the lack of the 70 kDa protein in the latter. This result suggests that the mutant is not expressing the protein, although it is possible that is is simply unable to secrete the protein.

Sequencing analysis of plasmid pFEN70 revealed a possible gene similar to the chitinases of a number of bacteria at the deduced amino acid level. It is interesting to hypothesize that a chitinase may be required for *Francisella* survival within ticks, when the bacteria is taken up in a blood meal. The blood meals of insects such as sandflies and mosquitoes are surrounded by a peritrophic matrix that is made of layers of chitin microfibrils enmeshed with proteins and glycoproteins. Microorganisms such as *Leishmania* spp. and *Plasmodium* spp. secrete chitinases which allow them to escape the peritrophic matrix (Shahabuddin, 1998; Schlein and Jacobson, 1994). It is possible that *Francisella* may also possess this capability, although it is not known if *F. novicida* can live in insects. Of course, this putative protein may serve an entirely different purpose in *Francisella* spp.

The presence of a genetic sequence that shows similarity to human endothelial-converting enzyme homologues is also interesting. In humans, this protein is a metalloproteinase that processes big endothelin, a potent vasoconstrictor peptide, into its active form. ECE-1 is a member of the NEP-ECE-Kell family of type II membrane-bound metalloproteases (Emoto and Yanagisawa, 1995). Possible homologues have been found in many bacteria. The putative *Francisella* homologue is most similar to proteins found in *M. tuberculosis*, *M. leprae*, and *Porphyromonas gingivalis*. In *Lactococcus lactis*, a 70 kDa protein called PepO was found to be a homologue of mammalian neutral endopeptidase (enkephilinase). This protein hydrolyzed peptides such as Met-enkephalin (5 aa) and the oxidized β-chain of insulin (30 aa). The *P. gingivalis* PepO protein hydrolysed big endothelin to its active form. It is also reported to cross-react with a monoclonal antibody against rat ECE-1 (Awano *et al.*, 1999). Although the functionality of these homologues has yet to be determined in these bacteria, they probably behave similarly to their mammalian counterparts, based upon their high degree of similarity.

Some pathogenic bacteria secrete proteases that play a role in virulence, and it is tempting to imagine these ECE homologues in such a role. A secreted 35 kDa zinc-dependent metalloprotease in *Listeria monocytogenes* processes a phosphotidylcholine phospholipase C virulence factor involved in cell-to-cell spread (Coffey *et al.*, 2000). *P. gingivalis* has been found to secrete over 20 proteases, some of which are thought to contribute to virulence (Curtis *et al.*, 1999).

The *F. tularensis* strain Schu 4 genome project was used to determine the sequences adjacent to these putative genes. The results of this search are shown in Figure 24. The presence of a possible transposase gene and a tRNA gene are of interest, as these are two hallmarks of pathogenicity islands. Pathogenicity islands are chromosomal gene clusters, containing genes that encode virulence factors, that can be acquired horizontally. They often integrate into the genome at tRNA loci. The percentage guanine and cytosine content of this region was approximately 32 %, which is within the normal range for *Francisella* DNA. Further analysis of this region is currently underway.

Conclusions and Future Research

Pathogenic bacteria possess numerous mechanisms that allow them to survive inside mammalian hosts. It is becoming increasingly apparent that these mechanisms are often common to many different bacteria, although they may be employed for diverse purposes. For example, the protein secretion system III (PSS III) has been found in a diverse group of bacteria, including well-studied pathogens such as *Salmonella* and *Shigella*, and plant pathogens such as *Erwinia* and *Pseudomonas* species. These commonalities are in part due to the mobile nature of the genetic elements, such as plasmids and pathogenicity islands, that encode them. It is, therefore, important to acknowledge that insights into the strategies employed by one pathogen can help in the study of unrelated organisms. However, despite the widespread prevalence of the PSS II, it is not found in all bacterial pathogens. *Legionella* and, reportedly, *Francisella* do not possess the PSS III, and must secrete virulence factors by other mechanisms. Presumably, studying many different types of bacterial pathogens will reveal additional common themes, as well as important differences.

Very little is known about the pathogenesis of *Francisella*, and few virulence factors have been identified prior to this study. This work describes the identification of eight genetic loci involved in intracellular survival of *F. novicida*. It demonstrates, for the first time, that large-scale mutagenesis and intracellular growth screening of *F. novicida* can be accomplished with the genetic tools and techniques available. Five of the eight genetic loci identified show similarity to database entries at the deduced amino acid level.

The identification of *fip*, which apparently encodes a protein identical to a 23 kDa protein involved in *F. tularensis* intracellular growth, is of special interest. In *F. tularensis* its expression has been shown to be induced two hours after bacterial entry into the host cells, as well as after exposure to hydrogen peroxide. This work has demonstrated that a *F. novicida fip* mutant showed decreased growth in macrophages, and was sensitive to hydrogen peroxide as compared to the wildtype strain. Further studies are required to elucidate the role of Fip in intracellular survival. Initial studies would determine whether this protein is secreted, although it appears as though it is not which precludes a direct role as an effector molecule. It is possible Fip is involved in the regulation of virulence effector molecules. Hydrogen peroxide is important for the induction of apoptosis, and it may be interesting to explore this connection.

Four of the *F. novicida* mutants had interrupted genetic regions similar to genes for a stress response protein, a purine biosynthesis protein, alanine racemase, and a protein of unknown function, respectively. Homology only suggests biochemical activity, however, and the role of these putative proteins is conjecture at this point. The genetic loci identified in the other three mutant strains showed no similarity to known genes. As the *F. tularensis* genome becomes available, further information about these possible genes and surrounding genes will provide clues as to their importance in virulence. The genome project may identify pathogenicity islands and regions of chromosomal DNA important in virulence.

This work presented growth assays of a *F. novicida* 70 kDa protein mutant and demonstrated decreased intracellular growth as compared to wildtype, which suggests this protein plays a role in virulence. Initial sequencing of the genetic locus containing the 70 kDa protein gene revealed a putative gene encoding bacterial chitinase, and a putative endopeptidase gene that showed homology to mammalian endothelin-converting enzyme, at the deduced amino acid level. This genetic region corresponds to a locus in *F. tularensis* which contains genes encoding a putative transposase and threonyl tRNA synthetase. These types of genes are often found in pathogenicity islands, but their purpose here is unknown. Further investigation would determine if this region may contain a mobile genetic element such as a pathogenicity island.

The genetic techniques developed for *F. novicida* in this study validate *Francisella* as a bacterial pathogen which can be studied with the aim of elucidating mechanisms of pathogenesis. The sequencing data presented here and elsewhere has confirmed the high degree of similarity between *F. novicida* and *F. tularensis*, and established the former as a viable mouse model for the investigation of tularemia. The availability of the *F. tularensis* genome sequencing data will greatly aid in future work of this nature.

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Appendix

GAATAACCCAAAAATAAGTTAGTTAGGAGATTATATAAATGCTACGTAAGCTGGTTAAAA 60

AACTGATTCCGAGTCCAAGATAAAATCTTTTTTGATTNAATGATAGAAGCGACCAGAGGC 120

TTGTTGAAGACTCGGGNTAGAATTCTTGATAAGTTAGTTAAAGAAGAAGATAATTTAACT 180

ATTTTTGAATTATCTGAAGAACTAAGATTAACAAGANCGGTANCAGTTGAAGTAGCTAAT 240

AAAATGGATCATGAATTAGCTCGCTACTTTGTAACTCCTGTTGATAGAGTAGAGATACAT 300

AATATCACTACTTTGCTTTTAAAACTAAATCGAAGAATTGTTAAGATTCATCGATATATG 360

CAGATCTTGATGGAAGAAGAGCGTGGCAATGTAAATATATACTTGGCTAATAGTGTTGAA 420

ACACTACGTAAAATGACAAAATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAG 480

GAAACAGCTATGACATGATTACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGA 540

CCTGCAGGCATGCAAGCTTTCATCATATCATCTAAAACCTTTGTCATTTTACGTAGTGTT 600

TCAACACTATTAGCCAAGTATATATTTACATTGCCACGCTCTTCTTCCATCAAGATCTGC 660

ATATATCGATGAATCTTAACAATTCTTCGATTTAGTTTTAAAAGCAAAGTAGTGATATTA 720

TGTATCTCTACTCTATCAACAGGAGTTACAAAGTAGCGAGCTAATTCATGATCCATTTTA 780

TTAGCTACTTCAACTGTTACCGTTCTTGTTAATCTTAGTTCTTCAGATAATTCAGAAATA 840

GTTAAATTATCTTCTTCTTTAACTAGCTTATCAAGAATTCTAGCCGAGTCTTCAACAGCC 900

TCTGTCGCTTCTATCATTAAATC 923

Figure 25. DNA sequence adjacent to the transposon from *F. novicida* strain CG45. Arrow indicates approximate region of transposon integration.

AGGATATTAGAACGTGGAAGACTTTAAAGAGATAGNAATTATTGTNGATATTATATCAAC 60

TACAAGAGAAATTATAGANGANANTGANTANGATAATGAGTAAATATCATNCCNTCGTAA 120

TAATANAAGAACAAGTATTTTCTTCCTACAAGAAGAGCTCTTTNGAAANATATANNGCTN 180

CCGTCTGGGAATATATAGTTTTTCCGTTACTTGCATATGTTGATGAAAAGNTGATGCNAC 240

ATTAGGGANCAGTCAGCAAGCAGATATTTCTTGGAGTCTACTACNATTAGAATATTATGA 300

TAGNAAAGATGGCGGTGAATATGTTTTTGAGATANCTGATAATATTTTATCAGAGAATAT 360

CTATCCACAAATTTGCTACCAGACAATATCACTTATCCTACACNATGACTTCTATGGCAA 420

ATANTATGACNATATATATAACCATAGCTTTCTTGNTTATAAGAAAGACNTTGAGTTCCC 480

CCATATAGAAAATTCGACAATTGATTCGGTTAATTTTATTGATATACCCGTTAATAGCCC 540

CCCACTGTCAAGAAAATATAGTAAGACGCTTAAATTCTTATTAAGAATANGCGTNCCTTT 600

AGGACTGTTTNTTTTAAGCTTACTAATTTTATTAAGCTGGNANTAAGGGGTGNGAGGAGA 660

NTTATATGAGTCAGATAATATCTACACTNANTAATANTGGTTTAGTAACTCACGAAAAAG 720

GAAATCTGGTTAGTAATATGCANNTCAAGCNAANTATGAAAACGCTNNNTNTAAGTCATT 780

TATTGATCNATTAATAGATAATCTTAATACTACTTACAGCATACATATATATAACTTANT 840

TCTATCTTTNNATCAGAAATANGCCTCCCTTATATCNGGCGNTAGATCTCTNAAAAAATT 900

ANCTCNTATTTATCTATCTAAATACTAACTTCACAACGAAATAGTNCNTCCGAATCAAAA 960

GCCAANAAAAANCAGCTTAGCTTGGNTGNGGGAATGGACCCAAACCCCTTTAGTCNCCTT 1020

TTGGNATGGCCAAAA 1035

Figure 26. DNA sequence adjacent to the transposon from *F. novicida* strain CG93. Arrow indicates approximate region of transposon integration.

GAGGTGATACNTGCTNGCNTGCAAACNNAAGGACCNGNGTTNAGNGGANTCAGTGNGCCG 60

AGGNAGCGAGAGGCGCGCCCAATATACACAGGGCGTNTNCCCANTGAGTTGNACCATNAA 120

CCNAATCAAGTTTTTTGGGGTNGAGGTNCCGTNAANCNCTAAATCGGAACCCTAAAGGGA 180

NCCCCCGATTTAGAGCTTGACGGGGAAANCCGGCGAATGTGGCGAGAAAGGAAGGGNAGA 240

AANNGAAAGGAGCGGGNGGTAGGGCGATGGCAAGTNTANCGGTCACATTGCGCGTNACCA 300

CCACACCCNCCNCGCNTAATGCNCCGCNNCNGGGCGCGTTCCATTCNCCATTCAGGCTGN 360

TCAACTGTTGGGAAGGNCNATCGTTGCGGGCCTTTTCGCTATTANGCCATNTNGCNAAAG 420

NGNNATGTGANCCAAACAGATTANANTGGCTTAGCGCAGGTNNTCTCAGTNATNACGTTN 480

TTNTATNACGACCATNGNATTCGTCACCATCGNACTCTAACTTTCCCATTTTTTGACACA 540

TGCCCGCGAAGGTTATAGATTTCAGCCTGACAGAAATGGGCTTTGAAGCTTAGCGTACGA 600

TTTTTTCCGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCA 660

AGCTTTCCCTATAGTGAGTCGTATTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCT 720

GTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGT 780

AAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCC 840

GCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGG 900

AGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCG 960

GTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACA 1020

GAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGGCAGGAAC 1080

CGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAAGCTCCGCCCCCTGACAGCATCACAA 1140

AAATCGACGCTCAAGTCAGAGGTGGNGAAACCGACAGGACTATTAAGATACAGCGTTTCC 1200

CCTGGAACTCCCTCGTGCGCTCTCTGTTCCAACCTGGCGCTTACGGATACTGTCCGCCTT 1260

TCTCCTTCGGGAACGTGGGC 1280

Figure 27. DNA sequence adjacent to the transposon from *F. novicida* strain CG124. Arrow indicates approximate region of transposon integration.

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