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Studies of the lipopolysaccharide from the intracellular pathogens *Francisella tularensis* and *Francisella novicida*

by

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B.Sc., University of Victoria, 1992

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

DOCTOR OF PHILOSOPHY

in the Department of Biochemistry and Microbiology

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ABSTRACT

Francisella tularensis and *Francisella novicida* are closely related facultative intracellular pathogens capable of survival and growth within macrophages. In this work we present evidence to show that *F. tularensis* uses phase variation to alter lipopolysaccharide (LPS) antigenicity, macrophage nitric oxide (NO) production, and microbial intramacrophage growth. The LPS and lipid A of *F. tularensis* LVS fail to stimulate production of significant levels of nitric oxide by rat macrophage monolayers. However, spontaneous variants of *F.tularensis* expressing an antigenically distinct LPS induce rat macrophages to produce increased levels of NO, thereby suppressing intracellular growth. This new form of LPS produced by *F. tularensis* is also the predominant form of LPS found normally in *F. novicida*. Rat macrophages infected with *F. novicida* produce high levels of NO and exhibit suppression of intracellular growth. LPS and lipid A isolated from *F. novicida* and variants of *F. tularensis* stimulate increased levels of NO production. In addition, a reverse phase shift can occur which returns the LPS of the *F. tularensis* variants to the original antigenic form, resulting in reduced macrophage NO production and restoration of intracellular growth. These results suggest that *F. tularensis* can modulate macrophage NO production through phase variation of its LPS.

It was of interest to initiate a study that would ultimately characterize the molecular mechanism of LPS phase variation in *Francisella tularensis*. To this end, we used shuttle mutagenesis to create a mutant library of *F. novicida*. We mutagenized a size-restricted plasmid library of *F. novicida* with the erythromycin-resistant transposon TnMax2. Putative *F. novicida* LPS mutants created by shuttle mutagenesis were screened visually for aberrant colony phenotypes on agar plates. Of 10464 mutants screened, 5 unique *F. novicida* LPS mutants were isolated which exhibit three distinct LPS phenotypes as determined by Western immunoblot. A single mutant from each of the three phenotypic groups was further characterized with respect to DNA sequence analysis, intramacrophage

growth, and sensitivity to detergent and serum complement. Furthermore, these three loci were shown to hybridize with a corresponding locus in *F. tularensis* LVS. However, there was no difference in the restriction pattern of the hybridizing bands between LVS and its LPS phase variants, thus indicating that no major genetic rearrangements or insertion/deletion of a large mobile genetic element occurs in these genes during the phase variation process of *F. tularensis*.

The *F. novicida valAB* locus has previously been cloned, sequenced, and shown to be functionally homologous to the *E. coli* genes *msbA/lpxK*. In order to investigate the hypothesis that *valAB* is involved in transport of LPS to the cell surface, an *E. coli* strain harboring an NTG-mutagenized temperature sensitive (t.s.) allele of *valAB*, a non-functional copy of *msbA/lpxK*, and an IPTG-inducible copy of the gene encoding the Chlamydia trachomatis genus-specific LPS epitope (*gseA*) was constructed. In this study, DNA sequencing was used to locate the temperature sensitive mutations in the *valAB* locus. Two C to T transitions were found in the *valA* coding region which result in a S to F change at amino acid 543 and a T to I change at amino acid 458. The ability of *E. coli* cells harboring this t.s. copy of *valAB* to transport the *Chlamydia* LPS epitope across the inner membrane at the permissive and non-permissive temperatures was determined using sucrose density gradient centrifugation and ELISA. It was determined that there was increased association of the LPS epitope with the inner membrane at the non-permissive temperature, thus suggesting that ValA is required for transport of an LPS precursor across the inner membrane.

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ACKNOWLEDGMENTS

I would like to thank all the individuals of the Department of Biochemistry and Microbiology who have helped me, guided me, and supported me during my stay at the University of Victoria. In particular, I would like to thank my supervisor, Dr. Francis Nano, for his patience and support. Special thanks go to Craig McDonald for his invaluable attention, support, and friendship. Best wishes go to the members of the Nano laboratory past and present in their future endeavors!

DEDICATION

I would like to dedicate this work to my parents, Owen and Maeve Cowley.

INTRODUCTION

(A.) *Francisella*

Francisella species are Gram negative cocco-bacilli which measure as small as 0.2-1.7 μm in length (Nano, 1992). Previously placed in a variety of genera, including *Pasteurella* and *Brucella*, the members of the genus *Francisella* have recently been classified in the γ subclass of Proteobacteria, and 16S rDNA sequence analysis reveals their closest relative to be the obligate intracellular pathogen *Wolbachia persica*. (Bell, 1981; Forsman *et al.*, 1994) Members of the genus *Francisella* may further be distinguished by their unusual fatty acid composition (Hollis *et al.*, 1989). The object of study in this thesis is *Francisella tularensis*, a facultative intracellular pathogen and the causative agent of a zoonotic febrile illness known as tularemia.

(1.) The disease manifestations of tularemia

Similar to a number of other pathogens, the outcome of a *Francisella* infection can vary depending on the route of inoculation and the natural resistance of the host. More specifically, tularemia can have both cutaneous and systemic manifestations, and the animal reservoirs of *Francisella* display a wide range of susceptibilities to infection. This section will briefly discuss the disease manifestations of tularemia.

The ulceroglandular (or cutaneous) form of tularemia generally arises as a result of entry via the skin through the bite of an arthropod vector or direct contamination of a wound (Bell, 1981). Human tularemia is commonly contracted in this manner during the dressing of hares following hunting, and thus has come to be known as "rabbit fever". This generally results in an ulcer at the site of inoculation followed by swelling of local lymph nodes accompanied by fever, headache, and malaise (Tarnvik, 1989). The systemic, fulminating manifestation of a *Francisella* infection is also known as the typhoidal form of tularemia, and may arise from inoculation via various routes. For

example, a *Francisella* infection initiated through any route (such as intradermal, intranasal, and intraperitoneal) in inbred laboratory mouse strains will ultimately result in dissemination of the bacteria, probably via the blood or lymphatic system, to reticuloendothelial organs such as the spleen, liver, and lungs (Fortier *et al.*, 1991). Bacteria may be cultured from these organs within 24 hours after infection, depending on the route of inoculation. Similarly, a typhoidal form of tularemia may also be initiated through ingestion of contaminated material or inhalation of an aerosol. Curiously, while the 50% lethal dose (LD_{50}) of laboratory mice infected with *Francisella* via the intravenous, intranasal, or intraperitoneal routes may be as low as a single bacterium, the LD_{50} of mice inoculated intradermally or subcutaneously is considerably higher (10^4 to 10^7 bacteria) (Elkins *et al.*, 1992; Fortier *et al.*, 1991). These findings are reflected in the disease severity of human *Francisella* infections. A primary pneumonic tularemia resulting from inhalation of a highly virulent strain of *F. tularensis* may result in a mortality rate of as much as 60% if left untreated, whereas infection resulting in the ulceroglandular form of tularemia has a mortality rate of only 5% (Evans *et al.*, 1985). The immune cells responsible for the enhanced resistance following intradermal inoculation are proposed to be dendritic in origin, but remain to be identified (Fortier *et al.*, 1991).

(2.) The Genus *Francisella* and Disease Severity

Multiple factors can influence the outcome of a *Francisella* infection. As described above, the route of inoculation can have a significant influence on disease severity. In addition, different animal species exhibit different levels of natural resistance to infection. Finally, there are several *Francisella* species, biotypes, and strains that exhibit different levels of virulence in the different animal models. Indeed, serial passage of *Francisella* strains on laboratory media or in animal hosts can significantly decrease or increase the relative virulence of the organism, respectively. This section will attempt to briefly

describe and underscore the importance of natural resistance to infection, as this concept is important to experiments described in Chapter 2.

First, it is necessary to describe the different species and biotypes of *Francisella* (summarized in Table 1). Currently, there are three recognized *Francisella* species, named *F. tularensis*, *F. novicida*, and *F. philomiragia*. *F. philomiragia* was initially isolated from water in Utah containing dead muskrats (Jensen *et al.*, 1969). *F. philomiragia* is poorly studied and thought to be avirulent for humans; it has been clinically encountered only 14 times since its discovery in 1959, and the majority of cases were either immunocompromised patients or near-drowning victims (Hollis *et al.*, 1989). Conversely, *F. tularensis* is relatively well studied and generally considered to be virulent for humans; however, it is classified into three biotypes (for simplicity, these biotypes will be referred to as type A, type B, and *F. novicida*) which exhibit widely different levels of virulence.

In the wild, *Francisella tularensis* strains have been cultured from a wide variety of animal species, including rabbits, hares, mice, rats, muskrats, and beavers (Bell, 1981). Additional laboratory isolates have come from ticks, humans, and water. Laboratory experiments have generally focussed on rabbits, monkeys, mice, and rats as models for *Francisella* infection, although the vast majority of the studies have used mice. The easiest method for describing the virulence of a pathogen is to cite the 50% lethal dose (LD_{50}) of the microbe for an animal model, as this provides a statistical representation of the number of organisms required to kill 50% of the animals tested in a given experiment. Unfortunately, this number does not provide much information regarding the subtleties of the disease progression, including dissemination of the organism to various organs and the time course of the infection. However, through examination of LD_{50}s it is possible to generalize that the animals models of *Francisella* may be classified from lowest to highest susceptibility to *Francisella* infection in the order rats, rabbits, guinea pigs, and mice.

Table 1 *Francisella* Species and Biotypes

***Francisella* species**	**Relative virulence (for humans)**	**Geographical location**	**Site of first recorded isolation**
Francisella tularensis biotype A	High	North America	Described as a plague in ground squirrels in 1911 (McCoy *et al.*, 1912)
Francisella tularensis biotype B	Medium	North America, Europe, and Asia	Described frequently under a variety of names (e.g. 'hare meat disease' in Japan, 1837) (Bell, 1981)
Francisella tularensis biotype *novicida*	Low	North America	Contaminated water in Utah (Larson *et al.*, 1965)
Francisella philomiragia	Low (immunocompromised individuals)	North America	Contaminated water in Utah (Jensen *et al.*, 1969)

The type A biotype of *F. tularensis* has been isolated only in North America, and is generally considered more virulent for animals and humans (Table 1). In humans, the type A biotype can cause a severe form of tularemia that may be fatal if left untreated. Conversely, the type B biotype of *F. tularensis* may be found in North America, Europe, and Asia, and is generally considered less virulent for animals and humans (Nano, 1992). These two biotypes may be distinguished biochemically only by differences in the ability to ferment glycerol and the presence of citrullineureidase activity, as both characteristics are possessed by type A strains but not type B strains (Bell, 1981). The live vaccine strain (LVS) of *Francisella tularensis* is an attenuated type B strain of *Francisella* developed in Russia for vaccination of the general public, although subsequent passage through mice in the United States has increased its virulence (Eigelsbach *et al.*, 1961). Although still avirulent for humans, LVS is highly virulent for mice and thus is frequently the current *Francisella* strain of choice for laboratory studies (Fortier et. al., 1991).

In contrast, *Francisella tularensis* biotype *novicida* has been poorly studied and was initially isolated from the environment in a contaminated water sample collected in Utah. *Francisella novicida* has recently been classified as a biotype or subspecies of *Francisella tularensis* based on high similarity (99.6%) between the 16S rDNA sequences of *F. tularensis* and *F. novicida* (Forsman *et al.*, 1994). In addition, the relatively high phenotypic similarity, DNA-DNA hybridization studies indicating DNA relatedness of 87-92% (Hollis et. al., 1989), and the ability to perform cross-species DNA transformation (Anthony et. al., 1991) also suggests a close relationship between these two bacteria. However, *F. novicida* is considered less virulent than *F. tularensis* due to a lower LD_{50} in mice of 10-100 bacteria (Owen *et al.*, 1964). Furthermore, *F. novicida* is considered avirulent in humans as there have been only two reported human clinical cases (Hollis *et al.*, 1989).

Despite the reduced virulence of *F. novicida* as compared to *F. tularensis*, the tularemia-like illness caused by *F. novicida* in mice is remarkably similar to that caused by

F. tularensis. More specifically, bacteria may be cultured from the reticuloendothelial organs of mice within 24 hours after intraperitoneal inoculation, and the bacterial burden in these organs increases exponentially over a 72 hour period (Anthony *et al.*, 1994). Post-mortem examination of the spleen and liver reveals necrotic foci similar to those seen following infection with *F. tularensis* (Anthony *et al.*, 1994).

Since *Francisella* is thought to survive and grow primarily within macrophages in the host, it is not surprising that numerous studies have sought to define the relationship between *Francisella* and the macrophage. Interestingly, in some instances, virulence of a particular *Francisella* strain for a given animal host may be correlated with the ability to grow *in vitro* within host macrophages. For example, in the rabbit model, Nutter *et al.* (1966) demonstrated that after 48 hours as many as 100% of rabbit alveolar macrophages were killed following infection with a highly virulent *F. tularensis* type A strain, whereas only 40% of the macrophages were killed in the same time period by the less virulent LVS. Given that the macrophage is an important link between the innate and acquired immune defenses, as well as a potent producer of immune modulating cytokines, it is not surprising that the ability of different *Francisella* strains to subvert macrophage defenses may have a significant effect on the outcome of disease. For example, studies by Anthony *et al.* (1991) reveal that *F. tularensis* LVS can proliferate exponentially in rat, mouse, and guinea pig macrophage monolayers *in vitro*. In contrast, *F. novicida* could only grow in mouse and guinea pig macrophages, and was unable to proliferate in rat macrophage monolayers. It is interesting to note that the rat model is more resistant to *F. novicida* infection than mice and guinea pigs (Owen *et al.*, 1964). Thus, further investigations of *Francisella*-macrophage interactions may reveal mechanisms used by intracellular pathogens to evade host defenses, as well as help elucidate important variations in the immune systems of different animal models.

(3.) *Francisella* Infection and Immunity - Major Concepts

Francisella tularensis is a Gram negative facultative intracellular pathogen and the causative agent of the disease tularemia. Within the host, *Francisella* survives and grows primarily within immune cells called macrophages, although there is evidence to indicate that *Francisella* can also invade and grow within hepatocytes (Conlan and North, 1992). In this section, I will briefly provide an overview of the host immune defenses relevant to a *Francisella* infection, and some of the mechanisms used by *Francisella* to avoid killing by the immune system.

(a) The Early Innate Defenses

The body has numerous natural defenses designed to attack and kill an invading microorganism. First, the skin presents an impenetrable physical barrier for many microbes. In the case of *Francisella*, infection is initiated when this barrier is broken through a tick bite or an open wound, or conversely, the organism may be ingested through the consumption of contaminated material or inhaled into the lungs as an aerosol. Once the organism has gained access to the internal tissues, a myriad of innate host defenses are available to attack the microrganism. For example, the serum complement cascade is a relatively non-specific innate host defense which may be activated by either microbial cell surface components or the binding of specific antibodies to the organism. However, although the lipopolysaccharide on the *Francisella* cell surface is known to activate the classical complement cascade (Fulop *et al.*, 1993), all wild type *Francisella* strains tested to date are resistant to nonimmune serum complement (Anthony *et al.*, 1994; Rhinehart-Jones *et al.*, 1994; Lofgren *et. al.*, 1983). This resistance to serum complement may at least partially be attributed to the protection provided by LPS O-antigen (see Chapter 4).

Soon after inoculation with *Francisella*, the short-lived phagocytic cells called neutrophils are drawn to the site(s) of infection. These cells are capable of unleashing a uniquely potent array of reactive oxygen intermediates (ROI) during phagocytosis in an

event called the oxidative burst, which includes the toxic metabolite hypochlorous acid (HOCl). Although some *Francisella* strains are susceptible to HOCl, the carbohydrate capsule surrounding the organism may be anti-phagocytic and help to protect *Francisella* from uptake and killing by neutrophils (Lofgren *et al.*, 1983). One study indicates that neutrophil phagocytosis and killing of *Francisella* can only occur efficiently in the presence of opsonizing immune serum, which is only available following a prior exposure to *Francisella* (Lofgren *et al.*, 1983). In addition, a neutrophil respiratory burst-inhibiting acid phosphatase has been identified in *F. tularensis* (Reilly *et al.*, 1996). However, several other studies indicate that neutrophils may play a critical role in host survival of a *Francisella* infection. For example, mice depleted of neutrophils and eosinophils by treatment with granulocyte-specific antibodies succumb to otherwise sublethal doses of *F. tularensis* (Sjostedt *et al.*, 1994; Elkins *et al.*, 1996). This protective role of neutrophils during a *Francisella* infection is proposed to be a result of neutrophil-mediated lysis of invaded hepatocytes in order to release and expose the proliferating bacteria to attack by macrophages (Conlan *et al.*, 1992). Neutrophils may also be instrumental in recruiting other immune cell types to the infectious foci through production of chemoattractants and activating cytokines.

The next main line of non-specific defense available early in an infection are macrophages and monocytes, which may either be fixed within the tissues or recruited to the site of infection. Similar to neutrophils, these phagocytic cells are also capable of generating an oxidative burst upon phagocytosis, although unlike neutrophils, the ROI produced by macrophages do not include HOCl. Once initially inside the macrophage, the organism resides within a membrane-bound vesicle called a phagosome. In a typical infection, phagosomes are short-lived, as lysosomal granules within the macrophage quickly fuse with the newly formed phagosome containing the microorganism, and deliver an array of microbiocidal agents which include lipases, proteases, and cationic peptides. However, *Francisella* is capable of evading these macrophage defenses. *Francisella* gains

entrance into the macrophage in an event which is mediated by currently unknown receptor-ligand interactions, and is apparently resistant to the ROI elaborated by the macrophage (Anthony *et al.*, 1994). Most *Francisella* strains tested to date have been shown to be relatively resistant to such toxic substances as superoxide anion, hydrogen peroxide, and hydroxyl radical (Anthony *et al.*, 1994; Lofgren *et al.*, 1984). In addition, electron microscopic evidence indicates that *Francisella* resides in the macrophage within a membrane bound phagosome that appears to remain unfused with secondary lysosomes. (Anthony *et al.*, 1992) However, the *Francisella*-containing phagosome matures sufficiently to allow for acidification, which apparently is necessary to release essential iron for growth from transferrin (Fortier *et al.*, 1995). The mechanism by which *Francisella* inhibits phago-lysosomal fusion remains unknown, but this is a survival tactic used by other intracellular pathogens such as *Mycobacterium tuberculosis* in order to avoid the toxic contents of the lysosome (Clemens, 1996).

Once inside the macrophage phagosome, *Francisella* apparently replicates freely, and *in vitro* observations of cultured murine macrophages infected with *Francisella* indicate that this organism will replicate until it lyses and kills the macrophage. Presumably, this provides the newly relased bacteria with the opportunity to infect adjacent cells. *In vitro*, it appears that in the absence of help from other immune cell types, the mouse macrophage is incapable of inhibiting *Francisella* growth. However, different animal reservoirs for *Francisella* display a range of levels of natural resistance which may at least be partially attributed to the ability of macrophages from the different animal species to limit intracellular replication of *Francisella* (Cowley *et al.*, 1997; Anthony *et al.*, 1991; also see Chapter 2).

(b) Macrophage Activation and Control of Early and Late *Francisella* Infection

In the animal host, macrophages are not isolated from the other cells of the immune system. The repertoire of macrophage killing mechanisms is greater than those described above, but typically the macrophage requires "help" from other immune cell types in order to activate these killing mechanisms. This help generally comes in the form of cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), which are secreted by immune cells such as the macrophage (TNF-α) or T cells and natural killer cells (IFN-γ). These cytokines can transform the macrophage into a spectrum of "activated" states with a new array of more potent killing mechanisms at its disposal. For example, *in vitro* cultured mouse macrophages infected with *Francisella* and treated with IFN-γ will produce the cytotoxic effector molecule nitric oxide (NO), which has been shown to be effective at inhibiting the growth of *F. tularensis* (Anthony *et al.*, 1992; Fortier *et al.*, 1992; Green *et al.*, 1993). This NO production is dependent on the autocrine action of macrophage-produced TNF-α (Fortier *et al.*, 1992). Macrophage production of NO in response to IFN-γ activation and subsequent killing of invading microbes is a common scenario described for other intracellular pathogens, including *Leishmania major* and *Toxoplasma gondii* (Adams *et al.*, 1990; Green *et al.*, 1990).

Although NO is effective at inhibiting *Francisella* growth both *in vivo* and *in vitro* (Anthony *et al.*, 1992; Green *et al.*, 1993), it remains unclear as to whether this molecule has a bactericidal or merely a bacteriostatic effect during an infection. It is interesting to note that IFN-γ-activated murine alveolar macrophages are capable of limiting *F. tularensis* growth by an undefined mechanism which is not dependent on NO production, thus suggesting that macrophages may have potent IFN-γ-activatable microbiocidal mechanisms which remain uncharacterized (Polsinelli *et al.*, 1994).

Activation of mouse macrophages during an infection to produce NO requires the production of IFN-γ by either the innate immune defense system through NK cells, or the

specific acquired immune defense system through T helper cells. However, in order to become activated to produce IFN-γ, both NK cells and T helper cells require signals from the infected macrophage or other immune cells. Since it takes several days for specific αβ T cells to become activated and proliferate to numbers effective for protection, this arm of the immune system is unlikely to be an early source of IFN-γ. Conversely, NK cells are a component of the innate immune defense system, which means that they are relatively non-specific and are good candidates for the control of the early stages of an infection. Several studies indicate that in order for NK cells to become activated to produce IFN-γ, one possible combination of activating signals are the cytokines TNF-α and IL-12 (Gazzinelli *et al.*, 1993; Tripp *et al.*, 1993). These two cytokines are typically produced by macrophages in response to various bacterial products, such as bacterial DNA. Indeed, one study has demonstrated that within the first 48 hours of murine tularemia, cytokine mRNA production in the liver of infected mice includes TNF-α, IL-12, and IFN-γ (Golovliov *et al.*, 1995). The T cell-independent nature of this response was inferred from the apparent lack of production of the autocrine T cell cytokine IL-2 in the livers over the same period. In addition, other studies have confirmed the requirement for IFN-γ and TNF-α during early resistance to *Francisella* infection (Anthony *et al.*, 1989; Leiby *et al.*, 1992; Elkins *et al.*, 1993). Not surprisingly, removal of the TH2-type cytokine IL-4 by administration of neutralizing antibodies to LVS-infected mice appears to have little effect on the course of infection (Leiby *et al.*, 1992).

Further studies implicate NK cells in the control of the early phase of a primary *F. tularensis* infection. T cell deficient mice such as athymic nu/nu mice and total lymphocyte deficient (B and T cell) *scid* mice are capable of controlling the early phase of an intradermally-inoculated infection (the first 2-3 weeks), but become moribund by day 30 post-infection (Elkins *et al.*, 1993; Elkins *et al.*, 1996). This is in comparison to normal control mice, which resolve the infection after 21 days. This is a common scenario for resistance to an intracellular pathogen infection, whereby there is an early non-specific T

cell-independent phase of resistance to infection (thought to be mediated by NK cells), followed by a later specific T cell-dependant phase required for final resolution of the infection. Despite abundant evidence implicating NK cells in this early T cell-independent resistance in the *Francisella* model, NK cell depletion studies remain to be performed. The requirement for a late T cell-dependant phase to resolve a *Francisella* infection suggests that early NO production by macrophages in response to putative NK cell-produced IFN-γ is not sufficient to eliminate a *Francisella* infection, and that a further (as yet unidentified) T cell-dependant function is necessary to completely eliminate the *Francisella* infection. Recent studies using either *in vivo* depletion of the T cell subsets or T cell receptor knockout mice indicate that either $CD4^+$ or $CD8^+$ T cells are sufficient to mediate late clearance of a *Francisella* infection (Yee *et al.*, 1996).

(c) B cell-mediated Immunity

Conversely, antibody (Ab)-mediated immunity appears to play only a minor role in a primary *Francisella* infection. Indeed, early T cell-independent survival to a *Francisella* infection in *scid* mice has been described, and long term survival of *scid* mice can be achieved by reconstitution with purified T lymphocytes lacking total B cells (depleted by treatment with anti-B220 Ab), thus suggesting that B cells are not required for either early or late survival of a primary *Francisella* infection (Elkins *et al.*, 1996). Furthermore, although significant passive immunity to *Francisella* could be generated in non-immune mice through administration of serum from infected mice (Foshay, 1946; Rhinehart-Jones *et al.*, 1994; Fortier *et al.*, 1991), further investigation demonstrated that this immunity was not actually passive but instead was dependant upon a host T cell response (Rhinehart-Jones *et al.*, 1994).

Despite the apparent lack of necessity for B cells in resolution of primary murine tularemia, a novel role for B cells in the generation of an unusual early protective immunity to an *F. tularensis* secondary challenge has been identified (Culkin *et al.*, 1997). As early

as three days after a sublethal *F. tularensis* infection, normal and athymic nu/nu mice exhibit strong protection against a lethal LVS challenge. Curiously, *scid* mice did not exhibit this early protective immunity, despite the fact that they possess a population of NK cells, which are generally thought to be responsible for early immunity. This protection was shown to be highly dependent on B cells and IFN-γ, but not on production of specific antibodies. The specificity of this protective response remains to be clearly defined (Elkins *et al.*, 1993; Culkin *et al.*, 1997). Although the aforementioned B cell depletion studies suggest that this early protective immunity does not play an essential role during a primary infection, this work suggests a novel effector mechanism for B cells during the early protective immune response to a secondary challenge with an intracellular pathogen.

(d) T cell-mediated Immunity

As previously mentioned, non-specific T cell-independent immune mechanisms are important for early defense during a *Francisella* infection, while specific T cell-dependant immunity is ultimately required for resolution of infection. Furthermore, T cell-dependant mechanisms are instrumental in protection from a secondary challenge. The events which lead to the establishment of T cell-mediated immunity are complex: macrophages or other antigen presenting cells (APCs) encounter *Francisella* antigen, and process this antigen for presentation on the APC cell surface in combination with MHC class II molecules. αβ T helper cells expressing a receptor specific for the antigen-MHC class II complex become activated and proliferate to produce a population of effector T cells. These effector T cells, in the case of a *Francisella* infection, have been shown to release cytokines such as IFN-γ and IL-2 (Tarnvik *et al.*, 1992). This results in macrophage activation as well as activation and proliferation of cytotoxic ($CD8^+$) T cells. Cytotoxic T cells may then be involved in lysis of intracellularly infected cells expressing specific MHC class I-antigen complexes.

T cell-dependant resistance to a *Francisella* infection appears to be mediated predominantly by αβ T cells of the $CD4^+$ or $CD8^+$ lineage (Anthony *et al.*, 1988; Yee *et al.*,

1996), and not $\gamma\delta$ T cells. Indeed, knockout mice with targeted gene disruptions lacking the $\gamma\delta$ T cell receptor readily survive sublethal infection with *Francisella* (Yee *et al.*, 1996). This is in contrast to other intracellular pathogens such as *Listeria monocytogenes* and *Mycobacterium tuberculosis*, where there is evidence to indicate that $\gamma\delta$ T cells play a role in immunity (Ladel *et al.*, 1996).

There is considerable evidence to indicate that both $CD8^+$ and $CD4^+$ T cells are activated during a *Francisella* infection in response to various *Francisella* antigens. Most of the studies investigating specific *Francisella* antigen-T cell interactions have focussed on human T cells acquired from vaccinated or infected individuals and membrane antigens isolated from *F. tularensis* LVS. The presence of antigen-specific T cells is usually assayed *in vitro* by measurement of lymphocyte proliferation or cytokine production in response to specific antigen presented by APCs. In the case of a *Francisella* infection, antigen-specific lymphocyte proliferation develops as early as a week or two after vaccination or infection (Karttunen *et al.*, 1991), and can remain detectable for decades (Tarnvik *et al.*, 1985; Ericsson *et al.*, 1994). All of the T cell clones isolated to date from humans vaccinated with *F. tularensis* LVS are $CD4^+$ $\alpha\beta$ T cells. There have been fewer studies aimed at investigating the role of $CD8^+$ T cells. Nonetheless, $CD8^+$ and $CD4^+$ T cell proliferative responses in response to various *F. tularensis* antigens have been demonstrated in peripheral blood lymphocytes taken from LVS-vaccinated or naturally infected individuals (Sjostedt *et al.*, 1992; Sjostedt *et al.*, 1990). Not surprisingly, both the primary and memory (CD45RO+) *Francisella*-specific $CD4^+$ T cell responses have indicated a predominance of the TH1-type of cytokines, including IFN$-\gamma$ and IL-2 (Karttunen *et al.*, 1987; Surcel *et al.*, 1989; Karttunen *et al.*, 1991; Surcel *et al.*, 1991; Sjostedt *et al.*, 1992). These cytokines are responsible for macrophage and $CD8^+$ T cell activation, and thus are ideally suited for defense against an intracellular pathogen. Indeed, it appears that the *in vitro* activation of *Francisella*-specific $CD8^+$ T cells is dependant upon co-cultivation with $CD4^+$ T cells or supplementation of the media with IL-2 (Sjostedt *et al.*,

1992). These studies demonstrating the development of *Francisella*-specifc CD4$^+$ and CD8$^+$ T cells in LVS-vaccinated individuals are in agreement with the previously described murine studies suggesting an essential role for either CD8$^+$ or CD4$^+$ T cells in the ultimate resolution of murine tularemia (Conlan *et al.*, 1994; Yee *et al.*, 1996). Redundancies in the immune system may allow for the functions of the cytotoxic (CD8$^+$) or helper (CD4$^+$) T cell subsets to be replaced in the artificially depleted murine systems described earlier: Surcel *et al.* (1991) have demonstrated the existence of *F. tularensis*-specific cytotoxic CD4$^+$ cells, while studies in murine *Leishmaniasis* have demonstrated the development of a class of helper T cells lacking CD4 in knockout mice defective for CD4 expression (Locksley *et al.*, 1993).

Immunity to a secondary *Francisella* challenge appears to be similar to that of a primary infection, in that there is an early T cell-independent phase followed by a later T cell dependant phase which may be mediated by either CD4$^+$ or CD8$^+$ T cells (Conlan *et al.*, 1994; Yee *et al.*, 1996). One significant difference is the previously mentioned novel role for B cells in protective immunity to an early secondary *Francisella* challenge. In both a primary and secondary exposure to *Francisella*, IFN-γ and TNF-α are the predominant cytokines expressed and are essential for resolution of the infection (Leiby *et al.*, 1992).

(4.) The Lipopolysaccharide (LPS) of *Francisella*

For a detailed description of bacterial lipopolysaccharide (LPS) structure, function, and effects on the immune system, please refer to Section B.

The LPS of *Francisella tularensis* does not exhibit the characteristics of a classical endotoxin. Numerous investigators have demonstrated that *Francisella* LPS is non-reactive in the limulus amaebocyte lysate assay, non-pyrogenic, and non-toxic for galactosamine-sensitized mice (Sandstrom *et al.*, 1992). Furthermore, production of the pro-inflammatory cytokines IL-1 or TNF-α from human monocytes and mouse macrophages in response to *F. tularensis* LPS is either absent or significantly reduced as compared to

Salmonella typhimurium LPS (Sandstrom *et al.*, 1992; Ancuta *et al.*, 1996). In addition, mouse macrophage production of the cytotoxic effector molecule nitric oxide (NO) in response to *F. tularensis* LPS is virtually undetectable (see Chapter 2). Not surprisingly, it appears that *F. tularensis* LPS does not interact with the classical LPS receptors, as it is unable to antagonize *Bordetella pertussis* LPS-induced effects on mouse macrophages (Ancuta *et al.*, 1996). Low toxicity of *Francisella tularensis* LPS may be an important requirement for subverting the defenses of the macrophage.

The type A and type B biotypes of *F. tularensis* apparently have no antigenic differences or serologically distinct strains, thus suggesting a conservation in the structure of the O-antigen of *F. tularensis* LPS . Chemical analyses performed by Vinogradov *et al.* (1991) revealed that *F. tularensis* O-antigen consists of repeating tetrasaccharide units composed of deoxy and dideoxy sugars derived from glucose and galacturonic acid. The structure of *Francisella* O-antigen was found to be similar to that of *Pseudomonas aeruginosa* and *Shigella dysenteriae* serotype O7.

In contrast, the carbohydrate composition of the core region and the structure of the lipid A of *Francisella* LPS has not been well studied, despite overwhelming evidence demonstrating an apparent lack of conventional LPS toxicity. There are reports of unusually low levels of 2-keto-3-deoxy octulosonic acid (Kdo) in the *Francisella* LPS inner core region as determined by biochemical assays, but this appears to be the limit of the information available (Sandstrom *et al.*, 1992). However, substitution of Kdo residues may influence the outcome of Kdo assays, providing a false measure of low Kdo levels.

Studies investigating the cellular fatty acid composition of *F. tularensis* have inadvertently revealed possible components of *F. tularensis* lipid A; unusual 2-hydroxydecanoate, 3-hydroxyhexadecanoate and 3-hydroxyoctadecanoate fatty acids were detected in whole cell lysates, but not in the phospholipids of *F. tularensis* (Jantzen *et al.*, 1979; Anderson *et al.*, 1986). These fatty acids may therefore be components of the lipid A. Indeed, structures such as these in a lipid A molecule could contribute to reduced

endotoxicity (please see Section B.2.c. for a discussion of the structures responsible for LPS toxicity).

(5.) *F. tularensis* Colony Variants and Phase Variation

The work to be described in this section is highly relevant to Chapters 2, and 3 of this thesis.

In 1951, Eigelsbach *et al.* isolated unusual colony variants from a culture of *Francisella tularensis* type A (Schu) and a less virulent type B strain (Jap). These colony variants exhibited differences in color and opacity from the parent strain which could be visualized with the aid of obliquely transmitted light and a dissecting microscope. Due to these differences in colony morphology, he referred to the parent strain as smooth (S) and the variant strains as gray or non-smooth (NS). Interestingly, although these variants are rarely seen in standard overnight cultures of *Francisella*, he discovered that prolonged incubation of broth cultures maintained at 37°C (pH 6.8) without agitation could significantly increase the numbers of NS variants. Indeed, after 8 days of incubation, cultures would consist of more than 60% NS variants.

Eigelsbach discovered that these findings closely resembled similar observations made with *Brucella abortus* (Braun, 1946). In the case of *B. abortus*, accumulation of NS variants in liquid culture correlated with increased resistance to a toxic metabolite (alanine) which accumulated in the medium. Similarly, incubation of fresh *Francisella* cultures with sterile filtrates from 8 day-old cultures resulted in faster establishment of high proportions of NS variants. Although Eigelsbach could not identify a toxic metabolite responsible for the establishment of NS *Francisella* variants, his hypothesis that the NS variants arise because they are better adapted to the environmental conditions arising from overpopulation, low pH, and low aeration remains well founded.

Although the NS variants initially appeared to be stable, frequent serial transfers were shown to result in the re-establishment of smooth colonies similar in morphology to

the original *F. tularensis* parent. Interestingly, colony morphology changes from smooth to non-smooth and back to smooth again correlated with variations in virulence in mice and the ability to generate protective immunity as a vaccine. Smooth Schu strains were shown to have a mouse LD_{50} of 1 -10 organisms, whereas the mouse LD_{50} of NS Schu variants is as much as 10^7 to 10^8 organisms. Surprisingly, although various smooth strains were protective, NS variants failed to protect mice challenged with 10-100 LD_{50}s of a smooth Schu strain (Moody *et al.*, 1955; Eigelsbach *et al.*, 1951). Thus, there is an obvious decrease in virulence for mice associated with the transition from a smooth to a non-smooth colony type, and this transition to a NS variant also coincides with a curious reduction in the protective capacity of this organism against re-challenge with a smooth strain. Indeed, in the 1955 control procedures for the production of the live *Francisella* vaccine, it was recommended that smooth colony forms must constitute at least 20-30% of the organisms in the vaccine (Tigertt, 1962). The demonstration that the appearance of NS variants is suppressed in smooth cultures which contain normal rabbit or guinea pig serum suggests that NS variants may have increased susceptibility to serum complement (Moody, 1955). However, direct measures of NS variant culture viabilities in the presence of serum were not performed.

Bacterial Lipopolysaccharide (LPS)

(1) Brief Introduction to LPS

Lipopolysaccharide (LPS) is the major constituent of the outer leaflet of the outer membrane of Gram negative bacteria. Figure 1 shows a simplified diagram of an LPS molecule. LPS is an amphipathic glycolipid, consisting of a hydrophobic lipid portion known as lipid A, covalently attatched to a hydrophilic complex polysaccharide. This carbohydrate portion is subdivided into two major regions consisting of the internal core region and the peripheral O side chain or O-antigen region. The core region may be further divided into the inner and outer core regions.

Since LPS comprises the Gram negative bacterial cell surface, this location provides LPS with a unique role in both bacterial physiology and bacterial infection. For example, LPS is often essential for the exclusion of a variety of potentially toxic molecules from the bacterial cell. Furthermore, the carbohydrate portion of LPS provides an immunodominant surface structure for immune recognition during infection, and in some cases this region may also be used for attachment and colonization within a host. Finally, the mammalian immune system has evolved to recognize most lipid A structures as a signal to indicate a

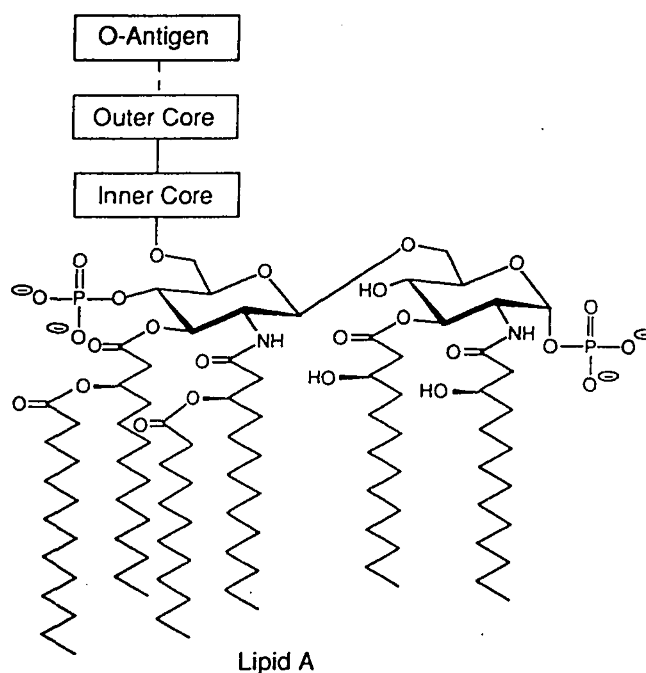


Figure 1. Schematic diagram of an LPS molecule.

bacterial invasion, and thus lipid A is often a trigger for the production of numerous cytokines. It is the ability of some lipid A structures to be potent stimulators of cytokine production from immune cells (primarily macrophages) that has resulted in the term endotoxin to be synonymous with lipid A. Endotoxic shock is a potentially lethal condition resulting from overproduction of cytokines in response to bacterial LPS. Thus, there are ongoing studies to characterize bacterial LPS structure, biosynthesis, and function. In addition, the presence of a minimal LPS structure at the bacterial cell surface is essential for bacterial cell viability and thus LPS is a potential target for antimicrobial therapy.

In this section, I will attempt to provide an overview of bacterial LPS structure and biosynthesis. Where appropriate, I will also describe the proposed importance of the various LPS structures in bacterial cell physiology as well as interactions with the host immune system. Since these topics comprise enormous and diverse areas of literature, in some cases the descriptions may be cursory in order to allow space for areas of research more relevant to this thesis. As will be described in this thesis, *Francisella* LPS appears to be interesting with respect to its biosynthesis as well as its interaction with the host immune system.

(2) Lipid A

(a) Structure

Although lipid A is the most conserved region of the LPS molecule, variations in lipid A structure may be found between and within bacterial species, and these variations frequently have profound effects on toxicity. Differences in lipid A structure and their effects will be described in Section B2c. The basic lipid A structure described here will be that of *Escherichia coli* and *Salmonella typhimurium*, as this lipid A is believed to be the most toxic and was the first to be characterized by Takayama *et al.* in 1983.

Figure 2 shows the minimal *E. coli* LPS structure previously thought to be required for bacterial cell viability (termed Re endotoxin), which consists of lipid A attatched to two

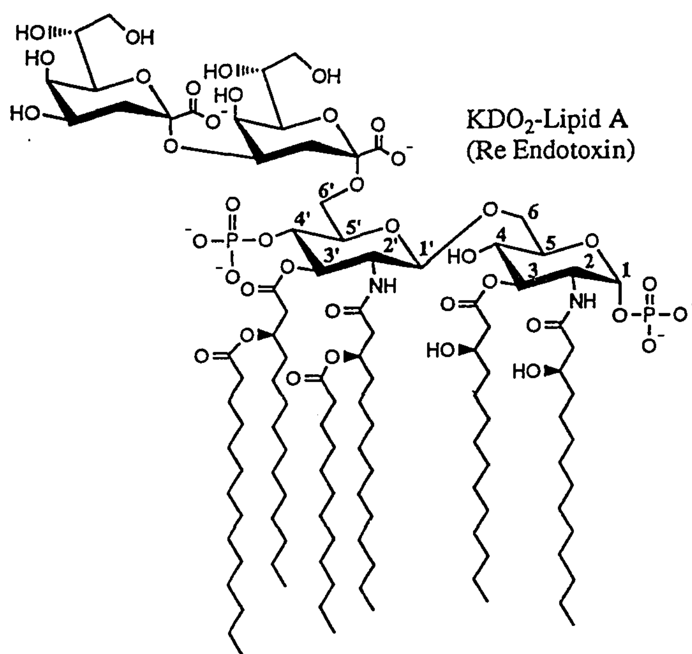


Figure 2. Chemical structure of Re endotoxin (KDO_2-lipid A).

carbohydrate residues of the inner core region (3-deoxy-d-manno-octulosonic acid or Kdo). Lipid A consists of a β-1-6 linked disaccharide of the sugar glucosamine that is phosphorylated at the 1 and 4' carbons of the sugars. A number for easy reference designates each of the glucosamine sugar carbons in Figure 2. These two central glucosamine residues are acylated with four short chain fatty acids (R-3-hydroxymyristate) attached to the carbons at the 2, 3, 2', and 3' positions via either amide or ester linkages. In addition, two of these fatty acids (at the 2' and 3' positions) are further esterified via their R-3-hydroxyl groups to an additional myristate or laurate moiety, to yield a lipid A molecule which contains six fatty acyl chains of 12 or 14 carbons in length. Finally, the inner core carbohydrate region is attached to lipid A via a Kdo moiety linked to the 6' glucosamine carbon. The branched LPS carbohydrate chain, which comprises the O-antigen and inner and outer core regions, is attached to the lipid A via this Kdo residue.

(b) Biological effects of lipid A in the host

Over 100 years ago, an unusual heat-stable and nonsecreted toxin in Gram negative bacteria was described which causes significant pathology in animals (reviewed in Raetz, 1993). This 'endotoxin', as it was described, could ellicit fever and result in an irreversible and lethal shock. It was not until the 1940's that endotoxin was proposed to consist of polymerized sugars, lipid, and phosphorus. Despite the early identification of endotoxin, it was only in 1983 that several laboratories converged on a complete structure for *E. coli* and *S. typhimurium* lipid A (Takayama *et al.*, 1983). Studies confirming that the lipid A portion of LPS was responsible for the observed toxicity in animals became possible following the chemical synthesis of a full lipid A molecule in 1984 by Shiba and Kusumoto. Despite the complexity of lipid A, further studies demonstrated that the entire lipid A structure, as opposed to merely some component, is necessary to achieve full toxicity in an animal model (Rietschel *et al.*, 1994). Ironically, although not entirely surprisingly, the very same potentially lethal effect induced by lipid A in the host was

discovered to actually be beneficial if limited to a local response of lower magnitude. The non-specific immune activation induced by LPS can have anti-bacterial, anti-viral, and anti-cancer effects.

Lipid A does not injure host tissue directly, but acts by inducing some host cell types to secrete mediators that when over-produced, such as during an over-whelming infection, can result in endotoxic shock (Rietschel *et al.*, 1994). These mediators act both locally and systemically to elicit a diversity of responses, which ultimately result in hypotension, reduced oxygen extraction by tissues, multiorgan failure, and death (Rietschel *et al.*, 1994). Although LPS can interact with a wide variety of cell types, the host cells proposed to be primarily responsible for production of these potentially toxic mediators are macrophages (Galanos *et al.*, 1986), and this section will therefore focus on lipid A-macrophage interactions.

The LPS-induced activation of macrophages results in the rapid production of a variety of lipids (prostaglandins, leukotrienes, and platelet activating factor), reactive oxygen and nitrogen intermediates, and peptide cytokines such as TNF-α, interleukin-1β (IL-1β), IL-6, IL-8, IL-10, and IL-12 (Luderitz *et al.*, 1989; Rietschel *et al.*, 1994; Hauschildt *et al.*, 1990; Loppnow *et al.*, 1989; Salkowski *et al.*, 1997). These bioactive molecules may then act to elicit either protective (at low concentrations) or harmful (at high concentrations) responses in the host (Parrillo *et al.*, 1993). High circulating levels of many of these cytokines may be found in humans and animals during endotoxemia.

TNF-α was first proposed to be the primary molecule responsible for instigating a cascade of events which results in endotoxic shock. Indeed, administration of anti-TNF-α mAbs prevents lethal endotoxemia in mice (Beutler *et al.*, 1985; Tracey *et al.*, 1987), and knockout mice deficient for the p55 TNF receptor are similarly resistant to endotoxic shock (Pfeffer *et al.*, 1993). Furthermore, administration of high levels of TNF-α can result in a condition similar to endotoxic shock in mice (Tracey *et al.*, 1986). Of course, further investigation reveals that multiple cytokines are essential for endotoxic shock. For

example, a receptor antagonist for IL-1 can reduce mortality from endotoxic shock in mice and rabbits (Ohlsson *et al.*, 1990;Alexander *et al.*, 1991; Wakabayashi *et al.*, 1991), and interferon-γ receptor deficient mice are resistant to endotoxic shock. Furthermore, depletion of IL-12 can also reduce the severity of endotoxemia in mice.

Thus, one possible scenario for development of endotoxemia would involve LPS-induced activation of macrophages resulting in production of IL-12 and TNF-α, which may then act in concert to induce IFN-γ production from NK cells. The combined action of LPS, IFN-γ, and TNF-α results in macrophage production of NO, the product of the enzyme inducible nitric oxide synthase (iNOS), which is proposed to be involved in hypotension during septic shock (Petros *et al.*, 1991). However, separate studies using iNOS knockout mice have both suggested and denied a role for NO in endotoxic shock (MacMicking *et al.*, 1995; Laubach *et al.*, 1995; Wei *et al.*, 1995).

Conversely, production of these cytokines at low concentrations results in a protective response to infection. For example, although TNF receptor deficient mice are resistant to endotoxic shock, they exhibit increased susceptibility to *Listeria monocytogenes* infection (Pfeffer *et al.*, 1993). Similarly, the essential role for IFN-γ-mediated induction of NO in resistance to infection with intracellular pathogens has been well documented (Adams *et al.*, 1990; Green *et al.*, 1990; Anthony *et al.*, 1992). Furthermore, IL-12 activation of NK cells and T cells is proposed to be important for the development of an appropriate immune response effective for the elimination of an intracellular infection (a TH1 response) (Hsieh *et al.*, 1993; Seder *et al.*, 1993; Tripp *et al.*, 1993), and IL-12-deficient mice exhibit increased susceptibility to the intracellular pathogen *Leishmania major* (Mattner *et al.*, 1996).

It is apparent that LPS-induced cellular responses are complex, and thus far attempts to block the interactions of LPS and the aforementioned cytokines with their target receptors as a therapy for endotoxic shock have been disappointing. The failure of this approach may be due, in part, to the essential nature of these cytokines during an infection

(Zisman *et al.*, 1997). Thus, investigations have focused on two other approaches: (1) the role of naturally-occurring cytokines involved in down-regulation of the proinflammatory immune response (such as IL-10 and IL-13) (Howard *et al.*, 1993; Muchamuel *et al.*, 1997), and (2) the search for LPS receptor antagonists coupled with investigation of the components of lipid A necessary for endotoxicity.

(c) Structure-Function Relationships of Lipid A

Despite the relatively conserved structure of lipid A among the various Gram negative bacteria, structural differences occur in some species that can profoundly effect endotoxicity. Variations in lipid A structure may include differences in the degree of phosphorylation, the nature of the glucosamine backbone, substitution of the phosphate groups, as well as differences in the length, number, structure, and location of the acyl chains. A few of these particular variations in structure have been associated with reduced toxicity relative to the prototypical *E. coli* LPS. However, when considering the following evidence, it is important to note that LPS 'toxicity' may be determined by a variety of methods, and different lipid A structures may not all contribute equally to the different measures of toxicity (Takada *et al.*, 1992).

Lipid A preparations from various bacteria as well as synthetic partial lipid A structures have been studied extensively in a variety of biological systems. It appears that both the hydrophilic and hydrophobic portions of lipid A are required for full toxicity (Kotani *et al.*, 1985). For example, studies evaluating cytokine production by murine macrophages and human monocytes have shown that lipid A structures lacking a phosphoryl group exhibit significantly reduced bioactivity (by a factor of 10^3) as compared to *E. coli* lipid A (Kotani *et al.*, 1985). Similarly, removal of an acyl group (to yield pentaacyl lipid A) or addition of an acyl group (heptaacyl lipid A) reduces bioactivity by a factor of 10^2 (Loppnow *et al.*, 1989; Kotani *et al.*, 1986). Interestingly, lipid A molecules lacking two acyl groups (tetraacyl lipid A, also known as lipid IV_A - see Section 2d) are

essentially non-toxic for human monocytes but not mouse macrophages (Golenbock *et al.*, 1991). Thus, it is important to note that macrophages and monocytes from different animal species may exhibit differential responses to the same LPS preparation (also see Chapter 2).

Rhodobacter sphaeroides lipid A was the first identified lipid A antagonist. Thus, when present in excess, this lipid A has been shown to block the effects of *E. coli* LPS in vitro and in vivo through presumed competitive inhibition of binding to an LPS receptor (Takayama *et al.*, 1989; Qureshi *et al.*, 1991). The four major differences between *E. coli* and *R. sphaeroides* lipid A (RSLA) includes: the presence of shorter (only 10 carbon) acyl chains, the presence of only one acyloxyacyl unit to result in a pentaacylated (5 chain) lipid A, and the presence of an unsaturated acyl chain. In particular, the reduction in chain length and number is proposed to be responsible for RSLA reduced toxicity (Raetz, 1993).

Another example of a lipid A that exhibits reduced toxicity is that of *Helicobacter pylori*, the causative agent of duodenal ulcers. *H. pylori* LPS exhibits 10^3-fold lower mitogenicity for B lymphocytes and pyrogenicity in rabbits as compared to *Salmonella* LPS. The reduced toxicity of this LPS is proposed to be due to the presence of long chain (16 and 18 carbon) 3-hydroxy fatty acids as well as the lack of 4' phosphorylation of the backbone glucosamine in the lipid A (Muotiala *et al.*, 1992). Similarly, *Porphyromonas gingivalis* lipid A has unique branched long-chain (15 to 17 carbon) acyl moieties and a substituted 4' phosphate group. This lipid A induces reduced NO and TNF-α production by mouse peritoneal macrophages as compared to *Salmonella* LPS (Tanamoto *et al.*, 1997). Interestingly, *P. gingivalis* lipid A induces increased levels of TNF-α production by the human monocytic cell line THP-1 as compared to enterobacterial LPS, again underscoring the difference between mouse and human macrophage responses to LPS.

As mentioned briefly above, some lipid A molecules may contain additional polar substituents at the 1 and 4' glucosamine positions. These substituents are commonly phosphoethanolamine or 4-amino-4-deoxy-L-arabinose, and are often linked via a

phosphate or pyrophosphate (Schnaitman *et al.*, 1993). Substitutions such as these may significantly change the properties of lipid A with respect to its interaction with the host immune system as well as its role in maintaining bacterial cell outer membrane integrity; for example, decreased endotoxicity has been noted for lipid As substituted with aminoarabinose, and furthermore, polymyxin resistant mutants of *Salmonella typhimurium* have been described which exhibit increased levels of aminoarabinose substitution (Vaara *et al.*, 1981). The reduction in negative charge resulting from the aminoarabinose substitution of lipid A phosphate is proposed to be responsible for the observed decreased association with the cationic antibiotic polymyxin.

Interestingly, a recent paper has shown that the PhoP-PhoQ proteins of *Salmonella typhimurium*, a two component regulatory system responsible for activation and repression of a variety of genes required for intracellular survival, is responsible for regulating structural changes of lipid A during infection (Guo *et al.*, 1997). Activation of the PhoP-PhoQ system results in addition of an aminoarabinose substituent as well as an additional 2-hydroxymyristate moiety to yield a less toxic heptaacylated lipid A (as measured by TNF-α production by monocytes). The concept of an intracellular pathogen altering LPS toxicity in order to modify the host response is relevant to work described in Chapter 2.

Thus, a fully toxic lipid A consists of a glucosamine disaccharide substituted with two phosphate residues located at the 1 and 4' positions, and six acyl chains of 12-14 carbons in length. These acyl chains must adhere to the specific substitution pattern and chain length observed in *E. coli* and *Salmonella* LPS to retain full toxicity (Kotani *et al.*, 1985). Changes in lipid A structure which result in acyl chains of longer or shorter length, substitution or absence of phosphate moieties, or alterations in the number and location of acyloxyacyl substitutions may decrease endotoxicity.

(d) Biosynthesis of Lipid A

This section is important for a complete understanding of the results presented in Chapter 1. The lipid A biosynthetic pathway has been characterized almost entirely by the laboratory of Christian Raetz. Early in the 1980's, Raetz proposed a pathway for lipid A biosynthesis that has subsequently been proven using multiple biochemical techniques. The biosynthetic pathway for lipid A was characterized primarily using *in vitro* systems consisting of radiolabelled precursors added either to *E. coli* cell extracts or purified biosynthetic enzymes. The products of these reactions were then identified by comparison to standards using thin layer chromatography and structurally characterized using mass spectrometry and nuclear magnetic resonance spectroscopy. Due to the essential nature of Kdo_2-lipid A for bacterial cell viability, studies involving the mutation and identification of these enzymes relied largely on the construction of temperature sensitive mutations. The present scheme for *E. coli* Kdo_2-lipid A biosynthesis, including the enzymes responsible for each step, is shown in Figure 3.

The lipid A biosynthetic pathway was initially believed to initiate with the formation of a glucosamine disaccharide that would eventually become acylated to form lipid A. However, attempts to demonstrate the presence of enzymes exhibiting this acylation activity in *E. coli* cell extracts failed (Taylor *et al.*, 1969). Some time later, molecular biology prevailed when lipid X (2,3-diacyl glucosamine phosphate) was discovered to accumulate in temperature sensitive mutants of the *pgsB* gene (subsequently re-named *lpxB*), and the structure of lipid X was noted to resemble both the reducing and non-reducing ends of lipid A (see Figure 3) (Nashijima *et al.*, 1979; Nashijima *et al.*, 1981). Thus, this suggested that lipid A biosynthesis occurs via a pathway that synthesizes and condenses two acylated glucosamine precursors.

Nine enzymes are required for the synthesis of *E. coli* Re-endotoxin or Kdo_2-lipid A (shown in Figure 3). In brief, it may be said that the first step of lipid A biosynthesis is the transfer of the acyl group from R-3-hydroxymyristoyl-acyl carrier protein to the 3-OH

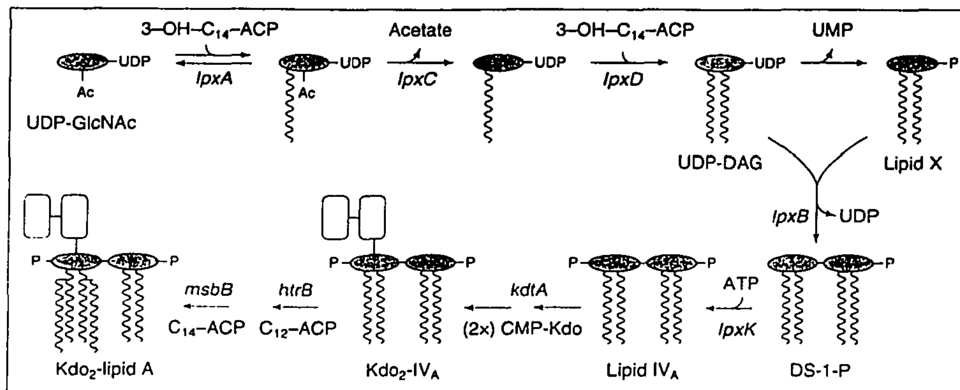


Figure 3. Biosynthetic pathway of Kdo_2-lipidA.

group of UDP-N-acetyl glucosamine (UDP-GlcNAc) by an acyltransferase encoded by the *lpxA* gene (Anderson *et al.*, 1985;.Anderson *et al.*, 1987; Galloway *et al.*, 1990) In *E. coli*, the *lpxA* gene is part of the macromolecular synthesis II operon, which consists of a cluster of 11 genes encoding a variety of proteins and enzymes (Schnaitman *et al.*, 1993; Crowell *et al.*, 1987). Also encoded within this operon are the lipid A biosynthesis genes *lpxB* and *lpxD*. The next step in lipid A biosynthesis involves the N-deacetylation of the O-acylated UDP-GlcNAc by the product of the *lpxC* gene, and the transfer of a second R-3-hyroxymyristoyl moiety to the resulting free 2-amino group by the gene product of *lpxD*. The *lpxC* gene is located in a cluster of genes involved in cell division and protein secretion (Schnaitman *et al.*, 1993). Conversion of UDP-GlcNac into UDP-2,3-diacyl glucosamine was performed in vitro using membrane-free *E. coli* cell extracts, thus suggesting that the gene products of *lpxA*, *lpxC*, and *lpxD* are cytoplasmic enzymes (Anderson *et al.*, 1985).

The actions of these enzymes results in the formation of UDP-2,3-diacylglucosamine, which is the precursor of the non-reducing end of lipid A. Removal of UDP from this molecular species by one of two pyrophosphatases yields lipid X, and the condensation of one molecule of lipid X with one molecule of UDP-2,3-diacyglucosamine by the gene product of *lpxB* results in the formation of the backbone of lipid A (Ray *et al.*, 1984; Radika *et al.*, 1988) This β1,6-linked glucosamine disaccharide carries a single phosphate at the 1 position and is tetraacylated with R-3-hydroxymyristate. As mentioned above, the *lpxB* gene is located within the macromolecular synthesis II operon, is transcriptionally coupled to expression of *lpxA*, and is proposed to be a peripheral membrane protein based on its amino acid sequence (Crowell *et al.*, 1987).

The subsequent action of a lipid A 4' kinase, the product of the *lpxK* gene (formerly known as *orfE*), generates a symmetrical bisphosphorylated tetraacylated disaccharide known as lipid IV_A (Ray *et al.*, 1984; Garrett *et al.*, 1997). Interestingly, the *lpxK* gene is organized in an apparent operon with the *msbA* locus, which encodes an ABC transporter protein proposed to be involved in LPS transport to the cell surface (McDonald

et al., 1997; Polissi *et al.*, 1996). The *Francisella* homolog of the *msbA/lpxK* operon, called *valAB*, is the subject of investigation in Chapter 1.

The next steps in Kdo_2-lipid A biosynthesis involve the attachment of the two Kdo components of the LPS inner core region to lipid IV_A prior to the synthesis of a complete lipid A molecule. The *kdtA* gene encodes a bifunctional enzyme responsible for the transfer of two molecules of Kdo (derived from CMP-Kdo) to the 6' carbon of lipid IV_A (Belunis *et al.*, 1992; Clementz *et al.*, 1991). In vitro Kdo-transferase assays using partially purified Kdo-transferase demonstrate addition of radiolabelled Kdo to lipid IV_A to create a new metabolite confirmed to consist of $(Kdo)_2$-lipid IV_A by mass spectroscopy and NMR analysis (Brozek *et al.*, 1989). KdtA activity in *E. coli* cell-free extracts has an absolute requirement for a substrate carrying the 4' phosphate moiety added by *lpxK* (Brozek *et al.*, 1989). Not surprisingly, lipid IV_A accumulates in temperature sensitive mutants of *kdtA* . The *kdtA* gene is located within the *rfa* cluster of genes involved in LPS core polysaccharide biosynthesis (Schnaitman *et al.*, 1993).

The final steps in lipid A biosynthesis involve the final acylation of $(Kdo)_2$ -lipidIV_A with laurate and myristate moieties to generate $(Kdo)_2$-lipid A (Brozek *et al.*, 1990). Early studies by Brozek *et al.* (1990) demonstrated that *E. coli* cell extracts were capable of converting $(Kdo)_2$-lipid IV_A into two more hydrophobic products. The membrane fraction of these extracts was shown to acylate $(Kdo)_2$-lipid IV_A when lauroyl or myristoyl-ACP were available as donors. Recent studies have shown that this occurs as a result of the action of the gene products of *htrB* and *msbB*. HtrB encodes a Kdo-dependant lauroyl transferase involved in the acylation of Kdo_2-lipid IV_A, resulting in the generation of an acyloxyacyl moiety at the 2' position (Clementz *et al.*, 1996). Similarly, MsbB is a myristoyl transferase which utilizes the resulting pentaacylated lipid A as a substrate for the addition of a 3' acyloxyacyl moiety, and thus completes the synthesis of acylate $(Kdo)_2$-lipid A (Clementz *et al.*, 1996). In *E. coli*, both the *msbB* and *htrB* genes appear to be unassociated with any operons or gene clusters involved in LPS biosynthesis, although the

promoter of the *htrB* gene overlaps a divergently transcribed gene with a currently unknown function (Karow *et al.*, 1991; Karow *et al.*, 1992). Interestingly, the genes *msbA* (described above) and *msbB* were initially identified as multicopy suppressors of mutations in *htrB* (Karow *et al.*, 1992; Karow *et al.*, 1993). Mutations in *htrB* exhibit an unusual phenotype which includes the inability to grow on rich media at temperatures above 33°C, and increased resistance to the anionic detergent deoxycholate (Karow *et al.*, 1991).

Thus, the genes involved in lipid A biosynthesis are located in several gene clusters and operons in the *E. coli* chromosome. The products of these genes require a number of activated acyl chain and nucleotide sugar precursors. Since nucleotide sugars are hydrophilic and synthesized in the cytoplasm, and the acyl chains require the hydrophobic environment of a membrane, it is believed that lipid A biosyntheisis occurs at the inner face of the cytoplasmic membrane. Indeed, all of the enzymes of lipid A and KDO biosynthesis, with the exception of KdtA, LpxK, HtrB, and MsbB, lack predicted transmembrane domains and are recovered from the cytosol. Correspondingly, the KdtA, LpxK, HtrB, and MsbB amino acid sequences exhibit putative transmembrane domains, and the activities of most of these proteins have been shown to be membrane-associated (Belunis *et al.*, 1992; Clementz *et al.*, 1991; Garrett *et al.*, 1997; Clementz *et al.*, 1996; Karow *et al.*, 1991; Karow *et al.*, 1992).

(3) Core Polysaccharide

(a) The Structure and Function of the LPS Core

The core polysaccharide of LPS is typically subdivided into inner and outer core subdomains. The inner core is the most conserved carbohydrate region of the LPS molecule, and is necessary for maintaining outer membrane integrity. The *E. coli* inner core region consists of two unique sugars that are characteristic of most LPS structures: two to three residues of the eight carbon sugar 2-keto-3-deoxy-octulosonic acid (KDO) and

three residues of the seven carbon sugar L-glycero-D-manno-heptose (see Figure 4). In contrast, the outer core is composed of hexoses, consisting primarily of glucose, galactose, and GlcNAc, and the combination of these sugars between and within bacterial species is subject to variability (see Figure 5). Substitutions of the inner core region are common and include the addition of phosphate, phosphoryl-ethanolamine and pyrophosphoryl-ethanolamine substituents (Schnaitman *et al.*, 1993) (shown in Figure 4).

The functional properties ascribed to the inner core region are due primarily to the negatively charged nature of this region. For example, phosphate substitutions of the heptoses of the inner core are frequent in *E. coli*. Furthermore, the carboxylate groups of Kdo contribute at least two negative charges to this region, and the temperature sensitivity of mutants defective in Kdo biosynthesis underscores the essential role for this sugar in lipid A biosynthesis and thus bacterial cell viability. Indeed, LPS isolated and analyzed from all bacterial species to date contain at least one Kdo molecule (Raetz, 1990). In addition, heptose-deficient mutants exhibit increased sensitivity to detergents and hydrophobic antibiotics and a decrease in the quantity of porins present in the outer membrane, thus suggesting an important role for heptose in the maintenance of outer membrane structure (Schnaitman *et al.*, 1993). These mutants are said to exhibit a deep rough phenotype, and the genes responsible for this mutant phenotype are described in the next section.

The variety of phenotypes associated with heptose-deficient inner core mutants is attributed to the involvement of this region in cross-linking adjacent LPS molecules through interactions with divalent and polyvalent cations. In particular, phosphate substituents on heptose residues are proposed to be predominant in binding of divalent cations, although the carboxylates of KDO residues may also be involved (Nikaido *et al.*, 1985). It is

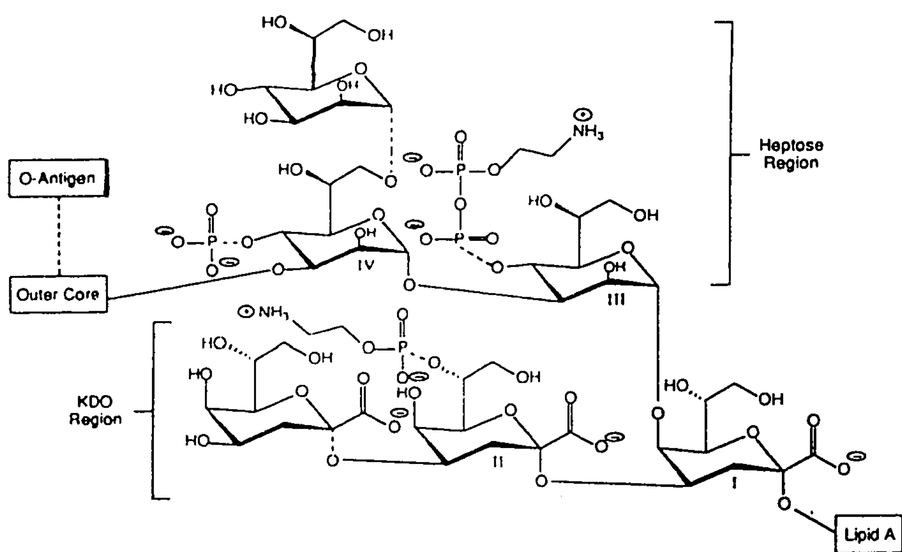


Figure 4. Chemical structure of the *E. coli* LPS inner core carbohydrate region.

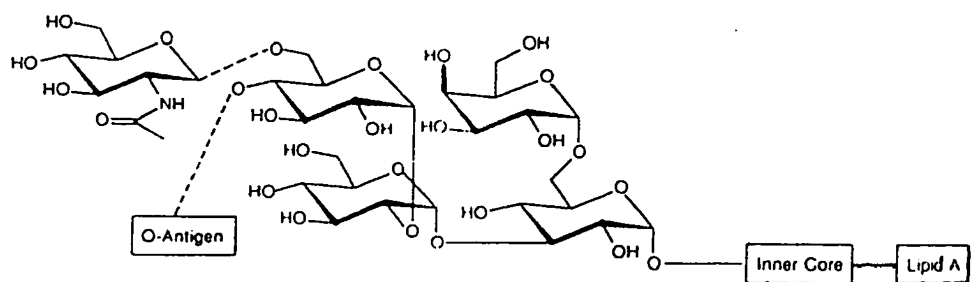


Figure 5. Chemical structure of the *E. coli* LPS outer core carbohydrate region.

observed that this region of LPS is required for an as yet undefined interaction with major outer membrane proteins, resulting in the decreased porin content of the outer membrane observed in deep rough mutants. Furthermore, the combined influence of the loss of outer membrane proteins with the reduction in LPS cross-linking is proposed to result in an increase in the phospholipid content of the outer membrane. Immunofluorescence studies of the cell surface of deep rough mutants confirms the presence of phospholipids in the outer membrane which are not typically present in wild type bacteria (Nikaido *et al.*, 1985). This results in the formation of large phospholipid zones in the outer membrane which are proposed to allow diffusion of detergents and hydrophobic antibiotics. Thus, the inner core region of LPS is instrumental in maintaining a tightly associated amphipathic barrier at the bacterial cell surface.

Studies investigating a role for the core region in LPS endotoxicity suggest that core carbohydrate may contribute significantly to lipid A toxicity. For example, Luderitz *et al.* (1989) demonstrated that *in vitro* mouse macrophage production of leukotrienes is triggered by rough LPS and that synthetic or natural lipid A has limited activity. Korvach *et al.* (1990) observed that Re LPS is more efficient than lipid A for induction of human monocyte TNF production. Similarly, Loppnow *et al.* (1989) showed that Re LPS induced significantly higher levels of TNF, IL-6, and IL-1 secretion by human monocytes in vitro as compared to synthetic lipid A (Cavaillon *et al.*, 1992).

Cumulative evidence indicates that the LPS inner core region, and in particular the KDO residues, contribute to LPS-induced cytokine production by human and mouse macrophages (Cavaillon *et al.*, 1992). Furthermore, isolated core oligosaccharides are capable of inducing human monocyte IL-1 production in the absence of lipid A, and this biological activity requires that the carboxylate group of Kdo remain unsubstituted (Cavaillon *et al.*, 1992). Thus, the core carbohydrate region may contribute to LPS-induced activation of macrophages as measured by cytokine production, and the Kdo residues appear to be instrumental in this process. However, it is important to note that

both complete LPS molecules and purified lipid A frequently exhibit similar abilities to stimulate macrophage activities, thus suggesting that lipid A remains one of the primary regions of the LPS molecule involved in stimulation of macrophage intracellular signalling.

(b) Genetics of the LPS Core

The genes involved in core polysaccharide biosynthesis may be broadly classified into three categories: (1) genes required for synthesis of activated sugar nucleotide precursors, (2) genes encoding enzymes involved in addition of a sugar moiety to the growing carbohydrate chain (called sugar transferases), and (3) genes encoding products responsible for modification of the core polysaccharide. In *E. coli* K-12, most of the genes involved in LPS core biosynthesis and modification are located within the *rfa* gene cluster, and are organized into overlapping transcriptional units (Austin *et al.*, 1990). This section will briefly summarize a selection of important genes involved in core biosynthesis.

Synthesis of the core region of LPS is proposed to occur by sequential addition of sugars to the non-reducing end of a lipid A acceptor at the cytoplasmic face of the inner membrane. The proposal for the cytoplasmic location of this process is primarily due to the hydrophilic nature of the activated sugar nucleotide precursors and the lack of transmembrane domains in the predicted amino acid sequences for most of the enzymes involved in core biosynthesis (Schnaitman *et al.*, 1993).

The exception to this rule is the KdtA protein, which is membrane-associated and acts prior to the final completion of lipid A biosynthesis. As previously described in Section II (d), the attachment of two Kdo residues to lipid IV_A by the product of the kdtA gene occurs prior to the final steps of acylation of lipid A (Brozek *et al.*, 1990; Belunis *et al.*, 1992). Mutants in Kdo biosynthesis and attachment to lipid IV_A are incapable of growth, thus demonstrating the essential nature of a minimal lipid A for bacterial cell viability.

In contrast, mutations affecting heptose biosynthesis, attachment, and modification are not lethal, but instead result in a deep rough mutant phenotype (Schnaitman *et al.*, 1993). In *E. coli*, deep rough mutants are characterized by an absence of O-antigen, increased sensitivity to detergents and hydrophobic antibiotics, an increase in the phospholipid content of the outer membrane, and a reduction in the porin content of the outer membrane (Austin *et al.*, 1990). Thus, null mutations in the heptose biosynthesis genes *rfaE* and *rfaD*, as well as the genes encoding the heptosyltransferases RfaC and RfaF, result in a deep rough phenotype (Austin *et al.*, 1990; Sirisina *et al.*, 1994). Similarly, mutants in the *rfaP* gene, proposed to be involved in modification of the heptose region, also exhibit a deep rough phenotype. In *S. typhimurium* and *E. coli*, the *rfaP* gene product is proposed to be involved in the attachment of a pyrophosphorylethanolamine (PPEA) moiety to the inner core (Parker *et al.*, 1992). Indeed, recent studies using NMR analysis has shown that the LPS of *S. typhimurium rfaP* mutants lack phosphate in the inner core region (Helander *et al.*, 1997). This finding agrees with the hypothesis that the negative charges of the inner core region are required for LPS-divalent cation cross-linking interactions which maintain outer membrane integrity (described in the previous section).

Synthesis of the hexose or outer core region of LPS is a result of sequential attachment of glucose and galactose residues to the lipid A-inner core (Figure 5 shows the structures of the outer core regions of *E. coli* and *S. typhimurium*). Nucleotide sugars function as the activated donors, and the genes encoding the sugar transferases are located within the *rfa* cluster (Shcnaitman *et al.*, 1992; Austin *et al.*, 1990). The genes encoding products proposed to be transferases involved in outer core biosynthesis include *rfaGBIJQ* The protein products of these genes share sequence homology, lack significant hydrophobic regions, and yield enzymatically active Tn*lacZ* fusions, thus suggesting they are cytoplasmic proteins (Pradel *et al.*, 1992). Indeed, the *rfaG* gene product, responsible for the transfer of glucose from UDP-glucose to the heptose region, has been purified from *S. typhimurium* and shown to form an enzymatically active complex with phospholipid and

partial LPS structures that can function as an acceptor for glucose (Schnaitman *et al.*, 1992). This combined evidence further suggests that core biosynthesis occurs at the cytoplasmic face of the inner membrane.

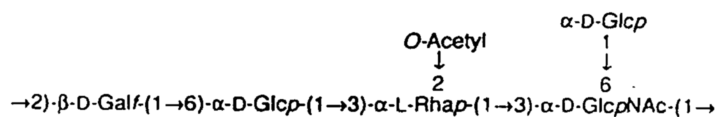
The phenotypes of outer core biosynthesis mutants can vary depending on the gene, the type of mutation, and the genetic background of the bacterial strain under investigation. For example, mutants in the *E.coli rfaQ* gene, proposed to encode a heptosyltransferase involved in substitution of the LPS core, exhibit low levels of O-antigen at the cell surface (MacLachlan *et al.*, 1992). Thus it has been suggested that in the absence of this branch residue, inefficient completion of LPS biosynthesis occurs. In contrast, mutations in *rfaIJ*, proposed to be responsible for the addition of backbone hexose residues, result in the production of truncated LPS molecules entirely lacking O-antigen (Pradel *et al.*, 1992).

(4) O-Antigen

(a) Structure of LPS O-antigen

The most variable portion of LPS is the O polysaccharide. Frequently, different O-antigen structures exist within and between species, and a single bacterium may simultaneously express more than one O-antigen (Raetz, 1990). Located at the non-reducing end of the LPS molecule, the O-antigen is surface-exposed, immunogenic, and responsible for numerous interactions with the host. Bacterial serotypes are frequently based upon different O-antigen structures, and in the *Salmonella* genus there are more than a thousand distinct O-antigen serotypes (Raetz, 1990). Antigenic variability among O-polysaccharides may be the result of large structural differences between O antigens or may simply arise due to more subtle changes in the carbohydrate backbone. For example, incorporation of different carbohydrate monomers, modifications of a basic backbone structure such as acetylation, or differences in carbohydrate linkages may provide new epitopes (Raetz, 1990). Variation of O-antigen structure is a virulence factor employed by numerous pathogens and is the subject of discussion in Section C.

A.



B.

Figure 6. Carbohydrate composition of (A) heteropolysaccharide O-antigen from *E. coli* K-12, and (B) homopolysaccharide O-antigen from *Klebsiella pneumoniae* O5.

O-antigen may be generally classified into two structural groups based on the carbohydrate composition of the polysaccharide (Schnaitman *et al.*, 1993; Whitfield, 1995). Heteropolysaccharide O-antigen is typically composed of up to 40 repeating polysaccharide structures called O units. Each O unit is identical in structure and is composed of up to 6 different sugar monomers which consists of a variety of unusual sugars, including deoxy and dideoxy sugars (Whitfield, 1995). An example of the carbohydrate composition of a heteropolysaccharide O unit from *Escherichia coli* K-12 is shown in Figure 6A. Conversely, homopolysaccharide O-antigen is not composed of discrete O-units but rather consists of multiple repeats of the same sugar monomer. The mannose-containing homopolymer of *Klebsiella pneumoniae* O5 is shown in Figure 6B. Not surprisingly, two such different O-polysaccharide structural groups arise from completely different biosynthetic mechanisms (Whitfield *et al.*, 1997).

(b) Biosynthesis of O-antigen

Similar to core polysaccharide, the synthesis of O-antigen requires a group of clustered genes (termed the *rfb* cluster) encoding sugar nucleotide biosynthetic enzymes and sugar transferases necessary for the addition of carbohydrate monomers to a growing polysaccharide chain (Schnaitman *et al.*, 1993). However, O-antigen biosynthesis is more complex in that O-antigen is not polymerized directly on a growing LPS molecule. Instead, O-antigen synthesis begins with the polymerization of either a long homopolysaccharide O antigen chain or a number of individual heteropolysaccharide O-units on a lipid carrier at the cytoplasmic face of the inner membrane (Whitfield, 1995). Furthermore, O-antigen synthesis terminates with the ligation of the O polysaccharide polymer to a complete lipid A-core at the periplasmic face of the inner membrane (Whitfield, 1995). Thus, O-antigen biosynthesis occurs in two different cellular compartments and is currently classified into two main groups based upon the components necessary for transport of the polysaccharide across the plasma membrane.

(i) Wzy-dependant Synthesis of Heteropolysaccharide O-antigen

Heteropolysacharide O-antigens are synthesized as individual O units at the cytoplasmic face of the inner membrane through the transfer of activated sugar nucleotides by specific sugar transferases to the acyl carrier lipid undecaprenol phosphate (ACL) (Osborn *et al.*, 1972; Weisberger *et al.*, 1982). Polymerization of the O units to create a longer ACL-linked O-antigen chain is proposed to occur at the periplasmic face of the cytoplasmic membrane by the O-antigen polymerase Wzy (formerly Rfc) (Morona *et al.*, 1994). The secondary structure of Wzy appears to be conserved between numerous bacterial species and indicates multiple membrane spanning domains. Not surprisingly, mutations in the *S. typhimurium wzy* gene result in the synthesis of a truncated LPS molecule consisting of lipid A-core ligated to a single O unit, termed smooth rough LPS (SR LPS) (Schnaitman *et al.*, 1993; Morona *et al.*, 1994). Electron microscopy experiments performed by Osborn *et al.* (1972) were the first to show that ACL-linked O-antigen accumulates at the periplasmic face of the inner membrane when LPS core biosynthesis is blocked. Subsequent pulse-chase experiments by McGrath *et al.* (1991) confirmed the periplasmic location of O-unit polymerization and the chase of these molecules into LPS located in the outer membrane.

Given that O-unit synthesis occurs at the cytoplasmic face of the inner membrane, and that O-unit polymerization occurs at the periplasmic face of the inner membrane, it is reasonable to expect that a transmembrane molecule involved in translocation of ACL-linked O-units across the inner membrane must exist. Indeed, the integral membrane protein Wzx (formerly RfbX) is currently proposed to fulfill this "flippase" function, although this remains to be conclusively proven biochemically (Liu *et al.*, 1996). In support of this hypothesis, mutations in the *wzx* gene result in the apparent cytoplasmic accumulation of ACL-linked O-units (Liu *et al.*, 1996).

Wzy-mediated polymerization of O-units on ACL is proposed to continue until a protein called the O-antigen ligase terminates polymerization by ligation of the nascent O-polysaccharide chain to the lipid A-core. This ligase is encoded by the *waaL* gene (formerly *rfaL*) and is an integral membrane protein with activity localized to the periplasmic face of the inner membrane (Whitfield *et al.*, 1997; MacLachlan *et al.*, 1991). Very little is known about the ligation reaction, and the ligase appears to be capable of addition of a variety of high and low molecular weight oligosaccharides to the lipid A-core. Theoretically, termination of polymerization and ligation of O-antigen to lipid A-core could occur after the polymerization of a random number of O-units, resulting in the expression of a heterogeneous mixture of LPS molecules at the bacterial cell surface. Although it is true that a heterogeneous mixture of LPS molecules is easily detectable at the bacterial cell surface by SDS-PAGE analysis (as seen in chapters 2 and 4), the length of the O side chains is not random but rather adheres to a specific distribution of chain lengths characteristic of each LPS biosynthetic locus. Regulation of O-antigen chain length is due to the product of the *wzz* gene (formerly *rol* for regulator of O-side-chain length). Although the precise mechanism of this chain length regulation remains to be characterized, Wzz is currently proposed to act as a 'molecular clock' which influences the activity of the O-unit polymerase to alternate between polymerization and transfer of lipid-linked O-antigens to WaaL for ligation to the lipid A-core (Batchelor *et al.*, 1991; Bastin *et al.*, 1993). Similar to Wzy and WaaL, the amino acid sequence of Wzz predicts a transmembrane protein and *phoA* fusions have localized a large hydrophilic domain of this protein to the periplasmic face of the inner membrane (Morona *et al.*, 1995). A putative *Francisella* homolog of this gene is the subject of further discussion in Chapter 3.

(ii) Wzy-independent Synthesis of Homopolysaccharide O-antigen

Homopolymer O antigens consist of a single repeating sugar monomer. Similar to the heteropolysaccharide O-antigens described above, homopolysaccharide O antigens are not directly synthesized on the lipid A core but are first added to ACL at the cytoplasmic face of the plasma membrane. However, in this system individual sugar monomers are rapidly added to the non-reducing terminus of the ACL acceptor one residue at a time in a processive mechanism until a full length O-antigen polymer has been completed (Jann *et al.*, 1985; Kopmann *et al.*, 1975). Consistent with this hypothesis, intermediates containing individual O units and mutants expressing SR LPS have not been identified (Schnaitman *et al.*, 1993). Polymerization of the complete O-antigen occurs in this manner through the action of specific sugar transferase(s) without the need for an O-antigen polymerase (Wzy). However, a regulator of chain length (Wzz) protein has recently been identified for one of the O-antigen forms expressed by *E. coli* strains O8 and O9 (Franco *et al.*, 1996). The Wzx flippase is also absent from this system because there is no requirement for the export of indidually synthesized O units (Whitfield *et al.*, 1997). Instead, transport of ACL-linked homopolymeric O-antigen from the cytoplasmic to the periplasmic face of the inner membrane is proposed to be due to an ATP- binding cassette (ABC) transporter. ABC transporters are integral membrane proteins which couple the energy of ATP hydrolysis to the import or export of a variety of substances across membranes in both prokaryotic and eukaryotic systems. Indeed, mutations in the *Yersinia entercolitica* and the *Klebsiella pneumoniae rfb* ABC transporters result in cytoplasmic accumulation of O-antigen (Zhang *et al.*, 1993; Bronner *et al.*, 1994). Once on the periplasmic side of the inner membrane, the WaaL protein ligates the complete O-antigen to the lipid A-core acceptor (Whitfield *et al.*, 1997).

(5) The Topology of LPS Biosynthesis

Despite the identification of numerous genes involved in LPS biosynthesis, some unanswered questions remain with respect to the export of LPS to the outer membrane (see Figure 7). Chapter 1 is concerned with one aspect of LPS biosynthesis and transport which remains undefined.

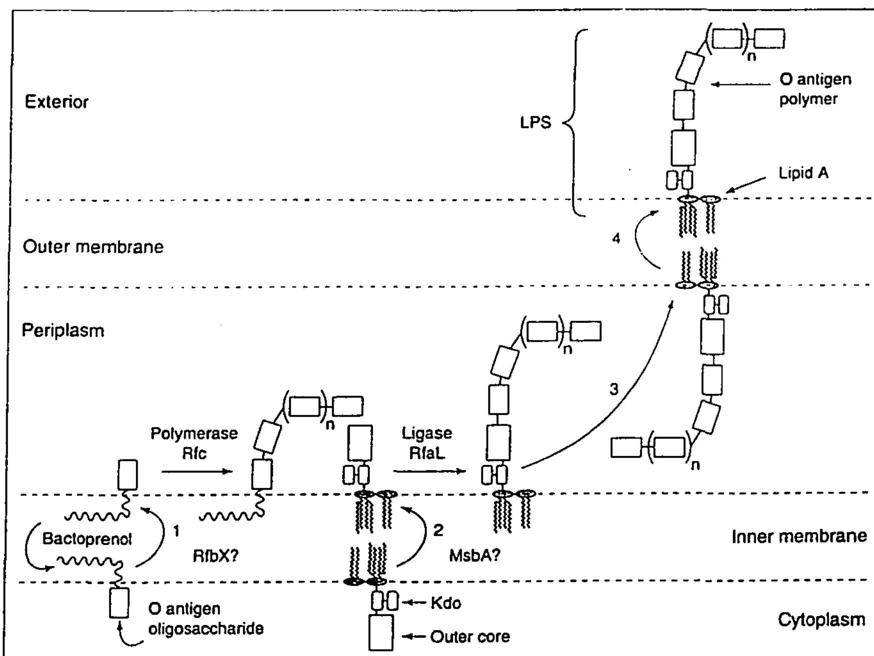


Figure 7. The topology of LPS biosynthesis in *E. coli.* Gray ovals represent glucosamine residues, and small white boxes represent KDO moieties. Core and O-antigen units are represented by white boxes. Nothing is known about LPS translocation across the periplasm (step 3) or outer membrane integration (step 4).

LPS biosynthesis is an unusual process involving hydrophilic sugars and hydrophobic lipid components, thus suggesting synthesis must always occur at the face of a membrane. Indeed, precursors to lipid A biosynthesis such as sugar nucleotides and R-3-hydroxymyristate are synthesized in the cytoplasm, and the amino acid sequences of many of the proteins involved in lipid A-core biosynthesis predict hydrophilic proteins lacking discernable transmembrane regions (Belunis *et al.*, 1992; Clementz *et al.*, 1991; Garrett *et al.*, 1997; Clementz *et al.*, 1996; Karow *et al.*, 1991; Karow *et al.*, 1992). Thus, lipid A-core biosynthesis is proposed to occur at the cytoplasmic face of the inner membrane.

At least two separate O-antigen biosynthetic pathways exist in Gram negative bacteria. These two pathways share several common features, including activated sugar nucleotides, sugar transferases predicted to be cytoplasmically located peripheral membrane proteins, and the initiation of O-antigen synthesis on a lipid carrier proposed to be at the cytoplasmic surface of the plasma membrane. Furthermore, electron microscopy and in vivo pulse-chase experiments have demonstrated that O-antigen is transferred to the completed lipid A-core molecule at the periplasmic face of the plasma membrane by an O-antigen ligase (WaaL) (Mulford *et al.*, 1983; McGrath *et al.*, 1991). Transport of homopolymeric O-antigen across the plasma membrane has been shown to be the result of an ABC transporter (Zhang *et al.*, 1993; Bronner *et al.*, 1994). Similarly, transport of heteropolymeric O-units across the plasma membrane is proposed to be due to the transmembrane protein Wzx (Liu *et al.*, 1996). However, the protein(s) involved in the transfer of lipid A-core to the periplasmic face of the plasma membrane remains to be determined, and the mechanism of transport of the completed LPS molecule to the outer leaflet of the outer membrane has remained little more than a subject of speculation for the last decade (Raetz, 1990).

(C.) Phase Variation

(1) Brief Introduction to Phase Variation

The variation of bacterial surface structures may arise from a reversible switching on and off of the expression of surface components (called phase variation) or from changes in the antigenic nature of a structure (called antigenic variation). Surface structures such as lipopolysaccharide, capsule, outer membrane proteins, flagella, and fimbriae have been shown to undergo phase variation in a variety of different bacteria. Numerous pathogenic bacteria use phase variation of surface structures as a strategy to evade or alter the host immune response, as well as to allow for colonization of multiple environmental niches within the host. The molecular mechanisms used to produce phase variation are diverse. In this section, I have chosen three examples of molecular mechanisms used to achieve phase variation of a surface structure. Where appropriate, I will also describe the biological significance of the phase variation with respect to effects on infection in the host. In Chapter 2, we describe a phase variation phenomenon in the lipopolysaccharide of *F. tularensis*.

(1) *Neisseria* and *Haemophilus* Surface Structures: Slipped-Strand Mispairing

The pathogenic *Neisseriae* are the etiologic agents of ghonorrhoea (*Neisseria gonorrhoeae*) and meningitis (*Neisseria meningitidis*). Similarly, *Haemophilus influenzae* is a major cause of meningitis and may also cause upper and lower respiratory tract infections. Significant and multiple similarities exist between *H. influenzae* and the *Neisseriae*. For example, both *Neisseriae* and *Haemophilus influenzae* exhibit a similar course of infection involving initial colonization of human mucous membranes, followed by invasion of mucosal cells, crossing of the mucosal cell barrier, and finally progression to a systemic form of disease. In both cases, phase variation is proposed to be instrumental in this process.

Commonly, bacterial structures, which are beneficial to a pathogen during one stage of disease, may become detrimental or unnecessary during another stage of disease, thus making advantageous a mechanism for varying the expression of these structures. In the case of *Neisseria* and *Haemophilus*, mucosal surface colonization and invasion of the bloodstream represent two vastly different host environments. In order to survive in both these environments, multiple protein and carbohydrate cell surface components of *Neisseria* and *Haemophilus* are subject to either antigenic or phase variation (De Vries *et al.*, 1996; Roche *et al.*, 1995). Thus, when considering the effect of phase variation of individual surface components on infection, it is important to understand that the expression of other components may also vary and thus contribute to the reported results. For example, host cell adhesion and invasion of *N. meningitis* is affected by the interplay of four phase variable surface structures (adhesins, opacity proteins, pili, and LPS) (DeVries *et al.*, 1996).

Both *Neisseria* and *Haemophilus* express an LPS molecule that is more correctly referred to as lipooligosaccharide (LOS). LOS is comprised of a lipid A moiety attatched to one or two KDO residues, heptose, as well as glucose and galactose, but lacks the traditional O-antigen region characteristic of most LPS molecules (van Putten *et al.*, 1995). Thus, LOS is similar in structure to rough LPS which contains only an inner and outer core polysaccharide attatched to lipid A. Interestingly, the LOS of both *Neisseriae* and *Haemophilus* undergoes high frequency gain and loss of oligosaccharide epitopes by a mechanism termed slipped-strand mispairing.

Neisserial LOS is commonly a bi- or tri-antennary structure due to the attatchment of various oligosaccharides to the inner core heptose residues. A single bacterium can produce a repertoire of different LOS molecules, and the variation between these structures occurs spontaneously at a frequency of 10^{-2} to 10^{-3} cells per generation (van Putten *et al.*, 1995). LOS variation is proposed to contribute to evasion of the host immune response by a variety of methods. The antigenic diversity which arises as a result of variable

oligosaccharide surface structures is thought to contribute to evasion of the humoral immune response (van Putten *et al.*, 1995). Furthermore, many of these oligosaccharide moieties mimic mammalian glycosphingolipids, thus combining poor immunogenicity with possible functional characteristics similar to the homologus host structures. Unfortunately, studies investigating the ability of these mammalian structural homologs to aid in Neisserial adherence and entry into host cells through the use of eukaryotic receptors which recognize such structures remain contradictory (Mandrell *et al.*, 1994; van Putten *et al.*, 1993).

The most commonly isolated LOS form expressed by *N. meningitidis* and *N. gonorrhoeae* carries the oligosaccharide lacto-N-neotetraose [Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)Glc], which is structurally similar to the mammalian glycolipid paragloboside. The terminal galactose of this structure is also an acceptor for the addition of a sialic acid moiety, and there is evidence to suggest that this sialylated LOS is involved in conferring serum resistance to the bacteria (Elkins *et al.*, 1992). Expression of sialylated LOS has also been shown to contribute to decreased host cell association and invasion, thus making expression of this structure detrimental during some stages of the disease (Virji *et al.*, 1995).

Variation in cell surface expression of lacto-N-neotetraose occurs at a frequency of 10^{-2} to 10^{-3} cells per generation. Jennings *et al.* (1995) identified three genes proposed to be responsible for lacto-N-neotetraose synthesis in *N. meningitidis*. These three genes, named *lgtA, lgtB,* and *lgtE* are arranged in an apparent operon and are predicted to encode proteins with high sequence identity to a group of homologous glycosyl transferases in *N. gonorrheae*. Mutagenesis and SDS-PAGE analysis suggests that *lgtABE* have the following roles in lacto-N-neotetraose synthesis: *lgtE* encodes a galactosyl transferase responsible for the addition of a β1,4-linked galactose to the core glucose, and thus creates an epitope referred to as the L8 meningococcal immunotype; *lgtA* is responsible for the addition of a β1,3-linked N-acetyl glucosamine to the L8 structure; and *lgtB* encodes a

transferase which catalyzes the addition of the β1,4-linked terminal galactose to GlcNAc, thus creating the L3 meningococcal immunotype.

Sequence analysis of *lgtABE* revealed the presence of a homopolymeric tract of 14 guanosine residues located within the 5' end of the *lgtA* gene. The length of the homopolymeric tract in *lgtA* correlates with switching between the L8 and L3 meningococcal immunotypes. Variants expressing the L8 immunotype contain 15 residues in the polyG tract, resulting in a frameshift in the *lgtA* coding region which is proposed to result in premature termination of translation. Conversely, variants of the L3 immunotype contained a 14 G residue homopolymeric region which is predicted to keep the downstream sequences in frame with the *lgtA* start codon, thus allowing for synthesis of an active LgtA protein. Variable control of LgtA expression would allow for conversion between the L3 and L8 immunotypes; in the absence of LgtA, synthesis of lacto-N-neotetraose would terminate following the addition of a Gal residue to the core oligosaccharide , thus creating the L8 epitope. However, in the presence of an active LgtA, the action of the LgtA and LgtB proteins would result in completion of the lacto-N-neotetraose moiety and the formation of the L3 epitope.

Variation of the number of G residues in the *lgtA* homopolymeric region is proposed to occur through a mechanism termed slipped-strand mispairing. According to this hypothesis, local denaturation occurs between DNA strands and results in mispairing of the repetitive sequences. This is thought to occur during DNA replication, and thus results in daughter cells containing insertions or deletions in the repeat region. In several cases, the upstream sequences are A-T rich and would favor denaturation that could promote slipped-strand mispairing of the repetitive DNA (Weiser *et al.*, 1989).

There are several other examples of genes that are subject to slipped-strand mispairing. In some cases, this mechanism is used to achieve a more subtle regulation of phase variable expression of a surface component (High *et al.*, 1993; Szabo *et al.*, 1992; Weiser *et al.*, 1989). For example, a *Haemophilus influenzae* locus required for synthesis

of the LOS epitope consisting of αGal(1,4)βGal is subject to different levels of phase variable expression in vivo (Weiser *et al.*, 1989; High *et al.*, 1993). Thus, instead of phase variation resulting in a simple on/off switch, different levels of expression (-,+, and ++++) of this epitope are detectable using monoclonal antibodies. Although the exact role of the *lic2A* gene product remains to be determined, deletion studies indicate that *lic2A* is essential for synthesis of the LOS structure αGal(1,4)βGal (High *et al.*, 1993; Weiser *et al.*, 1990). Amino acid sequence identity between Lic2A and galactosyl transferases from other bacteria has led to the suggestion that Lic2A is responsible for the synthesis of a LPS structure required for the subsequent assembly of αGal(1,4)βGal.

DNA sequence analysis of *lic2A* reveals multiple copies of the tetramer 5'-CAAT-3' within the 5' end of the coding sequence of this gene, located downstream from three possible translational start codons. Variation in the number of 5'-CAAT-3' repeats due to slipped-strand mispairing allows for translational frame-shifting by placing different upstream initiation codons either in or out of frame with the rest of the *lic2A* coding sequence, thus resulting in differential levels of expression of the αGal(1,4)βGal epitope. The reason for the different levels of expression of this epitope by variants that translate Lic2A from different initiation codons remains undefined. Studies examining the effect of the repetitive 5'-CAAT-3' region on the Lic2A protein structure and function reveal that this region, which is translated into a repetitive tetrapeptide motif (SINQ)n in the protein, is predicted to be a highly flexible random coil. Furthermore, deletion of this region reveals that it is unecessary for Lic2A activity. Thus, different numbers of repeats of the SINQ tetrapeptide in the protein are predicted to be unlikely to affect Lic2A activity, although the activity of this enzyme has not been directly measured (High *et al.*, 1996). Instead, it is proposed that the different levels of expression from the various start codons may due to differences in translation efficiency (Szabo *et al.*, 1992). In support of this hypothesis, this locus does not have an identifiable Shine-Dalgarno ribosome-binding sequence upstream from the transcription initiation codons, but instead has a downstream 16S rRNA-binding

consensus sequence within the repetitive (CAAT)n region (Weiser *et al.*, 1990). Distance between this putative ribosome-binding site and the different start codons may affect the rates of translation. Indeed, it has been observed that phase variants encoding the number of CAAT repeats required to place the closest start codon to this 16S rRNA consensus sequence in frame with the downstream coding sequence results in the strongest expression of the αGal(1,4)βGal epitope. A similar situation has been predicted to occur for the *H. influenzae lic3* locus, which is also involved in phase variable expression of LOS epitopes (Szabo *et al.*, 1992).

Finally, repetitive sequences subject to slipped-strand mispairing may not always be found within the coding region of a gene, but instead may located within the promoter region. Furthermore slipped-strand mispairing may regulate expression of a gene product at the level of transcription instead of at the level of translation. For example, the *opc* gene of *Neisseria meningitidis* encodes an outer membrane protein proposed to be involved in facilitating attatchment and invasion of the bacterium into endothelial cells (Virji *et al.*, 1993). This protein is subject to phase variation due to a homopolymeric tract of C residues located within the promoter region of the *opc* gene (Sakari *et al.*, 1994). The *opc* promoter consists of a -10 promoter consensus region located downstream from a stretch of contiguous C residues, lacking a -35 consensus promoter sequence. Different numbers of C residues correspond to three different levels of Opc protein expression, and the amount of Opc protein expressed correlates with levels of *opc* mRNA, thus suggesting transcriptional regulation of this locus. The authors propose that the length of the poly-C region is crucial for efficient transcription, and may be located between the -10 region and a binding site important for function of the *opc* promoter.

Thus, high frequency phase variation of a bacterial component may be achieved through slipped-strand mispairing of repetitive sequences within the coding region or promoter region of a gene. In addition to being a simple on/off switch for the expression of a protein product, it is also a mechanism that may allow for regulation of different levels

of expression of a product. Furthermore, slipped-strand mispairing may be used to achieve regulation of expression of a gene product at either the transcriptional or translational levels.

(2) *Neisseria meningitidis* Capsule and LOS Sialylation: Transposable Genetic Element

N. meningitidis serogroups B and C are the primary meningococcal strains associated with invasive disease. These strains are capable of synthesizing the nucleotide-activated sugar CMP-N-acetyl neuraminic acid (NeuNAc), also known as sialic acid. This CMP-NeuNAc precursor is used as the substrate for the synthesis of a polysialic acid capsule as well as for the modification of LOS. The meningococcal capsular polysaccharides are sialic acid homopolymers composed of either α-2,8 and α-2,9 linkages, while the LOS sialic acid modification consists of a NeuNAc residue attached to the terminal galactose of the variable lactose-N-neotetraose LOS moiety described above. Not surprisingly, capsule and LOS sialylation biosynthetic pathways share the same enzymes for synthesis of the CMP-NeuNAc precursor. However, mutants lacking the 2,8-sialyltransferase are capsule-deficient but retain the ability to sialylate their LPS, thus demonstrating that different enzymes are required to complete the synthesis of the capsule and sialylated LOS (Hammerschmidt *et al.*, 1994).

LOS sialylation and capsule biosynthesis are proposed to have a significant impact on neisserial pathogenesis. First, the sialylated LOS mimics a glycolipid found in human granulocytes, thus producing a surface structure which is proposed to lack immunogenicity (van Putten *et al.*, 1995). Similarly, the α-2,8 polysialic capsule is only weakly immunogenic and identical to the carbohydrate moiety of the eukaryotic neural cell adhesion molecule N-CAM (Bitter-Suermann and Roth, 1987). Furthermore, activation of the alternative complement pathway is inhibited by sialic acids on cell surfaces (Fearon, 1978). Indeed, the sialic acid capsule and LOS sialylation have both independently been shown to

increase resistance to human serum and to inhibit entry into several types of human cells (Hammerschmidt *et al.*, 1994). For example, meningococcal mutants defective in capsule biosynthesis adhere better to epithelial cells in vitro and invade these cells 100-fold better than encapsulated strains (Hammerschmidt *et al.*, 1996). Similarly, sialylation inhibits non-opsonic and antibody-mediated phagocytosis of *N. gonorrhoeae* (Rest *et al.*, 1992; Kim *et al.*, 1992). Thus, it is proposed that the sialylated *Neisseriae* cell surface prevents close association of bacteria with eukaryotic cells, and it also masks cell surface components involved in invasion of epitheliel cells and activation of the complement cascade. It has therefore been hypothesized that meningococcal penetration of mucosal barriers may require a non-sialylated bacterial cell surface, whereas bacteria in the systemic phase of disease would benefit from the serum and phagocytosis-resistant sialylated bacterial cell surface (van Putten *et al.*, 1995).

In vivo studies support this hypothesis. For example, organisms isolated from the nasopharynx of human meningococcal carriers are of the non-sialylatable LOS type, consistent with the colonization of a mucosal cell surface early in infection prior to invasion (Schneider *et al.*, 1991). In contrast, isolates from the blood and cerebrospinal fluid of infected individuals are of the sialylatable LOS immunotype, consistent with the need for resistance to immune defenses. Similar studies examining the expression of *N. meningitidis* sialic acid capsule confirm the presence of non-encapsulated invasive bacteria early in disease followed by encapsulated serum-resistant bacteria later in disease (van Putten *et al.*, 1995; Hammerschmidt *et al.*, 1996b). Thus, the ability to alter sialylation of LOS and capsule expression would allow the bacterial population to exploit new environments in the host.

Recent work by Hammerschmidt *et al.* (1996a) has demonstrated that phase variation of capsule expression and LOS sialylation is regulated by the reversible insertional inactivation of a gene shared by these two biosynthetic pathways. Southern blot and DNA sequencing analysis revealed that up to 20% of capsule-deficient meningococcal variants

contain an 844 base pair insertion sequence in the *siaA* gene, which encodes an epimerase essential for NeuNAc synthesis. This insertion sequence (named IS1301) exhibits the characteristics of a mobile genetic element, as it is flanked by inverted repeats and encodes two open reading frames proposed to be responsible for transposition. Insertion of this sequence into the *siaA* gene is thought to occur due to transposition of one of 12 other IS1301 sequences present in the meningococcal genome. Excision of IS1301 from the *siaA* gene and reversion to the encapsulated phenotype was calculated to occur at a frequency of 4×10^{-4}. Thus, insertion and excision of a transposable genetic element into the *N. meningitidis siaA* gene results in the reversible inactivation of an epimerase required for both capsule biosynthesis and LOS sialylation. The resulting capsule-deficient variants are more invasive than encapsulated strains.

However, only 20% of capsule-deficient variants contain a mobile genetic element insertion in the *siaA* gene, thus suggesting the existence of an additional mechanism for capsule phase variation. Recent evidence has shown that capsule phase variation is further controlled by slipped-strand mispairing of sequences within the *siaD* polysialyltransferase gene, a key enzyme in polysialic acid capsule synthesis (Hammerschmidt *et al.*, 1996b). Thus, unlike the mechanism described above, this phase variation mechanism controls capsule expression but does not affect LOS sialylation. Unencapsulated strains were shown to contain a stretch of six or eight C residues within the 5' coding region of *siaD*, resulting in a frameshift which is predicted to bring a translational stop codon into frame. Conversely, in encapsulated strains the poly dC stretch contains seven C residues and is predicted to allow for expression of the *siaD* gene product. Northern blot analyses confirms the absence of a full *siaD* mRNA transcript in capsule-negative variants.

The in vivo relevance of this mechanism of phase variation was confirmed by PCR amplification and sequencing of the *siaD* genes from nasopharyngeal and blood isolates taken from individuals suffering from meingococcal disease (Hammerschmidt *et al.*, 1996). The non-encapsulated nasopharyngeal isolates had a six or eight poly dC stretch, whereas

the encapsulated blood isolates had a seven residue poly dC stretch within the *siaD* gene. Thus, meningococcal polysialic acid capsule expression is subject to a phase variation phenomenon that is controlled by two different mechanisms in two separate genes essential for capsule biosynthesis.

(3)*Salmonella* Flagella: Site-Specific DNA Inversion

Over 70 years ago, it was discovered that mass cultures of *Salmonella typhimurium* derived from a single clone could be agglutinated by two different antiserums directed against their flagellar filaments (Zieg *et al.*, 1977). Subsequent studies have shown that this observation was the result of phase variation between the expression of two flagellar antigens which occurs at a frequency of 10^{-3} to 10^{-5} cells per generation. The flagellar antigens were postulated to be encoded by two genes, named *H1* and *H2*, which may only be expressed one gene at a time (Lederberg *et al.*, 1956).

The *H1* and *H2* loci are located far apart on the *Salmonella* chromosome (at 41 and 59 minutes, respectively). The ability to switch from the expression of one gene to another was shown by transduction analyses to be controlled by a genetic element linked to the *H2* gene (Zieg *et al.*, 1977). Electron microscopy of DNA heteroduplexes formed after denaturation and reannealing of DNA fragments encompassing the region encoding H2 revealed the formation of a "DNA inversion bubble". Thus, some of the clones encompassing the region of the *H2* gene had undergone a genetic rearrangement which, upon annealing with clones that had not undergone this rearrangement, resulted in the formation of a "bubble" consisting of two strands of non-compatible ssDNA. Formation of this bubble was correlated with phase variation of the flagella, and thus it was proposed that phase variation is controlled by the inversion of a DNA segment adjacent to the *H2* gene (Zieg *et al.*, 1977).

Further work by Silverman *et al.* (1979) demonstrated that the *H2* locus consisted of two cotranscribed genes, named *H2* and *rh1*. The expression of both of these genes

was shown to be regulated by the same DNA inversion event. Mutational analysis indicated that expression of the *rhl* gene product correlated with repression of *H1* gene activity, thus suggesting that *rhl* encodes a repressor of the *H1* gene. Thus, a model was proposed suggesting that inversion of a DNA segment located upstream of the *H2* and *rhl* genes results in a switch in the orientation of a promoter required for transcription of these genes. In the H2-on phase, the promoter is proximal to the *H2* gene, resulting in expression of H2 and repression of the *H1* gene by the Rh1 protein. In the H1-on phase, the promoter is in the opposite orientation, thus resulting in loss of transcription of *H2* and *rhl*, and de-repression of the *H1* gene.

Subsequent cloning and sequencing studies have confirmed this hypothesis. Flagella phase variation is mediated by the site-specific inversion of a 955 base pair (bp) DNA segment flanked by two 26 bp recombination sites (termed *hix* sequences) which are conserved among a number of DNA inversion systems (Johnson *et al.*, 1985). Primer extension analysis reveals that transcription of *H2* mRNA initiates 41 bp upstream of the right-hand *hix* sequence (termed *hixR*), and therefore is located within the DNA fragment subject to inversion (Osuna *et al.*, 1995). Correspondingly, this transcriptional start site was preceded by a promoter region for the *H2* gene with a consensus sequence specific for the σ28 form of RNA polymerase. Thus, inversion of the 955 bp DNA segment at the *hix* sequence boundaries relocates the promoter and transcriptional start site for the *H2* and *rhl* genes.

Within the 955 bp inversion sequence an open reading frame was found which shares high amino acid sequence identity to the site-specific recombinase family of proteins. The product of this gene, named Hin, is required for site-specific inversion of the 955 bp sequence. Purified Hin protein protects the two 26 bp recombination sites (*hixL* and *hixR*) from nuclease cleavage, thus suggesting that Hin mediates inversion by binding to *hixL* and *hixR*. *In vitro*, a supercoiled DNA molecule containing two copies of the *hix* sequences in inverted configuration can support a low rate of Hin-mediated inversion

(Johnson *et al.*, 1985). Interestingly, the *hix* sequences consist of two imperfect 12 bp half sites separated by a 2 bp spacer, which is proposed to be the site where Hin-mediated strand cleavage occurs. Further studies have confirmed this hypothesis (see below).

In addition to the requirement for the two inverted *hix* sequences and Hin, the in vitro DNA inversion reaction also requires a 63 bp enhancer sequence and two additional host proteins in order to acheive reaction levels similar to wild type. Interestingly, although the enhancer sequence is normally located within the inversion segment in *Salmonella*, it may still function if relocated several thousand base pairs away (Johnson *et al.*, 1985). The two host proteins required for efficient inversion have been identified as the *E .coli* histone-like proteins HU and Fis (factor for inversion stimulation) (Johnson *et al.*, 1986). Although the precise binding site of HU is unknown, DNA protection studies reveal that Fis binds to two sites within the recombinational enhancer sequence (Bruist *et al.*, 1987).

Thus, DNA inversion of the 955 bp sequence located upstream from the *Salmonella* H2 flagella protein subunit gene is proposed to occur following binding of Hin as a dimer to each of the two hix recombination sequences. Hin catalyzes a staggered double-stranded cleavage of the DNA at the central core nucleotides of the *hix* sequences to generate 2 bp 3'-OH overhangs (Johnson *et al.*, 1989). Hin remains covalently associated with the 5' end of the cleavage site until the DNA is resealed (Johnson *et al.*, 1989). Fis binds to two sites within the recombinational enhancer, and becomes colocalized with Hin bound at each recombination site to form a three-looped "invertasome" structural DNA intermediate which has been visualized by electron microscopy (Heichmann *et al.*, 1990). It is proposed that the two recombination sites are initially paired by Hin, followed by association of the Fis-bound enhancer region to form the invertasome structure where DNA cleavage and strand exchange occurs. The non-specific DNA-binding protein HU, normally involved in promoting DNA underwinding, is believed to help stabilize the invertasome by facilitating looping of the DNA (Johnson *et al.*, 1986).

Fis is capable of enhancing DNA inversion by 1000-fold *in vivo* (Finkel *et al.*, 1992). Interestingly, Fis is a multi-functional protein that is tightly regulated in the cell. For example, Fis has also been shown to function in transcriptional activation of rRNA and tRNA operons, repression of its own synthesis, and initiation of *oriC*-directed chromosomal DNA replication (Finkel *et al.*, 1992). Not surprisingly, cellular concentrations of Fis vary under different growth conditions in a manner which reflect its physiological roles. For example, Fis levels are very low or undetectable during slow growth or stationary phase when DNA replication and RNA synthesis would be detrimental, but levels of Fis rapidly increase when cells are shifted to rich medium. Indeed, *S. typhimurium fis* mutants lose Hin-mediated DNA inversion, exhibit reduced growth rates in rich medium, and also demonstrate cell filamentation (Osuna *et al.*, 1995). Thus, it is particularly interesting to note that phase variation of *Salmonella* flagella is subject to regulation by growth phase and culture conditions.

MATERIALS AND METHODS

Rats and mice (Chapters 2 and 4). Specific pathogen-free female Lewis rats and female BALB/c mice were purchased from Charles River. They were housed in barrier-topped cages under conventional conditions, and given food and water *ad libitum*.

Bacterial strains. The bacterial strains and plasmids used in Chapter 1 are described in Table 2. *E. coli* W3110, DH5α, DK1, and JM109 were used as host strains, and were cultured in LB broth supplemented with 30 μg/ml kanamycin sulfate (Km), 100 μg/ml ampicillin (Amp), 10 μg/ml chloramphenicol (Cm), 250 μg/ml erythromycin (Erm) and 30 μg/ml tetracycline (Tet) as required. For induction of expression of cloned genes, media were supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma).

F. tularensis LVS (live vaccine strain) (Eigelsbach and Downs, 1961) and *F. novicida* were obtained from the American Type Culture Collection and preserved in aliquots at -80°C. For each experiment described in Chapters 2 and 3, an aliquot was thawed and used to seed a fresh culture, which was grown overnight at 37°C with aeration in tryptic soy broth (Difco, Detroit, MI) supplemented with 0.1% cysteine hydrochloride (TSB-C). *Francisella* were enumerated by determining the number of colony forming units (CFU) in serial dilutions of the bacterial suspension after plating on cysteine-heart agar (Difco) containing 5% defibrinated horse blood (CHA-H) and incubating for 1 to 2 days at 37°C. *F. tularensis* LVSG is a spontaneous colony variant isolated from the parent strain *F. tularensis* LVS using a method previously described using obliquely transmitted light and a dissecting microscope (Eigelsbach *et al.*,1951). *F. tularensis* LVSR (Sandstrom *et al.*,1988) is a capsule negative mutant isolated after exposure of LVS to acridine orange. Strains LVSGB and LVSRB, which appear to have biochemical and biological phenotypes identical to the parental LVS strain emerged from rat macrophages infected with either LVSG or LVSR, respectively. During the study presented in Chapter 2 we discovered that different colony forms are distinguishible using fresh CHA-H plates incubated for 2 days at

37°C followed by 2 days at room temperature. *E. coli* strains were grown on Luria-Bertani medium and selected for antibiotic resistance at 250 μg/ml Erm.

E. coli strains used in chapter 4 include E131 (pTnMax2) and DH5α. *F. tularensis* LVS and *F. tularensis* biotype *novicida* were grown in TSB-C. Where necessary, *Francisella* broth cultures and CHA-H plates were supplemented with 25 μg/ml Erm.

***In vitro* assay of *Francisella* growth in macrophages (Chapters 2 and 3).** Dulbecco's modified essential medium (DMEM) (Gibco) supplemented with 2mM L-glutamine and 10% heat-inactivated defined fetal bovine serum (HyClone) was used for all intracellular growth assays. The endotoxin level of the serum was reported by the manufacturer to be 13 pg/ml. The assay for intracellular growth has been described (Anthony *et al.,* 1991). Briefly, rats were injected intraperitoneally with 1 ml of thioglycollate. After 4 days macrophages were harvested by lavage with DMEM. Cells were cultured for 1 hour in 96 well microtiter plates in DMEM at a density of 2 x 10^5 cells/well, after which the nonadherent cells were washed off. The monolayers were then infected by adding 150 μl *Francisella* culture, diluted in DMEM to give a multiplicity of infection of 2 to 5 bacteria/macrophage. In other experiments, the monolayers were treated with 150 μl LPS or lipid A diluted in DMEM to give the indicated concentrations. The plates were then centrifuged for 10 min at 600 x *g* and incubated for 1 hour at 37° C in a humidified atmosphere containing 5% CO_2. After this phagocytosis step, extracellular bacteria or LPS were removed by three washes with DMEM, and the cultures incubated for 1 to 3 days. The bacteria in the cultures were enumerated by lysing the monolayers with 0.1% sodium deoxycholate (or sterile distilled water) and plating serial dilutions on CHA-H. For macrophages with mixed infections of LVS and *F. novicida*, erythromycin (15 μg/ml) was used for antibiotic selection against *F. novicida* in order to count LVS numbers accurately. The indicated growth assays contained N^G-mono-methyl arginine (NMMA)

(Calbiochem) at a concentration of 500 mM added to the DMEM during both the phagocytosis step and the growth incubation step.

***In vivo* assay of *Francisella* growth (Chapter 2).** For infection studies, *Francisella* strains were grown in TSB-C to a density of approximately 1 x 10^7 bacteria per 100 µl and stored in aliquots at -76°C. Prior to infection, an aliquot was thawed and diluted in sterile physiological saline to a concentration of 1.3 x 10^4 LVS or 1.3 x 10^5 *F. novicida* per 200 µl. Rats were inoculated intraperitoneally with 200 µl of this suspension. At the indicated times following infection, rats were killed by CO_2 asphyxiation and the spleens were removed and homogenized in sterile 0.85% saline. Serial dilutions of organ homogenates were plated on CHA-H, and the number of colony forming units (CFU) per organ was determined after 1-2 days incubation at 37°C. For the mixed infections of LVS and *F. novicida*, dilutions were plated on CHA-H (to enumerate *F. novicida* growth), as well as on CHA-H plates containing Erm (15 µg/ml) in order to select against *F. novicida* and to count LVS numbers accurately.

Measurement of nitrite production (Chapter 2). The production of nitrite by macrophages was determined in the macrophage culture fluids 3 days after infection with *Francisella* or treatment with LPS or lipid A. Nitrite was assayed by the Griess reaction, as described elsewhere (Ding *et al.*, 1988). Briefly, 100 µl of culture supernatant were incubated with 100 µl of 0.5% sulfanilamide/0.05% naphthylethylene diamine dihydrochloride/2.5% H_3PO_4 at room temperature for 10 min. The absorbance at 540 nm was read using a Biotek microplate reader. The nitrite concentrations in the samples were determined by using sodium nitrite as the standard.

LPS and lipid A preparations (Chapter 2). A 1.5 L culture of each *Francisella* strain was grown in 4 L flasks at 37°C for 48 hours in TSB-C. The cultures were

centrifuged at 3 000 x *g* for 15 min, washed 3 times in phosphate buffered saline, once in methanol, once in acetone, and then lyophilized. The LPS was then extracted with the hot phenol method (Westphal and Luderitz, 1954). The crude LPS was purified by treatment with DNase, RNase, and proteinase K, followed by harvesting the pellet after centrifugation at 100 000 x *g* 3 times for 12 hours. The purified LPS concentration was determined by measuring the dry weight, followed by resuspension in sterile endotoxin-tested water. Lipid A was hydrolyzed from the LPS carbohydrate portion by resuspending the purified LPS in 2% acetic acid followed by incubation at 100° C for 2 hours. Insoluble lipid A was pelleted by centrifugation at 5 000 x *g* for 15 min. Lipid A was lyophilized and the concentration was determined by measuring dry weight, followed by resuspension in sterile endotoxin-tested water. Prior to treatment of macrophages, the lipid A samples were heated to 65°C briefly to facilitate solubilization. The *F. tularensis* LPS is abbreviated Ft-LPS and the *F. novicida* type of LPS is abbreviated Fn-LPS.

Mouse monoclonal antibody production (Chapter 2). Female BALB/c mice were immunized with 100 µl of 10^4 live, avirulent strains of *F. tularensis* and *F. novicida*, followed by a booster immunization 21 days later. Subsequent booster immunizations were performed every 2 weeks until LPS-reactive antibody was detectable in the serum by Western immunoblot assay. Fusion of spleen cells and cloning of hybridoma cells was carried out as described using the ClonaCell HY (Stem Cell Technologies, Inc., Vancouver, BC) protocol. Individual hybridomas were screened by Western immunoblot for reactivity with *F. tularensis* LVS and *F. novicida* LPS.

SDS-PAGE and Immunoblotting (Chapters 2 and 3). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (Laemmli, 1970) using a 12% separating gel and a 7.5% stacking gel, both containing 0.1% SDS. LPS samples (5 µg/ml) were diluted 1:1 with sample buffer, boiled for 10

min., and 10 μl of each sample was loaded to give a final concentration of 25μg of LPS loaded for each lane. Gels were electrophoresed for 50 min at 200 V using a Protean 2 mini gel system (Bio-Rad). Following electrophoresis, LPS was transferred to an Immobilon-P transfer membrane (Millipore) using a Tris (12.5 mM)/glycine (96 mM)/methanol (10%) buffer in a semi-dry immunoblotting apparatus (LKB) according to the manufacturer's instructions. Following transfer, the membrane was blocked with 10% skim milk in PBS with 0.05% Tween-80 overnight at room temperature. Detection of primary antibody was performed using the ECL chemiluminescent system (Amersham) according to the manufacturer's instructions. The primary antibody was either the anti-Ft-LPS mouse monoclonal antibody or the anti-Fn-LPS mouse monoclonal antibody described here. The secondary antibody was a horse radish peroxidase conjugated sheep anti-mouse IgG monoclonal antibody.

Molecular and genetic methods (Chapter 3). All enzymes used in this study were supplied by New England Biolabs (NEB, Beverly, MA) and used under conditions recommended by the manufacturer. Isolation of DNA from agarose gels was accomplished by electroelution using an IBI apparatus (for size-restricted chromosomal DNA) or the Quiaex Gel Extraction Kit (for plasmid subjected to alkaline phosphatase treatment). *E. coli* were transformed by electroporation using the Gene Pulser II Electroporation System (Bio-Rad) according to the manufacturer's instructions. *Francisella novicida* was transformed as described previously (Anthony et. al., 1992). Southern blots were performed using the ECL chemiluminescent system according to the manufacturer's instructions.

Construction of an *F. novicida* plasmid library and shuttle mutagenesis (Chapter 3). pUC18 was digested with Eco RI, treated with 1.5 μl (10 000 U/ml) of calf intestinal alkaline phosphatase (CIAP) (NEB) per 2-3 μg of DNA for

1hour, and linear vector was purified by agarose gel electrophoresis and gel extraction using Quiaex resin.

Partially Tsp509 I-digested *F. novicida* strain U112 chromosomal DNA was separated on 0.8% agarose TBE gels. Random 5-10 kb fragments were excised and purified by electoelution. The resulting size-restricted chromosomal DNA was ligated to linear, Eco RI cut, alkaline phosphatase-treated pUC18. Each ligation mix was tested for ligation of the expected size range of chromosomal DNA fragments by transformation into *E. coli* DH5α. Transformants were then picked and screened by isolation of plasmid DNA followed by agarose gel electrophoresis. Only samples which yielded greater than 95% 5-10 kb size-restricted chromosomal DNA inserts were used for shuttle mutagenesis.

Shuttle mutagenesis was performed as described by Haas *et al.* (1993) with a few modifications. Ligation mixtures produced and screened as described above were electroporated into *E. coli* E131 (pTn*Max2*) and selected on LB plates containing Erm and Amp. Transformants were picked and grown in pools of 100-150 colonies for 1hour at 37°C in LB liquid broth containing Erm and Amp. Cultures were then shifted to 30°C and grown for 2 hour in the presence of 100 μM IPTG to induce transposase production and transposition of the Tn*Max2* transposon. Following this 2hour incubation, a crude plasmid extraction was performed on each culture and the resulting DNA used to transform *F. novicida.* One hundred pools of one hundred colonies were tranformed into *F. novicida*, selected on CHA-H plates containing Erm, and screened visually on CHA-H plates for aberrant colony phenotypes which may represent LPS biosynthesis mutants.

In order to obtain DNA sequence of the regions adjacent to the Tn*Max2* transposon of plasmids pSC92-1, pSC66-1, and pSC4-1, it was necessary to subclone a Bam HI fragment of each of these plasmids into the pTZ19U phagemid vector. Two Bam HI sites are located close to the ends of the Tn*Max2* transposon. Restriction analysis revealed that these are the only Bam HI sites within the plasmids. Thus, plasmids pSC66-1, pSC92-1, and pSC4-1 were digested with Bam HI in order to remove the majority of the Tn*Max2*

transposon. The resulting fragments were subsequently subcloned into the Bam HI site of pTZ19U, such that the regions of DNA sequence that were previously adjacent to the Tn*Max2* transposon would be located next to the universal primer region of pTZ19U. DNA sequencing was performed using single-stranded DNA, the -40 universal primer, and Sequenase Version 2.0 according to the manufacturer's instructions.

Complement, deoxycholate and hydrogen peroxide sensitivity assays (Chapter 3). The susceptibility of *F. novicida* wild type, SC119, and the putative LPS mutants to serum, hydrogen peroxide, and deoxycholate killing were evaluated according to the following procedure. Bacteria were grown overnight in TSB-C, and the following day 1 ml of bacterial culture were washed in PBS three times to remove any residual medium. After the final wash, bacteria were resuspended in 100-200 µl PBS, and diluted 25,000-fold into 5ml of PBS. 100 µl of bacteria at a concentration of approximately 10^6 CFU per ml were inoculated into each well of a 96-well microtitre plate. 100 µl of either deoxycholate, guinea pig serum, or hydrogen peroxide diluted in PBS was added to each well to yield the indicated final concentrations. The number of viable bacteria was measured in the wells following a 3h incubation (serum and hydrogen peroxide) or 45 min incubation (deoxycholate) by serial dilution and plating on CHA-H. Percent survival was calculated by comparing the number of bacteria in the test wells to the number of bacteria incubated for the same period of time in PBS.

pH sensitivity assay. The survival of *F. novicida* wild type, SC119, and the putative LPS mutants in different pH environments was evaluated according to the following procedure. Bacteria were grown overnight in TSB-C, and the following day 1 ml of bacteria were washed in PBS three times to remove any residual medium. After the final wash, bacteria were resuspended in 100-200 µl PBS, and diluted 25,000-fold into 5 ml of PBS of the appropriate pH. 100 µl of bacteria at a concentration of approximately 10^6

CFU per ml were inoculated into each well of a 96-well microtitre plate. The number of viable bacteria was measured in the wells following a 3 hour incubation by serial dilution and plating on CHA-H. Percent survival was calculated by comparing the number of bacteria in the test wells to the number of bacteria in pH 7.0 PBS at the beginning of the experiment prior to incubation.

DNA manipulation and analysis. (Performed by Megan McDonald, reported in Chapter 1) Standard recombinant DNA techniques (Sambrook et. al., 1989) were used except where described. *E. coli* strains were transformed according to the procedure developed by Hanahan (Hanahan, 1983). PCR was performed using *Taq* polymerase (Perkin Elmer) according to the manufacturer's instructions. *msbA/orfE* were amplified using the primers 5'CGGGATCCAGTGGTTGGCGTGCCA3' and 5'CGGGATCCCGTAACTAGTTGCCAGA3' to initiate the amplification at the 5' and 3' ends of the operon, respectively. Generalized transduction using phage P1 was performed as previously described (Miller, 1971).

NTG mutagenesis. (Performed by Megan McDonald, reported in Chapter 1) Temperature sensitive mutations in the appropriate genes were induced using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis as previously described (Miller, 1971). Briefly, *E. coli* strains harboring plasmids containing the genes to be mutated were grown at 37°C in LB to exponential phase. Cells were then washed three times and resuspended in 0.1M citrate buffer (pH 5.5). NTG dissolved in citrate buffer was added to a final concentration of 50 μg/ml and the mixture was incubated at 37°C for 30 min. The mutagenized cells were then washed three times in phosphate buffered saline (PBS), resuspended in LB, and grown overnight at 30°C.

Deoxycholate sensitivity assay. (Performed by Megan McDonald, reported in Chapter 1) The susceptibility of temperature sensitive *valAB* mutants to deoxycholate was evaluated according to the following procedure. Cultures of bacteria were aliquoted into 1.5 ml centrifuge tubes, washed three times and resuspended in PBS. 100 µl of bacteria at a concentration of approximately 10^6 CFU per ml were added to wells of a 96-well microtiter plate. The indicated concentrations of sodium deoxycholate (Sigma) were added to the bacteria. Killing of bacteria was determined by measuring colony forming units (CFU) on LB agar from control wells (PBS) and from sodium deoxycholate wells after 30 min of incubation.

Temperature-shift growth assay. (Performed by Megan McDonald, reported in Chapter 1) The growth kinetics of various strains at 30°C and 42°C were assayed by growing cultures in LB media supplemented with the appropriate antibiotics at 30°C with agitation. Every hour turbidity readings were taken using a Klett-Summerson meter to measure cell mass. When the cultures reached early logrithmic phase at 2 hours, one flask for each strain was maintained at 30°C while another flask was shifted to 42°C. Turbidity measurements were then continued every hour post temperature-shift for 6 hours.

Immunofluorescence microscopy. (Performed by Megan McDonald, reported in Chapter 1) Expression of the *Chlamydia* genus-specific epitope by strains MKM5023 and MKM5523 was detected according to the following procedure. Both strains were grown as described in the temperature-shift growth assay. However, 15 min following the temperature shift to 42°C, the media of selected cultures were supplemented with 1 mM IPTG. 5 ml aliquots were taken 2 hours post-IPTG addition, pelleted by centrifugation at 8,000 x *g* for 15 min, and resuspended in 500 µl fresh 4% formaldehyde in PBS and allowed to incubate 1 hour at room temperature. Cells were then pelleted again, and resuspended in 500 µl PBS. Formaldehyde-treated cells were allowed to air dry

on microscope slides. Cells were fixed by immersion in acetone at -20°C for 20 min and allowed to air dry. Normal goat serum in PBS containing 5% fetal bovine serum (FBS, Hyclone) was added to the acetone-fixed cells for 30 min. Slides were then washed three times in PBS plus 5% FBS for 5 min each. The primary antibody was a mouse IgG monoclonal antibody CHL-888 (Pharmingen) reactive with the Chlamydial genus-specific LPS epitope, and the secondary antibody was a fluoro-isothiocyanate (FITC) conjugated goat $F(ab')_2$ anti-mouse IgG. Both antibodies were added at 1:50 dilutions and washed as outlined above. To prevent photo-bleaching, microscope slides were treated with the anti-fade reagent 1,4-diazabicyclo[2.2.2.]octane according to the manufacturer's procedure (Molecular Probes, Eugene, Oregon). Slides were viewed at 1,000 x using a Zeiss Axioscope microscope equipped with epifluorescence and camera. Excitation light was passed through a filter that transmits light in the 450 to 490 nm range. Fluorescent images were recorded on Kodak Kodacolor ISO 100 film exposed for 125 seconds.

Bacterial inner and outer membrane purification and detection of the *Chlamydia* genus-specific epitope (Chapter 1). Cultures of MKM5523 (200 ml) were grown at 30°C to an OD (600 nm) of 0.5 to 0.7. Cells were then either shifted to 42°C or maintained at 30°C for 15 min, and then grown for a further 2 hours in the presence of 1 mM IPTG. Equal amounts of cells were harvested, and inner and outer membranes were separated by sucrose density gradient centrifugation of the total membrane fraction as described (Osborn et. al., 1972). Fractions (400 µl) were collected, and 50 µl of each fraction was used to assay NADH oxidase activity (Osborn et. al., 1972). ELISA assays were used to determine the levels of the Chlamydial genus specific epitope in 200 µl of each fraction. The primary antibody was a mouse monoclonal anti-*Chlamydia* genus-specific epitope CHL-888 or EVI-HI (a kind gift of H. Caldwell) and the secondary antibody was an alkaline phosphatase-conjugated sheep anti-mouse IgG + IgM (Caltag,

South San Francisco, CA). Absorbence at 405 nm of hydrolyzed p-nitrophenyl phosphate (Sigma) was determined using a Biotech microplate reader.

Table 2

Bacterial strains and plasmids used in Chapter 1

Strain or Plasmid	Relevant Characteristics	Reference or Source
W3110	Wild type strain	Laboratory strain
DH5α	F-Φ80*lacZ*ΔM15*endA1recA1hsdR17supE44thi1gyrA96 relA1* Δ(*lacZYA-argF*)U169	Bethesda Research Laboratories
DK1	Δ(*srl-recA*)306 *ara*Δ*I*39Δ(*ara leu*)7697 Δ*lacX*74 *galU- galK hsdR strA mcrA mcrB*	(Kurnit et. al., 1989)
MLK1088	W3110 *msbA*::Δ*cam zbj*-1230::Tn*10*(pK-Cla)	(Karow et. al., 1993)
MCL30	Hfr P045 *thi*-1 Δ(*srl-recA*)306::Tn*10*	E. Ishiguro, University of Victoria
MKM1	W3110 *msbA*::Δ*cam* (pMM1)	This study
MKM2	W3110 *msbA*::Δ*cam* Δ(*srl-recA*)306::Tn*10* (pMM1)	This study
MKM50	W3110 *msbA*::Δ*cam* (pMM2)	This study
MKM55	W3110 *msbA*::Δ*cam* (pMM3)	This study
MKM5023	W3110 *msbA*::Δ*cam* (pMM2, pMM23)	This study
MKM5523	W3110 *msbA*::Δ*cam* (pMM3, pMM23)	This study
pBGS18	Km^R	(Spratt et. al., 1986)
pCM.301	temperature sensitive pSC101 replicon	(Tucker et. al., 1986)

pGEM-T	Ap^R	Promega
pK-Cla	pREG153::3.8 kb *Kpn* I-*Cla* I *msbA/orfE*, Ap^R	(Karow et. al., 1993)
pMMB66HE	Inc^Q *lacI*^{q} *bla*(Ap^R) *ptac rrnB*	(Morales et. al., 1991)
pKEM14-5	pTZ18U::5kb *Sal* I *valAB* fragment	(Mdluli et. al., 1994)
pMM1	pCM.301::*msbA/orfE*	This study
pMM2	pBGS18::5 *Sal* I-*Sal* I *valAB*, Km^R	This study
pMM3	pBGS18::5 *Sal* I-*Sal* I temperature sensitive allele of *valAB*, Km^R	This study
pMM23	pMMB666HE::mTn*10*::*gseA*	This study

Chapter 1

Temperature sensitive lesions in the Francisella *novicida valA* gene cloned into an *Escherichia coli msbA/lpxK* mutant affecting deoxycholate resistance and lipopolysaccharide assembly at the restrictive temperature*

* A version of this manuscript has been published in The Journal of Bacteriology (1997), vol 179 (24), pages 7638-7643.

INTRODUCTION

We have recently discovered a genetic locus in *Francisella novicida* that contains an apparent operon consisting of two open reading frames, *valAB* (Mdluli *et al.*, 1994). *F. novicida* strains thought to harbor mutations in *valAB* are defective for growth in macrophages and exhibit increased sensitivity to serum and deoxycholate, thus suggesting compromised outer membrane integrity. These two genes show high identity at the deduced amino acid level to the *E. coli* genes *msbA* and *orfE*, respectively.

Interestingly, the *msbA* gene was originally identified as a multicopy suppressor of the *E. coli htrB* gene (Karow *et al.*, 1991). Mutants having lesions in *htrB* exhibit an unusual phenotype whereby growth and viability of mutants at temperatures above 32.5° C in rich media are impaired. The gene product of *htrB* has been shown to be a Kdo-dependent acyl transferase involved in lipopolysaccharide (LPS) biosynthesis (Clementz *et al.*, 1991). Similar to *msbA*, we have previously shown that *valAB* can suppress mutations in *htrB*, thus suggesting that both gene products may have similar functions related to LPS expression. Recent work has demonstrated that *orfE* encodes a lipid A 4'-kinase, and has subsequently been renamed *lpxK* (Garrett *et al.*, 1997).

The presumptive gene products ValA and MsbA are members of the superfamily of ATP binding cassette (ABC) transporters. ABC transporters are a large family of integral membrane proteins that are responsible for the uptake or efflux of a wide variety of both proteinaceous and non-proteinaceous substrates (Fath *et al.*, 1993). In several bacterial systems, the involvement of ABC transporters in the export of capsular polysaccharide and the translocation of LPS O-antigen across the cytoplasmic membrane has been clearly demonstrated (Bronner *et al.*, 1993; Pavelka *et al.*, 1991). Interestingly, recent evidence suggests a role for MsbA in the translocation of LPS or its precursors across the cytoplasmic membrane (Polissi *et al.*, 1996). Given the similarity between *msbA* and *valA*, our objective in this study was to determine if the *valAB* gene products are involved in LPS transport.

Here we demonstrate that *valAB* can suppress the lethal phenotype of a *msbA/lpxK* mutation in *E. coli*. Furthermore, through the use of a temperature sensitive allele of *valAB*, we show that *E. coli* cells defective in both *valAB* and *msbA/lpxK* exhibit increased sensitivity to the detergent deoxycholate, and impaired cell surface expression of the LPS epitope synthesized by GseA, a *Chlamydia trachomatis* Kdo transferase. Thus, we hypothesize that *valAB* are involved in transport of LPS to the outer membrane.

RESULTS

Please note that the temperature sensitive *valA* mutant strain construction (*E. coli* strains MKM55 and MKM5523), as well as the temperature sensitive growth analysis, deoxycholate sensitivity assays, and immunofluoresence microscopy studies of these strains were done by Megan McDonald and are only included here in order to provide a complete story. My personal role in this study involved the DNA sequencing of the NTG mutagenized *valAB* and the sucrose density gradient centrifugations for separation of the inner and outer membranes of the temperature sensitive mutant MKM5523.

(I) Construction of a temperature sensitive locus of *valAB* (performed by Megan McDonald)

In order to study the function of *valAB* we chose to perform experiments in an *E. coli* genetic background, where we could perform many genetic manipulations. Given that *msbA*/*lpxK* --and presumably *valAB*-- are essential to the viability of the cell we decided to construct conditional lethal mutations in *valAB*.

To provide the proper background to study *valAB* we cloned *msbA*/*lpxK* into pCM.301, an ampicillin resistant plasmid that is temperature sensitive in its replication. *msbA*/*lpxK* was amplified from the plasmid pK-Cla via the polymerase chain reaction. After cloning into the pGEM-T vector the insert was removed by digestion with *Bam*H I and subcloned into the *Bam*H I site of pCM.301 to yield plasmid pMM1.

Plasmid pMM1 was transformed into W3110. Subsequently, bacteriophage P1 transduction from strain MKL1088 was used to introduce an allele of *msbA* containing a chloramphenicol resistance cassette into the chromosome of W3110(pMM1). As well, a *recA* locus that is interrupted by miniTn*10* was introduced by P1 transduction from strain MCL30 in order to prevent homologous recombination between the chromosomal copy and the plasmid-borne copy of *msbA*. The *msbA*::Ωcam *recA*::Tn*10*(pMM1) strain named

MKM2 was unable to grow at 42°C due to the loss of the temperature sensitive partition plasmid encoding *msbA/lpxK*.

A 5 kb *Sal* I-*Sal* I fragment containing *valAB* was subcloned from pKEM14-5 (Belunis *et al.*, 1992) into the kanamycin resistant plasmid pBGS18 to form pMM2. DK1(pMM2) was mutagenized with NTG, after which the plasmid was isolated and used to transform MKM2. One thousand transformants were picked to duplicate agar plates that were incubated at 30° and 42°C. At the restrictive temperature pMM1 (encoding *msbA/lpxK*) is lost, and the cells must rely on *valAB*, encoded by pMM2, for survival. Hence, cells containing temperature sensitive alleles of *valAB* should not be viable at 42°C. Two colonies were found to be stably temperature sensitive for growth, and plasmid pMM3 from one of these temperature sensitive strains was chosen for further study.

(II) DNA sequence analysis of the temperature sensitive *valAB* locus

The DNA insert in plasmid pMM3 was subjected to DNA sequence analysis. Three nucleotide changes were found in the *valAB* locus (Accession No. L17003). One change, a C to a T transition at bp 430 was 53 bp upstream of the translational start of ValA. As well, two changes in the *valA* cistron were found; both were C to T transitions that changed S543 to a F (bp 1841) and T458 to an I (bp 1856). These amino acid changes are next to the Walker B ATP binding motif, which spans amino acids 461 to 498. By analogy with other ABC transporter proteins, the C-terminus (amino acids 284-572) of ValA lies on the cytoplasmic side of the inner membrane.

(III) Phenotypic analysis of MKM55 (performed by Megan McDonald)

Plasmid pMM3 was transformed into W3110 to yield a Km^r, Am^s strain, and the Cm-interrupted *msbA* locus from strain MKL1088 was introduced into the chromosome as described above. The resulting strain, named MKM55, was unable to grow on LB agar plates at 42°C. Further, this strain ceased growth in broth culture 1 hour after a shift from

30° to 42° (Figure 8). A control strain, named MKM50, harboring a wild type copy of *valAB* showed continued growth at 42°C.

E. coli that have defects in the outer membrane, especially alterations in the lipopolysaccharide (LPS) composition, are often sensitive to detergents such as deoxycholate. Hence, we tested the relative sensitivity to deoxycholate of strains harboring a temperature sensitive *valAB* locus. Figure 9 shows that strain MKM50 harboring a wild type version of *valAB* is resistant to a 30 min exposure to 5% deoxycholate, regardless of whether it is grown at 30° (data not shown) or at 42 °C. Also, strain MKM55 harboring a temperature sensitive lesion of *valAB* and an interrupted *msbA* locus is insensitive to 5% deoxycholate when grown at 30°C, the permissive temperature (data not shown). However, when strain MKM55 is grown at 42°C it is significantly more sensitive to 5% deoxycholate as compared to strain MKM50.

(IV) Immunofluorescence analysis of LPS cell surface expression in MKM5523 (performed by Megan McDonald)

To test the hypothesis that ValA may be involved in the transport of LPS to the outer membrane we introduced a clone of *gseA* into strains MKM55 and MKM50 (Belunis *et al.*, 1992). The *Chlamydia* gene *gseA* encodes a Kdo transferase responsible for the addition of three Kdo residues to a lipid A precursor (Brade *et al.*, 1987). These three Kdo residues constitute the *Chlamydia* genus-specific epitope located within the inner core of the LPS molecule, and may be readily detected on the surface of *E. coli* cells expressing a clone of *gseA*. A 2.3 kb *Eco*R I - *Eco*R I fragment from pFEN212 was subcloned into the *Eco*R I site of the expression vector pMMB66HE, and subsequently transformed into MKM55 and MKM50 to yield the strains MKM5523 and MKM5023. Thus, strains MKM5523 and MKM5023 harbor a defective *msb A/lpx K* locus, an IPTG-inducible subclone of *gseA*, and either a temperature sensitive or wild type allele of *valAB*, respectively.

The IPTG-induced expression of the *Chlamydia* genus-specific epitope by both strains was determined at the restrictive and permissive temperatures by immunofluorescence microscopy using antibody reactive with the Chlamydial genus specific LPS epitope (Figure 10). Cultures of experimental and control strains were grown at 30°C and either maintained at 30°C or shifted to 42°C during mid-logarithmic growth. Fifteen minutes after the shift IPTG was added to induce expression of the Chlamydial epitope, and the ratio of immunofluorescent cells (expressing the epitope) to non-fluorescent cells was interpreted as an estimate of the transport of core LPS.

Strain MKM5523 harboring the temperature sensitive *valAB* locus exhibited a 10-fold lower expression of the *Chlamydia* genus-specific epitope after a shift to 42°C relative to cultures maintained at 30°C, and similarly showed approximately 10-fold less expression as compared to the control strain MKM5023 at both the permissive and non-permissive temperatures (Figure 10). In addition to the use of standard error as a criterion for the validity of the results we also employed the Mann-Whitney U test to examine the significance of the induction values. When the expression of the Chlamydial epitope was compared between different cultures using the Mann-Whitney U test the p-values were as follows: A vs. B, 0.036; C vs. D 0.003; E vs. F, 0.0003; G vs. H, 0.452. These results confirm that there is a significant increase in the expression of the Chlamydial genus-specific epitope between the IPTG-induced and non-induced cultures of strain MKM5523 at 30°C, and of the control strain MKM5023 at both temperatures. Conversely, there was no significant increase in epitope expression for strain MKM5523 at 42°C. Therefore, in an *E. coli* strain harboring a non-functional *msbA/lpxK* locus and a temperature sensitive allele of *valA*, expression of the *Chlamydia* genus-specific epitope at the cell surface is decreased at the non-permissive temperature, thus suggesting that ValA may be involved in the transport of LPS to the cell surface.

(V) Analysis of transport of lipid A-core polysaccharide across the inner membrane in MKM5523

We also measured the association of the Chlamydial genus-specific epitope with the inner membrane as an indicator of impaired translocation across the inner membrane at the restrictive temperature. Membranes from strain MKM5523 grown at the restrictive and permissive temperatures were prepared and separated on sucrose gradients according to the method of Osborne et. al. (1972). NADH oxidase activity was used as a marker for the inner membrane, and the amount of Chlamydial epitope was determined using an ELISA assay. The results of these assays are shown in Figure 11. In two separate experiments there is increased association of the Chlamydial epitope with the NADH oxidase fraction in membranes isolated from cultures grown at the restrictive temperature relative to membranes isolated from cultures grown at the permissive temperature. These results suggest the accumulation of LPS in the inner membrane at the restrictive temperature in ValA temperature sensitive mutants.

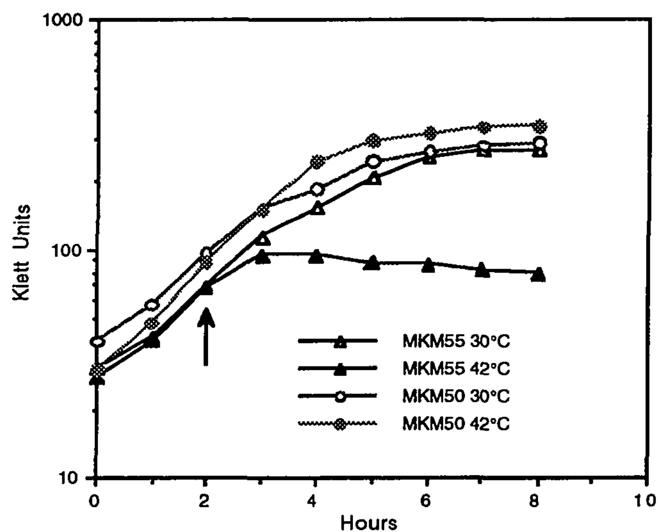


Figure 8. Growth characteristics of *E. coli* strains MKM50 and MKM55 at 30°C and 42°C. All bacterial cultures were initially grown at 30°C. Every hour the cell density was measured using a Klett-Summerson meter. After 2 hours (indicated by the arrow), select cultures were shifted to 42°C.

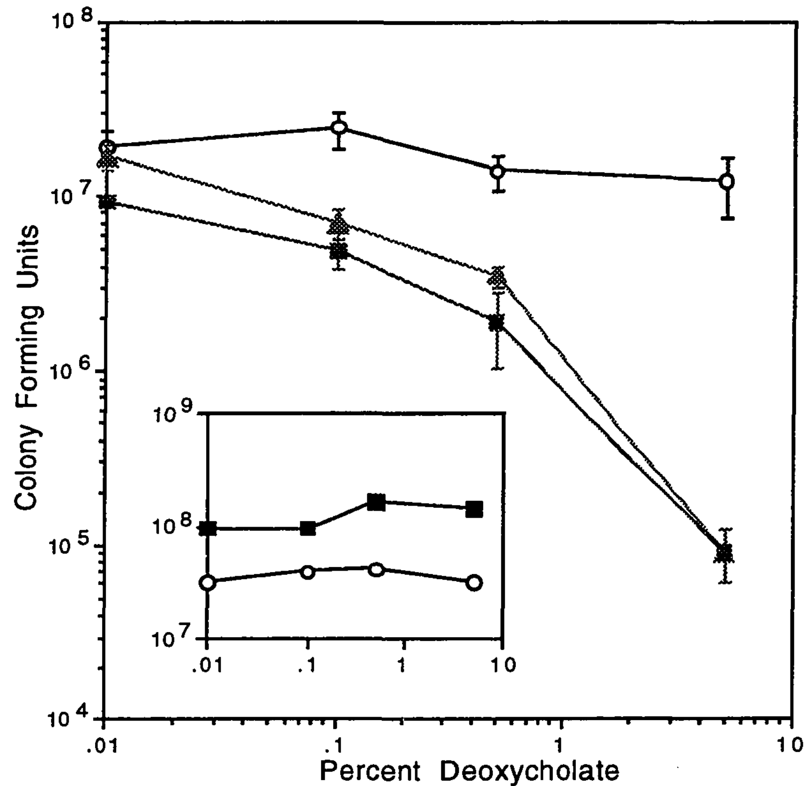


Figure 9. Deoxycholate sensitivity of *E. coli* strains MKM55 and MKM50 (inset). All bacterial cultures were and treated with deoxycholate for 0.5 hours at 30°C, then shifted to 42°C for the indicated period of time. MKM55 maintained at 42°C for 0 hours (open circles), 1 hour (closed triangles), and 1.5 hours (closed squares). Inset: MKM50 maintained at 42°C for 0 hours (open circles) and 1.5 hours (closed squares). Results are expressed as the averages of three determinations +/- standard error.

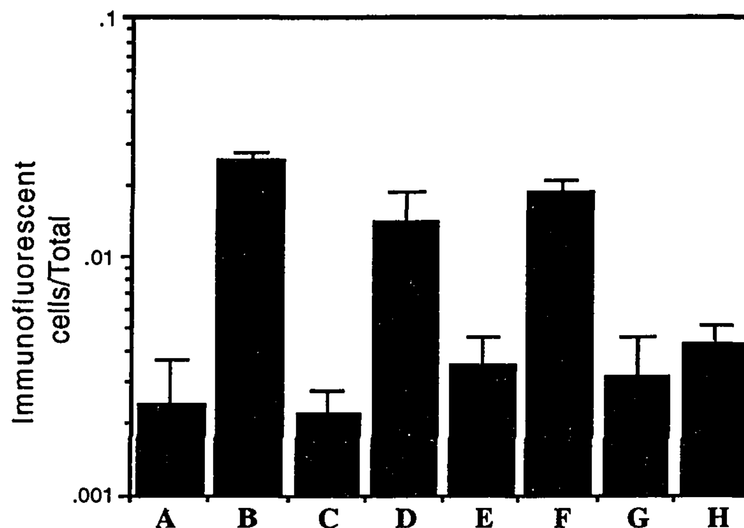


Figure 10. Immunofluorescence analysis of assembly of the Chlamydial LPS epitope in *E. coli* strains harboring a temperature sensitive *valAB* locus. Cultures were grown to mid-logarithmic phase at 30°C, and select cultures were shifted to 42°C. Fifteen minutes post-temperature shift IPTG was added to the appropriate cultures and after two hours samples were collected and reacted with monoclonal antibody specific for the genus-specific LPS epitope of *Chlamydia*. Cells reacting with monoclonal antibody were detected with a FITC-conjugated secondary antibody and counted. The values represent the number of fluorescent cells divided by the total number of cells in the cultures. (A) MKM5023 maintained at 30°C; (B) MKM5023 maintained at 30°C after IPTG induction; (C) MKM5023 post-shift to 42°C; (D) MKM5023 post-shift to 42°C after IPTG induction; (E) MKM5523 maintained at 30°C; (F) MKM5523 maintained at 30°C after IPTG induction; (G) MKM5523 post-shift to 42°C; (H) MKM5523 post-shift to 42°C after IPTG induction. Data is expressed as the mean +/- standard error of the ratio of fluorescent cells to the total number of cells per field. Cells were counted from a minimum of ten fields of view for each parameter, which included a minimum of 500 cells as seen by bright field microscopy

from a total of two experiments. For statistical purposes, each field of view was chosen in an unbiased manner and treated as a sample of the population.

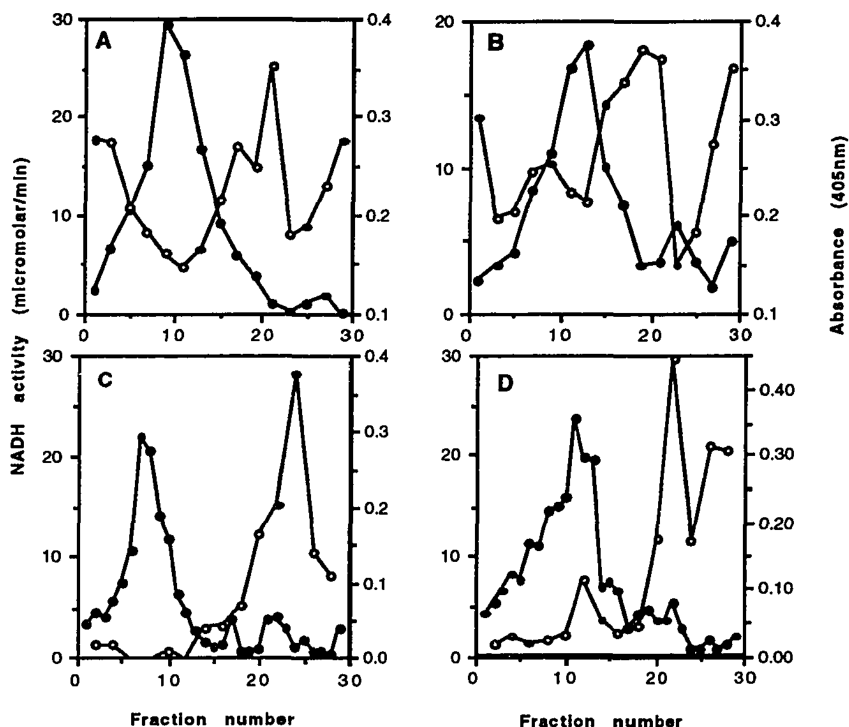


Figure 11. Analysis of the association of the Chlamydial genus-specific LPS epitope with NADH oxidase activity in sucrose density gradient profiles. Strain MKM5523 was grown at 30°C, A and C; or 42°C, B and D. Two separate experiments (upper two panels, and lower two panels) are shown. NADH activity (closed circles) : μmoles min^{-1} oxidized by 50 μl of each fraction. Fraction 1 represents the top of the gradient. Relative amounts of the Chlamydial genus-specific LPS epitope were determined in 200 μl of each fraction by ELISA (open circles) : absorbance at 405 nm.

DISCUSSION

We have previously demonstrated that in *F. novicida* mutants thought to contain a lesion in the *valAB* locus exhibited increased sensitivity to serum and were defective for growth in macrophages (Mdluli *et al.*, 1994). Since LPS is located in the outer membrane of Gram negative bacteria, it is essential for bacterial growth and survival and often confers protection against bactericidal agents such as serum complement and detergents. Therefore, if the transport of LPS to the outer membrane is reduced or abolished in the absence of *valAB*, then such a mutant may be expected to show increased sensitivity to membrane permeabilizing agents. Indeed, in this study we demonstrate that the expression of a temperature sensitive *valAB* locus in *E. coli* can suppress the lethal phenotype of a mutation in the *E. coli* homologue *msbA/lpxK*. At the permissive temperature, these cells are resistant to the detergent deoxycholate. However, at the non-permissive temperature ValA is inactivated and the cells become sensitive to deoxycholate. Furthermore, these mutants exhibited decreased cell surface expression of the *Chlamydia* genus-specific LPS epitope at the non-permissive temperature and increased association of the epitope with the inner membrane. Collectively, this evidence suggests that ValA is required for the optimal expression of LPS at the bacterial cell surface.

The role of ABC transporters in the export of LPS O-antigen and capsular polysaccharide has previously been demonstrated. For example, capsular polysaccharides in *E. coli* K1 and K5 are synthesized at the inner face of the cytoplasmic membrane, and subsequently require the action of a two component ABC transporter system (KpsT and KpsM) for transport across the cytoplasmic membrane (Bronner *et al.*, 1993; Kroncke *et al.*, 1990; Pavelka *et al.*, 1991). The presence of an ATP-binding protein is believed to couple the energy of ATP hydrolysis to the transport of the polysaccharide. Similarly, LPS O-antigen export in *Yersinia enterocolitica* serotype 0:3 and *Klebsiella pneumoniae* serotype 01 requires a two-component ABC transporter system encoded by the *rfb* locus (Bronner *et al.*, 1993; Zhang *et al.*, 1993). In *Yersinia*, mutations in these genes results in the

intracellular accumulation of O-antigen, thus suggesting a role for these ABC transporter systems in the transport of O-antigen across the cytoplasmic membrane. Similarly, *E. coli* K-12 harboring a clone of the *Klebsiella rfb* locus deleted in the genes for the ABC transporter system (*rfbA* and *rfbB*) accumulate cytoplasmic O-antigen but retain expression of rough LPS molecules on their cell surface (Bronner *et al.*, 1993).

Recent experiments performed by Polissi et. al. were designed to examine the role for the *E. coli msbA* gene in the translocation of LPS or its precursors across the cytoplasmic membrane (Polissi *et al.*, 1996). Through the use of sucrose density centrifugation for isolation of bacterial inner and outer membranes, they demonstrated that the LPS precursor N-acetyl [^{3}H]-glucosamine (GlcNAc) accumulates to high levels at the inner membrane in a strain in which the concentrations of MsbA and LpxK have been reduced by dilution of a recombinant plasmid harboring the only functional genes for these proteins. However, the recent discovery that LpxK is a lipid A 4' kinase explains their unequivocal results, as loss of LpxK in their experiments would result in the inability to complete lipid A biosynthesis (see the General Introduction). Incomplete lipid A structures are generally inefficiently expressed at the bacterial cell surface, thus rendering their results inconclusive. In this study, we created a temperature sensitive allele of the ABC transporter ValA, but left the LpxK homolog (ValB) intact. Our results suggest lipid A-core molecules may accumulate at the cytoplasmic membrane in ValA mutants, but further experiments need to be done to confirm this hypothesis.

In this study we evaluated the *E. coli* cell surface expression of the *Chlamydia* genus-specific LPS epitope encoded by *gseA* in the presence of a temperature sensitive ValA. Since we demonstrate that *E. coli* strains simultaneously lacking a functional ValA and MsbA/LpxK exhibit decreased cell surface expression of an epitope located within the inner core region of LPS, then it is unlikely that ValA is involved in the transport of O-antigen. Instead, ValA may be required for the transport of lipid A molecules linked to core polysaccharide across the cytoplasmic membrane. Conversely, ValA may be involved in

the transport of another unknown component essential for the export or synthesis of a complete LPS molecule.

Chapter 2

Phase variation in *Francisella tularensis* affecting intracellular growth, lipopolysaccharide antigenicity and nitric oxide production*

*Components of this chapter have been published in Molecular Microbiology (1996) vol. 20(4), pages 867-874 and FEMS Microbiology Letters (1997), vol. 153, pages 71-74.

INTRODUCTION

Growth inside macrophages is a strategy that many prokaryotic and eukaryotic pathogens use to evade the host immune responses (Moulder, 1985). Not only does growth inside macrophages protect microbes during the acute phase of disease it also serves as a niche for the long term survival of pathogens. Indeed, a large number of intracellular pathogens, including the causative agents of tuberculosis and typhoid fever, are well known for their ability to lie dormant in humans and re-emerge to cause disease years or decades after the initial infection. The carrier state promotes the survival of the pathogen by prolonging its association with its host and allowing recurring bouts of the disease to maximize its spread.

Another major virulence strategy employed by microbial pathogens is to vary antigenic determinants on their surface (Brunham *et al.*, . 1993). In many pathogens where this occurs, e.g. *Trypanosoma brucei brucei* (Cross, 1990) and *Borrelia hermsii* (Barbour *et al.*, 1982) it is clear that the evolutionary advantage of the variation is to avoid the consequences of the binding of specific antibody to the surface of the microbe. In other pathogens, e.g. *Neisseria gonorrhoeae* (Rudel *et al.*, 1992), it is thought that antigenic variation is a means of adapting the microbe to different enviromental conditions in the host. However, no system has yet been described in which antigenic variation adds to the biological advantages of intracellular growth.

In some animal species, nitric oxide (NO) is produced by macrophages via the inducible enzyme nitric oxide synthase, and is instrumental in limiting the growth of intracellular pathogens (Adams *et al.*, 1991; Adams *et al.*, 1990; Green *et al.*, 1990). This NO production is believed to require the presence of an activating signal such as bacterial lipopolysaccharide (LPS) (Ruco and Meltzer, 1978). The macrophage-activating capability of LPS varies among bacteria, but often it is a potent inducer of macrophage cytokine production and non-specific resistance.

F. tularensis segregates avirulent colony opacity variants at a frequency of 10^{-3} to 10^{-4} per generation (Eigelsbach *et al.*, 1951). For a more complete discussion of *F. tularensis* colony variation, please refer to the general introduction. In this work we show that the change in the colony phenotypes correlate with an antigenic shift in the LPS O-side chain and an enhanced ability of the LPS and lipid A to stimulate the production of NO. This new form of LPS acquired by *F. tularensis* is also the predominant form of LPS found normally in *F. novicida,* a species that is indistinguishable from *F. tularensis* by nucleic acid hybridization criteria (Forsman *et al.*, 1994; Hollis *et al.*, 1989). *Francisella* strains expressing this LPS exhibit the ability to induce NO production in rat macrophages which results in inhibition of intracellular growth.

RESULTS

(I) Growth of *Francisella* in rat and mouse macrophages

We isolated an opacity variant from the LVS strain of *F. tularensis*, named LVSG. During the course of this study we observed that macrophages infected with LVSG, and a previously isolated opacity variant named LVSR (Sandstrom *et al.*, 1988) produced colonies exhibiting the same morphological phenotype as the parental *F. tularensis* LVS (see Figure 12). We isolated one such revertant from each opacity variant, named LVSGB and LVSRB, respectively. We compared the growth of LVSGB in rat macrophages to that of *F. tularensis* LVS, LVSG, and *F. novicida.* (Fig. 13). After 72 hours of growth *F. tularensis* LVS and LVSGB had increased in numbers by 100-fold whereas *F. novicida* and *F. tularensis* LVSG exhibited no apparent growth. Mixing *F. novicida* with *F. tularensis* LVS during infection of rat macrophages resulted in suppression of growth of the LVS (Fig. 15). Interestingly, LVS, LVSG, and *F.novicida* proliferated in mouse macrophages to a similar extent (Fig. 16), indicating that they are all capable of intracellular survival and growth.

(II) Nitric oxide production by rat macrophages in response to a *Francisella* infection

Since NO is the major effector molecule for killing of intracellular pathogens, we investigated the possibility that NO production was responsible for the observed *Francisella* growth inhibition (Table 3). Growth of *F. tularensis* LVSG and *F. novicida* induced over a 10 and 20-fold increase, respectively, in NO production as compared to rat macrophage monolayers infected with *F. tularensis* LVS or LVSGB. Another opacity variant, LVSR, also induced high levels of NO. A "revertant" strain isolated from LVSR-infected macrophages, named LVSRB, induced negligible NO levels similar to that of LVS and LVSGB. This NO production did not require the addition of any cytokines and the levels induced by *F. novicida* were equivalent to that produced by a rat macrophage monolayer treated with 50 U/ml IFN-γ and 10 ng/ml *E.coli* LPS (data not shown). Conversely, mouse macrophage monolayers infected with LVS, LVSG, LVSR, or *F. novicida* failed to induce any significant production of NO (data not shown). These data indicate that the observed growth inhibition of the *Francisella* strains in macrophages correlates with the ability of these strains to induce NO production.

To demonstrate that the NO levels detected are a product of the L-arginine metabolic pathway of the macrophage and are responsible for the observed inhibition of *F. novicida* and *F. tularensis* LVSG growth, the nitric oxide synthase inhibitor N^G-mono-methyl arginine (NMMA) was used. In the presence of NMMA, NO production by the macrophage was inhibited and the LVSG and *F. novicida* growth rates were restored to normal levels with over 10^7 organisms present 72 hours post infection (Fig. 14 and Table 3).

We were interested to determine if the observed *in vitro* growth suppression of LVS in a co-infection with *F. novicida* would also occur *in vivo*. In a series of experiments, female Lewis rats were inoculated intraperitoneally with either LVS alone or a mixture of

LVS and *F. novicida* in a 1:10 ratio. After 72 hours the spleens were harvested and the bacterial load of LVS was enumerated (Figure 17). In a total of four separate experiments, the numbers of LVS in the spleens from co-infected animals was reduced by a maximum of 174-fold and a minimum of 18-fold as compared to animals infected with LVS alone. The reduced numbers of LVS in the co-infections are not simply due to the increased bacterial load, since rats inoculated with the same total number of LVS exhibited a correspondingly increased level of LVS in their spleens after 72 hours (Figure 17). Thus, LVS growth *in vivo* can be significantly inhibited by the rat macrophage-activating intracellular organism *F. novicida*. Further experiments need to be performed in order to determine if the observed growth suppression is due to *F. novicida*-induced NO production.

(III) LPS variation and its effect on nitric oxide induction

Specific monoclonal antibodies (mAb) to LPS from *F. tularensis* LVS and *F. novicida* were used to perform Western immunoblots on LPS purified from the six strains (Figure 18). As expected, lanes containing LPS isolated from *F. tularensis* LVS and *F. novicida* were recognized by their specific mAb to produce a banding pattern characteristic of bacterial LPS. In both cases, the LPS from each organism reacted with its cognate antibody but failed to be recognized by antibody directed to the LPS of the other organism, indicating that both *F. tularensis* LVS and *F. novicida* express antigenically distinct forms of LPS (these two LPS types will be referred to as Ft-LPS and Fn-LPS, respectively). However, the LPS isolated from the *F. tularensis* LVSG variant cross-reacted with both monoclonal antibodies, indicating that this variant has retained expression of the Ft-LPS while newly expressing Fn-LPS. Similarly, the rough variant LVSR produced less of the Ft-LPS and more of the Fn-LPS as compared to LVSG. Finally, LVSGB and LVSRB reacted only with the Ft-LPS mAb, indicating that these two "revertant" strains exhibiting the LVS colony phenotype have lost expression of the Fn-LPS.

Since bacterial LPS is a well known macrophage activator, we investigated the effects of LPS purified from *Francisella* on NO production by rat macrophage monolayers. *E. coli* LPS is provided for a frame of reference. Macrophage NO production in response to LPS isolated from *F. tularensis* LVS, LVSG, LVSR, and *F. novicida* was dose-dependant over a concentration range of 100 ng/ml to 200 mg/ml (Figure 19A). However, the levels of NO produced by the LPS from each strain over this concentration range exhibited significant differences; LPS isolated from LVS had the lowest stimulatory effect, whereas LPS isolated from LVSG, LVSR, and *F. novicida* showed increasing abilities to induce NO production. The NO produced by the LPS isolated from each strain correlates with the relative amounts of Fn-LPS detected for each strain on the Western blot (Figure 18). In mouse macrophage cultures, LPS purified from all four strains had no significant effect on NO production over the same dose range (less than 1 nanomole NO/well for all concentrations, data not shown). Thus the ability of *Francisella* strains to grow in rat macrophages correllates with their production of Fn-LPS, the amount of NO produced by macrophages in response to a live bacterial infection of the strains, and the ability of purified LPS from each strain to induce NO production by macrophages.

Since the lipid A moiety of LPS is believed to be responsible for the macrophage-activating properties, we isolated lipid A from the LPS of the four *Francisella* strains and tested its ability to induce NO production in rat macrophage monolayers (Figure 19B). Rat macrophage NO production in response to *Francisella* lipid A exhibited a similar profile to that seen with the *Francisella* LPS, thus indicating that *F. tularensis* LVS lipid A induces less NO production by rat macrophages than *F. novicida* lipid A. Rat macrophages treated with lipid A isolated from LVSG and LVSR produced intermediate levels of NO. This suggests that in addition to the changes in the antigenicity of the O-antigen, a portion of the lipid A moiety involved in macrophage activation may be changing during phase variation.

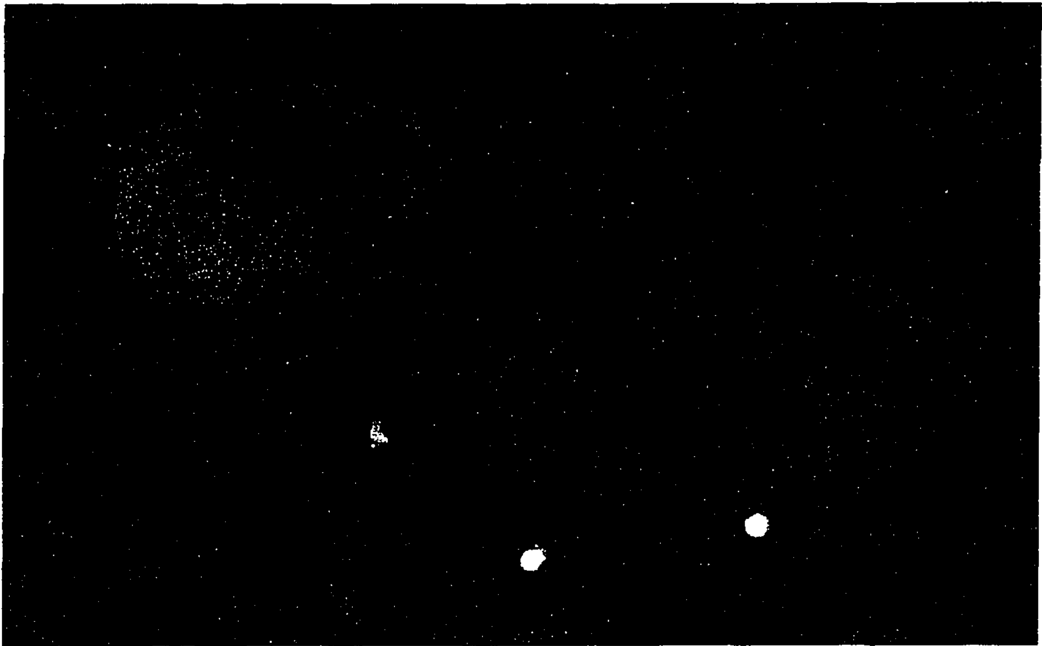


Figure 12. *F. tularensis* LVSR colonies (lower right) and a segmented LVSRB colony (upper left) recovered from an infected rat macrophage culture. Magnification x14.5.

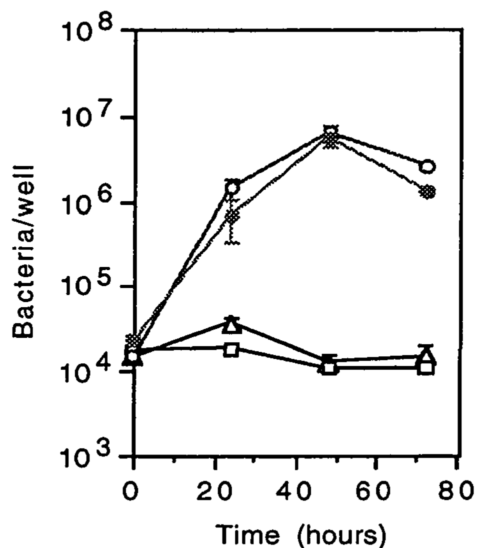


Figure 13. Growth and survival of *Francisella* species in thioglycollate-elicited peritoneal rat macrophages. Female Lewis rat macrophages were infected with *F. tularensis* LVS (open circles), LVSGB (closed circles), LVSG (open triangles), or *F. novicida* (open squares). Data are from representative experiments, and results are expressed as the average of three determinations ± standard error.

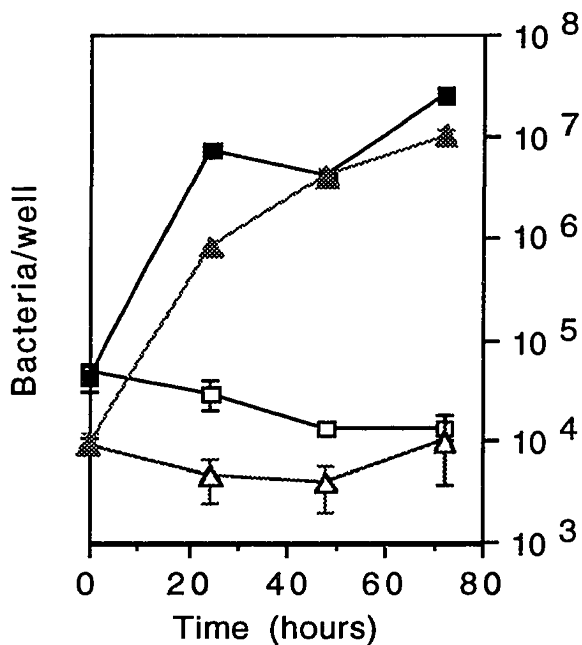


Figure 14. Reversal of NO growth inhibition of *Francisella* with NMMA. Thioglycollate-elicited rat peritoneal macrophages infected with *F. tularensis* LVSG (open triangles), *F. novicida* (open squares), *F. tularensis* LVSG with NMMA (filled triangles), *F. novicida* with NMMA (filled squares). Data are from representative experiments, and results are expressed as the average of three determinations ± standard error.

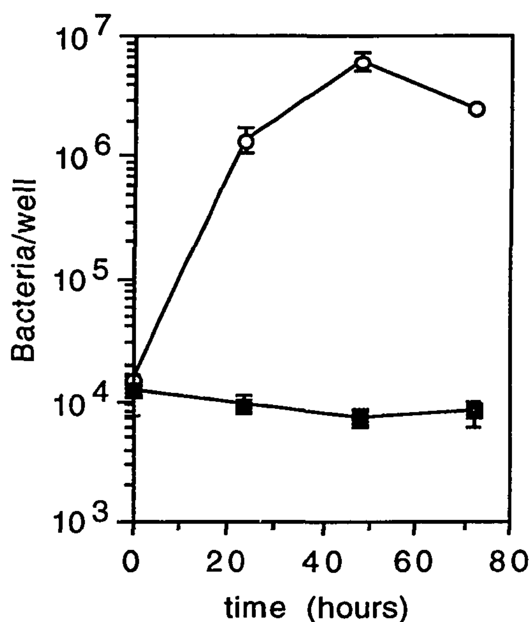


Figure 15. Inhibition of intramacrophage growth of *F. tularensis* LVS by *F. novicida* in vitro. Thioglycollate-elicited peritoneal Lewis rat macrophages were infected with LVS alone or a 1:1 mixture of *F. novicida* and *F. tularensis* LVS. The open circles represent LVS growth when introduced alone into macrophages. The closed circles represent LVS growth when introduced into macrophages along with *F. novicida.* To preclude the problem of *F. novicida* growth crowding the plates and influencing colony counts, the mixed infections were plated on agar both with and without erythromycin since *F. novicida* but not *F. tularensis* LVS is susceptible to this antibiotic. Data are from representative experiments, and results are expressed as the average of three determinations ± standard error.

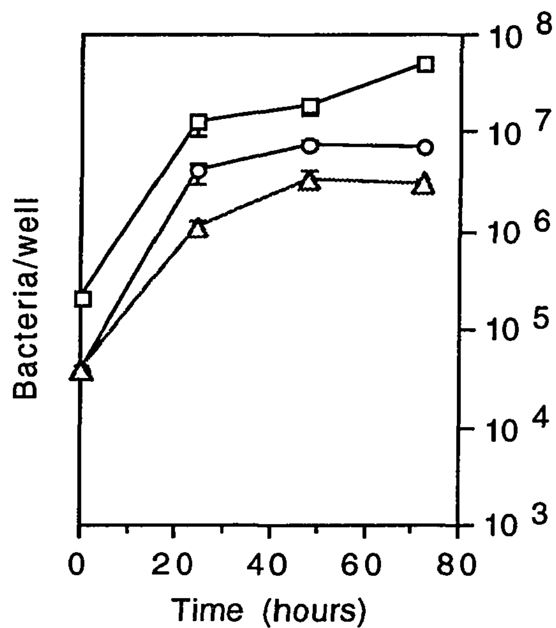


Figure 16. Thioglycollate-elicited peritoneal macrophages from BALB/c mice were infected with *F. novicida* (squares), *F. tularensis* LVS (circles), and *F. tularensis* LVSG (triangles). Data are from representative experiments, and results are expressed as the average of three determinations ± standard error.

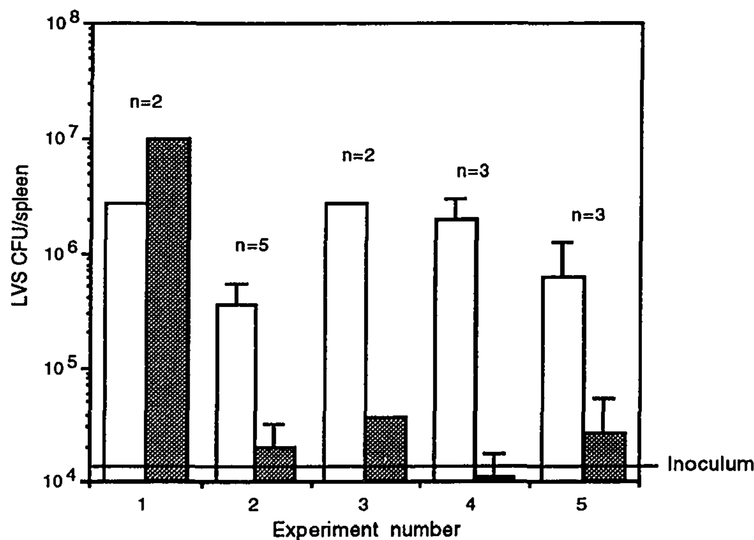
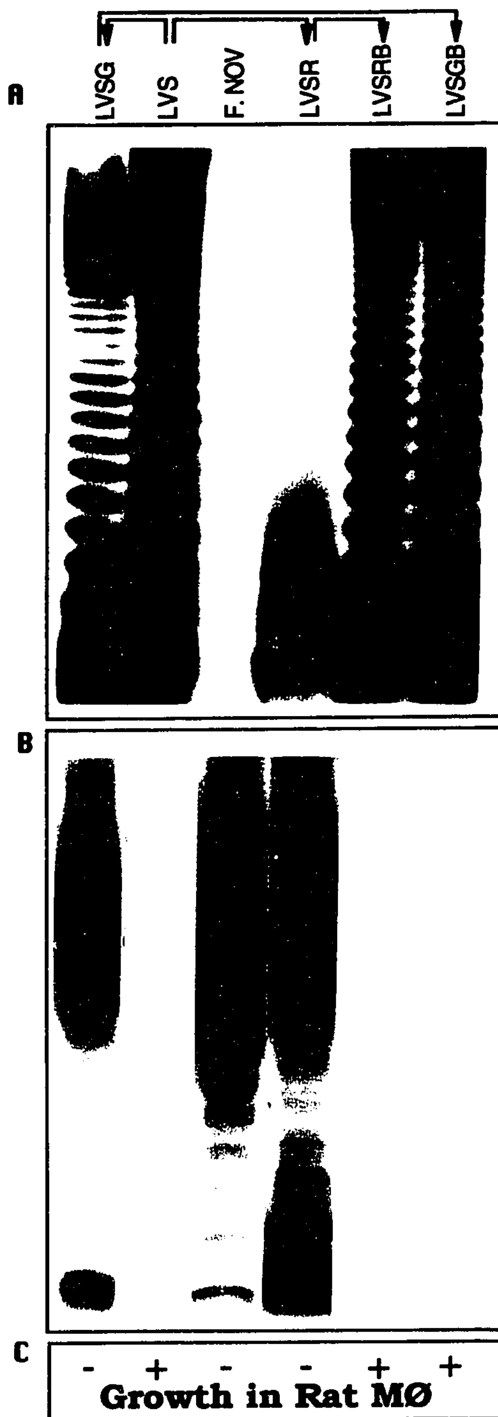


Figure 17. Survival of LVS in rat spleens during a co-infection with *F. novicida*. Rats were inoculated intraperitoneally with either 1.3 x 10^4 LVS (white bars, all experiments), 1.4 x 10^5 LVS (gray bar, experiment 1), or a co-infection of 1.3 x 10^4 LVS mixed with 1.3 x 10^5 *F. novicida* (gray bar, experiments 2-5). Numbers indicate the CFU in rat spleens for LVS only. LVS numbers in the spleens were determined after 72 h and are expressed as the averages of the number of determinations (indicated by n) +/- standard error when n≥3. The line at the bottom of the graph indicates the number of LVS cells used in the inoculum.

Figure 18 (located on the following page). Immunoblot analysis of LPS from *F. tularensis* strains, and *F. novicida.* LPS samples were purified and separated by SDS-PAGE and transferred to PVDF membranes. (A) Membrane probed with mAb reactive with Ft-LPS. Each lane contains 25 μg LPS as determined by dry weight. (B) Membrane probed with mAb reactive with Fn-LPS. Each lane contains 25 μg LPS. (C) Plus signs indicate the ability of a strain to grow in rat macrophages. Note the correlation of the ability to grow in rat macrophages with the absence of Fn-LPS. The arrows at the top of the figure indicate the first and second generations of strains that derived from LVS.

Figure 18.



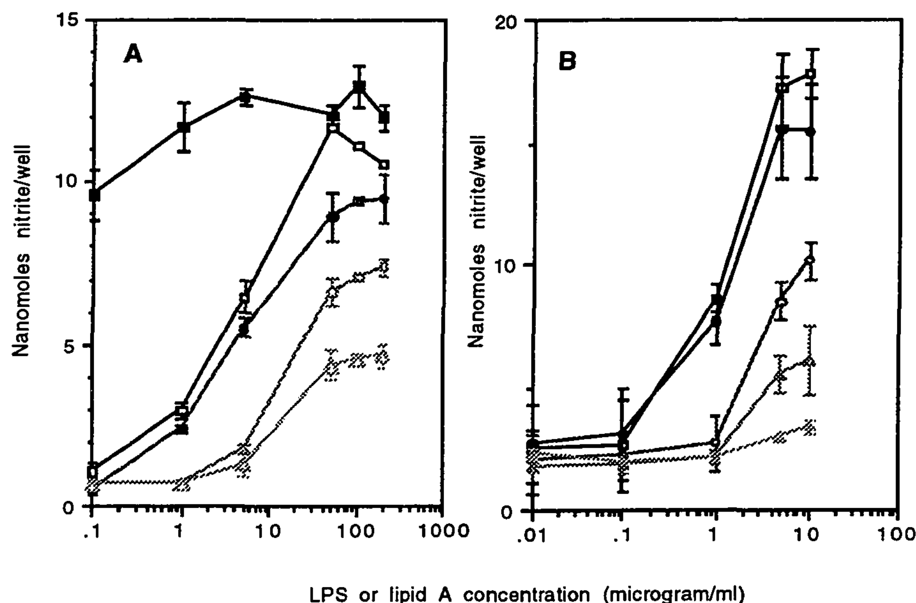


Figure 19. Effect of *F. tularensis* and *F. novicida* LPS or lipid A on nitrite production by rat macrophages. Thioglycollate-elicited peritoneal rat macrophages were incubated with the indicated concentrations of either (A) LPS or (B) lipid A purified from *F. tularensis* LVS (open triangles), LVSG (open circles), LVSR (filled circles), *F. novicida* (open squares), *E. coli* (filled squares), and LVSRB (filled triangles, only in (B)). Macrophages were incubated with LPS or lipid A for 1 hour at 37° C, followed by washing of the monolayers. After 72 hours at 37° C, macrophage supernatants were removed and assayed for nitrite by the Griess reaction. Data from a representative experiment are shown, and results are expressed as the average of three wells plus standard error. LPS and lipid A concentrations were calculated by measuring their dry weights and resuspending the sample in an appropriate volume of endotoxin-tested water.

TABLE 4

Induction of Nitric Oxide Production in Rat Macrophages Infected with *Francisella*

Strain	Nanomole NO_2^- per well*
F. tularensis LVS	0.59 ± 0.007†
F. tularensis LVSG	6.20 ± 0.073
F. tularensis LVSGB	0.48 ± 0.021
F. tularensis LVSR	8.80 ±0.132
F. tularensis LVSRB	0.54 ±0.047
F. novicida	12.57 ± 0.229
F. tularensis LVG + NMMA	0.54 ± 0.067
F. novicida + NMMA	0.60 ± 0.009

* NO determined by Griess reaction.

†Indicates standard error.

DISCUSSION

Our data shows that the intracellular pathogen *F. tularensis* is capable of a phase variation which alters the ability of the organism to induce rat macrophage NO production, and consequently, its growth in the rat macrophage. Furthermore, we have shown that *F. tularensis* can express two forms of LPS, one that induces NO production by rat macrophages and one that lacks this property. These two LPS forms differ both antigenically (at the O-antigen level) and functionally (at the lipid A level). We have demonstrated that this antigenic shift of *F. tularensis* LVS LPS correlates with the observed change in the organism's ability to induce macrophage NO production and grow in the rat macrophage.

The in vitro stimulation of macrophages to produce NO levels equivalent to those produced in response to the live bacteria required very high concentrations of *F. novicida* and LVSG LPS. *Francisella* may provide multiple signals for the induction of NO and LPS may be a necessary co-signal for NO induction. Alternatively, it may be that purified *Francisella* LPS in solution does not provide as effective a signal for NO induction as LPS on the bacterial surface, or that the LPS is rendered less potent during the isolation procedure due to changes such as dephosphorylation.

A number of recent discoveries regarding the regulatory role of NO in the immune system provides a possible explanation for the evolutionary role of the LPS variants. NO has been shown to inhibit macrophage surface Ia expression and hence antigen presentation (Sicher *et al.*, 1994). Furthermore, NO inhibits trafficking of leukocytes into inflammatory sites (Kubes *et al.*, 1991), and also limits the responsiveness and proliferation of T cells to alloantigens, mitogens, and superantigens (Isobe and Nakashima, 1992; Fu and Blankenhorn, 1992; Mills, 1991). In addition, NO has been shown to inhibit natural killer (NK) cell-mediated cytotoxicity against virally-infected cells (Ito *et al., 1996*). Hence, once a macrophage has been activated to produce NO and presumably kill an ingested organism, this NO may function to prevent excessive NK cell and T cell activation and

down-regulate the immune system. However, if an intracellular organism such as *Francisella* could induce the host macrophage to produce bacteriostatic levels of NO and survive the effects of this molecule, it may effectively exploit the immune system for its own benefit, by prolonging its existence in the host. The LVSG variants that have been found to segregate from *F. tularensis* LVS may act to slow the growth of bacteria in nearby infected macrophages; a new shift to LVSGB or a suppression of the host immune system may set off new growth and spread of *Francisella* to a new host.

There is some anecdotal evidence to indicate that *Francisella* may produce a longer protracted infection in some animal species. In Europe and Asia, human tularemia was reported to occur in cyclic epidemics of four to five years (Bell, 1981). Since tularemia is rarely transmitted between humans but almost exclusively contracted from animal reservoirs or ticks, this cycle is likely a reflection of epizootics in the indigenous wildlife. Similarly, studies in North America reveal that hares, voles, and lemmings exhibit regular cycles of tularemia. One common theory suggests that tularemia may proliferate in a host population until that population is decimated, thus resulting in the observed cyclic nature of the epidemics. However, further evidence suggests another possible explanation. Early studies by Bell and Green (1939) demonstrated that a "nonfatal" tularemia could be identified in grouse, hares, rabbits, and their ticks. Furthermore, this form of tularemia remained nonfatal in hares until they were afflicted with a "stress syndrome", at which time the tularemia assumed greater apparent severity in the hares. These observations suggest that *Francisella* infections may remain dormant in some animal populations until a trigger event alters the host's physiology and results in reactivation of the disease. Indeed, laboratory studies have shown that surviving rats which had previously been inoculated intraperitoneally with *F. tularensis* type A (Schu) carry persistent organisms in their spleens for as long as 46 days after inoculation (Downs *et al.*, 1949).

Since *Francisella* is well-known for its ability to infect a wide variety of animal species other evolutionary significances can be attributed to the LPS differences seen in *F.*

tularensis and *F. novicida* and among variants of *F. tularensis*. The lower virulence and restricted animal hosts for *F. novicida* may reflect the ability of different animals to produce NO in response to Fn-LPS; conceivably the differences in a few genes may dramatically alter the virulence and host range of *Francisella* strains.

This work suggests that research should be directed to discover similar phase variation phenomena in other pathogens. *Helicobacter pylori* (Muotiala *et al.*, 1992) and *Brucella abortus* (Goldstein *et al.*, 1992) both have LPS's that have very low immunostimulatory activities, and both pathogens clearly associate with their host for long periods. Indeed, *B. abortus* also undergoes a colony phase variation phenomenon very similar to *F. tularensis* which is attributed to changes in LPS antigenicity (Allen *et al.*, 1998). It is clear that several pathogens have the potential to genetically alter immuno-modulatory structures and affect the course of infection.

CHAPTER 3

Shuttle mutagenesis of *Francisella tularensis* biotype *novicida*: isolation and characterization of mutants defective in lipopolysaccharide biosynthesis

INTRODUCTION

Lipopolysaccharide (LPS) or endotoxin is a constituent of the outer membrane of Gram negative bacteria. LPS structure and toxicity varies among bacterial species, but it is often a potent inducer of proinflammatory cytokines. However, several reports have demonstrated that *F. tularensis* LPS possesses remarkably reduced toxicity as compared to the LPS of other Gram negative bacteria. Indeed, *F. tularensis* LPS exhibits reduced reactivity in the Limulus amebocyte lysate assay, induces low levels of TNF and IL-1 from human monocytes and mouse macrophages, and does not act as a pyrogen (Sandstrom *et al.*, 1992; Ancuta *et al.*, 1996). Furthermore, *F. tularensis* LPS fails to induce LPS desensitization and is unable to antogonize endotoxin-induced cellular responses, thus suggesting that this LPS does not interact with the classical LPS receptors (Ancuta *et al.*, 1996). The non-toxic nature of *F. tularensis* LPS may allow for the observed ability of *F. tularensis* to rapidly multiply within murine macrophages.

Over forty years ago, Eigelsbach (1951) reported that *Francisella tularensis* can produce colony variants following incubation of liquid broth cultures for several days without aeration. More recently, we have shown that this phase variation phenomenon is accompanied by an alteration in the toxicity of the LPS as measured by macrophage NO production, and thus also affects the ability of *F. tularensis* to grow within rat macrophages (Cowley *et al.*, 1996; Chapter 2). This change in LPS toxicity also correlates with the expression of a new O-antigenic form of LPS by *F. tularensis* which is also the predominant form of LPS found normally in *F. novicida* (called Fn-LPS). LPS and lipid A isolated from *Francisella* strains expressing Fn-LPS induce rat macrophage NO production, and this is proposed to result in reduced intracellular growth of *Francisella* strains expressing Fn-LPS.

In order to begin to characterize the molecular mechanism of the variable expression of the Fn-LPS O-antigen in *F. tularensis*, we sought to identify genes responsible for expression of Fn-LPS O-antigen. To this end, we performed shuttle mutagenesis using the

transposon Tn*Max2* on a size-restricted plasmid library of *F. novicida* DNA. A mutant *F. novicida* bank was created and used to screen for mutants defective in LPS biosynthesis. Five unique transposon insertion mutants were found which are defective in LPS biosynthesis, and exhibit three distinct phenotypes by Western immunoblot. A representative mutant from each of the three phenotypic groups was chosen and analyzed for changes in macrophage intracellular growth, serum sensitivity, and deoxycholate sensitivity. DNA sequence analysis was used to confirm that the inactivated loci are genes involved in LPS biosynthesis.

RESULTS

(I) Shuttle Mutagenesis and Identification of Putative *F. novicida* LPS Mutants

A size-restricted plasmid library was created by ligation of random 5-10 kb *F. novicida* chromosomal DNA fragments from strain U112 to vector pUC18. Preliminary transformation and screening experiments were done to determine if ligation of size-restricted preparations of *F. novicida* chromosomal DNA to pUC18 would result in *E. coli* transformants carrying a vector with an insert of the correct size. Once this had been confirmed, mutagenesis was performed using the erythromycin-resistant transposon Tn*Max2* according to the procedure shown in Figure 20. Plasmid was isolated from 110 pools of approximately 100 recombinant clones and transformed as pools into *F. novicida* U112 to create an erythromycin resistant (ErmR) mutant clone bank. *F. novicida* ErmR mutants were screened visually on blood agar plates for aberrant colony phenotypes. Out of 10,462 mutants screened, 36 mutants exhibiting transluscent or rough colony phenotypes were chosen and further screened by Western immunoblot in order to identify mutants which had lost or altered reactivity with an anti-*F. novicida* LPS monoclonal antibody (mAb). Out of the 36 mutants examined, 5 mutants were found to exhibit an altered LPS profile as determined by Western immunoblot (see Figure 21). Three distinct LPS mutant phenotypes may be distinguished by Western immunoblot, and include complete loss of reactivity with the anti-Fn LPS mAb (mutants SC2, SC4, SC79), a partial LPS ladder apparently lacking longer O-antigen chain lengths (SC66), and apparent diminished production of the O-antigen ladder (SC92).

(II) Genetic Characterization Of Putative LPS Mutants

A single mutant from each of the three LPS mutant phenotypic groups, as determined by Western immunoblot, was chosen for further study. Thus, rough *F.*

novicida mutants SC4, SC66, and SC92 were examined by Southern blot analysis. Each mutant contains a single insertion of the Tn*Max2* transposon, and two of these mutants (SC66 and SC92) appear to be the result of a transposon insertion in the same 6.0 kb Nde I *F. novicida* chromosomal DNA fragment (Figure 22). An Erm-resistant Nde I fragment from each of these 3 mutants was self-ligated and transformed into *E. coli* E131 to permit plasmid replication via the ori_{fd} origin of replication present in Tn*Max2*. The resulting plasmids, named pSC66-1, pSC4-1, and pSC92-1 were used to perform Southern blot analysis of chromosomal DNA isolated from wild type *F. novicida* U112 and the 3 putative LPS mutants (Figure 23). The results confirm that the mutants are the result of an allelic replacement event and the insertion of a single copy of the 1.6 kb ErmR transposon Tn*Max2*.

DNA sequence analysis of plasmids pSC92-1 and pSC4-1 was used to confirm that the predicted protein products of the DNA regions surrounding the Tn*Max2* insertions exhibit sequence similarity to proteins involved in LPS biosynthesis. DNA sequencing of pSC66-1 is currently in progress. The DNA sequence results are shown in Figure 24. The open reading frame (ORF) identified in pSC4-1 was shown to encode a protein having similarity to a number of enzymes involved in LPS and capsule biosynthesis, including the proposed dehydratase BplL of *Bordetella pertussis*, RfbU of *Vibrio cholerae*, and LpsB of *Rhizobium etli*. The interrupted ORF of pSC92-1 was shown to encode a protein with homology to glucosyl transferase II (GtrII) of the *Shigella flexneri* bacteriophage SfII.

(III) Phenotypic Characterization of Putative LPS Mutants

Mutants SC4, SC66, and SC92 were further analysed for a variety of phenotypes to evaluate virulence and outer membrane integrity, including growth in mouse macrophages, as well as deoxycholate and serum sensitivity. As a control, a randomly picked *F. novicida* mutant expressing the TnMax2 *ermC'* gene and exhibiting no obvious phenotypic defects (SC119) was also included in these experiments (data not shown).

We determined the growth rates of the three LPS mutants as compared to control SC119 and wild type *F. novicida* in liquid broth media. As seen in Figure 25, SC4 and SC92 exhibit a similar growth rate in vitro to the two control strains. In contrast, SC66 exhibits a slightly reduced growth rate as compared to wild type *F. novicida* and the control strain SC119.

We investigated the possibility that the LPS mutants may exhibit decreased growth in mouse macrophages. Figure 26 shows that mutants SC119, SC92, and SC4 are capable of exponential growth in mouse macrophage monolayers similar to wild type *F. novicida*. However, growth of mutant SC66 was decreased by as much as 1,000-fold as compared to wild type for the first 48 hours after infection of the monolayer. The increase in bacterial numbers at the 72 hr time point is attributed to the appearance of opaque ErmR colonies which have regained wild type expression of *F. novicida* LPS O-antigen (data not shown), probably due to chromosomal rearrangements of the ErmR cassette.

F. tularensis and *F. novicida* have been shown to reside in the mouse macrophage within an endosome that remains unfused with lysosomes. However, studies have shown that this vesicle must become acidified in order to support growth of *F. tularensis* LVS (Fortier *et al.*, 1995). Thus, the reduced survival of mutant SC66 in mouse macrophages could be attributed to a variety of factors, including: increased susceptibility to the oxidative burst, increased susceptibility to the low pH required for iron release from transferrin, or the loss of a component that is necessary for disruption of normal macrophage function for survival. To investigate the possibility that the reduced growth of SC66 is a result of early intracellular killing due to the bactericidal action of the products of the macrophage respiratory burst, we incubated SC66 with various concentrations of the reactive oxygen intermediate hydrogen peroxide (Figure 27A). It was observed that mutant SC66 exhibited a similar sensitivity profile as wild type and SC119 to hydrogen peroxide over the entire concentration range tested. Since the *Francisella*-containing phagosome must decrease to a mean pH of 5.5 in order to initiate the release of iron from transferrin, we tested the

viability of mutant SC66 over a pH range from 7.0 to 4.0. As shown in Figure 27B, mutant SC66, control strain SC119, and wild type *F. novicida* exhibit minimal sensitivity to low pH. Thus, these results suggest that the inability of mutant SC66 to grow in mouse macrophages is not a result of killing due to the respiratory burst or the low pH of an acidified phagosome.

The ability of Gram negative bacteria to exclude hydrophobic antibiotics and detergents depends largely of the strong association between LPS molecules at the bacterial cell surface. LPS mutants unable to maintain stable divalent cation cross bridging due to the loss of negatively charged components within the inner core region of LPS often exhibit increased sensitivity to detergents. Similarly, increased detergent sensitivity may occur with LPS mutants unable to associate strongly with outer membrane proteins. Thus, we analyzed the putative LPS mutants for increased sensitivity to the detergent deoxycholate. Bacterial strains to be tested were incubated with the indicated concentrations of deoxycholate in phosphate buffered saline (PBS) for 45 minutes and then plated and assessed for survival as compared to cells incubated for the same period of time with PBS alone. As seen in Figure 28B, wild type *F. novicida* and ErmR control SC119 exhibits significant killing in response to concentrations higher than 0.05% deoxycholate. Similarly, putative LPS mutants SC92 and SC4 are susceptible to concentrations higher than 0.05% deoxycholate. However, mutant SC66 is sensitive to concentrations as low as 0.005% dexycholate, and exhibits a consistently lower level of survival following exposure to all the concentrations of deoxycholate tested as compared to the other strains examined.

The O-antigen region of LPS is instrumental in protecting the cell from complement-mediated killing by serum. O-antigen is required to mask determinants within the core and lipid A region involved in activation of both the classical and alternative complement pathways. Furthermore, although O-antigen activates the alternative pathway, the steric hinderance of longer O-side chains is involved in protecting the membrane from the complement attack complex. We tested the susceptibility of the putative LPS mutants to

a three hour exposure to various concentrations of serum complement. As shown in Figure 28A, wild type *F. novicida* and ErmR control SC119 are resistant to all concentrations of complement tested. Similarly, mutant SC92 is resistant to serum complement. However, mutant SC4 shows a significant decrease in survival in response to 50% complement, and mutant SC66 is consistently more susceptible than control strains and the other LPS mutants to concentrations higher than 12.5% complement. Thus, mutants SC66 and SC4 exhibit increased sensitivity to serum complement as compared to wild type *F. novicida*, control strain SC119, and mutant SC92.

As previously described, *F. tularensis* is capable of undergoing a LPS phase variation phenomenon which involves the aquisiton and loss of O-antigen reactive with an anti-Fn-LPS mAb. One possible mechanism for this phase variation may involve the insertion or deletion of an insertion element in the *F. tularensis* locus responsible for Fn-LPS biosynthesis. In order to determine if there were any gross genetic rearrangements occuring in the *F. tularensis* chromosome during phase variation, we performed a Southern blot analysis on chromosomal DNA isolated from *F. tularensis* and two of its variants expressing Fn-LPS (LVSR and LVSG) using the cloned *F. novicida* mutant loci as probes. However, no such large genetic rearrangements were detectable by this analysis (data not shown).

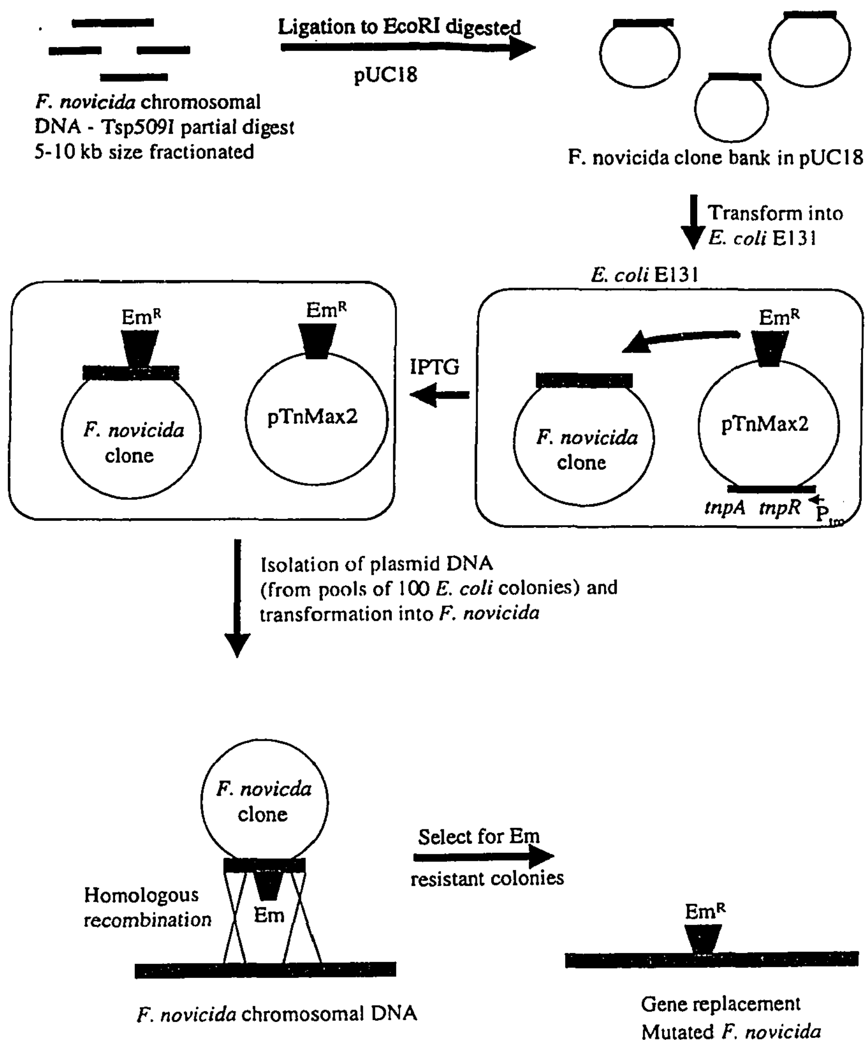


Figure 20. Schematic representation of the procedure used to perform shuttle mutagenesis of *Francisella novicida*. Details are described in the Materials and Methods section.

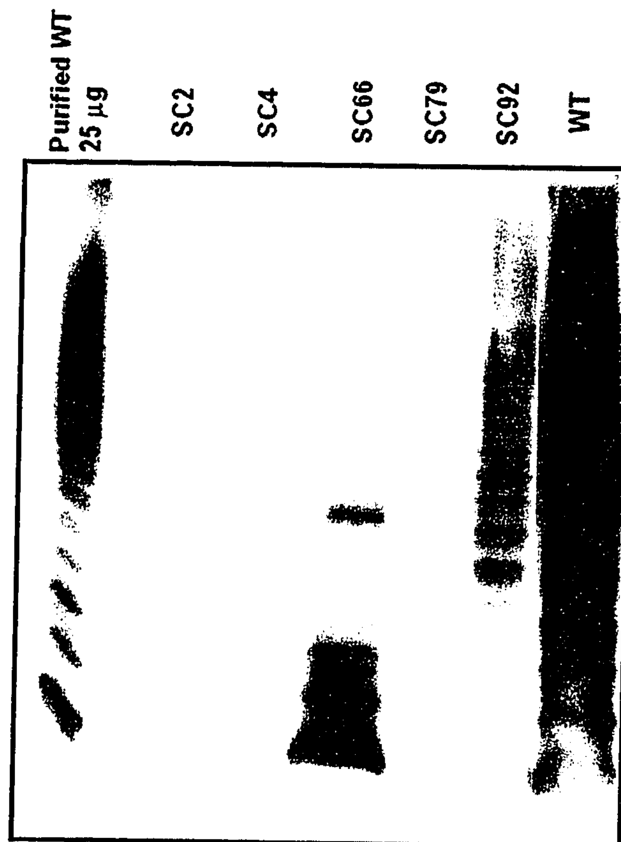


Figure 21. Western immunoblot of whole-cell lysates from *F. novicida* Tn*Max2* insertional mutants. Samples were electrophoresed on a 12% (wt/vol) SDS-PAGE gel and transferred to PVDF membrane. The membrane was probed with mAb reactive with *F. novicida* LPS.

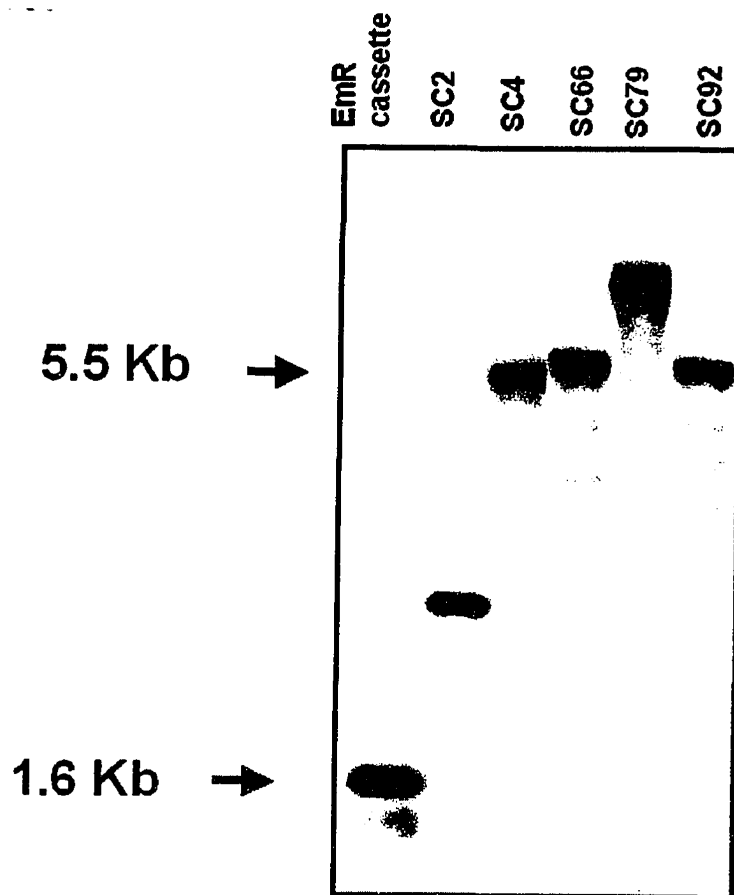


Figure 22. Southern blot analysis of *F. novicida* LPS mutants. Chromosomal DNA isolated from the indicated strains was digested to completion with Nde I and probed with the ErmR cassette from Tn*Max2*. The sizes were estimated based on the 1 kb ladder DNA standards.

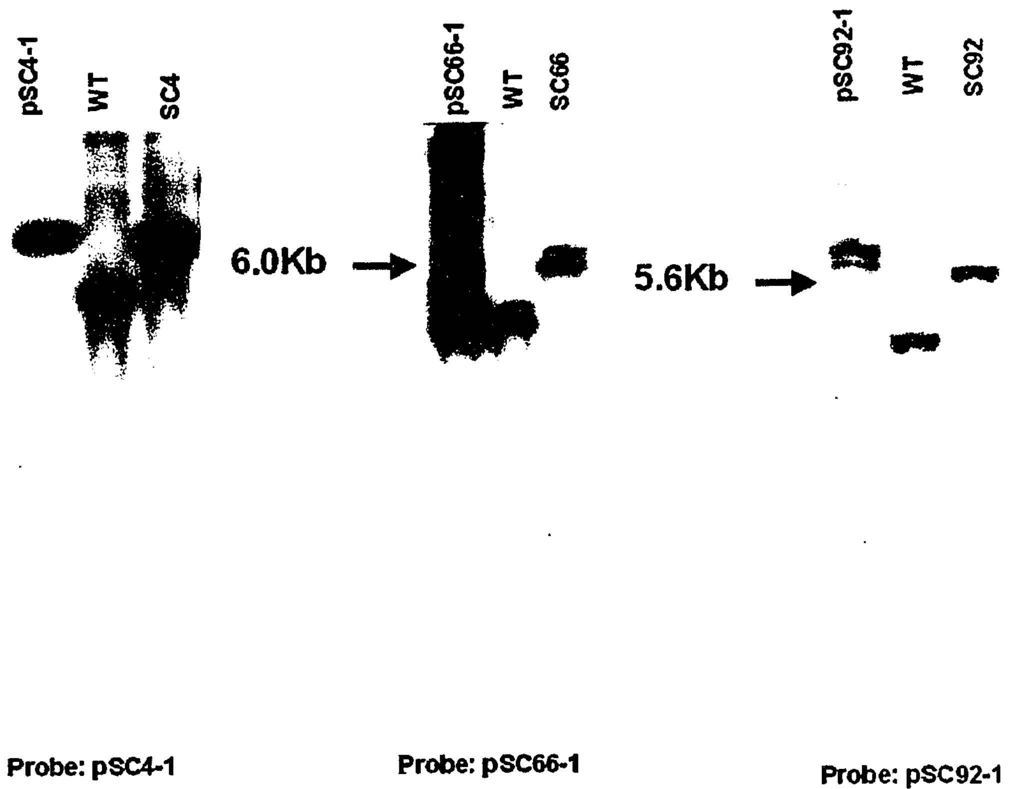


Figure 23. Southern blot analysis of *F. novicida* LPS mutants. Chromosomal DNA isolated from the indicated strains was digested to completion with Nde I and probed with (A) pSC4-1, (B) pSC66-1, or (C) pSC92-1. The arrows indicate the wild type bands and the mutant bands exhibiting a 1.6 kb increase in size due to the Tn*Max2* insertion. The sizes were estimated based on the 1 kb ladder DNA standards.

(A.)

```
     IDIKIVGLRPGEKLYEELLIEDDDVST
     I+I+I GLRPGEKLYEELLI +D   T
544  IEIRITGLRPGEKLYEELLIGEDSRET   570   BplL
```

70% identity (19/27 amino acids)
Smallest Sum Probability: 6.2×10^{-5}

(B.)

```
     IDIKIVGLRPGEKLYEELLIEDDDVSTDYK
     I+IK  GLRPGEKLYEELLI ++   T ++
543  IEIKFTGLRPGEKLYEELLIGENVEGTSHQ   572 RfbU
```

60% identity (18/30 amino acids)
Smallest Sum Probability: 3.0×10^{-4}

(C.)

```
     VMIFVGILIVFIDLFVNRSKAGYIIEFVVLIYILVIINTIVN
     ++IF GI++  + + + ++   YII FV+ + I  II    N
146  IVIFSGIVLSILSMAIYQTFVTYIIAFVIGLQINSIIRNEKN 187  Gtr II
```

27% identity (9/33 amino acids) and 60% similarity
Smallest Sum Probability: 0.69

```
    ILIVFIDLFVNRSKAGYIIEFVVLIYILVIINTIV
    ++++F   +  ++++ Y+I  ++ IYIL +I  +V
271 LILIFTLTYKLKTRSIYLISSIIFIYILPVIFIVV 305   Gtr II
```

30% identity (13/42 amino acids) and 57% similarity
Smallest Sum Probability: 0.69

Figure 27. Deduced amino acid sequence of Tn*Max2*-flanking DNA from the *F. novicida* rough mutants aligned with sequences from Genbank. Alignment of SC4 amino acid sequence with (A.) BplL protein of *Bordetella petussis*, and (B.) RfbU of *Vibrio cholerae*. (C.) Alignment of SC92 amino acid sequence with GtrII of *Shigella flexneri* bacteriophage II. In all alignments shown, the upper amino acid sequence corresponds to the putative *Francisella* protein, and the lower amino acid sequence corresponds to the similar protein coding sequence from Genbank. Identical amino acids are indicated as the letter code for each amino acid, and similar amino acids are represented as a (+) sign.

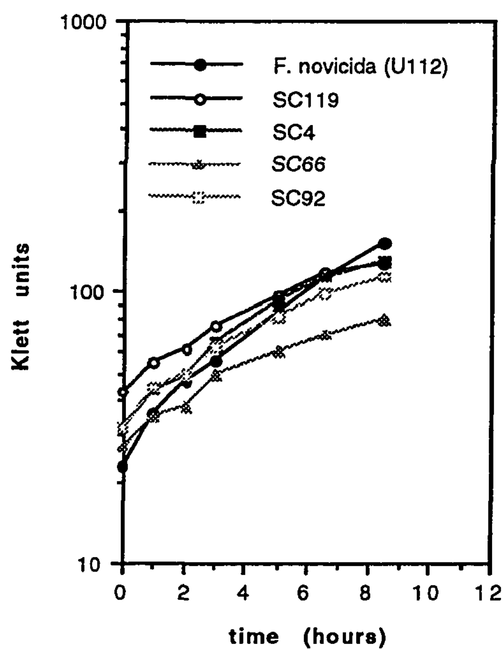


Figure 25. Growth of *F. novicida* LPS mutants in TSB-C. At the indicated times the cell density was measured with a Klett-Summerson meter. Data are from one of three representative experiments performed in triplicate.

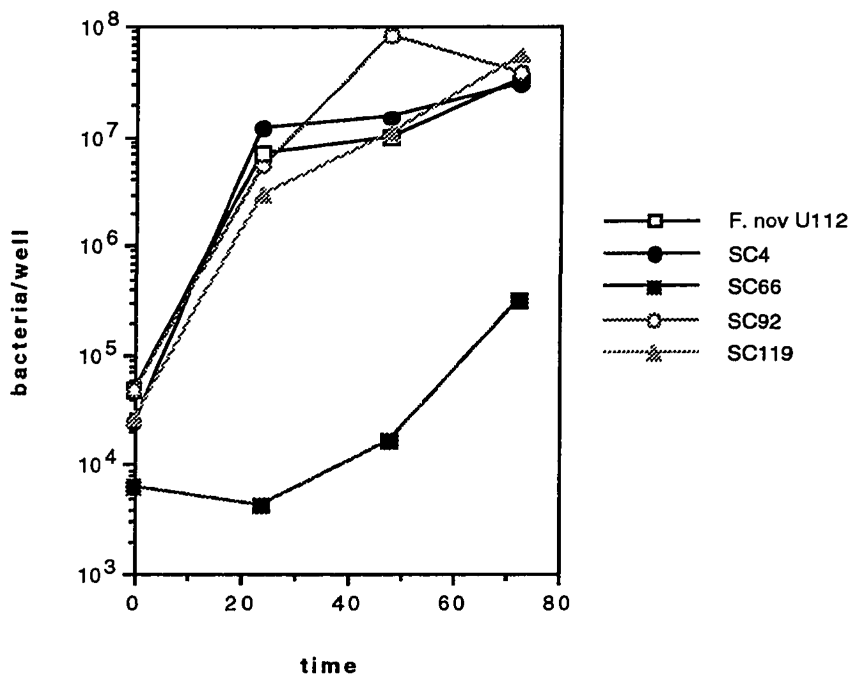


Figure 26. Growth and survival of *F. novicida* LPS mutants in thioglycollate-elicited C57/Bl6 mouse macrophages. Macrophage monolayers were infected with wild type *F. novicida* (open squares), SC119 (closed triangles), SC4 (closed circles), SC66 (closed squares), and SC92 (open circles). Data are from representative experiments, and results are expressed as the averages of three determinations +/- standard error.

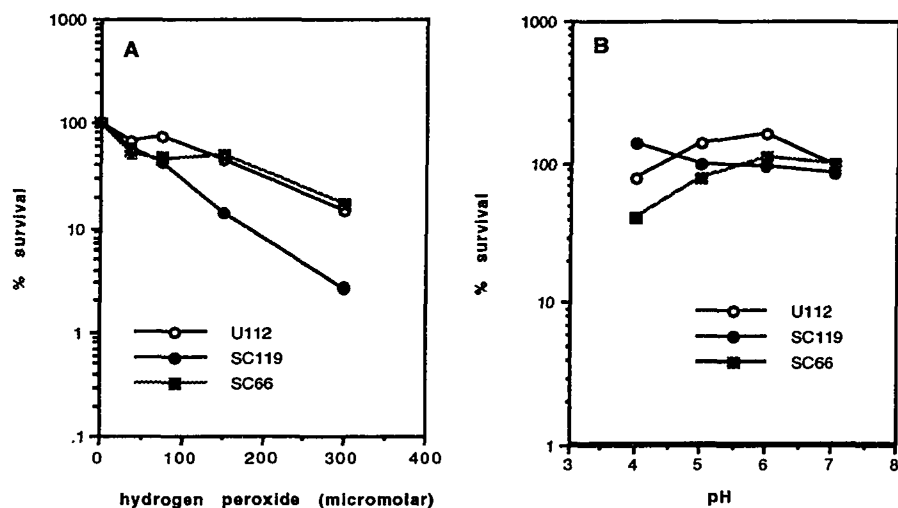


Figure 27. Survival of *F. novicida* LPS mutants following exposure to (A) hydrogen peroxide, or (B) low pH. The number of viable bacteria was measured in the wells following a 3h incubation in PBS containing various concentrations of hydrogen peroxide or adjusted to the indicated pH by serial dilution and plating on CHA-H. Percent survival was calculated by comparing the number of bacteria in the test wells to the number of bacteria incubated for the same period of time in PBS. Wild type *F. novicida* (open circles), SC119 (closed circles), and SC66 (closed squares). Data are from one of three representative experiments performed in triplicate.

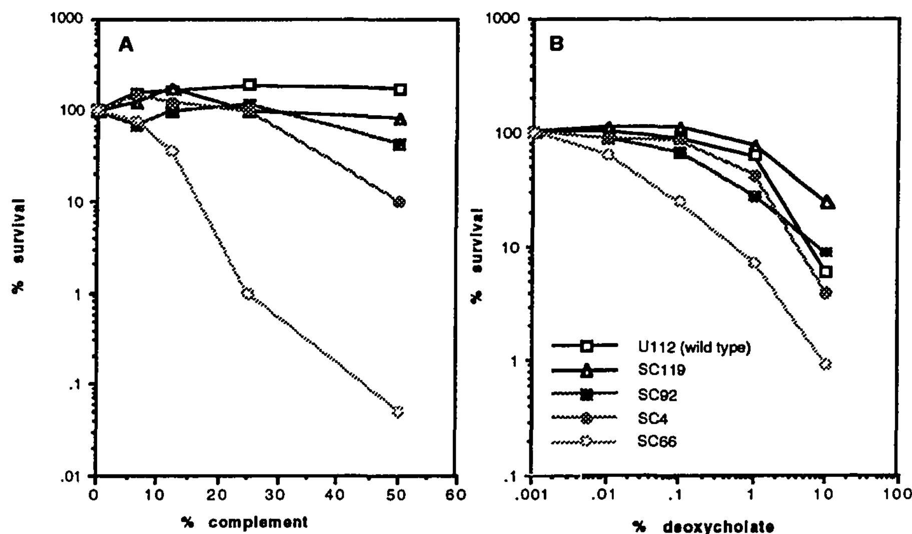


Figure 28. Survival of *F. novicida* LPS mutants following exposure to (A) guinea pig serum, or (B) deoxycholate. The number of viable bacteria was measured in the wells following a 3 h incubation (serum) or 45 min incubation (deoxycholate) by serial dilution and plating on CHA-H. Percent survival was calculated by comparing the number of bacteria in the test wells to the number of bacteria incubated for the same period of time in PBS. Wild type *F. novicida* (open squares), SC119 (open triangles), SC4 (closed circles), SC66 (open circles), and SC92 (closed squares). Data are from one of three representative experiments performed in triplicate.

DISCUSSION

In this study we performed shuttle mutagenesis on *F. novicida* using the erythromycin resistant transposon Tn*Max2*, and generated a mutant *F. novicida* clone bank in order to identify genes involved in LPS biosynthesis. This is the first step in a lengthy process which will ultimately lead to the characterization of the molecular mechanism of LPS phase variation in *F. tularensis*. Here, we identified 5 putative LPS biosynthesis mutants of *F. novicida* which exhibit three different phenotypes by Western immunoblot. A representative mutant from each of the three phenotypic groups was chosen for further characterization. One such mutant, named SC66, was particularly interesting; mutant SC66 exhibited increased sensitivity to the detergent deoxycholate and to serum complement as compared to wild type *F. novicida*. This mutant was also unable to grow in mouse macrophages.

Unexpectedly, SC66 does not exhibit increased susceptibility to conditions which may occur in the proposed *Francisella* intracellular compartment, an unfused acidified phagosome. Neither hydrogen peroxide, which is a component of the macrophage oxidative burst, or low pH, which is necessary for iron release from transferrin in the *F. tularensis* phagosome, resulted in reduced viability of SC66 as compared to wild type *F. novicida*. These results suggest two possibilities: first, the phagosome harboring mutant *F. novicida* may undergo aberrant processing as a result of the altered LPS molecules on the bacterial cell surface, resulting in the exposure of SC66 to bactericidal agents which reduce growth and viability. Conversely, the wild type *F. novicida* phagosome may not remain as isolated from the endocytic pathway as previously thought, and may naturally acquire some bactericidal agents to which SC66 is susceptible. Indeed, mutant SC66 is sensitive to membrane-permeabilizing agents such as detergents, suggesting that this mutant may also be susceptible to bactericidal agents found within the macrophage lysosomal compartments.

The LPS banding pattern observed on Western immunoblots reflects the heterogeneity of LPS found at the bacterial cell surface. Excluding a few notable

exceptions, LPS typically consists of a lipid A portion, an inner and outer core region composed of carbohydrate, and the O-antigen polysaccharide located at the non-reducing end of the molecule. In numerous bacterial systems, LPS O-antigen is composed of as many as 30 repeating O-units, with each O-unit consisting of up to six sugar monomers. During LPS biosynthesis, these O units are synthesized individually on an acyl lipid carrier (ACL). An O-antigen polymerase joins the O-units together to form a longer repeating carbohydrate chain attatched to the ACL. After the polymerization of a variable number of O-units, the O-antigen ligase attatches the O-antigen to the lipid A-core polysaccharide, thus terminating LPS biosynthesis. Consequently, each band on a SDS-PAGE gel represents an LPS molecule carrying a different number of O-units.

Typically, polymerization and ligation of the O-antigen is regulated to result in a modal distribution of O-antigen chain lengths. Frequently, as observed with *F. novicida* LPS, a bimodal distribution is apparent such that a strict number of shorter O-antigen side chains, as well as a defined number of longer O antigen chains, are favored for ligation to the lipid A-core. The product of the *rol* (or *wzz*) gene (regulator of chain length) is proposed to be a “molecular clock” or "molecular chaperone" responsible for regulating the interaction of the nascent O-antigen with either the O-antigen polymerase or the O-antigen ligase. Mutants lacking *rol* regulation typically express LPS molecules with a linear distibution of O-antigen chain lengths, such that the shortest chain lengths are the most abundant, followed by each increasing addition of an O unit having a lower probability of occurance.

Inspection of the Western immunoblot profile of mutant SC66 suggests that this unusual mutant may contain a genetic lesion in the *rol* gene, as the bimodal distribution of O antigen chain lengths appears to have been lost, while reactivity with the anti-Fn-LPS O-antigen mAb has been maintained. Unfortunately, the phenotypic susceptibility profile of *rol* mutants in other bacterial species has not been well characterized. It is interesting to note that O-antigen chain length regulation mutants of *Shigella flexneri* exhibit increased

sensitivity to serum complement as well as a decreased ability to promote F-actin tail formation within HeLa cells, and thus were incapable of intracellular spread between host cells (Van Den Bosch *et al.*, 1997; Hong *et al.*, 1997). Therefore, mutations affecting O-antigen chain length may have profound effects on the extracellular and intracellular survival of a pathogen. Indeed, studies with *Salmonella montevideo* have demonstrated that serum resistance can be correlated with O antigen chain length; in this bacterial species, serum resistance required greater than 20% of the bacterium to express O-antigen chain lengths consisting of at least 14 O-units (Grossman *et al.*, 1987). A *rol* mutation would similarly decrease the proportion of LPS molecules carrying longer O chain lengths at the bacterial cell surface.

However, in contrast, it is possible that SC66 harbors a mutation in one of the genes involved in core biosynthesis. Such a mutant would be expected to exhibit increased sensitivity to detergent and serum complement, and would be unable to ligate O-antigen to the lipid A-core polysaccharide due to the synthesis of an incomplete LPS core. Thus, the O-antigen observed in the Western immunoblot profile of whole cell lysates of this mutant may be due to the intracellular accumulation of O-antigen attached to the acyl carrier lipid prior to ligation to the lipid A-core polysaccharide. Furthermore, this O-antigen would be expected to lack regulation of O-antigen chain length in a manner similar to a *rol* mutant.

In contrast, Western blot analysis of SC4 reveals that this mutant has lost reactivity with the anti-Fn-LPS O-antigen mAb, thus suggesting either complete loss of O-antigen synthesis or loss of a component of the O-antigen which is necessary for binding of the mAb to its cognate O-antigen epitope. Indeed, DNA sequence analysis of SC4 showed the highest identity to the *bplL* gene of *Bordetella pertussis*, which is hypothesized to be a dehydratase involved in the synthesis of deoxy and dideoxy sugars (Allen *et al.*, 1996). SC4 also exhibits high identity to putative epimerases and dehydratases from a variety of different systems. Thus, it is likely that SC4 encodes an enzyme involved in the synthesis of an O-antigen sugar. Indeed, mutant SC4 is consistently and significantly more sensitive

to high concentrations of serum than wild type *F. novicida*, thus suggesting the absence of protective O-antigen.

The Western immunoblot profile of mutant SC92 reveals low levels of detectable O-antigen. The proposed amino acid sequence derived form the DNA region adjacent to the TnMax2 insertion of mutant SC92 exhibits limited similarity to the WbcE protein of *Yersinia entercolitica*, which is proposed to be involved in O-antigen synthesis (Zhang *et al.*, 1997). However, the precise function of WcbE remains unknown, and it does not show similarity with any known proteins in the databases. In addition, SC92 exhibits similarity to the *Shigella flexneri* bacteriophage glucosyl transferase II enzyme, involved in the glucosylation of O-antigen (Mavris *et al.*, 1997). Since O-antigens may be substituted with acetyl or glucosyl groups late in the assembly process, and these substituents may function as epitopes, it is possible that a relatively intact O-antigen is present on the surface of this mutant, but is poorly recognized by the *F. novicida* LPS mAb. Conversely, the Western immunoblot profile exhibited by SC92 may be the result of inefficient ligation of completed O-antigen molecules to the lipid A-core region during the final steps of synthesis of LPS. Such a situation may occur if either the O-antigen polymer or the lipid A-core region is not efficently recognized by O-antigen ligase. Indeed, mutations in the *rfaQ* gene of *E. coli* K-12 result in reduced levels of O-antigen ladder detectable on the surface of *E. coli* cells harboring the *S. dysenteriae rfb* region (Klena *et al.*, 1992). The amino acid sequence of RfaQ reveals similarity to various sugar transferases, and it has been suggested that RfaQ is responsible for a modification of the LPS core region which is necessary for a high rate of transfer of polymerized O-antigen to the lipid A-core. Similarly, it is possible that O-antigen modifications such as glucosylation may affect recognition by O-antigen ligase. Unfortunately, silver staining of *Francisella* LPS is difficult and uninformative with respect to the nature of the O-antigen from this mutant.

Despite the interesting nature of *Francisella* LPS, to our knowledge this is the first study to create mutations in *F.tularensis* that affect LPS biosynthesis. Here, we provide

evidence to demonstrate that regulation of O-antigen chain length in *Francisella* may be essential for serum resistance as well as macrophage intracellular growth. Furthermore, we have begun a study that will ultimately characterize the molecular mechanism of LPS phase variation in *F. tularensis*. Preliminary Southern blot analyses reveal that phase variation is not a result of large genetic rearrangements in the regions of the LPS biosynthetic genes examined in this study. However, it is possible that other genes not examined here may be subject to insertions or deletions. In addition, it is possible that phase variation is a result of smaller changes in DNA sequence within the LPS biosynthetic genes that may not be easily detected by Southern analysis. For example, the slipped-strand mispairing mechanism observed during the lipooligosaccharide (LOS) phase variation of *Neisseriae* species is the result of the insertion or deletion of only a few nucleotides within the coding region of a LOS biosynthetic gene. In contrast, variation of LPS structure may also be influenced by environmental conditions. For example, LPS phenotypes in *Neisseriae* have been shown to be influenced by glucose and oxygen availability; similarly, *Brucella* species exhibit LPS phenotypic switching in stationary phase cultures in a manner similar to *F. tularensis* (Allen *et al.*, 1998). Furthermore, *Salmonella typhimurium* lipid A structure is altered during infection due to the activation and repression of genes influenced by the PhoP-PhoQ environmental sensing system (Guo *et al.*, 1997). The coupling of a system which responds to environmental changes with a more random or spontaneous mechanism such as slipped-strand mispairing may allow for optimal control of *Francisella* LPS phase variation.

CONCLUSIONS AND FUTURE RESEARCH

LPS is a highly studied and important bacterial macromolecule for several reasons. First, LPS is surface-exposed, highly variable, and immunogenic. Thus, variation of the structure of LPS carbohydrate moieties is a mechanism commonly employed by pathogens such as *N. gonnhoroeae*, *N. meningitidis*, and *H. influenzae* in order to evade the host immune response during an infection. Numerous studies have sought to define the mechanisms used for phase variation of LPS biosynthetic gene expression in these and other pathogens. Here, we have shown that *Francisella tularensis* is also capable of phase variation of LPS carbohydrate structures, and we have begun a study that will allow for characterization of the molecular mechanism of phase variation. Future studies will attempt to define the *in vivo* relevance of this phenomenon.

In addition, LPS is the primary component of the bacterial outer membrane, which presents an efficient permeability barrier to numerous antibiotics such as rifampicin, rendering these antibiotics ineffective against numerous species of bacteria. It is interesting to note that lipid A biosynthetic mutants are more susceptible to a wide variety of antibiotics due to the decreased expression of LPS in the outer membrane. In addition, much of the lipid A biosynthetic pathway itself is essential for bacterial cell viability. Thus, a complete understanding of the LPS and lipid A biosynthetic pathways may allow for the development of drugs which target these essential enzymes. Such chemotherapeutic agents are expected to be particularly effective, as they may produce a synergistic effect due to the increased sensitivity of lipid A biosynthetic mutants to other antibiotics. In this study, we identified an essential gene encoding a protein potentially involved in lipid A-core polysaccharide transport across the bacterial inner membrane (*valA*). Until recently, LPS transport to the cell surface has been poorly characterized, but has been recognized as a potential target for antimicrobial therapy (Raetz, 1998). In addition, the *Francisella* LpxK functional homolog (*valB*) is currently under investigation to confirm lipid A 4' kinase activity. This enzyme is of particular interest due to its ability to label partial lipid A

structures with radioactive phosphate, thus allowing for the use of radiolabelled lipid A structures in biological assays (Garrett *et al.*, 1997).

The importance of lipid A is not limited to bacterial cell viability, since lipid A is also a primary causative agent of the pathogenesis encountered during Gram negative infection. Endotoxemia, a condition resulting from the release of lipid A molecules from Gram negative bacteria during an infection, may be the cause of 100 000 deaths per year in the United States alone. This is proposed to be primarily due to the interaction of lipid A with macrophages, which results in secretion of cytokines and inflammatory mediators by the macrophage. Although the interaction of toxic lipid A molecules with macrophages is the subject of intense interest, it has been the characterization and study of less toxic lipid A molecules which has lead to the development of putative LPS receptor antagonists which may be used as a clinical treatment for Gram negative sepsis. For example, a promising candidate currently undergoing clinical trials is the endotoxin antagonist E5531, which was modelled on the non-toxic *Rhodobacter capsulatus* lipid A. In this study, we further characterized the low toxicity LPS of *Francisella*, and noted an interesting phenomenon whereby *Francisella* can vary the toxicity of its lipid A (as measured by macrophage NO production). Indeed, a subsequent study examining *S. typhimurium* lipid A has revealed that this phenomenon may not be unique to *Francisella*; *S. typhimurium* lipid A is structurally modified to a less toxic form in response to the environmental conditions in the host (Guo *et al.*, 1997).

Future studies will attempt to examine the chemical structure of the lipid A and carbohydrate moieties of *Francisella* LPS. In addition, current studies are underway to further characterize the biological effects of *Francisella* LPS. A complete understanding of *Francisella* LPS structure and function may provide insight into the requirements for activation and stimulation of different cells of the immune system by LPS and other bacterial glycolipids. Furthermore, variation of lipid A toxicity may be a common strategy used by intracellular pathogens to manipulate the immune response.

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ABBREVIATIONS

Ab	antibody
ABC	ATP-binding cassette
Amp	ampicillin
APC	antigen presenting cell
ATP	adenosine triphosphate
CFU	colony forming units
Cm	chloramphenicol
CMP	cytidine-5'-monophosphate
DNA	deoxyribonucleic acid
Erm	erythromycin
Gal	galactose
Glc	glucose
GlcNAc	N-acetyl glucosamine
HOCl	hypochlorous acid
IFN-γ	interferon-gamma
IL-1	interleukin-1
IL-10	interleukin-10
IL-12	interleukin-12
IL-13	interleukin-13
IL-2	interleukin –2
IL-4	interleukin-4
IL-6	interleukin-6
IL-8	inteleukin-8
iNOS	inducible nitric oxide synthase
IPTG	isopropyl-1-thio-β-D-galactoside
Kdo	2-keto-3-deoxy-octulosonic acid

LD50	50% lethal dose
Lipid X	2,3-diacyl glucosamine phosphate
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LVS	*Francisella tularensis* live vaccine strain
LVSG	*Francisella tularensis* live vaccine strain gray
LVSGB	*Francisella tularensis* live vaccine strain gray blue
LVSR	*Francisella tularensis* live vaccine strain rough
LVSRB	*Francisella tularensis* live vaccine strain rough blue
mAb	monoclonal antibody
Man	mannose
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide, reduced from
N-CAM	neural cell adhesion molecule
NeuNAc	N-acetyl neuraminic acid
NK cell	natural killer cell
NO	nitric oxide
NS	non-smooth
PBS	phosphate buffered saline
PVDF	polyvinyl difluoride
Rha	rhamnose
ROI	reactive oxygen intermediates
RSLA	*Rhodobacter sphaeroides* lipid A
S	smooth
scid	severe combined immunodeficient
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Tet	tetracycline
TH1	T helper 1 cells
TH2	T helper 2 cells
TNF-α	tumor necrosis factor-alpha
TSB-C	trypticase soy broth supplemented with cysteine
UDP	uridine-5-diphosphate