The Mutagenesis and Characterization of *pdpC* in *Francisella novicida*

by

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B.Sc., University of Victoria, 2004

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Supervisory Committee

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ABSTRACT

Francisella tularensis is a highly infectious, Gram-negative coccobacillus that is the etiological agent of the acute, febrile, zoonotic disease tularemia. A ca. 35 kb *Francisella* pathogenicity island (FPI) was previously discovered. Two genes, *pdpA* and *pdpD* were shown to be required for virulence. The FPI gene *pdpC* encodes a protein that has no significant similarities to any motifs, domains, or homologues of known bacterial proteins. This gene of unknown function may encode a novel virulence factor involved in *Francisella* infection. The role of PdpC in *F. novicida* intracellular growth was investigated. Results from this study demonstrated that the erythromycin allelic replacement mutant of *pdpC* was more attenuated in intracellular growth in the murine macrophage-like J774A.1 cells than in bone marrow-derived macrophages from BALB/c mice and that complementation *in trans* partially complements this mutation. To further investigate the role of *pdpC* in virulence, partial deletion mutagenesis in the C-terminus of PdpC was performed which resulted in four mutants that showed slight attenuation in J774A.1 intramacrophage growth but behaved like wildtype *F. novicida* in bone marrow-

derived macrophages. Chicken embryos were infected to evaluate the virulence of these *pdpC* mutants. The virulence of the Em allelic replacement mutant was significantly more attenuated than wildtype *F. novicida* and complementation partially restored virulence. Partial deletion mutants of *pdpC* exhibited greater virulence than the Em^R mutant in chicken embryos and were able to cause 100% mortality at day 6. Furthermore, eukaryotic expression of triple FLAG-tagged PdpC in chicken embryo fibroblasts resulted in cells that exhibited different morphologies than uninfected fibroblasts which suggests that PdpC may play a role in cytoskeletal rearrangements by altering host cell signaling pathways.

Table of Contents

List of Tables

List of Figures

Abbreviations

aa	amino acid
Ap	Ampicillin
ATCC	American Type Culture Collection
bp	base pair(s)
CEF	chicken embryo fibroblast
CFU	colony forming units
cDMEM	Complete Dulbecco's Modified Eagle Medium
DAPI	4'-6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
Em	erythromycin
EHEC	enterohemorrhagic *E. coli*
EPEC	enteropathogenic *E. coli*
FPI	*Francisella* Pathogenicity Island
FTB	*Francisella* transformation buffer
IAHP	Icm-associated homologous proteins
IFN-γ	interferon gamma
Igl	intracellular growth locus
IL	interleukin
IPTG	isopropyl-β-D-thiogalactopyranoside
IS	insertion sequence
kb	kilobase pairs
kDa	kilodalton
Km	kanamycin
LB	Luria-Bertani broth
LPS	lipopolysaccharide
LVS	Live Vaccine Strain of *Francisella tularensis*
MCS	multiple cloning sites
Mgl	macrophage growth locus
mL	milliliter
MOI	multiplicity of infection
µg	microgram
µL	microlitre
µm	micrometer
µM	micromolar
mM	millimolar
ng	nanogram
NK	natural killer cell

NO	nitric oxide
ORF	open reading frame
PAI	pathogenicity island
PCF	primary chicken fibroblast
PCR	polymerase chain reaction
Pdp	pathogenicity determinant proteins
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
pI	isoelectric point
PMN	polymorphonuclear leukocyte
rRNA	ribosomal ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPI	*Salmonella* pathogenicity island
TAE	Tris-acetate EDTA
TNF-α	tumor necrosis factor alpha
tRNA	transfer ribonucleic acid
TSB/TSA	trypticase soy broth / agar
U	unit
UPEC	uropathogenic *Escherichia coli*
WT	wildtype
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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To Mommy and 老豆 for their undying love and support. Thanks for putting up with me and for believing in me. I am the worst at expressing myself and never quite know how to do so properly. I am glad you understand. And of course, a simple thanks to my twin, Kevin, for his silent support through the years. Love you all.

I will now look forward to the many more opportunities that life has to offer.
Farewell UVic!

kc

To my beloved grandmother who taught me never to take anything for granted,
I'll get there one day

Chapter 1 Introduction

1.1 The *Francisella* genus

1.1.1 History and Background

Francisella tularensis is a facultative intracellular pathogen and the etiological agent of the zoonotic disease, tularemia. Tularemia is a febrile illness in humans and can be fatal if not treated properly. Treatment with antibiotics streptomycin and tetracycline helps to clear the infection. Gentamicin and chloramphenicol have also proven effective, as well as doxycycline, and fluoroquinolones such as ciprofloxacin [114, 129]. This pathogen has been called *Bacterium tularensis* and *Pasteurella tularensis* in the past [83]. In 1911, the bacterium initially named *Bacterium tularensis* was first isolated by McCoy and Chapin while studying a plague-like epidemic in ground squirrels [87]. The pair of researchers named the bacterium after Tulare County, California where it was isolated. Cases of tularemia in humans and other mammals, birds, and amphibians have been discovered across North America, Japan, and the Soviet Union [67, 102]. In the 1920's and 30's, Edward Francis, an American pathologist, performed many of the initial experiments on the pathology of tularemia [52]. The bacterium was later renamed *Francisella tularensis* in his honor.

The genus *Francisella* is a part of the γ-subdivision of the Proteobacteria group. In addition to *F. tularensis* the other recognized *Francisella* species is *F. philomiragia* [47]. *F. philomiragia* was formerly named *Yersinia philomiragia* but was renamed a

Francisella species after 16S rRNA sequence analysis revealed its close relationship to *F. tularensis* [47, 65]. *F. philomiragia* has been isolated from dead muskrats and other sea mammals, and as well as human patients [69]. Humans infected with *F. philomiragia* had often experienced near-drowning and were immunocompromised after corticoid steroid treatment [92].

Early cases of tularemia in North America were found to be clinically more severe than those found in Europe and Asia despite identical antigenicity in the strains [93]. *F. tularensis* is further divided, based on the differences in virulence into two types: the type A biovar for the highly virulent strains of *F. tularensis* subsp. *tularensis* found exclusively in North America and the type B biovar for the less virulent strains of *F. tularensis* subsp. *holartica* found throughout the rest of the northern hemisphere [43, 92]. Metabolic differences in the two biovars include the ability of the type A biovar to ferment glycerol and produce citrulline ureidase, of which the role in virulence is unclear [47]. In 1955, a low virulence strain, later named *Francisella novicida* was isolated by Larson from swamp water in Utah [77]. *F. novicida* is highly related to *F. tularensis* based on the 16s rRNA sequence and deoxyribonucleic acid (DNA) hybridization studies and is now considered a separate species [47]. *F. novicida* is, however, avirulent in healthy individuals but is highly virulent in mice.

1.1.2 Morphology

Francisella tularensis is a Gram-negative coccobacillus which exhibits bipolar-staining when it is observed under light microscopy [92]. During the logarithmic phase of growth, *F. tularensis* subsp. *tularensis* is observed to be 0.2 μm X 0.2-0.7 μm by

electron microscopy. *F. novicida* is 0.7 μm X 1.7 μm. When cells are collected during stationary phase, *F. tularensis* appears as larger pleomorphic forms [92]. There is strong evidence that suggests that the highly virulent strains of *F. tularensis* possess a loosely associated capsule [66]. The encapsulation has been difficult to visualize by either light or electron microscopy because the capsule is easily dislodged from the cells [66].

F. tularensis is an obligate aerobe with an optimum growth temperature of 37°C. *F. tularensis* grows best on rich solid media supplemented with cysteine [11]. However, the addition of cysteine is not necessary for the growth of *F. philomiragia* and *F. novicida*. Cysteine heart agar supplemented with 5% defribrinated blood from horse, sheep, or rabbit supports the growth of all types of *Francisella* [92]. To differentiate colony morphology of the different *Francisella* species, peptone-cysteine agar can be used. For the liquid cultivation of *Francisella*, trypticase soy broth (TSB) supplemented with 0.1% cysteine, modified Mueller-Hinton broth, or the defined Chamberlain's medium can be used [8, 15]. *F. tularensis* has a more fastidious growth requirement and a longer incubation time than *F. novicida.* When *F. tularensis* is grown at 37°C on blood supplemented agar, smooth gray colonies surrounded by a green halo are observed in 2-4 days, whereas colonies of *F. novicida* appears within 24 hr [38]. Death of *Francisella* occurs if the incubation temperature exceeds 56°C for 10 minutes [87].

1.1.3 Epidemiology and Pathology

Tularemia is a disease found primarily in the northern hemisphere with most cases reported during the hunting season during the fall and winter [93]. However, some tick-associated infections do occur in the spring while deer-fly mediated infections occur in

the summer. Tularemia is most often transmitted by ingestion of infected meats, bites from infected arthropods, direct contact with contaminated soil or water, and inhalation of infectious aerosols created by lawn-mowing or brush cutting [43]. Infections may also occur through open cuts and soars by physical contact while handling wild meats from infectious animals. Small mammals such as lagomorphs (rabbits and hares), aquatic rodents (beaver and muskrats), water and field voles, rats, squirrels, lemmings, and field mice are the principal natural reservoirs for *F. tularensis* [103]. Domesticated animals such as cats, dogs, cattles, and some species of birds, fish, and amphibians are almost always the incidental hosts. The Rocky Mountain wood tick *Dermacentor andersoni* is one of the principal vectors in North America [96]. A number of other arthropod vectors that transmit *F. tularensis* have been identified. Some primary vectors include the lone star tick, American dog tick, mosquitoes, deer flies, and spiders [113].

Occasional localized outbreaks of tularemia have occurred. In 1979, people vacationing in Martha's Vineyard contracted the pneumonic form of tularemia [133]. During the late 1960's, there was an outbreak of tularemia among Swedish farmers exposed to contaminated hay [27]. In 1998, an outbreak in Castilla y Leon, Spain affected hundreds of people [123]. In late 1999, the first case of tularemia was confirmed in Kosovo [111]. In 2000, another outbreak of tularemia on Martha's Vineyard created panic after an employee at a vineyard died and many others became infected [45]. In 2002, there was an outbreak among wild-caught, commercially distributed prairie dogs in Texas. From this incident, the first case of prairie dog-to-human tularemia transmission was documented [105, 109]. Exposure in the laboratory setting by inhalation of infectious aerosols, handling cultures or other infectious materials, and accidental

exposure is not uncommon [11]. In 2004, three researchers working on *Francisella* in a laboratory at Boston University became infected after biosafety guidelines were violated [80].

The well documented outbreak in Martha's Vineyard suggests that *F. tularensis* has the ability to persist in the environment. However, its environmental reservoir has not been definitively identified; the ubiquitous protozoa *Acanthamoeba* has been suggested as one such reservoir [1]. Survival inside the protozoa may provide a mechanism for the persistence of *F. tularensis* in the environment.

Tularemia is an acute, febrile zoonotic disease which manifests differently in humans depending on the route of entry of the pathogen. The most common form is the ulcero-glandular form in which a granulomatous ulcer develops when organisms enter through breaks in the skin surface via the bite of infective arthropods [89]. During infection, *Francisella* proliferates and a papule is formed at the infection site within 3-5 days, followed by a localized inflammatory response that involves fibrin, neutrophils, macrophages, and T lymphocytes. The spread to regional lymph nodes causes lymph node swelling, fever, headache, nausea, and muscle ache. Subsequently, the liver and spleen of infected individuals show tiny grey focal lesions [89, 135]. This ulcero-glandular form of tularemia is rarely fatal with proper antibiotic treatment. In the absence of antibiotics, septicemia may develop leading to a systemic infection and multiple organ damage.

Other forms of tularemia include oculoglandular tularemia where organisms gain entry via the conjunctiva caused by the rubbing of eyes after handling infectious materials [128]. Superficial necrosis and ulceration of the conjunctiva may occur. Oropharyngeal

tularemia is a less common form whereby organisms enter the mucous membrane of the oropharynx following ingestion or inhalation of organisms causing exudative pharyngitis or tonsillitis [134]. Pneumonic tularemia occurs when organisms enter the lungs through inhalation of infectious aerosols. During the initial stages of infection, ulcerative bronchitis and bronchiolitis occur, followed by hemorrhagic edema, and eventually the necrosis of lung parenchyma and the alveolar [85]. If left untreated, septicemia may also occur and pneumonic tularemia may turn into typhoidal tularemia [56]. This form of tularemia is extremely fatal with a mortality rate of 30-60% [131].

1.1.4 Biochemistry

The cell wall of a Gram-negative bacterium is a complex, multilayer structure which consists of a cytoplasmic inner membrane, a thin layer of peptidoglycan, and an outer membrane containing lipopolysaccharide (LPS) in the outer leaflet. *Francisella tularensis* can undergo an unusual phase variation in the LPS that alters the form of the O-antigen and the toxicity of the endotoxin [25]. This variation affects the ability of *F. tularensis* to grow in macrophages of some animals and is a problem in the production of effective and consistent vaccines [25]. Although the O-antigen elicits an antibody response, the LPS acts neither as a stimulus nor a mitogen for lymphocyte multiplication [116]. Futhermore, unlike the LPS from most other Gram-negative bacteria the *F. tularensis* form is not endotoxic and does not trigger the production of interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) from either human or mouse cells [116].

Fully virulent *F. tularensis* were found to be encapsulated and the loss of the capsule in avirulent aged bacteria leads to a loss of virulence in aerosols [66]. Both the cell wall and

capsule are rich in lipids. Perhaps the best characterized structural constituents leading to the high pathogenicity of *F. tularensis* are the fatty acids of the organism. Gram-negative bacteria typically contain saturated and monoenoic straight-chain acids linked with one or several hydroxyl fatty acids. The fatty acid composition of *Francisella tularensis* was characterized as unusual, containing a high proportion of long chain, monounsaturated acids (C_{20}-C_{26}) and the hydroxyl fatty acids 2-hydroxy-decanoate, 3-hydroxy-hexadecanoate, and 3-hdyroxy-octadecanoate [68]. The unusual unsaturation in the monoenoic fatty acids in the cellular fatty acid profile of *F. tularensis* serves as an unique signature and represents a valuable taxonomic characteristic of this pathogen [95].

By total dry weight, cells of *F. tularensis* contain 21% lipid which is a high percentage for bacteria [3]. The two major lipids identified were phosphatidylglycerol (PG; 24%) and phosphatidylethanolamine (PE; 76%). Both were also found to contain the expected molar ratios of fatty acid/glycerol/phosphorus [3]. However, hydroxyl fatty acids were found lacking in the two major phospholipids PG and PE which are mainly found in the cytoplasmic membrane. This finding suggested that hydroxyl fatty acids could instead be constituents of the lipid A of LPS and the capsule [3].

1.1.5 Intracellular survival

Francisella tularensis is highly virulent for humans. As few as 10 organisms delivered by a subcutaneous route or 25 organisms delivered by inhalation can lead to a severe, potentially fatal infection [120]. Intracellular growth of *F. tularensis* was first discovered after the inoculation of the chorioallantois, a vascular membrane used in gas exchange in chick embryos [13]. Intracellular growth has also been described in experiments using

hepatic cells and endothelium from guinea pigs [130]. Both *F. tularensis* and *F. novicida* have been shown to grow in both professional phagocytes, such as macrophages and monocytes, and non-professional phagocytes, such as hepatocytes and HeLa cells [7, 125].

In vivo, the growth of *F. tularensis* appears to be primarily intracellular as it has been found mainly in macrophages. Evidence for this comes from studies using mice infected intraperitoneally with *Francisella*. *Francisella* cells were found exclusively in peritoneal macrophages and were not found within polymorphonuclear leukocytes (PMNs) [48]. Also, *F. tularensis* has been shown to invade and grow in specialized macrophages located in the liver. Mice inoculated with sublethal doses show *F. tularensis* localizing and replicating in Kupffer cells as well as vacuoles of hepatocytes [22]. Neutrophils and inflammatory macrophages appear at the site of infection later on. Numerous other studies using cell culture have demonstrated intracellular growth of *Francisella*. Cells used in these *in vitro* experiments include alveolar macrophages from rabbits and mice [101], peritoneal macrophages from mice, rats and guinea pigs [7, 50], and human monocytes [48].

Macrophages play an important role during the course of an infection. They function mainly to engulf pathogens or other infectious particles, limit the infection to a specific site, and destroy the pathogen by releasing reactive oxygen or nitrogen species through respiratory burst [118]. Macrophages also serve to establish specific immunity by secreting various inflammatory mediators such as cytokines that induce the killing activity of other effectors during an immune response. The uptake of bacteria by macrophages usually occurs through phagocytosis which involves the movement of

microfilaments. However, *Francisella* uptake does not require the mobilization of microfilaments. The ability of macrophages to phagocytose *Francisella* was not inhibited after treatment with cytochalasin B, a microfilament inhibitor, which suggests that they do not enter the cell by classic phagocytosis [48]. *F. tularensis* was found to enter macrophages by a novel process of engulfment involving spacious pseudopod loops[18]. In the same study, the adherence and uptake of *F. tularensis* was found to be dependent upon complement receptors and the complement factor C3 [18]. New findings from this study suggest that the uptake of *Francisella* does require the participation of actin microfilaments when macrophages treated with cytochalasin B exhibited a dramatic loss of pseudopod formation [18].

Upon internalization, *Francisella* is found to grow inside a membrane-enclosed vacuole that does not fuse with lysosomes [7]. Studies using LVS found that treatment of macrophages with chloroquine (NH_4Cl) which increases the intracellular pH prior to infection inhibited replication of LVS [49]. This confirmed that multiplication of *Francisella* requires an acidic environment. This seems logical as the processes of many metabolic pathways occur under acidic pH. In particular, the transport of iron coupled to transferrin occurs via the endocytic vacuole through receptor-mediated transport and releases ferric iron only when the vacuole is acidified [30]. The replication of LVS was restored in macrophages treated with NH_4Cl after the addition of ferric PP_i, a transferrin independent form of iron. The acidification of the bacteria-laden vacuoles is essential for the intracellular growth of *Francisella* [49].

Although it has the ability to grow inside both professional and non-professional phagocytes, *F. tularensis* has been found to replicate faster in inflammatory macrophages

than in resident macrophages [50]. This suggests that the bacterium has the ability to exploit inflammatory events that occur in inflammatory macrophages undergoing increased metabolic activity. However when these inflammatory macrophages are treated with lymphokines, nitric oxide is produced which inihibits the growth of *Francisella* more than resident macrophages [50]. Generally, inflammatory macrophages migrate to the site of infection later in the infection. Therefore, survival inside resident macrophages may help *Francisella* to evade phagocytosis by polymorphonuclear leukocytes (PMNs) which are the first cells to arrive at the site of infection. Phagocytosis of the pathogen by PMNs is inhibited in the absence of immune serum; however, high titres of both immune and non-immune serum have been shown to support PMN phagocytosis [107]. Once *Francisella* is phagocytosed, PMNs were found to be more efficient at killing the pathogen than macrophages. Thus, the evasion of phagocytosis and killing by PMNs help *Francisella* to migrate to different target tissues before the arrival of inflammatory macrophages to the infection site.

Fortier *et al.* have shown that both mouse inflammatory and resident macrophages treated with interferon gamma (IFN-γ) are capable of killing the phagocytosed *Francisella* [50]. However, inflammatory macrophages required less IFN-γ during treatment than resident macrophages to bring about the same degree of killing activity since inflammatory macrophages have a higher enzyme activity and oxidative capacity[50]. Treatment with IFN- γ was found to induce nitric oxide (NO) production which results in antimicrobial effects. *Francisella* was able to survive in macrophages activated by IFN-γ treatment when exogenous iron is supplied [50]. This suggested that NO serves to sequester free iron, forming nitrosyl-iron complexes, which inhibits the

activities of many iron-dependent enzymes important in many essential microbial processes such as DNA synthesis, the citric acid cycle, and respiration. It was also shown that IFN-γ can induce antimicrobial activity in human monocytes in a similar manner and the sequestration of iron by NO is reversed by the addition of iron pyrophosphate [48].

Recently, the intracellular survival and life cycle of *Francisella* were examined in murine primary macrophages. The modulation of phagosome maturation and the biogenesis of the membrane-bound replicative organelle were investigated. Phagosomal escape by *Francisella* occurred 1 h post infection after the bacteria transiently interact with the endocytic pathway shown by acquisition of the early endosome marker early endosome antigen-1 (EEA-1)[16]. By 4 – 20 h, bacterial replication in the cytoplasm occurs. Subsequently, a majority of *Francisella* is found enclosed in lysosomal-associated membrane protein 1 marker (LAMP-1) positive vacuoles called *Francisella*-containing vacuoles (FCVs). FCV formation is a post-replicative event [16]. Despite fusion of FCVs with lysosomes, intact bacteria were observed. Furthermore, FCVs were observed to be multimembranous which suggested that the biogenesis of these vacuoles was from the autophagic pathway [16]. Via autophagy, *Francisella* is able to re-enter the endocytic pathway after cytoplasmic replication. This trafficking of *Francisella* inside macrophages is a novel survival process which has not been found in other intracellular pathogens.

1.1.6 Immunology: Humoral and Cell-mediated immunity

The Live Vaccine Strain (LVS) of *F. tularensis* was developed in 1961 from a strain obtained in the Soviet Union. *F. tularensis* LVS was first used to immunize researchers

at a national laboratory in Fort Detrick, Maryland. This strain was proven to protect against respiratory tularemia in aerosol challenges, but subjects still developed the ulceroglandular form, however, the symptoms exhibited were milder than usual [14]. In humans and animals, long term specific protective immunity is established after recovery from the disease [14, 51]. *F. tularensis* LVS is extensively used for investigations of the immune response against *Francisella* infections as it provides a good model. This strain is lethal for mice but avirulent for humans and there is no cross-reactive immunity or tolerance in the mouse infection model [42]. The severity of the disease developed after wildtype or LVS infections in mice largely depends on the route of entry [51]. The 50% lethal dose (LD_{50}) for an intradermal injection of LVS is approximately 10^5 bacteria. Routes of entry by intranasal, intravenous, and intraperitoneal injections have much lower LD_{50}s in the range of 1 – 100 bacteria. Using this information, studies of the sublethal immune response in mice have shown that recovery after a sublethal injection intradermally provides resistance to inoculations from other routes [51].

When the innate immune response, the first line of defense, to a *Francisella* infection was studied, it was found that *Francisella* was resistant to the classical complement cascade normally activated by the lipopolysaccharide [4, 53, 112]. It was suggested that perhaps the capsule observed in *Francisella* or the LPS O-antigen may provide some sort of protection. Neutrophils have also been shown to play an important role in the defense against bacterial invasion. Neutrophils undergo oxidative burst to release reactive oxygen intermediates (ROI); therefore they are considered short-lived phagocytes. Mice depleted of neutrophils and eosinophils succumb to infection after sublethal primary injections with LVS [41, 127]. The depletion of neutrophils using granulocyte specific

antibodies is effective for only 2 days, after which neutrophils secrete cytokines to activate neighboring macrophages [127]. Thus, neutrophils do not play a direct role in killing *Francisella*. There is also evidence that efficient killing of *Francisella* happens only after opsonization [127].

As shown in the mouse model, vaccinations in humans also produce long term protection against tularemia. Both humoral and cell-mediated immune responses are elicited. The cell-mediated mechanisms are crucial for the ultimate clearing of the pathogen [36]. Despite a strong antibody response, humoral immunity does not contribute significantly to clearance. Vaccinations of humans with LVS showed that the appearance of specific antibodies occurred two weeks post infection [73]. However, the agglutinins did not reach diagnostically significant levels until 4 weeks post infection and did not peak until 2 months later [73].

When mice were infected with LVS intradermally, humoral immune factors appear to play a role. It was shown that the antibody titre produced can passively be transferred to offer limited protection to naïve mice during a LVS challenge. IgM and IgG_{2a} were the two classes of antibody induced [112]. The antibody helps to make LVS more susceptible to killing by PMNs via opsonization [112]. However, phagocytosis by macrophages does not require initial opsonization. The mice challenged with LVS after treatment with immune serum had a significantly higher bacterial burden in the liver and spleen than untreated control mice [112]. It was shown that IgM production in the challenged mice is detectable at 5 days and the titre appears to peak at two weeks whereas the IgG response is detectable at 10 days [112]. Humans vaccinated with LVS

show detectable levels of specific IgM, IgA, and IgG responses two weeks after vaccination and the titre remains one year later [73].

B cells have been shown to play a role in the control of initial *Francisella* infections. A study was performed in which mice were given sublethal doses of LVS, and resistance to a lethal injection 2 – 3 days later was observed. Only severe-combined immunodeficient (SCID) mice and B cell knockout mice were unable to survive the lethal challenge [26]. B cell knockout mice are unable to clear a primary intradermal infection of LVS completely, and upon a secondary lethal challenge, the mice are 100-fold less protected [39]. This demonstrated the importance of B cell in the contribution to secondary challenges that do not involve antibody production [39].

T lymphocytes are crucial for the generation of long term specific immunity to *Francisella* infections. Their role is to produce cytokines which help to activate macrophages that limit intracellular bacterial growth. The delayed-type hypersensitivity (DTH) response occurs after vaccination and has been shown to be correlated with cell-mediated immunity in rats, mice, and humans [74]. The DTH response has been used as a skin test for diagnosis. Mice immunized with LVS produced the DTH response when exposed to *Francisella* but survived a challenge with a lethal dose [21].

The differentiation and proliferation of CD4+ and CD8+ T cells are activated by sublethal infections of LVS in mice. Both types of T cells serve to transfer protection and terminate disease progression. The elimination of one of these subtypes has no effect on the level of protection [139]. The elimination of both types of T cells however will result in disease progression. The importance of T cells have been studied where nude (*nu/nu*) or α/β T cell knockout mice were killed one month post infection without

apparent signs of pronounced symptoms, and mice lacking only one subtype of T cell survived an intradermal infection [23, 40, 139]. The resistance to *F. tularensis* can be transferred by lymphocytes obtained from immune to non-immune syngeneic mice [5, 74]. The mechanism used in cell-mediated immunity by T cells remains unclear and it is possible that T cells eventually kill macrophages infected with the pathogen as it has been shown that CD8+ T cells, along with NK cells, from peripheral blood lymphocytes target monocyte-derived macrophages specifically and induces cytotoxicity that results in macrophage cell death [60].

1.2 Pathogenicity Islands

Genome evolution is the process by which the organization and content of genetic information of a species change over the course of time. The process normally includes four types of genetic changes: point mutations and gene conversions, rearrangements by inversion or translocation, deletions, and insertions of foreign DNA by plasmid integration or transposition [121]. Gene loss and acquisition can rapidly bring about changes in the adaptive lifestyle of a microbe in "quantum leaps" [121]. These latter mechanisms of change are primarily how bacteria have adapted genetically to survival in novel environments.

The loss and acquisition of foreign DNA are coupled events because genome growth is not unlimited. The major source of foreign DNA into the host DNA is horizontal gene flux which involves mobile genetic elements such as transposons, insertion elements, conjugative plasmids, bacteriophages, and genomic islands [121]. The pathogenicity islands (PAI) are a subgroup of genomic islands. PAI can contribute to the development

of diseases and to the virulence of bacterial pathogens harbouring them. The first PAI was discovered by Jörg Hacker, a German scientist, in the late 1980s. He observed the loss of two linked virulence gene clusters together with additional DNA segments greater than 30 kb apart in a single deletion event, which led to the definition of pathogenicity DNA islands [62].

1.2.1 Features of PAI

There are many genetic features of PAI. Briefly, PAI usually carry one or more genes associated with virulence. Genomic segments with characteristics of PAI lacking virulence genes are considered genomic or metabolic islands instead. Often a PAI will be present in the genome of a pathogen but will be absent in the genome of close relatives that are less virulent. PAI may be large genomic segments ranging from 10 to 200 kb. The base composition of a PAI is often different from the base composition of the host genome, as well as a different codon usage may be used. Base composition is the percentage of guanine and cytosine (G+C) bases. It is observed that the horizontally acquired PAI still has the base composition of the donor species. During evolution, the base composition will gravitate to that of the recipient's genome. PAI are often located adjacent to tRNA genes which may serve as anchor points for foreign DNA insertions during horizontal gene transfer.

PAI are often associated with mobile genetic elements. PAI are often flanked by direct repeats which serve as recognition sites for the integration of bacteriophages and for the excision of mobile genetic elements leading to instability of PAI flanked by direct repeats. Insertion sequences are often observed which result in the mobilization of larger DNA

segments. There is intrinsic genetic instability associated with PAI. Large portions of PAI or its entirety may be deleted. Virulence functions encoded by PAI are lost with a higher frequency than the normal rate of mutation. Lastly, PAI often represent mosaic structures of several DNA acquisitions rather than a single homogeneous segment. Some PAI may represent the single insertion of foreign genetic material, while others are more structurally complex consisting of several independent acquisitions from different hosts during evolution.[121]

1.2.2 Secretion systems

A large number of bacterial virulence determinants are located within PAI. Because most virulence factors are involved in the interaction with eukaryotic host cells, they are required to be exposed at the bacterial cell surface or transported out of the bacterial cell. Bacteria have developed at least five protein secretion systems for the export of pathogenicity factors.

The secretion of proteins is an important requirement for bacteria. The proteins secreted are required for cell envelop assembly, metabolism, and defense against immune mechanisms as well as interaction with host cells during pathogenesis [62]. In Gram positive bacteria, extracellular and surface proteins are secreted by the general secretion pathway. In Gram negative bacteria, the presence of an outer membrane led to the evolution of secretion systems that are structurally and functionally different.

Type I secretion systems (T1SS) contain a simple assembly of an ATP-binding cassette (ABC) transporter protein located within the inner membrane, a periplasmic protein, and an outer membrane protein that forms the secretion pore. This transporter is involved in

the transport of a specific substrate protein which is delivered into the extracellular medium. Common substrates of T1SS are toxins such as hemolysins.[62]

Type II secretion systems (T2SS) are also referred to as the main branch of the general secretion pathway. After the protein crosses the inner membrane, the signal sequence is cleaved by the signal protease and the cleaved substrate protein is transported across the outer membrane. T2SS consists of at least 12 subunits that are found in the periplasm, the inner membrane, and the outer membrane.[62]

Type III secretion systems (T3SS) require the function of more than 20 genes and are complex assemblies. Many of these subunits show similarity to the flagellum assembly machinery. The main function of T3SS is to translocate proteins across the bacterial membranes and the membrane of the eukaryotic host cell. Translocation of effector proteins leads to the specific interference of host functions such as host cell invasion, inactivation of phagocytes, apoptosis, as well as disruption of intracellular transport processes. Contact is required between the pathogen and the host cell for the function of T3SS. Gene clusters encoding T3SS have been found on virulence plasmids in *Yersinia* and *Shigella* sp. Components of T3SS are also encoded in PAI found in *Salmonella enterica* and enteropathogenic *E. coli*. [62]

Type IV secretion systems (T4SS) are also able to translocate proteins into eukaryotic host cells. The structure of T4SS is complex and requires at least 10 subunits. T4SS have similarities to the conjugation systems for DNA transfer. The system in *Agrobacterium tumefaciens* is the best studied T4SS which is involved in the translocation of DNA-protein complex into plant cells for the induction of tumor formation. Other T4SS have been discovered in human pathogens such as *Bordetella*

pertussis, *Bartonella* sp., *Legionella pneumophila*, *Brucella* sp., and *Helicobacter pylori*.[62]

Type V secretion systems (T5SS) are referred to as autotransporters and involve a secretion system and a substrate protein that are synthesized as a single preproprotein. The preproprotein contains an N-terminal signal sequence that directs its secretion via the Sec system into the periplasm. Proteolytic cleavage of the signal sequence results in the oligomerization of the transporter domains of the proprotein into a β-barrel which inserts into the outer membrane. The passenger domain of the proprotein can then pass through the pore formed by the β-barrel structure. Another proteolytic event releases the passenger domain into the extracellular space. [62]

1.2.3 Virulence factors

Virulence factors encoded on PAI vary widely and include factors such as adhesions and toxins. Adhesins are a major class of virulence factors which mediate the attachment of pathogens to specific receptors in the eukaryotic host [62]. Examples include the P fimbriae which represent adherence factors of UPEC and the S fimbria initially found in *Salmonella* sp. The locus of enterocyte effacement (LEE) of EPEC and EHEC encodes intimin, an intestinal adherence factor [62]. Secretion systems encompass a class of virulence associated genes that are essential for the secretion of virulence factors to the surface of or directly translocated into the host cell, or into the extracellular medium. Many pathogens also harbour genes that encode for toxins in their PAI. Heat labile and heat stable enterotoxins of enterotoxigenic *E. coli*, pore-forming toxins of other enterobacteria, cytolysins of enterococci *S. aureus*, and the neurotoxins of pathogenic

Clostridium and *Bacillus anthracis* have been well characterized [62]. Many pathogenic bacteria also produce enzymes with proteolytic activities. The LIPI1 in *Listeria monocytogenes* contains a locus encoding a metalloprotease. The Pic protease/mucinase of *S. flexneri* SHI-1 can degrade gelatin and mucin causing fluid accumulation in ileal loops of infected animals [62].

Iron uptake systems are encoded by both pathogenic and nonpathogenic species. The survival of microbes in certain niches is dependent on the availability of essential nutrients such as iron. The acquisition of iron is a necessity for the infectious process. The expression of receptors for iron carriers such as heme, hemoglobin, lactoferrin, and transferrin, and the production of siderophores which are high affinity iron-binding molecules are strategies employed by bacteria [62]. Overall, the expression of virulence genes by pathogenic bacteria helps to ensure their adaptability to particular environments as well as to their virulence.

1.2.4 Regulation of virulence functions

Similar to most virulence genes, genes encoded by PAI are not usually constitutively expressed. Instead this group of genes responds to environmental signals [62]. PAI are often part of complex networks that include regulators encoded by the PAI, regulators encoded by other PAI, and global regulators encoded on different loci in the chromosome or even by plasmids [121]. PAI regulators can regulate genes encoded on genetic elements outside the PAI.

Most regulators belong to the AraC/XylS family or to the two-component response regulator family. Interestingly, sigma factors and histone-like proteins are also found to

be involved in PAI regulation [121]. More complex regulatory networks have been found in the VPI of *Vibrio cholerae*, SPI-1 and SPI-2 of *Salmonella enterica*, the Yop virulon of pathogenic *Yersinia* sp., and the LEE of EPEC and EHEC whereby PAI-encoded regulators of PAI-encoded virulence genes are modulated by a system encoded outside of the PAI. [62, 121]

The regulation of SPI-1 and SPI-2 of *S. enterica* has been investigated extensively. SPI-1 invasion genes are expressed in response to the host microenvironment and are also regulated by genes encoded on the SPI. Such host conditions that trigger gene expression include oxygen level, osmolarity, pH changes, and even the presence of short-chained volatile fatty acids. In order to adapt to the microenvironmental changes, invasiveness is induced under conditions of low oxygen and high osmolarity whereas, the bacteria remain noninvasive under high oxygen conditions. The two-component global regulatory systems EnvZ/OmpR, BarA/SirA, PhoPQ and PhoRB, FliZ and Hha, all encoded by genes located on the core genome, function to transduce these signals.[121]

1.2.5 Integration sites of PAI

Most PAI have been found to be inserted at the 3' end of tRNA loci. This integration into the bacterial chromosome is a site-specific event [121]. Although, the molecular basis for the use of tRNA genes as integration sites is unknown, there exist several reasonable hypotheses. Specific tRNA genes associated with a PAI may be used to read codons of the associated PAI [54]. The presence of multiple copies of tRNA genes serves to provide multiple integration sites and amplification of pathogenicity factors[121]. The conserved structure of tRNA genes serves to provide structural motifs

which facilitate integration and excision of PAI by integrases. The conservation of tRNA genes helps to provide safety to the mobile genetic element for the ease of integration into any genome of any bacterial species in a dynamic population [54]. Thus, tRNA genes serve an important role in evolution in maintaining pathogenicity factors in a bacterial population.

1.2.6 Evolution and transfer of PAI

It has been observed that important virulence factors have orthologs in different bacteria. This commonality between bacterial species and strains may be explained by horizontal gene transfer [62]. Horizontal gene transfer of genomic islands is a powerful mechanism whereby the genotype of a bacterium can be permanently altered. The evolutionary success of a transfer event is governed by the need of the bacterium to increase its fitness to the selective pressures of the environment. [64]

Horizontal gene transfer can be accomplished in different ways. Transport systems are expressed at different stages of growth that promote the uptake of free DNA from the environment. During this natural transformation, most of this foreign DNA may be degraded but useful genes are integrated into the recipient chromosome. This form of DNA uptake can come from distantly related species [64]. It has been observed that in some pathogens, certain clusters of virulence genes are found in PAI whereas in other bacteria, they are encoded on virulence plasmids. For example, the T3SS of *Shigella* sp. is encoded by *mxi* and *spa* genes located on a virulence plasmid whereas a related gene cluster for epithelial cell invasiveness of *S. enterica* is found in SPI-1 within the chromosome[121]. The occurrence of related gene clusters on a plasmid or in the

chromosome may be a consequence of conjugation when plasmids are transferred between bacteria and, under certain conditions, integration of the plasmids into the chromosome occurs. Bacteriophages have been isolated in a wide range of bacterial species and are able to transfer bacterial virulence genes as passengers in the viral genome. However, many PAI are simply too large to be transferred by bacteriophages. Thus, during viral replication, the DNA is fragmented and are packaged into phage heads [121]. Bacteriophages capable of generalized transduction are able to transduce a fragment of the bacterial DNA packed in the phage head. Homologous recombination events may occur where the transduced DNA fragment integrates into the genome of the host. Horizontal gene transfer facilitated by plasmids, bacteriophages, or bacteria is evident throughout the bacterial world as PAI are not found exclusively only in human pathogens, but have also been discovered in animal and plant pathogens such as *hrp* islands of *Pseudomonas syringae* and *Xanthomonas campestris* and islands in the animal pathogenic strains of *Salmonellae* and *Staphylococci* [121].

1.3 *Francisella* Pathogenicity Island

F. tularensis is a facultative intracellular pathogen, however very little is known about the bacterial virulence factors needed for infection although it is clear that intracellular growth, especially in macrophages, is essential to the virulence of *F. tularensis*. A biochemical study of the LVS of *F. tularensis* showed that four proteins are induced after entry into macrophages [58]. The gene encoding the most prominently induced protein, the 23 kDa IglC protein, has been molecularly cloned and sequenced. The deletion of this

gene showed that the mutant was unable to grow in macrophages and unable to cause disease in mice [59].

Genetic approaches have also been used to discover other *F. tularensis* genes needed for optimal intracellular growth. The products of *mglA* and *mglB*, thought to be global regulators, are both required for intramacrophage growth and virulence in mice [10]. Random insertional mutagenesis revealed that inactivation of *F. tularensis* genes encoding homologues of glutamine phosphoribosylpyrophosphate amidotransferase (purine biosynthesis), alanine racemase (peptidoglycan biosynthesis), and the heat shock-inducible ClpB protease reduces the ability of *F. tularensis* to grow in mouse macrophages [61]. Transposon insertion into *iglB* and *iglC* also profoundly affects intramacrophage growth [61].

The sequencing of the *F. tularensis* genome [71, 106] has facilitated the analysis of virulence factors of *Francisella.* Two mutations in the linked genes *iglB* and *iglC* that reduce the ability of *F. tularensis* to grow in macrophages were previously isolated [61]. Bioinformatic analysis of this region of the genome surrounding the location of these insertions revealed an apparent FPI of approximately 30 kb (**Figure 1.1**). In the first 11 kb of the FPI are five open reading frames (ORFs). Four of which, *iglABCD* (for intracellular growth locus), appear to be organized into an operon. The deduced products of *iglA* and *iglB* have about 30% identity to hypothetical proteins found in several bacterial species, most of which are animal or plant pathogens or plant symbionts. However, the deduced proteins IglC and IglD show no significant similarity to other known proteins. In the right half of the FPI are three large ORFs, named *pdpABC* (for pathogenicity determinant protein). The region between *pdpB* and *pdpC* has eight

relatively short ORFs, seven of which are below 800 bp and one of which is 1,431 bp. None of the amino acid sequences deduced from *pdpABC* or the smaller ORFs show substantial similarity to those of known proteins. [94]

Pathogenicity islands are often recognized by the aberrant G+C content in their DNA, which differs from that for the rest of the resident genome. The *F. tularensis* genome has an overall G+C content of 33.2% [106]. The FPI has different regions that have variable G+C content. The region corresponding to *pdpD* through *iglD* has a G+C content of 31% (**Figure 1.1**). The region from the gene immediately downstream of *pdpC* to 204 bp upstream of the start codon of *pdpA* has a G+C content of 26.6%. Immediately to the right of the presumed promoter region of *pdpA* lies a 5,050 bp region that is 51% G+C and that encodes rRNA. The very different G+C content of this region is consistent with the need for conservation of the rRNA sequence, which permits changes in the G+C content primarily to adapt to the optimum growth temperature of the bacterium. [94]

The sequence data for the genome of *F. tularensis* LVS show that there are two copies of the FPI in this genome (ftp://bbrp.llnl.gov/pub/cbnp/F-tularensis/F.tularensis.html.) One of the LVS forms of the FPI is essentially identical to the Schu4 form from one set of inverted repeats on the left end to the other set on the right end. A second copy of the FPI in the LVS strain is identical from the inverted repeats on the left end through to the rRNA genes. Thus, the presence of two copies of this region in LVS suggests that the FPI region was capable of movement at one time and may still have the capacity to be mobile.[94]

1.3.1 Genes encoded on the FPI

In the same study by Nano *et al.* [94] several of the virulence-associated genes encoded on the FPI are described. The product of *iglC* has previously been shown [58] to be highly induced after entry of *F. tularensis* into mouse macrophages and has recently been shown by us and others to be needed for growth in macrophages [61]. Upstream of *iglABCD* is a large ORF named *pdpD*. The deduced amino acid sequence shows no significant similarity to that of any known protein. The *F. novicida* form of the PdpD protein is composed of 1,245 amino acids and is predicted to be 141 kDa with an pI of 6.84. DNA sequencing of the region around PdpD in the LVS strain showed that a 4,249-bp region is deleted from this region. This deletion is confirmed in the genome sequence of the LVS strain and it occurs in both copies of the FPI [94]. It was also shown that disruption of *pdpD* by allelic replacement with an erythromycin cassette resulted in a mutant defective for intramacrophage growth and virulence in mice. The inactivation of *pdpA* in *F. novicida* by transposon insertion also rendered the mutant unable to grow in macrophages and avirulent in mice. Complementation of *pdpA in trans* restored wildtype intramacrophage growth phenotype as well as virulence in mice [94].

Further characterization of the *pdpA* transposon insertion mutant indicated that *pdpA* is required to induce macrophage death [86]. It was shown that in contrast to wildtype *F. tularensis*, the *pdpA* mutant was incapable of phagosomal escape into the cytosol of the macrophage [86]. Thus, *pdpA* is required for intracellular bacterial replication. It was postulated that survival of *F. tularensis* within the cytosol is associated with the induction of apoptosis of the macrophage. Caspase-1, part of the complex assembly of proteins that make up the inflammasome in response to intracellular bacteria, signals for cell death and has been demonstrated to play an important role in *F. tularensis* induced macrophage

death [86]. The processing of caspase-1 did not occur in the *pdpA* mutant infected macrophages which suggests that casp-1 activation is dependent on the signals generated in response to the presence of cytosolic bacteria [86]. Essentially, all other virulence genes encoded on the FPI remain to be characterized in order to determine their role in the pathogenesis of *Francisella*.

1.3.2 Regulation of FPI genes

Recently, *iglA, iglC, pdpA,* and *pdpD* have been shown to be regulated by MglA which suggests that these FPI genes are coordinately regulated to produce a virulence phenotype in *F. tularensis* [31, 79, 86]. The FPI gene *iglC* has been the most studied of any of the genes encoded in the FPI. *iglC* is located within a putative operon consisting of *pdpDiglABCD* based on close or overlapping start and stop codons along with the lack of any transcriptional terminators [79]. Semiquantitative RT-PCR analysis suggested that *pdpDiglABCD* may be a single MglA-dependent transcriptional operon and that MglA is a positive transcriptional regulator of the iglC operon when lower levels of *pdpD, iglA, iglC, iglD,* and *iglA* transcripts were detected in the *mglA* mutant in comparison to wildtype [79]. IglC and MglA were also shown to be essential components in the modulation and biogenesis of *Francisella*-containing phagosomes and the subsequent phagosomal escape into the cytoplasm [117, 119]. A majority of phagosomes containing the *iglC* and *mglA* mutants acquired the late endosomal/lysosomal marker (LAMP-2) as well as the lysosomal enzyme Cathepsin D which marks the phagosomes for degradation through the endocytic pathway [119]. *pdpB* has also been shown to be positively regulated by *mglA* when transcriptional profiling of wildtype and *mglA* mutant was

performed to identify MglA-regulated genes [12]. Novel MglA-regulated genes were mutated and the *pdpB* mutant was found to be defective for growth in macrophages. The *pdpB* mutant also resulted in severe attenuation in the cytotoxicity of macrophages and virulence in mice [12].

1.3.3 Gene expression induced by iron-limitation

The role of iron in microbial physiology is important and pathogens that invade human cells encounter an environment of extreme iron limitation because free iron is not normally found in the bloodstream or tissues. Successful pathogens have evolved to express high-affinity iron uptake systems to obtain iron *in vivo*. Genes in *F. tularensis* LVS were examined for up-regulation under iron restricted conditions by the addition of the iron chelator desferal to the growth medium. All the major *pdp* and *igl* genes encoded by the FPI were found to be up-regulated at least two-fold under iron-restricted growth conditions by DNA microarray analysis and quantitative real-time reverse transcription PCR [33]. Increased level of protein expression of genes encoded by the *igl* operon during iron-limited growth has also been demonstrated in the LVS strain by another group [81]. Low abundance of iron encountered by the pathogen upon entry into a host cell may serve as a signal to increase the synthesis of virulence factors that aid in its survival in such an unfavorable environment.

1.3.4 Secretion system homologues

In the course of *Francisella* research, no Type III secretion system (T3SS) has ever been discovered. However, a genetic locus encoding genes with high similarity in their

deduced amino acid sequence to *msbA* and *orfE* of *E. coli* has been shown to be required for growth in macrophages [88]. These genes are part of the superfamily of ABC transporter proteins involved in T1SS. The presence of a functional T1SS has also been suggested when the allelic replacement mutant of *tolC* was found to cause significant attenuation of virulence in a mouse model [55]. The homolog of TolC in *E. coli* functions as the outer membrane channel component for both the T1SS and the multidrug efflux system. In addition to its role in multidrug resistance, TolC was demonstrated to be a critical virulence factor for the export of a toxin or other virulence factors critical for *Francisella* pathogenesis [55]. Also, using proteomic and bioinformatic approaches, it was revealed that Type IV pili secretion system homologues in *F. novicida* mediated the secretion of several proteins encoded outside of the FPI [63].

Recently, homologues of *iglAB* from *Vibrio cholerae, Salmonella enterica, Rhizobium leguminosarum* were found to be located in a gene cluster that encode proteins known as IcmF-associated homologous proteins (IAHP) [29, 46, 124]. Components of a proposed Type VI secretion system (T6SS) are encoded by this gene cluster in *V. cholerae* [108] and it was suggested that the FPI may encode a T6SS. The presence of genes encoding for a T6SS is likely upon the examination of FPI genes by bioinformatic analyses. A BLAST search of PdpB revealed significant alignment of its C-terminus third with regions of proteins belonging to the IcmF conserved orthologous group (COG) [31]. IglA and IglB were also found to share strong identity with two other COGs groups. Orthologs of *iglAB* were examined and found adjacent to each other on the chromosome [31]. Vgr proteins are hydrophilic proteins containing valine glycine repeats and are secreted by the T6SS in *V. cholera.* An ORF downstream of *pdpB* has weak similarity

(E-value 0.15) to the family of *vgr* products [31]. Another ORF further downstream of *pdpB* show some similarity (E-value 0.0005) to the IAHP-asssociated protein DotU [31]. The IcmF motif in PdpB, the strong identity shared by IglAB to COG3516 and COG3517 [31], and two genes with weak similarity to IAHP-associated proteins suggest that a secretion system similar to IAHP/T6SS may play a role in protein secretion by *Francisella*.

1.4 Research objectives

In the past, other laboratories have primarily focused their research on the host's immune response to infection by *Francisella* using the *Francisella tularensis* Live Vaccine Strain (LVS). Our laboratory has been focused on investigating the virulence properties of *Francisella* that enable its intramacrophage survival. Instead of conducting research with the highly infectious strain, our model strain is *Francisella novicida*. *F. novicida* shares a high degree of DNA homology by 16S rRNA hybridization studies with *F. tularensis*, and is avirulent in humans but remain virulent in mice. The pathology of tularemia developed in mice during infection is similar to that of infected humans. Advantages to using *F. novicida* include its amenability to basic genetic manipulations and its lack of fastidious growth requirements.

The purpose of this thesis is to investigate the role of the *pdpC* gene product in the virulence of *Francisella novicida* infections. The objectives are: (1) to characterize the phenotype of the erythromycin allelic replacement mutant of *pdpC* and its complement in intramacrophage growth and chicken embryos, (2) to create defined partial deletion mutants of *pdpC* and examine the mutants for intramacrophage growth and survival in

chicken embryos, (3) to conduct preliminary expression studies of PdpC in an eukaryotic system using chicken embryo fibroblast cells.

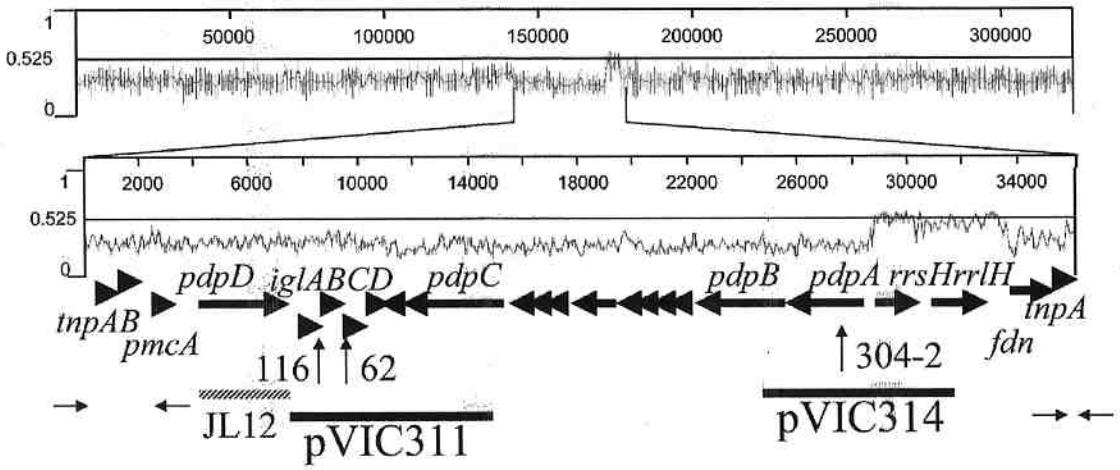


Figure 1.1 Gene organization and G+C content of the *Francisella* Pathogenicity Island (FPI). Top graph, fractional G+C content of the 300-kb region of the *F. tularensis* subsp. *tularensis* (strain Schu4) chromosome that encompasses the FPI. Bottom graph, G+C content of the FPI. The ORFs (arrows) from *pdpD* through *pdpA* are derived from the DNA sequence of *F. novicida* U112 strain and the remaining ORFs are derived from the Schu4 sequence. At the left end of the FPI are ORFs *tnpAB*, which show exact identity to genes encoding presumed transposases previously found in *F. tularensis*. The small opposing arrows indicate the approximate positions of 16-bp inverted repeats that have previously been shown to be associated with *tnpA*. The DNA sequences of the eight ORFs between *pdpB* and *pdpC* are highly conserved between *F. tularensis* subsp. *tularensis* and *novicida*. Arrows labeled 62 and 116 indicate the locations of the transposon insertions that originally indicated a cluster of virulence-associated genes; *rrsH* and *rrlH*, 16S and 23S rRNA genes, respectively; *fdn*, a subunit of formate dehydrogenase. The smaller ORFs are not drawn to scale. [94]

Chapter 2 Mutagenesis and Characterization of *pdpC*

2.1 Introduction

Our laboratory discovered the presence of a putative pathogenicity island in *Francisella* and have proven that the FPI is indeed functional by sequencing and analyses of the putative region and by deleting two of the encoded genes, *pdpA* and *pdpD*, which resulted in attenuation of growth in macrophages and virulence in a mouse model. Various other studies since the publication of our paper have shown other FPI genes to be required for bacterial replication in macrophages as well as virulence *in vivo*. Other studies have demonstrated that MglA, thought to be a global transcriptional regulator, positively regulates a majority of the FPI genes including *pdpD, iglABCD, pdpA and pdpB*, as well as other novel virulence-associated genes located outside the FPI. However, no mention has been made in any publication regarding *pdpC*.

Interests have re-emerged for the pathogen *Francisella tularensis* as it is classified by the U.S. CDC as a category A agent and a potential bioterrorist weapon, along with *Bacillus anthracis* and several other pathogens. In light of the discovery of a pathogenicity island in *Francisella*, the roles of such novel virulence or survival factors remain to be elucidated as relatively little is known about the genetic and molecular mechanisms of *Francisella* pathogenesis. *Francisella* can survive and replicate within macrophages *in vitro* and *in vivo* and this ability to replicate seems to be the primary

mechanism of pathogenesis. The exact function of the genes encoded on the FPI has yet to be determined.

The deduced amino acid sequence of PdpC has no definitive similarity to any known prokaryotic virulence protein and thus represents a novel factor. Its function in the pathogenesis of *Francisella* is unknown. In this study, mutations in *Francisella novicida pdpC* are demonstrated to attenuate both intramacrophage growth inside J774A.1 cells and virulence in chicken embryos. However, the same mutants show no growth defects when bone marrow-derived macrophages were used. The subcellular localization of PdpC in *F. novicida* will be examined to help predict a possible function for its role during *Francisella* infections.

2.2 Materials and Methods

Bacterial Strains and Plasmids

Francisella novicida (type strain U112; ATCC 15482) was used for all gene knockouts and virulence work in this study. *F. novicida* U112 and mutant strains were grown aerobically at 37°C in Trypticase soy broth supplemented with 0.1% cysteine (TSBC) or on Trypticase soy agar supplemented with 0.1% cysteine (TSAC) unless otherwise stated. Erythromycin (Em; 30 μg/mL) was added as needed. *Escherichia coli* DH5α was used for all molecular cloning and was cultured in Luria-Bertani broth (LB) with 250 μg/mL ampicillin (Ap) as needed. The pCR2.1-TOPO® vector (Invitrogen) was used to clone in PCR products. The low-copy-number plasmid pWSK29 was used to construct the clones for in-frame partial deletions of *pdpC*. All strains and plasmids used in this work are listed in **Table 2.1**.

Polymerase Chain Reaction (PCR) and Primer Design

PCR reactions were performed in volumes of 50 µL each. Amplification was carried out using Phusion High-Fidelity DNA Polymerase (New England Biolab) in a reaction containing nuclease-free water, 1X Phusion HF buffer, 200 µM dNTPs (Invitrogen), 0.5 µM each of the forward and reverse primers (Integrated DNA Technologies), 0.1 ng of template DNA, and 1.0 U of Phusion DNA Polymerase (NEB). PCR reactions were performed under the following cycling parameters: Initial denaturation at 98°C for 30 s; 35 cycles of 98°C for 30 s, 55°C for 30 s, 72°C for 2 min 30 s; final extension at 72°C for 10 min. All primers were designed based on the *F. novicida* U112 FPI sequence (GenBank accession no. AY293579) and are listed in **Table 2.2**.

Recombinant DNA Techniques

The blunt ended PCR products generated with Phusion DNA Polymerase were TA cloned into the pCR2.1-TOPO® vector by an initial clean-up with QIAquick® PCR Purification (QIAGEN) followed by the addition of 3'A-overhangs with *Taq* DNA Polymerase (NEB) at 72°C for 15 min in a reaction containing nuclease-free water, 1X PCR ThermoPol buffer (NEB), and 0.1 mM dATP (Invitrogen). TOPO ligation mixtures were electroporated into the *E. coli* DH5α strain using the Gene Pulser (Bio-Rad). Recombinant plasmids were purified using QIAprep® Spin Miniprep Kit (QIAGEN). Restriction endonucleases and T4 DNA Ligase (NEB) were used as per manufacturer's instructions. DNA fragments were purified from agarose gels using QIAquick® Gel Extraction Kits (QIAGEN). Unless otherwise stated, all cloning procedures including

plasmid DNA purification, digestion with restriction endonucleases, ligations, and agarose gel electrophoresis were performed according to the protocols described in *Molecular Cloning* [84].

Chemical Transformation of *F. novicida* U112

F. novicida U112 was grown in TSBC supplemented with 0.4% glucose until the exponential phase of growth. Cells were gently pelleted at 5,000 x *g* in a Beckman JA-20 rotor and resuspended in *Francisella* transformation buffer (FTB) [78] at room temperature (RT). Plasmid or linear DNA up to 100 µL was added to 200 µL of resuspended cells. The mixture was incubated at 37°C with slow shaking (95 RPM) for 1 hour. One mL of TSBC supplemented with 0.4% glucose was added and the mixture was further incubated at 37°C for 3 - 4 hours with vigorous shaking (220 RPM). Transformants were selected on TSAC with antibiotics as needed for 24 - 48 hours.

Conjugation

The triparental mating method for conjugation was used to introduce the pMMB207::*pdpC* complement into the erythromycin allelic replacement mutant *pdpC*::Em^R. One mL of each of the recipient strain *pdpC*::Em^R, the donor *E. coli* strain harbouring the pMMB207::*pdpC* plasmid, and the helper strain *E. coli* S17-1 which carries the *mob* gene used in broad host range conjugative transfer of mobilisable plasmids was grown overnight. The cultures were centrifuged in a microfuge and the cells were gently resuspended in a volume of 0.2 mL with LB broth and mixed in a ratio of 1:1:1. The mixture was spotted onto LB agar lacking any antibiotic and incubated

overnight at room temperature. The mating mixture was recovered from the agar by adding 1 ml of TSBC and scraping the cell mixture from the plate. The broth was collected and diluted appropriately before plating on TSAC containing kanamycin (15 μg/mL) and polymyxin B (30 μg/mL). The plates were incubated at 37°C for 24 – 48 h.

DNA Sequencing and Analysis

PCR products amplified with primers designed for the regions of the *F. novicida* chromosome containing the partial deletions of *pdpC* were purified using the QIAquick PCR purification kit to remove excess primers, dNTPs, salts, and Phusion DNA Polymerase. Sequencing of these amplicons was performed by the CMMT / CFRI DNA Sequencing Core Facility at the University of British Columbia. The sequences are included in **Appendix 2**. Comparisons of sequences obtained from sequencing to the PdpC sequence of the FPI were done using on-line BLAST (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Hydrophobicity analysis of PdpC was performed using the Vector NTI Advance 9.0 software (Invitrogen).

SDS-PAGE and Immunoblotting

The protein concentrations of samples were determined by the MCA assay (Pierce) and the amount of protein loaded was normalized to 5 μg / lane. Samples of whole cell lysates were mixed with SDS sample buffer containing 62.5 mM Tris (pH 6.8), 1% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue, and 10% glycerol; and boiled for 10 min prior to electrophoresis. SDS-PAGE was carried out according to the method of Laemmli [75] and samples were electrophoresed through 8 % SDS-PAGE gels and

transferred to Immobilon-FL membrane (Millipore) or Pure Nitrocellulose membrane (0.45μm) (Bio-Rad). Membranes were blocked with 5% skim milk (Difco) in PBS (0.2M NaCl, 4.2 mM KCl, 12.7 mM Na_2HPO_4, 2.3 mM KH_2PO_4). Immunoblots were detected using rabbit polyclonal immune serum raised against a PdpC peptide (CDDINVDRENRRELVAK) at a dilution of 1:10,000 obtained from J. Celli at the Rocky Mountain Laboratories, NIAID/NIH. After incubation overnight, blots were washed with PBS containing 0.1% Tween-20 for 15min three times and subsequently probed with IRDye800DX-conjugated goat anti-rabbit Immunoglobulin G (Rockland Immunochemicals, Gilbertsville, Pa.). The immunoblots were visualized using the LiCor Odyssey imaging system.

Macrophage Infection Assay

Macrophages were infected with wildtype U112, JL0, GB2 or *pdpC* mutants. Bone marrow-derived macrophages (BMDMs) were isolated from femurs of healthy BALB/c male mice and cultivated in 96-well cell culture plates at 4 x 10^5 cells / well (Costar, Corning, NY) for one week in complete Dulbecco's Modified Eagle Medium (cDMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% MEM non-essential amino acids, 1% HEPES buffer solution, and 10% conditioned L929 supernatant. BMDMs were infected with *F. novicida* strains at a multiplicity of infection (MOI) of 20:1 (bacterium-to-macrophage). Infected monolayers were incubated for 1 hr in cDMEM to allow for phagocytosis to occur, washed three times in Dulbecco's Phosphate Buffered Saline (DPBS), and incubated at 37°C in 5% CO_2. Murine macrophage-like J774A.1 cells were seeded into 96-well cell culture plates at 2 x 10^5 cells / well and

allowed to adhere overnight. Similarly, J774A.1 monolayers were infected at a MOI of 20:1 and incubated for 1 hr in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % FBS. To determine bacterial replication, infected macrophages were lysed in 0.1 % deoxycholate at 0, 24, and 48 h post infection. The lysates were serially diluted in DPBS containing 0.1 % gelatin and plated on TSAC. It has been previously demonstrated that *F. tularensis* extracellular growth in standard DMEM is not supported which makes the macrophage infection assay an appropriate determination of intracellular growth.

Chicken Embryo Infection

Fertilized White Leghorn chicken eggs were obtained from the University of Alberta Poultry Research Station. Seven-day old chicken embryos were injected with various doses of 100 µL of *F. novicida* strains diluted in DPBS just beneath the chorioallantoic membrane as previously described [98]. The chicken embryos were monitored for death for 6 days.

Subcellular Fractionation

F. novicida U112 (100 mL) was grown overnight at 37°C, pelleted, and resuspended in 30 mL of PBS. Cells were mechanically sheared by three passages through a French Pressure Cell (American Instruments Co., Silver Spring, MD) at 1250 PSI. The pressed cells were centrifuged at 10,000 x *g* for 15 min at 4°C to remove unbroken cells. A sample of the supernatant was taken as the total protein fraction. The lysate was further separated into the membrane fraction and the soluble protein fraction by ultracentrifugation for 1 hr at 100,000 x *g* at 4°C in a Beckman L8-70, rotor Type 45 Ti.

The membrane pellet was resuspended in 2 mL of 1% *N*-lauroyl sarcosine (Sigma). The sarkosyl soluble (inner membrane) and insoluble (outer membrane) fractions were separated by ultracentrifugation for 1 hr at 100,000 x *g* at 4°C in a Beckman TLA-100.3 micro-ultracentrifuge.

The osmotic shock procedure for isolation of periplasmic proteins was performed as previously described [99]. Briefly, a 10 mL culture of U112 was grown overnight at 37°C. The cells were pelleted at 5,000 x *g* for 10 min at 4°C. The pellet was resuspended in 20 mL 10 mM Tris-HCl (pH 7.3) + 4 mM EDTA at room temperature. An equal volume of 40 % sucrose in 10 mM Tris-HCl (pH 7.3) was added. The mixture was stirred at room temperature for 10 min before the plasmolyzed cells were pelleted by centrifugation at 5,000 x *g* for 10 min. The supernatant was collected as the plasmolysate fraction. The pellet was quickly resuspended in 10 mL of ice cold ddH_2O containing 1 mM $MgCl_2$ and protease inhibitor. The resuspension was kept on ice for 10 min followed by centrifugation at 5,000 x *g* for 10 min. The supernatant was collected as the shockate containing periplasmic proteins.

NADH Oxidase Assay

The enzymatic assay was performed to determine the activity of the inner membrane-associated enzyme NADH oxidase as described by Osborn *et al.* [104]. In a volume of 1 mL, incubation mixtures contained 50 mM Tris-HCl (pH 7.5), 0.12 mM NADH, 0.2 mM dithiothreitol, and the protein fraction (10 µg of protein). The rate of decrease in absorbance at 340 nm was measured at room temperature. The cross-contamination between the different cellular compartments from the subcellular fractionation was

determined as a measure of the relative enzyme activity per mg of protein in each of the fractions.

2.3 Results

2.3.1 Allelic gene replacement of *pdpC*

The Phusion DNA Polymerase-amplified PCR products of the two 1.5 kb flanking regions of *pdpC* using the primer pairs pdpCL-L/R and pdpCR-L/R were cloned into pCR2.1-TOPO® vector followed by restriction enzyme digestion with *Xho*I. Similarly, the TOPO clone of the erythromycin cassette, amplified using EmXhoF/R, was digested with *Xho*I. The three digested constructs were ligated in an equal molar ratio. The ligation mixture was transformed directly into wildtype U112 and the transformants were selected for erythromycin resistance. One of the transformants was chosen for further virulence studies (**Figure 2.1**). The allelic gene replacement of *pdpC* is illustrated in **Figure 2.2**.

2.3.2 Complementation of *pdpC*::Em^R mutant

To complement the erythromycin allelic replacement mutant of *pdpC*, *in trans* complementation was carried out. The complement was constructed by cloning amino acids 1-1218 of PdpC as well as 1.6 kb of the gene's upstream region into the pMMB207 vector containing a kanamycin resistance gene driven by the *Francisella* Omp26 promoter (performed by E. Nix of Dr. Nano's Laboratory). The outer membrane protein promoter was necessary for the strong expression of the kanamycin resistance gene. The schematic for the complement construction is illustrated in **Figure 2.3**. The complement

was introduced into the erythromycin allelic replacement mutant of *pdpC* by the triparental mating conjugative procedure. Conjugates resistant to polymyxin B, kanamycin, and erythromycin were selected for further analysis.

2.3.3 In-frame partial deletions of *pdpC*

To further investigate the role of PdpC in the virulence of macrophages, in-frame partial deletion mutants of *pdpC* were constructed (**Figure 2.4**). There has been no previous literature published on PdpC hence we had to devise a mutagenesis approach based on computer-generated information. Hydrophilic and hydrophobic regions of approximately 35 - 65 amino acids were identified using a hydrophobicity plot (**Figure 2.5**) generated with Vector NTI Advance 9.0 (Invitrogen), and these were knocked out by a Campbell-type double cross-over homologous recombination event. Regions flanking each deletion were amplified using primer pairs consisting of an internal deletion primer and an outer primer that is 2 kb either upstream or downstream of *pdpC*. The flanking regions were joined by a short annealing and extension step. Nested primers designed with *Xho*I linkers 1 kb upstream and downstream of *pdpC* were then used to amplify the partially deleted gene. The amplified PCR products were cloned into the low-copy-number plasmid pWSK29 and subsequently excised by digestion with restriction enzyme *Xho*I and ligated to an erythromycin resistance-*sacB* cassette. The ligation mixture was used to chemically transform the *F. novicida* JL0 strain. JL0 (created by J. Ludu of Dr. Nano's Laboratory) containing a deletion in the sucrose hydrolase gene is a derivative of the wildtype U112 strain and behaves like wildtype in virulence assays (data not shown). When *sacB* is expressed, this derivative strain is sensitive to sucrose and thus sacB acts as

a counter-selective marker. Erythromycin resistant transformants were grown and plated on TSAC containing 10% sucrose repeatedly to try to resolve co-integrates carrying the wildtype *pdpC* gene and the partially deleted gene in its chromosome. Colonies exhibiting sucrose resistance were examined by PCR for partial deletion of the intended region (**Figure 2.6**) and subsequently, sequencing of an internal *pdpC* region containing the deletion was performed to verify that an in-frame deletion was constructed (**Appendix 2**). Western immunoblotting analysis confirmed the expression of PdpC in the partial deletion mutants and the complemented erythromycin allelic replacement mutant, *pdpC*::Em^R(pEN1comp) (**Figure 2.7**).

2.3.4 Macrophage infection of *pdpC* mutants

The laboratory's previous publication on the FPI demonstrated that PdpA and PdpD are required for the growth of *F. tularensis* subsp. *novicida* in macrophages and virulence in mice; however, the role of PdpC has not been examined. In order to study the role of PdpC, *in vitro* studies in bone marrow-derived macrophages from BALB/c mice and the murine macrophage-like cell line J774A.1 were performed. In BMDMs, intracellular bacterial replication of JL0, *pdpC*::Em^R, and its complement increased about 2.5 logs by 48 hr post infection, whereas GB2, a *mglA* mutant defective in intramacrophage growth, failed to replicate (**Figure 2.8A**). Partial deletion mutants of PdpC in regions 9 – 12 exhibited the same pattern of intramacrophage growth as JL0 (**Figure 2.8B**). In contrast, bacterial replication of the erythromycin allelic replacement mutant *pdpC*::Em^R in J774A.1 cells was attenuated by 48 hr post infection in comparison to JL0 which increased 3 logs (**Figure 2.9A**). Complementation of *pdpC*::Em^R partially restored

intramacrophage growth capability inside J774A.1 (**Figure 2.9A**). Intracellular replication of all four of the partial deletion mutants was only very slightly attenuated (**Figure 2.9B**).

2.3.5 Chicken embryo infection of *pdpC* mutants

The laboratory's recently published paper on the virulence of *Francisella* sp. in chicken embryos demonstrated that the use of this infection model is inexpensive yet reproducible. The virulence of *pdpC*::Em^R and its complement, and the *pdpC* partial deletion mutants was tested in 7 day-old chicken embryos. When *pdpC*::Em^R was used for infection, it caused low mortality in comparison to JL0 (**Figure 2.10**). JL0 caused 100% mortality at day 4 with an infection dose of 2,900 CFU, whereas *pdpC*::Em^R caused 43% mortality at day 6 with an infection dose of 28,000 CFU (**Figure 2.10**). The *pdpC*::Em^R(pEN1comp) complement exhibited greater virulence than the allelic replacement mutant and caused 71% mortality at day 6 with an infection dose of 26,000 CFU (**Figure 2.10**). This is approximately 10,000-fold less virulent than JL0. GB2, which is completely incapable of intracellular replication inside BMDMs and J774A.1, caused only 14% mortality at day 6 with an infection dose of 11,000 CFU (**Figure 2.10**). The *pdpC* partial deletion mutants were about 1,000-fold less virulent than JL0 but all were still able to cause 100% lethality at day 6 with infection doses of about 30,000 CFU (**Figure 2.11**).

2.3.6 Expression of PdpC in broth culture of *F. novicida*

The growth of JL0 in TSBC was briefly examined to determine the optimal growth condition for its expression of PdpC. By western immunoblotting, it was determined that PdpC is optimally expressed in TSBC when *F. tularensis* subsp. *novicida* is grown to an optical density at 600 nm of 1.0 – 1.4 which corresponds to the late exponential phase of growth (**Figure 2.12**).

2.3.7 Subcellular localization of PdpC

PdpC of *F. novicida* U112 was found to be localized mainly in the sarkosyl soluble fraction containing largely inner membrane proteins (**Figure 2.13**). As a control the immunoblot was probed with anti-IglA antibody as IglA have been found to be localized to the cytoplasm [31]. It was observed that there is slight contamination of the cytoplasmic fraction into the inner membrane fraction (**Figure 2.13**) and measurement of the NADH oxidase activity, a marker for the inner membrane, indicates that there is approximately 29% cross-contamination of the inner membrane fraction into the cytoplasmic fraction. When an osmotic shock procedure was performed, PdpC was observed largely in the plasmolysate fraction containing cytoplasmic contents. However, it can not be excluded that a small fraction of PdpC might be localized to the periplasm as a faint band in the immunoblot can be seen in that fraction in **Figure 2.13**.

2.3.8 Bioinformatics

The Max-Planck Institute for Developmental Biology Bioinformatics Toolkit (http://toolkit.tuebingen.mpg.de/) was used to analyze PdpC. HHpred is a sensitive protein homology detection and structure prediction program. The Hidden Markov

Model (HMM) profile from the PdpC sequence was compared with a database of HMMs representing annotated protein families but yielded no significant results. SignalP-HMM which predicts the presence and localization of signal peptide cleavage sites was used and the results indicated that there is zero signal peptide probability within the PdpC amino acid sequence. Its prediction for PdpC is a non-secretory protein. TMHMM which predicts transmembrane helices and their topologies using the Hidden Markov Model predicted zero transmembrane helices within PdpC. Lastly, PSort was used to make predictions on the subcellular localization based on sorting signal motifs and correlative sequence features. The results predict the final localization of PdpC to be cytoplasmic with a final score of 8.96. The other scores were 0.51 for cytoplasmic membrane, 0.26 for periplasmic, 0.01 for outer membrane, and 0.26 for extracellular.

2.4 Discussion

The *pdpC* gene in *F. novicida* sequenced by us was initially reported to be a 3657 bp open reading frame located within the putative *pdpABC* operon, and it was predicted to encode a putative protein of 1218 amino acids. Recent sequence corrections suggest that *pdpC* encodes a 3978 bp open reading frame that would produce a protein with a molecular mass of 155 kDa. To determine whether a 155 kDa PdpC protein is produced by this open reading frame, western immunoblot analysis was performed and a product with an estimated molecular mass of 155 kDa was identified. The antibody used in the western blot was raised inside a rabbit against a synthetic peptide of PdpC. The reactive band in the immunoblots at approximately 175 kDa could not be explained, other than it

might be a non-specific protein antigen contamination during the antibody production process.

To determine whether the band of 155 kDa molecular mass was encoded by the *pdpC* gene in *F. novicida*, an allelic replacement mutant using an erythromycin cassette was constructed. In previous mutagenesis studies in our lab, the mini-transposon Tn*Max2* was used to disrupt genes by transposon insertion. However, stability issues with such insertions were noticed and genetic tools were created to help construct stable mutants using recombinant DNA approaches. The 155 kDa protein identified by the anti-PdpC antibody was not found in whole cell lysate prepared from the erythromycin replacement mutant of PdpC, however the non-specific 175 kDa was still present. These data demonstrate that the *pdpC* gene encodes the 155 kDa polypeptide indicating that the most recent *pdpC* sequence distributed by the University of Washington Genome Center is correct.

Previous studies suggest that the other *pdp* genes, *pdpABD,* are essential for *Francisella* pathogenesis. To determine the role of *pdpC* during macrophage infections, the capacity of the erythromycin mutant to grow intracellularly was measured. To complement the allelic replacement mutant, a fragment containing 3657 bp of the *pdpC* gene corresponding to our original sequence was amplified from the chromosome of *F. novicida* U112. The plasmid containing this amplified DNA fragment contains the entire *pdpC* gene transcribed by the endogenous *Francisella* promoter of the gene and was transferred by conjugation into the *pdpC* mutant strain.

The ability of the erythromycin replacement mutant and its complement to replicate in the mammalian phagocytic cells derived from BALB/c mice bone marrow was

investigated by measuring the CFUs recovered from infected macrophages over 48 h. The ability of the mutant to replicate inside BMDMs was unaltered and its growth paralleled that of the wildtype strain. To test the replication ability of the Em replacement mutant in another cell line, J774A.1 was used. The mutant was capable of replication but did not parallel that of wildtype growth. In contrast to previous allelic replacement mutants constructed using the erythromycin cassette, the intramacrophage growth of the *pdpC* mutant was not as severely attenuated as the *pdpA* and *pdpD* mutants. The complement was only able to partially restore wildtype phenotype in the *pdpC* mutant. These data demonstrate that *pdpC* is somewhat essential for intracellular growth of *F. novicida* within some macrophage cell lines. Interestingly, our collaborator J. Celli at the Rocky Mountain Laboratories in Montana observed that the *pdpC* Em replacement mutant was able to escape the *Francisella*-containing phagosome inside infected macrophages to half the extent of the wildtype strain (data not shown) which suggests that *pdpC* may not be required by *Francisella* for phagosomal escape and the subsequent replication inside the host cytosol.

In order to determine regions of *pdpC* required for intracellular growth inside macrophages, in-frame partial deletion mutants were constructed in the C-terminus half of *pdpC*. The in-frame partial deletion mutants gave similar results as the Em replacement mutant for the macrophage infection assays. The chicken embryo model for determining virulence was proven to be inexpensive and easy to manipulate in comparison to the mouse model and reasonable reproducibility could be obtained. These partial deletion mutants seemed to be less attenuated in virulence than the Em replacement mutant. The use of fibroblasts isolated from chicken embryos to study the

expression of PdpC and its effect on the host cell will be described in the following chapter.

To facilitate the study of the role of PdpC in *Francisella* infections, an interpretation can be drawn from the subcellular localization study performed. The PdpC protein was largely found in the cytoplasm where it might be synthesized, but may reside transiently in the periplasmic space. As observed by immunoblotting, the amount of PdpC localized to the inner membrane, despite some level of cross-contamination during the fractionation process, can be perhaps attributed to its potential translocation through the membrane by the help of a multi-subunit secretion complex to act as a putative effector virulence protein. Alternatively, it could not be excluded that PdpC itself may participate in the assembly of such a secretion complex, but this postulation is unlikely as bioinformatic analyses indicated a low probability for its localization within the inner or outer membrane.

Table 2.1 Bacterial strains and plasmids used in the *pdpC* mutagenesis study

Strains	Relevant characteristics	Source or Reference
Escherichia coli		
DH5α	*sup*E44 *lac*U169 (Φ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Invitrogen
S17-1	Conjugation helper strain	Simon *et al.* 1983 [126]
Francisella novicida		
U112	Wildtype *F. novicida* (Ap^R)	ATCC 15482
JL0	U112 Δsucrose hydrolase strain	Laboratory strain
pdpC::Em^R	U112, erythromycin replacement mutant (Em^R)	This study
pdpC::Em^R (pEN1comp)	*in trans* complementation of *pdpC*::Em^R	This study
Plasmids		
pCR2.1 TOPO	Cloning vector, Ap^R Km^R	Invitrogen
pWSK29	Low-copy-number, Ap^R, *lacZ*α gene	Wang and Kushner, 1991 [138]
pMMB207	Broad host range, Cm^R	Morales *et al.* 1991 [91]
pEN1comp	*in trans* complementation, Km^R, *Francisella omp*26 *promoter*, contains *pdpC* (3657 bp)	This study

Table 2.2 Primers used in mutant construction, complementation, and sequencing

Gene	Primer	Sequence 5' -> 3'	Tm
Allelic Replacement Primers			
downstream flank of *pdpC*	pdpCL-L	GCG GCC GCT GCT GAT AGA TTG TTA ACT CTT TGT G	64.7
	pdpCL-R	CTC GAG TTG AAG AAT TAA AAC CAA AAT GTA GTG	56.8
upstream flank of *pdpC*	pdpCR-L	CTC GAG TAT TTG TCG TTC ATA TGT ACC TCC TT	59.1
	pdpCR-R	GCG GCC GCA ATG ATA CGT ATT CCG ATA AAG AAG C	64.6
Erythromycin	EmXhoL	CTC GAG GGG TTA TAA TGA ACG AGA AAA	56.5
	EmXhoR	CTC GAG AAC AAG TTA AGG GGA TGC AGT	60.2
Complement Primers			
pdpC	UF_C_xhoF	CTC GAG CTT AGT CAC TAT GGA TGC	56.9
	Pdpc-r xho	CTC GAG TAA CTC GTT GCT TAG CTG GAA TT	60.0
In-frame Partial Deletion Primers			
*pdpC*Δ 9	9F	CGA ATG GAG TTT GAA TTA TTT AGC CTT AAT CTA ACA	57.4
	9R	TGT TAG ATT AAG GCT AAA TAA TTC AAA CTC CAT TCG	57.4
*pdpC*Δ 10	10F	GGA AAT TTA GAT CAA GCA GAG GTA AAG TAT CC	57.1
	10R	GGA TAC TTT ACC TCT GCT TGA TCT AAA TTT CC	57.1
*pdpC*Δ 11	11F	CAG ATA GAA AAA GAT TTC AAC ACT TTT TAT AAA ATA AAA GAT	55.4
	11R	ATC TTT TAT TTT ATA AAA AGT GTT GAA ATC TTT TTC TAT CTG	55.4
*pdpC*Δ 12	12F	CAA GAA GCT GCT AAT ACT TCA AAA CCA GTA ATA G	57.5
	12R	CTA TTA CTG GTT TTG AAG TAT TAG CAG CTT CTT G	57.5
2kb up/dn stream of *pdpC*	CPD up flank	GGC GTT CCT TTA GGA CTG TTT	55.2
	CPD dn flank	GAA AGG TTA AGC ACC GCA AG	54.5
1kb up/dn stream of *pdpC*	N1.0 CPDup Xho	CCG CTC GAG TTG AGA GAA TGC CCG AAA AT	63.8
	N1.0 CPDdn Xho	CCG CTC GAG GGA TAG TAG TGC GGT TTT	63.6
Partial Sequencing Primers			
*pdpC*Δ 9-10	Myc_C6F	GAG GTA CCC AAG GCG ATA TTA ATC CAA A	58.4
	Myc_C10R	CAC CAT GGA ATT AGG TGT TGC GAA ACT AT	59.1
*pdpC*Δ 11-12	Myc_C9F	GAG GTA CCT TTG CTA GGC CTG TAT TTG GA	61.3
	Myc 110R	CAT GCC ATG GTT GTA TTA CTA AAT GTT TGT TGG AAC G	61.0
*pdpC*Δ 9	Scr_9F	CCT GGC TTC TTG AGC TCT GT	57.1
	Scr_9R	TGC CTG AGT CAT TGC TTG AT	54.5
*pdpC*Δ 10	Scr_10F	TTG CCT CAA CAA CTG CTT TG	54.2
	Scr_10R	TCC AAA TAC AGG CCT AGC AA	53.8
*pdpC*Δ 11	Scr_11F	CAG TGG CTA TGC TGC GAT TA	55.1
	Scr_11R	TGT GAT AGC CCA ACC CAT ATC	54.3
*pdpC*Δ 12	Scr_12F	GGG TTG GGC TAT CAC ATC AA	54.9
	Scr_12R	GGA GAT AAG GAT AAG TTT TGA ACA TGA	53.2

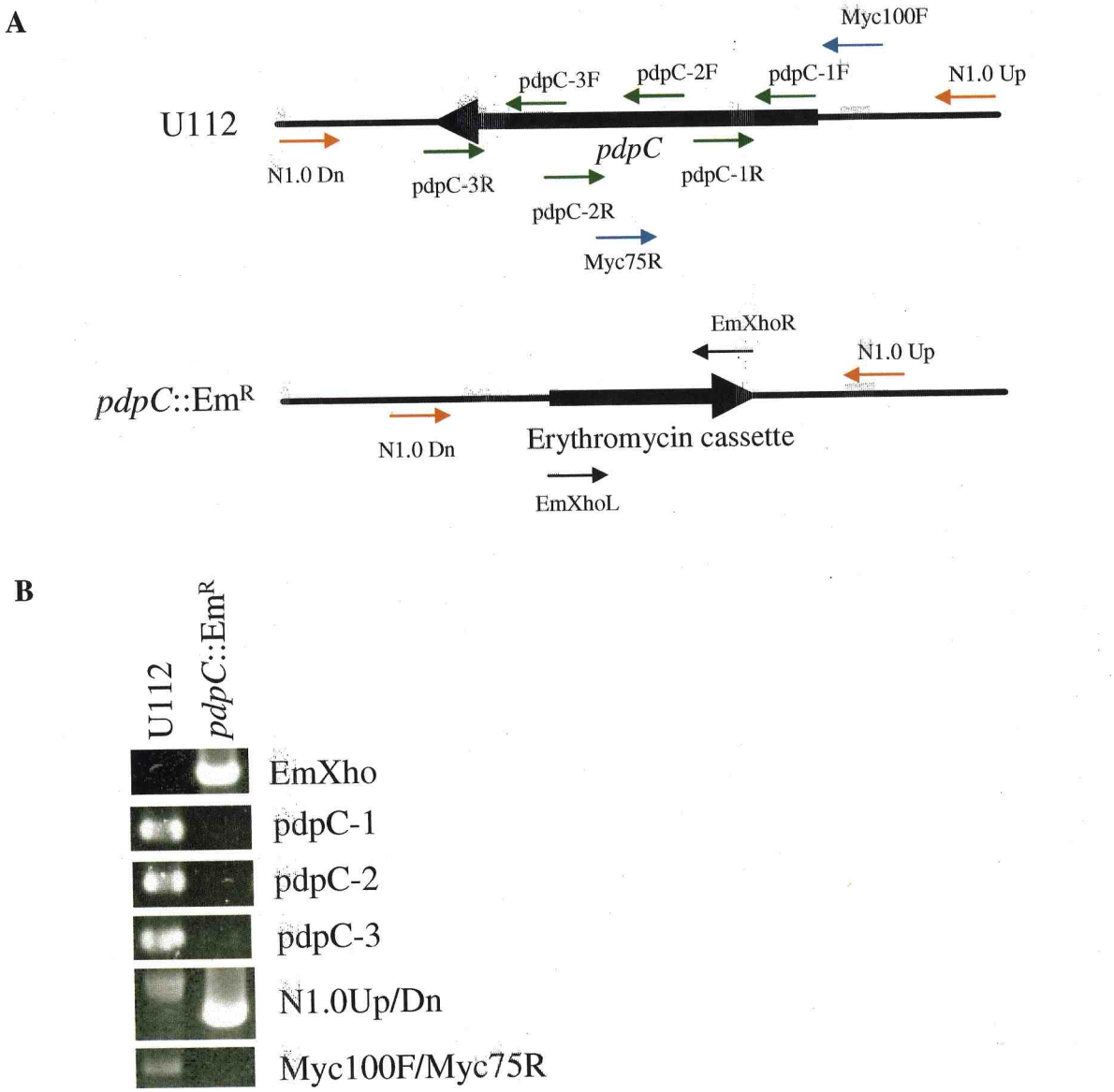


Figure 2.1 Agarose gel analysis of the *pdpC* allelic replacement mutant. (**A**) A map indicating the regions amplified by PCR. The broad arrow represents the *pdpC* gene in U112 and the erythromycin cassette in *pdpC*::Em^R; locations of the primers are indicated by small arrows. (**B**) Primers EmXhoL/R were used to show the presence of the erythromycin cassette in *pdpC*::Em^R. Three different pairs of internal primers were used to confirm the absence of *pdpC* in *pdpC*::Em^R. Primers N1.0Up/Dn were used to indicate a smaller amplicon size in *pdpC*::Em^R than in U112 from the replacement of *pdpC* by the Em cassette. An outer and internal primer pair Myc100F/Myc75R generated an amplicon in the U112 wildtype which was absent in *pdpC*::Em^R.

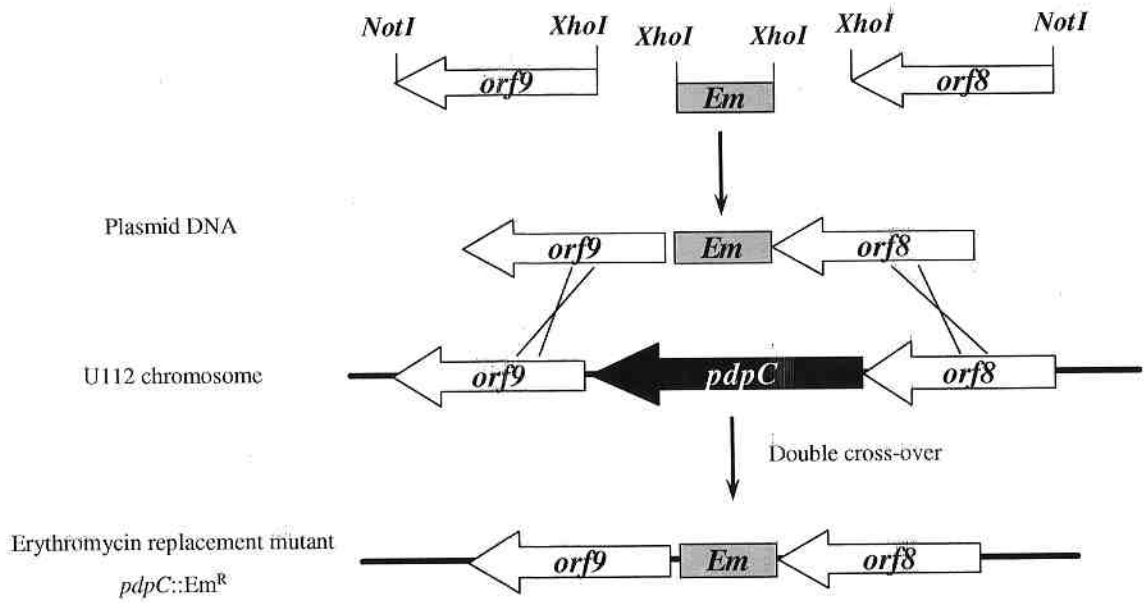


Figure 2.2 Allelic gene replacement. Schematic chart showing the recombinant constructs used to make the *pdpC*::Em^R allelic replacement mutant. The Em cassette was ligated with the two flanking regions of *pdpC*. The plasmid DNA construct was chemically transformed into U112 and via allelic exchange, the Em cassette replaced the *pdpC* gene.

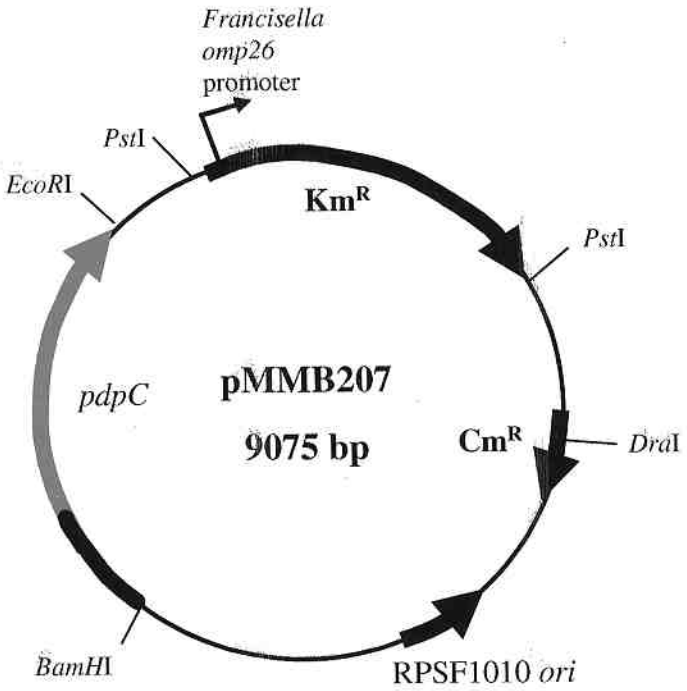


Figure 2.3 Cloning scheme in the construction of pEN1comp plasmid for *pdpC*::Em^R complementation. The chloramphenicol resistance gene (Cm^R) was truncated by digestion with *Dra*I and subsequent religation. The *Francisella omp*26 promoter- kanamycin gene (Km^R) was cloned into pMMB207 at *Pst*I. *pdpC* with 1.6 kb of its upstream sequence was directionally cloned as a *BamH*I – *EcoR*I fragment. The resulting complementation plasmid pEN1comp is Km^R.

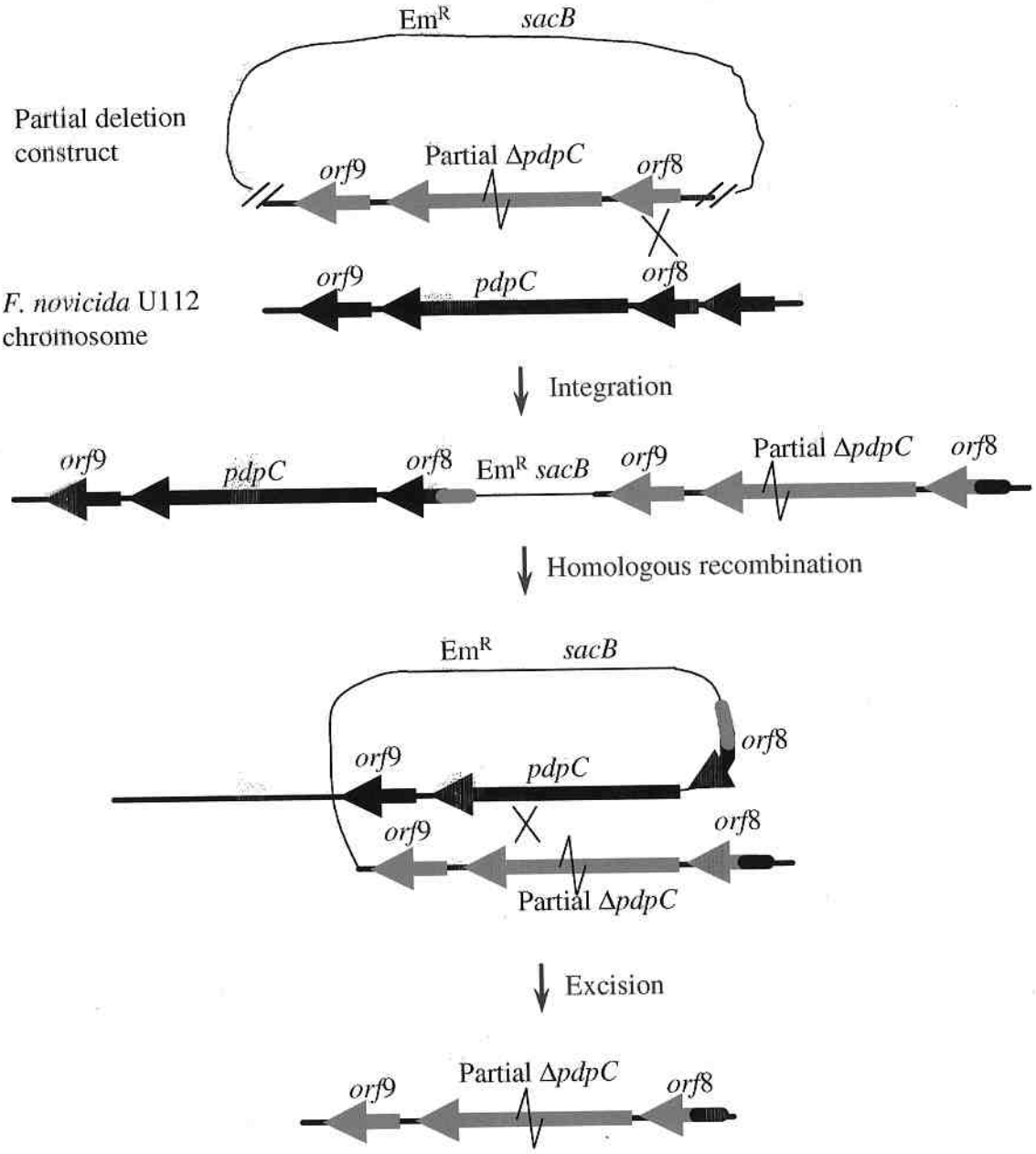
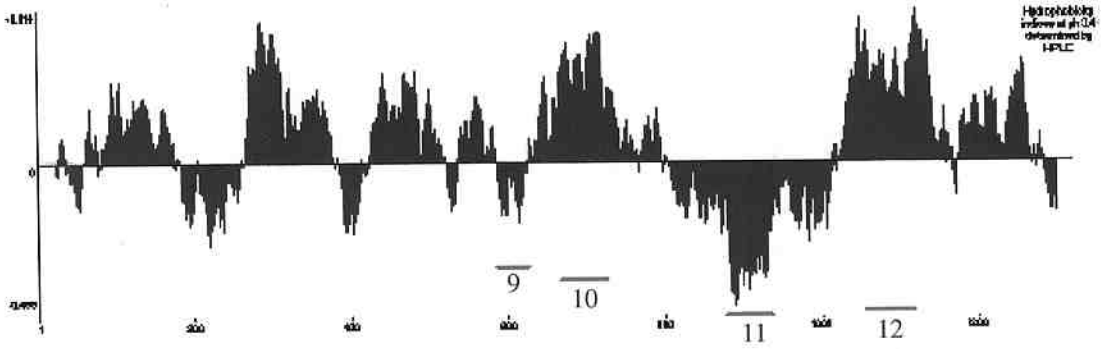


Figure 2.4 Partial deletion mutagenesis of *pdpC* by a Campbell-type double cross-over event. A schematic of the steps used to construct the four partial deletion mutants *pdpC*Δ9-12. *pdpC* was amplified using the partial deletion primers and was ligated to an Em^R-*sacB* cassette. After homologous recombination, the construct is integrated into the U112 chromosome. Plating on sucrose selected for strains that had excised out the *sacB* gene resulting in a partial deletion mutant of *pdpC*.



*pdpC*Δ9	aaΔ 589-625
*pdpC*Δ10	aaΔ 665-723
*pdpC*Δ11	aaΔ 880-942
*pdpC*Δ12	aaΔ 1053-1109

Figure 2.5 A Hydrophobicity plot analysis of PdpC generated by the Vector NTI Advance 9.0 software (Invitrogen). Regions of PdpC chosen for partial deletion are mapped to scale and the amino acids deleted are listed.

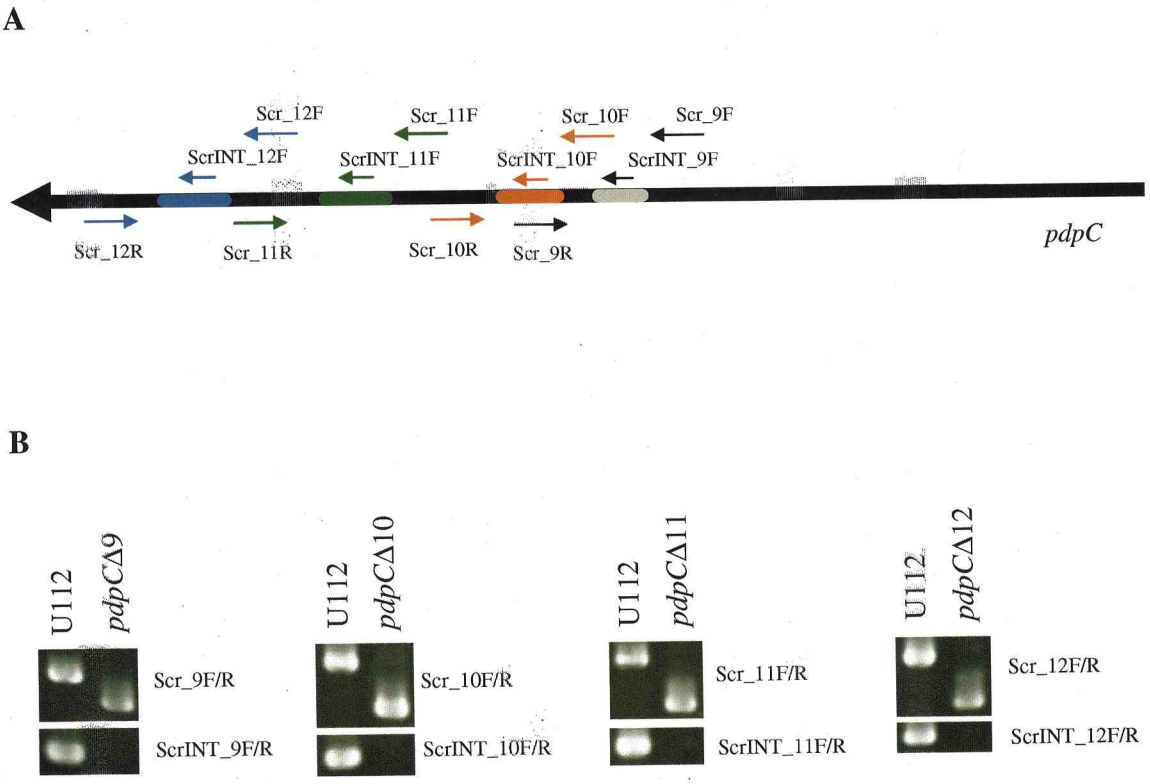


Figure 2.6 Agarose gel analysis of *pdpC* partial deletion mutants. (**A**) A map indicating the regions amplified by PCR. The broad arrow represents the *pdpC* gene in U112 and the regions in color indicate the *pdpC* deleted in each of the four partial deletion mutants. The locations of the primers are indicated by small arrows. (**B**) Screening primers Scr_F/R flanking the deleted regions were used to indicate a smaller amplicon size in the mutants than in U112. Primer pairs consisting of a flanking primer and an internal primer of the deleted region were used to show the absence of an amplicon in the partial deletion mutant.

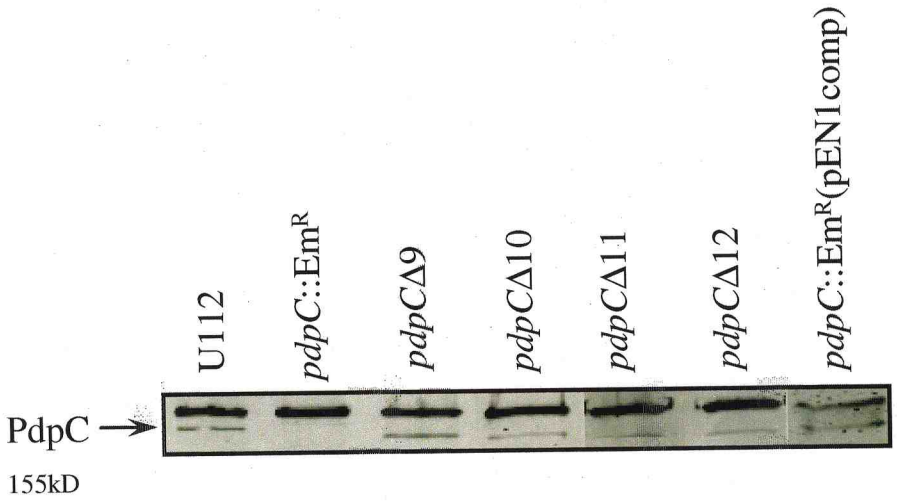
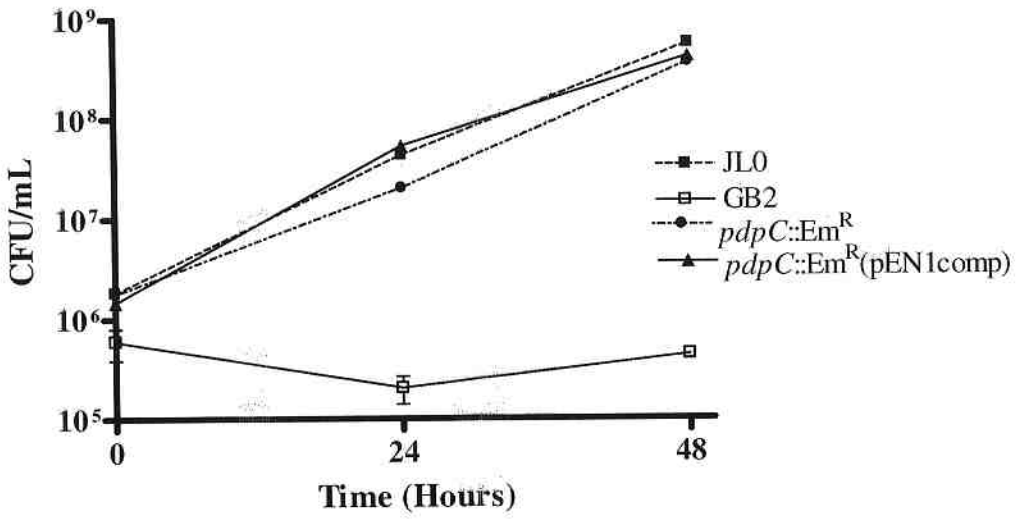


Figure 2.7 Western immunoblot analysis of *pdpC* mutants and the complement. The expression of wildtype PdpC was detected at ca. 155 kDa. The partially deleted PdpC in each of the partial deletion mutants was expressed at a slightly lower molecular weight. Expression of PdpC was detected in the *pdpC*::Em^R(pEN1comp) strain complemented *in trans*. The western blot shows the lack of a reactive 155 kDa protein in the *pdpC*::Em^R strain.

A



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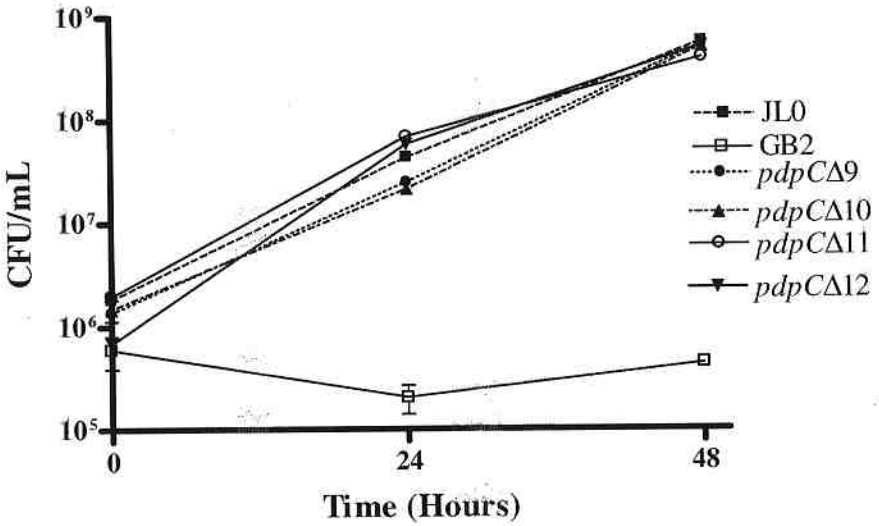
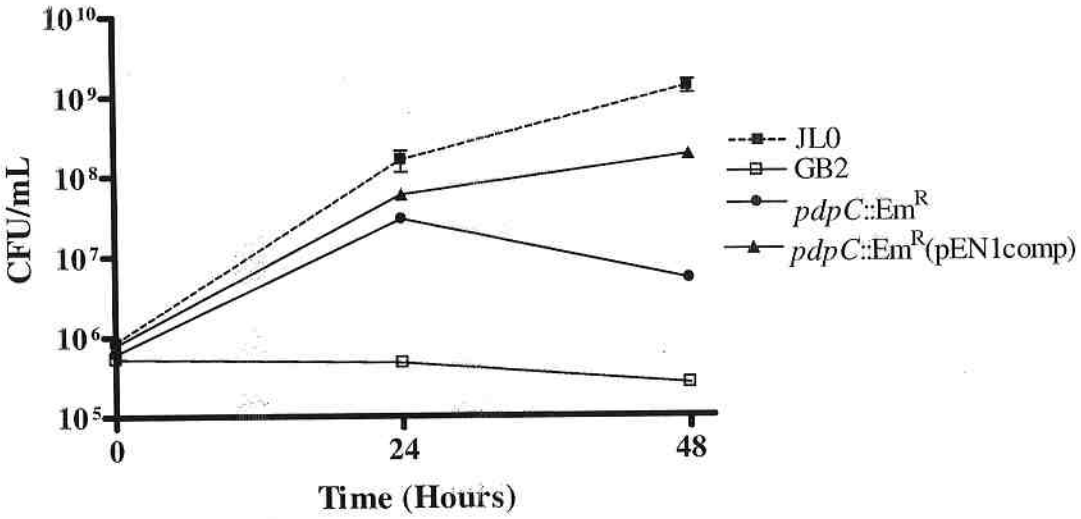


Figure 2.8 Growth of *F. novicida pdpC* mutants and control strains in bone marrow-derived macrophages from BALB/c mice. BMDMs infected with *Francisella* were lysed at 0, 24, and 48 h post infection to determine the number of viable bacteria (CFU) on agar medium. Standard errors are shown by bars. (**A**) *pdpC*::Em^R and *pdpC*::Em^R(pEN1comp) complemented strain with control strains, JL0 and GB2. (**B**) Partial deletion mutants of *pdpC* with control strains.

A



B

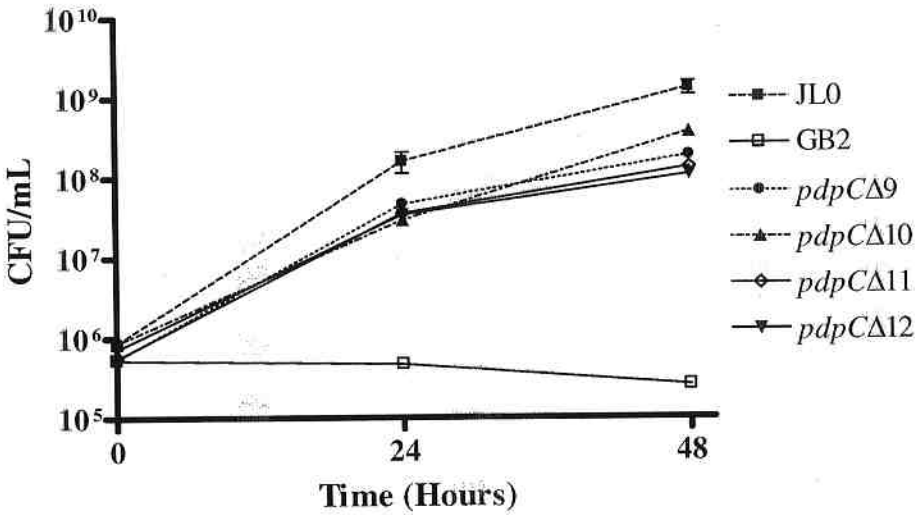


Figure 2.9 Growth of *F. novicida pdpC* mutants and control strains in the murine macrophage-like J774A.1 cells. J774A.1 cells infected with *Francisella* were lysed at 0, 24, and 48 h post infection to determine the number of viable bacteria (CFU) on agar medium. Standard errors are shown by bars. (**A**) *pdpC*::Em^{R} and *pdpC*::Em^{R}(pEN1comp) complemented strain with control strains, JL0 and GB2. (**B**) Partial deletion mutants of *pdpC* with control strains.

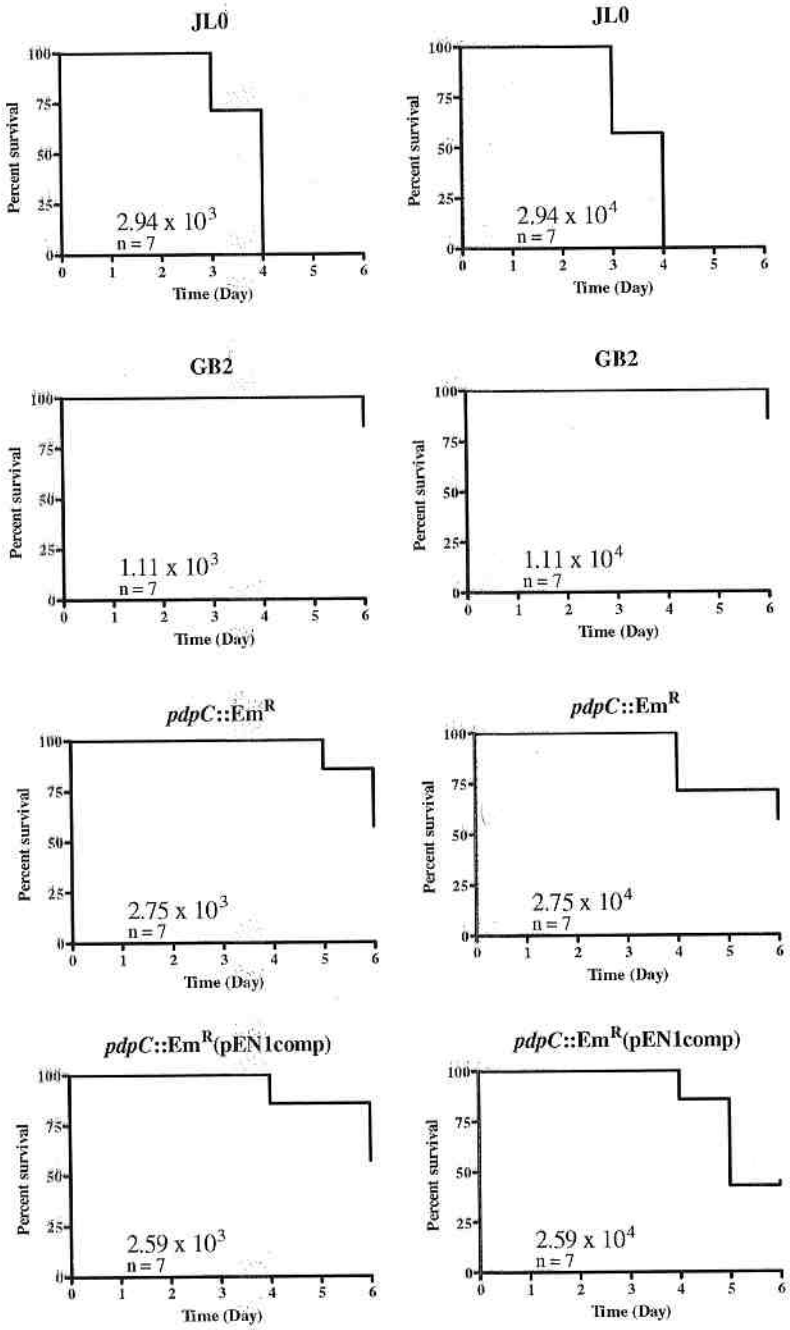


Figure 2.10 Virulence of *F. novicida pdpC*::Em^R and its complement in chicken embryos. A dilution series was prepared for each strain and seven embryos were infected with each infection dose. Embryonic death was monitored daily. The number in each graph represents the infection dose (CFU).

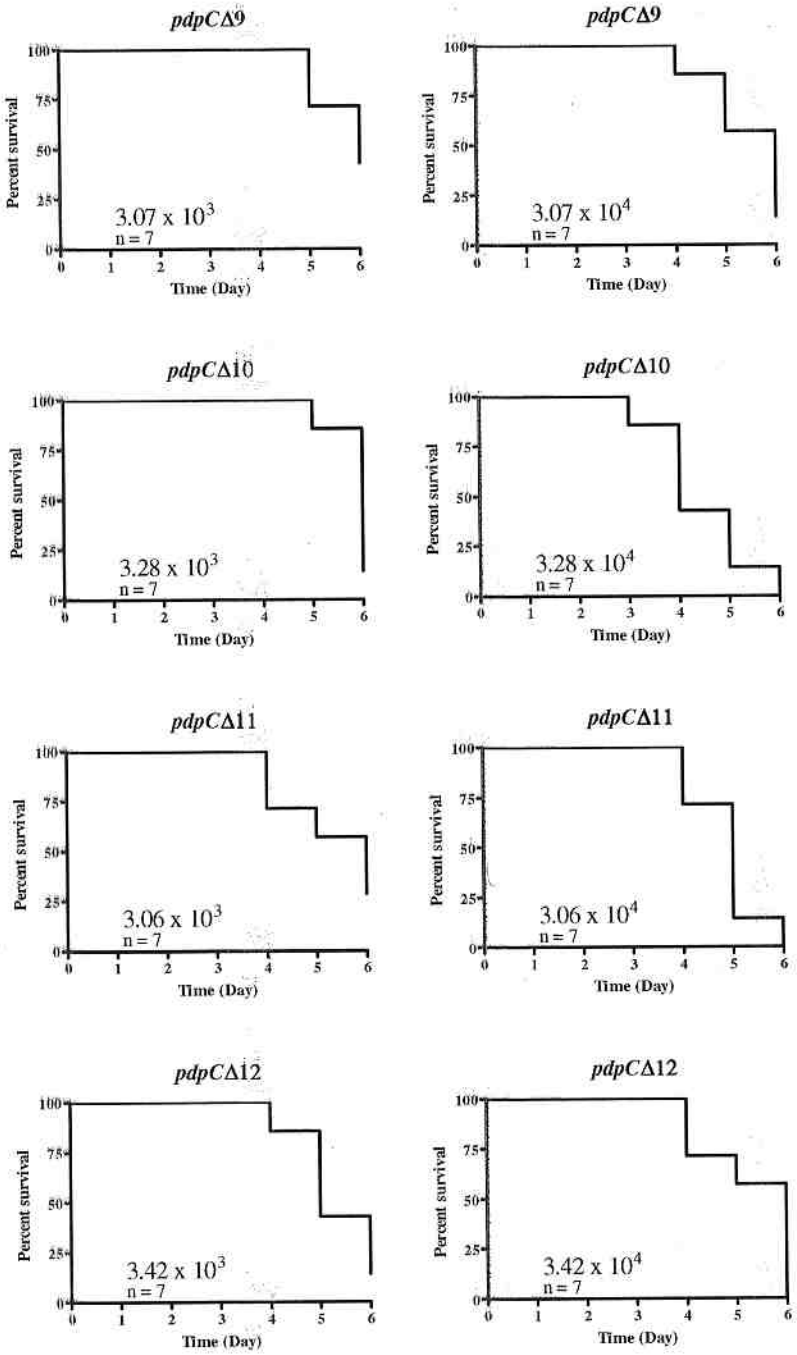


Figure 2.11 Virulence of *F. novicida pdpC* partial deletion mutants in chicken embryos. A dilution series was prepared for each strain and seven embryos were infected with each infection dose. Embryonic death was monitored daily. The number in each graph represents the infection dose (CFU).

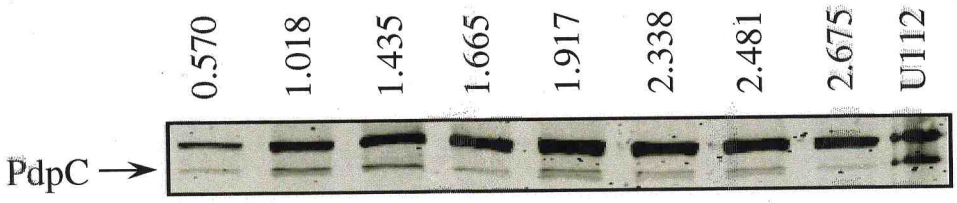


Figure 2.12 PdpC expression in TSBC broth culture at different optical density at 600nm. Western blot showing expression of PdpC from U112 harvested at different OD_{600nm} when normalized to the amount of total protein loaded per lane (12 µg).

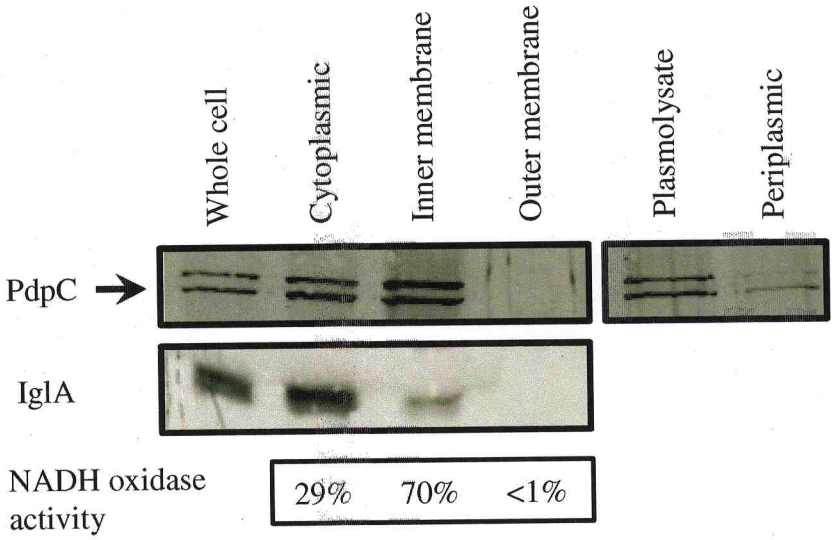


Figure 2.13 Subcellular localization of PdpC in *F. novicida* U112. Anti-PdpC was used to probe a western immunoblot of subcellular fractions of *F. novicida*. The sarkosyl insoluble fraction contains outer membrane (OM) protein and the sarkosyl soluble fraction contains inner membrane (IM) protein. The plasmolysate contains largely cytoplasmic protein. Samples were normalized to 5 µg protein per lane and were separated on an 8% SDS-PAGE gel. NADH oxidase assay was used to determine the amount of cross-contamination of the IM fraction into all of the other fractions. IglA, previously found to be localized to the cytoplasm, was used as a cytoplasmic protein control.

Chapter 3 Eukaryotic Expression of PdpC in Chicken Embryo Fibroblasts

3.1 Introduction

Chicken embryos were previously demonstrated to be an appropriate model to test for the virulence of *F. novicida* strains. Because intramacrophage growth was only slightly attenuated for the erythromycin allelic replacement *pdpC* mutant, it could be postulated that PdpC has other functions separate from growth inside macrophages. Other possible explanations could be that PdpC plays a role in the assembly of a secretion apparatus or the interference of the innate immune defense mechanism of Toll-like receptor signaling. The defined partial deletion mutants were also observed to cause macrophage cell death, so it is possible that PdpC could function in causing cytotoxicity to host cells by altering certain events in the host's apoptotic pathway. The effect of PdpC on host signaling pathways may be as explicit as disrupting normal microfilament reorganization.

To provide some preliminary data on the possible roles of PdpC in the virulence of host eukaryotic cells, the expression of PdpC was examined. In this study, chicken embryo fibroblasts were isolated from 10-day-old embryos and a clone of PdpC was constructed using an eukaryotic expression vector containing a N-terminal triple FLAG tag. The expression of PdpC was visualized using commercially purchased anti-FLAG antibody and the host cell morphology of fibroblasts expressing PdpC exhibited significant differences compared with fibroblasts transfected with the empty vector

control. The isolation, culturing, and transfection of chicken embryo fibroblasts and microscopy were performed by B. Duplantis at Dr. Nano's laboratory.

3.2 Materials and Methods

Bacterial Strains and Plasmids

Escherichia coli DH5α was used for all molecular cloning and was cultured in Luria-Bertani broth (LB) with 250 μg/mL ampicillin (Ap), 30 μg/mL kanamycin (Km), or 30 μg/mL chloramphenicol (Cm) as needed. The low-copy-number plasmid pWSK29 was used to clone blunt ended PCR products amplified with Phusion DNA polymerase. The V37 pDNR MCS SA donor vector and V180 pLP-Triple FLAG SD acceptor vector were used to construct the eukaryotic expression clone of *pdpC* [20]. G418 (400 μg/mL) (Sigma) was used as a neomycin analogue when V180 pLP-Triple FLAG SD was used in tissue cell culture studies. All bacterial strains and plasmids used in this work are listed in **Table 3.1**.

Polymerase Chain Reaction (PCR) and Primer Design

PCR reactions were performed in volumes of 50 μL each. Amplification was carried out using Phusion High-Fidelity DNA Polymerase (New England Biolab) in a reaction containing nuclease-free water, 1X Phusion HF buffer, 200 μM dNTPs (Invitrogen), 0.5 μM each of the forward and reverse primers (Integrated DNA Technologies), 0.1 ng of template DNA, and 1.0 U of Phusion DNA Polymerase (NEB). PCR reactions were performed under the following cycling parameters: Initial denaturation at 98°C for 30 s; 35 cycles of 98°C for 30 s, 50°C for 30 s, 72°C for 2 min 30 s; final extension at 72°C for

10 min. For the purpose of screening for positive clones, *Taq* DNA polymerase (NEB) was used. All primers were designed based on the *F. novicida* U112 *pdpC* sequence and are listed in **Table 3.2**.

Recombinant DNA Techniques

All cloning procedures including plasmid DNA purification, digestion with restriction endonucleases, ligations, and agarose gel electrophoresis were performed according to the protocols described in *Molecular Cloning* [84]. QIAquick® PCR Purification Kit, QIAquick® Gel Extraction Kit, and QIAprep® Spin Miniprep Kit (QIAGEN) were used according to manufacturer's instructions. Restriction endonucleases, T4 DNA Ligase, and Cre Recombinase (NEB) were used as per manufacturer's instructions. Inactivation of enzymes was carried out at 70°C for 10 min. Ligation reactions were electroporated into the *E. coli* DH5α strain using the Gene Pulser (Bio-Rad). The ChargeSwitch® Plasmid ER (Endotoxin Reduced) Mini Kit (Invitrogen) was used to purify plasmid DNA from the eukaryotic expression construct for transfection of chicken embryo fibroblasts.

DNA Sequencing and Analysis

Sequencing of the V37 pDNR MCS SA::*pdpC* construct was performed by the CMMT / CFRI DNA Sequencing Core Facility at the University of British Columbia. The sequences were analyzed using on-line BLAST (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) and are included in **Appendix 3**.

Chicken Embryo Fibroblast Culture

White Leghorn chicken eggs were shipped from the University of Alberta Poultry Research Station. The aortae from 10-day-old chicken embryos was dissected into an Eppendorf tube on ice and rinsed twice with DPBS. The aortae was homogenized to <1mm^3 pieces with fine scissors and the tissue pieces were rinsed twice with DPBS. The aortae pieces were then incubated in 900 µL of trypsinization solution containing 0.25% Trypsin (Gibco BRL, Eggenstein, Germany) and 0.5% Collagenase D (Sigma) in DPBS without calcium or magnesium at 37°C for 10 min. The mixture was vortexed vigorously and the non-solubilized tissues were allowed to settle to the bottom of the Eppendorf tube. The dissociated fibroblast cells were transferred to a fresh tube with 100 µL of FBS (10% final concentration) as the Trypsin inhibitor. The cells were centrifuged at 400 x *g* for 2 min. The cell pellet was resuspended in 200 µL DPBS. The chicken embryo fibroblasts (CEF) were cultured in 100 mm tissue culture Petri dishes with 10 mL of Primary Chicken Fibroblast (PCF) growth medium containing 1 mM sodium pyruvate (Sigma), 2 mM glutamine (Sigma), 5% FBS (Gibco), and 1xOPI in complete DMEM/F12. The cell culture was incubated at 37°C with 5% CO_2. The culture medium was changed the next day and the CEFs were transfected with the eukaryotic intron splicing vector *pdpC* construct at 75% confluency.

Transfection of Chicken Embryo Fibroblasts

Chicken embryo fibroblasts were cultured to 75% confluency before transfection. Control plasmid V180 and *pdp* V180 construct in amounts of 0.75, 1, 1.5, and 2 µg were each added to 100 µL of serum-free media. In a separate tube, 5 µL of lipofectamine

(Invitrogen) was added to 100 µL of serum-free media for each DNA amount. The DNA was combined with the lipofectamine and incubated at room temperature for 45 min to allow for complexation. The CEF in the slide chambers were changed to 0.2 mL of serum-free media. The lipofectamine/DNA complex was added to the chambers and the slide was incubated at 37°C for 5 h. The chambers were then rinsed in serum-free media once and for the remainder of the experiment, the transfected CEF were cultured in complete media at 37°C for overnight.

Microscopy

The complete media was removed from the slide chambers. The slide was fixed in 2% paraformaldehyde for 12 min and washed with PBS, followed by a brief submersion in PBS at 4°C. The slide was then blocked with 5% lamb serum-PBS containing 0.05% Tween-20 at room temperature for 30 min and then rinsed with PBS for 5 min three times. The primary mouse monoclonal anti-FLAG antibody (Sigma) was diluted 1:20,000 in PBS + 0.05% Tween-20. The slide was incubated in the primary anti-FLAG antibody at 4°C overnight. After three washes for 5 min each in PBS, the slide was incubated at room temperature for 1 h with the secondary antibody, goat anti-mouse-Alexa 568 (Red; Molecular Probes) diluted 1:1,200. The slide was washed with PBS for 5 min and then counterstained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) for 5 min, followed by several washes with PBS. The fixed CEFs were imaged with a Leica DM-6000 compound microscope. Digital images were captured and color was added with OpenLab (version 4.04) and Scion Image (version 4.03).

3.3 Results

3.3.1 Construction of the V37 pDNR MCS SA clone of *pdpC*

The blunt ended PCR product of *pdpC* amplified with Phusion DNA polymerase was cloned into *EcoR*V digested pWSK29 plasmid. The pWSK29 clone was digested with the restriction endonucleases *Kpn*I and *Xho*I to excise the *pdpC* fragment, which was subcloned into the V37 pDNR MCS SA donor vector. Several pools of 20 transformants were screened for positive clones of V37 pDNR MCS SA::*pdpC* (**Figure 3.1A**). A positive clone was chosen for restriction digestion (**Figure 3.1B**) and DNA sequencing analyses (**Appendix 3**).

3.3.2 Construction of the V180 pLP-Triple FLAG SD clone of *pdpC*

Plasmid DNA of the V37 pDNR MCS SA::*pdpC* clone was added to the V180 pLP-Triple FLAG SD plasmid in a Cre Recombinase reaction. The Cre Recombinase catalyzes the site-specific recombination of *pdpC*-Cm^R between the *loxP* sites in V37 pDNR MCS SA. The excision and integration in the Cre-mediated recombination of *pdpC*-Cm^R between the directly repeated *loxP* sites is shown in **Figure 3.2**.

The Cre Recombinase reaction was used to transform *E. coli* DH5α. The transformants were selected on LB agar containing chloramphenicol and 10% sucrose for counter selection. Transformants containing V37 pDNR MCS SA::*pdpC* plasmid DNA carry the *sacB* gene in the construct and will be sensitive to sucrose. Chloramphenicol- and sucrose-resistant colonies were chosen for further PCR analysis to confirm the presence of *pdpC* (**Figure 3.3**). Clones of V180 pLP-Triple FLAG SD::*pdpC* were

grown in LB containing Cm and Km for plasmid isolation in the remainder of the experiment.

The acceptor vector V180 pLP-Triple FLAG SD contains an intron sequence which puts the triple FLAG tag out of frame. Upon successful transfection into chicken embryo fibroblasts, the splicing mechanism in the eukaryotic cells will excise out the intron sequence and the triple FLAG will be expressed in-frame with *pdpC*. The *pdpC* gene has been difficult to clone using high-copy number plasmids in previous cloning experiments and often caused lethality to *E. coli*. This lethality to the cloning host is prevented by using V180 pLP-Triple FLAG SD which is a high-copy number plasmid because the intron sequence puts *pdpC* out of frame before it is expressed by the eukaryotic cell.

3.3.3 Expression of PdpC in chicken embryo fibroblasts

Chicken embryo fibroblasts (CEF) transiently transfected with the V180 pLP-Triple FLAG SD::*pdpC* construct show expression of PdpC tagged with triple FLAG in red (**Figure 3.4**). DAPI was used as a counter-stain. In **Figure 3.4**, it is observed that the cytochemical properties of CEFs expressing PdpC exhibit drastically different cellular morphologies than the CEFs transfected with the empty V180 pLP-Triple FLAG SD vector as a negative control. Fibroblasts expressing PdpA also behave differently than the negative control.

3.4 Discussion

An eukaryotic vector containing a triple FLAG tagged *pdpC* was constructed for the expression inside chicken embryo fibroblasts. The expression of PdpC demonstrated that

the morphology of transfected host cells exhibited changes when compared with cells transfected with an empty vector control. The cells expressing PdpC were elongated and branched at numerous locations. The expression of PdpA served as a control for the morphologies exhibited by CEFs expressing PdpC in comparison to another Pdp protein. The expression of PdpC was not localized to foci in the fibroblast cells as the FLAG tagged protein was visualized to be diffused throughout the CEFs. As this study was meant to provide only preliminary data, the results obtained are inconclusive and further investigations using different cellular markers need to be performed to determine how else host compartments are being affected.

As *pdpA* mutants have been shown to be defective for macrophage growth [94], the role of PdpA may be in the modulation and biogenesis of the *Francisella*-containing phagosome inside phagocytic cells. As mentioned in the previous chapter, *pdpC* mutants are only slightly attenuated in growth inside macrophages and may have a function unrelated to that of PdpA. From the data of this expression study, PdpC must somehow interfere with normal host cell mechanisms, possibly by mimicking host proteins and their functions in various signaling pathways.

The mimicry of host proteins by the Yop proteins of pathogenic *Yersinia* sp. has been well studied. Invasion strategies employed by *Yersinia* to hijack the cellular signaling pathways of the host involves the function of the Type III secretion system. Once inside the host cell, the secreted Yop effector proteins interfere with signaling pathways involved in the regulation of actin cytoskeleton, phagocytosis, apoptosis, and the inflammatory response, all of which favor the survival of the pathogen [136]. Although no T3SS have been discovered in *Francisella*, homologues of the T6SS have been

reported by bioinformatic analyses of the FPI [31]. Virulence proteins of *Francisella* may participate in similar events that promote its survival. In addition, *pdpB* contains an *icmF* motif and another FPI gene has similarity to *dotU* found in the T4SS of *Legionella pneumophila* and *Agrobacterium tumefaciens* [31]. *L. pneumophila* uses the Dot/Icm T4SS to inject a large number of effector proteins into its host which alters phagosome trafficking [9, 97]. A. *tumefaciens* uses the T4SS to translocate DNA-protein complexes into plant cells to induce tumor formation causing crown galls [32]. The roles of the Pdp proteins remain to be discovered. However, the use of affinity tagged proteins in macrophage infections and subsequent immunoprecipitation using monoclonal antibodies against the tag can help to reveal the components that are interacting with the target protein and to elucidate molecularly its role in the mechanism of *Francisella* pathogenesis.

Table 3.1 Bacterial strains and plasmids used in the construction of a *pdpC* clone in an eukaryotic expression vector

Strains	Relevant characteristics	Source or Reference
Escherichia coli		
DH5α	*sup*E44 *lac*U169 (Φ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Invitrogen
Francisella novicida		
U112	Wildtype *F. novicida* (Ap^R)	ATCC 15482
Plasmids		
pWSK29	Low-copy-number, Ap^R, *lacZ*α gene	Wang and Kushner, 1991 [138]
V37 pDNR MCS SA	Donor vector, Ap^R Cm^R, directly repeated *loxP* sites, 3' splice acceptor sequence	Colwill *et al.*, 2006 [20]
V180 pLP-3xFLAG SD	Acceptor vector, intron sequence, Km^R/Neomycin, single *loxP* site, 5' splice donor sequence	Colwill *et al.*, 2006 [20]

Table 3.2 Primers used in PCR amplification of *pdpC* for eukaryotic intron splicing vector cloning and screening

Gene	Primer	Sequence 5' -> 3'	Tm
Cloning Primers			
pdpC	newC-int-F	GGG GTA CCC ATA TGA ACG ACA AAT ATG AAC	58.9
	newC-int-R	CCG CTC GAG CTG ACG ATA TTT TTT TAA AAA AGT CTG	60.8
Screening Primers			
internal *pdpC*	C1-F	CTC GAG ATC CCT CAA AAA TAG CGATGC	66.1
	C1-R	CTC GAG CTA GCC TTG GTC AAT AAG TTC A	66.1
internal *pdpC*	C2-F	CTC GAG ACC AAG GCG ATA TTA ATC CAA A	64.6
	C2-R	CTC GAG CTA TGC CTG AGT CAT TGC TGT AT	67.4
internal *pdpC*	C3-F	CTC GAG TAC TTG GGA TGG CAA CTA CAA A	66.1
	C3-R	CTC GAG CTA TGA ATT AGG TGT TGC GAA AC	66.0
internal *pdpC*	C4-F	CTC GAG AAC AAA ATA CGC GAT ATT GCT AC	64.6
	C4-R	CTC GAG CTA ACT ACA TTT TGG TTT TAA TTC	61.9

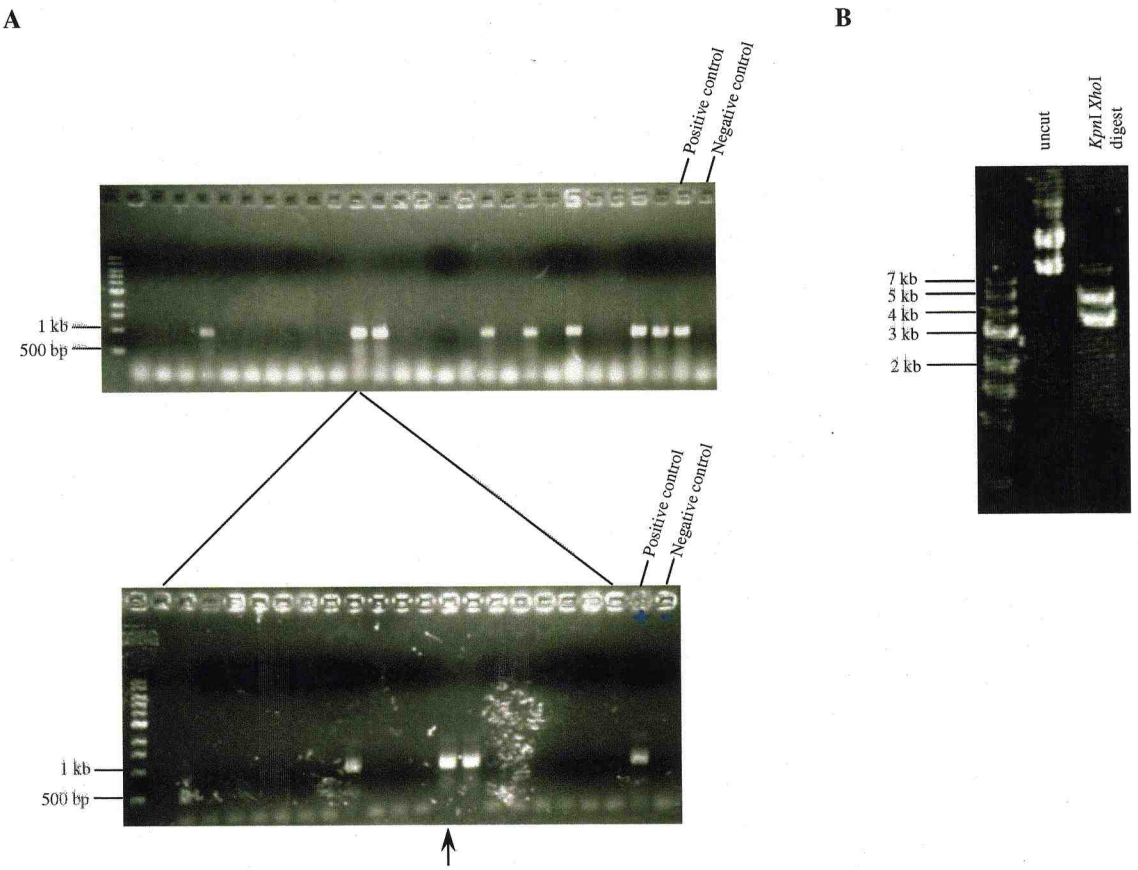


Figure 3.1 Agarose gel analysis of the V37 pDNR MCS SA clone of *pdpC*. (**A**) Positive clones were identified by PCR screening transformants with the internal *pdpC* primer pair C1-F/R in pools of 20 colonies. Individual colonies were screened within a positive pool. Small black arrow indicates the positive clone of V37 pDNR MCS SA containing *pdpC* used in this experiment. (**B**) Restriction digestion analysis of the positive clone. Double digestion with the restriction enzymes *Kpn*I and *Xho*I resulted in two bands, 3.6 kb represents the *pdpC* gene and 4.9 kb represents the V37 pDNR MCS SA donor vector.

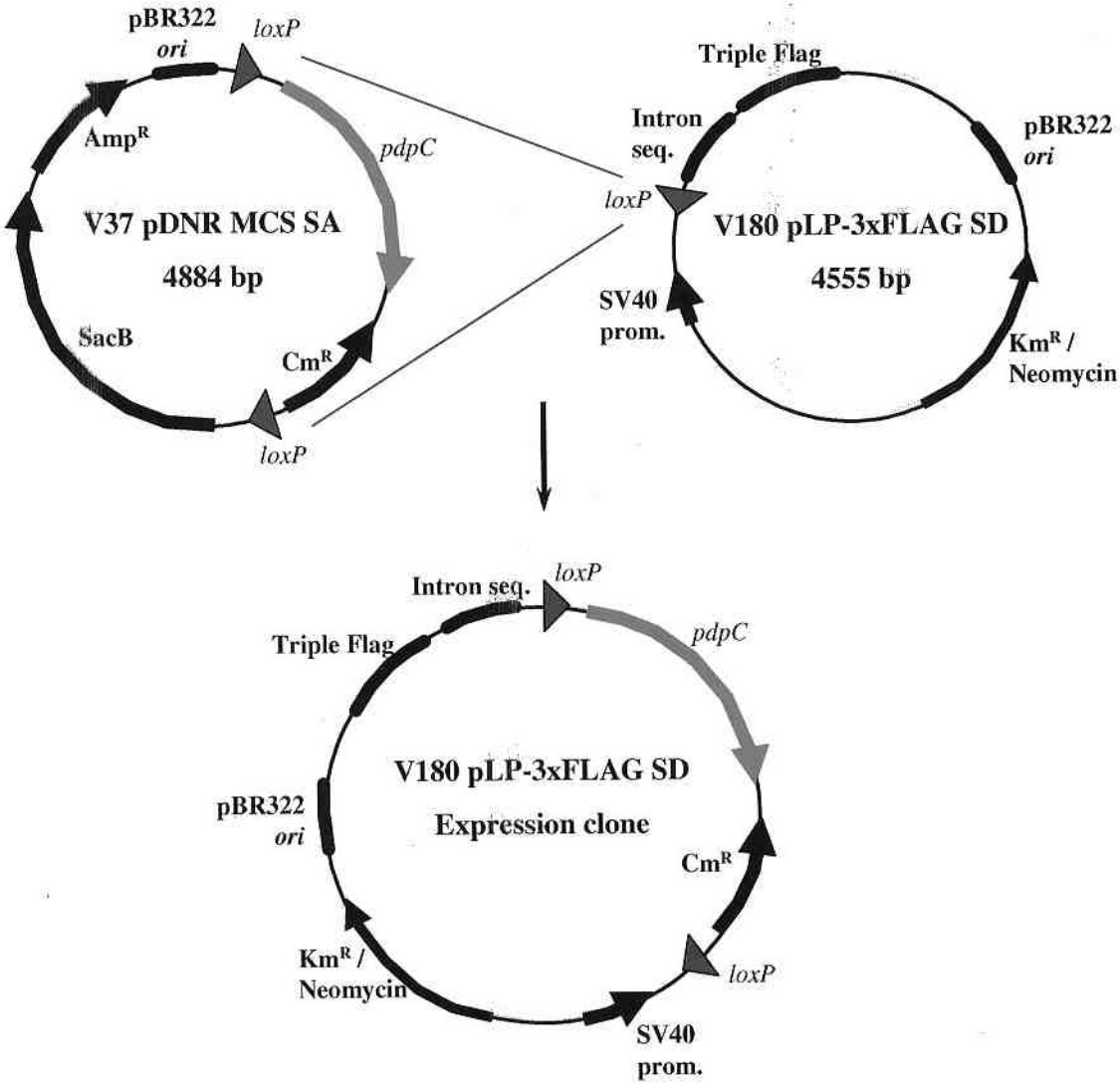


Figure 3.2 Construction of V180 pLP-Triple FLAG SD::*pdpC*. A schematic for the cloning used to make the eukaryotic expression clone of *pdpC*. In a Cre-mediated recombination event, the *pdpC* gene between two directly repeated *loxP* sites in the donor vector is excised and integrated into the *loxP* site of the acceptor vector. The resulting expression clone contains an intron sequence that splices out when the construct is expressed in eukaryotic cells, leaving the 5' triple FLAG tag in-frame with *pdpC*.

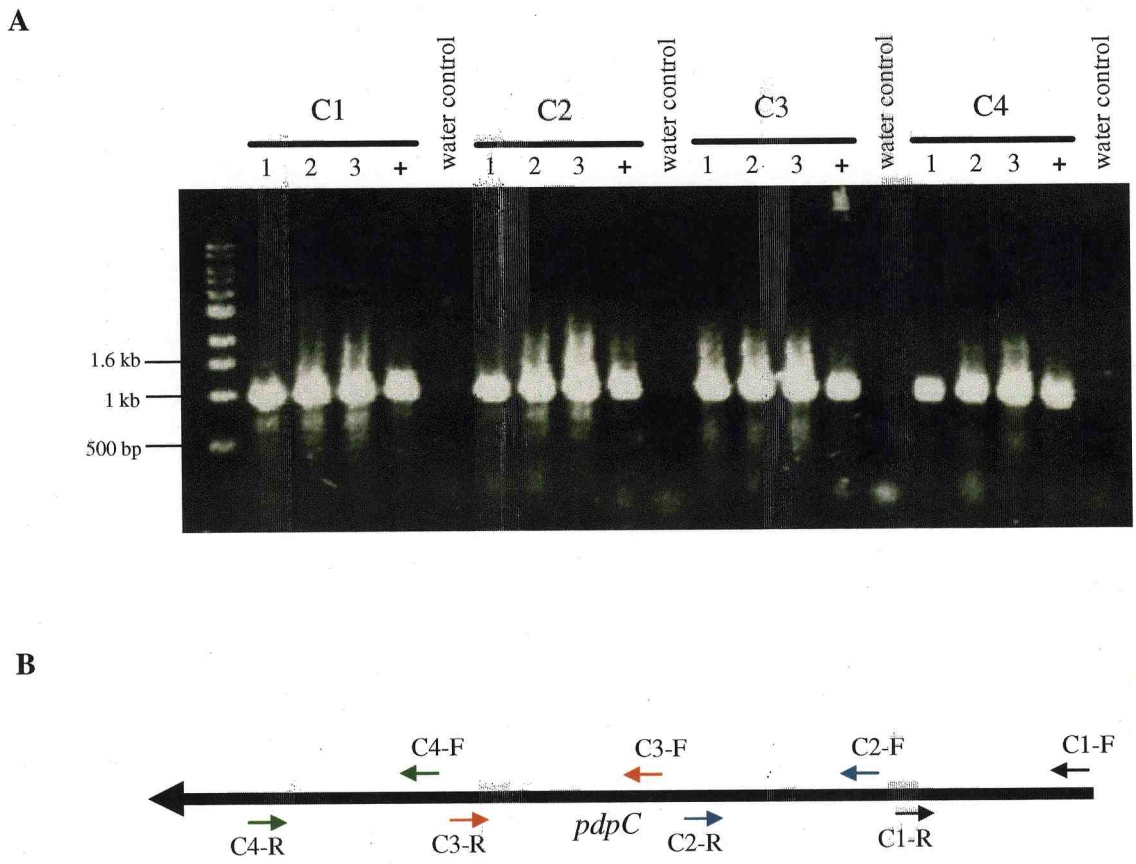


Figure 3.3 Agarose gel analysis of the V180 pLP-Triple FLAG SD clone of *pdpC*. (**A**) Four pairs of internal *pdpC* screening primers were used to confirm the presence of *pdpC* in three (1 – 3) potential V180 pLP-Triple FLAG SD::*pdpC* clones. (**B**) A map indicating the regions amplified by PCR. The broad arrow represents the *pdpC* gene; locations of the primers are indicated by the small arrows.

Figure 3.4 Expression of PdpA and PdpC in chicken embryo fibroblasts. *pdpA* and *pdpC* were cloned into the eukaryotic intron splicing vector, V180 pLP-Triple FLAG SD and transfected for transient expression. Cells were fixed and stained using anti-FLAG (red) and DAPI (blue), as described in Materials and Methods; observed at 100X magnification. (**A**) Expression of PdpA; (**B**) Expression of PdpC; (**C**) Empty V180 pLP-Triple FLAG SD vector as negative control.

Chapter 4 Conclusions and Future Research

4.1 Conclusions

Pathogenic bacteria have evolved to possess a variety of mechanisms that enable their survival inside the mammalian host cells that they infect. Bacterial genome sequencing and bioinformatic analyses have revealed increasingly apparent mechanisms that are commonly shared by many different species of bacteria. The similarities shared among species of a bacterial population may be due to the mobile nature of genetic elements such as transposons, bacteriophages, plasmids, and pathogenicity islands.

In recent years, the genomes of several *Francisella* species have been made available to fellow researchers by sequencing groups. The Type III secretion system has been found in a diverse group of bacteria such as *E. coli, Salmonella, Shigella,* and *Pseudomonas*. However, no homologues for such a secretion system have ever been reported for *Francisella*. Very little is known about the pathogenesis of *Francisella* and only a small number of virulence factors have been identified previously, due in part to the fact that the research into the virulence associated genes has been hindered by the high infectivity of this pathogen. Currently, there is still no effective vaccine for the disease tularemia that is caused by this organism.

Using the tools of bioinformatics, a *Francisella* pathogenicity island was deduced and the genes encoded by the FPI represented novel proteins that could give insight into the molecular mechanism of *Francisella* virulence. The focus of our laboratory's research is

to use *F. novicida* as our model strain because it is avirulent for humans but virulent for mice to investigate the possible roles of the FPI encoded proteins during infection of macrophages. The putative protein named *pdpC* was the focus of my research for this thesis. This work demonstrated that PdpC, unlike the PdpA and PdpD, may have a separate function other than to enable intramacrophage growth of the pathogen. *pdpC* mutants were found to replicate inside bone marrow derived macrophages from BALB/c mice as competently as the wildtype strain. However, *pdpC* virulence was found to be attenuated in the chicken embryo model. These data led to the preliminary study of the effects of PdpC expression in chicken embryo fibroblasts. Indeed, the expression of this protein altered the host fibroblast morphology. The alteration of morphology may be due to the interference of various factors involved in host cell signal transduction pathways.

4.2 Future Research

It has been reported that *Fransicella* infections can induce the cytotoxicity of macrophages [76] and also alter Toll-like receptor signaling in host cell defense mechanisms [132]. However, it is not plausible to try to investigate where along the host signaling pathways *Francisella* virulence proteins may exert their functions on because the number of pathways and the components of each of them are too numerous. It was recently reported that the FPI contains loci encoding proteins with similarity to the IAHP/T6SS. Therefore, it is more reasonable to conduct research on the nature of the FPI proteins. By studying the protein biochemistry of the FPI genes, protein-protein interactions, localizations of the proteins, and their subsequent fates during *Francisella* infections can be examined. Such data can then help to clarify their precise functions in

promoting virulence and survival of *Francisella* inside host cells. It can be foreseen that bioinformatics will be a major tool in the prediction of FPI orthologs in other bacterial species that share similar mechanism of pathogenesis during infection. The knowledge gained from such studies may help in the design of approaches to manage the consequences of *Francisella* infections in humans and ultimately help in the search for a good vaccine candidate in the prevention against tularemia.

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Appendices

The following journal article included as **Appendix 1** is an 100% reproduction of an article titled "Virulence of *Francisella* spp. in chicken embryos" published in *Infection and Immunity* 2006 **74**(8): 4809-16 by Nix, EB; Cheung, KK; Wang, D; Zhang, N; Burke, RD; Nano, FE from the Department of Biochemistry and Microbiology at the University of Victoria. The article is reproduced with permission from the authors.

My participation in the publication of this paper involved the generation of data for the growth of *F. tularensis* LVS and *F. novicida* in chicken embryos (Figure 1).

ABSTRACT

We examined the utility of infecting chicken embryos as a means of evaluating the virulence of different *Francisella* sp. strains and mutants. Infection of 7-day-old chicken embryos with a low dose of *F. novicida* or *F. tularensis* subsp. *holarctica* live vaccine strain (LVS) resulted in sustained growth for 6 days. Different doses of these two organisms were used to inoculate chicken embryos to determine the time to death. These experiments showed that wild-type *F. novicida* was at least 10,000-fold more virulent than the LVS strain. We also examined the virulence of several attenuated mutants of *F. novicida*, and they were found to have a wide range of virulence in chicken embryos. Fluorescent microscopic examination of infected chicken embryo organs revealed that *F. tularensis* grew in scattered foci of infections, and in all cases the *F. tularensis* appeared to be growing intracellularly. These results demonstrate that infection of 7-day-old chicken embryos can be used to evaluate the virulence of attenuated *F. tularensis* strains.

Appendix 1 Virulence of *Francisella* spp. in Chicken Embryos

by Eli B. Nix,[1] Karen K. M. Cheung,[1] Diana Wang,[2] Na Zhang,[1] Robert D. Burke,[1,2] and Francis E. Nano[1] published in *Infection and Immunity*, August 2006, Vol. 74, No. 8, p. 4809-4816.

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Introduction

Francisella tularensis is a highly infectious, gram-negative bacterial pathogen with a 50 % infectious dose of less than 10 cells for most animals [43]. Although all strains are highly infectious, there is great variety in the morbidity and mortality that each strain is able to induce in different host animals. *F. tularensis* subsp. *tularensis* biovar "type A" is clearly the most virulent biovar in humans and laboratory animals. The type A biovar is found naturally only in North America, where it caused high mortality rates before the availability of antibiotics [44]. In one survey 19% of tularemia patients died [34], but it is not known what proportion of these patients were infected with a biovar type A strain. Even with antibiotic treatment infection of humans with this subspecies results in some deaths. Recently, the type A biovar has been separated into two clades [70], but the relative levels of virulence of the two groups have not been studied. *F. tularensis* subsp. *holarctica* is found throughout the Northern Hemisphere. Although it is highly infectious in all of the animals that it infects and is fatal to mice [17], this subspecies rarely causes death in humans, although it can cause considerable morbidity. The live vaccine strain (LVS) of *F. tularensis* subsp. *holarctica* and *F. novicida* have been widely used as models

of *F. tularensis* infection, primarily because these bacteria have low virulence in humans and can be handled in BSL2 facilities [5, 36, 41, 51]. In the mouse model of infection the LVS has an intradermal 50% lethal dose (LD_{50}) of about 3×10^5 CFU, and *F. novicida* has an LD_{50} of about 2×10^3 CFU [72]; however, both strains have an intraperitoneal LD_{50} of less than 10 organisms. Hence, mouse infections with LVS and *F. novicida* may be approximations of *F. tularensis* type B and type A infections in humans, respectively.

F. tularensis is thought to grow primarily inside cells during infection of animals. In vitro studies of intramacrophage growth have shown that initially *F. tularensis* resides in a phagosome, from which it largely escapes between 2 and 4 h after cell entry [19, 57]. The *F. tularensis*-laden phagosome has a relatively neutral pH and accumulates some markers of late endosomes, such as LAMP1 and CD63, while it excludes another late endosome marker, cathepsin D [19]. Expression of the *F. tularensis* protein IglC is required for escape of *F. tularensis* from the phagosome, but its role is unknown [82]. The live vaccine strain of *F. tularensis* has been shown to induce apoptosis in the J774 mouse macrophage cell line [76] and to inhibit secretion of tumor necrosis factor alpha and interleukin-1 [132]. Although the suppression of cytokines probably represents an *F. tularensis* virulence strategy, the induction of apoptosis likely reflects a defensive response of the host, as caspase-1 knockout mice are more susceptible to *F. tularensis* infection [86].

A small number of virulence factors have been identified in *F. tularensis*, and most of these factors affect intramacrophage growth. Inactivation of the *mglAB* global regulatory genes results in strains whose growth is severely hampered in macrophages [10].

Presumably, MglA and MglB are required for transcription of genes encoding effector proteins, especially genes found in the *Francisella* pathogenicity island [79]. There is genetic evidence that the *Francisella* pathogenicity island-associated genes *iglA*, *iglC*, *pdpA*, and *pdpD* are required for intramacrophage growth [59, 61, 94]. There is biochemical [66] and genetic [115] evidence that a capsule exists and is needed for infectivity and virulence. Defects in the production of lipopolysaccharide can affect intracellular growth [24, 88]. The observed in vitro intracellular growth and the requirement for cell-mediated immunity for clearance [2, 6, 23, 36] of an *F. tularensis* infection suggest that intracellular growth is required for virulence in animals. The observation that mutants defective for growth in macrophages are also less virulent in animals supports this notion [88, 94].

F. tularensis infects a wide variety of animals, and several animals, including rabbits, guinea pigs, primates, hamsters, rats, and mice, have been used as models of infection [7, 36, 37, 41, 90, 100, 122]. Chicken embryos have also been used to test *F. tularensis* virulence and pathology [13, 110]. Recently, researchers have begun to use simple biological systems, such as the nematode *Caenorhabditis elegans* [28], flies, and insect larvae [137], to examine the virulence properties of bacterial pathogens. Such systems permit large-scale testing that is humane and relatively inexpensive. Our objective in this work was to develop an assay system that allowed us to evaluate the virulence of *F. tularensis* strains without having to infect animals that have fully developed nervous systems.

Materials and Methods

Bacterial strains and growth conditions

The *F. tularensis* strains used in this study are listed in Table 2.1. All of the *F. novicida* strains were derived from the prototype strain U112 (ATCC 15482), which had been passaged through a mouse and aliquoted for subsequent experiments. The LVS (ATCC 29684) was obtained from the American Type Culture Collection. Strains were grown aerobically at 37°C in either tryptic soy broth or on tryptic soy agar (TSA) supplemented with 0.1% L-cysteine.

Chicken embryo infections

F. tularensis strains were grown to the late log phase (optical density at 600 nm, 0.9 to 1.0) and diluted in phosphate-buffered saline (PBS) (Gibco) for injection. The inoculating dose was calculated retrospectively by determining the CFU following dilution and plating on TSA. Fertilized White Leghorn eggs were obtained from the University of Alberta Poultry Research Station. Chicken embryos were incubated at 37°C with high humidity for 7 days prior to infection and throughout the experiment were mechanically tilted to a 45° angle every 40 min. After the initial 7-day incubation the eggs were examined in order to discard those that lacked a viable embryo, a phenomenon that occurred in between 5 and 10 % of the fertilized eggs. For inoculation the tops of the egg shells were disinfected with 70 % ethanol. A 1-cm-diameter window was made in the air sac end of an egg, and the egg shell membrane was reflected. With a tuberculin syringe, 100 µl of inoculum was injected under the chorioallantoic membrane. After injection the shells were sealed with clear packing tape. Eggs were candled to detect signs of death

every 24 h for 6 days. This process consisted of shining a focused bright light at one end of an egg to determine if the network of blood capillaries was intact. Embryos that died within 24 h of inoculation were assumed to have suffered lethal trauma during the inoculation and were removed from the experiment. For the study of the time course of *F. tularensis* growth, the chicken embryos were killed by incubating the eggs in a -20°C freezer for 1 h, followed by blending of the eggs and plating dilutions of the homogenized egg contents on TSA to determine the bacterial load. All experiments were terminated by the time the embryos were 14 days old. Chicken embryos that were less than 17 days old are not subject to regulatory control in many countries, including Canada.

Microscopy

Embryos were removed from shells and rinsed with PBS, and organs of interest were removed by dissection and fixed overnight with PBS containing 4% paraformaldehyde. The organs were rinsed with PBS, infiltrated with OCT compound (Tissue-Tek 4583), and snap frozen with liquid nitrogen, and then they were transferred to –80°C for storage. Ten-micrometer cryostatic sections were cut and mounted on gelatin-coated slides. The tissue sections were blocked with 5% lamb serum-PBS containing 0.05% Tween 20 at room temperature for 45 min. Primary antibodies were diluted 1:1,000 in 5% lamb serum-PBS and incubated overnight at 4°C with the sections. After several washes in PBS, sections were incubated with Alexa 488 or 568-conjugated (Molecular Probes) goat anti-rabbit or goat anti-mouse secondary antibodies for 2 h at room temperature, rinsed with PBS, and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes).

After mounting with a coverslip, specimens were examined with a Leica DM-6000 compound microscope.

Digital images were collected, and color was added with OpenLab (version 4.04) or Scion Image (version 4.03). Figures were prepared with Photoshop (Adobe 6.0) by cropping and adjusting the brightness and contrast.

Results and Discussion

Growth of *F. tularensis* in 7-day-old chicken embryos

When a small inoculum of *F. tularensis* subsp. *holarctica* LVS or *F. novicida* was introduced into chicken embryos, exponential growth occurred for 4 to 5 days, and the concentration reached about 5×10^8 bacteria per g of egg mass (Fig. 1). *F. novicida* and LVS appeared to grow at approximately the same rate, and the concentrations leveled off at similar total numbers of bacteria by day 5 postinfection.

Virulence of *F. novicida*

The wild-type strain of *F. novicida* is highly virulent in mice and also appears to be highly virulent in chicken embryos, and 100% lethality was observed with 30 to 200 CFU (Fig. 2). Infections with different doses and repetitions of experiments with different lots of eggs demonstrated that the time to death due to infection generally correlated with the infectious dose and was consistent for different experiments. Some aberrations were seen, like the results obtained with the intermediate dose given in experiment 2, and these aberrations may have been due to the natural variation in embryos or to the inexact nature

of the inoculation. All of the embryos inoculated with phosphate-buffered saline lived until the termination of the experiment on day 6 postinfection (data not shown).

Virulence of *F. tularensis* LVS

In the mouse *F. novicida* is about 100-fold more virulent than LVS when both organisms are delivered via intradermal injection. Infection of chicken embryos revealed an even larger difference in virulence between the two organisms, and *F. novicida* was at least 10,000-fold more lethal than *F. tularensis* LVS (Fig. 2 and 3).

Virulence of mutants of *F. novicida*

The virulence of the strains listed in Table 1 was evaluated by performing a series of infection experiments using different numbers of bacteria as inocula and determining the time to death of the chicken embryos. First, a pilot experiment was performed for each mutant to determine the approximate lethal dose, and then at least two repetitions of experiments were carried out using inoculum concentrations in the sublethal to lethal range. For each inoculating dose seven eggs were infected. Mutants of *F. novicida* U112 which had previously been shown to have defects in virulence or growth in macrophages displayed a range of virulence levels in chicken embryos. In total, the mutant strains were about 100-fold less virulent to more than 100,000-fold less virulent (Fig. 4) than the wild-type *F. novicida* U112 strain. Mutant GB2, which has a defect in the global regulator MglA, and mutant NZ9, which has a substitution in the pathogenicity island gene *pdpA*, are both avirulent in mice and were found to be unable to kill any of the six chicken embryos that were infected with inocula larger than 10^6 CFU. The previously described

mutants SC66, CG57, and CG69 exhibited intermediate levels of virulence. Strain SC66, which has a defect in O-antigen production, required an inoculum that was about 100 times that of its parent strain, strain U112, to induce 100% lethality. A *purA* mutant, CG57, was shown to be attenuated about 10,000-fold compared to wild-type *F. novicida*. Finally, mutant CG69, which has an insert in the gene encoding the heat shock-induced protease ClpB, was shown to be attenuated about 10^6-fold compared to the wild-type strain.

F. tularensis in chicken embryonic tissue

Infected chicken embryos were surveyed by microscopy to localize tissues that support *F. tularensis* growth. Embryos that had been infected with the U112 strain or LVS were sectioned, and bacteria were visualized with strain-specific antisera or monoclonal antibodies (Fig. 5 and 6). Two days after infection of the embryos, the immunoreactive foci were small and widely dispersed (Fig. 6A to C). Immunoreactive material was found in all of the tissues examined, including the heart, liver, kidney, and bone marrow. For both the U112 strain and LVS immunoreactivity was found inside individual chicken cells in which distinctive granular DAPI staining dominated the cytoplasm. Four days after infection, immunoreactive foci were abundant and were present throughout embryonic tissues. Bacterial cells appeared to be contained within chicken cells, and the infected cells were typically in clusters (Fig. 5A to F and 6D to I). Often, lesions had necrotic tissue at the center that was surrounded by a ring of infected cells. In some instances the host cell nucleus was pyknotic, which is typical of an apoptotic response (Fig. 5G).

These studies indicate that the chicken embryo system is a useful system for evaluating the virulence of *F. tularensis* strains. *F. tularensis* grew vigorously in chicken embryos and induced death within 1 week after inoculation. The bacteria were intracellular, and the structure of the lesions suggested that the initial infections spread by localized infection of adjacent cells. The use of chicken embryo infection is not intended to replace mammalian models of tularemia, which involve complex immune responses and approximate human tularemia. However, despite its flaws, *F. tularensis* infection of chicken embryos provides a rapid, inexpensive test to determine differences in levels of bacterial strain virulence that does not induce pain in animals. This procedure could be combined with in vitro intracellular assays to screen mutants, allowing researchers to plan subsequent animal experiments so that the minimal number of animals is used.

Table 1. *Francisella* sp. Strains

Strain	Genotype or phenotype	Reference
LVS	Live vaccine strain of *F. tularensis* subsp. *holarctica*	[35]
U112	Wild-type *F. novicida*	[77]
SC66	U112 derivative; lacks complete O-antigen; delayed growth in thioglycolate-induced C57BL/6 peritoneal macrophages	[24]
CG57	U112 derivative with insertion in *purA* gene affecting purine biosynthesis; suppressed growth in thioglycolate-induced C57BL/6 peritoneal macrophages	[61]
CG69	U112 derivative with insertion in *clpB* gene which encodes a heat shock response protease; suppressed growth in thioglycolate-induced C57BL/6 peritoneal macrophages	[61]
NZ9	U112 derivative in which the *pdpA* gene is replaced with an erythromycin resistance cassette; unable to grow in macrophages	[94]
GB2	U112 derivative; point mutation in *mglA* which encodes a global regulator of virulence; unable to grow in macrophages and avirulent in mice	[10]

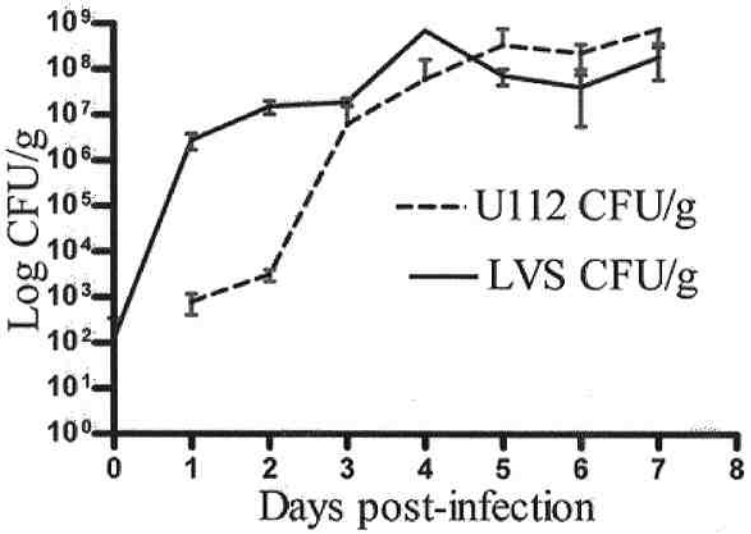

Figure 1. Growth of *F. tularensis* LVS and *F. novicida* in chicken embryos. Twenty-one chicken embryos were infected with 168 CFU of LVS or 159 CFU of *F. novicida* U112. Immediately after infection and every 24 h after infection the bacterial burden of three embryos was determined. At zero time for the U112 strain there were too few bacteria to count.

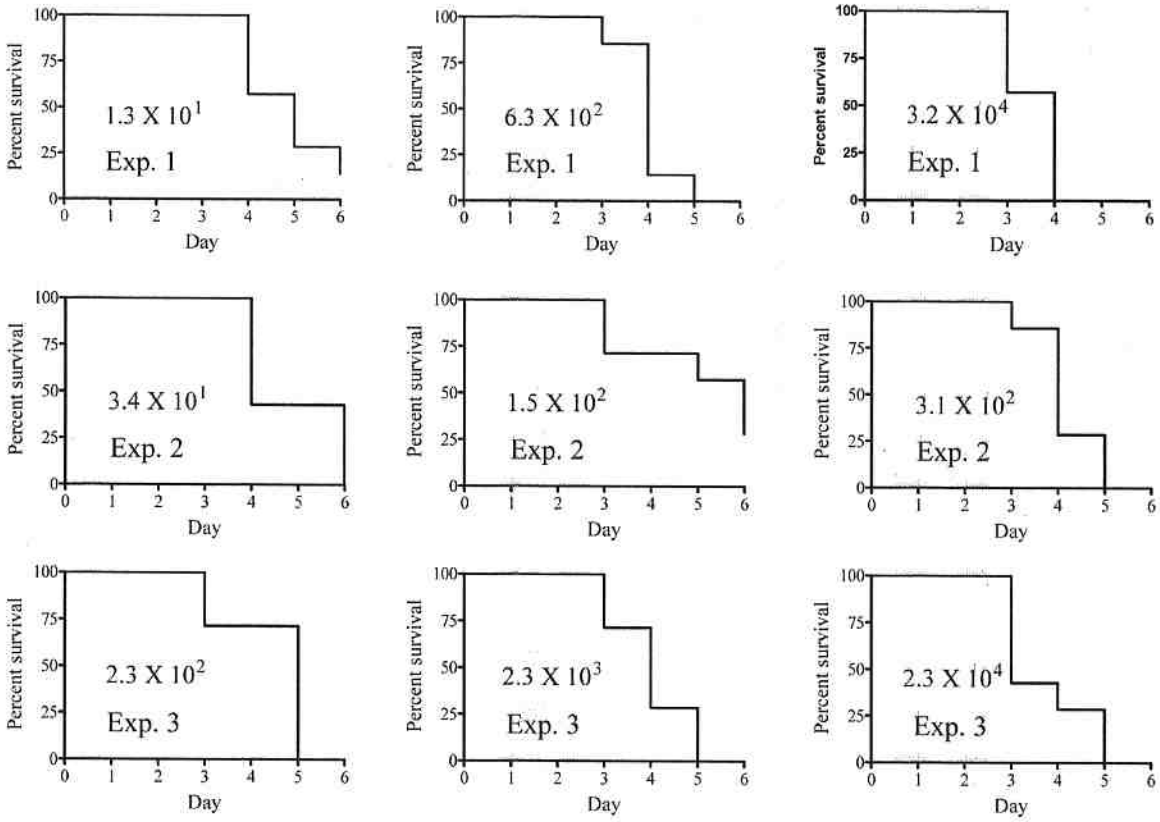

Figure 2. Reproducibility of the time to death induced by *F. novicida* U112. Three inocula that were different sizes were each used for infection of seven fertilized eggs. The inoculum used for infection (in CFU) is indicated on each graph. In each experiment eggs were infected on separate days and different lots of fertilized eggs were used.

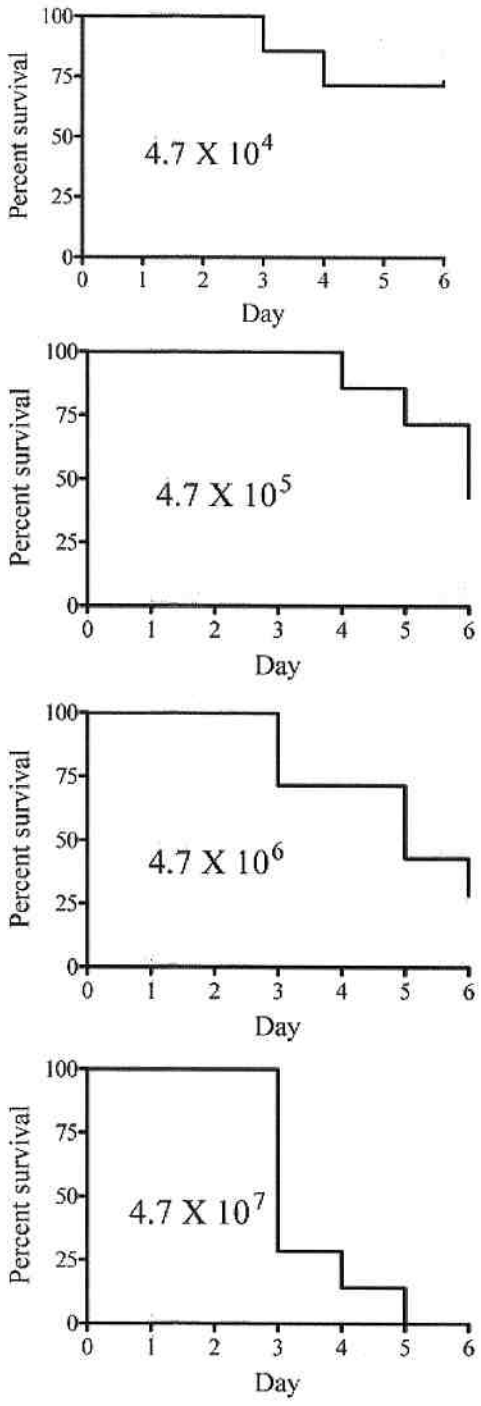

Figure 3. Virulence of *F. tularensis* LVS in chicken embryos. Seven fertilized eggs were infected using each inoculation dose, and the doses (in CFU) are indicated on the graphs. The infection series was repeated two times, and similar results were obtained.

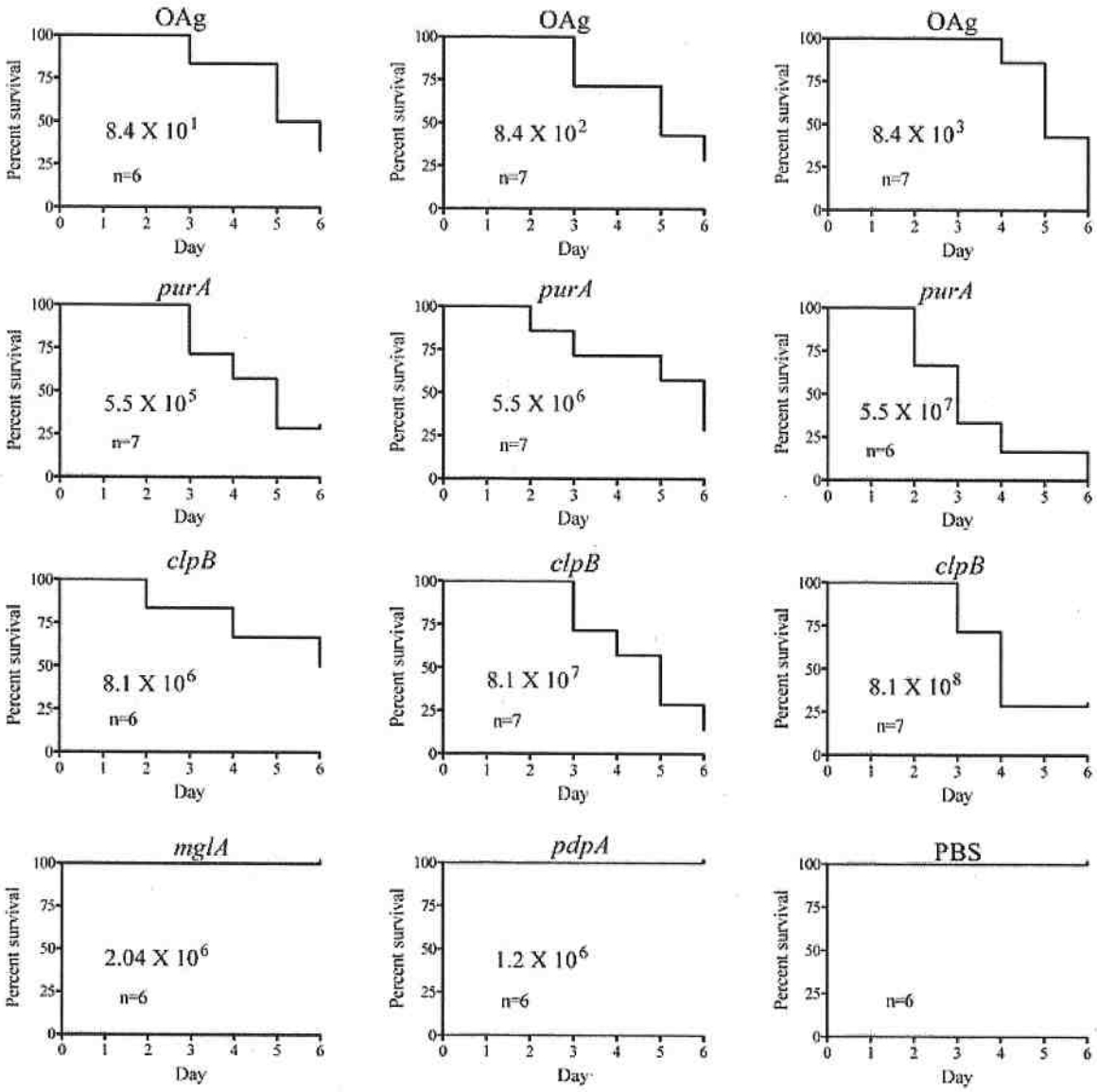

Figure 4. Levels of virulence of *F. tularensis* strains in chicken embryos. A dilution series was prepared for each *F. tularensis* strain, and seven embryos were infected with each inoculating dose. The death of the embryos was monitored each day. In some instances the embryos died within 24 h of inoculation (indicated by $n = 6$), and such deaths were attributed to injury induced during the inoculation and thus were discounted. The number in each graph indicates the inoculating dose (in CFU). The graphs labeled OAg, *purA*, *clpB*, *mglA*, and *pdpA* indicate the results for infections with strains SC66, CG57, CG69, GB2, and NZ9, respectively. The virulence of each strain was tested at least twice, and similar results were obtained in all trials.

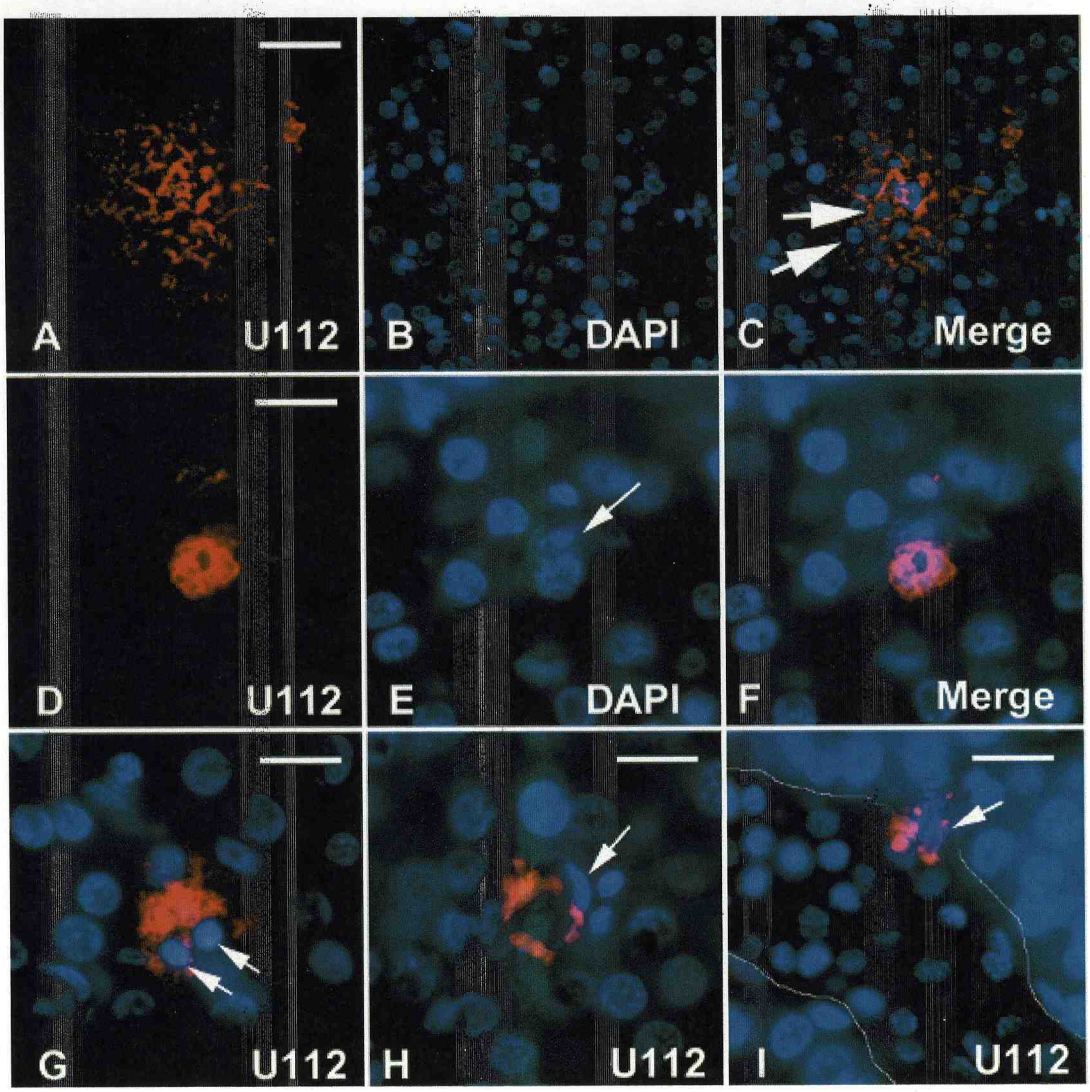

Figure 5. Immunofluorescence of *F. novicida* U112 in chicken embryonic tissues. (A to C) Focal infections in livers resulted in intense immunoreactivity in the cytoplasm of the central cells and a gradation of immunoreactive material in surrounding cells. The arrows indicate individual nuclei that were surrounded by immunoreactive cytoplasm. (D to F) Higher magnification, showing that the immunoreactive material in the cytoplasm also stained with DAPI in a granular pattern consistent with intracellular accumulation of bacterial cells (arrow). (G) In some liver cells the nuclei of infected cells were pyknotic and fragmented (arrows), which was indicative of an apoptotic response. (H) In some instances cells associated with sites of infection had the flattened nuclear morphology of liver macrophage cells. (I) Infected cells (arrow) were also associated with the walls of blood vessels (indicated by a thin white line). Nucleated erythrocytes are present in the blood vessels. All images are images of tissue from embryos at 3 days postinfection, and all tissues were reacted with anti-*F. novicida* rabbit serum. (A to C) Bar = 25 μm. (D to I) Bars = 15 μm.

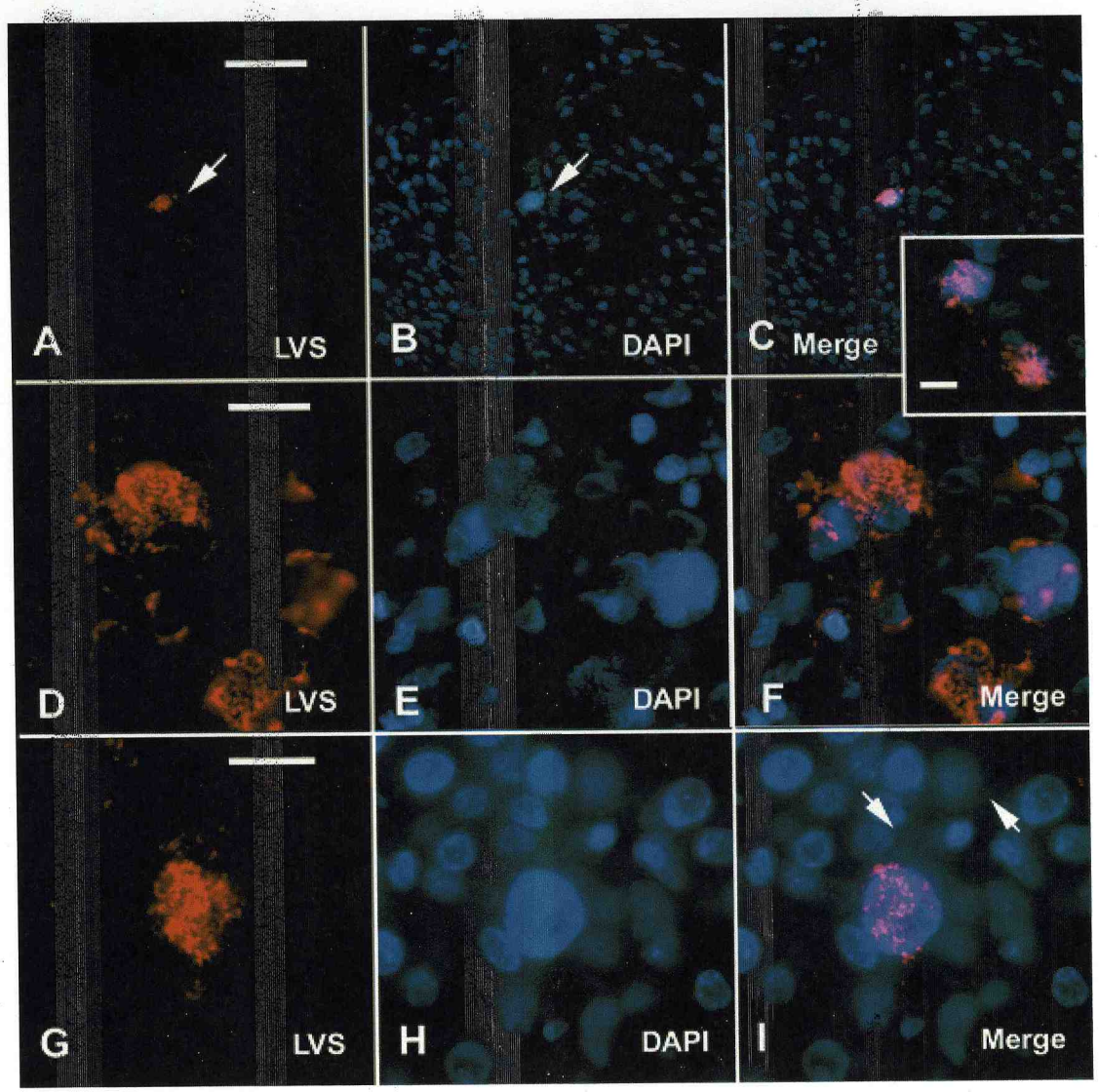

Figure 6. Immunofluorescent localization of *F. tularensis* LVS in chicken embryonic tissues. (A to C) In embryos 2 days after inoculation, immunoreactive cells were scattered throughout embryonic tissues. In sections through the heart immunoreactivity and characteristic granular DAPI staining were tightly clustered around individual heart cell nuclei (arrows and inset). (D to F) In embryos 4 days after inoculation, large foci of immunoreactive material were present in most tissues. Details of infected cells suggested that bacterial cells were in individual heart cells. (G to I) Focal infections also occurred in the liver 6 days after infection, and in individual cells there was granular immunoreactive material in the cytoplasm that also stained with DAPI. There was scattered immunoreactive material in the surrounding cells (arrows). Panels A to F show tissues that were reacted with anti-*F. tularensis* LVS monoclonal antibody that is reactive with O-antigen. Panels G to I show tissues that were reactive with anti-*F. tularensis* LVS rabbit serum. (A to C) Bar = 25 μm. (D to I) Bars = 15 μm. (Inset) Bar = 5 μm.

Appendix 2

BLAST results comparing the sequences of the deleted regions in each of the *pdpC* partial deletion mutants with the wildtype U112 *pdpC* sequence.

*pdpC*Δ9

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U112pdpC  1509  CTTTATAGACTGGTATTCTAATAAGAAAGAAAATCAAGAATTACTCAAAAACTCTTATTT  1568
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9     2    CTTTATAGACTGGTATTCTAATAAGAAAGAAAATCAAGAATTACTCAAAAACTCTTATTT  61

U112pdpC  1569  GAACAATTTATATAAGTCAAAGCAGAAATATATAAAAGATTTTATAGATCTTGATATTAT  1628
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9     62   GAACAATTTATATAAGTCAAAGCAGAAATATATAAAAGATTTTATAGATCTTGATATTAT  121

U112pdpC  1629  TAAACTTCTAAAACAGACATCATCTCAAATCAAGCCAAGCTATATACCAGTAGCTTTTAG  1688
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9     122  TAAACTTCTAAAACAGACATCATCTCAAATCAAGCCAAGCTATATACCAGTAGCTTTTAG  181

U112pdpC  1689  ATATGGTGCATTTGCCTCAACAACTGCTTTGATAAGATCAAATGCAAATGTTTCTAATCG  1748
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9     182  ATATGGTGCATTTGCCTCAACAACTGCTTTGATAAGATCAAATGCAAATGTTTCTAATCG  241

U112pdpC  1749  AATGGAGTTTGAATTAT  1765
                ||||||||||||||||
pdpCΔ9     242  AATGGAGTTTGAATTAT  258
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U112pdpC  1873  TTATTTAGCCTTAATCTAACAAATGAAAAAGAAGATGGACTTCGAAAAGTTATGTTGAAA  1932
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9     255  TTATTTAGCCTTAATCTAACAAATGAAAAAGAAGATGGACTTCGAAAAGTTATGTTGAAA  314

U112pdpC  1933  GTTGCACAACTATATAATCTTGATTTCAAGGTAGGTATCTCAGGAAATTTAGATCAAGCA  1992
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9     315  GTTGCACAACTATATAATCTTGATTTCAAGGTAGGTATCTCAGGAAATTTAGATCAAGCA  374

U112pdpC  1993  ATGACTCAGGCATTAATACTTGGGATGGCAACTACnnnnnnnnnTGGCGATATCTTACTT  2052
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9     375  ATGACTCAGGCATTAATACTTGGGATGGCAACTACAAAAAAAAATGGCGATATCTTACTT  434

U112pdpC  2053  GATGAAGATCAAATGCTATATATGACATACTTATACTGCATTTTCATGGCACATAGTGTC  2112
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9     435  GATGAAGATCAAATGCTATATATGACATACTTATACTGCATTTTCATGGCACATAGTGTC  494

U112pdpC  2113  GATCATACTGTAGATGAAATACTAATGTCTGCAAATACTTATCTGTTAAATTCTGAAGAG  2172
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9     495  GATCATACTGTAGATGAAATACTAATGTCTGCAAATACTTATCTGTTAAATTCTGAAGAG  554

U112pdpC  2173  GTAAAGTATCCTATATTTAATATAGCTGAnnnnnnnnGCTAGGCCTGTATTTGGACTCTCT  2232
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9     555  GTAAAGTATCCTATATTTAATATAGCTGATTTTTTTGCTAGGCCTGTATTTGGACTCTCT  614

U112pdpC  2233  AAGGATAAAGAGTTTAAATCATTAGTTGAAAAGTATGAAAATAGCCTAAAACCTAATTCA  2292
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9     615  AAGGATAAAGAGTTTAAATCATTAGTTGAAAAGTATGAAAATAGCCTAAAACCTAATTCA  674
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U112pdpC  2293  AAAATTCTCAAAGAGAATTATATAAGTAGAGTAGTCACACTATCTGAAGTATATGAAGAT  2352
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9    675   AAAATTCTCAAAGAGAATTATATAAGTAGAGTAGTCACACTATCTGAAGTATATGAAGAT  734

U112pdpC  2353  ATATATAACTTAAATTGTTTATATAGTTCTCTCTCAGAAGGATCCTTGTATAATCTACTA  2412
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9    735   ATATATAACTTAAATTGTTTATATAGTTCTCTCTCAGAAGGATCCTTGTATAATCTACTA  794

U112pdpC  2413  TCAACTCACAGTGAAAGACACTGTACTTTATTAGAGCAGTATTCTCGCAAGAAGAAAGCT  2472
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ9    795   TCAACTCACAGTGAAAGACACTGTACTTTATTAGAGCAGTATTCTCGCAAGAAGAAAGCT  854
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U112PdpC  504  FIDWYSNKKENQEXXXXXXXXXXXXXXQKYXXXXXXXXXXXXXXQTSSQIKPSYIPVAFR  563
               FIDWYSNKKENQELLKNSYLNNLYKSKQKYIKDFIDLDIIKLLKQTSSQIKPSYIPVAFR
PdpCΔ9     1   FIDWYSNKKENQELLKNSYLNNLYKSKQKYIKDFIDLDIIKLLKQTSSQIKPSYIPVAFR  60

U112PdpC  564  YGAFASTTALIRSNANVSNRMEFELYDSPERLHNQYSEKEEMIMPKSVNNPKDHSIDNGI  623
               YGAFASTTALIRSNANVSNRMEFEL
PdpCΔ9     61  YGAFASTTALIRSNANVSNRMEFEL----------------------------------  85

U112PdpC  624  SLFSLNLTNEKEDGLRKVMLKVAQLYNLDFKVGISGNLDQAMTQALILGMATTKKNGDIL  683
                 FSLNLTNEKEDGLRKVMLKVAQLYNLDFKVGISGNLDQAMTQALILGMATTKKNGDIL
PdpCΔ9     86  --FSLNLTNEKEDGLRKVMLKVAQLYNLDFKVGISGNLDQAMTQALILGMATTKKNGDIL  143

U112PdpC  684  LDEDQMLYMTYLYCIFMAHSVDHTVDEILMSANTYLLNSEEVKYPIFNIADFFARPVFGL  743
               LDEDQMLYMTYLYCIFMAHSVDHTVDEILMSANTYLLNSEEVKYPIFNIADFFARPVFGL
PdpCΔ9     144 LDEDQMLYMTYLYCIFMAHSVDHTVDEILMSANTYLLNSEEVKYPIFNIADFFARPVFGL  203

U112PdpC  744  SKDKEFKSLVEKYENSLKPNSKILKENYISRVVTLSEVYEDIYNLNCLYSSLSEGSLYNL  803
               SKDKEFKSLVEKYENSLKPNSKILKENYISRVVTLSEVYEDIYNLNCLYSSLSEGSLYNL
PdpCΔ9     204 SKDKEFKSLVEKYENSLKPNSKILKENYISRVVTLSEVYEDIYNLNCLYSSLSEGSLYNL  263
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*pdpC*Δ10

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U112pdpC  1735  AATGTTTCTAATCGAATGGAGTTTGAATTATATGACTCACCTGAAAGACTACATAATCAA  1794
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ10   4     AATGTTTCTAATCGAATGGAGTTTGAATTATATGACTCACCTGAAAGACTACATAATCAA  63

U112pdpC  1795  TACTCAGAAAAAGAGGAAATGATTATGCCAAAATCGGTTAATAACCCCAAGGATCACTCA  1854
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ10   64    TACTCAGAAAAAGAGGAAATGATTATGCCAAAATCGGTTAATAACCCCAAGGATCACTCA  123

U112pdpC  1855  ATTGACAATGGAATATCTTTATTTAGCCTTAATCTAACAAATGAAAAAGAAGATGGACTT  1914
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ10   124   ATTGACAATGGAATATCTTTATTTAGCCTTAATCTAACAAATGAAAAAGAAGATGGACTT  183

U112pdpC  1915  CGAAAAGTTATGTTGAAAGTTGCACAACTATATAATCTTGATTTCAAGGTAGGTATCTCA  1974
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ10   184   CGAAAAGTTATGTTGAAAGTTGCACAACTATATAATCTTGATTTCAAGGTAGGTATCTCA  243

U112pdpC  1975  GGAAATTTAGATCAAGCA  1992
                ||||||||||||||||||
pdpCΔ10   244   GGAAATTTAGATCAAGCA  261


U112pdpC  2169  AGAGGTAAAGTATCCTATATTTAATATAGCTGAnnnnnnnnGCTAGGCCTGTATTTGGACT  2228
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ10   261   AGAGGTAAAGTATCCTATATTTAATATAGCTGATTTTTTTTGCTAGGCCTGTATTTGGACT  320

U112pdpC  2229  CTCTAAGGATAAAGAGTTTAAATCATTAGTTGAAAAGTATGAAAATAGCCTAAAACCTAA  2288
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ10   321   CTCTAAGGATAAAGAGTTTAAATCATTAGTTGAAAAGTATGAAAATAGCCTAAAACCTAA  380
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U112pdpC   2289  TTCAAAAATTCTCAAAGAGAATTATATAAGTAGAGTAGTCACACTATCTGAAGTATATGA  2348
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ10    381   TTCAAAAATTCTCAAAGAGAATTATATAAGTAGAGTAGTCACACTATCTGAAGTATATGA  440

U112pdpC   2349  AGATATATATAACTTAAATTGTTTATATAGTTCTCTCTCAGAAGGATCCTTGTATAATCT  2408
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ10    441   AGATATATATAACTTAAATTGTTTATATAGTTCTCTCTCAGAAGGATCCTTGTATAATCT  500

U112pdpC   2409  ACTATCAACTCACAGTGAAAGACACTGTACTTTATTAGAGCAGTATTCTCGCAAGAAGAA  2468
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ10    501   ACTATCAACTCACAGTGAAAGACACTGTACTTTATTAGAGCAGTATTCTCGCAAGAAGAA  560

U112pdpC   2469  AGCTGAGATAGGATTAGTCCAAGATGGAGAGAAAATAAAAGTCGTAAACAACTACAGTGG  2528
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ10    561   AGCTGAGATAGGATTAGTCCAAGATGGAGAGAAAATAAAAGTCGTAAACAACTACAGTGG  620

U112pdpC   2529  CTATGCTGCGATTAATCAATATCAACGTTTTGTATCACTAGGCAGAATGTATGATAGTGG  2588
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ10    621   CTATGCTGCGATTAATCAATATCAACGTTTTGTATCACTAGGCAGAATGTATGATAGTGG  680

U112pdpC   2589  TGCAAAATACTCCTCTTCTCATAAAAAACAGATAGAAAAAGATTTCAACTTATACTTACC  2648
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ10    681   TGCAAAATACTCCTCTTCTCATAAAAAACAGATAGAAAAAGATTTCAACTTATACTTACC  740

U112pdpC   2649  AGATAAAGAACAGATAAATTATCTAAAATATAACTTTAAAGATAATAAATTTGATGCTGT  2708
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ10    741   AGATAAAGAACAGATAAATTATCTAAAATATAACTTTAAAGATAATAAATTTGATGCTGT  800
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U112PdpC   579  NVSNRMEFELYDSPERLHNQYSEKEEMIMPKSVNNPKDHSIDNGISLFSLNLTNEKEDGL  638
                NVSNRMEFELYDSPERLHNQYSEKEEMIMPKSVNNPKDHSIDNGISLFSLNLTNEKEDGL
PdpCΔ10    2    NVSNRMEFELYDSPERLHNQYSEKEEMIMPKSVNNPKDHSIDNGISLFSLNLTNEKEDGL  61

U112PdpC   639  RKVMLKVAQLYNLDFKVGISGNLDQAMTQALILGMATTKKNGDILLDEDQMLYMTYLYCI  698
                RKVMLKVAQLYNLDFKVGISGNLDQA
PdpCΔ10    62   RKVMLKVAQLYNLDFKVGISGNLDQA----------------------------------  87

U112PdpC   699  FMAHSVDHTVDEILMSANTYLLNSEEVKYPIFNIADFFARPVFGLSKDKEFKSLVEKYEN  758
                                        EVKYPIFNIADFFARPVFGLSKDKEFKSLVEKYEN
PdpCΔ10    88   -------------------------EVKYPIFNIADFFARPVFGLSKDKEFKSLVEKYEN  122

U112PdpC   759  SLKPNSKILKENYISRVVTLSEVYEDIYNLNCLYSSLSEGSLYNLLSTHSERHCTLLEQY  818
                SLKPNSKILKENYISRVVTLSEVYEDIYNLNCLYSSLSEGSLYNLLSTHSERHCTLLEQY
PdpCΔ10    123  SLKPNSKILKENYISRVVTLSEVYEDIYNLNCLYSSLSEGSLYNLLSTHSERHCTLLEQY  182

U112PdpC   819  SRKKKAEIGLVQDGEKIKVVNNYSGYAAINQYQRFVSLGRMYDSGAKYSSSHKKQIEKDF  878
                SRKKKAEIGLVQDGEKIKVVNNYSGYAAINQYQRFVSLGRMYDSGAKYSSSHKKQIEKDF
PdpCΔ10    183  SRKKKAEIGLVQDGEKIKVVNNYSGYAAINQYQRFVSLGRMYDSGAKYSSSHKKQIEKDF  242
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*pdpC*Δ11

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U112pdpC   2554  CGTTTTGTATCACTAGGCAGAATGTATGATAGTGGTGCAAAATACTCCTCTTCTCATAAA  2613
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ11    2     CGTTTTGTATCACTAGGCAGAATGTATGATAGTGGTGCAAAATACTCCTCTTCTCATAAA  61

U112pdpC   2614  AAACAGATAGAAAAAGATTTCAAC  2637
                 |||||||||||||||||||||||||
pdpCΔ11    62    AAACAGATAGAAAAAGATTTCAAC  85


U112pdpC   2827  ACTTTTTATAAAATAAAAGATAAATCAGGAAACTATTTAGTAAACTTACATAATGAAAAA  2886
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ11    86    ACTTTTTATAAAATAAAAGATAAATCAGGAAACTATTTAGTAAACTTACATAATGAAAAA  145
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U112pdpC  2887  TATAGTTTCGCAACACCTAATTCAGATTCTAAAATATATAGAGTTTCACCAGAATTACTT  2946
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ11   146   TATAGTTTCGCAACACCTAATTCAGATTCTAAAATATATAGAGTTTCACCAGAATTACTT  205

U112pdpC  2947  AACAATAGAGATGATTTTAAAAGAGTATCTAAAGATATTATAAAATCATATAAATATATT  3006
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ11   206   AACAATAGAGATGATTTTAAAAGAGTATCTAAAGATATTATAAAATCATATAAATATATT  265

U112pdpC  3007  AGTTTTGATAAACAAAAGGAAGATATAGTTAAAAATTTTGGTAAAAACTTATATCATACT  3066
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ11   266   AGTTTTGATAAACAAAAGGAAGATATAGTTAAAAATTTTGGTAAAAACTTATATCATACT  325

U112pdpC  3067  AATTATGAGATATGGGTTGGGCTATCACATCAAGCTATATCATGTTTTTCAGTTTTAGAT  3126
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ11   326   AATTATGAGATATGGGTTGGGCTATCACATCAAGCTATATCATGTTTTTCAGTTTTAGAT  385

U112pdpC  3127  AACATAGATACACAAGAAGCTGCTAATACTTTTATCGATGCTTTATATTATGTTAGATTA  3186
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ11   386   AACATAGATACACAAGAAGCTGCTAATACTTTTATCGATGCTTTATATTATGTTAGATTA  445

U112pdpC  3187  ATGCAATTATATTATGGCAAAACTATACCTTTTTTGTGGATTAGTAGCGAAATTGTAAGA  3246
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ11   446   ATGCAATTATATTATGGCAAAACTATACCTTTTTTGTGGATTAGTAGCGAAATTGTAAGA  505

U112pdpC  3247  TATTCTTCATCAGAAACTCACTACTATATACCAACAGAAGAAAACTTTGAAAATATACTT  3306
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ11   506   TATTCTTCATCAGAAACTCACTACTATATACCAACAGAAGAAAACTTTGAAAATATACTT  565

U112pdpC  3307  AAAATTGCTCTTAATAATGCCTCAAAACCAGTAATAGAAAGATTCTTAATTATTCTTAAC  3366
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ11   566   AAAATTGCTCTTAATAATGCCTCAAAACCAGTAATAGAAAGATTCTTAATTATTCTTAAC  625

U112pdpC  3367  ATATATAATAGTACTATTGATAATAATGCTTTAATTTTGATACGATGCCGTTTACCAATA  3426
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ11   626   ATATATAATAGTACTATTGATAATAATGCTTTAATTTTGATACGATGCCGTTTACCAATA  685

U112pdpC  3427  ATATTATTTGAATATGAAGAGCAGAAACTTAAATTAAAAGCTTTGCAAAAGTGTGCTGAT  3486
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ11   686   ATATTATTTGAATATGAAGAGCAGAAACTTAAATTAAAAGCTTTGCAAAAGTGTGCTGAT  745

U112pdpC  3487  TCTTATnnnnnnnnTAATTATAAAAATGAAATTAACTTTAGTGCTTGGTTTGAATCTATA  3546
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ11   746   TCTTATAAAAAAAATAATTATAAAAATGAAATTAACTTTAGTGCTTGGTTTGAATCTATA  805
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U112PdpC  852   RFVSLGRMYDSGAKYSSSHKKQIEKDFNLYLPDKEQINYLKYNFKDNKFDAVYKNKKSKE  911
                RFVSLGRMYDSGAKYSSSHKKQIEKDFN
PdpCΔ11   1     RFVSLGRMYDSGAKYSSSHKKQIEKDFN--------------------------------  28

U112PdpC  912   KNQSHIVYAKKQNTRYCYGYFNDFFVKNRITTFYKIKDKSGNYLVNLHNEKYSFATPNSD  971
                                              TFYKIKDKSGNYLVNLHNEKYSFATPNSD
PdpCΔ11   29    --------------------------------TFYKIKDKSGNYLVNLHNEKYSFATPNSD  57

U112PdpC  972   SKIYRVSPELLNNRDDFKRVSKDIIKSYKYISFDKQKEDIVKNFGKNLYHTNYEIWVGLS  1031
                SKIYRVSPELLNNRDDFKRVSKDIIKSYKYISFDKQKEDIVKNFGKNLYHTNYEIWVGLS
PdpCΔ11   58    SKIYRVSPELLNNRDDFKRVSKDIIKSYKYISFDKQKEDIVKNFGKNLYHTNYEIWVGLS  117

U112PdpC  1032  HQAISCFSVLDNIDTQEAANTFIDALYYVRLMQLYYGKTIPFLWISSEIVRYSSSETHYY  1091
                HQAISCFSVLDNIDTQEAANTFIDALYYVRLMQLYYGKTIPFLWISSEIVRYSSSETHYY
PdpCΔ11   118   HQAISCFSVLDNIDTQEAANTFIDALYYVRLMQLYYGKTIPFLWISSEIVRYSSSETHYY  177

U112PdpC  1092  IPTEENFENILKIALNNASKPVIERFLIILNIYNSTIDNNAXXXXXXXXXXXXFEYEEXX  1151
                IPTEENFENILKIALNNASKPVIERFLIILNIYNSTIDNNALILIRCRLPIILFEYEEQK
PdpCΔ11   178   IPTEENFENILKIALNNASKPVIERFLIILNIYNSTIDNNALILIRCRLPIILFEYEEQK  237
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pdpCΔ12

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U112pdpC  3111  GTTTTTCAGTTTTAGATAACATAGATACACAAGAAGCTGCTAATACTT  3158
                ||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ12   4     GTTTTTCAGTTTTAGATAACATAGATACACAAGAAGCTGCTAATACTT  51

U112pdpC  3322  TAATGCCTCAAAACCAGTAATAGAAAGATTCTTAATTATTCTTAACATATATAATAGTAC  3381
                ||||  | ||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ12   44    TAATACTTCAAAACCAGTAATAGAAAGATTCTTAATTATTCTTAACATATATAATAGTAC  103

U112pdpC  3382  TATTGATAATAATGCTTTAATTTTGATACGATGCCGTTTACCAATAATATTATTTGAATA  3441
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ12   104   TATTGATAATAATGCTTTAATTTTGATACGATGCCGTTTACCAATAATATTATTTGAATA  163

U112pdpC  3442  TGAAGAGCAGAAACTTAAATTAAAAGCTTTGCAAAAGTGTGCTGATTCTTATnnnnnnnn  3501
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ12   164   TGAAGAGCAGAAACTTAAATTAAAAGCTTTGCAAAAGTGTGCTGATTCTTATAAAAAAAA  223

U112pdpC  3502  TAATTATAAAAATGAAATTAACTTTAGTGCTTGGTTTGAATCTATATTTCATGTTCAAAA  3561
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ12   224   TAATTATAAAAATGAAATTAACTTTAGTGCTTGGTTTGAATCTATATTTCATGTTCAAAA  283

U112pdpC  3562  CTTATCCTTATCTCCTAATTATATTGGCAATAATATTTTAATCTTGAGTTTAGTTGAAGA  3621
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ12   284   CTTATCCTTATCTCCTAATTATATTGGCAATAATATTTTAATCTTGAGTTTAGTTGAAGA  343

U112pdpC  3622  ATTAAAACCAAAATGTAGTGAATATnnnnnnnnCATGATTAACCAACAAATTACAGCTAA  3681
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ12   344   ATTAAAACCAAAATGTAGTGAATATAAAAAAAACATGATTAACCAACAAATTACAGCTAA  403

U112pdpC  3682  AGCAAACGAGTTAAACTTTTATCAACTTCTAATAAGACTTATTGAAATATCAACCTATCA  3741
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ12   404   AGCAAACGAGTTAAACTTTTATCAACTTCTAATAAGACTTATTGAAATATCAACCTATCA  463

U112pdpC  3742  TAGAATACTTACGAAATCAATCATAACTAATACAACCTCGTTACTTTATGACATATTAAA  3801
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ12   464   TAGAATACTTACGAAATCAATCATAACTAATACAACCTCGTTACTTTATGACATATTAAA  523

U112pdpC  3802  TAAACCAGAATTTCAAACAATAAATAAATTAATAAATAAGCTATTTATTTATAAAAACAA  3861
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ12   524   TAAACCAGAATTTCAAACAATAAATAAATTAATAAATAAGCTATTTATTTATAAAAACAA  583

U112pdpC  3862  GGATCTAAATACTGATAAATATAAAGCCTTTCACACTAAGCTTATAACTATTGAAAGTAC  3921
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ12   584   GGATCTAAATACTGATAAATATAAAGCCTTTCACACTAAGCTTATAACTATTGAAAGTAC  643

U112pdpC  3922  CTATAAAAAAAATAAATAGCCTATATAAATCAGACTTTTTTAAAAAAATATCGTCA  3976
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||
pdpCΔ12   644   CTATAAAAAAAATAAATAGCCTATATAAATCAGACTTTTTTAAAAAAATATCGTCA  698
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U112PdpC  1037  CFSVLDNIDTQEAANTFIDALYYVRLMQLYYGKTIPFLWISSEIVRYSSSETHYYIPTEE  1096
                CFSVLDNIDTQEAANT
PdpCΔ12   1     CFSVLDNIDTQEAANT----------------------------------------------  16

U112PdpC  1097  NFENILKIALNNASKPVIERFLIILNIYNSTIDNNAXXXXXXXXXXXXXFEYEEXXXXXXX  1156
                          SKPVIERFLIILNIYNSTIDNNALILIRCRLPIILFEYEEQKLKLKA
PdpCΔ12   17    -------------SKPVIERFLIILNIYNSTIDNNALILIRCRLPIILFEYEEQKLKLKA  63

U112PdpC  1157  XXXCADSYKKNNYKNEINFSAWFESIFHVQNLSLSPNYIGNNILILSLVEELKPKCSEYK  1216
                LQKCADSYKKNNYKNEINFSAWFESIFHVQNLSLSPNYIGNNILILSLVEELKPKCSEYK
PdpCΔ12   64    LQKCADSYKKNNYKNEINFSAWFESIFHVQNLSLSPNYIGNNILILSLVEELKPKCSEYK  123

U112PdpC  1217  KNMINQQITAKANELNFYQLLIRLIEISTYHRILTKSIITNTTSLLYDILNKPEFQTINK  1276
                KNMINQQITAKANELNFYQLLIRLIEISTYHRILTKSIITNTTSLLYDILNKPEFQTINK
PdpCΔ12   124   KNMINQQITAKANELNFYQLLIRLIEISTYHRILTKSIITNTTSLLYDILNKPEFQTINK  183

U112PdpC  1277  LINKLFIYKNKDLNTDKYKAFHTKLITIESTYKKINSLYKSDFFKKISS  1325
                LINKLFIYKNKDLNTDKYKAFHTKLITIESTYKKINSLYKSDFFKKISS
PdpCΔ12   184   LINKLFIYKNKDLNTDKYKAFHTKLITIESTYKKINSLYKSDFFKKISS  232
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Appendix 3

BLAST results comparing the amino acid sequences translated from DNA sequencing of regions of *pdpC* in the V37 pDNR MCS SA donor vector with the wildtype U112 *pdpC* sequence.

cintF

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U112pdpC  10   YFEKIELPKTADFNMISKHDIKELRVDANLKKKIHLLQFDEDYLAYMRSLRAIHPSKIAM  69
               Y  +IELPKTADFNMISKHDIKELRVDANLKKKIHLLQFDEDYLAYMRSLRAIHPSKIAM
pdpCint   1    YLFEIELPKTADFNMISKHDIKELRVDANLKKKIHLLQFDEDYLAYMRSLRAIHPSKIAM  60

U112pdpC  70   QKIKSIRNKEDSFIIAIFSLDKIIHKTKFISFGHKTVIFDFKKLWGLVDFVIVHTSNKTW  129
               QKIKSIRNKEDSFIIAIFSLDKIIHKTKFISFGHKTVIFDFKKLWGLVDFVIVHTSNKTW
pdpCint   61   QKIKSIRNKEDSFIIAIFSLDKIIHKTKFISFGHKTVIFDFKKLWGLVDFVIVHTSNKTW  120

U112pdpC  130  VNHKLTSFMPSITYCNQNIIHLAYHSDFLYIYHTPEFMDDINVDRENRRELVAKIPDPYW  189
               VNHKLTSFMPSITYCNQNIIHLAYHSDFLYIYHTPEFMDDINVDRENRRELVAKIPDPYW
pdpCint   121  VNHKLTSFMPSITYCNQNIIHLAYHSDFLYIYHTPEFMDDINVDRENRRELVAKIPDPYW  180

U112pdpC  190  VRADTXXXXXXXXXXXXXXXDLKKITKLKNFEISANDIFFSKAIKAAPRLQHKNKSKLF  249
               VRADTKENKININSEEENIEKDLKKITKLKNFEISANDIFFSKAIKAAPRLQHKNKSKLF
pdpCint   181  VRADTKENKININSEEENIEKDLKKITKLKNFEISANDIFFSKAIKAAPRLQHKNKSKLF  240

U112pdpC  250  NSLAIENNEKIKRDIIDYAISNAWYKNEGLLENLMTFLDALVVRHLYLIAVYSVYEIEIG  309
               NSLAIENNEKIKRDIIDYAISNAWYK EGLLENLMTFLDALVVRHLYLIAVYSVYEIEIG
pdpCint   241  NSLAIENNEKIKRDIIDYAISNAWYKTEGLLENLMTFLDALVVRHLYLIAVYSVYEIEIG  298
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cint 4

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U112pdpC  282  NLMTFLDALVVRHLYLIAVYSVYEIEIGIKSVKPEYSKLLKAGLLNKDIQNQLIYDQKKI  341
               NLMTFLDALVVRHLYLIAVYSVYEIEIGI+SVKPEYSKLLKAGLLNKDIQNQLIYDQKKI
pdpCint   1    NLMTFLDALVVRHLYLIAVYSVYEIEIGIQSVKPEYSKLLKAGLLNKDIQNQLIYDQKKI  60

U112pdpC  342  SNIIWLGETFHGLDIEEAEDLCELIDQGDINPKINPINPEKLYRAYKENYGNEALSFREY  401
               SNIIWLGETFHGLDIEEAEDLCELIDQGDINPKINPINPEKLYRAYKENYGNEALSFREY
pdpCint   61   SNIIWLGETFHGLDIEEAEDLCELIDQGDINPKINPINPEKLYRAYKENYGNEALSFREY  120

U112pdpC  402  KEKYNFLDNDKLREEHANKLSSILEDPKFRVLSYINAFLCSTKNYLVPYGYLGSNPLTYY  461
               KEKYNFLDNDKLREEHANKLSSILEDPKFRVLSYINAFLCSTKNYLVPYGYLGSNPLTYY
pdpCint   121  KEKYNFLDNDKLREEHANKLSSILEDPKFRVLSYINAFLCSTKNYLVPYGYLGSNPLTYY  180

U112pdpC  462  NCMLETGKRRTSKE  475
               NCMLETGKRRTSKE
pdpCint   181  NCMLETGKRRTSKE  194
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cint 7

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U112pdpC  476  AYFADIRNKLFMVCYLPGFLSSVIADDDFIDWYSNKKENQEXXXXXXXXXXXXXXXQKYXX  535
               AYFADIRNKLFMVCYLPGFLSSVIADDDFIDWYSNKKENQELLKNSYLNNLYKSKQKYIK
pdpCint   1    AYFADIRNKLFMVCYLPGFLSSVIADDDFIDWYSNKKENQELLKNSYLNNLYKSKQKYIK  60

U112pdpC  536  XXXXXXXXXXXXQTSSQIKPSYIPVAFRYGAFASTTALIRSNANVSNRMEFELYDSPERL  595
               DFIDLDIIKLLKQTSSQIKPSYIPVAFRYGAFASTTALIRSNANVSNRMEFELYDSPERL
pdpCint   61   DFIDLDIIKLLKQTSSQIKPSYIPVAFRYGAFASTTALIRSNANVSNRMEFELYDSPERL  120

U112pdpC  596  HNQYSEKEEMIMPKSVNNPKDHSIDNGISLFSLNLTNEKEDGLRKVMLKVAQLYNLDFKV  655
               HNQYSEKEEMIMPKSVNNPKDHSIDNGISLFSLNLTNEKEDGLRKVMLKVAQLYNLDFKV
pdpCint   121  HNQYSEKEEMIMPKSVNNPKDHSIDNGISLFSLNLTNEKEDGLRKVMLKVAQLYNLDFKV  180
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U112pdpC  656  GISGNLDQAMTQALILGMATTKKNGDILLDEDQMLYMTYLYCIFMAHSVDHTVDEILMSA  715
               GISGNLDQAMTQALILGMATTKKNGDILLDEDQMLYMTYLYCIFMAHSVDHTVDEILMSA
pdpCint   181  GISGNLDQAMTQALILGMATTKKNGDILLDEDQMLYMTYLYCIFMAHSVDHTVDEILMSA  240

U112pdpC  716  NTYLLNSEEVKYPIFNIADFFA  737
               NTYLLNSEEVKYPIF  + F A
pdpCint   241  NTYLLNSEEVKYPIFKYSFFCA  262


end of cint7F
U112pdpC  747  KEFKSLVEKYENSLKPNSKILKENYISRVVTLSEVYEDIYNLNCL  791
               K  KSLVEKYENSLKPNSKILKENYISRVVTLSEVYEDIYNLNCL
pdpCint   22   KSLKSLVEKYENSLKPNSKILKENYISRVVTLSEVYEDIYNLNCL  66


cint 9
U112pdpC  751    SLVEKYENSLKPNSKILKENYISRVVTLSEVYEDIYNLNCLYSSLSEGSLYNLLSTHSER  810
                 SLVEKYENSLKPNSKILKENYISRVVTLSEVYEDIYNLNCLYSSLSEGSLYNLLSTHSER
pdpCint   1      SLVEKYENSLKPNSKILKENYISRVVTLSEVYEDIYNLNCLYSSLSEGSLYNLLSTHSER  60

U112pdpC  811    HCTLLEQYSRKKKAEIGLVQDGEKIKVVNNYSGYAAINQYQRFVSLGRMYDSGAKYSSSH  870
                 HCTLLEQYSRKKKAEIGLVQDGEKIKVVNNYSGYAAINQYQRFVSLGRMYDSGAKYSSSH
pdpCint   61     HCTLLEQYSRKKKAEIGLVQDGEKIKVVNNYSGYAAINQYQRFVSLGRMYDSGAKYSSSH  120

U112pdpC  871    KKQIEKDFNLYLPDKEQINYLKYNFKDNKFDAVYKNKKSKEKNQSHIVYAKKQNTRYCYG  930
                 KKQIEKDFNLYLPDKEQINYLKYNFKDNKFDAVYKNKKSKEKNQSHIVYAKKQNTRYCYG
pdpCint   121    KKQIEKDFNLYLPDKEQINYLKYNFKDNKFDAVYKNKKSKEKNQSHIVYAKKQNTRYCYG  180

U112pdpC  931    YFNDFFVKNRITTFYKIKDKSGNYLVNLHNEKYSFATPNSDSKIYRVSPELLNNRDDFKR  990
                 YFNDFFVKNRITTFYKIKDKSGNYLVNLHNEKYSFATPNSDSKIYRVSPELLNNRDDFKR
pdpCint   181    YFNDFFVKNRITTFYKIKDKSGNYLVNLHNEKYSFATPNSDSKIYRVSPELLNNRDDFKR  240

U112pdpC  991    VSKDIIKSYKYISFDKQKEDIVKNFGKXLYHTNYEIWVGLSHQAISCF  1039
                 VSKDIIKSYKYISFDKQKEDIVKNFGKXLYHTNYEIWVGLSHQAISCF
pdpCint   241    VSKDIIKSYKYISFDKQKEDIVKNFGKXLYHTNYEIWVGLSHQAISCF  288


cintR
U112pdpC  1039   SVLDNIDTQEAANTFIDALYYVRLMQLYYGKTIPFLWISSEIVRYSSSETHYYIPTEENF  1098
                 SVLDNIDTQEAANTFIDALYYVRLMQLYYGKTIPFLWISSEIVRYSSSETHYYIPTEENF
pdpCint   1      SVLDNIDTQEAANTFIDALYYVRLMQLYYGKTIPFLWISSEIVRYSSSETHYYIPTEENF  60

U112pdpC  1099   ENILKIALNNASKPVIERFLIILNIYNSTIDNNAXXXXXXXXXXXXXFEYEEXXXXXXXXX  1158
                 ENILKIALNNASKPVIERFLIILNIYNSTIDNNALILIRCRLPIILFEYEEQKLKLKALQ
pdpCint   61     ENILKIALNNASKPVIERFLIILNIYNSTIDNNALILIRCRLPIILFEYEEQKLKLKALQ  120

U112pdpC  1159   XCADSYKKNNYKNEINFSAWFESIFHVQNLSLSPNYIGNNILILSLVEELKPKCSEYKKN  1218
                 KCADSYKKNNYKNEINFSAWFESIFHVQNLSLSPNYIGNNILILSLVEELKPKCSEYKKN
pdpCint   121    KCADSYKKNNYKNEINFSAWFESIFHVQNLSLSPNYIGNNILILSLVEELKPKCSEYKKN  180

U112pdpC  1219   MINQQITAKANELNFYQLLIRLIEISTYHRILTKSIITNTTSLLYDILNKPEFQTINKLI  1278
                 MINQQITAKANELNFYQLLIRLIEISTYHRILTKSIITNTTSLLYDILNKPEFQTINKLI
pdpCint   181    MINQQITAKANELNFYQLLIRLIEISTYHRILTKSIITNTTSLLYDILNKPEFQTINKLI  243

U112pdpC  1279   NKLFIYKNKDLNTDKYKAFHTKLITIESTYKKINSLYKSDFFKK  1322
                 NKLFIYKNKDLNTDKYKAFHTKLITIESTYKKINSLYKSDFFKK
pdpCint   244    NKLFIYKNKDLNTDKYKAFHTKLITIESTYKKINSLYKSDFFKK  287
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