Stony Brook University

OFFICIAL COPY

The official electronic file of this thesis or dissertation is maintained by the University Libraries on behalf of The Graduate School at Stony Brook University.

© All Rights Reserved by Author.

Characterization of Outer Membrane Vesicles and Nanotubes in *Francisella*

A Dissertation Presented

by

William D. McCaig

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Genetics

Stony Brook University

December 2012

Stony Brook University

The Graduate School

William Daniel McCaig

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend

acceptance of this dissertation.

Dr. David G. Thanassi – Dissertation Advisor
Professor Department of Molecular Genetics and Microbiology

Dr. Adrianus van der Velden - Chairperson of Defense
Assistant Professor Department of Molecular Genetics and Microbiology

Dr. Martha B. Furie
Professor Department of Pathology

Dr. Erwin London
Professor Department of Biochemistry and Cell Biology

Dr. Adam Ratner
Assistant Professor of Pediatrics
Columbia University

This dissertation is accepted by the Graduate School

Charles Taber
Interim Dean of the Graduate School

Abstract of the Dissertation

Characterization of Outer Membrane Vesicles and Nanotubes in *Francisella*

by

William Daniel McCaig

Doctor of Philosophy

in

Genetics

Stony Brook University

2012

Francisella spp. are highly infectious and virulent bacteria that cause the zoonotic disease tularemia. The identification of virulence factors and mechanisms of virulence factor secretion by *Francisella* spp. are not well understood. Gram-negative bacteria constitutively release vesicles from their cell surface, and these outer membrane vesicles (OMV) may function in the delivery of virulence factors to host cells. In addition, prokaryotic and eukaryotic cells have been shown to produce membrane-enclosed projections, termed nanotubes (NT), which appear to function in cell-cell communication and exchange of molecules. Examination of *Francisella* bacteria revealed the presence of NT extending out from the bacterial surface, and purification of OMV resulted in a heterogeneous mixture of OMV and NT. Proteomic analysis of gradient-purified OMV and NT identified 292 protein constituents, including known *Francisella* secreted proteins and virulence factors.

Francisella produced the OMV and NT in a regulated manner. In contrast to previously characterized NT, the *F. novicida* NT were produced by bacteria grown in liquid as well as on solid medium, and were derived from the outer membrane rather than the cytoplasmic

membrane. An increase in the number of OMV and NT was observed when bacteria are grown in brain heart infusion (BHI) medium, a growth condition which has been shown to more closely resemble infection of host cells. In addition, infection of host cells stimulated the production of NT by *F. novicida*. The OMV/NT are effective at shielding cargo proteins from extracellular proteases and the NT structure is resistant to numerous forms of chemical disruption. NT appear to be sensitive to treatment with high levels of heat, as evidenced by disruption of these structures when so treated.

The effects of purified OMV/NT on host cells were examined and their use as a potential subunit vaccine explored. Purified OMV/NT incubated with primary murine macrophages show a minor cytotoxic effect at high doses over long periods of time. Interestingly, at earlier time points and lower doses, proinflammatory cytokines are released when purified OMV/NT are incubated with macrophages. The OMV/NT must be intact for the majority of this cytokine response, as OMV/NT disrupted by heat treatment showed a marked reduction in levels of cytokines released by host cells. Mice vaccinated intranasally with purified OMV/NT and subsequently challenged with high doses of wild-type *F. novicida* were delayed in time to death or survived the challenge entirely.

This work shows that *Francisella* produces OMV and NT in a regulated manner and reveals a novel class of bacterial NT. The presence of known virulence factors and effects of the vesicles on host cells suggests roles for the OMV and NT in the pathogenesis of tularemia and opens up the possibility for generation of an effective component-based vaccine.

Table of Contents

ABSTRACT OF THE DISSERTATION iii
LIST OF FIGURES viii
LIST OF TABLES ix
ABBREVIATIONS x
ACKNOWLEDGEMENTS xi
INTRODUCTION 1
I. *Francisella tularensis* 1
II. *Francisella* virulence 3
III. *Francisella* secretion 9
IV. Outer membrane vesicles 11
V. Nanotubes 16
VI. Figures 18
MATERIALS AND METHODS 20
I. Bacterial strains, media and growth conditions 20
II. Protein profiles of cell-free culture supernatants 20
III. Purification of OMV and NT 21
IV. Electron microscopy 22
V. Sample preparation for TEM 23
VI. Protein quantification 23
VII. Fractionation of *F. novicida* 23
VIII. Protease accessibility assays 25
IX. Heat treatment of OMV/NT 25
X. Lysozyme treatment of OMV/NT and whole bacteria 26
XI. Multidimensional chromatography and tandem mass spectrometry 26
XII. Mass spectrometry data analysis 27
XIII. Preparation of macrophages 29
XIV. Macrophage co-incubation with *F. novicida* 29
XV. Cytotoxicity assays 30
XVI. Detection of cytokine secretion 31
XVII. Mouse vaccination 31

XVIII. Statistical analysis 32
XIX. Whole proteome analysis 32
XVIII. Chemical treatment of OMV/NT 33
XVIII. Cryo-EM tomography 33
RESULTS 34
Isolation of outer membrane vesicles in *F. novicida* 34
I. Introduction 34
II. Results 36
A. *Francisella* produces OMV and NT in response to growth phase and medium 36
B. Purification and initial characterization of *Francisella* OMV and NT 39
C. Identification of *F. novicida* OMV and NT-associated proteins 41
D. *F. novicida* OMV/NT are minimally cytotoxic to host cells 45
E. *F. novicida* OMV/NT produce a dose-dependent cytokine response 46
F. *F. novicida* produces NT during infection of host cells 47
G. Vaccination with *F. novicida* OMV/NT provides protection against bacterial challenge ..48
III. Figures 50
IV. Tables 62
Characterization of nanotubes in *F. novicida* 98
I. Introduction 98
II. Results 99
A. Whole proteome analysis of *Francisella novicida* 99
B. *Francisella novicida* mutant screen 100
C. Separation of OMV from NT 101
D. NT structure 102
E. Cryo-EM tomography 103
III. Figures 105
IV. Tables 109
F. tularensis OMV and NT 123
I. Introduction 123

II. Results 124
A. *F. tularensis* produces NT in addition to OMV 124
B. *F. tularensis* OMV and NT 124
III. Figures 127
IV. Tables 129
DISCUSSION 148
I. *Francisella novicida* outer membrane vesicle isolation and characterization 148
II. *Francisella novicida* nanotube characterization 155
III. *Francisella tularensis* outer membrane vesicle isolation and characterization 157
IV. Conclusions and future directions 159
BIBLIOGRAPHY 162

List of Figures

FIGURE 1-1. INTRACELLULAR LIFE CYCLE OF *F. TULARENSIS.* 18
FIGURE 1-2. MODEL OF VESICLE BIOGENESIS. 18
FIGURE 3-1. *F. TULARENSIS SUBSP. NOVICIDA* GROWTH IN BHI MEDIA. 50
FIGURE 3-2. OPTIPREP FLOTATION OF OMV/NT. 51
FIGURE 3-3. TEM IMAGES OF *F. TULARENSIS* SUBSP. *NOVICIDA* PURIFIED OMV/NT. 52
FIGURE 3-4. TEM IMAGES OF *F. TULARENSIS SUBSP. NOVICIDA* WHOLE BACTERIA AND THIN-SECTIONS. 53
FIGURE 3-5. TEM IMAGES OF *F. TULARENSIS* SUBSP. *NOVICIDA* WHOLE BACTERIA. 54
FIGURE 3-6. PROTEIN PROFILES DIFFER AMONG OMV/NT, SECRETED PROTEINS AND OTHER FRACTIONS. 55
FIGURE 3-7. PROTEINASE ACCESSIBILITY ASSAY. 56
FIGURE 3-8. DISRUPTION OF OMV/NT. 57
FIGURE 3-9. PREDICTED LOCALIZATION OF OMV/NT-ASSOCIATED PROTEINS. 58
FIGURE 3-10. OMV/NT CYTOTOXICITY AND CYTOKINE RELEASE. 59
FIGURE 3-11. THIN-SECTION TEM IMAGES OF *F. NOVICIDA* DURING INFECTION OF MUBMDM. 60
FIGURE 3-12. C3H/HEN MOUSE VACCINATION STUDY. 61
FIGURE 4-1. TEM IMAGES OF DIFFERENTIAL CENTRIFUGATION ATTEMPTED SEPARATION OF OMV/NT. 105
FIGURE 4-2. TEM IMAGES OF DENSITY FLOTATION ATTEMPTED SEPARATION OF OMV/NT. 106
FIGURE 4-3. TEM IMAGES OF CHEMICAL TREATMENT OF PURIFIED OMV/NT. 107
FIGURE 4-4. CRYO-EM IMAGES OF *F. NOVICIDA* AND PURIFIED NT. 108
FIGURE 5-1. TEM IMAGES OF *F. TULARENSIS* WHOLE BACTERIA. 127
FIGURE 5-2. TEM IMAGES OF *F. TULARENSIS* OMV/NT. 128

List of Tables

TABLE 3-1. *F. NOVICIDA* EXPONENTIAL PHASE OMV/NT-ASSOCIATED PROTEINS. . 62
TABLE 3-2. *F. NOVICIDA* STATIONARY PHASE OMV/NT-ASSOCIATED PROTEINS. 68
TABLE 3-3. DIFFERENTIAL PROTEIN CONTENT BETWEEN EXPONENTIAL AND STATIONARY PHASE OMV/NT. 84
TABLE 3-4. *FRANCISELLA* OUTER MEMBRANE-ASSOCIATED PROTEINS. 86
TABLE 3-5. GRAM-NEGATIVE OMV-ASSOCIATED PROTEINS. 90
TABLE 3-6. OMV/NT-ASSOCIATED PROTEINS IDENTIFIED PREVIOUSLY AS SECRETED OR VIRULENCE FACTORS. 95
TABLE 4-1. DIFFERENTIALLY REGULATED PROTEINS DETERMINED BY WHOLE PROTEOME ANALYSIS. 109
TABLE 4-2. *F. NOVICIDA* MUTANTS SCREENED FOR NT PRODUCTION DEFECTS. .. 120
TABLE 5-1. *F. TULARENSIS* STATIONARY PHASE OMV/NT-ASSOCIATED PROTEINS. 129
TABLE 5-2. *F. TULARENSIS* OMV/NT-ASSOCIATED PROTEINS IDENTIFIED PREVIOUSLY AS VIRULENCE FACTORS. 140
TABLE 5-3. DIFFERENTIAL PROTEIN CONTENT BETWEEN *F. TULARENSIS* AND *F. NOVICIDA* OMV/NT. 142
TABLE 5-4. SIMILAR PROTEIN CONTENT BETWEEN *F. TULARENSIS* AND *F. NOVICIDA* OMV/NT. 145

Abbreviations

BHI	Brain Heart Infusion Medium
BSL	Biosafety Level
CDM	Chamberlain's Defined Medium
CFU	Colony Forming Units
ELISA	Enzyme-Linked Immunosorbent Assay
FCP	*Francisella* Containing Phagasome
FPI	*Francisella* Pathogenicity Island
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
LVS	Live Vaccine Strain
MHB	Mueller-Hinton Broth
MudPIT	Multidimensional Protein Identification Technology
NSAF	Normalized Spectral Abundance Factor
NT	Nanotubes
OM	Outer Membrane
OMV	Outer Membrane Vesicles
PAMP	Pathogen-associated Molecular Pattern
PRR	Pattern Recognition Receptors
TCA	Trichloroacetic Acid
TEM	Transmission Electron Microscopy
TLR	Toll-like Receptor
T4P	Type IV Pili
TSB	Tryptic Soy Broth

Acknowledgements

For support and guidance throughout my graduate career, I would like to thank my dissertation committee: Martha Furie, Erwin London, Adam Ratner and Ando van der Velden. Their suggestions and encouragement have been very helpful in shaping the progress of my work. I would like to thank my thesis advisor, David Thanassi for allowing me to perform my graduate work in his lab, for guiding and encouraging me over the years and for listening to my ideas, no matter how rambling and incoherent they may have been. I would like to thank Dr. Jorge Benach, Director of the CID, for providing the best working environment I could have hoped for, for fostering research and collaboration and for always making time for students. I would like to thank all the members of the CID for their help and hard work. In particular, I would like to thank Galina Romanov and Patricio Mena for help with experiments, advice and encouragement. I would like to thank Nadine Henderson for keeping the Thanassi lab running, for her help with everything over the course of my graduate career and for being such a kind person. I would like to thank Timothy LaRocca and Sarit Lilo for help, advice, encouragement and friendship. I want to thank my brother Padric for always being there for me and for being the kind of brother I could always look up to. Lastly, and most importantly, I would like to thank my significant other, Anastasia. I never would have been able to finish my degree without her support, her love and her understanding. She has been my constant companion, my best friend and my pillar throughout my endeavors.

Chapter 1: Introduction

I. *Francisella tularensis*

Francisella tularensis is the causative agent of the zoonotic disease tularemia, also known as rabbit fever. The first authenticated report of tularemia was published in 1911 by McCoy and describes a plague-like disease amongst ground squirrels (McCoy 1911). Original reports called the organism *Bacterium tularense,* after Tulare County in central California, the site of the original discovery. The organism was eventually renamed *Francisella tularensis* in honor of Edward Francis, a researcher that extensively studied the bacterium. *F. tularensis* is a Gram-negative, non-motile, facultative intracellular bacterial pathogen, capable of invading a number of host cell types. *F. tularensis* persists in nature in mammalian reservoirs, arthropod vectors and freshwater amoeba, and can be acquired by humans via several routes of infection (Oyston, Sjostedt et al. 2004). A number of small mammals, including rabbits, voles, squirrels, hares and water rats, are natural reservoirs for this organism. The bacterium can be transmitted by the bite of ticks, flies, or mosquitos or by contact with contaminated environments. Humans can acquire the disease by handling of infected animal carcasses, ingestion of contaminated food or water, bites from infected arthropods and breathing in infected dirt or plant material. The most serious infections result from inhalation of aerosolized bacteria, a route that, if untreated, leads to a pneumonic form of tularemia with mortality rates as high as 60% (Dennis, Inglesby et al. 2001). As a result of its high infectivity, low infectious dose (as few as 10 organisms), and the ability to cause widespread public panic, *F. tularensis* has been classified as a category A agent of bioterrorism by the

Centers for Disease Control and Prevention (http://www.bt.cdc.gov/agent/agentlist-category.asp).

There is historical precedent for concerns over the use of *F. tularensis* as a biological weapon. During World War II, Japanese biological warfare units utilized human subjects in their experiments to study tularemia and its potential use as a weapon (Harris 1992). In addition, programs existed in both the former Soviet Union and the United States for study of *F. tularensis* and its ability to be used as a weapon (Dennis, Inglesby et al. 2001). In the 1950s and 1960s, the United States developed aerosol delivery systems capable of disseminating *F. tularensis* (Christopher, Cieslak et al. 1997). The US military also had a stockpile of biological weapons, including *F. tularensis,* in the 1960s. The former Soviet Union continued a similar program into the 1990s. While these programs have since been abolished, worries over the ease with which this organism can be aerosolized, availability of already developed weapons and potential use of this organism as a bioweapon remain.

There are four related subspecies of *Francisella: tularensis, holarctica, mediasiatica* and *novicida.* There are two clinically relevant subspecies of *F. tularensis*: subsp. *tularensis* (also known as type A), which is highly virulent, and subsp. *holarctica* (type B), which causes a milder disease (Oyston, Sjostedt et al. 2004). *F. tularensis* type A strains are found primarily in North America and can be transmitted by ticks from rabbits to humans or by handling of infected animal carcasses. *F. tularensis* type B strains are found in the Northern Hemisphere and can be transmitted in a similar manner. An attenuated live vaccine strain (LVS) was derived from a subsp. *holarctica* strain, but the basis for its attenuation is not fully understood (Dennis, Inglesby et al. 2001). The

LVS causes a lethal infection in mice that closely mimics the human disease, making it useful as an experimental strain. An additional strain of *Francisella*, *F. novicida* (also referred to as *F. tularensis* subsp. *novicida* (Huber, Escudero et al. 2010; Johansson, Celli et al. 2010)), has low virulence in humans, but has also proven highly useful as an experimental strain. *F. novicida* infection of host cells and pathogenesis in mice shares many similarities with *F. tularensis*, and the *Francisella* strains are greater than 98% similar at the genomic level (Rohmer, Fong et al. 2007). Use of both the LVS and *F. novicida* as model organisms is in part due to their ability to be worked with under Biosafety Level 2 (BSL2) conditions, while the fully virulent *F. tularensis* strain must be worked with under Biosafety Level 3 (BSL3) conditions.

II. *Francisella* virulence

The molecular mechanisms underlying the extreme virulence of *F. tularensis* are just beginning to be understood. *Francisella* has a complex infection cycle, with numerous defenses against host immune cells (Fig. 1-1). *Francisella* has a number of methods for dealing with the extracellular defenses of host cells, including a non-stimulatory lipopolysaccharide (LPS) (Gunn and Ernst 2007) and an extracellular polysaccharide capsule (Bandara, Champion et al. 2011). Once taken up by host macrophages, *Francisella* is able to survive within the harsh environment of the phagosome and to suppress certain intracellular signals (Jones, Napier et al. 2012). *Francisella* eventually escapes the phagosome and replicates within the cytosol of the

host. Host cell death is then achieved through activation of apoptotic or pyroptotic pathways and leads to release of the bacteria.

A wide variety of microbial pathogens are recognized and defended against by the innate immune system. Key to the host defense against pathogens are pattern recognition receptors (PRRs) which are capable of recognizing pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are one such family of PRRs present on numerous cell types of the innate immune system. TLRs recognize a wide variety of microbial PAMPs, including lipopolysaccharide (LPS), bacterial lipoproteins, flagella and CpG DNA (Takeda and Akira 2004). Once these PAMPs are detected, host signaling cascades lead to the activation of transcription factors and production of proinflammatory cytokines. The innate immune response to *Francisella* has been shown to primarily be through activation of TLR2, which recognizes bacterial lipoproteins. Researchers have identified two lipoproteins, FTT1103 and Tul4, which are recognized by TLR2 and result in production of proinflammatory cytokines (Thakran, Li et al. 2008). In another study, researchers showed that TLR2 signaling in response to *Francisella* resulted in rapid inflammasome activation, increased cell death and release of the cytokine interleukin-18 (IL-18) (Jones and Weiss 2011).

Francisella is capable of invading numerous cells of the host, though its main replicative niche appears to be macrophages. Researchers have shown that *Francisella* can invade erythrocytes (Horzempa, O'Dee et al. 2011), hepatocytes (Law, Lin et al. 2011), epithelial cells (Craven, Hall et al. 2008), dendritic cells (Bosio and Dow 2005) and macrophages (Thorpe and Marcus 1964). In non-phagocytic cells, *Francisella* has been shown to utilize cholesterol and clathrin dependent means to gain entry to the

cytosol (Law, Lin et al. 2011). Much of the research has, however, focused on invasion of and replication within host macrophages (Fig. 1-1). *Francisella* has been shown to be engulfed by macrophages within asymmetrical, spacious pseudopod loops (Clemens, Lee et al. 2005), forming a *Francisella*-containing phagosome (FCP). *Francisella* prevents the acidification and maturation of the FCP, which eventually degrades, leading to escape into the cytosol of the macrophage where bacterial replication occurs (Clemens, Lee et al. 2004).

Part of the virulence of this organism stems from its ability to passively evade or actively suppress the host response to its presence. LPS is a key component of the outer membrane in Gram-negative bacteria that can be recognized by immune cells of the host. The LPS of *Francisella* poorly activates proinflammatory responses in host cells (Gunn and Ernst 2007). This unique property of *Francisella* LPS results from lack of recognition of the molecule by TLR4 (Hajjar, Harvey et al. 2006), which readily recognizes LPS from other Gram-negative bacteria. This lack of recognition is the result of modifications in the LPS by *Francisella* which make this molecule 1000-fold less stimulatory than the LPS of other enteric bacteria (Barker, Weiss et al. 2006). Thus, *Francisella* is capable of avoiding recognition by host cells of one of its primary outer membrane constituents.

Francisella creates a capsule that surrounds the organism and protects the bacterium from host complement and antimicrobial peptides (Jones, Napier et al. 2012). Mutants lacking a capsule are attenuated for virulence in a mouse model of infection and are readily killed by non-immune human serum (Sandstrom, Lofgren et al. 1988). Bacteria that have lost their capsule have low virulence in mice, and culturing *Francisella*

in synthetic medium is sufficient to increase encapsulation and pathogenicity (Cherwonogrodzky, Knodel et al. 1994). Researchers identified two genes, FTL_1422 and FTL_1423, in the LVS which contributed to production of a capsule-like complex (Bandara, Champion et al. 2011). Deletion of these genes resulted in an attenuated strain capable of protecting mice against challenge with high doses of the wild-type bacteria.

Francisella is capable of suppressing TLR activation of intracellular signaling (Telepnev, Golovliov et al. 2003; Lopez, Duckett et al. 2004; Bosio and Dow 2005). A number of bacterial pathogens are capable of modulating host cell signaling pathways to facilitate invasion and survival within host cells (Heussler, Rottenberg et al. 2002; Park, Greten et al. 2002; Yoon, Liu et al. 2003; Kim, Butcher et al. 2004). Researchers showed that the macrophage response, specifically production of both tumor necrosis factor-α (TNF-α) and IL-1β, to *E. coli* LPS was inhibited in a macrophage-like cell line when infected with *F. tularensis* LVS (Telepnev, Golovliov et al. 2003). Similar results were seen in airway dendritic cells, which failed to produce TNF-α and IL-6 in response to infection or stimulation with TLR agonists (Bosio and Dow 2005). In addition, the interferon-γ (IFN-γ) response of mononuclear phagocytes is suppressed by *Francisella* (Parsa, Butchar et al. 2008). Taken together, these results show that *Francisella* is capable of suppressing numerous cell types and intracellular signals.

Bacteria in the cytosol can activate the inflammasome, leading to cell death by pyroptosis (Henry and Monack 2007) or apoptosis (Santic, Pavokovic et al. 2010), which leads to release of *Francisella*. The inflammasome is a multi-protein complex involved in sensing cytosolic bacterial molecules and leading to caspase-1 activation. Caspase-1 is a cysteine protease, activation of which results in release of the proinflammatory

cytokines IL-1β, IL-18 and IL-33. Caspase-1 is also involved in triggering cell death through formation of pores in the host plasma membrane, which leads to osmotic lysis in a process called pyroptosis (Fink and Cookson 2006). *F. novicida* and the LVS were shown by researchers to cause release of IL-1β and IL-18 from murine peritoneal macrophages in a caspase-1 dependent manner (Mariathasan, Weiss et al. 2005). Similarly, infection with *Francisella* causes IL-1β release in murine bone marrow-derived macrophages (Henry, Brotcke et al. 2007), human monocytes (Gavrilin, Bouakl et al. 2006) and dendritic cells from both humans and mice (Ben Nasr, Haithcoat et al. 2006; Li, Nookala et al. 2006). Some bacterial pathogens are capable of activating components of the apoptotic pathway and inducing apoptosis in host cells (Navarre and Zychlinsky 2000). Researchers have shown that a murine macrophage-like cell line underwent apoptosis in response to infection by *Francisella tularensis* LVS (Lai, Golovliov et al. 2004). Similarly, *Francisella tularensis* is capable of activating numerous caspases and inducing apoptosis in a human B cell line (Zivna, Krocova et al. 2010). Conversely, some researchers have shown that *Francisella* is capable of inhibiting apoptosis in neutrophils and conclude that this is another form of innate immune evasion by this organism (Schwartz, Barker et al. 2012).

Much of the virulence of *Francisella* is mediated by genes residing within the *Francisella* pathogenicity island (FPI), a conserved genomic region of ~30 kb (de Bruin, Ludu et al. 2007). This region is duplicated in *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*, while only a single region exists in *F. novicida*. It has been speculated that this duplication may account for the increased virulence of these subspecies. The FPI consists of a conserved cluster of 17 genes, which have been found

to be essential for virulence, survival and growth inside macrophages. The pathogenicity determinant proteins (Pdp) have been shown to be important for virulence. The FPI PdpA protein was examined for virulence and shown to be a soluble protein that is upregulated under iron-limiting conditions (Schmerk, Duplantis et al. 2009). The FPI PdpD protein has been similarly shown to be required for full virulence (though not intramacrophage growth) and localizes to the outer membrane of *Francisella* (Ludu, de Bruin et al. 2008). The intracellular growth locus (Igl) genes are important for growth inside macrophages. The FPI IglA protein interacts with IglB in the bacterial cytoplasm and is required for growth in macrophages (de Bruin, Ludu et al. 2007). The FPI gene *iglC* has been shown to play a role in disruption of the phagosome in macrophages and subsequent escape into the cytosol (Santic, Molmeret et al. 2005). Likewise, an *iglD* mutant is defective in replication within the macrophage cytosol (Santic, Molmeret et al. 2007). Genes in the FPI have been shown to be under the control of numerous virulence regulators, including MglA, PmrA and FevR (Baron and Nano 1998; Brotcke, Weiss et al. 2006; Mohapatra, Soni et al. 2007; Brotcke and Monack 2008).

There are a number of virulence regulators that exist in *Francisella* which have been shown to control both FPI and other genes important for pathogenicity. The transcription factor MglA regulates numerous genes important for virulence in *Francisella* (Brotcke, Weiss et al. 2006) and is required for intramacrophage growth (Baron and Nano 1998). Mutations in another regulator, PmrA, resulted in complete attenuation of an *F. novicida* strain in mice and defects in macrophage growth (Mohapatra, Soni et al. 2007). PmrA is capable of regulating some of the same FPI genes as MglA, though the two are distinct in that they do not regulate each other. The

Francisella *e*ffector of *v*irulence *r*egulation protein, FevR, was identified as working in parallel with MglA to positively regulate virulence gene expression (Brotcke and Monack 2008).

III. *Francisella* secretion

The composition of the Gram-negative bacterial cell envelope is complex, consisting of an inner membrane (IM), outer membrane (OM), a periplasmic space in between and a peptidoglycan cell wall located in the periplasmic space. As a result of this complexity, Gram-negative bacteria have developed a number of strategies to move proteins across their two membranes and into the extracellular milieu or directly into host cells (Thanassi and Hultgren 2000; Filloux, Hachani et al. 2008). A number of canonical secretion systems exist, numbered type I through VI. These systems vary in complexity, from a simple membrane spanning pore to a needle-like apparatus capable of direct injection of bacterial effectors into host cells. Bacteria use these secretion systems to transfer proteins across the inner membrane to the periplasmic space or across both inner and outer membranes to the outside of the cell. These bacterial secretion products are capable of interacting with host cells and regulating specific processes from uptake of bacteria to host cell death. Utilizing these systems, larger structures such as pili and adhesins can be assembled on the outside of the bacteria to facilitate bacterial adhesion to and invasion of the host cell (Thanassi, Bliska et al. 2012). These systems can also be used defensively to secrete drugs and other harmful products out of the interior of the

bacterial cell. Many of these systems are required for bacterial pathogenicity, and have been shown to secrete key virulence factors.

Francisella spp. lack secretion pathways typically used by intracellular, Gram-negative pathogens to deliver virulence factors to host cells, such as the type III and type IV secretion systems that are capable of directly injecting virulence factors into the host cell cytoplasm (Wallden, Rivera-Calzada et al. 2010; Izore, Job et al. 2011). *F. tularensis* contains a type I secretion system (T1SS), which contributes to the virulence of the LVS and subsp. *tularensis* in the mouse infection model (Gil, Platz et al. 2006; Platz, Bublitz et al. 2010). T1SS function in the delivery of proteins from the bacterial cytoplasm to the extracellular environment (Holland, Schmitt et al. 2005). Composed of an outer membrane pore, an inner membrane ATPase and a periplasmic adapter protein, this system has been shown to be important for multi-drug resistance in *Francisella.* However, factors secreted by the T1SS in *Francisella* have not yet been identified.

Francisella spp. encode a type IV pili (T4P) biogenesis system, which also functions in the secretion of soluble proteins to the extracellular medium (Hager, Bolton et al. 2006; Chakraborty, Monfett et al. 2008; Zogaj, Chakraborty et al. 2008). Mutations in T4P genes attenuate the virulence of *F. novicida*, the LVS, and subsp. *tularensis* (Chakraborty, Monfett et al. 2008; Forslund, Salomonsson et al. 2010; Ark and Mann 2011). Researchers previously identified seven proteins secreted through this system in *F. novicida*, two chitinases (ChiA and ChiB), a chitin binding protein (CbpA), a protease (PepO), a β-glucosidase (BglX) and two proteins of unknown function (Fsp53 and Fsp58) (Hager, Bolton et al. 2006). The transcription of both BglX and PepO was shown to be regulated by the MglA virulence regulator. Surprisingly, researchers found that mutants

in PepO or T4P machinery were capable of enhanced spread of *F. novicida* to systemic sites (Hager, Bolton et al. 2006). The human pathogenic strains of *Francisella* contain mutations in *pepO,* and the authors speculate that loss of this secreted protein increases the virulence of the organism. This is in contrast to experiments by other research groups which showed mutants in T4P components to be attenuated.

The Type VI secretion system (T6SS) is a complex, multi-component system that has recently been discovered in a number of organisms (Filloux, Hachani et al. 2008). A number of proteins encoded by the FPI share homology with Type VI secretion components identified in *V. cholerae* (Pukatzki, Ma et al. 2006) and *P. aeruginosa* (Mougous, Cuff et al. 2006). Experimental evidence supports a role for the FPI in the delivery of *Francisella* proteins to host cells (Nano, Zhang et al. 2004; de Bruin, Ludu et al. 2007; Ludu, de Bruin et al. 2008; Broms, Lavander et al. 2009). The FPI has been shown to be required for secretion of effectors into the macrophage cytosol, phagosomal escape, intramacrophage growth and virulence in mice (Barker, Chong et al. 2009). Despite this, only two proteins were identified as being secreted via the putative FPI T6SS. Researchers showed that the VgrG and IglI proteins were secreted into the cytosol of infected macrophages and that VgrG secretion did not require the other FPI genes. In contrast, IglI required VgrG and numerous other FPI genes for secretion into the cytosol of macrophages.

IV. Outer Membrane Vesicles

Gram-negative bacteria have been shown to secrete proteins through the formation of outer membrane vesicles (OMV) (Fig. 1-2). These OMV, or blebs, are shed

during all phases of growth of Gram-negative bacteria and consist of OM proteins, phospholipids, lipopolysaccharide (LPS), peptidoglycan, and periplasmic proteins. They are small spherical structures, 20-300 nm in diameter, and are produced when the outer membrane of the bacteria bulges away from the cell and is released to form an enclosed sphere. These structures are then capable of floating away from the bacteria to deliver their contents to other cells. Studies have shown that these structures are capable of performing a variety of roles, including horizontal gene transfer, pathogenesis, quorum signaling and nutrient acquisition (Mayrand and Grenier 1989; Yaron, Kolling et al. 2000; Mashburn and Whiteley 2005). Because production of OMV is ubiquitous in Gram-negative bacteria and there is a definite cost to an organism to produce these structures, it is believed that this process confers a distinct advantage to an organism. There is some confusion in the literature regarding the term OMV, as some researchers use this term to refer to structures that are created through detergent treatment of whole bacterial cells. Other researchers may refer to OMV which are created through normal growth of a bacterial cell as nOMV, for natural or native. During the course of this dissertation, the term OMV will be used exclusively to refer to naturally produced structures and not detergent extracted membranes.

The production of OMV by Gram-negative bacteria has been reported for nearly 40 years (Beveridge 1999); however, research into their role as secretory vehicles has dramatically increased in recent years (Horstman and Kuehn 2000; Wai, Lindmark et al. 2003; Kuehn and Kesty 2005; Bauman and Kuehn 2006; Alaniz, Deatherage et al. 2007; Lee, Bang et al. 2007; Bomberger, Maceachran et al. 2009; Deatherage, Lara et al. 2009; Furuta, Tsuda et al. 2009; Parker, Chitcholtan et al. 2010; Tashiro, Ichikawa et al. 2010;

Nakao, Hasegawa et al. 2011). Numerous virulence factors have been identified as being associated with OMV (Wensink, Gankema et al. 1978; Gankema, Wensink et al. 1980; Nowotny, Behling et al. 1982; Grenier and Mayrand 1987; Shoberg and Thomas 1993; Kadurugamuwa and Beveridge 1995; Rosen, Naor et al. 1995; Wai, Takade et al. 1995; Patrick, McKenna et al. 1996; Kadurugamuwa and Beveridge 1998; Li, Clarke et al. 1998; Fiocca, Necchi et al. 1999; Kolling and Matthews 1999; Horstman and Kuehn 2000; Keenan and Allardyce 2000; Negrete-Abascal, Garcia et al. 2000; Yokoyama, Horii et al. 2000; Kato, Kowashi et al. 2002; Chi, Qi et al. 2003; Kamaguchi, Nakayama et al. 2003; Khandelwal and Banerjee-Bhatnagar 2003; Wai, Lindmark et al. 2003; Duncan, Yoshioka et al. 2004; Dutta, Iida et al. 2004), including cytolysin A, leukotoxin, shiga toxin, proteases, and chitinases. Researchers have shown that heat-labile enterotoxin (LT) is secreted through the general secretory pathway and associates with the outside of OMV in enterotoxigenic *E. coli* (Horstman and Kuehn 2002). In *Actinobacillus actinomycetemcomitans,* an enrichment of leukotoxin in OMV was shown as being responsible for cytotoxicity in host cells in the absence of bacteria (Kato, Kowashi et al. 2002). In uropathogenic *E. coli,* cytotoxic necrotizing factor type 1, a secreted virulence factor, was shown to be enriched in OMV and capable of exerting its effects on HeLa cells (Kouokam, Wai et al. 2006). A role for OMV in signaling amongst a population of bacteria has been shown for the pathogenic *Pseudomonas aeroginosa,* which packages the quorum sensing molecule PQS within these structures (Mashburn and Whiteley 2005). Likewise, a role for transfer of virulence genes amongst populations of bacteria has been shown in *P. aeroginosa* (Renelli, Matias et al. 2004) and *E. coli* (Yaron, Kolling et al. 2000). By far, the most research has been performed on the use of

OMV by pathogenic bacteria to package toxins and other virulence factors for export out of the cell.

OMV are capable of gaining entry to host cells in a number of ways. OMV can fuse with the host cell plasma membrane and delivering cargo directly to the host cell cytoplasm. There is evidence of a role for surface exposed outer membrane molecules in uptake of OMV by host cells. In enterotoxigenic *E. coli* (ETEC) heat-labile enterotoxin plays a role in binding and uptake of OMV to host cells (Kesty, Mason et al. 2004). The vacuolating cytotoxin VacA facilitates uptake of OMV derived from *H. pylori* (Parker, Chitcholtan et al. 2010). *P. aeruginosa* OMV are able to fuse with cholesterol rich host lipid rafts and to deliver virulence factors via N-WASP-mediated actin trafficking (Bomberger, Maceachran et al. 2009). Similarly, researchers studying ETEC or *P. gingivalis* OMV showed that binding to lipid rafts was the method by which factors were delivered to host cells (Kesty, Mason et al. 2004; Furuta, Tsuda et al. 2009). In a separate study, OMV from *Shigella flexneri* were able to deliver antibiotic to host cells in a process involving phagocytosis of the OMV and subsequent release of these structures from the phagosome (Kadurugamuwa and Beveridge 1998).

Studies have demonstrated the potential of OMV to serve as protective vaccines in diverse organisms. In *S.* Typhimurium*,* researchers found that mice vaccinated with OMV were capable of generating *Salmonella*-specific immunoglobulin and CD4+ T cell responses and were subsequently protected from challenge with wild-type bacteria (Alaniz, Deatherage et al. 2007). There exists a fully licensed OMV based vaccine against *N. meningitidis,* though the production of this vaccine generally involves the use of detergents to extract the outer membrane and should be differentiated from naturally

occurring OMV (Vipond, Suker et al. 2006). Other researchers have prepared native OMV vaccines against *N. meningitidis* in order to preserve the structure of particular antigens in the outer membrane (Koeberling, Seubert et al. 2008). Researchers working with *P. aeruginosa* and *S. flexneri* showed that it was possible to fuse OMV from these strains into the membranes of *E. coli* and *S.* Typhimurium, effectively incorporating antigenic proteins into other bacteria (Kadurugamuwa and Beveridge 1999). They went on to propose that this might be a method for creation of possible vaccine candidates. A number of other researchers have either proposed methods for creation of vaccines from OMV, or have done research in diverse organisms to characterize the immune response to OMV based vaccines (Kadurugamuwa 2005; Roy, Hamilton et al. 2011; Avila-Calderon, Lopez-Merino et al. 2012; Bishop, Tarique et al. 2012).

There are several proposed models for the biogenesis of OMV (Fig. 1-2) (Lee, Choi et al. 2008); however, the mechanisms by which they are created and by which proteins are targeted to them have yet to be determined. In one model, OMV are shed from the cell surface due to missing peptidoglycan-associated lipoproteins as a result of a faster expansion of the outer membrane as compared to the peptidoglycan layer (Wensink and Witholt 1981). An additional model proposes that the turgor pressure of the cell envelope can change due to the accumulation of peptidoglycan fragments and that this is responsible for formation of OMV (Zhou, Srisatjaluk et al. 1998). A final model is that the salt bridges in the outer membrane are destabilized by certain quinolone signaling molecules and that this process causes the membrane to bud off (Mashburn and Whiteley 2005). The formation of OMV is thought to be integral to the survival of Gram-negative organisms, as no null mutants have been identified (McBroom, Johnson et al. 2006).

Both pathogenic and non-pathogenic strains produce OMV, though more virulent strains tend to produce higher amounts of these structures (Wai, Takade et al. 1995; Kuehn and Kesty 2005)

V. Nanotubes

In addition to OMV, tube-like extracellular structures, or nanotubes (NT), have recently been observed in Gram-negative and Gram-positive bacteria (Dubey and Ben-Yehuda 2011; Galkina, Romanova et al. 2011). These structures are approximately 30-130 nm in diameter and can reach several microns in length. Researchers showed that these structures were formed between *Salmonella enterica* bacterial cells and between bacteria and host neutrophils (Galkina, Romanova et al. 2011). Similar bridging NT structures have also been described in eukaryotic cells (Rustom, Saffrich et al. 2004), and bacterial NT may allow direct bridging between bacteria and eukaryotic cells (Galkina, Romanova et al. 2011). The previously described NT were observed only in bacteria grown on solid surfaces and appeared to be an extension of the cytoplasmic membrane, connecting neighboring bacterial cells to allow the transfer of cytoplasmic constituents, including protein and nucleic acids.

In contrast to these previously described NT, *Francisella* creates tube-like structures when grown in liquid as well as on solid medium. The *Francisella* NT appear to be extensions of the bacterial OM, rather than the cytoplasmic membrane, and are released into the extracellular medium along with the OMV. These NT had been previously observed in the LVS of *F. tularensis* subspecies *holarctica* (Gil, Benach et al. 2004) when grown on solid media. The purpose of the NT was not determined, and there

are relatively few publications which even mention their existence in *Francisella* (Gerasimov, Dolotov et al. 1997).

Pierson and colleagues recently demonstrated the production of OMV by *F. novicida*, identified protein constituents of these OMV, characterized their effects on host cells, and showed vaccination with the OMV provides limited protection against bacterial challenge (Pierson, Matrakas et al. 2011). Despite this, additional research on production of OMV in *Francisella* is necessary. This dissertation will describe the isolation and subsequent characterization of *Francisella* OMV and NT. Experiments will show that production of the *Francisella* OMV and NT are coordinately regulated and responsive to growth medium and growth phase. I will list the proteins associated with OMV and NT, many of which have previously been shown to be secreted or associated with virulence in *Francisella.* I will describe the effects that purified OMV and NT have on host cells, including cytotoxicity and production of proinflammatory cytokines. Lastly, I will explore the use of OMV and NT as a subunit vaccine for protection against *Francisella* infection.

VI. Figures

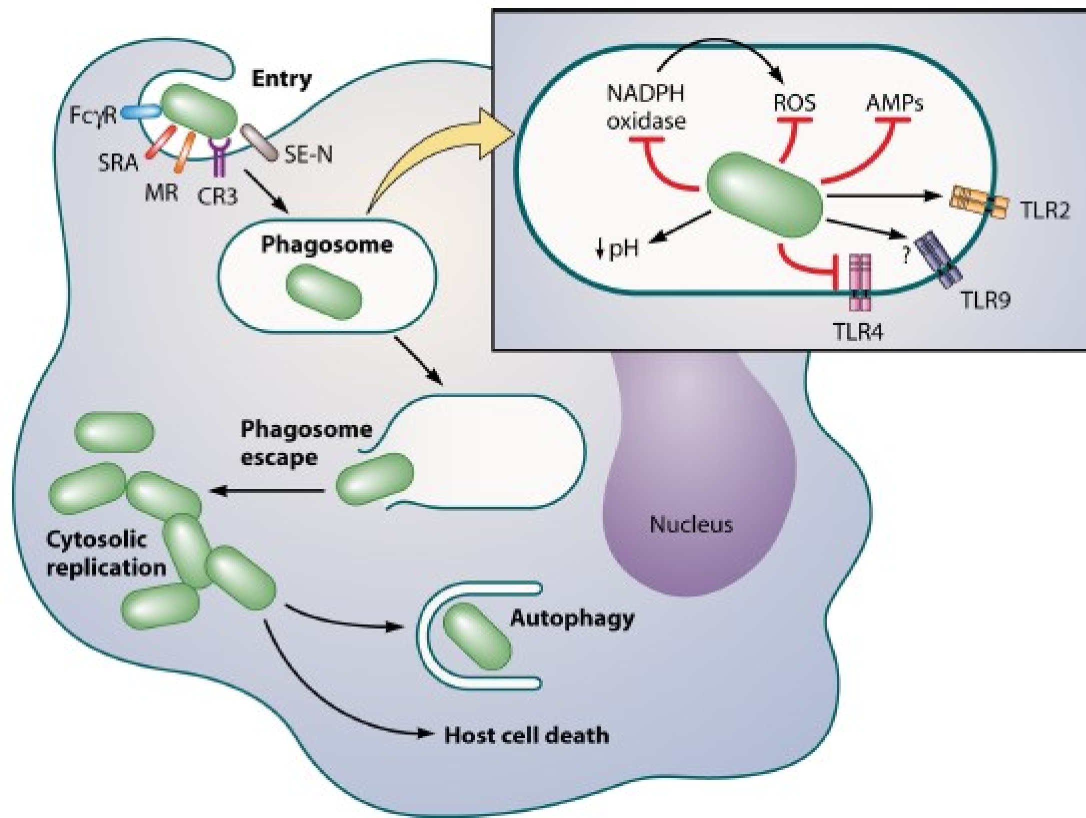


Figure 1-1. Intracellular life cycle of *F. tularensis.*

Francisella can be detected by numerous macrophage receptors and is engulfed by macrophages through looping phagocytosis. *Francisella* then forms a *Francisella*-containing phagosome (FCP). While in this harsh environment, *Francisella* is able to employ multiple mechanisms to evade host defenses (inset). *Francisella* escapes the FCP to replicate within the cytosol of the infected cell. The organism then induces apoptosis or pyroptosis, killing the cell and releasing the bacteria. Figure reprinted from (Jones, Napier et al. 2012), with permission.

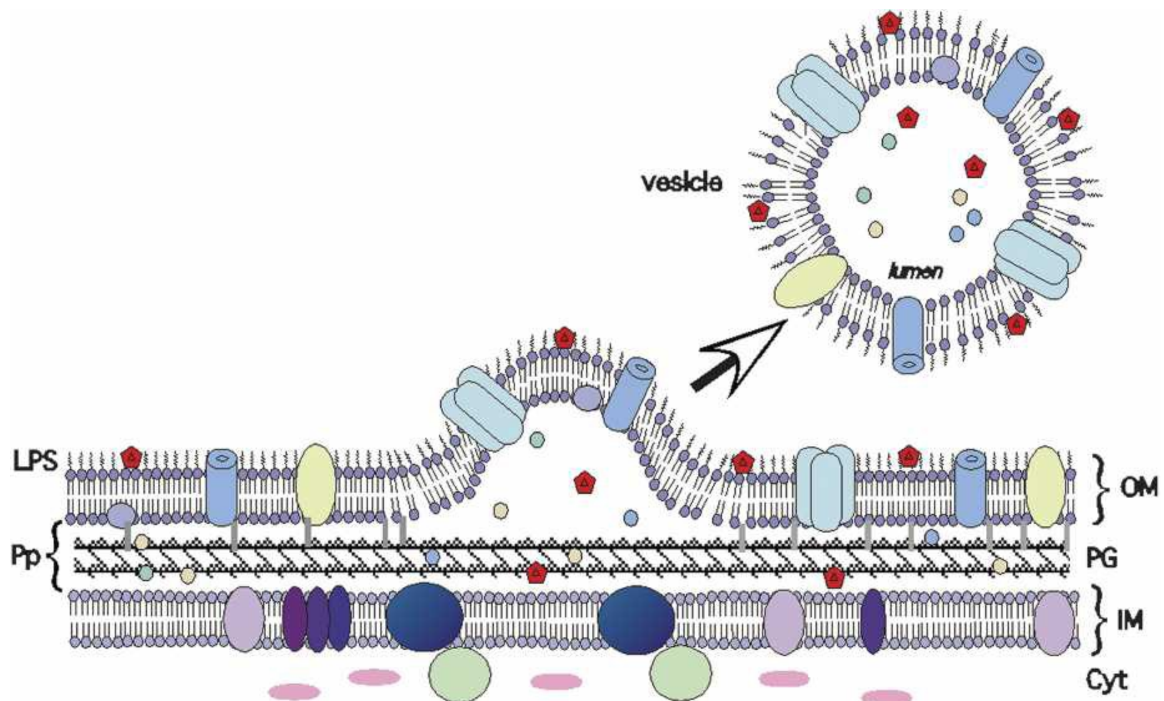


Figure 1-2. Model of Vesicle Biogenesis

Outer membrane vesicles consist of OM proteins, phospholipids, LPS and periplasmic proteins. Proteins are enriched in vesicles through unknown mechanisms. Some proteins are capable of associating with the outside of vesicles, as in the case of LT (red), after secretion through other pathways. (LPS) Lipopolysaccharide; (Pp) periplasm; (OM) outer membrane; (PG) peptidoglycan; (IM) inner membrane; (Cyt) cytosol. Figure reprinted from (Kuehn and Kesty 2005), with permission.

Chapter 2: Materials and Methods

I. Bacterial strains, media and growth conditions

F. novicida strain U112 (BEI Resources, ATCC 15482) was grown in TS [tryptic soybean powder (30 g/l), supplemented with 0.1% cysteine], BHI [brain heart infusion powder (37 g/l), adjusted to pH 6.8], MHB [Mueller-Hinton II broth powder (22 g/l), supplemented with 625 µM $CaCl_2 \cdot 2H_2O$, 530 µM $MgCl_2 \cdot 6H_2O$, 335 µM ferric pyrophosphate (= 0.025% w/v), 5.6 mM D-glucose (= 0.1% w/v) and 2% IsoVitaleX] (all media from BD Biosciences) and CDM [Chamberlain Defined Medium (Chamberlain 1965)]. *F. tularensis* Schu S4 (Biodefense and Emerging Infections Research Resources Repository) was grown in BHI medium. For plates, bacto-agar (BD Biosciences) was added to 15 g/l. Bacteria streaked on plates were incubated at 37ºC in the presence of 5% CO_2. Liquid media were incubated in the presence of 5% CO_2 for one hour prior to inoculation with bacteria, and the cultures were grown at 37ºC with aeration (shaking at 100 rpm). Starter liquid cultures were inoculated directly from frozen stocks, grown overnight and diluted 1:100 to an OD_{600} of ~0.01. Day cultures of *F. novicida* were grown to exponential (OD_{600} 0.5-0.8, ~4 hours) or stationary (OD_{600} 1.2-1.4, ~9 hours) phase as indicated. Cultures of *F. tularensis* were grown to late stationary phase (OD_{600} 0.8-0.9, ~70 hours).

II. Protein profiles of cell-free culture supernatants

Bacterial cultures were grown in BHI medium to the indicated OD_{600}, and aliquots of 3 ml were removed. To create cell free supernatants, the aliquots were centrifuged (10,000 × *g*, 5 minutes), supernatants were filtered through 0.22 µm syringe filters (Sarstedt) and sodium azide was added to a final concentration of 0.05%. Trichloroacetic acid (TCA) was added to 1 ml of cell free supernatants to a final

concentration of 9%. Samples were placed on ice for 30 minutes, then centrifuged at 16,000 × *g,* 4ºC for 5 minutes to pellet proteins. Supernatants were removed, and pellets were washed twice with ice cold acetone followed by 5 minute spins at 16,000 × *g*. Final pellets were resuspended in 20 µl SDS-PAGE sample buffer, heated at 95ºC for 10 minutes and loaded onto 12% SDS-polyacrylamide gels and stained with Coomassie blue.

III. Purification of OMV and NT

For *F. novicida,* bacterial cultures (400 ml culture in 2 liter flasks with baffles) were grown in BHI to exponential (OD_{600} ~ 0.6) or stationary phase (OD_{600} ~ 1.4). For *F. tularensis*, bacterial cultures (200 ml culture in 1 liter flasks with baffles) were grown in BHI to stationary phase (OD_{600} ~ 0.9). Bacteria were removed by successive low speed centrifugation (5000 × *g* and 7500 × *g,* 30 minutes each), followed by filtration through a 0.2 µm MF75 filter unit (Nalgene). Sodium azide was added to the cleared culture medium to a final concentration of 0.05%, and vesicles were harvested by ultracentrifugation (100,000 × *g*, 1 h, 4ºC). For bacteria grown to exponential phase, prior to ultracentrifugation, 1 l of cell-free medium was concentrated to ~50 ml using a tangential flow filtration unit (Pall) with a 100 kDa molecular weight cutoff membrane. For bacteria grown to stationary phase, 60 ml of cell-free medium per ultracentrifuge tube was directly centrifuged. The pelleted OMV and NT were resuspended in 20 mM HEPES (pH 7.5), 0.05% sodium azide, and pellets from multiple tubes were combined and subjected to an additional centrifugation step (100,000 × *g*, 1 h, 4ºC). The final vesicle pellet was resuspended in 20 mM HEPES (pH 7.5), 1% streptomycin/penicillin, 10 µg/ml gentamicin and stored at 4ºC.

To purify the OMV and NT further, the resuspended vesicle pellets were adjusted to 40% (vol/vol) OptiPrep (Axis-Shield) in 20 mM HEPES (pH 7.5), 0.05% sodium azide in a total volume of 2 ml. Samples were loaded into a 13.2 ml ultracentrifuge tube, and lower concentration OptiPrep solutions were layered on top (2 ml 35%, 2 ml 30%, 2 ml 25%, 2 ml 20%, 1 ml 15% and 0.5 ml 0%). Tubes were centrifuged (100,000 × *g*, 16 h, 4ºC) in a swinging-bucket rotor, and 1 ml fractions were collected from the top. Fractions were examined by SDS-PAGE and Coomassie blue staining for protein content. Adjacent fractions, with similar protein profiles, were combined, diluted with 20 mM HEPES (pH 7.5) and recovered via ultracentrifugation (100,000 × *g*, 1 h, 4º C). The final pellet was resuspended in 20 mM HEPES, pH 7.5 (containing 1% penicillin/streptomycin, 10μg/ml gentamicin), and aliquots were flash-frozen in liquid nitrogen and stored at -80ºC.

IV. Electron microscopy

For transmission electron microscopy (TEM), samples were adsorbed onto polyvinyl formal-carbon-coated grids (EMS) for 2 min, fixed with 1% gluteraldehyde for 1 min, washed twice with PBS and twice with water, and then negatively stained with 0.5% phosphotungstic acid (Ted Pella) for 30 s. Samples for thin-sectioning were fixed with 2.5% EM grade glutaraldehyde in 0.1 M PBS, pH 7.4, for at least 1 h. Samples were then placed in 1% osmium tetroxide in 0.1 M PBS, dehydrated in a graded series of ethyl alcohol, and embedded in Durcupan resin. Ultrathin sections of 80 nm were cut with a Reichert-Jung UltracutE ultramicrotome and placed on formvar coated slot copper grids. Sections were then counterstained with uranyl acetate and lead citrate. All grids were viewed in a FEI Tecnai12 BioTwinG^2 electron microscope at 80 kV accelerating voltage,

and images were obtained using an AMT XR-60 charge-coupled device digital camera system and compiled using Adobe Photoshop.

V. Sample Preparation for TEM.

For whole cell samples grown in liquid culture, 1 ml of culture supernatant was centrifuged (8,000 × *g*, 5 minutes, 4ºC) and then resuspended in 200 µl sterile PBS. For whole cells grown on solid media, 5 colonies were resuspended in 50 µl sterile PBS. Samples were then adsorbed onto grids as noted above.

VI. Protein quantification

Total protein concentration of the OMV and NT samples was determined via bicinchoninic acid (BCA) assay (Sigma Aldrich), according to manufacturer's instructions, with the addition of 2% sodium dodecyl sulfate (Morton and Evans 1992).

VII. Fractionation of *F. novicida*

Bacterial cultures were grown in BHI to early stationary phase (OD_{600} ~ 1.4). For whole cell lysates, 1 ml of culture was centrifuged (10,000 × *g*, 5 min, 4ºC), and the pellet was resuspended in 100 µl SDS-PAGE sample buffer and heated at 95ºC for 10 min. For bacterial fractionation, 100 ml of culture was centrifuged (10,000 × *g*, 5 min, 4ºC) and the supernatant was removed and saved for analysis of secreted proteins as described below. The bacterial pellet was resuspended in 10 ml 20 mM Tris-HCl (pH 8.0), moved to a fresh tube, and centrifuged again. The pelleted bacteria were resuspended in 1 ml 20 mM Tris-HCl (pH 8.0) plus 20% sucrose, and 1 ml was moved to a clean tube. EDTA was added to 15 mM, lysozyme was added to 200 µg/ml, and the suspension was incubated on ice for 40 min. $MgCl_2$ was then added to 26 mM, 4 µl DNAse I (10,000 units/ml; Thermo Scientific) was added, and the suspension was

incubated for an additional 20 min on ice. The spheroplasted bacteria were pelleted by centrifugation (10,000 × *g*, 20 min, 4ºC), and the supernatant was collected (equals periplasm sample). The bacterial pellet was resuspended in 1 ml 20 mM Tris-HCl (pH 8.0) plus Complete protease inhibitor cocktail (Roche), and 1 ml was transferred to a clean tube. The sample was sonicated (Misonix Microson model XL-2000; power level 5) on an ice water bath, 15 seconds on and 15 seconds off, for 2 min. The sonicated bacteria were then centrifuged (8,000 × *g*, 10 min, 4ºC) to remove unbroken cells, and the supernatant was then ultracentrifuged (100,000 × *g*, 1 h, 4ºC) to pellet membranes. The membrane pellet was resuspended in 1 ml 20 mM Tris-HCl (pH 8.0) plus Complete protease inhibitor cocktail, and sarkosyl (sodium-N-lauroryl-sarcosinate; Fisher) was added to a final concentration of 0.5% to solubilize the cytoplasmic membrane. The tube was rocked at room temperature for 5 minutes and then ultracentrifuged (100,000 × *g*, 1 h, 4ºC) to pellet the OM. The final pellet was resuspended in 20 mM Tris-HCl (pH 8.0), 0.3 M NaCl (equals OM sample). Protein concentrations were determined by the BCA assay, and aliquots were mixed with SDS-PAGE sample buffer and heated at 95ºC for 10 min.

For analysis of secreted proteins, 3 ml aliquots of the saved culture supernatants were filtered through 0.22 µm syringe filters (Sarstedt), and sodium azide was added to 0.05%. Trichloroacetic acid (TCA) was then added to 1 ml of the filtered supernatants to 9% final concentration. Samples were placed on ice for 30 minutes and centrifuged (16,000 × *g*, 4ºC, 5 min) to pellet precipitated proteins. The pellets were washed twice with ice-cold acetone, followed each time by centrifugation (16,000 × *g*, 4ºC, 5 min).

Final pellets were resuspended in 20 µl SDS-PAGE sample buffer and heated at 95ºC for 10 min.

All samples were subjected to SDS-PAGE and stained with Coomassie blue.

VIII. Protease accessibility assay

Purified OMV/NT were left untreated or treated with proteinase K (10 µg/ml), SDS (0.02%), or proteinase K plus SDS for 1 h at room temperature. Phenylmethanesulfonyl fluoride (PMSF, 0.1 mM) was added to inhibit the protease, and samples were processed for TEM as described above or heated at 95ºC for 10 min in SDS sample buffer for subsequent SDS-PAGE analysis. For immunoblotting, proteins separated by SDS-PAGE were transferred to a PVDF (Osmonics) membrane and probed with 1:10,000 anti-FopA (Savitt, Mena-Taboada et al. 2009) or anti-FipB (Qin, Scott et al. 2011) antibodies. Immunoblots were developed with alkaline phosphatase-conjugated secondary antibodies and BCIP (5-bromo-4-chloro-3-indolylphosphate)-NBT (nitroblue tetrazolium) substrate (KPL).

IX. Heat treatment of OMV/NT

Purified OMV/NT were left at room temperature, heated at 60ºC for 5, 15 or 30 min, or 80ºC for 1 h, and then left to cool at room temperature for 1 h. Samples were processed for TEM as described above. For quantification of OMV/NT at different time points during the 60ºC heat treatment, ten random TEM fields at each time point were chosen and the numbers of OMV or nanotubes were determined by visual inspection. The values for the 10 fields were then averaged.

X. Lysozyme treatment of OMV/NT and whole bacteria

Treatment of bacteria to generate spheroplasts was performed as described above for bacterial fractionation, except that after the 40 min incubation in the presence of lysozyme/EDTA, a 20 µl aliquot was removed and processed for TEM as described above. For treatment of purified OMV/NT, samples were pelleted and resuspended in 20 mM Tris-HCl (pH 8.0), EDTA was added to 15 mM, lysozyme was added to 200 µg/ml, and the suspension was incubated on ice for 40 min before processing for TEM.

XI. Multidimensional chromatography and tandem mass spectrometry

Purified vesicle pellets were resuspended in 20 µl 8 M urea and diluted to 2 M urea with 0.1 M ammonium bicarbonate. The proteins were reduced with 5 mM dithiothreiotol and alkylated with 10 mM iodoacetamide. Two µg of trypsin was added to the proteins and incubated overnight at 37ºC. The digestion reaction was stopped with formic acid (5% final concentration), the peptides were purified on C18 columns (Supel-Tips C18, Supelco) and the dried peptides were resuspended in 30 µl 5% formic acid, 2% acetonitrile. Peptide mixtures were pressure-loaded onto a 250 µm inner diameter (i.d.) fused-silica capillary packed first with 3 cm of 5 µm strong cation exchange material (Partisphere SCX, Whatman), followed by 3 cm of 10 µm C18 reverse phase (RP) particles (Magic, Michrom). Loaded and washed microcapillaries were connected via a 2 µm filtered union (UpChurch Scientific) to a 100 µm i.d. column, which had been pulled to a 5 µm i.d. tip using a P-2000 CO_2 laser puller (Sutter Instruments), then packed with 13 cm of 3 µm C18 reverse phase (RP) particles (Magic, Michrom) and equilibrated in 2% acetonitrile, 0.1 % formic acid (Buffer A). This split column was then installed in-line with a NanoLC Eskigent HPLC pump. For the organic gradient, the flow rate of

channel 2 was set at 300 nl/min. The flow rate of channel 1 was set to 0.5 μl/min for the salt pulse. Fully automated 13-step chromatography runs were carried out. Three different elution buffers were used: Buffer A; 98% acetonitrile, 0.1% formic acid (Buffer B); and 0.5 M ammonium acetate, 2% acetonitrile, 0.1% formic acid (Buffer C). In such sequences of chromatographic events, peptides are sequentially eluted from the SCX resin to the RP resin by increasing salt steps (increase in Buffer C concentration), followed by organic gradients (increase in Buffer B concentration). The last chromatography step consists of a high salt wash with 100% Buffer C followed by an acetonitrile gradient. The application of a 1.8 kV distal voltage electrosprayed the eluting peptides directly into a LTQ-Orbitrap XL mass spectrometer equipped with a nano-LC electrospray ionization source. Full MS spectra were recorded on the peptides over a 400 to 2000 m/z range by the Orbitrap, followed by five tandem mass (MS/MS) events sequentially generated by LTQ in a data-dependent manner on the first, second, third, and fourth most intense ions selected from the full MS spectrum (at 35% collision energy). Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (ThermoFinnigan, San Jose, CA).

XII. Mass spectrometry data analysis

MS/MS spectra were extracted from the RAW file with ReAdW.exe (http://sourceforge.net/projects/sashimi). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using X! Tandem (The GPM, thegpm.org; version 2006.06.01.1). X! Tandem was set up to search the F_tularensis_U112_humanbovine database (123104 entries) assuming the digestion enzyme trypsin. X! Tandem was searched with a fragment ion mass tolerance of 0.40 Da

and a parent ion tolerance of 10.0 PPM. Iodoacetamide derivative of cysteine was specified in X! Tandem as a fixed modification. Deamidation of asparagine, oxidation of methionine, sulphone of methionine, tryptophan oxidation to formylkynurenin of tryptophan and acetylation of the N terminus were specified in X! Tandem as variable modifications. Scaffold (version Scaffold_2_06_01, Proteome Software) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller, Nesvizhskii et al. 2002). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Keller et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Spectral count normalization (Paoletti, Parmely et al. 2006) was applied to spectra identified through mass spectrometric analysis for each independent OMV/NT sample isolated, to generate a normalized spectral abundance factor (NSAF) value. Three independent experiments were performed at each time point, and only proteins identified in all experiments at their respective time points were considered as vesicle associated.

Localization of *F. tularensis* subsp. *novicida* U112 proteins was determined by using the pSORTb v 3.0 (Yu, Wagner et al. 2010) precomputed proteome. Comparison of individual *F. novicida* proteins to *F. tularensis* Schu S4, *F. tularensis* LVS or to the OMV-associated content of other organisms was accomplished using the Basic Local Alignment Search Tool (Altschul, Gish et al. 1990) (http://blast.ncbi.nlm.nih.gov/) .

XIII. Preparation of macrophages

Murine bone marrow-derived macrophages (muBMDM) were obtained as previously described (Celada, Gray et al. 1984) from C3H/HeN mice (Charles River) and resuspended in bone marrow medium [BMM_{HI}; DMEM (Invitrogen) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 20% heat-inactivated FBS (HyClone), and 30% medium previously conditioned by L929 cells]. The muBMDM were allowed to differentiate for 5 days before being seeded in 24-well plates at a concentration of 1.5×10^5 cells per well in bone marrow assay medium [BMAM; DMEM (Invitrogen) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 1% heat-inactivated FBS (HyClone), 1% penicillin/streptomycin and 4 µg/ml gentamicin], incubated at 37ºC, 5% CO_2 and used for experiments the next day. The L-cell conditioned medium was obtained by plating 2×10^5 L929 cells in 75-cm^2 culture flasks in Minimum Essential Medium (Invitrogen) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids (Invitrogen), and 10% FBS, and collecting the medium after 10 days.

All protocols involving animals were approved by the Institutional Animal Care and Use Committee of Stony Brook University.

XIV. Macrophage co-incubation with *F. novicida* for TEM

Co-incubation experiments were performed as previously described for phagocytic uptake analysis with minor modifications (Clemens, Lee et al. 2011). C3H/HeN muBMDM were obtained as described above and resuspended at a concentration of 6×10^6 in BMM_{HI}. Cells were pelleted (1000 × g, 10 min, 4ºC), and the supernatant was removed. One ml *F. novicida*-containing BMM_{HI} was added to the

pelleted macrophages at an approximate multiplicity of infection (MOI) of 2000:1. The tube was centrifuged twice (200 × *g*, 800 × *g*, 10 min each, 4ºC), and the supernatant was removed. The tube containing pelleted bacteria and cells was placed in a 37ºC water bath for 5 minutes. Cells and bacteria were then fixed with 1 ml 2.5% gluteraldehyde at 37ºC for 2 minutes, then on ice for 30 minutes. The tube was centrifuged (10,000 × *g*, 10 mins, 4ºC), and the pellet was resuspended in 1 ml ice cold PBS. The sample was then processed for thin-sectioning and pictures were taken as described above.

XV. Cytotoxicity assays

Purified OMV/NT were resuspended in room temperature BMAM at concentrations of 0.1, 1, 10 and 20 μg/ml. The supernatant was removed from muBMDM previously seeded into 24-well plates, cells were washed twice with room temperature PBS, and 1 ml of vesicle-containing medium was added to the wells. Plates were incubated at 37ºC, 5% CO_2 for 24 or 48 h, supernatants were collected, and a lactate dehydrogenase (LDH) assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega) was performed according to manufacturer's instructions. Background LDH release was measured in medium lacking vesicles, while total LDH release (= 100%) was measured from uninfected cells that were lysed by freezing and thawing. The percentage of LDH release was calculated by subtracting the background LDH release value from the LDH release value of the samples, and this number was then divided by the total LDH release value and multiplied by 100. The values for each experiment were determined from the average of triplicate wells; three independent experiments were performed.

XVI. Detection of cytokine secretion

OMV/NT were added to muBMDM as described above. After 24 h incubation, conditioned media from the wells were clarified by centrifugation (200 × *g*, 5 min) and stored at -20°C until assayed. Quantikine ELISA kits (R&D Systems) were used to detect TNF-α, CCL2 or CXCL2 release from the macrophages according to manufacturer's instructions. Heat and protease treatment of the OMV/NT was performed as described above. A sham OMV/NT preparation was created by incubation of 400 ml BHI medium (without bacteria) in a 2 l flask, followed by all steps as done for purification of OMV/N. The final ultracentrifugation tubes containing the sham "pellet" were washed with 20 mM HEPES buffer containing 1% penicillin/streptomycin, 10 µg/ml gentamicin, and aliquots were flash frozen and stored at -80ºC. The values for each experiment were determined from the average of triplicate wells; three independent experiments were performed for the dose response experiments, and two independent experiments were performed for the experiments involving heat and proteinase K treatment.

XVII. Mouse Vaccination

Groups of seven BALB/c mice (6 to 8-weeks old, Charles River) were intranasally inoculated with 20 µg OMV/NT in PBS or PBS alone. Six weeks after vaccination, mice were challenged intranasally with 620 (n=3) or 960 (n=4) CFU of *F. novicida* grown in BHI medium to exponential phase. The infectious doses were determined by retrospective CFU counts. The LD_{50} for intranasal infection of mice by *F. novicida* U112 is 10 or fewer CFU (Lauriano, Barker et al. 2004; Pierson, Matrakas et al. 2011). Mice were monitored for 21 days following bacterial challenge.

XVIII. Statistical analysis

Cytotoxicity results were analyzed for significance using data obtained from three independent experiments with multiple replicates. *P* values were calculated by one-way analysis of variance and Bonferroni's multiple-comparison posttest against the negative control value. The log-rank test was used to calculate the *P* value for the mouse challenge experiments, using the combined survival data. Statistical calculations were performed using Prism 4.0 (GraphPad Software). P values < 0.05 were considered significant.

XIX. Whole Proteome Analysis

F. novicida cultures were grown in 50 ml BHI or TS in 250 ml flasks with baffles to OD_{600} ~1.4. Twenty-five ml of each culture was centrifuged (10,000 × *g,* 5 minutes, 4ºC). Pellets were resuspended in 10 ml PBS, and 25 µl was saved for TEM analysis. Resuspended pellets were centrifuged (10,000 × *g,* 5 minutes, 4ºC). Pellets were resuspended in 1 ml PBS then centrifuged (10,000 × *g,* 5 minutes, 4ºC). Supernatant was removed, and pellets were resuspended in 500 µl 100 mM ammonium bicarbonate containing 1% Triton X-100. Samples were sonicated in an ice water bath (power level 5), 15 seconds on and 15 seconds off, for 2 minutes. The sample was centrifuged (10,000 × *g*, 10 minutes, 4ºC) to pellet unbroken cells. Supernatants were stored at -20ºC until analysis by mass spectrometry. TEM grids were made from 25 µl aliquots and checked for presence or absence of NT.

XX. Chemical treatment of OMV/NT

Purified OMV/NT were treated with 6 M guanidine-HCl or 6 M urea for one hour at room temperature. Purified OMV/NT were resuspended in 0.1 M Tris-EDTA and heated at 37˚C for one hour. Samples were then processed for TEM as described above.

XXI. Cryo-EM tomography

F. novicida was grown in 400 ml BHI medium in a 2L flask with baffles to OD_{600} ~1.4. One ml aliquots were centrifuged at 8,000 × *g* for 2 minutes and resuspended in PBS containing 10 μg/ml chloramphenicol. Samples were then plunge frozen by the Huilin Li laboratory and examined by EM.

Chapter 3. Isolation of Outer Membrane Vesicles in *F. novicida*

I. Introduction

We report here the production of outer membrane vesicles (OMV) and nanotubes (NT) by *F. novicida*. OMV are spherical structures ranging from 50 to 250 nm in diameter and composed of phospholipids, proteins and lipopolysaccharide, all components commonly found in the outer membrane of bacteria. OMV are produced by numerous bacteria and function as secretory vehicles for toxins, DNA and signaling molecules in these organisms (Ellis and Kuehn 2010). OMV are continuously shed from the membrane of bacteria during all stages of growth, and their ubiquity suggests an important role in bacterial survival. The exact method of OMV biogenesis is not known, though a number of models have been proposed (Mashburn-Warren and Whiteley 2006). NT have been described in eukaryotic cells and select bacteria as tube-like structures, 50-200 nm in diameter and as long as several cell lengths. In eukaryotic cells, tunneling nanotubes are capable of transferring cytoplasmic molecules, organelles and even viruses between cells (Belting and Wittrup 2008; Schara, Jansa et al. 2009; Hurtig, Chiu et al. 2010). In bacteria, a role in transferring cytoplasmic molecules between organisms has been demonstrated (Dubey and Ben-Yehuda 2011; Galkina, Romanova et al. 2011).

In numerous pathogenic organisms OMV have been shown to be enriched in toxins and other molecules which can adversely affect host cells (Wai, Lindmark et al. 2003; Bartruff, Yukna et al. 2005; Kouokam, Wai et al. 2006; Berlanda Scorza, Doro et al. 2008; Bomberger, Maceachran et al. 2009; Ellis and Kuehn 2010; Kim, Lee et al.

2010; Vidakovics, Jendholm et al. 2010; Pierson, Matrakas et al. 2011). As a result of the cargo carried by these structures, OMV are capable of causing numerous effects in the absence of the bacteria from which they are generated. OMV from other organisms are capable of causing a cytotoxic effect when applied to host cells (Jin, Kwon et al. 2011; Maldonado, Wei et al. 2011). A number of studies have shown that OMV are capable of stimulating the host immune response through activation of cytokines (Bauman and Kuehn 2006; Alaniz, Deatherage et al. 2007; Prados-Rosales, Baena et al. 2011; Avila-Calderon, Lopez-Merino et al. 2012). The stimulatory properties of OMV have prompted researchers to explore their use as subunit vaccines for some pathogenic organisms (Alaniz, Deatherage et al. 2007; Holst, Martin et al. 2009; Schild, Nelson et al. 2009; Roy, Hamilton et al. 2011).

In this study we have identified specific growth conditions under which *Francisella* produces OMV and NT in abundance, have analyzed the protein content, and examined the host response to these structures. We have found that the proteins associated with OMV/NT change over the course of the organism's growth and vary between strains of *Francisella.* Many of the associated proteins have previously been described as secreted or virulence factors of *Francisella,* or OMV-associated in other bacteria. We examined the response of host cells when OMV/NT isolated from *F. novicida* are applied to them. We show that the cytotoxic effect normally seen with other pathogenic OMV is minimally observed with OMV/NT from *Francisella.* The cytokine response from incubation of OMV/NT with host cells is robust, dose dependent and requires these structures to be intact for full effect. Finally, we show that immunization

of mice with OMV/NT affords protection against challenge with high doses of the *F. novicida* organism.

II. Results

Francisella produces OMV and NT in response to growth phase and medium

A thorough examination of the production of outer membrane vesicles by *Francisella* begins with determining the ideal growth conditions for production of these structures. OMV production occurs through all stages of an organism's growth, though increased cell death at later phases would raise concerns about cytosolic contaminants. The *F. novicida* OMV characterized by Pierson *et al.* were isolated from bacteria grown to very late stationary phase (40 h of growth), a time when many bacteria are dying and releasing contents due to cell lysis (Pierson, Matrakas et al. 2011). The protein composition and other properties of OMV may change with growth phase (Tashiro, Ichikawa et al. 2010), and vesicles produced by dying bacteria may be very different from vesicles produced during bacterial growth. Therefore, we examined *F. novicida* strain U112 for the production of OMV at earlier stages of growth. After dilution of an overnight culture to an OD_{600} of 0.01, strain U112 grows exponentially in Brain Heart Infusion medium (BHI) for approximately 8 h, until reaching an OD_{600} of ~1.0 (Fig. 3-1). The bacteria then enter stationary phase and remain at an OD_{600} of ~1.4 for an additional 16 h before beginning to decrease in optical density, indicating cell death (Fig. 3-1). We chose mid-exponential phase (OD_{600} = 0.6, ~4 h growth) and early stationary phase

(OD_{600} = 1.4, ~9 h growth) time points to examine strain U112 for the production of OMV.

Cell-free culture supernatant fractions from *F. novicida* U112 grown in BHI to the two time points were subjected to ultracentrifugation to harvest OMV. The OMV pellets were then further purified by floatation through a discontinuous OptiPrep density gradient. The majority of proteins in the pellets floated to the top, lower-density region of the gradient, as expected for vesicle-associated proteins (Fig. 3-2). We recovered vesicles from this same lower-density region of the gradient, but not from other fractions, confirming flotation of the vesicles up the gradient. OMV pellets were obtained from both the exponential and stationary phase BHI cultures. However, the exponential phase bacteria produced many fewer vesicles compared to the stationary phase cultures; 2 L of exponential phase supernatant yielded ~0.25 mg purified vesicles, whereas 2 L of stationary phase supernatant yielded ~1-2 mg purified vesicles. In addition, we were unable to isolate OMV from strain U112 grown to either exponential or early stationary phase in a different rich medium, Tryptic Soy (TS). Thus, vesicle production by *F. novicida* is responsive to both growth phase and growth media.

Examination of the gradient purified vesicles by transmission electron microscopy (TEM) revealed the presence of typical, spherical OMV for both the exponential and stationary phase BHI cultures (Fig. 3-3). Surprisingly, elongated, tube-shaped structures, or NT, were also present in the purified samples from both growth phases (Fig. 3-3). The spherical OMV ranged from ~50 to 300 nm in diameter, and the NT were ~40 nm in diameter and ranged from ~300 nm to 1.5 μm in length. Previous studies noted the presence of large protrusions on the surface of *Francisella* spp., similar in appearance to

the NT present in the purified vesicles (Gerasimov, Dolotov et al. 1997; Gil, Benach et al. 2004). In addition, recent publications have described morphologically similar NT extending from the surface of bacteria grown on solid medium (Dubey and Ben-Yehuda 2011; Galkina, Romanova et al. 2011). However, production of NT by liquid-grown bacteria or the release of NT into the culture medium has not been reported.

To determine if *F. novicida* produced NT on its cell surface, we examined U112 bacteria grown to early stationary phase in BHI broth. TEM imaging of whole bacteria revealed the presence of NT projecting out from the bacterial surface, similar in diameter and appearance to the NT present in the cell-free culture supernatants (Fig. 3-4a). NT produced by *Bacillus subtilis* were shown to be extensions of the cytoplasmic membrane and to connect neighboring bacteria to allow the exchange of cytoplasmic constituents (Dubey and Ben-Yehuda 2011). In contrast, as revealed by thin section TEM, the *F. novicida* NT appear to be formed by extensions of the OM (Fig. 3-4b). In addition, we did not observe evidence of direct bacterial-bacterial bridging, as most of the NT were not in contact with neighboring bacteria (Figs. 3-4 and 3-5). As found for the OMV, production of NT on the surface of strain U112 was greater for bacteria grown to early stationary phase compared to exponential phase, and for bacteria grown in BHI broth compared to TSB (data not shown). Plate grown bacteria also produced NT (Fig. 3-5). NT produced by plate-grown bacteria were more numerous and longer than those seen in broth-grown cultures. This suggests that the NT may be sensitive to shear forces generated during the growth and processing of liquid cultures. However, many of the NT produced by the plate-grown bacteria were also detached from the bacterial surface, suggesting that release of the NT into the surrounding medium may be an active process

and not solely driven by shear forces (Fig. 3-5). Similar to broth grown *F. novicida*, we observed a dramatic decrease in the amount of NT produced by bacteria grown on TSB compared to BHI agar (Fig. 3-5). Bacteria grown on Mueller-Hinton (MH) or Chamberlain's Defined Media (CDM) agar also had markedly fewer NT compared to BHI-grown bacteria (data not shown). Taken together, these results show that production of NT on the bacterial surface and the release of NT and OMV into the culture medium are similarly regulated processes and responsive to both growth medium and growth phase.

Purification and initial characterization of *Francisella* OMV and NT

As shown in Fig. 3-3, the gradient-purified vesicles contained both OMV and NT. We were unable to separate the OMV from the NT using either density gradient flotation or velocity sedimentation, suggesting that the OMV and NT are similar in composition. The purified OMV/NT had a distinct protein profile compared to *F. novicida* total cell lysates, periplasm, OM, or total secreted proteins (Fig. 3-6). This differential protein profile is in keeping with OMV from other bacteria, which are enriched in a subset of OM and periplasmic proteins (Horstman and Kuehn 2000; Kato, Kowashi et al. 2002; Lee, Bang et al. 2007; Haurat, Aduse-Opoku et al. 2011). Notably, there were substantial differences in the protein profiles for OMV/NT isolated from exponential versus stationary phase cultures (Fig. 3-6). A similar dynamic protein content has been reported for OMV isolated from *P. aeruginosa*, which upregulates the OMV-associated signaling molecule PQS upon entry into stationary phase (Tashiro, Ichikawa et al. 2010).

To obtain a qualitative measure of luminal versus surface-exposed proteins present in the *F. novicida* OMV/NT, we incubated purified vesicles with proteinase K in the absence or presence of 0.02% SDS. Addition of 0.02% SDS disrupts the integrity of both the OMV and NT, allowing access of the protease to the interior content of the vesicles. Incubation of purified vesicles with proteinase K alone resulted in the loss of a number of presumably surface exposed proteins, but the overall protein profile was mostly unchanged (Fig. 3-7a). Incubation of the samples with SDS alone had no effect on the protein profile; however, incubation with proteinase K in the presence of 0.02% SDS resulted in a dramatic loss of protein bands (Fig. 3-7a), showing that a large number of proteins are protected by the intact vesicles. Of note, incubation of the vesicles with proteinase K alone did not cause changes in the appearance or number of NT, indicating that whatever is structuring these tubes is not a surface-accessible protein (data not shown).

The NT are formed by extension of the bacterial OM (Fig. 3-4b) and thus could be structured by an internal peptidoglycan backbone. To test this, we incubated the purified OMV/NT with lysozyme in Tris-EDTA buffer, to allow access of the lysozyme to the lumen of these structures, and also examined spheroplasted whole bacteria for the presence of NT. Lysozyme treatment had no effect on the purified NT, and while treatment of whole bacteria was clearly effective in digesting the peptidoglycan and generating spheroplasts, the NT remained intact on the spheroplasted bacteria (Fig. 3-8a). Thus, the NT are not structured by peptidoglycan. We next examined sensitivity of the vesicles to heat treatment. Purified OMV/NT were held at room temperature, incubated at 80ºC for 1 h, or incubated at 60ºC for 5, 15 or 30 min, and then left to cool at room

temperature. OMV/NT were stable at room temperature, but incubation at 80ºC caused a nearly complete disruption of the vesicles (data not shown). The nanotubes were sensitive to heat treatment, as no tubular vesicles remained after heating to 60ºC for as little as 5 min (Fig. 3-8b). In contrast, the number of spherical vesicles increased (Fig. 3-8c), suggesting denaturation of a factor responsible for structuring the nanotubes and conversion to spherical shape. Consistent with this, some vesicles at the 5 min time point appeared to be transitioning from a tubular to a spherical shape (Fig. 3-8b). The total numbers of remaining spherical vesicles decreased with longer incubation (Fig. 3-8b and c), demonstrating a general sensitivity to lysis by heat. Taken together, these results indicate that a heat-sensitive factor, presumably a protein(s), is responsible for structuring the nanotubes.

Identification of *F. novicida* OMV and NT-associated proteins

To identify OMV- and NT-associated proteins, gradient-purified vesicles were analyzed by mass spectrometry, using the MudPIT (multidimensional protein identification technology) method (Delahunty and Yates 2007). Three independent analyses were performed for each time point (exponential or early stationary phase), and only proteins appearing in all three analyses were considered as vesicle-associated for that time point. A normalized spectral abundance factor (NSAF, see Materials and Methods) was used to quantify the relative amounts of individual proteins in each sample. The MudPIT analysis identified 99 proteins from the exponential phase vesicles and 286 proteins from the stationary phase vesicles (Tables 3-1 and 3-2), with a combined identification of 292 unique OMV/NT-associated proteins. Consistent with the different

protein profiles of the exponential and stationary phase OMV/NT (Fig. 3-6), there were a number of changes in the proteins identified by mass spectrometry between the two time points. Most notably, although 90 of the 94 proteins present in exponential phase vesicles are also found in stationary phase vesicles, the stationary phase vesicles contain almost 200 additional proteins. This likely reflects the upregulation in OMV and NT production upon entry of cultures into stationary phase, as well as changes in protein expression associated with stationary phase. The relative abundance of most of the 90 shared proteins remained consistent at both time points; however, 28 proteins exhibited greater than 2 fold changes in abundance between samples, with 24 being found in lower abundance and 4 in greater abundance in the stationary compared to exponential phase OMV/NT (Table 3-3).

The 292 unique OMV/NT-associated proteins comprise ~17% of the *F. novicida* genome and are distributed among multiple functional categories and cellular locations (Fig. 3-9). Approximately 16% of the vesicle-associated proteins were previously shown to be OM-associated in *Francisella* spp. (Table 3-4) (Pavkova, Hubalek et al. 2005; Huntley, Conley et al. 2007; Janovska, Pavkova et al. 2007) and 20% have homologs that are OMV-associated in other bacteria (Table 3-5) (Post, Zhang et al. 2005; Nevot, Deroncele et al. 2006; Vipond, Suker et al. 2006; Lee, Bang et al. 2007; Berlanda Scorza, Doro et al. 2008). OM-associated proteins are prominent among the most abundant vesicle-associated proteins (comprising ~15% of the NSAF values), consistent with their derivation from the OM, and include the major *Francisella* antigens and T cell epitopes FopA, FopB and LpnA (Huntley, Conley et al. 2007; Yu, Goluguri et al. 2010). FopA is an integral OM protein that is highly immunogenic and serves as a protective antigen for

tularemia (Nano 1988; Savitt, Mena-Taboada et al. 2009; Hickey, Hazlett et al. 2011). Immunoblotting with anti-FopA antibodies confirmed its presence in the purified OMV/NT (Fig. 3-7b). Furthermore, FopA was insensitive to digestion by proteinase K and only minimally sensitive to digestion in the presence of 0.02% SDS (Fig. 3-7b), indicating maintenance of proper protein structure and membrane integrity in the purified vesicles.

Approximately 22% of the vesicle-associated proteins were previously shown to be secreted, extracellular, or associated with virulence in *Francisella* spp. (Table 3-6) (Nano, Zhang et al. 2004; Hager, Bolton et al. 2006; Lee, Horwitz et al. 2006; Qin and Mann 2006; Tempel, Lai et al. 2006; Su, Yang et al. 2007; Qin, Scott et al. 2011). Four proteins, PepO, BglX, ChiA and Fsp53, are secreted by strain U112 in a type IV pili-dependent manner (Tables 3-1 and 3-2) (Hager, Bolton et al. 2006; Zogaj, Chakraborty et al. 2008). Notably, Fsp53 is the most abundant protein present in the exponential phase OMV/NT (Table 3-1) and appears as the most abundant band in the protein profile of the exponential phase vesicles (Fig. 3-6). The identity of this band was confirmed by mass spectrometry (data not shown). These proteins might associate with the OMV and NT following secretion by the type IV pilus pathway, similar to the secretion of heat-labile enterotoxin in enterotoxigenic *E. coli* (Horstman and Kuehn 2000; Ellis and Kuehn 2010). Alternatively, the proteins might enter the vesicles from the periplasm, prior to their secretion across the OM. Lee *et al*. identified twelve major extracellular proteins in cell-free culture supernatants of *F. tularensis* LVS and a fully virulent clinical isolate (Lee, Horwitz et al. 2006). Five of these proteins are present in the purified OMV/NT, including the peroxidase/catalase KatG, the succinyl-CoA synthetase SucD and subunit

SucC, the peroxiredoxin Ahp1, and the chaperonin GroEL (Tables 3-1 and 3-2) (Noah, Malik et al. 2010). A number of FPI-associated proteins were also detected in the purified vesicles: IglB, IglC, IglI, PdpB, and PdpD. All of these proteins have been shown to be essential for intramacrophage growth and *Francisella* virulence (Santic, Molmeret et al. 2005; Tempel, Lai et al. 2006; de Bruin, Ludu et al. 2007; Ludu, de Bruin et al. 2008; Cong, Yu et al. 2009; Schmerk, Duplantis et al. 2009).

Additional abundant OMV/NT-associated proteins known to be virulence factors of *Francisella* spp. include FipB and the hypothetical proteins FTN_0714, FTN_0340, FTN_0429 and FTN_0643 (Tables 3-1, 3-2, 3-6) (Tempel, Lai et al. 2006; Weiss, Brotcke et al. 2007; Kraemer, Mitchell et al. 2009; Qin, Scott et al. 2011). Each of the hypothetical proteins was identified in transposon mutant screens of strain U112 as defective for colonization of mice following either pulmonary or intraperitoneal infection (Tempel, Lai et al. 2006; Weiss, Brotcke et al. 2007; Kraemer, Mitchell et al. 2009). All have predicted signal sequences and therefore are likely to be exported outside the cytoplasm where they could associate with the OMV/NT. FipB was identified as an essential virulence factor in the fully virulent *F. tularensis* Schu S4 strain (Qin, Scott et al. 2011). FipB is a predicted lipoprotein with a DsbA periplasmic disulfide isomerase domain and a domain homologous to the surface-exposed Mip host cell invasion protein of *Legionella pneumophila* (Riboldi-Tunnicliffe, Konig et al. 2001). *F. novicida* FipB is 98.9% homologous to the Schu S4 protein. In addition to FipB, stationary phase OMV/NT contained high levels of the FipA protein (Table 3-1). FipA is encoded immediately upstream of *fipB* and also has homology with Mip proteins. FipA is not required for the virulence of *F. tularensis* in mice, but contributes to intracellular survival

and may influence the activity of FipB (Qin, Scott et al. 2011). We confirmed the presence of FipB in the purified vesicles by immunoblotting with anti-FipB antibody (Fig. 3-7c). FipB was largely protected from proteinase K digestion when vesicles were incubated with proteinase K alone, although a small amount of a ~30 kDa cleavage product appeared (Fig. 3-7c). In contrast, FipB was completely degraded by proteinase K in the presence of 0.02% SDS (Fig. 3-7c). This indicates a primarily luminal location for FipB, consistent with a periplasmic location, but suggests that at least some FipB may be surface-exposed, similar to Mip proteins (Riboldi-Tunnicliffe, Konig et al. 2001). The presence of multiple virulence factors and secreted proteins supports a role for the OMV/NT in the pathogenesis of tularemia.

F. novicida OMV/NT are minimally cytotoxic to host cells

OMV are enriched in immunostimulatory molecules such as LPS and lipoproteins, and may contain toxins and other proteins that are active against host cells (Wai, Lindmark et al. 2003; Bartruff, Yukna et al. 2005; Kouokam, Wai et al. 2006; Berlanda Scorza, Doro et al. 2008; Bomberger, Maceachran et al. 2009; Ellis and Kuehn 2010; Kim, Lee et al. 2010; Vidakovics, Jendholm et al. 2010; Pierson, Matrakas et al. 2011) (Bauman and Kuehn 2006; Alaniz, Deatherage et al. 2007; Prados-Rosales, Baena et al. 2011; Avila-Calderon, Lopez-Merino et al. 2012). Although *Francisella* LPS is not proinflammatory, the *F. novicida* OMV and NT contain numerous lipoproteins as well as known *Francisella* virulence factors and antigenic proteins (Table 3-6). To determine effects of the *F. novicida* OMV/NT on host cells, we first examined cytotoxicity using a lactate dehydrogenase (LDH) release assay. Increasing amounts of purified OMV/NT

isolated from stationary phase cultures were incubated with primary murine bone marrow-derived macrophages (muBMDM) isolated from C3H/HeN mice and LDH release was measured at 24 and 48 h. We observed no significant cell death after 24 h incubation, but the vesicles triggered an apparent dose-dependent cytotoxic response after 48 h (Fig. 3-10a). However, only the 20% cell death achieved with the highest vesicle dose (20 μg/ml) was significantly different from the untreated cells ($P < 0.05$). Given the extended incubation time required and general lack of significant effect, we conclude that the *F. novicida* vesicles have minimal cytotoxicity to host cells.

F. novicida OMV/NT produce a dose dependent cytokine response

We next examined the ability of the *F. novicida* OMV/NT to stimulate proinflammatory responses of host cells. We incubated purified OMV/NT with muBMDM for 24 h and measured levels of released cytokines TNFα, CXCL2 and CCL2 by ELISA. TNFα is primarily produced by activated macrophages and is involved in systemic inflammation. CXCL2 is a chemokine secreted by macrophages which functions as a chemoattractant for polymorphonuclear leukocytes. CCL2 is another chemokine which can recruit monocytes, memory T cells and dendritic cells to the sites of infection. A robust, dose dependent increase for each of the cytokines was observed, with significantly increased release for the 1 and 10 μg/ml vesicle doses compared to buffer only or sham vesicle preparation controls (Fig. 3-10b). To examine the mechanism by which the *F. novicida* OMV/NT trigger proinflammatory responses, we incubated the vesicles with proteinase K to digest surface-accessible proteins, as shown in Fig. 3-7a, prior to adding the vesicles to the muBMDM. The proteinase K treatment of

intact OMV/NT had no effect on release of the three cytokines compared to untreated vesicles (Fig. 3-10c), indicating surface-exposed proteins are not required. It has been shown that OMV must be intact to deliver their cargo to host cells (Bomberger, Maceachran et al. 2009). Therefore, we next pre-incubated the *F. novicida* OMV/NT at 80ºC for 1 h to disrupt the vesicles prior to addition to the muBMDM. Disruption of the OMV/NT significantly decreased the levels for each of the cytokines by more than half compared to untreated vesicles (Fig. 3-10c) (representative experiment of two). Treatment of the heat-disrupted vesicles with proteinase K did not result in further changes in cytokine release compared to heat-treatment alone (Fig. 3-10c). Thus, the OMV/NT must be intact to fully trigger proinflammatory responses from host cells.

F. novicida produces NT during infection of host cells

The OMV and NT released into the surrounding medium by *F. novicida* would need to diffuse away from the bacteria to interact with host cells. In contrast, the NT extending from the bacterial surface could mediate direct contact with host cells. To determine if NT are produced during infection of host cells, we examined *F. novicida* U112 during early stages of infection of muBMDM. The bacteria were grown in BHI to early log phase (OD_{600} = 0.4, ~2 h growth), a time point where very few bacteria express NT, and placed in suspension with muBMDM on ice. Samples were heated to 37ºC to initiate phagocytosis, then fixed after 5 minutes and processed for TEM imaging by thin-sectioning as previously described (Clemens, Lee et al. 2011). NT were seen extending from bacteria in close proximity to macrophages as well as from those that had been taken up by phagocytosis (Fig. 3-11). In some cases, the NT extended out from the

bacteria toward the macrophage plasma membrane and appeared to be initiating contact with the host cells (Fig. 3-11). These images show that production of NT by *F. novicida* is stimulated by interaction with host cells, and suggests roles for the NT in bacterial uptake. Of note is that addition of muBMDM medium (BMM_{HI}; see Materials and Methods) to the bacteria in the absence of host cells did not induce the production of NT (data not shown). Therefore, a host cell-derived signal presumably triggers NT production.

Vaccination with *F. novicida* OMV/NT provides protection against bacterial challenge

OMV have proved effective as vaccines for a number of bacterial pathogens (Alaniz, Deatherage et al. 2007; van de Waterbeemd, Streefland et al. 2010; Roy, Hamilton et al. 2011; Avila-Calderon, Lopez-Merino et al. 2012; Bishop, Tarique et al. 2012), and Pierson *et al.* showed that vaccination of mice with OMV isolated from late-stage *F. novicida* cultures provided limited protection against subsequent bacterial challenge (Pierson, Matrakas et al. 2011). We vaccinated mice by intranasal administration of 20 μg purified OMV/NT isolated from early stationary phase U112 cultures, or PBS as a control. We then challenged the mice 6 weeks later with highly lethal doses of *F. novicida* by the intranasal route (620-960 CFU; the LD_{50} of U112 is less than 10 CFU (Pierson, Matrakas et al. 2011)). As shown in Fig. 3-12, the vaccinated mice had significantly increased survival compared to the control mice ($P = 0.0053$). All mice infected at the lower challenge dose of 620 CFU (n = 3) survived the entire course of infection, whereas mice challenged at the higher dose of 960 CFU (n = 4) exhibited

significantly delayed time-to-death, with two mice surviving until day 17. This demonstrates that the *F. novicida* OMV/NT are capable of eliciting a protective immune response in vivo.

III. Figures

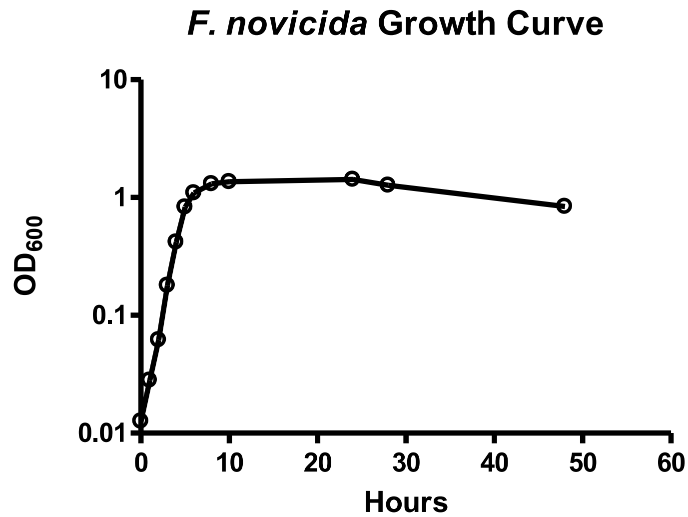


Figure 3-1. ***F. tularensis subsp. novicida*** **growth in BHI media.** Cultures (400 ml) were grown in BHI media for 48 hours.

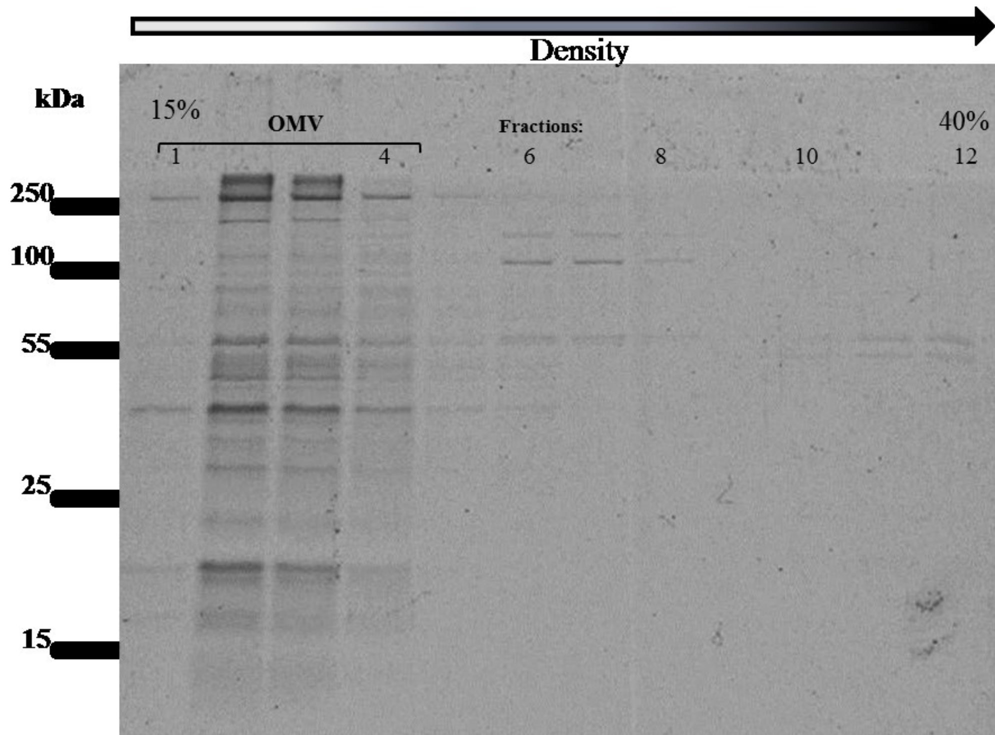


Figure 3-2. OptiPrep flotation of OMV/NT.

OMV/NT isolated from *F. novicida* were further purified through a discontinuous density gradient. Equal volume fractions were collected after flotation through the gradient, and aliquots were run on SDS-PAGE to examine protein content.

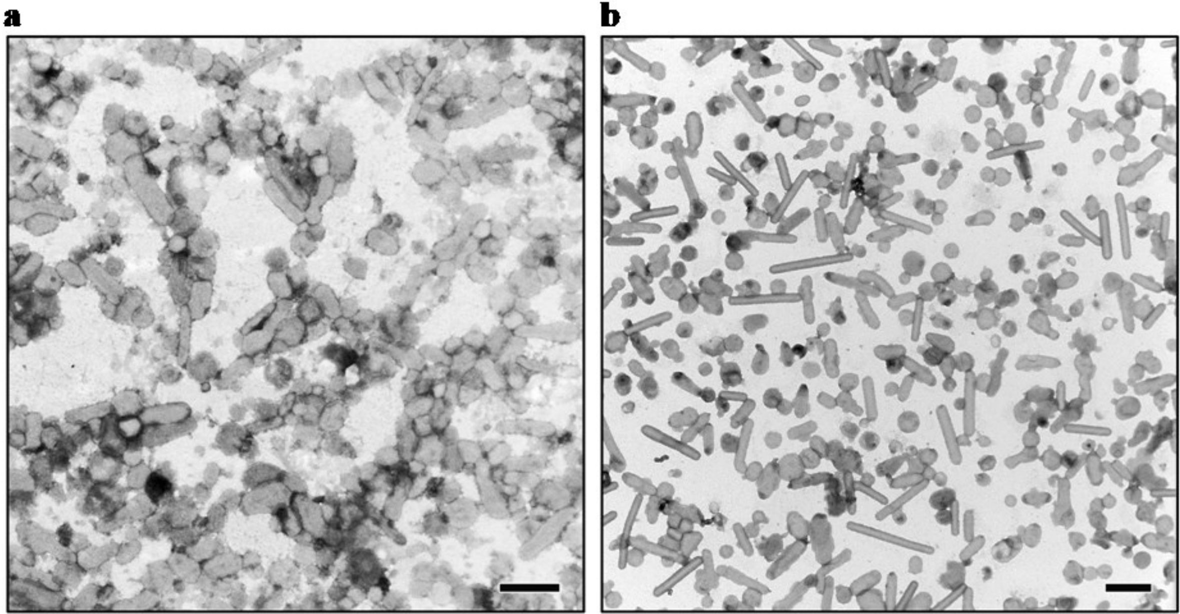


Figure 3-3. TEM Images of *F. tularensis* subsp. *novicida* purified OMV/NT.

(a) OMV/NT isolated using tangential flow filtration. (b) OMV/NT isolated by high-speed centrifugation of stationary phase cultures (black bars = 100 nm).

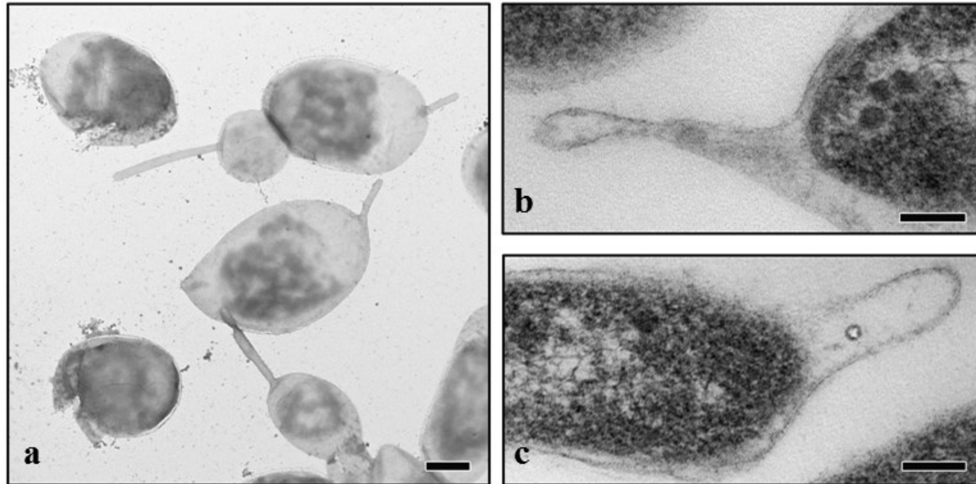


Figure 3-4. TEM Images of *F. tularensis subsp. novicida* whole bacteria and thin-sections.

(a) Bacteria grown in BHI to early stationary phase show production of nanotubes. (b, c) Bacteria grown in BHI were subjected to thin-sectioning, and nanotubes appear to be continuous with the periplasmic space (black bars = 100 nm).

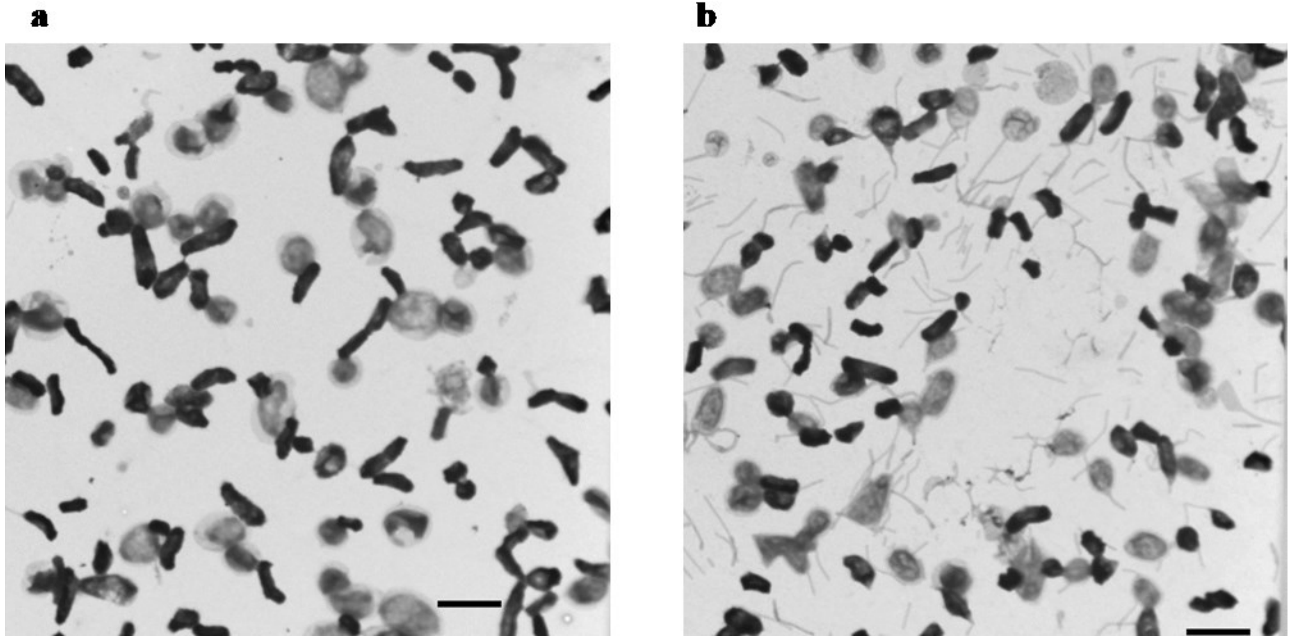


Figure 3-5. TEM Images of *F. tularensis* subsp. *novicida* whole bacteria.

Single colonies were isolated from (a) tryptic soy agar or (b) brain heart infusion agar. An increase in nanotubes is observed when bacteria are cultured with BHI medium (black bars = 2 microns).

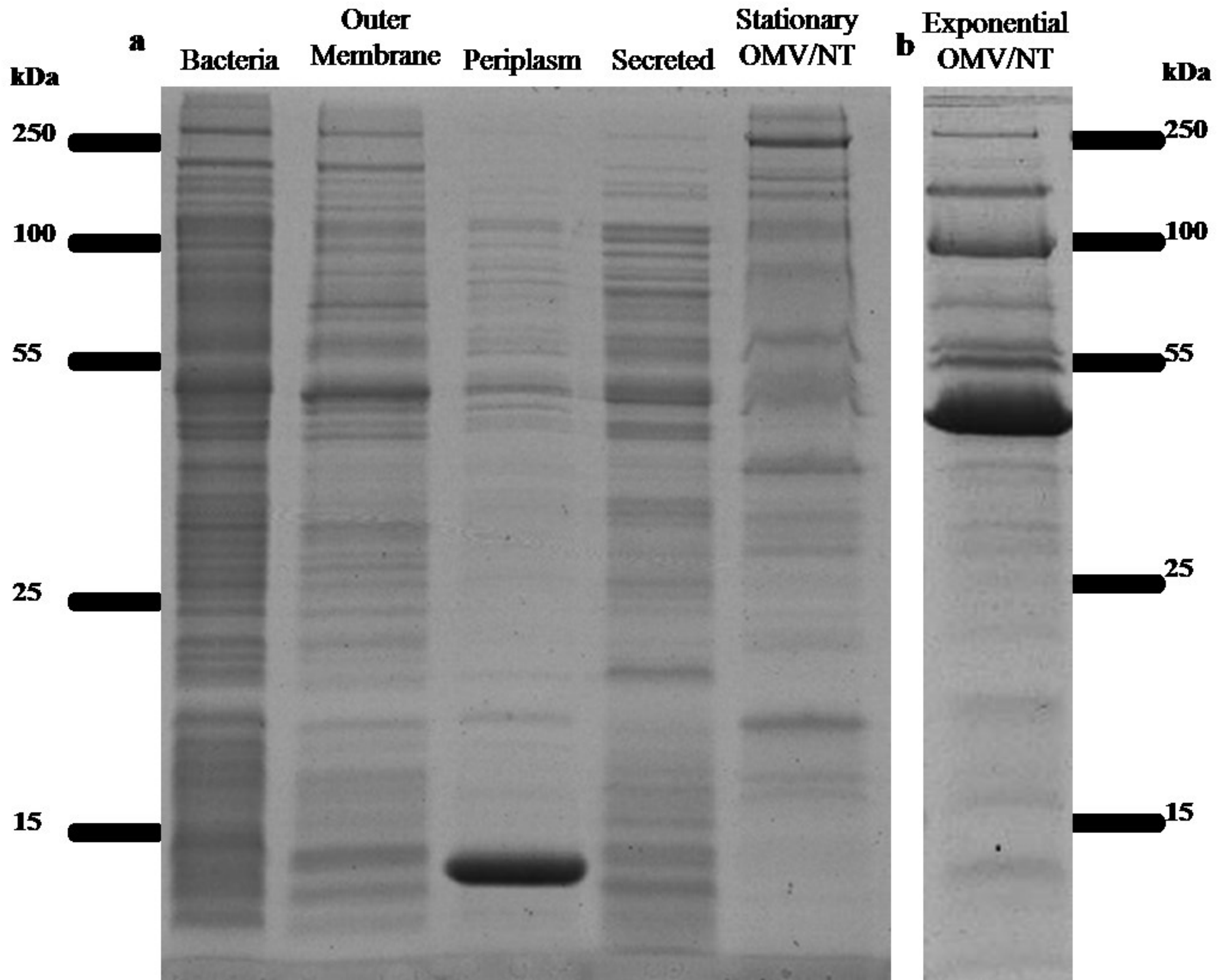


Figure 3-6. Protein profiles differ among OMV/NT, secreted proteins and other fractions.

Proteins isolated from stationary phase grown (a) whole bacteria, outer membrane (5 µg), periplasm (18 µg), TCA precipitated bacteria-free supernatant and density purified OMV/NT (6 µg) run on SDS-PAGE. (b) Concentrated and density purified OMV/NT (10 µg) run on SDS-PAGE.

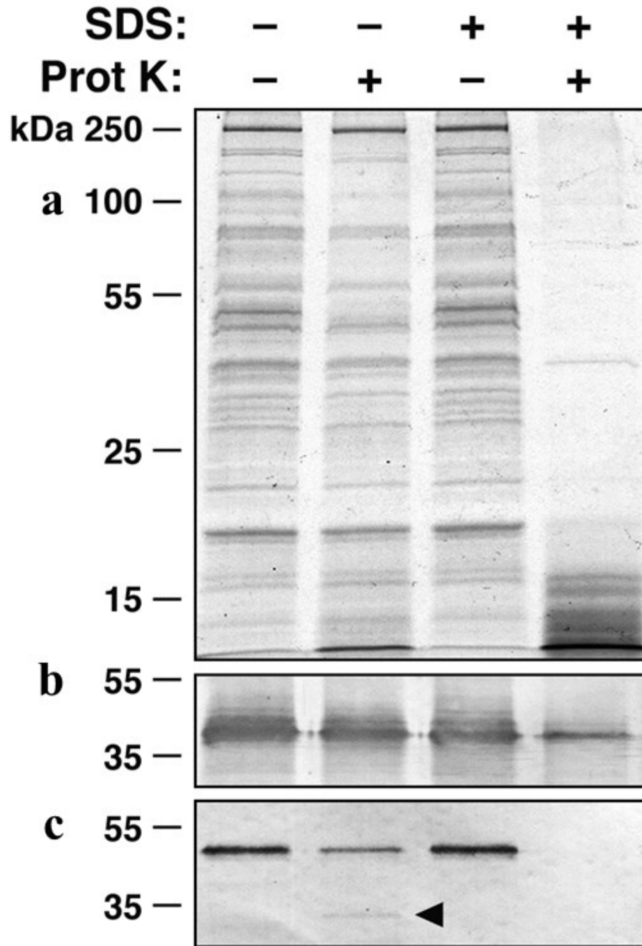


Figure 3-7. Proteinase accessibility assay.

OMV/NT purified from U112 grown to early stationary phase were treated with proteinase K in the presence or absence of 0.02% SDS to disrupt vesicle integrity. (a) The vesicles were subjected to SDS-PAGE and (a) stained with Coomassie blue, (b) blotted with anti-FopA, or (c) blotted with anti-FipB. A portion of FipB is sensitive to proteinase K digestion in the intact vesicles (filled arrowhead).

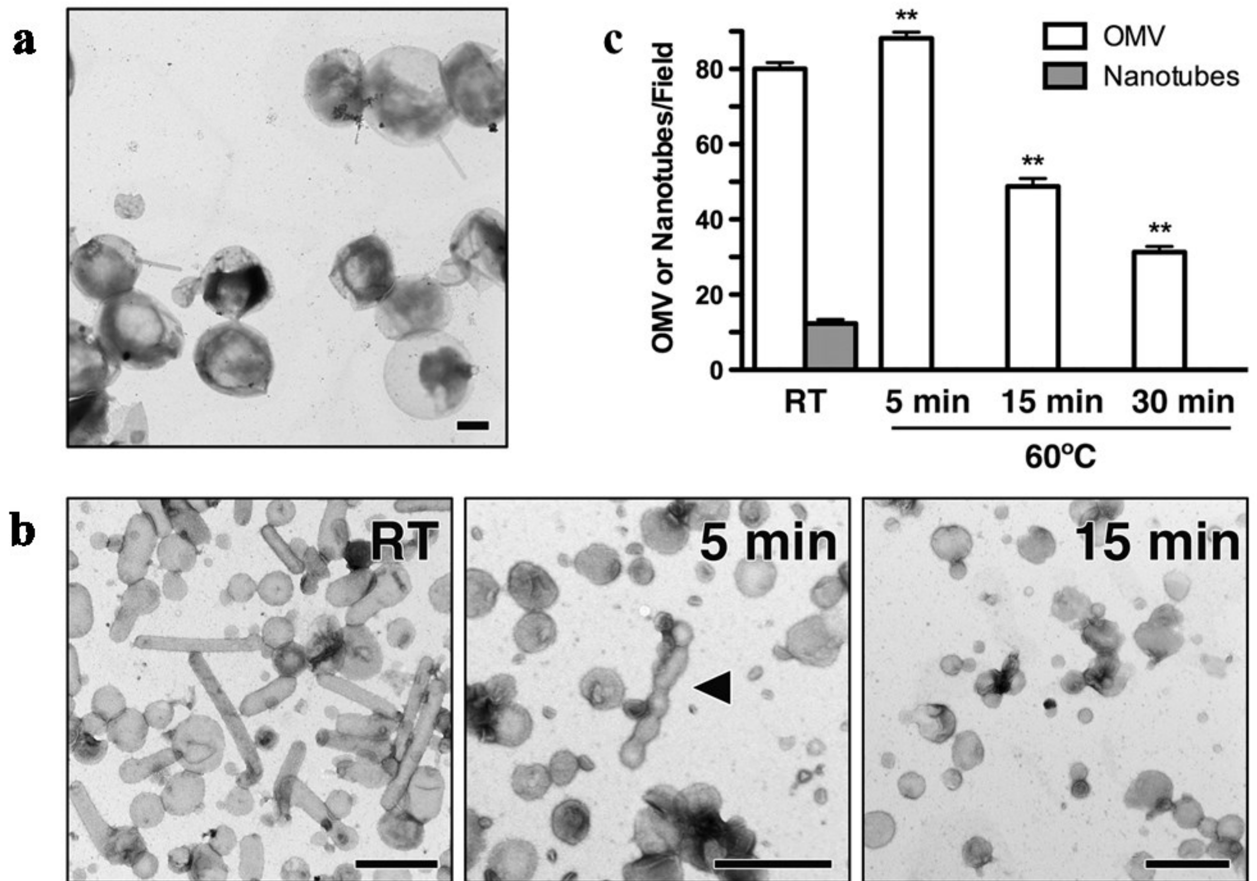


Figure 3-8. Disruption of OMV/NT.

TEM image of U112 whole bacteria grown in BHI broth to early stationary phase and treated with lysozyme to generate spheroplasts (a) (black bar = 500 nm). (b and c) OMV/NT purified from early stationary phase U112 were held at room temperature (RT) or incubated at 60ºC for 5, 15 or 30 min. TEM images of OMV/NT heated as indicated (b). The filled arrowhead notes a vesicle that appears to be transitioning from tubular to spherical shape (black bars = 500 nm). Quantitation of nanotubes and spherical OMV per TEM field at each time point (c). Bars = means ± standard errors of the means (SEM) from 10 fields. **, $P < 0.01$ for OMV in heated versus room temperature samples.

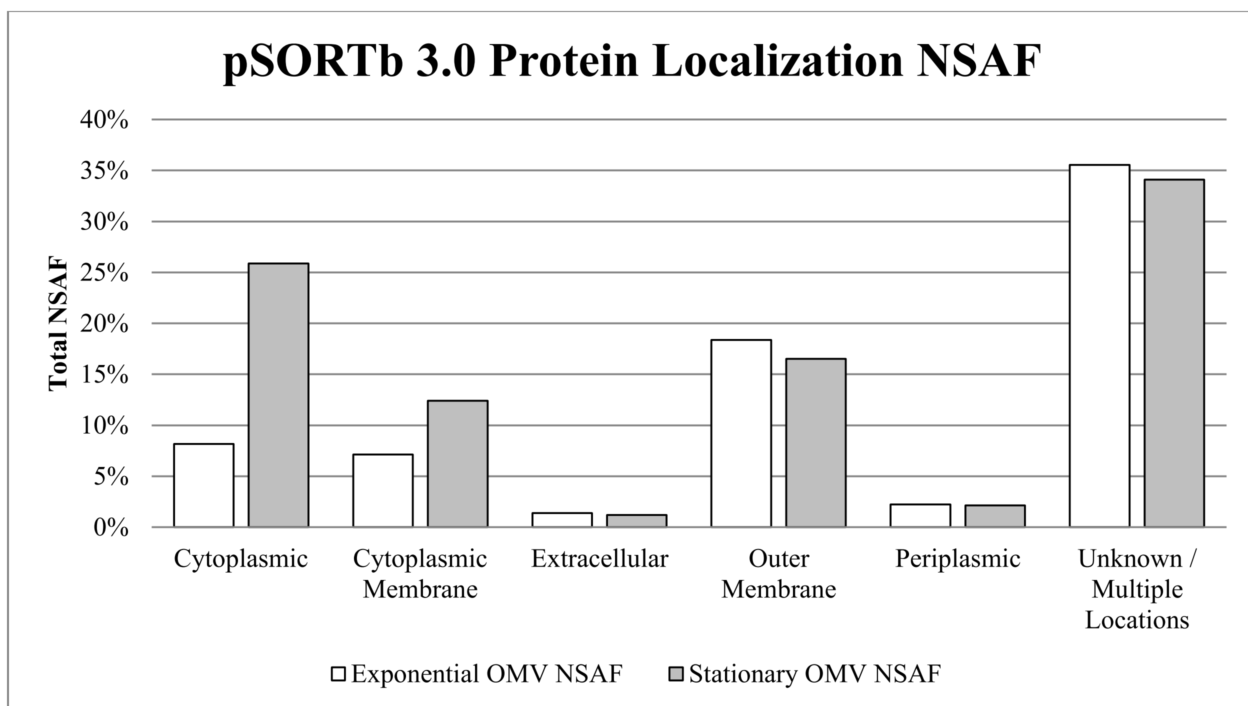


Figure 3-9. Predicted localization of OMV/NT-associated proteins.

pSORTb 3.0 predicted localization shows that the OMV/NT-associated protein content differs between different growth phases (NSAF = normalized spectral abundance factor).

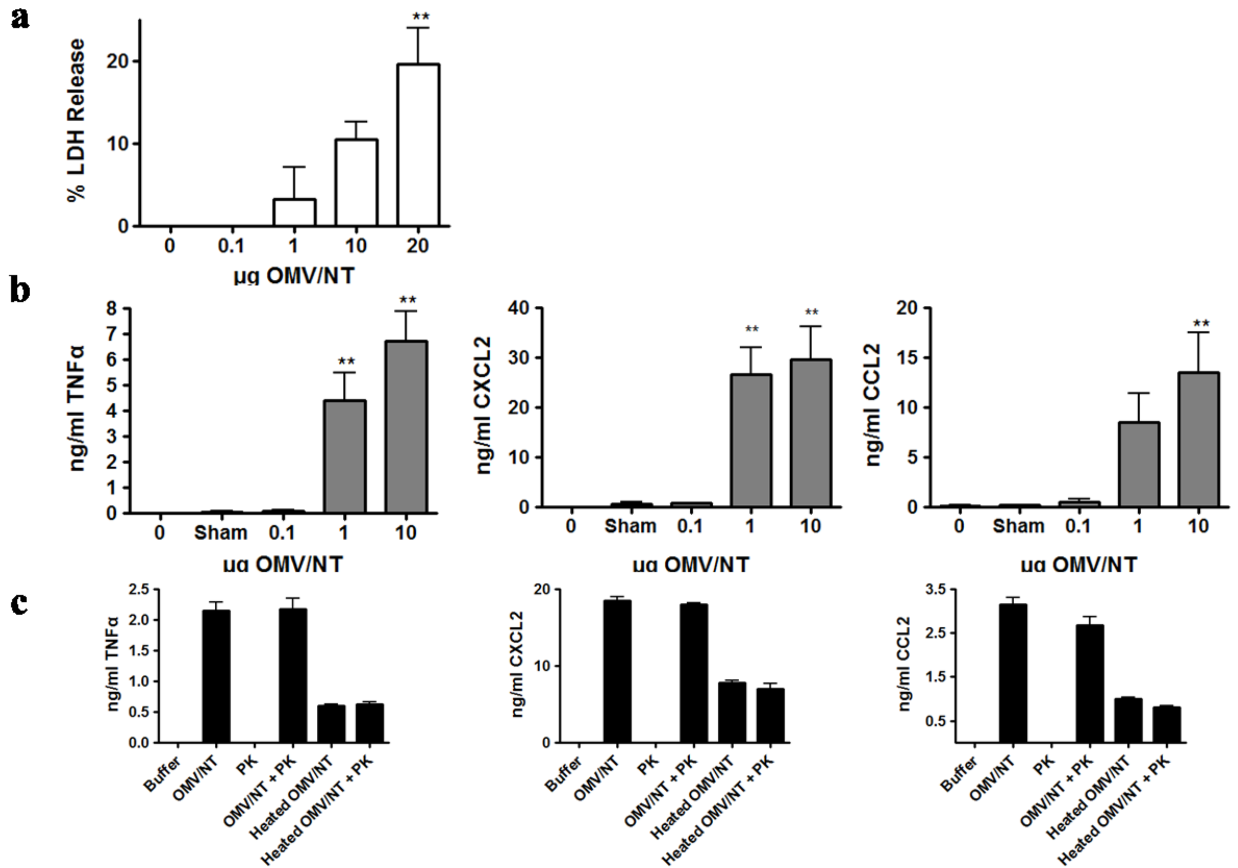


Figure 3-10. OMV/NT cytotoxicity and cytokine release.

(a) The indicated amounts of OMV/NT purified from early stationary phase U112 were incubated with muBMDM for 48 h and cytotoxicity was quantified by measuring LDH release. (b) The indicated amounts of OMV/NT or a sham vesicle preparation were incubated with muBMDM for 24 h and the release of TNFα, CXCL2 and CCL2 was quantified by ELISA of conditioned media. (c) Buffer only, 1 µg OMV/NT, proteinase K (PK) only, or 1 µg OMV/NT treated with proteinase K, heated to 80ºC for 1 h, or heated and then treated with proteinase K were added to muBMDM and cytokine release was quantified as in (b). Bars = means ± SEM for (a and b) three independent experiments or (c) a representative of two experiments. **, $P < 0.01$ for comparison with the buffer-only (0 µg OMV/NT) control.

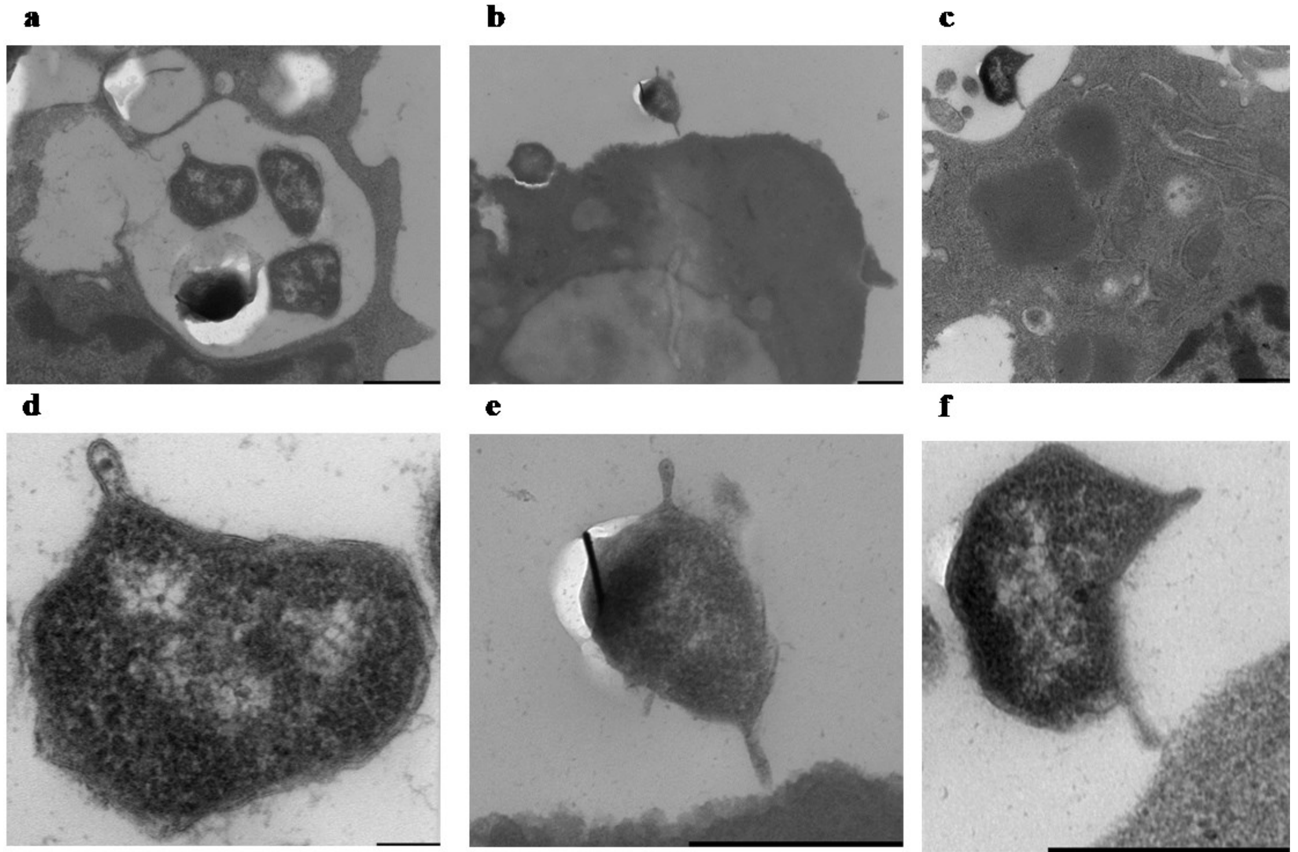


Figure 3-11. Thin-section TEM images of *F. novicida* during infection of muBMDM.

Bacteria were co-cultured with C3H/HeN murine bone-marrow derived macrophages and subjected to thin-sectioning. NT can be seen budding from bacteria located within phagosomes (a) or interacting with the membranes of macrophages (b, c), (d-f enlargements of a-c, all black bars = 500 nm except d, d black bar = 100 nm).

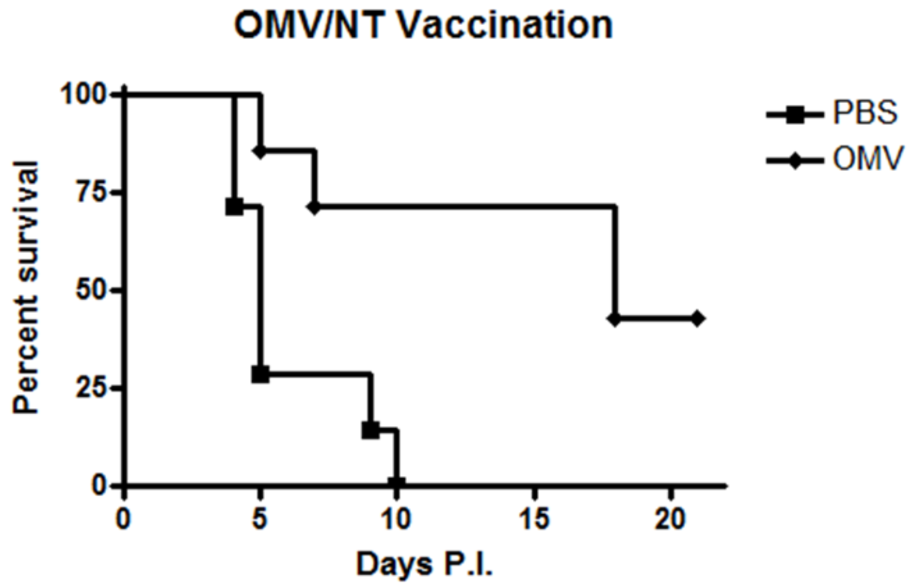


Figure 3-12. C3H/HeN Mouse Vaccination Study.

C3H/HeN mice were inoculated intranasally with purified OMV/NT (or a PBS control) and challenged after six weeks with high doses (60-90 LD_{50}) of *F. novicida* wild-type bacteria.

IV. Tables

Table 3-1. *F. novicida* exponential phase OMV/NT-associated proteins.

Gene	Locus	MW	Description	NSAF Average	pSORTb Localization
fsp53	FTN_1261	55 kDa	hypothetical protein	0.0974779	Unknown
fopB	FTN_0119	19 kDa	outer membrane protein of unknown function	0.047168	Periplasmic
lpnA	FTN_0427	16 kDa	lipoprotein of unknown function	0.038744	Outer Membrane
unknown	FTN_1451	20 kDa	hypothetical protein	0.0377439	Unknown
unknown	FTN_1734	14 kDa	hypothetical protein	0.028588	Unknown
unknown	FTN_0714	197 kDa	hypothetical protein	0.02779	Outer Membrane
unknown	FTN_0340	12 kDa	hypothetical protein	0.0236886	Unknown
fopA	FTN_0756	41 kDa	OmpA family protein	0.0221124	Outer Membrane
atpF	FTN_1650	17 kDa	F0F1 ATP synthase subunit B	0.0199808	Cytoplasmic Membrane
unknown	FTN_0429	19 kDa	hypothetical protein	0.0177096	Unknown
unknown	FTN_0428	18 kDa	hypothetical protein	0.0171688	Unknown
ompH	FTN_1481	19 kDa	outer membrane protein OmpH	0.0144517	Periplasmic
pal	FTN_0357	23 kDa	OmpA family peptidoglycan-associated lipoprotein	0.0127318	Outer Membrane
fipB	FTN_0771	39 kDa	protein-disulfide isomerase	0.012717	Unknown
unknown	FTN_1448	52 kDa	hypothetical protein	0.0110081	Unknown
dacD	FTN_0907	48 kDa	D-alanyl-D-alanine	0.0100713	Unknown/Multipl

			carboxypeptidase		e Localizations
unknown	FTN_0033	21 kDa	chorismate mutase	0.0097631	Cytoplasmic
unknown	FTN_0643	18 kDa	hypothetical protein	0.0088472	Unknown
tufA	FTN_1576	43 kDa	elongation factor Tu	0.0086381	Cytoplasmic
unknown	FTN_1449	22 kDa	hypothetical protein	0.0084314	Unknown
unknown	FTN_0921	31 kDa	FKBP-type peptidyl-prolyl cis-trans isomerase	0.0083011	Outer Membrane
unknown	FTN_1260	52 kDa	hypothetical protein	0.0082485	Unknown
unknown	FTN_0855	26 kDa	hypothetical protein	0.0076836	Unknown
unknown	FTN_0346	47 kDa	OmpA family protein	0.0072064	Unknown
unknown	FTN_0191	28 kDa	polar amino acid uptake transporter	0.0071796	Periplasmic
pcp	FTN_0211	24 kDa	pyrrolidone carboxylylate peptidase	0.0066218	Unknown/Multipl e Localizations
atpD	FTN_1646	50 kDa	F0F1 ATP synthase subunit beta	0.0066031	Cytoplasmic
unknown	FTN_0203	16 kDa	hypothetical protein	0.0064281	Extracellular
unknown	FTN_0183	34 kDa	periplasmic solute binding family protein	0.0064098	Cytoplasmic Membrane
pepO	FTN_1186	79 kDa	M13 family metallopeptidase	0.0063888	Cytoplasmic
bglX	FTN_1474	43 kDa	glycosyl 4hydrolase family protein	0.0060959	Cytoplasmic
sdhA	FTN_1637	66 kDa	succinate dehydrogenase flavoprotein	0.0054497	Cytoplasmic Membrane
tolB	FTN_0355	48 kDa	group A colicin translocation; tolB protein	0.0053847	Periplasmic
unknown	FTN_0109	37 kDa	hypothetical protein	0.0053743	Cytoplasmic

unknown	FTN_0381	38 kDa	hypothetical protein	0.0050469	Unknown
unknown	FTN_0782	21 kDa	hypothetical protein	0.0048514	Extracellular
unknown	FTN_0595	70 kDa	hypothetical protein	0.0048101	Cytoplasmic Membrane
katG	FTN_0633	82 kDa	peroxidase/catalase	0.004777	Unknown/Multiple Localizations
unknown	FTN_1433	33 kDa	hypothetical protein	0.0043567	Cytoplasmic Membrane
rpsA	FTN_0159	62 kDa	30S ribosomal protein S1	0.0040643	Cytoplasmic
atpA	FTN_1648	55 kDa	F0F1 ATP synthase subunit alpha	0.0039849	Cytoplasmic
unknown	FTN_1072	32 kDa	beta-lactamase class A	0.003955	Periplasmic
ftsZ	FTN_0164	40 kDa	cell division protein FtsZ	0.0038862	Cytoplasmic
acnA	FTN_1623	103 kDa	aconitate hydratase	0.0038579	Cytoplasmic
fabF	FTN_1341	44 kDa	beta-ketoacyl-ACP synthase II	0.0036751	Cytoplasmic
unknown	FTN_0597	28 kDa	protein-disulfide isomerase	0.0035855	Unknown
unknown	FTN_1367	60 kDa	hypothetical protein	0.0034999	Unknown
unknown	FTN_1372	40 kDa	hypothetical protein	0.0034568	Unknown
aceF	FTN_1493	67 kDa	dihydrolipoamide acetyltransferase	0.0033468	Cytoplasmic
unknown	FTN_1447	36 kDa	hypothetical protein	0.0032604	Unknown
unknown	FTN_0282	34 kDa	hypothetical protein	0.0032585	Cytoplasmic Membrane
pyk	FTN_1330	52 kDa	pyruvate kinase	0.0030421	Cytoplasmic
msbA	FTN_1606	67 kDa	lipid exporter (LipidE) family protein	0.0030331	Cytoplasmic Membrane

unknown	FTN_0322	40 kDa	VacJ like lipoprotein	0.0029597	Outer Membrane
pilF	FTN_0946	35 kDa	Type IV pili	0.0027028	Unknown
tolQ	FTN_0352	26 kDa	TolQ protein	0.0026219	Cytoplasmic Membrane
cyoB	FTN_0196	76 kDa	cytochrome bo terminal oxidase subunit I	0.0025443	Cytoplasmic Membrane
unknown	FTN_1276	38 kDa	membrane fusion protein	0.0025205	Cytoplasmic Membrane
unknown	FTN_0449	33 kDa	hypothetical protein	0.0024487	Unknown
unknown	FTN_0917	51 kDa	serine-type D-Ala-D-Ala carboxypeptidase	0.0024174	Periplasmic
fusA	FTN_0237	78 kDa	elongation factor G	0.0023773	Cytoplasmic
unknown	FTN_0022	39 kDa	histidine acid phosphatase	0.0023605	Extracellular
fimV	FTN_1596	49 kDa	Type IV pili	0.0023348	Unknown
unknown	FTN_1692	40 kDa	membrane fusion protein	0.0023264	Unknown
unknown	FTN_0715	135 kDa	hypothetical protein	0.0022658	Outer Membrane
kdpB	FTN_1717	73 kDa	potassium-transporting ATPase B chain	0.0022592	Cytoplasmic Membrane
accA	FTN_1508	35 kDa	acetyl-CoA carboxylase	0.0022328	Cytoplasmic
unknown	FTN_0545	36 kDa	glycosyl transferase	0.0022313	Cytoplasmic Membrane
tolR	FTN_0353	16 kDa	TolR protein	0.0021988	Cytoplasmic Membrane
unknown	FTN_0925	38 kDa	hypothetical protein	0.0021653	Cytoplasmic Membrane
fadE	FTN_1437	83 kDa	acyl-CoA dehydrogenase	0.0020788	Unknown
unknown	FTN_1268	27 kDa	hypothetical protein	0.0020569	Cytoplasmic

					Membrane
pdpB	FTN_1310	127 kDa	hypothetical protein	0.0019779	Unknown/Multiple Localizations
unknown	FTN_0073	62 kDa	membrane protein of unknown function	0.0019205	Cytoplasmic Membrane
aceE	FTN_1494	100 kDa	pyruvate dehydrogenase subunit E1	0.001912	Cytoplasmic
unknown	FTN_0482	36 kDa	hypothetical protein	0.0018871	Unknown
nuoG	FTN_1674	87 kDa	NADH dehydrogenase subunit G	0.001636	Cytoplasmic
putA	FTN_1131	150 kDa	bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	0.0016181	Cytoplasmic
unknown	FTN_1053	55 kDa	hypothetical protein	0.0016086	Cytoplasmic Membrane
nuoD	FTN_1677	48 kDa	NADH dehydrogenase subunit D	0.001602	Cytoplasmic
mltA	FTN_1286	45 kDa	membrane-bound lytic murein transglycosylase	0.0015854	Unknown/Multiple Localizations
ftsK	FTN_0294	92 kDa	cell division protein	0.0015711	Cytoplasmic Membrane
tolC	FTN_1703	57 kDa	outer membrane protein tolC precursor	0.0015357	Outer Membrane
rne	FTN_1246	101 kDa	ribonuclease E	0.0014988	Cytoplasmic
unknown	FTN_1610	113 kDa	RND efflux transporter	0.001492	Cytoplasmic Membrane
rpoB	FTN_1568	151 kDa	DNA-directed RNA polymerase subunit beta	0.0014533	Cytoplasmic
ilvC	FTN_1040	38 kDa	ketol-acid	0.0014444	Cytoplasmic

			reductoisomerase		
ggt	FTN_1159	65 kDa	gamma-glutamyltranspeptidase	0.0014003	Periplasmic
unknown	FTN_1644	105 kDa	hypothetical protein	0.0013537	Unknown
slt	FTN_0496	77 kDa	soluble lytic murein transglycosylase	0.0013204	Periplasmic
wbtA	FTN_1431	66 kDa	dTDP-glucose 4	0.0012862	Cytoplasmic Membrane
unknown	FTN_0103	88 kDa	hypothetical protein	0.0012229	Unknown
wbtE	FTN_1426	49 kDa	UDP-glucose/GDP-mannose dehydrogenase	0.0011936	Cytoplasmic
kdtA	FTN_1469	50 kDa	3-deoxy-D-manno-octulosonic-acid transferase	0.0011738	Cytoplasmic
infB	FTN_1660	92 kDa	translation initiation factor IF-2	0.0010941	Cytoplasmic
ostA1	FTN_0558	98 kDa	organic solvent tolerance protein	0.0010505	Outer Membrane
clpB	FTN_1743	96 kDa	chaperone clpB	0.0010438	Cytoplasmic
leuA	FTN_0062	58 kDa	2-isopropylmalate synthase	0.000974	Cytoplasmic
rpoC	FTN_1567	157 kDa	DNA-directed RNA polymerase	0.0009598	Cytoplasmic

Table 3-2. *F. novicida* stationary phase OMV/NT-associated proteins.

Gene	Locus	MW	Description	NSAF Average	pSORTb localization
fopB	FTN_0119	19 kDa	outer membrane protein of unknown function	0.057833	Periplasmic
unknown	FTN_1451	20 kDa	hypothetical protein	0.045424	Unknown
lpnA	FTN_0427	16 kDa	lipoprotein of unknown function	0.031957	Outer Membrane
fipA	FTN_0772	10 kDa	hypothetical protein	0.026185	Unknown
ompH	FTN_1481	19 kDa	outer membrane protein OmpH	0.025742	Periplasmic
fipB	FTN_0771	39 kDa	protein-disulfide isomerase	0.021712	Unknown
tufA	FTN_1576	43 kDa	elongation factor Tu	0.020572	Cytoplasmic
unknown	FTN_1448	52 kDa	hypothetical protein	0.016441	Unknown
ahp1	FTN_0973	22 kDa	AhpC/TSA family peroxiredoxin	0.016305	Cytoplasmic
unknown	FTN_1734	14 kDa	hypothetical protein	0.016023	Unknown
pal	FTN_0357	23 kDa	OmpA family peptidoglycan-associated lipoprotein	0.015458	Outer Membrane
ugpQ	FTN_0637	39 kDa	glycerophosphoryl diester phosphodiesterase	0.010331	Unknown/Multiple Localizations
unknown	FTN_0120	16 kDa	rhodanese-related sulfurtransferase	0.009824	Unknown
atpF	FTN_1650	17 kDa	F0F1 ATP synthase subunit B	0.009561	Cytoplasmic Membrane
unknown	FTN_0643	18 kDa	hypothetical protein	0.009394	Unknown
unknown	FTN_0275	40 kDa	hypothetical protein	0.009121	Unknown
sdhA	FTN_1637	66 kDa	succinate dehydrogenase flavoprotein	0.008755	Cytoplasmic Membrane

unknown	FTN_0340	12 kDa	hypothetical protein	0.008287	Unknown
dacD	FTN_0907	48 kDa	D-alanyl-D-alanine carboxypeptidase	0.008248	Unknown/Multiple Localizations
unknown	FTN_0183	34 kDa	periplasmic solute binding family protein	0.007887	Cytoplasmic Membrane
rplL	FTN_1569	13 kDa	50S ribosomal protein L7/L12	0.007409	Unknown/Multiple Localizations
atpD	FTN_1646	50 kDa	F0F1 ATP synthase subunit beta	0.007187	Cytoplasmic
metN	FTN_1106	40 kDa	methionine uptake transporter (MUT) family protein	0.006424	Cytoplasmic Membrane
unknown	FTN_0157	21 kDa	hypothetical protein	0.00641	Cytoplasmic Membrane
rplK	FTN_1572	15 kDa	50S ribosomal protein L11	0.006359	Cytoplasmic
ftsZ	FTN_0164	40 kDa	cell division protein FtsZ	0.006269	Cytoplasmic
unknown	FTN_0921	31 kDa	FKBP-type peptidyl-prolyl cis-trans isomerase	0.006255	Outer Membrane
rplI	FTN_0949	16 kDa	50S ribosomal protein L9	0.006167	Cytoplasmic
acnA	FTN_1623	103 kDa	aconitate hydratase	0.006078	Cytoplasmic
unknown	FTN_1433	33 kDa	hypothetical protein	0.005911	Cytoplasmic Membrane
fsp53	FTN_1261	55 kDa	hypothetical protein	0.005881	Unknown
pcp	FTN_0211	24 kDa	pyrrolidone carboxylylate peptidase	0.005836	Unknown/Multiple Localizations
unknown	FTN_0782	21 kDa	hypothetical protein	0.005472	Extracellular
unknown	FTN_0565	25 kDa	hypothetical protein	0.005456	Unknown
ilvC	FTN_1040	38 kDa	ketol-acid reductoisomerase	0.005246	Cytoplasmic

rpsC	FTN_0245	25 kDa	30S ribosomal protein S3	0.005213	Cytoplasmic
unknown	FTN_0855	26 kDa	hypothetical protein	0.004949	Unknown
rpsA	FTN_0159	62 kDa	30S ribosomal protein S1	0.004807	Cytoplasmic
msbA	FTN_1606	67 kDa	lipid exporter (LipidE) family protein	0.004794	Cytoplasmic Membrane
katG	FTN_0633	82 kDa	peroxidase/catalase	0.004653	Unknown/Multiple Localizations
unknown	FTN_0391	22 kDa	LemA-like protein	0.0045	Cytoplasmic
iglC	FTN_1322	22 kDa	intracellular growth locus protein C	0.004495	Unknown
fopA	FTN_0756	41 kDa	OmpA family protein	0.004396	Outer Membrane
unknown	FTN_0065	12 kDa	hypothetical protein	0.004394	Unknown
atpH	FTN_1649	19 kDa	F0F1 ATP synthase subunit delta	0.004378	Cytoplasmic
unknown	FTN_0022	39 kDa	histidine acid phosphatase	0.004327	Extracellular
unknown	FTN_0828	16 kDa	hypothetical protein	0.004309	Unknown
unknown	FTN_1101	30 kDa	hypothetical protein	0.004294	Unknown
rplQ	FTN_0265	17 kDa	50S ribosomal protein L17	0.004184	Cytoplasmic
unknown	FTN_0109	37 kDa	hypothetical protein	0.004085	Cytoplasmic
unknown	FTN_1476	22 kDa	hypothetical protein	0.003976	Unknown
unknown	FTN_1627	16 kDa	hypothetical protein	0.003897	Unknown
rplA	FTN_1571	24 kDa	50S ribosomal protein L1	0.003878	Cytoplasmic
unknown	FTN_0428	18 kDa	hypothetical protein	0.003805	Unknown
unknown	FTN_0033	21 kDa	chorismate mutase	0.003733	Cytoplasmic
atpA	FTN_1648	55 kDa	F0F1 ATP synthase subunit alpha	0.003632	Cytoplasmic

pepO	FTN_1186	79 kDa	M13 family metallopeptidase	0.003575	Cytoplasmic
unknown	FTN_0325	25 kDa	membrane protein of unknown function	0.003392	Unknown
unknown	FTN_1382	15 kDa	hypothetical protein	0.003344	Unknown
accA	FTN_1508	35 kDa	acetyl-CoA carboxylase	0.003324	Cytoplasmic
unknown	FTN_0346	47 kDa	OmpA family protein	0.003285	Unknown
unknown	FTN_1367	60 kDa	hypothetical protein	0.003263	Unknown
rplC	FTN_0239	22 kDa	50S ribosomal protein L3	0.003257	Cytoplasmic
unknown	FTN_1072	32 kDa	beta-lactamase class A	0.003255	Periplasmic
unknown	FTN_0429	19 kDa	hypothetical protein	0.003186	Unknown
rpsG	FTN_0236	18 kDa	30S ribosomal protein S7	0.003166	Cytoplasmic
rplJ	FTN_1570	19 kDa	50S ribosomal protein L10	0.003143	Cytoplasmic
unknown	FTN_1093	19 kDa	hypothetical protein	0.003113	Unknown
rne	FTN_1246	101 kDa	ribonuclease E	0.003084	Cytoplasmic
uspA	FTN_0085	30 kDa	universal stress protein	0.003081	Cytoplasmic
rpsB	FTN_0227	26 kDa	30S ribosomal protein S2	0.003031	Cytoplasmic
iglB	FTN_1323	58 kDa	intracellular growth locus protein B	0.002989	Cytoplasmic
unknown	FTN_1609	50 kDa	membrane fusion protein	0.002957	Cytoplasmic Membrane
sdhB	FTN_1636	27 kDa	succinate dehydrogenase iron-sulfur subunit	0.002954	Cytoplasmic Membrane
unknown	FTN_0077	17 kDa	hypothetical protein	0.002932	Unknown
unknown	FTN_0714	197 kDa	hypothetical protein	0.002884	Outer Membrane
rpsM	FTN_0261	13 kDa	30S ribosomal protein S13	0.002864	Cytoplasmic

hflB	FTN_0668	71 kDa	ATP-dependent metalloprotease	0.002828	Cytoplasmic Membrane
minD	FTN_0330	30 kDa	septum site-determining protein MinD	0.002826	Cytoplasmic
unknown	FTN_0869	73 kDa	hypothetical protein	0.002762	Cytoplasmic
sucB	FTN_1634	53 kDa	2-oxoglutarate dehydrogenase complex	0.002761	Cytoplasmic
unknown	FTN_0545	36 kDa	glycosyl transferase	0.002757	Cytoplasmic Membrane
unknown	FTN_0595	70 kDa	hypothetical protein	0.002742	Cytoplasmic Membrane
rplF	FTN_0254	19 kDa	50S ribosomal protein L6	0.002715	Cytoplasmic
unknown	FTN_0575	26 kDa	hypothetical protein	0.002698	Unknown
unknown	FTN_0191	28 kDa	polar amino acid uptake transporter	0.002694	Periplasmic
unknown	FTN_0715	135 kDa	hypothetical protein	0.002676	Outer Membrane
unknown	FTN_1447	36 kDa	hypothetical protein	0.002628	Unknown
nusG	FTN_1573	20 kDa	transcription antitermination protein nusG	0.002615	Cytoplasmic
fimV	FTN_1596	49 kDa	Type IV pili	0.002471	Unknown
unknown	FTN_0282	34 kDa	hypothetical protein	0.002465	Cytoplasmic Membrane
tig	FTN_1058	50 kDa	trigger factor	0.002458	Cytoplasmic
rplE	FTN_0251	20 kDa	50S ribosomal protein L5	0.002403	Cytoplasmic
sucC	FTN_0594	42 kDa	succinyl-CoA synthetase subunit beta	0.002403	Cytoplasmic
unknown	FTN_0765	37 kDa	choloylglycine hydrolase family protein	0.002376	Cytoplasmic Membrane

unknown	FTN_1449	22 kDa	hypothetical protein	0.002348	Unknown
fusA	FTN_0237	78 kDa	elongation factor G	0.00233	Cytoplasmic
htpG	FTN_0266	72 kDa	heat shock protein 90	0.002298	Cytoplasmic
lpxA	FTN_1478	28 kDa	UDP-N-acetylglucosamine acyltransferase	0.00228	Cytoplasmic
rpsI	FTN_1289	15 kDa	30S ribosomal protein S9	0.002271	Cytoplasmic
rpsD	FTN_0263	23 kDa	30S ribosomal protein S4	0.002255	Cytoplasmic
unknown	FTN_1750	28 kDa	acyltransferase	0.002252	Cytoplasmic Membrane
unknown	FTN_1517	23 kDa	hypothetical protein	0.00223	Unknown
unknown	FTN_1372	40 kDa	hypothetical protein	0.002229	Unknown
sohB	FTN_0550	38 kDa	putative periplasmic protease	0.002112	Cytoplasmic Membrane
tolQ	FTN_0352	26 kDa	TolQ protein	0.002072	Cytoplasmic Membrane
nuoD	FTN_1677	48 kDa	NADH dehydrogenase subunit D	0.002023	Cytoplasmic
kdpB	FTN_1717	73 kDa	potassium-transporting ATPase B chain	0.001955	Cytoplasmic Membrane
cyoB	FTN_0196	76 kDa	cytochrome bo terminal oxidase subunit I	0.001907	Cytoplasmic Membrane
cyoA	FTN_0195	35 kDa	cytochrome bo terminal oxidase subunit II	0.001845	Cytoplasmic Membrane
tolB	FTN_0355	48 kDa	group A colicin translocation; tolB protein	0.001839	Periplasmic
fopC	FTN_0444	59 kDa	membrane protein of unknown function	0.001828	Unknown/Multipl e Localizations
unknown	FTN_1749	29 kDa	acyltransferase	0.001821	Cytoplasmic Membrane

metIQ	FTN_1107	53 kDa	methionine uptake transporter (MUT) family protein	0.001807	Cytoplasmic Membrane
ggt	FTN_1159	65 kDa	gamma-glutamyltranspeptidase	0.001803	Periplasmic
yhbG	FTN_0902	27 kDa	ABC transporter	0.001789	Cytoplasmic
potG	FTN_0739	42 kDa	ATP-binding cassette putrescine uptake system	0.00172	Cytoplasmic Membrane
unknown	FTN_1109	28 kDa	rhodanese-like family protein	0.00172	Unknown
fadE	FTN_1437	83 kDa	acyl-CoA dehydrogenase	0.001705	Unknown
tsf	FTN_0228	31 kDa	elongation factor Ts	0.001692	Cytoplasmic
atpG	FTN_1647	33 kDa	F0F1 ATP synthase subunit gamma	0.001669	Cytoplasmic
aceF	FTN_1493	67 kDa	dihydrolipoamide acetyltransferase	0.001667	Cytoplasmic
fabI	FTN_1228	28 kDa	enoyl-ACP reductase I	0.001652	Cytoplasmic Membrane
unknown	FTN_0032	23 kDa	hypothetical protein	0.001648	Unknown
unknown	FTN_0141	49 kDa	ABC transporter	0.001639	Cytoplasmic Membrane
nuoI	FTN_1672	19 kDa	NADH dehydrogenase subunit I	0.00162	Cytoplasmic
unknown	FTN_0449	33 kDa	hypothetical protein	0.001617	Unknown
oppA	FTN_1593	63 kDa	ABC-type oligopeptide transport system	0.001606	Periplasmic
ilvE	FTN_0063	33 kDa	branched-chain amino acid aminotransferase protein (class IV)	0.001593	Cytoplasmic
capB	FTN_1201	45 kDa	capsule biosynthesis protein CapB	0.001593	Cytoplasmic Membrane

leuA	FTN_0062	58 kDa	2-isopropylmalate synthase	0.00157	Cytoplasmic
lolA	FTN_0293	23 kDa	lipoprotein releasing system	0.001569	Periplasmic
rpoB	FTN_1568	151 kDa	DNA-directed RNA polymerase subunit beta	0.001569	Cytoplasmic
recA	FTN_0122	39 kDa	recombinase A protein	0.001568	Cytoplasmic
nuoG	FTN_1674	87 kDa	NADH dehydrogenase subunit G	0.001559	Cytoplasmic
aroG	FTN_0842	41 kDa	phospho-2-dehydro-3-deoxyheptonate aldolase	0.001556	Cytoplasmic
rplB	FTN_0242	30 kDa	50S ribosomal protein L2	0.001514	Cytoplasmic
nuoF	FTN_1675	46 kDa	NADH dehydrogenase I	0.001513	Cytoplasmic
rpoC	FTN_1567	157 kDa	DNA-directed RNA polymerase	0.001507	Cytoplasmic
clpB	FTN_1743	96 kDa	chaperone clpB	0.0015	Cytoplasmic
glnA	FTN_0172	38 kDa	glutamine synthetase	0.001498	Cytoplasmic
unknown	FTN_0381	38 kDa	hypothetical protein	0.001481	Unknown
unknown	FTN_0471	20 kDa	NADPH-dependent FMN reductase	0.001459	Unknown
unknown	FTN_0871	21 kDa	rare lipoprotein B family protein	0.001455	Unknown
glpD	FTN_1584	58 kDa	glycerol-3-phosphate dehydrogenase	0.001428	Unknown/Multiple Localizations
pyk	FTN_1330	52 kDa	pyruvate kinase	0.001401	Cytoplasmic
unknown	FTN_0917	51 kDa	serine-type D-Ala-D-Ala carboxypeptidase	0.001384	Periplasmic
rpsN	FTN_0252	12 kDa	30S ribosomal protein S14	0.00138	Cytoplasmic
accD	FTN_0272	33 kDa	acetyl-CoA carboxylase	0.001378	Cytoplasmic
unknown	FTN_0523	29 kDa	hypothetical protein	0.001377	Periplasmic

grxB	FTN_1033	25 kDa	glutaredoxin 2	0.001365	Cytoplasmic
unknown	FTN_0131	51 kDa	hypothetical protein	0.001365	Cytoplasmic
unknown	FTN_1535	31 kDa	short chain dehydrogenase	0.001363	Cytoplasmic
unknown	FTN_0439	18 kDa	hypothetical protein	0.001326	Unknown
unknown	FTN_1412	35 kDa	DNA-directed RNA polymerase subunit alpha	0.001311	Cytoplasmic
unknown	FTN_1001	32 kDa	hypothetical protein	0.001281	Cytoplasmic Membrane
unknown	FTN_0802	15 kDa	hypothetical protein	0.001274	Unknown
atpC	FTN_1645	16 kDa	F0F1 ATP synthase subunit epsilon	0.001274	Unknown/Multiple Localizations
aceE	FTN_1494	100 kDa	pyruvate dehydrogenase subunit E1	0.001254	Cytoplasmic
secD	FTN_1095	70 kDa	preprotein translocase subunit SecD	0.001243	Cytoplasmic Membrane
wbtP	FTN_1429	24 kDa	galactosyl transferase	0.001242	Cytoplasmic Membrane
pdpB	FTN_1310	127 kDa	hypothetical protein	0.001176	Unknown/Multiple Localizations
unknown	FTN_0893	22 kDa	hypothetical protein	0.001154	Cytoplasmic
unknown	FTN_1547	24 kDa	hypothetical protein	0.00115	Unknown
unknown	FTN_0436	33 kDa	hypothetical protein	0.001147	Extracellular
unknown	FTN_1692	40 kDa	membrane fusion protein	0.001142	Unknown
apt	FTN_1633	19 kDa	adenine phosphoribosyltransferase	0.001141	Cytoplasmic
sucA	FTN_1635	106 kDa	alpha-ketoglutarate decarboxylase	0.001125	Cytoplasmic
unknown	FTN_1199	44 kDa	hypothetical protein	0.001123	Unknown

lpdA	FTN_1492	50 kDa	dihydrolipoamide dehydrogenase	0.001111	Cytoplasmic
rho	FTN_1416	47 kDa	transcription termination factor Rho	0.001106	Cytoplasmic Membrane
unknown	FTN_1369	27 kDa	hypothetical protein	0.001104	Unknown
aroK	FTN_1136	20 kDa	shikimate kinase I	0.001097	Cytoplasmic
lysU	FTN_0168	66 kDa	lysyl-tRNA synthetase	0.00109	Cytoplasmic
unknown	FTN_1610	113 kDa	RND efflux transporter	0.001088	Cytoplasmic Membrane
pnp	FTN_0609	75 kDa	polynucleotide phosphorylase/polyadenylase	0.001084	Cytoplasmic
unknown	FTN_0890	29 kDa	hypothetical protein	0.00108	Cytoplasmic
lepA	FTN_0107	66 kDa	GTP-binding protein LepA	0.001078	Cytoplasmic Membrane
secA	FTN_0672	104 kDa	preprotein translocase subunit SecA	0.001059	Cytoplasmic
bcp	FTN_1756	18 kDa	bacterioferritin comigratory protein	0.001057	Cytoplasmic
cphB	FTN_1209	29 kDa	cyanophycinase	0.001055	Unknown
bglX	FTN_1474	43 kDa	glycosyl 4hydrolase family protein	0.00103	Cytoplasmic
nusA	FTN_1661	55 kDa	transcription elongation factor NusA	0.001027	Cytoplasmic
unknown	FTN_0410	44 kDa	aspartate aminotransferase	0.001023	Cytoplasmic
unknown	FTN_1053	55 kDa	hypothetical protein	0.000982	Cytoplasmic Membrane
leuC	FTN_0061	52 kDa	isopropylmalate isomerase large subunit	0.000973	Cytoplasmic

oppF	FTN_1589	37 kDa	peptide/opine/nickel uptake transporter (PepT) family protein	0.000962	Cytoplasmic Membrane
unknown	FTN_1122	14 kDa	hypothetical protein	0.000961	Unknown
ffh	FTN_0843	50 kDa	signal recognition particle GTPase	0.000961	Cytoplasmic Membrane
wbtA	FTN_1431	66 kDa	dTDP-glucose 4	0.00095	Cytoplasmic Membrane
unknown	FTN_0103	88 kDa	hypothetical protein	0.000945	Unknown
unknown	FTN_0222	36 kDa	hypothetical protein	0.000925	Unknown
mdh	FTN_0980	34 kDa	malate dehydrogenase	0.000922	Cytoplasmic
unknown	FTN_1049	44 kDa	hypothetical protein	0.000895	Unknown
galP2	FTN_0688	51 kDa	major facilitator superfamily galactose-proton symporter	0.000891	Cytoplasmic Membrane
rpoA	FTN_0264	35 kDa	DNA-directed RNA polymerase subunit alpha	0.000877	Cytoplasmic
unknown	FTN_0620	45 kDa	major facilitator transporter	0.00087	Cytoplasmic Membrane
ftsA	FTN_0163	45 kDa	cell division protein FtsA	0.000859	Cytoplasmic
slt	FTN_0496	77 kDa	soluble lytic murein transglycosylase	0.000855	Periplasmic
tolC	FTN_1703	57 kDa	outer membrane protein tolC precursor	0.000855	Outer Membrane
unknown	FTN_1617	55 kDa	two-component regulator	0.000847	Cytoplasmic Membrane
unknown	FTN_1644	105 kDa	hypothetical protein	0.000841	Unknown
glpK	FTN_1585	55 kDa	glycerol kinase	0.000833	Cytoplasmic
ilvB	FTN_1042	62 kDa	acetolactate synthase large	0.000831	Cytoplasmic

			subunit		
cphA	FTN_1112	104 kDa	cyanophycin synthetase	0.000822	Cytoplasmic
unknown	FTN_0482	36 kDa	hypothetical protein	0.000817	Unknown
gltB	FTN_1360	58 kDa	glutamate synthase domain-containing 2	0.000804	Cytoplasmic
unknown	FTN_0073	62 kDa	membrane protein of unknown function	0.000786	Cytoplasmic Membrane
accC	FTN_0564	50 kDa	acetyl-CoA carboxylase	0.000785	Cytoplasmic
leuB	FTN_0059	40 kDa	3-isopropylmalate dehydrogenase	0.000781	Cytoplasmic
kdpD	FTN_1715	101 kDa	two component regulator	0.000773	Cytoplasmic Membrane
unknown	FTN_0903	32 kDa	hypothetical protein	0.000772	Unknown
unknown	FTN_0559	53 kDa	peptidyl-prolyl cis-trans isomerase (PPIase)	0.000761	Periplasmic
unknown	FTN_0604	79 kDa	AMP-binding protein	0.000747	Cytoplasmic Membrane
wbtG	FTN_1423	42 kDa	glycosyl transferase	0.000745	Cytoplasmic
lpcC	FTN_1253	41 kDa	glycosyl transferase	0.000733	Cytoplasmic
unknown	FTN_1693	59 kDa	ATP-binding cassette (ABC) superfamily protein	0.000725	Cytoplasmic Membrane
mltA	FTN_1286	45 kDa	membrane-bound lytic murein transglycosylase	0.00072	Unknown/Multiple Localizations
aspS	FTN_0129	67 kDa	aspartyl-tRNA synthetase	0.000711	Cytoplasmic
infB	FTN_1660	92 kDa	translation initiation factor IF-2	0.000708	Cytoplasmic
putA	FTN_1131	150 kDa	bifunctional proline dehydrogenase/pyrroline-5-	0.000698	Cytoplasmic

			carboxylate dehydrogenase		
unknown	FTN_1695	24 kDa	hypothetical protein	0.000695	Unknown
serA	FTN_1249	45 kDa	D-3-phosphoglycerate dehydrogenase	0.000691	Cytoplasmic
unknown	FTN_1276	38 kDa	membrane fusion protein	0.000677	Cytoplasmic Membrane
groEL	FTN_1538	57 kDa	chaperonin GroEL	0.000676	Cytoplasmic
rpoD	FTN_0913	68 kDa	RNA polymerase sigma-70 factor	0.000669	Cytoplasmic
feoB	FTN_0066	82 kDa	ferrous iron transport protein B	0.000666	Cytoplasmic Membrane
cydA	FTN_0193	64 kDa	cytochrome bd-I terminal oxidase subunit I	0.000656	Cytoplasmic Membrane
gyrB	FTN_0600	90 kDa	DNA gyrase subunit B	0.000647	Cytoplasmic
pheT	FTN_0882	88 kDa	phenylalanine tRNA synthetase	0.000641	Cytoplasmic
kdtA	FTN_1469	50 kDa	3-deoxy-D-manno-octulosonic-acid transferase	0.000637	Cytoplasmic
unknown	FTN_1268	27 kDa	hypothetical protein	0.000625	Cytoplasmic Membrane
unknown	FTN_1762	63 kDa	putative ABC transporter ATP-binding protein	0.000624	Cytoplasmic
hflK	FTN_1048	40 kDa	HflK-HflC membrane protein complex	0.000614	Cytoplasmic
sucD	FTN_0593	30 kDa	succinyl-CoA synthetase	0.000613	Cytoplasmic
unknown	FTN_0394	78 kDa	heavy metal cation transport ATPase	0.000587	Cytoplasmic Membrane
unknown	FTN_1772	27 kDa	peptide methionine sulfoxide reductase	0.000585	Unknown

unknown	FTN_1172	54 kDa	hypothetical protein	0.000572	Cytoplasmic
unknown	FTN_0322	40 kDa	VacJ like lipoprotein	0.000564	Outer Membrane
fumA	FTN_0337	55 kDa	fumerate hydratase	0.00056	Cytoplasmic
tyrS	FTN_0992	45 kDa	tyrosyl-tRNA synthetase	0.000558	Cytoplasmic
gshB	FTN_0804	37 kDa	glutathione synthetase	0.000553	Cytoplasmic
maeA	FTN_0443	67 kDa	malate dehydrogenase	0.000549	Cytoplasmic
pheS	FTN_0883	38 kDa	phenylalanyl-tRNA synthetase subunit alpha	0.000549	Cytoplasmic
eno	FTN_0621	50 kDa	enolase (2-phosphoglycerate dehydratase)	0.000549	Cytoplasmic
ftsK	FTN_0294	92 kDa	cell division protein	0.000546	Cytoplasmic Membrane
unknown	FTN_0597	28 kDa	protein-disulfide isomerase	0.000537	Unknown
trpS	FTN_1499	38 kDa	tryptophanyl-tRNA synthetase	0.000536	Cytoplasmic
unknown	FTN_0827	34 kDa	carbon-nitrogen hydrolase family protein	0.000536	Cytoplasmic
dnaA	FTN_0001	56 kDa	chromosomal replication initiator protein	0.000526	Cytoplasmic
nuoC	FTN_1678	25 kDa	NADH dehydrogenase I	0.000522	Cytoplasmic
unknown	FTN_0502	46 kDa	ABC transporter	0.000482	Cytoplasmic Membrane
ispG	FTN_1076	44 kDa	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	0.000478	Cytoplasmic
pilQ	FTN_1137	65 kDa	Type IV pili secretin component	0.000473	Outer Membrane
unknown	FTN_0835	28 kDa	hypothetical protein	0.000467	Unknown
purT	FTN_1745	42 kDa	phosphoribosylglycinamide formyltransferase 2	0.000453	Cytoplasmic Membrane

iglI	FTN_1317	45 kDa	hypothetical protein	0.00045	Cytoplasmic
unknown	FTN_0975	56 kDa	hypothetical protein	0.000444	Extracellular
wbtH	FTN_1421	72 kDa	glutamine amidotransferase/asparagine synthase	0.000437	Cytoplasmic
pilF	FTN_0946	35 kDa	Type IV pili	0.000436	Unknown
sufB	FTN_0851	53 kDa	cysteine desulfurase activator complex subunit SufB	0.000426	Cytoplasmic
ubiB	FTN_0459	64 kDa	2-octaprenylphenol hydroxylase	0.000408	Cytoplasmic Membrane
guaB	FTN_0661	52 kDa	IMP dehydrogenase/GMP reductase	0.000403	Cytoplasmic
ndh	FTN_0912	48 kDa	NADH dehydrogenase	0.000371	Cytoplasmic Membrane
lldD	FTN_0991	42 kDa	L-lactate dehydrogenase	0.000368	Cytoplasmic
nrdA	FTN_0981	67 kDa	ribonucleotide-diphosphate reductase subunit alpha	0.000362	Cytoplasmic
pilC	FTN_1116	45 kDa	Type IV pili polytopic inner membrane protein	0.000358	Cytoplasmic Membrane
unknown	FTN_1277	54 kDa	outer membrane efflux protein	0.000358	Outer Membrane
lon	FTN_1055	86 kDa	DNA-binding	0.000354	Cytoplasmic
unknown	FTN_0040	125 kDa	hypothetical protein	0.000332	Unknown
ostA1	FTN_0558	98 kDa	organic solvent tolerance protein	0.000306	Outer Membrane
unknown	FTN_0861	47 kDa	hypothetical protein	0.000306	Cytoplasmic
unknown	FTN_0200	37 kDa	UDP-3-O-[3-fatty acid] glucosamine N-	0.000298	Cytoplasmic

			acyltransferase		
unknown	FTN_1192	66 kDa	chitin-binding protein	0.000296	Extracellular
valS	FTN_0214	105 kDa	valyl-tRNA synthetase	0.00029	Cytoplasmic
unknown	FTN_0649	114 kDa	4Fe-4S ferredoxin	0.000279	Cytoplasmic
alaS	FTN_0778	96 kDa	alanyl-tRNA synthetase	0.000251	Cytoplasmic
unknown	FTN_0983	47 kDa	bifunctional gluaredoxin/ribonucleoside-diphosphate reductase subunit beta	0.00025	Cytoplasmic
unknown	FTN_1024	55 kDa	RmuC family protein	0.000215	Cytoplasmic
unknown	FTN_0140	67 kDa	ABC-type anion transport system	0.000215	Cytoplasmic Membrane
chiA	FTN_0627	96 kDa	glycosyl hydrolase family chitinase	0.000195	Periplasmic
gyrA	FTN_1484	97 kDa	DNA gyrase	0.000174	Cytoplasmic
unknown	FTN_0043	58 kDa	hypothetical protein	0.000164	Cytoplasmic
spoT	FTN_1198	81 kDa	GDP diphosphokinase/guanosine-3'	0.000156	Cytoplasmic
pdpD	FTN_1325	141 kDa	hypothetical protein	0.000148	Outer Membrane
leuS	FTN_0870	93 kDa	leucyl-tRNA synthetase	0.00014	Cytoplasmic

Table 3-3. Differential protein content between exponential and stationary phase OMV/NT.

Gene	Locus	Description	Exponential nSAF	Stationary nSAF	Fold Change
fsp53	FTN_1261	hypothetical protein	0.097478	0.005881	-16.5744
unknown	FTN_0714	hypothetical protein	0.02779	0.002884	-9.63711
unknown	FTN_0597	protein-disulfide isomerase	0.003585	0.000537	-6.68201
pilF	FTN_0946	Type IV pili	0.002703	0.000436	-6.19499
bglX	FTN_1474	glycosyl 4hydrolase family protein	0.006096	0.00103	-5.91782
unknown	FTN_0429	hypothetical protein	0.01771	0.003186	-5.55805
unknown	FTN_0322	VacJ like lipoprotein	0.00296	0.000564	-5.24363
fopA	FTN_0756	OmpA family protein	0.022112	0.004396	-5.03023
unknown	FTN_0428	hypothetical protein	0.017169	0.003805	-4.51247
unknown	FTN_1276	membrane fusion protein	0.002521	0.000677	-3.7253
unknown	FTN_1449	hypothetical protein	0.008431	0.002348	-3.5912
ostA1	FTN_0558	organic solvent tolerance protein	0.001051	0.000306	-3.42773
unknown	FTN_0381	hypothetical protein	0.005047	0.001481	-3.40866
unknown	FTN_1268	hypothetical protein	0.002057	0.000625	-3.29041
tolB	FTN_0355	group A colicin translocation; tolB protein	0.005385	0.001839	-2.92806
ftsK	FTN_0294	cell division protein	0.001571	0.000546	-2.87727
unknown	FTN_0340	hypothetical protein	0.023689	0.008287	-2.85844
unknown	FTN_0191	polar amino acid uptake transporter	0.00718	0.002694	-2.66526
unknown	FTN_0033	chorismate mutase	0.009763	0.003733	-2.61527
unknown	FTN_0073	membrane protein of unknown	0.00192	0.000786	-2.4448

		function			
putA	FTN_1131	bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	0.001618	0.000698	-2.31948
unknown	FTN_0482	hypothetical protein	0.001887	0.000817	-2.31099
mltA	FTN_1286	membrane-bound lytic murein transglycosylase	0.001585	0.00072	-2.20201
unknown	FTN_0346	OmpA family protein	0.007206	0.003285	-2.19386
pyk	FTN_1330	pyruvate kinase	0.003042	0.001401	-2.17109
atpF	FTN_1650	F0F1 ATP synthase subunit B	0.019981	0.009561	-2.08972
unknown	FTN_1692	membrane fusion protein	0.002326	0.001142	-2.03786
aceF	FTN_1493	dihydrolipoamide acetyltransferase	0.003347	0.001667	-2.0073
rne	FTN_1246	ribonuclease E	0.001499	0.003084	2.057486
tufA	FTN_1576	elongation factor Tu	0.008638	0.020572	2.381474
ilvC	FTN_1040	ketol-acid reductoisomerase	0.001444	0.005246	3.632161

Table 3-4. *Francisella* Outer Membrane-Associated proteins.

Gene	Locus	Description	References
unknown	FTN_0109	hypothetical protein	(Janovska, Pavkova et al. 2007)
fopB	FTN_0119	outer membrane protein of unknown function	(Pavkova, Hubalek et al. 2005)
ftsZ	FTN_0164	cell division protein FtsZ	(Janovska, Pavkova et al. 2007)
pcp	FTN_0211	pyrrolidone carboxylylate peptidase	(Pavkova, Hubalek et al. 2005)
htpG	FTN_0266	heat shock protein 90	(Janovska, Pavkova et al. 2007)
unknown	FTN_0275	hypothetical protein	(Pavkova, Hubalek et al. 2005)
unknown	FTN_0322	VacJ like lipoprotein	(Janovska, Pavkova et al. 2007)
unknown	FTN_0346	OmpA family protein	(Pavkova, Hubalek et al. 2005; Huntley, Conley et al. 2007; Janovska, Pavkova et al. 2007)
pal	FTN_0357	OmpA family peptidoglycan-associated lipoprotein	(Pavkova, Hubalek et al. 2005; Huntley, Conley et al. 2007)
unknown	FTN_0391	LemA-like protein	(Janovska, Pavkova et al. 2007)
lpnA	FTN_0427	lipoprotein of unknown function	(Pavkova, Hubalek et al. 2005; Huntley, Conley et al. 2007; Janovska, Pavkova et al. 2007)
fopC	FTN_0444	membrane protein of unknown function	(Huntley, Conley et al. 2007)
katG	FTN_0633	peroxidase/catalase	(Pavkova, Hubalek et al. 2005; Huntley,

			Conley et al. 2007; Janovska, Pavkova et al. 2007)
ugpQ	FTN_0637	glycerophosphoryl diester phosphodiesterase	(Pavkova, Hubalek et al. 2005; Janovska, Pavkova et al. 2007)
guaB	FTN_0661	IMP dehydrogenase/GMP reductase	(Pavkova, Hubalek et al. 2005; Janovska, Pavkova et al. 2007)
potG	FTN_0739	ATP-binding cassette putrescine uptake system	(Pavkova, Hubalek et al. 2005)
fopA	FTN_0756	OmpA family protein	(Pavkova, Hubalek et al. 2005; Huntley, Conley et al. 2007; Janovska, Pavkova et al. 2007)
fipB	FTN_0771	protein-disulfide isomerase	(Pavkova, Hubalek et al. 2005; Huntley, Conley et al. 2007; Janovska, Pavkova et al. 2007)
unknown	FTN_0828	hypothetical protein	(Pavkova, Hubalek et al. 2005)
unknown	FTN_0871	rare lipoprotein B family protein	(Pavkova, Hubalek et al. 2005)
dacD	FTN_0907	D-alanyl-D-alanine carboxypeptidase	(Pavkova, Hubalek et al. 2005)
unknown	FTN_0917	serine-type D-Ala-D-Ala carboxypeptidase	(Gilmore, Bacon et al. 2004; Pavkova, Hubalek et al. 2005)
unknown	FTN_0921	FKBP-type peptidyl-prolyl cis-trans isomerase	(Pavkova, Hubalek et al. 2005)
pilQ	FTN_1137	Type IV pili secretin component	(Huntley, Conley et al. 2007)
ggt	FTN_1159	gamma-glutamyltranspeptidase	(Gilmore, Bacon et

			al. 2004)
unknown	FTN_1268	hypothetical protein	(Pavkova, Hubalek et al. 2005)
unknown	FTN_1276	membrane fusion protein	(Pavkova, Hubalek et al. 2005)
unknown	FTN_1277	outer membrane efflux protein	(Pavkova, Hubalek et al. 2005)
iglC	FTN_1322	intracellular growth locus protein C	(Janovska, Pavkova et al. 2007)
iglA	FTN_1324	intracellular growth locus protein A	(Janovska, Pavkova et al. 2007)
unknown	FTN_1367	hypothetical protein	(Janovska, Pavkova et al. 2007)
unknown	FTN_1448	hypothetical protein	(Janovska, Pavkova et al. 2007)
unknown	FTN_1449	hypothetical protein	(Pavkova, Hubalek et al. 2005)
unknown	FTN_1451	hypothetical protein	(Pavkova, Hubalek et al. 2005)
ompH	FTN_1481	outer membrane protein OmpH	(Pavkova, Hubalek et al. 2005)
lpdA	FTN_1492	dihydrolipoamide dehydrogenase	(Janovska, Pavkova et al. 2007)
aceF	FTN_1493	dihydrolipoamide acetyltransferase	(Pavkova, Hubalek et al. 2005; Janovska, Pavkova et al. 2007)
aceE	FTN_1494	pyruvate dehydrogenase subunit E1	(Gilmore, Bacon et al. 2004; Huntley, Conley et al. 2007)
accA	FTN_1508	acetyl-CoA carboxylase	(Janovska, Pavkova et al. 2007)
groEL	FTN_1538	chaperonin GroEL	(Huntley, Conley et al. 2007; Janovska,

			Pavkova et al. 2007)
rplL	FTN_1569	50S ribosomal protein L7/L12	(Janovska, Pavkova et al. 2007)
tufA	FTN_1576	elongation factor Tu	(Janovska, Pavkova et al. 2007)
acnA	FTN_1623	aconitate hydratase	(Janovska, Pavkova et al. 2007)
sucB	FTN_1634	2-oxoglutarate dehydrogenase complex	(Janovska, Pavkova et al. 2007)
sdhA	FTN_1637	succinate dehydrogenase flavoprotein	(Janovska, Pavkova et al. 2007)
atpD	FTN_1646	F0F1 ATP synthase subunit beta	(Huntley, Conley et al. 2007)
clpB	FTN_1743	chaperone clpB	(Janovska, Pavkova et al. 2007)

Table 3-5. Gram-negative OMV-Associated proteins.

Gene	Locus	Description	References
unknown	FTN_0105	outer membrane lipoprotein	(Post, Zhang et al. 2005)
rpsA	FTN_0159	30S ribosomal protein S1	(Vipond, Suker et al. 2006; Lee, Bang et al. 2007)
glnA	FTN_0172	glutamine synthetase	(Lee, Bang et al. 2007)
unknown	FTN_0183	periplasmic solute binding family protein	(Vipond, Suker et al. 2006)
unknown	FTN_0191	polar amino acid uptake transporter	(Post, Zhang et al. 2005; Berlanda Scorza, Doro et al. 2008)
rpsB	FTN_0227	30S ribosomal protein S2	(Lee, Bang et al. 2007)
fusA	FTN_0237	elongation factor G	(Vipond, Suker et al. 2006; Lee, Bang et al. 2007)
rplB	FTN_0242	50S ribosomal protein L2	(Lee, Bang et al. 2007)
rpsC	FTN_0245	30S ribosomal protein S3	(Lee, Bang et al. 2007)
rplP	FTN_0246	50S ribosomal protein L16	(Lee, Bang et al. 2007)
rplE	FTN_0251	50S ribosomal protein L5	(Lee, Bang et al. 2007)
rplF	FTN_0254	50S ribosomal protein L6	(Lee, Bang et al. 2007)
rpsD	FTN_0263	30S ribosomal protein S4	(Lee, Bang et al. 2007)
rplQ	FTN_0265	50S ribosomal protein L17	(Lee, Bang et al.

			2007)
unknown	FTN_0282	hypothetical protein	(Vipond, Suker et al. 2006)
lolA	FTN_0293	lipoprotein releasing system	(Berlanda Scorza, Doro et al. 2008)
unknown	FTN_0325	membrane protein of unknown function	(Berlanda Scorza, Doro et al. 2008)
minD	FTN_0330	septum site-determining protein MinD	(Vipond, Suker et al. 2006)
unknown	FTN_0346	OmpA family protein	(Berlanda Scorza, Doro et al. 2008)
tolB	FTN_0355	group A colicin translocation; tolB protein	(Nevot, Deroncele et al. 2006; Lee, Bang et al. 2007; Berlanda Scorza, Doro et al. 2008)
pal	FTN_0357	OmpA family peptidoglycan-associated lipoprotein	(Post, Zhang et al. 2005; Lee, Bang et al. 2007; Berlanda Scorza, Doro et al. 2008)
slt	FTN_0496	soluble lytic murein transglycosylase	(Post, Zhang et al. 2005; Berlanda Scorza, Doro et al. 2008)
ostA1	FTN_0558	organic solvent tolerance protein	(Nevot, Deroncele et al. 2006; Lee, Bang et al. 2007; Berlanda Scorza, Doro et al. 2008)
unknown	FTN_0559	peptidyl-prolyl cis-trans isomerase (PPIase)	(Nevot, Deroncele et al. 2006; Berlanda Scorza, Doro et al. 2008)
pnp	FTN_0609	polynucleotide phosphorylase/polyadenylase	(Vipond, Suker et al.

			2006)
fopA	FTN_0756	OmpA family protein	(Lee, Bang et al. 2007)
alaS	FTN_0778	alanyl-tRNA synthetase	(Lee, Bang et al. 2007)
pheT	FTN_0882	phenylalanine tRNA synthetase	(Lee, Bang et al. 2007)
pheS	FTN_0883	phenylalanyl-tRNA synthetase subunit alpha	(Lee, Bang et al. 2007)
unknown	FTN_0921	FKBP-type peptidyl-prolyl cis-trans isomerase	(Berlanda Scorza, Doro et al. 2008)
rplI	FTN_0949	50S ribosomal protein L9	(Lee, Bang et al. 2007)
unknown (ahp1)	FTN_0973	AhpC/TSA family peroxiredoxin	(Berlanda Scorza, Doro et al. 2008)
tig	FTN_1058	trigger factor	(Vipond, Suker et al. 2006)
pilQ	FTN_1137	Type IV pili secretin component	(Post, Zhang et al. 2005; Berlanda Scorza, Doro et al. 2008)
ggt	FTN_1159	gamma-glutamyltranspeptidase	(Post, Zhang et al. 2005)
fabI	FTN_1228	enoyl-ACP reductase I	(Vipond, Suker et al. 2006)
comL	FTN_1263	competence lipoprotein ComL	(Vipond, Suker et al. 2006; Berlanda Scorza, Doro et al. 2008)
unknown	FTN_1277	outer membrane efflux protein	(Post, Zhang et al. 2005)
mltA	FTN_1286	membrane-bound lytic murein transglycosylase	(Lee, Bang et al. 2007)

rpsI	FTN_1289	30S ribosomal protein S9	(Lee, Bang et al. 2007)
fabF	FTN_1341	beta-ketoacyl-ACP synthase II	(Vipond, Suker et al. 2006)
lpdA	FTN_1492	dihydrolipoamide dehydrogenase	(Lee, Bang et al. 2007; Berlanda Scorza, Doro et al. 2008)
aceF	FTN_1493	dihydrolipoamide acetyltransferase	(Lee, Bang et al. 2007)
aceE	FTN_1494	pyruvate dehydrogenase subunit E1	(Lee, Bang et al. 2007)
groEL	FTN_1538	chaperonin GroEL	(Vipond, Suker et al. 2006; Lee, Bang et al. 2007; Berlanda Scorza, Doro et al. 2008)
rpoC	FTN_1567	DNA-directed RNA polymerase	(Lee, Bang et al. 2007)
rpoB	FTN_1568	DNA-directed RNA polymerase subunit beta	(Lee, Bang et al. 2007)
rplL	FTN_1569	50S ribosomal protein L7/L12	(Lee, Bang et al. 2007)
rplJ	FTN_1570	50S ribosomal protein L10	(Lee, Bang et al. 2007)
rplA	FTN_1571	50S ribosomal protein L1	(Lee, Bang et al. 2007)
tufA	FTN_1576	elongation factor Tu	(Vipond, Suker et al. 2006; Berlanda Scorza, Doro et al. 2008)
glpD	FTN_1584	glycerol-3-phosphate dehydrogenase	(Lee, Bang et al. 2007)
glpK	FTN_1585	glycerol kinase	(Lee, Bang et al.

			2007)
oppA	FTN_1593	ABC-type oligopeptide transport system	(Berlanda Scorza, Doro et al. 2008)
unknown	FTN_1609	membrane fusion protein	(Post, Zhang et al. 2005)
unknown	FTN_1610	RND efflux transporter	(Nevot, Deroncele et al. 2006)
sucB	FTN_1634	2-oxoglutarate dehydrogenase complex	(Lee, Bang et al. 2007; Berlanda Scorza, Doro et al. 2008)
sucA	FTN_1635	alpha-ketoglutarate decarboxylase	(Lee, Bang et al. 2007)
sdhB	FTN_1636	succinate dehydrogenase iron-sulfur subunit	(Berlanda Scorza, Doro et al. 2008)
sdhA	FTN_1637	succinate dehydrogenase flavoprotein	(Berlanda Scorza, Doro et al. 2008)
atpA	FTN_1648	F0F1 ATP synthase subunit alpha	(Berlanda Scorza, Doro et al. 2008)
tolC	FTN_1703	outer membrane protein tolC precursor	(Vipond, Suker et al. 2006; Lee, Bang et al. 2007; Berlanda Scorza, Doro et al. 2008)

Table 3-6. OMV/NT-associated Proteins Identified Previously as Secreted or Virulence Factors.

Gene	Locus	Description	Secreted	Virulence	References
unknown	FTN_0109	hypothetical protein		X	(Su, Yang et al. 2007)
fopB	FTN_0119	outer membrane protein of unknown function		X	(Su, Yang et al. 2007; Yu, Goluguri et al. 2010)
cyoB	FTN_0196	cytochrome bo terminal oxidase subunit I		X	(Weiss, Brotcke et al. 2007)
Pcp	FTN_0211	pyrrolidone carboxylylate peptidase		X	(Weiss, Brotcke et al. 2007)
rplQ	FTN_0265	50S ribosomal protein L17		X	(Weiss, Brotcke et al. 2007)
htpG	FTN_0266	heat shock protein 90		X	(Tempel, Lai et al. 2006; Weiss, Brotcke et al. 2007)
unknown	FTN_0325	membrane protein of unknown function		X	(Su, Yang et al. 2007)
mind	FTN_0330	septum site-determining protein MinD		X	(Anthony, Cowley et al. 1994)
fumA	FTN_0337	fumerate hydratase		X	(Tempel, Lai et al. 2006)
unknown	FTN_0340	hypothetical protein		X	(Kraemer, Mitchell et al. 2009)
lpnA	FTN_0427	lipoprotein of unknown function		X	(Su, Yang et al. 2007)
unknown	FTN_0429	hypothetical protein		X	(Weiss, Brotcke et al. 2007)
unknown	FTN_0436	hypothetical protein		X	(Weiss, Brotcke et al. 2007)
maeA	FTN_0443	malate dehydrogenase		X	(Tempel, Lai et al. 2006)
fopC	FTN_0444	membrane protein of unknown		X	(Su, Yang et al. 2007)

		function			
unknown	FTN_0545	glycosyl transferase		X	(Weiss, Brotcke et al. 2007; Yu, Goluguri et al. 2010)
unknown	FTN_0559	peptidyl-prolyl cis-trans isomerase (PPIase)		X	(Weiss, Brotcke et al. 2007)
sucD	FTN_0593	succinyl-CoA synthetase	X		(Lee, Horwitz et al. 2006)
sucC	FTN_0594	succinyl-CoA synthetase subunit beta	X		(Lee, Horwitz et al. 2006)
unknown	FTN_0597	protein-disulfide isomerase		X	(Yu, Goluguri et al. 2010)
pnp	FTN_0609	polynucleotide phosphorylase/polyadenylase		X	(Kraemer, Mitchell et al. 2009)
chiA	FTN_0627	glycosyl hydrolase family chitinase	X		(Hager, Bolton et al. 2006)
katG	FTN_0633	peroxidase/catalase	X		(Lee, Horwitz et al. 2006)
unknown	FTN_0643	hypothetical protein		X	(Weiss, Brotcke et al. 2007)
unknown	FTN_0714	hypothetical protein		X	(Tempel, Lai et al. 2006)
fopA	FTN_0756	OmpA family protein		X	(Su, Yang et al. 2007; Yu, Goluguri et al. 2010)
fipB	FTN_0771	protein-disulfide isomerase		X	(Su, Yang et al. 2007; Qin, Scott et al. 2011)
unknown	FTN_0855	hypothetical protein		X	(Su, Yang et al. 2007)
unknown	FTN_0869	hypothetical protein		X	(Brotcke, Weiss et al. 2006)
unknown	FTN_0893	hypothetical protein		X	(Su, Yang et al. 2007)
unknown	FTN_0925	hypothetical protein		X	(Weiss, Brotcke et al.

					2007)
ahp1	FTN_0973	AhpC/TSA family peroxiredoxin	X		(Lee, Horwitz et al. 2006)

Chapter 4. Characterization of Nanotubes in *F. novicida*

I. Introduction

Nanotubes have recently been described in both eukaryotic and prokaryotic organisms as long, tube-like structures capable of transferring cytosolic components between organisms (Drecktrah, Levine-Wilkinson et al. 2008; Gerdes and Carvalho 2008; Mullineaux, Mariscal et al. 2008; Hurtig, Chiu et al. 2010; Dubey and Ben-Yehuda 2011; Galkina, Romanova et al. 2011). In eukaryotic cells, these structures have been termed tunneling nanotubes and have been observed in immune, neuronal and primary cells, allowing transfer of cytosolic molecules and organelles and the spread of pathogens (Gerdes and Carvalho 2008; Gousset and Zurzolo 2009). Similar structures have been observed in bacteria, and a role for transfer of DNA and small molecules has been demonstrated between organisms of the same and different species (Dubey and Ben-Yehuda 2011). The exact methods of biogenesis, structural protein composition and specific purpose of these tubes have yet to be discovered. In addition, NT observation in bacterial cells has only been reported when the organisms are grown on solid surfaces.

The NT observed in *Francisella* appear to be novel structures, possessing unique characteristics that set them apart from previously reported NT. They are readily observed free-floating or attached to bacteria in cultures grown in liquid media. Unlike previous reports that place a role for cytosolic transfer of components between cells, *Francisella* NT appear to be continuous with the periplasmic space. Furthermore, these NT are hardy structures, resistant to degradation by proteases, chemical denaturation and other forms of biochemical manipulation. This is in contrast to the structures observed in

other bacteria, which are hypothesized to be too fragile to exist when not grown on solid surfaces.

In this study, we attempted to determine what factors contribute to formation of *Francisella* NT and what is responsible for structuring them. We performed whole proteomic analysis of *F. novicida* under NT-producing and non-producing conditions and found a large number of differentially regulated proteins. We examined numerous mutants in OMV/NT-associated proteins, as well as mutants in differentially regulated proteins, for defects in production of NT. We attempted to separate OMV from NT by differential centrifugation and velocity sedimentation gradients. We also treated the OMV/NT with various chemical reagents in an attempt to denature what is structuring the NT. Lastly, we have performed cryo-electron tomography on whole bacteria and purified OMV/NT in an attempt to visualize NT formation and structure.

II. Results

Whole proteome analysis of *Francisella novicida*

To identify which proteins are differentially regulated when *F. novicida* is producing NT, we performed whole proteomic analysis on bacterial cultures grown under NT-producing and non-producing conditions. Cultures were grown in BHI or TS broth to an OD_{600} of ~1.3, a time when numerous NT are observed associated with bacteria in BHI cultures, but not in TS cultures. MudPIT analysis was performed 5 times on each sample to obtain a statistically significant set of relative protein amounts. We found 400 proteins that were differentially regulated between the two growth conditions (Table 4-1). Of these proteins, 117 had previously been identified as OMV/NT associated.

Interestingly, 13 of the 17 FPI proteins were found to be differentially regulated (12 increased and only 1 decreased), which mimics observations published by other researchers (Hazlett, Caldon et al. 2008) on growth of *Francisella* in BHI media. These researchers had previously shown that BHI and macrophage-grown *Francisella* showed similar expression of MglA-dependent and independent proteins. The FPI genes are known to be highly regulated by MglA, a master virulence regulator in *Francisella* (Brotcke, Weiss et al. 2006). In fact, one of the most highly upregulated proteins in our whole proteome analysis is IglE, an uncharacterized FPI protein. A number of proteins involved in metabolic pathways are also differentially regulated in our whole proteome analysis. Proteins involved in leucine biosynthesis and pantothenic acid production are highly upregulated. Proteins involved in the production of biotin are some of the most down regulated. This could be the result of differences in nutrient requirements resulting from the different growth media. We also found five regulator proteins to be differentially regulated in the whole proteome analysis. Three of these were upregulated, while two of them were down regulated.

F. novicida mutant screen

In an attempt to examine NT production in *Francisella,* we utilized deletion mutants and an available defined transposon mutant library (Gallagher, Ramage et al. 2007) to screen mutants in specific proteins via electron microscopy. Additional deletion mutants in pilus machinery or OMV/NT-associated proteins were also screened for defects in NT production. Mutants were selected from the transposon insertion library based on relative abundance of OMV/NT-associated proteins, or those proteins determined to be differentially regulated by whole proteome analysis under NT-

producing and non-producing conditions. We examined mutants in T4P components, including *pilF*, the ATPase responsible for pilus biogenesis and protein secretion. We also examined a number of FPI proteins, as well as *mglA*, the master virulence regulator responsible for regulation of numerous genes. We looked at mutants in the five regulators shown by whole proteome analysis to be differentially regulated. Mutants were either grown in liquid BHI medium or plated on BHI agar plates and screened visually for defects in NT production by TEM. Of the 292 OMV/NT associated proteins, 51 have been screened by TEM for NT defects, with no clear candidates identified (Table 4-2). Of the 400 differentially regulated proteins identified by whole proteome analysis, 40 have been screened by TEM for NT defects, with no clear candidates identified (Table 4-2).

Separation of OMV from NT

Separation of OMV from NT would allow identification of NT-associated proteins, including structural proteins, and begin the process of identifying factors responsible for biogenesis of NT. In an attempt to separate *F. novicida* OMV from NT, we have employed several previously utilized experimental methods. Differential centrifugation is frequently performed to separate larger-sized OMV and cellular debris from smaller-sized OMV. In this method, cell free supernatants are spun in successively faster centrifugation steps, with larger structures pelleting out at slower speeds and smaller OMV at higher speeds. We applied this method to *F. novicida* cell-free supernatants, spinning them at 20,000 × *g*, 50,000 × *g,* and 100,000 × *g*. Unfortunately, we were not able to separate larger OMV, NT or smaller OMV from each other using this

method. Pellets were obtained at each speed and each pellet contained a heterogeneous mixture of all structures that are normally observed at the highest speed spins (Fig. 4-1).

Density gradient centrifugation is often used to separate cellular debris and other contaminants from genuine OMV. This method involves the use of a viscous medium, such as a sucrose solution, formed into a gradient of increasing density within a centrifuge tube. Samples to be separated are placed in the densest fraction and the tube is spun for long periods of time at high *g*-force. Individual particles will rise through the gradient and settle at their natural buoyant density, effectively removing contaminants such as pili, flagella and cellular debris from intact OMV. An alternative method, velocity sedimentation, involves placing the sample in the lowest density portion of the tube and spinning for shorter periods of time. In this manner, objects move through the density fractions slower or quicker, depending on their size and shape. In an attempt to separate OMV from NT, we subjected OMV/NT samples to density gradient centrifugation and velocity sedimentation. We did not achieve separation of OMV and NT by either of these methods (Fig. 4-2).

NT structure

In an attempt to determine what is giving shape to the *F. novicida* NT, we treated purified OMV/NT with various chemicals to disrupt proteins associated with these structures. Samples were treated with 0.1 M Tris-EDTA (Fig. 4-3), which weakens OM structure and has been published as a method to disrupt OMV (Horstman and Kuehn 2000); however, no effect was observed on the OMV/NT of *Francisella*. Similarly, samples were treated with 6 M guanidine-HCl to denature proteins, with no loss of OMV

or NT structure (Fig. 4-3). Treatment with 6 M urea also had no effect on OMV or NT structure (Fig. 4-3). As mentioned previously (Chapter 3, Fig. 3-7 & 3-8), only heat treatment or use of SDS effectively disrupted the OMV/NT. This seems to suggest that the OMV/NT from *Francisella* are resistant to chemical degradation, since the published method for OMV disruption had no effect on either OMV or NT structure. Additionally, since neither guanidine-HCl nor urea had an effect on NT structure, it is tempting to speculate that the factor structuring NT is not a protein. However, since heat treatment was successful in denaturing NT, clearly something that is sensitive to an increase in temperature is being affected.

Cryo-EM tomography

To better visualize *Francisella* NT structure, we performed preliminary cryo-EM tomography analysis of whole bacteria and purified OMV/NT. This technique allows one to observe samples in their native environment, without any staining of bacteria or structures they may produce. Through collaboration with Huilin Li's group at Brookhaven National Laboratory, we performed cryo-EM tomography on *F. novicida.* We observed some denser structural formations within tubes which may explain their characteristic shape (Fig. 4-4). This denser matter contained within the OMV/NT was reminiscent of the appearance of cytosolic components (Fig. 4-4). It is possible that these internal vesicles are giving shape to some of the nanotubes, though this presumably cytosolic material was not seen in all NT, arguing that another factor may be responsible. As all images seem to indicate that NT are continuous with the periplasmic space the presence of this dense material inside these structures is surprising. These images may explain why we see such a high percentage of cytoplasmic proteins in purified OMV/NT

samples examined by mass spectrometry. It is interesting to note the unusually large and distended periplasmic space of these bacteria, though there does not seem to be an internal structure that can be observed by EM which would account for this large space between the outer membrane and the inner membrane of the bacteria. Perhaps whatever factor is forming the tubes is also pushing the outer membrane away from the inner membrane of the organism.

III. Figures

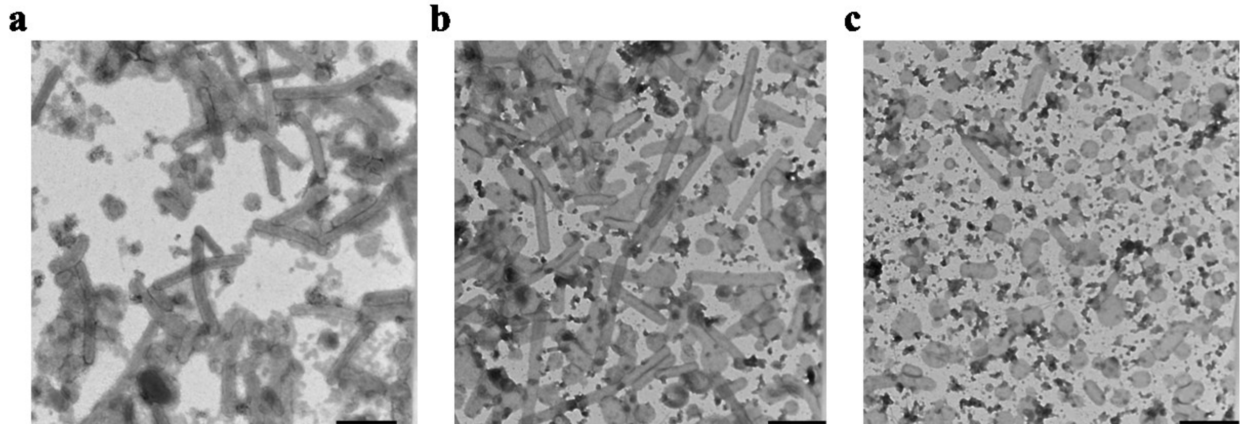
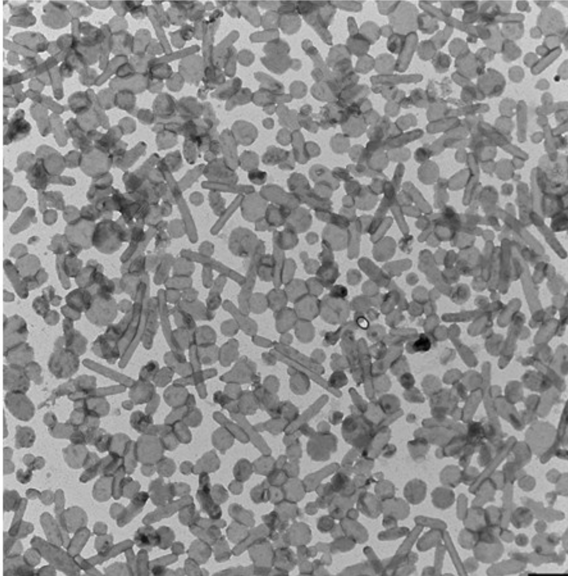


Figure 4-1. TEM Images of differential centrifugation attempted separation of OMV/NT. Cell-free supernatant was centrifuged at (a) 20,000 × *g*, (b) 50,000 × *g* and (c) 100,000 × *g*. Resultant pellets were collected and visualized by TEM (black bars = 500 nm).

a



b

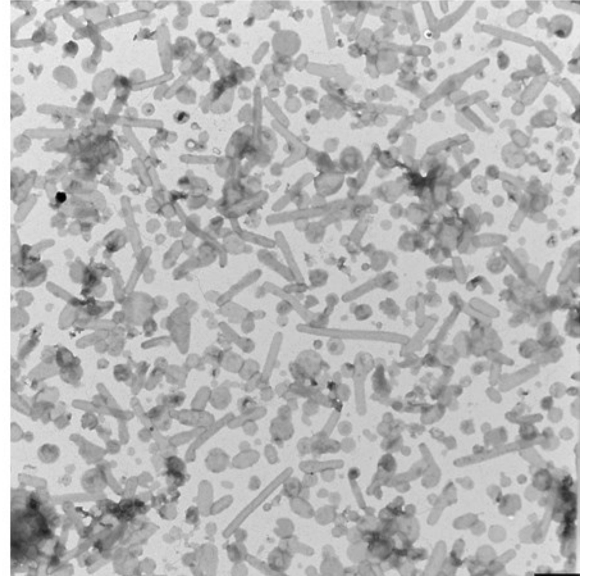


Figure 4-2. TEM Images of density flotation attempted separation of OMV/NT.

Pelleted OMV/NT were subjected to OptiPrep discontinuous density gradient centrifugation. Bands were observed in fractions 1-4 (a) and fractions 5-6 (b) of the gradient. Fractions were collected, pooled, recovered and visualized by TEM (black bars = 500 nm).

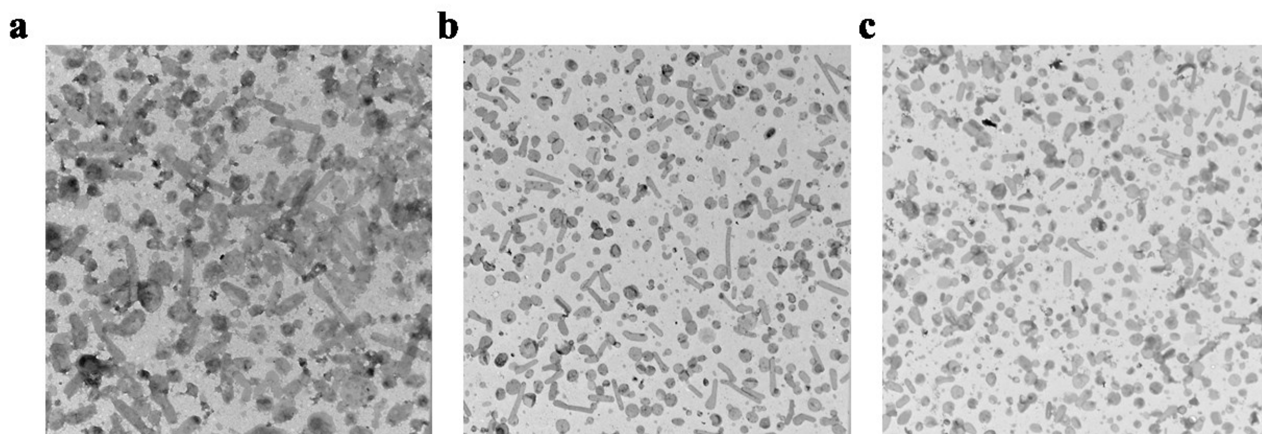


Figure 4-3. TEM Images of chemical treatment of purified OMV/NT.

Purified *F. novicida* OMV/NT were treated with (a) 0.1 M Tris-EDTA, (b) 6 M guanidine-HCl or (c) 6 M urea before being visualized by TEM (black bars = 500 nm).

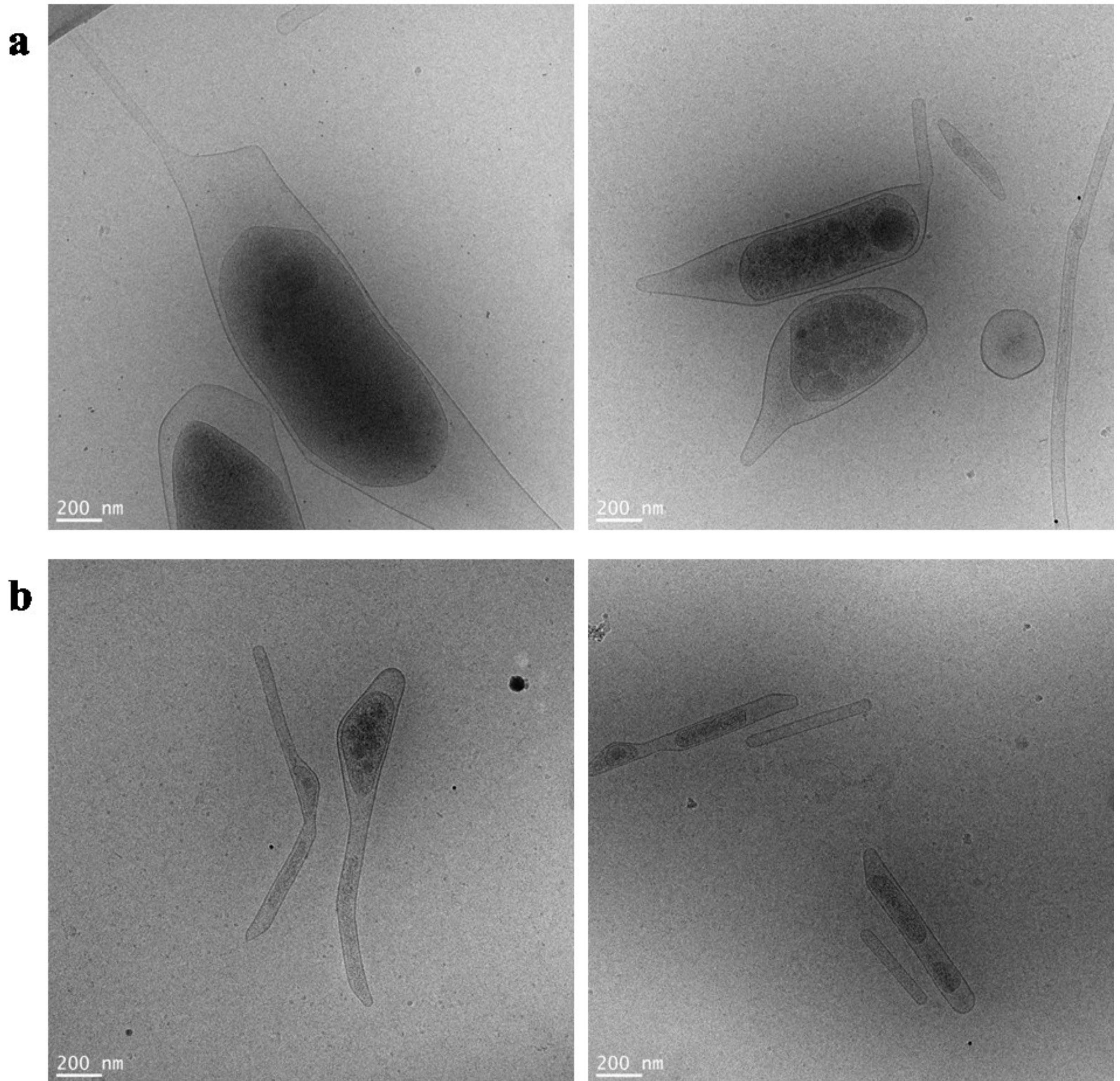


Figure 4-4. Cryo-EM Images of *F. novicida* and purified NT.

Whole bacteria were visualized by Cryo-EM (a). Individual NT appear to contain denser material in some cases (b).

IV. Tables

Table 4-1. Differentially regulated proteins determined by whole proteome analysis.

Gene	Locus	Description	BHI/TSB
unknown	FTN_0359	short-chain alcohol dehydrogenase-like dehydrogenase	50.64145
iglE	FTN_1311	hypothetical protein	46.96412
unknown	FTN_1351	hypothetical protein	38.52679
panB	FTN_1352	3-methyl-2-oxobutanoate hydroxymethyltransferase	24.9247
unknown	FTN_1272	proton-dependent oligopeptide transporter (POT) family protein	21.06332
unknown	FTN_0004	aspartate/glutamate transporter	20.56989
unknown	FTN_1362	hypothetical protein	17.34938
unknown	FTN_0923	hypothetical protein	16.00256
unknown	FTN_1616	hypothetical protein	15.7843
panC	FTN_1353	pantoate-beta-alanine ligase	15.7165
leuB	FTN_0059	3-isopropylmalate dehydrogenase	11.27183
unknown	FTN_1355	pantothenate kinase	10.07984
unknown	FTN_1757	D-isomer specific 2-hydroxyacid dehydrogenase	9.781945
leuD	FTN_0060	isopropylmalate isomerase small subunit	9.028674
unknown	FTN_0431	hypothetical protein	8.332711
pyrE	FTN_0529	orotate phosphoribosyltransferase	7.155154
relA	FTN_1518	GDP pyrophosphokinase/GTP pyrophosphokinase	7.130796
unknown	FTN_1080	phosphosugar binding protein	6.701988
sodC	FTN_0405	superoxide dismutase (Cu-Zn) precusor	6.12162
iglI	FTN_1317	hypothetical protein	5.976433
dotU	FTN_1316	hypothetical protein	5.693769
unknown	FTN_0643	hypothetical protein	5.13666
unknown	FTN_1265	hypothetical protein	4.987272
unknown	FTN_0822	para-aminobenzoate synthase component I	4.982846
ispA	FTN_1470	geranyl diphosphate synthase/farnesyl diphosphate synthase	4.55802
ansB	FTN_0555	periplasmic L-asparaginase II precursor	4.438756
bfr	FTN_1410	bacterioferritin	4.265548
unknown	FTN_0740	hypothetical protein	4.212561
xseA	FTN_1168	exodeoxyribonuclease VII large subunit	4.116673
ilvE	FTN_0063	branched-chain amino acid aminotransferase protein (class IV)	4.108849
unknown	FTN_1240	hypothetical protein	3.8876
unknown	FTN_1169	M20 family peptidase	3.78607

unknown	FTN_0290	hypothetical protein	3.762737
chaB	FTN_1126	cation transport regulator	3.717051
unknown	FTN_0065	hypothetical protein	3.706425
nfnB	FTN_0218	dihydropteridine reductase	3.706351
unknown	FTN_0269	hypothetical protein	3.656969
cfa	FTN_1456	cyclopropane fatty acid synthase	3.420419
unknown	FTN_0282	hypothetical protein	3.393119
unknown	FTN_0721	hypothetical protein	3.38517
unknown	FTN_1032	proton-dependent oligopeptide transporter (POT) family protein	3.336443
unknown	FTN_1239	5-formyltetrahydrofolate cycloligase	3.304213
iglA	FTN_1324	intracellular growth locus protein A	3.260365
recA	FTN_0122	recombinase A protein	3.254058
lolB	FTN_0145	outer membrane lipoprotein LolB	3.186101
mutT	FTN_0865	mutator protein	3.169367
unknown	FTN_0391	LemA-like protein	3.157404
unknown	FTN_0850	transcriptional regulator	3.137776
unknown	FTN_1266	ABC transporter membrane protein	3.108972
unknown	FTN_0791	hypothetical protein	3.02675
pssA	FTN_0350	CDP-alcohol phosphatidyltransferase	3.025835
unknown	FTN_0702	YjeF-related protein	3.015671
unknown	FTN_1369	hypothetical protein	2.966205
unknown	FTN_1618	hypothetical protein	2.965341
unknown	FTN_1769	HSP20 family protein	2.950799
leuA	FTN_0062	2-isopropylmalate synthase	2.931491
unknown	FTN_0381	hypothetical protein	2.884841
iglH	FTN_1315	hypothetical protein	2.874471
pip	FTN_1731	proline iminopeptidase	2.87217
unknown	FTN_1105	hypothetical protein	2.865513
glpF	FTN_1583	glycerol uptake facilitator protein	2.853872
unknown	FTN_1772	peptide methionine sulfoxide reductase	2.844912
fadE	FTN_1437	acyl-CoA dehydrogenase	2.841795
unknown	FTN_1020	hypothetical protein	2.754959
leuC	FTN_0061	isopropylmalate isomerase large subunit	2.729159
pheA	FTN_0748	prephenate dehydratase	2.714281
unknown	FTN_1458	hypothetical protein	2.711425
unknown	FTN_1021	hypothetical protein	2.706009
unknown	FTN_1771	hypothetical protein	2.698777
iglF	FTN_1313	hypothetical protein	2.672702
iglB	FTN_1323	intracellular growth locus protein B	2.661253
unknown	FTN_1438	fusion product of 3-hydroxacyl-CoA dehydrogenase and acyl-CoA-binding protein	2.631106

unknown	FTN_1109	rhodanese-like family protein	2.615185
unknown	FTN_0450	hypothetical protein	2.605162
fadD	FTN_1436	long chain fatty acid CoA ligase	2.597962
unknown	FTN_1184	hypothetical protein	2.575686
unknown	FTN_1617	two-component regulator	2.563023
deoB	FTN_1602	phosphopentomutase	2.550159
unknown	FTN_0697	hypothetical protein	2.509453
katG	FTN_0633	peroxidase/catalase	2.508916
galE	FTN_1219	UDP-glucose 4-epimerase	2.457014
unknown	FTN_1448	hypothetical protein	2.452858
unknown	FTN_0449	hypothetical protein	2.447683
betT	FTN_0767	betaine/carnitine/choline transporter (BCCT) family protein	2.443486
recG	FTN_0335	ATP-dependent DNA helicase RecG	2.397849
fadA	FTN_1439	acetyl-CoA acetyltransferase	2.347892
ssb	FTN_0124	single-strand DNA binding protein	2.318172
unknown	FTN_1233	haloacid dehalogenase-like hydrolase	2.296457
spoU	FTN_0766	rRNA methyltransferase	2.287904
unknown	FTN_1468	HAM1-like protein	2.256303
gpsA	FTN_0397	glycerol-3-phosphate-dehydrogenase-(NAD+)	2.227833
unknown	FTN_0832	proton-dependent oligopeptide transporter (POT) family protein	2.222369
ggt	FTN_1159	gamma-glutamyltranspeptidase	2.207691
unknown	FTN_1267	ABC transporter ATP-binding protein	2.196554
unknown	FTN_1615	hypothetical protein	2.179512
unknown	FTN_0088	hypothetical protein	2.168468
sufC	FTN_0852	sufS activator complex	2.148797
unknown	FTN_1082	hypothetical protein	2.144324
sufB	FTN_0851	cysteine desulfurase activator complex subunit SufB	2.139588
mutS	FTN_1509	DNA mismatch repair protein	2.137522
oppA	FTN_1593	ABC-type oligopeptide transport system	2.134024
unknown	FTN_0225	hypothetical protein	2.128276
unknown	FTN_1413	ATPase	2.117084
unknown	FTN_1273	long chain fatty acid CoA ligase	2.095156
unknown	FTN_1613	U61 family peptidase	2.082072
unknown	FTN_1765	hypothetical protein	2.081029
unknown	FTN_0632	dGTP triphosphohydrolase	2.079892
gltA	FTN_1640	citrate synthase	2.079055
unknown	FTN_1488	prophage maintenance system killer protein (DOC)	2.072932
yajC	FTN_1096	preprotein translocase family protein	2.071117
unknown	FTN_1343	hypothetical protein	2.070046

ampG	FTN_1641	major facilitator transporter	2.069066
yjfH	FTN_0531	tRNA/rRNA methyltransferase	2.059399
blc	FTN_0174	outer membrane lipoprotein	2.048812
pdpB	FTN_1310	hypothetical protein	2.048575
unknown	FTN_0983	bifunctional gluaredoxin/ribonucleoside-diphosphate reductase subunit beta	2.041804
unknown	FTN_0635	serine-type D-Ala-D-Ala carboxypeptidase	2.015796
unknown	FTN_1083	hypothetical protein	1.983573
unknown	FTN_0183	periplasmic solute binding family protein	1.95128
iglD	FTN_1321	intracellular growth locus protein D	1.951162
ubiC	FTN_0386	chorismate pyruvate lyase	1.945092
unknown	FTN_1466	hypothetical protein	1.944298
unknown	FTN_1684	diaminopimelate decarboxylase	1.922439
unknown	FTN_0033	chorismate mutase	1.920076
fopB	FTN_0119	outer membrane protein of unknown function	1.903236
unknown	FTN_0081	hypothetical protein	1.891776
unknown	FTN_1066	HlyC/CorC family transporter-associated protein	1.879071
fumC	FTN_0220	fumarate hydratase	1.87678
gcvT	FTN_0505	glycine cleavage system aminomethyltransferase T	1.867042
trpA	FTN_1740	tryptophan synthase subunit alpha	1.858132
glgA	FTN_0516	glycogen synthase	1.856489
fumA	FTN_0337	fumerate hydratase	1.849107
unknown	FTN_0855	hypothetical protein	1.843166
unknown	FTN_1271	hypothetical protein	1.83998
trpS	FTN_1499	tryptophanyl-tRNA synthetase	1.837167
unknown	FTN_1692	membrane fusion protein	1.826769
unknown	FTN_1491	adenine specific DNA methylase	1.814247
unknown	FTN_0149	hypothetical protein	1.797776
pdpD	FTN_1325	hypothetical protein	1.782352
pepN	FTN_1768	aminopeptidase N	1.781306
rpsO	FTN_0608	30S ribosomal protein S15	1.779398
uspA	FTN_0085	universal stress protein	1.775574
unknown	FTN_0404	methionine sulfoxide reductase B	1.774984
unknown	FTN_0839	hypothetical protein	1.768828
unknown	FTN_1666	conserverd protein of unknown function	1.762251
uppS	FTN_0231	undecaprenyl pyrophosphate synthetase	1.762089
hisS	FTN_1658	histidyl-tRNA synthetase	1.747697
unknown	FTN_1405	ABC transporter ATP-binding protein	1.746756
wbtF	FTN_1425	NAD dependent epimerase	1.744759
trpB	FTN_1739	tryptophan synthase subunit beta	1.735937
murF	FTN_0522	UDP-N--acetylmuramoylalanyl-D-glutamyl-2	1.732739

pgk	FTN_1331	phosphogylcerate kinase	1.720095
unknown	FTN_1750	acyltransferase	1.71768
unknown	FTN_0118	S49 family serine peptidase	1.714326
unknown	FTN_0911	alpha-glucosidase	1.706565
lpxC	FTN_0165	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	1.698669
unknown	FTN_0346	OmpA family protein	1.695976
ilvC	FTN_1040	ketol-acid reductoisomerase	1.678133
purT	FTN_1745	phosphoribosylglycinamide formyltransferase 2	1.673564
manB	FTN_1417	phosphomannomutase	1.672761
unknown	FTN_0595	hypothetical protein	1.671737
minE	FTN_0329	cell division topological specificity factor protein	1.654
nrdA	FTN_0981	ribonucleotide-diphosphate reductase subunit alpha	1.645165
unknown	FTN_1012	small conductance mechanosensitive ion channel family protein	1.63271
unknown	FTN_0103	hypothetical protein	1.632141
iscS	FTN_1245	cysteine desulfarase	1.616707
unknown	FTN_1770	bifunctional indole-3-glycerol phosphate synthase/phosphoribosylanthranilate isomerase	1.609964
unknown	FTN_1522	subunit of DnaJ/DnaK/GrpE: chaperone with DnaK; heat shock protein	1.607055
msbA	FTN_1606	lipid exporter (LipidE) family protein	1.604896
unknown	FTN_1542	hypothetical protein	1.597962
unknown	FTN_0504	lysine decarboxylase	1.59469
unknown	FTN_0509	hypothetical protein	1.593773
glpD	FTN_1584	glycerol-3-phosphate dehydrogenase	1.58821
rbsK	FTN_1767	ribokinase	1.58518
kdpC	FTN_1716	potassium-transporting ATPase C chain	1.576614
kdpB	FTN_1717	potassium-transporting ATPase B chain	1.568805
hupB	FTN_1054	DNA-binding protein HU-beta	1.566822
rng	FTN_1782	ribonuclease G	1.560029
cscK	FTN_0646	ROK family protein	1.54903
serS	FTN_0647	seryl-tRNA synthetase	1.53869
pcp	FTN_0211	pyrrolidone carboxylylate peptidase	1.53007
unknown	FTN_1472	hypothetical protein	1.525549
dcd	FTN_0873	deoxycytidine triphosphate deaminase	1.516798
clpB	FTN_1743	chaperone clpB	1.513386
unknown	FTN_1113	hypothetical protein	1.50513
pdpA	FTN_1309	hypothetical protein	1.499529
unknown	FTN_1547	hypothetical protein	1.498824
gabD	FTN_0127	succinate semialdehyde dehydrogenase (NAD(P)+ dependent)	1.490438

unknown	FTN_1447	hypothetical protein	1.489584
pyrC	FTN_0024	dihydroorotase	1.488871
ispG	FTN_1076	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	1.484569
unknown	FTN_0032	hypothetical protein	1.479306
unknown	FTN_1644	hypothetical protein	1.477763
gyrA	FTN_1484	DNA gyrase	1.469785
infB	FTN_1660	translation initiation factor IF-2	1.468766
appC	FTN_1619	cytochrome bd-II terminal oxidase subunit I	1.464773
glgC	FTN_0515	glucose-1-phosphate adenylyltransferase	1.464556
unknown	FTN_1372	hypothetical protein	1.464439
fimV	FTN_1596	Type IV pili	1.462544
unknown	FTN_0962	hypothetical protein	1.462306
sucB	FTN_1634	2-oxoglutarate dehydrogenase complex	1.459752
secB1	FTN_0121	preprotein translocase subunit SecB	1.459181
ftsZ	FTN_0164	cell division protein FtsZ	1.456987
ubiG	FTN_0321	3-demethylubiquinone-9 3-methyltransferase	1.450957
unknown	FTN_0958	AhpC/TSA family protein	1.445159
unknown	FTN_0500	peptide deformylase	1.44154
unknown	FTN_1741	hypothetical protein	1.441265
gcvP2	FTN_0508	glycine dehydrogenase subunit 2	1.438273
pdpC	FTN_1319	hypothetical protein	1.436482
unknown	FTN_0429	hypothetical protein	1.435029
unknown	FTN_1624	hypothetical protein	1.428382
unknown	FTN_1459	short chain dehydrogenase	1.42239
unknown	FTN_0387	ribonuclease PH	1.417742
queA	FTN_1234	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	1.41088
aceF	FTN_1493	dihydrolipoamide acetyltransferase	1.403062
unknown	FTN_1444	ornithine cyclodeaminase	1.401203
sdhD	FTN_1638	succinate dehydrogenase hydrophobic membrane anchor protein	1.391044
unknown	FTN_1074	X-prolyl aminopeptidase 2	1.390411
unknown	FTN_1049	hypothetical protein	1.38999
guaB	FTN_0661	IMP dehydrogenase/GMP reductase	1.381309
unknown	FTN_1697	galactose mutarotase	1.365615
dnaX	FTN_0166	DNA polymerase III	1.351352
cydD	FTN_0642	cysteine/glutathione ABC transporter membrane/ATP-binding component	1.346745
pgm	FTN_0514	phosphoglucomutase	1.325613
unknown	FTN_1476	hypothetical protein	1.324283
unknown	FTN_1072	beta-lactamase class A	1.323893

unknown	FTN_1053	hypothetical protein	1.31996
unknown	FTN_1557	oxidoreductase iron/ascorbate family protein	1.319792
lpdA	FTN_1492	dihydrolipoamide dehydrogenase	1.308409
unknown	FTN_0131	hypothetical protein	1.308223
unknown	FTN_0782	hypothetical protein	1.294621
icd	FTN_1434	isocitrate dehydrogenase	1.291677
unknown	FTN_0022	histidine acid phosphatase	1.281358
unknown	FTN_1465	two-component response regulator	1.267346
yhbG	FTN_0902	ABC transporter	1.256968
ileS	FTN_0441	isoleucyl-tRNA synthetase	1.256388
nuoD	FTN_1677	NADH dehydrogenase subunit D	1.252865
gcvP1	FTN_0507	glycine dehydrogenase subunit 1	1.247733
accA	FTN_1508	acetyl-CoA carboxylase	1.238327
unknown	FTN_0833	hypothetical protein	1.234057
gshA	FTN_0277	glutamate-cysteine ligase	1.232904
cysK	FTN_1302	cysteine synthase	1.232367
unknown	FTN_0893	hypothetical protein	1.225441
metlQ	FTN_1107	methionine uptake transporter (MUT) family protein	1.222545
hemE	FTN_1664	uroporphyrinogen decarboxylase	1.219701
ppdK	FTN_0064	pyruvate phosphate dikinase	1.214076
plsX	FTN_1336	putative glycerol-3-phosphate acyltransferase PlsX	1.197485
unknown	FTN_0109	hypothetical protein	1.191592
prfB	FTN_0167	peptide chain release factor 2	1.190594
putA	FTN_1131	bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	1.187028
acnA	FTN_1623	aconitate hydratase	1.160569
upp	FTN_0628	uracil phosphoribosyltransferase	1.159499
lysU	FTN_0168	lysyl-tRNA synthetase	1.110902
atpH	FTN_1649	F0F1 ATP synthase subunit delta	-1.11484
gcp	FTN_1565	O-sialoglycoprotein endopeptidase	-1.13485
rpmA	FTN_0676	50S ribosomal protein L27	-1.14164
purF	FTN_1700	amidophosphoribosyltransferase	-1.17069
gdhA	FTN_1532	glutamate dehydrogenase	-1.17733
carA	FTN_0021	carbamoyl phosphate synthase small subunit	-1.19015
rplB	FTN_0242	50S ribosomal protein L2	-1.19062
unknown	FTN_0575	hypothetical protein	-1.19385
lytB	FTN_0348	1-hydroxy-2-methyl-2-(E)-butenyl 4- diphosphate reductase	-1.20592
pepB	FTN_0780	cytosol aminopeptidase	-1.20687
nuoF	FTN_1675	NADH dehydrogenase I	-1.23461
gatB	FTN_1689	aspartyl/glutamyl-tRNA amidotransferase subunit B	-1.2389

atpG	FTN_1647	F0F1 ATP synthase subunit gamma	-1.26949
unknown	FTN_0477	hypothetical protein	-1.2712
fipB	FTN_0771	protein-disulfide isomerase	-1.28114
rpmG	FTN_0332	50S ribosomal protein L33	-1.28164
unknown	FTN_1117	ATP binding protein	-1.282
unknown	FTN_1170	hypothetical protein	-1.28392
unknown	FTN_0925	hypothetical protein	-1.28938
fabl	FTN_1228	enoyl-ACP reductase I	-1.2954
ppiC	FTN_0689	parvulin-like peptidyl-prolyl isomerase domain-containing protein	-1.29893
rpoB	FTN_1568	DNA-directed RNA polymerase subunit beta	-1.32577
unknown	FTN_1346	inositol monophosphatase family protein	-1.32633
greA	FTN_0665	transcriptional elongation factor	-1.3298
groEL	FTN_1538	chaperonin GroEL	-1.33303
unknown	FTN_1294	rRNA methylase	-1.33608
purB	FTN_1694	adenylosuccinate lyase	-1.34238
carB	FTN_0020	carbamoyl phosphate synthase large subunit	-1.35046
rpoD	FTN_0913	RNA polymerase sigma-70 factor	-1.35418
cphA	FTN_1112	cyanophycin synthetase	-1.35797
rpoC	FTN_1567	DNA-directed RNA polymerase	-1.35842
rpsH	FTN_0253	30S ribosomal protein S8	-1.36104
unknown	FTN_0920	ATPase	-1.37266
nuoG	FTN_1674	NADH dehydrogenase subunit G	-1.385
tufA	FTN_1576	elongation factor Tu	-1.39841
add	FTN_0695	deoxyadenosine deaminase/adenosine deaminase	-1.39873
sdhB	FTN_1636	succinate dehydrogenase iron-sulfur subunit	-1.41375
unknown	FTN_1165	ATPase	-1.41631
pgi	FTN_0663	glucose-6-phosphate isomerase	-1.43509
potG	FTN_0739	ATP-binding cassette putrescine uptake system	-1.43608
hslV	FTN_0995	ATP-dependent protease peptidase subunit	-1.44025
unknown	FTN_1252	choloylglycine hydrolase family protein	-1.45292
unknown	FTN_0073	membrane protein of unknown function	-1.45727
ahp1	FTN_0973	AhpC/TSA family peroxiredoxin	-1.46623
unknown	FTN_1412	DNA-directed RNA polymerase subunit alpha	-1.4675
unknown	FTN_0841	ThiJ/PfpI family protein	-1.47631
unknown	FTN_0034	hypothetical protein	-1.47811
unknown	FTN_0437	HD superfamily hydrolase	-1.48536
rpsF	FTN_0951	30S ribosomal protein S6	-1.48922
fopC	FTN_0918	hypothetical protein	-1.49956
rho	FTN_1416	transcription termination factor Rho	-1.50451
nuoI	FTN_1672	NADH dehydrogenase subunit I	-1.51092

unknown	FTN_1143	4Fe-4S ferredoxin	-1.51137
fbaA	FTN_1329	fructose-1	-1.51234
unknown	FTN_0921	FKBP-type peptidyl-prolyl cis-trans isomerase	-1.52847
unknown	FTN_0043	hypothetical protein	-1.53881
rpsU	FTN_0487	30S ribosomal protein S21	-1.54027
tig	FTN_1058	trigger factor	-1.54499
fabD	FTN_1338	malonyl-CoA:ACP transacylase	-1.57608
lpnA	FTN_0427	lipoprotein of unknown function	-1.5968
unknown	FTN_0789	putative rhodanese	-1.61872
udhA	FTN_0999	soluble pyridine nucleotide transhydrogenase	-1.62123
oppF	FTN_1589	peptide/opine/nickel uptake transporter (PepT) family protein	-1.62885
unknown	FTN_0117	ferredoxin	-1.63663
rplI	FTN_0949	50S ribosomal protein L9	-1.64228
iglC	FTN_1322	intracellular growth locus protein C	-1.65289
unknown	FTN_0566	mechanosensitive ion channel protein	-1.6629
dnaG	FTN_0914	DNA primase	-1.67352
dnaK	FTN_1284	heat shock protein DnaK	-1.68322
tpiA	FTN_1631	triosephosphate isomerase	-1.68431
unknown	FTN_0715	hypothetical protein	-1.69247
chiA	FTN_0627	glycosyl hydrolase family chitinase	-1.70727
unknown	FTN_0649	4Fe-4S ferredoxin	-1.72343
unknown	FTN_0827	carbon-nitrogen hydrolase family protein	-1.75363
fabG	FTN_1339	3-oxoacyl-(acyl-carrier-protein) reductase	-1.76795
coaE	FTN_1496	dephospho-CoA kinase	-1.78701
unknown	FTN_0765	choloylglycine hydrolase family protein	-1.80283
tdh	FTN_0625	L-threonine 3-dehydrogenase	-1.80412
ychF	FTN_1004	translation-associated GTPase	-1.82109
rplW	FTN_0241	50S ribosomal protein L23	-1.85251
unknown	FTN_1014	nicotinamide ribonucleoside (NR) uptake permease (PnuC) family protein	-1.86044
unknown	FTN_0601	pyridoxine biosynthesis protein	-1.87088
ksgA	FTN_0560	dimethyladenosine transferase	-1.90728
cydA	FTN_0193	cytochrome bd-I terminal oxidase subunit I	-1.917
blaA	FTN_1002	beta-lactamase class A	-1.91895
nth	FTN_1035	endonuclease III	-1.94992
rpsC	FTN_0245	30S ribosomal protein S3	-1.95255
holC	FTN_0213	DNA polymerase III (chi subunit) protein	-1.95872
clpX	FTN_1056	ATP-dependent protease ATP-binding subunit	-1.98856
gapA	FTN_1332	glyceraldehyde-3-phosphate dehydrogenase/erythrose-4-phosphate dehydrogenase	-1.99531

pilP	FTN_1138	Type IV pili periplasmic component	-2.01187
deaD	FTN_0690	DEAD-box subfamily ATP-dependent helicase	-2.01415
unknown	FTN_0339	arsenate reductase	-2.02859
ppa	FTN_0906	inorganic pyrophosphatase	-2.05718
ilvB	FTN_1042	acetolactate synthase large subunit	-2.0583
unknown	FTN_1103	hypothetical protein	-2.09638
unknown	FTN_0714	hypothetical protein	-2.11259
mdaB	FTN_0840	NADPH-quinone reductase (modulator of drug activity B)	-2.13056
unknown	FTN_0831	ATP-dependent RNA helicase	-2.1558
unknown	FTN_0776	DNA/RNA helicase superfamily I protein	-2.28925
unknown	FTN_1178	short-chain dehydrogenase	-2.30041
unknown	FTN_0620	major facilitator transporter	-2.30809
unknown	FTN_0313	acetyltransferase	-2.31366
lipA	FTN_1030	lipoyl synthase	-2.34038
unknown	FTN_0861	hypothetical protein	-2.45109
unknown	FTN_0007	hypothetical protein	-2.5444
accB	FTN_0563	acetyl-CoA carboxylase	-2.55565
rimK	FTN_0154	glutathione synthase/ribosomal protein S6 modification enzyme	-2.60193
unknown	FTN_0937	hypothetical protein	-2.93404
unknown	FTN_0773	4Fe-4S ferredoxin	-3.03341
unknown	FTN_0367	phage integrase	-3.07865
unknown	FTN_1500	hypothetical protein	-3.19993
unknown	FTN_1063	tRNA-methylthiotransferase MiaB protein	-3.22349
unknown	FTN_0044	hypothetical protein	-3.40107
bglX	FTN_1474	glycosyl 4hydrolase family protein	-3.41034
unknown	FTN_1392	rhodanese-related sulfurtransferase	-3.58272
unknown	FTN_1161	hypothetical protein	-3.58927
unknown	FTN_0047	hypothetical protein	-3.60492
rnfB	FTN_1034	iron-sulfur cluster-binding protein	-3.98167
unknown	FTN_0965	metal-dependent exopeptidase	-4.00285
unknown	FTN_0300	glycosyl transferase	-4.11596
unknown	FTN_0568	birA-like protein	-4.40195
unknown	FTN_0801	ArsR family transcriptional regulator	-4.44529
unknown	FTN_0583	LysR family transcriptional regulator	-4.47557
unknown	FTN_0880	hypothetical protein	-4.8694
unknown	FTN_0878	hypothetical protein	-4.88465
unknown	FTN_0638	sulfate permease family protein	-4.91434
unknown	FTN_0467	major facilitator superfamily sugar transporter	-5.01048
unknown	FTN_1748	4Fe-4S ferredoxin	-5.11359
unknown	FTN_0867	hypothetical protein	-5.83417

unknown	FTN_0722	hypothetical protein	-6.31626
unknown	FTN_1708	ATP-binding cassette (ABC) superfamily protein	-6.78442
rplR	FTN_0255	50S ribosomal protein L18	-6.95223
unknown	FTN_0090	acid phosphatase	-7.08421
unknown	FTN_0987	tRNA-dihydrouridine synthase	-8.17849
unknown	FTN_0287	type I restriction-modification system	-9.20073
unknown	FTN_0362	deoxyribodipyrimidine photolyase-related protein	-10.9207
unknown	FTN_0217	L-lactate dehydrogenase	-10.9512
bioF	FTN_0814	8-amino-7-oxononanoate synthase	-14.1519
bioC	FTN_0813	biotin synthesis protein BioC	-17.2012
unknown	FTN_0151	ABC transporter ATP-binding protein	-18.9198
unknown	FTN_1363	prophage repressor protein	-19.4079
unknown	FTN_0866	hypothetical protein	-19.5029
bioD	FTN_0812	dethiobiotin synthetase	-20.1939
bioA	FTN_0816	adenosylmethionine-8-amino-7-oxononanoate aminotransferase	-23.7628
unknown	FTN_0050	hypothetical protein	-43.2677
bioB	FTN_0815	biotin synthase	-61.1457

Table 4-2. ***F. novicida*** **mutants screened for NT production defects.**

Gene	Locus	Description
unknown	FTN_0004	aspartate/glutamate transporter
unknown	FTN_0065	hypothetical protein
pilE	FTN_0070	Type IV pili
unknown	FTN_0109	hypothetical protein
fopB	FTN_0119	outer membrane protein of unknown function
recA	FTN_0122	recombinase A protein
unknown	FTN_0183	periplasmic solute binding family protein
cyoB	FTN_0196	cytochrome bo terminal oxidase subunit I
pcp	FTN_0211	pyrrolidone carboxylylate peptidase
ftsK	FTN_0294	cell division protein
unknown	FTN_0322	VacJ like lipoprotein
unknown	FTN_0340	hypothetical protein
unknown	FTN_0346	OmpA family protein
tolB	FTN_0355	group A colicin translocation; tolB protein
pal	FTN_0357	OmpA family peptidoglycan-associated lipoprotein
unknown	FTN_0389	Type IV pili
sodC	FTN_0405	superoxide dismutase (Cu-Zn) precusor
pilV	FTN_0413	Type IV pili
unknown	FTN_0414	Type IV pili
pilA	FTN_0415	Type IV pili
lpnA	FTN_0427	lipoprotein of unknown function
unknown	FTN_0428	hypothetical protein
unknown	FTN_0429	hypothetical protein
unknown	FTN_0431	hypothetical protein
unknown	FTN_0449	hypothetical protein
slt	FTN_0496	soluble lytic murein transglycosylase
sspA	FTN_0549	stringent starvation protein A
unknown	FTN_0583	LysR family transcriptional regulator
unknown	FTN_0595	hypothetical protein
katG	FTN_0633	peroxidase/catalase
unknown	FTN_0643	hypothetical protein
unknown	FTN_0702	YjeF-related protein
unknown	FTN_0714	hypothetical protein
unknown	FTN_0715	hypothetical protein
unknown	FTN_0740	hypothetical protein
fopA	FTN_0756	OmpA family protein
unknown	FTN_0782	hypothetical protein
unknown	FTN_0791	hypothetical protein

unknown	FTN_0801	ArsR family transcriptional regulator
unknown	FTN_0822	para-aminobenzoate synthase component I
unknown	FTN_0850	transcriptional regulator
unknown	FTN_0871	rare lipoprotein B family protein
dacD	FTN_0907	D-alanyl-D-alanine carboxypeptidase
unknown	FTN_0917	serine-type D-Ala-D-Ala carboxypeptidase
unknown	FTN_0921	FKBP-type peptidyl-prolyl cis-trans isomerase
unknown	FTN_0923	hypothetical protein
unknown	FTN_1053	hypothetical protein
unknown	FTN_1072	beta-lactamase class A
unknown	FTN_1080	phosphosugar binding protein
pilB	FTN_1115	Type IV pili ATPase
ggt	FTN_1159	gamma-glutamyltranspeptidase
unknown	FTN_1169	M20 family peptidase
unknown	FTN_1240	hypothetical protein
unknown	FTN_1260	hypothetical protein
fsp53	FTN_1261	hypothetical protein
unknown	FTN_1265	hypothetical protein
unknown	FTN_1266	ABC transporter membrane protein
unknown	FTN_1268	hypothetical protein
mltA	FTN_1286	membrane-bound lytic murein transglycosylase
mglA	FTN_1290	macrophage growth locus
pdpB	FTN_1310	hypothetical protein
iglE	FTN_1311	hypothetical protein
iglF	FTN_1313	hypothetical protein
iglH	FTN_1315	hypothetical protein
dotU	FTN_1316	hypothetical protein
iglI	FTN_1317	hypothetical protein
iglD	FTN_1321	intracellular growth locus protein D
iglB	FTN_1323	intracellular growth locus protein B
iglA	FTN_1324	intracellular growth locus protein A
pdpD	FTN_1325	hypothetical protein
unknown	FTN_1355	pantothenate kinase
unknown	FTN_1362	hypothetical protein
unknown	FTN_1372	hypothetical protein
unknown	FTN_1433	hypothetical protein
unknown	FTN_1447	hypothetical protein
unknown	FTN_1448	hypothetical protein
unknown	FTN_1449	hypothetical protein
unknown	FTN_1451	hypothetical protein
cfa	FTN_1456	cyclopropane fatty acid synthase

unknown	FTN_1465	two-component response regulator
unknown	FTN_1465	two-component response regulator
relA	FTN_1518	GDP pyrophosphokinase/GTP pyrophosphokinase
unknown	FTN_1616	hypothetical protein
unknown	FTN_1617	two-component regulator
pilT	FTN_1622	Type IV pili nucleotide-binding protein
unknown	FTN_1644	hypothetical protein
fur	FTN_1681	ferric uptake regulation protein
unknown	FTN_1692	membrane fusion protein
tolC	FTN_1703	outer membrane protein tolC precursor
unknown	FTN_1734	hypothetical protein
unknown	FTN_1769	HSP20 family protein

Chapter 5. *Francisella tularensis* OMV and NT

I. Introduction

F. tularensis subsp. *tularensis* is the highly virulent human pathogenic strain of *Francisella* responsible for naturally occurring infections in healthy individuals. Though there are only ~120 reported cases of tularemia per year in the United States, concerns about the use of this organism as a bioweapon have led to increased research of *Francisella* virulence. Many of the mechanisms of *Francisella* virulence are being elucidated by researchers; however, much about this organism remains to be discovered. While *F. novicida* and the *F. tularensis* LVS strains are excellent models, there are some differences between them and the fully virulent *F. tularensis* which requires that experiments be performed in this strain.

In this study, we report the production of OMV and NT in *F. tularensis* subsp. *tularensis* strain Schu S4. All experiments done with this strain were performed under BSL3 conditions, in keeping with requirements for experimentation with this pathogen. We have analyzed the Schu S4 OMV/NT by mass spectrometry and compared the results to what was observed in *F. novicida* samples. Similar to what we observed in *F. novicida,* we found a large number of proteins associated with these structures, many of them virulence factors not yet shown to be secreted by any conventional means. We found similar levels of specific proteins in samples from both strains and a number of differences in the *F. tularensis* OMV/NT which may account for the increased pathogenicity of this strain.

II. Results

F. tularensis produces NT in addition to OMV

We first examined whole bacteria of *F. tularensis* Schu S4 to determine if NT were created under the same conditions as observed in *F. novicida.* We compared colonies grown on Mueller Hinton Chocolate (MHC) and BHI agar plates for production of NT by TEM. We readily observed free floating and attached NT in the samples from the BHI plates and saw no signs of NT from the MHC plate samples (Fig. 5-1). This is consistent with what was observed under the same conditions with *F. novicida*.

F. tularensis OMV and NT

We next attempted to isolate OMV/NT from *F. tularensis* cultures grown in BHI medium. In order to accomplish this, we again needed to determine the ideal growth conditions necessary to minimize cell death and maximize OMV/NT yields. Due to differences in growth between *F. novicida* and *F. tularensis*, we found that it was necessary to grow *F. tularensis* cultures much longer in order to reach similar levels of optical density. To reach OD_{600} ~1.0 required approximately 18-24 hours of growth, in comparison to the 4-5 hours required for *F. novicida* to reach similar levels. To reliably obtain an OMV/NT pellet from *F. tularensis*, a culture diluted to OD_{600} ~0.01 was grown for ~70 hours before generation of cell free supernatant. Lack of cell death at this time point suggests that the bacteria are still in stationary phase. Even at this time point a minimal vesicle pellet was obtained, though large enough for further purification through a discontinuous OptiPrep gradient. Similar to *F. novicida* samples, the pellets obtained

from *F. tularensis* cell free supernatants contained both OMV and NT, as observed in TEM analysis of density gradient purified pellets (Fig. 5-2).

We used mass spectrometry to identify OMV/NT-associated proteins in the purified Schu S4 vesicle pellets. We performed two independent experiments to isolate OMV/NT from strain Schu S4 and had these samples analyzed by mass spectrometry. *F. tularensis* OMV/NT-associated protein content is distinctly different from that of *F. novicida* OMV/NT-associated proteins. Of the 411 identified proteins from *F. tularensis* samples (Table 5-1), only 182 were previously seen in *F. novicida* OMV/NT samples. The remaining 229 proteins are specific to *F. tularensis* OMV/NT and consist of numerous virulence factors, including 14 of the 17 FPI proteins. Specifically, 37 *F. tularensis* OMV/NT-associated proteins (comprising ~25.6% of the NSAF) were previously shown to be virulence factors in *Francisella* (Table 5-2). In comparison, only 23 OMV/NT-associated proteins (comprising ~16.2% of the NSAF) in *F. novicida* were known virulence factors (Table 3-6). In addition, 91 of the 182 proteins found in both samples have 2 fold or greater altered levels in *F. tularensis* when compared to *F. novicida* (Table 5-3). Taken together, this suggests that the protein content of OMV/NT isolated from *F. tularensis* bacteria contains unique protein content, distinctly different than OMV/NT isolated from *F. novicida.*

F. tularensis OMV/NT-associated protein content shares some similarities with *F. novicida* OMV/NT-associated proteins. Of the 182 proteins found in both samples, 91 of these are found at similar levels in both *F. tularensis* and *F. novicida* (Table 5-4). Most notable amongst these proteins are FipB, FopB, LpnA, Pal and OmpH. These are primarily structural/outer membrane proteins, though the presence of FipB, a known

virulence factor, in OMV/NT from both strains of *Francisella* is interesting. This suggests a secretion of this protein through OMV/NT from multiple strains of *Francisella,* and its enrichment in these structures could be indicative of a larger role for this virulence factor.

IV. Figures

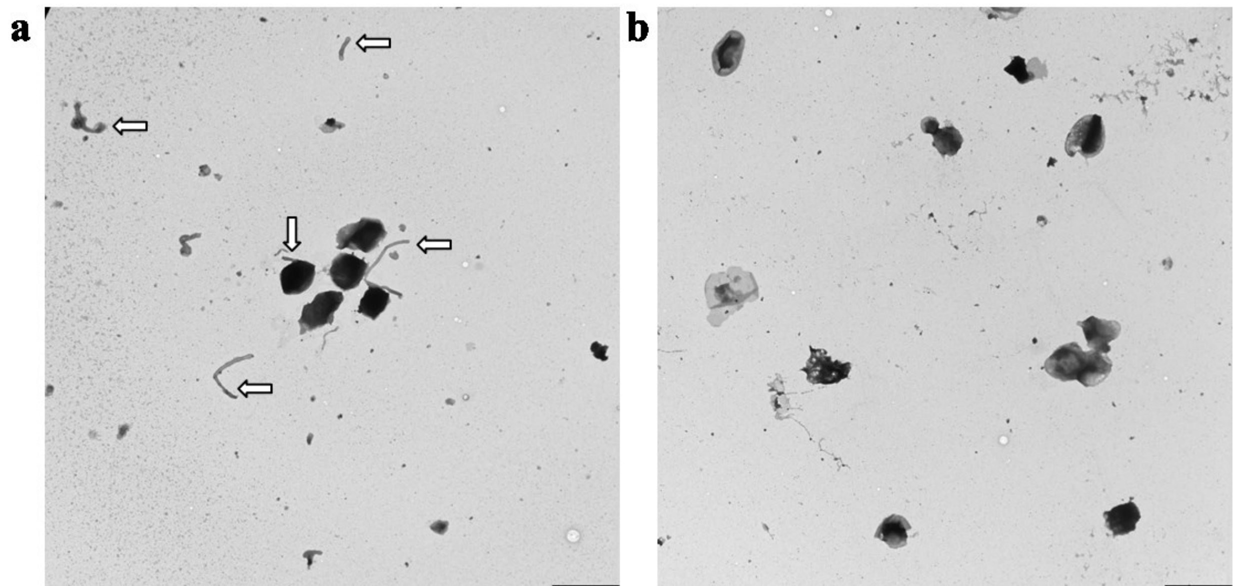


Figure 5-1. TEM Images of *F. tularensis* whole bacteria.

Colonies of *F. tularensis* were grown on BHI plates (a) or MHC plates (b) and processed for TEM analysis. Nanotubes can be seen free floating or attached to whole bacteria (arrows) (black bars = 2 μm).

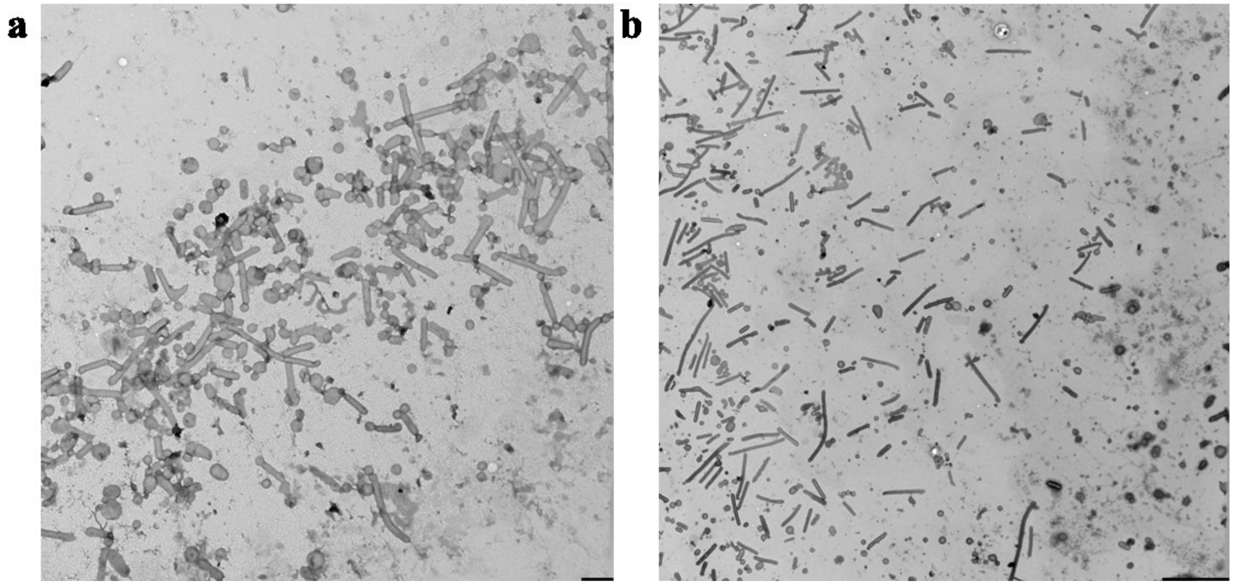


Figure 5-2. TEM Images of *F. tularensis* OMV/NT.

OMV/NT were isolated from BHI grown *F. tularensis* subsp. *tularensis* bacteria and purified by density gradient centrifugation (black bars (a) 500 nm, (b) 2 μm).

IV. Tables

Table 5-1. ***F. tularensis*** **stationary phase OMV/NT-associated proteins.**

Gene	Locus	MW	Description	NSAF
unknown	FTT_1416	15 kDa	lipoprotein	0.059952
lpnA	FTT_0901	16 kDa	lipoprotein	0.047393
katG	FTT_0721	83 kDa	peroxidase/catalase	0.046482
fopB	FTT_1747	21 kDa	outer membrane protein	0.030078
omp26	FTT_1542	20 kDa	hypothetical protein	0.022247
fipB	FTT_1103	39 kDa	lipoprotein	0.021939
unknown	FTT_1043	29 kDa	FKBP-type peptidyl-prolyl cis-trans isomerase family protein	0.021789
unknown	FTT_1539	52 kDa	hypothetical protein	0.01908
unknown	FTT_0369	40 kDa	hypothetical protein	0.01851
unknown	FTT_1778	14 kDa	hypothetical protein	0.018464
ompH	FTT_1572	19 kDa	outer membrane protein OmpH	0.017608
unknown	FTT_0842	23 kDa	peptidoglycan-associated lipoprotein	0.016129
dacD	FTT_1029	48 kDa	D-alanyl-D-alanine carboxypeptidase (penicillin binding protein) family protein	0.014394
dotU	FTT_1351	25 kDa	hypothetical protein	0.013285
unknown	FTT_0991	21 kDa	lipoprotein	0.013274
unknown	FTT_0831	47 kDa	OmpA family protein	0.011252
yajC	FTT_1116	13 kDa	preprotein translocase family protein	0.0108
unknown	FTT_1540	22 kDa	hypothetical protein	0.010643
iglE	FTT_1346	15 kDa	hypothetical protein	0.009839
fopA	FTT_0583	41 kDa	outer membrane associated protein	0.009494
unknown	FTT_0611	32 kDa	beta-lactamase	0.00919
unknown	FTT_1676	37 kDa	hypothetical protein	0.009074
atpD	FTT_0064	50 kDa	F0F1 ATP synthase subunit beta	0.008714
pdpB	FTT_1345	128 kDa	hypothetical protein	0.008536
unknown	FTT_1777	15 kDa	hypothetical protein	0.007939
unknown	FTT_1653	15 kDa	hypothetical protein	0.007495
unknown	FTT_1092	17 kDa	hypothetical protein	0.007434
unknown	FTT_1651	23 kDa	hypothetical protein	0.007167
unknown	FTT_0613	16 kDa	hypothetical protein	0.006869
tolB	FTT_0840	49 kDa	TolB protein precursor	0.006662
unknown	FTT_0101	38 kDa	hypothetical protein	0.006591
unknown	FTT_0704	21 kDa	hypothetical protein	0.006413
sdhA	FTT_0074	66 kDa	succinate dehydrogenase	0.006232
unknown	FTT_1334	18 kDa	hypothetical protein	0.005529

unknown	FTT_1402	60 kDa	hypothetical protein	0.005509
unknown	FTT_0890	13 kDa	Type IV pili fiber building block protein	0.00544
unknown	FTT_0726	39 kDa	glycerophosphoryl diester phosphodiesterase family protein	0.005234
unknown	FTT_0209	34 kDa	periplasmic solute binding family protein	0.005148
unknown	FTT_0903	19 kDa	hypothetical protein	0.005145
acpA	FTT_0221	58 kDa	acid phosphatase (precursor)	0.005106
unknown	FTT_0628	55 kDa	hypothetical protein	0.005021
groES	FTT_1695	10 kDa	co-chaperonin GroES	0.004831
unknown	FTT_0505	70 kDa	hypothetical protein	0.004783
unknown	FTT_0165	50 kDa	lipoprotein	0.004772
unknown	FTT_0825	12 kDa	hypothetical protein	0.004737
sodC	FTT_0879	20 kDa	superoxide dismuate (Cu-Zn) precusor	0.004716
unknown	FTT_0474	25 kDa	hypothetical protein	0.0046
unknown	FTT_1045	16 kDa	hypothetical protein	0.004532
fopC	FTT_0918	59 kDa	hypothetical protein	0.004515
msrB	FTT_0878	20 kDa	methionine sulfoxide reductase B	0.004477
acnA	FTT_0087	103 kDa	aconitate hydratase	0.004472
pdpE	FTT_1355	22 kDa	hypothetical protein	0.004277
unknown	FTT_1538	36 kDa	hypothetical protein	0.00427
accA	FTT_1498	35 kDa	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	0.004006
plsX	FTT_1372	38 kDa	putative glycerol-3-phosphate acyltransferase PlsX	0.003996
yfiO	FTT_1244	32 kDa	lipoprotein	0.003867
rplJ	FTT_0142	19 kDa	50S ribosomal protein L10	0.003783
unknown	FTT_1639	21 kDa	hypothetical protein	0.003781
unknown	FTT_0816	33 kDa	chitin binding protein	0.003686
lolA	FTT_1636	23 kDa	lipoprotein releasing system	0.003654
unknown	FTT_0237	17 kDa	hypothetical protein	0.003641
cyoA	FTT_0281	35 kDa	cytochrome O ubiquinol oxidase subunit II	0.003609
unknown	FTT_1567	22 kDa	hypothetical protein	0.003454
unknown	FTT_1407	40 kDa	hypothetical protein	0.003416
unknown	FTT_0902	18 kDa	hypothetical protein	0.0034
unknown	FTT_1419	12 kDa	lipoprotein	0.003396
metN	FTT_1124	40 kDa	D-methionine transport protein	0.003364
dacB	FTT_1039	51 kDa	D-alanyl-D-alanine carboxypeptidase (penicillin binding protein) family protein	0.00332
unknown	FTT_0484	26 kDa	hypothetical protein	0.003314
slt	FTT_0400	77 kDa	soluble lytic murein transglycosylase	0.003208
mltA	FTT_1271	45 kDa	membrane-bound lytic murein transglycosylase A (MLT) family protein	0.003145
unknown	FTT_1794	17 kDa	heat shock protein	0.003031
wbtA	FTT_1464	66 kDa	dTDP-glucose 4	0.002985

unknown	FTT_1525	33 kDa	hypothetical protein	0.002902
tolC	FTT_1724	57 kDa	outer membrane protein tolC precursor	0.002886
glpe	FTT_1748	16 kDa	thiosulfate sulfurtransferase	0.002886
iglC	FTT_1357	22 kDa	intracellular growth locus	0.002879
unknown	FTT_0507	28 kDa	lipoprotein	0.002814
atpF	FTT_0060	17 kDa	F0F1 ATP synthase subunit B	0.002779
sohB	FTT_0459	38 kDa	putative periplasmic protease	0.002777
lpnB	FTT_0904	17 kDa	lipoprotein	0.002639
unknown	FTT_1258	54 kDa	outer membrane efflux protein	0.002638
unknown	FTT_1109	37 kDa	choloylglycine hydrolase family protein	0.002625
unknown	FTT_0807	44 kDa	hypothetical protein	0.00261
yfdH	FTT_0454	36 kDa	glycosyl transferase	0.002549
unknown	FTT_1137	10 kDa	hypothetical protein	0.00252
unknown	FTT_1493	15 kDa	hypothetical protein	0.002454
sdhB	FTT_0075	27 kDa	succinate dehydrogenase iron-sulfur subunit	0.002438
groEL	FTT_1696	57 kDa	chaperonin GroEL	0.002409
wbtB	FTT_1463	23 kDa	galactosyl transferase	0.00238
unknown	FTT_0166	24 kDa	hypothetical protein	0.002365
unknown	FTT_0989	73 kDa	hypothetical protein	0.002298
pcp	FTT_0296	25 kDa	pyrrolidone-carboxylate peptidase	0.002281
unknown	FTT_0261	15 kDa	hypothetical protein	0.002279
secD	FTT_1115	70 kDa	preprotein translocase subunit SecD	0.002235
unknown	FTT_0106	50 kDa	RND efflux transporter	0.002207
unknown	FTT_0924	15 kDa	hypothetical protein	0.002142
unknown	FTT_1040	23 kDa	lipoprotein	0.00214
olmA	FTT_1680	13 kDa	outer membrane lipoprotein	0.002111
valA	FTT_0109	67 kDa	Lipid A transport protein	0.0021
atpH	FTT_0061	19 kDa	F0F1 ATP synthase subunit delta	0.002075
unknown	FTT_1507	23 kDa	hypothetical protein	0.00204
unknown	FTT_0485	25 kDa	hypothetical protein	0.001981
gtrB	FTT_1433	36 kDa	glycosyl transferase	0.00198
unknown	FTT_1206	15 kDa	hypothetical protein	0.001976
unknown	FTT_0956	28 kDa	hypothetical protein	0.001957
rpsE	FTT_0342	18 kDa	30S ribosomal protein S5	0.001945
unknown	FTT_0360	31 kDa	Short-chain dehydrogenase/reductase	0.001939
emrA1	FTT_1257	38 kDa	HlyD family secretion protein	0.001929
unknown	FTT_0732	13 kDa	hypothetical protein	0.001918
lepB	FTT_1556	33 kDa	signal peptidase I	0.001917
msrA2	FTT_1797	27 kDa	peptide methionine sulfoxide reductase msrA	0.001903
unknown	FTT_0385	36 kDa	hypothetical protein	0.001903
metIQ	FTT_1125	53 kDa	D-methionine binding transport protein	0.001896

unknown	FTT_1746	34 kDa	peptidase	0.001872
unknown	FTT_1650	21 kDa	chorismate mutase	0.001865
unknown	FTT_0083	17 kDa	hypothetical protein	0.001845
tolR	FTT_0838	16 kDa	TolR protein	0.001842
unknown	FTT_0018	40 kDa	secretion protein	0.001827
unknown	FTT_1406	44 kDa	hypothetical protein	0.00178
unknown	FTT_0308	37 kDa	hypothetical protein	0.001779
unknown	FTT_1611	26 kDa	hypothetical protein	0.001778
tufA	FTT_0137	43 kDa	elongation factor Tu	0.001736
unknown	FTT_0066	105 kDa	hypothetical protein	0.001695
hflK	FTT_0633	40 kDa	SPFH domain-containing protein/band 7 family protein	0.001676
unknown	FTT_1303	33 kDa	hypothetical protein	0.001659
fadE	FTT_1529	84 kDa	acyl-CoA dehydrogenase	0.001637
cydA	FTT_0279	65 kDa	cytochrome d terminal oxidase	0.001624
hflB	FTT_1310	70 kDa	ATP-dependent metalloprotease	0.001609
unknown	FTT_1573	88 kDa	outer membrane protein	0.001604
unknown	FTT_0768	39 kDa	hypothetical protein	0.001585
unknown	FTT_1113	20 kDa	hypothetical protein	0.00157
unknown	FTT_0863	22 kDa	LemA-like protein	0.00156
unknown	FTT_0610	41 kDa	DNA/RNA endonuclease family protein	0.001542
unknown	FTT_0119	49 kDa	hypothetical protein	0.001526
rplQ	FTT_0351	17 kDa	50S ribosomal protein L17	0.001488
unknown	FTT_0835	30 kDa	CDP-alcohol phosphatidyltransferase	0.001478
minD	FTT_1606	30 kDa	septum site-determining protein MinD	0.001471
unknown	FTT_1158	22 kDa	Type IV pili glycosylation protein	0.00143
unknown	FTT_0550	54 kDa	hypothetical protein	0.001421
wbtD	FTT_1461	42 kDa	galacturonosyl transferase	0.001411
unknown	FTT_0609	69 kDa	peptidase	0.001372
unknown	FTT_0181	21 kDa	hypothetical protein	0.001366
yhbG	FTT_1024	27 kDa	ABC transporter	0.001363
emrA2	FTT_1654	37 kDa	HlyD family secretion protein	0.001359
unknown	FTT_0540	56 kDa	hypothetical protein	0.001351
unknown	FTT_0742	73 kDa	lipoprotein	0.001334
glpT	FTT_0725	48 kDa	glycerol-3-phosphate transporter	0.001316
unknown	FTT_1591	42 kDa	lipoprotein	0.001305
atpA	FTT_0062	55 kDa	F0F1 ATP synthase subunit alpha	0.001299
lpcC	FTT_1235	41 kDa	glycosyl transferase group 1 family protein	0.00129
ftsI	FTT_0697	63 kDa	penicillin binding protein (peptidoglycan synthetase)	0.001286
hfq	FTT_0630	13 kDa	host factor I for bacteriophage Q beta replication	0.001265
unknown	FTT_0128	22 kDa	hypothetical protein	0.00126
unknown	FTT_0211	16 kDa	outer membrane lipoprotein	0.001255

unknown	FTT_0756	43 kDa	cation-efflux family protein	0.001252
unknown	FTT_1097	17 kDa	hypothetical protein	0.001246
atpG	FTT_0063	33 kDa	F0F1 ATP synthase subunit gamma	0.001244
unknown	FTT_1249	27 kDa	cell entry (mce) related family protein	0.001238
pilQ	FTT_1156	65 kDa	Type IV pilin multimeric outer membrane protein	0.001237
blc	FTT_0198	18 kDa	outer membrane lipoprotein	0.001232
unknown	FTT_1025	32 kDa	hypothetical protein	0.001229
unknown	FTT_1153	8 kDa	hypothetical protein	0.001228
accD	FTT_0372	34 kDa	Acetyl-CoA carboxylase beta subunit	0.001228
atpC	FTT_0065	16 kDa	F0F1 ATP synthase subunit epsilon	0.001218
unknown	FTT_1506	22 kDa	hypothetical protein	0.001218
unknown	FTT_0105	113 kDa	AcrB/AcrD/AcrF family transporter	0.001206
unknown	FTT_0913	18 kDa	hypothetical protein	0.001202
unknown	FTT_0557	20 kDa	AhpC/TSA family protein	0.001198
unknown	FTT_0014	15 kDa	hypothetical protein	0.001167
pdpC	FTT_1354	156 kDa	hypothetical protein	0.001114
lolB	FTT_0270	24 kDa	lipoprotein releasing system	0.001108
ssb	FTT_1752	18 kDa	single-strand binding protein	0.001106
unknown	FTT_1157	23 kDa	Type IV pili lipoprotein.	0.001103
unknown	FTT_1246	33 kDa	hypothetical protein	0.0011
ftsK	FTT_1635	92 kDa	cell division protein	0.001096
unknown	FTT_0289	16 kDa	hypothetical protein	0.001067
unknown	FTT_1763	29 kDa	acetyltransferase protein	0.001064
unknown	FTT_1015	22 kDa	hypothetical protein	0.001056
unknown	FTT_1170	67 kDa	lipoprotein	0.001053
unknown	FTT_1057	35 kDa	Type IV pili lipoprotein	0.001028
rplE	FTT_0337	20 kDa	50S ribosomal protein L5	0.001023
pdpD	FTT_1360	135 kDa	hypothetical protein	0.001013
unknown	FTT_0017	59 kDa	hypothetical protein	0.00101
potG	FTT_0562	42 kDa	polyamine transporter	0.000998
glpA	FTT_0132	58 kDa	anaerobic glycerol-3-phosphate dehydrogenase	0.000996
fabG	FTT_1375	26 kDa	3-oxoacyl-(acyl-carrier-protein) reductase	0.00099
rpsC	FTT_0331	25 kDa	30S ribosomal protein S3	0.000978
tolQ	FTT_0837	27 kDa	TolQ protein	0.000976
yidC	FTT_0233	62 kDa	inner-membrane protein	0.000976
rpsH	FTT_0339	14 kDa	30S ribosomal protein S8	0.000971
feoB	FTT_0249	81 kDa	ferrous iron transport protein	0.000963
kdtA	FTT_1561	50 kDa	3-deoxy-D-manno-octulosonic-acid transferase	0.000961
ggt	FTT_1181	65 kDa	gamma-glutamyltranspeptidase	0.00096
unknown	FTT_1392	28 kDa	pantothenate kinase	0.00095
rplB	FTT_0328	30 kDa	50S ribosomal protein L2	0.000938

potF	FTT_0481	45 kDa	putrescine-binding periplasmic protein	0.0009
unknown	FTT_0749	36 kDa	hypothetical protein	0.000884
unknown	FTT_0399	43 kDa	BNR/Asp-box repeat-containing protein	0.000884
htrB	FTT_0231	36 kDa	acyltransferase	0.000882
unknown	FTT_0067	12 kDa	glutaredoxin-like protein	0.000876
accB	FTT_0472	16 kDa	Acetyl-CoA carboxylase	0.000872
lpcA	FTT_1681	21 kDa	phosphoheptose isomerase	0.000862
unknown	FTT_1234	43 kDa	choloylglycine hydrolase family protein	0.000862
hflC	FTT_0634	35 kDa	SPFH domain-containing protein/band 7 family protein	0.000861
fabI	FTT_0782	28 kDa	enoyl-[acyl-carrier-protein] reductase (NADH)	0.000853
qseC	FTT_0094	55 kDa	sensor histidine kinase	0.000847
secF	FTT_1114	34 kDa	preprotein translocase subunit SecF	0.000846
ampG	FTT_0070	47 kDa	major facilitator superfamily tranporter	0.000846
cydD	FTT_1335	66 kDa	cysteine/glutathione ABC transporter membrane/ATP-binding component	0.000841
unknown	FTT_1248	29 kDa	ABC transporter	0.000835
unknown	FTT_1537	52 kDa	hypothetical protein	0.000835
sdhC	FTT_0072	17 kDa	succinate dehydrogenase	0.000829
lpd	FTT_1483	50 kDa	dihydrolipoamide dehydrogenase	0.00082
rplF	FTT_0340	19 kDa	50S ribosomal protein L6	0.000814
unknown	FTT_1242	46 kDa	hypothetical protein	0.000809
unknown	FTT_0354	39 kDa	hypothetical protein	0.000807
kdpD	FTT_1736	101 kDa	two component sensor protein kdpD	0.000805
nuoI	FTT_0039	19 kDa	NADH dehydrogenase subunit I	0.000803
lpxB	FTT_1568	43 kDa	Lipid-A-disaccharide synthase	0.000802
sdhD	FTT_0073	14 kDa	succinate dehydrogenase hydrophobic membrane anchor protein	0.000798
nuoA	FTT_0031	15 kDa	NADH dehydrogenase I	0.000797
iglB	FTT_1358	59 kDa	intracellular growth locus	0.000789
unknown	FTT_1064	22 kDa	hypothetical protein	0.000788
rplL	FTT_0143	13 kDa	50S ribosomal protein L7/L12	0.000786
ubiB	FTT_1298	64 kDa	2-polyprenylphenol 6-hydroxylase	0.000773
unknown	FTT_0747	30 kDa	hypothetical protein	0.000772
putA	FTT_1150	150 kDa	bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	0.000772
unknown	FTT_1247	40 kDa	ABC transporter	0.000767
gapA	FTT_1368	37 kDa	glyceraldehyde-3-phosphate dehydrogenase	0.000765
unknown	FTT_0954	11 kDa	hypothetical protein	0.000757
nuoC	FTT_0033	25 kDa	NADH dehydrogenase I	0.000747
unknown	FTT_0715	83 kDa	chitinase family 18 protein	0.000743
dnaK	FTT_1269	69 kDa	heat shock protein DnaK	0.000728
nuoG	FTT_0037	87 kDa	NADH dehydrogenase subunit G	0.000719

unknown	FTT_1122	17 kDa	lipoprotein	0.000718
unknown	FTT_0910	33 kDa	hypothetical protein	0.000713
rne	FTT_1227	101 kDa	ribonuclease E	0.000706
ostA1	FTT_0467	98 kDa	organic solvent tolerance protein	0.000706
unknown	FTT_0364	17 kDa	hypothetical protein	0.000699
secY	FTT_0345	48 kDa	preprotein translocase subunit SecY	0.000694
unknown	FTT_0839	35 kDa	hypothetical protein	0.000691
pheA	FTT_0575	32 kDa	prephenate dehydratase	0.00069
ffh	FTT_0964	50 kDa	signal recognition particle protein	0.00068
cydC	FTT_1336	62 kDa	cysteine/glutathione ABC transporter membrane/ATP-binding component	0.000678
wbtK	FTT_1452	33 kDa	glycosyltransferase	0.000677
rplT	FTT_0820	13 kDa	50S ribosomal protein L20	0.000676
lolC	FTT_0404	46 kDa	lipoprotein releasing system	0.000673
rpsK	FTT_0348	14 kDa	30S ribosomal protein S11	0.000672
ygiH	FTT_1123	22 kDa	hypothetical protein	0.000669
unknown	FTT_0558	22 kDa	short chain dehydrogenase	0.000668
unknown	FTT_0793	64 kDa	ABC transporter	0.000662
ftsZ	FTT_0188	40 kDa	cell division protein FtsZ	0.000654
unknown	FTT_1496	38 kDa	hypothetical protein	0.000643
unknown	FTT_0708	46 kDa	major facilitator transporter	0.000643
nuoD	FTT_0034	48 kDa	NADH dehydrogenase subunit D	0.000638
unknown	FTT_1602	12 kDa	hypothetical protein	0.000635
mdh	FTT_0535	34 kDa	lactate dehydrogenase	0.000635
unknown	FTT_0555	28 kDa	hypothetical protein	0.000622
unknown	FTT_0243	29 kDa	hypothetical protein	0.000621
unknown	FTT_1404	27 kDa	cell division protein	0.00062
capB	FTT_0805	45 kDa	capsule biosynthesis protein capB	0.000618
pdpA	FTT_1344	95 kDa	hypothetical protein	0.000608
unknown	FTT_1666	34 kDa	3-hydroxyisobutyrate dehydrogenase	0.0006
ansB	FTT_0464	38 kDa	periplasmic L-asparaginase II precursor	0.000597
unknown	FTT_1159	22 kDa	Type IV pili associated protein	0.000597
surA	FTT_0468	54 kDa	peptidyl-prolyl cis-trans isomerase (PPIase)	0.000596
aceF	FTT_1484	67 kDa	dihydrolipoamide acetyltransferase	0.000594
fabF	FTT_1377	44 kDa	3-oxoacyl-[acyl-carrier-protein] synthase II	0.000593
usp	FTT_0245	30 kDa	universal stress protein	0.000576
unknown	FTT_0694	79 kDa	hypothetical protein	0.000575
unknown	FTT_0748	26 kDa	hypothetical protein	0.000573
gshB	FTT_0926	37 kDa	glutathione synthetase	0.000571
rpsB	FTT_0313	26 kDa	30S ribosomal protein S2	0.000567
lpxA	FTT_1569	28 kDa	UDP-N-acetylglucosamine acyltransferase	0.000559
rpsI	FTT_1274	15 kDa	30S ribosomal protein S9	0.000552

iglD	FTT_1356	47 kDa	intracellular growth locus	0.000541
unknown	FTT_1319	41 kDa	permease YjgP/YjgQ family protein	0.000534
iglI	FTT_1352	45 kDa	hypothetical protein	0.000527
hemH	FTT_1138	39 kDa	ferrochelatase	0.000526
rplP	FTT_0332	16 kDa	50S ribosomal protein L16	0.000525
unknown	FTT_0293	38 kDa	hypothetical protein	0.000524
cyoB	FTT_0282	76 kDa	cytochrome O ubiquinol oxidase subunit I	0.000523
rplA	FTT_0141	24 kDa	50S ribosomal protein L1	0.000521
fur	FTT_0030	16 kDa	ferric uptake regulation protein	0.000521
unknown	FTT_1250	27 kDa	hypothetical protein	0.000515
unknown	FTT_0291	34 kDa	hypothetical protein	0.000508
unknown	FTT_0295	61 kDa	hypothetical protein	0.000504
fbaB	FTT_1365	38 kDa	fructose-1	0.000503
unknown	FTT_0208	26 kDa	ABC transporter	0.0005
unknown	FTT_1251	26 kDa	hypothetical protein	0.000491
lgt	FTT_1228	31 kDa	prolipoprotein diacylglyceryl transferase	0.000482
lldD	FTT_0303	43 kDa	L-lactate dehydrogenase	0.000482
unknown	FTT_0682	32 kDa	hypothetical protein	0.000455
unknown	FTT_0511	31 kDa	pyridoxine biosynthesis protein	0.00045
unknown	FTT_1127	28 kDa	rhodanese-like family protein	0.000448
unknown	FTT_1185	16 kDa	hypothetical protein	0.000448
unknown	FTT_0512	20 kDa	glutamine amidotransferase subunit PdxT	0.000441
unknown	FTT_0455	69 kDa	dolichyl-phosphate-mannose-protein mannosyltransferase family protein	0.000441
sucD	FTT_0503	30 kDa	succinyl-CoA synthetase	0.00044
unknown	FTT_1022	69 kDa	hypothetical protein	0.000439
clpP	FTT_0624	22 kDa	ATP-dependent Clp protease subunit P	0.000438
galP2	FTT_1473	51 kDa	major facilitator superfamily galactose-proton symporter	0.000434
gdh	FTT_0380	49 kDa	glutamate dehydrogenase	0.00043
htpX	FTT_0862	41 kDa	heat shock protein HtpX	0.000424
nuoH	FTT_0038	38 kDa	NADH dehydrogenase I	0.000413
unknown	FTT_1198	32 kDa	hypothetical protein	0.000408
eno	FTT_0709	50 kDa	enolase (2-phosphoglycerate dehydratase)	0.000408
aceE	FTT_1485	100 kDa	pyruvate dehydrogenase subunit E1	0.000405
unknown	FTT_0490	46 kDa	phospholipase D family protein	0.0004
psd	FTT_0384	32 kDa	phosphatidylserine decarboxylase proenzyme	0.000394
unknown	FTT_1762	28 kDa	acetyltransferase protein	0.000394
trpE	FTT_1802	58 kDa	anthranilate synthase component I	0.000393
fadD2	FTT_1528	63 kDa	long chain fatty acid CoA ligase	0.000387
unknown	FTT_1557	26 kDa	two-component response regulator	0.000384
sodB	FTT_0068	22 kDa	superoxide dismutase (Fe)	0.00038
unknown	FTT_0968	53 kDa	amino acid antiporter	0.000378

wbtI	FTT_1455	41 kDa	sugar transamine/perosamine synthetase	0.000378
nuoF	FTT_0036	46 kDa	NADH dehydrogenase I	0.000376
lpxH	FTT_0436	28 kDa	UDP-2	0.000372
unknown	FTT_1621	32 kDa	hypothetical protein	0.00037
ppx	FTT_1444	35 kDa	exopolyphosphatase	0.000366
topA	FTT_0906	87 kDa	DNA topoisomerase I	0.00036
accC	FTT_0473	50 kDa	Acetyl-CoA carboxylase	0.000356
sucC	FTT_0504	42 kDa	succinyl-CoA synthetase subunit beta	0.000353
unknown	FTT_0546	23 kDa	hypothetical protein	0.000352
unknown	FTT_1016	23 kDa	GDSL-like lipase/acylhydrolase family protein	0.000346
rplC	FTT_0325	22 kDa	50S ribosomal protein L3	0.000343
dedA1	FTT_1223	24 kDa	DedA family protein	0.000332
tsf	FTT_0314	31 kDa	elongation factor Ts	0.000332
moxR	FTT_0290	36 kDa	methanol dehydrogenase regulatory protein	0.000329
unknown	FTT_0888	22 kDa	Type IV pili fiber building block protein	0.000324
unknown	FTT_1495	74 kDa	hypothetical protein	0.000319
unknown	FTT_1374	34 kDa	malonyl CoA-acyl carrier protein transacylase	0.000317
tet	FTT_0444	45 kDa	multidrug transporter (tetracycline resistance protein)	0.000313
rpsD	FTT_0349	23 kDa	30S ribosomal protein S4	0.000309
suhB	FTT_1382	29 kDa	inositol-1-monophosphatase	0.000308
wbtC	FTT_1462	30 kDa	UDP-glucose 4-epimerase	0.000305
iglH	FTT_1350	55 kDa	hypothetical protein	0.000304
unknown	FTT_0443	40 kDa	hypothetical protein	0.000298
unknown	FTT_1302	32 kDa	hypothetical protein	0.000292
pgk	FTT_1367	42 kDa	phosphogylcerate kinase	0.000287
pepA	FTT_1318	52 kDa	cytosol aminopeptidase	0.000283
gpmI	FTT_1329	58 kDa	phosphoglyceromutase	0.000283
lolD	FTT_0405	26 kDa	lipoprotein releasing system	0.00028
unknown	FTT_1349	18 kDa	hypothetical protein	0.000277
unknown	FTT_0981	44 kDa	hypothetical protein	0.000277
unknown	FTT_0256	39 kDa	lipopolysaccharide protein	0.000274
unknown	FTT_0676	47 kDa	hypothetical protein	0.000271
unknown	FTT_0266	50 kDa	ABC transporter	0.000261
rpiA	FTT_1208	24 kDa	ribose-5-phosphate isomerase A	0.000251
unknown	FTT_0598	46 kDa	Sodium-dicarboxylate symporter family protein	0.000249
glyA	FTT_1241	45 kDa	serine hydroxymethyltransferase	0.000249
ddg	FTT_0232	35 kDa	acyltransferase	0.000242
clpB	FTT_1769	96 kDa	ClpB protein	0.000242
msc	FTT_0475	42 kDa	mechanosensitive ion channel protein	0.000241
lpxD	FTT_1571	35 kDa	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase	0.00024
dgt	FTT_0720	50 kDa	deoxyguanosinetriphosphate triphosphohydrolase	0.000234

nusA	FTT_0049	55 kDa	transcription elongation factor NusA	0.000232
secA	FTT_0769	104 kDa	preprotein translocase subunit SecA	0.000231
metK	FTT_0149	42 kDa	S-adenosylmethionine synthetase	0.000227
gltA	FTT_0071	47 kDa	citrate synthase	0.000226
unknown	FTT_1589	29 kDa	hypothetical protein	0.000224
fabH	FTT_1373	35 kDa	3-oxoacyl-[acyl carrier protein] synthase III	0.00022
unknown	FTT_1730	69 kDa	amino acid transporter	0.000219
blaA	FTT_0681	33 kDa	Beta-lactamase class A	0.000218
wbtL	FTT_1451	32 kDa	glucose-1-phosphate thymidylyltransferase	0.000218
dnaJ	FTT_1268	42 kDa	heat shock protein DnaJ	0.000215
unknown	FTT_0614	56 kDa	apolipoprotein N-acyltransferase	0.00021
unknown	FTT_1297	23 kDa	hypothetical protein	0.0002
maeA	FTT_0917	67 kDa	malate dehydrogenase	0.000199
fusA	FTT_0323	78 kDa	elongation factor G	0.000193
trkA	FTT_0969	52 kDa	potassium transporter peripheral membrane component	0.000192
unknown	FTT_0129	47 kDa	major facilitator transporter	0.00019
unknown	FTT_1320	39 kDa	permease YjgP/YjgQ family protein	0.000183
sucB	FTT_0077	53 kDa	dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex	0.000181
unknown	FTT_1342	41 kDa	hypothetical protein	0.00018
lepA	FTT_1678	66 kDa	GTP-binding protein LepA	0.000178
unknown	FTT_0980	47 kDa	hypothetical protein	0.000175
galP1	FTT_1474	51 kDa	major facilitator superfamily galactose-proton symporter	0.000174
unknown	FTT_1629	64 kDa	hypothetical protein	0.000174
valB	FTT_0110	36 kDa	tetraacyldisaccharide 4'-kinase	0.000172
ftsA	FTT_0187	45 kDa	cell division protein FtsA	0.00017
unknown	FTT_0659	54 kDa	DNA recombination protein RmuC family protein	0.00017
dfp	FTT_1147	43 kDa	4'-phosphopantothenoylcysteine decarboxylase	0.000165
wbtH	FTT_1456	72 kDa	asparagine synthase	0.000164
unknown	FTT_0602	55 kDa	hypothetical protein	0.000162
unknown	FTT_1129	63 kDa	hypothetical protein	0.000158
pnp	FTT_0699	75 kDa	polynucleotide phosphorylase/polyadenylase	0.00015
unknown	FTT_1253	55 kDa	proton-dependent oligopeptide transport (POT) family protein	0.000145
unknown	FTT_0148	44 kDa	fatty acid desaturase	0.000145
yajR	FTT_0280	50 kDa	major facilitator transporter	0.000144
unknown	FTT_0361	51 kDa	amino acid transporter	0.000144
yjhB	FTT_1148	45 kDa	major facilitator transporter	0.000142
fadD1	FTT_1254	64 kDa	Acyl-CoA synthetase (long-chain-fatty-acid--CoA ligase)	0.00014
ispG	FTT_0607	44 kDa	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	0.000139
pilC	FTT_1134	45 kDa	Type IV pili polytopic inner membrane protein	0.000139
unknown	FTT_1328	114 kDa	FAD-binding family protein	0.000137

visC	FTT_1217	46 kDa	monooxygenase family protein	0.000136
unknown	FTT_0180	34 kDa	acetyltransferase	0.000134
unknown	FTT_1348	68 kDa	hypothetical protein	0.000127
fadB/acbP	FTT_1530	101 kDa	fusion product of 3-hydroxacyl-CoA dehydrogenase and acyl-CoA-binding protein	0.000125
unknown	FTT_0953	56 kDa	proton-dependent oligopeptide transport (POT) family protein	0.000124
ybhO	FTT_0997	55 kDa	cardiolipin synthetase	0.000119
idh	FTT_1526	83 kDa	isocitrate dehydrogenase	0.000109
unknown	FTT_0265	67 kDa	ABC transporter	0.000106
htpG	FTT_0356	72 kDa	heat shock protein 90	0.000101
unknown	FTT_0268	69 kDa	Sodium/hydrogen exchanger (antiporter) family protein	0.000101
pyk	FTT_1366	52 kDa	pyruvate kinase	0.0001
rnr	FTT_1553	88 kDa	ribonuclease R	8.26E-05
cphA	FTT_1130	104 kDa	cyanophycin synthetase	6.82E-05

Table 5-2. *F. tularensis* OMV/NT-associated Proteins Identified Previously as Virulence Factors.

Gene	Locus	Description	References
unknown	FTT_1416	lipoprotein	(Su, Yang et al. 2007)
lpnA	FTT_0901	lipoprotein	(Su, Yang et al. 2007)
fopB	FTT_1747	outer membrane protein	(Su, Yang et al. 2007; Yu, Goluguri et al. 2010)
fipB	FTT_1103	lipoprotein	(Su, Yang et al. 2007; Qin, Scott et al. 2009)
dotU	FTT_1351	hypothetical protein	(Barker, Chong et al. 2009)
iglE	FTT_1346	hypothetical protein	(Barker, Chong et al. 2009)
fopA	FTT_0583	outer membrane associated protein	(Su, Yang et al. 2007; Yu, Goluguri et al. 2010)
unknown	FTT_1676	hypothetical protein	(Su, Yang et al. 2007)
pdpB	FTT_1345	hypothetical protein	(Barker, Chong et al. 2009)
unknown	FTT_0101	hypothetical protein	(Su, Yang et al. 2007)
fopC	FTT_0918	hypothetical protein	(Su, Yang et al. 2007)
pdpE	FTT_1355	hypothetical protein	(Barker, Chong et al. 2009)
wbtA	FTT_1464	dTDP-glucose 4	(Su, Yang et al. 2007)
unknown	FTT_1525	hypothetical protein	(Su, Yang et al. 2007)
iglC	FTT_1357	intracellular growth locus	(Barker, Chong et al. 2009)
unknown	FTT_0507	lipoprotein	(Yu, Goluguri et al. 2010)
unknown	FTT_0807	hypothetical protein	(Su, Yang et al. 2007)
yfdH	FTT_0454	glycosyl transferase	(Yu, Goluguri et al. 2010)
unknown	FTT_1040	lipoprotein	(Yu, Goluguri et al. 2010)
unknown	FTT_1611	hypothetical protein	(Su, Yang et al. 2007)
unknown	FTT_0742	lipoprotein	(Tempel, Lai et al. 2006)
pdpC	FTT_1354	hypothetical protein	(Barker, Chong et al. 2009)
unknown	FTT_1015	hypothetical protein	(Su, Yang et al. 2007)
pdpD	FTT_1360	hypothetical protein	(Barker, Chong et al. 2009)
lpxB	FTT_1568	Lipid-A-disaccharide synthase	(Su, Yang et al. 2007)
iglB	FTT_1358	intracellular growth locus	(Barker, Chong et al. 2009)

dnaK	FTT_1269	heat shock protein DnaK	(Tempel, Lai et al. 2006)
capB	FTT_0805	capsule biosynthesis protein capB	(Su, Yang et al. 2007)
pdpA	FTT_1344	hypothetical protein	(Barker, Chong et al. 2009)
iglD	FTT_1356	intracellular growth locus	(Barker, Chong et al. 2009)
iglI	FTT_1352	hypothetical protein	(Barker, Chong et al. 2009)
iglH	FTT_1350	hypothetical protein	(Barker, Chong et al. 2009)
unknown	FTT_0443	hypothetical protein	(Su, Yang et al. 2007)
iglG	FTT_1349	hypothetical protein	(Barker, Chong et al. 2009)
unknown	FTT_1589	hypothetical protein	(Yu, Goluguri et al. 2010)
iglF	FTT_1348	hypothetical protein	(Barker, Chong et al. 2009)
htpG	FTT_0356	heat shock protein 90	(Tempel, Lai et al. 2006)

Table 5-3. Differential protein content between *F. tularensis* and *F. novicida* OMV/NT.

Gene	Locus	Description	F.t. NSAF	F.n. NSAF	Fold Change
unknown	FTT_1416	lipoprotein	0.059952	0.003344	17.93
unknown	FTT_0816	chitin binding protein	0.003686	0.000296	12.46
katG	FTT_0721	peroxidase/catalase	0.046482	0.004653	9.99
unknown	FTT_0991	lipoprotein	0.013274	0.001455	9.12
unknown	FTT_1258	outer membrane efflux protein	0.002638	0.000358	7.36
pdpB	FTT_1345	hypothetical protein	0.008536	0.001176	7.26
pdpD	FTT_1360	hypothetical protein	0.001013	0.000148	6.83
unknown	FTT_0507	lipoprotein	0.002814	0.000537	5.24
unknown	FTT_0628	hypothetical protein	0.005021	0.000982	5.12
iglB	FTT_1358	intracellular growth locus	0.000789	0.000164	4.81
unknown	FTT_1540	hypothetical protein	0.010643	0.002348	4.53
mltA	FTT_1271	membrane-bound lytic murein transglycosylase A (MLT) family protein	0.003145	0.00072	4.37
unknown	FTT_1651	hypothetical protein	0.007167	0.001648	4.35
unknown	FTT_0956	hypothetical protein	0.001957	0.000467	4.19
unknown	FTT_0715	chitinase family 18 protein	0.000743	0.000195	3.81
slt	FTT_0400	soluble lytic murein transglycosylase	0.003208	0.000855	3.75
tolB	FTT_0840	TolB protein precursor	0.006662	0.001839	3.62
groEL	FTT_1696	chaperonin GroEL	0.002409	0.000676	3.56
unknown	FTT_1043	FKBP-type peptidyl-prolyl cis-trans isomerase family protein	0.021789	0.006255	3.48
unknown	FTT_0831	OmpA family protein	0.011252	0.003285	3.43
tolC	FTT_1724	outer membrane protein tolC precursor	0.002886	0.000855	3.38
msrA2	FTT_1797	peptide methionine sulfoxide reductase msrA	0.001903	0.000585	3.25
wbtA	FTT_1464	dTDP-glucose 4	0.002985	0.00095	3.14
unknown	FTT_0540	hypothetical protein	0.001351	0.000444	3.04
emrA1	FTT_1257	HlyD family secretion protein	0.001929	0.000677	2.85
unknown	FTT_0611	beta-lactamase	0.00919	0.003255	2.82
hflK	FTT_0633	SPFH domain-containing protein/band 7 family protein	0.001676	0.000614	2.73
pilQ	FTT_1156	Type IV pilin multimeric outer membrane protein	0.001237	0.000473	2.61
cydA	FTT_0279	cytochrome d terminal oxidase	0.001624	0.000656	2.48
fopC	FTT_0918	hypothetical protein	0.004515	0.001828	2.47

dacB	FTT_1039	D-alanyl-D-alanine carboxypeptidase (penicillin binding protein) family protein	0.00332	0.001384	2.40
unknown	FTT_1057	Type IV pili lipoprotein	0.001028	0.000436	2.36
unknown	FTT_0385	hypothetical protein	0.001903	0.000817	2.33
lolA	FTT_1636	lipoprotein releasing system	0.003654	0.001569	2.33
unknown	FTT_0807	hypothetical protein	0.00261	0.001123	2.32
unknown	FTT_1591	lipoprotein	0.001305	0.000564	2.31
ostA1	FTT_0467	organic solvent tolerance protein	0.000706	0.000306	2.30
unknown	FTT_1676	hypothetical protein	0.009074	0.004085	2.22
fopA	FTT_0583	outer membrane associated protein	0.009494	0.004396	2.16
unknown	FTT_0166	hypothetical protein	0.002365	0.00115	2.06
unknown	FTT_0369	hypothetical protein	0.01851	0.009121	2.03
unknown	FTT_0066	hypothetical protein	0.001695	0.000841	2.02
ftsK	FTT_1635	cell division protein	0.001096	0.000546	2.01
unknown	FTT_1650	chorismate mutase	0.001865	0.003733	-2.00
nuoI	FTT_0039	NADH dehydrogenase subunit I	0.000803	0.00162	-2.02
unknown	FTT_0265	ABC transporter	0.000106	0.000215	-2.02
unknown	FTT_1525	hypothetical protein	0.002902	0.005911	-2.04
omp26	FTT_1542	hypothetical protein	0.022247	0.045424	-2.04
atpH	FTT_0061	F0F1 ATP synthase subunit delta	0.002075	0.004378	-2.11
unknown	FTT_0083	hypothetical protein	0.001845	0.003897	-2.11
tolQ	FTT_0837	TolQ protein	0.000976	0.002072	-2.12
unknown	FTT_0742	lipoprotein	0.001334	0.002884	-2.16
nuoG	FTT_0037	NADH dehydrogenase subunit G	0.000719	0.001559	-2.17
accC	FTT_0473	Acetyl-CoA carboxylase	0.000356	0.000785	-2.21
valA	FTT_0109	Lipid A transport protein	0.0021	0.004794	-2.28
rplE	FTT_0337	50S ribosomal protein L5	0.001023	0.002403	-2.35
pcp	FTT_0296	pyrrolidone-carboxylate peptidase	0.002281	0.005836	-2.56
capB	FTT_0805	capsule biosynthesis protein capB	0.000618	0.001593	-2.58
wbtH	FTT_1456	asparagine synthase	0.000164	0.000437	-2.66
maeA	FTT_0917	malate dehydrogenase	0.000199	0.000549	-2.76
atpA	FTT_0062	F0F1 ATP synthase subunit alpha	0.001299	0.003632	-2.80
aceF	FTT_1484	dihydrolipoamide acetyltransferase	0.000594	0.001667	-2.81
rplQ	FTT_0351	50S ribosomal protein L17	0.001488	0.004184	-2.81
unknown	FTT_0682	hypothetical protein	0.000455	0.001281	-2.82
unknown	FTT_0863	LemA-like protein	0.00156	0.0045	-2.88
aceE	FTT_1485	pyruvate dehydrogenase subunit E1	0.000405	0.001254	-3.09
nuoD	FTT_0034	NADH dehydrogenase subunit D	0.000638	0.002023	-3.17
glpe	FTT_1748	thiosulfate sulfurtransferase	0.002886	0.009824	-3.40
atpF	FTT_0060	F0F1 ATP synthase subunit B	0.002779	0.009561	-3.44
cyoB	FTT_0282	cytochrome O ubiquinol oxidase	0.000523	0.001907	-3.65

		subunit I			
nuoF	FTT_0036	NADH dehydrogenase I	0.000376	0.001513	-4.02
lpxA	FTT_1569	UDP-N-acetylglucosamine acyltransferase	0.000559	0.00228	-4.08
rne	FTT_1227	ribonuclease E	0.000706	0.003084	-4.37
secA	FTT_0769	preprotein translocase subunit SecA	0.000231	0.001059	-4.58
unknown	FTT_1762	acetyltransferase protein	0.000394	0.001821	-4.62
ftsA	FTT_0187	cell division protein FtsA	0.00017	0.000859	-5.05
tsf	FTT_0314	elongation factor Ts	0.000332	0.001692	-5.10
rpsC	FTT_0331	30S ribosomal protein S3	0.000978	0.005213	-5.33
rpsB	FTT_0313	30S ribosomal protein S2	0.000567	0.003031	-5.34
usp	FTT_0245	universal stress protein	0.000576	0.003081	-5.35
clpB	FTT_1769	ClpB protein	0.000242	0.0015	-6.19
sucC	FTT_0504	succinyl-CoA synthetase subunit beta	0.000353	0.002403	-6.81
pnp	FTT_0699	polynucleotide phosphorylase/polyadenylase	0.00015	0.001084	-7.24
unknown	FTT_1242	hypothetical protein	0.000809	0.005881	-7.27
rpsD	FTT_0349	30S ribosomal protein S4	0.000309	0.002255	-7.30
rplA	FTT_0141	50S ribosomal protein L1	0.000521	0.003878	-7.44
rplL	FTT_0143	50S ribosomal protein L7/L12	0.000786	0.007409	-9.42
ftsZ	FTT_0188	cell division protein FtsZ	0.000654	0.006269	-9.59
tufA	FTT_0137	elongation factor Tu	0.001736	0.020572	-11.85
fusA	FTT_0323	elongation factor G	0.000193	0.00233	-12.06
htpG	FTT_0356	heat shock protein 90	0.000101	0.002298	-22.68

Table 5-4. Similar protein content between *F. tularensis* and *F. novicida* OMV/NT.

Gene	F.t. Locus	Description	F.t. NSAF	F.n. NSAF	F.n. Locus
lpnA	FTT_0901	lipoprotein	0.047393	0.031957	FTN_0427
fopB	FTT_1747	outer membrane protein	0.030078	0.057833	FTN_0119
fipB	FTT_1103	lipoprotein	0.021939	0.021712	FTN_0771
unknown	FTT_1539	hypothetical protein	0.01908	0.016441	FTN_1448
unknown	FTT_1778	hypothetical protein	0.018464	0.016023	FTN_1734
ompH	FTT_1572	outer membrane protein OmpH	0.017608	0.025742	FTN_1481
pal	FTT_0842	peptidoglycan-associated lipoprotein	0.016129	0.015458	FTN_0357
dacD	FTT_1029	D-alanyl-D-alanine carboxypeptidase (penicillin binding protein) family protein	0.014394	0.008248	FTN_0907
atpD	FTT_0064	F0F1 ATP synthase subunit beta	0.008714	0.007187	FTN_1646
unknown	FTT_1092	hypothetical protein	0.007434	0.005472	FTN_0782
sdhA	FTT_0074	succinate dehydrogenase	0.006232	0.008755	FTN_1637
unknown	FTT_1334	hypothetical protein	0.005529	0.009394	FTN_0643
unknown	FTT_1402	hypothetical protein	0.005509	0.003263	FTN_1367
unknown	FTT_0726	glycerophosphoryl diester phosphodiesterase family protein	0.005234	0.010331	FTN_0637
unknown	FTT_0209	periplasmic solute binding family protein	0.005148	0.007887	FTN_0183
unknown	FTT_0903	hypothetical protein	0.005145	0.003186	FTN_0429
unknown	FTT_0505	hypothetical protein	0.004783	0.002742	FTN_0595
unknown	FTT_0825	hypothetical protein	0.004737	0.008287	FTN_0340
unknown	FTT_0474	hypothetical protein	0.0046	0.005456	FTN_0565
acnA	FTT_0087	aconitate hydratase	0.004472	0.006078	FTN_1623
unknown	FTT_1538	hypothetical protein	0.00427	0.002628	FTN_1447
accA	FTT_1498	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	0.004006	0.003324	FTN_1508
rplJ	FTT_0142	50S ribosomal protein L10	0.003783	0.003143	FTN_1570
unknown	FTT_0237	hypothetical protein	0.003641	0.002932	FTN_0077
cyoA	FTT_0281	cytochrome O ubiquinol oxidase subunit II	0.003609	0.001845	FTN_0195
unknown	FTT_1567	hypothetical protein	0.003454	0.003976	FTN_1476
unknown	FTT_1407	hypothetical protein	0.003416	0.002229	FTN_1372
unknown	FTT_0902	hypothetical protein	0.0034	0.003805	FTN_0428
metN	FTT_1124	D-methionine transport protein	0.003364	0.006424	FTN_1106
unknown	FTT_0484	hypothetical protein	0.003314	0.002698	FTN_0575

iglC	FTT_1357	intracellular growth locus	0.002879	0.004495	FTN_1322
sohB	FTT_0459	putative periplasmic protease	0.002777	0.002112	FTN_0550
unknown	FTT_1109	choloylglycine hydrolase family protein	0.002625	0.002376	FTN_0765
yfdH	FTT_0454	glycosyl transferase	0.002549	0.002757	FTN_0545
sdhB	FTT_0075	succinate dehydrogenase iron-sulfur subunit	0.002438	0.002954	FTN_1636
wbtB	FTT_1463	galactosyl transferase	0.00238	0.001242	FTN_1429
unknown	FTT_0989	hypothetical protein	0.002298	0.002762	FTN_0869
secD	FTT_1115	preprotein translocase subunit SecD	0.002235	0.001243	FTN_1095
unknown	FTT_0106	RND efflux transporter	0.002207	0.002957	FTN_1609
unknown	FTT_0924	hypothetical protein	0.002142	0.001274	FTN_0802
unknown	FTT_1507	hypothetical protein	0.00204	0.00223	FTN_1517
unknown	FTT_0360	Short-chain dehydrogenase/reductase	0.001939	0.001363	FTN_1535
metIQ	FTT_1125	D-methionine binding transport protein	0.001896	0.001807	FTN_1107
unknown	FTT_0018	secretion protein	0.001827	0.001142	FTN_1692
unknown	FTT_0308	hypothetical protein	0.001779	0.000925	FTN_0222
unknown	FTT_1611	hypothetical protein	0.001778	0.003392	FTN_0325
unknown	FTT_1303	hypothetical protein	0.001659	0.001617	FTN_0449
hflB	FTT_1310	ATP-dependent metalloprotease	0.001609	0.002828	FTN_0668
unknown	FTT_1113	hypothetical protein	0.00157	0.003113	FTN_1093
unknown	FTT_0119	hypothetical protein	0.001526	0.002471	FTN_1596
minD	FTT_1606	septum site-determining protein MinD	0.001471	0.002826	FTN_0330
yhbG	FTT_1024	ABC transporter	0.001363	0.001789	FTN_0902
lpcC	FTT_1235	glycosyl transferase group 1 family protein	0.00129	0.000733	FTN_1253
atpG	FTT_0063	F0F1 ATP synthase subunit gamma	0.001244	0.001669	FTN_1647
unknown	FTT_1249	cell entry (mce) related family protein	0.001238	0.000625	FTN_1268
unknown	FTT_1025	hypothetical protein	0.001229	0.000772	FTN_0903
accD	FTT_0372	Acetyl-CoA carboxylase beta subunit	0.001228	0.001378	FTN_0272
atpC	FTT_0065	F0F1 ATP synthase subunit epsilon	0.001218	0.001274	FTN_1645
unknown	FTT_0105	AcrB/AcrD/AcrF family transporter	0.001206	0.001088	FTN_1610
unknown	FTT_0913	hypothetical protein	0.001202	0.001326	FTN_0439
unknown	FTT_0014	hypothetical protein	0.001167	0.000695	FTN_1695
unknown	FTT_1763	acetyltransferase protein	0.001064	0.001821	FTN_1749
unknown	FTT_1015	hypothetical protein	0.001056	0.001154	FTN_0893

unknown	FTT_0017	hypothetical protein	0.00101	0.000725	FTN_1693
potG	FTT_0562	polyamine transporter	0.000998	0.00172	FTN_0739
glpA	FTT_0132	anaerobic glycerol-3-phosphate dehydrogenase	0.000996	0.001428	FTN_1584
yidC	FTT_0233	inner-membrane protein	0.000976	0.000786	FTN_0073
feoB	FTT_0249	ferrous iron transport protein	0.000963	0.000666	FTN_0066
kdtA	FTT_1561	3-deoxy-D-manno-octulosonic-acid transferase	0.000961	0.000637	FTN_1469
ggt	FTT_1181	gamma-glutamyltranspeptidase	0.00096	0.001803	FTN_1159
rplB	FTT_0328	50S ribosomal protein L2	0.000938	0.001514	FTN_0242
fabI	FTT_0782	enoyl-[acyl-carrier-protein] reductase (NADH)	0.000853	0.001652	FTN_1228
qseC	FTT_0094	sensor histidine kinase	0.000847	0.000847	FTN_1617
lpd	FTT_1483	dihydrolipoamide dehydrogenase	0.00082	0.001111	FTN_1492
kdpD	FTT_1736	two component sensor protein kdpD	0.000805	0.000773	FTN_1715
ubiB	FTT_1298	2-polyprenylphenol 6-hydroxylase	0.000773	0.000408	FTN_0459
putA	FTT_1150	bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	0.000772	0.000698	FTN_1131
nuoC	FTT_0033	NADH dehydrogenase I	0.000747	0.000522	FTN_1678
unknown	FTT_0910	hypothetical protein	0.000713	0.001147	FTN_0436
ffh	FTT_0964	signal recognition particle protein	0.00068	0.000961	FTN_0843
lolC	FTT_0404	lipoprotein releasing system	0.000673	0.000482	FTN_0502
unknown	FTT_0708	major facilitator transporter	0.000643	0.00087	FTN_0620
mdh	FTT_0535	lactate dehydrogenase	0.000635	0.000922	FTN_0980
unknown	FTT_1404	cell division protein	0.00062	0.001104	FTN_1369
surA	FTT_0468	peptidyl-prolyl cis-trans isomerase (PPIase)	0.000596	0.000761	FTN_0559
unknown	FTT_0694	hypothetical protein	0.000575	0.000747	FTN_0604
gshB	FTT_0926	glutathione synthetase	0.000571	0.000553	FTN_0804
iglI	FTT_1352	hypothetical protein	0.000527	0.00045	FTN_1317
sucD	FTT_0503	succinyl-CoA synthetase	0.00044	0.000613	FTN_0593
eno	FTT_0709	enolase (2-phosphoglycerate dehydratase)	0.000408	0.000549	FTN_0621
unknown	FTT_0659	DNA recombination protein RmuC family protein	0.00017	0.000215	FTN_1024

Discussion

I. *Francisella novicida* Outer Membrane Vesicle Isolation and Characterization

In this study we report the isolation and characterization of OMV and NT from *F. novicida*. We show that *F. novicida* creates NT when grown in BHI medium and that these structures can be free floating or attached to whole bacteria, are continuous with the periplasmic space (Fig. 3-4) and are distinctly different from similar structures observed in other bacteria. A role for NT in bridging bacteria during biofilm formation and sharing of cytosolic components has been shown in *B. subtilis, S. aureus, E. coli* (Dubey and Ben-Yehuda 2011) and *S.* Typhimurium (Galkina, Romanova et al. 2011). The *Francisella* NT are unique in that they are readily observed in cultures growing in liquid media, a phenomenon not observed for the other organisms in which these structures have been reported. We also show that these NT are produced when bacteria interact with murine bone marrow derived macrophages (Fig. 3-11), evidence that these structures are not simply artifacts of *in vitro* cultivation. We observed similar production of NT in both *F. tularensis* subspecies *tularensis* (Schu S4, Chapter 5) and *F. tularensis* subspecies *holarctica* LVS (Gil, Benach et al. 2004). Biogenesis of these structures is dependent upon the growth environment as evidenced by the observations that the NT production increases in cultures grown in BHI or when grown on solid surfaces (Fig. 3-5). The inability to isolate OMV/NT from cultures grown in TS under the same conditions further supports this conclusion.

F. novicida appears to produce fewer OMV during exponential growth phase than other Gram-negative bacteria. Standard OMV isolation protocols require removal of the

bacteria, through low-speed centrifugation and filtration, followed by high-speed centrifugation of the resulting cell-free supernatant. Our attempts to isolate OMV from cell-free supernatants of exponentially growing *F. novicida* resulted in limited sample pellets. To recover these structures in sufficient quantities, it was necessary to concentrate cell-free supernatants via a tangential flow filtration device. This step is often employed to increase the yield of OMV in isolation protocols, but was a necessity to obtain these structures in *F. novicida* at exponential phase. Growth of cultures to stationary phase (OD_{600} 1.2-1.4) resulted in large quantities of OMV/NT able to be isolated via standard high-speed centrifugation. The exponential phase bacteria produced fewer vesicles compared to the stationary phase cultures; 2 L of exponential phase supernatant yielded ~0.25 mg purified vesicles, whereas 2 L of stationary phase supernatant yielded ~1-2 mg purified vesicles. Thus, there was a 4-to-8 fold increase in vesicle yield despite an only ~2 fold increase in bacterial numbers. This increase in OMV/NT yield at stationary phase may result from an accumulation of structures over the course of the natural growth period. Alternatively, more structures may be produced during the stationary growth phase due to stress, lack of nutrients or differential gene expression.

Isolation of OMV was recently reported in the *Francisella* subspecies *novicida* and *philomiragia*, their proteomic content analyzed by mass spectrometry and their cytotoxicity assessed (Pierson, Matrakas et al. 2011). Despite this, there are distinct differences in our methods and results. Pierson and colleagues determined that the optimal time to isolate OMV was after 44 hours of growth (OD_{600} ~0.7), while in the present study we were successful in isolating OMV/NT as early as 3-4 hours (exponential

phase, OD_{600} ~0.6) utilizing a tangential flow filtration device to concentrate bacteria-free supernatants. Additional samples analyzed in the current study were grown no longer than 9 hours (stationary phase, OD_{600} ~1.3-1.4) when OMV/NT were isolated without concentration of the supernatant. The tube-like structures we observe when cultures are grown in BHI were not reported in the previous study (Pierson, Matrakas et al. 2011) and the protein content was vastly different. Furthermore, the OMV were not subject to additional purification steps after harvesting from the culture medium, increasing the likelihood of contaminating proteins.

Previous studies demonstrated that *in vitro* cultivation of *F. tularensis* results in differential regulation of genes depending on the medium employed. Specifically, gene expression of *F. tularensis* grown in BHI medium more closely resembled that of organisms grown in murine bone marrow derived macrophages (Hazlett, Caldon et al. 2008). This is why we chose to focus on *F. novicida* cultures grown in this medium and why choice of media or growth conditions is so critical to these experiments. The fact that OMV/NT production is increased during growth in BHI medium suggests a role for these structures during infection. We observe numerous changes in the OMV/NT associated proteins after 5-6 hours of growth, with a 300% increase in the number of proteins, a similar increase in the average spectral counts (Exponential: 6150; Stationary: 19144), changes in the predicted localization of proteins associated with these structures (Fig. 3-9), and numerous changes in the spectral abundance of specific proteins (Tables 3-1 and 3-2). Given our observations with our own experiments, it is a distinct possibility that the protein content observed by Pierson and colleagues has changed drastically over the course of 44 hours of growth and was influenced by their choice of

medium. This is not to say that the OMV isolated in the previous study are not valid structures produced by *F. novicida* under the conditions employed by Pierson, et al., but production of these structures is a dynamic process and highly dependent on the growth conditions employed.

Hager and colleagues identified seven proteins secreted by the type IV pilus system in *F. novicida* (Hager, Bolton et al. 2006), four of which we found to be associated with the purified OMV/NT in high abundance: PepO, BglX, ChiA and Fsp53 (Tables 3-1, 3-2 and 3-6). These proteins might associate with the OMV and NT following secretion by the type IV pilus pathway, similar to the secretion of heat-labile enterotoxin in enterotoxigenic *E. coli* (Horstman and Kuehn 2000; Ellis and Kuehn 2010). Alternatively, the proteins might enter the vesicles from the periplasm, prior to their secretion across the OM. Of note, Fsp53, the most abundant protein found at exponential phase, is more than 16-fold decreased in the stationary phase vesicles (Table 3-3). This suggests a role for Fsp53 during exponential growth of *F. novicida*. The function of Fsp53 is unknown; however, a *F. novicida* strain deleted for both Fsp53 and the homologous upstream gene FTN_1260 (which is also present in the exponential phase OMV/NT; Table 3-1) is attenuated for replication in macrophages and virulence in mice (N.P. Mohapatra and J.S. Gunn; personal communication). Other proteins that have been associated with virulence or secretion and are also found in lesser abundance include FTN_0714, BglX and FopA.

There are a number of OMV/NT-associated proteins which have been identified in this study that make further research into these structures important for elucidation of *Francisella* virulence. In other organisms, OMV are generally enriched in a subset of

proteins distinct from those found in the outer membrane or periplasm of the bacteria in question. These include, but are not limited to: toxins, highly abundant periplasmic proteins, virulence factors, secreted proteins and specific outer membrane proteins. Purification through density gradient centrifugation enabled us to remove proteins which may pellet through the ultracentrifugation process or become peripherally associated with these structures through non-specific interactions. The reproducibility of the data obtained through MudPIT analysis, in combination with our protease accessibility assay (Fig. 3-7) and protein profile comparisons (Fig. 3-6), demonstrates that the proteins we are observing are genuinely associated with OMV/NT. These results also indicate that the OMV/NT are capable of protecting cargo proteins from degradation by extravesicular proteinases found in the host cytosol or extracellular milieu. There are several proteins previously identified as secreted or extracellular (Table 3-6) which are found in OMV/NT samples. There are also a number of virulence-associated proteins (Table 3-6) which are apparently being packaged into these structures. There are a large number of proteins which are hypothetical or lack a known function, any one of which may offer a wealth of new information regarding *Francisella* virulence. Given the lack of secreted virulence factors (and functional secretion systems), the number of OMV/NT associated proteins seems to indicate that this is a viable alternative secretion pathway in *F. novicida*.

F. novicida OMV/NT are proinflammatory, similar to OMV observed in other bacteria; however, they have only a minor cytotoxic effect. Cytotoxicity of OMV has been seen for numerous bacteria (Bomberger, Maceachran et al. 2009; Furuta, Tsuda et al. 2009; Prados-Rosales, Baena et al. 2011), but at lower concentrations of vesicles. For *F. novicida,* cytotoxicity was only significantly achieved when using large quantities of

OMV/NT (20 µg), while production of proinflammatory cytokines happened at much lower levels (1 µg). It is difficult to determine the concentration of OMV/NT produced during an infection, and it has previously been shown that different organisms produce more or less OMV (Horstman and Kuehn 2000). Additionally, since protein content appears to be dynamic in nature, these structures could serve numerous roles depending on when they are produced. *Francisella* has been shown to down regulate the immune response of host cells, and OMV/NT could be involved in preventing a normal reaction to the full organism. Alternatively, the OMV/NT could be used to recruit new macrophages to the location of existing bacteria for subsequent infection. The observed production of the proinflammatory chemokine CCL2 would support the latter hypothesis. It is possible that the proinflammatory, but not the cytotoxic, effects of the OMV/NT may be physiologically relevant during infection. Innate immune responses to *Francisella* are primarily mediated by TLR2, with recognition of lipoproteins being a major part of this response (Thakran, Li et al. 2008). The *F. novicida* OMV/NT are enriched in lipoproteins, including the known TLR2 agonists LpnA and FipB (Thakran, Li et al. 2008), and delivery of the lipoproteins to host cells may underlie the inflammatory activity of the vesicles. Although the immunostimulatory component of lipoproteins is heat-resistant (Jones, Sampson et al. 2012), disruption of the OMV/NT by heat treatment resulted a significant decrease in proinflammatory activity, indicating that the vesicles must be intact for greatest potency and arguing against non-specific activation of the BMDM due to components released from the vesicles or from contaminating molecules. An alternative explanation is that a heat-sensitive factor such as a protein may be responsible for the proinflammatory activity of the OMV/NT. A role for the immune-

modulatory activity of the *F. novicida* OMV/N during infection remains to be determined; however, a recent study demonstrated that a TLR2-dependent inflammatory response conferred by membrane vesicles contributes to the virulence of mycobacteria (Prados-Rosales, Baena et al. 2011).

The OMV/NT protect proteins from degradation and must be intact to elicit their full effect upon host cells. The observed cytokine response to OMV/NT incubation with muBMDM is unaffected by pre-incubation of these structures with proteinase K (Fig. 3-10c). Heat treatment effectively disrupts these structures (Fig. 3-8b) and lessens the observed cytokine response by approximately two-thirds (Fig. 3-10c). The remaining observed response to the heat treated OMV/NT could be the result of intact OMV/NT that survive the heat treatment (Fig. 3-8b) or liposome formation incorporating cytokine stimulating proteins. These experiments demonstrate that there is a dose-dependent immune response to OMV/NT (Fig. 3-10b); these structures must be intact to deliver their cargo to host cells, and exposure of host cells to the heat-treated luminal contents is not enough to produce a full response.

There exists no currently licensed vaccine which might deter the use of *F. tularensis* as a bioweapon. Usage of OMV as vaccines has risen in recent years (Kadurugamuwa 2005; Findlow, Taylor et al. 2006; Vipond, Suker et al. 2006; Boutriau, Poolman et al. 2007; Koeberling, Seubert et al. 2008; Kim, Kim et al. 2009; Schild, Nelson et al. 2009; Chen, Osterrieder et al. 2010), and the method for OMV/NT isolation detailed in the current study could greatly aid attempts to create an effective subunit vaccine for use in preventing infection with *F. tularensis*. Mice inoculated with a single dose of OMV/NT were afforded significant protection against challenge with wild type

F. novicida (Fig. 3-11). The combination of OMV and NT may elicit more of a protective immune response than OMV alone. Further study needs to be done to determine the nature of the novel tube-like structures observed in isolated samples; however, their presence suggests an unknown mechanism by which *Francisella* can interact with its environment. It remains to be seen whether the OMV and NT can be separated or if their properties are such that they will only be found together under the majority of circumstances.

II. *F. novicida* Nanotube Characterization

In this study we attempted to gain further insight into the regulation of *Francisella* NT. These novel structures are produced by *F. novicida* in a regulated manner and can be isolated along with OMV. Using whole proteome analysis of *F. novicida* cultures grown under tube producing (BHI medium) and non-tube producing (TS medium) conditions identified 400 statistically significant differentially regulated proteins. This list, in combination with the 292 OMV/NT-associated proteins, has greatly increased the number of potential targets for NT structure and regulation. We have already screened a number of potential mutants visually by TEM, with no clear candidates. Unfortunately, we do not yet possess a rapid method for screening mutants for OMV/NT production defects. We are therefore relegated to screening each mutant individually and in a non-quantitative manner. Development of an assay for OMV/NT production, in combination with the available defined transposon mutant library, would greatly speed the process of identifying NT regulatory and structural proteins.

We have attempted to separate OMV from NT by several previously published methods. Differential centrifugation has successfully been used to separate larger OMV and contaminants from smaller OMV of interest (Lee, Bang et al. 2007). Density gradient centrifugation has been successfully used to purify OMV from contaminating cellular debris, flagella and pili. Unfortunately, all attempts to separate NT from OMV by centrifugation and density flotation were unsuccessful (Figs. 4-1 and 4-2). Once OMV/NT have been isolated from cell-free supernatants, it becomes possible to pellet these structures at relatively low speeds (~16,000 × *g*), without the need for ultracentrifugation. This may indicate that aggregation is occurring between these structures, which would also complicate any efforts to isolate one population from another by differential or density gradient centrifugation.

Disruption of OMV/NT with commonly used reagents does not seem to be possible. A previously published method to disrupt OMV involves the use of 0.1 M EDTA and heating the OMV at 37ºC for 1 hour. When we did this with *Francisella* OMV/NT, we did not observe any changes in NT or disruption of OMV (Fig. 4-3a). Similar attempts to denature proteins associated with NT by addition of 6 M guanidine-HCl or urea also had no effect (Fig. 4-3b and c). It is possible that the nature of *Francisella* OMV/NT is such that they are resistant to chemical disruption or that the proteins involved in structuring NT are shielded from chemical denaturation. Our experiments with heat treatment of OMV/NT indicates that there is a heat modifiable element to NT structure, most likely a protein, though why chemical treatment has failed we cannot say with certainty.

Cryo-EM experiments give further insight into the nature of *Francisella* NT. Our previous thin-section experiments demonstrate that the NT forming on whole bacteria are continuous with the periplasmic space (Fig. 3-4). This is confirmed in our cryo-EM pictures, as we can readily observe NT formation and distinguish between the outer membrane and inner cytosolic material (Fig. 4-4a). What is more intriguing is the presence of dense material in free-floating NT and what this may signify for the purpose of these structures (Fig. 4-4b). This dense material more closely resembles the cytosolic material observed in the whole bacteria and would explain the presence of so many cytosolic proteins in OMV/NT-associated protein samples (Table 3-1 & 3-2). Why the material is being packaged into these structures, what methods are being used to target this denser matter to NT and whether this internal material may provide structure to the tubes are important questions, worthy of further study.

III. *Francisella tularensis* Outer Membrane Vesicle Isolation and Characterization

In this study we successfully isolated OMV and NT from *F. tularensis* subsp. *tularensis.* Similar to what was observed with *F. novicida*, *F. tularensis* also produces NT in a regulated manner, and these structures readily detach from whole bacteria (Fig. 5-1). Growth in BHI medium increases the number of NT observed when Schu S4 whole bacteria are visualized by TEM (Fig. 5-1). Isolation of OMV/NT was possible by culture in BHI medium, generation of cell-free supernatant and subsequent high speed ultracentrifugation to pellet these structures. We observed numerous NT in addition to

the expected OMV in density gradient purified samples (Fig. 5-2), further evidence of production of NT by *F. tularensis*. Production of NT by *F. tularensis* is also evidence that this phenomenon is not just specific to *F. novicida*, but may in fact occur in all strains of *Francisella.* Further experiments with additional strains of *Francisella* would be necessary to confirm this hypothesis.

The OMV/NT-associated protein content of *F. tularensis* is distinctly different from that observed in *F. novicida*. There are numerous proteins found in *F. tularensis* OMV/NT samples at higher levels (Table 5-3) than in *F. novicida. F. tularensis* OMV/NT samples also consist of an additional 229 proteins not found in *F. novicida* OMV/NT. Some of these proteins are known virulence factors, such as the FPI proteins, while others, such as KatG, may aid in the survival of *Francisella* (Lindgren, Shen et al. 2007). Still, there are some similarities between specific proteins in both *F. tularensis* and *F. novicida* OMV/NT. FopB, FipB, LpnA, Pal and OmpH are all found in similar levels in samples from both of these strains. These proteins are mainly structural in nature, and their incorporation into OMV/NT may be due to the intrinsic mechanisms by which these structures are created. The presence of FipB in both strains of *Francisella* could simply be because of the protein's high abundance in the periplasm, though we have observed at least partial outer membrane localization for this protein (Fig. 3-7c), which hints at an additional role beyond disulfide bond formation in the periplasm. FipB has already been shown to be an essential *F. tularensis* virulence factor, though secretion of this protein has not been shown. There are also numerous hypothetical proteins whose role in *Francisella* virulence has yet to be determined. Whatever the role of the

OMV/NT-associated proteins, it is clear that they are being secreted by this novel mechanism and that this process is important for *Francisella*.

IV. Conclusions and Future Directions

Discovery of the production of OMV and NT by *Francisella* as an alternative secretory pathway is informative and encouraging. Given the relatively few secreted proteins that have been identified in this organism (Hager, Bolton et al. 2006; Qin, Scott et al. 2009; Dai, Mohapatra et al. 2012), an alternate pathway containing hundreds of secreted factors is a gold mine of information. These factors are important for understanding how *Francisella* interacts with its environment, infects host cells and produces an immune response. That OMV/NT protein content is dynamic is not surprising, though only one other research group has demonstrated this phenomenon (Tashiro, Ichikawa et al. 2010). OMV are produced throughout the life of the bacterium, which is itself capable of reacting to external stimuli and changing protein production based on environment (Hazlett, Caldon et al. 2008). What is more surprising is the production of NT by this organism and what their purpose might be. Since *Francisella* NT appear to be novel structures, not conforming to previously published reports of similar structures in other bacteria, it is difficult to say what their purpose might be. Cryo-EM images of whole bacteria seem to indicate that at least some NT are encapsulating cytosolic material, so it may be that these structures are responsible for sharing of material amongst a population of bacteria. However, the *Francisella* NT are not transient (like other published NT), are hardy structures resistant to degradation and appear to readily detach from bacteria. In addition, our results with macrophage co-

incubation suggest that NT may be used to initiate contact with host cells. These facts hint at a larger role for these structures beyond bridging bacteria and sharing material.

The use of naturally produced OMV/NT as a subunit vaccine has broad implications for defense against *Francisella* infection. Since no currently licensed vaccine exists for protection against *Francisella*, the discovery that a subunit vaccine composed of OMV/NT provides protection against challenge with high doses of bacteria is important. We demonstrated here that the *F. novicida* OMV/NT can elicit an immune response and does provide protection against challenge with wild-type bacteria in a mouse model of infection. Future experiments should focus on identifying growth conditions in *F. tularensis* to increase production of OMV/NT in this strain and use of the resultant samples to determine their potential use as a vaccine against the human virulent strains of *Francisella.* We have already begun to examine an alternative method for OMV/NT production through isolation of these structures from plate grown bacteria. This has resulted in a significant increase in the yield of OMV/NT in the *F. novicida* strain and should increase the yield in the *F. tularensis* Schu S4 strain. Further examination of the observed immune response against OMV/NT would also be a profitable area of research. We have examined only a few of the cytokines produced by host cells in response to OMV/NT incubation and have not looked at the mechanisms by which these cytokines are produced. Examining whether the response to OMV/NT is mainly through lipoprotein recognition in a Toll-like receptor 2 dependent manner would be advantageous. This could also help to identify other methods of recognition by which OMV/NT are causing production of cytokines from the host.

Identification of proteins involved in NT formation in *Francisella* will increase our knowledge of *Francisella* virulence and should be a priority. These regulated structures are being produced in multiple strains of *Francisella* and have numerous virulence factors associated with them. Determining the proteins involved in structuring them is an important first step in identifying their function and what role they play in virulence. We have already attempted separation of NT from OMV utilizing a number of methods. Gel filtration chromatography is a method which has successfully separated OMV on the basis of size and one which should be applied to OMV/NT separation. In combination with the *F. novicida* mutant library, a screen for rapid detection of OMV/NT production would allow us to identify proteins involved in biogenesis of these structures. We have already experimented with lipophilic dyes in an attempt to identify OMV/NT in cell-free supernatants of *Francisella.* Optimizing this screen for detection of OMV/NT production in numerous mutant strains is the next step. Once mutants deficient in NT production are identified, we can examine them for defects in virulence and altered OMV protein content.

Bibliography

Alaniz, R. C., B. L. Deatherage, et al. (2007). "Membrane vesicles are immunogenic facsimiles of Salmonella typhimurium that potently activate dendritic cells, prime B and T cell responses, and stimulate protective immunity in vivo." J Immunol **179**(11): 7692-7701.

Altschul, S. F., W. Gish, et al. (1990). "Basic local alignment search tool." Journal of Molecular Biology **215**(3): 403-410.

Anthony, L. S., S. C. Cowley, et al. (1994). "Isolation of a Francisella tularensis mutant that is sensitive to serum and oxidative killing and is avirulent in mice: correlation with the loss of MinD homologue expression." FEMS Microbiol Lett **124**(2): 157-165.

Ark, N. M. and B. J. Mann (2011). "Impact of Francisella tularensis pilin homologs on pilus formation and virulence." Microbial Pathogenesis **51**(3): 110-120.

Avila-Calderon, E. D., A. Lopez-Merino, et al. (2012). "Characterization of Outer Membrane Vesicles from Brucella melitensis and Protection Induced in Mice." Clinical & Developmental Immunology **2012**: 352493.

Bandara, A. B., A. E. Champion, et al. (2011). "Isolation and mutagenesis of a capsule-like complex (CLC) from Francisella tularensis, and contribution of the CLC to F. tularensis virulence in mice." PLoS One **6**(4): e19003.

Barker, J. H., J. Weiss, et al. (2006). "Basis for the failure of Francisella tularensis lipopolysaccharide to prime human polymorphonuclear leukocytes." Infect Immun **74**(6): 3277-3284.

Barker, J. R., A. Chong, et al. (2009). "The Francisella tularensis pathogenicity island encodes a secretion system that is required for phagosome escape and virulence." Mol Microbiol **74**(6): 1459-1470.

Baron, G. S. and F. E. Nano (1998). "MglA and MglB are required for the intramacrophage growth of Francisella novicida." Mol Microbiol **29**(1): 247-259.

Bartruff, J. B., R. A. Yukna, et al. (2005). "Outer membrane vesicles from Porphyromonas gingivalis affect the growth and function of cultured human gingival fibroblasts and umbilical vein endothelial cells." J Periodontol **76**(6): 972-979.

Bauman, S. J. and M. J. Kuehn (2006). "Purification of outer membrane vesicles from Pseudomonas aeruginosa and their activation of an IL-8 response." Microbes Infect **8**(9-10): 2400-2408.

Belting, M. and A. Wittrup (2008). "Nanotubes, exosomes, and nucleic acid-binding peptides provide novel mechanisms of intercellular communication in eukaryotic cells: implications in health and disease." The Journal of Cell Biology **183**(7): 1187-1191.

Ben Nasr, A., J. Haithcoat, et al. (2006). "Critical role for serum opsonins and complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in phagocytosis of Francisella tularensis by human dendritic cells (DC): uptake of Francisella leads to activation of immature DC and intracellular survival of the bacteria." Journal of Leukocyte Biology **80**(4): 774-786.

Berlanda Scorza, F., F. Doro, et al. (2008). "Proteomics characterization of outer membrane vesicles from the extraintestinal pathogenic Escherichia coli DeltatolR IHE3034 mutant." Molecular & Cellular Proteomics : MCP **7**(3): 473-485.
Beveridge, T. J. (1999). "Structures of gram-negative cell walls and their derived membrane vesicles." J Bacteriol **181**(16): 4725-4733.
Bishop, A. L., A. A. Tarique, et al. (2012). "Immunization of mice with vibrio cholerae outer-membrane vesicles protects against hyperinfectious challenge and blocks transmission." The Journal of Infectious Diseases **205**(3): 412-421.
Bomberger, J. M., D. P. Maceachran, et al. (2009). "Long-distance delivery of bacterial virulence factors by *Pseudomonas aeruginosa* outer membrane vesicles." PLoS Pathog **5**(4): e1000382.
Bosio, C. M. and S. W. Dow (2005). "Francisella tularensis induces aberrant activation of pulmonary dendritic cells." Journal of Immunology **175**(10): 6792-6801.
Boutriau, D., J. Poolman, et al. (2007). "Immunogenicity and safety of three doses of a bivalent (B:4:p1.19,15 and B:4:p1.7-2,4) meningococcal outer membrane vesicle vaccine in healthy adolescents." Clin Vaccine Immunol **14**(1): 65-73.
Broms, J. E., M. Lavander, et al. (2009). "A conserved alpha-helix essential for a type VI secretion-like system of Francisella tularensis." J Bacteriol **191**(8): 2431-2446.
Brotcke, A. and D. M. Monack (2008). "Identification of fevR, a novel regulator of virulence gene expression in Francisella novicida." Infect Immun **76**(8): 3473-3480.
Brotcke, A., D. S. Weiss, et al. (2006). "Identification of MglA-regulated genes reveals novel virulence factors in Francisella tularensis." Infect Immun **74**(12): 6642-6655.
Celada, A., P. W. Gray, et al. (1984). "Evidence for a gamma-interferon receptor that regulates macrophage tumoricidal activity." The Journal of Experimental Medicine **160**(1): 55-74.
Chakraborty, S., M. Monfett, et al. (2008). "Type IV pili in Francisella tularensis: roles of pilF and pilT in fiber assembly, host cell adherence, and virulence." Infect Immun **76**(7): 2852-2861.
Chamberlain, R. E. (1965). "Evaluation of Live Tularemia Vaccine Prepared in a Chemically Defined Medium." Applied Microbiology **13**: 232-235.
Chen, D. J., N. Osterrieder, et al. (2010). "Delivery of foreign antigens by engineered outer membrane vesicle vaccines." Proc Natl Acad Sci U S A **107**(7): 3099-3104.
Cherwonogrodzky, J. W., M. H. Knodel, et al. (1994). "Increased encapsulation and virulence of Francisella tularensis live vaccine strain (LVS) by subculturing on synthetic medium." Vaccine **12**(9): 773-775.
Chi, B., M. Qi, et al. (2003). "Role of dentilisin in Treponema denticola epithelial cell layer penetration." Res Microbiol **154**(9): 637-643.
Christopher, G. W., T. J. Cieslak, et al. (1997). "Biological warfare. A historical perspective." JAMA : the Journal of the American Medical Association **278**(5): 412-417.
Clemens, D. L., B. Y. Lee, et al. (2004). "Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages." Infect Immun **72**(6): 3204-3217.

Clemens, D. L., B. Y. Lee, et al. (2005). "Francisella tularensis enters macrophages via a novel process involving pseudopod loops." Infect Immun **73**(9): 5892-5902.
Clemens, D. L., B. Y. Lee, et al. (2011). "O-Antigen Deficient Francisella tularensis Live Vaccine Strain Mutants Are Ingested Via an Aberrant Form of Looping Phagocytosis and Show Altered Kinetics of Intracellular Trafficking in Human Macrophages." Infection and Immunity.
Cong, Y., J. J. Yu, et al. (2009). "Vaccination with a defined Francisella tularensis subsp. novicida pathogenicity island mutant (DeltaiglB) induces protective immunity against homotypic and heterotypic challenge." Vaccine **27**(41): 5554-5561.
Craven, R. R., J. D. Hall, et al. (2008). "Francisella tularensis invasion of lung epithelial cells." Infection and Immunity **76**(7): 2833-2842.
Dai, S., N. P. Mohapatra, et al. (2012). "The acid phosphatase AcpA is secreted in vitro and in macrophages by Francisella spp." Infection and Immunity **80**(3): 1088-1097.
de Bruin, O. M., J. S. Ludu, et al. (2007). "The Francisella pathogenicity island protein IglA localizes to the bacterial cytoplasm and is needed for intracellular growth." BMC Microbiol **7**: 1.
Deatherage, B. L., J. C. Lara, et al. (2009). "Biogenesis of bacterial membrane vesicles." Mol Microbiol **72**(6): 1395-1407.
Delahunty, C. M. and J. R. Yates, 3rd (2007). "MudPIT: multidimensional protein identification technology." Biotechniques **43**(5): 563, 565, 567 passim.
Dennis, D. T., T. V. Inglesby, et al. (2001). "Tularemia as a biological weapon: medical and public health management." JAMA **285**(21): 2763-2773.
Drecktrah, D., S. Levine-Wilkinson, et al. (2008). "Dynamic behavior of Salmonella-induced membrane tubules in epithelial cells." Traffic **9**(12): 2117-2129.
Dubey, G. P. and S. Ben-Yehuda (2011). "Intercellular nanotubes mediate bacterial communication." Cell **144**(4): 590-600.
Duncan, L., M. Yoshioka, et al. (2004). "Loss of lipopolysaccharide receptor CD14 from the surface of human macrophage-like cells mediated by Porphyromonas gingivalis outer membrane vesicles." Microb Pathog **36**(6): 319-325.
Dutta, S., K. Iida, et al. (2004). "Release of Shiga toxin by membrane vesicles in Shigella dysenteriae serotype 1 strains and in vitro effects of antimicrobials on toxin production and release." Microbiol Immunol **48**(12): 965-969.
Ellis, T. N. and M. J. Kuehn (2010). "Virulence and immunomodulatory roles of bacterial outer membrane vesicles." Microbiol Mol Biol Rev **74**(1): 81-94.
Filloux, A., A. Hachani, et al. (2008). "The bacterial type VI secretion machine: yet another player for protein transport across membranes." Microbiology **154**(Pt 6): 1570-1583.
Findlow, J., S. Taylor, et al. (2006). "Comparison and correlation of neisseria meningitidis serogroup B immunologic assay results and human antibody responses following three doses of the Norwegian meningococcal outer membrane vesicle vaccine MenBvac." Infect Immun **74**(8): 4557-4565.
Fink, S. L. and B. T. Cookson (2006). "Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages." Cellular Microbiology **8**(11): 1812-1825.

Fiocca, R., V. Necchi, et al. (1999). "Release of Helicobacter pylori vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and vesicles by gastric epithelium." J Pathol **188**(2): 220-226.
Forslund, A. L., E. N. Salomonsson, et al. (2010). "The type IV pilin, PilA, is required for full virulence of Francisella tularensis subspecies tularensis." BMC Microbiology **10**: 227.
Furuta, N., K. Tsuda, et al. (2009). "Porphyromonas gingivalis outer membrane vesicles enter human epithelial cells via an endocytic pathway and are sorted to lysosomal compartments." Infect Immun **77**(10): 4187-4196.
Galkina, S. I., J. M. Romanova, et al. (2011). "Membrane tubules attach Salmonella Typhimurium to eukaryotic cells and bacteria." FEMS Immunology and Medical Microbiology **61**(1): 114-124.
Gallagher, L. A., E. Ramage, et al. (2007). "A comprehensive transposon mutant library of Francisella novicida, a bioweapon surrogate." Proc Natl Acad Sci U S A **104**(3): 1009-1014.
Gankema, H., J. Wensink, et al. (1980). "Some characteristics of the outer membrane material released by growing enterotoxigenic Escherichia coli." Infect Immun **29**(2): 704-713.
Gavrilin, M. A., I. J. Bouakl, et al. (2006). "Internalization and phagosome escape required for Francisella to induce human monocyte IL-1beta processing and release." Proceedings of the National Academy of Sciences of the United States of America **103**(1): 141-146.
Gerasimov, V. N., V. I. Dolotov, et al. (1997). "[Morphology, ultrastructure and populational features of bacteria francisella]." Vestn Ross Akad Med Nauk(6): 24-30.
Gerdes, H. H. and R. N. Carvalho (2008). "Intercellular transfer mediated by tunneling nanotubes." Curr Opin Cell Biol **20**(4): 470-475.
Gil, H., J. L. Benach, et al. (2004). "Presence of pili on the surface of Francisella tularensis." Infect Immun **72**(5): 3042-3047.
Gil, H., G. J. Platz, et al. (2006). "Deletion of TolC orthologs in Francisella tularensis identifies roles in multidrug resistance and virulence." Proc Natl Acad Sci U S A **103**(34): 12897-12902.
Gilmore, R. D., Jr., R. M. Bacon, et al. (2004). "Identification of Francisella tularensis genes encoding exported membrane-associated proteins using TnphoA mutagenesis of a genomic library." Microb Pathog **37**(4): 205-213.
Gousset, K. and C. Zurzolo (2009). "Tunnelling nanotubes: a highway for prion spreading?" Prion **3**(2): 94-98.
Grenier, D. and D. Mayrand (1987). "Functional characterization of extracellular vesicles produced by Bacteroides gingivalis." Infect Immun **55**(1): 111-117.
Gunn, J. S. and R. K. Ernst (2007). "The structure and function of Francisella lipopolysaccharide." Annals of the New York Academy of Sciences **1105**: 202-218.
Hager, A. J., D. L. Bolton, et al. (2006). "Type IV pili-mediated secretion modulates *Francisella* virulence." Mol Microbiol **62**(1): 227-237.

Hajjar, A. M., M. D. Harvey, et al. (2006). "Lack of in vitro and in vivo recognition of Francisella tularensis subspecies lipopolysaccharide by Toll-like receptors." Infection and Immunity **74**(12): 6730-6738.
Harris, S. (1992). "Japanese biological warfare research on humans: a case study of microbiology and ethics." Annals of the New York Academy of Sciences **666**: 21-52.
Haurat, M. F., J. Aduse-Opoku, et al. (2011). "Selective sorting of cargo proteins into bacterial membrane vesicles." The Journal of Biological Chemistry **286**(2): 1269-1276.
Hazlett, K. R., S. D. Caldon, et al. (2008). "Adaptation of Francisella tularensis to the mammalian environment is governed by cues which can be mimicked in vitro." Infect Immun **76**(10): 4479-4488.
Henry, T., A. Brotcke, et al. (2007). "Type I interferon signaling is required for activation of the inflammasome during Francisella infection." The Journal of Experimental Medicine **204**(5): 987-994.
Henry, T. and D. M. Monack (2007). "Activation of the inflammasome upon Francisella tularensis infection: interplay of innate immune pathways and virulence factors." Cell Microbiol **9**(11): 2543-2551.
Heussler, V. T., S. Rottenberg, et al. (2002). "Hijacking of host cell IKK signalosomes by the transforming parasite Theileria." Science **298**(5595): 1033-1036.
Hickey, A. J., K. R. Hazlett, et al. (2011). "Identification of *Francisella tularensis* outer membrane protein A (FopA) as a protective antigen for tularemia." Vaccine **29**(40): 6941-6947.
Holland, I. B., L. Schmitt, et al. (2005). "Type 1 protein secretion in bacteria, the ABC-transporter dependent pathway (review)." Molecular Membrane Biology **22**(1-2): 29-39.
Holst, J., D. Martin, et al. (2009). "Properties and clinical performance of vaccines containing outer membrane vesicles from Neisseria meningitidis." Vaccine **27 Suppl 2**: B3-12.
Horstman, A. L. and M. J. Kuehn (2000). "Enterotoxigenic Escherichia coli secretes active heat-labile enterotoxin via outer membrane vesicles." J Biol Chem **275**(17): 12489-12496.
Horstman, A. L. and M. J. Kuehn (2002). "Bacterial surface association of heat-labile enterotoxin through lipopolysaccharide after secretion via the general secretory pathway." J Biol Chem **277**(36): 32538-32545.
Horzempa, J., D. M. O'Dee, et al. (2011). "Invasion of erythrocytes by Francisella tularensis." The Journal of Infectious Diseases **204**(1): 51-59.
Huber, B., R. Escudero, et al. (2010). "Description of *Francisella hispaniensis* sp. nov., isolated from human blood, reclassification of *Francisella novicida* (Larson *et al*. 1955) Olsufiev *et al*. 1959 as *Francisella tularensis* subsp. *novicida* comb. nov. and emended description of the genus *Francisella*." Int J Syst Evol Microbiol **60**(Pt 8): 1887-1896.
Huntley, J. F., P. G. Conley, et al. (2007). "Characterization of *Francisella tularensis* outer membrane proteins." J Bacteriol **189**(2): 561-574.

Hurtig, J., D. T. Chiu, et al. (2010). "Intercellular nanotubes: insights from imaging studies and beyond." Wiley Interdisciplinary Reviews. Nanomedicine and Nanobiotechnology **2**(3): 260-276.

Izore, T., V. Job, et al. (2011). "Biogenesis, regulation, and targeting of the type III secretion system." Structure **19**(5): 603-612.

Janovska, S., I. Pavkova, et al. (2007). "Identification of immunoreactive antigens in membrane proteins enriched fraction from *Francisella tularensis* LVS." Immunol Lett **108**(2): 151-159.

Jin, J. S., S. O. Kwon, et al. (2011). "Acinetobacter baumannii secretes cytotoxic outer membrane protein A via outer membrane vesicles." PLoS One **6**(2): e17027.

Johansson, A., J. Celli, et al. (2010). "Objections to the transfer of *Francisella novicida* to the subspecies rank of *Francisella tularensis*." Int J Syst Evol Microbiol **60**(Pt 8): 1717-1718.

Jones, C. L., B. A. Napier, et al. (2012). "Subversion of host recognition and defense systems by Francisella spp." Microbiology and Molecular Biology Reviews : MMBR **76**(2): 383-404.

Jones, C. L., T. R. Sampson, et al. (2012). "Repression of bacterial lipoprotein production by *Francisella novicida* facilitates evasion of innate immune recognition." Cell Microbiol **14**(10): 1531-1543.

Jones, C. L. and D. S. Weiss (2011). "TLR2 signaling contributes to rapid inflammasome activation during F. novicida infection." PLoS One **6**(6): e20609.

Kadurugamuwa, J. L. and T. J. Beveridge (1995). "Virulence factors are released from Pseudomonas aeruginosa in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion." J Bacteriol **177**(14): 3998-4008.

Kadurugamuwa, J. L. and T. J. Beveridge (1998). "Delivery of the non-membrane-permeative antibiotic gentamicin into mammalian cells by using Shigella flexneri membrane vesicles." Antimicrob Agents Chemother **42**(6): 1476-1483.

Kadurugamuwa, J. L. and T. J. Beveridge (1999). "Membrane vesicles derived from Pseudomonas aeruginosa and Shigella flexneri can be integrated into the surfaces of other gram-negative bacteria." Microbiology **145 (Pt 8)**: 2051-2060.

Kadurugamuwa, J. L. V., NJ, US), Beveridge, Terry J. (Elora, CA) (2005). Vaccines and pharmaceutical compositions using membrane vesicles of microorganisms, and methods for preparing same. United States, University of Guelph (Ontario, CA).

Kamaguchi, A., K. Nakayama, et al. (2003). "Effect of Porphyromonas gingivalis vesicles on coaggregation of Staphylococcus aureus to oral microorganisms." Curr Microbiol **47**(6): 485-491.

Kato, S., Y. Kowashi, et al. (2002). "Outer membrane-like vesicles secreted by Actinobacillus actinomycetemcomitans are enriched in leukotoxin." Microb Pathog **32**(1): 1-13.

Keenan, J. I. and R. A. Allardyce (2000). "Iron influences the expression of Helicobacter pylori outer membrane vesicle-associated virulence factors." Eur J Gastroenterol Hepatol **12**(12): 1267-1273.

Keller, A., A. I. Nesvizhskii, et al. (2002). "Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search." Analytical Chemistry **74**(20): 5383-5392.

Kesty, N. C., K. M. Mason, et al. (2004). "Enterotoxigenic Escherichia coli vesicles target toxin delivery into mammalian cells." EMBO J **23**(23): 4538-4549.

Khandelwal, P. and N. Banerjee-Bhatnagar (2003). "Insecticidal activity associated with the outer membrane vesicles of Xenorhabdus nematophilus." Appl Environ Microbiol **69**(4): 2032-2037.

Kim, L., B. A. Butcher, et al. (2004). "Toxoplasma gondii interferes with lipopolysaccharide-induced mitogen-activated protein kinase activation by mechanisms distinct from endotoxin tolerance." Journal of Immunology **172**(5): 3003-3010.

Kim, S. H., K. S. Kim, et al. (2009). "Structural modifications of outer membrane vesicles to refine them as vaccine delivery vehicles." Biochim Biophys Acta **1788**(10): 2150-2159.

Kim, S. H., S. R. Lee, et al. (2010). "Shiga toxin A subunit mutant of Escherichia coli O157:H7 releases outer membrane vesicles containing the B-pentameric complex." FEMS Immunol Med Microbiol **58**(3): 412-420.

Koeberling, O., A. Seubert, et al. (2008). "Bactericidal antibody responses elicited by a meningococcal outer membrane vesicle vaccine with overexpressed factor H-binding protein and genetically attenuated endotoxin." J Infect Dis **198**(2): 262-270.

Kolling, G. L. and K. R. Matthews (1999). "Export of virulence genes and Shiga toxin by membrane vesicles of Escherichia coli O157:H7." Appl Environ Microbiol **65**(5): 1843-1848.

Kouokam, J. C., S. N. Wai, et al. (2006). "Active cytotoxic necrotizing factor 1 associated with outer membrane vesicles from uropathogenic Escherichia coli." Infect Immun **74**(4): 2022-2030.

Kraemer, P. S., A. Mitchell, et al. (2009). "Genome-wide screen in *Francisella novicida* for genes required for pulmonary and systemic infection in mice." Infect Immun **77**(1): 232-244.

Kuehn, M. J. and N. C. Kesty (2005). "Bacterial outer membrane vesicles and the host-pathogen interaction." Genes Dev **19**(22): 2645-2655.

Lai, C. H., M. A. Listgarten, et al. (1981). "Comparative ultrastructure of leukotoxic and non-leukotoxic strains of Actinobacillus actinomycetemcomitans." J Periodontal Res **16**(4): 379-389.

Lai, X. H., I. Golovliov, et al. (2004). "Expression of IglC is necessary for intracellular growth and induction of apoptosis in murine macrophages by Francisella tularensis." Microbial Pathogenesis **37**(5): 225-230.

Lauriano, C. M., J. R. Barker, et al. (2004). "MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intraamoebae and intramacrophage survival." Proc Natl Acad Sci U S A **101**(12): 4246-4249.

Law, H. T., A. E. Lin, et al. (2011). "Francisellatularensis uses cholesterol and clathrin-based endocytic mechanisms to invade hepatocytes." Scientific Reports **1**: 192.

Lee, B. Y., M. A. Horwitz, et al. (2006). "Identification, recombinant expression, immunolocalization in macrophages, and T-cell responsiveness of the major extracellular proteins of Francisella tularensis." Infect Immun **74**(7): 4002-4013.

Lee, E. Y., J. Y. Bang, et al. (2007). "Global proteomic profiling of native outer membrane vesicles derived from *Escherichia coli*." Proteomics **7**(17): 3143-3153.

Lee, E. Y., D. S. Choi, et al. (2008). "Proteomics in gram-negative bacterial outer membrane vesicles." Mass Spectrom Rev **27**(6): 535-555.
Li, H., S. Nookala, et al. (2006). "Innate immune response to Francisella tularensis is mediated by TLR2 and caspase-1 activation." Journal of Leukocyte Biology **80**(4): 766-773.
Li, Z., A. J. Clarke, et al. (1998). "Gram-negative bacteria produce membrane vesicles which are capable of killing other bacteria." J Bacteriol **180**(20): 5478-5483.
Lindgren, H., H. Shen, et al. (2007). "Resistance of Francisella tularensis strains against reactive nitrogen and oxygen species with special reference to the role of KatG." Infection and Immunity **75**(3): 1303-1309.
Lopez, M. C., N. S. Duckett, et al. (2004). "Early activation of NK cells after lung infection with the intracellular bacterium, Francisella tularensis LVS." Cellular Immunology **232**(1-2): 75-85.
Ludu, J. S., O. M. de Bruin, et al. (2008). "The *Francisella* pathogenicity island protein PdpD is required for full virulence and associates with homologues of the type VI secretion system." J Bacteriol **190**(13): 4584-4595.
Ludu, J. S., O. M. de Bruin, et al. (2008). "The Francisella pathogenicity island protein PdpD is required for full virulence and associates with homologues of the type VI secretion system." Journal of Bacteriology **190**(13): 4584-4595.
Maldonado, R., R. Wei, et al. (2011). "Cytotoxic effects of Kingella kingae outer membrane vesicles on human cells." Microbial Pathogenesis **51**(1-2): 22-30.
Mariathasan, S., D. S. Weiss, et al. (2005). "Innate immunity against Francisella tularensis is dependent on the ASC/caspase-1 axis." The Journal of Experimental Medicine **202**(8): 1043-1049.
Mashburn-Warren, L. M. and M. Whiteley (2006). "Special delivery: vesicle trafficking in prokaryotes." Mol Microbiol **61**(4): 839-846.
Mashburn, L. M. and M. Whiteley (2005). "Membrane vesicles traffic signals and facilitate group activities in a prokaryote." Nature **437**(7057): 422-425.
Mayrand, D. and D. Grenier (1989). "Biological activities of outer membrane vesicles." Canadian journal of Microbiology **35**(6): 607-613.
McBroom, A. J., A. P. Johnson, et al. (2006). "Outer membrane vesicle production by Escherichia coli is independent of membrane instability." J Bacteriol **188**(15): 5385-5392.
McCoy, G. W. (1911). "Some Features of the Squirrel Plague Problem." California State Journal of Medicine **9**(3): 105-109.
Mohapatra, N. P., S. Soni, et al. (2007). "Identification of an orphan response regulator required for the virulence of Francisella spp. and transcription of pathogenicity island genes." Infect Immun **75**(7): 3305-3314.
Morton, R. E. and T. A. Evans (1992). "Modification of the bicinchoninic acid protein assay to eliminate lipid interference in determining lipoprotein protein content." Anal Biochem **204**(2): 332-334.
Mougous, J. D., M. E. Cuff, et al. (2006). "A virulence locus of Pseudomonas aeruginosa encodes a protein secretion apparatus." Science **312**(5779): 1526-1530.
Mullineaux, C. W., V. Mariscal, et al. (2008). "Mechanism of intercellular molecular exchange in heterocyst-forming cyanobacteria." The EMBO Journal **27**(9): 1299-1308.

Nakao, R., H. Hasegawa, et al. (2011). "Outer Membrane Vesicles of Porphyromonas gingivalis Elicit a Mucosal Immune Response." PLoS One **6**(10): e26163.
Nano, F. E. (1988). "Identification of a heat-modifiable protein of *Francisella tularensis* and molecular cloning of the encoding gene." Microb Pathog **5**(2): 109-119.
Nano, F. E., N. Zhang, et al. (2004). "A *Francisella tularensis* pathogenicity island required for intramacrophage growth." J Bacteriol **186**(19): 6430-6436.
Navarre, W. W. and A. Zychlinsky (2000). "Pathogen-induced apoptosis of macrophages: a common end for different pathogenic strategies." Cellular Microbiology **2**(4): 265-273.
Negrete-Abascal, E., R. M. Garcia, et al. (2000). "Membrane vesicles released by Actinobacillus pleuropneumoniae contain proteases and Apx toxins." FEMS Microbiol Lett **191**(1): 109-113.
Nesvizhskii, A. I., A. Keller, et al. (2003). "A statistical model for identifying proteins by tandem mass spectrometry." Analytical Chemistry **75**(17): 4646-4658.
Nevot, M., V. Deroncele, et al. (2006). "Characterization of outer membrane vesicles released by the psychrotolerant bacterium Pseudoalteromonas antarctica NF3." Environmental Microbiology **8**(9): 1523-1533.
Noah, C. E., M. Malik, et al. (2010). "GroEL and lipopolysaccharide from Francisella tularensis live vaccine strain synergistically activate human macrophages." Infection and Immunity **78**(4): 1797-1806.
Nowotny, A., U. H. Behling, et al. (1982). "Release of toxic microvesicles by Actinobacillus actinomycetemcomitans." Infect Immun **37**(1): 151-154.
Oyston, P. C., A. Sjostedt, et al. (2004). "Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*." Nat Rev Microbiol **2**(12): 967-978.
Paoletti, A. C., T. J. Parmely, et al. (2006). "Quantitative proteomic analysis of distinct mammalian Mediator complexes using normalized spectral abundance factors." Proceedings of the National Academy of Sciences of the United States of America **103**(50): 18928-18933.
Park, J. M., F. R. Greten, et al. (2002). "Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition." Science **297**(5589): 2048-2051.
Parker, H., K. Chitcholtan, et al. (2010). "Uptake of Helicobacter pylori outer membrane vesicles by gastric epithelial cells." Infection and Immunity **78**(12): 5054-5061.
Parsa, K. V., J. P. Butchar, et al. (2008). "Francisella gains a survival advantage within mononuclear phagocytes by suppressing the host IFNgamma response." Mol Immunol **45**(12): 3428-3437.
Patrick, S., J. P. McKenna, et al. (1996). "A comparison of the haemagglutinating and enzymic activities of Bacteroides fragilis whole cells and outer membrane vesicles." Microb Pathog **20**(4): 191-202.
Pavkova, I., M. Hubalek, et al. (2005). "*Francisella tularensis* live vaccine strain: proteomic analysis of membrane proteins enriched fraction." Proteomics **5**(9): 2460-2467.
Pierson, T., D. Matrakas, et al. (2011). "Proteomic characterization and functional analysis of outer membrane vesicles of Francisella novicida suggests possible role in virulence and use as a vaccine." J Proteome Res **10**(3): 954-967.

Platz, G. J., D. C. Bublitz, et al. (2010). "A tolC mutant of Francisella tularensis is hypercytotoxic compared to the wild type and elicits increased proinflammatory responses from host cells." Infection and Immunity **78**(3): 1022-1031.
Post, D. M., D. Zhang, et al. (2005). "Biochemical and functional characterization of membrane blebs purified from Neisseria meningitidis serogroup B." The Journal of Biological Chemistry **280**(46): 38383-38394.
Prados-Rosales, R., A. Baena, et al. (2011). "Mycobacteria release active membrane vesicles that modulate immune responses in a TLR2-dependent manner in mice." The Journal of Clinical Investigation **121**(4): 1471-1483.
Pukatzki, S., A. T. Ma, et al. (2006). "Identification of a conserved bacterial protein secretion system in Vibrio cholerae using the Dictyostelium host model system." Proc Natl Acad Sci U S A **103**(5): 1528-1533.
Qin, A. and B. J. Mann (2006). "Identification of transposon insertion mutants of *Francisella tularensis tularensis* strain Schu S4 deficient in intracellular replication in the hepatic cell line HepG2." BMC Microbiol **6**: 69.
Qin, A., D. W. Scott, et al. (2011). "Requirement of the CXXC motif of novel Francisella infectivity potentiator protein B FipB, and FipA in virulence of F. tularensis subsp. tularensis." PLoS One **6**(9): e24611.
Qin, A., D. W. Scott, et al. (2009). "Identification of an essential *Francisella tularensis* subsp. *tularensis* virulence factor." Infect Immun **77**(1): 152-161.
Renelli, M., V. Matias, et al. (2004). "DNA-containing membrane vesicles of Pseudomonas aeruginosa PAO1 and their genetic transformation potential." Microbiology **150**(Pt 7): 2161-2169.
Riboldi-Tunnicliffe, A., B. Konig, et al. (2001). "Crystal structure of Mip, a prolylisomerase from Legionella pneumophila." Nature Structural Biology **8**(9): 779-783.
Rohmer, L., C. Fong, et al. (2007). "Comparison of Francisella tularensis genomes reveals evolutionary events associated with the emergence of human pathogenic strains." Genome Biol **8**(6): R102.
Rosen, G., R. Naor, et al. (1995). "Proteases of Treponema denticola outer sheath and extracellular vesicles." Infect Immun **63**(10): 3973-3979.
Roy, K., D. J. Hamilton, et al. (2011). "Outer Membrane Vesicles Induce Immune Responses to Virulence Proteins and Protect against Colonization by Enterotoxigenic Escherichia coli." Clinical and Vaccine Immunology : CVI **18**(11): 1803-1808.
Rustom, A., R. Saffrich, et al. (2004). "Nanotubular highways for intercellular organelle transport." Science **303**(5660): 1007-1010.
Sandstrom, G., S. Lofgren, et al. (1988). "A capsule-deficient mutant of Francisella tularensis LVS exhibits enhanced sensitivity to killing by serum but diminished sensitivity to killing by polymorphonuclear leukocytes." Infection and Immunity **56**(5): 1194-1202.
Santic, M., M. Molmeret, et al. (2007). "A Francisella tularensis pathogenicity island protein essential for bacterial proliferation within the host cell cytosol." Cell Microbiol **9**(10): 2391-2403.
Santic, M., M. Molmeret, et al. (2005). "The Francisella tularensis pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome

biogenesis and subsequent bacterial escape into the cytoplasm." Cell Microbiol **7**(7): 969-979.
Santic, M., G. Pavokovic, et al. (2010). "Regulation of apoptosis and anti-apoptosis signalling by Francisella tularensis." Microbes and Infection / Institut Pasteur **12**(2): 126-134.
Savitt, A. G., P. Mena-Taboada, et al. (2009). "*Francisella tularensis* infection-derived monoclonal antibodies provide detection, protection, and therapy." Clin Vaccine Immunol **16**(3): 414-422.
Schara, K., V. Jansa, et al. (2009). "Mechanisms for the formation of membranous nanostructures in cell-to-cell communication." Cellular & Molecular Biology letters **14**(4): 636-656.
Schild, S., E. J. Nelson, et al. (2009). "Characterization of Vibrio cholerae outer membrane vesicles as a candidate vaccine for cholera." Infect Immun **77**(1): 472-484.
Schild, S., E. J. Nelson, et al. (2009). "Characterization of Vibrio cholerae outer membrane vesicles as a candidate vaccine for cholera." Infection and Immunity **77**(1): 472-484.
Schmerk, C. L., B. N. Duplantis, et al. (2009). "A Francisella novicida pdpA mutant exhibits limited intracellular replication and remains associated with the lysosomal marker LAMP-1." Microbiology **155**(Pt 5): 1498-1504.
Schmerk, C. L., B. N. Duplantis, et al. (2009). "Characterization of the pathogenicity island protein PdpA and its role in the virulence of Francisella novicida." Microbiology **155**(Pt 5): 1489-1497.
Schwartz, J. T., J. H. Barker, et al. (2012). "Francisella tularensis inhibits the intrinsic and extrinsic pathways to delay constitutive apoptosis and prolong human neutrophil lifespan." Journal of Immunology **188**(7): 3351-3363.
Shoberg, R. J. and D. D. Thomas (1993). "Specific adherence of Borrelia burgdorferi extracellular vesicles to human endothelial cells in culture." Infect Immun **61**(9): 3892-3900.
Su, J., J. Yang, et al. (2007). "Genome-wide identification of *Francisella tularensis* virulence determinants." Infect Immun **75**(6): 3089-3101.
Takeda, K. and S. Akira (2004). "TLR signaling pathways." Seminars in Immunology **16**(1): 3-9.
Tashiro, Y., S. Ichikawa, et al. (2010). "Variation of physiochemical properties and cell association activity of membrane vesicles with growth phase in Pseudomonas aeruginosa." Applied and Environmental Microbiology **76**(11): 3732-3739.
Telepnev, M., I. Golovliov, et al. (2003). "Francisella tularensis inhibits Toll-like receptor-mediated activation of intracellular signalling and secretion of TNF-alpha and IL-1 from murine macrophages." Cell Microbiol **5**(1): 41-51.
Telepnev, M., I. Golovliov, et al. (2003). "Francisella tularensis inhibits Toll-like receptor-mediated activation of intracellular signalling and secretion of TNF-alpha and IL-1 from murine macrophages." Cellular Microbiology **5**(1): 41-51.
Tempel, R., X. H. Lai, et al. (2006). "Attenuated *Francisella novicida* transposon mutants protect mice against wild-type challenge." Infect Immun **74**(9): 5095-5105.

Thakran, S., H. Li, et al. (2008). "Identification of *Francisella tularensis* lipoproteins that stimulate the toll-like receptor (TLR) 2/TLR1 heterodimer." J Biol Chem **283**(7): 3751-3760.

Thanassi, D. G., J. B. Bliska, et al. (2012). "Surface organelles assembled by secretion systems of Gram-negative bacteria: diversity in structure and function." FEMS Microbiology Reviews **36**(6): 1046-1082.

Thanassi, D. G. and S. J. Hultgren (2000). "Multiple pathways allow protein secretion across the bacterial outer membrane." Curr Opin Cell Biol **12**(4): 420-430.

Thorpe, B. D. and S. Marcus (1964). "Phagocytosis and Intracellular Fate of Pasteurella Tularensis. I. In Vitro Studies with Rabbit Peritoneal Mononuclear Phagocytes." Journal of Immunology **92**: 657-663.

van de Waterbeemd, B., M. Streefland, et al. (2010). "Improved OMV vaccine against Neisseria meningitidis using genetically engineered strains and a detergent-free purification process." Vaccine **28**(30): 4810-4816.

Vidakovics, M. L., J. Jendholm, et al. (2010). "B cell activation by outer membrane vesicles--a novel virulence mechanism." PLoS Pathog **6**(1): e1000724.

Vipond, C., J. Suker, et al. (2006). "Proteomic analysis of a meningococcal outer membrane vesicle vaccine prepared from the group B strain NZ98/254." Proteomics **6**(11): 3400-3413.

Wai, S. N., B. Lindmark, et al. (2003). "Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin." Cell **115**(1): 25-35.

Wai, S. N., A. Takade, et al. (1995). "The release of outer membrane vesicles from the strains of enterotoxigenic Escherichia coli." Microbiol Immunol **39**(7): 451-456.

Wallden, K., A. Rivera-Calzada, et al. (2010). "Type IV secretion systems: versatility and diversity in function." Cellular Microbiology **12**(9): 1203-1212.

Weiss, D. S., A. Brotcke, et al. (2007). "In vivo negative selection screen identifies genes required for *Francisella* virulence." Proc Natl Acad Sci U S A **104**(14): 6037-6042.

Wensink, J., H. Gankema, et al. (1978). "Isolation of the membranes of an enterotoxigenic strain of Escherichia coli and distribution of enterotoxin activity in different subcellular fractions." Biochim Biophys Acta **514**(1): 128-136.

Wensink, J. and B. Witholt (1981). "Outer-membrane vesicles released by normally growing Escherichia coli contain very little lipoprotein." European Journal of Biochemistry / FEBS **116**(2): 331-335.

Yaron, S., G. L. Kolling, et al. (2000). "Vesicle-mediated transfer of virulence genes from Escherichia coli O157:H7 to other enteric bacteria." Appl Environ Microbiol **66**(10): 4414-4420.

Yokoyama, K., T. Horii, et al. (2000). "Production of shiga toxin by Escherichia coli measured with reference to the membrane vesicle-associated toxins." FEMS Microbiol Lett **192**(1): 139-144.

Yoon, S., Z. Liu, et al. (2003). "Yersinia effector YopJ inhibits yeast MAPK signaling pathways by an evolutionarily conserved mechanism." The Journal of Biological Chemistry **278**(4): 2131-2135.

Yu, J. J., T. Goluguri, et al. (2010). "Francisella tularensis T-cell antigen identification using humanized HLA-DR4 transgenic mice." Clin Vaccine Immunol **17**(2): 215-222.

Yu, N. Y., J. R. Wagner, et al. (2010). "PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes." Bioinformatics **26**(13): 1608-1615.

Zhou, L., R. Srisatjaluk, et al. (1998). "On the origin of membrane vesicles in gram-negative bacteria." FEMS Microbiology Letters **163**(2): 223-228.

Zivna, L., Z. Krocova, et al. (2010). "Activation of B cell apoptotic pathways in the course of Francisella tularensis infection." Microbial Pathogenesis **49**(5): 226-236.

Zogaj, X., S. Chakraborty, et al. (2008). "Characterization of the *Francisella tularensis* subsp. *novicida* type IV pilus." Microbiology **154**(Pt 7): 2139-2150.