AEM Accepts, published online ahead of print on 30 November 2009
Appl. Environ. Microbiol. doi:10.1128/AEM.02037-09

Francisella tularensis subspecies *novicida* chitinases and Sec secretion system contribute to biofilm formation on chitin

Jeffrey J. Margolis[1], Sahar El-Etr[2], Lydia-Marie Joubert[3], Emily Moore[4], Richard Robison[4], Amy Rasley[2], Alfred M. Spormann[5], and Denise M. Monack[1*]

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305, USA[1]. Bioscience and Biotechnology Division, Lawrence Livermore National Laboratory, Livermore, CA 94550, USA[2]. Cell Sciences Imaging Facility, Stanford University School of Medicine, Stanford, CA 94305[3]. Department of Microbiology and Molecular Biology, Brigham Young University, Provo, UT 84602, USA[4]. Department of Civil and Environmental Engineering, Stanford University, Stanford, CA 94304, USA[5].

Running Title: *Francisella* biofilm formation on chitin

* Corresponding Author: Mailing address: 299 Campus Dr., Fairchild Bldg. D347, Stanford, CA 94305. Phone: (650) 725-1756. Fax: (650) 723-1837. E-mail: dmonack@stanford.edu

ABSTRACT

Francisella tularensis, the zoonotic cause of tularemia, can infect numerous mammals and other eukaryotes. Although studying *F. tularensis* pathogenesis is essential to comprehending disease, mammalian infection is just one step in the ecology of *Francisella* species. *F. tularensis* has been isolated from aquatic environments and arthropod vectors, environments in which chitin could serve as a potential carbon source and as a surface for attachment and growth. We show that *F. tularensis* subsp. *novicida* forms biofilms during colonization of chitin surfaces. The ability of *F. tularensis* to persist using chitin as a sole carbon source is dependent on chitinases, since mutants lacking *chiA* or *chiB* are attenuated for chitin colonization and biofilm formation in the absence of exogenous sugar. A genetic screen for biofilm mutants identified the Sec translocon export pathway and 14 secreted proteins. We show that these genes are important for initial attachment during biofilm formation. We generated defined deletion mutants in two chaperone genes (*secB1* and *secB2*) involved in Sec-dependent secretion and in four genes that encode for putative secreted proteins. All mutants were deficient for attachment to polystyrene and chitin surfaces and for biofilm formation compared to wild-type *F. novicida*. In contrast, mutations in the Sec translocon and secreted factors did not affect virulence. Our data suggest biofilm formation by *F. tularensis* promotes persistence on chitin surfaces. Further study of the interaction of *F. tularensis* with the chitin microenvironment may provide insight into environmental survival and transmission mechanisms of this pathogen.

INTRODUCTION

Francisella tularensis is a Gram-negative facultative intracellular pathogen that causes the zoonotic disease tularemia (53). Although researchers have focused on various aspects of *F. tularensis* infections in mammalian hosts, this organism can survive and grow in one of the widest environmental ranges of any studied pathogen. Indeed, *F. tularensis* has been isolated from a variety of sources including lagomorphs, arthropods, amoeba and fresh water (3, 57, 62, 63). Mammals either succumb to infection or clear the bacterium (71) suggesting that mammals may not support prolonged persistence of *F. tularensis* in nature. Understanding the environmental lifestyle of *F. tularensis* will help elucidate the survival mechanisms of this pathogen outside of a host and identify risks for human exposure. Recently, outbreaks of tularemia were associated with fresh water, particularly outbreaks of *F. tularensis* subspecies *holarctica* (Type B) in Eurasia (11, 81). While the most virulent subspecies, *F. tularensis* subsp. *tularensis* (Type A), was historically linked with the arid climates of North America, a recent epidemiological study found that 100% of tularemia mortality was associated with Type A1 strains found in moist climates of the United States (40), suggesting that water may serve as an environmental reservoir for *F. tularensis*.

The survival of some bacteria in an aquatic environment is associated with their ability to utilize chitin as a carbon source. Chitin is the second most abundant biopolymer in nature and provides structure to many organisms, including the cell wall of fungi (5) and the exoskeleton of arthropods and insects (51). This oligomer of *N*-acetyl-D-glucosamine (GlcNAc) is hydrolyzed by a family of enzymes, termed chitinases (6). These enzymes serve a variety of roles and are conserved from bacteria to mammals. Bacterial chitinases provide environmental organisms the ability to acquire carbon in otherwise nutrient-limiting conditions (37). For example, *Vibrio*

cholerae, the etiological agent of cholera, utilizes chitinases to persist in marine environments on copepod molts (54). The interaction of *V. cholerae* with chitin influences various metabolic and physiologic responses in this microorganism. For example, Meibom *et al.* demonstrated that association with chitin and chitin-derivatives led to a specific expression profile in *V. cholerae* that included two chitinase genes and the pili genes required for colonization and subsequent biofilm formation on nutritive and non-nutritive surfaces (49). Environmental studies have clearly shown that attachment to chitin surfaces is an integral part of the aquatic lifestyle of *V. cholerae*, and these surface-attached bacterial communities constitute a successful survival mechanism (66).

Formation of biofilms is associated with enhanced survival during environmental stress (1) and increased resistance to antibiotics (13). Biofilms formed by many pathogenic bacteria play an important role in environmental persistence and disease transmission. For instance, *Yersinia pestis* biofilms are reported to function in transmission of plague bacteria via colonization of the proventriculus of fleas and the mouth of nematodes (15, 32). We hypothesized that biofilm formation by *F. tularensis* may represent a mechanism of persistence and transmission, as well.

A review by Hassett *et al*. (30) indicated that the *F. tularensis* subsp. *holartica* live vaccine strain (LVS) can form biofilms on glass coverslips (30). However, the environmental relevance and molecular mechanisms of *F. tularensis* biofilm formation were not characterized. *F. tularensis* subspecies encode for 2 conserved putative chitinases, *chiA* and *chiB* (http://www.biohealthbase.org). Various *F. tularensis* subspecies have been isolated from chitin-exoskeletoned arthropods (57) and from fresh water, where outbreaks have been associated with chitinous crustaceans (2, 17). We therefore investigated the interaction of *F. tularensis* subsp.

novicida (*F. novicida*) with chitin. We show that *F. novicida* forms biofilms on natural and synthetic chitin surfaces. Formation of these bacterial communities was dependent on two chitinase genes when exogenous sugar was not present. Attachment to chitin was dependent on factors that are secreted by the Sec translocon protein export system. This mechanism of colonization is specific for environmental surfaces, because deletion of genes that facilitate attachment to chitin did not result in defects in virulence.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Francisella novicida* strain U112 and *F. tularensis* subsp. *holarctica* live vaccine strain (LVS), (18, 61), as well as *F. novicida Francisella* Pathogenecity Island (FPI) and *hspX* deletion mutants (80), have been previously described. *F. tularensis* subsp. *tularensis* strains, SchuS4 and AS2058 (FT-10), were provided by Jean Celli and the New Mexico Department of Health, respectively, and handled under biosafety level-3 (BSL-3) precautions per Centers for Disease Control and Prevention protocol. Unless otherwise noted, strains were grown in modified Mueller Hinton media (MMH), (Difco, Corpus Christi, TX) supplemented with 0.025% ferric pyrophosphate, 0.02% IsoVitaleX (Becton Dickinson, Franklin Lakes, NJ) as a cysteine source, and 0.1% glucose as a carbon source. For some experiments, *F. tularensis* strains were grown in Chamberlain's Defined Medium (CDM), (47) with or without glucose. For enumeration studies, bacteria were grown on MMH agar plates.

Imaging *F. novicida* colonization on chitin films and sterile crab shell pieces. Wild-type *F. novicida* was allowed to attach to either synthetic chitin films (82) or sterile crab shell pieces for 1 h. After 1 h, surfaces were washed 3X with phosphate buffered saline to remove non-adhered bacteria and samples were incubated at 30°C in CDM without glucose. After one hour and one week of incubation, respectively, crab shell and chitin film samples were processed for scanning electron microscopy (SEM) investigation. Substrates with attached cells were fixed for 3 days at 4°C in 4% paraformaldehyde with 2% glutaraldehyde in 0.1M NaCacodylate Buffer (pH 7.3) (EM grade, EMS, Hatfield, PA). After primary fixation, samples were rinsed in the same buffer, post-fixed in 1% aqueous OsO_4 for 1 h, and dehydrated in an ascending ethanol series (30, 50, 70, 80, 90, 100%; for 20min each), followed by critical point drying with liquid CO_2 using a Tousimis SAMDRI-VT-3B apparatus (Tousimis, Rockville, MD). Samples were mounted on

adhesive carbon film on 15mm aluminum stubs, and sputter-coated with 100Å gold/palladium using a Denton Desk 11 TSC Sputter Coater. Visualization was carried out with a Hitachi S-3400N VP SEM (Hitachi Ltd, Pleasanton, CA) operated at 10-15kV, working distance 8-10mm, and secondary electron detector. Images were capture in TIF format.

Growth in CDM broth. *F. novicida* was grown overnight in CDM at 37° with aeration. The culture was then diluted to Optical density 600_{nm} (OD_{600}) using an Ultropec 2100 Pro spectrophotomer (Amersham Biosciences, Pittsburgh, PA) 0.05 in either CDM with no sugar, CDM with 10mM glucose or 10mM GlcNAc. Optical density and colony forming units (CFU) were monitored over time for each media condition. The doubling time for each culture was calculated.

Imaging of flow cell grown biofilms. Flow cells were assembled as previously described (12, 76). The flow system apparatus was sterilized and pre-conditioned with MMH plus 5µg/mL tetracycline (Tet^5) overnight at ambient temperature (20-22°C). *F. novicida* harboring the pKK219-GFP plasmid (26, 41) was grown overnight at 26° C in MMH Tet^5 with aeration. Overnight-grown bacteria were diluted 1:50 in fresh media and grown to optical density 600 (OD_{600}) 1.0. The culture was then diluted to OD_{600} 0.1. Flow was stopped on the flow system and 1ml of culture was inoculated into each channel of the flow cell. Flow cells were inverted for 1 h to allow the bacteria to adhere. Flow cells were then uprighted and flow was initiated at 0.1 ml/minute. Biofilm progression at ambient temperature was imaged by confocal microscopy (Bio-Rad, Hercules, CA) every 24h over the course of 5 days. Z-sections were taken with 0.1 μm steps and 3-D renderings of the z-stacks were generated using Volocity imaging software (Improvision, Lexington, MA).

Crystal violet assaying for biofilm formation. Crystal violet assaying for biofilm formation was performed as previously described (59). Briefly, *Francisella* strains were grown overnight at the appropriate temperature. Cultures were diluted into fresh media to OD_{600} 0.05 and 200µl aliquoted per well in a 96-well polystyrene plate in at least triplicate. The bacteria were allowed to grow statically and sampled at various time points. The OD_{570} was read in a 96-well microplate reader (BioTek, Winooski, VT). At each time point non-adhered bacteria were removed from the well and 30µl of 0.1% crystal violet was added to each well for 15 minutes. Wells were washed three times with distilled water and the remaining biomass-absorbed crystal violet was solubilized with 95% ethanol. Staining was then quantified at OD_{570} in a 96-well microplate reader (labeled CV_{570}). All OD readings for the assay comparing relative crystal violet staining between lab strains of *Francisella* and Type A *Francisella* were obtained at 600nm (CV_{600}) using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Transposon library screen for biofilm-deficient mutants. A sequenced two-allele transposon mutant library was used to test for *F. novicida* transposon mutants that were deficient in biofilm formation (the following reagent was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: *F. tularensis* subsp. *novicida*, "Two-Allele" Transposon Mutant Library Plates 1-14, 16-32). The library represents two or more transposon insertions in all non-essential genes. At the time of screening, Plate 15 of the library was unavailable due to quality control issues, resulting in a library size of 2,954 mutants. The two-allele library was received frozen in 96-well format. MMH media was inoculated in 96-well plates with the library and mutants grown overnight to stationary phase at 37°C shaking at 200 rpm. Overnight cultures were diluted 1:50 in 200µl of fresh MMH in 96-well plates. Plates were grown statically for 10h in a 37°C incubator and the ability of each transposon-mutant to

form a biofilm was assessed as described above. Mutants exhibiting lower potential for biofilm formation were classified by crystal violet staining more than two standard deviations lower than the plate average. Wild-type *F. novicida* was included on each plate as a positive control and a well of MMH only was used as a blank. To account for small differences in culture growth, crystal violet staining was normalized to each mutant culture at OD_{570}. Wells where significant growth defects were observed were excluded. Biofilm-deficient transposon-mutants were retested in triplicate.

Secondary screening for attachment. Overnight cultures of biofilm mutants identified in our screen were grown in triplicate with shaking (200 rpm) at 37°C. Stationary phase cultures (200µl) were transferred to new 96-well plates and allowed to adhere statically for 1 h at 37°C. Crystal violet staining was assayed as before. Attachment-deficiency was defined as crystal violet staining two standard deviations below that of wild-type.

Bacterial Mutagenesis. Targeted deletions were generated in the U112 strain as previously described (9) using the primers in Table A1. Briefly, the regions of the chromosome 5' and 3' to the gene of interest were amplified by PCR. Using splicing by overlap extension (SOE) PCR (44), a kanamycin resistance cassette expressed by the *groEL* promoter was introduced between these regions of homology. Briefly, ~500bp sequences flanking the targeted gene were amplified and spliced to either end of the *gro* promoter-resistance cassette construct. The resulting PCR product was transformed into *F. novicida* strain U112 by chemical transformation and transformants were selected on MMH agar with 30µg/ml kanamycin. Gene deletions were confirmed by sequencing. *ΔsecB1, ΔFTN_1750,* and *chiA* targeted deletion strains were subsequently complemented in cis by re-introducing the wild-type gene into the chromosome at the original locus, along with the CAT cassette chloramphenicol resistance marker, again by

SOE and homologous recombination of a spliced PCR construct. *ΔsecB2, ΔostA2*, *ΔFTN_0308,* and *chiB* deletion mutants were complemented in trans by introducing the wild-type gene, as well as the CAT cassette, into *gro-gfp* pFNLTP6 (46). ~500bp regions flanking the *gfp* gene of pFNTLTP were amplified and spliced to the wild-type copy of the gene for complementation with the CAT resistance cassette on the 3' end. SOE PCR complementation constructs were introduced by homologous recombination with the pFNLTP6 at the NdeI and BamHI sites, removing the *gfp* gene. The resulting plasmid expressed the complementing gene under the regulation of the constitutive *groEL* promoter. Complemented strains were selected for growth on 3μg/ml chloramphenicol and also confirmed by sequencing. Complementation plasmids were then chemically transformed into deletion strains. All complementation primers are listed in Table A1. The Δ*chiA*Δ*chiB* double mutant was constructed using the same method as the single deletion strains, except the *chiB* gene was replaced with the CAT cassette instead of the kanamycin resistance cassette.

RAW264.7 macrophage infections. RAW264.7 macrophages were seeded at $2.5x10^5$ cells per well in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) and incubated overnight at 37°C incubation with 5% CO_2. Wild-type and mutant U112 strains were grown overnight to stationary phase at 37°C with aeration and diluted to $5x10^6$ colony forming units (CFU) per ml in Dulbecco's Modified Eagle Medium (Gibco, Carlsbad, CA) with 10% fetal bovine serum. For each strain, 1 ml inocula were added to triplicate wells and centrifuged at 730 x *g* for 15 min to mediate attachment. Infected plates were incubated at 37°C with 5% CO_2 (time zero) for 0.5h and washed three times with warm media. Three wells per strain were harvested at this time using 0.1% saponin to lyse the cells. CFU were enumerated by serial dilution and

percent recovered was calculated by normalizing to the inocula. Fresh warm media was added to the remaining wells and wells were harvested in triplicate, as above, at 8h and 24h post-infection.

Mouse infections. Competitive index (CI) mouse infections were performed as previously described (80) in 6-8 week old female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). Mice were infected intradermally or intraperitoneally with equal amounts ($5x10^3$ CFU) of wild type and mutant *F. novicida* in 0.05 ml. Mice were monitored for morbidity and mortality during the course of infection. Mice were sacrificed 2 d post-infection and the spleens were removed and homogenized for CFU enumeration. Competitive indices were calculated as the ratio of mutant to wild type of the output, normalized for the input, and significance was calculated by comparing the CI to 1 (CI of gene with no role in virulence) using one sample *t*-tests. All animal infection experiments were approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of Stanford University. Deletion mutants for the entire *Francisella* Pathogenecity Island (FPI) and negative control, *hspX* chaperone gene were described previously (80).

Crab shell attachment. Overnight cultures were grown at 30°C in MMH medium. Approximately $1cm^2$ pieces of sterile crab shell were inoculated with 2ml of stationary phase cultures in 12-well plates. After 1 h at 30°C, the shells were washed to remove unattached bacteria. Attached bacteria were recovered by vortexing and enumerated for colony forming units (CFUs). All strains were tested in triplicate. Unpaired t-tests were used to determine statistical differences between wild type and mutant counts.

Statistical analysis.

Statistical analysis was performed using Prism4 software (GraphPad, La Jolla, CA). Unless otherwise stated, unpaired Student's *t* tests were applied, and two-tailed *P*-values are shown. For

mouse CI data, one-sample *t*-test was used to compare mutant:wild-type bacteria ratio to an expected value of 1.

RESULTS AND DISCUSSION

***F. novicida* forms a biofilm on chitin surfaces.** We hypothesized that chitin may be an environmentally relevant surface for the persistence of *F. tularensis* in nature based on the presence of two well-conserved chitinase genes in the sequenced *F. tularensis* genomes (Table 1). Maintenance of the *chiA* and *chiB* genes in *F. tularensis* subspecies and the related but divergent fish pathogen, *Francisella philomiragia*, suggested that chitinases provide a selective advantage for *Francisella* species in nature. *F. tularensis* subsp. *novicida* (*F. novicida*) is a close relative of the highly virulent Type A *F. tularensis* subsp. *tularensis* and encodes for both chitinase enzymes. Because *F. novicida* is genetically tractable, we use this subspecies here as a model to study the molecular aspects of *F. tularensis* ecology.

To test the ability of *F. tularensis* species to adhere to a chitin-containing surface, we incubated *F. novicida* with crab shell pieces. Crab shells are rich in chitin, a constituent of various surfaces *Francisella* species may encounter and subsequently colonize in their natural habitats. These surfaces include copepod and zooplankton shells in fresh water environments and the exoskeletons of arthropod vectors. After 1 h at 30°C, individual and small groups of adhered bacteria were present on the shell surface as visualized by scanning electron microscopy (SEM) (Fig. 1A,B). After one week on the crab shells in the presence of minimal Chamberlain's defined medium (CDM), without exogenous sugar, three-dimensional bacterial communities were present on the chitin-based surface (Fig. 1C). At higher magnification (Fig. 1D), we saw microcolonies to consist of individual bacteria surrounded by a matrix of extracellular polymeric substance (EPS). The observed community structure suggests *F. novicida* can attach and proliferate as biofilms on the environmentally relevant surface, chitin.

Although crab shells consist mainly of chitin, they contain additional components, such as other carbohydrates and protein (56). To test if chitin is sufficient to support *F. novicida* colonization and proliferation, we visualized bacterial attachment and biofilm formation on synthetic chitin films (82). *F. novicida* attached to lower levels on smooth chitin films compared to the topographically varied crab shells after one hour (Fig. 1A,E). At one week after shift to minimal medium, the surface of the chitin films contained *F. novicida* microcolonies and EPS extensions (Fig. 1G,H), indicating the initiation biofilm formation. However, the architecture of the bacterial communities on chitin films was not as developed as the communities on the crab shell pieces (Fig. 1A-D), which may be explained by the lower starting population on this surface (Fig. 1C,G). More likely, additional components in the crab shell, like protein, may allow for more rapid expansion of the adhered population. We conclude that chitin is necessary, but not necessarily sufficient for wild-type levels of *F. novicida* biofilm maturation in the absence of exogenous sugar.

F. novicida can utilize GlcNAc as a carbon source for growth. *F. novicida* persistence and proliferation on chitin surfaces in the absence of exogenous sugar suggested that this pathogen was able to utilize the chitin component of the surface as a nutrient source. To test this, we grew *F. novicida* in CDM either without added sugar, supplemented with 10mM glucose (a known metabolic substrate for *Francisella* species), or with 10mM GlcNAc (the monosaccharide end product of chitin hydrolysis) in aerated batch culture. *F. novicida* growth was negligible in CDM in the absence of an added sugar (doubling time 11.25h). In contrast, *F. novicida* grew in CDM supplemented with 10mM glucose with a doubling time of 63 min. Similarly, *F. novicida* grew in CDM supplemented with 10mM GlcNAc (doubling time 76 min.). The high proliferation of *F. novicida* on chitin surfaces (Fig. 1) may therefore be explained by the ~11-

fold increase in growth rate between *F. novicida* grown in CDM with GlcNAc compared to CDM without sugar. We conclude that *F. novicida* can metabolize GlcNAc, suggesting that hydrolysis of chitin by chitinases to generate GlcNAc (36) may provide a local nutrient source for persistence and growth.

Chitinase genes facilitate *F. novicida* growth on chitin surfaces. To further address the importance of chitin as a non-host niche for *Francisella* in nature, we constructed *F. novicida* mutants lacking either of the chitinase genes, *ΔchiA* and *ΔchiB*, and a mutant lacking both chitinases. Hager *et al*. demonstrated that the *F. novicida* homologs of these enzymes that contain chitin-binding domains are secreted and bind to chitin beads (29). We expected these deletion mutant strains to be attenuated for persistence and biofilm formation on chitin surfaces if *F. tularensis* species have evolved to form biofilms on chitin surfaces to scavenge carbon. Indeed, the *ΔchiA* and *ΔchiB* deletion mutants were attenuated for colonization of crab shells when incubated in CDM without sugar. Although the chitinase mutant bacteria attached to chitin to the same extent as wild-type *F. novicida* at 1 h (data not shown), we recovered 16- and 15-fold fewer *ΔchiA* and *ΔchiB* mutant bacteria compared to wild-type *F. novicida* ($P<0.001$), respectively, after 2 days colonization on crab shells (Fig. 2A). Furthermore, we recovered the same number of *ΔchiAΔchiB* double chitinase mutant bacteria compared to the *ΔchiA* or *ΔchiB* single chitinase mutant strains (Fig. 2A), suggesting that the two chitinase genes act in the same metabolic pathway, as predicted by KEGG pathway analysis (34). The abilities of the *ΔchiA* and *ΔchiB* mutant bacterial strains to grow on chitin was restored by the reintroduction of wild-type copies of each chitinase gene into the coinciding mutant strain as measured by increased crab shell colonization to near wild-type *F. novicida* levels (Fig. 2A). The ability of the chitinase mutant strains to persist at low levels could be due to the utilization of the amino acids present in

the CDM medium. Alternatively, natural degradation of the crab shell during the experiment could liberate enough free GlcNAc to enable the bacteria to persist, but not replicate. Regardless, the highly significant difference between wild-type and chitinase mutant bacteria suggests that chitinase activity strongly contributes to *F. novicida* persistence on chitin in otherwise carbon-limiting conditions.

We postulated that the inability of chitinase mutant bacteria to convert chitin to the useable metabolite, GlcNAc, explains their attenuated colonization on chitin. Indeed, the inability of the chitinase mutants to colonize crab shells was alleviated by the addition of 10mM GlcNAc to the exogenous medium (Fig. 2B), indicating that the chitinase mutant bacteria possess the determinants required to colonize a chitin surface, but lack the ability to generate a useable carbon source in order to proliferate. The 13-fold decrease in recovered wild-type *F. novicida* when GlcNAc was added (Fig. 2) is consistent with microarray data published for *V. cholerae* demonstrating that when this pathogen was grown in the presence of excess GlcNAc, the pili and chitinases required to colonize this surface were repressed (49).

We next compared the architecture of the communities formed by the chitinase mutants on crab shells or chitin films in the absence of exogenous sugar for 1 week by scanning electron microscopy. In contrast to wild-type *F. novicida*, the chitinase mutants were present as single bacteria or small, mostly monolayer, clusters of bacteria (Fig. 3). We conclude that *F. novicida* biofilm formation on chitin in the absence of exogenous sugar requires functional chitinase enzymes. Unlike motile *V. cholerae* that can chemotax towards nutrients, *F. tularensis* species are non-flagellated and non-motile under laboratory conditions (14). Therefore, the ability of *Francisella* species to adhere and colonize chitin may represent a single mechanism for survival in nutrient poor non-host environments. Growth on chitin may trigger a specific biofilm

program of genes that promote the retention of scavenged GlcNAc in the local microenvironment for use by *F. tularensis*.

Beyond scavenging carbon in the environment, the secreted chitinases that are vital for biofilm formation on chitin could be important for the establishment of the arthropod infection, similar to the malaria parasite *Plasmodium falciparum* (79). The *P. falciparum* chitinase allows the parasite to penetrate the chitin-containing peritrophic matrix surrounding the blood meal in the mosquito midgut and establish the infection. Efforts to target this chitinase to block transmission of malaria are ongoing (69, 73). We are currently working to discern the role(s) of *F. tularensis* chitinases in arthropod vectors.

Characterization of biofilm development by *Francisella* species. *F. tularensis* chitin utilization provides insight into potential persistence mechanisms of this highly virulent pathogen. The missing piece to our model was the proteins that promote attachment to chitin surfaces. We established in vitro systems for studying *F. tularensis* biofilm formation to aid in identifying attachment determinants. In vitro biofilms on abiotic surfaces provided a model system to characterize and genetically dissect *F. novicida* biofilm formation and test the ability of other pathogenic *F. tularensis* strains to similarly attach and proliferate on a surface.

We incubated GFP-labeled *F. novicida* in the flow cell system (12) to confirm in vitro formation of these bacterial communities under flow conditions. Bacterial attachment and surface growth at ambient temperature (20-22°C) and a flow rate of 0.1ml/min was analysed by confocal laser scanning microscopy (CLSM) at various timepoints (24h, 48h, 72h, 96h and 120h) (Fig. 4). We observed the formation of a matt-like biofilm with an average depth of approximately 15μm. This architecture of flow cell grown *F. novicida* biofilms was similar to that reported for other Gram-negative species, including the related γ-proteobacterium

Shewanella oneidensis (76) and α-proteobacterium *Caulobacter crescentus* (20). Our results indicate that *F. novicida* is able to form biofilms on an abiotic surface, such as glass, with similar architecture to that observed on chitin (Fig. 1). These results are consistent with the report by Hassett *et al*. indicating that LVS can form biofilms on glass coverslips in the absence of flowing media (30).

A modified O'Toole and Kolter microtiter assay (59) was utilized to establish a high throughput model for *F. tularensis* biofilm formation. This assay measures adhered biomass under static conditions by crystal violet stain. *F. novicida* and *F. tularensis* subsp. *holarctica* live-vaccine (LVS) strains were grown at 26°C and 37°C in 96-well microtiter plates. Recent work by Horzempa *et al*. found that the LVS strain demonstrated different expression profiles at these two temperatures (31). The OD_{570} (Fig. 5A,B) and crystal violet staining (CV_{570}), (Fig. 5C,D) were measured over 152 h. Both *F. novicida* and LVS strains showed increased crystal violet staining over time when grown at 26°C and 37°C, indicating increased accumulation of adhered biomass. This result is consistent with our finding that *F. novicida* forms biofilms when adherent to an abiotic surface (Fig 4). At both temperatures assayed, we observed a decrease in crystal violet staining (Fig. 5C,D) concurrent with *F. novicida* and LVS entering stationary phase (Fig. 5A,B). This result suggested that the biofilms were undergoing dispersion (75), a process of biofilm dissolution and re-seeding occurring during decreased oxygen tension and nutrient deprivation. Similar dispersal did not occur in the flow cell system grown *F. novicida* biofilms (Fig. 4), presumably because the population was constantly provided an undepleted carbon and oxygen source under flow conditions.

Type A *Francisella* strains form biofilms in the microtiter plate assay. A high percentage of tularemia morbidity and mortality is caused by infection with *F. tularensis* subsp. *tularensis*

(Type A) strains (22). These strains have a very low infectious dose, and as few as ten organisms can cause a lethal infection in humans (19). Molecular subtyping of Type A strains has identified two distinct subtypes (A1 and A2) with specific geographic distributions (40). Type A1 strains are primarily found in the Eastern United States, while Type A2 strains are almost exclusively isolated in the West. The O'Toole and Kolter assay demonstrated that these highly virulent strains were able to form biofilms to similar levels as *F. novicida* and LVS strains (Fig. 6). SchuS4 (Type A1) and FT-10 (Type A2) *F. tularensis* subsp. *tularensis* strains reached similar optical densities as LVS (Type B) when grown under static conditions (Fig. 6), while SchuS4 and FT-10 exhibited higher crystal violet staining at 24 h ($P<0.05$), implying increased biofilm formation of Type A strains (Fig. 6). *F. novicida* CV_{600} staining was approximately two-fold higher than the other strains tested ($P<0.001$). However, the optical density of the *F. novicida* culture was 2.5-fold higher than the other strains at 24h. Similar crystal violet staining by Type A1 and Type A2 strains compared to the Type B LVS strain suggests that biofilm formation may be pertinent to the survival of pathogenic *F. tularensis* strains in the environment.

Screen for biofilm-deficient mutants identifies novel genes important for *F. novicida* biofilm formation. We screened a two-allele transposon library (BEI Resources, Manassas, VA) that represented two or more transposon-insertion mutants per non-essential gene in the *F. novicida* genome to elucidate the genetic determinants of *F. novicida* interaction with abiotic and biotic surfaces. To facilitate high-throughput screening, individual insertion mutants were assayed for biofilm formation in the microtiter assay established above rather than on chitin. We defined biofilm-deficient mutants as strains where crystal violet staining was two standard deviations below the mean of the plate. We eliminated mutant strains that exhibited a significant growth defect from further characterization. In total, we identified 98 *F. novicida* transposon-

insertion mutants, representing 88 genes that were attenuated for biofilm formation (Table A2). To elucidate pathways important for *F. novicida* surface attachment and growth, we assigned gene ontology classifications to the genes identified in the biofilm screen (www.biohealthbase.org) (Fig. A1). Roles for the 64 annotated genes included protein secretion, various metabolic pathways, signal transduction, protein transport, and cell envelope biogenesis. Although many Gram-negative and Gram-positive bacteria can form biofilms, the bacterial mechanisms utilized to facilitate these communities vary (4, 45, 55, 59, 78). For example, the role of Type-IV pili and flagella in biofilm formation is well documented (58, 65). However, little is known about attachment and surface growth during biofilm maturation of non-motile bacteria. By characterizing the roles of the genes we identified in this study, including the approximately 25% with no annotated function, we aim to elucidate alternate methods of environmental persistence by non-motile bacteria.

Sec-dependent secretion functions in initial attachment during *F. novicida* biofilm formation on abiotic and biotic surfaces. We were particularly interested in the four transposon-insertion mutants we identified in the Sec translocon complex involved in protein export from the cytoplasm (25). The core components of the Sec translocon in *Escherichia coli* are the SecYEG protein channel and the SecA ATPase motor protein (10). Due to the pleiotropic roles of general protein secretion in bacteria, components in this apparatus are considered essential in other Gram-negative organisms (24, 43). The Sec translocon in *F. novicida* is comprised of 13 proteins, but only the four genes we identified in our screen were represented in the two-allele library; the *secA* motor ATPase and *secG* pore genes, as well as the *secB1* and *secB2* genes that encode for chaperones which specifically target pre-proteins to SecA (77). Additionally, we identified 18 transposon biofilm mutant clones, representing 14 genes

that are predicted to encode for proteins with secretion signals based on the signal sequence detection algorithm, SignalP (7) (Table 2).

We hypothesized from the results of our genetic biofilm screen and a secondary attachment assay that proteins secreted by the Sec translocon may represent novel mediators of *F. novicida* adhesion, a process that has not been characterized. We confirmed that transposon mutants in the secretion apparatus were deficient for biofilm formation (Fig. 7A) and attachment (Fig. 7B). Deletion mutants in *secB1* and *secB2* were constructed while deletions in *secA* and *secG* could not be generated; suggesting that these genes are essential and the transposon-insertion mutations present in the library represent an incomplete loss of gene function. Additionally, attempts to construct a double deletion of *secB1* and *secB2* did not yield viable colonies. Growth curves performed with the *ΔsecB1* and *ΔsecB2* mutants showed no growth defect in batch culture compared to wild-type *F. novicida* and microscopic analysis of cell morphology revealed no alternations in bacterial shape (data not shown). Both the *ΔsecB1* and *ΔsecB2* mutants were deficient in biofilm formation (Fig 7C) and attachment (Fig 7D) when grown in MMH. The *ΔsecB1* and *ΔsecB2* mutant phenotypes were restored to wild-type attachment and biofilm formation levels when wild-type copies of *secB1* and *secB2* were added back to the deletion mutants (Fig. 7C,D). These experiments were also performed in CDM to confirm that the role of Sec-dependent secreted factors in biofilm formation was not limited to growth in a nutrient-rich environment (Fig. 7E,F). Our data indicate that Sec-dependent secretion is important for *F. novicida* attachment to abiotic surfaces and biofilm formation. We therefore postulated that Sec-secreted proteins represent novel mediators of *F. novicida* adherence.

The 18 Sec-dependent transposon-insertion mutants (Table 2) were all defective for biofilm formation (Fig. 7A) and initial attachment (Fig. 7B) based on crystal violet staining, confirming our screen results. Surprisingly Type-IV pili genes, known mediators of biofilm formation in Gram negative bacteria, were not among the Sec-secreted factors identified and were found to be dispensible for *F. novicida* biofilm formation upon further study (data not shown).

We focused on four of the secreted factors with homologs in all *F. tularensis* subspecies and were highly attenuated for biofilm formation when deleted; FTN_0308, FTN_0713, FTN_0714 and FTN_1750. FTN_0713 (*ostA2*), FTN_0714 and FTN_1750 were all identified at least twice in the biofilm screen. We selected FTN_0308 due to the strong biofilm phenotype of the one transposon-insertion mutant that was identified in the genetic screen (Fig. 7A,B). We constructed deletion mutants in each of these genes and tested for attachment and biofilm formation. All four mutants were defective in initial attachment and biofilm formation in both rich and defined media (Fig 7C-F). The *ΔostA2*, *ΔFTN_1750* and *ΔFTN*_0308 mutants were complemented for attachment and biofilm attenuation by re-introduction of the deleted genes in *cis* into the chromosome or in *trans* by expressing the gene in pFNLTP6 using the constitutive *gro* promoter. The *ΔFTN_0714* mutant could not be complemented for technical reasons, likely due to the length of the complementation PCR product (~8kb). Taken together, our data indicate that initial attachment during *Francisella* biofilm formation is facilitated by proteins secreted by the Sec-dependent secretion system.

The protein encoded by FTN_0713 (*ostA2*) has significant homology (E-value $6e^{-64}$) to organic solvent tolerance proteins involved in lipopolysaccharide (LPS) modification (8). Although *ostA2* homologs have not been implicated in biofilm formation, LPS chemistry has

been shown to influence attachment during biofilm formation in other bacteria (4, 16, 23). The unique structure of *Francisella* species LPS (28) could contribute to adhesion of *F. tularensis* to non-mammalian surfaces. FTN_1750 is a putative acyltransferase with strong homology (E-value $4e^{-27}$) to acylhomoserine-lactone biosynthesis enzyme, HdtS, suggesting that this protein may function in quorum sensing, a cell-cell communications process that regulates biofilm formation under certain conditions (38).

While *F. novicida* biofilm genes were identified by screening for mutants defective for adherence and biofilm formation on polystyrene, we identified two novel putative chitin-binding proteins, FTN_0308 and FTN_0714. The protein encoded by FTN_0714 is annotated as a hypothetical lipoprotein (BioHealthBase). The SMART domain prediction algorithm (42, 68) indicates that FTN_0714 contains repeating polycystic kidney disease-family domains conserved from archae through mammals that facilitate adhesion (33). This domain-family plays a role in the binding and hydrolysis of chitin by the *chiA* chitinase of aquatic bacterial strain *Alteromonas 0-7* (60). FTN_0308 is annotated to encode for a hypothetical protein with unknown function (www.biohealthbase.org). However, the Phyre protein-folding prediction algorithm (35) indicates a structural homology to the *Streptomyces* chitinase C chitin-binding domain and the C-terminus contains homology to F17c-family bacterial adhesins. We are currently determining the specific roles that these two gene products may have in attachment to both abiotic and chitin surfaces.

F. tularensis species genomes contain an annotated chitin-binding protein, *cbpA*, that was not identified by our biofilm screen. This gene product may specifically mediate association with chitin. Additionally, we did not identify *chiA* and *chiB* in our screen despite their conserved Sec-dependent secretion signals and role in biofilm formation on chitin. We would not expect

chitinases to mediate biofilm formation on polystyrene, however. This result was confirmed using clean deletion mutant stains (data not shown).

Sec secretion mutants are not attenuated in murine models of infection. While no evidence of *F. tularensis* biofilm formation inside of mammalian hosts exists, bacteria often utilize proteins to attach to both environmental and host surfaces (65, 67, 70). Attachment of *F. tularensis* in any context is poorly understood. We, therefore, tested if the biofilm attachment factors we describe also mediate host tissue association using in vitro and in vivo infection models. *F. tularensis* species are primarily found within macrophages in a mammalian host (27). Therefore, RAW264.7 macrophage-like cells were infected at a multiplicity of infection of 20:1 with wild-type *F. novicida* or the Sec-dependent secretion mutants. At 0.5 h post-infection, non-cell associated *F. novicida* were washed away and the remaining bacteria were recovered and enumerated. No statistical differences in CFU counts were observed (Fig. 8A), suggesting that the mutants that are defective for attachment to polystyrene and chitin were still able to efficiently associate with eukaryotic cells. Intracellular replication was monitored in the presence of extracellular gentamicin for 8- and 24h (Fig. 8B). Wild-type *F. novicida* and all mutants showed approximately 100–fold replication at 24h compared to the initial 0.5 h counts. Thus, the mutants successfully entered and replicated within macrophages, demonstrating that the Sec secretion biofilm mutants that we characterized are not deficient for attachment to, or replication within macrophages.

To test the potential role of *secB1, secB2, ostA2*, *FTN_0308, FTN_0714* and *FTN_1750* during a systemic mouse infection, we infected C57BL/6J mice with a 1:1 mixture of $5x10^3$ colony forming units of wild-type and deletion mutant bacteria. Competitive indices (CI's) for each wild-type/mutant combination were obtained for both intradermal (ID) and intraperitoneal

(IP) routes of infection. Mutants that are not attenuated in mice should have a CI of one, *i.e.*, equal numbers of wild-type and mutant bacteria are recovered in the tissue at the time of harvest, as is observed for the previously described Δ*hspX* strain (80). As a positive control, we included a *F. novicida* ΔFPI mutant that lacks the entire *Francisella* pathogenecity island (80). As expected, this mutant was severely attenuated in mice (Fig. 8C,D). However, none of the Sec secretion biofilm mutants demonstrated a CI value statistically different from one via either route of infection (Fig. 8C,D). Additionally, no defect was observed in the spread of Sec secretion mutants to systemic tissues such as the liver and spleen after ID inoculation (data not shown). Our data indicate that these genes that are crucial for association to non-mammalian surfaces do not contribute to local or systemic colonization of mammalian hosts. FTN_0713 (*ostA2*), the putative LPS-modification gene, was identified by Kraemer *et al*. in a negative selection screen for *F. novicida* mutants attenuated for infection via intranasal inoculation of mice, indicating that this mutant may be more sensitive to the innate immune response in the lung, (e.g., antimicrobial peptides) due to an altered LPS (39, 80). Transposon-mutants for *secA* and *secE* were identified by Su *et al*. to be involved in lung colonization (72). These attenuated phenotypes for the non-redundant Sec translocon genes imply that Sec secreted proteins other than those characterized here do influence host colonization. The lack of attenuation for the deletion mutants in *secB1* and *secB2* in the virulence assays tested here supports the idea that these two genes encode for redundant function.

***F. novicida* biofilm determinants also play a role in attachment to chitin-based surfaces.** Given *Francisella* species induce biofilm formation on both abiotic and chitin surfaces, we hypothesized that the attachment determinants we identified for association with polystyrene may also facilitate attachment to chitin. After a 1 h incubation at 30°C, an average of 3.33×10^7

CFU/ml wild-type *F. novicida* were attached to the crab shell pieces (Fig. 9). Sec secretion mutants were 5.6- to 16.2-fold attenuated for attachment to this chitin-based surface compared to wild-type bacteria ($P<0.01$), confirming that Sec-secreted proteins contribute to attachment to chitin-based surfaces. The specificity of these adherence factors for non-mammalian surfaces further supports our suggestion that *F. tularensis* biofilm formation in nature has evolved to promote this pathogen's survival outside of a host; potentially by facilitating chitin utilization.

From our collective data we propose a model in which these early determinants of biofilm formation allow for association with chitin surfaces in nature. Through this interaction, *F. tularensis* chitinases have access to this substrate and provide bacteria with GlcNAc, which is utilized for growth in nutrient-limiting environments. Biofilm maturation on chitin would then create a local microenvironment enriched for this carbon source, providing a non-host niche for this zoonotic pathogen.

The ability of *F. tularensis* to form biofilms on chitin may also provide the bacterium resistance to grazing by fresh water protozoa. For chitin-colonizer *V. cholerae*, biofilm formation was shown to reduce grazing by flagellate organisms compared to planktonic bacteria (48). Thelaus *et al*. found that *F. tularensis* subsp. *holarctica* had increased resistance to both ciliate and flagellate protozoa compared to *E. coli* (74). Although the role of biofilm formation on predation was not addressed, this observation suggests that *F. tularensis* may actively prevent protozoal grazing in nature. Coupled with the ability to survive in nutrient-limited aquatic environments, biofilm-mediated resistance to predation could contribute to *F. tularensis* persistence in the environment and allow for prolonged transmission of this pathogen.

We provide here the first extensive characterization of *F. tularensis* biofilm formation and explore how these communities may promote environmental persistence and transmission on

chitin surfaces. The *F. novicida* biofilm genes we describe contribute to the ability of this pathogen to colonize a surface it may encounter in nature. Very little is known about how and where *Francisella* species persist in nature when not replicating within a host. Our findings may help explain how tularemia outbreaks that have been attributed to fresh water crustaceans (2, 17) occur. Additionally, chitin utilization may support *F. tularensis* persistence on other arthropods such as zooplankton, copepods, and biting arthropod vectors. A study of *F. tularensis* survival in artificial water found that the presence of chitinous fresh water shrimps, mullosks, diatoms, or zooplankton promoted sustained viability of this pathogen for an additional week to one month in nutrient-poor water (52). Survival on environmental chitin may therefore serve as a reservoir for disease transmission during seasonal tularemia outbreaks. Palo *et al*. identified a strong epidemiological correlation between areas with low water turnover and human cases of tularemia (64). These researchers postulated low water turnover as an environmental cue for a burst of *F. tularensis* replication. As with cholera outbreaks (21), conditions that promote interaction of *F. tularensis* with chitin surfaces on which the bacteria can replicate may seed infection. Further study of *F. tularensis* biofilm formation and the role of these communities in chitin colonization could clarify the open question of the location of the *F. tularensis* environmental reservoir.

ACKNOWLEDGEMENTS

We thank Melanie Blokesch for her generous gifts of reagents and technical assistance, Gary K. Schoolnik for his thoughtful discussions and for providing the chitin films. Jonathan W. Jones and Thomas Henry for help with mouse experiments and Carmen D. Cordova for assistance with the biofilm CLSM imaging. Jean Celli graciously provided the SchuS4 strain. J.J.M was supported by National Science Foundation and Department of Homeland Security graduate fellowships, as well as a National Institutes of Health Cell and Molecular Biology training grant.

FIGURE LEGENDS

Figure 1. *F. novicida* biofilm formation on chitin surfaces. Images display SEM visualization of F. novicida colonization of crab shell pieces (A-D) and synthetic chitin films (E-H). Individual attached bacteria and small attached microcolonies were observed on the crab shell pieces at one hour (A,B). After one week, typical 3D biofilm architecture was observed, consisting of bacteria surrounded by an EPS matrix (C,D). Similar results were obtained after one hour (E,F) and one week (G,H) on synthetic chitin. Scale bar is 20μm for lower magnification images (left column) and 5μm for higher magnification images (right column).

Figure 2. Chitinase mutants are attenuated for chitin colonization in the absence of exogenous sugar. Stationary phase wild-type and chitinase mutant bacteria were allowed to adhere for 1 h to crab shell pieces. Equivalently adhered strains were allowed to colonize these chitin surfaces in CDM with or without GlcNAc at 30°C. Triplicate samples were harvested 2 d post-inoculation and enumerated for CFU. Chitinase mutant *F. novicida* (white) were recovered at statistically lower levels than wild-type bacteria (black), ($P<0.001$) when incubated in CDM (A), but in equivalent numbers in CDM with GlcNAc (B). Addition of wild-type *chiA* and *chiB* genes to deletion mutant strains (grey) complemented the chitin colonization defects observed during colonization in CDM without GlcNAc (A).

Figure 3. Chitinase genes are required for biofilm architecture on chitin surfaces during nutrient stress. Images show representative colonization by wild-type and chitinase mutant strains on crab shells (A-D) or synthetic chitin films (E-H). Bacteria were allowed to attach for 1 h and then incubated for one week at 30°C before being processed for SEM. In contrast to

extensive 3D biofilm development in wild-type *F. novicida*, the chitinase mutants were present as single bacteria or small clusters of bacteria on both natural and synthetic chitin. Scale bar is 10μm.

Figure 4. *Francisella* forms a matt-like biofilm under flow conditions. GFP expressing *F. novicida* grown at room temperature (20-22°C) were imaged daily in flow cells run at 0.1ml/min using confocal laser scanning microscopy. Representative images from triplicate experiments are shown. At 24h (A), small groups of bacteria are present. Over the next 48h (B,C), a uniform monolayer of bacteria is observed on the surface. By 96h (D), depth in the biofilm is observed and at 120h (E) the biofilm reached an average thickness of 15μm. Scale bar is 15.2μm.

Figure 5. Kinetics of *F. tularensis* biofilm formation under static conditions. A modified O'Toole and Kolter assay was performed to compare the kinetics and relative levels of biofilm formation for *F. novicida* (solid circles) and LVS (open circles). Bacterial growth (A,B) and crystal violet staining (C,D) were determined over time at 26°C (A,C) and 37°C (B,D) by OD_{570} readings. Both *F. tularensis* strains were found to acquire crystal violet stain at both temperatures. Growth and crystal violet staining were faster at 37°C for both strains.

Figure 6. Biofilm formation by virulent *F. tularensis* subspecies *tularensis* strains. *F. novicida*, LVS and Type A strains SchuS4 and FT-10 were assayed for growth and crystal violet staining at 24h post-inoculation. Culture OD_{600} (A) and crystal violet staining (B) were determined after static growth at 37°C. *F. novicida* demonstrated increased growth kinetics and

crystal violet staining compared to the other strains (P>0.001). Virulent SchuS4 and FT-10 strains exhibited significantly higher crystal violet staining compared to LVS.

Figure 7. Sec-secreted factors mediate initial attachment during biofilm formation. transposon-insertions representing mutants in 4 genes in the Sec translocon (grey) and 18 transposon-insertions representing mutants in 14 genes in putative secreted factors (white) identified in the forward genetic screen were tested in triplicate compared to wild-type *F. novicida* (black) for 8h biomass accumulation (A) and 1 h initial attachment (B). Multiple transposon-mutants were tested for genes identified more than once in the screen. Adherence of biomass at 8h was used a measurement for biofilm formation. Attachment was assessed by crystal violet stain 1 h post-inoculation of stationary phase cultures. Targeted mutants in selected representative genes (white) showed similar defects in biofilm formation (C, E) and attachment (D, F) compared to wild-type *F. novicida* (black) when grown in MMH and CDM, respectively, based on crystal violet staining. Complementation of deleted genes (grey) restored mutants to wild-type levels in all cases. Bars represent the mean and the lines indicate standard deviation calculated from triplicate samples of a representative experiment. Each experiment was repeated in triplicate. No data (ND) was obtained for FTN_0714 complementation due to technical difficulties.

Figure 8. The Sec translocon and secreted factors do not influence *F. novicida* virulence. Sec secretion targeted deletion mutants were assessed in *in vitro* and *in vivo* models for *F. tularensis* virulence. Entry efficiency of *F. novicida* strains into RAW264.7 macrophage-like cells was measured as the percent of inocula recovered from inside the cells 30min post-infection

(A). Intracellular replication of wild-type and mutant bacteria was assessed as fold-replication compared to 30 min counts at 8 h and 24 h post-infection (B). The ability of mutants to colonize the skin after intradermal (C) and the spleen after intraperitoneal (D) routes of inoculation was determined by competitive indices in C57BL/6J mice 2 d post-infection. For all virulence assays, no difference was observed between the Sec secretion biofilm mutants and wild-type *F. novicida*.

Figure 9. Biofilm mutants are attenuated for attachment to chitin-based crab shell pieces. Stationary phase cultures of *secB1, secB2, ostA2,* FTN_0308, FTN_0714, and FTN_1750 deletion mutants were allowed to attach for 1 h to sterile crab shell pieces. Attached bacteria were enumerated for CFU in triplicate samples. Sec secretion biofilm mutants were found to attach statistically lower ($P>0.01$) than wild-type *F. novicida* by unpaired *t*-test.

Table 1 – *Francisella* species chitinase genes

Strain	*chiA* homolog (E-value)*	*chiB* homolog (E-value)*
F. tularensis subsp. *tularensis* SchuS4	FTT0715 (9e-66)	FTT_1768c (2e-15)
F. tularensis subsp. *tularensis* FSC198	FTF0715 (3e-70)	FTF_1768c (2e-15)
F. tularensis subsp. *holarctica* LVS	FTL_1521 (9e-66)	FTL_0093 (1e-15)
F. tularensis subsp. *holarctica* OSU18	FTH_1471 (2e-68)	FTH_0088 (1e-15)
F. tularensis subsp. *novicida* U112	FTN_0627 (4e-69)	FTN_1744 (4e-14)
Francisella philomiragia	Fphi_0215 (1e-66)	Fphi_0864 (1e-15)

* E-value based on comparison to glycosyl hydrolase 18 family chitinases

Table 2- Sec translocon and Sec-dependent secreted proteins involved in biofilm formation

FTN	Well ID[a]	Gene	Gene Product	Biological Process	Sec Secretion[b]
FTN_0090	4F07	*acpA*	acid phosphatase	fatty acids and lipids metabolism	Secreted
FTN_0100	20C12		hypothetical membrane protein	hypothetical - novel	Secreted
FTN_0109	14G06		protein of unknown function	unknown function - novel	Secreted
FTN_0121	26G09	*secB1*	preprotein translocase, subunit B	motility, attachment and secretion structure	Translocon
FTN_0121	4F06	*secB1*	preprotein translocase, subunit B	motility, attachment and secretion structure	Translocon
FTN_0191	19 E06		polar amino acid uptake transporter	transport - amino-acid	Secreted
FTN_0304	20C11		pilus assembly protein	motility, attachment and secretion structure	Secreted
FTN_0308	19H06		membrane protein of unknown function	unknown function - novel	Secreted
FTN_0357	21B08	*pal*	peptidoglycan-associated lipoprotein, OmpA family	transport - drugs / antibacterial compounds	Secreted
FTN_0429	14G12		conserved protein of unknown function	unknown function - conserved	Secreted
FTN_0635	25C04		serine-type D-Ala-D-Ala carboxypeptidase	cell wall / LPS / capsule	Secreted
FTN_0672	12G03	*secA*	preprotein translocase, subunit A (ATPase, RNA helicase)	motility, attachment and secretion structure	Translocon
FTN_0713	14C04	*ostA2*	organic solvent tolerance protein OstA	cell wall / LPS / capsule	Secreted
FTN_0713	21 h10	*ostA2*	organic solvent tolerance protein OstA	cell wall / LPS / capsule	Secreted
FTN_0713	26 E07	*ostA2*	organic solvent tolerance protein OstA	cell wall / LPS / capsule	Secreted
FTN_0714	12G01		protein of unknown function	unknown function - novel	Secreted
FTN_0714	27C09		protein of unknown function	unknown function - novel	Secreted
FTN_1093	18A05		protein of unknown function	unknown function - novel	Secreted
FTN_1476	26A03		protein of unknown function	unknown function - novel	Secreted
FTN_1503	26A08		protein of unknown function	unknown function - novel	Secreted
FTN_1510	1 E01	*secB2*	preprotein translocase, subunit B	motility, attachment and secretion structure	Translocon
FTN_1630	13C11	*secG*	preprotein translocase, subunit G, membrane protein	motility, attachment and secretion structure	Translocon
FTN_1750	19H02		acyltransferase	fatty acids and lipids metabolism	Secreted
FTN_1750	23D04		acyltransferase	fatty acids and lipids metabolism	Secreted

[a] Well ID annotation from BEI Resources *F. novicida* Two-Allele Transposon Library.
[b] Genes labeled translocon are structural components of Sec-dependent secretion. Secreted proteins were predicted using SignalP algorithm.

REFERENCES

1. **Adams, J. L., and R. J. C. McLean.** 1999. Impact of rpoS Deletion on Escherichia coli Biofilms. Appl. Environ. Microbiol. **65:**4285-4287.
2. **Anda P., J. S. d. P., J. M. Díaz García, R. Escudero, F. J. García Peña, M. C. López Velasco, R. E. Sellek, M. R. Jiménez Chillarón, L. P. Sánchez Serrano, and J. F. Martínez Navarro.** 2001. Waterborne Outbreak of Tularemia Associated with Crayfish Fishing. Emerging Infectious Diseases **7:**575-582.
3. **Anders, S.** 2007. Tularemia: History, Epidemiology, Pathogen Physiology, and Clinical Manifestations. Annals of the New York Academy of Sciences **1105:**1-29.
4. **Balestrino, D., J. M. Ghigo, N. Charbonnel, J. A. Haagensen, and C. Forestier.** 2008. The characterization of functions involved in the establishment and maturation of Klebsiella pneumoniae in vitro biofilm reveals dual roles for surface exopolysaccharides. Environ Microbiol **10:**685-701.
5. **Bartnicki-Garcia, S.** 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annu Rev Microbiol **22:**87-108.
6. **Bassler, B. L., C. Yu, Y. C. Lee, and S. Roseman.** 1991. Chitin utilization by marine bacteria. Degradation and catabolism of chitin oligosaccharides by Vibrio furnissii. J Biol Chem **266:**24276-86.
7. **Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak.** 2004. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol **340:**783-95.
8. **Braun, M., and T. J. Silhavy.** 2002. Imp/OstA is required for cell envelope biogenesis in Escherichia coli. Mol Microbiol **45:**1289-302.
9. **Brotcke, A., and D. M. Monack.** 2008. Identification of fevR, a novel regulator of virulence gene expression in Francisella novicida. Infect Immun **76:**3473-80.
10. **Brundage, L., J. P. Hendrick, E. Schiebel, A. J. Driessen, and W. Wickner.** 1990. The purified E. coli integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. Cell **62:**649-57.
11. **Celebi, S., M. Hacimustafaoglu, and S. Gedikoglu.** 2008. Tularemia in children. Indian Journal of Pediatrics **75:**1129-1132.
12. **Christensen, B. B., C. Sternberg, J. B. Andersen, R. J. Palmer, A. Toftgaard Nielsen, M. Givskov, S. Molin, and J. D. Ron.** 1999. [2] Molecular tools for study of biofilm physiology, p. 20-42, Methods in Enzymology, vol. Volume 310. Academic Press.
13. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial Biofilms: A Common Cause of Persistent Infections. Science **284:**1318-1322.
14. **Cowan S.T., G. I. B., Kenneth John Steel, R. K. A. Feltham.** 2004. Cowan and Steel's manual for the identification of medical bacteria, 3rd ed. Cambridge Press.
15. **Darby, C., J. W. Hsu, N. Ghori, and S. Falkow.** 2002. Caenorhabditis elegans: Plague bacteria biofilm blocks food intake. Nature **417:**243-244.
16. **de Lima Pimenta, A., P. Di Martino, E. Le Bouder, C. Hulen, and M. A. Blight.** 2003. In vitro identification of two adherence factors required for in vivo virulence of Pseudomonas fluorescens. Microbes Infect **5:**1177-87.
17. **Díaz de Tuesta A.M., C.-Q., M.P. Geijo Martínez, J. Dimas Núñez a, F.J. Díaz de Tuesta, C.R. Herranz, E. Val Pérez** 2001. Tularemia outbreak in the province of Cuenca associated with crab handling. Rev Clin Esp **201:**385-9.

18. **Eigelsbach, H. T., and C. M. Downs.** 1961. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. J Immunol **87:**415-25.
19. **Ellis, J., P. C. F. Oyston, M. Green, and R. W. Titball.** 2002. Tularemia. Clin. Microbiol. Rev. **15:**631-646.
20. **Entcheva-Dimitrov, P., and A. M. Spormann.** 2004. Dynamics and Control of Biofilms of the Oligotrophic Bacterium Caulobacter crescentus. J. Bacteriol. **186:**8254-8266.
21. **Epstein, P. R.** 1993. Algal blooms in the spread and persistence of cholera. Biosystems **31:**209-21.
22. **Farlow, J., D. M. Wagner, M. Dukerich, M. Stanley, M. Chu, K. Kubota, J. Petersen, and P. Keim.** 2005. Francisella tularensis in the United States. Emerging Infectious Diseases **11:**1835-1841.
23. **Fujise, O., Y. Wang, W. Chen, and C. Chen.** 2008. Adherence of Aggregatibacter actinomycetemcomitans via serotype-specific polysaccharide antigens in lipopolysaccharides. Oral Microbiol Immunol **23:**226-33.
24. **Gold, V. A. M., F. Duong, and I. Collinson.** 2007. Structure and function of the bacterial Sec translocon (Review). Molecular Membrane Biology **24:**387-394.
25. **Gold, V. A. M., F. Duong, and I. Collinson.** 2007. Structure and function of the bacterial Sec translocon (Review). Molecular Membrane Biology **24:**387-394.
26. **Golovliov, I., V. Baranov, Z. Krocova, H. Kovarova, and A. Sjostedt.** 2003. An attenuated strain of the facultative intracellular bacterium Francisella tularensis can escape the phagosome of monocytic cells. Infect Immun **71:**5940 - 5950.
27. **Golovliov, I., M. Ericsson, G. Sandstrom, A. Tarnvik, and A. Sjostedt.** 1997. Identification of proteins of Francisella tularensis induced during growth in macrophages and cloning of the gene encoding a prominently induced 23-kilodalton protein. Infect. Immun. **65:**2183-2189.
28. **Gunn, J. S., and R. K. Ernst.** 2007. The structure and function of Francisella lipopolysaccharide. Ann N Y Acad Sci **1105:**202-18.
29. **Hager, A. J., D. L. Bolton, M. R. Pelletier, M. J. Brittnacher, L. A. Gallagher, R. Kaul, S. J. Skerrett, S. I. Miller, and T. Guina.** 2006. Type IV pili-mediated secretion modulates Francisella virulence. Mol Microbiol **62:**227-37.
30. **Hassett, D. J., P. A. Limbach, R. F. Hennigan, K. E. Klose, R. E. W. Hancock, M. D. Platt, and D. F. Hunt.** 2003. Bacterial biofilms of importance to medicine and bioterrorism: proteomic techniques to identify novel vaccine components and drug targets. Expert Opinion on Biological Therapy **3:**1201-1207.
31. **Horzempa, J., P. Carlson, D. O'Dee, R. Shanks, and G. Nau.** 2008. Global transcriptional response to mammalian temperature provides new insight into Francisella tularensis pathogenesis. BMC Microbiology **8:**172.
32. **Jarrett, ClaytonÂ O., E. Deak, KarenÂ E. Isherwood, PetraÂ C. Oyston, ElizabethÂ R. Fischer, AdelineÂ R. Whitney, ScottÂ D. Kobayashi, FrankÂ R. DeLeo, and B. Â J. Hinnebusch.** 2004. Transmission of Yersinia pestis from an Infectious Biofilm in the Flea Vector. The Journal of Infectious Diseases **190:**783-792.
33. **Jing, H., J. Takagi, J.-h. Liu, S. Lindgren, R.-g. Zhang, A. Joachimiak, J.-h. Wang, and T. A. Springer.** 2002. Archaeal Surface Layer Proteins Contain [beta] Propeller, PKD, and [beta] Helix Domains and Are Related to Metazoan Cell Surface Proteins. Structure **10:**1453-1464.

34. **Kanehisa, M., M. Araki, S. Goto, M. Hattori, M. Hirakawa, M. Itoh, T. Katayama, S. Kawashima, S. Okuda, T. Tokimatsu, and Y. Yamanishi.** 2008. KEGG for linking genomes to life and the environment. Nucleic Acids Res **36:**D480-4.
35. **Kelley, L. A., and M. J. Sternberg.** 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc **4:**363-71.
36. **Keyhani, N. O., and S. Roseman.** 1999. Physiological aspects of chitin catabolism in marine bacteria. Biochim Biophys Acta **1473:**108-22.
37. **Keyhani, N. O., and S. Roseman.** 1999. Physiological aspects of chitin catabolism in marine bacteria. Biochimica et Biophysica Acta (BBA) - General Subjects **1473:**108-122.
38. **Kirisits, M. J., and M. R. Parsek.** 2006. Does Pseudomonas aeruginosa use intercellular signalling to build biofilm communities? Cell Microbiol **8:**1841-9.
39. **Kraemer, P. S., A. Mitchell, M. R. Pelletier, L. A. Gallagher, M. Wasnick, L. Rohmer, M. J. Brittnacher, C. Manoil, S. J. Skerett, and N. R. Salama.** 2009. Genome-Wide Screen in Francisella novicida for Genes Required for Pulmonary and Systemic Infection in Mice. Infect. Immun. **77:**232-244.
40. **Kugeler, K. J., P. S. Mead, A. M. Janusz, J. E. Staples, K. A. Kubota, L. G. Chalcraft, and J. M. Petersen.** 2009. Molecular Epidemiology of Francisella tularensis in the United States. Clin Infect Dis **48:**863-70.
41. **Kuoppa, K., Å. Forsberg, and A. Norqvist.** 2001. Construction of a reporter plasmid for screening in vivo promoter activity in Francisella tularensis. FEMS Microbiology Letters **205:**77-81.
42. **Letunic, I., T. Doerks, and P. Bork.** 2009. SMART 6: recent updates and new developments. Nucl. Acids Res. **37:**D229-232.
43. **Lill R., K. C., L.A. Brundage, K. Ito, D. Oliver, and W. Wickner.** 1989. SecA protein hydrolyzes ATP and is an essential component of the protein translocation ATPase of Escherichia coli. EMBO J **8:**961–966.
44. **Liu J., X. Z., J.R. Barker, K.E. Klose.** 2007. Construction of targeted insertion mutations in Francisella tularensis subsp. novicida. Biotechniques **43:**487-90.
45. **Loo, C. Y., D. A. Corliss, and N. Ganeshkumar.** 2000. Streptococcus gordonii Biofilm Formation: Identification of Genes that Code for Biofilm Phenotypes. J. Bacteriol. **182:**1374-1382.
46. **Maier, T. M., A. Havig, M. Casey, F. E. Nano, D. W. Frank, and T. C. Zahrt.** 2004. Construction and Characterization of a Highly Efficient Francisella Shuttle Plasmid. Appl. Environ. Microbiol. **70:**7511-7519.
47. **Maier, T. M., R. Pechous, M. Casey, T. C. Zahrt, and D. W. Frank.** 2006. In vivo Himar1-based transposon mutagenesis of Francisella tularensis. Appl Environ Microbiol **72:**1878-85.
48. **Matz, C., D. McDougald, A. M. Moreno, P. Y. Yung, F. H. Yildiz, and S. Kjelleberg.** 2005. Biofilm formation and phenotypic variation enhance predation-driven persistence of Vibrio cholerae. Proc Natl Acad Sci U S A **102:**16819-24.
49. **Meibom, K. L., X. B. Li, A. T. Nielsen, C.-Y. Wu, S. Roseman, and G. K. Schoolnik.** 2004. The Vibrio cholerae chitin utilization program. Proc Natl Acad Sci U S A **101:**2524-2529.
50. **Melillo, A., D. D. Sledjeski, S. Lipski, R. M. Wooten, V. Basrur, and E. R. Lafontaine.** 2006. Identification of a Francisella tularensis LVS outer membrane protein that confers adherence to A549 human lung cells. FEMS Microbiol Lett **263:**102-8.

51. **Merzendorfer, H., and L. Zimoch.** 2003. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J Exp Biol **206:**4393-412.
52. **Mironchuk I.V., A. V. M.** 2002. Viability and virulence of Francisella tularensis subsp. Holarctica in water ecosystems (experimental study). Zh Mikrobiol Epidemiol Immunobiol **March-April:**9-13.
53. **Morner, T.** 1992. The ecology of tularaemia. Rev Sci Tech **11:**1123-30.
54. **Mueller, R. S., D. McDougald, D. Cusumano, N. Sodhi, S. Kjelleberg, F. Azam, and D. H. Bartlett.** 2007. Vibrio cholerae Strains Possess Multiple Strategies for Abiotic and Biotic Surface Colonization. J. Bacteriol. **189:**5348-5360.
55. **Munoz-Elias, E. J., J. Marcano, and A. Camilli.** 2008. Isolation of Streptococcus pneumoniae biofilm mutants and their characterization during nasopharyngeal colonization. Infect Immun **76:**5049-61.
56. **Naczk, M., J. Williams, K. Brennan, C. Liyanapathirana, and F. Shahidi.** 2004. Compositional characteristics of green crab (Carcinus maenas). Food Chemistry **88:**429-434.
57. **Nigrovic, L. E., and S. L. Wingerter.** 2008. Tularemia. Infectious Disease Clinics of North America **22:**489-504.
58. **O'Toole, G. A., and R. Kolter.** 1998. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol Microbiol **30:**295-304.
59. **O'Toole, G. A., and R. Kolter.** 1998. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol **28:**449-61.
60. **Orikoshi, H., S. Nakayama, C. Hanato, K. Miyamoto, and H. Tsujibo.** 2005. Role of the N-terminal polycystic kidney disease domain in chitin degradation by chitinase A from a marine bacterium, Alteromonas sp. strain O-7. J Appl Microbiol **99:**551-7.
61. **Owen, C. R., E. O. Buker, W. L. Jellison, D. B. Lackman, and J. F. Bell.** 1964. COMPARATIVE STUDIES OF FRANCISELLA TULARENSIS AND FRANCISELLA NOVICIDA. J. Bacteriol. **87:**676-683.
62. **Oyston, P. C., A. Sjostedt, and R. W. Titball.** 2004. Tularaemia: bioterrorism defence renews interest in Francisella tularensis. Nat Rev Microbiol **2:**967 - 978.
63. **Oyston, P. C. F.** 2008. Francisella tularensis: unravelling the secrets of an intracellular pathogen. J Med Microbiol **57:**921-930.
64. **Palo, R., C. Ahlm, and A. Tärnvik.** 2005. Climate variability reveals complex events for tularemia dynamics in man and mammals. Ecology and Society **10:**22.
65. **Paranjpye, R. N., and M. S. Strom.** 2005. A Vibrio vulnificus Type IV Pilin Contributes to Biofilm Formation, Adherence to Epithelial Cells, and Virulence. Infect. Immun. **73:**1411-1422.
66. **Pruzzo, C., L. Vezzulli, and R. R. Colwell.** 2008. Global impact of Vibrio cholerae interactions with chitin. Environ Microbiol **10:**1400-10.
67. **Rosen, D. A., J. S. Pinkner, J. M. Jones, J. N. Walker, S. Clegg, and S. J. Hultgren.** 2008. Utilization of an Intracellular Bacterial Community Pathway in Klebsiella pneumoniae Urinary Tract Infection and the Effects of FimK on Type 1 Pilus Expression. Infect. Immun. **76:**3337-3345.
68. **Schultz, J. r., F. Milpetz, P. Bork, and C. P. Ponting.** 1998. SMART, a simple modular architecture research tool: Identification of signaling domains. Proceedings of the National Academy of Sciences of the United States of America **95:**5857-5864.

69. **Shahabuddin, M., and J. M. Vinetz.** 1999. Chitinases of human parasites and their implications as antiparasitic targets. EXS **87:**223-34.
70. **Shanks, R. M. Q., N. A. Stella, E. J. Kalivoda, M. R. Doe, D. M. O'Dee, K. L. Lathrop, F. L. Guo, and G. J. Nau.** 2007. A Serratia marcescens OxyR Homolog Mediates Surface Attachment and Biofilm Formation. J. Bacteriol. **189:**7262-7272.
71. **Sjostedt, A.** 2007. Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. Ann N Y Acad Sci **1105:**1-29.
72. **Su, J., J. Yang, D. Zhao, T. H. Kawula, J. A. Banas, and J.-R. Zhang.** 2007. Genome-Wide Identification of Francisella tularensis Virulence Determinants. Infect. Immun. **75:**3089-3101.
73. **Takeo, S., D. Hisamori, S. Matsuda, J. Vinetz, J. Sattabongkot, and T. Tsuboi.** 2009. Enzymatic characterization of the Plasmodium vivax chitinase, a potential malaria transmission-blocking target. Parasitol Int **58:**243-8.
74. **Thelaus, J., A. Andersson, P. Mathisen, A. L. Forslund, L. Noppa, and M. Forsman.** 2009. Influence of nutrient status and grazing pressure on the fate of Francisella tularensis in lake water. FEMS Microbiol Ecol **67:**69-80.
75. **Thormann, K. M., S. Duttler, R. M. Saville, M. Hyodo, S. Shukla, Y. Hayakawa, and A. M. Spormann.** 2006. Control of formation and cellular detachment from Shewanella oneidensis MR-1 biofilms by cyclic di-GMP. J Bacteriol **188:**2681-91.
76. **Thormann, K. M., R. M. Saville, S. Shukla, D. A. Pelletier, and A. M. Spormann.** 2004. Initial Phases of Biofilm Formation in Shewanella oneidensis MR-1. J. Bacteriol. **186:**8096-8104.
77. **Valent, Q. A., P.A. Scotti, S. High, J.W. de Gier, G. von Heijne, G. Lentzen, W. Wintermeyer, B. Oudega, and J. Luirink.** 1998. The Escherichia coli SRP and SecB targeting pathways converge at the translocon. EMBO J **19:**2504–2512.
78. **Vilain, S., J. M. Pretorius, J. Theron, and V. S. Brozel.** 2009. DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilm. Appl. Environ. Microbiol.**:**AEM.01317-08.
79. **Vinetz, J. M., S. K. Dave, C. A. Specht, K. A. Brameld, B. Xu, R. Hayward, and D. A. Fidock.** 1999. The chitinase PfCHT1 from the human malaria parasite Plasmodium falciparum lacks proenzyme and chitin-binding domains and displays unique substrate preferences. Proc Natl Acad Sci U S A **96:**14061-6.
80. **Weiss, D. S., A. Brotcke, T. Henry, J. J. Margolis, K. Chan, and D. M. Monack.** 2007. In vivo negative selection screen identifies genes required for Francisella virulence. Proc Natl Acad Sci U S A **104:**6037-42.
81. **Willke, A., M. Meric, R. Grunow, M. Sayan, E. J. Finke, W. Splettstosser, E. Seibold, S. Erdogan, O. Ergonul, Z. Yumuk, and S. Gedikoglu.** 2009. An outbreak of oropharyngeal tularaemia linked to natural spring water. J Med Microbiol **58:**112-116.
82. **Yusof, N. L., L. Y. Lim, and E. Khor.** 2004. Flexible chitin films: structural studies. Carbohydr Res **339:**2701-11.

Figure 1.

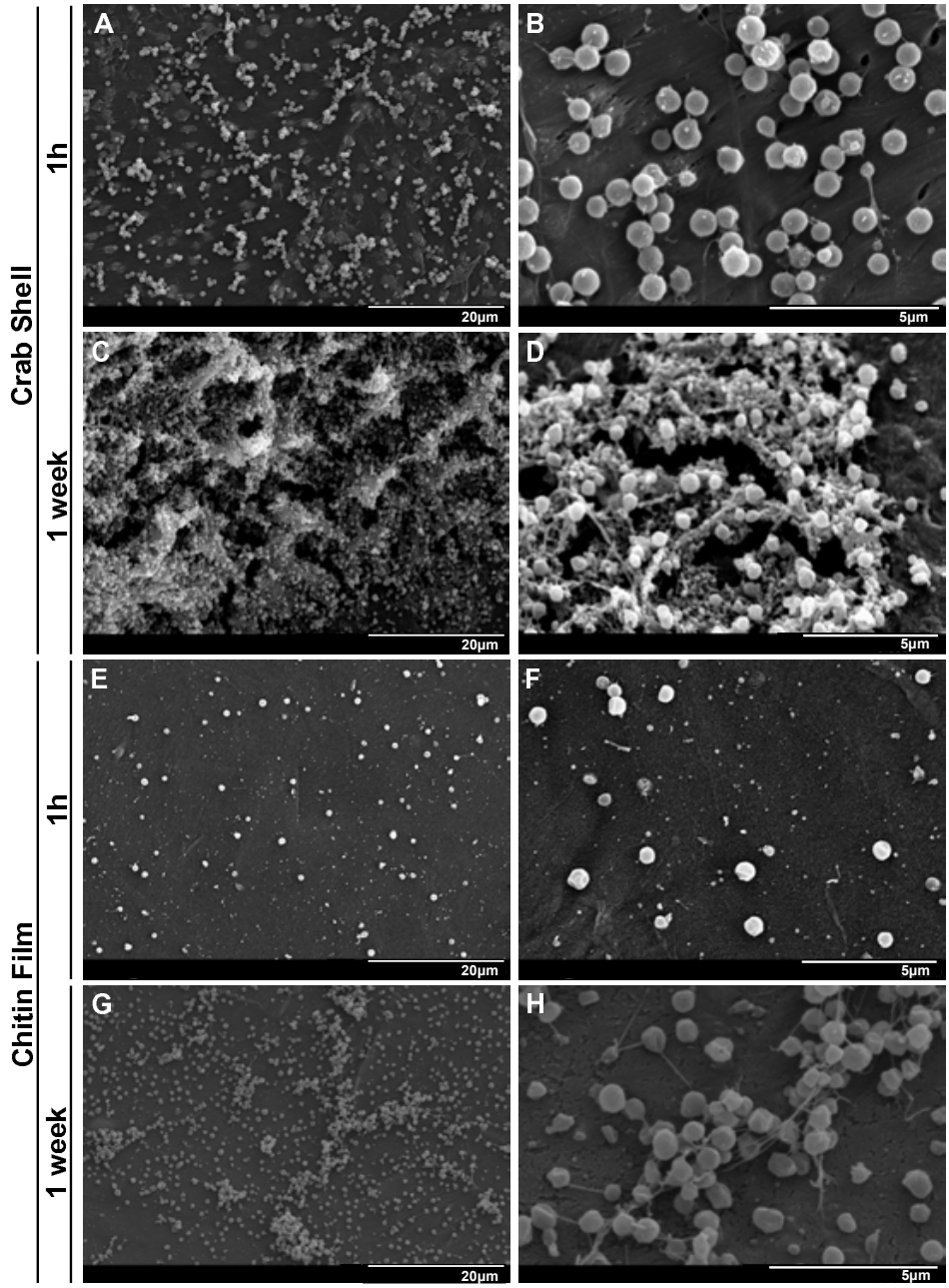

Figure 2.

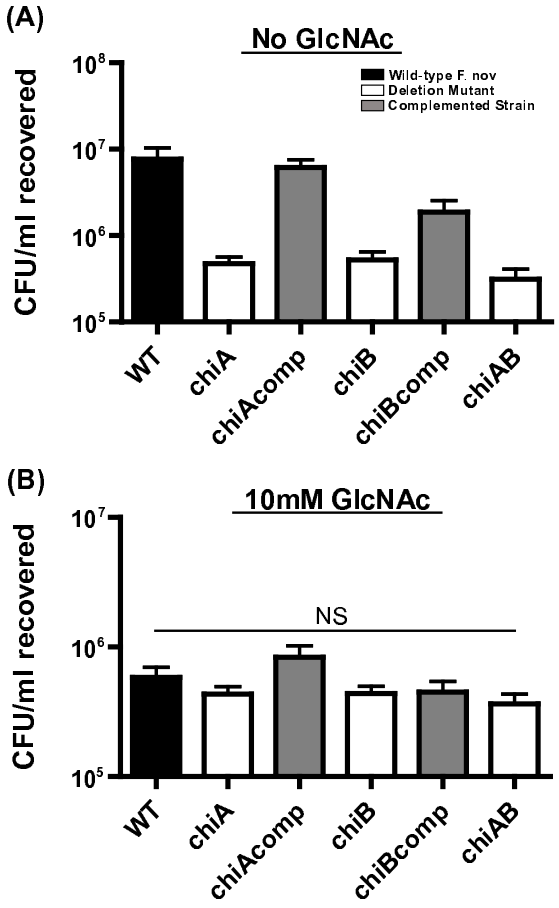

Figure 3.

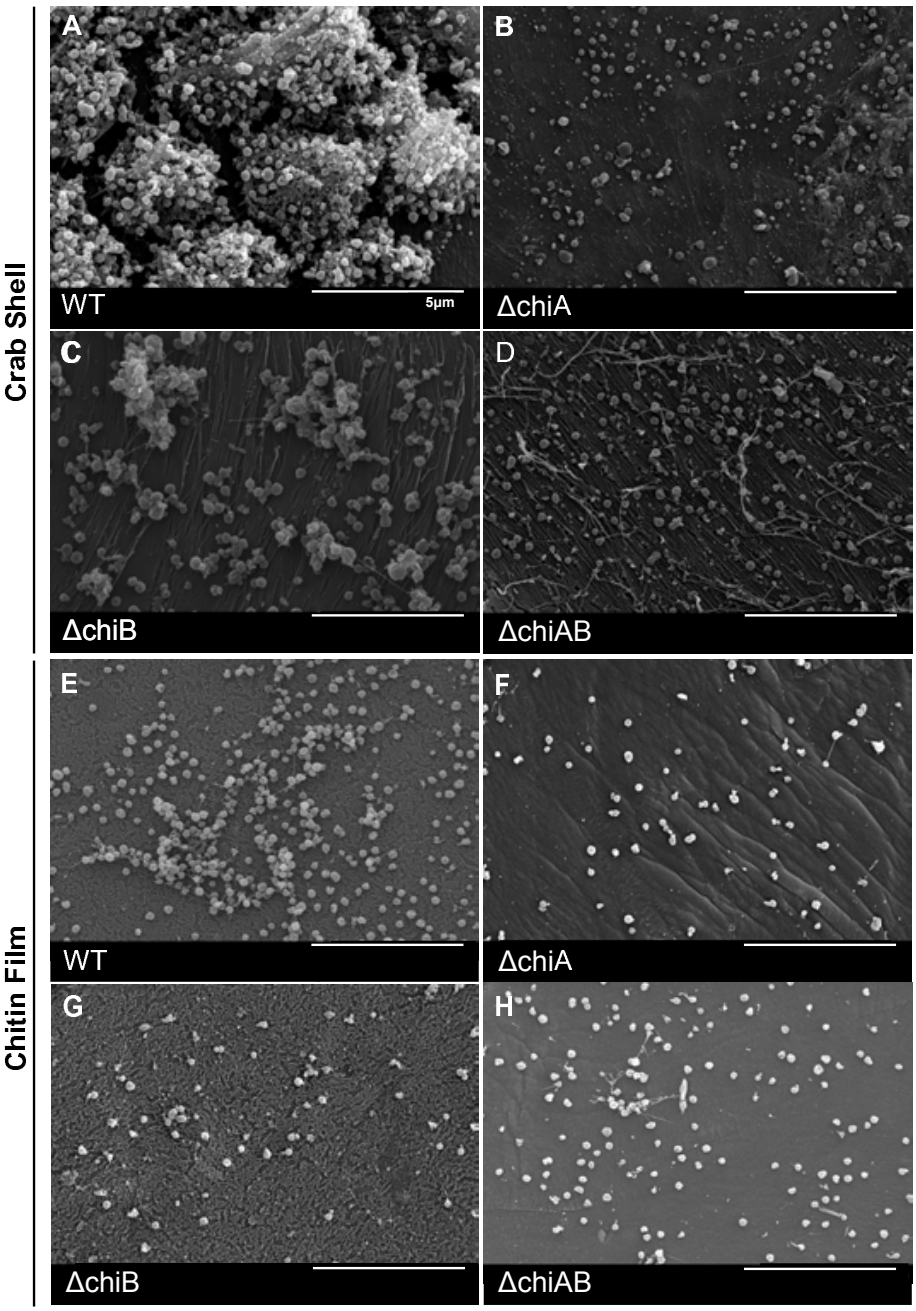

Figure 4.

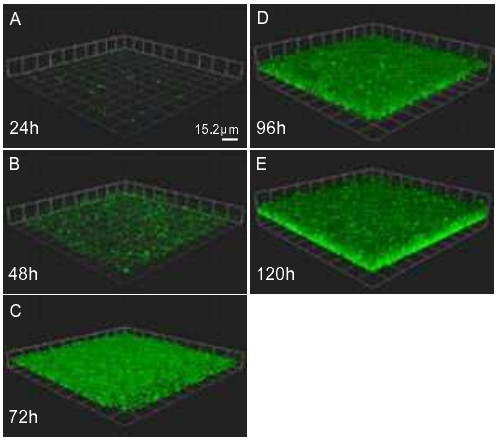

Figure 5.

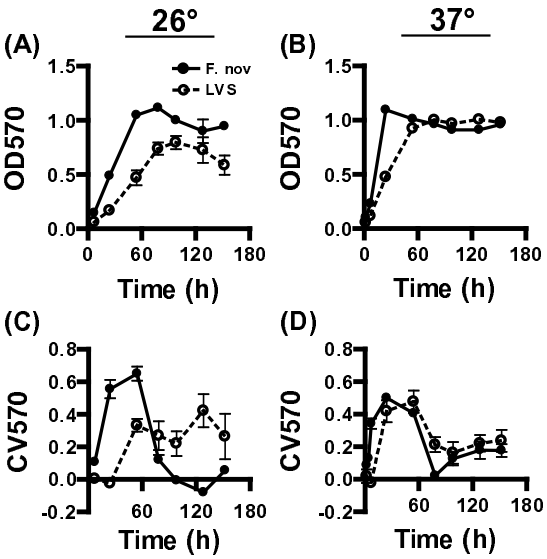

Figure 6.

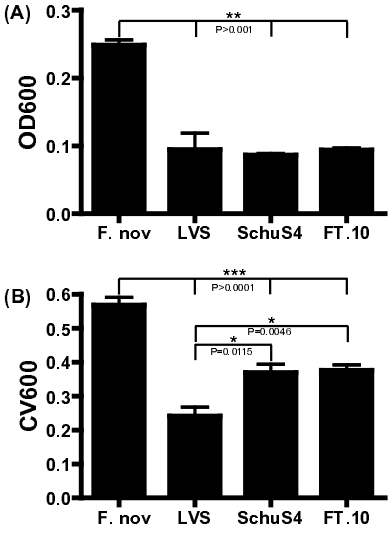

Figure 7.

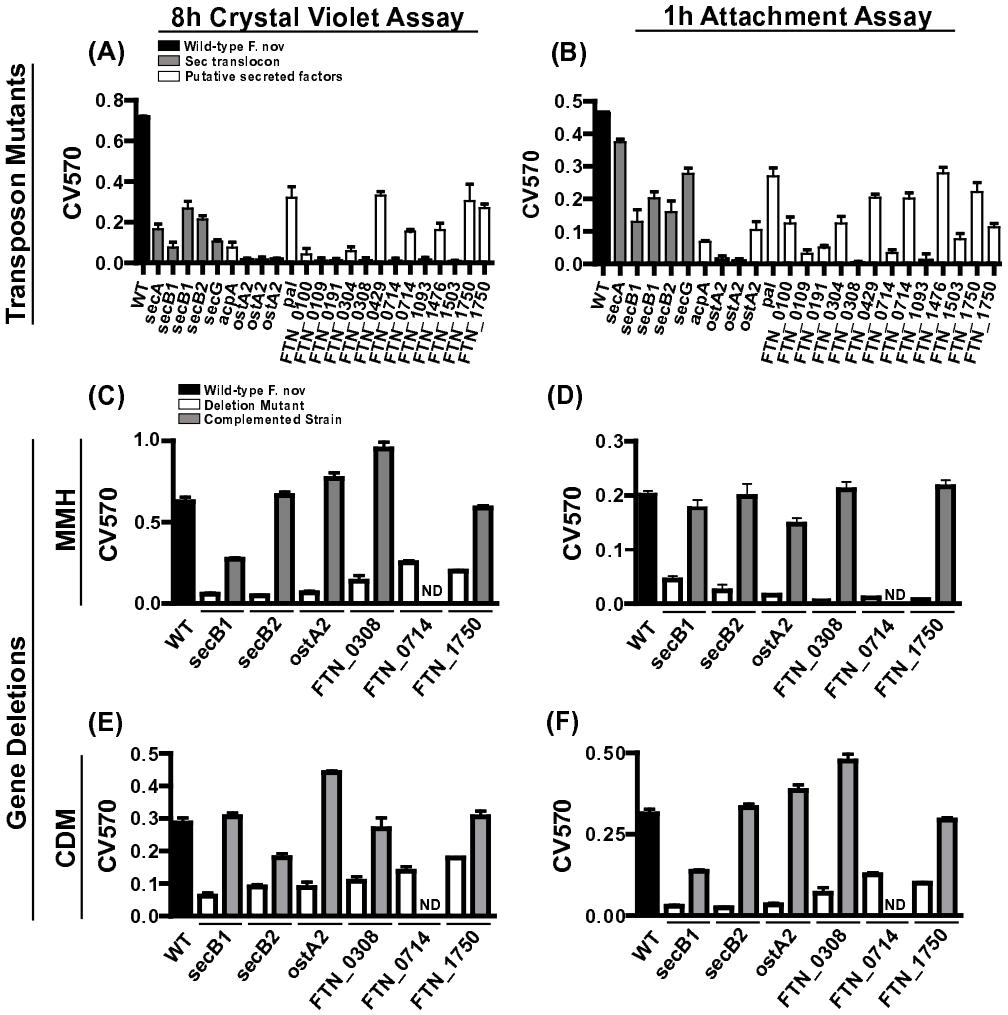

Figure 8.

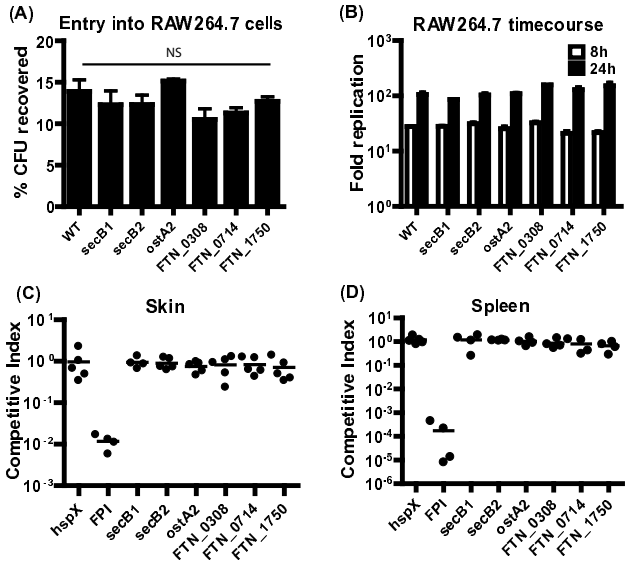

Figure 9.