Chitinases Are Negative Regulators of *Francisella novicida* Biofilms

Myung-Chul Chung*, Scott Dean, Ekaterina S. Marakasova, Albert O. Nwabueze, Monique L. van Hoek

School of Systems Biology and the National Center for Biodefense and Infectious Diseases, George Mason University, Manassas, Virginia, United States of America

**Abstract**

Biofilms, multicellular communities of bacteria, may be an environmental survival and transmission mechanism of *Francisella tularensis*. Chitinases of *F. tularensis* ssp. *novicida* (*Fn*) have been suggested to regulate biofilm formation on chitin surfaces. However, the underlying mechanisms of how chitinases may regulate biofilm formation are not fully determined. We hypothesized that *Fn* chitinase modulates bacterial surface properties resulting in the alteration of biofilm formation. We analyzed biofilm formation under diverse conditions using chitinase mutants and their counterpart parental strain. Substratum surface charges affected biofilm formation and initial attachments. Biophysical analysis of bacterial surfaces confirmed that chitinase cleavage of its substrates could have exposed the concanavalin A-binding epitope. *Fn* biofilm was sensitive to chitinase, proteinase and DNase, suggesting that *Fn* biofilm contains exopolysaccharides, proteins and extracellular DNA. Exogenous chitinase increased the drug susceptibility of *Fn* biofilms to gentamicin while decreasing the amount of biofilm. In addition, chitinase modulated bacterial adhesion and invasion of A549 and J774A.1 cells as well as intracellular bacterial replication. Our results support a key role of the chitinase(s) in biofilm formation through modulation of the bacterial surface properties. Our findings position chitinase as a potential anti-biofilm enzyme in *Francisella* species.

**Introduction**

Many bacteria including bacterial pathogens live in multicellular communities, called biofilms, on abiotic and biotic surfaces [1–3]. Biofilms have characteristic architectural and phenotypic properties including the creation of sticky extracellular matrix, consisting of proteins, lipids, extracellular DNA (eDNA), and exopolysaccharides (EPS) to mediate surface attachment, intercellular adhesion, biofilm resistance, and immune evasion [4]. Biofilm matrix alters bacterial sensitivity to chemical attack [5], causing phenotypic antibiotic resistance.

*Francisella tularensis* is a Gram negative, facultative intracellular pathogen that causes tularemia. It is considered a category A agent by the Centers for Disease Control and Prevention (CDC) due to its high infectivity, dissemination by aerosol and high mortality to humans. In environmental conditions, *F. tularensis* Type B (*holarctic*) is associated with water and waterways and infects many species of animals, insects, and protists. Our previous study showed that *F. tularensis* ssp. *novicida* (*Fn*), a model strain for *Francisella*, is associated with water and waterways and infects many species of animals, insects, and protists. Our previous study showed that *Fn* forms biofilms on chitin surfaces, and this activity is dependent on chitinases, the Sec secretion system, and several Sec-dependent secreted proteins, some of which are predicted to bind to and/or degrade chitin [8]. Since *Fn* is associated with water-borne transmission, biofilm formation is likely linked to its environmental persistence in aquatic habitats [7,9,10], as well as possibly within tick and mosquito vectors that have chitin in their exoskeletons [8,11]. However, the role of chitinases in *Francisella* biofilm formation is not known.

Chitinases are glycosyl hydrolases that hydrolyze chitin, a linear β-1,4-linked polymer of N-acetyl-D-glucosamine (GlcNAc), the second most abundant polysaccharide in nature after cellulose. Chitinases are found in a wide range of species [12–14], including those that are known not to synthesize chitin, such as bacteria, viruses, higher plants as well as mammals. Based on the cleavage site on chitin of the chitinolytic enzymes, chitinases are divided into exo-chitinases and endo-chitinases [15]. Endo-chitinases cleave chitin randomly at internal sites, generating soluble oligomers (2~4 units of GlcNAc). Exo-chitinases such as chitobiosidases and β-(1,4)-N-acetyl-glucosaminidases act on the non-reducing end of chitin to digest into (GlcNAc)2 and GlcNAc, respectively [15]. In *Francisella*, four putative chitinases (ChiA, ChiB, ChiC, and ChiD) were identified and characterized *in vitro* using biochemical studies coupled with bioinformatics analyses [16]. Enzymatic analyses revealed that chitinases ChiA and ChiB possessed both endo- and exo-chitinase activity. *Fn* thus has two functional chitinases ChiA and ChiB, despite having all four chitinase genes in the genome [16]. Although biofilm formation of *Fn* on chitin was shown to be dependent on the two chitinase genes, *chiA* and *chiB* [8], the underlying mechanisms of how chitinases regulate biofilms are not fully determined.

In this study, we hypothesized that *Fn* chitinase changes the contents and/or composition of its EPS, resulting in altered biofilm formation. Studies using transposon-inserted *chi* mutants

---

**Citation:** Chung M-C, Dean S, Marakasova ES, Nwabueze AO, van Hoek ML (2014) Chitinases Are Negative Regulators of *Francisella novicida* Biofilms. PLoS ONE 9(3): e93119. doi:10.1371/journal.pone.0093119

**Copyright:** © 2014 Chung et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This research was supported by the DOD Defense Threat Reduction Agency (DTRA; grant No. 421 HDTRA1-12-C0039). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: mchung3@gmu.edu
and exogenous chitinase showed that chitinase is a negative regulator of *Francisella* biofilm formation and causes dispersion of pre-formed biofilms, and alters bacterial surface properties. Our results provide a basis for understanding the mechanism of biofilm dispersion that may be applicable to a large number of biofilm-forming pathogenic species. Insights into the mechanism of chitinase function have implications for the control of biofilm-related infections.

**Results**

**Effect of chitinases on biophysical properties of the bacterial surface**

To examine a role of *Fn* chitinases on biofilm formation, we analyzed the biophysical properties of the bacterial surfaces of WT and transposon insertion mutants in *chiA* and *chiB* gene. In the Hydrophobic Interaction Chromatography (HIC) and Microbial Adhesion To Hydrocarbon (MATH) analysis [17,18], the *chi* mutants had a lower adsorption activity to the phenyl-sepharose and to the nonpolar solvent hexadecane than WT, respectively (Fig. 1A). The *chi* mutants always precipitated faster than WT cells in the autoaggregation study. After 48 h, the autoaggregation of the *chi* mutants reached 60%, while that of WT was 43% (Fig. 1B). The size tunable pore sensor qNano utilizes a non-optical detection principle to determine the size, concentration, dynamics and charge of a wide range of particle types [19–21]. To analyze bacterial sizes and surface charges, we used the qNano nanoparticle characterization system with planktonic cultures of the strains in PBS measured by qNano analysis. (D) Particle translocation time (fwhm). The *chi* mutants had a longer fwhm duration than that observed for WT, indicating that the lower charge *chi* mutants took longer to traverse the pore. Mean presented in dots was calculated from every 100 data points.

doi:10.1371/journal.pone.0093119.g001

**Effect of substratum surface charges on biofilm formation and initial attachment**

We examined the effect of substratum surface properties on *Fn* biofilm formation of wild type *Fn* (WT) and *chi* mutants to substrata with different surface charge properties. Different types of microplates including tissue-culture treated (TC), non-treated (PS), amine treated and Primaria surface-modified polystyrene plates were used for negative, hydrophobic neutral, positive and positive/negative-charges, respectively. Biofilm formation of WT on a positively-charged amine microplate was significantly higher than on a negatively-charged tissue-culture plate with *P* < 0.01 (Fig. 2A). There were no significant differences of bacterial growth in different types of microplates (data not shown). However, biofilm formation of WT on non-treated PS and Primaria plate
was comparable to that on TC plate, suggesting that only a positive-charged substratum surface affected WT Fn biofilm formation.

The first step of biofilm formation is adhesion to a surface. This is mediated by many factors including the charge of the substratum surface and the charge of the bacteria. To determine the potential effect of surface charges on bacterial attachment, we determined the capacity of WT and chi mutants for attachment to different surface charged plates using a 1-h attachment assay. Initial attachment of Fn WT was very low to the TC (−) and Primaria (+/−) plates, but high to the amine (+) and PS (0), indicated by CV staining (Fig. 2B). The chi mutants showed higher initial attachment to the TC, amine, and PS, but not to the Primaria plate. Relative initial attachment of chi mutants was higher than that of WT in a negatively-charged TC plate (Fig. S1), suggesting that in wild-type Fn, chitinases are involved in increasing charge of the bacterial surface, and promoting attachment to negatively-charged surfaces (Fig. 1D). For chitinase mutants, attachment appears to be independent of surface charge. Therefore, we hypothesize that altered production of EPS may also be contributing to the differences in chi mutant biofilm production through increase in hydrophobicity.

**COMSTAT2 analysis of biofilms**

When compared to WT biofilm formation, chiA and chiB mutants showed a significant increase in biofilm formation for both the TC and non-treated PS plate. In microscopic analysis with CV staining, WT did not show prominent 3D bacterial communities on the TC plate the surface (Fig. S1). Chi mutants displayed significant 3D biofilm architectures on the negative charged borosilicate glass (Fig. 3A, Fig. S1D) and in the TC plate (Fig. S1C). COMSTAT2 analysis confirmed that mutation of chi genes resulted in a significant increase in mean thickness and biomass of biofilms (Fig. 3B and 3C). The ratio of surface to biovolume for WT biofilms was 2.5- or 3.6-fold higher than the biomass of biofilms (Fig. 3B and 3C). The ratio of surface to biovolume for WT biofilms was 2.5- or 3.6-fold higher than the ratio for induced chiA or chiB biofilms, respectively (Fig. 3D). This indicated that WT Fn formed flat, undifferentiated biofilms that covered 2.6 or 3.5 times more surface (with the same amount of biomass) than chi mutant biofilms. These results suggest a chi-dependent regulation of Fn biofilm formation, such that the ability to produce chitinase leads to an overall decrease in biofilm structure and architecture (i.e. chitinase mutants produce a hyper-biofilm structure). This effect may be partly due to altered attachment, suggesting that EPS acts as an adhesin involved in cell-to-surface interactions [24,25].

**Lectin binding assay for identification of potential chitinase substrate**

To determine if chitinase is involved in the EPS production, the EPS contents of cells and culture supernatants of the three strains were determined by a phenol-sulfuric acid method. Total EPS contents of the chi mutant cells were higher than that of WT cells, while total EPS content in WT culture supernatants was higher than those of the mutant culture supernatants (Fig. 4A and 4B). These results support the findings that the chi mutants have a thick EPS, contributing to the bigger size of the cells as observed in Fig. 1C.

The structure or composition of biofilm EPS can be partly deduced on the basis of the specific binding of lectins to different sugar residues [26]. To identify a potential chitinase substrate, binding of the FITC-labeled lectin concanavalin A (ConA) to the biofilms of three strains was fluorospectrometrically analyzed in a TC microplate. The binding of ConA to chiA and chiB mutants, but not to WT (Fig. 4), demonstrates that fluorescence from ConA is closely associated with the cells. Fluorescence microscopic analysis further supported the binding of ConA to the mutants. These results suggest that chitinase cleavage of its substrates could have exposed the ConA-binding epitope (e.g. mannose α1-3- or α1-6-containing EPS) in Fn biofilms.

**Requirement of chitinase activity for its anti-biofilm property**

Since chitinase appeared to modulate biofilm formation, we tested whether chitinase activity itself is responsible for the anti-biofilm activity. To test the effect of exogenous chitinase on biofilm formation, bacteria were incubated with Streptomyces griseus chitinase, both because S. griseus chitinase is comparable to Fn chitinase in three chitinase activities, e.g. chitobiosidase, β-N-acetylglucosaminidase, and endochitinase activity (Fig. S2) [16], and because enzymatic activity of chitinase contributes to its antibiofilm activity as demonstrated below. Fn WT showed slightly higher chitinase EC50 (0.65 μg/ml) in the TC (−) plate compared with the chi mutants (0.18 and 0.21 μg/ml for chiA and chiB, respectively) (Fig. 5A) consistent with the increased biofilm formation in chiA. On the other hand, on amine (+) plate, EC50 of exogenous chitinase to WT was dramatically increased (87.46 μg/ml), while EC50 of exogenous chitinase to the chi mutants did not show significant changes (0.17 and 0.15 μg/ml for...
chiA and chiB, respectively) (Fig. 5B). The chi mutants showed high biofilm formation with no significant difference (Fig. 2A) on TC and amine plates. Exogenous chitinase also significantly affected biofilm formation of the Gram-negative pathogen *P. aeruginosa*. Exogenous chitinase could affect biofilm formation of *S. aureus*, but only if much higher concentration was used (Fig. S3).

We next examined whether pre-formed biofilms can be detached from plastic surfaces by enzymatic degradation of the matrix polymers. Biofilms were grown in the wells of 96-well TC plates and then treated with different test enzymes for 2 h at a final enzyme concentration of 50 μg/ml. Treatment with proteinase K, chitinase, and DNase I resulted in a significant decrease in remaining biofilms of all three strains assayed by CV staining (Fig. 5C), suggesting these enzymes partially degraded matrix materials. Interestingly, chitinase had by far the greatest effect on biofilm of the chi mutants, causing over 90% reduction in CV staining, suggesting that chitinase substrate polysaccharides are a major structural component of the biofilm. However, this finding suggests that proteins and eDNA are also important components of the *Fn* biofilms.

**Effect of chitinase inhibitors on biofilm formation**

To further examine whether chitinase activity itself is responsible for the anti-biofilm activity, we utilized potent family-18 chitinase inhibitors sanguinarine (SAN) and dequalinium (DEQ) which have inhibitory activity to chitinase and an antimicrobial activity to the bacteria [27]. The antimicrobial activity of SAN and DEQ was first tested against *Fn* WT, and the EC50s for SAN and DEQ were found to be 4.00 and 0.89 μM, respectively (Fig. 6). The chi mutants showed a slightly higher EC50 values (P<0.05) for SAN and DEQ, suggesting that the chi mutants with different surface charges responded differently to positive-charged SAN and DEQ in their drug susceptibility. To examine the effect of chitinase inhibitors on biofilm formation, we determined the anti-biofilm activity of the inhibitors on *Fn*. Fig. 6C demonstrates that biofilms of chitinase-positive WT, but not the chi mutants, were increased by treatment with chitinase inhibitors SAN and DEQ. This indicated that chitinase activity itself might be degrading the biofilms produced by *Fn* WT.

**Increased drug susceptibility of biofilms treated with exogenous chitinase**

Bacteria within biofilms are inherently resistant to antimicrobial agents. We therefore determined whether chitinase regulates the resistance of *Fn* biofilms to antimicrobial agents through its regulation of biofilm production. To examine drug susceptibility of the different strains, we cultured WT and chiA mutant in TC or amine plates. After 48 h of incubation, the wells were washed to remove planktonic bacteria and treated with gentamicin (10 μg/ml) for 24 h. The remaining live bacteria were quantified by resazurin reduction assays that detect cellular metabolic activity. WT *Fn* biofilms, which were thin in the TC plates (Fig. 2A), were highly sensitive (EC50 = 0.69 μg/ml), while the chiA mutant bacteria having thick biofilm formation on TC plates were highly resistant to gentamicin (EC50 = 13.77 μg/ml) (Fig. 7A). The EC50 of WT for gentamicin was 3-fold increased in the amine plates (EC50 = 2.2 μg/ml) compared with that in the TC plates (Fig. 7B) corresponding to the thicker biofilm (Fig. 2). The chiA biofilms grown on the TC and the amine plates did not exhibit significant differences in susceptibility to gentamicin. Of importance, difference of gentamicin sensitivity (4.7-fold in EC50) between WT and chiA mutants in the amine plates, despite showing no

**Figure 3. COMSTAT2 analysis of WT and chi mutants.** Biofilms were grown in LabTek II glass chambers for 24 h and representative images were taken using a Nikon TE2000-U confocal microscope. (A) 3D structures of biofilms were analyzed by CLS9 z-stacks and z-stacks were rendered using Bitplane Imaris software. The images shown are representative of three independent experiments. (B) Mean thickness, (C) biomass, (D) surface to volume ratio, and (E) roughness coefficient of biofilms. *P<0.05 (n = 3) by unpaired Student’s t-test.

doi:10.1371/journal.pone.0093119.g003
protected from gentamicin. However, exogenous chitinase treatment resulted in a drastic decrease of the remaining bacteria in the biofilm (Fig. 7E). This suggests that chitinase alters bacterial properties for drug susceptibility during biofilm formation.

**Chitinase modulates bacterial adhesion and invasion to A549 cells**

Differential expression of extracellular matrix materials in biofilms alters adhesion and invasion of pathogens to host cells [29]. In *in vitro* experiments including biophysical properties and drug susceptibility assays as described above suggested that chitinase changed the surface properties of *Francisella* in biofilms. We therefore investigated whether the matrix produced by the chi mutant bacteria could also promote bacterial adherence to and invasion of A549 human lung cell monolayers. As shown in Fig. 8A, the thick biofilm-forming chi mutants were able to adhere to the A549 cells ~100-fold more than WT. Invasion of the bacteria as determined by gentamicin protection assay showed similar results as adhesion assays (Fig. 8B). To determine whether this activity was chitinase dependent, we performed the same experiment using chitinase-treated WT and chi mutant bacteria, which reduced biofilm formation. Treatment with chitinase before infection of the A549 cells resulted in a drastic decrease of adhesion ability of the chi mutants, while WT displayed only a slight decrease of adhesion in chitinase-treated bacteria compared to untreated control (Fig. 8C). Chitinase mutant bacteria showed a 1.5–2.3 fold increase in adhesion in biofilm vs. planktonic form (Fig. 8D). Within 24 h post infection, there were no drastic changes in A549 cell viability (Fig. 8A). These results suggested that chitinase activity may modulate bacterial adhesion and invasion to the A549 cells through the change of surface matrix materials, which may be targets for chitinase.

In order to test the functional role of *Fn* chitinase, we determined intracellular replication of the mutants in A549 cells. The initial invasion of the chi mutants was significantly higher than that of WT *Fn*; however, there was no significant difference in intracellular bacteria at 18 h post infection (Fig. 8E). Calculation of replication rates from Fig. 8E implied that the chi mutants may have a severe defect (3.7–5.7-fold of decrease) in replication rates compared with WT 18 h post infection (Fig. 8F). Invasion activity and intracellular replication rates of the mutants in J774A.1 cells had a similar pattern to the A549 cells (Fig. 8G and 8H), supporting the conclusion obtained from A549 cells. Overall, these data suggested that *Fn* chitinase might be involved in some pathogenic function of the pathogen, although its overall contribution to virulence is not clearly observed in murine model [8,30,31].

**Discussion**

In previous studies, we demonstrated that *Fn*, a model organism of highly virulent *F. tularensis*, forms a biofilm *in vitro*, mediated by an orphan response regulator [7]. We also reported that *F. philomiragia*, which causes francisellosis of farmed and wild fish, can form a biofilm in a co-culture with *Acanthamoeba castellanii*, an aquatic amoeba [9], and suggested these biofilms may be ‘lures’ for environmental amoebae and other protists. Margolis et al. [8] showed that *Fn* forms biofilms during the colonization of chitin surfaces (i.e. crab shells) by using chitin as a sole carbon source. They demonstrated that mutants lacking *chiA* or *chiB* were attenuated for chitin colonization and biofilm formation in the absence of exogenous sugar. *Fn* secretes proteins including chitinases (ChiA and ChiB), a chitin binding protein (CbpA), a protease (PepO), and a beta-glucosidase (BglX) [32]. In the present

---

**Figure 4.** (A) EPS contents of the cells and (B) culture supernatants of the strains. EPS contents were determined by phenol extraction followed by phenol-sulfuric acid method for carbohydrates as described in Materials and Methods. (C) Lectin binding assay to biofilms. FITC-Con A and FITC-WGA lectins were used for biofilm binding. Lectin binding capacity to biofilms was measured by a fluorescence plate reader and calculated relative fold to WT binding. Fluorescence microscopic images of biofilms of WT, chiA and chiB grown in TC plate are shown in the top panel. Biofilms in the TC plate were shown by CV staining (Fig. S1C). Scale bar, 100 μm. doi:10.1371/journal.pone.0093119.g004
study, we show that chitinase modulates attachment and biofilm formation on abiotic material and host cell surfaces. Bacterial surface characteristics are important in bacterial attachment to substrates [17,33]. To understand which surface properties might play an important role in the initial attachment of \(Fn\) to substrates, we compared fundamental surface properties (i.e., hydrophobicity and surface charges) of WT and \(chi\) mutants. Results indicated that WT bacteria are more hydrophobic and less charged than \(chiA\) or \(chiB\) mutants. However, autoaggregation rate are higher in \(chi\) mutants compared to WT bacteria, suggesting that a more charged bacterial surface may contribute to cell-to-cell interaction for aggregation. These properties might partly account for the increased resistance of \(chiA\) mutants to cationic antimicrobial gentamicin. Surface charges significantly affect initial attachment and biofilm formation. Different surface charged microplates were used to demonstrate the relationship between WT and \(chi\) mutant initial attachment and subsequent biofilm formation. There was no difference between WT and mutants in the positively-charged (amine plate), uncharged hydrophobic (polystyrene plate), and net zero-charged surface (Primaria plate). In contrast, \(chi\) mutants exhibited high biofilm formation on the negatively-charged tissue-culture plates compared to WT bacteria. Exogenous addition of chitinase protein could explain, in part, the effect of chitinase gene on biofilm formation. In addition, since Margolis et al. [8] showed that addition of the \(chiA\) and \(chiB\) genes to deletion mutant strains complemented the chitin colonization defects, these results suggest that chitinase modulates surface charge of bacteria, resulting in high attachment and biofilm formation to the negatively-charged surface. This charge-dependent biofilm formation may contribute to defining the natural environments for \(Francisella\) biofilm formation.

Furthermore, such surface properties might be linked to bacterial adhesion and invasion to host cells [34,35]. \(Chi\) mutants showed a dramatic increase of adhesion and invasion to human lung epithelial A549 cells compared to WT. Our data showed that replication rate of \(chi\) mutants, on the other hand, was decreased in A549 cells. One may speculate that positively-charged \(chi\) mutant bacteria are able to more efficiently bind negatively-charged A549 cell membranes [36], resulting in an increased invasion activity. However, Mellio et al. [34] have reported that \(F.\) tularensis surface protein FsaP was able to bind A549 cells. Although we did not examine whether chitinase mutation induced FsaP expression to investigate a link between them, changes of surface charges by chitinase may contribute to adhesion and invasion changes. This explanation is supported by our finding that addition of exogenous chitinase to WT bacteria decreased bacterial adhesion to A549 cells.

Our data also showed that chitinase is involved in the detachment of pre-formed biofilms by its enzymatic activity. This implies that \(Francisella\) biofilms include a substrate for chitinase in extracellular matrix. The chitinase substrate chitin is the second most abundant natural polysaccharide consisting of \(\beta\) (1→4)-linked N-acetyl-D-glucosamine (GlcNAc) units in a linear form. There are no reports of chitin production in \(Francisella\) species; however, chitinase is required for providing carbon source under nutrient-limiting conditions [37]. Nevertheless, our detachment studies of pre-formed \(Francisella\) biofilm with chitinase imply a possibility for existence of a yet-unrecognized chitinase substrate in

Figure 5. Enzymatic activity of chitinase is required for regulation of \(Fn\) biofilm formation. (A) Effect of exogenously added chitinase on biofilm formation in the negatively-charged TC plates. EC\(_{50}\) of exogenous chitinase to WT, \(chiA\) and \(chiB\) mutants were determined to be 6.55, 0.18, and 0.21 \(\mu g/ml\), respectively (\(n = 6\)). (B) Effect of exogenous chitinase on biofilm formation in the positively-charged amine plates. EC\(_{50}\) of chitinase to WT, \(chiA\) and \(chiB\) mutants were determined to be 87.46, 0.17, and 0.15 \(\mu g/ml\), respectively (\(n = 6\)). (C) Detachment of \(Fn\) biofilms after exposure to proteinase K, chitinase and DNase I (50 \(\mu g/ml\)) in the TC plates. Untreated control CV570 values were 0.149±0.032, 0.588±0.012, and 0.585±0.017 for \(Fn\) WT, \(chiA\) and \(chiB\) mutants, respectively. *\(P<0.01\) and **\(P<0.001\) compared to control without enzyme treatment (\(n = 6\)).

doi:10.1371/journal.pone.0093119.g005.
biofilms. The content and composition of the potential substrates could be different between WT and chi mutants based on the differential susceptibility of the bacteria to exogenous chitinase (Fig. 5A and 5B). This speculation is also supported by resistance of WT biofilms to gentamicin compared to that of chi mutants (Fig. 7).

Recently, Margolis et al. [8] showed that ChiA and ChiB are important for Fn biofilm formation on biotic chitinous crab shell surfaces. This finding was also confirmed on abiotic glass surfaces [38]. In another study, however, chiA and chiB mutants showed no defects in the ability to colonize ticks [39], which have chitin in their exoskeleton. Our study using abiotic, different-charged polystyrene microplates showed that, unlike on glass or crab shells, there was no difference between WT and chi mutants; however, negatively-charged tissue-culture plate and human lung epithelial cells showed increased biofilm formation in chi mutants. This suggests that regulation of biofilm formation by Fn chitinase is sensitive to environmental conditions, i.e. charges of substrata and bacterial surfaces.

In summary, we have shown that chitinase plays a pivotal role in biofilm formation by Fn. Substratum surface charges affect Fn biofilm formation. Changes of biophysical properties (hydrophobicity, surface charge, and autoaggregation) by chi mutation increased Fn biofilm formation. Preformed Fn biofilms were degraded by treatment with protease K, chitinase and DNase. Chitinase-treated preformed biofilms became susceptible to gentamicin killing. In addition, mutation of chi genes enhanced bacterial adhesion and invasion to A549 and J774A.1 cells; however, intracellular replication rate was decreased in chi mutants. We propose that regulation of EPS may be involved in chitinase-mediated biofilm formation and bacterial invasion of the host.

Materials and Methods

Bacterial strains and growth conditions

*Francisella* tullarensis novicida type strain U112 (Fn), *Francisella* novicida (NR-5007) and *chiA* (NR-6005) and *chiB* (NR-6005) transposon mutants, *Pseudomonas aeruginosa* (Schroeter) Migula R. Hugh 813 and *Staphylococcus aureus* Rosenbach 502A were obtained from American Type Culture Collection (Manassas, VA). *Francisella* strains were cultured at 37°C in tryptic soy broth containing 0.1% cysteine (TSBC). Kanamycin (20 µg/ml) was used to select for Fn mutants. *P. aeruginosa* and *S. aureus* were cultured at 37°C in nutrient broth.

To validate the *Tn* mutation of the *chi* gene, we performed PCR with genomic DNA and qRT-PCR with total RNAs isolated from each strain. The results confirmed the mutation of *chiA* and *chiB* gene by a transposon insertion using primers of the outside of flanking region (Fig. S5A and S5B). Chitinase activity in culture supernatants was also decreased by a transposon insertion, especially in chitobiosidase activity (Fig. S5C). Of note, there was no significant β-N-acetyl-glucosaminidase activity in *Fn*...
culture supernatants and the endochitinase activity was increased in both chi mutants. There was no growth defect in the chi mutants compared with WT (Fig. S5D).

Crystal violet assays for biofilms

Biofilm was measured as previously described [7] with the following modifications. Bacteria (1 x 10^6 per well) in 100 μl of TSBC were incubated without and with antibiotics for 24–48 h at 37°C in different types of 96-well microplate: negatively-charged carboxyl group-containing, tissue-culture (TC)-treated polystyrene (PS); nontreated, hydrophobic PS; positively-charged, amine group-containing PS; and both negatively and positively-charged, carboxyl and amine group-containing Primaria PS (BD Biosciences). Optical density of the cultures (OD_{600}) was determined prior to staining as a measure of bacterial growth. Biofilm production was measured using the crystal violet (CV) stain technique with absorbance measurements at 570 nm (CV570) [6]. For bacterial attachment assays, overnight culture (OD_{600} = 1.0) of bacteria in 100 μl of TSBC was incubated in different types of plates for 1 h at 37°C. Attached bacteria were measured using the CV stain technique [7,8].

Confocal Scanning Laser Microscopy (CSLM)

Bacteria were grown on the Lab-Tek II chamber slide (Thermo Scientific) for 24 h and biofilms attached to the glass were fixed with methanol followed by DAPI staining (920 ng/ml). Biofilm structure was observed using a Nikon TE2000-U confocal laser scanning microscope equipped with an argon ion laser. Sections through the XY, YZ and XZ planes were obtained using the Nikon EZ-C1 Confocal Software program. Each strain was examined on at least three separate occasions. Quantitative analysis of the CLSM z-stacks was performed using established protocols for the image analysis tool COMSTAT2 [40,41]. The biofilm parameters, biomass (biovolume), mean thickness, and roughness coefficient (an indicator of biofilm heterogeneity) were assessed using a minimum of 3 different images per plate from 2 independent experiments for each strain.

Hydrophobic interaction chromatography (HIC)

The cell surface hydrophobicity of WT and chi mutants was determined using a Pasteur pipette with 1 ml of phenyl sepharose fast flow resin (GE Healthcare), washed with 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl. Bacteria (cultured overnight) were diluted 10-fold in TSBC, incubated for 4 h for mid-log phase culture. Bacteria were washed and resuspended with 50 mM phosphate buffer (pH 7.2), 150 mM NaCl. Bacteria (0.3 ml) were loaded onto column, washed with 0.9 ml of the same buffer. OD_{600} was measured (PowerWave X microplate reader, BioTek Instruments), and the percentage of bacteria retained in the hydrophobic column was calculated from the absorbance of a 1/4 dilution of the original bacterial suspension as follows. Equation 1: % adsorption = [(A_{0} - A_{1})/A_{0}] x 100, where A_{0} = OD of 1/4 diluted bacterial suspension, and A_{1} = OD of the eluted bacterial suspension.
Microbial adhesion to hydrocarbon test (MATH)

Relative cell-surface hydrophobicity was measured by microorganism adhesion to hydrocarbon hexadecane (Sigma-Aldrich). Mid-log phase culture (50 mM phosphate buffer, pH 7.2) as prepared above was added to equal volume of hexadecane, vortexed for 30 s, and incubated for 20 min at room temperature. OD_{600} of the aqueous phase was measured and percentage adsorption was determined using Eq. 1.

Autoaggregation

Autoaggregation was measured according to published method [42]. Briefly, cells harvested at stationary phase were washed twice with PBS (pH 7.4), resuspended in PBS (5 ml) to OD_{600} ~1.0. The tubes were stored at room temperature and OD_{600} of the upper 0.5 ml culture was measured at 0, 24 and 48 h. Percentage of autoaggregation was calculated as described above (Eq. 1).
qNano analysis of bacteria

Relative surface charge and size distribution analysis of WT, and mutants chiA and chiB was performed using a qNano (Izon Science). The qNano utilizes Tunable Resistive Pulse Sensing technology to allow for a high-throughput, particle-by-particle, analysis of particle size, surface charge, and electrophoretic mobility [20,43]. All qNano experiments were performed using the manufacturer’s established protocols [19–21,43]. Briefly, overnight cultures of WT, chiA, and chiB were pelleted (five minutes at 4,500 × g) and washed three times with sterile PBS. For each measurement, 40 μl of the washed bacterial suspension was added to the top fluid cell and a minimum of 1,000 blockade events were recorded. Measurements were taken at 48.49 mm of applied stretch with an applied voltage of 0.10 V. An applied pressure of 5 cm H2O was applied to the top fluid cell using the IZON proprietary variable pressure module. The size distribution and relative surface charge analysis was performed using IZON Science technology to allow for a high-throughput, particle-by-particle, analysis of particle size, surface charge, and electrophoretic mobility [20,43].

EPS determination

For EPS extraction, 30 ml of culture was pelleted and resuspended in 1/5 volume of TNE (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl) containing 0.1% SDS (final). The samples were stirred for 5 min at room temperature, passed 5 times through 18-G needle, and then centrifuged at 17,000 × g for 15 min. The pellets were washed 5 times with 10 mM Tris-HCl (pH 7.5) and resuspended in 3 ml 10 mM Tris-HCl (pH 7.5). Carbohydate content was determined by phenol-sulfuric acid method with glucose standard [44]. Briefly, 50 μl of sample or standard was added to 150 μl conc. H2SO4 and 30 μl of 5% phenol in water. The samples were heated at 90°C for 5 min in a hot plate and cooled down to RT. The absorbance values at 490 nm were used to determine released carbohydrate (in mg per g cells or ml of culture supernatants).

Lectin binding assays

Bacteria were cultured overnight in TSBC medium in a 96 well or a 6-well TC plate. The wells were washed 3 times with PBS and incubated with FITC-labeled ConA (Invitrogen; a final concentration of 50 μg/ml in PBS) for 30 min. After washing 5 times with PBS, fluorescence of the well in a 96 well plate was measured by Tecan Safire II microplate reader (Tecan) with excitation of 488 nm and emission of 532 nm (bandwidth = 10 nm) and images of the well in a 6-well plate were taken by EVOS FL Cell Imaging System (Life Technologies) using green channel, respectively.

Enzymatic detachment of biofilms

Proteinase K from Trichoderma album, chitinase from Streptomyces griseus, and DNG A1 (Sigma-Aldrich) were used. Biofilm disruption by proteinase K and chitinase was assayed in 50 mM sodium phosphate buffer (pH 7.2); that of DNG A1 was in 10 mM Tris-HCl (pH 7.6), 2.5 mM MgCl2 and 0.5 mM CaCl2. Pre-formed biofilms (2 days in the TC plates) were incubated with 50 μg/ml of each enzyme and control received an equal volume of buffer (2 days in the TC plates) were incubated with 50 μg/ml of each enzyme and control received an equal volume of buffer. The plates were incubated for 2 h at 37°C, biofilms stained by CV as described above.

Antimicrobial susceptibility

Antimicrobial susceptibility of biofilm was performed by the resazurin reduction assay. Bacterial biofilm was formed (1×10^6 CFU per well in the TC or amine plate, incubated for 48 h at 37°C). The wells were gently washed twice with PBS. 100 μl TSBC containing serially diluted from 10 μg/ml gentami-
is the yz plane. White lines indicate the particular locations of the xz, yz, and xy planes depicted. (TIF)

Figure S2  Chitinase activity from F. novicida (recombinant ChiA), S. griseus, and Trichoderma viridae. The assay was based on the release of 4-methylumbelliferoine (4MU) by enzymatic hydrolysis from chitinase substrates, 4-methylumbelliferyl N, N′-diacetyl-β-D-chitobiose for chitinobiose activity, 4- methylumbelliferol N-acetyl-β-D-glucosaminide for β-N-acetylglucosaminidase activity, and 4-methylumbelliferyl β-D-N,N',N'- triacetylchitotriose for endochitinase activity. (TIF)

Figure S3  Effect of chitinase on biofilm formation of S. aureus and P. aeruginosa. S. aureus and P. aeruginosa were cultured in the TC plates for 2 days in the presence of different concentrations of chitinase. Biofilm production was measured using the CV stain technique with absorbance measurements at 570 nm (CV570). Relative biofilm formation of each condition was calculated by dividing CV570 of untreated control. (TIF)

Figure S4  No cytotoxic effect of F. novicida infection on A549 cells for 24 h. Lactate dehydrogenase (LDH) released into the supernatant was measured using a commercially available kit (Promega CytoTox 96 Non-Radioactive Cytotoxicity Assay).

Cytotoxicity was expressed relative to LDH release from whole cell lysates in controls (n = 6). (TIF)

Figure S5  Confirmation of Fn chi mutants. (A) Colony PCR of mutants. Single colony of each strain was suspended in water (100 μl) and 2 μl of cell suspension was subjected to a typical PCR reaction [20 μl] with forward primer (5′-ACAGCAC- CAATTGTGAGCA-3′) and reverse primer (5′-CAATAAC- GACTTTCCGCAACCA-3′) for chiA and forward primer (5′- TCTGTTAAATCTAACTGGTATA-3′) and reverse primer (5′- ACTTAATCATTTGCTTATTGTT A-3′) for chiB mutants. (B) qRT-PCR of total RNAs isolated from WT, chiA and chiB mutants using SYBR green dye and the same primers. (C) Chitinase assays of culture supernatants prepared from overnight cultures using fluorimetric chitinase assay kit (Sigma). *P<0.01 and **P<0.001 compared to WT (n = 3). (D) Growth kinetics of WT and chi mutants in TSBC media using a PowerWave X microplate reader (BioTek Instruments) with kinetic mode. (TIF)

Author Contributions
Conceived and designed the experiments: MC MvH. Performed the experiments: MC SD EM AON. Analyzed the data: MC MvH. Wrote the paper: MC MvH.

References
1. Bryers JD (2008) Medical biofilms. Biotechnol Bioeng 100: 1–18.
2. O'Toole G, Kaplan HB, Koher R (2000) Biofilm formation as microbial development. Annu Rev Microbiol 54: 49–79.
3. Hall-Stoodley L, Costerton JW, Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2: 95–108.
4. Flemming HC, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8: 103–111.
5. Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284: 1318–1322.
6. Liu J, Zhou X, Barker JR, Klose KE (2007) Construction of targeted insertion mutations in Francisella tularemia subsp. novicida. BioTechniques 43: 487–492.
7. Kasprzewska A (2003) Plant chitinases—regulation and function. Cell Mol Life Sci 60: 201–216.
8. Naqvi HS, Baig A, Yarman MC (2004) Inhibition of biofilm growth and dispersion in the freshwater bacterium Pseudomonas fluorescens using bioactive natural plant extracts. J Sci Food Agric 84: 1866–1876.
9. Verhoeven AB, Durham-Colleran MW, Pierson T, Boswell WT, van Hoek ML (2011) Francisella tularemia subsp. novicida F. novicida ChiA and ChiB are essential for biofilm production. J Bacteriol 193: 2962–2970.
10. van Hoek ML (2013) Biofilms: An advancement in our understanding of the Francisella system to biofilm formation on chitin. Appl Environ Microbiol 76: 596–608.
11. Mahajan UV, Gravgaard J, Turnbull M, Jacobs DB, McNealy TL (2011) Larval exposure to Francisella tularemia LVS affects fitness of the mosquito tules. Antimicrob Agents Chemother 55: 1111–1119.
12. Messerschmidt JF, El-Etr S, Jouhat LM, Moore E, Robison R, et al. (2010) Contributions of Francisella tularemia subsp. novicida chitinases and Sec secretion system to biofilm formation on chitin. Appl Environ Microbiol 76: 596–608.
13. Verhoeven AB, Durham-Colleran MW, Pierson T, Boswell WT, van Hoek ML (2010) Francisella tularemia subsp. novicida biofilm formation and interaction with the aquatic protozoan Acanthamoeba castellanii. J Bact Pathol 219: 170–180.
14. van Hoek ML (2013) Biofilms: An advancement in our understanding of Francisella species. Virulence 4: 833–846.
15. Lee CG, Da Silva CA, Della Cruz CS, Ahangari F, Ma B, et al. (2011) Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. Annu Rev Physiol 73: 479–501.
16. Dahiya N, Tewari R, Hoondal GS (2006) Biotechnological aspects of chitinolytic enzymes. a review. Appl Microbiol Biotechnol 71: 773–782.
17. Chandler JC, Molins CR, Petersen JM, Belisle JT (2011) Differential chitinase activity and production within Francisella species, subspecies, and subpopulations. J Bacteriol 193: 2962–2970.
18. Handeland KI, Haugen H, Moen JD, Sorensen H, Skandsen T (2003) Francisella tularemia subsp. novicida and Francisella tularemia subsp. holarctica: distinct species or subspecies? J Bacteriol 185: 2578–2587.
19. Pomatto S, Verrier IR, Prinz H, Suginta W (2011) Potent family-18 chitinases inhibitors: x-ray structures, affinities, and binding mechanisms. J Biol Chem 286: 24312–24323.
20. Walters MC 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS (2003) Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Francisella tularemia biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother 47: 317–323.
21. Hager AJ, Bolton DL, Pelletier MR, Gallagher LA, Wasnick M, et al. (2006) Francisella novicida subsp. novicida LVS affects fitness of the mosquito tules. Antimicrob Agents Chemother 50: 4997–5001.
22. Kruger PS, Mitchell A, Pelletier MR, Gallagher LA, Wasnick M, et al. (2009) Genome-wide screen in Francisella novicida for genes required for pulmonary and systemic infection in mice. Infect Immun 77: 232–244.
23. Weiss DS, Bresticke A, Henry T, Margelis J, Chan K, et al. (2007) In vivo negative selection screen identifies genes required for Francisella virulence. Proc Natl Acad Sci USA 104: 6037–6042.
24. Kozak D, Anderson W, Vogel R, Chen S, Antaw F, et al. (2012) Simultaneous size and z-pattern measurements of individual nanoparticles in dispersion using size-tunable pore sensors. ACS Nano 6: 6990–7.
25. Watanabe M, Iwata S, Shinoda S, Hori T, Nishino H, et al. (2011) Remodeling of extracellular matrix protein of Pseudomonas aeruginosa LWS affects fitness of the mosquito tules. PLoS Pathog 8: e1002843.
26. Parish DJ, O’Toole G (2009) Noninvasive analyses of bacterial biofilm formation and function. Nat Rev Microbiol 7: 25–36.
27. Møller J, Stenholm SP, Poulsen L, Knudsen OM, Madsen J, et al. (2006) In vivo studies of the biofilm forming ability of Pseudomonas aeruginosa in early and late stage chronic lung infections of CF patients. J Bacteriol 188: 1423–1432.
28. Kraemer PS, Mitchell A, Pelletier MR, Gallagher LA, Wasnick M, et al. (2009) Genome-wide screen in Francisella novicida for genes required for pulmonary and systemic infection in mice. Infect Immun 77: 232–244.
29. Sly WS (1974) Biofilm formation as microbial development. Annu Rev Microbiol 28: 223–243.
30. Kraemer PS, Mitchell A, Pelletier MR, Gallagher LA, Wasnick M, et al. (2009) Genome-wide screen in Francisella novicida for genes required for pulmonary and systemic infection in mice. Infect Immun 77: 232–244.
31. Sly WS (1974) Biofilm formation as microbial development. Annu Rev Microbiol 28: 223–243.
32. Hager AJ, Bolton DL, Pelletier MR, Gallagher LA, Wasnick M, et al. (2006) Francisella novicida subsp. novicida LVS affects fitness of the mosquito tules. Antimicrob Agents Chemother 50: 4997–5001.
33. Bos R, van der Mei HC, Busscher HJ (1999) Physio-chemistry of initial microbioal adhesive interactions—its mechanisms and methods for study. FEMS Microbiol Rev 23: 179–210.
34. Melillo A, Sledjeski DD, Lipski S, Wooten RM, Basrur V, et al. (2006) Identification of a Francisella tularemia LWS outer membrane protein that confers adherence to A349 human lung cells. FEMS Microbiol Lett 263: 102–108.
35. Hall JD, Craven RR, Fuller JR, Pickles RJ, Kawula TH (2007) Francisella tularemia replicates within alveolar type II epithelial cells in vitro and in vivo following inhalation. Infect Immun 75: 1034–1039.
36. Thiel K, Duszyk M (1997) Decreased cell surface charge in cystic fibrosis epithelia. Cell Biochem Funct 15: 35–38.
37. Keyhani NO, Roseman S (1999) Physiological aspects of chitin catabolism in marine bacteria. Biochim Biophys Acta 1473: 108–122.
38. Zogaj X, Wyatt GC, Klose KE (2012) Cyclic di-GMP stimulates biofilm formation and inhibits virulence of Francisella novicida. Infect Immun 80: 4239–4247.
39. Reif KE, Palmer GH, Ueti MW, Scoles GA, Margolis JJ, et al. (2011) Dermacentor andersoni transmission of Francisella tularensis subsp. novicida reflects bacterial colonization, dissemination, and replication coordinated with tick feeding. Infect Immun 79: 4941–4946.
40. Heydorn A, Erbsoll BK, Hentzer M, Parsek MR, Givskov M, et al. (2000) Experimental reproducibility in flow-chamber biofilms. Microbiology 146: 2409–2415.
41. Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, et al. (2000) Quantification of biofilm structures by the novel computer program COM-STAT. Microbiology 146: 2395–2407.
42. Rahman MM, Kim WS, Kimura H, Shimazaki K (2008) Autoaggregation and surface hydrophobicity of bifidobacteria. World J Microbiol Biotechnol 24: 1593–1596.
43. Vogel R, Anderson W, Eldridge J, Glossop B, Willmott G (2012) A variable pressure method for characterizing nanoparticle surface charge using pore sensors. Anal Chem 84: 3125–3131.
44. Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S, et al. (2005) Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal Biochem 339: 69–72.