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Biofilms
An advancement in our understanding of *Francisella* species

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Abbreviations: BHI, brain heart infusion; EPS, extracellular polymeric substances; c-di-GMP, cyclic di-guanosine monophosphate or cyclic diguanylate; OMV, outer membrane vesicle; LPS, lipopolysaccharide; LVS, live vaccine strain

Our understanding of the virulence and pathogenesis of *Francisella* spp. has significantly advanced in recent years, including a new understanding that this organism can form biofilms. What is known so far about *Francisella* spp. biofilms is summarized here and future research questions are suggested. The molecular basis of biofilm production has begun to be studied, especially the role of extracellular carbohydrates and capsule, quorum sensing and two-component signaling systems. Further work has explored the contribution of amoebae, pili, outer-membrane vesicles, chitinases, and small molecules such as c-di-GMP to *Francisella* spp. biofilm formation. A role for *Francisella* spp. biofilm in feeding mosquito larvae has been suggested. As no strong role in virulence has been found yet, *Francisella* spp. biofilm formation is most likely a key mechanism for environmental survival and persistence. The significance and importance of *Francisella* spp.'s biofilm phenotype as a critical aspect of its microbial physiology is being developed. Areas for further studies include the potential role of *Francisella* spp. biofilms in the infection of mammalian hosts and virulence regulation.

Overview

The last decade has seen a tremendous increase in the number of publications and laboratories that study the bacteria of the genus *Francisella*. One important advancement has been to understand that some species of this organism can form biofilms. The molecular basis of biofilm formation has been studied, and may include pili, two-component systems and extracellular carbohydrates and capsule. Further work has explored the contribution of chitinases, small molecules such as c-di-GMP, and outer-membrane vesicles to *Francisella* spp. biofilm formation. New knowledge of the role of co-dwelling eukaryotes such as amoebae, and the interaction of biofilm with mosquito larvae has also been demonstrated, suggesting interactions with potential vectors of transmission. *Francisella* spp. biofilm formation in aquatic habitats is likely a key mechanism of environmental survival and persistence. However, the significance and importance of this finding especially with respect to the microbial physiology and virulence of this organism has not yet been fully developed. Areas of possible future research include the potential role for biofilm in the infection of mammalian hosts by *Francisella* spp. and a potential regulation of virulence. This review will summarize the current knowledge of *Francisella* spp. biofilms, discuss its potential role in *Francisella* virulence and environmental persistence and suggest areas for future research.

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Introduction

Tularemia, or "rabbit fever", is caused by the gram-negative bacterium *Francisella tularensis*. Tularemia is considered a "zoonotic" disease; that is, it normally affects animal populations, but can infect humans with direct contact. Infrequent but regularly occurring cases of human and domestic animal tularemia occur worldwide. Tularemia was a prevalent public health issue in the early 1900s, with around 2000 cases per year in US. Hunters contracted the disease when they cut themselves skinning animals such as rabbits or squirrels.[1] Today, only around 200 cases per year occur in the US, and these cases are usually tick-borne. Ticks employ a "transstadial mechanism" of transmission, in which the tick acquires the bacterium as a larva or nymph and retains it into adulthood, when it can infect humans. CDC categorizes the virulent form of *F. tularensis* as a Tier 1 threat agent due to its high infectivity when inhaled by the human lung.[1] The historical development of *Francisella* spp. as a biological weapon merits detailed understanding of its microbial physiology.

The diversity of *Francisella* species is continuously increasing, especially with the use of genomic analysis of environmental samples,[2] and thus the taxonomy has changed in the last decades. The genus *Francisella* historically contains two species (*F. tularensis* and *F. philomiragia*), with four subspecies of *F. tularensis*: *F. tularensis tularensis* (Type A), *F. tularensis holarctica* (Type B), *F. tularensis mediasiatica*, and *F. tularensis novicida*. Recently, the nomenclature of the genus *Francisella* has undergone significant revision, reducing *F. novicida* to a subspecies,[2-4] and the suggested promotion of a *F. philomiragia* subspecies to a new species, *F. noatunensis*.[5] Bacteria of *F. tularensis* ssp. are facultative pathogens of land and water-associated mammals, especially rodents and lagamorphs.[6] There is a strong association of environmental species such as *F. tularensis holarctica*, *F. novicida*, and *F. philomiragia* with waterways.[7-11] The causative agent of

franciselloisis (an infection of fish), *F. noatunensis* is found in marine environments.[5,12] For the purpose of this review, we will focus on *F. tularensis* species and subspecies, with some mention of *F. philomiragia*, where there is relevant biofilm information. Not enough is known about the biofilm capability of the other newly described strains and species of *Francisella* at this time.

The European form of tularemia (Holarctic, Type B) is less virulent overall for humans than the American form (Type A), but is more common in humans. Despite this lower virulence, in northern Europe, especially Sweden, there are thousands of human cases each year in active years. Natural zoonotic epidemics of tularemia occur during the summer months in animal populations throughout Europe, the US, and Russia. These epidemics are commonly spread by arthropod vectors such as mosquitoes, biting flies, and ticks.[1] The Swedish human tularemia cases are suspected to be mosquito-borne, and are closely associated with the afflicted patients having been near water and having mosquito bites.[13] Between epidemics, *F. tularensis* strains (Type A in the US and Type B in Europe) can also be routinely found in the environment by molecular sequencing of environmental samples (e.g., water and mud).[14,15]

Francisella Forms Biofilms

It is unclear how *Francisella* spp. survive in the natural environment given that laboratory growth of this nutritionally fastidious organism requires supplementation with bio-available iron, cysteine, and up to 12 other nutrients.[16,17] Our working hypothesis is that the ability of *Francisella* spp. to form biofilms allows it to achieve environmental persistence, similar to the closely related pathogen *Legionella* (*L.*) *pneumophila*.[18]

Biofilms are defined as naturally formed adherent communities of bacteria within an extracellular polymeric matrix.[19-21] The formation of a biofilm community allows for bacteria to resist shear stress in a flowing stream or water system and to increase the capture of nutrients. Additionally, bacteria embedded within biofilms show increased resistance to antibiotics and disinfectants due to slow diffusion rate and decreased metabolic activity.[22,23] Biofilm formation has also been shown to increase the survival of microorganisms by enhancing resistance to antimicrobials, oxidative radicals, and phagocytosis by amoeba or immune cells.[23,24] Biofilm production has been demonstrated in many infectious bacteria such as *Escherichia coli* and *Pseudomonas* but has not been demonstrated in *Francisella* until recently.[25-28]

The current literature contains examples of in vitro biofilm formation in the following species and subspecies: *F. novicida* has been shown to form biofilms on a variety of surfaces, including plastics, crab shells, and glass.[25,27,28] *F. tularensis holarctica* LVS has been shown to form biofilms on plastic 96-well plates.[26,29] *F. tularensis tularensis* SchuS4 has also been shown to form biofilms in vitro, more than LVS and less than *F. novicida*.[29] *F. philomiragia* has been shown to form biofilms on plastic 96-well plates and on glass slides and forms biofilms preferentially at 25 °C than 37 °C, perhaps reflecting the environmental niche of this species.[30]

The genus *Francisella* has several characteristics which have been hypothesized could aid in biofilm formation.[25] First, *Francisella* genomes all encode proteins necessary for the production of type IV pili.[31,32] In *Pseudomonas*, Type IV pili have been shown to be important for attachment to surfaces during biofilm formation.[33] Second, it produces a variety of extracellular carbohydrates, including a capsule, recently identified as an O-antigen capsule,[34] and at least in *F. tularensis* subsp. *tularensis* a capsule-like complex (CLC).[35] These extracellular carbohydrates potentially could contribute to the extracellular polymeric substance (EPS) that forms the biofilm. Third, there is an unlinked two-component system in *F. tularensis* and *F. novicida* that is quite similar to QseBC which has been shown in *E. coli* to aid in biofilm formation through quorum sensing.[36] Finally, there are some species-specific factors that may contribute to biofilm formation in those species uniquely, such as the c-di-GMP system in *F. novicida*. These factors will each be addressed individually below.

Molecular Mechanisms of Biofilm Formation

Bacteria (both pathogenic and environmental) form water-associated biofilms that promote their survival under challenging environmental conditions including nutrient limitation, protozoan predation, and other stressors. Well studied examples include *L. pneumophila*,[18,37] *Helicobacter pylori*,[38] *Pseudomonas aeruginosa*,[39] and *Vibrio cholera*.[21] Detailed studies of the regulation of biofilm formation in these organisms have shown that they use multiple molecular mechanisms to integrate varied environmental signals (such as nutrient limitation) and signals from other bacteria (such as quorum sensing molecules) to regulate their physiological status between biofilm vs. planktonic phenotype. Studies of *Francisella* biofilm formation at the molecular level have primarily been done in *F. novicida* to date, and are more limited in scope due to the newness of this area of research.

Type IV pili in *Francisella* biofilm production

Type IV pili are composed of pilin proteins, such as those encoded by the *F. novicida pilE* genes. The role of Type IV pili in *Francisella* spp. has been the subject of recent study,[31,32,40-49] and current results suggest that they may play a role in adhesion to host or surfaces. *Francisella* spp. encode multiple pilin genes. The pilE4 gene is important for fiber formation in *F. tularensis*, *F. novicida*, and LVS.[32,42,50,51] Transposon mutants in *F. novicida pilE4* were not defective for *F. novicida* biofilm production,[25] and thus it was concluded that *pilE4* is not essential to biofilm production in *F. novicida*. This result was surprising because of the association of Type IV pili with biofilms in other organisms.[51,52] Type IV pili are required in *Pseudomonas* for full formation of biofilm due to their role in initial attachment and colonization of surfaces.[33] The role of Type IV pili in *Francisella* spp. bacterial physiology is still not fully understood,[24,31,45] and the role for Type IV pili in *Francisella* spp. biofilm production has not been definitively addressed.

Extracellular carbohydrates in *Francisella* spp. biofilm production

Francisella spp. have long been reported to be capsulated,[53] and this capsule has been thought to play a critical role in

virulence.[54] The precise composition and even the existence of this capsule has been a matter of investigation for many years. *Francisella* spp. do not exhibit a tightly-associated capsule, as is seen for example on *Staphylococcus aureus* in the presence of India Ink.[55] Extracellular carbohydrate, perhaps in capsule, may also represent a potential vaccine target for tularemia.[56] Recent studies have suggested that there is an O-antigen capsule for *F. tularensis* and *F. holarctic LVS*, which appears to be important for virulence.[57-59] In addition, a capsule-like complex (CLC) in *F. tularensis* has recently been reported.[35] This may be the same as the HMW carbohydrate that was recently identified as separate from the *F. tularensis* O-antigen capsule.[60] It is not yet known whether CLC, HMW carbohydrate and/or capsule have not yet been demonstrated in *F. novicida*. Antibody responses to *Francisella* spp. carbohydrates are frequently reported.[61] Vaccination has been attempted with "capsule" material.[54,62] More recently, renewed vaccine efforts that focus the immune response to the carbohydrate and polysaccharides suggest that this may be an effective approach.[62,63]

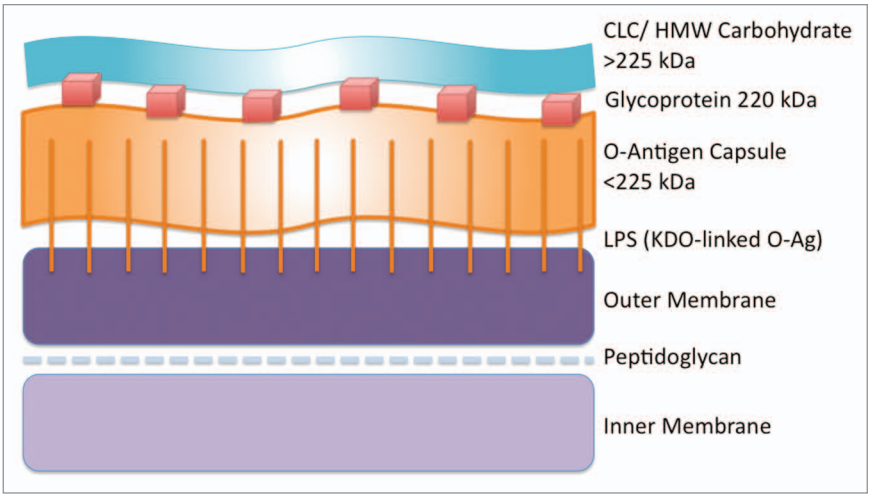

Figure 1. Schematic of proposed capsule organization in *Francisella* spp. The CLC/HMW carbohydrate is shown as an outer layer for illustration purposes. This layer contains carbohydrates >225 kDa. The next layer shown is the O-Ag capsule. This O-Ag capsule may be intermixed with KDO-linked O-Ag (i.e., LPS), thus attaching it to the outer membrane, shown in purple. The CLC/HMW layer and the O-Ag layers may not be actually separate as shown here, but rather are likely to be intermixed. The peptidoglycan layer is shown as a blue dotted line, and then the light purple inner membrane of this gram-negative organism. (Adapted from Zarrella et al., 2011[60]).

Capsule-like complex, CLC

Recently, it has been demonstrated that *F. tularensis* produces a capsule-like complex (CLC), an electron-dense surface material resembling a capsule, consisting of glucose, galactose and mannose.[35] CLC is shown to be distinct from LPS and contributes to the virulence of *F. tularensis*. A glycoprotein was also identified with a MW of 220 kDa. The authors also suggest a polysaccharide locus at FTL_1432-1421 that may be responsible for the production of CLC.[35] The role of CLC in biofilm matrix or biofilm formation is not yet determined.

HMW carbohydrates

F. tularensis grown in BHI pH 6.8 produces a high-molecular weight (HMW) carbohydrate,[60,64] which is independent of O-antigen, as shown by its presence in a *wbtA* mutant (although they may also produce large polymers of O-antigen).[60] HMW carbohydrate is defined as the material >225 kDa, and is found in wild-type (WT), as well as *wbtA* mutants, which clearly separates it from O-antigen. In these studies, additional material between 100 and 225 kDa is seen in WT bacteria that is not considered the HMW carbohydrate, and is likely the O-antigen capsule. This HMW carbohydrate material can interfere with antibody binding to OMP components, complement deposition and pro-inflammatory cytokine production in mouse macrophages, suggesting its capsule-like nature.[60] A schematic of the proposed organization of the multiple layers of capsule in *F. tularensis* is shown below in **Figure 1**.

What is the role of capsule and carbohydrate-like-complex in biofilm? This question has been challenging to answer clearly. Recently, it was shown that the surface carbohydrates of *F. tularensis* are altered upon growth in the host, leading to a so-called host-adapted phenotype.[60] This phenotype can also be induced by growth in BHI pH 6.8,[60,64] leading to HMW carbohydrate production. This host-adapted phenotype and the altered bacterial cell surface may also change the ability of the bacteria to induce Th1-immunity and to cause disease when delivered via aerosol to animal model hosts.[65] The capsule of *Francisella* spp. can be altered upon culturing,[53,66] suggesting that this phenotype of encapsulation may be modulated by environmental conditions.

Capsule genes

Despite an unclear biochemical function, mutants in capB in *F. tularensis tularensis or F. tularensis LVS* are significantly attenuated, including defective intracellular replication, suggesting that this gene product may play some yet undefined role in virulence.[62,67-72] In addition, according to Bergey's manual, *F. novicida* is reported to be uncapsulated (although this may vary with growth conditions).[53,73]

The role of CapBC genes as part of a potential capsule biosynthesis locus was investigated in biofilm formation in *F. novicida*.[74] Transposon mutants in the capC gene (FTN_1200, capsule biosynthesis protein), and the capB gene (FTN_1201, capsule biosynthesis protein) were tested for their biofilm forming activity. *F. novicida capC* mutants exhibited both a ~20% inhibition of biofilm production compared with the wild-type strain but were also ~20% inhibited in their growth, likely accounting for the apparent biofilm defect. The *capB* mutants had no significant effect on biofilm production, yet were equally inhibited in their in vitro growth (~20%). The small extent of the *F. novicida* biofilm defect with the *capBC* mutants suggests that the extracellular polysaccharide components of the biofilm may be comprised of components other than those produced by the

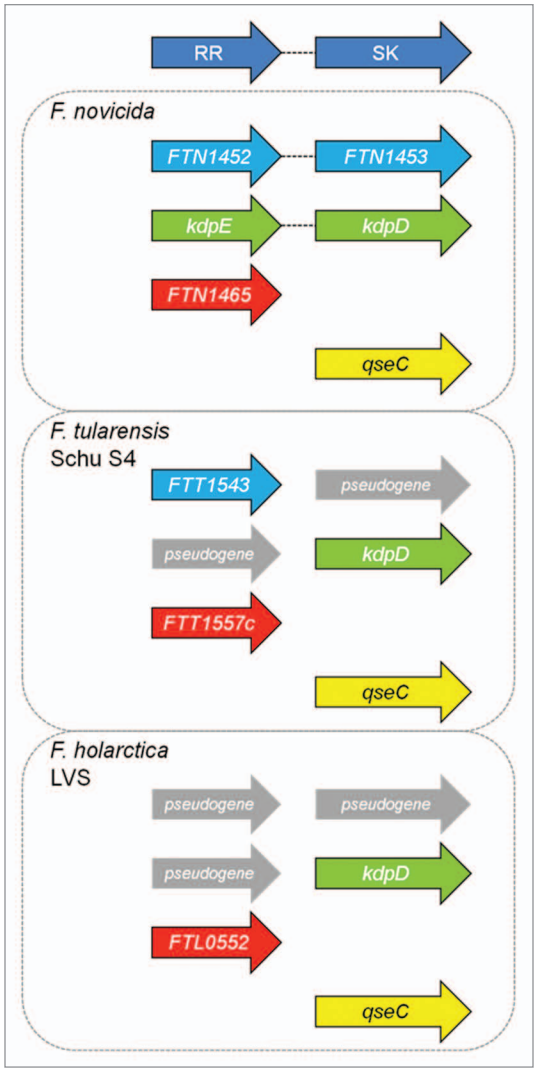

Figure 2. Sensor kinases and response regulators in *Francisella* species. (**A**) There are two complete and one incomplete TCS in *F. novicida*. *FTN1452/FTN1453* and *kdpDE* form the two complete TCS, while *FTN1465* (*pmrA/qseB*) and *qseC* are orphans members. (**B**) There are no complete TCS in *F. tularensis* Schu S4. Both the sensor kinase *FTT1544* and response regulator *FTT1735c* (*kdpE*) appear to be pseudogenes. As in *F. novicida* and LVS, *pmrA/qseB* and *qseC* appear to be orphan TCS components. (**C**) There are no complete TCS in *F. holarctica* LVS. Other than the additional mutation of *FTN0568,* the TCS make up of LVS is the same as found in *F. tularensis* Schu S4. This results in one undisrupted response regulator *FTL0552* (*pmrA/qseB*), and two sensor kinase encoding genes *kdpD* and *qseC*.

capBC genes. From these studies, it was concluded that *capB* and *capC* are not essential to biofilm production in *F. novicida*. *F. tularensis* SchuS4 Cap locus is FTT_0807- FTT_0805 (FtLVS Cap locus is FTL_1414–FTL_1416). The genes FTN_1199–1201 are annotated as genes related to the *Bacillus anthracis capBCADE* locus, proteins which produce a poly-D-glutamic acid capsule in that organism.[75] However, there is no evidence of a poly-D-glutamic acid capsule in *Francisella*, so the physiological role of the Cap locus in *Francisella* is unclear. Nonetheless, the frequent identification of the cap locus in *Francisella* virulence studies suggests that the Cap locus genes are playing some significant role in virulence.[68,71,72]

F. novicida biofilm formation is dependent on the two-component sensor kinase QseC and an orphan response regulator

Quorum sensing systems contribute to bacterial biofilm formation by controlling a phenotypic change in response to sufficient numbers of bacteria. In the case of *P. aeruginosa*, a LasI/LasR, RhlI/RhlR quorum sensing system is required to switch from free-living bacteria to sessile bacteria and biofilm formation within the lungs of cystic fibrosis patients.[51,76] Quorum sensing typically involves a "quorum-sensing signal production and sensing system" in gram-negative bacteria such as LuxI/LuxR, LasI/LasR, RhlI/RhlR, or auto-inducer peptide (AIP) sensing two-component regulatory systems in gram-positive bacteria. However, it has been shown that the gram-negative pathogen *E. coli* 0157:H7 actually also uses a two-component quorum sensing system QseBC for motility and biofilm formation.[36,77] *Francisella* spp. do not encode any genes that resemble known "quorum-sensing signal production and sensing system", such as LuxI/LuxR or the other well-known systems. For example, the luxI/luxR system recognizes acyl-homoserine lactones, and there are no obvious acyl homoserine lactone synthase genes or luxI/luxR genes in *Francisella* so these are not likely to be involved in *Francisella* quorum sensing.

Sensor kinase

An *E. coli* QseC homolog which is involved in a two-component system quorum sensing system has been identified in *Francisella*,[78] and these genes are also commonly found in other biofilm-forming bacteria. The QseBC two-component system in *E. coli* regulates flagellar motility, toxin expression, and a type iii secretion system (T3SS)[79] but, as *Francisella* does not produce flagella, toxins, or a T3SS, the downstream systems regulated by the homolog of QseBC are unknown for *Francisella*. It was found that transposon mutations in the putative *qseC* mutants had a significant effect on biofilm formation. *F. tularensis* subsp. *tularensis* Schu S4 encodes the gene for QseC which is 99% identical to the *F. novicida* gene FTN_1617. Its cognate response regulator QseB has not been formally identified as there are no transcriptionally linked response regulators to the QseC sensor kinase in a co-transcribed operon. Thus, QseC is an orphan sensor kinase with no linked response regulator. There are only three sensor kinases and three response regulators found among *Francisella* species, as shown in **Figure 2** and **Table 1**. Thus, *Francisella* spp have very limited two-component systems compared to other gamma-proteobacteria, and multiple dysfunctional genes (due to pseudogenes).

Response regulators

In *F. novicida* there are a total of 3 response-regulators, two of which are transcriptionally linked to sensor kinases (**Fig. 2**; **Table 1**). FTN_1465 has been identified as an orphan two-component response regulator in *F. novicida* that has high sequence similarity to QseB in *E. coli* by BLAST and is also present in the various other strains of *Francisella*.[28] The putative *qseB* gene had a significant effect on biofilm production, without affecting growth.[25] This gene has previously been suggested to

Table 1. Francisella two-component systems (role in virulence, if any, is indicated in the references)

Strain	RR1	SK1	KdpE (RR)	KdpD (SK)	QseB/pmrA (RR)	QseC (SK)
Ftt SchuS4	FTT1543			FTT1736c[71,160]	FTT1557c	FTT0094c[71]
Ft LVS	FTL0552		FTL1879	FTL1878		FTL1762
F. novicida	FTN1452	FTN1453	FTN1714	FTN1715	FTN1465[81]	FTN1617[107]

be called pmrA by Mohapatra et al. as it has a similar sequence, but not function, to *Salmonella enterica* serovar Typhimurium *pmrA*.[80] The PmrA–PmrB system functions in many gram-negative organisms to modify LPS, but Mohapatra and coworkers found no defect in LPS production using a FTN_1465 mutant.[80] In vitro biofilm formation of *F. novicida* is dependent upon this orphan response regulator,[25] suggesting that it may be acting as a biofilm mediating response-regulator. Therefore, the orphan response regulator FTN_1465 is potentially acting as a QseB-type molecule. Clearly, FTN_1465 pmrA/qseB is critical to the regulation of gene expression of many genes, including the genes of the *Francisella* pathogenicity island, and its activity is required for intramacrophage replication and mouse virulence.[81] However, QseC has not been demonstrated to be the sensor kinase responsible for phosphorylation and activation of FTN_1465 pmrA/qseB. The sensor kinases appear to be more promiscuous in their partnering than just phosphorylating their "cognate" response regulators. For example, Bell and Gunn have shown that the KdpD sensor kinase can phosphorylate the QseB/PmrA orphan response regulator in the absence of its "own" response regulator KdpE in *F. novicida*;[81] and in enterohemorrhagic *E. coli,* QseC can phosphorylate KdpE, a non-cognate response-regulator.[82] The nature of quorum sensing and the signaling molecules required to carry it out in *Francisella* remain undefined; however, clearly there are as yet unidentified extracullular signals that are being integrated by the bacterium to regulate its function through these two-component systems.

Other factors that may contribute to *Francisella* biofilm production

Outer membrane vesicles (OMVs) may contribute to biofilm formation by contributing to biofilm matrix

OMVs are subcellular vesicles which many gram-negative bacteria bleb off during all stages of growth, especially during times of stress.[83] OMVs have many functions, including a role in biofilm formation, enzyme delivery, and antibiotic resistance. Importantly, they have also been associated with pathogenesis.[83] *Francisella* has been shown to produce OMVs, and that these OMVs are effective as an intranasal vaccine against subsequent intranasal infection.[83] In addition, preparations of native outer membrane proteins of *F. tularensis* were found to be protective as an intraperitoneal vaccine against Type A challenge[84] further supporting the idea that preparations of multiple membrane proteins presented in a native (membranous) context may provide protection as a *Francisella* vaccine. Schooling and Beveridge suggested that OMVs associated with biofilms may be capable of binding antibiotics, thus further affording organisms in biofilms insulation against antimicrobial agents.[85] Beveridge also hypothesized that OMVs released from one species in a biofilm may also be able to lyse neighboring bacteria, thus releasing nutrients for growth and eDNA for the biofilm matrix.[86] Furthermore, Schooling and Beveridge also suggested that OMVs themselves may form part of the biofilm matrix.[85] The novel discovery that *Francisella* forms OMVs[83] and biofilms[28] and the work of Beveridge and Schooling[85,86] suggest a possible role for *Francisella* OMVs within *Francisella* biofilms.

Role of chitinase in Francisella *biofilm*

There are no reports of chitin production in *Francisella* species; however, chitinase is required for providing a carbon source under nutrient-limiting conditions.[87] Chitinases are glycosyl hydrolases that hydrolyze chitin, a linear β-1,4-linked polymer of N-acetyl-D-glucosamine (GlcNAc) that is the second most abundant polysaccharide in nature after cellulose. Chitinases are found in a wide range of species from all kingdom of life,[88,89] including those that are known not to synthesize chitin, such as bacteria, viruses, and higher plants as well as mammals. A recent study has demonstrated that *F. novicida* forms biofilms on biotic chitin surfaces such as crab shells in a chitinase-dependent manner, also demonstrating a role for the Sec secretion system and several Sec-dependent secreted proteins, some of which are predicted to bind and/or degrade chitin.[29]

In *Francisella,* four putative chitinases (ChiA, ChiB, ChiC, and ChiD) were identified and characterized in vitro using biochemical studies coupled with bioinformatic analyses.[90] Enzymatic analyses revealed these different chitinases possess dissimilar chitinase activities against substrates for endo- and exo-chitinase. *F. novicida* has two functional chitinases ChiA and ChiB, although it has all four genes of chitinases in the genome.[90] Biofilm formation of *F. novicida* on chitin is reported to be regulated by two chitinase genes *chiA* and *chiB*.[29] ChiA and ChiB, along with a chitin binding protein CbpA, are known to be secreted from *Francisella*.[44] Margolis et al. (2010) showed that *F. novicida* forms biofilms during the colonization of chitin surfaces (i.e., crab shells) by using chitin as a sole carbon source. In their study, they demonstrated that mutants lacking *chiA* or *chiB* were attenuated for chitin colonization and biofilm formation in the absence of exogenous sugar. This finding was also confirmed on abiotic glass surfaces.[28] In another study, however, *chiA* and *chiB* mutants showed no defects in the ability to colonize ticks,[91] which have chitin in their exoskeleton. Microarray analyses revealed that c-di-GMP stimulated the transcription of ChiA and ChiB,[28] which is likely contributing to biofilm formation as a result of c-di-GMP treatment. We recently demonstrated that chitinase expression is a negative regulator of biofilm production in *F. novicida* (Chung et al., in press), similar to the situation in *Yersinia*.[92] However, the underlying mechanisms of precisely how chitinases regulate *Francisella* biofilms are not fully determined, especially with regards to the potential substrate of these enzymes. Although all *Francisella* lack the chitin synthesis genes, and so

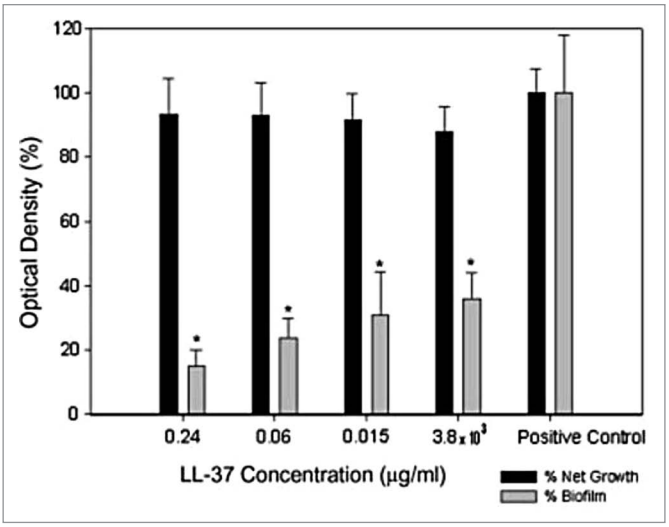

Figure 3. *F. novicida* biofilm inhibition by LL-37. Biofilm detection on polystyrene (PS) 96-well plate at 37 °C (PS 37 °C) after 48 h of growth in TSB-C is expressed as the absorbance at 570 nm. Growth is indicated in black bars with control set to a 100% and percent biofilm is indicated in gray bars with $n = 6$. This experiment is a representative of three independent trials. *Indicates P value less than 0.01 compared with control. (Figure is from Amer et al., 2010,[104] used with permission).

are not predicted to make chitin, chitinases are members of the broader glycosyl hydrolase family, and thus could potentially cleave other complex EPS substrates than chitin.

ciDiGMP regulates biofilm only in F. novicida

In most bacteria that form biofilm, including the gamma-proteobacteria and select agent *Yersinia pestis*,[93] cyclic di-GMP is a major regulator molecule that stimulates biofilm formation and inhibits virulence. However, the situation in *Francisella* is perhaps unique. While *F. novicida*, *F. tularensis LVS*, and *F. philomiragia* can all form biofilms, only *F. novicida* appears to regulate biofilm production through the classical c-di-GMP pathway.[28] The gene cluster for c-di-GMP production appears to be *F. novicida*-specific (FTN_0451 to FTN_0456) and these genes are not present in *F. tularensis* LVS or *F. tularensis* SchuS4 strains. So, while there appears to be a role for c-di-GMP in regulating *F. novicida* biofilm production (perhaps through chitinase as described above), this mechanism does not address the larger question of how biofilm production is regulated in other *Francisella* species that lack c-di-GMP.[28]

Antibiofilm host factors

While *Francisella* is not normally a respiratory pathogen, the most severe infections by *Francisella* species occur via inhalation or direct inoculation of the lungs leading to pneumonic tularemia.[1,94] Antimicrobial peptides represent an ancient host defense mechanism for combating infection as part of the innate immune response[95] to which relatively little bacterial resistance has emerged. Antimicrobial peptides are small (3–6 kDa) cationic peptides that can exert a direct antimicrobial effect on microbes.[95] These peptides are produced by almost all higher organisms and have specificity toward targeting the cellular membranes of microbes without attacking eukaryotic membranes.[95] Interest in antimicrobial peptides has grown with the increasing resistance of bacteria to commonly used antibiotics and the potential therapeutic applications of these peptides and their synthetic analogs.[96]

The cathelicidin family is a large and diverse collection of cationic antimicrobial peptides found in variety of vertebrate hosts.[95] In humans, only one cathelicidin (LL-37) has been characterized. LL-37 is derived by proteolysis from the C-terminal end of the human CAP18 protein (hCAP18).[96] This peptide can be found in the lung and in broncho-alveolar lavage fluid.[96,97] LL-37 is a 37-residue cationic peptide that forms α-helical structures when in association with the bacterial cell membrane.[95,98] LL-37 has been shown to exert broad-spectrum antimicrobial activity against a wide range of gram-positive and gram-negative bacteria and protects the host from endotoxic shock.[96] LL-37 is antimicrobial in the phagolysosomes of immune cells and at the sites of inflammation, but it plays a broader role in immunomodulation in systemic settings such as the lung[96,99] and has been reported to play a major role in protecting humans against naturally occurring respiratory diseases.[100] Several groups including ours have demonstrated that *Francisella* directly infects the human lung Type II alveolar epithelial cell line A549 in vitro[101-103] and this infection was found to strongly induce the expression of the antimicrobial peptides, including human β-defensins 2 (hBD-2), hBD-3, and LL-37, forming part of the lungs' innate immune mechanism to respond to this and other inhaled pathogens.[101]

The capacity of LL-37 to inhibit *Francisella* biofilm formation was recently tested.[104] It has been described that the LL-37 cathelicidin can inhibit the formation of *P. aeruginosa* biofilms at a concentration well below that required to kill or inhibit growth in broth microdilution assays.[105] While *F. novicida* growth was not inhibited by LL-37 peptide in TSB-C broth even at the highest peptide concentration tested (0.24 μg/ml), due to the high amount of salt in this bacterial growth media, a significant inhibition of *Francisella* biofilm formation was observed at sub-antimicrobial peptide concentrations (**Fig. 3**). The anti-biofilm targets of LL-37 in *Pseudomonas* include the Rhl and Las, quorum sensing systems that control biofilm production.[105] However, these quorum-sensing systems are not present in *Francisella*, and thus the *Francisella* target of LL-37 remains to be defined.

Biofilm and Virulence

QseC is an orphan sensor kinase with no linked response regulator. *F. tularensis* subsp. *tularensis* Schu S4 encodes a gene for QseC which is 99% identical to the *F. novicida* gene FTN_1617. Its cognate response regulator QseB is likely a pseudogene in *F. tularensis* subsp. *tularensis* Schu S4, as shown in **Figure 2** and **Table 1**. Rasko et al.[106] performed an interesting experiment using an antagonist of QseC, LED209, which has been shown to block autoinducer-3 signaling in *E. coli*. In an experiment that tests the hypothesis that QseC may be important for *Francisella* virulence, they demonstrated that LED209 was able to block *F. tularensis* SchuS4 infection in mice (**Fig. 4**), suggesting that signaling through QseC was critical in some manner for *Francisella*

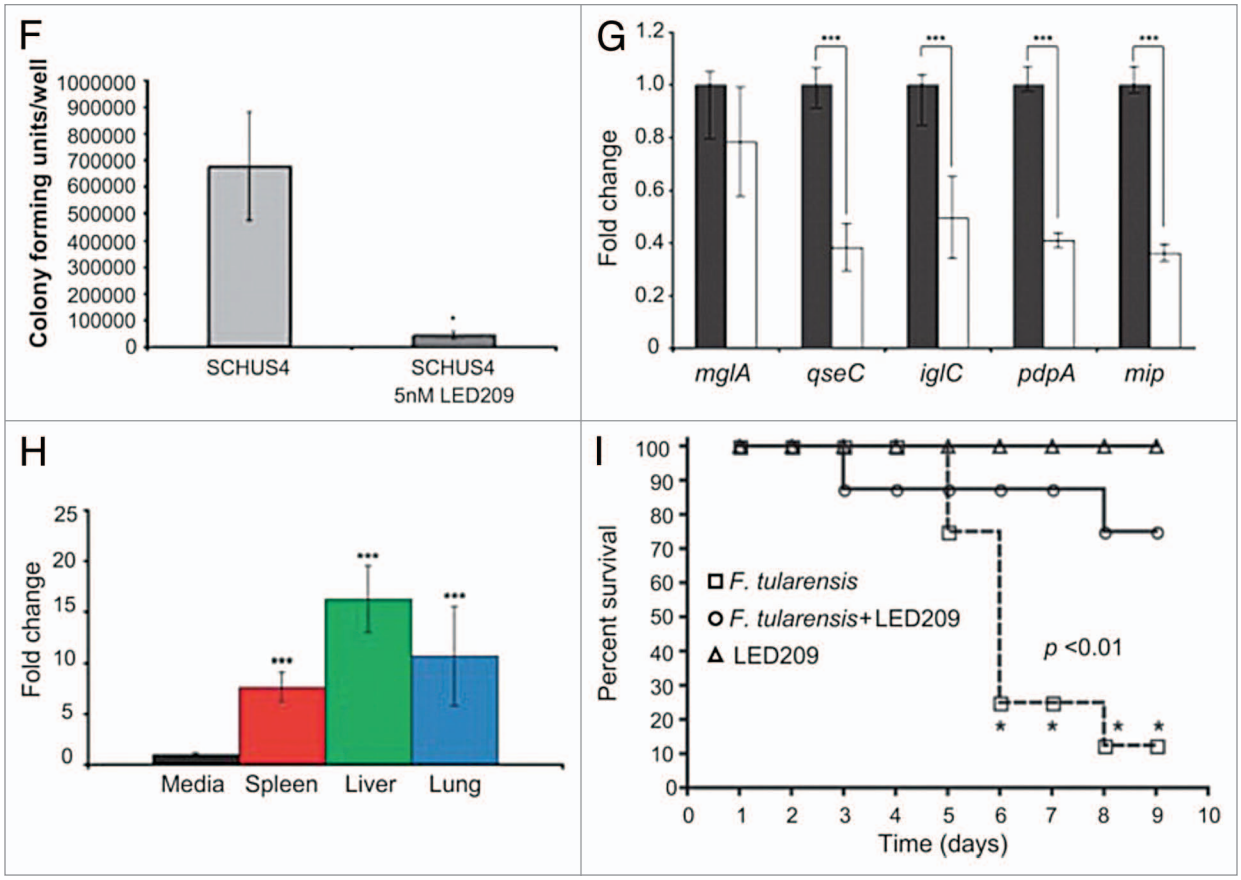

Figure 4. LED209 inhibits *F. tularensis* virulence in vivo and in vitro. Showing only panels (**F–I**) of the original figure. (**F**) Infection of J774 murine macrophages with *F. tularensis* SCHU S4 in the absence and presence (5 nM) of LED209. (**G**) QPCR of *F. tularensis* virulence genes in the absence (gray bars) and presence (white bars) of LED209 (5 pM). (**H**) QPCR measuring expression of qseC in SCHU S4 during growth in vitro and in vivo (spleen, liver, and lungs). These data were collected from 5 C3H HeN mice intranasally infected with 30 CFUs of SCHU S4. QPCR of qseC was normalized against rpoA. (**I**) Survival plot of mice (C3H HeN) upon oral treatment with LED209 (20 mg/kg) alone, intranasal infection with 30 CFUs of SCHU S4, and intranasal infection with 30 CFUs of SCHU S4 plus LED209 (20 mg/kg). *$P < 0.01$; **$P < 0.001$; ***$P < 0.0001$. From Rasko et al.[106] with permission.

infection.[106] In a screen in *Drosophila*, the *F. novicida* QseC sensor kinase was identified as a virulence factor.[107] These are some of the first indirect evidence suggesting a role of *Francisella* quorum sensing in virulence, but direct evidence of the role of quorum sensing in virulence has yet to be established.

In a survey of virulence screens, no two-component system gene (histidine kinase or response regulator) is consistently implicated as a virulence factor in *Francisella*.[108-116] KdpD and PmrA/QseB are most frequently identified as having a role in virulence (**Table 1**).[25,113,117-120]

Biofilm and Environmental Persistence

Some species of *Francisella* are water-associated

The persistence of *Francisella* spp. in the environment has been a topic of great interest with respect to the epidemiology of tularemia outbreaks, and the potential for these strains to form biofilms. Although Type A tularemia strains (especially *F. tularensis tularensis* Schu S4) are commonly associated with dry, arid habitats and may not have a strong connection with water (except in Martha's Vineyard, see below), Type B and the other environmental species of *Francisella* spp. have been closely associated with water and water-systems (**Fig. 5**).[121-124] Indeed, *Francisella* spp. DNA has been identified in surface water and sediment samples in endemic sites in Sweden,[13] even in years with little tularemia activity in humans, suggesting that environmental persistence (defined as the continued presence of *Francisella* independent of infected vertebrate hosts) may be a regular feature of Type B tularemia.[15] In support of this fact, *Francisella* spp.-contaminated mud and silt have been found to remain infectious for up to 8–10 weeks.[7] Additional sources of *Francisella* spp. could come from the carcasses of dead infected animals, or from the excreta of immune or sick animals.[125-127]

In addition, it has been demonstrated that brackish water can promote the survival of multiple *Francisella* species. In particular, Type A *Francisella tularensis* was demonstrated to have increased time of survival in brackish water vs. fresh water (although brackish water alone does not support proliferation of bacteria), and outperformed Type B LVS and *F. novicida* in this regard.[128] It has been suggested that the persistence of Type A tularemia on Martha's Vineyard may be supported by the multiple brackish water ponds that exist there. From the point of view of potential

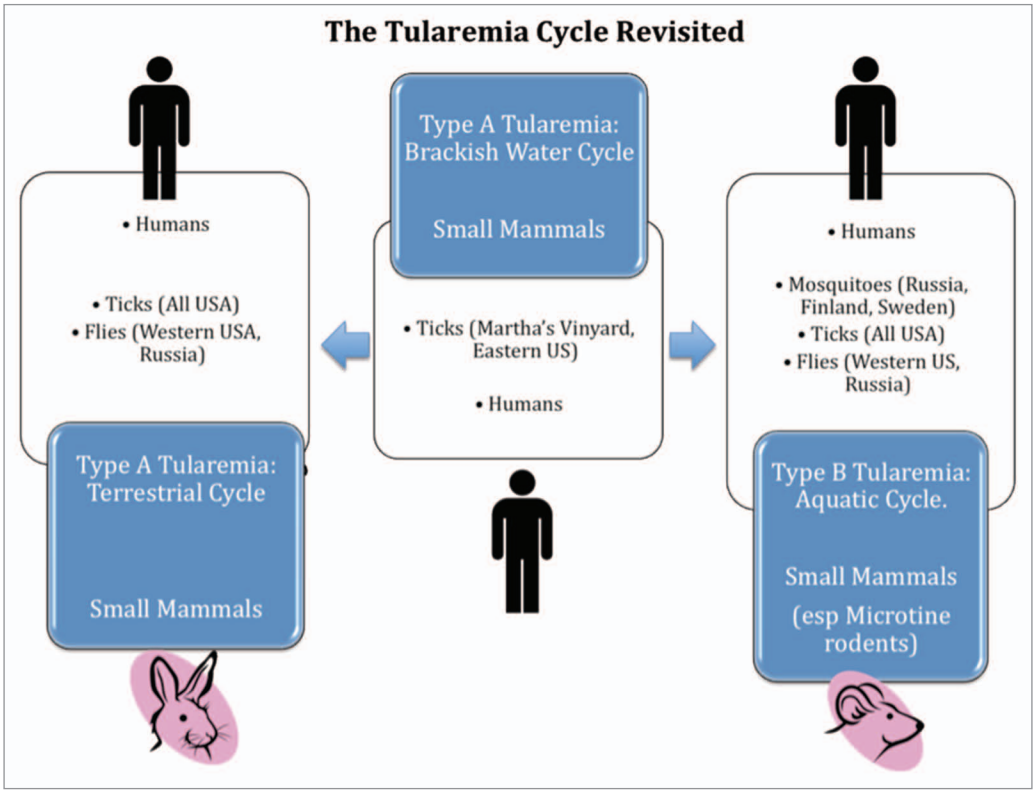

Figure 5. The tularemia cycle revisited. The figure illustrates a new adaptation of the tularemia zoonosis cycle from Akimana et al., 2011[129] illustrating the American (Type A) and European (Type B) tularemia cycles and their relationship to animal hosts, human hosts, vectors, and water. In addition to the standard Type A~terrestrial (dry) loop, and Type B~aquatic (wet) loop mediated by the appropriate vectors, a third loop has been added representing the special case of Type A~brackish water (wet) conditions, such as on Martha's Vineyard.

biofilm formation, aquatic environments are supportive of biofilm formation,[21] unlike dry, arid environments. This suggests that there could be an amendment to the previously published tularemia cycle by Akimana et al.,[129] which describes a two-part schematic, with a terrestrial (dry) cycle for Type A and an aquatic cycle for Type B tularemia. The increasing reports of *F. tularensis* survival in brackish water[128,130,131] imply that a third loop in the tularemia cycle could be an aquatic cycle for Type A tularemia, potentially mediated by brackish water conditions enabling biofilm formation in this species (**Fig. 5**). This suggests interesting research questions regarding the ability of *F. tularensis* to form biofilms in situ, potential additional vectors for transmission such as protozoans, and whether mosquitoes could transmit Type A tularemia under these "aquatic conditions".

F. tularensis in open water has been characterized as entering a viable but not culturable (VBNC) state in which the bacteria are not infectious.[10,128] However, using molecular methods, *Francisella* species have been detected in many natural water samples throughout the Northern Hemisphere,[15,132,133] although the "culturability" of these samples was not always demonstrated. Historically, many older publications regarding the natural history of tularemia also included reference to natural water sources,[7,125,134-136] and water is named as the source of several of the well-studied strains, including *F. philomiragia* and *F. novicida*.

There is also an emerging group of *Francisella* species or subspecies associated with francisellosis infection in fish, particularly in farmed fish. These strains have recently been reclassified from a subspecies of *F. philomiragia* to their own species, *F. noatunensis*. These are completely marine-associated organisms whose known hosts include a wide variety of farmed fish across the globe.[12] Their ability to form biofilms has not yet been studied.

Our hypothesis is that *Francisella* spp. cannot thrive (i.e., replicate) in open water, but is instead found in biofilms or harbored inside aquatic eukaryotes such as amoebae within the water column. This would be in alignment with the lifestyles of many other bacteria.[21] As part of this concept, it was further hypothesized that *Francisella* spp. persist in water (seawater, brackish, or fresh) within the protection of a biofilm instead of in a planktonic state. This biofilm could be the product of *Francisella* bacteria alone or, more likely, a complex, polymicrobial mixture that includes *Francisella* spp. and other organisms, such as amoebae.[23,25,137,138] As *F. tularensis* is a Tier 1 bioweapon, it is important to understand its ability to form biofilm in the context of its persistence mechanisms in the natural environment.[138,139] Biofilms formed within mud, sediment and waterways could be one way that *Francisella* spp. persists in the environment and could contribute to outbreaks of this disease in animals and humans. Alternatively, in the context of bioremediation after an event, understanding that *Francisella* may be present in biofilms (which are inherently more resistant to disinfection) is critical.

F. novicida is associated with water-borne tularemia of animals (it was first isolated from water in Utah), and in at least

two documented cases, it has caused a tularemia-like disease in outdoor workers.[140-143] Using this species (*F. novicida*, or *Francisella tularensis novicida)*, which is a less virulent strain that is widely used as a model organism for the more virulent *F. tularensis*,[144] it has been shown that *F. novicida* (as well as *F. philomiragia*) is able to form biofilms in vitro.

***Francisella* interaction with single celled eukaryotes**

The second part of our hypothesis is that *Francisella* may be harbored inside aquatic eukaryotes such as amoebae within the water column. In trying to understand how *Francisella* can survive in the environment, several groups have proposed that it finds protection and achieves persistence by infecting water-associated eukaryotic hosts such as single-celled protists, including *Acanthamoeba* (*A.*) *castellanii*.[30,138,145,146] It has recently been demonstrated that *Francisella* species (including live vaccine strain [LVS], SchuS4, and *F. novicida*) can infect the water-dwelling amoeba *A. castellanii*.[30,138,145,147,148] Our group has recently demonstrated that the environmental organism *F. philomiragia* can also infect *A. castellanii* amoeba (**Fig. 6**).[30] *Francisella* can also survive within another protist *Hartmonella* (*H.*) *vermiformis*,[149] and an interaction with *Tetrahymena* (*T.*) *pyriformis* was also reported.[150]

In addition, it has been shown that *Francisella* LVS grown in the presence of *A. castellanii*-conditioned medium has an increased overall growth rate, suggesting that the bacteria benefited from a close association with the amoebae.[30,138,147] For other pathogens such as *Legionella,* the interaction of bacteria with amoebae has been demonstrated to promote persistence in aquatic systems and increase virulence, i.e., the ability of *Legionella* to invade mammalian host cells.[151] *Legionella* and amoebae have been identified together in both artificial and naturally occurring biofilms, and this may represent a replication niche for *Legionella*, even within a eukaryotic host.[152]

In conjunction with our hypothesis that *Francisella* persists in natural water within the protection of a biofilm instead of in a planktonic state,[28] we wondered if this biofilm could be polymicrobial and the product of a complex microbial ecology that includes *Francisella* and other organisms, such as amoebae.[23,137,138] We recently demonstrated that *F. philomiragia* is capable of growing, surviving, and producing a mixed biofilm in the presence of *A. castellanii* (**Fig. 6**).[25] In the mixed biofilm, *A. castellanii* appears to reside on the outskirts of the biofilm, possibly grazing on its edges. It has been suggested that *F. tularensis* biofilms may act as "lures" for attracting environmental amoebae and other protists which can then be hosts for further *Francisella* infection.[25,153] Thus, when co-cultured with *Francisella*, amoebas are found in the mixed biofilm, and may secrete factors that promote biofilm formation.[25]

This new understanding of the persistence of the *Francisella* organism in aquatic systems potentially through complex-biofilm formation and interaction with water-dwelling protists such as *Acanthamoeba* may be important in developing prevention strategies for this pathogen.[153] This may especially apply in northern Europe and Asia, where the environmental conditions (aquatic, brackish environments) may favor biofilm formation for the Type B Holarctic strains.

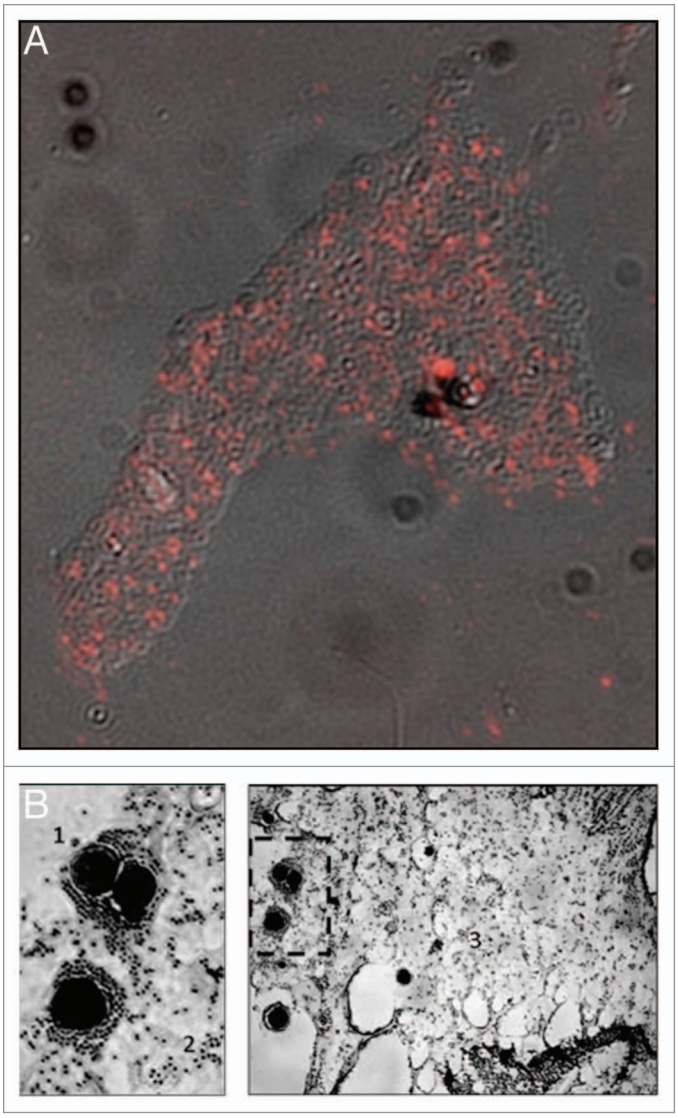

Figure 6. *Francisella philomiragia* interaction within amoeba (**A**) *Francisella philomiragia* internalized within amoeba. Immunofluorescence microscopy is shown with DIC overlay. Amoebae were infected with *F. philomiragia* for 6 d. DIC image of amoeba overlaid with red-channel image of *F. philomiragia* (stained with Tetracore anti-*Francisella* antibody and AlexaFluor-568 secondary antibody, red). (Figure is from Verhoeven et al.[30] with permission).(**B**) *Francisella* (*F.*) *philomiragia* biofilm formation when grown in the presence of *Acanthamoeba castellanii*. *F. philomiragia* at a concentration of 1×10^7 was co-incubated with *A. castellanii* at a concentration of 1×10^6 at 25 °C for 48 h in a six-well polystyrene plate. (**1**) Following crystal violet staining, darkly stained amoebae surrounded by bacteria can be observed at the edge of the biofilm but are not observed throughout the rest of the biofilm. (**2**) *F. philomiragia* in the fixed and crystal-violet stained biofilm. (**3**) *F. philomiragia* biofilm extracellular matrix. Image was taken at 40×. (From Verhoeven et al.[30] with permission).

Mosquito larvae and *Francisella* biofilm

It has also been suggested that mosquito larvae may acquire *Francisella* infection by ingesting such protozoa from their aquatic habitat or from feeding on the biofilms of *Francisella*.[26,154-156] A recent study by Mahajan et al. demonstrated that *F. tularensis*

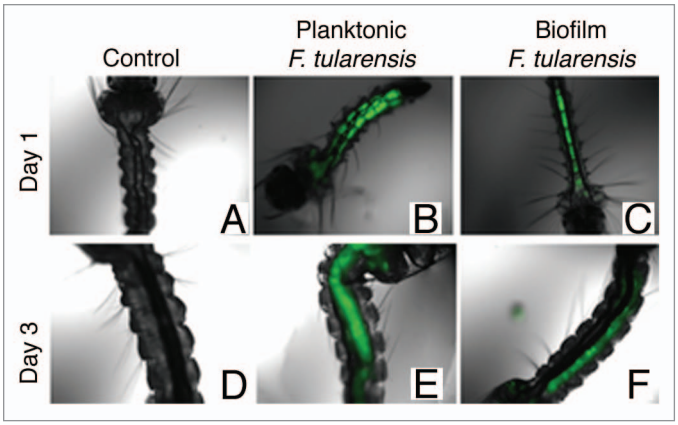

Figure 7. *Culex quinquefasciatus* larvae feed on planktonic and biofilm *F. tularensis* LVS. *Culex quinquefasciatus* feeds on planktonic and biofilm *F. tularensis* LVS resulting gut fluorescence lasting at least 72 h post feeding. (**A–C**): 24 h post feeding: (**A**) control; (**B**) planktonic *F. tularensis* LVS; (**C**) biofilm *F. tularensis* LVS; (**D–F**): 72 h post feeding (**D**) control; (**E**) planktonic *F. tularensis* LVS; (**F**) biofilm *F. tularensis* LVS. (Figure from Mahajan et al.,[26] with permission).

LVS can form biofilms in natural water.[26] Furthermore, that the mosquito larvae of *Culex* (*C.*) *quinquefasciatus* are able to feed on both biofilm and planktonic forms of *F. tularensis* LVS (**Fig. 7**).[26] These *C. quinquefasciatus* larvae exhibited defects in growth and fertility attributed to feeding on *Francisella*, so the relevance to their "fitness" as vectors is unclear. Additional data has been presented for mosquito larvae acquiring infection from water, perhaps by the ingestion of predatory protozoa suggesting that *Francisella*–biofilm–protozoan interactions could be important for vectors as well as for persistence.[15] Unlike *Yersinia pestis* biofilm and fleas, there does not appear to be a phenotype of *Francisella* biofilm increasing the ability of infected vectors such as ticks[157] to transmit tularemia.

***Francisella* interaction with other organisms of the marine environment**

Finally, reports of *F. novicida* and *F. tularensis tularensis* forming biofilm on crab shells in a chitinase-dependent manner[29] suggests that *Francisella* can grow on chitin-rich surfaces that might be widely found in the natural aquatic environment.[158] In support of this concept, a Russian study demonstrated that the addition of shrimp, mollusks, diatoms and zooplankton all increased the survival of *F. tularensis holarctica* in nutrient-depleted water samples.[159]

Conclusion

Biofilms may represent a previously unrecognized physiological state of *Francisella*. Understanding the microbial physiology of *Francisella* as being related to biofilm formation may allow the testing of new hypotheses, and may lead to the development of new prevention strategies for this organism. Overall, the current data suggest that biofilms are likely to be a key mechanism of environmental persistence in the natural environment for this fastidious and delicate organism. A role for *Francisella* biofilm in pathogenesis and infection of the mammalian host should be the focus of future studies.

Outstanding Questions

Q1) How is biofilm matrix produced in *Francisella*? What is the precise composition of the biofilm matrix? Is the composition of the biofilm different between the species of *Francisella?*

Q2) How are biofilm and biofilm matrix related to the capsule or to high molecular weight carbohydrate/CLC in *Francisella*?

Q3) What is the role of Type IV pili in *Francisella* biofilm formation?

Q4) What is the quorum sensing system in *Francisella*?

Q5) Is there an alternate system in "non-*novicida*" *Francisella* strains that functionally replaces c-di-GMP system found in *F. novicida*?

Q6) Are the bacteria found within mosquito larvae in biofilm or planktonic form?

Q7) Is there a role for brackish water in the outbreaks of fully virulent Type A tularemia?

Q8) What is the role for biofilm for different *Francisella* species and subspecies in virulence and environmental persistence?

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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