Cyclic Di-GMP Stimulates Biofilm Formation and Inhibits Virulence of *Francisella novicida*

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Francisella tularensis is a Gram-negative bacterium that is highly virulent in humans, causing the disease tularemia. *F. novicida* is closely related to *F. tularensis* and exhibits high virulence in mice, but it is avirulent in healthy humans. An *F. novicida*-specific gene cluster (FTN0451 to FTN0456) encodes two proteins with diguanylate cyclase (DGC) and phosphodiesterase (PDE) domains that modulate the synthesis and degradation of cyclic di-GMP (cdGMP). No DGC- or PDE-encoding protein genes are present in the *F. tularensis* genome. *F. novicida* strains lacking either the two DGC/PDE genes (*cdgA* and *cdgB*) or the entire gene cluster (strain KKF457) are defective for biofilm formation. In addition, expression of CdgB or a heterologous DGC in strain KKF457 stimulated *F. novicida* biofilms, even in a strain lacking the biofilm regulator QseB. Genetic evidence suggests that CdgA is predominantly a PDE, while CdgB is predominantly a DGC. The *F. novicida qseB* strain showed reduced *cdgA* and *cdgB* transcript levels, demonstrating an *F. novicida* biofilm signaling cascade that controls cdGMP levels. Interestingly, KKF457 with elevated cdGMP levels exhibited a decrease in intramacrophage replication and virulence in mice, as well as increased growth yields and biofilm formation *in vitro*. Microarray analyses revealed that cdGMP stimulated the transcription of a chitinase (ChiB) known to contribute to biofilm formation. Our results indicate that elevated cdGMP in *F. novicida* stimulates biofilm formation and inhibits virulence. We suggest that differences in human virulence between *F. novicida* and *F. tularensis* may be due in part to the absence of cdGMP signaling in *F. tularensis*.

Tularemia is a serious infectious disease of humans that is caused by the facultative intracellular bacterium *Francisella tularensis*. Infection with *F. tularensis* can occur through exposure to infected animals, bites of infected insects or arthropods, or contaminated meat or water or by inhaling airborne bacteria (30, 43). Pneumonic tularemia can lead to high mortality (12, 44). However, the various subspecies of *F. tularensis* exhibit differing levels of virulence in humans.

F. tularensis subsp. *tularensis* is highly virulent for humans, with an infectious dose of <10 bacteria (11, 23, 36, 38). *F. tularensis* subsp. *tularensis* can be disseminated in an aerosolized form to cause pneumonic tularemia and has been developed into a bioweapon by various countries (9). At the opposite extreme of infectious capability for humans is *F. novicida* (depending on the classification system, *F. novicida* is also classified as a subspecies, *F. tularensis* subsp. *novicida*) (47). *F. novicida* is highly attenuated for virulence in humans and is known to cause disease only in immunocompromised individuals (12). However, both *F. tularensis* subsp. *tularensis* and *F. novicida* are highly virulent in mice and utilize a shared set of virulence genes within the *Francisella* pathogenicity island (FPI) that encode a type VI-like secretion system to escape the phagosome within macrophages and cause disease (2, 29). Thus, the tropisms responsible for the high and low virulence, respectively, of these subspecies in humans are still unclear.

The complete genome sequences of several representative *F. tularensis* subsp. *tularensis* and *F. novicida* strains have revealed a high degree of homology between them, and also highlighted important differences. In comparison to the *F. novicida* genome, the *F. tularensis* subsp. *tularensis* genome is undergoing reduction resulting from rearrangements and the accumulation of pseudogenes (7, 21, 35, 39). Relatively few elements are present in the *F. tularensis* subsp. *tularensis* genome that are absent from *F. novicida*; rather, a number of genes and gene clusters can be found in *F. novicida* that are absent or disrupted in *F. tularensis* subsp. *tularensis*. We have identified a gene cluster present in *F. novicida* but absent in *F. tularensis* subsp. *tularensis* that is involved in the synthesis and degradation of the secondary messenger molecule bis-(3′-5′)-cyclic dimeric GMP (cdGMP).

Elevated cdGMP levels within a large number of bacteria are generally used to control the switch from a free-living planktonic lifestyle to a sedentary biofilm-associated lifestyle (19, 41). Proteins with diguanylate cyclase (DGC) activity, encoded within domains containing a GGDEF motif, synthesize cdGMP, whereas those with phosphodiesterase (PDE) activity, encoded within domains containing an EAL motif, degrade cdGMP; these domains can be found in separate proteins as well as in composite proteins that contain both motifs (GGDEF-EAL) (20). cdGMP signaling generally controls exopolysaccharide expression associated with biofilm development but has also been shown to control a variety of other behaviors in different bacteria, including motility and virulence (17, 41).

F. novicida forms biofilms *in vitro*, and this behavior is likely linked to its environmental persistence in aquatic habitats as well as possibly within tick and mosquito vectors (10, 26). An orphan response regulator, QseB (FTN1465), is required for normal biofilm development (10), as is the Sec secretion system and several Sec-dependent secreted proteins, some of which are predicted to

bind and/or degrade chitin (26). In the current study, we show that high cdGMP levels stimulate *F. novicida* biofilm formation and inhibit virulence, suggesting that differential cdGMP signaling within *Francisella* species may contribute in part to their relative virulence in humans.

MATERIALS AND METHODS

Strains, plasmids, and media. *F. novicida* strains are isogenic with strain U112 (ATCC 15482) and are listed in Table S1 in the supplemental material. *Vibrio cholerae* smooth strain A1552 (49) was used for some biofilm studies. *F. novicida* strains were grown on TSAP broth/agar (24) or Chamberlain's defined medium (CDM) (6), supplemented with antibiotics as appropriate. The concentrations of antibiotics used were as follows: kanamycin, 50 µg ml^{-1}; tetracycline, 10 µg ml^{-1}; ampicillin, 100 µg ml^{-1}; and erythromycin, 150 µg ml^{-1}. *F. novicida* mutant strain KKF457 [Δ(FTN0451-FTN0456)] was created by splicing by overlap extension (SOE) as described by Liu et al. (24), using the PCR primers listed in Table S2. The *cdgA cdgB* mutant (KKF539) was constructed by first removing the kanamycin resistance marker from the *cdgB* transposon mutant tnfn1_pw060323p04q138 (ISFn2/FRT), obtained from a comprehensive *F. novicida* transposon library (13) (www.beiresources.org/), by FLP-mediated recombination as described previously (2), resulting in strain KKF535. The *cdgA* transposon mutant tnfn1_pw060328p03q151 (ISFn2), obtained from the comprehensive *F. novicida* transposon library, was then recombined into the chromosome of this strain via cryotransformation with genomic DNA (22), resulting in strain KKF539. The *qseB chiA chiB* mutant was constructed in the same manner, utilizing the transposon mutants tnfn1_pw060328p07q138 (*qseB*), tnfn1_pw060323p01q146 (*chiA*), and tnfn1_pw060328p03q184 (*chiB*) from the comprehensive *F. novicida* transposon library. The transposon insertions in all strains were verified by sequencing. Various experiments with the complementation plasmid pKEK1524 (expresses *cdgB*) demonstrated a lack of polarity of the *cdgB* transposon insertion, as shown in Results. To demonstrate a lack of polarity of the *cdgA* transposon insertion, reverse transcription-PCR (RT-PCR) was performed on the wild-type U112 and KKF538 (*cdgA*) strains, which showed that transcription of the downstream gene FTN0450 appeared unaffected by the presence of the transposon (see Fig. S1).

Plasmids were constructed using the expression vector pKEK894 as described previously (50) and are listed in Table S1 in the supplemental material. *Escherichia coli* strain DH5α (15) was used for cloning procedures. Primers used in plasmid construction are listed in Table S2. PCR fragments were digested with NcoI and EcoRI and ligated into similarly digested pKEK894.

Biofilm formation assay. Overnight cultures of *F. novicida* strains were used to inoculate 1-ml aliquots of CDM (1:100) in 5-ml borosilicate glass tubes (Falcon), and the cultures were incubated statically at 37°C. At the indicated time points, the liquid medium was removed, and tubes were washed three times with phosphate-buffered saline (PBS) and allowed to air dry. Tubes were then incubated with 1.5 ml 0.4% crystal violet for 30 min, rinsed with distilled water, and air dried. Crystal violet was extracted with 30% acetic acid and quantitated by measuring the optical density at 570 nm (OD$_{570}$).

RNA isolation and quantitative RT-PCR (qRT-PCR). Total RNAs were isolated from mid-log-phase (OD$_{600}$, ~0.6) *F. novicida* cultures grown in CDM at 37°C by use of TRIzol reagent (Ambion) and were further purified using an RNeasy miniprep kit (Qiagen). RNA concentrations were determined using a Nano Drop 1000 spectrophotometer (Thermo Scientific). The isolated RNAs were treated with DNase I (Turbo DNA-free kit; Ambion) and reverse transcribed using random primers to produce cDNAs (Invitrogen). cDNAs were amplified with an Applied Biosystems ABI 7500 real-time PCR system, using Sybr green PCR master mix (Applied Biosystems). Gene-specific primers (see Table S2 in the supplemental material) were designed to amplify 120- to 150-bp fragments of each gene. Relative expression values (R) were calculated by using the formula $R = 2^{-(\Delta C_T \text{ target} - \Delta C_T \text{ endogenous control})}$, where C_T is the fractional threshold cycle (25). The constitutively expressed *F. novicida* *rpoB* gene was used as an endogenous control, and a value of 1 was used to standardize the gene expression of the wild-type strain.

Microarray expression analysis. A whole-genome NimbleGen microarray was developed for multiple *Francisella* subspecies, using shared probes for common genes as well as unique probes for subspecies-specific genes. Included were probes representing the *F. novicida* U112 genome, with a maximum of 6 probes/gene. RNA was isolated from bacteria and purified as described above. Double-stranded cDNA was generated and labeled with Cy3 according to the NimbleGen gene expression array protocol, using a SuperScript double-stranded cDNA synthesis kit (Invitrogen) and a DNA labeling kit (NimbleGen). Microarray results were analyzed using ArrayStar software (DNASTAR).

Intramacrophage survival assay. *F. novicida* strains were used to infect the J774.1 macrophage cell line (ATCC) at a multiplicity of infection (MOI) of 10:1. The intramacrophage survival assay was performed as previously described (22).

Mouse virulence assays. Groups of five female 4- to 6-week-old BALB/c mice (Charles River Laboratories) were inoculated intranasally with *F. novicida* strains in 20 µl PBS. Actual bacterial numbers delivered were determined by plate counts of the inocula, and an approximate 50% lethal dose (LD$_{50}$) was determined based on the number of surviving mice. Mice were monitored for 30 days after infection. The Institutional Animal Care and Use Committee (IACUC) at UT San Antonio approved all animal procedures.

Microarray data accession number. Microarray data have been submitted to the NCBI Gene Expression Omnibus (GEO) in accordance with MIAME standards, under accession number GSE39647.

RESULTS

***F. novicida* contains a cdGMP modulatory gene cluster that is absent in *F. tularensis* subsp. *tularensis*.** A gene cluster (FTN0451 to FTN0456 [FTN0451-FTN0456]) was identified in the *F. novicida* U112 genome that contains six genes that are missing from the *F. tularensis* subsp. *tularensis* Schu S4 genome (Fig. 1A and B). The genes are organized into two divergently transcribed operons, FTN0451 and FTN0452 to FTN0456. FTN0451 and FTN0456 encode composite GGDEF-EAL proteins (i.e., containing both DGC and PDE domains) (Fig. 1C), and because evidence below indicates that these proteins modulate cdGMP levels, we have renamed the genes *cdgA* (cyclic *di*guanylate modulatory protein *A*) and *cdgB*, respectively. CdgA and CdgB also contain PAS domains, which are found in bacterial signaling proteins. CdgA and CdgB are predicted to be localized to the cytoplasm, because they lack obvious signal peptides or transmembrane domains.

Alignment of the amino acid (aa) sequences of the DGC domains of CdgA and CdgB with those of well-characterized DGCs, i.e., VCA0956 from *V. cholerae* (45, 46), PleD from *Caulobacter crescentus* (16, 31), and WspR from *Pseudomonas aeruginosa* (14, 18, 28), revealed the conservation of critical residues required for nucleotide binding and catalytic activity (see Fig. S2 in the supplemental material). CdgA has a Y substitution (Y331) at a position that normally has a histidine residue important for indirect binding of substrate, as well as substitutions in residues identified as primary and secondary inhibitory sites (48) important for cdGMP inhibition of activity. These changes may correlate with our evidence below suggesting that CdgA is predominantly a PDE rather than a DGC.

Alignment of the aa sequences of the PDE domains of CdgA and CdgB with those of well-characterized PDEs, i.e., VieA from *V. cholerae* (42, 45, 46), BlrP1 from *Klebsiella pneumonia* (1), and YkuI from *Bacillus subtilis* (27), revealed conservation of the crit-
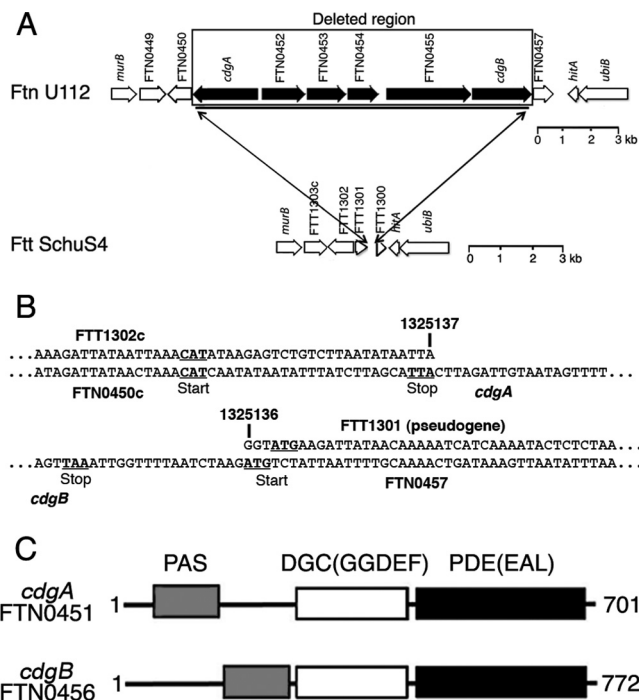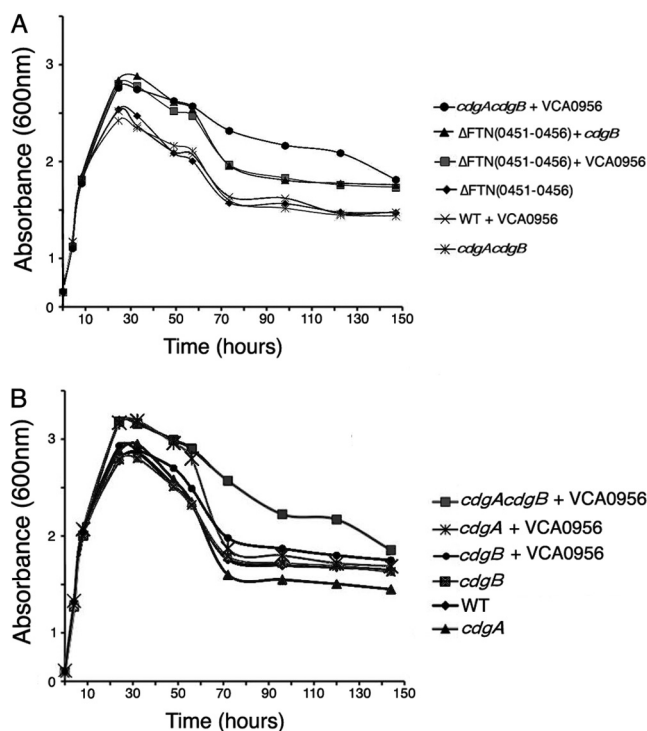
FIG 1 *F. novicida* FTN0451-FTN0456 gene cluster. (A and B) The 11.3-kbp *F. novicida* FTN0451-FTN0456 gene cluster is missing in *F. tularensis* subsp. *tularensis*. In panel B, the numbers refer to the nucleotides within the Schu S4 genome (GenBank accession no. NC_006570), which are contiguous and missing all coding sequences corresponding to FTN0451 to FTN0456 in *F. novicida* U112 (GenBank accession no. CP000439). (C) CdgA and CdgB are composite GGDEF-EAL (DGC-PDE) proteins; PAS is a signal transduction domain.

FIG 2 Growth rates and yields of *F. novicida* with high and low cdGMP levels. (A and B) *F. novicida* growth curves. *F. novicida* strains U112 (WT), KKF457 [ΔFTN(0451–0456)], KKF535 (*cdgB*), KKF538 (*cdgA*), and KKF539 (*cdgA cdgB*), carrying plasmid pKEK1524 (+ *cdgB*) or pKEK1525 (+ VCA0956), were grown at 37°C in CDM.

ical aspartate and most of the loop 6 residues found in active PDE domains (32), as well as conserved glutamate and lysine residues required for phosphodiesterase activity (33, 37) (see Fig. S3 in the supplemental material). Interestingly, CdgB has an S substitution for T in the highly conserved loop 6 motif DDFGTG (32), as well as a Q substitution for a highly conserved E residue (37), which may correlate with our evidence below suggesting that this protein is primarily a DGC rather than a PDE.

The FTN0452 gene encodes a conserved hypothetical protein with a domain of unknown function (DUF814); however, HHPred analysis (40) identified potential structural homology with DGC domains in the C terminus (aa 314 to 466). Alignment of the FTN0452 protein's aa sequence with those of other DGC domains revealed that many of the critical residues for DGC activity are lacking, including the entire GGDEF motif (see Fig. S4 in the supplemental material), so we suggest that this protein may represent a novel cdGMP binding protein. The FTN0453 gene encodes a protein with homology to the cellulose synthase superfamily (cd06423), likely involved in the synthesis of polysaccharide chains (e.g., cellulose and chitin). The FTN0454 gene encodes a protein with homology to membrane fusion proteins of multidrug resistance pumps (COG1566). The FTN0455 gene encodes a protein with a CheR methyltransferase domain and a CheB methylesterase domain, normally involved in methylation and demethylation, respectively, of chemoreceptors in motile bacteria.

Elevated cdGMP stimulates *F. novicida* growth yield. To determine the role of cdGMP signaling in *F. novicida*, the entire FTN0451-FTN0456 gene cluster was removed and replaced with an erythromycin resistance gene, resulting in strain KKF457 [Δ(FTN0451-FTN0456)]. This strain lacks any DGC- or PDE-encoding genes, similar to *F. tularensis* subsp. *tularensis*, and thus can be considered to have no cdGMP. In order to elevate cdGMP levels in this strain, we introduced a well-characterized heterologous DGC from *V. cholerae*, namely, VCA0956 (45, 46), expressed from an *F. novicida* promoter. VCA0956 expression is known to elevate intracellular cdGMP levels (4, 45).

The KKF457 strain lacking cdGMP exhibited a growth curve similar to that of the wild-type U112 strain in CDM (Fig. 2A). However, high levels of cdGMP in KKF457 (expressing VCA0956) stimulated a higher OD_{600} in stationary phase, and this was caused by an ~100-fold larger number of bacterial cells as determined by plate counts. The growth rate and decline of the strain with high cdGMP appeared similar to those of the strain with low cdGMP, and thus it appears that the stationary-phase yield (cell number) is specifically stimulated by elevated cdGMP. We also introduced a plasmid expressing CdgB into KKF457, and the resulting strain exhibited a growth curve similar to that of KKF457 expressing VCA0956 (Fig. 2A), suggesting that CdgB has predominantly DGC activity (similar to VCA0956) that is able to elevate cdGMP levels in this strain.

Interestingly, expression of VCA0956 in the wild-type U112 strain did not stimulate the stationary-phase yield (Fig. 2A), indicating lower cdGMP levels and suggesting that a gene(s) within the FTN0451-FTN0456 cluster has PDE activity capable of reducing cdGMP levels. We created an *F. novicida* strain lacking only

cdgA and *cdgB*, rather than the entire gene cluster, and this strain grew similarly to the wild-type and KKF457 strains. However, expression of VCA0956 in the *cdgA cdgB* strain stimulated an increase in growth yield similar to that with expression of VCA0956 in KKF457 (Fig. 2A). These results indicate high levels of cdGMP in this strain, consistent with PDE activity associated with CdgA and/or CdgB.

We further investigated cdGMP stimulation of the growth yield by measuring the growth of *F. novicida* strains lacking CdgA and/or CdgB (Fig. 2B). As seen previously, the *F. novicida cdgA cdgB* strain grew similarly to the wild-type U112 strain, but elevating cdGMP in this strain by expression of VCA0956 stimulated the growth yield. However, expression of VCA0956 stimulated the growth yield only in the *cdgA* mutant, not the *cdgB* mutant, consistent with CdgA possessing predominantly PDE activity that reduces cdGMP levels. A lack of both CdgA and CdgB was required to see sustained elevated cell counts during the decline phase (>70 h), suggesting that CdgB may possess some PDE activity during this phase. Unfortunately, despite repeated attempts, we were unable to construct a plasmid that expresses CdgA, suggesting that there may be some toxic effect associated with overexpression of this protein. Determination of viable cell counts by plating of samples from several time points during the growth curve analysis revealed that bacterial cell numbers were correlated with optical density. Cumulatively, our results indicate that cdGMP levels are modulated mostly positively by CdgB and mostly negatively by CdgA and that elevated cdGMP levels stimulate the *F. novicida* growth yield.

Elevated cdGMP stimulates *F. novicida* biofilm formation. *F. novicida* forms biofilms on abiotic and chitinous surfaces (10, 26), and an orphan response regulator, QseB, has been shown to be important for biofilm formation. To investigate the contribution of cdGMP levels to *F. novicida* biofilm formation, we measured biofilms formed by various *F. novicida* strains with high or low cdGMP levels (Fig. 3A). As shown previously (10, 26), the wild-type U112 strain formed biofilms on borosilicate glass tubes. A strain lacking the entire FTN0451-FTN0456 cluster (KKF457) or a strain lacking just CdgA and CdgB (*cdgA cdgB* strain) was defective for biofilm formation, demonstrating that cdGMP signaling contributes to *F. novicida* biofilm formation. Expression of VCA0956 or CdgB in either KKF457 or the *cdgA cdgB* strain stimulated more biofilm formation than that of the wild-type strain, demonstrating that high cdGMP levels stimulate *F. novicida* biofilm formation. These results also further confirm the DGC activity associated with CdgB. Finally, biofilm formation of the individual *cdgA* and *cdgB* mutants revealed that a lack of CdgA actually stimulates biofilm formation, whereas a lack of CdgB decreases biofilm formation. These results are consistent with CdgA possessing predominantly PDE activity and CdgB possessing predominantly DGC activity.

Further confirmation of the DGC activity of CdgB was obtained by transforming *V. cholerae* strain A1552 with the plasmid expressing CdgB and then measuring biofilm formation (see Fig. S5 in the supplemental material). It has been shown previously that elevated cdGMP levels stimulate *V. cholerae* biofilm formation (3, 5). As anticipated, A1552 expressing either VCA0956 or CdgB stimulated enhanced biofilm formation, providing additional evidence that CdgB possesses DGC activity.

QseB regulates *cdgA* and *cdgB* transcription. Durham-Colleran et al. (10) previously demonstrated a role for the response
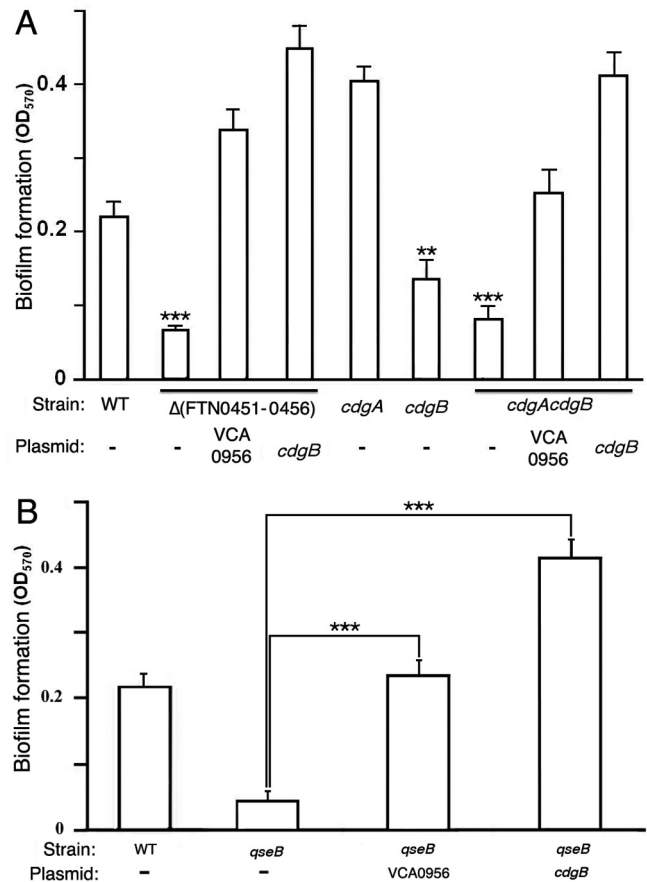
FIG 3 cdGMP stimulates *F. novicida* biofilm formation. (A) *F. novicida* strains U112 (WT), KKF457 [Δ(FTN0451-0456)], KKF535 (*cdgB*), KKF538 (*cdgA*), and KKF539 (*cdgA cdgB*), either with no plasmid (−) or carrying plasmid pKEK1524 (*cdgB*) or pKEK1525 (VCA0956), were assayed for biofilm formation as described in Materials and Methods. **, $P < 0.005$; ***, $P < 0.0005$. (B) *F. novicida* strains U112 (WT) and KKF552 (*qseB*), either with no plasmid (−) or carrying plasmid pKEK1524 (*cdgB*) or pKEK1525 (VCA0956), were assayed for biofilm formation. ***, $P < 0.0005$.

regulator QseB in *F. novicida* biofilm formation. To determine if QseB regulates biofilm formation by regulating cdGMP levels, a *qseB* mutant was transformed with plasmids expressing either VCA0956 or CdgB, and biofilm formation was measured (Fig. 3B). As shown previously, the *F. novicida qseB* mutant was defective for biofilm formation compared to the wild-type U112 strain. However, elevation of cdGMP levels in the *qseB* strain by expression of VCA0956 stimulated biofilm formation, demonstrating that the requirement for QseB can be bypassed by elevated cdGMP levels. This suggests that QseB functions to elevate cdGMP levels in *F. novicida* and thereby stimulates biofilm formation. Expression of CdgB in the *qseB* mutant stimulated biofilm formation to a greater extent, further confirming the DGC activity associated with CdgB.

To determine if QseB regulates transcription of *cdgA* and/or *cdgB*, qRT-PCR measuring the *cdgA* and *cdgB* transcripts was performed with the wild-type and *qseB* mutant strains (Fig. 4). There was a significant decrease in *cdgA* and *cdgB* transcripts in the *qseB* mutant compared to the wild-type strain, indicating that QseB positively influences *cdgA* and *cdgB* transcription.

High cdGMP levels decrease *F. novicida* intramacrophage replication. Intramacrophage replication is an essential compo-
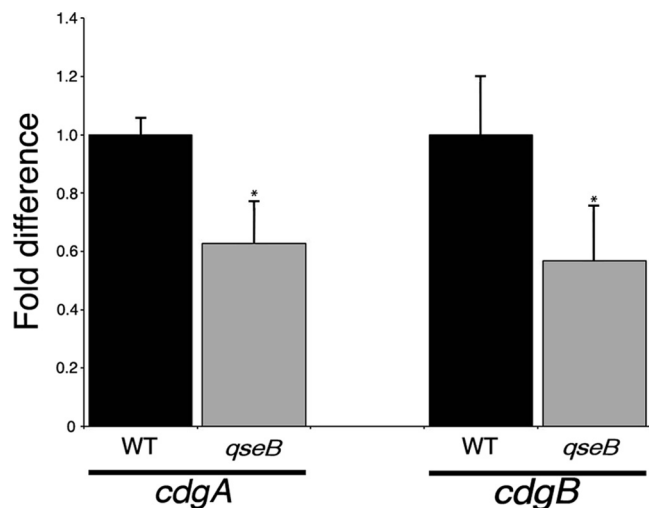
FIG 4 QseB regulates *cdgB* transcription. *F. novicida* strains U112 (WT) and KKF552 (*qseB*) were grown in CDM, and mRNA abundances for *cdgA* and *cdgB* were determined by quantitative reverse transcription-PCR (see Materials and Methods for details). Results shown represent three biological replicates consisting of three technical replicates for each strain. *, $P < 0.05$.

FIG 6 cdGMP inhibits *F. novicida* virulence in mice. *F. novicida* strains U112 (WT) and KKF457 [ΔFTN(0451–0456)], either without plasmid or carrying plasmid pKEK1525 (+ VCA0956), were inoculated intranasally into groups of 5 female BALB/c mice at the indicated inocula, and mouse survival was monitored.

nent of *Francisella* virulence. The ability of *F. novicida* strains with low and high cdGMP levels to replicate intracellularly was measured in the mouse macrophage-like J774 cell line (Fig. 5). Strains lacking cdGMP [*cdgA cdgB* or Δ(FTN0451-FTN0456) strain] replicated similarly to the wild-type U112 strain within the J774 cells, indicating that low cdGMP levels do not inhibit intramacrophage replication. In contrast, strains with high cdGMP levels, achieved by expression of VCA0956 or CdgB in the *cdgA cdgB* or Δ(FTN0451-FTN0456) strain, were defective for intramacrophage replication, exhibiting an approximately 2-log reduction in CFU at 48 h. These results demonstrate that high cdGMP levels inhibit intramacrophage replication.

Interestingly, expression of VCA0956 in the wild-type U112 strain had no effect on intramacrophage replication. We hypothe-
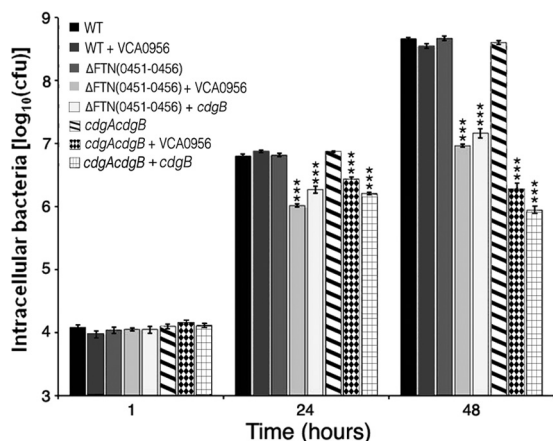

FIG 5 cdGMP inhibits *F. novicida* intramacrophage replication. *F. novicida* strains U112 (WT), KKF457 [ΔFTN(0451–0456)], and KKF539 (*cdgA cdgB*), either without a plasmid or carrying plasmid pKEK1524 (*cdgB*) or pKEK1525 (VCA0956), were inoculated at an MOI of ~10:1 into J774 cells, and intracellular bacteria were enumerated at 1, 24, and 48 h (see Materials and Methods for further details). The assay was performed in triplicate. *, $P < 0.0005$.
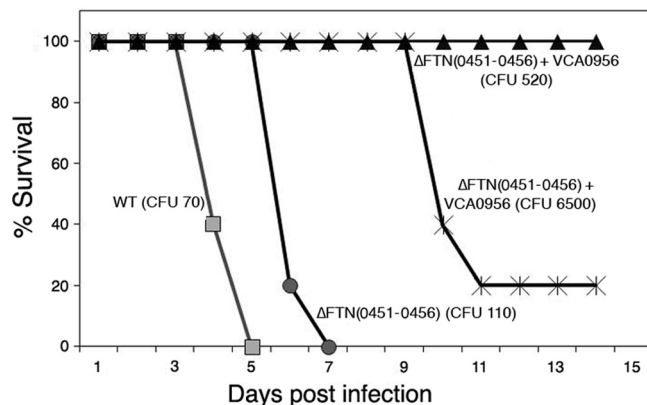
sized that this was likely because of the PDE activity associated with *cdgA* reducing intracellular cdGMP levels (see above). To confirm that the PDE activity of CdgA inhibits the elevation of cdGMP levels, we tested the ability of *F. novicida cdgA* and *cdgB* strains expressing the DGC VCA0956 to replicate within the J774 cell line (see Fig. S6 in the supplemental material). The *cdgA* strain expressing VCA0956 was defective for intramacrophage replication at 48 h postinfection, whereas the *cdgB* strain expressing VCA0956 replicated similarly to the wild-type strain. These results are consistent with CdgA possessing a PDE activity that inhibits elevated cdGMP levels.

High cdGMP levels decrease *F. novicida* virulence in mice. To determine the effect of cdGMP levels on *F. novicida* virulence via the pulmonary route, *F. novicida* strains with low and high cdGMP levels were inoculated into BALB/c mice intranasally at various inocula, and survival was monitored. Strains lacking either *cdgA*, *cdgB*, or both *cdgA* and *cdgB* exhibited similarly high levels of virulence as the wild-type strain at relatively low inocula (60 to 115 CFU) (see Fig. S7 in the supplemental material), demonstrating that low levels of cdGMP are not inhibitory to *F. novicida* virulence. Likewise, the KKF457 strain lacking the entire cdGMP modulatory cluster [Δ(FTN0451-FTN0456)] showed a similarly high level of virulence as the wild-type strain at a relatively low inoculum (110 CFU), with a slightly delayed time to death (Fig. 6), providing further evidence that low levels of cdGMP have little inhibitory effect on *F. novicida* virulence.

In contrast, the high cdGMP levels achieved by expression of VCA0956 in KKF457 attenuated *F. novicida* virulence (Fig. 6). Strain KKF457 expressing VCA0956 was unable to cause the death of any mice at 520 CFU, whereas 4 of 5 mice succumbed to disease when inoculated with 6,500 CFU of this strain. This result suggested that the apparent LD_{50} of *F. novicida* with high cdGMP levels is ~5,000 CFU. However, because the elevated cdGMP levels were driven by the plasmid-expressed VCA0956 protein in this strain, we tested *F. novicida* colonies recovered from the spleens of mice that succumbed to infection and discovered that the bacteria recovered from diseased animals had lost the VCA0956 plasmid. This result indicates that the LD_{50} of *F. novicida* with high cdGMP levels is likely >6,500 CFU. Since the LD_{50} of the wild-type *F.*

TABLE 1 cdGMP-regulated genes in *F. novicida*

Gene	Annotation of gene product	Fold change	Presence in strain Schuh S4
Upregulated genes			
FTN0010	Phage terminase	+2.091	Missing
FTN0286	Transposase	+2.047	Missing
FTN0324	Anti-sigma factor antagonist	+2.030	FTT1612
FTN0627	ChiA (chitinase)	+2.238	Functional ChiA (44- and 66-aa deletions)
FTN0793	Hypothetical protein	+2.145	FTT1078; 78-aa C-terminal truncation
FTN0879	Zinc-iron permease	+3.689	Pseudogene
FTN0880	Cobalamin synthase	+4.379	CobS
FTN1328	Trehalase	+2.298	Pseudogene
FTN1485	Chitin-binding protein	+3.438	Split: FTT1576-FTT1577
FTN1744	ChiB (chitinase)	+2.983	ChiB with 124-aa N-terminal truncation
FTN1758	Hypothetical protein	+2.407	Missing
Downregulated genes			
FTN0313	Acetyltransferase	−2.234	Pseudogene
FTN0714	Conserved hypothetical protein	−3.299	Missing
FTN0715	Conserved hypothetical protein	−2.472	FTT0742; 594-aa C-terminal truncation
FTN1021	Conserved hypothetical protein	−2.423	FTT0662; 43-aa C-terminal truncation
FTN1101	Lipoprotein, unknown function	−2.721	Missing
FTN1102	Hypothetical protein	−2.070	Missing
FTN1103	Conserved hypothetical protein	−2.438	Missing
FTN1104	Hypothetical protein	−2.293	FTT1122 (only N-terminal 66 aa)

novicida strain is <10 CFU by the pulmonary route, this demonstrates the strong attenuation of virulence (>650-fold) caused by high cdGMP levels.

cdGMP modulates *F. novicida*-specific genes. Our cumulative data indicate that cdGMP positively affects growth yield and biofilm formation and negatively affects intramacrophage replication and virulence in *F. novicida*. In order to determine which gene(s) modulated by cdGMP might account for these phenotypes, we performed whole-genome microarray transcriptome analysis of *F. novicida* strains with either a low cdGMP (KKF457) or high (KKF457 expressing VCA0956) level of cdGMP. We utilized a custom-designed NimbleGen array that incorporated probes for *F. novicida*-specific genes, in addition to the genes shared with other *F. tularensis* strains, in order to accurately identify transcription changes in these *F. novicida* strains. The experiment was performed with 2 biological replicates grown in CDM (Table 1).

Surprisingly few genes showed 2-fold or greater changes in transcript levels between the *F. novicida* strain with high cdGMP and the strain with low/no cdGMP. This analysis identified 11 genes that were upregulated and 8 genes that were downregulated by high levels of cdGMP (Table 1). Margolis et al. (26) previously demonstrated that the chitinases ChiA and ChiB contribute to *F. novicida* biofilm formation on chitinous surfaces. Our microarray results indicate that transcription of *chiA*, *chiB*, and the gene for a chitin-binding protein (FTN1485) is positively regulated by cdGMP, consistent with the role of these genes in biofilm formation. Other genes positively regulated by cdGMP encode cobalamin synthase, trehalase, zinc-iron permease, and hypothetical proteins.

Interestingly, Margolis et al. (26) identified FTN0714 as a secreted protein that contributes to *F. novicida* biofilm formation; our microarray analysis identified FTN0714 as being down- rather than upregulated by cdGMP. The microarray analysis provides a snapshot of transcription at a distinct time point, whereas biofilm

formation is a dynamic developmental process, so it may be that cdGMP represses some biofilm components that are otherwise upregulated at different time points during this process. Virtually all the other genes downregulated by cdGMP encode proteins of unknown function.

Given that the FTN0451-FTN0456 gene cluster that modulates cdGMP levels is found specifically in *F. novicida* and absent in *F. tularensis* subsp. *tularensis*, it was of interest to determine whether the cdGMP-modulated genes are present in *F. tularensis* subsp. *tularensis*. Interestingly, only three of the genes positively regulated by cdGMP in *F. novicida* (the FTT1612 gene, *cobS*, and *chiA*) appear to be fully functional in *F. tularensis* subsp. *tularensis* (Table 1); all 8 other genes positively regulated by cdGMP appear to be compromised (pseudogenes, genes with truncations in the coding sequence, or split genes) or completely missing in *F. tularensis* subsp. *tularensis*. Likewise, all the genes downregulated by cdGMP in *F. novicida* are compromised or missing in *F. tularensis* subsp. *tularensis*. The ChiA protein from *F. tularensis* subsp. *tularensis* Schu S4 contains 44-aa and 66-aa deletions within the C terminus in comparison to the ChiA protein from *F. novicida*, but Chandler et al. (8) showed that this protein is expressed and functional in Schu S4.

We measured transcript levels of the *chiA* and *chiB* genes in the *F. novicida* strains with high cdGMP [Δ(FTN0451-FTN0456) strain plus VCA0956] or no cdGMP [Δ(FTN0451-FTN0456) strain], as well as the wild-type U112 strain, by qRT-PCR to validate the results of the microarray analysis (Fig. 7). Transcript levels of *chiB* were significantly lower in the strain with no cdGMP than in the strain with high cdGMP, demonstrating that high cdGMP levels positively regulate *chiB* transcription. The cdGMP-mediated increase in *chiA* transcript levels was not statistically significant. Interestingly, the transcript levels for both *chiA* and *chiB* were elevated in the KKF457 strain with both high and low cdGMP levels compared to transcript levels in the wild-type strain. We hypothesize that one of the genes in the FTN0451-
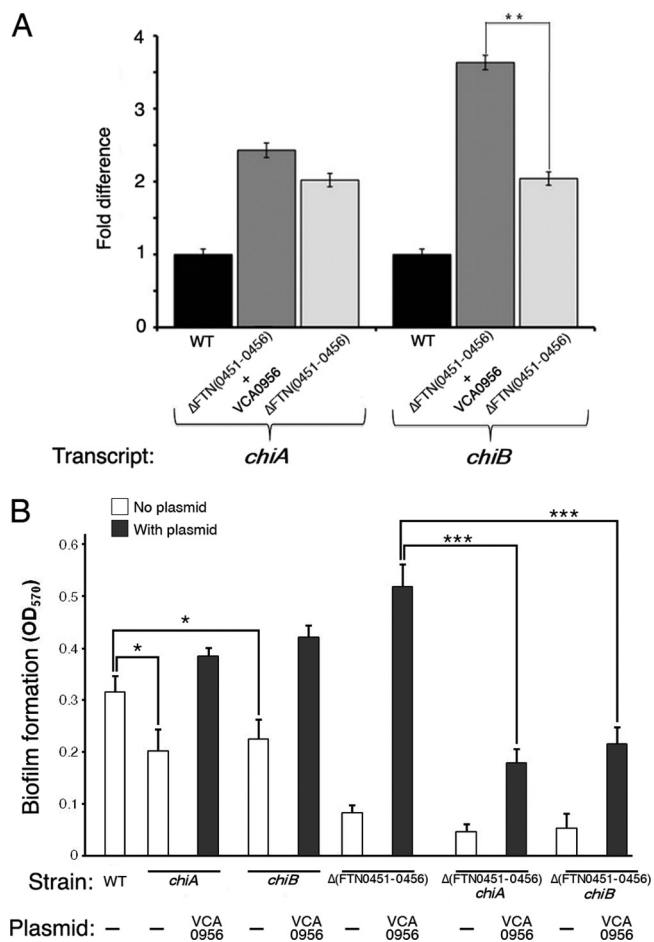
FIG 7 cdGMP controls *chiB* transcription, which enhances biofilm formation. (A) *F. novicida* strains U112 (WT) and KKF457 [Δ FTN(0451–0456)], either without plasmid or carrying plasmid pKEK1525 (+ VCA0956), were grown in CDM, and mRNA abundances for *chiA* and *chiB* were determined by quantitative reverse transcription-PCR (see Materials and Methods for details). Results shown represent three biological replicates consisting of three technical replicates for each strain. **, $P < 0.005$. (B) *F. novicida* strains U112 (WT), KKF555 (*chiA*), KKF556 (*chiB*), KKF457 [Δ(FTN0451-0456)], KKF557 [Δ(FTN0451-0456) *chiA*], and KKF558 [Δ(FTN0451-0456) *chiB*], either with no plasmid (−) or carrying plasmid pKEK1525 (VCA0956), were assayed for biofilm formation as described in Materials and Methods. *, $P < 0.05$; ***, $P < 0.0005$.

FTN0456 gene cluster (absent in the KKF457 strain) negatively regulates *chiA* and *chiB* transcription.

To determine if cdGMP-modulated *chiA* and/or *chiB* transcription contributes to *F. novicida* biofilm development on glass surfaces, we first created *F. novicida* strains containing either *chiA* or *chiB* mutations in both the wild-type and Δ(FTN0451-FTN0456) backgrounds. The heterologous DGC VCA0956 was then introduced into these strains, and biofilm formation was assessed (Fig. 7B). Disruption of either *chiA* or *chiB* in the wild-type background reduced the ability of *F. novicida* to form biofilms. As expected, the Δ(FTN0451-FTN0456) strain combined with the *chiA* or *chiB* mutations led to significant decreases of biofilm formation. Importantly, raising cdGMP levels in these strains by the introduction of VCA0956 could not fully complement biofilm formation. The Δ(FTN0451-FTN0456) *chiA* and Δ(FTN0451-FTN0456) *chiB* strains with VCA0956 showed significant de-

creases in biofilm formation in comparison to the parental Δ(FTN0451-FTN0456) strain with VCA0956. Thus, cdGMP-dependent regulation of ChiB facilitates biofilm formation on glass surfaces.

DISCUSSION

Although *F. novicida* and *F. tularensis* subsp. *tularensis* are closely related, they exhibit vastly different virulence capabilities in humans, despite both sharing the critical secretion-related virulence genes localized in the FPI. *F. tularensis* subsp. *tularensis* is highly virulent in humans, which has resulted in this bacterium being considered a select biothreat agent requiring high-level biocontainment. In contrast, *F. novicida* is considered avirulent in healthy humans and is exempt from select agent regulations due to its low level of virulence. Comparison of their genomes reveals that in contrast to the *F. novicida* genome, the *F. tularensis* subsp. *tularensis* genome is undergoing decay and is replete with insertion sequence (IS) elements, genomic rearrangements, and pseudogenes (21). This suggests that elements present in the *F. novicida* genome but absent from the *F. tularensis* subsp. *tularensis* genome may have been lost from the *F. tularensis* subsp. *tularensis* genome to enhance its virulence (35).

We showed here that the FTN0451-FTN0456 gene cluster, which is found in *F. novicida* but missing from *F. tularensis* subsp. *tularensis*, modulates cdGMP levels. This gene cluster is also missing from the human-pathogenic organism *F. tularensis* subsp. *holarctica*. High cdGMP levels positively influence *F. novicida* biofilm formation and negatively influence virulence, a paradigm seen in other bacterial pathogens that utilize cdGMP signaling (e.g., *V. cholerae* [45, 46]). cdGMP levels are increased by DGC-containing proteins and decreased by PDE-containing proteins; *F. novicida* encodes two proteins within the FTN0451-FTN0456 cluster, i.e., CdgA and CdgB, that have both DGC and PDE domains.

Several lines of evidence suggest that CdgB is predominantly a DGC: (i) expression of CdgB stimulates *V. cholerae* biofilm formation, (ii) expression of CdgB in the absence of CdgA stimulates *F. novicida* biofilm formation as well as growth yields, and (iii) expression of CdgB in the absence of CdgA inhibits *F. novicida* intramacrophage growth and virulence in mice. All of these phenotypes could be replicated by replacing CdgB with VCA0956, a well-characterized DGC with no PDE domain. We suggest that the PDE domain of CdgB has very little or no activity and that this may be due to a substitution in the well-conserved loop 6 region; substitutions in this region have been shown to differentiate active from inactive PDE domains (32).

Although we were unable to clone *cdgA* into an expression vector, for unknown reasons, our evidence still suggests that CdgA is predominantly a PDE. The cdGMP-mediated effects on *F. novicida* caused by CdgB (increased growth yields and intramacrophage replication) occurred only in the absence of CdgA, indicating that CdgA has an opposing enzymatic activity to that of CdgB. Also, the *cdgA* mutant exhibited enhanced biofilm formation compared to the wild-type strain, suggesting higher cdGMP levels when CdgA is absent. Finally, elevating cdGMP in *F. novicida* by the introduction of the heterologous DGC VCA0956 reduced intramacrophage growth only in the absence of CdgA, suggesting that CdgA's PDE activity opposes the activity of VCA0956. The DGC domain of CdgA contains a substitution in a highly conserved histidine residue as well as in inhibitory sites, suggest-

ing that this domain may have decreased or absent enzymatic activity.

Stimulation of biofilm formation is normally associated with persistence within the environment, and the *F. novicida* genome appears to have a more robust repertoire of genes, which may indicate it is more adept at environmental persistence than *F. tularensis* subsp. *tularensis*. Microarray analysis revealed that cdGMP positively regulates genes known to be involved in *F. novicida* biofilm formation, including the chitinase genes *chiA* and *chiB* (26). We confirmed by qRT-PCR that the *F. novicida chiB* gene is positively regulated by cdGMP. Margolis et al. showed that ChiA and ChiB are important for *F. novicida* biofilm formation on chitinous surfaces that would be encountered outside the mammalian host. In the current study, we found that ChiA and ChiB also contribute to *F. novicida* biofilm formation on glass surfaces. In a study by Reif et al., the *chiA* and/or *chiB F. novicida* mutant showed no defects in the ability to colonize ticks (34), so perhaps the encoded chitinases facilitate biofilm formation in the environment rather than in insect/arthropod vectors.

ChiA from *F. tularensis* subsp. *tularensis* is missing two internal portions, corresponding to aa 663 to 706 and aa 747 to 812 of *F. novicida* ChiA. Despite this difference, the ChiA proteins from both *F. novicida* and *F. tularensis* subsp. *tularensis* Schu S4 have been shown to possess similar endochitinase activities (8) and to be expressed in both strains. In contrast, *F. tularensis* subsp. *tularensis* ChiB is missing the N-terminal 124 aa found in *F. novicida* ChiB and could not be detected in lysates of *F. tularensis* subsp. *tularensis*, unlike *F. novicida* ChiB. Most of the other cdGMP-regulated genes in *F. novicida* are either missing or compromised in some manner in *F. tularensis* subsp. *tularensis*. This suggests that after the loss of the cdGMP regulatory region, *F. tularensis* subsp. *tularensis* is in the process of losing the genes regulated by cdGMP.

The loss of the cdGMP regulon in *F. tularensis* subsp. *tularensis* indicates that the absence of cdGMP provides an advantage to this bacterium. We showed that high levels of cdGMP inhibit *F. novicida* intramacrophage replication and virulence in mice. We suggest that the cdGMP-mediated inhibition of intramacrophage replication and virulence may have driven the loss of the cdGMP regulon in *F. tularensis* subsp. *tularensis*. From the microarray analysis, there was no obvious downregulation of known virulence genes in the strain with high cdGMP, and thus the inhibition of intramacrophage replication may be the result of inhibition of a novel virulence gene(s) or, alternately, enhanced expression of a gene(s) that is deleterious to virulence.

Our results have uncovered a regulatory cascade controlling biofilm formation in *F. novicida* (Fig. 8). The orphan response regulator QseB positively regulates *cdgB* transcription, presumably upon phosphorylation in response to environmental conditions. As discussed above, CdgB is predominantly a DGC that raises cdGMP levels within the cell. cdGMP, in turn, positively stimulates the transcription of genes that enhance biofilm formation, including *chiB*. Elevated cdGMP levels also inhibit intramacrophage growth and virulence by an unknown mechanism. QseB is also present in *F. tularensis* subsp. *tularensis* Schu S4, but the downstream components are missing or defective. Given that most of the cdGMP-regulated genes in *F. novicida* are missing or defective in *F. tularensis* subsp. *tularensis*, it will be interesting to determine whether induction of cdGMP synthesis in this bacte-
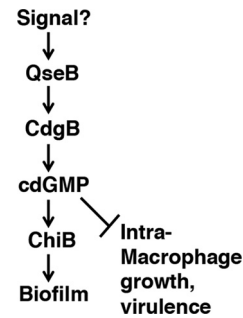

FIG 8 cdGMP signaling cascade in *F. novicida*.

rium will have similar deleterious effects on intramacrophage growth and virulence.

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