In many bacteria, the ability to modulate biofilm production relies on specific signaling molecules that are either self-produced or made by neighboring microbes within the ecological niche. We analyzed the potential interspecies signaling effect of the \textit{Burkholderia} diffusible signal factor (BDSF) on \textit{Francisella novicida}, a model organism for \textit{Francisella tularensis}, and demonstrated that BDSF both inhibits the formation and causes the dispersion of \textit{Francisella} biofilm. Specificity was demonstrated for the cis versus the trans form of BDSF. Using transcriptome sequencing, quantitative reverse transcription-PCR, and activity assays, we found that BDSF altered the expression of many \textit{F. novicida} genes, including genes involved in biofilm formation, such as chitinases. Using a chitinase inhibitor, the antibiofilm activity of BDSF was also shown to be chitinase dependent. In addition, BDSF caused an increase in ReA expression and increased levels of (p)ppGpp, leading to decreased biofilm production. These results support our observation that exposure of \textit{F. novicida} to BDSF causes biofilm dispersal. Furthermore, BDSF upregulated the genes involved in iron acquisition (fgaABCD), increasing siderophore production. Thus, this study provides evidence for a potential role and mechanism of diffusible signal factor (DSF) signaling in the genus \textit{Francisella} and suggests the possibility of interspecies signaling between \textit{Francisella} and other bacteria. Overall, this study suggests that in response to the interspecies DSF signal, \textit{F. novicida} can alter its gene expression and regulate its biofilm formation.
has been postulated to be involved in its environmental persistence (22). Virulent strains of *F. tularensis* share many of the genes required for biofilm formation (although they do not have genes encoding proteins involved in c-di-GMP turnover [21]) and are currently understood to produce biofilm under laboratory conditions (19). Based on our interest in *Franciscella* biofilms, we sought to determine if DSF-family molecules could affect this process.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *Franciscella novicida* strain Utah 112 (catalog number NR-13) and transposon Tn20 (ISFtu2/FLP recombination target) mutants with mutations in *fgA*, *chiA*, *chiB*, or *redA* were obtained from BEI Resources, NIAID, NIH. The strains and primers used in this study are listed in Table S1 in the supplemental material. Bacteria were grown at 37°C in tryptic soy broth with 0.1% (wt/vol) cysteine (TSBC). When necessary, kanamycin was added at 20 µg/ml.

**Biofilm assays.** Overnight cultures of bacteria were diluted 1:30 into 20 ml of TSBC, and 200 µl was added to each well of a 96-well plate (Falcon 353072; BD). After 24 h at 37°C, the optical densities (ODs) at 600 nm of the wells were taken to normalize for growth, and then the liquid was removed by washing as previously described (23, 24). The plates were then incubated at 70°C for 1 h and stained with 0.1% (wt/vol) crystal violet for 15 min. The stain was removed, and the previous wash step was repeated. The stain was solubilized out of the biofilm by adding 200 µl of 30% (vol/vol) acetic acid, and the OD at 590 nm was read with a microplate reader. The minimum concentration of compound required for 50% inhibition of biofilm (MBIC<sub>50</sub>) was obtained using the same microtiter plate conditions.

**Observation of biofilm formation in test tubes** was similarly performed. First, 1:30 dilutions of overnight cultures were carried out in 3 ml of TSBC. After growth at 24 h at 37°C, the OD at 600 nm was taken. Bacteria were removed from the tubes, which were then washed and stained with 0.1% (wt/vol) crystal violet. Unbound crystal violet was removed, and images were captured with a Perfection 2480 photo scanner (Epson, Nagano, Japan).

For confocal microscopy, bacteria were grown on a Lab-Tek II chamber slide (Thermo Scientific, Waltham, MA) for 24 h to allow attachment to the glass. Biofilms were observed with a 40× objective using a Nikon TE2000-U confocal laser scanning microscope (Nikon, Tokyo, Japan) equipped with an argon ion laser at 510 nm. The inhibitor dequalinium (DEQ) was purchased from Sigma-Aldrich (St. Louis, MO), dissolved in dimethyl sulfoxide, and added to the biofilm assays as necessary.

**Liquid CAS assay.** Wild-type (WT) *F. novicida* and the *fgA* mutant were grown overnight in TSBC to mid-logarithmic phase with various concentrations of BDSF (Sigma-Aldrich, St. Louis, MO). The bacteria were centrifuged at 10,000 × g. The supernatant (100 µl) was collected and added to equal parts of chrome azurol S (CAS) solution. After 30 min of incubation at room temperature, the OD at 625 nm was read on a Safire 2 fluorescence spectrophotometer (Tecan, Männedorf, Switzerland). CAS activity was normalized to the OD at 600 nm of the bacterial cultures read prior to centrifugation. All strains were tested in triplicate.

**(p)pGpp quantification.** (p)pGpp quantification was carried out as previously described (25). Bacteria were harvested by centrifugation when the cultures (25 ml) reached an OD at 600 nm of 0.5. Following the addition of methanol (500 µl), vigorous vortexing, and centrifugation, the supernatant was collected and concentrated by lyophilization overnight. The dried extracts were then resuspended in 20 mM phosphate buffer at pH 6.6 to final concentrations of 3 µM and 18 µM, respectively; this mixture was then kept away from light at room temperature for 20 min. Next, NaBH<sub>4</sub> (Sigma-Aldrich, St. Louis, MO) was added to the DNA–Ag mixture to a final concentration of 18 µM, and the components were mixed; the resultant solution was kept in the dark at 4°C overnight to form nanoclusters. The bacterial lysate prepared as described above was then added to the nanocluster solution. After 15 min, Cu<sup>2+</sup> (copper sulfate) was added to a final concentration of 800 nM at room temperature. The fluorescence of the mixture was measured using the Safire 2 fluorescence spectrophotometer with an excitation wavelength of 585 nm and an emission wavelength of 635 nm, allowing the relative (p)pGpp concentration to be determined.

**Chitinase assay.** The chitinase activity of *F. novicida* with BDSF and trans-BDSF (Sigma-Aldrich, St. Louis, MO) was measured using a fluorometric chitinase assay kit (Sigma-Aldrich, St. Louis, MO) per the manufacturer’s instructions. Briefly, mid-log-phase bacteria and supernatants were separated by centrifugation at 10,000 × g for 10 min at 4°C. An appropriate volume of each sample was incubated with a substrate working solution (4-methylumbelliferyl-<i>N</i>-<i>N</i>′-diacetetyl-<i>B</i>-<i>B</i>-chitobioside) at 37°C, and readings were taken at exponential intervals (10, 20, 40, 80, 160, 320, 640, and 1,280 min) overnight. Fluorescence was then quantified at 450 nm after excitation at 360 nm. An appropriate positive control (chitinase from *Trichoderma viride* [Sigma-Aldrich]) and negative control (medium alone) were included in each plate. The results were plotted as relative fluorescence units versus time.

**RNA-seq.** *F. novicida* cultures were grown overnight in TSBC in the presence or absence of 30 µM BDSF. On the next day, the cultures were centrifuged, and RNA was prepared using a Qiagen RNeasy minikit. DNase I treatment was performed according to the manufacturer’s instructions. RNA samples were sent to Otagenetics (Norcross, GA, USA) for sequencing. Sequencing data (fastq) for each of the samples were mapped to the *F. novicida* strain Utah 112 genome (GenBank accession number NC_008601, available from the National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov/genome?term=NC_008601]). Mapped data sets were input into the BEDTools tool set (http://bedtools.readthedocs.org/en/latest/) to determine the hit counts on each of the regions defined in the NC_008601 sequence. The hit counts of the samples were used as the input into the edgeR package (26) for differential gene expression analysis, including calculations of the count per million (CPM), <i>P</i> value, and false discovery rate (FDR), using Fisher’s exact test with the biological coefficient of variation (BCV) set to 0.1. An FDR of <0.05 was considered significantly different. The ID mapping tool at the UniProt database (www.uniprot.org) was used for functional (KEGG) categorization by inputting *F. novicida* GeneID numbers (27). These protein function assignments were then paired with the transcriptome sequencing (RNA-seq) data.

**qRT-PCR.** Real-time quantitative reverse transcription-PCR (qRT-PCR) was used to validate the RNA-seq results. *F. novicida* cultures were grown overnight in TSBC in the presence or absence of 30 µM BDSF. On the next day, the cultures were centrifuged and RNA was prepared using an Qiagen RNeasy minikit. DNase I treatment was performed according to the manufacturer’s instructions. The purified RNA was then reverse transcribed into cDNA with reverse transcriptase (SuperScript III reverse transcriptase; Invitrogen, Carlsbad, CA). The protocol was performed as previously described (28). The primers used in the study are found in Table S1 in the supplemental material. *rpoA* was used as the housekeeping gene for the normalization of expression. The fold change in gene expression was calculated using the standard 2<sup>−ΔΔCT</sup> formula, where ΔCT<sub>T</sub> is the difference in the threshold cycle (C<sub>T</sub>) values of the untreated and treated target and control genes, and ΔΔCT<sub>T</sub> is the difference in the ΔCT<sub>T</sub> value of the untreated target and control genes and the ΔCT<sub>T</sub> values of the untreated target and control genes.

**Statistical analysis.** All statistical tests were performed using GraphPad Prism software (version 6) for the Mac operating system (GraphPad Software, San Diego, CA). Student’s <i>t</i> test was used for determining significance, unless stated otherwise.

**Microarray data accession number.** All RNA-seq data were deposited in the Gene Expression Omnibus (GEO) database under accession number GSE72142.
RESULTS
BDSF inhibits the formation of F. novicida biofilm. The ability of BDSF (cis-2-dodecenoic acid) to inhibit biofilm formation in F. novicida was first assessed on a borosilicate cover glass using confocal laser scanning microscopy. These data show that BDSF inhibits biofilm formation by F. novicida, as it does in other bacteria (Fig. 1A). To confirm that BDSF has antibiofilm activity against F. novicida, we performed a titration of BDSF and trans-BDSF (trans-2-dodecenoic acid) and stained for remaining biofilm on tissue culture plates (see Table 1 for the structures of the compounds). The results demonstrate that BDSF (but not trans-BDSF) has antibiofilm activity in a concentration-dependent manner, significantly inhibiting biofilm formation starting at 5 μM (Fig. 1B).

We then tested the ability of the trans-BDSF isomer to compete with BDSF for its activity. We coincubated various concentrations of the trans and cis forms and stained for biofilm remaining on the tube wall. These data show that equimolar concentrations of the cis and trans isomers compete, in that the addition of trans-BDSF could block the antibiofilm effect of BDSF (Fig. 1C).

To determine whether the antibiofilm activity that we observed for BDSF was related to its structure and was specific, we tested a panel of fatty acids (Table 1). For the assay, we determined the minimum concentration of compound required for 50% inhibition of biofilm (MBIC50). We found that BDSF inhibited biofilm by 50% at 4 μM, a concentration 25-fold lower than that of trans-DSF. Since BDSF and DSF have similar MBIC50s, this may suggest that the methyl group at the 11th position of DSF does not greatly impact sensing by F. novicida.

In order to assess the importance of the cis double bond location for signaling, we tested cis-5-dodecenoic acid. The results showed that for biofilm inhibition a double bond location is needed at the 2 position, as cis-5-dodecenoic acid was 125-fold less active than BDSF. To determine the role of carbon chain length, we tested several saturated and unsaturated fatty acids. The 10-carbon cis-2-decenoic acid displayed very low activity that was 125-fold higher than that of BDSF. In general, fatty acids that have the same chain length as BDSF exhibited higher activities than those with longer chains (e.g., compare the activity of lauric acid to that of palmitic acid).

BDSF induces the dispersal of F. novicida biofilm. DSF-family molecules are known to disperse biofilm in several bacterial species (2, 6); thus, we tested the dispersion activity of BDSF against F. novicida biofilm. In contrast to the biofilm inhibition assays, biofilm dispersion assays were carried out on preformed biofilms, where treatment causes the release of surface-adhered bacteria into the medium. The results showed that high concentrations of BDSF (50 μM) disrupted the preformed biofilm and bacteria were released into the medium, causing a significant increase in the absorbance at 600 nm (Fig. 1D). As expected, trans-BDSF had no effect on preformed biofilm and did not induce the release of bacteria into the medium (data not shown).
BDSF alters gene expression in *F. novicida*. To investigate the effect of BDSF on *F. novicida* gene expression, we performed RNA-seq and validated the results by qRT-PCR. For comparison with the gene expression of an overnight culture of untreated *F. novicida*, we grew an identical culture in the presence of 30 μM BDSF, extracted the RNA, and subjected the RNA to analysis. Using the average of two replicate RNA-seq runs, we found that the change in expression of 278 out of 1,720 genes (16%) registered above or below a 2-fold cutoff. To narrow down our analysis, we focused on genes whose expression changed 4-fold. Using the 4-fold criterion, we selected 44 out of 1,720 genes (3%) (see Table S2 in the supplemental material for the complete data set).

As part of the analysis, we categorized each gene into KEGG pathways (Fig. 2). Focusing on the most changed 44 genes, we found that a disproportionate number of the BDSF-affected genes fell into certain KEGG pathway categories. Immediately noticeable were chitinase-related genes: chitinase A (*chiA*), chitinase B (*chiB*), and chitinase-binding protein A (*cbpA*). The expression of each was increased greater than 20-fold compared to the level of expression of the untreated control (Table 2; Fig. 2). Confirmation of the results by qRT-PCR showed similar results, with *chiA*, *chiB*, and *cbpA* each being upregulated (Table 2). Each of these chitinase-related genes fell into the amino sugar and nucleotide sugar metabolism KEGG category. Chitinases are negative regulators of biofilm production in *F. novicida* and are important for detachment or dispersal from abiotic surfaces (18).

Since 1,124 of the total of 1,720 genes were categorized as unknown, a large number of genes whose expression was changed ≥4-fold were not well annotated. Nevertheless, by analyzing gene

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Mol wt</th>
<th>MBIC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
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<tr>
<td>cis-2-Decenoic acid</td>
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<td>500</td>
</tr>
<tr>
<td>cis-2-Dodecenoic acid (BDSF)</td>
<td></td>
<td>198.3</td>
<td>4</td>
</tr>
<tr>
<td>trans-2-Dodecenoic acid (trans-BDSF)</td>
<td></td>
<td>198.3</td>
<td>2,500</td>
</tr>
<tr>
<td>cis-5-Dodecenoic acid</td>
<td></td>
<td>198.3</td>
<td>500</td>
</tr>
<tr>
<td>Lauric acid</td>
<td></td>
<td>200.3</td>
<td>500</td>
</tr>
<tr>
<td>cis-11-Methyl-2-dodecenoic acid (DSF)</td>
<td></td>
<td>212.3</td>
<td>20</td>
</tr>
<tr>
<td>trans-11-Methyl-2-dodecenoic acid (trans-DSF)</td>
<td></td>
<td>212.3</td>
<td>500</td>
</tr>
<tr>
<td>Myristoleic acid</td>
<td></td>
<td>226.4</td>
<td>500</td>
</tr>
<tr>
<td>cis-2-Hexadecenoic acid</td>
<td></td>
<td>254.4</td>
<td>500</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td></td>
<td>254.4</td>
<td>&gt;2,500</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td></td>
<td>256.4</td>
<td>&gt;2,500</td>
</tr>
<tr>
<td>Stearic acid</td>
<td></td>
<td>284.5</td>
<td>&gt;2,500</td>
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</tbody>
</table>

*Table 1: Antibiofilm activity of DSFs and other fatty acids*  
*a The compounds were diluted, and the MBIC<sub>50</sub> of each compound is presented. The data are the averages of two repeats.*
location and the homology of the most changed genes, we found that the expression of a single operon (FTN1682 to FTN1685) averaged an increase of 85-fold (10-fold by qRT-PCR). The four genes in this cluster are (by homology) figABCD, recently described to be involved in iron acquisition in Francisella tularensis. The high level of expression of this locus suggests that F. novicida may be increasing its ability to acquire iron in response to BDSF, perhaps in preparation for growth outside the biofilm.

Finally, another category significantly altered by BDSF treatment was the Francisella pathogenicity island (FPI). Out of the 17 FPI genes recorded in the RNA-seq analysis, the expression of 16 was decreased ≥2-fold and the expression of 8 was decreased ≥3-fold. The genes downregulated included iglC, a virulence factor known to be necessary for escape from host phagosomes into the cytoplasm (30). Assessing the expression of iglC by qRT-PCR, we found that it was downregulated 8-fold, confirming its decreased expression in response to BDSF treatment (Table 2).

Role of chitinases in BDSF-induced biofilm dispersal. Chitinases in F. novicida were recently shown to negatively affect biofilm production (18). Expression of active chitinases de-
To analyze the effect of this inhibitor on BDSF-dependent antibiofilm activity of BDSF, we used a family 18 chitinase previously found that chitinase activity is needed for iron acquisition in both F. novicida (29, 31) and F. tularensis (32, 33) and may be critical for Francisella growth under iron-limiting conditions. By mutation analysis, figA, figB, and figC mutants have each been shown to be deficient in siderophore production using the chrome azurol S (CAS) assay (31). Since the figABCD operon was shown to be dramatically upregulated in the RNA-seq and qRT-PCR experiments, we utilized the CAS assay to determine whether siderophore production increases with BDSF treatment. The results showed that, indeed, the production of siderophore increased in a BDSF dose-dependent manner in wild-type F. novicida, but the response was absent in the figA mutant (Fig. 4). These results suggest that BDSF induces F. novicida to increase siderophore production in preparation for growth in a planktonic state.

RelA and (p)ppGpp are altered by BDSF treatment. A previous study by Dean et al. on the role of RelA in F. novicida showed the ability of wild-type F. novicida bacteria to produce (p)ppGpp, while the relA mutant did not produce (p)ppGpp in detectable quantities (20). We first replicated the results of Dean et al. (20); the relA mutant was observed to produce significantly more biofilm than wild-type F. novicida (Fig. 5A). We also showed that the antibiofilm activity of BDSF is absent in the relA mutant, even at high concentrations (Fig. 5B). Together, these data suggest that BDSF leads to the increased expression of relA, resulting in an increase in (p)ppGpp production, and that the antibiofilm activity of BDSF is at least partially dependent on relA.

The production of (p)ppGpp has been associated with biofilm regulation in many bacteria (34). Because relA gene expression was shown to be upregulated 4-fold following treatment with

![FIG 3 BDSF-induced chitinase activity.](aem.asm.org/...)

**TABLE 2 qRT-PCR confirmation of RNA-seq transcriptomic results on effect of 30 μM BDSF treatment on gene expression**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Fold change in expression by RNA-seq</th>
<th>qRT-PCR result</th>
<th>Fold change in expression</th>
<th>95% CI</th>
<th>qRT-PCR result for relA mutant</th>
<th>Fold change in expression</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdpA</td>
<td>FTN_1485</td>
<td>20.61</td>
<td>13.36</td>
<td>3.22</td>
<td>1.03</td>
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<tr>
<td>chiA</td>
<td>FTN_1744</td>
<td>52.62</td>
<td>26.08</td>
<td>1.45</td>
<td>3.41</td>
<td>1.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chiB</td>
<td>FTN_0627</td>
<td>23.22</td>
<td>9.29</td>
<td>2.77</td>
<td>2.76</td>
<td>1.20</td>
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<tr>
<td>figA</td>
<td>FTN_1682</td>
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<td>20.75</td>
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<td>2.29</td>
<td>0.98</td>
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<tr>
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<td>11.96</td>
<td>0.16</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>figC</td>
<td>FTN_1322</td>
<td>0.56</td>
<td>0.13</td>
<td>2.41</td>
<td>6.28</td>
<td>2.18</td>
<td></td>
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</tr>
<tr>
<td>relA</td>
<td>FTN_1518</td>
<td>4.76</td>
<td>17.94</td>
<td>1.30</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 95% CI, confidence interval.

**FIG 3 BDSF-induced chitinase activity. (A) Results of time course fluorescence chitinase activity assay with 30 μM BDSF and trans-BDSF. The cis isomer but not the trans isomer increased chitobiosidase activity. RFU, relative fluorescence units. (B) DEQ (200 nM) blocks BDSF-mediated biofilm inhibition. Crystal violet staining was used to measure biofilm biomass. The biofilm/growth ratio is shown. * and **, P < 0.01 and P < 0.001, respectively.**
BDSF in the RNA-seq analysis and ~18-fold by qRT-PCR analysis (Table 2), we tested whether this increased expression would result in an increase in the amount of (p)ppGpp. Using a DNA-silver nanocluster complex previously shown to specifically detect low concentrations of (p)ppGpp (25), we extracted (p)ppGpp from wild-type *F. novicida* and the *relA* mutant treated with a titration of BDSF under normal culture conditions. The assay demonstrated the increased production of (p)ppGpp with increasing BDSF concentrations in wild-type *F. novicida*, while no upward trend in the level of production was detected in the *relA* mutant samples (Fig. 5C).

To better assess the dependence of *F. novicida* on *relA* for BDSF signaling, we performed qRT-PCR using the *relA* mutant untreated and treated with 30 μM BDSF. We found that the fold changes in expression of chitinase-related genes *chpA*, *chiA*, and *chiB*, siderophore production gene *figA*, and virulence factor *iglC* were not significant in the *relA* mutant, whereas the changes in the WT were significant (Table 2). Since the *relA* mutant is relatively unresponsive, this result suggests that *relA* may be necessary for the response to BDSF.

**DISCUSSION**

How *Francisella* survives in between epizootic episodes is of great interest (35–39). *Francisella* has been found throughout the natural environment in water, mud, and soil. Recent work has shown that type A species, such as *F. tularensis* subsp. *tularensis* Schu S4, are soil associated, while type B species within the genus *Francisella* are more likely to be water associated (40). Pyrosequencing has been performed on soil samples throughout the world (35, 41–45). In several studies, the most prevalent organisms found in soil and sediments are *Pseudomonas* and *Burkholderia*, specifically, the BCC (39, 46); therefore, we hypothesized that *Francisella* in the soil may share the same environmental niche with these other organisms and could potentially be exposed to interspecies DSF signals from the other organisms present.

*F. novicida*, a model strain for *F. tularensis*, responds to the cell–cell communication signal BDSF. As *Burkholderia* is a genus within the betaproteobacterial subdivision of proteobacteria, which is distant from the gammaproteobacterial subdivision, which contains *Francisella*, this result bolsters the idea that the DSF family is an important class of signals in bacterial interspecies communication. Moreover, this broad signaling is consistent with reports of both Gram-negative and Gram-positive bacteria and fungi responding to DSF, BDSF, and *cis*-2-decenolic acid (3). Significantly, this study brings *Francisella*, a genus containing a class A select agent, into the group of microbes responsive to DSF signaling.

The relatively high concentrations of BDSF (5 to 50 μM) used in this study likely exceed the levels of BDSF that would be found in the natural environment. However, this may be partially a result of the insensitivity of the biofilm assay, since a significant response was observed at lower concentrations in the CAS assay. It is also possible that BDSF is not an optimal, native DSF molecule for *Francisella* and thus requires the higher concentrations to induce a strong effect. However, in our testing of different fatty acids, *Francisella* appeared to be the most sensitive to BDSF. Fatty acids with different conformations or locations of the double bond were significantly less active, as were *cis*-2-decenolic acid and *cis*-2-hexadecenoic acid. Together, these findings suggest that *F. novicida* preferably responds to 12-carbon DSF signals rather than other fatty acid signals.

Chitinase-related proteins (including chitinases and chitinase-binding proteins) have recently been described to be virulence factors in bacteria (47) but are more generally known to be important for biofilm formation and maintenance of bacteria within aquatic or moist environments (48). The ChiA of *F. novicida* is annotated as a member of glycoside hydrolase family 18 of the
endo-β-N-acetylglucosaminidases and likely controls biofilm by cleaving the biofilm matrix as it is formed. ChiB is not annotated for its specific chitinase class. Three of the top 10 most upregulated genes following BDSF treatment were chitinase related: chaA, chaB, and chpA. We and others have previously shown that chitinases modulate biofilm (19, 21) and are necessary for biofilm detachment or dispersal from abiotic surfaces (18). This implies that Francisella biofilms may include a substrate for chitinases ChiA and ChiB in the exopolysaccharide (EPS). There is no evidence that chitin is found in Francisella biofilm or is produced by bacteria of this genus. However, our previous (18) and current studies suggest the presence of a chitinase (glycosyl hydrolase) substrate in the EPS, and through enzymatic cleavage, the BDSF-induced overexpression of these enzymes leads to a significant decrease in the EPS matrix. Thus, the upregulation of these genes is consistent with a chitinase-regulated biofilm being one of the downstream targets of the BDSF signal.

Another interesting result was the upregulation of the ribosome-associated (p)ppGpp synthetase I (relA) above our 4-fold cutoff to a level placing it in the top 3% of genes with the most altered expression. Dean et al. (20) determined that relA is associated with biofilm: the relA mutant formed significantly more biofilm than wild-type F. novicida strain Utah 112, and (p)ppGpp synthesis was completely abolished in the mutant. This result was significant for two reasons: (i) mutation of relA alone was sufficient for stopping (p)ppGpp production. In most other studied bacteria, deletion of both spot and relA is required to create (p)ppGpp-deficient bacteria. (ii) In most other bacteria in which this has been studied, relA and (p)ppGpp are positive regulators of biofilm, where more biofilm is formed as a response to increased relA expression and (p)ppGpp; this does not appear to be the case in F. novicida (20). Together, the study by Dean et al. (20) and our current study show the negative relationship between the levels of RelA and (p)ppGpp and the levels of biofilm. Altogether, the induction of chitinase-related genes and relA and the resulting increase in chitinase activity suggest that BDSF may act as an escape signal, where induction of a stringent response and increased chitinase activity promote the escape of the bacterium away from the current environment. In this aspect, biofilm dispersal may represent the coordinated evacuation of a location, where bacteria actively release from the biofilm matrix through changes in gene expression (2), possibly in response to an unfavorable signal.

Consistent with the dysregulation of relA and chitinase-related genes was the upregulation of the siderophore cluster figABCD. Iron acquisition is known to be crucial for the growth of Francisella species (49, 50). Studies in P. aeruginosa have previously shown that both high and low levels of environmental iron can directly lead to biofilm dispersal (2). Specifically, iron-deficient environments lead to increased rhamnolipid synthesis, which has been shown to induce biofilm dispersion. In previous studies, the authors suggested that the level of iron is important for the ability of P. aeruginosa to form biofilm in vivo (51, 52). In our study, since figABCD was highly upregulated and siderophore production was increased with BDSF treatment, we can postulate that iron may also be important for the dispersal of F. novicida biofilm.

Also of interest was the overall downregulation of genes within the FPI with BDSF treatment. Specifically, gIC, a known virulence factor necessary for phagosomal escape (30), was confirmed by qRT-PCR to be significantly downregulated. In the context of the other effects that BDSF has on F. novicida, the decrease in virulence factor expression is surprising. The biofilm dispersal of other pathogens is known to be necessary for the full virulence of Vibrio cholerae, enteropathogenic Escherichia coli, DSF-producing X. campesiris, and Yersella fastidiosa (1, 53). The sensing of an interspecies DSF signal by Francisella may signal that the bacteria are no longer within a host and, thus, the expression of virulence factors is unnecessary. Further research into the effect of DSF on virulence in Francisella pathogenesis is required.

Although the target of BDSF in F. novicida is unknown, all known targets of DSF-family signals in other bacteria are proteins; these are predominantly periplasmic domains of membrane-associated sensor kinases (5), but cyttoplasmic sensors have also been demonstrated to be targets in some organisms (8, 54). Using the DELTA-BLAST program (55) to attempt to identify homologs of DSF sensors RpfC (X. campesiris), PA1396 (P. aeruginosa), and BCAM0227 (B. cenocepacia) in F. novicida or other Francisella species, we found that there were no significant matches using the sensory input domains to search. Thus, we cannot suggest which protein in Francisella is responsible for the sensing of BDSF, and our future work will be to experimentally identify the target.

There are no published dispersal signals or DSF systems in the genus Francisella. This study reports on the ability of Francisella to respond to a Burkholderia DSF-family molecule BDSF with significant phenotypic alterations. In conclusion, we have elucidated small-molecule-based interspecies DSF signaling in Francisella.

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