

RESEARCH ARTICLE

Characterization of a Diffusible Signaling Factor from *Xylella fastidiosa*

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ABSTRACT Cell-cell signaling in *Xylella fastidiosa* has been implicated in the coordination of traits enabling colonization in plant hosts as well as insect vectors. This cell density-dependent signaling has been attributed to a diffusible signaling factor (DSF) produced by the DSF synthase RpfF. DSF produced by related bacterial species are unsaturated fatty acids, but that of *X. fastidiosa* was thought to be different from those of other taxa. We describe here the isolation and characterization of an *X. fastidiosa* DSF (XfDSF) as 2(Z)-tetradecenoic acid. This compound was isolated both from recombinant *Erwinia herbicola* expressing *X. fastidiosa* rpfF and from an *X. fastidiosa* rpfC deletion mutant that overproduces DSF. Since an rpfF mutant is impaired in biofilm formation and underexpresses the hemagglutinin-like protein-encoding genes *hxfA* and *hxfB*, we demonstrate that these traits can be restored by ca. 0.5 μ M XfDSF but not by myristic acid, the fully saturated tetradecenoic acid. A *phoA*-based *X. fastidiosa* biosensor that assesses DSF-dependent expression of *hxfA* or *hxfB* revealed a high level of molecular specificity of DSF signaling.

IMPORTANCE *X. fastidiosa* causes diseases in many important plants, including grape, where it incites Pierce's disease. Virulence of *X. fastidiosa* for grape is coordinated by cell-cell signaling molecules, designated DSF (Diffusible Signaling Factor). Mutants blocked in DSF production are hypervirulent for grape, suggesting that virulence is suppressed upon DSF accumulation and that disease could be controlled by artificial elevation of the DSF level in plants. In this work, we describe the isolation of the DSF produced by *X. fastidiosa* and the verification of its biological activity as an antivirulence factor. We also have developed *X. fastidiosa* DSF biosensors to evaluate the specificity of cell-cell signaling to be investigated.

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The xylem-limited plant pathogen *Xylella fastidiosa* causes serious diseases of several important agricultural crop plants, including Pierce's disease (PD) of grapevine and variegated chlorosis in citrus (CVC). The pathogen is obligately transmitted from one plant to another by xylem sap-feeding insects (1, 2). Infected plants exhibit progressive leaf scorching or other foliar symptoms consistent with the water stress that is associated with the large numbers of xylem vessels that are occluded by bacterial cells. The virulence of *X. fastidiosa* is thus linked with its ability to migrate and proliferate within xylem vessels, and disease symptoms may largely be an inadvertent effect caused by successful colonization that interferes with xylem sap flow. *X. fastidiosa*, like the related pathogens *Xanthomonas* and *Stenotrophomonas* species, utilizes small molecules known as diffusible signaling factors (DSF) to regulate its behavior in a cell density-dependent manner (3–5). DSF is also implicated in interspecies signaling, where they exert antagonistic activity against competitors in a given niche (6) and regulate antimicrobial drug resistance in *Pseudomonas aeruginosa* (7).

In those taxa in which they have been characterized, DSF species are generally monounsaturated fatty acids of medium chain length containing a Z alkene α to the carboxylic acid. Pathogens synthesize and respond to DSF using genes in the *rpf* cluster (regulation of pathogenicity factor). In this cell-cell communication pathway, RpfF is the DSF synthase, annotated as a homologue of crotonase, the first enzyme in fatty acid degradation pathways (8). Upon reaching a threshold level outside the cell, DSF is sensed by the hybrid membrane sensor kinase RpfC that, in turn, phosphorylates the intracellular response regulator RpfG which then converts the intercellular signal into an intracellular signal through its cyclic di-GMP phosphodiesterase activity (9).

RpfF-dependent signaling involving DSF accumulation has been associated with regulation of motility, biofilm formation, and virulence in several *Xanthomonas* species and in *X. fastidiosa* (4, 9–14). While promoting the expression of genes encoding secreted hydrolytic enzymes, flagella, and chemotaxis in *X. campestris* pv. *campestris* (Xcc) and *X. citri* subsp. *citri* (Xac) (3, 10, 11), DSF accumulation leads to suppression of those genes in the vas-

cular pathogen of rice *X. oryzae* pv. *oryzae* (*Xoo*). Instead, in this organism, accumulated DSF promotes the expression of genes contributing to biofilm formation (12). DSF-dependent gene expression patterns in *X. fastidiosa* share many similarities with that in *X. oryzae* pv. *oryzae*, perhaps because it also colonizes a vascular habitat. In *X. fastidiosa*, DSF suppresses genes involved in motility (e.g., *pil* genes encoding type IV pili) and in hydrolytic enzymes that disrupt pit membranes (e.g., *pglA*, which encodes a pectinase), enabling cell movement from one xylem vessel to another. Instead, DSF promotes the expression of genes involved in cell-cell aggregation and surface attachment (e.g., *fimA* encoding type I pili and *hxfA* and *hxfB* encoding hemagglutinin-like proteins) and those that drive biofilm formation (e.g., *gum* genes involved in exopolysaccharide synthesis) (13, 14). However, despite the similarities in DSF-dependent regulation of such genes, an *rpjF* mutant of *X. oryzae* pv. *oryzae* is attenuated in virulence to rice (15), while an *rpjF* mutant of *X. fastidiosa* is hypervirulent in grape (4). Since an *rpjF* mutant of *X. fastidiosa* was unable to colonize and to thus be transmitted by insect vectors, it has been hypothesized that DSF signaling is used as a context-dependent switch that enables a subset of *X. fastidiosa* cells to become adhesive and thus able to be acquired by insects, a phenotype incompatible with movement through the plant (16). The accumulation of DSF thus favors insect transmission but suppresses the virulence of *X. fastidiosa*. As such, modulation of the levels of *X. fastidiosa* DSF (here designated *XfDSF*) in plants could provide a control strategy for PD by altering the behavior of this pathogen.

Three different chemical species that function as DSFs in both *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* have been characterized: 2(Z)-11-methyldodecenoic acid (designated DSF) (17), 2(Z)-dodecenoic acid (BDSF), and (2Z, 5Z)-11-methyldodecadienoic acid (CDSF) (18). Eight different DSF-like molecules, including DSF and BDSF, were found in the human pathogen *Stenotrophomonas maltophilia*; of the other six, two were saturated fatty acids and the rest were 2(Z)-mono-unsaturated fatty acids (19). While the fatty acids found in these six chemical species differ in their carbon chain lengths (C12 to C14) and in the position of the branched methyl group, their exact structure and biological activity were not determined. The DSF produced by another human pathogen, *Burkholderia cenocepacia*, was also characterized as BDSF (6), and its production by RpfF involved a novel dual process consisting of the dehydration of a 3-hydroxydodecanoyl-ACP to 2(Z)-dodecenoyl-ACP and the cleavage of the thioester bond to yield a free acid (8). It has been reported that *XfDSF* isolated from the CVC 9a5c strain of *X. fastidiosa* is 12-methyltetradecanoic acid (20). However, while culture extracts of both a CVC and PD strain of *X. fastidiosa* were found to induce expression of DSF-responsive genes in *X. campestris* pv. *campestris* (4, 20, 21), 12-methyltetradecanoic acid was inactive when assayed with *X. campestris* pv. *campestris*-based DSF biosensors (data not shown). Since the putative *XfDSF* from the CVC strain of *X. fastidiosa* was characterized only by high-resolution gas chromatography-mass spectrometry (HR-GC-MS) and was not shown by this group to have bioactivity or to be RpfF dependent in its production, it may represent either a component of an orthogonal signaling system or a compound unlinked to DSF signaling. We thus explored the isolation and characterization of *XfDSF* from *X. fastidiosa* strain temecula1, the causative agent of PD, by performing bioassay-guided fractionation of culture supernatants using a previously reported *X. campe-*

stris pv. *campestris*-based DSF biosensor strain (4). Further, we demonstrate that the *XfDSF* species, 2(Z)-tetradecenoic acid (Fig. 1A), alters gene expression in newly developed *Xylella* DSF biosensor strains and affects biofilm phenotypes at concentrations as low as 500 nM.

RESULTS

***XfDSF* isolation and structural elucidation from recombinant *E. herbicola* and *E. coli*.** To obtain sufficient amounts of *XfDSF* for characterization, we introduced the *X. fastidiosa* *rpjF* gene from strain temecula1 under the control of the *kan* promoter into *Escherichia coli* and *Erwinia herbicola* on a high-copy-number plasmid. The recombinant bacteria released sufficient amounts of *XfDSF* into the culture medium to be readily detected by the *X. campestris* pv. *campestris* DSF (*XccDSF*) reporter. Since the cell yield of *E. coli* and *E. herbicola* was high, greater amounts of *XfDSF* were isolable from these strains than from *X. fastidiosa* cultures. These recombinant strains were thus used in initial isolation and characterization of *XfDSF*. After 36 h of growth, cells were pelleted from 3-liter cultures, and the supernatant was extracted with ethyl acetate (EtOAc). The crude isolate was purified by flash column chromatography (FCC), and fractions were purified using reverse-phase high-performance liquid chromatography (HPLC). Each fraction was assayed for DSF activity using the *XccDSF* reporter (4).

From both *E. coli* and *E. herbicola* cultures, we isolated and characterized a single active molecule. The ¹H nuclear magnetic resonance (NMR) and ¹³C NMR spectra indicated that this compound was an enoic acid with 14 carbons (see Fig. S1A and S1B in the supplemental material). H-H correlation spectroscopy (COSY; see Fig. S1C in the supplemental material) of the active fraction showed that it contained a single compound where each peak in the spectrum correlated to at least one other signal. The proton spectrum of this acid (see Fig. S1A in the supplemental material) contains a triplet at 0.89 ppm that integrates into three protons consistent with a terminal methyl group. To determine whether the acid had any branch points on the chain, distortionless enhancement by polarization transfer (DEPT) NMR spectroscopy was used (see Fig. S2A in the supplemental material). This experiment revealed that the active compound had two CH signals corresponding to each olefinic carbon, 10 aliphatic CH₂ carbons, and one CH₃ signal correlating to the triplet at 0.89 ppm, consistent with a single terminal methyl group. There were no aliphatic quaternary carbons in the full ¹³C NMR spectrum. These results confirmed that the enoic acid did not contain any branching. The geometry of the olefin was determined using two-dimensional (2D) nuclear Overhauser effect spectroscopy (NOESY) (see Fig. S2B in the supplemental material), which showed a correlation between the olefinic protons. This observation is consistent with a Z olefin geometry. The spectral data indicated that the putative *XfDSF* isolated from the recombinant strains was 2(Z)-tetradecenoic acid, a straight-chained enoic acid with 14 carbons (Fig. 1A). The mass spectral data from the methyl ester of the isolated acid confirmed this assignment (See Figure S6).

The structure of the putative *XfDSF* was verified by its chemical synthesis (Fig. 1A). The compound was synthesized using a Still-Gennari olefination (22) followed by saponification of the ethyl ester to afford the enoic acid (see Materials and Methods in the supplemental material). The natural and synthetic 2(Z)-tetradecenoic acids were shown to be identical by spectroscopic

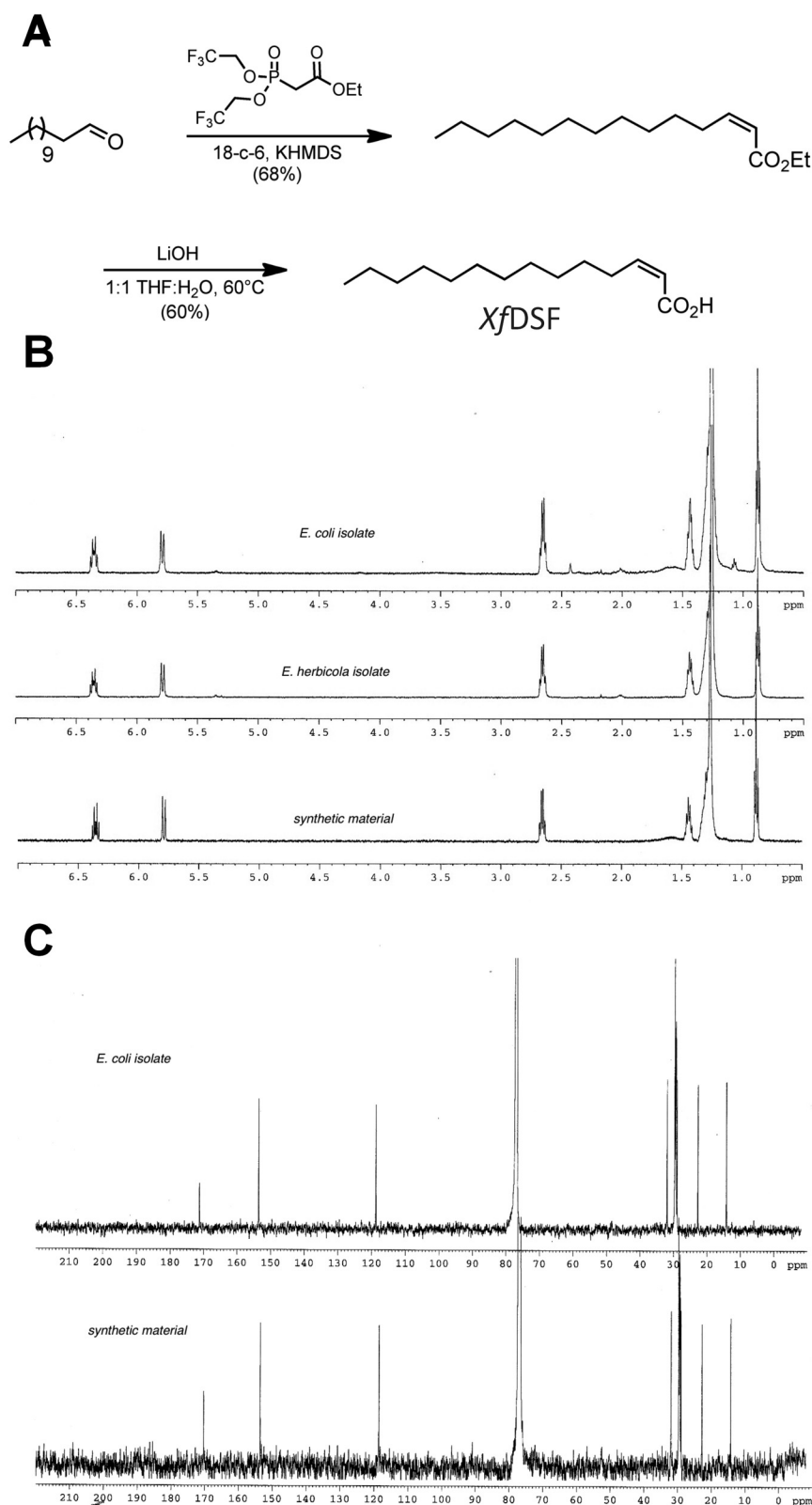


FIG 1 Synthesis and structural confirmation of putative XfDSF. (A) Synthetic scheme for XfDSF featuring a Still-Gennari olefination followed by saponification to afford 2(Z)-tetradecenoic acid. (B and C) Comparative ^1H (B) and ^{13}C (C) NMR results determined for active isolates from recombinant expression compared to those determined for the synthetic acid.

methods (Fig. 1B and C; Table 1). See Figure S5 for NMR spectra of synthetic compounds.

Isolation and structural elucidation of XfDSF from a *Xylella fastidiosa* *rpfC* deletion mutant. To verify that 2(Z)-tetradecenoic acid is produced in *X. fastidiosa* itself and not only in surrogate hosts harboring an *rpfF* gene from this species, we also isolated active compounds from *X. fastidiosa*. Since it was shown previously that RpfC is a negative regulator of RpfF in *X. fastidiosa* (13), an *rpfc* mutant hyperexpresses *rpff* and therefore overproduces XfDSF. An *rpfc* mutant of strain temecula1 was thus used for XfDSF isolation from *X. fastidiosa*. While growth of the *rpfc* mutant in periwinkle wilt broth medium did not result in appreciable yields of the XfDSF compound, substantial amounts could be recovered from 300 plates of this medium solidified with gelrite using the same extraction scheme used with surrogate hosts. From this round of isolation, we obtained 0.6 mg of an enoic acid. The ^1H NMR spectrum of this acid aligned with that of the XfDSF isolated from *E. herbicola* (see Fig. 2SC in the supplemental material).

Unfortunately, we were unable to obtain a ^{13}C spectrum of the *X. fastidiosa* isolate due to the paucity of material. Instead, we turned to electron ionization low-resolution MS (EI LRMS) to validate our structural assignment of the putative XfDSF isolated from *X. fastidiosa*. We used EI because this is a relatively harsh ionization technique that promotes a large amount of fragmentation from the molecular ion. Consequently, EI LRMS spectra have complex fragmentation patterns with a unique signature for the analyte being tested. The EI LRMS spectrum for the isolate from *X. fastidiosa* displayed a molecular ion peak for the acid at 226 m/z . However, there was an additional peak at 270 m/z which was not associated with the analyte of interest. High-sensitivity mass spectrometry as an analytical technique can exaggerate trace impurities, since the ion intensity does not necessarily correlate with quantity in the analyte. Since there were baseline peaks in the ^1H NMR spectrum of the *X. fastidiosa* isolate, we did not attribute the peak at 270 m/z to our compound. The rest of the LRMS spectrum had a fragmentation pattern identical to that of the synthetic 2-(Z)-tetradecenoic acid (see Fig. S2D in the supplemental mate-

TABLE 1 Chemical shift data for synthetic 2(Z)-tetradecenoic acid versus the natural *Xf*DSF isolate

¹ H NMR result(s) 500 MHz, CDCl ₃		¹³ C NMR shifts 125 MHz, CDCl ₃	
Natural	Synthetic	Natural	Synthetic
6.36, m, 1 H	6.36, m, 1 H	171.2	171.4
5.79, d (<i>J</i> = 11.5 Hz), 1 H	5.79, d (<i>J</i> = 11.4 Hz), 1 H	153.6	153.6
2.66, m, 2 H	2.66, m, 2 H	31.9	31.9
1.45, m, 2 H	1.45, m, 2 H	29.6 (2C)	29.6 (2C)
1.20–1.39, m, 16 H	1.20–1.39, m, 16 H	29.5	29.5
0.89, t (<i>J</i> = 6.9 Hz), 3 H	0.89, t (<i>J</i> = 6.9 Hz), 3 H	29.4	29.4
		29.23	29.23
		29.17	29.18
		28.9	28.9
		22.7	22.7
		14.1	14.1

rial). The calculated mass is 226.1933 for an empirical formula of C₁₄H₂₆O₂. An EI high-resolution MS (HRMS) analysis of the molecular ion at 226 *m/z* found a mass of 226.1929. These data strongly suggested that heterologous expression did yield the RpfF protein product and that *Xf*DSF was 2-(Z)-tetradecenoic acid. We did not isolate 2(Z)-tetradecenoic acid from extracts of an *rpfF* mutant of *X. fastidiosa*, verifying the function of RpfF as the *Xf*DSF synthase (see Fig. S3A in the supplemental material).

Construction of *X. fastidiosa*-based DSF reporters as a secondary assay. To characterize the biological activity of putative DSF species, we developed two *X. fastidiosa* bioreporter strains that respond to *Xf*DSF (which we refer to here as *Xf*DSF reporters). Since there was evidence that the *X. campestris* pv. *campestris*-based DSF reporter strain in which an *rpfF* mutant strain harbors an *engXCA'::gfp* transcriptional fusion was not very responsive to *Xf*DSF (4), we produced an *X. fastidiosa* bioreporter in which known DSF-responsive genes were linked to a promoterless *phoA* reporter gene. The low-codon-usage bias of *X. fastidiosa* (23) reflects an atypical ratio of tRNA abundances and thus altered translational parameters (24), leading to the apparently low efficiency of production of heterologous proteins. Previous attempts to express transcriptional fusions with *gfp* and *inaZ* reporter genes in *X. fastidiosa* failed (data not shown). The endogenous *phoA* was an effective reporter gene in *X. fastidiosa* since it conferred substantial

alkaline phosphatase (AP) activity when driven by the *E. coli lacZ* promoter upon induction with isopropyl-β-D-thio-galactoside (IPTG) (data not shown). An *X. fastidiosa phoA* mutant was constructed by allelic replacement, and the *phoA* gene of *X. fastidiosa* was cloned into the broad host vector pBBR1MCS-2 (25). Promoters of the DSF-dependent genes encoding the hemagglutinin-like proteins HxfA and HxfB (13, 14) were then cloned upstream of a promoterless *phoA* and introduced into the *phoA* mutant to yield the resultant *Xf*DSF reporters termed *Xf*HA and *Xf*HB, respectively. The *rrsH* promoter driving the expression of one of the two 16S rRNA-encoding genes (PD0133) was also fused with a promoterless *phoA* to yield a constitutive AP control termed *Xf*R. The *Xf*DSF reporters all harbored a wild-type *rpfF* since DSF-responsive transcription in *X. fastidiosa* was found to be dependent on the presence of the DSF synthase RpfF by mechanisms to be described in a subsequent report.

DSF produced by *X. fastidiosa* induces the *hxfA* and *hxfB* promoters. To assess the temporal patterns of DSF production in *X. fastidiosa* and to determine the appropriate incubation times to assess DSF-dependent gene expression from exogenous application, the AP activity of *Xf*HA and *Xf*HB as well as *Xf*R and a promoterless control was assayed over 5 days of growth on PD3 plates. The relative levels of AP activity of the various bioreporters at a given time differed greatly (Fig. 2A). While little AP activity was observed in *Xf*HA or *Xf*HB for about 30 h after inoculation, activity increased substantially after this time; the activity exhibited by *Xf*HA was always about 4-fold greater than that of *Xf*HB. *Xf*R, reflecting the expression of the rRNA genes, exhibited earlier and stronger activation for up to 60 h, but its AP activity decreased greatly after this time as cells stopped multiplying. The strain harboring only a promoterless *phoA* fusion exhibited no detectable AP activity, confirming that the *phoA* knockout was complete and that no transcriptional activity derived from other promoters present on the plasmid induced expression of the *phoA* reporter gene. Assuming that expression of the *hxfA* and *hxfB* promoters is induced only by DSF, endogenous *Xf*DSF accumulates to a detectable level only after ca. 24 h of cell growth, yielding an amply long time during which the effect of exogenous *Xf*DSF on their expression can be assessed (see Fig. S4A in the supplemental material).

The specificity of the response of the *Xf*DSF reporters to the signal molecules produced by *X. fastidiosa* RpfF was assayed. The *Xf*HA or *Xf*HB bioreporters were grown on media that were over-

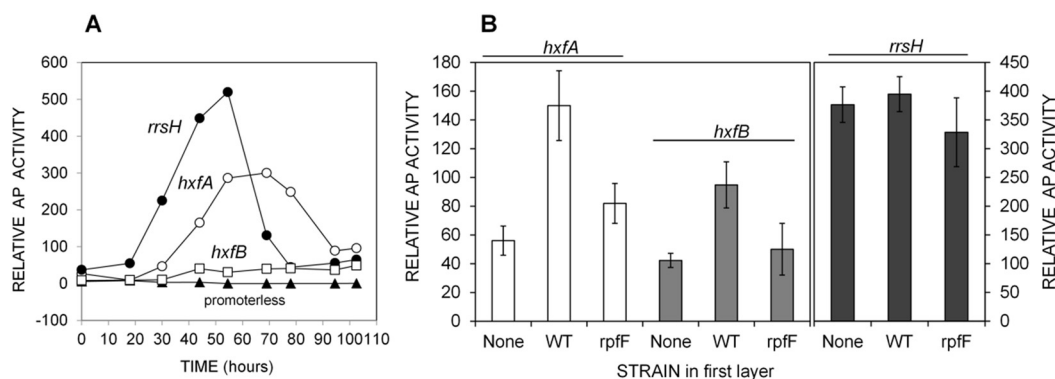


FIG 2 (A) Activity of *hxfA*, *hxfB*, and *rrsH-phoA* transcriptional fusions (designated *Xf*HA, *Xf*HB, and *Xf*R) during growth of *X. fastidiosa* on solid PD3 medium. (B) Induction of *Xf*HA and *Xf*HB in response to *Xf*DSF. *X. fastidiosa* wild-type (WT) cells or an *rpfF* mutant were grown for 5 days on PD3 plates, and then the plates were overlaid with a fresh PD3 layer onto which the bioreporter strains were inoculated. AP (alkaline phosphatase) activity was measured after 24 to 48 h.

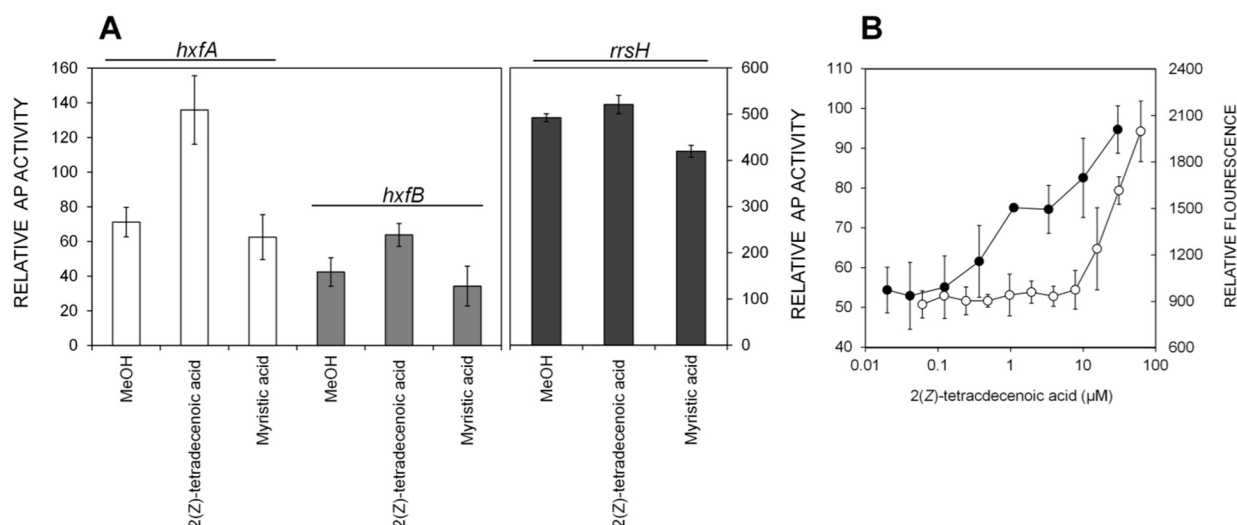


FIG 3 (A) Alkaline phosphatase activity exhibited by *XfHA*, *XfHB*, and *XfR* in response to 10 μ M 2(Z)-tetradecenoic acid or myristic acid. AP activity was measured after 24 to 48 h of growth on PD3 plates. (B) Alkaline phosphatase activity exhibited by *XfHA* (filled circles) and GFP fluorescence exhibited by the *XccDSF* reporter (open circles) in response to increasing amounts of 2(Z)-tetradecenoic acid. *XfHA* was assayed in a 48-well microtiter plate after 24 to 48 h of growth in PD3 broth.

laid on cultures of either the wild-type *X. fastidiosa* strain or an *rpfF* mutant. AP activity would be associated with compounds from the producing strains that had diffused through the medium. The AP activity of both *XfHA* and *XfHB* was about 2-fold higher in overlays of the wild-type culture than in overlays of cultures of either an *rpfF* mutant or uninoculated plates (Fig. 2B), indicating that the increased AP activity of the bioreporters cultured over that of the wild-type strain was solely attributable to excreted *XfDSF*. The levels of AP activity of *XfR* were similar under all conditions, suggesting that *XfDSF* did not greatly affect translation in this species.

2(Z)-Tetradecenoic acid induces *hxfA* and *hxfB* promoter activity. Since the isolation of the putative *XfDSF* was guided by the *XccDSF* reporter, the biological activity of 2(Z)-tetradecenoic acid in *X. fastidiosa* was verified by using the newly developed *XfDSF* reporters. Substantial induction of AP activity was observed within 24 h after exposure of *XfHA* and *XfHB* to 10 μ M synthetic 2(Z)-tetradecenoic acid on solid PD3 media (Fig. 3A). As observed for crude extracts, *XfHA* exhibited higher AP activity than did *XfHB* upon exposure to 2(Z)-tetradecenoic acid. A similar concentration of myristic acid did not induce any AP activity in these bioreporters. Likewise, *XfR* exhibited similar levels of AP activity irrespective of the presence or absence of these molecules. *XfHA* exhibited a dose-dependent increase in AP activity in PD3 broth containing more than about 0.5 μ M 2(Z)-tetradecenoic acid (Fig. 3B). In contrast, the green fluorescent protein (GFP) fluorescence exhibited by the *XccDSF* reporter, induced by its cognate DSF (11-methyl-cis-2-dodecenoic acid) at concentrations as low as 0.5 μ M (17), was induced only by concentrations of 2(Z)-tetradecenoic acid higher than about 10 μ M (Fig. 3B), suggesting that the Rpf system of *X. fastidiosa* is much more sensitive to 2(Z)-tetradecenoic acid than that of *X. campestris*. The putative *XfDSF* reported in the CVC strain of *X. fastidiosa* (20) 2-methyl-tetradecenoic acid did not induce any detectable AP activity in either the *XfHA* or *XfHB* bioreporter (see Fig. S4C in the supplemental material) or induce GFP fluorescence in the *XccDSF* reporter strain.

2(Z)-Tetradecenoic acid induces biofilm formation in *X. fastidiosa*. To further test the biological activity of 2(Z)-tetradecenoic acid in *X. fastidiosa*, its ability to induce biofilm formation at the liquid-air interface of shake cultures in glass tubes was quantified by crystal violet staining. *rpfF* mutants of *X. fastidiosa* are substantially impaired in the formation of biofilm, and thus exogenous *XfDSF* should induce this trait. Crude culture extracts from the wild-type strain do induce biofilm formation in this assay. Biofilm formation in a wild-type strain, *temecula1*, was induced in a dose-dependent manner within 24 h by addition of 2(Z)-tetradecenoic at concentrations greater than about 1.0 μ M (Fig. 4A).

To more directly link *XfDSF* exposure with changes in gene expression in *X. fastidiosa*, the abundance of transcripts of *hxfB* was assessed by real-time PCR analysis in cells grown with and without 10 μ M 2(Z)-tetradecenoic acid. In the presence of 2(Z)-tetradecenoic acid, transcripts of *hxfB* were 4.5-fold higher than the transcript of *rpsO*, which was invariant. Relative transcription levels were normalized to *rpoD* transcript levels (Fig. 4B). This demonstrates that *XfDSF*-mediated increases in biofilm formation are accompanied by increases in the expression of *hxfB*.

2(Z)-Tetradecenoic acid accounts for only part of the induction of *hxfA* in culture extracts of *X. fastidiosa*. Since several related chemical species with possible DSF activity are produced by *Xanthomonas* and *Stenotrophomonas* species (18, 19), we explored the extent to which 2(Z)-tetradecenoic acid could account for the induction of AP activity in the *XfHA* bioreporter by crude culture extracts of *X. fastidiosa*. *XfHA* was thus exposed to serial dilutions of crude extract of plate cultures added to PD3 broth in a microtiter well plate assay. A concentration-dependent increase in AP activity was observed in samples containing as little as 10% of a culture plate from which the extracts were made (Fig. 5; see also Fig. S4B in the supplemental material). Extracts containing 10-fold more than this threshold amount of material conferred apparent toxicity, resulting in decreased growth of the bioreporter, and reduced or complete suppression of AP activity was observed. Quantitative HPLC (using synthetic *XfDSF* as a standard) was

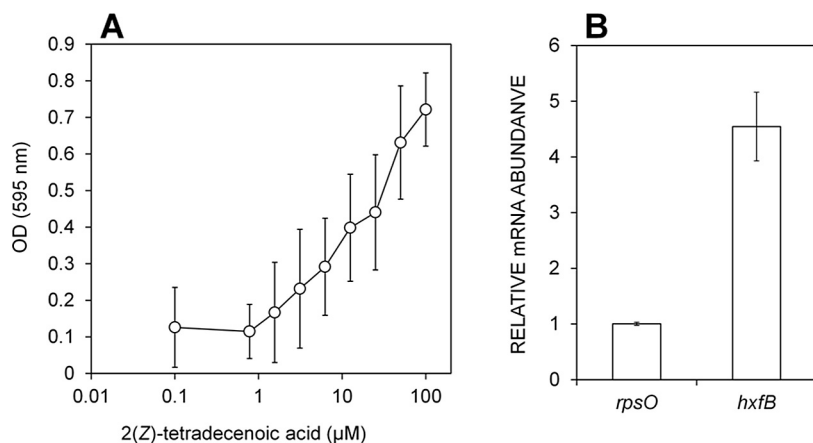


FIG 4 (A) 2(Z)-Tetradecenoic acid dose-dependent increase in biofilm formation in the liquid-air interface of a wild-type *X. fastidiosa* strain grown in a shaken broth culture. Biomass attached to the glass wall of a culture tube was measured after 24 to 48 h by crystal violet staining. (B) qPCR analysis of the relative levels of expression of *hxfB* in *X. fastidiosa* wild-type cells, in a shaken broth culture in the presence of 10 μM 2(Z)-tetradecenoic acid or MeOH only. RNA was extracted after 24 to 48 h; *rpsO* and *rpoD* served as endogenous controls.

used to estimate the concentration of 2(Z)-tetradecenoic acid in the extract. The concentration of 2(Z)-tetradecenoic acid in samples in which AP activity of the *Xf*HA bioreporter was maximally induced was only about 100 nM (Fig. 5). These concentrations of 2(Z)-tetradecenoic acid are lower than the minimum concentration required for detection by *Xf*HA (ca. 500 nM; Fig. 3B), suggesting that other active molecules that can induce *hxfA* were also

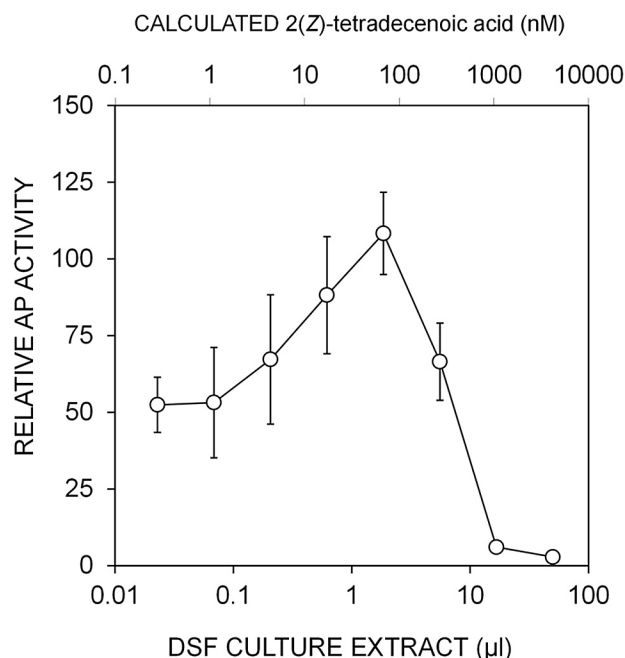


FIG 5 Alkaline phosphatase activity exhibited by *Xf*HA harboring an *hxfA*::*phoA* transcriptional fusion incubated in PD3 broth containing increasing amounts of DSF-containing culture extract of PD3 plate cultures of a wild-type *X. fastidiosa* strain. The concentration of 2(Z)-tetradecenoic acid in the extract determined by HPLC (high-performance liquid chromatography) is shown by the upper x axis.

present in the extract or that other compounds that can synergize with 2(Z)-tetradecenoic acid, thereby enhancing signal, were present in the cell extracts.

DISCUSSION

While *X. fastidiosa* is one of several plant-pathogenic bacteria that coordinate their gene expression in a density-dependent fashion using small signaling molecules known as DSF, its signal molecule apparently differs from those of the other taxa. Here we isolated and characterized by *Xcc*DSF reporter-guided fractionation a novel DSF molecule, 2(Z)-tetradecenoic acid, from the PD strain of *X. fastidiosa* that both enhances the expression of genes contributing to biofilm formation (*hxfA* and *hxfB*) and promotes biofilm formation. This result provides further support for the idea that a clear structural specificity exists in various bacteria that conduct DSF-based signaling. Studies

have shown that an *Xcc*DSF reporter reacts differentially to DSF extracts from other *X. campestris* pv. *campestris* strains and from the closely related pathogen *X. oryzae* pv. *oryzae* (17), suggesting that these strains produce different chemical species, or different ratios of a common group of related DSF molecules. In addition, the *X. campestris* pv. *campestris*-based DSF bioreporter responded quite differently to structurally diverse DSF-like fatty acids; a strong response to *Xcc*DSF was seen at concentrations as low as 0.5 μM whereas the minimum inducing concentrations of the *E* and the saturated isomers were 100 μM and 10 mM, respectively (17). Similarly, the 12-carbon, unbranched molecule with a 2(Z)-double bond (BDSF) had a minimum inducing concentration of 30 μM (17). Even within *X. oryzae* pv. *oryzae*, the three forms of DSF apparently have different biological activities (18). The response to such molecules appears species specific, since DSF and BDSF both restored exopolysaccharide (EPS) production and xylanase activity to an *rpfF* mutant of *X. oryzae* pv. *oryzae* at a concentration of ca. 1 μM (18). Likewise, in *S. maltophilia*, DSF and its saturated isomer appeared to be equally effective in the stimulation of flagellum-independent translocation (19). Curiously, a CVC strain of *X. fastidiosa* was reported to produce a potential DSF-like molecule (12-methyl-tetradecanoic acid) (20). However, while the DSFs from *X. campestris* pv. *campestris* and the PD strain of *X. fastidiosa* were verified through demonstration of the activity of synthetic material in their respective bioreporters (17, 18), this was not shown for the putative DSF from the CVC strain. Moreover, we have not been able to observe activity of this compound in the *Xf*HA or *Xf*HB bioreporters or in the *X. campestris* pv. *campestris* GFP reporter strains. This diversity of responses to related molecules within this group of bacteria suggests that even though they are closely related, their cognate signaling molecules (to which they are most responsive) can differ. Also, though the RpfC sensor machinery is conserved across taxa and can sense DSFs from related organisms, there is specificity associated with the sensor that gives differential responses of DSF signals in the DSF bioreporters. Therefore, although the putative *Xf*DSF described in a CVC strain of *X. fastidiosa* was unable to activate either

the *Xf*HA bioreporter or the *Xcc*DSF reporter, it is possible that it could differ from that of the PD strain. For similar reasons, it is not surprising that our *Xcc*DSF reporter was not as proficient at sensing *Xf*DSF in culture extracts (4) or synthetic 2(Z)-tetradecenoic acid (Fig. 3B).

*Xf*DSF accumulation has been strongly linked with the induction of genes associated with attachment and biofilm formation. These processes, in turn, are associated with upregulation of several adhesins, including the hemagglutinin-like protein-encoding genes *hxfA* and *hxfB* that are involved in cell-cell aggregation and surface adhesion (13). Upregulation of these factors apparently attenuates the virulence of *X. fastidiosa* by increasing its adhesiveness, reducing its ability to spread throughout the plant (26). We have shown that 2(Z)-tetradecenoic acid is sufficient to upregulate the expression of these genes (Fig. 3). More importantly, the relatively low concentrations of 2(Z)-tetradecenoic acid that are sufficient to both induce gene expression and modulate biofilm formation (500 nM) support the idea of this molecule as being a biologically relevant signal molecule, since it is as active in the *X. fastidiosa* system as the cognate DSF species tested in *Xanthomonas* species (17). Although its DSF-dependent modulation of gene expression operates through an RpfCG phosphorelay pathway in *X. fastidiosa*, as in those other species (27), the genes that are regulated and their modes of regulation appear to differ in these taxa, reflecting the coordination of different lifestyles of these pathogens in their various environmental niches by similar regulatory mechanisms and structurally similar signal molecules (27). It remains to be seen if the distinctive DSF species produced by *X. fastidiosa* reflects a functional difference in its ability to act as an extracellular signal molecule in the high-flow, vascular environments that it colonizes or whether it might avoid cross-talk with other taxa that might co-occur in these settings.

Our finding that *X. fastidiosa* can sense exogenous DSF and alter gene expression and group behavior within a relatively short time of exposure is noteworthy in that it seems to differ from findings determined with other species that use DSF as a signaling molecule. Unlike the results seen in *X. fastidiosa*, DSF-mediated cell-cell signaling could not be induced in *X. campestris* pv. *campestris* in low-density cells by the addition of exogenous DSF (3). It was suggested that an additional factor(s) needs to accumulate to a functional level in order to allow cell-cell signaling to occur. Likewise, in some species such as *Pseudomonas aeruginosa* that utilize acyl homoserine lactones (AHLs) as signaling molecules, induction of expression at least some of the quorum-regulated genes is dependent on the expression of *rpoS*, and thus on the growth phase, in addition to accumulation of AHLs (28). The expression of *hxfA*, *hxfB*, and apparently other genes needed for biofilm formation is induced rapidly upon the addition of 2(Z)-tetradecenoic acid to cultures, independently of their cell concentration (Fig. 2 to 5). Since DSF accumulation tends to suppress the virulence of *X. fastidiosa* (27), it might be possible to alter the temporal patterns by which the pathogen would experience DSF in plants by applying this signal molecule to plants before or shortly after inoculation by the sharpshooter vector. Such cells would be expected to increase their adhesiveness and suppress their motility and secretion of extracellular enzymes, thereby reducing their ability to move and multiply within the plant. Therefore, the pathogen should aggregate before ever producing a quorum and would then be blocked in its ability to colonize the plant and cause disease. Disease control through this mechanism,

termed “pathogen confusion,” would be facilitated by the cell density-independent response to DSF.

In this study, DSF identification in *X. fastidiosa* was initially guided by an *Xcc*DSF reporter. The isolated *Xf*DSF molecule, 2(Z)-tetradecenoic acid, is thus one that can be sensed by the *Xcc*DSF reporter. While this molecule can modulate *rpfF*-dependent traits in both *X. campestris* pv. *campestris* and *X. fastidiosa*, it clearly is more active in *X. fastidiosa*, suggesting that other compounds that show an even higher preferential activity in *X. fastidiosa* might exist that may have been overlooked by the *Xcc*-based primary screen. There is evidence for the existence of such compounds, given that there is more biological activity sensed using the *Xf*HA bioreporter than can be accounted for by the amount of 2(Z)-tetradecenoic acid isolated by HPLC (Fig. 5). The novel *Xf*DSF reporter strains described here should be very useful in identification of any other DSF-like molecules produced by *X. fastidiosa*.

MATERIALS AND METHODS

Analytical methods. Unless otherwise noted, starting materials and analytical grade solvents were obtained from commercial suppliers and used without further purification. Tetrahydrofuran (THF) was freshly distilled from sodium using benzophenone as an indicator. Both reaction mixtures and chromatography fractions were analyzed on Merck silica gel 60 F254 TLC plates. Flash column chromatography was carried out with ICN Silitech silica gel (32 to 63 D, 60 Å). Fourier transform infrared (FT-IR) spectra were obtained as thin films on NaCl plates with an ATI Mattson Gemini spectrometer. Preparative reverse-phase HPLC purification was conducted using Agilent 1100 series preparative HPLC with an Alltech Econosil C₁₈ column (250 mm by 22 mm) with acetonitrile and water and with 0.1% formic acid as the eluent. Proton and carbon NMR spectra (1H-NMR and 13C-NMR) were recorded on a Bruker DRX-500 spectrometer and calibrated to the residual solvent peak. Unless otherwise noted, spectra were recorded at room temperature (294.9 K). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, m = multiplet, dd = doublet of doublet. The prefix b stands for broad signals. Low-resolution mass spectra were recorded using a VGProSpec (EI) mass spectrometer. Unless otherwise noted, reactions were conducted with oven-dried glassware under an inert N₂ atmosphere.

Construction of *E. coli* and *E. herbicola* strains for heterologous expression. The *rpfF* gene from *X. fastidiosa* strain temecula1 (4, 29, 30) (see Table S1 in the supplemental material) was expressed from the promoter of the kanamycin resistance gene, *kan2*. *rpfF* was amplified from the *X. fastidiosa* genome by PCR using the *rpfF* primers (see Table S1 in the supplemental material). The *rpfF* ribosomal binding site (RBS) was included in the forward primer to enable translation. The P_{kan} promoter (137 bp) was amplified from the transposon *kan2* Tn5 (Epicenter) with the *kan*Promoter primers (see Table S1 in the supplemental material). The *rpfF* forward and *kan*Promoter reverse primers included a HindIII restriction site, while the *rpfF* reverse and the *kan*Promoter forward primers included an EcoRI site. The two PCR fragments were digested with HindIII and then ligated to each other and subsequently amplified with the primers *kan*Promoter forward and *rpfF* reverse, gel purified, and then cloned into PCR2.1 (Invitrogen). The insertion was then subcloned into the EcoRI site of pVSP61 to yield pVSP61-*rpfF*. DSF production in *E. coli* (pVSP61-*rpfF*) was confirmed using the *Xcc*DSF reporter strain (see below) and pVSP61-*rpfF* transformed also into *E. herbicola* 299R electrocompetent cells; transformants were selected on Kirby-Bauer (KB) plates (31) supplemented with 50 µg/ml kanamycin and verified for DSF production as before.

Isolation of *Xf*DSF from heterologous expression. *E. coli* or *E. herbicola* harboring pVSP61-*rpfF* was cultured in 3 liters of KB broth (31) containing kanamycin. Cultures were incubated at 28°C for 36 h. Cells were formed into pellets, and the supernatant was extracted three times

with equal volumes of EtOAc. The combined organics were washed once with brine (500 ml) and dried over Na_2SO_4 , and the volatiles were removed *in vacuo*. The crude extract was purified using flash column chromatography (10:1 $\rightarrow$ 5:1 $\rightarrow$ 1:1 $\rightarrow$ 1:5 hexane:EtOAc $\rightarrow$ EtOAc). The active fraction from elution with 5:1 hexane:EtOAc was further purified with preparative reverse-phase HPLC on an Alltech Econosil C_{18} column (250 mm by 22 mm) with the gradient depicted in Table S2 in the supplemental material.

Isolation from *rpjC* mutant of *X. fastidiosa*. The inoculum of the *rpjC* mutant was harvested from PWG Periwinkle Gelrite (PWG) plates into 10 mM phosphate buffer (optical density at 600 nm [OD_{600}] of ~ 1), and then 50 μl was plated onto each of 300 PWG plates. Cells were allowed to grow for 12 days at 28°C, and the medium containing the cells was then sliced into 2-mm² cubes and sonicated in an equal volume of EtOAc for 2 h. The EtOAc was decanted, and the volatiles were removed *in vacuo*. The crude extract was purified using flash column chromatography (10:1 $\rightarrow$ 5:1 $\rightarrow$ 1:1 $\rightarrow$ 1:5 hexane:EtOAc $\rightarrow$ EtOAc). The active fraction from elution with 5:1 hexane:EtOAc was further purified with preparative reverse-phase HPLC on an Alltech Econosil C_{18} column (250 mm by 22 mm) with the gradient depicted in Table S2 in the supplemental material. Using this method, 0.6 mg of putative *Xf*DSF was isolated.

XccDSF reporter assay. *rpjF* mutant 8523 of *X. campestris* pv. *campestris* (3) harboring an *engXCA'::gfp* transcriptional fusion (highly stable plasmid pKLN55; here designated the *Xcc*DSF reporter) (4) was employed for assays of DSF. A 2-day-old culture grown on KB plates without antibiotics was suspended in 10 mM phosphate buffer (pH 7.4) to an OD_{600} of 0.1. Cells were then sprayed on a KB plate onto which a DSF-producing strain was spotted at the center. The plates were incubated at 28°C for 36 h, and relative DSF abundance was estimated from the radius of cells exhibiting GFP fluorescence.

Alkaline phosphatase (AP) assays. AP activity was quantified after cells were permeabilized and disrupted (32). Cells were grown on either PD3 plates or PD3 broth supplemented with 15 $\mu\text{g}/\text{ml}$ gentamicin; much lower AP activity was seen in cells grown on PWG plates. Cells of *Xf*HA, *Xf*HB, *Xf*R, or *Xf*PL were grown for 5 to 6 days at 28°C on PWG plates supplemented with 15 $\mu\text{g}/\text{ml}$ gentamicin and 50 $\mu\text{g}/\text{ml}$ kanamycin prior to inoculation into the assay plates. When the assays were performed using solid media, cells were incubated either on PD3 plates supplemented with 10 μM 2(Z)-tetradecenoic acid (added from a stock of 100 mM dissolved in MeOH directly to the medium; MeOH alone was used as a control) or on a fresh layer of PD3 applied over 5-day cultures of various strains in PD3. Uncultured plates were overlaid as well for a negative control. To enable harvest of cells after only short incubation times, a high initial cell concentration ($>10^9$ cells/ml) was used. *Xf*DSF reporter strains (10 μl) were plated side by side on a single plate, and cells were collected and assayed for AP activity after 24 to 48 h of incubation. Cells were collected from assay plates using a loop and suspended in 1 ml of 10 mM Tris-Base (pH 8.0) containing 10 mM MgSO_4 in a 2-ml Eppendorf tube. The cells were formed into pellets by centrifugation for 4 min at 10,000 $\times g$ and resuspended in 2 ml 1 M Tris-Base (pH 8.0). The samples were transferred to glass assay tubes, and cell density (measured as OD_{600}) was adjusted to ca. 0.15. Then, 1 ml of 1 M Tris-Base (pH 8.0) containing 0.1 mM ZnCl_2 was added to each tube and the final OD_{600} was recorded. The cells were then disrupted by adding 50 μl of 0.1% SDS and 50 μl of chloroform followed by vigorous mixing by vortexing and incubated for 5 min at room temperature, and 3 μl of a 10 mM fluorescein diphosphate (Sigma) stock solution was then added to the tube and mixed briefly by vortexing. Fluorescence measurements (excitation, 485 nm; emission, 515 nm) were taken at 2-min intervals for 30 min using TD-700 fluorimeter (Turner Design). Enzyme activity was calculated as the rate of fluorescence signal accumulation with time divided by the cell density. For assays using 48-well Falcon microtiter plates (Becton Dickinson), PD3 broth was supplemented with either DSF analogs or DSF-containing crude extract (in MeOH) or an equal volume of MeOH. The two solutions (with and without DSF) were then mixed to produce various concentra-

tions of DSF, and 400 μl was distributed into wells with six replicates. The *Xf*HA reporter was collected into PD3 broth, and 400 μl of the suspension was applied to each well (final $\text{OD}_{600} = \sim 0.05$). At various time intervals, the AP activity of the microtiter plate was determined; the plate was centrifuged for 10 min at 4,000 rpm (Eppendorf), the growth medium was removed by aspiration, the cells were resuspended in 400 μl of 10 mM Tris-Base (pH 8.0) containing 10 mM MgSO_4 , and AP activity was then measured using a method similar to that described above.

Biofilm formation assays. The effect of *Xf*DSF on the biofilm formation capacity of the wild-type *X. fastidiosa* strain was determined in PIM6 broth (a medium developed in the laboratory of Michele Igo, University of California, Davis; personal communication) in glass culture tubes at 28°C and 200 rpm. Cells were grown on PWG plates for 7 days, resuspended in PIM6 broth to an $\text{OD}_{600} = 0.2$, and added to PIM6 broth containing either 10 μM 2(Z)-tetradecenoic acid (from a 100 mM MeOH stock solution) or an equal volume of MeOH only (0.1% [vol/vol] final concentration). The tubes were incubated for 24 to 48 h, during which a visible biomass was observed at the liquid-air interface. The medium, containing unattached cells, was then removed by aspiration, and the biomass attached to the glass wall was quantified by staining with 1% crystal violet for 10 min.

Quantitative PCR (qPCR). *hxfB* expression was assessed in cultures of the wild-type strains grown in PIM6 broth containing either 10 μM 2(Z)-tetradecenoic or an equal volume of MeOH only. Total RNA was isolated using an RNeasy RNA extraction kit (Qiagen); DNA was eliminated using an on-column RNase-Free DNase kit (Qiagen). RNA samples were stored at -80°C , and 1- μg aliquots were used to initiate cDNA synthesis using 3 μg of random hexamer primers and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

Quantitative PCR was performed in an ABI Prism 7100 sequence detector system (Applied Biosystems), and primers listed in Table S1 in the supplemental material. PCR products were detected by measuring the increase in fluorescence produced upon binding of SYBR green dye (Qiagen) to double-stranded DNA (33). Two endogenous control genes (*rpoD* and *rpsO*) were employed to normalize gene expression. To ensure that the threshold cycle (C_t) values obtained were from a single PCR product, melting curve analysis was run at the end of each expression analysis. Relative expression (RQ) was calculated from the threshold cycle (C_t) as follows (33): $\text{d}C_t = C_t(\text{target gene}) - C_t(\text{endogenous control})$; $\text{dd}C_t = \text{d}C_t(\text{treatment}) - \text{d}C_t(\text{reference})$; $\text{RQ} = 2^{(-\text{dd}C_t)}$.

Additional Material and Methods are described in the supplemental material in Text S1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00539-12/-/DCSupplemental>.

Text S1, PDF file, 0.1 MB.

Figure S1, PDF file, 1 MB.

Figure S2, PDF file, 0.7 MB.

Figure S3, PDF file, 1 MB.

Figure S4, PDF file, 0.3 MB.

Figure S5, PDF file, 0.5 MB.

Figure S6, PDF file, 0.5 MB.

Table S1, PDF file, 0.1 MB.

Table S2, PDF file, 0.1 MB.

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