

Phenotype Overlap in *Xylella fastidiosa* Is Controlled by the Cyclic Di-GMP Phosphodiesterase *eal* in Response to Antibiotic Exposure and Diffusible Signal Factor-Mediated Cell-Cell Signaling

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***Eal* is an EAL domain protein in *Xylella fastidiosa* homologous to one involved in resistance to tobramycin in *Pseudomonas aeruginosa*. EAL and HD-GYP domain proteins are implicated in the hydrolysis of the secondary messenger bis-(3'-5')-cyclic dimeric GMP (cyclic di-GMP). Cell density-dependent communication mediated by a Diffusible Signal Factor (DSF) also modulates cyclic di-GMP levels in *X. fastidiosa*, thereby controlling the expression of virulence genes and genes involved in insect transmission. The possible linkage of *Eal* to both extrinsic factors such as antibiotics and intrinsic factors such as quorum sensing, and whether both affect virulence, was thus addressed. Expression of *eal* was induced by subinhibitory concentrations of tobramycin, and an *eal* deletion mutant was more susceptible to this antibiotic than the wild-type strain and exhibited phenotypes similar to those of an *rpfF* deletion mutant blocked in DSF production, such as hypermotility, reduced biofilm formation, and hypervirulence to grape. Consistent with that, the *rpfF* mutant was more susceptible than the wild-type strain to tobramycin. Therefore, we propose that cell-cell communication and antibiotic stress can apparently lead to similar modulations of cyclic di-GMP in *X. fastidiosa*, resulting in similar phenotypes. However, the effect of cell density is dominant compared to that of antibiotic stress, since *eal* is suppressed by RpfF, which may prevent inappropriate behavioral changes in response to antibiotic stress when DSF accumulates.**

The plant pathogen *Xylella fastidiosa* colonizes xylem vessels of many economically important crop plants, including grape, almond, and citrus, often blocking water flow and thus causing disease symptoms. The pathogen is transmitted from plant to plant by xylem-feeding leafhoppers (1), where it attaches to the precibarium in the mouthpart. In order to colonize both plant and insect hosts, *X. fastidiosa* has evolved a “bipolar” lifestyle, either movement within the plant or acquisition by insects, whereby different subpopulations within a plant express traits that allow bacterial behaviors that appear incompatible (2–4). In the plant, most cells exhibit an initial “exploratory” phase, enabling spread both within and between xylem vessels, in which genes involved in active movement and plant cell wall degradation are expressed while genes involved in attachment are suppressed. Only when the cell density in some colonized vessels becomes high do those cells increase the expression of genes for the production of adhesins and exopolysaccharide (EPS) and suppress the expression of gene products involved in active movement such as extracellular enzymes and type IV pili, thereby transitioning to an “insect acquisition phase,” in which biofilm-related features allow the adherence of the bacterium to the insect’s precibarium upon acquisition (5).

The behavioral transitions of *X. fastidiosa* are regulated by signal transduction pathways involving bis-(3'-5')-cyclic dimeric GMP (cyclic di-GMP)-modulating proteins. Cyclic di-GMP is an intracellular secondary messenger exclusively found in bacteria that is produced by enzymes containing GGDEF domains (diguanylate cyclases [DGCs]) and is hydrolyzed by those containing either EAL or HD-GYP domains (phosphodiesterases [PDEs]) (6–10). Cyclic di-GMP-mediated signaling is often involved in the transition between the motile and sessile bacterial lifestyles. *X. fastidiosa* harbors five genes encoding proteins with the capacity to modulate cyclic di-GMP levels; PD1671 and PD1994 encode

GGDEF/EAL dual-domain proteins, *cgsA* (PD0279) encodes a GGDEF protein, *rpfG* (PD0405) encodes a HD-GYP protein, and PD1617 encodes an EAL protein. These proteins also have N-terminal sensory input domains; PD1671 and RpfG have a CheY-like REC domain typical of response regulators in phosphorelay systems (11), PD1994 has a Per-Arnt-Sim (PAS) domain that typically binds to a specific ligand (12), and CgsA and PD1617 have transmembrane helices that place their sensory domains in the periplasm.

RpfG is the response regulator of the RpfCG phosphor-relay system that receives and converts a quorum-sensing signal (Diffusible Signal Factor [DSF]) to the cyclic di-GMP currency. RpfC, a hybrid membrane sensor kinase, phosphorylates RpfG upon interacting with DSF, which is synthesized by RpfF (9, 13). The activated RpfG hydrolyzes cyclic di-GMP, thereby reducing its availability to Clp, a transcriptional regulator (14). An *rpfF* mutant (blocked in the production of DSF and therefore in the hydrolysis of cyclic di-GMP by RpfG) is hypervirulent to grape and poorly adherent and thus attenuated in biofilm formation (3, 15). Consistent with this, a *cgsA* mutant (attenuated in cyclic di-GMP synthesis) is hypovirulent to grape and hyperadhesive (16), sug-

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TABLE 1 Strains and plasmids

Strain or plasmid	Genotype or description	Reference or source
<i>X. fastidiosa</i> Temecula1 strains	Wild type	ATCC 700964
Dif $rpfF$	<i>X. fastidiosa</i> Temecula1 $\Delta rpfF$ (markerless)	2
ADS1	<i>X. fastidiosa</i> Temecula1 Δeal (<i>kanR</i>)	This study
MIX1	<i>X. fastidiosa</i> Temecula1 Δclp (<i>gentR</i>)	This study
<i>P. aeruginosa</i> PAO1 cyclic di-GMP bioreporter	$\Delta pelA \Delta pslBCD$ mini-Tn7- <i>P_{cdrA}</i> -RBSII- <i>gfp</i>	29
Plasmid		
pUC19	Cloning vector, <i>ampR</i> (<i>bla</i>)	Invitrogen
pBBR1MCS-2	<i>kanR</i> gene [<i>aph</i> (3')III]	25
pBBR1MCS-5	<i>gentR</i> gene (<i>aaaC1</i>)	25
pFXF kan	pUC19 <i>kanR</i> [<i>aph</i> (3')III]	This study
pFXF $gent$	pUC19 <i>gentR</i> (<i>aaaC1</i>)	This study
pFXF1	pFXF kan <i>eal</i> knockout vector	This study
pFXF2	pBBR1MCS-5 <i>eal</i> complementation vector	This study
pFXF3	pFXF $gent$ <i>clp</i> knockout vector	This study
pEAL	pBBR1MCS-2 <i>lacZ'</i> :: <i>eal</i> (aa 275–522) ^a	This study

^a aa, amino acids.

gesting that low levels of cyclic di-GMP in *X. fastidiosa* are associated with the “insect acquisition phase” whereas high levels of cyclic di-GMP are associated with the “plant exploratory phase.” This was unexpected, since the activity of diguanylate cyclases in other bacteria facilitates biofilm formation (reviewed in reference 17). RpfF was also reported to suppress the expression of *cgsA* (2), suggesting that signaling through the Rpf system reduces cyclic di-GMP levels in more than one manner.

In addition to the role of cyclic di-GMP-modulating proteins in modulating surface features of cells and their virulence, there is evidence that they control responses to a wide variety of environmental factors such as nutrient limitation (18) and antibiotic exposure (19–21). In *Pseudomonas aeruginosa*, subinhibitory concentrations of the aminoglycoside antibiotic tobramycin trigger biofilm formation. This trait requires Arr (for Aminoglycoside Response Regulator), a membrane-associated EAL domain protein (19). PD1617 encodes a protein with 43% identity to Arr and has similar EAL, transmembrane, and periplasmic domains (hereby designated Eal). In this work, we address the possible role of *eal* in drug resistance in *X. fastidiosa* and explore whether cross talk involving the common intracellular signal cyclic di-GMP could lead to common responses of this pathogen to the different input cues of cell density and antibiotics.

MATERIALS AND METHODS

Bacterial strains and culture media. The strains and plasmids that were employed in this study are listed in Table 1. *X. fastidiosa* strains were routinely grown on PW broth (22), PWG plates (PW with 8 g/liter gelrite), or XFMP broth (XFM plus 1% pectin) (23, 24) at 28°C. *Escherichia coli* strains were grown on LB media (10 g/liter Bacto tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) at 37°C. Antibiotics were used at the following concentrations: kanamycin at 50 μ g/ml and gentamicin at 15 μ g/ml.

Deletion of *eal* (PD1617) and *clp* (PD0755). Deletion of *eal* (Δeal) and *clp* (Δclp) was carried out by transforming *X. fastidiosa* strain Temecula1 with a pUC19-based suicide vector harboring a kanamycin [*aph*(3')III] or a gentamicin (*aaaC1*) resistance gene, flanked by two 1,000-bp genomic fragments which adjoin the target gene in the *X. fastidiosa* genome. The kanamycin and gentamicin resistance genes were amplified from pBBR1MCS-2 and pBBR1MCS-5, respectively (25), using Phusion DNA polymerase (Finnzymes, Finland) and the primers listed in

Table S1 in the supplemental material. The PCR products and pUC19 were digested with BamHI (NEB) and ligated with T4 DNA ligase (Roche, Switzerland) to generate pFXF kan and pFXF $gent$. The orientation of the resistance gene was determined by sequencing. Regions flanking the target genes were amplified and cloned into the pFXF kan vector using the primers listed in Table S1 in the supplemental material to generate pFXF1 (*eal*::*kanR*) and pFXF3 (*clp*::*gentR*). These suicide vectors were transformed into *X. fastidiosa* Temecula1 by exploiting its natural competence (26). Transformed cells were cultured on PWG plates supplemented with kanamycin or gentamicin and incubated for 2 to 3 weeks at 28°C. Colonies were screened for deletion of *eal* or *clp* by PCR using the relevant primers listed in Table S1 in the supplemental material. The presence of a single insertion of the kanamycin gene in the *eal* mutant genome (see Fig. S1 in the supplemental material) was confirmed by Southern blotting (27) using a PCR DIG (digoxigenin) probe synthesis kit (Roche, Switzerland) and the primers employed to amplify the *kanR* cassette from pBBR1MCS-2 (see Table S1 in the supplemental material).

Complementation of Δeal . *eal* was cloned into pBBR1MCS-5 using the primers listed in Table S1 in the supplemental material and introduced into the *eal* mutant by using a previously described transformation protocol (28). The restoration of some phenotypes was verified (see Fig. S2 in the supplemental material).

Verification of Eal cyclic di-GMP phosphodiesterase activity. The EAL domain of *eal* (amino acids 275 to 522) was amplified from the *X. fastidiosa* genome with the primers listed in Table S1 in the supplemental material. The forward primer had a tail harboring the ribosome binding site of *P. aeruginosa* PAO1 *rpsO* followed by an ATG sequence. The PCR product was cloned into pBBR1MCS-2 (25) at the HindII and EcoRI restriction sites as described above to generate pEAL (Table 1). pEAL was transformed by electroporation into the (nonaggregative) *P. aeruginosa* PAO1 $\Delta pel \Delta psl$ double mutant, which harbors a chromosomal *cdrA'*::*gfp* transcriptional fusion, previously shown to respond to cyclic di-GMP levels (29). The green fluorescent protein (GFP) fluorescence of this sensor harboring either pEAL or the empty vector pBBR1MCS-2 was assessed. Colonies were collected from LB plates supplemented with 150 μ g/ml kanamycin and 60 μ g/ml gentamicin and suspended in ABTG-casA broth (29) to an optical density at 600 (OD₆₀₀) of 0.05. Aliquots (500 μ l) were then incubated for 48 h in a clear 48-well microtiter plate at 37°C without shaking. GFP fluorescence (excitation wavelength of 485 nm and emission wavelength of 528 nm) and the OD₆₀₀ were recorded using a Synergy 2 plate reader (BioTek). *cdrA* promoter activity was expressed as the fluorescence divided by the OD₆₀₀.

Tobramycin MIC determination and biofilm induction. The MIC of tobramycin for the wild-type *X. fastidiosa* strain was determined with cells collected from PWG plates and suspended in PW broth to $OD_{600} = 0.1$ in glass tubes. The tubes were shaken in a rotary shaker at 120 rpm and 28°C, and different concentrations of tobramycin (ranging from 0 to 30 µg/ml) were added at days 1, 4, 7, and 10 after inoculation. A 100-µl aliquot of undiluted suspension was plated onto fresh PWG plates after 24 h of exposure to the antibiotic. The MIC was determined as the lowest concentration of tobramycin that completely inhibited cell viability (fewer than 2 colonies obtained from triplicate plating) (30). Testing of sensitivity to subinhibitory doses was performed in a similar manner, using 0, 0.3, 0.5, and 1 µg/ml tobramycin. To assess the induction of biofilm formation by tobramycin, 0, 0.3, 0.5, or 1.0 µg/ml was added to cell suspensions after 4 days of incubation; the biofilm biomass at the liquid-air interface was stained and quantified with 1% crystal violet 24 h after antibiotic addition as described below.

Biofilm assay. Cells were collected from PWG plates and suspended in PW broth to $OD_{600} = 0.1$ (approximately 10^8 CFU/ml), and 300-µl aliquots were transferred to 12-well polystyrene culture plates or glass tubes containing 3 ml of XFMP broth and incubated at 28°C without shaking (plates) or with shaking at 200 rpm (tubes). After 7 days of growth, the medium was discarded and the wells were gently washed with double-distilled water (DDW) to remove planktonic and loosely attached cells. The attached biofilm was quantified by staining the remaining cells with 1% crystal violet for 20 min at room temperature. Excess crystal violet was removed by washing the wells several times with DDW. Crystal violet bound to the attached cells was recovered in 1 ml of 90% ethanol and quantified by measuring absorbance at 595 nm.

Biofilm was also formed on coverslips at the bottom of 6-well polystyrene culture plates containing 1 ml of XFMP broth. To each well, 100 µl of cell suspension ($OD_{600} = 0.1$) was added, and the plate was incubated at 28°C without shaking. After 10 days, the medium was removed and the coverslips were washed with DDW. Cells were stained with Syto-9 (Invitrogen) in a 1:600 dilution in MilliQ autoclaved H₂O (Millipore). Fluorescence images were captured using a Zeiss 510 confocal laser scanning microscope (Zeiss, Germany) (excitation wavelength of 488 nm and emission wavelengths of 505 to 550 nm) at the University of California College of Natural Resources Biological Imaging Facility.

Determination of the exopolysaccharides (EPS). Cells were collected from PWG plates and resuspended in phosphate-buffered saline (PBS) to $OD_{600} = 0.1$, and 40-µl aliquots were spread on 6 plates of XFMP medium. The plates were incubated on 28°C for 10 days, and the cells were collected and resuspended in PBS. Two different methods were used to quantify EPS: (i) a protein A double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) according to a protocol described before (31) and (ii) quantification of total carbohydrate content using Anthrone reagent (32). The protein A double-antibody sandwich ELISA was performed using an antibody raised against EPS of an *X. campestris* pv. *campestris* *gumI* mutant (31) (provided by B. Kirkpatrick, University of California, Davis). Briefly, the wells of a MaxiSorp microtiter plate (Nalge Nunc) were coated with 5 µg/ml anti-EPS F(ab)₂ fragments in 0.015 M Na₂CO₃ (0.035 M)–NaHCO₃ (pH 9.6). The wells were blocked with 1% nonfat milk for 1 h at room temperature and washed three times with phosphate-buffered saline–0.05% Tween 20 (PBST). Cell were suspended in PBS ($OD_{600} = 0.1$), added to the antibody-coated wells, and incubated for 1.5 h at 37°C, and then the wells were washed three times with PBST. Anti-EPS IgG was then added to the wells at a concentration of 5 µg/ml, and the plate was incubated for 1.5 h at 37°C followed by three washes with PBST. Protein A-alkaline phosphatase conjugate (Sigma-Aldrich) was diluted 1:1,500 in PBS, added to the wells, and incubated for 1 h at 37°C. Sigma Fast p-nitrophenyl phosphate (Sigma-Aldrich) was used as the substrate. The substrate color was allowed to develop for 1 h, and the absorbance was measured at 405 nm using a SpectraMax M2 Microplate Reader (Molecular Devices).

Colony morphology. Differences in the morphologies of individual colonies of mutants of similar ages grown on PWG plates were assessed. The plates were incubated at 28°C for 10 days, and the morphology of the fringe of the colonies was examined using a Lumar dissecting microscope (Zeiss, Germany). Colonies with an obvious peripheral fringe were designated motile (34). These colonies were further examined by scanning electronic microscopy at the Electron Microscope Laboratory at the University of California, Berkeley. The samples were prepared as previously described (16).

Pathogenicity assays. Cell suspensions ($OD_{600} = 1.0$) of various strains recovered after growth for 10 days on PWG were mechanically inoculated into 15 Thompson seedless grapevines using a droplet puncture method as described before (33). The number of symptomatic leaves was counted weekly starting 10 weeks after inoculation. Symptomatic leaves were scored for the presence of typical Pierce's disease symptoms (loss of chlorophyll and death of the leaf margins). The population size and migration distance were determined 15 weeks postinoculation in petioles collected at 7 different locations (0, 30, 60, 120, 150, 180, and 210 cm from the inoculation point). The petioles were surface sterilized and weighed, and cells were extracted as described before (35). Serial dilutions of tissue macerates were cultured on PWG plates, and the number of CFU obtained was normalized by the weight of the petiole.

RNA isolation, cDNA synthesis, and quantitative PCR (qPCR). The abundance of transcripts of genes associated with biofilm formation and motility was measured for wild-type and mutant cells collected after 10 days of growth on XFMP plates. Cells were washed and collected by centrifugation at $8,000 \times g$ for 5 min at 4°C with diethylpyrocarbonate-treated water. Total RNA was isolated using an RNeasy RNA extraction kit (Qiagen, Germany); DNA was eliminated using an on-column RNase-Free DNase apparatus (Qiagen, Germany). RNA samples were stored at –80°C, and new 1-µg aliquots were used for cDNA synthesis before each analysis, using 3 µg of random hexamers and Superscript II reverse transcriptase (Life Technologies) according to the manufacturer's instructions.

Quantitative PCR was performed in an ABI PRISM 7100 sequence detector system (Applied Biosystems). Detection of PCR products was done by measuring the increase in fluorescence produced upon binding of SYBR green dye (Qiagen, Germany) to double-stranded DNA. Both *rpoD* and *rpsO* were used as endogenous control genes to normalize gene expression. The specificity of primers (listed in Table S1 in the supplemental material) for the target genes was verified by DNA sequencing of PCR products at the University of California Biological Sequence Facility. 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 C_T values (36) as follows: $dC_T = C_T$ (target gene) – C_T (endogenous control); $ddC_T = dC_T$ (treatment) – dC_T (reference); $RQ = 2^{(-ddC_T)}$. To estimate the abundance of *eal* and *rpfG* transcripts in young (1-day-old) and old (7-day-old) wild-type cultures, a semiquantitative analysis was performed in which the transcript level was normalized to the total RNA level as follows: $dC_T = C_T$ (7 days) – C_T (1 day); $RQ = 2^{(-dC_T)}$ (36).

RESULTS

***X. fastidiosa eal* is a phosphodiesterase involved in the response to tobramycin.** The phosphodiesterase activity of *X. fastidiosa* Eal (PD1617) was verified using a *P. aeruginosa*-based cyclic di-GMP bioreporter (29) in which GFP fluorescence is dependent upon cytoplasmic cyclic di-GMP levels. Heterologous expression of the EAL domain of Eal in this bioreporter resulted in a (ca. 30%) reduction in the GFP fluorescence of this bioreporter (see Fig. S3 in the supplemental material), suggesting that cyclic di-GMP levels had decreased due to the expression of the EAL domain.

Eal has an organization of transmembrane, periplasm, and EAL domains similar to that of Arr (Fig. 1), suggesting that it might also confer antibiotic resistance in *X. fastidiosa*. To test this

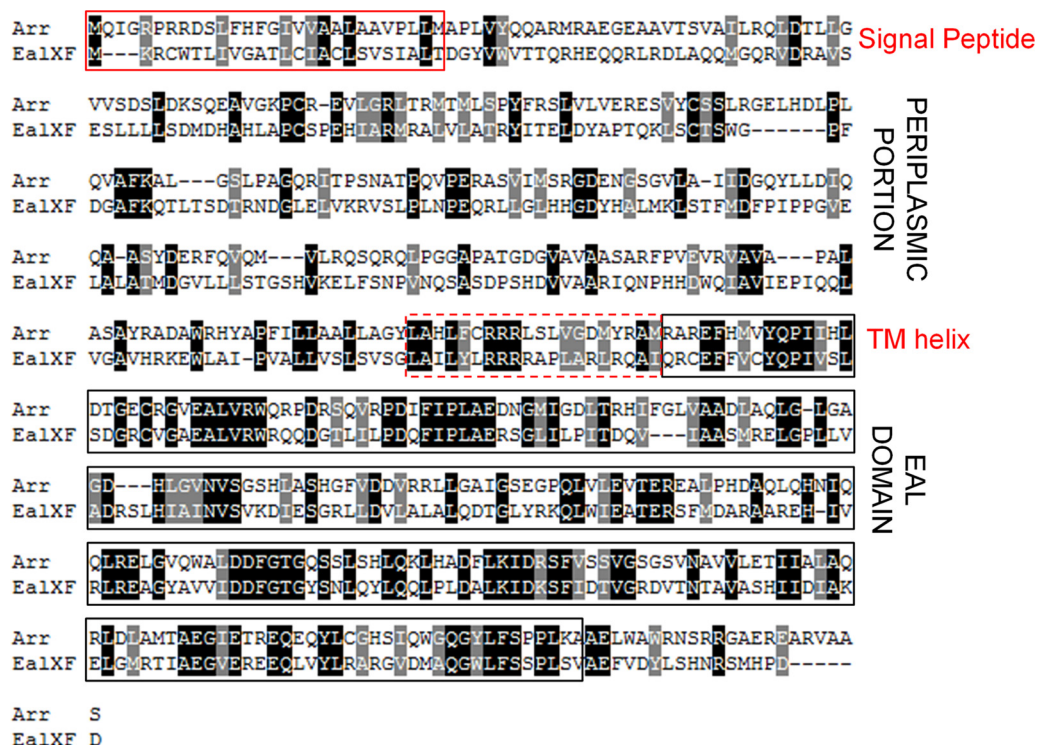


FIG 1 Pairwise alignment comparison of *Pseudomonas aeruginosa* PAO1 Arr and *Xylella fastidiosa* Eal. The EAL domain is boxed in a black frame, the signal peptide in a solid red frame, and the transmembrane (TM) helix in a dashed red frame. The comparison was performed using the EBI server, the image was generated using the BOXSHADE server, the EAL domain was located using the NCBI CDD database, and signal peptides and transmembrane helices were predicted using the Tmpred server (56).

conjecture, we assessed *eal* expression in cells exposed to sub-inhibitory concentrations of tobramycin. The wild-type strain exhibited considerable resistance to tobramycin, and survival (not shown) and growth (see Fig. S4 in the supplemental material) were reduced significantly at concentrations higher than 5 µg/ml. After incubation for 7 days in the presence of 0.5 and 1 µg/ml tobramycin, *eal* expression was 5- and 10-fold higher, respectively, than in control cells grown without this antibiotic (Fig. 2A). Ex-

pression of the *rpfG* gene, encoding a protein also having cyclic di-GMP hydrolysis activity such as Eal apparently does, was induced by only 1.5-fold following exposure to 1 µg/ml tobramycin (Fig. 2A). The induction of *eal* expression by tobramycin suggested that elevated cyclic di-GMP hydrolysis is linked to tobramycin resistance and that an *eal* deletion mutant would exhibit hypersensitivity to this antibiotic. The number of viable cells in cultures of the *eal* mutant and the wild-type strain to which vari-

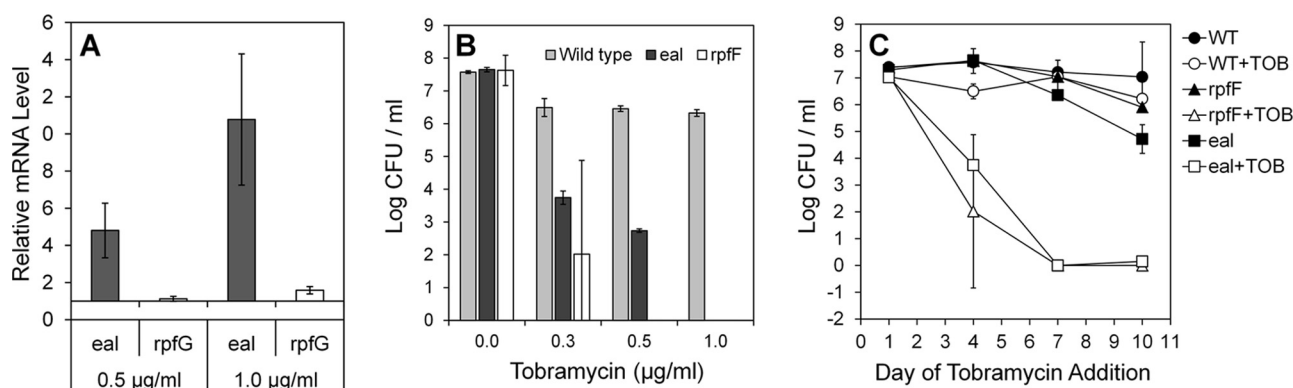


FIG 2 Eal is required for tobramycin resistance. (A) Relative expression of *eal* and *rpfG* in wild-type cells of *Xylella fastidiosa* grown for 7 days in the presence of 0.5 or 1 µg/ml tobramycin; expression values are relative to the expression of each gene in cells grown without tobramycin. (B) Loss of culturability of cells exposed to different concentrations of tobramycin added at day 4 of growth to cultures of the wild-type strain, an *eal* mutant, or an *rpfF* mutant. (C) Loss of culturability of cells exposed to 0.3 µg/ml added at day 1, 4, 7, or 10 of growth to cultures of the wild-type (WT) strain, an *eal* mutant, or an *rpfF* mutant. Viable cells were enumerated 24 h after the addition of the antibiotic. Vertical bars represent the standard errors of the means ($n = 5$ in panel A and $n = 3$ in panels B and C).

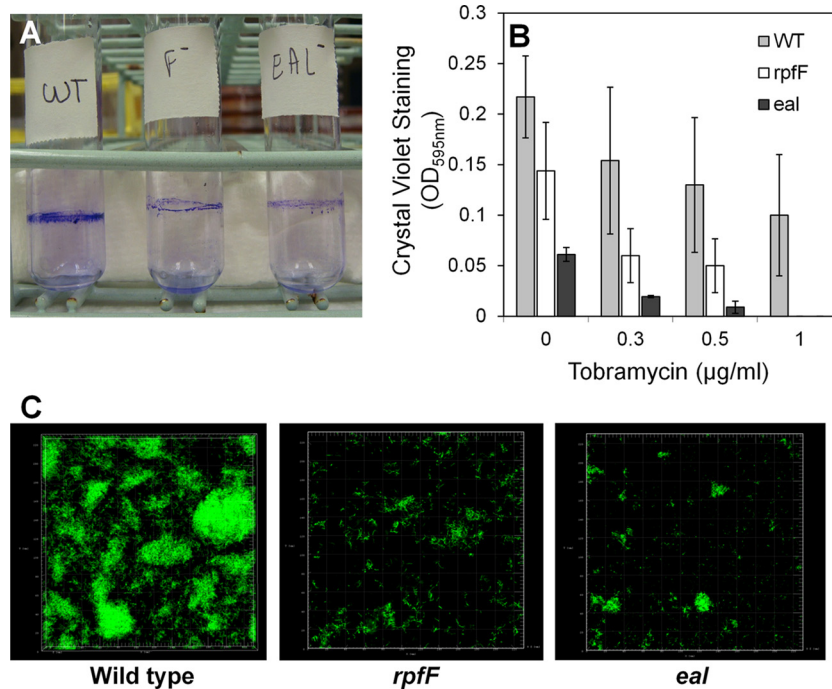


FIG 3 *eal* and *rpfF* contribute to biofilm formation. (A) Crystal violet staining of biofilm formed at the liquid-air interface of cultures of various strains of *Xylella fastidiosa* in glass culture tubes. (B) Quantification of crystal violet staining of cells attached to the bottom of wells of polystyrene microtiter plates after 10 days of growth. (C) Confocal fluorescence imaging of Syto 9-stained biofilms formed on glass coverslips placed in the bottom of 6-well microtiter plates in which various strains of *Xylella fastidiosa* were grown for 10 days. Vertical bars represent the standard deviations of the means ($n = 6$).

ous concentrations of tobramycin were added at various times was therefore assessed. The *eal* mutant was highly susceptible to concentrations of tobramycin that were only slightly inhibitory to the wild-type strain (Fig. 2B). For example, while the viable cell concentration in cultures of the mutant to which 0.3 µg/ml tobramycin was added after 4 days of growth was reduced 1,000-fold or more, such an addition had a modest effect on the culturability of the wild-type strain (Fig. 2B and C). Furthermore, the susceptibility increased with the age of the culture (Fig. 2C); no viable cells were recovered from cultures of the mutant in the presence of even as little as 0.3 µg/ml tobramycin when it was added after either 7 or 10 days of growth (Fig. 2C). This suggests that Eal, presumably through its cyclic di-GMP hydrolysis activity, is involved in the regulation of the cellular response to tobramycin stress. The increased antibiotic susceptibility of cells lacking Eal in older cultures suggests that another factor(s) is responsible for stress tolerance in young cultures and that Eal becomes essential only in later growth stages.

***eal* is involved in biofilm formation.** Tobramycin exposure induces biofilm formation in *P. aeruginosa* PAO1 whereas an *arr* mutant is attenuated in this process (19). We did not observe such an increase in biofilm formation in the wild-type strain of *X. fastidiosa* in response to any concentration of tobramycin (Fig. 3B; see also Fig. S2 in the supplemental material). In fact, a slight decrease in biofilm formation was observed. Similarly, it was reported (37) that the biofilm formed in the presence of the aminoglycoside antibiotic streptomycin by *X. fastidiosa* 9a5c did not differ from that formed by nontreated cells, suggesting that responses to antibiotic stress in *X. fastidiosa* do not necessarily depend upon traits linked with biofilm formation. It was clear, how-

ever, that both *eal* and *rpfF* deletion mutants formed less biofilm than the wild-type strain even in the absence of the antibiotic (Fig. 3A and B) as measured by three different methods: crystal violet staining of cells attached at the liquid-air interface in glass culture tubes (Fig. 3A), crystal violet staining of cells attached on the bottom of polystyrene microtiter plates (Fig. 3B), and examination of biofilms that had formed on glass coverslips immersed within cultures using confocal microscopy (Fig. 3C). Crystal violet staining revealed that the *eal* mutant exhibited only 25% of the attachment of the wild-type strain (Fig. 3A and B). *eal* mutants were mostly attached as single cells or as small aggregates, while the wild-type strain formed a thicker and more structured biofilm (Fig. 3C). In repeated experiments, it was also observed that the *eal* mutant was more impaired in biofilm formation than the *rpfF* mutant ($P < 0.05$), suggesting either that Eal and RpfF might both control the same traits in quantitatively different manners or that they control separate traits that each contribute to this process. These results clearly indicate that Eal is involved in the regulation of fundamental processes such as attachment to surfaces in *X. fastidiosa*. Since Eal can control both biofilm formation and antibiotic resistance whereas tobramycin-dependent elevation of its expression did not confer an increase in biofilm formation, we posit that it controls a tobramycin-dependent set of traits contributing to antibiotic resistance independently of traits contributing to biofilm formation.

***eal* and *rpfF* mutants exhibit similar *in vitro* and *in planta* phenotypes.** Given that expression of both Eal and RpfF might be expected to lead to decreases in cyclic di-GMP levels, the phenotypes of the *eal* and *rpfF* mutants were compared to determine the extent to which they overlapped. The *rpfF* mutant was more susceptible to tobramycin than the wild-type strain and even slightly

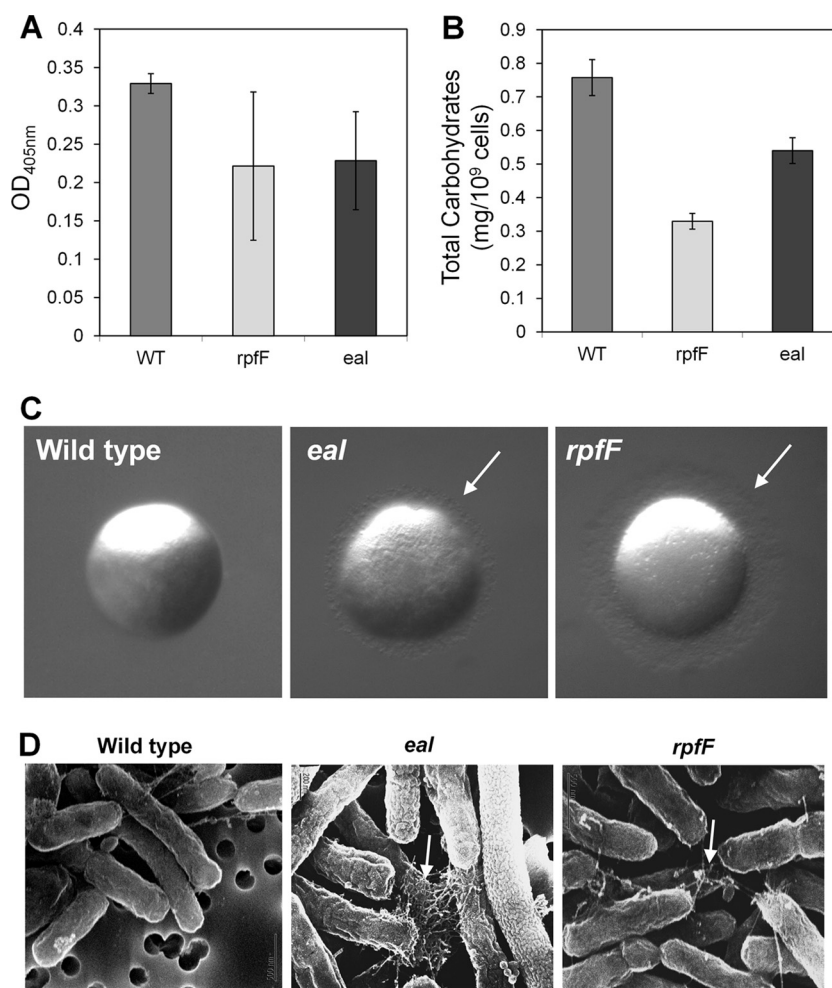


FIG 4 *eal* and *rpfF* mutants of *Xylella fastidiosa* exhibit similar phenotypes. (A) EPS production of different strains assessed with protein A double-antibody sandwich ELISA with anti-EPS antibodies. (B) Quantification of total carbohydrates with Anthrone reagent. (C) Colony morphology of colonies grown for 10 days. (D) Scanning electron microscopy of cells collected from the colonies shown in panel C. White arrows point toward pilus-like structures. Vertical bars represent the standard deviations of the means ($n = 3$).

more susceptible than the *eal* mutant (Fig. 2B). As with the *eal* mutant, its susceptibility increased with the age of the culture (Fig. 2C), indicating that accumulation of DSF in late growth stages regulates traits involved in tobramycin resistance that seem to be different from those regulated by Eal. Both mutants were also attenuated in EPS production as measured with either a protein A double-antibody sandwich ELISA using antibodies raised against EPS produced by the *Xanthomonas campestris gumI* mutant (31) or a colorimetric carbohydrate assay using Anthrone reagent. The amounts of EPS produced by the *rpfF* and *eal* mutants were significantly ($P < 0.01$) lower than that produced by the wild-type strain (Fig. 4A and B). Since EPS accumulation contributes to biofilm formation (39), this deficiency may partly account for the reduced levels of biofilm produced by these mutants. Both mutants were also hypermotile, having fringed colony margins not seen in the wild-type strain on a medium that tends to suppress motility (Fig. 4C; see also Fig. S2 in the supplemental material). The *eal* and *rpfF* mutants both also expressed more polar pili that might have been type IV pili that were observable with scanning electron microscopy than the wild-type strain (Fig. 4D). Type IV

pili have been associated with the ability of *X. fastidiosa* to move along xylem vessels by twitching (34, 40). Taken together, these observations suggest that RpfF and Eal independently negatively control the expression of traits associated with movement but induce traits involved in attachment, biofilm formation, and resistance to subinhibitory concentrations of tobramycin.

We further compared the abilities of the wild-type strain and the *eal* and *rpfF* mutants of *X. fastidiosa* strains to cause Pierce's disease in grapevine after mechanical inoculation into stems. The virulence of the strains was assessed by determining the rate of change of the incidence of symptomatic leaves in vines inoculated with a given strain with time after inoculation. The incidence of symptomatic leaves increased linearly with time in all treatments. Disease progression was significantly more rapid in plants inoculated with the *eal* mutant (1.36 leaves per plant/week) than in plants inoculated with the wild-type strain (0.69 leaves per plant/week) ($P < 0.0001$). Likewise, disease progression in plants inoculated with the *rpfF* mutant (1.43 leaves per plant/week) was higher than that in plants infected with the wild-type strain ($P < 0.0002$). The levels of virulence of *eal* and *rpfF* mutants did not

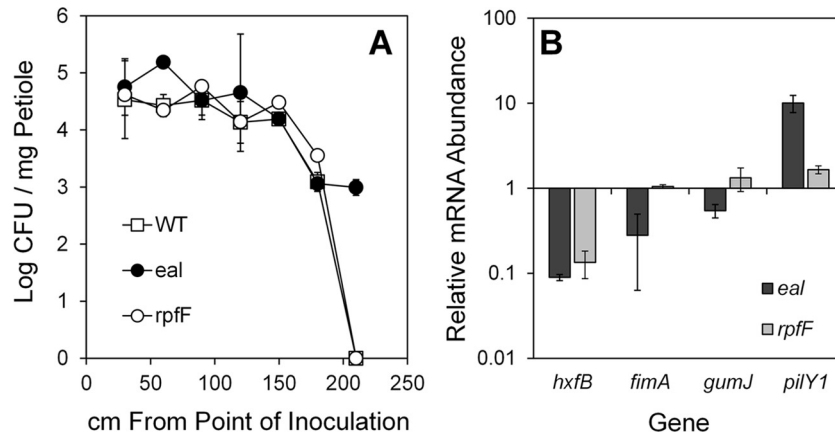


FIG 5 *eal* mutants of *Xylella fastidiosa* are hypermotile. (A) Population sizes of cells of various strains in petioles collected 11 weeks after inoculation at various distances from the point of inoculation. (B) Transcript abundance of various genes influencing biofilm formation, movement, and virulence determined by quantitative reverse transcriptase PCR in cells after 10 days of growth on XFMP plates. Vertical bars represent the standard deviations of the means ($n = 3$).

differ significantly ($P = 0.68$). Enumeration of bacterial population size in petioles at different distances from the point of inoculation revealed that the *eal* mutant moved further than the wild-type strain; by 15 weeks after inoculation, both the wild-type strain and the *rpfF* mutant were detected in all petioles at up to 180 cm away from the point of inoculation whereas the *eal* mutant was detected also in petioles at a distance of up to 210 cm (Fig. 5A). The population sizes of both mutants were either similar to or larger than that of the wild-type strain in all petioles at the distances at which all of the strains were present (Fig. 5A).

Eal controls the expression of virulence genes. The levels of expression of four key genes involved in movement, biofilm formation, and virulence were compared in the *X. fastidiosa* wild-type strain and the *eal* and *rpfF* mutants to determine if they could be associated with the higher virulence of the mutants. The expression of *hxfB*, encoding a hemagglutinin-like protein that is an antivirulence factor that reduces cell movement within plants (38), was 11- and 7-fold lower in the *eal* and *rpfF* mutants, respectively, than in the wild-type strain (Fig. 5B). The expression of *fimA*, an attachment factor encoding type I pili, a mutant of which was reported to move longer distances in the plant than the wild-type strain (33), was 3.5-fold lower in the *eal* mutant than in the wild-type strain, while its expression in the *rpfF* mutant was similar to that in the parental strain (Fig. 5B). Likewise, the expression of *gumJ*, involved in exopolysaccharide production, was unchanged in the *rpfF* mutant but 2-fold lower in the *eal* mutant than in the parental strain (Fig. 5B). Transcripts of *pilY1*, encoding the adhesion protein of the type IV pilus enabling twitching motility (40), were 10-fold more abundant in the *eal* mutant than in the wild-type strain. This gene was also more highly expressed in the *rpfF* mutant, although its expression level was less than that in the *eal* mutant (Fig. 5B). The lower expression of the adhesins HxfB and FimA and the higher expression of PilY1, all of which would aid active movement, is consistent with the hypermotile phenotype exhibited by both *eal* and *rpfF* mutants *in vitro* and *in planta*, despite the fact that the two mutants differ quantitatively in expression of these genes.

***eal* is negatively controlled by RpfF and DSF.** Our results suggest that *X. fastidiosa* employs two different cyclic di-GMP hydro-

lyzing pathways to control similar traits but in response to different cues. RpfF was reported to repress *cgsA* and thus to suppress cyclic di-GMP synthesis (16). We thus hypothesized that RpfF might also induce the expression of *eal*, thus increasing cyclic di-GMP degradation, to confer a large and consistent effect of cell density on the abundance of this second messenger. Surprisingly, the expression of *eal*, like that of *cgsA*, was higher in the *rpfF* mutant than in the wild-type strain when assessed in older cultures. The levels of expression of *cgsA* and *eal* were 5 ± 2.2 - and 8.6 ± 5.9 -fold higher in the *rpfF* mutant than in the wild-type strain, respectively, while the levels of expression of a control gene, *rpsO*, did not differ (0.99 ± 0.48). This suggested that DSF represses *eal* (Fig. 6A).

To test this model, the levels of expression of *eal* were compared in young cultures of the wild-type strain that had not accumulated DSF (1-day-old cultures) and older cultures in which DSF had accumulated (7 days). The abundance of *eal* transcript in older cells was about 10-fold lower than that in younger cells (ratio, 0.09 ± 0.19), supporting the conjecture that DSF-mediated signaling suppresses *eal* (Fig. 6B). In contrast, *rpfG* transcript abundance in the old cells was similar to that of the young cells (ratio, 1.07 ± 0.08). It is expected that *rpfG* is regulated by DSF at the protein level, and not at the transcriptional level, via phosphorylation by RpfC upon DSF sensing.

DISCUSSION

We demonstrate here that whereas *eal*, encoding a cyclic di-GMP PDE, plays a significant role in antibiotic resistance and biofilm formation in *X. fastidiosa*, it also controls motility and virulence, traits that are also controlled by the Rpf signaling system which regulates another cyclic di-GMP PDE, RpfG. A large number of different cyclic di-GMP-modifying enzymes containing GGDEF, EAL, and HD-GYP domains are found in many bacteria (6–8). The multiplicity of cyclic di-GMP-modulating proteins permits the integration of many signals, enabling several cellular processes to be regulated by separate pathways in parallel, but raises the issue of how they can exhibit specificity in outputs (41). It is intriguing that *X. fastidiosa* possesses only 5 such enzymes, many fewer than *P. aeruginosa* PAO1, which contains 38 (19), *E. coli* K-12, which

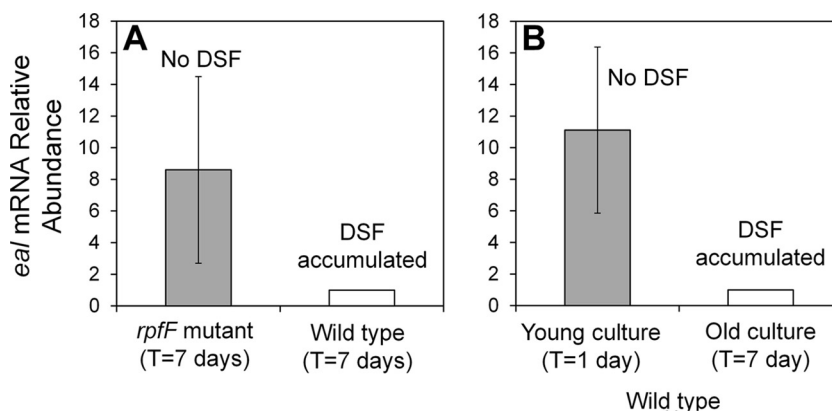


FIG 6 Regulation of *eal* by RpfF and DSF. (A) Relative expression of *eal* in the wild-type strain and the *rpfF* mutant of *X. fastidiosa* after 7 days of growth, during which DSF is expected to accumulate. (B) Relative expression of *eal* in wild-type cells after 1 day of growth (young culture), during which DSF had not yet accumulated, and after 7 days of growth (old culture), during which DSF is expected to have accumulated. The vertical bars represent the standard deviations of the means ($n = 7$ for panel A and $n = 5$ for panel B).

contains 29 (41), or even the close relative *X. campestris* pv. *campestris* 8004, which contains 37 (42). The small number of such players in *X. fastidiosa* should facilitate understanding of how such a redundant system is buffered against stochastic noise and prevents excessive cross talk in regulation of target traits. However, to date, we have not observed substantial differences in the traits controlled by the different cyclic di-GMP modules tested in *X. fastidiosa*; all of the phenotypes tested (biofilm formation, motility, and virulence) were generally consistent with the cyclic di-GMP levels predicted to occur in the *rpfF*, *cgsA*, and *eal* mutants; and the similar canonical EAL/GGDEF domains found in PD1671 and PD1994 make it difficult to discern distinct roles of these two enzymes.

In other bacteria, DGCs and PDEs that harbor a single domain and thus have similar effects on cyclic-di-GMP levels can either similarly regulate a given trait or, unexpectedly, differ in such regulation. Of 19 predicted DGCs in *P. fluorescens* Pf0-1, mutations in only four genes caused significant reductions in biofilm formation, indicating that, despite their common enzymatic function, they have different roles (43). Mutants with mutations in the PDE-encoding genes *rpfG* and XC2161 of *X. campestris* pv. *campestris* 8004, which have a single HD-GYP and EAL domain, respectively, were attenuated in biofilm formation, whereas a mutant with a mutation in XC0362, also having a single HD-GYP domain, formed more biofilm than the wild-type strain (42). Likewise, it was previously reported (44) that two different DGC proteins, when abolished, conferred equally reduced levels of cyclic di-GMP and similar levels of impairment in biofilm formation in *P. aeruginosa* PA14 but had distinct regulatory targets, one controlling EPS production and the other flagellar motility. These discrete outputs at the same level of cyclic di-GMP were suggested to be due to distinct subcellular localizations of these proteins. Consistent with this model, other cyclic di-GMP-modulating proteins have been shown to be localized to the cell poles upon activation by phosphorylation (45). In fact, RpfG has been suggested to be spatially localized in *X. campestris* pv. *campestris* 8004 (46). In addition, enzymes such as HD-GYP proteins that are capable of modulating cyclic di-GMP levels regulate certain traits via direct interactions with other proteins (47). There are thus many possible reasons why *rpfF* and *eal* mutants of *X. fastidiosa* might have

been expected to differ somewhat in their phenotypes. Indeed, while these mutants had similar defects in biofilm formation and antibiotic resistance and both were hypermotile and hypervirulent to plants, they differed quantitatively in many phenotypes and in the expression of certain target genes. For example, while the *eal* mutant was more impaired in biofilm formation than the *rpfF* mutant (Fig. 3), the *rpfF* mutant was more susceptible to tobramycin (Fig. 2); the decrease in expression of *hxfB*, *fimA*, and *gumJ*, which contribute to biofilm formation, was greater in the *eal* mutant (Fig. 5B), consistent with its lower biofilm formation ability. These results suggest that gene expression in the *eal* mutant was somewhat different from that of *rpfF*. Therefore, it is possible that *eal* and the Rpf/DSF system could differentially contribute to biofilm formation and tobramycin resistance by regulating different sets of genes or by differentially regulating a common set of genes. RpfG and Eal might differ in enzymatic efficiencies or be present at different levels or in different locations in the cell, thus affecting discrete cyclic di-GMP pools, or might confer different total cyclic di-GMP levels in the cell, thereby expressing subsets of common or distinct target genes.

There are apparently two main behavioral traits that are controlled by cyclic di-GMP in *X. fastidiosa*: traits contributing to the plant-exploratory phase which are induced by high levels of cyclic di-GMP and the converse phenotypes that enable insect acquisition which are induced by low levels of cyclic di-GMP (Fig. 7A). *X. fastidiosa* harbors at least four genes encoding proteins predicted to be directly regulated by cyclic di-GMP levels (cyclic di-GMP effector proteins): *clp*, PD1311, PD1497, and PD0726. Those proteins mediate the regulation of the opposing phenotypes, and it is expected that at least one would also control the response to antibiotic stress. We can eliminate *Clp* from that list, since a *clp* mutant assayed for tobramycin susceptibility did not show increased susceptibility (data not shown) such as the *eal* and *rpfF* mutants did. Tobramycin would not have been expected to induce biofilm formation if Eal controls biofilm formation and antibiotic resistance via two different cyclic di-GMP effector proteins. An increase in expression of *eal* upon tobramycin exposure might also increase the expression of a particular cyclic di-GMP effector protein, establishing an alternative signaling cascade that favors traits for antibiotic resistance rather than biofilm formation. Such a

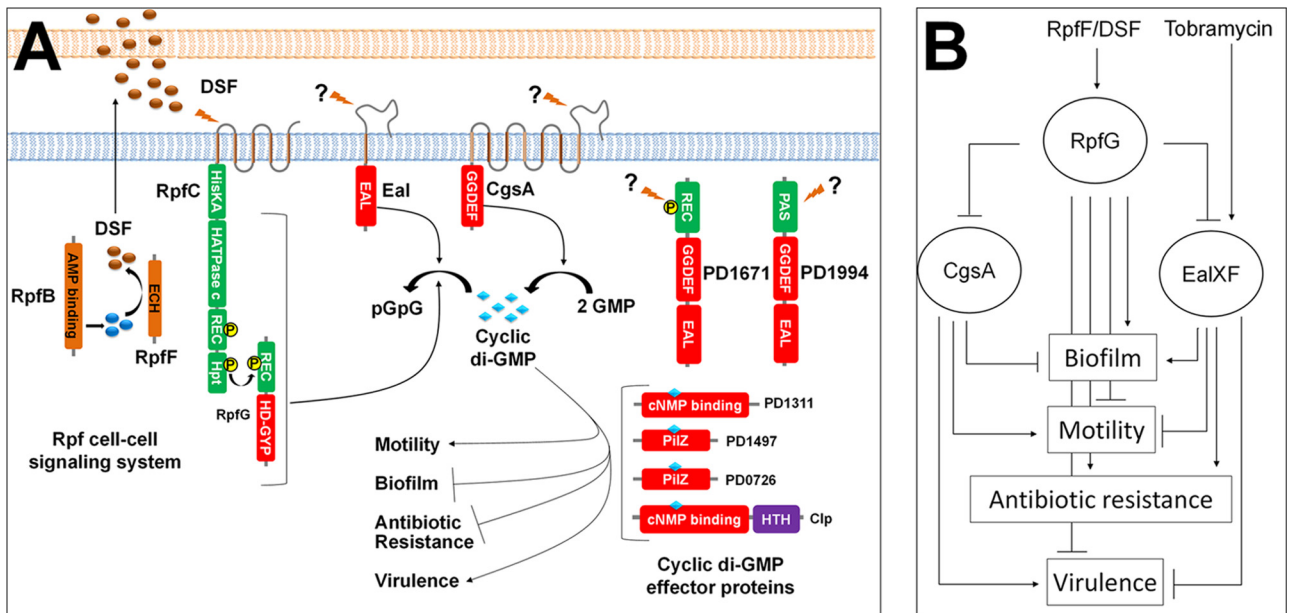


FIG 7 (A) A mechanistic model of the cyclic di-GMP regulatory network in *Xylella fastidiosa*. *X. fastidiosa* possesses 5 proteins (RpfG, CgsA, Eal, PD1671, and PD1994) implicated in cyclic di-GMP metabolism and 4 proteins (Cip, PD1311, PD1497, and PD0726) predicted to be cyclic di-GMP effectors. RpfB produces a DSF precursor (57) that is modified by RpfF to produce DSF. DSF accumulates outside the cell and, upon reaching a threshold level, interacts with RpfC, which in turn phosphorylates RpfG. High levels of cyclic di-GMP induce motility and virulence, and low levels induce biofilm formation and antibiotic resistance. (B) Hierarchical regulatory model for DSF and tobramycin signaling: tobramycin induces *eal* expression and Eal and CgsA expression is suppressed by RpfF, presumably through RpfG. Both the Eal and the Rpf systems contribute to cyclic di-GMP reduction and thus to an increase in antibiotic resistance and biofilm formation. However, their discrete contributions are expected to overlap only partially.

scenario can explain the reduction in biofilm formation seen in wild-type cells subjected to tobramycin exposure, if the signaling cascade favoring biofilm formation would then be weakened.

Although tobramycin did not induce biofilm formation and since both mutants with mutations in *eal* and *rpff*, like a *P. aeruginosa arr* mutant (19), were attenuated in biofilm formation, it is likely that their susceptibility to subinhibitory concentrations of tobramycin is at least partially due to their impairment in biofilm formation. It is well established that cells in biofilms are more resistant to antibiotics than planktonic cells (48, 49), but the basis for this resistance is not fully understood. In fact, an *ndvB* (encoding a glucosyltransferase involved in the formation of cyclic glucans) mutant of *P. aeruginosa* that formed biofilms with a normal architecture was still sensitive to tobramycin (50), indicating that physical features of biofilms do not confer resistance but rather that the physiological state of cells in biofilms (having altered patterns of gene expression) leads to resistance. Furthermore, planktonic aggregates of *P. aeruginosa*, but not planktonic solitary cells, exhibited an enhanced capacity to survive otherwise lethal antibiotic exposure (51), further indicating that surface attachment is not essential for resistance. We presume that one or more of the many genes regulated by DSF signaling (52) contribute to antibiotic resistance in *X. fastidiosa*. Likewise, Eal presumably also affects the expression of a large number of genes, some of which are involved in antibiotic resistance, and some (but not all) are also regulated by the Rpf/DSF system. Several traits such as multidrug transporters (53) or factors that prevent the antibiotics from reaching their site of action such as sequestration of them in the periplasm with glucose polymers (50) that are distinctly controlled by Eal and/or RpfF, and are expressed in a biofilm, may contribute to resistance.

It might be expected, given the apparent role of the Rpf/DSF signaling system in resistance to antibiotics and the fact that DSF signaling is more prominent in denser cultures, that the *rpff* mutant would be more susceptible than the wild-type strain to antibiotics as the cells age. However, it was surprising that the *eal* mutant also exhibited such a pattern. The age-dependent susceptibility of both mutants indicates that both Eal and RpfF are functional in batch cultures with a high cell density in which DSF has already accumulated. Since each could not compensate for the loss of the other, each is probably regulating different traits. The considerable resistance seen in young cultures (1 day of growth) is likely due to factors that are expressed in early stages of growth. It is noteworthy that the *rpff* mutant was more susceptible to antibiotics at all ages than the *eal* mutant. While Eal clearly controls traits that contribute to antibiotic resistance, additional traits that are controlled by RpfF but not Eal might contribute to the higher resistance of the *eal* mutant.

The attachment to surfaces and thus biofilm formation is a major life strategy required for dissemination of *X. fastidiosa* by insects (54, 55). Once inside xylem vessels of host plants, *X. fastidiosa* proliferates and spreads, apparently mostly as planktonic cells, although some can attach to the plant. *X. fastidiosa* reaches high cell densities in only a small proportion of vessels where it forms a biofilm along the xylem wall (reviewed in reference 3). Such cells are presumably more readily acquired by sharpshooter vectors due to their higher adhesiveness, and they form a biofilm on the cuticular lining of the insect foregut (5). Induction of biofilms and of cell-cell aggregation is thus a common behavior of bacteria unrelated to antibiotic exposure, although the changes in gene expression associated with biofilm formation often lead to higher antibiotic tolerance, as discussed above. Many of the cells

that experience toxic compounds *in planta* are thus likely to be in a biofilm or an aggregated form that is more tolerant of the toxic compounds to which they may be exposed.

Given that DSF signaling suppresses Eal expression, the innate antibiotic resistance seen in wild-type cells might be due predominantly to those traits that are under RpfF but not Eal control. As cells become more abundant with age, DSF is expected to accumulate and reduce *eal* expression, thus enabling RpfG to function in a cellular environment without the influence of cyclic di-GMP degradation that would occur because of external events such as antibiotic-induced expression of Eal. Nevertheless, when cells experience tobramycin, *eal* expression seems to be maintained at a high level despite the presence of DSF, since both DSF-regulated traits and Eal-regulated traits are required for tobramycin resistance to be achieved. DSF-mediated suppression of Eal may occur to prevent excessive depletion of cyclic di-GMP and to avoid unnecessary expression of Eal in the absence of antibiotics. It might be expected that antimicrobial factors from which *X. fastidiosa* might need protection occur mostly in plants that are heavily colonized by this pathogen and thus are most symptomatic. This would explain why the RpfF system influences tolerance of antibiotics (and other toxic compounds) in addition to the more specific contextual regulator Eal. A common process that would link to both antibiotic resistance and biofilm formation is the modulation of the level of cyclic di-GMP. Cyclic di-GMP levels might be different in subcellular compartments, depending on the total cellular DGC and PDE activities which, in turn, depend on the levels and specific activities of each of the GGDEF, EAL, and HD-GYP domain proteins. These parameters are apparently dynamic and interconnected. RpfF temporally regulates at least 3 of the 5 cyclic di-GMP control modules: RpfG at the protein activity level (9) and both CgsA (16) and Eal at the transcriptional level (Fig. 6A and 7). Our results strongly suggest that the cell density-dependent Rpf cell-cell communication system of *X. fastidiosa* is dominant over the more contextual CgsA and Eal signaling systems and that intracellular signaling mediated by cyclic di-GMP is coordinated in a way to maximize pathogen fitness. Such complex contextual coordination of motility and biofilm formation is needed to enable the sequential colonization of host plants and insect vectors while at the same time allowing appropriate responses to toxic compounds and other stresses to which the cell might be exposed during any life stage.

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