The Putative Enoyl-CoA Hydratase DspI is Required for Production of the Pseudomonas aeruginosa Biofilm Dispersion Autoinducer, cis-2-Decenoic Acid

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ABSTRACT

In the present study we report the identification of a putative enoyl-CoA hydratase/isomerase that is required for synthesis of the biofilm dispersion autoinducer \textit{cis}-2-decenoic acid in the human pathogen \textit{Pseudomonas aeruginosa}. The protein is encoded by PA14_54640 (PA0745), named \textit{dspI} for dispersion inducer. The gene sequence for this protein shows significant homology to RpfF in \textit{Xanthomonas campestris}. Inactivation of \textit{dspI} was shown to abolish biofilm dispersion autoinduction in continuous cultures of \textit{P. aeruginosa}, and resulted in biofilms that were significantly greater in thickness and biomass compared to the parental wild type strain. Dispersion was shown to be inducible in \textit{dspI} mutants by the exogenous addition of synthetic \textit{cis}-2-decenoic acid or by complementation of \textit{ΔdspI} in trans under the control of an arabinose inducible promoter. Mutation of \textit{dspI} was also shown to abolish \textit{cis}-2-decenoic acid production, as revealed by GC-MS analysis of cell-free spent culture medium. Transcript abundance of \textit{dspI} correlated with cell density as determined by quantitative RT-PCR. This regulation is consistent with the characterization of \textit{cis}-2-decenoic acid as a cell-to-cell communication molecule that regulates biofilm dispersion in a cell density-dependent manner.
INTRODUCTION

Biofilm dispersion is the terminal stage of the biofilm developmental cycle, where bacteria regulate their escape from a biofilm and transition to a mobile planktonic lifestyle (1, 2). Induction of biofilm dispersion in *Pseudomonas aeruginosa* occurs naturally when biofilm microcolonies attain a critical size, releasing bacteria as free cells into the surrounding environment (2, 3). Recently, we have reported that the small messenger fatty acid molecule, *cis*-2-decenoic acid (*cis*-DA), produced by *P. aeruginosa* in batch and continuous cultures, acts as the autoinducer of biofilm dispersion for *P. aeruginosa* (3). This molecule has also been shown to induce biofilm dispersion in a range of Gram-negative and Gram-positive bacteria and in the fungal pathogen *Candida albicans* (3).

The autoinducer *cis*-DA is a fatty acid cell-to-cell communication molecule with structural homology to *cis*-11-methyl-2-dodecenoic acid (DSF), isolated from *Xanthomonas campestris* (4, 5). Analogs of DSF have been identified in *Burkholderia cenocepacia*, (*cis*-2-dodecenoic acid or BDSF), *Streptococcus mutans* (*trans*-2-decenoic acid or SDSF), and *Xylella fastidiosa* (*trans*-2-tetradecenoic acid or *Xy*DSF) (6-8). Additional, structurally related, fatty acid signals have been identified in the genera *Burkholderia*, *Xanthomonas* and *Stenotrophomonas* (6, 8-13). Fatty acid signals have been shown to regulate a wide range of bacterial behaviors including virulence, motility, biofilm development and dispersion (4, 8-10, 12, 14-23).

The mechanism of fatty acid signal biosynthesis appears to be widely conserved. DSF biosynthesis in *X. campestris* is dependent on the gene *rpfF*, which encodes a putative enoyl-CoA hydratase (4, 24). The role of Rpf homologues in fatty acid signal biosynthesis has subsequently been established in *B. cenocepacia* (6), *Xanthomonas oryzae* pv. oryzae (10), *X.
fastidiosa (18), Stenotrophomonas maltophilia (13), and Xanthomonas axonopodis pv. glycines (20).

In the present work, we report that the gene PA14_54640 (PA0745) named dspI (dispersion inducer), is required for production of the dispersion-inducing cell-to-cell signal cis-DA, synthesized by P. aeruginosa. The gene dspI encodes a putative enoyl-CoA hydratase/isomerase, responsible for catalyzing the formation of α,β-unsaturated fatty acids. We further demonstrate that expression of dspI is correlated with cell density during planktonic and biofilm growth.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and culture conditions. All bacterial strains and plasmids used in this study are listed in Table 1. Pseudomonas aeruginosa strain PA14 was used as a parental strain for all work in the present study. Planktonic cultures were grown aerobically at 22°C in modified EPRI medium containing 0.005% ammonium nitrate, 0.00019% KH₂PO₄, 0.00063% K₂HPO₄ (pH 7.0), and 0.001% Hutner salts (25), supplemented with 0.2% glucose, or in Luria-Bertani (LB) broth (BD, Sparks, MD) in flasks with shaking at 220 rpm. Continuous culture biofilms were grown at 22°C in modified EPRI medium or 5% LB broth in tube reactors. Semi-batch culture biofilms were grown in 20% LB broth in 24-well culture plates. Gene complementation experiments were performed in modified EPRI medium or 5% LB broth with or without 0.1% arabinose. Antibiotics were used at the following concentrations: 75 μg/mL gentamicin (Gm), 250 μg/mL carbenicillin (Cb), and 50 μg/mL tetracycline (Tet) for P. aeruginosa; 50 μg/mL ampicillin (Amp), 25 μg/mL kanamycin (Km) and 20 μg/mL Tet for E. coli.
coli. Cb at a concentration of 10 μg/mL was used for maintenance of the pMJT1 plasmid in P. aeruginosa continuous culture biofilm reactors.

Strain construction. Complementation of Δdspl (26) was accomplished by placing the gene under the control of an arabinose-inducible P_{BAD} promoter in the pMJT1 vector (27). Briefly, the open reading frame of dspl was amplified by PCR using primers listed in Table S1, and cloned into pMJT1 at restriction sites indicated in Table S1. Plasmids were mobilized into P. aeruginosa from E. coli via electroporation and transformants were selected by growth on LB with 250 μg/mL Cb. Strains were confirmed to contain vector constructs following amplification by PCR using MCS primers for pMJT1 listed in Table S1.

Reporter strain construction. A transcriptional reporter for dspl was constructed by placing the promoter region of dspl upstream of the lacZ gene in the mini-CTX-lacZ vector (28). We found that dspl was co-transcribed with the upstream genes, PA14_54620 and PA14_54630 (Fig. S1). A 500 bp region of DNA upstream of the gene PA14_54620, was selected as including the putative promoter region of dspl based on the observation that most promoters are between 100 and 200 base pairs long, and recognizing that multiple promoters are possible in P. aeruginosa such as is the case for algD (29, 30). This sequence was amplified by PCR using primers listed in Table S1, cloned into the mini-CTX-lacZ vector at restriction sites indicated in Table S1, and introduced into P. aeruginosa via tri-parental mating (31). Transformants were selected by growth on Vogel-Bonner minimal medium (VBMM) containing 0.3% citrate as the sole carbon source (32) supplemented with Tet. Chromosomal vector integration was confirmed via PCR amplification using primers for the attB integration site listed in Table S1.
Dispersion phenotype screen. Biofilms were grown in semi-batch on the submerged surfaces of twenty-four-well cell culture plates inoculated with 250 µl/well overnight *P. aeruginosa* culture diluted 1:100 in 20% LB growth medium and incubated at 37°C with shaking at 220 rpm for 24 hr. Plates were incubated at a 45° angle to allow biofilm development within each well. Medium in the wells was replaced every 24 hr for 6 days to promote biofilm growth and remove planktonic cells. Images of biofilm microcolonies were viewed by transmitted light using an Olympus BX60 microscope and a 20X and 50X magnification UPlanF Olympus objective. Images used to evaluate biofilm dispersion in wild type and mutant bacterial strains were recorded using a ProgRes® CF camera (Jenoptik, Jena, Thuringia) and processed via ProgRes® CapturePro 2.7.7 software.

Microscopic analysis. A continuous culture once-through flow cell (BioSurface Technologies, Bozeman, MT) was configured to observe biofilm growth, architecture and development on a glass substratum as described previously (3). Biofilms grown in flow cells were observed by transmitted light as described above. Biofilms were also analyzed by confocal scanning laser microscopy (CSLM) using a Leica TCS SP5 confocal microscope and SYTO 40 nucleic acid stain (Invitrogen Corp.). The CLSM images were processed using the LAS AF software v. 2.4.1 and quantitative analysis was performed by COMSTAT using MATLAB software to determine biofilm biomass, average thickness, and total thickness (33). All microscopy experiments were performed in triplicate.
Biofilm dispersion assays. *P. aeruginosa* biofilm cultures were grown in continuous flow tube reactors as described previously (2, 3, 34). Briefly, the interior surfaces of silicone tubing (81.5 cm length by 14 mm inner diameter) (Masterflex; Cole Parmer, Inc.) of a continuous once-through reactor system were used to culture biofilms. Tubing was connected to an influent medium reservoir and effluent waste reservoir. Medium was pumped through the closed and sterilized reactor system using an eight head peristaltic pump (Cole Parmer, Inc.) at a flow rate of 0.2 mL/min. Silicone tubes were inoculated with 6 mL of log-phase cultures of *P. aeruginosa* by syringe through a rubber septum immediately upstream from each reactor tube. Bacterial cells were allowed to attach to the surface of the tubing for 1 hr under static conditions prior to initiation of medium flow. Biofilms were grown at 22°C for a period of 6 days.

Biofilm dispersion assays were performed on 6-day biofilm cultures by addition of cis-DA or sterile medium under static flow conditions. Synthetic cis-DA (310 nM) or sterile media was added to test or control tubes, respectively, via a rubber septum upstream of the biofilm reactor. Following 2 hr incubation, both the liquid fraction, containing released bacterial cells, and the remaining biofilm from each tube were collected separately on ice. CFU were determined by serial dilution and plating. Dispersion efficacy was calculated as follows:

\[
\text{dispersion efficacy} = \frac{(\text{CFU in liquid fraction} \times 100)}{(\text{CFU in liquid fraction} + \text{CFU in biofilm fraction})}.
\]

Preparation of spent medium. Preparation of cell-free spent culture medium was performed as described previously (3) with the following modifications. Batch cultures of *P. aeruginosa* wild type PA14 or dspI mutant were grown in 4 L of modified EPRI medium for 10 days at 22°C with stirring at 220 rpm. Batch cultures of *P. aeruginosa* ΔdspI/pMJT-dspI or ΔdspI/pMJT were
grown in 4 L of arabinose-supplemented LB medium for 10 days at 22°C with stirring at 220 rpm. Cell free spent culture medium was prepared by centrifugation (16,000 x g, 20 min, 4°C) followed by pre-filtration using a 0.45 μm nitrile filter (Millipore, Billerica, MA), and secondary filtration using a 0.2 μm syringe filter (Millipore). Cell-free spent culture medium was stored at 4°C.

**Preparation of chloroform-extracted spent medium (CSM).** Chloroform-extracted spent medium (CSM) samples were prepared as described previously (3) with the following modifications: The organic compounds contained within 3 L of spent medium were extracted in 0.96 L of chloroform. Chloroform was evaporated using a Rotavapor® R-3000 rotovap (Buchi, Switzerland) and the remaining organic material resuspended in 2 mL of 18 mΩ water. CSM contained a final concentration of cis-DA 250-fold greater than in cell-free spent culture medium.

**GC-MS.** Preparation of CSM and synthetic cis-DA (Carbosynth Limited, Compton-Berkshire, United Kingdom) samples for GC-MS and MS/MS analyses was carried out as previously described (3). Spectra were obtained with a Shimadzu (Columbia, MD) QP5050A gas chromatograph-mass spectrometry (GC-MS) system and analysis performed using the Lab Solutions program GC-MS solution (version 1.2).

**Batch Culture Growth Curve.** To determine cell density throughout planktonic growth, a growth curve of *P. aeruginosa* PA14 was performed on cultures grown in shake flasks at 23°C. Overnight cultures were grown in LB, OD 600 adjusted, and diluted 1:100 into fresh LB.
Absorbance readings (OD 600) were taken at 12 time points throughout 32 hr of growth. Experiments were carried out in triplicate from independent overnight cultures.

**qRT-PCR.** Quantitative reverse transcriptase PCR (qRT-PCR) was used to determine expression levels of *dspI* using 1 ug of total RNA isolated from wild type *P. aeruginosa* cells grown as planktonic cultures (6.5 hr, 10 hr, 12.5 hr, 15 hr, and 24 hr) and as biofilm cultures (1-, 3-, and 5-day cultures). qRT-PCR was also used to determine the effect of the exogenous addition of 310 nM *cis*-DA on *dspI* transcript abundance in 12.5 hr-old PA14 planktonic cultures. Isolation of mRNA and subsequent cDNA synthesis were performed as described previously (35-38). Transcript amplification by qRT-PCR was performed according to manufacturer’s specifications using an Eppendorf Mastercycler ep realplex instrument (Eppendorf AG, Hamburg, Germany) and a KAPA SYBR FAST qPCR Kit (KAPA Biosystems, Woburn, MA), using oligonucleotide primers listed in Table S1. The gene *mreB* was used as a housekeeper control. Relative transcript abundances were determined using the ep realplex software (Eppendorf AG). Transcript quantification was normalized (based on the threshold cycle [*C*<sub>T</sub>] value) to *mreB* transcripts, followed by determination of transcript abundance ratios. Fold change in *dspI* abundance for planktonic and biofilm cells was determined relative to *dpsI* abundance of early-exponential planktonic samples. Melting curve analyses were performed to ensure specific single-product amplification.

**dspI transcription assays.** β-Galactosidase specific activity of strains harboring the *dspI* reporter construct was determined using the Miller assay (39) modified to determine specific β-galactosidase activity and normalized to cell protein extracts, as described previously (38, 40).
An extinction coefficient for o-nitrophenyl-β-galactoside (ONPG) cleavage at 420 nm of 4,500 nM/nmol/cm was used. Specific activity values were calculated following subtraction of background levels of β-galactosidase activity in a promoter-less lacZ control strain. β-Galactosidase activity was also assessed by fluorescent microscopy (2). Microscopic analysis of dspI expression during planktonic and biofilm growth was performed for cultures grown in medium containing 0.02 g/L methylumbelliferyl β-D-galactopyranoside (MUG) dissolved in N,N-di-methylformamide. β-Galactosidase activity was assessed via microscopy by examination under long-wave UV excitation (2, 41). Samples were analyzed using an exposure time of 1500 ms, with UV illumination only during image collection. β-Galactosidase activity was determined for both planktonic (early-exponential, mid-exponential, late-exponential, early- and late-stationary) and biofilm (1-, 3-, and 5-day) cells grown in batch culture or continuous culture flow cells, respectively.

Statistical analysis. A Student’s t-test was performed for pairwise comparisons of groups and multivariant analyses were performed using a one-way analysis of variance (ANOVA).

RESULTS

Identification of P. aeruginosa fatty acid synthase gene required for native biofilm dispersion. We have previously described that the cell-to-cell communication molecule cis-DA was capable of inducing a biofilm dispersion response in P. aeruginosa (3). To identify a key enzyme required for the production of cis-DA, we focused on novel enoyl-CoA hydratase proteins with potential to be required for native biofilm dispersion. A query of the P. aeruginosa database (www.pseudomonas.com) revealed 15 putative enoyl-CoA hydratases encoded within...
the genome. Knockout mutations were found to be non-lethal in 8 of these genes (PA14_19740/PA3426, PA14_26690/PA2890, PA14_28310/PA2767, PA14_40640/PA1846, PA14_40980/PA1821, PA14_43440/PA1629, PA14_51110/PA1021, and PA14_54640/PA0745). Transposon mutants of these genes were selected for analysis by dispersion phenotype screen (described in the methods) to determine whether they naturally formed central voids within microcolonies following 6 days of biofilm growth. Central void formation in biofilm microcolonies is a characteristic consequence of natural dispersion, inducible with endogenously produced cis-DA. These voids result from the release of bacteria from the interior of mature biofilm microcolonies (3, 42). All of the mutants tested, with the exception of PA14_54640/PA0745 (dspI), formed a central void in the majority of microcolonies observed by microscopic analysis (Fig. 1A). Void formation was observed in only 5% of microcolonies of dspI mutant biofilms compared to 63% of wild type biofilm microcolonies (Fig. 1B). The presence of void spaces in dspI mutant biofilms may have resulted from P. aeruginosa dispersion in response to factors other than cis-DA. Thus, complete loss of the dispersion phenotype may not be possible with a single mutation. Interestingly, of all the enoyl-CoA hydratase proteins investigated, DspI had the highest homology (5.0E-14) to RpfF, the synthase for DSF in X. campestris (4).

**dspI is required for native biofilm dispersion and is restored by complementation of dspI in trans.** To further investigate the native dispersion phenotype of dspI mutant biofilms, the architecture of 6-day biofilms was analyzed using a microscope mounted continuous culture flow cell reactor system. Previous work in our laboratories has shown the dispersion stage of *P. aeruginosa* biofilm development to occur at day 6, under the continuous culture conditions used.
in this work. We hypothesized that loss of native dispersion by mutation of \textit{dspI}, would result in increased biomass and microcolony size of 6-day biofilms, compared to the wild type. Both the wild type and \textit{dspI} mutant formed biofilm microcolonies with distinct three-dimensional architecture, however the \textit{dspI} mutant biofilm displayed microcolonies with greater thickness and diameter in both modified EPRI and 5% LB media (Fig. 2 A,B). Quantitative analysis of biofilm architecture using COMSTAT confirmed these observations, indicating that the \textit{dspI} mutant 6-day biofilms had greater average and maximum thickness as well as greater total biomass compared to the wild type strain (Table 2).

We investigated whether activation of a plasmid-born \textit{dspI} gene in \textit{ΔdspI} biofilms would result in restoration of the dispersion phenotype. Complementation of \textit{dspI} for the duration of biofilm development resulted in biofilm architecture not observably different from the wild type, and restored void formation associated with the natural dispersion phenotype (Fig. 2 A,B; Table 2). To determine the effect of \textit{dspI} induction in mature \textit{ΔdspI} biofilms, \textit{dspI} gene expression was induced between days 5 and 6 in the complemented mutant strain. Quantitative analysis of biofilm architecture using COMSTAT was performed for 5-day (pre-induction) biofilms and again for 6-day (post-induction) biofilms for the wild type, \textit{dspI} mutant, and \textit{dspI}-inducible complement, grown in flow cell reactors. At 5 days, both the \textit{dspI} mutant and \textit{dspI} inducible complement formed biofilms with greater average and maximum thickness as well as greater total biomass compared to the wild type. However, at 6 days, induction of \textit{dspI} resulted in biofilms with average and maximum thickness and total biomass more similar to wild type biofilms than biofilms of the uninduced \textit{dspI} mutant (Table 2).
Mutation of *dspI* does not impair growth. We tested whether mutation of *dspI* would have an impact on cellular growth rates to ensure that there was no difference in growth between wild type and mutant strains in this study. Growth curves of both wild type and *dspI* mutant were found to be superimposable, indicating no difference in growth kinetics between the two strains (Fig. S2).

Exogenous addition of *cis*-2-decenoic acid restores dispersion in *dspI* mutant biofilms. To investigate whether *dspI* mutants (deficient in native dispersion) disperse in response to exogenous addition of synthetic *cis*-DA, 6-day biofilms grown in continuous culture tube reactors were exposed to medium containing synthetic *cis*-DA (310 nM) or carrier control for a period of 2 hr. Exogenous addition of *cis*-DA to *dspI* mutant cultures resulted in the release of 51% of the total biofilm population into the bulk liquid. This number is comparable to the release of 47% of biofilm cells by the wild type strain and 55% by the complemented *dspI* mutant strain. Carrier controls showed a 4%, 3% and 3% cell release, respectively (Fig. 3A). When viewed by CLSM, biofilms of the *dspI* mutant were observed to become significantly reduced in biomass following treatment for 1 hr with synthetic *cis*-DA. Results from a representative experiment are illustrated in Fig. 3B.

dspl is essential for production of *cis*-2-decenoic acid. The *dspI* gene encodes a 272 amino acid peptide harboring a crotonase/enoyl-CoA hydratase-like conserved domain (www.ncbi.nlm.nih.gov), similar to other enoyl-CoA hydratase/isomerase proteins involved in fatty acid metabolism (Fig. 4A). Enoyl-CoA hydratase/isomerase proteins are known to catalyze the dehydration reaction in short-chain fatty acids resulting in the formation of a double bond at
the 2,3 carbon (Fig. 4B). These proteins are also known to catalyze the cis/trans isomerization of double bonds. To investigate whether *dspl* mutants produce the cis-DA signal, samples of cell-free chloroform-extracted spent medium (CSM) were chemically analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). A single major peak with a retention time of 7.0 min was detected for CSM of the wild type and complemented *dspl* mutant, identical to that of synthetic cis-DA compound, but was absent in the *dspl* mutant (Fig. 4C). The presence of cis-DA in the samples was confirmed by MS/MS; the fragmentation patterns for all three peaks were shown to be identical at the 95% confidence interval (Fig. 4D). These findings indicated that *dspl* is required for production of cis-DA in *P. aeruginosa*, and that DspI is most likely the terminal enzyme in the synthesis pathway, responsible for double bond formation and cis/trans isomerization.

**Transcription of *dspl*** **is correlated to cell density during planktonic and biofilm growth.** We sought to characterize the transcriptional regulation of *dspl* throughout planktonic and biofilm growth. During planktonic growth, cellular *dspl* transcript abundance was observed to increase throughout the 24 hr period of batch growth (Fig. 5A). A 7-fold increase was detected between 10 hr and 12.5 hr, followed by a 4-fold increase between 12.5 hr and 15 hr. Finally, a 14-fold increase between 15 hr and 24 hr was observed (Fig. 5B). Similarly, under biofilm conditions, *dspl* transcript levels increased from 1 to 5 days, with a 5-fold increase between day 1 and day 3, and a 1-fold increase between day 3 and day 5. These findings suggested that expression of *dspl*, under both planktonic and biofilm growth conditions, is correlated to cell density.
A transcriptional reporter for \textit{dspI}. A chromosomal transcriptional \textit{lacZ}-fusion for the \textit{dspI} promoter was used to determine whether \textit{dspI} promoter activity supported the finding of increasing \textit{dspI} transcript levels with increasing cell density. \(\beta\)-Galactosidase activity of the \textit{dspI} reporter construct was determined for planktonic cells at 6.5 hr, 10 hr, 12.5 hr, 15 hr, and 24 hr, and for biofilm cells at 1 day, 3 days, and 5 days. \(\beta\)-Galactosidase activity was also monitored by bright-field and fluorescent microscopy to determine when and where in the biofilm \textit{dspI} promoter activity occurred. The \(\beta\)-galactosidase specific activity of 6.5 hr, 10 hr and 12.5 hr planktonic cells was below the level of detection, but increased significantly in 15 hr, and 24 hr cells (Fig. 5B; Fig. 6). Cell numbers in the images of Fig. 6 do not reflect the actual cell density in the sample. Similarly, \(\beta\)-galactosidase specific activity increased throughout biofilm development, with the highest level being observed at 5 days (Fig. 5B). Microscopic observation of biofilm cells carrying the \textit{dspI} reporter revealed \(\beta\)-galactosidase activity as early as 1 hr after attachment to a glass substratum, with continued expression though 6 days of growth (Fig. 7). Individual cell reporter activity was not observed to be location-specific within the biofilm.

Control biofilms carrying the \textit{lacZ} transcriptional reporter without an upstream \textit{dspI} promoter did not show fluorescence at the single cell level (Fig. S3). Detectable fluorescence in cell clusters at 3 and 5 days was likely due to either autofluorescence of the cells, or readthrough of RNA polymerase into the \textit{lacZ} structural gene downstream from the \textit{attB} integration site of the chromosome.

\textbf{DISCUSSION}

The structure of many fatty acid signals has been elucidated, however, the synthase genes for these signals are in many cases not identified. For those fatty acid signals whose synthase has
been identified, all have been shown to be dependent on enoyl-CoA hydratase enzymes encoded by rpfF or rpfF-like homologs. In X. campestris, mutation of rpfF abolishes DSF production and results in reduced virulence of the plant pathogen. The gene rpfF is encoded by the rpf operon, which also encodes the genes rpfC (sensor kinase) and rpfG (response regulator) (4).

Homologs of RpfF have been identified in B. cenocepacia (6), X. oryzae pv. oryzae (10), X. fastidiosa (18), S. maltophilia (13), and X. axonopodis pv. glycines (20). Thus, synthesis of fatty acid signaling molecules appears to be widely conserved. A BLAST search (www.pseudomonas.com) reveals homologs of RpfF (greater than 35% identity) in over ten Pseudomonas species, indicating that production of small fatty acid signaling molecules may be widespread among members of this genus. Here we report that DspI is a previously uncharacterized enoyl-CoA hydratase/isomerase, which is required for production of the communication molecule cis-DA in P. aeruginosa (3). DspI is homologous (>30%) to the synthase RpfF in X. campestris, X. oryzae, S. maltophilia, and X. fastidiosa as well as the synthase Bcam0581 in B. cenocepacia (Fig. 9A).

DspI is a putative member of the crotonase superfamily (Fig. 4A), which includes enzymes that catalyze the reversible addition of water to α,β-unsaturated enoyl-CoA thioesters. Previous work has determined that rat mitochondrial enoyl-CoA hydratase contains two conserved catalytic glutamate residues, Glu$^{144}$ and Glu$^{164}$, that are required for complex formation between the enzyme, a catalytic water and the bound substrate at the active site (43-45). Sequence alignment of DspI with rat mitochondrial enoyl-CoA hydratase (NCBI accession #CAA34080) reveals that Glu$^{144}$ and Glu$^{164}$ align with Glu$^{126}$ and Glu$^{146}$ of DspI, supporting the role of DspI as a putative enoyl-CoA hydratase (Fig. 7B). These catalytic Glu residues are also
conserved in RpfF of *X. campestris*, and mutation of either Glu residue abolished DSF synthesis (46).

Recently, Reddy et al. (47) identified a putative active site for the RpfF protein in *X. oryzae* pv. *oryzae*, which elucidated 29 amino acid residues involved in ligand binding: Leu$^{84}$, Gly$^{85}$, Gly$^{86}$, Leu$^{88}$, Phe$^{91}$, Ile$^{95}$, Tyr$^{106}$, Ala$^{107}$, Cys$^{110}$, Val$^{111}$, Leu$^{136}$, Gly$^{137}$, Gly$^{138}$, Glu$^{141}$, Pro$^{160}$, Glu$^{161}$, Leu$^{163}$, Leu$^{164}$, Leu$^{166}$, Pro$^{168}$, Gly$^{169}$, Met$^{170}$, Thr$^{255}$, Trp$^{258}$, Ala$^{262}$, Leu$^{265}$, Thr$^{272}$, Met$^{273}$, Leu$^{276}$. Interestingly, the catalytic Glu$^{144}$ and Glu$^{164}$ residues for enoyl-CoA hydratase activity are included in those predicted to be involved in the active site (Fig 7A). An alignment with *X. oryzae* RpfF reveals that *P. aeruginosa* DspI contains 15/29 of the predicted active site amino acid residues, indicating that these are related enzymes that bind similar substrates and produce structurally related products (Fig 7A).

It has been shown that mutation of *rpfC* results in increased DSF production and a significant increase in *rpfF* transcription (24, 48). This indicates that DSF detection by RpfC results in negative regulation of *rpfF*. This is in contrast to other quorum sensing (QS) signaling systems in which signal detection exhibits transcriptional positive feedback on the signal synthase. For example, in the *P. aeruginosa* QS LasR/I system, the 3-oxo-C12-homoserine lactone (3OC12-HSL) autoinducer molecule is produced by the LasI acylhomoserine lactone (AHL) synthase (49). Transcription of the *lasI* gene is positively regulated when the LasR regulator is bound to the signal, 3OC12-HSL (50). Interestingly, in *P. aeruginosa* we have found no evidence of the cis-DA signal auto-regulating the expression of its cognate synthase, dspI. Addition of synthetic cis-DA to late exponential stage planktonic cultures resulted in less than a 2-fold change in *dspI* transcript levels. These results do not support that autoregulation of cis-DA synthesis occurs at the transcriptional level.
We have previously demonstrated the role of cis-DA as an autoinducer of biofilm dispersion in P. aeruginosa and that dispersion of mature biofilms is inducible by the exogenous addition of naturally or synthetically produced cis-DA (3). However, the full range of phenotypes regulated by this signaling molecule in P. aeruginosa has not been characterized. Recently, Feinbaum et al., have shown that production of the virulence factor pyoverdine is reduced in P. aeruginosa when the gene PA14_54640 (dspl) is mutated (51). These mutants were also shown to be defective in swarming motility, a phenotype inversely related with biofilm formation (51). Together these findings suggest that loss of cis-DA-induced dispersion may be associated with reduced pathogenicity and loss of swarming motility.

The discovery that DspI is required for cis-DA production has important implications for the future characterization of cis-DA signal transduction in P. aeruginosa. A two-component regulatory system for signal transduction of cis-DA has not yet been identified, however several homologs of the DSF two-component regulatory system, RpfC/G, exist in P. aeruginosa. Elucidation of the signal transduction of cis-DA poses a significant challenge considering the P. aeruginosa genome encodes many predicted sensor kinase and response regulator proteins (63 and 64, respectively). A dspl mutant strain, in which cis-DA production is abrogated yet still disperses upon exogenous addition of cis-DA, may be a useful tool for future work to characterize cis-DA signal transduction and dispersion-related phenotypes, including acute virulence and antimicrobial tolerance.

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signal factor influences biofilm formation and polymyxin tolerance in *Pseudomonas aeruginosa*. Mol Microbiol 68:75-86


Table 1. Bacterial Strains and Plasmids

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<td>(26)</td>
</tr>
<tr>
<td>PA14Δ26690</td>
<td>PA14 26690::MAR2xT7; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(26)</td>
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<tr>
<td>PA14Δ28310</td>
<td>PA14 28310::MAR2xT7; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(26)</td>
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<tr>
<td>PA14Δ40640</td>
<td>PA14 40640::MAR2xT7; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(26)</td>
</tr>
<tr>
<td>PA14Δ40980</td>
<td>PA14 40980::MAR2xT7; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(26)</td>
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<tr>
<td>PA14Δ43440</td>
<td>PA14 43440::MAR2xT7; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(26)</td>
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<tr>
<td>PA14Δ51110</td>
<td>PA14 51110::MAR2xT7; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(26)</td>
</tr>
<tr>
<td>PA14ΔdspI</td>
<td>PA14 54640::MAR2xT7; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(26)</td>
</tr>
<tr>
<td>PA14ΔdspI/pMJT</td>
<td>PA14 $\Delta dspI$ bearing empty pMJT-1 vector; Gm&lt;sup&gt;+&lt;/sup&gt; Cbr&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>PA14ΔdspI/pMJT-dspI</td>
<td>Complementation of $\Delta dspI$; Gm&lt;sup&gt;+&lt;/sup&gt; Cbr&lt;sup&gt;+&lt;/sup&gt; arabinose-inducible</td>
<td>This study</td>
</tr>
<tr>
<td>PA14/dspI-lacZ</td>
<td>pCTX-$\Delta dspI$-lacZ conjugated into PA14, Tet&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>PA14/lacZ</td>
<td>pCTX conjugated into PA14, Tet&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>PCR2.1-TOPO</td>
<td>TA cloning vector; Km&lt;sup&gt;+&lt;/sup&gt; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Invitrogen Corp.</td>
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<tr>
<td>pRK2013</td>
<td>Helper plasmid for triparental mating; mob tra; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(31)</td>
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<tr>
<td>pMJT1</td>
<td>araC-P&lt;sub&gt;BAD&lt;/sub&gt; cassette of pJN105 cloned into pUCP18, Amp&lt;sup&gt;+&lt;/sup&gt; Cbr&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(27)</td>
</tr>
<tr>
<td>pCTX-dspI</td>
<td>mini-CTX-lacZ; Tet&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(28)</td>
</tr>
<tr>
<td>pMJT-dspI</td>
<td>dspI cloned into pMJT1 using primers dspI&lt;sub&gt;NheI&lt;/sub&gt;_for/dspI&lt;sub&gt;SacI&lt;/sub&gt;_rev; Cbr</td>
<td>This study</td>
</tr>
<tr>
<td>pCTX-dspI-lacZ</td>
<td>dspI promoter reporter construct in mini-CTX-lacZ using primers dspI-PROM&lt;sub&gt;XhoI&lt;/sub&gt;_for/dspI-PROM&lt;sub&gt;EcoRI&lt;/sub&gt;_rev; Tet&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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</table>
Table 2. Quantitative analysis of biofilm architecture using COMSTATa

<table>
<thead>
<tr>
<th>Strains and Conditions</th>
<th>Total Biomass (µm^3/µm^2)</th>
<th>Average Biofilm Thickness (µm)</th>
<th>Maximum Biofilm Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Continuous dspI induction (5% LB; 6-day-old biofilms)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA14</td>
<td>9.4 (±3.8)</td>
<td>13.4 (±5.0)</td>
<td>61.9 (±21.8)</td>
</tr>
<tr>
<td>ΔdspI</td>
<td>22.0 (±7.0)^1</td>
<td>35.5 (±11.5)^1</td>
<td>127.1 (±35.6)^1</td>
</tr>
<tr>
<td>ΔdspI/pMJT-dspI</td>
<td>6.5 (±3.9)^2,3</td>
<td>10.4 (±5.1)^2,3</td>
<td>64.2 (±23.0)^2,3</td>
</tr>
<tr>
<td>ΔdspI/pMJT</td>
<td>25.4 (±10.8)^1</td>
<td>39.3 (±16.8)^1</td>
<td>97.4 (±35.7)^1</td>
</tr>
<tr>
<td><strong>Before dspI induction (EPRI; 5-day-old biofilms)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA14</td>
<td>9.3 (±2.9)</td>
<td>9.7 (±3.4)</td>
<td>25.7 (±7.8)</td>
</tr>
<tr>
<td>ΔdspI</td>
<td>14.8 (±2.4)^1</td>
<td>16.0 (±3.6)^1</td>
<td>43.0 (±14.0)^1</td>
</tr>
<tr>
<td>ΔdspI/pMJT-dspI</td>
<td>14.5 (±5.6)^1,4</td>
<td>16.4 (±6.0)^1,4</td>
<td>38.7 (±18.0)^1,4</td>
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<tr>
<td>ΔdspI/pMJT</td>
<td>16.7 (±3.8)^1</td>
<td>18.3 (±4.4)^1</td>
<td>38.8 (±12.1)^1</td>
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<tr>
<td><strong>After dspI induction (EPRI; 6-day-old biofilms)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA14</td>
<td>9.2 (±4.7)</td>
<td>10.5 (±5.4)</td>
<td>30.23 (±13.4)</td>
</tr>
<tr>
<td>ΔdspI</td>
<td>20.2 (±3.5)^1</td>
<td>22.5 (±3.7)^1</td>
<td>53.8 (±16.3)^1</td>
</tr>
<tr>
<td>ΔdspI/pMJT-dspI</td>
<td>6.0 (±3.8)^2,3</td>
<td>7.0 (±4.3)^2,3</td>
<td>20.4 (±8.3)^2,3</td>
</tr>
<tr>
<td>ΔdspI/pMJT</td>
<td>21.8 (±7.3)^1</td>
<td>23.5 (±8.1)^1</td>
<td>40.0 (±12.4)^1</td>
</tr>
</tbody>
</table>

**dspI expression induced via growth in arabinose-containing culture medium for 24 hours

COMSTAT analysis was carried out from biofilms grown in replicate (n = 2) from at least 8 images per replicate

1 Significantly different from the wild type PA14 (P < 0.01), as determined by single variant ANOVA
2 Significantly different from the dspI mutant (P < 0.01), as determined by single variant ANOVA
3 Not significantly different from the wild type (P > 0.01), as determined by single variant ANOVA
4 Not significantly different from the dspI mutant (P > 0.01), as determined by single variant ANOVA
FIGURE LEGENDS

FIG. 1. Microcolonies of *P. aeruginosa* PA14 biofilms grown in twenty-four-well cell culture plates demonstrating native dispersion response. Transmitted light image (A) showing presence and absence of interior voids formed within microcolonies of wild type PA14 and 8 putative enoyl-CoA hydratase mutants. Biofilms of *dspI* (PA14_54640) mutant showed no evidence of void formation. All images are shown at same relative size at ×200 magnification. Scale bar, 50 µm. (B) Quantification number of microcolony voids formed as a percent of total microcolonies observed for biofilms of PA14 and Δ*dspI*.

FIG. 2. *DspI* is required for native biofilm dispersion. Transmitted light images (A,B) and confocal scanning laser microscopy images (C) at a magnification of ×500 of *P. aeruginosa* wild type and *dspI* mutant biofilms. Photomicrographs show microcolonies of biofilms grown in modified EPRI medium (A) or 5% LB medium (B,C) for 6 days, with continuous *dspI* induction in the complemented *dspI* mutant strain. Microcolonies of the *dspI* mutant remained solid, whereas wild type and complemented mutant biofilms showed dispersion. Experiments were completed in triplicate. Scale bar, 50 µm.

FIG. 3. *DspI* mutant biofilms disperse in the presence of exogenous *cis*-DA. (A) Biofilms of wild type PA14, *dspI* mutants, or complemented *dspI* mutants were grown in continuous culture tube reactors for 6 days and switched to fresh medium or *cis*-DA for 2 hr under static conditions. The number of released cells in the bulk liquid of each tube and remaining biofilm cells in each tube were determined by viable count (CFU). Percent dispersion was calculated as a function of released cells (CFU) divided by the total number of CFU from each tube (released cells plus remaining biofilm cells). (B) CLSM images of mature *dspI* mutant biofilm microcolonies grown
in continuous culture in a microscope-mounted flow cell before and after the addition of cis-DA. Microcolony disaggregation is shown following treatment under static conditions for 1 hr. Control biofilms treated with fresh medium show no disaggregation (not shown). Images are the same relative size at 500× magnification. Scale bar, 50 µm. Experiments were completed in triplicate. *, values significantly different from respective negative control (P < 0.01).

FIG. 4. DspI is required for synthesis of cis-2-decenoic acid in P. aeruginosa. (A) DspI contains a conserved domain (grey) belonging to the crotonase/enoyl-Coenzyme A (CoA) hydratase family, which includes many diverse enzymes involved in fatty acid metabolism. (B) The predicted enzymatic reaction performed by the enoyl-CoA hydratase dspI includes the formation of a double bond at the β-carbon of small fatty acids. (C) Spectral analysis of synthetic cis-DA, and CSM prepared from P. aeruginosa PA14 wild type and mutants inactivated in or complimented in dspI, was performed using Gas Chromatography-Mass Spectrometry. The y axis indicates intensity; the x axis indicates time in min. (D) MS/MS fragmentation patterns of the 7.0 min peak from the GC-MS spectrum of cis-DA, PA14 CSM and CSM of the complimented dspI mutant. The y axis indicates intensity; the x axis indicates m/z.

FIG. 5. Expression and transcript abundance levels of dspI in planktonic and biofilm cells. (A) Growth curve of P. aeruginosa PA14 in LB medium. Curve represents average of 3 replicates. Error bars indicate standard deviation. (B) Fold change in dspI mRNA levels in P. aeruginosa planktonic and biofilm cells compared to lag phase planktonic cells. Experiments were performed in triplicate. (C) Transcriptional reporter fusion assay for dspI expression in P. aeruginosa wild-type 6.5 hr, 10 hr, 12.5 hr, 15 hr, and 24 hr-old planktonic cells, and 1-day, 3-
day, and 5-day-old biofilm cells. Values indicated by an asterisk (*), differ significantly from values of the preceding growth phase (P < 0.05).

FIG. 6. Microscopic analysis of dspI transcriptional reporter activity during planktonic and biofilm growth. *P. aeruginosa* PA14 harboring a dspI-lacZ reporter construct was grown in batch or continuous culture in medium supplemented with MUG. The following planktonic conditions are shown: (A) 6.5 hr; (B) 10 hr; (C) 12.5 hr; (D) 15 hr; and (E) 24 hr. The following biofilm conditions are shown: (F) 1 hr; (G) 1-day; (H) 2-day; (I) 3-day; (J) 5-day. Bright field image (left panel) and fluorescent cells displaying β-galactosidase activity (right panel). Scale bar, 20 μm.

FIG. 7. Multiple sequence alignment of DspI, RpfF homologs, and rat mitochondrial enoyl-CoA hydratase. The sequences were obtained from the National Center for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov/ and were aligned using ClustalW. Fully conserved (*), strongly conserved (:), and weakly conserved (.) amino acid residues are shown. (A) The 29 amino acid residues of the predicted ligand binding site for RpfF in *X. oryzae* pv. *oryzae* are indicated in boxes (47). DspI contains 15 out of 29 conserved amino acid residues of the predicted DSF ligand binding site of RpfF. (B) Conserved glutamate residues at the enoyl-CoA active site of rat mitochondrial enoyl-CoA hydratase, Glu$^{144}$ and Glu$^{164}$, align Glu$^{126}$ and Glu$^{146}$ of DspI (shown in red boxes, designated by (∆)).
Figure 1.
FIG. 2.

A  PA14  ΔdspI  ΔdspI/pMJT-dspI  ΔdspI/pMJT

B  

C  

FIG. 2.
Figure 3
Figure 4
Figure 5
Figure 6.