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A Global Regulator of Secondary Metabolite Production in *Pseudomonas fluorescens* Pf-5

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Mutations in the *apdA* (for antibiotic production) gene of the plant root-colonizing bacterium *Pseudomonas fluorescens* Pf-5 pleiotropically abolish the production of an array of antibiotics, including pyrrolnitrin, pyoluteorin, and 2,4-diacetylphloroglucinol, as well as the production of tryptophan side chain oxidase, hydrogen cyanide, and an extracellular protease. The lack of production of secondary metabolites by ApdA$^-$ mutants was correlated with the loss of inhibition of the phytopathogenic fungus *Rhizoctonia solani* in culture. Sequencing of the *apdA* region identified an open reading frame of 2,751 bp. The predicted amino acid sequence of the *apdA* gene contains conserved domains of the histidine kinases that serve as sensor components of prokaryotic two-component regulatory systems. The *apdA* nucleotide and predicted amino acid sequences are strikingly similar to the sequences of *lemA* and *repA*, genes encoding putative sensor kinases that are required for the pathogenicity of *Pseudomonas syringae* pv. syringae and *Pseudomonas viridiflava*, respectively. Introduction of the cloned *apdA*$^+$ gene restored the wild-type phenotype to both LemA$^-$ mutants of *P. syringae* and ApdA$^-$ mutants of Pf-5. The 101-kDa ApdA protein reacted with an anti-LemA antiserum, further demonstrating the similarity of ApdA to LemA. These results show that *apdA* encodes a putative sensor kinase component of a classical two-component regulatory system that is required for secondary-metabolite production by *P. fluorescens* Pf-5.

Fluorescent pseudomonads are ubiquitous soil microorganisms and common inhabitants of the rhizosphere. Certain strains suppress plant diseases by protecting seeds or roots from infection by soilborne fungal and bacterial plant pathogens (6, 30). Exploiting the potential of fluorescent pseudomonads to act as crop protectants has become a focus of many research groups. There are, however, obstacles that impede the widespread application of pseudomonads as biocontrol agents in agriculture. Chief among these is the variable performance of fluorescent pseudomonads under field conditions (44). An understanding of how biocontrol bacteria regulate the expression of genes involved in the inhibition of pathogens is likely to be prerequisite for predicting the environmental conditions under which these bacteria will perform optimally.

The biological control agent *Pseudomonas fluorescens* Pf-5 inhabits the spermosphere and rhizosphere, where it suppresses the soilborne fungi *Rhizoctonia solani* (13) and *Pythium ultimum* (14). These fungi infect seeds and roots, thereby reducing seedling emergence and survival. Strain Pf-5 also colonizes wheat straw residue, in which it suppresses ascocarp formation by *Pyrenophora tritici-repentis* (32), the tan spot pathogen of wheat. Pf-5 produces the antifungal antibiotics pyrrolnitrin (13), pyoluteorin (14), and 2,4-diacetylphloroglucinol (28); hydrogen cyanide (22); and a noncharacterized pyoverdine siderophore. Each antibiotic has a unique spectrum of activity against fungal pathogens suppressed by Pf-5. Pyrrolnitrin inhibits *R. solani* (13) and *Pyrenophora tritici-repentis* (32), pyoluteorin inhibits *Pythium ultimum* (14), and 2,4-diacetylphloroglucinol inhibits all three fungi (19, 28). The profile of secondary metabolites produced by Pf-5 is remarkably similar to that of *P. fluorescens* CHA0, a biological control organism in which secondary-metabolite production is required for effective suppression of black root rot of tobacco (25, 43), *Pythium* damping-off of cress (27), and take-all of wheat (19). In strain CHA0, the *gacA* gene (for global antibiotic and cyanide control) encodes a regulatory protein (GacA) that is required for antibiotic and hydrogen cyanide production (25) and tryptophan side chain oxidase (29) and protease activities (35). Similarly, GacA$^-$ mutants of *P. fluorescens* BL915 are pleiotropic; they fail to synthesize several antifungal factors, including hydrogen cyanide and pyrrolnitrin (11). GacA is a response regulator in the FixJ-DegU family of two-component regulatory systems (11, 25). Prokaryotic two-component regulatory systems are typically composed of a transmembrane protein that functions as an environmental sensor and a cytoplasmic response regulatory protein that mediates changes in gene expression in response to sensor signals (31, 40).

In a previous study, 6,286 mutants of *P. fluorescens* Pf-5, each with a single genomic Tn*5* insertion, were screened for the production of pyoluteorin and pyrrolnitrin by thin-layer chromatography (22). Five ApdA$^-$ mutants (previously termed Apd$^-$ group I for antibiotic production [22]) had pleiotropic phenotypes similar to those of GacA$^-$ mutants of other strains of *P. fluorescens*; they did not produce pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol, or hydrogen cyanide. In this report, we describe further functional characterization of the ApdA$^-$ phenotype and the sequence of the *apdA* gene. We present evidence that the *apdA* gene (i) is distinct from *gacA*, (ii) encodes a protein that contains domains conserved among the sensor kinases of two-component regulatory proteins, (iii) is responsible for the global regulation of secondary metabolites in strain Pf-5, and (iv) is the functional equivalent of *lemA*, which encodes a putative sensor kinase of a two-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Relevant characteristic(s)[a]	Reference
P. fluorescens			
Pf-5	Field isolate	$ApdA^+$	13
JL4097	Derivative of Pf-5; *apdA*::Tn*5*	$ApdA^-$ Km^r	22
JL4106	Derivative of Pf-5; *apdA*::Tn*5*	$ApdA^-$ Km^r	22
JL4135	Derivative of Pf-5; *apdA*::Tn*5*	$ApdA^-$ Km^r	22
JL4209	Derivative of Pf-5; *apdA*::Tn*5*	$ApdA^-$ Km^r	22
JL4210	Derivative of Pf-5; *apdA*::Tn*5*	$ApdA^-$ Km^r	22
P. syringae pv. syringae			
B728a	Field isolate causing brown spot of bean	$LemA^+$	15
NPS3136	Derivative of B728a; *lemA*::Tn*5*	$LemA^-$ Km^r	15
E. coli DH5α	F^- *endA1 hsdR17* ($r_K^-m_K^+$) *supE44 thi-1 recA1 gyrA96 relA1* ϕ80*dlacZ*Δ M15 λ^-		36
Plasmids			
pUC18	ColE1 replicon	Ap^r	36
pRK2013	Mobilizing plasmid	Tra^+ Km^r	10
pRK415	IncP1 replicon; polylinker of pUC19	Mob^+ Tc^r	20
pBR322	ColE1 replicon	Ap^r Tc^r	36
pME3066	1.65-kb *Bam*HI-*Bgl*II fragment containing the *gacA* gene of *P. fluorescens* CHA0 cloned into pLAFR3	Tc^r	25
pJEL1657	13.5-kb Tn*5* containing *Eco*RI fragment from JL4106 cloned into pBR322	Ap^r Km^r Tc^r	This study
pJEL5591	6.5-kb *Kpn*I fragment from Pf-5 cloned into pRK415	Tc^r $ApdA^+$	This study
pJEL5771	3.5-kb *Kpn*I-*Sun*I fragment from Pf-5 cloned into pRK415	Tc^r $ApdA^+$	This study
pJEL5680	6.5-kb *Kpn*I fragment from Pf-5 cloned into pUC18	Ap^r $ApdA^+$	This study

[a] Ap^r, Km^r, and Tc^r, resistance to ampicillin, kanamycin, and tetracycline, respectively.

component regulatory system required for the pathogenicity of *Pseudomonas syringae* pv. syringae (15, 45).

(Portions of this work were published earlier [5].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* strains were cultured in Luria-Bertani broth (36) or on Luria-Bertani agar plates incubated at 37°C. *Pseudomonas* strains were routinely cultured at 27°C in King's medium B (KMB) (21) broth or on KMB agar plates. Antibiotic concentrations were as follows, unless otherwise specified: 100 μg of ampicillin per ml, 50 μg of kanamycin per ml, and 20 μg of tetracycline per ml.

Recombinant DNA techniques. Transformation of *E. coli*, restriction digests, ligations, electrophoresis in Tris-phosphate-EDTA, and plasmid DNA isolation were performed by standard methods (36). Enzymes were from Gibco BRL Life Technologies (Gaithersburg, Md.). Southern blots of plasmid and genomic DNAs were prepared with nylon membranes (Nytran; Schleicher and Schuell, Keene, N.H.) according to the supplier's directions. The DNA probes were *apdA*::Tn*5* (the 13.5-kb Tn*5*-containing *Eco*RI fragment of pJEL1657) and the *gacA* gene of *P. fluorescens* CHA0 (the 1.65-kb *Bam*HI-*Bgl*II fragment of pME3066) (25), labelled with biotinylated dATP or [^{32}P]dCTP by using a nick translation kit (Gibco BRL Life Technologies) and purified with a D50 column (International Biotechnologies, Inc., New Haven, Conn.). Plasmids pLAFR3 and pRK415 and their derivatives were mobilized from *E. coli* DH5α donors into *P. fluorescens* and *P. syringae* in triparental matings with helper plasmid pRK2013 (10). Because of the high degree of natural tetracycline resistance exhibited by *P. fluorescens* Pf-5, transconjugants were selected on KMB agar containing 200 μg of tetracycline per ml and 100 μg of ampicillin per ml.

Cloning of the *apdA* gene of Pf-5. An existing genomic library of Pf-5 (32), constructed in cosmid vector pLAFR3, was screened by colony hybridization to identify clones that hybridized to the *apdA*::Tn*5* probe or the *gacA* probe. Filters for colony hybridization were prepared with Whatman 541 paper (Whatman International, Ltd., Maidstone, England) and hybridized with the [^{32}P]dCTP-labelled probe as previously described (12).

DNA sequencing and sequence analysis. DNA sequencing and oligonucleotide syntheses were performed at the Center for Gene Research and Biotechnology at Oregon State University, Corvallis. The sequencing of double-stranded templates was performed on an ABI 373A automated DNA sequencer with a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.) according to the manufacturer's protocol. Oligonucleotide primers were synthesized on an ABI 380B DNA synthesizer by phosphoramidite chemistry (2). Initial sequencing of the *apdA* locus was performed on regions flanking the Tn*5* inserts of three $ApdA^-$ mutants (JL4106, JL4135, and JL4210). *Eco*RI fragments containing Tn*5* and the flanking genomic DNA from each of these mutants were cloned into pUC18 in both orientations. Each derivative of these plasmids, constructed by deleting a fragment bordered by the *Bam*HI site internal to Tn*5* and the *Bam*HI site of the pUC18 polylinker, contained a single inverted repeat of Tn*5* and contiguous genomic DNA. These plasmids were sequenced by using an oligonucleotide primer complementary to bases 37 through 18 in the inverted repeat of Tn*5* (5′-GGTTCCGTTCAGGACGCTAC-3′) (34). Further sequencing of the *apdA* gene cloned in pJEL5680 was directed by oligonucleotide primers designed from previous sequence determinations of the *apdA* region. DNA sequence analysis and comparisons with sequences contained in the GenBank and EMBL databases were accomplished with software from the Genetics Computer Group, Inc., Madison, Wis. (9).

Characterization of the ApdA phenotype. The ApdA phenotypes of Pf-5 and derivatives were characterized by protease and tryptophan side chain oxidase activities, hydrogen cyanide and antibiotic production, and inhibition of *R. solani* in culture. Protease production was determined qualitatively by streaking strains on Bacto litmus milk agar (Difco Laboratories, Detroit, Mich.). After cultures were incubated for 36 h at 27°C, the presence of a cleared zone surrounding bacterial growth indicated protease production. Tryptophan side chain oxidase activity was determined by the method of Takai and Hayaishi (41). The production of hydrogen cyanide was assessed by the method originally described by Castric and Castric (4), as modified by Sarniguet et al. (38).

Concentrations of the antibiotics pyrrolnitrin, pyoluteorin, and 2,4-diacetylphloroglucinol were quantified by high-pressure liquid chromatography

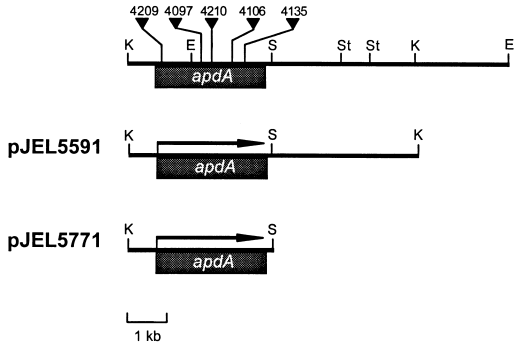

FIG. 1. Schematic representation of the *apdA* locus. Numbered triangles indicate Tn*5* insertion sites in specified $ApdA^-$ mutants. pJEL5591 and pJEL5771 contain functional *apdA* genes. An overhead arrow indicates the direction of *apdA* transcription. Restriction sites: E, *Eco*RI; K, *Kpn*I; St, *Sst*I; S, *Sun*I.

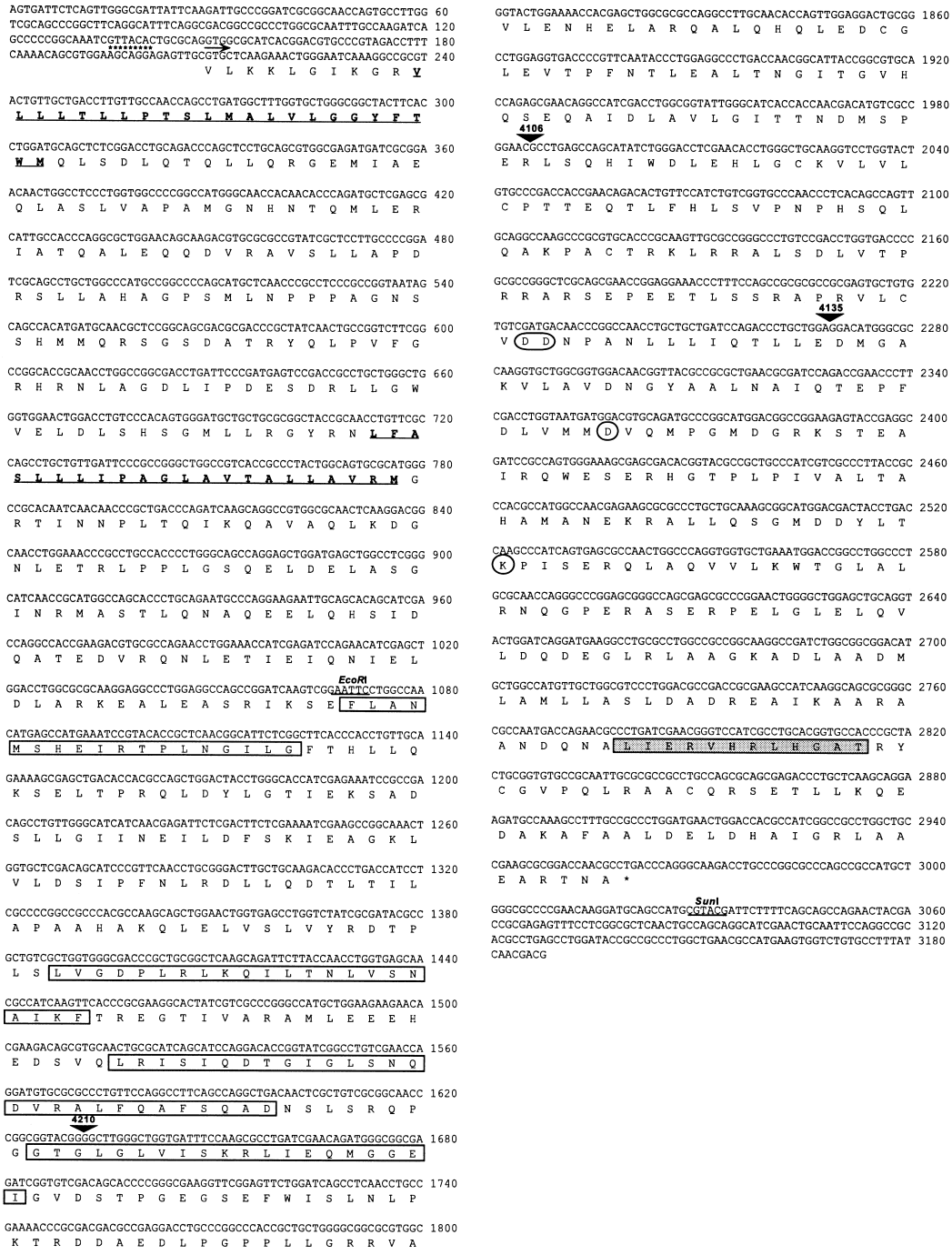

FIG. 2. Complete nucleotide and predicted amino acid sequences of *apdA*. Restriction sites are underlined. The putative translational start codon is indicated by an arrow; the most probable ribosome binding site is indicated by a series of asterisks. Numbered triangles are sites of Tn*5* insertion. Bold underlined regions are hydrophobic regions. Conserved sensor kinase domains (40) are indicated by open boxes. Conserved residues of the receiver module are circled. A region in the C terminus that is conserved among certain sensor kinases (42) is indicated by a shaded box. A stop codon is indicated by a single asterisk at the end of the amino acid sequence.

(HPLC). *P. fluorescens* was grown at 20°C either for 48 h in 5 ml of nutrient broth (Difco) supplemented with 1% (wt/vol) glycerol, a medium in which pyoluteorin and pyrrolnitrin are produced preferentially, or for 96 h in 5 ml of nutrient broth supplemented with 2% (wt/vol) glucose, a medium in which 2,4-diacetylphloroglucinol is produced preferentially (28). Cells and supernatants of 5-ml cultures were separated by centrifugation. Cells were extracted once in 1 ml of acetone. Cell extracts were taken to dryness, resuspended in MeOH, and analyzed by HPLC as described below. Culture supernatants were adjusted to pH 2 with 1 M HCl and extracted three times with 1 ml of ethyl acetate. Organic phases were combined and back extracted once with 1 ml H_2O. The organic-phase extracts were dried, dissolved in MeOH, and analyzed on a Waters Nova-pak C_{18} reverse-phase column (0.8 by 10 cm; 4 μm) eluted isocratically (45% H_2O, 30% acetonitrile, 25% MeOH) at a flow rate of 1.5 ml/min. Antibiotics were detected quantitatively with a UV photodiode array detector at 225 (pyrrolnitrin), 310 (pyoluteorin), and 278 nm (2,4-diacetylphloroglucinol) and compared with authentic samples of these compounds, as described previously (28).

Inhibition of *R. solani*. *P. fluorescens* was grown overnight in KMB broth. Cells were collected by centrifugation, washed, and adjusted to an optical density of 0.2 at 600 nm. Ten microliters of the cell suspension was applied to the centers of petri plates containing nutrient agar (Difco) supplemented with 1% (wt/vol) glycerol or 2% (wt/vol) glucose. Following 3 days of incubation at 27°C, bacterial cells were killed by exposure to chloroform vapors for 30 min. Plates were vented for 30 min, and the medium was overlaid with 5 ml of molten potato dextrose agar (Difco) containing hyphal fragments of *R. solani* J1 (obtained from C. R. Howell, Cotton Research Laboratory, USDA Agricultural Research Service, College Station, Tex.). Hyphal suspensions were prepared by growing isolate J1 in 20 ml of potato dextrose broth (Difco) for 3 days at 27°C with shaking and then homogenizing the hyphal mat and spent broth. The inhibition zone diameters from duplicate plates were measured after incubation for 2 days at 27°C. The experiment was repeated.

Protein analysis. Cells from cultures grown overnight in KMB broth were pelleted and suspended in ice-cold phosphate-buffered saline (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 [pH 7.5]) to an optical density of 0.2 at 600 nM. Cells were concentrated 10-fold by centrifugation, resuspended in ice-cold saline, mixed with 2× Laemmli sample buffer (36), and boiled for 5 min. Lysates were separated on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Proteins were stained with Coomassie blue or transferred to reinforced nitrocellulose membranes for Western blot (immunoblot) analysis (36). Immunoblotting was performed with polyclonal anti-LemA antiserum (raised in New Zealand White rabbits) (33), which was supplied generously by T. Kitten and D. K. Willis (University of Wisconsin, Madison). The secondary antibody, goat anti-rabbit immunoglobulin G (Fc) conjugated to alkaline phosphatase, was detected with 5-bromo-4-chloro-3-indolyl-1-phosphate and nitro blue tetrazolium (Promega Corp., Madison, Wis.).

Nucleotide sequence accession number. The GenBank accession number for the DNA sequence of the *apdA* gene of *P. fluorescens* Pf-5 is U30858.

RESULTS

Localization of Tn*5* insertions in the genome of ApdA$^-$ mutants. Five ApdA$^-$ mutants, each of which has a single Tn*5* insertion in the genome, were described previously (22). Southern analysis of genomic DNAs of these mutants probed with the Tn*5*-containing *Eco*RI fragment isolated from ApdA$^-$ mutant JL4106 (i.e., the *apdA*::Tn*5* probe) revealed that four of the mutants (JL4097, JL4106, JL4135, and JL4210) contained a single Tn*5* insertion within the same 13.5-kb *Eco*RI fragment of genomic DNA. The 13.5-kb *Eco*RI fragment, consisting of 7.8 kb of genomic DNA and Tn*5*, from each of these mutants was cloned, and the Tn*5* insertions were mapped to a 1.5-kb region by restriction analysis (Fig. 1). The single Tn*5* insertion in the genome of ApdA$^-$ mutant JL4209 was localized to the adjacent *Eco*RI fragment by Southern analysis. A 1.6-kb *Bam*HI-*Bgl*II fragment that hybridized to the *gacA* gene of *P. fluorescens* CHA0 (25) was present in the genomic DNAs of Pf-5 and the five ApdA$^-$ mutants, indicating that the ApdA$^-$ mutants did not have a Tn*5* insertion within *gacA*.

Cloning of the *apdA* gene. Three cosmids, each containing the wild-type *apdA* gene, were identified from a genomic library of *P. fluorescens* Pf-5 by hybridization to the *apdA*::Tn*5* probe. Southern hybridization indicated that each cosmid contained a 7.8-kb *Eco*RI fragment and a 6.5-kb *Kpn*I fragment that hybridized to the *apdA*::Tn*5* probe. By probing the Pf-5 cosmid library with the *gacA* gene, we identified two other cosmids, each of which contained a *gacA* homolog. On Southern blots, the *gacA* probe hybridized to a 1.6-kb *Bam*HI-*Bgl*II fragment in both *gacA*-containing cosmids but it did not hybridize to any of the *apdA*-containing cosmids. The two cosmids that hybridized to the *gacA* probe did not hybridize to the *apdA*::Tn*5* probe. Together, these data present convincing evidence that the *apdA* and *gacA* loci of *P. fluorescens* Pf-5 are distinct and unlinked loci.

Sequence analysis of the *apdA* gene. A 3,188-bp region of DNA flanking the Tn*5* insertions of ApdA$^-$ mutants and present within the 6.5-kb *Kpn*I fragment of pJEL5591 was sequenced. The Tn*5* insertions of JL4210, JL4106, and JL4135 were localized within a 2,751-bp open reading frame identified as the *apdA* locus (Fig. 2). The open reading frame, beginning at bp 209 with a GTG initiation codon and terminating at bp 2960 with a TAG stop codon, was predicted to encode a protein of 917 amino acids with a molecular mass of 101 kDa. Upstream from the GTG codon, there is an AG-rich region from bp 194 to bp 203 containing two overlapping sequences

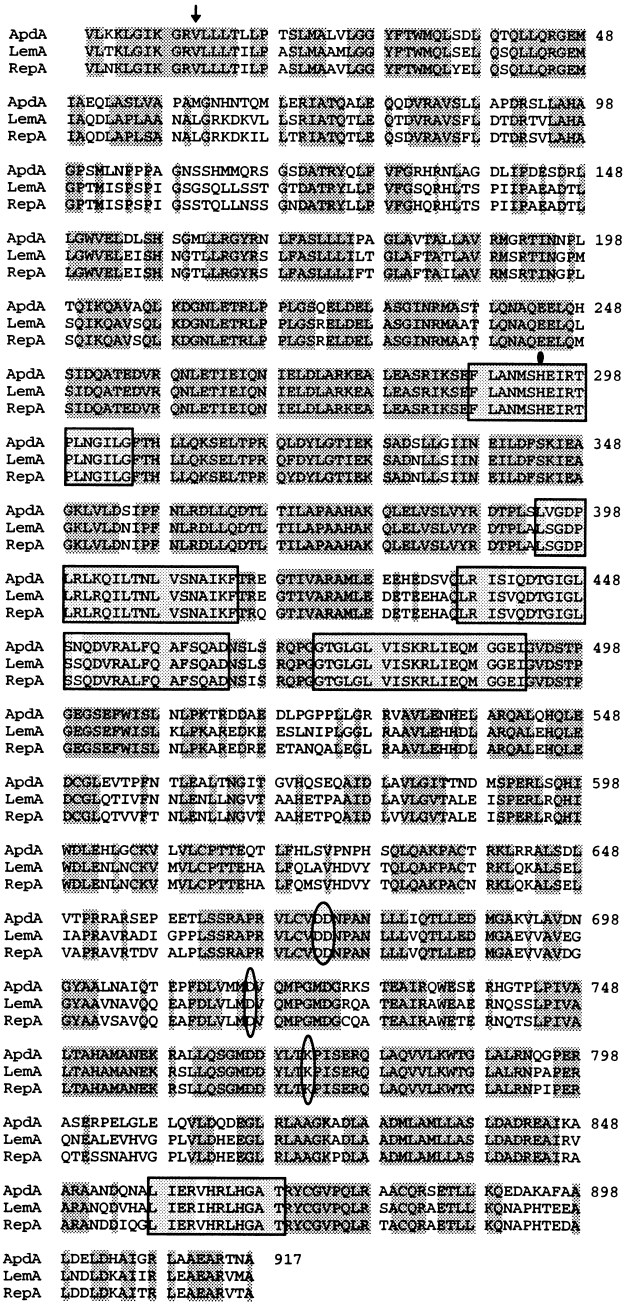

FIG. 3. Amino acid sequence alignments of ApdA, LemA, and RepA. Residues that are identical in all three sequences are shaded. The sequence comparison begins at the predicted first amino acid of ApdA. An arrow indicates the predicted first amino acids of LemA and RepA. Boxed residues indicate the following conserved domains, which are named according to their signature amino acids: H box, amino acids 288 to 305; N box, amino acids 394 to 415; D/F box, amino acids 437 to 464; G box, amino acids 473 to 492 (40). A conserved C-terminal domain (amino acids 858 to 869 [42]) is also boxed. A black dot indicates the autophosphorylated histidine residue. Conserved residues typical of a receiver domain are circled.

TABLE 2. Phenotypic characterization and complementation of an ApdA$^-$ mutant of *P. fluorescens* Pf-5

Strain	Secondary-metabolite production[a]						Inhibition of *R. solani* (cm)[b]
	Pn (μg/ml)	Plt (μg/ml)	Phl (μg/ml)	HCN	Protease	TSO	
Pf-5(pRK415)	0.7 ± 0.1	17.1 ± 0.5	37.7 ± 1.8	+	+	+	2.6
JL4135(pRK415)	−	−	−	−	−	−	−
JL4135(pJEL5771)	1.2 ± 0.1	9.9 ± 0.5	21.5 ± 10.3	+	+	+	2.6

[a] Data are the averages of two replications ± standard deviations. Pn, pyrrolnitrin; Plt, pyoluteorin; Phl, 2,4-diacetylphloroglucinol; HCN, hydrogen cyanide; TSO, tryptophan side chain oxidase; +, metabolite detected; −, no metabolite detected.

[b] Each value is the diameter of the inhibition zone minus the diameter of the bacterial colony grown on nutrient agar supplemented with 1% glycerol. −, no inhibition detected.

similar (five of six nucleotides) to Shine-Dalgarno sites for ribosome binding (39). Several methionine codons downstream from the putative translational start site (bp 209) could also serve as translational start sites. However, only one of these alternate start sites (the two consecutive methionine codons from bp 548 to bp 553) is preceded by a possible ribosome binding site.

The sequence of the *apdA* gene is similar to those of a class of regulatory proteins. The *apdA* nucleotide and predicted amino acid sequences are similar to those of sensor kinase components of two-component regulatory systems (31, 40). All of the conserved domains associated with the kinase portions of sensor kinase genes (1, 31, 40) were identified in the *apdA* gene (Fig. 2). The N-terminal region of ApdA contains two long hydrophobic regions (Fig. 2) which could constitute transmembrane regions typical of sensor kinases. The predicted ApdA amino acid sequence contains conserved aspartate and lysine residues (Fig. 2) that are common to response regulators and also are found in some sensor kinases of two-component regulatory systems (31, 40).

The most striking sequence similarities are those with *lemA* (15) and *repA* (26), genes encoding putative sensor kinases in phytopathogenic *Pseudomonas* species. ApdA, LemA, and RepA are 77% identical in a 917-amino-acid overlap (Fig. 3). Like *lemA* and *repA*, the *apdA* gene contains no recognizable consensus sequences for σ^{70}-, σ^{54}-, or σ^{32}-type pseudomonad promoters (7).

Complementation of ApdA$^-$ and LemA$^-$ mutants with the cloned *apdA*$^+$ gene. ApdA$^-$ mutants of Pf-5 are deficient in the production of pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol, and hydrogen cyanide and fail to inhibit *R. solani* in culture (22). ApdA$^-$ mutants also fail to produce an extracellular protease and tryptophan side chain oxidase in culture. Plasmid pJEL5591, containing a 6.5-kb *Kpn*I fragment encompassing the *apdA*$^+$ gene (Fig. 1), restored cyanide synthesis, extracellular protease production, and tryptophan side chain oxidase activity to ApdA$^-$ mutants JL4097, JL4106, JL4135, JL4209, and JL4210. The function of the *apdA* region was further localized to a 3.5-kb *Kpn*I-*Sun*I fragment containing the 2,751-bp *apdA*$^+$ locus (Fig. 1), which restored the ApdA$^+$ phenotype to JL4135 (Table 2). We established that the products of the *apdA* and *lemA* loci are cross-functional by restoring extracellular protease production to a LemA$^-$ mutant of *P. syringae* pv. syringae upon the introduction of pJEL5771 (data not shown) or pJEL5591 (Fig. 4).

Plasmid pME3066, containing the *gacA* gene of *P. fluorescens* CHA0 (25), did not restore protease, cyanide, or tryptophan side chain oxidase activity to any ApdA$^-$ mutant.

Western analysis. Western analysis of total protein extracted from bacterial cells identified a 101-kDa protein that reacted with the anti-LemA antiserum in strain Pf-5 but not in JL4135, an ApdA$^-$ derivative strain (Fig. 5). When the wild-type *apdA*$^+$ gene cloned in pJEL5771 or pJEL5591 was introduced into JL4135, production of the 101-kDa protein was restored. The anti-LemA antiserum also reacted with a 75-kDa protein in mutant JL4135 and the complemented mutants JL4135(pJEL5771) and JL4135(pJEL5591). On the basis of the location of the Tn*5* insertion, the predicted size of the truncated ApdA protein in JL4135 is 75 kDa.

DISCUSSION

A mutation in the locus that we have termed *apdA* abolished the biosynthesis of an array of secondary metabolites produced by the biological control bacterium *P. fluorescens* Pf-5. Although the phenotype of ApdA$^-$ mutants of Pf-5 is remarkably similar to that of GacA$^-$ mutants of the biological control bacterium *P. fluorescens* CHA0 (25), *apdA* is distinct from the *gacA* homolog of Pf-5. Sequence analysis of the *apdA* gene showed that it is closely related to genes from two plant pathogens, (i) the *lemA* gene of *P. syringae*, which is a regulatory gene controlling the production of protease and syringomycin (16) and lesion formation (15), and (ii) the *repA* gene of *P. viridiflava*, which is required for the production of pectate lyase and other metabolites (26). The *lemA* and *repA* genes have been proposed to be the sensor kinase components of two-component regulatory systems controlling the pathogenicity genes in these strains (15, 26). Anti-LemA antiserum cross-reacted with the 101-kDa ApdA protein of Pf-5. Taken together, the sequence data, results from Western analysis, and the capacity of *apdA* to restore protease activity to a LemA$^-$ mutant of *P. syringae* provide convincing evidence that *apdA* is a homolog of *lemA*. Because ApdA$^-$ and GacA$^-$ mutants have similar phenotypes and because the sequences of *apdA* and *gacA* are similar to the sequences of genes encoding known sensor kinases and response regulators, respectively, we infer that these two loci constitute a two-component system in control of antibiotic production by *P. fluorescens*. This conclusion is in agreement with reports that *lemA* interacts with *gacA* to

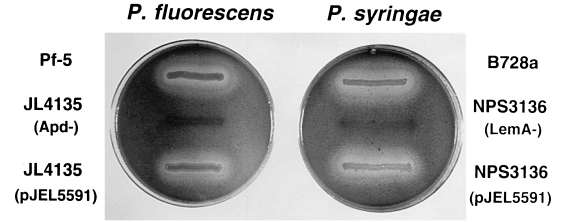

FIG. 4. Protease production on Bacto litmus milk agar showing complementation of JL4135, an ApdA$^-$ mutant of *P. fluorescens* Pf-5, and NPS3136, a LemA$^-$ mutant of *P. syringae* pv. syringae, by a plasmid containing the *apdA*$^+$ gene of Pf-5, pJEL5591. Clear zones around bacterial colonies indicate protease production.

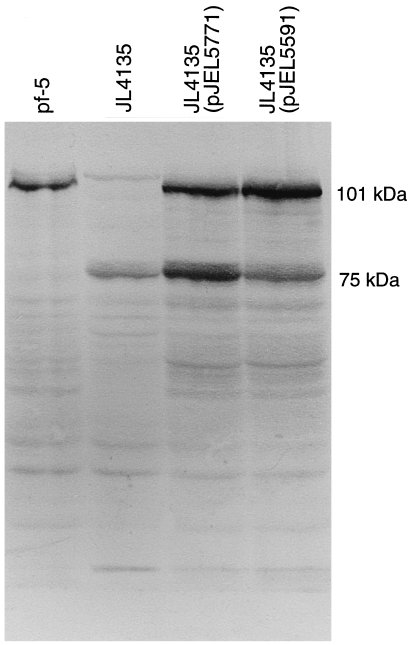

FIG. 5. Western blot analysis of proteins isolated from strains of *P. fluorescens* Pf-5 with anti-LemA antiserum. Lanes: 1, Pf-5(pRK415); 2, JL4135(pRK 415); 3, JL4135(pJEL5771); 4, JL4135(pJEL5591). The positions of the 101-kDa ApdA protein and the truncated (75-kDa) ApdA protein are indicated on the right.

regulate antibiotic biosynthesis by the biocontrol agent *P. fluorescens* BL915 (24) and the expression of pathogenicity genes in *P. syringae* (33, 45).

Sensor components of two-component regulatory systems are defined by the presence of a transmitter domain, which contains an ATP-binding site and a conserved histidine residue (31, 40). After autophosphorylation at the conserved histidine residue, the sensor protein typically serves as a kinase, transferring a phosphoryl group to the receiver domain of the cognate response regulatory protein; then the phosphorylated response regulator functions as a transcriptional activator of target genes (31). According to this model, sensor kinases control gene expression by controlling the concentration of the phosphorylated response regulator, which can be achieved through regulation of the autophosphorylation rate or kinase-versus-phosphatase activities of the sensor (40). Certain sensor proteins, including ApdA, LemA, ArcB, and BvgS, contain both transmitter and receiver domains (31) but lack a helix-turn-helix DNA binding domain (3), which is characteristic of response regulators. For at least two of these sensor proteins, ArcB and BvgS, conserved amino acids in both the transmitter and receiver domains are essential for efficient phosphorylation of the cognate response regulators (ArcA and BvgA) (18, 42). Intramolecular transphosphorylation, in which a phosphate group is transferred from the histidine in the transmitter domain to an aspartic acid residue in the receiver domain, is thought to cause conformational changes in the sensor protein that facilitate phosphorylation of the cognate response regulator (17, 42). A conserved histidine residue in the C-terminal region of ApdA, LemA, ArcB, and BvgS may serve as an additional site of phosphorylation; deletion of the C-terminal region destroys the function of BvgS (42). Thus, ApdA is a member of a family of complex sensor proteins which contain multiple cytoplasmic domains that are believed to participate in a phosphorylation cascade involved in signal transduction.

The transmembrane sensor kinases of two-component regulatory systems are thought to be autophosphorylated in response to a signal molecule(s), thereby mediating changes in gene expression in response to environmental signals (31). Although extracellular signals may trigger the autophosphorylation of these proteins directly, it may be just as common for sensor kinases to perform an intermediate role in signal transduction rather than to act as the receptor for an environmental signal. For example, at least some of the signals triggering autophosphorylation of the sensor protein ArcB are in the cytosol; these may be metabolites produced by *E. coli* under anaerobic conditions, which trigger the phosphorylation cascade regulating an array of genes responsive to O_2 tension (18). The natures of environmental or physiological signals to which ApdA responds have not been established. Because phenotypes under the control of *apdA* are expressed by Pf-5 in culture media (28) and the rhizosphere (23), autophosphorylation of ApdA is likely to be prompted by multiple signals or a single signal molecule that is commonly produced or encountered by *Pseudomonas* spp. in these diverse habitats. The identification of ApdA as a putative sensor kinase that serves as a global regulator of antibiotic biosynthesis genes provides an opportunity to identify the environmental cues to which *P. fluorescens* responds. Such information may have predictive value, allowing the identification of environmental conditions conducive to antibiotic production, and may also lead to the genetic improvement of biological control agents. For example, as more two-component systems are identified, it may be possible to exchange an input domain from a sensor with a known signal for the wild-type input domain of ApdA, thereby providing greater control over the expression of genes required for antifungal metabolite production by *P. fluorescens.*

It must be recognized, however, that bacterial signalling systems may rarely be as simple as a system composed of two proteins. In *Pseudomonas* spp., several regulatory cascades in which two-component systems play a role have been partially characterized. For example, alginate biosynthesis by *Pseudomonas aeruginosa* is coordinated by a complex regulatory cascade consisting of at least two response regulators, the alternate sigma factor AlgU, and regulators of AlgU; the sensor kinases for this system have yet to be identified (8). In *P. syringae*, the regulation of *hrp* (for hypersensitive response and pathogenicity) and avirulence (*avr*) genes is controlled by a multicomponent cascade involving HrpR, HrpS, and HrpL (46). HrpR and HrpS are believed to be response regulators that interact with σ^{54} to promote the transcription of *hrpL*, which codes for an alternate sigma factor similar to AlgU of *P. aeruginosa*. Then the RNA polymerase-HrpL holoenzyme activates the transcription of *hrp* and *avr* genes (46).

The regulation of secondary-metabolite biosynthesis by *P. fluorescens* may be as complicated as the systems mentioned above. For example, a regulatory locus coding for a stationary-phase sigma factor similar to *rpoS* has recently been identified in *P. fluorescens* Pf-5 (37). A mutation in this locus abolished the production of pyrrolnitrin and enhanced the production of pyoluteorin and 2,4-diacetylphloroglucinol. Such information leads us to speculate that antibiotic production by Pf-5 is controlled by a complex cascade of genetic regulation in which the *apdA-gacA* regulatory system may be only one layer.

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