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Autoinduction of 2,4-Diacetylphloroglucinol Biosynthesis in the Biocontrol Agent *Pseudomonas fluorescens* CHA0 and Repression by the Bacterial Metabolites Salicylate and Pyoluteorin†

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The antimicrobial metabolite 2,4-diacetylphloroglucinol (2,4-DAPG) contributes to the capacity of *Pseudomonas fluorescens* strain CHA0 to control plant diseases caused by soilborne pathogens. A 2,4-DAPG-negative Tn*5* insertion mutant of strain CHA0 was isolated, and the nucleotide sequence of the 4-kb genomic DNA region adjacent to the Tn*5* insertion site was determined. Four open reading frames were identified, two of which were homologous to *phlA*, the first gene of the 2,4-DAPG biosynthetic operon, and to the *phlF* gene encoding a pathway-specific transcriptional repressor. The Tn*5* insertion was located in an open reading frame, tentatively named *phlH*, which is not related to known *phl* genes. In wild-type CHA0, 2,4-DAPG production paralleled expression of a *phlA′-′lacZ* translational fusion, reaching a maximum in the late exponential growth phase. Thereafter, the compound appeared to be degraded to monoacetylphloroglucinol by the bacterium. 2,4-DAPG was identified as the active compound in extracts from culture supernatants of strain CHA0 specifically inducing *phlA′-′lacZ* expression about sixfold during exponential growth. Induction by exogenous 2,4-DAPG was most conspicuous in a *phlA* mutant, which was unable to produce 2,4-DAPG. In a *phlF* mutant, 2,4-DAPG production was enhanced severalfold and *phlA′-′lacZ* was expressed at a level corresponding to that in the wild type with 2,4-DAPG added. The *phlF* mutant was insensitive to 2,4-DAPG addition. A transcriptional *phlA-lacZ* fusion was used to demonstrate that the repressor PhlF acts at the level of transcription. Expression of *phlA′-′lacZ* and 2,4-DAPG synthesis in strain CHA0 was strongly repressed by the bacterial extracellular metabolites salicylate and pyoluteorin as well as by fusaric acid, a toxin produced by the pythopathogenic fungus *Fusarium*. In the *phlF* mutant, these compounds did not affect *phlA′-′lacZ* expression and 2,4-DAPG production. PhlF-mediated induction by 2,4-DAPG and repression by salicylate of *phlA′-′lacZ* expression was confirmed by using *Escherichia coli* as a heterologous host. In conclusion, our results show that autoinduction of 2,4-DAPG biosynthesis can be countered by certain bacterial (and fungal) metabolites. This mechanism, which depends on *phlF* function, may help *P. fluorescens* to produce homeostatically balanced amounts of extracellular metabolites.

Certain root-associated strains of fluorescent *Pseudomonas* spp. produce and excrete metabolites that are inhibitory to soilborne plant pathogens (13, 24, 52). Among these metabolites, 2,4-diacetylphloroglucinol (2,4-DAPG) has received particular attention because of its production by a wide range of pseudomonads used for the biological control of root diseases (13, 26, 50, 52). 2,4-DAPG is a phenolic compound with broad-spectrum antifungal, antibacterial, antihelminthic, and phytotoxic activity (13, 25, 52). A 2,4-DAPG biosynthetic gene cluster is conserved among numerous 2,4-DAPG-producing pseudomonads isolated from soils that are naturally suppressive to take-all of wheat, black root rot of tobacco, and tomato wilt caused by the fungal pathogens *Gaeumannomyces graminis*, *Thielaviopsis basicola*, and *Fusarium oxysporum*, respectively (26, 40). Indigenous populations of 2,4-DAPG-producing pseudomonads occurring at high densities in take-all suppressive soils are a key component of the natural biological control operating in these soils (38–40). Evidence for an important role of 2,4-DAPG in plant protection comes from studies on 2,4-DAPG-negative mutants of *Pseudomonas fluorescens* and nonproducing strains into which 2,4-DAPG biosynthetic plasmids have been transferred. By this approach, it has been demonstrated that 2,4-DAPG contributes to the control of black root rot of tobacco (25, 27), take-all of wheat (25, 52), *Pythium* damping-off of sugarbeet (18), bacterial soft rot of potato (10), and potato cyst nematodes (11). In situ detection of 2,4-DAPG in the rhizosphere, i.e., at the site of disease suppression, of plants treated with producing strains further supports the role of the metabolite in plant protection (6, 25, 32, 38).

The production of 2,4-DAPG by fluorescent *Pseudomonas* spp. is stimulated by glucose in many strains (15) or by sucrose or ethanol in a few strains (15, 49, 59). In addition, zinc sulfate and ammonium molybdate have been reported to favor 2,4-DAPG production in some strains, whereas inorganic phosphate in general has an inhibitory effect (15). The differential

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influence of carbon and mineral sources on 2,4-DAPG production in different *Pseudomonas* strains may reflect various degrees of adaptation to the nutrient palette available in a given root environment. Recently, Duffy and Défago (14) have identified fusaric acid, which is produced by the phytopathogen *F. oxysporum*, as an effector repressing 2,4-DAPG production in a biocontrol strain of *P. fluorescens*. This fungal toxin can lead to repression of bacterial 2,4-DAPG production on tomato roots, thereby abolishing biocontrol of *Fusarium* crown and root rot (14).

Four global regulators are known to control 2,4-DAPG production. A conserved two-component regulatory system composed of the sensor kinase GacS (formerly designated LemA) and the cognate response regulator GacA is essential for the synthesis of 2,4-DAPG, and other secondary metabolites and exoenzymes in root-colonizing biocontrol pseudomonads (5, 9, 30, 43, 57). The relative levels of the housekeeping sigma factor RpoD and of the stationary-phase and stress sigma factor RpoS also influence 2,4-DAPG synthesis. Amplification of the *rpoD* gene or mutational inactivation of the *rpoS* gene in *P. fluorescens* results in an overproduction of the antibiotics 2,4-DAPG and pyoluteorin and in improved control of certain root diseases (32, 45, 46, 57).

In the biological control agent *P. fluorescens* Q2-87, Bangera and Thomashow (2) have identified a gene cluster that comprises the 2,4-DAPG biosynthetic genes *phlACBD* flanked by genes encoding, respectively, a regulator (*phlF*) and an efflux protein (*phlE*). The *phlACBDE* operon is indispensable for the production of 2,4-DAPG and monoacetylphloroglucinol (MAPG), a potential precursor of 2,4-DAPG (2, 48). The divergently oriented *phlF* gene encodes a pathway-specific repressor (2), but little is known about the mechanism by which PhlF regulates the *phl* biosynthetic genes.

P. fluorescens strain CHA0 used in this study is an effective biocontrol agent of plant diseases caused by soilborne pathogenic fungi (24, 55). 2,4-DAPG, pyoluteorin, and hydrogen cyanide (HCN) are major biocontrol determinants in strain CHA0 (25, 27, 29, 31, 32, 46). Here we report on the identification of a genomic region encompassing the 2,4-DAPG biosynthetic gene *phlA* and the regulatory gene *phlF* in this strain. We demonstrate that expression of *phlA* is autoinduced by 2,4-DAPG and strongly repressed by the bacterial extracellular metabolites salicylate and pyoluteorin as well as by the fungal metabolite fusaric acid. Finally, we present evidence that this regulatory mechanism is mediated, at least in part, by the transcriptional repressor PhlF.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. *P. fluorescens* and *Escherichia coli* strains were routinely cultivated on nutrient agar plates (51), in Luria-Bertani broth (LB) (44), and in nutrient yeast broth (NYB) (51) with aeration. *P. fluorescens* and *E. coli* cultures were incubated, respectively, at 30 and 37°C, if not mentioned otherwise. For gene expression studies, *P. fluorescens* was grown in a minimal glucose-ammonium medium (OSG), containing 0.5% (wt/vol) D-glucose, 0.1% (wt/vol) ammonium sulfate, and 0.01% (wt/vol) Triton X-100, in addition to the salt solutions described by Ornston and Stanier (34). Production of DAPG, MAPG, and pyoluteorin by *P. fluorescens* was determined after growth of the bacteria in OSG or in a yeast-malt extract (YME) medium containing, per liter: yeast extract (Difco), 3 g; malt extract (Oxoid), 3 g; Bacto Peptone (Difco), 5 g; sucrose, 10 g; and L-asparagine, 1 g. Production of salicylate and pyochelin was assessed in glycerol-Casamino Acids medium (GCM) (33). For extraction of the signal compound inducing early *phl* gene expression, *P. fluorescens* was cultivated in GCM supplemented with 100 μM $FeCl_3 \cdot 6H_2O$ and 100 μM $ZnCl_2$ (GCM-Fe-Zn). Antimicrobial compounds, when required, were used at the following concentrations (micrograms per milliliter): ampicillin, 100; chloramphenicol, 25; gentamicin, 10; $HgCl_2$, 20; kanamycin sulfate, 25; and tetracycline hydrochloride, 25 for *E. coli* and 125 for *P. fluorescens* strains. When appropriate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was incorporated into solid media to monitor β-galactosidase expression (44). *F. oxysporum* f. sp. *radicis-lycopersici* strain FORL22 (obtained from C. Alabouvette, Institut National de la Recherche Scientifique Dijon, France) was routinely cultivated on malt agar (per liter, 15 g of Difco malt extract and 17 g of Serva agar) at 24°C.

DNA manipulations. Small-scale plasmid DNA preparations from *P. fluorescens* strains and isolation of pME3049- and pME3088-based plasmids from *E. coli* were done by alkaline lysis (44). All other plasmid preparations from *E. coli* were performed according to the method of Del Sal et al. (12). Qiagen columns (Qiagen Inc.) were used for large-scale plasmid DNA preparations. Chromosomal DNA of *P. fluorescens* was isolated as described by Gamper et al. (20). Standard techniques were used for restriction, agarose gel electrophoresis, dephosphorylation, generation of blunt ends with the Klenow fragment of *E. coli* DNA polymerase I or T4 DNA polymerase (Boehringer), isolation of DNA fragments from low-melting-point agarose gels, ligation, and transformation of *E. coli* by $CaCl_2$ treatment (44). Restriction fragments were purified from agarose gels using the Geneclean II kit (Bio 101). Electroporation of bacterial cells with plasmid DNA was done as described by Farinha and Kropinski (16). Southern blotting with Hybond N membranes (Amersham), random-primed DNA labeling with digoxygenin-11-dUTP, hybridization, and detection (Boehringer) were performed according to the protocols of the suppliers. For nucleotide sequence determination, subclones of pME6250S and pME6251H (Table 1; Fig. 1) were constructed in pBluescript II KS+ (Stratagene) or pUK21 (54). Nucleotide sequences reported here were determined on both strands with a Dye terminator kit (Perkin-Elmer product no. 402080) and an ABI PRISM 373 sequencer and, in part, by Genome Express (Grenoble, France). Nucleotide and deduced amino acid sequences were analyzed with programs of the University of Wisconsin Genetics Computer Group package (version 9.1).

Plasmid mobilization and transposition. Derivatives of the suicide plasmids pME3049 and pME3088 were mobilized from *E. coli* to *P. fluorescens* with the helper plasmid pME497 in triparental matings as described (47). Transposon mutagenesis using *E. coli* W3110 (1) containing the Tn*5* suicide plasmid pLG221 (7) as the donor strain and *P. fluorescens* CHA0 as the recipient strain was carried out as previously described (31).

To complement strain CHA638 (*phlF*::Ω-Km; construction described below), the *phlF* gene was introduced as a single copy into the chromosome using a Tn*7* delivery system. For this purpose, a gentamicin-resistant derivative of pUX-BF5 (3, 28), pME3280a, was constructed which carries translational and transcriptional termination signals from Ω-Sm (37) and the multiple cloning site of pUK21 (54). A 2.3-kb *Nru*I-*Sma*I fragment (Fig. 1) was then cloned into the above mini-Tn*7*-Gm carrier plasmid. The construct obtained, pME6825, and the helper plasmid pUX-BF13 (3) were coelectroporated (23) into the recipient strain CHA638. The single Tn*7*-*phlF* insertion in strain CHA638.*phlF*$^+$ was checked by Southern blotting (data not shown).

Construction of *P. fluorescens* mutants by gene replacement. To obtain the *phlA* in-frame mutant CHA631 (Fig. 1), the 639-bp *Bgl*II fragment within the *phlA* gene was deleted. The flanking genomic DNA, consisting of a 1.1-kb *Bgl*II fragment of pME6250S and a 5.8-kb *Bgl*II-*Bam*HI fragment of pME6250S, was cloned into the suicide vector pME3088 (55), which carries a tetracycline resistance determinant. For the construction of the *phlF* mutant CHA638 (Fig. 1), the 2.9-kb *Eco*RV-*Sma*I fragment of pME6250S was cloned into pME3088, followed by insertion of the Ω-Km fragment into the unique *Bam*HI site within the *phlF* gene. The pME3088 derivatives were mobilized with the helper plasmid pME497 to wild-type strain CHA0. Cells with a chromosomally integrated plasmid were selected for tetracycline resistance. Excision of the vector by a second homologous recombination event was carried out by enrichment for tetracycline-sensitive cells (41). Selection for kanamycin resistance ensured the presence of the Ω-Km insertion in the mutant strain CHA638. Both mutations were checked by Southern blotting (data not shown).

Extraction of a signal compound inducing expression of the *phlA* gene. To detect signal compounds in culture supernatants of *P. fluorescens* CHA0, the strain was grown in 2-liter Erlenmeyer flasks containing 500 ml of GCM-Fe-Zn medium to an optical density (OD) at 600 nm of 2.5. The culture supernatants were filtered through 0.45-μm-pore-size filters (Millipore), acidified to pH 5.0, and extracted three times with 1/3 volume of dichloromethane. The combined organic phases were dried over anhydrous $MgSO_4$, and the solvent was removed by rotary evaporation. The extract was dissolved in methanol-dichloromethane (2:98) and loaded on a silica gel column (ICN TSC 60Å; bed volume, 32 ml; ICN Biomedicals GmbH, Eschwege, Germany) equilibrated with the same solvent mixture. During stepwise elution with methanol-dichloromethane (2:98, 10:90, 20:80, 30:70, 40:60, 50:50, 100:0 [vol/vol]) at a flow rate of 1 ml min^{-1}, 14 fractions of 32 ml were collected; fractions 1 to 3, 4 to 6, 7 to 10, and 11 to 14 were pooled for further analysis, and solvents were removed by evaporation. The dry residues were dissolved in 50% (vol/vol), acetonitrile, and aliquots were tested for induction of *phlA* gene expression in strain CHA0 (see below). The extract of the positive fraction (pooled fractions 7 to 10) was further separated by high-pressure liquid chromatography (HPLC) with a Hewlett-Packard 1050 liquid chromatograph equipped with a diode array detector, using a reversed-phase column (250 by 4 mm) packed with Nucleosil 100-5-C18 (Bischoff, Leonberg, Germany) and protected by a precolumn (40 by 4 mm) packed with the same material. The extract was eluted at a flow rate of 1 ml min^{-1} by using a binary gradient of solvent A (0.1% [vol/vol] trifluoracetic acid) and solvent B (95% [vol/vol] acetonitrile in 0.1% [vol/vol] trifluoracetic acid) as follows: 0 to 13 min with 0 to 20% solvent B, 13 to 29 min with 20 to 46% solvent B, and 29 to 50 min

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype[a]	Reference or source
Strains		
P. fluorescens		
CHA0	Wild type	55
CHA630	*phlH*::Tn*5* Km^r	This study (Fig. 1)
CHA631	Δ*phlA*	This study (Fig. 1)
CHA638	*phlF*::Ω-Km Km^r	This study (Fig. 1)
CHA638.*phlF*$^+$	CHA638::miniTn*7*-Gm-*phlF*$^+$ Km^r Gm^r	This study
CHA89	*gacA*::Ω-Km Km^r	30
CHA207	Chromosomal *hcnA′-′lacZ* fusion	5
CHA805	Chromosomal *aprA′-′lacZ* fusion	5
CHA901	Chromosomal constitutive promoter fused to *lacZ*	23
E. coli		
DH5α	*recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* (ϕ80d*lacZ*Δ*M15*)	44
W3110	Prototroph, *sup*° λ$^-$	1
Plasmids		
pBluescript II KS+	Cloning vector; ColE1 replicon; Ap^r	Stratagene
pHP45 Ω-Km	ColE1 replicon; Ap^r	17
pLG221	Suicide vector; ColIb*drd-1*::Tn*5*; IncIα; Km^r	7
pME497	Mobilizing plasmid; IncP-1; Tra; RepA (Ts); Ap^r	56
pME3049	Suicide vector; ColE1 replicon; RK2-Mob; Hg^r Km^r	47
pME3088	Suicide vector; ColE1 replicon; RK2-Mob; Tc^r	55
pME3219	pME6010 with a 410-bp *hcnA* promoter fragment and an *hcnA′-′lacZ* translational fusion at the *Pst*I site in *hcnA*	29
pME3280a	Carrier plasmid for Tn*7*; containing the mini-Tn*7*-Gm transposon with translational and transcriptional signals; pUC19*ori*; Ap^r Gm^r	This study
pME6010	Cloning vector; pACYC177-pVS1 shuttle vector; Tc^r	22
pME6014	Cloning vector for construction of translational *lacZ* fusions; derived from pME6010; Tc^r	This study
pME6016	Cloning vector for construction of transcriptional *lacZ* fusions; derived from pME6010; Tc^r	This study
pME6030	Cloning vector; pACYC177-pVS1 shuttle vector; Tc^r	22
pME6250B	pME3049 with a 1.7-kb genomic fragment of *P. fluorescens* CHA630	This study (Fig. 1)
pME6250S	pME3049 with a 11.1-kb genomic fragment of *P. fluorescens* CHA630	This study (Fig. 1)
pME6251H	pME3049 with a 9.8-kb genomic fragment of *P. fluorescens* CHA0	This study (Fig. 1)
pME6256	pUK21 with a 2-kb *Bam*HI-*Sph*I fragment of pME6250S	This study
pME6259	pME6014 with a 1.1-kb *Bgl*II fragment of pME6256, containing a fragment of 0.7 kb upstream of *phlA* and a *phlA′-′lacZ* translational fusion at the *Bgl*II site in *phlA*	This study (Fig. 1)
pME6261	pME6030 with a 2-kb *Bam*HI-*Xho*I fragment of pME6256, containing *phlA*	This study (Fig. 1)
pME6284	pBluescript II KS+ with a 2.9-kb *Eco*RV-*Sma*I fragment of pME6250S	This study
pME6710	pME6016 with a 1.1-kb *Bgl*II fragment of pME6256, containing a *phlA-lacZ* transcriptional fusion at the *Bgl*II site in *phlA*	This study
pME6824	pUC18 with a 2.3-kb *Nru*I-*Spe*I fragment of pME6284, containing *phlF*	This study
pME6825	pME3280a with a 2.3-kb *Kpn*I-*Hin*dIII fragment of pME6824, containing *phlF*	This study (Fig. 1)
pUC18	pBR322- and M13mp18-derived cloning vector; *lacZ*α; Ap^r	58
pUK21	pUC118/pUC119-derived cloning vector; *lacZ*α; Km^r	54
pUX-BF13	Helper plasmid for Tn*7*-based transposon mutagenesis containing the transposition functions; R6K replicon; Ap^r	3

[a] Ap, ampicillin; Hg, mercury; Km, kanamycin; Gm, gentamicin; Tc, tetracycline.

with 46 to 80% solvent B. Fractions were collected between 0 to 13 min, 13 to 22 min, 22 to 29 min, and 29 to 50 min and monitored by UV absorption at 210 and 280 nm. The retention time and UV spectrum of the peak showing *phlA*-inducing activity (see below) were found to be identical to those of authentic 2,4-DAPG (25).

Assay for induction and repression of *phlA′-′lacZ* expression. *P. fluorescens* CHA0, its mutant derivatives, and *E. coli* strains carrying a *phlA′-′lacZ* translational fusion on plasmid pME6259 (Table 1; Fig. 1) were grown in 20 ml of OSG medium or NYB without selective antibiotics in 100-ml Erlenmeyer flasks sealed with cellulose stoppers. For inoculation, 40-μl aliquots of exponential-growth-phase LB cultures of the bacterial strains diluted to an OD at 600 nm of 0.05 were used. Cultures were incubated with rotational shaking at 180 rpm. When appropriate, NYB was supplemented with crude or purified dichloromethane extracts corresponding to a volume of 50 ml of culture supernatant of strain CHA0. Extracts were dissolved in 100 μl of 50% (vol/vol) acetonitrile. Likewise, effectors added to OSG medium (2,4-DAPG, MAPG, pyoluteorin, salicylate, fusaric acid, benzoate, and acetophenone) were dissolved in ethanol, except for fusaric acid, which was dissolved in 20% (vol/vol) ethanol (pH 7). Controls received the same amount of the respective solvent. In all experiments, β-galactosidase specific activities of at least three independent cultures were determined throughout the exponential and stationary growth phases by the method of Miller (44).

Isolation of the 2,4-DAPG-negative mutant CHA630. Each of 1,500 Tn*5*-induced mutants was cultivated at 24°C for 24 h with shaking (150 rpm) in 25-ml bottles containing 10 ml of YME medium. The cultures were acidified to pH 2.0 and extracted with the same volume of ethyl acetate. The ethyl acetate phase was analyzed by HPLC for 2,4-DAPG and MAPG as described previously (25, 31, 32). 2,4-DAPG-negative candidates were checked for the production of pyoluteorin, HCN, extracellular protease, tryptophan side chain oxidase (TSO), pyoverdine-dependent fluorescence, auxotrophic defects, and growth characteristics in different media by using established methods (25, 27, 31).

Quantification of antibiotic and siderophore production. Production of 2,4-DAPG, MAPG, and pyoluteorin was assessed for bacteria grown in 100-ml Erlenmeyer flasks with 20 ml of OSG medium. Bacteria were inoculated as described above (see assay for induction and repression of *phlA′-′lacZ*). To monitor degradation of 2,4-DAPG by *P. fluorescens*, 100 μM synthetic 2,4-DAPG

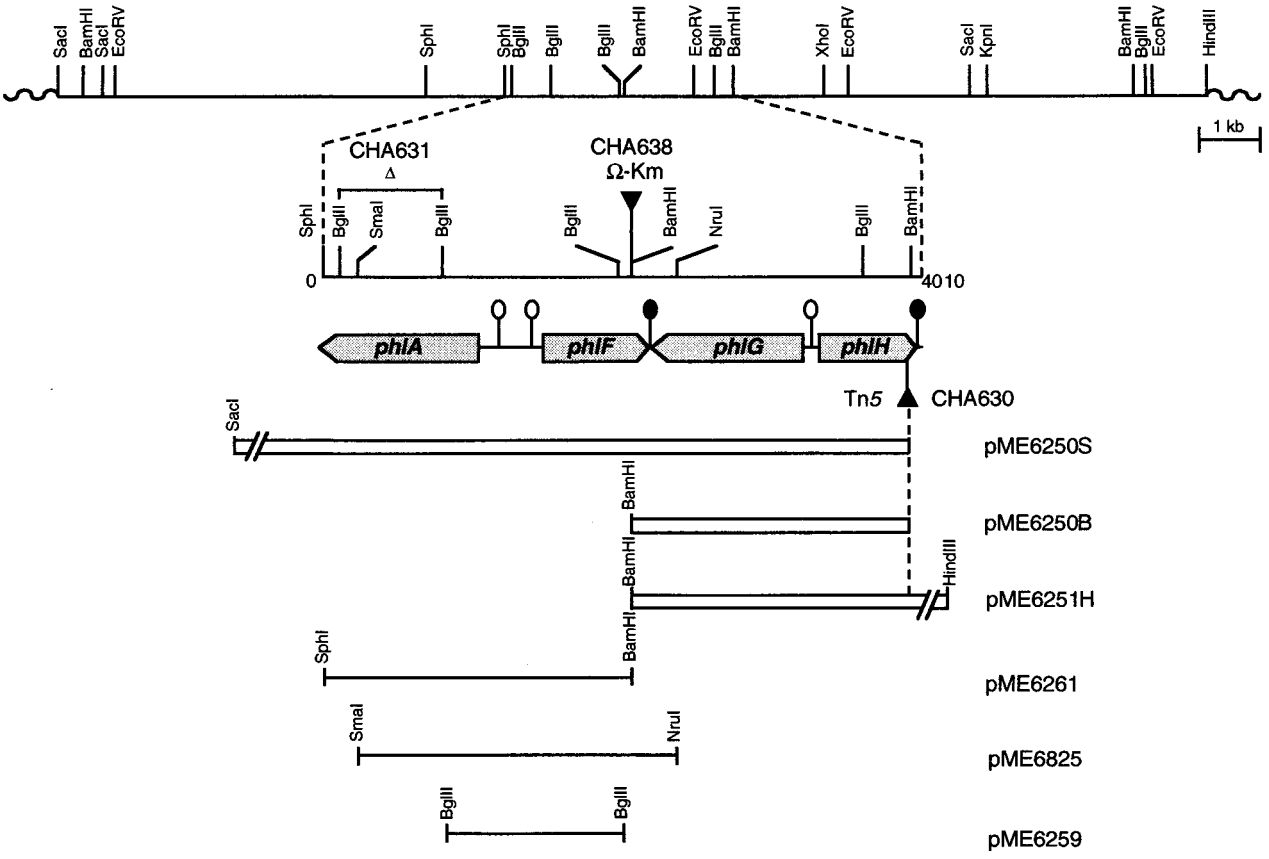

FIG. 1. Physical location of the *phlA*, *-F*, *-G*, and *-H* genes in *P. fluorescens* CHA0. ▼, Tn*5* insertion in the chromosome of strain CHA630 and Ω-Km fragment insertion in strain CHA638; ♀, inverted repeats; ●, putative rho-independent transcription terminators; △, region deleted in strain CHA631. The shaded arrows show the genes sequenced. The open boxes below indicate the genomic inserts in the ColE1-based plasmids pME6250S, pME6250B, and pME6251H. The bars designate the fragments cloned into the vectors pME6030, pME3280a, and pME6014 to give pME6261, pME6825, and pME6259, respectively.

was added to the OSG medium and incubated for up to 170 h in presence or absence of the 2,4-DAPG- and MAPG-negative mutant CHA631 or CHA630. Cultures were incubated at 24 or 30°C with rotational shaking at 180 rpm. 2,4-DAPG, MAPG, and pyoluteorin were extracted with acetonitrile using silica C_{18} cartridges (Sep-Pak; Waters) as described elsewhere (4). Pyochelin and salicylate were extracted with ethyl acetate from acidified culture supernatants (42) of bacteria grown in 30 ml of GCM for 72 h. Extracted compounds were identified and quantified by established HPLC procedures (25, 31, 32, 42).

Effect of *Fusarium* on 2,4-DAPG production by *P. fluorescens*. The plate assay was performed on malt agar, which promotes 2,4-DAPG production, and with *Bacillus subtilis* as a highly 2,4-DAPG-sensitive indicator organism (25). A 0.5-mm circular plug from a 3-week-old culture of *F. oxysporum* f. sp. *radicis-lycopersici* was placed at the edge of the plate and incubated at 24°C for 5 days, prior to addition of the bacteria. Suspensions of washed bacterial cells were prepared from exponential-growth-phase LB cultures and adjusted to an OD at 600 nm of 1.5. Aliquots (5 μl) of the cell suspensions were spotted on the plates (two spots per plate) at 1.5 cm from the edge of the fungal colony. Plates without addition of the fungus were used as controls. Plates were then further incubated at 24°C for 18 h. Thereafter, bacteria were killed by exposure to UV light and overlaid with 4 ml of soft agar (NYB with 0.5% [wt/vol] agar) mixed with 400 μl of an LB overnight culture of *B. subtilis*. After overnight incubation at 28°C, inhibition of *B. subtilis* was assessed by measuring the diameter of growth inhibition and subtracting the diameter of the *P. fluorescens* spot. Fusaric acid produced by the fungus was quantified as described by Duffy and Défago (14).

Nucleotide sequence accession number. The nucleotide sequence of the *phlA*, *-F*, *-G*, and *-H* genes of *P. fluorescens* CHA0 has been assigned GenBank accession no. AF207529.

RESULTS

Isolation and characterization of the 2,4-DAPG-negative mutant CHA630. Following Tn*5* mutagenesis of *P. fluorescens* strain CHA0, one out of 1,500 mutants tested was unable to produce detectable amounts of 2,4-DAPG and MAPG in YME and OSG media (Table 2). The mutant strain, CHA630, was compared with the wild-type strain CHA0 for HCN, extracellular protease, salicylate, pyochelin, and fluorescent pigment production, TSO activity, prototrophy, and growth characteristics in different media, and no difference was found (data not shown). However, the mutant produced small amounts of pyoluteorin in OSG medium, whereas strain CHA0 grown to the same cell density did not (Table 2). Southern hybridization showed that the mutant CHA630 contained a single Tn*5* insertion (data not shown). The Tn*5* insertion was located to position 3617 in an open reading frame (ORF) termed *phlH* (see below and Fig. 1).

Cloning of the wild-type genes surrounding the Tn*5* insertion in strain CHA630. The genomic region adjacent to Tn*5* in strain CHA630 was obtained by using the two-step cloning-out procedure described by Schnider et al. (47). The suicide plasmid pME3049, which carries a mercury resistance and the kanamycin resistance determinant of Tn*5*, was mobilized into strain CHA630. The integration of pME3049 into the chromosome occurred at a frequency of 10^{-6} to 10^{-7} per donor. Genomic DNA of a recombinant with a chromosomally integrated plasmid was digested with *Bam*HI, ligated, and used to transform *E. coli* DH5α. The resulting plasmid pME6250B (Fig. 1) carries a 1.7-kb genomic DNA insert. The chromosome of strain CHA630::pME3049 was also digested with *Sac*I. Self-ligation and transformation of *E. coli* led to plasmid

TABLE 2. Antibiotic production by *P. fluorescens* CHA0 and its derivatives

Strain	Relevant genotype	Antibiotic production (μM)[a]		
		2,4-DAPG	MAPG	Pyoluteorin
CHA0	Wild type	24.0 ± 2.4	3.7 ± 0.9	BD[b]
CHA630	*phlH*::Tn*5*	BD	BD	3.5 ± 0.6
CHA631	Δ*phlA*	BD	BD	3.9 ± 0.2
CHA631/pME6030	Δ*phlA*/−	BD	BD	4.5 ± 0.4
CHA631/pME6261	Δ*phlA*/*phlA*$^+$	22.5 ± 3.8	24.5 ± 4.9	0.1 ± 0.1
CHA638	*phlF*::Ω-Km	90.4 ± 25.4	172.0 ± 10.9	BD
CHA638.*phlF*$^+$	*phlF*::Ω-Km/*phlF*$^+$	8.2 ± 1.3	1.3 ± 0.3	BD
CHA89	*gacA*::Ω-Km	BD	BD	BD

[a] Bacteria were grown in OSG medium at 24°C to an OD at 600 nm of 3.5. Each value is the mean ± standard deviation from three experiments.
[b] BD, below detection limit (<0.08 μM for 2,4-DAPG, <1.2 μM for MAPG, and <0.1 μM for pyoluteorin).

pME6250S (Fig. 1) carrying an extended insert of 11.1 kb downstream of the Tn*5* insertion site. For the isolation of the wild-type genes, plasmid pME6250B was transferred into the wild-type strain CHA0. The chromosomal DNA of CHA0::pME6250B was digested with *Hin*dIII and ligated. Plasmid pME6251H (Fig. 1), containing a 9.8-kb genomic fragment, was selected.

Sequence analysis of the *phlA*, *-F*, *-G*, and *-H* genes. The nucleotide sequence of 4,010 bp (expanded region shown in Fig. 1) revealed two ORFs designated *phlA* and *phlF*, by analogy with homologous genes of *P. fluorescens* Q2-87 (2), as well as two new ORFs named *phlG* and *phlH* (Fig. 1). This sequence contained 57.9% G+C. The deduced product (362 amino acids, 38.5 kDa) of the *phlA* gene of strain CHA0 showed 83.3% identity with its homolog PhlA, the first gene product of the 2,4-DAPG biosynthetic operon in strain Q2-87 (2). The intergenic region upstream of *phlA*, encompassing 462 nucleotides, is relatively AT rich (56.0%) and contains two inverted repeats (Fig. 1). The divergently oriented *phlF* gene appears to start at a GTG codon, and its product (200 amino acids, 22.9 kDa) is 84.5% identical over the entire sequence with PhlF, a repressor protein involved in the regulation of 2,4-DAPG biosynthesis, of *P. fluorescens* Q2-87 (2) and F113 (GenBank accession no. AF129856). An identical, putative helix-turn-helix motif ($^{34}GYX_3SIX_2VX_5ASXPXIYXWWX NKX_2L^{64}$), which is typical of DNA-binding regulatory proteins, exists near the PhlF N terminus of strains CHA0, Q2-87, and F113. The average amino acid change per codon score (8) was 0.76; scores of >0.8 are considered to be highly indicative of a helix-turn-helix motif (8). Downstream of *phlF* there is an inverted repeat that could function as a transcription terminator (ΔG, −21.9 kcal mol^{-1}) (Fig. 1). The adjacent ORF, tentatively named *phlG*, could encode a protein, PhlG (320 amino acids, 36.3 kDa), which shows no similarities to protein sequences with known functions deposited in the SwissProt and the EMBL GenBank. The deduced product PhlH (223 amino acids, 24.3 kDa) of the adjacent ORF shows similarities to regulatory proteins, e.g., IfeR of *Agrobacterium tumefaciens* (AF039653; 34% identity), a putative transcriptional regulator of *Streptomyces coelicolor* (AL035569; 29% identity), AcrR, a potential AcrAB operon repressor of *E. coli* (P34000; 21% identity), and members of the TetR/AcrR family of a series of other microorganisms (data not shown). The similarities in the deduced amino acid sequences of these proteins are concentrated within approximately 100 residues of the N termini; beyond this region, the proteins bear little resemblance to one another (data not shown). The ORF *phlH* is followed by an inverted repeat (ΔG, −17.0 kcal mol^{-1}) (Fig. 1) likely to be a transcription terminator.

Construction of *phlA* and *phlF* mutants. A *phlA* in-frame deletion mutation was created in the chromosome of strain CHA0 (Fig. 1) as described in Materials and Methods. In contrast to the wild type, the *phlA* mutant CHA631 obtained did not produce measurable amounts of 2,4-DAPG and MAPG in OSG medium (Table 2). 2,4-DAPG and MAPG production in strain CHA631 could be restored by complementation with pME6261 (Table 2), a plasmid which carries *phlA* under its own promoter (Fig. 1), whereas introduction of the cloning vector pME6030 alone had no effect. These results are in accordance with the findings of Bangera and Thomashow (2) and confirm the requirement of the biosynthetic gene *phlA* for 2,4-DAPG production.

A chromosomal *phlF* mutant, CHA638, was constructed by insertion of the transcription termination element Ω-Km. Compared with the wild-type strain CHA0, the mutant CHA638 overproduced 2,4-DAPG about fourfold when grown in OSG medium at 24°C (Table 2) or 30°C (data not shown) to early stationary phase at an OD at 600 nm of 3.5. For restoration of the disrupted gene, *phlF*$^+$ was inserted as a single copy via a Tn*7*-based system into the chromosome of strain CHA638. In the resulting strain CHA638.*phlF*$^+$ the formation of 2,4-DAPG and MAPG was repressed to levels that were lower than those in the wild type, for reasons that are not understood (Table 2). Nevertheless, these results provide experimental evidence for PhlF acting as a repressor of 2,4-DAPG biosynthesis.

The *phlA* mutant CHA631, but not the wild-type strain CHA0 and the *phlF* mutant CHA638, excreted the antibiotic pyoluteorin (about 4 μM) when grown in OSG medium (Table 2). In the iron-poor GCM medium, the wild-type and both mutant strains produced the same amounts of the siderophores salicylate (about 60 μM) and pyochelin (about 40 μM). In addition, the mutants were indistinguishable from the wild type in terms of HCN production, fluorescence, production of extracellular enzymes, and growth in different media (data not shown).

Growth-dependent *phlA* expression and 2,4-DAPG production, and bacterial degradation of 2,4-DAPG to MAPG. Expression of a *phlA'-'lacZ* translational fusion carried by pME6259 (Table 1; Fig. 1) was monitored in strain CHA0 growing in OSG medium (Fig. 2). Biosynthesis of 2,4-DAPG and MAPG was determined in parallel. Expression of *phlA* occurred from the mid-exponential to the early stationary growth phase (Fig. 2). Thereafter, β-galactosidase activity slowly declined, as expected for the long half-life of the enzyme. The kinetics of 2,4-DAPG and MAPG production paralleled expression of the *phlA'-'lacZ* reporter construct in strain CHA0 (Fig. 2). 2,4-DAPG and MAPG synthesis started

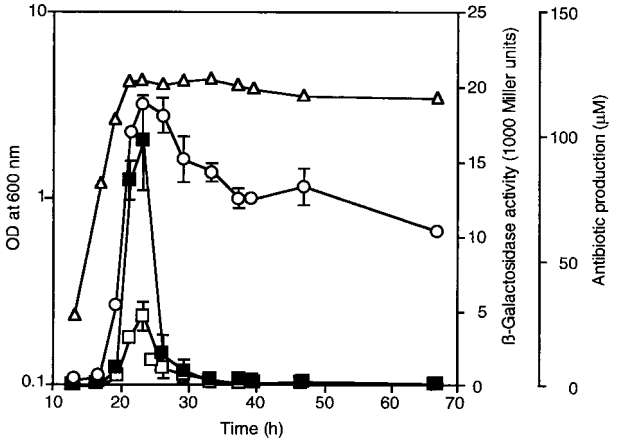

FIG. 2. Production of 2,4-DAPG (■) and MAPG (□) and expression of a *phlA'-'lacZ* translational fusion in a growing culture of *P. fluorescens* CHA0. Strain CHA0 carrying the *phlA* reporter construct on plasmid pME6259 was cultivated in OSG medium at 30°C. At different ODs at 600 nm (△), antibiotic production and specific β-galactosidase activities (○) were determined as described in Materials and Methods. Means ± standard deviations from three experiments are shown. Some of the error bars are too small to be shown.

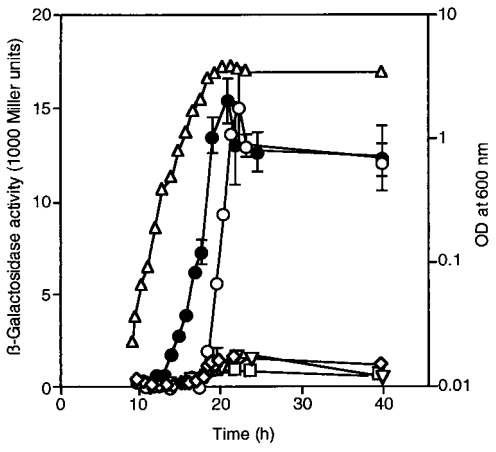

FIG. 4. Induction by 2,4-DAPG and repression by salicylate, pyoluteorin, and fusaric acid of *phlA'-'lacZ* expression in growing cultures of *P. fluorescens* CHA0 harboring pME6259. Specific β-galactosidase activities were determined for strain CHA0/pME6259 cultivated in OSG medium supplemented with 100 μM 2,4-DAPG (●), 100 μM pyoluteorin (□), 500 μM salicylate (◇), 500 μM fusaric acid (▽), or no effector (○). ODs at 600 nm (△) are shown for CHA0/pME6259 grown in OSG without effector added. Addition of effectors to the medium had no effect on cell growth (data not shown). Means ± standard deviations from at least nine experiments are shown. Some of the error bars are too small to be shown.

at mid-exponential growth phase, and the compounds were accumulated until the beginning of stationary growth phase, with 2,4-DAPG reaching a maximum concentration of about 100 μM (Fig. 2). Thereafter, 2,4-DAPG and MAPG concentrations in the medium steadily decreased, due to degradation by the bacterium. This was confirmed by the observation that 100 μM synthetic 2,4-DAPG added to OSG medium remained stable for at least 1 week in the absence of bacteria but was no longer detectable after a 30-h incubation in the presence of the 2,4-DAPG- and MAPG-negative mutants CHA631 (Fig. 3) or CHA630 (data not shown). Interestingly, degradation of 2,4-DAPG by strain CHA631 was succeeded by a temporary accumulation of MAPG in the growth medium (Fig. 3), suggesting that MAPG can be a breakdown product of 2,4-DAPG.

Expression of *phlA* is induced by 2,4-DAPG. To identify signals inducing *phlA* expression, late-exponential-phase, cell-free culture supernatants from strain CHA0 were extracted with dichloromethane. The extract enhanced expression of the *phlA'-'lacZ* reporter 15- to 20-fold in a CHA0 background growing in NYB, a medium which does not support 2,4-DAPG and MAPG production. The inducing signal was purified by chromatography on silica gel and by preparative HPLC. A single active peak was isolated with a retention time (19.5 min) and a UV spectrum identical to that of authentic 2,4-DAPG (25). Addition of 300 μM synthetic 2,4-DAPG to an NYB culture induced *phlA'-'lacZ* expression in CHA0 to a similar level as did the crude dichloromethane extract (data not shown), thus confirming the identification of the signal.

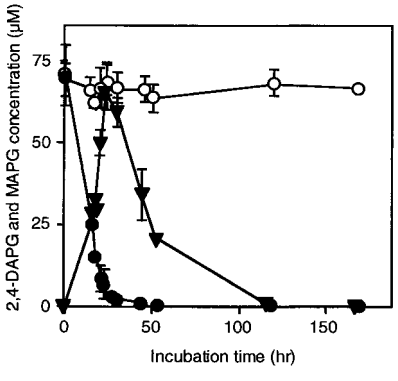

FIG. 3. Degradation of 2,4-DAPG to MAPG by a growing culture of the 2,4-DAPG- and MAPG-negative *phlA* mutant CHA631. Before bacterial inoculation, 100 μM 2,4-DAPG was added to OSG medium. After different incubation periods at 24°C, concentrations of 2,4-DAPG in absence of bacteria (○) and of 2,4-DAPG (●) and MAPG (▼) in the presence of CHA631 were determined. Means ± standard deviations from four experiments are shown.

The effect of added 2,4-DAPG on *phlA'-'lacZ* expression was further analyzed in OSG, a medium which supports production of up to 100 μM 2,4-DAPG by strain CHA0 (Fig. 2). Addition of 100 μM synthetic 2,4-DAPG to OSG medium inoculated with CHA0/pME6259 advanced the expression of *phlA'-'lacZ* to the early exponential growth phase, and *phlA'-'lacZ* expression during exponential growth was induced sixfold by 2,4-DAPG (Fig. 4; Table 3). However, *phlA'-'lacZ* expression at the end of the exponential growth phase was the same with or without addition of 2,4-DAPG (Fig. 4). A five-fold-higher concentration of 2,4-DAPG did not further promote *phlA'-'lacZ* expression (Table 3). Remarkably, 100 or 500 μM MAPG also induced *phlA'-'lacZ* expression, although to a significantly lesser extent than did 2,4-DAPG (Table 3). In NYB amended with 2,4-DAPG, the maximal level of *phlA'-'lacZ* expression in CHA0 was similar to that observed in OSG medium (data not shown), suggesting that exogenously added 2,4-DAPG is an effective signal. To test whether induction of *phlA* expression by 2,4-DAPG was specific, we also assayed derivatives of strain CHA0 carrying a chromosomal *lacZ* fusion either in the HCN biosynthetic gene *hcnA* (strain CHA207) or fused to an artificial constitutive promoter (strain CHA901). No differences in β-galactosidase specific activities could be detected throughout exponential and stationary growth of these strains in OSG medium amended or not with 100 μM 2,4-DAPG (data not shown). In conclusion, it appears that 2,4-DAPG induction is specific for *phlA* expression.

TABLE 3. Influence of PhlF and different effectors on the expression of a *phlA'-'lacZ* fusion in *P. fluorescens* CHA0 and in *E. coli* DH5α

Strain[a]	Effector added	β-Galactosidase activity (Miller units)[b]	Relative expression[c]
CHA0 (wild type)	None	2,087 ± 144	1.0
	2,4-DAPG (100 μM)	12,092 ± 454	5.8
	2,4-DAPG (500 μM)	11,884 ± 394	5.7
	MAPG (100 μM)	6,463 ± 557	3.1
	MAPG (500 μM)	5,540 ± 805	2.6
	Salicylate (50 μM)	753 ± 55	0.4
	Salicylate (500 μM)	973 ± 325	0.5
	Pyoluteorin (100 μM)	756 ± 4	0.4
	Fusaric acid (500 μM)	599 ± 171	0.3
	Acetophenone (500 μM)	2,293 ± 822	1.1
	Benzoate (500 μM)	2,595 ± 257	1.2
CHA638 (*phlF*::ΩKm)	None	13,989 ± 1124	1.0
	2,4-DAPG (100 μM)	12,090 ± 628	0.9
	Salicylate (500 μM)	11,907 ± 554	0.9
	Fusaric acid (500 μM)	11,353 ± 182	0.8
CHA638.*phlF*$^+$ (*phlF*::ΩKm/*phlF*$^+$)	None	756 ± 19	1.0
	2,4-DAPG (100 μM)	4,687 ± 243	6.2
	Salicylate (500 μM)	271 ± 36	0.4
DH5α	None	1,396 ± 35	1.0
	2,4-DAPG (100 μM)	1,350 ± 54	0.9
	Salicylate (500 μM)	1,397 ± 93	1.0
DH5α/pME6824 (*phlF*$^+$)	None	110 ± 6	1.0
	2,4-DAPG (100 μM)	174 ± 14	1.6
	Salicylate (500 μM)	70 ± 13	0.6

[a] *P. fluorescens* strains were grown in OSG medium at 30°C. *E. coli* strains were cultured in NYB medium at 37°C. All bacterial strains carried the reporter construct pME6259 containing a translational *phlA'-'lacZ* fusion.

[b] Determined in bacterial cultures grown to an OD at 600 nm of 3.0 ± 0.1, i.e., at late exponential growth phase. Data represent means ± standard deviation from at least three experiments.

[c] Expression of *phlA'-'lacZ* in the relevant bacterial strain grown in the presence of a given effector relative to the expression of the reporter construct with no effector applied.

Salicylate and pyoluteorin, extracellular metabolites of *P. fluorescens* CHA0, repress *phlA* expression and 2,4-DAPG production. To evaluate the specificity of induction by 2,4-DAPG further, we tested other extracellular metabolites of *P. fluorescens* CHA0, i.e., salicylate and pyoluteorin, for their effect on the expression of *phlA'-'lacZ*. Addition of 500 μM salicylate or 100 μM pyoluteorin to OSG medium repressed *phlA* expression throughout exponential and stationary growth of CHA0/pME6259 up to 10-fold, without affecting bacterial growth (Fig. 4; Table 3). Addition of salicylate also nearly completely abolished 2,4-DAPG and MAPG production by CHA0/pME6259 at any growth stage in OSG medium (data not shown). Salicylate added at a 10-fold-lower concentration had the same repressive effect on *phlA'-'lacZ* expression (Table 3) and on the production of the two metabolites (data not shown). In a control experiment, 500 μM salicylate had no effect on the expression of chromosomal *lacZ* fusions in *hcnA* or in the protease biosynthetic gene *aprA* in the CHA0 derivatives CHA207 and CHA805 grown under the same experimental conditions. Likewise, salicylate did not affect expression of an *hcnA'-'lacZ* fusion carried by plasmid pME3219 (Table 1) in a CHA0 background (data not shown). As additional controls, benzoate and acetophenone, two compounds having some structural resemblance to 2,4-DAPG and MAPG, were shown to have no significant effect on the expression of the *phlA'-'lacZ* reporter (Table 3). Taken together, these results suggest that *phl* biosynthetic genes as well as 2,4-DAPG and MAPG production in *P. fluorescens* CHA0 can be repressed by the bacterium's own extracellular metabolites salicylate and pyoluteorin.

Autoinduction of *phlA* by 2,4-DAPG requires the repressor PhlF. To study the regulation of the *phl* biosynthetic genes, the *phlA'-'lacZ* reporter construct pME6259 was transferred to several mutants of *P. fluorescens* CHA0, which were grown in OSG medium. In the 2,4-DAPG- and MAPG-negative mutant CHA631 (Δ*phlA*), *phlA'-'lacZ* expression was about 10-fold lower than in wild-type strain CHA0 in the exponential growth phase (Fig. 4 and 5A). Addition of 100 μM 2,4-DAPG to the medium led to induction of *phlA* expression in CHA631 in the exponential growth phase, although the final level was lower than that observed for the wild type (Fig. 5A). In the 2,4-DAPG- and MAPG-negative *phlH* mutant CHA630, the same partial restoration of *phlA* expression by added 2,4-DAPG could be observed (data not shown), suggesting that additional factors may be required for wild-type-level expression of *phlA*.

In the *phlF* mutant CHA638, *phlA'-'lacZ* was expressed earlier than in the wild type (Fig. 5B), confirming the role of PhlF as a repressor and explaining the fourfold increased 2,4-DAPG production in strain CHA638 (Table 2). Expression of *phlA'-'lacZ* in the *phlF* mutant was not influenced by the addition of 2,4-DAPG (Fig. 5B). Intriguingly, *phlA* expression in the *phlF* mutant CHA638 was not repressed by the addition of 500 μM salicylate (Table 3), and production of 2,4-DAPG and MAPG was diminished only by 20 to 25% at any growth stage (data not shown). Growth of strain CHA638 was not affected by 2,4-DAPG or salicylate. In the complemented mutant

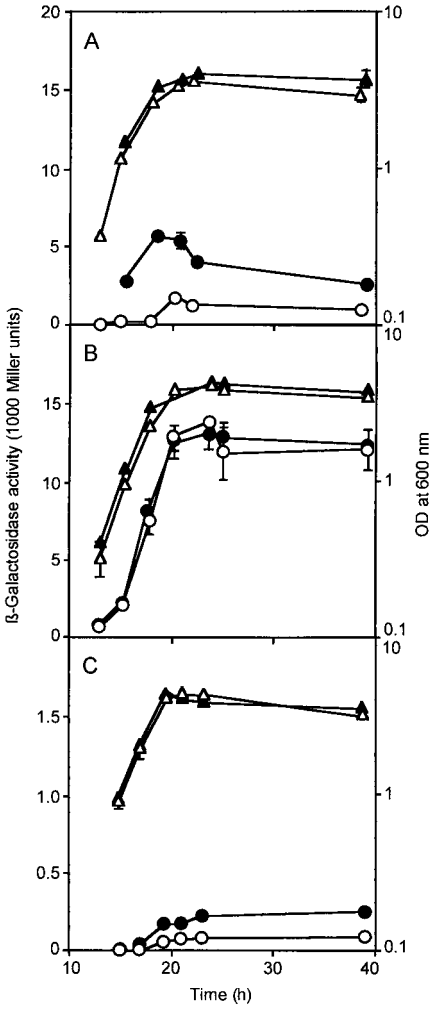

FIG. 5. Effect of 2,4-DAPG on the expression of a *phlA'-'lacZ* translational fusion carried by pME6259 in the *P. fluorescens phlA* mutant CHA631 (A), in the *phlF* mutant CHA638 (B), and in the *gacA* mutant CHA89 (C). Specific β-galactosidase activities were determined for strains grown in OSG medium at 30°C without (○) or with (●) addition of 100 μM 2,4-DAPG ODs at 600 nm for cultures grown in the absence (△) and presence (▲) of 2,4-DAPG are shown. Means ± standard deviations from at least three experiments are shown. Some of the error bars are too small to be shown.

CHA638.*phlF*$^+$, induction by 2,4-DAPG and repression by salicylate of *phlA'-'lacZ* expression was similar to that observed in the wild-type strain CHA0, although for unknown reasons the reporter construct was expressed at a threefold-lower level (Table 3).

To test whether *phlA* gene expression is regulated at the level of transcription, a transcriptional *phlA-lacZ* fusion carried by pME6710 (Table 1) was introduced into the wild-type strain CHA0, the *phlA* mutant CHA631, and the *phlF* mutant CHA638. As in the case of the translational *phlA'-'lacZ* fusion (Fig. 4, 5A, and 5B; Table 3), the expression of the transcriptional *phlA-lacZ* fusion was fourfold lower in the *phlA* mutant (1,440 ± 163 Miller units) and sixfold higher in the *phlF* mutant (34,810 ± 1,240 Miller units) than in wild-type strain CHA0 (5,690 ± 140 Miller units) during the late exponential growth phase (OD at 600 nm of 3.0). These results confirm that *phlA* expression is regulated positively by 2,4-DAPG and negatively by PhlF at the transcriptional level.

PhlF-mediated regulation was confirmed in a heterologous host, *E. coli* DH5α, expressing the *phlA'-'lacZ* fusion on pME6259, with or without the *phlF* gene on pME6824 (Table 1). In strain DH5α carrying the *phlA'-'lacZ* reporter alone, addition of 100 μM 2,4-DAPG or 500 μM salicylate had no effect on *phlA* gene expression (Table 3). When the strain carried also the *phlF* gene, the expression of the *phlA* reporter was repressed about 10-fold (Table 3), confirming the role of PhlF as a *phl* repressor. The strong repression was probably due to the high copy number of plasmid pME6824. Expression of *phlA* was significantly induced (1.6-fold) by 2,4-DAPG and repressed (1.6-fold) by salicylate when *phlF* was present in DH5α (Table 3). Poor uptake of the effectors by *E. coli* might be the reason for the low induction and repression factors.

P. fluorescens CHA0 requires the global response regulator GacA for 2,4-DAPG production (Table 2) (30). Accordingly, the expression of the *phlA'-'lacZ* fusion on pME6259 was strongly lowered in the *gacA* mutant CHA89 and was only marginally induced by 2,4-DAPG (Fig. 5C).

Fusaric acid, a fungal metabolite, represses *phlA* gene expression and 2,4-DAPG production. Fusaric acid, a mycotoxin produced by *Fusarium* spp., represses 2,4-DAPG production in strain CHA0 in vitro and on tomato roots (14). The effect of added fusaric acid (500 μM) on *phlA'-'lacZ* expression in strain CHA0/pME6259 was similar to that observed for salicylate and pyoluteorin. Fusaric acid repressed the expression of *phlA* by a factor of up to 10, depending on the growth stage (Fig. 4; Table 3). Likewise, biosynthesis of 2,4-DAPG and MAPG was diminished to levels close to the detection limit when the strain was grown in presence of 50 or 500 μM fusaric acid (data not shown). Fusaric acid appears to act specifically on *phl* biosynthetic genes, since CHA0 derivatives carrying *hcnA'-'lacZ* fusions in the chromosome (CHA207) or on a plasmid (pME3219) were not affected by the compound (data not shown). The *phlF* mutant CHA638 was largely insensitive to fusaric acid with respect to *phlA* gene expression (Table 3) and 2,4-DAPG production (data not shown), and the fungal metabolite did not affect bacterial growth.

In a plate assay, *F. oxysporum* f. sp. *radicis-lycopersici* interfered with 2,4-DAPG production in *P. fluorescens* CHA0 but not in a *phlF* mutant. This was shown by growing bacterial strains in the presence or absence of *Fusarium* on malt agar plates, a medium which promotes 2,4-DAPG production by *P. fluorescens* CHA0 (25). 2,4-DAPG production by *P. fluorescens* strains was monitored as clear inhibition zones in the growth of *B. subtilis*, used as 2,4-DAPG-sensitive indicator (25). In the absence of *Fusarium*, inhibition zones obtained with the *phlF* mutant CHA638 were 18% ± 4% larger than those produced by the wild-type strain CHA0, thus illustrating derepression of 2,4-DAPG production in the *phlF* mutant. When the fungal pathogen was present on the same plate, inhibition zones produced by strain CHA0 were significantly smaller (17% ± 6%), whereas no reduction of inhibitory activity could be observed for the *phlF* mutant. Controls showed that the fungus had produced 4.3 μM fusaric acid on this medium. In summary, fusaric acid represses *phlA* expression via PhlF, and this fungal interference can be demonstrated in vivo.

DISCUSSION

Autoinduction of 2,4-DAPG biosynthesis. This study shows, for the first time, that expression of *phlA*, the first gene of the 2,4-DAPG biosynthetic operon (2), is specifically autoinduced by 2,4-DAPG (Fig. 4; Table 3) and to a lesser extent by MAPG (Table 3). This positive autoregulation was evident in a *phlA* mutant, CHA631, in which exogenously added 2,4-DAPG

compensated for the lack of 2,4-DAPG and MAPG production and partially restored *phlA* expression (Fig. 5A). The inducing effect of exogenous 2,4-DAPG was most pronounced in a medium (NYB) which does not promote the production of 2,4-DAPG by strain CHA0. Other examples of autoinduction in bacteria are known. The best-documented mechanism is the autoinduction of the biosynthesis of *N*-acyl-homoserine lactones, i.e., diffusible molecules that mediate cell-to-cell communication, commonly known as quorum sensing, in many gram-negative bacteria (19). Positive autoregulation of biosynthetic genes has also been described for the siderophores pyochelin in *P. aeruginosa* (42) and yersiniabactin in *Yersinia enterolitica* (35). Furthermore, indole-3-acetic acid was found to upregulate the expression of the *ipdC* gene encoding a key enzyme of its own biosynthetic pathway in *Azospirillum brasilense* (53). Finally, early work by Gutterson (21) proposed positive feedback regulation as a mechanism controlling the biosynthesis of oomycin A, an antifungal compound produced by *P. fluorescens* Hv37a.

Our work confirms the proposed role of PhlF as a pathway-specific repressor (2) acting at the transcriptional level and establishes PhlF as a mediator of autoinduction by 2,4-DAPG. Two observations support our findings. First, a *phlF* mutant, CHA638, was insensitive to 2,4-DAPG addition and kinetics of *phlA* expression corresponded to those in the wild-type CHA0 with 2,4-DAPG added (Fig. 4 and 5B). Second, *phlF* was required for 2,4-DAPG-induced *phlA* expression in the heterologous host *E. coli* (Table 3). PhlF may bind to the promoter(s) of *phlA*, thereby repressing the expression of the 2,4-DAPG biosynthetic operon. 2,4-DAPG might bind to PhlF and thereby prevent the interaction of the repressor protein with the promoter, leading to increased expression of the 2,4-DAPG biosynthetic genes. This autoinduction circuit can be boosted by the global response regulator GacA (5, 30), as shown by the strong GacA dependence of *phlA* expression (Fig. 5C).

Repression of 2,4-DAPG biosynthesis. The 2,4-DAPG autoinduction circuit in *P. fluorescens* CHA0 was shown to be blocked by salicylate or pyoluteorin (Fig. 4; Table 3), both extracellular metabolites produced by this strain and numerous other pseudomonads (24, 55). Moreover, fusaric acid, a pathogenicity factor of *F. oxysporum*, also strongly repressed *phlA* expression (Fig. 4; Table 3), confirming, at a molecular level, earlier observations by Duffy and Défago (14) that the fungal metabolite abolishes 2,4-DAPG production by strain CHA0 in vitro and in the rhizosphere of tomato plants. PhlF seems to have a mediator role, since the presence of an intact *phlF* gene was required for repression of *phlA* expression by salicylate, pyoluteorin, or fusaric acid (Table 3). One possible explanation could be that the repressing compounds might directly or indirectly compete with 2,4-DAPG for binding sites on PhlF.

Based on our observations, it is tempting to speculate that relative concentrations of 2,4-DAPG, salicylate, pyoluteorin, and perhaps other extracellular metabolites might be sensed by *P. fluorescens* and maintained at homeostatically balanced levels. For instance, the data in Table 2 indicate that 2,4-DAPG levels are inversely correlated with pyoluteorin concentration and pyoluteorin-negative mutants of strain CHA0 overproduce 2,4-DAPG and MAPG (31). Positive feedback regulation and repression by extracellular metabolites might allow the bacterium to adapt to and fine-tune levels of these extracellular metabolite in response to environmental changes and to avoid escalation of the autoinduction loop.

Kinetics of 2,4-DAPG biosynthesis. In *P. fluorescens* CHA0, 2,4-DAPG and MAPG were accumulated until the beginning of stationary growth phase (Fig. 2), as is typical for secondary metabolites. Surprisingly, thereafter the concentrations of the two metabolites in the growth medium steadily decreased. Initially 2,4-DAPG appears to be degraded to MAPG, which could then be further metabolized to compounds that have not been identified. At a temperature (i.e., 18°C) that is closer to that found in the natural habitat, accumulation and degradation rates of 2,4-DAPG were slowed down and the period of maximal concentrations was doubled compared to that at 30°C (data not shown). Why should maximal concentrations of a major biocontrol compound be available to the bacterium only during a limited period? As long as the physiological functions of 2,4-DAPG remain unknown, it is difficult to provide a good answer. Maybe 2,4-DAPG-mediated self-defense against competitors and predators is more effective when it does not operate permanently in the biocontrol bacterium. Our findings have two important implications. First, they illustrate that kinetic studies, rather than point measurements, can be important in studies on the regulation of antibiotic biosynthesis in *P. fluorescens*. Second, degradation of 2,4-DAPG to MAPG by strain CHA631 adds a further level of complexity to positive autoregulation and kinetics of 2,4-DAPG biosynthesis. Previously, MAPG has been proposed to be a direct precursor of 2,4-DAPG. A genomic region encoding an acetyltransferase activity capable of converting MAPG to 2,4-DAPG has been described for *P. fluorescens* strain F113 (13, 48). In *P. fluorescens* strain Q2-87, the products of the 2,4-DAPG biosynthetic genes *phlACB* are necessary for the conversion of MAPG to 2,4-DAPG (2).

Implications for biocontrol. From an ecological point of view, rapid accumulation of the biocontrol compound 2,4-DAPG via positive autoregulation may be advantageous since it may allow the bacterium to respond promptly to competition with other microorganisms, including plant pathogens, in the rhizosphere. At a population level, the capacity of *P. fluorescens* to perceive exogenous 2,4-DAPG as a signal inducing 2,4-DAPG biosynthesis potentially implies a novel way of communication within or between populations of 2,4-DAPG producers. In this way, the 2,4-DAPG pool within a homogenous or mixed *P. fluorescens* population could rapidly be boosted to levels that are relevant to pathogen control (38). The importance of extracellular signal molecules for the communication within and between populations of fluorescent pseudomonads has recently been demonstrated for *N*-acyl-homoserine lactone-mediated expression of phenazine antibiotic genes in the rhizosphere of wheat (36). The potential for negative cross talk is illustrated by the fact that the fungal pathogen *F. oxysporum* was shown to break the autoregulatory mechanism (Fig. 4; Table 3) (14).

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