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Characterization of PhlG, a Hydrolase That Specifically Degrades the Antifungal Compound 2,4-Diacetylphloroglucinol in the Biocontrol Agent *Pseudomonas fluorescens* CHA0

Mélanie Bottiglieri and Christoph Keel*

Department of Fundamental Microbiology, University of Lausanne, CH-1015 Lausanne, Switzerland

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The potent antimicrobial compound 2,4-diacetylphloroglucinol (DAPG) is a major determinant of biocontrol activity of plant-beneficial *Pseudomonas fluorescens* CHA0 against root diseases caused by fungal pathogens. The DAPG biosynthetic locus harbors the *phlG* gene, the function of which has not been elucidated thus far. The *phlG* gene is located upstream of the *phlACBD* biosynthetic operon, between the *phlF* and *phlH* genes which encode pathway-specific regulators. In this study, we assigned a function to PhlG as a hydrolase specifically degrades DAPG to equimolar amounts of mildly toxic monoacetylphloroglucinol (MAPG) and acetate. DAPG added to cultures of a DAPG-negative Δ*phlA* mutant of strain CHA0 was completely degraded, and MAPG was temporarily accumulated. In contrast, DAPG was not degraded in cultures of a Δ*phlA* Δ*phlG* double mutant. To confirm the enzymatic nature of PhlG in vitro, the protein was histidine tagged, overexpressed in *Escherichia coli*, and purified by affinity chromatography. Purified PhlG had a molecular mass of about 40 kDa and catalyzed the degradation of DAPG to MAPG. The enzyme had a k_{cat} of 33 s^{-1} and a K_m of 140 μM at 30°C and pH 7. The PhlG enzyme did not degrade other compounds with structures similar to DAPG, such as MAPG and triacetylphloroglucinol, suggesting strict substrate specificity. Interestingly, PhlG activity was strongly reduced by pyoluteorin, a further antifungal compound produced by the bacterium. Expression of *phlG* was not influenced by the substrate DAPG or the degradation product MAPG but was subject to positive control by the GacS/GacA two-component system and to negative control by the pathway-specific regulators PhlF and PhlH.

Among the variety of microorganisms inhabiting the rhizosphere, certain strains of fluorescent pseudomonads have received particular attention because of their potential to function as biological agents for the control of soilborne pathogenic fungi and oomycetes that attack plants roots and cause considerable damage to crops worldwide (17, 21). Root-colonizing fluorescent pseudomonads produce a diversity of extracellular metabolites with antimicrobial activity, some of which have a determinative role in disease suppression (16, 46). Important antibiotic compounds for which a major contribution to biocontrol has been demonstrated include 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, phenazines, pyrrolnitrin, cyclic lipopeptides, and hydrogen cyanide (16, 17). In general, effective biocontrol pseudomonads produce at least one of these diffusible or volatile antibiotics. Some strains, such as *Pseudomonas fluorescens* CHA0 and Pf-5, produce multiple antibiotics with overlapping or different degrees of activity against specific pathogens (16, 30).

DAPG is one of the *Pseudomonas* antibiotics that have been intensively studied during the past years. DAPG is a phenolic compound (Fig. 1) displaying a remarkably broad spectrum of toxic activity against bacteria, fungi, nematodes, and, at higher concentrations, plants (7, 23, 24, 34, 46). DAPG is produced by numerous fluorescent *Pseudomonas* strains that have been isolated from diverse soils worldwide and have the capacity to control one or several soilborne diseases (22, 30, 32, 45). For instance, DAPG has been shown to be a major determinant in the protection by *P. fluorescens* CHA0 of tobacco against black root rot (23, 24), by *P. fluorescens* strains Q2-87 and CHA0 of wheat against take-all (24, 46), and by *P. fluorescens* F113 of sugar beet against *Pythium* damping-off (14). Recent work has established indigenous populations of DAPG-producing pseudomonads as a key biological factor of the natural suppressiveness of certain agricultural soils to take-all of wheat and black root rot of tobacco (11, 22, 32, 33, 49). In addition to its direct antimicrobial activity, DAPG may also function as a signal inducing systemic plant resistance against pathogens (20). Besides DAPG, other phloroglucinol compounds have been isolated from fluorescent pseudomonads, including monoacetylphloroglucinol (MAPG) and triacetylphloroglucinol (TAPG) (Fig. 1) (4, 7, 34, 39, 44) and condensation products of DAPG (13, 25). It has been proposed that MAPG, which exhibits only mild toxic activity (7, 25, 34), may be a direct precursor (4, 44) and/or a degradation product (39) of DAPG.

DAPG is synthesized and accumulated until the early stationary growth phase, as is typical for secondary metabolites (1, 3, 8, 31, 39). Thereafter, the metabolite appears to be degraded by the producing bacterium, with MAPG temporarily accumulating as an intermediary product of the degradation process (39). The chromosomal region required for the biosynthesis of DAPG has been identified in several pseudomonads, including *P. fluorescens* strains Q2-87, F113, CHA0, and Pf-5 (4, 10, 30, 39). The DAPG locus comprises the four biosynthetic genes *phlACBD* that are transcribed as a single operon and are required for the synthesis of both MAPG and DAPG (4, 10, 39). The products of the *phlACB* genes also mediate the conversion

* Corresponding author. Mailing address: Département de Microbiologie Fondamentale, Bâtiment de Biologie, Université de Lausanne, CH-1015 Lausanne-Dorigny, Switzerland. Phone: 41 21 692 56 36. Fax: 41 21 692 56 05. E-mail: christoph.keel@unil.ch.

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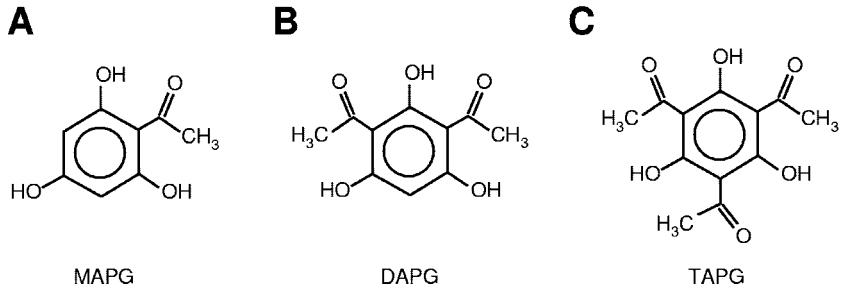

FIG. 1. Structures of three acylphloroglucinols produced by fluorescent pseudomonads. (A) Monoacetylphloroglucinol (MAPG); (B) 2,4-diacetylphloroglucinol (DAPG); (C) triacetylphloroglucinol (TAPG).

of MAPG to DAPG (4). The *phlE* gene located immediately downstream of the *phlACBD* operon encodes a putative transmembrane permease (4) which appears to be implicated in DAPG resistance (1). The divergently transcribed *phlF* gene located adjacent to *phlA* codes for a pathway-specific transcriptional repressor of the DAPG biosynthetic operon (4, 10, 39). The repression by the TetR-like regulator PhlF is due to its interaction with specific binding sites in the *phlA* promoter region (2, 16). DAPG acts as the signal that dissociates PhlF from the *phlA* promoter, thereby autoinducing its own biosynthesis (2, 16, 39). In *P. fluorescens* CHA0, the *phlH* gene located downstream of *phlF* encodes a second TetR-like regulator involved in pathway-specific control of DAPG synthesis (39). However, the precise role of PhlH remains to be elucidated.

Besides the pathway-specific regulators PhlF and PhlH, a number of global regulatory elements may directly or indirectly influence DAPG biosynthesis in response to environmental signals and to the physiological status of the bacterial cell (16). The two-component system GacS/GacA positively controls the production of DAPG and a series of other exoproducts via a regulatory cascade involving regulatory RNAs and RNA-binding proteins (16, 17, 50). Relative cellular levels of several σ factors may also profoundly affect DAPG synthesis (16). They include the housekeeping σ factor RpoD, the stationary-phase and stress response σ factor RpoS, and the alternative σ factor RpoN (31, 36, 38). In addition, many abiotic and biotic environmental factors may modulate levels of DAPG production, including different carbon and nitrogen sources, transition metal ions and other minerals, and metabolites released by bacteria, fungi, and plants (12, 27, 28). For instance, DAPG biosynthesis in *P. fluorescens* strains CHA0 and Pf-5 is negatively affected by pyoluteorin, another antifungal compound synthesized by these bacteria (3, 8, 39). In the same vein, the bacterial and plant metabolite salicylate, as well as the fungal pathogenicity factor fusaric acid, strongly inhibits DAPG production (3, 39).

Previously, we have identified *phlG* as a further gene contained within the DAPG biosynthetic locus of *P. fluorescens* CHA0 (39), a strain that serves as a model biocontrol bacterium in our laboratory. The *phlG* gene is located between *phlF* and *phlH* (Fig. 2), and its role in DAPG biosynthesis has not been elucidated thus far. In the present study, we demonstrate that *phlG* encodes an enzyme that catalyzes the conversion of the potent antibiotic DAPG to less-toxic MAPG, with a high specificity for its substrate. We report on the factors that influence the activity of the PhlG enzyme. Finally, we illustrate that *phlG* expression is controlled by the pathway-specific regulators PhlF and PhlH and by the two-component system GacS/GacA.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. The bacterial strains and plasmids used in the present study are described in Table 1. *P. fluorescens* strains were routinely cultivated on nutrient agar (NA), in nutrient yeast broth (NYB), and in Luria-Bertani broth (LB), at 30°C (3, 31, 39). Production and degradation of antifungal compounds was monitored in King's medium B broth supplemented with 0.75% (wt/vol) malt extract (KMBmalt) (3). A minimal glycerol-ammonium (OSGly) medium (3) was used for gene expression studies. *Escherichia coli* strains were grown on NA and in NYB at 37°C. Antibiotics were added to the growth media at the following concentrations: chloramphenicol at 10 μg/ml, kanamycin sulfate at 25 μg/ml, and tetracycline hydrochloride at 25 μg/ml for *E. coli* and at 125 μg/ml for *P. fluorescens* strains.

DNA manipulations and sequencing. Chromosomal DNA of *P. fluorescens* was prepared as described elsewhere (39). Small- and large-scale plasmid preparations were performed with the cetyltrimethylammonium bromide method (35) and the Jetstar 2.0 kit (Genomed, Basel, Switzerland), respectively. Standard techniques were used for restriction, agarose gel electrophoresis, isolation of DNA fragments from low-melting-point agarose gels, and ligation (35, 39, 40). Restriction fragments were purified from agarose gels using the QIAGEN gel extraction kit (QIAGEN, Hombrechtikon, Switzerland). Bacterial cells were transformed with plasmid DNA by electroporation (39). PCRs were carried out according to amplification protocols described in detail previously (3, 31). Nucleotide sequences of PCR-derived constructs were determined on both strands by Microsynth (Balgach, Switzerland). Nucleotide and deduced amino acid sequences were analyzed with programs of the European Molecular Biology Open Software Suite (http://www.ch.embnet.org/EMBOSS).

Construction of *phlG* in-frame deletion mutants of *P. fluorescens* and complementation by monocopy *phlG*$^+$. For the construction of the Δ*phlG* mutant CHA1091 and the Δ*phlA* Δ*phlG* double mutant CHA1092, a 900-bp fragment was deleted in-frame in the *phlG* gene (Fig. 2) of strain CHA0 and its *phlA* mutant CHA631, respectively, as follows. A 597-bp fragment, including the first five codons of *phlG* and the adjacent upstream region, was amplified by PCR with primers MBP22 and MBP23. A 603-bp fragment including the last three codons of *phlG* and the neighboring downstream region was amplified by PCR with primers MBP20 and MBP26. The resulting upstream and downstream fragments were digested with HindIII and XbaI and with XbaI and EcoRI, respectively, and cloned by a triple ligation into pUK21 digested with EcoRI and HindIII, yielding plasmid pME8019 (Table 1). The 1,200-bp EcoRI-HindIII insert in pME8019 was checked by sequencing, excised, and cloned into the suicide plasmid pME3087. The resulting plasmid pME8020 (Fig. 2) was then integrated into the chromosome of strains CHA0 or CHA631 by triparental mating using *E. coli* HB101/pME497 as the mobilizing strain, with selection for tetracycline- and chloramphenicol-resistant recombinants. Excision of the vector by a second crossing-over occurred after enrichment for tetracycline-sensitive cells (39, 40).

Strain CHA1092 (Δ*phlA* Δ*phlG*) was complemented for the *phlG* defect by introduction of a single copy of intact *phlG*$^+$ into the chromosome using a mini-Tn*7* delivery system (39, 50). For this purpose, a 1,303-bp fragment, encompassing *phlG* and a 226-bp upstream region (Fig. 2), was amplified by PCR

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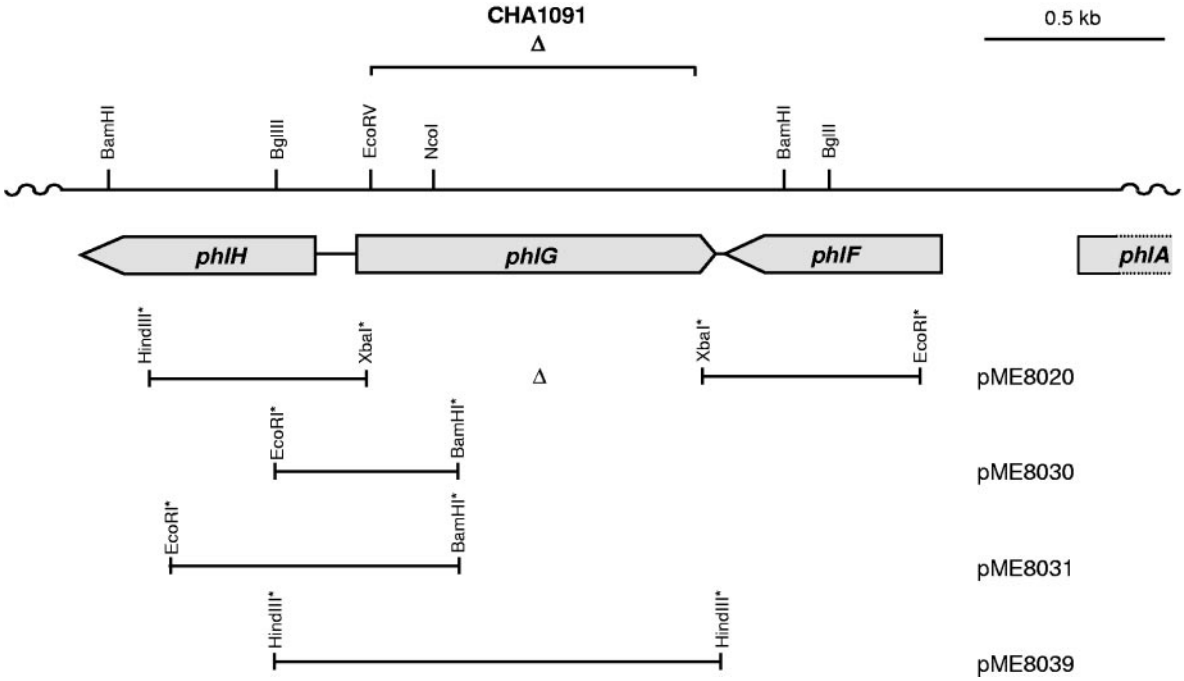

FIG. 2. Physical location of *phlG* in the DAPG biosynthetic locus of *P. fluorescens* strain CHA0. The *phlG* gene is located upstream of *phlA*, i.e., the first DAPG biosynthetic gene, and is flanked by *phlH* and *phlF* which encode pathway-specific transcriptional regulators (16, 39). △, Region deleted in strains CHA1091 and CHA1092 and in plasmid pME8020. The genes are indicated by shaded arrows. For *phlA*, only the 5′ end is shown. The horizontal bars designate the fragments cloned into vector pME3087 to give pME8020, into pME6015 to give pME8030 and pME8031, and into pME6182 to give pME8039.

from *P. fluorescens* CHA0 with primers MBP52 and MBP53, digested with HindIII, and cloned into the mini-Tn*7*-Gm carrier plasmid pME6182 (31). The construct obtained, pME8039 (Fig. 2), and the Tn*7* transposition helper plasmid pUX-BF13 (5) were coelectroporated into the recipient strain CHA1092 to produce strain CHA1094.

Construction of translational *lacZ* fusions to the *phlG* gene. A first *phlG′-'lacZ* fusion was constructed as follows. Primers MBP37 and MBP38 containing artificial restriction sites for EcoRI and BamHI, respectively, served to amplify the 226-bp upstream region and the first 83 codons of the *phlG* gene. The PCR product was cloned into pUK21. The 474-bp EcoRI-BamHI fragment from the resulting plasmid was fused in-frame with the *'lacZ* gene in vector pME6015 (Table 1), to produce the *phlG′-'lacZ* reporter pME8030 (Fig. 2). A second *phlG′-'lacZ* fusion with a 495-bp upstream region and the first 83 codons of *phlG* was constructed in the same way using primers MBP41 and MBP38, yielding plasmid pME8031 (Fig. 2). Both fusions were checked by sequencing.

Assay for monitoring *phlG* gene expression. *P. fluorescens* CHA0 and its derivatives carrying *phlG′-'lacZ* fusions on plasmids pME8030 and pME8031 were grown in 100-ml Erlenmeyer flasks containing 20 ml of OSGly broth. For inoculation, aliquots of 20 μl of cell suspensions prepared from exponential-growth-phase LB cultures of the bacterial strains and adjusted to an optical density (OD) at 600 nm of 0.1 were added per flask. When appropriate, OSGly medium was supplemented with synthetic DAPG or MAPG dissolved in acetonitrile. Cultures were incubated at 30°C with rotational shaking at 180 rpm. β-Galactosidase specific activities were determined by the method of Miller (35).

Quantification of DAPG, MAPG, and pyoluteorin. Production of DAPG, MAPG, and pyoluteorin was assessed for bacteria grown in 100 ml of KBMmalt broth in 300-ml flasks. Each flask was inoculated with a single colony of the bacterial strains grown on NA for 24 h. To monitor bacterial degradation of DAPG, 100 μM synthetic DAPG was added to KBMmalt broth and incubated for up to 65 h in presence or absence of the 2,4-DAPG- and MAPG-negative mutants CHA631 (Δ*phlA*) or CHA1092 (Δ*phlA* Δ*phlG*). Cultures were incubated at 27°C with rotational shaking at 150 rpm. DAPG, MAPG, and pyoluteorin were extracted with ethyl acetate from acidified culture supernatants and quantified by established high-performance liquid chromatography (HPLC) procedures as described before (24, 27, 39).

Overexpression of histidine-tagged PhlG and preparation of cell extracts. For histidine-tagging of PhlG, the *phlG* gene was amplified by PCR using primers MBP39 and MBP40 with CHA0 DNA as the template. The PCR product was digested with XbaI and HindIII, and the resulting 968-bp fragment was cloned into the expression vector peT28a (Novagen, Dietikon, Switzerland), giving plasmid pME8032 (Table 1). This placed *phlG* under the control of the T7 promoter and added six histidine codons to the 3′ end of the gene. The insert was checked by sequencing. For overexpression of the PhlG-His6 protein, pME8032 was electroporated into *E. coli* strain BL21(DE3) and the transformant was grown in NYB at 37°C with orbital shaking at 180 rpm. When the culture reached an OD at 600 nm of 0.8, PhlG-His6 expression was induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 1 mM. After further incubation for 2 h, cells were harvested by centrifugation and washed twice with an 0.1 M potassium phosphate resuspension buffer (pH 7.0) containing 5 mM KCl, 10 mM $MgCl_2$, 10% (vol/vol) glycerol, and 5 mM β-mercaptoethanol. Approximately 1 g of the cell pellets were resuspended in 5 ml of buffer and sonicated six times for 30 s. The cell debris and the soluble fraction in the crude cell extracts were then separated by centrifugation for 30 min at 12,000 × *g* and 4°C.

Purification of histidine-tagged PhlG. For purification of PhlG-His6, cell extracts were prepared from IPTG-induced cultures of *E. coli* strain BL21(DE3)/pME8032 as described above, except that cell pellets (approximately 1 g per 5 ml of buffer) were resuspended in an 0.5 M potassium phosphate buffer (pH 7.0) containing 300 mM KCl, 5% glycerol, 5 mM β-mercaptoethanol, and 20 mM imidazole. Prior to sonication, 1 mM Pefabloc SC serine protease inhibitor (Roche) was added to the suspensions. After centrifugation of the cell extracts, PhlG-His6 was purified from the supernatants by Ni-nitrilotriacetic acid (NTA)-agarose chromatography as recommended by the manufacturer (QIAGEN). Briefly, 4-ml portions of the supernatants were mixed with 1 ml of Ni-NTA resin by gentle agitation during 30 min. The mixtures were loaded on mini-columns for gravity-flow chromatography. For washing the resins and for elution of PhlG-His6 from the Ni-NTA columns, imidazole concentrations in the potassium phosphate buffer were increased to 40 and 250 mM, respectively. The eluate was dialyzed against 10 mM Tris-acetate (pH 7.0) and used for enzymatic assays. All purification steps were carried out at 4°C. Expression and purification of PhlG-

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TABLE 1. Bacterial strains, plasmids and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristics[a] or sequence (5′ → 3′)	Reference or source
Strains		
P. fluorescens		
CHA0	Wild type	48
CHA19	Δ*gacS*	50
CHA630	*phlH*::Tn*5*; Km^r	39
CHA631	Δ*phlA*	39
CHA638	*phlF*::ΩKm; Km^r	39
CHA1091	Δ*phlG*	This study
CHA1092	Δ*phlA* Δ*phlG*	This study
CHA1094	CHA1092::*att*Tn*7*-*phlG*$^+$; Gm^r	This study
E. coli		
BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-)*gal dcm* (λDE3)	Novagen
DH5α and HB101	Laboratory strains	35
Plasmids		
peT28a	Expression vector designed to produce His_6 tag fusions	Novagen
pME497	Mobilizing plasmid; IncP-1, Tra; RepA(Ts); Ap^r	48
pME3087	Suicide vector; ColE1 replicon; RK2-Mob; Tc^r	48
pME6010	Cloning vector; pACYCI77-pVS1 shuttle vector; Tc^r	18
pME6015	Cloning vectors for construction of translational *lacZ*-fusions; derived from pME6010; Tc^r	39
pME6182	Carrier plasmid for Tn*7* containing the mini-Tn*7*-Gm transposon; Ap^r Gm^r	31
pME6259	*phlA′-′lacZ* fusion; Tc^r	39
pME8019	pUK21 carrying a 1,200-bp EcoRI-HindIII insert with a deletion in *phlG*; Km^r	This study
pME8020	pME3087 carrying a 1,200-bp EcoRI-HindIII insert with a deletion in *phlG*; Tc^r	This study
pME8030	pME6015 with a 226-bp *phlG* upstream fragment and a translational *phlG′-′lacZ* fusion containing the first 83 *phlG* codons; Tc^r	This study
pME8031	pME6015 with a 495-bp *phlG* upstream fragment and a translational *phlG′-′lacZ* fusion containing the first 83 *phlG* codons; Tc^r	This study
pME8032	peT28a containing the *phlG* gene flanked by a C-terminal His_6 tag under the control of the T7 promoter; Tc^r	This study
pME8039	pME6182 with a 1,303-bp HindIII fragment containing *phlG* of CHA0; Ap^r Gm^r	This study
pUK21	Cloning vector; *lacZ*α; Km^r	47
pUX-BF13	Helper plasmid for Tn*7*-based transposon mutagenesis containing the transposition functions; R6K-replicon; Ap^r	5
Oligonucleotides		
MBP20	GGAATTCGCCATTCTCACTTCCACCATC, EcoRI	
MBP22	GCTCTAGAACAGATAGCCGCCATTTCGCT, XbaI	
MBP23	CCCAAGCTTCGATAGATCCATGATCTGGCC, HindIII	
MBP26	GCTCTAGAGCGGCCTGAAACAACAGCCAT, XbaI	
MBP37	GGAATTCTGTTGGCGTACCCCAGCTCAG, EcoRI	
MBP38	CGGGATCCGCGATTGATGTCCTCGATGCT, BamHI	
MBP39	GCTCTAGAATAATTTTGTTTTAACTTTAAGAAGGAGATATACCATGGCGGCTATCTGTCAATTCACGCCAAAG, XbaI	
MBP40	CCCAAGCTTGGCCGCGGGGGTGCCGAACTC, HindIII	
MBP41	GGAATTCGTGACAACAGTTCGCGGGTCA, EcoRI	
MBP52	CCCAAGCTTTGCTGGTATCGGCTGTTGACT, HindIII	
MBP53	CCCAAGCTTTGTTGGCGTACCCCAGCTCAG, HindIII	

[a] Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance. Restriction sites specified for oligonucleotides are underlined.

His6 was followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (26). Myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa) (Bio-Rad, Reinach, Switzerland) were used as molecular mass standards. Protein bands were stained with Coomassie brilliant blue G250 (Serva, Catalys, Wallisellen, Switzerland). Protein concentrations were determined with the method of Bradford (6) with bovine serum albumin as the standard.

Enzymatic assays. The DAPG-degrading activity of PhlG-His6 was assayed in the 0.1 M potassium phosphate resuspension buffer described above. The buffer was amended with DAPG at final concentrations ranging from 50 to 200 μM and the reaction was started by the addition of cell extracts (about 24 μg of protein per ml) or purified enzyme (0.65 μg of protein per ml). The degradation of DAPG to MAPG was monitored by HPLC as follows. Samples were taken immediately before as well as 2, 5, 15, 30, and 60 min after the start of the reaction. The reaction was stopped by mixing the samples with 1 volume of methanol-H_2O-acetic acid (50:45:5 [vol/vol/vol]). The mixtures were centrifuged for 5 min at 12,000 × *g* and 20°C, and the supernatants were analyzed by HPLC. An enzymatic test kit (catalog no. 10-148-261-035; R-Biopharm, Murten, Switzerland) was used for quantification of acetate in the samples immediately prior to stopping the reaction. The K_m value of the purified enzyme for DAPG was determined at concentrations ranging from 50 to 200 μM. The initial velocity and the substrate concentration were used as input for iterative fitting of curve parameters to the Michaelis-Menten equation (43) with the program Kaleidagraph (Synergy Software, Reading, PA). Three independent kinetic determinations were made to calculate means and standard deviations for the reported K_m and k_{cat} values. The enzyme activity was investigated at pH values of 6.0, 6.6, 7.0, 7.6, and 8.0 and at temperatures ranging from 16 to 42°C. Substrate specificity of PhlG was evaluated by testing compounds with structural similarities to DAPG, including MAPG, TAPG, salicylate, and 2,6-dihydroxyacetophenone at concentrations of 60 to 100 μM. Concentrations of the potential substrates in the reaction mix were monitored for 60 min by HPLC analysis. The latter compounds and pyoluteorin were also tested as potential inhibitors of PhlG activity. For this purpose, they were preincubated with the purified enzyme for 10 min at 4°C, before the reaction was started by the addition of DAPG. PhlG-His6 activity was then determined after a 30-min incubation as described above.

RESULTS

Construction and characterization of a *phlG* mutant of *P. fluorescens*. In previous work, we have identified a 924-bp open reading frame termed *phlG* that is contained within the DAPG biosynthetic locus of *P. fluorescens* CHA0 (39) and whose function thus far has not been determined. The deduced product (307 amino acids; 35.1 kDa) of the *phlG* gene shows some similarities to phloretin hydrolase (Phy) of *Eubacterium ramulus* (GenBank accession no. AF548616; 24% identity and 43% similarity in a 256-amino-acid overlap), an enzyme involved in flavonoid degradation (41). PhlG also shares limited similarities (26 to 34% identity and 41 to 48% similarity in 259- to 283-amino-acid overlaps) with hypothetical proteins of *Mycobacterium tuberculosis* (NP_336281, locus tag MT1825), *M. bovis* (NP_855457, locus tag Mb1804), *Archaeoglobus fulgidus* (NP_070295, locus tag AF1466), and the rice blast fungus *Magnaporthe grisea* (EAA57329, locus tag MG08298.4). No conserved motif could be detected in the amino acid sequence of PhlG.

To investigate the function of PhlG, a chromosomal *phlG* in-frame deletion was created in *P. fluorescens* CHA0, leaving only the first five codons and the last three codons of the *phlG* gene (Fig. 2). In assay systems described in detail previously (31, 39), the *phlG* mutant (CHA1091) did not differ from the parental strain CHA0 in its growth characteristics in rich and minimal media, its carbon source utilization profiles, and its in vitro inhibitory activity against the highly DAPG-sensitive indicator organism *Bacillus subtilis* (data not shown). We then tested whether loss of PhlG function in *P. fluorescens* CHA0 may affect the biosynthesis of antifungal compounds. Interestingly, no major differences in kinetics of DAPG, MAPG, and pyoluteorin production and expression of a *phlA′-′lacZ* reporter fusion carried on pME6259 (39) could be detected between wild-type CHA0 and its *phlG* mutant CHA1091 growing in KMBmalt broth (data not shown).

PhlG is required for DAPG degradation in *P. fluorescens*. In an earlier study, we obtained evidence that strain CHA0 can degrade the potent antimicrobial phenolic DAPG to the mildly toxic compound MAPG (39). To test whether PhlG is involved in this mechanism, we deleted the *phlG* gene in strain CHA631 which carries a deletion in *phlA* and thus is unable to produce DAPG and MAPG (39). DAPG added to KMBmalt medium at a concentration of 100 μM was then incubated during 65 h in the presence or absence of the *phlA* mutant CHA631 and the *phlA phlG* double mutant CHA1092. DAPG concentrations rapidly declined in cultures of the *phlA* mutant CHA631 and the compound was no longer detectable after a 45-h incubation (Fig. 3A). Degradation of DAPG was accompanied by a temporary accumulation of MAPG in the growth medium. In contrast, no such degradation of DAPG and no formation of MAPG could be observed in cultures of the *phlA phlG* double mutant CHA1092 (Fig. 3B) and in medium without bacteria (data not shown). The similar slight decrease of DAPG concentrations in cultures of CHA1092 (Fig. 3B) and in the absence of bacteria (data not shown) is in agreement with a previously reported certain instability of this compound in solutions (9, 39). Kinetics of DAPG degradation could be restored to those observed for CHA631 by complementation of the double mutant CHA1092 with a single copy of intact *phlG*$^+$ introduced into the chromosomal Tn*7* attachment site, resulting in strain CHA1094 (data not shown). Taken together, these results suggest that the product of the *phlG* gene is required for the degradation of DAPG to MAPG in *P. fluorescens* CHA0.

Purification and characterization of PhlG as a hydrolase that specifically degrades DAPG to MAPG. To further investigate the role of PhlG, the protein was histidine tagged and overexpressed in *E. coli*. For this purpose, the *phlG* gene was cloned under the control of the T7 promoter in the expression vector pET28a. After IPTG induction of cultures of *E. coli* BL21(DE3) carrying the resulting plasmid pME8032, a protein of approximately 40 kDa was overexpressed (Fig. 4, lane 3), which is consistent with the predicted molecular mass of 35.1 kDa of PhlG. The 40-kDa band could not be detected in cell extracts of *E. coli* carrying the empty vector control pET28a (data not shown). The overexpressed PhlG-His6 protein was localized in the soluble cell fraction (Fig. 4, lane 4) and was purified by affinity chromatography on Ni-NTA columns (Fig. 4, lane 5).

To determine whether PhlG is an enzyme which can convert DAPG into MAPG, cell extracts, and the purified PhlG-His6 protein were used for enzymatic assays. Incubation of DAPG with cell extracts from *E. coli* BL21(DE3)/pME8032 or with purified PhlG-His6 resulted in a complete conversion of DAPG to equimolar amounts of MAPG and acetate. A typical reaction in which 50 μM DAPG was entirely degraded to similar concentrations of MAPG within 30 min in the presence of 0.65 μg of purified PhlG-His6 per ml of reaction buffer (pH 7.0; 30°C) is shown in Fig. 5. Concentrations of MAPG, once

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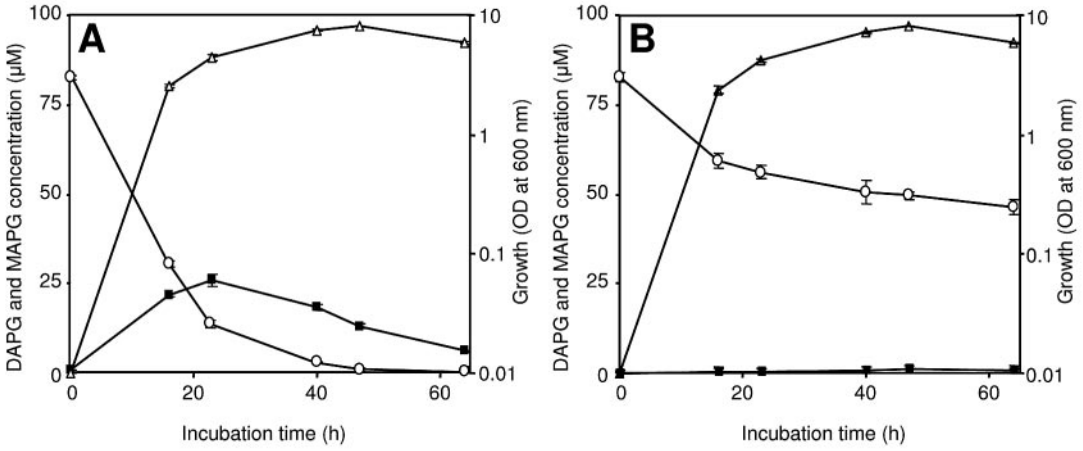

FIG. 3. Requirement of PhlG function for degradation of DAPG to MAPG by *P. fluorescens*. The DAPG- and MAPG-negative mutants CHA631 (Δ*phlA*) (A) and CHA1092 (Δ*phlA* Δ*phlG*) (B) were grown at 27°C in KMBmalt broth supplemented with 100 μM DAPG. After different incubation periods, bacterial growth (ODs at 600 nm) (△) and concentrations of DAPG (○) and MAPG (■) were determined. Means ± the standard deviations from three independent cultures are shown. The experiment was repeated twice with similar results.

formed, remained stable, suggesting that PhlG acts on DAPG only (Fig. 5). In control treatments, the reaction buffer alone and cell extracts of *E. coli* BL21(DE3) carrying the empty vector did not lead to degradation of DAPG to MAPG (data not shown). DAPG alone remained stable for at least 1 h in the reaction buffer. The K_m value for DAPG at 30°C and pH 7.0 was 140 ± 48 μM, and the k_{cat} was 33 ± 9 s^{-1}. PhlG-His6 activity was slightly enhanced at pH 6.6 and pH 6.0 (by ca. 15 and 5%, respectively). Higher pH values were found to strongly reduce PhlG-His6 activity: the enzyme was virtually inactive at pH 8.0 and retained only 10% of its maximal activity at pH 7.6. The effect of temperature on PhlG-His6 activity was tested in a range from 16 to 42°C, and the enzymatic activity of the protein was found to be gradually reduced by up to 50% at temperatures below or above 30°C (data not shown).

PhlG appears to be an enzyme that is highly specific for its substrate DAPG since purified PhlG-His6 (at 0.65 μg ml^{-1} reaction buffer) was unable to degrade or modify several compounds showing a structure similar to DAPG, i.e., in particular the degradation product MAPG (see above), TAPG (Fig. 1), salicylate, and 2,6-dihydroxyacetophenone. Levels of these compounds added to the reaction mix at concentrations of 60 to 100 μM remained stable during the entire 60-min incubation period (data not shown). Finally, we also tested whether the latter four compounds could function as inhibitors of PhlG-His6 activity. For this purpose, we preincubated 16 nM PhlG-His6 enzyme for 10 min with 100 μM samples of the potential inhibitors before adding the substrate DAPG. However, none of these structurally similar compounds was found to inhibit or

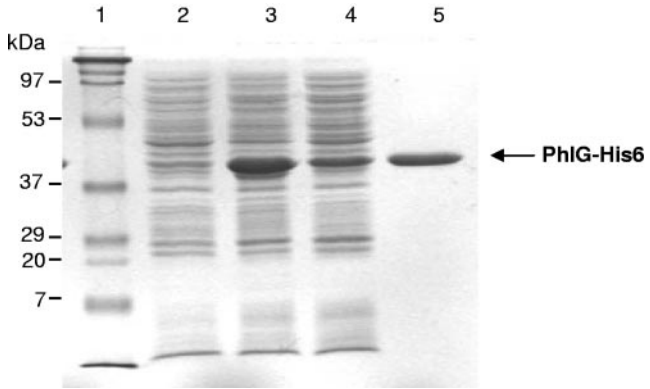

FIG. 4. SDS-PAGE analysis of PhlG-His6 expression and purification. Lanes: 1, molecular mass standards; 2, crude cell extract (10 μg of protein) before induction; 3, crude cell extract 2 h after induction with 1 mM IPTG (12 μg of protein); 4, soluble fraction from the IPTG-induced cell extract (8 μg of protein); 5, purified PhlG-His6 (4 μg of protein). Cell extracts were prepared from *E. coli* BL21(DE3)/pME8032. The arrow indicates PhlG-His6. The SDS–15% PAGE gel was stained with Coomassie brilliant blue.

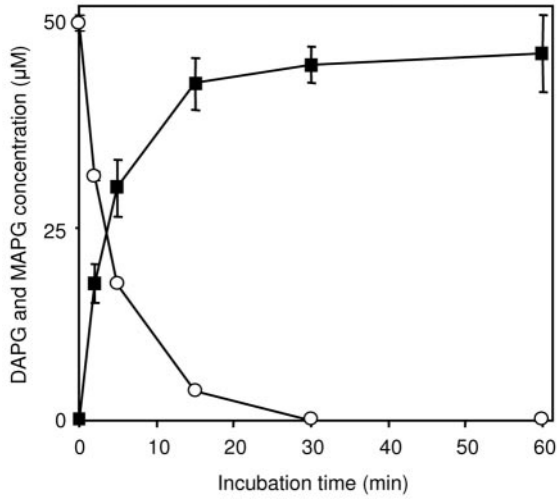

FIG. 5. Time course of DAPG degradation by the purified PhlG-His6 protein. Portions of 50 μM DAPG were incubated with 16 nM purified PhlG-His6 enzyme in a reaction buffer at pH 7.0 and 30°C as detailed in Materials and Methods. DAPG (○) and MAPG (■) concentrations were determined by HPLC at different time points after the start of the reaction. Means ± the standard deviations from three independent experiments are shown.

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slow down DAPG degradation (data not shown). Remarkably, pyoluteorin, which is known to act as a potent antagonist of DAPG production in *P. fluorescens* (3, 8, 39), reduced PhlG-His6 activity to 45% ± 4% when added at a concentration of 100 μM. Methanol, which served as the solvent for the tested compounds, had no significant effect on enzyme activity. In conclusion, these findings illustrate that *P. fluorescens* possesses an active mechanism for DAPG degradation that is mediated by PhlG, a highly specific hydrolase that catalyzes the degradation of DAPG to MAPG and acetate.

Regulation of *phlG* expression. To study the regulation of the *phlG* gene, we used a plasmid-based *phlG'-'lacZ* reporter construct (pME8030) that contains a 226-bp *phlG* upstream fragment encompassing the intergenic region between *phlG* and *phlH* and the presumable *phlG* promoter (Fig. 2). The *phlG'-'lacZ* fusion was expressed in strain CHA0 throughout the exponential and early stationary growth phases, reaching maximal levels of 4,300 to 6,000 Miller units (Fig. 6). We first tested whether the expression of the *phlG* gene could be affected by the PhlG substrate DAPG or by the degradation product MAPG. Interestingly, neither of the two compounds significantly affected *phlG* expression in strain CHA0 when added to the growth medium at physiological concentrations (Fig. 6). Likewise, the lack of DAPG and MAPG in the *phlA* mutant CHA631 resulted in *phlG* expression levels that did not differ from those in the parental strain CHA0 (Fig. 6A).

We next assessed whether the expression of *phlG* was controlled by PhlF and PhlH, which are known to act as pathway-specific transcriptional regulators of DAPG biosynthetic gene expression (2, 39). For this purpose, we followed kinetics of expression of the *phlG'-'lacZ* fusion carried on pME8030 in a *phlF* mutant (CHA638) and a *phlH* mutant (CHA630) of strain CHA0. Remarkably, *phlG* expression was enhanced up to threefold in both the *phlF* and *phlH* mutants during the late exponential growth phase (OD at 600 nm of 2.5 to 3) (Fig. 7), suggesting that both regulators act as repressors of *phlG* expression. During early exponential growth (OD at 600 nm of 0.5 to 2.0), *phlG* expression was significantly advanced in the *phlH* mutant compared to the wild-type CHA0 and the *phlF* mutant CHA638 (Fig. 7). Since the expression of DAPG biosynthetic genes is tightly controlled by the GacS/GacA two-component regulatory system (16, 39), we monitored *phlG* expression also in a *gacS* mutant of CHA0. As expected, expression of the *phlG'-'lacZ* reporter was virtually abolished in the *gacS* mutant CHA19 (Fig. 7). In a series of control experiments, a second *phlG'-'lacZ* reporter construct (pME8031) containing a 495-bp *phlG* upstream fragment (Fig. 2) was used to check whether regulation of *phlG* expression could be influenced by an extended leader region. However, results corresponded to those obtained with the pME8030-based construct (data not shown), suggesting that this reporter is equipped with the relevant *phlG* leader sequence. In summary, *phlG* expression strongly depends on the DAPG pathway-specific regulators PhlF and PhlH and the global regulatory system GacS/GacA but is influenced by neither the substrate nor the product of the enzymatic reaction catalyzed by PhlG.

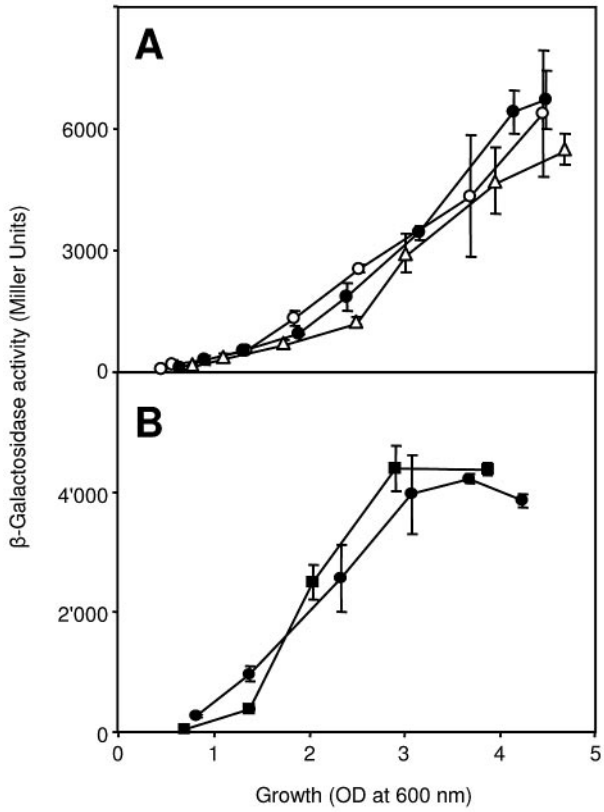

FIG. 6. Effect of DAPG and MAPG on *phlG* expression in *P. fluorescens* CHA0. (A) β-Galactosidase expression of a *phlG—lacZ* translational fusion carried by pME8030 was determined in wild-type CHA0 with (△) or without (●) addition of 100 μM DAPG and in the *phlA* mutant CHA631 (○). (B) Expression of *phlG—lacZ* in CHA0 with (■) or without (●) addition of 100 μM MAPG. Bacteria were grown in OSGly medium at 30°C. DAPG and MAPG were dissolved in acetonitrile. Acetonitrile did not affect *phlG* expression (data not shown). Means ± the standard deviations from three replicate cultures are shown. The experiment was repeated twice with similar results.

DISCUSSION

In the present study, we have identified the function of the *phlG* gene that is located in the DAPG biosynthetic cluster of *P. fluorescens* CHA0. Mutational analysis of the *phlG* gene (Fig. 3) and examination of the purified PhlG protein (Fig. 5) allowed us to demonstrate that *phlG* encodes a hydrolase that degrades DAPG to MAPG and acetate. Remarkably, the enzymatic activity of PhlG appears to be highly specific for the substrate DAPG. This was best illustrated by the fact that PhlG was unable to degrade MAPG or TAPG, i.e., two acylphloroglucinols commonly detected in cultures of DAPG-producing pseudomonads (4, 7, 22, 34, 39, 44). Since the three phloroglucinol molecules display high structural similarity (Fig. 1), our finding suggests that the number and/or position of the acetyl groups may be crucial for substrate recognition by PhlG. Substrate specificity of PhlG was further corroborated by our observation that related phenolics such as salicylate or 2,6-dihydroxyacetophenone did not serve either as substrates for the enzyme. An examination of available genomic data from other DAPG-producing pseudomonads, including *P. fluorescens* strains Pf-5, Q2-87, F113, and 2P24 (GenBank accession numbers CP000076, U41818, AF497760, and DQ083928, respectively) indicates that the *phlG* gene is not unique to strain CHA0 but appears to be commonly associated with the DAPG

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biosynthetic locus. Moreover, a steady decrease of DAPG levels at later growth stages that is indicative of DAPG degradation occurs in cultures of strains CHA0 (Fig. 3) (3, 31, 39), Pf-5, Q2-87, and F113 (8, 10, 27). Together, these observations suggest that an active mechanism of DAPG degradation is a conserved feature among DAPG-producing pseudomonads.

The amino acid sequence of PhlG of *P. fluorescens* CHA0 displays some similarities to several hypothetical proteins of archeal, actinobacterial, and fungal origin and, interestingly, to Phy, a phloretin hydrolase that has recently been purified from the gut bacterium *E. ramulus* (41). Phloretin is a dihydrochalcone that has been described as an intermediate in the degradation of the flavonoids apigenin and naringenin (37). Phloretin hydrolase of *E. ramulus* catalyzes the hydrolysis of phloretin to equimolar amounts of phloroglucinol and 3-(4-hydroxyphenyl)propionic acid by cleaving the carbon-carbon (C-C) bond adjacent to the carbonyl moiety of the molecule (41). Likewise, PhlG of *P. fluorescens* CHA0 needs to cleave the C-C bond adjacent to the carbonyl of the acetyl group of DAPG in order to catalyze the conversion of DAPG to MAPG. The hydrolytic cleavage of a C-C bond adjacent to a carbonyl is considered to be a rare class of reaction in biochemistry and is carried out by enzymes belonging to the α/β-hydrolase-fold superfamily (15, 19, 29). However, both phloretin hydrolase and DAPG hydrolase show no sequence similarity to other known C-C hydrolase enzymes and contain no motifs typical of the α/β-hydrolase-fold superfamily as determined by a search of the ESTHER database (19, 41; C. Keel and M. Bottiglieri, unpublished findings). Based on these observations, it is probable that the DAPG hydrolase groups in a distinct enzyme family, as has been proposed for the phloretin hydrolase (41).

Among the factors that affected PhlG activity, the negative effect exerted by pyoluteorin probably was most surprising. In fact, pyoluteorin, at physiological concentrations, reduced PhlG activity by more than 50%, whereas a series of compounds that are structurally related to DAPG such as MAPG, TAPG, and salicylate had no such inhibitory effect. Pyoluteorin is another antifungal compound synthesized by a distinct class of DAPG-producing pseudomonads (22) and complex regulatory mechanisms help keep the two antibiotics at balanced levels (39). In *P. fluorescens* strains CHA0 and Pf-5, DAPG and pyoluteorin are known to be involved in an inverse relationship in which each metabolite, while activating its own production, represses the biosynthesis of the other metabolite (3, 8, 39). Our finding that pyoluteorin markedly inhibited PhlG activity, thereby at least partially preventing DAPG degradation, provides the compound with an additional role in its interference with the DAPG biosynthetic pathway. The dual role of pyoluteorin as an inhibitor of DAPG production and degradation adds a further level of complexity to the molecular cross talk between the DAPG and pyoluteorin biosynthetic pathways in *P. fluorescens*.

We also investigated which factors affect *phlG* expression in *P. fluorescens* CHA0. Somewhat unexpectedly, neither the PhlG substrate DAPG nor the degradation product MAPG had an effect on *phlG* expression (Fig. 6). However, expression of the *phlG′-′lacZ* reporter construct was virtually abolished in a Δ*gacS* mutant of strain CHA0 (Fig. 7), thus further underlining the strong dependence of the DAPG biosynthetic locus

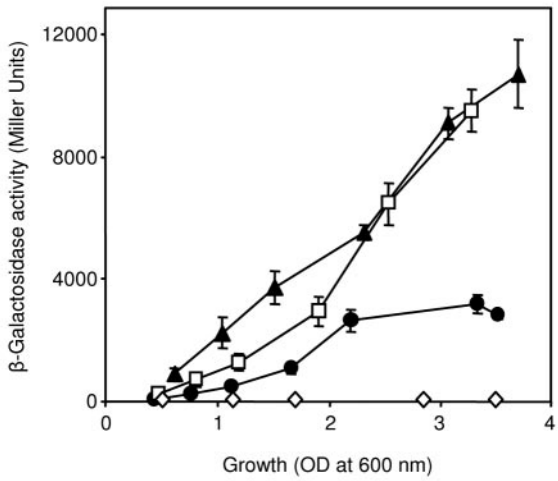

FIG. 7. PhlF, PhlH, and GacS control of *phlG* expression in *P. fluorescens* CHA0. β-Galactosidase expression of a *phlG′-′lacZ* translational fusion carried by pME8030 was determined in the wild-type CHA0 (●), the *phlF* mutant CHA638 (□), the *phlH* mutant CHA630 (▲), and the *gacS* mutant CHA19 (◇). Strains were grown in OSGly medium at 30°C. Means ± the standard deviations from three replicate cultures are shown. The experiment was repeated twice with similar results.

on the GacS/GacA regulatory cascade (16, 39, 50). Expression of *phlG* was also subject to negative control by PhlF (Fig. 7), which is known to act as a pathway-specific transcriptional repressor of DAPG gene expression (2, 4, 10, 39), suggesting that the negative effects of PhlF on DAPG biosynthesis and degradation are coupled. Specific PhlF binding sites have been identified in the *phlA* promoter regions of *P. fluorescens* strains F113 and CHA0 (2, 16). However, similar plausible PhlF recognition sites appear to be absent from the *phlG* leader region (unpublished observations). PhlH, the second TetR-like regulator associated with the DAPG biosynthetic locus also negatively affected *phlG* expression in strain CHA0 (Fig. 7), whereas it previously has been reported to exert a positive effect on *phlA* expression and DAPG production (39). The reason for the apparent divergence in PhlH function is not known at the present stage. Different DAPG levels did not affect *phlG* expression (Fig. 6), suggesting that PhlF and PhlH exert their negative effects on *phlG* expression directly or via an intermediate but not through their modulation of DAPG production.

Previously, MAPG has been proposed to function as a direct precursor of DAPG synthesis. An acetyltransferase activity converting MAPG to DAPG occurs in *P. fluorescens* F113 (44), and the products of the *phlACB* genes are required for this transacetylation reaction in *P. fluorescens* Q2-87 (4). Here, we demonstrate that *P. fluorescens* possesses a hydrolase that specifically converts DAPG to MAPG, confirming our previous hypothesis that MAPG can also be a degradation product of DAPG (39). Why should DAPG-producing pseudomonads dispose of this highly specific DAPG-degrading activity? The dual mechanism of conversion of MAPG to DAPG and, vice versa, degradation of DAPG to MAPG may provide pseudomonads with an additional means of fine-tuning levels of this antibiotic. Moreover, by acting on the metabolite itself, PhlG

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offers the bacterium an effective alternative for modulating DAPG levels, in addition to a series of pathway-specific and global regulators and microbial metabolites that act on the expression of the DAPG biosynthetic operon (2, 3, 8, 10, 16, 31, 36, 38, 39). Alternatively, by degrading DAPG to mildly toxic MAPG, PhlG may help avoid accumulation of a metabolite that at high levels may become toxic to the producing bacterium, as has been observed for strains CHA0 and F113 (1, 24; M. Bottiglieri and C. Keel, unpublished data). In this context, it is noteworthy that detoxification of DAPG by deacetylation to MAPG and phloroglucinol is used by the phytopathogenic fungus *F. oxysporum* as a defense mechanism against the antifungal action of biocontrol pseudomonads (42). Another observation may provide an additional clue about the physiological role of the PhlG-mediated enzymatic mechanism. Degradation of DAPG added to cultures of a *phlA* mutant of *P. fluorescens* is followed by a temporary accumulation of MAPG. This phenomenon can be observed in rich (Fig. 3A), as well as in minimal (39), growth media. What exactly happens then to MAPG remains unknown as yet. Since at that stage MAPG is not reused for DAPG synthesis (Fig. 3A), it is possible that it is further degraded to compounds that have not been identified thus far. In a more exciting alternative scenario, MAPG could serve as a substrate for a hitherto unknown pathway, suggesting that the compound could be more than an intermediate in DAPG synthesis.

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