Polymorphism of the Polyketide Synthase Gene *phlD* in Biocontrol Fluorescent *Pseudomonads* Producing 2,4-Diacetylphloroglucinol and Comparison of PhlD with Plant Polyketide Synthases

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Many biocontrol fluorescent pseudomonads can protect plants from soilborne fungal pathogens through production of the antifungal secondary metabolite 2,4-diacetylphloroglucinol (Phl). One of the *phl* biosynthetic genes, *phlD*, encodes a polyketide synthase similar to plant chalcone synthases. Here, restriction analysis of *phlD* from 39 *Phl*⁺ biocontrol fluorescent pseudomonads yielded seven different banding patterns. The gene was sequenced in seven strains, representing the different restriction patterns. Cluster analysis of *phlD* restriction data or *phlD* sequences indicated that *phlD* polymorphism was high, and two main clusters were obtained when predicted PhlD sequences were compared. When the seven PhlD sequences were studied with those of other prokaryotic polyketide synthases (gram-positive bacteria) and plant chalcone synthases, however, *Phl*⁺ pseudomonads, gram-positive bacteria, and plants clustered separately. Yet, sequence analysis of active site regions for PhlD and plant chalcone synthases revealed that PhlD can be considered a member of the chalcone synthase family, which may be interpreted as convergent evolution of key enzymes involved in secondary metabolism. For the 39 *Phl*⁺ pseudomonads, a relationship was found among *phlD* restriction patterns, phylogenetic groups defined by 16S rDNA restriction analysis (confirmed by 16S rDNA sequencing), and production levels of Phl in vitro.

Additional keywords: diversity, phylogeny, plant protection.

Certain root-associated fluorescent *Pseudomonas* spp. capable of protecting plants from soilborne fungal pathogens produce antifungal secondary metabolites (Dowling and O'Gara 1994; Thomashow and Weller 1996). The implication of these bacterial antifungal compounds in disease suppression has been substantiated by comparing wild-type pseudomonads with mutant derivatives in which production of antifungal compound(s) was altered (Keel and Défago 1997). Among antifungal compounds synthesized by pseudomonads,

2,4-diacetylphloroglucinol (Phl) is a key secondary metabolite associated with disease suppression in several pathosystems (Fenton et al. 1992; Harrison et al. 1993; Keel et al. 1992). In addition, Phl was found by chemical means in the rhizosphere of plants inoculated with *Phl*⁺ strains (Bonsall et al. 1997; Keel et al. 1992) or colonized by naturally occurring *Phl*⁺ pseudomonads in disease-suppressive soil (Raaijmakers et al. 1999).

Six *phl* genes are documented in the *Phl*⁺ strain *Pseudomonas fluorescens* Q2-87, and one of them, *phlD*, is essential for the synthesis of the Phl precursor monoacetylphloroglucinol (Bangera and Thomashow 1999). The predicted PhlD protein was identified as a polyketide synthase (PKS) and shares homologies with chalcone synthases (CHSs) and stilbene synthases from plants. Furthermore, amino acid sequence alignments indicate that the putative active site region of PhlD in strain Q2-87 (QLGC¹³⁸VAG) corresponds well with the unique active site region found in plant CHS (QQGC¹⁶⁹FAG). Bangera and Thomashow (1999) proposed that Phl synthesis was mediated by a novel type of prokaryotic PKS, designated PKS type III. The function of the PhlD PKS is to catalyze the condensation of the probable starter molecule acetoacetyl-CoA and the subsequent cyclization step to form the Phl precursor monoacetylphloroglucinol.

In addition, the similarities between PhlD and plant CHS raises the possibility of a common evolutionary origin, probably as a result of horizontal gene transfer from plants to the bacteria colonizing them (Bangera and Thomashow 1999; Cook et al. 1995). This hypothesis is in agreement with the facts that i) bacterial transformation can take place inside plant tissues (Bertolla et al. 1999), ii) *P. fluorescens* can acquire DNA by transformation (Troussieux 2000), and iii) *Phl*⁺ pseudomonads can display endophytic behavior (Troxler et al. 1997). For the three other biosynthetic genes of the *phlACBD* operon, however, homologs were found only in archaeobacteria (Bangera and Thomashow 1999). A 4.8-kb DNA fragment carrying *phl* biosynthetic genes from Q2-87 hybridized under high-stringency conditions with genomic DNA from other *Phl*⁺ biocontrol pseudomonads originating from the United States (Oklahoma, Texas, and Washington), Europe (Ireland, Italy, and Switzerland), and Africa (Ghana) (Keel et al. 1996).

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This led these authors to propose that the *phl* biosynthetic locus is well conserved in *Phl*⁺ pseudomonads, although more discriminating methods were not used to strengthen this hypothesis.

In contrast, when other strain properties were considered, the collection of *Phl*⁺ pseudomonads studied by Keel et al. (1996) displayed considerable genotypic and phenotypic diversity. For example, as many as three amplified 16S ribosomal DNA restriction analysis (ARDRA) groups and eight randomly amplified polymorphic DNA (RAPD) groups were found among 45 *Phl*⁺ pseudomonads. The diversity of *Phl*⁺ pseudomonads has also been characterized for isolates obtained from a single host species in the case of wheat (McSpadden Gardener et al. 2000) and maize (Picard et al. 2000), with a combination of ARDRA, whole-cell BOX-polymerase chain reaction (PCR), enterobacterial repetitive intergenic consensus (ERIC)-PCR, RAPD markers, and/or carbon source utilization profiles. Similarly, large differences exist in the ability of *Phl*⁺ pseudomonads to produce *Phl* under in vitro conditions (Keel et al. 1996). Whether this is linked with strain properties unrelated to the *phl* locus and/or features of the *phl* locus remains to be assessed.

Our goal was to determine whether or not *phlD* was polymorphic. In order to carry this out, we used a worldwide collection of *Phl*⁺ biocontrol fluorescent *Pseudomonas* spp. In light of the postulated plant origin of *phlD* in pseudomonads, the gene products deduced from *phlD* sequences were compared with other members of the type III PKS superfamily from bacteria and plants to establish their evolutionary relationships. In addition, the relationships were assessed among *phlD* characteristics, *Phl* production in vitro, and broad genotypic features of the *Phl*⁺ pseudomonads because of the importance of these bacteria in biocontrol and *Phl*-mediated antagonism as a mechanism in plant protection.

RESULTS

Amplification and restriction analysis of *phlD*.

The 745-bp *phlD* amplicons obtained from 39 *Phl*⁺ biocontrol pseudomonads were analyzed by restriction with *Hae*III, *Msp*I, *Cfo*I, *Sau*3A, or *Nde*II. The results obtained with these five enzymes were subjected to cluster analysis (Fig. 1). Restriction analysis with *Hae*III yielded seven restriction patterns (designated *Hae*III-1 to *Hae*III-7), and pseudomonads exhibiting a same *Hae*III restriction pattern were undistinguishable when analysis was carried out with any of the four other endonucleases (data not shown). The seven clusters were very different from one another (Fig. 1), except for clusters corresponding to *Hae*III-3 and *Hae*III-4 (79% similarity) or *Hae*III-6 and *Hae*III-7 (73% similarity).

Several *Hae*III restriction patterns only comprised pseudomonads from the same geographic location (Table 1), e.g., *Hae*III-2 (Quincy, WA, U.S.A.), *Hae*III-4 (Morens, Switzerland), or *Hae*III-6–*Hae*III-7 (Albenga, Italy). Different *Hae*III restriction patterns could be observed, however, when all bacteria from Quincy (*Hae*III-2 and *Hae*III-3), Morens (*Hae*III-1, *Hae*III-3, and *Hae*III-4) or Albenga (*Hae*III-1, *Hae*III-6, and *Hae*III-7) were considered. The nine tobacco isolates (from three different countries) were all within *Hae*III-1, but four *Hae*III restriction patterns were found with wheat isolates (*Hae*III-1, *Hae*III-2, *Hae*III-3, and

*Hae*III-6), and three with tomato isolates (*Hae*III-3, *Hae*III-4, and *Hae*III-7).

Analysis of *phlD* sequences.

Seven *Phl*⁺ strains, each displaying a different *Hae*III restriction pattern, were chosen to sequence *phlD*. After *phlD* sequence alignment, a maximum-likelihood tree was inferred (Fig. 2A). Three main clusters corresponding to CHA0, Q2-87, and the five other strains were obtained. Within the latter cluster, three subclusters consisting of F113, (Q65c-80, CM1'A2) and (PITR2, PILH1) were found. Alternative subclusters such as F113 (Q65c-80, CM1'A2) (Fig. 1) and F113, (PITR2, PILH1) were supported by low bootstrap values (34 and 49%, respectively). The same tree topology also was obtained by maximum parsimony or distance-based methods (Neighbor-Joining [NJ] tree, constructed according to the Kimura 2-parameter or the Jukes-Cantor models), except for F113, which was clustered with either Q65c-80 and CM1'A1 or PITR2 and PILH1, although with low bootstrap values of 52 to 66%.

The base composition for *phlD* is $T = 21.5\%$ ($\pm$ standard deviation 0.4), $C = 26.5\%$ ($\pm$ 0.3), $A = 21.3\%$ ($\pm$ 0.6), and $G = 30.8\%$ ($\pm$ 0.6). No statistical difference was found between the strains, regardless of whether base composition was considered overall or at each of the three codon positions. The rate of nucleotide substitutions in *phlD* was assessed with the gamma distribution, whose shape is determined by the alpha parameter. The alpha parameter was estimated to be 0.29 on the basis of the topology presented in Figure 2A, the equilibrium frequencies of the four bases, and a transition rate (Ti)–transversion rate (Tv) ratio estimated by the same model to be 1.66. A value below 1 for the alpha parameter indicates that the gamma distribution is highly skewed, which means that most sites in *phlD* have low substitution rates but that substitutional hot spots can exist (Swofford et al. 1996).

Because selection pressure does not have the same effect on the nonsynonymous (leading to amino acid replacement; dN) and synonymous (silent; dS) rates of nucleotide substitution (Yang 1998), the average dN and dS were calculated, with the approximation of the Jukes–Cantor formula. dN ($0.100 \pm$ standard deviation 0.072) was lower statistically (Student *t* test, with infinite degrees of freedom; $P < 0.001$) than dS (0.230 ± 0.146), and the dN–dS ratio was 0.41 ± 0.09 . Similar results (average dN–dS ratio of 0.40 ± 0.11) were obtained by the Nei and Gojobori (1986) method, implemented with phylogenetic analysis by the maximum-likelihood program (PAML; Z. Yang, University College London, U.K.). This means that bacteria in which mutations of *phlD* resulted in actual modification of *PhlD* characteristics were not selected during evolution, i.e., a lack of adaptive protein evolution.

Analysis of predicted *PhlD* sequences.

A NJ tree was inferred from the predicted *PhlD* protein sequences (Fig. 2B). Strain CHA0 clustered separately from the six other pseudomonads. Among the latter, four subclusters consisting of Q2-87, (Q65c-80, CM1'A2), F113 and (PITR2, PILH1) were found. The same topology also was obtained when the sequence of bacterial type III PKS (Table 2) was included or a heuristic tree search with maximum parsimony criterion (data not shown) was chosen. The heuristic tree search yielded nine trees, but the 50%-majority-rule consensus

tree displayed bootstrap values of only 53 and 73% for the clusters (CM1'A2, Q65c-80) and (PITR2, PILH1), respectively. Other distance methods, which were calculated on the basis of Poisson correction, the number of differences, or gamma-corrected distances implemented in the molecular evolutionary genetics analysis program (MEGA; S. Kumar, K. Tamura, and M. Nei, Pennsylvania State University, U.S.A.), gave the same topology.

Not surprisingly, the strongest differences in *PhlD* amino acid sequences were obtained between CHA0 and the other pseudomonads, with an average of 85.6% amino acid identity,

whereas the other pairwise comparisons gave 96 to 99.6% identity.

Comparison of *PhlD* with type III PKS from other bacteria and from plants.

When compared with other type III PKSs such as those from gram-positive bacteria and plants, the seven *PhlD* proteins clustered separately from the former (Fig. 3). The tree distinguished gram-positive bacteria from plants, with the exception of *Nicotiana sylvestris*, which appeared in an intermediate position. Amino acid sequence similarity between

Fig. 1. Comparison of *phlD* restriction patterns (unweighed pair group method with averages [UPGMA] tree with Jaccard's pairwise similarity coefficient) for 39 *Phl*⁺ biocontrol fluorescent pseudomonads and relationships with broad genotypic properties (ARDRA and RAPD groups) (Keel et al. 1996) of the strains. Polymerase chain reaction-restriction fragment length polymorphism analysis of *phlD* was carried out with five enzymes, although one of them (*Hae*III) was enough to yield the seven *phlD* restriction groups (termed *Hae*III-1–*Hae*III-7). The seven strains for which *phlD* was subsequently sequenced are underlined. The dotted line corresponds to a 40% similarity level.

PhlD (on the basis of the seven deduced sequences) and other bacterial type III PKS was 42 to 64% (identity from 25 to 49%). The highest values obtained were with *Streptomyces griseus* (Table 2). Similarity between PhlD and plant type III PKS was 43 to 50% (identity was 22 to 31%), when CHA0, Q2-87, or F113 were considered.

The functional role of key amino acid residues is documented for certain plant type III PKSs (Lanz et al. 1991). Some of these residues also were found in PhlD proteins and other bacterial type III PKSs (Fig. 4) such as plant C¹⁶⁹, a cysteine residue that is essentially responsible for the catalytic activity of plant CHSs. In addition to this active cysteine, the conservation of a serine subunit contact site (S¹⁵⁸ in plants) and a glutamine residue (Q¹⁶⁶ in plants) could lead to a similar catalytic pocket in PhlD proteins and most plant type III PKSs. Nevertheless, cysteines in positions 135 and 195 (both implicated in substrate specificity) and lysine K¹⁸⁰ (important for enzymatic structure and function) in plant type III PKSs were not present in bacterial counterparts. Threonine, serine, and asparagine, respectively, were found in place of these amino acids in PhlD sequences. Noticeably, lysine and asparagine codons differ only at the third nucleotide position (purine and pyrimidine, respectively) and, after a single transversion, there is enough to yield an asparagine instead of a lysine.

Kyte–Doolittle hydropathy plots derived from predicted PhlD sequences were very similar from one to the other, yet CHA0 could be differentiated from other Phl⁺ pseudomonads such as Q2-87 (Fig. 5A) or F113 (Fig. 5B), especially when its hydrophobic peak in position 240 was considered. The similarity between PhlD proteins and PKSs from gram-positive

bacteria or plants was less pronounced, as illustrated when comparing CHA0 with *S. griseus* (Fig. 5C) or *Lycopersicon esculentum* (Fig. 5D).

Relationship between *phlD* polymorphism and broad genotypic features for 39 Phl⁺ pseudomonads.

Among the 39 Phl⁺ biocontrol fluorescent pseudomonads, a good relationship was found between groups defined by *phlD* PCR-restriction fragment length polymorphism (RFLP) and those defined by broad genotypic features (ARDRA or RAPD fingerprints) (Fig. 1). Indeed, HaeIII-1 corresponded to group ARDRA1, HaeIII-5 to ARDRA3, and the five other *phlD* restriction patterns were all found in ARDRA2 strains (Fig. 1). Additional subdivisions were made within ARDRA2 by comparing RAPD groups. HaeIII-2 was found in RAPD groups 4 and 6, HaeIII-3 and HaeIII-4 in RAPD group 3, and HaeIII-6 and HaeIII-7 in RAPD group 5.

The 16S rRNA genes of reference strains CHA0 (group ARDRA1), Q2-87 (ARDRA2), and F113 (ARDRA3) were sequenced. Sequence identity was 97.6% between CHA0 and Q2-87 (1,530 nucleotides overlap), 97.8% between CHA0 and F113 (1,529 nucleotides overlap), and 99.5% between Q2-87 and F113 (1,527 nucleotides overlap). The three sequences were aligned with other *Pseudomonas* spp. 16S rRNA sequences (Ribosomal Database Project database), and a NJ tree was inferred with *Pseudomonas putida* IAM1236 as the out group (Fig. 6). Noticeably, CHA0 clearly clustered away from Q2-87 and F113 because it was found in the cluster containing *Pseudomonas syringae*, *Pseudomonas caricapapayae*, and *Pseudomonas chlororaphis*. Strains Q2-87 and F113 clustered close to *P. corrugata* ATCC29736 (sequence identity was

Table 1. Origin and biocontrol properties of Phl⁺ fluorescent *Pseudomonas* spp. strains used in the study

Strain	Biocontrol activity ^a	Source ^b	Reference
Reference strains			
CHA0	Tobacco (Tb), wheat (Ggt), cucumber (Pu)	Tobacco; Morens, Switzerland	Stutz et al. 1986
Q2-87	Wheat (Ggt)	Wheat; Quincy, WA, U.S.A.	Vincent et al. 1991
Q65c-80	Wheat (Ggt)	Wheat; Quincy, WA, U.S.A.	Harrison et al. 1993
Pf-5	Cotton (Pu, Rs), Cucumber (Pu)	Cotton; Texas, U.S.A.	Howell and Stipanovic 1979
F113	Sugar beet (Pu)	Sugar beet; Ireland	Fenton et al. 1992
PF	Wheat (St)	Wheat; Oklahoma, U.S.A.	Levy et al. 1992
Isolates from Quincy, WA, U.S.A.			
Q1-87, Q4-87, Q5-87, Q6-87, Q7-87, Q8-87, Q9-87, Q12-87, Q13-87, Q37-87, Q86-87, Q95-87, Q139-87	Wheat (Ggt)	Wheat	Keel et al. 1996
Q88-87, Q128-87	Wheat (Ggt)	Wheat	Harrison et al. 1993
Isolates from Morens, Switzerland			
Pf1	Tobacco (Tb), cucumber (Pu)	Tobacco	Keel et al. 1996
C*1A1, CM1'A2	Cucumber (Pu, Ps), cotton (Rs)	Cucumber	Fuchs and Défago 1991
TM1A3, TM1A5, TM1B2	Cucumber (Pu, Ps), cotton (Rs)	Tomato	Fuchs and Défago 1991
TM1'A4, TM1'A5	Cucumber (Pu, Ps), cotton (Rs)	Tomato	Keel et al. 1996
Isolates from Albenga, Italy			
PINR2, PINR3	Cucumber (Pu), tomato (Forl)	Tobacco	Keel et al. 1996
PITR2, PITR3	Cucumber (Pu), tomato (Forl)	Wheat	Keel et al. 1996
PILH1	Cucumber (Pu), tomato (Forl)	Tomato	Keel et al. 1996
Isolates from IITA soil (Ghana)			
PGNR1, PGNR2, PGNR3, PGNR4, PGNL1	Cucumber (Pu), tomato (Forl)	Tobacco	Keel et al. 1996

^a Tb, *Thielaviopsis basicola*; Ggt, *Gaeumannomyces graminis* var. *tritici*; Pu, *Pythium ultimum*; Rs, *Rhizoctonia solani*; Ps, *Phomopsis sclerotoides*; St, *Septoria tritici*; Forl, *Fusarium oxysporum* f. sp. *radicis-lycopersici*.

^b *Pseudomonas* spp. strains were isolated from roots, with the exception of strain PF, which was isolated from wheat leaves. Root samples consisted of macerated roots or roots previously washed of soil.

99.3% for Q2-87 and 99.6% for F113, compared with 97.5% for CHA0) but were not distantly separated from the two *P. fluorescens* strains included in the tree. Comparison of *Phl*⁺ pseudomonads with *P. fluorescens* DSM50090 and IAM12022 (98.2% sequence identity between the two) yielded 97.5 and 98.2% sequence identity, respectively, for Q2-87; 97.7 and 98.2%, respectively, for F113; and only 96.2 and 96.6%, respectively, for CHA0.

Relationship between *phlD* polymorphism and production of *Phl* in vitro.

The relationship between *phlD* PCR-RFLP groups and *Phl* production was assessed on malt agar (Keel et al. 1992) and King's B agar (King et al. 1954) (Fig. 7A and B). Raw data of *Phl* production were obtained by Keel et al. (1996). Because several *phlD* PCR-RFLP groups comprise only a few bacteria each, the statistical significance of the relationship investigated for *phlD* clusters, which were defined at an arbitrarily chosen similarity level of 40% (Fig. 1) and were congruent with clusters derived from *phlD* sequence data (Fig. 2A). Thus, *Phl* production for HaeIII-1 ($n = 11$), HaeIII-2 ($n = 13$), and the cluster gathering HaeIII-3 to HaeIII-7 ($n = 15$) was 3.8 ($\pm$ standard deviation of 2.3), 18.1 ($\pm$ 8.5) and 27.8 ($\pm$ 12.1) μ g, respectively, per ml of malt agar and 1.2 ($\pm$ 3.9), 14.3 ($\pm$ 8.6), and 30.7 ($\pm$ 14.0) μ g, respectively, per ml of King's B agar. Each of the three groups was statistically different from the others ($P < 0.05$), regardless of the growth medium considered.

DISCUSSION

phlD is polymorphic.

Hybridization experiments carried out under high-stringency conditions suggest that the *phl* biosynthetic locus

was well conserved among *Phl*⁺ biocontrol fluorescent pseudomonads (Keel et al. 1996). Here, however, PCR-RFLP analysis revealed that one of the structural genes involved in *Phl* production, *phlD*, displayed substantial polymorphism as i) several different restriction patterns were obtained and ii) similarity levels between clusters were low (Fig. 1). *phlD* PCR-RFLP patterns agreed with those predicted by virtual restriction analysis of *phlD* sequences. Furthermore, the tree topology obtained for *phlD* with restriction data was congruent with that derived from *phlD* sequences, indicating that restriction analysis could represent a valuable tool in ecological studies of *Phl*⁺ *Pseudomonas* spp. populations. In the current work, certain *phlD* PCR-RFLP patterns appeared to be associated with particular soils or plant hosts, whereas others were not. Sampling biases were probably important, however, and thus the relationship between the *phlD* PCR-RFLP pattern and soil or plant host will have to be assessed with a larger collection of *Phl*⁺ pseudomonads. Indeed, other *Phl*⁺ fluorescent pseudomonads have been isolated from plants, especially monocots (McSpadden Gardener et al. 2000; Picard et al. 2000).

phlD characteristics suggest absence of positive selection on *PhlD*.

Despite the fact that *phlD* displayed significant polymorphism, the analysis of synonymous and nonsynonymous substitutions and the estimated value of the alpha parameter of the gamma distribution suggests a lack of adaptively driven amino acid substitutions in *PhlD* (absence of positive selection). Indeed, many synonymous substitutions, i.e., not leading to amino acid replacement, were observed. The dN–dS ratio was well below 1, which means that advantageous nonsynonymous mutations were much less frequent than synony-

Fig. 2. Phylogenetic relationships on the basis of **A**, *phlD* nucleotide and **B**, predicted *PhlD* amino acid sequences between *Phl*⁺ biocontrol fluorescent pseudomonads representing the seven *phlD* polymerase chain reaction–restriction fragment length polymorphism groups (HaeIII-1–HaeIII-7) (Fig. 1). Strains studied include CHA0 (group HaeIII-1), Q2-87 (HaeIII-2), Q65c-80 (HaeIII-3), CM1'A2 (HaeIII-4), F113 (HaeIII-5), PITR2 (HaeIII-6), and PILH1 (HaeIII-7). **A**, The *phlD* tree (depicted as midpoint rooted) was constructed by maximum likelihood (DNAML) and the robustness of the inferred tree was assessed by 100 bootstrap replicates. Nodal support is indicated when higher than 50%. Scale bar indicates the number of nucleotide substitutions per site. **B**, The *PhlD* tree (strict consensus tree; 1,000 bootstrap replicates) was constructed by the Neighbor-Joining method, with the Kimura formula.

mous (silent) mutations. In the absence of positive selection, most nonsynonymous substitutions in proteins are deleterious and result in purifying selection (Kimura 1983; Miyata and Yasugana 1980; Nielsen and Yang 1998), which apparently was the case for PhlD. A direct consequence of the relative importance of synonymous substitutions is that fewer clusters were obtained for PhlD than for *phlD* (Fig. 2). The relationship observed between strains when considering *phlD* sequences, however, also was found, to a large extent, in the PhlD tree. In both clusters, CHA0 clearly was different from the six other pseudomonads, whereas Q65c-80 clustered with CM1'A2 and P1TR2 with PILH1.

The lack of positive selection for PhlD agrees with an alpha parameter lower than 1, which indicates that most sites in the sequence have low substitution rates or are almost invariable. In fact, as many as 523 of the 746 nucleotides (70.1%) in *phlD* were constant, whereas 152 were parsimony uninformative and 71 parsimony informative.

PhlD differs from type III PKSs found in gram-positive bacteria and plants.

CHSs (EC 2.3.1.74) are plant members of the type III PKS superfamily (Bangera and Thomashow 1999) and implicated in the biosynthesis of flavonoids (Martin 1993; Schröder and Schröder 1990; Tropf et al. 1995). Although CHS-type reactions also can be catalyzed by enzymes of other plant secondary metabolism pathways such as valerophenone synthase (Zuurbier et al. 1998), CHSs display little similarity to other eukaryotic-condensing enzymes and, therefore, represent an independent development (Tropf et al. 1995). In this work, PhlD (predicted from *phlD* sequences) and plant CHS displayed common features, as indicated by the comparison of amino acid sequences (Table 2 and Fig. 4) and Kyte–Doolittle hydropathy plots (Fig. 5D). Cluster analysis clearly distin-

guished between PhlD and plant CHS (Fig. 3), however, and comparison of the active site region indicated differences that are likely to influence their substrate specificity (Fig. 4).

Type III PKSs also are known in other microorganisms, all of which correspond to gram-positive bacteria (Funa et al. 1999; Sorokin et al. 1996; Ueda et al. 1995). In *S. griseus*, production of a red-brown pigment associated with the PKS RppA (Ueda et al. 1995) was lost after site-directed mutagenesis of *rppA* targeting the central cysteine residue (Funa et al. 1999). This indicates that the cysteine had the same catalytic function as in plant CHSs, where it binds to the substrate (Lanz et al. 1991). Interestingly, type III PKSs from gram-positive bacteria were found between PhlD and plant type III PKS following cluster analysis (Fig. 3). The PKS closest to PhlD was from *S. griseus*, based on cluster analysis (Fig. 3) and an amino acid sequence comparison (Table 2). In cluster analysis, *S. griseus* was found at a distance of approximately 0.3 amino acid substitutions per site from other gram-positive bacteria. In addition, one of the plants (*Nicotiana sylvestris*) was found between the other plants and gram-positive bacteria. Therefore, it is possible that type III PKSs from fluorescent pseudomonads, gram-positive bacteria, and higher plants arose independently in each of the three phyla and may represent a case of convergent evolution of key enzymes involved in the biosynthesis of secondary metabolites such as pigments, flavonoids, and antimicrobial compounds rather than the result of horizontal gene transfer events between those phyla, as speculated earlier based on a smaller number of available sequences (Bangera and Thomashow 1999; Cook et al. 1995). This is in accordance with the observation that, at the scale of the 39 Phl⁺ pseudomonads studied in our report, each *phlD* PCR-RFLP pattern was associated strongly with particular ARDRA and RAPD groups (Fig. 1).

Table 2. Comparison of PhlD from *Pseudomonas* strain CHA0 with type III PKSs from Gram-positive bacteria and plants, based on the percentages of amino acid sequence identity and similarity. Gram-positive and plant sequences were first aligned with PhlD and then subjected to the calculations (the sequence from CHA0 was introduced first in the program BLAST 2 sequences)

Organism	Accession number ^b	CHA0 ^a	
		Identity ^c	Similarity
Gram-positive bacteria			
<i>Streptomyces griseus</i> ^d	BAA33495	45% (249)	59%
<i>Bacillus subtilis</i>	P54157	26% (225)	46%
<i>Deinococcus radiodurans</i>	A75315 (strain R1)	28% (233)	42%
<i>Mycobacterium tuberculosis</i> (1)	C70985 (pks11)	28% (233)	44%
<i>Mycobacterium tuberculosis</i> (2)	G70621 (pks10)	30% (240)	45%
Plants			
<i>Gerbera hybrida</i> (1)	CAA62683.1	28% (238)	45%
<i>Gerbera hybrida</i> (2)	P48391	26% (241)	43%
<i>Vitis vinifera</i> (1)	P51090	28% (236)	45%
<i>Vitis vinifera</i> (2)	BAA31259	28% (236)	45%
<i>Hordeum vulgare</i>	Q96562	27% (236)	43%
<i>Solanum tuberosum</i>	Q43188	28% (236)	44%
<i>Petunia hybrida</i>	P22928	27% (236)	45%
<i>Lycopersicon esculentum</i>	S12224	28% (226)	45%
<i>Nicotiana sylvestris</i>	T15054 (anther)	22% (235)	44%

^a Percentages of identity (and similarity) differed by 5% units or less when gram-positive and plant polyketide synthases (PKS) were compared with any of the other six Phl⁺ pseudomonads sequenced (rather than CHA0).

^b A GenBank data search was carried out via the ENTREZ service from the National Center for Biotechnology Information (Bethesda, MD, U.S.A.). Details in parentheses were used.

^c Number of amino acids considered in the calculations is given in parentheses.

^d Highest values were obtained with *S. griseus*. PKS identity between the six other Phl⁺ pseudomonads and *S. griseus* ranged from 45 to 49% (59 to 64% similarity).

Phylogenetic diversity of *Phl*⁺ pseudomonads.

The 39 *Phl*⁺ pseudomonads studied here have been characterized genetically with ARDRA (Keel et al. 1996), and three phylogenetic groups were identified. ARDRA patterns were in agreement with those (Fig. 6) obtained by virtual restriction analysis of the 16S rRNA sequences of CHA0, Q2-87, and F113. ARDRA groups 1, 2, and 3, defined by Keel et al. (1996), corresponded to ARDRA groups A, B, and C in the *phlD*⁺ fluorescent pseudomonad study by McSpadden Gardener et al. (2000). *AluI* was not discriminative in the two investigations, but Picard et al. (2000) found as many as four ARDRA profiles with this enzyme when comparing 167 *phlD*⁺ fluorescent pseudomonads from soil and maize roots. In the latter work, *Phl*⁺ strains also producing pyoluteorin, which are only known in ARDRA1 (Keel et al. 1996) and *phlD* group HaeIII-1 (Fig. 1), were not found. Here, the comparison of 16S rRNA sequences from strains CHA0 (ARDRA1), Q2-

87 (ARDRA2), and F113 (ARDRA3) indicate that ARDRA3 is closer to ARDRA2 than to ARDRA1 (Fig. 6) and confirms previous results of ARDRA clustering (Keel et al. 1996) as well as subsequent findings (McSpadden Gardener et al. 2000).

Strains CHA0 (Stutz 1985; Stutz et al. 1986), Q2-87 (Pier-son and Weller 1994), and F113 (Shanahan et al. 1992) were originally classified as *P. fluorescens*, largely on the basis of phenotypic tests. When considering 16S rRNA sequences, the three strains would belong to the "*P. fluorescens* lineage," which gathers *P. fluorescens*, *Pseudomonas aureofaciens*, *P. chlororaphis*, *Pseudomonas marginalis*, *Pseudomonas tolaasi*, and *Pseudomonas viridiflava*, proposed by Moore et al. (1996). The 16S rRNA sequences of Q2-87 and F113 are compatible with the *P. fluorescens* species, based on criterion (a threshold homology level of $\leq 97\%$) determined by Stackebrandt and Goebel (1994), whereas CHA0 is not (only 96.2 and 96.6% homology with *P. fluorescens* DSM50090 and

Fig. 3. Relationships between predicted amino acid sequences of *PhlD* from *Phl*⁺ biocontrol fluorescent pseudomonads and type III polyketide synthase from gram-positive bacteria and plants. The distance tree was generated by the Neighbor-Joining method implemented in PAUP. Nodal robustness was assessed by 100 bootstrap replicates. The 50%-majority-rule consensus tree is represented. Bootstrap values within the *Pseudomonas* spp. and plant clusters are not shown. Arrows indicate the nodes to which bootstrap values must be attributed. Scale bar represents the number of amino acid substitutions per site. Only genus names are given (Table 2 includes full names and accession numbers).

IAM12022, respectively). This raises the possibility that strain CHA0 should be considered to belong to a different species compared with Q2-87 and F113, in accordance with the observation that CHA0 clustered next to the *P. syringae* group (Fig. 6), as defined in Anzai et al. (2000). It should be remembered, however, that the current taxonomy of the genus *Pseudomonas sensu stricto* remains very unsatisfactory because of the large genomic diversity found within most *Pseudomonas* species described so far (Bossis et al. 2000). *P. fluorescens*, for instance, is expected to be subdivided into several different species once it is fully reassessed by DNA–DNA hybridization studies (Bossis et al. 2000; Palleroni 1992). In this context, it could be that the subdivisions made within ARDRA groups on the basis of RAPD clustering (Fig. 1) or spacer ARDRA (Sharifi-Tehrani et al. 1998) are more relevant than ARDRA groups in phylogenetic terms.

Patterns of Phl production linked with *phlD* polymorphism and phylogenetic diversity.

Individual Phl⁺ pseudomonads differ in their ability to produce Phl in vitro (Keel et al. 1996). Some of these differences

could be explained by distinguishing Phl⁺ strains capable of producing pyoluteorin (group ARDRA1) from ARDRA2 strains (Sharifi-Tehrani et al. 1998). We followed this approach further by grouping Phl⁺ pseudomonads according to their *phlD* restriction profile (Fig. 7), which resulted in additional subdivisions compared with the mere distinction between groups ARDRA1 and ARDRA2. Indeed, statistical differences were found when comparing the *phlD* clusters HaeIII-1, HaeIII-2, and HaeIII-3 to HaeIII-7 (defined at 40% similarity), which enabled us to discriminate strains within group ARDRA2. Unfortunately, only a small number of strains exhibiting *phlD* restriction patterns HaeIII-4, HaeIII-5, HaeIII-6, or HaeIII-7 have been identified so far, but this number is likely to increase with *phlD* restriction analysis of other Phl⁺ pseudomonads, which will allow a comparison to be made at the restriction pattern level rather than with broad *phlD* clusters. Nevertheless, *phlD* restriction patterns may be useful as markers in ecological studies or when selecting Phl⁺ pseudomonads for specific biocontrol applications.

Whether *phlD* polymorphism is of any relevance in terms of Phl production is unknown. Indeed, these differences in Phl

Plants	(1)	(2)	(3)	(4)	(5)	(6)
<i>Lycopersicon</i>	VVF C TTS...RP S VK...Y Q Q GC FAG...LA K DLA...VV C SEITAVTF					
<i>Solanum</i>	VVF C TTS...RP S VK...Y Q Q GC FAG...LA K DLA...VV C SEITAVTF					
<i>Petunia</i>	LVF C TTS...RS S VK...Y Q Q GC FAG...LA K DLA...VV C SEITAVTF					
<i>Vitis</i> 1	LVF C TTS...KP S VK...Y Q Q GC FAG...LA K DLA...VV C SEITAVTF					
<i>Vitis</i> 2	LVF C TTS...KP S VK...Y Q Q GC FAG...LA K DLA...VV C SEITAVTF					
<i>Gerbera</i> 1	LIF C TTA...SP S IS...Y Q Q GC SAG...LA K DLA...VV C CEITAIIF					
<i>Gerbera</i> 2	LIF C TTA...SP S VK...Y Q Q GC AAG...LA K DLA...IV C SEITAILF					
<i>Hordeum</i>	LVF C TTS...SP T VK...Y Q Q GC FGG...LA K DIA...VV C SEITAMAF					
<i>Nicotiana</i>	IVY V SSS...RN D IG...Y F L GC YGG...VA K DIA...LT T SETTILGF					
Gram-positive bacteria						
<i>Mycobacterium</i> 1	IAT A TVT...RP D VR...F G L GC VAG...RL R DYL...LV S VELC ¹³⁵ SLTY					
<i>Mycobacterium</i> 2	LIT A TVT...RA D VR...F G L GC VAG...RL H DYL...LV S VELC ¹³⁵ SLTY					
<i>Streptomyces</i>	IVY V SCT...RP E TR...A Q L GC AAG...RA H DFC...IV S CEFCS ¹³⁵ LCY					
<i>Deinococcus</i>	VVV V NTS...NP H AA...W G L GC AGG...RA A DLV...YV A VELC ¹³⁵ SLTL					
<i>Bacillus</i>	VFF V SST...SP Y TK...W G L GC AGG...RA A EYC...VI S AELC ¹³⁵ SLTF					
Phl⁺ pseudomonads						
CHA0	VIV T SCT...PT S TV...A Q L GC VAG...RA N DFA...IV S LEFSSSL ¹³⁵ CY					
Q2-87	VAV T SCT...RT S TV...A Q L GC VAG...RA N DFA...IV S LEFSSSL ¹³⁵ CY					
Q65c-80	VAV T SCT...RT S TV...A Q L GC AAD...RA N DFA...IV S LEFSSSL ¹³⁵ CY					
CM1'A2	VAV T SCT...RA S TV...A Q L GC VAG...RA N DFA...IV S LEFSSSL ¹³⁵ CY					
F113	VAV T SCT...RT S TV...A Q L GC VAG...RA N DFA...IV S LEFSSSL ¹³⁵ CY					
PITR2	VAV T SCT...RT S TV...A Q L GC VAG...RA N DFA...IV S LEFSSSL ¹³⁵ CY					
PILH1	VAV T SCT...RT S TV...A Q L GC VAG...RA N DFA...IV S LEFSSSL ¹³⁵ CY					

Fig. 4. Alignment of predicted amino acid sequences for the putative active site region of PhlD from Phl⁺ biocontrol fluorescent pseudomonads and type III polyketide synthase (PKS) from other bacteria and the active site region from plant chalcone synthases (CHS; Table 2). Conserved residues of particular interest in plant CHS are indicated by boxes. (1) Cysteine (C¹³⁵) from plant CHS implicated in substrate specificity and corresponding to threonine (T¹⁰⁴) in PhlD. (2) Serine (S¹³⁸) subunit contact site corresponding to S¹²⁷ in PhlD. (3) Glutamine (Q¹⁶⁶) residue conserved in most plant CHSs and corresponding to Q¹⁶⁶ in the PKS of *Streptomyces griseus* and Q¹³⁵ in PhlD proteins. (4) The glycine cysteine (GC) box corresponds to the conserved region with its putative (formally established for *S. griseus* and all plants, except *Nicotiana sylvestris*) catalytic cysteine residue (C¹³⁸ for Q2-87 and C¹⁶⁹ for the other bacteria and plants). (5) Lysine (K¹⁸⁰) residue, which corresponds to asparagine (N¹⁴⁹) in PhlD, conserved strictly in plant. (6) C¹⁹⁵ involved in product specificity in plant CHS. C¹⁷¹ for PhlD proteins and C¹⁹⁹ for other bacterial PKSs are underlined to indicate potential binding sites for the substrate.

production may be explained by the link between *phlD* characteristics and other strain properties, as illustrated by ARDRA and RAPD groups (Fig. 1). Furthermore, whether data obtained in vitro with the use of two laboratory media is of any relevance when considering actual Phl production in situ, i.e., in the rhizosphere, remains to be established. In this work, no clear relationship was found between Phl production in vitro for a given *phlD* restriction group and the origin (plant host or geographic location) of the strains, but perhaps Phl⁺ biocontrol strains from a given *phlD* restriction group are par-

ticularly suited for production of Phl when colonizing a specific host plant or in a given soil type. This issue deserves further attention because of its potential implications in terms of microbial ecology and plant protection.

MATERIALS AND METHODS

Organisms, culture conditions, and storage.

Phl⁺ fluorescent *Pseudomonas* spp. strains used in this study are listed in Table 1. All strains hybridized to a 4.8-kb DNA probe harboring the *phl* biosynthetic genes from strain

Fig. 5. Kyte–Doolittle hydropathy plots of polyketide synthase sequences (indicated by thick lines) of **A**, Q2-87; **B**, F113; **C**, *Streptomyces griseus*; and **D**, *Lycopersicon esculentum* compared with CHA0 (indicated by a thin line). Sequence position refers to plant chalcone synthase (CHS) numbering. The hydrophobicity index (positive and negative values indicate hydrophobic and hydrophilic properties, respectively) was calculated for a window of seven residues. Arrows indicate the C¹⁶⁹ in plant CHS and the corresponding cysteines in bacterial counterparts.

Q2-87 (Keel et al. 1996). They were routinely cultivated on King's medium B (King et al. 1954) at 27°C and stored at -80°C in 40% glycerol.

PCR amplification and restriction analysis of *phlD*.

Two bacterial colonies were suspended in 100 µl of lysis buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.1% Tween 20) and then heat lysed for 10 min at 99°C. PCR amplification was performed with forward primer Phl2a (20-mer 5'-GAG GACGTCGAAGACCACCA-3') and reverse primer Phl2b (20-mer 5'-ACCGCAGCATCGTGTATGAG-3'), which were developed from sequences present within *phlD* (1,047 bp) (Banger and Thomashow 1999) in *P. fluorescens* Q2-87 (Raaijmakers et al. 1997). Primers were synthesized by MWG Biotech (Basel, Switzerland). PCR amplification was carried out in 20-µl reaction mixtures containing 5 µl of heat-lysed bacterial suspension, 1× PCR buffer (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), 100 µM (each) dATP, dCTP, dGTP, and dTTP (Amersham Pharmacia), 0.20 µM (each) primer, and 1.1 U of *Taq* DNA polymerase (Amersham Pharmacia). Amplifications were performed with a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA, U.S.A.). The initial denaturation (2 min at 94°C) was followed by 30 PCR cycles (94°C for 40 s, 63°C for 30 s, and 72°C for 30 s) and a final extension at 72°C for 10 min.

Restriction analysis was performed with 5 µl of amplified product and 10 µl of restriction buffer containing 1.5 U of either *Hae*III, *Sau*3A, *Nde*II, *Cfo*I, or *Msp*I (Boehringer Mannheim, Mannheim, Germany). After a 2-h digestion at 37°C, restriction fragments were separated by gel electrophoresis (2.5% agarose) with standard protocols (Sambrook et al.

1989). A 100-bp ladder (Life Technologies, Gaithersburg, MD, U.S.A.) was used as a molecular size marker. For each *Pseudomonas* spp. strain, PCR amplification and restriction analysis were performed at least three times. The different patterns obtained with each of the five endonucleases were readily distinguishable from one another and highly reproducible from one experiment to the next.

Calculation of the Jaccard coefficient of pairwise similarity (Jaccard 1908), which was determined by the presence or absence of bands and cluster analysis with the unweighed pair group method with averages (UPGMA) algorithm (Nei and Li 1979), were performed with NTSYS-pc numerical taxonomy and multivariate analysis software (Applied Bio-statistics, Setauket, NY, U.S.A.). Only band sizes over 80 bp were evaluated.

Sequencing of *phlD*.

phlD sequences were already available for strains CHA0 (GenBank accession no. AF214456), Pf-5 (AF214457), Q8r1-96 (AF207693), M1-96 (AF207692), and UP61 (AF214108). In the current work, PCR amplicons were purified with the Qiaquick Extraction Kit (Qiagen, Basel, Switzerland) and cloned in pPCR-Script Amp SK(+) cloning vector, according to the manufacturer's recommendations (Stratagene, Zurich, Switzerland). They were directly sequenced with primers M13 and M13rev (Microsynth, Balgach, Switzerland), and *phlD* sequences were deposited in the EMBL database for strains CHA0 (accession no. AJ278806), Q65c-80 (AJ278807), CM1'A2 (AJ278808), PITR2 (AJ278809), PILH1 (AJ278810), and F113 (AJ278811).

Fig. 6. Phylogenetic relationships between *Phl*⁺ *Pseudomonas* spp. strains CHA0 (group ARDRA1), Q2-87 (group ARDRA2), F113 (group ARDRA3), and other *Pseudomonas* spp. based on 16S rDNA sequences. The Neighbor-Joining tree was constructed according to Jukes and Cantor (1969), with *Pseudomonas putida* IAM1236 as the outgroup (1,000 bootstrap replicates). Scale bar represents the number of nucleotide substitutions per site. Virtual restriction analysis of the 16S rDNA sequences of CHA0, Q2-87, and F113 was performed with six enzymes: *Cfo*I, *Taq*I, *Alu*I, *Rsa*I, *Hpa*II, and *Hae*III. Fragment sizes with *Cfo*I: 203, 250, 281, 357, and 439 bp for Q2-87 and F113 and 63, 91, 250, 281, 409, and 436 bp for CHA0; with *Taq*I: 53, 223, 361, and 893 bp for CHA0 and Q2-87 and 53, 223, 315, 361, and 578 bp for F113; with *Alu*I: 15, 106, 162, 196, 207, 209, 230, and 403 bp for the three strains; with *Rsa*I: 146, 153, 357, and 874 bp for Q2-87 and F113 and 6, 70, 146, 150, 357, and 801 bp for CHA0. With *Hpa*II: 11, 81, 110, 130, 163, 486, and 549 bp for Q2-87 and F113 and 11, 81, 110, 130, 142, 160, 347, and 549 bp for CHA0. With *Hae*III: 9, 34, 37, 59, 157, 161, 171, 220, and 682 bp for Q2-87 and F113 and 9, 34, 37, 59, 156, 171, 220, and 844 bp for CHA0.

Sequencing of 16S rRNA genes.

Twenty microliters of heat-lysed cell suspension was used for PCR amplification with a pair of universal primers (FGPF5-255 and FGPS1509'-153) corresponding to *Escherichia coli* (GenBank accession no. J01859) at positions 5 to 24 and 1,524 to 1,540 (Normand 1995). Additional primers within the sequences were designed to carry out double-stranded coverage of the whole 16S rRNA gene (Normand et al. 1996). PCR was performed in 100 µl of reaction volume with the Expand High Fidelity PCR system (Roche Molecular Biochemicals, Mannheim, Germany). PCR amplicons were purified with the Qiaquick Extraction Kit and sequenced by Genome Express (Grenoble, France). Complete 16S rRNA sequences were determined for strains CHA0 (1,531 bp), Q2-87 (1,528 bp), and F113 (1,527 bp) and are available at the EMBL database at accession nos. AJ278812 (CHA0), AJ278813 (Q2-87), and AJ278814 (F113).

DNA sequence analyses.

Consensus DNA sequences were assembled with the Genetic Computer Group (GCG) fragment assembly system, version 10 (University of Wisconsin, Madison, U.S.A.). Virtual restriction analysis also was carried out with GCG. Multiple sequences were aligned with the CLUSTAL W computer program (Thompson et al. 1994); sites presenting alignment gaps were excluded from analysis (alignments available upon request).

For *phlD* nucleotide sequences, the maximum-likelihood tree was constructed with the DNA Maximum Likelihood program (DNAML) of the Phylogeny Inference Package (PHYLIP; J. Felsenstein, University of Washington, Seattle, U.S.A.). The strict consensus tree from out of 100 bootstrap replicates (global rearrangements, jumbled five times) was obtained with PHYLIP's CONSENSE program, and tree plotting was performed with TreeExplorer 2.12 (K. Tamura, Penn-

Fig. 7. Phl production on **A**, malt agar and **B**, King's B agar, in accordance with *phlD* polymerase chain reaction-restriction fragment length polymorphism patterns for 39 Phl⁺ biocontrol pseudomonads. Means and standard deviations (error bars) were obtained from raw data presented in Keel et al. (1996). The number of bacteria sharing a same restriction pattern is indicated in parentheses.

sylvania State University, University Park, U.S.A.). Base composition was obtained by the PAML package, and statistical differences between strains in base composition (overall and at each of the three codon positions) were assessed with a Chi-square test at $P < 0.05$. The alpha parameter of the gamma distribution and the Ti–Tv ratio were estimated with the model F84 (program BASEML, implemented in the PAML package). The numbers of synonymous (dS) and non-synonymous substitutions (dN) between each pair of *phlD* sequences were estimated in accordance with Nei and Gjobori (1986) and implemented in the MEGA program by applying the Jukes–Cantor formula. Statistical comparison between dN and dS was performed in MEGA with the use of a Student *t* test incorporating infinite degrees of freedom. Parsimony analyses were performed with PAUP (implemented in GCG), PHYLIP, or MEGA.

The 16S rRNA sequences were aligned with the most similar sequences of the Ribosomal Database Project database. Only complete sequences that yielded the highest similarity scores were kept for subsequent analysis. The corresponding *Pseudomonas* spp. strains were *P. fluorescens* IAM12022 (GenBank accession no. D84013) and DSM 50090 (Z76662), *Pseudomonas caricapapayae* ATCC33615 (D84010), *P. corrugata* ATCC29736 (D84012), *P. syringae* ATCC19310 (D84026) and LMG1247 (Z76669), *P. chlororaphis* LMG5004 (Z76657), and *P. putida* IAM1236 (D84020). The sequence alignment was checked manually and corrected. The NJ tree was constructed with MEGA, in accordance with Jukes and Cantor (1969). *P. putida* IAM1236 was used as the out group. Nodal support of the inferred tree was evaluated by 1,000 bootstrap replicates.

Amino acid sequence analyses.

Predicted PhlD amino acid sequences were aligned (CLUSTAL W) by taking into account the PhlD sequence of strain Q2-87 (accession no. AAB48106). NEIGHBOR, implemented in PHYLIP package, was used to generate a NJ tree, according to the 2-parameter model of Kimura (1980), and statistical support of a given node was evaluated by 1,000 bootstrap replicates. The strict consensus tree was obtained with CHA0 as the outgroup. Parsimony analyses were performed with PAUP (implemented in GCG), PHYLIP, or MEGA. The identity and similarity between PhlD sequences were calculated with BLAST 2 program sequences, version 2.0.14 (National Center for Biotechnology Information, Bethesda, MD, U.S.A.), with a BLOSUM62 default matrix gap opening and gap extension penalties of 10 and 1, respectively.

Sequences similar to PhlD were searched in the GenBank and EMBL databases with the BLAST, Fasta3t, and MOTIF programs. The ExPASy molecular tool server was used to scan PROSITE and SWISS-PROT for active site and protein domain similarities. Sequences were aligned with CLUSTAL W software, and sites presenting alignment gaps were removed from analysis. The NJ tree was generated with 100 bootstrap replicates to assess nodal support with PAUP in the GCG package or 1,000 bootstrap replicates with PHYLIP (Kimura formula). Both methods gave the same tree topology. The 50%-majority-rule consensus tree was presented unrooted. The identity and similarity between PhlD and other PKSs from plants and gram-positive bacteria were determined as described above.

Kyte–Doolittle (1982) hydropathy plots were determined with the PepPlot program included in the GCG package. The hydrophobicity index was calculated over a window of seven residues.

Phl production.

Raw data of Phl production (Keel et al. 1996) were used to calculate means and standard deviations (Fig. 7) for strains sharing a same *phlD* restriction pattern. The raw data did not fit a normal distribution because of skewness and kurtosis of the data, but this could be corrected with a $\log(x + 1)$ transformation. When comparing clusters HaeIII-1, HaeIII-2, and HaeIII-3 to HaeIII-7 (Fig. 1) for a given medium, data were $\log(x + 1)$ transformed prior to performing analysis of variance and subsequent Tukey “Honestly Significantly Different” tests ($P < 0.05$). Calculations were performed with SYSTAT for Windows, version 7 (SPSS Science, Chicago, IL, U.S.A.).

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