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Evolutionary History of the *phl* Gene Cluster in the Plant-Associated Bacterium *Pseudomonas fluorescens*▽†

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***Pseudomonas fluorescens* is of agricultural and economic importance as a biological control agent largely because of its plant association and production of secondary metabolites, in particular 2,4-diacetylphloroglucinol (2,4-DAPG). This polyketide, which is encoded by the eight-gene *phl* cluster, has antimicrobial effects on phytopathogens, promotes amino acid exudation from plant roots, and induces systemic resistance in plants. Despite its importance, 2,4-DAPG production is limited to a subset of *P. fluorescens* strains. Determination of the evolution of the *phl* cluster and understanding the selective pressures promoting its retention or loss in lineages of *P. fluorescens* will help in the development of *P. fluorescens* as a viable and effective inoculant for application in agriculture. In this study, genomic and sequence-based approaches were integrated to reconstruct the phylogeny of *P. fluorescens* and the *phl* cluster. It was determined that 2,4-DAPG production is an ancestral trait in the species *P. fluorescens* but that most lineages have lost this capacity through evolution. Furthermore, intragenomic recombination has relocated the *phl* cluster within the *P. fluorescens* genome at least three times, but the integrity of the cluster has always been maintained. The possible evolutionary and functional implications for retention of the *phl* cluster and 2,4-DAPG production in some lineages of *P. fluorescens* are discussed.**

The *Pseudomonas* genus includes species that live in close association with eukaryotic hosts such as insects, plants, and humans. Some of these, for example, *P. entomophila*, *P. syringae*, and *P. aeruginosa*, are pathogens, whereas others, such as *P. fluorescens*, may be beneficial to the host. It is emerging that there is often a fine line separating pathogenic and beneficial interactions, which makes the determination of the genes that drive interactions between different pseudomonads and their hosts fundamental to understanding microbe-host interactions (49). In this regard, gene clusters (for example, the type three secretion loci in *P. syringae* and the insecticidal locus in *P. entomophila*) appear to be very important in pathogenic pseudomonads (17, 49, 63). In contrast, the molecular basis for beneficial associations between pseudomonads and eukaryotic hosts is poorly understood. *P. fluorescens* is the archetypical beneficial plant-associated bacterium and has been isolated from the rhizospheres of diverse plants, making this an ideal model to study the evolution of beneficial plant-microbe interactions. Gene clusters in *P. fluorescens* are known, and one of the best studied is the *phl* locus, which encodes the biosynthetic and regulatory genes for production of 2,4-diacetylphloroglucinol (2,4-DAPG). This low-molecular-weight polyketide inhibits a variety of phytopathogens, including fungi, helminths, and nematodes, and is responsible for the biocontrol capabilities of many *P. fluorescens* isolates (8, 16, 22, 30, 42, 64, 65). 2,4-DAPG also directly affects plants and is reported to trigger induced systemic resistance, leading to enhanced plant protection (26, 54), and to promote exudation of amino acids from the plant root (45). Intriguingly, there are a number of reports that some plant genotypes actually select for 2,4-DAPG-producing strains, suggesting that 2,4-DAPG may be involved in mediating some type of association with plant hosts (39, 46).

Despite the potential benefits of 2,4-DAPG production, the percentage of *P. fluorescens* isolates that produce 2,4-DAPG is likely to be in single figures (12, 42; J. A. Moynihan and J. P. Morrissey, unpublished data). The 8-kb cluster involved in the biosynthesis, regulation, export, and degradation of 2,4-DAPG consists of eight genes, *phlHGFACBDE*, and is conserved at the organizational level in 2,4-DAPG-producing strains (1, 2, 5, 7, 13, 31, 57). The key biosynthetic gene is *phlD*, which displays interesting similarity to genes for plant chalcone synthases. This type III polyketide synthase is unusual in prokaryotes, and it was suggested that the gene may have been acquired by horizontal gene transfer (5, 6, 12). In contrast, phylogenetic analysis of *phlD* does not support this theory, so the provenance of 2,4-DAPG production remains uncertain (52, 53). Understanding the evolution of the *phl* biosynthetic genes offers a route to determining the precise role of 2,4-DAPG in beneficial plant-microbe interactions. In this study, we exploited genomic resources to reconstruct the evolutionary history of the *phl* cluster in *P. fluorescens*.

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TABLE 1. Strains used in this study

Species and strain (accession no.)	Source	Location	*phl* status	Reference
P. brassicacearum NFM421	*A. thaliana*	France	+	4
P. fluorescens				
F113 (AJ278811)	Sugar beet	Ireland	+	58
5AT2	Sugar beet	Ireland	+	This study
4A3	Sugar beet	Ireland	+	This study
MVP1-6 (AY928637)	Pea	United States	+	33
CM1'A2 (AJ278808)	Cucumber	Switzerland	+	20
7MA12 (AY548378)	Pea	United States	+	33
7MA20 (AY928660)	Pea	United States	+	33
6WSU4 (AY928656)	Pea	United States	+	33
6MA17 (AY928658)	Pea	United States	+	33
STAD384-97 (AY928633)	Wheat	United States	+	38
PITR2 (AJ2788090)	Wheat	Italy	+	31
PILH1 (AJ278810)	Tomato	Italy	+	31
Q2-1 (AY928632)	Wheat	United States	+	40
Q1-87 (AY928631)	Wheat	United States	+	31
Pf-5 (AF214457)	Cotton	United States	+	24
PGNL1	Tobacco	Ghana	+	31
MVP1-3 (AY928628)	Pea	United States	+	33
PINR2 (AY928630)	Tobacco	Italy	+	31
PGNR2	Tobacco	Ghana	+	31
CHAO (AJ278806)	Tobacco	Switzerland	+	61
I5B	Wheat	United States	−	41
SBW25	Sugar beet	England	−	51
E206	Wheat	United States	−	41
dI1	Wheat	United States	−	41
Pf0-1	Loam soil	United States	−	11
PFCA3	Sugar beet	Ireland	−	This study
PFCA5	Sugar beet	Ireland	−	This study
PFTC1	Sugar beet	Ireland	−	This study
PFTC9	Sugar beet	Ireland	−	This study
PFCC11	Sugar beet	Ireland	−	This study
PFTA11	Sugar beet	Ireland	−	This study
PFCA11	Sugar beet	Ireland	−	This study
PFCC2	Sugar beet	Ireland	−	This study
PFTA1	Sugar beet	Ireland	−	This study
PFTA5	Sugar beet	Ireland	−	This study

MATERIALS AND METHODS

Genome sequences. The complete nucleotide sequences and annotations of the *P. fluorescens* Pf-5, *P. fluorescens* Pf0-1, *P. putida* KT2440, *P. aeruginosa* PAO1, *P. syringae* pv. tomato DC3000, *P. syringae* pv. phaseolicola 1448A, *P. syringae* pv. syringae B728a, and *P. entomophila* L48 genomes used in this project were retrieved and downloaded from the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/genomes) (9, 15, 28, 43, 44, 60, 63). Relevant sequences from *P. fluorescens* SBW25 were retrieved from the Sanger Institute (http://www.sanger.ac.uk/Projects/P_fluorescens/). A 170-kb bacterial artificial chromosome (BAC) library clone from *P. fluorescens* F113, which contained the *phl* cluster, was also examined. For examination of the regions flanking the *phl* cluster, BLAST analysis was first performed (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Approximately 10 of the open reading frames (ORFs) flanking the cluster in Pf-5 were analyzed against *P. fluorescens* Pf0-1, *P. putida* KT2440, *P. aeruginosa* PAO1, *P. syringae* pv. tomato DC3000, *P. syringae* pv. phaseolicola 1448A, *P. syringae* pv. syringae B728a, and *P. entomophila* L48. In addition to this, bioinformatic analysis was also performed using WebACT, an Artemis comparison tool based on BLAST scores that allows for the comparison of complete genomes (www.webact.org/WebACT/home) (3).

F113 annotation and mapping. Annotation of an approximately 170-kb BAC clone was performed using Artemis. Predicted ORFs were identified using two gene prediction programs, Glimmer and Orpheus (14, 19). BLASTP analysis was performed to determine the putative function of the predicted gene. To search for the presence of tRNA in the F113 sequence, tRNAscan-SE was used (35). Following the identification of putative genes, mapping of the 170-kb region against other sequenced *Pseudomonas* species was performed to identify core regions present in *P. fluorescens* sequenced strains and regions present in other *Pseudomonas* species. BLAST identity values as well as gene numbers were noted to identify regions of interest. Genes were scored as positive if the identity values were above 70%. Genes with the identity values close to 70% were also acceptable if these genes were contiguous with other genes that had identity values of 70% or greater.

Bacterial strains. A total of 36 bacterial strains, comprising 21 2,4-DAPG-producing and 15 nonproducing strains, were used in this study (Table 1). These strains were isolated from different plant rhizospheres in diverse geographical locations. With the exception of *P. brassicacearum* NFM421, all are classified as *P. fluorescens*. Standard *Pseudomonas* growth and storage conditions were used.

DNA isolation. Chromosomal DNA was extracted from a 5-ml overnight culture of each strain in LB broth using the G-nome DNA isolation kit following the manufacturer's protocol (Bio 101, Cambridge, United Kingdom). Following extraction, DNA was resuspended in 200 μl of 10 mM Tris buffer (pH 8), quality was verified on a 0.6% agarose gel, and DNA was stored at −20°C.

Molecular analysis. The oligonucleotide primers used in this study are listed in Table 2. Standard PCR mixtures and PCR conditions were employed: typically, an initial denaturation step at 96°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 s, 30 s of primer annealing with temperatures dependent on the primer pairs used, and an extension of 1 kb per min at 72°C, in a PTC-200 Peltier thermal cycler (MJ Research). PCR using the primer pair Phl2a and Phl2b (50) was used to identify *phlD*-positive strains. PCR assays were also performed to examine the genomic context of the *phl* cluster, based on primers from Pf-5 and F113, among our collection of *phl*-positive isolates. For two *phl*-positive strains, Q2-1 and Q1-87, additional analysis of the genomic context was performed using a semirandom, two-step PCR protocol (ST-PCR)

TABLE 2. Primers used in this study

Function and primer	Sequence (5′ to 3′)	Product size (kb)	Reference
Genomic contexts			
F113 5′ end			
HPF	CCTGTTTACCCACGGCTTGC	1.8	This study
PhlGR	GCTCATCAGGGCACTGACG		
F113 3′end			
PhlDF	CGCCGGACAACCATGTCCT	2.8	This study
CHPR	CTGAGCCAATCACCGTGCTG		
Pf-5 5′end			
5950F	GACACCTTCTGGCGTAACGTC	1.7	This study
5952R	TCAACGACACCGGCACCTCA		
Pf-5 3′end			
5957F	GATGCCCGCAACCACGTACTG	4.5	This study
5959R	CTGTGACGAAAGCACCGTGAC		
SBW25 and Pf0-1			
5950F	CCGC(GC)ATGCAGAAAGTCC	3.2 (Pf0-1)	This study
5961R	GGATCTTCAAGCTCAAGC	2.3 (SBW25)	
ST-PCR			
Degenerate primers			
2a	GGCCACGCGTCGACTAGTACN$_{(10)}$AGAG		36
2b	GGCCACGCGTCGACTAGTACN$_{(10)}$ACGCC		36
2c	GGCCACGCGTCGACTAGTACN$_{(10)}$GATAT		10
4	GGCCACGCGTCGACTAGTAC		10
Specific primers			
phlH1r(ST)	AGTGCTGACACGTGAACTGC		This study
phlH3r(ST)	TTTCCCCAAGGCACACATAC		This study
phlE1f(ST)	CATACTGACGTGCCAATTCG		This study
phlE3f(ST)	CCTGTCGTTTCCATCTGCTT		This study
phlE3f2(ST)	AGTTTACTGGCCACCGCTAC		This study
phlE3f3(ST)	GACCCGTTGCAGATTCCTTA		This study
phlE3f4(ST)	CAATCCGTGCAGCGAACATC		This study
phlE3f5(ST)	TAGGCCAATACGCCAACAAT		This study
phlE3f6(ST)	CGGGCTGATACCGAACAATA		This study
Multilocus sequence typing analysis			
Housekeeping			
gyrBF	CGGTAAGTT(CT)GACGACAACTC	0.75	This study
gyrBR	CAGGAAGTCGGAGAAGTAC		
leubF	CTGGGCTTCGAGCTGAGCC	0.56	This study
leubR	GCACGCACCAGTTGCATGG		
groELF	GATGCCCATTTCGATCATG	0.67	This study
groELR	GAACAACATGCGTGGCATC		
rpoDF	GAAATCGCCAAGCGTATCG	0.74	This study
rpoDR	ATCGACATGCGACGGTTG		
phlD			
Phl2a	GAGGACGTCGAAGACCACCA	0.745	50
Phl2b	ACCGCAGCATCGTGTATGAG		

(10). The *phl*-negative insertion site was examined using primers designed to Pf0-1 and SBW25 (Table 2).

Multilocus sequence analysis. Multilocus sequence analysis was performed using primer pairs designed for PCR amplification of four housekeeping (HK) genes (*gyrB*, *leuB*, *groEL*, and *rpoD*) and one gene from the *phl* cluster, *phlD* (Table 2). Many of the *phlD* sequences from the *phl*-positive isolates were obtained through the accession numbers given in Table 1. Following PCR product amplification, PCR purification was performed using the JetQuick protocol from GenoMed (Löhne, Germany) or the gel extraction kit protocol from Qiagen (West Sussex, United Kingdom), and sequencing was performed by MWG Biotech (Martinsried, Germany).

Phylogenetic analysis. Sequences for the four HK genes and *phlD* were aligned using the ClustalW multiple-sequence alignment program (http://www.ebi.ac.uk/clustalw/) (62). Further sequence analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA) version 3.1 program (32). The rates per site of synonymous (K_S) and nonsynonymous (K_N) substitutions were calculated. The K_N/K_S ratios, which provide information on whether purifying (ratio, <1), neutral (ratio, 1), or diversifying (ratio, >1) selection is acting on the gene under study, were determined. Tranversion and transition changes were also examined. Total nucleotide diversity (π) was measured for each gene between the two main lineages, *phl* positive and *phl* negative. The concatenated sequence was used to measure π within the *phl*-positive strains and within the *phl*-negative lineage.

Four different statistical tests to test for recombination in each gene sequence were employed. These were the maximum chi-square test (http://www.lifesci.sussex.ac.uk/CSE/test/maxchi.php) (48, 59), LD (r^2 versus distance), LD (D' versus distance) (http://www.lifesci.sussex.ac.uk/CSE/test/ld.php) (23, 34, 47), and the PHI test (SplitsTree4, version 4.8) (25). Phylogenetic trees for the *phlD* gene and concatenated HK genes were constructed using the neighbor-joining method with the Jukes-Cantor distance method for all sites (29, 55). Bootstrap tests using 1,000 replicates were performed to test the robustness of each phylogeny.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers FJ012172 (F113 BAC clone), FJ012173 to FJ012205 (*groEL*), FJ012206 to FJ012238 (*gyrB*), FJ012239 to FJ012271 (*leuB*), FJ012277 to FJ012309 (*rpoD*), FJ012272 to FJ012276 (*phlD*), FJ012310 and FJ012311 (Q2-1 and Q1-87 3′ region before the 2,4-DAPG gene cluster), and FJ012312 and FJ012313 (Q2-1 and Q1-87 5′ region after the 2,4-DAPG gene cluster).

RESULTS

The *phl* gene cluster is located in several genomic loci. To understand the evolution of the 2,4-DAPG biosynthetic and regulatory genes, we first looked at the genomic context of the *phl* cluster (PFL_5951-PFL_5958) in the sequenced strain *P. fluorescens* Pf-5. We compared the region flanking the *phl* cluster in Pf-5 with genomes of other sequenced *Pseudomonas* species (Fig. 1a; see Table S1 in the supplemental material). This analysis showed that although these other *Pseudomonas* strains do not carry the *phl* cluster, the genes 5′ and 3′ of the cluster in Pf-5 have clear orthologues in all the sequenced *Pseudomonas* strains. Furthermore, with only minor variations, these orthologous genes are contiguous in all species, indicating that the *phl* cluster in Pf-5 is located in a region of the ancestral *Pseudomonas* genome. To determine whether the *phl* cluster genomic location was a hot spot for integration of foreign DNA in *P. fluorescens*, we analyzed this genomic region in *phl*-negative strains using primers designed from Pf0-1 and SBW25 gene sequences (Fig. 1b). Some variation in the size of the product at this site was seen, but no evidence for large DNA insertions was found in Pf0-1 and SBW25 or any of the other 13 *phl*-negative strains examined (data not shown). This demonstrates that this genomic region is not an insertional hot spot. As expected, a band was not amplified from the *phl*-positive strain Pf-5 under these conditions, but unexpectedly, another *phl*-positive strain, *P. fluorescens* F113, resembles the *phl*-negative strain SBW25 at this locus, which therefore indicates that a large DNA insertion is not present at this locus in strain F113 (Fig. 1b). This finding means that the *phl* cluster has different locations in the Pf-5 and F113 genomes and raised the possibility that the cluster could be on a mobile genetic element.

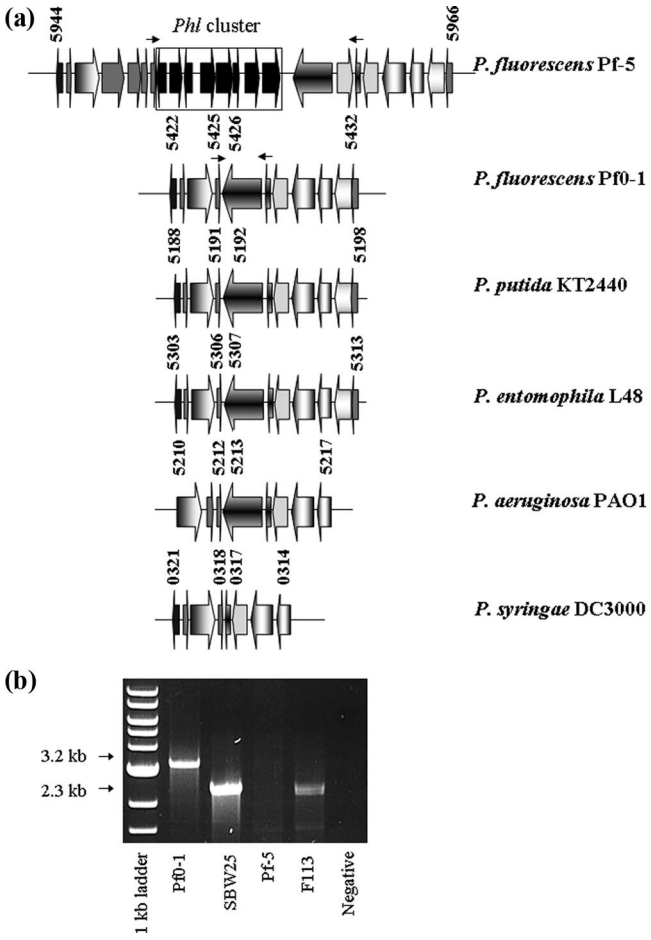

FIG. 1. The *P. fluorescens* Pf-5 *phl* cluster is located within the core *Pseudomonas* genome. The *phl* cluster genes are surrounded by a box. The arrows on either side represent the genes flanking the cluster in *P. fluorescens* Pf-5 and indicate ORF orientation. Also shown above the arrows are gene numbers. (a) Comparison of *P. fluorescens* Pf-5 with the sequenced *Pseudomonas* species. In this image *P. syringae* 1448a and B728a are not included, as both have the same result as for DC3000. Identical shaded patterns indicate homologous genes, which are found both 5′ and 3′ of the *phl* cluster. Homologous genes are contiguous in each of the species examined. (b) PCR results using primers designed to *P. fluorescens* Pf-5 flanking genes, PFL_5950 and PFL_5961. The positions of the primers are indicated by arrows in both the *P. fluorescens* Pf-5 and Pf0-1 diagrams in panel a. Shown here are the PCR results for two *phl*-positive strains (F113 and Pf-5) and two *phl*-negative strains (Pf0-1 and SBW25).

To determine the genomic context of the *phl* locus in F113, we annotated and analyzed a 170-kb F113 BAC clone that carried the *phl* genes to establish the genomic context of the *phl* cluster in strain F113. Along with the eight genes of the *phl* cluster, we identified 109 putative genes on this BAC clone. There were two interesting features to these genes: first, most did not have clear orthologues in other sequenced *Pseudomonas* species and thus constitute a large genomic region that appeared to be unique to strain F113, and second, there were several examples of gene clusters with orthologues in *P. syringae* or *P. entomophila* but not in other *P. fluorescens* strains (see Tables S2 and S3 in the supplemental material). The *phl* cluster had the same gene order in Pf-5 and F113 and showed a high level of sequence similarity, averaging 77% at the nucleotide level and 81% at the amino acid level over the eight genes. Comparison of the flanking genes showed that the *phl* cluster in F113 was not located in the same genomic context as in Pf-5, which led us to investigate the location of the *phl* cluster among a collection of 21 *phl*-positive isolates by PCR and Southern blotting using primer pairs specific for either the Pf-5 or F113 locations (Table 2). Employing these strain-specific primers, PCR was used to amplify the sequences between the first gene before the cluster and the *phlG* gene (5′ region) and between the *phlD* gene and a gene downstream of the cluster (3′ region). Of the 19 additional strains, 5 strains resembled Pf-5, 12 strains resembled F113, and 2 strains, Q2-1

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F113    MTHYVMLLDSPVP-RGGLGVFDFKSGQSREVRPPQGNLYEALAHCADRNMDWESYLKDLPSTPYPLT--PAAVKYRPPLKPDNNLLVQIS 87
Q2-1    MTHYVMLLDSPQP-LRGLGVFDFKSGQSLEVRSPQGNLYEALSHCARLNMDWESYLRSLPSTPYPLS--PTGAKYRPPLKPDDNLAVQVS 87
Pf-5    MDDYLLLLDVCHKDQRAIGLFDFHNGRSVRAYSEHGGLYETLLFCSRRHLGWSEHLRSLTPHPQLPADGPQQGPLRPPLKPRDNLLVQVS 90
        * .*::***       .:*:***:.*:* .. . :*.***:* .*:  ::.*..:*:.*.. *   :  *     ****** :** **:*

F113    EITLCTAKTEQAWYYKGNGRLLKTDGDPLQVPYHSQSISSEPCIVCLYWVDPFGALRFIGFTLGNDIHDHVLHSRDAISSTHAHLRTCAI 177
Q2-1    EITLCTAKTEQAWYYKGNGRLLKTDGDPLQIPYHAQSIS-EPCIVCLYWVDHFGALRFIGFTLGTDIQDHVLHSRDAVSSTHARLRTCAI 176
Pf-5    EVTLNTPHTRQGWFYKGNGSLLKTDGEPLMIPYHAQSISSQPGVVCVYLVDPFGQLRFVGFSLGHDIHDPLLSHQDASSCAQSRLRQCAI 180
        *:** *.:*.*.*:***** ******:** :***:**** :* :**:* ** ** ***:**:** **:* :*  :** *.::::** ***

F113    APALIVGELQADLAINVQIERDGQPLTASHHRMSLHRWQSLRRYSEAFLGKHEQFLEPGLVHYVFHSLSHRTANVPLQHGDWLSIDCPEL 267
Q2-1    APALILGELQADLAINVQIERDGLPLAASHHRMSVHRWQSLRRYSQGFLEKHEQFLEPGLVHYVFHSLSHRTTPVPLQHGDWLSIDCPEL 266
Pf-5    APALILGELQNSLSLNVRVERQGTPLPQRSYRLDLQNWWAIRKYSQAFLEQHEQFLQPGMVHYVFHSASRRESDLQLQHGDWLDLDCPEL 270
        *****:**** .*::**::**:* **.   :*:.::.* ::*:**:.** :*****:**:******* *:* : : *******.:*****

F113    ELAMSNQIVEEELVSLVPLPERRAPDHDCIDRPLPVPKKF* 308
Q2-1    ELTMSNQIVEEELASLLPLPKPHASHNPCSEHPLSAPKKF* 307
Pf-5    ELGLSNQVIEEELLHLYPLETQPARASTHGNQPVRTRHHY* 311
        ** :***::****  * **    *      ::*: . :::
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FIG. 2. Alignment of the protein sequences of the putative gene identified in the Pf-5, F113, and Q2-1 lineages. This gene is located in the region following the *phlE* gene from the *phl* cluster in all lineages and may constitute an additional *phl* cluster gene.

and Q1-87, returned inconclusive results (data not shown). To further analyze strains Q2-1 and Q1-87 and to identify the regions flanking the *phl* cluster, we performed ST-PCR. This established that the 5′ region flanking the cluster in Q2-1/Q1-87 resembles the F113 context but the 3′ region is distinct, with the second putative gene downstream of the cluster showing most similarity to an oligopeptide transporter from *P. syringae*. The DNA region immediately 3′ of the cluster (downstream of the *phlE* gene) displayed interesting characteristics that led to further analysis. Examination of the sequence indicated that a putative gene of 921 nucleotides was present. This region was highly homologous to a putative gene that we identified in our annotation of the F113 BAC clone using Glimmer, and when identified, it had an E value of 1e−15 (see Table S3 in the supplemental material). In addition, however, the DNA showed homology to sequence within the 947-bp intergenic region found between the *phlE* gene (PFL_5958) and the glycine dehydrogenase gene (PFL_5959) in Pf-5. This putative coding sequence was not annotated as a gene in Pf-5; therefore, we carried out sequence alignment of the translated amino acid sequences of this putative gene from the three strains (Fig. 2). The alignment clearly showed that there is a highly conserved sequence in this location with levels of identity and similarity comparable to those for the genes in the *phl* cluster. The presence of this putative gene in all three strains, coupled with the lack of a homologue in other sequenced strains of *Pseudomonas* (see Table S3 in the supplemental material) or in other sequenced bacteria, indicates that this putative gene may constitute an additional gene in the *phl* cluster, which is involved in some way in 2,4-DAPG production. In conclusion, based on the location of the *phl* locus, the strains divided into three groups: those similar to Pf-5, those similar to F113, and two strains, Q2-1 and Q1-87, that resemble F113 at the 5′ end of the cluster but differ at the 3′ end (Fig. 3).

Sequence analysis of HK genes and the *phlD* gene. The low frequency of phloroglucinol-producing strains within the species *P. fluorescens* and the presence of the *phl* cluster in different genomic loci is reminiscent of horizontally transferred genetic elements. We addressed this possibility by comparing the

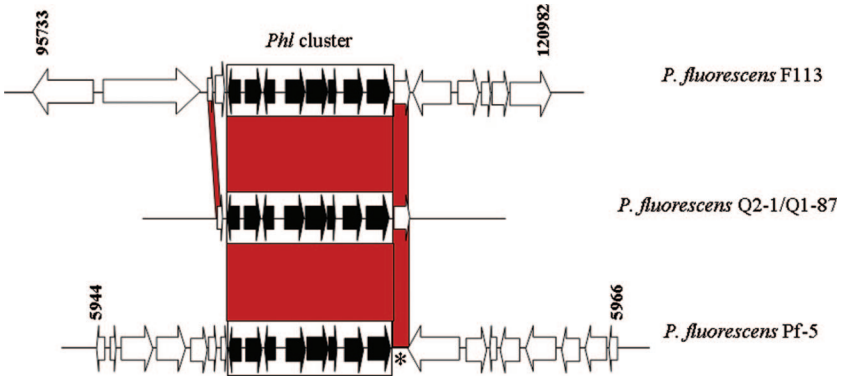

FIG. 3. The genomic locations of the *phl* gene cluster differ between *P. fluorescens* strains. Shown here are representatives of the main genomic location groups, F113, Q2-1/Q1-87, and Pf-5. The white arrows indicate genes flanking the *phl* cluster. The black arrows surrounded by a box represent the eight genes of the *phl* cluster. Arrows also indicate the orientation of the ORF. Homology between the three strains is indicated by red shading. It is seen that the *phl* cluster and the sequence immediately 3′ are homologous. Overall the 3′ flanking region differs among the three representative strains. In the 5′ region, strains F113 and Q2-1/Q1-87 are similar to each other but are distinct from strain Pf-5.

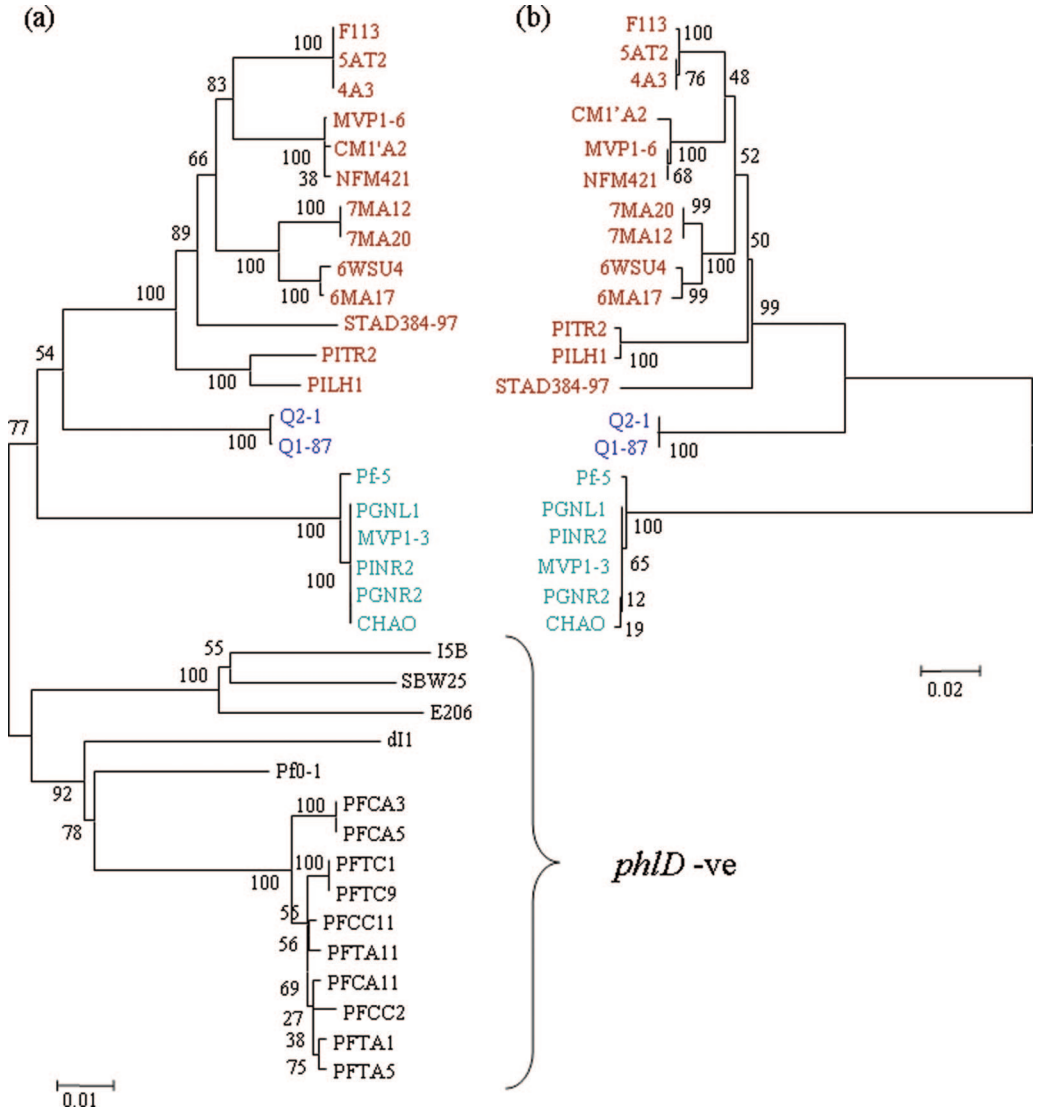

FIG. 4. Evolutionary relationships using all sites in the concatenated HK tree consisting of 21 *phl*-positive strains (shown in color) and 15 *phl*-negative strains (in black) (a) and in the *phlD* gene tree among our collection of 21 *phl*-positive strains (shown in color) (b). The three colors for the *phl*-positive strains on both phylogenetic trees represent the three different genomic context groups identified. Phylogenetic trees were constructed using the neighbor-joining method based on the Jukes-Cantor distance method (29, 55). Bootstraps of 1,000 computer-generated replicates were used and are shown at the branch nodes on the two phylogenetic trees.

phl cluster phylogeny with the species phylogeny of our strains. Four HK loci, *gyrB*, *leuB*, *groEL*, and *rpoD*, were partially sequenced from 21 *phl*-positive and 15 *phl*-negative isolates and used to reconstruct the phylogenetic history of the species. The number of polymorphic sites in each gene among the isolates was determined. A total of 623 polymorphic sites were present, with *leuB* (124 sites) being the least polymorphic gene and *rpoD* (195 sites) the most variable gene. The 623 polymorphic nucleotide sites resulted in 112 amino acid replacements, which ranged from 17 replacements for *gyrB* and *leuB* to 42 replacements for *rpoD* (see Table S4 in the supplemental material). To determine the *phl* cluster phylogeny, we obtained sequences for the *phlD* genes from all *phl*-positive strains. At the nucleotide level, *phlD* had 187 polymorphic sites, resulting in 36 amino acid replacements (see Table S4 in the supplemental material). The K_N/K_S ratio was <1 for all five loci, indicating that each of these genes is subject to purifying selection. We also analyzed the sequences for recombination using four statistical tests and found no evidence for recombination in the *phlD*, *groEL*, or *leuB* gene but inconclusive data for *gyrB* and *rpoD* (data not shown).

The *phl* cluster is ancestral in *P. fluorescens*. To determine relationships among the 36 *P. fluorescens* isolates, we first constructed a phylogenetic tree using the 2.3-kb concatenated sequence of our four HK genes (*gyrB*, *leuB*, *groEL*, and *rpoD*) (Fig. 4a). From the concatenated HK tree, two major lineages were observed, which correspond precisely to the 21 *phl*-positive and the 15 *phl*-negative isolates (Fig. 4a). It was unexpected that the *phl*-positive and -negative isolates should have such distinct divergent lineages and indicates an evolutionary

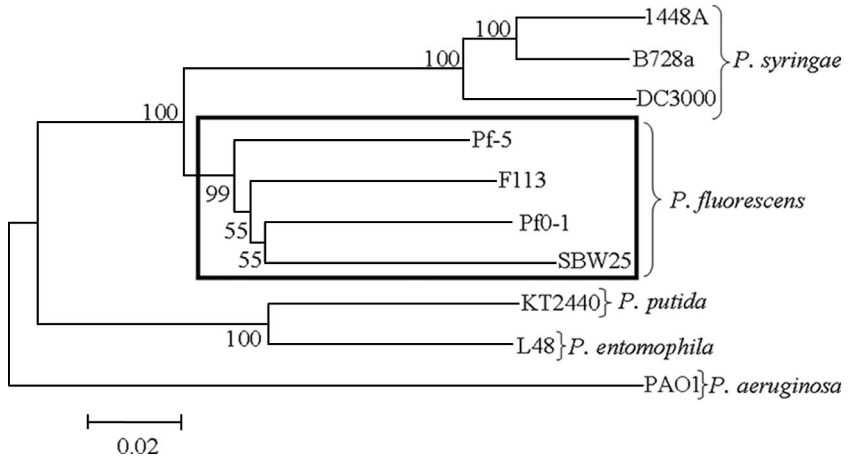

FIG. 5. Genus tree demonstrating the phylogenetic relationships among *Pseudomonas* species. Shown here are representatives of *Pseudomonas* species, i.e., *P. syringae*, *P. fluorescens*, *P. putida*, *P. entomophila*, and *P. aeruginosa*. Highlighted by a box are the *P. fluorescens* species with two *phl*-positive strains (F113 and Pf-5) and two *phl*-negative strains (Pf0-1 and SBW25). This concatenated HK tree was constructed using the neighbor-joining method based on the Jukes-Cantor distance method (29, 55). Bootstraps of 1,000 computer generated replicates were used and are shown at the branch nodes on the tree.

split soon after the species emerged. We examined nucleotide polymorphism and diversity within each lineage and found that the *phl*-negative lineage is more diverse (0.051) than the *phl*-positive lineage (0.014) (see Table S5 in the supplemental material). This suggests that the *phl*-positive lineage may be under selective constraints. Next, we constructed a phylogenetic tree using the *phlD* gene sequences of our 21 *phl*-positive isolates (Fig. 4b). Both trees have an almost identical branching pattern, showing that the trees are highly congruent. These data demonstrate that the *phlD* gene has an evolutionary history identical to that of the species and is ancestral in *phl*-positive *P. fluorescens*. Close examination of the *phl*-positive strains on both trees shows three identical subgroupings (clades). The first comprises 12 strains related to F113; the second comprises two strains, Q2-1 and Q1-87; and the third comprises five isolates related to Pf-5. Crucially, these three distinct clades correlate to the three genomic locations groups of the *phl* cluster (F113, Pf-5, and Q2-1). Integrating the molecular phylogeny and distribution data, it is now established that the entire *phl* cluster is ancestral in *phl*-positive strains. The final question was whether the last common ancestor to give rise to *P. fluorescens* strains contained the *phl* cluster. To answer this, we reconstructed the phylogeny of the genus using the same four HK fragments as for the species tree (Fig. 5). This tree included two *phl*-positive strains, F113 and Pf-5; two *phl*-negative strains, Pf0-1 and SBW25; and representatives of *P. syringae*, *P. putida*, *P. entomophila*, and *P. aeruginosa*. Within the *P. fluorescens* lineage, Pf-5 and F113 formed the most divergent branches, suggesting that the *phl* cluster was present in the last common ancestor to give rise to *P. fluorescens*.

DISCUSSION

Although it has been known for some time that 2,4-DAPG production is an important trait in plant-associated strains of *P. fluorescens*, this is the first study to look at the evolution of the *phl* cluster. Most previous work focused on *phlD*, which encodes the key biosynthetic enzyme, a polyketide synthase. Those data were somewhat conflicting, with sequence homologies suggesting that PhlD may have a relationship to plant chalcone synthases but molecular phylogeny indicating that *phlD* was an ancestral gene. By comparing *phlD* phylogeny to a robust strain phylogeny, we confirmed the ancestral nature of *phlD* in *P. fluorescens*. Furthermore, we showed that *phlD* is part of a gene cluster that is also ancestral in the species and is not a recent acquisition. One of the most striking findings was that there was a clear bifurcation in the species phylogeny, with only one major branch retaining the *phl* cluster. Within the *phl*-positive lineage, our data show that the cluster has relocated within the genome at least three times. This is perfectly plausible since it is known that intragenomic recombination and rearrangements are common in *Pseudomonas*, and it will not be surprising if further lineages with additional *phl* locations are found. Indeed, another recent study of *phlD*-positive strains proposed that six subgroups of strains could be identified, though these data were based exclusively on gene sequences and did not consider genomic data (18). The identification of the putative gene at the 3′ end of the cluster in all strains is also intriguing. Even though the genomic context of the cluster varies among the strains, the *phl* cluster and this putative gene are retained in the same order each time, and like the *phl* genes, this sequence is also not present in the other *Pseudomonas* species. This supports an idea that this putative gene may be a bona fide member of the *phl* cluster, but this requires experimental testing and alternative explanations are also plausible. Analysis of the particular locations for the *phl* cluster indicates that they do not show any of the typical signatures for targeted insertion (e.g., tRNA genes or insertion sequence elements) and are likely to arise from random recombination (56). The number of different locations for *phl* and the finding that in the F113 lineage it is situated in a large lineage-specific genomic region that also carries genes that are ancestral in the genus support the premise that the cluster dates at least to the origin of the species. These data also make it clear that F113 and Pf-5 are representatives of major lin-

eages of *P. fluorescens* and illustrate the value of examining genomic organization as well as sequences of individual genes when reconstructing species phylogenies. Evolutionary relationships and species identification are sometimes problematic for the fluorescent pseudomonads (as seen by the location of *P. brassicacearum* NFM421 in a *P. fluorescens* lineage), and this genomic approach may help resolve some of these conflicts.

Detailed analysis of the HK and the *phlD* gene sequences and the cluster structure identify some interesting points. First, the *phlD* locus is subject to purifying selection, indicating that it is functionally important. Second, whereas the strains that we selected for this study were chosen simply on the basis that they were *phlD* positive (typically by PCR), all 21 strains carried the entire cluster in one of the three locations. This suggests a selective pressure to maintain all the genes in the cluster together even in times when the genome as a whole underwent extensive rearrangement. Third, there is less diversity in the *phl*-positive lineage, indicating that these strains are evolving under some constraint. As outlined earlier, there are separate data that point to plant selection of 2,4-DAPG-producing strains of *P. fluorescens* in the rhizosphere (39, 46). Since *Pseudomonas* is predominantly a plant-associated bacterium, it is tempting to speculate that 2,4-DAPG plays a role in an intimate interaction between *P. fluorescens* and (some) plants that confers benefits to the plant (e.g., protection against phytopathogens). Although this would explain the selective pressure to retain the *phl* locus, this idea remains a speculative hypothesis at this stage. It must also be noted that some *phl*-negative strains also intimately associate with plant hosts.

Despite the widespread belief that intimate signaling and regulated gene expression underpin interactions between plants and associated bacteria, this has proved very difficult to establish. Specifically for *Pseudomonas*, some success has been obtained using in vitro expression technology (21, 27) and transcriptome analysis (37) to identify niche (plant)-specific or plant-induced regulatory genes. The data from the study reported here identify another strategy that may prove useful in dissecting intimate plant microbe interactions. By focusing on the phylogenetic and evolutionary aspects, we have identified specific *P. fluorescens* lineages that appear to have been selected to maintain 2,4-DAPG production and possibly plant association. The advent of inexpensive bacterial genome sequencing opens the possibility of sequencing entire genomes of strains from different lineages and undertaking global comparative genomics to identify the loci that are lineage specific and therefore likely to be of functional importance. The *phl* cluster itself is one such locus, and the ongoing challenge is to understand the full biological role of 2,4-DAPG in interactions between *P. fluorescens* and plant hosts.

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