Characterization of a Genomic Locus Required for Synthesis of the Antibiotic 2,4-diacetylphloroglucinol by the Biological Control Agent *Pseudomonas fluorescens* Q2-87

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The antibiotic 2,4-diacetylphloroglucinol (Phl) is an important factor in the biological control by fluorescent *Pseudomonas* spp. of many soilborne diseases including take-all disease of wheat. A 6.5-kb genomic DNA fragment from *Pseudomonas fluorescens* Q2-87 conferred production of Phl and of a red pigment distinct from Phl, but which typically is present when Phl is produced, upon all of 13 Phl-nonproducing recipient *Pseudomonas* strains into which it was introduced. Larger fragments that included flanking DNA sequences did not transfer this capability, suggesting that they contain negative regulatory element(s). Analysis of the 6.5-kb fragment by Tn3HoHo1 mutagenesis further localized the sequences required for Phl production to a segment of approximately 5 kb and revealed the presence of at least two divergently oriented transcriptional units. Insertions within the smaller unit or within about 3 kb of the 5' end of the larger unit caused loss of production of both Phl and the red pigment. Other insertions within the distal 1.5 kb of the larger transcriptional unit abolished production of only the red pigment. Pleiotropic changes in secondary metabolism or colony morphology were not observed in *Pseudomonas* strains containing the 6.5-kb fragment, although some Phl-producing derivatives grew more slowly and gave rise to smaller colonies than did the wild-type parental strains. The size of the genomic region involved in Phl production, and the consistency and specificity with which these sequences transferred Phl biosynthetic capability, support the conclusion that the 6.5-kb fragment contains the Phl biosynthetic locus.

Additional keywords: biocontrol, *Gaeumannomyces graminis* var. *tritici*.

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The antibiotic 2,4-diacetylphloroglucinol (Phl) is a phenolic compound of probable polyketide origin with antifungal, antibacterial, antiviral, antihelminthic, and phytotoxic properties (Bowden et al. 1965; Broadbent et al. 1976; Harrison et al. 1993; Kataryan and Torgashova 1976; Keel et al. 1992; Reddi et al. 1969; Reddi and Borovkov 1970; Shanahan et al. 1992; Tada et al. 1990; Vincent et al. 1991). Phl is produced by fluorescent *Pseudomonas* spp. of diverse geographic origin that have in common the ability to suppress one or more root and seedling diseases of crop plants caused by soilborne pathogens. These include root rot of wheat, caused by *Fusarium oxysporum* Schlechtend.:Fr. (Garagulya et al. 1974; Pidoplichko and Garagulya 1974); black root rot of tobacco, caused by *Thielaviopsis basicola* (Berk. & Broome) Ferraris (Défago et al. 1990; Keel et al. 1990, 1992); damping-off of sugar beet, caused by *Pythium ultimum* Trow (Fenton et al. 1992; Shanahan et al. 1992); damping-off of cotton, caused by *P. ultimum* and *Rhizoctonia solani* Kühn (Kraus and Loper 1992; Nowak-Thompson et al. 1994); blotch of wheat, caused by *Septoria tritici* Roberge in Desmaz. (Levy et al. 1992); and take-all of wheat, caused by *Gaeumannomyces graminis* (Sacc.) Arx and D. Olivier var. *tritici* J. Walker (Défago et al. 1990; Keel et al. 1992; Vincent et al. 1991). The strains that produce Phl therefore have considerable agricultural potential, and an understanding of the genetics and regulation of Phl biosynthesis will facilitate their exploitation as effective and reliable biocontrol agents.

Among the genes affecting Phl biosynthesis that have been cloned from *Pseudomonas* spp. are *gacA* and *lemA* (*apdA*), which are global regulators required for the production of secondary metabolites including Phl (Corbell et al. 1995; Gaffney et al. 1994; Laville et al. 1992; Willis et al. 1994). A similar positive regulatory locus has been reported in *Pseudomonas* sp. strain F113, which suppresses damping-off of sugar beet caused by *P. ultimum* (Fenton et al. 1992, 1993). Another locus from strain F113 was contained in a 6-kb *Bam*HI fragment that transferred the ability to produce Phl to one of eight other *Pseudomonas* strains into which it was introduced. The fragment subsequently was shown to carry monoacetylphloroglucinol (MAPG) transacetylase activity, which may be required for the conversion of MAPG to the

diacetyl compound (Shanahan et al. 1993). Two loci that affect Phl production have been cloned from *P. fluorescens* CHA0, which produces MAPG, pyoluteorin, salicylic acid, and hydrogen cyanide (HCN) in addition to Phl, and is effective against a wide range of fungal plant pathogens (Défago et al. 1990; Keel et al. 1992; Maurhofer et al. 1992). The first is contained on an 11-kb fragment that complemented the Phl⁻ Tn*5* mutant CHA625 to Phl⁺ (Keel et al. 1992); and the second contains the housekeeping sigma factor *rpoD* and caused increased production of both Phl and pyoluteorin when introduced into wild-type CHA0 (Maurhofer et al. 1992, 1995; Schnider et al. 1995).

Pseudomonas fluorescens (formerly *aureofaciens*) strain Q2-87, when applied to seeds or soil, protects wheat against take-all (Pierson and Weller 1994). Strain Q2-87 produces Phl, MAPG, HCN, and a diffusible red pigment distinct from Phl, but which is correlated with Phl production (Keel et al., in press). Phl is the primary determinant of biological control in strain Q2-87 (Harrison et al. 1993; Vincent et al. 1991); thus, Q2-87 provides an ideal system for the study of Phl biosynthesis, uncomplicated by the presence of additional, biochemically unrelated antibiotics. Vincent et al. (1991) previously cloned a 35-kb region from Q2-87 on the plasmid pMON5118 that was able to restore the Phl⁻ Tn*5* mutant Q2-87::Tn*5*-1 to Phl production and transferred Phl biosynthetic activity to two Phl-nonproducing strains of *Pseudomonas*, 2-79 and 5097. The purpose of the present study was to further characterize the locus described by Vincent et al. (1991). Subclones from the 35-kb region were introduced into the Phl-nonproducing *P. fluorescens* strain M4-80R and 12 other recipient strains to identify a region that was able to consistently transfer Phl biosynthetic capability. Mutagenesis with the transposon Tn*3*HoHo1 then was used to reveal the size of the region required for Phl production, the number and orien-

tation of transcriptional units present, and the existence of a linked locus required for production of the red pigment typically seen in cultures that produce Phl.

RESULTS

Identification of a fragment sufficient to transfer Phl synthesis.

Plasmids pMON5118 and pMON5117, the deletion derivatives pMON5120, pMON5122, and pMON5123, and the Tn*5*-containing 20-kb genomic *Eco*RI fragment from Q2-87::Tn*5*-1 in pMON5116 were introduced into *P. fluorescens* M4-80R to determine which clone(s) were able to transfer Phl production to nonproducing strains. Rifʳ, Tcʳ transconjugants obtained from triparental matings were evaluated for production of Phl and MAPG by thin-layer chromatography (TLC) of cell extracts and bioassay with *Clavibacter michiganensis* subsp. *michiganensis*. Neither Phl nor MAPG was detected by TLC of cell extracts or by bioassay with *C. michiganensis* subsp. *michiganensis* in strain M4-80R containing pMON5116, pMON5117, pMON5118, pMON5120, or pMON5123, nor were such cultures red in color. On the other hand, Phl, MAPG, and the red pigment were present in broth cultures of M4-80R(pMON5122), and this strain inhibited *C. michiganensis* subsp. *michiganensis* (Fig. 1).

Sequences capable of activating or transferring antibiotic production in fluorescent *Pseudomonas* spp. may function in a limited number of recipient strains (Fenton et al. 1992). To determine whether Phl production mediated by the fragment in pMON5122 was broadly transferable, the fragment was cloned into pVSP41, which can be maintained without selection in many fluorescent *Pseudomonas* spp. The resulting plasmid, pPHL5122, was introduced into 12 Phl-nonproducing strains and into wild-type Q2-87. Cultures of the transconjugant derivatives shown in Table 1 were red in color and produced Phl as detected by TLC of cell extracts and the bioassay with *C. michiganensis* subsp. *michiganensis*. Concentrations ranged from barely detectable (M7z-80) to up to 2,000 μg/ml (Q26a-80), depending on the recipient strain. Strain Q2-87(pPHL5122) produced from 30 to 200 μg/ml of Phl, in contrast to Q2-87 or Q2-87(pVSP41), which produced less than 1 μg/ml in the broth cultures used for these assays. Cultures of the remaining seven strains (*P. fluorescens* Q48a-80, Q58aw-80, M12z-80, M14dz-80, M32z-80, 2-79, and 13-79) containing pPHL5122 exhibited the red phenotype consistent with Phl production, whereas the respective wild-type strains or strains containing pVSP41 did not. No Phl was detected in cultures of *E. coli* DH5α(pPHL5122), nor were such cultures red in color.

Phenotypic analysis of mutant and transconjugant strains.

Mutation or transfer of certain regulatory genes can give rise to pleiotropic phenotypic changes or activate expression of previously silent biosynthetic pathways such as that for production of HCN in alternate host strains (Gaffney et al. 1994). In contrast, the effects of mutation or transfer of Phl biosynthesis genes should be specific to a limited number of biosynthetically related products including MAPG, Phl, and the red pigment associated with the presence of Phl. To determine the complexity of the phenotype associated with the locus contained in pMON5122, wild-type, mutant, and trans-

Plasmids	Q2-87::Tn*5*-1		M4-80R	
	Phl	Red	Phl	Red
pMON 5118	+	4	-	0
pMON 5117	+	3	-	0
pMON 5120	+	3	-	0
pMON 5122	+	4	+	4
pMON 5123	+	N.D.	-	0

Fig. 1. Effect of pMON5118 and its deletion derivatives on production of 2,4-diacetylphloroglucinol (Phl) and red pigment by *Pseudomonas fluorescens* strains Q2-87::Tn*5*-1 and M4-80R. Plasmids were introduced into the Phl⁻, nonpigmented recipients by triparental mating. Transconjugants were evaluated for Phl production by thin-layer chromatography and bioassay with *Clavibacter michiganensis* subsp. *michiganensis*. Amount of red pigment accompanying production of Phl was rated on a 0 to 6 scale in which 0 is no visible pigment and 6 is a dark red color. Double slashes in pMON5118 indicate the fragment has not been drawn to scale. Vertical arrow indicates site of Q2-87::Tn*5*-1. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I; S, *Sau*3A. ND, not determined.

conjugant derivative strains were evaluated for colony morphology, pigmentation, and production of HCN. HCN was selected for these studies because its production is controlled by global regulators of secondary metabolism (Gaffney et al. 1994; Laville et al. 1992) and it is the only such metabolite other than Phl known to be produced by strain Q2-87. Colony morphology was examined because it can be influenced by mutation or introduction of global regulatory genes.

Colonies of Q2-87 and the Phl⁻ mutant Q2-87::Tn5-1 were circular, opaque, convex, and virtually indistinguishable from each other on KMB, YM, and LB agar except that Q2-87 produced the diffusible red pigment after 4 to 7 days. Similarly, no gross differences in colony morphology were observed between the 12 wild-type, Phl-nonproducing *Pseudomonas* spp. and their respective pPHL5122-containing derivatives. However, colonies of the plasmid-containing derivatives that produced substantial amounts of Phl (e.g., Q26a-80(pPHL5122) and Q2-87(pPHL5122)) typically were smaller and grew more slowly than those of the respective wild-type strains. Interestingly, the intensity of the red pigmentation of Q2-87::Tn5-1 complemented to Phl⁺ by pMON5118 or its deletion derivatives varied depending upon the identity of the complementing plasmid. The red color was most intense with pMON5118 and pMON5122, and was progressively lighter with pMON5117 and pMON5120.

HCN was assayed in cultures of Q2-87, Q2-87::Tn5-1, and seven recipient *Pseudomonas* strains and their derivatives

Table 1. Phenotypes of recipient strains of *Pseudomonas* spp. and transconjugant derivatives containing the vector pVSP41 or the plasmid pPHL5122

Strains	Phl synthesis (μg/ml culture)[a]	Red pigment[b]	HCN[c]	Plasmid stability[d]
M4-80R	0	0	0	...
M4-80R(pPHL5122)	1.0 to 2.5	3	0	0.93
M7z-80	0	0	0	...
M7z-80(pVSP41)	0	0	0	0.76
M7z-80(pPHL5122)	0.25	2	0	1.10
Q26a-80	0	0	0	...
Q26a-80(pVSP41)	0	0	0	0.71
Q26a-80(pPHL5122)	600 to 2,000	4	0	0.93
Q29z-80	0	0	0	...
Q29z-80(pVSP41)	0	0	0	0.88
Q29z-80(pPHL5122)	20 to 35	4	0	0.92
M23dz-80	0	0	1	...
M23dz-80(pVSP41)	0	0	1	0.93
M23dz-80(pPHL5122)	10 to 20	5	1	1.10
Q69c-80	0	0	4	...
Q69c-80(pVSP41)	0	0	3	1.10
Q69c-80(pPHL5122)	1.0 to 3.8	4	3	0.30
Q2-87	0.5	5	5	...
Q2-87(pVSP41)	0	5	5	0.61
Q2-87(pPHL5122)	30 to 200	5	2	ND

[a] Phl was extracted from 4-ml cultures grown in yeast-malt broth. The concentration was estimated by visual comparison of Phl spots produced by strains with spots from a range of concentrations of authentic Phl standard on thin-layer chromatography plates.

[b] Red pigment production on King's medium B (KMB) agar was rated on a 0 to 6 scale in which 0 is no visible pigment and 6 is a dark red color.

[c] HCN production on KMB agar supplemented with glycine was rated on a 0 to 7 scale in which 0 indicates no change in color of indicator paper color and 7 indicates change in the paper to a dark red color.

[d] Plasmid stability was evaluated as the ratio of the number of Km^r to total colonies after 7 consecutive daily transfers in Luria-Bertani broth without kanamycin selection. ND = not determined.

containing pMON5122 or pPHL5122. Three wild-type recipient strains produced HCN (M23dz-80, Q69c-80, and Q2-87) whereas four others did not (M4-80R, M7z-80, Q26a-80, and Q29z-80) (Table 1). Both Q2-87 and Q2-87::Tn5-1 produced similar amounts of HCN (data not shown). HCN production was not activated upon introduction of pPHL5122 into the HCN-nonproducing strains, nor was the amount of HCN produced by M23dz-80 changed by pPHL5122. However, HCN production was slightly reduced in the plasmid-bearing derivatives of Q69c-80 and was markedly lessened in Q2-87(pPHL5122) (Table 1).

In transconjugant strains, loss of Phl production could occur by elimination of pPHL5122 during growth in the absence of kanamycin. To determine the tolerance of recipient strains to pPHL5122, plasmid stability was evaluated after seven consecutive daily transfers of cultures in LB broth. The plasmid was maintained without selection in all strains except for Q69c-80(pPHL5122), in which only about 30% of colonies remained kanamycin-resistant (Table 1). Colony morphology and pigmentation were unchanged in kanamycin-resistant colonies following serial transfer.

Size, number, and orientation of transcriptional units.

To localize the sequences necessary for Phl production, a restriction map of pMON5122 was generated and the plasmid was mutagenized with the transposon Tn3HoHo1. The XbaI-EcoRI fragment in pMON5122 was determined to be 6.5 kb in size, in contrast to a previous estimate (Vincent et al. 1991) of 9.5 kb. Of 846 mutants analyzed, 19% contained insertions within the 6.5-kb fragment. Insertions mapping to similar or identical positions within the left half of the fragment were obtained with high frequency, whereas fewer insertions were obtained within the right end (Fig. 2). Of 42 unique insertions
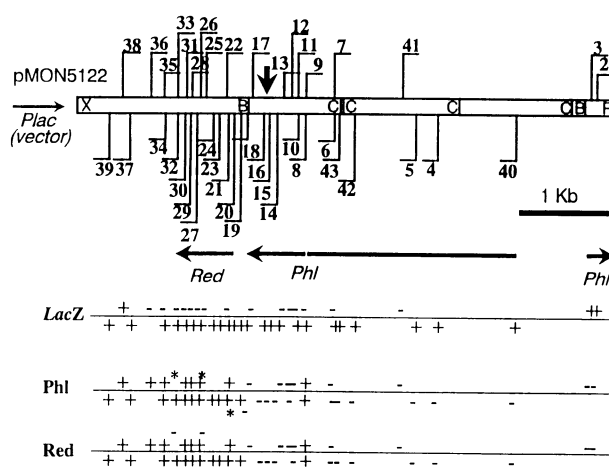

Fig. 2. Location of Tn3HoHo1 insertions in the 6.5-kb fragment contained in pMON5122. Unique insertions (1 to 42) were mapped as described in text. Crossbars on each insertion indicate transcriptional orientation of the *lacZ* gene in Tn3HoHo1. Horizontal arrows delineate location and transcriptional orientation of regions required for production of Phl and the red pigment. Also indicated for each insertion are β-galactosidase activity as determined by production of blue or white colonies on X-Gal plates (+ or −); Phl production as determined by bioassay with *Clavibacter michiganensis* subsp. *michiganensis*; and red pigment production on King's medium B. * mark positions of insertions 20, 25, and 33, which caused reduced Phl production and loss of the red pigment. Vertical arrow indicates site of Q2-87::Tn5-1. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; X, *Xba*I.

in the cloned fragment, all but three of those giving rise to Lac$^+$ colonies in strain M4-80R were similarly oriented from right to left. Only insertions 2 and 3 at the right end of the fragment and insertion 38 at the left end were Lac$^+$ and oriented from left to right.

The ability of the mutated plasmids to direct synthesis of Phl in strain M4-80R was determined by TLC of cell extracts and bioassay with *C. michiganensis* subsp. *michiganensis*. Insertions 2 through 7 and 10 through 18, spanning a 5-kb region at the right end of the cloned fragment, caused a loss of Phl production (Fig. 2). Furthermore, cultures of M4-80R containing these mutated plasmids were not red. Insertions 8 and 9 contained oppositely oriented transposons at approximately the same site in pMON5122. M4-80R cultures containing these plasmids produced Phl and exhibited the red phenotype. Insertion derivative 8 was also Lac$^+$, indicating no break in transcription. Most insertions to the left of number 18 did not affect Phl production or red pigmentation. However, insertions 20, 25, and 33 caused reduced Phl production to a level intermediate between the positive and negative controls, and loss of the red phenotype (Fig. 2).

The region in which Tn*3*HoHo1 insertions 8 and 9 are located resides within a 1.0-kb *Bam*HI-*Cla*I fragment that also contains the site of Tn*5* insertion in Q2-87::Tn*5*-1. To determine whether this region encodes a transcriptional unit sufficient to complement the Tn*5* mutation, it was cloned in both orientations in pRK415 to generate pPHL5124.35 and pPHL5124.36 and then introduced into Q2-87::Tn*5*-1. Q2-87::Tn*5*-1 containing either plasmid was unable to inhibit *C. michiganensis* subsp. *michiganensis* and did not produce MAPG, Phl, or the red pigment.

DISCUSSION

The results reported here provide several lines of evidence that a 6.5-kb fragment cloned from *P. fluorescens* Q2-87 contains the 2,4-diacetylphloroglucinol biosynthetic locus. The fragment enabled Phl production by six Phl-nonproducing *Pseudomonas* strains into which it was introduced, and conferred the production of a red pigment indicative of Phl production in seven other strains in which Phl itself was not assayed directly. These 13 recipient strains originated from three different soils and had no known common characteristics that would have predisposed them to express the introduced locus. Secondly, the Phl fragment differed phenotypically from known global regulatory loci that affect the expression of genes involved in antibiotic production. Transfer or mutation of regulatory loci such as *gacA* (global activator of cyanide; Laville et al. 1992) generally results in complex phenotypic changes that can include changes in colony morphology and activation or loss, respectively, of cyanide production (Gaffney et al. 1994; Laville et al., 1992). In contrast, the 6.5-kb fragment affected only the production of Phl and the red pigment associated with Phl production, but not colony morphology or the synthesis of HCN. Thus, Q2-87::Tn*5*-1 remained HCN$^+$ and, conversely, introduction of the fragment into various *Pseudomonas* strains did not qualitatively change their HCN phenotype (Table 1). Reduced HCN levels observed in Q2-87(pPHL5122) may have been due to the additional demand on carbon resources imposed by the plasmid, or to diminished biomass and metabolic activity

resulting from slower growth of the plasmid-containing derivative as opposed to the wild type. Finally the locus required to transfer Phl biosynthetic capability was substantially larger than global regulatory genes such as *gacA* (639 bp; Laville et al. 1992; Gaffney et al. 1994) and *lemA* (*apdA*) (2.7 kb; Corbell et al. 1995; Willis et al. 1994) and had no detectable sequence similarity to these regulatory genes (M. G. Bangera and L. S. Thomashow, manuscript in preparation).

The structure of Phl is consistent with synthesis via the polyketide pathway, with MAPG a probable biosynthetic intermediate (Shanahan et al. 1993). Polyketides are synthesized via successive condensation reactions of short-chain carboxylic acids in a process similar to fatty acid biosynthesis (Hopwood and Sherman 1990). Three types of polyketide synthases (PKSs) have been described. Type I PKSs, encoded by several contiguous 10-kb open reading frames, are complex proteins with domains that catalyze the individual steps of the pathway (Donadio et al. 1991). Type II PKSs consist of four to six mono- or bifunctional proteins that are used repeatedly for the synthesis of the polyketide molecule (Fernández-Moreno et al. 1992). The third type of PKS is exemplified by plant chalcone synthases, which consist of a single homodimeric protein (monomers of 41 kDa) that performs the condensation and cyclization steps needed to produce the final phenolic product (Martin 1993; Schröder and Schröder 1990).

Polyketide antibiotics known to be produced by *Pseudomonas* spp. include coronatine and pyoluteorin. The coronatine biosynthetic locus from *P. syringae* occupies about 30 kb, only part of which is required for synthesis of the polyketide component of the toxin (Liyanage et al. 1995; Ullrich et al. 1994). The pyoluteorin biosynthetic locus from *P. fluorescens* Pf-5 is carried on a 21-kb DNA fragment of which at least 15 kb is necessary for pyoluteorin synthesis (Kraus and Loper 1995). If the genes present in pMON5122 are indeed sufficient for Phl production, as indicated by the efficiency with which they transfer biosynthetic capability, then the smaller size of the Phl locus suggests that the enzymes involved in Phl synthesis will differ from those involved in production of coronatine and pyoluteorin. Whether gene(s) encoding the MAPG acetyltransferase described by Shanahan et al. (1993) are present within the 6.5-kb fragment also remains to be determined. None of the Phl$^-$ transposon derivatives of pMON5122 produced MAPG, suggesting that a common precursor was not produced, that the two compounds might be products of the same multienzyme complex, or that the genes in this region are cotranscribed, with insertions in the upstream region exerting polar effects on downstream genes. Cotranscription of genes occurs in the type II PKS pathway of *Streptomyces coelicolor*, in which six open reading frames within a 5.3-kb region form the actinorhodin biosynthetic operon (Fernández-Moreno et al. 1992).

The Phl locus consisted of at least two divergently transcribed units. The promoters responsible for expression of the *lacZ* genes in Tn*3*HoHo1 insertion derivatives 2 and 3, and in the remaining Lac$^+$ derivatives (except for number 38, which probably is expressed from the *lac* promoter in the vector) presumably reside within the 0.75-kb region between insertions 3 and 40. However, additional promoters also may be present to the right of insertion 42 (Fig. 2). That very few insertions were recovered in this right-hand region, and only

about 19% of the 864 insertions analyzed mapped to the 6.5-kb fragment, as opposed to the 38% anticipated based on the relative sizes of the vector and the insert, indicates that these sequences were recalcitrant to mutagenesis. Whether this is due to the function of the encoded gene products or to the physical nature of the DNA (Tn3 transposes by formation of a cointegrate and is not completely random in its insertion) (Kretschmer and Cohen 1977) remains to be determined. Further structural analysis also will be needed to understand why the oppositely oriented insertions 8 and 9 had no significant effect on production of Phl or the red pigment, even though the lacZ gene in insertion 8 was expressed and both insertions are located within the portion of pMON5122 required for production of Phl.

Cultures of M4-80R containing Tn3HoHo1 insertion derivatives 20, 25, and 33 produced reduced levels of Phl as indicated by bioassay with *C. michiganensis* subsp. *michiganensis* but were not pigmented. These insertions identify a region designated Red (Fig. 2) that, while not strictly required for production of Phl, is genetically linked to other *phl* genes and may be involved in the processing, derivatization, or export of Phl to form the red pigment. Pigment formation could occur either before, during, or after transport of Phl from the cell, but it is unlikely that the Red region codes for an enzyme directly responsible for derivatization because the three insertions that caused loss of the red phenotype were interspersed with other insertions that had no effect on pigmentation. More likely is the possibility that the red pigment results from a reaction between Phl and component(s) of the medium. Phloroglucinol (unacetylated) can condense with the reactive carbonyl of the coniferaldehyde moiety in lignin compounds from plants to give a reddish color (Allan 1971). Similar reactive carbonyl groups may be present on compounds in media or produced in media by growth of the Phl-producing bacteria. A pivotal step in this case would be the export of Phl. If the Red region coded for a membrane-associated trans-

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Pseudomonas fluorescens		
Q2-87	Phl+ Rif r HCN+	Vincent et al. 1991
Q2-87::Tn5-1	Phl- Rif r HCN+Km r	Vincent et al. 1991
Q26a-80	Phl- HCN-	Pierson and Weller 1994
Q29z-80	Phl- HCN-	Pierson and Weller 1994
Q48a-80	Phl- HCN-	Pierson and Weller 1994
Q58aw-80	Phl- HCN-	Pierson and Weller 1994
M4-80R	Phl- Rif r HCN-	Hamdan et al. 1991
M7z-80	Phl- HCN-	D. M. Weller
M12z-80	Phl-	D. M. Weller
M14dz-80	Phl-	D. M. Weller
M23dz-80	Phl- HCN+	D. M. Weller
M32z-80	Phl-	D. M. Weller
13-79	Phl-	Weller and Cook 1983
2-79	Phl- HCN-	Weller and Cook 1983
Pseudomonas fluorescens-putida		
Q69c-80	Phl- HCN+	Pierson and Weller 1994
Escherichia coli		
DH5α	F- φ80dlac ZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 phoA hsdR17 (r$_K^-$,m$_K^+$) supE44 λ-thi-1 gyrA96 relA1	Bethesda Research Laboratory (BRL), Gaithersberg, MD
HB101	F- mcrB mrr hsdS20(r$_B^-$,m$_B^-$) recA1 supE44 ara14 galK2 lacY1 proA2 rpsL20 (Sm r) xyl5 λ- leu mtl1	BRL
C2110	Nal r, polA-	Stachel et al. 1985
Clavibacter michiganensis subsp. *michiganensis*		
13-3		D. Gross
Plasmids		
pIC19H	pUC8-derived αlac,Ap r,	Marsh et al. 1984
pRK415	RK2-derived broad host range vector, Tc r	Keen et al. 1988
pVSP41	pVS1 replicon and mobilization, p15A replicon,Km r	W. Tucker
pHoHo/pSSHE	Tn3 donor/tnpA	Stachel et al. 1985
pRK2013	IncP Km r TraRK2+repRK2 repEl+ (mobilization helper)	Ditta et al. 1980
pMON5116	pBR325 vector with 20-kb EcoRI mutant genomic fragment from Q2-87::Tn5-1	Vincent et al. 1991
pMON5117	pCP13/B with 25-kb genomic fragment from Q2-87, Tc r	Vincent et al. 1991
pMON5118	pCP13/B with 35-kb genomic fragment from Q2-87, Tc r	Vincent et al. 1991
pMON5120	pRK415 with 15-kb genomic fragment from Q2-87, Tc r	Vincent et al. 1991
pMON5122	pRK415 with 6.5-kb genomic fragment from Q2-87, Tc r	Vincent et al. 1991
pMON5123	pRK415 with 4.8-kb genomic fragment from Q2-87, Tc r	Vincent et al. 1991
pPHL5122	pVSP41 with 6.5-kb fragment from pMON5122, Km r	This study
pPHL5124.35	pRK415 with 1-kb fragment from pMON5122, Tc r	This study
pPHL5124.36	pRK415 with 1-kb fragment from pMON5122 in reverse orientation, Tc r	This study

[a] Ap r, Km r, Rif r, and Tc r indicate resistance to ampicillin, chloramphenicol, rifampicin, and tetracycline, respectively. Phl+, produces 2,4-diacetylphloroglucinol and monacetylphloroglucinol; Phl-, does not produce either compound.

port protein, insertions 20, 25, and 33 might have interfered specifically with domains involved in Phl export, accounting for diminished activity in the bioassay and loss of the red phenotype. Membrane leakiness or increased permeability resulting from other insertions in the region could have favored the red phenotype and remained undetected in our assays.

Our observation that plasmids containing additional DNA contiguous with the sequences in pMON5122 did not enable Phl production suggests that the flanking DNA may contain negative regulator(s) of Phl production. To our knowledge this would be the first report of negative regulatory element(s) linked to an antibiotic biosynthetic locus in *Pseudomonas*. The absence of a functional repressor in pMON5122 might also account for the comparatively large amounts of Phl produced in some recipient strains. Interestingly, whereas Vincent et al. (1991) reported transfer by pMON5118 and pMON5117 of Phl synthesis to *P. fluorescens* 2-79 and 5097 (which produce phenazine compounds), we were unable to detect Phl production directed by these plasmids in M4-80R. M4-80R may be deficient in a global regulatory element such *gacA* or *lemA* (*apdA*) that is necessary for the induction of the Phl locus when the linked negative regulator is present. In any case, it is clear, from the differences in the levels of Phl production directed by pPHL5122 in different genetic backgrounds (Table 1) and from the difference in the degree of pigmentation of Q2-87::Tn5-1 complemented by plasmids containing the Phl locus plus additional flanking sequences, that regulation of this locus will be complex.

MATERIALS AND METHODS

Organisms and culture conditions.

Plasmids and bacterial strains used in this study are described in Table 2. Cultures of fluorescent *Pseudomonas* strains were grown at 28°C in King's medium B (KMB) (King et al. 1954) for detecting fluorescence, yeast malt (YM) medium (3 g of Difco yeast extract, 3 g of malt extract, 5 g of peptone, 10 g of glucose, and 1 liter of deionized distilled water) for Phl extraction, or Luria-Bertani (LB) medium (Sambrook et al. 1989) for other manipulations. Ampicillin, kanamycin, rifampicin, and tetracycline were used at 100 μg, 50 μg, 100 μg, and 25 μg per ml, respectively. The production of the red pigment that accompanies Phl production (Keel et al., in press) was assessed visually after 4 to 7 days of growth in YM broth or on KMB agar plates. *Escherichia coli* DH5α was routinely used as a host for the various plasmid constructions and was grown in LB medium at 37°C.

Nucleic acid methods.

Plasmid isolation was performed by the method of Birnboim and Doly (1979). Restriction and ligation procedures, agarose gel electrophoresis, and DNA isolation from agarose were performed by standard methods (Sambrook et al. 1989) or as directed by the suppliers. Enzymes used to generate a physical map of the fragment carried on pMON5122 included *Bam*HI, *Bst*EII, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Pvu*II, and *Sal*I. Competent cells were prepared for transformation according to Morrison (1979). Triparental matings were performed as described by Thomashow and Weller (1988) with the modification that the donor, helper, and recipient cultures were suspended in 500 μl of fresh LB broth prior to spotting on nitrocellulose to increase the cell density on the mating filters. The *Hin*dIII-*Eco*RI fragment from pMON5122 was cloned into the stable plasmid vector pVSP41 to generate pPHL5122. To generate pPHL5124.35 and pPHL5124.36, the 1-kb *Bam*HI-*Cla*I fragment from pMON5122 was cloned into pIC19H and then introduced as a *Hin*dIII fragment into pRK415 in both orientations.

Extraction and detection of metabolites.

For extraction of Phl, cultures were grown for 4 to 7 days in YM broth, cells were pelleted by centrifugation, and the antibiotic was extracted from the cell pellet as described by Keel et al. (1992) except that the final sample was dissolved in 100% methanol. Samples or standards consisting of purified Phl and MAPG were separated on Uniplate Silica Gel GHLF TLC plates (Alltech Associates Inc., Deerfield, IL) with chloroform/acetone (9:1, vol/vol). Spots were visualized by UV absorption at 254 nm. The detection limit by this method was about 1 μg for both Phl and MAPG.

Hydrogen cyanide production was detected in cultures grown on modified KMB agar containing glycine (4.4 g per liter) as described by Pierson and Thomashow (1992). HCN indicator paper (Whatman 3 MM paper saturated with a solution of 2% sodium carbonate and 0.5% picric acid and then air dried) was taped inside the lids and the plates were sealed with Parafilm and incubated for 2 to 4 days at 28°C. HCN production was detected by the yellow to red color change of the indicator paper. *Pseudomonas fluorescens* strain CHA0, which produces copious amounts of HCN, and strain M4-80R, which does not produce HCN, were included as positive and negative controls, respectively.

Phl bioassay.

Phl also was detected using *C. michiganensis* subsp. *michiganensis* as an indicator organism. A spontaneous tetracycline-resistant mutant of *C. michiganensis* subsp. *michiganensis* was selected by growth on LB agar amended with 25 μg of tetracycline per ml. YM plates were overlaid with 3 ml of water agar containing 100 μl of a 48-hr-old culture of *C. michiganensis* subsp. *michiganensis* and incubated for 4 to 6 hr to allow solidification of the agar and growth of *C. michiganensis* subsp. *michiganensis*. To establish detection limits for Phl and MAPG, filter paper disks (Whatman 3MM paper) were prepared by adding Phl or MAPG stock solutions in methanol to provide concentration ranges of 100 ng to 25 μg or 50 μg to 1 mg per disk, respectively. The disks were air dried, placed on the plates, and incubated at 28°C for 2 to 4 days, after which inhibition zones were measured. Disks impregnated with methanol served as negative controls. Detection limits were 250 ng and 75 μg for Phl and MAPG, respectively. The procedure was modified to assay Phl production by transconjugant bacterial strains; tetracycline was added to the YM agar and strains were inoculated by toothpick onto the solidified overlayer containing *C. michiganensis* subsp. *michiganensis*. Strains were assayed in sets of three per plate, with each set replicated three times. On each plate M4-80R(pMON5122) and M4-80R(pRK415) were included as positive and negative controls, respectively. The diameter of a zone of inhibition was measured after 2 to 4 days and zone sizes of strains on the same plate were compared statistically by analysis of variance and Fisher's protected least significant

different test. The experiment was repeated and the results were similar.

Plasmid maintenance test.

Transconjugant strains containing pPHL5122 were grown on LB agar with kanamycin. A single colony was transferred to 5 ml of LB broth and seven consecutive daily transfers of 25 µl were made to fresh LB broth. On the seventh day cultures were serially diluted and plated on LB agar with and without kanamycin. Colonies were counted and the ratio of the number of kanamycin-resistant colonies to total colonies was calculated for each strain. The experiment was repeated and similar results were obtained; the results of one experiment are presented.

Tn3HoHo1 mutagenesis and mutant characterization.

Insertions of the transposon Tn3HoHo1 in pMON5122 were recovered in *E. coli* C2110 after triparental mating with *E. coli* HB101(pHoHo/pSShe/pMON5122) and *E. coli* HB101 (pRK2013) (Stachel et al. 1985). Transconjugants (Tc^r, Nal^r, Ap^r) were cultured in 18 sets of 48 on a grid. The site and orientation of insertions into pMON5122 were analyzed by digestion of plasmid DNA isolated from each transconjugant with *Eco*RI alone and in combination with *Hin*dIII and *Bam*HI. Plasmids containing insertions within the cloned 6.5-kb fragment were then introduced into *P. fluorescens* M4-80R by triparental mating. The effect of the insertion on Phl production was analyzed by TLC and by bioassay with *C. michiganensis* subsp. *michiganensis*. Results from the two methods correlated but the bioassay was more sensitive. Expression of the *lacZ* reporter gene in the transposon, an indicator of transcriptional activity at the site of the insertion, was detected by the appearance of blue colonies on LB agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

ACKNOWLEDGMENTS

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