Tn5-Directed Cloning of *pqq* Genes from *Pseudomonas fluorescens* CHA0: Mutational Inactivation of the Genes Results in Overproduction of the Antibiotic Pyoluteorin

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Pseudomonas fluorescens **CHA0 produces several secondary metabolites, e.g., the antibiotics pyoluteorin (Plt) and 2,4-diacetylphloroglucinol (Phl), which are important for the suppression of root diseases caused by soil-borne fungal pathogens. A Tn5 insertion mutant of strain CHA0, CHA625, does not produce Phl, shows enhanced Plt production on malt agar, and has lost part of the ability to suppress black root rot in tobacco plants and take-all in wheat. We used a rapid, two-step cloning-out procedure for isolating the wild-type genes corresponding to those inactivated by the Tn5 insertion in strain CHA625. This cloning method should be widely applicable to bacterial genes tagged with Tn5. The region cloned from *P. fluorescens* contained three complete open reading frames. The deduced gene products, designated PqqFAB, showed extensive similarities to proteins involved in the biosynthesis of pyrroloquinoline quinone (PQQ) in *Klebsiella pneumoniae*, *Acinetobacter calcoaceticus*, and *Methylobacterium extorquens*. PQQ-negative mutants of strain CHA0 were constructed by gene replacement. They lacked glucose dehydrogenase activity, could not utilize ethanol as a carbon source, and showed a strongly enhanced production of Plt on malt agar. These effects were all reversed by complementation with *pqq*+ recombinant plasmids. The growth of a *pqqF* mutant on ethanol and normal Plt production were restored by the addition of 16 nM PQQ. However, the Phl− phenotype of strain CHA625 was due not to the *pqq* defect but presumably to a secondary mutation. In conclusion, a lack of PQQ markedly stimulates the production of Plt in *P. fluorescens*.**

Certain strains of the genus *Pseudomonas* have the ability to suppress a range of plant diseases caused by soil-borne plant-pathogenic fungi (8, 10, 12, 38, 67, 70). Evidence that antibiotic compounds play an important role in disease suppression comes from *Pseudomonas* mutants which are defective in the production of antibiotics and have lost part or all of their biocontrol properties. The antibiotics 2,4-diacetylphlorogluci-nol (Phl) (28, 30, 59, 66), pyoluteorin (Plt) (34, 40), pyrrolnitrin (24, 51), and phenazine-1-carboxylic acid (52, 63) are produced by various *Pseudomonas* strains, and each of these compounds has been found to contribute to disease suppression. Genes involved in the biosyntheses of these antibiotics have been localized by transposon mutagenesis and cloned. However, in general, little is known about the genetic and biochemical organization of antibiotic pathways in *Pseudomonas* spp.

The production of antimicrobial metabolites by pseudomonads depends on environmental factors, such as the carbon source. For instance, the synthesis of Phl in *Pseudomonas* sp. strain F113 is stimulated by mannitol or sucrose but not by glucose (59). The synthesis of Plt by *Pseudomonas fluorescens* Pf-5 is lowered in the presence of glucose (34, 48), whereas in *P. fluorescens* HV37a the production of oomycin A is enhanced when glucose is the carbon source (21, 27).

We are interested in the mechanisms by which *P. fluorescens* CHA0 suppresses root diseases of several crop plants. The antifungal metabolites Phl, Plt, and hydrogen cyanide produced by strain CHA0 are involved in the suppression of diseases caused by *Thielaviopsis basicola*, *Gaeumannomyces graminis* var. *tritici*, and *Pythium ultimum*. Depending on the host plant and the pathogen, the relative importance of Phl, Plt, and cyanide may vary (22, 28, 40, 58, 68). We have genetically identified three proteins which regulate the production of antimicrobial compounds in strain CHA0. The global response regulator GacA and its putative cognate sensor LemA together control the expression of the secondary metabolites Phl, Plt, and cyanide, as well as the expression of extracellular protease and tryptophan side chain oxidase (TSO) (7, 36, 56). The anaerobic regulator Anr is required for the production of cyanide (67).

We have previously described a Tn5 insertion mutant of *P. fluorescens*, CHA625, which is blocked in the synthesis of Phl and shows a reduced capacity to suppress black root rot of tobacco and take-all of wheat (28, 30). This mutant also overproduces Plt. The aim of this study was to identify the gene(s) responsible for these effects in *P. fluorescens*. To this end, we have used a rapid, two-step method to clone from strain CHA0 those genes which are inactivated by Tn5 in the mutant CHA625. We show that the cloned genes are necessary for the biosynthesis of pyrroloquinoline quinone (PQQ), a cofactor of different dehydrogenases, and that the inactivation of the *pqq* genes leads to enhanced Plt production in *P. fluorescens* CHA0.

MATERIALS AND METHODS

Microorganism and plasmids. *P. fluorescens* CHA0 (62), its mutant CHA625 (*pqqF*::Tn5 Phl− [30]), and other CHA0 derivatives are listed in Table 1. *Esch-*

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TABLE 1. Characteristics of PQQ⁻ mutants of *P. fluorescens* CHA0 and complementation analysis

Strain	Phenotype	Utilization of ethanol[a]	Production of acid on glucose-EMB plates	Glucose dehydrogenase activity[b]	Antibiotic production on malt agar (μg/ml medium)[c]	
					Phl	Plt
CHA0	Wild-type	+	+	+	5.4 ± 0.2	<0.1
CHA625	PQQ⁻ Phl⁻	−	−	−	0.1 ± 0.1	3.1 ± 1.3
CHA625/pME3186	PQQ⁺	+	+	+	1.2 ± 0.4	<0.1
CHA625/pME3188	PQQ⁺	+	+	+	1.1 ± 0.3	<0.1
CHA120	PQQ⁻	−	−	−	4.4 ± 1.1	2.8 ± 0.4
CHA120/pME3186	PQQ⁺	+	+	+	5.7 ± 1.6	<0.1
CHA120/pME3188	PQQ⁺	+	+	+	5.5 ± 1.4	<0.1
CHA177	PQQ⁻	−	−	−	4.9 ± 0.6	5.0 ± 0.6
CHA177/pME3186	PQQ⁻	−	−	−	3.9 ± 0.5	4.2 ± 1.7
CHA177/pME3188	PQQ⁺	+	+	+	4.5 ± 0.7	<0.1
CHA178	PQQ⁻	−	−	−	4.0 ± 0.5	4.5 ± 0.6
CHA178/pME3186	PQQ⁺	+	+	+	4.9 ± 1.1	<0.1
CHA178/pME3188	PQQ⁺	+	+	+	4.9 ± 0.3	<0.1
CHA179	PQQ⁻	−	−	−	2.9 ± 0.8	3.0 ± 0.6
CHA179/pME3186	PQQ⁻	−	−	−	3.9 ± 0.6	3.9 ± 1.3
CHA179/pME3188	PQQ⁺	+	+	+	3.9 ± 1.1	<0.1
CHA181	PQQ⁺	+	+	+	3.3 ± 1.0	<0.1
CHA121	PQQ⁺	+	+	+	3.7 ± 0.6	<0.1

[a] Growth was determined in liquid medium (see Materials and Methods).
[b] By enzyme assays. +, wild-type levels; −, not detectable (<0.02 nmol of 2,6-dichlorophenol-indophenol reduced min^{-1} mg of $protein^{-1}$).
[c] The bacteria were grown on malt agar plates at 24°C for 3 days. The agar plates were extracted as described in Materials and Methods. The average numbers of bacteria per plate at the time of extraction did not vary significantly among different strains and reached ≈ 3×10^{10} cells per malt agar plate. Each value is the mean (± standard error) of three independent experiments with two plates per experiment.

erichia coli SK1592 (35), JM108 (71), and ED8767 (46) served as plasmid hosts for cloning experiments. The IncP plasmid pVK100 (33), the suicide plasmids pME3049 (Fig. 1B) and pME3087 (67), and pBluescript II KS+ (60) were used as cloning vectors. The Ω-Km (kanamycin) fragment from plasmid pHP45Ω-Km (13) and the Km resistance gene cassette of pUC4KIXX (Pharmacia, Uppsala, Sweden) were used for gene disruption experiments. Plasmids were mobilized with the helper plasmids pME497 (69) and R64*drd*-11 (44).

Media and growth conditions. Strains of *E. coli* were routinely cultured on nutrient agar plates (61) and in nutrient yeast broth with aeration at 37°C. M13 phages mp18 and mp19 (47) were propagated on *E. coli* JM108 in 2× YT medium (23). *P. fluorescens* was routinely cultivated at 30°C on nutrient agar or on a modified eosin-methylene blue (EMB) agar (Difco), containing, per liter, glucose, 100 mmol; proteose peptone, 10 g; K_2HPO_4, 2.0 g; eosin yellowish, 0.4 g; methylene blue, 0.06 g; and agar (Serva), 16 g. The utilization of ethanol as the carbon source was tested in a minimal medium containing, per liter, ethanol, 5 ml; Na_2HPO_4, 7.1 g; KH_2PO_4, 6.8 g; $MgSO_4 \cdot 7 H_2O$, 1.2 g; $(NH_4)_2$ SO_4, 1.0 g; $CaCl_2 \cdot 2 H_2O$, 0.09 g; $FeSO_4 \cdot 7 H_2O$, 2 mg; and $(NH_4)_6Mo_7O_{24} \cdot 4$ H_2O, 0.2 mg. When required, PQQ (Methoxatin; Fluka) was added as an aqueous solution sterilized by filtration. For measurements of glucose dehydrogenase (GDH) activity, *P. fluorescens* cells were cultivated in a medium (1 liter, pH adjusted to 7.3) consisting of gluconate, 9.9 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7 H_2O$, 0.5 g; and acid-hydrolyzed, vitamin-free Casamino Acids (Difco), 5 g. Liquid cultures of *P. fluorescens* were incubated in 500-ml Erlenmeyer flasks containing 200 ml of medium on a rotary shaker (120 rpm). Antibiotic production by *P. fluorescens* was assessed after growth at 24°C on malt agar (29) or King's medium B agar (31). Antimicrobial compounds, when added, were used at the following concentrations (in micrograms per milliliter of medium): ampicillin, 100; kanamycin sulfate, 25; $HgCl_2$, 20; and tetracycline hydrochloride, 25 for *E. coli* and 125 for *P. fluorescens* strains.

DNA manipulations. Plasmid DNA from *E. coli* and *P. fluorescens* was isolated by alkaline lysis (57) for IncP derivatives and by the cetyl trimethyl ammonium bromide method (11) for ColE1-based plasmids. Large-scale plasmid preparations were performed with Qiagen Tip 100 (Diagen GmbH, Hilden, Germany). Restriction enzyme digestions, DNA fragment isolation from low-melting-point agarose gels, ligation, agarose gel electrophoresis, and transformation of *E. coli* were done according to standard procedures (57). Partial digestions of plasmid DNA were performed as described by Ausubel et al. (2). Chromosomal DNA of *P. fluorescens* was isolated by the method described by Gamper et al. (16). Southern blotting with Hybond N membranes (Amersham), random-primed DNA labeling with digoxigenin-11-dUTP, and hybridization and detection (Boehringer, Mannheim, Germany; kits 1,175,033 and 1,093,657) were performed according to the protocols of the manufacturers. For nucleotide sequence determination, subclones of the 4.4-kb *Bgl*II-*Eco*RI fragment of pME3141 were constructed in either pBluescript II KS+ (60), M13mp18, or M13mp19 (71). Both strands were sequenced by the dideoxy-chain termination method with 7-deaza-dGTP and Sequenase (version 2.0; United States Biochemical Corp.). The software (FastA, Bestfit, Motif, and Pileup) from the Genetics Computer Group,

Inc. (Madison, Wis.) was used to analyze nucleotide and deduced amino acid sequences.

Bacterial matings. Triparental matings of *P. fluorescens* recipients with *E. coli* containing a mobilizable plasmid (pVK100, pME3087, or pME3049) and *E. coli* harboring a mobilizing plasmid (pME497 or R64*drd*-11) were performed as previously described (68, 69), with a minor modification: mating mixtures were incubated on nutrient agar at 37°C instead of 30°C, for 3 h; this modification enhanced plasmid transfer from *E. coli* to *P. fluorescens* about 10-fold.

Construction of *P. fluorescens* mutants by gene replacement. Genomic fragments of the plasmids pME3122K and pME3141 were cloned into the suicide vector pME3087, which carries a tetracycline resistance determinant. The kanamycin resistance gene cassette from pUC4KIXX or the Ω-Km fragment from pHP45Ω-Km was cloned into specific restriction sites for the disruption of *pqq* genes. The plasmids were mobilized with the helper plasmid R64*drd*-11 to the wild-type strain CHA0 (68). Cells with a chromosomally integrated plasmid were selected for tetracycline and kanamycin resistance. Strains from which the vector had been excised via a second homologous recombination event could be obtained by enrichment for tetracycline sensitivity (54), and selection for kanamycin resistance ensured the presence of the insertion mutation.

Enzyme assays. Membrane fractions were prepared and solubilized according to the protocol of Biville et al. (6), with minor modifications. Bacterial cells were harvested in the mid-exponential phase and washed twice with buffer A (5 mM potassium phosphate buffer, pH 7.3, 2.5 mM $MgSO_4$). Cells were resuspended in buffer A containing 1% (wt/vol) Triton X-100, stirred at 4°C for 2 h, disrupted by sonication at 4°C, and centrifuged at $80,000 \times g$ for 30 min. The supernatant containing the solubilized membrane fraction was used for the GDH assay according to the protocol of Matsushita and Ameyama (39). TSO activity was assessed qualitatively as described by Oberhänsli and Défago (49).

Extraction and measurements of antibiotics. *P. fluorescens* strains were cultivated on malt agar or King's B agar at 24°C for 72 h. Extraction, detection, and quantification of the antibiotics Phl and Plt have been previously described (28, 42). The extracts were analyzed in a Hewlett-Packard 1090 liquid chromatograph equipped with a diode-array detector, using a column (4 × 100 mm) packed with Nucleosil 120-5-C18 (Macherey-Nagel, Oensingen, Switzerland) and a three-step linear gradient (28, 42). Phl and Plt were detected by UV absorption at 270 nm and 313 nm, respectively.

Nucleotide sequence accession number. The nucleotide sequence of the *pqqFABC* region reported here has been assigned GenBank accession number X87299.

RESULTS

Cloning of the wild-type genes (*pqq*) corresponding to those inactivated by a Tn*5* insertion in *P. fluorescens* CHA625. *P. fluorescens* CHA625, a Tn*5* insertion mutant of the wild-type strain
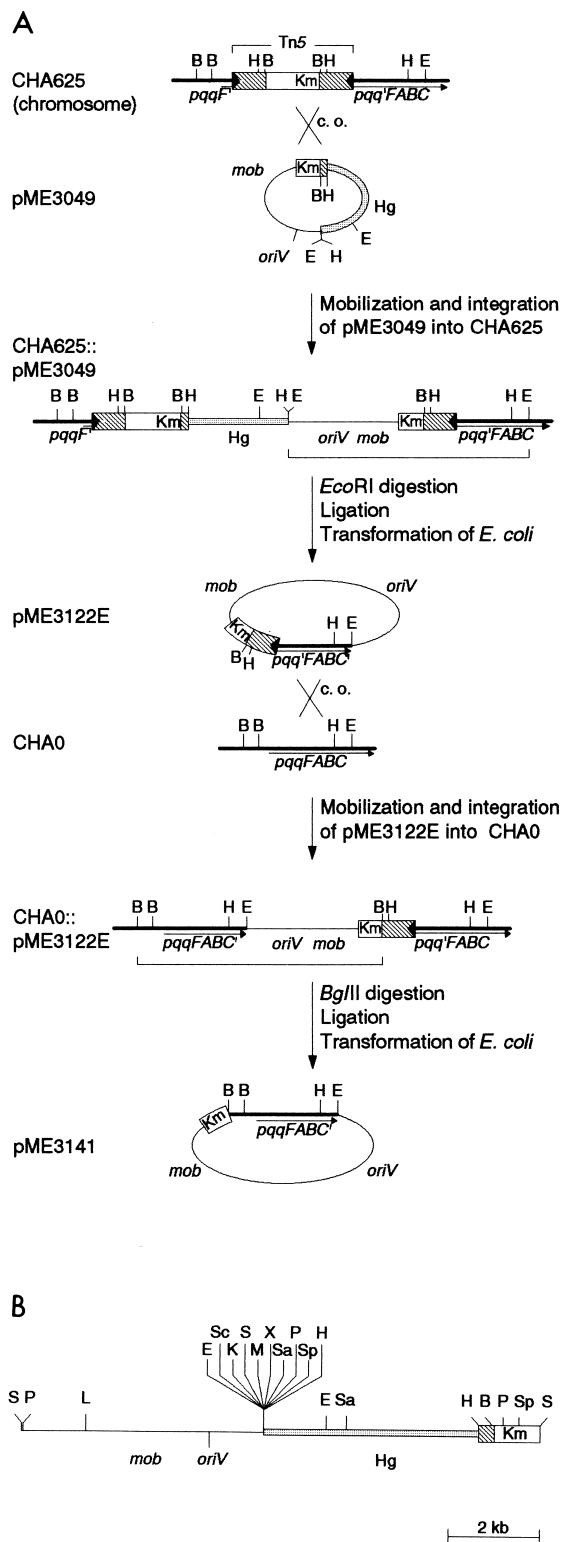
FIG. 1. (A) Two-step, Tn5-directed cloning of the *P. fluorescens* wild-type genes corresponding to those inactivated by the Tn5 insertion in strain CHA625. The location of Tn5 in *pqqF* and the extent of the *pqq* region were determined by sequencing (Fig. 3). For further details, see the text. (B) Restriction map of the vector pME3049. The vector pME3049, a ColE1-based plasmid (thin line), carries the IS*50*L element (hatched box) with the kanamycin resistance gene (Km) of Tn5 (open box), the mercury resistance genes (Hg) of Tn5-259 (stippled box) (69), and the *Eco*RI-*Hin*dIII polylinker of pJF118EH (15). Abbreviations: c. o., crossover due to homologous recombination; *oriV*, origin of ColE1 replication;

CHA0 (30), did not produce detectable amounts of Phl on malt agar, whereas the wild-type strain CHA0 excreted 5.4 μg of Phl per ml during incubation for 3 days. The mutant CHA625, but not the wild-type CHA0, produced Plt on the same medium (Table 1). On King's medium B, the mutant produced significantly more Plt (24 μg/ml) than did the wild-type strain (3.5 μg/ml). To test whether Plt overproduction and the lack of Phl in strain CHA625 are due to the same mutation, we cloned the wild-type genes which are located at and near the Tn5 insertion site in the mutant. We used an efficient two-step cloning strategy proposed by Voisard et al. (67). The principal aim of this strategy is to clone the wild-type allele of a gene inactivated by Tn5, without the need for a genomic library or shotgun cloning. The rationale and cloning steps are shown in Fig. 1A. The ColE1-based suicide plasmid pME3049 (Fig. 1B), which carries the kanamycin resistance gene of Tn5 and a HgCl$_2$ resistance determinant from plasmid pVS1 (67), was mobilized with the conjugative helper plasmid R64*drd*-11 into the mutant CHA625, with selection for kanamycin and mercury resistance. Homologous recombination occurred between the kanamycin resistance genes of pME3049 and the chromosomal Tn5 element at frequencies of 10^{-7} to 10^{-8}, resulting in the chromosomal integration of pME3049. Genomic DNA of strain CHA625::pME3049 was digested with *Eco*RI, religated, and used to transform *E. coli* ED8767, with selection for kanamycin-resistant clones. The resulting plasmid pME3122E carried the kanamycin resistance gene with the IS*50*L element of Tn5 and an adjacent 3.4-kb genomic fragment (Fig. 1A). To clone the wild-type genes, plasmid pME3122E was transferred with the helper plasmid R64*drd*-11 into *P. fluorescens* wild-type strain CHA0. Kanamycin-resistant cells had a chromosomally integrated plasmid, because of a single crossover between the chromosomal insert of pME3122E and the homologous region in the chromosome. The digestion of genomic DNA from this recombinant with *Bgl*II, self-ligation, and transformation of *E. coli* ED8767 resulted in the plasmid pME3141. Analysis of this plasmid showed that it contained two *Bgl*II fragments, probably because the chromosomal DNA of strain CHA0::pME3122E had been partially digested with *Bgl*II. Thus, the plasmid pME3141 contained a 4.4-kb *Bgl*II-*Eco*RI and an additional 0.7-kb *Bgl*II fragment of the chromosomal DNA from *P. fluorescens* CHA0. As will be shown below, the 4.4-kb fragment contains three complete open reading frames (ORFs) and the 5′ end of a fourth ORF, all of which show homology with *pqq* genes of other bacteria. The 4.4-kb insert of pME3141 was confirmed as an authentic fragment from the CHA0 chromosome by Southern hybridization (data not shown).

Previously, we have isolated plasmid pME3128 (Fig. 2), which partially restores Phl production in the mutant CHA625 (28). Restriction map analysis and Southern hybridization (data not shown) indicated that the genomic DNA inserts of pME3128 and pME3141 overlapped with each other in a region of approximately 4.3 kb (Fig. 2).

The suicide vector pME3049 (Fig. 1B) offers several possibilities to carry out the first cloning step. Instead of *Eco*RI, other restriction enzymes (*Kpn*I, *Bam*HI, *Sac*I, *Sal*I, *Xba*I) can be used to excise the integrated plasmid. All enzymes cut pME3049 between the HgCl$_2$ resistance determinant and the *oriV* region but not in the kanamycin resistance fragment (Fig. 1B). The choice of the restriction enzyme depends on

mob, region required for mobilization by, e.g., IncI or IncP plasmids; B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; L, *Mlu*I; M, *Bam*HI; P, *Pst*I; S, *Sma*I; Sa, *Sal*I; Sc, *Sac*I; Sp, *Sph*I; X, *Xba*I.
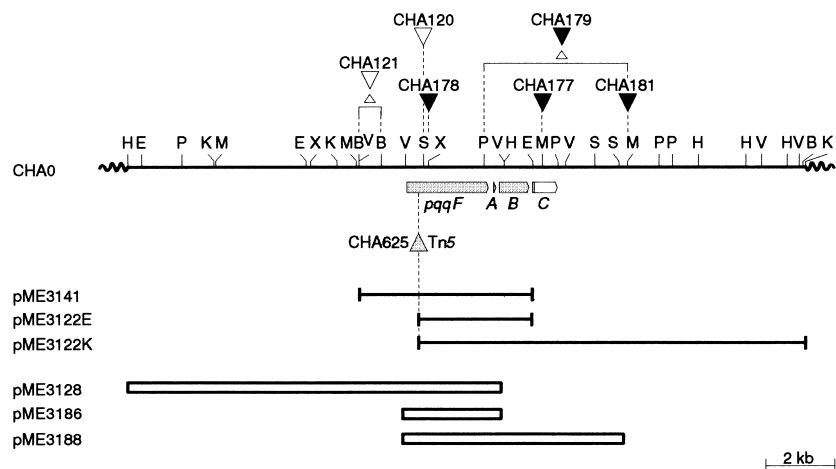
FIG. 2. Genetic organization and restriction map of the *pqq* cluster in the chromosome of *P. fluorescens*. Chromosomal insertions are indicated as follows: △, Tn*5* in strain CHA625; ▼, Ω-Km fragment; and ▽, Km resistance gene cassette from pUC4KIXX. The regions deleted in the mutant strains CHA179 and CHA121 are indicated by △. Stippled arrows indicate the *pqq* genes sequenced. The bars below indicate the genomic inserts in the ColE1-based plasmids pME3122E and pME3141 as outlined in Fig. 1A. The plasmid pME3122K was obtained after *Kpn*I digestion of the chromosomal DNA of strain CHA0::pME3049 (Fig. 1A), ligation, and transformation of *E. coli*. The open boxes designate fragments cloned into pVK100. Abbreviations: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Bam*HI; P, *Pst*I; S, *Sac*I; V, *Eco*RV; X, *Xba*I.

appropriately located sites in the genomic region next to the Tn*5* insertion. In the context of this study, the chromosome of strain CHA0::pME3049 was also digested with *Kpn*I, since Southern blots had indicated that a *Kpn*I site was located approximately 11 kb downstream of the Tn*5* insertion in strain CHA625. Self-ligation and cloning in *E. coli* produced pME3122K, containing an 11.3-kb chromosomal insert (Fig. 2). In this way, an extended region downstream of the site of the Tn*5* insertion could be cloned.

Nucleotide sequence of the *pqqFAB* genes of *P. fluorescens* and comparison with *pqq* genes of other microorganisms. The nucleotide sequence of the 4.4-kb *Bgl*II-*Eco*RI genomic fragment in pME3141 was determined (Fig. 3). The analysis of the 4,408-bp fragment revealed three ORFs, each of which contains (at least) one putative ribosome-binding site adjacent to the proposed ATG translational start codons. The average G + C content of the predicted ORFs is 64.9%, which corresponds roughly to the G + C content expected for the *P. fluorescens-P. putida* group (50). The average G + C content at the third codon position is 76% in the first ORF, 79% in the second ORF, and 82% in the third ORF. These values are close to those predicted by computer analysis (5). The ORFs have the same orientation and might form an operon. However, transcription start and termination sites have not been determined.

The deduced amino acid sequences of the three ORFs show extended sequence similarities with proteins involved in the synthesis of PQQ in *Klebsiella pneumoniae* (43), *Acinetobacter calcoaceticus* (18), and *Methylobacterium extorquens* (45). We designated the ORFs of *P. fluorescens* CHA0 *pqqF*, *pqqA*, and *pqqB* (Fig. 3), according to the nomenclature used for *K. pneumoniae*. The large PqqF protein of *P. fluorescens* has a predicted molecular mass of 91.3 kDa and 829 amino acids. The *pqqF* gene product of *K. pneumoniae*, which consists of 761 amino acids, giving a molecular mass of 83.6 kDa (43), shows 53.7% similarity and 35.2% identity, over the whole sequence, with PqqF of strain CHA0. Homologous proteins in *M. extorquens* and *A. calcoaceticus* have not been described. The second ORF (*pqqA*) of *P. fluorescens* encodes a small protein of 24 or 39 amino acids (Fig. 4A), depending on which of the

two possible translation start sites of this ORF (Fig. 3) are used. However, the 24-amino-acid version is very similar to PqqIV of *A. calcoaceticus* (24 amino acids, 79.2% identity), PqqA of *K. pneumoniae* (23 amino acids, 56.5% identity), and PqqD of *M. extorquens* (29 amino acids, 33.3% identity) (Fig. 4A). The third ORF of *P. fluorescens* has a deduced molecular mass of 33.2 kDa (303 amino acid residues) and corresponds to *pqqB* of *K. pneumoniae*, *pqqV* of *A. calcoaceticus*, and *pqqG* of *M. extorquens*. These genes have similar lengths (Fig. 4B). The identities between their products range between 41.9 and 63.6%, calculated over the entire length of the proteins.

A fourth ORF, tentatively designated *pqqC*, in the gene cluster of *P. fluorescens* CHA0 was sequenced only in its 5′ region (Fig. 3). The deduced 11 N-terminal amino acids show sequence identities with the N termini of PqqI of *A. calcoaceticus* (54.5%), PqqC of *K. pneumoniae* (45.4%), and PqqC of *M. extorquens* (27.3%). From these sequence comparisons, it appears that the order of the *pqqABC* genes and their homologs is conserved in the four bacterial species (Fig. 4B).

Localization of the Tn*5* insertion in the mutant CHA625, construction of *pqq* mutants by gene replacement, and effects on Plt production. The chromosomal Tn*5* insertion in *P. fluorescens* CHA625 had previously been mapped to a position lying approximately 2.9 kb from the *Hin*dIII restriction site now known to be in *pqqB* (Fig. 2) (28). The Tn*5* insertion could be localized precisely by sequencing the genomic region adjacent to IS*50*L of Tn*5* in the plasmid pME3122E (Fig. 2). Thus, Tn*5* in strain CHA625 was found to be located at position 954 in the *pqqF* gene (Fig. 3).

To determine whether the lack of Phl synthesis and enhanced Plt production in strain CHA625 were both due to the *pqqF* mutation, we constructed a *pqqF* mutant (CHA178) (Fig. 2) by inserting the transcription termination element Ω-Km (13) into the CHA0 chromosome. Similarly, the Ω-Km element was also inserted at a site downstream of *pqqF* (resulting in strain CHA177) and used to replace a 4.0-kb segment encompassing *pqqABC* (resulting in strain CHA179) (Fig. 2). Further, a kanamycin resistance cassette without transcriptional stop sites was integrated into the chromosomal *pqqF* gene (resulting in strain CHA120) (Fig. 2). The *pqq* mutants thus constructed
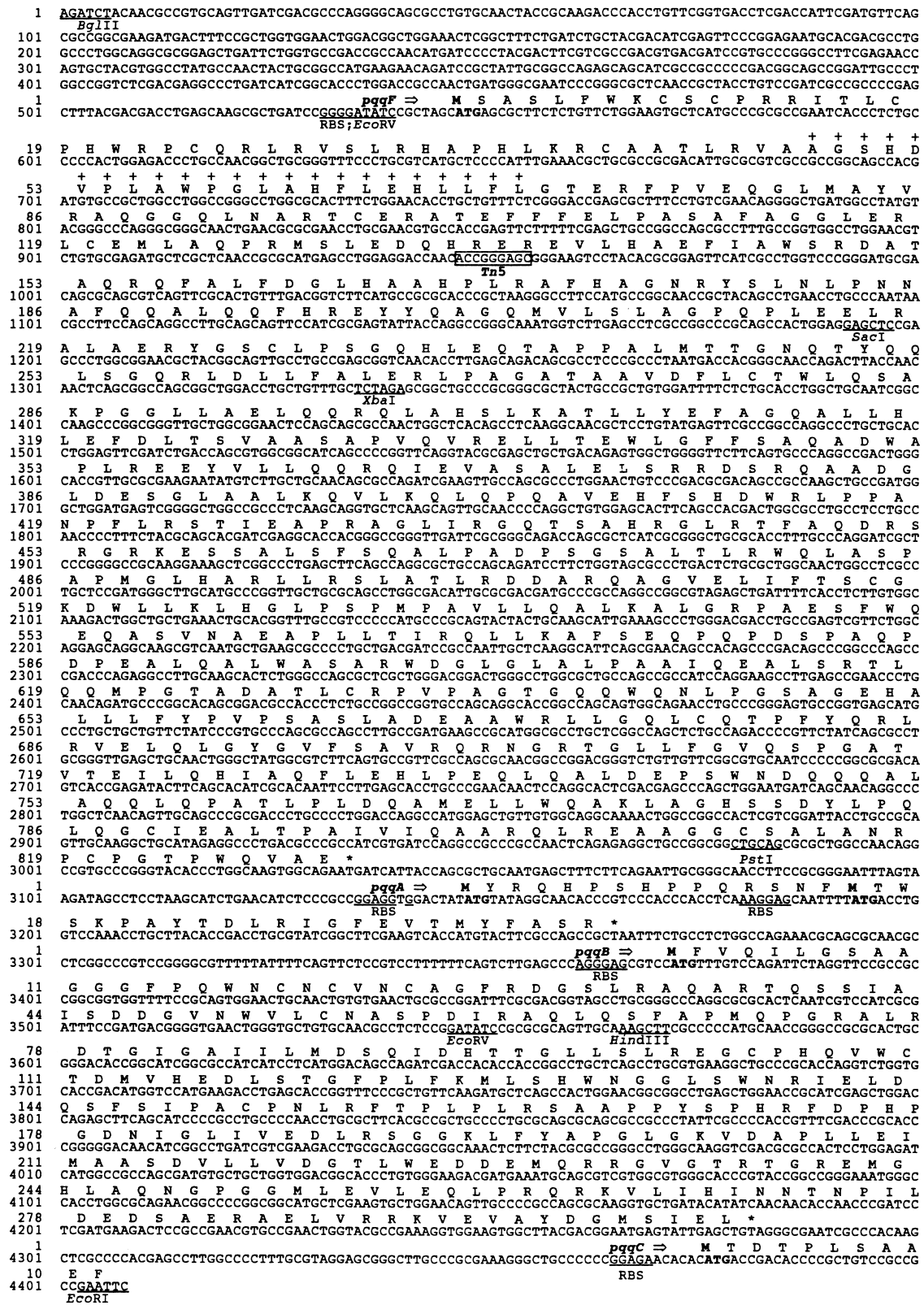
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  1 AGATCTACAACGCCGTGCAGTTGATCGACGCCCAGGGGCAGCGCCTGTGCAACTACCGCAAGACCCACCTGTTCGGTGACCTCGACCATTCGATGTTCAG
    BglII
101 CGCCGGCGAAGATGACTTTCCGCTGGTGGAACTGGACGGCTGGAAACTCGGCTTTCTGATCTGCTACGACATCGAGTTCCCGGAGAATGCACGACGCCTG
201 GCCCTGGCAGGCGCGGGAGCTGATTCTGGTGCCGACCGCCAACATGATCCCCTACGACTTCGTCGCCGACGTGACGATCCGTGCCCGGGCCTTCGAGAACC
301 AGTGCTACGTGGCCTATGCCAACTACTGCGGCCATGAAGAACAGATCCGCTATTGCGGCCAGAGCAGCATCGCCGCCCCCGACGGCAGCCGGATTGCCCT
401 GGCCGGTCTCGACGAGGCCCTGATCATCGGCACCCTGGACCGCGAATCCGATGGGCGAATCCCGGGCGGCTCAACCGCTACCTGTCCGATCGCGCCCCGAG
                                                      pqqF ⇒  M  S  A  S  L  F  W  K  C  S  C  P  R  R  I  T  L  C
501 CTTTACGACGACCTGAGCAAGCGCTGATCCGGGGATATCCGCTAGCATGAGCGCTTCTCTGTTCTGGAAGTGCTCATGCCCGCGCCGAATCACCCTCTGC
                                      RBS;EcoRV
                                                                      +  +  +  +  +
 19 P  H  W  R  P  C  Q  R  L  R  V  S  L  R  H  A  P  H  L  K  R  C  A  A  T  L  R  V  A  A  G  S  H  D
601 CCCCACTGGAGACCCTGCCAACGGCTGCGGGTTTCCCTGCGTCATGCTCCCCATTTGAAACGCTGCGCCGCGACATTGCGCGTCGCCGCCGGCAGCCACG
       +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +
 53 V  P  L  A  W  P  G  L  A  H  F  L  E  H  L  L  F  L  G  T  E  R  F  P  V  E  Q  G  L  M  A  Y  V
701 ATGTGCCGCTGGCCTGGCCGGGCCTGGCGCACTTTCTGGAACACCTGCTGTTTCTCGGGACCGAGCGCTTTCCTGTCGAACAGGGGCTGATGGCCTATGT
 86 R  A  Q  G  G  G  Q  L  N  A  R  T  C  E  R  A  T  E  F  F  F  E  L  P  A  S  A  F  A  G  G  L  E  R
801 ACGGGCCCAGGGCGGGCAACTGAACGCGCGAACCTGCGAACGTGCCACCGAGTTCTTTTTCGAGCTGCCGGCCAGCGCCTTTGCCGGTGGCCTGGAACGT
119 L  C  E  M  L  A  Q  P  R  M  S  L  E  D  Q  H  R  E  R  E  V  L  H  A  E  F  I  A  W  S  R  D  A  T
901 CTGTGCGAGATGCTCGCTCAACCGCGCATGAGCCTGGAGGACCAACACCGGGAGCGGGAAGTCCTACACGCGGAGTTCATCGCCTGGTCCCGGGATGCGA
                                                Tn5
153 A  Q  R  Q  F  A  L  F  D  G  L  H  A  A  H  P  L  R  A  F  H  A  G  N  R  Y  S  L  N  L  P  N  N
1001 CAGCGCAGCGTCAGTTCGCACTGTTTGACGGTCTTCATGCCGCGCACCCGCTAAGGGCCTTCCATGCCGGCAACCGCTACAGCCTGAACCTGCCCAATAA
186 A  F  Q  Q  A  L  Q  Q  F  H  R  E  Y  Y  Q  A  G  Q  M  V  L  S  L  A  G  P  Q  P  L  E  E  L  R
1101 CGCCTTCCAGCAGGCCTTGCAGCAGTTCCATCGCGAGTATTACCAGGCGGGCAAATGGTCTTGAGCCTCGCCGGCCCGCAGCCACTGGAGGAGCTCCGA
                                                                              SacI
219 A  L  A  E  R  Y  G  S  C  L  P  S  G  Q  H  L  E  Q  T  A  P  P  A  L  M  T  T  G  N  Q  T  Y  Q  Q
1201 GCCCTGGCGGAACGCTACGGCAGTTGCCTGCCGAGCGGTCAACACCTTGAGCAGACAGCGCCTCCCGCCCTAATGACCACGGGCAACCAGACTTACCAAC
253 L  S  G  Q  R  L  D  L  F  A  L  E  R  L  P  A  G  A  T  A  A  V  D  F  L  C  T  W  L  Q  S  A
1301 AACTCAGCGGCCAGCGGCTGGACCTGCTGTTTGCTCTAGAGCGGCATCGCCGCGGGCGCTACTGCCGCTGTGGATTTTCTCTGCACCTGGCTGCAATCGGC
                                      XbaI
286 K  P  G  G  L  L  A  E  L  Q  Q  R  R  Q  L  A  H  S  L  K  A  T  L  L  Y  E  F  A  G  Q  A  L  L  H
1401 CAAGCCCGGCGGGTTGCTGGCGGAACTCCAGCAGCGCCAACTGGCTCACAGCCTCAAGGCAACGCTCCTGTATGAGTTCGCCGGCCAGGCCCTGCTGCAC
319 L  E  F  D  L  T  S  V  A  A  S  A  P  V  Q  V  R  E  L  L  T  E  W  L  G  F  F  S  A  Q  A  D  W  A
1501 CTGGAGTTCGATCTGACCAGCGTGGCGGCATCAGCCCCGGTTCAGGTACGCGAGCTGCTGACAGAGTGGCTGGGGTTCTTCAGTGCCCAGGCCGACTGGG
353 P  L  R  E  E  Y  V  L  L  Q  Q  R  Q  I  E  V  A  S  A  L  E  L  S  R  R  D  S  R  Q  A  A  D  G
1601 CACCGTTGCGCGAAGAATATGTCTTGCTGCAACAGCGCCAGATCGAAGTTGCCAGCGCCCTGGAACTGTCCCGACGCGACAGCCGCCAAGCTGCCGATGG
386 L  D  E  S  G  L  A  A  L  K  Q  V  L  K  Q  L  P  Q  A  V  E  H  F  S  H  D  W  R  L  P  P  A
1701 GCTGGATGAGTCGGGGCTGGCCGCCCTCAAGCAGGTGCTCAAGCAGTTGCAACCCCAGGCTGTGGAGCACTTCAGCCACGACTGGCGCCTGCCTCCTGCC
419 N  P  F  L  R  S  T  I  E  A  P  R  A  G  L  I  R  G  Q  T  S  A  H  R  G  L  R  T  F  A  Q  D  R  S
1801 AACCCCTTTCTACGCAGCACGATCGAGGCACCACGGGCCGGGTTGATTCGCGGGCAGACCAGCGCTCATCGCGGGCTGCGCACCTTTGCCCAGGATCGCT
453 R  G  R  K  E  S  S  A  L  S  F  S  Q  A  L  P  A  D  P  S  G  S  A  L  T  L  R  W  Q  L  A  S  P
1901 CCCGGGGCCGCAAGGAAAGCTCGGCCCTGAGCTTCAGCCAGGCGCTGCCAGCAGATCCTTCTGGTAGCGCCCTGACTCTGCGCTGGCAACTGGCCTCGCC
486 A  P  M  G  L  H  A  R  L  L  R  S  L  A  T  L  R  D  D  A  R  Q  A  G  V  E  L  I  F  T  S  C  G
2001 TGCTCCGATGGGCTTGCATGCCCGGTTGCTGCGCAGCCTGGCGACATTGCGCGACGATGCCCGCCAGGCCGGCGTAGAGCTGATTTTCACCTCTTGTGGC
519 K  D  W  L  L  K  L  H  G  L  P  S  P  M  P  A  V  L  L  Q  A  L  K  A  L  G  R  P  A  E  S  F  W  Q
2101 AAAGACTGGCTGCTGAAACTGCACGGTTTGCCGTCCCCCATGCCGCAGTACTACTGCAAGCATTGAAAGCCCTGGGACGACCTGCCGAGTCGTTCTGGC
553 E  Q  A  S  V  N  A  E  A  P  L  L  T  I  R  Q  L  L  K  A  F  S  E  Q  P  Q  P  D  S  P  A  Q  P
2201 AGGAGCAGGCAAGCGTCAATGCTGAAGCGCCCCTGCTGACGATCCGCCAATTGCTCAAGGCATTCAGCGAACAGCCACAGCCCGACAGCCCGGCCGAGCC
586 D  P  E  A  L  Q  A  L  W  A  S  A  R  W  D  G  L  G  L  A  L  P  A  A  I  Q  E  A  L  S  R  T  L
2301 CGACCCAGAGGCCTTGCAAGCACTCTGGGCCAGCGCTCGCTGGGACGGCAGTGGGCCTGGCGCTGCCAGCCGCCATCCAGGAAGCCTTGAGCCGAACCCTG
619 Q  Q  M  P  G  T  A  D  A  T  L  C  R  P  V  P  A  G  T  G  Q  Q  W  Q  N  L  P  G  S  A  G  E  H  A
2401 CAACAGATGCCCGGCACAGCGGACGCCCACCCTCTGCCGGCCGGTGCCAGCAGGCACCGGCCAGCAGTGGCAGAACCTGCCCGGGAGTGCCGGTGAGCATG
653 L  L  L  F  Y  P  V  P  S  A  S  L  A  D  E  A  A  W  R  L  L  G  Q  L  C  Q  T  P  F  Y  Q  R  L
2501 CCCTGCTGCTGTTCTATCCCGTGCCCAGCGCCAGCCTTGCCGATGAAGCCGCATGGCGCCTGCTCGGCCAGCTCCTGCCAGACCCCGTTCTATCAGCGCCT
686 R  V  E  L  Q  L  G  Y  G  V  F  S  A  V  R  Q  R  N  G  R  T  G  L  L  F  G  V  Q  S  P  G  A  T
2601 GCGGGTTGAGCTGCAACTGGGCTATGGCGTCTTCAGTGCCGTTCGCCAGCGCAACGGCCGGAGCGGGTCTGTTGTTCGGCGTGCAATCCCCCGGCGCGACA
719 V  T  E  I  L  Q  H  I  A  Q  F  L  E  H  L  P  E  Q  L  Q  A  L  D  E  P  S  W  N  D  Q  Q  Q  A  L
2701 GTCACCGAGATACTTCAGCACATCGCACAATTCCTTGAGCACCTGCCCGAACAACTCCAGGCACTCGACGAGCCCAGCTGGAATGATCAGCAACAGGCCC
753 A  Q  Q  L  Q  P  A  T  L  P  L  D  Q  A  M  E  L  L  W  Q  A  K  L  A  G  H  S  S  D  Y  L  P  Q
2801 TGGCTCAACAGTTGCAGCCCGCGACCCTGCCCCTGGACCAGGCCATGGAGCTGTTGTGGCAGGCAAAACTGGCCGGCCACTCGTCGGATTACCTGCCGCA
786 L  Q  G  C  I  E  A  L  T  P  A  I  V  I  Q  A  A  R  Q  L  R  E  A  A  G  G  C  S  A  L  A  N  R
2901 GTTGCAAGGCTGCATAGAGGCCCTGACGCCCGCCATCGTGATCCAGGCCGCCCGCCAACTCAGAGAGGCTGCCGGCGGCCTGCAGCGCGCTGGCCAACAGG
819 P  C  P  G  T  P  W  Q  V  A  E  *
3001 CCGTGCCCGGGTACACCCTGGCAAGTGGCAGAATGATCATTACCAGCGCTGCAATGAGCTTTCTTCAGAATTGCGGGCAACCTTCCGCGGGAATTTAGTA
                                                              PstI
                                          pqqA ⇒  M  Y  R  Q  H  P  S  H  P  P  Q  R  S  N  F  M  T  W
3101 AGATAGCCTCCTAAGCATCTGAACATCTCCCGCCGGGAGGTGGACTATATGTATAGGCACACCCGTCCCACCCACCTCAAAGGAGCAATTTTATGACCTG
                                          RBS                                        RBS
 18 S  K  P  A  Y  T  D  L  R  I  G  F  E  V  T  M  Y  F  A  S  R  *
3201 GTCCAAACCTGCTTACACCGACCTGCGTATCGGCTTCGAAGTCACCATGTACTTCGCCAGCCGCTAATTTCTGCCTCTGGCCAGAAACGCAGCGCAACGC
                                          pqqB ⇒  M  F  V  Q  I  L  G  S  A  A
3301 CTCGGCCCGTCCGGGGCGTTTTTATTTTCAGTTCTCCGTCCTTTTTTCAGTCTTGAGCCCAGGGAGCGTCCATGTTTGTTCAGATTCTAGGTTCCGCCGC
                                          RBS
 11 G  G  G  F  P  Q  W  N  C  N  C  V  N  C  A  G  F  R  D  G  S  L  R  A  Q  A  R  T  Q  S  S  I  A
3401 CGGCGGTGGTTTTCCGCAGTGGAACTGCAACTGTGTGAACTGCGCCGGATTTCGCGACGGTAGCCTGCGGGCCCAGGCGCGCACTCAATCGTCCATCGCG
 44 I  S  D  D  G  N  W  V  L  C  N  A  S  P  D  I  R  A  Q  L  Q  S  F  A  P  M  Q  P  G  R  A  L  R
3501 ATTTCCGATGACGGGGTGAACTGGGTGCTGTGCAACGCCTCTCCGGATATCCGCGCGCAGTTGCAAAGCTTCGCCCCCATGCAACCGGGCCGCGCACTGC
                                          EcoRV              HindIII
 78 D  T  G  I  G  A  I  I  L  M  D  S  Q  I  D  H  T  T  G  L  L  S  L  R  E  G  C  P  H  Q  V  W  C
3601 GGGACACCGGCATCGGCGCCATCATCCTCATGGACAGCCAGATCGACCACACCACCGGCCTGCTCAGCCTGCGTGAAGGCTGCCCGCACCAGGTCTGGTG
111 T  D  M  V  H  E  D  L  S  T  G  F  P  L  F  K  M  L  S  H  W  N  G  G  L  S  W  N  R  I  E  L  D
3701 CACCGACATGGTCCATGAAGACCTGAGCACCGGTTTCCCGCTGTTCAAGATGCTCAGCCACTGGAACGGCGGCCTGAGCTGGAACCGCATCGAGCTGGAC
144 Q  S  F  S  I  P  A  C  P  N  L  R  F  T  P  L  P  L  R  S  A  A  P  P  Y  S  P  H  R  F  D  P  H  P
3801 CAGAGCTTCAGCATCCCCGCCTGCCCCAACCTGCGCTTCACGCCGCTGCCCCTGCGCAGCGCAGCGCCGCCCTATTCGCCCCACCGTTTCGACCCGCACC
178 G  D  N  I  G  L  I  V  E  D  L  R  S  G  G  K  L  F  Y  A  P  G  L  G  K  V  D  A  P  L  L  E  I
3901 CGGGGGACAACATCGGCCTGATCGTCGAAGACCTGCGCAGCGGCGGCAAACTCTTCTACGCGCCGGGCCTGGGCAAGGTCGACGCGCCACTCCTGGAGAT
211 M  A  A  S  D  V  L  L  V  D  G  T  L  W  E  D  D  E  M  Q  R  R  G  V  G  T  R  T  G  R  E  M  G
4010 CATGGCCGCCAGCGATGTGCTGCTGGTGGACGGCCACCCTGTGGGAAGACGATGAAATGCAGCGTCGTGGCGTGGGCACCCGTACCGGCCGGGAAATGGGC
244 H  L  A  Q  N  G  P  G  G  M  L  E  V  L  E  Q  L  P  R  Q  R  K  V  L  I  H  I  N  N  T  N  P  I  L
4101 CACCTGGCGCAGAACGGCCCCGGCGGCATGCTCGAAGTGCTGGAACAGTTGCCCCGCCAGCGCAAGGTGCTGATACATATCAACAACACCAACCCGATCC
278 D  E  D  S  A  E  R  A  E  L  V  R  R  K  V  E  V  A  Y  D  G  M  S  I  E  L  *
4201 TCGATGAAGACTCCGCCGAACGTGCCGAACTGGTACGCCGAAAGGTGGAAGTGGCTTACGACGGAATGAGTATTGAGCTGTAGGGCGAATCGCCCACAAG
                                          pqqC ⇒  M  T  D  T  P  L  S  A  A
4301 CTCGCCCCACGAGCCTTGGCCCCTTTGCGTAGGAGCGGGCTTGCCCGCGAAAGGGCTGCCCCCCGGAGACACACATGACCGACACCCCGCTGTCCGCCGCG
                                          RBS
 10 E  F
4401 CCGAATTC
    EcoRI
```

FIG. 3. Nucleotide sequence of the *pqqFAB* cluster of *P. fluorescens* and deduced amino acid sequences. The 4,408-bp *Bgl*II-*Eco*RI fragment represents the insert in pME3141 (Fig. 1A). The putative ribosome binding sites (RBS) are underlined, and termination codons are designated with an asterisk. The insulin-degrading protease motif is indicated with plus signs. The site of the Tn*5* insertion in strain CHA625 is indicated by a box representing the nine base pairs that were duplicated upon Tn*5* insertion.
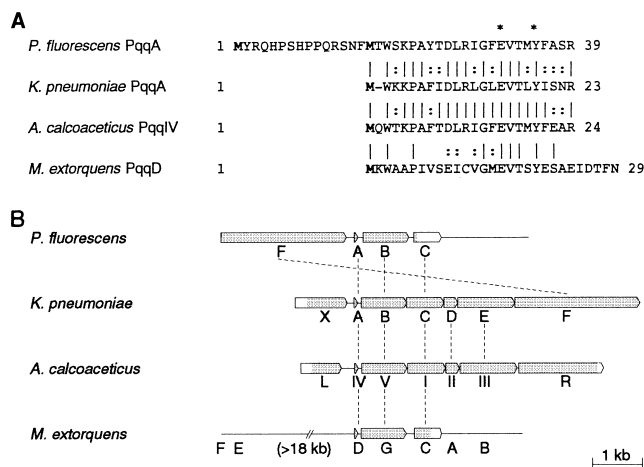
FIG. 4. (A) Alignment of the predicted PqqA amino acid sequence of *P. fluorescens* CHA0 with those of the corresponding proteins of *K. pneumoniae*, *A. calcoaceticus*, and *M. extorquens*. Two N-terminal methionine residues (1 or 16) seem possible in PqqA of *P. fluorescens* (Fig. 3). Identical amino acids are connected with solid lines, and similar amino acids are connected with dotted lines. The conserved essential glutamate (E) and tyrosine (Y) residues are designated with asterisks. (B) Alignment of the *pqq* gene clusters from *P. fluorescens*, *K. pneumoniae*, *A. calcoaceticus*, and *M. extorquens*. Sequenced parts of ORFs are shown by stippling. Letters and Roman numerals refer to *pqq* genes. The dashed lines designate equivalent genes (19, 43, 45).

(CHA120, CHA178, CHA177, and CHA179) showed Plt synthesis on malt agar, on which the wild-type CHA0 did not synthesize this antibiotic (Table 1). However, all *pqq* mutants except the original strain CHA625 produced wild-type levels of Phl on malt agar (Table 1). This suggests that strain CHA625 carries at least one spontaneous mutation affecting Phl synthesis, in addition to the *pqqF*::Tn5 mutation.

It is known that spontaneous mutations affecting secondary metabolism arise at high frequencies when strain CHA0 is incubated in rich media, and the level of activity of TSO can be used as a cytochemical marker for this genetic instability (49). All derivatives of strain CHA0 constructed here showed wild-type TSO activity on King's medium B. The exception was strain CHA625, which appeared to have a low level of TSO activity, suggesting that this mutant had undergone a secondary change(s).

To test complementation of the *pqq* mutants, we constructed two new plasmids, pME3186 and pME3188 (Fig. 2). Plasmid pME3186 contains the 3.0-kb *Eco*RV-*Hin*dIII fragment of plasmid pME3141 carrying *pqqFA* in the vector pVK100. Plasmid pME3188 is similar but in addition contains the 3.6-kb *Hin*dIII-*Bam*HI fragment of pME3122K, which includes *pqqBC* and possibly additional *pqq* genes downstream of *pqqC* (Fig. 2). The *pqq* mutants CHA625, CHA120, CHA177, CHA178, and CHA179 were all complemented by pME3188 in that this plasmid reduced Plt production on malt agar to undetectable levels (Table 1). This suggests that pME3188 carries the entire *pqq* cluster of *P. fluorescens*. The Phl levels in the complemented mutants were unaffected or slightly elevated compared with the levels in the mutants (Table 1). Plasmid pME3186 (*pqqFA*⁺) complemented the *pqqF* mutants CHA625, CHA120, and CHA178 but had no effect on the other *pqq* mutants CHA177 and CHA179 (Table 1), as expected from the locations of the Ω-Km insertions (Fig. 2). The extent of the *pqq* cluster of *P. fluorescens* CHA0 was estimated to be ≤6 kb, as deduced from strains CHA121 and CHA181 (Fig. 2), which each carry an insertion mutation not affecting PQQ synthesis (Table 1).

We have previously observed that pME3128 (*pqqFA*⁺) partially complements strain CHA625 for Phl production (28). Similarly, both plasmids pME3186 and pME3188 partially restored Phl production in strain CHA625 (Table 1), suggesting that under certain conditions, the availability of PQQ can also have an impact on Phl synthesis. However, the mechanism by which this partial restoration occurs is not clear.

Further characterization of the *pqq* mutants: role of PQQ in glucose and ethanol catabolism of *P. fluorescens* CHA0. PQQ is an essential cofactor of GDH and alcohol dehydrogenases in a variety of gram-negative bacteria (17, 32, 64). GDH-deficient mutants of pseudomonads can be distinguished from the wild-type strains on EMB plates containing glucose (37). The conversion of glucose to gluconate by the periplasmic enzyme GDH lowers the pH. Colonies of the wild-type CHA0 and of strains CHA121 and CHA181 (Fig. 2) appeared dark purple with a bright green sheen, whereas the colonies of the *pqq* mutants CHA625, CHA120, CHA177, CHA178, and CHA179 were all pink (Table 1). This phenotype correlated with GDH activities in crude membrane fractions from cells grown on gluconate medium. The levels of GDH activity of the wild type and of strain CHA181 were low but detectable, whereas no GDH activity could be measured in membrane fractions of the mutants CHA625, CHA120, CHA177, CHA178, and CHA179 (Table 1). The addition of PQQ (16 nM) to EMB-glucose plates restored the wild-type colony appearance. In the GDH-negative mutants glucose can still serve as a carbon source, after intracellular conversion to glucose-6-phosphate (37).

In *P. aeruginosa*, an NADP-dependent and a PQQ-dependent ethanol dehydrogenase occur simultaneously (20). In *P. fluorescens* CHA0 ethanol appears to be oxidized only via a PQQ-dependent enzyme(s), since none of the PQQ-deficient mutants was able to utilize 0.5% (vol/vol) ethanol as the sole carbon source (Table 1). Methanol dehydrogenase (potentially another PQQ-dependent enzyme) could not be detected in *P. fluorescens*; the wild-type CHA0 did not grow in minimal medium containing methanol (0.5% vol/vol) instead of ethanol (data not shown).

The recombinant plasmid pME3188 (*pqqFABC*⁺) complemented all *pqq* mutants for growth on ethanol, acid production on EMB-glucose, and GDH activity (Table 1). Plasmid pME3186 (*pqqFA*⁺) complemented the *pqqF* mutants but not the other mutants for the same phenotypes (Table 1).

The growth of strain CHA120 in ethanol medium was fully restored after the addition of 16 nM PQQ. Smaller amounts of PQQ resulted in reduced growth on ethanol. The growth curve of the *pqq* mutant CHA120 supplemented with 16 nM PQQ was identical to the curves obtained with the wild-type CHA0 or with the complemented strain CHA120/pME3188 without added PQQ (data not shown). The mutant CHA120 produced 2.8 µg of Plt per ml on malt agar (Table 1). The addition of 16 nM PQQ reduced Plt production to the wild-type level (≤0.1 µg/ml).

In conclusion, a PPQ-negative phenotype strictly correlates with enhanced Plt production.

DISCUSSION

A two-step, Tn5-dependent cloning strategy has been developed by Voisard et al. (67) to isolate wild-type genes complementing Tn5 insertion mutations in gram-negative bacteria. The feasibility and the practical aspects of the method are illustrated here by the isolation of the *pqq* gene cluster from strain CHA0 (Fig. 1A). The first step of the cloning procedure yields a piece of genomic DNA flanking a Tn5 insertion. Several other methods exist that achieve the same goal (55). The

second step of the cloning procedure yields the intact wild-type gene(s), owing to the special features of the suicide plasmid used, pME3049 (Fig. 1B). This plasmid contains several restriction sites (e.g., *Bam*HI, *Sac*I, *Sal*I, and *Xba*I) within a polylinker, in addition to the two sites (*Eco*RI and *Kpn*I) used in this study. All these restriction sites can be used to carry out the first cloning step (Fig. 1A). The second cloning step has to be performed with *Bgl*II (as shown in Fig. 1A) or with another restriction enzyme that cuts IS*50* but not the ColE1 moiety or the kanamycin resistance gene: *Bgl*I, *Hin*dIII, *Hpa*I, or *Xho*I. The appropriate restriction enzymes for both cloning steps can be chosen after an analysis of the restriction sites flanking the chromosomal Tn*5* insertion by Southern blotting with a Tn*5* probe. In this study, we have applied the two-step method to extend and further characterize a genomic segment (28) involved in the regulation of antibiotic biosynthesis in *P. fluorescens*.

Little is known about the biosynthetic pathway of Plt and its regulation. Proline and acetyl-coenzyme A are assumed to be precursors of this antibiotic (9), and both the presence of glycerol (34) and poor aeration (4) have stimulative effects in one Plt producer strain, Pf-5, of *P. fluorescens*. Strain CHA0 synthesizes Plt in the rhizosphere (41) and on complex media such as King's medium B, which contains glycerol as the major carbon source. However, we have yet to find a defined synthetic medium that supports the antibiotic production of strain CHA0. For instance, the sucrose-asparagine minimal medium used for *Pseudomonas* sp. F113 (59) does not stimulate strain CHA0 to produce Phl or Plt.

Mutations that enable strain CHA0 to produce Plt on malt agar or to overproduce Plt on King's medium B have now been localized to a gene cluster required for PQQ synthesis, in particular to the *pqqF* gene (Fig. 2). This is a novel phenotype of *pqq* mutants. Two functions of PQQ previously recognized in pseudomonads—cofactor activity in alcohol and GDHs (17, 64, 65)—can also be seen in *P. fluorescens* CHA0 (Table 1). Since glucose is known to have a negative effect on Plt production in *P. fluorescens* Pf-5 (34), it is tempting to speculate that this effect might involve the GDH reaction. There is a precedent for a GDH involvement in antibiotic biosynthesis in *P. fluorescens*: in strain HV37a glucose stimulates the synthesis of oomycin and mutants deficient in GDH activity are also deficient in oomycin synthesis (21, 27). Therefore, James and Gutterson (27) have postulated that glucose and gluconate might have an important role in the regulation of antibiotic biosynthesis in *P. fluorescens*. In strain CHA0, we have shown that a lack of PQQ causes both GDH deficiency and Plt overproduction. How these phenotypes are connected is not known. One possibility is that the lack of PQQ might enhance the flux of precursors (acetyl-coenzyme A?) into the Plt pathway, but in the absence of detailed biochemical and genetic information this hypothesis is difficult to test.

The arrangement of the *pqqABC* genes of *P. fluorescens* appears to be the same as that of *K. pneumoniae*, *A. calcoaceticus*, and *M. extorquens*, whereas the relative location of the *pqqF* gene differs in *P. fluorescens* and *K. pneumoniae* (Fig. 4B). Sequence analysis suggests that the PqqF proteins of these two organisms are related to a family of proteases (43) which require divalent cations for their activity (53). Members of this family include protease III of *E. coli* (which has 27% identity with PqqF of *P. fluorescens*) (14) and a human insulin-degrading enzyme (1) (which has 24.7% identity with PqqF). Sequence conservation is strongest in a 23-amino-acid stretch corresponding to residues 46 to 69 in PqqF of *P. fluorescens* (Fig. 3), with a His-X-X-X-Glu-His motif (positions 62 to 66). In protease III of *E. coli*, the two histidine residues binding

Zn^{2+} and glutamate are necessary for catalytic activity (3). It has been speculated that PqqF might be involved in releasing PQQ (or an intermediate of PQQ biosynthesis) from a peptide precursor (43). Glutamate and tyrosine are building blocks for PQQ (25, 26), and in the small PqqIV polypeptide of *A. calcoaceticus* the glutamate and tyrosine residues (Fig. 4A) are essential for PQQ synthesis (19). The PqqA polypeptide of *P. fluorescens*, like its homologs PqqIV of *A. calcoaceticus*, PqqA of *K. pneumoniae*, and PqqD of *M. extorquens*, contains these two essential residues, which are present in a conserved motif, Glu*-Val-Thr-X-Tyr* (Fig. 4A). Thus, the possibility that the PqqA polypeptide might be the PQQ precursor (43) is further supported by the sequence of the *P. fluorescens ppqA* gene.

The cloning strategy described in this work should prove generally useful when genes have been tagged with Tn*5* in a gram-negative bacterium and when a genomic library of the wild-type organism is either incomplete or unavailable.

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