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Identification and Characterization of Genes for a Second Anthranilate Synthase in *Pseudomonas aeruginosa*: Interchangeability of the Two Anthranilate Synthases and Evolutionary Implications

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Two anthranilate synthase gene pairs have been identified in *Pseudomonas aeruginosa*. They were cloned, sequenced, inactivated in vitro by insertion of an antibiotic resistance gene, and returned to *P. aeruginosa*, replacing the wild-type gene. One anthranilate synthase enzyme participates in tryptophan synthesis; its genes are designated *trpE* and *trpG*. The other anthranilate synthase enzyme, encoded by *phnA* and *phnB*, participates in the synthesis of pyocyanin, the characteristic phenazine pigment of the organism. *trpE* and *trpG* are independently transcribed; homologous genes have been cloned from *Pseudomonas putida*. The phenazine pathway genes *phnA* and *phnB* are cotranscribed. The cloned *phnA phnB* gene pair complements *trpE* and *trpE(G)* mutants of *Escherichia coli*. Homologous genes were not found in *P. putida* PPG1, a non-phenazine producer. Surprisingly, PhnA and PhnB are more closely related to *E. coli* TrpE and TrpG than to *Pseudomonas* TrpE and TrpG, whereas *Pseudomonas* TrpE and TrpG are more closely related to E. coli PabB and PabA than to *E. coli* TrpE and TrpG. We replaced the wild-type *trpE* on the *P. aeruginosa* chromosome with a mutant form having a considerable portion of its coding sequence deleted and replaced by a tetracycline resistance gene cassette. This resulted in tryptophan auxotrophy; however, spontaneous tryptophan-independent revertants appeared at a frequency of 10^{-5} to 10^{-6}. The anthranilate synthase of these revertants is not feedback inhibited by tryptophan, suggesting that it arises from PhnAB. *phnA* mutants retain a low level of pyocyanin production. Introduction of an inactivated *trpE* gene into a *phnA* mutant abolished residual pyocyanin production, suggesting that the *trpE trpG* gene products are capable of providing some anthranilate for pyocyanin synthesis.

We have found that *Pseudomonas aeruginosa* has two anthranilate synthase enzymes; they are homologous, and both have conventional α and β subunits (9b). The two anthranilate synthases have different functions. One participates in tryptophan synthesis; its genes have been designated *trpE* and *trpG* (9b). These genes are quite similar to those of *Pseudomonas putida* (9a). In both pseudomonads, *trpG*, which encodes the β subunit, is cotranscribed with *trpD* and *trpC* in a three-gene operon.

The second anthranilate synthase, previously misidentified as the product of a *trp* gene pair, was obtained on a R-prime plasmid by mating *P. aeruginosa* PAC174 carrying R68.44 with *Escherichia coli* W3110 *tna* Δ*trpE5* (8). This R-prime plasmid complements only those *E. coli* auxotrophs blocked in anthranilate synthase, the first enzyme in the tryptophan synthetic pathway. Enzyme assays indicated that both the α and β subunits of anthranilate synthase were produced. The anthranilate synthase-encoding segment of the R-prime plasmid was subcloned into pBR322 (8). DNA sequencing of one of these subclones, pIA14, showed that the genes for the α and β anthranilate synthase subunits were indeed present on the cloned DNA. These genes are adjacent, and their coding sequences overlap by 23 base pairs (bp) (7). The deduced amino acid sequence for this *P. aeruginosa* small (β, glutamine amidotransferase) subunit differed to a surprising extent from the directly determined amino acid sequence of the glutamine amidotransferase subunit of *P. putida* anthranilate synthase (14). We found that insertional inactivation of the chromosomal version of the cloned β-subunit gene in *P. aeruginosa* did not result in a requirement for tryptophan in low-ammonium medium but instead resulted in cells defective in pyocyanin production. The genes encoding this second anthranilate synthase in *P. aeruginosa* have been designated *phnA* (α subunit) and *phnB* (β subunit) for their role in phenazine synthesis, specifically pyocyanin production in *P. aeruginosa*.

Pyocyanin (Fig. 1) is the characteristic blue-green phenazine pigment produced by *P. aeruginosa*. Pyocyanin is the most thoroughly studied of the phenazine pigments (13, 33). Phenazines are classified as secondary metabolites, i.e., compounds formed during the stationary phase and often having antibiotic properties. Shikimic acid and chorismate have been established as precursors for the phenazines in all cases investigated (33). The hypothetical scheme for pyocyanin biosynthesis (18) is represented in Fig. 1. In this pathway, phenazine-1,6-dicarboxylate is the common precursor of the phenazines. Until now, the identity of the intermediate between chorismate and phenazine-1,6-dicarboxylate has been uncertain; our evidence indicated that this intermediate is anthranilate and that the hypothetical first step is catalyzed by anthranilate synthase.

In this report, the cloning and sequencing of *phnA* and *phnB* from *P. aeruginosa* PAO1 are presented, as well as the

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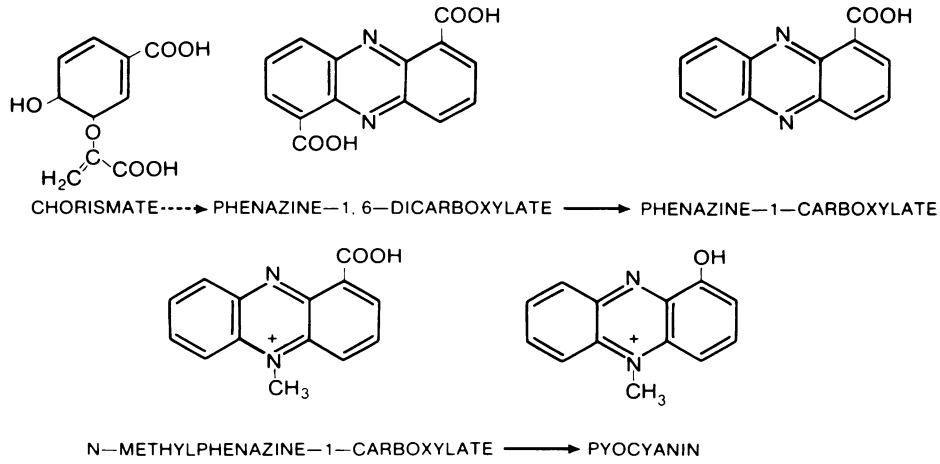

FIG. 1. Hypothetical scheme for pyocyanin biosynthesis in *P. aeruginosa*. In the synthesis of pyocyanin, phenazine-1,6-dicarboxylate is decarboxylated to yield phenazine-1-carboxylate. N-methylation of phenazine-1-carboxylate yields 5-methylphenazine-1-carboxylate. Oxidative decarboxylation of 5-methylphenazine-1-carboxylate yields pyocyanin (1-hydroxy-5-methylphenazine).

completion of the sequencing of the *P. aeruginosa* PAC174 *phnA* and *phnB* genes. Experiments that implicate this anthranilate synthase in the production of pyocyanin are presented. Evidence is also presented to support the interchangeability of the two *P. aeruginosa* anthranilate synthases.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used are listed in Table 1. Plasmids are listed in Table 2.

Media and antibiotics. Complete medium was Luria broth (LB) (24), and minimal medium was Vogel-Bonner minimal salts medium E (35). For solid LB or Vogel-Bonner minimal medium, 15 g of agar (Difco Laboratories, Detroit, Mich.) was added per liter. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates were used to screen recombinant pUC plasmid derivatives (34, 36). Pseudomonas isolation agar was obtained from Difco. Pseudomonas isolation agar was used to select against *E. coli* donors in bacterial matings with *Pseudomonas* species and to examine pyocyanin production by *P. aeruginosa*. Pseudomonas broth (PB) is a medium developed in this laboratory to maximize pyocyanin production in liquid culture. The composition of PB is 20 g of Bacto-Peptone (Difco), 1.4 g of $MgCl_2$, and 10 g of K_2SO_4 per liter of distilled water.

Antibiotic concentrations used for *E. coli* were ampicillin at 100 μg/ml, chloramphenicol at 25 μg/ml, kanamycin at 50 μg/ml, tetracycline at 25 μg/ml, mercuric chloride at 15 μg/ml, and cefazolin at 100 μg/ml; for *P. aeruginosa* PAO4290, kanamycin was used at 250 μg/ml; for *P. aeruginosa* PAO1, mercuric chloride was used at 15 μg/ml and tetracycline was used at 100 μg/ml.

DNA isolation, Southern hybridization, and colony hybridizations. Procedures for DNA isolation and Southern hybridization were as previously described (9b). Colony hybridization (12) was used to identify recombinant plasmids carrying the DNA sequences of interest. Stringency washes were the same as for Southern hybridizations.

DNA sequencing. Fragments were usually labeled with ^{32}P by 3′ fill-in labeling with the large fragment of *E. coli* DNA polymerase I and the appropriate α-^{32}P-labeled radioactive deoxynucleotide triphosphate (4) or by 3′ end labeling with terminal transferase and [2′,3′-α-^{32}P]ddATP (37). Occasionally, the 5′ ends were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and then reduced by restriction enzyme cleavage to smaller fragments labeled at one end (23). Labeled fragments were electroeluted from polyacrylamide gels and sequenced by the method of Maxam and Gilbert (23); reaction mixtures were developed by 8% urea–polyacrylamide gel electrophoresis by the method of Sanger and Coulson (25), except the gels contained 25% formamide to minimize compressions. Sequence data were analyzed with the aid of the PCS computer program (17).

Low-melting-temperature agarose ligation. Several of the subcloning experiments were done with DNA fragments isolated from low-melting-temperature agarose gels (31) and as described by Bae et al. (2).

TABLE 1. Bacterial strains

Strain	Genotype or phenotype[a]	Source or reference
Pseudomonas aeruginosa		
PAO1	Wild type	B. Holloway
PAO4290	*aphA argF leu-10*	H. Matsumoto
PADE E1	TrpE$^-$ Tc^r	9b
PADE G2	TrpGDC$^-$ Hg^r	9b
PADE B1	PhnB$^-$ Km^r exconjugant of PAO4290 with pDE1443	This study
PADE A47	PhnA$^-$ Tc^r exconjugant of PAO1 with pDE1560	This study
PADE A47E1	PhnA$^-$ TrpE$^-$ Tc^r Hg^r exconjugant of PADE A47 with pDE1567	This study
PADE A48	PhnA$^-$ Hg^r exconjugant of PADE A47 with pDE1595	This study
PADE E1r	Trp$^+$ revertant of PADE E1	This study
Escherichia coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 rel* λ$^-$ Δ(*lac-proAB*)(F′ *traD36 proAB lacI^{q}ZΔM15*)	36
S17-1	[RP4-2 (Tc::Mu)(Km::Tn*7*) Tra (IncP)] *pro hsdR recA* Tp^r Sm^r	A. Puhler (28)

[a] Abbreviations: Hg, mercuric chloride; Km, kanamycin; Sm, streptomycin; Tc, tetracycline; Tp, trimethoprim.

TABLE 2. Plasmids

Plasmid	Relevant characteristics[a]	Source or reference
pUC8	Amp^r, cloning vector	34
pUC9	Amp^r, cloning vector	34
pUC12	Amp^r, cloning vector	34
pUC13	Amp^r, cloning vector	34
pUC18	Amp^r, cloning vector	36
pUC18-*Xho*I	Amp^r, pUC18 with a *Xho*I adaptor inserted into the *Eco*RI site	9b
pUC19	Amp^r, cloning vector	36
pDG106	Hg^r Km^r	B. D. Gambill (10)
pDE-Km	Amp^r Km^r, 2.5-kb *Xho*I-Km^r fragment from Tn*5* inserted into the *Xho*I site of pUC18-*Xho*I, high-copy-number source of the *Xho*I-Km^r cassette	9b
pDE-Tc	Amp^r Tc^r, 2.7-kb *Bgl*II-Tc^r fragment from Tn*5-132* inserted into pUC18-*Bgl*II, high-copy-number source of the *Bgl*II-Tc^r cassette	9b
pDE-Hg	Amp^r Hg^r 5.2-kb *Bam*HI-*Pst*I Hg^r fragment from pDG106 inserted into pUC18-*Xho*I	9b
pDE-HgB	Amp^r Hg^r, pDE-Hg digested with *Hin*dIII, Klenow treated, and a *Bam*HI linker inserted, high-copy-number source of the *Bam*HI-Hg^r cassette	This study
pSUP205	Cm^r Tc^r Mob, mobilizable vector	A. Puhler (29)
pSUP205-*Xho*I	Cm^r Mob, pSUP205 with a *Xho*I adaptor inserted into the *Bam*HI site	This study
pIA10	Tc^r, *P. aeruginosa* PAC174 *phnAB*	8
pIA20	Amp^r, *P. aeruginosa phnB*, subclone of pIA10, 1.2-kb *Kpn*I fragment in pUC18	7
pIA14	Amp^r, *P. aeruginosa phnB*, subclone of PIA10, 1.2-kb *Bam*HI-*Bgl*II fragment in pBR322	8
p1256	Amp^r, subclone of pIA10, 1,053-bp *Pst*I-*Eco*RI *phnA* fragment inserted into pUC13	This study
p1257	Amp^r, subclone of pIA10, 827-bp *Bam*HI-*Eco*RI *phnA* fragment inserted into pUC12	This study
p1433	Amp^r, subclone of pIA10, 1,161-bp *Pst*I *phnA* fragment inserted into pUC8	This study
p1460	Amp^r, *P. aeruginosa trpE*	9b
pDE1530	Cm^r Mob, *P. aeruginosa trpE-Bgl*II, 3.0-kb *Hin*dIII-*Sma*I *trpE-Bgl*II fragment from pDE1528 inserted into *Hin*dIII- and *Eco*RV-digested pSUP205	9b
pDE1567	Cm^r Hg^r Mob, *P. aeruginosa trpE*-Hg, pDE1530 digested with *Bgl*II and the *Bam*HI-Hg^r cassette inserted	This study
p1326	Amp^r, 965-bp *Pst*I-*Cla*I fragment containing the 5′ end of *P. aeruginosa* PAO1 *phnA* and approximately 400 bp of upstream DNA inserted into pUC18 digested with *Pst*I and *Acc*I	This study
p1514	Amp^r, 4.0-kb *Kpn*I-*Eco*RI fragment containing the 5′ end of *P. aeruginosa* PAO1 *phnA* and approximately 3.5 kb of upstream DNA inserted into pUC19	This study
p1580	Amp^r, 2.0-kb *Eco*RI-*Bgl*II fragment containing most of *P. aeruginosa* PAO1 *phnA*, all of *phnB*, and 600 bp of downstream DNA inserted into pUC19	This study
p1515	Amp^r, 2.0-kb *Eco*RI-*Bgl*II fragment from pIA10 inserted into *Eco*RI- and *Bam*HI-digested pUC19, similar in construction to p1580	This study
pIA20-*Xho*I	Amp^r, *P. aeruginosa phnB-Xho*I, pIA20 digested with *Sac*II and a *Sac*II-*Xho*I adaptor inserted into *phnB*	This study
pIA1006	Tc^r, pIA10 digested with *Kpn*I, the 1.2-kb *Kpn*I fragment removed and replaced with the 1.2-kb *Kpn*I fragment from *Kpn*I-digested pIA20-*Xho*I	This study
pDE1412	Amp^r, 3.7-kb *Eco*RI *phnB-Xho*I fragment from pIA1006 inserted into *Eco*RI-digested pUC9	This study
pDE1413	Amp^r, *P. aeruginosa phnB*-Km, pDE1412 digested with *Xho*I and the *Xho*I-Km^r cassette from pDE-Km inserted	This study
pDE1443	Tc^r Km^r Mob, *P. aeruginosa phnB*-Km, 6.2-kb *Eco*RI *phnB*-Km fragment inserted into *Eco*RI-digested pSUP205	This study
pDE1518	Amp^r, *P. aeruginosa phnAB*, 4.0-kb *Kpn*I-*Eco*RI fragment from p1514 and the 2.0-kb *Eco*RI-*Hin*dIII fragment from p1515 ligated into pUC18 digested with *Kpn*I and *Hin*dIII	This study
pDE1523	Amp^r, *P. aeruginosa phnAB*, 6.0-kb *Sac*I-*Hin*dIII *phnAB* fragment from pDE1518 inserted into pUC18-*Xho*I digested with *Sac*I and *Hin*dIII	This study
pDE1529	Amp^r, *P. aeruginosa phnA-Bgl*II, pDE1523 digested with *Eco*RI-*Bgl*II adaptor inserted into *phnA*	This study
pDE1559	Cm^r Mob, *P. aeruginosa phnAB*, 6.0-kb *Hin*dIII-*Xho*I *phnAB* fragment from pDE1523 inserted into pSUP205-*Xho*I digested with *Xho*I and *Hin*dIII	This study
pDE1560	Cm^r Tc^r Mob, *P. aeruginosa phnA*-Tc, pDE1559 digested with *Bam*HI and the *Bgl*II-Tc^r cassette from pDE-Tc inserted near the 3′ end of *phnA*	This study
pDE1585	Amp^r Hg^r, *P. aeruginosa* Δ*phnA*-Hg, pDE1529 digested with *Bgl*II and *Bam*HI, the 830-bp fragment internal to *phnA* removed, and the *Bam*HI-Hg^r cassette from pDE-Hg inserted	This study
pDE1595	Cm^r Hg^r Mob, *P. aeruginosa* Δ*phnA*-Hg, 10.4-kb *Xho*I-*Hin*dIII Δ*phnA*-Hg-containing fragment from pDE1585 inserted into *Xho*I- and *Hin*dIII-digested pSUP205-*Xho*I	This study

[a] Abbreviations: Amp, ampicillin; Cm, chloramphenicol; Hg, mercuric chloride; Km, kanamycin; Tc, tetracycline.

Construction of *Bam*HI-Hg^r cassette. The plasmid pDE-Hg (9b) was digested with *Hin*dIII and treated with the large fragment of DNA polymerase I and the appropriate deoxyribonucleotides, and a *Bam*HI (CGGGATCCCG) linker was inserted; this plasmid is a high-copy-number source of the *Bam*HI-Hg^r cassette and is designated pDE-HgB.

Construction of pDE1433. The construction of pDE1433 used to create a PhnB$^-$ Km^r *P. aeruginosa* mutant is diagrammed in Fig. 2. pIA20 was digested with *Sac*II, and a *Sac*II-*Xho*I adapter (CCTCGAGGGC) was inserted into *phnB* to yield pIA20-*Xho*I. pIA10 was then digested with *Kpn*I, and the 1.2-kilobase (kb) *Kpn*I fragment was removed and replaced with the 1.2-kb *Kpn*I fragment purified from *Kpn*I-digested pIA20-*Xho*I to yield pIA1006. pIA1006 was digested with *Eco*RI, and the purified 3.7-kb *Eco*RI fragment was inserted into *Eco*RI-digested pUC9 to create pDE1412. pDE1412 was then digested with *Xho*I, and the 2.5-kb *Xho*I-Km^r cassette from pDE-Km was inserted to yield the Amp^r Km^r plasmid pDE1413. pDE1413 was subsequently digested with *Eco*RI, and the purified 6.2-kb fragment was inserted into *Eco*RI-digested pSUP205 to create the Km^r Tc^r Mob plasmid pDE1443.

Construction of pDE1560. The construction of the Cm^r Tc^r Mob plasmid pDE1560 used to create a PhnA$^-$ Tc^r *P. aeruginosa* mutant is illustrated in Fig. 3. p1514 was digested with *Kpn*I and *Eco*RI, and the 4.0-kb fragment was isolated and purified. p1515 (the 2.0-kb *Eco*RI-*Bgl*II fragment from pIA10 inserted into *Eco*RI- and *Bam*HI-digested pUC19, similar in construction to p1580) was digested with *Hin*dIII and *Eco*RI, and the 2.0-kb fragment was isolated and purified. The 4.0-kb *Kpn*I-*Eco*RI fragment from p1514 and the 2.0-kb *Eco*RI-*Hin*dIII fragment from p1515 were then ligated into pUC18 that had been digested with *Kpn*I and *Hin*dIII to create pDE1518. pDE1518 was digested with *Sac*I and *Hin*dIII, and the 6.0-kb *phnA phnB* fragment was inserted into pUC18-*Xho*I digested with *Sac*I and *Hin*dIII to yield pDE1523. pDE1523 was then digested with *Xho*I and *Hin*dIII, and the 6.0-kb *phnA phnB* fragment was inserted into *Hin*dIII- and *Xho*I-digested pSUP205-*Xho*I (pSUP205 digested with *Bam*HI and a *Bam*HI-XhoI adaptor [GATCT CGA] inserted by ligation) to yield the Cm^r Mob plasmid pDE1559. pDE1559 was subsequently digested with *Bam*HI, and the 2.7-kb *Bgl*II-Tc^r cassette from pDE-Tc was inserted into the *phnA* gene to create the Cm^r Tc^r Mob plasmid pDE1560.

Construction of pDE1567. The plasmid pDE1567 was used to create a *trpE* mutant of *P. aeruginosa* PADE A47. The construction of pDE1567 is outlined in Fig. 4. pDE1530 (9b) was digested with *Bgl*II, and the 5.2-kb *Bam*HI-Hg^r cassette from pDE-HgB was inserted to yield the Cm^r Hg^r Mob plasmid pDE1567.

Construction of pDE1595. The plasmid pDE1595 was used to create the Δ*phnA* Hg^r derivative of PADE A47. The construction of pDE1595 is outlined in Fig. 5. pDE1523 was digested with *Eco*RI, and an *Eco*RI-*Bgl*II adaptor (AATTA GATCT) was inserted to yield pDE1529. pDE1529 was subsequently digested with *Bgl*II and *Bam*HI to remove an 830-bp fragment internal to *phnA*, and the 5.2-kb *Bam*HI Hg^r cassette from pDE-HgB was inserted to yield the Amp^r Hg^r plasmid pDE1585. pDE1585 was then digested with *Xho*I and *Hin*dIII, and the 10.4-kb fragment was inserted into *Xho*I- and *Hin*dIII-digested pSUP205-*Xho*I to create the Cm^r Hg^r Mob plasmid pDE1595.

Transformation and conjugation. *E. coli* transformations and bacterial matings were done as previously described (9b). We used the method of Simon et al. (28) for the

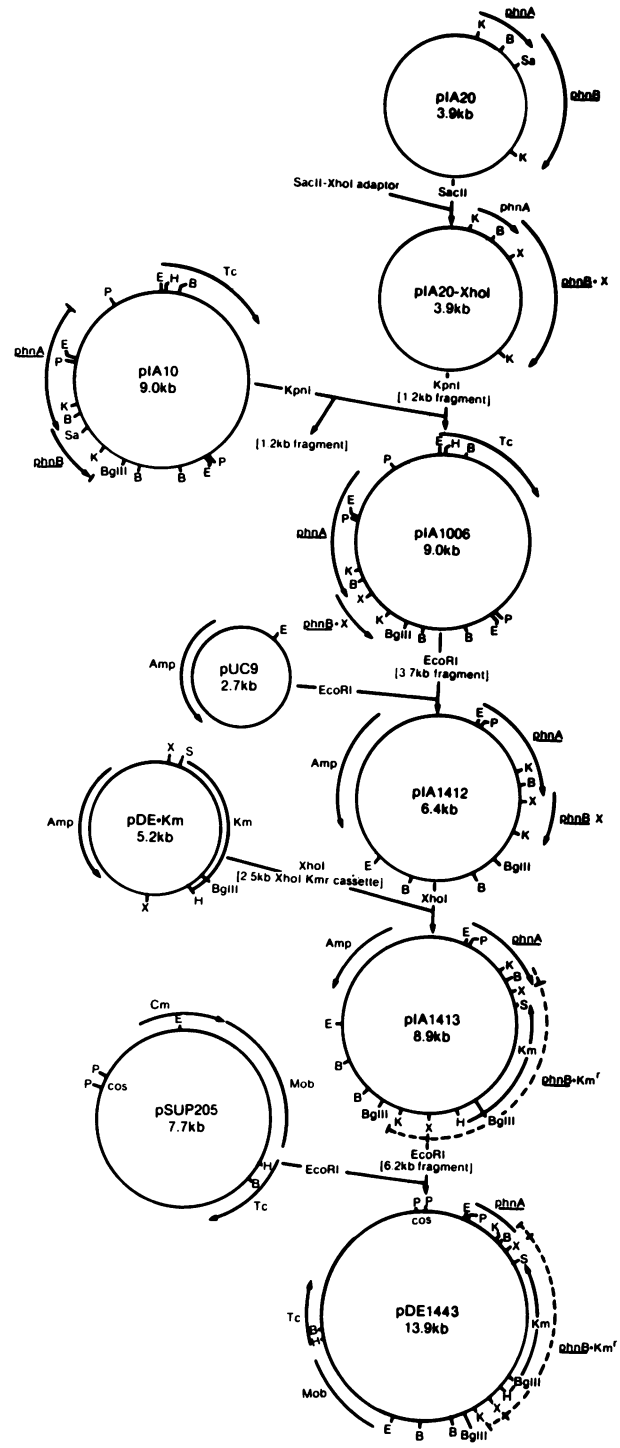

FIG. 2. Construction of pDE1443. The following letters are used to denote different restriction enzyme sites on the maps: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sa, *Sac*II; and X, *Xho*I.

construction of *P. aeruginosa* gene replacement mutants. The insertionally inactivated gene is cloned into a pSUP plasmid vector (28,29) that carries selectable antibiotic resistances and the RP-4-*mob* site. The RP-4 transfer functions are integrated into the chromosome of the mobilizing *E. coli* donor strain, S17-1. These integrated transfer functions

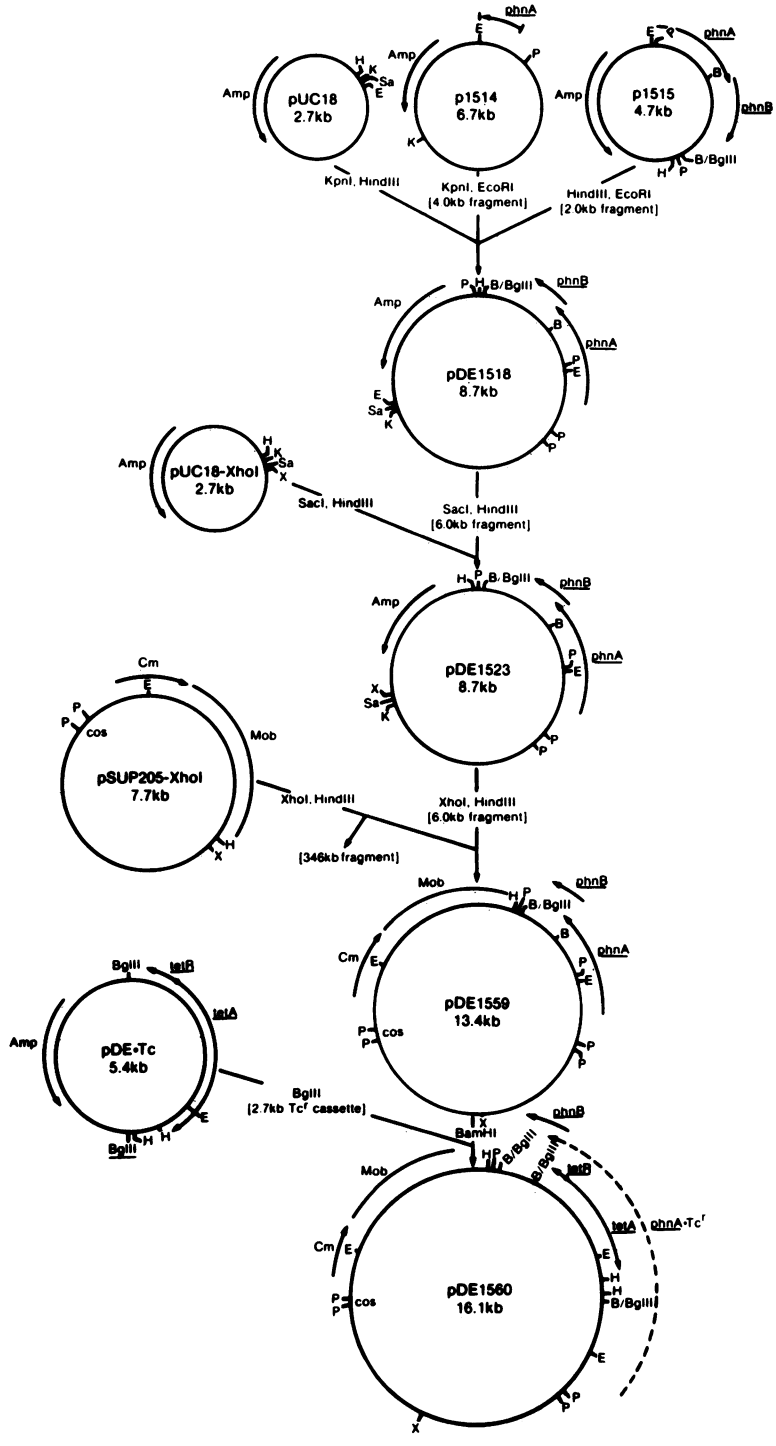

FIG. 3. Construction of pDE1560. The following letters are used to denote different restriction enzyme sites on the maps: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sac*I; and X, *Xho*I.

recognize the *mob* site on the plasmid vector and perform the conjugative transfer into the recipient *Pseudomonas* cells. Donor and recipient cells were grown in liquid culture to the log phase, mixed (1:1) in a sterile Eppendorf tube, centrifuged, and suspended in 200 μl of LB. Mating mixtures were then spread onto nitrocellulose filters (13-mm diameter, 0.45-μm pore size) placed on a prewarmed LB agar plate. Matings were incubated for 16 to 20 h at 37°C. The cells were then suspended and diluted in LB and spread onto selective media. Exconjugants were selected for antibiotic resistance on either pseudomonas isolation agar or cefazolin-containing LB agar medium. Since the vector cannot replicate in *Pseudomonas* species, the antibiotic resistance gene must be rescued by homologous recombination. A single crossover leads to insertion of the entire vector into the chromosome and results in a merodiploid having the antibiotic resistances

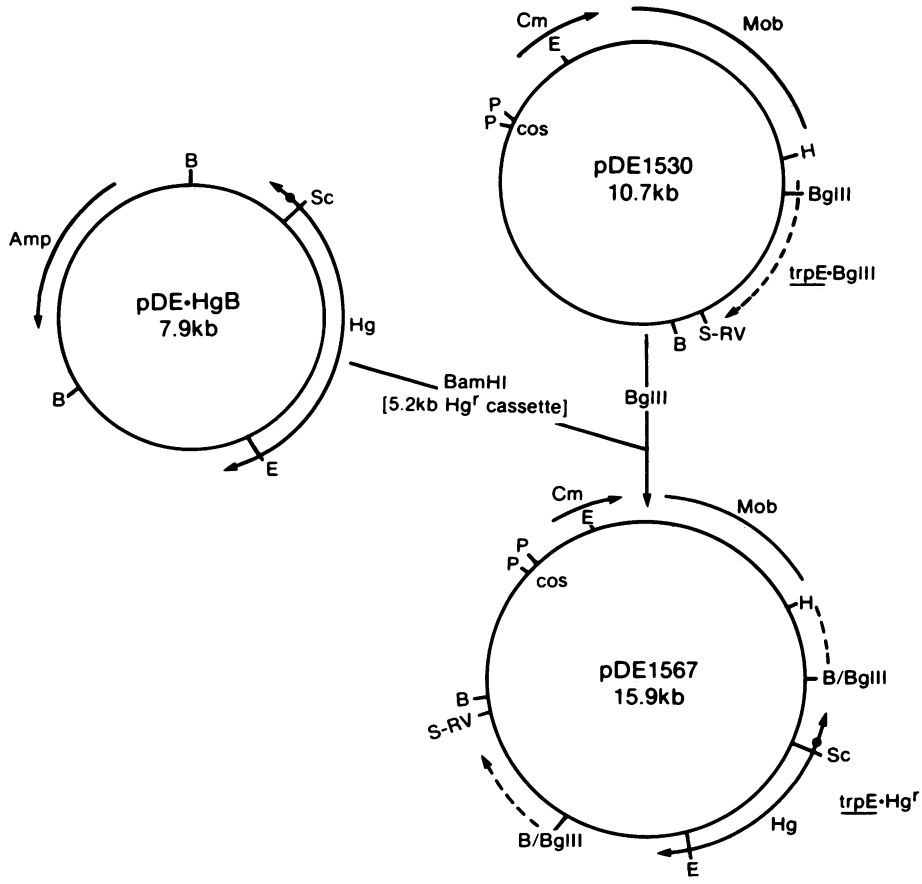

FIG. 4. Construction of pDE1567. The following letters are used to denote different restriction enzyme sites on the maps: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; RV, *Eco*RV; S, *Sma*I; and Sc, *Sca*I.

encoded by the vector and the insertional antibiotic cassette. A double crossover (or subsequent resolution of the merodiploid) gives rise to marker exchange. The product strain loses the vector sequences and shows resistance only to the antibiotic inserted into the mutant gene. Southern hybridization (30) was used to show that only the insertionally inactivated gene is present.

Cell extracts and enzyme assays. Preparation of cell extracts by sonication and assays for enzyme activity and protein concentration were as described previously (8).

Pyocyanin quantitation assay. The pyocyanin assay is based on the absorbance of pyocyanin at 520 nm in acidic solution (15, 20). A 5-ml sample of culture grown in PB to maximize pyocyanin production was extracted with 3 ml of chloroform and then reextracted into 1 ml of 0.2 N HCl to give a pink to deep red solution. The absorbance of this solution was measured at 520 nm. Concentrations, expressed as micrograms of pyocyanin produced per milliliter of culture supernatant, were determined by multiplying the optical density at 520 nm (OD_{520}) by 17.072 (16).

Preparation of RNA. Total cellular RNA from *P. aeruginosa* was prepared by the method of Aiba et al. (1) with some modifications (2).

RNA slot-blot analysis. RNA samples (30 to 50 μg) were dissolved in 75 μl of 50% deionized formamide–6% formaldehyde. Samples were incubated for 1 h at 50°C to denature the RNA and then placed on ice. Gene Screen Plus hybridization transfer membrane (Dupont, NEN Research Products, Boston, Mass.) and a filter pad were then cut to fit a Minifold II Slot-Blotter (Schleicher & Schuell, Inc., Keene, N.H.). The membrane and filter pad were floated on deionized water for 15 min and then placed in the manifold and clamped tightly. Wells of the manifold were washed with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), RNA samples were added to the wells, followed by light suction for 30 to 60 s. The membrane was removed and allowed to air dry. The membrane with the immobilized RNA was then baked at 80°C for 2 h to remove the formaldehyde and placed in a Seal-A-Meal bag.

The hybridization conditions used were the same as for Southern hybridization except that both the prehybridization and hybridization incubations were done at 60°C instead of 65°C.

Analysis of protein sequence data and normalized alignment scores. For the analysis of protein sequence data, we utilized the FATSP program (19) as described by Chang et al. (5). The alignments used were extracted from ones containing more sequences published elsewhere (6). To estimate similarity between homologous or paralogous sequences, we used the method of Doolittle (9). This method is direct, using only identical residues, with a double value given to Cys residues; it imposes a gap penalty (2.5 times the weight of a residue identity) and provides a simple test of homology based on the likelihood of this degree of similarity having occurred by chance (9). The normalized alignment score of Doolittle (9) is approximately 10 times the percentage of identical residues less the gap penalty. For sequences 200 residues long, similarity values greater than 160 indicate that

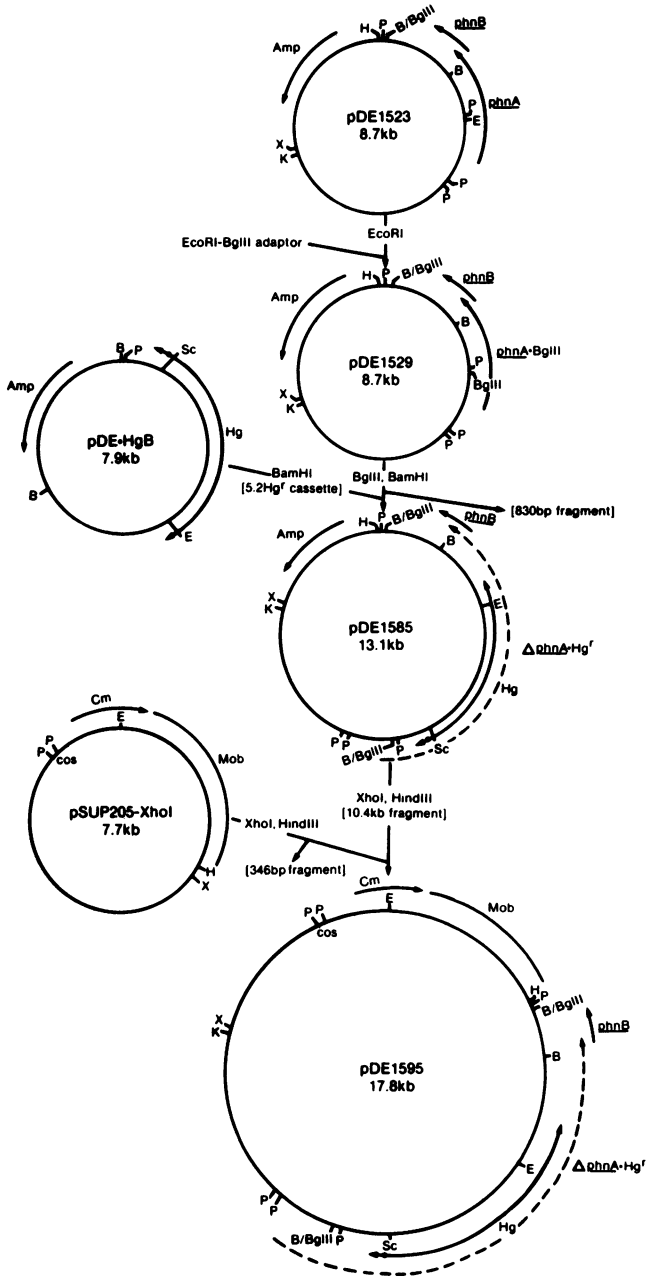

FIG. 5. Construction of pDE1595. The following letters are used to denote different restriction enzyme sites on the maps: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sc, *Sca*I; and X, *Xho*I.

common ancestry is probable, and values above 280 make it certain; for longer sequences, somewhat lower values are significant (9).

Enzymes and chemicals. All DNA manipulations were performed essentially as described by Maniatis et al. (22) or as recommended by the supplier. Restriction endonucleases were purchased from New England BioLabs, Inc. (Beverly, Mass.), Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), and Boehringer Mannheim Biochemicals (Indianapolis, Ind.). DNA polymerase I, DNA polymerase I Klenow fragment, mung bean nuclease, and calf intestinal alkaline phosphatase were purchased from New England BioLabs. Bacterial alkaline phosphatase was obtained from Bethesda Research Laboratories. RNase-free DNase I was purchased from Boehringer Mannheim. *N*-lauroyl-sarcosine pronase, X-Gal, and lysozyme were purchased from Sigma Chemical Co. (St. Louis, Mo.). Radiolabeled deoxynucleotide triphosphates were obtained from Amersham Corp. (Arlington Heights, Ill.). Linkers and adaptors were obtained from the University of Iowa Recombinant DNA Core Facility or New England BioLabs. Low-melting-temperature agarose was purchased from FMC Corp. (Philadelphia, Pa.).

RESULTS

Cloning *P. aeruginosa phnA* and *phnB*. The *phnA* and *phnB* genes from *P. aeruginosa* PAC174 were cloned, and the DNA sequence of the β-subunit gene, *phnB*, was obtained earlier (7, 8). We cloned the corresponding genes from *P. aeruginosa* PAO1 for the following reasons. (i) *P. aeruginosa* PAO1 is used as the wild-type strain in this and most other laboratories, not *P. aeruginosa* PAC174, and (ii) the cloned PAC174 *phnAB* gene pair lacked sufficient DNA upstream from *phnA* for gene replacement analyses.

We first purified and used as a probe a 1,165-bp *Pst*I fragment containing the 5′ end of *P. aeruginosa* PAC174 *phnA*, as well as upstream DNA, from plasmid pIA10 (8). This fragment, labeled with ^{32}P by nick translation, hybridized strongly to *P. aeruginosa* PAO1 chromosomal DNA digests.

We cloned a 4.0-kb *Kpn*I-*Eco*RI fragment from *P. aeruginosa* PAO1 after electroelution from agarose and ligation into pUC19 DNA that had been digested with *Kpn*I and *Eco*RI. The ligation mixture was transformed into *E. coli* JM109, and cells containing the desired plasmid, designated p1514, were identified by colony hybridization. This insert contained the 5′ end of *phnA* and approximately 3.5 kb of upstream DNA.

We next purified and used as a probe a 1,227-bp *Kpn*I fragment from plasmid pIA20 containing the 3′ end of *P. aeruginosa* PAC174 *phnA* and most of *phnB* (7). This fragment was labeled with ^{32}P by nick translation. The probe showed strong hybridization to a 2.0-kb *Eco*RI-*Bgl*II fragment from *P. aeruginosa* PAO1 chromosomal DNA digests. This fragment was isolated after electroelution from agarose and cloned into pUC19 digested with *Bam*HI and *Eco*RI in the manner described above for p1514. This plasmid was designated p1580; its insert contained the remainder of *phnA*, all of *phnB*, plus approximately 600 bp of DNA downstream from *phnB*.

The plasmid p1326 had been isolated earlier in this work by shotgun cloning of a *Cla*I-*Pst*I digest of PAO1 chromosomal DNA into pUC18 digested with *Acc*I and *Pst*I. Cells containing the desired plasmid were identified by colony hybridization with the 1,165-bp *Pst*I fragment of pIA10 as a probe. This plasmid was used in the initial sequence comparisons of *phnA* in *P. aeruginosa* PAO1 and PAC174.

Relevant portions of p1326, p1514, and p1580 were sequenced, as well as previously unsequenced portions of pIA10 (subclones p1256, p1257, and p1515). All restriction sites were bridged, and complementary sequences were obtained from both strands.

Sequences of *P. aeruginosa phnA* and *phnB*. The DNA sequences of the *phnA* and *phnB* genes of *P. aeruginosa* PAO1 and PAC174 and their deduced amino acid sequences are presented in Fig. 6. The sequences are nearly identical; nucleotide differences are indicated. The designated start codons were assigned based on alignment with 22 other

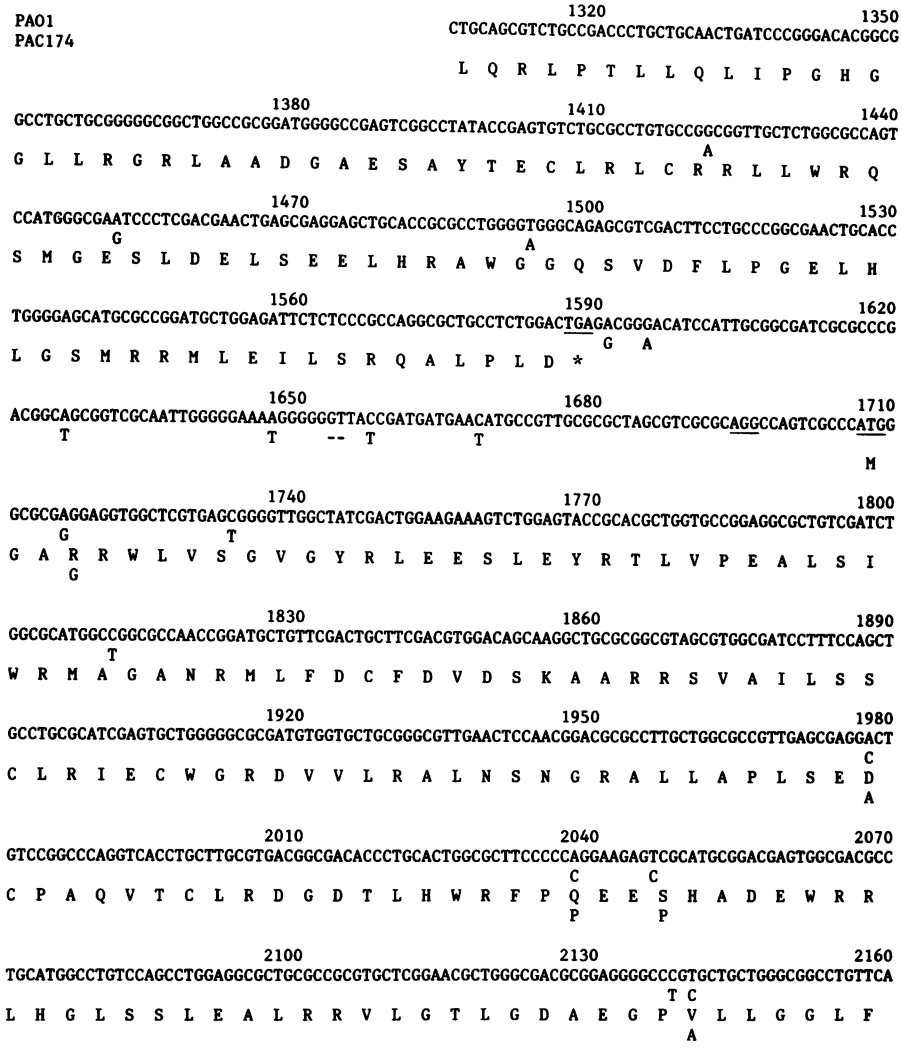

FIG. 6. DNA sequence and deduced amino acid sequence of *P. aeruginosa* PAO1 and PAC174 *phnA* and *phnB* and flanking DNA. The nucleotide and amino acid differences are indicated. Start and stop codons and the presumed Shine-Dalgarno sequences are underlined. *phnA* and *phnB* overlap by 23 bp, not counting the *phnB* Shine-Dalgarno region.

anthranilate synthases (6). The initial 20 amino acids are highly conserved in the small subunit, with four of these residues absolutely conserved in every small-subunit sequence. Several other areas of strong conservation are seen in the small-subunit proteins. *phnA* and *phnB* overlap by 23 bp, not including the *phnB* Shine-Dalgarno region (26). Preliminary results implicate this overlap in translational coupling (data not presented).

Figure 6 also presents the sequences of 285 bp of the 3′ end of an upstream open reading frame (ORF), designated ORF1, from *P. aeruginosa*. The entire DNA sequence of ORF1 was obtained in *P. aeruginosa* PAO1, as well as 690 bp of another upstream ORF, designated ORF2. Figure 7 presents the DNA sequences and deduced amino acid sequences for ORF1 and ORF2. A good Shine-Dalgarno sequence (26) exists 10 bp upstream from the suggested start codon for ORF1. The two ORFs overlap by 7 bp, suggesting the possibility of coupled translation. ORF1 has the codon usage characteristic of moderately expressed genes in *Pseudomonas* species (68% of the codons ending in either G or C). Both the DNA and amino acid sequences of ORF1 and ORF2 were used in database searches for similar sequences without finding a significant match.

Analysis of relatedness of the two *P. aeruginosa* anthranilate synthases to homologous enzymes in diverse organisms. In alignment of the amino acid sequences of the β subunits of 17 anthranilate synthase and 5 *p*-aminobenzoate synthase enzymes from a variety of bacteria and fungi, similarity values range from 206 to 1,000 (6). Table 3 presents the normalized alignment scores (9) derived and condensed from alignments published elsewhere (6) for the β subunits of a selected group of bacteria. TrpG sequences of the enteric bacteria, including *Vibrio parahaemolyticus*, form a noticeably similar cluster (normalized alignment scores, 591 to 965), with *P. aeruginosa* PhnB possibly weakly allied to it. Enteric bacterial PabA sequences form a second cluster with TrpG of *Bacillus subtilis* (Slock et al., manuscript in preparation), *P. putida*, *P. aeruginosa*, and *Acinetobacter calcoaceticus* (similarity values from 570 to 877).

It is interesting that the two *P. aeruginosa* anthranilate synthase β subunits, TrpG and PhnB, are less closely related to each other than to β subunits in other organisms. Both the normalized alignment scores presented in Table 3 and the size and location of gaps used to achieve alignment (data provided on request) indicate that the TrpG proteins of *P. aeruginosa*, *P. putida*, and the somewhat more distantly

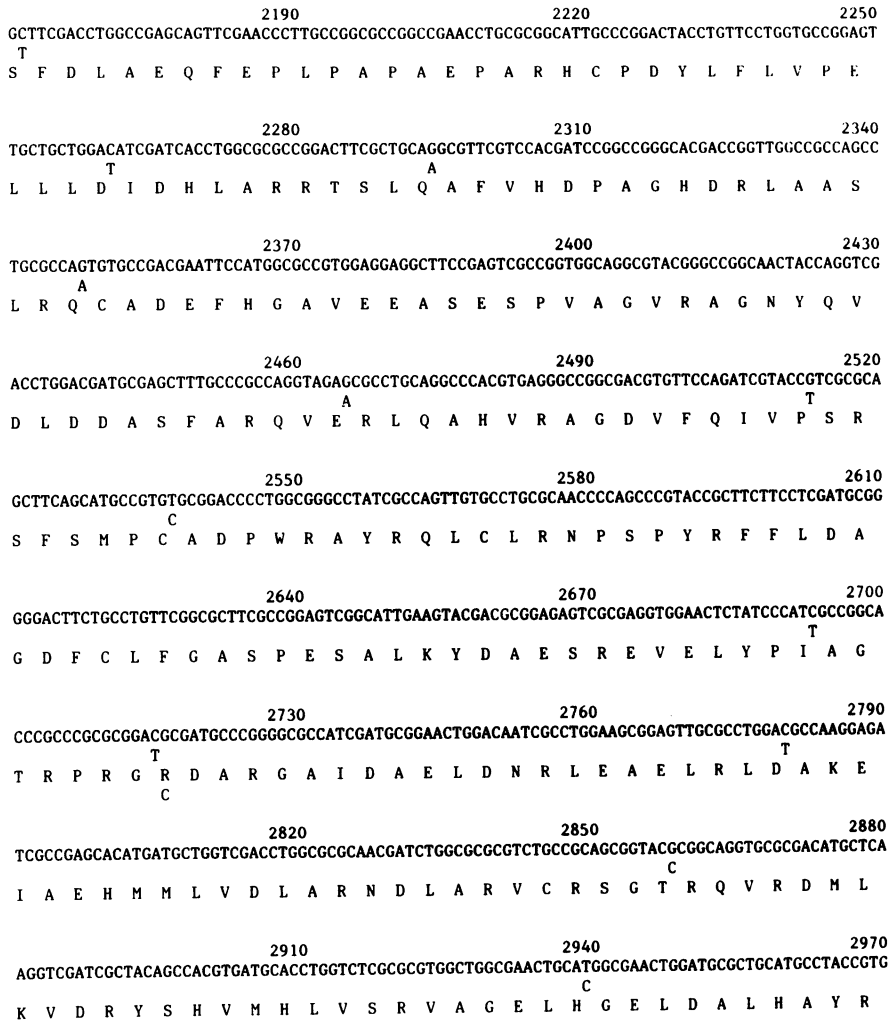

FIG. 6—*Continued*

related *A. calcoaceticus* more closely resemble the enterobacterial PabA than the enterobacterial TrpG sequence. In contrast, *P. aeruginosa* PhnB seems closer to the enterobacterial TrpG than to enterobacterial PabA.

Comparing sequence data for the α subunit of anthranilate synthase and *p*-aminobenzoate synthase is considerably more difficult than for the β subunit (6). Table 3 presents the normalized alignment scores for these α subunits, based on the best current alignment (6). Since these molecules have approximately 500 amino acid residues, all values are within the range indicative of homology (9). Alignment of the carboxyl-terminal half of the molecules poses no problems. In fact, between position 280 and the end there are 29 locations where all sequences have the same amino acids. Alignment of the amino-terminal half is more arbitrary; there are many gaps, and there is only one position between 1 and 279 where the residues are identical (6). *P. aeruginosa* and *P. putida* TrpE are very similar (normalized alignment score of 866); however, among the others, only the first four sequences are clearly related.

Insertion of pDE1443 into *P. aeruginosa* PAO4290 chromosome. This experiment provided the first indication that the anthranilate synthase genes obtained on the R-prime plasmid were not involved in tryptophan synthesis. These anthranilate synthase genes had been originally designated *trpE* and *trpG* because of their ability to complement *trpE* auxotrophs of *E. coli* (8). We wanted to create a *trpG* mutant of *P. aeruginosa* and examine its phenotype. Therefore, the presumed *trpG* was insertionally inactivated with a Km^r cassette, and the insertionally inactivated gene used to replace the normal chromosomal gene. Inactivation of this small-subunit gene on the *P. aeruginosa* chromosome did not result in the expected requirement for tryptophan in low-ammonium media but instead resulted in a strain defective in pyocyanin production. Plasmid pDE1443 was used to create this mutant *P. aeruginosa*.

Replacement of *phnB* on the *P. aeruginosa* chromosome with the Km^r mutant *phnB* gene was demonstrated directly by subjecting DNA from the parental strain PAO4290 and its derivative $PhnB^-$ Km^r strain PADE B1 to restriction and hybridization analysis. Southern hybridization analyses with appropriate probes containing either segments of the *phnB* region or Km^r cassette demonstrated that the Km^r cassette was indeed inserted into the mutant *phnB* (data not shown).

Insertion of pDE1560 into *P. aeruginosa* PAO1 chromosome. The *phnB* mutant, PADE B1, was constructed in *P. aeruginosa* PAO4290 because of the kanamycin sensitivity of this strain. PAO4290 is a poor pyocyanin producer. A difference between pyocyanin production in the parent strain and its $PhnB^-$ Km^r mutant is discernible, but for further

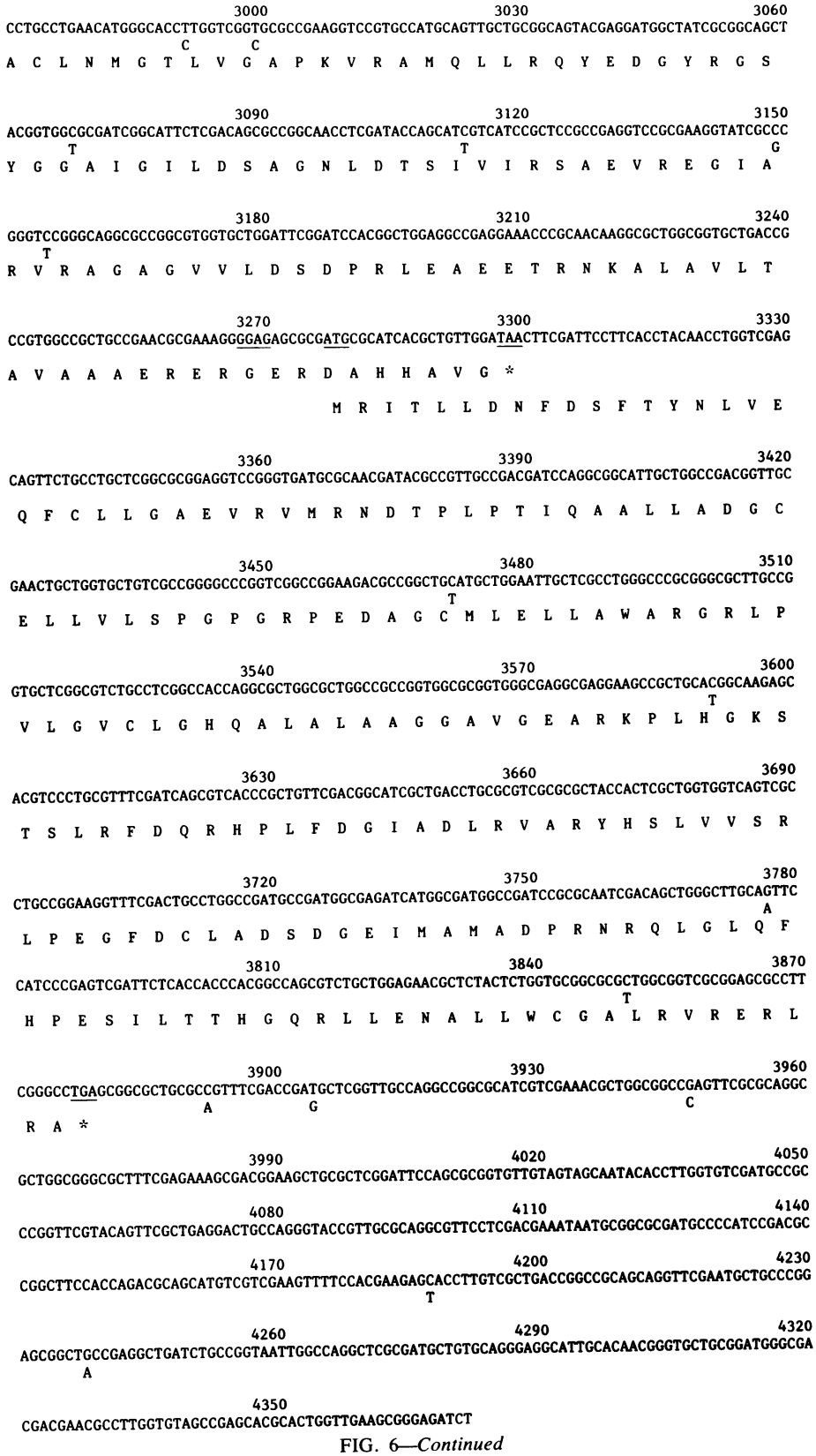

FIG. 6—*Continued*

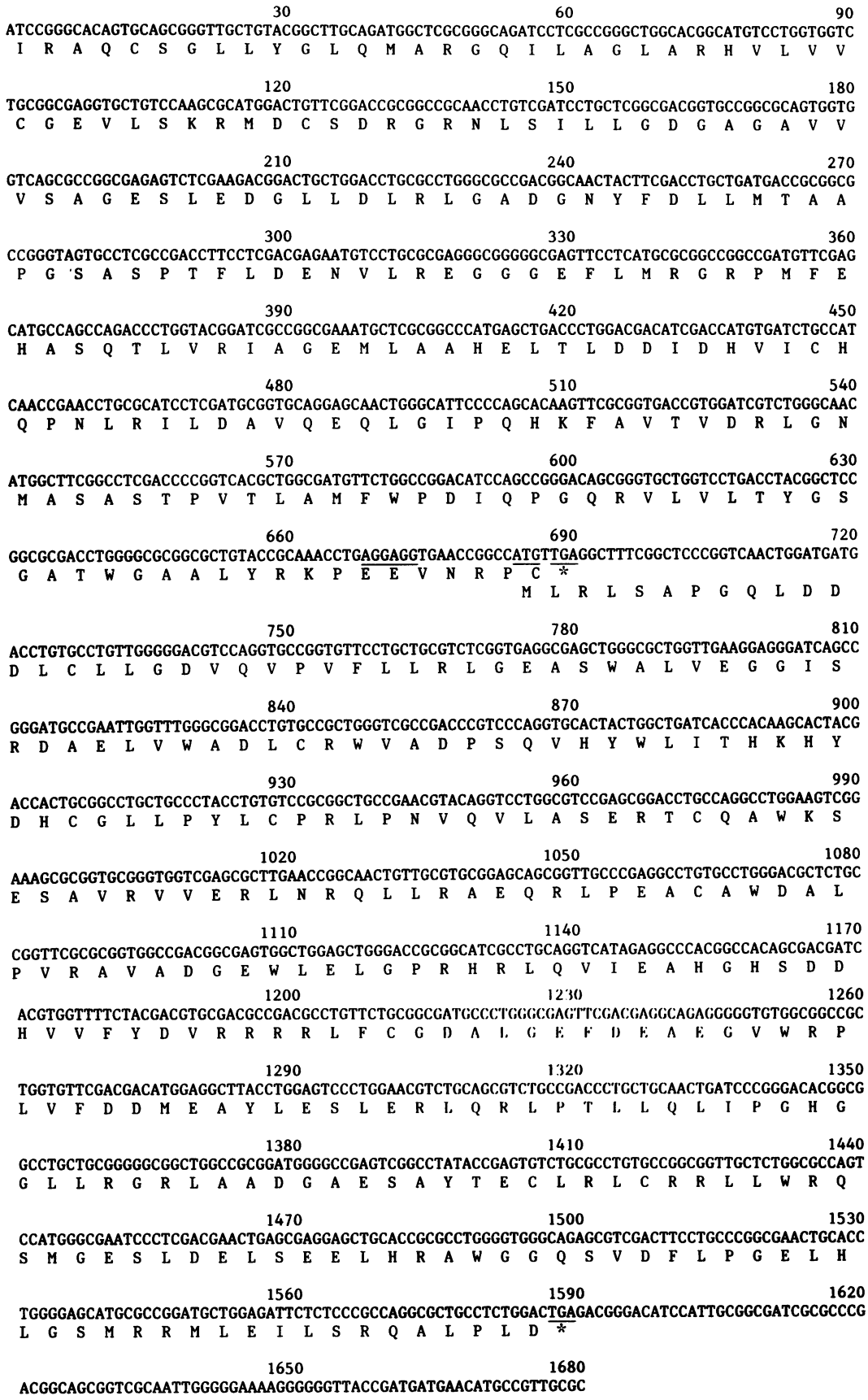

FIG. 7. DNA sequence and deduced amino acid sequence of ORF1 and ORF2 upstream from *P. aeruginosa phnA*. Start and stop codons and presumed Shine-Dalgarno sequences are underlined.

TABLE 3. Normalized alignment scores for anthranilate synthase and *p*-aminobenzoate synthase α and β subunits from various organisms[a]

Organism	Normalized alignment score																			
	S.t. TrpG	S.m. TrpG	V.p. TrpG	P.a. PhnB	E.c. PabA	S.t. PabA	S.m. TrpG	B.s. TrpG	P.p. TrpG	P.a. TrpG	A.c. TrpG	V.p. TrpE	P.a. PhnA	B.l. TrpE	R.m. TrpE	S.a. TrpE	E.c. PabB	P.p. TrpE	P.a. TrpE	B.s. TrpE	S.c. TrpE
β-Subunit similarities																					
E.c. TrpG	965	839	622	475	398	366	406	399	383	396	317										
S.t. TrpG		834	627	470	409	372	395	384	363	381	302										
S.m. TrpG			591	396	409	356	395	401	380	401	327										
V.p. TrpG				415	356	350	340	369	367	379	362										
P.a. PhnB					301	312	304	225	270	316	278										
E.c. PabA						877	773	626	676	687	586										
S.t. PabA							762	610	676	671	575										
S.m. PabA								607	707	718	593										
B.s. TrpG									633	585	563										
P.p. TrpG										843	655										
P.a. TrpG											649										
α-Subunit similarities																					
E.c. TrpE												580	456	426	236	269	198	255	257	240	235
V.p. TrpE													431	415	208	275	209	253	260	230	220
P.a. PhnA														392	208	247	184	244	260	225	204
B.l. TrpE															198	222	208	233	237	205	192
R.m. TrpE																208	151	214	213	201	217
S.a. TrpE																	213	305	305	261	251
E.c. PabB																		265	269	270	217
P.p. TrpE																			866	325	299
P.a. TrpE																				319	286
B.s. TrpE																					255

[a] Scores obtained from Crawford (6). Organisms: A.c., *A. calcoaceticus*; B.l., *Brevibacterium lactofermentum*; B.s., *B. subtilis*; E.c., *E. coli*; P.a., *P. aeruginosa*; P.p., *P. putida*; R.m., *Rhizobium meliloti*; S.a., *Spirochaeta aurantia*; S.c., *Saccharomyces cerevisiae*; S.m., *Serratia marcescens*; S.t., *Salmonella typhimurium*; V.p., *V. parahaemolyticus*.

studies on the *phnA* and *phnB* genes, we chose to work with *P. aeruginosa* PAO1, an excellent pyocyanin producer and the wild-type strain used in this and most other laboratories. We constructed a *phnA* mutant of PAO1 by replacing the wild-type chromosomal gene with a mutant *phnA* containing an insertion of a 2.7-kb Tc^r cassette near the 3′ end of the gene. The plasmid pDE1560 was used to create this $PhnA^-$ Tc^r *P. aeruginosa* mutant.

Replacement of *phnA* on the *P. aeruginosa* chromosome with the Tc^r mutant *phnA* gene was demonstrated directly by subjecting DNA prepared from the parental strain PAO1 and its derivative $PhnA^-$ Tc^r strain PADE A47 to restriction and hybridization analysis. Appropriate probes were used in Southern hybridization analyses to verify replacement of the chromosomal *phnA* with the insertionally inactivated mutant *phnA* (data not shown).

Insertion of pDE1567 into *P. aeruginosa* PADE A47 chromosome. We constructed a double mutant altered in both large-subunit genes, *trpE* and *phnA*. We used pDE1567 to create a *trpE* mutant of *P. aeruginosa* PADE A47.

Replacement of *trpE* on the *P. aeruginosa* PADE A47 chromosome with the Hg^r mutant *trpE* was demonstrated directly by subjecting DNA from PAO1, the parental strain *P. aeruginosa* PADE A47, and its derivative $TrpE^-$ Hg^r strain PADE A47E1 to restriction and hybridization analysis (data not shown).

Insertion of pDE1595 into *P. aeruginosa* PADE A47 chromosome. Because *P. aeruginosa* PADE A47 retained some pyocyanin production, we wanted to create a more seriously disabled derivative of this strain by replacing its Tc^r mutant *phnA* with a Δ*phnA* containing a substantial deletion in its coding region. This Δ*phnA* construct has an Hg^r cassette inserted into the deleted region to allow selection. Thus, selection for the desired haploid strain could be detected by the loss of Tc^r and the acquisition of Hg^r. The plasmid pDE1595 was used to create the Δ*phnA* Hg^r derivative of PADE A47.

The replacement of the Tc^r mutant *phnA* with the Hg^r mutant Δ*phnA* on the *P. aeruginosa* chromosome was demonstrated directly by subjecting DNA from PAO1, the parental strain PADE A47, and its derivative Δ*phnA* Hg^r strain PADE A48 to restriction and hybridization analysis (data not shown).

Phenotypic characterization of PADE A47, PADE A48, and PADE A47E1. PADE A47 and PADE A48 are both prototrophs; they grow without supplementation on Vogel-Bonner minimal medium and on low- or high-ammonia M9 medium. The $PhnA^-$ $TrpE^-$ mutant PADE A47E1 requires supplementation with either anthranilate of L-tryptophan for growth on minimal medium.

Cultures of PAO1, PADE E1, PADE A47, PADE A48, and PADE A47E1 were grown for 16 to 20 h in 5 ml of PB for quantitation of pyocyanin production. Pyocyanin production by the five strains is shown in Table 4. The results showed that pyocyanin production is greatly reduced in the *phnA* mutants PADE A47 and PADE A48 and eliminated in the double mutant PADE A47E1.

Characterization of PADE E1r. During the characterization of PADE E1 (9b), spontaneous tryptophan-independent colonies appeared at a frequency of 10^{-5} to 10^{-6}; one of these Trp^+ colonies was isolated and designated PADE E1r. PADE E1r grew without supplementation on Vogel-Bonner minimal medium and on low- or high-ammonia medium. Its parent, PADE E1, required supplementation with either anthranilate or L-tryptophan for growth on minimal medium.

PADE E1r produced more pyocyanin than either PAO1 or PADE E1. Pyocyanin production by the three strains was analyzed spectrophotometrically. Table 5 shows the results of this analysis. Pyocyanin production was greatest in PADE E1r, approximately 25% more than PAO1 and 40% more than PADE E1.

TABLE 4. Spectrophotometric quantitation of pyocyanin production by *P. aeruginosa* PAO1 and its derived mutants[a]

Strain	OD_{520}	Pyocyanin production (μg/ml)[b]	% Wild-type production
PAO1	0.369	6.30	100
PADE E1	0.319	5.44	86
PADE A47	0.125	2.13	34
PADE A48	0.085	1.45	22
PADE A47E1	0.001	0.02	2

[a] Cells were grown for 16 to 20 h in 5 ml of PB at 37°C with aeration. Extraction procedures are outlined in Materials and Methods.
[b] Pyocyanin concentrations are reported as micrograms of pyocyanin produced per milliliter of PB culture.

Anthranilate synthase (with either ammonia or glutamine as the nitrogen source) and TrpD-derived phosphoribosyl transferase enzymatic activities were measured in extracts from tryptophan-starved cultures (3) of PAO1, PADE E1, PADE E1r, and PADE G2 (9b). The results are shown in Table 6. Anthranilate synthase activity in PAO1 and PADE G2 is completely inhibited by either 150 or 750 μM tryptophan (data not shown), whereas the anthranilate synthase activity of PADE E1r showed no inhibition upon the addition of either concentration of tryptophan. Addition of either 75 or 150 μM pyocyanin to the reaction mixtures also had no effect on anthranilate synthase activity. The anthranilate synthase activity of the cloned *phnAB* on pIA10 expressed in *E. coli* is also not feedback inhibited by tryptophan (data not shown), suggesting that the anthranilate synthase activity of PADE E1r arises from *phnAB*.

To verify that the Trp^+ strain PADE E1r was not a merodiploid that had somehow survived the isolation of PADE E1, to show that the Tc^r cassette was still inserted into the mutant *trpE*, and to prove that the prototoph isolated was not a contaminant, we subjected DNA from PAO1, PADE E1, and PADE E1r to restriction and hybridization analyses. These analyses demonstrated that the Tc^r cassette was still inserted into the mutant *trpE*.

Measurement of *trpG* and *phnA* mRNA levels in PAO1, PADE E1, and PADE E1r. mRNA production corresponding to *trpE* and *phnA* was measured in PAO1, PADE E1, and PADE E1r during different growth phases. *trpE* of PADE E1 and PADE E1r contains a deletion/insertion. A preliminary experiment showed little or no *trpE* RNA transcript present in the *trpE* mutants PADE E1 and PADE E1r compared with

TABLE 5. Spectrophotometric quantitation of pyocyanin production by *P. aeruginosa* PAO1, PADE E1, and PADE E1r[a]

Strain	OD_{520}	Pyocyanin production (μg/ml)[b]	% Wild-type production
PADE E1r	0.453	7.73	123
PAO1	0.369	6.30	100
PADE E1	0.319	5.44	86

[a] Cells were grown for 16 to 20 h in 5 ml of PB at 37°C with aeration. Extraction procedures are outlined in Materials and Methods.
[b] Pyocyanin concentrations are reported as micrograms of pyocyanin produced per milliliter of PB culture.

TABLE 6. Anthranilate synthase and phosphoribosyl transferase activities of *P. aeruginosa* PAO1, PADE E1, PADE E1r, and PADE G2[a]

P. aeruginosa cell extract	Enzymatic activity[b] with:		Phosphoribosyl transferase activity[b]
	Ammonia	Glutamine	
PAO1	0.014	0.058	0.400
PADE E1	0	0	1.07
PADEE1r	0.064	0.086	1.37
PADE G2	0.55	0	0

wild-type PAO1, possibly due to degradation of the incomplete transcript. Accordingly, we chose to monitor *trpG* mRNA production because of its known coordinate regulation with *trpE* (3).

We prepared *phnA*- and *trpG*-specific probes for use in hybridization studies against crude mRNA samples. The *phnA*-specific probe was a 205-bp *Eco*RV-*Pst*I fragment from p1433 (p1433 contains the 1,161-bp *Pst*I *phnA* fragment from pIA10 inserted into pUC8.) The *trpG*-specific probe was a 1.0-kb *Eco*RV fragment from p1395. These probes were first tested by Southern hybridization against the cloned *phnA*, *phnB*, *trpE*, and *trpG* genes and PAO1 chromosomal DNA digests to confirm their specificity. Under high-stringency conditions, no cross-hybridizations were seen. These probes were then hybridized to crude mRNA preparations made from cells in various phases of growth.

RNA was prepared from cultures harvested in the early log (OD_{560} = 0.30), late log (OD_{560} = 0.60), and stationary (OD_{560} = 0.75) phases. Samples containing equal amounts of RNA were used undiluted and at 1:10 and 1:50 dilutions. These samples were applied to nylon hybridization membranes with a slot-blot apparatus. The slot blots were then hybridized with the ^{32}P-labeled 205-bp *Eco*RV-*Pst*I fragment from p1433 or the ^{32}P-labeled 1.0-kb *Eco*RV fragment from p1395. After hybridization, the blots were washed under high stringency.

The slot-blot autoradiographs were subjected to densitometric analysis (Table 7). The results showed that in PADE E1r the quantity of *phnA* mRNA is at least eight times greater than in PAO1 and PADE E1 in the early log phase but that this difference diminishes in the late log and stationary phases. The *trpG* probe showed that *trpG* mRNA levels are greatest in the early log phase and that they diminish markedly by the stationary phase.

DISCUSSION

We showed that *P. aeruginosa* has two anthranilate synthases, that they are homologous, and that both have conventional α and β subunits. One anthranilate synthase, encoded by *trpE* and *trpG*, catalyzes the first step in the tryptophan synthetic pathway. The other anthranilate synthase, encoded by *phnA* and *phnB*, functions in phenazine synthesis. *P. aeruginosa* is unique in this respect, for until now no other procaryote or eucaryote has been shown to have two anthranilate synthases.

The existence of two anthranilate synthases in *P. aeruginosa* explains the differences seen earlier between the two sequences of the small (β, glutamine amidotransferase) subunits of anthranilate synthase in *P. putida* and *P. aeruginosa* (7). The sequence from *P. putida* had been obtained by sequencing the purified protein from the tryptophan synthetic enzyme (14). The other was a DNA sequence obtained from a *P. aeruginosa* PAC174 R-prime plasmid capable of complementing *trpE* mutants of *E. coli* (7, 8). It is now clear that these two genes do not specify the enzyme catalyzing the first reaction of the tryptophan synthetic pathway but in fact encode a second anthranilate synthase in *P. aeruginosa*. These genes are now designated *phnA* and *phnB* for their involvement in the synthesis of phenazines, specifically pyocyanin, in *P. aeruginosa*.

We established the existence of two distinct anthranilate synthases by inactivating the chromosomal version of the cloned *P. aeruginosa* small-subunit gene. Inactivation did not result in a requirement for tryptophan in low-ammonia medium but instead gave cells defective in the production of pyocyanin. Using the small-subunit gene from the R-prime plasmid as a probe, a second, cross-reacting chromosomal gene in *P. aeruginosa* PAO1 was identified by Southern hybridization at low stringency. This second gene was cloned and sequenced. The deduced protein sequence of this small-subunit gene proved to be very similar to that obtained earlier by Kawamura et al. (14) from the purified *P. putida* anthranilate synthase small subunit. This gene is now correctly designated *trpG*; when insertionally inactivated and inserted into the *P. aeruginosa* chromosome, replacing the wild-type gene, the predicted phenotype was found, a requirement for tryptophan or anthranilate in low (1 mM NH_4^+)-ammonia medium but not in high (50 mM NH_4^+)-ammonia media (9b). This *trpG* mutant produces pyocyanin normally.

The *phnA* and *phnB* genes from *P. aeruginosa* PAC174 had been cloned and the DNA sequence of *phnB* had been obtained (7, 8). We cloned the corresponding genes from *P. aeruginosa* PAO1; the sequences were nearly identical (Fig. 4). We identified two ORFs upstream from *phnA*. A complete DNA sequence was obtained for ORF1, while only a partial DNA sequence was obtained for ORF2. The two ORFs overlapped by 7 bp. We have no clue as to the function of these ORFs, and a database search for similar sequences found no significant matches.

Comparison of TrpE, TrpG, TrpD, and TrpC sequences in *P. putida* and *P. aeruginosa* showed them to be 79 to 87% identical (9a, 9b). PhnA and PhnB are clearly homologous to *P. aeruginosa* TrpE and TrpG but at a much lower level (26 to 39% identity). A detailed analysis of all known anthranilate and *p*-aminobenzoate synthases (6) shows, surprisingly, that PhnA and PhnB are more closely related to *E. coli*

TABLE 7. Slot-blot analysis of *trpG* and *phnA* mRNA production by PAO1, PADE E1, and PADE E1r: densitometric quantitation[a]

Probe	Early-log-phase RNA (OD_{560} = 0.30)			Late-log-phase RNA (OD_{560} = 0.60)			Stationary-phase RNA (OD_{560} = 0.75)		
	PAO1	PADE E1	PADE E1r	PAO1	PADE E1	PADE E1r	PAO1	PADE E1	PADE E1r
trpG	36.3	25.1	34.6	17.05	5.32	12.41	7.06	1.09	4.43
phnA	8.0	7.5	64.95	14.02	12.7	65.5	84.5	64.96	66.36

[a] Values presented are area × mean OD and are an average of the undiluted, 1:10, and 1:50 dilution values.

TrpE and TrpG (46 and 48% identity) than to *Pseudomonas* TrpE and TrpG (26 and 39% identity), whereas *Pseudomonas* TrpE and TrpG are more closely related to *E. coli* PabB and PabA, the genes for the paralogous enzyme synthesizing *p*-aminobenzoate (11) (28 and 68% identity), than to *E. coli* TrpE and TrpG (25 and 40% identity).

The analysis of the evolution of anthranilate synthase genes is complicated by their similarity to the *p*-aminobenzoate synthase genes and the likelihood that some switching of function may have occurred. Such switching may explain why TrpG in the fluorescent pseudomonads, which does not seem to be involved in folate synthesis, is most similar to enteric bacterial PabA and to the *B. subtilis* and *A. calcoaceticus* amphibolic TrpG proteins. Further complicating this matter is the second anthranilate synthase gene pair in *P. aeruginosa*, *phnA* and *phnB*, which is more closely related to the enteric bacterial *trpE-trp(G)* gene pair but is not normally used in the synthesis of tryptophan. This relationship of the *trp* enzymes in the fluorescent pseudomonads has some elements needed to make a case for reticulate evolution at the molecular level. The occasional need for two anthranilate synthase genes (one for tryptophan synthesis and the other for secondary metabolism) might increase the likelihood of such role switching.

Clearly, the genes for the two anthranilate synthase enzymes are not the result of a recent duplication. Their sequences are much less alike than the homologous TrpE and TrpG sequences from *P. aeruginosa* and *P. putida*. The *phnA* and *phnB* reading frames overlap by 23 bp, whereas the *trpE* and *trpG* genes are on different transcriptional units separated by 25 kb of DNA (27). It is reasonable that the genes for these two anthranilate synthases arose by duplication of an ancestral gene and began to diverge in sequence much earlier in evolution than the time of speciation of *P. aeruginosa* and *P. putida*.

The PhnAB enzyme is interesting because of its apparent role in secondary metabolism. Our studies of *phnAB* mutants provide the first genetic evidence that anthranilate is the intermediate between chorismate and phenazine-1,6-dicarboxylate in the phenazine synthetic pathway. Earlier evidence had indicated that anthranilate is not a phenazine precursor (reviewed in reference 33). These studies based their conclusions on the inability of radiolabeled anthranilate added exogenously to be incorporated into phenazines and the fact that an anthranilate synthase mutant (TrpE) still produced phenazines. The latter finding is now explained. One hypothesis to explain the lack of incorporation of exogenous anthranilate into phenazines is that the *phnAB*-encoded anthranilate synthase forms a multienzyme complex with the other *phn* genes and that chorismate, but not anthranilate, can enter this complex. Chorismate is converted to anthranilate, which is then channelled to the next active site. Such multienzyme complexes with catalytic subunits arranged to catalyze successive steps of synthesis are known to be involved in the biosynthesis of some secondary metabolites (21; V. S. Malik, personal communication).

Transplacement experiments clearly implicate *phnA* and *phnB* in pyocyanin production. Inactivation of *phnA* or *phnB* results in defective pyocyanin production. PhnA$^-$ mutants PADE A47 and PADE A48 produce pyocyanin at levels 34 and 22%, respectively, that of the wild type, PAO1.

Thomashow and Weller (32) previously identified five unlinked loci required for phenazine biosynthesis in *Pseudomonas fluorescens* 2-79. Further examination of the mutants created in their study has also led to the tentative conclusion that anthranilate is an intermediate in phenazine synthesis (L. Thomashow, personal communication).

The first indication that the two *P. aeruginosa* anthranilate synthase genes may interchangeable under certain circumstances came during the phenotypic characterization of *phnA* mutants. The two PhnA$^-$ strains PADE A47 and PADE A48 both retain a low level of pyocyanin production. PADE A48 contains a substantial deletion of *phnA* and an insertion of the Hg^r cassette into this region, whereas PADE A47 has an insertion of the Tc^r cassette near the 3′ end of *phnA*; in either case, *phnA* should not be functional. Pyocyanin production is not completely abolished unless both *phnA* and *trpE* are inactivated, as in strain PADE A47E1. PADE E1, a *trpE* mutant, shows a 20% reduction in pyocyanin synthesis when compared with the wild-type strain PAO1. Apparently, the tryptophan-specific anthranilate synthase is capable of providing some anthranilate for pyocyanin production. Possibly the *trpE-trpG*-encoded anthranilate synthase may also combine with the other *phn* gene products in a multienzyme complex. This complex may allow the residual pyocyanin production seen in the PhnA mutants. It may be less effective, since this is not the normal enzyme, but the two enzymes are definitely homologous and such a complex is not impossible.

The second indication of interchangeability came when the *trpE* mutant PADE E1 showed reversion to Trp$^+$. The inactivated form of *trpE* in the PADE E1 chromosome has a significant portion of its coding sequence deleted, yet during its characterization, spontaneous tryptophan-independent colonies appeared at a frequency of 10^{-5} to 10^{-6}. Southern hybridization analysis showed no differences in the *trpE* region between PADE E1 and the Trp$^+$ strain, PADE E1r, and confirmed that PADE E1r was not a merodiploid. Several lines of evidence lead to the conclusion that the anthranilate synthase activity in PADE E1r arises from the *phnAB* gene products. (Similar *trpE* mutants of *P. putida*, a non-phenazine producer, do not revert. Hybridizations at low stringency with *phnAB* DNA as a probe showed no cross-hybridization with *P. putida* chromosomal DNA digests [data not shown].) The anthranilate synthase activity of PADE E1r is not feedback inhibited by tryptophan, whereas that of the *trpE-trpG*-encoded enzyme is. The anthranilate synthase activity of the cloned *phnAB* gene product expressed in *E. coli* is also not feedback inhibited by tryptophan. PADE E1r produces more pyocyanin than either PAO1 or PADE E1. But the most definitive experiment was a quantitative analysis of mRNA from PAO1, PADE E1, and PADE E1r. RNA samples were prepared from the three strains during the early log, late log, and stationary growth phases and hybridized with either a *phnA*-specific or a *trpG*-specific probe. The results of these hybridizations showed that PADE E1r produced at least eight times more *phnA* transcript in the early log phase than PAO1 and PADE E1. This experiment also showed that normally the production of *phnA* transcript increases throughout the growth phase and is maximal during the stationary phase, whereas the production of *trpG* transcript is maximal in the early log phase and has decreased sharply by the stationary phase. The high level of production of *phnAB* mRNA by PADE E1r in the early log phase may explain the Trp$^+$ phenotype. Excess production of the PhnAB anthranilate synthase early in growth, more than can enter into enzyme complexes with the other Phn-specific enzymes, may leave functional enzyme free in the cytoplasm capable of producing anthranilate for tryptophan synthesis.

Why is there no production of the PhnAB anthranilate

synthase in the early and late log phases by PAO1 and PADE E1? mRNA analysis indicates that a low level of *phnAB* transcript is produced in both the early and late log phases by the two strains. However, anthranilate synthase activity is totally feedback inhibited by tryptophan in enzyme assays in PAO1 in the late exponential phase; no residual anthranilate synthase activity, as expected if a fraction of the activity arose from the *phnA* and *phnB* genes, is observed. Moreover, PADE E1 shows no anthranilate synthase activity in enzyme assays. The possibility exists that this *phnAB* message is not translated in PAO1 and PADE E1, perhaps because additional control mechanisms exist specific for genes of secondary metabolism.

It is now clear that *P. aeruginosa* has two anthranilate synthases. One anthranilate synthase, encoded by *trpE* and trpG, catalyzes the first step in the tryptophan synthetic pathway; *trpE* and *trpG* are independently transcribed. The other anthranilate synthase, encoded by *phnA* and *phnB*, participates in the synthesis of the phenazine pigment pyocyanin; *phnA* and *phnB* are cotranscribed. These two anthranilate synthase gene pairs are regulated differently transcriptionally; a difference in feedback inhibition by tryptophan is seen in the two anthranilate synthases produced. This case illustrates how biosynthetically essential genes are exploited via duplication and divergence for secondary metabolism.

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