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Cloning of a Phenazine Biosynthetic Locus of *Pseudomonas aureofaciens* PGS12 and Analysis of Its Expression In Vitro with the Ice Nucleation Reporter Gene

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***Pseudomonas aureofaciens* PGS12 produces three phenazine antibiotics, in addition to siderophores, hydrogen cyanide, pyrrolnitrin, and indoleacetic acid. Tn*5-259.7* transposon mutagenesis was carried out to identify and clone a chromosomal locus involved in phenazine biosynthesis. Three classes of mutants were obtained: mutants deficient in phenazine production (Phz^-), mutants deficient in hydrogen cyanide production (HCN^-), and mutants deficient in the production of both compounds. *Eco*RI DNA fragments that contained the transposon and flanking regions were cloned from three mutants with single-transposon insertions, one from each phenotypic class. Phenazine and hydrogen cyanide production was restored by complementation of Phz^- or HCN^- mutants with selected cosmids from a PGS12 genomic library. No cosmids that complemented the doubly deficient Phz^-HCN^- mutant were obtained. A promoterless ice nucleation reporter gene was inserted in a phenazine biosynthetic locus by Tn*3*-spice transposon mutagenesis of a cosmid which complemented a phenazine-minus mutant. Reporter gene fusions that expressed the ice nucleation phenotype and no longer complemented phenazine production were introduced into the PGS12 chromosome by marker exchange. The expression of this locus was then monitored under different culture conditions. Expression decreased at pH levels below 7, and it was not affected by iron. Shikimic acid and phenylalanine favored higher expression levels. Expression was reduced in media with low substrate concentrations, indicating the importance of nutrient availability.**

Numerous soil-inhabiting pseudomonads colonize the surface of seeds and roots and inhibit infection by soilborne plant pathogens; some enhance plant growth and have been termed plant growth-promoting rhizobacteria (17). Many of these strains produce antibiotics and other compounds (siderophores, volatile substances) that are thought to account in part for their antimicrobial properties (5, 9, 12, 17, 32, 33, 45). Siderophores have been widely isolated in soils (1, 29), and considerable work has been done on their role in microbial interactions (17–19, 35, 48, 49). In contrast, the role of antibiotics in affecting the population dynamics of microorganisms in the rhizosphere is not clear. However, Mazzola et al. (25) reported that the production of phenazine antibiotics by *Pseudomonas fluorescens* 2-79 and *Pseudomonas aureofaciens* 30-84 contributes to their ecological competence in soil in the rhizosphere of wheat by enhancing their ability to compete with indigenous microflora. Broadbent et al. (4) found that although 40% of 3,500 soilborne bacteria expressed in vitro inhibition against one or more plant pathogens, only 4% were effective inhibitors of the same pathogens in soil. Although phenazine-1-carboxylic acid (41) and 2,4-diacetylphloroglucinol (12) have been isolated from the rhizosphere of wheat, antibiotics have, in general, only rarely been isolated from soil, presumably because of sporadic production, rapid inactivation, lack of sensitive methods (46), or adsorption by soil colloids (37).

The most critical work on the role of antibiotics in the soil-root environment has been done with the use of mutants that do not produce antibiotics. Thomashow et al. (39–41) related the inhibition of wheat root infections from *Gaeumannomyces graminis* var. *tritici* by *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 to antibiotic production. They showed that Tn*5* transposon mutants of these strains deficient in production of the antibiotic phenazine lost much of the ability to control the fungus. When phenazine production was restored by wild-type DNA introduced as a cosmid clone, the strains regained the ability to prevent the fungal infections. These researchers further detected phenazine on healthy and *G. graminis* var. *tritici*-infected wheat roots that were colonized by strain 2-79, and they related this to inhibition of the pathogen (41).

However, the importance of antibiotic production by root-colonizing microorganisms may not necessarily be confined to the rhizosphere or rhizoplane. A study by Kempf et al. (15) suggests that antibiotics may prevent infections by being absorbed into plant tissues. The antibiotic herbicolin, which inhibits *Fusarium culmorum* infections on wheat, was detected in the crown and contiguous roots of wheat seedlings that had high populations of *Erwinia herbicola* in the spermosphere and rhizosphere.

Assuming that microbial metabolites play an important role in influencing the dynamics of seed and root colonization, there is a need for a much better understanding of the factors that affect their production in situ. In general, antibiotics accumulate during the mid-exponential and stationary phases of growth in culture and their biosynthesis is affected by substrates. James and Gutterson (14) showed that antibiotics produced in vitro by a strain of *P. fluorescens* were differentially regulated by glucose. Shanahan et al. (34) reported that in vitro production of an antibiotic by a *Pseudomonas* sp. was influ-

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enced by temperature, medium composition, and oxygen availability. In strains *P. fluorescens* 2-79 and *P. aureofaciens* 30-84, phenazine biosynthesis was influenced by the source of nitrogen. Higher amounts of antibiotics were synthesized when nitrate was the nitrogen source; in contrast, ammonium nitrogen did not favor phenazine biosynthesis (38).

P. aureofaciens PGS12 is a root-colonizing bacterium originally isolated from roots of corn and is inhibitory to numerous plant pathogens in vitro. This strain produces a number of compounds with antimicrobial properties such as phenazine and pyrrolnitrin antibiotics, hydrogen cyanide, siderophores, indoleacetic acid, and proteases. Phenazines appear as a pigment around red bacterial colonies, and in older cultures they form red and yellow crystals embedded in the agar medium. In culture PGS12 often loses the ability to produce phenazine, hydrogen cyanide, and proteases simultaneously, because of a spontaneous mutation (13). PGS12 in greenhouse tests caused growth promotion of celery cultivars and was inhibitory to celery wilt caused by *Fusarium oxysporum* f. sp. *apii*. However, in fields infested with the pathogen, PGS12 populations increased early plant growth and yield on only one of three cultivars grown at the same location (3). Although the mechanism of growth promotion was not investigated, it raised the question of whether the production of the antifungal factor was influenced by the celery cultivar.

The purpose of this study was to clone a chromosomal locus involved in phenazine biosynthesis in PGS12, to create reporter gene insertions into this locus, and to monitor the culture conditions that influence the expression of this locus. The ice nucleation reporter gene system (22, 23) was chosen because it affords high sensitivity, since, theoretically, one cell expressing the gene can act as one ice nucleus. The ice nucleation assay is a rapid, simple technique, suitable for experiments in culture and in planta, and does not require tissue processing, other than grinding or washing and serial dilutions.

(Some of this work has already been reported [10].)

MATERIALS AND METHODS

Chemical supplies. Chemicals were from Fisher Scientific (Pittsburgh, Pa.) and Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted. Agarose was from BioRad Laboratories (Richmond, Calif.); agar, proteose peptone, and yeast extract were from Difco Laboratories (Detroit, Mich.); and antibiotics were from Sigma Chemical Co.

Bacteria, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise noted, *P. aureofaciens* PGS12 was grown in King's B (KB) (16) or Luria-Bertani medium (LB) (24) at 28°C. *Escherichia coli* ED8654 and DH5α were grown in LB at 37°C. For selection purposes, unless otherwise noted, antibiotics were used at the following concentrations (in micrograms per milliliter) from stock solutions: kanamycin, 100; tetracycline, 20 (for selection of *E. coli*) and 150 (for selection of PGS12); streptomycin, 25; and ampicillin, 50. The high concentration of tetracycline used for PGS12 was because PGS12 is naturally resistant to at least 100 μg of tetracycline per ml, as well as to ampicillin, streptomycin, spectinomycin, and nalidixic acid.

Molecular and genetic techniques. All conjugations were performed in triparental matings with *E. coli* HB101 which harbored the helper plasmid pRK2013 (8). Chromosomal DNA from *P. aureofaciens* was isolated by the method of Chesney et al. (7). Small-scale plasmid DNA isolation from *E. coli* was done by using the Circleprep kit (BIO101, La Jolla, Calif.) according to the instructions of the supplier, or by the alkaline lysis method (24). Small-scale cosmid DNA was isolated from *E. coli* and *P. aureofaciens* by the alkaline lysis method. Large-scale plasmid and cosmid DNA preparations were performed by alkaline lysis followed by cesium chloride density gradient centrifugation (24). Restriction enzyme digestions were performed as recommended by the enzyme suppliers (Gibco-BRL, Gaithersburg, Md., and Amersham Corporation, Arlington Heights, Ill.). Gel electrophoresis (24) was performed in agarose gels (1% [wt/vol]), and Southern trans-

TABLE 1. Bacterial strains and plasmids used in this study and relevant characteristics

Strain or plasmid	Characteristics[a]	Source or reference(s)
E. coli		
DH5α	F⁻ *recA1 gyrA lac*ZDM15	Gibco BRL
ED8654	*metB hsdR hsdM*⁺ *supE supF*	31
HB101	*hsdR recA proA leu-6 ara-14 galK2 lacY1 xyl-5 mtl-1 str-20 thi-1 supE44*	24
JM101	*lacI*^{q} Δ(*lacZ*)*M15 proAB supE thi*	24
P. aureofaciens		
PGS12	Wild type	3, 13
02A1	PGS12::Tn*5-259.7* Phz⁻ HCN⁺	This study
41B6	PGS12::Tn*5-259.7* Phz⁺ HCN⁻	This study
15E5	PGS12::Tn*5-259.7* Phz⁻ HCN⁻	This study
EH5, FB8, FH1	PGS12::Tn*3*-spice Phz⁻ HCN⁻ *inaZ*⁺ marker exchange derivatives	This study
Plasmids		
pLAFR3	IncP-I *lacZ cos*⁺ *rlx*⁺ Tc^r	27
pRK2013	IncP *traRK2*⁺ *repRK2 repE1* Km^r	8
pGEM3Zf(+)	Ap^r	Promega
pME12	IncP-1 RepA(ts) Tra Tc^r Hg^r Km^r Tn*5-259.7*	31
pME495	ColE1-replicon, Km^r Hg^r Tn*5-259.7*	31
pPHG3	pGEM3Zf(+) carrying a PGS12 genomic DNA insert; Ap^r Km^r	This study
pCNG3	pGEM3Zf(+) carrying a PGS12 genomic DNA insert; Ap^r Km^r	This study
pPCNG3	pGEM3Zf(+) carrying a PGS12 genomic DNA insert; Ap^r Km^r	This study
pPHE12	pLAFR3 carrying PGS12 genomic DNA; Tc^r, complements Phz⁻ mutant	This study
pPHE15	pLAFR3 carrying PGS12 genomic DNA; Tc^r, complements Phz⁻ mutant	This study
pPHE22	pLAFR3 carrying PGS12 genomic DNA; Tc^r, complements Phz⁻ mutant	This study
pHCN27	pLAFR3 carrying PGS12 genomic DNA; Tc^r, complements HCN⁻ mutant	This study
pHCN35	pLAFR3 carrying PGS12 genomic DNA; Tc^r, complements HCN⁻ mutant	This study
pHCN40	pLAFR3 carrying PGS12 genomic DNA; Tc^r, complements HCN⁻ mutant	This study
pTn*3*-spice	pMB8 replicon carrying transposon Tn*3*-spice, Ap^r Str^r Spc^r	22
pICE1	pLAFR1 carrying the *iceC* gene from *P. syringae* Cit7	26

[a] Tc^r, Km^r, Ap^r, Str^r, Spc^r, and Hg^r indicate resistance to tetracycline, kanamycin, ampicillin, streptomycin, spectinomycin, and mercury, respectively. Phz and HCN indicate production, or coding for, phenazine and hydrogen cyanide, respectively.

fers were done on Nytran membranes according to the instructions of the supplier (Schleicher and Schuell, Inc., Keene, N.H.). For colony hybridization, colonies of a PGS12 genomic DNA library were transferred with a replicator with metal prongs onto circular Nytran membranes (diameter, 8.2 cm; pore size, 0.45 μm; Schleicher and Schuell). Membranes were treated according to standard methods (24). Probe DNA was labeled with the nonradioactive Genius labeling kit, according to the instructions of the supplier (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) or with [α-^{32}P]dCTP (10^6 cpm), using the multiprime labeling system (Amersham Corp., Arlington Heights, Ill.). Autoradiography was carried out at −80°C with Kodak X-Omat AR film in a cassette with one intensifying screen. Ligations using T4 DNA ligase (Gibco-BRL) were performed according to standard methods (24). Alkaline phosphatase (Boehringer Mannheim) treatments were done according to the instructions of the supplier. Transformations were performed by the calcium chloride procedure as described by Maniatis et al. (24).

Construction of PGS12 genomic library. A genomic library of PGS12 was constructed as described by Peet et al. (27) by using the cosmid vector pLAFR3. Recombinant molecules were packaged in vitro and transduced into strain DH5α.

Detection of hydrogen cyanide. Bacterial strains were transferred to 96-well microtiter dishes (Becton Dickinson and Co., Oxnard, Calif.) containing 0.3 ml of KB agar, supplemented with 50 mM glycine and 0.01 mM ferric chloride. Filter paper (Whatman No. 1) cut to the dimensions of the microtiter dish and impregnated with the HCN detection solution (6), was suspended over the wells of the dish. The HCN detection solution consisted of copper(II)-ethyl-acetoacetate (5 mg) and 4,4′-methylenebis-(*N*,*N*-dimethylaniline) (5 mg) (Eastman Kodak Co., Rochester, N.Y.) diluted in 1 to 2 ml of chloroform. A positive reaction, indicating production of HCN by the respective strain, was determined by the formation of a dark blue spot on the filter paper above the strain, after the microtiter dishes were incubated for 24 h at 28°C (6).

Transposon mutagenesis of strain PGS12 and characterization of mutants. The suicide plasmid pME12 (31) was used to generate transposon insertions in the chromosome of PGS12. This plasmid harbors the transposon Tn*5-259.7*, a derivative of Tn*5* that carries the kanamycin and mercury resistance genes (31). Mating was performed by mixing mid-log-phase cultures of *E. coli* ED8654, the host of pME12, with PGS12 Sm^r in a 1:1 ratio, along with the appropriate controls. After incubation at 28°C for 24 h, transconjugants were selected on LB with streptomycin and kanamycin. Transconjugant colonies were visually examined for production of phenazine and HCN. The number of auxotrophs and transconjugants still carrying the plasmid pME12 was determined. Genomic DNA was isolated from a few selected phenazine-nonproducing mutants, digested with *Eco*RI, and transferred onto a Nytran membrane. The blot was hybridized with Tn*5-259.7* (28) labeled with [α-^{32}P]dCTP, as described previously (24), to estimate the size of the PGS12 genomic *Eco*RI fragment that contained the transposon in each mutant.

Lack of phenazine production by selected mutants was further confirmed by paper chromatography analysis of phenazine extracts by the method of Toohey et al. (42).

Molecular cloning and complementation of mutants. Chromosomal DNA from selected Tn*5-259.7* mutants was cloned into the *Eco*RI site of pGEM3Zf(+) vector DNA (Promega Corp., Madison, Wis.). Vector and insert DNA were ligated and transformed into competent DH5α cells. Transformants were plated on LB agar containing ampicillin and kanamycin.

The plasmids constructed as described above were used as probes to screen by colony hybridization a PGS12 genomic DNA library for clones that carried phenazine and HCN biosynthetic loci. Plasmid DNA was labeled with [α-^{32}P]dCTP. Cosmid clones that hybridized with these probes were mobilized into phenazine- and HCN-nonproducing mutants of PGS12. The phenotype of the transconjugants was scored visually for restoration of phenazine and HCN production. Cosmid clones were mapped according to standard methods (24).

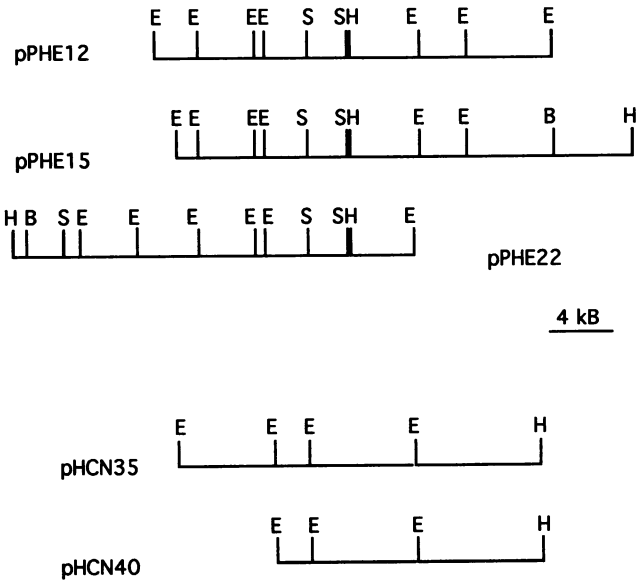

FIG. 1. Restriction endonuclease maps of cosmids that complemented phenazine production in strain 02A1 (pPHE12, pPHE15, pPHE22) and HCN production in strain 41B6 (pHCN35, pHCN40). E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Sst*I. The rightmost *Eco*RI site in the pPHE22 insert is that of the vector.

Tn*3*-spice insertion mutagenesis. The construction and properties of transposon Tn*3*-spice have been described previously (22). This transposon is a modified Tn*3-lac* element (36) in which the *lacZYA* gene sequences have been replaced with a promoterless *inaZ* ice nucleation gene (46). Tn*3*-spice allows the construction of transcriptional (not translational) target gene-*inaZ* fusions by insertional mutagenesis. The ice nucleation gene serves as a reporter of target gene transcriptional activity (22). The element also contains a spectinomycin-streptomycin resistance cassette as an additional marker.

Plasmid pPHE12 (Fig. 1), which complements a Phz$^-$ mutant, was chosen for further study. Tn*3*-spice derivatives of plasmid pPHE12 were constructed by random mutagenesis according to Rahme et al. (30).

To identify Tn*3*-spice insertions into phenazine-coding regions of pPHE12, all Tn*3*-spice derivatives of pPHE12 were tested for complementation of phenazine production in strain 02A1, a Phz$^-$ mutant of PGS12 (Table 1). Mutated plasmids were transferred to strain 02A1 using the helper HB101 (pRK2013) (8). Transconjugants were selected on KB agar with kanamycin, tetracycline (150 μg/ml), and spectinomycin (1,000 μg/ml). Plasmids which did not complement phenazine production were further analyzed. The positions of insertion of Tn*3*-spice in mutant plasmids were mapped with single and double digestions with *Eco*RI and *Hin*dIII.

Construction of marker exchange haploids. The wild-type PGS12 is not a good recipient in matings with *E. coli* HB101,

DH5α, or SF800 (36). Therefore, marker exchange of Tn*3*-spice derivatives of pPHE12 was carried out in strain 02A1 (Table 1), which is a better recipient. Merodiploids were grown in KB broth without tetracycline selection and were subcultured twice a day for 5 days to allow loss of the cosmid and recombination of homologous sequences into the chromosome. The cultures then were diluted and plated onto KB supplemented with spectinomycin. Presumed marker exchange haploids were tested by plating onto KB agar with tetracycline and KB agar with kanamycin. Sensitivity to tetracycline indicated loss of the cosmid, and sensitivity to kanamycin indicated loss of the transposon Tn*5-259.7* from the chromosome of 02A1, suggesting that phenazine-coding sequences with Tn*3*-spice insertions from pPHE12 had integrated into the chromosome of strain 02A1. The presence of Tn*3*-spice in the chromosomal DNA of marker exchange derivatives was confirmed by Southern analysis using the *Eco*RI fragment containing the ice nucleation gene *inaZ* (47) as the hybridization probe. The fragment was isolated from plasmid pMSW10 (47) by digestion with *Eco*RI and elution from an agarose gel with the Geneclean kit (BIO101) according to the manufacturer's instructions.

Gene expression assays. The ice nucleation activity of *inaZ* fusions into strain 02A1 was quantified by a droplet freezing assay on paraffin-coated aluminum foil sheets floated on an ethanol-water cooling bath at −9°C, as described by Lindow (23). Bacteria were grown in 5-ml broth cultures at 24°C for 24 h. Cells grown in Ayers' minimal broth (2) with 10 mM glutamine were used as the starter inoculum, and all cultures had an initial optical density at 600 nm of 0.05 to 0.1. Cell concentrations were determined by plating on KB with spectinomycin. Twenty 10-μl droplets from each dilution were placed on the aluminum foil sheet, and the number of droplets that froze was scored after 3 to 5 min. The ice nucleation frequency is determined as $N(T) = 1n\,[1/(1 - f)] \cdot [10^D/V] \cdot [1/C]$, where D is the number of 10-fold serial dilutions, f is the fraction of frozen droplets, V is the volume of droplets (in milliliters), and C is the cell concentration of each sample (22, 23, 44).

Effect of culture conditions on gene expression. The effect of different substrates found in root and seed exudates, iron concentration, pH, and substrate concentration on ice nucleation activity of *inaZ* fusion haploids was investigated. Treatments were replicated three times, and all experiments were repeated at least twice. Sugars, organic acids, and amino acids were added at 10 mM in Ayers' minimal broth amended with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and micronutrients, from a Huntner's stock solution (40 ml/liter) (11). The effect of selected combinations of substrates was also investigated. Ferric chloride was incorporated in KB broth at 2, 10, 50, and 100 mM from a filter-sterilized stock solution. The pH of KB broth was adjusted to various levels ranging from pH 5 to pH 8.5 by adding solutions of either 10% potassium hydroxide or 10% hydrochloric acid in the medium. To determine the effect of nutrient level on gene expression, marker exchange derivatives were grown in KB broth diluted in sterile distilled water at the following rates: 1/2, 1/5, 1/10, 1/20, and 1/50.

Statistical analysis. Analysis of variance combined with Fisher's least significance multiple comparison procedure was used to analyze data from the ice nucleation experiment (MSTAT program, Michigan State University, 1987).

RESULTS

Characterization of phenazine and HCN mutants. Three phenotypic classes of mutants were obtained from 10 separate matings: mutants defective in production of both phenazine and hydrogen cyanide (Phz^- HCN^-, 5%); mutants defective in phenazine production (Phz^-, 0.2%); and mutants defective in hydrogen cyanide production (HCN^-, 0.04%). One percent of the mutants were auxotrophic, and 17% retained the suicide vector.

Mutants 02A1 (Phz^-), 41B6 (HCN^-), and 15E5 (Phz^- HCN^-) were selected for further investigation. Tn*5-259.7* had inserted into a 10-kb *Eco*RI fragment in 02A1, a 12-kb *Eco*RI fragment in 41B6, and a 7.2-kb *Eco*RI fragment in 15E5 (data not shown). Mutants 02A1 and 15E5 produced none of the phenazines synthesized by PGS12, as determined by paper chromatography (42).

Molecular cloning of chromosomal loci involved in the production of phenazine and HCN and complementation of the mutants. Transposon Tn*5-259.7* carries kanamycin and mercury resistance genes. The transposon contains a single *Eco*RI site within the mercury resistance gene. PGS12 chromosomal DNA flanking one side of Tn*5-259.7* was cloned from Tn*5-259.7* insertion mutants by digesting chromosomal DNA with *Eco*RI, ligating the DNA with *Eco*RI-digested pGem3Zf (+) DNA, and selection on medium containing kanamycin and ampicillin after transformation into JM101. Plasmid pPHG3 contained chromosomal DNA from strain 02A1 (Phz^-), which is involved in phenazine biosynthesis by PGS12. Similarly, plasmids pCNG3 and pPCNG3 contained chromosomal DNA flanking Tn*5-259.7* from mutants 41B6 and 15E5, respectively. A total of 3,990 cosmid clones from the PGS12 library were analyzed by colony hybridization for homology with plasmids pPHG3, pCNG3, and pPCNG3 as probes. Cosmids pPHE12, pPHE15, and pPHE22 were identified by using pPHG3 as a probe; these cosmids fully restored phenazine production when mobilized into 02A1. Similarly, cosmids pHCN27, pHCN35, and pHCN40 were identified by probing with pCNG3, and all three restored HCN production in 41B6. The pPCNG3 probe identified two cosmids in the library. However, none of these complemented the phenazine and HCN mutations in 15E5. This mutation was also not complemented when pPHE12, pPHE15, pPHE22, pHCN27, pHCN35, or pHCN40 was introduced into strain 15E5.

Restriction endonuclease maps for pPHE12, pPHE15, and pPHE22 are shown in Fig. 1. These cosmids are overlapping, as is evident from their restriction patterns. Maps for cosmids pHCN35 and pHCN40 also are shown in Fig. 1. These cosmids overlap each other, and their restriction pattern showed no similarity to the pattern of the cosmids which complement the Phz^- mutant. The restriction pattern of the two cosmids identified with probe pPCNG3 (not shown) had no similarities to those of any of the cosmids in Fig. 1.

Tn*3*-spice insertion mutagenesis. Transposon insertions in pPHE12 were mapped and are shown in Fig. 2. Tn*3*-spice insertions inside a 6-kb region of the 10-kb *Eco*RI fragment of pPHE12 abolished complementation of phenazine production in strain 02A1. Insertions in other areas of the cosmid did not affect complementation of phenazine; however, two insertions within ≈100 bp of each other in the leftmost 2.8-kb *Eco*RI fragment of the pPHE12 insert (Fig. 2) also abolished phenazine complementation in 02A1(pPHE12:Tn*3*-spice) merodiploids. The ice nucleation activity of all insertions that affected phenazine complementation was determined when conjugated into 02A1. Insertions that were ice nucleation active (Ina^+) also determined the orientation of the transcription in this locus (Fig. 2).

Selected Tn*3*-spice insertions in pPHE12 which expressed high levels of ice nucleation activity and which did not complement phenazine production were recombined into the corresponding regions of the 02A1 chromosome by marker

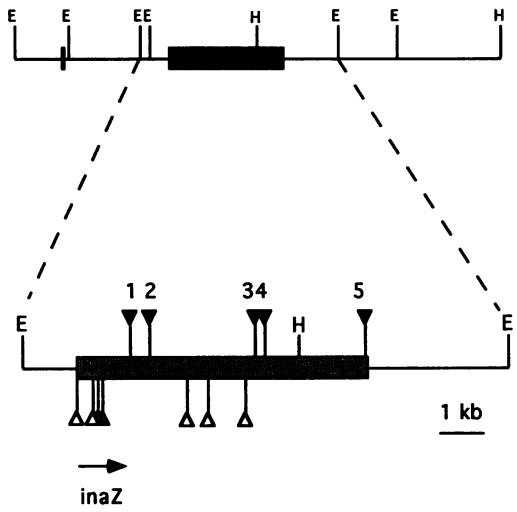

FIG. 2. Mapping of Tn*3*-spice insertions in pPHE12. The shaded box in the 10-kb *Eco*RI fragment and the vertical bar in the leftmost 2.8-kb *Eco*RI fragment represent the area of the cosmid that contains DNA involved in phenazine biosynthesis, based on complementation analysis of each insertion. Tn*3*-spice insertions outside of the shaded box in the 10-kb *Eco*RI fragment did not affect complementation of strain 02A1. Triangles represent ice nucleation reporter gene insertions. The ice nucleation activity of the insertions was tested in plasmid-borne *inaZ* fusions. ▲, ice nucleation was expressed; △, ice nucleation was not expressed. The orientations of these two sets of insertions were left to right and right to left, respectively. The arrow shows the orientation of transcription in the region, deduced from the ice nucleation phenotype of the plasmid-borne *inaZ* fusions. 1, AC2; 2, FH1; 3, FE3; 4, EH5; 5, FB8.

exchange to yield strains EH5, FB8, and FH1. These strains carried a fusion of the *inaZ* reporter gene in phenazine-coding or regulatory regions of 02A1, by replacement of the Tn*5-259.7* insertion with the Tn*3*-spice insertion. For strain FH1, EH5, and FB8, 95, 96, and 84%, respectively, of recombinant colonies that were selected on KB with spectinomycin were marker exchange haploids. Either the rest of the colonies retained the cosmid or the reporter gene had inserted into the chromosome without simultaneous loss of Tn*5-259.7*. The ice nucleation frequencies (no. of log nuclei per cell) of marker exchange haploids of FH1, EH5, and FB8 were -2.28 ± 0.17, -1.95 ± 0.12, and -2.04 ± 0.09, respectively. Growth rates of marker exchange derivatives were the same as that of the PGS12 wild type in KB and Ayers media. Marker exchange haploids were not generated for Tn*3*-spice insertions AC2 and FE3 (Fig. 2), which expressed low ice nucleation activity, or for Tn*3*-spice insertions in the leftmost 2.8-kb *Eco*RI fragment (Fig. 2), which had negligible ice nucleation activity (data not shown).

Expression levels of haploid derivatives with reporter gene fusions in the chromosome were slightly higher than the levels measured in merodiploids when the reporter gene was in a plasmid. Expression levels of the *iceC* gene transcribed from its native promoter in plasmid pICE1 (26) were lower than those measured when the *inaZ* reporter gene was transcribed from the phenazine locus promoter (data not shown).

Effect of culture conditions on gene expression. The marker exchange derivative strain EH5 was used in all of the following experiments. The expression levels induced by individual substrates (commonly found in root exudates) ranged from −6.51 (glycerol) to −9.08 (malic acid) log nuclei per cell (Table 2).

TABLE 2. Effect of carbohydrates, organic acids, and amino acids on reporter gene expression[a]

Substrate	Nucleation frequency (log nuclei/cell)[b]
Glycerol	−6.51 ± 0.59 (a)
Sucrose	−6.83 ± 0.48 (ab)
Arginine	−7.19 ± 0.09 (bc)
Galactose	−7.19 ± 0.21 (bcd)
γ-Aminobutyric acid	−7.33 ± 0.12 (bcde)
Arabinose	−7.44 ± 0.08 (bcdef)
Mannose	−7.56 ± 0.50 (cdefg)
Fructose	−7.63 ± 0.23 (cdefg)
Glucose	−7.83 ± 0.10 (cdefgh)
Histidine	−7.87 ± 0.17 (defgh)
Alanine	−7.89 ± 0.13 (efghi)
Succinic acid	−8.01 ± 0.09 (fghi)
Glutamine	−8.07 ± 0.27 (fghi)
Shikimic acid	−8.12 ± 0.09 (ghi)
Citric acid	−8.32 ± 0.06 (hij)
Anthranilic acid	−8.44 ± 0.33 (hijk)
Propionic acid	−8.56 ± 0.32 (ijk)
Glutamic acid	−8.92 ± 0.23 (jk)
Aspartic acid	−9.01 ± 0.08 (k)
Malic acid	−9.08 ± 0.06 (k)

[a] Each substrate was added to Ayers' minimal broth at 10 mM. Cultures of strain EH5 were grown for 24 h.

[b] Values represent means ± standard errors. Values followed by the same letter are not significantly different when compared with Fisher's least significant difference at $P = 0.05$.

Slightly higher expression levels (−5.15 to −9.36 log nuclei per cell) were induced when a combination of a carbohydrate or an organic acid and an amino acid were added to the medium (Table 3). Substrate combinations that included phenylalanine, tryptophan, and shikimic acid induced the highest expression of the reporter gene. In contrast, combinations with anthranilic acid induced significantly lower expression levels. In most cases, higher expression levels correlated with production of phenazine by cultures of PGS12 grown in the same substrate combinations. In one exception, phenazine was produced with substrate combinations that included histidine, although the expression of the reporter gene in these cases was low (Table 3).

Concentrations of iron ranging from 2 to 100 mM did not affect the expression levels of the reporter gene. The highest expression was achieved at pH 7.50 to 8.52 and decreased slightly at pH levels ranging from 5.76 to 7.07. Expression decreased significantly at pH 4.99 (Table 4).

Strain EH5 was grown in various dilutions of KB broth with progressively lower nutrient availability. Expression decreased dramatically as the dilution factor of KB broth increased. After 48 h, the expression in undiluted KB broth was −0.65 log nuclei per cell, whereas the expression in KB broth diluted 1/50 was −5.27 log nuclei per cell (Table 5). Cell growth was not affected as much as *inaZ* expression. The cell count in the 1/50 dilution was approximately 10 times lower than that in the undiluted KB broth (1 log CFU/ml) (data not shown). The expression levels observed in KB broth were always several orders of magnitude higher than the expression in defined media (Tables 2, 3, and 5).

DISCUSSION

The high percentage of mutants that were deficient in both phenazine and hydrogen cyanide biosynthesis after Tn*5-259.7* transposon mutagenesis of *P. aureofaciens* PGS12 complicated the efforts to clone a locus involved in phenazine biosynthesis.

TABLE 3. Effect of combinations of substrates on reporter gene expression[a]

Substrate	Nucleation frequency (log nuclei/cell)[b]
Sucrose/phenylalanine	−5.15 ± 0.25 (a) (P)
γ-Aminobutyric acid/phenylalanine	−5.24 ± 0.36 (a) (P)
Shikimic acid/histidine	−5.28 ± 0.06 (a) (P)
Glycerol/phenylalanine	−5.56 ± 0.24 (ab) (P)
Shikimic acid/tryptophan	−5.87 ± 0.04 (bc)
Shikimic acid/phenylalanine	−6.22 ± 0.02 (cd) (P)
Shikimic acid/Casamino acids	−6.25 ± 0.05 (cde) (P)
Sucrose/tryptophan	−6.50 ± 0.21 (def)
Glycerol/tryptophan	−6.68 ± 0.43 (efg)
Glucose/phenylalanine	−6.79 ± 0.12 (fgh) (P)
γ-Aminobutyric acid/Casamino acids	−7.02 ± 0.22 (gh)
γ-Aminobutyric acid/tryptophan	−7.06 ± 0.07 (gh)
Anthranilic acid/phenylalanine	−7.17 ± 0.13 (h)
Sucrose/glutamine	−7.19 ± 0.07 (h)
γ-Aminobutyric acid/glutamine	−7.20 ± 0.07 (h)
Glycerol/glutamine	−7.20 ± 0.04 (h)
Sucrose/histidine	−7.73 ± 0.03 (i) (P)
Shikimic acid/glutamine	−7.74 ± 0.02 (i)
Glucose/glutamine	−7.78 ± 0.14 (i)
Anthranilic acid/glutamine	−7.93 ± 0.05 (ij)
Anthranilic acid/histidine	−8.15 ± 0.24 (ijk)
Glucose/tryptophan	−8.29 ± 0.10 (jkl)
Sucrose/Casamino acids	−8.37 ± 0.06 (jkl)
γ-Aminobutyric acid/histidine	−8.38 ± 0.16 (kl) (P)
Glucose/histidine	−8.45 ± 0.10 (klm) (P)
Glycerol/histidine	−8.52 ± 0.18 (klm) (P)
Anthranilic acid/Casamino acids	−8.68 ± 0.05 (lm)
Glucose/Casamino acids	−8.68 ± 0.11 (lm)
Anthranilic acid/tryptophan	−8.86 ± 0.02 (m)
Glycerol/Casamino acids	−9.36 ± 0.24 (n)

[a] Combinations included one carbohydrate or organic acid with one amino acid. Each substrate was added to Ayers' minimal broth at 10 mM. Cultures of strain EH5 were grown for 24 h.

[b] P, production of phenazine by PGS12 grown under identical conditions. Values represent means ± standard errors. Values followed by the same lowercase letter are not significantly different when compared with Fisher's least significant difference at $P = 0.05$.

Moreover, this mutation was not complemented by mobilizing cosmids from a PGS12 genomic library that contained the corresponding wild-type genomic DNA. Doubly deficient mutants that lose both phenazine and hydrogen cyanide biosynthesis occur frequently in PGS12 (13). It is possible that a large number of the doubly deficient mutants seen after transposon mutagenesis simultaneously contain a transposon insertion and a spontaneous mutation(s) in unrelated regions of the chromosome. Therefore, individual cosmids may not contain genes that could complement both phenazine and hydrogen cyanide production in these mutants. It is also possible that the region affected in the (doubly deficient) mutants was not present in the genomic library, that the mutation in 15E5 resulted in deletion of large regions that cannot be cloned intact in a single cosmid, or that the mutation occurred in a *cis*-acting regulatory element. The mutation(s) responsible for the Phz^-HCN^- phenotype in strain 15E5 is not in the same locus as the mutations in either 02A1 (Phz^-) or 41B6 (HCN^-), since cosmids that contained this locus had no similarity to any of the cosmids that complemented phenazine production in 02A1 and hydrogen cyanide production in 41B6. The nature of the mutation(s) leading to the double phenotypic defect in 15E5 or other similar mutants is unknown.

TABLE 4. Effect of pH on phenazine locus expression in EH5

pH	Nucleation frequency (log nuclei/cell)[a]
4.99	−7.03 ± 0.24 (d)
5.76	−2.98 ± 0.16 (c)
6.53	−2.65 ± 0.16 (c)
7.07	−2.14 ± 0.15 (b)
7.50	−1.62 ± 0.10 (a)
8.02	−1.67 ± 0.08 (ab)
8.52	−1.65 ± 0.09 (a)

[a] Values represent means ± standard errors. Values followed by the same letter are not significantly different when compared with Fisher's least significant difference at $P = 0.05$.

TABLE 5. Effect of substrate concentration on reporter gene expression

Dilution	Nucleation frequency (log nuclei/cell)[b]
1/1	−0.65 ± 0.20 (a)
1/2	−2.03 ± 0.13 (b)
1/5	−2.95 ± 0.13 (c)
1/10	−3.37 ± 0.16 (c)
1/20	−4.70 ± 0.26 (d)

[a] Cultures of strain EH5 were grown for 24 h in various dilutions of KB broth with sterile distilled water.

[b] Values represent means ± standard errors. Values followed by the same letter are not significantly different when compared with Fisher's least significant difference at $P = 0.05$.

Strain 02A1 lacked the ability to synthesize any of the phenazine pigments produced by PGS12. This suggests that the mutation affected a regulatory gene or an early step(s) of the phenazine biosynthetic pathway. Cloning of phenazine biosynthetic loci in *P. aureofaciens* 30-84 indicated that there is a core region essential to the production of all phenazine pigments, phenazine-1-carboxylic acid, 2-hydroxyphenazine-1-carboxylic acid, and 2-hydroxyphenazine (28). Mapping of the Tn*5-259.7* insertion in the chromosome of 02A1 and of cosmids pPHE12, pPHE15, and pPHE22 suggested that the phenazine biosynthetic locus of PGS12 cloned in this study is contained in a 10-kb *Eco*RI DNA fragment. This 10-kb *Eco*RI fragment was found by Southern hybridization to share homology with the phenazine biosynthetic regions of *P. aureofaciens* 30-84 (28) and *P. fluorescens* 2-79 (38) (results not shown). Further mapping of Tn*3*-spice insertions in pPHE12 located a 6-kb region inside the 10-kb *Eco*RI fragment which was essential for complementation of phenazine biosynthesis in strain 02A1. In addition, a small region within the 2.8-kb *Eco*RI fragment upstream of the one characterized above was also involved in phenazine biosynthesis. The ice nucleation activities of Tn*3*-spice insertion mutants in this region were low, suggesting a regulatory role for this region.

The ice nucleation reporter gene fused to the cloned phenazine biosynthetic locus in *P. aureofaciens* PGS12 provided useful information about the effects of various nutrients and other culture parameters on the expression of this locus. This method is a good, although indirect, alternative to quantification of phenazines by chemical extraction because of its simplicity and accuracy. The most critical factor that affected the expression of this locus appeared to be nutrient availability. In cultures with progressively fewer nutrients, expression decreased several orders of magnitude whereas cell growth was not much affected. The expression of this locus also was affected by different substrates commonly found in seed and root exudates and substrate combinations. The substrates alanine, aspartic acid, citric acid, glutamic acid, malic acid, and succinic acid have previously been shown not to support

phenazine-1-carboxylic acid (20, 21) in *P. aureofaciens* 517. Our results indicate that expression of the phenazine biosynthetic locus in *P. aureofaciens* PGS12 in the presence of these substrates was low.

In fluorescent pseudomonads, precursors for phenazine biosynthesis are derived from the shikimic acid pathway, with chorismic acid as the probable branchpoint (43). Glutamine is the probable origin of nitrogen in the phenazine molecule. However, when shikimic acid and glutamine were combined in a mineral salts medium, the expression levels were low. In contrast, the aromatic amino acids tryptophan and phenylalanine induced higher expression levels. A similar observation was made during an analysis of phenazine-1-carboxylic acid in *P. aureofaciens* (20, 21) and pyocyanine biosynthesis in *Pseudomonas aeruginosa* (43). Phenylalanine is not a precursor of pyocyanine (a compound that belongs in the phenazine family), but it stimulated its production. It was suggested (43) that when phenylalanine is available, its biosynthesis from the chorismic acid pathway is repressed and the pathway is then diverted to phenazine biosynthesis.

Substrate combinations that supported greater expression of the reporter gene correlated with phenazine synthesis in identical cultures of PGS12. One exception to this finding was with combinations involving histidine. PGS12 synthesized phenazine when histidine was present, although the expression of the reporter gene was low. It is possible that histidine is involved at a later step of phenazine biosynthesis than the one controlled by the locus with the reporter gene fusion.

Expression of the phenazine locus in strain EH5 was only slightly affected by iron concentration and pH levels of 6 to 8.5. At pH 5, expression was significantly reduced. This is in contrast to the biosynthetic locus of *P. aureofaciens* 30-84 in which expression at pH 5 was delayed, before achieving the same level at 26 h as at pH 6 to 8.

Engineering for early expression of antibiotic genes (disengaging the control mechanism from secondary metabolism) may be feasible once the regulation of antibiotic biosynthesis is fully understood. Evaluation of the physiological factors that are involved in antibiotic biosynthesis in culture should help to gain a better understanding of the factors that affect antibiotic production in nature. In the soil environment, lack of nutrients is recognized as the most important factor limiting antibiotic production by microorganisms (46). These results suggest that antibiotic production should be inherently greater at sites with good nutrient availability. Reporter gene fusions offer the opportunity to accurately monitor antibiotic biosynthesis in the soil and provide new insights in the biology of soil microorganisms.

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