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Phenazine Antibiotic Biosynthesis in *Pseudomonas aureofaciens* 30-84 Is Regulated by PhzR in Response to Cell Density

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We have identified a gene that acts in *trans* to activate the expression of the phenazine biosynthetic genes in the biological control organism *Pseudomonas aureofaciens* 30-84. This gene, *phzR* (phenazine regulator), is located upstream of and divergently transcribed from the phenazine biosynthetic genes. Thus, the phenazine biosynthetic locus consists of at least two divergently transcribed operons. A functional *phzR* gene is required for phenazine production. The nucleotide sequence of *phzR* revealed an open reading frame of 723 nucleotides encoding a protein of ca. 27 kDa. The predicted amino acid sequence of PhzR has homology with other bacterial positive transcriptional activators, including LasR of *Pseudomonas aeruginosa*, LuxR of *Vibrio fischerii*, and TraR of *Agrobacterium tumefaciens*. The addition of cell-free supernatants from late-exponential-phase cultures of strain 30-84 resulted in expression of a genomic *phzB*::*lacZ* reporter strain at a lower cell density than normal, indicating the possible presence of an autoinducer. These results indicate that PhzR is a member of a two-component sensor-regulator family with known or predicted carboxy-terminal DNA-binding domains which regulates gene expression in response to environmental and cell density signals.

Pseudomonas aureofaciens 30-84, when applied to wheat seeds, is capable of protecting wheat from take-all, a disease caused by the fungus *Gaeumannomyces graminis* var. *tritici* (1, 20). Strain 30-84 produces three phenazine antibiotics, phenazine-1-carboxylic acid, 2-hydroxy-phenazine-1-carboxylic acid, and 2-hydroxy-phenazine. Production of these phenazine antibiotics by strain 30-84 is primarily responsible for disease suppression (20).

In addition to inhibiting fungal pathogenesis, phenazine antibiotics play important roles in microbial competition and rhizosphere survival (16). Populations of Phz^- mutants of either *Pseudomonas fluorescens* 2-79 or *P. aureofaciens* 30-84 decline more rapidly in nonsterile soil than their isogenic Phz^+ strains. Thus, phenazine production may be important for the long-term survival of these fluorescent pseudomonads in the wheat rhizosphere.

The mechanisms regulating phenazine antibiotic biosynthesis in fluorescent pseudomonads are unknown. Because phenazine production is important for both pathogen inhibition and bacterial survival, understanding the regulation of phenazine antibiotic production has important practical implications for improving biological control of soilborne plant diseases. Phenazine antibiotics are produced only during late-exponential and stationary growth phases (28). In a previous work, Tn*5* mutants of *P. aureofaciens* 30-84 unable to produce phenazine antibiotics were identified (20). Loss of antibiotic production in these mutants resulted in loss of the ability to inhibit the fungal pathogen. Restoration of phenazine production in these mutants by a single cosmid from a genomic library of strain 30-84 resulted in restoration of pathogen inhibition both in vitro and in vivo. This cosmid, pLSP259, contained contiguous *Eco*RI fragments of 11.2 and 9.2 kb. The phenazine biosynthetic region was localized to a region of ca. 5 kb in the 9.2-kb *Eco*RI fragment.

In this paper, we report the identification, cloning, and DNA sequence of a positive regulator of phenazine antibiotic production in *P. aureofaciens* 30-84. This gene, which we have named *phzR* (phenazine regulator), activates expression of *phzB*, a gene involved in phenazine-1-carboxylic acid biosynthesis, severalfold in *trans*. A functional *phzR* is required for phenazine antibiotic production. The deduced amino acid sequence of the PhzR protein and the effect of cell-free culture supernatants on the expression of a *phzB*::*lacZ* reporter strain indicate that PhzR is a member of a family of prokaryotic transcriptional two-component activators that positively regulate gene expression in response to cell density signals (reviewed in reference 6).

MATERIALS AND METHODS

Bacteria and plasmids. Bacterial strains and plasmids are described in Table 1. A spontaneous rifampin-resistant derivative of strain 30-84 (20) was used in all studies. Strain 30-84 and its derivatives were grown at 28°C in nutrient broth yeast extract (29), pigment production medium (PPM) (11), or AB minimal medium (22). *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium (LB) containing 5 g of NaCl per liter (14). *E. coli* LE392 containing Tn*5*-B20 (25) was grown in LB and maltose (0.2%). *p*-Aminobenzoic acid was used for triparental matings at a concentration of 10 μg/ml in order to suppress phenazine antibiotic production which otherwise killed the donor bacteria (20). The antibiotics used included kanamycin sulfate (50 μg/ml), ampicillin (100 μg/ml), nalidixic acid (50 μg/ml), and rifampin (75 μg/ml). Tetracycline was used at a concentration of 25 μg/ml for *E. coli* strains and of 40 μg/ml for selecting *P. aureofaciens* exconjugants.

Triparental matings. Triparental matings into strain 30-84 or its derivatives were performed with *E. coli* DH5α as the donor and HB101(pRK2013) as the helper. Approximately equal numbers of donor, helper, and recipient were spotted onto sterile nitrocellulose filters on a plate with LB plus *p*-aminobenzoic acid and incubated at 28°C for 24 to 48 h. The filters were resuspended in 2 ml of sterile water, and an appropriate amount was spread on plates with LB plus rifampin and tetracycline and incubated at 28°C.

Tn*5lacZ* mutagenesis of cosmid pLSP259. Lysates of lamb-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or transposon	Relevant characteristics	Source or reference
P. aureofaciens		
30-84	Phz$^+$ Rif^r wild type	W. W. Bockus
30-84.28-11	Phz$^-$ Tn*5*::*phzB* Rif^r	20
30-84Z	Phz$^-$ Rif^r *phzB*::*lacZ* genomic fusion	This study
30-84R	Phz$^-$ Rif^r *phzR*::Tn*5lacZ* genomic fusion	This study
E. coli		
S17-1	*thi pro hsdR hsdM recA rpsL* RP4-2 (Tet^r::Mu) (Kan^r::Tn*7*)	24
DH5α	F$^-$ *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*argF-lacZYA*) I169 ϕ80*lacZ* ΔM15/-	GIBCO-BRL
HB101	F$^-$ *hsdS20* ($r_B^-m_B^-$) *supE44 recA1 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-5*/-	GIBCO-BRL
ET8556	*ntr*$^+$ *rbs lacZ*::IS1 *gyrA hutC*^c_k	13
LE392	F$^-$ *hsdR514* (r_k^- m_k^-) *supE44 supF58 lacY1 galK2 galT22 metB1 trpR55* λ$^-$	19
Plasmids		
pLAFR3	IncP1 Tet^r *cos*$^+$ *rlx*$^+$	26
pRK2013	IncP1 *tra oriE1* Kan^r	3
pUC18	ColE1 Amp^r	33
pIC20H	ColE1 Amp^r	15
pLSP259	pLAFR3 containing 22 kb of 30-84 chromosomal DNA	20
pLSP10-21	pLAFR3 containing the 11.2-kb *Eco*RI fragment of pLSP259	This study
pLSP10-30	pLAFR3 containing the 9.2-kb *Eco*RI fragment of pLSP259	This study
pLSP259Tn*5lac*#42	Tn*5lacZ* insertion within *phzR* in pLSP259	This study
pLSP10-21*Pst*#25 through -#39	pLAFR3 containing *Pst*I fragments from pLSP10-21	This study
pLSP20H-2.7#7	pIC20H containing the 2.7-kb *Pst*I fragment of pLSP10-21	This study
pLSP20H-2.7#12	pIC20H containing the 2.7-kb *Pst*I fragment of pLSP10-21 (opposite orientation)	This study
pLSP20H-2.7RVdel	*Eco*RV deletion of pLSP20H-2.7#7	This study
pLSP20H-2.7Bgldel	*Bgl*II deletion of pLSP20H-2.7#12	This study
pLSP2.7#11	pLAFR3 containing the 2.7-kb *Pst*I *phzR* fragment	This study
pLSP2.7#20	pLAFR3 containing the 2.7-kb *Pst*I *phzR* fragment (opposite orientation)	This study
pLSP2.7RVdel	pLAFR3 containing the *Eco*RV deletion of the 2.7-kb *Pst*I fragment containing *phzR*	This study
pLSP2.7Bgldel	pLAFR3 containing the *Bgl*II deletion of the 2.7-kb *Pst*I fragment containing *phzR*	This study
pLSP2.7#20exo#7 through -#21	Exonuclease III deletions into the 3′ end of *phzR*	This study
Transposon		
Tn*5*-B20	pSUP102::Tn*5lac*ZB20	25

da::Tn*5*-B20 were prepared from *E. coli* LE392(Tn*5*-B20) by the confluent lysis method (14). These lysates were used to introduce Tn*5*-B20 into DH5α(pLSP259) as described by Simon et al. (25). Kanamycin-resistant colonies were washed from plates with 2 ml of LB, centrifuged briefly, and resuspended in 0.2 ml of LB. To select for insertions of Tn*5*-B20 in pLSP259, the resulting cells were conjugated with ET8556 (Nal^r) for 4 h, spread on plates with LB plus nalidixic acid, tetracycline, and kanamycin, and incubated at 37°C. Alternatively, a spontaneous rifampin-resistant derivative of DH5α was used and plated on plates with LB plus rifampin, tetracycline, and kanamycin. Plasmid DNA was isolated from the exconjugants, and the location of the Tn*5*-B20 insert was determined by restriction enzyme analysis.

DNA manipulations. DNA isolations, restriction enzyme digestions, agarose gel electrophoresis, ligations, and transformation were all performed as described previously (20). Exonuclease III deletion derivatives were constructed with the Erase-A-Base kit, supplied by Promega Corporation, according to the manufacturer's instructions.

Construction of *Eco*RI deletions in pLSP259. Cosmid pLSP259 contains 11.2- and 9.2-kb *Eco*RI fragments carrying the phenazine biosynthetic region plus adjacent chromosomal DNA (Fig. 1). The phenazine biosynthetic region was localized previously to ca. 5 kb in the 9.2-kb *Eco*RI fragment (20). Cosmid pLSP259 was digested with *Eco*RI, and the resulting products were ligated and transformed into *E. coli* DH5α. Tetracycline-resistant transformants were screened for the presence or loss of either the 11.2- or 9.2-kb *Eco*RI fragment, respectively. Plasmid derivative pLSP10-21 contains the 11.2-kb *Eco*RI fragment while pLSP10-30 contains the 9.2-kb *Eco*RI fragment.

Cloning pLSP10-21 *Pst*I fragments into pLAFR3. Digestion of cosmid pLSP10-21 with *Pst*I resulted in the identification of six *Pst*I fragments in the 11.2-kb *Eco*RI fragment. These fragments were cloned into the unique *Pst*I site in pLAFR3, transformed into *E. coli* DH5α, and plated on media containing tetracycline and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Tetracycline-resistant white transformants were introduced into 30-84Z, and their effect on β-galactosidase activity was determined.

Localization of *phzR* to a ca. 0.8-kb fragment. A 2.7-kb *Pst*I

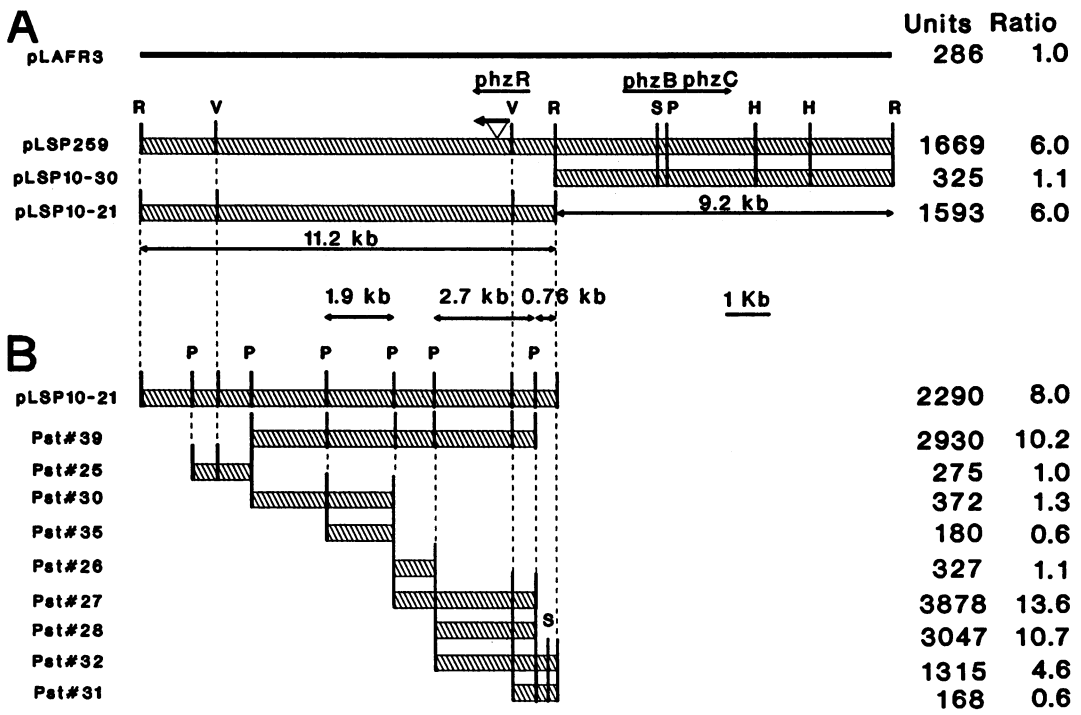

FIG. 1. Localization of the region containing *phzR*. The location of the region resulting in maximum β-galactosidase activation was determined by measuring the effects of the indicated subclones of cosmid pLSP259 on β-galactosidase expression in the *phzB*::*lacZ* reporter strain 30-84Z. Strains were grown overnight as described previously. Effect of *Eco*RI deletions of pLSP259 (A) and of *Pst*I subclones of the 11.2-kb *Eco*RI fragment of pLSP10-21 (B) on β-galactosidase expression. Thick lines indicate 30-84 genomic DNA. Thin line indicates pLAFR3 DNA. The inverted triangle above pLSP259 indicates the location and orientation of the Tn*5lacZ* insert in pLSP259Tn*5lac*#42. Units indicates average β-galactosidase units (Miller units) after 24 h (OD_{620} of ca. 0.7 to 1.0). Ratio indicates the ratio of β-galactosidase units in the presence of the introduced DNA to the β-galactosidase units in the presence of the vector alone. Restriction site abbreviations are as follows: R, *Eco*RI; V, *Eco*RV; S, *Sal*I; P, *Pst*I; H, *Hin*dIII.

fragment from pLSP10-21 was shown to confer a 10-fold increase in β-galactosidase levels in *trans* in strain 30-84Z (Fig. 1). This *Pst*I fragment was cloned into the unique *Pst*I site in vector pIC20H, and two of the resulting constructs, pLSP20H-2.7#7 and pLSP20H-2.7#12, contained the 2.7-kb *Pst*I fragment in opposite orientations. Restriction mapping indicated the 2.7-kb fragment contained a single *Eco*RV site and a single *Bgl*II site (Fig. 2A). Plasmid pLSP20H-2.7RVdel was constructed by digestion of pLSP20H-2.7#7 with *Eco*RV followed by ligation. Similarly, plasmid pLSP20H-2.7Bgldel was constructed by digestion of pLSP20H-2.7#12 with *Bgl*II. Each of these deletion derivatives was digested with *Hin*dIII and reintroduced into the *Hin*dIII site in pLAFR3. The resulting tetracycline-resistant transformants (pLSP2.7RVdel and pLSP2.7Bgldel) were screened for loss of the desired fragments, and the correct clones were introduced into strain 30-84Z by triparental mating. Plasmids pLSP2.7#11 and pLSP2.7#20, which contain the 2.7-kb *Pst*I fragment in pLAFR3 in opposite orientations, were used to construct a series of exonuclease III deletions in order to localize the ends of *phzR* (Fig. 2B).

Transactivation assays. For each strain to be tested, 3 ml of AB plus tetracycline culture was grown with shaking for 24 h at 25°C to an average optical density at 620 nm (OD_{620}) of ca. 0.7 to 0.9. Each culture was sampled and assayed for β-galactosidase activity according to the method of Miller (18). Each experiment was repeated three to five times, with strain 30-84Z or 30-84Z(pLAFR3) included as a control.

DNA sequence of *phzR*. The DNA sequence of *phzR* was determined with Sequenase 2.0 from U.S. Biochemical Corporation or was carried out by the University of Arizona Biotechnology Center on an Applied Biosystems automated DNA sequencer. DNA sequence analysis was performed with University of Wisconsin Genetics Computer Group software packages.

Expression of PhzR. The DNA sequence of *phzR* indicated that a *Nco*I restriction site was located 59 nucleotides downstream of the first possible start codon of *phzR* (Fig. 3). A second *Nco*I site was located ca. 0.9 kb further downstream by restriction mapping (not shown in Fig. 3). This 0.9-kb *Nco*I fragment was introduced into the *Nco*I site in the prokaryotic expression vector pRSETB (Invitrogen, Inc.) in both orientations. Expression of the *phzR* gene fragment was induced according to the protocol supplied with the vector, and the products were analyzed by discontinuous 10% polyacrylamide gel electrophoresis (14).

Biological assay for autoinducer. Cultures (10 ml) of the strain to be tested for the presence of an autoinducer were grown in PPM medium with aeration for 15 to 18 h at 28°C to an average OD_{620} of ca. 0.7 to 1.0. Cells were removed by centrifugation (5,820 × *g*), and the supernatants were passed through a 0.22-μm-pore-size filter. The supernatants were adjusted to pH 7.5. The indicator *phzB*::*lacZ* reporter strain 30-84Z was grown in PPM broth with aeration at 28°C to an OD_{620} of ca. 0.1 (ca. 10^8 CFU/ml). The culture was centrifuged (5,820 × *g*) and resuspended in 90% supernatant–10% fresh PPM medium. β-Galactosidase activity was measured over time as described above.

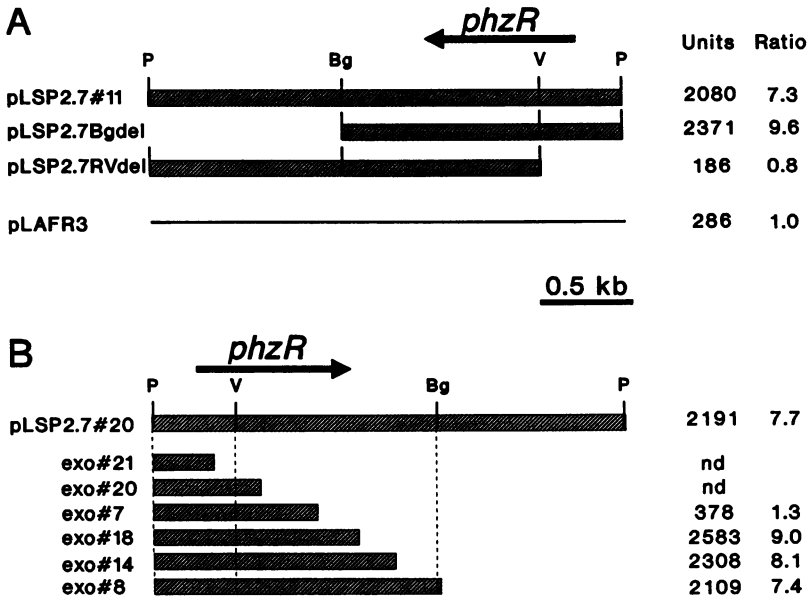

FIG. 2. Localization of *phzR*. (A) Effect of specific deletions in the 2.7-kb *Pst*I fragment containing *phzR* on transactivation. (B) Transactivation by *phzR* is orientation independent. Localization of one end of *phzR* by exonuclease III digestion. Thick lines indicate 30-84 DNA. Thin lines indicate pLAFR3 DNA. β-Galactosidase units and ratios are as defined in the legend to Fig. 1. Restriction site abbreviations are as before; Bg, *Bgl*II. nd, plasmids not assayed.

Nucleotide sequence accession number. The DNA sequence of *phzR* has been submitted to the GenBank data base under the accession number L32729.

RESULTS

Cloning of *phzR* from *P. aureofaciens* 30-84. To identify the regions of the *P. aureofaciens* genome involved in regulation of phenazine gene expression, cosmids from a genomic library of strain 30-84 were introduced in *trans* into the reporter derivative strain 30-84Z by triparental mating (Fig. 1). Strain 30-84Z contains a single-copy genomic *phzB*::*lacZ* fusion and a wild-type copy of *phzR*. The gene *phzB* is involved in the production of phenazine-1-carboxylic acid, the first of three phenazines produced by strain 30-84 (27). In strain 30-84Z, β-galactosidase activity replaces phenazine-1-carboxylic acid production. Introduction of cosmid pLSP259, which contains the 9.2-kb *Eco*RI fragment carrying the phenazine biosynthetic locus plus the 11.2-kb *Eco*RI fragment, resulted in a sixfold increase in the level of β-galactosidase expression in strain 30-84Z compared with the activity obtained with the vector pLAFR3 alone (Fig. 1A). Deletion analysis indicated that all subclones capable of activating β-galactosidase activity contained a common 2.7-kb *Pst*I fragment located in the right end of the 11.2-kb *Eco*RI fragment. The presence of additional copies of this 2.7-kb *Pst*I fragment was sufficient to result in the transactivation of *phzB* (Fig. 1B). Since this 2.7-kb *Pst*I fragment activates β-galactosidase expression in *trans*, we named the gene contained in this fragment *phzR* for phenazine regulator.

Localization of *phzR*. Deletion of the 0.5-kb *Pst*I-*Eco*RV region of pLSP2.7#11 destroyed its ability to stimulate *phzB* expression in strain 30-84Z (Fig. 2A). Deletion of the 1.0-kb *Pst*I-*Bgl*II region of pLSP2.7#20 did not affect transactivation. This localized the *phzR* coding region to a ca. 1.7-kb region. Transactivation by the 2.7-kb *Pst*I fragment was orientation independent in pLAFR3, indicating that expression of *phzR* was not dependent on an exogenous plasmid promoter (Fig. 2B). Unidirectional deletions in the region believed to contain *phzR* indicated that one end of *phzR* was located ca. 550 bp upstream from the *Bgl*II site (Fig. 2B).

Additional regions influencing *phzB* activation by PhzR. Introduction of the adjoining 0.76-kb *Pst*I fragment in addition to the 2.7-kb *Pst*I fragment containing *phzR* (pLSP10-21*Pst*#32) attenuated but did not abolish activation of the *phzB*::*lacZ* fusion by *phzR* (Fig. 1B). Introduction of the 0.76-kb *Pst*I fragment alone (pLSP10-21*Pst*#31) resulted in a decrease in the basal level of β-galactosidase expression in strain 30-84Z, as did the presence of a 1.9-kb *Pst*I fragment (pLSP10-21*Pst*#35) located to the left of the 2.7-kb *Pst*I fragment (Fig. 1A).

DNA sequence analysis and expression of *phzR* in *E. coli*. The nucleotide sequence of 1,075 bp containing the *phzR* coding region was determined by the Sanger dideoxy method (Fig. 3). Computer analysis revealed a single open reading frame consisting of 723 nucleotides which we designated *phzR*. A potential Shine-Dalgarno sequence was located 7 nucleotides upstream from the putative start of translation. The translated protein has a deduced molecular mass of 27,426 Da. The amino acid sequence of PhzR predicts a relatively hydrophilic protein, with no transmembrane-spanning domains (12). The frequency of codon usage is similar to those of other *Pseudomonas* spp., and the GC content (57%) is high throughout the *phzR* open reading frame, indicating that *phzR* represents an actual *Pseudomonas* gene (10, 32). Additionally, an A/T-rich region, located upstream of the translation start site, may be involved in transcription initiation (8).

To determine whether the proposed open reading frame could encode a protein, we tested its expression in *E. coli*. We placed the 0.9-kb *Nco*I fragment which contained all but the proposed first 20 residues of PhzR under control of the T7 promoter in pRSETB. This construction should fuse a 24-kDa peptide from *phzR* in frame with a 4.5-kDa peptide from the vector. In this construct, a fusion protein of the predicted size would be produced only if the proposed reading frame and

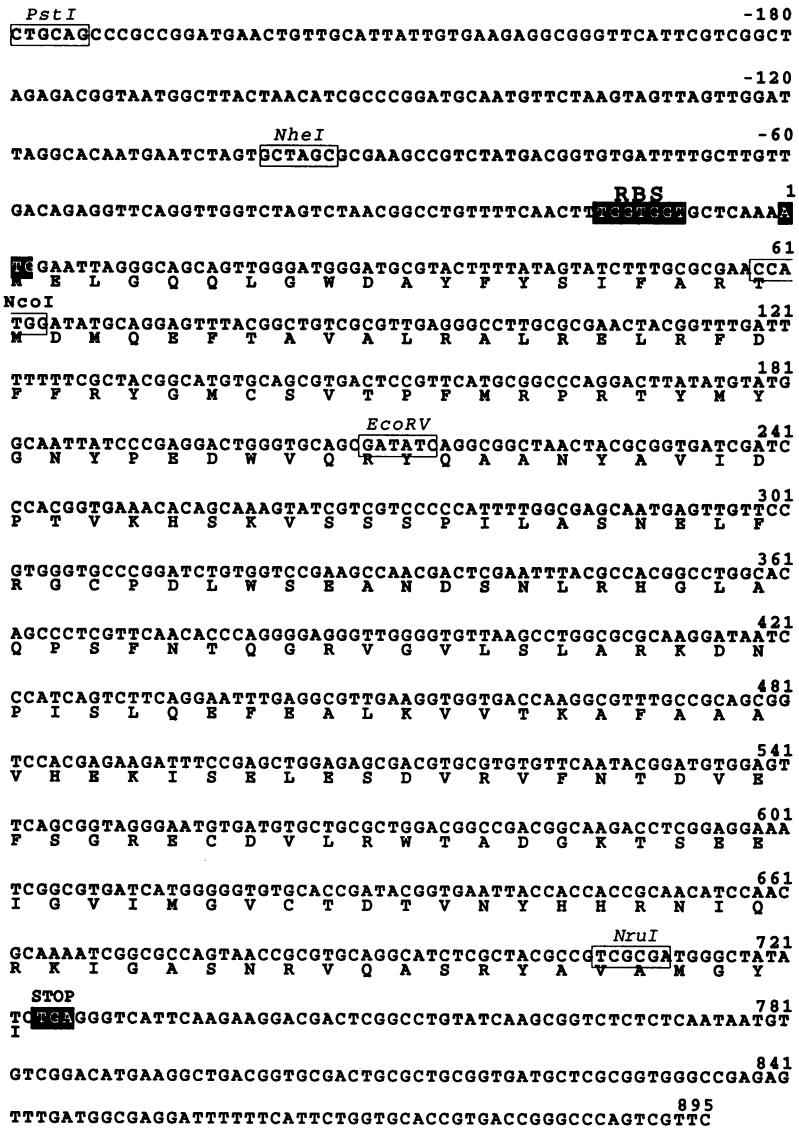

FIG. 3. Nucleotide sequence of *phzR*. Nucleotide sequence of the 1,075-bp region that contains the *phzR* open reading frame. The deduced amino acid sequence (single-letter code) of PhzR is shown below the nucleotide sequence. A putative ribosomal start site (RBS), possible start codon, and termination codon (STOP) are shown in shaded blocks. The sequence is numbered from the first potential start codon.

orientation were correct. A protein of ca. 29 kDa was detected only when the *NcoI* insert was in the orientation predicted to yield a fusion peptide (Fig. 4).

Comparison of PhzR with other bacterial regulatory proteins. The predicted amino acid sequence of PhzR was compared with those of other regulatory proteins with the TFASTA program contained in the University of Wisconsin Genetics Computer Group software package. PhzR is homologous to a family of five proteins involved in cell density-dependent transcriptional activation: the 28-kDa LasR protein of *Pseudomonas aeruginosa* (7), the 28-kDa LuxR protein of *Vibrio fischerii* (5), the 28-kDa SdiA protein of *E. coli* (9, 30), the RhiR protein of *Rhizobium leguminosarum* bv. *viciae* (2), and the 27-kDa TraR protein of *Agrobacterium tumefaciens* (21) (Fig. 5). The 28-kDa SdiA protein upstream of *uvrC* regulates the *ftsQAZ* operon involved in cell division in *E. coli* (30). LasR and LuxR are known to be positive activators of elastase production in *P. aeruginosa* and of bioluminescence in *V. fischerii*, respectively. TraR is involved in the activation of conjugation in *A. tumefaciens*. The proposed amino acid sequence of PhzR shares 49, 45, 60, and 46% similarity and 31, 23, 35, and 23% identity with LasR, LuxR, SdiA, and TraR, respectively. Among the four protein sequences, there are two regions that contain the highest homology. In one region, located near the carboxy terminus of each protein, PhzR has 58, 53, 79, and 47% similarity and 47, 37, 51, and 26% identity with LasR, LuxR, SdiA, and TraR, respectively. In a second region, located toward the N-terminal third of each protein, PhzR has 56, 51, 56, and 52% similarity and 38, 38, 39, and 32% identity with LasR, LuxR, SdiA, and TraR, respectively.

Phenazine gene expression requires an autoinducer. Phenazine antibiotic production in *P. aureofaciens* 30-84 and other pseudomonads occurs during late exponential and stationary growth (17, 27, 28). When strain 30-84 is streaked on solid

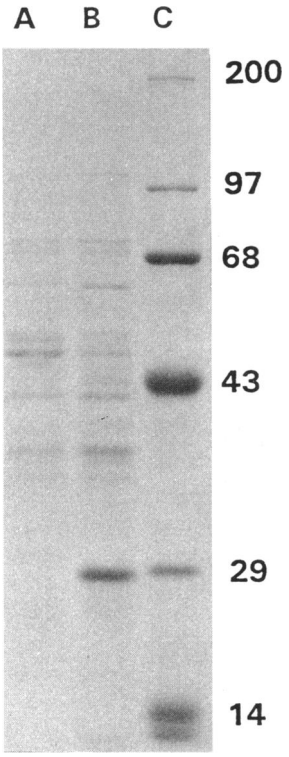

FIG. 4. Expression of the *phzR* open reading frame. Lanes: B, polyacrylamide gel indicating the presence of a ca. 29-kDa fusion peptide produced only when the *phzR* open reading frame is fused to the T7 promoter in the orientation predicted to result in gene expression; A, identical experiment with the *phzR* open reading frame in the opposite orientation; C, molecular weight standards.

media, the small colonies are colorless and phenazines are produced (as judged by colony pigmentation) only after a specific size is reached, suggesting cell density-dependent regulation (data not shown). The predicted amino acid homology between PhzR and other positive transcriptional activators dependent on the buildup of an autoinducer in culture media suggested a mechanism to explain these observations. To test the hypothesis that autoinduction is involved in phenazine gene regulation, culture supernatants from wild-type strain 30-84, the *phzB* mutant 30-84.28-11, and *E. coli* DH5α were screened for the presence of a diffusible molecule capable of activating phenazine gene expression. The assay is based on the observation that the presence of a diffusible autoinducer in a culture supernatant activates expression of a target gene at a lower cell density than is required normally for gene activation. Cell-free culture supernatants of strains 30-84 and 30-84.28-11 induced expression of the *phzB*::*lacZ* genomic fusion in strain 30-84Z at an OD_{620} of ca. 0.1, in comparison to the normal cell density of activation of ca. 0.6 (Fig. 6). The control strain, *E. coli* DH5α, had no ability to activate strain 30-84Z, as expected.

Construction of a *phzR* mutant in *P. aureofaciens* 30-84. Several Tn*5*ZB20 inserts were isolated in *phzR* on cosmid pLSP259 as judged by restriction enzyme analysis and complementation studies (data not shown). All of the *phzR*::Tn*5*-B20 inserts in pLSP259 isolated were oriented in the direction predicted by the DNA sequence to result in a Lac^+ fusion. No inserts oriented in the opposite direction were obtained. When these fusion plasmids were introduced into strain 30-84 in *trans* and plated on medium containing the chromogenic indicator X-Gal, all colonies were blue, suggesting that transcription was initiated from the *phzR* gene (data not shown). To confirm that the gene the insert was located in was *phzR*, the *phzR*::*lacZ* insert carried on pLSP259::Tn*5lac*#42 was introduced into the genome of strain 30-84 by marker exchange. The resulting strain containing the putative *phzR*::*lacZ* genomic fusion was named 30-84R. Inactivation of *phzR* resulted in loss of phenazine antibiotic production as determined by colony color, UV-visible spectroscopy, and thin-layer chromatography (data not shown). The orientation of the inserts and the DNA sequence indicated that *phzR* is transcribed in the opposite direction from the phenazine biosynthetic genes (Fig. 3). Thus, phenazine expression requires at least two divergently oriented operons, one containing *phzR* and the other the phenazine biosynthetic genes.

Introduction of *phzR* in *trans* activates *phzR*. Plasmids pLSP2.7Bgldel and pLSP2.7RVdel were introduced in *trans* into strain 30-84R. Introduction of the functional copy of *phzR* carried on pLSP2.7Bgldel complemented the genomic mutation as evidenced by restoration of phenazine antibiotic production. Introduction of pLSP2.7RVdel, in which the N-terminal end of *phzR* has been deleted, failed to complement the genomic mutation as evidenced by the lack of restoration of antibiotic production (data not shown). Additionally, introduction of a functional *phzR* into strain 30-84R resulted in a ca. 3.5-fold increase in the level of β-galactosidase expression of the genomic *phzR*::*lacZ* fusion (Table 2).

DISCUSSION

We have identified a gene, *phzR* (phenazine regulator), that encodes a positive activator required for the expression of the phenazine biosynthetic locus in *P. aureofaciens* 30-84. Computer analysis of the predicted PhzR coding sequence revealed homology with other bacterial regulatory proteins, including LasR of *P. aeruginosa*, LuxR of *V. fischerii*, SdiA of *E. coli*, RhiR of *R. leguminosarum* bv. *viciae*, and TraR of *A. tumefaciens* (Fig. 5). It has been proposed that these proteins positively activate expression of their target genes in response to the accumulation of a freely diffusible autoinducer molecule.

Two regions among these proteins are highly conserved. One region, located near the C-terminal end of the proteins, is thought to be involved in DNA binding in LasR and LuxR (6, 23). On the basis of this similarity among PhzR, LasR, LuxR, and TraR, it is probable that the PhzR protein activates the phenazine biosynthetic genes in *P. aureofaciens* 30-84 by binding to a DNA sequence upstream of the phenazine biosynthetic genes or to an intermediate gene which, in turn, interacts directly with the phenazine biosynthetic genes.

The mechanism by which PhzR activates *phzB* expression is currently unknown. One hypothesis is that PhzR activates *phzB* expression directly by binding to the phenazine promoter region. Although the exact location of the phenazine biosynthetic promoter is unclear, several lines of evidence suggest that it may be located between *phzR* and *phzB* and may be present at the right end of the cloned 11.2-kb *Eco*RI fragment (Fig. 1). The phenazine biosynthetic genes contained in the 9.2-kb *Eco*RI fragment in pLAFR3 are expressed only when cloned in a particular orientation in strain 30-84Z, indicating expression in pLAFR3 is being driven by an exogenous cosmid promoter, probably the Lac promoter (data not shown). This suggests that the endogenous phenazine biosynthetic promoter is not carried on the 9.2-kb *Eco*RI fragment but may be located within the right end of the 11.2-kb *Eco*RI fragment. This

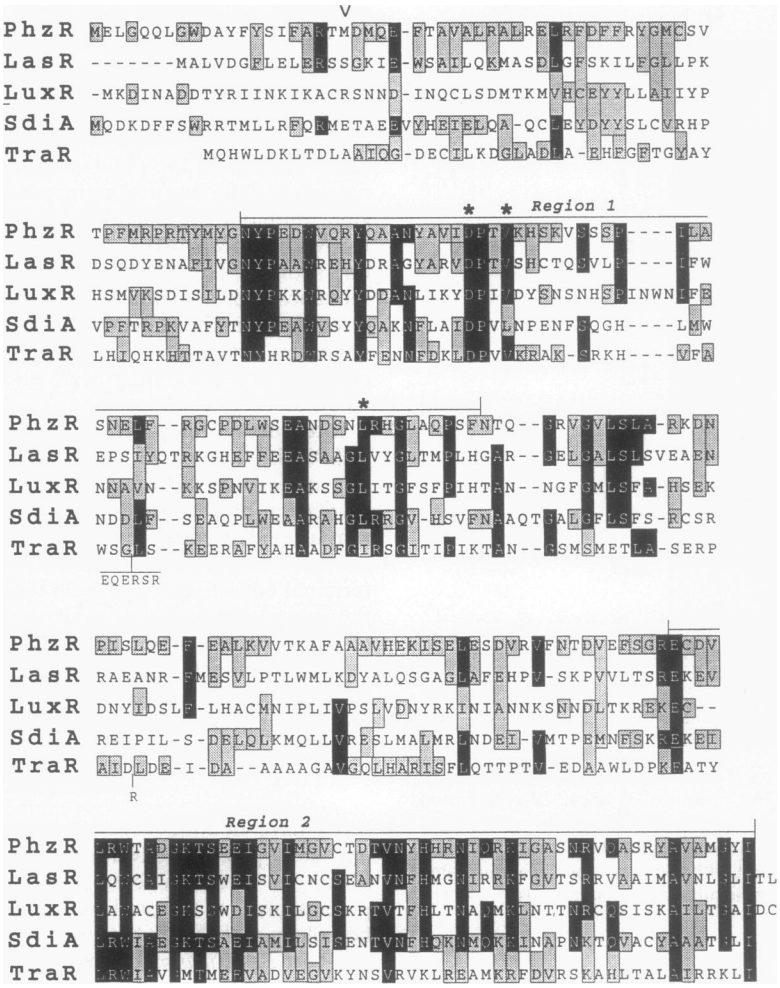

FIG. 5. Comparison of the predicted amino acid sequences of *P. aureofaciens* PhzR, *P. aeruginosa* LasR, *V. fischerii* LuxR, *E. coli* SdiA, and *A. tumefaciens* TraR. Areas of identity among PhzR and at least three of the other activators are indicated by solid boxes. Conservative amino acid replacements between PhzR and at least one other activator are indicated by shaded boxes. Region 1, the autoinducer recognition region; Region 2, the proposed C-terminal DNA-binding domain. The arrowhead at the top indicates the site where the *phzR* open reading frame was fused to the T7 promoter in pRSETB.

orientation-dependent expression is not due to a lack of PhzR or autoinducer since strain 30-84Z is altered only in the *phzB* structural gene. In addition, the presence in *trans* of plasmid pLSP10-21*Pst*#31, which contains the 1.1-kb region between *phzR* and the 9.2-kb *Eco*RI fragment containing the phenazine biosynthetic genes, reduces β-galactosidase expression in strain 30-84Z by a factor of 0.6 (Fig. 1B). Additional subclones of this 1.1-kb region result in even larger reductions in β-galactosidase expression (data not shown). These results suggest that this region may contain the phenazine biosynthetic promoter and may be titrating the PhzR activator.

It is intriguing that the presence of a 1.9-kb *Pst*I fragment in the 11.2-kb *Eco*RI fragment also results in reduced levels of β-galactosidase expression compared with those of the vector alone (Fig. 1B). In *P. aeruginosa*, LasR is known to regulate several genes involved in bacterial virulence (7). Whether this region in *P. aureofaciens* represents another genetic region regulated by PhzR is currently being studied.

In addition to the region of homology among PhzR, LasR, and LuxR proposed to be involved in DNA binding, a second

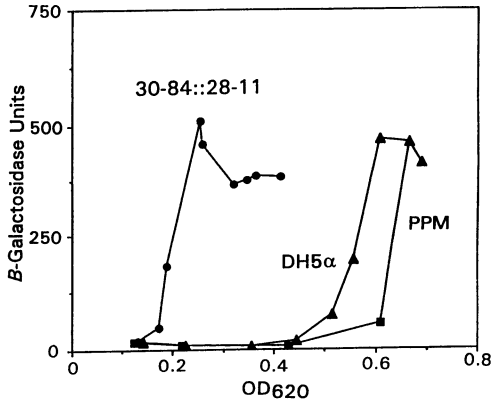

FIG. 6. Induction of *phzB* gene expression by cell-free culture supernatants. The assay for the presence of an autoinducer is described in Materials and Methods. The effects of supernatants prepared from 15- to 18-h cultures of strains 30-84.28-11, DH5α, and fresh PPM media on β-galactosidase activity in strain 30-84Z are indicated.

TABLE 2. Activation of the genomic *phzR::lacZ* fusion by *phzR*[a]

Strain	β-Galactosidase units[b]	Ratio[c]
30-84R	459	1.0
30-84R(pLSP2.7#11)	1,654	3.6
30-84R(pLSP2.7#20)	1,537	3.3

[a] Cultures were grown as described in Materials and Methods.
[b] β-Galactosidase activity in Miller units is as described in the legend to Fig. 1.
[c] Ratio of activity is as described in the legend to Fig. 1.

region of homology near the N-terminal end of the three proteins was identified. This region, called the autoinducer recognition region (23), has been implicated in binding a low-molecular-weight autoinducer in both LasR and LuxR. In *V. fischerii* and other systems, this autoinducer has been shown to be a small homoserine lactone derivative (4; reviewed in reference 6). Binding of this autoinducer molecule is thought to be required in order for these activators to bind DNA and activate gene expression. In these systems, it is proposed that the amount of autoinducer produced by individual cells is small enough that expression of elastase production and bioluminescence in *P. aeruginosa* and *V. fischerii*, respectively, occurs only at a specific cell density when a sufficient amount of autoinducer has accumulated. *P. aureofaciens* may employ an analogous cell density-dependent mechanism of gene regulation for the phenazine biosynthetic genes. Cell culture supernatants from strain 30-84.28-11, which contains a Tn*5* insertion in the phenazine biosynthetic region (20), induce β-galactosidase expression in strain 30-84Z at a lower cell density than usual, indicating the presence of a diffusible molecule (Fig. 6). Although the structure of the *P. aureofaciens* autoinducer is unknown, it is probably similar to the small homoserine lactone derivatives utilized by *P. aeruginosa* and *V. fischerii* (6). Consistent with this hypothesis is the observation that an autoinducer extract from culture supernatants of *P. aeruginosa* induces expression of strain 30-84Z (data not shown). Thus, PhzR appears to be a member of the LuxR/LuxI family of cell density-responsive transcriptional activators (6). To our knowledge, this is the first example of the regulation of genes in a free-living, root-associated bacterium being analogous to the regulation of genes involved in bacterial virulence in human, animal, and plant pathogens.

Cell density-dependent regulation of genes responsible for pathogen inhibition in the rhizosphere has important practical implications for biological control. The major limitation to the use of microbial strains for biological control is their inconsistent performance in the field (reviewed in reference 31). Inconsistent control of take-all by strain 30-84 in the field may be explained by this regulatory mechanism. Specific environmental conditions may limit *phzR* expression and/or autoinducer production or accumulation, resulting in little or no phenazine gene expression. Since phenazine production is required for both pathogen inhibition and rhizosphere survival, the lack of phenazine gene expression could affect the population of strain 30-84 and its ability to inhibit disease. Studies to determine the environmental factors that regulate the expression of *phzR* and to identify the diffusible autoinducer that interacts with PhzR to activate the phenazine biosynthetic genes in *P. aureofaciens* are in progress.

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