APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2001, p. 4305–4315 Vol. 67, No. 9
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.9.4305–4315.2001

A Second Quorum-Sensing System Regulates Cell Surface Properties but Not Phenazine Antibiotic Production in *Pseudomonas aureofaciens*

ZHONGGE ZHANG AND LELAND S. PIERSON III*

Department of Plant Pathology, University of Arizona, Tucson, Arizona 85721

Received 29 March 2001/Accepted 27 June 2001

The root-associated biological control bacterium *Pseudomonas aureofaciens* 30-84 produces a range of exoproducts, including protease and phenazines. Phenazine antibiotic biosynthesis by *phzXYFABCD* is regulated in part by the PhzR-PhzI quorum-sensing system. Mutants defective in *phzR* or *phzI* produce very low levels of phenazines but wild-type levels of exoprotease. In the present study, a second genomic region of strain 30-84 was identified that, when present in *trans*, increased β-galactosidase activity in a genomic *phzB*::*lacZ* reporter and partially restored phenazine production to a *phzR* mutant. Sequence analysis identified two adjacent genes, *csaR* and *csaI*, that encode members of the LuxR-LuxI family of regulatory proteins. No putative promoter region is present upstream of the *csaI* start codon and no *lux* box-like element was found in either the *csaR* promoter or the 30-bp intergenic region between *csaR* and *csaI*. Both the PhzR-PhzI and CsaR-CsaI systems are regulated by the GacS-GacA two-component regulatory system. In contrast to the multicopy effects of *csaR* and *csaI* in *trans*, a genomic *csaR* mutant (30-84R2) and a *csaI* mutant (30-84I2) did not exhibit altered phenazine production in vitro or in situ, indicating that the CsaR-CsaI system is not involved in phenazine regulation in strain 30-84. Both mutants also produced wild-type levels of protease. However, disruption of both *csaI* and *phzI* or both *csaR* and *phzR* eliminated both phenazine and protease production completely. Thus, the two quorum-sensing systems do not interact for phenazine regulation but do interact for protease regulation. Additionally, the CsaI *N*-acylhomoserine lactone (AHL) signal was not recognized by the phenazine AHL reporter 30-84I/Z but was recognized by the AHL reporters *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* A136(pCF240). Inactivation of *csaR* resulted in a smooth mucoid colony phenotype and formation of cell aggregates in broth, suggesting that CsaR is involved in regulating biosynthesis of cell surface components. Strain 30-84I/I2 exhibited mucoid colony and clumping phenotypes similar to those of 30-84R2. Both phenotypes were reversed by complementation with *csaR-csaI* or by the addition of the CsaI AHL signal. Both quorum-sensing systems play a role in colonization by strain 30-84. Whereas loss of PhzR resulted in a 6.6-fold decrease in colonization by strain 30-84 on wheat roots in natural soil, a *phzR csaR* double mutant resulted in a 47-fold decrease. These data suggest that gene(s) regulated by the CsaR-CsaI system also plays a role in the rhizosphere competence of *P. aureofaciens* 30-84.

Numerous plant- and animal-associated bacteria regulate the expression of specific sets of genes in response to their own population densities, a phenomenon termed quorum sensing (10, 33). Most quorum-sensing systems thus far identified in gram-negative bacteria employ *N*-acylhomoserine lactones (AHL) as signaling molecules. AHL signals, which differ in the length and substitution of their acyl side chains, are generated by a single enzyme (a member of the LuxI protein family) (11, 25, 30). These signals accumulate with increasing cell density and upon reaching a threshold concentration bind a transcriptional regulator that in turn activates or represses target gene expression. Over 30 bacterial species have been shown to use quorum-sensing circuits to regulate diverse functions, including bioluminescence, virulence factor production, plasmid conjugal transfer, biofilm formation, motility, symbiosis, and antibiotic production (7).

Pseudomonas aureofaciens strain 30-84, isolated from the wheat rhizosphere, is a biological control agent effective in inhibiting *Gaeumannomyces graminis* var. *tritici*, the causal agent of take-all disease of wheat (34). The production of three phenazine antibiotics by strain 30-84 is responsible for its suppressive capacity (34) and its ability to persist on wheat roots (21). In addition to phenazines, this bacterium has been found to produce exoprotease, siderophores, and hydrogen cyanide (6). However, the specific roles of these compounds (all of which were reported to be responsible for disease suppression by other bacterial biocontrol agents [40]) in the antagonism of strain 30-84 against plant pathogens are unknown. Phenazine antibiotic biosynthesis in strain 30-84 is regulated at multiple levels. The PhzR-PhzI quorum-sensing system regulates phenazine production in a cell density-dependent manner (35, 42). The *phzR* gene encodes a transcriptional regulator of the phenazine operon, and *phzI* encodes an AHL synthase that directs the synthesis of the signal hexanoylhomoserine lactone (HHL). Upon binding HHL, PhzR becomes activated, thereby inducing transcription of the phenazine genes. The GacS-GacA two-component signal transduction system is also involved in controlling phenazine production, partly via regulating transcription of *phzI* and partly via other regulatory elements (6). Mutation of *gacS* or *gacA* has pleiotropic effects, eliminating production of HHL, phenazines, exoprotease, and HCN and increasing fluorescence (6). However, *phzI* and *phzR* null mutants produced wild-type levels of protease, HCN, and siderophores (unpublished data). Production of these compounds is

* Corresponding author. Mailing address: Department of Plant Pathology, Forbes Building, Room 104, University of Arizona, P.O. Box 210036, Tucson, AZ 85721-0036. Phone: (520) 621-9419. Fax: (520) 621-9290. E-mail: lsp@u.arizona.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
P. aureofaciens		W. Bockus
30-84	Wild type, plant disease biocontrol agent, Rif^r	W. W. Bockus
30-84Z	*phzB*::*lacZ* genomic fusion	35
30-84Ice	*phzB*::*inaZ* genomic fusion	43
30-84I	*phzI*::*npt* genomic fusion, Km^r	42
30-84Z/I	*phzB*::*lacZ* and *phzI*::*npt* genomic fusion, Km^r	42
30-84Ice/I	*phzB*::*inaZ* and *phzI*::*npt* genomic fusion, Km^r	43
30-84R	*phzR*::Tn*5lacZ* genomic fusion, Km^r	35
30-84I2	*csaI*::*uidA-Gm* genomic fusion, Gm^r	This study
30-84Ice/I2	*phzB*::*inaZ* and *csaI*::*uidA-Gm* genomic fusion, Gm^r	This study
30-84R2	*csaR*::*uidA-Gm* genomic fusion, Gm^r	This study
30-84Ice/R2	*phzB*::*inaZ* and *csaR*::*uidA-Gm* genomic fusion, Gm^r	This study
30-84I/I2	*phzI*::*npt* and *csaI*::*uidA-Gm* genomic fusion, Km^r Gm^r	This study
30-84I/R2	*phzI*::*npt* and *csaR*::*uidA-Gm* genomic fusion, Km^r Gm^r	This study
30-84I2/R	*csaI*::*uidA-Gm* and *phzI*::*npt* genomic fusion, Gm^r Km^r	This study
30-84R/R2	*phzR*::Tn*5lacZ* and *csaR*::*uidA-Gm* genomic fusion, Km^r Gm^r	This study
30-84 *gacA*	*gacA*::*npt* genomic fusion, Km^r	6
30-84Z/*sgacA*	*phzB*::*lacZ* genomic fusion, spontaneous *gacA* mutant of 30-84Z	6
C. violaceum CV026	Double mini-Tn*5* mutant from *C. violaceum* ATCC 31532, AHL biosensor	16
A. tumefaciens A136	Ti plasmid-less, pCF240*traA*::*lacZ113*, pCF251	9
E. coli		
DH5α	F$^-$ *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*argE-lacZYA*)*169* ϕ*80lacZ*Δ*M15*	Gibco-BRL
MC1061	F$^-$ *araD139* Δ(*araABC-leu*)*7679 galU galK* Δ(*lac*) *X74 rpsL thi*	Gibco-BRL
Plasmids		
pLAFR3	IncP1, Tc^r	39
pMGm	ColE1, Gm^r	26
pWM3	ColE1, Ap^r, *uidA* transcriptional fusion cassette	23
pUC18	ColE1, Ap^r	44
pLSP5-5	pLAFR3 carrying a 29-kb fragment of 30–84 chromosomal DNA that contains *csaI* and *csaR*	This study
pZZG5-5-2	pLAFR3 carrying the 7.15-kb *Eco*R1 fragment from pLSP5-5 that contains *csaI* and *csaR*	This study
PZZG3	pLAFR3 carrying the 5-kb *Pst*I-*Hin*dIII fragment from pIC20H5-5-2 that contains *csaI* and *csaR*	This study
pZZG11	pLAFR3 carrying the 1-kb *Sph*I-*Eco*RV fragment that contains *csaR*	This study
pZZGP18-1	pUC18 carrying the 5-kb *Pst*I-*Hin*dIII fragment that contains *csaI* and *csaR*	This study
pZZGP18-2	pZZGP18-1 containing the introduced *Sac*I site in *csaI*	This study
pZZGP18-3	pZZGP18-1 containing the introduced *Sac*I site in *csaR*	This study
pWM3-*Gm*	pWM3 carrying the 2-kb *Sal*I fragment from pMGm, Gm^r	This study
pZZGI-*Sac*	pLAFR3 carrying the 5-kb *Pst*I-*Hin*dIII fragment from pZZGP18-2	This study
pZZGR-*Sac*	pLAFR3 carrying the 5-kb *Pst*I-*Hin*dIII fragment from pZZGP18-3	This study
pZZGI-*uidAGm*	pZZGI-Sac carrying the 4-kb *Sac*I fragment from pWM3-*Gm* that contains *uidA-Gm*	This study
pZZGR-*uidAGm*	pZZGR-Sac carrying the 4-kb *Sac*I fragment from pWM3-*Gm* that contains *uidA-Gm*	This study
pUC18-*csaI*	pUC18 containing the 2.5-kb *Sac*I-*Sph*I fragment from pZZGP18–4	This study
pLSP*phzB-inaZ*	pLAFR3 containing the *phzB*::*inaZ* fusion	43

regulated in a cell density-dependent manner in a number of other bacterial species (5, 17, 22). Interestingly, *phzI* and *phzR* null mutants produced phenazines at low levels on a certain medium. Recently, several bacteria were shown to harbor two or more quorum-sensing systems that regulate expression of the same or different factors (12, 15, 37). Taken together, the above results suggested that strain 30-84 might contain an additional regulatory system acting independently or cooperatively with the PhzR-PhzI system to mediate secondary metabolite production.

In this study, we report the identification of a second quorum-sensing system, CsaR-CsaI, in *P. aureofaciens* strain 30-84. The nature of the interaction between CsaR-CsaI and PhzR-PhzI in regulating phenazine and exoprotease production and rhizosphere colonization was examined. In addition, several phenotypes regulated specifically by the CsaR-CsaI system were identified.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. *P. aureofaciens* strain 30-84, a spontaneous rifampin-resistant mutant of the wild-type strain (35), and its derivatives were grown at 28°C in Luria-Bertani (LB) medium (19), King's B medium (KMB) (14), M9 minimal medium (19), AB minimal medium (38), skim milk-water agar (6), or pigment production medium (PPM-D) (42). *Chromobacterium violaceum* CV026 (16) and *Agrobacterium tumefaciens* A136(pCF240) (9) were grown at 28°C in LB or AB medium. *Escherichia coli* strains were cultured in LB medium at 37°C. Where applicable, antibiotics were used at the following concentrations (in micrograms per milliliter): for *E. coli*, ampicillin at 100, gentamicin (GM) at 25, kanamycin (KM) at 50, and tetracycline (TC) at 25; for *P. aureofaciens*, KM at 50, rifampin at 100, TC at 50, and GM at 50; for *A. tumefaciens*, KM at 150, spectinomycin at 50, and TC at 10.

Screening for the presence of a new *luxR-luxI* homologue. A cosmid library of strain 30-84 was mobilized into the indicator *phzB*::*lacZ* reporter 30-84Z or the *phzR* mutant 30-84R through triparental mating as described previously (35). In the case of 30-84Z, the Rif^r and Tc^r transconjugants were inoculated on M9 agar containing TC and 4% (wt/vol) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). After incubation (24 h), β-galactosidase activity of the transcon-

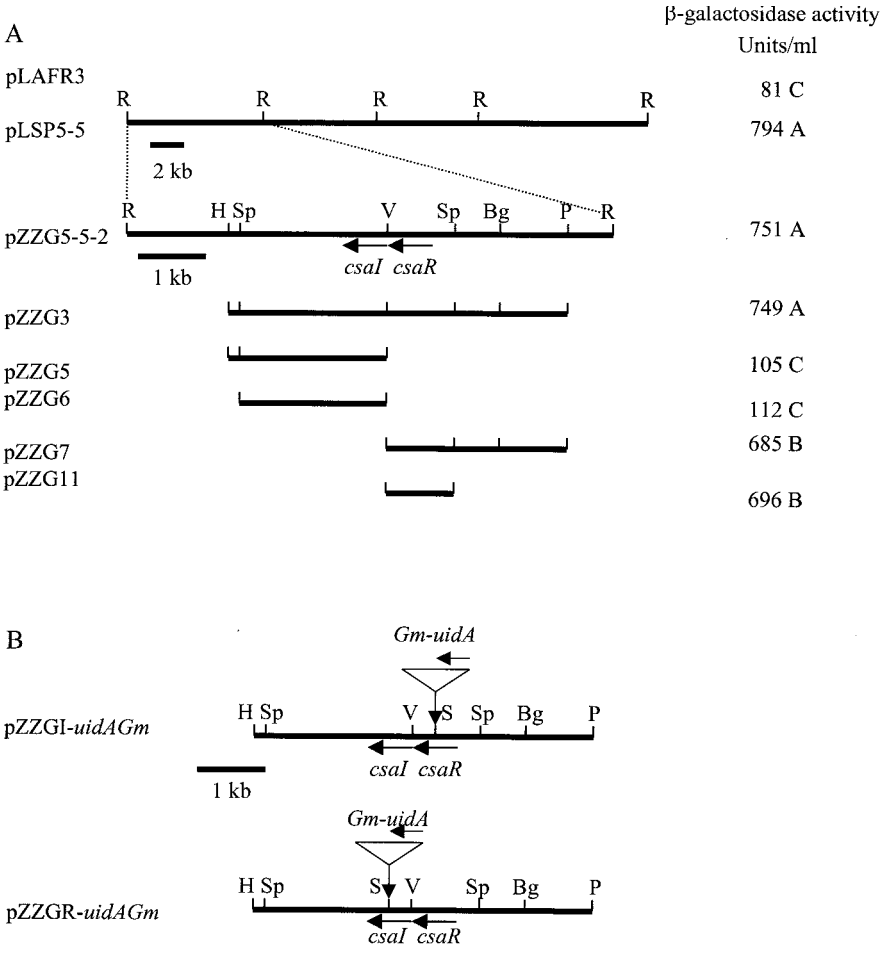

FIG. 1. Localization and physical map of the regions containing *csaI* or *csaR* and construction of disrupted versions of *csaI* and *csaR*. Arrows represent the location and orientation of the genes. (A) The location of the regions leading to elevated β-galactosidase activity was determined by measuring the effects of the indicated subclones of pLSP5-5 on β-galactosidase expression in the *phzB*::*lacZ* reporter strain 30-84Z. Letters indicate significant differences between values. (B) *csaR* and *csaI* loci from pZZG3 and insertional mutation of *csaR* and *csaI*. Restriction enzyme abbreviations: R, *Eco*RI; H, *Hin*dIII; Sp, *Sph*I; V, *Eco*RV, Bg, *Bgl*II, P, *Pst*I; S, *Sac*I.

jugants was determined by examining the blue intensity and presence or absence of a blue halo around the colonies. When strain 30-84R was used, transconjugants were assessed for phenazine production on LB agar supplemented with TC by the orange intensity on and around the colonies.

DNA manipulations. DNA isolations, restriction enzyme digestions, agarose gel electrophoresis, ligations, transformations, and Southern hybridizations were carried out as described previously (6, 34).

Oligonucleotides for PCR and DNA sequencing were synthesized by Gibco-BRL (Gaithersburg, Md.). DNA sequencing was performed at the University of Arizona Biotechnology Center using an Applied Biosystems automatic DNA sequencer (model 373A, version 1.2.1.). Sequence analysis was performed with the University of Wisconsin Genetics Computer Group Software packages (version 9.1).

Construction of 30-84I2, 30-84R2, and double mutants. Since Km^r and *lacZ* were used previously to construct strains 30-84I (*phzI*::*npt*) and 30-84R (*phzR*::Tn*5lacZ*), mutants with mutations in *csaR* and *csaI* were constructed using a β-glucuronidase-and-gentamicin resistance (*uidA-Gm*) cartridge constructed in our laboratory. A 2-kb *Sal*I fragment from pMGm (26) was inserted into the *Sal*I site of pWM3 (23), resulting in pWM3-*Gm*. The 5-kb *Pst*I-*Hin*dIII fragment from pZZG5-5-2 (Fig. 1A) was ligated into pUC18, resulting in pZZGP18-1. PCR primers were designed to create a *Sac*I restriction site in either the *csaI* or *csaR* coding region in pZZGP18-1, as described in the QuickChange site-directed mutagenesis kit (Stratagene), yielding pZZGP18-2 and pZZGP18-3, respectively. The primers for introduction of the *Sac*I site (underlined) in *csaI* are 5′-CACGCCGGCGCTGGAGCTCGTTATTTCCTGC-3′ (forward) and 5′-GCAGGAAATAACGAGCTCCAGCGCCGGCGTG-3′ (reverse), and those for *csaR* are 5′-GCCTTGTTCGGCAAGAGCTCGGTGTTGTG-3′ (forward) and 5′-CACAACACCGAGCTCTTGCCGAACAAGGC-3′ (reverse). The 5-kb *Pst*I-*Hin*dIII fragment with the engineered *Sac*I site in *csaI* or in *csaR* was ligated into pLAFR3 to create pZZGI-Sac or pZZGR-Sac. The *csaI* or *csaR* genes were disrupted by insertion of the 4-kb *Sac*I *uidA-Gm* cartridge from pMW3-*Gm* into the engineered *Sac*I site in *csaI* contained in pZZGI-Sac and in *csaR* contained in pZZGR-Sac. The resultant plasmids, pZZGI-*uidAGm* and pZZGR-*uidAGm* (Fig. 1B), were mobilized into *P. aureofaciens* via triparental mating and introduced into the genome via homologous recombination. Mutants resistant to GM but sensitive to TC were selected and verified by Southern hybridization (data not shown). Two mutants, named 30-84I2 (*csaI*) and 30-84R2 (*csaR*), were selected. Double mutants 30-84I/I2 (*phzI csaI*), 30-84R/R2 (*phzR csaR*), 30-84I/R2 (*phzI csaR*), and 30-84I2/R (*csaI phzR*) were constructed analogously as described above and confirmed by Southern hybridization (data not shown).

Assays for exoproducts. Exoprotease activity was assessed qualitatively on skim milk agar (6) and quantitatively by a modified Lowry method (41) using the substrate 1% (wt/vol) casein in 50 mM Tris-acetate (pH 7.5), prepared as described by Belew and Porath (4). One enzyme unit of exoprotease activity was defined as the amount of enzyme that liberated 1 μg of tyrosine per min at 30°C.

Phenazine production was qualitatively determined by examining orange color on PPM-D medium and quantified from cell extracts as described previously (34). HCN production and cell fluorescence indicative of siderophores were determined as described previously (6).

Colony morphology of strain 30-84 and its mutant derivatives was determined on KMB, AB, LB, or PPM-D plates. After 2 days, cultures were examined for various characteristics, such as mucoidy, shininess, and roughness. To determine

whether bacterial cells clumped, strains were cultured in KMB for 24 h. Three microliters of culture was spotted onto a slide, air dried, and stained with Congo red. The cells were examined for distribution, the presence of capsules, and aggregation under light microscopy. For each culture, three slides (five fields per slide) were examined.

AHL detection and quantification. An AHL donor strain, 30-84Ice/I2 (*phzI*$^+$ *csaI*::*uidA-Gm phzB*::*inaZ*), was constructed by introduction of pLSP*phzB-inaZ* (42) into 30-84I2 via triparental mating and was verified via Southern hybridization (data not shown). To determine whether *csaI* can direct synthesis of a diffusible signal, the 3.2-kb *Sph*I fragment containing *csaI* was excised from pZZGP18-3 and ligated into pUC18 (44) in the same orientation as in pZZGP18-3. The resultant plasmid was digested with *Sac*I and religated, generating pUC18-*csaI*, which carries *csaI* under the control of the vector *lacZ* promoter. *E. coli* DH5α(pUC18-*csaI*) was used as a CsaI AHL donor strain.

The AHL-dependent reporter strains 30-84I/I2 (*phzI*::*npt csaI*::*uidA-Gm*), 30-84I/Z (*phzI*::*npt phzB*::*lacZ*), and *C. violaceum* CV026 (*cviI* mutant) were tested for their ability to respond to exogenously added AHL compounds in a cross-feeding assay. Strains 30-84Ice/I (*phzB*::*inaZ phzI*::*npt csaI*$^+$) and 30-84Ice/I2 (*phzB*::*inaZ csaI*::*uidA-Gm phzI*$^+$) were used as the AHL donor strains. AHL donor strains were streaked onto one side of a plate, while the reporter strain was streaked onto the other side of the plate. When 30-84I/I2 was used as the AHL recipient, the assay was performed on PPM-D agar to examine phenazine production. Skim milk plates and KMB plates were used for examination of exoprotease activity and colony surface roughness of the double I mutant, respectively. When strain 30-84I/Z was used, the recipient and donor were streaked onto PPM-D plus X-Gal. When CV026 was the recipient, the assay was performed on LB broth. The plates were incubated 1 to 2 days before being evaluated for cross-communication.

To quantify AHL production, culture supernatants of *P. aureofaciens* or *E. coli* strains were extracted with acidified ethyl acetate as described previously (32). AHL extracts added to LB broth were tested for activation of β-galactosidase activity in strains 30-84I/Z and *A. tumefaciens* A136(pCF240). β-Galactosidase activity was measured as described by Miller (24). Strain 30-84Z/s*gacA* (6), a spontaneous *gacA* mutant of 30-84Z, was used as a negative reporter, and the extract from *E. coli* DH5α(pUC18) was used as a negative control.

Growth chamber assays for rhizosphere colonization and phenazine production. Wheat seeds (cv. Penewawa) were surface sterilized and treated with bacterial cultures as described previously (32). Briefly, treated seeds were sown in 30- by 30-cm plastic cones (two seeds per cone) containing 25 cm of steam-pasteurized or natural field soil mixed with vermiculite and sand in equal volumes and moistened with 10 ml of sterile 1/3 Hoagland's solution (13) prior to planting. Each treatment was repeated in at least four cones, which were arranged in a randomized complete block design. The seeds were covered with a 1-cm layer of the potting medium and incubated in a Conviron growth chamber (20°C during the light period and 15°C during the dark period, ~75% relative humidity, 12-h photoperiod). Four weeks after emergence, the roots were aseptically excised, and bacterial populations were determined on KMB plus rifampin as described previously (32).

The effect of the CsaR-CsaI system on phenazine operon expression in situ was determined by comparing ice nucleation activities of the isogenic reporter strains 30-84Ice/R2 (*csaR*::*uidA-Gm phzB*::*inaZ*) and 30-84Ice/I2 with that of 30-84Ice (*phzB*::*inaZ*) in the wheat rhizosphere. Wheat seeds were prepared, treated, and grown as described above. Twenty days after emergence, bacterial populations and ice nucleation activities were determined. Ice nucleation activity was expressed as ice nucleation frequency calculated on a per-cell level, as described by Lindgren et al. (18).

Statistical analysis. All experiments described above were repeated at least once. Data from the two experiments were pooled, and analysis of variance was used to analyze data. Fisher's least significant difference and Duncan's multiple range tests were used to compare means.

Nucleotide sequence accession number. The nucleotide sequences of the *csaI* and *csaR* genes have been deposited in GenBank (accession no. AY040629).

RESULTS

Identification of CsaR and CsaI. A cosmid library of strain 30-84 was mobilized into the reporter strains 30-84Z and 30-84R to search for genomic regions able to enhance phenazine production. Strain 30-84Z and strain 30-84R transconjugants were tested for β-galactosidase activity and for phenazine production, respectively. A single cosmid (pLSP5-5) when present in *trans* in strain 30-84Z resulted in a dark blue colony surrounded by a blue halo (data not shown). When cultured in M9 broth, 30-84Z(pLSP5-5) expressed ninefold-higher β-galactosidase activity than 30-84Z(pLAFR3) (Fig. 1A). The same cosmid restored phenazine production to strain 30-84R, as indicated by an orange colony compared to the white 30-84R (pLARF3) colony (data not shown). Analysis of pLSP5-5 indicated the presence of a 29-kb insert comprised of *Eco*RI fragments of 5, 7.2, 7.5, and 10 kb, respectively. Only the 7.2-kb fragment in pZZG5-5-2 was effective in enhancing β-galactosidase activity in strain 30-84Z (Fig. 1A). Further deletion analysis revealed that the 2.2-kb *Sph*I-*Eco*RV fragment and the 1-kb *Eco*RV-*Sph*I fragment resulted in elevated β-galactosidase activity.

DNA sequence analysis of the 1,034-bp *Eco*RV-*Sph*I fragment revealed the presence of one 723-nucleotide open reading frame (ORF), designated CsaR. A putative ribosome binding site (RBS), AGGA, is located eight nucleotides upstream from the CsaR start codon. Potential promoter sequences for *csaR* include a −10 region (TAGATT) and −35 region (TTG ACA). This 723-nucleotide ORF can encode a 241-amino-acid protein with a predicted molecular mass of 27.2 kDa. BLAST searches revealed similarity between the deduced amino acid sequence of CsaR and diverse members of the LuxR family (Fig. 2A). It has 67, 37, 36, 35, 35, and 33% identity (80, 52, 53, 52, and 51% similarity) with *Pseudomonas aeruginosa* RhlR (27), *Salmonella enterica* serovar Typhimurium SdiA (GenBank accession number U88651), *P. aureofaciens* 30-84 PhzR (35), *Pseudomonas fluorescens* PhzR (20), *Pseudomonas chlororaphis* PhzR (GenBank accession number AF195615), and *Burkholderia cepacia* CepR (17), respectively.

A second ORF was found in the 2,204-bp *Sph*I-*Eco*RV fragment adjacent to *csaR* and was designated *csaI*. The CsaI ORF contains 657 nucleotides and potentially encodes a 219-amino-acid protein of 24.4 kDa. Since the translational start codon is located only five nucleotides downstream from the *Eco*RV site, the RBS and promoter region are probably outside the fragment. When the sequences from both fragments were assembled, the CsaI ORF was found to be located only 30 bp downstream from the CsaR ORF and contained a putative RBS, TAAGGA, 9 bp upstream from the start codon. No promoter region was identified in the 30-bp region, indicating that *csaI* may be cotranscribed with *csaR*. No consensus *lux* box sequence (12) was found in either the 30-bp intergenic region or the *csaR* promoter region. CsaI shared significant homology with numerous AHL synthases (Fig. 2B). It has 53, 43, 39, 39, and 39% identity (69%, 56, 58, 55, 57, and 56% similarity) to *P. aeruginosa* RhlI (28), *B. cepacia* CepI (17), *P. aureofaciens* PhzI (42), *P. fluorescens* PhzI (20), and *P. chlororaphis* PhzI (AF195615), respectively.

CsaR-CsaI is not required for phenazine gene expression. To determine whether the CsaR-CsaI quorum-sensing system is involved in regulating production of phenazines or other factors, we individually disrupted the genomic *csaI* locus and *csaR* locus by inserting a *uidA-Gm* cartridge in the genes, resulting in strains 30-84I2 (*csaI*::*uidA-Gm*) and 30-84R2 (*csaR*::*uidA-Gm*). Similarly, four double mutants, 30-84I/I2 (*phzI*::*km csaI*::*uidA-Gm*), 30-84I/R2 (*phzI*::*npt csaR*::*uidA-Gm*), 30-84I2/R (*csaI*::*uidA-Gm phzR*::Tn*5lacZ*), and 30-84R/R2 (*phzR*::Tn*5lacZ csaR*::*uidA-Gm*), were constructed. All mutants were verified

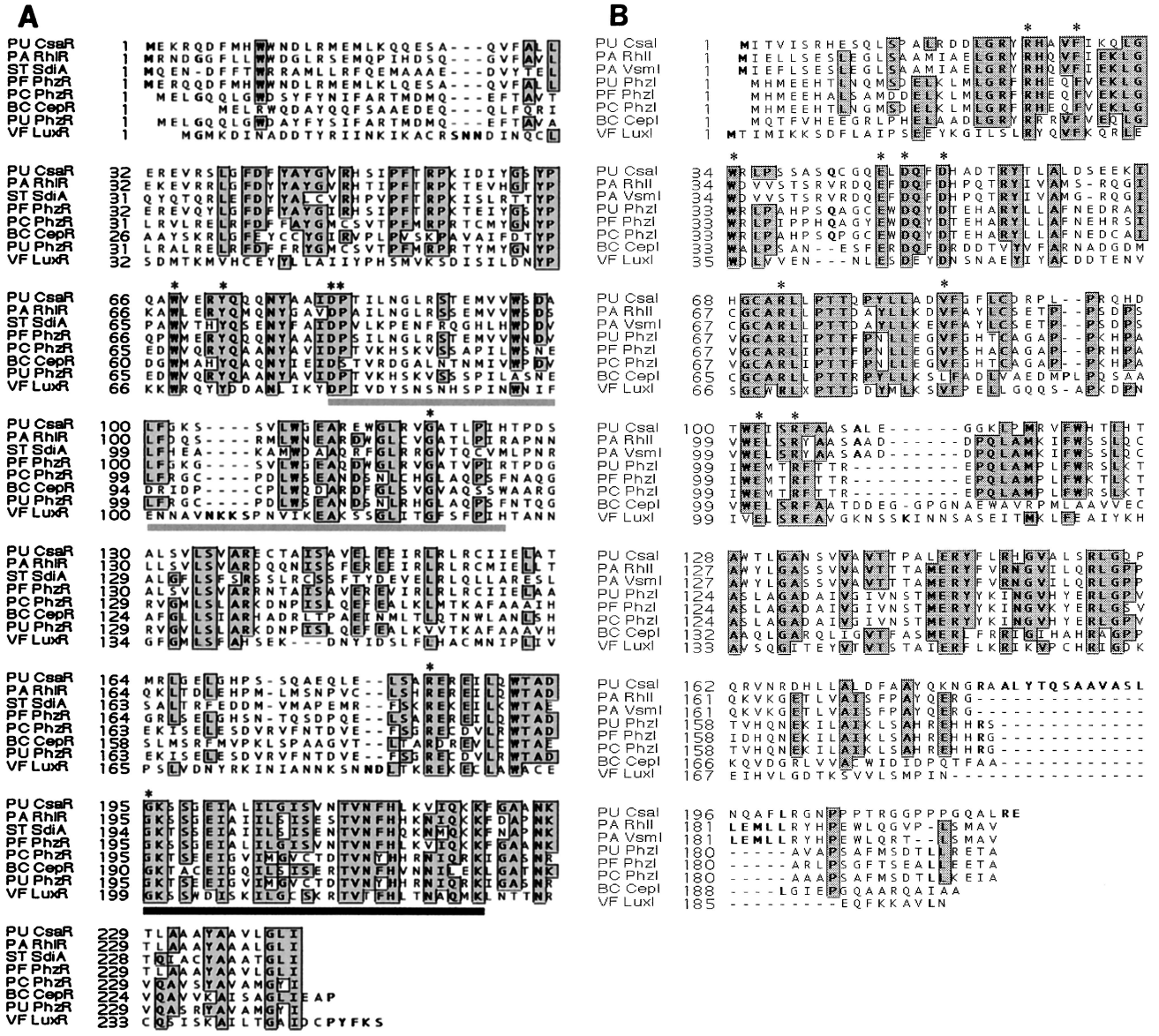

FIG. 2. Multiple alignment of CsaR and CsaI with other LuxR-LuxI family members. Regions in which amino acids are identical in at least five proteins are boxed and shaded. (A) Alignment of CsaR with seven other LuxR family members. The gray bar below LuxR residues 81 to 129 represents the conserved autoinducer-binding domain (10). The black bar below LuxR residues 199 to 226 represents the putative helix-turn-helix region of the DNA-binding domain. The seven invariant amino acids of LuxR homologs are indicated with asterisks (8). Abbreviations: PA RhlR, *P. aeruginosa* PAOI RhlR (L08962); PA VsmR, *P. aeruginosa* VsmR (U15644); ST SdiA, *S. enterica* serovar Typhimurium SdiA (U88651); PF PhzR, *P. fluorescens* 2-79 PhzR (L48616); BC CepR, *B. cepacia* CepR (AF01954); PU PhzR, *P. aureofaciens* PU PhzR (L32729); VF LuxR, *Vibrio fischeri* LuxR (Y00509). (B) Alignment of CsaI with seven representative LuxI family members. The 10 invariant amino acids characteristic of LuxI homologs are labeled with asterisks (29). Abbreviations: PA RhlI, *P. aeruginosa* PAOI RhlI (AE004768); PA VsmI, *P. aeruginosa* VsmI (U15644); PU PhzI, *P. aureofaciens* 30-84 PhzI (L33724); PF PhzI, *P. fluorescens* 2-79 PhzI (L48616); PC PhzI, *P. chlororaphis* PhzI (AF195615); BC CepI, *B. cepacia* CepI (AF019654); VF LuxI, *V. fischeri* LuxI (Y00509).

by Southern hybridization using a 2-kb DNA fragment containing *csaI* and *csaR* as a probe, with all *csaR* mutants displaying fragments of 4.9 and 6.2 kb and all *csaI* mutants displaying fragments of 5.3 and 5.8 kb.

When cultured on agar medium, loss of either *csaR* or *csaI* had no effect on phenazine production compared to that of strain 30-84 (data not shown). Both mutants initially produced slightly lower levels of the antibiotics than 30-84 in PPM-D broth after 24 h, but no significant differences in the amount of phenazines was detected among the three strains after 72 h (Table 2). As found previously, loss of *phzR* or *phzI* resulted in ca. 10% of the wild-type levels of phenazines after 72 h on PPM-D medium. In contrast to the single mutants, all double mutants (*phzI csaI*, *phzI csaR*, *csaI phzR*, and *phzR csaR* strains) failed to produce detectable phenazines on PPM-D at any time, as judged by colony color (data not shown) and the absence of detectable absorbance (optical density at 467 nm) of culture extracts (Table 2).

To determine if *csaR* in *trans* restored phenazine production, pZZG11 (Fig. 1A) was introduced into strains 30-84I/R2

TABLE 2. Phenotypes of *P. aureofaciens* 30-84 and its derivatives

Strain	Genotype	Absorbance at 367 nm[a,c]		Protease activity[b,c] (U/ml)	Colony morphology[d]	Clumping phenotype[e]
		After 24 h	After 72 h			
30-84	Wild type	6.46 ± 0.09 A	3.05 ± 0.14 A	19.80 ± 1.83 A	Semidry and rough	−
30-84I	*phzI*::*npt*	0.04 ± 0.00 C	0.30 ± 0.02 B	21.13 ± 2.91 A	Semidry and rough	−
30-84R	*phzR*::Tn*5lacZ*	0.04 ± 0.00 C	0.24 ± 0.01 B	18.59 ± 2.68 A	Semidry and rough	−
30-84I2	*csaI*::*uidA-Gm*	5.96 ± 0.18 B	2.80 ± 0.28 A	19.06 ± 1.56 A	Semidry and rough	−
30-84R2	*csaR*::*uidA-Gm*	5.99 ± 0.33 B	2.89 ± 0.22 A	19.72 ± 1.98 A	Shiny and mucoid	+
30-84I/I2	*phzI*::*npt csaI*::*uidA-Gm*	0.02 ± 0.00 C	0.03 ± 0.00 C	0.71 ± 0.00 B	Shiny and mucoid	+
30-84I/R2	*phzI*::*npt csaR*::*uidA-Gm*	0.03 ± 0.01 C	0.05 ± 0.01 C	18.64 ± 1.78 A	Shiny and mucoid	+
30-84I2/R	*csaI*::*uidA-Gm phzR*::Tn*5lacZ*	0.03 ± 0.01 C	0.05 ± 0.01 C	21.04 ± 2.75 A	Semidry and rough	−
30-84R/R2	*phzR*::Tn*5lacZ csaR*::*uidA-Gm*	0.02 ± 0.01 C	0.02 ± 0.01 C	0.35 ± 0.00 B	Shiny and mucoid	+
30-84.*gacA*	*gacA*::*npt*	0.02 ± 0.01 C	0.03 ± 0.01 C	0.00 ± 0.00 B	Shiny and mucoid	+

[a] Phenazines were extracted from supernatants of 24- and 72-h-old bacterial cultures grown in PPM-D and quantified by measuring absorbance at 367 nm.
[b] Exoprotease activity in supernatants of 24-h-old broth cultures was determined by a modified Lowry method using casein as the substrate.
[c] All values are means ± standard deviations of two experiments, with three replicates per experiment. Values followed by the same letter in a column are not significantly different ($P = 0.05$).
[d] Bacterial strains were cultured on KMB agar, and 2 days later the colonies were examined for roughness and mucoidy.
[e] Bacterial strains were cultured on KMB broth, and 1 day later the cultures were stained with Congo red and observed for the aggregation of bacterial cells.

(*phzI csaR*), 30-84R/R2 (*phzR csaR*), and 30-84I/I2 (*phzI csaI*). When cultured in PPM-D, 30-84I/R2(pZZG11) and 30-84R/R2(pZZG11) produced phenazines at ca. 40% of the wild-type level. Strain 30-84I/I2(pZZG11) did not produce detectable phenazine after 24 h (Table 3) but did produce low levels after 48 h (data not shown). Strains 30-84I/I2(pZZG3) and 30-84I2/R(pZZG3) were assayed for phenazine production. The presence of *csaR csaI* in *trans* also restored production to ca. 40% of wild-type levels (Table 3). This partial complementation also occurred in LB medium and KMB (data not shown).

The ice nucleation reporter strains 30-84Ice/I2 (*phzB*::*inaZ csaI*::*uidA-Gm*) and 30-84Ice/R2 (*phzB*::*inaZ csaR*::*uidA-Gm*) were used to determine whether *csaI* or *csaR* is required for phenazine gene expression in the wheat rhizosphere. These strains, together with strain 30-84Ice, contain a genomic *phzB*::*inaZ* fusion and synthesize ice nucleation protein under the control of the phenazine operon promoter. The amount of ice nucleation activity (InaZ) is proportional to the amount of phenazines produced by the *csaI* or *csaR* mutation. Twenty days after emergence, high InaZ activity (−1.6 to −1.8 log nuclei/cell) was detected from each bacterial strain isolated from roots in comparison to the negative control strain 30-84.*gacA* (6), which showed no activity. However, there was no significant difference in ice nucleation activity (−1.61, −1.78, and −1.59 log nuclei per cell for 30-84Ice, 30-84Ice/I2, and 30-84Ice/R2, respectively) among the three strains. These results are consistent with the effect of mutations in *csaI* or *csaR* in strain 30-84 on phenazine production in vitro, further indicating that the CsaR-CsaI system is not required for phenazine biosynthesis by strain 30-84.

CsaR and CsaI are involved in exoprotease activity. Exoprotease activity was compared between strain 30-84 and mutant derivatives. Strain 30-84 and derivatives with single null mutations in *csaI*, *csaR*, *phzI*, or *phzR* or with *phzI csaR* or *csaI phzR* double mutations produced extracellular protease, as shown by the presence of indistinguishable clear zones on skim milk agar (data not shown). However, *phzI csaI* and *phzR csaR* double mutants failed to produce any clear zone. Quantitative assays of culture supernatants showed that all single mutants and the *csaI phzR* and *phzI csaR* double mutants exhibited levels of proteolysis similar to those of strain 30-84 (Table 2). The double mutants in which both AHL synthases (PhzI and CsaI) or both transcriptional activators (PhzR and CsaR) were inactivated had significantly diminished protease activity com-

TABLE 3. Complementation of *P. aureofaciens* 30-84 derivatives with *csaR* or *csaR-csaI* in *trans*

Strain[f]	Genotype (host/plasmid)	Absorbance at 367 nm[a,c]	Protease activity[b,c]	Colony morphology[d]	Clumping phenotype[e]
30-84(pLAFR3)	Wild type	5.91 ± 0.29 A	+	Semidry and rough	−
30-84R(pLAFR3)	*phzR*::Tn*5lacZ*	0.03 ± 0.01 C	+	Semidry and rough	−
30-84R2(pZZG11)	*csaR*::*uidA-Gm*/*csaR*$^+$	5.98 ± 0.39 A	+	Semidry and rough	−
30-84I/I2(pZZG11)	*phzI*::*npt csaI*::*uidA-Gm*/*csaR*$^+$	0.04 ± 0.01 C	−	Semidry and rough	−
30-84I/I2(pZZG3)	*phzI*::*npt csaI*::*uidA-Gm*/*csaR*$^+$ *csaI*$^+$	2.67 ± 0.11 B	−	Semidry and rough	−
30-84I/R2(pZZG11)	*phzI*::*npt csaR*::*uidA-Gm*/*csaR*$^+$	2.36 ± 0.20 B	+	Semidry and rough	−
30-84I2/R(pZZG3)	*csaI*::*uidA-Gm phzR*:Tn*5lacZ*/*csaR*$^+$ *csaI*$^+$	2.57 ± 0.20 B	+	Semidry and rough	−
30-84R/R2(pZZG11)	*phzR*::Tn*5lacZ, csaR*::*uidA-Gm*/*csaR*$^+$	2.42 ± 0.19 B	−	Semidry and rough	−
30-84.*gacA*(pZZG3)	*gacA*::*npt*/*csaR*$^+$ *csaI*$^+$	0.03 ± 0.00 C	−	Shiny and mucoid	+

[a] Phenazines were extracted from supernatants of 24-h-old bacterial cultures grown in PPM-D and quantified by measuring absorbance at 367 nm.
[b] Exoprotease activity in supernatants of 24-h-old broth cultures was determined by a modified Lowry method using casein as the substrate.
[c] All values are means ± standard deviations of two experiments, with three replicates per experiment. Values followed by the same letter in a column are not significantly different ($P = 0.05$).
[d] Bacterial strains were cultured on KMB agar, and 2 days later the colonies were examined for roughness and mucoidy.
[e] Bacterial strains were cultured on KMB broth for 24 h, and the cultures were stained with Congo red and observed for the presence of the clumping phenotype.
[f] The presence of the vector pLAFR3 alone had no effect on any of the strains listed (data not shown).

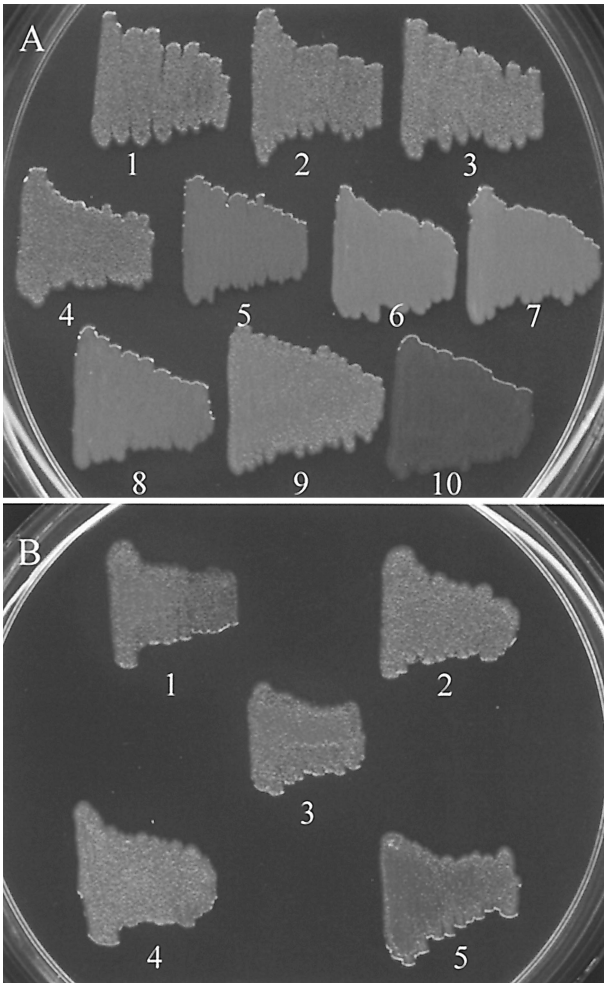

FIG. 3. Colony morphology of 30-84 and derivatives on KMB agar. Photographs were taken after 48 h. (A) 30-84 and various mutants. Bacterial strains: 1, 30-84; 2, 30-84I (*phzI*); 3, 30-84R (*phzR*); 4, 30-84I2 (*csaI*); 5, 30-84R2 (*csaR*); 6, 30-84R/R2 (*phzR csaR*); 7, 30-84I/I2 (*phzI csaI*); 8, 30-84I/R2 (*phzI csaR*); 9, 30-84I2/R (*csaI phzR*); and 10, 30-84.*gacA*. (B) Complementation of 30-84I/I2 and *csaR* mutants. Bacterial strains: 1, 30-84R2(pZZG11); 2, 30-84R/R2(pZZG11); 3, 30-84I/I2(pZZG3); 4, 30-84I/I2(pZZG11); and 5, 30-84I/R2(pZZG11). Plasmid pZZG11 contains a functional *csaR*, while pZZG3 contains both *csaR* and *csaI*.

parable to that of a GacA null mutant (30-84.*gacA*). Surprisingly, introduction of *csaI-csaR* into the *phzI csaI* double mutant or introduction of *csaR* in *trans* into the *phzR csaR* double mutant failed to restore protease activity (Table 3).

No differences in HCN production or fluorescence between strain 30-84 and any of the single or double mutants were detected (data not shown).

CsaR and the AHL signal are required for expression of two surface traits. Mutations in *csaR*, regardless of other alterations, exhibited a shiny, mucoid phenotype on KMB agar in contrast to the rough, semidry phenotype of strain 30-84 (Fig. 3A). Like the *csaR* mutants, strain 30-84I/I2 also showed a mucoid phenotype (Fig. 3A). Strains that contained a functional *csaR* and at least one AHL synthase gene, such as 30-84I, 30-84R, 30-84I2, and 30-84I2/R, produced bacterial colonies with a rough, semidry surface (Fig. 3A). Each of the above strains also showed the same phenotype on AB agar (data not shown). In contrast, all the mutant strains and strain 30-84 displayed the same mucoid phenotype when grown on PPM-D or LB agar (data not shown). Strain 30-84(pZZG11) containing multiple copies of *csaR* showed a rough phenotype not only on KMB and AB agar but also on PPM-D and LB agar (data not shown). Complementation of the *csaR* mutation in 30-84R2, 30-84R/R2, and 30-84I/R2 by pZZG11 resulted in colonies with surface morphologies indistinguishable from that of strain 30-84 on KMB agar (Fig. 3B). These strains maintained the rough morphology on PPM-D agar (data not shown). In addition, the presence of multiple copies of *csaI csaR* or *csaR* alone in the *phzI csaI* double mutant also resulted in a rough phenotype (Fig. 3B).

All strains produced capsules when grown in KMB, as indicated by the presence of a white envelope surrounding the cell (data not shown). Both the *csaR* and *csaI phzI* mutants displayed a clumping phenotype with cell aggregates, in contrast to cells of strain 30-84, which were uniformly distributed (Fig. 4). Introduction of functional copies of *csaR* or *csaI csaR* into these mutants restored the wild-type phenotype. Interestingly, the *csaI* mutant did not clump, suggesting that *phzI* may complement this defect. The addition of AHL-containing ethyl acetate extracts from strain *E. coli* DH5α(pUC18-*csaI*) reversed the clumping phenotype in the *csaI phzI* mutant, unlike an equivalent amount of strain DH5α(pUC18) extract (data not shown).

CsaI directs the synthesis of a diffusible signal. Several methods were employed to determine whether CsaI is responsible for the production of a diffusible AHL signal. The AHL-specific reporter strains 30-84I/I2, 30-84I/Z, A136(pCF240), and CV026 and the differential AHL donor strains 30-84Ice/I and 30-84Ice/I2 were used in a cross-feeding assay, in which an AHL donor strain and an AHL reporter strain were "V" streaked onto the same agar plate. Diffusion of AHL signals from 30-84Ice/I2 (*phzI*$^+$ *csaI*) but not from strain 30-84Ice/I (*phzI csaI*$^+$) or DH5α(pUC18-*csaI*) stimulated phenazine production in 30-84I/I2 (Fig. 5A) or β-galactosidase activity in 30-84I/Z (data not shown). These data indicate that the PhzI-generated signal, not the CsaI-generated signal, specifically activates PhzR to induce phenazine operon expression. When *C. violaceum* CV026 was used as the AHL sensor, production of violacein was restored by the presence of either signal. To further verify the presence and specificity of the *csaI* signal, *E. coli* DH5α(pUC18-*csaI*) was used as an AHL donor. This strain did not effectively induce phenazine production by 30-84I/I2 but did rapidly induce violacein production by CV026 (Fig. 5B) in comparison to strain DH5α(pUC18), which did not affect either strain (data not shown).

In a separate cross-feeding assay, the double *phzI csaI* mutant was restored to the wild-type semidry, rough colony morphology on KMB or AB agar when cross-streaked with 30-84Ice/I (*phzI*), 30-84Ice/I2 (*csaI*), or DH5α(pUC18-*csaI*) (Fig. 5C).

AHL signal production by 30-84Ice/I (*csaI*$^+$) and 30-84Ice/I2 (*phzI*$^+$) was quantified by determining their effects on β-galactosidase activity in 30-84I/Z and *A. tumefaciens* A136(pCF240). The amount of β-galactosidase activity in the reporters is correlated to the specificity and amount of the AHL signal present. AHL extracts from 30-84Ice/I2 (*phzI*$^+$) significantly

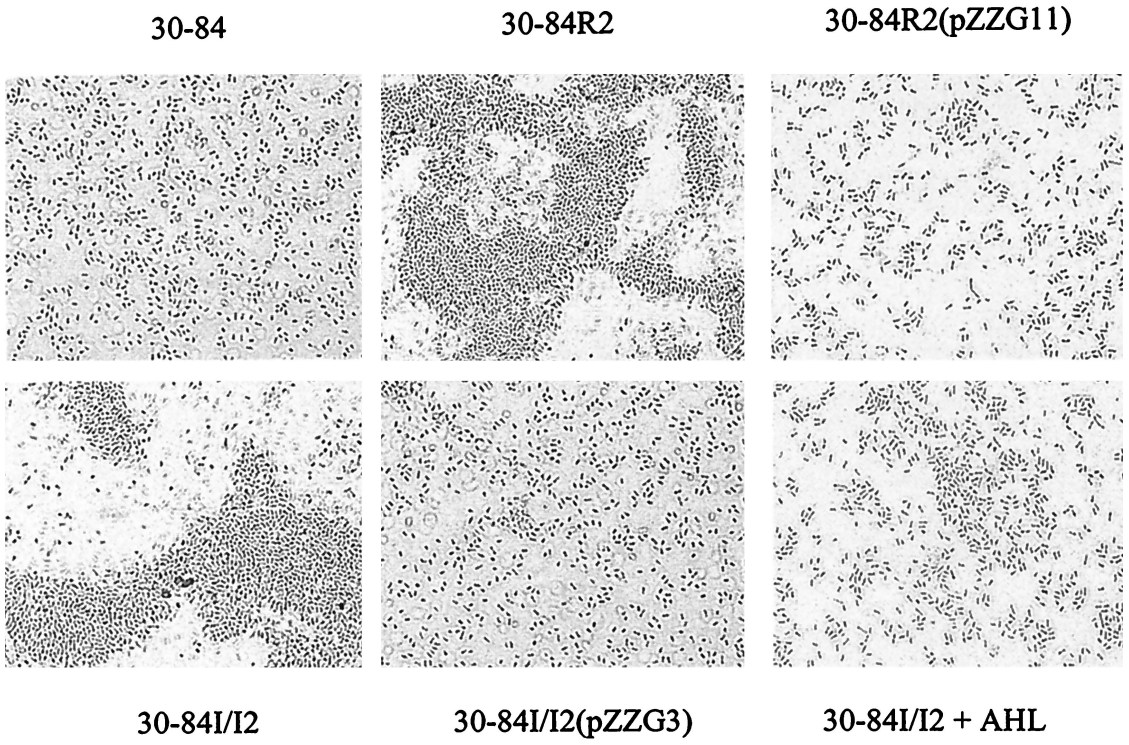

FIG. 4. Micrographs of *P. aureofaciens* 30-84 and derivatives. Bacteria were grown in KMB broth for 24 h and stained with Congo red prior to microscopic observation.

stimulated *phzB*::*lacZ* expression in 30-84I/Z compared to that of 30-84I/Z without extract (796 ± 32 versus 45 ± 8 U/ml). In contrast, the 30-84Ice/I (*csaI*$^+$) AHL extract only slightly elevated β-galactosidase activity in 30-84I/Z (108 ± 12 U/ml). When A136(pCF240) was used as the reporter, AHL extracts from both strains markedly improved *traA*::*lacZ* expression, with their effects on β-galactosidase activity in A136(pCF240) being virtually equal (3,743 ± 316 versus 3,693 ± 260 U/ml). As expected, addition of extracts of either signal had no effect on β-galactosidase activity in 30-84Z/s*gacA* (11 ± 3 U/ml for each).

Consistent with the above observations, the presence of AHL extracts from *E. coli* DH5α(pUC18-*csaI*) caused a 10-fold increase in *traA*::*lacZ* expression in A136(pCF240) but had little effect on *phzB*::*lacZ* expression in 30-84I/Z, although the above data suggest that DH5α(pUC18-*csaI*) synthesizes high levels of AHL (data not shown).

Colonization of the wheat rhizosphere. The various mutants were compared to strain 30-84 for survival and colonization of the wheat rhizosphere. When the seeds were sown in potting mix containing pasteurized soil, the *phzI*, *csaI*, *phzR*, and *csaR* single null mutants colonized the roots at levels comparable to that of strain 30-84. However, fivefold-lower population levels were detected on the roots colonized by the double *phzI csaI* and *phzR csaR* mutants (Table 4).

When plants were grown in potting soil containing natural soil, overall bacterial rhizosphere populations were lower than those from roots grown in pasteurized soil (Table 4). Except for the *csaR* mutant, which established population numbers similar to those of the wild type, all the other mutants showed significantly lower root colonization abilities than 30-84. Similar to observations seen in the pasteurized soil, the double I and double R mutants were the least effective in colonizing and surviving in the wheat rhizosphere, with their population densities being at least 47-fold lower than that of 30-84 isolated from roots in the same soil (Table 4).

DISCUSSION

Our previous research identified the PhzR-PhzI quorum-sensing system responsible for controlling phenazine antibiotic production in *P. aureofaciens* strain 30-84 (35, 42). In the present study, we identified a second quorum-sensing regulatory system, termed CsaR-CsaI (for "cell surface alterations"), which is only marginally involved in phenazine regulation. The primary function of the CsaR-CsaI system appears to be the regulation of exoprotease production in conjunction with the PhzR-PhzI system and also the regulation of cell surface properties.

CsaI and CsaR were most similar to RhlI and RhlR, respectively, the second quorum-sensing system discovered in *P. aeruginosa* (16, 31). However, these two quorum-sensing systems differ from each other. While *rhlI* and *rhlR* are separated by 181 bp and *rhlI* has its own promoter (28), *csaR* and *csaI* are separated by only 30 bp and *csaI* has an RBS but no promoter, suggesting that *csaI* expression is dependent on *csaR*. The RhlR-RhlI system is primarily responsible for regulating rhamnolipid production in *P. aeruginosa*, but *P. aureofaciens* strain 30-84 does not synthesize rhamnolipids. Finally, in *P. aeruginosa* the LasR-LasI and RhlR-RhlI systems exist in a hierarchical relationship, while PhzR-PhzI and CsaR-CsaI appear to function independently.

The CsaR-CsaI system is responsible for the low but detect-

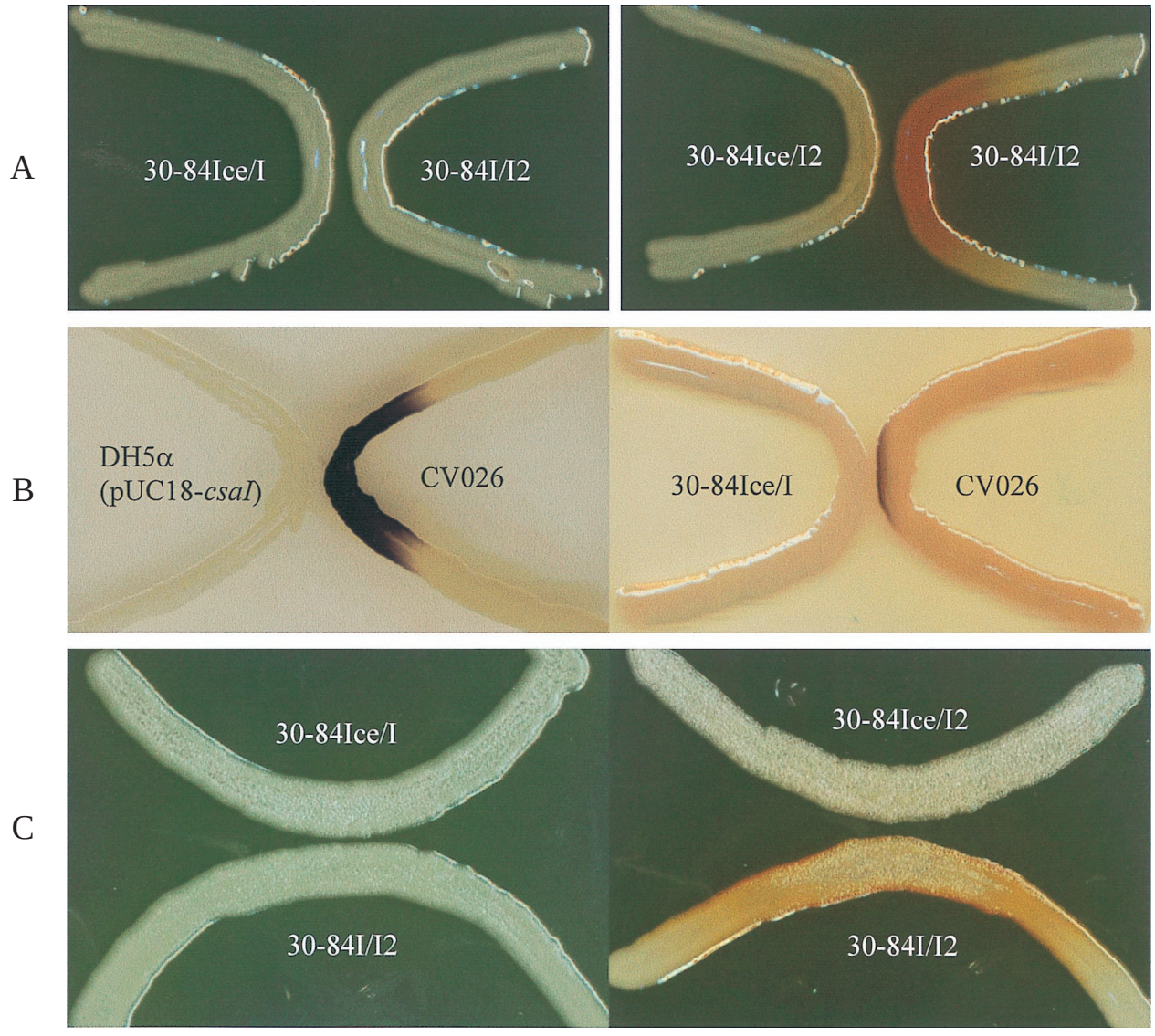

FIG. 5. Assay for the presence and specificity of AHL signals generated by CsaI and PhzI. (A) Activation of phenazine biosynthesis in 30-84I/I2 (*phzI csaI*) on PPM-D agar by AHL signal diffused from 30-84Ice/I (*phzI*) or 30-84Ice/I2 (*csaI*) cultures. (B) Activation of violacein biosynthesis in *C. violaceum* CV026 on LB agar by AHL signal from *E. coli* DH5α(pUC18-*csaI*) or 30-84Ice/I. (C) Restoration of 30-84I/I2 to a rough phenotype on KMB agar by exogenous AHL from 30-84Ice/I or 30-84Ice/I2 cultures.

able phenazine production observed in *phzI* and *phzR* null mutants in PPM-D medium. However, *csaI* or *csaR* null mutants produced phenazines in only slightly smaller amounts in PPM-D than the wild-type strain, and disruption of *phzI* and *csaI* or *phzR* and *csaR* completely eliminated antibiotic production in this medium. The presence of multiple copies of *csaR-csaI* in *trans* in 30-84I/I2 (Table 3) and 30-84R/R2 (data not shown) only partially restored phenazine production, indicating that the CsaR-CsaI system cannot substitute for the PhzR-PhzI system for phenazine production. Evidence that CsaI is an AHL synthase includes activation of the AHL-specific reporters *C. violaceum* CV026 and *A. tumefaciens* A136(pCF240) by AHL extracts of the *phzI* mutant 30-84I and *E. coli* DH5α(pUC18-*csaI*) (Fig. 5). The AHL signals generated by CsaI and PhzI cannot activate PhzR and CsaR, respectively, to induce phenazine biosynthesis, as evidenced by the fact that phenazine production in double mutants containing one functional AHL synthase and the noncognate regulator was abolished. These data suggest that unlike for PhzR-PhzI, phenazine regulation is not the primary role of CsaR-CsaI.

Analogous to the PhzR-PhzI system, while multiple copies of *csaR-csaI* in *trans* did enhance β-galactosidase activity and phenazine production in strains 30-84Z and 30-84R, respectively, neither restored detectable phenazine production in strain 30-84.*gacA* (Table 3). These data indicate that both quorum sensing systems require GacS-GacA in order to function.

Mutation of either *phzI* or *csaI* or of either *phzR* or *csaR* had no effect on exoprotease production by strain 30-84, indicating the two quorum-sensing systems may interact to regulate exoprotease production (Table 2). However, disruption of *phzI* and *csaI* or *phzR* and *csaR* abolished exoprotease activity, in-

TABLE 4. Bacterial populations of 30-84 and its derivatives recovered from the wheat rhizosphere[a]

Strain	Genotype	Log CFU/g of roots[b]	
		Pasteurized soil	Natural soil
30-84	Wild type	7.68 ± 0.27 A	7.14 ± 0.26 A
30-84I	*phzI*::*npt*	7.60 ± 0.25 A	6.42 ± 0.23 B
30-84R	*phzR*::Tn*5lacZ*	7.54 ± 0.26 A	6.32 ± 0.23 B
30-84I2	*csaI*::*uidA-Gm*	7.54 ± 0.24 A	6.20 ± 0.29 B
30-84R2	*csaR*::*uidA-Gm*	7.63 ± 0.21 A	6.89 ± 0.28 A
30-84I/I2	*phzI*::*npt csaI*::*uidA-Gm*	6.98 ± 0.29 B	5.44 ± 0.28 C
30-84R/R2	*phzR*::Tn*5lacZ csaR*::*uidA-Gm*	6.94 ± 0.30 B	5.46 ± 0.23 C

[a] Pregerminated wheat seeds were treated with a bacterial suspension and sown in potting mix containing pasteurized or natural soil contained in plastic cones. The cones were placed in a growth chamber. Twenty days after emergence, root samples were collected and bacteria were isolated.

[b] All values are means ± standard deviations of two experiments, with four replicates per experiment. Values followed by the same letter in each column are not significantly different ($P = 0.05$).

dicating that exoprotease in strain 30-84 is under AHL-mediated regulation. It is interesting that the two quorum-sensing systems appear to be able to interact for exoprotease production while they are unable to interact for phenazine production. This suggests that both CsaI and PhzI signals are capable of activating their noncognate R proteins to induce protease activity. This is different from *P. aeruginosa*, in which both *las* and *rhl* systems are involved in regulating exoprotease activity (5, 31). Although the *las* system regulated *lasB* and *lasA* and the *rhl* system regulated *lasB*, the R proteins were not significantly activated by their noncognate AHL to induce transcription of the respective protease genes (31).

A further complication in exoprotease regulation was the observation that AHL produced by CsaI or PhzI (or both) failed to restore exoprotease activity in 30-84I/I2. Furthermore, multiple copies of *csaR* in *trans* failed to restore proteolysis in 30-84R/R2, and so did the introduction of *csaR-csaI* in *trans* in 30-84I/I2. This is in contrast to the phenazine phenotype discussed above and the colony surface phenotype discussed below, and it differs from the situation in other bacteria, in which exoprotease activity in an I or R mutant can be restored by the respective I or R gene (5, 17). This may reflect additional as-yet-unknown levels of exoprotease regulation in strain 30-84. A similar observation was reported in that lipase activity was not restored in a *B. cepacia cepI* or *cepR* mutant by addition of AHL signal or introduction of the appropriate gene (17).

Unlike strain 30-84, mutants defective in *csaR*, regardless of *csaI*, *phzR*, or *phzI*, exhibited a mucoid colony morphology and a clumping phenotype in KMB. This suggested that *csaR* mutants are altered in some cell surface property. Additionally, a *csaI phzI* double mutant demonstrated a similar smooth and clumping phenotype, whereas variants with mutations in *csaI* or *phzI* alone did not. This suggest that colony smoothness and the clumping phenotype are controlled by CsaR and that either the CsaI or PhzI signal can interact with CsaR to regulate these traits. It is currently unclear whether the smooth phenotype is due to the *csaR* mutation allowing a trait to be expressed or, more likely, the loss of a trait. In *Pantoea stewartii*, EsaR represses transcription of extracellular polysaccharide (2, 3), in *B. cepacia* the CepR-CepI system represses siderophore production (17), and in *Rhodobacter sphaeroides* a *cepI* mutant overproduces exopolysaccharide (3, 36), while a *ypsR* mutant of *Yersinia pseudotuberculosis* exhibits increased cell aggregation, motility, and flagellin production (1). Microscopic observation did not reveal any obvious differences in cell capsules between the *csaR* mutant and wild-type strain 30-84 (data not shown). Fatty acid analysis (Microbial ID, Inc.) indicated that the *csaR* mutant had alterations in fatty acid composition or relative percentages of some fatty acids compared to 30-84, although both strains were still clearly *P. aureofaciens* (data not shown). Comparison of the *csaR* mutant with 30-84 did not reveal motility differences (data not shown).

Disruption of *phzI*, *phzR*, *or csaI* resulted in reduced colonization of the wheat rhizosphere in natural soil, with the most notable reduction being seen in the *phzI csaI* and *phzR csaR* double mutants. These results indicate that quorum sensing plays an important role in the survival of strain 30-84 and competition with other microorganisms in situ. Interestingly, the *csaR* mutant still colonized roots to levels similar to those of strain 30-84. The reason for this is unclear, but one possible hypothesis is that aggregation of the *csaR* mutants may have enabled them to persist over the course of the experiment. In potting soil containing pasteurized soil, all single mutants colonized wheat roots to the same level as the wild type. This may reflect the lack of competition with other rhizosphere microflora. However, mutations in *phzI csaI* or *phzR csaR* resulted in lower bacterial survival in this pasteurized soil, demonstrating that both quorum-sensing systems play a role in rhizosphere survival.

This work provides a new example of a microorganism that employs two unrelated AHL-mediated quorum-sensing circuits to regulate multiple functions. The existence of two nonhierarchical regulatory systems that interact to control some behaviors but not others has important implications for the spatial and temporal control of gene expression in the bacterium. Future studies will focus on determining how the CsaR-CsaI and PhzR-PhzI systems interact with each other and their effect on rhizosphere colonization and biocontrol activity.

ACKNOWLEDGMENTS

We thank Francoise Blachere, Scott Chancey, Patricia Figuli, and Cheryl Whistler for technical assistance. We also thank Christina Kennedy and Elizabeth Pierson for critical reviews of the manuscript.

This work was supported by USDA NRI-CGP grant 98-02129.

REFERENCES

1. **Atkinson, S., J. P. Throup, G. S. A. B. Stewart, and P. Williams.** 1999. A hierarchical quorum-sensing system in *Yersinia pseudotuberculosis* is involved in the regulation of motility and clumping. Mol. Microbiol. **33:**1267–1277.
2. **Beck von Bodman, S., and S. K. Farrand.** 1995. Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an *N*-acylhomoserine lactone autoinducer. J. Bacteriol. **177:**5000–5008.
3. **Beck von Bodman, S., D. R. Majerczak, and D. L. Coplin.** 1998. A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. stewartii. Proc. Natl. Acad. Sci. USA **95:**7687–7692.
4. **Belew, M., and J. Porath.** 1970. Extracellular proteinase from *Penicillium notatum*. Methods Enzymol. **19:**576–581.
5. **Brint, J. M., and D. E. Ohman.** 1995. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under control of RhlR-RhlI, another set of regulators in strain PAOI with homology to the autoinducer-response LuxR-LuxI family. J. Bacteriol. **177:**7155–7163.
6. **Chancey, S. T., D. W. Wood, and L. S. Pierson III.** 1999. Two-component transcriptional regulation of *N*-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. Appl. Environ. Microbiol. **65:**2294–2299.
7. **Eberl, L.** 1999. *N*-Acyl homoserine lactone-mediated gene regulation in

Gram-negative bacteria. Syst. Appl. Microbiol. **22:**493–506.
8. **Flavier, A. B., L. M. Ganova-Raeva, M. A. Schell, and T. P. Denny.** 1997. Hierarchical autoinduction in *Ralstonia solanacearum*: control of *N*-acyl-homoserine lactone production by a novel autoregulatory system responsive to 3-hydroxypalmitic acid methyl ester. J. Bacteriol. **179:**7089–7097.
9. **Fuqua, W. C., and S. C. Winans.** 1994. A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. J. Bacteriol. **176:**2796–2806.
10. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the Lux-LuxI family of quorum sensing transcriptional regulators. Annu. Rev. Microbiol. **50:**727–751.
11. **Gilson, L., A. Kuo, and P. V. Dunlop.** 1995. AinS and a new family of autoinducer synthesis proteins. J. Bacteriol. **177:**6946–6951.
12. **Gray, K. M., L. Passador, B. H. Iglewski, and E. P. Greenberg.** 1994. Interchangeability and specificity of components from the quorum-sensing regulatory systems of *Vibrio fischeri* and *Pseudomonas aeruginosa*. J. Bacteriol. **176:**3076–3080.
13. **Hoagland, D. R., and K. I. Arnon.** 1950. The water-culture method for growing plants without soil. UC Agricultural Experiment Station circular 347, revised ed. UC Agricultural Experiment Station, Davis, Calif.
14. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. **44:**301–307.
15. **Kuo, A., N. V. Blough, and P. V. Dunlap.** 1994. Multiple *N*-acyl-L-homoserine lactone autoinducer of luminescence in the marine symbiotic bacterium *Vibrio fischeri*. J. Bacteriol. **176:**7558–7565.
16. **Latifi, A., M. K. Winson, M. Foglino, B. W. Bycroft, G. S. A. B. Stewart, A. Lazdunski, and P. Williams.** 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. Mol. Microbiol. **17:**333–343.
17. **Lewenza, S., B. Conway, E. P. Greenberg, and P. A. Sokol.** 1999. Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. J. Bacteriol. **181:**748–756.
18. **Lindgren, P. B., R. Frederick, A. G. Govindaragan, N. J. Panopoulos, B. J. Staskawicz, and S. E. Lindow.** 1989. An ice nucleation reporter gene system: identification of inducible pathogenicity genes in *Pseudomonas syringae* pv. phaseolicola. EMBO J. **8:**1291–1301.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Mavrodi, D. V., V. N. Ksenzenko, R. I. Bonsall, R. J. Cook, A. M. Boronin, and L. S. Thomashow.** 1998. A seven-gene locus for synthesis of phenazine-1-carboxylic acid by *Pseudomonas fluorescens* 2-79. J. Bacteriol. **180:**2541–2548.
21. **Mazzola, M., R. J. Cook, L. S. Thomashow, D. M. Weller, and L. S. Pierson.** 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Appl. Environ. Microbiol. **58:**2616–2624.
22. **McLean, K. H., M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. Microbiology **143:**3703–3711.
23. **Metcalf, W. M., and B. L. Wanner.** 1993. Construction of new β-glucuronidase cassettes for making transcriptional fusions and their use with methods for allele replacement. Gene **129:**17–25.
24. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Morè, M. I., L. D. Finger, J. L. Stryker, C. Fuqua, A. Eberhard, and S. C. Winans.** 1996. Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science **272:**1655–1658.
26. **Murillo, J., H. Shen, D. Gerhold, A. Sharma, D. Cooksey, and N. T. Keen.** 1994. Characterization of pPT23B, the plasmid involved in syringolide production by *Pseudomonas syringae* pv. tomato PT23. Gene **31:**275–287.
27. **Ochsner, U. A., A. K. Koch, A. Fiechter, and J. Reiser.** 1994. Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **176:**2044–2054.
28. **Ochsner, U. A., and J. Reiser.** 1995. Autoinducer-mediated regulation of rhamnolipid biosynthesis in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **92:**6424–6428.
29. **Parsek, M. R., A. L. Schaefer, and E. P. Greenberg.** 1997. Analysis of random and site-directed mutations in *rhlI*, a *Pseudomonas aeruginosa* gene encoding an acylhomoserine lactone synthase. Mol. Microbiol. **26:**301–310.
30. **Parsek, M. R., D. L. Val., B. L. Hanzelka, J. E. Cronan, Jr., and E. P. Greenberg.** 1999. Acyl homoserine-lactone quorum-sensing signal generation. Proc. Natl. Acad. Sci. USA **96:**4360–4365.
31. **Pearson, J. P., E. C. Pesci, and B. H. Iglewski.** 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J. Bacteriol. **179:**5756–5767.
32. **Pierson, E. A., D. W. Wood, J. A. Cannon, F. M. Blachere, and L. S. Pierson III.** 1998. Interpopulation signaling via *N*-acyl-homoserine lactone among bacteria in the wheat rhizosphere. Mol. Plant-Microbe Interact. **11:**1078–1084.
33. **Pierson, L. S., III, D. W. Wood, and E. A. Pierson.** 1998. Homoserine lactone-mediated gene regulation in plant-associated bacteria. Annu. Rev. Phytopathol. **36:**207–225.
34. **Pierson, L. S., III, and L. S. Thomashow.** 1992. Cloning and heterologous expression of phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84. Mol. Plant-Microbe Interact. **5:**330–339.
35. **Pierson, L. S., III, V. D. Keppenne, and D. W. Wood.** 1994. Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. J. Bacteriol. **176:**3966–3974.
36. **Puskas, A., E. P. Greenberg, S. Kaplan, and A. L. Schaeffer.** 1997. A quorum-sensing system in the free-living photosynthetic bacterium *Rhodobacter sphaeroides*. J. Bacteriol. **179:**7530–7537.
37. **Rosemeyer, V., J. Michiels, C. Verreth, and J. Vanderleyden.** 1998. *luxI*- and *luxR*-homologous genes of *Rhizobium etli* CNPAF-52 contribute to synthesis of autoinducer molecules and nodulation of *Phaseolus vulgaris*. J. Bacteriol. **180:**815–821.
38. **Schleif, R. F., and P. C. Wensink.** 1981. Practical methods in molecular biology. Springer-Verlag, New York, N.Y.
39. **Staskawicz, B., D. Dahlbreck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. glycinea. J. Bacteriol. **169:**5789–5794.
40. **Weller, D. M.** 1988. Biological control of soilborne pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. **26:**379–407.
41. **Whooley, M. A., J. A. O'Callaghan, and A. J. McLoughlin.** 1983. Effect of substrate on the regulation of exoprotease production by *Pseudomonas aeruginosa* ATCC 10145. J. Gen. Microbiol. **129:**981–988.
42. **Wood, D. W., and L. S. Pierson III.** 1996. The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. Gene **168:**49–53.
43. **Wood, D. W., F. Gong, M. M. Daykin, P. Williams, and L. S. Pierson III.** 1997. *N*-Acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. J. Bacteriol. **179:**7603–7670.
44. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.