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

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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 *phzYFABCD* 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/Z exhibited a rough 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 conjugation, 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
regulated in a cell density-dependent manner in a number of other bacterial species (5, 17, 22). Interestingly, phzI regulated in a cell density-dependent manner in a number of W. Bockus P. aureofaciens 4306 ZHANG AND PIERSON APPL. ENVIRON. MICROBIOL.

phenotypes regulated specifically by the CsaR-CsaI system PhzI in regulating phenazine and exoprotease production and The nature of the interaction between CsaR-CsaI and PhzR-P. aureofaciens or more quorum-sensing systems that regulate expression of medium. Recently, several bacteria were shown to harbor two null mutants produced phenazines at low levels on a certain C. violaceum CV026 Double mini-Tn5 mutant from C. violaceum ATCC 31532, AHL biosensor 16 A. tumefaciens A136 Ti plasmid-less, pCF240traA lacZ113, pCF251

E. coli DH5α F- recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 Δ(argE-lacZYA)169 Δ80lacZΔM15 Gibco-BRL MC1061 F- araD139 Δ(araABC-lev)7679 galU galK Δ(lac) X74 rpsL thi Gibco-BRL

TABLE 1. Bacterial strains and plasmids used in this study

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<td>Wild type, plant disease biocontrol agent, Rif’</td>
<td>W. Bockus</td>
</tr>
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<td>phzB:lacZ genomic fusion</td>
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Plasmids

pLAFLR3 IncP1, Te’ 39
pPMgM ColE1, Gm’ 26
pWM3 ColE1, Ap’, uidA transcriptional fusion cassette 23
pUC18 ColE1, Ap’ 44
pLSP5-5 pLAFLR3 carrying a 29-kb fragment of 30-84 chromosomal DNA that contains csal and csar This study
pZZG5-5-2 pLAFLR3 carrying the 7.15-kb EcoRI fragment from pLSP5-5 that contains csal and csar This study
pZZG3 pLAFLR3 carrying the 5-kb PstI-HindIII fragment from PC20/H5-5-2 that contains csal and csar This study
pZZG11 pLAFLR3 carrying the 1-kb SphI-EcoRV fragment that contains csar This study
pZZG18-1 pLAFLR3 carrying the 5-kb PstI-HindIII fragment that contains csal and csar This study
pZZG18-2 pZZG18-1 containing the introduced SacI site in csal This study
pZZG18-3 pZZG18-1 containing the introduced SacI site in csar This study
pWM3 pWW3 carrying the 2.5-kb SacI fragment from pWM3-Gm, Gm’ This study
pZZG11-Sac pLAFLR3 carrying the 5-kb PstI-HindIII fragment from pZZG18-2 This study
pZZG- Sac pLAFLR3 carrying the 5-kb PstI-HindIII fragment from pZZG18-3 This study
pZZG11-uidAGm pZZG11-Sac carrying the 4-kb SacI fragment from pWM3-Gm that contains uidA-Gm This study
pZZG-uidAGm pZZG-Sac carrying the 4-kb SacI fragment from pWM3-Gm that contains uidA-Gm This study
pUC18-csal pUC18 containing the 2.5-kb SacI-SphI fragment from pZZG18-4 This study
pLSPphzB-inaZ pLAFLR3 containing the phzB-inaZ fusion 43

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 pigm 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 luxZ-luxB 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’ and Te’ 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-
jugglers 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 Kmr and Xmr were used previously to construct strains 30-84I (phzI::pstI), 30-84R (phzR::pstR), TxSacZ), mutants with mutations in csaR and csaI were constructed using a β-glucuronidase-and-gentamicin resistance (uidA-Gm) cartridge constructed in the University of Wisconsin Genetics Computer Group Software packages (version 9.1). 30-84I2 (phzI csaR csaI phzR csaR), 30-84R2 (phzI csaR csaI phzR csaI), and 30-84I2/R2 (phzI csaI phzR csaR) were constructed analogously as described above and confirmed by Southern hybridization (data not shown). Two mutants, named 30-84I2 (csaI::phzI) and 30-84R2 (csaR::phzR), were selected. Double mutants 30-84I2 (phzI csaI), 30-84R2 (phzR csaR), 30-84I2/R2 (phzI csaI), and 30-84I2/R2 (csaI csaR) were constructed qualitatively determined by examining orange color on PPM-D medium and quantified from cell extracts as described previously (6).

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).

Colonies 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

**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 pLS5-5 on β-galactosidase expression in the phzR::lacZ reporter strain 30-84Z. Letters indicate significant differences between values. (B) csaR and csaI loci from pZZG3 and insertional mutation of csaI and csaR. Restriction enzyme abbreviations: R, EcoRI; H, HindIII; Sp, SphI; V, EcoRV, Bg, BglII, P, PstI, S, SacI.
whether bacterial cells clumped, strains were cultured in KMB for 24 h. Three microtiter plates 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, 30-84iecI2 (p251 csaI::uidA-Gm phzI::mazZ) was constructed by introduction of pLSphzI::mazZ (42) into 30-84iecI via triparental mating and verified via Southern hybridization (data not shown). To determine whether csaI can direct synthesis of a diffusable signal, the 3.2-kb SpIil fragment containing csaI was excised from pZZGP18-3 and ligated into pUC18 (44) in the same orientation as in pZZGP18-3. The resulting plasmid was digested with SacI and religated, generating pUC18-csaI, which carries csaI under the control of the vector lacZ promoter. E. coli DH5a(pUC18-csaI) was used as a CaAl AHL donor strain.

The AHL-dependent reporter strains 30-84iecI2 (p251::ntpI::uidA-Gm), 30-84iecI (p251::ntpI::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 30-84iecI2 (p251::ntpI::lacZ), 30-84iecI (p251::ntpI::lacZ) and 30-84iecI2 (p251::mazZ csaI::uidA-Gm phzI::mazZ) 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 30-84iecI2 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 30-84iecI was used, the recipient and donor were streaked onto PPM-D plus X-Gal. When CV026 was the recipient, the assay was performed on PPM-D agar to examine phenazine production. Strain 30-84iecI and strain 30-84iecI2 transconjugants in strain 30-84iecI2 resulted in a dark blue colony surrounded by a blue halo (data not shown). When cultured in M9 broth, 30-84iecI2(pLSphzI-5) expressed ninefold-higher β-galactosidase activity than 30-84iecI2(pLARF3) (Fig. 1A). The same cosmid restored phenazine production to strain 30-84iecI, as indicated by an orange colony compared to the white 30-84iecI2 (pLARF3) colony (data not shown). Analysis of pLSphzI-5 indicated the presence of a 29-kb insert comprised of EcoRI 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 30-84iecI2 (Fig. 1A). Further deletion analysis revealed that the 2.2-kb SpIil-EcoRV fragment and the 1-kb EcoRV-SpIil fragment resulted in elevated β-galactosidase activity.

DNA sequence analysis of the 1,034-bp EcoRV-SpIil 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. aeruginosa 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 SpIil-EcoRV 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 EcoRV 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 RhlR (28), B. cepacia CepI (17), P. aeruginosa 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 30-84iecI2 (ca5I::uidA-Gm) and 30-84iecI2 (c5I::uidA-Gm). Similarly, four double mutants, 30-84iecI2 (phzI::km csaI::uidA-Gm), 30-84iecI2 (phzI::zpt csaI::uidA-Gm), 30-84iecI2 (ca5I::uidA-Gm phzI::zpt csaI::uidA-Gm), and 30-84iecI2 (phzI::zpt csaI::uidA-Gm) were constructed. All mutants were verified in trans in strain 30-84iecI2 in a dark blue colony surrounded by a blue halo (data not shown). When cultured in M9 broth, 30-84iecI2(pLSphzI-5) expressed ninefold-higher β-galactosidase activity than 30-84iecI2(pLARF3) (Fig. 1A). The same cosmid restored phenazine production to strain 30-84iecI, as indicated by an orange colony compared to the white 30-84iecI2 (pLARF3) colony (data not shown). Analysis of pLSphzI-5 indicated the presence of a 29-kb insert comprised of EcoRI 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 30-84iecI2 (Fig. 1A). Further deletion analysis revealed that the 2.2-kb SpIil-EcoRV fragment and the 1-kb EcoRV-SpIil fragment resulted in elevated β-galactosidase activity.

**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...
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

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 PA01 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 (L32724); 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 PA01 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 Cepl (AF019654); VF LuxI, V. fischeri LuxI (Y00509).
2 days after emergence, high InaZ activity (phenazines produced by the nucleation activity (InaZ) is proportional to the amount of control of the phenazine operon promoter. The amount of ice fusion and synthesize ice nucleation protein under the phenazine gene expression in the wheat rhizosphere. These nuclei/cell) was detected from each bacterial strain isolated P significantly different (P < 0.05).

When cultured in PPM-D, 30-84R/R2 (phzI csaI), and 30-84I/csaI (phzI csaI) produced phenazines at ca. 40% of the wild-type levels (Table 3). This partial complementation R(pZZG3) were assayed for phenazine production. The presence of the vector pLAFR3 alone had no effect on any of the strains listed (data not shown).

Bacterial strains were cultured on KMB agar, and 2 days later the colonies were examined for roughness and mucoidy. Exoprotease activity in supernatants of 24-h-old broth cultures was determined by a modified Lowry method using casein as the substrate. Phenazines were extracted from supernatants of 24-h-old bacterial cultures grown in PPM-D and quantified by measuring absorbance at 367 nm.

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

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<th>Strain</th>
<th>Genotype</th>
<th>Absorbance at 367 nm$^{ac}$</th>
<th>Protease activity$^{bc}$ (U/ml)</th>
<th>Colony morphology$^{d}$</th>
<th>Clumping phenotype$^{e}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-84</td>
<td>Wild type</td>
<td>6.46 ± 0.09 A</td>
<td>3.05 ± 0.14 A</td>
<td>Semidry and rough</td>
<td>–</td>
</tr>
<tr>
<td>30-84I</td>
<td>phzI:nap</td>
<td>0.04 ± 0.00 C</td>
<td>0.30 ± 0.02 B</td>
<td>Semidry and rough</td>
<td>–</td>
</tr>
<tr>
<td>30-84R</td>
<td>phzR:Tn5lacZ</td>
<td>0.04 ± 0.00 C</td>
<td>0.24 ± 0.01 B</td>
<td>Semidry and rough</td>
<td>–</td>
</tr>
<tr>
<td>30-84I2</td>
<td>csaI:uidA-Gm</td>
<td>5.96 ± 0.18 B</td>
<td>2.89 ± 0.22 A</td>
<td>Shiny and mucoid</td>
<td>+</td>
</tr>
<tr>
<td>30-84R2</td>
<td>csaI:uidA-Gm</td>
<td>5.99 ± 0.33 B</td>
<td>2.89 ± 0.22 A</td>
<td>Shiny and mucoid</td>
<td>+</td>
</tr>
<tr>
<td>30-84I2</td>
<td>phzI:nap csaI:uidA-Gm</td>
<td>0.02 ± 0.00 C</td>
<td>0.03 ± 0.00 C</td>
<td>Shiny and mucoid</td>
<td>+</td>
</tr>
<tr>
<td>30-84R2</td>
<td>phzI:nap csaI:uidA-Gm</td>
<td>0.03 ± 0.01 C</td>
<td>0.05 ± 0.01 C</td>
<td>Shiny and mucoid</td>
<td>+</td>
</tr>
<tr>
<td>30-84I2/R</td>
<td>csaI:uidA-Gm phzR:Tn5lacZ</td>
<td>0.03 ± 0.01 C</td>
<td>0.05 ± 0.01 C</td>
<td>Shiny and mucoid</td>
<td>+</td>
</tr>
<tr>
<td>30-84R/R2</td>
<td>phzR:Tn5lacZ csaR:uidA-Gm</td>
<td>0.02 ± 0.01 C</td>
<td>0.02 ± 0.01 C</td>
<td>Shiny and mucoid</td>
<td>+</td>
</tr>
<tr>
<td>30-84, gacA</td>
<td>gacA:nap</td>
<td>0.02 ± 0.01 C</td>
<td>0.03 ± 0.01 C</td>
<td>Shiny and mucoid</td>
<td>+</td>
</tr>
</tbody>
</table>

$^{a}$ 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.

$^{e}$ The presence of the vector pLAFR3 alone had no effect on any of the strains listed (data not shown).

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

<table>
<thead>
<tr>
<th>Strain/</th>
<th>Genotype (host/plasmid)</th>
<th>Absorbance at 367 nm$^{ac}$</th>
<th>Protease activity$^{bc}$ (U/ml)</th>
<th>Colony morphology$^{d}$</th>
<th>Clumping phenotype$^{e}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-84(pLAFR3)</td>
<td>Wild type</td>
<td>5.91 ± 0.29 A</td>
<td>+</td>
<td>Semidry and rough</td>
<td>–</td>
</tr>
<tr>
<td>30-84R(pLAFR3)</td>
<td>phzR:Tn5lacZ</td>
<td>0.03 ± 0.01 C</td>
<td>+</td>
<td>Semidry and rough</td>
<td>–</td>
</tr>
<tr>
<td>30-84R2(pZZG11)</td>
<td>csaI:uidA-Gm/csaR$^+$</td>
<td>5.98 ± 0.39 A</td>
<td>+</td>
<td>Semidry and rough</td>
<td>–</td>
</tr>
<tr>
<td>30-84I2(pZZG11)</td>
<td>phzI:nap csaI:uidA-Gm/csaR$^+$</td>
<td>0.04 ± 0.01 C</td>
<td>+</td>
<td>Semidry and rough</td>
<td>–</td>
</tr>
<tr>
<td>30-84I2(pZZG33)</td>
<td>phzI:nap csaI:uidA-Gm/csaR$^+$ csaI$^+$</td>
<td>2.67 ± 0.11 B</td>
<td>–</td>
<td>Semidry and rough</td>
<td>–</td>
</tr>
<tr>
<td>30-84R2(pZZG11)</td>
<td>phzI:nap csaI:uidA-Gm/csaR$^+$</td>
<td>2.36 ± 0.20 B</td>
<td>+</td>
<td>Semidry and rough</td>
<td>–</td>
</tr>
<tr>
<td>30-84I2/R(pZZG11)</td>
<td>csaI:uidA-Gm phzR:Tn5lacZ/csaR$^+$ csaI$^+$</td>
<td>2.57 ± 0.20 B</td>
<td>+</td>
<td>Semidry and rough</td>
<td>–</td>
</tr>
<tr>
<td>30-84R/R2(pZZG11)</td>
<td>phzR:Tn5lacZ csaR:uidA-Gm/csaR$^+$</td>
<td>2.42 ± 0.19 B</td>
<td>–</td>
<td>Semidry and rough</td>
<td>–</td>
</tr>
<tr>
<td>30-84,gacA1(pZZG33)</td>
<td>gacA:nap/csaR$^+$ csaI$^+$</td>
<td>0.03 ± 0.00 C</td>
<td>+</td>
<td>Shiny and mucoid</td>
<td>–</td>
</tr>
</tbody>
</table>
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, 30-84/I2 (phzI csaI); 8, 30-84R2 (phzI csaR); 9, 30-84I2R (csaI phzR); and 10, 30-84.gacA. (B) Complementation of 30-84I/I2 and csaR mutants. Bacterial strains: 1, 30-84R2(pZZG11); 2, 30-84I2(pZZG11); 3, 30-84I2(pZZG3); 4, 30-84I/I2(pZZG11); and 5, 30-84/I2(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 csaR 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 30-84/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 30-84I2/R 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 csaR 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 phzI csaR 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 (phzl’ 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 csaR mutant was restored to the wild-type semidity, rough colony morphology on KMB or AB agar when cross-streaked with 30-84Ice/I (phzI), 30-84Ice/I2 (csaR), 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
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/sngacA (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 rhII and rhII are separated by 181 bp and rhII 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 csaI. 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-RhlII 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-
able phenazine production observed in \textit{phzI} and \textit{phzR} null mutants in PPM-D medium. However, \textit{csaI} or \textit{csaR} null mutants produced phenazines in only slightly smaller amounts in PPM-D than the wild-type strain, and disruption of \textit{phzI} and \textit{csaI} or \textit{phzR} and \textit{csaR} completely eliminated antibiotic production in this medium. The presence of multiple copies of \textit{csaR-csaI} in \textit{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 \textit{C. violaceum CV026} and \textit{A. tumefaciens A136(pCF240)} by AHL extracts of the \textit{phzI} mutant 30-84I and \textit{E. coli DH5\alpha(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 \textit{csaR-csaI} in \textit{trans} did enhance \(\beta\)-galactosidase activity and phenazine production in strains 30-84Z and 30-84R, respectively, neither restored detectable phenazine production in strain 30-84\textit{gacA} (Table 3). These data indicate that both quorum sensing systems require GacS-GacA in order to function.

Mutation of either \textit{phzI} or \textit{csaI} or of either \textit{phzR} or \textit{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 \textit{phzI} and \textit{csaI} or \textit{phzR} and \textit{csaR} abolished exoprotease activity, in-

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**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 (\textit{phzI csaI}) on PPM-D agar by AHL signal diffused from 30-84Ice/I (\textit{phzI}) or 30-84Ice/I2 (\textit{csaI}) cultures. (B) Activation of violacein biosynthesis in \textit{C. violaceum CV026} on LB agar by AHL signal from \textit{E. coli DH5\alpha(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.
indicating 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 lasI and the rhl system regulated lasB, the R proteins were not significantly activated by their noncognate AHL to induce transcription. 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 lasI and the rhl system regulated lasB, the R proteins were not significantly activated by their noncognate AHL to induce transcription.

A further complication in exoprotease regulation was the observation that AHL produced by CsaI or PhzI (or both) failed to restore exoprotease activity in 30-84/R2. Furthermore, multiple copies of lasA in trans failed to restore proteolysis in 30-84/R2, and so did the introduction of csaR csaI in trans in 30-84/R2. 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 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 non-hierarchical 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.

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