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A Quorum-Sensing System in the Free-Living Photosynthetic Bacterium *Rhodobacter sphaeroides*

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***Rhodobacter sphaeroides* is a free-living, photoheterotrophic bacterium known for its genomic and metabolic complexity. We have discovered that this purple photosynthetic organism possesses a quorum-sensing system. Quorum sensing occurs in a number of eukaryotic host-associated gram-negative bacteria. In these bacteria there are two genes required for quorum sensing, the *luxR* and *luxI* homologs, and there is an acylhomoserine lactone signal molecule synthesized by the product of the *luxI* homolog. In *R. sphaeroides*, synthesis of a novel homoserine lactone signal, 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone, is directed by a *luxI* homolog termed *cerI*. Two open reading frames immediately upstream of *cerI* are proposed to be components of the quorum-sensing system. The first of these is a *luxR* homolog termed *cerR*, and the second is a small open reading frame of 159 bp. Inactivation of *cerI* in *R. sphaeroides* results in mucoid colony formation on agar and formation of large aggregates of cells in liquid cultures. Clumping of CerI mutants in liquid culture is reversible upon addition of the acylhomoserine lactone signal and represents a phenotype unlike those controlled by quorum sensing in other bacteria.**

Rhodobacter sphaeroides 2.4.1 is a free-living, photoheterotrophic member of the α-3 subdivision of the *Proteobacteria* and has been widely studied due to its remarkable genomic and physiologic complexity. It has a unique genome architecture, possessing both a large (chromosome I, 3.0 Mbp) and a smaller (chromosome II, 0.9 Mbp) circular chromosome (42, 43) in addition to five other endogenous replicons (15). It can grow aerobically as a chemoheterotroph, and it can grow anaerobically by using photosynthetic electron transport (8, 24) or anaerobic respiration (13).

We report that *R. sphaeroides* produces an acylhomoserine lactone. *N*-Acylhomoserine lactones (commonly called autoinducers) are produced by many gram-negative bacteria and serve as intercellular signals that facilitate a phenomenon termed quorum sensing (17, 18, 34, 40). Quorum sensing allows a bacterial species to monitor its population density and activate specific sets of genes at sufficiently high cell numbers. Regulation of luminescence in *Vibrio fischeri* was the first reported example of *N*-acylhomoserine lactone-mediated quorum sensing (9, 25) and continues to serve as a model system (17, 18). In *V. fischeri*, the *luxI* gene encodes an autoinducer synthase that catalyzes the production of *N*-(3-oxohexanoyl) homoserine lactone (9). The *luxR* gene encodes a transcriptional regulator that activates expression of the luminescence genes in the presence of a sufficient concentration of *N*-(3-oxohexanoyl)homoserine lactone (11).

This type of regulation is involved in the expression of genes encoding extracellular virulence factors in *Pseudomonas aeruginosa* (27, 28), conjugal transfer in *Agrobacterium tumefaciens* (16, 30, 46), and antibiotic synthesis and extracellular enzyme and exopolysaccharide production in *Erwinia* species (2, 31) to name a few. In these quorum-sensing systems there is a LuxR homolog that responds to the acylhomoserine lactone autoinducer and a LuxI homolog that catalyzes the synthesis of the autoinducer signal. Depending upon the LuxI homolog the acyl group can vary from 4 to 14 carbons in length, can possess either a hydroxyl, a carbonyl, or no substitution on the third carbon, and can be fully saturated or contain a carbon-carbon double bond (17). The nature of the acyl group provides signal specificity (10, 36). Over a dozen LuxI and a dozen LuxR homologs have been identified (17, 18, 34, 40).

In most LuxR-LuxI-type quorum-sensing systems studied to date, the lifestyle of the bacterium includes a host-associated state, either as a pathogen or a mutualistic symbiont of an animal or plant. For *R. sphaeroides*, such interactions with a eukaryotic host have not been described. Nevertheless we have discovered that it produces an acylhomoserine lactone. We describe the structure of the acylhomoserine lactone and the cloning and characterization of the *luxI* homolog responsible for production of this molecule and a linked *luxR* homolog. We further describe a novel phenotype associated with a mutation in the *luxI* homolog, in which mutants secrete large quantities of an extracellular polysaccharide that appears to result in growth of large aggregates of *R. sphaeroides*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 1. Photoheterotrophic cultures of *R. sphaeroides* 2.4.1 were grown anaerobically in Sistrom's succinic acid minimal medium A (SIS) (6) or Luria-Bertani broth (LB) (35) and illuminated at 100 W/m^2. Anaerobic chemoheterotrophic cultures of *R. sphaeroides* 2.4.1 were grown in the dark in LB with 0.5% dimethylsulfoxide as an electron acceptor. For aerobic chemoheterotrophic growth, *R. sphaeroides* 2.4.1 cultures were grown in LB with shaking. *Paracoccus denitrificans* was grown in SIS, and *Escherichia coli* strains were grown in LB. Media for plating contained 1.5% agar. In all cases growth was at 30°C. For selection of transformants and transconjugants and for plasmid maintenance, media were supplemented with the appropriate antibiotics at the following concentrations: tetracycline, 1 μg/ml (10 μg/ml for *E. coli* strains); spectinomycin, 50 μg/ml; streptomycin, 50 μg/ml; kanamycin, 25 μg/ml; and ampicillin, 100 μg/ml.

Extraction, purification, and identification of autoinducer activity. Extraction of late-exponential-phase *R. sphaeroides* culture fluid with acidified ethyl acetate was done as previously described for extraction of *P. aeruginosa* autoinducers (28). The autoinducer was purified from the ethyl acetate extracts by using C_{18} reverse-phase high-performance liquid chromatography (HPLC) as described previously (28) except that the isocratic elution was in 70% methanol in water.

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Reference or source
Bacterial strains		
R. sphaeroides		
2.4.1	Wild type, 2 circular chromosomes, 5 endogenous replicons	45
AP3	*cerI*::ΩKm	This study
P. denitrificans		ATCC 17741
E. coli		
DH5α-*phe*	F^- ϕ80d*lac*ZΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44*λ$^-$*thi-1 gyrA relA1 phe*::Tn*10*d Cm	12
HB101	*lacY1 galK2 supE44 ara14 proA2 rspL20 recA13 xyl-5 mtl-1 hsdS20 mcrB*	3
MG-4	Δ(*argF-lac*)*U169 zah-*735::Tn*10 recA56 srl*::Tn*10*	33
S17-1	Pro^- Res^- Mod^+ *recA*	39
Plasmids		
pBS II SK	Ap^r, with *lac* promoter	Stratagene
pKDT17	*lasB::lacZ plac-lasR* Ap^r	28
pHP45ΩKm	Source of ΩKm	32
pRK2013	ColE1 Tra(RK2)$^+$ Km^r	14
pRK415	Tc^r	23
pSUP202	pBR325 derivative, Mob^+ Ap^r Cm^r Tc^r	39
pUI8166	pLA2917-derived cosmid from *R. sphaeroides* 2.4.1 library	1
pUI2501	7-kb insert from pUI8166 with 14.1-kb *Bgl*II fragment removed	This study
pUI2502	5.9-kb *Hin*dIII fragment from pUI2501 in pRK415	This study
pUI2503	1.1-kb *Pst*I fragment of pUI2502 in pBS II SK	This study
pUI2504	2.3-kb *Not*I-*Hin*dIII fragment of pUI2502 in pBS II SK	This study
pUI2505	pUI2504 with 1.1-kb *Pst*I fragment deletion, ΩKm inserted into *Bam*HI site	This study
pUI2506	pUI2505-derived 3.4-kb *Not*I-*Xho*I fragment in pSUP202Δ*Sca*I	This study

The purified material was analyzed by proton nuclear magnetic resonance (NMR) spectroscopy, chemical ionization mass spectrometry (MS), and fast atom bombardment (FAB) MS. NMR spectroscopy was performed with a Varian Unity 500-MHz instrument, chemical ionization MS was performed with a VG Trio-1 quadrapole mass spectrometer using methane as the reagent gas, and high-resolution FAB MS was performed at the Nebraska Center for Mass Spectrometry. The symmetrized two-dimensional correlated spectroscopy (COSY) NMR spectrum was generated by techniques described elsewhere (37).

Autoinducer bioassays. Several bioassays were used to screen for acylhomoserine lactone autoinducers in *R. sphaeroides* culture fluid or in fractions of material separated by HPLC. These assays included the *V. fischeri* autoinducer assay (28), which detects molecules with six to eight carbons in the acyl groups; the *P. aeruginosa rhl (vsm)* assay (29), which detects molecules with four to six carbons in acyl groups unsubstituted at the third carbon; the *Vibrio harveyi* autoinducer assay (4), which detects *N*-(3-hydroxybutyryl)homoserine lactone; and the *P. aeruginosa las* assay (28), which detects molecules with acyl groups of 8 to 14 carbons.

Identification and subcloning of a *luxI* homolog. A cosmid library of *R. sphaeroides* 2.4.1(pLA2917) DNA (1) was mobilized into *P. denitrificans* as previously described (7). In biparental matings the donor to recipient ratio was 1:10, and in triparental matings the helper to donor to recipient ratio was 1:10:100. Late-exponential-phase cultures of the exconjugants were screened for their ability to activate the *lacZ* reporter in the *P. aeruginosa las* bioassay (see above). The gene for autoinducer synthesis was subcloned from pU18166, and plasmids containing the subcloned DNA are described in Table 1.

DNA sequencing and DNA sequence analysis. The DNA sequence of the 2.3-kb *Not*I-*Hin*dIII fragment, which contained the gene for autoinducer synthesis, was determined at the DNA Core Facility of the Department of Microbiology and Molecular Genetics, University of Texas Health Science Center, Houston, Tex., on the ABI 373A automatic DNA sequencer and with the *Taq* dideoxy terminator sequencing kit (Perkin-Elmer, Applied Biosystems Division, Foster City, Calif.). We used pUI2502, pUI2503, and pUI2504 as template DNAs. The sequences were analyzed by using DNA Strider (Institut de Researche Fondamentale, Commissariat a l'Energie Atomique, Paris, France) and GCG (Genetics Computer Group, Wisconsin Package, Madison, Wis.) software.

ΩKm disruption of the autoinducer biosynthesis gene. After removal of a 1.1-kb *Pst*I fragment from pUI2504, the insert DNA contained a unique *Bam*HI site located 557 bp from the *Not*I and 636 bp from the *Hin*dIII sites. An ΩKm cartridge excised from pHP45ΩKm was cloned into this *Bam*HI site. The resulting construct, pUI2505, contained the ΩKm cartridge 149 bp from the predicted start codon of the *luxI* homolog described in Results. The 3.4-kb *Not*I-*Xho*I fragment of pUI2505 was subcloned by blunt-end ligation into *Sca*I-digested pSUP202 and mobilized from *E. coli* S17-1 into *R. sphaeroides* 2.4.1. The mating was performed on LB agar plates, and the exconjugants were selected on SIS-Km agar plates. Several tetracycline-sensitive exconjugants were selected for further study and shown to contain the ΩKm cartridge, but not vector DNA, by Southern hybridization (Stratagene, La Jolla, Calif.) of genomic DNA digests and by pulsed-field agarose gel electrophoresis (43).

Nucleotide sequence accession number. The nucleotide sequence shown in Fig. 5 has been deposited in GenBank under accession no. AF016298.

RESULTS

***R. sphaeroides* produces a compound that can substitute for the autoinducer synthesized by the *P. aeruginosa* LasI protein.** We screened for production of acylhomoserine lactone autoinducers by preparing an ethyl acetate extract of the extracellular fluid from a culture of *R. sphaeroides* grown in LB with

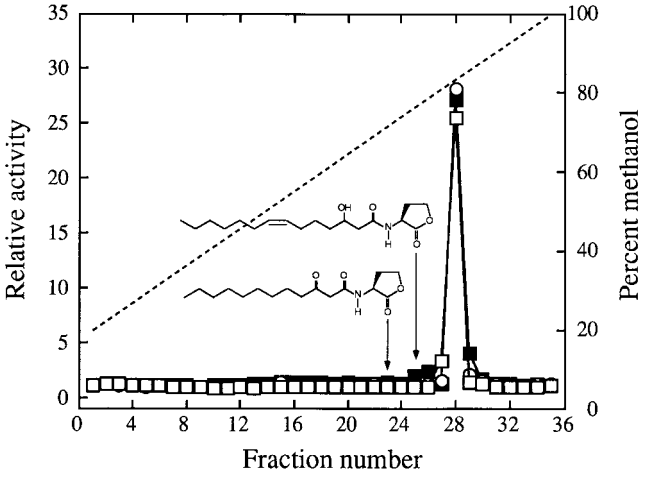

FIG. 1. HPLC analysis of acylhomoserine lactone from LB culture fluid extracts of *R. sphaeroides* grown in air with shaking (○), anaerobically in the dark (■), and anaerobically with light (□). Equal amounts of culture fluid were extracted and analyzed. The arrows indicate the positions at which *N*-(3-oxodecanoyl)homoserine lactone (*P. aeruginosa* autoinducer) and 7,8-*cis*-*N*-(3-hydroxytetradecenoyl)homoserine lactone (*R. leguminosarum* autoinducer) elute. Methanol concentration is indicated by the dashed line.

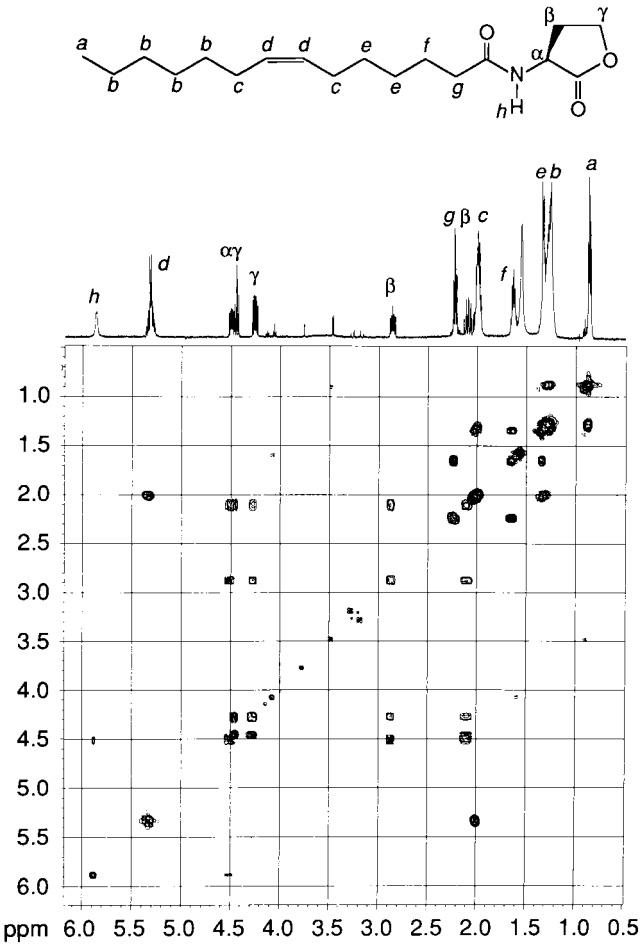

FIG. 2. Predicted structure, proton NMR, and COSY spectrum of the purified *R. sphaeroides* autoinducer. Protons are indicated by italicized letters corresponding to peaks in the ^{1}H-NMR ($CDCl_3$, 500 MHz) spectrum: δ_H 0.88 (3H, t, *a*), 1.27 (8H, m, *b*), 1.34 (4H, m, *e*), 1.54 (H_2O), 1.65 (2H, m, *f*), 2.01 (4H, m, *c*), 2.11 (1H, m, β), 2.24 (2H, m, *g*), 2.87 (1H, m, β), 4.27 (1H, m, γ), 4.46 (1H, m, γ), 4.52 (1H, m, α), 5.33 (2H, m, *d*), 5.89 (1H, broad d, *h*). The signals at 5.33 ppm were coupled to each other with a coupling constant of 10 Hz, indicating a *cis* configuration of the double bond. COSY was used to determine the position of the double bond in the acyl group. The methyl signal (*a*) at 0.88 ppm was coupled to the signal at 1.27 ppm (*b*), which was coupled to the 2.01-ppm signal (*c*). This indicates that there are 4 methylenes (8H) between the terminal methyl group and the $CH_2CH{=}CHCH_2$ group (*c*) at 2.01 ppm. Thus, we conclude that the double bond is between carbons 7 and 8.

shaking. Among the several assays employed in our screen (see Materials and Methods) the only positive response was with the *P. aeruginosa las* bioassay. This bioassay can be used to detect autoinducers with acyl groups ranging from 8 to 14 carbons in length (28). Because we obtained negative results with the *V. fischeri* bioassay, which can detect molecules with acyl groups of five to eight carbons, our results suggested that *R. sphaeroides* produced an acylhomoserine lactone with an acyl group greater than eight carbons in length.

We obtained similar results with photoheterotrophic and anaerobic chemoheterotrophic cultures. When cultures were grown in SIS rather than LB, there was about 10-fold-less acylhomoserine lactone produced (data not shown). Ethyl acetate extracts of cultures grown under different metabolic conditions were fractionated by C_{18} reverse-phase HPLC (Fig. 1). In each case a peak of activity was eluted in approximately 80% methanol in water. This indicates the *R. sphaeroides* factor is more hydrophobic than any autoinducer of a known structure. However, a compound of unknown structure with activity in the *P. aeruginosa las* bioassay and similar HPLC elution behavior is produced by *Rhizobium meliloti* (20).

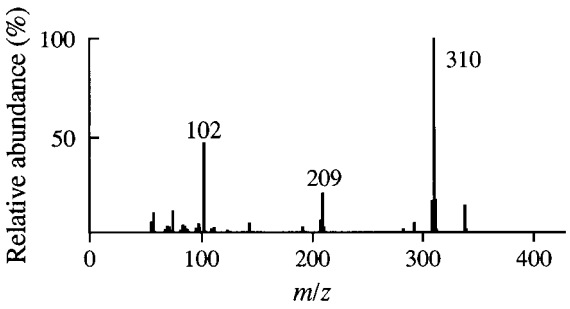

FIG. 3. Chemical ionization mass spectrum of the purified *R. sphaeroides* autoinducer. The *m*/*z* of the $(M + 1)^+$ was 310. This is consistent with that expected for 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone.

Structure of the acylhomoserine lactone produced by *R. sphaeroides*. Because SIS-grown *R. sphaeroides* cultures contained approximately 10-fold less of the acylhomoserine lactone than LB-grown cultures, we purified the acylhomoserine lactone from LB-grown cultures. Approximately 400 μg of the active compound was purified from a 3-liter LB culture grown with shaking. The proton NMR spectrum of the purified material (Fig. 2) was similar to that of other acylhomoserine lactones (20, 28, 37). The structure predicted from the NMR spectrum is of a molecule containing an acyl group 14 carbons in length with one unsaturated carbon-carbon (*cis* configuration) bond and with two protons on the carbon at position 3. As determined by COSY, the double bond in the acyl chain was between carbons 7 and 8 (Fig. 2). Chemical ionization MS showed a strong quasimolecular $(M + H)^+$ ion with an *m*/*z* of 310 (Fig. 3). This is consistent with the structure deduced from NMR spectroscopy, 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone (Fig. 2). High-resolution FAB MS showed the *m*/*z* of the $(M + H)^+$ to be 310.2379. This corresponded to a chemical composition of $C_{18}H_{32}O_3N$, which is consistent with the structure shown in Fig. 2. We measured the concentration of the acylhomoserine lactone autoinducer, by using known amounts of purified 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone to generate a standard curve, in the *P. aeruginosa las* bioassay. We found that late-exponential-phase LB-grown *R. sphaeroides* cultures (optical density at 660 nm of about 1) contained 4 to 6 μM 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone whether grown chemoheterotrophically or photoheterotrophically.

Cloning the gene required for production of the acylhomoserine lactone. There is not sufficient sequence identity between LuxI family members to allow design of oligonucleotide primers or probes that are useful for heterologous cloning. Therefore, we cloned the gene responsible for production of the acylhomoserine lactone signal by screening a cosmid library of *R. sphaeroides* DNA in the heterologous host, *P. denitrificans*, for acylhomoserine lactone production by using the *P. aeruginosa las* bioassay (see Materials and Methods). *P. denitrificans* was chosen because it has proven useful for expression of *R. sphaeroides* genes (7) and because we found no evidence of acylhomoserine lactone synthesis by this bacterium in the *las* bioassay. Three exconjugants, containing the cosmids pUI8166, pUI8374, and pUI8595, produced an active factor. The *R. sphaeroides* DNA in these plasmids overlaps (data not shown) and had been previously mapped to the *Ase*I G fragment of chromosome I (43). One cosmid, pUI8166, was chosen

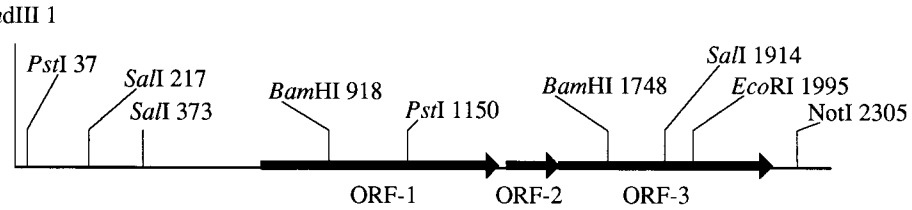

FIG. 4. Physical and genetic map of the ~2.3-kb *Not*I-*Hin*dIII fragment designated pUI2504. This fragment contains three ORFs designated ORF-1, ORF-2, and ORF-3. The ORF-2 stop codon overlaps the putative start site of ORF-3.

for further study and was subcloned as described in Materials and Methods. *P. denitrificans* containing pUI2504, which possesses a 2.3-kb *Not*I-*Hin*dIII fragment of *R. sphaeroides* DNA, produced an acylhomoserine lactone. An HPLC analysis indicated that the active molecule produced by *P. denitrificans* containing pUI2504 comigrated with that produced by *R. sphaeroides* (data not shown).

The region of *R. sphaeroides* DNA shown in Fig. 4 was sequenced and found to contain three open reading frames (ORF-1, -2, and -3), each with the same apparent transcriptional orientation. The sequences of these ORFs are shown in Fig. 5.

The polypeptide predicted from the sequence of ORF-1 is 233 amino acids (Fig. 5), has a predicted molecular weight of 26,082, and showed significant similarity to LuxR family members (Fig. 6A). For example, it showed 30% identity (54% similarity) with AhyR from *Aeromonas hydrophila*, 30% identity (49% similarity) with LasR from *P. aeruginosa*, 28% identity (45% similarity) with LuxR from *V. fischeri*, and 27% identity (50% similarity) with RhiR from *Rhizobium leguminosarum*. These are in the range of values previously obtained for pairwise comparisons of LuxR family members from different bacteria. Furthermore, the regions of greatest sequence similarity between ORF-1 and other LuxR family members corresponded to the conserved autoinducer-binding and DNA-binding regions of LuxR (Fig. 6A).

ORF-2 consisted of 159 bp (Fig. 5). The codon usage of this small ORF was not characteristic of *R. sphaeroides* genes in that the percentage of rarely used codons was approximately 34% and the overall G+C content was 55%, which is lower than the 67% expected for an *R. sphaeroides* gene (5). This ORF is preceded by a good Shine-Delgarno sequence, and its translation stop codon overlaps the start codon of the third ORF. The translation product of this small ORF is 52 amino acids with a predicted molecular weight of 5,906, and it does not show significant similarity to any gene products in the sequence databases.

ORF-3 (Fig. 5) showed significant similarity to LuxI family

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                ACGGCTCCCA AAGGTGTTTG GAACGTGCTA AATAAGCAGG ACGGCTGCGC GGTGGCCGGGG GGAGCCGGC  70
ATGGACATCATCGACCTCAGCACAGTTGCCACGGACGATGCGAGCTTCCTCGACTACATCGACCAGCTCTGCCAGAAGCTGGGCTTCGACTATGCC 166
M  D  I  I  D  L  S  T  V  A  T  D  D  A  S  F  L  D  Y  I  D  Q  L  C  Q  K  L  G  F  D  Y  A   32
TCCTATGCGACAACGAGTCCGATGACGGGCGCGGTCCAGGGCTATGCCAACTATCCCGACAGCTGGAAGATGCACTACATGCGGCGCAACCTGCAC 262
S  Y  A  T  T  S  P  M  T  G  A  V  Q  G  Y  A  N  Y  P  D  S  W  K  M  H  Y  M  R  R  N  L  H   64
CGCGTGGATCCCACCATCCACAAGTCGGCGCTGAGCATCGCCCCCGTGGACTGGAGCCGCTTCGAGCGCGACGAGCGTTTCCGGGCCGTGTTCTTT 358
R  V  D  P  T  I  H  K  S  A  L  S  I  A  P  V  D  W  S  R  F  E  R  D  E  R  F  R  A  V  F  F   96
GCGGCCGAGGATTTCGGCATCACCCCTCAGGGGCTGACGGTGCCCGTCCGCGGCCCCTACGGCGACCGGGGCCTTCTGTCGGTGACGCGCAACTGC 454
A  A  E  D  F  G  I  T  P  Q  G  L  T  V  P  V  R  G  P  Y  G  D  R  G  L  L  S  V  T  R  N  C  128
GCCCGCCCGGAATGGGAGAAGCACAAGCGCGCCGTGATCGGCGAGCTGCAGGTTGCGGCCGTCCACCTGCACGATGCGGTCATGCGGTCGGACGTG 550
A  R  P  E  W  E  K  H  K  R  A  V  I  G  E  L  Q  V  A  A  V  H  L  H  D  A  V  M  R  S  D  V  160
ATCTCACGCGCCCTGCGCCAGCCGAGGCTCTCCACGCGCGAGATCGAGGTCCTGCAATGGGCGGCGGCGGGCAAGTCGCAGACGGACATCGGCGAC 646
I  S  R  A  L  R  Q  P  R  L  S  T  R  E  I  E  V  L  Q  W  A  A  A  G  K  S  Q  T  D  I  G  D  192
ATCCTCGGCATCTCGCACCGCACGGTCGAGGTGCACCTGCGCTCGGCGCGCGAGAAGCTCGGCACCCTCTCGACGGTGCAGGCCGTGGGCCGTGCG 742
I  L  G  I  S  H  R  T  V  E  V  H  L  R  S  A  R  E  K  L  G  T  L  S  T  V  Q  A  V  G  R  A  224
ATCGGGCTGGGTCTGGTCTATCCGAGGTGA GAGTGCTCCG CC GGAAGGCGTATGAGGCACTTTGAGCCCTATCCCTGCCCCAGAGGACCGAAA 835
I  G  L  G  L  V  Y  P  R  *                        M  R  H  F  E  P  Y  P  C  P  R  G  P  K   14
TTCAACCGGAAATTGCCAAAGGAAGCCTTCCGGCTACGGGATTCCCCTCATAGCCGAAACGGGTCGGGATGCGCAACTATAGCCGTATGTTACGCA 931
F  N  R  K  L  P  K  E  A  F  R  L  R  D  S  P  H  S  R  N  G  S  G  C  A  T  I  A  V  C  Y  A   46
ACCTTGGGGATTGAAGAATGATCTTCATTATCGACTCGCTCAACCTCCGCGAGCACGCAGACATCGTGAAGGACATGTTCCGGTTGCGCAAGCGC 1026
T  L  G  I  E  E  *
                  M  I  F  I  I  D  S  L  N  L  R  E  H  A  D  I  V  K  D  M  F  R  L  R  K  R   26
GTTTTTGCGGACCGTCTCGGCTGGGACGTTCAGATTTCGCAGGGCATGGAGCGCGACCGCTTCGACGACCTGGATCCGGCTCACGTCGTGAGCGTG 1122
V  F  A  D  R  L  G  W  D  V  Q  I  S  Q  G  M  E  R  D  R  F  D  D  L  D  P  A  H  V  V  S  V   58
GATGACGAGGGCCGCGTGGTCGGCTGCATGCGCCTGCTTCAGACGACGGGCCCGCACATGCTCTCCGACGTGTTCAGCTCGATCCTGGACGGAGAG 1218
D  D  E  G  R  V  V  G  C  M  R  L  L  Q  T  T  G  P  H  M  L  S  D  V  F  S  S  I  L  D  G  E   90
CCTCCGCTGCGCAGCGCAACTCTCTGGGAAGCCACGCGCTTCTGTGTCGACACCGACCGCCTGGTTTCGGGCCGCGCCCGCAATTCGATCGCCTAT 1314
P  P  L  R  S  A  T  L  W  E  A  T  R  F  C  V  D  T  D  R  L  V  S  G  R  A  R  N  S  I  A  Y  122
GTCACGAGCGAAGTGATGATCGGCGCCTTCGAATTCGCCATGTCGGCGGGTGTGACCGATGCGGTCGCCGTGATCGATCCGGTGATGGACCGCGTG 1410
V  T  S  E  V  M  I  G  A  F  E  F  A  M  S  A  G  V  T  D  A  V  A  V  I  D  P  V  M  D  R  V  154
CTGAAGCGGTCGGGCAATGCGCCGCAGGGCTATGTCGGCACGCCGAAGCCGATGGGCAAGGTGACGGCTCTGGCCGCGCTGATGGACTGCTCGGAA 1506
L  K  R  S  G  N  A  P  Q  G  Y  V  G  T  P  K  P  M  G  K  V  T  A  L  A  A  L  M  D  C  S  E  186
GAGCGTGTGAAGCGCATCCGCGATTTCGCCGGCATCTACCACGACGTGACGCAACCGCAGACGGTCATCGCCTGA                    1581
E  R  V  K  R  I  R  D  F  A  G  I  Y  H  D  V  T  Q  P  Q  T  V  I  A  *                     210
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ORF-1 ORF-2 ORF-3

FIG. 5. Nucleotide sequence and polypeptide translation of ORF-1, ORF-2, and ORF-3. Putative Shine-Delgarno sequences are underlined, and the bold type indicates putative translational start codons.

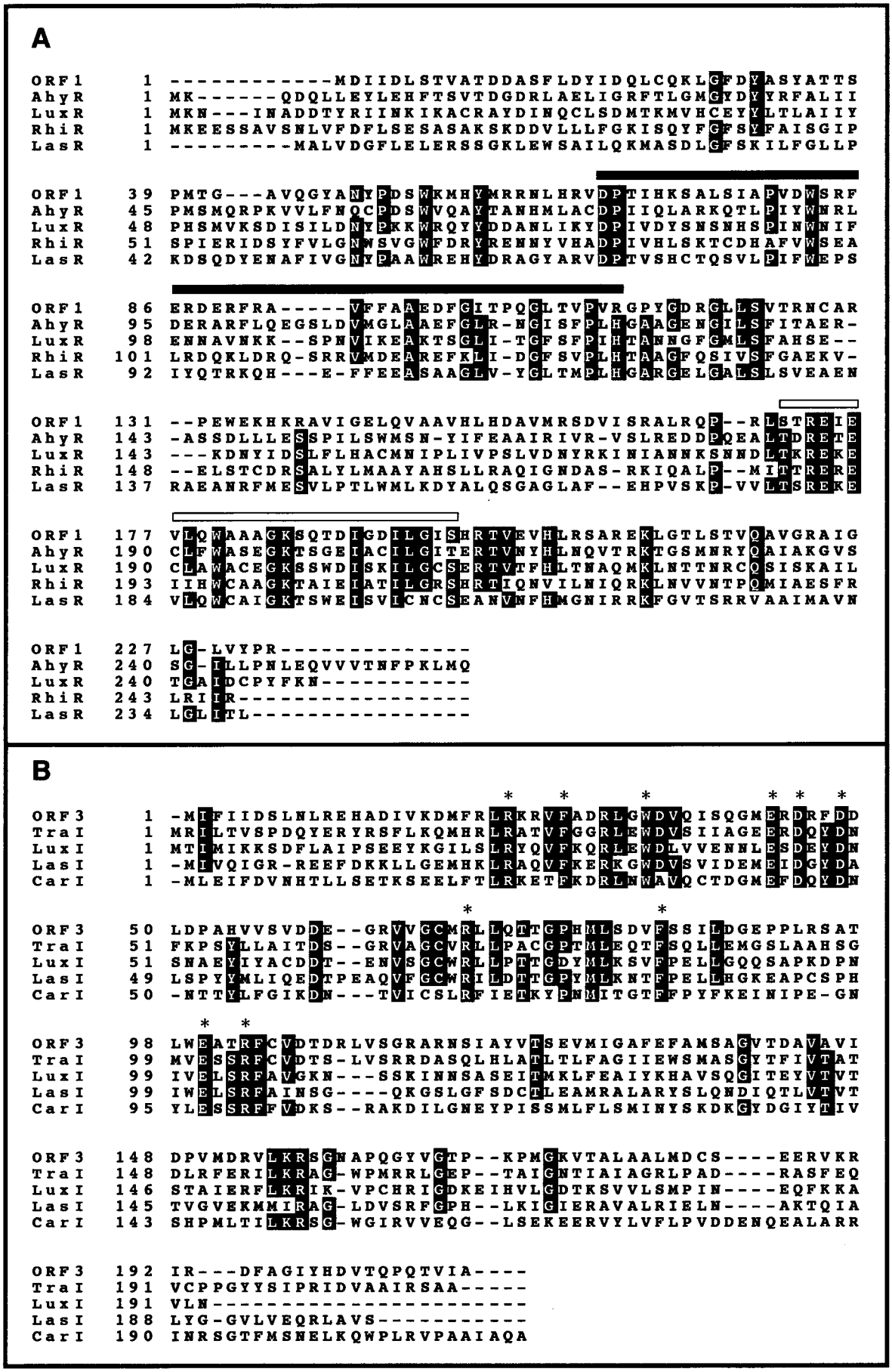
A
ORF1 1 ----------MDIIDLSTVATDDASFLDYIDQLCQKLGFDYASYATTS
AhyR 1 MK-------QDQLLEYLEHFTSVTDGDRLAELIGRFTLGMGYDYYRFALII
LuxR 1 MKN---INADDTYRIINKIKACRAYDINQCLSDMTKMVHCEYYLTLAIIY
RhiR 1 MKEESSAVSNLVFDFLSESASAKSKDDVLLLFGKISQYFGFSYFAISGIP
LasR 1 ---------MALVDGFLELERSSGKLEWSAILQKMASDLGFSKILFGLLP
ORF1 39 PMTG---AVQGYANYPDSWKMHYMRRNLHRVDPTIHKSALSIAPVDWSRF
AhyR 45 PMSMQRPKVVLFNQCPDSWVQAYTANHMLACDPIIQLARKQTLPIYWNRL
LuxR 48 PHSMVKSDISILDNYPKKWRQYYDDANLIKYDPIVDYSNSNHSPINWNIF
RhiR 51 SPIERIDSYFVLGNWSVGWFDRYRENNYVHADPIVHLSKTCDHAFVWSEA
LasR 42 KDSQDYENAFIVGNYPAAWREHYDRAGYARVDPTVSHCTQSVLPIFWEPS
ORF1 86 ERDERFRA------VFFAAEDFGITPQGLTVPVRGPYGDRGLLSVTRNCAR
AhyR 95 DERARFLQEGSLDVMGLAAEFGLR-NGISFPLHGAAGENGILSFITAER-
LuxR 98 ENNAVNKK--SPNVIKEAKTSGLI-TGFSFPIHTANNGFGMLSFAHSE--
RhiR 101 LRDQKLDRQ-SRRVMDEAREFKLI-DGFSVPLHTAAGFQSIVSFGAEKV-
LasR 92 IYQTRKQH---E-FFEEASAAGLV-YGLTMPLHGARGELGALSLSVEAEN
ORF1 131 --PEWEKHKRAVIGELQVAAVHLHDAVMRSDVISRALRQP--RLSTREIE
AhyR 143 -ASSDLLLESSPILSWMSN-YIFEAAIRIVR-VSLREDDPQEALTDRETE
LuxR 143 ---KDNYIDSLFLHACMNIPLIVPSLVDNYRKINIANNKSNNDLTKREKE
RhiR 148 --ELSTCDRSALYLMAAYAHSLLRAQIGNDAS-RKIQALP--MITTREKE
LasR 137 RAEANRFMESVLPTLWMLKDYALQSGAGLAF--EHPVSKP-VVLTSREKE
ORF1 177 VLQWAAAGKSQTDIGDILGISHRTVEVHLRSAREKLGTLSTVQAVGRAIG
AhyR 190 CLFWASEGKTSGEIACILGITERTVNYHLNQVTRKTGSMNRYQAIAKGVS
LuxR 190 CLAWACEGKSSWDISKILGCSERTVTFHLTNAQMKLNTTNRCQSISKAIL
RhiR 193 IIHWCAAGKTAIEIATILGRSHRTIQNVILNIQRKLNVVNTPQMIAESFR
LasR 184 VLQWCAIGKTSWEISVICNCSEANVNFHMGNIRRKFGVTSRRVAAIMAVN
ORF1 227 LG-LVYPR---------------
AhyR 240 SG-ILLPNLEQVVVTNFPKLMQ
LuxR 240 TGAIDCPYFKN------------
RhiR 243 LRIIR-----------------
LasR 234 LGLITL----------------
B
ORF3 1 -MIFIIDSLNLREHADIVKDMFRLRKRVFADRLGWDVQISQGMERDRFDD
TraI 1 MRILTVSPDQYERYRSFLKQMHRLRATVFGGRLEWDVSIIAGEERDQYDN
LuxI 1 MTIMIKKSDFLAIPSEEYKGILSLRYQVFKQRLEWDLVVENNLESDEYDN
LasI 1 -MIVQIGR-REEFDKKLLGEMHKLRAQVFKERKGWDVSVIDEMEIDGYDA
CarI 1 -MLEIFDVNHTLLSETKSEELFTLRKETFKDRLNWAVQCTDGMEFDQYDN
ORF3 50 LDPAHVVSVDDE--GRVVGCMRLLQTTGPHMLSDVFSSILDGEPPLRSAT
TraI 51 FKPSYLLAITDS--GRVAGCVRLLPACGPTMLEQTFSQLLEMGSLAAHSG
LuxI 51 SNAEYIYACDDT--ENVSGCWRLLPTTGDYMLKSVFPELLGQQSAPKDPN
LasI 49 LSPYYMLIQEDTPEAQVFGCWRILDTTGPYMLKNTFPELLHGKEAPCSPH
CarI 50 -NTTYLFGIKDN---TVICSLRFIETKYPNMITGTFFPYFKEINIPE-GN
ORF3 98 LWEATRFCVDTDRLVSGRARNSIAYVTSEVMIGAFEFAMSAGVTDAVAVI
TraI 99 MVESSRFCVDTS-LVSRRDASQLHLATLTLFAGIIEWSMASGYTFIVTAT
LuxI 99 IVELSRFAVGKN---SSKINNSASEITMKLFEAIYKHAVSQGITEYVTVT
LasI 99 IWELSRFAINSG----QKGSLGFSDCTLEAMRALARYSLQNDIQTLVTVT
CarI 95 YLESSSRFFVDKS--RAKDILGNEYPISSMLFLSMINYSKDKGYDGIYTIV
ORF3 148 DPVMDRVLKRSGNAPQGYVGTP--KPMGKVTALAALMDCS----EERVKR
TraI 148 DLRFERILKRAG-WPMRRLGEP--TAIGNTIAIAGRLPAD----RASFEQ
LuxI 146 STAIERFLKRIK-VPCHRIGDKEIHVLGDTKSVVLSMPIN----EQFKKA
LasI 145 TVGVEKMMIRAG-LDVSRFGPH--LKIGIERAVALRIELN----AKTQIA
CarI 143 SHPMLTILKRSG-WGIRVVEQG--LSEKEERVYLVFLPVDDENQEALARR
ORF3 192 IR---DFAGIYHDVTQPQTVIA----
TraI 191 VCPPGYYSIPRIDVAAIRSAA-----
LuxI 191 VLN----------------------
LasI 188 LYG-GVLVEQRLAVS-----------
CarI 190 INRSGTFMSNELKQWPLRVPAAIAQA

FIG. 6. Multiple alignments of several LuxR and LuxI family members with ORF-1 and ORF-3, respectively. Conserved amino acid residues are highlighted in black. (A) Representative LuxR family members. The solid bar above the residues corresponding to LuxR amino acids 79 to 127 represents the conserved autoinducer binding region (21, 38, 41), and the open bar above the residues corresponding to LuxR amino acids 184 to 210 indicates the conserved helix-turn-helix region of the DNA-binding domain. (B) Representative LuxI family members. The asterisks indicate the residues conserved among all 15 LuxI family members in the databases at the time of our search (26). Each of these 10 residues occurs in the aligned sequence of ORF-3. Clustal-W (44) was used to align the sequences and Boxshade (0.8 setting) was used to determine the degree of residue shading. The sequences used in the alignments are *A. hydrophila* AhyR (GenBank accession no. X899143), *V. fischeri* LuxR (Y00509), *R. leguminosarum* RhiR (M98835), *P. aeruginosa* LasR (M59425), *A. tumefaciens* TraI (L17024), *V. fischeri* LuxI (Y00509), *P. aeruginosa* LasI (L04681), and *E. carotovora* CarI (X74299).

members (Fig. 6B), suggesting that it may encode an acylhomoserine lactone synthase. The encoded polypeptide is 210 amino acids with a predicted molecular mass of 23,336 daltons. It showed 33% identity (52% similarity) to TraI from *A. tumefaciens*, 28% identity (52% similarity) to LuxI from *V. fischeri*, 28% identity (54% similarity) to LasI from *P. aeruginosa*, and 26% identity (54% similarity) to CarI from *Erwinia carotovora*. These numbers are comparable to those for pairwise comparisons between other LuxI homologs. Furthermore, there is a region between residues 25 and 104 in LuxI which contains all of the amino acids that are conserved throughout the LuxI family (22, 26) and all of these residues are found in ORF-3 (Fig. 6B).

We insertionally inactivated ORF-3 with an ΩKm cartridge to determine whether the suspected acylhomoserine lactone synthase was required for production of 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone by *R. sphaeroides* and to investigate the nature of the functions that might be controlled by quorum sensing in *R. sphaeroides*. The ΩKm cartridge in the ORF-3 knockout mutant was 2,918 bp clockwise from the arbitrary origin of chromosome I. The ORF-3 knockout mutant did not produce an acylhomoserine lactone that was detected with the *P. aeruginosa las* bioassay. This is consistent with the hypothesis that ORF-3 represents an autoinducer synthase gene required for production of 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone in *R. sphaeroides*.

7,8-*cis*-*N*-(Tetradecenoyl)homoserine lactone is involved in the regulation of extracellular material accumulation. The acylhomoserine lactone synthesis mutant described above exhibited a striking phenotype (Fig. 7). As the mutant achieved high densities in broth cultures, cells aggregated and formed a floc that settled to the bottom of the culture tube (Fig. 7A). The aggregation in broth was prevented by addition of purified 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone (final concentration of about 3 μM) or by providing ORF-3 on pUI2504 (Fig. 7A). After 10 days on agar plates, colonies of mutant strains but not the wild type exhibited a mucoid appearance consistent with hyperproduction of an extracellular polysaccharide. When pUI2504 was used to complement the ORF-3 mutation in strain AP-3, colonies were indistinguishable from those of the wild type (Fig. 7B).

DISCUSSION

Our results demonstrate that the purple nonsulfur photosynthetic bacterium *R. sphaeroides* 2.4.1 possesses a quorum-sensing system. This organism produces an acylhomoserine lactone autoinducer with a structure not previously described, 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone (Fig. 2). The most closely related known autoinducer is 7,8-*cis*-*N*-(3-hydroxytetradecenoyl)homoserine lactone from *R. leguminosarum* (20, 37). To date these two autoinducers are the only ones reported to contain a double bond in the acyl moiety. It is interesting that a presumptive acylhomoserine lactone of unknown structure from *R. meliloti* (20) shows an identical elution profile to that of the *R. sphaeroides* autoinducer in C_{18} reverse-phase HPLC. This suggests that the *R. meliloti* compound may be 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone. The concentrations of the *R. sphaeroides* autoinducer in late-exponential-phase anaerobic photosynthetic, anaerobic chemoheterotrophic, and aerobic chemoheterotrophic cultures were roughly equivalent (4 to 6 μM in LB-grown cultures). This is similar to autoinducer concentrations in *V. fischeri* and *P. aeruginosa* cultures (9, 28).

We identified three ORFs believed to be involved in quorum sensing. For reasons discussed below we have termed these ORFs *cerI*, ORF-2, and *cerR*. We believe that the *cerI* gene

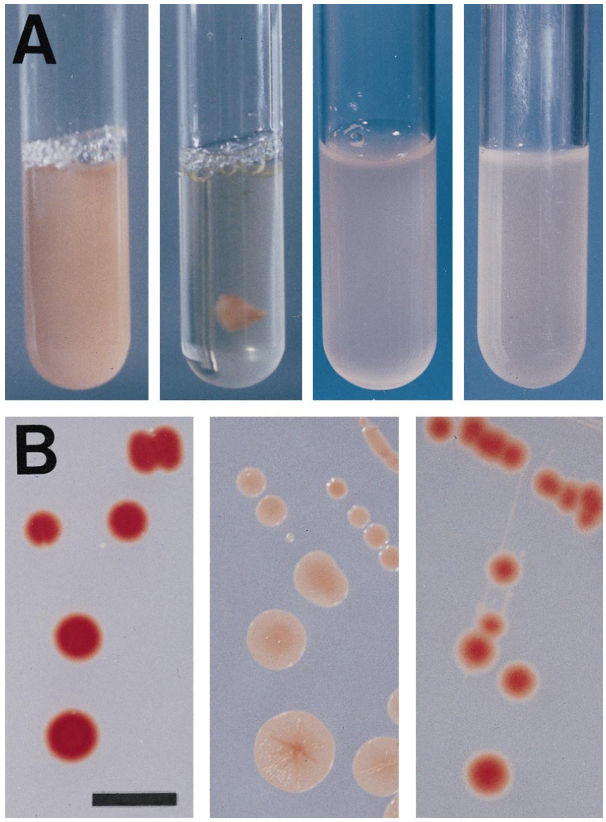

FIG. 7. Phenotypes of an ORF-3 mutant. (A) Left to right: SIS broth cultures of the wild-type *R. sphaeroides* 2.4.1; the ORF-3$^-$ mutant, AP3; AP3 with exogenously added purified 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone (10 nmol/5-ml culture just prior to inoculation and again at 48 h); and AP3 containing pUI2504. When grown in broth, the mutant cells aggregate and form a floc that settles to the bottom of the tube. For this photograph the cultures were agitated so that the mutant floc could be observed more easily. The cultures were grown for 96 h. (B) Mucoid colonies of *R. sphaeroides* AP3. From left to right: the wild-type strain 2.4.1; the ORF-3$^-$ mutant, AP3; and AP3 containing pUI2504. Photographs were taken after an incubation period of 10 days at 30°C on SIS agar plates. Bar, 5 mm.

encodes an autoinducer synthase for the following reasons: (i) the *cerI* product is a member of the LuxI protein family (Fig. 6B); (ii) *cerI* directs the *R. sphaeroides* cloning vehicle, *P. denitrificans*, to synthesize a molecule with biological and chromatographic properties consistent with 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone; and (iii) an *R. sphaeroides cerI* mutant does not make any detectable acylhomoserine lactone. Although another bacterial species, *R. leguminosarum*, produces an acylhomoserine lactone with a carbon-carbon double bond in the acyl moiety (20, 37), the gene responsible for synthesis of this signal has not yet been identified. Thus, *cerI* is the only gene known to direct the synthesis of an autoinducer containing an unsaturated double bond.

On the basis of sequence comparisons alone we believe *cerR* encodes a LuxR-type transcriptional regulator protein (Fig. 6A) and speculate that it responds to 7,8-*cis*-*N*-(tetradecenoyl) homoserine lactone, but further study is required to test this hypothesis. The deduced amino acid sequence of the 159-bp ORF, designated ORF-2, shows no significant similarity to any known protein. The observations that ORF-2 contains numerous rarely used codons, that its translation terminator overlaps the ATG start site of *cerI*, and that *cerI* does not have a good Shine-Delgarno sequence suggest that ORF-2 may play a role in posttranscriptional regulation of *cerI*. Previously we have shown that in *pufK*, a small ORF preceding the *pufB* gene, the abundance of rare codons plays an important role in translation of downstream genes (19).

As discussed above, we created a *cerI* null mutation in *R. sphaeroides* and confirmed that this gene is required for production of 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone. Mutations in *cerI* result in cell aggregation in broth cultures (Fig. 7A) and formation of mucoid colonies on agar plates (Fig. 7B). Furthermore, CerI mutants produce 20- to 30-fold more of an exopolysaccharide than do wild-type cells (8a). In the plant pathogen *Erwinia stewartii*, production of an extracellular heteropolysaccharide capsule is regulated by a quorum-sensing system. However, in *E. stewartii* the acylhomoserine lactone signal serves as an inducer of extracellular polysaccharide production (2), whereas the *R. sphaeroides* signal appears to repress production of the extracellular polysaccharide (Fig. 7). It appears that in *R. sphaeroides* quorum sensing is required to prevent cellular aggregates from forming. The gene designation *cer* for community escape response is based upon our finding that CerI mutant cells grow in large masses rather than as individuals in liquid media (Fig. 7A) and that the *R. sphaeroides* autoinducer blocks formation of these aggregated community structures. We presume that the aggregation is directly related to overproduction of the extracellular polysaccharide in CerI mutants.

The role of the *cer* quorum-sensing system in the ecology of *R. sphaeroides* is unknown, and it is not clear as to the significance of quorum-sensing control in a switch from aggregated to dispersed growth. As we continue to learn about the *cer* regulatory system in *R. sphaeroides* our understanding of its significance in this free-living purple nonsulfur photosynthetic bacterium should increase. One might imagine that in sufficiently large aggregates of cells, light could become a limiting nutrient and escape from a community aggregate could be advantageous. However, there are certainly other possible explanations for the involvement of a quorum-sensing and response system in dispersal of *R. sphaeroides* aggregates. Future studies of quorum sensing in *R. sphaeroides* should be informative.

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