Long-Chain Acyl-Homoserine Lactone Quorum-Sensing Regulation of *Rhodobacter capsulatus* Gene Transfer Agent Production

Amy L. Schaefer,^{1†} Terumi A. Taylor,² J. Thomas Beatty,² and E. P. Greenberg^{1*}

Department of Microbiology and W. M. Keck Foundation Microbial Communities & Cell Signaling Laboratory, University of Iowa, Iowa City, Iowa 52242,¹ and Department of Microbiology & Immunology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3²

Received 29 May 2002/Accepted 1 September 2002

Many proteobacteria use acyl-homoserine lactones as quorum-sensing signals. Traditionally, biological detection systems have been used to identify bacteria that produce acyl-homoserine lactones, although the specificities of these detection systems can limit discovery. We used a sensitive approach that did not require a bioassay to detect production of long-acyl-chain homoserine lactone production by *Rhodobacter capsulatus* and *Paracoccus denitrificans*. These long-chain acyl-homoserine lactones are not readily detected by standard bioassays. The most abundant acyl-homoserine lactone was *N*-hexadecanoyl-homoserine lactone. The long-chain acyl-homoserine lactones were concentrated in cells but were also found in the culture fluid. An *R. capsulatus* gene responsible for long-chain acyl-homoserine lactone synthesis was identified. A mutation in this gene, which we named *gtaI*, resulted in decreased production of the *R. capsulatus* gene transfer agent, and gene transfer agent production was restored by exogenous addition of *N*-hexadecanoyl-homoserine lactone. Thus, long-chain acyl-homoserine lactones serve as quorum-sensing signals to enhance genetic exchange in *R. capsulatus*.

Many proteobacteria use acyl-homoserine lactone (acyl-HSL) signals in cell density-dependent gene regulation (9, 11, 45). Acyl-HSLs act as intercellular signals that allow bacterial species to monitor their population density and activate specific sets of genes at high cell densities. This type of cell density-dependent gene regulation, also called quorum sensing, was first described in the marine bacterium *Vibrio fischeri* (6, 21), which uses an acyl-HSL to activate luminescence gene expression. The *V. fischeri* quorum-sensing regulatory elements are LuxI and LuxR (8). The LuxI protein is the acyl-HSL synthase responsible for production of the *N*-3-oxohexanoyl-HSL. LuxR is a transcription factor that activates luminescence gene expression when bound by the acyl-HSL signal (8, 9, 11, 12, 23).

Acyl-HSL signaling controls a number of bacterial processes, including virulence factor production, secondary metabolite production, and biofilm development in *Pseudomonas aeruginosa* (25, 27, 46) and conjugal transfer in *Agrobacterium tumefaciens* (10, 29, 49). Generally, LuxR and LuxI homologs serve as signal receptors and signal generators, respectively. Depending on the system, the signal varies in acyl group length and substitution (11), and these acyl side chain differences confer signal specificity (7, 35). Differences in acyl chain lengths are also a factor in signal permeability. Short-chain acyl-HSLs, like *N*-3-oxohexanoyl-HSL (3OC6-HSL) and butanoyl HSL, can diffuse freely through the cell membrane (14,

26). While still diffusible, long-chain acyl-HSLs like the *P. aeruginosa* signal *N*-3-oxododecanoyl-HSL (3OC12-HSL) appear to partition to the cell membrane. The MexAB-OprD efflux pump and perhaps other efflux pumps can aid in 3OC12-HSL export (26).

Rhodobacter capsulatus and *Paracoccus denitrificans* are members of the α group of the *Proteobacteria*. These free-living organisms are closely related and have been well studied for reasons related to their physiological flexibility (1, 20). We assessed their ability to synthesize acyl-HSLs by using a previously described radiotracer technique (3, 34, 38). This allowed us to discover that *R. capsulatus* and *P. denitrificans* made a long-chain acyl-HSL that was not readily detected by any available bioassay. We show that the most abundant acyl-HSL produced by these bacteria is *N*-hexadecanoyl-HSL (C16-HSL).

Because the *R. capsulatus* genomic sequence is available, we studied acyl-HSL signaling in this organism further. We identified *luxI* and *luxR* homologs in the *R. capsulatus* genome. We demonstrate that cells concentrate this hydrophobic acyl-HSL. We also show it activates production of the gene transfer agent when added to an *R. capsulatus* acyl-HSL synthesis mutant. Gene transfer agent is a bacteriophage-like particle that transfers random 4.5-kb fragments of host genomic DNA between *R. capsulatus* cells (16, 40, 47). This type of genetic exchange is a biological process that we show is related to quorum sensing.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The plasmids and strains used are listed in Table 1. *R. capsulatus* cultures were grown aerobically or phototrophically in either yeast extract-peptone-salts (YPS) (44) or RCV minimal medium (2) at 30°C. *P. denitrificans* was grown aerobically in Sistrom's succinic acid minimal medium A (SIS) (4) or Luria broth (LB) (33) at 30°C. *V. fischeri* was grown at 23°C in morpholinepropanesulfonic acid (MOPS) minimal medium (22) modified to contain 0.3% (vol/vol) glycerol and 75% (vol/vol) artificial seawater. *E. coli* was grown at 37°C in LB. Antibiotic concentrations

* Corresponding author. Mailing address: Department of Microbiology, University of Iowa, 540 EMRB, Newton Road, Iowa City, IA 52242. Phone: (319) 335-7775. Fax: (319) 335-7949. E-mail: everett-greenberg@uiowa.edu.

† Present address: W. M. Keck Microbial Communities & Cell Signaling Laboratory, Kewalo Marine Laboratories, Pacific Biomedical Research Center, University of Hawaii–Manoa, Honolulu, HI 96813.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference or source
<i>R. capsulatus</i>		
SB1003	Parental strain for <i>gtaI</i> mutant, Rif ^r	48
B10	Wild-type GTA indicator strain, Rif ^r	19
ALS1	<i>gtaI</i> :: Ω Sp ^r derivative of SB1003, Rif ^r Sp ^r	This work
<i>P. denitrificans</i> ATCC 177441	Wild type	
<i>V. fischeri</i> MJ-1	Wild type	32
<i>E. coli</i> S17- λ pir	Donor in interspecies conjugations	28
Plasmids		
pGtaI-1	pCR2.1 containing TA-cloned 2.7-kb RCC03805 PCR fragment, Ap ^r	This work
pHP45 Ω Sp	Ω Sp ^r cassette source	30
pGtaIKO	pGtaI-1 with Ω Sp ^r cassette inserted in RCC03805 ORF, Ap ^r Sp ^r	This work
pJP5603	Mobilizable suicide vector, Km ^r	28
pJPGtaIKO	4.7-kb <i>MfeI</i> - <i>XbaI</i> fragment of pGtaIKO cloned into pJP5603, Km ^r Sp ^r	This work
pBBRMCS-2	Broad-host-range cloning vector, Km ^r	15
pBBRGtaI	RCC03805 cloned in pBBRMCS-2, Km ^r	This work
pYP	GTA gene fusion <i>orf2</i> :: <i>lacZ</i> , Tc ^r	18
pYNP	pYP construct lacking promoter, Tc ^r	18

^a GTA, gene transfer agent.

used for *R. capsulatus* were as follows: rifampin, 100 μ g/ml; kanamycin, 5 μ g/ml; spectinomycin, 10 μ g/ml; and tetracycline, 0.5 μ g/ml. For *E. coli*, antibiotic concentrations were kanamycin, 30 μ g/ml; spectinomycin, 50 μ g/ml; tetracycline, 12.5 μ g/ml; and ampicillin, 100 μ g/ml.

Detection and identification of *R. capsulatus* and *P. denitrificans* acyl-HSLs. Previously, discovery of acyl-HSLs has relied on bioassays, which are limited by signal specificity constraints (36). Novel acyl-HSLs might not be detected by any of the available bioassays. We adapted an assay that detects the incorporation of ¹⁴C label into acyl-HSLs (3, 34, 38) to screen *R. capsulatus*, *P. denitrificans*, and other bacteria for acyl-HSL production regardless of whether the molecules could be readily detected in any bioassay. Late-logarithmic-phase cultures (5-ml volume) grown in methionine-free medium (RCV or SIS) were labeled for 30 to 60 min with 5 μ Ci of carboxy[¹⁴C]methionine, 55 mCi/mmol (American Radiochemical Company, St. Louis, Mo.). Unless indicated, total cultures were extracted twice with an equal volume of acidified ethyl acetate (100 μ l of glacial acetic acid per liter). Extracts were combined and dried under a stream of N₂ gas. The residue was suspended in 200 μ l of methanol and separated by C₁₈ reverse-phase high-performance liquid chromatography (HPLC) in a 20 to 100% methanol-in-water gradient (27). Four milliliters of scintillation cocktail 3a70b (Research Products Inc., Mount Prospect, Ill.) was added to each of the 70 1-ml fractions collected, and radioactivity was determined by scintillation counting.

To determine C16-HSL concentrations, *R. capsulatus* ALS1 harboring pYP (18), a plasmid that produces β -galactosidase in response to added C16-HSL (see Results), was used. The procedure was identical to that described for the 3OC12-HSL bioassay described elsewhere (27, 36) except for the different reporter and that cultures were grown aerobically in YPS medium for 22 h. Synthetic C16-HSL was used to generate a standard curve.

To identify acyl-HSLs, we extracted late-logarithmic-phase culture fluid with acidified ethyl acetate. The acyl-HSLs were purified from extracts of LB-grown cultures by C₁₈ reverse-phase HPLC as described above. The purified material was analyzed by chemical ionization mass spectrometry (CI-MS) with a VG Trio-1 quadrupole mass spectrometer with methane as the reagent gas. We restricted our isolation of material for mass spectrometry to material from the culture fluid. As described in the Results, this represented about half of the total for each of the acyl-HSLs. This facilitated purification because the bulk of the cellular lipids were not included in the extract. Alternatively, identification was done by coelution of radiolabeled material from whole cultures (cells plus culture fluid) with chemically synthesized acyl-HSLs.

Acyl-HSL association experiments. To determine if the long-chain acyl-HSLs are associated with cells, we compared the amount of radiolabeled acyl-HSL associated with the cell-free culture fluid and cell pellet for *R. capsulatus*, *P. denitrificans*, and, as a control, *V. fischeri*. Radiolabeling was performed as described above except that prior to extraction, the cultures were centrifuged (10,000 $\times$ g, 4°C), and the culture fluid was separated from the cell pellet. The cell pellet was resuspended in 5 ml of fresh medium (the original culture volume). Acyl-HSLs from the culture fluid and suspended cell pellet were extracted and separated by HPLC as described above. The total amounts of [¹⁴C]3OC6-HSL (fraction 10), [¹⁴C]C16-HSL (fractions 65 to 67), and [¹⁴C]C14-HSL (frac-

tions 60 to 62) in the culture fluid and cell pellet were determined by scintillation counting.

Acyl-HSLs. C16-HSL was synthesized in a manner similar to that described elsewhere (5). Hexadecanoic acid (Sigma Chemical Company, St. Louis, Mo.) was mixed with L-homoserine lactone hydrochloride in dry dichloromethane with activation by dicyclohexylcarbodiimide and 1-hydroxybenzotriazol. The crude synthetic product was washed with 5% NaHCO₃, and the C16-HSL was purified by C₁₈ reverse-phase HPLC. As shown by CI-MS, the product showed the expected molecular mass. All other synthetic acyl-HSLs were purchased from Aurora Biosciences.

Identification and genetic analysis of *R. capsulatus* acyl-HSL synthase. We performed a Blast search of the *R. capsulatus* genome (<http://ergo.integrated-genomics.com/ERGO>; Integrated Genomics, Chicago, Ill.) for translation products showing similarity to the *Rhodobacter sphaeroides* CerI protein (accession number AAC46022). One putative *R. capsulatus* gene, open reading frame (ORF) RCC03805, coded for a polypeptide that showed significant similarity to CerI and was adjacent to a gene coding for a probable LuxR-type regulatory protein, ORF RCC03806. A 2.7-kb fragment of *R. capsulatus* SB1003 chromosomal DNA containing ORF RCC03805 was amplified with the Expand long-template PCR system (Boehringer Mannheim) and the following primers: forward, 5'-CAATTGGGGTACCGCCGTCTGAACCG-3', and reverse, 5'-TCTA GACGGGTCCGATCCGCGGACGG-3'.

The PCR fragment was used to create pGtaI-1 with the Original TA cloning kit (Invitrogen, Carlsbad, Calif.). A 2-kb *SmaI* fragment of pHP45 Ω Sp (containing the spectinomycin resistance [Sp^r] cassette) was cloned into a unique *PshAI* site of pGtaI-1, disrupting RCC03805. This construct was designated pGtaIKO. A pGtaIKO *MfeI*-*XbaI* fragment containing the inactivated RCC03805 gene was ligated to *EcoRI*- and *XbaI*-digested pJP5603 to create pJPGtaIKO, which contains the Sp^r cassette 178-bp downstream of the predicted translational start codon of RCC03805. The Sp^r cassette in pJPGtaIKO is flanked by 1.4 kb of upstream and 1.3 kb of downstream *R. capsulatus* DNA. *E. coli* S17-1 λ pir was used to mobilize pJPGtaIKO into *R. capsulatus* SB1003 (28). We selected spectinomycin-resistant colonies and screened for a kanamycin-sensitive mutant. One mutant, ALS1, contained an Sp^r cassette insertion in RCC03805, as shown by Southern blot analysis with RCC03805 and Sp^r cassette probes.

For complementation studies, pBBRGtaI was constructed by cloning the RCC03805 ORF into the *SalI* and *EcoRI* sites of pBBRMCS-2, a broad-host-range plasmid (15). The resulting plasmid was introduced into *R. capsulatus* ALS1 by conjugation as described above. A kanamycin-resistant exconjugant was selected for subsequent studies.

Assessment of gene transfer agent transcription and production. Plasmid pYP, which contains the gene transfer agent promoter, *orf1*, and an in-frame translational fusion of the gene transfer agent structural gene *orf2* to *lacZ* (18), was introduced into *R. capsulatus* strains SB1003 and ALS1 by conjugation. Exconjugants were grown phototrophically in YPS at 30°C, and β -galactosidase activity was measured in stationary-phase cells ($\approx$ 22 h) (46). Where indicated, synthetic C16-HSL (2 μ M) was added to cultures. As a control, similar experi-

FIG. 1. HPLC analysis of radiolabeled acyl-HSLs from (A) *R. capsulatus* and (B) *P. denitrificans* culture extracts. Acyl-HSLs from *P. aeruginosa* (3OC12-HSL) and *R. sphaeroides* (7,8-*cis*-C14-HSL) eluted at fractions 50 and 56, respectively, as shown by the solid and open arrows, respectively. C14-HSL eluted at fraction 61 and C16-HSL at fractions 65 and 66. These are indicated as references. The void volume was in fractions 1 to 5. The radioactivity in the void volume likely represents a small amount of methionine in the ethyl acetate. The methanol gradient is shown by the solid line. No radioactive material was eluted after fraction 70.

ments were performed with pYNP, which contains a promoterless *orf2::lacZ* fusion (18). With pYNP, β -galactosidase activity was less than 0.1 U.

Transducing particle production was measured as gene transfer agent-mediated transfer of a rifampin resistance (Rif^R) marker from the gene transfer agent donor strains (SB1003 and ALS1) to the Rif^R gene transfer agent recipient strain (B10) as described previously (18). *R. capsulatus* SB1003 and ALS1 were grown as described above, and synthetic C16-HSL (2 μ M) was added to cultures where indicated.

RESULTS

***R. capsulatus* and *P. denitrificans* synthesize acyl-HSLs.** Acyl-HSL discovery has relied on detection with bioassays employing heterologous reporter constructs (36). These bioassays have certain limitations: they are labor intensive, require construction of an appropriate indicator strain, and are biased towards detection of acyl-HSLs close enough in structure to the natural signal to be recognized by specific LuxR homologs. The development of a radiotracer assay to detect acyl-HSLs overcomes these limitations. We have developed such an assay and used it with success to monitor the relative abundance of multiple acyl-HSLs produced by *P. aeruginosa* (34, 38). The radiotracer assay depends upon incorporation of radiolabel from carboxyl- 14 C-methionine into acyl-HSLs. Radiolabeled acyl-HSL can be separated from methionine or *S*-adenosylmethionine by solvent extraction.

We screened *R. capsulatus* and *P. denitrificans* for acyl-HSL synthesis with the radiotracer assay. These were both organisms in which we had failed to detect acyl-HSLs with a variety of bioassays (31; unpublished results). *P. denitrificans* incorporated 14 C label into a product that was ethyl acetate extractable and was eluted with approximately 95% methanol in our C₁₈ reverse-phase HPLC (Fig. 1B). *R. capsulatus* appeared to

make two acyl-HSLs, one that coeluted with the *P. denitrificans* product and an additional product that was eluted with 90% methanol in water (Fig. 1A). These acyl-HSLs were more hydrophobic than any previously described naturally occurring acyl-HSLs. Our results demonstrate one significant advantage of the nonbiological detection approach, that acyl-HSLs exhibiting little or no activity in existing bioassays can be detected.

Identification of *R. capsulatus* and *P. denitrificans* acyl-HSLs. The *R. capsulatus* product from fraction 60 coeluted with synthetic tetradecanoyl-HSL (C14-HSL), but the identities of the more hydrophobic compounds produced by *R. capsulatus* and *P. denitrificans* (fractions 65 and 66) were unknown. The material in fractions 65 and 66 was purified from *P. denitrificans* culture fluid as described in Materials and Methods and analyzed by CI-MS, which showed a quasimolecular ($M + H$)⁺ ion with an m/z of 340 (Fig. 2A). There were also peaks at 102 and 143 m/z , corresponding to homoserine lactone and the homoserine lactone ring with two of the acyl carbons, respectively, which are characteristic peaks found in CI-MS spectra of acyl-HSLs (36). The spectrum was consistent with the conclusion that the compound in fractions 65 and 66 was C16-HSL. As a confirmation, we chemically synthesized C16-HSL. Synthetic C16-HSL showed an elution profile identical to that of the *P. denitrificans* material and had a CI-MS spectrum similar to that of the *P. denitrificans* material (Fig. 2B). It was not possible to obtain sufficiently pure material from *R. capsulatus* for further analysis, but we conclude that the *R. capsulatus* material that coeluted with C16-HSL in HPLC is also C16-HSL.

***R. capsulatus* acyl-HSL synthase gene.** We chose to study C16-HSL production further in *R. capsulatus*, an organism for

FIG. 2. Chemical ionization mass spectra of acyl-HSLs. (A) Spectrum of material purified from a *P. denitrificans* culture and (B) spectrum of synthetic C16-HSL.

A

```

RhII      1  -MELLSESLEGLSAAMIAELGRYRHQVFIEKLGWDVVSTSRVRDQEFDO
SolI      1  --MRTFVHGGGRLPEGIDAALAHYRHQVFVGRIGWQLPMADGTF--ERDO
LuxI      1  MTIMIKKSDFLAIPSEEYKGILSLRYQVEKQRLWDLVVENNL---ESDE
CerI      1  -MIFIIDSLNLRHADIVKDMFRLKRVFADRLGWDVQISOQM---ERDR
RRC03805  1  MQTTTSLFANMHNHGELEFANILRARRQSEIIQNRWELPEALGM---EEDO

RhII      50 FHPQTRYIVAMSROG-ICGCARLLPTTD-----AYLLKDVFAYLCS---
SolI      47 YDRDDTVYVVARDEGGTICGCARLLPTTR-----PYLLKDVFAILLMH--
LuxI      48 YDNSNAEYIYACDDTENVSGCWRLPTTG-----DYMLKSVFPPELLGQ--
CerI      47 FDDLPAHVVSVDDEGRVVGCMRLLOITG-----PHMLSDVFSSTLDG--
RRC03805  48 YDTPQSRWVAVHDDGQ-VHAGLRITPSIARCGIYSYMRDAQKGTLLGGSI

RhII      91 -----ETPPSDESVWELSRYAASAADDPQLAMKIFWSS-----IQ
SolI      90 -----GMPPPESEVWELSRFAARSGAPCPRSGRADWAVRPMLASV---VQ
LuxI      91 -----QSAPKDENIVELSRFAVGKNSKINNSASEITMK-LFEAI--YK
CerI      90 -----EPHLRSATLWEATRFCDTDLVSGRARNIAYVTSEVMIGAFE
RRC03805  97 PQDLLWEFAFVDFQVWENSRVFSHDI PMQDRRKVHFQLV---EAM--VQ

RhII      126 CAWYLGASSVVAVTTTAMERYFVRNGVILQRLG--PPQVKVGETLVAITSF
SolI      133 CAAQRGARRLIGATEVSMVRLFRIGVRAHRAG--PVRCIGGRPVVACWI
LuxI      132 HAVSQGITTEYVTVTSTAIFERFLKRIKVPCHRIQDKEIHVLGDTKSVLISM
CerI      134 FAMSAGVTDAAVVIDPVMIDRVLKRSGNAPOGYVG-TKPMGKVTAALM
RRC03805  142 AARDQGATRLLAISAASWFRWYRRCGLVAOPIC--FVVFIGDGYQCVSI

RhII      174 PAYQERGLEMLLRYHPEWLAEP-----
SolI      181 DIDASTCAALGIPSASAAPGPVLQ----
LuxI      182 PINEQFKKAVLN-----
CerI      183 DCSEERVKRIRDFAGIYHDVTQPQTIVA
RRC03805  190 DLMAKLH-----

```

B

```

CerR      1  -----MDIIDLSTVATDDASFLDYIDQLCQKLGFDYASYATTSP
RRC02401  1  -----MLDHLEQLLVADDVATIWSMHCAQMAKEGFDRLIYGFTRF
LuxR      1  MGMKNINADDTYRIINKIKACRSNNDINQCLSDMTKMVHCEYYLLAIYP
RRC04617  1  -MASSAAHHATPRPGPEAAGASGLGREVDRFETARGFGASALIYDYTPR
RRC03806  1  -----MSIHTINKCLREIGRVATDGYFIGLHIR

CerR      40 MTGAVQG-----YANYPDSWKMHVMRNLHRVDPTIHKSAISIAFVD
RRC02401  41 RATQNLGSLD-DTLILSNHDPAYLNSFLGAGLFSEAPMVKWLDSVSGACS
LuxR      41 HSMVKSDIS-----ILDNYPKKTRQYVDANLIKYPDIVDYSNSNHSEIN
RRC04617  50 HRETDTGTVTAPAVMMVRNLDPGMIDYWCGRGYAHVDPVQAVAVNSVAFV
RRC03806  30 FAAPIMQ-----FQTYPEAMTDHYTRQAYALRDETIAGWFSEEGACR

CerR      82 WSRFERDERFRVFFAAEDFGITPQGLT-----VVRGPGYDGRGLLSVT
RRC02401  90 WR--VVAERMAAGELTEGERVIDLNRR-----FOVLAGYSIGFPDTS
LuxR      96 WNIFFENAVNKKSPNVIKEAKTSGIITG-----FSFPIHTANNFGMLS
RRC04617  100 WSYDAEAGDLSASFELTRNSRPVVEFISASGFVNGITVPIHLPGGGYVTN
RRC03806  72 WS----EFTLPDPFNILGEARRFGLNYG-----VTVSCGPVTSRTI

CerR      126 RN-----CARPEWEKHKRAVIGELQVAHVHLDAVMRSVISRALRQPR
RRC02401  132 RAKGAIGLCAQGLTQADVDAIWEHGRDLRVLNSIMHLKISALPFSTTR
LuxR      140 FA-----HSEKDNIDSLFLHACMNIPLIVPSLVDNYRKINIANNKSN
RRC04617  150 GLWN----GPGRAFAAFAAQVLPGFALMAQAFQNDIGAPAPADPAPQPFQ
RRC03806  109 AS-----FTLRDREFRDDEIDLISQIRRLHDLAQPEE

CerR      170 ----LSTREIEVLQWAAAAGKSQTDIGDILGISHRTVEVHLRSAREKLGT
RRC02401  182 R---PLTSRQREVLEWVGDKTSADIAQLLGPTTATVEKHLRLAREALEV
LuxR      183 N---DLTKREKECLAWACEGKSSWDISKILGCSERTVTFHLTNAQMKLNT
RRC04617  196 PRTPALSARERECLDHAQGRSAKETSRLIERSVPTVVMHLNAVRKLG
RRC03806  142 G----LTKAQIEALRCIAAGDRHAAAAAKLGITQSAFKARLLSARERLMA

CerR      215 LSTVQAVGRAIGLGLVYPR----
RRC02401  229 ETTAQALLKASMQQLFITSAP-
LuxR      230 TNRCQISKAILTCAIDCPYFKN
RRC04617  246 RNRSHAVAEAVRRGLI-----
RRC03806  188 RTTAAEIQRAKEYRLL-----

```


which the genome has been sequenced. We identified an ORF, RRC03805, that encoded a product that showed 26% identity (43% similarity) to the *R. sphaeroides* CerI and contained each of the nine completely conserved amino acid residues found in acyl-HSL synthases (Fig. 3A) (24).

To verify that RRC03805 coded for an acyl-HSL synthase, we created an RRC03805 Ω SP⁺ insertion mutant, *R. capsulatus* ALS1 (Table 1). Strain ALS1 did not synthesize C16-HSL or C14-HSL (Fig. 4A), and we detected no other acyl-HSLs. This suggested that the RRC03805 gene codes for an acyl-HSL synthase. Unlike the *R. sphaeroides* cerI mutant, which overproduces exopolysaccharide (31), *R. capsulatus* ALS1 had no obvious phenotype. When the RRC03805 mutation in strain ALS1 was complemented with pBBRGtaI, acyl-HSL synthesis was restored (Fig. 4A). Interestingly, *E. coli* containing pBBRGtaI synthesized C14-HSL, additional putative acyl-HSLs (fractions 57 to 58), but not C16-HSL (Fig. 4B). To our knowledge, this appears to be the first acyl-HSL synthase that fails to direct the synthesis of all cognate acyl-HSL molecules in *E. coli*.

A Blast search of the *R. capsulatus* translation products with the *R. sphaeroides* CerR (AAC47021) revealed three related polypeptides, RRC03806, RRC04617, and RRC02401. These translation products were 26%, 21%, and 23% identical (37%, 31%, and 36% similar) to CerR, respectively. Two of the three, RRC03806 and RRC04617, contained all seven of the most highly conserved residues found among LuxR family members (Fig. 3B) (42). RRC02401 possessed only three of the seven conserved residues, but did show similarity to other LuxR-related proteins along what is thought to be the acyl-HSL binding region (Fig. 3B). RRC03806 was 53 bp upstream of the acyl-HSL synthase, and the two genes were in the same orientation. The ORFs encoding the other two LuxR-like proteins were unlinked.

C16-HSL induction of *R. capsulatus* gene transfer agent. The *R. capsulatus* genome harbors a 15-kb cluster of genes that code for production of a phage-like particle known as the gene transfer agent (17, 18). Each gene transfer agent particle can deliver approximately 4.5 kb of randomly packaged genomic DNA to a recipient cell, where recombination may occur (40, 47). Because gene transfer agent production is maximal in stationary phase (18, 41), when cell densities are high, and because a gene transfer system in another bacterium has been reported to be controlled by quorum sensing (10, 49), we tested whether the gene transfer agent is regulated by C16-HSL.

First, we examined transcription of a gene transfer agent structural gene. Plasmid pYP (Table 1), which carries the gene transfer agent gene *orf2* fused to *lacZ* (18), was introduced into the wild-type and acyl-HSL synthase mutant *R. capsulatus* strains. Measurements of *lacZ*-encoded β -galactosidase activity indicated that *orf2* expression was reduced sevenfold in the acyl-HSL synthase mutant compared to the wild-type strain (Table 2). Addi-

FIG. 4. HPLC analysis of radiolabeled acyl-HSLs from culture extracts. (A) HPLC profile of extract of *R. capsulatus* ALS1 (○) and ALS1 containing pBBRGtaI (●). (B) HPLC profile of extract of *E. coli* containing pBBRGtaI.

tion of C16-HSL restored wild-type β -galactosidase activity to the mutant (Table 2). This sevenfold reduction in β -galactosidase activity was observed in early, mid-, and late logarithmic phase and in stationary phase (data not shown). Second, we assessed the number of gene transfer agent particles produced by the wild-type and acyl-HSL synthase mutant *R. capsulatus* strains. In agreement with the *lacZ* reporter data, gene transfer agent production was fivefold less in the acyl-HSL-deficient strain, and the number of transductants was increased by addition of C16-HSL (Table 2). These data indicate that gene transfer agent production is controlled by C16-HSL.

Both C16-HSL and C14-HSL activated gene transfer agent gene expression in a concentration-dependent manner (Fig. 5). The half-maximal response occurred at 2.5 to 5 nM C16-HSL and 7.5 to 10 nM C14-HSL. This is consistent with information about other acyl-HSL systems, in which responses generally require nanomolar concentrations of signals (14, 27, 49). C16-HSL appeared to be the preferred signal for gene transfer agent gene expression (Fig. 5). It is clear from the available *R. capsulatus* genomic sequencing data (assembled into nine contigs) that the acyl-HSL synthase gene and the *gta* genes are not tightly linked; the minimum distance between them is 570 kbp (www.integratedgenomics.com).

Concentrations and locations of long-chain acyl-HSLs in *R.*

FIG. 3. Multiple alignments of several LuxI and LuxR family members with homologs found in the *R. capsulatus* genome. (A) Representative LuxI family members. The asterisks indicate residues conserved among most LuxI family members (24). Each of these nine residues occurs in the aligned sequence of RRC03805. ClustalW (43) was used to align the sequences, and Boxshade (0.6 setting) was used to determine the degree of residue shading. (B) Representative LuxR family members. The solid bar above the residues corresponding to LuxR amino acids 79 to 127 represents the conserved signal-binding region (13, 37, 39), and the open bar above the residues corresponding to LuxR amino acids 184 to 210 indicates the conserved helix-turn-helix-containing region in the DNA-binding domain. The sequences used in the alignments were *R. sphaeroides* CerI (accession number AAC46022) and CerR (AAC47021), *V. fischeri* LuxI (CAA68562) and LuxR (B33538), *Ralstonia solanacearum* SolI (030920), and *P. aeruginosa* RhlI (AAC44037).

TABLE 2. Effect of hexadecanoyl-HSL on gene transfer agent transcription and particle production

Strain	β -Galactosidase activity (U) from pYP ^a	No. of gene transfer agent transductants ^b
Wild type	69.2	28.0
ALS1	9.9	5.3
ALS1 plus 2 μ M C16-HSL	61.2	37.3

^a Average of two samples.^b Average of three samples.

capsulatus cultures. One limitation of the ¹⁴C-labeled acyl-HSL assay is that it does not provide information on how much of an acyl-HSL accumulates during culture growth (34). Thus, we needed to perform bioassays to determine the absolute level of C16-HSL. Our discovery that a gene transfer agent structural gene-lacZ fusion is expressed in response to C16-HSL (Fig. 5) provided us with a bioassay tool (see Materials and Methods). Cell-free late-logarithmic-phase *R. capsulatus* and *P. denitrificans* culture fluids contained approximately 325 to 390 nM and 350 to 400 nM C16-HSL, respectively. Unlike the case with *V. fischeri*, for example, where almost all of the 3OC6-HSL is found in the culture supernatant fluid (14), a significant fraction of C14 and C16-HSL remained with the cells.

Ethyl acetate extracts of *P. denitrificans* cell pellets contained 48 to 50% of the total ¹⁴C-labeled C16-HSL. Extracts of *R. capsulatus* cells contained 33 to 34% and 49 to 52% of the total ¹⁴C-labeled C16-HSL and putative ¹⁴C-labeled C14-HSL signals, respectively. For comparison, we measured the fraction of 3OC6-HSL in *V. fischeri* cells. With *V. fischeri*, 2 to 4% of the total ¹⁴C-labeled 3OC6-HSL remained with cells, consistent with a previous report (14). These data suggest that the long-chain acyl-HSLs produced by *P. denitrificans* and *R. capsulatus* are concentrated in cells. Presumably, they partition with the membranes, where they may be unavailable as signals.

DISCUSSION

We have used a radiotracer technique rather than bioassays to show that *R. capsulatus* and *P. denitrificans* synthesize the long-chain C16-HSL (both organisms) and material that co-elutes with C14-HSL in HPLC (*R. capsulatus*). This extends the range of known acyl-HSL signals. Other bacteria also produce

relatively long-chain acyl-HSLs, for example, *R. sphaeroides*, which produces 7,8-*cis*-3-tetradecenoyl-HSL (31). It is interesting that, as a rule, long-chain acyl-HSLs are produced by alpha-proteobacteria. The hydrophobic nature of the *R. capsulatus* and *P. denitrificans* long-chain acyl-HSLs may explain why about half of the total acyl-HSLs in cultures were associated with cells. Because the cells constitute only a small percentage of the total culture volume, the cellular concentrations of these acyl-HSLs were estimated to be quite high, on the order of 50 times higher than the extracellular concentrations.

We assume that the bulk of the cellular acyl-HSLs was associated with the membranes. The implications of this are that although there would be significant levels of acyl-HSLs in membranes, the cytoplasmic concentrations might be similar to the environmental concentrations. Thus, the acyl-HSLs could still serve a signaling function. Short-chain acyl-HSLs, like 3OC6-HSL and butanoyl HSL, are freely diffusible and not concentrated in cells (14, 26). Longer-chain signals like 3OC12-HSL in *P. aeruginosa* are concentrated in cells, and an efflux pump has been shown to aid 3OC12-HSL export (26).

We studied acyl-HSL production by *R. capsulatus* further because of the availability of the genome sequence for this organism. We identified a single ORF that showed significant sequence similarity with LuxI family members, and we showed that this ORF directed the synthesis of acyl-HSLs. Insertion of an Sp^r cassette in this ORF generated a strain that did not produce acyl-HSLs. Introduction of a wild-type copy of this gene into the *R. capsulatus* ALS1 mutant or into *E. coli* resulted in acyl-HSL synthesis. In the complemented *R. capsulatus* mutant, the acyl-HSLs produced were identical to those produced by the wild-type strain, but *E. coli* did not produce C16-HSL. To our knowledge, this is the first example of an *I* gene-containing recombinant *E. coli* that does not produce the native acyl-HSL. Adjacent to this acyl-HSL synthase gene is a gene coding for a LuxR homolog. However, there are other LuxR homologs in the *R. capsulatus* genome database, and we do not know if the adjacent gene is a transcription factor that responds to C16-HSL.

Although we have not identified the receptor experimentally, we have shown that C16-HSL can serve as an environmental signal for activation of gene transfer agent structural genes in *R. capsulatus*. This demonstrates that even though C16-HSL is concentrated in cells, it is a signal for gene expression when added externally. Of course, it is possible that long-chain acyl-HSLs play an additional role in *R. capsulatus* and *P. denitrificans* membranes.

The *R. capsulatus* long-chain acyl-HSL induced gene transfer agent gene expression five- to sevenfold, whereas expression of gene transfer agent and flagellar genes was reduced 10-fold or more in *cckA* and *ctrA* mutants (16). It will be interesting to evaluate the possibility of connections between the *cckA/ctrA* system and the acyl-HSL-dependent regulation described here. Perhaps there are independent but overlapping signals that cooperatively induce maximal production of gene transfer agent.

ACKNOWLEDGMENTS

We thank R. Haselkorn for sharing *R. capsulatus* genomic sequence data and A. S. Lang for comments.

FIG. 5. Dose-response curves for activity of C16-HSL (●) and C14-HSL (○), measured as β -galactosidase (β -gal) activity in *R. capsulatus* ALS1(pYP).

This work was supported by grants from the W. M. Keck Foundation, the National Institutes of Health (GM59026), the National Science Foundation (MCB 9808308), and the Natural Sciences and Engineering Research Council (Canada). A.L.S. was supported by U.S. Public Health Service Training Grant T32-AI07343.

ADDENDUM IN PROOF

After acceptance of our paper, Marketon et al. (M. M. Marketon, M. R. Gronquist, A. Eberhard, and J. E. Gonzalez, *J. Bacteriol.* **184**:5686–5695, 2002) showed with radiotracers that *Sinorhizobium meliloti* produces long-chain acyl-HSLs (14- to 18-carbon acyl groups).

REFERENCES

- Baker, S. C., S. J. Ferguson, B. Ludwig, M. D. Page, O. M. Richter, and R. J. V. Spanning. 1998. Molecular genetics of the genus *Paracoccus*: metabolically versatile bacteria with bioenergetic flexibility. *Microbiol. Mol. Biol. Rev.* **62**:1046–1078.
- Beatty, J. T., and H. Gest. 1981. Generation of succinyl-coenzyme A in photosynthetic bacteria. *Arch. Microbiol.* **129**:335–340.
- Blosser-Middleton, R. D., and K. M. Gray. 2001. Multiple *N*-acyl homoserine lactone signals of *Rhizobium leguminosarum* are synthesized in a distinct temporal pattern. *J. Bacteriol.* **183**:6771–6777.
- Cohen-Bazire, G., W. R. Sistrom, and R. Y. Stanier. 1956. Kinetic studies of pigment synthesis by non-sulfur purple bacteria. *J. Cell. Comp. Phys.* **49**:25–68.
- Dekhane, M., K. T. Douglas, and P. Gilbert. 1996. A novel convenient route to the naturally occurring 3-oxoacyl-L-homoserine lactones and related bacterial autoinducers. *Tetrahedron Lett.* **37**:1883–1884.
- Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Neilson, and N. J. Oppenheimer. 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry* **20**:2444–2449.
- Eberhard, A., C. A. Widrig, P. McBath, and J. B. Schineller. 1986. Analogs of the autoinducers of bioluminescence in *Vibrio fischeri*. *Arch. Microbiol.* **146**:35–40.
- Engbrecht, J., K. H. Neilson, and M. Silverman. 1983. Bacterial bioluminescence: isolation and genetic analysis of the functions from *Vibrio fischeri*. *Cell* **32**:773–781.
- Fuqua, C., M. R. Parsek, and E. P. Greenberg. 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu. Rev. Gen.* **35**:439–468.
- Fuqua, W. C., and S. C. Winans. 1993. 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.
- Fuqua, W. C., S. C. Winans, and E. P. Greenberg. 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu. Rev. Microbiol.* **50**:727–751.
- Fuqua, W. C., S. C. Winans, and E. P. Greenberg. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* **176**:269–275.
- Hanzelka, B. L., and E. P. Greenberg. 1995. Evidence that the N-terminal region of the *Vibrio fischeri* LuxR protein constitutes an autoinducer-binding domain. *J. Bacteriol.* **177**:415–417.
- Kaplan, H. B., and E. P. Greenberg. 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. *J. Bacteriol.* **163**:1210–1214.
- Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II, and K. M. Peterson. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS carrying different antibiotic-resistance cassettes. *Gene* **166**:175–176.
- Lang, A., and J. Beatty. 2002. Bacterial signal transduction system simultaneously control genetic exchange and motility. *J. Bacteriol.* **184**:913–918.
- Lang, A. S., and J. T. Beatty. 2001. The gene transfer agent of *Rhodobacter capsulatus* and “constitutive transduction” in prokaryotes. *Arch. Microbiol.* **175**:241–249.
- Lang, A. S., and J. T. Beatty. 2000. Genetic analysis of a bacterial genetic exchange element: the gene transfer agent of *Rhodobacter capsulatus*. *Proc. Natl. Acad. Sci. USA* **97**:859–864.
- Marrs, B. L. 1974. Genetic recombination in *Rhodopseudomonas capsulata*. *Proc. Natl. Acad. Sci. USA* **71**:971–973.
- McEwan, A. G. 1994. Photosynthetic electron transport and anaerobic metabolism in purple non-sulfur phototrophic bacteria. *Antonie Van Leeuwenhoek* **66**:151–164.
- Neilson, K. H., T. Platt, and J. W. Hastings. 1970. Cellular control of the synthesis and activity of the bacterial luminescence system. *J. Bacteriol.* **104**:313–322.
- Neidhardt, F. C., P. L. Bloch, and D. F. Smith. 1974. Culture medium for enterobacteria. *J. Bacteriol.* **119**:736–747.
- Parsek, M. R., and E. P. Greenberg. 2000. Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. *Proc. Natl. Acad. Sci. USA* **97**:8789–8793.
- 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.
- Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski. 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* **260**:1127–1130.
- Pearson, J. P., C. V. Delden, and B. H. Iglewski. 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J. Bacteriol.* **181**:1203–1210.
- Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg. 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc. Natl. Acad. Sci. USA* **91**:197–201.
- Penfold, R. J., and J. M. Pemberton. 1992. An improved suicide vector for construction of chromosomal insertion mutations in bacteria. *Gene* **118**:145–146.
- Piper, K. R., S. Beck v. Bodman, and S. K. Farrand. 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature (London)* **362**:21670–21676.
- Prentki, P., and H. M. Krusch. 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29**:303–313.
- Puskas, A., E. P. Greenberg, S. Kaplan, and A. L. Schaefer. 1997. A quorum-sensing system in the free-living photosynthetic bacterium *Rhodobacter sphaeroides*. *J. Bacteriol.* **179**:7530–7537.
- Ruby, E. G., and K. H. Neilson. 1976. Symbiotic association of *Photobacterium fischeri* with the marine luminous fish *Monocentris japonica* L., a model of symbiosis based on bacterial studies. *Biol. Bull.* **151**:574–596.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
- Schaefer, A. L., E. P. Greenberg, and M. R. Parsek. 2001. Acylated homoserine lactone detection in *Pseudomonas aeruginosa* biofilms by radiolabel assay. *Methods Enzymol.* **336**:41–47.
- Schaefer, A. L., B. L. Hanzelka, A. Eberhard, and E. P. Greenberg. 1996. Quorum sensing in *Vibrio fischeri*: probing autoinducer-LuxR interactions with autoinducer analogs. *J. Bacteriol.* **178**:2897–2901.
- Schaefer, A. L., B. L. Hanzelka, M. R. Parsek, and E. P. Greenberg. 2000. Detection, purification, and structural elucidation of the acylhomoserine lactone inducer of *Vibrio fischeri* luminescence and other related molecules. *Methods Enzymol.* **305**:288–301.
- Shadel, G. S., R. Young, and T. O. Baldwin. 1990. Use of regulated cell lysis in a lethal genetic selection in *Escherichia coli*: identification of the autoinducer-binding region of the LuxR protein from *Vibrio fischeri* ATCC 7744. *J. Bacteriol.* **172**:3980–3987.
- Singh, P. K., A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh, and E. P. Greenberg. 2000. Quorum sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407**:762–764.
- Slock, J., D. VanRiet, D. Kolibachuk, and E. P. Greenberg. 1990. Critical regions of the *Vibrio fischeri* LuxR protein defined by mutational analysis. *J. Bacteriol.* **172**:3974–3979.
- Soliz, M., and B. Marrs. 1977. The gene transfer agent of *Rhodopseudomonas capsulata*: purification and characterization of its nucleic acid. *Arch. Biochem. Biophys.* **181**:300–307.
- Soliz, M., H. C. Yen, and B. Marrs. 1975. Release and uptake of gene transfer agent by *Rhodopseudomonas capsulata*. *J. Bacteriol.* **123**:651–657.
- Stevens, A. M., and E. P. Greenberg. 1999. Transcriptional activation by LuxR, p. 231–242. In G. Dunny and S. Winans (ed.), *Cell-cell signaling in bacteria*. American Society for Microbiology, Washington, D.C.
- Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.
- Wall, J. D., P. F. Weaver, and H. Gest. 1975. Gene transfer agents, bacteriophages, and bacteriocins of *Rhodopseudomonas capsulata*. *Arch. Microbiol.* **105**:217–224.
- Whitehead, N. A., A. M. L. Barnard, H. Slater, N. J. L. Simpson, and G. P. C. Salmond. 2001. Quorum sensing in gram-negative bacteria. *FEMS Microbiol. Rev.* **25**:365–404.
- Whiteley, M., K. M. Lee, and E. P. Greenberg. 1999. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **96**:13904–13909.
- Yen, H. C., N. T. Hu, and B. L. Marrs. 1979. Characterization of the gene transfer agent made by an overproducer mutant of *Rhodopseudomonas capsulata*. *J. Mol. Biol.* **131**:157–168.
- Yen, H. C., and B. Marrs. 1976. Map of genes for carotenoid and bacteriochlorophyll biosynthesis in *Rhodopseudomonas capsulatus*. *J. Bacteriol.* **126**:619–629.
- Zhang, L., P. J. Murphy, A. Kerr, and M. E. Tate. 1993. *Agrobacterium* conjugation and gene regulation by *N*-acyl-homoserine lactones. *Nature (London)* **362**:446–448.