

Genes of *Rhodobacter sphaeroides* 2.4.1 Regulated by Innate Quorum-Sensing Signal, 7,8-*cis*-N-(Tetradecenoyl) Homoserine Lactone

Hwang, Won¹, Ko-Eun Lee¹, Jeong Kug Lee¹, Byoung Chul Park², and Kun-Soo Kim^{1*}

¹Department of Life Science and Interdisciplinary Program of Integrated Biotechnology, Sogang University, Seoul 121-742, Korea

²Translational Research Center, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Daejeon 305-600, Korea

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The free-living photoheterotrophic Gram-negative bacterium *Rhodobacter sphaeroides* possesses a quorum-sensing (QS) regulatory system mediated by CerR-CerI, a member of the LuxR-LuxI family. To identify the genes affected by the regulatory system, random *lacZ* fusions were generated in the genome of *R. sphaeroides* strain 2.4.1 using a promoter-trapping vector, pSG2. About 20,000 clones were screened and 23 showed a significantly different level of β -gal activities upon the addition of synthetic 7,8-*cis*-N-tetradecenoyl-homoserine lactone (RAI). Among these 23 clones, the clone showing the highest level of induction was selected for further study, where about a ten-fold increase of β -gal activity was exhibited in the presence of RAI and induction was shown to be required for *cerR*. In this clone, the *lacZ* reporter was inserted in a putative gene that exhibited a low homology with *catD*. A genetic analysis showed that the expression of the *catD* homolog was initiated from a promoter of another gene present upstream of the *catD*. This upstream gene showed a strong homology with *luxR* and hence was named *qsrR* (quorum-sensing regulation regulator). A comparison of the total protein expression profiles for the wild-type cells and *qsrR*-null mutant cells using two-dimensional gel electrophoresis and a MALDI-TOF analysis allowed the identification of sets of genes modulated by the *luxR* homolog.

Keywords: Reporter vectors, *Rhodobacter sphaeroides*, quorum sensing

Quorum-sensing regulation, which is a cell-to-cell communication system among bacteria, depends on the cell density. Quorum-sensing bacteria detect their cell density via monitoring the accumulation of the signal molecules, termed autoinducers, produced by such bacteria. Several types of signal molecules have already been identified, and

the most common signal molecules produced by Gram-negative bacteria are *N*-acyl homoserine lactones (AHLs). These molecules are diffusible and accumulate extracellularly. When the concentration of the accumulated signal molecules reaches a critical threshold, the signal molecules then trigger the expression of certain target genes associated with cell-to-cell interactions [3, 4, 9–11, 22].

The regulation of luminescence gene expression in *Vibrio fischeri* is an established example of an AHL-mediated quorum-sensing system [20]. When living planktonically in seawater (at a low cell density), this bacterium is non-luminescent. However, when the cell density is high, the bacterium expresses luminescence genes (for review, [7]). The luminescence gene cluster of *V. fischeri* consists of eight genes (*luxA-E*, *luxG*, *luxI*, and *luxR*) arranged as bidirectional operons. The *luxI* gene encodes an AHL synthase, whereas the *luxR* gene encodes a transcription factor that activates luminescence gene expression when bound by the AHL signal [7]. *V. fischeri* cells express *luxI* at a basal level when in a low cell density, and the concentration of AHL remains low within the medium. However, as cell density increases within the light organ, the concentration of AHL in the medium also increases [6, 10]. When the concentration of AHL reaches a threshold level, the signal molecules diffuse back into the cells. The signal molecules bind to LuxR, which then binds to 20-bp inverted repeat sequences, known as a *lux* box [6]. The *lux* box is located around 40-bp upstream of the transcriptional start site of *luxI* [33]. The binding of the activated LuxR to the *lux* box activates the transcription of the *lux* operon, plus cAMP-CRP (cAMP receptor protein) also activates the *lux* operon [33]. LuxR is a 250-amino-acid polypeptide that contains two domains: a regulator domain and an activator domain [31]. The regulator domain located in the N-terminal contains an AHL-binding region, whereas the activator domain located in the C-terminal contains a DNA binding region, including a helix-turn-helix motif [31].

Rhodobacter sphaeroides 2.4.1 is a Gram-negative purple nonsulfur bacterium that is a free-living, photoheterotrophic

*Corresponding author

Phone: 82-2-705-8460; Fax: 82-2-704-3601;

E-mail: kskim@sogang.ac.kr

member of the α -3 subdivision of proteobacteria [15]. *R. sphaeroides* 2.4.1 produces an AHL, 7,8-*cis*-*N*-(tetradecenoyl) homoserine lactone (RAI), and harbors *cerR* (*luxR* homolog) and *cerI* (*luxI* homolog) [24]. The mutation of *cerI* results in cell aggregation in broth, and this aggregation can be resolved by the addition of purified RAI. It is thought that the aggregation is directly related to the overproduction of extracellular polysaccharide in *cerI* mutants. In the case of sufficiently large cell aggregates, it has been suggested that, since the supply of light is likely to be limited, escape from a community aggregate may be advantageous [24]. Although *R. sphaeroides* is known to contain a quorum-sensing system, the number and types of genes controlled by quorum sensing have not yet been studied systematically.

Accordingly, to identify the genes controlled by quorum sensing in this photosynthetic bacterium, a genomic library of the bacterium was constructed in pSG2, a suicide vector containing a promoterless *lacZ* (*pl-lacZ*) as a reporter [1]. The quorum-sensing-related genes were identified by monitoring the expression of β -galactosidase in the presence and absence of synthetic RAI. As a result, it was found that RAI induced the expression of a *luxR* homolog that modulated certain sets of genes.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Culture Conditions, and Chemicals

The bacterial strains and plasmids used in this study are listed in Table 1. A Luria-Bertani (LB) medium (Sigma, St. Louis, U.S.A.) was used as a rich medium for growing the *Escherichia coli* and *R.*

sphaeroides. Additionally, the *R. sphaeroides* was also grown in Sistrof's succinate-based (Sis) minimal medium, as described previously [14, 30]. The *R. sphaeroides* was grown at 28°C, whereas the *E. coli* was grown at 37°C. Antimicrobial agents were used at the following concentrations: trimethoprim (Tp) at 50 μ g/ml, streptomycin (Sm) at 25 μ g/ml, and spectinomycin (Sp) at 25 μ g/ml for *R. sphaeroides*, plus ampicillin at 50 μ g/ml for *E. coli*. Exogenous synthetic *Rhodobacter* AHL (RAI) was chemically synthesized, as previously described [28], and used at a concentration of 4 μ M. The 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) was used at 40 μ g/ml.

DNA Manipulations and Transformation

The large-scale and small-scale isolations of the plasmid DNA were performed using an alkaline lysis procedure. When required, these DNA samples were further purified by cesium chloride-gradient centrifugation [26] and using Wizard Plus SV Minipreps (Promega, Madison, U.S.A.). The restriction enzymes (MBI Fermentas, Amherst, U.S.A.) and DNA ligase (New England Biolab, Beverly, U.S.A.) were used in accordance with the manufacturer's instructions. The plasmids were introduced into *R. sphaeroides* by biparental mating using *E. coli* S17-1 [29].

Isolation of *R. sphaeroides* Genomic DNA

The total DNA from *R. sphaeroides* was isolated as described previously [18]. Briefly, cells were grown in a Sis medium. TE (10 mM Tris, 1 mM EDTA)-washed cells were then resuspended in 5 M NaCl and the cell membranes weakened with 5% sarkosyl. Next, the cells were collected by centrifugation, resuspended in TE, and lysed with 5% sarkosyl and proteinase K (5 mg/ml) for 1–3 h at 37°C. This DNA was then purified by extraction with 3% NaCl-saturated phenol, chloroform:isoamyl alcohol (24:1), and diethyl ether, followed by ethanol precipitation.

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant genotype and characteristics	Source/reference
Strains		
<i>Escherichia coli</i>		
S17-1	<i>recA</i> , <i>thi</i> , <i>pro</i> , <i>hsdR</i> ⁻ <i>M</i> [RP4-2Tc::Mu::Kmr::Tn7], Tp ^r , Sm ^r	[29]
<i>Rhodobacter sphaeroides</i>		
2.4.1.	Wild type	Our collection
AP3	Derivative of 2.4.1. containing Km ^r cassette insertion in <i>cerI</i>	[24]
AP3T	Derivative of AP3 with mutation in <i>cerI</i>	This study
AP3T-pSG2	Clone in which pSG2 is integrated into genome of AP3T by homologous recombination, Tp ^r Sm ^r Sp ^r	This study
2.4.1- <i>qsrR</i> ⁻	Derivative of 2.4.1 with Ω Sm ^r /Sp ^r cassette inserted in <i>qsrR</i>	This study
Plasmids		
pSG2	Suicide promoter-trapping vector, ColE1 derivative with <i>oriT</i> , <i>pl-aph</i> , <i>pl-lacZ</i> , Ap ^r Sm ^r Sp ^r	[1]
pCF1010	Derivative of transcriptional fusion vector, pLV106, modified to contain unique <i>PstI</i> , <i>NotI</i> , <i>NsiI</i> , <i>AvrII</i> , <i>SstI</i> , <i>BspMII</i> , and <i>XbaI</i> restriction sites between 2.0-kb Ω Sm ^r Sp ^r and 5.1-kb <i>lacZYA'</i> , <i>IncQ/IncP4</i>	[17]
pLO1	Suicide vector for allelic exchange, <i>sacB</i> , RP4 <i>oriT</i> , ColE1 <i>ori</i> , Km ^r	[13]
pSG2-#1ret	Derivative of pSG2 containing <i>SaII</i> fragment retrieved from AP3T-pSG2#1, Ap ^r	This study
pCF::#1pvu	pCF1010 containing 1.9-kb <i>PvuII/XbaI</i> fragment from pSG2-#1ret, Tc ^r Sm ^r Sp ^r	This study
pCF::#1pst	pCF1010 containing 579bp <i>PstI/XbaI</i> fragment from pSG2-#1ret, Tc ^r Sm ^r Sp ^r	This study
pCF::#1pvu/pst	pCF1010 containing 479-bp <i>PvuII/PstI</i> fragment from pSG2-#1ret, Tc ^r Sm ^r Sp ^r	This study

Construction of *R. sphaeroides* 2.4.1 Genomic Library in pSG2

The DNA library of *R. sphaeroides* 2.4.1 was constructed in pSG2 [1]. The *R. sphaeroides* 2.4.1 genomic DNA was partially digested with *Sau*3AI, and the DNA fragments size-fractionated in a 5–40% (w/v) sucrose gradient by centrifugation at 26,000 rpm at 20°C for 24 h in a Beckman SW41 rotor. The fractions containing DNA fragments of 3–5 kb were pooled and precipitated using cold 95% ethanol. The purified DNA fragments were then ligated into the unique *Bam*HI site of pSG2, and the ligated DNA was introduced into *E. coli* S17-1 by electroporation. The library clones were mobilized into AP3T, a *cer*I-null mutant derivative of *R. sphaeroides* 2.4.1, and the transformants selected at 28°C on a *Sis* solid medium supplemented with Tp at 50 µg/ml, Sm at 25 µg/ml, and Sp at 25 µg/ml.

Screening for 2.4.1-pSG2 Clones Showing Different β-Galactosidase Activity by RAI

Each of the isolated transformants was patched on two kinds of *Sis* solid medium: one containing X-gal at 40 µg/ml, Tp at 50 µg/ml, Sm at 25 µg/ml, Sp at 25 µg/ml, and synthetic RAI at 4 µM dissolved in methanol, and the other containing the same chemicals and same amount of methanol, but without synthetic RAI. The cells were grown at 28°C. After 5 days, the clones showing a different color development between the two media were selected, inoculated into 3 ml of a *Sis* broth containing Tp at 50 µg/ml, Sm at 25 µg/ml, and Sp at 25 µg/ml, and cultured overnight at 28°C. When the OD at 600 nm reached about 0.3, 1-ml culture aliquots were transferred to two tubes, where one tube contained 4 µM synthetic RAI and the other contained diluted methanol as the control. After allowing the cells to grow for 6 h at 28°C with shaking, 100 µl of each culture was transferred to a microtube and the β-galactosidase activity measured using a previously reported method [1].

Determination of DNA Nucleotide Sequence and Analysis

The DNA nucleotide sequence was determined by automated sequencing (ABI PRISM; Perkin Elmer). The PCR amplification was performed as follows: denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, and an extension at 60°C for 4 min, while the amplification was carried out based on 25 cycles. The amplified DNA was ethanol-precipitated twice using anhydrous ethanol with 3 M sodium acetate, and then washed with 70% ethanol. The dried DNA pellet was dissolved in 20 µl of a template suppression reagent (TSR), boiled at 96°C for 2 min, and stored in ice for 2 min before being injected into the automated sequence machine. The sequencing analysis and homology search were performed using the BLAST programs at the National Center for Biotechnology Information (Bethesda, MD, U.S.A.).

Construction of Transcriptional *lacZ* Fusions to Subclones of AP3T-pSG2 Clone #1

A 1.9-kb *Xba*I-*Pvu*II fragment from AP3T-pSG2 clone #1, containing the upstream region and 5' end of RSP6131, was cloned into pUC19W-*Sma*I. From the resulting plasmid, the *Xba*I-*Eco*RI fragment, in which the Ω cassette was located upstream of the *Pvu*II site, was eluted and cloned into pCF1010 [17] digested with *Xba*I and *Eco*RI. The resulting construction was named pCF::#1pvu. Constructions pCF::#1pst and pCF::#1pvu/pst were also constructed using the same strategy, where a 950-bp *Pst*I-*Xba*I fragment and 579-bp *Pvu*II-*Pst*I fragment were transcriptionally fused to the promoterless *lacZ* of pCF1010, respectively.

Construction of *qsrR* Mutation in Strain 2.4.1

A 976-bp *Pvu*II-*Nru*I DNA fragment from pSG2#1-ret was inserted into *Sma*I-digested pUC19. A 227-bp fragment from the resulting construct was then deleted by digesting with *Xma*III and the overhang filled by treatment with a Klenow fragment. The resulting linear DNA was then ligated with a *Sma*I fragment containing the transcription and translation stop site Ω*Sm*^r/*Sp*^r cassette [23] to construct pUC19::Ω*qsrR*. The *Sac*I-*Xba*I fragment from the construction, containing the *qsrR* gene inserted by the Ω cassette, was then cloned into the suicide shuttle vector pLO1 [13], and the resulting construction pLO1::Ω*qsrR* introduced into *R. sphaeroides* 2.4.1 by biparental mating via *E. coli* S17-1 [29] to construct a *qsrR*-insertion mutation by allelic exchange. The *qsrR*-insertion mutant, named 2.4.1-*qsrR*, was confirmed by Southern hybridization (data not shown).

Preparation of Soluble Cell-Free Extract for Two-Dimensional Polyacrylamide Gel Electrophoresis

Cells were harvested by centrifugation for 15 min at 8,000 rpm (4°C), resuspended in a PBS buffer, and broken by two passages through a French press. The unbroken cells were then removed by centrifugation at 8,000 rpm (4°C) for 15 min. A modified Lowry method was used to determine the protein concentrations, with bovine serum albumin as the standard.

2D-PAGE and MALDI-TOF Analysis

The two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed using an immobiline/polyacrylamide system and 18-cm IPG strips (pH range, 4 to 7) (Amersham Pharmacia Biotech, Sweden). The isoelectric focusing (IEF) was conducted at 20°C for 58,000 Vh (maximum voltage of 8,000 V) on an IPGphor (Amersham Pharmacia Biotech, Sweden). After the IEF, each strip was equilibrated in equilibration buffer I (6 M urea, 0.5% DTT, 30% glycerol, 50 mM Tris-Cl, pH 8.8) and buffer II (6 M urea, 4.5% iodoacetamide, 30% glycerol, 50 mM Tris-Cl, pH 8.8) for 15 min each. For the second dimension, vertical slab SDS-PAGE (12.5%) was used (Bio-Rad Protean II Xi; Bio-Rad Laboratories, U.S.A.). The gels were stained using Colloidal Coomassie Blue G-250, and each gel was repeated at least three times and analyzed using an Image-Master 2 D Elite (Amersham Pharmacia Biotech, Sweden). The relative volume of each spot was determined from the spot

Table 2. List of genes whose expression was modulated by synthetic RAI, as determined by measuring the β-galactosidase activity of pSG2-induced *lacZ* fusions.

Clone number	Gene designation ^b	Predicted product
1 ^a	RSP6131	Hypothetical protein
57	RSP1611	Sensor histidine kinase
74	RSP0339	Hypothetical protein
193	RSP3684	<i>solR</i> (transcriptional regulator)
198	RSP1576	<i>trxB</i> (thioredoxin reductase)
209	RSP1536	Acyl dehydratase
779	RSP1533	Putative alginate <i>o</i> -acetyltransferase

^aSixteen additional clones were isolated with the reporter gene fused at the same spot.

^bProteins predicted from genome of *R. sphaeroides* 2.4.1 in the GenBank database.

intensities in pixel units and normalized to the sum of the intensities for all the spots in the gel. Proteins displaying at least 2-fold volume variations in comparison were considered as comparative proteins. The protein spots of interest were excised from the gels. After destaining, the gel pieces were digested with trypsin (Roche, Germany) for 12 h at 37°C, and then the extracts were dried and resolubilized in 2 µl of 0.5% TFA. The PMF measurements were performed on a Bruker Reflex III MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) working in the reflectron mode with an accelerating voltage of 20 kV and reflecting voltage of 23 kV, as described previously [12]. A saturated solution of CHCA in 50% acetonitrile and 0.1% TFA was used for the matrix. A total of 2 µl of the matrix solution and sample solution were mixed in a 1:1 (v/v) ratio and applied to a Score 384 target well. The mass accuracy of the PMF analysis was 0.1–0.2 Da with external calibration, and the internal calibration was carried out using enzyme autolysis peaks with a resolution of 12,000. To process the MALDI results, peaks were assigned using XTOF software (Bruker), and PeakClean (<http://www.proteomics.com.cn/tools/PkClean/>) was used to remove contaminant peaks, including matrix peaks, solvent peaks, and enzyme autolysis peaks. Database searches were performed using the Mascot v1.7.02 software (Matrix Science Ltd.) licensed in-house (<http://www.proteomics.com.cn>). No protein was found with more than one name and one accession number. The research results were also checked against the in-house database of NCBI and using Mascot with free access on the Internet (<http://www.matrixscience.com>). Mono-isotopic peptide masses were used to search the databases, allowing a peptide mass accuracy of 100 ppm (300 ppm in some cases) and one partial cleavage. The oxidations of methionine and carbamidomethyl modification of cysteine were also considered. Scores greater than 48 that included more than five matching peptides and a sequence coverage greater than 15% were considered significant ($p < 0.05$).

RESULTS

Identification of Genes in *R. sphaeroides* 2.4.1 Regulated by Exogenous Synthetic RAI

To identify the genes under the control of RAI-mediated quorum-sensing regulation, a genomic library was constructed using a promoter-trapping vector, pSG2, which contained a promoterless *lacZ* as the reporter [1]. The genomic library of *R. sphaeroides* 2.4.1 in pSG2 was then introduced into a *R. sphaeroides* *cerI*-null mutant strain, AP3T. About 20,000 exoconjugants were obtained that included pSG2-derived random *lacZ* fusions in the genome generated by a single crossover. The fusion lines were tested for a modulated expression of the reporter gene upon the addition of exogenous synthetic 7,8-*cis*-*N*-tetradecenoyl-homoserine lactone (RAI) by measuring the β-galactosidase activity in solid media containing X-gal. In this screening, 23 clones showed a different blue color when RAI was supplemented in the media, where 21 clones revealed an RAI-dependent induction of β-galactosidase activities, whereas the remaining 2 clones exhibited an RAI-dependent reduction of β-galactosidase activities. Thus, for these 23

clones, the β-galactosidase activities were quantitatively measured in the presence and absence of exogenous RAI (Fig. 2). As a result, clone #1 showed about a ten-fold increase in β-galactosidase activity in the presence of RAI, clone #209 showed a four-fold increase, and clones #57, #198, and #779 showed about a two-fold increase, whereas, clones #74 and #193 exhibited a decrease in activity.

The genes inserted by the *lacZ*-fusion in the seven abovementioned clones were retrieved for further study. Thus, the total genomic DNA was isolated from the clones and digested with *S*alI, making a single cut in pSG2 (Fig. 1). The digested DNA fragments were then self-ligated and transformed into *E. coli* S17-1 and the ampicillin-resistant clones selected. As each of the clones harbored a pSG2 derivative as an episome, including an insert derived from the genome linked just next to the 5' end region of the *pl-lacZ* [1], the DNA nucleotide sequence of the insert was determined by employing a primer complementary to the region. For clone #1, the highest activated clone, the *lacZ* reporter was inserted in a predicted acyltransferase; for clone #57, the *lacZ* reporter was inserted in a hypothetical protein that did not have significant homology with any

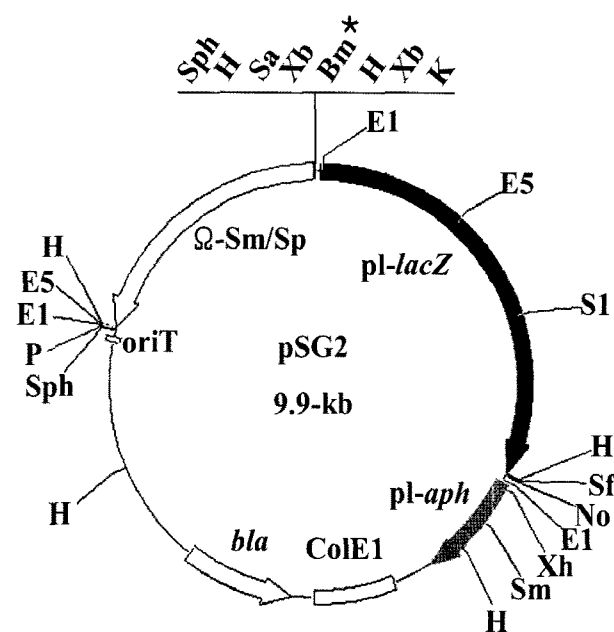


Fig. 1. Structure of promoter-trapping suicide vector pSG2.

The vector pSG2 contains a promoterless *lacZ* (*pl-lacZ*) and promoterless *aph* (*pl-aph*) as the reporters, which can be randomly fused in AP3T genomic DNA by homologous recombination. The vector is a ColE1 derivative, and hence cannot replicate in *R. sphaeroides*. The genomic DNA fragments are inserted in the BamHI enzyme site (*), allowing a primer complementary to the 5' end region of *pl-lacZ* to be employed for sequencing the inserted fragments. Abbreviations: *oriT*, origin of conjugal transfer; *bla*, β-lactamase; Ω-Sm/Sp, Sm-/Sp-resistant gene in Ω cartridge that contains translation/transcription stop sequences; A, ApaI; Bm, BamHI; Bg, BglII; E1, EcoRI; E5, EcoRV; H, HindIII; K, KpnI; No, NotI; P, PstI; S1, SacI; Sa, SalI; Sf, SfiI; Sm, SmaI; Sph, SphI; Xb, XbaI; Xh, XhoI.

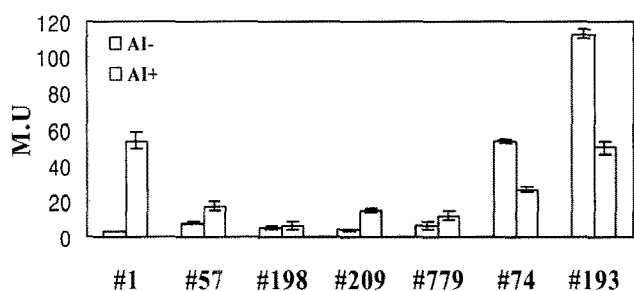


Fig. 2. Quantitative measurement of β -galactosidase activities of selected clones in the presence and absence of RAI.

The cells were grown in 3 ml of a Sis broth medium overnight. When the A_{600} value reached 0.2–0.3, the culture was divided into two portions, where one was added to synthetic RAI dissolved in methanol at a final concentration of 4 mM, and the other was added to diluted methanol as the negative control. After 6 h incubation, the β -galactosidase activity was quantitatively measured. This experiment was performed twice independently.

genes in the databases; for clone #198, the *lacZ* was inserted in a thioredoxin reductase gene; for clone #209, the *lacZ* reporter was inserted upstream of a gene encoding a putative protein that had homology with an (*R*)-specific enoyl-CoA hydratase that catalyzes the hydration of trans-2-enoyl-CoA to (*R*)-3-hydroxyacyl-CoA as part of the polyhydroxy alkanolate biosynthetic pathway; and for clone #779, the *lacZ* reporter was inserted in an alginate *o*-acetylase gene. For clones #74 and #193 that were negatively regulated by RAI, the *lacZ* reporter was fused to a putative methyltransferase gene and acyl homoserine lactone-dependent transcriptional regulator, respectively.

The β -galactosidase activities of these clones were also quantitatively measured in the presence and absence of RAI (Fig. 2). Consistent with the qualitative assessment based on the blue-color development in media containing X-gal, clones #1, #57, and #209 showed a significant induction of the reporter expression with the addition of RAI, whereas clones #74 and #193 showed decreased activities with RAI. However, clones #198 and #779 did not show a significant change in β -galactosidase activity in the presence of RAI. Further study then focused on the genes in clone #1 that showed the highest level of transcriptional activation in the presence of exogenous synthetic RAI.

Expression of *luxR* Homolog, *qsR*, Induced by RAI

A DNA sequence analysis of clone #1 showed that the acyltransferase (GenBank Accession No. YP_353847 RSP6131), inserted by the *lacZ* fusion in clone #1, was preceded by another open reading frame (GenBank Accession No. YP_353848 RSP0768). This putative gene included a DNA-binding H-T-H domain and was annotated as a *luxR* family gene. This gene exhibited homology with the transcriptional regulator *luxR* family in *Roseovarius* sp. 217 (identity/similarity=32%/45%). The *luxR* homolog

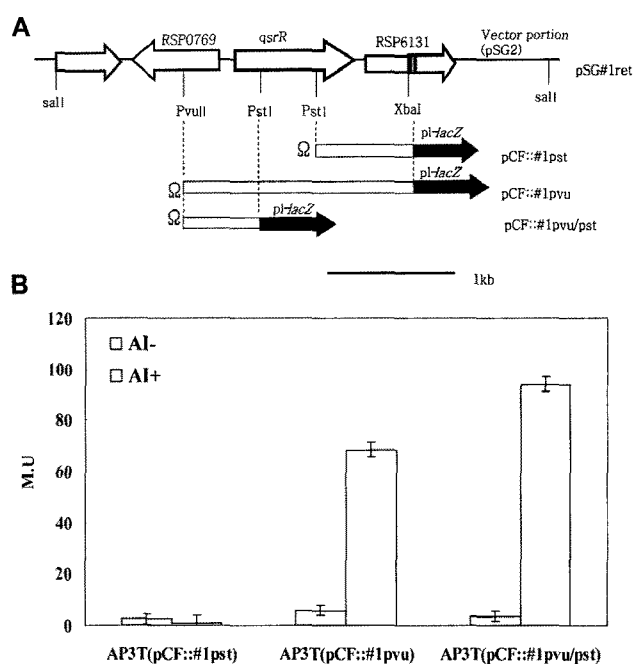


Fig. 3. Expression of *luxR* homolog is induced by RAI.

A. Organization of genes inserted by the reporter gene in pSG2 in clone #1, and genetic map of three subclones, AP3T(pCF::#1pst), AP3T(pCF::#1pvu), and AP3T(pCF::#1pvu/pst). B. β -Galactosidase activities from three subclones in the presence and absence of RAI.

was oriented in the same direction as RSP6131, suggesting that the expression of the *lacZ* fusion in clone #1 was initiated from a promoter upstream of the *luxR* homolog. To verify this hypothesis, reporter fusions were constructed, as described in Fig. 3. In pCF::#1pst, a fragment spanning the C-terminal end part of the *luxR* homolog to the middle part of RSP6131 was transcriptionally fused to the *lacZ*, in pCF::#1pvu, a fragment spanning the region upstream of *qsR* to the N-terminal half region of the RSP6131 region was fused to the *lacZ*, and in pCF::#1pvu/pst, a fragment spanning the region upstream of *qsR* to the N-terminal end part of the *luxR* homolog was fused to the report. These constructs were then introduced into AP3T, a *cerI*-null mutant derivative of strain 2.4.1, and the β -galactosidase activities quantitatively measured in the presence and absence of exogenous RAI. As shown in Fig. 3B, no significant level of β -galactosidase activity was detected from any of the three clones in the absence of RAI. However, with the supplement of RAI, the β -galactosidase activities from AP3T(pCF::#1pvu) and AP3T(pCF::#1pvu/pst) were significantly enhanced, yet no detectable level of β -galactosidase activity was detected from AP3T(pCF::#1pst), indicating that the expression of β -galactosidase activity in clone #1 was derived from a promoter located in the region upstream of the homolog and induced by RAI. Therefore, these results suggest that the *luxR* homolog upstream of RSP6131 was modulated by RAI and likely involved in the RAI-mediated regulation of

the genes in *R. sphaeroides* 2.4.1. Consequently, the *luxR* homolog was named *qsrR* (quorum-sensing regulation regulator). The expression of *qsrR* was also detected in a *cerR*-null mutant (unpublished data). Thus, the present data showed that *qsrR* is part of a quorum-sensing regulatory circuit.

qsrR is Involved in Regulation of Numerous Genes

Assuming that *qsrR* is a regulator involved in RAI-mediated gene regulation, the genes modulated by *qsrR* were investigated. Thus, an insertion mutation of *qsrR* was constructed in *R. sphaeroides* 2.4.1 by allelic exchange, as described in Materials and Methods. To screen the proteins regulated by *qsrR*, 2-DE was performed and a comparison made of the protein spot patterns for the wild type and the *qsrR* mutant (Fig. 4). According to the 2-DE analysis, 12 spots were differentially expressed between the wild type and the isogenic *qsrR* mutant, where 7 spots showed a reduced density and 5 spots showed an increased density for the *qsrR* mutant. The protein spots showing differential expression between the two strains were then digested with trypsin and subjected to mass spectrometric analyses (Fig. 4 and Table 3).

For the spots that were down-regulated in the *qsrR* mutant strain, spot #1 (19.7 kDa, pI=5.2) showed homology to the molybdopterin-guanine dinucleotide biosynthesis protein A of *R. sphaeroides* (Accession No. P95645) (Table 3), spot #2 (16.7 kDa, pI=5.7) showed homology to the CheW of *R. sphaeroides* (Accession No. Q60251), spot #3 (31.4 kDa, pI=7.0) showed homology to the reaction center protein L chain of *R. sphaeroides* (Accession No. P02954), spot #8 (34.6 kDa, pI=6.2) showed homology to the CbbX protein of *R. sphaeroides* (Accession No.

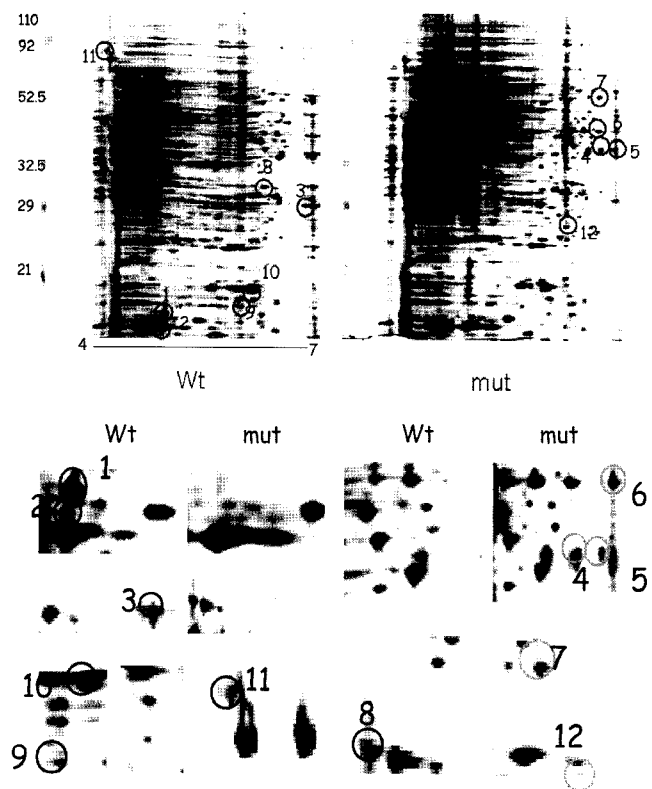


Fig. 4. 2D gel electrophoresis of total soluble proteins from wild-type and *qsrR*-null mutant cells. Circled spots represent proteins differentially expressed between wild-type and *qsrR*-mutant cells. The numbers of spots correspond to those in Table 3.

P95648), spot #9 (16.9 kDa, pI=5.9) showed homology to the diheme cytochrome *c* napB precursor (Accession No. Q53177), and spots #10 (20.4 kDa, pI=6.9) and #11

Table 3. Protein spots expressed differentially between wild-type cells and *qsrR*-null mutant cells as determined by MALDI-TOF analysis.

Protein spot number ^a	Relative expression ^b	MW/pI ^c	GenBank Accession No.	Matched proteins
1	-	19,782/5.2	P95645	Molybdopterin-guanine dinucleotide biosynthesis protein A
2	-	16,672/5.7	Q60251	Chemotaxis protein CheW
3	-	31,457/7.0	P02954	Reaction center protein L chain
4	+	36,362/6.3	P54933	ATP-binding transport protein SmoK
5	+	39,635/6.5	O33558	Chemotaxis response regulator protein-glutamate methyltransferase CheB
6	+	41,228/6.2	Q01179	Cysteine desulfurase NifS
7	+	51,034/5.7	Q53068	Sensor histidine kinase RegB (PrrB protein)
8	-	34,685/6.2	P95648	CbbX protein
9	-	16,908/5.9	Q53177	Diheme cytochrome <i>c</i> NapB precursor
10	-	20,484/6.9	Q53228	Response regulator PrrA
11	+	89,208/5.1	Q57366	DMSO reductase (DMSOR)
12	-	25,532/6.0	Q53178	Cytochrome <i>c</i> type protein NapC

^aThese numbers correspond to the numbers of protein spots shown in Fig. 4.

^b'+' and '-' denote up- and down-expressions, respectively, in *qsrR*-null mutant cells compared with wild-type cells.

^cMolecular weight/Iso-electric point.

(89.2 kDa, pI=5.1) exhibited homology with the response regulator PrrA (Accession No. Q53228) and DMSO reductase (Accession No. Q57366), respectively.

For the spots that were upregulated in the *qsrR* mutant strain, spot #4 (36.3 kDa, pI=6.3) corresponded to the ATP-binding transport protein SmoK (Accession No. P54933), spot #5 (39.6 kDa, pI=6.5) was the chemotaxis response regulator protein (Accession No. O33553) glutamate-methylesterase, spot #6 was a 41.2-kDa protein identified as NifS (Accession No. Q01179), spot #7 (51 kDa, pI=5.7) matched the sequence of a 51-kDa sensor histidine kinase RegB (PrrB protein) (Accession No. Q53068), and spot #12 (25.5 kDa, pI=6.0) was identified as a cytochrome *c* type NapC (Accession No. Q53178).

DISCUSSION

R. sphaeroides possesses a quorum-sensing system that is mediated by the *luxR/luxI* homologs, *cerR/cerI*, and the structure of the quorum-sensing signal molecule is 7,8-*cis*-N-(tetradecenoyl)-homoserine-lactone [24]. However, the genes modulated by the signal molecule remain to be elucidated. Most microorganisms that employ quorum-sensing modulation have interactions with other multicellular organisms, at least as a part of their life cycles. In contrast, the photosynthetic bacterium *R. sphaeroides* has no obvious interaction with other organisms during its life cycle. Hence, the biological meaning of quorum-sensing in this apparent free-living organism remains a mystery. Thus, a study on the nature of the target genes regulated by the quorum-sensing regulation in this bacterium may provide a clue to the biological role of the regulation. Therefore, the present study attempted to identify the genes modulated by the quorum-sensing regulatory system using a promoter-trapping suicide vector.

Among ten clones that exhibited different expression levels of the *lacZ* reporter from pSG2 fusions upon the addition of synthetic RAI, clone #1 showed the highest increase in β -gal activity (Fig. 2). A genetic study revealed that the expression of the gene in clone #1 was apparently driven by a promoter of another gene present upstream of the gene inserted by the reporter. This upstream gene showed a high homology with the *luxR* homologs of *R. sphaeroides* ATCC17029 (99% similarity), *R. sphaeroides* ATCC17025 (70% similarity), and *Oceanicola batsensis* HTCC2597 (42% similarity), leading to the speculation that the *luxR* homolog, named *qsrR*, was associated with quorum-sensing regulation, and prompting a search for the genes modulated by *qsrR*.

In 2D-PAGE and a MALDI-TOF analysis of the protein expression profiles for the wild type and 2.4.1-*qsrR*⁻, a derivative of 2.4.1 with an insertion in *qsrR*, sets of protein spots were expressed on significantly different levels between

the two strains. Among 12 such spots, spots #2 and #5 were matched as CheW and CheB proteins, respectively, which are key components of chemotaxis signal transduction [27], suggesting that *qsrR* is related with the chemotaxis of the bacterium. In addition, spot #1 showed a homology with MobA (Molybdopterin-guanine dinucleotide biosynthesis protein A), spot #4 with SmoK, and spot #6 with NifS, where these proteins are involved in biosynthetic and metabolic functions [16, 19, 32, 36]. MobA links a guanosine 5'-phosphate to MPT forming molybdopterin-guanine dinucleotide, which is an essential enzyme for DMSOR (DMSO reductase) activity. MobA (spot #1) and DMSOR (spot #11) were both down-expressed in the *qsrR* mutant. In addition, MobA is an important protein for the NapA (nitrate reductase catalytic subunit) protein, which contains a molybdopterin-guanine dinucleotide cofactor [8]. Spots #9 and #12 corresponding to NapB and NapC proteins, respectively, which are related to bacterial periplasmic nitrate reductases (Nap), and spot #11 showing homology to DMSOR, would all seem to be involved in redox transfer functions. In *R. sphaeroides* DSM158, Nap activity is stimulated by nitrate and is present under both oxia and anoxia states, although the activity is higher under aerobic conditions [8].

It was also noticeable that spots #7 and #10 matched PrrB and PrrA proteins, respectively, which comprise a two-component regulatory system. In *R. sphaeroides* and *R. capsulatus*, this system is involved in controlling the expression of the photosynthesis genes *puf*, *puhA*, *puc*, and *cycA*, which encode the photosynthetic reaction center, two light-harvesting antenna subunits, and cytochrome *c*2, respectively [21]. Therefore, this would seem to suggest that the biosynthesis of the photosynthetic apparatus is regulated by a quorum-sensing circuit in *R. sphaeroides*. Supporting this idea, spot #3 also showed homology to the reaction center (RC) protein L chain, which is associated with the photosynthetic system [25, 34].

The RegB (PrrB homolog) and RegA (PrrA homolog) regulon includes genes involved in nonphotosynthesis genes, such as carbon fixation, nitrogen fixation, hydrogen utilization, several terminal oxidases, DMSO reductase, and nitrate respiration [2, 5]. Therefore, this system constitutes a signal transduction system that not only is responsible for the regulation of photosynthesis, but also functions as a global regulator of many metabolic pathways [2]. PrrA functions as a response regulator, whereas PrrB functions as a sensor histidine kinase/phosphatase [21]. The sensor PrrB is believed to detect changes in oxygen tensions by responding to a change in the flow of reductants or redox carriers [21]. Thus, when taken together, one common theme of the genes that are controlled by PrrB and PrrA is that they are all related with the redox state of the cell. Therefore, in this photosynthetic bacterium, the results would seem to suggest that quorum sensing plays a role in

orchestrating the physiology of the cells according to their nutritional state and possibly their intracellular redox level. *R. sphaeroides* is a free-living bacterium and can grow either aerobically or anaerobically. Thus, to adapt to these diverse environmental conditions, the bacterium must continuously monitor the light quality and intensity, as well as the oxygen tension. Any reduction in the oxygen levels is detected by a sensor protein, such as PrrB, and the signal then induces a signal transduction cascade *via* PrrA. As a result, the genes that are sensitive to the redox state of the cell and regulated by the PrrA and PrrB system are repressed or activated by the signal. The cell density is also an important component of an environmental condition, as increasing the cell density in limited environmental conditions induces a reduction in the oxygen levels. Thus, the QsrR-mediated quorum-sensing system may be a cell-density monitoring system, based on allowing the cells to monitor their environmental conditions, such as the oxygen levels. Puskas and colleagues [24] previously suggested that quorum sensing in a free-living photosynthetic bacterium may help cells capture more light by preventing aggregation. However, the present study discovered that the oxygen supply may be a more important key factor for *R. sphaeroides* to employ quorum-sensing regulation, but evaluation of this model awaits future physiology studies.

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