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Identification and characterization of genes regulated by AqsR, a LuxR-type regulator in *Acinetobacter oleivorans* DR1

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Abstract The complete genome of *Acinetobacter oleivorans* DR1 contains AqsR and AqsI genes, which are LuxR and LuxI homolog, respectively. In a previous study, we demonstrated that quorum sensing (QS) signals play an important role in biofilm formation and hexadecane biodegradation. However, the regulation of genes controlled by the QS system in DR1 remains unexplored. We constructed an *aqsR* mutant and performed RNA sequencing analysis to understand the QS system. A total of 353 genes were differentially expressed during the stationary phase of wild-type cells compared to that of the *aqsR* mutant. AqsR appears to be an exceptionally important regulator because knockout of *aqsR* affected global gene expression. Genes involved in posttranslational modification, chaperones, cell wall structure, secondary metabolites biosynthesis, and stress defense were highly upregulated only in the wild type. Among upregulated genes, both the AOLE_03905 (putative surface adhesion protein) and the AOLE_11355 (L-asparaginase) genes have putative LuxR binding sites at their promoter regions. Soluble AqsR proteins were successfully purified in *Escherichia coli* harboring both *aqsR* and *aqsI*. Comparison of QS signals in an AqsI–AqsR co-overexpression strain with *N*-acyl homoserine lactone standards showed that the cognate *N*-acyl homoserine lactone binding to AqsR might be 3OH C12HSL. Our electrophoretic mobility shift assays with purified AqsR revealed direct binding of AqsR to those promoter regions. Our data showed that AqsR functions as an important regulator and is associated with several phenotypes, such as hexadecane utilization, biofilm formation, and sensitivity to cumene hydroperoxide.

Keywords Bacteria · Quorum sensing · Transcriptional regulation · Soil · *N*-acyl homoserine lactone · Oxidative stress

Introduction

A cell-to-cell communication mechanism known as quorum sensing (QS) plays an important role in many bacteria, in which the QS system is mediated by an autoinducer produced and released by the bacterial cell (Fuqua et al. 1994). The detection of a threshold concentration of an autoinducer alters gene expression. The QS system reportedly controls various physiological traits, such as motility (Atkinson et al. 2006; Rader et al. 2007; Weiss et al. 2008), biofilm formation (Stanley and Lazazzera 2004; Waters et al. 2008), virulence (Hentzer et al. 2003; Zhu et al. 2002), and antibiotic production (Duerkop et al. 2009).

The genus *Acinetobacter* comprises aerobic, gram-negative, nonfermentative bacteria that are isolated from many environments (Bhargava et al. 2010; Jung et al. 2010). Currently, 14 complete genomes of *Acinetobacter* species are available, whereas only three environmental isolates have been sequenced. Most genomic and transcriptomic research on *Acinetobacter* species has focused chiefly on *Acinetobacter baumannii* strains because of their clinical importance as pathogenic bacteria. Thus, many studies have been carried out to examine the relationship between QS and virulence factors in *Acinetobacter* species. QS systems in soil-borne *Acinetobacter* species, which have been only rarely explored, are therefore a valuable line of study. *A. baumannii* forms biofilms on both abiotic and biotic surface. Pili and surface adhesion proteins are involved in biofilm initiation and maturation after initial attachment to abiotic surfaces (Gaddy and Actis 2009). QS signals may be sensed by the BfmRS two-component regulatory system that activates the expression of the usher–chaperone assembly system, which produces pili (Gaddy and Actis 2009).

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Acyl homoserine lactones (AHLs) of *A. baumannii* strain M2 have been identified as *N*-(3-hydroxydodecanoyl)-$_L$-HSL (HSL, homoserine lactone) (Niu et al. 2008). The lengths of the acyl-chain and substitution at position C3 in AHLs produced by various organisms differ: 3-oxo, 3-hydroxy, and 3-unsubstituted (Bhargava et al. 2010). When unclassified genomic *Acinetobacter* species have been analyzed for the production of QS signals, most have displayed QS molecules and produced more than one quorum signal (González et al. 2009).

Diesel-degrading *Acinetobacter oleivorans* DR1 has been isolated from a rice paddy (Jung et al. 2010). DR1 has a QS system consisting of a LuxI-type AHL synthase protein and a LuxR-type regulator, designated AqsI and AqsR, respectively (Kang and Park 2010b). QS signals play an important role in biofilm formation and hexadecane biodegradation, and the loss of QS signal-producing activity had a clear effect on protein expression compared to that in wild-type DR1 (Kang and Park 2010b). Conversely, acquisition of antibiotic resistance reduces QS signal production (Kang and Park 2010a). Commensal interaction between *Pseudomonas* sp. AS1 and *A. oleivorans* DR1 demonstrates that AHLs in DR1 decrease in the AS1 and DR1 co-culture system owing to quorum quenching activity in the AS1 strain (Seo et al. 2011). These phenomena alter biofilm formation physiologically. Our previous studies have demonstrated that the QS system of DR1 has strong effects on various aspects of the biological physiology of *A. oleivorans* DR1. Although QS has recently become widely studied, the regulation of genes controlled by the QS system in *Acinetobacter* species has received little attention. The goal of this study was to identify the target genes regulated by AqsR. Thus, we focused on transcriptional profiling using RNA sequencing (RNA-seq) analysis during which the QS system was switched on and direct binding of AqsR to the target promoters was examined using a DNA–protein binding affinity assay.

Materials and methods

Bacterial strains, culture conditions, and DNA manipulation

The bacterial strains and plasmids used in this study are shown in Table S1. *A. oleivorans* DR1 was grown at 30 °C in nutrient broth or minimal salts basal media (MSB) with aeration via shaking. MSB media were supplemented with 2 % hexadecane (*v*/*v*; Sigma). *Escherichia coli* was grown at 37 °C in Luria–Bertani medium. When required, antibiotics were added at the following concentrations: 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 50 μg/ml gentamicin. Synthesized HSLs—*N*-dodecanoyl-DL-HSL (C12HSL, Sigma), *N*-3-Oxo-dodecanoyl HSL (3oxo C12HSL), and *N*-(3-hydroxydodecanoyl)-DL-HSL (3OH C12HSL)—were added to the growth medium. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) of the cultures using a BioPhotometer (Eppendorf). The complete genome sequence of strain DR1 can be found in GenBank (accession no. CP002080). Strain DR1 was deposited in Korea Collection for Type Cultures (KCTC 23045) and Japan Collection of Microorganisms (JCM 16667).

Cloning procedures and AqsR mutant construction

The primers used in this study are listed in Table S2. A 941-bp fragment of the internal region of *aqsR* that had been amplified using *aqsR* OE-F and *aqsR* OE-R primers was cloned into a pGEM-T Easy vector (Promega, USA) via TA cloning. pGEM-*aqsR* was digested with *Bam*HI, and the kanamycin (*km*) cassette (1,264 bp) from pUC4K (Oka et al. 1981) was inserted. The *aqsR*::*km* cartridge was cloned into the *Eco*RI and *Sal*I cloning site of pCVD442 (Philippe et al. 2004), creating pCVD442-*aqsR*::*km*. The constructed plasmid was then introduced via electroporation into *E. coli* S17-1 λpir. pCVD442-*aqsR*::*km* was transformed into *A. oleivorans* DR1. The *aqsR* mutant stain was confirmed via both PCR verification using *aqsR* OE-F/R primer pairs and sequencing (Macrogen) of the PCR product.

RNA extraction, library construction, and sequencing

The cells were grown to the stationary phase (OD_{600}>2.0). Total RNA was isolated from 5 ml stationary cells using an RNeasy Mini kit (QIAGEN, USA) according to manufacturer's instructions. All procedures for RNA sequencing and alignment of the transcriptome were conducted by Chunlab (Seoul, South Korea). The RNA was subjected to a subtractive hybridization-based rRNA removal process using a MICROBExpress Bacterial mRNA Enrichment Kit (Ambion). This process, including library construction, was carried out as described previously (Yi et al. 2011). RNA sequencing was performed with two runs of an Illumina Genome Analyzer IIx to generate single-ended 36-bp reads. The genome sequence and annotation information of *A. oleivorans* DR1 was obtained from the National Center for Biotechnology Information (NCBI) database (accession number NC_014259.1). Quality-filtered reads were aligned to the reference genome sequence using CLC Genomics Workbench 4.0 (CLC Bio). Mapping was based on a minimal length of 32 bp with an allowance of up to two mismatches. The relative transcript abundance was measured in reads per kilobase of transcript per million mapped sequence reads (RPKM) (Mortazavi et al. 2008). The genes that showed a fold change (RPKM of DR1-sta/RPKM of *ΔaqsR*-sta [sta, stationary phase]) larger than 2.0 and lower than 0.5 were regarded as up- and downregulated genes, respectively. Fold change values less than 0.5 were replaced

by the negative of its inverse (e.g., 0.5-fold change value was represented by −2.0). Mapped reads were visualized using the BamView (Camarena et al. 2010) and Artemis (Rutherford et al. 2000) programs. The RNA-seq data were deposited in the NCBI Gene Expression Omnibus site under accession number GSE44347.

AqsR purification

To demonstrate the direct regulation of candidate genes by AqsR, we overproduced and purified AqsR. The *aqsR* region was amplified via PCR using the *aqsR* OE-F and *aqsR* OE-R primers (yielding a 941-bp fragment). The fragment was cloned into the *Eco*RI and *Sal*I cloning sites of the pET-28a(+) vector. The constructed vector was conjugated to *E. coli* Top10 and introduced via transformation into *E. coli* BL21 for overexpression. The expression of AqsR in *E. coli* BL21 (DE3) resulted in the formation of inclusion bodies. To enhance the production of soluble AqsR, we co-expressed AHL synthase, which is encoded by *aqsI*, in *E. coli* harboring *aqsR* (Fig. S3a). The *aqsI* fragment was cloned into the *Hin*dIII and *Bam*HI cloning sites of the pBBR1MCS4 vector. pBBR1MCS4-*aqsI* was transformed into *E. coli* BL21(pET-28a[+]-*aqsR*). The recombinant strains were confirmed with PCR using the primer pairs (*aqsR* OE-F/R and *aqsI*-F/R; Fig. S3b). Both the 914- and 656-bp PCR products were observed in the AqsI–AqsR co-overexpression strain. For AqsR purification, *E. coli* BL21(DE3) cells harboring both pET-28a(+)-*aqsR* and pBBR1MCS4-*aqsI* were grown to the exponential phase (OD_{600} ~0.4) at 37 °C with aeration. The cell cultures were grown at 20 °C for 12 h after induction with 0.25 mM IPTG and then harvested. All purification steps were performed at 4 °C using an FPLC system (AKTA FPLC, Unicorn 4.0, Amersham Bioscience). *E. coli* cell pellets were resuspended in buffer A (50 mM Tris–Cl and 1 mM DTT, pH 7.5) and disrupted by sonication. After removal of cell debris via centrifugation at 12,000×*g* for 30 min, the soluble fraction was loaded onto an anion exchange column (1 ml, DEAE-cellulose, Amersham Bioscience) equilibrated with buffer A, and the proteins were eluted with a 20-ml linear gradient of 0–1 M NaCl in 50 mM Tris–Cl (pH 7.5). The fractions (0.5 ml each) were collected and concentrated via ultrafiltration using a Centricon (2 ml, YM-10, Amicon). The concentrates were applied to a nickel-nitriloacetic acid (Ni-NTA) column (1 ml, His-trap, Amersham Bioscience) equilibrated with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 40 mM imidazole, pH 7.4), and the proteins were eluted with 20 ml elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 250 mM imidazole, pH 7.4). The fractions were applied to the Ni-NTA column again, and the eluted fractions were dialyzed via ultrafiltration with the Centricon (Amicon) and stored at −80 °C in 10 % glycerol. SDS-PAGE was carried out using 10 % polyacrylamide gels to check the level of expression and purification.

Detection of quorum sensing signal

AHLs in the DR1 and recombinant *E. coli* strain were detected using the *Agrobacterium tumefaciens* C58 (pZLR4) indicator strain (Cha et al. 1998). The supernatant of the cell culture from the stationary phase was collected via centrifugation (1,000×*g*, 15 min) at 4 °C, and the pellets were removed. Cell-free supernatant (CFS) was prepared via filter sterilization with 0.22-μm pore filters, and 500 ml of the obtained supernatants were extracted with 100 ml ethyl acetate. Subsequently, the extracts were evaporated and resuspended with 0.5 ml ethyl acetate. Concentrated extracts of each sample were loaded on TLC silica gel 60 RP-18 (5×7.5 cm, Merck, Germany) and developed with a mobile phase (methanol:distilled water = 8:2, *v*/*v*). The loaded plate was dried at room temperature, and the TLC plate was overlaid with AB medium (Cangelosi et al. 1991) containing the indicator strain, *A. tumefaciens* C58 (pZLR4). The plates were incubated at room temperature, and the spots indicating AHLs on the TLC plate turned blue. The extracted CFSs containing AHLs were added individually to media in which the indicator strain was inoculated. The cultures were incubated for 24 h until they achieved the stationary phase. The presence of AHLs in the strains was quantified via measurement of β-galactosidase activity using ONPG as a substrate.

Gene expression analysis by quantitative reverse transcriptase PCR

Total RNA was isolated from 5 ml of stationary phase (OD_{600}>2.0) cells, using the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. cDNA was synthesized from 1 μg of RNA with primers of target genes (Table S1) and used as templates for quantitative reverse transcriptase PCR (qRT-PCR). The PCR mixture contained 12.5 μl of the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 1 μl of each primer (0.5 μM), and 2 μl of cDNA, in a total volume of 25 μl. The PCR conditions were 95 °C for 3 min, followed by 40 cycles of 45 s at 95 °C, 45 s of 60 °C, and 45 s of 72 °C. To normalize the expression of each gene, the expression level of 16S rDNA was quantified with primers used previously (Watanabe et al. 2001). The quantification results were calculated from triplicate experiments.

Identification of the consensus AqsR binding sequence

To identify AqsR target genes in *Acinetobacter* DR1, we determined the consensus AqsR binding sequence using the

LuxR homolog binding sequences of other bacteria. The sequences were constructed using well-known LuxR binding sequences: the LuxR binding site of *Vibrio fischeri* (Antunes et al. 2008; Devine et al. 1989), the CepR binding sites of *Burkholderia cepacia* (Lewenza et al. 1999), the SolR binding sites of *Ralstonia solanacearum* (Flavier et al. 1997), the RhlR binding sites of *Pseudomonas aeruginosa* (Latifi et al. 1995), and the TraR binding sites of *A. tumefaciens* (White and Winans 2007). The AqsR binding consensus sequence was constructed as 5′-TRTNRRANYTRNYADKW-3′ (where R indicates G or A; Y indicates C or T; D indicates A, G, or T; K indicates G or T; W indicates A or T; and N indicates any nucleotide).

Electrophoretic mobility shift assay (EMSA)

An EMSA was conducted as described previously (Kim et al. 2008). The DNA probe was generated using the primer pairs listed in Table S2. The PCR product was dephosphorylated and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The reaction mixture (20 ml final volume) containing the probe, crude extract, and loading buffer in 5× binding buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 50 % glycerol *v*/*v*, 10 mM DTT, and 375 mM KCl) was incubated for 20 min at room temperature. The resulting complexes were analyzed with electrophoresis on 5 % polyacrylamide gels in 0.5× Tris-borate/EDTA buffer (1.1 M Tris, 900 mM borate, 25 mM EDTA, pH 8.3).

Biofilm formation

Biofilm formation of DR1 strains was analyzed as described elsewhere (Jackson et al. 2002). Bacterial cells were inoculated into nutrient broth and incubated for 24 h at 30 °C. The cultures were then diluted 100-fold in nutrient broth and grown in 96-well polystyrene microtiter plates (Costar, USA) for 48 h at 30 °C under static conditions. Biofilm formation was measured by staining attached cells with crystal violet. After staining, attached cells were resuspended with ethanol, and absorbance was measured at 595 nm. A microplate spectrophotometer (PowerWave XS; Bio-Tek, USA) was used to measure optical density.

Microbial adherence to *n*-hexadecane (MATH) test

Cell hydrophobicity was estimated using the MATH test (Rosenberg et al. 1982). Two milliliters of bacterial suspension in PBS (pH 7.5) at an OD_{600} of 0.5 was overlaid with 10 % *n*-hexadecane (*v*/*v*; Sigma). After vortexing for 1 min, the phases were permitted to separate for 20 min. Absorbance at 600 nm of the aqueous phase was then measured. The results were determined using this equation: $\{1-(A_0-A)\}\times 100$, where A_0 and A are, respectively, the initial and the final OD of the aqueous phase.

Oxidative stress sensitivity assay

The wild-type and mutant strains were grown overnight in nutrient broth and subsequently diluted 100-fold. The cells had reached exponential phase (OD_{600} ~0.5) and serially diluted cells were spotted on nutrient agar with or without cumene hydroperoxide (CHP).

Results

Construction and characterization of the *aqsR* mutant

To compare the wild-type and QS system-deficient strain accurately, we constructed an *aqsR* mutant. Disruption of the *aqsR* gene was achieved using a double-crossover recombination method with pCVD442 (Fig. S1a). PCR with an *aqsR* OE-F/R primer pair was used to amplify the *aqsR* region. Detailed procedures for the mutant construction are described in the "Materials and methods" section. To ensure that homologous recombination occurred in the recipient strain, we conducted PCR verification. When the *aqsR* OE-F/R primer pair was used, a 941-bp PCR product was observed only in wild-type cells, and a 2,205-bp PCR product, which was shifted up via insertion of the *km* cassette, was found in the *aqsR* mutant (Fig. S1b). The *aqsR* mutant was confirmed via the sequencing of the 2,205-bp PCR product (Fig. S1c).

DR1 is capable of degrading *n*-hexadecane (Jung et al. 2010; Kang and Park 2010b). Thus, we tested the *aqsR* mutants for their growth on and attachment to hexadecane. Wild-type and *aqsR* mutant cells were incubated with MSB media containing 2 % *n*-hexadecane. The *aqsR* mutant had reduced hexadecane degradation capability and had a longer lag phase (~12 h) compared with those of the wild type (~5 h; Fig. 1a). The growth rates (per hour) of DR1 and the *aqsR* mutant were 1.42±0.11 and 0.55±0.04, respectively. We evaluated the effects of bacterial cell surface hydrophobicity via the MATH test. MATH values for DR1 and the *aqsR* mutant were 41.55±0.49 and 85.10±2.40, respectively, which demonstrated that the hydrophobicity of *aqsR* mutant was diminished compared with that of the wild type. In our previous study, we showed that the *aqsI* mutant, which was disrupted using DR1R (a rifampin-resistant variant) as a parental strain, has poor hydrophobicity owing to a decrease of hydrophobicity in the DR1R strain (Kang and Park 2010a). Therefore, the reduction of hydrophobicity in both mutant stains is correlated with QS. The *aqsR* mutant displayed significantly reduced biofilm formation compared to that of wild type (Fig. 1b). The *aqsI* mutant displayed

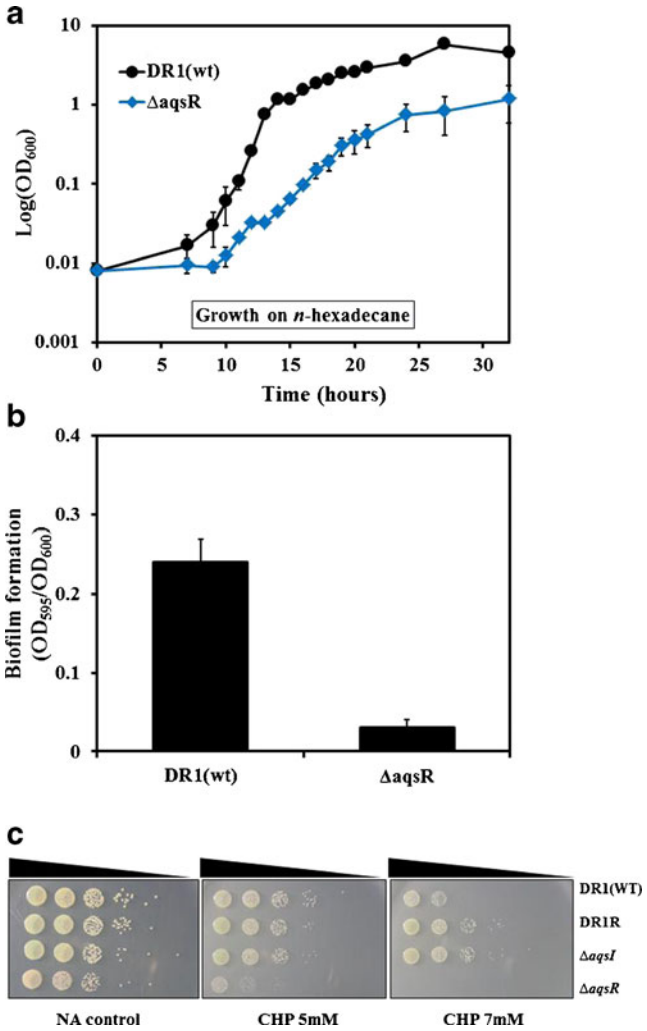


Fig. 1 Characteristics of the *aqsR* mutant. **a** Growth on *n*-hexadecane in the DR1 and the *aqsR* mutant. Cells were grown at 30 °C in minimal salts basal medium. The growth of each strain was monitored by measuring the optical density (OD) of the cultures at 600 nm. **b** Biofilm formation assay. **c** Sensitivity test of DR1 derivatives to cumene hydroperoxide (CHP). *DR1R* rifampin-resistant variant, *WT* wild type

decreased biofilm formation compared to that of DR1 wild type, and it increased after the addition of CFS or synthesized AHLs (Kang and Park 2010b). Therefore, bacterial biofilm formation might depend on QS signals and, by extension, the QS system in DR1. Exponentially grown cells were serially diluted and spotted on agar with and without CHP (Fig. 1c). The *aqsR* mutant proved more sensitive compared with the wild-type and *aqsI* mutants.

Transcriptional profiling between the wild-type and the *aqsR* mutants

To identify genes regulated by AqsR in *A. oleivorans* DR1, we conducted RNA sequencing. In our previous study, the AHL from DR1 increased via β-galactosidase activity as the culture reached a high cell density (OD_{600}>2.0) (Kang and Park 2010a). Considering this tendency, we isolated total RNA from stationary phase cells (OD_{600}>2.0) of both the wild-type and *aqsR* mutants. The number of reads for two samples—which were designated DR1-sta for stationary phase wild-type cells and *ΔaqsR*-sta for stationary phase cells of the *aqsR* mutant—were 20,796,801 and 20,476,650, respectively. To confirm the expression of genes, which were expressed in a cell-density-dependent manner in DR1, we compared transcript profiling of the stationary phase cells to those of the DR1 exponential phase cells. RNA-seq data presented as RPKM values were organized according to a cutoff of 50 RPKM value. A total of 1,156 genes had an RPKM value of 50 or higher, and the expression of these genes was compared. Our analysis demonstrated that 353 genes were expressed differentially by more than 2-fold (111 genes upregulated and 242 genes downregulated) in the DR1-sta cells compared with expression in the *ΔaqsR*-sta cells (Fig. 2 and Tables S3).

To compare differentially expressed transcripts between the wild-type and AqsR-deficient strains of DR1, we classified genes using the clusters of orthologous groups (COGs) categories of proteins. At the transcriptome level of COGs, genes involved in posttranslational modification, protein turnover, chaperones (category O), cell wall structure and biogenesis and outer membrane maintenance (category M), secondary metabolite biosynthesis transport and catabolism (category Q), and defense mechanisms (category V) were highly upregulated in the stationary phase of

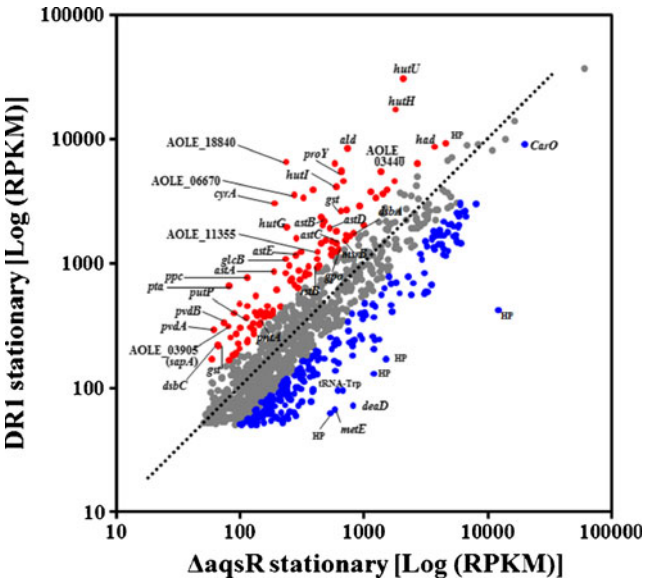
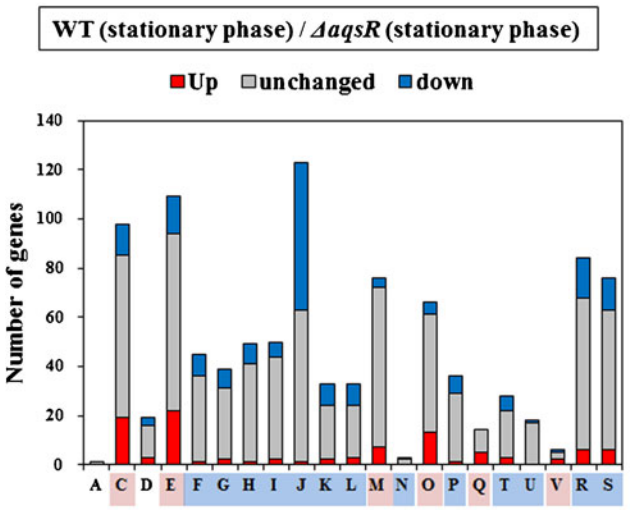
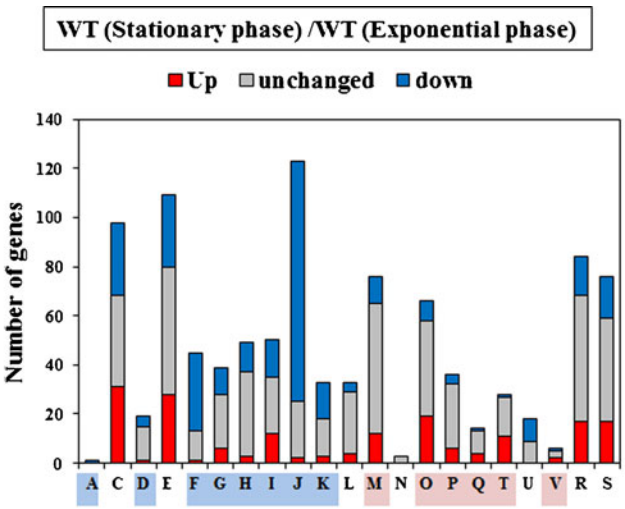


Fig. 2 Comparison of gene expression patterns between wild-type and *aqsR* mutant cells. The *y*-axis shows reads per kilobase of transcript per million mapped sequence reads (RPKM) values of wild-type cells, and the *x*-axis represents RPKMs of *aqsR* mutant cells during the stationary phase according to the genome locus tag. *Red dots* represent upregulated genes, and *blue dots* represent downregulated genes in wild-type cells. *HP* hypothetical proteins

wild-type cells (Fig. 3 and Table S4). Interestingly, expression of peroxiredoxin hydrogen peroxide resistance protein 1 (*gpo*, encoded by AOLE_11335) and peptide methionine sulfoxide reductase (*msrB*, encoded by AOLE_11340) belonging to category O was increased more than 2-fold by the presence of AqsR (see Figs. 2 and 3 and Table S4). These results were consistent with those of the CHP sensitivity test, which showed increased sensitivity in the absence of AqsR (see Fig. 1c). Many oxidative stress-related genes, such as glutathione S-transferase (*gst*, encoded by AOLE_13420 and AOLE_16495), thiol–disulfide isomerase and thioredoxin (*dsbA*, encoded by AOLE_19220), and thiol/disulfide interchange protein dsbC precursor (*dsbC*, AOLE_03095), involved in thiol–disulfide exchange reactions were highly expressed in the stationary phase of wild-type cells compared with that of the *aqsR* mutant (see Fig. 2 and Table S3). Thus, we speculated that AqsR directly or indirectly participates in oxidative stress defense mechanisms.

Two pyoverdine biosynthesis proteins (encoded by AOLE_01050 and AOLE_01055) belonging to the COGs category M were 2.07-fold and 2.11-fold upregulated in the stationary phase of wild-type cells (see Fig. 2 and Table S4). Pyoverdine is a fluorescent siderophore produced by *Pseudomonas* species, and 14 pyoverdine genes are involved in its synthesis (Lamont and Martin 2003). The amino acid sequence similarity of the two pyoverdine biosynthesis proteins was 40.7 and 32.2 % compared to pyoverdine biosynthesis protein (Pvc) A (encoded by PA2254) and PvcB (encoded by PA2255), respectively, in *P. aeruginosa* PAO1. However, no other related genes, such as PvcC and PvcD, appeared in the *A. oleivorans* DR1 genome. Our observation also showed yellow pigment





UP regulated COGs	
M	Cell wall structure and biogenesis and outer membrane
O	Posttranslational modification, protein turnover, chaperones
Q	Secondary metabolites biosynthesis, transport and catabolism
V	Defense mechanisms

DOWN regulated COGs	
F	Nucleotide metabolism and transport
G	Carbohydrate metabolism and transport
H	Coenzyme metabolism
I	Lipid metabolism
J	Translation, including ribosome structure and biogenesis
K	Transcription

Fig. 3 A summary of up- and downregulated genes sorted according to general clusters of orthologous groups (COGs). To confirm the expression of genes expressed in AqsR-dependent and cell-density-dependent manners in DR1, we compared transcript profiling of stationary phase wild-type cells with those of stationary phase *ΔaqsR* cells (*left bar graph*) and those of exponential phase wild-type cells (*right bar graph*). In both graphs, *red* and *blue shadows* at the *x*-axis under one-letter abbreviations represents upregulated (*red*) and downregulated (*blue*) categories. Tables located under the graphs list up- and downregulated COGs common under both conditions. A, RNA processing and modification; C, energy production and conversion; D, cell division and chromosome partitioning; E, amino acid metabolism and transport; L, replication, recombination and repair; N, secretion, motility, and chemotaxis; P, inorganic ion transport and metabolism; R, general functional prediction only; S, no functional prediction; T, signal transduction; U, Intracellular trafficking, secretion, and vesicular transport

production in long-term-cultured wild-type and *aqsI* mutant cells, whereas the *aqsR* mutant produced no pigment (Fig. S2). Thus, we considered that *A. oleivorans* DR1 can produce iron-chelating pigments such as pyoverdine and that this phenotype may be controlled by AqsR. This phenomenon is worthy of further investigation because pyoverdine-like pigment production has not been reported in any *Acinetobacte*r species. Our COGs analysis also suggested that the QS regulator of *A. oleivorans* DR1 might control accessory and secondary metabolite genes (COGs category Q), which might be required for adaptation in harsh environments. Many genes involved in metabolisms for nucleotides (category F), carbohydrates (COGs category G), coenzymes (category H), and lipids (category I) were downregulated in the wild-type strain (see Fig. 3 and Table S4). Membrane protein (eight genes) and transporter (nine genes) were more than 2-fold downregulated in the wild type compared to *aqsR* mutant (Table S3). Genes classified as translation (category J) and transcription (category K) appeared to show lower expression in the stationary phase of wild-type cells compared with that of the *aqsR* mutant cells. Among differentially expressed genes, there were 18 and 34 hypothetical proteins that were up- and downregulated in the wild type. It is worth investigating the function of these unknown proteins to understand the complete QS system in DR1.

The QS system plays important roles in hexadecane degradation in *A. oleivorans* DR1 (see Fig. 1a) (Kang and Park 2010b). We have previously demonstrated that proteins involved in gluconeogenesis, fatty acid metabolism, and oxidative stress defense appear to be highly expressed during hexadecane degradation (Jung et al. 2011). Most genes that encoded differentially expressed proteins in the presence of hexadecane displayed no significant differences in mRNA expression in our RNA-seq analysis owing to differences in the carbon sources in the culture media (Table S5). Nevertheless, four genes involved in fatty acid metabolism, the glyoxylate pathway, and gluconeogenesis were upregulated in stationary phase wild-type cells compared with that of the *aqsR* mutant (see Fig. 2): NAD(P) transhydrogenase subunit alpha (*pntA*, AOLE_16625, 2.30-fold), malate synthase G (*glcB*, AOLE_10740, 4.60-fold), putative alcohol dehydrogenase (AOLE_06670, 12.88-fold), and multifunctional fatty acid oxidation complex (*fadB*, AOLE_17905, 2.07-fold). Thus, these genes might have critical functions in fundamental metabolism pathways regardless of carbon sources, and AqsR might control these basic essentials.

Construction of the AqsR expression vector and overproduction of AqsR

To demonstrate the direct regulation of candidate genes by AqsR, we overproduced and purified AqsR. To enhance the production of soluble AqsR, we co-expressed AHL synthase, which is encoded by *aqsI*, in *E. coli* harboring *aqsR* (Fig. S3a). To confirm the production of AHLs in the *E. coli* strains, we used the indicator strain, *A. tumefaciens* C58 (pZLR4) to detect the AHLs. The indicator strain was activated by AHLs from cells of DR1 wild type and two types of *E. coli* harboring *aqsI*. To quantify AHL production among the strains further, we cultured the indicator in modified M9 medium supplemented with CFS from each strain. The β-galactosidase activities of *aqsI*-harboring *E. coli* were similar to those of DR1. Conversely, *E. coli*, which has a two-component QS system, showed a low level of β-galactosidase activity (Fig. 4a). Detection of the AHLs from each strain showed that the level of AHL signals produced by *E. coli* harboring *aqsR* and *aqsI* was lower than that of DR1 wild type and *E. coli* harboring only *aqsI*. The DR1 strain generated three putative AHLs with R_f values of 0.72, 0.53, and 0.38 (Kang and Park 2010b). TLC assays of *E. coli* BL21 (*aqsR*, *aqsI*) without IPTG treatment yielded results similar to previous results in the DR1 strain, shown in lane 1 (Fig. 4b). However, the lowest spot disappeared when IPTG was added to *E. coli* harboring both *aqsR* and *aqsI* for AqsR protein induction (Fig. 4b). We assumed that the AHL corresponding to this spot is cognate AHL of AqsR. To characterize this signal, we analyzed synthesized HSLs on TLC plates developed under the same conditions. Comparison of the lowest spot with AHL standards (C12HSL, 3oxo C12HSL, 3OH C12HSL) showed that the cognate AHL binding to AqsR might be 3OH C12HSL. The 3oxo-HSL derivatives displayed a comet tailing pattern (Shaw et al. 1997). The cognate signal produced no tailing spot, suggesting that it was not 3oxo C12HSL. We speculated that 3OH C12HSL binds to AqsR inside cells, lowering the secretion of AHLs. Autoinducers may be required for AqsR protein folding or activation instead of outward dispersion. All of the purification steps were performed using an FPLC system. Detailed AqsR purification procedures are described above in the "Materials and methods" section. To obtain high purity protein, we tried to pass the proteins through the Ni-NTA column twice. More soluble AqsR proteins were successfully overproduced in *E. coli* with both *aqsR* and *aqsI* (Fig. S4).

Binding of AqsR to the promoter region of target genes

To identify AqsR target genes in *Acinetobacter* DR1, we searched the consensus sequence of AqsR binding sites (5′-TRTNRRANYTRNYADKW-3′, where R indicates G or A; Y indicates C or T; D indicates A, G, or T; K indicates G or T; W indicates A or T; and N indicates any nucleotide) in the complete genome of DR1 (Jung et al. 2010). Among the differentially expressed genes from the RNA-seq analysis, two genes with consensus sequences on their promoter regions were selected and subjected to qRT-PCR and EMSA:

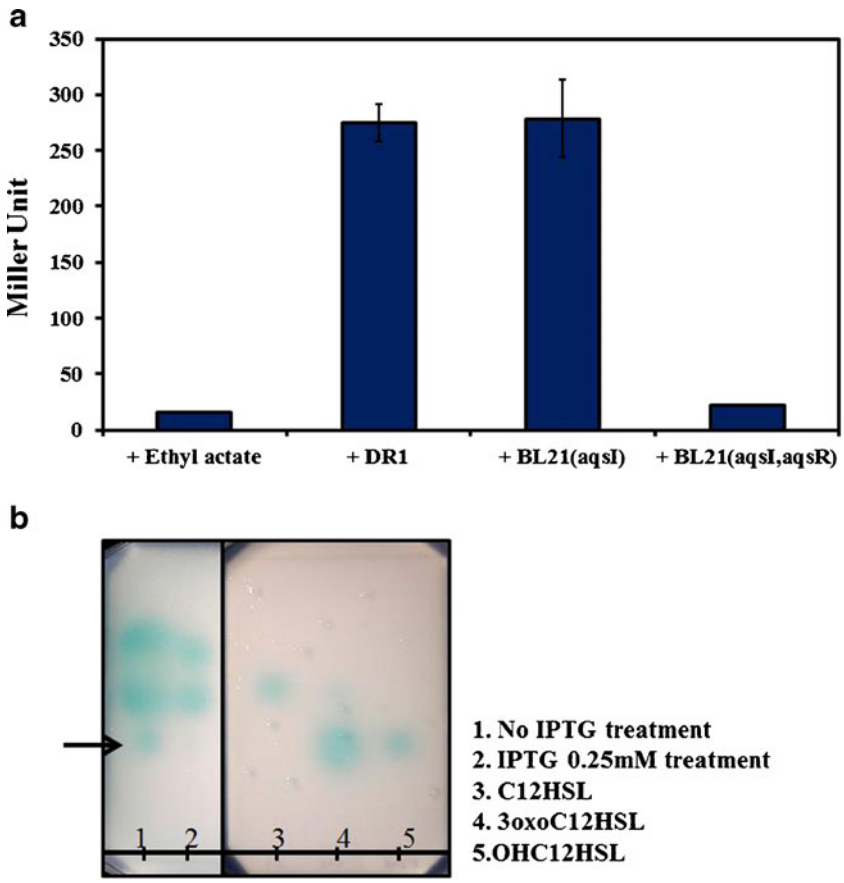


Fig. 4 Visualization and quantification of autoinducer synthase in *Escherichia coli* BL21: **a** β-galactosidase assay. Experiments confirmed acyl homoserine lactone (AHL) production by *E. coli* strains. *Agrobacterium tumefaciens* C58(pZLR4) was used to indicate the AHLs. Ethyl acetate was used as a negative control because it was used for AHL extraction. **b** Differences in AHL production occurred after IPTG induction in *E. coli* BL21 (*aqsR*, *aqsI*). *Arrow* indicates cognate AHL. Part **b** shows the TLC results of 5 nmol C12HSL, 0.1 nmol 3oxo C12HSL, and 2 nmol 3OH C12HSL. *HSL* homoserine lactone

sapA and AOLE_11355 (L-asparaginase/Glu-tRNAGln amidotransferase subunit D). Our qRT-PCR data also confirmed that these two genes appeared to be regulated by the AqsR (Fig. 5a). To investigate whether AqsR binds to the promoter regions of the target genes, we conducted EMSA using P_{sapA} and P_{AOLE_11355} DNA probes with purified AqsR. The EMSA results demonstrated that AqsR retarded the migration of the probes (Fig. 5b) and indicated that the AqsR binding site might lie in two promoter-specific regions of the target genes. To ensure that the binding of AqsR to the DNA probe was specific, we added nonspecific competitors poly(di-dc) to all binding reactions, and the addition of excess unlabeled probe completely abolished AqsR binding to the labeled fragments. The expressions of *sapA* and AOLE_11355 were truly regulated by the QS system according to our experiments.

A group of surface adhesion proteins share several structural and functional features. Most of these proteins have high molecular weights, core domains of tandem repeats, and the capacity to form biofilm (Lasa and Penadés 2006). SapA showed low amino acid identity with well-known surface-associated proteins: 21.1 % with LapF and 14.6 % with LapA in *Pseudomonas putida* KT2440 (Martínez-Gil et al. 2010), 16.0 % with BapA in *A. baumannii* (Loehfelm et al. 2008), 15.8 % with BapA in *Salmonella enterica* (Latasa et al. 2005), and 16.7–19.9 % with BapA in *Staphylococcus* species (Tormo et al. 2005). However, SapA encoded by AOLE_03905 is 3,367 amino acids long and shows fundamental structure similarity with other surface-associated proteins. We speculated that SapA presents three domains, as previously determined in LapF of *P. putida* KT2440 (Martínez-Gil et al. 2010). Domain 1, comprising the first 180 amino acids, is followed by a repetitive region (domain 2) covering more than 80 % of the protein. Domain 3 corresponds to the C-terminal portion of the protein and contains a calcium-binding region designated as a GGXGXD motif (GGNGHD).

L-Asparaginase/Glu-$tRNA^{Gln}$ amidotransferase subunit D is involved in many metabolic pathways, such as various amino acids, nitrogen metabolism, and biosynthesis of secondary metabolites. L-Asparaginase is known to catalyze the hydrolysis of L-asparagine/glutamine to L-aspartic/L-glutamic acid and ammonia (Kelo et al. 2002). The majority of bacteria cannot attach glutamine directly to $tRNA^{Gln}$ owing to a lack of functional glutaminyl-tRNA synthetase (Feng et al. 2005). Thus, alternative enzymes exist. Glutamate is joined with $tRNA^{Gln}$ by a nondiscriminating glutamyl-tRNA synthetase and converted to glutamine by a Glu-$tRNA^{Gln}$ amidotransferase (Feng et al. 2004). The function of this protein in *Acinetobacter* species has not yet been studied. Studies on the exact function

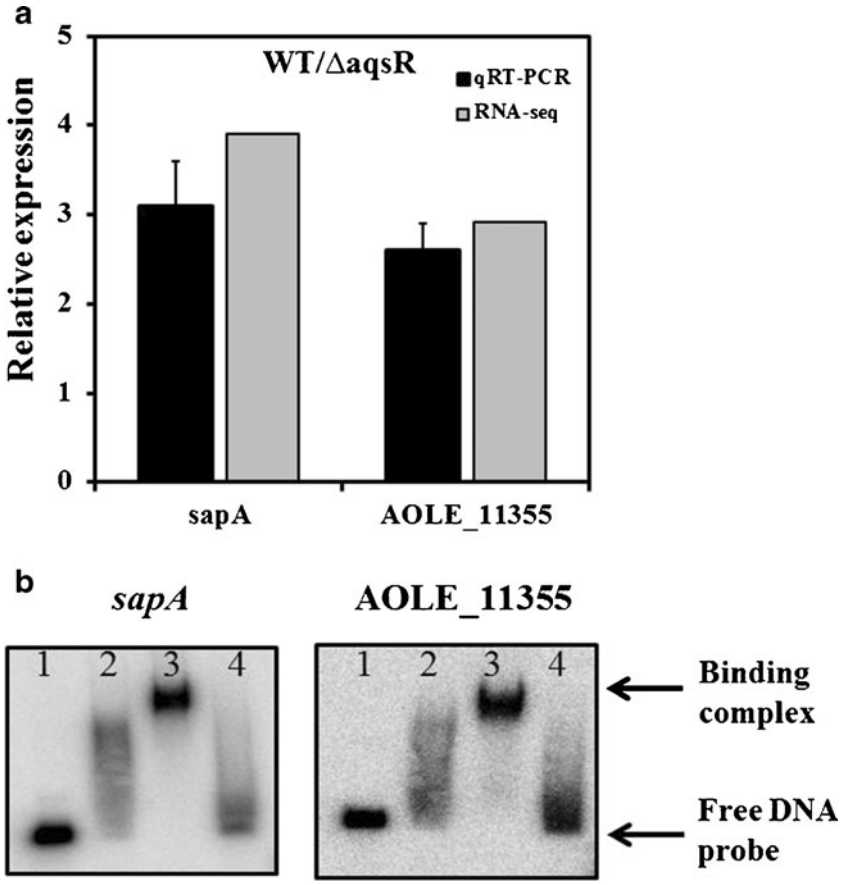


Fig. 5 Expression analysis and binding of AqsR to the promoter region of target genes: **a** relative expression of *sapA* and AOLE_11355 in wild type and *aqsR* mutant by quantitative reverse transcriptase PCR. Expression levels from qRT-PCR (*black bar*) are consistent with RNA-seq data (*gray bar*). Total RNA was isolated from stationary cells (OD_{600}>2.0). **b** Binding of AqsR to the promoter region of target genes: surface adhesion protein (*sapA*), AOLE_11355: *lane 1*, free DNA (no protein); *lane 2*, 5 μg purified AqsR; *lane 3*, 10 μg purified AqsR; *lane 4*, 10 μg purified AqsR with nonprobing DNA. The nonspecific competitors poly(di-dc) were added to all binding reactions

of these AqsR-targeted genes in *A. oleivorans* DR1 and why AqsR regulates them will continue.

Discussion

Performing an in vitro LuxR activity assay presents several challenges. One challenge is that LuxR forms insoluble inclusion bodies when overexpressed in *E. coli* (Kaplan and Greenberg 1987). *A. tumefaciens*, which is a plant pathogen, has a TraR/TraI QS system. TraR is an autoinducer-dependent transcriptional regulator, and TraI is a synthetase for the autoinducer *N*-3-oxo-octanoyl-$_L$-HSL (3-oxo-C8-HSL) (Fuqua and Winans 1996). Overexpressed TraR in *E. coli* is proteolyzed or forms inclusion bodies in the absence of 3-oxo-C8-HSL. However, if 3-oxo-C8-HSL is added before the induction of overexpression, TraR exits the soluble state (Zhu and Winans 2001). 3-Oxo-C8-HSL added after induction is ineffective. Like TraR in *A. tumefaciens*, the *E. coli* QS protein SdiA was produced in a soluble form in the presence of C8-HSL (Yao et al. 2006). Therefore, during the purification of some LuxR-type regulators, cognate AHLs are necessary for protein stability and solubility (Swem et al. 2009; Urbanowski et al. 2004). As described above, we conducted AqsR purification in the presence of exogenous C12HSL, but AqsR was still insoluble. Therefore, we concluded that AHL synthase, which is encoded by *aqsI*, is co-expressed in the AqsR overexpression strain to enhance the production of soluble protein. More soluble AqsR proteins were successfully overproduced when *E. coli* had *aqsR* and *aqsI*. GroESL, the chaperone that assists many proteins in attaining their native tertiary structure, might also have a role in TraR folding (Chai and Winans 2009).

A. oleivorans DR1 had a lower biofilm forming capability in the absence of AqsI (Kang and Park 2010b) and AqsR (see Fig. 1b). A surface adhesion protein (SapA) encoded by AOLE_03905 (hereafter *sapA*) was 3.91-fold upregulated in the presence of AqsR and 3.70-fold increased at the stationary phase compared with levels in exponentially growing cells (see Fig. 2 and Table S3). Interestingly, SapA is the second largest protein (3,367 amino acids) in *A. oleivorans* DR1. Most bacterial biofilms have similarities, including the contribution of extracellular polysaccharides, signaling mechanisms induced by small molecules, and large surface adhesive proteins (Loehfelm et al. 2008). Large surface protein was first identified in *Staphylococcus aureus* (Lasa 2006) and called biofilm-associated protein (Bap). Some Baps have been shown to affect biofilm formation and colonization in diverse organisms, such as *S. aureus* (Cucarella et al. 2001), *Salmonella enteritidis* (Latasa et al. 2005), *A. baumannii* (Loehfelm et al. 2008), and *P. putida*

(Martínez-Gil et al. 2010). Thus, we speculated that SapA of DR1 might play important roles in the development of biofilm and be regulated in both cell-density-dependent and AqsR-dependent manners.

In this study, we constructed the *aqsR* mutant and conducted phenotype experiments to determine its properties compared with those of the *aqsI* mutant and the wild type. Similar to the *aqsI* mutant (Kang and Park 2010b), the *aqsR* mutant reduced hexadecane utilization (see Fig. 1a) and biofilm formation (see Fig. 1b) by disrupting QS regulation. Therefore, both traits are clearly related to the QS system. Our results demonstrated different tendencies between *aqsI* and *aqsR* mutants in terms of oxidative stress response (see Fig. 1c), suggesting that some LuxR-type regulators operate without their cognate AHL signals or regulate the expression of specific target genes via AHL dependence. EsaR, a LuxR homolog of *Pantoea stewartii* subsp. *stewartii*, functions in the absence of AHL by binding to DNA and inhibiting the transcription of genes related to exopolysaccharide production (Minogue et al. 2002, 2005). ExpR controls the production of pectic enzymes in *Erwinia chrysanthemi* as a regulator protein (Reverchon et al. 1998). ExpR represses its target gene expression in the absence of AHL, and the addition of AHL promotes concentration-dependent de-repression (Castang et al. 2006). Our data also suggested that AHL-independent AqsR regulation could be present in DR1 cells. In our transcriptomic analysis, AOLE_17580 (general secretion pathway protein G, COG category N) and AOLE_17460 (multidrug ABC transporter, COG category V) appeared to be regulated by AHL-independent AqsR. Stationary phase cells have similar level of those gene expression with exponential phase cells. It is of interest to note that many ribosomal genes (43 genes) were more downregulated in the wild type, which suggested that AqsR might be important for physiological adaptation at the stationary phase. We speculated that these differentially expressed genes were directly or indirectly regulated by AqsR in *A. oleivorans* DR1.

In our previous study, proteomics analysis revealed that several proteins including guanosine 3′,5′-polyphosphate pyrophosphokinase synthase, BaeS- and GacS-like histidine kinase sensors, S-adenosyl-L-methionine methyltransferase, and glucose-sensitive porin might be controlled by the QS system (Kang and Park 2010b). We compared the results of proteomics and RNA-seq analyses (Table S6). Sensory histidine kinase in a two-component regulatory system (*rstB*, encoded by AOLE_15940) was upregulated during the stationary phase and showed AqsR-dependent expression. Sensor protein GacS (encoded by AOLE_16585) was expressed at higher levels in non-AHL strains at the protein expression level and showed the greatest RPKM values in the *aqsR* mutant strain, which also displayed lower AHL production compared with that in the wild type. Many two-component systems are related to multidrug resistance and QS signaling systems (George Cisar et al. 2009; Gotoh et al. 2010), thus indicating that histidine kinase may also be regulated by the QS system of *A. oleivorans* DR1. Guanosine 3′,5′-polyphosphate pyrophosphokinase (encoded by AOLE_01575), S-adenosyl methionine methyltransferase (encoded by AOLE_01430), and porin B precursor (outer membrane protein D1, encoded by AOLE_02980) showed lower protein expression in the absence of *aqsI* compared with that in the wild type. However, these genes were not differentially expressed at the mRNA expression level. We speculated that these observations might reflect differences in transcriptional and posttranscriptional regulation by the QS system in *A. oleivorans* DR1. Aminotransferase (encoded by AOLE_00535), SnoaL-like polyketide cyclase family protein (encoded by AOLE_18870), and acyl-coenzyme A (CoA) synthetase (encoded by AOLE_11835) were highly expressed in wild-type cells at the protein expression level and showed extremely high RPKM values in stationary phase cells. Ferric siderophore receptor protein (encoded by AOLE_17040) had the opposite tendency. We suggested that these genes were expressed in a cell-density-dependent or AHL-dependent manner.

Under our experimental conditions, the most actively expressed transcript region was that from AOLE_18790 to AOLE_18850 (Fig. S5). In this region, the expression of 12 of the 13 genes was extremely increased in the wild type compared to that in the *aqsR* mutant. Among these genes, methylmalonate-semialdehyde dehydrogenase (encoded by AOLE_18820), 3-hydroxyisobutyrate dehydrogenase (encoded by AOLE_18815), acetyl-CoA synthetase (encoded by AOLE_18810), and enoyl-CoA hydratase (encoded by AOLE_18800) were involved in propanoate metabolism, a carbohydrate metabolism. Five genes from AOLE_18830 to AOLE_18850 were responsible for alanine metabolic processes and transport.

RNA-seq analysis also revealed that several aspects of histidine and arginine/proline metabolism were regulated in an AqsR-dependent manner at the transcriptional level (see Figs. 2 and S6). L-Histidine might be actively converted to L-glutamate by highly expressed histidine ammonia-lyase (*hutH*, AOLE_00360, 9.60-fold), urocanate hydratase (*hutU*, AOLE_00355, 14.82-fold), imidazolonepropionase (*hutI*, AOLE_00370, 6.92-fold), and formimidoylglutamase (*hutG*, AOLE_00375, 8.22-fold). AqsR-targeted AOLE_11355 encoding L-asparaginase is involved in arginine and proline metabolism and catalyzes the hydrolysis of glutamine to L-glutamic acid and ammonia. Glutamate synthesis might be regulated by AqsR through indirect induction of glutamate synthase but through circuitous induction of genes involved in the transformation of the other amino acids. Glutamate is produced by AqsR-dependent expression in arginine succinyltransferase (*astA*, AOLE_01805, 4.51-fold), succinylarginine dihydrolase (*astB*, AOLE_01815, 4.57-fold),

bifunctional succinylornithine transaminase/acetylornithine transaminase (*astC*, AOLE_01800, 2.42-fold), succinylglutamic semialdehyde dehydrogenase (*astD*, AOLE_01810, 3.63-fold), and succinylglutamate desuccinylase (*astE*, AOLE_01820, 4.12-fold). L-Proline may also be converted into glutamate by bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase (encoded by AOLE_11005), which showed a 21.01-fold increase in the presence of AqsR. Because proline and arginine, acting as compatible solutes, were actively involved in the metabolic process, the expression of genes encoding relevant transport systems was increased through AqsR dependence; proline-specific permease ProY (*proY*, encoded by AOLE_00365) and sodium/proline symporter (*putP*, encoded by AOLE_10995) were 8.24-fold and 3.27-fold increased, respectively (see Fig. 2 and Table S3). Thus, we suggest that intracellular mechanisms such as amino acid utilization and carbohydrate metabolism are also regulated in QS regulator-dependent and cell-density-dependent manners, as are visible phenotypes such as surface attachment, biofilm formation, and pigment production.

Our results indicated that AqsR plays an important role in hexadecane degradation and biofilm formation, as demonstrated by our preceding study, and regulates other cellular mechanisms such as oxidative stress defense and amino acid/carbohydrate metabolism. Further studies of the function of QS-controlled genes in DR1 are warranted to extend understanding of the QS regulation system in *Acinetobacter* species.

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