

## Characterization of Quorum-Quenching Bacteria Isolated from Biofouled Membrane Used in Reverse Osmosis Process

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### Biofouling이 일어난 역삼투막에서 분리한 퀴럼 저해 세균의 특성

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Acyl homoserine lactone (AHL) lactonase has been proved to be the AHL-degrading enzyme with the highest substrate specificity for AHL molecules and has shown a considerable potential as low-cost and efficient quorum quenching (QQ) technique. However, few studies focused on its inhibitory effect on biofilm formation which is also a quorum sensing (QS)-regulated phenomenon. In this study, QQ activity of six isolates from biofouled reverse osmosis membranes was studied using *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NTL4 as biosensors under various conditions. All of the isolates belonged to the genus *Bacillus* and showed QQ activity regardless of the acyl chain length or substitution of AHL molecule. The isolates were capable of significantly inhibiting biofilm formation (46.7–58.3%) by *Pseudomonas aeruginosa* PAO1 and produced heat-sensitive extracellular QQ substances. The LC-MS analysis of the QQ activity of a selected isolate, ROIS-5, revealed the degradation of *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12 AHL) and the production of corresponding acyl homoserine (3-oxo-C12-HS), which indicated the activity of AHL lactonase. The broad AHL substrate range and high substrate specificity suggested that the isolate would be useful for the control of biofilm-related pathogenesis and biofouling in industrial processes.

**Keywords:** *Pseudomonas aeruginosa*, AHL lactonase, biofilm, quorum quenching, quorum sensing

Quorum sensing (QS) allows bacteria to perceive the density of the surrounding bacterial populations using signal molecules such as *N*-acyl homoserine lactone (AHL) and to coordinately respond to this information by regulating expression of various genes (Smith and Iglewski, 2003a). QS has been known to play significant roles in many biological processes such as virulence factor production, bioluminescence, antibiotic production, sporulation, and biofilm formation (Novick and Geisinger, 2008; Ng and Bassler, 2009; Williams and Cámara, 2009).

Among these, biofilm formation is one of the major contributors to the pathogenesis of many clinically important bacteria, and the loss of efficiency in medical, industrial, and environmental processes such as medically-implanted devices, oil drilling, paper production, food processing, fish farming, and membrane

separation processes (Donlan, 2001; Defoirdt *et al.*, 2004; Adonizio *et al.*, 2008; Ammor *et al.*, 2008; Wevers *et al.*, 2009; Van Houdt and Michiels, 2010). Biofilm is not only ubiquitous and costly but also endows the cells with resistance to many antimicrobial agents, which has made it difficult to suggest prevalent techniques to control biofilm development (Davies *et al.*, 1998; Stewart and Costerton, 2001; Hoffman *et al.*, 2005). Therefore, there has been a high demand for effective strategies to control biofilm formation, and targeting the QS regulatory mechanism has been a highly considerable candidate due to the critical role of QS in biofilm formation (Davies *et al.*, 1998; Smith and Iglewski, 2003b).

Such regulation works through specific disruption mechanisms of QS system such as inhibition of AHL synthesis by *S*-adenosyl methionine analogues (Parsek *et al.*, 1999); blocking the transport of AHLs across the cell membrane (Whitehead *et al.*, 2001); signal turnover by enzymatic degradation of AHLs

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(Dong *et al.*, 2000); sequestration of AHLs (Whitehead *et al.*, 2001); inhibition of QS signal transduction by antagonistic AHL analogues competing for AHL receptors (Smith and Iglewski, 2003b). The clearest QS-control strategy could be quorum quenching (QQ) through enzymatic degradation of QS signal molecules by AHL acylase or AHL lactonase. AHL acylase degrade AHL molecules at the amide linkage in the acyl side chain and produces homoserine lactone and free fatty acid (Chen *et al.*, 2013). AHL lactonase is widely conserved among bacteria (Whitehead *et al.*, 2001; Dong and Zhang, 2005) and degrade AHL molecules by hydrolyzing the lactone bond in the homoserine lactone ring and releasing acyl homoserine. Unlike AHL acylase that shows variable substrate spectra, AHL lactonase was known to be the AHL-degrading enzyme showing the highest substrate specificity for AHL molecules without being influenced by the acyl chain length of the AHL (Wang *et al.*, 2004; Dong *et al.*, 2007; Chen *et al.*, 2013). The first expression of AHL-lactonase coding gene (*AiiA*) from *Bacillus* sp. 240B1 in a plant pathogen, *Erwinia carotovora*, and the successful attenuation of virulence of *E. carotovora* has shown a considerable promise for the efficient and low-cost QQ technique (Dong *et al.*, 2000). Following the discovery of AHL-lactonase *AiiA* in *Bacillus* sp. 240B1, a variety of AHL-lactonases have been identified in many bacteria and their QQ potential has been successfully evaluated (Dong *et al.*, 2002; Lee *et al.*, 2002; Carlier *et al.*, 2003; Park *et al.*, 2003, 2005, 2006; Ulrich, 2004).

Most of these findings, however, highlighted the potential of AHL lactonases to control bacterial infections with regard to the expression of the lactonase coding gene in pathogenic bacteria such as *E. carotovora* (plant), *Pseudomonas fluorescens* (human), and *Bacillus thuringiensis* (insect), and plants such as potato and tobacco (Dong *et al.*, 2001; Reimann *et al.*, 2002; Molina *et al.*, 2003; Ulrich, 2004; Zhang *et al.*, 2007). Moreover, little has been studied about the inhibitory effect of AHL lactonase on biofilm formation. Thus, we screened QQ bacteria among the isolates from biofouled reverse osmosis (RO) membranes and investigated the QQ characteristics (e.g., QQ against various AHLs, localization of QQ substance, heat and protease susceptibility) and biofilm inhibition potential. Among the 6 strains, RO1S-5 was selected for its high efficiency of QQ and biofilm reduction, and its QQ mechanism was further identified using ultra performance liquid chromatography (UPLC)-MS/MS analysis.

## Materials and Methods

### Bacterial strains and media

Among a total of 505 bacteria isolated from fouled RO

membranes (Veolia Water Solutions & Technologies, Korea), six isolates designated as strains RO1S-2, RO1S-5, RO1S-10, RO1S-14, RO2S-1, and RO2S-2, were selected based on their high QQ activity and cultivated in Tryptic Soy Broth (TSB, Difco Laboratories, USA). *Chromobacterium violaceum* CV026 (ATCC 31532, ATCC, USA) was used as the biosensor strain to test QQ activity of the isolates against C4–C8 AHLs and cultivated in Luria-Bertani (LB) medium (McClellan *et al.*, 1997; Steindler and Venturi, 2007). *Agrobacterium tumefaciens* NTL4 (pZLR4, Luo *et al.*, 2003) served as the biosensor for C8–C12 AHLs and cultivated in AB mannitol medium (ATCC medium 1691). When necessary, 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal; Promega, USA) and gentamycin (Sigma-Aldrich, USA) were added to AB mannitol medium at 40 and 15 µg/ml, respectively. *Pseudomonas aeruginosa* PAO1 (ATCC BAA-47) was used as QS signal (AHLs) producer and biofilm former (Pesci *et al.*, 1997). *Bacillus cereus* (ATCC 14579) and *Escherichia coli* DH5α were used as QQ positive and negative control, respectively (Morohoshi *et al.*, 2009). All of these three strains were cultivated in LB medium at 35°C with shaking (150 rpm).

Proteinase K, ONPG (2-nitrophenyl β-D-galactopyranoside), and the AHLs, *N*-butanoyl-homoserine lactone (C4, BHL), *N*-hexanoyl-homoserine lactone (C6, HHL), *N*-octanoyl-homoserine lactone (C8, OHL), *N*-decanoyl-homoserine lactone (C10, DHL), *N*-dodecanoyl-homoserine lactone (C12, dDHL), and *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12, OdDHL) were purchased from Sigma-Aldrich with the highest grade available. All AHL stock solutions were prepared in DMSO (Sigma-Aldrich) and stored at -30°C until use.

### Detection of QS and QQ activities of isolates

QS and QQ activities of the 505 isolates obtained from the biofouled RO membrane were screened by parallel streaking method using overnight grown cells (Ravn *et al.*, 2001). To test QS induction activity, the isolates were streaked on LB agar plate in parallel to AHL biosensors, CV026 or NTL4, and PAO1 and *B. cereus* were used as QS positive and negative control, respectively. For QQ activity test, isolates were streaked on the center of the LB or AB plate and the biosensor strain (CV026 or NTL4) and PAO1 were streaked on the top and the bottom of the isolate streak, respectively. *B. cereus* and *E. coli* were used instead of the isolate as QQ positive and negative control, respectively.

Subsequently, the QQ activity of the pre-screened QQ isolates by the parallel streaking method was confirmed by a disc-diffusion assay (Mani *et al.*, 2012) which was a modified well-diffusion method. For BHL and HHL detection, overnight grown CV026 (200 µl) was added to 10 ml fresh LB agar at

50°C and the agar-culture mixture was immediately poured into a Petri dish. The prepared BHL (6.8 µg) or HHL (0.2 µg) mixed with 20 µl of 24 h cultured isolate in fresh LB medium ( $A_{600} = 2.6 \pm 0.1$ ) was loaded into a sterile paper disc (8 mm in diameter; Adventec, Toyo Roshi Kaisha, Ltd., Japan) which was placed on the solidified medium. The plates were incubated for 24 h, and the diameter of colored circle was measured. For dDHL and OdDHL detection, the procedure was same as in the BHL and HHL detection except using 36 h cultured NTL4 as the bioassay strain and AB mannitol agar supplemented with X-Gal and gentamycin. The QQ activity of each isolate was determined based on the standard curves created from the relationship between AHL amount and the diameter of the colored zone (Ravn *et al.*, 2001).

#### Identification of QQ isolates

Strains having relatively high QQ activity were screened and their phylogenetic analysis was performed. The 16S rRNA genes of the isolates were amplified by PCR with universal primers: 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). After purification (SolGent PCR purification kit, SolGent Co., Ltd., Korea), the PCR products were sequenced and analyzed on an ABI Prism® 3730xl DNA Analyzer (Applied Biosystems, USA). The sequences were compared with those sequences available in the GenBank database using BLAST programs of the National Center for Biotechnology Information (NCBI) to identify their closest phylogenetic relatives. GenBank accession numbers for the 6 isolates are from KJ413086 to KJ413091.

#### Inhibition of biofilm formation by QQ isolates

Quantification of biofilm formation was performed by microtiter plate assay as described previously (O'Toole and Kolter, 1998). Overnight culture of PAO1 diluted to  $A_{600}$  of 0.2 with fresh LB medium was mixed with 24 h culture supernatant of each isolate (5:1, v/v), and then 1 ml aliquots of the mixture was loaded into the 48-wells of polystyrene microtiter plate (Falcon, BD, USA). After incubation for 24 h at 30°C, the medium was carefully removed and 1 ml of 1% crystal violet solution was added. The dye solution was removed after 20 min and the wells were rinsed thoroughly with filtered (0.20 µm pore size) distilled water. To quantify the attached cells, crystal violet was solubilized in 1 ml of 95% ethanol and  $A_{595}$  was measured. PAO1 culture mixed with *E. coli* spent medium served as a negative control for QQ. All samples were tested in triplicates.

#### Assessment of relative QS expression

QQ activity of the six isolates was determined by  $\beta$

-galactosidase assay which was measured according to Tang *et al.* (2013) with some modification. The overnight cultured isolates on Tryptic Soy Agar (Difco) were inoculated in TSB and cultivated at 30°C with shaking at 150 rpm for 12 h. Three aliquots (270 µl) of each culture were transferred to three wells of 48-well microtiter plate (Falcon, BD, USA) and each well received one of the three AHLs (DHL, dDHL, or OdDHL) dissolved in 30 µl of DMSO to make a final concentration of  $10^{-7}$ ,  $10^{-7}$ ,  $10^{-6}$  M, respectively. Cultures received 30 µl of DMSO (no AHL) served as QS-expression negative controls and 270 µl TSB with 30 µl of corresponding AHL (no cells) served as QS positive controls. All samples were prepared in triplicates and the 48-well plate was incubated at 30°C with shaking for 24 h. After incubation, the cultures were centrifuged at  $10,000 \times g$  for 5 min, and then the supernatants were filter sterilized (0.20 µm pore size; Whatman, UK). The prepared supernatants were mixed with 1 ml of the NTL4 culture (1/50 dilution of 36 h culture in AB medium) and incubated at 30°C with shaking for 16 h. A 200 µl aliquot of each of the 16 h cultures was transferred to the wells of a honeycomb plate (Oy Growth Curves Ab Ltd, USA), and then 20 µl of ONPG (8 mg/ml in PBS) was added to each well. The honeycomb plate was incubated in Bioscreen C (Oy Growth Curves Ab Ltd) for 23 h at 25°C with measuring  $A_{420}$  every 30 min.

To test the heat stability of the QQ compounds, the cell-free supernatants prepared as described above were subjected to heat inactivation (autoclaved for 15 min). The supernatants were also treated with proteinase K (final concentration 100 µg/ml; at 37°C for 24 h) and all the treated supernatants were used in the ONPG test for AHL residue acquisition.

#### Identification of QQ mechanism of strain RO1S-5

To identify the QQ mechanism, OdDHL was incubated with the strain RO1S-5 having the highest QQ activity, and the resulting products were analyzed by UPLC (Waters, Acquity UPLC) and a negative electrospray ionization (ESI)-MS/MS (Waters, Quattro Premier MS). Fifty-microliter of OdDHL ( $1.0 \times 10^{-2}$  M in DMSO) was mixed with 450 µl of 12 h culture of RO1S-5. After 24 h incubation at 30°C, the incubation mixture was extracted three times with acidified ethyl acetate (500 µl) and the combined organic phase was evaporated to dryness under nitrogen flow. The residue was reconstituted in 500 µl of methanol:water (1:1; v/v) mixture and then analyzed by UPLC using a  $C_{18}$  reversed-phase column (100 mm  $\times$  0.2 mm  $\times$  0.17 µm, Waters). Water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) were used as eluents for fractionation at flow rate of 0.4 ml/min under following conditions: 90% of A at 0 min to 0% at 3 min, maintained to 4

min, to 90% of A at 5 min, and maintained to 6 min. The fractionated sample was analyzed via ESI-MS/MS at Center for Zero Emission Technology, Suwon, Korea. *N*-(3-oxododecanoyl)-L-homoserine (3-oxo-C12-HS), the lactonolysis product of OdDHL was prepared by incubating OdDHL in 200  $\mu$ l DMSO (25 mM) mixed 300  $\mu$ l of 1 M NaOH for 6 h at 37°C. The pH of the mixture was adjusted to 6.0 with H<sub>3</sub>PO<sub>4</sub> and then the lactonolysis product was extracted three times with ethyl acetate (Mei *et al.*, 2010). After evaporating the solvent under nitrogen flow to dryness, the residue was reconstituted in 500  $\mu$ l of a methanol:water (1:1) and used as a standard lactonolysis product of OdDHL.

## Results and Discussion

### Detection of QQ activities

Among the AHL biosensors, *C. violaceum* CV026 and *A. tumefaciens* NTL4 were chosen in this study as they have been most commonly used for the detection of C4 to C12 AHLs, which are the lengths of acyl chains of AHLs produced by most bacterial QS systems. CV026 which was used for C4 to C8 AHL detection develops purple pigmentation when exposed to exogenous AHLs with acyl chains shorter than C10 except C4-3-oxo-BHL (McClellan *et al.*, 1997; Steindler and Venturi, 2007). NTL4 was used for the detection of AHLs with acyl chains longer than C10 as it can respond to 3-unsubstituted and substituted AHLs with exception of C4-AHL (Cha *et al.*, 1998; Farrand *et al.*, 2002; Zhu *et al.*, 2003; Kawaguchi *et al.*, 2008).

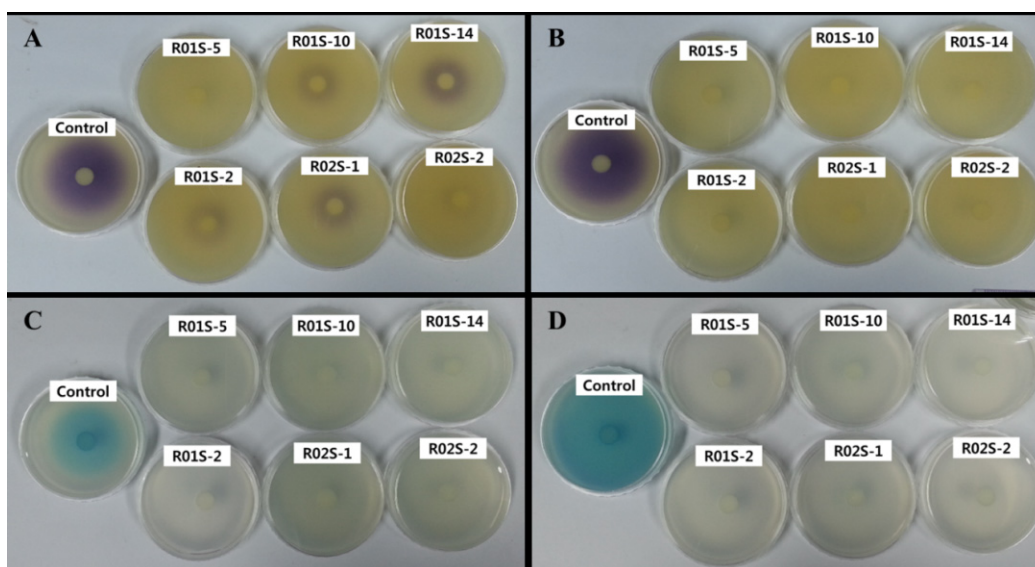
Among a total of 505 bacteria which were isolated from

**Table 1.** Identification of QQ bacteria isolated from fouled RO membranes collected from a full-scale water treatment plant

Isolate	Closest match		
	Strain	Homology (%)	Source
RO1S-2 (KJ413086)	<i>Bacillus</i> sp. A-BT-nw (JF901711)	99.8	Smelting factory effluent
RO1S-5 (KJ413087)	<i>Bacillus</i> sp. cp-h45 (EU584544)	99.6	Rolling wastewater
RO1S-10 (KJ413088)	<i>Bacillus cereus</i> S45 (GQ462533)	99.8	Mulberry rhizosphere
RO1S-14 (KJ413089)	<i>Bacillus</i> sp. A-BT-nw (JF901711)	99.2	Smelting factory effluent
RO2S-1 (KJ413090)	<i>Bacillus</i> sp. cp-h48 (EU584546)	100.0	Rolling wastewater
RO2S-2 (KJ413091)	<i>Bacillus</i> sp. B26 (2010) (HM852450)	99.4	Soil

fouled RO membranes collected from a full-scale water treatment plant (Veolia Water Solutions & Technologies Korea), Daesan, Chungbuk, Korea (Kim *et al.*, 2009), 34 strains presented QQ activity in parallel streaking assay. Based on the disc-diffusion assay, 6 isolates were chosen to have high QQ activity against BHL (C4), HHL (C6), DHL (C10), and OdDHL (3-oxo-C12) (Fig. 1).

The 16S rDNA sequences of the isolates were compared using BLAST programs of NCBI to identify their closest

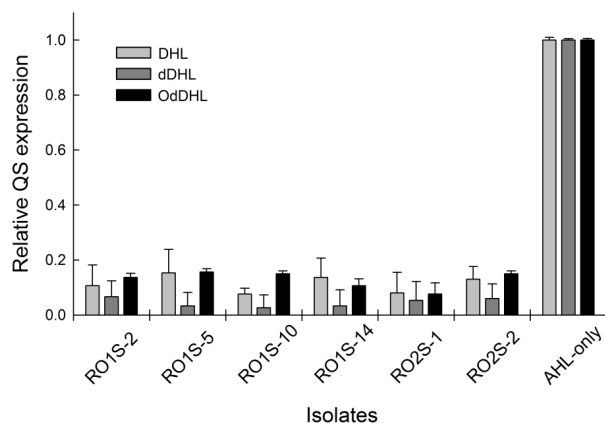


**Fig. 1.** Disc-diffusion assay for the detection of QQ activity of each strain using CV026 and NTL4 as biosensor for (A) BHL (6,848 ng loaded on the disc), (B) HHL (199.2 ng), (C) DHL (283.4 ng), (D) OdDHL (29.7 ng). Narrower colored zone indicates higher QQ activity. In case of the control, only AHL was loaded onto the disc.

phylogenetic relatives (Table 1). All of the QQ isolates belonged to the genus *Bacillus* with a high sequence homology (99.2–100.0%) including the *B. cereus* type strain ATCC 14579. None of them produced AHL signal molecules which could be detected by CV026 and NTL4 (data not shown). The QQ activities of the 6 strains were compared with each other by the  $\beta$ -galactosidase assay using NTL4 as biosensor, and DHL (C10), dDHL (C12), and OdDHL (3-oxo-C12) as QS-inducing AHL molecules (Fig. 2). Compared with the AHL-only control (100% QS expression), all of the isolates caused a noticeable decrease in QS-regulated  $\beta$ -galactosidase expression, which suggested that the level of available AHLs was reduced by the QQ activity of the isolates. Reductions in the  $\beta$ -galactosidase expression by the QQ activity of the isolates against DHL, dDHL, and OdDHL were in the range of 85–92%, 93–97%, and 84–92%, respectively. These results suggested that QQ by the isolates was not influenced by acyl chain length or substitution of AHL molecules.

#### Inhibition of biofilm formation by QQ isolates

*P. aeruginosa* has been one of the best model organisms in screening QQ organisms and compounds as they produce QS signal molecules (BHL and OdDHL) and the QS has a critical role in their biofilm formation (Hentzer *et al.*, 2002; Smith and Iglewski, 2003b; Kim *et al.*, 2008; Shetye *et al.*, 2013). After verifying the decrease in QS induction by lowering AHL levels in the presence of the isolates, we examined if the QQ activity of the isolates could inhibit the biofilm formation by *P. aeruginosa* PAO1. All of the tested isolates efficiently inhibited the biofilm formation as judged based on the crystal violet



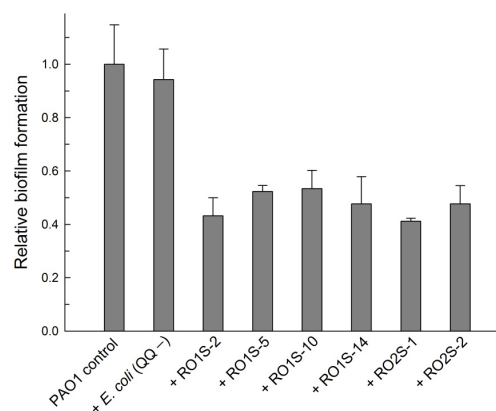
**Fig. 2.** QQ activity of each isolate was determined by  $\beta$ -galactosidase assay using NTL4 as biosensor. Relative  $\beta$ -galactosidase activity induced in the presence of AHL-only (=1.0) was compared with the activity in the presence of AHL and each isolate. The final concentrations of DHL, dDHL, and OdDHL used in the study were  $10^{-7}$ ,  $10^{-7}$ ,  $10^{-6}$  M, respectively.

microtiter assay (Fig. 3). After 24 h of co-cultivation, PAO1 formed less biofilm in the presence of individual isolates by 46.7–58.3% compared to the control (PAO1-only). The significance of the QQ activity of *Bacillus* spp. associated with the inhibition of biofilm formation is further discussed later following the identification of the inhibition mechanism.

#### Characterization of QQ mechanism

QQ mechanisms in bacteria have been known to work through specific interferences with the QS signaling system such as enzymatic degradation of AHLs (Reimann *et al.*, 2002); inhibition of AHL synthesis (Parsek *et al.*, 1999); sequestration of AHLs (Whitehead *et al.*, 2001); inhibition of QS signal transduction (Smith and Iglewski, 2003b). Among these mechanisms, QQ at the level of AHL synthesis could be excluded as the isolates showed QQ activity against preformed AHLs (Fig. 2).

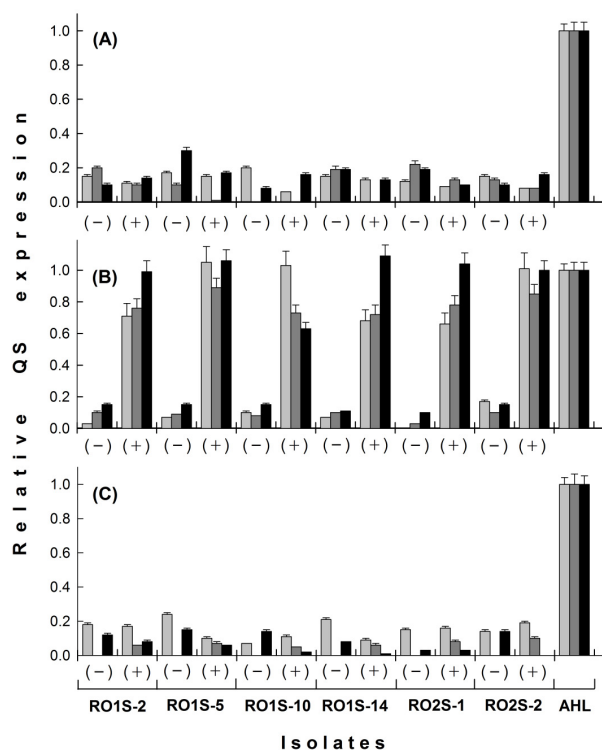
As all of the isolates were *Bacillus* spp. that have been known to produce QQ enzymes (Dong *et al.*, 2000; Lee *et al.*, 2002; Chowdhary *et al.*, 2007; Nithya *et al.*, 2010; Romero *et al.*, 2011), we attempted to test if the QQ mechanism of the isolates was enzymatic. When the QQ activity of the cell-free spent medium was tested, all the isolates were found to have extracellular QQ activities against DHL, dDHL, and OdDHL as high as those of the spent medium with cells (Fig. 4A). Cheong *et al.* (2013) also reported that two *Pseudomonas* spp. and one *Stenotrophomonas* sp. isolated from lab-scale membrane bioreactor presented higher extracellular QQ activities than intracellular ones by the production of AHL-acylase. The QS expression levels of the heat-treated supernatants were



**Fig. 3.** Relative biofilm formation by *P. aeruginosa* PAO1 in the presence of the QQ isolates. Biofilm formation by PAO1 only (=1.0, PAO1 control) was compared with the biofilm formation by PAO1 in the presence of each isolate. ‘PAO1 + *E. coli*’ were used as QQ negative control.

significantly higher than those of untreated ones, which indicated the QQ compound in the supernatant was not heat-stable (Fig. 4B). These results highly suggested that the QQ activities of the isolates could be enzymatic activity. However, the QQ activity of the isolates was found to be still remained in the proteinase K-treated spent medium (Fig. 4C). As there have been several reports about protease-resistant QQ enzymes (Chen *et al.*, 2010; Christiaen *et al.*, 2011; Cao *et al.*, 2012), we decided to confirm if the QQ of the isolates relied on an enzymatic process by analyzing the reaction products using UPLC-MS/MS.

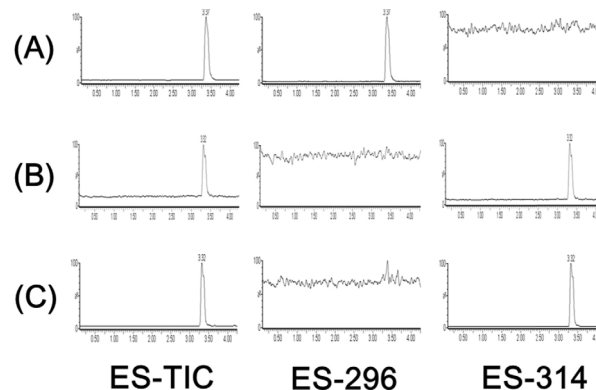
AHL lactonase and acylase are known to degrade AHL molecules by hydrolyzing the lactone bond in the homoserine lactone ring and the amide linkage in the acyl side chain, respectively (Dong and Zhang, 2005). Therefore, release of corresponding acyl homoserine molecule or homoserine lactone with free fatty acid as the degradation products suggest whether it is by the activity of lactonase or acylase,



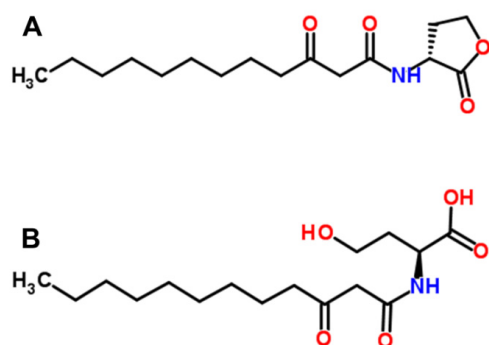
**Fig. 4.** Characterization of the QQ compound of the isolates. Relative QS expression in the (A) presence (+)/absence (-) of intact cells, (B) heat treatment (+, -), and (C) proteinase K treatment (+, -) of the QQ compound was determined by  $\beta$ -galactosidase assay using NTL4 as biosensor. The  $\beta$ -galactosidase activity induced in the presence of AHL-only (=1.0) was compared with the activity in the presence of AHL and each isolate. The final concentrations of DHL (■), dDHL (■), and OdDHL (■) used in the study were  $10^{-7}$ ,  $10^{-7}$ ,  $10^{-6}$  M, respectively.

respectively. If none of the aforementioned degradation products are detected, it means that the QQ activity is due to sequestration or antagonistic one.

Although strains RO1S-5 and RO1S-10 showed the highest QQ activity against DHL, dDHL, and OdDHL among the six isolates (Fig. 2), RO1S-5 was selected for LC-MS/MS analysis as RO1S-10 shared a high degree of 16S rDNA sequence identity with *B. cereus* which is a well-known species having QQ activity (Dong *et al.*, 2002; Zamani *et al.*, 2013). The selective ion recording (SIR) profiles of OdDHL standard by the LC-MS/MS analysis are shown in Fig. 5A. OdDHL generated a single molecular ion peak at  $m/z$  (mass to charge ratio) of 296 at retention time (RT) of 3.37 min, which corresponded to 97.5% of the total ion current (TIC). When OdDHL was incubated with RO1S-5 for 24 h, there was only one ion peak generated in TIC plot at RT of 3.32 min which is slightly different from that of OdDHL (Fig. 5B). There was no ion peak detected at  $m/z$  of 296 (RT 3.37 min) in the SIR 296 scan, but one ion peak was detected at  $m/z$  of 314 (RT 3.32 min) in the SIR 314 scan (Fig. 5B). The results indicated that OdDHL was almost completely degraded by RO1S-5 to a compound having an  $m/z$  of 314, which corresponds to that of acyl homoserine of OdDHL, e.g. 3-oxo-C12-HS. To confirm this, we produced synthetic 3-oxo-C12-HS from OdDHL by alkaline hydrolysis of the lactone ring (Yates *et al.*, 2002) and compared the generated ion molecule with the one produced by RO1S-5. As shown in Fig. 5C, the SIR profiles of the synthetic 3-oxo-C12-HS were identical to those of the degradation product of OdDHL by RO1S-5 (Fig. 5B). These results suggested a lactonase action of RO1S-5 on OdDHL ( $m/z$  of 296) resulted in the production of 3-oxo-C12-HS ( $m/z$  of 314) with a mass increase of 18, corresponding to a water molecule (Fig. 6). The



**Fig. 5.** UPLC and negative ESI-MS/MS analysis of OdDHL treated with the QQ strain, RO1S-5. Selective ion recording (SIR) chromatogram of (A) OdDHL, (B) degradation product of OdDHL by RO1S-5, (C) synthetic 3-oxo-C12-HS with respect to the total ion current (TIC) and molecular ion peaks at  $m/z$  of 296 and 314.



**Fig. 6.** Chemical structures of (A) OdDHL and (B) 3-oxo-C12-HS, a proposed degradation product of OdDHL by lactonase.

3-oxo-C12-HS was not detected in the negative control (OdDHL in TSB medium without RO1S-5), which indicated that nonenzymatic cleavage of the lactone ring such as alkaline hydrolysis and ethyl acetate effect during the extraction were not plausible (data not shown). A possibility of the pH-dependent lactone hydrolysis during the incubation could be also ruled out by confirming that the pH of the medium during 48 h cultivation of RO1S-5 was remained in the range of 6.3–7.0. Altogether, these results strongly suggested that the QQ by RO1S-5 was attributed to the lactonase activity on AHL molecules.

Although biofilm formation is one of the major QS-regulated virulence in a wide range of pathogenic bacteria such as *P. aeruginosa* and *Serratia marcescens* (Adonizio *et al.*, 2008; Estrela and Abraham, 2010; Chen *et al.*, 2013), few studies have shown the inhibitory effect of AHL lactonase from *Bacillus* sp. on biofilm formation. To our knowledge, the only available reports about biofilm study with respect to AHL lactonase were: eradication of preformed biofilm (24 h-old) of PAO1 by an exposure to AiiA lactonase for 6 h at 37°C (Kiran *et al.*, 2011); reduction in biofilm formation by *P. fluorescens* strain 2P24 (pR8C-1) harboring *aidH* gene (Mei *et al.*, 2010). In contrast to lactonase, inhibition of biofilm by AHL acylase has been extensively studied: acylase I-mediated inhibition of the biofilm formation by *Aeromonas hydrophila* and *Pseudomonas putida* (23–73%) and biofilms on RO membrane (20–24%) (Paul *et al.*, 2009); acylase from *Bacillus pumilus* on the biofilm formation by PAO1 (20–87%) and *S. marcescens* (~61%) (Nithya *et al.*, 2010); application of immobilized acylase in membrane bioreactors for biofouling control (Yeon *et al.*, 2009; Cheong *et al.*, 2013) and nanofiltration membrane (Kim *et al.*, 2011).

Acylases have been known to show variable substrate spectra and a narrow AHL substrate range in terms of acyl chain length and substitution (Lin *et al.*, 2003; Sio *et al.*, 2006; Dong *et al.*, 2007). Hence, it has been a challenge to search for

QQ enzymes having broad substrate range as more than one bacterial species as well as multiple AHL-based QS system could be a target of QQ strategy (Case *et al.*, 2008; Liu *et al.*, 2011). Lactonases, on the other hand, are known to have a broad AHL substrate range and high substrate specificity showing little activity to non-acyl lactones and non-cyclic esters (Park *et al.*, 2003; Wang *et al.*, 2004; Dong *et al.*, 2007). Therefore, a significant reduction of biofilm formation by RO1S-5 in the present study suggested that this isolate would be useful for future application for the control of biofilm-related pathogenesis and biofouling in industrial processes.

## 적요

Acyl homoserine lactone (AHL) 분해효소인 lactonase는 높은 기질 특이성을 지니기 때문에 경제적이고 효율적인 쿼럼 저해 기술로 이용될 가능성을 지니고 있다. 본 연구에서는 *Chromobacterium violaceum* CV026과 *Agrobacterium tumefaciens* NTL4를 바이오센서로 이용하여 biofouling이 일어난 역삼투막 시료로부터 쿼럼 센싱과 관련된 생물막 형성을 억제하는 6종의 균주를 분리 연구하였다. 분리된 균주는 모두 *Bacillus* 속으로 동정되었으며, AHL 분자의 acyl 사슬 길이나 치환 종류에 상관 없이 쿼럼 저해 활성을 보여주었다. 균주들은 *Pseudomonas aeruginosa* PAO1에 의한 생물막 형성을 46.7–58.3% 정도 감소시켰으며 이 때 저해물질은 열처리에 민감한 특성을 보여주었다. 분리 균주 중 RO1S-5를 이용하여 *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12 AHL)과 반응시킨 결과, 상응하는 acyl homoserine (3-oxo-C12-HS)이 생성되는 것을 LC-MS로 확인하여 쿼럼 저해가 lactonase 활성에 의한 것임을 규명하였다. AHL 물질에 대한 높은 특이성 등을 감안할 때 분리 균주 RO1S-5는 생물막 형성과 관련된 질병이나 산업공정 중 발생하는 biofouling을 해결하는데 유용하게 쓰일 수 있을 것으로 기대된다.

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