Genetic and Biochemical Analyses of Cell-to-Cell Communication System in Multidrug Resistant-Bacterium *Acidovorax* sp. Strain MR-S7

January 2014

Hiroyuki KUSADA

Genetic and Biochemical Analyses of Cell-to-Cell Communication System in Multidrug Resistant-Bacterium *Acidovorax* sp. Strain MR-S7

A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Agricultural Science
(Doctoral Program in Life Sciences and Bioengineering)

Hiroyuki KUSADA

TABLE OF CONTENTS

Acknowledgements

I would like to express my deepest appreciation to Prof. Satoshi Hanada for his valuable advices, sound suggestions, generous guidance and constant encouragements during the entire course of my graduate study at the Univ. of Tsukuba. I would also like to thank Prof. Seigo Sato and Prof. Nobuhiko Nomura for their helpful comments to this thesis as my final dissertation advisory committee members.

I feel very grateful to Dr. Nobutada Kimura for his considerable guidance, critical discussion, generous helps and introduction to experimental techniques. My heartfelt appreciation goes to Dr. Yoichi Kamagata and Dr. Hideyuki Tamaki for their helpful suggestions, insightful direction and powerful encouragements. Without their generous supports and encouragements, this thesis would not have been completed.

I'm also thankful to Dr. Yu Zhang for provision of newly isolated bacterium *Acidovorax* sp. strain MR-S7 presented in Chapter 1. My greatly thanks are due to Dr. Yoshiaki Yasutake for constructive advices, introduction to structural genomics and experimental support of crystallographic analysis of MacQ protein presented in Chapter 2. I also deeply grateful to Dr. Takamasa Miura for generous understandings, constant encouragements, technical advices and experimental support of genomic and transcriptomic analyses presented in Chapter 3. Without their experimental supports, this dissertation would not have been possible.

Special thanks go to Dr. Kazunori Nakamura, Dr. Yasuo Mitani, Dr. Shigeyuki Kakizawa, Dr. Ryo Miyazaki, Mr. Manabu Kanno, Dr. Kyosuke Yamamoto, Dr. Satoshi Tamazawa, Dr. Kazuto Takasaki, Mr. Masahiro Tagawa, Dr. Takashi Narihiro, Dr. Daisuke Mayumi, Dr. Tomoyuki Hori, Dr. Eri Nasuno, Mr. Danilo T. do Amaral, Mr. Koji Yamada, Mr. Sho Morohoshi, Ms. Mizuho Muramatsu and Ms. Yukiko Tadamatsu for their invaluable support and worm words of encouragement during my Ph.D. student period. My profound gratitude to all members belonged to Microbial and Genetic Resources Research Group for their helps, supports and sharing precious times.

This thesis is dedicated to my parents, Toshihiko and Chiaki Kusada, in rural Saitama prefecture for their continual supports, generous understandings and constant encouragements throughout my life.

Chapter 4

The Effects of *N*-Acylhomoserine Lactones, β-Lactam Antibiotics and Adenosine on Biofilm Formation in the Multidrug Resistant Bacterium *Acidovorax* sp. Strain MR-S7

4.1 Abstract

Bacteria in the natural ecosystem frequently live as adherent communities called biofilms. Some chemical compounds are known to affect biofilm formation. The effects of exogenous small molecules, *N*-acylhomoserine lactones (AHLs), β-lactam antibiotics, and adenosine, on biofilm formation in the β-lactam antibiotic-resistant bacterium *Acidovorax* sp. strain MR-S7 were investigated. Biofilm formation was induced by the addition of various types of AHL isomers and β-lactam antibiotics, whereas the addition of adenosine strongly interfered with the biofilm formation. A gene (*macP*) encoding adenosine deaminase (that converts adenosine to inosine controlling intracellular adenosine concentration) was successfully cloned from MR-S7 genome and heterologously expressed in *Escherichia coli*. The purified MacP protein clearly catalyzed the deamination of adenosine to produce inosine. A transcriptional analysis revealed that biofilm-inducing molecules, an AHL and a β-lactam antibiotic, strongly induced not only biofilm formation but also adenosine deaminase gene expression, suggesting that an elaborate gene regulation network for biofilm formation is present in the β-lactam antibiotic-resistant bacterium studied here.

4.2 Introduction

Bacterial biofilms are generally described as surface-attached bacterial communities comprised of mixed microorganisms surrounded by a self-produced extracellular polymeric substance (EPS) that contains polysaccharides, DNAs and proteins (De Kievit, 2009). Biofilms are widely distributed in natural environments and are frequently found in a variety of places. The biofilm formation can be regarded as a basic life path among bacteria. Lerchner *et al.* estimated that 90% of bacteria dwell in biofilm communities rather than planktonic states (Lerchner et al., 2008). An advantage of biofilm formation is that it eases environmental stresses and fluctuations such as temperature, pH and UV light (Cotton et al., 2009). In pathogenic bacteria, biofilm formation helps avoid an attack by the biofilm host's immune system (Donlan

and Costerton, 2002), as an effective survival strategy.

Various small molecules work as a trigger to form biofilms (De Kievit, 2009). These signaling molecules are mainly produced and released by a biofilm-forming bacterium itself, and these events prompt the biofilm formation. Biofilm formation is known to be closely associated with cell density and the concentration of signal molecules. This population density-dependent cell-to-cell communication mechanism is referred to as the quorum sensing system. Among the molecules that work as a trigger to form biofilms, *N*-acylhomoserine lactones (AHLs) are the most studied signal molecules in the quorum sensing system for biofilm formation among Gram-negative bacteria (De Kievit et al., 2001; Singh et al., 2000).

In the common bacterium *Pseudomonas aeruginosa*, biofilm formation is under the control of AHLs, and it was found that AHLs induce the production of the main constituents of the biofilm matrix, e.g., exopolysaccharides and extracellular DNA (Allesen-Holm et al., 2006; Sakuragi and Kolter, 2007) and rhamnolipids (Pamp and Tolker-Nielsen, 2007). AHLs also promote the swarming motility that is essential to adhesion in the early stage of biofilm formation (Shrout et al., 2006).

Some antibiotics are also known to affect biofilm formation (Linares et al., 2006). The addition of various β-lactam antibiotics at sub-inhibitory concentrations induced biofilm formation in *Staphylococcus aureus* (Haddadin et al., 2010; Kaplan et al., 2012; Mirani and Jamil, 2011). In addition, β-lactam antibiotics at sub-inhibitory concentrations induced biofilm formation in the Gram-negative bacteria *Salmonella enterica* serotype Typhimurium (Majtan et al., 2008) and *Acinetobacter baumannii* (Nucleo et al., 2009). Biofilm formation clearly seems to be advantageous to the cells within it, because the surrounding matrix, i.e., the EPS, prevents antibiotic agents from penetrating into the cells. However, the manner in which antibiotics induce biofilm formation is largely unknown.

Small molecules that are neither an AHL nor an antibiotic can have significant effects on biofilm formation. Sheng *et al*. (Sheng et al., 2012) found that exogenous adenosine nearly abolished static biofilm formation in *P. aeruginosa* and that exogenous inosine, a structural analogue of adenosine, did not interfere with it. Adenosine, one of the common components essential to life, is constantly synthesized in

the cells and occasionally converted to inosine by adenosine deaminase (Heurlier et al., 2006). Little is known about the mechanism underlying the inhibition of biofilm formation by adenosine, and questions remain regarding role of adenosine in biofilm formation and what controls the adenosine concentration in cells.

Even in a β-lactam antibiotic-resistant bacterium, *Acidovorax* sp. strain MR-S7, recently isolated from a penicillin-containing wastewater treatment process (Zhang et al., unpublished data), such biofilm formation is occasionally observed and AHLs, antibiotics and adenosine likely affects it. In this chapter, the effects of these small molecules on the biofilm formation of the strain were minutely investigated. In addition, adenosine deaminase gene was also cloned and its expression examined by a transcriptional assay to know the mechanisms underlying biofilm formation in strain MR-S7.

4.3 Materials and Methods

4.3.1 Bacterial strains, culture media, and growth conditions.

The bacterium *Acidovorax* sp. strain MR-S7 was isolated from an activated sludge sample in a treatment system for penicillin G-polluted wastewater (Zhang et al., unpublished data). *Escherichia coli* strain DH5α (TaKaRa, Tokyo) was used as the host strain for DNA manipulation. *E. coli* strain Origami™ 2 (DE3) (Novagen, Madison, WI) was used as the host strain for expression of the cloned gene. Strain MR-S7 and *E. coli* strains were cultured on Luria-Bertani (LB) agar or in LB broth at 30°C and 37°C, respectively. When necessary, kanamycin was added to the LB media at the final concentration of 100 mg/L.

4.3.2 Bioassay of AHLs-production activity.

The AHL-production assay of strain MR-S7 was performed using GFP-based

biosensor strains, *E.coli* strain MT102 harboring plasmid pJBA132 and *Pseudomonas putida* F117 harboring plasmid pKR-C12 (Andersen et al., 2001; Steidle et al., 2001). These biosensor strains yield GFP fluorescence when AHL-like compound is present. Briefly, the overnight culture fluid of MR-S7 was harvested by centrifugation at 1,500 ×g for 1min and washed with potassium phosphate buffer (pH 7.0). In addition, the overnight culture fluid of MR-S7 was extracted with equal volumes of ethyl acetate for three times and the combined organic phases were resuspended in sterilized water. These liquid solutions were dispensed into a well of a 96-well microtitre plate (Becton Dickinson, Franklin Lakes). Then, 50 μl of a five-fold-diluted overnight culture of the reporter strains were added in equivalent numbers into each well of the 96-well microtiter plates and statically incubated at 30°C for 4h to induce detectable GFP expression from the reporter cells. Sterilized water was mixed with biosensor strains and used as a non-GFP-fluorescence control. A SpectraMax Gemini XS Microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) was used to measure GFP fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

4.3.3 Biofilm formation assay.

Strain MR-S7 was grown in LB broth overnight, and 8 μL of culture was inoculated to fresh LB broth (1% inoculation) with or without small molecules, i.e., *N*-acylhomoserine lactones (AHLs), β-lactam antibiotics, adenosine and inosine, in 48-well polystyrene tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ). After static incubation for 5 days at 30°C, all culture fluid was removed and each well was carefully washed with sterilized distilled water. The culture plates were then stained with 1% crystal violet (Wako, Osaka, Japan) in 33% acetic acid. After static incubation for 20min at room temperature, the crystal violet solution was removed and the culture plates were carefully washed twice with sterilized distilled water as described previously (Irie et al., 2004; Irie et al., 2005; Pedersen, 1982). For the quantification of attached cells, the dye bound to the adherent cells was solubilized with

95% ethanol. To quantify the biofilm mass remaining after treatment, the absorbance of the crystal violet solution was measured on a spectrophotometer (Beckman Instruments, Fullerton, CA) at 595 nm. The concentrations of small molecules in the cultures were as follows: AHLs (1, 5, 10, 20 and 50 μM), β-lactam antibiotics (1, 5, 10, 50 and 100 mg/L), adenosine (1, 5 and 10 mM) and inosine (1, 5 and 10 mM).

For the confocal laser scanning microscopy (CLSM) analysis, bacterial biofilm was grown on glass surfaces (76 × 26 mm, Matsunami, Osaka, Japan) for 5 days in LB medium at 30°C. After the incubation, the glasses were washed and stained with 1% crystal violet solution. Confocal images were collected using an LSM 5 PASCAL microscope (Carl Zeiss Microscopy, Thornwood, NY). A He-Ne laser (633 nm) was used for sample excitation.

4.3.4 Antibiotics susceptibility testing.

The minimum inhibitory concentrations (MICs) of non-supplemented, OC_8-HSL-supplemented and penicillin G-supplemented strain MR-S7 were determined by an agar dilution technique with an inoculum of 10^4 colony-forming units (CFU) per plate. Strain MR-S7 was cultivated in LB broth with OC_8-HSL or penicillin G. After cultivation, each culture of MR-S7 cells was equated and inoculated on an LB agar plate with the selected antibiotic. All of the plates were incubated at 30°C for 3 days at ambient atmosphere. The MICs were defined as the lowest concentration of antibiotics at which the strains showed no visible growth on the agar. The tested antibiotics were neomycin and gentamicin, which are classified as aminoglycoside antibiotics, and tetracycline and chloramphenicol. The tested concentrations of neomycin were 1, 5, 10, 25, 50 and 100 mg/L; those of gentamicin were 2.5, 6.25, 12.5, 18.75, 25 and 50 mg/L, tetracycline 2, 5, 10, 15, 20 and 40 mg/L, and chloramphenicol 1.25, 6.25, 12.5, 25, 50 and 75 mg/L.

4.3.5 Cloning and protein expression of the putative adenosine deaminase gene (macP).

The genomic DNA of strain MR-S7 was purified according to the method described previously (Kimura et al., 2006) and used it as the template for the polymerase chain reaction (PCR)-based cloning. The *macP* gene coding region was amplified using the following PCR primers. For_adaP, 5'-GGAATTCCATATGTACAAGGTCCCGCCCATC-3' (*NdeI* site underlined) and Rev_adaP 5'-CGGAATTCCTAGTCGTTCTCGACGTAGCG-3' (*EcoRI* site underlined) with PrimeSTAR HS DNA Polymerase (TaKaRa). The PCR amplification was performed with initial denaturation at 98°C for 5min, followed by 40 cycles at 98°C for 10s, 68°C for 1min. The PCR product was then gel-purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA), digested by each restriction enzyme, and cloned into expression vector pET-28b (Novagen, Madison, WI). The resulting plasmid, termed pAD28, was transformed into *E. coli* Origami™ 2 (DE3).

Protein expression was induced by adding 0.1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). After 18h of cultivation at 18°C, the cells were harvested by centrifugation at 5,800 ×g for 10min, washed with suspension buffer (50 mM Na_2PO_4, 300 mM NaCl, 10% glycerol, pH 7.5), resuspended in the same buffer, and disrupted using an ultrasonic disintegrator (Sonicator BRANSON sonifer 250). Cell debris was removed by centrifugation at 5,800 ×g for 10min. The supernatant was applied to HIS-Select Nickel Affinity Gel (Sigma-Aldrich, Saint Louis, MO), and the His-tagged protein was purified based on the manufacturer's instructions.

4.3.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

His-tagged crude cell extract was treated with marker dye (1% SDS, 1% 2-mercaptoethanol, 10 mM Tris-HCl (pH 6.8), 20% glycerin, and 1 mg/ml bromophenol blue), and then heated for 5min at 98°C. For the SDS-PAGE analysis, the samples

were subjected to 10% PAGE gels under 30mA for 80min using 10 × SDS-PAGE running buffer (1% SDS, 3% Tris, and 14.4% glycine). Proteins were detected by staining with a Rapid Stain CBB kit (Nacalai Tesque, Kyoto, Japan). The resulting His-tagged MacP protein was dialyzed to the ten-thousandth by dialysis buffer (20 mM Tris, 300 mM NaCl, 30% glycerol, pH 8.0).

4.3.7 Enzymatic activity assay of MacP protein.

The ammonia concentration was determined using an Ammonia-Test-Wako kit (Wako Pure Chemical Industries, Osaka, Japan) in accord with the manufacturer's instructions. Briefly, 10 mM adenosine solution was mixed with 100 μg MacP protein and incubated statically for 24h at 30°C. After the addition of deproteinizing solution, the solution was mixed with coloring reagent A (containing phenol and sodium pentacyanonitrosylferrate [III] dihydrate), coloring reagent B (containing potassium hydroxide) and coloring reagent C (containing potassium carbonate and sodium hypochlorite). After allowing the mixture to stand at 37°C for 20min, the absorbance of the reaction mixtures were measured on a spectrophotometer (Beckman Instruments) at 630 nm wavelength. Indophenol was produced after ammonia was mixed with coloring reagents A, B and C. As a negative control, 10 mM adenosine solutions was mixed with 100 μg heat-denatured MacP protein (98°C for 30min) and with dialysis buffer, respectively.

4.3.8 Reverse transcriptase PCR (RT-PCR) analyses of **macP** *gene.*

Strain MR-S7 was grown in LB medium with or without OC_8-HSL and penicillin G to exponential phase, and the total RNAs were isolated using the RNeasy Mini Kit (Qiagen). The concentration and quality of the RNA samples were determined by using the Agilent 2100 Bioanalyzer Nano Chip (Agilent Technologies, Santa Clara, CA). Reverse transcription reactions were performed using 1 μg DNase

I-treated total RNA samples, random hexamers, dNTPs, and ReverTra Ace in a 20-μL reaction volume. Reverse transcription reactions were performed with the following cycling parameters: 30°C for 10min, 42°C for 50min, 98°C for 5min. The resulting cDNA samples were used as the PCR template with the relevant PCR primer set, For_adaP and Rev_adaP, amplified by the 1,053 bp of *macP* gene (see above). The presence of chromosomal DNA was checked by PCR using the 16S rRNA gene universal PCR primers 8F and 1492R.

4.3.9 Chemicals.

The following chemicals were purchased from Sigma-Aldrich: *N*-decanoyl-L-homoserine lactone (C_{10}-HSL), *N*-dodecanoyl-L-homoserine lactone (C_{12}-HSL), *N*-3-oxo-hexanoyl-L-homoserine lactone (OC_6-HSL), *N*-3-oxo-octanoyl-L-homoserine lactone (OC_8-HSL), ampicillin sodium salt, adenosine and inosine. *N*-3-oxo-tetradecanoyl-L-homoserine lactone (OC_{14}-HSL) was purchased from Nottingham University, University Park, Nottingham, UK. The following antibiotics were purchased from Wako: penicillin G sodium salt, amoxicillin trihydrate, carbenicillin sodium salt and cefalexin.

4.4 Results

4.4.1 Strain MR-S7 does not produce AHLs.

GFP-based biosensor strains were used to confirm whether strain MR-S7 produce AHL-isomers. The ethyl acetate extracts and culture fluid of MR-S7 showed low values of GFP fluorescence as well as negative control. Based on the draft genome sequence data of *Acidovorax* sp. strain MR-S7 (Miura et al., 2013), *luxI*-like homologue gene (which encodes the AHL synthase) did not present in the MR-S7 genome, whereas four *luxR*-like homologue genes (which encode the transcriptional

regulator) were found in MR-S7 genome (locus_tags; AVS7_01953, AVS7_03407, AVS7_03923 and AVS7_04716). These low values of GFP fluorescence and genomic information indicated that strain MR-S7 does not possess the ability to produce biosensors-detectable AHL-like compounds.

4.4.2 Effect of small molecules on biofilm formation in strain MR-S7.

To investigate the effect of one of the most common and available AHL isomers, *N*-3-oxo-octanoyl-L-homoserine lactone (OC_8-HSL), on biofilm formation in strain MR-S7, the biofilm mass of strain MR-S7 culture in the presence of OC_8-HSL was quantified. According to the effect of several concentrations of OC_8-HSL on biofilm formation in strain MR-S7, the largest amount of biofilm formation occurred when the cells were treated with OC_8-HSL at 5 μM (Fig. 4.1 A). When several concentrations of penicillin G were supplemented to the culture of wild-type strain MR-S7, the largest amount of MR-S7 biofilm was observed at the penicillin G concentration 5 mg/L (Fig. 4.1 A). The following experiments were thus conducted to unify the OC_8-HSL concentration at 5 μM and the penicillin G concentration at 5 mg/L, respectively.

The CLSM analysis revealed biofilm-like aggregation on glass surfaces in the culture of OC_8-HSL- and penicillin G-supplemented strain MR-S7 (Fig. 4.1 B, center and right), whereas a mature aggregation was not observed in non-supplemented strain MR-S7 (Fig. 4.1 B, left). In addition, the crystal violet absorbance of strain MR-S7 supplemented with the β-lactam antibiotics (ampicillin, amoxicillin, carbenicillin and cefalexin) and that of the AHLs with different acyl side chain lengths and 3'-carbon substitutions (OC_4-HSL, OC_6-HSL, OC_8-HSL, C_{10}-HSL, C_{12}-HSL and OC_{14}-HSL) showed approximately 2.5- to 5-fold higher values than that with non-supplemented strain MR-S7 (Fig. 4.1 C). These spectroscopic results indicated that the various β-lactam antibiotics and a broad range of AHLs act as biofilm formation inducers in this strain.

4.4.3 Effect of small molecules on antibiotics resistance.

The MICs of biofilm-forming strain MR-S7 against four selected non-β-lactam antibiotics (neomycin, gentamicin, tetracycline and chloramphenicol) were determined to test whether biofilm formation affects the antibiotic resistance in strain MR-S7. As shown in Table 4.1, the MICs of OC_8-HSL-supplemented strain MR-S7 showed 5- to 10-fold higher resistance to all four tested antibiotics compared to that of non-supplemented strain MR-S7. In addition, the MICs of penicillin G-supplemented strain MR-S7 developed 2.5-fold higher tolerance to gentamicin compared to non-biofilm-forming strain MR-S7 (Table 4.1).

4.4.4 Adenosine inhibits biofilm formation.

To confirm the effects of adenosine and inosine on the MR-S7 biofilm formation, the biofilm masses induced by 5 μM OC_8-HSL or 5 mg/L penicillin G were quantified. The concentrations of adenosine and inosine (each 1, 5 and 10 mM) were established according to the Sheng's experiments (Sheng et al., 2012). As shown in Figure 4.2, adenosine markedly reduced the crystal violet absorbance of both OC_8-HSL- and penicillin G-supplemented strain MR-S7 biofilm formation in a concentration dependent manner in 48-well plates (Fig. 4.2, white bar). These spectroscopic decreases indicated that adenosine works as a biofilm formation inhibitor in strain MR-S7. In addition, it was examined whether inosine (an adenosine metabolite) could also affect biofilm formation, and found that 1, 5 and 10 mM inosine did not interfere with the biofilm formation (Fig. 4.2, gray bar).

4.4.5 Cloning and heterologous expression of the adenosine deaminase gene **(macP).**

Using the draft genome sequence data of *Acidovorax* sp. strain MR-S7 (Miura

et al., 2013), the putative adenosine deaminase gene (terms *macP* gene; locus_tag AVS7_00616) was cloned and heterologously expressed. The *macP* gene was cloned into the pET-28b vector (pAD28) and constructed the heterologous expression system using *E. coli* strain Origami™ 2 (DE3). The SDS-PAGE analysis revealed a single band for His_6-MacP fusion protein (Fig. 4.3A). The molecular weight for MacP was estimated as approx. 40.0 kDa, consistent with the predicted molecular mass of MacP protein (40.5 kDa) based on the deduced amino acid sequence (351 amino acids).

4.4.6 **macP** *gene codes for a adenosine deaminase.*

To determine whether MacP enzyme is capable of converting adenosine to inosine, the ammonia concentration in the reaction mixture was quantified. Adenosine deaminase enzyme catalyzes the hydrolytic breakdown of adenosine to inosine and free ammonia. As shown in Figure 4.3 B, the absorbance unit of 10 mM adenosine solution mixed with 100 μg MacP protein was 0.0149, and the ammonia concentration was 0.2224 μg/μL according to the value of the absorption wavelength, whereas the absorbance unit of 10 mM adenosine solution mixed with heat-denatured MacP was 0.0007, and the ammonia concentration was 0.0010 μg/μL.

4.4.7 Transcriptional analyses of **macP** *gene.*

To confirm how *macP* gene was transcribed in strain MR-S7, RT-PCR analysis was performed with total RNA extracted from strain MR-S7 cells from OC_8-HSL- and penicillin G-supplemented LB medium cultures, respectively. Total RNA extracted from MR-S7 cells grown on LB medium was used as a control. With the use of a PCR primer set, For_adaP and Rev_adaP, 1,053-bp fragments were amplified from the total RNA originated in both OC_8-HSL- and penicillin G-supplemented cells (Fig. 4.4, lanes 3 and 4). A similar band was also observed in control using genomic DNA (Fig. 4.4, lane 1). However, this fragment was not amplified from total RNA extracted from

LB-grown cells (Fig. 4.4, lane 2).

4.5 Discussion

According to the draft genome sequencing of the multiple β-lactam antibiotic-resistant bacterium *Acidovorax* sp. strain MR-S7, several gene candidates that appear to be involved in multiple β-lactam antibiotic-resistant activity were found; that is, β-lactamase domain-containing proteins, multidrug efflux pump proteins, and penicillin-binding proteins were annotated (Miura et al., 2013). Strain MR-S7 can degrade β-lactam antibiotics, and therefore shows resistance to these agents. In addition, the strain seems to have another counterplot to antibiotics.

It was found here that penicillin G at concentrations between 5 and 50 mg/L caused the formation of biofilm-like aggregates in strain MR-S7 (Fig. 4.1 A and B). The culture forming a biofilm supplemented with penicillin G showed 2.5 times higher tolerance to gentamicin compared to the non-biofilm-forming culture (Table 4.1). Since gentamicin in an aminoglycoside but not a β-lactam antibiotic, this tolerance would be due to the biofilm formation as a result of the response to penicillin G. Similar biofilm formation was also found in the presence of several β-lactam antibiotics other than penicillin G, i.e., cephem-related β-lactam antibiotics with a fundamental skeleton that differs from those of penicillin-related β-lactam antibiotics (Fig. 4.1C).

Biofilm formation is known to be induced by *N*-acylhomoserine lactones (AHLs) in a large number of bacteria (De Kievit, 2009; Huber et al., 2001; Lynch et al., 2002). In the present study, the addition of *N*-3-oxo-octanoyl-L-homoserine lactone (OC_8-HSL) at concentrations from 1 to 50 μM caused biofilm formation in strain MR-S7 (Fig. 4.1A). Not only OC_8-HSL but also various AHLs with different acyl side chain lengths with or without 3-oxo substitutions also worked as a trigger to form biofilms in strain MR-S7 (Fig. 4.1C). Biofilm-grown strain MR-S7 in the presence of OC_8-HSL developed 5- to 10-fold tolerance to non-β-lactam antibiotics, neomycin, gentamicin, tetracycline and chloramphenicol (Table 4.1).

These results indicate that biofilm formation mediated by AHLs is also an

effective defense against multiple antibiotic agents in strain MR-S7. The biofilm formation by AHLs appears to be driven by a quorum sensing system. AHLs-LuxR complex directly induce EPS synthesis and promote biofilm formation. In contrast, it is still unclear whether β-lactam antibiotics directly regulate the transcription of EPS synthesis, although β-lactam antibiotic-mediated biofilm formation is widely observed among Gram-positive and -negative bacteria (Haddadin et al., 2010; Kaplan et al., 2012; Majtan et al., 2008; Mirani and Jamil, 2011; Nucleo et al., 2009).

Sheng *et al*. (Sheng et al., 2012) reported that adenosine strongly inhibited biofilm formation in *P. aeruginosa*, and as shown in the present study using strain MR-S7, an exogenous adenosine prevented biofilm formation, and an inosine (an adenosine metabolite) did not inhibit the formation (Fig. 4.2). Despite their phylogenetic distance, the inhibition by adenosine was evident in both *P. aeruginosa* (which belongs to the class *Gammaproteobacteria*) and the *Acidovorax* sp. strain MR-S7 (which belongs to the class *Betaproteobacteria*). These findings suggest that adenosine may act as a biofilm formation inhibitor in all *proteobacteria*. Adenosine is one of the general constituents involved in energy transfer and signal transduction, and the intracellular adenosine concentrations are controlled by the biosynthetic/metabolic pathways. Adenosine deaminase is one of the enzymes involved in those pathways; it catalyzes the conversion of adenosine to inosine (the deamination of adenosine) and ammonia.

A putative adenosine deaminase gene (*macP*) found in the strain MR-S7 genome was cloned and heterologously expressed in *E. coli* Origami™ 2 (DE3). The molecular weight for purified MacP protein was coincident with that based on the deduced amino acid sequence (Fig. 4.3 A), and the protein showed activity to release ammonia from adenosine, showing that it is adenosine deaminase (Fig. 4.3 B). RT-PCR analysis disclosed that the transcription of *macP* gene was remarkably induced in the presence of OC_8-HSL and penicillin G (Fig. 4.4). This finding strongly suggests that intracellular adenosine suppresses the biofilm formation in the planktonic state of strain MR-S7. In addition, AHL and penicillin G each directly induced the expression of *macP* (adenosine deaminase), which released the suppression of biofilm formation by adenosine.

Based on the present findings, a new biofilm formation mechanism in strain

MR-S7 was proposed (Fig. 4.5). Both AHLs and β-lactam antibiotics induce biofilm formation in several bacteria such as *P. aeruginosa* and *S. aureus*. Generally, each induction has been thought to be driven by different mechanisms: AHLs, an auto-inducer in the quorum sensing system, combines with a regulatory protein, LuxR, and the AHL-LuxR complex directly switches on the synthetic genes for extracellular polymeric substances (Allesen-Holm et al., 2006; Sakuragi and Kolter, 2007) and changes the gene expression involved in swarming motility (Shrout et al., 2006) in order to form a biofilm. By contrast, biofilm formation caused by β-lactam antibiotics occurs as a result of various responses to the antibiotic agents (Haddadin et al., 2010; Kaplan et al., 2012; Majtan et al., 2008; Mirani and Jamil, 2011; Nucleo et al., 2009). The most important finding in this study, however, is that both AHLs and β-lactam antibiotics induce the expression of adenosine deaminase. Adenosine deaminase catalyzes the deamination of adenosine and can decrease the concentration of intracellular adenosine. It is very likely that the intracellular level of adenosine, a potent inhibitor of biofilm formation (Sheng et al., 2012), suppresses the formation in the planktonic state and the concentration is controlled by expressing adenosine deaminase. AHLs and β-lactam antibiotics strongly increase the expression level of adenosine deaminase. This is a previously unknown induction pathway for biofilm formation by AHLs as well as β-lactam antibiotics via the regulation of adenosine deaminase. In conclusion, these findings demonstrate that AHLs and β-lactam antibiotics induced not only biofilm formation but also adenosine deaminase gene expression, suggesting that a previously unknown gene regulation network for biofilm formation is present in β-lactam antibiotic-resistant bacteria, and perhaps in other bacteria.

Table 4.1 Antibiotics resistant assay in *Acidovorax* sp. MR-S7.

Strains	Minimum Inhibitory Concentrations (mg/L)			
	Antibiotics			
	Nm	Gm	Tc	Cm
Non-supplemented	10	2.5	<2	1.25
OC_8-HSL-supplemented	50	12.5	2	12.5
Penicillin G-supplemented	10	6.25	<2	1.25

Abbreviated as Nm: neomycin, Gm: gentamicin, Tc: tetracycline, Cm: chloramphenicol. The tested concentrations of Nm was 1, 5, 10, 25, 50 and 100 mg/L, Gm was 2.5, 6.25, 12.5, 18.75, 25 and 50 mg/L, Tc was 2, 5, 10, 15, 20 and 40 mg/L, and Cm was 1.25, 6.25, 12.5, 25, 50 and 75 mg/L. Note, increased MICs by supplemented OC_8-HSL and penicillin G are indicted by underlined.

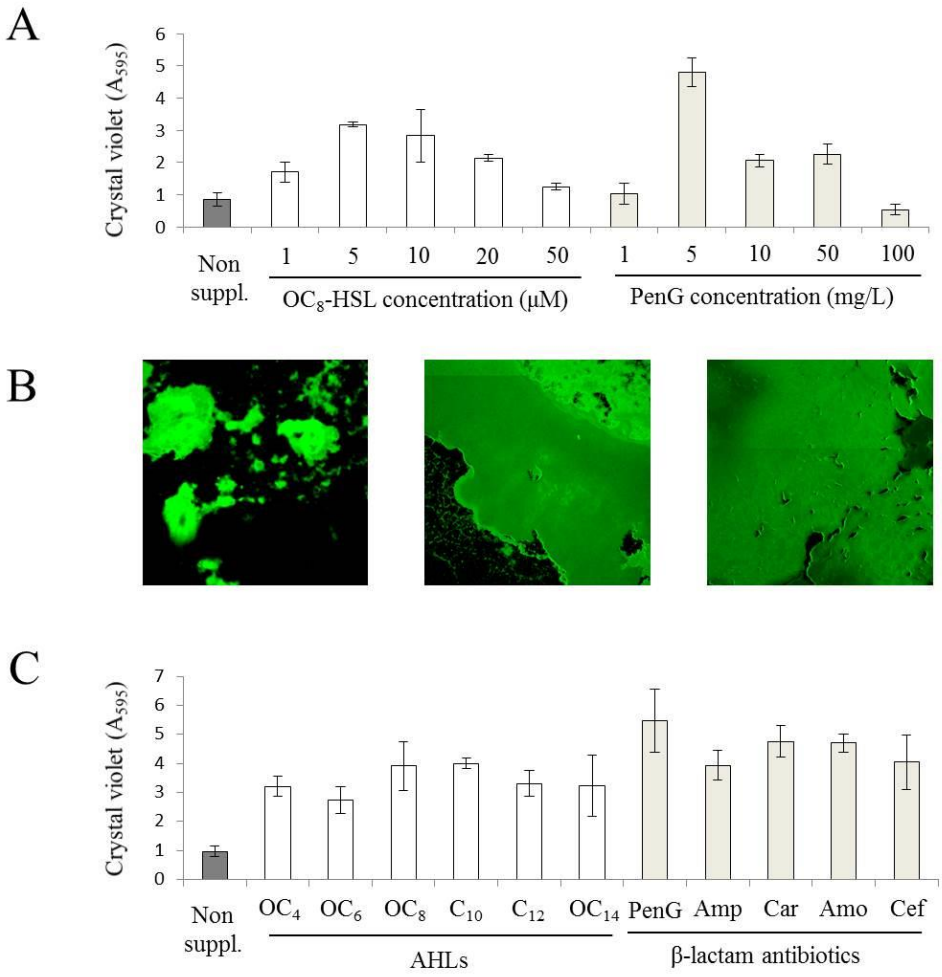

Figure 4.1 Effects of small molecules on biofilm formation in *Acidovorax* sp. MR-S7.

(A) Total biofilm formation was assayed as described in the Materials and Methods section with 1, 5, 10, 20 and 50 μM OC_8-HSL (white bar), and 1, 5, 10, 50 and 100 mg/L penicillin G (gray bar) in LB media. (B) The CLSM analysis of biofilms which were constructed by non-supplemented (left), OC_8-HSL-supplemented (middle) and penicillin G-supplemented strain MR-S7 (right) at 30°C after 5 days in glass surfaces without shaking after stained by crystal violet solution. A He-Ne laser (633 nm) was used for sample excitation. (C) Total biofilm formation was assayed as described with 5 μM AHLs with different acyl side chain lengths with or without 3-oxo substitutions (white bar) and 5 mg/L various β-lactam antibiotics (gray bar) in LB media. Dark gray bars indicate non-supplemented with AHLs or β-lactam antibiotics.

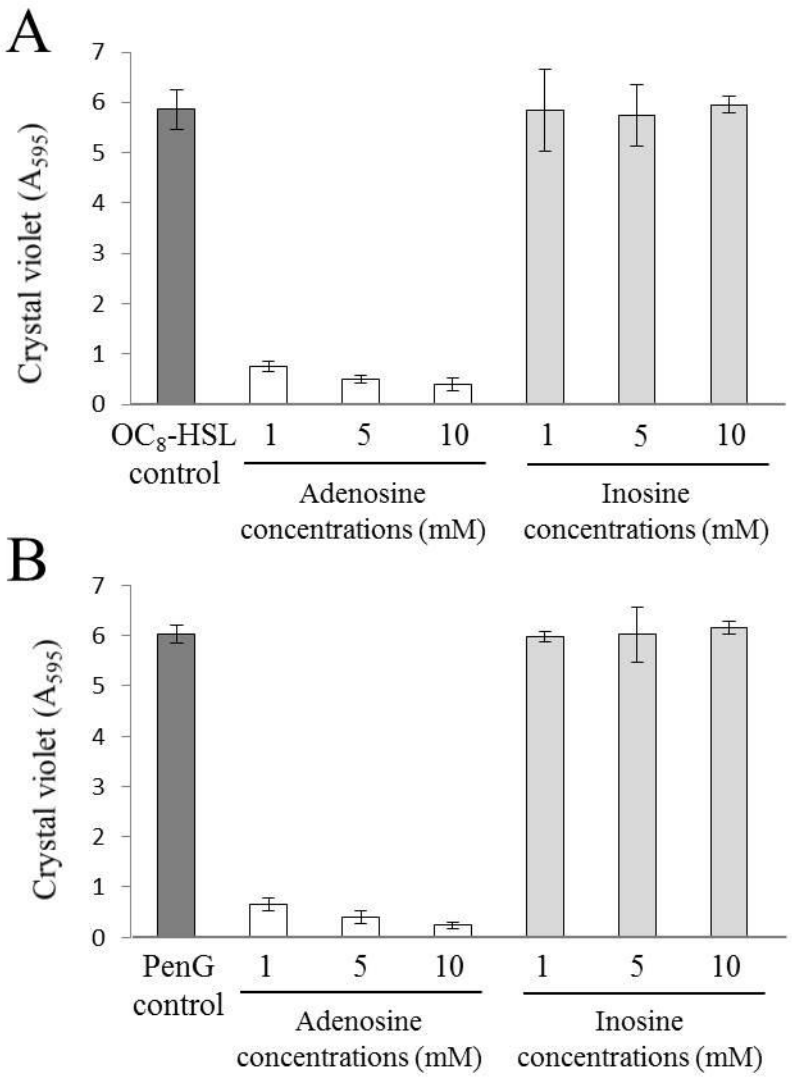

Figure 4.2 Biofilm formation with adenosine and inosine.

Total biofilm formation was assayed at 30°C after 5 days in 48-well plates without shaking with 5 μM OC_8-HSL (A), 5 mg/L penicillin G (B), 1, 5 and 10 mM adenosine (white bar) or 1, 5 and 10 mM inosine (gray bar) in LB media. Dark gray bars indicate non-supplemented with adenosine or inosine.

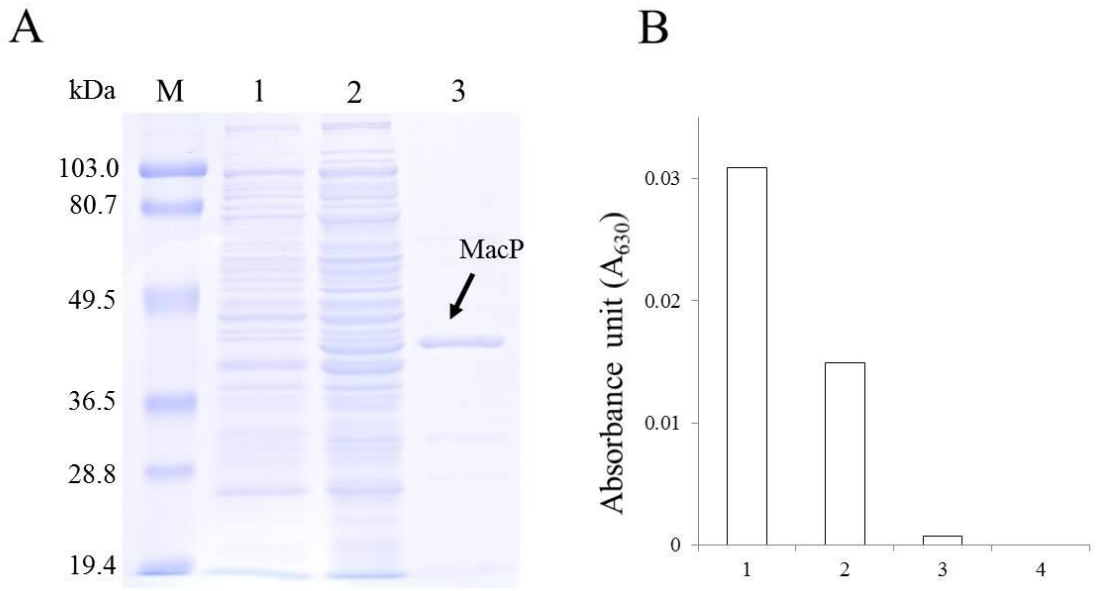

Figure 4.3 SDS-PAGE analysis and enzymatic property of MacP protein.

(A) Lane 1 and lane 2 are soluble cell extracts of *E. coli* Origami™ 2 (DE3) (pET-28b vector) and *E. coli* Origami™ 2 (DE3) (pAD28), respectively. A fraction after Ni-NTA affinity purification of soluble extract of *E. coli* Origami™ 2 (DE3) (pAD28) is indicated in lane 3. A single band for His_6-MacP fusion protein is indicated by an arrow. The sizes of the molecular marker are indicated at the left, Lanes: M. Molecular size-marker (BIO-RAD). (B) The absorbance of indophenol produced by ammonia which was converted from adenosine by MacP protein. The wavelength of each solution was measured at 630 nm (absorbance maxima of indophenol). 1: 0.5 μg ammonia solution used as a positive control, 2: 10 mM adenosine solution mixed with 100 μg MacP protein, 3: 10 mM adenosine solution mixed with heat-denatured MacP, and 4: 10 mM adenosine solution mixed with dialysis buffer used as a negative control.

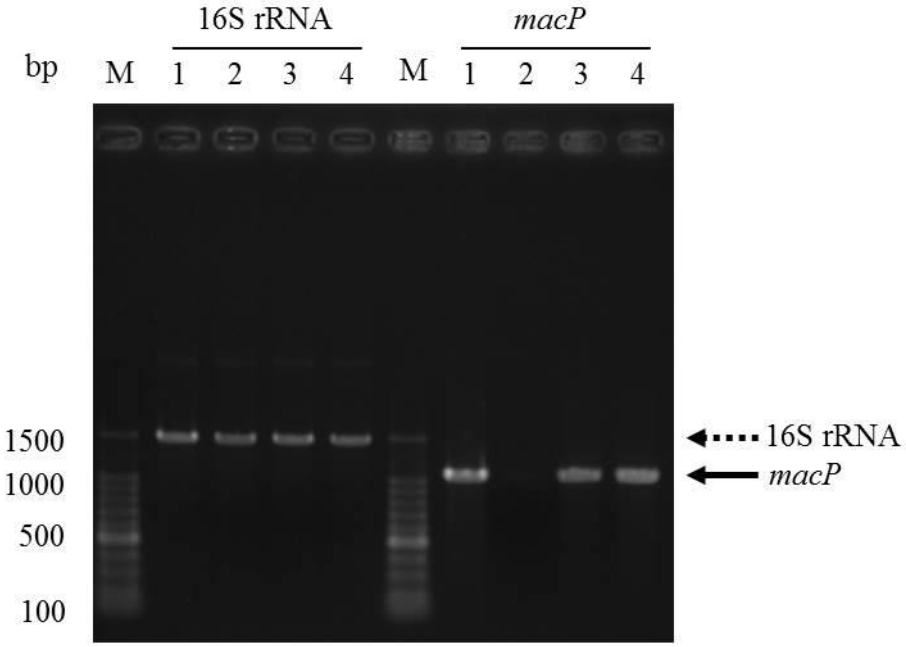

Figure 4.4 RT-PCR analysis of adenosine deaminase gene in strain MR-S7.

Genomic DNA of strain MR-S7 was used as a positive control (Lane 1). A cDNA from total RNA isolated from LB grown strain MR-S7 cell was used as the template (Lane 2). The product of reverse transcription from total RNA of penicillin G-supplemented strain MR-S7 cells were used as the templates (lanes 3). The product of reverse transcription from total RNA of OC_8-HSL-supplemented strain MR-S7 cells was used as the templates (lanes 4). The objective PCR-amplified gene fragment (right; 1,053 bp) was indicated by arrow. The PCR-amplified internal standard (16S rRNA) gene fragment (left; 1,484 bp) was indicated by dashed arrow. Lane M, molecular size markers.

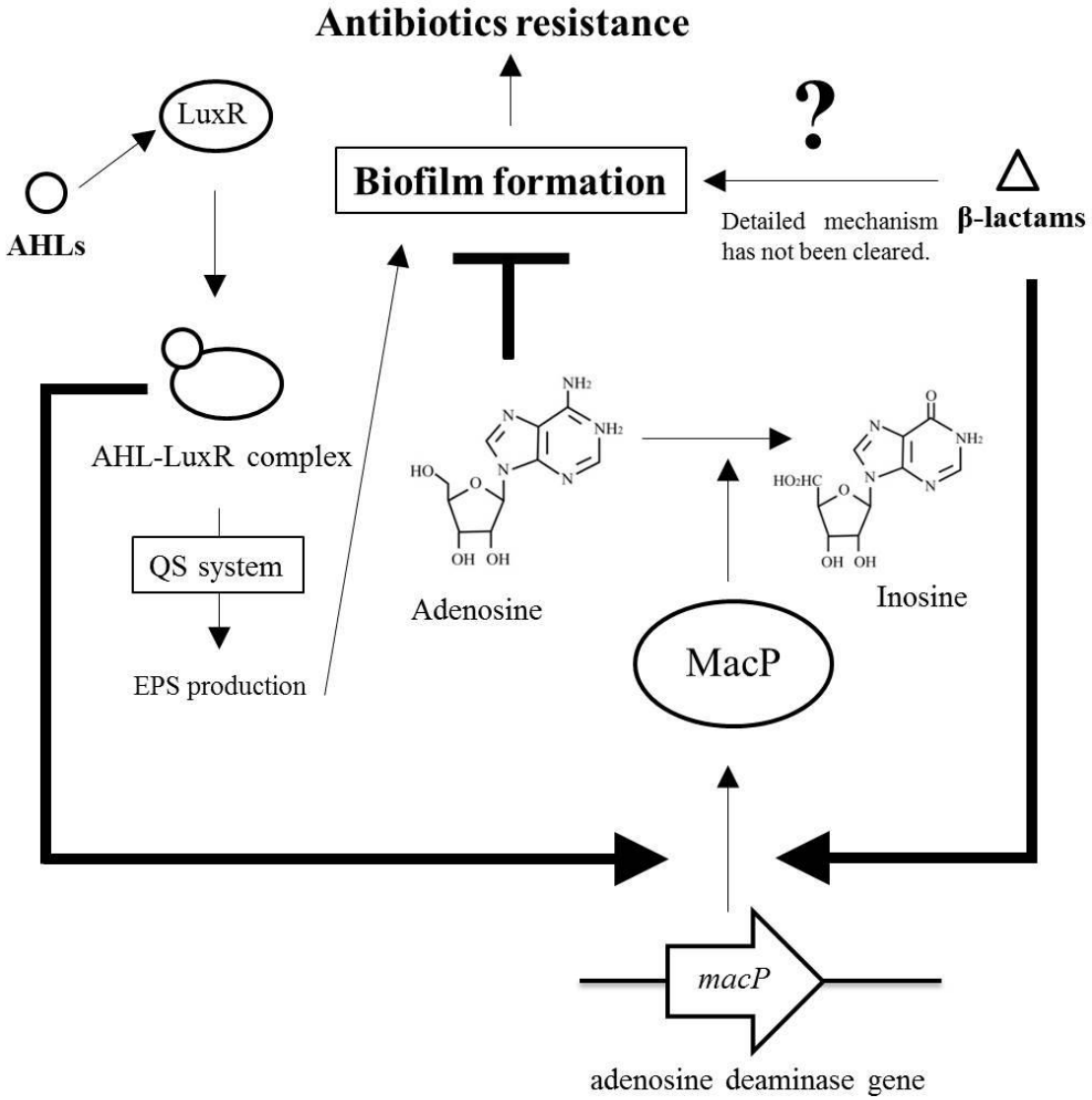

Figure 4.5 A hypothesized diagram of biofilm formation and the antibiotic resistant mechanism in strain MR-S7 inferred from this study.

Genes and proteins are represented as open arrows and ovals, respectively. Experimentally-verified positive and negative regulations are represented by solid arrows and blunt-ended lines, respectively. In particular, new insights into adenosine metabolism are indicated by heavy arrows.

References

Allesen-Holm, M., Barken, K.B., Yang, L., Klausen, M., Webb, J.S., Kjelleberg, S., Molin, S., Givskov, M. and Tolker-Nielsen, T. (2006) A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol Microbiol*, 59, 1114-1128.

Andersen, J.B., Heydorn, A., Hentzer, M., Eberl, L., Geisenberger, O., Christensen, B.B., Molin, S. and Givskov, M. (2001) *gfp*-based *N*-acyl homoserine-lactone sensor systems for detection of bacterial communication. *Appl Environ Microbiol*, 67, 575-585.

Cotton, L.A., Graham, R.J. and Lee, R.J. (2009) The role of alginate in *P. aeruginosa* PAO1 biofilm structural resistance to gentamicin and ciprofloxacin. *J. Exp. Microbiol. Immunol*, 13, 58-62.

De Kievit, T.R. (2009) Quorum sensing in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol*, 11, 279-288.

De Kievit, T.R., Gillis, R., Marx, S., Brown, C. and Iglewski, B.H. (2001) Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: their role and expression patterns. *Appl Environ Microbiol*, 67, 1865-1873.

Donlan, R.M. and Costerton, J.W. (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev*, 15, 167-193.

Haddadin, R.N., Saleh, S., Al-Adham, I.S., Buultjens, T.E. and Collier, P.J. (2010) The effect of subminimal inhibitory concentrations of antibiotics on virulence factors expressed by *Staphylococcus aureus* biofilms. *J Appl Microbiol*, 108, 1281-1291.

Heurlier, K., Denervaud, V. and Haas, D. (2006) Impact of quorum sensing on fitness of *Pseudomonas aeruginosa*. *Int J Med Microbiol*, 296, 93-102.

Huber, B., Riedel, K., Hentzer, M., Heydorn, A., Gotschlich, A., Givskov, M., Molin, S. and Eberl, L. (2001) The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. *Microbiology*, 147, 2517-2528.

Irie, Y., Mattoo, S. and Yuk, M.H. (2004) The Bvg virulence control system regulates biofilm formation in *Bordetella bronchiseptica*. *J Bacteriol*, 186, 5692-5698.

Irie, Y., O'Toole G, A. and Yuk, M.H. (2005) *Pseudomonas aeruginosa* rhamnolipids disperse *Bordetella bronchiseptica* biofilms. *FEMS Microbiol Lett*, 250, 237-243.

Kaplan, J.B., Izano, E.A., Gopal, P., Karwacki, M.T., Kim, S., Bose, J.L., Bayles, K.W. and Horswill, A.R. (2012) Low levels of beta-lactam antibiotics induce extracellular DNA release and biofilm formation in *Staphylococcus aureus*. *MBio*, 3, e00198-00112.

Kimura, N., Kitagawa, W., Mori, T., Nakashima, N., Tamura, T. and Kamagata, Y. (2006) Genetic and biochemical characterization of the dioxygenase involved in lateral dioxygenation of dibenzofuran from *Rhodococcus opacus* strain SAO101. *Appl Microbiol Biotechnol*, 73, 474-484.

Lerchner, J., Wolf, A., Buchholz, F., Mertens, F., Neu, T.R., Harms, H. and Maskow, T. (2008) Miniaturized calorimetry - a new method for real-time biofilm activity analysis. *J Microbiol Methods*, 74, 74-81.

Linares, J.F., Gustafsson, I., Baquero, F. and Martinez, J.L. (2006) Antibiotics as intermicrobial signaling agents instead of weapons. *Proc Natl Acad Sci U S A*, 103, 19484-19489.

Lynch, M.J., Swift, S., Kirke, D.F., Keevil, C.W., Dodd, C.E. and Williams, P. (2002) The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. *Environ Microbiol*, 4, 18-28.

Majtan, J., Majtanova, L., Xu, M. and Majtan, V. (2008) In vitro effect of subinhibitory concentrations of antibiotics on biofilm formation by clinical strains of *Salmonella enterica* serovar *Typhimurium* isolated in Slovakia. *J Appl Microbiol*, 104, 1294-1301.

Mirani, Z.A. and Jamil, N. (2011) Effect of sub-lethal doses of vancomycin and oxacillin on biofilm formation by vancomycin intermediate resistant *Staphylococcus aureus*. *J Basic Microbiol*, 51, 191-195.

Miura, T., Kusada, H., Kamagata, Y., Hanada, S. and Kimura, N. (2013) Genome sequence of the multiple-β-lactam-antibiotic-resistant bacterium *Acidovorax* sp. strain MR-S7. *Genome Announc*, 1, e00412-00413.

Nucleo, E., Steffanoni, L., Fugazza, G., Migliavacca, R., Giacobone, E., Navarra, A., Pagani, L. and Landini, P. (2009) Growth in glucose-based medium and exposure to subinhibitory concentrations of imipenem induce biofilm formation in a multidrug-resistant clinical isolate of *Acinetobacter baumannii*. *BMC Microbiol*, 9, 270.

Pamp, S.J. and Tolker-Nielsen, T. (2007) Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa*. *J Bacteriol*, 189, 2531-2539.

Pedersen, K. (1982) Method for studying microbial biofilms in flowing-water systems. *Appl Environ Microbiol*, 43, 6-13.

Sakuragi, Y. and Kolter, R. (2007) Quorum-sensing regulation of the biofilm matrix genes (*pel*) of *Pseudomonas aeruginosa*. *J Bacteriol*, 189, 5383-5386.

Sheng, L., Pu, M., Hegde, M., Zhang, Y., Jayaraman, A. and Wood, T.K. (2012) Interkingdom adenosine signal reduces *Pseudomonas aeruginosa* pathogenicity. *Microb Biotechnol*, 5, 560-572.

Shrout, J.D., Chopp, D.L., Just, C.L., Hentzer, M., Givskov, M. and Parsek, M.R. (2006) The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. *Mol Microbiol*, 62, 1264-1277.

Singh, P.K., Schaefer, A.L., Parsek, M.R., Moninger, T.O., Welsh, M.J. and Greenberg, E.P. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature*, 407, 762-764.

Steidle, A., Sigl, K., Schuhegger, R., Ihring, A., Schmid, M., Gantner, S., Stoffels, M., Riedel, K., Givskov, M., Hartmann, A., Langebartels, C. and Eberl, L. (2001) Visualization of

N-acylhomoserine lactone-mediated cell-cell communication between bacteria colonizing the tomato rhizosphere. *Appl Environ Microbiol*, 67, 5761-5770.