Regulation of Phenotypic Variations in *Pseudomonas aeruginosa*

May 2013

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Regulation of Phenotypic Variations in *Pseudomonas aeruginosa*

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 Environmental Studies

(Doctoral Program in Sustainable Environmental Studies)

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Chapter 1 General Introduction

1.1 *Pseudomonas aeruginosa* and its clinical significance

Pseudomonas aeruginosa is a ubiquitous microorganism existing in various environments, and it could be isolated from a variety of living sources such as plants, soil, animals and humans. *P. aeruginosa* has the ability of surviving limited nutritional habitats and has the tolerance for various conditions, allowing to persist in both community and hospital devices. In the hospital, *P. aeruginosa* can live on respiratory therapy settings, soap, sinks, mops, medicines, as well as hydrotherapy pools (1). In fact, *P. aeruginosa* is a non-dominant member of the normal microbial flora in humans. Representative colonization rates for specific sites in humans are 0 to 2% for skin, 0 to 3.3% for the nasal mucosa, 0 to 6.6% for the throat, and 2.6 to 24% for fecal samples (2) while they may exceed 50% during hospitalization (1) among patients who have suffered wound or damage in cutaneous and mucosal barriers by ventilation, tracheostomy, catheters, surgery, or severe burns (3-7). Immunocompromised patients have higher risks that are colonized by *P. aeruginosa* (1, 2), and disruption in the normal microbial flora as a result of antimicrobial therapy

has also been shown to increase colonization by *P. aeruginosa* (3, 8-9).

Despite *P. aeruginosa* distributed widely in the natural environments and the potential for the acquisition of community infections, the most serious infections with *P. aeruginosa* are obtained in hospital. *P. aeruginosa* was identified as the fifth most frequently isolated nosocomial pathogen, occupying 9% of all the nosocomial infections in the United States (10-11). Recent studies reveal *P. aeruginosa* as the second most common reason for nosocomial pneumonia, health care-associated pneumonia, and ventilator-associated pneumonia (12-13) and the main cause of pneumonia among pediatric patients in the intensive care unit (ICU) (14).

P. aeruginosa is very troublesome for seriously sick patients in ICUs. It was shown that *P. aeruginosa* was responsible for 21% of pneumonias, 13% of eye, ear, nose, and throat infections, 10% of urinary tract infections, and 3% of bloodstream infections within ICUs in the United States from 1992 to 1997 (15). Europe also executed a similar study, suggesting *P. aeruginosa* as the second most frequently isolated microorganism in reported cases of ICU-obtained infections (16). In this study, *P. aeruginosa* was responsible for 30% of pneumonias, 19% of urinary tract

infections, and 10% of bloodstream infections.

1.2 Current policies on the therapy of *P. aeruginosa* caused infections

One of the most serious chronic infections that caused by *P. aeruginosa* is cystic fibrosis (CF). There are thousands of different strains of *P. aeruginosa*, which can often be eliminated or kept at bay with early antibiotic treatment. However, there is some concern that people with CF may pick up strains of *P. aeruginosa* from each other that are more difficult to treat than those exist in the natural environment (17). Therefore, until now the developed countries such as England, Canada, and the United States have developed many specific policies for the treatment of CF. As a representative one, recently, England has passed a policy of inhaled therapy for adults and children with CF (18). This policy detailed the circumstances in which four named drugs (Aztreonam lysine, Colistimethate sodium, Dornase alpha and Tobramycin) to treat CF would be routinely funded. The policy specifically addressed the nebulized forms of these drugs. In this representative policy, the policy implementation guidance includes technology, provider type, current service currency, contracting implications, leader for implementation and recommended

implementation date.

In general, the policies for treating Pseudomonas aeruginosa caused CF infection are comprehensive and effective. However, there still exist some problems. First, most of the policies are scattered, which means until now there is no uniform standard for the treatment of CF between countries or even between different places in one country. This kind of scatter may lead to a mass and misguiding of doctors when they are carrying out the treatment for CF patients. Second, some policies' implementations are still not intact. For instance, the four Specialised Commissioning Groups (SCGs) in England agreed the policy for inhaled CF therapy (18), the implementation of which lack of current providers, impact of change, and financial implications. This kind of lack will lead to some possible hidden dangers in the future such as regulatory failure and the hospital may escape responsibility when medical dispute happens. Third, some detailed rules such as evidence summary should be more elaborate so that there is no leak to be exploited.

Therefore, for improving current policies for the treatment of CF patients, the developed countries could initiate and lead the countries all over the world to

formulate the uniform standards and different detailed policies for the treatment of CF that caused by *P. aeruginosa*. In the meanwhile, all the countries could build cooperated database and share the relative information from the network. In addition, some special centers such as basic infection research center, drug discovery center, and clinical trail center could be founded for better therapy, especially for the patient that got serious infections. In correspondence, supervision mechanism should be developed and improved continually in these non-profit organizations.

1.3 Quorum sensing and its regulation of virulence factors in *P. aeruginosa*

P. aeruginosa is a highly environment-adaptable bacterium with a large dynamic genome (18), approximately 10% of which is appropriate for regulatory factors including a sophisticated cell-to-cell signaling system in a cell density-dependent manner known as quorum sensing (QS) (19). QS describes the phenomenon whereby the accumulation of signaling molecules in the surrounding circumstance enables a single cell to sense the cell density, and therefore the population as a whole can make a coordinated response. The signal molecule regulates its own production

(autoinduction), leading to a positive feedback and greatly increased signal production. At critical cell densities, the binding of a regulator protein to the signal results in the switch on of genes controlled by QS and a coordinated population response (21). In *P. aeruginosa*, QS is essential for regulating swarming motility, virulence determinants production, biofilm formation, and the expression of antibiotic efflux pumps while the QS signal molecules involved also directly contribute to the outcome of host pathogen interactions.

There are two important QS systems in *P. aeruginosa*: the *N*-acyl-$_{L}$-homoserine lactones (AHLs) based system and the 2-alkyl-4-quinolone (AQ)-dependent system. The core of AHLs system consists of the *lasRI* and *rhlRI* genes where LasR and RhlR are members of the LuxR family of transcriptional regulators that specifically bind to *N*- (3-oxododecanoyl) homoserine lactone (3-oxo-C12-HSL) and N-butanoylhomoserine lactone (C4-HSL), respectively (Fig. 1) (22-23). The *las* and *rhl* systems regulate over 10% of the *P. aeruginosa* genome (23) and are organized as a hierarchy in which LasR/3-oxo-C12-HSL drives the expression of *lasI* as well as *rhlRI* (22). In addition, AQ-dependent QS system employs

2-heptyl-3-hydroxy-4-quinolone (PQS) and its precursor 2-heptyl-4-quinolone (HHQ) as signal molecules (Fig. 1) (24-25). Both AHLs based and AQ-dependent QS systems are involved in the regulation of a large number of genes, many of which code for virulence factors. For example, the *las* system controls the production of multiple virulence factors, including the LasA and LasB elastases, exotoxin A, and alkaline protease (26-29). The *rhl* system could induce the expression of several genes that regulating rhamnolipids production, and inhibiting those responsible for assembly and function of the type III secretion system (T3SS), a major virulence factor that allows the release of toxic proteins into the cytoplasm of eukaryotic cells (30). The AQ-dependent system controls the production of pyocyanin, lectin, rhamnolipids, and hydrogen cyanide (HCN) (31-32).

1.4 Aim of this study

Since *P. aeruginosa* is refractory especially during chronic infections, *P. aeruginosa* PAO1 as a typical laboratory strain has been studied for many years, providing many insights for therapy. However, the *P. aeruginosa* clinical isolates may possess different genomic information from PAO1 and consequently lead to

variations of phenotypes. Therefore, it is significative to study the regulation pathway of virulence determinants in the *P. aeruginosa* clinical isolates, which may quite differ from the PAO1 strain.

By using approaches of molecular microbiology, the aim of this study is to investigate if QS system functions variously in *P. aeruginosa* clinical isolates due to diverse phenotypes. In addition, as some chronic infections such as CF was reported to harbor regions with a steep oxygen gradient ranging from aerobic to anaerobic (33-34), and given the fact that *P. aeruginosa* is able to live anaerobically in the presence of alternative electron acceptors such as nitrate (NO_3^-) (35), virulence determinants and its regulation is also valuable to be researched under anaerobic conditions. My study will provide new theoretical basis for *P. aeruginosa* therapy and prompt the development of environmental and public health.

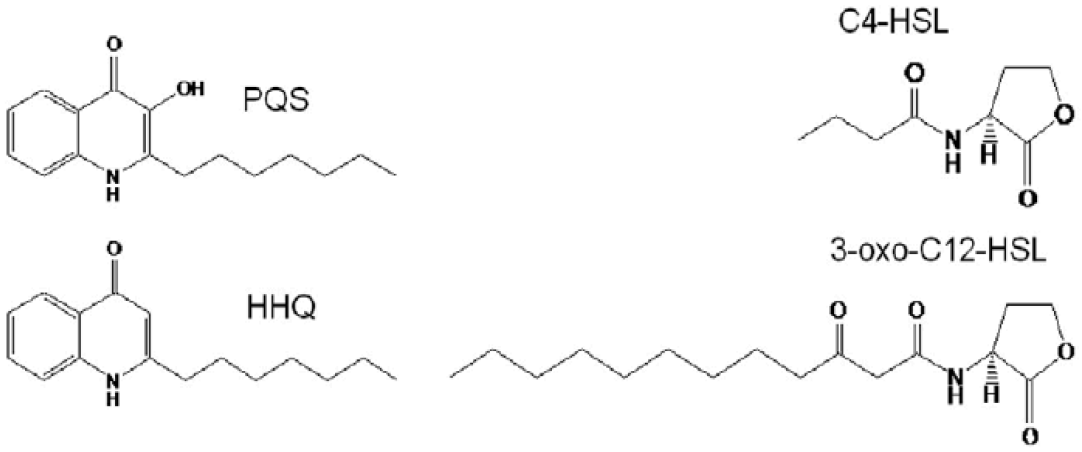


Figure 1. Structure of the *P. aeruginosa* QS signal molecules and related compounds.

Chapter 2 A New Perspective of *Pseudomonas* Quinolone Signaling

2.1 Introduction

P. aeruginosa employ sophisticated QS systems such as AQs-dependent system for regulating many social behaviors and phenotypes. In the AQs-dependent system, both PQS and its precursor HHQ play a role in cell-to-cell communication: after a certain threshold concentration of these two signal molecules in the extracellular medium is reached, they could bind their cognate receptor, PqsR (also named MvfR). However, PQS is shown significantly more potent than HHQ for both PqsR binding and activation; and PQS, but not HHQ is required for full pyocyanin production (25). In addition, PQS appears to have a number of other biologically important functions (Fig. 2.1) (36) such as iron delivery mediation (37) and biofilm formation (31) in *P. aeruginosa.* From these perspectives, PQS is more active and plays more important role in AQs-dependent system than HHQ does. Nevertheless, HHQ was reported highly produced in vivo, where it is not fully converted into PQS (36). This finding

may further indicate the importance of HHQ in *P. aeruginosa* pathogenesis. Although recently there is a report that HHQ modulates swarming motility in *P. aeruginosa* (38), the pathogenic role of HHQ is still not fully elucidated especially when anaerobically grown since PQS is not produced under such conditions (39-40).

Given that only *P. aeruginosa* produces PQS, while other *Pseudomonas* spp. and *Burkholderia* spp. rely on HHQ and other methylated 4-hydroxy-2-alkylquinoline analogues for 4-quinolone signaling (41-42), the role of HHQ in cell-to-cell signaling would become more evident and considerable. To this end, in this chapter several *P. aeruginosa* clinical isolates were used for the investigation of *Pseudomonas* quinolone signaling compared with laboratory strain PAO1. In my results, a *P. aeruginosa* clinical strain D4, which was isolated from mouse blood, showed much higher response to HHQ and less response to PQS compared with PAO1, due to the variations of PqsR and PqsH. My data suggested HHQ might possess a more clinical significance in *P. aeruginosa*, and this is a strain-dependent manner.

2.2 Materials and Methods

2.2.1 Bacteria strains, plasmids, and culture conditions

The strains and plasmids used in this chapter are listed in Table 2. Bacteria strains were grown routinely at 37°C in Luria-Bertani (LB) medium or on LB agar plates. When necessary, gentamicin was added at the concentration of 10 μg/ml for Escherichia coli and 80 μg/ml for *P. aeruginosa*. PQS and HHQ were synthesized and purchased from NARD institute, Ltd. (Hyogo, Japan). Before starting experimental cultures, *P. aeruginosa* was grown aerobically in 24-ml test tubes containing 4 ml of LB medium and was used to inoculate cultures at a starting optical density at 600 nm (OD600) of 0.01. For anaerobic cultures, strains were grown in 17-ml Hungate tubes containing 5 ml LBN medium (LB medium supplemented with 100 mM KNO_3) with shaking at 200 rpm and an initial OD600 of 0.001. The Hungate tubes were sealed with rubber stoppers, and the air was replaced with argon by flushing gas through a needle. pG19pqsA, pG19pqsH, and pG19pqsR plasmids carrying deletion cassettes of *pqsA*, *pqsH*, *pqsR* were transformed into PAO1, D4, 6-1, 6-2, and 6-3 by conjugating with *E. Coli* S17-1 (43), followed by homologous recombination previously described

(44). The mutants were analyzed by PCR.

2.2.2 Measurement of pyocyanin production

Pyocyanin was extracted from 48 h aerobically static culture supernatants and measured by a previously reported method (45-46). Briefly, 0.6 ml of chloroform was added to 1 ml of culture supernatant. After extraction, the chloroform layer was transferred to a fresh tube and mixed with 200 µl of 0.2 N HCl. After centrifugation, 100 µl of the top layer (0.2 N HCl) was measured its absorbance at 520 nm.

2.2.3 PQS assay

PQS was collected from the supernatant and detected by thin-layer chromatography (TLC) analysis, following the method previously described (39). 12 h shaking or 48 h static aerobic cultures were centrifuged for 3 min at 13,200 rpm to collect the supernatants. PQS was extracted from 1 ml of supernatants with 0.6 ml acidified ethyl acetate. The ethyl acetate portion was collected into a new tube and air-dried. Extracts were resuspended in 30 µl of 1:1 acidified ethyl acetate/acetonitrile. Aliquots of extracts were loaded on TLC plates (silica gel 60 F254; Merck) that had

been soaked in 5% KH2PO4 for 30 min and activated at 100℃ for 1 h. The extracts were separated using 17:2:1 methylene chloride/acetonitrile/1,4-dioxane as the solvent. 50 μM synthetic PQS was used as a control. Photographs were taken under UV light at 366 nm.

2.2.4 PQS and HHQ semi-quantification

The plasmids pMpqsAG and pMEXpqsA were transformed into PAO1, D4 and their *pqsA* and *pqsH* mutants. All reporter strains were cultured aerobically or anaerobically for 12 h with shaking at 200 rpm and appropriate antibiotic (40 μg/ml of gentamicin). The strains contained pMEXGFP or pMEX9 were used as aerobic or anaerobic negative control. For the aerobic cultures, 200 μl of each culture was pipetted into 96-well microplate (Iwaki, Japan) and a Varioskan Flash spectral scanning multimode reader (Thermo Scientific) was used for reading the fluorescence at an excitation wavelenth of 488 nm and emission wavelenth of 509 nm as well as OD_{600}. For the anerobic cultures, Catechol 2,3-dioxygenase (C23O) specific activity (the *xylE* gene product) was determined by reading the absorbance change at OD_{375} per min (44). When measuring the PQS or HHQ response to PAO1 and D4, PQS or

HHQ were added into *pqsA* muants of PAO1 and D4 cultures with a final concentration range from 10 nM to 50 μM.

2.2.5 Quantitative real-time PCR

P. aeruginosa PAO1, D4, 6-1, 6-2, 6-3 and their *pqsH* or *pqsR* mutants were grown aerobically or anaerobically for 12 h. Total RNA were extracted by using RNeasy Mini Kit (QIAGEN) and all the procedures were followed by the instruction of the manufacturer. Residual DNA was eliminated by DNase treatment using 20 U Recombinant DNase I (TAKARA) at 37℃ for 30 min. cDNA was synthesized by using SuperScript^{TM} III Reverse Transcriptase (Invitrogen). The primers pqsARTFw and pqsARTRv were used for quantitative real-time PCR. The procedures of real-time PCR are as follows: holding stage 95°C, 10 min; cycling stage 95°C 15', 60 °C 1 min for 40 cycles; melt curve stage 95 °C 15', 60 °C 1 min, (+0.3 °C) 95 °C 15'. Data represented relative *rplU* expressions.

2.2.6 Sequencing and protein blast

The genes of *pqsR* in PAO1 and D4 were sequenced by Hokkaido System

Science Co., Ltd. By using the primers cpqsRF and cpqsRR. Protein blast was carried out on the website of National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blastho me).

2.3 Results

During the 48 h aerobically static cultures, *pqsH* mutant of D4 produces much higher pyocyanin than *pqsH* mutant of PAO1 (Fig. 2.2). As PQS is not produced in a *pqsH* mutant in *P. aeruginosa* (Fig. 2.3A), this result indicates that the strain of D4 may respond to HHQ within the AQ-dependent system. Therefore, the aerobic *pqsA* promoter activities were investigated in PAO1 and D4 after 12 h culture (late stationary phase). The result clearly showed that pqsA promoter activity is higher in *pqsH* mutant of D4 than that in *pqsH* mutant of PAO1 (Fig. 2.4A). According to the previous report, PQS is not produced under anaerobic conditions (39). This may lead to my speculation that D4 triggers higher *pqsA* transcription than PAO1 when anaerobically grown. Indeed, The result of C23O activities revealed that *pqsH* mutant

of D4 induces more *pqsA* expression than *pqsH* mutant of PAO1 does (Fig. 2.4B). These two *pqsA* promoter activities results were confirmed by using quantitative real-time PCR (Fig. 2.5A and 2.5B). However, no similar result was observed in other clinical isolates: *pqsH* mutants of 6-1, 6-2, and 6-3 revealed that pqsA expressions are the same level as *pqsH* mutant of PAO1 (Fig. 2.5C), indicating that high *pqsA* expression in clinical isolate is in a strain-dependent manner. In addition, *pqsR* gene seldom expresses in both D4 and PAO1 under aerobic or anaerobic conditions, suggesting that PqsR indeed involved in inducing the expression of *pqsA*, which is accordance with previous study (25). Given this truth, there may exist a PqsR mutation in the strain of D4. The result of PqsR protein blast between PAO1 and D4 exhibited that there is a variation in PqsR of D4 compared with PAO1 (Fig. 2.6). From this result, I speculate that the variation in PqsR of D4 leads to a less response to PQS and higher HHQ response in triggering *pqsA* expression in D4 than PAO1, and this speculation was confirmed by adding PQS into *pqsA* mutant and HHQ into *pqsApqsH* double mutant (Fig. 2.7A and 2.7B). Another reason for the less PQS response in D4 lies in that the ability of PqsH converts HHQ to PQS is lower in D4

than PAO1. 50 μM and 100 μM HHQ were added into *pqsA* mutants of PAO1 and D4, PQS assay results showed that PQS production was less in *pqsA* mutant of D4 (Fig. 2.3B). All the results indicated that HHQ plays a more important role in cell-to-cell communication in *P. aeruginosa* clinical isolate D4.

2.4 Discussion

Pyocyanin is a very important virulence factor that produced by *P. aeruginosa* and regulated by the AQ-dependent system. It was reported that PQS, but not HHQ, is responsible for the full pyocyanin production (25). Until now, people believe that pyocyanin was seldom produced in a *pqsH* mutant in *P. aeruginosa*. Surprisingly, it was observed that when grown aerobically in static LB cultures, the *pqsH* mutant of D4 still produces high level of pyocyanin, indicating that HHQ response may be higher in this strain compared with other *P. aeruginosa* such as PAO1.

Although both PQS and HHQ were demonstrated dual ligands of PqsR, PQS is 100-fold more potent than HHQ in activating *pqsA-E* transcription (25), which means PQS is a dominant signal molecule within AQ-dependent QS system. However, both the promoter reporter assay and quantitative real-time PCR results revealed *pqsA*

expression in a *pqsH* mutant of D4 is unexpectedly higher than its counterpart of PAO1 (Fig. 2.4A and 2.5A). Further investigation demonstrated that HHQ is still much more active for autoinduction in D4 under anaerobic conditions (Fig. 2.4B and 2.5B). It was reported HHQ was produced more than PQS *in vivo* (25), indicating its pathogenesis of *P. aeruginosa* infections. Interestingly, the localizations of PQS and HHQ seem to be different, as PQS, but not HHQ, is primarily found in membrane vesicles (47). Hence, HHQ may follow a different pathway in regulating virulence factors such as pyocyanin *in vivo*. Although it is still hard to say this is due to adaptation for host environments, my observation gives a new perspective on the *Pseudomonas* quinolone signaling system that HHQ could play a more important role in triggering *pqsA* transcription in *P. aeruginosa* through a strain-dependent manner. Variation of PqsR and low PQS converting capability of PqsH in D4 strain also revealed PQS may not always play a main signal molecule role in some *P. aeruginosa* clinical isolates. In the case of D4 strain, HHQ may occupy a very important position compared with PQS and other AQs.

Strains, plasmids, and primers	Relevant characteristics	Source or reference
Strains		
E. coli		
S17-1	Mobilizer strain	43
P. aeruginosa		
PAO1	WT	39
Δ*pqsA*-PAO1	PAO1 mutant with a deletion in the *pqsA* gene	39
Δ*pqsH*-PAO1	PAO1 mutant with a deletion in the *pqsH* gene	39
Δ*pqsR*-PAO1	PAO1 mutant with a deletion in the *pqsR* gene	39
Δ*pqsA*Δ*pqsH*-PAO1	PAO1 mutant with a deletion in the *pqsA* and *pqsH* genes	This study
D4	WT	This study
Δ*pqsA*-D4	D4 mutant with a deletion in the *pqsA* gene	This study
Δ*pqsH*-D4	D4 mutant with a deletion in the *pqsH* gene	This study
Δ*pqsR*-D4	D4 mutant with a deletion in the *pqsR* gene	This study
Δ*pqsA*Δ*pqsH*-D4	D4 mutant with a deletion in the *pqsA* and *pqsH* genes	This study
6-1	WT	This study
Δ*pqsH*-6-1	6-1 mutant with a deletion in the *pqsH* gene	This study
6-2	WT	This study
Δ*pqsH*-6-2	6-2 mutant with a deletion in the *pqsH* gene	This study
6-3	WT	This study
Δ*pqsH*-6-3	6-3 mutant with a deletion in the *pqsH* gene	This study
Plasmids		
pG19II	pK19mobsac derived suicide vector; *sacB* Gm^r	44
pG19pqsA	*pqsA* deletion cassette in pG19II	39
pG19pqsH	*pqsH* deletion cassette in pG19II	39
pG19pqsR	*pqsR* deletion cassette in pG19II	39
pMEXGFP	pME4510 derived promoter-probe vector; *egfp* Gm^r	This study
pMpqsAG	*pqsA* promoter region in pMEXGFP	This study
pMEX9	pME4510 derived promoter-probe vector; *xylE* Gm^r	39
pMEXpqsA	*pqsA* promoter region in pMEX9	39
Primers		
ΔpqsAF1	5’-GGTCTAGAGGCAAGGTGCAACAATGGACAGTGG-3’	39
ΔpqsAR2	5’-GCGAAGCTTGGAAGTTCACAGGTGATCGCTGCC-3’	39
pqsHF	5’-CCCAAGCTTCTTGTCCTGCAGGTCGATATCC-3’	39
pqsHR	5’-GCTCTAGATCGAGAGCTTCTCGAAGATGCG-3’	39
cpqsRF	5’-GCTCTAGAACCCAATAAAAGGAATAAGGGATGC-3’	39
cpqsRR	5’-CCCAAGCTTGAACGCTCTACTCTGGTGCGG-3’	39
pqsARTFw	5’-CCTGGTGGTGCGTGAAGCC-3’	This study
pqsARTRv	5’-CGTCGAGCAAAGGGCGTCC-3’	This study

Table 2. Bacteria strains, plasmids, and primers

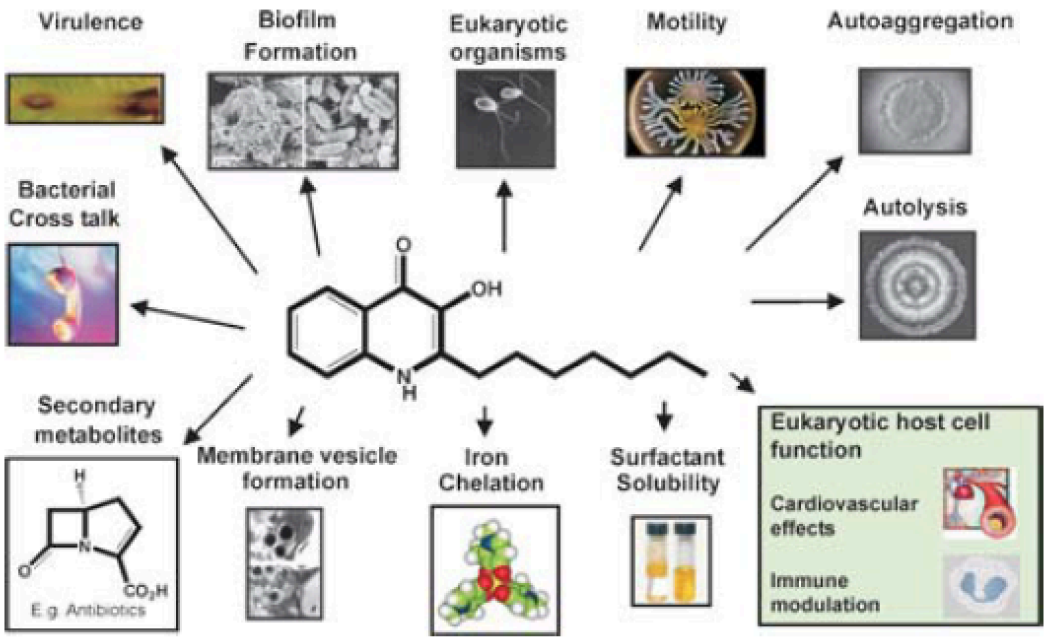


Figure 2.1. Biological roles of PQS and phenotypes known to be regulated by PQS in *P. aeruginosa*

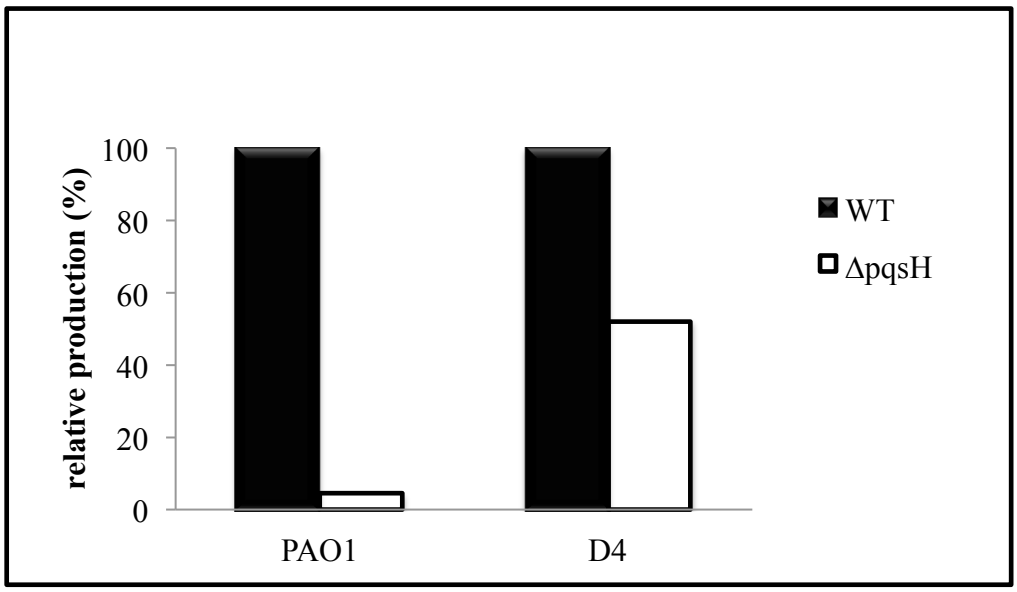
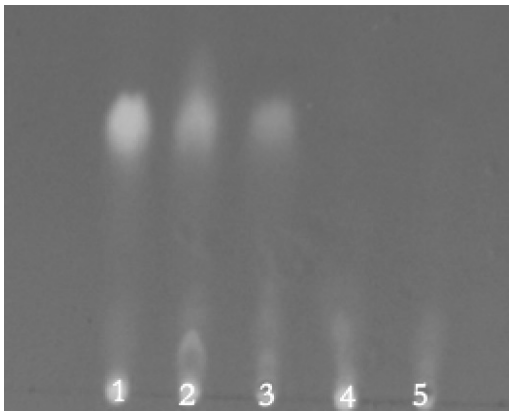


Figure 2.2. Pyocyanin production in *pqsH* mutants of PAO1 and D4 after 48 h static cultures

A



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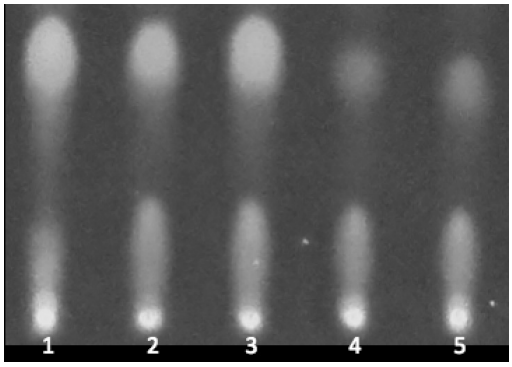
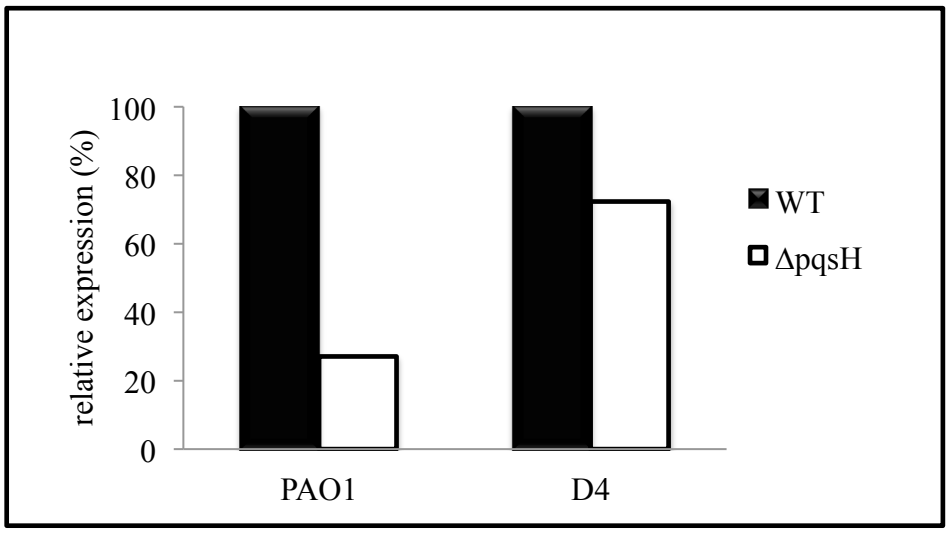


Figure 2.3. A) PQS production under 48 h static cultures. 1, 50 μM PQS control; 2, PAO1; 3, D4; 4, *pqsH* mutant of PAO1; 5, *pqsH* mutant of D4 **B)** PQS production after 12 h 200 rpm shaking cultures. 1, 50 μM PQS control; 2, 50 μM HHQ added into *pqsA* mutant of PAO1; 3, 100 μM HHQ added into *pqsA* mutant of PAO1; 4, 50 μM HHQ added into *pqsA* mutant of D4; 5, 100 μM HHQ added into *pqsA* mutant of D4

A



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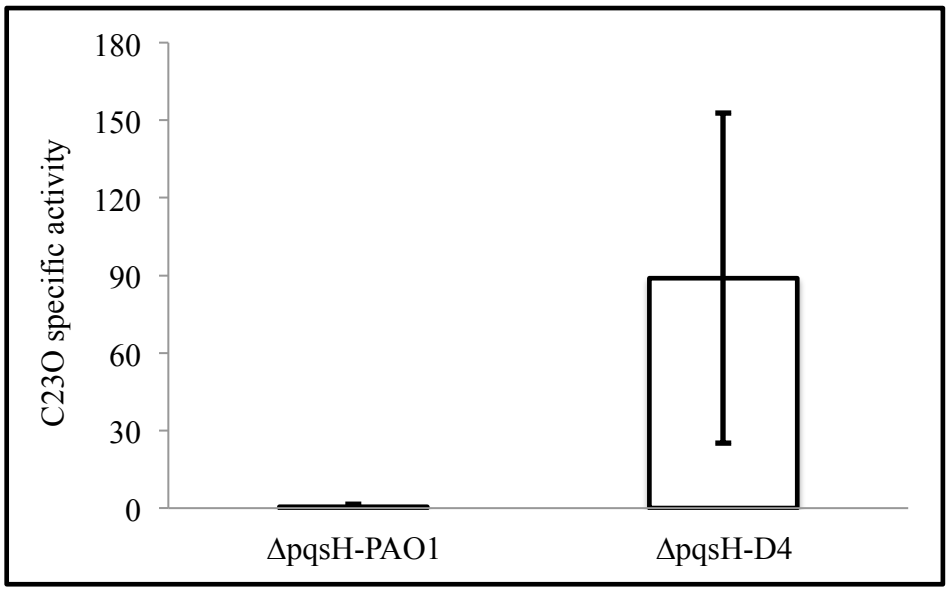
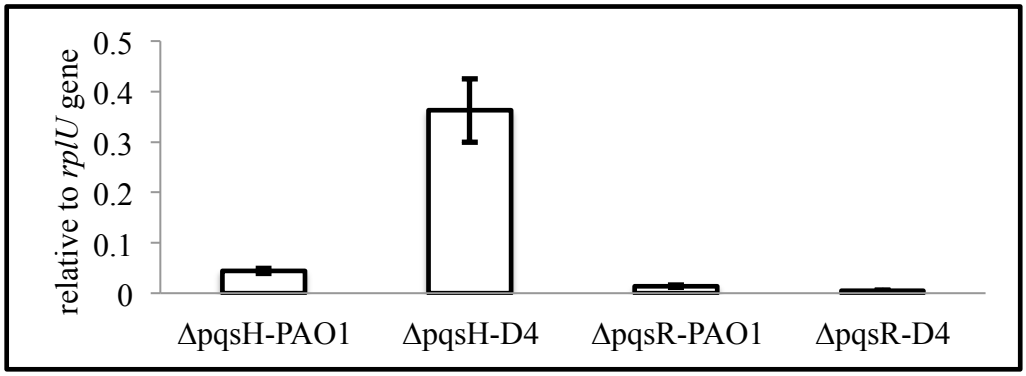


Figure 2.4. A) *pqsA* promoter activities in *pqsH* mutants of PAO1 and D4 under 12 h aerobic culture conditions; **B)** *pqsA* promoter activities in *pqsH* mutants of PAO1 and D4 under 12 h anaerobic culture conditions

A



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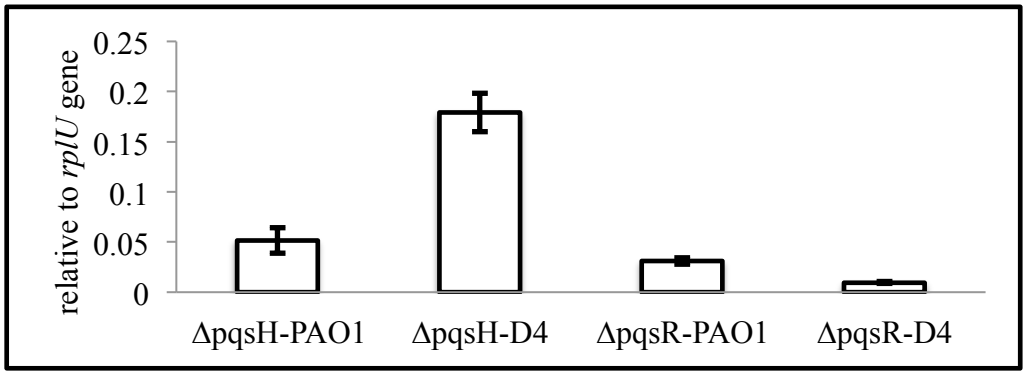

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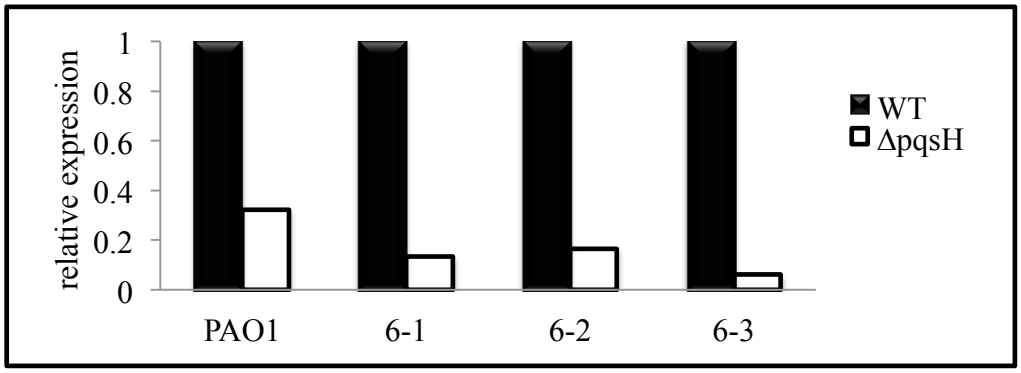


Figure 2.5. Quantitative real-time PCR results: **A)** *pqsA* expression in *pqsH* and *pqsR* mutants of PAO1 and D4 under 12 h aerobic conditions; **B)** *pqsA* expression in *pqsH* and *pqsR* mutants of PAO1 and D4 under 12 h anaerobic conditions; **C)** *pqsA* expression in *pqsH* mutants of PAO1, 6-1, 6-2 and 6-3 under 12 h aerobic conditions

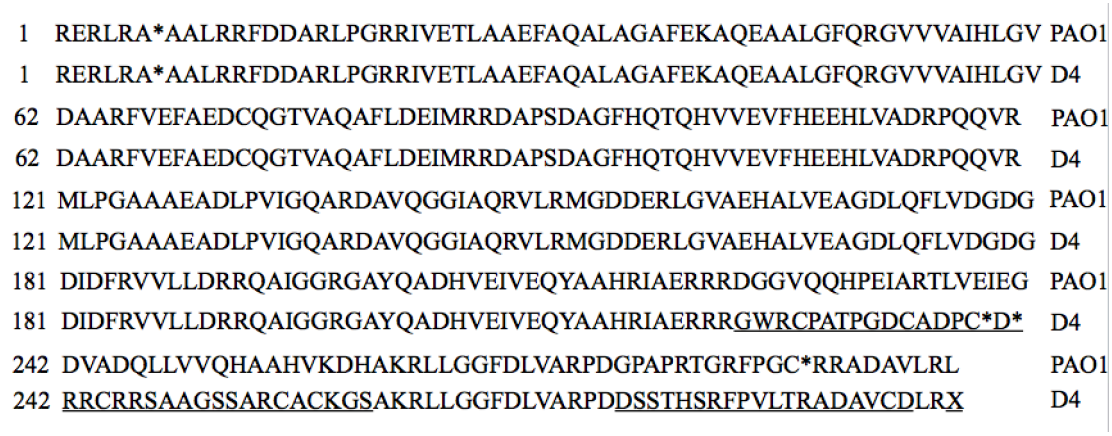
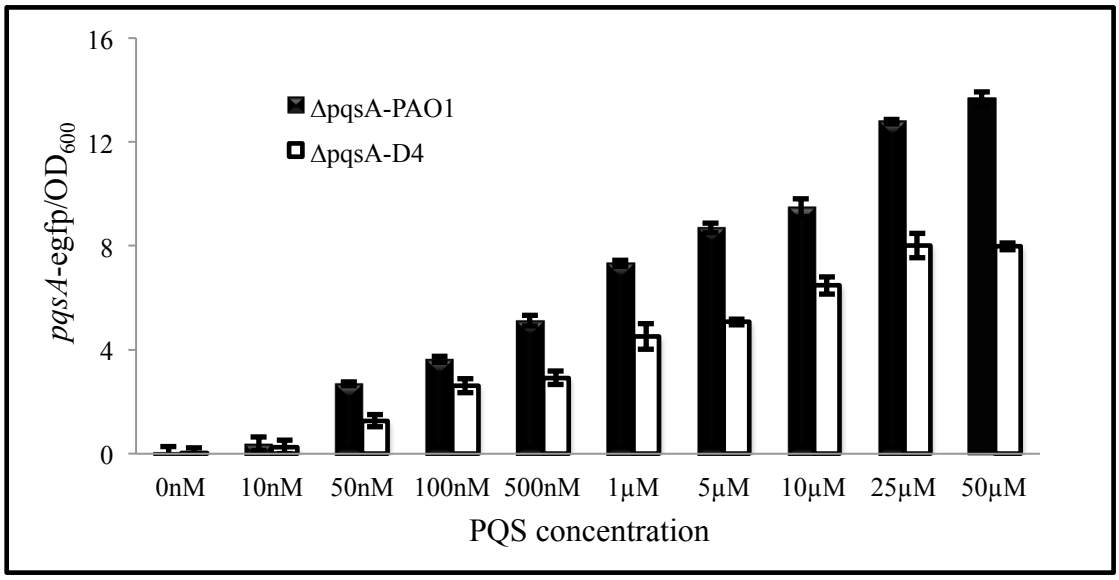


Figure 2.6. PqsR protein blast between PAO1 and D4, variances are underlined.

A



B

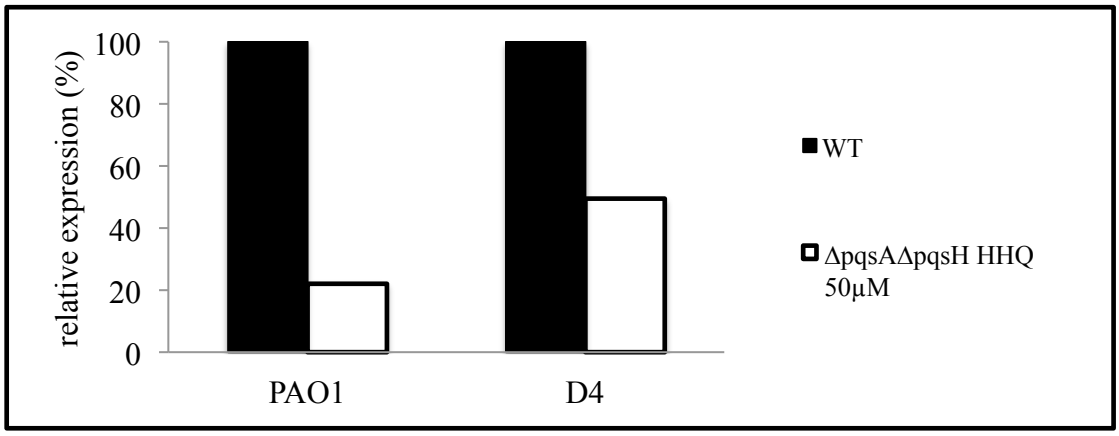


Figure 2.7. A) Series of PQS response to PAO1 and D4; **B)** 50 μM HHQ response to PAO1 and D4

Chapter 3 The Impact of Anaerobiosis on Strain-dependent Phenotypic Variations in *P. aeruginosa*

3.1 Introduction

P. aeruginosa is a denitrifying bacterium capable of anaerobic growth by utilizing N-oxides as terminal electron acceptors under low-oxygen conditions (48). It is reported that denitrification is crucial for the pathogenicity of *P. aeruginosa* (49-50), while how *P. aeruginosa* exhibit pathogenicity under anaerobic conditions remains poorly understood. Under aerobic conditions, extracellular virulence factors such as elastase, protease, pyocyanin, rhamnolipids, exotoxin A, and siderophores (24, 51-55) are produced that are mainly regulated by a cell density-dependent regulatory manner termed as QS (56).

So far, three QS systems in *P. aeruginosa* have been well characterized: the *las*, *rhl* and *pqs* systems. The *las* system is comprised of the transcriptional regulator LasR and its cognate AHL signal, 3-oxo-C_{12}-HSL, synthesized by the AHL synthase LasI (28, 57). The *rhl* system is comprised of RhlR and its cognate AHL, C_4-HSL,

synthesized by the RhlI synthase (58-59). Besides, *P. aeruginosa* produces a third signaling molecule, 2-heptyl-3-hydroxy-4(1*H*)-quinolone, termed *Pseudomonas* Quinolone Signal (PQS) (24). While these QS systems regulate the virulence factors under aerobic conditions, PQS synthesis requires oxygen, and AHL dependent QS systems are reported to be attenuated by an unknown mechanism under anaerobic conditions (60). Consistent with these observations, the productions of QS regulated virulence factors are significantly reduced under anaerobic conditions and *P. aeruginosa* PAO1 becomes avirulent compared to aerobic conditions (61).

Another important phenotype in the pathogenicity of *P. aeruginosa* is the formation of biofilms. Biofilms are highly organized microbial communities that are embedded in a self-produced extracellular matrix. Usually biofilms confer high antibiotic resistant compared to their planktonic counterpart (62). During infections such as cystic fibrosis, *P. aeruginosa* is exposed to a microaerobic to anaerobic environment (63-64), while little is known about biofilm formation under anaerobic conditions. Interestingly, *P. aeruginosa* forms more robust biofilm when grown anaerobically than formed during aerobic conditions (65). A recent study revealed

that biofilm formation undergoes a different process from aerobic conditions when grown anaerobically where cell elongation is critical (66).

By comparing several clinical isolates as well as PAO1, here I demonstrate that the impact of anaerobiosis vary greatly among the strains. Cell growth, morphology, extracellular virulence production, AHL production as well as biofilm formation under anaerobic conditions were strain-dependent showing diverse phenotypes among the strains. Interestingly some clinical isolates formed thick biofilms under anaerobic conditions that do not depend on the cell elongation process. Hence, social behaviors of *P. aeruginosa* could be regulated by several pathways in a strain-dependent manner and may impact their adaptation to such conditions. The data presented here will provide a better understanding of anaerobiosis in *P. aeruginosa* as well as their pathogenicity.

3.2 Materials and Methods

3.2.1 Bacterial strains, plasmids, and culture conditions

Six *P. aeruginosa* clinical strains, which were isolated from Toho University Omori Hospital, together with *P. aeruginosa* PAO1 and its Δ*lasI*Δ*rhlI* double mutant, were grown at 37℃ aerobically in 24-ml test tubes containting 4 ml of LB medium or anaerobically in 17-ml Hungate tubes containing 5 ml LBN medium with shaking at 200 rpm. For anaerobic cultures, the Hungate tubes were sealed with rubber stoppers, and the air was replaced with argon by flushing gas through a needle. The *lasI* and *rhlI* transcriptional fusion plasmid were constructed by cloning the promoter region of *lasI* and *rhlI* into the multicloning site of pMEXGFP reproter plasmid, using the primers as follows: lasI F (5'- CCGGAATTCCAGAAAGTTTCCTGGCTTTCC-3'), lasI R (5'- TTCAAGCTTCACTTGAGCACGCAACTTGT-3'), rhlI F (5'- CCGGAATTCGAACATCCAGAAGAAGTTCGA-3'), rhlI R (5'- CCCAAGCTTAAAAGGCGGCATCCCTAC-3') (Restriction sites were underlined) (67). The constructed plasmids, named pMlasIG and pMrhlIG, were transformed into Δ*lasI* and Δ*rhlI* mutants of *P. aeruginosa* PAO1 by electroporation (39). All

experiments were carried out at an initial optical density at 600 nm (OD_{600}) of 0.01.

3.2.2 Microscopy

The *P. aeruginosa* PAO1 and clinical isolates were grown aerobically or anaerobically for 12 h at 37°C with shaking at 200 rpm. 5 µl of each culture was fixed and stained on a glass slide (VWR, USA) and observed using a Zeiss Axio Observer. Z1 microscope. Images were captured using an AxioCam digital camera and processed with Zeiss Axiovision 4.8 software.

3.2.3 Enzyme activity assays

Elastolytic activity was measured by elastin Congo red (ECR) assay (68) with some modifications. Briefly, 12 h aerobic or anaerobic *P. aeruginosa* culture supernatants were filtered (0.2 µm pore-size filter). 1-ml cell-free supernatants were added to 15-ml centrifuge tubes containing 10 mg of ECR (Sigma) and 2 ml of buffer (0.1 M Tris, pH 7.2, 1 mM $CaCl_2$). After 18 h incubation at 37°C with rotation, tubes were placed on ice and 0.1 ml of 0.12 M EDTA was added into each tube to stop the reaction. Insoluble ECR was removed by centrifugation and units of elastase activity

were expressed as the increase at OD_{495} per milligram of protein. Proteolytic activity was determined as preiously described (69) with azocasein (Sigma) as the substrate, and units of protease activity were expressed as the increase at OD_{400} per milligram of protein. Protein concentraion was measured in triplicate by the Bradford method (70).

3.2.4 AHL semi-quantification

1 ml *P. aeruginosa* cell-free supernatants were collected from aerobic or anaerobic cultures incubated for 12 h, and mixed with 3 ml of LB medium where AHL reporter strains were inoculated. AHL reporter strains Δ*lasI* (containing pMlasIG) and Δ*rhlI* (containing pMrhlIG) were grown in the mixture above for 12 h at 37°C with appropriate antibiotic (40 μg/ml of gentamicin). The strain of Δ*lasI* or Δ*rhlI* which contained pMEXGFP vector was used as negtive control, respectively. 200 μl of each culture was pipetted into 96-well microplate (Iwaki, Japan) and a Varioskan Flash spectral scanning multimode reader (Thermo Scientific) was used for reading the fluorescence at an excitation wavelenth of 488 nm and emission wavelenth of 509 nm as well as OD_{600}.

3.2.5 Biofilm assay

For biofilm formation assay, *P. aeruginosa* were inoculated (1:100) in LB or LBN medium with an overnight culture of each strain, and the diluted bacteria suspensions were added into 96-well sterile flat-bottom polystyrene tissue culture plates (Costar, USA) (71). After 8 h aerobic incubation or 16 h anaerobic incubation at 37℃, the nonattached and loosely adherent bacteria were removed by discarding the medium and the cells, and then washed 3 times with distilled water. 100 μl of a 0.1% solution of crystal violet (Sigma) was added to each well and the plates were incubated at room temperature for 15 min to stain the biofilm. Excess stain was removed by distilled water washing and the plates were air-dried. The crystal violet staining the attached cells was solubilized with 200 μl of 95% ethanol, and 150 μl was removed and added in a fresh polystyrene microtiter plate to determine the A_{595}.

3.3 Results

3.3.1 Anaerobic growth and cell morphology variations

When grown aerobically in LB medium, strains 6-1, 6-2, and 6-3 revealed approximately 20% less growth than the other strains in the stationary phase (Fig.

3.1A). However, when grown anaerobically, the differences in growth expanded where the growth yields of strains 6-1, 6-2, and 6-3 were apparently lower than that of PAO1, and strains D4, 7-3 and 7-17 grew better than PAO1 (Fig. 3.1B). While *P. aeruginosa* is a rod-shaped cell under aerobic conditions, PAO1 was reported to be highly elongated when grown anaerobically (66). This result was also verified in our microscopic analysis (Fig. 3.2A and 3.2B). In contrast to PAO1, strain 7-17 (Fig 3.2C and 3.2D) and the other five clinical isolates remained rod cells when grown anaerobically. Thus, high diversity of growth and cell morphology was observed under anaerobic conditions.

3.3.2 Comparison of extracellular virulence factors

P. aeruginosa could produce many virulence factors such as elastase and protease under aerobic environments, that is controlled by QS (59, 72-73). Under anaerobic conditions, it was reported that elastase production is significantly repressed while anaerobiosis is explained to be important for infecting the host (60). In order to better understand the capability of this bacterium to express virulence factors under anaerobic conditions, I investigated the elastase and protease activities

of the clinical isolates and PAO1 (Fig. 3.3). A Δ*lasI*Δ*rhlI* double mutant of PAO1 was used as a negative control. Consistent with previous reports (60-61), when grown under anaerobic conditions, elastase activity was significantly reduced in PAO1. In addition, protease activity decreased to approximately 20 % compared to aerobic conditions. Interestingly, strain 7-17 possessed comparable activity of the virulence factors under anaerobic conditions and aerobic conditions. The other five clinical isolates had low activities in these virulence factors under both conditions. As expected, elastase and protease activity was hardly detected in the PAO1 Δ*lasI*Δ*rhlI* double mutant confirming that QS regulate these extracellular virulence factors in PAO1. Taken that these virulence factors are regulated by QS, the high production of these virulence factors in strain 7-17 indicated that this strain has a high ability of QS under anaerobic conditions, which was further examined.

3.3.3 AHL production under anaerobic conditions

Since PQS can not be produced in the absence of oxygen (39), I focused on the production of two AHLs, 3-oxo-C_{12}-HSL and C_4-HSL. AHL production was measured by examining promoter activity of QS regulated genes in an AHL reporter

strain (74). To this end, a promoter region of *lasI* or *rhlI* was fused to enhanced green florescent protein (eGFP) on the plasmid. The supernatant of each culture was collected and added to the reporter strains in which eGFP expression was measured. The Δ*lasI*Δ*rhlI* double mutant of PAO1 was used as a negative control. Consistent with the above results (Fig. 3.3), high levels of 3-oxo-C_{12}-HSL and C_4-HSL production were observed in the strain 7-17 under both aerobic and anaerobic conditions (Fig. 3.4A and 3.4B). Particularly, C_4-HSL production under anaerobic conditions was 3.12 fold higher in 7-17 than PAO1, and was only reduced to 71.9% in 7-17 whereas it was reduced to 26.7 % in PAO1 compared to aerobic conditions (Fig 3.4B). The other clinical isolates seldom produced AHLs under anaerobic conditions though AHLs were detected form aerobic supernatants. Hence, QS activity is strongly reduced in these five strains under anaerobic conditions. These results demonstrate that anaerobiosis impact QS activity in a strain-dependent manner.

3.3.4 Strain-dependent diversity of biofilm formation

It is well known that biofilm formation is involved in *P. aeruginosa* chronic infection since biofilm can resist antibiotic therapy, host immune responses, and

biocide treatment (75). It was reported that cell-elongation contribute to biofilm formation under anaerobic conditions (66), however, as shown above, the clinical isolates used in our study exhibited rod-shaped cell morphology under anaerobic conditions (Fig. 3.2). Therefore, I further examined biofilm formation of these strains. Similar to the observation in planktonic growth, the clinical isolates were rod-shaped in biofilms under anaerobic conditions (Fig. 3.5). Interestingly, 7-3 and 7-17 produced more biofilm than PAO1 under anaerobic conditions, although these strains except PAO1 do not elongate (Fig. 3.6). Strain 6-13 produced less biofilm than PAO1 and cell elongation may contribute to biofilm formation in this strain. The strains also showed varied biofilm formation under aerobic conditions suggesting that some of the strain have a high ability to form biofilm. The high ability of these strains to form biofilm may be the reason why cell elongation does not take part in the biofilm formations of these strains. These result demonstrate that biofilm formation vary among the strains and further suggest that the biofilm formation process differ among the cells under anaerobic conditions.

3.4 Discussion

P. aeruginosa can exist under anaerobic conditions and I demonstrate here that anaerobiosis has a great impact on the strain-dependent variation.

Most strikingly, although it was believed that in *P. aeruginosa* QS is attenuated under anaerobic conditions, and therefore becomes avirulent compared to aerobic conditions (60), my data demonstrate that the extracellular virulence factors are produced anaerobically in a clinical isolate 7-17. This strain exhibited high AHL production under anaerobic conditions compared to the other strains, suggesting that high amount of AHL production induced the expression of the virulence factors. This hypothesis is in accordance that the exogenous addition of AHL signaling molecules can restore the transcription of target genes under anaerobic conditions in PAO1 (39, 60). Interestingly, a number of clinical isolates scarcely produced AHLs when grown anaerobically. This is not due to mutation in the QS system as reported previously in clinical isolates (76) since AHL was detected when grown aerobically. Thus, there is a large variation in AHL production under anaerobic conditions, suggesting a complex regulatory system under these conditions.

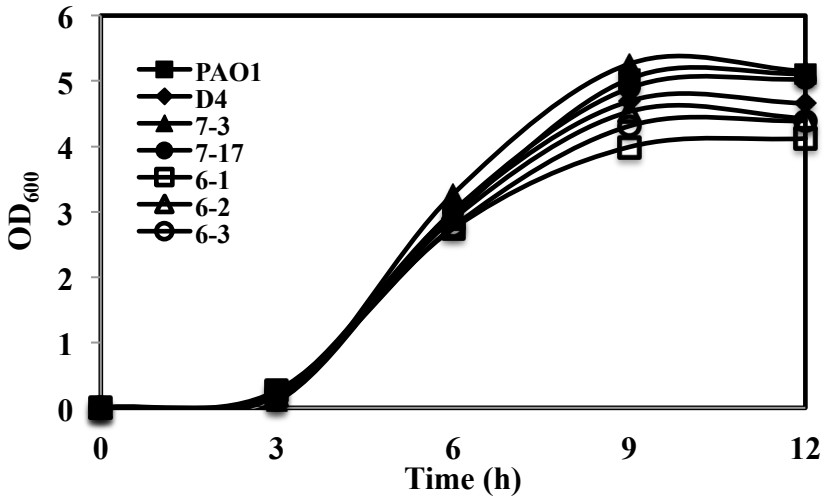
Although *P. aeruginosa* is a rod-shaped cell, the PAO1 strain becomes filamentous under anaerobic conditions. NO induces elongation of the cell where it inhibits DNA synthesis (66). It was reported that the cell elongation contribute to biofilm formation under anaerobic conditions in PAO1 and biofilm formation of a non-elongated *nirS* mutant is significantly reduced (66). To my surprise, unlike PAO1, clinical isolates remained rod-shaped cells under anaerobic conditions. When biofilm formation was examined among these strains, the rod-shaped clinical isolates were able to form biofilms. These data demonstrate that the clinical isolates depend on another mechanism than cell-elongation in biofilm formation under anaerobic conditions. The involvement of extracellular polysaccharides, as well as flagella and pili in biofilm formation under aerobic conditions is well studied (77-78) and these components could be the main factors determining biofilm formation in the rod-shaped clinical isolates under anaerobic growth.

Because NO induce cellular elongation under anaerobic conditions in PAO1 (66), the difference in the cell morphology under this condition may due to different NO accumulation among the strains. NO is an intermediate of denitrification and the

growth variation among the isolates suggest that they have different denitrifying activity. Compared to aerobic respiration, denitrification is a relatively complex process where four terminal electron acceptors are utilized (79). In addition to the master regulators (ANR and DNR) of denitrification (80-81), QS is reported to affect denitrifying activity that leads to the control of NO accumulation (82). Hence, the different QS ability among the strains could be one of the reasons that lead to the variation of anaerobic growth. Obviously, other factors could be involved since a clear correlation of AHL production and growth was not observed.

Strain-dependent variation in a bacterial population has become of interest in term of evolution as well as pathogenicity. *P. aeruginosa* is known to have a highly diverse genome structure mainly caused by accessory DNA elements (83). A recent report demonstrated that the QS regulon is strain-dependent suggesting an important role for QS in niche adaptation (84). Most of the works comparing *P. aeruginosa* strains are limited to aerobic conditions and my study would extend the studies to how anaerobiosis impacts the phenotypic variation.

A



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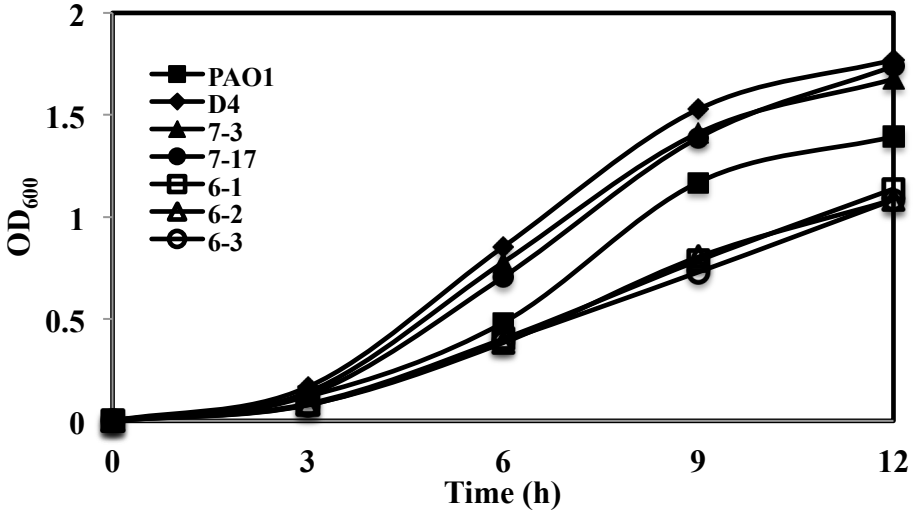


Figure 3.1. Growth of *P. aeruginosa* PAO1 and the clinical isolates under **A)** aerobic conditions and **B)** anaerobic conditions. Three independent experiments were conducted, and representative data are shown

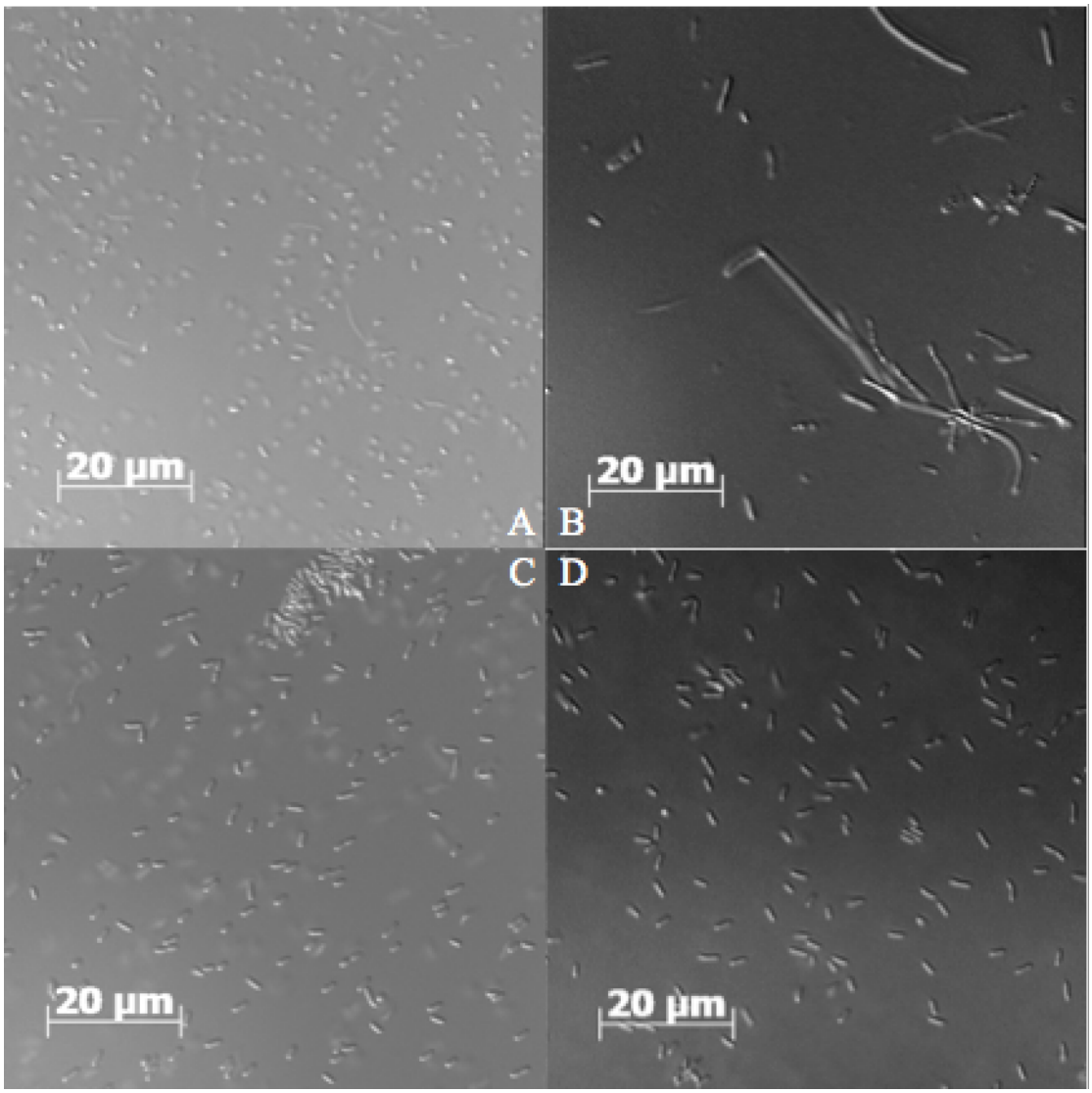
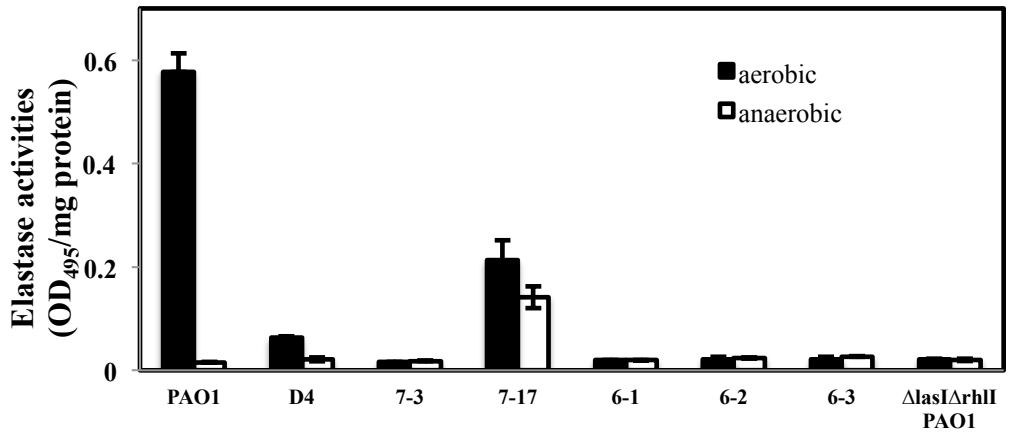


Figure 3.2. Microscopic observations between *P. aeruginosa* PAO1 and 7-17. **A)** PAO1 under aerobic conditions; **B)** PAO1 under anaerobic conditions; **C)** 7-17 under aerobic conditions; **D)** 7-17 under anaerobic conditions

A



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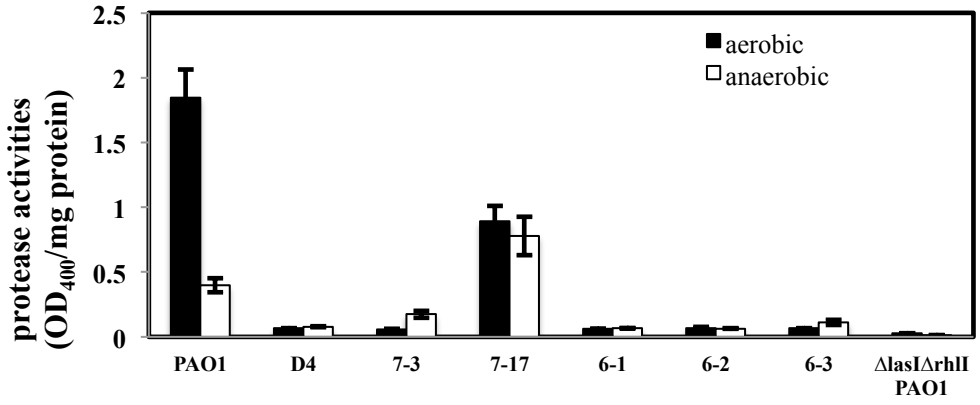
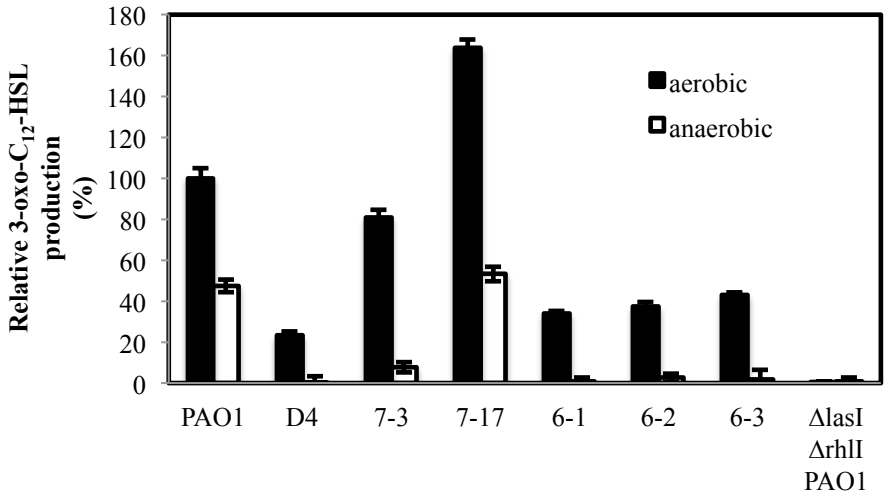


Figure 3.3. Extracellular virulence productions: **A)** elastase activities and **B)** protease activities in *P. aeruginosa* PAO1 and clinical isolates under 12 h aerobic and anaerobic conditions. Data were expressed as means ± standard deviations (error bars) of three replicates

A



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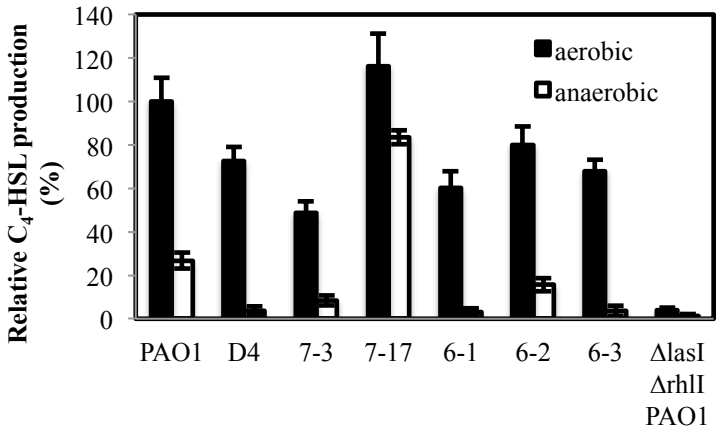
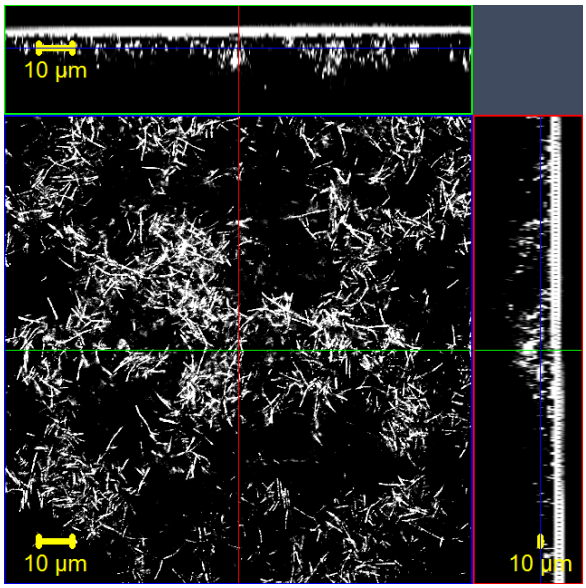


Figure 3.4. 3-oxo-C_{12}-HSL **A)** and C_4-HSL **B)** production in aerobic and anaerobic supernatants of *P. aeruginosa* PAO1 and clinical isolates. Supernatants of each strain were collected and AHL production was measured by using AHL reporter strains as described in materials and methods. Data represent relative AHL productions to PAO1 aerobic conditions. Data were expressed as means ± standard deviations (error bars) of three replicates.

A



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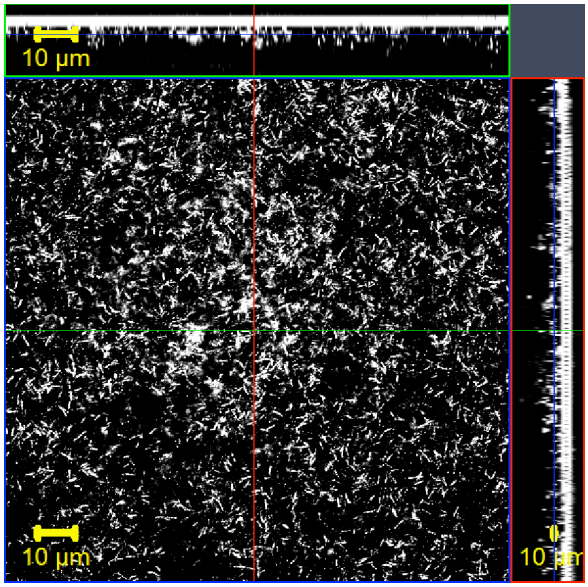

Figure 3.5. Biofilms observation after 16 h anaerobic culture conditions between **A)** PAO1 and **B)** 7-17 strains.

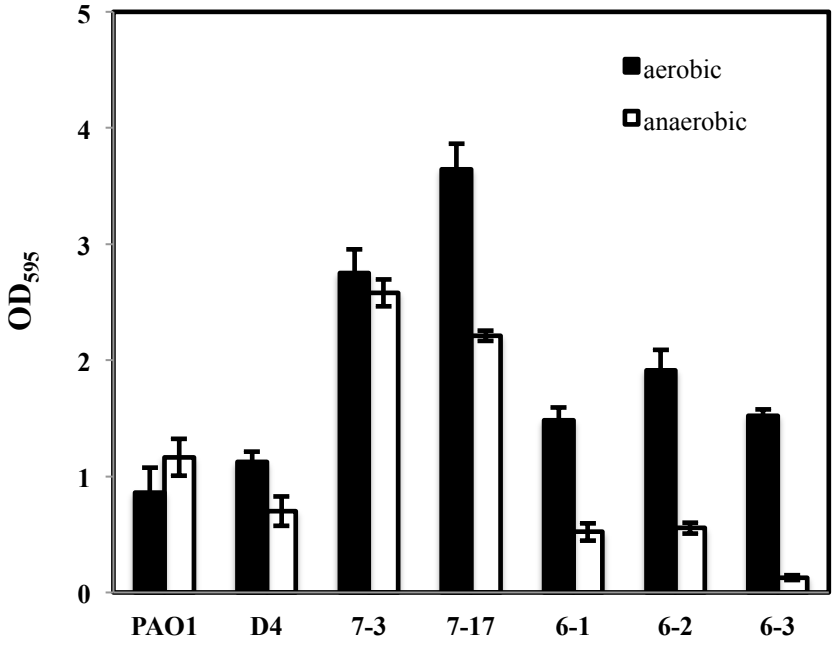

Figure 3.6. Aerobic and anaerobic biofilm formations in *P. aeruginosa* PAO1 and clinical isolates. Biofilm formations were measured at 8 h for aerobic cultures and 16 h for anaerobic cultures. Data were expressed as means ± standard deviations (error bars) of eight replicates.

Chapter 4 Conclusions

As a notorious human pathogen, *P. aeruginosa* produces many virulence determinants, most of which could be regulated by complicated QS systems. To elucidate the regulating mechanism of virulence determinants by QS will help to develop better means to control the pathogenic *P. aeruginosa* that use social interactions to affect humans and provide new insights for the therapy of *P. aeruginosa* related infections.

In this study, PQS and HHQ, as two main signal molecules within AQs-dependent QS system, were investigated with respect to their response to *P. aeruginosa* PAO1 and other clinical isolates under both aerobic and anaerobic culture conditions. In addition, cell growth, morphology, extracellular virulence production, AHL production as well as biofilm formation between PAO1 and these clinical isolates under anaerobic conditions were also explored.

In chapter 1, my results suggest that the *P. aeruginosa* clinical isolate D4 *pqsH* mutation strain shows high *pqsA* expression under aerobic and anaerobic conditions, indicating its response to HHQ as a prevailing *Pseudomonas* quinolone signal

molecule for cell-to-cell communication. Variation of PqsR and low PQS converting capability of PqsH in D4 strain also revealed HHQ, but not PQS, may play a main signal communication role in some *P. aeruginosa* clinical isolates. This divergence of signaling provides a new perspective of QS systems and HHQ may contribute more for the pathogenesis of *P. aeruginosa.*

In Chapter 2, my data revealed that when grown anaerobically, growth and cell morphology greatly differ among the strains. 7-17 strain produced comparable amount of QS signaling molecules and extracellular virulence factors under aerobic and anaerobic conditions, while the other strains had low production under anaerobic conditions. Biofilm formation also exhibited strain-dependent variations suggesting that there are several mechanisms that lead to biofilm formation under anaerobic conditions. Taken together, this chapter demonstrates that the anaerobiosis impact on social interactions of *P. aeruginosa* is strain dependent and implies that multiple regulatory mechanisms may be involved in the regulation of QS and biofilm formation under anaerobic conditions.

On the whole, in this study *P. aeruginosa* clinical isolates exhibit diversity of QS

signal molecules response and virulence factors as well as other phenotypes, suggesting *P. aeruginosa* may vary with the changing of survival circumstances.

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Abstract

Pseudomonas aeruginosa is an oppotunanistic human pathogen, which could secrete many extracellular virulence determinants that regulated by a cell density dependent quorum sensing (QS). The 2-alkyl-4-quinolone (AQ)-dependent system regulates pyocyanin production, a blue redox-active secondary metabolite that related to some chronic infections such as cystic fibrosis. In this study, unlike the *pqsH* mutant of PAO1, the *pqsH* mutant of *P. aeruginosa* clinical isolate D4 still produce high level of pyocyanin, indicating its response to HHQ. To investigate the PQS and HHQ differential between the clinical isolate and wild type in activating PqsR function in *P. aeruginosa*, *pqsA* expression was measured under both aerobic and anaerobic conditions. Accordance with the pyocyanin production, *pqsA* expression was higher in the *pqsH* mutant of D4. Variation of PqsR and low PQS converting capability of PqsH in D4 strain were also observed. All these results suggest that HHQ may occupy a very important position compared with PQS and other AQs in a strain dependent manner.

Bacteria perform social behaviors by communicating with each other and

forming surface-associated biofilms. In *P. aeruginosa,* such social behaviors are affected greatly by the environment. Although *P. aeruginosa* survive under anaerobic conditions, previous studies indicate that QS is attenuated under anaerobic conditions, which will lead to less production of extracellular virulence factors compared to the aerobic conditions. Hence it has become a question whether *P. aeruginosa* are virulent under anaerobic conditions or not. Here, I compared different phenotypes between the PAO1 and clinical isolates under anaerobic conditions. My data revealed that when grown anaerobically, growth and cell morphology greatly differ among the strains. One of the clinical isolates produced comparable amount of QS signaling molecules and extracellular virulence factors under aerobic and anaerobic conditions, while the other strains had low production under anaerobic conditions. Biofilm formation also exhibited strain-dependent variations suggesting that there are several mechanisms that lead to biofilm formation under anaerobic conditions.

On the whole, in this study *P. aeruginosa* clinical isolates exhibit diversity of QS signal molecules response and virulence factors as well as other phenotypes, suggesting *P. aeruginosa* may vary with the changing of survival circumstances. This

study will provide new insights of theoretical basis for *P. aeruginosa* therapy and prompt the development of environmental and public health.

Acknowledgments

I would like to express my sincere thanks and appreciation to the following who in one way or another, have contributed to the completion of this study:

- To Professor **Nobuhiko NOMURA**, my supervisor, for his valuable insights, kind help and support during the whole period of study, not only the academic issues, but also my daily life in Japan.
- To Assistant Professor **Masanori TOYOFUKU**, for his direction, guidance, suggestion and fruitful discussion during the whole study. Without his patient guidance, I could not accomplish this study.
- To Professor **Hiroo UCHIYAMA** and Professor **Zhenya ZHANG**, for their kind and sincere help in my study and daily life.
- To Professor **Maki TSUJIMURA**, Professor **Naomi WAKASUGI**, Associate Professor **Xiaogang SUN**, and all the members of **Environmental Diplomatic Leader Program**. They made me more confident and active during the three years' study. I really learned many skills not only technologies but also social communications, which are useful for the whole of my life.
- To Dr. **Yuanbai PAN**, my tutor in the University of Tsukuba. He supported me a lot during the whole life in Japan.
- To **China Scholarship Council**, for the scholarship that makes this study possible.
- To **Ryosuke SAKAI**, **Kei OOURA**, **Satoshi ITO**, **Keiji NAGATA**, **Jiayue YANG**, and all other members of my laboratory, for their daily support and guidance on my study.
- To my family, for their love and encouragement all through these years.

Publication

Fang H, Toyofuku M, Kiyokawa T, Ichihashi A, Tateda K, and Nomura N. The impact of anaerobiosis on strain-dependent phenotypic variations in *Pseudomonas aeruginosa*. *Biosci. Biotech. Bioch.* 2013 (accepted on 21st, May, 2013)