The biofilm lifestyle of comamonas in environmental applications : mechanistic studies and environmental implications

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NANYANG
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**The Biofilm Lifestyle of *Comamonas* in
Environmental Applications: Mechanistic
Studies and Environmental Implications**

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Abstract

Bacteria of the *Comamonas* genus are often present in various engineered and natural ecosystems, in particular, the aromatic compounds-contaminated and denitrifying environments. Intriguingly, they are usually found to be associated with surface or interface as multicellular aggregates known as biofilms. Extensive previous research efforts have been focused on the versatile catabolic capabilities of *Comamonas*; in contrast, little is known about their biofilm lifestyle and biofilm-mediated environmental processes. The objective of this study was to understand the biofilm lifestyle of *Comamonas* in environmental processes and the influence of environmental conditions. Specifically, using *C. testosteroni* as a model organism, this thesis work focuses on elucidating the influences of (i) a model aromatic pollutant 3-chloroaniline (3-CA), (ii) nitrate, and (iii) extracellular biomacromolecules in wastewater on the biofilm lifestyle of *Comamonas* and their environmental implications.

Bioaugmentation of *C. testosteroni* has been frequently proposed in wastewater and soil treatment to remove toxic aromatic compounds. The performance of bioaugmentation is affected by a number of biological and environmental factors including the interaction between the target pollutant and the augmented bacterial cells. The first part of this thesis work explored the influence of a toxic aromatic pollutant 3-CA on the biofilm lifestyle of *C. testosteroni* capable of degrading the aromatic compound toward a better understanding of cell-pollutant interaction in bioaugmentation. The results showed that the exposure to 3-CA greatly reduced the retention of *C. testosteroni* cells in packed-bed bioreactors, which could be attributed to the altered bacterial motility and cell surface hydrophobicity. Through an integrated genomic and transcriptomic analysis, we found that exposure to 3-CA reduced the intracellular c-di-GMP level by downregulating the expression of genes encoding c-di-GMP synthases and induced massive cell dispersal from the biofilms. As the reduced c-di-GMP caused biofilm dispersal, we attempted to develop robust biofilm processes by elevating c-di-GMP level. To achieve this objective, the *C. testosteroni* strain was constructed which constitutively expressing a c-di-GMP synthase YedQ. Under batch growth

conditions with a high surface to volume ratio, an elevated c-di-GMP concentration in *C. testosteroni* significantly increased the contribution of biofilms in 3-CA biodegradation. In continuous submerged biofilm reactors, *C. testosteroni* with an elevated c-di-GMP level exhibited an enhanced 3-CA biodegradation and a decreased cell detachment rate.

In wastewater microbial communities, bacteria of the *Comamonas* genus were often reported to be among the most abundant microorganisms. In addition, a high abundance of *Comamonas* was also found in nitrate-removing microbial communities presented in river wetlands, soil, and constructed wetlands. However, why they are there and what roles they may play remain largely unknown. In this thesis work, we demonstrated the involvement of *Comamonas* biofilms in denitrification under bulk aerobic conditions and elucidated the influence of nitrate respiration on its biofilm lifestyle. Our results showed that *C. testosteroni* could use nitrate as sole electron acceptor for anaerobic growth. Under bulk aerobic condition, biofilms of *C. testosteroni* were capable of reducing nitrate and, intriguingly, nitrate reduction significantly enhanced viability of the biofilm-cells and reduced cell detachment from the biofilms. Nitrate respiration was further shown to play an essential role in maintaining high cell viability in the biofilms. RNA-seq analysis, quantitative polymerase chain reaction (qPCR), and liquid chromatography-mass spectrometry (LC-MS) revealed a higher level of bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) in cells respiring on nitrate than those grown aerobically (1.3×10^{-4} fmol/cell vs. 7.9×10^{-6} fmol/cell; $P < 0.01$). C-di-GMP is one universal signaling molecule that regulates the biofilm mode of life and a higher c-di-GMP concentration reduces cell detachment from biofilms. Taken together, this study reveals that nitrate reduction occurs in mature biofilms of *C. testosteroni* under bulk aerobic conditions and the respiratory reduction of nitrate is beneficial to the biofilm lifestyle by providing more metabolic energy to maintain high viability and a higher level of c-di-GMP to reduce cell detachment.

Wastewater bacteria of the *Comamonas* genus were often found to form cell aggregates in activated sludge-based wastewater treatment. However, the isolates rarely form flocs in synthetic wastewater, a chemically defined medium that is formulated based on key physicochemical characteristics of real wastewater, *e.g.*,

pH, ionic strength, chemical oxygen demand (COD), and total nitrogen/phosphorus ($\text{NH}_4\text{-N}$ and $\text{PO}_4\text{-P}$). The discrepancy observed for bacterial floc formation in real wastewater and synthetic wastewater suggests an important role of neglected, unknown components, *i.e.*, “dark matter”, in real wastewater. In this study, using *C. testosteroni* as a model organism, we examined the effect of microfiltered (pore size of 0.22 μm) and ultrafiltered (molecular weight cut-off (MWCO) of 225 kDa) wastewater on bacterial floc formation. Combining the floc formation observations with the Fourier Transform Infrared Spectroscopy (FTIR) analyses and enzymatic treatment, we reported for the first time that high molecular weight fraction of the soluble EPS, in particular, proteins and extracellular DNA, are the major floc-forming inducers in wastewater. The underlying mechanism was further characterized by using the DLVO theory. In the presence of soluble EPS, cell surface was found to become more hydrophobic and the energy barrier disappeared, leading to irreversible attachment and floc formation. Therefore, the physicochemical effect of soluble EPS in real wastewater should not be neglected in wastewater biofilm processes which facilitated the initial biofilm formation.

List of Publications

- **Yichao Wu**, Anee Mohanty, Wu Siang Chia, and Bin Cao (2016) Influence of 3-Chloroaniline on the Biofilm Lifestyle of *Comamonas testosteroni* and its Implications on Bioaugmentation. *Applied and Environmental Microbiology* 82: 4401-4409.
- **Yichao Wu**, Sudha Shukal, Manisha Mukherjee, and Bin Cao (2015) Involvement in Denitrification is Beneficial to the Biofilm Lifestyle of *Comamonas testosteroni*: A Mechanistic Study and its Environmental Implications. *Environmental Science & Technology* 49: 11551-11559.
- **Yichao Wu** and Bin Cao (2015) Assessment of Bacterial Survival in the Presence of Nanomaterials: Is Colony Forming Unit Count Sufficient? *Environmental Engineering Science* 32.12: 977-977. **(Featured Articles)**
- **Yichao Wu**, Krithika Arumugam, Martin Qi Xiang Tay, Hari Seshan, Anee Mohanty, and Bin Cao (2015) Comparative genome analysis reveals genetic adaptation to versatile environmental conditions and importance of biofilm lifestyle in *Comamonas testosteroni*. *Applied Microbiology and Biotechnology* 99: 3519-3532.
- **Yichao Wu**, Yuanzhao Ding, Yehuda Cohen, and Bin Cao (2015) Elevated level of the second messenger c-di-GMP in *Comamonas testosteroni* enhances biofilm formation and biofilm-based biodegradation of 3-chloroaniline. *Applied Microbiology and Biotechnology* 99: 1967-1976.
- Yidan Hu, **Yichao Wu**, Manisha Mukherjee, and Bin Cao (2017) Near-infrared light responsive c-di-GMP module-based AND logic gate in *Shewanella oneidensis*. *Chemical Communications* in press.
- Zhe Yang, **Yichao Wu**, Jianqiang Wang, Bin Cao, Chuyang Tang (2016) In situ reduction of silver by polydopamine: a novel antimicrobial modification of thin-film composite polyamide membrane. *Environmental Science & Technology* 50.17: 9543-9550.
- Yang Yun, **Yichao Wu**, Yidan Hu, Yingxiu Cao, Chueh Loo Poh, Bin Cao, and Hao Song (2015) Engineering electrode-attached microbial consortia for

- high-performance xylose-fed microbial fuel cell. *ACS Catalysis* 5: 6937–6945.
- Yangyang Yu, **Yichao Wu**, Bin Cao, Yong-Gui Gao, and Xiaoli Yan (2015) Adjustable bidirectional extracellular electron transfer between *Comamonas testosteroni* biofilms and electrode via distinct electron mediators. *Electrochemistry Communications* 59: 43-47.
 - Anee Mohanty, **Yichao Wu**, and Bin Cao (2014). Impacts of engineered nanomaterials on microbial community structure and function in natural and engineered ecosystems. *Applied Microbiology and Biotechnology* 98: 8457-8468.

Oral Presentations

- **Yichao Wu**, Xinhua Wang, Seungdae Oh, Chuyang Tang, Bin Cao (2016) Influence of Elevated Salinity and the Presence of Cytostatic Drugs on Microbial Community in FO Anaerobic Membrane Bioreactors, 2016 American Institute of Chemical Engineers (AIChE) Annual Meeting, Nov 13-18, San Francisco, America.
- Manisha Mukherjee, **Yichao Wu**, Nishanth V Menon, Yuejun Kang, Bin Cao (2016) Revealing membrane biofouling dynamics using a novel fluidic membrane biofilm flow cell, 2016 International Conference of Microfluidics, Nanofluidics and Lab-on-a-Chip, June 10-12, Dalian, China (**Invited talk**).
- **Yichao Wu**, Bin Cao (2015) Nitrate enhances cell viability and structure stability of *Comamonas testosteroni* biofilms, 2015 American Institute of Chemical Engineers (AIChE) Annual Meeting, Nov 8-13, Salt Lake City, America.
- **Yichao Wu**, Bin Cao (2015) Involvement in Denitrification is Beneficial to the Biofilm Lifestyle of *Comamonas testosteroni*, 2015 Asia-Oceania Top University League on Engineering (AOTULE) Student Conference, Nov 2-3, Singapore (**Session Chair**).
- **Yichao Wu**, Yehuda Cohen, Bin Cao (2014) Biodegradation of 3-chloroaniline by *Comamonas testosteroni*: Genomic and transcriptomic

analyses and the role of cyclic-di-GMP signalling, the 15th International Society for Microbial Ecology Symposia (ISME), Aug 24-29, Seoul, South Korea.

Awards

- 1st Runner Up (team mentored by Yichao Wu) (2015), The Energy Innovation Challenge Competition, Singapore
- Travel Award (2014), the 15th International Society for Microbial Ecology Symposium

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Chapter 1. Introduction

1.1 Research background and problem statements

Bacteria from the *Comamonas* genus are a group of widely distributed Gram-negative, non-pigmented, rod-shaped bacteria often found in various natural and engineered environments (Willems and Vos 2006). Due to their high abundance in aromatic compound-polluted environments, these organisms are featured by their catabolic versatility mineralizing a wide variety of aromatics (Hurtubise, Barriault et al. 1995, Nishino and Spain 1995, Goyal and Zylstra 1996, Goyal and Zylstra 1997, Hollender, Hopp et al. 1997). Recent meta-omics data showed that they are also present in many other environments without aromatic pollutants, suggesting their wide adaptation to a variety of environments. In various natural and engineered environments, bacteria of the *Comamonas* spp. are often found to form cell aggregates (Andersson, Kuttuva Rajarao et al. 2008, Juang, Adav et al. 2010, Luo, Xu et al. 2012, Kim, Pagaling et al. 2014), resulting in multicellular structures known as biofilms.

The biofilm lifestyle is ubiquitous for environmental bacteria. Cells in a biofilm matrix are embedded in self-produced extracellular polymeric substances and closely associated with other residents (Flemming and Wingender 2010, Flemming, Wingender et al. 2016). With the unique physical, chemical and biological features, cells in a biofilm exhibit distinct biological and physiological characteristics compared with their planktonic counterparts. For example, a number of studies have shown that the biofilm lifestyle provides survival advantages to the embedded cells under unfavourable conditions such as exposure to toxic contaminants, high salinity and antimicrobial agents (Singh, Paul et al. 2006, Cao, Majors et al. 2012). Although *Comamonas* commonly exists as biofilms in diverse environments, most previous studies focused on exploring the catabolic activities of *Comamonas* in planktonic cultures. Therefore, understanding the biofilm lifestyle of *Comamonas* in different environmental processes and elucidating the influence of environmental conditions on the biofilm lifestyle are key to better understanding

environmental biofilm processes and developing high performance, robust biofilm-based environmental biotechnologies.

1.2 Objective

The objective of this study is to understand the biofilm lifestyle of *Comamonas* in environmental processes and the influence of environmental conditions. Specifically, using *C. testosteroni* as a model organism, this thesis work aims to:

1. Elucidate the influence of a toxic aromatic pollutant 3-CA on the biofilm lifestyle of *C. testosteroni* and its implications on bioaugmentation in biofilm bioreactors.
2. Demonstrate the use of key findings in 1 for the development of a more robust *Comamonas* biofilm for biodegradation of 3-CA.
3. Reveal the role of nitrate reduction in *C. testosteroni* biofilms.
4. Examine the biofilm lifestyle of *C. testosteroni* in synthetic and real wastewater and identify key floc-forming inducers in real wastewater and the mechanism of flocculation.

1.3 Thesis outline

This thesis includes 7 chapters, which are highlighted as follows:

Chapter 1. Introduction - This chapter provides the background information and research gaps and states the objective of this study.

Chapter 2. Literature review - This chapter summarizes previous studies on biofilm biology of *Comamonas* and the ecological role of *Comamonas* in different environments.

Chapter 3. Influence of 3-chloroaniline on the biofilm lifestyle of *C. testosteroni* and implications in bioaugmentation - The influence of 3-CA on biofilm lifestyle of *C. testosteroni* and its bioaugmentation in packed-bed biofilm reactors were elucidated.

Chapter 4. Elevated level of the secondary messenger c-di-GMP in *Comamonas testosteroni* enhances biofilm formation and biofilm-based biodegradation – In Chapter 3, we found that 3-CA reduces the c-di-GMP level in *C. testosteroni* and induces cell detachment. In this Chapter, *C. testosteroni* was genetically modified to elevate the intracellular c-di-GMP level and a more robust biofilm for 3-CA biodegradation was demonstrated.

Chapter 5. Involvement in denitrification is beneficial to the biofilm lifestyle of *C. testosteroni* – This chapter reveals the role of nitrate reduction in the biofilm lifestyle of *C. testosteroni*.

Chapter 6. Soluble, high-molecular-weight extracellular polymeric substances in wastewater enhance floc formation of *Comamonas* - This chapter examines the biofilm lifestyle of *C. testosteroni* in synthetic and real wastewater and identifies key floc-forming inducers in real wastewater and the mechanism of flocculation.

Chapter 7. Conclusions and recommendations - This chapter summarizes main findings of this thesis work and proposes future recommendations.

Chapter 2. Literature review

2.1 Introduction

The biofilm lifestyle is ubiquitous for bacteria living in environmental and engineered systems. Biofilm cells are embedded in a matrix of self-produced extracellular polymeric substances and closely associated with other residents. With unique physical, chemical and biological features, cells in biofilms exhibit distinctive metabolic and physiological states from planktonic ones. Therefore, the understanding of biofilm biology and physiological roles of biofilm-forming microbes in environmental and engineered systems are vital for a deep understanding of biofilm processes and potential applications.

With the development of omics techniques, there is increasing evidence that bacteria belonging to the genus *Comamonas* are the major members of biofilm communities in different engineered and natural environments, including soil, sediment, wastewater, marine and water supply system (Table 2.1). They are a widely distributed group of Gram-negative, non-pigmented and rod-shaped bacteria (Stampi, Zanetti et al. 1999). In various environments, *Comamonas* spp. were commonly found to form aggregates in activated sludge and colonize on substratum, indicating the importance of the biofilm lifestyle for these organisms (Boon, Goris et al. 2000, Andersson, Kuttuva Rajarao et al. 2008, Kim, Pagaling et al. 2014). Meanwhile, previous research efforts were focused primarily on the species *C. testosteroni* and their biodegradation and poly-beta-hydroxybutyrate (PHB) metabolism. Little is known about the biofilm biology and physiological roles at the genus level. Taking advantages of advanced genomic sequencing techniques, more genomes of different species in this genus have been sequenced. This enables us to reveal the core genomic properties of *Comamonas* at the genus level. In this review, we analysed the physiological features of *Comamonas* core genome (Figure 2.1) and summarized the current state of the art in biofilm biology of *Comamonas*.

Figure 2.1 Core genome analysis of *Comamonas* genus. The number of common gene clusters is plotted as a function of the numbers of genome by Pan-Genome Profile Analyze Tool (PanGP) (Zhao, Jia et al. 2014). Genes were clustered by pan-genome analysis pipeline (PGAP) using MultiParanoid (MP) method (Zhao, Wu et al. 2012). The resulting core genome consists of 1461 gene clusters with an average of $42.0 \pm 7.3\%$ of genes in each *Comamonas* strain. The pan genome of 9 *Comamonas* strains contains 9866 different gene clusters.

2.2 Biofilm biology of *Comamonas*

Comamonas spp. are known to degrade various aromatic compounds and have always been isolated from contaminated systems. Moreover, there are lots of quantitative evidence to show the omnipresence of *Comamonas* in different environments without aromatic pollutants, such as microbial fuel cells, bioreactors, soil and wetlands (Table 2.1). Under aerated or intermittently aerated conditions, their abundance can reach more than 20% of total community which indicates a high competitive advantage of *Comamonas* spp. in these certain environments. Moreover, in these processes, they are also identified as primary colonizer and form aggregates on surfaces and interfaces. Therefore, to understand their abundance in these processes, it is crucial to examine their biofilm biology at the genus level. The following parts will discuss the nature of the *Comamonas* biofilm matrix and regulatory factors in biofilm development.

Table 2.1 The abundance of *Comamonas* in different environments without aromatic pollutants.

Reactor type	Samples	<i>Comamonas</i> abundance	Quantification method	Comments	Reference
Sewage treatment plants	Activated sludge	1.0 %	16s rDNA amplification		(Zhang, Shao et al. 2012)
Sequencing batch reactor	Phosphate-removing activated sludge	3.5 %	16s rDNA amplification		(Liu, Zhang et al. 2005)
Sequencing batch reactor	Phosphate-removing activated sludge	5.8 %	16s rDNA amplification		(Zhang, Liu et al. 2005)
Sequencing batch reactor	Activated sludge	43.8 %	16s rDNA amplification	Without accumulation of polyphosphate and glycogen	(Fang, Zhang et al. 2002)
Sequencing batch reactor	Phosphate-removing Activated sludge	1.0 %	16s rDNA amplification		(Zengin, Artan et al. 2011)

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Granular sludge reactor	Sulfate-reducing sludge	5.8 %	16s rDNA amplification	Detected in the substratum zone	(Hao, Luo et al. 2016)
Anaerobic rising main pipes	Biofilm on plastic carriers	6.4%	16s rDNA amplification	Increased after nitrate dosage	(Auguet, Pijuan et al. 2015)
Biological aerated filter reactors	Biofilm community on substratum	3.6-40.1 %	16s rDNA amplification		(Kim, Pagaling et al. 2014)
Water lifting and aeration system	Water sample	29.4 %	16s rDNA amplification	Nitrogen removing system	(Zhou, Huang et al. 2016)
Activated sludge system	Activated sludge	4.4-4.3 %	16s rDNA amplification	Low ammonium and oxygen condition	(Sui, Liu et al. 2016)
Single-chamber microbial fuel cells	Anode biofilm	>5 %	16s rDNA amplification		(Jiang, Xing et al. 2016)
Cr(VI)-contaminated Soil	Surface soil	4 %	16s rDNA amplification		(Wang, Peng et al. 2015)

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Wetland	Typha rhizosphere community	8.8 %	16s rDNA amplification		(Guo, Gong et al. 2015)
Microbial fuel cell cascade	Anodic biofilm	5.4 %	16s rDNA amplification		(Hodgson, Smith et al. 2016)
Microbial fuel cell	Anodic biofilm	7.0 %	16s rDNA amplification		(Borole, Hamilton et al. 2009)
Microbial fuel cell	Cathodic microbial community	13.2 %	16s rDNA amplification	Intermittent aerated cathode	(Sotres, Cerrillo et al. 2016)
Biofilm development on glass slide	Biofilm	>10 %	16s rDNA amplification	Slime on river stones as inoculum	(Nakamura, Yamamoto et al. 2016)

Table 2.2 Genomes of *Comamonas* used in core genome analysis.

Strain	Origin	Isolation-site characteristics	Reference
<i>C. aquatica</i> CJG	China	Freshwater river	(Dai, Zhu et al. 2016)
<i>C. badia</i> DSM 17552	Japan	Phenol-adapted activated sludge	(Tago and Yokota 2004)
<i>C. composti</i> DSM 21721	China	Food waste compost	(Young, Chou et al. 2008)
<i>C. granuli</i> NBRC 101663	Korea	Granule from bioreactor treating industrial wastewater	(Kim, Ten et al. 2008)
<i>C. kerstersii</i> J29	China	Isolated from intra-abdominal infection	NCBI Accession: PRJNA288932
<i>C. nitratorans</i> DSM 13191	Uruguay	Sludge from denitrifying reactor treating landfill leachate	(Etchebehere, Errazquin et al. 2001)
<i>C. testosteroni</i> I2	Belgium	Sludge from domestic wastewater treatment plant	(Boon, Goris et al. 2000)
<i>C. testosteroni</i> CNB-1	China	Sludge from industrial wastewater treatment facility	(Ma, Zhang et al. 2009)
<i>C. thiooxydans</i> DSM 17888	India	Sediment from a sulfur spring	(Pandey, Narayan et al. 2009)

2.2.1 Biofilm matrix

Biofilms do not only consist of bacterial cells, but also the extracellular polymeric substance (EPS) secreted by themselves. These biopolymers constitute over 90% of the biofilm dry biomass which are mainly composed of proteins, polysaccharides and DNA (Flemming and Wingender 2010). This EPS matrix constitutes the scaffold and also plays important roles in maintaining the physical-chemical properties of biofilm.

The production of EPS is a prerequisite of biofilm formation and *Comamonas* strains are well-known EPS producers. *C. denitrificans* biofilm development on polystyrene initiated as scattered microcolonies with little EPS and gradually developed into multilayer biofilm matrix with filamentous network and high protein and nucleic acids content (Andersson, Dalhammar et al. 2009). When grown on agar plates, *Comamonas* spp. formed mucoid colonies, as a result of secreted polysaccharides secreted by the cells (Bossier and Verstraete 1996, Bossier, Kersters et al. 1997). In the extracellular polysaccharides secreted by *Comamonas* strains, more than two different polymers were identified, with the larger one in capsular EPS and dominant in biofilms (Tago and Aida 1977, Andersson, Dalhammar et al. 2009). Based on monomer analysis, ribose, rhamnose, glucosamine, galactose, arabinose, mannose and glucose are the most abundant in exopolysaccharides of *Comamonas* (Tago and Aida 1977, Bossier and Verstraete 1996, Andersson, Dalhammar et al. 2011). Correspondingly, one Lpt lipopolysaccharide assembly and export system (LptABCEFG) were conserved in the core genome of *Comamonas*.

The features of extracellular DNA and protein have only been examined in the EPS produced by *C. denitrificans* (Andersson, Dalhammar et al. 2009). Besides polysaccharide, eDNA also plays a critical role in the biofilm development, especially initial attachment. When treated with DNase, biofilm formation of *C. denitrificans* was inhibited, indicating that eDNA may function as adhesin in its biofilm formation. Distinct from the genome-length eDNA in *Pseudomonas aeruginosa* and *P. putida* biofilms (Steinberger and Holden 2005), the highly abundant small DNA fragments in *Comamonas* biofilm indicate that they may originate from the active secretions rather

than cell lysates. In *C. denitrificans* biofilm, the extracellular protein was found to occupy about 3-37% of the EPS. One major extracellular protein (~36 kDa) was found in both biofilm and planktonic cultures.

Remarkably, *Comamonas* strains also secrete some extracellular proteins with specific enzymatic activities. These enzymes which are secreted into the surrounding or located on cell surface, were found to digest exogenous macromolecules, like polyhydroxyalkanoates (PHA) and polyurethane (PUR) (Kasuya, Doi et al. 1994, Nakajima-Kambe, Onuma et al. 1997, Akutsu, Nakajima-Kambe et al. 1998). With these depolymerases, insoluble polymers can be hydrolysed into soluble monomers or dimers that can be further used as carbon and nitrogen sources for bacterial growth.

2.2.2 Biofilm regulation systems

The formation of biofilm is regulated by two universal signalling systems, C-di-GMP signalling and quorum sensing (QS). A previous study found that a wastewater consortium containing *Comamonas* has a correlation between the total c-di-GMP level and granule size (Wan, Yang et al. 2014). However, there is no direct experimental result to show either the role of c-di-GMP or QS in *Comamonas* biofilm regulation.

C-di-GMP is a secondary signalling molecule which is produced by GGDEF domain-containing proteins and hydrolysed by EAL or HD-GYP domain-containing proteins (Hengge 2009). Generally, high c-di-GMP level promotes biofilm formation by stimulation of EPS secretion and inhibition of bacterial motility. The basic c-di-GMP signalling module is initiated by the binding of c-di-GMP with an effector component. Subsequently, the effector can affect corresponding downstream target components, such as promoter DNA, enzymes and other cellular structures. Through core genomic analysis, 4 putative gene clusters were found to be involved in c-di-GMP controlling (Figure 2.2). About 10 conserved genes in each genome were assigned in these clusters and most of the putative proteins contain a PAS domain. With the sensor domain, bacteria can directly detect environmental signals and control intracellular c-di-GMP levels. Therefore, it indicates that c-di-GMP may also play a central role in *Comamonas*

biofilm regulation which contributes to the competitive advantage of *Comamonas* spp. in changing environments.

QS is another important signalling system in biofilm regulation in which bacteria communicate through secreted signal molecules. Although there is no putative QS signal synthase detected in the core genome, two gene clusters were found to code for anthranilate synthase (*phnA* and *phnB*). This pathway is exclusively to synthesis of the precursor of QS signals in *Pseudomonas* (Farrow and Pesci 2007). Therefore it indicates that *Comamonas* strains may be able to produce QS signals via a similar pathway.

Figure 2.2 Conserved clusters for c-di-GMP controlling. (A) Domain of proteins in different clusters. (B) Abundance of genes in conserved clusters in different genomes.

2.2.3 Environmental factors influencing biofilm lifestyle

The regulation of biofilm formation by environmental signals was also found to differ from one species to another (Stanley and Lazazzera 2004). This is likely to account for the different biofilm development efficiencies of each species in the environments they prefer. The composition of carbon and energy sources are the most studied environmental factors in *Comamonas* biofilm. Among the different carbon sources, acetate and isobutyric acid (IBA) were shown to be able to promote *Comamonas* biofilm formation (Andersson, Kuttuva Rajarao et al. 2008, Andersson, Dalhammar et al. 2009). This is consistent with the phenomenon in mixed-culture system, where *Comamonas* was always enriched in acetate-fed processes (Fang, Zhang et al. 2002, Osaka, Yoshie et al. 2006, Xing, Cheng et al. 2009). However, sugars like galactose, glucose and mannose, can only support planktonic growth of *C. denitrificans* with less biofilm formation (Andersson, Dalhammar et al. 2009). Furthermore, many other *Comamonas* species are unable to utilize sugar as carbon source (Willems and Vos 2006). Therefore, it suggested rather than directly attacking sugar, organic acids produced in fermentation processes are likely to be the favoured carbon sources for *Comamonas* biofilm at the genus level.

Electron acceptors are also required for biofilm formation. *Comamonas* biofilms were found to be enriched in bioprocesses with intermittent aeration (e.g. EBPR reactors) (Liu, Zhang et al. 2005, Zhang, Liu et al. 2005, Zengin, Artan et al. 2011), indicating that *Comamonas* strains can easily adapt to environment with varying oxygen levels. Previous genomic analysis found *C. testosteroni* CNB1 encodes different types of putative terminal oxidase complexes including *cbb₃*-type, *bo₃*-type, *aa₃*-type and *bd*-type complexes (Ma, Zhang et al. 2009). At the genus level, most *Comamonas* strains harbor putative genes for three different types of oxygen-dependent terminal oxidases among which *cbb₃*-type and *bd*-type are the most abundant (Table 2.3). As *cbb₃*-type and *bd*-type oxidases have higher oxygen affinity, this combination of different cytochromes facilitates the respiration of *Comamonas* under both oxygen-rich and oxygen-limited environments.

Although *Comamonas* spp. are considered as aerobic microorganism, some *Comamonas* strains were also found to be able to respire on nitrate, solid mineral and electrode (Gumaelius, Magnusson et al. 2001, Wu, Zhuang et al. 2010, Yu, Wu et al. 2015). In microbial fuel cells, *Comamonas* was enriched and dominant on anode electrodes when fed with acetate and aniline (Xing, Cheng et al. 2010, Cheng, Liang et al. 2015, Miyahara, Kouzuma et al. 2015, Jiang, Xing et al. 2016). When operated under denitrifying condition, apparent enrichment of *Comamonas* (>10 %) was observed in cathode chamber (Sotres, Cerrillo et al. 2016). Therefore, *Comamonas* spp. are a group of electrochemically active bacteria, like *Geobacter* and *Shewanella*, which capable of extracellular electron transfer to/from electrode and simultaneously chemical transformation. Recent electrochemical characterization of *C. testosteroni* found the existence of two different redox species (Yu, Wu et al. 2015). These two independent redox species are found as membrane-bound and extracellular soluble mediators which facilitates outward and inward electron transfer respectively. Different from *Shewanella* strains (Kim, Ikeda et al. 1999), the redox species from *C. testosteroni* can facilitate outward electron transfer under both aerobic and anaerobic conditions. The ability of using alternative solid electron acceptors, like electrode and Fe(III) minerals, was also found to benefit the associated *Comamonas* biofilms by enhancing their growth and viability (Wu, Zhuang et al. 2010, Xing, Cheng et al. 2010, Yu, Wu et al. 2015).

Table 2.3 Number of putative genes for different terminal oxidases

	<i>cbb₃</i>	<i>bd</i>	<i>bo₃</i>	<i>aa₃</i>
<i>C. aquatica</i> CJG	1	1	1	1
<i>C. badia</i> DSM 17552	1	1		1
<i>C. composti</i> DSM 21721	1	1	1	1
<i>C. granuli</i> NBRC 101663	1			1

<i>C. kerstersii</i> J29	1	2	1	
<i>C. nitratorans</i> DSM 13191	1			
<i>C. testosteroni</i> I2	1	1	1	1
<i>C. testosteroni</i> CNB-1	1	1	1	1
<i>C. thiooxydans</i> DSM 17888	1	2	1	1

Besides nutrient levels, stress inducers and toxic compounds also affect biofilm formation. Generally, when exposed to heavy metals and toxic aromatic compounds, bacterial metabolism is inhibited and biofilms start to disperse (Boon, Top et al. 2003, Ding, Peng et al. 2014). Similar phenomenon was also observed for *Comamonas* spp., as its flocculation ability was found to be correlated with the state of cells. When exposed to sublethal stress, including H₂O₂, SDS and starvation, the exopolysaccharide excretion of *C. testosteroni* A20 decreased (Bossier and Verstraete 1996). The stressed bacterial surface became more hydrophobic which increased cell attachment on hydrophobic surfaces like polystyrene and air bubbles. Thus, environmental stresses can influence the biofilm development by affecting its cell surface properties and EPS secretion.

Conversely, certain stress factors can induce bacterial biofilm formation as a stress response. Because biofilm cells embedded in EPS are less susceptible to the external toxicity. Pathogens like *Vibrio cholera* was shown an enhanced biofilm formation when exposed to bile acids, which is a strategy to evade this defence and establish infection (Hung, Zhu et al. 2006). For *Comamonas*, alkylbenzenesulfonate (LAS), a common surfactant inhibiting bacterial metabolism, was found to induce biofilm formation of *C. testosteroni* KF-1 (Mösche and Meyer 2002, Schleheck, Knepper et al. 2004). As the strain was isolated from a LAS-mineralizing community, the induced biofilm formation is presumably an evolved stress response mechanism to enhance their fitness.

2.3 Metabolic capabilities of *Comamonas*

2.3.1 General carbon assimilation

Based on physiological characterization, *Comamonas* strains can grow well on various amino acids and organic acids, while rarely attack sugars (Willems and Vos 2006). With the same inoculum in microbial fuel cells, enrichment of *Comamonas* were occurred in acetate-fed system instead of glucose-fed (Xing, Cheng et al. 2009). So far, only *C. denitrificans*, *C. koreensis* and some other isolates were found to grow on glucose (Wang, Yamazoe et al. 2004, Andersson, Dalhammar et al. 2009, Wu, Zhuang et al. 2010). Genomic analysis of other strains found they lack key enzymes like hexokinase and glucokinase for the phosphorylation step of glycolysis (Ma, Zhang et al. 2009, Liu, Zhu et al. 2015). Conserved acetate and pyruvate metabolic pathways are found in the core genome (Figure 2.3). And without putative glucokinase or hexokinase, it contains other glycolysis genes to metabolize phosphorylated glucose.

The assimilated carbon not only supports general metabolism but also accumulates as carbon reserve in the form of poly-beta-hydroxybutyrate (PHB). With versatile catabolism, *Comamonas* strains were also found to convert other carbon sources like oil (Thakor, Trivedi et al. 2005) and naphthalene (Thakor, Patel et al. 2003) to PHB. This ability to produce PHB was conserved in the core genome with key enzymes to convert acetyl-CoA to PHB. *Comamonas* can also utilize exogenous PHB derived from other PHB producers or secretors as carbon source. To exploit extracellular water-insoluble PHB, *Comamonas* strains produce type II extracellular depolymerases to hydrolyse them to soluble dimers and monomers (Kasuya, Doi et al. 1994, Kasuya, Inoue et al. 1997). The ability to store substrates and utilize exogenous PHB may also be a reason for their survival in wastewater treatment processes operated under feast/famine conditions.

Figure 2.3 Central carbon metabolism of *Comamonas* core genome. *GPI*, glucose-6-phosphate isomerase; *FBP*, fructose-1,6-bisphosphatase; *pfkA*, 6-phosphofructokinase; *FBA*, fructose-bisphosphate aldolase; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *PGK*, phosphoglycerate kinase; *gpmB*, probable phosphoglycerate mutase; *PGAM*, 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase; *ENO*, enolase; PEP, phosphoenolpyruvate; *PPC*, phosphoenolpyruvate carboxykinase; *PK*, pyruvate kinase; *pps*, pyruvate, water dikinase; *tkt*, transketolase; *rpe*, ribulose-phosphate 3-epimerase; *rpiA*, ribose 5-phosphate isomerase A; *DLAT*, dihydrolipoamide acetyltransferase; *DLD*, dihydrolipoamide dehydrogenase; *aceE*, pyruvate dehydrogenase E1 component; *ackA*, acetate kinase; *ACSS*, acetyl-CoA synthetase; *pta*, phosphate acetyltransferase.

2.3.2 Aromatic compound degradation

Comamonas strains are widely found in aromatic compound-contaminated environments, including wastewater reactors, groundwater and soil (Nishino and Spain 1995, Dejonghe, Berteloot et al. 2003, Wu, Sun et al. 2005). Due to the potential applications in organic pollutant removal, their corresponding catabolic ability to mineralize aromatic compounds has been well characterized, especially for the species *C. testosteroni*. This species got its name by its capability of testosterone decomposition. They can also grow on C19 and C21 steroid hormones (Horinouchi, Hayashi et al. 2003). Besides steroids, they can degrade a number of aromatics, ranging from polycyclic aromatic hydrocarbons, like biphenyl, phenanthrene, naphthalene and anthracene, to single phenyl ring compounds, like chlorophenol, benzoate, nitrobenzene, gentisate, phenol, hydroxybenzoate, protocatechuate, vanillate and aniline (Hurtubise, Barriault et al. 1995, Nishino and Spain 1995, Goyal and Zylstra 1996, Goyal and Zylstra 1997, Hollender, Hopp et al. 1997, Boon, Goris et al. 2000, Ma, Zhang et al. 2009). Remarkably, although many of these degradation steps are driven by oxygen-dependent oxygenases, some catabolism can take place under anoxic conditions. For example, *C. koreensis* CY01 was found to be able to utilize Fe(III) mineral like ferrihydrite, goethite, lepidocrocite or hematite as electron acceptors to dechlorinate of 2,4-Dichlorophenol (Wu, Zhuang et al. 2010). If humic substances, like anthraquinone-2,6-disulfonate, are present in this process, they can act as electron shuttles between *Comamonas* and chlorinated aromatics which enhances the dechlorination efficiency.

Therefore, when *Comamonas* are present in aromatic compound-contaminated environments, they can alleviate the detrimental effects of pollutants on the community by removing toxic compounds. When co-inoculated with *Variovorax* sp. strain WDL1 and *Hyphomicrobium sulfonivorans*, *C. testosteroni* WDL7 stimulated linuron degradation in mixed species biofilms by consuming the aromatic intermediates (Dejonghe, Berteloot et al. 2003). Also, when *Comamonas* strains were augmented in aromatic contaminated bioreactors and soil, complete removal of aromatics was observed (Bokhamy, Deront et al. 1997, Boon, Goris et al. 2000, Gentry, Newby et al. 2001). The augmented system also exhibited a higher community stability and a faster recovery of microbial metabolism against pollutants (Boon, Top et al. 2003). Moreover,

Comamonas strain CNB-1 was also used to establish a plant-microbe system to degrade 4-chloronitrobenzene within the soil and eliminate the phytotoxicity to alfalfa for phytoremediation (Liu, Jiang et al. 2007).

Besides protecting indigenous communities from mineralizing deleterious pollutants, another important role that *Comamonas* plays is transferring the catabolic capability to other members via horizontal gene transfer. Based on genomic analysis, the IncP-1 β plasmids of *C. testosteroni* harbour xenobiotic-catabolic and heavy-metal resistant genes (Chen, Xu et al. 2016). The existence of plasmids was closely associated with catabolic capability and heavy metal resistance of their host strain (Wu, Jiang et al. 2006, Zhang, Ma et al. 2007). Due to the broad host range of IncP-1 plasmids, catabolic capability and heavy metal resistance of *Comamonas* can be transferred and spread in bacterial community. After activated sludge augmented with *Comamonas* strains containing catabolic plasmids, the IncP-1 β plasmids were found to transfer to other community members to achieve aromatic removal (Bathe, Schwarzenbeck et al. 2005, Bathe, Schwarzenbeck et al. 2009).

2.3.3 Denitrification and ammonification

Due to the high abundance in denitrifying microbial communities and denitrification capability of specific isolates, *Comamonas* spp. are considered to be potential denitrifiers (Lemmer, Zaglauer et al. 1997, Etchebehere, Errazquin et al. 2001, Gumaelius, Magnusson et al. 2001). Through genomic analysis, almost every *Comamonas* strains have one *nas* assimilatory nitrate reductase gene located downstream of the nitrite reductase *nirBD* gene and one gene cluster for dissimilatory nitrate reduction (Figure 2.4). Therefore, most of them are capable of nitrate ammonification and assimilative reduction nitrate to nitrite, while some of them are capable of complete denitrification. This indicates *Comamonas* may mainly be involved in ammonification processes under nitrate-reducing conditions. Previous studies have found that the increased ratio of electron donors to electron acceptors can stimulate the ammonification relative to denitrification (King and Nedwell 1987). Therefore, when the competition for nitrate become more severe, the decreased nitrate levels would

favour nitrate-ammonifying bacteria over denitrifying bacteria (Dong, Smith et al. 2009). Moreover, in growth yield analysis, the ATP synthesis in denitrification was also lower than ammonification (Strohm, Griffin et al. 2007). Therefore, ammonification may gain *Comamonas* strains a competitive advantage over other denitrifiers in nitrate-reducing conditions.

Comamonas strains harbour either putative *nar* or *nap* nitrate reductase for the dissimilatory nitrate reduction. Although previous studies consider *Comamonas* as aerobes, the strains with Nar nitrate reductase are facultative anaerobes which are capable of respiring under anoxic conditions (Etchebehere, Errazquin et al. 2001). Nap nitrate reductase is known to balance the redox state, especially when grown on reduced carbon sources (Dietrich, Okegbe et al. 2013). The enzyme was also shown to have higher affinity to nitrate at low level which outcompetes the Nar system under nitrate-limited conditions (Potter, Millington et al. 1999). In biofilm mode of life, it was found to affect biofilm morphology of *Pseudomonas* by aiding in redox homeostasis (Dietrich, Okegbe et al. 2013). Meanwhile, *Pseudomonas* biofilms respiring on nitrate with Nar nitrate reductase were found to have an enhanced viability than that respiring on oxygen (Yoon, Hennigan et al. 2002). Similar beneficial effects of nitrate reduction on *Comamonas* biofilm are expected which would explain the abundance of *Comamonas* in denitrifying environments.

Figure 2.4 Comparison of nitrogen-related metabolism in *Comamonas* spp. *nap*, periplasmic nitrate reductase; *nir*, nitrite reductase; *nas*, assimilatory nitrate reductase; *nar*, respiratory nitrate reductase; *nor*, nitric oxide reductase; *nos*, nitrous-oxide reductase.

2.3.4 Microbial interactions

When grown in mixed communities, *Comamonas* strains are found to secrete products which benefit the local population and close neighbours. For example, *Comamonas* strains isolated from activated sludge, agricultural soil and root surfaces were capable of producing the iron-scavenging molecule siderophores (Elo, Maunuksela et al. 2000, Das, Jean et al. 2014). As iron is a major limiting nutrient for microbial growth, the secretion of siderophore under iron-limiting conditions was beneficial to both the producer and nearby relatives for iron uptake. A number of genes coding for siderophore transporters such as the Ton-B receptor also conserved in the core genome which indicated its capability to produce siderophores and uptake similar agents secreted by other community members.

Besides iron-chelators, *Comamonas* strains also benefit their neighbours by synthesizing micronutrients. A previous study found that *C. aquatica* and *C. testosteroni* were capable of vitamin B12 synthesis (Watson, MacNeil et al. 2014). Its predator *Caenorhabditis elegans* was benefited from this metabolite which preventing toxin buildup. Vitamin B12 is also an important growth supplement for cyanobacteria and microalgae in various natural environment which mainly retrieved from the symbiotic relationship with bacteria (Croft, Lawrence et al. 2005). Rhizospheric *Comamonas* strains were also found to secrete phytohormone like indole-3-acetic acid (IAA) (Elo, Maunuksela et al. 2000). The synthesis genes, *cbiB* and *cbiB* for vitamin B12 synthesis and nitrilase for IAA synthesis, were conserved in the core genome. Therefore, *Comamonas* cells in the environment not only have a close relationship with other bacteria, but also exhibit great potential to be involved in cross-kingdom interactions.

QS system is known to play a central role in biofilm development by which bacteria communicate via signalling molecule (Hall-Stoodley, Costerton et al. 2004). On the contrary, the degradation of signal compounds — quorum quenching is one key mechanism to inhibit quorum sensing. AHL-degrading *Comamonas* strains were isolated from rhizosphere and activated sludge (Uroz, D'Angelo-Picard et al. 2003, Christiaen, Brackman et al. 2011, Ochiai, Morohoshi et al. 2013). These isolates were

found to have a very different degradation efficiency towards different types of AHL molecules. Further characterization found that *Comamonas* strain D1 exhibited broad N-AHSL degradation via a cell-associated amidohydrolase activity (Uroz, Oger et al. 2007). Therefore, *Comamonas* cells may also be involved in quorum quenching in mixed community which can gain *Comamonas* spp. competitive advantages over others in environment to interfere cell communication and biofilm formation (Rendueles and Ghigo 2015).

Chapter 3. Influence of 3-chloroaniline on biofilm lifestyle and implication in bioaugmentation

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3.1 Introduction

Aromatic compounds are widely used in chemical and pharmaceutical industries (Key, Howell et al. 1997). These organic compounds contain chemically stable benzene ring structures and hence, their degradation by naturally occurring microbial communities in the contaminated environments is often slow (Spain 1995, Kulkarni and Chaudhari 2007). This is because the indigenous microbial community usually lacks efficient catabolic capabilities for these anthropogenic compounds. To efficiently remove these toxic compounds from contaminated water or soil, bioaugmentation has been a frequently investigated bioremediation approach, in which active bacteria capable of degrading recalcitrant organic compounds are introduced into the bioremediation system (El Fantroussi and Agathos 2005, Mrozik and Piotrowska-Seget 2010). However, the performance of bioaugmentation remains difficult to predict and in many cases bioaugmentation failed because of the complex interactions among the augmented organisms (Thompson, Van Der Gast et al. 2005), indigenous microbial community (Bouchez, Patureau et al. 2000), and environmental factors (Goldstein, Mallory et al. 1985).

Previous studies have shown that bioavailability, concentration and toxicity of the target pollutant are important characteristics influencing the performance of bioaugmentation (Vogel 1996). Further molecular level mechanistic studies on responses of bacteria to the pollutants have improved our understanding on the

interaction between augmented population and the pollutants and its potential effects on bioaugmentation. Most such studies used planktonic bacterial cultures and focused on the pollutant-induced changes in catabolic activity responsible for biodegradation of the pollutant and detoxification mechanisms employed by the bacteria at the gene, mRNA, or protein levels (Domínguez-Cuevas, González-Pastor et al. 2006, Tam, Eymann et al. 2006, Zhang, Wu et al. 2009). However, bacterial cells that are introduced into bioaugmentation systems often exist in the form of biofilms by attaching onto biotic and abiotic surfaces (Boon, Goris et al. 2000, Ivanov, Wang et al. 2006). Bacterial cells in biofilms differ physiologically and genetically from their planktonic counterparts. Compared with cells in planktonic cultures, the biofilm lifestyle provides cells with a number of advantages under unfavourable conditions including exposure to toxic chemicals (Hall-Stoodley, Costerton et al. 2004, Singh, Paul et al. 2006, Cao, Majors et al. 2012), although toxic chemicals may compromise biofilm stability by inducing detachment of cells from the biofilms and result in a loss of the bioaugmented function (Ding, Peng et al. 2014, Wu, Ding et al. 2015). Hence, the influence of toxic aromatic pollutant on the biofilm lifestyle of the introduced bacterial population is a critical aspect contributing to the overall performance of bioaugmentation, which however remains largely unexplored.

The objective of this study was to reveal the influence of toxic aromatic pollutant on the biofilm lifestyle of bacteria capable of degrading aromatic compounds toward a better understanding of cell-pollutant interaction in bioaugmentation. *Comamonas testosteroni* and 3-chloroaniline (3-CA) were used as the model organism and the industrial pollutant, respectively, because biodegradation of 3-CA by *C. testosteroni* and bioaugmentation with *C. testosteroni* for the treatment of wastewater containing 3-CA have been well documented (Boon, Goris et al. 2000, Boon, De Gelder et al. 2002, Boon, Top et al. 2003). By using a packed bed biofilm reactor, we quantified the effect of 3-CA exposure on the retention of *C. testosteroni* cells in the bioreactor. To further understand the mechanisms, we employed an integrated genomic and transcriptomic analysis to examine the influence of 3-CA on the expression of genes important to the biofilm lifestyle of *C. testosteroni*.

3.2 Materials and methods

3.2.1 Bacterial Strain and Culture Conditions.

C. testosteroni WDL7, a Gram-negative, aerobic organism capable of degrading 3-CA (Wu, Ding et al. 2015), was pre-cultured in LB medium at 30°C. The overnight seed cultures were then inoculated in M9 minimal medium which was supplemented with 10 mM pyruvic acid sodium salt as the sole carbon source (Wu, Ding et al. 2015). Bacterial growth was monitored by measuring the optical density at 600 nm (OD₆₀₀).

3.2.2 Retention of *C. testosteroni* Cells in Biofilm Reactors.

Packed bed column reactors were used to characterize bacterial retention under continuous flow conditions. The column reactors were set up as previously described by using 10-mL syringes (Luer-Lok tip, latex free; BD) packed with glass beads (diameter, 2.0 mm) (solid-glass beads; Sigma-Aldrich) (Wu, Ding et al. 2015). Each packed bed reactor contains 1520±18 beads with a total surface area of 19,091±305 mm². The reactors were sterilized with 70% ethanol and then saturated with PBS buffer (pH 7.4) with or without 200 ppm 3-CA at an interstitial rate of 5.99 cm/h against gravity. For each condition, replicate experiments in three independent reactors were conducted. *C. testosteroni* cells were cultivated in M9 minimal medium with pyruvate as carbon source. At the mid-exponential phase, one set of the cultures were treated with 3-CA at the final concentration of 200 ppm, while the other set of cultures with no 3-CA addition served as controls. After 2-h treatment, bacterial cells were centrifuged and resuspended in PBS buffer with or without 200 ppm of 3-CA to obtain a cell density of 3.2×10^9 cells/mL. About 0.4 mL of the cell suspensions were introduced into the bioreactors and effluent samples were collected periodically. Cell density in the effluent samples was determined using a drop-plate method as previously described (Wu, Ding et al. 2015). Three technical replicates were performed to assess the average cell density for each biological replicate.

3.2.3 Mathematical Analysis.

The breakthrough curves of the treated and control groups were obtained based on cell density in the effluents. The bacterial transport process was modeled using the one-dimensional advection-dispersion equation (Wang and Ford 2009, Adadevoh, Triolo et al. 2015):

$$R \frac{\partial b}{\partial t} = D \left(\frac{\partial^2 b}{\partial z^2} \right) - v \frac{\partial b}{\partial z} - k_m b \quad (1)$$

Where R represents the retardation factor, b is the cell density in the aqueous phase (normalized by inoculum density), D is the longitudinal hydrodynamic dispersion coefficient, z is the longitudinal distance, k_m represents the irreversible retention coefficient. R, D and k_m were determined by fitting the advection-dispersion model to the experimental breakthrough curve data via optimizing the least-square error by using CXTFIT/EXCEL code (Tang, Mayes et al. 2010). Student's *t* test was performed to compare the kinetic coefficients in different groups (<http://www.physics.csbsju.edu/stats/t-test.html>).

3.2.4 Motility Assay

The swimming motility of the *C. testosteroni* cells were determined on agar (0.3%) (Murray and Kazmierczak 2008, Wu, Ding et al. 2015) containing M9 minimal medium supplemented with 10mM pyruvic acid sodium salt in the presence and absence of 200 ppm of 3-CA. Briefly, 1 μ L of overnight culture ($OD_{600} \sim 1.4$) was inoculated into the agar plate using a sterile needle (25G, Braun) by half penetration method.

3.2.5 Cell Surface Hydrophobicity Assay

Cell surface hydrophobicity was determined by bacterial adhesion to hydrocarbons (BATH) (Prabhu and Phale 2003). Cells grown in M9 media with 10 mM pyruvate in the presence or absence of 3-CA (200 ppm) were harvested and resuspended in PBS buffer to a final OD_{600} of 0.20. Two ml of the cell suspensions were mixed with 2 ml of hexane. After 2 min vortex, the mixture was left for 15 min at room temperature and then the OD_{600} of the aqueous phase was measured.

The hydrophobicity was quantified by calculating the percentage of cells transferred to the hexane phase.

3.2.6 DNA and RNA Extraction.

After overnight cultivation in LB medium, culture was harvested and DNA was extracted by Qiagen Genomic- Kit (Qiagen K.K., Tokyo, Japan). DNA concentration was monitored by using Nanodrop (Thermoscientific, DE, USA) while the integrity and quality was examined by loading DNA on agarose gel. For the total RNA extraction, cells grown in M9 media with or without 3-CA exposure (1 h exposure at late exponential phase) were harvested by centrifugation and RNA was extracted by using Qiagen mini RNA prep kits. The concentration and quality of extracted total RNA were determined by Nanodrop (Thermoscientific, DE, USA). For each experimental condition, there were three independent biological replicates.

3.2.7 Genome Sequencing, Draft Genome Assembly and Annotation

Genomic DNA was sequenced on Illumina's MiSeq platform (Illumina, CA, USA) with 250-bp paired-end sequences (5,733,600 reads). The quality filtering and adapter removal were performed by using cutadapt 1.2.1 (Martin 2011). Filtered reads were assembled using Velvet 1.2.08 (k=153) (Zerbino and Birney 2008). The assembled sequence was deposited in the NCBI's Assembly database (accession: ASM126683v1). All contigs were annotated on the Integrated Microbial Genomes Expert Review (IMG ER) platform (Markowitz, Mavromatis et al. 2009, Hyatt, Chen et al. 2010). The genome sequence and annotation information were available on IMG database (analysis project ID: Ga0022323). Prophages and transposases containing regions were analyzed by Phast and ISSaga (Varani, Siguier et al. 2011, Zhou, Liang et al. 2011). Membrane-bound transporter genes was predicted in IMG using Transport Classification Database (Enright, Iliopoulos et al. 1999, Markowitz, Chen et al. 2014). Protein subcellular localization prediction was done by using PSORTb 3.0 (Nancy, Wagner et al. 2010).

3.2.8 RNA-seq Analysis.

After RNA isolation, paired-end sequencing was performed on Illumina Hi-Seq 2500 platform with a read length of 100 nucleotides. The RNA-seq raw data

was deposited in the NCBI GEO Short Read Archives: SRX1124987 and SRX1124990. The sequence reads were assembled and analyzed in the RNA-Seq and expression analysis application of CLC genomic Workbench 6.0 (CLC Bio, Aarhus, Denmark). The sequence of strain WDL7 genome and plasmid pWDL7::*rfp* (accession: GQ495894.1) were used as the reference for assembly. Data were normalized by calculating the reads per kilobase per million mapped reads. Significant changes in genes expression was determined using selection criteria of more than 2-fold change and p-value<0.05 (Wu, Shukal et al. 2015).

3.2.9 Quantification of 3-CA.

The concentration of 3-CA in culture supernatant was determined by using a reverse-phase HPLC after filtering through 0.2 µm Acrodisc polyethersulfone membrane syringe filters (Pall Corporation, Singapore). In the HPLC system, an Acsentis C18 column (100mm×2.1mm, 5 µm particle size), a mobile phase of methanol-water (50/50 v/v), a flow rate of 0.6 ml/min and a UV detector set at 254 nm were used (Wu, Ding et al. 2015).

3.2.10 Extraction and Quantification of Bis-(3'-5')-cyclic Dimeric Guanosine Monophosphate (c-di-GMP).

The extraction and quantification of c-di-GMP followed the methods described in the previous paper (Wu, Ding et al. 2015). Cells were grown at 30°C in M9 media using pyruvate as sole carbon source. At the late exponential phase, 3-CA was added into the culture to a final concentration of 200 ppm. After the cells were harvested by centrifugation, c-di-GMP was extracted using organic solvent mixture (Kuchma, Ballok et al. 2010). C-di-GMP concentration was analyzed by using a Thermo Accela 1250 series LC system fitted with a Thermo Velos Pro Orbitrap mass spectrometer as earlier described (Spangler, Böhm et al. 2010). The c-di-GMP concentration was normalized to total protein.

3.2.11 Flow Cell Biofilms.

Response of *C. testosteroni* biofilms to 3-CA was examined using multi-channel flow cells (BioCentrum-DTU, Denmark) (Ding, Peng et al. 2014, Mohanty, Wei et al. 2015, Wu, Shukal et al. 2015). Each channel was inoculated using 1.5 mL

diluted overnight culture in M9 medium ($OD_{600} \sim 0.1$). Air-saturated M9 medium containing 10 mM pyruvate was supplied with a flow rate of 3.0 mL/h for biofilm growth in each channel. 3-CA was introduced into the medium to a final concentration of 200 ppm after 4-day growth. The experiments were conducted in triplicate. Cell detachment rate was determined by a drop-plate method as previously described (Wu, Ding et al. 2015).

3.2.12 Confocal Laser Scanning Microscopy and Image Processing.

Biofilms were stained with LIVE/DEAD BacLight Bacterial Viability Kit L7012 (Molecular Probes, Inc.) (Teal, Lies et al. 2006). Diluted SYTO 9 (11.1 μ M) and propidium iodide (66.7 μ M) solution (~ 0.7 mL) was injected into each flow channel. After staining for 15 min in dark, the flow cell biofilms were imaged by using a confocal laser scanning microscopy (CLSM) (Carl Zeiss Microscopy LSM 780). Five images for each biofilm were collected by CSLM and analyzed with IMARIS software (version: 8.0.0, Bitplane, Zurich, Switzerland).

3.3 Results and discussion

3.3.1 Exposure to 3-CA Reduced Retention of *C. testosteroni* Cells in Bioreactors.

Compared with the control, *C. testosteroni* cells exposed to 3-CA exhibited a higher migration rate through the packed bed biofilm reactors (Figure 3.1). Cells from the control group began to elute at 0.71 pore volume and the normalized cell density in the effluent reached a peak value of 0.085 ± 0.006 at 1.18 pore volume. While for the 3-CA-treated group, cells began to elute from the bioreactor after 0.56 pore volume and the highest normalized cell density in the effluent (0.122 ± 0.009) was reached rapidly within 0.86 pore volume.

Figure 3.1 Breakthrough curves of *C. testosteroni* WDL7 with the absence or presence of 3-CA in packed bed reactors. Experimental data for cell concentration in the effluent from the reactor with 3-CA (empty triangle) and without 3-CA (empty circle) were normalized by cell density of the influent cell suspension. Simulated data based on one-dimensional advection-dispersion model was represented by the solid line for the group with 3-CA and dash line for the group without 3-CA.

After fitting the experimental data with the advection-dispersion model, we obtained the kinetic parameters for the breakthrough curves (Table 3.1). Comparing with the control, the cells exposed to 3-CA has a lower dispersion coefficient ($1.406 \pm 0.033 \text{ cm}^2/\text{h}$ vs $1.487 \pm 0.168 \text{ cm}^2/\text{h}$), a lower retardation factor R (1.003 ± 0.023 vs 1.245 ± 0.017 , $P < 0.05$) and a lower irreversible retention k_m ($0.151 \pm 0.032 \text{ h}^{-1}$ vs $0.237 \pm 0.043 \text{ h}^{-1}$, $P < 0.05$). Lower R and k_m values for cells exposed to 3-CA suggest that both the reversible and irreversible retention of *C. testosteroni* cells in the bioreactors were reduced (Adadevoh, Triolo et al. 2015). The 3-CA-treated cells also exhibited a shorter mean travel time which suggested 3-CA would decrease the residence time of bacterial cells in the bioreactors. Compared with control cells, the treated cells retained in the reactors over three pore volumes decreased from $22.4 \pm 3.2\%$ to $15.4 \pm 3.2\%$.

Table 3.1 Fitted kinetics obtained from advection-dispersion model and parameters for simulated breakthrough curves. C/C_0 represents the normalized cell density in the effluent (normalized by the initial cell density in the influent). Retention rate means the proportion of the cells retained in the bioreactors.

	R	k_m (h^{-1})	D (cm^2/h)	$(C/C_0)_{max}$	Mean travel time (Pore volume)	Retention rate (%)
Control group	1.245 ± 0.017	0.237 ± 0.043	1.487 ± 0.168	0.085 ± 0.006	1.25 ± 0.02	22.4 ± 3.2
Treated group	1.003 ± 0.023	0.151 ± 0.032	1.406 ± 0.033	0.116 ± 0.005	1.02 ± 0.02	15.4 ± 3.2

Oxygen availability in the medium could affect the transport and biofilm formation of the bacterial cells. However, the effect of oxygen availability can be ruled out in this study because the presence of 3-CA did not affect the dissolve oxygen concentration in the medium (6.89 ± 0.04 mg/L (medium only) vs. 6.96 ± 0.05 mg/L (medium containing 3-CA)). The reduction in cell retention in the bioreactors could be caused by a decrease in bacterial motility that might result in a low effective motility coefficient and a low hydrodynamic dispersion coefficient (Camper, Hayes et al. 1993). When growing in or on the agar containing 200 ppm of 3-CA, *C. testosteroni* cells formed much smaller halo or colonies, confirming a repressed swimming motility in the presence of 3-CA (Figure 3.2). In addition, the hydrophobicity assay showed that about 40% of the 3-CA-treated cells and only 28% of the control cells were transferred to the hexane phase, suggesting an increase in cell surface hydrophobicity upon exposure to 3-CA. An increased hydrophobicity of the cells after 3-CA treatment may loosen the interaction between bacteria and the hydrophilic surfaces of borosilicate glass beads, which could also contribute to the observed lower retention in the bioreactors.

Figure 3.2 Repressed swimming motility with presence of 3-CA. (A) Colony morphology in M9 agar in the presence or absence of 3-CA. (B) Average diameter of motility halos (swimming) (n=6).

3.3.2 Genome Analysis Suggested Versatile Environmental Controls of Biofilm Lifestyle.

To explore molecular mechanisms of the influence of 3-CA on the retention of WDL7 cells in the bioreactors, we conducted an integrated genomic and transcriptomic analysis. The draft genome of *C. testosteroni* WDL7 was 5.49 Mbp in length and comprised 65 contigs with a GC content of 61.47%. A total of 5068 genes with 4972 protein-coding genes were predicted, whereas 4130 (81.49% of total genes) protein-encoding genes were associated with predicted functions. Compared with the genome of other *Comamonas* strains, it shared similar genomic feature with other *Comamonas* strains (Table 3.2).

Table 3.2 General feature of *Comamonas* strains

			ATCC			NBRC			DSM	DSM
	WDL7	I2	11996	CNB-2	KF-1	100989	S44	B-9	17552	21721
Size (bp)	5494070	5778710	5410155	5373643	6026527	5589358	5534155	5217447	3681020	4639907
GC content (%)	61.47	61.88	61.48	61.40	61.79	61.90	61.42	63.6	65.96	63.24
DNA coding number of bases (%)	87.40	87.47	86.82	86.84	87.54	87.28	86.82	88.43	92.18	87.95
Predicted CDS	5068	5365	5056	4893	5606	5169	5218	5249	3550	4165
Protein coding genes	4972	5263	4984	4805	5492	5079	5154	5165	3489	4075
	(98.11%)	(98.10%)	(98.58%)	(98.20%)	(97.97%)	(98.26%)	(98.77%)	(98.40%)	(98.28%)	(97.84%)
rRNA genes	6	6	3	9	18	4	3	5	7	9
	(0.12%)	(0.11%)	(0.06%)	(0.18%)	(0.32%)	(0.08%)	(0.06%)	(0.10%)	(0.20%)	(0.22%)
tRNA genes	81	79	69	79	96	68	61	67	41	64
	(1.60%)	(1.47%)	(1.36%)	(1.61%)	(1.71%)	(1.32%)	(1.17%)	(1.28%)	(1.15%)	(1.54%)
Protein coding genes	4130	4324	3776	3762	4009	4275	3831	4103	3082	3551
with function prediction	(81.49%)	(80.60%)	(74.68%)	(76.88%)	(71.51%)	(82.70%)	(73.42%)	(78.17%)	(86.82%)	(85.26%)

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Prophages	149 (2.94%)	126 (2.35%)	96 (1.90%)	129 (2.64%)	213 (3.80%)	16 (0.31%)	224 (4.29%)	-	0	25 (0.60%)
Transposase	37 (0.73%)	76 (1.42%)	43 (0.85%)	70 (1.43%)	121 (2.16%)	47 (0.91%)	29 (0.56%)	-	30 (0.84%)	60 (1.44%)
Protein coding genes with COGs	3572 (70.48%)	3692 (68.82%)	3564 (70.49%)	3116 (63.68%)	3746 (66.82%)	3636 (70.34%)	3520 (67.46%)	3293 (62.74%)	2651 (74.68%)	3060 (73.47%)
Protein coding genes connected to KEGG pathways	1391 (27.45%)	1405 (26.19%)	1400 (27.69%)	1387 (28.35%)	1440 (25.69%)	1378 (26.66%)	1415 (27.12%)	1249 (23.80%)	1202 (33.86%)	1304 (31.31%)
Protein coding genes connected to Transporter Classification	857 (16.91%)	891 (16.61%)	838 (16.57%)	816 (16.68%)	905 (16.14%)	878 (16.99%)	857 (16.42%)	904 (17.22%)	556 (15.66%)	759 (18.22%)

In previous genomic analysis, *C. testosteroni* strains were found to harbor a high number of putative c-di-GMP controlling enzymes and many of which containing a sensor domain (Wu, Arumugam et al. 2015). The c-di-GMP is one of the most important signaling molecules in biofilm regulatory networks that coordinate biofilm formation and detachment (Hengge 2009). Previous study showed that c-di-GMP signaling not only plays an important role in biofilm formation, but also influences catabolic activity of *C. testosteroni* (Wu, Ding et al. 2015). The c-di-GMP level is controlled by synthases (diguanylate cyclases) and hydrolases (phosphodiesterases). In the genome of *C. testosteroni* WDL7, 51 putative c-di-GMP controlling proteins were identified (Figure 3.3A & Table 3.3). Among these putative proteins, 23 contain a sensor domain (Figure 3.3B & Table 3.3). Remarkably, 38 of the 51 putative c-di-GMP controlling proteins were predicted to locate on the cytoplasmic membranes, while 20 of them contain a sensor domain. The high abundance of sensor domain-containing c-di-GMP controlling proteins on the inner membranes suggests versatile environmental controls of the intracellular c-di-GMP concentration.

Figure 3.3 Specific genes involved in c-di-GMP controlling. (A) Numbers of putative c-di-GMP-controlling enzymes in different organisms; (B) Putative c-di-GMP-controlling enzymes binding with sensor domains

Table 3.3 Putative genes involved in intracellular c-di-GMP concentration regulation

Feature ID	Description	C-di-GMP controlling domain	Signalling domain	Predicted location
WDL7contig_00979	EAL domain	EAL	-	Cytoplasmic membrane
WDL7contig_05079	EAL domain	EAL	GAF	Cytoplasmic membrane
WDL7contig_00489	Predicted signal transduction protein containing EAL and modified HD-GYP domains	EAL-HD-GYP	-	Unknown
WDL7contig_03287	Predicted signal transduction protein containing EAL and modified HD-GYP domains	EAL-HD-GYP	-	Cytoplasm
WDL7contig_03288	Predicted signal transduction protein containing EAL and modified HD-GYP domains	EAL-HD-GYP	-	Unknown
WDL7contig_00113	PAS domain S-box/diguanylate cyclase (GGDEF) domain	GGDEF	PAS	Cytoplasmic membrane
WDL7contig_00190	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane
WDL7contig_00192	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane
WDL7contig_00443	Diguanylate cyclase (GGDEF) domain	GGDEF	CACHE	Cytoplasmic membrane
WDL7contig_00546	Diguanylate cyclase (GGDEF) domain	GGDEF	MASE	Cytoplasmic membrane

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WDL7contig_00724	Diguanylate cyclase (GGDEF) domain	GGDEF	PAS	Cytoplasm
WDL7contig_00725	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane
WDL7contig_00747	Response regulator containing a CheY-like receiver domain and a GGDEF domain	GGDEF	-	Cytoplasm
WDL7contig_00769	Diguanylate cyclase with PAS/PAC sensor	GGDEF	PAS	Cytoplasmic membrane
WDL7contig_00775	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane
WDL7contig_00893	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasm
WDL7contig_01038	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane
WDL7contig_01800	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane
WDL7contig_01803	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane
WDL7contig_01842	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane
WDL7contig_01963	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasm
WDL7contig_01982	PAS domain S-box/diguanylate cyclase (GGDEF) domain	GGDEF	PAS	Cytoplasmic membrane
WDL7contig_02013	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane
WDL7contig_02055	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane

Influence of 3-chloroaniline on biofilm lifestyle and implication in bioaugmentation

WDL7contig_02218	Diguanylate cyclase (GGDEF) domain	GGDEF	CACHE	Cytoplasmic membrane
WDL7contig_02402	PAS domain S-box/diguanylate cyclase (GGDEF) domain	GGDEF	PAS	Cytoplasm
WDL7contig_02488	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane
WDL7contig_02743	Diguanylate cyclase (GGDEF) domain	GGDEF	CACHE	Cytoplasm
WDL7contig_02791	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane
WDL7contig_03415	Response regulator receiver modulated diguanylate cyclase	GGDEF	-	Cytoplasm
WDL7contig_03907	Diguanylate cyclase (GGDEF) domain	GGDEF	MASE	Cytoplasmic membrane
WDL7contig_04387	Diguanylate cyclase (GGDEF) domain	GGDEF	CACHE	Cytoplasmic membrane
WDL7contig_04949	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane
WDL7contig_05029	Diguanylate cyclase (GGDEF) domain	GGDEF	-	Cytoplasmic membrane
WDL7contig_00230	PAS domain S-box/diguanylate cyclase (GGDEF) domain	GGDEF-EAL	PAS	Cytoplasmic membrane
WDL7contig_00447	Diguanylate cyclase/phosphodiesterase	GGDEF-EAL	-	Cytoplasmic membrane
WDL7contig_00679	Diguanylate cyclase/phosphodiesterase with	GGDEF-EAL	PAS	Cytoplasmic membrane

Influence of 3-chloroaniline on biofilm lifestyle and implication in bioaugmentation

	PAS/PAC sensor(s)			
WDL7contig_00927	Diguanylate cyclase/phosphodiesterase with PAS/PAC sensor(s)	GGDEF-EAL	PAS	Cytoplasmic membrane
WDL7contig_01961	PAS domain S-box/diguanylate cyclase (GGDEF) domain	GGDEF-EAL	PAS	Cytoplasmic membrane
WDL7contig_02014	Diguanylate cyclase (GGDEF) domain	GGDEF-EAL	-	Cytoplasmic membrane
WDL7contig_02312	Diguanylate cyclase/phosphodiesterase	GGDEF-EAL	MYHT	Cytoplasmic membrane
WDL7contig_02724	PAS domain S-box/diguanylate cyclase (GGDEF) domain	GGDEF-EAL	PAS	Cytoplasmic membrane
WDL7contig_02793	PAS domain S-box/diguanylate cyclase (GGDEF) domain	GGDEF-EAL	PAS	Cytoplasmic membrane
WDL7contig_03117	PAS domain S-box/diguanylate cyclase (GGDEF) domain	GGDEF-EAL	PAS	Cytoplasmic membrane
WDL7contig_03518	Diguanylate cyclase (GGDEF) domain	GGDEF-EAL	PAS	Cytoplasmic membrane
WDL7contig_04012	Diguanylate cyclase/phosphodiesterase with PAS/PAC and Chase sensor(s)	GGDEF-EAL	PAS	Cytoplasmic membrane
WDL7contig_04492	Diguanylate cyclase (GGDEF) domain	GGDEF-EAL	-	Cytoplasmic membrane

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WDL7contig_04686	Diguanylate cyclase/phosphodiesterase with PAS/PAC sensor(s)	GGDEF-EAL	PAS	Cytoplasmic membrane
WDL7contig_01116	HD-GYP domain	HD-GYP	-	Cytoplasm
WDL7contig_01960	Uncharacterized domain HDIG	HD-GYP	-	Unknown
WDL7contig_02643	Uncharacterized domain HDIG	HD-GYP	-	Cytoplasm

3.3.3 Transcriptomic Analysis Suggested an Elevated Catabolic Activity but Decreased C-di-GMP Synthesis in *C. testosteroni* Exposed to 3-CA.

Figure 3.4 Inhibited growth and decreased intracellular c-di-GMP concentration by exposure to 3-CA (200 ppm). (A) The growth of *C. testosteroni* WDL7 on pyruvate and inhibition by addition of 3-CA. (B) Decreased intracellular c-di-GMP after treated by 3-CA for different length of time. (*) Significant difference ($P < 0.05$).

By using the draft genome as reference, we further investigated the global transcriptomic changes after 3-CA treatment. Due to the toxicity, a reduced growth was observed after 3-CA was added (Figure 3.4A). By using filtering criteria ($p \text{ value} \leq 0.05$ and $\log_2 \text{ fold change} \geq 1$), 1696 genes were significantly downregulated and only 104 genes were upregulated. According to the COG classification, various metabolic activities were repressed. More than 30% of genes in amino acid, carbohydrate, coenzyme, energy, inorganic, lipid and nucleotide metabolisms were significantly downregulated (Figure 3.5). Other energy-intensive activities were also highly inhibited, especially motility. More than 50% of motility-related genes were downregulated (Figure 3.5), which is consistent with bacterial motility assay (Figure 3.2). The oil/water partition coefficient of 3-CA is 1.88, suggesting its potential effects on cell membrane integrity and function (Zok, Gorge et al. 1991, Ramos, Duque et al. 2002). Based on the transcriptomic data, 395 of 853 membrane-bound transporter genes exhibited a decreased expression level. The altered expression of membrane proteins may contribute partially to the changes in surface

hydrophobicity. When exposed to 3-CA, one catabolic pathway for the degradation of 3-CA was induced (Figure 3.6). *C. testosteroni* WDL7 was originally isolated from herbicide-treated soil and is capable of mineralizing 3-CA (Dejonghe, Berteloot et al. 2003). Based on the induced expression of catabolic enzymes, WDL7 degraded 3-CA through the ortho-cleavage pathway, which is in contrast to the meta-cleavage pathway employed by other strains such as *C. testosteroni* I2 (Wu, Arumugam et al. 2015). In addition, two exopolysaccharide biosynthesis genes and six exopolysaccharide export genes were found significantly downregulated in cells exposed to 3-CA (Table 3.4). In Congo red assay, the colonies formed by *C. testosteroni* cells in the presence of 3-CA were lighter in red color (Figure 3.7). As Congo red dye binds EPS components cellulose and fimbriae, a lighter color suggested a less EPS production by the cells exposed to 3-CA (Friedman and Kolter 2004, Hickman, Tifrea et al. 2005, Ueda and Wood 2009).

Table 3.4 3-CA treatment downregulated the expression of exopolysaccharide genes.

Feature ID	Description	log2 Ratio (3-CA/control)
WDL7contig_02641	Capsular polysaccharide biosynthesis protein	-2.31
WDL7contig_05072	Lipid A export permease/ATP-binding protein MsbA	-2.25
WDL7contig_00291	Periplasmic protein involved in polysaccharide export	-2.08
WDL7contig_04091	Capsule polysaccharide export protein	-1.95
WDL7contig_00289	ABC-type polysaccharide/polyol phosphate transport system, ATPase component	-1.67
WDL7contig_00290	Capsule polysaccharide export protein	-1.49
WDL7contig_00288	ABC-type polysaccharide/polyol phosphate export systems, permease component	-1.33
WDL7contig_04190	Glycosyltransferase involved in LPS biosynthesis	-1.17

Figure 3.5 The percentage of genes differentially expressed (p-value<0.05, fold-change>2) after 3-CA treatment in each COG categories.

A

B

Figure 3.6 The upregulation of 3-CA degradation pathway. (A) The ortho-cleavage degradation pathway. (B) The upregulated expression of catabolic genes. The transcript level was normalized to the corresponding genes in control group. *dcaQ*, glutamine synthesis; *catA*, catechol 1,2-dioxygenase; *catB*, muconate cycloisomerase; *CMBL*, carboxymethylenebutenolidase; *tfdF*, maleylacetate reductase; *pcaI*, 3-oxoadipate CoA-transferase, alpha subunit; *pcaJ*, 3-oxoadipate CoA-transferase, beta subunit; *pcaF*, 3-oxoadipyl-CoA thiolase.

Figure 3.7 Colony morphology on M9 Congo red agar plates with or without 200 ppm 3-CA

The above genomic analysis revealed that *C. testosteroni* has a sophisticated signaling system to regulate biofilm lifestyle. After exposed to 3-CA, 28 c-di-GMP controlling genes were downregulated (fold change > 1.5) and 24 of them are predicted to be c-di-GMP synthases (Table 3.5). The downregulation of this group of genes implies a decrease in the c-di-GMP level after exposure to 3-CA.

Table 3.5 3-CA treatment decreased the expression of putative c-di-GMP-synthases. Ratio indicates a relative gene expression level in cells treated by 3-CA to those in control group

Feature ID	Description	log ₂ Ratio (3-CA/control)
WDL7contig_02402	PAS domain S-box/diguanylate cyclase (GGDEF) domain	-2.24
WDL7contig_01842	Diguanylate cyclase (GGDEF) domain	-2.11
WDL7contig_03518	Diguanylate cyclase (GGDEF) domain	-1.81
WDL7contig_00113	PAS domain S-box/diguanylate cyclase (GGDEF) domain	-1.40
WDL7contig_03907	Diguanylate cyclase (GGDEF) domain	-1.36

WDL7contig_02218	Diguanylate cyclase (GGDEF) domain	-1.31
WDL7contig_00230	PAS domain S-box/diguanylate cyclase (GGDEF) domain	-0.99
WDL7contig_00747	Response regulator containing a CheY-like receiver domain and a GGDEF domain	-0.98
WDL7contig_01803	Diguanylate cyclase (GGDEF) domain	-0.98
WDL7contig_02055	Diguanylate cyclase (GGDEF) domain	-0.97
WDL7contig_02791	Diguanylate cyclase (GGDEF) domain	-0.87
WDL7contig_00546	Diguanylate cyclase (GGDEF) domain	-0.83
WDL7contig_01961	PAS domain S-box/diguanylate cyclase (GGDEF) domain	-0.78
WDL7contig_02793	PAS domain S-box/diguanylate cyclase (GGDEF) domain	-0.72
WDL7contig_04387	Diguanylate cyclase (GGDEF) domain	-0.67
WDL7contig_02014	Diguanylate cyclase (GGDEF) domain	-0.65
WDL7contig_02488	Diguanylate cyclase (GGDEF) domain	-0.65
WDL7contig_00724	Diguanylate cyclase (GGDEF) domain	-0.64
WDL7contig_00769	Diguanylate cyclase with PAS/PAC sensor	-0.64
WDL7contig_03415	Response regulator receiver modulated diguanylate cyclase	-0.62

3.3.4 Exposure to 3-CA Decreased Intracellular c-di-GMP Level in *C. testosteroni* and Induced Biofilm Dispersal.

Intracellular c-di-GMP level was quantified before and after exposure to 3-CA (Figure 3.4A). For the control cultures with no 3-CA exposure, the c-di-GMP concentration gradually increased when the cultures were growing from the exponential phase to the stationary phase (Figure 3.4B).

When exposed to 3-CA, the intracellular c-di-GMP concentration was lower than that of the control cultures (Figure 3.4B). At the stationary phase, the c-di-GMP level for the control cultures was about 2.5-fold of that of the 3-CA-treated cultures. Previous studies found the decreased c-di-GMP would downregulate the expression of adhesin and exopolysaccharide which would negatively influence the irreversible attachment (Kuchma, Brothers et al. 2007, Ueda and Wood 2009). Therefore, the decreased c-di-GMP level could explain the reduced cell retention observed for the packed bed bioreactors.

Flow cell biofilms were used to further investigate the consequences of a reduced c-di-GMP upon 3-CA exposure. The WDL7 biofilms were grown using pyruvate as sole carbon source. After 4-day growth, the towering biofilm structure, a typical feature of mature biofilms in flow cells, was observed (Figure 3.8A). Upon exposure to 3-CA, massive detachment of cells from the mature biofilms occurred (Figure 3.8B). The cell detachment rate increased from 6.07×10^8 cells/h to 1.11×10^9 cells/h after 3-CA was introduced to the biofilms (Figure 3.8C). Quantitative image analysis showed a loss of 86% of the biofilm biomass after 5-h exposure to 3-CA (Figure 3.8D). The massive detachment also resulted in a thinner and evened biofilm, which has a decreased mean thickness (from 13.3 ± 2.2 μm to 8.4 ± 2.0 μm , $P < 0.05$) and roughness (from 6.8 ± 1.3 to 4.9 ± 0.9 , $P < 0.05$).

Figure 3.8 (A) Biofilms developed in flow cells after 4-day growth. (B) Biofilms after treatment by 3-CA (200 ppm) for 5 h. Scale bar represents 20 μm . (C) Cell detachment rate from biofilms before and after 5-h exposure to 3-CA. (D) Biovolume of the biofilms before and after 3-CA treatment (n=5). (*) Significant difference ($P < 0.05$).

Previous studies on *P. putida* and *B. subtilis* found that when exposed to aromatic hydrocarbons, cells minimize energy expenditure by repressing growth phase-dependent genes rather than pursuing a better environment (Domínguez-Cuevas, González-Pastor et al. 2006, Tam, Eymann et al. 2006). Similar inhibition of 3-CA on cell motility and basic metabolisms of *C. testosteroni* was found in this study. Although in most Gram-negative bacteria a low level of c-di-GMP often leads to an increased motility via the activation of the c-di-GMP-binding effectors (Hengge 2009), the influence of 3-CA on the motility could not be predicted solely by the reduced level of c-di-GMP because a lower energy metabolism was also observed. Meanwhile, 3-CA treatment also results in biofilm dispersal. It suggested, when exposed to toxic chemicals, bacterial cells would prefer the planktonic form to the biofilm lifestyle so that they could move passively with the aqueous phase.

On community level, toxic contaminants such as 3-CA were found to have a negative impact on the whole wastewater treatment system (Boon, Top et al. 2003, Falk and Wuertz 2010). In addition to inhibiting metabolic activities, exposure to 3-CA also decreased the settleability of activated sludge (Boon, Top et al. 2003). Previous study showed that granulation process was correlated with an increase in the intracellular c-di-GMP level (Wan, Yang et al. 2014). When the intracellular c-di-GMP level was reduced, a decrease in EPS content and a loss of granular stability were observed (Wan, Zhang et al. 2013). Hence, the negative influence on the biofilm lifestyle and a decrease in the c-di-GMP concentration in *C. testosteroni* caused by the exposure to 3-CA would have important implications on bioaugmentation.

3.3.5 Environmental Implications.

In bioaugmentation bioprocesses for the treatment of wastewater or soil containing toxic industrial pollutants, the organisms to be augmented are usually prepared in media containing the targeted pollutants to induce desirable catabolic activities and to allow the cells adapt well to the toxic conditions (Boon, Goris et al. 2000, Gentry, Newby et al. 2001). In this study, we demonstrate that although the catabolic activities can be induced, the pre-exposure to the pollutants has a great impact to the biofilm lifestyle of the bacteria including decreases in bacterial

motility and the intracellular c-di-GMP level. Consequently, when introducing the treated cultures into the bioaugmentation systems, cells would have a lower dispersion and retention coefficient which leads to a rapid flow-out and a less biomass retention. In addition, the reduced c-di-GMP level would destabilize biofilms and cause massive cell dispersal, which may eventually leads a bioaugmentation failure. For example, in the real bioaugmentation cases, the augmented organism with a weak biofilm formation ability resulted in a failed bioaugmentation (Songzhe, Hongxia et al. 2009), while enhanced biofilm formation capability improved the bioaugmentation performance (Wang, Zheng et al. 2014). As a decreased c-di-GMP level leads to less initial attachment and biofilm dispersal, approaches to elevating or maintaining a high c-di-GMP level may be promising to establish and maintain a sustainable bioaugmentation activity. In previous study, an elevated intracellular c-di-GMP concentration for *C. testosteroni* WDL7 was achieved by constitutively expressing a c-di-GMP synthase (Wu, Ding et al. 2015). The high c-di-GMP level was found to alleviate inhibition by 3-CA at high concentration and enhance biofilm-based degradation. Genetic engineering may not be favored in environmental application; alternative approaches may be explored to maintain a high c-di-GMP level to enhance overall biofilm robustness and reduce cell detachment under unfavorable condition. For example, when nitrate was present in aqueous phase, *C. testosteroni* I2 cells residing at the bottom of biofilm maintain metabolic active by respiring on nitrate and also have a high c-di-GMP level to reduce biofilm dispersal (Wu, Shukal et al. 2015).

In bioaugmentation, pure cultures of bacteria capable of degrading a targeted pollutant to be remediated are usually introduced into the bioremediation system. Therefore, in this study, we used pure cultures of a model organism (*C. testosteroni*) and one targeted pollutant (3-CA) to investigate the influence of the pollutant on the transport and biofilm lifestyle of the augmented organism. The findings from this model system provide an improved understanding of the transport and biofilm development of the augmented bacteria, which is essential for the development of novel strategies to achieve more robust bioaugmentation. When multiple pollutants are present in the bioremediation system, pollutants with similar chemical structures would likely display similar effects on the augmented bacteria;

otherwise, a combined effect of different pollutants needs to be considered. When multiple organisms, for example, microbial consortia, are augmented, we would expect that both the organism-pollutant interaction and the organism-organism interaction contribute to the overall performance. The influence of the targeted pollutants on the transport and biofilm development of the augmented microbial consortia would be an intriguing research topic for future exploration.

3.4 Conclusions

In bioaugmentation bioprocesses for the treatment of wastewater or soil containing toxic industrial pollutants, the organisms to be augmented are usually prepared in media containing the targeted pollutants to induce desirable catabolic activities and to allow the cells adapt well to the toxic conditions. In this study, we demonstrate that although the catabolic activities can be induced, the pre-exposure to the pollutants has a great impact to the biofilm lifestyle of the bacteria including decreases in bacterial motility and the intracellular c-di-GMP level. Consequently, when introducing the treated cultures into the bioaugmentation systems, cells would have a lower dispersion and retention coefficient which leads to a rapid flow-out and a less biomass retention. In addition, the reduced c-di-GMP level would destabilize biofilms and cause massive cell dispersal, which may eventually leads a bioaugmentation failure.

Chapter 4. Elevated level of the second messenger c-di-GMP in *C. testosteroni* enhances biofilm formation and biofilm-based biodegradation

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4.1 Introduction

In most natural and engineered environments, microbes are commonly found closely associated with surfaces or interfaces in the form of multicellular aggregates known as biofilms, where cells are embedded in a matrix of self-produced extracellular polymeric substances (EPS) (Hall-Stoodley, Costerton et al. 2004, Singh, Paul et al. 2006, Flemming and Wingender 2010, Ding, Peng et al. 2014, Zhang, Ng et al. 2014). A number of studies have suggested that the biofilm mode of growth provides a range of advantages to the cells, especially under unfavorable conditions such as exposure to toxic metals, high salinity and antimicrobial agents (Singh, Paul et al. 2006, Cao, Majors et al. 2012). Hence, biofilm-based bioprocesses have been widely applied for industrial and municipal wastewater treatment (Nicolella, van Loosdrecht et al. 2000, Singh, Paul et al. 2006). One of the important molecular determinants for biofilm formation and detachment is a signaling molecule bis-(3' -5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (Hengge 2009). Generally, elevated c-di-GMP levels stimulate the synthesis of EPS components and inhibit various forms of motility, favoring biofilm formation (Hengge 2009). In the previous chapter, we already demonstrated that when exposed to toxic aromatic, the reduced c-di-GMP level is correlated with decreased EPS secretion and biofilm dispersal. Therefore, a robust

biofilm processes may be able to construct by elevating intracellular c-di-GMP level.

In this study, we used *C. testosteroni* WDL7 as a model organism and investigated the impacts of elevating c-di-GMP level on biodegradation of 3-CA. We expressed a c-di-GMP synthase YedQ from *Escherichia coli* in *C. testosteroni* WDL7, quantified the c-di-GMP concentration and biofilm formation capability of the wild-type and the transconjugant, and compared their performance in 3-CA biodegradation.

4.2 Materials and methods

4.2.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *C. testosteroni* strains were pre-cultured in lysogeny broth (LB) medium at 30°C. When appropriate, gentamicin (20 µg/mL) was added for plasmid maintenance. The overnight seed cultures were subsequently inoculated in M9 minimal medium which was supplemented with 10 mM pyruvic acid sodium salt or 200 mg/L 3-chloroaniline as the sole carbon source. Growth was monitored by measuring optical density at 600 nm. Colony morphology was assayed on Congo red agar plate (M9 minimal medium, 1.5% agar, 20mM pyruvic acid sodium salt, 15 µg/mL Congo red) (Kirisits, Prost et al. 2005).

4.2.2 Conjugative plasmid transfer

The construction of *C. testosteroni* WDL7 harboring the pYedQ2 plasmid was performed by a triparental mating (Haagensen, Hansen et al. 2002). The helper plasmid RK600 was used to mobilize the plasmid pYedQ2 into the recipient *C. testosteroni* WDL7. LB agar plate containing 50 µg/mL rifampicin and 50 µg/mL gentamicin was used to select for *C. testosteroni* WDL7 derivative carrying the pYedQ2 plasmid, which is designated WDL7/pYedQ2.

4.2.3 Motility assay

Swarming and swimming motility of the *C. testosteroni* strains was tested by spot- and point- incubation, respectively, on M9 minimal medium agar containing 20 mM pyruvic acid sodium salt. For swarming activity, 0.5% agar was used; while 0.3% agar was used for swimming assay (Murray and Kazmierczak 2008).

4.2.4 Quantitative biofilm assay

Biofilm assay was conducted based on a 96-well plate method with slight modifications (Wakimoto, Nishi et al. 2004, Ding, Peng et al. 2014). 150 μ L of M9 minimal medium containing 10 mM pyruvic acid sodium salt was inoculated by 1.5 μ L of overnight LB culture ($OD_{600} \sim 1.8$). The plate was incubated at 30°C with shaking. The growth of planktonic biomass was monitored by measuring OD_{600} in a Tecan microplate reader Infinite 200 PRO (Tecan, Männedorf, Switzerland). After 12 h and 24 h incubation, planktonic cells were removed. Surface-associated biomass was stained by 1% crystal violet for 15 min. After adding 200 μ L of 95% ethanol, the amount of biofilm biomass was quantified by OD_{590} . For each strain, 16 replicates were carried out.

4.2.5 Biodegradation of 3-CA

Biodegradation of 3-CA by *C. testosteroni* WDL7 and WDL7/pYedQ2 was carried out aerobically at 30°C. For planktonic cultures, cells were grown in conical flasks with a low surface to volume ratio of $\sim 0.83 \text{ cm}^2/\text{mL}$. About 2 mL of an overnight LB culture ($OD_{600} \sim 1.8$) was transferred to a 500-mL flask with 200 mL M9 minimal medium containing 200 mg/L 3-CA. To check cell aggregation, particle size of the cultures at the early-stationary phase was measured using a SALD-3101 particle analyzer (Shimadzu, Japan). For cultures with both planktonic and sessile cells, 24-well Nunc polystyrene titer plates with a high surface to volume ratio of $\sim 4.52 \text{ cm}^2/\text{mL}$ were used. About 15 μ L overnight LB culture ($OD_{600} \sim 1.8$) was mixed with 1.5 mL of M9 minimal medium containing 200 mg/L 3-CA as the sole carbon source followed by incubation at 30°C with shaking. Control experiments without cells were also conducted. Biomass yield was estimated directly from experimental data as biomass produced per total amount of

3-CA consumed. Biomass concentration was indicated by total protein concentration. Planktonic culture was centrifuged and hydrolyzed in 0.1 M NaOH at 70°C for 1 h. The final protein concentration was determined by Qubit protein assay kit (Invitrogen) following manufacturer's instructions. The percentage of 3-CA consumed by planktonic cells were determined using biomass yield and the amount of biomass of the planktonic cells.

4.2.6 Quantification of 3-CA

Samples from biodegradation experiments were taken at predetermined time points and filtered through 0.20 µm filters. The supernatant was analyzed for 3-CA concentration using a reverse-phase HPLC, where an Acsentis C18 column (100mm×2.1mm, 5 µm particle size), a mobile phase of methanol/water (50/50), a flow rate of 0.6 mL/min and a UV detector set at 254 nm were used (Król, Penrod et al. 2012).

4.2.7 Tolerance to 3-CA at high concentrations

The tolerance of the wild type and the high c-di-GMP transconjugant to 3-CA at high concentrations was examined by measuring growth inhibition. Cells were grown in 30 ml M9 medium containing 10 mM pyruvate in 150 ml flasks with shaking (150 rpm) at 30°C. At the early stationary phase (OD₆₀₀ ~0.75), the cultures were spiked with 3-CA to a final concentration of 400 or 800 mg/L. The stock solution of 3-CA was prepared in methanol and the volume of stock solution added to the cultures was less than 1% v/v. The same amount of methanol was added into control cultures.

4.2.8 3-CA degradation in submerged biofilm reactors

Milliliter-scale submerged biofilm reactors were set up in triplicates using 3-mL syringes (Luer-Lok tip, latex free; BD) packed with glass beads (diameter, 2.0 mm) (solid-glass beads; Sigma-Aldrich) for biofilm growth (Ding, Peng et al. 2014). Total surface area in each reactor was 6283 mm². Each reactor was inoculated using diluted overnight LB cultures (OD₆₀₀ ~0.1) followed by a 2-h stop-flow to allow cells to attach onto the glass beads. M9 medium containing 10 mM pyruvate was then continuously fed with a flow rate of 4.6 mL/h for biofilm growth. After 72 h,

3-CA was introduced to the M9 medium containing 10 mM pyruvate at a final concentration of 200 mg/L. The concentration of 3-CA in the effluent from the submerged biofilm reactors was quantified at predetermined time points. Cell detachment rate induced by 3-CA was quantified using a drop-plate method following previously reported protocols (Chen, Nace et al. 2003, Ding, Peng et al. 2014, Sivakumar, Wang et al. 2014).

4.2.9 Extraction and quantification of c-di-GMP

Cells were grown at 30°C in M9 medium containing 10 mM pyruvic acid sodium salt or 200 mg/L 3-CA as sole carbon source. About 15 mL culture was harvested at the late-exponential growth phase by centrifugation at 10,000 g for 5 min at 4°C. The c-di-GMP extraction was carried out by using organic solvent mixture (Kuchma, Ballok et al. 2010). Cell pellet were resuspended in 0.5 mL of cold (-20°C) solvent mixture acetonitrile/methanol/water (40:40:20 v/v/v) containing 0.1 M formic acid to quench enzyme activities. After incubation of 30 min at -20°C, the mixture was transferred to a 1.5-mL Eppendorf tube and centrifuged at 20,000g for 5 min at 4°C. The supernatant was retained and the pellet was extracted again by adding 0.2 mL of extraction solvent and incubating at -20 °C for 15 min. Cell debris were removed by centrifugation and the supernatant was combined with the previous extract. The extract was neutralized using 15% NH₄HCO₃, vacuum-dried, and then re-constituted in 200 µL 1 mM ammonium acetate. Cellular concentration of c-di-GMP was analyzed using a Thermo Accela 1250 series LC system fitted and a Thermo Velos Pro Orbitrap mass spectrometer based on a previously published method (Spangler, Böhm et al. 2010). Separation was carried out on a NUCLEODUR C18 Pyramid column (2 mm x 50 mm, 3 µm particle size, Macherey-Nagel GmbH, Düren, Germany) at 35°C. A gradient elution system was used. Mobile phase A was 10 mM ammonium acetate buffer with 0.1% acetic acid, and mobile phase B was acetonitrile with 0.1% acetic acid. Solvent gradient conditions were as follows: 0% B from 0 to 3 min, 10 % at 3 min, 90% at 4 min, hold until 5 min, then return to 0% at 5.5 min and equilibrated for 4.5 min. Total run time was 10 min. The flow rate was 0.3 mL/min and injection volume was 10.0 µL. Detection of c-di-GMP was carried out in positive ion electrospray ionization (ESI+) mode. For quantitation, a scan range of m/z 675-725 was used in

high-resolution mode with AGC target 5×10^5 . The c-di-GMP concentration was normalized to total protein concentration, which was determined by Qubit protein assay kit (Invitrogen) as described above.

4.3 Results and discussion

4.3.1 Elevated c-di-GMP level enhances biofilm formation of *C. testosteroni*

Figure 4.1 Planktonic growth of *C. testosteroni* WDL7 (WT) and *C. testosteroni* WDL7/pYedQ2 in M9 minimal medium containing 10 mM pyruvate.

The WDL7 wild type and WDL7/pYedQ2 grew comparably well in M9 medium containing pyruvate as the sole carbon source (Figure 4.1). Constitutively expressing a c-di-GMP synthase YedQ in *C. testosteroni* WDL7 resulted in an elevated c-di-GMP level. The c-di-GMP concentration the WDL7/pYedQ2 cells was determined to be 37.1 ± 2.0 pmol/mg protein, which was significantly higher ($p = 0.009$) than that of the wild type (20.2 ± 5.9 pmol/mg protein) (Figure 4.2A). Further, compared with the wild type, the high c-di-GMP transconjugant WDL7/pYedQ2 bound more Congo red dye and exhibited a lower swarming and swimming motility (Figure 4.2B and C).

Figure 4.2 (A) Intracellular c-di-GMP concentration of the *C. testosteroni* WDL7 wild type (WT) and the pYedQ2-harboring transconjugant when using pyruvate and 3-CA as sole carbon source. The concentration of c-di-GMP was expressed as the amount of c-di-GMP per mg of total cellular protein. (B) Colony morphology on M9 Congo red agar plates, swarming and swimming motility assay of the WDL7/pYedQ2 and the WT. (C) Average diameter of motility halos (swimming) and colonies (swarming) (n=5). (D) Quantitative analysis of biofilm formation of wide type and WDL7/pYedQ2 during growth in M9 minimal medium containing 10 mM pyruvate. The data values are the averages of 8 replicates, and the bars represent the standard deviation.

Congo red is a dye that binds EPS components cellulose and fimbriae (Friedman and Kolter 2004, Hickman, Tifrea et al. 2005, Ueda and Wood 2009). The colonies of WDL7/pYedQ2 bound more Congo red, suggesting that a higher c-di-GMP level enhances EPS production in *C. testosteroni*. In swarming and swimming motility assays, WDL7/pYedQ2 formed significantly smaller colonies,

which implies that elevating c-di-GMP level in *C. testosteroni* represses cell motility. Both the EPS production and cell motility have been shown to play critical roles in biofilm formation (Donlan 2002). Because WDL7/pYedQ2 showed an enhanced EPS production and a decreased motility, we further quantified its biofilm formation capability and found that, under growth conditions with a large surface to volume ratio (802.07 cm²/mL), the fraction of biofilm biomass in WDL7/pYedQ2 cultures was ~10-fold higher than the wild type (Figure 4.2D).

4.3.2 Elevated c-di-GMP level increases biofilm contribution to 3-CA degradation

To address whether c-di-GMP level influences biodegradation activity of *C. testosteroni*, we analyzed 3-CA degradation by the wild type and the high c-di-GMP transconjugant WDL7/pYedQ2.

Figure 4.3 Planktonic growth of *C. testosteroni* WDL7 (circle) and *C. testosteroni* WDL7/pYedQ2 (triangle) in M9 minimal medium containing 200 mg/L 3-chloroaniline.

Under planktonic culture conditions with a low surface to volume ratio (about 0.83 cm²/mL), the wild type and WDL7/pYedQ2 grew comparably well (Figure 4.3) and, intriguingly, both cultures did not contain observable cell aggregates, which was confirmed by a quantitative particle size analysis (Figure

4.4). In cultures using 3-CA as sole carbon source, the transconjugant WDL7/pYedQ2 also exhibited an elevated c-di-GMP concentration (44.0 ± 2.3 pmol/mg protein) than wild type (17.6 ± 3.4 pmol/mg protein) in the degradation of 3-CA (Figure 4.2A). Both the wild type and the transconjugant cultures completely degraded 200 mg/L 3-CA within 20 h (Figure 4.3). The degradation rate for the high c-di-GMP transconjugant was slightly lower than the wild type (Figure 4.3). For example, at 8 h and 11 h, ~34% and 72% of total 3-CA were consumed by the transconjugant, while about 39% and 89% were consumed by the wild type. However, the difference in biomass yield coefficient $Y_{x/s}$ for both strains were marginal (0.343 vs. 0.349 mg/mg), suggesting that a high c-di-GMP level in *C. testosteroni* does not influence its biomass yield in 3-CA degradation.

Figure 4.4 Particle size distribution of (A) the wild type and (B) the transconjugant WDL7 (pYedQ2) in planktonic cultures grown in culture flasks with a low surface to volume ratio.

Under culture conditions with a high surface to volume ratio, cultures of the high c-di-GMP strain WDL7/pYedQ2 would be expected to contain a significant fraction of biofilm cells, which may influence the biodegradation efficiency. To test this, biodegradation of 3-CA by *C. testosteroni* growing in 24-well microtiter plates with a high surface to volume ratio ($4.52 \text{ cm}^2/\text{mL}$) was conducted and the results are shown in Figure 4.5. There was a significant fraction of biofilm cells for both the wild type and the high c-di-GMP transconjugant cultures, in particular, the latter.

At the mid-exponential phase (12 h) and early stationary phase (15 h), the OD590 value of stained biofilm biomass in the transconjugant cultures was about 10- to 14-fold higher than that of the wild type. In contrast, the biomass concentration of planktonic cells for the wild type reached up to ~60 mg/L, while only about ~35 mg/L could be obtained for the transconjugant. We further examined the effect of the observed difference in relative abundance of planktonic and biofilm cells in the cultures on overall biodegradation activity. The wild type cultures consumed all the 3-CA in 15 h, while ~29 mg/L of 3-CA (~15% of total) still remained in the transconjugant cultures, suggesting a slightly lower biodegradation rate in the transconjugant cultures, which could be because of the slow growth rate and reduced metabolic activities of biofilm cells (Mah and O'Toole 2001). Using the yield coefficients obtained above for the planktonic cells, relative contribution of the planktonic and biofilm cells in degrading 3-CA was estimated (Figure 4.5B and C). At the early exponential stage of growth (9 h), ~25% and 30% of total 3-CA was consumed by biofilm cells for the wild type and for the transconjugant, respectively. While at the early stationary phase (15 h), the degradation of ~53% of total 3-CA in the transconjugant cultures could be attributed to biofilm cells, while biofilm cells in the wild type cultures only contributed to the degradation of ~15% of total 3-CA. These results demonstrated that, in batch culture conditions with a high surface to volume ratio, an elevated c-di-GMP level increases significantly the contribution of biofilm cells to biodegradation.

Figure 4.5 (A) Degradation of 3-CA by *C. testosteroni* WDL7 (filled circle) and WDL7/pYedQ2 (filled triangle) in relation to the concentration of planktonic biomass measured as total protein concentration of WDL7 (empty circle) and WDL7/pYedQ2 (empty triangle). The percentage of 3-CA consumed by planktonic cells and biofilm cells after growing for 9 h (B) and 15 h (C).

4.3.3 Elevated c-di-GMP level enhances tolerance to 3-CA

To examine whether the transconjugant with an elevated c-di-GMP level has a higher tolerance to 3-CA at high concentrations, the inhibitory effects of 3-CA at 400 mg/L or 800 mg/L on early stationary cultures of the wild type and the high c-di-GMP transconjugant were examined (Figure 4.6). 3-CA was spiked in the culture to a different final concentration when it reached stationary phase. When exposed to 400 mg/L of 3-CA, the growth of wild type culture was marginally decreased. In contrast, the high c-di-GMP transconjugant culture exhibited a slightly better growth than the control (no 3-CA), suggesting a rapid adaptation of the transconjugant cells to the presence of 3-CA. In the presence of 800 mg/L of 3-CA, a marked inhibition on growth of the wild type could be observed, while the transconjugant culture showed a comparable growth with the control (no 3-CA). These results demonstrated that an elevated c-di-GMP level enhances bacterial tolerance to 3-CA.

Figure 4.6 Influence of 3-CA at a concentration of 400 or 800 mg/L on planktonic cultures of the wild type and the high *c*-di-GMP tranconjugant in M9 medium containing 10 mM pyruvate. Experimental data were presented as the means of at least 3 replicates. In cases of no more than 10% variation among replicates, error bars were omitted for a better clarity.

4.3.4 Elevated *c*-di-GMP level enhances biofilm-based biodegradation

To quantify the influence of elevated *c*-di-GMP level on biodegradation in biofilm reactors, we compared the performance of 3-CA degradation by biofilms of the wild type and the high *c*-di-GMP transconjugant in submerged biofilm reactors. Pre-grown 3-day biofilms were supplied continuously with the same growth medium containing 3-CA. The concentration of 3-CA in effluent from the reactors of the wild type was significantly higher than that of the high *c*-di-GMP transconjugant (Figure 4.7A), suggesting a higher degradation efficiency of the transconjugant biofilms.

Figure 4.7 Biodegradation of 3-CA in submerged biofilm reactors: (A) 3-CA concentration in effluents from the reactors; (B) Cell detachment rates from the biofilm of WDL7 and WDL7/pYedQ2 before exposed to 3-CA and after 3-h exposure to 3-CA

Previous studies showed that xenobiotic shocks could cause cell detachment and disrupt biofilm structure and function (Boon, Top et al. 2003, Suja, Nancharaiah et al. 2012). To address whether the elevated intracellular *c-di-GMP* level would enhance the robustness of the biofilms, we quantified the cell detachment rate before and after the biofilms were exposed to 3-CA. Before 3-CA exposure, cell detachment from the WDL7/pYedQ2 biofilms was at a rate comparable with that of the wild type biofilms (6.0×10^4 vs. 3.5×10^4 cells/h/mm²) (Figure 4.7B). In contrast, in the presence of 3-CA, cell detachment rate for the wild type biofilms was significantly higher than that from the WDL7/pYedQ2 biofilms (1.4×10^5 vs. 4.8×10^4 cells/h/mm²; $p < 0.01$). The exposure to 3-CA caused a ~2.4-fold increase in cell detachment for the wild type biofilms, while only ~1.4-fold induction was observed for the WDL7/pYedQ2 biofilms. These results suggest that the high *c-di-GMP* strain WDL7/pYedQ2 is capable of maintaining more robust biofilms during 3-CA biodegradation in continuous reactors. The enhanced 3-CA biodegradation achieved by the biofilms of the high *c-di-GMP* transconjugant could be due to a higher retention of cells in the biofilm reactors in the presence of 3-CA. Consistent with this hypothesis, after 72-h exposure to 3-CA, our results show that total protein content for the transconjugant biofilms was 601.4 ± 8.5 ng/mm², which was significantly higher than that of the wild type biofilms ($430.5 \pm$

6.1 ng/mm²). Hence, an elevated c-di-GMP level in *C. testosteroni* alleviated cell detachment induced by toxic 3-CA and improved cell retention in the biofilm reactors to achieve a higher biodegradation capability.

4.4 Discussion

At cellular level, c-di-GMP is synthesized by diguanylate cyclases (DGCs, containing a GGDEF domain) and degraded by phosphodiesterases (PDEs, containing an EAL domain) (Simm, Morr et al. 2004). In *E. coli*, YedQ is a GGDEF domain protein that has been shown to stimulate the production of c-di-GMP by its DGC activity (Da Re and Ghigo 2006, Sanchez-Torres, Hu et al. 2011). Previous studies have demonstrated that the expression of *E. coli* YedQ in *Salmonella Typhimurium* and *P. putida* increased the intracellular c-di-GMP concentration, induced biofilm formation by stimulating the synthesis of biofilm matrix components and inhibiting cell motility (Simm, Morr et al. 2004, Gjermansen, Ragas et al. 2006, Wolfe and Visick 2008, Gjermansen, Nilsson et al. 2010). Although the genomes of *C. testosteroni* encode at least 55 proteins containing GGDEF and/or EAL domain with potential DGC and PDE activities (Ma, Zhang et al. 2009), there have been no reports on the role of c-di-GMP in this organism. Here, we show that constitutively expressing YedQ in WDL7 resulted in an elevated c-di-GMP level in this organism. And it is also the first time to report that constitutively expressing YedQ in *C. testosteroni* WDL7 elevates c-di-GMP concentration and the high c-di-GMP level enhances EPS production, inhibits cell motility, and induces biofilm formation.

Recent studies reported that an elevated c-di-GMP level in *P. aeruginosa* contributes to antimicrobial resistance and improves its fitness in the presence of imipenem through regulating porins and multidrug efflux pump gene expression (Gupta, Liao et al. 2014, Nicastro, Kaihami et al. 2014). Here, we show that an elevated c-di-GMP level in *C. testosteroni* improves its tolerance to 3-CA, and hence, biodegradation of 3-CA at high concentrations. The exact molecular mechanism on how the elevated c-di-GMP increases bacterial tolerance to 3-CA remains elusive and requires further investigations.

Under unfavorable conditions such as exposure to toxic pollutants, bacterial cells often change to biofilm mode of life because biofilm cells protected by the EPS matrix are more tolerant. For example, Bossier et al. (Bossier and Verstraete 1996) reported that *C. testosteroni* A20 changed its morphology to dense flocs in unfavorable conditions. Boon et al. (Boon, Goris et al. 2000) found that, when augmenting *C. testosteroni* I2 into the activated sludge, the I2 cells were not randomly distributed but only found as biofilms. It has also been shown that long-term exposure of bacterial cells to toxic compounds was detrimental to their metabolic activities and the protection by extracellular matrix could be effective for maintaining biodegradation activities (Boon, De Gelder et al. 2002). In addition, biofilms have a high cell density and quorum sensing (QS) systems are often activated (Parsek and Greenberg 2005). In many cases, catabolic genes for biodegradation are regulated by QS (Valle, Bailey et al. 2004, Yong and Zhong 2013). For example, TeiR, a LuxR-type QS regulator in *C. testosteroni* ATCC 11996 was found to regulate the bacterial steroid degradation through interacting with the steroid catabolic gene promoters (Pruneda-Paz, Linares et al. 2004, Linares, Pruneda-Paz et al. 2008). Hence, although the metabolic activities of cells in biofilms are often lower than their planktonic counterparts (Mah and O'Toole 2001), biofilm-based biodegradation is highly desirable, especially for toxic pollutants. Here, we demonstrated that an elevated c-di-GMP level in *C. testosteroni* enhances biofilm formation, increases the contribution of biofilms in biodegradation, and improves biodegradation in continuous biofilm reactors, suggesting that manipulating c-di-GMP signaling may be a promising approach to enhance biofilm-based biodegradation. The exact mechanism of whether and how c-di-GMP regulates the expression of catabolic genes in 3-CA biodegradation in *C. testosteroni* biofilms remains unclear and warrants further investigations.

Together, transconjugant harboring pYedQ2 can play an important role in many environmental and biotechnological application, especially in wastewater treatment process. First, the increased c-di-GMP can stabilize granular structure by secreting more adhesins and exopolysaccharide (Wan, Zhang et al. 2013). The loss of granular structure is a main challenge to its successful field application in long term. Stable aggregates can maintain a high biomass concentration in reactor due to

good settling characteristics from the bulk liquid. Beside stabilization of physical properties, bioaugmentation of bioreactor by aromatic compound-tolerant strains like *P. putida* and *C. testosteroni* can also protect the microbial structure and function against the toxic shock (Boon, Top et al. 2003, McLaughlin, Farrell et al. 2006, Yu, Ali et al. 2010). Second, biofilm embedded cells are experiencing a slow growth rate. Less sludge would be produced in long-term application. Third, biofilms of strain with elevated c-di-GMP level would have a better performance by maintaining more robust biofilm in continuous reactor.

4.5 Conclusions

In summary, the impacts of elevating c-di-GMP level in *C. testosteroni* on its biofilm formation and biodegradation activity were investigated. Here are the main conclusions can be drawn from this study: (i) constitutively expressing YedQ, a c-di-GMP synthase from *E. coli*, in *C. testosteroni* increases c-di-GMP concentration significantly, (ii) the high c-di-GMP level in *C. testosteroni* enhances EPS production, inhibits cell motility, and induces biofilm formation, (iii) compared with the wild type, the high c-di-GMP transconjugant exhibits a higher tolerance to 3-CA at high concentrations, (iv) under conditions with a high surface to volume ratio, elevating c-di-GMP level in *C. testosteroni* significantly increases the contribution of biofilms in 3-CA biodegradation, and (v) in continuous biofilm reactors, an elevated c-di-GMP level in *C. testosteroni* alleviates cell detachment from biofilms and enhances 3-CA biodegradation. Taken together, this study provides a novel strategy to enhance biofilm-based biodegradation of toxic xenobiotic compounds through manipulating bacterial c-di-GMP signaling.

Chapter 5. Involvement in denitrification is beneficial to the biofilm lifestyle of *C. testosteroni*

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5.1 Introduction

In microbial communities driving domestic wastewater treatment, bacteria of the *Comamonas* genus were often reported to be among the most abundant microorganisms (Andersson, Kuttuva Rajarao et al. 2008, Juang, Adav et al. 2010, Luo, Xu et al. 2012, Kim, Pagaling et al. 2014). In addition, a high abundance of *Comamonas* was also found in nitrate-removing microbial communities presented in river wetlands (Guo, Gong et al. 2015), soil (Patureau, Zumstein et al. 2000), and constructed wetlands (Zhong, Wu et al. 2014). However, why they are there and what roles they may play remain largely unknown. To address these questions, a comprehensive understanding of the physiology and metabolic capabilities of this group of organisms is required and *Comamonas testosteroni* is often used as a model organism for such studies.

C. testosteroni is a bacterium that is commonly found in natural and engineered environments including bioreactors for the treatment of domestic and/or industrial wastewater (Willems and Vos 2006). It has a versatile catabolic capabilities in degrading a variety of organic compounds and has been demonstrated to play an important role in bioremediation (Boon, Goris et al. 2000, Dejonghe, Berteloot et al. 2003, Horinouchi, Hayashi et al. 2003). In various natural and engineered ecosystems, *C. testosteroni* is often found to exist as surface- or interface-associated microbial aggregates, *i.e.*, biofilms, the predominant mode of

life on earth (Flemming and Wingender 2010). Comparative genome analyses suggested a sophisticated regulatory system integrating a number of proteins to sense environmental conditions for the biofilm lifestyle of *C. testosteroni* (Ma, Zhang et al. 2009, Wu, Arumugam et al. 2015). Although bacteria in the *Comamonas* genus were usually considered strictly aerobic organisms, the genomes of certain strains of *C. testosteroni* have been shown to contain a gene cluster encoding the membrane-bound NAR-type nitrate reductase system (Wu, Arumugam et al. 2015), suggesting that they may be capable of catalyzing nitrate reduction to nitrite under oxygen-limiting conditions. Reduction of nitrate to nitrite is a critical step in microbial denitrification, which plays an important role in wastewater treatment and biogeochemical cycles (Jetten 2008). Highly dynamic and heterogeneous physicochemical microenvironments are often featured in mature biofilms (Stewart and Franklin 2008, Cao, Majors et al. 2012, Nguyen, Cao et al. 2012). Hence, even in the presence of oxygen in the bulk liquid phase, nitrate reduction may occur in *C. testosteroni* biofilms. However, previous studies about the nitrate reduction by *C. testosteroni* are focused on phenotypic characteristics based on physiological experiment (Willems and Vos 2006, Ma, Zhang et al. 2009). There has been no experimental evidence to support nitrate reduction by *C. testosteroni* biofilms under bulk aerobic conditions and, further, how does nitrate reduction influence the biofilm lifestyle has never been explored. Elucidating whether *C. testosteroni* biofilms are involved in denitrification under bulk aerobic condition and how does nitrate reduction influence the biofilm lifestyle of *C. testosteroni* is an important step toward a comprehensive understanding of the role of *Comamonas* in natural and engineered ecosystems.

The objectives of this study were to demonstrate the involvement of *C. testosteroni* biofilms in denitrification under bulk aerobic condition and to mechanistically investigate the influence of nitrate reduction by *C. testosteroni* on its biofilm lifestyle. Specifically, we grew biofilms of *C. testosteroni* strain I2 under bulk aerobic condition in the presence and absence of nitrate and quantitatively compared the biofilm structure, viability of the biofilm-cells, and cell detachment from the biofilms. We further conducted RNA-seq analysis to examine the expression profiles of genes that are critical to nitrate metabolism and the biofilm

mode of life. This study provides mechanistic insights into the links between nitrate respiration and the biofilm lifestyle of *C. testosteroni*, which is critical toward a comprehensive understanding of the role of *Comamonas* in natural and engineered ecosystems.

5.2 Materials and methods

5.2.1 Bacterial Strains and Growth Conditions.

The preculture of *C. testosteroni* strain I2 (LMG19554 from BCCM/LMG) was prepared by inoculating a single colony in 10 mL sterile lysogeny broth (LB) medium and incubated on a rotary shaker at 30°C. After overnight growth, cells were harvested through a 10-min centrifugation at 7,000 g and then resuspended in PBS buffer (pH 7.4). The cell suspension was used to inoculate M9 minimal medium supplemented with 10 mM sodium acetate. For growth under anoxic conditions, 4 mM potassium nitrate was supplemented as the sole electron acceptor and the culture was flushed with N₂ gas for 10 min to remove oxygen and then sealed with butyl rubber stoppers (Ng, Sivakumar et al. 2013). All the cultures were incubated at 30°C on a rotary shaker (200 rpm). The experiments were conducted in triplicates.

5.2.2 Flow Cell Biofilms.

Biofilms of *C. testosteroni* I2 were grown using multi-channel flow cells (BioCentrum-DTU, Denmark) at room temperature (23°C). Each channel of the flow cell was inoculated using 1.5 mL diluted overnight preculture of *C. testosteroni* I2 in M9 medium (OD₆₀₀ ~0.1) under sterile condition. Air-saturated M9 medium containing 10 mM acetate and 4 mM nitrate was supplied to the flow cells at a flow rate of 3.5 mL/h for biofilm growth in each channel. When needed, 0.1 mM sodium azide was added to the medium to inhibit nitrate reduction. To examine the resilience of the biofilms, a toxic compound 3-chloroaniline (3-CA) was introduced into the medium after the biofilms have been grown for 5 days. Biofilm experiments were conducted in triplicates. Cell detachment from the

biofilms was quantified using a drop-plate method as previously described (Chen, Nace et al. 2003, Wu, Shukal et al. 2015).

5.2.3 Confocal Laser Scanning Microscopy (CLSM) and Image Analysis.

To examine biofilm structure and cell viability, the flow cell biofilms were stained with the LIVE/DEAD BacLight Bacterial Viability Kit L7012 (Molecular Probes, Inc.) (Teal, Lies et al. 2006). Diluted SYTO 9 (11.1 μ M) and propidium iodide (66.7 μ M) solution (~0.7 mL) was injected into each flow channel (Ding, Peng et al. 2014). After staining for 15 min in dark, the flow cell biofilms were imaged using a confocal laser scanning microscopy (CLSM) (Carl Zeiss Microscopy LSM 780). The CLSM images were then analyzed with the IMARIS software (version: 8.0.0, Bitplane, Zurich, Switzerland).

5.2.4 Quantification of Acetate and Nitrate.

Samples were filtered through 0.20 μ m Acrodisc polyethersulfone membrane syringe filters (Pall Corporation, Singapore). Acetate in the filtrate was quantified using a HPLC (Shimadzu, Singapore) equipped with an Aminex HPX-87H column (300 mm $\times$ 7.8 mm; Bio-Rad, USA) using 5 mM sulfuric acid as the mobile phase at a flow rate of 0.6 mL/min at 50°C (Klinke, Thomsen et al. 2001, Sivakumar, Wang et al. 2014). Concentrations of nitrate and nitrite were determined using HACH kits with a HACH DR 3900 photometer according to manufacturer's instructions (HACH, USA).

5.2.5 RNA-seq Analysis.

Cells were harvested at the late exponential phase from planktonic cultures with oxygen or nitrate as the sole electron acceptor. The same cultivation method mentioned above was used. Total RNA was extracted using Qiagen mini RNA prep kits (Qiagen, Germany) following the manufacturer's instruction. All experiments were conducted in triplicates. Paired-end sequencing was performed on an Illumina Hi-Seq 2500 platform with a read length of 100 nucleotides (San Diego, CA, USA). The RNA-seq raw data was deposited in the NCBI GEO Short Read Archives with accession numbers of SRX951273, SRX951880, SRX956913, SRX956914, SRX956916 and SRX956918. The sequence reads were assembled and analyzed on

a CLC genomic Workbench 6.0 (CLC Bio, Aarhus, Denmark) with the genome of strain I2 (IMG database analysis project ID: Ga0022151) as the reference genome. Data were normalized by calculating the reads per kilobases per million mapped reads (RPKM). Significant changes in gene expression were defined using the criterion of at least a two-fold change with P-value < 0.05.

5.2.6 Quantitative Polymerase Chain Reaction (qPCR).

The differential expression of 3 diguanylate cyclase (DGC) genes obtained in RNA-seq analysis was validated using qPCR. The genes and the primers used in this study are listed in Table 5.1. The RNA samples for RNA-seq were used to synthesize cDNA by using a cDNA synthesis kit (Fermentas Life Sciences, Glen Burnie, MD). The resultant cDNA was used in qPCR reactions on an ABI StepOnePlus system (Life Technologies, Foster City, CA). PCR reactions and analyses were performed as previously described (Mohanty, Kathawala et al. 2014). The relative expression data of each target gene was normalized to a reference gene - transcriptional regulator *lysR* and was compared with control samples.

Table 5.1 List of primers used in qPCR analysis.

Feature ID	Gene	Forward primer	Reverse primer
I2contig_00961	<i>dgc-2</i>	CTGGCCGTGCGTGACTTGTG	GCGCCTTTCCCGACTGCTC
I2contig_04562	<i>dgc-1</i>	GTGAGTATCGGCGGGGTCTATG	CTTGCGGCGTCGGTGTTTAC
I2contig_05141	<i>dgc-3</i>	TCTGCGTGCACTCTGCTGTGTTTT	ACCGGCATAGGACGACTGAAGAAG

5.2.7 Extraction and Quantification of bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP).

The methods for extraction and quantification of c-di-GMP have been previously described (Wu, Ding et al. 2015). Briefly, cells were harvested at the late exponential growth phase in planktonic cultures and the c-di-GMP was extracted using an organic solvent mixture (Kuchma, Ballok et al. 2010). C-di-GMP concentration was quantified using a Thermo Accela 1250 series LC system fitted with a Thermo Velos Pro Orbitrap mass spectrometer (Thermo Scientific, USA).

5.3 Results and discussions

5.3.1 Nitrate Reduction by *C. testosteroni*.

Figure 5.1 Anaerobic growth of *C. testosteroni* I2 in M9 medium with acetate and nitrate as the electron donor and acceptor, respectively. (A) Profiles of cell growth (CFU) and acetate consumption. (B) Profiles of nitrate reduction and nitrite accumulation.

Although a gene cluster encoding the membrane-bound nitrate reductase (NAR) system has been identified in the genome of *C. testosteroni* I2 (Wu, Arumugam et al. 2015), there has been no experimental evidence on anaerobic nitrate reduction in this organism. Under anoxic condition with acetate and nitrate as the electron donor and electron acceptor, respectively, an apparent growth of *C.*

testosteroni I2 was observed (Figure 5.1A). The cell density increased from 2.5×10^6 cells/mL to 6.0×10^6 cells/mL within 27 h and about 1.5 mM acetate was consumed, while no acetate consumption or cell growth occurred in media without nitrate. Nearly all nitrate in the medium (~ 54.6 mg/L NO_3^- -N) was reduced to nitrite and a stoichiometric amount of nitrite (~ 54.8 mg/L NO_2^- -N) was accumulated in the culture (Figure 5.1B). Our results demonstrate that *C. testosteroni* I2 is capable of utilizing nitrate as an electron acceptor and coupling growth to the reduction of nitrate to nitrite. To the best of our knowledge, this is the first experimental evidence in the literature on anaerobic nitrate respiration in *C. testosteroni*. Nitrate reduction is an important step in microbial denitrification; hence, the capability of anaerobic nitrate respiration by *Comamonas* suggests a possible involvement of this organism in denitrification. However, *Comamonas* has been traditionally considered to be strictly aerobic and is mostly found in aerobic bioprocesses (Willems and Vos 2006). So, one would question whether the anaerobic nitrate reduction in *Comamonas* is relevant or not. Previous studies have suggested that *Comamonas* in wastewater treatment is often present in the form of biofilms or aggregates (Boon, Goris et al. 2000, Andersson, Kuttuva Rajarao et al. 2008, Kim, Pagaling et al. 2014). One typical feature of a biofilm or cell aggregate is the presence of a sticky matrix formed by self-produced extracellular polymeric substances (EPS) (Flemming and Wingender 2010), which may create physicochemical gradients and heterogeneity at the micrometre scale (Stewart and Franklin 2008). For example, even under bulk aerobic conditions, microenvironments with low or no oxygen have been reported in biofilms or cell aggregates (Xu, Stewart et al. 1998, Wessel, Arshad et al. 2014). Hence, it is plausible that anaerobic nitrate reduction occurs in biofilms of *C. testostearni* I2 under bulk aerobic conditions. To confirm it, we grew I2 biofilms using air-saturated medium in the presence of nitrate. Figure 5.2A shows the concentration profiles of nitrate and nitrite in the effluent from the flow cell biofilms. Intriguingly, the reduction of nitrate to nitrite occurred only after 55 h of biofilm growth, which correlated well with the development of 3-dimensional structures in the biofilms (Figure 5.2B). The results suggest that a mature biofilm with a certain extent of structural heterogeneity is required for nitrate reduction under bulk aerobic conditions.

Figure 5.2 Nitrate reduction in *C. testosteroni* I2 biofilms growing in flow chambers with air-saturated medium containing nitrate as an alternative electron acceptor. (A) Nitrate, nitrite and acetate concentration in the effluent from the flow chambers (n=3). (B) CLSM images of representative biofilms at 24 h and 60 h during biofilm growth in flow chambers. Scale bar 20 μ m.

5.3.2 Nitrate Reduction in *C. testosteroni* Biofilms under Bulk Aerobic Condition Enhanced Cell Viability and Biofilm Stability.

Figure 5.3 (A-C) Biofilm architectures and cell viability of mature biofilms developed in flow chambers with air-saturated medium in the presence or absence of nitrate as an alternative electron acceptors. (D-F) Biofilm architectures and cell viability of the mature biofilms after exposure to 3-chloroaniline (3-CA). Scale bar 20 μ m. (G) Cell detachment from the mature biofilms before and after 3-CA exposure. Asterisks represent significant different ($P < 0.05$).

We have shown that nitrate reduction could occur in mature biofilms of *C. testosteroni* I2 under bulk aerobic conditions. To further understand how nitrate reduction influences the biofilm lifestyle of *C. testosteroni*, we grew I2 biofilms with air-saturated medium in the absence and presence of nitrate. Biofilm architecture and cell viability of the biofilms grown under both conditions were compared using CLSM imaging analysis. After biofilm growth under both conditions for 120 h, *C. testosteroni* I2 developed into similar towering biofilm architecture (Figure 5.3A and B), a typical feature of mature biofilms in flow cells (Webb, Givskov et al. 2003). In the biofilms, cells in green and red fluorescence represent live and dead cells, respectively. Quantitative analysis showed a cell viability of $92\pm 2\%$ for the biofilms grown with nitrate, while only $75\pm 6\%$ for the biofilms grown without nitrate (Figure 5.3C). The presence of nitrate in the biofilm growth media significantly enhanced cell viability in the mature biofilms ($P < 0.05$), which could be because cells residing in oxygen-limiting microenvironments in the mature biofilms obtain energy from nitrate respiration for survival which would otherwise die.

To examine whether nitrate respiration is key to maintaining high cell viability in mature biofilms, we grew *C. testosteroni* biofilms with the nitrate-containing growth media supplemented with azide at a concentration of 0.1 mM, which inhibited nitrate respiration by up to 85% but did not exhibit an apparent inhibition on aerobic respiration in *C. testosteroni* (Figure 5.4). With the presence of azide in growth medium, reduction of nitrate to nitrite by *C. testosteroni* biofilms was negligible during 5-day biofilm growth as evidenced by a constant concentration of nitrate and nearly no nitrite was detected in the effluent (Figure 5.4). Cell viability in the biofilms grown in the presence of nitrate and azide was significantly lower than that without azide ($65\pm 9\%$ vs. $92\pm 2\%$; $P = 0.03$) (Figure 5.3C and 5.5C) and was at a level comparable with those without nitrate ($65\pm 9\%$ vs. $61\pm 7\%$; $P = 0.71$) (Figure 5.5A-C). These results confirmed that nitrate respiration is key to maintaining high cell viability in mature biofilms of *C. testosteroni* under bulk aerobic conditions. Similar observations have been reported in *Pseudomonas aeruginosa* where this organism formed biofilms with a higher viability when respiring on nitrate instead of oxygen (Yoon, Hennigan et al. 2002).

Figure 5.4 Inhibitory effect of azide on aerobic growth and nitrate reduction in planktonic cultures and biofilms of *C. testosteroni* I2. (A) Aerobic growth in M9 medium containing 4 mM nitrate in the presence of azide at different concentrations. (B) Aerobic growth in M9 medium without nitrate in the presence of azide at different concentrations. (C) Inhibitory effect of 0.1 mM azide on nitrate reduction by planktonic culture grown in M9 medium with nitrate as sole electron acceptor. (D) Inhibitory effect of 0.1 mM azide on nitrate reduction by biofilms grown in M9 medium with nitrate as sole electron acceptor. All experiments were conducted in triplicates.

Figure 5.5 The influence of azide-inhibited nitrate reduction on biofilm cell viability and stability. (A-C) Biofilm architecture and cell viability of mature biofilms developed in flow chambers with 0.1 mM azide-containing air-saturated medium in the presence or absence of nitrate as an alternative electron acceptor. (D-F) Biofilm architectures and cell viability of the mature biofilms after exposure to 3-chloroaniline (3-CA). Scale bar 20 μm . (G) Cell detachment from the mature biofilms before and after 3-CA exposure.

Previous studies have shown that metabolic energy is critical for bacterial cell to maintain its stability against environmental stress (Tan, Sequeira et al. 2010, Mak, Rao et al. 2012). Hence, in the presence of nitrate as an alternative electron acceptor, the *C. testosteroni* biofilms would have a higher stability. To test this hypothesis, we exposed mature biofilms grown in the presence and absence of nitrate to a model toxic compound 3-chloroaniline (3-CA) which has been reported to be able to induce cell detachment from *C. testosteroni* biofilms (Wu, Ding et al. 2015). Upon exposure to 3-CA, the biofilms grown in the absence of nitrate lose the typical towering structures because of massive cell detachment (detachment rate $\sim 1.8 \times 10^9$ cells/h) and the remaining cells were present in the form of cell clusters with a low cell viability ($\sim 27\%$) (Figure 5.3D-G). In contrast, after 3-CA exposure, the biofilms grown in the presence of nitrate exhibited a slower cell detachment ($\sim 5.5 \times 10^8$ cells/h) and maintained the towering structures with a high cell viability ($\sim 72\%$) (Figure 5.3D-G). When nitrate respiration was inhibited, upon exposure to 3-CA, mature biofilms grown in the presence and absence of nitrate did not exhibit any significant difference in cell viability and biofilm stability (Figures 5.3D-G and 5.5D-G). Taken together, our results demonstrate that, under bulk aerobic conditions, nitrate respiration in the mature biofilms of *C. testosteroni* I2 enhanced viability of cells in the biofilms and biofilm stability.

5.3.3 Differential Gene Expression in *C. testosteroni* with Oxygen and Nitrate as the Electron Acceptor.

To further explore the mechanism underlying the enhancement of cell viability and biofilm stability, we conducted RNA-seq-based transcriptomics analysis to elucidate the influences of nitrate respiration on the physiology of *C. testosteroni*. Comparison between the cells grown with nitrate or oxygen as sole electron acceptor revealed that a total of 392 genes ($\sim 7.4\%$ of all genes in I2 genome) exhibited a differential expression and among them 372 showed an increased expression in cells grown with nitrate. In particular, the genes involved in nitrogen and energy metabolism as well as those responsible for the synthesis of c-di-GMP were highly expressed in cells respiring on nitrate than those respiring on oxygen.

Nitrogen and Energy Metabolism. The nitrogen and energy metabolism were found significantly increased when nitrate was used as the electron acceptor (Figure 5.6). In cells respiring on nitrate, the genes encoding nitrate reductase NarGHI, nitrate/nitrite sensor protein NarX, nitrate/nitrite transporter NarK, and 3 ABC-type nitrate transporter proteins were highly expressed. The high expression of the energy-coupled NAR-type nitrate reductase system in I2 cells grown with nitrate confirmed the presence of a similar nitrate reduction system in *C. testosteroni* I2 with those in facultative anaerobes such as *Escherichia coli* and *Bacillus subtilis* (Ramos, Hoffmann et al. 2000, Bertero, Rothery et al. 2003) and the capability in coupling growth to nitrate reduction. Intriguingly, the expression level of nitrite reductase genes *nirBD* remained the same for both oxygen and nitrate respiration conditions, which is consistent with the observed accumulation of nitrite in culture (Figure 5.1B). In cells respiring on nitrate, the genes responsible for acetate oxidation were also highly expressed (Figure 5.6). The electron generated in acetate metabolism was transferred to electron carriers NADH and FADH₂. The reductases for other electron mediators including ferredoxin, quinone and flavin also induced to facilitate electron transfer. Finally, nitrate was reduced by NarGHI with electron from quinol pool coupled to the generation of proton motive force (Bertero, Rothery et al. 2003). In particular, genes encoding formate dehydrogenase FdnGHI were highly expressed. A respiratory chain in *E. coli* has been identified to consist of formate dehydrogenase N (FdnGHI) and nitrate reductase (NarGHI) where the electrons from formate oxidation was transferred to nitrate reduction through the quinol pool (Bertero, Rothery et al. 2003). The *fdnGHI* operon in *E. coli* was reported to be induced by nitrate, which is consistent with the findings of this study (Berg and Stewart 1990, Li and Stewart 1992).

Figure 5.6 A summary of key highly expressed genes in the metabolic pathways in *C. testosteroni* I2 cells respiring on nitrate vs. oxygen (reconstructed from KEGG pathway and annotation). The color gradient represents the fold-change in gene expression levels.

The c-di-GMP Signalling. Eight out of 30 predicted diguanylate cyclases (DGCs) in *C. testosteroni* I2 (Wu, Arumugam et al. 2015) were found highly expressed when nitrate was used as the electron acceptor (Table 5.2). DGCs are enzymes responsible for the synthesis of c-di-GMP, which is one of the most important molecular determinants for the biofilm mode of life (Singh, Paul et al. 2006, Hengge 2009, Wu, Ding et al. 2015). The higher expression level of genes encoding DGCs in *C. testosteroni* cells respiring on nitrate than those respiring on oxygen was further confirmed using qPCR (Figure 5.7A): 1.75-fold, 2.82-fold, and 2.42-fold in expression for genes *dgc-1*, *dgc-2*, and *dgc-3*, respectively. A higher expression level of DGCs suggested that nitrate respiration may increase the intracellular concentration of c-di-GMP.

Figure 5.7 (A) The qPCR analysis of the expression of 3 key genes involved in c-di-GMP synthesis. Values were normalized to that of the housekeeping gene *lysR*. (B) Intracellular c-di-GMP concentration of *C. testosteroni* I2 cells respiring on nitrate and oxygen determined using LC-MS/MS.

Table 5.2 Nitrate respiration-induced expression of genes responsible for the synthesis of c-di-GMP. Ratio indicates a relative gene expression level in cells respiring on nitrate to those respiring on oxygen.

Feature ID	Description	Log ₂ Ratio	P value (%)
I2contig_00961	PAS domain S-box/diguanylate cyclase domain (DGC-2)	2.46	0.97
I2contig_05141	Diguanylate cyclase domain (DGC-3)	2.41	3.38
I2contig_04562	Diguanylate cyclase domain (DGC-1)	2.01	2.82
I2contig_00763	Diguanylate cyclase domain	1.59	3.17
I2contig_02275	Diguanylate cyclase/phosphodiesterase	1.50	3.70
I2contig_04913	Diguanylate cyclase	1.25	4.46
I2contig_01952	Response regulator containing a CheY-like receiver domain and a GGDEF domain	1.04	4.08
I2contig_04723	Diguanylate cyclase domain	1.02	2.19
I2contig_01917	Diguanylate cyclase domain	1.00	4.87
I2contig_02501	Diguanylate cyclase domain	0.88	4.34
I2contig_03397	Diguanylate cyclase domain	0.60	2.44

5.3.4 Nitrate Respiration Increased Intracellular C-di-GMP Level.

The intracellular c-di-GMP was extracted from cells respiring on nitrate and oxygen, respectively, and quantified using LC-MS/MS. Figure 5.7B shows the intracellular c-di-GMP level of the cells grown under both conditions. Compared with the oxygen-respiring cells, the nitrate-respiring cells exhibited a significantly higher concentration of intracellular c-di-GMP (1.3×10^{-4} fmol/cell vs. 7.9×10^{-6} fmol/cell; $P < 0.01$), which is consistent with the higher expression level of DGC genes in nitrate-respiring cells observed in the RNA-seq analysis. In bacteria, c-di-GMP is one universal signaling molecule that regulates the formation and detachment of biofilms (Gjermansen, Ragas et al. 2006, Jenal and Malone 2006, Wolfe and Visick 2008, Spurbeck, Tarrien et al. 2012). Generally, a high concentration of c-di-GMP inhibits various forms of motility, stimulates EPS production, and favors the biofilm mode of life (Hengge 2009). For example, a high c-di-GMP concentration in *C. testosteroni* decreased toxicity-induced cell detachment from its biofilms (Wu, Ding et al. 2015). An elevated c-di-GMP level in *P. aeruginosa* was found to improve bacterial fitness and enhance antimicrobial resistance (Gupta, Liao et al. 2014, Nicastro, Kaihami et al. 2014). In this study, we found that nitrate respiration increased c-di-GMP level in *C. testosteroni* I2. Hence, cells respiring on nitrate in the internal region of biofilms would have a higher c-di-GMP level than those respiring on oxygen. This would lead to a higher c-di-GMP level for biofilm grown with air-saturated medium in the presence of nitrate compared to those grown in the absence of nitrate. Consistent with the effect of elevated c-di-GMP level on biofilm sustainability, the higher concentration of this biofilm determinant could be the major reason for enhanced biofilm stability by internal nitrate respiration, in particular, when the mature biofilms were exposed to 3-CA.

5.4 Conclusions

Denitrification plays a critical role in the nitrogen biogeochemical cycle and in engineering bioprocesses for nitrogen removal from wastewater. Microbial reduction of nitrate to nitrite is an important step in denitrification process that often

occurs under oxygen-limiting conditions. *Comamonas* is a group organism that is often found in various natural and engineered ecosystems including nitrate removal processes. We know little about their physiology and their possible roles except that this group of organisms are well known to have versatile catabolic activities for efficient degradation of organic compounds. Here, we show that *C. testosteroni*, an organism that is traditionally considered strictly aerobic, can reduce nitrate to nitrite and derive metabolic energy from nitrate reduction anaerobically. In particular, biofilms of *C. testosteroni* are capable of reducing nitrate under bulk aerobic conditions and nitrate reduction in the biofilms is beneficial to the biofilm mode of life by providing more metabolic energy to maintain high cell viability and a higher level of c-di-GMP to reduce cell detachment from the biofilms. It suggested a potential role of *Comamonas* in biofilm-mediated denitrification in aerobic bioprocesses and oxic natural environments and the availability of nitrate on its biofilm lifestyle.

Chapter 6. Soluble, high-molecular-weight extracellular polymeric substances in wastewater enhance floc formation of *Comamonas*

6.1 Introduction

Activated sludge (AS) process is the most widely applied bioprocess for wastewater treatment. In AS process, bacterial flocs and granules, *i.e.*, aggregated bacterial cells and extracellular polymeric substances (EPS), are crucial for the overall performance of wastewater treatment. And the development of this multicellular structure is initiated from bacterium-to-bacterium contact and then stabilized by different attractive forces (Liu and Tay 2004). Therefore, floc-forming capability is the key feature in the flocculation process.

In microbial communities driving AS process-based wastewater treatment, *Comamonas* has been often reported to be the one of the primary colonizers that readily form cell aggregates and colonizes on biotic and abiotic substrata in wastewater (Andersson, Kuttuva Rajarao et al. 2008, Luo, Xu et al. 2012, Kim, Pagaling et al. 2014, Hao, Luo et al. 2016). Interestingly, *Comamonas* isolates from wastewater treatment plants and bioreactors rarely form flocs in synthetic wastewater media. For example, *C. testosteroni* I2 formed flocs in activated sludge reactors (Boon, Goris et al. 2000), but no floc formation was observed when growing in synthetic wastewater (Wu, Shukal et al. 2015). Synthetic wastewater is a chemically defined medium that has been widely used in environmental engineering. It is formulated based on key physicochemical characteristics of real wastewater, *e.g.*, pH, ionic strength, chemical oxygen demand (COD), and total nitrogen/phosphorus (NH₄-N and PO₄-P). The discrepancy observed for bacterial floc formation in real wastewater and synthetic wastewater suggests an important role of neglected, unknown components, *i.e.*, “dark matter”, in real wastewater.

The objective of this study was to reveal the “dark matter” components that facilitate bacterial floc formation in domestic wastewater and to elucidate the

underlying mechanism using the DLVO theory. Specifically, using *C. testosteroni* as a model organism, we examined the effect of microfiltered (pore size of 0.22 µm) and synthetic wastewater on bacterial floc formation and development. Fourier Transform Infrared Spectroscopy (FTIR) analyses and enzymatic treatment suggested soluble EPS, in particular, proteins and extracellular DNA, as the major floc-forming inducers in wastewater. The underlying mechanism was further characterized by using the DLVO theory. This study reports, for the first time, an important role of soluble EPS in floc formation and development in AS-based wastewater treatment processes.

6.2 Materials and methods

6.2.1 Wastewater Sampling, Sterilization, and Characterization.

Wastewater effluent from the primary settling tank was collected at Ulu Pandan Water Reclamation Plant, Singapore. After centrifugation at 4000 g for 10 min, the supernatant was filtered through a microfiltration polyethersulfone (PES) membrane filter (pore size: 0.22 µm) (Corning, USA). The filter-sterilized wastewater (designated as MF-WW) was further filtered through an ultrafiltration polyacrylonitrile (PAN) membrane filter synthesized following previous approaches (Liu, Qi et al. 2013, Liu, Foo et al. 2016). The molecular weight cut-off (MWCO) of the ultrafiltration membrane is 225±5 kDa and the filtrate was designated as UF-WW. The concentrations of ammonium, phosphate and COD were determined using HACH assay kits as per manufacturer's instructions (Wu, Shukal et al. 2015).

6.2.2 Bacterial Strain and Growth Conditions.

C. testosteroni I2 (LMG19554 from BCCM/LMG) (Boon, Goris et al. 2000, Wu, Mohanty et al. 2016) was pre-cultivated in lysogeny broth (LB) medium at 30 °C before being used in subsequent experiments using MF-WW, UF-WW and synthetic wastewater as growth media. Synthetic wastewater (designated as SynWW) was prepared based on the chemical characteristics of filter-sterilized wastewater, *i.e.*, MF-WW (Table 6.1). Each liter of the SynWW contained 0.195 g

CH₃COONa, 0.156 g NH₄Cl, 0.031 g K₂HPO₄, 0.03 g MgSO₄, 0.40 g NaHCO₃ and 0.02 g CaCl₂. The pH of the SynWW was adjusted to 7.0.

6.2.3 Floc Formation of *C. testosteroni* in MF-WW.

Overnight LB culture of *C. testosteroni* I2 was centrifuged and washed twice with 0.9 % NaCl solution and then resuspended in the same solution. About 0.5 mL of the cell suspension was inoculated in 50 mL MF-WW or SynWW to a final optical density (OD₆₀₀) of ~0.20 and incubated with shaking (200 rpm) under room temperature (25±2 °C). To quantify bacterial growth, the culture was centrifuged at 12000 g for 5 min. The resulting pellets were incubated in 0.1 M NaOH at 70 °C for 1 h and the concentration of total protein was quantified by using the BCA method (Wu, Ding et al. 2015). The particle size distribution of the bacterial flocs was determined using a SALD-3101 particle analyzer (Shimadzu). To examine the role of extracellular DNA and proteins in the floc formation, DNase (RQ1 RNase free DNase; Promega) and proteinase (Proteinase K; Qiagen) were used to hydrolyze the extracellular DNA and proteins in the bacterial flocs. After an incubation with DNase (50 U/mL) or proteinase K (12 mAU/mL) at 37 °C for 30 min, the flocs was observed under an optical microscope (Zeiss Primo Star) for morphological changes.

6.2.4 Flow Cell Biofilm Formation of *C. testosteroni* in MF-WW.

A red fluorescence-tagged *C. strain* I2::*rfp* was used to characterize the biofilm formation of *C. testosteroni* in MF-WW (Boon, De Gelder et al. 2002). Biofilms of strain I2::*rfp* were grown in multichannel flow cells at 25 °C (Wu, Shukal et al. 2015). MF-WW was supplied at a flow rate of 3 mL/h and the control group was supplied with SynWW at the same flow rate. The biofilms were imaged using Confocal Laser Scanning Microscopy (CLSM) (Carl Zeiss Microscopy LSM 780) and the CLSM images were analyzed using the IMARIS software (version 8.2.0; Bitplane) (Mohanty, Wei et al. 2015, Wu, Shukal et al. 2015, Mukherjee, Menon et al. 2016).

6.2.5 Flocculating of *C. testosteroni* with Floccs in MF-WW.

Flocs were collected from one lab-scale wastewater reactor. After centrifuged and washed with 0.9 % NaCl solution twice, flocs was resuspended into MF-WW and SynWW to a final concentration around 225 mg/L. And strain I2::*rfp* was subsequently inoculated at the final concentration about 200 mg/L. The resulting liquor was shaking (200 rpm) under room temperature. To differentiate the non-flocculated and flocculated biomass, the mixed liquor was settling for 40 min and the supernatant was considered as non-flocculated fraction. The biomass concentration for non-flocculated and total liquor were quantified by using the total protein approach as mentioned above. The biomass of flocculated fraction was determined by subtracting the non-flocculated fraction from total biomass. The abundance of strain I2::*rfp* in supernatant was estimated based on the red fluorescent intensity and corresponding protein concentration in pure culture. To estimate the relative abundance of strain I2::*rfp* in flocs, all the cells were stained with SYTO 9 (20 μ m) and biovolume ratio of red cells was determined by IMARIS software (version 8.2.0; Bitplane) (Mohanty, Wei et al. 2015, Wu, Shukal et al. 2015, Mukherjee, Menon et al. 2016). Three independent biological replicates were conducted and 10 flocs were examined for each replicate at one time point.

6.2.6 Soluble EPS Characterization.

To quantify soluble EPS components, MF-WW and UF-WW were concentrated 10 times using a CentriVap vacuum concentrator (Labconco) at 4 °C. Proteins and polysaccharides were determined using the BCA and the phenol-sulfuric acid methods (Masuko, Minami et al. 2005, Ding, Peng et al. 2014). DNA concentration was measured using the Qubit dsDNA HS Assay Kit (Invitrogen) (Zhang, Ng et al. 2014). The biomolecules retained on the ultrafiltration PAN membrane filter surface were characterized by Fourier Transform Infrared Spectroscopy (FTIR) spectroscopy (Cao, Shi et al. 2011) on the FTIR Spectrophotometer IRPrestoge-21 (Shimadzu) in attenuated total reflection mode with 4 cm^{-1} resolution. In total, 45 scans were collected between 4000 cm^{-1} and 400 cm^{-1} . The assignment of the absorption peaks was based on specific vibrations

reported previous studies (Schmitt and Flemming 1998, Eboigbodin and Biggs 2008, Cao, Shi et al. 2011).

6.2.7 Scanning Electron Microscopy (SEM) Analysis.

The ultrafiltration PAN membrane filter after the filtration of MF-WW was dried and coated with platinum using a JFC-1300 vacuum electric sputter coater (JEOL Asia Pte Ltd) (Ng, Sivakumar et al. 2013). The foulant on the membrane was visualized by using a JSM-7600 field emission SEM (JEOL Asia Pte Ltd) at a voltage of 2.0 kV.

6.2.8 Derjaguin-Landau-Verwey-Overbeek (DLVO) Analysis.

The total interaction energy (W^{TOT}) was composed of van der Waals energy (W^{LW}) and electric double layer energy (W^{EL}) (Liu, Sheng et al. 2007).

These two interactions were expressed as following equations:

$$W^{LW} = -\frac{A_{BLB}R}{12H} \quad (1)$$

$$W^{EL} = 2\pi\epsilon R\psi^2 \ln(1 + e^{-\kappa H}) \quad (2)$$

Where A_{BLB} is the effective Hamaker constant; R is the radius of a bacterial cell; H is the distance between two cells; ϵ is dielectric constant; ψ is surface potential; κ^{-1} is the thickness of double layer. Surface potential was approximated by zeta-potential, which measured by Zetasizer Nano ZS (Malvern Instruments Ltd).

κ and A_{BLB} were given as:

$$A_{BLB} = -12\pi l_0^2 \Delta G_{adh}^{LW} \quad (3)$$

$$\kappa = \sqrt{\frac{4N_A e^2 I}{\epsilon k_B T}} \quad (4)$$

Where ΔG_{adh}^{LW} is free energy generated by van der Waals interaction; N_A is the Avogadro constant; e is the electron charge; I is the ionic strength; k_B is the Boltzmann constant; T is the temperature.

The free energy generated by van der Waals interaction were calculated as:

$$\Delta G_{adh}^{LW} = -2(\sqrt{\gamma_B^{LW}} - \sqrt{\gamma_L^{LW}})^2 \quad (5)$$

Where γ_i^{LW} is Lifshitz-van der Waals component; B and L refer to the bacterial cells and wastewater.

All the surface tension component of bacterial cells were calculated from the contact angles of three liquid (water, glycerol and dimethyl sulfoxide) using equation 6. After culturing in wastewater for 30 min, cells were filtered on a 0.45 μm cellulose nitrate membrane filter (Sartorius). Filter was dried in the air for 30 min and contact angle was measured by using VCA optima (AST Products).

$$(1 + \cos \theta)\gamma_l = 2(\sqrt{\gamma_B^{LW}\gamma_l^{LW}}) + \sqrt{(\gamma_B^+\gamma_l^-)} + \sqrt{(\gamma_B^-\gamma_l^+)} \quad (6)$$

Where θ is the contact angle; γ_i refers to the total surface tension; γ_i^+ and γ_i^- are the electron-donor parameter and electron-acceptor; l refers to the liquid used in this study.

6.3 Results and discussion

6.3.1 Floc Formation in MF-WW

Figure 6.1 (A) The floc formation of *C. testosteroni* in filter-sterilized wastewater (MF-WW) and synthetic wastewater (SynWW). Numbers in brackets, *i.e.*, 0.25, 0.50 and 0.75, represent the size of the first quartile, the median, and the third quartile of the flocs. (B) The batch biomass growth and COD consumption in MF-WW and SynWW.

When *C. testosteroni* was inoculated into MF-WW, visible flocs formed within 2 h. Particle size analysis showed a rapid increase of the mean diameter of the flocs formed in the MF-WW from $0.74 \pm 0.07 \mu\text{m}$ to $23.57 \pm 0.56 \mu\text{m}$ in the first 2 h, which further increased to an average size of $73.77 \pm 7.02 \mu\text{m}$ after 10 h (Figure 6.1A). In contrast, no flocs could be observed when *C. testosteroni* was growing in the SynWW with similar chemical characteristics (Table 6.1). Based on total protein quantification, after 10 h growth, the biomass in MF-WW increased by about 67% (from 190 to 317 mg/L) while the biomass in SynWW more than doubled (from 190 to 478 mg/L) (Figure 6.1B). The difference in bacterial growth in MF-WW and SynWW could be attributed to different biodegradability of the available COD. Although the initial COD in MF-WW and SynWW was comparable (127.6 ± 0.4 vs 115.7 ± 0.7 mg/L), only ~40 mg/L of the COD in MF-WW was consumed, while 106 mg/L of the COD in SynWW (primarily acetate) was used (Figure 6.1B). Since SynWW supported more bacterial biomass growth than MF-WW, the floc formation observed in MF-WW but not in SynWW was not caused by high cell densities. Intriguingly, this phenomenon is not exclusive to the model organism; other bacterial isolates from wastewater, for example, *Aeromonas* and *Klebsiella* (Tan, Koh et al. 2015), were also found to form aggregates in MF-WW but not in SynWW (Figure 6.2).

Figure 6.2 Particle size distribution of *Aeromonas caviae* (A) and *Klebsiella sp.* (B) in filter-sterilized wastewater (MF-WW) and synthetic wastewater (SynWW) after 10-hour growth.

Table 6.1 Chemical composition of wastewater

	COD (mg/L)	NH₄-N (mg/L)	PO₄-P (mg/L)	Ionic strength (mM)	Protein (mg/L)	Polysaccharide (mg/L)	DNA (µg/L)
Sterilized wastewater (MF-WW) ^a	127.55±0.35	37.15±0.78	5.24±0.07	8.15	29.8±1.1	9.73±2.01	53.03±0.71
Synthetic wastewater (SynWW)	115.71±0.71	40.20±0.28	5.03±0.02	9.93	0	0	0
Ultrafiltered WW(UF- WW) ^b	113.10±0.14	36.82±0.30	4.91±0.03	7.85	25.1±0.8	7.57±0.54	2.43±0.02
SYN with retentate (SynWW+R) ^c	122.72±0.57	40.13±0.38	5.23±0.03	9.46	3.3±0.1	1.19±0.10	21.70±0.51

^a filtered through 0.22 µm PS filter; ^b filtered through ultrafiltration membrane with a molecular cutoff of 225 kDa; ^c retentate from ultrafiltration of WW

To test whether MF-WW also promoted biofilm growth under continuous flow conditions, we conducted biofilm development studies using flow cells as previously described (Wu, Shukal et al. 2015). After 24 h of biofilm development, only one thin layer of bacterial cells formed in the flow cells supplied with the SynWW, while 3-dimensional (3-D) heterogeneous biofilm structure was observed in the flow cells supplied with the MF-WW (Figure 6.3). After 48 h, the biovolume of the biofilms developed in MF-WW increased to $7.69 \pm 1.41 \mu\text{m}^3/\mu\text{m}^2$ with a roughness of 15.68 ± 3.13 . In contrast, the biofilms developed in SynWW only had a biovolume of $3.90 \pm 1.13 \mu\text{m}^3/\mu\text{m}^2$ with a roughness of 3.45 ± 0.37 . Hence, MF-WW also promoted biofilm development under continuous flow conditions. The results obtained in this study confirmed the floc-forming capability of *C. testosteroni* in wastewater.

Figure 6.3 Biofilm morphology of strain I2::rfp developed in flow chambers. Thin lay of biofilm formed in synthetic wastewater (SynWW) at 24 (A) and 48 h (C). Heterogeneous biofilm structure developed in sterilized wastewater (MF-WW) at 24 (B) and 48 h (D). Scale bar represents 20 μm .

6.3.2 Enhanced attachment of *Comamonas* on flocs in MF-WW

Figure 6.4 The relative abundance of *C. testosteroni* I2::rfp and total biomass concentration in supernatant (A) and settled flocs (B).

In a previous study, *C. testosteroni* was found to form aggregates when augmented in a activated sludge bioreactor (Boon, Goris et al. 2000). To further investigate how wastewater matrix affects this process, we added red fluorescent *Comamonas* (strain I2::rfp) into SynWW and MF-WW with the presence of flocs. At time 0, cells in supernatant were mainly consisted by *Comamonas* cells (Figure 6.4A). However, after 3 h, the total biomass in supernatant of MF-WW decreased by 71.1%, while that in SynWW increased by 35.5%. Compensated to the decrease in supernatant, biomass of settled flocs in MF-WW increased (Figure 6.4B), which indicated the decreased cell concentration in supernatant was caused by the attachment of planktonic cells on floc surface. To verify whether *Comamonas* cells shifted from supernatant to flocs surface, the flocs were visualized by CLSM. According to CLSM images (Figure 6.5F), most surface area of flocs were covered by *Comamonas* cells after 3-h shaking. Meanwhile, only small amount of *Comamonas* cells were colonized on floc surface in SynWW (Figure 6.5B). And based on imaging analyses, the proportion of *Comamonas* cells in flocs was increased from 0.8% to 69.1% in 3 h and this high ratio was maintained afterwards

which resulted in a low abundance of *Comamonas* cells remained in supernatant (Figure 6.4A). And in SynWW, the ratio of *Comamonas* only increased to 6.1% in 3 h (Figure 6.4B). And the portion didn't further increase even though more *Comamonas* cells were in supernatant.

Figure 6.5 Flocs attached by *C. testosteroni* I2::rfp in SynWW (A-D) and MF-WW (E-H). *Comamonas* cells appear as red and all the cells in flocs stained with Syto 9 show green fluorescent. Scale bars represent 20 μ m.

Therefore, *Comamonas* cells in real wastewater have an enhanced floc-forming capability which enables them to colonize floc surface. And this colonization process also affected floc development as *Comamonas* abundance increased significantly. In conventional activated sludge system, the hydraulic retention time is usually around 4-8 h (Grady Jr, Daigger et al. 2011). The rapid attachment to the existing flocs enables them to be retained in wastewater system and also explained the omnipresence of *Comamonas* biofilm in AS processes.

6.3.3 Physical Separation Soluble EPS by Ultrafiltration.

The different phenotype of *Comamonas* in SynWW and MF-WW indicated the existence of certain floc-forming inducers in MF-WW. Since one of the most significant differences between SynWW and MF-WW is the prior presence of activated sludge, the wastewater-facilitated floc formation may be attributed to the soluble EPS produced by the activated sludge microbial community. To test this hypothesis, ultrafiltration (MWCO of 225 ± 5 kDa) was applied to physically remove the high molecular weight fraction of the soluble EPS from MF-WW. To characterize the retentate on the membrane, SEM was used to observe the membrane before and after filtration of MF-WW (Figure 6.6). After filtration, fibrous long chain polymeric substances longer than $2 \mu\text{m}$ were found on the membrane. The retentate was further analyzed using FTIR and exhibited typical absorbance peaks for key components of EPS (Figure 6.6C). The major absorbance peaks were attributed to lipids ($820\text{-}920 \text{ cm}^{-1}$ and $2600\text{-}3100 \text{ cm}^{-1}$), nucleic acid ($970\text{-}1430 \text{ cm}^{-1}$) and the amide I and II groups ($1500\text{-}1700 \text{ cm}^{-1}$) of proteins.

Figure 6.6 The SEM images of ultrafiltration membrane (A) before and (B) after filtration of MF-WW. (C) FTIR spectra of the high molecular weight fraction (MWCO ~225 kDa) of soluble EPS retained on the ultrafiltration membrane. Peak assignments: 1 (water); 2, 3, 9 (lipid); 4, 5, 6 (protein); 7 (nucleic acid); 8 (nucleic acid and polysaccharides). The detailed peak assignments are listed in Table 6.2.

Table 6.2 Surface tension components and zeta potential for strain I2 cells in sterilized wastewater (MF-WW), synthetic wastewater (SynWW), ultrafiltered wastewater (UF-WW) and synthetic wastewater with soluble EPS (SynWW+R) (mJ/m²).

	MF-WW	SynWW	UF-WW	SynWW+R
γ^{LW}	30.78	25.94	27.17	28.26
γ^+	0.39	1.39	1.35	0.79
γ^-	56.19	60.10	55.27	52.81
ζ (mV)	-3.18±0.15	-3.37±27	-3.55±0.38	-3.45±0.52

Compared with MF-WW, the filtrate, *i.e.*, UF-WW, exhibited a much lower capability in causing floc formation (Figure 6.7). The mean diameter of the flocs formed in UF-WW and MF-WW increased to 23.58±2.37 μm and 73.77±7.02 μm , respectively, in 10 h. Intriguingly, supplementing the retentate from the UF filter to the SynWW (designated as SynWW+R) increased the soluble EPS content (Table 6.1) and restored the capability of facilitating floc formation (Figure 6.7).

Figure 6.7 The floc-formation of *C. testosteroni* in SynWW+R and UF-WW. Numbers in brackets, i.e., 0.25, 0.50 and 0.75, represents the size of the first quartile, the median, and the third quartile of the flocs.

Due to the high MWCO of the ultrafiltration membrane filter, the low molecular weight chemical components in the filtrate (UF-WW) were comparable with that of MF-WW (Table 6.1). After ultrafiltration of the MF-WW, the concentration of DNA and protein decreased drastically (Figure 6.8 and Table 6.1). About 95% of DNA and 16% of protein in MF-WW were removed by the ultrafiltration. Since DNA and proteins are the components that differ greatly before and after the ultrafiltration, enzymatic treatment was used to further confirm the role of DNA and proteins in the floc formation. After 30-min incubation with DNase or proteinase, most of the flocs were disassembled (Figure 6.9), suggesting an indispensable role of DNA and proteins in the floc formation process. Previous studies found that EPS of the suspended flocs in wastewater treatment processes mainly consisted of polysaccharide and proteins (McSwain, Irvine et al. 2005, Sheng, Yu et al. 2010). When comparing with that in attached biofilm, the EPS content in flocs were much higher. It was attributed to high amount of protein and

polysaccharide in flocs, while the fraction of eDNA was higher in attached biofilm (Mahendran, Lishman et al. 2012, Zhang, Fang et al. 2014). Therefore, although eDNA was not the main component in wastewater flocs, it can still trigger bacterial flocculation.

Figure 6.8 Relative abundance of different EPS components before (MF-WW) and after ultrafiltration (UF-WW). * Significant different ($P < 0.05$).

Figure 6.9 Disassociation of floc structure after enzymatic treatment.

6.3.4 Influence of Soluble EPS on Floc Formation by DLVO Analysis.

To further investigate how soluble EPS, in particular eDNA and proteins, facilitate bacterial floc formation, we conducted the DLVO analysis. The contact angle between bacterial cells and the media was measured to determine the surface tension components. Cells harvested from MF-WW were more hydrophobic than that in SynWW ($33.1 \pm 1.5^\circ$ vs $26.8 \pm 1.7^\circ$). After the portion of soluble EPS was removed from the MF-WW, the bacterial surface became more hydrophilic ($30.5 \pm 1.7^\circ$). When the retentate on the microfiltration membrane was resuspended into the SynWW, the cell surfaces became more hydrophobic ($35.6 \pm 0.8^\circ$). Therefore, the soluble EPS may increase bacterial floc-forming capability by increasing cell surface hydrophobicity. Surface hydrophobicity is also well

correlated with the changes of γ^- which decreased or increased in the absence or presence of soluble EPS, respectively (Table 6.2) (Grasso, Smets et al. 1996). Moreover, a higher γ^{LW} value was obtained for the culture with soluble EPS, which indicated a more apolar surface, thus contributing to a higher attractive W^{LW} force in bacterial adhesion (Figure 6.10A). For the major repulsive interaction, the electrical double layer energy W^{EL} did not differ much for all the cultures in different wastewater matrices (Figure 6.10B) because the ionic strength and the zeta potential of the wastewater matrices were comparable (Table 6.1).

Figure 6.10 DLVO analyses: (A) van der Waals energy, (B) electrical double layer energy and (C) total interaction energy as a function of separation distance between cells in MF-WW, SynWW, UF-WW and SynWW+R.

The total interaction between cells was expressed as the sum of W^{LW} and W^{EL} . With a strong van der Waals attraction, the primary minimum existed for all four cultures in wastewater (Figure 6.10C). However, an energy barrier existed for cultures grown in the SynWW (1.92 KT) and the UF-WW (-0.98 KT). Since the kinetic energy for most bacteria does not exceed 1.5 KT (van Loosdrecht, Lyklema et al. 1989), bacteria growing in the SynWW was not likely to overcome the energy barrier for floc formation. No energy barriers could be observed for the media containing soluble EPS, *i.e.*, MF-WW and SynWW+R, suggesting that bacterial cells in these media could approach each other easily and established irreversible attachment and formed flocs. These modeling results are consistent with our experimental observations.

The changes in bacterial surface thermodynamic parameters caused by soluble EPS are similar to the sludge cells in the presence or absence of bound EPS (Xu, Yu et al. 2016). Without bound EPS, the hydrophobicity of the sludge surface increased and the energy barrier of interaction decreased. And among bound EPS, the loosely-bound EPS (LB-EPS) was found to contribute more than the tightly-bound EPS in floc formation (Xu, Yu et al. 2016). Due to similar EPS components, the flocculation test based on kaolin suspension also showed similar flocculation ability between supernatant, slime and LB-EPS (Yu, He et al. 2009). The effect of soluble EPS on bacterial floc formation could share a similar mechanism with flocculation by the LB-EPS.

6.4 Conclusions

In this study, using *C. testosteroni* as a model organism, we examined its floc formation in real and synthetic wastewater. Combining the floc formation observations with the FTIR analyses and enzymatic treatment, we report for the first time that the high-molecular-weight fraction of the soluble EPS, in particular, proteins and extracellular DNA, are the major floc-forming inducers in wastewater. We show that soluble, high-molecular-weight EPS in wastewater enhances the attachment of planktonic cells onto bacterial flocs by increasing the hydrophobicity of bacterial surfaces and reducing the energy barrier for cell-cell interaction.

Chapter 7. Conclusions and Recommendations

7.1 Overall conclusions

Bacteria of the *Comamonas* genus are widely distributed environmental bacteria, which commonly found in a broad spectrum of bioprocesses. Although previous observations show the omnipresence of *Comamonas* biofilm in different environments, only their planktonic state of life rather than biofilm lifestyle was characterized. Therefore, to better understand their biofilm processes and the role in different environmental and engineered systems, it is crucial to study physiological response of *Comamonas* biofilm to toxic aromatics (Chapter 3), improve biofilm robustness for biodegradation (Chapter 4), evaluate the role of *Comamonas* biofilm in denitrification processes (Chapter 5) and identify the cause for *Comamonas* flocculation in real wastewater system (Chapter 6). The major findings and conclusions are summarized as follows (Figure 7.1):

- Although the catabolic activities can be induced, the pre-exposure to the pollutants has a great impact to the biofilm lifestyle of the bacteria including decreases in bacterial motility and the intracellular c-di-GMP level. Consequently, when introducing the treated cultures into the bioaugmentation systems, cells would have a lower dispersion and retention coefficient which leads to a rapid flow-out and a less biomass retention. The reduced c-di-GMP level would destabilize biofilms and cause massive cell dispersal, which may eventually lead a bioaugmentation failure.
- Constitutively expressing YedQ, a c-di-GMP synthase from *E. coli*, in *C. testosteroni* increases c-di-GMP concentration significantly. The high c-di-GMP level in *C. testosteroni* enhances EPS production, inhibits cell motility, and induces biofilm formation.
- Compared with the wild type, the high c-di-GMP transconjugant exhibits a higher tolerance to 3-CA at high concentrations. Under conditions with a high surface to volume ratio, elevating c-di-GMP level in *C. testosteroni* significantly increases the contribution of biofilms in 3-CA biodegradation. In continuous

biofilm reactors, an elevated c-di-GMP level in *C. testosteroni* alleviates cell detachment from biofilms and enhances 3-CA biodegradation. Taken together, this study provides a novel strategy to enhance biofilm-based biodegradation of toxic xenobiotic compounds through manipulating bacterial c-di-GMP signalling.

- Although *C. testosteroni* is traditionally considered strictly aerobic, it is found to reduce nitrate to nitrite and derive metabolic energy from nitrate reduction anaerobically. In particular, biofilms of *C. testosteroni* are capable of reducing nitrate under bulk aerobic conditions.
- Nitrate reduction in the biofilms is beneficial to the biofilm mode of life by providing more metabolic energy to maintain high cell viability and a higher level of c-di-GMP to reduce cell detachment from the biofilms. It suggests a potential role of *Comamonas* in biofilm-mediated denitrification in aerobic bioprocesses and oxic natural environments and the availability of nitrate on its biofilm lifestyle.
- The high-molecular-weight fraction of the soluble EPS, in particular, proteins and extracellular DNA, are the major floc-forming inducers in wastewater. They enhance the attachment of planktonic cells onto bacterial flocs by increasing the hydrophobicity of bacterial surfaces and reducing the energy barrier for cell-cell interaction.

Figure 7.1 Schematic diagram summarizing major findings.

7.2 Recommendations for future research

The following tasks are recommended as future researches to further understand the ecological role of *Comamonas* biofilm in environments and broaden the potential applications of *Comamonas* in biotechnology.

7.2.1 Characterization the genomic feature of *Comamonas* community in various environments

In the real environments, *Comamonas* strains are not existed as pure culture. The local community consisted of mixed population must share some common feature to facilitate their survival in specific conditions. Therefore, it's essential to characterize their genomic feature which provide sights on their role and function. Previous researches used ribosomal RNA-targeted fluorescence *in situ* hybridization (FISH) to tag specific bacterial population in mixed community and fluorescence activated cell sorting (FACS) technique to separate the targeted cells from community (Yilmaz, Haroon et al. 2010, Lee, Men et al. 2015). And then isolated

culture was subject to subsequent genomic sequencing and analysis. In our preliminary experiments, we designed one FISH probe (COM0142) based on the available 16s rRNA gene sequences of *Comamonas*. Combining with another formerly designed FISH probe COM1424 (Amann, Ludwig et al. 1996), the *Comamonas* strains in activated sludge were visualized. Further studies can be conducted by applying these probes in different communities to extract *Comamonas* strains and reveal their genomic features at the genus level.

Figure 7.2 CLSM images of activated sludge hybridized with two *Comamonas*-specific probes (COM1424: cy5-labelled; COM0142: cy3-labelled). The scale bar represents 20 μm .

7.2.2 Exploration biocatalytic potential of *Comamonas*

Presence and enrichment of *Comamonas* cells in microbial fuel cell (MFC) system have been observed (Xing, Cheng et al. 2010). Interestingly, *Comamonas* was found to colonize on both anodic and biocathodic electrode which indicate a bidirectional extracellular electron transfer (Yu, Wu et al. 2015). As cathodic strains can consume electrode from anode, biocathode with *Comamonas* can be used to replace the abiotic catalyst or electron mediator which reducing the cost and increasing operational sustainability (He and Angenent 2006). Moreover, its

versatile catabolic abilities can also be applied in this biochemical-catalyzed system. High abundance of *Comamonas* was found in MFC system for the dechlorination and mineralization of recalcitrant aromatics (Huang, Gan et al. 2012, Huang, Shi et al. 2014). Therefore, exploration and characterization of their catabolic and bioelectric properties may expand the application scope of MFC system in pollutant treatment.

Besides the bioelectrochemical properties, their diverse capability towards steroid can be utilized for steroid-based pharmaceutical production. *C. testosteroni* strains were found to initiate testosterone degradation via dehydrogenation (Donova, Egorova et al. 2005). The biotransformation of hydroxysteroid to ketosteroid is an important step to produce different kinds of bioactive steroid drugs, like progesterone, androgens and estrogens (Tong and Dong 2009). Currently, the toxicity of steroid products and organic solvent is one of the main limiting factors in whole-cell biocatalyst (Donova and Egorova 2012). Meanwhile, biofilm cells having enhanced tolerance against toxic substrates could be a potential alternative to address these challenges. The direct substrate-to-cell contact and excreted biosurfactants also increased bioavailability of pollutants which reduced the usage of organic solvent (Johnsen and Karlson 2004). Therefore, *Comamonas* biofilms are worth further study to develop cost-efficient catalytic biofilm processes for pharmaceutical production.

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