Involvement in Denitrification is Beneficial to the Biofilm Lifestyle of *Comamonas testosteroni*: A Mechanistic Study and Its Environmental Implications

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2015

Wu, Y., Shukal, S., Mukherjee, M., & Cao, B. (2015). Involvement in Denitrification is Beneficial to the Biofilm Lifestyle of *Comamonas testosteroni*: A Mechanistic Study and Its Environmental Implications. *Environmental Science & Technology*, 49(19), 11551-11559.

<https://hdl.handle.net/10356/80969>

<https://doi.org/10.1021/acs.est.5b03381>

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[<http://dx.doi.org/10.1021/acs.est.5b03381>].

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1 **Involvement in Denitrification is Beneficial to the Biofilm Lifestyle of *Comamonas***
2 ***testosteroni*: A Mechanistic Study and its Environmental Implications**

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21 **ABSTRACT**

22 *Comamonas* is one of the most abundant microorganisms in biofilm communities driving
23 wastewater treatment. Little has been known about the role of this group of organisms and
24 their biofilm mode of life. In this study, using *Comamonas testosteroni* as a model organism,
25 we demonstrated the involvement of *Comamonas* biofilms in denitrification under bulk
26 aerobic conditions and elucidated the influence of nitrate respiration on its biofilm lifestyle.
27 Our results showed that *C. testosteroni* could use nitrate as sole electron acceptor for
28 anaerobic growth. Under bulk aerobic condition, biofilms of *C. testosteroni* were capable of
29 reducing nitrate and, intriguingly, nitrate reduction significantly enhanced viability of the
30 biofilm-cells and reduced cell detachment from the biofilms. Nitrate respiration was further
31 shown to play an essential role in maintaining high cell viability in the biofilms. RNA-seq
32 analysis, quantitative polymerase chain reaction (qPCR), and liquid chromatography-mass
33 spectrometry (LC-MS/MS) revealed a higher level of bis-(3'-5')-cyclic dimeric guanosine
34 monophosphate (c-di-GMP) in cells respiring on nitrate than those grown aerobically
35 (1.3×10^{-4} fmol/cell vs. 7.9×10^{-6} fmol/cell; $P < 0.01$). C-di-GMP is one universal signaling
36 molecule that regulates the biofilm mode of life and a higher c-di-GMP concentration reduces
37 cell detachment from biofilms. Taken together, this study reveals that nitrate reduction occurs
38 in mature biofilms of *C. testosteroni* under bulk aerobic conditions and the respiratory
39 reduction of nitrate is beneficial to the biofilm lifestyle by providing more metabolic energy
40 to maintain high viability and a higher level of c-di-GMP to reduce cell detachment.

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42 INTRODUCTION

43 In microbial communities driving domestic wastewater treatment, bacteria of the *Comamonas*
44 genus were often reported to be among the most abundant microorganisms¹⁻⁴. In addition, a
45 high abundance of *Comamonas* was also found in nitrate-removing microbial communities
46 presented in river wetlands⁵, soil⁶, and constructed wetlands⁷. However, why they are there
47 and what roles they may play remain largely unknown. To address these questions, a
48 comprehensive understanding of the physiology and metabolic capabilities of this group of
49 organisms is required and *Comamonas testosteroni* is often used as a model organism for
50 such studies.

51 *C. testosteroni* is a bacterium that is commonly found in natural and
52 engineered environments including bioreactors for the treatment of domestic and/or
53 industrial wastewater⁸. It has versatile catabolic capabilities in degrading a variety of
54 organic compounds and has been demonstrated to play an important role in
55 bioremediation⁹⁻¹¹. In various natural and engineered ecosystems, *C. testosteroni* is
56 often found to exist as surface- or interface-associated microbial aggregates, *i.e.*,
57 biofilms, the predominant mode of life on earth¹². Comparative genome analyses
58 suggested a sophisticated regulatory system integrating a number of proteins to sense
59 environmental conditions for the biofilm lifestyle of *C. testosteroni*^{13,14}. Although
60 bacteria in the *Comamonas* genus were usually considered strictly aerobic organisms,
61 the genomes of certain strains of *C. testosteroni* have been shown to contain a gene
62 cluster encoding the membrane-bound NAR-type nitrate reductase system¹³,
63 suggesting that they may be capable of catalyzing nitrate reduction to nitrite under
64 oxygen-limiting conditions. Reduction of nitrate to nitrite is a critical step in
65 microbial denitrification, which plays an important role in wastewater treatment and

66 biogeochemical cycles¹⁵. Highly dynamic and heterogeneous physicochemical
67 microenvironments are often featured in mature biofilms¹⁶⁻¹⁸. Hence, even in the presence of
68 oxygen in the bulk liquid phase, nitrate reduction may occur in *C. testosteroni* biofilms.
69 However, previous studies on nitrate reduction by *Comamonas* mostly focused on phenotypic
70 observation in planktonic cultures under aerobic conditions^{8, 14, 19, 20}. There has been no
71 experimental evidence to support nitrate reduction by *C. testosteroni* biofilms under bulk
72 aerobic conditions and, further, how does nitrate reduction influence the biofilm lifestyle has
73 never been explored. Elucidating whether *C. testosteroni* biofilms are involved in
74 denitrification under bulk aerobic condition and how does nitrate reduction influence the
75 biofilm lifestyle of *C. testosteroni* is an important step toward a comprehensive
76 understanding of the role of *Comamonas* in natural and engineered ecosystems to inform
77 biofilm-mediated bioprocess design for environmental applications such as wastewater
78 treatment.

79 The objectives of this study were to demonstrate the involvement of *C. testosteroni*
80 biofilms in denitrification under bulk aerobic condition and to mechanistically investigate the
81 influence of nitrate reduction by *C. testosteroni* on its biofilm lifestyle. Specifically, we grew
82 biofilms of *C. testosteroni* strain I2 under bulk aerobic condition in the presence and absence
83 of nitrate and quantitatively compared the biofilm structure, viability of the biofilm-cells, and
84 cell detachment from the biofilms. We further conducted RNA-seq analysis to examine the
85 expression profiles of genes that are critical to nitrate metabolism and the biofilm mode of
86 life. This study provides mechanistic insights into the links between nitrate respiration and
87 the biofilm lifestyle of *C. testosteroni*, which is critical toward a comprehensive
88 understanding of the role of *Comamonas* in natural and engineered ecosystems.

89 MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. The preculture of *C. testosteroni* strain I2 (LMG19554 from BCCM/LMG) was prepared by inoculating a single colony from a plate culture into 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, potassium nitrate was supplemented as the sole electron acceptor at a final concentration of 4 mM (~56 mg/L NO₃⁻-N) and the culture was flushed with N₂ gas for 10 min to remove oxygen and then sealed with butyl rubber stoppers²¹. All the cultures were incubated at 30°C on a rotary shaker (200 rpm). The experiments were conducted in triplicates.

Flow Cell Biofilms. Biofilms of *C. testosteroni* I2 were grown using multi-channel flow cells (BioCentrum-DTU, Denmark) at room temperature (~25±2°C). A schematic illustration of the experimental system for biofilm growth is shown in SI (Figure S1). The experimental system including the multi-channel flow cells, tubing, and connectors were sterilized by autoclaving prior to inoculation. 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). 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^{22, 23}.

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.)²⁴. Diluted SYTO 9 (11.1 μ M) and propidium iodide (66.7 μ M) solution (~0.7 mL) was injected into each flow channel²⁵. 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).

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^{26,27}. Concentrations of nitrate and nitrite were determined using HACH kits with a HACH DR 3900 photometer according to manufacturer's instructions (HACH, USA).

RNA-seq Analysis. Cells were cultivated in planktonic cultures with oxygen or nitrate as the sole electron acceptor and harvested at the late exponential phase. Growth conditions were same as those described above. 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 in RPKM with P-value < 0.05.

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 S1. 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 described²⁸. The relative expression level (copy number of mRNA transcript) of each target gene was normalized to a reference gene - transcriptional regulator *lysR* and was compared with control samples.

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 described²². Briefly, cells were harvested at the late exponential growth phase in planktonic cultures and the c-di-GMP was extracted using an organic solvent mixture²⁹. 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).

RESULTS AND DISCUSSION

Nitrate Reduction by *C. testosteroni*. Although a gene cluster encoding the membrane-bound nitrate reductase (NAR) system has been identified in the genome of *C. testosteroni* I2¹³, 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,

161 respectively, an apparent growth of *C. testosteroni* I2 was observed (Figure 1A). The cell
162 density increased from 2.5×10^6 cells/mL to 6.0×10^6 cells/mL within 27 h and acetate
163 concentration decreased by ~ 1.5 mM, while no acetate consumption or cell growth occurred
164 in media without nitrate. Nearly all nitrate in the medium (~ 54.6 mg/L out of 56.0 mg/L NO_3^-
165 $-N$) was reduced to nitrite and a stoichiometric amount of nitrite (~ 54.8 mg/L NO_2^- -N) was
166 accumulated in the culture (Figure 1B). Our results demonstrate that *C. testosteroni* I2 is
167 capable of utilizing nitrate as an electron acceptor and coupling growth to the reduction of
168 nitrate to nitrite. To the best of our knowledge, this is the first experimental evidence in the
169 literature on anaerobic nitrate respiration in *C. testosteroni*. Nitrate reduction is an important
170 step in microbial denitrification; hence, the capability of anaerobic nitrate respiration by
171 *Comamonas* suggests a possible involvement of this organism in denitrification. However,
172 *Comamonas* has been traditionally considered to be strictly aerobic and is mostly found in
173 aerobic bioprocesses⁸. So, one would question whether the anaerobic nitrate reduction in
174 *Comamonas* is relevant or not. Previous studies have suggested that *Comamonas* in
175 wastewater treatment is often present in the form of biofilms or aggregates^{1, 3, 10}. One typical
176 feature of a biofilm or cell aggregate is the presence of a sticky matrix formed by self-
177 produced extracellular polymeric substances (EPS)¹², which may create physicochemical
178 gradients and heterogeneity at the micrometre scale¹⁶. For example, even under bulk aerobic
179 conditions, microenvironments with low or no oxygen have been reported in biofilms or cell
180 aggregates^{30, 31}. Hence, it is plausible that anaerobic nitrate reduction occurs in biofilms of *C.*
181 *testosteon*i I2 under bulk aerobic conditions. To confirm it, we grew I2 biofilms using air-
182 saturated medium in the presence of nitrate. Figure 2A shows the concentration profiles of
183 nitrate and nitrite in the effluent from the flow cell biofilms. Intriguingly, the reduction of
184 nitrate to nitrite occurred only after 55 h of biofilm growth, which correlated well with the
185 development of 3-dimensional structures in the biofilms (Figure 2B). The results suggest that

186 a mature biofilm with a certain extent of structural heterogeneity is required for nitrate
187 reduction under bulk aerobic conditions.

188 **Nitrate Reduction in *C. testosteroni* Biofilms under Bulk Aerobic Condition Enhanced**
189 **Cell Viability and Biofilm Stability.** We have shown that nitrate reduction could occur in
190 mature biofilms of *C. testosteroni* I2 under bulk aerobic conditions. To further understand
191 how nitrate reduction influences the biofilm lifestyle of *C. testosteroni*, we grew I2 biofilms
192 with air-saturated medium in the absence and presence of nitrate. Biofilm architecture and
193 cell viability of the biofilms grown under both conditions were compared using CLSM
194 imaging analysis. After biofilm growth under both conditions for 120 h, *C. testosteroni* I2
195 developed into similar towering biofilm architecture (Figure 3A and B), a typical feature of
196 mature biofilms in flow cells³². The thickness of the biofilms in our experimental systems
197 were about 40-50 μm . In biofilms grown in similar flow cells, anoxic zones often occur at a
198 depth of 10-40 μm ³³. In the biofilms, cells in green and red fluorescence represent live and
199 dead cells, respectively. Quantitative analysis showed a cell viability of $92\pm 2\%$ for the
200 biofilms grown with nitrate, while only $75\pm 6\%$ for the biofilms grown without nitrate
201 (Figure 3C). The presence of nitrate in the biofilm growth media significantly enhanced cell
202 viability in the mature biofilms ($P < 0.05$), which could be because cells residing in oxygen-
203 limiting microenvironments in the mature biofilms obtain energy from nitrate respiration for
204 survival which would otherwise die. To examine whether nitrate respiration is key to
205 maintaining high cell viability in mature biofilms, we grew *C. testosteroni* biofilms with the
206 nitrate-containing growth media supplemented with azide at a concentration of 0.1 mM,
207 which inhibited nitrate respiration by up to 85% but did not exhibit an apparent inhibition on
208 aerobic respiration in *C. testosteroni* (Figure S2). With the presence of azide in growth
209 medium, reduction of nitrate to nitrite by *C. testosteroni* biofilms was negligible during 5-day
210 biofilm growth as evidenced by a constant concentration of nitrate and nearly no nitrite was

detected in the effluent (Figure S2). 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 3C and 4C) and was at a level comparable with those without nitrate ($65\pm 9\%$ vs. $61\pm 7\%$; $P = 0.71$) (Figure 4A-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 oxygen³⁴.

Previous studies have shown that metabolic energy is critical for a biofilm to maintain its stability^{25, 35}. 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* biofilms²². 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 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 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 3D-G and 4D-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.

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 (~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). 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*^{36, 37} 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 1B). In cells respiring on nitrate, the genes responsible for acetate oxidation and the electron transport chain were also highly expressed (Figure 5). In particular, genes encoding formate dehydrogenase FdnGHI were highly expressed. A respiratory chain in *E. coli* has been identified to consist of formate dehydrogenase FdnGHI and nitrate reductase NarGHI where the electrons from formate oxidation were transferred to nitrate

reduction through the quinol pool³⁶. The *fdnGHI* operon in *E. coli* was reported to be induced by nitrate, which is consistent with the findings of this study^{38, 39}.

The c-di-GMP Signalling. Eight out of 30 predicted diguanylate cyclases (DGCs) in *C. testosteroni* I2¹³ were found highly expressed ($P < 0.05$) when nitrate was used as the electron acceptor (Table 1). 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^{22, 40, 41}. 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 S3A): 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.

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 S3B 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 biofilms⁴²⁻⁴⁵. Generally, a high concentration of c-di-GMP inhibits various forms of motility, stimulates EPS production, and favors the biofilm mode of life⁴⁰. For example, *C. testosteroni* biofilms formed by cells with an elevated c-di-GMP level were more stable and exhibited a decreased toxicity-induced cell detachment from the biofilms²². An elevated c-di-GMP level in *P. aeruginosa* was found to improve bacterial fitness and enhance antimicrobial resistance^{46, 47}.

283 In this study, we found that nitrate respiration increased c-di-GMP level in *C. testosteroni* I2.
284 Hence, biofilms grown with air-saturated medium in the presence of nitrate would be able to
285 maintain a higher c-di-GMP level than those grown in the absence of nitrate. The higher
286 concentration of c-di-GMP is consistent with the observation that nitrate respiration enhanced
287 biofilm stability, in particular, when the mature biofilms were exposed to 3-CA.

288 **Environmental Implications.** Denitrification plays a critical role in the nitrogen
289 biogeochemical cycle and in engineering bioprocesses for nitrogen removal from wastewater.
290 Microbial reduction of nitrate to nitrite is an important step in denitrification process that
291 often occurs under oxygen-limiting conditions. *Comamonas* is a group organism that is often
292 found in various natural and engineered ecosystems. We know little about their possible roles
293 in environmental bioprocesses except that this group of organisms are well known to have
294 versatile catabolic activities for efficient degradation of organic compounds. Here, we show
295 that *C. testosteroni*, an organism that is traditionally considered strictly aerobic, can reduce
296 nitrate to nitrite and derive metabolic energy from nitrate reduction anaerobically. In
297 particular, biofilms of *C. testosteroni* are capable of reducing nitrate under bulk aerobic
298 conditions and nitrate reduction in the biofilms is beneficial to the biofilm mode of life by
299 providing more metabolic energy to maintain high cell viability and a higher level of c-di-
300 GMP to reduce cell detachment from the biofilms. This study suggests a potential role of
301 *Comamonas* in biofilm-mediated denitrification in aerobic bioprocesses in natural
302 environments and engineered bioreactor systems. In addition, this study also demonstrated an
303 approach to increase the c-di-GMP level to establish more robust biofilms of *C. testosteroni*,
304 which provides a more feasible alternative to genetic manipulation approaches for
305 environmental applications such as biofilm-mediated biodegradation of toxic organic
306 compounds²².

307

308 **ASSOCIATED CONTENT**309 **Supporting Information**

310 Additional information on primers used in qPCR analysis (Table S1), experimental system
311 illustration (Figure S1), inhibitory effect of azide on planktonic cultures and biofilms (Figure
312 S2), and qPCR analysis of genes involved in c-di-GMP synthesis and intracellular c-di-GMP
313 concentration (Figure S3).

314

315 **ACKNOWLEDGEMENTS**

316 The c-di-GMP quantification was carried out with the help of Dr. Peter Imre Benke and
317 Professor Sanjay Swarup in the Metabolomics Laboratory of the Singapore Centre on
318 Environmental Life Sciences Engineering (SCELSE). RNA sequencing was carried out with
319 the help of Dr. Daniela Moses and Professor Stephan Schuster using the sequencing facilities
320 at SCELSE. We thank Yifang Bi for assistance in sample preparation. This research was
321 supported by the National Research Foundation and Ministry of Education Singapore under
322 its Research Centre of Excellence Programme, Singapore Centre on Environmental Life
323 Sciences Engineering (SCELSE) (M4330005.C70) and a Start-up Grant (M4080847.030)
324 from College of Engineering, Nanyang Technological University, Singapore. The authors
325 thank the Singapore Ministry of Education (MOE2011-T2-2-035, ACR 3/12) for the research
326 scholarship to Yichao Wu.

327 **The authors declare no competing financial interest.**

328

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455

456 **Table 1. Nitrate respiration-induced expression of genes responsible for the synthesis of c-di-GMP. Ratio indicates a relative gene**
457 **expression level in cells respiring on nitrate to those respiring on oxygen (P < 0.05).**

Feature ID	Description	Log ₂ Ratio
I2contig_00961	PAS domain S-box/diguanylate cyclase domain (DGC-2)	2.46
I2contig_05141	Diguanylate cyclase domain (DGC-3)	2.41
I2contig_04562	Diguanylate cyclase domain (DGC-1)	2.01
I2contig_00763	Diguanylate cyclase domain	1.59
I2contig_02275	Diguanylate cyclase/phosphodiesterase	1.50
I2contig_04913	Diguanylate cyclase	1.25
I2contig_01952	Response regulator containing a CheY-like receiver and a GGDEF domain	1.04
I2contig_04723	Diguanylate cyclase domain	1.02
I2contig_01917	Diguanylate cyclase domain	1.00
I2contig_02501	Diguanylate cyclase domain	0.88
I2contig_03397	Diguanylate cyclase domain	0.60

Figure Captions

Figure 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.

Figure 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 represents 20 μm .

Figure 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 alternative electron acceptors. (D-F) Biofilm architectures and cell viability of the mature biofilms after exposure to 3-chloroaniline (3-CA). Scale bar represents 20 μm . (G) Cell detachment from the mature biofilms before and after 3-CA exposure. * Significant different ($P < 0.05$).

Figure 4. 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 represents 20 μm . (G) Cell detachment from the mature biofilms before and after 3-CA exposure.

Figure 5. 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.

Figure 1
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Figure 2
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Figure 3
252x302mm (150 x 150 DPI)

Figure 4
249x308mm (150 x 150 DPI)

Figure 5
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TOC art
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