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<th>Involvement in Denitrification is Beneficial to the Biofilm Lifestyle of Comamonas testosteroni: A Mechanistic Study and Its Environmental Implications (Main article)</th>
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<td><strong>Author(s)</strong></td>
<td>Wu, Yichao; Shukal, Sudha; Mukherjee, Manisha; Cao, Bin</td>
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Involvement in Denitrification is Beneficial to the Biofilm Lifestyle of *Comamonas testosteroni*: A Mechanistic Study and its Environmental Implications

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Comamonas is one of the most abundant microorganisms in biofilm communities driving wastewater treatment. Little has been known about the role of this group of organisms and their biofilm mode of life. In this study, using Comamonas testosteroni as a model organism, 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/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.
INTRODUCTION

In microbial communities driving domestic wastewater treatment, bacteria of the *Comamonas* genus were often reported to be among the most abundant microorganisms \(^1\)\(^-\)\(^4\). In addition, a high abundance of *Comamonas* was also found in nitrate-removing microbial communities presented in river wetlands \(^5\), soil \(^6\), and constructed wetlands \(^7\). 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 \(^8\). It has versatile catabolic capabilities in degrading a variety of organic compounds and has been demonstrated to play an important role in bioremediation \(^9\)\(^-\)\(^11\). 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 \(^12\). Comparative genome analyses suggested a sophisticated regulatory system integrating a number of proteins to sense environmental conditions for the biofilm lifestyle of *C. testosteroni* \(^13\), \(^14\). 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 \(^13\), 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. Highly dynamic and heterogeneous physicochemical microenvironments are often featured in mature biofilms. Hence, even in the presence of oxygen in the bulk liquid phase, nitrate reduction may occur in C. testosteroni biofilms. However, previous studies on nitrate reduction by Comamonas mostly focused on phenotypic observation in planktonic cultures under aerobic conditions. 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 to inform biofilm-mediated bioprocess design for environmental applications such as wastewater treatment.

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.

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 stoppers. 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.
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.)\textsuperscript{24}. Diluted SYTO 9 (11.1 µM) and propidium iodide (66.7 µM) solution (~0.7 mL) was injected into each flow channel\textsuperscript{25}. 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 µm 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 × 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\textsuperscript{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 described. The relative expression level (copy number of mRNA transcript) of each target gene was normalized to a reference gene - transcriptional regulator $\text{lysR}$ 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 described. 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. 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 $C.\ testosteroni$ [12,13], 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 1A). The cell
density increased from $2.5 \times 10^6$ cells/mL to $6.0 \times 10^6$ cells/mL within 27 h and acetate
concentration decreased by ~1.5 mM, while no acetate consumption or cell growth occurred
in media without nitrate. Nearly all nitrate in the medium (~54.6 mg/L out of 56.0 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 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.$^8$ 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.$^1,^3,^10$ One typical
feature of a biofilm or cell aggregate is the presence of a sticky matrix formed by self-
produced extracellular polymeric substances (EPS)$^{12}$, which may create physicochemical
gradients and heterogeneity at the micrometre scale.$^6$ For example, even under bulk aerobic
conditions, microenvironments with low or no oxygen have been reported in biofilms or cell
aggregates.$^{30,^31}$ Hence, it is plausible that anaerobic nitrate reduction occurs in biofilms of *C.
testosteoni* I2 under bulk aerobic conditions. To confirm it, we grew I2 biofilms using air-
saturated medium in the presence of nitrate. Figure 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-dimentional structures in the biofilms (Figure 2B). The results suggest that
a mature biofilm with a certain extent of structural heterogeneity is required for nitrate reduction under bulk aerobic conditions.

**Nitrate Reduction in C. testosteroni Biofilms under Bulk Aerobic Condition Enhanced Cell Viability and Biofilm Stability.** 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 3A and B), a typical feature of mature biofilms in flow cells. The thickness of the biofilms in our experimental systems were about 40-50 µm. In biofilms grown in similar flow cells, anoxic zones often occur at a depth of 10-40 µm. In the biofilms, cells in green and red fluorescence represent live and dead cells, respectively. Quantitative analysis showed a cell viability of 92±2 % for the biofilms grown with nitrate, while only 75±6 % for the biofilms grown without nitrate (Figure 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 S2). 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 S2). Cell viability in the biofilms grown in the presence of
nitrate and azide was significantly lower than that without azide (65±9% vs. 92±2%; P = 0.03)
(Figure 3C and 4C) and was at a level comparable with those without nitrate (65±9% vs.
61±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 oxygen

Previous studies have shown that metabolic energy is critical for a biofilm to maintain
its stability. 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. 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
~1.8×10⁹ cells/h) and the remaining cells were present in the form of cell clusters with a low
cell viability (~27%) (Figure 3D-G). In contrast, after 3-CA exposure, the biofilms grown in
the presence of nitrate exhibited a slower cell detachment (~5.5×10⁸ cells/h) and maintained
the towering structures with a high cell viability (~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* 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 pool. The fdhGHI operon in *E. coli* was reported to be induced by nitrate, which is consistent with the findings of this study.

*The c-di-GMP Signalling.* Eight out of 30 predicted diguanylate cyclases (DGCs) in *C. testosteroni* 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. 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 (1.3×10⁴ fmol/cell vs. 7.9×10⁶ 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. Generally, a high concentration of c-di-GMP inhibits various forms of motility, stimulates EPS production, and favors the biofilm mode of life. 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 biofilms. An elevated c-di-GMP level in *P. aeruginosa* was found to improve bacterial fitness and enhance antimicrobial resistance.
In this study, we found that nitrate respiration increased c-di-GMP level in *C. testosteroni* I2. Hence, biofilms grown with air-saturated medium in the presence of nitrate would be able to maintain a higher c-di-GMP level than those grown in the absence of nitrate. The higher concentration of c-di-GMP is consistent with the observation that nitrate respiration enhanced biofilm stability, in particular, when the mature biofilms were exposed to 3-CA.

**Environmental Implications.** 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. We know little about their possible roles in environmental bioprocesses 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. This study suggests a potential role of *Comamonas* in biofilm-mediated denitrification in aerobic bioprocesses in natural environments and engineered bioreactor systems. In addition, this study also demonstrated an approach to increase the c-di-GMP level to establish more robust biofilms of *C. testosteroni*, which provides a more feasible alternative to genetic manipulation approaches for environmental applications such as biofilm-mediated biodegradation of toxic organic compounds.²²
ASSOCIATED CONTENT

Supporting Information

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

ACKNOWLEDGEMENTS

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

The authors declare no competing financial interest.
REFERENCES


Table 1. 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 (P < 0.05).

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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-chloroanine (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-chloroanine (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
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