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Full Paper

Nitrogen Removal with Energy Recovery Through N_2O Decomposition Supplementary Information

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This supplementary information contains the following:

(1) Materials and Methods for quantitative-PCR (qPCR) calibration, qPCR of 16S rDNA and phaC, and pyrosequencing of 16S rDNA and microbial community analysis

(2) Supplementary Table 1. Retrieved standard DNA sequences for qPCR calibration

(3) Supplementary Table 2. Comparison of DNA sequences to a known organisms

(4) Supplementary Table 3. Dominant bacterial species populations (>1% relative abundance) and best-matched type strains.

Materials and Methods

Standard DNA sequences for Quantitative-PCR (qPCR) calibration

Bacterial and archaeal 16s rDNA genes were retrieved from activated sludge at the Palo Alto Regional Water Quality Control Plant (PARWQCP). Genomic DNA (gDNA) was extracted from the biomass using the MoBio Ultraclean Soil DNA extraction kit (Carlsbad, CA) per the manufacturer's protocol. Polymer chain reaction (PCR) amplification was performed in a PCR reaction containing 0.5 µM each primer, 1X Fail-Safe PCR Premix F (Epicentre Technologies), 1.25 units AmpliTaq LD (Applied Biosystems), and approximately 20 ng of template genomic DNA. For bacterial 16s rDNA, primers 1055F and 1392R[1] were used. The PCR thermocycling steps were: (i) 95°C for 10 min; (ii) 35 cycles consisting of 95°C for 30 s, 50°C for 60 s, 72°C for 20 s; and (iii) an extension at 72°C for 10 min. For archaeal 16s rDNA, primers Arch21F and Arch958R[2] were used. The PCR thermocycling steps were: (i) 94°C for 5 min; (ii) 35 cycles consisting of 94°C for 90 s, 55°C for 90 s, 72°C for 90 s; and (iii) an extension at 72°C for 7 min.

Acaligenes eutrophus was used for PCR amplification and cloning to prepare standard DNA of *pha*C [poly-3-hydroxyalanoate (PHA) synthase] gene for qPCR calibration. Genomic DNA (gDNA) was extracted from *A. eutrophus* using the MoBio Ultraclean Soil DNA extraction kit (Carlsbad, CA) per the manufacturer's protocol. For PCR amplification, primers phaCF2 and phaCR4[3] were used. The PCR thermocycling steps were: (i) 95°C for 5 min; (ii) 35 cycles consisting of 95°C for 20 s, 57°C for 45 s, 72°C for 1 min; and (iii) an extension at 72°C for 10 min.

The cloning method is previously described.[4] Retrieved DNA sequences and the results of sequence comparison using the Basic Local Alignment Search Tool (BLAST) are listed in supplementary table 1 and 2, respectively.

Quantitative-PCR (qPCR) of 16S rDNA and phaC 1

Two-milliliter samples were periodically removed from the well-mixed bioreactor. After each sample was centrifuged to a cell pellet and washed with 1 ml Tris-EDTA buffer (1 mM, pH=7.0), genomic DNA (gDNA) was extracted in triplicate using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH) per the manufacturer's protocol. The copy numbers for bacterial and archeal 16S rDNA and bacterial *pha*C [poly-3-hydroxyalanoate (PHA) synthase] genes were determined by qPCR using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). PCR amplifications were conducted in 25 µl reactions consisting of 20-50 ng gDNA of template DNA, 0.4 µM of the appropriate forward and reverse primers, 1.25 U AmpliTaq LD DNA Polymerase (Applied Biosystems, Foster City, CA), 0.8 µl of passive reference dye, and 12.5 µl of Failsafe Green Premix F (Epicentre, Madison, WI).

For qPCR of bacteraial 16S rDNA, primers 1055F and 1392R1 were used. The thermocycling steps were: (i) 95°C for 5 min; (ii) 45 cycles at 95°C for 30 s, 50°C for 1 min, 72°C for 20 s; and (iii) a detection step for 15 s at 80°C. For qPCR of archaeal 16S rDNA, primers Arch349F and Arch806R[5] were used. The thermocycling steps were: (i) 95°C for 5 min; (ii) 40 cycles at 95°C for 30 s, 50°C for 30 s, 72°C for 40 s; and (iii) a detection step for 15 s at 80°C.

For qPCR of *pha*C, primers *pha*CF1and *pha*CR43 were used. The PCR thermocycling steps were: (i) 95°C for 5 min; (ii) 45 cycles at 95°C for 30 s, 57°C for 30 s, 72°C for 20 s; and (iii) a detection step for 15 s at 80°C. After the termination of qPCR thermocycling steps, melting curves were obtained to verify the specificity of amplification. For calibration, standard curves were constructed using plasmids containing cloned PCR amplicons of each target gene.

Pyrosequencing of 16S rDNA and Microbial Community Analysis

The following 16S rDNA primers were used for the PCR reactions: F563/16 (AYTGGGYDTAAAGNG) and BSR926/20 (CCGTCAATTYYTTTRAGTTT.[6] Primers were barcoded with different nucleotides for sorting individual samples. Each PCR reaction was carried out with 3 of the 25-μL reaction mixtures containing 60 ng of template DNA, 10 µM of each primer (Macrogen, Seoul, Korea), 1.25 U of *Taq* polymerase, 50 mM of $MgSO_4$, and 10X of the PCR buffer of AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen, WI, USA). A C1000TM Thermal Cycler (BIO-RAD, CA, USA) was used for PCR according to the following steps: (i) denaturation at 94°C for 1 min; (ii) 35 cycles of denaturation, annealing, and extension (94°C for 1 min followed by 55°C for 1 min, and an extension step at 72°C for 2 min); and (iii) extension at 72°C for 5 min. After PCR amplification, amplicons were purified using a QIAquick Gel Extraction Kit and a QIAquick PCR Purification Kit (Qiagen). All amplicons were pooled and sequenced using a 454/Roche GS-FLX Titanium instrument (Roche, Nutley, NJ, USA). Sequencing data were analyzed using the Pyrosequencing Pipeline at Ribosomal Database Project (RDP) II.[7] Low-quality sequences (shorter than 250 nucleotides and of an average quality score of less than 20) were eliminated using the initialization process of the RDP Pyrosequencing Pipeline.[8] Chimera sequences were removed by UCHIME.[9] Pyrosequenced reads were aligned and clustered at 0.03 distances using the Infernal Aligner and complete-linkage clustering of RDP. The RDP Classifier assigned representative operational taxonomic unit (OTU) reads with a confidence threshold of 50% at genus level. The EzTaxon database of type strains was used to identify phylogenetic neighbors.[10]

PHB imaging

PHB granule images were obtained by Transmission Electron Microscopy (JEOL-1230) at 80kV, using a Gatan Orius digital camera.

Results

At the species level (> 97% sequence similarity), the decoupled feed strategy selected for OTUs that best matched *Pseudoxanthomonas daejeonensis* TR6-08(T), *Paracoccus versutus* ATCC 25364(T), *and Diaphorobacter nitroreducens* NA10B(T),. Decoupled feeding appeared to select against species with best matches to *Chryseobacterium hominis* NF802(T) and *Pseudomonas stutzeri* ATCC 17588(T) (Table S3).

Table S1. Retrieved standard DNA sequences for qPCR calibration.

Gene	Sequence (5' – 3')	Amplicon Length
16S rDNA bacteria 1055F 1392R	ATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTCCTATAACGAGCGCAACCCC TGTTGTTAGTTGCCAGCGAGTAATGTCGGGAACTCTAGCAAGACTGCCAGTGCAAACTGTG AGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACGTCCTGGGCTACACACGTGCT ACAATGGTGGGGACAGAGAGCAGCCACTGGGTGACCAGGAGCGAATCTACAAACCCCATC ACAGTTCGGATCGGAGGCTGCAACTCGACTCCGTGAAGCTGGAATCGCTAGTAATCGCAG ATCAGCCATGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT	353
16 rDNA archaea Arch21F Arch958R	TTCCGGTTGATCCCGCCGGAAGTCACTGCTATCGGTGTTCGACTAAGCCATGCGAGTCAAA TGTTCTTCGTGAACATGGCGTACTGCTCAGTAACACGTGGATAACCTGCCCTTAGGTCTGG CATAACCCCGGGAAACTGGGGATAATTCCGGATAGATCATGGATGCTGGAATGCACCGTG GTCGAAAGCTTTTGTGCCTAAGGATGGGTCTGCGGTCTATCAGGTTGTAGTGGGTGTAACG TTCCTACTAACCTACGACGGATACGGGTTGTGGGAGCAAGAGCCCGGAGATGGATTCTGA GACATGAATCCAGGCCCTACGGGGCGCAGCAGGCGCGAAAACTTTACAATGCTGGCAACA GCGATAAGGGAACCTCGAGTGCCAGGTTACAAATCTGGCTGTCGAGATGCCTAAAAAGCA TTTCATAGCAAGGGCCGGGCAAGACCGGTGCCAGCCGCCGCGGTAACACCGGCGGCTCGA GTGGTAACCGTTATTATTGGGTCTAAAGGGTCTGTAGCCGGCCGGATAAGTCTCTTGAGAA ATCTGGCAGCTTAACTGTCAGGCTTTCAGGAGATACTGTCTGGCTCGAGGCCGGGAGAGGT GAGAGGTACTTCAGGGGTAGGGGTGAAATCTTGTAATCCCTGTGGGACCACCAGTGGCGA AGGCGTCTTACCAGAACGGGTCCGACGGTGAGGGACGAAAGCTGGGGGCACGAACCGGA TTAGATACCCGGGTAGTCCCAGCTGTAAACTATGCTCGCTAGGTGTCAGGGACGGTGCGAC CGTTTCTGGTGCCGCAGGGAAGCCGTGAAGCGAGCCACCTGGGAAGTACGGTCGCAAGGC TGAAACTTAAAGGAGTTGGCGGGGGAGCACTACAACGGGTGGAGCCTGCGGTTTAATTGG ATTCAACGCCGGA	918
phaC phaCF2 phaCR4	GTGTTCATCGTCTCCTGGCGCAACCCCGACGCCTCCGTCGCCGGCAAGACCTGGGACGACT ACGTGGAGCAGGGCGTGATCCGCGCCATCCGCGTGATGCAGCAGATCACGGGGCACGAGA AGGTCAACGCGCTGGGCTTCTGCGTCGGCGGCACCATCCTGAGCACGGCGCTGGCGGTGC TGGCCGCGCGCGGCGAGCAGCCCGCGGCGAGCCTGACGCTGCTGACCACGCTGCTGGACT TCAGCAACACCGGCGTGCTGGACCTGTTCATCGACGAGGCCGGCGTGCGCCTGCGCGAGA TGACCATCGGCGAGAAGGCGCCCAACGGCCCGGGCCTGCTCAACGGCAAGGAGCTGGCCA CCACCTTCAGCTTCCTGCGCCCGAACGACCTGGTCTGGAACTACTTCGTCAACAACTACCT	422

Table S2. Comparison of DNA sequences to known organisms.

Sequence	Accession number	Species	e-value
16S rDNA bacteria	FR691443.1	*Gelidibacter algens* partial 16S rRNA gene	1.00E-172
16 rDNA archaea	EF174501.1	*Methanomethylovorans sp.* 16S rRNA gene	0.0
*pha*C	AF004933.1	*Alcaligenes latus* poly-deta-hydroxybutyric acid synthase (*phb*C) gene	0.0

*indicates relative abundance.

Table S3. Dominant bacterial species populations (>1% relative abundance) and best-matched type strains.

OTU	Coupled R.A.* (%)	Decoupled R.A. (%)	Best Matched Type Strain (Ez-Taxon DB)	Identity (%)
1	7.0	12.41	*Pseudoxanthomonas daejeonensis* TR6-08(T)	95
2	6.1	10.81	*Paracoccus versutus* ATCC 25364(T)	99
3	22.2	8.79	*Chryseobacterium hominis* NF802(T)	95
4	1.3	8.10	*Diaphorobacter nitroreducens* NA10B(T)	99
5	3.7	6.49	*Pseudoxanthomonas daejeonensis* TR6-08(T)	95
6	1.9	4.55	*Subsaxibacter broadyi* P7(T)	96
7	24.3	4.25	*Pseudomonas stutzeri* ATCC 17588(T)	99
8	1.5	2.81	*Pseudoxanthomonas daejeonensis* TR6-08(T)	95
9	3.3	2.11	*Stenotrophomonas nitritireducens* L2(T)	99
10	5.5	2.09	*Castellaniella ginsengisoli* DCY36(T)	99
11	1.7	1.93	*Thermomonas koreensis* KCTC 12540(T)	96

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