ENVIRONMENTAL BIOTECHNOLOGY

Microbial community of acetate utilizing denitrifiers in aerobic granules

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Abstract The aerobic sludge granules cultivated at high organic loading rates could effectively convert 100–700 mg l^{-1} nitrite to nitrogen gas with 400 or 1,200 mg l^{-1} dosed acetate. The denitrifying microbial community structure of the so-cultivated granules was investigated by 16S rRNA gene sequences and localized using fluorescence in situ hybridization (FISH). The 16S rRNA gene phylotypes in the clone library and FISH probes used exhibited high diversity among the bacteria and denitrifying communities, with the members of *Betaproteobacteria* predominant that were closely related to families *Comamonadaceae*, *Nitrosomonadaceae*, *Alcaligenaceae*, and *Rhodocyclaceae*. The confocal laser scanning microscope and staining test revealed that active microbial community principally distributed at 200–250 μm beneath the outer surface, embedded in extracellular polymeric substances.

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Introduction

Denitrifying bacteria converts nitrite into nitrogen gas by enzymatic pathway consisting three successive steps involving nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (Mellor et al. 1992; Shapeligh et al. 1987).

$$NO_3^- \xrightarrow{\text{Nitrate reductase}} NO_2^- \xrightarrow{\text{Nitrate reductase}} [NO]$$
$$\xrightarrow{\text{Nitric oxide reductase}} N_2O \xrightarrow{\text{Nitrous oxide reductase}} N_2$$
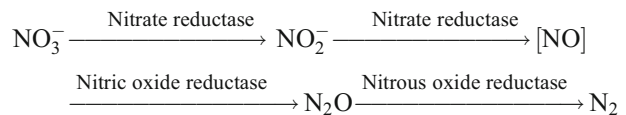
The dissolved oxygen level affects the expression of nitrite reductase and nitrous oxide reductase, hence the denitrification efficiency of nitrite, too. High levels of nitrite inhibit denitrification process in wastewater treatment (Glass et al. 1997). A biological treatment process that can denitrify nitrite at high concentrations is desirable.

The aerobic sludge granulation technology had several advantages over conventional wastewater treatment systems, including possessing a dense and strong microbial structure, good settleability, high biomass retention, tolerance to high organic loading rate (OLR), and to high levels of toxic substances (Liu and Tay 2004; Adav et al. 2008). Aerobic granules were used to conduct simultaneous nitrification–denitrification process for wastewaters (Beun et al. 2001; Tsuneda et al. 2003; de Kreuk et al. 2005; Mosquera-Corral et al. 2005; Adav et al. 2009). Denitrifying bacteria thus present in excess quantity in cultivated aerobic granules. The microbial diversity in acetate fed aerobic sludge granules is seldom reported.

This work aims at cultivating stable aerobic granules that can degrade high concentrations of nitrite and at analyzing microbial community of acetate utilizing denitrifiers using 16S rRNA gene amplification and cloning. The spatial

distributions of typical denitrifiers were probed using fluorescent in situ hybridization (FISH) probes.

Materials and methods

Reactor start-up

Aerobic-activated sludge was obtained from a local municipal wastewater treatment plant in Taipei, Taiwan. The sludge sample (1 l in each reactor) was seeded in column-type sequential batch reactors (120×6 cm). The column reactor was fed with synthetic wastewater containing acetate as the sole carbon source with the following media composition (in gl^{-1}): sodium acetate, 0.4; $(NH_4)_2SO_4$, 1.0; NaCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3$, 0.02; $CaCl_2 \cdot 2H_2O$, 0.01; K_2HPO_4, 1.65; KH_2PO_4, 1.35; pH 6.8±0.2; and micronutrients, 1.0 ml l^{-1} (Moy et al. 2002). The chemical oxygen demand (COD) concentration of this initial feed was 292±17 mgl^{-1} and was increased to attain higher OLR by proportionally adjusting the concentration of each chemical ingredient except buffer constitutes (K_2HPO_4 and KH_2PO_4). Fine air bubbles for aeration and mixing were fed through the reactor bottom at superficial velocity of 3.0 cm s^{-1}. The reactors were operated sequentially in 4-h cycles with 5-min settling, 10-min effluent withdrawal, and 5-min filling, and the remaining time in a cycle was the reaction time. The volumetric exchange ratio of liquid was 50%.

PCR amplification of 16S rRNA gene sequences and library construction

The DNA from granules were extracted via enzymatic lysis using extraction buffer (100 mM Tris–HCl at pH 8.0, 100 mM EDTA, and 1.5 M NaCl) containing Proteinase K (10 mg ml^{-1}; Amresco Inc., Solon, OH, USA), as described previously (Adav et al. 2007). The isolated DNA was purified by Gene-Spin™ (Protect Technology Co. Ltd, USA). PCR amplification of 16S rRNA gene sequences was carried out using the primers F27 (5′-CCA GAG TTT GAT CMT GGC TCA G-3′) and R1492 (5′-TAC CTT GTT ACG ACT T-3). PCR amplification was performed as recommended by Polz and Cavanaugh (1998) to reduce bias in amplification. Briefly, 50-μl reaction volumes contained PCR buffer with 1.5 mM $MgCl_2$, 200 μM dNTPs, 100 ng genomic DNA, 0.5 U Taq DNA polymerase, and 1 μM of each primer. PCR amplification were performed in Eppendorf master-cycler (Eppendorf AG, Hamburg, Germany) under the following conditions: one cycle of 95°C for 3 min; 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s;

and one cycle of 10 min at 72°C. PCR products were purified, cloned using the Qiagen PCR cloning kit (Qiagen, Valencia, CA) as per the manufacturer's instructions, and transformed into Escherichia coli TOP10F cells (Invitrogen, Carlsbad, CA).

In total, 102 clones were sequenced using the ABI Prism model 3730 (version 3.2). The 16S rRNA gene sequences were aligned by the multiple alignments ClustalW. Phylogenetic trees based on 16S rRNA gene sequences were constructed by the neighbor-joining method using the MEGA software. To estimate the confidence of the tree topologies, bootstrap re-sampling analysis for 1,000 replicates was performed. The gene sequences of the clones were deposited in GenBank under accession numbers GQ891759 to GQ891860.

FISH and CLSM imaging

Sludge granule samples were washed with deionized water and fixed with 4% paraformaldehyde. The fixed sludge granule samples were then dehydrated by successive passages through 50%, 80%, and 100% ethanol (10 min each) and were subsequently embedded in Shandon cryomatrix (Thermo Scientific, Pittsburg, PA, USA) and frozen at −20°C. The frozen sludge granules were then cut into 40-μm-thick sections with a cryotome (model CS-3306, Thermo Shandon, Runcorn, UK). The sections of sludge samples were immobilized on glass slides coated with polysine for FISH experiments. The hybridization was performed as described previously (Amann et al. 1990). The probes and formamide concentration used in this study were listed in Table 1. The samples were hybridized using hybridization buffer (0.9 M NaCl, 100 mM Tris–HCl at pH 7.4, 0.01% sodium dodecyl sulfate) containing 50 ng $μl^{-1}$ of fluorescently labeled probes and incubated for 30 min. The samples were washed with washing buffer (0.9 M NaCl, 100 mM Tris–HCl at pH7.4, 0.01% sodium dodecyl sulfate) and ringed with Milli-Q water. The cells hybridized with the probes were observed with a confocal laser scanning microscope (Leica TCS SP5 Confocal Spectral Microscope Imaging System, Germany).

Analytical methods

The dry weight of granules, volatile suspended solids (VSS), sludge volume index (SVI), and nitrate was measured according to Standard Methods (APHA 1998). The granules were sampled, washed with distilled water, and inoculated in 21 serum bottles (bottle volume, 200 ml) containing 100 ml synthetic wastewater supplemented with 400 mgl^{-1} acetate at different NO_2–N concentrations. The test bottles were sealed with a butyl rubber stopper and incubated at room temperature (25±2ºC). At fixed time

Table 1 The probes used in this study

Probe	Sequence (5′–3′)	rRNA target site	Specificity	Formamide (%)	Reference
EUB338	GCTGCCTCCCGTAGGAGT	16S, 338–355	Most bacteria	20	Amann et al. 1990
DEN220	GGCCGCTCCGTCCGC	16S, 220–234	Acetate-denitrifying cluster and some members of the *Comamonadaceae* family	40	Ginige et al. 2005
DEN220a	TCGGCCGCTCCGGAAGC	16S, 220–236	Acetate-denitrifying cluster and some members of the *Rhodocyclaceae* family	45	Ginige et al. 2005
DEN581	TGTCTTACTAAACCGCCTGC	16S, 581–600	Acetate-denitrifying cluster	45	Ginige et al. 2005
DEN1454	CCGTGGCAATCGCCCCCC	16S, 1454–1471	Acetate-denitrifying cluster	40	Ginige et al. 2005
AT1458	GAATCTCACCGTGGTAAGCGC	16S, 1458–1478	*Azoarcus–Thauera* cluster in the *Betaproteobacteria*	50	Rabus et al. 1999

interval, concentration of NO_2–N in filtered samples was determined by high-performance liquid chromatography (Ecom LCP 4100 Pump, C18 column, LCD 2083) with 10 mM NaH_2PO_4 in acetonitrile/water (150:850 v/v) as described by Tsikas et al. (1999). Similarly, experiments were performed with different acetate concentrations. The size of sludge granules was measured by a laser particle size analysis system (Mastersizer Series 2600; Malvern, Instruments, Worcestershire, UK) or an image analysis system. Washed granules were prepared for scanning electron microscopic (SEM; Jeol JSM-5310, Tokyo, Japan) observation via fixing with 2.5% glutaraldehyde for 2 h and dehydration via successive passages through 30%, 50%, 75%, 85%, 90%, 95%, and 100% ethanol followed by critical drying in critical point dryer (HCP-2, Hitachi Co. Ltd., Tokyo, Japan).

Results

Reactor performance and granules characteristics

During the first week of reactor operation, most biomass was floc-like and small aggregates approximately 400–600 μm in size emerged in second week. With the beginning of sludge granulation, the COD removal rate increased from 60% to 75–80% in the test reactors. The granule size, SVI (Fig. 1) and settling velocity increased with reactor operation period and remain stable. Further reactor operation for 90 days, the COD removal rate ranged between 95.8% and 96.5%.The matured sludge granules had smooth surfaces without surface filaments and distinct-shaped various cell morphotypes, including rods and cocci (Fig. 2). At the end of first month of rector operation, COD removal rate was 95.5%. The settling velocity of the granule was 47.2±7.3 mh^{-1}.

The batch test of the granules showed nitrification–denitrification potential. At the initial concentration of 300 mg l^{-1} NO_2^-–N and the supplement of 1,200 mg l^{-1} acetate, NO_2^-–N removal rate was 2.35±0.05 mg NO_2^-–N g^{-1} VSS h^{-1}. While the corresponding value for NO_2^-–N removal at 400 mg l^{-1} acetate supplement was 1.18± 0.02 mg NO_2^-–N g^{-1} VSS h^{-1} (Fig. 3). The granules could denitrify 600 mg NO_2^-–N in 105 h when supplied with acetate 1,200 mgl^{-1}. In denitrification test with the initial concentration of 100–700 mgl^{-1} NO_3^-–N, complete deni-trification without accumulation of nitrite was achieved (data not shown).

Bacterial diversity in aerobic sludge granule

The matured granules of size 3.5–5.0 mm from the SBR reactor that exhibited >95% COD removal rate at OLR 19.5 kg m^{-3}day^{-1} were used to analyze the microbial diversity. In total, 102 randomly selected bacterial clones
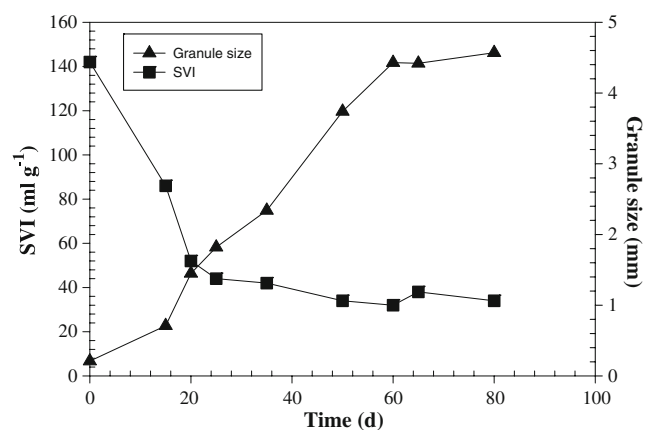

Fig. 1 Granule mean size and mixed liquor SVI values during granulation process. Sequencing batch reactor
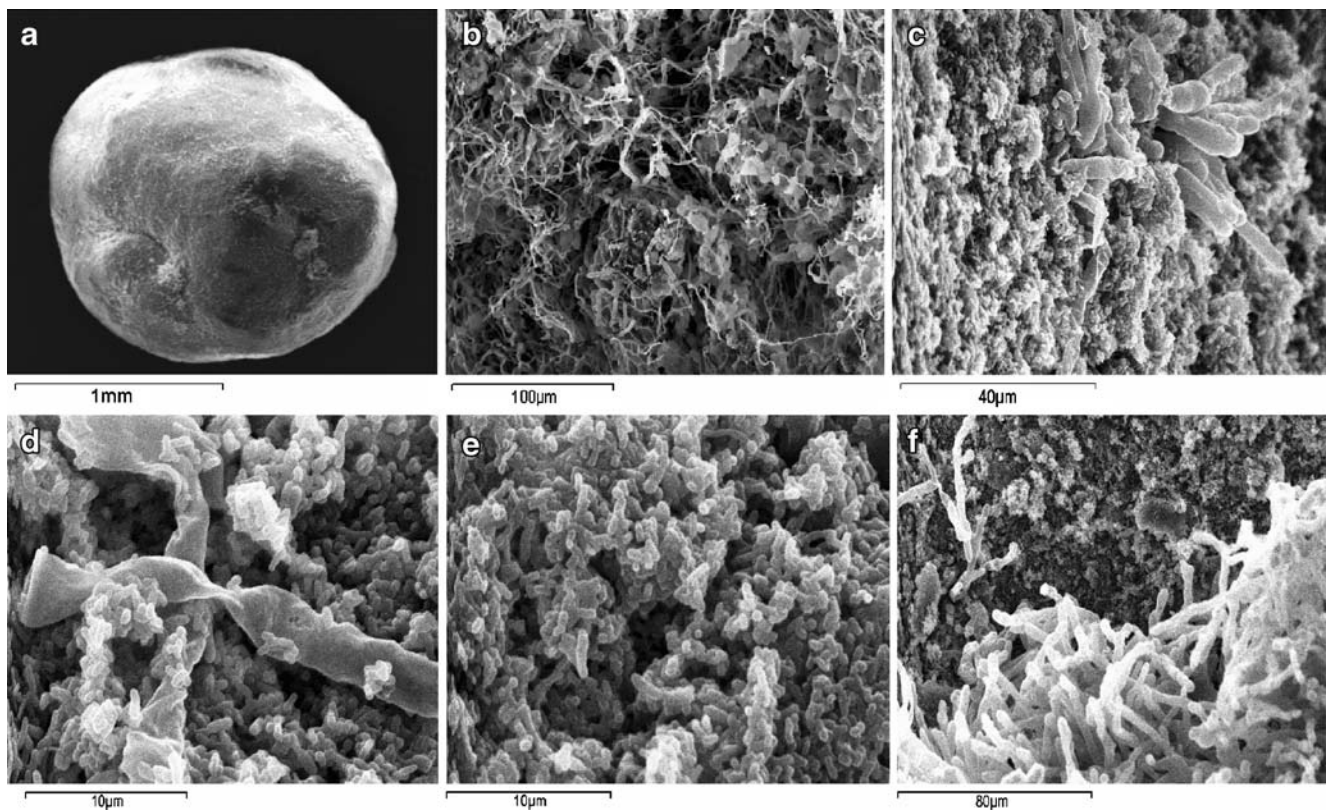
Fig. 2 Scanning electron micrograph of sludge aerobic granule (a) and different cell morphotypes (b–f)

from bacterial 16S rRNA gene clone libraries were analyzed. The compositions of the bacterial 16S rRNA gene libraries for the aerobic granules showed many sequences with high sequence similarity (97–99%) to sequences of uncultivated microorganisms. The microbial community composition in aerobic sludge granules demonstrated dominancy of *Proteobacteria* and exhibited 21 phylotypes. Of 102 clones, 66 clone sequences belong to phylum *Proteobacteria* (64.7%), 34 clones to *Bacteroidetes*

(33.3%), and only two clones affiliated to *Firmicutes* phyla (2.0%). The *Proteobacteria*-related clone sequences were further grouped into *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*.

Proteobcteria

Of the 53 clone sequences that related to the *Betaproteobacteria*, mostly belong to members of families that include
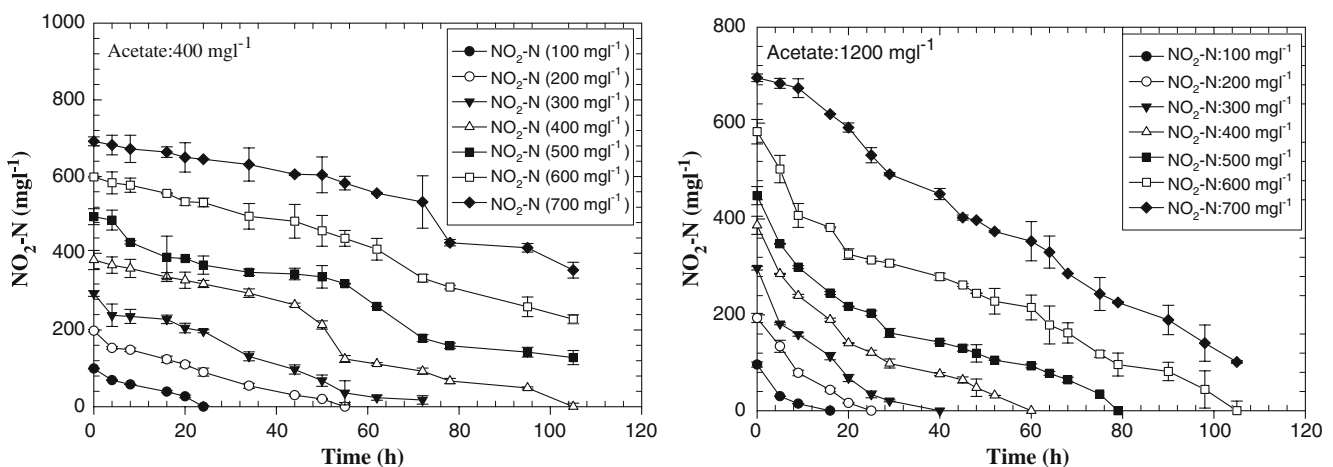

Fig. 3 Batch testing of denitrification by aerobic sludge granules under anoxic condition with acetate

denitrifying bacterial community, such as *Rhodocyclaceae* (one), *Comamonadaceae* (44), *Nitrosomonadaceae* (three), *Alcaligenaceae* (two), and *Incertae sedis* (two), while one sequence was unclassified *Betaproteobacteria*. The clone sequences (48) belonging to *Burkholderiales* showed dominancy of acetate-denitrifying clusters of members belonging to *Comamonadaceae* family (44), two clone sequences (G-23 and G-64) belong to genus *Pelomonas*; one each was affiliated to *Alcaligenes* (clone G-27) and *Castellaniella* genus (G-78). Phylotypes G-50 clustered within family *Rhodocyclaceae* (99% bootstrap) with *Azoarcus* sp. by 95% bootstrap. The clone sequences G-65, G-18, G-61, G-77, and G-58 clustered within *Comamonadaceae* family and were similar to *Comamonas* sp. The clone sequence G-14 affiliated with *Delftia acidovorans* (92%), while the other two clone sequences (G-64 and G-23) formed a separate sub-cluster with iron-reducing bacterium by 96% bootstrap value. Table 2 and Fig. 3 indicate the relative abundances of acetate utilizing members of the family *Comamonadaceae* in the acetate fed aerobic granule. Three clone sequences (G-10, G-67, and G-22) were closely related with members of family *Nitrosomonadaceae* and had 96% and 99% sequence similarity with *Nitrosomonas* sp. Phylotype G-78 clustered with the members of the family *Alcaligenaceae*.

The *Alphaproteobacteria* represents 2.9% of clone library. Three sequences belonged to *Alphaproteobacteria*

with 97–99% similarity to strains affiliated to family *Hyphomicrobiaceae*. Clone G-90 had sequence similarity of 99% with strain *Hyphomicrobium denitrificans* strain DSM 1869, while clone G-21 and G-26 exhibited 97% sequence similarity with *Devosia* sp.

Ten sequences were affiliated with the *Gammaproteobacteria*. Seven sequences were related to family *Pseudomonadaceae* (*Chryseomonas*, 14.3%; *Pseudomonas*, 71.4%; and unclassified *Pseudomonadaceae* genus, 14.3%); six clones were related to strain *Pseudomonas* and one clone to *Chryseomonas*; and three belonged to *Xanthomonadaceae* family. The clone sequence G-24 and G-11clustered within *Xanthomonadaceae* family, G-24 clustered with *Thermomonas* sp. by 98% bootstrap while G-11 with *Rhodonobacter* sp.

Non-proteobacteria

Of the total 102 clone sequences, 34 grouped within *Bacteroidetes* and two under *Firmicute*. The *Bacteroidetes* were further categorized under class *Flavobacteria* (two), *Bacteroidetes* (four), *Sphingobacteria* (27), and unclassified *Bacteroidetes* (one). The two clone sequences (G-38 and G-54) clustered within *Lactococcus* with 100% bootstrap value. The three phylotypes grouped within *Dysgonomonas gadei* group. The clone sequence G-103 clustered with *Cryomorphaceae* bacterium with 92% bootstrap. The analysis of the clone sequences belonging to *Sphingobac-*

Table 2 Clone library of 16S rRNA genes constructed from the aerobic sludge granules

| Phylum (95% confidence threshold)	Family	No. of clones	Best match in GenBank	Range of % similarity	GenBank accession no.
Firmicutes	*Streptococcaceae*	2	*Lactococcus* sp. YM05004	97–98	EU689105
Bacteroidetes	*Flavobacteriaceae*	1			
Bacteroidetes	*Cryomorphaceae*	1	*Chryseobacterium* sp. OS11	96	EF491961
Bacteroidetes	*Porphyromonadaceae*	4	Uncultured Dysgonomonas sp.	95–99	AM268127
Bacteroidetes	*Crenotrichaceae*	1	*Terrimonas ferruginea*; type strain: DSM 30193	94	AM230484
Bacteroidetes	*Sphingobacteriaceae*	1	*Pedobacter* sp. PhyCEm-147	96	AM921632
Bacteroidetes	*Flexibacteraceae*	2	*Leadbetterella byssophila* strain 4M15	98–99	AY854022
Bacteroidetes	*Saprospiraceae*	3	Uncultured Saprospiraceae bacterium clone Epr33	95–98	EU177727
Bacteroidetes	*Unclassified Sphingobacteriales*	13	Uncultured bacterium	95–98	FM174351
Proteobacteria	*Hyphomicrobiaceae*	3	*Devosia* sp. 64 V 16 S	97–97	EF540476
Proteobacteria	*Pseudomonadaceae*	7	*Pseudomonas fluorescens* SBW25	95–99	AM181176
Proteobacteria	*Xanthomonadaceae*	3	*Thermomonas fusca* strain R-10289	95–98	NR025577
Proteobacteria	*Rhodocyclaceae*	1	*Azoarcus* sp. XQ1	99	EU878233
Proteobacteria	*Nitrosomonadaceae*	3	*Nitrosomonas eutropha* C91	97–99	NR027566
Proteobacteria	*Alcaligenaceae*	2	*Alcaligenes* sp. F78	96–98	EU443097
Proteobacteria	*Comamonadaceae*	44	*Acidovorax* sp. C1 1	96–100	AJ457191
Proteobacteria	Incertae sedis	2	*Pelomonas saccharophila* strain del-2	97–99	FJ513082
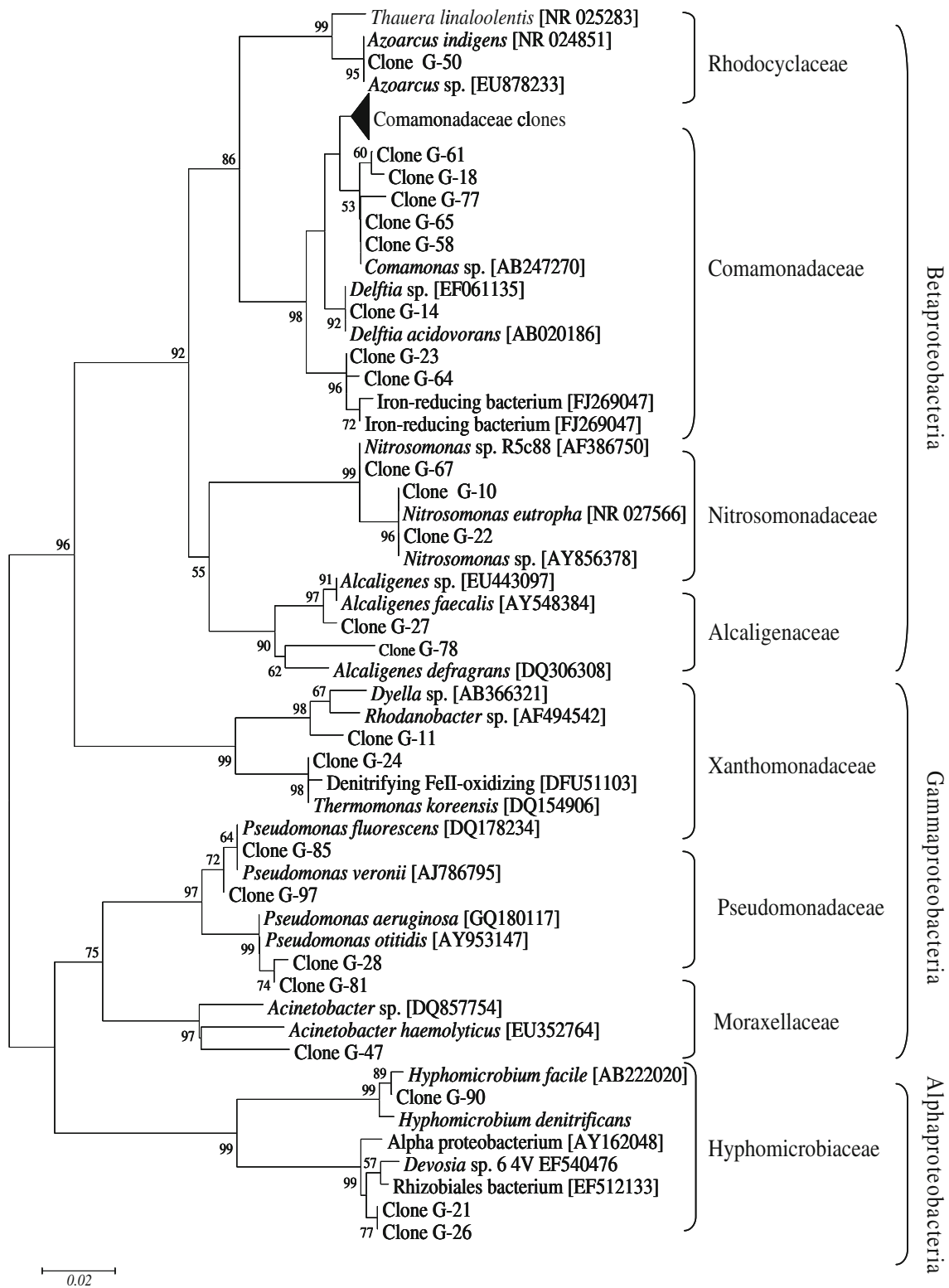
Fig. 4 Phylum Proteobacteria neighbor-joining phylogentic tree of 16S rRNA gene sequences. The sequences from the present study and close relatives were aligned by multiple alignments ClustalW. Bootstrap analyses were conducted on 1,000 samples, and percentage greater than 50% are indicated at the nodes. Scale bar, 0.02 changes per nucleotide position
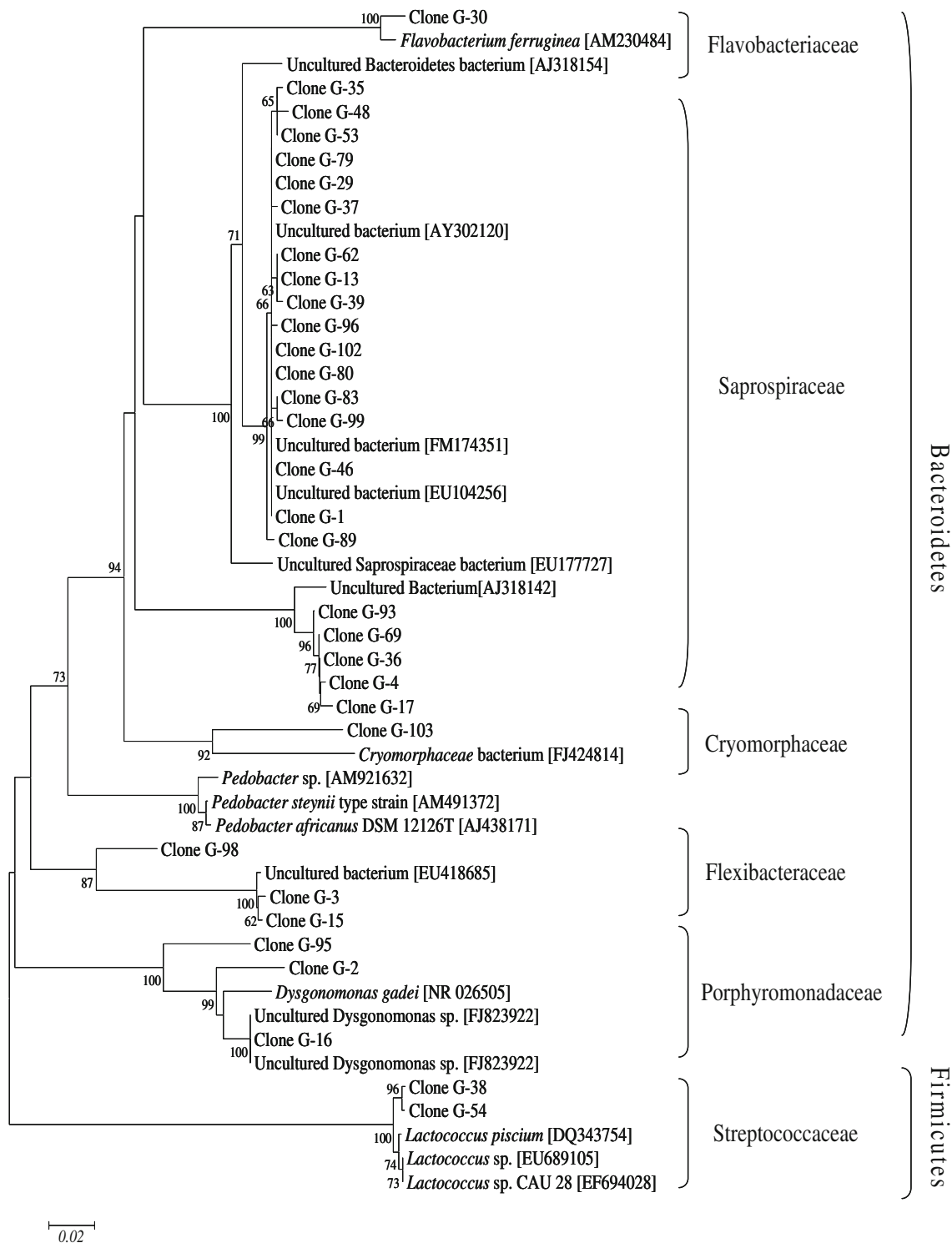
Fig. 5 Non-Proteobacteria neighbor-joining phylogentic tree of 16S rRNA gene sequences. The sequences from the present study and close relatives were aligned by multiple alignments ClustalW.

Bootstrap analyses were conducted on 1,000 samples, and percentage greater than 50% are indicated at the nodes. Scale bar, 0.02 changes per nucleotide position

teriales (27) revealed 3.7% *Crenotrichaceae*, 3.7% *Sphingobacteriaceae*, 7.4% *Flexibacteraceae*, and 74.1% *Saprospiraceae* family members, while others (11.1%) were unclassified (Table 2 and Fig. 4).

Spatial distribution of microorganisms in granules

FISH, using EUB and Cy3-labeled DEN581 probes, revealed that the outer layer of 250 µm (Fig. 5a) was

Fig. 6 CLSM image of in situ functional analyses of denitrifying bacteria in the SBR sludge granule. **a** Hybridized with both the EUB (*red*) and DEN581 (*yellow*) probe targeting acetate-denitrifying cluster, bar=200 μm; **b** hybridized with EUB (*red*) and DEN220 probe targeting *Comamonadacea*e family members(*yellow*), bar=300 μm; **c** granule from denitrification batch test under anoxic condition, dual-hybridized for total bacteria (*red*) and *Rhodocyclaceae* family members (*pink*), bar=75 μm; **d** granule from denitrification batch test under anoxic condition hybridized with EUB (*red*), probe DEN220 targeting *Comamonadaceae* family members (*yellow*), and probe DEN220a targeting *Rhodocyclaceae* family members (*pink*), bar=75 μm
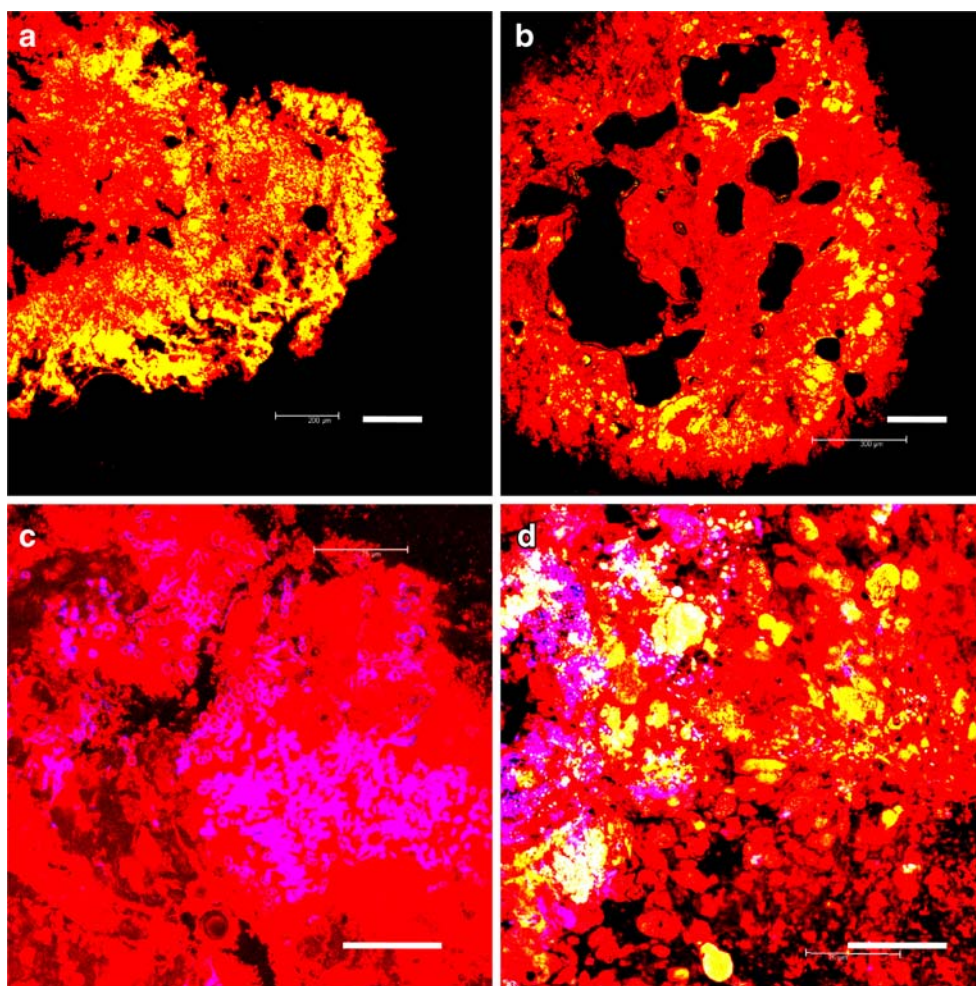
dominated by acetate-denitrifying bacterial community. The hybridization with probe DEB220 specific for acetate-denitrifying cluster and some members of the *Comamonadaceae* family demonstrated that members of *Comamonadaceae* family clustered within 100–200 μm granule depth (Fig. 5b). Some members of acetate-denitrifying community with *Rhodocyclaceae* and *Comamonadaceae* family members were clustered very close to each other at the granule interior within 100–200 μm granule depth (Fig. 5c, d). The layered structure of acetate-denitrifying cluster and members of *Rhodocyclaceae* and *Comamonadaceae* families was repeatedly observed in all the granular sections analyzed. The cells hybridized with probe AT1458, specific for *Azoarcus–Thauera*, were numerically important bacteria and were located in core region of the granule (Figs. 6 and 7).

Discussion

The abundance and dominancy of the denitrifying bacterial community in cultivated aerobic granules was

likely due to the denitrification activity within the granules at high dosed level of ammonium and sufficient electron donor for denitrification. Majority of non-proteobacterial clone sequences belong to *Bacteroidetes*,
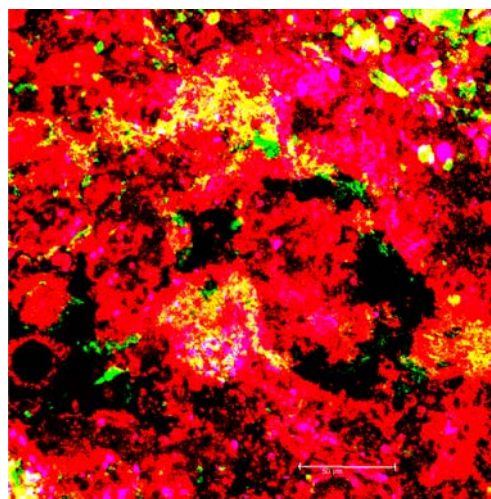

Fig. 7 FISH-CLSM image of granule from denitrification batch test under anoxic condition, hybridized with EUB (*red*), probe AT1458 (*pink*), and DEN 1454 (*yellow*), bar=75 μm

which are obligate anaerobes, indicating the presence of anoxic core in the granule (Adav et al. 2009). The majorities (64.7%) of the 16S rRNA gene sequences from clone analyzed were denitrifying microbial community within *Proteobacteria* phylum, and 51.9% were from the families *Comamonadaceae*. Denitrifying representatives of *Alpha-*, *Beta-*, and *Gammaproteobacteria*, *Firmicutes*, and *Bacteroidetes* were found in the sludge granules, and apart from genera known to harbor denitrifiers, such as *Pseudomonas*, *Comamonas*, and *Acidovorax*, genera less frequently observed in cultivation studies of denitrifiers were encountered prominently in this study. The major strains assigned for the clones in *Comamonadaceae* family were *Comamonas*, *Alicycliphilus*, *Diaphorobacter*, and *Delftia.* Similarly, Kim et al. (2006) reported members of *Acidovorax*, *Alicycliphilus*, *Comamonas*, and *Diaphorobacter* strain in denitrifying activated sludge, while Park et al. (2005) reported denitrifying *Thauera* sp. with dominancy of *Hydrogenophaga* sp. in autohydrogenotrophic biofilm reactor. The other clone sequences within protobacteria phylum were affiliated with the members of families *Nitrosomonadaceae* under *Betaproteobacteria* and *Hyphomicrobiaceae*, *Pseudomonadaceae*, and *Xanthomonadaceae.* A significant positive correlation between the SBR denitrification rates and the abundance of the denitrifiers was demonstrated. According to Otlanabo (1993), various species of *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Chromobacterium*, *Flavobacterium*, *Hyphomicrobium*, *Pseudomonas*, and *Vibrio* are responsible for denitrification.

Researchers attempted to identify denitrifiers in activated sludge (Drysdale et al. 2001; Etchebehere et al. 2001, 2002; Khan and Hiraishi 2002; Khan et al. 2002; Ginige et al. 2005). Our finding and that of Ginige et al. (2005) suggested that the members of the *Comamonadaceae* family play a major role in denitrification processes in the presence of acetate. The present study also identified members of *Nitrosomonadaceae* and *Alcaligenaceae* within *Betaproteobacteria*, which was not reported in related works. Members of denitrifying Fe(II) oxidizers that belong to *Xanthomonadaceae* and denitrifying *Pseudomonas* sp. of *Pseudomonadaceae* family were identified in the present study that may be attributable to the strong binding strength of EPS matrix of aerobic granules. The present study also identified members of families *Flavobacteriaceae*, *Porphyromonadaceae*, *Flexibacteraceae*, and *Saprospiraceae* within *Bacteroidetes* phylum that also involved in the processes of denitrification (Mills et al. 2008). The clone sequences belonging to *Azoarcus* were identified in aerobic sludge granule, corresponding to the works by Wagner and Loy (2002) on activated sludge.

The clone analysis from our acetate fed SBR differs from the reactor fed with methanol (Ginige et al. 2009).

The denitrifying microbial community depends on the fed major carbon sources and electron donor. The acetate can be degraded by microbes using tricarboxylic acid cycle and is one of the best carbon sources for denitrification (Grabinskaloniewska 1991). The granules cultivated at high OLR are composed of diverse denitrifying microbial community and are able to degrade high-level nitrogenous compound from wastewaters.

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