Microbiological and structural aspects of granular sludge from autotrophic denitrifying reactors

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ABSTRACT

Denitrification is applied in the tertiary treatment of wastewater to reduce N-pollutants. Fluorescence *in situ* hybridisation (FISH), CARD (catalyzed reporter deposition)-FISH, cloning, and scanning electron microscopy (SEM) were applied to follow the evolution of the microbial composition and structure of granular sludge in autotrophic denitrifying bioreactors fed with nitrate and thiosulfate. With this goal, FISH oligonucleotide probes for the autotrophic denitrifiers, *Thiobacillus denitrificans* and *Thiomicrospira denitrificans*, were designed and their utility tested. CARD-FISH and cloning data showed that bacterial diversity changed with bioreactor operation time. After 110 days of operation, the abundance of Thiobacillus denitrificans cells increased considerably: from 1 to 35% of total DAPI-stained cells and from no isolated clones to 30% of the total positives clones. This fact strongly suggests that this microorganism played a dominant role in the autotrophic denitrification. The Archaeal diversity remained almost unchanged and it was mainly represented by *Methanosaeta soehngenii*. Scanning electron microscopy results indicated a considerable loss in the integrity of the sludge granules during the operation, with risk of sludge buoyancy.

KEYWORDS:

Autotrophic denitrification; denitrifying bacteria; granular sludge; molecular ecology;

Thiobacillus denitrificans; Thiomicrospira denitrificans

INTRODUCTION

Denitrification is a microbial redox process by which nitrate is transformed into benign dinitrogen gas (N_2). Denitrifying microorganisms can utilize organic or inorganic compounds (i.e. hydrogen or reduced sulfur compounds) as electron-donors. Heterotrophic denitrifiers are widespread and phylogenetically diverse. In contrast, few microorganisms appear to be capable of coupling the reduction of nitrate to N_2 gas to the oxidation of reduced sulfur compounds to sulfate. Among the obligate autotrophs (i.e. use CO_2 as carbon source) only two species that grow at neutral pH have been described, *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* (Schedel and Truper, 1980; Timmer-Ten Hoor, 1981; Kuenen et al., 1992).

Heterotrophic denitrification (also called nitrate respiration) is widely applied in wastewater treatment (Green et al., 1995) and other water treatment technologies (Robertson and Anderson, 1999). In contrast, autotrophic denitrification is still underdeveloped for fullscale application. Significant advantages of autotrophic denitrification, such as its ability to attain simultaneous removal of nitrate and reduced sulfur compounds (e.g. H_2S), and the fact that the process does not require supplementation of organic substrates (e.g. methanol, glucose) as carbon and energy source, suggest that the process could be attractive for application in the removal of nitrate from groundwater (Wang and Qun, 2003) and in the treatment of nitrogen and sulfur contamination in effluents poor in organic matter (e.g. petrochemical wastewaters) (Nugroho et al., 2002; Reyes-Avila et al., 2004; Sierra-Alvarez et al., 2005), among others.

Knowledge of the structure of microbial communities in bioreactor biomass is extremely important to understand any biotechnological process and improve the operation parameters. Microbiologists have used classical culture-based techniques to achieve this goal. However, culture-dependent techniques have many shortcomings and, as a consequence, less than 1% of the microbial diversity has been characterized (Amann et al., 1995). Culturing-independent molecular biology techniques based on 16S rRNA/rDNA such as fluorescence *in situ* hybridisation (FISH) and denaturing gradient gel electrophoresis (DGGE) have become essential tools to study structure and biodiversity of both natural and engineered complex microbial ecosystems (Muyzer et al., 1996; Amann et al., 2001; Wagner et al., 2003).

In the present work, we have applied CARD (catalyzed reporter deposition)-FISH and cloning, together with scanning electron microscopy (SEM) to follow the evolution of the microbial composition and structure of granular sludge in laboratory scale autotrophic denitrifying bioreactors. Given that oligonucleotide probes targeting autotrophic denitrifying microorganisms are not currently available, specific probes to detect *Thiomicrospira denitrificans* and *Thiobacillus denitrificans* were designed in this study.

METHODS

Biomass and scanning electron microscopy.

Biomass samples were obtained at different times of operation from two different denitrifying bioreactors operated under autotrophic conditions. The electron donor for both reactors (R1 and R2) was thiosulfate ($S_2O_3^{2-}$). R1 and R2 were inoculated with methanogenic granular sludge obtained from a full-scale upflow anaerobic sludge bed reactor (Eerbeek, The Netherlands). R2 was also supplied with granular activated carbon. Sludge granules were studied by SEM (Alphenaar et al., 1994). DNA was extracted using FastDNA Spin kit for Soil (BIO101 Inc, Q-Biogene) according to the manufacturer's protocol.

Clone libraries.

The 16S rRNA genes from mixed microbial DNA were amplified by PCR. To obtain almost complete 16S rRNA gene sequences, two oligonucleotide primer pairs were used: 27F and 1492R (annealing T: 56 ºC) for the Bacteria domain and 25F and 1492R (annealing T: 52 ºC) for the Archaea domain (Lane, 1991). The thermal profile for amplification included 10 min at 94 ºC for denaturing, 30 cycles of elongation (94 ºC for 1 min, primer annealing at specific temperature for 1 min, and 3 min at 72 ºC of elongation), and, finally, 10 min at 72 ºC to complete the elongation. The 16S rRNA gene amplificates (length 1465–1467 bp) were cloned using TOPO Cloning Kit (Invitrogen, San Diego, CA) and then transformed into competent *E. coli* cells. Subsequently, representatives of each group were amplified by PCR, using the M13 primer set (Invitrogen), and sequenced. Sequences were compared with the NCBI databases by using the Basic Local Alignment Search Tool (BLAST) program to identify the closest sequence.

FISH and CARD-FISH.

Oligonucleotide probes design (ARB program, package available at http://www.mikro.biologie.tu-muenchen.de/; Fuchs et al., 1998), FISH (Amann et al., 1995, 2001), CARD-FISH (Pernthaler et al., 2002) and cell counts were performed as previously described. The probes EUB338, ARC915, NON338, ALF968, BET42a, GAM42a, SRB385, LGC354a, MEB859, MSSH859, MC1109, MG1200 (described elsewhere) and TBD121 and TMD131 (this work) were tested.

RESULTS AND DISCUSSION

Oligonucleotide design

Specific oligonucleotide probes for FISH were designed using the ARB package. TBD121 (5'-CTCGGTACGTTCCGACGC-3'), a probe targeting both *Tb. denitrificans* and *Tb. thioparus*, was chosen due to satisfactory brightness in FISH. Another probe, TMD131 (5'-TCCCAGTCTTTGAGGTAC-3'), was developed for hybridising with *Tm. denitrificans*. The hybridisation conditions of all probes were optimized using pure cultures of the target microorganisms.

FISH and CARD-FISH

The biomass samples were analyzed by FISH and CARD-FISH with the aim to compare both techniques. The higher signal intensity in the hybridisation using CARD-FISH allowed a better cell detection and quantification by means of image analysis. Few changes in the microbial composition of the sludge in the two reactors were detected in samples obtained at different times of operation. The microbiota of both reactors was found to be very similar. Bacteria was the most abundant Domain (40–60% of the total DAPI-stained cells). γ-*Proteobacteria* (25–45%), in combination with α- and β-*Proteobacteria* (less than 10% each), represented the Bacteria almost in its entirety. It is noteworthy that in the methanogenic granular sludge used as inoculum, α-*Proteobacteria* was the predominant group, with only 1% of γ-*Proteobacteria*.

The specific oligonucleotides developed in this study showed the presence of both autotrophic denitrifies. *Tb. denitrificans* was the dominant species, whereas *Tm. Denitrificans* only represented a minor fraction of the microorganisms (2–3%). Phylogenetically, Thiobacillus is

grouped in the β-*Proteobacteria*. Surprisingly, the number of *Tb. denitrificans* cells detected was very similar to the number of γ-*Proteobacteria* cells. Further tests with pure cultures showed that *Tb. denitrificans* could hybridise with the γ-*Proteobacteria* probe but not with the β-*Proteobacteria* probe. This fact can explain the high relative abundance of cells in the γ-*Proteobacteria* class. In the methanogenic inoculum, *Tb. denitrificans* was detected occasionally but after 110 days of operation its number increased significantly, from 1% to 35% of the total DAPI-stained cells. These results suggest that *Tb. denitrificans* was enriched during the operation of the bioreactors and that this microorganism probably played a dominant role in the autotrophic denitrification.

Microorganisms in the *Archaea* domain were less abundant and only represented about 10% of the total DAPI-stained cells, both in the inoculum and in the biomass of the autotrophic reactors. Only cells from the orders *Methanobacteriales* (1% of the total DAPI-stained cells) and *Methanosarcinales* (the number significantly decreased from the inoculum to the autotrophic reactors) were found. The unspecific hybridisation of the *Thiobacillus* cells with the Arc915 probe (González-Toril, personal comm.) could explain the difference between the numbers of cells hybridised with the *Archaea* probe and the *Methanobacteriales* plus *Methanosarcinales* probes.

Cloning

Table 1 summarizes the phylogenetic affiliations of 16S clones corresponding to the inoculum and to biomass obtained at the end of the operation of R1. The Gram-positive phyla, *Firmicutes* and *Actinobacteria*, were dominant in the inoculum. Clones from the phylum *Bacteroidete* (clones E-J-36, E-G-12 and E-Q-101) resemble clone populations found at sites contaminated with chlorinated solvents. All genera detected include strict or facultative fermentative anaerobes which is in agreement with the source of the microbial culture, an anaerobic reactor.

Analysis of the R1 clone library showed that although the diversity of Gram-positive microorganisms was maintained, species most closely related to the *Proteobacteria* became dominant. A considerable number of clones (42%) corresponded to former “Pseudomonas”, currently known as *Burkholderiales*, *Xhantomonadales* and *Pseudomonadales*, which are all microbes well known for their ability to degrade xenobiotic compounds. The only autotrophic

denitrifier was *Thiobacillus denitrificans*, which was not detected in the inoculum. Although cloning is not a quantitative technique, it is interesting to note that the percent *Tb. denitrificans* clones in the 16S clone library (30%) corresponded closely to the relative abundance of this bacterium as determined by CARD-FISH (35%). For the *Archaea* domain, 85 clones were obtained which after restriction digestion with the enzyme Sau3A1 could be grouped into 7 different restriction patterns. Surprinsingly, all the clone sequences were closely related (97–99% similarity) to a single methanogenic species, *Methanosaeta soehngenii*. In the autotrophic reactors, the methanogens must be likely resting forms from the inoculum (methanogenic sludge).

SEM

The structure of representative granules from the inoculum and sludge obtained from both reactors at the end of the continuous experiment was studied by SEM. Figures 1A and B show that the methanogenic inoculum had a compact structure with a fluffy surface. With time, the structure of granules from the denitrifying bioreactors became looser, showing increased voids (Figures 1C–F). Some granules were hollow (Figures 1F and C detail). Possibly the growth of microorganisms was restricted to the outer side, giving the granule surface a continuous, dense, smooth aspect (Figure 1G). Channels perpendicular to the surface were observed (Figures 1C and D). These effects could cause operation problems in the reactor by increasing the buoyancy of the sludge, which may result in sludge flotation and/or wash-out problems.

CONCLUSIONS

FISH oligonucleotide probes for the autotrophic denitrifiers *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* were designed and their utility was tested. Cloning and CARD-FISH data showed that bacterial diversity in the two autotrophic denitrifying bioreactors operated with thiosulfate as electron donating substrate and bicarbonate as carbon source changed during the first period of operation and later became nearly constant. Bacterial sequences most closely related to Gram-positives were dominant in the sludge used as inoculum. α-, β- and γ-*Proteobacteria* were dominant in the mature autotrophic reactor. The archaeal diversity remained almost unchanged and was represented by *Methanosaeta soehngenii*.

241 *Thiobacillus denitrificans* appeared to be the dominant denitrifier in the two autotrophic
242 bioreactors examined in this study based on FISH-CARD cell counts and high abundance in the
243 16S clone library. Some microbial granules became less dense and lost their integrity and
244 structure during the operation, turning into a shell probably due to the superficial growth of
245 microorganisms.
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329 **TABLES**

330 **Table 1** 16S rDNA sequence-based phylogenetic affiliations of the Bacteria clones

Clone	Phylum/class/order	Closest species or taxon (NCBI access no.)	Similarity (%)	Abundance (%)
Inoculum (methanogenic sludge				
E-N-108	δ-*Proteobacteria*	*Desulfovibrio alcoholovorans*	97	2.8
E-E-50	*Firmicutes*	Uncultured anaerobic reactor AY426453	98	5.6
	Clostridiales	*Acidaminococcus fermentans*	93	
E-P-33	*Firmicutes*	Uncultured granular sludge AY426451	98	2.8
E-B-76	*Actinobacteria*	Uncultured anaerobic reactor AB195904	99	39.1
	Actinomycetales	*Propionibacterium*	92	
E-I-30	*Actinobacteria*	*Cellulomonas fermentans*	99	8.4
	Actinomycetales			
E-M-93	*Actinobacteria*	Uncultured granular sludge AY426443	98	2.8
	Actinomycetales	Uncultured *Rubrobacteridae* AY395379		
E-D-59	*Chloroflexi*	Uncultured anaer. consortium AJ009475	95	5.6
		Uncultured AY304372	94	
E-J-36	*Bacteroidete*	Uncultured from aquifer AF050541	100	22.3
E-G-12	*Sphingobacteriales*	*Cytophaga* AB015525	94-97	8.4
Reactor biofilm (alter 186 days of operation)				
R1C-M-20	α-*Proteobacteria*	*Ochrobactrum*	99	9
	Rhizobiales	*(O. tritici)*		
R1C-A-5	β-*Proteobacteria*	*Thiobacillus denitrificans*	94-98	30
R1C-C-27	*Hydrogenophilales*			
R1C-E-91				
R1C-G-86	β-*Proteobacteria,*	*Comamonas*	97	6
	Burkholderiales			
R1C-L-1	γ-*Proteobacteria*	*Rhodanobacter*	95-98	30
R1C-D-73	*Xhantomonadales*	(*R. lindaniclasticus*)		
R1C-B-96				
R1C-J-90	γ-*Proteobacteria*,	*Pseudomonas*	95	6
	Pseudomonadales			
R1C-H-6	*Firmicutes*,	*Clostridium*	95	6
	Clostridiales			
R1C-K-79	*Firmicutes, Bacillales*	*Exiguobacterium (E. aestuari)*	98	3
R1C-P-24	*Firmicutes*	Uncultured anaer. digestor UEU81756	98	3
	Lactobacillales	*Alkalibacterium*	91	
R1C-O-10	*Cloroflexi*	Uncultured AY921924	97	3
	Anaerolinaeles	*Caldilinea*	93	
R1C-N-108		Uncultured intestinal microflora AY916338	97	3
		Uncultured bioreactor AY050603	97	

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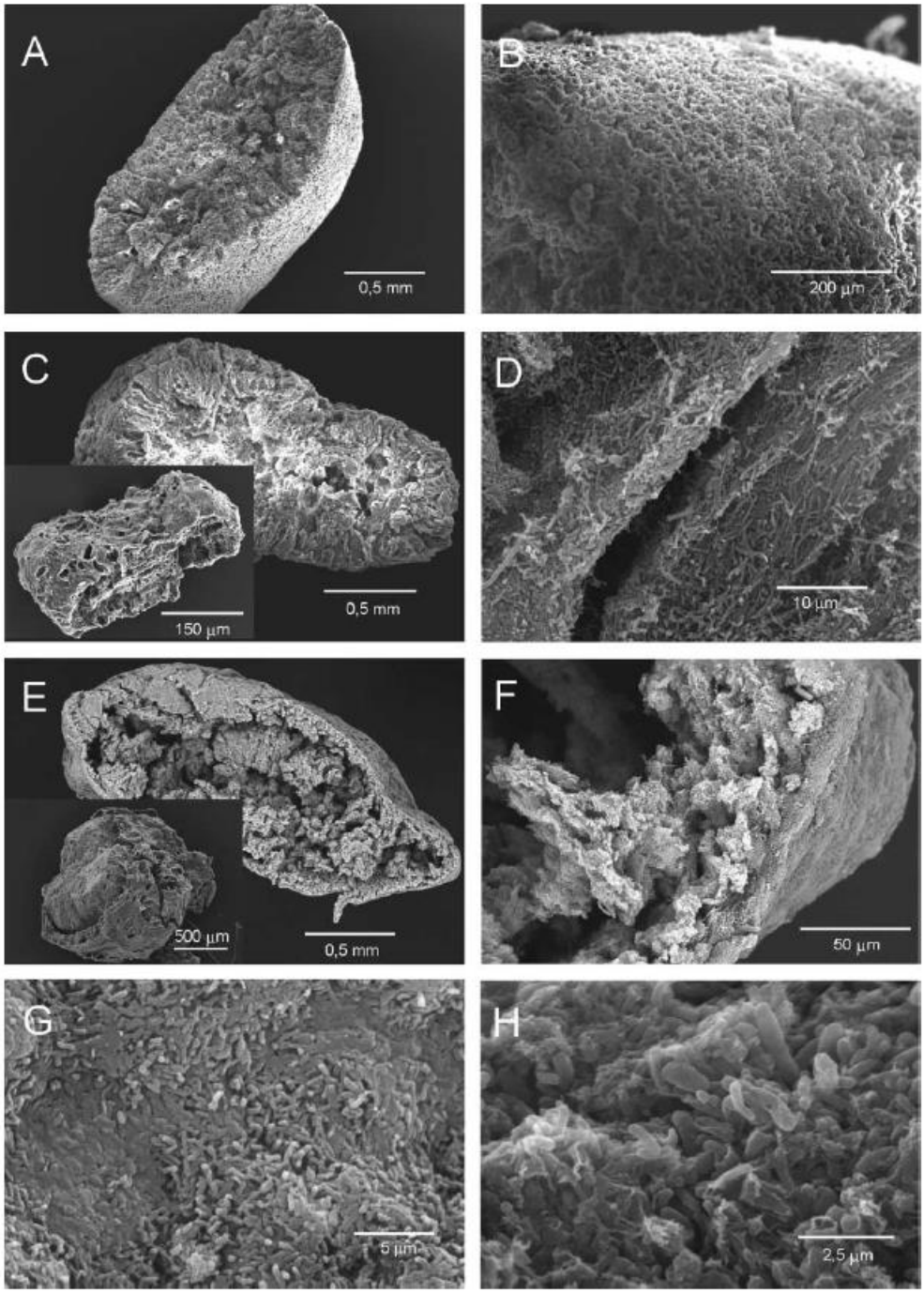
FIGURE LEGENDS

Figure 1 Scanning electron microphotographs of granules. A: Methanogenic granular sludge used as inoculum; B: Detail of the surface of the inoculum; C: Granule from the autotrophic denitrifying reactor R1 after 186 d of operation. Detail in the bottom-left corner shows a hollow granule; D: Detail of the radial structures formed in the granule showed in C; E: Granule from the autotrophic denitrifying reactor R2 at the end of operation; F: Outer shell of these granules; G and H: Details of the surface and inside of granules from R2. Morphologies resembling *Thiobacillus* can be observed. Note the densely packed biofilm on the surface and the heterogeneity inside the granule. Detail in E shows a sludge granule from R2 established on an active carbon particle

373 **FIGURES**

374 **Figure 1**



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