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Microbial community and biochemistry process in autosulfurotrophic denitrifying biofilm

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Abstract

The 16S rDNA-based molecular technique was applied to analyze the microbial community of autotrophic denitrification bacteria in a biofilm developed on the surface of sulfur particles and then the biochemistry process involved in this biofilm was discussed based on the microbial community analysis. Six key operational taxonomy units were identified, which were all unknown species belonging to a wide range of bacteria from four major subdivisions (α, β, γ and δ) of the kingdom *Proteobacteria* and from the kingdom *Chlorobia* (green sulfur bacteria). One species was chemoautotrophic and related to *Thiobacillus denitrificans*, two species were photoautotrophic, and three were chemoheterotrophic. Contrary to expectation, *T. denitrificans*-like bacteria constituted only 32% of the microbial community. As a result of the study, the entire microbiology of the autosulfurotrophic denitrification process as well as the interactions between the different microbial groups in the biofilm may need to be reconsidered.

Keywords: Autotrophic denitrification; Biofilm; Nitrogen cycle; Sulfur; *Thiobacillus*; Wastewater

1. Introduction

A wide variety of bacteria can use nitrate as a terminal electron acceptor. These bacteria are called nitrate-reducing bacteria, which includes heterotrophs using organic compounds as electron donors and autotrophs using inorganic compounds such as sulfide, sulfur, thiosulfate or ferrous iron as electron donors. Products of nitrate reduction include NO_2^-, NO, N_2O, N_2, and NH_4^+. When N_2 is produced, the process is called denitrification.

Biological denitrification has become an integral part of modern wastewater treatment. For wastewater with a low BOD_5/N ratio, autotrophic denitrification is an interesting alternative to heterotrophic denitrification. Autotrophic denitrifying bacteria (ADB) include autohydrogenotrophic denitrifiers (Lee and Rittmann, 2000) as well as autosulfurotrophic denitrifiers, which reduce sulfur compounds, such as elemental sulfur or thiosulfate, to sulfate while reducing nitrate to elemental nitrogen gas. Contrary to heterotrophic denitrification, autotrophic denitrification eliminates the need for addition of organic carbon sources, consumes alkalinity and, in addition, generates high concentrations of sulfate.

Autotrophic denitrification using elemental sulfur has been investigated to remove nitrate from polluted groundwater (Kruithof et al., 1988) and some selected

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wastewaters with high nitrate concentrations such as nitrified leachate (Koenig and Liu, 1996, 2001, 2002). For *Thiobacillus denitrificans*, the stoichiometric reaction of autotrophic denitrification using elemental sulfur can be represented by the following equation (Batchelor and Lawrence, 1978):

$$NO_3^- + 1.10S + 0.40CO_2 + 0.76H_2O + 0.08NH_4^+ \xrightarrow{Thiobacillus\ denitrificans} 0.5N_2 + 1.10SO_4^{2-} + 1.28H^+ + 0.08C_5H_7O_2N \quad (1)$$

Although autotrophic denitrification using elemental sulfur has been extensively studied since 1978 (Batchelor and Lawrence, 1978), knowledge about the microbial community in the biofilm on the sulfur particle surface is still very limited. It is generally assumed that *T. denitrificans* is the microbial species responsible for autotrophic denitrification (Batchelor and Lawrence, 1978). This bacterium can reduce nitrate to nitrogen gas while oxidising elemental sulfur or reduced sulfur compounds to sulfate. Other autotrophic denitrifiers in addition to *T. denitrificans*, such as *Thiomicrospira denitrificans,* could also perform the sulfur-based autotrophic denitrification. However, no detailed analyses on the autotrophic denitrification microbial community have been reported.

Traditionally, microbes are identified by isolating individual cultures and examining their physiological, biochemical and morphological characteristics. These methods are often unreliable, because some microbes are syntrophically associated with others and thus cannot be isolated and cultured individually (Wagner et al., 1993). Furthermore, many microbes share similar physiological, biochemical and morphological characteristics, and cannot be distinguished from one another based on these characteristics. However, microbial communities may be analyzed using advanced molecular techniques. Among them, 16S rDNA-based methods have been extensively applied in various studies (Zhang and Fang, 2001; Fang et al., 2004).

In this study, the 16S rDNA-based method was applied to analyze the microbial community of the ADB in a biofilm developed on the surface of sulfur particles.

2. Materials and methods

2.1. Reactor operation

The sulfur particles were obtained from batch experiments carried out to study the use of limestone for pH control in autotrophic denitrification (Liu and Koenig, 2002). In these experiments, 150 ml of synthetic wastewater (100 mg l^{-1} NO_3^--N, 1000 mg l^{-1} $NaHCO_3$, 120 mg l^{-1} K_2HPO_4, 12 mg l^{-1} NH_4Cl, 2 mg l^{-1} $MgCl_2 \cdot 6H_2O$ and 0.1 mg l^{-1} $FeSO_4 \cdot 7H_2O$) were incubated in 250 ml Erlenmeyer flasks at 25 °C. Each flask contained a total of 200 ml elemental sulfur and limestone (2.8–5.6 mm diameter) at a volumetric ratio between 1:0 and 1:4. The batch experiments lasted about 5 h each, with the specific denitrification rate in all batch experiments amounting to approximately 0.030 mg NO_3^--N g^{-1} S h^{-1}, independent of the sulfur to limestone ratio. The initial cultures were prepared from sludge collected from tidal flats of the Mai Po marshes in Hong Kong. The steps for forming the biofilm on the sulfur particles were as follows: the reactors were inoculated with 50 ml enrichment solution and 100 ml medium according to the enrichment method for *T. denitrificans* as previously described (Koenig and Liu, 1996). Every 8 h, 120 ml solution in the reactors was decanted and 120 ml fresh medium was added. After 10 d incubation, the medium was changed to synthetic wastewater. After a further two weeks of incubation, the denitrification rate became stable and the batch experiments were started.

2.2. DNA extraction and amplification

The microstructure of the biofilm was examined using scanning electron microscopy (SEM, Stereoscan 360, Cambridge, USA). Biofilm was removed from the surface of the sulfur particles by sonification in phosphate buffer saline (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.4). Then the genomic DNA in the biofilm was extracted (Zhang and Fang, 2001). For denaturing gradient gel electrophoresis (DGGE) screening, the extracted DNA was amplified by polymerase chain reaction (PCR) using an automated thermal cycler (GeneAmp® PCR 9700, Perkin-Elmer, Foster City, CA) as follows: an initial denaturation at 94 °C for 7 min; 35 cycles of denaturation (1 min at 92 °C), annealing (1 min at 54 °C) and extension (1 min at 72 °C); a final extension at 72 °C for 10 min and then stored at 4 °C. The same procedures were also applied for DNA cloning, but only 25 cycles of denaturation, annealing and extension were carried out. All PCR amplifications were conducted in 30 μl of a pH 8.3 buffer (Pharmacia Biotech Inc., Piscataway, NJ) containing 200 μM each of the four deoxynucleotide triphosphates, 15 mM $MgCl_2$, 0.1 μM of individual primers and 1U of *Taq* polymerase (Pharmacia Biotech Inc. Piscataway, NJ). The purity of PCR products were checked by electrophoresis profile using 1% agarose gel.

2.3. Cloning and sequencing

The extracted DNA was PCR-amplified using the primer set of EUB8F and EUB1509R (Table 1) (Zhang et al., 2003). Cloning of these fragments was conducted using the TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA). A total of 72 colonies were selected for inoculation in a 1.0 ml LB medium containing 50 mg l^{-1}

Table 1
DNA sequences of primers and GC-clamp

Primer	Sequence	Specificity	Target position	Reference
EUB8F	5′-AGA GTT TGA TCM TGG CTC AG	Bacteria	8–27	Lane (1991)
EUB1509R	5′-GGT TAC CTT GTT ACG ACT T	Prokaryote	1491–1509	Lane (1991)
M13F	5′-GTA AAA CGA CGG CCA G	pCR® 2.1Vector	–	Invitrogen (1999)
M13R	5′-CAG GAA ACA GCT ATG AC	pCR® 2.1Vector	–	Invitrogen (1999)

kanamycin. After 18 h incubation at 37 °C, the plasmids were recovered. The primer set of M13F and M13R (Table 1) was used to amplify the inserted rDNA fragments carried on the plasmid.

Judging from the DGGE profiles of the PCR products, a total of 72 clones were selected. They were composed of 12 unique DNA sequences, commonly referred as operational taxonomy units (OTUs). Six major OTUs were shared by three or more clones. The DNA of these OTUs were then sequenced using an autosequencer (ABI model 377A, Perkin-Elmer Ltd., Foster City, CA) and dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer Ltd., Foster City, CA).

2.4. Phylogenetic analysis

Each DNA sequence was compared with the reference microorganisms available in the GenBank by BLAST search (Altschul et al., 1990). The obtained DNA sequences and their closest 16S rDNA sequences of reference microorganisms retrieved from the GenBank were aligned by BioEdit (Hall, 1999) to construct phylogenetic trees using the neighbor-joining method (Saitou and Nei, 1987) by MEGA 2.1 (Kumar et al., 1993). Bootstrap re-sampling analysis (Felsenstein, 1985) for 500 replicates was performed to estimate the confidence of the tree topologies.

2.5. Accession number

The nucleotide sequence data reported in this paper have been submitted to the GenBank, EMBL and DDBJ databases and assigned the accession numbers *AY261821-AY261826*.

3. Results and discussions

3.1. Microbial community analysis

Fig. 1 illustrates the denitrifying biofilm formed on the sulfur particle surface. The morphology of the bacterial cells appears to exhibit low diversity. However, further analysis using PCR-cloning method revealed that the denitrification biofilm is more diverse than that implied by the SEM image.

Table 2 summarizes the sequence length, number of clones and relative abundance, plus the closest species found in the GenBank and the degree of similarity by Blast analysis. Fig. 2 is the phylogenetic tree of the OTUs obtained in this study and their close related species.

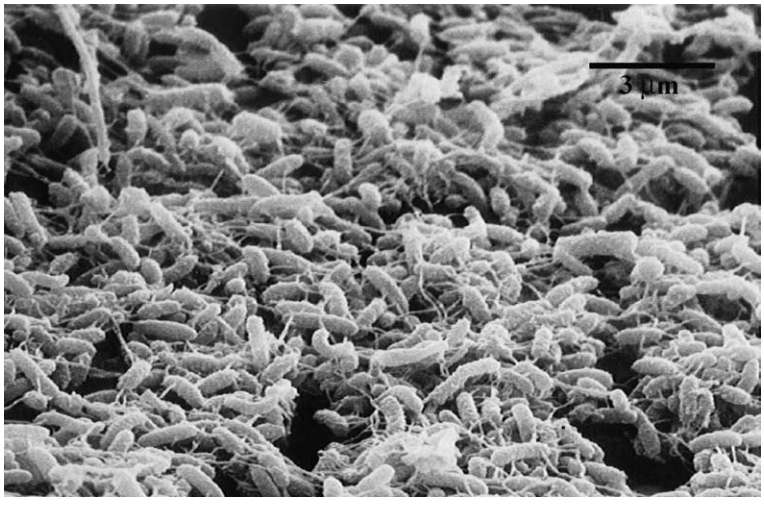

Fig. 1. SEM image of microbial community on the surface of sulfur particle.

Table 2
Six key OTUs and their closest species

OTU	Sequence length	Closest species in GenBank	Similarity (%)	Clone no.	Abundance (%)
ADB-2	334	*Thiobacillus denitrificans*	95	23	32
ADB-67	321	*Trichlorobacter thiogenes*	87	16	22
ADB-66	399	Uncultured bacterium PHOS-HE36	97	12	17
ADB-13	400	*Xanthomonas maltophilia*	93	6	8
ADB-3	305	*Rhodobacter sphaeroides*	94	4	6
ADB-12	317	*Sinorhizobium sp.*	97	3	4
Six OTUs with the clone number of 1 or 2				8	11
Total				72	100

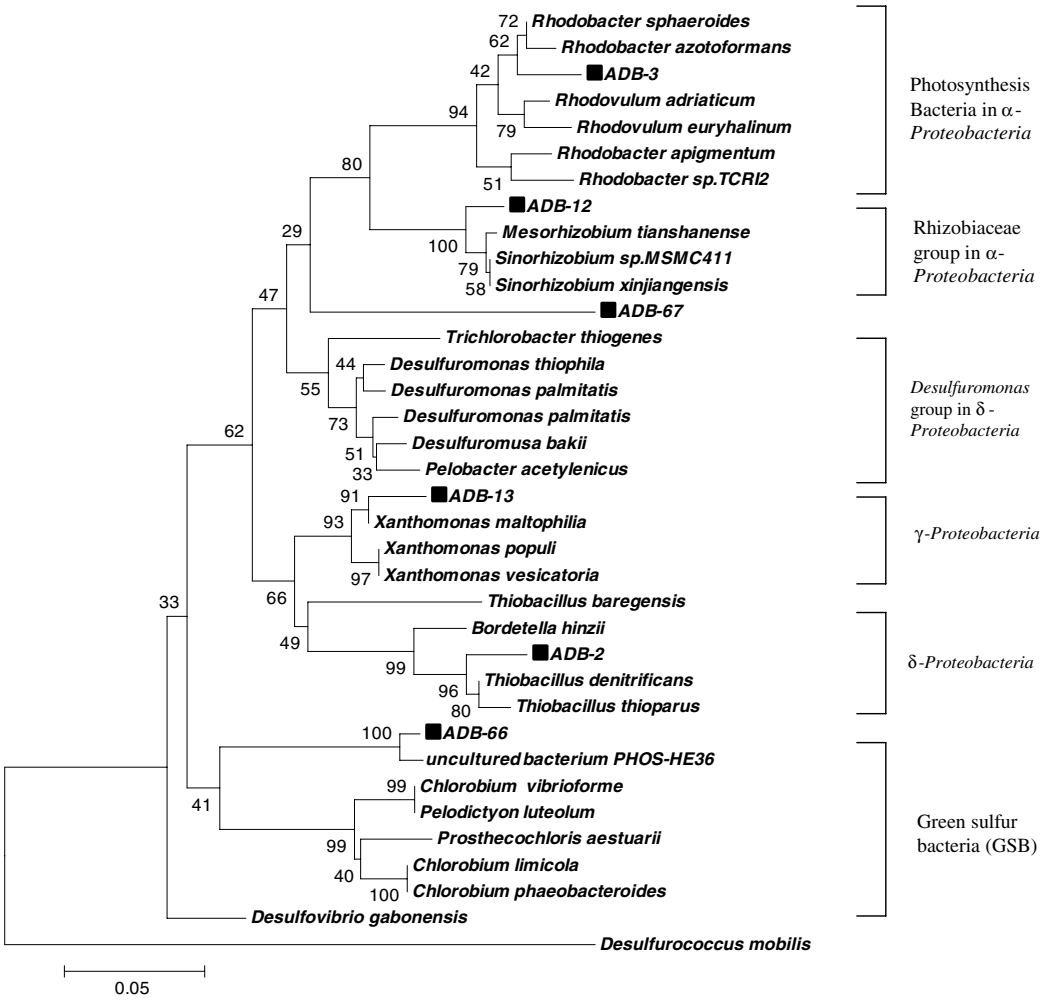

Fig. 2. Phylogenetic tree of six key OTUs in autosulfurotrophic denitrification biofilm and their close relatives based on 310 nucleotides in 16S rDNA sequence. The tree based on Jukes-Cantor distance was constructed using the neighbor-joining algorithm with 500 bootstrappings. *Desulfurococcus mobilis* in the *Archaea* domain was selected as the outgroup species. The scale bar represents 0.05 substitutions per nucleotide position. Numbers at the nodes are the bootstrap values. (■) OTUs obtained in this study.

Table 2 shows that the OTU ADB-2 (*AY261822*, comprising 23 clones) is most closely related to the known species *T. denitrificans* of β-*Proteobacteria* with 95% similarity. *T. denitrificans* is a well-known autotrophic denitrifier using elemental sulfur as the energy source producing SO_4^{2-} (Batchelor and Lawrence, 1978; Koenig and Liu, 1996). Fig. 2 illustrates that ADB-2 formed a cluster, at a node bootstrap value of 96%, with two species of genus *Thiobacillus*, i.e. *T. denitrificans* and *T. thioparus,* but distantly related to another *Thiobacillus* species*, T. baregensis*. It is thus likely that ADB-2, comprising 32% of total clones, might be an uncultured new species with the physiological characteristics of *T. denitrificans*.

OTU ADB-67 (*AY261824*, comprising 16 clones) is most closely (87% similarity) related to *Trichlorobacter thiogenes*, the only species in a new genus in the *Desulfuromonas* group of δ-*Proteobacteria*. Due to the lack of similarity to any known sequences or species, ADB-67 cannot be assigned to any known taxonomic group in the *Desulfuromonas* group. It may constitute a special species, quite different from other species in the *Desulfuromonas* group, which reduces sulfur to hydrogen sulfide using acetate, propionate and ethanol as the electron donor, but never reduces sulfate or other oxoanions of sulfur (Holt et al., 1994).

OTU ADB-66 (*AY261826*, comprising 12 clones) is most closely related to an uncultured photosynthetic bacterium PHOS-HE36 (97% similarity) previously found in a denitrification community (Dabert et al., 2001). Fig. 2 also shows that OTU ADB-66 clusters with PHOS-HE36 at the bootstrap value of 100% and groups together with the representative species of the green sulfur bacteria (GSB), such as *Chlorobium phaeobacteroides*, *Pelodictyon luteolum*, *Pelodictyon aestuarii*, *Chlorobium vibrioforme*, and *Chlorobium limicola*. Thus ADB-66 is most likely a member of the kingdom GSB, which is photoautotrophic, using CO_2 as sole carbon source but also able to use acetate as a carbon source, and capable of oxidizing H_2S into elemental S or further to sulfate. It is frequently found in hydrogen sulfide containing mud and water of freshwater, brackish water, and marine environments (Holt et al., 1994). Sometimes thiosulfate or hydrogen can also be used as electron donor, besides elemental sulfur.

OTU ADB-13 (*AY261823*, comprising six clones) is most closely related to *Xanthomonas maltophilia* at the similarity of 93%. Fig. 2 illustrates that they cluster together at the node bootstrap value of 91% and cluster with *Xanthomonas populi* and *Xanthomonas vesicatoria* with a 93% node bootstrap value. It is thus likely that ADB-6 is a member of *Xanthomonas*, which is a heterotrophic denitrifier, but may also be a parasite of GSB (Holt et al., 1994).

OTU ADB-3 (*AY261825*, comprising four clones) is closely related (94% similarity) to *Rhodobacter sphaeroides*. This OTU likely belongs to the photosynthetic bacteria in α-*Proteobacteria* as it clusters with the reference photosynthetic bacterial species as illustrated in Fig. 2. *Rhodobacter* species could be photoautotroph (anaerobic), photoheterotroph (anaerobic/light) or chemoheterotroph (aerobic) (Holt et al., 1994).

Table 2 shows that OTU ADB-12 (*AY261821*, comprising three clones) is closely related (97% similarity) to *Sinorhizobium sp.* and might be assigned to the *Rhizobiaceae* group in α-*Proteobacteria* as illustrated in Fig. 2. Thus OTU ADB-12 might comprise heterotrophic bacteria using the dead and lysed bacterial cells as their energy source.

3.2. Diversity of the biofilm microbial community

Based on the wastewater composition, autosulfurotrophic denitrifiers, such as *T. denitrificans*, would be expected to be predominant in the biofilm. Yet, the *T. denitrificans*-like ADB-2 represented only 32% of the microbial community. The remaining microbes in the biofilm consisted of a wide range of bacteria from four major subdivisions (α, β, γ, and δ) of the kingdom *Proteobacteria* and from the kingdom *Chlorobia* (the GSB). One OTU was chemoautotrophic, two were phototrophic (at anaerobic condition), and three were heterotrophic. The dead and lysed cells of the autotrophs were likely the carbon source for the heterotrophic bacteria. This may indicate that even so-called pure autotrophic microbial communities will always contain a certain proportion of heterotrophs, feeding on the organic matter (autotrophs, intermediate and degradation products) produced. Table 3 indicates the possible biochemical reactions taking place in the autosulfurotrophic denitrification biofilm based on the classification of the key OTUs and the relationships between them.

The proposed relationships among the major species and their possible distribution in the autosulfurotrophic denitrification biofilm on the sulfur particle are schematically illustrated in Fig. 3. As stated by Batchelor and Lawrence (1978), the microbial activity in the biofilm depends mainly on two steps, (i) sulfur (electron donor) must be transported from the surface of the sulfur particle through the biofilm while being microbially oxidized, and (ii) nitrate (electron acceptor) must be transported from the bulk liquid through the biofilm while being microbially reduced. It is suggested that, initially, ADB-2 forms a single layer biofilm on the sulfur surface. With the thickness of the biofilm increasing, the ADB-2 cells next to the sulfur surface are not able to obtain sufficient NO_3^- for denitrification, become inactive and lyse. The organic matter from the lysed cells provides the food for the sulfur reducing chemoheterotrophs ADB-67 which, in the absence of NO_3^-, reduce S to H_2S and inhabit the biofilm bottom layer. Meanwhile, the H_2S formed by ADB-67 is utilized by the autotrophs ADB-3 and ADB-66, which

Table 3
Possible biochemical reactions of the major OTUs in this study

Trophic type	OTU	Electron donor	Electron acceptor	Oxic state
Autotrophs (54% of the total)	ADB-2	$\mathbf{S} \rightarrow SO_4^{2-}$	$\mathbf{NO_3^-} \rightarrow \mathbf{NO_2^-} \rightarrow N_2$	Anaerobic
	ADB-66	$\mathbf{H_2S} \rightarrow \mathbf{S} \rightarrow SO_4^{2-}$	$\mathbf{CO_2} + H_2O \rightarrow OM$	Anaerobic
	ADB-3	$\mathbf{H_2S} \rightarrow \mathbf{S} \rightarrow SO_4^{2-}$	$\mathbf{CO_2} + H_2O \rightarrow OM$	Facultative
Heterotrophs (35% of the total)	ADB-67	$\mathbf{OM} \rightarrow CO_2 + H_2O$	$\mathbf{SO_4^{2-}} \rightarrow \mathbf{S} \rightarrow H_2S$	Anaerobic
	ADB-13	$\mathbf{OM} \rightarrow CO_2 + H_2O$	$\mathbf{NO_3^-} \rightarrow NO_2^-$	Facultative
	ADB-12	$\mathbf{OM} \rightarrow CO_2 + H_2O$	$\mathbf{NO_3^-} \rightarrow \mathbf{NO_2^-} \rightarrow N_2$	Facultative

Note: OM = organic matter ($C_nH_aO_b$). The electron donors or acceptors are in bold.

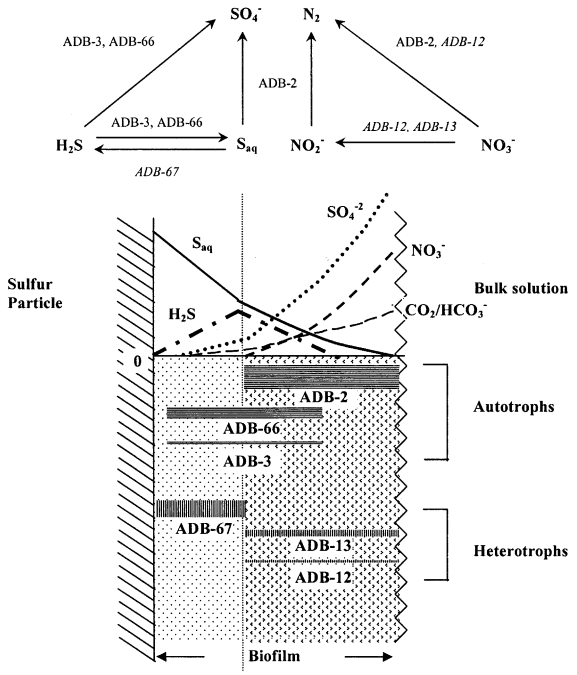

Fig. 3. Suggested relationships among the major OTUs and their possible distribution in the autosulfurotrophic denitrification biofilm as a function of substrate concentration and depth (modified from the schematic of sulfur–biofilm system with zero-order nitrate rate limitation by Batchelor and Lawrence, 1978).

probably occupy the middle layer of the biofilm, to form S or further SO_4^-. The chemoheterotrophs ADB-12 and ADB-13 also depend on organic matter from lysed cells, but appear to inhabit mostly the middle and outer layer of the biofilm, where both organic matter and NO_3^- are available. As the biofilm grows over a longer duration of time, its makeup could change from a single layer to two layers or even three layers, depending on the (i) transport and reaction rate limitations of electron donor/acceptor, and, (ii) the varying environmental conditions within the biofilm. This could explain the presence of so many microbial groups. But their proposed distribution in the biofilm could not be verified as no further investigations were carried out to determine the layered structure and microbial composition of the biofilm. Evidence of incomplete penetration of nitrate in the biofilm and hence for the formation of two layers was demonstrated in sulfur-packed bed reactors, when autotrophic denitrification rates followed a half-order kinetic model (Koenig and Liu, 2001). The theoretical basis for this model was first established by Harremoes (1978), as a consequence of partly penetrated pores in biofilms.

The results demonstrate, however, that the biofilm has a much more complex microbial community and metabolic diversity than originally thought. Although the composition of the microbial community might reflect the source of the bacteria, it is believed that the well-defined experimental conditions must have led to the development of a stable, specialized community of ADBs.

One has to be cautious in interpreting results of this study because of certain intrinsic limitations of the molecular technique applied. Using this method, the microbial DNA has to be extracted, followed by PCR amplification and cloning. The accuracy of this method depends on the reliability of each individual step. It is possible that the DNA of some species could not be effectively extracted because they are difficult to lyse even by vigorous bead beating (Curtis and Craine, 1998). Also, some DNA fragments could be amplified more efficiently than others, so that the former would be overrepresented in the PCR products (Muyzer et al., 1993).

The detailed biochemical interactions between the different microbial groups discovered could not yet be fully established, qualitatively or quantitatively, and merit further investigation, including the determination of the environmental conditions within the biofilm as a function of distance from the sulfur surface (concentrations of S and N compounds, pH, oxidation reduction potential, etc.).

4. Conclusion

The results obtained by application of the 16S rDNA-based molecular technique indicate that the

microbial community of an autotrophic denitrification biofilm developed on the surface of sulfur particles was much more complex than previously thought. Contrary to expectation, *T. denitrificans*-like bacteria constituted only 32% of the microbial community. Other populations included at least two autotrophs and three heterotrophs. A tentative scheme of the interactions among the identified microbial groups was proposed. As a result of this study, the entire microbiology of the autosulfurotrophic denitrification process may need to be reconsidered.

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