Evolution of the bacterial community during granules formation in denitrifying reactors followed by molecular, culture-independent techniques.

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Abstract
The community in two acetate-fed denitrifying reactors, inoculated with methanogenic sludge, was monitored by 16S rDNA-based methods (SSCP and FISH). Both reactors converged to similar, stable communities. The predominant organisms belonged to the genera Thauera, Paracoccus and Denitrobacter, detected both by molecular and culture-based methods. Isolated strains belonging from the genus Thauera and Paracoccus presented high adhesion properties, suggesting that these strains were selected during granule formation because of the ability to adhere

INTRODUCTION
Anoxic UASB reactors have been successfully used for nitrate removal in integrated systems (anaerobic, aerobic, anoxic) treating effluents with high organic and nitrogen content (Borzacconi et al., 1999; Morgan-Sagastume et al., 1994). However, there are few reports on the parameters that affect granulation of the denitrifying biomass (Cuervo-López et al., 1999; Etchebehere et al, 2002a). The effect of inocula on sludge granulation in denitrifying reactors was studied in two laboratory scale UASB denitrifying reactors, fed with acetate and nitrate and seeded with methanogenic granules (Etchebehere et al, 2002a). The evolution of the microflora was studied by culture-dependent methods, and organisms belonging to the same species within the genus Thauera were isolated from both reactors, suggesting a role for such organism in acetate-fed denitrifying reactors. However, in order to evaluate the denitrifying community, it is necessary to apply methods that detect both culturable and non-culturable organisms. Recently, several 16S rDNA-based methods have been developed. Among them, Fluorescent in situ hybridization (FISH) reveals whole cells and has been used to analyze the microbial community structure of many ecosystems (Amann et al., 1990). Single Strand Conformation polymorphism (SSCP) is based on the different electrophoretic mobility of 16SrDNA molecules with different sequences (Orita et al, 1989) and was successfully applied to study population dynamics in anaerobic digestors (Zumstein et al, 2000) and in phosphorus removal SBR (Dabert et al, 2000). The aim of the present work was to study the evolution of the microflora developed in both reactors by culture-independent methods.

MATERIALS AND METHODS

Two upflow reactors (4.6 L) were seeded with intact (R1) and ground granules (R2) from an anaerobic UASB reactor treating brewery wastewater. Both reactors were fed with acetate and nitrate (COD/N-NO₃ = 4) during more than one year (Etchebehere et al, 2002a). The nitrate loading rates was gradually
increased up to values of 0.9 g N-NO3-/l.d at the end of the study. Samples were taken from the inoculum and from the both reactors at 3, 4, 10 and 11 months of operation.

**Sludge SSCP analysis**

DNA was extracted from sludge samples using a commercial kit (UltraClean soil DNA kit, Mo Bio). The V3 region of the bacterial 16S rDNA was amplified by PCR using specific primers according to Zumstein *et al.* (2000), one of the primers were fluorescent labelled. The PCR products were heat denaturated and separated according to their size and structure by capillary electrophoresis in an automatic sequencer (Abi prism 310 Perkin Elmer, Applied Biosystems). Data processing was performed with Genescan software (Applied Biosystems).

The characterization of the predominant peaks were performed by cloning and SSCP analysis of the clones as was describes previously (Dabert *et al.*, 2001). Two libraries were performed, library B from sample R1 (t=4 months) and library C from R1 (t=11 months) using TOPO DNA cloning kit (Invitrogen). Clones were analysed by SSCP, a single pattern was obtain for each clone. Clones with profiles matching to DNA SSCP profile were selected to sequence. Sequencing was performed as described by Zumstein *et al.*, (2000). The sequences were compared with all sequences available in databases using BLAST Search at National Center for Biotechnology Information (NCBI).

**SSCP analysis of isolated strains.**

16 denitrifying strains were isolated from both reactors (Etchebehere *et al.*, 2001a) and grouped according to the ARDRA profile. 16S rDNA of the most frequently isolated strains was analysed by SSCP. Strains showing different SSCP profiles were selected and the 16S rDNA partial sequences were determined as previously described (Etchebehere *et al.* 2001b). The sequences were compared with all sequences available in databases using BLAST Search at NCBI.

**Fluorescent In Situ Hybridisation (FISH).**

In order to quantify the proportion of alpha and beta Proteobacteria in the total bacterial population, FISH was performed on sludge samples as described by Amann *et al.* (1990). The following probes were used: Alf968 to detect alpha *Proteobacteria* (Neef, 1997); BET42a and GAM42a (Manz, 1992) to detect beta and gamma *Proteobacteria*, and Eub338 direct to the *Bacteria* Domain (Amann *et al.*, 1990). The probes were fluorescent labelled with Cy3 or Cy5. For quantitative analysis cells targeted by a probe were expressed as a percentage of total cells counterstained with 4’,6-diamidino-2-phenylindole (DAPI).

**RESULTS AND DISCUSSION**

**Evolution of the microbial community in denitrifying reactors**

The results of the sludge SSCP analysis at different times of the denitrifying reactors (R1 and R2) are shown in Fig 1. As expected, important shifts in the community structure were detected during the first period of reactor operation, suggesting adaptation of the methanogenic population to denitrifying conditions. This was in accordance with reactor performance (Etchebehere *et al.* 2001a). The high number of peaks detected in the inoculum reflects the high diversity of Bacterial organisms generally found in this kind of community, capable of degrading complex substrates in methanogenic conditions (Zumstein *et al.*, 2000). A clearly less diverse community was selected during operation in both reactors, as shown by the smaller number of peaks. Furthermore, the community in both reactors
evolved in the same way. Seven dominant peaks were observed: Peaks A, D, F and G were detected at different times, indicating that the organisms represented by such peaks persisted during reactor operation. Moreover, Peaks E and G increased during time, suggesting that organisms represented by these peaks became dominant.

**Identification of the SSCP peaks, comparison with clones and strains SSCP profiles.**

In order to identify the predominant peaks two strategies were followed: cloning and SSCP analysis of the clones, and SSCP analysis of the strains isolated from the same reactors samples. For the cloning approach two sludge samples were selected and a library was performed for each one, library B from sample R1 (t=4 months) and library C from R1 (t=11 months). The SSCP analysis of the clones showed that 19 clones out of forty matched with peaks present in the sludge SSCP profile. Eleven out of sixteen strains isolated from both reactors at different times presented SSCP profiles matching with peaks of the sludge SSCP analysis (Table 1).

The 16S rDNA partial sequence analysis of the clones and strains revealed a prevalence of organisms belonging to the genera *Thauera* and *Paracoccus* in the beta and alpha subgroup of *Proteobacteria*. The SSCP profiles of these clones and strains matched with peaks from the SSCP analysis of all the sludge samples, indicating that these organisms were dominant in both reactors and persisted during operation. Several clones and one strain presented SSCP profiles matching with peak G present in the sludge SSCP, which increased during reactors operation. These clones and strain showed sequences closely related to the genus *Denitromonas*, suggesting that this organism was selected in the conditions of the reactors.

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**Fig 1.** SSCP profiles of the reactors sludge samples, at different operation time. R1: reactor inoculated with intact granules, R2: reactor inoculated with ground granules.
Table 1. Comparison of clones and strains SSCP profiles with sludge SSCP peaks. Organisms identification according to 16S rDNA partial sequence comparison (BLAST Search)

<table>
<thead>
<tr>
<th>SSCP Profile from clones and strains</th>
<th>Number of clones from library C</th>
<th>Number of clones from library B</th>
<th>Strains</th>
<th>Sludge SSCP peak</th>
<th>Sequence identification of representative clones and strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2</td>
<td>6</td>
<td>F</td>
<td>Thauera</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>2</td>
<td></td>
<td>G</td>
<td>Denitromonas</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>2</td>
<td>A</td>
<td></td>
<td>Paracoccus</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>2</td>
<td>D</td>
<td></td>
<td>Thauera</td>
</tr>
</tbody>
</table>

Fluorescent in situ hybridisation

In both reactors a high proportion of the Bacteria cells hybridised with the alpha and beta Proteobacteria probes (Fig. 2 and 3). No organisms hybridising with the gamma subgroup were detected.

Figure 2. Fluorescent in situ hybridisation in sludge from R2 (sample time=11 months), (a) hybridization with Bacteria probe Cy5 labelled (blue) and with beta Proteobacteria probe Cy3 labelled (red); (b) hybridisation with beta Proteobacteria probe Cy3 labelled (red).

As expected, organisms hybridising with the Archaea probe were detected in low percentage of the total cells (Fig. 3), previous work showed a decrease of the Archaea population during reactors operation ( ), this may be due to the decrease of methanogenic population. High proportion of Bacteria organisms were detected in both reactors (Fig. 3), indicating that the population was enriched in organisms from the Bacteria domain, probably denitrifiers. Similar percentages of alpha and beta-Proteobacteria were detected, this was in accordance to the SSCP analysis that shows a predominance of this organisms in the microbial community.
CONCLUSIONS

In both denitrifying reactors the community evolved in the same way, independently of the physical structure of the inoculum. A specialised denitrifying community was selected in both reactors with a predominance of organisms from the genus *Thauera* and *Paracoccus*, as detected by culture and non-culture methods. In spite of the high diversity of denitrifiers, these genera prevailed in both UASB denitrifying reactors, suggesting that they present a selective advantage under the operation conditions. Several strains isolated during reactor operation and characterised as belonging to these genera, presented adhesion properties, as reflected by aggregates formation in liquid media and colony adhesion to solid media. It can be speculated that these strains were selected during granule formation because of the ability to adhere. Sludge flotation problems were reported in these reactors (Etchebehere *et al.*, 2001a), probably due to bubble retention inside the aggregates as was also detected in pure cultures of the isolates.

Organism pertaining to the genus *Denitromonas* became dominant and increased during reactor operation. Pure cultures of these strains did not form aggregates during growth. Further work is necessary to investigate the role of this organism in UASB denitrifying reactors.

The results from FISH analysis showed that a community enriched in alpha and beta Proteobacteria was selected; in accordance with the SSCP analysis and with previous culture-based analysis of these communities (Etchebehere *et al.*, 2001b).

By a combination of SSCP, FISH and culture dependent techniques it was possible to analyse the evolution of the microbial community during reactor operation; a correlation of these data with reactor operation will permit to improve reactor start up and performance.

REFERENCES


