Characterization of the bacterial community in a biotrickling filter treating high loads of H₂S by molecular biology tools

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ABSTRACT

The diversity and spatial distribution of bacteria in a lab-scale biotrickling filter treating high loads of hydrogen sulfide (H₂S) were investigated. Diversity and community structure were studied by terminal-restriction fragment length polymorphism (T-RFLP). A 16S rRNA gene clone library was established. Near full-length 16S rRNA gene sequences were obtained, and clones were clustered into 24 operational taxonomic units (OTUs). Nearly 74% and 26% of the clones were affiliated with the phyla Proteobacteria and Bacteroidetes, respectively. Beta-, epsilon- and gamma-proteobacteria accounted for 15, 9 and 48%, respectively. Around 45% of the sequences retrieved were affiliated to bacteria of the sulfur cycle including *Thiothrix* spp., *Thiobacillus* spp. and *Sulfurimonas denitrificans*. Sequences related to *Thiothrix lacustris* accounted for a 38%. Rarefaction curve demonstrated that clone library constructed can be sufficient to describe the vast majority of the bacterial diversity of this reactor operating under strict conditions (2,000 ppmv of H₂S). A spatial distribution of bacteria was found along the length of the reactor by means of the T-RFLP technique. Although aerobic species were predominant along the reactor, facultative anaerobes had a major relative abundance in the inlet part of the reactor, where the sulfide to oxygen ratio is higher.

Key words | bacteria, biotrickling filter, clone library, community diversity, hydrogen sulfide, T-RFLP

INTRODUCTION

Air pollution is currently one of the most prominent environmental issues. Industrial processes generate a variety of gas contaminants jeopardizing human health and well-being. Moreover, with increasingly strict regulations, the necessity for treating gas pollutants is critical. Among other pollutants, the acid rain caused by sulfur dioxide emissions is produced when burning of coal, petroleum or biogas. Biogas is produced in multiple industrial processes like the anaerobic treatment of wastewater treatment plant sludge. Unfortunately, biogas contains not only methane but also secondary components that make it technically difficult and economically expensive to use for electric power production. One of them is hydrogen sulfide (H₂S), a corrosive, toxic and odorous gas, typically representing 0.1 to 2% (vv⁻¹) of the biogas.

Biological processes have proven to be suitable, environmental-friendly, and cost-effective alternatives for low H₂S concentration treatment (Devinny et al. 1999; Gabriel & Deshusses 2003), but only a few references address biological treatment of high concentrations of
H₂S in biotrickling filters (Fortuny et al. 2008). In biofiltration, the gas to be treated is forced through a filter bed where microorganisms are attached. Gaseous pollutants are transferred to a moisturized biofilm, where the biodegradation of the compounds takes place, involving several physical, chemical and biological interactions.

Although bioreactors for odors or, in general, for waste gas treatment have been frequently treated as “black boxes” due to the complexity of the underlying biochemical processes, a thorough study of the microbial community in these bioreactors has been shown to be critical to the understanding of how these bioreactors work. Traditional cultivation-dependent methods of assessing the microbial community are often biased since it has been estimated that as many as 99% of all microorganisms are not cultivable by standard methods. However, with the advent of novel molecular biology techniques targeting the small subunit rRNA-encoding genes, this bias can be overcome and improved knowledge of the populations present in these kinds of reactors can be obtained. Terminal restriction fragment-length polymorphism (T-RFLP) is a molecular approach for characterizing environmental microbial communities and for studying changes in their structure and diversity (Liu et al. 1997). T-RFLP is the most appropriate fingerprinting molecular method for frequent bioreactor monitoring.

The objective of this study was to study the microbial diversity in a biotrickling filter treating H₂S using a combination of T-RFLP and clone libraries of the 16S rRNA gene. This combination allows more accurate identification of individual T-RFLP peaks. A 16S rRNA clone library was constructed by using Bacterial universal primers. The spatial distribution of the microbial community attached to the packing material in the reactor was assessed by T-RFLP and a clone library created. To our knowledge, although a few studies have characterized the microbial community of such gas-phase bioreactors, mainly biofilters, using molecular techniques (Sakano & Kerkhof 1998; Friedrich et al. 2002), this work is the first to characterize the populations in a biotrickling filter treating H₂S by constructing an extensive clone library and using T-RFLP as the fingerprinting technique for studying H₂S-degrading communities.

**MATERIALS AND METHODS**

**Experimental setup**

In this work, an experimental reactor based on a conventional biotrickling filter was used. The reactor was continuously operated for three months in upflow mode at an empty bed residence time of 180 s, an average liquid retention time of 54 ± 7 h, an inlet H₂S concentration of 2,000 ppmv (55.6 g H₂S m⁻³ h⁻¹) and a liquid recirculation velocity of 3.6 m h⁻¹. Under these conditions, elimination capacities of 55 ± 1 g H₂S m⁻³ h⁻¹ were achieved for the whole experimental period. Metered amounts of H₂S, N₂ and air (0.2, 77.3 and 22.4% respectively) were used to simulate a controlled biogas inflow. Details of the reactor, mineral medium, other operational conditions and analytical methods can be found elsewhere (Fortuny et al. 2008).

**DNA isolation and 16S rRNA gene amplification**

Biomass samples were withdrawn from two sampling ports situated at different heights in the reactor, 17 cm and 34 cm. The first one will be referred as inlet part and the second one as outlet part. For library construction biomass samples from inlet and outlet part of the reactor were mixed in a 1:1 ratio (w/w). The DNA was extracted and tested and preserved as described elsewhere (Chi-hua & Banks 2006).

The small subunit (SSU) rRNA gene was polymerase chain reaction (PCR) amplified from genomic DNA extracted from the environmental samples and two isolates using universal primers for the Bacterial domain. These two isolates were obtained from the same reactor (data not shown). For isolate identification and clone library generation, the PCR reactions were performed as described elsewhere (Chi-hua & Banks 2006) but containing 100 ng of template DNA. After amplification, the PCR products were purified using the High Pure PCR Product Purification Kit (Roche Molecular Biochemicals, Germany). For isolate identification, PCR products were sent for direct sequencing. For clone libraries, the 16S rRNA gene fragments were cloned with the TOPO TA Cloning® Kit for Sequencing (Invitrogen, CA, USA) according to the manufacturer’s instructions.
Genomic DNA of *Thiobacillus denitrificans*, used as positive control, was obtained from Dr. Christiane Dahl’s laboratory in the Institut für Mikrobiologie & Biotechnologie, Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany.

**T-RFLP analysis**

For T-RFLP analysis, the labeled forward primer Fam-8F and the unlabeled reverse primer 926R (Molbiol, Germany) were used. T-RFLP was performed as described in Chi-hua & Banks (2006) with some modification as described by Egert & Friedrich (2005) to reduce partially single-stranded amplicon. Some parameters and conditions differed from Chi-hua and Banks procedure: for PCR 200 ng of template were used. Approximately 200 ng of product were digested. The T-RFLP digests were analyzed on an ABI automated sequencer (Model 3,100). Duplicates from the initial step of DNA extraction were performed through the whole process to evaluate the reproducibility of the methodology. The distribution of the peaks from the collected profiles was performed using ABI Genemarker 1.51.71 (Softgenetics, PA, USA). A level of 80 fluorescence units was used as a minimum threshold value for all peaks in the selected size range. The profiles were visually inspected and aligned based on the relative peak distribution. To assess community similarity, terminal restriction patterns were standardized based on T-RF peak area (Kaplan et al. 2001). Once the samples were normalized, peaks below the threshold were deleted and new peak area percentages were calculated. Duplicate sample results were grouped, and consensus terminal-restriction fragments (T-RFs) and average peak areas were obtained by using the web tool T-align (http://inismon.ucd.ie/~talign/) developed by Smith et al. (2005). Each T-RF represents an OTU. Samples from the inlet and outlet parts of the bioreactor as well as eight selected clones from the clone library and two isolates were subjected to T-RFLP analysis.

**Sequencing and phylogenetic reconstruction**

One hundred clone inserts were sequenced in full with pUC/T4-T7 primers by Macrogen (Korea). All sequences were checked for chimeric artifacts by using the CHIMERA_CHECK program of the Ribosomal Database Project (Maidak et al. 2000) and for similar sequences with the ClustalW program. Near full-length sequences (approximately 1,500 bp) were compared with similar sequences of the reference organisms by a BLAST search (Altschul et al. 1997). Sequences with the same first accession number were grouped into clusters. Thus, the clustering process was based on the BLAST results obtained for every clone. Similarity between clones within the same cluster was studied by sequence comparison using ClustalW.

**Phylogenetic trees method by ARB and rarefaction analysis**

Sequences were subjected to a BLAST search (Altschul et al. 1997) to get a first suggestion of the phylogenetic affiliation. Sequences were aligned using the automatic alignment tool in the ARB program package (http://www.mikro.biologie.tu-muenchen.de; Ludwig et al. 1998). The optimized maximum parsimony tree obtained from the ARB software and derived from complete sequence data was used to add one clone sequence of each OUT from this study, according the “Quick add using parsimony” tool, which does not affect the initial tree topology. The 24 clones added were aligned applying the “Positional Variability by Parsimony” filter resulting 1,302 valid columns. The resulting tree was trimmed to save space and only the closest relatives of the clone sequences of interest were retained. The rarefaction analysis was calculated using the defined OTUs. The software Analytic Rarefaction (version 1.3, S.M. Holland, http://www.uga.edu/~strata/software/) was used to perform rarefaction analysis. The 16S rRNA gene sequences of the clones have been submitted to the Genbank/EMBL/DDBJ to obtain the corresponding accession numbers. The Genbank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the clones used for the phylogenetic analysis are FM165203 to FM165231 and FM174321 to FM174366.

**RESULTS AND DISCUSSION**

**Bacterial diversity**

In this study, a lab-scale biotrickling filter degrading high loads of H2S was investigated. These high loads of hydrogen...
sulfide may constrain the bacterial diversity of the biofilter. Moreover, the nature of the packing material, inert plastic grid, decreases the microenvironments available for bacterial communities. On the other hand, the presence of a biofilm enhances bacterial diversity due to the different microenvironments that exist in the biofilm.

A 16S rRNA gene clone library was constructed with near-full-length 16S rRNA gene sequences were obtained for all the clones. After eliminating putative chimeric sequences, the remaining 75 clones were clustered into 24 groups. Rarefaction analysis demonstrated that the clone library constructed was suitable to describe the vast majority of the bacterial diversity of the reactor since the rarefaction curve almost achieved a plateau (data not shown). It was found that similarities of the clones within a cluster were always above 90%, indicating that clustering based on BLAST results was suitable for results synthesis. Similarities among clones between clusters with the same cultured closest match accession were also subjected to sequence comparison, and were clustered together when presenting similarities higher than 98%. The 75 clone sequences were phylogenetically analyzed using RDP II; this analysis indicates that 74.0% of the sequences were related to the phylum Proteobacteria and 26.0% to Bacteroidetes. 49.4% of the clones were affiliated with the γ-proteobacteria, and 15.6 and 9.1% with the β- and ε-proteobacteria, respectively. No members of other classes were found.

Among clones analyzed and clustered, 10 clusters, including 45 clones, were closely related to known sulfur-oxidizing bacteria (SOB) species: *Thiothrix lacustris*...
(γ-proteobacteria), *Thiothrix* sp. CT3 (γ-proteobacteria), *Sulfurimonas denitrificans* (ε-proteobacteria), *Thiobacillus denitrificans* (β-proteobacteria), *Thiobacillus sajanensis* (β-proteobacteria) and *Thiobacillus plumbophilus* (β-proteobacteria) (Figure 1).

Among γ-proteobacteria, Thiotrichales was the most predominant order (almost 90% of γ-proteobacteria). The remaining orders of this class represented in the clone library were related to Xanthomonadales (clones were affiliated with *Dokdonella koreensis* and *Xanthomonas axonopodis*). All clones belonging to ε-proteobacteria were exclusively related to Campilobacterales (clones were affiliated with *Sulfurimonas denitrificans*). Regarding class β-proteobacteria, the order most frequently represented was Hydrogenophilales (clones were related to *Thiobacillus* spp.). The orders Nitrosonomadales (*Nitrosospira* and *Nitrosomonas* spp.), Burkholderiales (*Ottowia* spp.) and Rhodocyclales (*Denitrasoma* spp.) were scarcely represented with only. Concerning the phylum Bacteroidetes, class Sphingobacteria comprised more than 20% of the clone library. The clones included in this class were affiliated with *Haliscomenobacter hydrossis*.

Molecular phylogenetic analysis performed can suggest what metabolic roles are being played by the organisms present in the reactor. *Thiothrix* is a filamentous genus in the γ-proteobacteria. It can grow under heterotrophic, chemolithoautotrophic, and mixotrophic conditions. This could explain the predominance of *Thiothrix* in the bioreactor since it had been operated for 6 months, and there was a considerable quantity of biomass accumulated in the packing material *Thiothrix* spp. have been described as a component of biofilms in sulfide-containing flowing water in natural systems and wastewater treatment plants. These filamentous bacteria are believed to be one of the major causes of activated sludge bulking and foaming.

The genera *Thiobacillus* and *Sulfurimonas*, both potentially found in the library, have been described previously as sulfur-oxidizers (*Kelly et al. 1997*). The genus *Thiobacillus* is represented in the clone library by the chemolithoautotrophic species *T. denitrificans*, *T. sajanensis* and *T. plumbophilus*. These species are facultative anaerobic, in the case of *T. denitrificans* or aerobic in the case of *T. sajanensis* and *T. plumbophilus*. Also, the genus *Sulfurimonas* is represented in the collection by the chemolithoautotroph facultative anaerobe *Sulfurimonas denitrificans*. Although both genera represent less than 20% of the clones in the collection, they are presumably playing an active role in the sulfur oxidation process, mostly in microenvironments where oxygen levels are extremely low. These microenvironments are present in thick biofilms like the existing in this type of bioreactor.

### Microbial spatial distribution through T-RFLP

T-RFLP is not considered a quantitative methodology, but a comparison between samples can be performed based on the areas of the peaks representing different operational taxonomic units. T-RFLP was used for the samples of biomass withdrawn at two different heights of the reactor. Since the most significant clones of the clone library were subjected to T-RFLP analysis, it was possible to identify the populations represented by some of the OTUs found in the T-RFLP patterns (Table 1). Clones affiliated with *Thiothrix lacustris* which were digested with HhaI yielded a terminal restriction fragment of 173bp as is shown in Table 1. Two species isolated from the reactor on the second month of operation were also studied by T-RFLP. Genomic DNA from *Thiobacillus denitrificans* strain DSMZ 807 was used through the whole T-RFLP process as a positive control. This organism has been described as an SOB previously (*Beller et al. 2006*). When it was subjected to T-RFLP analysis, it showed a T-RF of 563bp which corresponds to the same OTU as *Thiobacillus plumbophilus* (OTU 9).

<table>
<thead>
<tr>
<th>Cultured closest match</th>
<th>T-RF (bp)</th>
<th>OTU id.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dokdonella koreensis</em></td>
<td>58</td>
<td>OTU 1</td>
</tr>
<tr>
<td><em>Thiomonas intermedia</em></td>
<td>63</td>
<td>OTU 2</td>
</tr>
<tr>
<td><em>Thiobacillus neapolitanus</em></td>
<td>74</td>
<td>OTU 3</td>
</tr>
<tr>
<td><em>Haliscomenobacter hydrossis</em></td>
<td>78</td>
<td>OTU 4</td>
</tr>
<tr>
<td><em>Sulfurimonas denitrificans</em></td>
<td>97</td>
<td>OTU 5</td>
</tr>
<tr>
<td><em>Thiothrix lacustris</em></td>
<td>173</td>
<td>OTU 6</td>
</tr>
<tr>
<td><em>Nitrosomonas</em> sp. Is425</td>
<td>207</td>
<td>OTU 7</td>
</tr>
<tr>
<td><em>Thiobacillus denitrificans</em></td>
<td>446</td>
<td>OTU 8</td>
</tr>
<tr>
<td><em>Thiobacillus plumbophilus</em></td>
<td>563</td>
<td>OTU 9</td>
</tr>
</tbody>
</table>

*Two species isolated from the reactor.*
In Figure 2, electropherograms corresponding to outlet (a) and inlet (b) samples are shown. Samples were normalized based on area. OTUs under study are highlighted.

Species abundance in the sample is related to peak area percentage. It is possible to observe that the OTU 6 with 173 bp of fragment length, which was confirmed to comprise the clone affiliated with Thiothrix lacustris, is the most abundant OTU found in the T-RFLP patterns, in accordance with the results obtained in the clone library. It was found that the clones and isolates used for OTU identification by T-RFLP only comprised 50% of the total peak area of the patterns, meaning that there is at least 70% of peak area representing other OTUs that remains unknown. However, the clones used for OTUs identification represented 65% of the clone library diversity. The distribution 30% peak area studied is represented in Figure 3. The OTU 6, related to Thiothrix lacustris, is predominant among the species studied in both parts of the reactor. This result was confirmed by using the enzyme AluI (data not shown). It could be hypothesized that this species would be the most adapted to the operational conditions throughout the reactor, most probably because of its mixotrophy. In the inlet part of the bioreactor, the rest of OTUs studied gained in significance at the expense of OTU 6. This means that the physiological advantage of Thiothrix lacustris over the rest of species diminishes in the inlet part of the reactor where there is higher sulfide to oxygen ratio. Here, it can be highlighted that OTUs that increment its significance in the inlet part of the reactor are related to mainly facultative anaerobic species (Thiobacillus denitrificans, Sulfurimonas denitrificans and Haliscomenobacter hydrossis) so they could substitute the lack of oxygen with other electron acceptor. The OTU 1, affiliated with the aerobic Dokdonella koreensis, increased its relative abundance in the inlet part as well.

It is remarkable that OTUs 2 and 3, linked to Thiomonas intermedia and Thiobacillus neapolitanus, represents a low proportion of peak area. This result was confirmed by using the enzyme AluI (data not shown). These results agree with those obtained through the clone library where clones affiliated to these species were not even found. This could

Figure 2 | Electropherograms showing T-RFLP patterns in (a) outlet part, and (b) inlet part of the reactor. Main OTUs studied are labeled with their respective fragment lengths. The molecular weight ladder is shown in red color.

Figure 3 | Distribution of studied OTUs in microbial communities of A) outlet part of the reactor and B) inlet part of the reactor. Distribution is based on % peak area from T-RFLP.
indicate either clone library should be more extend to cover a higher portion of the bacterial diversity and/or that behind these OTUs there could be other species that we have not analyzed individually by T-RFLP.

CONCLUSIONS

In this study, the microbial diversity in a biotrickling filter treating H$_2$S is characterized by creating a clone library potentially representing most abundant species present and using T-RFLP to study the differential microbial distribution between two areas of the reactor sited at different heights. The use of both novel molecular tools improves the knowledge about the microbiology of gas-phase bio-reactors. To our knowledge, this is the first research to retrieve an extensive number of near full-length 16S rRNA gene sequences from a biotrickling filter treating H$_2$S and constructing a collection representative of the existing diversity. Around 45% of the sequences retrieved were affiliated with bacteria of the sulfur cycle including Thiothrix spp., Thiobacillus spp. and Sulfurimonas denitrificans. Although clones related to aerobic species were predominant along the reactor, facultative anaerobes had a major relative abundance in the inlet part of the reactor, where the sulfide to oxygen ratio is higher. Even though these results may help to break new ground in the biofiltration field, further research about the microbial composition and distribution in biotrickling filters is needed in order to fully understand the underlying processes that take place in these systems. Quantification of populations would help to hypothesize about the causes of changes in the microbial community composition and in particular to understand the relation between phylogeny and reactor efficiency.

REFERENCES


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