Genetic Diversity and Expression of the [NiFe] Hydrogenase Large-Subunit Gene of Desulfovibrio spp. in Environmental Samples

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The genetic diversity and expression of the [NiFe] hydrogenase large-subunit gene of Desulfovibrio spp. in environmental samples were determined in order to show in parallel the existing and active members of Desulfovibrio populations. DNA and total RNA were extracted from different anaerobic bioreactor samples; RNA was transcribed into cDNA. Subsequently, PCR was performed to amplify a ca. 440-bp fragment of the [NiFe] hydrogenase large-subunit gene and its mRNA. Denaturing gradient gel electrophoresis analysis was used to separate the PCR products according to their sequence and thereby to visualize the individual community members. Desulfovibrio strains corresponding to amplified [NiFe] hydrogenase transcripts were regarded as metabolically active, because in pure cultures transcripts were detectable in exponentially growing cells but not in cultures in the stationary phase. DNA sequencing and comparative sequence analysis were used to identify the detected organisms on the basis of their [NiFe] hydrogenase sequences. The genes of characterized Desulfovibrio spp. showed a considerable extent of divergence (ca. 30%), whereas sequences obtained from bacterial populations of the bioreactors showed a low level of variation and indicated the coexistence of closely related strains probably belonging to the species Desulfovibrio sulfoxismutans. Under methanogenic conditions, all detected populations were active; under denitrifying conditions, no [NiFe] hydrogenase mRNA was visible. Changes in activity and composition of Desulfovibrio populations caused by changes in the environmental conditions could be monitored by using the approach described in this study.

Diversity and metabolic activity of organisms play an important role in ecological studies of microbial communities. Molecular techniques can be used to analyze composition and genetic diversity without the need for cultivation of individual species (1, 29). Targeting DNA and the detection and analysis of specific genes can reveal the existing microbes within an environmental sample, including both dormant and inactive organisms. Identification of the organisms by comparative sequence analysis can lead to conclusions about their potential activity but not about the actual in situ activity. Thus, rRNA sequences are now being employed more frequently to study microbial communities. Because of the higher number of ribosomes in metabolically active cells than in dormant cells, it is assumed that analysis of rRNA instead of genomic DNA reflects the metabolically active organisms (12, 40, 48). A more specific approach to monitor microbial activity and to understand the function of the community members, however, is the analysis of mRNA. Detection of mRNA can ensure that the target gene, and therefore the corresponding microorganism, is active.

There exist probes and specific PCR assays for functional genes of a variety of bacterial groups, such as the gene encoding formyltetrahydrofolate synthase in acetylogenic bacteria (25), nifD and nifH in N2-fixing bacteria (49, 62), the dissimilatory nitrite reductase genes in denitrifying bacteria (55), the ammonium mono-oxygenase gene in ammonium-oxidizing bacteria (45), the methane mono-oxygenase gene in methano-}


trophs (27), and the hydrogenase genes in aerobic H2-oxidizing bacteria (19) and sulfate-reducing bacteria (54, 56). Nevertheless, the detection of mRNA in natural populations has been demonstrated for only a few genes. These include, for instance, the ribulose-1,5-bisphosphate carboxylase large-subunit gene (rbcL) in phytoplankton populations (35, 39, 61), genes responsible for the detoxification of mercurial compounds from a microbial community of a mercury-contaminated pond (16), and the nifH gene in natural populations of an N2-fixing marine cyanobacterium (60).

We are interested in the ecological role of sulfate-reducing bacteria. Sulfate-reducing bacteria occur in a variety of anaerobic habitats, where they are involved in the terminal degradation of organic matter, the sulfur cycle, and the anaerobic corrosion of metals (57). Recently, we developed a PCR-based detection system which specifically amplifies [NiFe] hydrogenase large-subunit gene sequences of the sulfate-reducing genus Desulfovibrio (56). Furthermore, it allows the discrimination of different strains by denaturing gradient gel electrophoresis (DGGE) of amplified gene fragments. The main objective of this study was to extend this assay to the analysis of mRNA. The [NiFe] hydrogenase plays an important role in the energy metabolism of Desulfovibrio spp. (14). Therefore, its expression should be an excellent marker for the metabolic activity of those bacteria in the environment.

Here, we describe a molecular approach that attempts to differentiate the active from the dormant members within populations of Desulfovibrio spp. in environmental samples.

MATERIALS AND METHODS

Cultivation of bacteria. Strains of Desulfovibrio spp. were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany, D-38045 Braunschweig, Germany). These strains were grown anaerobically in TYM broth (yeast extract, 10 g; tryptone, 10 g; magnesium sulfate heptahydrate, 0.5 g; and sodium chloride, 3 g) at 37°C.

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Germany). Strain PIB2 ("Desulfovibrio oceanicum" [21]) was isolated from the microbial mat of Solar Lake (Sinai, Egypt) by Yehuda Cohen (The Moshe Shilo Center for Marine Biogeochemistry, Jerusalem, Israel).

The bacteria were grown anaerobically in a defined, bicarbonate-buffered, sulfide-reducing medium at 28°C (58). The culture density was maintained by periodic feeding of 20 mM lactate. Cultivation with hydrogen was performed with 2 mM acetate as carbon source under an atmosphere of H₂-CO₂ (80:20 [vol/vol]).

Growth experiments. Different strains of Desulfovibrio spp. were grown as batch cultures in a lactate-sulfate medium. A large volume of medium was inoculated with a fresh culture (1 to 5% [vol/vol]) and divided into 10- or 50-ml portions to facilitate sampling. Growth was measured by monitoring the optical density of the cultures at 660 nm. Sulfide was determined colorimetrically by the method of Knoop and the reaction method (5) with a final reaction volume of 4 ml containing 2 to 20 µl of culture.

Isolation of RNA from bacterial cultures. RNA was isolated from bacterial cultures by the method of Oelmüller et al. (32). Cells of 10- to 50-ml cultures were harvested, washed in 1 ml of SSC (150 mM NaCl, 15 mM sodium citrate [pH 7]), and resuspended in 500 µl of ice-cold AE buffer (20 mM sodium acetate [pH 5.5], 1 mM EDTA). The solution was incubated with 1 ml of hot phenol-chloroform-isomyl alcohol (25:24:1) and 10 µl of 25% [wt/vol] sodium dodecyl sulfate (SDS) for 10 min at 60°C, cooled on ice, and centrifuged at 4°C. The aqueous phase was mixed with 62.5 µl of 2 M sodium acetate (pH 5.5) and 1 ml of phenol-chloroform-isomyl alcohol for 5 min. The aqueous phase was again extracted with 1 ml of phenol-chloroform-isomyl alcohol. After ethanol precipitation of the pellets obtained in 100 µl of DNase buffer (40 mM Tris-HCl [pH 7.5], 6 mM MgCl₂, 2 mM CaCl₂) and incubated with 10 to 40 U of RNase-free DNase (Boehringer) for 30 min at 37°C. The enzyme was removed by phenol-chloroform extraction followed by ethanol precipitation. The resulting RNA pellet was dissolved in 20 to 40 µl of water.

Western blotting and immunodetection of hydrogenase subunits. Cell lysates were prepared by direct lysis of bacteria in a modified sample solution for SDS-polyacrylamide gel electrophoresis (2% [wt/vol] SDS, 10% [vol/vol] glycerol, 100 mM Tris-Cl [pH 6.8], 0.1% [wt/vol] 2-mercaptoethanol). The protein content was determined by the method of Bradford (3) with bovine serum albumin used as a standard. Cell lysates were electrophoresed in 15% [wt/vol] polyacrylamide gels containing 0.2% [wt/vol] SDS (23). Proteins were transferred from the gel to a nitrocellulose membrane (Millipore) for 1 h at 200 mA with a Trans-Blot SD Semi Dry Transfer Cell (Bio-Rad Laboratories, Inc.). The membranes were washed with Tris-buffered saline (TBS) (Tris-HCl [pH 7.4], NaCl, KCl), treated with 10% [vol/vol] horse serum in TBS-0.05% (vol/vol) Tween 20 for at least 1 h, and incubated with antiserum raised against the [NiFe] hydrogenase of D. gigas (E. C. Hatchikian, Centre National de la Recherche Scientifique, Marseille, France) diluted 1:1,000 in TBS–1% (vol/vol) Tween 20. Immunodetection of bound antibodies was done by using anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma no. A3687), diluted 1:3,000, and a subsequent color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt.

Bioreactor samples. Samples were obtained from two experimental bioreactors (Delft University of Technology, Delft, The Netherlands). The methanogenic bioreactor was fed with wastewater from a baker's yeast production plant and oxygen-free inerted biogas from the wastewater treatment plant of a Dutch sugar factory. The denitrifying fluidized bed reactor (28) has been in operation for 5 years and was originally inoculated with methanogenic sludge from the wastewater plant of the Moshe Shilo Foundation (Marseille, France) diluted 1:1,000 in TBS–1% (vol/vol) Tween 20 and 1% (wt/vol) Triton X-100, and 0.5 U of SuperTag DNA polymerase (HT Biotechnology, Ltd.) as previously described (56). The amplification products were analyzed by electrophoresis in 2% (wt/vol) agarose gels.

Confirmation that PCR products obtained after amplification of the RT mix were derived from mRNA and not from contaminating DNA within the RNA preparation was performed as follows: 2 µl of total RNA or 5 µl of the RT mix without reverse transcriptase added was used for PCR.

DGGE analysis. PCR products were separated in 6% (wt/vol) polyacrylamide gels with gradients ranging from 17 to 60% acrylamide containing 15% formamide, 200 V and a temperature of 60°C for 4 h by using a Bio-Rad Protein II system as described previously (56).

Determination of nucleotide sequences. PCR products of pure bacterial cultures were obtained by genomic DNA isolation as previously described (56) using primers Hyd1F without the GC clamp and Hyd5R. Selected DGGE bands were punched from the gel. The DNA was eluted as described elsewhere (30) and reamplified with primer pair Hyd1F-Hyd5R. Prior to sequencing, the PCR products were purified with a Quiquick Spin PCR purification kit (Quaien Inc.). The DNA fragments were directly sequenced with primer Hyd1F and a truncated form of Hyd5R (Hyd5-18, 5'-CGGCCCCGCGCCGGCGCGCGCGCCGCCGCGCGCGGCGGCGCGC-3’) by using the Taq Dye Deoxy Terminator Cycle Sequencing kit and the DNA sequencer 3735S of Applied Biosystems (Foster City, Calif.) in accordance with the manufacturer's directions.

Sequence analysis. The deduced amino acid sequences were obtained from the nucleotide sequence data by using the software program DNA Star Strider 1.2 (26) and corrected by each other manually by using the CLUSTAL W (11). The alignment was improved by using the Genetics Computer Group analysis software package (Biotechnology Center, University of Wisconsin, Madison). The nucleotide sequences were aligned according to the alignment of the deduced amino sequences. Ambiguities in the alignment were obtained by using the “show distance matrix” option in the software program PAUP 3.1 (47).

In estimates of evolutionary relationships, regions with missing data were omitted from the alignment. Matrices of evolutionary distances were computed by using DNA Dist (Kimura option) for nucleotide sequences and PROTDIST (Dayhoff PAM option) for amino acid sequences. Evolutionary trees were constructed from these evolutionary distances with the program FITCH. All programs are implemented in the software package PHYLIP 3.5 (11). The reliability of tree nodes was determined by bootstrap analysis (100 replicates) using SEQBOOT implemented in PHYLIP 3.5 (11) and PAUP 3.1 (47).

Nucleic acid extraction from bioreactor samples. Nucleic acids were recovered directly from the bioreactor samples by the method described above, which was adapted as follows. Ca. 10 g (wet weight) of frozen sludge was mixed with 8 ml of AE buffer, 20 ml of hot phenol-chloroform-isomyl alcohol (25:24:1), and 200 µl of 25% [wt/vol] SDS, shaken for 30 min at 60°C, and cooled on ice. After centrifugation, 200 µl of 0.2 M NaCl was added to a final volume of 0.25 M to the aqueous phase, which was repeatedly extracted with 1 volume of phenol-chloroform-isomyl alcohol (25:24:1) until no protein precipitate was observed at the aqueous-organic interface. Nucleic acids were precipitated with ethanol by either with DNase-free RQase or with RNase-free DNase (Boehringer) to obtain DNA and RNA, respectively.

The quality of the nucleic acid preparations was tested in 2% (wt/vol) agarose gels with MOPS (morpholinepropanesulfonic acid) buffer; their concentration and purity were determined by absorption spectrophotometry (43).

RT of RNA. Prior to PCR amplification, total RNA preparations were transcribed into cDNA in a final volume of 20 µl as follows. A 6.5- to 1-µg sample of RNA and 10 pmol of hexanucleotides (Boehringer) were denatured for 10 min at 70°C and immediately placed on ice. Subsequently, 4 µl of deoxyadenosine triphosphates (2.5 mM each) and 4 µl of 5× reverse transcription (RT) buffer (250 mM Tris-HCl [pH 8.3], 150 mM KCl, 5 mM dithiothreitol) were added. The RT mix was incubated for 2 min at 37°C before 1 µl of Moloney murine leukemia virus H’ reverse transcriptase (200 U/µl; Promega) was added. After incubation for 1 h at 37°C, the samples were heated to 95°C for 5 min and immediately cooled on ice to start the reaction.

PCR amplification. Primers complementary to conserved regions in the [NiFe] hydrogenase large-subunit gene of Desulfovibrio spp. were used to amplify a ca. 440-bp DNA fragment (56) corresponding to positions 1441 to 1879 in the D. vulgaris sequence (8). The nucleotide sequence of these sequences was determined as described previously (56). The amplification products were analyzed by electrophoresis in 2% (wt/vol) agarose gels.

RESULTS

Expression of the [NiFe] hydrogenase gene in pure cultures. [NiFe] hydrogenase mRNA was detected by RT-PCR in exponentially growing cells of Desulfovibrio desulfuricans DSM 1926, as an electron donor in the presence of sulfate (Table 1). However, D. desulfuricans DSM 1926 did not grow with hydrogen as an energy source, as was also demonstrated by Devereux et al. (9). Thus, mRNA was not analyzed.

In addition, D. vulgaris DSM 644, D. desulfuricans DSM...
1926, and *D. baculatus* DSM 2555 were grown in lactate-sulfate medium. Growth, sulfide production, and the expression of the [NiFe] hydrogenase gene were monitored until the cultures reached the stationary phase. Figure 1 shows the results obtained with *D. baculatus*, which are representative for all tested strains. The cultures reached the stationary phase 35 h after inoculation (Fig. 1A). [NiFe] hydrogenase mRNA was detectable in exponentially growing cells at 5 to 30 h after inoculation, as determined by RT-PCR amplification of a ca. 440-bp fragment from equal amounts of total RNA and subsequent agarose gel electrophoresis. As the culture approached the stationary phase, the mRNA level decreased, and mRNA was no longer visible after 45 h (Fig. 1B). The large and the small subunits of the [NiFe] hydrogenase were detected by Western immunoblotting. Antisera raised against the [NiFe] hydrogenase of *D. gigas* (31) cross-reacted with proteins of the correct size for the large (60-kDa) and the small (30-kDa) subunits. In contrast to the RT-PCR-amplified mRNA, no difference in the intensity of stained proteins was observed, indicating that the amount of [NiFe] hydrogenase relative to the total protein content of the cultures did not change during growth over 55 h.

**Detection of [NiFe] hydrogenase gene fragments and mRNA in anaerobic bioreactors.** The presence and expression of the [NiFe] hydrogenase gene of *Desulfovibrio* spp. were investigated in a denitrifying and a methanogenic bioreactor. In parallel, DNA and RNA were isolated and used as templates for PCR and RT-PCR, respectively. The PCR products were analyzed by DGGE to visualize the individual members of the *Desulfovibrio* populations. As previously demonstrated (56), DGGE analysis of amplified [NiFe] hydrogenase gene fragments allows the discrimination of different *Desulfovibrio* strains due to the different migration behavior caused by sequence variations within their PCR products.

The gel electrophoresis analysis of PCR products obtained from samples of the denitrifying bioreactor is shown in Fig. 2. [NiFe] hydrogenase gene fragments were amplified from DNA isolated from a sample taken directly out of the reactor. Positive results were also found for DNA obtained from subsamples that were incubated with hydrogen and oxygen to induce and inhibit gene expression, respectively. The corresponding mRNA was detectable in only the bioreactor subsample which was incubated with hydrogen. Agarose gel electrophoresis of the PCR products revealed that the sizes of all amplified fragments were as expected for the [NiFe] hydrogenase of *Desulfovibrio* spp. (Fig. 2A). DGGE analysis (Fig. 2B) of the amplified gene fragments showed several bands with identical electrophoresis patterns. Only one band in the denaturing gradient gel was observed for the PCR products derived from mRNA. This band showed the same position in the gel as a band in the DGGE pattern derived from amplified DNA. Samples from the methanogenic bioreactor were analyzed as described above. Enrichment cultures of sulfate-reducing bac-

### Table 1. Expression of the [NiFe] hydrogenase gene in *Desulfovibrio* spp. under different growth conditions

| Strain                  | Result with the indicated electron donor
<table>
<thead>
<tr>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>20 mM lactate</td>
</tr>
<tr>
<td><em>D. baculatus</em> DSM 2555</td>
<td>+</td>
</tr>
<tr>
<td><em>D. gigas</em> DSM 1382</td>
<td>+</td>
</tr>
<tr>
<td><em>D. desulfuricans</em></td>
<td></td>
</tr>
<tr>
<td>DSM 1924</td>
<td>+</td>
</tr>
<tr>
<td>DSM 1926</td>
<td>+</td>
</tr>
<tr>
<td><em>D. vulgaris</em> DSM 644</td>
<td>+</td>
</tr>
<tr>
<td>DSM 1744</td>
<td>+</td>
</tr>
</tbody>
</table>

* +, PCR products after RT-PCR; ND, not determined; −, no growth.
The genetic diversity of [NiFe] hydrogenase in anaerobic bioreactors was examined. Amplified [NiFe] hydrogenase gene fragments and mRNA were detected in all tested samples. The DGGE analysis of the PCR products is shown in Fig. 3. The amplified gene fragments from a sample taken directly out of the bioreactor (original sample) and samples of the enrichment cultures harvested 1 day after inoculation produced three distinct bands (Fig. 3). The same electrophoresis pattern was observed for PCR products derived from the [NiFe] mRNA in the original sample and in the enrichment with hydrogen. Conversely, mRNA amplificates from the lactate enrichment showed a weak but obviously different pattern. This pattern became more visible after analysis of PCR products obtained from nucleic acids of cultures that were incubated longer with lactate. Two additional DGGE bands appeared (Fig. 3), while other bands disappeared. The latter enrichment cultures showed an increased production of sulfide compared to cultures harvested earlier.

**Genetic diversity of the [NiFe] hydrogenase of Desulfovibrio spp.** The [NiFe] hydrogenase gene fragments defined by primers Hyd1F and Hyd5R of a variety of characterized *Desulfovibrio* strains were sequenced. On average, 360 nucleotides were determined. The aligned nucleotide sequences (not shown) as well as the deduced amino acid sequences (Fig. 4) were compared. The calculated distance-similarity matrix is given in Table 2. The sequences of most strains showed 70 to 75% similarity. Higher similarities were found only for strain DSM 644 and strain Miyazaki of the species *D. vulgaris*, with 87.5% similarity on the DNA level and 95.5% similarity for the deduced amino acid sequence. *D. sulfodismutans* and *D. fructosovorans* also showed high similarities of 85.9% on the DNA level and 84.8% for the deduced amino acid sequence.

**Genetic diversity of the [NiFe] hydrogenase in anaerobic bioreactors.** Sequence data were also obtained from PCR products of the bioreactor samples that were previously separated by DGGE. The DGGE pattern of amplified [NiFe] gene fragments obtained from samples of the denitrifying bioreactor exhibited five distinct bands (Fig. 2B). They were designated K6S1, K6S2, K6S4, K6S8, and K6S6 (bands in electrophoresis direction from top to bottom). The PCR product derived from mRNA corresponds to K6S8. By sequencing the DNA fragments of several bands, it could be confirmed that PCR products with the same position in the denaturing gradient gels have identical nucleotide sequences.

The nucleotide and deduced amino acid sequences of K6S1, K6S2, K6S4, K6S8, and K6S6 were analyzed by comparative sequence analysis (Table 2). They showed high similarity val-
ues ranging from 88 to 97.6% on the DNA level; similarities of the deduced amino acid sequences were above 90%. The highest similarity with sequences of characterized *Desulfovibrio* spp. was found for *D. sulfodismutans*. The sequence of K6S6 was almost identical to the sequence of that species and differed by only 1 amino acid (99.1% similarity).

For the methanogenic bioreactor, sequences were obtained from PCR products of four different DGGE bands. The selected bands (Fig. 3) were designated as follows: K5S18 in lane 4, K5S14 in lane 6, K5S12 in lane 7, and K5S5 in lane 10. Bands K5S14 and K5S12 showed the same position within the denaturing gradient gel, and identical sequences (named K5S12/14) were determined for those DNA fragments.

The results of the comparative sequence analysis are presented in Table 2. K5S12/14 and K5S18 showed 91.7% similarity with each other. A very low level of variation was observed by comparison with the sequences obtained from the denitrifying bioreactor and therefore also with the [NiFe] hydrogenase sequence of *D. sulfodismutans*. For K6S6 and K5S12/14, identical amino acid sequences were determined as well. However, K5S5, a sequence which was present exclusively in the lactate enrichment cultures, showed only 76.5% similarity to the nucleotide sequence of *D. sulfodismutans* but 87.7% similarity to *D. baculatus* DSM 2555 (Table 2).

**Phylogenetic analysis.** Evolutionary relationships were calculated from the nucleotide and amino acid sequences by using different algorithms, i.e., the distance method and parsimony. Although the constructed trees varied slightly depending on the algorithm and depending on whether DNA (Fig. 5A) or amino acid sequences (Fig. 5B) were used, some general features could be observed. As shown in Fig. 5, the sequences of *D. vulgaris* strains Miyazaki and DSM 644 always grouped...
Sequences named K6 were obtained from a bacterial community within a denitrifying bioreactor; sequences named K5 were obtained from a bacterial community within a methanogenic bioreactor.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Similarity (%)</th>
</tr>
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<tbody>
<tr>
<td>K6</td>
<td>86.7</td>
</tr>
<tr>
<td>K5</td>
<td>86.7</td>
</tr>
</tbody>
</table>

**TABLE 2. Comparison of partial [NiFe] hydrogenase gene sequences amplified from nucleic acids of two different experimental bioreactors and from characterized desulfovibrio strains.**

<table>
<thead>
<tr>
<th>Bioreactor</th>
<th>Similarity (%)</th>
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<tbody>
<tr>
<td>Denitrifying</td>
<td>86.7</td>
</tr>
<tr>
<td>Methanogenic</td>
<td>86.7</td>
</tr>
</tbody>
</table>

The sequences were obtained from a bacterial community within a denitrifying bioreactor; sequences were obtained from a bacterial community within a methanogenic bioreactor. The sequences were determined in this work.

The sequence similarity was performed using the CLUSTALW program. The first value for each sequence is the nucleotide sequence; the second value is the deduced amino acid sequence (in boldface)
together. The same was found for the sequences of *D. vulgaris* DSM 1744 and PIB2 ("*D. oxyclinae*") as well as for *D. baculatus* DSM 2555 and one of the sequences obtained from the methanogenic bioreactor, i.e., sequence K5S5. Sequences K5S12 and K5S18 and those obtained from the denitrifying bioreactor (K6 sequences) consistently formed a cluster with the sequences of *D. sulfodismutans* and *D. fructosovorans*. These groupings, determined with two different algorithms (parsimony and distance matrix), were validated by high bootstrap values for both nucleic acid and amino acid sequences (Fig. 5).

**DISCUSSION**

Expression of the [NiFe] hydrogenase gene in *Desulfovibrio* spp. We investigated the expression of the [NiFe] hydrogenase gene in pure cultures to determine if metabolically active strains, such as growing *Desulfovibrio* spp., can be identified by the detection of the [NiFe] hydrogenase mRNA. Hydrogen metabolism is a central bioenergetic pathway in *Desulfovibrio* spp. that involves hydrogen consumption and production under both sulfate-reducing and fermentative conditions (14). Different classes of hydrogenases, viz., [NiFe], [NiFeSe], and [Fe] hydrogenases, have been identified within the genus (10). Their genes are unevenly distributed among *Desulfovibrio* strains (54). The complexity of the hydrogen metabolism and the hydrogenase system in *Desulfovibrio* spp. makes it difficult to elucidate the physiological roles for the different hydrogenases. However, the [NiFe] hydrogenase gene is present in all *Desulfovibrio* strains tested so far (54). In *Desulfovibrio* spp. in which this is the only hydrogenase gene, the [NiFe] hydrogenase is responsible for all aspects of hydrogen metabolism (15, 31).

We tested various *Desulfovibrio* spp. containing different numbers of hydrogenase genes, including the following: *D. vulgaris* DSM 644, which possesses at least three different hydrogenase genes; *D. desulfuricans* DSM 1926, containing two hydrogenase genes; and *D. gigas*, which contains only the [NiFe] hydrogenase gene (54). Transcription of the [NiFe] hydrogenase gene was demonstrated in all strains growing in hydrogen-sulfate and lactate-sulfate media.

In addition, experiments performed to monitor [NiFe] hydrogenase expression during growth on lactate showed a decrease in [NiFe] hydrogenase mRNA content until no amplified mRNA sequences were visible when the cultures reached the stationary phase. The same expression pattern has been observed by using Northern blotting for the [Fe] hydrogenase of *D. vulgaris* growing with organic substrates; the decrease of the mRNA was accompanied by a decrease in hydrogenase activity (52). In contrast to the mRNA, we found that the amount of the [NiFe] hydrogenase protein remained constant during growth. This might indicate a very stable protein. Studies with *D. vulgaris* showed that even in aged cultures contain-
ing nonviable cells, the hydrogenases could be reactivated by addition of the reducing agent dithionite (4).

We did not measure the hydrogenase activity, since the enzyme assays reflect the total hydrogenase activity and not the activities of the different types of hydrogenases. It has been shown by others that the extent of [NiFe] hydrogenase activity can vary in different Desulfovibrio spp. from less than 5% of the total hydrogenase activity, as demonstrated for D. vulgaris by expression of antisense mRNA (51), to 90% of the total, as shown by marker exchange mutagenesis in D. fructosovorans (42). Furthermore, it has been previously demonstrated that the total hydrogenase activity is correlated with bacterial growth (31, 51, 52).

In summary, our results indicate that the transcription of the [NiFe] hydrogenase gene is correlated with bacterial growth and therefore with the metabolic activity, i.e., sulfate reduction rate, in various Desulfovibrio spp. Thus, the detection of the [NiFe] hydrogenase mRNA appears to be a suitable tool with which to determine metabolically active populations in environmental samples.

Presence and expression of [NiFe] hydrogenase genes in anaerobic bioreactors. We were able to amplify [NiFe] hydrogenase gene fragments from samples of the denitrifying bioreactor as well as from subsamples incubated with H₂ and O₂. Different bands obtained after DGGE analysis of the PCR products suggest the presence of as many Desulfovibrio strains in these samples. Identical electrophoresis patterns for all tested DNA samples indicate that the composition of the Desulfovibrio population was constant over 20 h after addition of H₂ or O₂. However, from the DGGE analysis of PCR products derived from mRNA and their positions in the gel, we conclude that only one strain within the Desulfovibrio population actively transcribed the [NiFe] hydrogenase gene after incubation with H₂.

The presence of different Desulfovibrio spp. within the bacterial community of the methanogenic bioreactor was also shown. In contrast to the strains from the denitrifying bioreactor, all detected Desulfovibrio strains actively transcribed the [NiFe] hydrogenase gene, as demonstrated after DGGE analysis of PCR products derived from DNA and RNA. However, the electrophoresis pattern of the original bioreactor sample differed from the pattern of subsamples that were incubated in lactate-sulfate medium. This indicates that the composition of the Desulfovibrio population had changed during enrichment with lactate. The change of the population and thus the changing environmental conditions could already be observed after 1 day of incubation with lactate, since those samples showed different patterns for amplified gene fragments and mRNA.

Genetic diversity and identity of Desulfovibrio spp. in anaerobic bioreactors. Determination of the genetic relationship and identification of bacteria based on DNA sequences are possible only if enough sequence data are available. Only three [NiFe] hydrogenase sequences, viz., those of D. vulgaris (8), D. fructosovorans (41), and D. gigas (24, 53), were available from the EMBL nucleotide database. Thus, [NiFe] hydrogenase gene fragments from a variety of characterized Desulfovibrio spp. were sequenced to create a framework for the identification of uncultivated Desulfovibrio strains by comparative sequence analysis.

In accordance with the results of Wu and Mandrand (59), who examined the sequences of 30 microbial hydrogenases of various bacterial groups, the [NiFe] hydrogenases of Desulfovibrio spp. form a distinct cluster of related sequences. However, our comparison of partial gene sequences of 16 strains and of the three previously published sequences revealed a great genetic diversity. One branch containing deep lineages of Desulfovibrio spp. was also observed in phylogenetic trees based on 16S rRNA sequences (9). While the [NiFe] hydrogenase sequences of two strains belonging to the species D. vulgaris showed a high percent similarity and formed an evolutionarily related group, which was supported by high bootstrap values, different strains of D. desulfuricans did not. Devereux et al. (9) also found that on the basis of 16S rRNA sequences, some strains of D. desulfuricans were not closely related. They concluded that those strains are misclassified. Thus, the relationship of [NiFe] hydrogenase sequences is largely consistent with the relationship of Desulfovibrio spp. derived from 16S rRNA sequences (9).

[NiFe] hydrogenase sequences obtained from the PCR products of the denitrifying bioreactor showed very high similarities to each other. Such closely related sequences could be due to multiple copies of the gene in one organism. However, there is no evidence for such an organization of [NiFe] hydrogenase operons. Our results indicate the coexistence of closely related strains within the denitrifying bioreactor. On the basis of comparative sequence analysis, they are closely related to the species D. sulfodismutans. The type strain of this species was isolated from freshwater mud (2). D. sulfodismutans is characterized by its ability to conserve energy for growth under anaerobic conditions by disproportionation of thiosulfate or sulfite to sulfate and sulfide. Growth by sulfate reduction is slower than by disproportionation. The species also differs from the classical Desulfovibrio spp. by its inability to utilize pyruvate and its very slow growth on hydrogen. Interestingly, the [NiFe] hydrogenase gene sequence corresponding to the Desulfovibrio strain which actively transcribed the gene after incubation with H₂ (K6S8) showed a high similarity to the sequence of D. sulfodismutans (95.5% for the deduced amino acids). However, K6S8 also showed a high similarity (86.9%) to the sequence of D. fructosovorans. D. fructosovorans, isolated from an estuarine sediment, is a typical H₂ oxidizer, further characterized by its ability to utilize fructose (33).

Since the main metabolic process occurring in the denitrifying bioreactor concerns the conversion of nitrate to dinitrogen gas, it is not surprising that sulfate-reducing bacteria were presumably not active in a sample taken directly from the reactor. Besides, it is well known that nitrate inhibits sulfate reduction (17, 44), and it has been shown that sulfate reduction takes place within the denitrifying bioreactor when nitrate is exhausted (28). On the other hand, several Desulfovibrio spp. can reduce nitrate or nitrite to ammonia (7, 20, 44). However, the closest relatives to the detected strains, D. sulfodismutans and D. fructosovorans, were unable to reduce nitrate (2, 33). According to Dalsgaard and Bak (7), the ability to reduce nitrate might be underestimated for sulfate-reducing bacteria because medium containing more than 0.1 mM sulfide inhibits nitrate reduction. However, sulfide concentrations of more than 2 mM were measured in the influent of the denitrifying bioreactor (28), and thus it is unlikely that the Desulfovibrio populations were directly involved in nitrogen metabolism.

In contrast, within methanogenic bioreactors sulfate-reducing bacteria and methanogenic archaea usually live in close association (34). The wastewater of the baker’s yeast production plant contains high concentrations of sulfate, which is partly converted to sulfide in the methanogenic reactor (28). Hence, it is not surprising that the Desulfovibrio spp. present in a sample taken directly from a methanogenic bioreactor were actively transcribing the [NiFe] hydrogenase gene. Members of the detected population were also identified as D. sulfodismutans on the basis of their partial [NiFe] hydrogenase sequences. An additional hint that those strains were indeed similar to D. sulfodismutans arises from the inability to enrich
them by using lactate-sulfate medium. Within the enrichments, strains containing [NiFe] hydrogenase sequences similar to that of D. baculatus DSM 2555 were found. DSM 2555 was isolated from an anaerobic intertidal sediment of the Ems-Dollard estuary on the border between The Netherlands and Germany (22). Our results present another example demonstrating the limitation of culture-dependent approaches to describing the structure of microbial communities.

In summary, closely related strains probably belonging to the species D. sulfosidismutans were found in the denitrifying bio-reactor as well as in the methanogenic bio-reactor, where they were metabolically active. The coexistence of closely related bacterial strains in environmental samples was also suggested by Ueda et al. (49) in a study of N2-fixing bacteria. They obtained nifD sequences from the rice rhizosphere and found a low level of genetic diversity within sequence clusters. The same was also demonstrated by Xu and Tabita (61) for the rbcL gene of CO2-fixing phytoplanktonic organisms in Lake Erie.

The concept of mRNA detection for the study of microbial activity. The detection of mRNA is a promising tool for monitoring the activity of microbes in the environment. The idea of this approach is that the abundance of a transcript may be related to the state of microbial activity. Such molecular approaches might serve to resolve microbial processes at the levels of specific populations, species, or even strains, whereas traditional methods, for example, radiotracer techniques, reflect microbial activity only at the community level. Northern blotting and mRNA probing as well as RT-PCR have been applied for this purpose (37, 60, 61). In consideration of the main problems arising in studies of gene expression in the environment, Pichard and Paul (38) developed the concept of gene expression per gene dose. In order to account for changes in activity caused by increased transcription or changes in relative gene abundance caused, for example, by changes in cell population size, they suggested a hybridization approach to determine the levels of both specific mRNA and DNA.

Accordingly, we also used RNA as well as DNA as starting material for PCR amplification of [NiFe] hydrogenase gene nucleotide sequences. After DGGE analysis of the PCR products, we could distinguish the Desulfovibrio strains that expressed the gene, which we regarded as metabolically active, from the inactive strains. However, a disadvantage of a PCR-based method concerns the quantitation of transcript and gene abundances. Although quantitative PCR has been applied in the environmental context (36), a cautious use of PCR for quantitative purposes is indicated. Preferential amplification has been described, and factors determining the efficiency of PCR in complex DNA mixtures, as are common in the environment, are less well understood (46). Thus, we did not quantify the mRNA or target DNA in environmental samples explicitly and interpreted our results only in a semiquantitative way. Assuming a constant PCR bias for a specific sequence, the appearance and disappearance of a DGGE band should reflect the increase or decrease of the corresponding mRNA or gene(s).

In defining the function of sulfate-reducing bacteria in natural populations, it would be interesting to study in addition to the [NiFe] hydrogenase gene the expression of genes directly involved in sulfate reduction. Recently, a PCR assay for the dissimilatory sulfite reductase of sulfate-reducing bacteria was described (18). Transcripts of the gene encoding this enzyme could indicate organisms performing sulfate reduction, whereas transcripts of the [NiFe] hydrogenase gene probably indicate a general metabolic activity.

Examination of the presence and expression of genes in parallel, as described in this paper, is well suited to investigation of the dynamics of bacterial populations in the environment and their response to changing conditions. In addition to the application of more-classical approaches in microbial ecology, such as the use of radiotracer techniques and microelectrodes and the isolation of bacteria, the molecular approach will enable a better understanding of the structure and function of microbial communities.

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