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Genetic Diversity of *Desulfovibrio* spp. in Environmental Samples Analyzed by Denaturing Gradient Gel Electrophoresis of [NiFe] Hydrogenase Gene Fragments

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The genetic diversity of *Desulfovibrio* species in environmental samples was determined by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified [NiFe] hydrogenase gene fragments. Five different PCR primers were designed after comparative analysis of [NiFe] hydrogenase gene sequences from three *Desulfovibrio* species. These primers were tested in different combinations on the genomic DNAs of a variety of hydrogenase-containing and hydrogenase-lacking bacteria. One primer pair was found to be specific for *Desulfovibrio* species only, while the others gave positive results with other bacteria also. By using this specific primer pair, we were able to amplify the [NiFe] hydrogenase genes of DNAs isolated from environmental samples and to detect the presence of *Desulfovibrio* species in these samples. However, only after DGGE analysis of these PCR products could the number of different *Desulfovibrio* species within the samples be determined. DGGE analysis of PCR products from different bioreactors demonstrated up to two bands, while at least five distinguishable bands were detected in a microbial mat sample. Because these bands most likely represent as many *Desulfovibrio* species present in these samples, we conclude that the genetic diversity of *Desulfovibrio* species in the natural microbial mat is far greater than that in the experimental bioreactors.

Our knowledge of the diversity of sulfate-reducing bacteria (SRB) has increased during the last decade (41). It was long thought that sulfate reduction was the only metabolic process for SRB to oxidize organic compounds in anoxic environments. However, it was recently shown that dissimilatory metal reduction might be another important process for SRB to obtain energy for growth (7, 20, 21). In addition to this novel recognized form of metabolic activity, it has been suggested by several researchers that SRB, which are considered obligate anaerobes, might even respire under aerobic conditions (5, 16). Moreover, it was shown that some SRB use hydrocarbons as a nutritional carbon source (2, 28). So, apart from the phylogenetic diversity of SRB, their metabolic versatility is also much greater than previously realized. However, so far, neither the ecological significance of this metabolic flexibility nor a possible relationship between metabolic and phylogenetic diversity has been investigated. Parallel with our increasing comprehension of physiological diversity, our view of the phylogeny of the SRB has changed also. Sulfate-reducing microorganisms can be found within the *Archaea* domain as well as in different lineages of the *Bacteria* domain, i.e., the proteobacteria and gram-positive bacteria (13).

The use of molecular techniques, combined with the geochemical analysis of environmental parameters and the physiological characterization of isolated strains, might help us to obtain a better understanding of the importance of the different metabolic activities of SRB in nature and might allow us to perceive the niche differentiation for the different members of this important group of bacteria. rRNA-targeted oligonucleotide probes specific for SRB in general or subgroups of the SRB have been used successfully in the detection of these microorganisms in a variety of environments (3, 4, 11, 29, 30). They have been used to determine the distribution of different SRB groups in microbial mats from Baja California (30) and in photosynthetic biofilms isolated from a wastewater trickling filter (29). In the latter study, the researchers combined the molecular approach with the application of microelectrodes to profile oxygen and sulfide within the biofilms.

Apart from these rRNA-targeted probes, functional gene probes have been used to detect SRB. Voordouw et al. (37) used hydrogenase gene probes to detect *Desulfovibrio* species in environmental samples. In later studies, Voordouw et al. described a technique called reverse sample genome probing to identify different SRB in oil field samples (38–40).

Here, we present another approach to detect SRB in environmental samples and to perceive their ecological role. This approach consists of a combination of PCR (32), to amplify the [NiFe] hydrogenase gene from *Desulfovibrio* species, and denaturing gradient gel electrophoresis (DGGE) (1, 15, 25). We have chosen the [NiFe] hydrogenase gene, because it encodes an enzyme which plays an important role in the hydrogen metabolism of SRB (36) and in the dissimilatory metal reduction by SRB (20, 21) and because it is present in all *Desulfovibrio* species (37). This makes it an excellent molecular marker to study the distribution of *Desulfovibrio* species in natural samples, as well as their metabolic activity. We have applied DGGE of the PCR products of this gene to discriminate between different *Desulfovibrio* species in environmental samples. In DGGE, DNA fragments of the same length but with different base pair sequences, such as PCR fragments obtained from a mixture of target DNAs, can be separated. The technique was recently introduced in the field of molecular microbial ecology to analyze the genetic diversity of bacterial populations (23) and to identify the phylogenetic affiliation of individual population members (22). This report describes the first step in our goal to perceive the niche differentiation of *Desulfovibrio* species in natural samples. It describes the application of DGGE analysis of PCR-amplified [NiFe] hydroge-

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nase fragments to determine the genetic diversity of *Desulfovibrio* species in natural samples.

MATERIALS AND METHODS

Bacteria and environmental samples. A wide variety of hydrogenase-containing and hydrogenase-lacking bacteria were used in this study (see Table 2). LTK4, a *Desulfobulbus*-like bacterium, was isolated from a sediment along the Danish coast by Mai Isaksen (Århus University, Århus, Denmark). The SRB PIB2 was isolated from the microbial mat of Solar Lake (Sinai, Egypt) by Yehuda Cohen (The Moshe Shilo Center for Marine Biogeochemistry, Jerusalem, Israel). A microbial mat sample was obtained from the Slufter sediment on the island of Texel (The Netherlands). Bacterial biofilms isolated from experimental bioreactors, which were kept under aerobic or anaerobic conditions, were provided by Luc Tijhuis (Delft University of Technology, Delft, The Netherlands).

DNA extraction. Bacterial DNA was obtained either by freeze-thawing of bacterial cell pellets or by using the following procedure. Bacterial cells, resuspended in 500 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8), were incubated with lysozyme at 37°C for 30 min. Subsequently, sodium dodecyl sulfate (SDS) and proteinase K were added and incubated at 55°C for 2 h. An equal volume of Tris-saturated phenol was added and mixed carefully. After centrifugation, the aqueous phase was transferred to a clean tube and extracted with an equal volume of a mixture of phenol-chloroform-isoamyl alcohol (25:24:1 [by volume]). This process was repeated until no protein precipitate was observed at the aqueous-organic interface. Then 0.1 volume of 3 M sodium acetate (pH 5.5) and 2.5 volumes of ice-cold ethanol were added to the aqueous phase and incubated for 30 min at −80°C. The precipitated DNA was rinsed with 70% (vol/vol) ethanol, dried under vacuum, and dissolved in TE buffer. The concentration and purity of the DNA preparations were determined by absorption spectrophotometry. These preparations were subsequently used as template DNAs in the PCR (32) to amplify the [NiFe] hydrogenase gene.

PCR amplification of the [NiFe] hydrogenase gene. PCR amplifications were performed with a Techne PHC-3 Temperature Cycler (Techne, Cambridge, United Kingdom) as follows: 10 to 100 ng of purified genomic DNA or 1 to 5 μl of cell lysate, 25 pmol each of the appropriate primers, 200 μmol of each deoxyribonucleoside triphosphate, and 5 μl of 10× PCR buffer (100 mM Tris-HCl [pH 9], 15 mM $MgCl_2$, 500 mM KCl, 0.1% [wt/vol] gelatin, 1% [vol/vol] Triton X-100) were added to a 0.5-ml volume test tube which was filled to a volume of 50 μl with sterile water (Sigma Chemicals Co., Ltd.) and overlaid with 2 drops of mineral oil (Sigma Chemicals Co., Ltd.). To minimize nonspecific annealing of the primers to nontarget DNA, the SuperTaq DNA polymerase (HT Biotechnology, Ltd.) was added to the reaction mixture after the denaturing step (94°C, 5 min), at a temperature of 80°C. In addition, to increase the specificity of the amplification and to reduce the formation of spurious byproducts, a "touchdown" PCR (14) was performed. A touchdown PCR is PCR in which the annealing temperature is set 10°C above the expected annealing temperature (70°C) and decreased by 1°C every second cycle until a touchdown of 60°C, at which temperature 10 additional cycles were carried out. Primer extension was carried out at 72°C for 3 min.

The amplification products were analyzed by electrophoresis in 2% (wt/vol) Nusieve agarose (FMC) gels containing ethidium bromide (0.5 μg/ml).

DGGE analysis. DGGE was performed with a Bio-Rad Protean II system, as described previously (23, 24). PCR samples were applied directly onto 6% (wt/vol) polyacrylamide gels in 0.5× TAE (20 mM Tris-acetate [pH 7.4], 10 mM acetate, 0.5 mM Na_2EDTA) with gradients which were formed with 6% (wt/vol) acrylamide stock solutions (acrylamide-*N*,*N*′-methylene-bis-acrylamide, 37:1) and which contained 0 and 100% denaturant (7 M urea and 40% [vol/vol] formamide, deionized with AG501-X8 mixed bed resin [Bio-Rad Laboratories, Inc.]). Electrophoresis was performed at a constant voltage of 200 V and a temperature of 60°C. After electrophoresis, the gels were incubated for 15 min in Milli-Q water containing ethidium bromide (0.5 mg/liter), rinsed for 10 min in Milli-Q water, and photographed with UV transillumination (302 nm) with Cybertech CS1 equipment.

Electroblotting of agarose gels. After electrophoresis, the gels were allowed to equilibrate in 0.5× TBE (45 mM Tris-borate [pH 8], 45 mM boric acid, 1 mM Na_2EDTA) for 15 min. The electrophoresis patterns were transferred to a nylon membrane (Hybond N^+; Amersham, Amersham, United Kingdom) with a Trans-Blot SD Semi Dry Transfer Cell (Bio-Rad Laboratories, Inc.). Electrotransfer was performed for 10 min at a constant current of 3.55 mA/cm^2. Immediately after being transferred, the membrane was incubated for 15 min in 0.4 M NaOH–0.6 mM NaCl solution to denature the DNA. It was neutralized by rinsing twice in a large volume of 2.5× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate) and was subsequently exposed for 45 s to 302-nm-wavelength UV light to cross-link the DNA fragments to the membrane.

Hybridization analysis. The membrane was preincubated for approximately 3 h at the hybridization temperature with 25 ml of hybridization solution (5× SSC, 2% [wt/vol] blocking reagent [Boehringer Mannheim Biochemicals], 0.1% [wt/vol] *N*-lauroylsarcosine, 0.02% [wt/vol] SDS). Oligonucleotide Hyd4 (100 pmol) (Fig. 1) was labelled with digoxigenin at its 3′ end by incorporation of a single digoxigenin (DIG)-labelled nucleotide, using the enzyme terminal transferase. The chemicals for this labelling reaction were obtained from Boehringer Mannheim Biochemicals, and the reaction was performed according to the manufacturer's instructions.

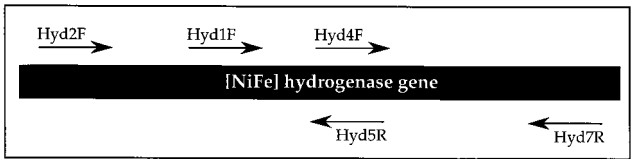

FIG. 1. Schematic diagram of the primer positions in the [NiFe] hydrogenase gene of *D. vulgaris*. The following primer pairs were used to amplify the [NiFe] hydrogenase gene: Hyd2F-Hyd7R, Hyd1F-Hyd7R, Hyd4F-Hyd7R, Hyd2F-Hyd5R, Hyd1F-Hyd5R. In addition, Hyd4F was labelled with DIG and used as a probe in hybridization experiments to identify amplified hydrogenase sequences.

The labelled probe was added to 6 ml of hybridization solution and incubated overnight at 50°C. After hybridization, the membrane was washed twice at the hybridization temperature for 15 min with 50 ml of a solution containing 2× SSC–0.1% (wt/vol) SDS and twice with 50 ml of a solution containing 0.1× SSC–0.1% (wt/vol) SDS. The membrane was rinsed with washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% [vol/vol] Tween 20) and incubated for 30 min with blocking solution containing 1% (wt/vol) blocking reagent, 0.1 M maleic acid, and 0.15 M NaCl (pH 7.5). The DIG-labelled oligonucleotide probe was subsequently detected by an enzyme-linked immunoassay with 4 μl of anti-DIG alkaline phosphatase conjugate in 40 ml of the blocking solution. After 30 min of incubation, the membrane was washed twice for 15 min with washing buffer and equilibrated for 5 min with 50 ml of a solution containing 0.1 M Tris-HCl, 0.1 M NaCl, and 50 mM $MgCl_2$ (pH 9.5). A subsequent enzyme-catalyzed reaction with the chemiluminescent reagent CSPD (Tropix, Inc.) allows the detection of the hybrids with X-ray film (Kodak) (17).

RESULTS

Design of PCR primers. Three [NiFe] hydrogenase sequences, viz., those of *Desulfovibrio vulgaris* (8), *Desulfovibrio fructovorans* (31), and *Desulfovibrio gigas* (19) which were available from the EMBL nucleotide database, were aligned to each other by using the BESTFIT program in the Sequence Analysis Software Package of the University of Wisconsin Genetics Computer Group (9). From these aligned sequences, seven consensus sequences were retrieved, five of which were used in this study. Figure 1 shows a schematic drawing of the positions of the primers. Table 1 gives the primer sequences and the exact locations. The primer sequences were compared with all of the sequences stored in the EMBL database by using the FASTA search program (26, 27). Significant similarity values were found with hydrogenase sequences only.

PCR of the [NiFe] hydrogenase gene from pure cultures. Subsequently, these primers were used to amplify the [NiFe] hydrogenase gene from genomic DNA of *D. vulgaris* DSM 644. Figure 2 shows an ethidium bromide-stained agarose gel with PCR products obtained with primer pairs Hyd2F-Hyd7R, Hyd1F-Hyd7R, Hyd4F-Hyd7R, Hyd2F-Hyd5R, and Hyd1F-Hyd5R (see Fig. 1 for the relative positions of the primers). The sizes of the products are in good agreement with the sizes calculated from the published sequence (8), namely, 2,070, 1,440, 1,090, 1,070 and 440 bp, respectively.

To explore the specificity of the primer pairs, we used them in a PCR to amplify the hydrogenase gene from genomic DNAs of a variety of hydrogenase-containing as well as hydrogenase-lacking bacteria. In this experiment, we tested 10 *Desulfovibrio* spp., 11 representatives from the six other SRB groups (10), as well as the gram-positive SRB *Desulfotomaculum orientis*. Furthermore, we tested 2 new SRB isolates and 12 non-SRB from which several have a homologous [NiFe] hydrogenase gene. For a positive control for the presence of DNA, we amplified the 16S rRNA genes of the bacteria (results not shown). The results of the hydrogenase PCR exper-

TABLE 1. Primer sequences and positions

Primer[a]	Position[b]	Sequence
Hyd2F	811–829	5′-CCGG(C/T)TGCCCGCC(G/C)AACCC-3′
Hyd1F	1441–1467	5′-CGCGACGCCCAGCACTTCACCCAGCGC-3′
Hyd4F	1786–1815	5′-TTCACCAA(C/T)GCCTACTTCCT(G/C)GGCGGCCAC-3′
Hyd5R	1844–1879	5′-GCAGGGCTTCCAGGTAGTGGGCGGTGGCGATGAGGT-3′
Hyd7R	2859–2878	5′-CGCAGGCGATGCA(G/C)GGGTC-3′
GC clamp[c]		5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′

[a] The forward and reverse primers are indicated by the last letters F and R, respectively.
[b] Positions in the [NiFe] hydrogenase gene of *D. vulgaris* (8).
[c] The GC clamp is attached to the 5′ end of the Hyd1F primer.

iments are summarized in Table 2. Amplification products were mainly found for all primer pairs and the *Desulfovibrio* species. PCR products were also obtained with the new isolate PIB2, while no product was obtained with isolate LTK4. One primer pair, i.e., Hyd1F-Hyd5R, was most specific; it gave PCR products only with genomic DNAs of *Desulfovibrio* spp. and not with those of other bacteria. The other primer pairs also amplified DNA sequences from some of the other bacteria, even from bacteria which do not use hydrogen, such as *Desulfococcus multivorans* and *Desulfoarculus baarsii*. Voordouw et al. (37) also found a weak, but reproducible hybridization signal for these species using hydrogenase gene probes.

To reduce the formation of spurious by-products, we used a so-called touchdown PCR protocol (14), whereby the annealing temperature is set at 70°C, 10°C above the expected annealing temperature of 60°C, and decreased for 1°C every second cycle until a touchdown of 60°C, at which temperature 10 additional cycles are carried out. However, by using this highly stringent PCR protocol and primer pair Hyd1F-Hyd5R, we obtained a low yield of PCR product for *D. vulgaris* DSM 1744 and no product for *Desulfovibrio longus* and *Desulfovibrio salexigens*. A better result, i.e., a higher yield of PCR product for *D. vulgaris* DSM 1744 and a positive signal with *D. longus*, was obtained when we used a less stringent PCR protocol, i.e., a touchdown from 65 to 55°C. However, at this annealing temperature, PCR products with other bacteria were also found, although they never had the same size as those obtained from the *Desulfovibrio* species. With this low stringent PCR protocol, it was not possible to obtain a PCR product for *D. salexigens* with this primer pair. A positive signal for this species was obtained only with primer pair Hyd2F-Hyd7R.

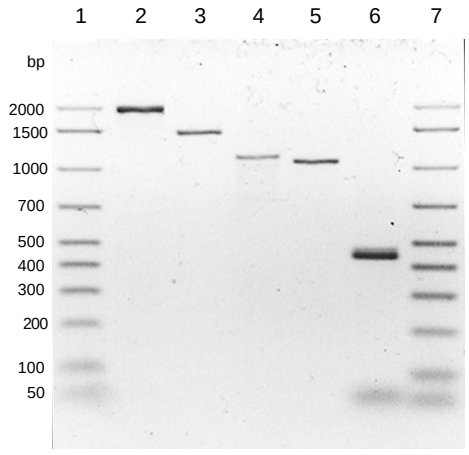

FIG. 2. Negative image of an ethidium bromide-stained agarose gel with DNA fragments obtained after enzymatic amplification of the [NiFe] hydrogenase gene from genomic DNA of *D. vulgaris* DSM 644 using the following primer pairs: Hyd2F-Hyd7R (lane 2), Hyd1F-Hyd7R (lane 3), Hyd4F-Hyd7R (lane 4), Hyd2F-Hyd5R (lane 5), and Hyd1F-Hyd5R (lane 6). DNA size markers (Bio-Rad) were applied to lanes 1 and 7.

Amplification of [NiFe] hydrogenase genes from environmental DNA. Further experiments were performed with primer pair Hyd1F-Hyd5R only and a touchdown PCR protocol from 70 to 60°C. These primers were used to amplify the [NiFe] hydrogenase gene from environmental samples. Figure 3A shows an ethidium bromide-stained agarose gel with PCR products obtained from bacterial genomic DNA isolated from different environmental samples, such as a microbial mat, and experimental wastewater treatment reactors. Positive results were found with genomic DNAs from three different anaerobic biofilms (Fig. 3, lanes 3, 6, and 7) and from the microbial mat sample (Fig. 3, lane 5); DNA isolated from a bacterial biofilm grown under aerobic conditions (Fig. 3, lane 4) gave no PCR product. All of the PCR-amplified fragments had the same size, i.e., 440 bp, which was identical to the PCR product amplified from the genomic DNAs from two pure cultures, *D. vulgaris* DSM 644 (Fig. 3, lane 1) and *D. desulfuricans* DSM 1926 (Fig. 3, lane 9), respectively.

Hybridization analysis with a [NiFe] hydrogenase probe. To support the specificity of this hydrogenase gene amplification, we performed a hybridization analysis of the agarose separation pattern using the DIG-labelled probe Hyd4F, for which the target sequence is located within the amplified fragment (Fig. 1). Figure 3B shows a photograph of the chemiluminescence results of this hybridization analysis. All of the expected hydrogenase bands were stained, while the DNA size standards were not.

Theoretical melting behavior. DGGE was used to separate the putative hydrogenase gene fragments amplified from the environmental samples. To optimize this DGGE analysis, we used a software program called Melt87, developed by L. S. Lerman and E. Hovig. This program determines a theoretical melting map for a known sequence (18). Figure 4 shows a melting map for the 440-bp [NiFe] hydrogenase sequence of *D. vulgaris* (solid line). From this map we could discern three melting domains: one from positions 1 to 200 with a melting temperature of about 81°C, one from positions 201 to 300 with a melting temperature of about 84°C, and one from positions 301 to 440 with a mean melting temperature of about 78°C. The melting temperatures of these three domains are so close to each other that they might melt one after the other very quickly, resulting in two single-stranded molecules, which will not stop in the denaturing gradient gel. The dashed line shows the melting map for the hydrogenase sequence with an attached 40-bp GC-rich sequence at its 5′ end (33). Here we also notice three melting domains; one from positions −40 to 1 with a melting temperature of about 95°C, created by the

TABLE 2. Specificity of the [NiFe] hydrogenase PCR[a]

Species or isolate	Source	Hydrogen metabolism[b]	PCR products[c] determined					
			Hyd2F-Hyd7R	Hyd1F-Hyd7R	Hyd4F-Hyd7R	Hyd2F-Hyd5R	Hyd1F-Hyd5R	Hyd1F-Hyd5R[d]
Desulfovibrio vulgaris	DSM 1744	+	+	+	+	±	±	+
	DSM 644	+	+	+	+	+	+	+
Desulfovibrio baculatus	DSM 2555	+	+	+	+	+	+	+
Desulfovibrio desulfuricans	DSM 1926	+	+	+	+	+	+	+
	DSM 1924	+	+	+	+	+	+	+
	Vosjan	+	−	−	−	−	+	+
Desulfovibrio gigas	DSM 1382	+	+	+	+	+	+	+
Desulfovibrio sulfodismutans	DSM 3696	+	+	+	+	+	+	+
Desulfovibrio salexigens	DSM 2638	+	+	−	−	−	−	−
Desulfovibrio longus	DSM 6739	+	+	+	+	±	−	±
Desulfobacterium autotrophicum	DSM 3382	+	−	−	−	−	−	−
Desulfobacterium vacuolatum	DSM 3385	+	−	−	−	−	−	−
Desulfobulbus sp.	DSM 2058	+	−	−	−	−	−	−
Desulfosarcina variabilis	DSM 2060	+	+	−	*	*	−	−
Desulfotomaculum orientis	DSM 765	+	−	−	−	−	−	−
Desulfobacter curvatus	DSM 3379	+	−	−	−	−	−	−
Desulfobacter postgatei	DSM 2553	−	−	−	−	−	−	−
Desulfobacter latus	DSM 3381	−	−	−	−	−	−	−
Desulfobotulus sapovorans	DSM 2055	−	−	−	−	−	−	−
Desulfoarculus baarsii	DSM 2075	−	+	+	*	−	−	*
Desulfococcus multivorans	DSM 2059	−	*	−	−	*	−	*
LTK4	Isaksen	−	−	−	−	−	−	−
PIB2	Cohen	+	+	+	±	+	+	+
Rhodocyclus gelatinosus	DSM 1709	+	*	−	*	+	−	*
Rhodopseudomonas palustris	DSM 123	+	−	−	−	−	−	−
Rhodobacter capsulatus	DSM 1709	+	*	−	+	−	−	*
Escherichia coli	Goosens	+	+	−	−	−	−	*
Alcaligenes eutrophus	Friedrich	+	*	−	−	−	−	−
Pseudomonas facilis	DSM 649	+	−	−	−	−	−	−
Xanthobacter autotrophicus	DSM 432	+	+	−	−	−	−	*
Paracoccus denitrificans	DSM 1404	+	+	−	−	−	−	−
Pseudomonas stutzeri Zobell	Zumft	−	*	−	*	*	−	*
Bacillus cereus	Thauer	−	−	ND	−	−	−	−
Bacillus sp.	de Vrind	−	−	−	−	−	−	*
Leptothrix discophora	Ghiorsi	−	−	−	ND	−	−	−

[a] Touchdown reaction from 70 to 60°C used.
[b] +, yes; −, no.
[c] +, PCR product of the expected size: −, no PCR product; ±, low yield of PCR product; *, several PCR products or bands with a different size; ND, not determined.
[d] Touchdown reaction from 65 to 55°C used.

GC-rich sequence, one from positions 1 to 300 with a melting temperature of 82°C, and a melting domain from positions 301 to 440, with an identical melting temperature as for the sequence without the GC clamp. Here the actual hydrogenase sequence (positions 1 to 440) is melted rapidly, but the two strands will not dissociate completely, because they are held together by the GC clamp, which has a melting temperature of about 95°C. Only these partially melted fragments will stop in the denaturing gradient gel.

From these maps we can also see that the pattern of melting domains is a property of the entire fragment: adding a GC clamp to one end of the fragment alters the melting in much of the fragment (1).

Perpendicular DGGE analysis of the [NiFe] hydrogenase gene fragment. The experimental evidence for this melting behavior was established by a so-called perpendicular DGGE analysis (25). Figure 5 shows an ethidium bromide-stained polyacrylamide gel of a perpendicular DGGE analysis of two PCR-amplified [NiFe] hydrogenase gene fragments from *D. vulgaris* DSM 644. One fragment had a GC clamp attached to its 5′ end, the other had no clamp. At low concentrations of DNA denaturants, from 0% to approximately 50% denaturants, the fragment still has its double-stranded helical conformation and migrates according to its size. At a concentration of about 50% denaturants, the fragment starts to melt, resulting in a reduction in mobility. However, the fragments without GC clamp show no stable melting behavior; they melt in two single-stranded DNA molecules, which gave a fuzzy appearance in the perpendicular gel (Fig. 5). To obtain a stable melting behavior, we attached a 40-bp GC-rich sequence to the forward PCR primer Hyd1F. A melting curve similar to that for the fragment amplified without the GC clamp primer was visible; however, the two DNA strands did not melt apart completely at high denaturant concentrations (above 50% denaturants), because the GC clamp held them together. From this perpendicular DGGE analysis, we were able to define a narrower gradient of denaturants, i.e., 30 to 70%, to obtain a better resolution in the separation of different sequences in parallel denaturing gradient gels.

Determination of the optimal electrophoresis time. To determine the length of electrophoresis time, we performed a so-called "time travel" experiment (23). Figure 6 shows the results of this experiment. Two samples, PCR fragments of *D. gigas*, one with and one without a GC clamp, were loaded at

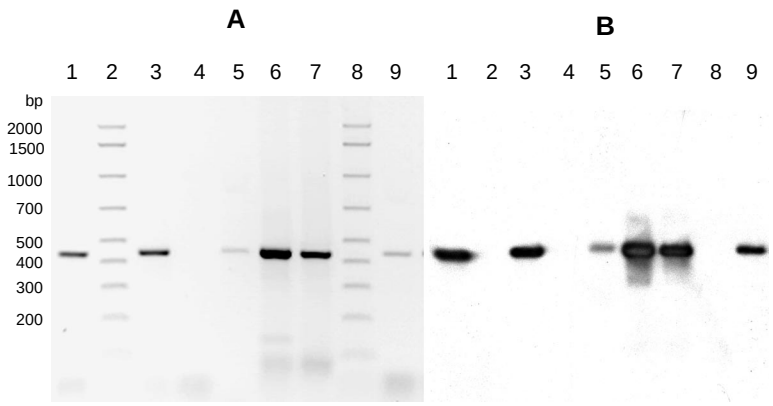

FIG. 3. Detection of *Desulfovibrio* spp. in environmental samples by PCR amplification of the [NiFe] hydrogenase gene using primer pair Hyd1F-Hyd5R. (A) Negative image of an ethidium bromide-stained agarose gel with PCR products obtained from DNAs from bacteria isolated from a microbial mat (lane 5), bacteria grown under anaerobic conditions in wastewater treatment reactors (lanes 3, 6, and 7), bacteria grown under aerobic conditions in a wastewater treatment reactor (lane 4). Lanes 1 and 9 represent the PCR products obtained after amplification of the genomic DNAs from *D. vulgaris* DSM 644 and *D. desulfuricans* DSM 1926, respectively. Lanes 2 and 8 contain DNA size markers (Bio-Rad). (B) Results after hybridization analysis using the DIG-labelled oligonucleotide probe Hyd4F, whose target sequence is located within the amplified region.

30-min intervals for up to 5 h onto a polyacrylamide gel containing a 30 to 70% linear gradient of denaturants. Between 120 and 150 min of electrophoresis, the PCR fragment with GC clamp starts to melt and stopped abruptly in the electrophoresis, while the PCR fragment without the GC clamp melts completely into two single strands and did not stop. Further experiments were therefore performed with the GC clamp PCR primer, a gradient of 30 to 70% denaturants, and an electrophoresis time of 4 h to obtain good separation between PCR fragments of different *Desulfovibrio* species.

DGGE analysis of [NiFe] hydrogenase gene fragments from pure cultures. The resolution of this hydrogenase DGGE analysis was first tested with amplified PCR fragments obtained from pure cultures of *Desulfovibrio* species. The [NiFe] hydrogenase genes of seven different *Desulfovibrio* species, i.e., *D. sulfodismutans*, *D. gigas*, *D. vulgaris* DSM 644, *D. desulfuricans* DSM 1924, *D. baculatus*, SRB isolate PIB2, and *D. desulfuricans* DSM 1926, were amplified with primer pair Hyd1F-Hyd5R with GC clamp, and loaded onto a polyacrylamide gel containing a linear increasing gradient from 30 to 70% denaturants. As is shown in Fig. 7, we were able to separate the amplified hydrogenase fragments from these species on a polyacrylamide gel containing a gradient of DNA denaturants.

Genetic diversity analysis of environmental samples. In addition, we analyzed the [NiFe] hydrogenase sequences amplified from four different environmental samples, i.e., from three different wastewater treatment reactors and from a microbial mat. For two of the bioreactors, we obtained one band (Fig. 7, lanes 8 and 10, respectively); for the other bioreactor sample, we found two bands (Fig. 7, lane 9). There were at least five bands present in the microbial mat sample (Fig. 7, lane 11).

DISCUSSION

Here we describe a molecular approach to analyze the genetic diversity of *Desulfovibrio* species in environmental samples. Other molecular methods have also been attempted to

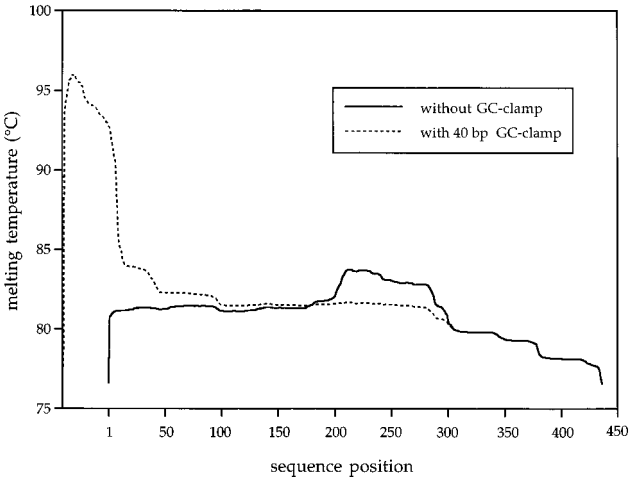

FIG. 4. Theoretical melting map for the *D. vulgaris* [NiFe] hydrogenase sequence located between primers Hyd1F and Hyd5R. The solid line represents the melting map of the sequence without an attached 40-bp GC-rich sequence. The dashed line represents the melting map of the same sequence but with a 40-bp GC-rich sequence attached to the 5′ end.

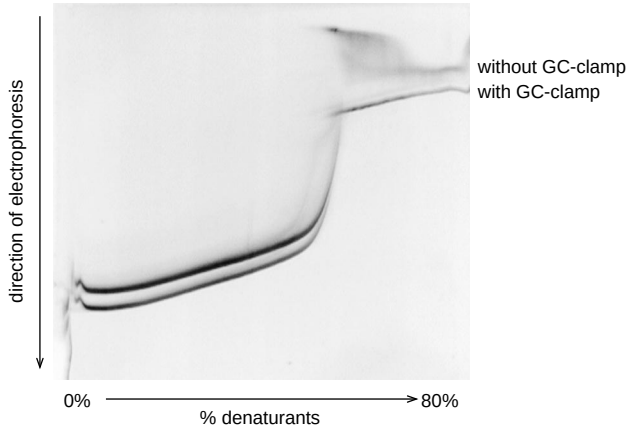

FIG. 5. Negative image of an ethidium bromide-stained perpendicular DGGE separation pattern of PCR-amplified [NiFe] hydrogenase gene fragments from *D. vulgaris* DSM 644 obtained with primer pair Hyd1F-Hyd5R without GC clamp and with GC clamp. The fragment without GC clamp shows no stable melting behavior; it falls apart in two single-stranded molecules at high denaturant concentrations.

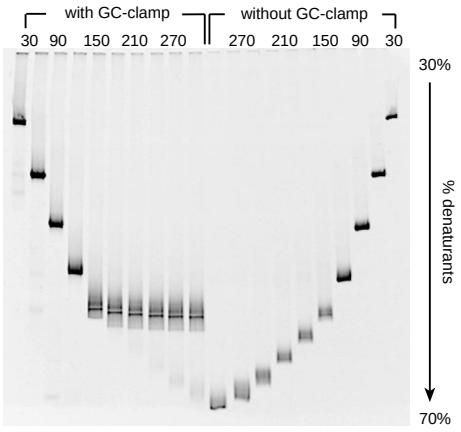

FIG. 6. "Time travel" experiment. The figure shows a negative image of an ethidium bromide-stained DGGE separation pattern of PCR-amplified [NiFe] hydrogenase fragments from *D. gigas* with GC clamp and without GC clamp. The different samples were loaded onto the gel every 30 min (30′) for a total of 5 h. Note that the fragments with GC clamp stop after about 2.5 h and do not move further into the gel even after 5 h of electrophoresis. The fragments without GC clamp, however, slow down but do not stop.

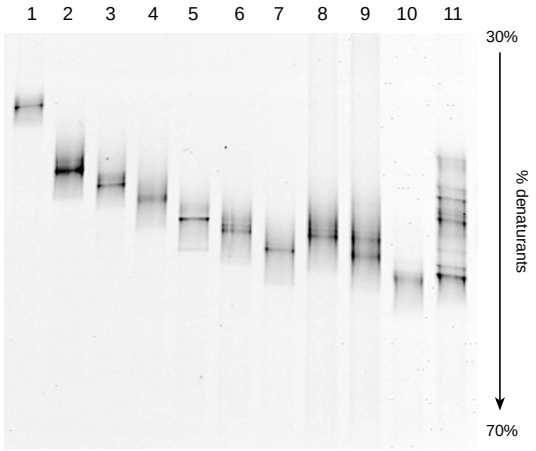

FIG. 7. DGGE analysis of PCR-amplified [NiFe] hydrogenase gene fragments from uncharacterized environmental samples and individual bacteria. The figure shows a parallel DGGE separation pattern of gene fragments obtained from *D. desulfuricans* DSM 1926 (lane 1), isolate PIB2 (lane 2), *D. baculatus* (lane 3), *D. desulfuricans* DSM 1924 (lane 4), *D. vulgaris* DSM 644 (lane 5), *D. gigas* (lane 6), *D. sulfodismutans* (lane 7), anaerobic bioreactor sample A (lane 8), anaerobic bioreactor sample B (lane 9), anaerobic bioreactor sample C (lane 10), and microbial mat sample (lane 11).

fulfil this purpose. 16S rRNA-targeted oligonucleotide probes have been used successfully to detect SRB in environmental samples (4, 29, 30). However, these probes were designed to detect all (3) or certain subgroups (11) of SRB. So far, no species-specific probes have been described for any of the SRB, including members of the genus *Desulfovibrio*. Furthermore, no new sequence information was obtained by this approach.

Voordouw et al. (37) used a combination of four DNA probes for different hydrogenase genes to detect *Desulfovibrio* species in environmental samples, but they had to enrich the bacteria prior to molecular identification. In later studies, a technique called reverse sample genome probing was described to identify distinct populations of SRB in oil fields (38–40). In reverse sample genome probing analysis, DNA is extracted from an environmental sample, labelled, and hybridized with a "master filter," which contains denatured genomic DNAs from bacteria isolated from the target environment. In this way, the researchers were able to identify which of the bacterial genomes spotted on the master filter were most prevalent in the samples. This approach has been successfully applied to discriminate between two distinct bacterial communities, i.e., saltwater and freshwater SRB (39). An advantage of this method is that it is not restricted to one phylogenetic group of microorganisms, such as the *Desulfovibrio* species, but that it can be used to detect every bacterium isolated from a particular environment. However, as indicated by the researchers (39), isolation of the ecologically important organisms might be a problem. Furthermore, phylogenetic inference of the detected community members is not possible, because no nucleotide sequences from the natural DNA samples are retrieved by this approach.

PCR amplification of particular genes to identify bacteria does not depend on the isolation of the bacteria prior to detection but is only possible for those bacteria for which enough sequence information is available. However, although not completely free of biases, the advantages of DGGE analysis of PCR-amplified gene fragments are manifold; the method is rapid (about 10 h for PCR and DGGE), simple (just casting a polyacrylamide gel), and inexpensive. Sample-to-sample comparison is possible, because multiple samples (up to 20 samples for our system) can be analyzed simultaneously on one gel. However, the most important advantage is that the DGGE-separated bands can be sequenced directly, without the need of cloning (22, 24), which will allow us to build an accumulating database of nucleotide sequences from which we can infer the phylogenetic affiliation of new species and from which species-specific probes can be designed. A limitation of the DGGE analysis is that only relatively small fragments, up to 500 bp, can be analyzed.

Here we have used this molecular approach to analyze the genetic diversity of *Desulfovibrio* species in different environments. It is based on the separation of PCR-amplified fragments of the [NiFe] hydrogenase gene by DGGE. We have chosen this gene because (i) it is present in all *Desulfovibrio* species, and (ii) it might help us to understand the importance of metal reduction by SRB. Voordouw and coworkers (37) have investigated the distribution of three types of hydrogenase genes among 22 members of the genus *Desulfovibrio* and found that the [NiFe] hydrogenase gene was present in all *Desulfovibrio* species, while the genes for [Fe] hydrogenase and [NiFeSe] hydrogenase had a more limited distribution. Lovley et al. (20, 21) have found that hydrogenases of *D. vulgaris* play an indirect role in the reduction of uranium. They even claimed that SRB might prefer the reduction of metals above the reduction of sulfate under certain conditions, such as low hydrogen concentrations. As mentioned before, the reduction of oxidized metals, such as iron and manganese, might be another important metabolic process in the oxidation of organic carbon in anoxic marine sediments (6, 34). However, from these studies it was unclear which organisms play a role in this process. Some researchers have suggested that SRB might be the key players (20, 21). Therefore, in order to understand the importance of metal reduction by SRB, we need to determine the hydrogenase activity of SRB in those environments where metal reduction occurs. Preliminary experiments performed by us (results not shown) indicate that the expression of the [NiFe] hydrogenase gene can be detected by using the reverse transcriptase PCR (35) and that the identity of the active *Desulfovibrio* species can be determined by sub-

sequent DGGE analysis of the reverse transcriptase PCR products.

By using different sets of primers, we were able to amplify the [NiFe] hydrogenase gene from all *Desulfovibrio* species tested. The positive result for the new SRB isolate PIB2 indicates that it is a *Desulfovibrio* species. This result was supported by phylogenetic analysis of its 16S rDNA (results not shown); it groups with other *Desulfovibrio* species, while isolate LTK4, which did not give a positive result in our PCR assay, was related to *Desulfobulbus* species (34a). For some species it was possible to obtain a PCR product with only one primer pair. Application of other primer pairs did not give a PCR product. This might indicate that the target sequences for some of the PCR primers might differ in these species. For two species, *D. vulgaris* DSM 1744 and *D. longus*, we obtained a specific product only with primer pair Hyd1F-Hyd5R, when a less stringent amplification protocol was used. For *D. salexigens*, we obtained only one PCR product when primer pair Hyd2F-Hyd7R was used. This primer pair also gave PCR products with some of the other bacteria, such as *D. variabilis*, *D. baarsii*, *Escherichia coli*, *Xanthobacter autotrophicus*, and *Pseudomonas denitrificans*. Therefore, the target sites for these primers were probably more conserved than those for the other primer pairs.

By using primer pair Hyd1F-Hyd5R, we were able to amplify the [NiFe] hydrogenase gene from different environmental samples and hence to detect the presence of *Desulfovibrio* species in these samples. However, only after DGGE analysis of these PCR products were we able to determine the genetic diversity of *Desulfovibrio* species within these samples. In two of the bioreactor samples, we found only one band, which would indicate the presence of one *Desulfovibrio* species. In one bioreactor sample, we detected two bands, indicating the presence of two different *Desulfovibrio* species. In the microbial mat sample, we detected at least five dominant bands, which would indicate the presence of at least five different species constituting this community. This high number of different *Desulfovibrio* species in the microbial mat might be surprising; however, Devereux and Mundfrom (12) recently found a high degree of genetic diversity of SRB in a sandy marine sediment sample. Thirteen unique sequences, obtained from cloned 16S rDNA PCR products, grouped with SRB within the delta subclass of proteobacteria. From these results we conclude that the genetic diversity of *Desulfovibrio* species within the natural microbial mat is far greater than within the experimental bioreactors.

In summary, DGGE analysis of PCR products obtained after amplification of the [NiFe] hydrogenase gene from genomic DNAs isolated from environmental samples reveals the genetic diversity of *Desulfovibrio* species in these samples. Together with the DGGE analysis of PCR products obtained after amplification of the mRNA, we hope to determine the niche differentiation of the different *Desulfovibrio* species and to relate for the first time community structure to community function.

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