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Molecular Identification of Bacteria from a Coculture by Denaturing Gradient Gel Electrophoresis of 16S Ribosomal DNA Fragments as a Tool for Isolation in Pure Cultures

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Molecular information about the bacterial composition of a coculture capable of sulfate reduction after exposure to oxic and microoxic conditions was used to identify and subsequently to isolate the components of the mixture in pure culture. PCR amplification of 16S ribosomal DNA fragments from the coculture, analyzed by denaturing gradient gel electrophoresis, resulted in two distinct 16S ribosomal DNA bands, indicating two different bacterial components. Sequencing showed that the bands were derived from a *Desulfovibrio* strain and an *Arcobacter* strain. Since the phylogenetic positions of bacteria are often consistent with their physiological properties and culture requirements, molecular identification of the two components of this coculture allowed the design of specific culture conditions to separate and isolate both strains in pure culture. This approach facilitates the combined molecular and physiological analysis of mixed cultures and microbial communities.

Analysis of rRNA has been widely used to determine the bacterial-species composition of microbial communities. The methodology encompasses such different approaches as direct analysis of rRNA by chromatography and membrane blotting (27, 29), PCR amplification and subsequent cloning and sequencing of reverse-transcribed environmental rRNA (35), and denaturing gradient gel electrophoresis (DGGE) of PCR products obtained from extracted bacterial DNA and rRNA (22–24). In nearly all cases, rRNA analysis of microbial communities has been a one-way approach to infer the composition of a microbial ecosystem from rRNA sequence and base composition. Nevertheless, molecular information from RNA sequences also provides a starting point for classical, culture-dependent microbiological investigations and a guideline to identify and subsequently to isolate specific microorganisms from natural bacterial communities or from cocultures (16). One prerequisite of this approach is that the phylogenetic position of a bacterium, as inferred from sequence data, be consistent with its physiology and culture requirements. Phylogeny and physiology are congruent for many major prokaryotic groups, such as the gram-negative sulfate reducers, the methanogens, and the *Campylobacter-Helicobacter* group (1, 5, 33). We investigated a bacterial coculture of unknown composition that was capable of conserving sulfate reduction after oxygen exposure by DGGE of PCR-amplified 16S ribosomal DNA (rDNA) fragments. DGGE can separate PCR products of identical length on the basis of primary sequence and base composition (22, 25). Two different DGGE bands, indicating two different bacteria as components of the culture, were found and sequenced. The bands were identified as being derived from a *Desulfovibrio* strain and from an *Arcobacter* strain; the genus *Arcobacter* is related to *Campylobacter*, *Helicobacter*, *Wolinella*, and *Thiovulum*. After molecular identification of these two components by partial 16S rDNA sequences, this information was used to design selective culture conditions to isolate both bacterial strains in pure culture for subsequent physiological studies of the individual isolates.

Sampling and culture conditions. Cyanobacterial mats from Solar Lake (Sinai) were introduced to an experimental hypersaline pond established at the Interuniversity Institute of Eilat in 1987 and were developed under evaporated Red Sea seawater at a constant total salinity of 9% + 0.5% for 5 years prior to this research (17). The 3-mm surface layers of these mats, characterized by strong sulfate-reducing activity and extreme diurnal oxic-anoxic shifts (11), were used for the inoculation of redox-gradient agar shake enrichments in an attempt to isolate sulfate-reducing bacteria specifically adapted to oxygen exposure in shifting oxygen gradients. One milliliter of the homogenized mat surface layer was mixed with 9 ml of filtered pond water supplemented with 20 mM sodium acetate, 0.1% (wt/vol) yeast extract, and 1.5% (wt/vol) agar. This mixture was placed on top of 2 ml of reducing 2% (wt/vol) agar at the bottom of a 18-ml test tube to create a redox gradient within the agar shakes. A duplicate series of five sequential 10-fold dilutions of the overlaid enrichment was then carried out. The enrichment shake agar series were later overlaid by a 2% (wt/vol) agar suspension of an axenic culture of a halophilic cyanobacterium, *Synechococcus* sp. (3), and were incubated at 35°C under illumination of 200 $\mu E\ m^{-2}\ s^{-1}$ for several days. Oxygen profiles established in the enrichment shake agar were measured with oxygen microelectrodes (26) in one of the dilution series. Slices (2 mm thick) of the oxic layers of the other series were transferred to 2% (wt/vol) agar plates of semisynthetic growth medium containing 10 mM sodium thiosulfate as the sulfur source and 20 mM lactate as the carbon source and electron donor. After a week of incubation at 25°C, black colonies were developed. Repeated plate transfers and incubation under anaerobic conditions at room temperature yielded black colonies consisting of uniformly shaped bacteria resembling vibrio and spirillum bacteria. One of the colonies, originating from the upper slice of a 10^{-3} dilution and grown on thiosulfate, was chosen for further study.

Semisynthetic growth medium contained, per liter of filtered pond water, the following: NH_4Cl, 1.0 g; K_2HPO_4, 0.5 g; so-

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dium citrate dihydrate, 1.0 g; vitamin solution, vitamin B_{12} solution, and thiamine solution, 1 ml each (38); mineral solution SL7, 1 ml (2); resazurin, 0.001 g; reductant solution, 10 ml (38); 5% (wt/vol) $FeSO_4$ solution, 5 ml; yeast extract, 1 ml; and agar, 20 g. Synthetic growth medium contained, per liter of double-distilled water, the following: NaCl, 50.0 g; KCl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 2.5 g; NH_4Cl, 1.0 g; K_2HPO_4, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.08 g; sodium citrate dihydrate, 1.0 g; resazurin, 0.001 g; vitamin solution, vitamin B_{12} solution, and thiamine solution, 1 ml each; mineral solution SL7, 1 ml; reductant solution, 10 ml. Agarized media in petri dishes were supplemented with 5 ml of 5% $FeSO_4$ solution and 1 g of yeast extract per liter. Carbon and sulfur sources were added to the autoclaved media as sterile 1 M solutions. If not specified otherwise, 20 mM sodium lactate and 10 mM sodium sulfate were used as the carbon and sulfur sources.

The continuous-culture experiments were carried out in an apparatus manufactured at the Biological Center of the University of Groningen, Haren, The Netherlands, and included controls for temperature, pH, and dissolved oxygen. The 1,200-ml growth vessel was inoculated with 120 ml of a late-exponential-phase (3- to 4-day-old) culture. Growth temperature was maintained at 35°C. pH was maintained at 7.0 to 7.5. Dissolved oxygen was measured by a 900 series New Brunswick (Edison, N.J.) oxygen electrode. The stirring rate was 1,500 rpm. For batch precultivation and for anaerobic continuous-culture experiments, the medium was bubbled with pure nitrogen at the rate of 0.1 liter/min. After 24 h of batch incubation, the flow of the synthetic medium with 20 mM sodium lactate and 10 mM sodium sulfate was commenced at a dilution rate of 0.1 h^{-1}. A gas mixture containing 90% N_2, 5% O_2, and 5% CO_2 was used for aerobic continuous-culture experiments. Samples were taken daily and plated on synthetic medium with 20 mM lactate, with and without a sulfur source. The plates were incubated anaerobically and aerobically.

For isolation of the two components of the coculture, the defined multipurpose medium with a nonchelated trace element mixture and 0.01% (wt/vol) yeast extract was used as described by Widdel and Bak (38). Agar shakes with the following substrate combinations were used for the analysis of the mixed culture and for further strain purification: (i) 10 mM lactate; (ii) 5 mM lactate, 5 mM fumarate, and 2 mM acetate; and (iii) 10 mM fumarate and 2 mM acetate.

The ability of both strains of the coculture to grow under various oxygen concentrations on agar plates was tested under increasing oxygen partial pressures of 0, 5, and 20% (vol/vol) oxygen in the gas phase. The agar plates for the oxygen growth tests were prepared with 1% (wt/vol) Bacto agar and defined multipurpose medium (38), with the following modifications. For growth under 5 and 20% oxygen, sulfide was omitted from the medium, since the oxidation of sulfide forms toxic by-products, and was replaced by 0.5 mM $Na_2S_2O_3$ as a sulfur source. Gas mixtures were checked by gas chromatography to be the following (values are vol/vol): (i) 10% CO_2 and 90% N_2; (ii) 5% O_2, 10% CO_2, and 85% N_2; and (iii) 20% O_2, 5% CO_2, and 75% N_2. To compensate for the lower CO_2 content of the 20% oxygen gas phase, the medium was modified to contain 10 ml of 1 M $NaHCO_3$ and 0.5 g of KH_2PO_4 per liter.

Molecular techniques. From several batch culture samples of the coculture and from pure cultures of the two components, DNA was isolated by phenol extraction (28) and then subjected to PCR, DGGE, and sequencing. The primer combination GM5F (with GC-clamp) and DS907R amplified a 550-bp fragment of the 16S rDNA, which was suitable for subsequent DGGE analysis. For phylogenetic analysis of pure cultures, the nearly complete 16S rRNA sequences were amplified with the primer combination GM3 and GM4. The primer sequences and their location on the 16S rRNA gene have been published by Muyzer et al. (23, 24). PCR amplifications were performed as described previously (23, 24) by using a hot-start and touchdown annealing reaction (6) to increase the specificity of the amplification and to reduce the formation of spurious by-products.

DGGE was performed with a Bio-Rad Protean II system (22–24). Electrophoresis was performed for 4 h at a constant voltage of 200 V and at a temperature of 60°C. After electrophoresis the gels were stained in aqueous ethidium bromide solution (0.5 mg/liter) and photographed on a UV (302 nm) transillumination table with a CS1 digital camera (Cybertech, Berlin, Germany). Small pieces of selected DGGE bands were punched from the DGGE gel, eluted, and reamplified with the same primers, but without GC-clamp (23). Purification and sequencing of the purified PCR products were done as described previously (23, 24).

The 16S rRNA sequences were aligned with those of other bacteria obtained from the Ribosomal Database Project database (20). The SIMILARITY_RANK tool of the Ribosomal Database Project database was used to search for close evolutionary relatives. Sequence alignments were prepared with the sequence alignment editor SEQAPP (13). Distance matrixes were calculated with DNADIST, as implemented in the software package PHYLIP (version 3.5) developed by Felsenstein (8), by using the Jukes-Cantor model, which assumes independent change at all sites with equal probability (15). Phylogenetic trees were constructed from evolutionary distances calculated with the algorithm of Fitch and Margoliash (10), as implemented in the program FITCH in the software package PHYLIP (version 3.5).

Growth characteristics of the coculture. The coculture was maintained at steady state with 10 mg of protein $liter^{-1}$ under an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 supplied to the chemostat at a rate of 0.1 liters min^{-1} at a dilution rate of 0.1 h^{-1}. Under these conditions all oxygen was removed in the growth vessel and no sulfate or thiosulfate reduction could be detected. Sulfate or thiosulfate reduction was demonstrated in the chemostat immediately after removal of oxygen from the atmosphere by the stoichiometric production of sulfide from sulfate or thiosulfate. Samples obtained from the chemostat coculture and incubated anaerobically in culture tubes with reduced synthetic growth medium were able to reduce both sulfate and thiosulfate. Also, chemostat samples which had been transferred several times over plates of aerobic, sulfate-free synthetic growth medium resumed sulfate reduction after several days of anaerobic incubation. These contradictory findings, i.e. viability in the oxygen-limited chemostat and on plates exposed to oxygen and sulfate-reducing activity after transfer to anaerobic conditions, suggested further work to clarify the composition of this culture. The vibrio-shaped and curved cells of this culture could not be readily identified by light microscopy and required a combined molecular-microbiological approach.

Molecular and microbiological analysis of the coculture. DGGE of PCR-amplified 16S rDNA fragments was used for molecular identification of the components of the coculture. After DNA extraction, PCR amplification of 16S rDNA fragments, and DGGE, two PCR products were separated in the DGGE pattern (Fig. 1, lane 8), suggesting that the culture consisted of two different bacterial types. The PCR products were excised from the gel, reamplified, and sequenced. The similarity search option of the Ribosomal Database Project database (20) was used to search for bacteria phylogenetically affiliated with the DGGE-separated PCR products derived

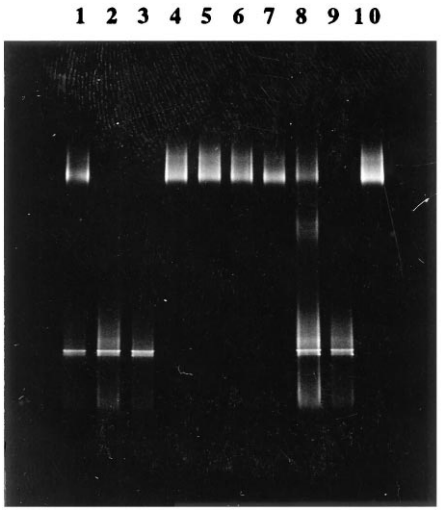

FIG. 1. DGGE gel with 16S rDNA fragments derived from the mixed culture and from its two components in pure culture, the *Desulfovibrio* and the *Arcobacter* strains, as follows: Lane 1, both pure cultures; lanes 2 and 3, two independent isolates of the *Desulfovibrio* strain; lanes 4 through 7 and 10, five independent isolates of the *Arcobacter* strain, either picked from agar shakes with 5 mM lactate–5 mM fumarate–2 mM acetate before purification in 10 mM fumarate–2 mM acetate agar shakes (lanes 4 and 5) or isolated and purified directly in 10 mM fumarate–2 mM acetate agar shakes (lanes 6, 7, and 10); lane 8, original mixed culture; lane 9, same as lane 2, provided for ease of comparison with lane 8.

from the mixed culture. The 16S rDNA fragment in the upper position of the DGGE gel was derived from an epsilon subdivision proteobacterium related to the spirilloid sulfur reducers *Campylobacter*, *Helicobacter*, *Wolinella*, and *Arcobacter* spp. (33). The 16S rDNA fragment in the lower gel position was derived from a *Desulfovibrio* strain. A more detailed phylogenetic analysis was postponed at this stage, since only partial 16S rDNA sequences were available. After isolation of the coculture components in pure culture and cross-checking of their DGGE pattern with the original culture (Fig. 1), nearly complete 16S rDNA sequences (approximately 1,400 bp) of both pure strains were determined for phylogenetic analysis. The *Desulfovibrio* strain of this mixed culture was related to the salt-requiring or salt-tolerant species *Desulfovibrio salexigens* ATCC 14822^T, *Desulfovibrio desulfuricans* El Agheila Z, and *Desulfovibrio africanus* Benghazi NCIB 8401^T and to the freshwater species *Desulfovibrio longus* SEBR 2582^T (Fig. 2). A recently isolated, physiologically versatile, oxygen-respiring *Desulfovibrio* strain from the same mat environment, *Desulfovibrio oxyclini* PIB, is, by its 16S rRNA sequence, closely affiliated with this coculture *Desulfovibrio* strain (18). The epsilon subdivision bacterium belongs to the recently established genus *Arcobacter* (Fig. 2). *Arcobacter* consists of former *Campylobacter* species which were reclassified on the basis of phenotypic characteristics and 16S rRNA sequences (33, 34). The nitrogen-fixing species *Arcobacter nitrofigilis* ATCC 33309^T, isolated from *Spartina* roots in a salt marsh, is the closest relative of the strain obtained from the coculture (21).

The phylogenetic affiliation of the components of the coculture determined our choice of selective culture conditions to separate both target organisms, the *Desulfovibrio*-related bacterium and the epsilon subdivision bacterium related to *Campylobacter*, *Helicobacter*, *Wolinella*, and *Arcobacter* spp. The *Desulfovibrio* strain of this bacterial mixture was isolated and purified in anaerobic agar shakes with defined sulfate-reducing-bacterium multipurpose medium with 10 mM lactate as substrate (38), under a gas mixture of 10% CO_2 and 90% N_2. The pure culture was grown in a batch culture on the same medium. The cells showed the characteristic morphology of *Desulfovibrio* spp. (Fig. 3A).

Culture conditions for the epsilon subdivision bacterium were adjusted with *Campylobacter* and *Wolinella* spp., the spirilloid sulfur reducers, as model organisms. Spirilloid sulfur reducers catalyze, under anaerobic conditions, the oxidation of H_2S to elemental sulfur, with fumarate being converted to succinate (37). *Wolinella succinogenes* has been shown to grow by this reaction (19). Therefore, sulfate-reducing-bacterium multipurpose medium was supplemented with 10 mM fumarate as the electron acceptor and 2 mM acetate as the carbon source, since the spirilloid sulfur reducers do not oxidize succinate via an operating citric acid cycle and cannot grow autotrophically (37). Agar shakes with this substrate combination favored growth of the *Arcobacter* strain. Colonies were surrounded by a halo of sulfur grains formed by the oxidation of sulfide, which was present in the medium at 1.5 mM. The shape of the cells ranged from curved to spirilloid, characteristic of *Arcobacter*, *Campylobacter*, *Helicobacter*, and *Wolinella* spp. and morphologically similar to *Desulfovibrio* spp. (Fig. 3B).

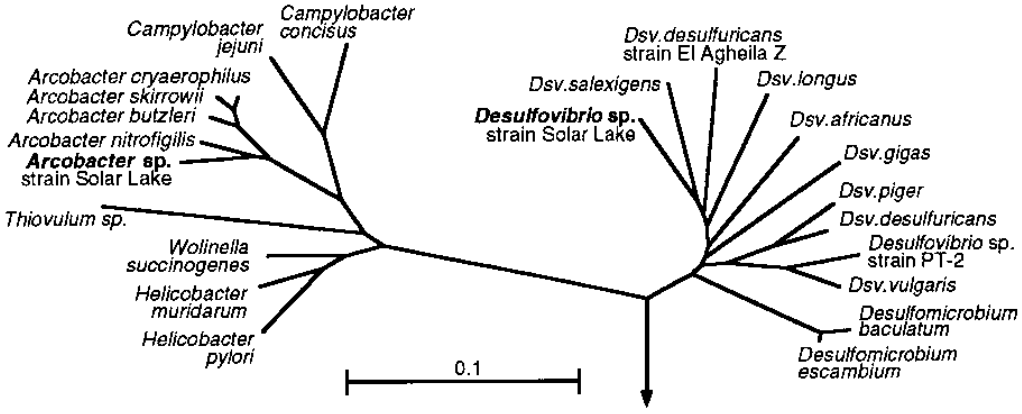

FIG. 2. 16S rRNA distance tree of *Desulfovibrio* (*Dsv.*), *Arcobacter*, and related genera of the delta and epsilon proteobacterial subdivisions based on 16S rRNA sequence positions 24 to 1384 (*Escherichia coli* numbering). The tree was rooted with *Desulfotomaculum ruminis* as outgroup. The scale bar corresponds to 0.1 substitutions per nucleotide position. Sequences used in preparing this figure were derived from the Ribosomal Database Project on the anonymous ftp server at the University of Illinois in Urbana, updated on 19 June 1994 (20).

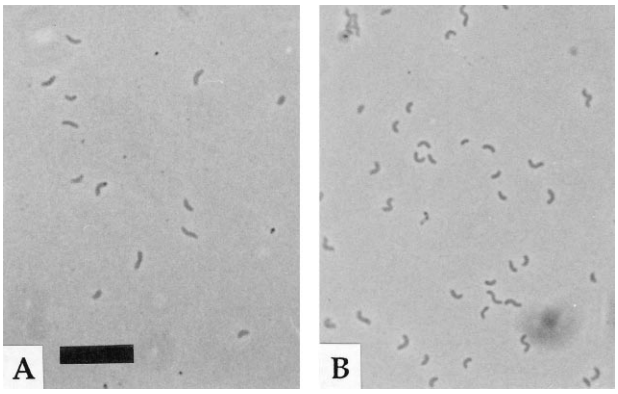

FIG. 3. (A) Phase-contrast microphotograph of the *Desulfovibrio* strain. Bar, 10 μm. (B) Phase-contrast microphotograph of the *Arcobacter* strain.

Colonies were picked and purified by further agar shakes and cross-checked with lactate medium for growth of *Desulfovibrio* contaminations. The *Arcobacter* strain was able to grow anaerobically on agar plates with a gas mixture of 10% CO_2 and 90% N_2, as well as aerobically with 5% O_2, 10% CO_2, and 85% N_2 and with 20% O_2, 5% CO_2, and 75% N_2. The *Desulfovibrio* strain grew only anaerobically with 10% CO_2 and 90% N_2. Samples of the *Arcobacter* strain and of the *Desulfovibrio* strain were scratched off the agar plates and checked by PCR and DGGE, showing again the respective 16S rDNA DGGE bands and thus confirming the identity of the samples. The *Desulfovibrio* and *Arcobacter* strains were checked in batch culture growth experiments for their range of electron donors and acceptors. The ability of the *Arcobacter* strain to reduce nitrate corresponds to that of the related species *A. nitrofigilis*, *Arcobacter butzleri*, and *Arcobacter skirrowii* (34). The physiological characteristics of the *Desulfovibrio* and *Arcobacter* isolates are summarized in Table 1.

In order to verify that these isolates were actually the two components of the investigated mixed culture, the two strains were checked by DGGE. PCR-amplified 16S rDNA fragments of the *Desulfovibrio* and *Arcobacter* strains were analyzed side by side with the original sample by DGGE. The DGGE bands of the pure cultures matched the two DGGE bands of the original coculture (Fig. 1). The DGGE fragments of the two pure cultures were resequenced and found to be identical to their counterparts in the coculture.

Similar associations of sulfate-reducing bacteria or methanogenic archaea with facultative aerobes are stable in oxygen-limited chemostat cocultures, indicating that anaerobic bacteria may survive and even grow in partially oxic habitats, as long as oxygen input does not exceed the potential rate of consumption (12, 14). Large aerobic-anaerobic fluctuations dominate the cyanobacterial mat surface layer from which the *Desulfovibrio-Arcobacter* coculture was isolated. The 3-mm surface layer exhibited marked diurnal fluctuations from up to 1,200 μM oxygen and no detectable sulfide at noon to 100 μM sulfide and no oxygen at midnight. High sulfate reduction rates of a maximum of 1 to 2 μmol of SO_4^{2-} cm^{-3} day^{-1} were found in the diurnally oxic zone (11). The organisms responsible for aerobic sulfate reduction in this oxic-anoxic interface could be either sulfate-reducing bacteria capable of reducing sulfate under aerobic conditions or oxygen-sensitive sulfate-reducing bacteria in close association with aerobic, oxygen-scavenging organisms. Although isolation strategies involving frequent oxic-anoxic shifts have yielded oxygen-respiring *Desulfovibrio* species from the Solar Lake cyanobacterial mat surface layer, pure-culture isolates obtained so far do not grow by this metabolic mode and do not reduce sulfate under aerobic conditions (18). Therefore, activity and growth of sulfate reducers in this habitat probably depend on their close association and coculture formation with facultative aerobes.

TABLE 1. Electron donors and acceptors for the *Arcobacter* and *Desulfovibrio* spp.[a]

Electron donor or acceptor	*Arcobacter* sp.	*Desulfovibrio* sp.
Electron donors (20 mM)		
Lactate	+	+
Formate	+	−
Succinate	−	−
Fumarate	−	−
Malate	+	−
Citrate	−	−
Acetate	+	−
Glycolate	−	−
Glutamate	+	−
Methanol	−	−
Ethanol	−	+
Glucose	−	−
Fructose	−	−
Electron acceptors (10 mM)		
Sulfate	−	+
Sulfite	−	+
Thiosulfate	−	+
Nitrate	+	−
Fumarate	+	−
2% oxygen[b]	+	−
5% oxygen[b]	+	−
20% oxygen[b]	+	−
Air	−	−

[a] Electron acceptors used to determine the electron donor spectrum were 10 mM Na_2SO_4 for the *Desulfovibrio* sp. and 5% oxygen (with 5% CO_2 and 90% N_2) for the *Arcobacter* sp. The electron donor for both isolates was 20 mM lactate. For the *Arcobacter* sp., fumarate also served as the electron acceptor with 1.5 mM sulfide, which was oxidized to sulfur. Yeast extract was excluded from the medium. None of the isolates was capable of autotrophic growth on hydrogen and bicarbonate or of fermentation with lactate, pyruvate, and succinate.

[b] Gas mixtures for testing oxygen tolerance contained 5 to 10% CO_2, since the *Arcobacter* sp. required elevated CO_2 levels for growth under oxygen.

Linking sequence data to culture strategies. The molecular analysis of this coculture allowed us to choose specific culture conditions to isolate both components in pure culture. This approach does not depend exclusively on 16S rRNA similarity, but more on coherence of phenotype and phylogeny within a bacterial group. In our case, *Desulfovibrio* spp. and the *Arcobacter-Campylobacter-Helicobacter-Wolinella* group both show considerable phylogenetic depth (>10% Jukes-Cantor 16S rRNA sequence divergence) but are nevertheless phylogenetically separated from each other and belong to different proteobacterial subdivisions, with each group being physiologically coherent and mutually exclusive. This provided the basis for isolating the lactate-utilizing sulfate reducer and the fumarate-reducing, sulfide-oxidizing spirillum by using two general media for these physiological types. Medium specificity on this level of major physiological groups would probably not suffice to separate closely related and physiologically similar organisms, i.e., those within the same genus. In this more detailed frame, more specific information about the culture requirements of the respective closest phylogenetic relatives is required. The congruence of phylogeny and physiology certainly approaches practicability limits, depending on the particular case. Physiologically dissimilar bacteria may have only small

16S rRNA differences, e.g., the nitrite-oxidizing genus *Nitrobacter* and the phototrophic genus *Rhodopseudomonas* (30), or can be phylogenetically intertwined, such as the sulfur-reducing genus *Desulfuromonas* and the fermentative, anaerobic genus *Pelobacter* (7).

Important methodological caveats of this approach concerning the choice of the sample, the selectivity determined by the PCR primers, the resolution of the DGGE gel, and the problem of unidentifiable, uncultured molecular isolates have to be considered. Samples with a limited number of cultivable bacterial species (24), preenriched cultures and cocultures, defined syntrophic associations (4, 39), and possibly consortia (32) are most promising for this approach. DGGE patterns of natural samples are very complex, often because of the abundance of uncultured or—by present techniques—uncultivable bacteria (31). To simplify DGGE patterns, specific primer sets for 16S rRNA genes or for other functional genes of selected bacterial groups which focus the DGGE on accessible subsets of the natural bacterial population can be designed (36). This approach can be extended to monitoring gene expression (36). For the analysis of multiple, neighboring DGGE bands, the resolution of DGGE gels is an important limiting factor. Closely related organisms, differing by only a few nucleotides in the DGGE band sequence, can be discriminated with proper technique in the absence of background and smear (9). Finally, DGGE analysis and sequence identification of novel or previously uncultured groups of bacteria give no hints as to the culture conditions and physiology of the respective bacteria (31). However, this problem may become less severe in the future with increasing cross-connections between molecular and physiological data.

Nucleotide sequence accession numbers. The sequences obtained in this study have been assigned GenBank accession numbers L42994 and L42995.

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