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ORIGINAL PAPER

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Desulfovibrionales-related bacteria in a paper mill environment as detected with molecular techniques and culture

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Abstract The aim of the present study was to evaluate the suitability of a nested PCR-DGGE (denaturing gradient gel electrophoresis) method for the detection of Desulfovibrionales-related sulfate-reducing bacteria (SRB) from paper mill samples. The samples were also analyzed with culturing. SRB cause/enhance industrial problems, namely creation of foul-smelling gases (hydrogen sulfide) and biological corrosion, and so far there has not been a simple method to study these bacteria in paper mill laboratories. In our study, culturing was able to detect Desulfovibrionales-related bacteria from two different white waters, two different brokes, pulp, clay, and slime. Out of the isolated Desulfovibrionales, 23 enrichment cultures were further characterized with Desulfovibrionales-selective PCR-DGGE. An identical *Desulfovibrio* species sequence was found from paper machine I (broke I, slime, and pulp) and from paper machine II (broke II and white water II), suggesting an in-house contamination with the same strain. Desulfovibrionales-selective PCR-DGGE was also performed from DNA templates extracted directly from the paper mill samples. The DGGE profiles derived from the samples without prior enrichment were more diverse and the sequenced amplicons proved to belong to the Desulfovibrionales order. Moreover, molecular techniques were able to detect Desulfovibrionales-related bacteria from calcium carbonate samples whereas culture did not. Altogether, the nested PCR-DGGE method used in this study was suitable for the detection of Desulfovibrionales-related SRB directly from different paper mill samples and it could be used for the rapid identification of SRB-contaminated industrial sites and, when combined with sequencing, for tracing of the contamination routes.

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Introduction

The industrial problems created and/or enhanced by sulfate-reducing bacteria (SRB), namely biological corrosion and creation of foul-smelling gases (hydrogen sulfide, H_2S), have been known for decades. H_2S is generated in both anaerobic and aerobic environments [14], and being a rather volatile compound, it easily escapes from the aqueous phase and may become dangerous to humans under poorly ventilated conditions [23]. In addition, gaseous and dissolved sulfides cause physical (corrosion, odor, increased effluent chemical oxygen demand, COD) and biological (toxicity) constraints that may lead to process failure [14]. Therefore, perhaps the single most important factor stimulating the upsurge of interest in SRB in recent years has been their considerable, albeit largely negative, ecological, and economic impact [13, 28].

SRB form a phylogenetically diverse and heterogeneous group of microorganisms, which however share common physiological and ecological characteristics. SRB communities are well suited to rRNA-based studies, since the classical physiological based taxonomy is in good agreement with the small subunit rRNA derived phylogeny [8]. SRB may be divided phylogenetically into four groups: Gram-negative mesophilic SRB (families Desulfovibrionaceae and Desulfobacteriaceae); Gram-positive spore-forming SRB (genus *Desulfotomaculum*); bacterial thermophilic SRB; and archeal thermophilic SRB [3]. SRB are mostly obligate anaerobes [19]. However, observations of sulfate reduction occurring in the anaerobic environment reported previously have demonstrated a much larger ecological range of SRB than previously thought [5]. Complete oxidizing acetate-utilizing SRB, such as *Desulfobacter* species, are much more sensitive to oxygen than robust species such as *Desulfovibrio* species, which are able to survive under

oxic conditions due to their oxygen-scavenging system involving superoxide dismutase and catalase [4]. SRB are not only able to coexist in a complex microbial population but are also capable of utilizing the end-products of other bacteria as a carbon source and benefit from the resulting low redox potential [10, 28]. SRB are also able to survive a hostile environment at an extreme pH until conditions become such that they are able to resume activity [10]. Development of SRB in biofilms can be expected whenever environmental conditions are suitable. SRB exist in aerobic water, anaerobic microniches and in the anoxic microlayers of biofilms. Thus, an effort should be made to prevent biofilm build-up in industrial systems [15].

The paper machine environment is favorable for microbial growth due to the suitable pH (4–10) and temperature (30–50°C). In addition, paper-making chemicals provide nutrients for microbes, and therefore microbes will always be present in paper-making machines [26]. The paper-making process has been reviewed by, for example, Sjöberg et al. [22]. The activity of sulfate reducers in the paper-making industry can easily be explained by the abundant presence of cellulose fibers that via fermentative bacteria lead to electron donors for sulfate reduction. Attempts to avoid contamination of water with SRB are mostly difficult and may only be successful in completely closed water systems after sterilization. Open water systems probably receive sulfate reducers also from entering water, soil particles, dust, and animal excreta [28]. SRB may be controlled by maintaining the environmental conditions unfavorable for these bacteria [19].

Our aim was to evaluate the suitability of the nested Desulfovibrionales-selective PCR-DGGE (denaturing gradient gel electrophoresis) for the paper mill environment and to investigate the occurrence, diversity, and identity of the Desulfovibrionales-related SRB from paper mill samples using different detection methods, namely cultivation, PCR-DGGE coupled with sequencing, and cultivation coupled with PCR-DGGE and sequencing.

Materials and methods

Sampling

Samples, each of 1000 ml, were taken aseptically from nine different sites from a paper mill: two different white waters (I and II from paper machines I and II, respectively), two different brokes (I and II from paper machines I and II, respectively), pulp, clay slurry, calcium carbonate ($CaCO_3$) slurry, slime, and starch slurry. All the samples were in the form of liquid or slurry. Samples were placed in plastic vials with pierced lids for gas exchange in sterile containers containing an Anaerocult-bag (Anaerocult A; Merck, Germany), which develops an oxygen-free environment. The temperature, redox potential, pH, and conductivity of each sample were measured (Mettler Toledo portable device; Beaumont Leys, Leicester, UK) at the time of sampling. The temperature during transportation remained between 8 and 12°C. The samples for culture were analyzed within the same day. The rest of the samples for DNA-based studies were kept at –20°C until analysis.

Culture

All the samples were thoroughly mixed manually. The samples were serially diluted in prereduced peptone-saline (LabM, Bury, UK) containing 0.5 g/l L-cysteine-HCl (Merck), and further plated on prereduced plate count agar (Difco, USA) to determine the total anaerobic count and on prereduced trypticase soy agar (TSA; BBL, USA) supplemented with 0.5 g/l $FeSO_4 \cdot 7H_2O$ [20] to determine the SRB count. The samples were cultured in an anaerobic cabinet (Don Whitley Scientific, Shipley, UK) under an atmosphere of $H_2/CO_2/N_2$ (10/10/80%, respectively). The plates were thereafter incubated anaerobically in Anoxomat containers (Mart, Lichtenwoorde, The Netherlands) under an atmosphere of $H_2/CO_2/N_2$ (10/5/85%, respectively) for 10 days at 30°C and 55°C.

DNA isolation

Colonies with slightly different morphology on TSA agar (three from broke I, one from white water I, six from slime, four from pulp, five from clay, five from broke II, and five from white water II) were separately plated on $FeSO_4$-supplemented TSA (one colony per plate) and incubated anaerobically as stated above. At that time it was decided not to continue identification by conventional techniques due to poor regrowth of the cultures on TSA. The whole cell mass was scraped from the second TSA plate and transferred to Postgate's medium B [19] and thereafter incubated as above. The cell mass from one plate was transferred to one bottle of Postgate's medium B. After growth occurred on Postgate's medium (i.e. enrichment culture), which could be observed visually by the formation of the black ferrous precipitate, the samples were stored at –20°C until analysis.

The bacterial reference strains (Table 1) were obtained from the VTT culture collection (Espoo, Finland) and they were grown according to the directions from DSMZ (www.dsmz.de). The DNA was isolated from the reference strains, enrichment cultures, and original frozen samples with a FastDNA Spin kit for Soil (Bio101, Carlsbad, Calif.) according to manufacturer's instructions with a few modifications; the bacterial cells were broken with a Fast Prep instrument (Bio101 Savant, Holbrook, N.Y.) at 6.0 m/s for 60 s four times and the samples were centrifuged for 45 min at 4°C at 19,000 *g* to pellet the precipitate. The isolated DNA was stored at –20°C until analysis. The DNA was extracted in duplicate from the frozen industrial samples.

Table 1 Reference strains used for optimization of the PCR protocol for Desulfovibrionales-related bacteria and the results obtained after optimization of Desulfovibrionales-selective PCR (+ positive amplification signal, – no amplification signal)

Reference strains	Strain number	Results after Desulfovibrionales-selective PCR[a]
Desulfovibrio desulfuricans subsp. *desulfuricans*	VTT E-95573 (=DSM 642)	+
Desulfovibrio desulfuricans (isolated from an industrial sample, partially 16S rDNA sequenced)	VTT E-022206	+
Desulfovibrio vulgaris subsp. *vulgaris*	VTT E-001447 (=DSM 644)	+
Desulfomicrobium escambiense	VTT E-001445 (=DSM 10707)	+
Desulfotomaculatum nigrificans	VTT E-001654 (=DSM 574)	–
Desulfosarcina variabilis	VTT E-001656 (=DSM 2060)	–
Desulfobacter curvatus	VTT E-001657 (=DSM 3379)	–
Desulfobacterium autotrophicum	VTT E-001658 (=DSM 3382)	–
Lactobacillus plantarum	VTT E-78076	–
Hafnia alvei	VTT E-022142 (=DSM 30163)	–

[a]Desulfovibrionales-selective PCR was performed with primers DSV230 and DSV838 [6]

PCR-DGGE

Desulfovibrionales-related bacteria-specific PCR was performed with DSV230f–DSV838r according to the method of Daly et al. [6] with a few modifications. The PCR mixture (50 µl) contained 20 µ*M* of each primer, 4 µl of the four deoxynucleoside trisphosphates (2.5 µ*M*), 1 µl bovine serum albumin (10 mg/ml), 2 µl magnesium chloride (50 m*M*), 2 U Dynazyme II enzyme (Finnzymes, Espoo, Finland), 5 µl 10x PCR buffer (Finnzymes), and 2 µl template DNA. Thermocycling consisted of 40 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 1 min in a UNOII thermocycler system (Biometra, Göttingen, Germany). For DGGE analysis of Desulfovibrionales-related bacteria, a nested PCR amplification was used, in which the DSV230f–DSV838r PCR product was used as a template for PCR amplification of 16S rDNA gene fragments with primers 341f+GC and 534r (see below) [17]. For nested PCR of DNA extracted directly from paper industrial samples, various purification procedures were tested for the first PCR product. The Desulfovibrionales product used as a template for the universal bacterial PCR was purified with a Qiaquik PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions or diluted to 1:10, 1:100, and 1:1000 prior to the second PCR step.

PCR amplification of the predominant bacterial population of enrichment cultures and DNA extracted directly from the samples was performed from V_3 [17] and V_6–V_8 [18] hypervariable regions of the 16S rRNA gene. For PCR amplification of V_3 hypervariable region, thermocycling consisted of 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min using 2 U Dynazyme II enzyme and 20 µ*M* of each of the forward primer 341f+GC and reverse primer 534r in a UNOII thermocycler system [17]. For PCR amplification of the V_6–V_8 hypervariable region, thermocycling consisted of 35 cycles of 94°C for 30 s, 50°C for 20 s and 72°C for 40 s using 3 U Dynazyme II enzyme and 20 pmol of each of the forward primer U968f+GC and reverse primer U1401 [18] in a UNOII thermocycler system. All the amplified DNA fragments were visualized by 1% agarose gel electrophoresis, which also allowed determination of the size of the PCR product.

DGGE analysis and sequencing of the amplicons

DGGE analysis was performed using a Dcode universal mutation detection system (BioRad, Hercules, Calif.) maintained at 60°C and 85 V for 16 h in 0.5x TAE buffer (20 m*M* Tris-acetate, 0.5 m*M* EDTA, pH 8.0). Samples were loaded onto 8% acrylamide-bisacrylamide (37.5:1) gels with linear denaturing gradients from 38% to 60% (where 100% is 7 *M* urea and 40% vol/vol deionized formamide). The gels were stained with SYBR Green I (Molecular Probes, Leiden, The Netherlands) for 20 min at room temperature and the images were captured with a Gel Doc 2000 gel documentation system (BioRad).

The DGGE bands of interest from the Desulfovibrionales-selective PCR-DGGE were carefully excised and incubated in 36 µl sterile water at 80°C for 1 h and thereafter overnight at 4°C. The DNA obtained from the excised bands was reamplified with the same primer pair. A second DGGE was run to confirm that the amplified band had the same position in the gel as the excised band. Excision DGGE confirmation rounds were repeated until a single band was obtained for each amplicon. Amplification products were purified using a Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions. DNA purity and yield were estimated by electrophoresis in 1% agarose gels. Sequencing reactions of the PCR amplicons were performed with an ABI PRISM BigDye terminator cycle sequencing kit v.3.1 (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions using a primer 534-r. Sequences were analyzed with an ABI PRISM 3100 automated capillary DNA cycle sequencer (Applied Biosystems) and checked and edited with the Chromas program (Technelysium, Helensvale, Australia) and thereafter identified through the GenBank database (www.ncbi.nlm.nih.gov) using the BLAST (basic local alignment search tool) algorithm [1].

DNAMAN 4.1 (Lynnon BioSoft) was used for sequence alignment. The sequences obtained in this study are available from the GenBank database under the accession numbers DQ189058–DQ189076.

Results

Process conditions

Temperature, redox potential, pH, and conductivity were measured from each sample at the time of sampling. The results are presented in Table 2.

Culture

The results from culturing are presented in Fig. 1. The numbers of anaerobically growing bacteria also contain the numbers of facultative anaerobic bacteria. The samples containing high numbers of mesophilic (growth at 30°C) anaerobically growing bacteria were slime (3.7×10^7 cfu/ml), clay (1.8×10^7 cfu/ml), and broke II (6.9×10^7 cfu/ml). A high number of thermotolerant anaerobically growing bacteria (growth at 55°C) was found in pulp (4.0×10^7 cfu/ml). Furthermore, no thermotolerant anaerobically growing bacteria were found in $CaCO_3$ and starch slurries.

The numbers of culturable SRB are also presented in Fig. 1. Rather high numbers of mesophilic (growth at 30°C) SRB were found from clay (1.4×10^5 cfu/ml) and broke II (1.9×10^5 cfu/ml). Thermophilic SRB were found only from slime (6.0×10^3 cfu/ml) and clay (2.0×10^2 cfu/ml).

Desulfovibrionales-selective PCR-DGGE

During SRB colony isolation and purification, it was decided to discontinue traditional purification methods due to poor regrowth of the isolates to be purified. When cultures (one plateful of colonies originating from a colony growing on TSA on which the sample was plated) were transferred to Postgate's medium B, some of the cultures (six) ceased to grow. Two out of three cultures from broke I, two out of six from slime, three out of four from pulp and all the cultures from other samples showed growth on Postgate's medium B. DNA was isolated from all these grown enrichment cultures (23 out of 29). Before Desulfovibrionales PCR was performed with enrichment cultures and industrial samples, the PCR procedure was optimized with reference strains. The results are presented in Table 1. Combined results of the Desulfovibrionales-selective PCR are presented in Table 3. All the 23 enrichment cultures were positive by Desulfovibrionales PCR.

After PCR, DGGE was performed from the nested PCR products. It was noted that if dilutions 1:1000 and 1:100 were used prior to the second PCR step, the images of the DGGE gels were more in focus (data not shown). Some of the DGGE results are presented in Fig. 2, where the excised and sequenced amplicons are marked with arrows. Partial 16S rRNA was sequenced from 19 amplicons, 12 from enrichment cultures and 7 from the DGGE profiles of industrial samples from which the DNA was directly extracted (Table 4). According to the sequence data, an identical *Desulfovibrio* species sequence (sequence numbers 2, 8, 9, 11, and 12 in Fig. 2) was found for broke I (from paper machine I), slime (paper machine I), pulp (paper machine I), broke II (paper machine II) and white water II (paper machine II). Other identical sequences are presented in Table 4. Desulfovibrionales-related bacteria were also found from the samples without prior enrichment and the sequenced amplicons proved to belong to the Desulfovibrionales order (Table 4). The DGGE profiles derived from the samples without prior enrichment were more diverse than what would have been the combined DGGE-profile of the enrichment cultures of that same sample (Fig. 3). In addition, the amplicons which were seen in the DGGE profiles of the enrichment cultures, were not necessarily seen in the DGGE profiles of the samples without prior enrichment (i.e. the amplicons migrated differently; Figs. 2 and 3). The DNA was extracted from the industrial samples in duplicate and parallel samples gave similar profiles except that the DGGE profiles of parallel samples of white water II and $CaCO_3$ were somewhat different (Fig. 3).

PCR-DGGE of predominant bacteria

The SRB enrichment cultures were subjected to universal PCR of V_3 and V_6–V_8 hypervariable regions of

Table 2 Process parameters measured at the sampling sites during sampling

Sample	Temperature (°C)	pH	Conductivity (mS/cm)	Redox potential (V)
Broke I	42.0	7.5	0.945	0.145
White water I	45.6	7.0	1.26	0.090
Pulp	47.4	6.6	1.49	0.675
Clay	25.3	7.1	0.76	0.090
$CaCO_3$	30.3	8.6	1.44	0.095
Starch slurry	59.5	6.5	0.779	0.312
Broke II	32.5	6.2	2.28	−0.239
White water II	45.0	7.0	1.03	−0.002

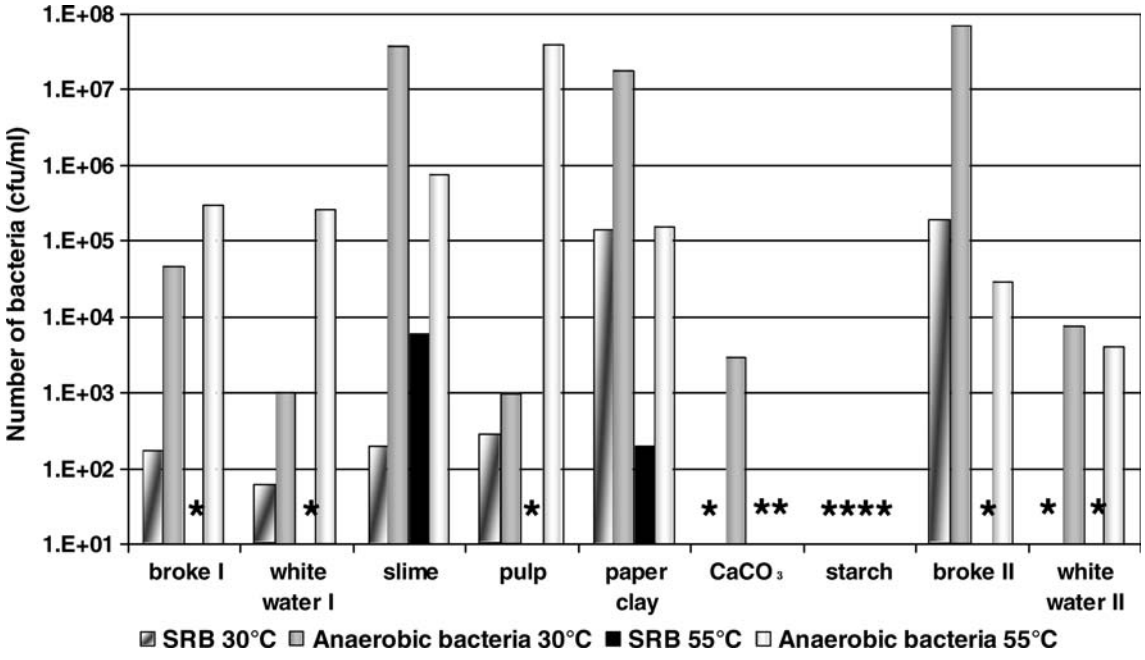

Fig. 1 Number of culturable anaerobic bacteria (including facultative anaerobic bacteria) and SRB as detected with plate count agar and $FeSO_4$ supplemented TSA, respectively, at 30°C and 55°C. *Samples in which the number of bacteria was below the detection limit (20 cfu/ml)

the 16S rRNA gene (according to the methods of Muyzer et al. [17] and Nübel et al. [18], respectively) to detect possible differences in amplification efficiency with different primer pairs. There were major differences in amplification signal intensities as detected by gel electrophoresis after universal PCRs. PCR of the V_3 hypervariable region amplified both the enrichment cultures and reference strains more efficiently than the PCR of the V_6–V_8 hypervariable region (data not shown).

Universal PCR-DGGE of the V_3 region [17] was performed on enrichment cultures to determine their purity. Only 4 out of 23 enrichment cultures showed identical banding patterns (one amplicon) with Desulfovibrionales-selective PCR-DGGE and universal PCR-DGGE (data not shown). Since the same universal PCR of the V_3 region of 16S rDNA was also used as the latter PCR in the nested Desulfovibrionales PCR, the banding patterns of Desulfovibrionales DGGE and universal DGGE were comparable. The amplicons which had the highest intensity in the banding pattern of universal PCR-DGGE of the enrichment cultures were also seen in the banding pattern of the Desulfovibrionales PCR-DGGE of the enrichment cultures (data not shown).

Both universal PCRs (V_3 [17] and V_6–V_8 [18]) were also performed from the DNA extracted directly from the frozen industrial samples. The DGGE profiles of the V_3 region were extremely diverse (data not shown). In contrast, the DGGE profiles obtained after universal PCR of the hypervariable region V_6–V_8 did not show as many bands as the same samples after PCR of the V_3 region (data not shown).

Discussion

The aim of this study was to evaluate the suitability of a nested PCR-DGGE method for the detection of Desulfovibrionales-related SRB from paper-making environment. The culture-based detection of SRB has been hampered by the inability of culture techniques to detect many anaerobic SRB. In addition, culture-based detection may take up to 10 days, which is a too long time period for the industrial quality control. During the past decade, SRB have been studied in great detail by fluorescent in situ hybridization (FISH) from various environments (e.g. Ref. 21). Unlike FISH, PCR-DGGE is suitable for a population analysis of SRB without exact prior knowledge of the species present in the sample. Furthermore, PCR results are obtained within one working day, which would be beneficial in the quality control of paper mills. The Desulfovibrionales-selective PCR used had already been validated with a great number of SRB [6] and the PCR procedure was optimized with a smaller panel of SRB for this study (Table 1). The present nested DGGE procedure enabled obtaining a general picture of the Desulfovibrionales diversity of the samples to be obtained. Furthermore, the possibility to sequence the amplicons of interest enabled the identification of the bacteria of interest (Table 4).

Desulfovibrionales was chosen as a target group since they are able to survive in the presence of oxygen [4, 5], which is a prerequisite for causing major problems in paper mills, since oxygen will inevitably be present in

Table 3 Results of the *Desulfovibrionales* PCR and universal PCR performed directly from samples and from cultures obtained after cultivation of the samples on TSA. The results from cultures are the combined results from all parallel cultures (+ weak amplification product as detected by gel electrophoresis or a medium number of culturable bacteria, + + strong amplification product or high number of culturable bacteria, − no amplification product or no culturable bacteria, * PCR of the cultures was not performed, since no cultures were obtained after cultivation)

	Broke I	White water I	Slime	Pulp	Paper clay	$CaCO_3$	Starch	Broke II	White water II
Universal PCR (M)[a] from cultures	+ +	+	+ +	+ +	+ +	*	*	+ +	+ +
Universal PCR (M)[a] from samples	+	+ +	+ +	+ +	+ +	+	−	+ +	+
Universal PCR (N)[b] from cultures	+	−[d]	+ +	+ +	+	*	*	+ +	+ +
Universal PCR (N)[b] from samples	−	+ +	+ +	+	+	−	−	+	−
Desulfovibrionales PCR[c] from cultures	+ +	+ +	+ +	+ +	+ +	*	*	+ +	+ +
Desulfovibrionales PCR[c] from samples	+	+ +	+ +	+ +	+ +	−	−	+ +	+
Nested *Desulfovibrionales* PCR from cultures	+ +	+ +	+ +	+ +	+ +	*	*	+ +	+ +
Nested *Desulfovibrionales* PCR from samples	+ +	+ +	+ +	+ +	+ +	+	−	+ +	+ +
SRB culture results (30°C)	+	+	+	+	+ +	−	−	+ +	−
SRB culture results (55°C)	−	−	+ +	−	+	−	−	−	−
Anaerobic culture results (30°C)	+	+	+ +	+	+ +	+	−	+ +	+
Anaerobic culture results (55°C)	+ +	+ +	+ +	+ +	+ +	−	−	+	+

[a]M: Universal PCR of V_3 hypervariable region of the rRNA gene performed according to the method of Muyzer et al. [17]
[b]N: Universal PCR of V_6–V_8 hypervariable region of the rRNA gene performed according to Nübel et al. [18]
[c]*Desulfovibrionales* PCR according to Daly et al. [6]
[d]Only one isolate was obtained from white water, compared to three to six isolates from other samples

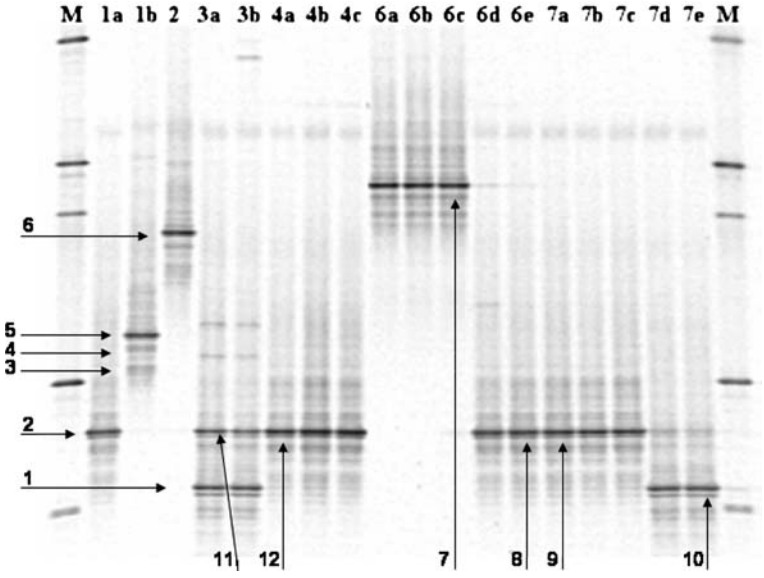

Fig. 2 Desulfovibrionales-selective PCR-DGGE profiles of different enrichment cultures from samples from paper machine I (*lanes 1* broke I, *lanes 2* white water I, *lanes 3* slime, *lanes 4* pulp) and from paper machine II (*lanes 6* broke II, *lanes 7* white water II). *M* denotes marker. The excised and sequenced bands are marked with *arrows*. The sequencing results are shown in Table 4

most parts of the paper-making process. In addition, Desulfovibrionales is one of the few orders within the SRB that is capable of growing quite adequately on artificial solid media and has therefore been shown to be present in the paper-making environment (e.g. Refs. 12 and 23). Furthermore, *Desulfovibrio* species have been conventionally regarded as the main causative bacteria of anaerobic corrosion [9]. A study with *Desulfovibrio*-specific PCR-DGGE targeting the [NiFe] hydrogenase gene was reported by Wawer and Muyzer in 1995 [27].

Table 4 Closest relatives of the PCR-DGGE amplicons obtained with Desulfovibrionales-selective PCR-DGGE as determined by comparative sequence analysis

Sequence number	Sample	Closest sequence found in the GenBank database	Similarity (%)[a]	Alignment: identical sequences[b]
1	Slime (enrichment culture)	Uncultured bacterium	100	Sequence 10
		Desulfovibrio longreachii	99	
		Desulfovibrio termitidis	99	
		Desulfovibrio oryzae	99	
		Desulfovibrio vulgaris subsp. *oxamicus*	99	
2	Broke I (enrichment culture)	*Desulfovibrio* sp.	100	Sequences 8, 9, 11, and 12
		E.g. *Desulfovibrio vulgaris* subsp. *oxamicus*	100	
		Desulfovibrio longreachii	100	
		Desulfovibrio termitidis	100	
		Desulfovibrio oryzae	100	
3	Broke I (enrichment culture)	*Desulfovibrio* sp.	97	
		Desulfovibrio alcoholovorans	96	
4	Broke I (enrichment culture)	*Desulfovibrio* sp.	95	
		E.g. *Desulfovibrio burkinensis*	94	
		Desulfovibrio carbinolicus	94	
		Desulfovibrio alcoholovorans	94	
		Desulfovibrio fructosovorans	94	
		Desulfovibrio sulfodismutans	94	
		Desulfovibrio magneticus	94	
5	Broke I (enrichment culture)	*Desulfovibrio alcoholovorans*	98	
6	White water I (enrichment culture)	*Desulfovibrio* sp.	99	Sequence E
		E.g. *Desulfovibrio vulgaris* subsp. *oxamicus*	99	
		Desulfovibrio longreachii	99	
		Desulfovibrio termitidis	99	
		Desulfovibrio oryzae	99	
		Desulfovibrio cavernae	99	
7	Broke II (enrichment culture)	*Desulfovibrio desulfuricans*	100	
8	Broke II (enrichment culture)	*Desulfovibrio* sp.	100	Sequences 2, 9, 11, and 12
		E.g. *Desulfovibrio vulgaris* subsp. *oxamicus*	100	
		Desulfovibrio longreachii	100	
		Desulfovibrio termitidis	100	
		Desulfovibrio oryzae	100	

Table 4 (Contd.)

Sequence number	Sample	Closest sequence found in the GenBank database	Similarity (%)[a]	Alignment: identical sequences[b]
9	Broke II (enrichment culture)	*Desulfovibrio* sp.	100	Sequences 2, 8, 11, and 12
		E.g. *Desulfovibrio longreachii*	100	
		Desulfovibrio termitidis	100	
		Desulfovibrio oryzae	100	
		Desulfovibrio vulgaris subsp. *oxamicus*	100	
10	White water II (enrichment culture)	Uncultured bacterium	100	Sequence 1
		Desulfovibrio longreachii	99	
		Desulfovibrio termitidis	99	
		Desulfovibrio oryzae	99	
		Desulfovibrio vulgaris subsp. *oxamicus*	99	
11	Slime (enrichment culture)	*Desulfovibrio* sp.	100	Sequences 2, 8, 9, and 12
		E.g. *Desulfovibrio longreachii*	100	
		Desulfovibrio termitidis	100	
		Desulfovibrio oryzae	100	
		Desulfovibrio vulgaris subsp. *oxamicus*	100	
12	Pulp (enrichment culture)	*Desulfovibrio* sp.	100	Sequences 2, 8,9, and 11
		E.g. *Desulfovibrio longreachii*	100	
		Desulfovibrio termitidis	100	
		Desulfovibrio oryzae	100	
		Desulfovibrio vulgaris subsp. *oxamicus*	100	
A	White water I (directly from the sample)	*Desulfocaldus terraneus*	98	Sequences C and D
B	Slime (directly from the sample)	*Desulfovibrio* sp.	98	
		E.g. *Desulfovibrio vulgaris* subsp. *oxamicus*	98	
		Desulfovibrio longreachii	98	
		Desulfovibrio termitidis	98	
		Desulfovibrio oryzae	98	
		Desulfovibrio cavernae	98	
C	Pulp (directly from the sample)	*Desulfocaldus terraneus*	98	Sequences A and D
D	White water II (directly from the sample)	*Desulfocaldus terraneus*	98	Sequences C and D
E	Broke II (directly from the sample)	*Desulfovibrio* sp.	99	Sequence 6
		E.g. *Desulfovibrio vulgaris* subsp. *oxamicus*	99	
		Desulfovibrio longreachii	99	
		Desulfovibrio termitidis	99	
		Desulfovibrio oryzae	99	
		Desulfovibrio cavernae	99	
F	Broke II (directly from the sample)	*Desulfovibrio alcoholovorans*	95	
G	Broke II (directly from the sample)	*Desulfovibrio* sp.	97	
		Desulfovibrio alcoholovorans	96	

[a]Comparative sequence analysis was performed using the BLAST (basic local alignment search tool) algorithm from the GenBank database (www.ncbi.nlm.nih.gov)
[b]DNAMAN 4.1 (Lynnon BioSoft) was used for sequence alignment

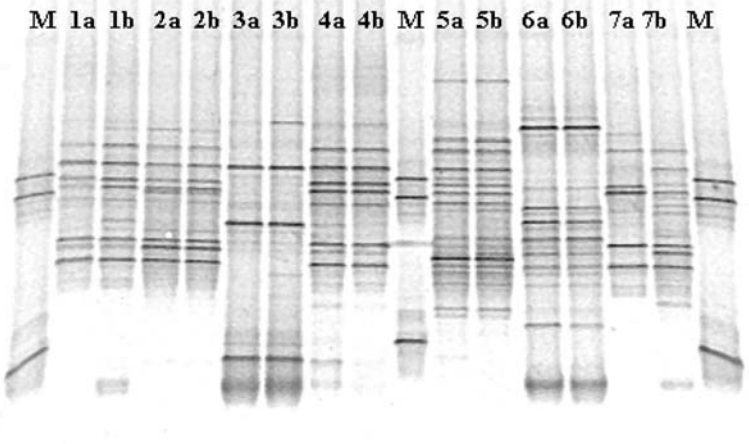

Fig. 3 Desulfovibrionales-selective PCR-DGGE profiles of parallel samples from paper machine I (*lanes 1* broke I, *lanes 2* white water I, *lanes 3* slime, *lanes 4* pulp, *lanes 5* paper clay), and from paper machine II (*lanes 6* broke II, *lanes 7* white water II). *M* denotes marker. The DNA was extracted directly from the frozen industrial samples in duplicate

We did not find this approach useful, since analysis of the [NiFe] hydrogenase sequences would not have yielded as many species identifications as with 16S rDNA sequences due to the abundance of the latter sequences in databases. Therefore, a Desulfovibrionales-selective PCR-DGGE targeting 16S rDNA was used instead. A similar nested PCR-DGGE technique for SRB analysis from upflow anaerobic sludge bed waste-water treatment reactors has been reported recently [7].

In our study, culturing was able to detect Desulfovibrionales-related bacteria from two different white waters, two different brokes, pulp, clay, and slime. Desulfovibrionales-selective PCR-DGGE was used to further characterize 23 enrichment cultures. Sequencing of the PCR-DGGE amplicons indicated that the current nested protocol correctly targeted the Desulfovibrionales group. Same sequence of *Desulfovibrio* sp. (identical DGGE migrations and 16S rRNA sequences in the sampling places) was found from paper machine I (broke I, slime, and pulp) and from paper machine II (broke II and white water II), suggesting an in-house contamination with the same strain. In addition, the same *Desulfocaldus terraneus* sequence was found in white water I (from paper machine I), pulp (paper machine I), and white water II (paper machine II). Desulfovibrionales-selective PCR-DGGE was also performed from the DNA templates extracted directly from the samples. Desulfovibrionales-related bacteria were found from all the same samples directly subjected to PCR-DGGE from which Desulfovibrionales were also found by the culturing method. In addition, Desulfovibrionales-related bacteria were detected in $CaCO_3$ slurry by only molecular techniques. Furthermore, the DGGE profiles derived from the directly extracted DNA (Fig. 3) were more diverse than the DGGE profiles derived from enrichment cultures of a given sample (Fig. 2), showing preferential growth of some SRB on the agar and/or in the enrichment culture.

The pH and temperature values detected in the samples (Table 2) were mostly suitable for the survival of SRB. The redox potential of the broke II sample (−0.239 V) indicated that the environmental conditions in broke II favored anaerobic growth. In fact, the highest numbers of culturable anaerobic bacteria (6.9×10^7 cfu/ml) and SRB (1.9×10^5 cfu/ml) at 30°C were found from broke II (Fig. 1). It has also been shown that SRB can become a problem in large storage vessels (e.g. broke towers) where residence times are long and the bottom of the tower may become completely deoxygenated. Depending on the contamination of dilution waters, original contamination, and especially storage time, the total microbial numbers in broke can be quite high. Microbiologically the most critical raw material in paper mills is coated broke, because the coating material (containing e.g. starch) is often a good nutrient for microorganisms [2, 11, 16, 26]. In this study, the second highest number of SRB with culturing at 30°C was detected from paper clay (1.4×10^5 cfu/ml; Fig. 1), which has also been reported to be a microbiologically critical material [26]. The other samples had only low numbers of culturable SRB. However, a possible methodological error should be taken into account because when a synthetic growth medium is used, as in our study, the number of viable SRB is underestimated [25]. In addition, sampling (without immediate hydrogen flow) and the agar medium used both favored Desulfovibrionales in our study.

In the present study we also investigated whether the predominant bacterial PCR-DGGE of the V_3 region [17] of 16S rDNA would reflect the bacterial diversity of a paper machine better than the V_6–V_8 region [18], or vice versa. Our DGGE results showed that the predominant bacterial populations of all the paper industrial samples were diverse with both V_3 and V_6–V_8 primers. However, V_3 primers clearly generated more amplicons separable in the DGGE than V_6–V_8 primers. Vanhoutte et al. [24] found that the similarity values of amplicons generated with V_6–V_8 primers were usually higher than those of the amplicons generated with V_3 primers. This result suggests that V_3 primers are less discriminatory as universal primers for DGGE than V_6–V_8 primers. This was earlier evidenced by some difficulties of the V_6–V_8 primers to generate an amplicon from some type strains of *Bacteroides* species [24]. The same phenomenon was detected in this study, since the type strains of *Desulfovibrio desulfuricans* and *Desulfomicrobium escambiense* did not amplify with V_6–V_8 primers.

In conclusion, the PCR–DGGE method used in this study was suitable for the detection of Desulfovibrionales-related SRB directly and with pre-enrichment from the different paper mill samples. Furthermore, the newly developed technique could be used for the rapid detection of Desulfovibrionales-contaminated industrial sites and, combined with sequencing, for tracing the contamination routes.

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