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Comparison of CE-SSCP and DGGE for monitoring a complex microbial community remediating mine drainage

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Abstract

Capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) is a promising high-throughput tool for profiling complex bioremediation communities, but has not been well characterized with respect to other methods such as denaturing gradient gel electrophoresis (DGGE). The purpose of this study was to compare CE-SSCP with DGGE with respect to: 1) overall representation of the community in terms of the dominant species identified and corresponding Shannon diversity indices; 2) reproducibility and resolution; and 3) artifacts, using a complex sulfate-reducing community remediating mine drainage as a model system. Some of the dominant microorganisms were detected by both methods, but there were also differences in the reported community compositions, and more phylogenetic groups were detected by CE-SSCP. CE-SSCP Shannon diversity indices were slightly higher than those determined from DGGE data, and differed in terms of the time point at which the community was reported to have the highest diversity. Both methods had high reproducibility, but CE-SSCP resolution was higher in terms of the total number of peaks resolved, reduced co-migration of distinct DNA sequences, and length and legibility of the DNA sequencing data of clones used to identify peaks. Ten double bands in the same lane representing the same species were found by DGGE, whereas only one such artifact was observed by CE-SSCP. Finally, less overall sample preparation and analysis time was required for CE-SSCP than for DGGE. The results suggest that CE-SSCP offers several advantages over DGGE, especially for high-throughput monitoring.
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Keywords: DGGE; CE-SSCP; Microbial community fingerprint; Mine drainage remediation

1. Introduction

There is significant need for high-throughput tools for monitoring microbial communities involved in bioremediation. Nucleic acid "fingerprinting" techniques using universal polymerase chain reaction (PCR) primers targeting 16S rRNA genes, such as denaturing gradient gel electrophoresis (DGGE), offer a major advantage in that they provide a convenient snapshot of the overall community that can be compared with time (Muyzer et al., 1996). Furthermore, fingerprinting techniques are relatively less labor-intensive than other profiling approaches, such as cloning and sequencing, and thus many samples can be processed and compared in parallel. This is a critical attribute for bioremediation monitoring, which typically has a restricted budget and requires continued measurements of several parameters with time.

Since DGGE was first reported by Muyzer et al. (1993), the original work has been cited in over 2000 studies (Web of Science "DGGE" keyword search, November, 2006). Since then, several other fingerprinting techniques have been described and applied, including terminal restriction fragment length polymorphism (tRFLP) (Clement et al., 1998), length heterogeneity PCR (LH-PCR) (Suzuki et al., 1998), and single-strand conformation polymorphism (SSCP) (Lee et al., 1996). Although these fingerprinting methods have had a clear impact on the field of molecular microbial ecology, there still is no clear "gold standard" community fingerprinting method. Each method carries with it various advantages and disadvantages in terms of specificity, resolution, throughput, and corresponding sequence length and

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quality available for identifying dominants. Artifacts, such as one species being represented by more than one band or peak, are also commonly cited problems.

Capillary electrophoresis SSCP (CE-SSCP) (Delbes et al., 2000) may offer several advantages over existing methods. However, this method has not been well characterized with respect to more commonly applied fingerprinting approaches, such as DGGE. In CE-SSCP, fluorescently labeled blunt-ended PCR products are obtained from the highly variable V3 region of the 16S rRNA gene, denatured at high temperature, and then rapidly cooled to form unique single-strand conformations, which are separated based on their electrophoretic mobility in a polymer-filled capillary. The identity of peaks is determined by analyzing a clone library generated from the same sample in parallel and sequencing clones that correspond to peaks in the community. A major advantage of CE-SSCP over gel-based techniques is the high-throughput nature that allows parallel processing of multiple samples. Also, problems with gel-to-gel variation are avoided by using a sensitive capillary and standardized reagents (Hebenbrock et al., 1995). When compared to other high-throughput capillary-based techniques, such as tRFLP, a significant improvement is that CE-SSCP does not require restriction digestion of the templates, which has been reported to result in incomplete digestion and subsequent formation of multiple peaks per operational taxonomic unit (OTU) (Clement et al., 1998; Mills et al., 2003). CE-SSCP has recently been applied in the study of complex communities (Dabert et al., 2005; Peu et al., 2006), but its potential for such applications has not been established. In a recent study by King et al. (2005), the resolution capability of CE-SSCP was explored using pure cultures and mixtures of pure cultures, however, there is a need to better characterize the method for applications to complex communities.

The purpose of this study was to compare CE-SSCP with DGGE for profiling a complex microbial community. A sulfate-reducing mine drainage treatment community was considered to be a particularly suitable model for this study because of its known complexity, and the requirement of multiple microbial functional groups for its overall success in sulfate reduction and precipitation of heavy metals. Columns were used to simulate sulfate-reducing permeable reactive zones (SR-PRZs), which are typically provided with a complex slow-release carbon substrate, such as wood chips (Hulshof et al., 2006). A fully-functioning SR-PRZ community of the type studied here includes anaerobic cellulose degraders, cellobiose degraders, fermenters, and sulfate reducers (Logan et al., 2005).

CE-SSCP and DGGE were used to monitor SR-PRZ column communities at three time points and the following aspects of the results were compared: 1) overall representation of the community in terms of the dominant species identified and corresponding Shannon diversity indices; 2) reproducibility and resolution; and 3) artifacts. The expected results were that the two approaches would provide complementary information in terms of the species identified and diversity, but that CE-SSCP would have higher resolution and be less susceptible to artifacts, with less labor and overall analysis time.

2. Materials and methods

2.1. Sample collection and characteristics

Samples were collected from SR-PRZ columns constructed and operated at the Colorado School of Mines (Golden, Colorado). Pairs of duplicate columns were sacrificed at different times (Logan et al., 2005) and column samples from each pair were combined in equal proportions, thus yielding three samples for this study: B17, collected after 41 days of operation when sulfate-reducing activity was high ("performance"); B34, collected after 99 days of operation when the sulfate-reduction rate had decreased by about half ("decline"); and B56, collected after about a year of operation when flow became hindered due to clogging ("hindered").

2.2. DNA extraction

DNA extraction was carried out using the FastDNA Spin Kit for Soil (Q-BIOGene, Irvine, CA) according to the manufacturer's protocol using approximately 500 mg (wet weight) of column material. The concentration of extracted DNA was measured by absorbance at 260 nm using an 8452A diode array spectrophotometer (Hewlett-Packard, Houston, TX).

2.3. Denaturing gradient gel electrophoresis (DGGE)

Two DGGE analyses were performed in this study on the variable V3 region of the bacterial 16S rDNA. The first DGGE (DGGE1) was carried out on samples B17 and B34 with nested PCR products, and the second (DGGE2) was carried out on all three samples with un-nested PCR products. Conducting both a nested and an un-nested DGGE provided a means to identify any potential effects of PCR primers and protocols on the results obtained. Also, the effect of nested PCR on community fingerprints remains a topic of significant interest, and in some cases nested PCR protocols have been found to result in a broader detection of bacterial groups (Mahmood et al., 2006).

For the nested DGGE, the bacterial forward primer 8F and universal reverse primer 1492R (Table 1) were used for the first step. Universal bacterial primers I-341-FGC and I-533R (Table 1) were then used to obtain products for both DGGE analyses. PCR conditions, including denaturing, annealing and extension temperatures, number of cycles, and polymerase applied, are summarized in Table 1. For the un-nested PCR, the number of cycles was kept at 25 for direct comparison to CE-SSCP, whereas for the nested PCR, the number of cycles was chosen as recommended by Watanabe et al. (2001).

DGGE was carried out at 57 °C with a D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). Eight percent (w/v) polyacrylamide gels with a denaturing gradient from 20 to 55% (100% = 40% (v/v) deionized formamide and 42% (w/v) urea) were loaded with 23 μL of PCR product, and a voltage of 45 V was applied for 20 h. In the second DGGE, the gradient was narrowed to the range of 35 to 50% to improve band resolution. The gels were stained with 1X SYBR Gold (Molecular Probes, Eugene, OR) and documented

Table 1
Primers and PCR conditions applied in this study[a]

Primer[b]	Sequence 5′-3′ [c, d]	Method	Anneal temp. (°C)	Prod. size (bp)	# Cycles[e]	Reference
8-F	AGAGTTTGATCCTGGCTCAG	Cloning/Nested DGGE	50	1485	35/35	Weisburg et al. (1991)
1492-R	GGTTACCTTGTTACGACTT					Weisburg et al. (1991)
I-341-FGC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-CCTACGGGIGGCIGCA	Nested/ Un-nested DGGE	47–52	193	10 touchdown followed by 35/25	Watanabe et al. (2001)
I-533-R	TTACCGIIICTICTGGCAC					Watanabe et al. (2001)
329-F(w49)	ACGGTCCAGACTACGGGTCCAGACTCCTACGGG	CE-SSCP	61	205	25	(Delbes et al., 2001; Peu et al., 2006)
533-R (w104)	6FAM–TTACCGCGGCTGCTGGCAC					(Delbes et al., 2001; Peu et al., 2006)

[a]Eppendorf (Hamburg, Germany) Master*Taq* polymerase and buffers used for all PCRs, except CE-SSCP, in which Stratagene (La Jolla, CA) *Pfu* blunt-ended polymerase and buffer was used.
[b]Primer names incorporate number corresponding 16S gene position for *Escherichia coli*.
[c]Ambiguities: R (G or A); Y (C or T); K (G or T); M (A or C); S (G or C); W (A or T).
[d]Overlapping regions are highlighted for forward and reverse primers, respectively.
[e]All protocols implemented 2 min initial denaturing at 94 °C and 7 min final incubation at the extension temperature (68–72 °C).

using the UVP BioChemi System and Labworks software (UVP, Upland, CA). Visible bands were excised, stored in 35 μL sterile water, and re-amplified using I-341-FGC and I-533-R primers using the same conditions described above. PCR products were purified using a GeneClean Spin Kit (Q-BIOGene) and sequenced off-site by Davis Sequencing (Davis, CA) or SeqWright (Houston, TX).

2.4. Capillary electrophoresis single-strand conformation polymorphism (CE-SSCP)

CE-SSCP PCR amplification was carried out using the primers described in Table 1. As was the case in DGGE, these primers targeted the variable V3 region of the 16S rRNA gene. The reverse primer was identical to that applied in DGGE, except that it carried 6-carboxyfluorescein (6FAM) at the 5′ end, and the primer in DGGE had several inosine base substitutions, which have been reported to improve the range of detection (Watanabe et al., 2001). The CE-SSCP and DGGE forward primers had 8 bp of overlap (Table 1), with the DGGE primer carrying a 40 bp GC clamp and two inosine base substitutions. The *Pfu* DNA polymerase applied in CE-SSCP provides blunt-ended PCR products, which avoids interference of random adenylation in mutation analyses (Hu, 1993). The template was either microbial community DNA extract or M13 PCR product obtained from clones from sample B17 (see Section 2.5).

Because of the high sensitivity of CE-SSCP, it was necessary to dilute all PCR products before analysis. The dilutions applied resolved the highest peak within the range of 85–95% of the upper detection limit (peak saturation) for CE-SSCP. One microliter of the diluted PCR product was mixed with 18.8 μL deionized formamide and 0.2 μL GeneScan-400 ROX internal size standard (Applied Biosystems, Foster City, CA). The samples were denatured for 5 min at 95 °C and placed directly on a mixture of ice and water for 15 min. The single-stranded DNA conformations were separated by capillary electrophoresis using an ABI Prism 310 Genetic Analyzer (Applied Biosystems) equipped with a capillary (47 cm × 50 μm) filled with a polymer consisting of 5.6% GeneScan Polymer (Applied Biosystems), 10% glycerol, and 1× TBE (Tris-Borate-EDTA). The results obtained were analyzed using GeneMapper v3.0 (Applied Biosystems) (Chackhiani et al., 2004). The analysis temperature was kept constant at 37 °C. Because the ABI 310 is only equipped with a warmer and not a chiller, it was necessary to maintain ambient temperature at 70 °F or less for the analysis to proceed without an error warning.

To identify peaks, the microbial community PCR products were analyzed in parallel with PCR products obtained from a clone library of sample B17 (described in Section 2.5). For consistency, the community DNA from sample B17 was included with every run, and the average and standard deviation of the relative migration distance (RMD) of all peaks were determined. The purified M13 PCR products from clones that matched community peaks were sequenced at the CSU Macromolecular Resources Facility (Colorado State University, Fort Collins, CO). In the case of multiple matching clones per peak, a conservative approach was taken by selecting the

clones with the highest and lowest RMDs in order to identify possible mixed sequences per peak. For example, if two clones matched a peak, then both were sequenced, if three or four matched, then the two with the highest and lowest RMD were sequenced, if five or six matched, then the clones with the highest and lowest and the median RMD were sequenced (three total). In the remaining cases of 9, 17, and 31 matches, 4, 5, and 6 clones were sequenced, respectively, representing the highest, lowest, and median RMDs. An evolutionary distance dendrogram of the resulting sequences was constructed (as described in Section 2.6) in order to verify clustering of clones corresponding to the same peak and to identify peaks with multiple matching clones.

2.5. Construction of clone libraries

Near full-length 16S rDNA was amplified with bacterial forward primer 8F and universal reverse primer 1492R (Table 1) from column B17 DNA extract, which had the most peaks according to CE-SSCP and was obtained from columns with the highest level of sulfate-reducing activity. The PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The inserts were PCR amplified from random colonies using M13 forward (−20) and M13 reverse primers. A library of 159 M13 PCR products was obtained from the clones and 54 were sequenced according to the above criteria. Legible data were obtained from 46 of these; in the remaining cases, the lack of quality data was likely due to insufficient PCR product available for sequencing. Legibility refers generally to the relative infrequency of ambiguous (N) base pairs. The length of the legible sequence region was defined by the number of base pairs that aligned when analyzed using the BLAST online alignment tool (defined in the following section).

2.6. DNA sequence analysis and assignment to functional groups

Phylogenetic identities of sequences were estimated using the BLAST (Altschul et al., 1997) and RDP-II (Cole et al., 2005) online alignment tools. DGGE, CE-SSCP, and reference sequences were aligned using ClustalX (Thompson et al., 1997) and edited using ProSeq (Filatov, 2002). Phylogenetic distances between the sequences were calculated using the neighbor-joining method (Saitou and Nei, 1987) as implemented by the ClustalX program, with a bootstrap analysis of 1000 replicates. The parameters for the ClustalX program were set according to Hall (Hall, 2004).

The identification of functional groups was also important to this study because information about the capabilities of the organisms present may be of more interest than phylogenetic identity in situations such as applied bioremediation studies. To assign sequences to functional groups of interest (cellulose degradation, cellobiose/saccharide degradation, fermentation, and sulfate reduction), a literature review was conducted for the highest match(es) obtained for each sequence.

2.7. Relative diversities from DGGE and CE-SSCP

There is significant interest in obtaining biodiversity information from fingerprints in the absence of DNA sequence data (Hori et al., 2006; Loisel et al., 2006), and the Shannon

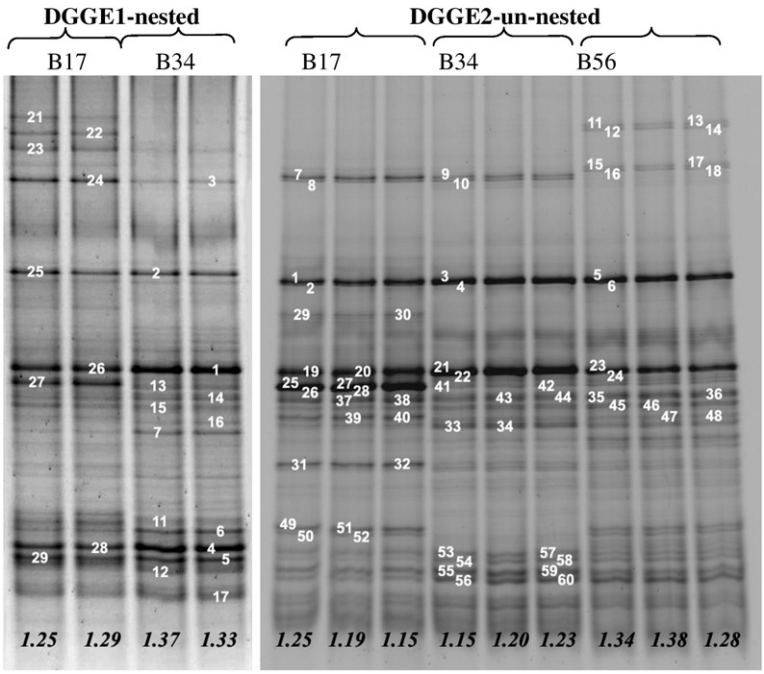

Fig. 1. DGGE gel profiles from columns with time. B17 was collected on day 41 (performance), B34 on day 99 (decline), and B56 after approximately one year (hindered). The DNA present in numbered bands was sequenced, the highest matches are summarized in Table 2. The average Shannon diversity indices (H) are shown at the bottom of each lane in *italics*.

Table 2
Characterization of bacterial DGGE bands from DGGE1 and DGGE2

Sample	Band	Highest matches[a]	Putative functional category[b]	GenBank accession number	Matching CE-SSCP peak[c]
B17 on DGGE1	21	*Eubacterium* sp. clone BU061 (Unknown)		AF385567	
	22	Uncultured Clostridia BREC56 (Unknown)		AY338353	
	23	Butyrate-producing Clostridia L2-50	C	AJ270491	
		Uncultured cattle rumen Clostridia		AF018559, AB034032, AB034025	
		Uncultured non-ruminant Clostridia		AF153856, AF052416	
	24	*Eubacterium halii*	C	L34621	
		Butyrate-producing Clostridia L2-7	C	AJ270490	
		Rumen Clostridia RF5		AF001738	
		Non-ruminant Clostridia		AB064719, AB064718, AF371667, AF371666, AF371660, AF371659, AF371658	
	25	Cattle rumen Clostridia		AB034116, AB034115, AB034056	
		Non-ruminant Clostridia		AB099740, AB064880, AF371941, AF371671, AF371656, AF371649	
	26, 27	Clostridia clone p-1594-c5		AF371648	Peak12-2
	28	Mixed ruminal Clostridia PAD27		AF070466	
	29	Various bacteria in genus *Clostridium*	A B C	X68180, X68179, X71850, Y18171, X81021, U16165, U16122, X76164, Y15984, AJ458417-AJ458419, AJ229244, AB020190, AB020188, AB020187	Peak8-4
		Non-ruminant Clostridia		AF371842	Peak12-1
B17 on DGGE2	7, 8	Elbe River Bacteroidetes snow isolate 9_3	C	AF150715	Peak14
	19, 20	Uncultured non-ruminant Clostridia		AY916219, AF371648, AY457848, AY986051, AY985370, AY985008, AY984650, AY984644, AY977826, AF371648, AY816875, AY457848	
	25	*Paenibacillus* sp. TERI 1018 found at oily sludge contaminated site		AY269226	
	27	Bacteria in genus *Acinetobacter*, aerobic nonfermentative bacteria capable of growing on a large range of organic compounds		X81662, Z93437, AY586400, AF467306, AF467305, AF467302, AF467300, AF467298, AF467295, AF467294, AY639376, AJ247201, AJ247200	Peak23
	29, 30	Non-ruminant Clostridia clone C20_a22		AY991724	
	31	Non-ruminant Clostridia clone C13_H12		AY991279	Peak8-4
	32	Non-ruminant Clostridia clone K319		AY976726	
	37, 38	Non-ruminant Clostridia A12		AF052413	
		Non-ruminant manure pit Clostridia P3OTU1		AF261794	
	39, 40	Non-ruminant Clostridia clone C15_N08		AY993895	
	49, 50, 51, 52	*Clostridium mangenotii*	C D	AJ318905, AJ318904	
B34 on DGGE1	1		Same as Band 26 on DGGE1		
	2	Bacilli MOL361	C	AF349724	
		Soil Bacilli	C	AY242740, AY242739	
		Uncultured pig intestinal Bacilli		AF371534-AF371536	
	3		Same as Band 24 on DGGE1		
	4	Various bacteria in genus *Clostridium*	C	X73451, Y18787, X73437, X73447, M59107, AB075769, AF320283	
		Eubacterium tenue	C	M59118	
		Uncultured Clostridia	C	AF150692, AJ229220, AF018038, AJ409003, AB064872, AB099796, AB075658, AY167932	
	5	Various bacteria in genus *Clostridium*	A B C	X73446, X77842, U16147, X76164, X71850, X68170, X68171, X68173-X68180, Y18175, Y18170-Y18172, AJ289704-AJ289706, AE007815, AE007612, AE007543, AE007541, AE007536, AE007528, AE007527, AE007513, AF270501, AF270502, AJ229244, AJ420008	Peak8-4
		Sarcina maxima and *Sarcina ventriculi*	C	X76650, X76649, AF110272	Peak12-1
		Non-ruminant Clostridia MPN-isolate group 26		AF357574	
	6	*Clostridium puniceum*	C	X71857	Peak8-4
		Clostridium sp. DSM 5906 and 5907	C	X76742, X76749	Peak12-1
	7	Butyrate-producing Clostridia L2-50	C	AJ270491	
		Uncultured cattle rumen Clostridia		AB034025, AF018559	
		Uncultured non-ruminant Clostridia		AF371640, AF052416, AF153856	

Table 2 (*continued*)

Sample	Band	Highest matches[a]	Putative functional category[b]	GenBank accession number	Matching CE-SSCP peak[c]
B34 on DGGE1	11	Various bacteria in genus *Clostridium*	A B C	X68180, X68179, Y18175, Y18171, X71857, X81021, U16165, U16122, X71850, X76740, X76164, X76742, X76749, AJ458417-AJ458419, AF069742, AJ229244	Peak8-4
		Anaerobacter polyendosporus	B C	Y18189	Peak12-1
		Sarcina maxima and *Sarcina ventriculi*		X76650, X76649, AF110272	
		Uncultured pig intestinal Clostridia		AF445291-AF445293, AF445272-AF445275, AF445216-AF445221, AF445210-AF445214, AF371846, AF371842	
	12	Various bacteria in genus *Clostridium*	B C	X73451, X76750, Y18787, X73437, X73447, X73450, M59107, AB075769, AF072474, AF072473, AB075770, AJ310756, AY244773, AJ291746, AY007244, AF320283	
		Eubacterium tenue	C	M59118	
		Uncultured Clostridia	C	AF150692, AF150690, AJ229220, AF018038, AY328361, AY328358, AJ409003, AB099796, AB075658, AF371689-AF371691, AY167932	
	13		Same as Band 26 on DGGE1		
	14, 15	*Eubacterium xylanophilum*	A B	L34628	
		Uncultured non-ruminant Clostridia		AF153857, AJ409006, AF371674-AF371677, AF371657, AF371653, AF371599, AF371575, AJ400272	
	16	*Clostridium herbivorans*	A	L34418	
		Eubacterium xylanophilum	A B C	L34628	
		Oribacterium sinus		AY323228	
		Uncultured non-ruminant Clostridia		AJ400272, AF153857, AF365665, AF371674-AF371677, AF371657, AF371654, AF371653, AF371599, AF371575	
	17	Unidentified swine feces Clostridia FOTU3		AF261778	
B34 on DGGE2	3, 4	Ruminant Bacilli clone 1-H07		AB107581	Peak20-1
		Bighorn sheep Bacilli		AY939152, AY939151, AY939139, AY938739, AY938736, AY938732, AY938728, AY938721, AY938708, AY938690, AY938689	
	21, 22	Unidentified rumen Clostridia RC7		AF001700	
		Uncultured ruminant Clostridia Thompsons108		AY854304	
	33	Uncultured non-ruminant Clostridia Eldhufec261		AY920136	
	41, 42	Uncultured non-ruminant Clostridia		AY919983, AY816875, AY916219, AF371648, AY457848, AY986051, AY985370, AY985008, AY984650, AY984644, AY977826	
	43	*Oribacterium sinus*	C	AY323228	
		Uncultured Clostridia		AY854354, AY854284, AF001716	
	44		Same as Band 37, 38 on DGGE2		
	53, 54, 58	Ruminant Clostridia clone 2-B03		AB107491	Peak5-4
	55	Various bacteria in genus *Clostridium*	C	X73451, X73447, X73437, AF320283, AY458851	Peak5-4
		Uncultured cattle rumen Clostridia		AB185612, AB185550, AB107434, AB107433	
		Uncultured non-ruminant Clostridia		AY916153, AB064872, AB189898, AB099796, AB075658, DQ015599, DQ015509, DQ015428, DQ015318, DQ015099, DQ015075	
	56	Uncultured Clostridia clone KR12 found from glucose biofuel cells		AY483171	Peak5-4
	57	Uncultured swine feces Clostridia F2		AF261779	Peak5-4
	59, 60		Same as Band 55 on DGGE2		
B56 on DGGE2	5, 6		Same as Band 3 on DGGE2		Peak20-1
	11, 12	Non-ruminant Clostridia clone NM47		AY983520	
	13	*Clostridium butyricum*	C	AB075768	
		Non-ruminant *Clostridium* sp.		AJ243511	
	14	Unidentified actinomycete found from rhizosphere of a green alder		L43598	
	23, 24		Same as Band 21 on DGGE2		
	35	Non-ruminant Clostridia clone C11_L151		AY991640	
	36, 45		Same as Band 37, 38 on DGGE2		
	46	Non-ruminant Clostridia clone LE36		AY816906	
		Non-ruminant Clostridia clone HuDI84		AY684365	

[a]When more than one sequence was listed as the highest match, all the results are presented.

[b]When the matching sequence had >97% similarity to a sequence corresponding to an organism with one of the four functional categories, it was assigned to that category: A: Cellulose degrader; microorganisms with the ability to degrade cellulose and ferment other saccharides. B: Cellobiose degrader; microorganisms with ability to ferment cellobiose and other saccharides. C: Fermenter. D: Sulfate reducer. When the functional/environmental property of a sequence match did not correspond to one of these four categories, a basic description of the source and/or characteristics is provided.

[c]Based on a comparison of GenBank accession numbers obtained from BLAST searches.

diversity index is a common approach for accomplishing this. In this study, the Shannon diversity index were calculated as:

$$H = -\sum \left[\frac{n_i}{N} \log\left(\frac{n_i}{N}\right) \right] \quad (1)$$

where H is the Shannon diversity index, n_i is the intensity or height of the individual DGGE bands or CE-SSCP peaks, and N is the sum of intensity or height of all the bands or peaks (Cox, 1972; Xia et al., 2005). CE-SSCP peaks were defined using ABI GeneMapper Software (Foster City, CA) with the minimum value for the width of a peak at half the maximum height set to a value of 5. This same definition was also applied in defining DGGE bands by converting the gel image in each lane to a saturation profile using UVP Labworks Software (Upland, CA). This approach provided a means of directly comparing the numbers of peaks and bands detected by the two methods.

2.8. Cluster analysis

The reproducibility of DGGE and CE-SSCP profiles was compared using cluster analysis. Intensity data from each lane

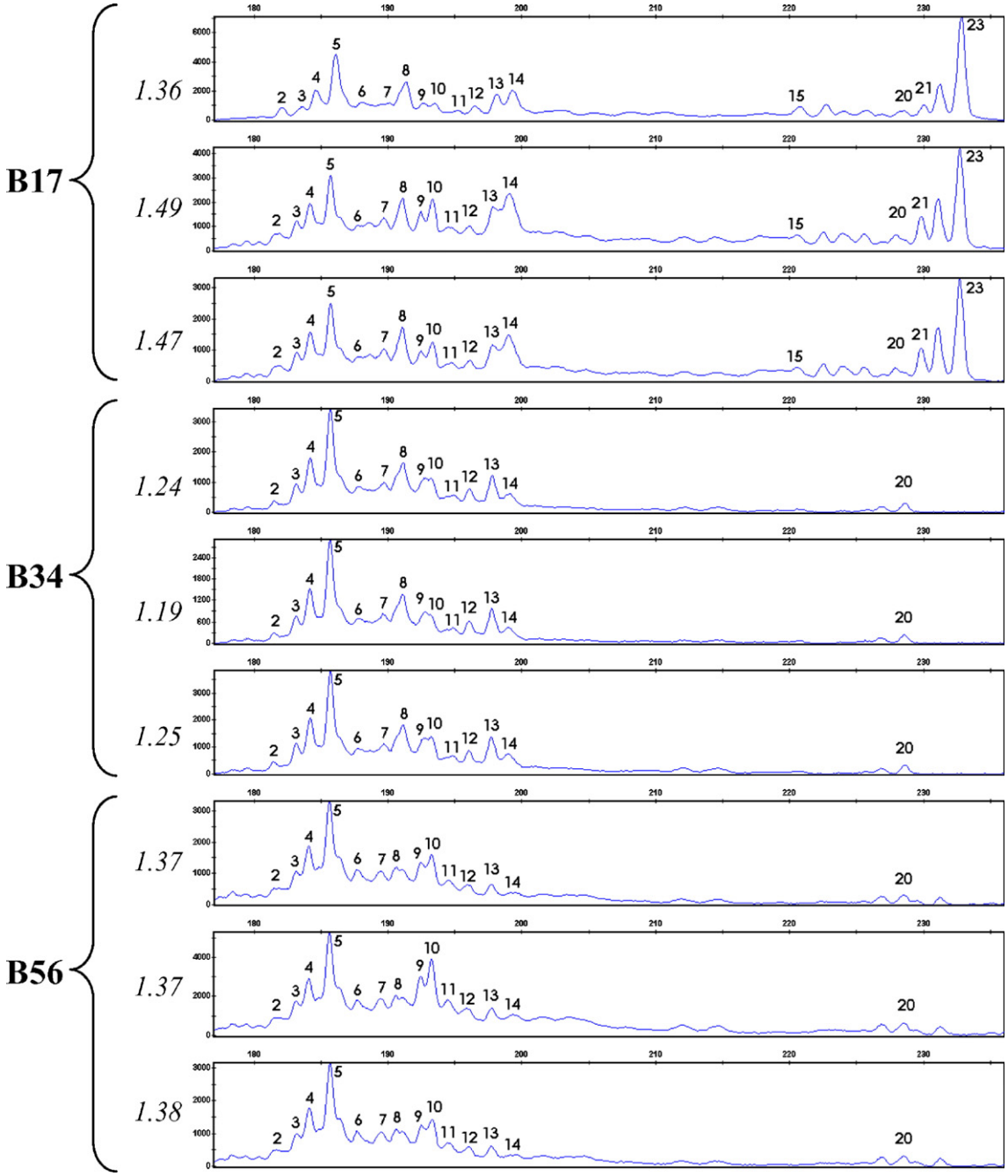

Fig. 2. CE-SSCP community profiles with time. Each sample was analyzed in triplicate. B17 was collected on day 41 (performance), B34 on day 99 (decline), and B56 after approximately one year (hindered). The *Y*-axes of each electropherogram was scaled to its maximum to compare relative peak heights. Only distinguishable peaks for which matching clones were found are numbered (identified in Table 3). The values of Shannon diversity index (H) are shown at the left of each in *italics*.

or electropherogram was obtained using Labworks (UVP, Upland, CA) for DGGE or extracted from GeneMapper using DigitizeIt (Ingo Bormann, Braunschweig, Germany) for CE-SSCP. Cluster analyses were performed using SPSS for Windows (SPSS Inc., Chicago) and the dendrograms were drawn using the squared Euclidean distance and Ward's method.

3. Results and discussion

3.1. Overall representation of the community

The community profiles obtained by DGGE (Fig. 1) include a few distinct bands and a high background signal, an indication of community complexity. For sample B17, 16 distinguishable bands were observed in the nested DGGE and 15 were observed in the un-nested DGGE. From excised bands across all samples and gels, it was possible to assign an identity to 80 of 97 total sequences (Table 2). Judging by the number of peaks and the relative height of the baseline, the CE-SSCP patterns also revealed a complex community (Fig. 2). However, for sample B17, 23 distinct peaks were observed and matching clones were found for 17 of these (Table 3). The average sequence similarity to the highest matches was 94.9%±4.7 for the DGGE bands and 93.6%±3.8 for CE-SSCP peaks, and the length of legible sequence ranged from 49 to 169 base pairs (average = 103 bp) for DGGE bands and 408 to 705 base pairs (average = 652 bp) for clones matching CE-SSCP peaks. It was also noted generally that the DNA sequences obtained from the nested DGGE were of higher quality in terms of overall legibility than those obtained from the un-nested DGGE.

There were some similarities and some differences observed between the two methods in comparing the community compositions. Only six CE-SSCP peaks were found to have the same identity as DGGE bands (Tables 2, 3). Both methods detected several Clostridia and a few Bacilli, Bacteroidetes, and Gamma Proteobacteria. However, DGGE failed to detect Alpha Proteobacteria, Beta Proteobacteria, and Delta Proteobacteria, which were all detected by CE-SSCP (Table 4). Both methods revealed that Bacteria belonging to the class Clostridia were the most common in all columns (Table 4). In terms of functional groups, both methods detected bacteria with high similarities to cellulose degraders, fermenters, cellobiose degraders, sulfate reducers, or which had unrelated or unknown function. Interestingly, although sulfate reduction was the main function of the columns, each method detected only one sulfate reducer (Table 4).

Peak heights were also compared as representative of relative dominance. Fig. 3 is a plot of the number of matching clones versus peak height, with an R^2 value of 0.727. Therefore, cloning results, which are commonly used to obtain semi-quantitative information about communities, were in good agreement with CE-SSCP results. The fact that there were six CE-SSCP peaks that could not be identified after screening 159 random clones (e.g., Peak 22) is likely a result of the different primers applied in cloning and CE-SSCP (Table 1). However, applying different primer sets is a useful way to provide validation between the two methods and also to obtain longer DNA sequences. A comparison of the CE-SSCP and DGGE, results reveals that the most intense band and peak for both methods corresponded to *Acinetobacter* sp., and was only present in sample B17 according to both methods. CE-SSCP peaks 5 and 8 had the next highest intensities, and all matches to these peaks corresponded to Clostridia. Sequences from several high intensity DGGE bands were also found to have high matches with various Clostridia.

There is currently interest in estimating diversity directly from community fingerprints without the requirement for DNA sequencing (Hori et al., 2006; Loisel et al., 2006). In this study, the Shannon diversity index revealed differences between the relative diversities estimated by CE-SSCP and DGGE. The Shannon indices determined from the un-nested DGGE community profiles indicated that column B56 had the highest diversity (1.33±0.05) and that the diversity in columns B17 and B34 were not significantly different (1.19±0.05 and 1.19±0.04, respectively). The nested DGGE yielded a slightly higher diversity estimate for column B34, likely because more bands were detected in the nested PCR product. Diversity indices determined for B34 from CE-SSCP profiles (1.23±0.03) were not significantly different from those determined from DGGE profiles, but were higher for B17 (1.44±0.07) and B56 (1.38±0.01). The higher resolution of CE-SSCP, which resulted in the detection of more peaks than DGGE bands, is the likely cause of the higher diversity estimates obtained from CE-SSCP data. It is also of interest to note that DGGE diversity estimates were lower than those of CE-SSCP in spite of the fact that sequencing data revealed the presence of several double bands in the DGGE profiles with the same identity, whereas this was not an issue with CE-SSCP (discussed further in Section 3.3).

3.2. Reproducibility and resolution

The reproducibility of the DGGE and CE-SSCP methods was determined using cluster analysis. For DGGE, PCR replicates run on the same gel were highly reproducible (Fig. 4). While the nested and un-nested DGGE profiles visually appeared to be similar (Fig. 1), the cluster analysis showed that B17 and B34 were more similar to each other on the nested DGGE gel than they were to the same sample on the un-nested gel (Fig. 4). In fact, on the un-nested gel, B34 was distinct from B17 and was more similar to B56. Therefore, though there were more bands detected and the sequence data was of higher quality, the nested PCR protocol had a clear impact on the character of the DGGE profile.

Cluster analysis of the CE-SSCP profiles also suggested that B34 was more similar to B56, and that there is some reduction in reproducibility when the sample is not analyzed using the same capillary (e.g., sample B17-1), although the profiles still cluster together. Though it was not the focus of this study, it has been noted in our broader analyses with the ABI 310 that while most capillaries are consistent with respect to overall mobility, different capillary lot numbers differ with respect to their relative sensitivities. Therefore, the PCR product dilutions

Table 3
Characterization of bacterial 16S rDNA cloned for CE-SSCP from sample B17

Clone[a]	Matching peaks	Highest matches[b]	Putative functional category[c]	GenBank accession number	Matching DGGE Bands[d]
Clone2-8 Clone2-9	Peak2	*Brevundimonas diminuta* found from contaminant of *Bacillus cereus*, food poisoning bacteria, culture		X87274	
Clone3-1	Peak3	Clostridia clone C19		DQ088208	
Clone4-2	Peak4	Clostridia clone C15_G08		AY993790	
Clone4-6		Clostridia clone C20_e05		AY991781	
Clone5-1	Peak5-1	Clostridia clone AKAU3868 found from process of uranium reduction and re-oxidation		DQ125755	
Clone5-12	Peak5-2	Same as peak3			
Clone5-13	Peak5-3	Low G+C Gram-positive bacterium clone LV60-CY1-10	C	AY642581	
Clone5-35	Peak5-4	Clostridia clone AKIW694 found from air of city in Texas		DQ129301	Bands 53, 54, 55, 56, 57, 58, 59, 60 on DGGE2
Clone5-36	Peak5-5	*Clostridium* sp. SP3 (C)	C	AF005092	
Clone6-3	Peak6	Clostridia clone AKIW439 found in air of city in Texas		DQ129393	
Clone6-5 Clone7-1	Peak7	*Azospirillum* sp. DA2-3-1 and LOD4 in association with plant roots		AY118224, AY283791	
Clone7-3 Clone8-3	Peak8-1	Clostridia clone AKAU3585 found from process of uranium reduction and re-oxidation		DQ125588	
Clone8-2 Clone8-6 Clone9-1	Peak8-2	Clostridia clone 1013-28-CG46 from a uranium-contaminated aquifer		AY532583	
Clone8-11	Peak8-3	Clostridia clone AKIW390 found in air of city in Texas		DQ129396	
Clone8-12	Peak8-4	*Clostridium* sp. L1/8 and F7/7	A	AY188845, AY188843	Bands 5, 6, 11, 29, 50 on DGGE1, 31 on DGGE2
		Uncultured *Clostridium* RSb47 and RSb24 found from colonies on rice straw		AJ289216, AJ289213	
Clone8-19	Peak8-5	Clostridia clone Z45 found in river sediment		AY754834	
Clone9-2 Clone9-9	Peak9-1	*Eubacterium* clone BSV61 found from anoxic rice paddy soil		AJ229210	
Clone9-7	Peak9-2	*Rhizobium daejeonense* strain L61T and L22 found from cyanide treatment bioreactor		AY341343, DQ089696	
Clone10-1	Peak10-1	Clostridia clone e10 found from slurry-composting process for degradation of sewage sludge		AB241562	
Clone10-2 Clone11-1	Peak10-2	*Rhizobiales* bacterium RR47 found from rice plant roots		AB174816	
Clone11-3	Peak11	*Novosphingobium subarctica,* polychlorophenol degrader		X94102-X94104	
Clone12-1	Peak12-1	Various bacteria in genus *Clostridium*	B C	X68179, X68176, Y18171, X81021, U16165, AJ458417-AJ458419	Bands 5, 6, 11, 29 on DGGE1
Clone12-3	Peak12-2	Uncultured Clostridia from pig intestine		AF371643, AF371642	Bands 1, 13, 26, 27 on DGGE1
Clone12-6	Peak12-3	*Desulfovibrio desulfuricans*	D	M34113, AF192153	
Clone13-1	Peak13-1	*Escherichia coli* C2 Non-ruminant clone p-4395-4Wa2	C	AF403733 AF371931	
Clone13-3	Peak13-2	Uncultured human intestinal Clostridia		AY984066, AY977894	
Clone14-5	Peak14	Bacteroidetes clone PL-26B10 from production waters of a low-temperature biodegraded oil reservoir		AY570561	Bands 7, 8 on DGGE2
Clone15-1	Peak15	*Stenotrophomonas maltophilia* strain e-a21 found in human infection, soil, and water		AJ293470	
		Gamma Proteobacterium PL_GH2.1.C4 found from sea-water sample		AY162042	
Clone20-1	Peak20-1	Anaerobic Bacilli clone A-3L found in anaerobic swine lagoons		AY953239	Bands 3, 4, 5, 6 on DGGE2
Clone20-4	Peak20-2	Bacilli clone AKIW775 and AKIW620 found from air of city in Texas		DQ129407, DQ129405	Bands 3, 4, 5, 6 on DGGE2
Clone21-1	Peak21-1	Beta Proteobacterium clone TD12 found from carbon tetrachloride contaminated soil		DQ248265	
Clone21-4	Peak21-2	Clostridia clone LJ6		AY756595	
Clone23-1 Clone23-2 Clone23-7	Peak23	*Acinetobacter* sp. isolate LY1 in association with toluene degradation in a compost biofilter, 98.2% similarity to *Acinetobacter haemolyticus*		AJ007008	Band 27 on DGGE2

Table 3 (*continued*)

Clone[a]	Matching peaks	Highest matches[b]	Putative functional category[c]	GenBank accession number	Matching DGGE Bands[d]
Clone23-8					
Clone23-30					
Clone23-31					

[a]The first number of each clone name indicates the peak of community DNA to which the clone was initially assigned, and the second number indicates the order of the clone in the same peak group.

[b]When more than one sequence was listed as the highest match, all the results are presented.

[c]When the matching sequence had >97% similarity to a sequence corresponding to an organism with one of the four functional categories, it was assigned to that category: A: Cellulose degrader; microorganisms with the ability to degrade cellulose and ferment other saccharides. B: Cellobiose-degrader; microorganisms with ability to ferment cellobiose and other saccharides. C: Fermenter. D: Sulfate-reducer. When the functional/environmental property of a sequence match did not correspond to one of these four categories, a basic description of the source and/or characteristics is provided.

[d]Based on a comparison of GenBank accession numbers obtained from BLAST searches.

required prior to analysis differed widely among different capillary lots. However, it is still possible with CE-SSCP to analyze a much greater number of samples under the same conditions than it is with DGGE. In this study, CE-SSCP results were reproducible on a much larger scale, considering the total number of parallel CE-SSCP runs (540 total, including clone and community replicates, data not shown) versus DGGE runs (13 total).

Table 4
Summary of microbial community composition obtained from DGGE and CE-SSCP

		Number of bands or peaks[b]							
		DGGE1		DGGE2			CE-SSCP		
		B17	B34	B17	B34	B56	B17	B34	B56
Phylogenetic class	Clostridia	9	13	7	9	6	19	18	18
	Bacilli		1	1	1	1	2	2	2
	Alpha Proteobacteria						5	5	5
	Beta Proteobacteria						1		
	Gamma Proteobacteria			1			3	1	1
	Delta Proteobacteria						1	1	1
	Actinobacteria					1			
	Bacteroidetes			1			1	1	1
Putative Functional Group	Cellulose Degrader	1	5				1	1	1
	Cellobiose-Degrader			1			2	2	2
	Fermenter	2	5	1	3	1	3	3	3
	Sulfate-reducer		1				1	1	1
	Unrelated or Unknown Function	6	3	8	8	8	26	22	22
	Total bands or peaks[a]	16	18	15	13	16	32	28	28

[a]For DGGE, total unique bands observed, not necessarily sequenced. For CE-SSCP, total unique sequenced clones matching peaks.

[b]Bands and peaks were assigned to the highest upstream functional groups even if the bands or peaks matched with several different functions, except when a band or peak corresponded to a sulfate-reducer. DGGE bands at the same horizontal position were considered to represent the same organism.

CE-SSCP resolution was better than that of DGGE based on three main indicators. First, more distinguishable peaks were resolved by CE-SSCP than by DGGE (e.g., 23 versus 15 respectively for sample B17). This was determined using the same criteria to define bands and peaks in terms of signal to noise ratio. Also, several of the DGGE bands that were resolved were determined to be artifacts (described in Section 3.3), which should have artificially increased the estimate of diversity by DGGE. Second, DNA sequences from clones used to identify CE-SSCP peaks were of superior quality in terms of length and legibility than those obtained from DGGE bands (average 652 bp versus 103 bp), which allowed for more precise identification (Table 3 versus Table 2). While it could be stated that this is a property of cloning rather than CE-SSCP, it is the high-throughput nature of CE-SSCP that makes identification of peaks by cloning feasible. In the case of DGGE, only a limited number of clones may be analyzed in parallel under the same conditions with the corresponding community DNA. Because of significant gel-to-gel variation, even with analyzing multiple clones on multiple gels, the fingerprint of the community DNA itself may change significantly from gel\gel such that it is unclear whether or not a particular band has already been matched with a clone based on migration pattern. Third, more phylogenetic groups were detected by CE-SSCP (Table 4). If this indicator were not affected by

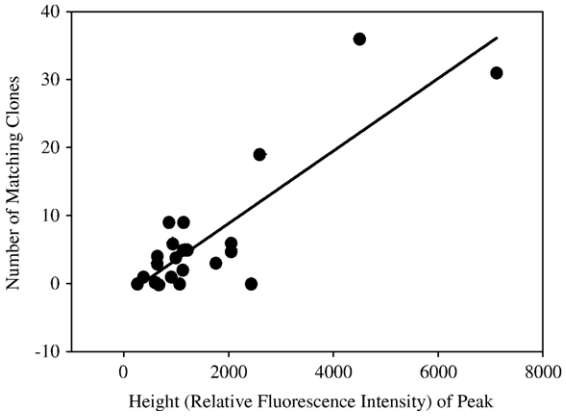

Fig. 3. Relationship between the relative fluorescence intensity (RFI) of CE-SSCP peaks and number of corresponding matching clones (R^2=0.727).

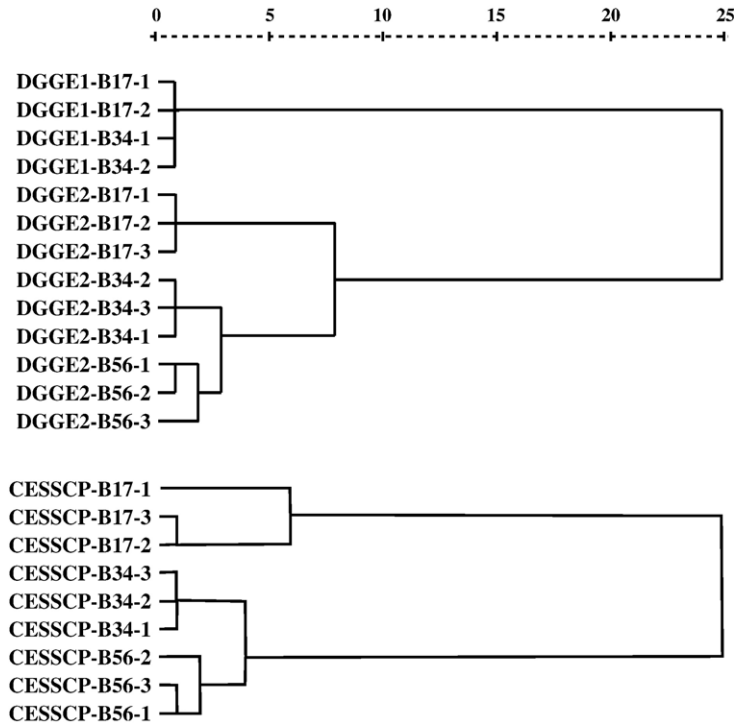

Fig. 4. Cluster analyses of DGGE lanes and CE-SSCP electropherograms: DGGE1 = DGGE gel run with nested PCR products, DGGE2 = DGGE gel run with un-nested PCR products, B17 = composite sample from columns 1 and 7 (performance), C34 = composite sample from columns 3 and 4 (decline), C56 = mixed sample from columns 5 and 6 (hindered). Numbered suffixes indicate the order of lanes from the left to right for DGGE (Fig. 1) and patterns from the top to the bottom for CE-SSCP (Fig. 2). Note that CE-SSCP-B17-1 was analyzed on a separate capillary than the other samples. The dashed scale bar indicates relative distance for each cluster.

resolution, then a correlation between the number of clones or DGGE bands sequenced and the number of phylogenetic groups detected would be expected. In this study, the opposite trend was observed, and though more DGGE bands were analyzed, fewer phylogenetic groups were detected. Although the sequence quality of the DGGE bands was poorer, 80 of 97 sequenced could readily be assigned to phylogenetic class, yet fewer classes were identified. This difference could be an effect of the primers (Table 1); however, the DGGE primers were designed incorporating inosine base pairs in order to provide a broader range of detection (Watanabe et al., 2001). Therefore, the differences in detection of phylogenetic groups are likely due to the actual separation mechanism. Also, fewer phylogenetic groups were detected by DGGE regardless of the nested or un-nested protocol, suggesting that it was not a result of PCR conditions, but rather the separation mechanism itself. Interestingly, Hori et al. (2006) also noted higher resolution when comparing gel-based SSCP to DGGE, which supports these results. The main effect of the nested PCR conditions appeared to be that the dominant bands increased in intensity and that DNA sequence quality was generally better (data not shown), suggesting enrichment of dominants, but also the likelihood of PCR bias effects.

A comparison of resolution in terms of co-migration of bands and peaks was more difficult to make. While DGGE bands yielding illegible sequence data were likely due to the presence of multiple co-migrating sequences (e.g., 17 of 90 sequenced), the sequence quality for remaining DGGE bands was generally poor and required significant editing of ambiguous base pairs compared to sequences used to identify CE-SSCP peaks. Recently, a method termed gel-expansion DGGE (DGGEGE) (Gafan and Spratt, 2005) has been described for re-resolving excised DGGE bands. However, it was considered that this approach would defeat the purpose of a high-throughput comparison of the methods. Since that the vast majority of DGGE bands were closely related and matched Clostridia, it is highly possible that many of the bands were in fact the result of co-migration of similar species that manifested as a noisy, but legible sequence. This may help to explain the common observation that bands at the same horizontal position in the DGGE gels only represented the same organism in about half of the cases. In contrast, though just under half of the dominant CE-SSCP peaks were the result of co-migration (Table 2), these could be clearly characterized because the sequence information was obtained from clones. In order to definitively identify multiple sequences present in DGGE bands, it would be necessary to clone each band or to apply the DGGEGE approach, either of which would greatly add to the cost and labor.

3.3. Artifacts

A common critique of community fingerprinting techniques is the potential for artifacts, such as one organism yielding more than one band or peak (Gelsomino et al., 1999; Tiedje et al., 1999). This kind of artifact was clearly more problematic for DGGE than CE-SSCP. Ten pairs of DGGE bands from DGGE2 were observed as two separate bands in the same lane, but had the same sequencing results. Interestingly, all of these appeared as “double bands”

adjacent to each other, rather than having distinct migration patterns (all entries in Table 2 in which two bands are listed on the same line). In the CE-SSCP results there was one sequence that matched with more than one peak (peak 3 and 5-2, Table 3), but these were not present as "double peaks" as observed in DGGE. This suggests that CE-SSCP is less likely to produce artifacts in the form of double peaks. One potential reason why more double bands were observed in DGGE was because *Taq* polymerase was used, which does not have 100% efficiency in adenylating PCR products. Thus, partial adenylation could have yielded two bands from one microorganism with highly similar migration patterns. The blunt-ended polymerase applied in CE-SSCP may have helped to avoid this problem. In this study, sequences with the same DNA sequence identity found in different bands or peaks were assumed to be artifacts. However, strictly speaking, it is also possible that two closely related strains could legitimately form distinct bands or peaks that were not distinguished based on the DNA sequence information. Therefore, future studies are suggested to investigate the migration behavior of 16S rRNA genes from pure cultures.

Another artifact that has been cited is that bands and peaks from different samples at the same position do not always represent the same microorganism. In this study, only about half of the DGGE bands that were at the same position from different samples collected with time revealed the same results from the databases (13 of 25 bands that were sequenced at the same horizontal position). This confirmed that for complex communities, it cannot always be assumed that bands appearing at the same horizontal position across samples represent the same organism, and therefore it is good protocol to sequence multiple bands for each position in a complex community. In CE-SSCP, sequences were only obtained from B17, so this type of artifact was not characterized. However, as discussed above, CE-SSCP peaks containing sequences with multiple identities can be readily characterized.

A third artifact typically associated with DGGE is the effect of heteroduplexes. The mismatched base pairs of heteroduplexes cause the strands to denature readily, and thus, these can typically be observed at the top of the DGGE gel and are distinguishable from true bands. In this study, it was noted that there were several bands at the top of the DGGE gel when nested products were analyzed, but not when un-nested products were analyzed. These suspect heteroduplexes were therefore avoided in downstream analyses (this section of the gel is not shown in Fig. 1). Because single strands of DNA are analyzed using CE-SSCP, heteroduplex artifacts are entirely avoided.

3.4. Labor and analysis time

The actual labor time per sample, including any necessary PCR, cloning, or electrophoresis preparation steps, was estimated to be similar between CE-SSCP and DGGE. In terms of total analysis time per sample, which was determined as the time that passed starting when the first PCR reaction was prepared and ending when samples were processed for sequencing, CE-SSCP required 25% less time (27 h) than DGGE (36 h). For more than 16 samples, DGGE gel preparation time increases because two gels have to be prepared. For more than 32 samples, DGGE total analysis time doubles because only two gels can be run at one time, while CE-SSCP analysis time increases only linearly in this range. It is also important to consider that gel-to-gel variation is likely to be a problem for DGGE with more than 16 samples, and also that the two outer wells of each gel tend to have skewed migration patterns. DNA sequence analysis time for clones was significantly shorter than for DGGE bands because of the quality and length of the sequences obtained.

4. Conclusions

This study provides a detailed comparison of DGGE versus CE-SSCP for profiling a complex sulfate-reducing community involved in bioremediation of mine drainage. Overall, CE-SSCP was found to be superior to DGGE in terms of the detection range of different phylogenetic groups, resolution, susceptibility to artifacts, and labor and analysis time. Thus, CE-SSCP holds promise for high-throughput monitoring of bioremediation communities.

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