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Development of oligonucleotide probes and PCR primers for detecting phylogenetic subgroups of sulfate-reducing bacteria

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PCR primer sets for the 16S rRNA gene of six phylogenetic groups of sulfate-reducing bacteria (SRB) were designed. Their application in conjunction with group-specific internal oligonucleotide probes was used to detect SRB DNA in samples of landfill leachate. Six generic/suprageneric groups could be differentiated: *Desulfotomaculum*; *Desulfobulbus*; *Desulfobacterium*; *Desulfobacter*; *Desulfococcus–Desulfonema–Desulfosarcina*; *Desulfovibrio–Desulfomicrobium*. The predicted specificities of the PCR primer and oligonucleotide probe combinations were confirmed with DNA from reference strains. In all cases, the PCR primers and probes were specific, the only exception being that the *Desulfococcus–Desulfonema–Desulfosarcina* (group 5) PCR primers were able to amplify DNA from *Desulfobacterium* (group 3) reference strains but these groups could nevertheless be differentiated with the internal oligonucleotide probes. The proliferation of SRB in landfill sites interferes with methanogenesis and waste stabilization, but relatively little is known about the composition of SRB populations in this environment. DNA was extracted from samples of landfill leachate from several municipal waste landfill sites and used as template in PCR reactions with SRB group-specific primer sets. Group-specific oligonucleotide probes were then used to confirm that the PCR products obtained contained the target SRB 16S rDNA. Both 'direct' and 'nested' PCR protocols were used to amplify SRB 16S rDNA from landfill leachates. Three of the six SRB groups could be detected using the 'direct' PCR approach (*Desulfotomaculum*, *Desulfobacter* and *Desulfococcus–Desulfonema–Desulfosarcina*). When 'nested' PCR was applied, an additional two groups could be detected (*Desulfobulbus* and *Desulfovibrio–Desulfomicrobium*). Only *Desulfobacterium* could not be detected in any leachate samples using either direct or nested PCR. The SRB-specific 16S rDNA primers and probes described here can be applied to investigations of SRB molecular ecology in general, and can be further developed for examining SRB population composition in relation to landfill site performance.

Keywords: sulfate-reducing bacteria, 16S rDNA, landfill, PCR primers, oligonucleotide probes

INTRODUCTION

The application of molecular biological methods to investigate the occurrence and distribution of bacteria in the environment has the advantage of providing direct information on community structure. Not only do culture-based methods recover merely a fraction of the natural population, but for sulfate-reducing bacteria (SRB), the isolation of axenic cultures from environmental samples is not straightforward. 16S rRNA-targeted oligonucleotide probes have been designed to detect groups of SRB (Devereux *et al*., 1992) and used successfully to demonstrate the presence of SRB in such

Abbreviations: DIG, digoxigenin; RDP, ribosomal database project; SRB, sulfate-reducing bacteria.

diverse habitats as anaerobic biofilms (Kane *et al*., 1993; Raskin *et al*., 1996), marine, estuarine and freshwater sediments (Sahm *et al*., 1999; Devereux *et al*., 1996b; Purdy *et al*., 1997; Trimmer *et al*., 1997), activated sludge flocs (Manz *et al*., 1998) and salt marshes (Devereux *et al*., 1996a; Rooney-Varga *et al*., 1997).

Landfill sites are essentially bioreactors in which anaerobic bacterial communities mediate the mineralization and stabilization of organic matter (Barlaz, 1997). They have long been overlooked as important habitats for SRB due to the fact that methanogenesis predominates as the key terminal process of carbon mineralization in the absence of significant concentrations of sulfate. Our knowledge of the occurrence and distribution of SRB in landfill is therefore extremely limited. The SRB are a diverse group of anaerobic bacteria that have the ability to use sulfate as a terminal electron acceptor in the consumption of organic matter, with the concomitant production of H_2S. They are ubiquitous in the environment and have pivotal roles in the biogeochemical cycling of carbon and sulfur. Sulfate reduction could be responsible for up to 50% of organic matter degradation in high-sulfate environments such as estuarine and marine sediments (Jorgensen, 1982); however, active sulfate reduction has also been reported in low-sulfate environments such as soils and freshwater sediments (Postgate, 1984; Jones & Simon, 1984; Bak & Pfennig, 1991a, b). In landfill sites, the breakdown of waste material ultimately to methane is a complex process involving a series of microbially driven transformations that harness the co-ordinated activity of several trophic groups of bacteria. While the key terminal process is methanogenesis, SRB can compete with methanogenic bacteria for available electron donors such as acetate and H_2, and have the potential to inhibit the methanogenic decomposition of waste organic matter, resulting in the increased production of H_2S and the phenomenon of 'souring' (Gurijala & Suflita, 1993; Harvey *et al*., 1997). Conventional wisdom suggests that the low availability of sulfate outside the marine environment will limit sulfate reduction and therefore SRB populations, but this may not be true of landfill sites. Exogenous sources of sulfate, e.g. gypsum from construction and demolition debris, have been thought to be responsible for sulfate levels as high as 80 mmol per kg dry weight waste material in particular landfill sites (Suflita *et al*., 1992; Gurijala & Suflita, 1993). Cellulosic material can account for over 40% of the volume of a landfill site and act as a reservoir of sulfate that originates from other waste fractions (Suflita *et al*., 1992; Gurijala & Suflita, 1993). Consequently sulfate may be present in landfills in significant amounts.

Inhibition of methanogenesis by sulfate has been observed in a range of environments (Oremland & Polcin, 1982; Beeman & Suflita, 1987; Raskin *et al*., 1996) and so could clearly occur in landfill (Gurijala & Suflita, 1993). The SRB are therefore one of a number of important functional bacterial groups whose structure and activity in landfill sites needs to be directly addressed. Data on their occurrence and distribution should ultimately enable the development of detection protocols that can be used to monitor the microbiology of landfill sites in order to provide information for site management. For example, molecular biological methods could give SRB population profiles that provide an early warning of interference with methanogenesis by sulfate reduction.

Phylogenetic analysis based on 16S rRNA sequence comparisons has classified the major SRB genera into a number of distinct lineages (Devereux *et al*., 1989) and this was used as the starting point for the study reported here. Oligonucleotide probes designed by Devereux *et al*. (1992) target a number of these groups; however, the suite of probes currently available in the literature does not encompass all of the main groups of SRB, nor have specific PCR amplification primers for SRB detection in environmental samples been described or applied. In this paper, we describe and evaluate combinations of PCR primers and oligonucleotide probes for the six major phylogenetic groups of SRB, and apply these to DNA extracted from samples of landfill leachate to provide baseline information on the occurrence and distribution of SRB taxa.

METHODS

Reference strains and environmental samples. The SRB comprise six main phylogenetic groups; these are labelled G1–G6 in Fig. 1. The strains used as controls in this study were as follows: *Desulfotomaculum nigrificans* NCIMB 8395 (group 1); *Desulfobulbus propionicus* DSM 2032 (group 2); *Desulfobacterium autotrophicum* DSM 3382 (group 3); *Desulfobacter curvatus* DSM 3379 (group 4); *Desulfosarcina variabilis* DSM 2060 (group 5); *Desulfovibrio desulfuricans* DSM 642 (group 6). For each of the groups, oligonucleotide probe target regions that contained 1, 2 or 3 bp mismatches were identified in the Ribosomal Database Project (RDP) aligned SSU_rRNA database (Maidak *et al*., 1997). The following strains were therefore used as additional appropriate controls for evaluating the specificity of the SRB probes: *Zymomonas mobilis* NCIMB 8938; *Clostridium aurantibutyricum* NCIMB 10695; '*Desulfobacterium vacuolatum*' DSM 3385; *Pelobacter carbinolicus* DSM 2380.

Samples of fresh, pooled landfill leachates were collected by the landfill operators of seven conventional municipal landfill sites in the north-west of England (designated P, B, S, R, C, H and W). Only one sample was provided from each landfill site. However, as the leachates were pooled from run-off collected on site they were regarded as representative of each landfill site as a whole.

The leachate samples were processed immediately upon receipt. Each 1 litre sample was concentrated by centrifugation (27000 ***g***, 40 min) and the pellet resuspended in 20 ml 0·1 M K_2HPO_4. Aliquots (1·5 ml) of this concentrated sample were centrifuged (22000 ***g***, 5 min) and the pellets stored at −80 °C until required.

Nucleic acid extraction and purification. Pellets of concentrated leachate stored at −80 °C were thawed on ice and resuspended in 200 µl sterile distilled H_2O to give a final 375-fold concentration of the leachate solids. DNA was extracted and purified from this concentrated leachate using the FastDNA SPIN kit (Bio 101) and a Hybaid Ribolyser according

Table 1. 16S rDNA-targeted PCR primer sequences specific for SRB subgroups

Primer	Target site*	Sequence 5′–3′†	Specificity	Annealing temp. (°C)	Expected size of product (bp)
DFM140	140–158	TAG MCY GGG ATA ACR SYK G	Group 1	58	700
DFM842	842–823	ATA CCC SCW WCW CCT AGC AC			
DBB121‡	121–142	CGC GTA GAT AAC CTG TCY TCA TG	Group 2	66	1120
DBB1237‡	1237–1215	GTA GKA CGT GTG TAG CCC TGG TC			
DBM169	169–183	CTA ATR CCG GAT RAA GTC AG	Group 3	64	840
DBM1006	1006–986	ATT CTC ARG ATG TCA AGT CTG			
DSB127‡§	127–148	GAT AAT CTG CCT TCA AGC CTG G	Group 4	60	1150
DSB1273‡	1273–1252	CYY YYY GCR RAG TCG STG CCC T			
DCC305	305–327	GAT CAG CCA CAC TGG RAC TGA CA	Group 5	65	860
DCC1165	1165–1144	GGG GCA GTA TCT TYA GAG TYC			
DSV230‡	230–248	GRG YCY GCG TYY CAT TAG C	Group 6	61	610
DSV838	838–818	SYC CGR CAY CTA GYR TYC ATC			

* 16S rDNA positions; *E. coli* numbering.

† 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).

‡ Primer sequences DBB121, DBB1237, DSB127, DSB1273, DSV230 were provided by Dr Mark Munson, University of Essex (personal communication).

§ Primer DSB127 was derived from probe DSB129 described by Devereux *et al.* (1992).

Table 2. 16S rDNA-targeted oligonucleotide probe sequences specific for SRB subgroups

Probe	Target site*	Sequence 5′–3′†	Specificity	Hyb. temp. (°C)	Reference
DFM228	228–242	GGG ACG CGG AYC CAT	Group 1	48	This study
DBB660	660–679	GAA TTC CAC TTT CCC CTC TG	Group 2	50	Devereux *et al.* (1992)
DBM221	221–240	TGC GCG GAC TCA TCT TCA AA	Group 3	56	Devereux *et al.* (1992)
DSB623	623–644	TGT TTC AAG TGC WCT TCC GGG G	Group 4	56	This study
DCC868	868–885	CAG GCG GAT CAC TTA ATG	Group 5	46	This study
DSV687	687–702	TAC GGA TTT CAC TCC T	Group 6	45	Devereux *et al.* (1992)

* 16S rRNA positions; *E. coli* numbering.

† 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).

to the manufacturer's instructions. DNA was extracted from control strains by resuspending freeze-dried cultures in 200 µl sterile distilled H_2O and applying the Bio 101 kit and Hybaid Ribolyser protocol described above. DNA recovery, purity and yield were evaluated by agarose gel electrophoresis as described below.

Design of 16S rDNA-targeted PCR primers and internal 16S rRNA-targeted oligonucleotide probes. A phylogenetic tree showing the lineage of the six main groups of SRB was constructed from aligned 16S rRNA sequences obtained from the GenBank, EMBL and RDP (Maidak *et al.*, 1997) databases using the neighbour-joining method of Jukes & Cantor (1969) and produced by the TREEVIEW program (PHYLIP 3.4) (Felsenstein, 1993). Bootstrap analysis consisting of 100 resamplings of the data was performed using SEQBOOT (PHYLIP 3.4) and a consensus phenogram was generated using CONSENSE (PHYLIP 3.4). 16S rDNA-targeted PCR primers and internal 16S rRNA-targeted oligonucleotide probes were designed from a collection of 60 SRB 16S rRNA sequences obtained from the databases. *Escherichia coli* and *Bacillus subtilis* 16S rRNA sequences were used as reference points for the alignment of the SRB sequences. Regions of variability between sequences representing each SRB group and the reference sequences were located by eye. Potential candidates for PCR primers and internal oligonucleotide probes were compared to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility.

PCR amplification of 16S rDNA with SRB group-specific primers. 'Direct' PCR amplification with the group-specific PCR primers (Table 1) was attempted on the DNA extracted from each landfill site. Reactions were carried out as follows: 95 °C for 1 min, annealing for 1 min and 72 °C for 1 min for 30 cycles. Each reaction tube (100 µl) contained: 2 µl each primer (10 pmol μl^{-1}), 2 µl dNTP (10 mM each), 85 µl distilled H_2O, 10 µl 10 × PCR buffer (HT Biotech), 0·2 µl 10 % (w/v) BSA, 1 U Super*Taq* polymerase (HT Biotech) and DNA template (approx. 100–150 ng). The amplifications were carried out using a 'hot-start' PCR protocol whereby each reaction, without *Taq* polymerase, was heated at 95 °C for 5 min to fully denature the DNA template. The tubes were

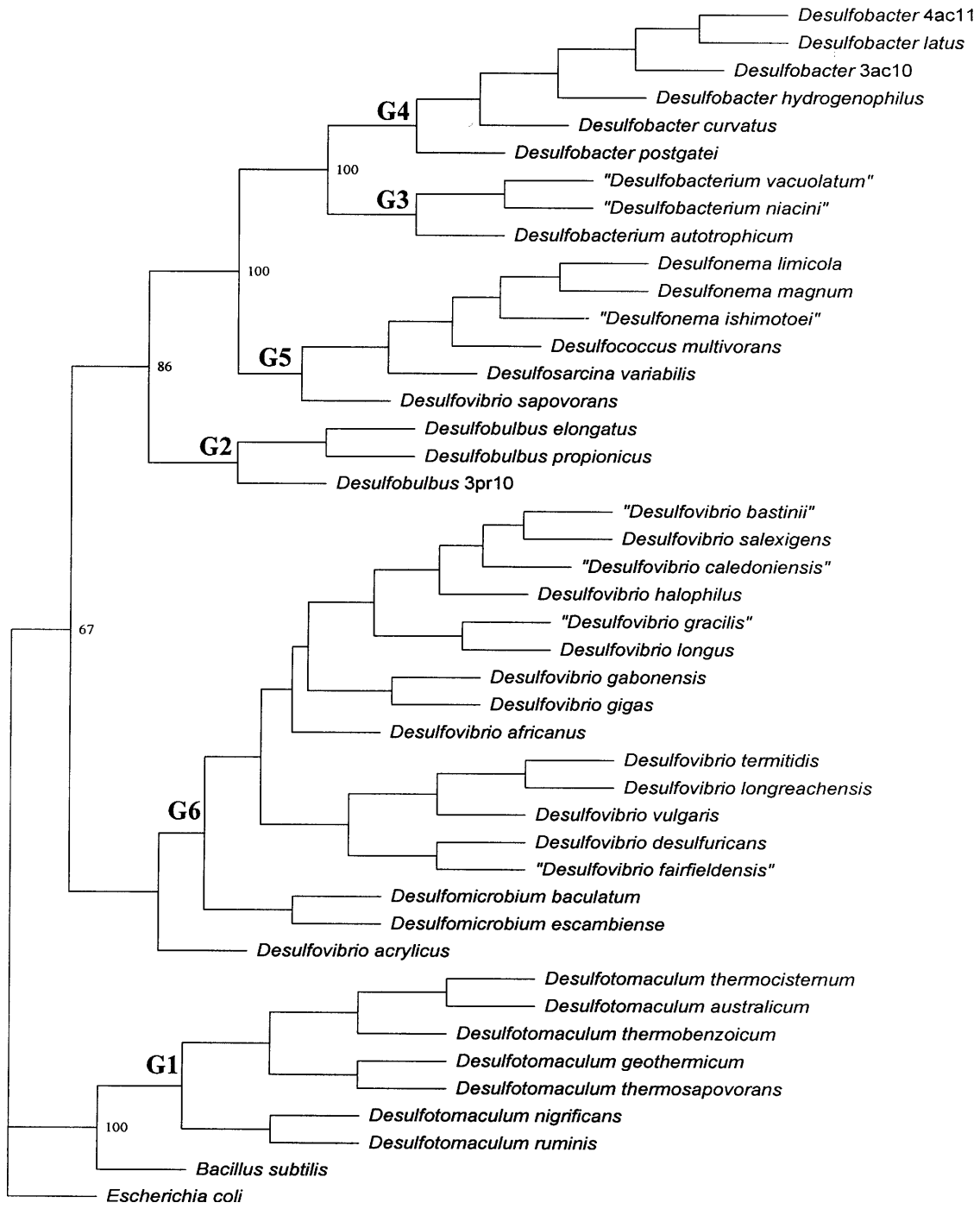

Fig. 1. 16S rDNA phylogenetic tree showing the lineages of the six main subgroups of SRB. The tree was constructed using the neighbour-joining method of Jukes & Cantor (1969) and analysis was based on 1351 nucleotides. Bootstrap values (out of 100 trees) are shown adjacent to nodes. SRB subgroups marked G1–G6.

then cooled to 80 °C and maintained at this temperature while the enzyme was added. Each reaction was then overlaid with mineral oil prior to cycling. In addition to the 'direct' amplification of landfill DNA, 'nested' amplification was also applied. DNA extracted from landfill leachate was first amplified with the eubacterial primers pA and pH′ (Edwards *et al*., 1989) at low stringency (annealing temperature 45 °C), then aliquots of these eubacterial PCR amplification products were diluted 100-fold into fresh reaction mixtures containing a pair of SRB group-specific primers.

PCR products were electrophoresed through a 1% (w/v) agarose gel in 1 × Tris/acetate/EDTA containing ethidium bromide (0·2 μg ml^{-1}). DNA bands were visualized by UV illumination. Marker pBR322 DNA/*Alw*441/*Mva*1 (MBI

Table 3. Theoretical cross-specificity analysis of SRB group-specific PCR primers and oligonucleotide probes compared to SRB reference sequences

+, Sequence match in target region; 1 bp, 1 bp mismatch in target region.

		PCR PRIMERS												PROBES					
		Group 1		Group 2		Group 3		Group 4		Group 5		Group 6		1	2	3	4	5	6
	SPECIES	DFM140	DFM842	DBB121	DBB1237	DBM169	DBM1006	DSB127	DSB1273	DCC305	DCC1165	DSV230	DSV838	DFM228	DBB660	DBM221	DSB623	DCC868	DSV687
Group 1	*Dfm.austra*	+	+									+		+					
	Dfm.thbenz	+	+									+		+					
	Dfm.geothe	+	+									+		+					
	Dfm.thacid	+	+									+		+					
	Dfm.nigrif	+	+											+					
	Dfm.rumin2	+	+											+					
Group 2	*Dbb.3pr10*			+	+										+				
	Dbb.elonga			+	+										+				
	Dbb.propio			+	+										+				
Group 3	*Dbm.auicum*					+	+			+	1bp					+			
	Dbm.niacin					+	+			+	1bp					+			
	Dbm.vacuol					+	+			+	1bp					+			
Group 4	*Dsb.3ac10*							+	+								+		
	Dsb.latus							+	+								+		
	Dsb.hyphil							+	+								+		
	Dsb.curvat							+	+								+		
	Dsb.postga							+	+								+		
Group 5	*Dcc.multiv*									+	+							+	
	Dnm.ishim1										+							+	
	Dnm.limico									1bp	1bp							+	
	Dnm.magnum									1bp	1bp							+	
	Dss.variab									+	+							+	
	Dsv.sapovo									+	+	+						+	
Group 6	*Dsv.acryli*											+							+
	Dsv.salexi											+	+						+
	Dsv.desulf											+	+						+
	Dsv.fairfi											+	+						+
	Dsv.lonrch											+	+						+
	Dsv.termit											+	+						+
	Dsv.vulgar											+	+						+
	Dsv.africa											+	+						+
	Dsv.gigas											+	+						
	Dsv.haloph											+	+						+
	Dsv.bastin											+	+						+
	Dmb.bacul											+	+						+
	Dmb.escamb											+	+						+

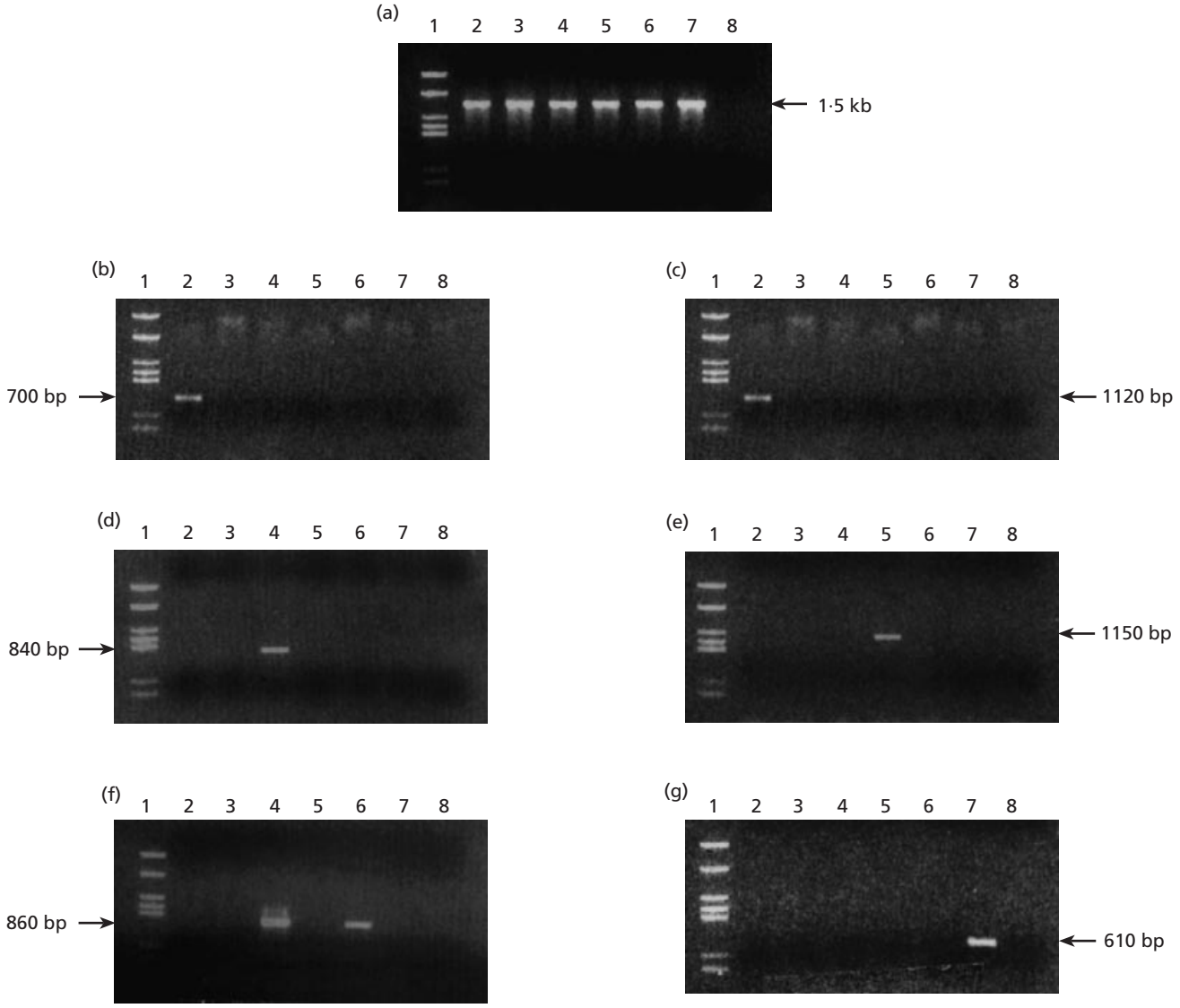

Fig. 2. PCR amplifications of SRB 16S rDNA with eubacterial and SRB group-specific primers. (a) pA and pH′ (Edwards *et al.*, 1989), 55 °C; (b) DFM140 and DFM842 (group 1), 58 °C; (c) DBB121 and DBB1237 (group 2), 66 °C; (d) DBM169 and DBM1006 (group 3), 64 °C; (e) DSB127 and DSB1273 (group 4), 60 °C; (f) DCC305 and DCC1165 (group 5), 65 °C; (g) DSV230 and DSV838 (group 6), 61 °C. Lanes: 1, pBR322 DNA/*Alw*441/*Mva*1 (MBI Fermentas); 2, *Desulfotomaculum nigrificans*; 3, *Desulfobulbus propionicus*; 4, *Desulfobacterium autotrophicum*; 5, *Desulfobacter curvatus*; 6, *Desulfosarcina variabilis*; 7, *Desulfovibrio desulfuricans*; 8, PCR negative control.

Fermentas) was included to enable estimation of the molecular mass of the DNA bands amplified.

Oligonucleotide probing. For the optimization of hybridization conditions, DNA extracted from each control strain was diluted in an equal volume of denaturing solution (1 M NaOH, 3 M NaCl) and transferred to positively charged nylon membrane (Boehringer Mannheim) using a dot-blot apparatus (Minifold, Schleicher and Schuell). DNA was then fixed to membranes by UV cross-linking. For the environmental samples, PCR amplification products were transferred and fixed to positively charged nylon membrane (Boehringer Mannheim) by Southern blotting and UV cross-linking. Membranes were first incubated in standard prehybridization solution [5 × SSC, 0·1% (w/v) *N*-lauroyl sarcosine, 0·02% (w/v) SDS, 1% (w/v) blocking reagent [Boehringer Mannheim)] at the appropriate hybridization temperature for 1 h to prevent non-specific binding of the probe.

Oligonucleotides specific for each of the six main groups of SRB (Table 2) were 3′-end labelled with non-radioactive DIG-11-ddUTP (1 mM) using terminal transferase (Boehringer Mannheim) according to the manufacturer's instructions. The concentrated labelled probes were diluted in prehybridization solution and membranes incubated overnight at a temperature appropriate to the melting temperature (T_m) of the probe used (Table 2). After hybridization, two 15 min high-stringency washes were performed at the hybridization temperature. DIG-labelled DNA was then detected using the standard DIG luminescent detection procedure (Boehringer Mannheim) and membranes were exposed to X-ray film at room temperature.

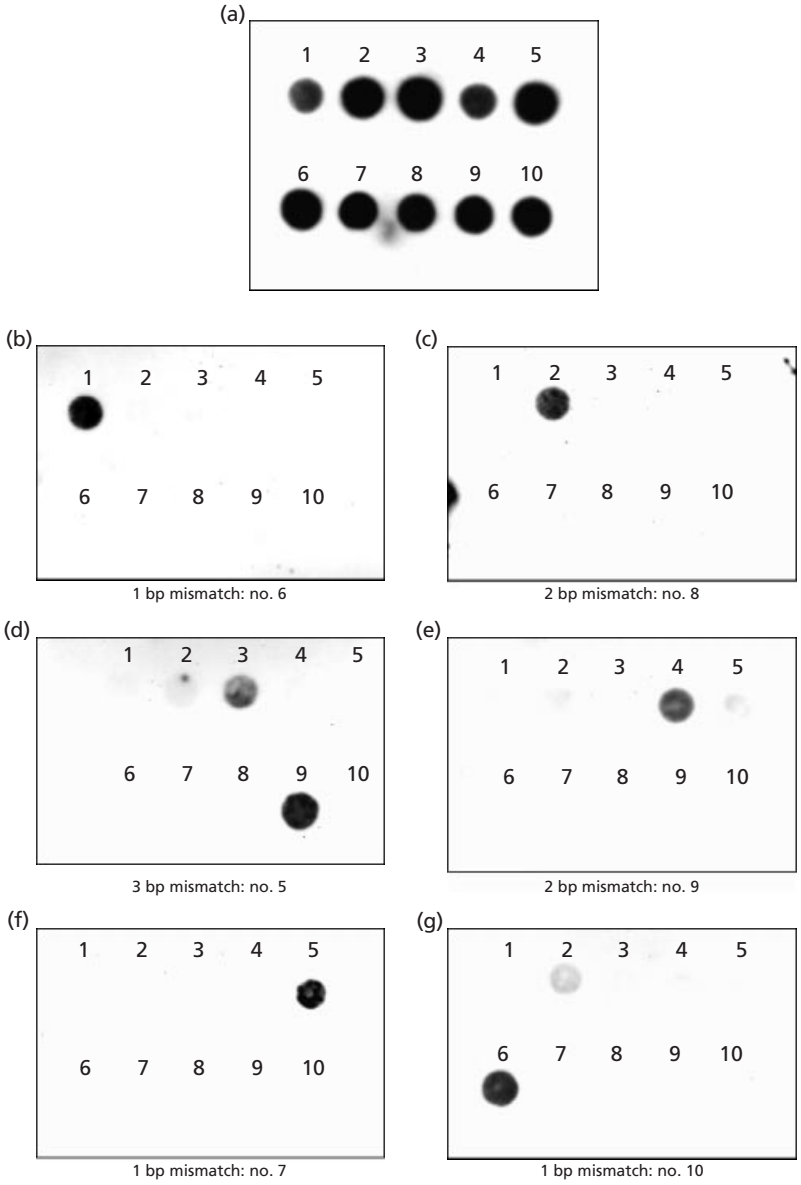

Fig. 3. Dot-blot hybridizations of 16S rDNA demonstrating the specificity of SRB group-specific oligonucleotide probes. (a) EUB338 (Amann *et al.*, 1989), 45 °C; (b) DFM228 (group 1), 48 °C; (c) DBB660 (group 2) (Devereux *et al.*, 1992), 50 °C; (d) DBM221 (group 3) (Devereux *et al.*, 1992), 56 °C; (e) DSB623 (group 4), 56 °C; (f) DCC868 (group 5), 46 °C; (g) DSV687 (group 6) (Devereux *et al.*, 1992), 45 °C. 1, *Desulfotomaculum nigrificans*; 2, *Desulfobulbus propionicus*; 3, *Desulfobacterium autotrophicum*; 4, *Desulfobacter curvatus*; 5, *Desulfosarcina variabilis*; 6, *Desulfovibrio desulfuricans*; 7, *Zymomonas mobilis*; 8, *Clostridium aurantibutyricum*; 9, *'Desulfobacterium vacuolatum'*; 10, *Pelobacter carbinolicus*.

RESULTS

Design and evaluation of SRB group-specific PCR primers

A phylogenetic tree was constructed using alignments of the full length 16S rRNA sequences of named SRB strains on the GenBank, EMBL and RDP databases (Fig. 1). This confirmed the described phylogeny (Devereux *et al.*, 1989) and identified the following six main groups of SRB: group 1, *Desulfotomaculum* (DFM); group 2, *Desulfobulbus* (DBB); group 3, *Desulfobacterium* (DBM); group 4, *Desulfobacter* (DSB); group 5, *Desulfococcus–Desulfonema–Desulfosarcina* (DCC-DNM-DSS); group 6, *Desulfovibrio–Desulfomicrobium* (DSV-DMB).

16S rDNA-targeted PCR primers were designed from this collection of 60 SRB 16S rRNA sequences and potential candidates for PCR primers were compared to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility. Dr Mark Munson (University of Essex, Colchester, UK) kindly provided five potentially specific PCR primer sequences, one of which was derived from an oligonucleotide probe designed by Devereux *et al.* (1992) (see Table 1). The results of this comprehensive cross-specificity check on the complete RDP database enabled the final selection of six 16S rDNA-targeted PCR primer pairs theoretically specific for each of the six main groups of SRB (Table 1). In some cases, one or both of the primers were not completely specific, but no single non-target organism matched both primers and, in any case, Southern hybridization was always used for verification. The theoretical cross-specificity of the primers and probes with SRB as predicted by the RDP database is presented in Table 3. The specificity of the primers was confirmed by screening the current release of the RDP database.

This specificity was further confirmed by amplifying

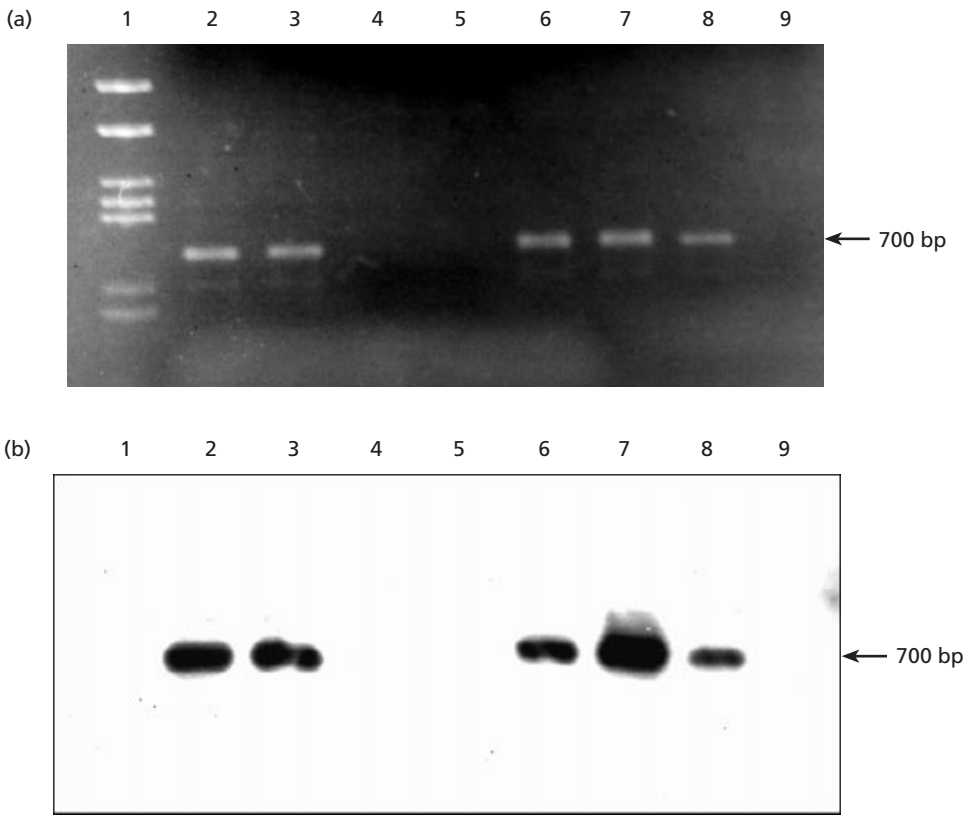

Fig. 4. (a) 'Direct' PCR amplification of 16S rDNA extracted from landfill leachate with primers DFM140 and DFM842 (group 1); (b) Southern blot hybridized against probe DFM228 (group 1). Lanes: 1, pBR322 DNA/*Alw*441/*Mva*1 (MBI Fermentas); 2 and 3, landfill P; 4 and 5, landfill B; 6 and 7, landfill S; 8, *Desulfotomaculum nigrificans* (positive control); 9, PCR negative control.

DNA from target and non-target SRB strains with each of the six sets of primers (Fig. 2). The highest annealing temperature for each primer pair was determined empirically and applied throughout. All six primer pairs were specific for their target groups at the appropriate annealing temperatures and yielded PCR products of the expected size (Table 1, Fig. 2). None of the primer sets amplified DNA from single representatives of non-target SRB groups, with the exception of the DCC-DNM-DSS (group 5) primers, which gave amplification products of the expected size (860 bp) from DBM (group 3) template DNA (Fig. 2), as predicted by the theoretical cross-specificity check between the two groups (Table 3).

Design and evaluation of group-specific oligonucleotide probes

16S rRNA-targeted oligonucleotide probes were designed from the collection of full-length SRB sequences obtained from the GenBank, EMBL and RDP databases. Candidates were compared to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility. The results of this cross-specificity check enabled the design of three group-specific 16S rRNA-targeted oligonucleotide probes to complement those already described by Devereux *et al*. (1992) (Table 2). The specificities of the probes were confirmed by screening the current release of the RDP database. DIG-labelled oligonucleotide probes were used in hybridization experiments with DNA extracted from a range of SRB and non-SRB strains (Fig. 3). Probe EUB338 (Amann *et al*., 1990) was used as a control. Melting temperatures (T_m) for each probe were calculated and optimum hybridization temperatures were determined empirically (Table 2). At these temperatures, target strains could be unambiguously and reproducibly discriminated from non-target strains that contained 1, 2 or 3 bp mismatches. Probe EUB338 gave strong positive signals for all of the strains (Fig. 3).

'Direct' PCR amplification of SRB 16S rDNA from landfill leachate

PCR amplification of DNA extracted from landfill leachate samples was attempted with the primers specific for each of the six SRB groups. The amplification products obtained were transferred to positively charged nylon membrane by Southern blotting. DNA fixed to membranes was then hybridized against the appropriate group-specific oligonucleotide probe.

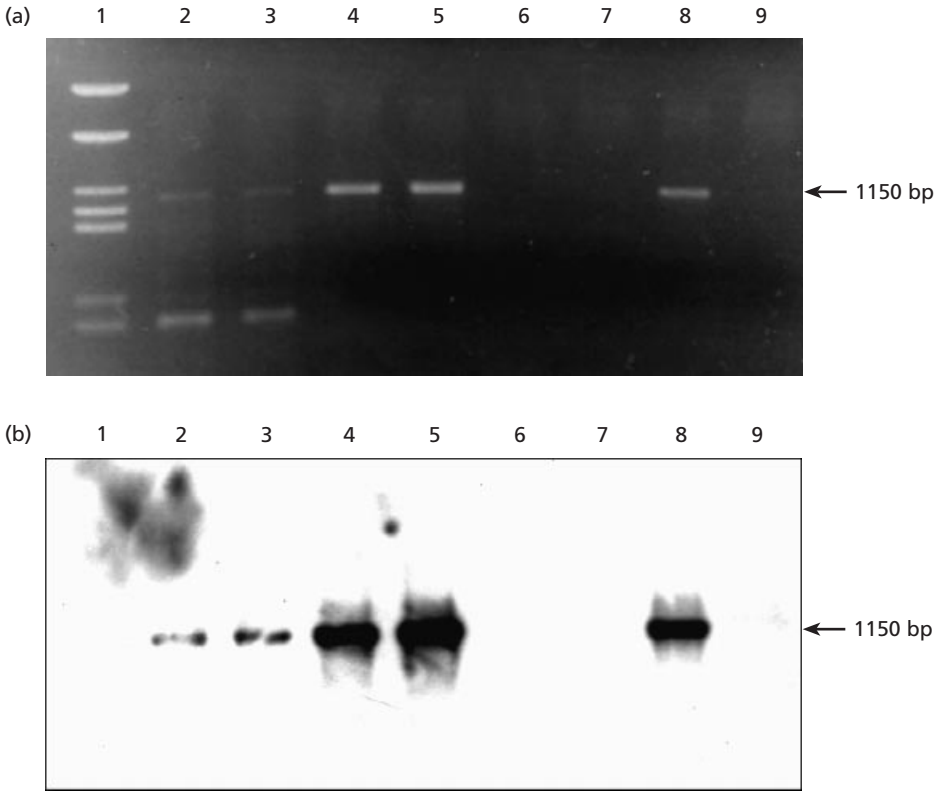

Fig. 5. (a) 'Direct' PCR amplification of 16S rDNA extracted from landfill leachate with primers DSB127 and DSB1273 (group 4); (b) Southern blot hybridized against probe DSB623 (group 4). Lanes: 1, pBR322 DNA/*Alw*441/*Mva*1 (MBI Fermentas); 2 and 3, landfill P; 4 and 5, landfill B; 6 and 7, landfill S; 8, *Desulfobacter curvatus* (positive control); 9, PCR negative control.

Desulfotomaculum-like (group 1) amplification products were obtained from three of the seven landfill sites (P, S and C) and shown to contain the target 16S rDNA by hybridization against probe DFM228 (Fig. 4 and not shown).

Desulfobacter-like (group 4) amplification products were obtained from two landfill sites (P and B) and these hybridized to probe DSB623 (Fig. 5).

Desulfococcus–Desulfonema–Desulfosarcina-like (group 5) amplification products were obtained from four of the seven landfill sites (P, B, C and W), confirmed by hybridization against probe DCC868 (data not shown), while no *Desulfobulbus*-like (group 2) (Fig. 6), *Desulfobacterium*-like (group 3) (data not shown) or *Desulfovibrio*-like (group 6) (data not shown) amplification products were obtained from any of the landfill sites using this 'direct' PCR approach.

'Direct' PCR amplification with all six sets of SRB group-specific primers failed to yield any products from the R or H landfill sites.

These results are summarized in Table 4(a).

'Nested' PCR amplification of SRB 16S rDNA from landfill leachate

Eubacterial 16S rDNA PCR products obtained from landfill leachate samples were used as templates for a second round of specific amplification with primers for all six SRB groups. The amplification products obtained were transferred to nylon membrane and hybridized against the appropriate oligonucleotide probe.

Desulfotomaculum-like (group 1) amplification products were obtained from all seven landfill sites, confirmed by hybridization against probe DFM228 (data not shown).

Desulfobulbus-like (group 2) amplification products were obtained from four landfill sites (P, B, R and W) and these hybridized with probe DBB660 (Fig. 6 and not shown).

Desulfobacter-like (group 4) amplification products were obtained from four landfill sites (P, B, S and W) with hybridization against probe DSB623 (data not shown).

Desulfococcus–Desulfonema–Desulfosarcina-like (group 5) and *Desulfovibrio*-like (group 6) amplification

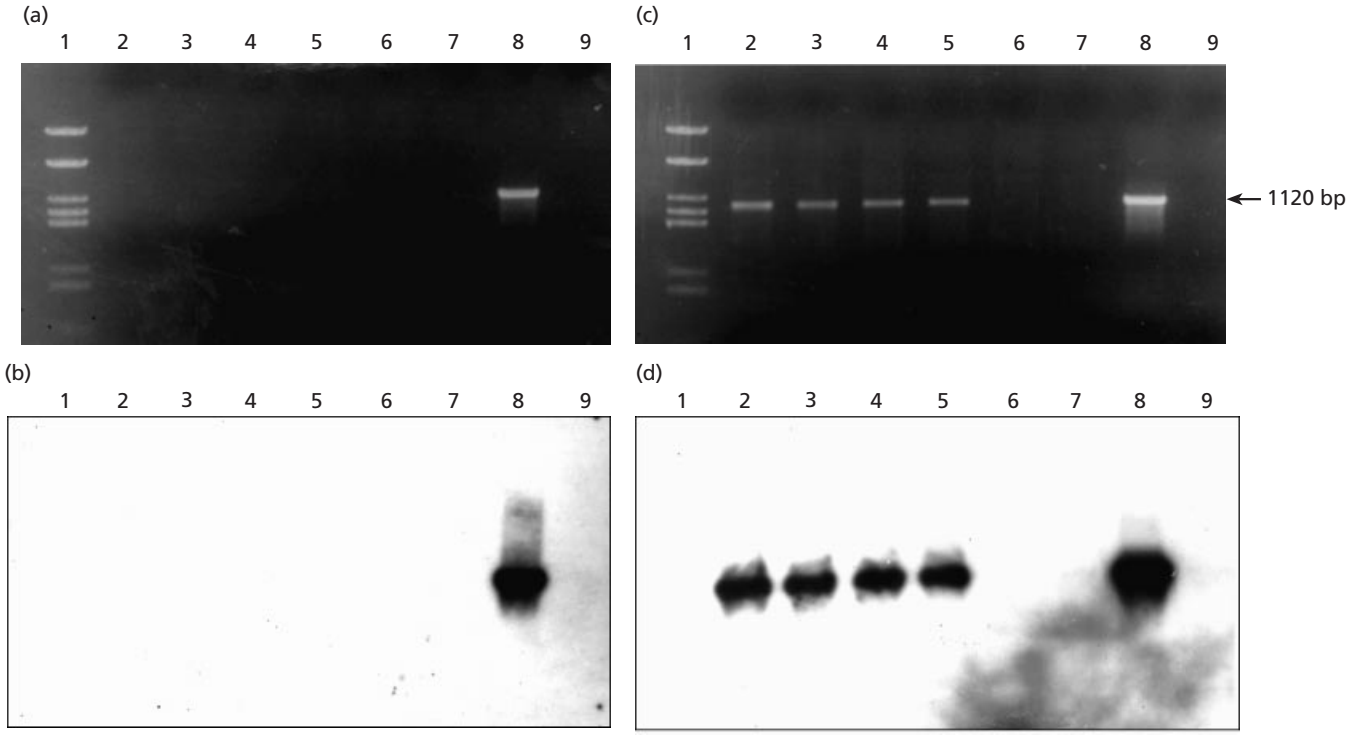

Fig. 6. Comparison of results for 'direct' and 'nested' PCR with DBB (group 2)-specific primers. (a) 'Direct' PCR amplification of 16S rDNA extracted from landfill leachate with primers DBB121 and DBB1237 (group 2); (b) Southern blot hybridized against probe DBB660 (group 2); (c) 'nested' PCR amplification of 16S rDNA extracted from landfill leachate with primers DBB121 and DBB1237 (group 2); (d) Southern blot hybridized against probe DBB660 (group 2). Lanes: 1, pBR322 DNA/*Alw*441/*Mva*1 (MBI Fermentas); 2 and 3, landfill P; 4 and 5, landfill B; 6 and 7, landfill S; 8, *Desulfobulbus propionicus* (positive control); 9, PCR negative control.

products were obtained from six of the seven landfill sites (all except site S), confirmed by hybridization against probes DCC868 and DSV687 respectively (data not shown).

Desulfobacterium-like (group 3) amplification products were never obtained from any of the landfill site leachate samples using this 'nested' PCR approach. Although the direct PCR amplification of 16S rDNA demonstrated that SRB were present in five of the seven landfill sites sampled, application of 'nested' PCR was able to show that SRB 16S rDNA could be detected in all seven leachate samples.

These data are summarized in Table 4(b).

DISCUSSION

Analysis of SRB 16S rRNA sequences has enabled the phylogeny of SRB to be described (Fowler *et al*., 1986; Devereux *et al*., 1989) and major SRB groups to be identified. This in turn has enabled the design and development of group-specific 16S rDNA-RNA-targeted oligonucleotide probes, and now PCR primers also. 16S rDNA-targeted PCR primers specific for SRB have not been described previously and they provide a reproducible means of routinely screening environmental samples for the presence of SRB. 16S rRNA-targeted oligonucleotide probes specific for SRB have been described previously (Devereux *et al*., 1992) and used extensively in environmental studies (Kane *et al*., 1993; Ramsing *et al*., 1993; Risatti *et al*., 1994; Devereux *et al*., 1996a, b; Raskin *et al*., 1996; Purdy *et al*., 1997; Trimmer *et al*., 1997; Rooney-Varga *et al*., 1997; Manz *et al*., 1998; Sahm *et al*., 1999). However, not all of the six main SRB groups are encompassed by these probes, and those described here (Table 2) can now be added to provide a complete suite. Degenerate PCR primers were used in this study (Table 1) in order to broaden the specificity within each group. As each primer set was designed using 16S rRNA sequences from axenic SRB strains, it is possible that non-target species, as yet uncharacterized, could be amplified from environmental samples. Therefore, only PCR products that subsequently gave a positive signal upon hybridization with the appropriate group-specific oligonucleotide probe were recorded as SRB positives. Although it has been reported that probe DSV687 (SRB group 6) (Devereux *et al*., 1992) hybridizes to several non-SRB species, for example some members of the family *Geobacteriaceae* (Lonergan *et al*., 1996), the DSV (group 6)-specific PCR primer sequences used in this study do not occur in these non-SRB species. Conse-

Table 4. Summary of results for 'direct' and 'nested' PCR amplification of 16S rDNA extracted from landfill leachate using SRB group-specific primers and hybridization against group-specific oligonucleotide probes

+, Positive signal when amplification products were hybridized against the group-specific oligonucleotide probe; −, negative hybridization signal in the presence or absence of a visible band of PCR products on an agarose gel.

SRB group	Detection in landfill sites:							
	P	B [97]*	S	R	C	H	W	B [98]*
	(a) 'Direct' PCR amplification							
1. DFM	+	−	+	−	+	−	−	−
2. DBB	−	−	−	−	−	−	−	−
3. DBM	−	−	−	−	−	−	−	−
4. DSB	+	+	−	−	−	−	−	+
5. DCC-DNM-DSS	+	+	−	−	+	−	+	−
6. DSV-DMB	−	−	−	−	−	−	−	−
	(b) 'Nested' PCR amplification							
1. DFM	+	+	+	+	+	+	+	+
2. DBB	+	+	−	+	−	−	+	−
3. DBM	−	−	−	−	−	−	−	−
4. DSB	+	+	+	−	−	−	+	+
5. DCC-DNM-DSS	+	+	−	+	+	+	+	+
6. DSV-DMB	+	+	−	+	+	+	+	+

* Two samples were taken from landfill site B, in consecutive years (1997 and 1998).

quently, application of the group 6-specific primers with confirmation by hybridization to probe DSV687 provides firm evidence that these SRB are present. Although this application of primers and probes in combination provides good evidence for the presence of an SRB group, sequence analysis of cloned PCR products provides final confirmation. We can report (data not shown) that of 21 sequenced clones (600–1150 bp) of environmental DNA amplified using the group-specific primers and containing the respective target oligonucleotide probe sequence, all aligned with the predicted SRB group based on a FASTA search of the GenBank and EMBL databases.

Theoretical cross-specificity analysis of the primers and probes designed in this study indicated that primer–probe combinations would provide highly specific molecular tools for unequivocal detection of each of the six SRB groups in environmental samples. This was confirmed experimentally, providing further confidence in the data on SRB group detection in the landfill leachate samples. The data showed that populations of SRB were detectable in landfill leachate by PCR amplification and probing, and that their occurrence would appear to be widespread. SRB 16S rDNA was successfully amplified from five out of seven landfill sites using the 'direct' PCR approach and from all seven sites sampled using 'nested' PCR.

The results obtained using the 'direct' PCR amplification approach suggest that there are one or two dominant groups of SRB in each of the landfill sites: *Desulfotomaculum* (group 1) in landfills S and C; *Desulfotomaculum* (group 1) and *Desulfococcus–Desulfonema–Desulfosarcina* (group 5) in landfill P; *Desulfobacter* (group 4) in landfill B; *Desulfococcus–Desulfonema–Desulfosarcina* (group 5) in landfill W. Only in two landfill sites (R and H) were no SRB detected using this 'direct' PCR approach.

However, 'nested' PCR amplification revealed the presence of other groups not detected by the 'direct' PCR: *Desulfotomaculum* (group 1) in landfills B, R, H and W; *Desulfobulbus* (group 2) in landfills P, B, R and W; *Desulfobacter* (group 4) in landfills S and W; *Desulfococcus–Desulfonema–Desulfosarcina* (group 5) in landfills R and H; *Desulfovibrio* (group 6) in all except landfill S.

It is presumed that SRB groups that can only be detected in landfill leachates when a second round of amplification is employed ('nested' PCR) are present in lower numbers than members of the dominant groups detectable by 'direct' PCR. Therefore, the dual application of 'direct' and 'nested' PCR can permit a rapid estimate of the relative predominance of SRB groups in landfill leachate. However, this is only a qualitative estimation of relative numbers based on detection through one round of PCR ('direct') compared to two rounds of PCR ('nested') and bears no statistical significance.

It is possible that the requirement for 'nested' PCR to detect members of group 2 (DBB) and group 6 (DSV-DMB) in any leachate sample could be a feature of the PCR efficiency of these specific primers, rather than

reflection of a relatively small population size. However, PCR amplifications of DNA extracted from pure cultures using all six group-specific primer sets yielded approximately equivalent amounts of PCR product, i.e. no significant differences in performance of the primer pairs were noted. *Desulfobacterium*-like (group 3) amplification products were never obtained from any of the landfill sites by either 'direct' or 'nested' PCR; this would appear to correlate with the association of most of the known species of the genus *Desulfobacterium* with the marine environment (Postgate, 1984; Fauque, 1995).

Nevertheless, the results obtained from the 'nested' PCR (Table 4) suggest that there is a high level of SRB diversity in landfill, as a distribution of the other five main groups was observed. This correlates with investigations of SRB occurrence and distribution in other environments in which most of the main groups have been detected by oligonucleotide probing (Kane *et al*., 1993; Ramsing *et al*., 1993; Risatti *et al*., 1994; Devereux *et al*. 1996a, b; Raskin *et al*., 1996; Purdy *et al*., 1997; Trimmer *et al*., 1997; Rooney-Varga *et al*., 1997; Manz *et al*., 1998; Sahm *et al*., 1999). This is, to our knowledge, the first investigation of SRB occurrence in landfill using molecular biological techniques, and the only study of SRB molecular ecology described to date in which DNA extracts have been amplified by specific PCR prior to confirmation by oligonucleotide hybridization.

This apparent diversity of SRB, at least at the generic/suprageneric level, in landfill sites is not unexpected. The extremely high and varied organic carbon load together with long retention times encourages large and active populations of fermentative micro-organisms, which in turn produce various volatile fatty acids that serve as substrates for SRB. The scale of landfill sites and their extreme heterogeneity would promote microbial diversity. Also, as leachate results from the percolation of water through the site, high diversity would be expected even though SRB distribution is undoubtedly non-uniform throughout the site. While it would be of interest to study SRB populations in solid landfill material, leachate is going to be the only practical sample material for routine analysis and SRB monitoring. Thus, the argument that SRB populations in leachate may be a poor representation of SRB population size and distribution in the landfill does not preclude their use as a practical source of useful information on the landfill site as a whole.

It is now well established that SRB and methanogens compete for fermentation products such as acetate and H_2 and that, in the presence of non-limiting levels of sulfate, SRB generally outcompete methanogenic bacteria (Oremland & Polcin, 1982; Beeman & Suflita, 1987; Raskin *et al*., 1996), with sulfate reduction being the key process of carbon mineralization in these environments. However, in landfill it is usually methanogenic bacteria that dominate, with methanogenesis, not sulfate reduction, as the key terminal process of carbon mineralization. This suggests that SRB populations in landfill are limited by the availability of sulfate, thereby allowing methanogenesis to dominate. However, the detection of SRB in these landfill sites suggests that the potential for sulfate reduction and the possible inhibition of methane production is present (Suflita *et al*., 1992; Gurijala & Suflita, 1993). It is important to be able to monitor SRB populations in landfill sites because their proliferation can potentially affect site performance via the inhibition of methanogenesis.

This study provides 16S rRNA-based methods for detecting SRB in landfill and also provides the first insight into SRB diversity in landfill sites.

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