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A Culture-Independent Survey of the Bacterial Community in a Radon Hot Spring

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ABSTRACT

Paralana is an active, radon-containing hot spring situated in a region of South Australia's Flinders Ranges with a long history of hydrothermal activity. Our aim was to determine the bacterial composition of Paralana using a culture-independent, 16S rRNA-based technique. The presence of a diverse bacterial community was strongly suggested by the large number (~180) of different ribotypes obtained upon analysis of nine hot spring samples. DNA sequencing of Paralana 16S rRNA genes corroborated this observation, identifying representatives of seven confirmed and two candidate divisions of the domain Bacteria. These included Cyanobacteria, Proteobacteria (both β and δ subdivisions), the Cytophaga–Flexibacter–Bacteroides group, Low G+C Gram-positives, Nitrospira, green non-sulfur bacteria, green sulfur bacteria, OP8, and OP12. No known ionizing radiation-resistant Bacteria were identified. Only one Paralana 16S rRNA sequence type (recombinant B5D) was homologous to a sequence previously identified from a radioactive environment. Key Words: Hot spring—Radon—Hydrothermal—Bacteria—16S rRNA. Astrobiology 2, 255–270.

INTRODUCTION

HYDROTHERMAL REGIONS ON EARTH support a rich and varied biota. Recent interest has focused on the microbial composition of these ecosystems, such as those present in subaerial hot springs and submarine vents (Stetter, 1996; Jeanthon, 2000). For example, studies have indicated that hot springs support a high diversity of Bacteria and Archaea. The most thoroughly studied modern examples are Obsidian Pool and Octopus Spring in Yellowstone National Park in the United States, which contain a flourishing community of thermophilic (heat-loving) microbes (Barns *et al.,* 1994; Hugenholtz *et al.,* 1998a; Ferris *et al.,* 2001). Microorganisms inhabiting hydrothermal systems have generally been identified using both traditional culture as well as DNA-based, culture-independent methods (Theron and Cloete, 2000). Apart from establishing the microbial community diversity, studies like those at Yellowstone have also contributed to our current understanding of early life on Earth. For example, evolutionary phylogenetic trees constructed from sequences encoding the small 16S subunit (SSU) of rRNA suggest that extant thermophiles are descended from the earliest terrestrial life forms. This has, in turn, led to the theory that life

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originated in a hydrothermal environment like that found at deep-sea vents (Woese, 1987; Weigel and Adams, 1998).

Investigations of the microbiota inhabiting modern hydrothermal systems are also important for interpreting presumed biomarkers (microfossils, lipids, isotopes, etc.) found in ancient systems on Earth (Walter and Des Marais, 1993; Reysenbach and Cady, 2001). Finally, since it is highly probable that thermal springs were present for a large part of Martian history, both modern and ancient hydrothermal systems can provide information relevant to the search for extant or extinct microbial life on Mars (Brakenridge, 1990; Walter and Des Marais, 1993; Christensen *et al.*, 2000). Despite these benefits, little research has been published on thermal springs in Australia.

Paralana is the only currently active hot spring in the Mt. Painter province of South Australia's Flinders Ranges. This region is highly mineralized as a result of ancient hydrothermal activity. There are large deposits of quartz in which possible fossil evidence of microbial life has been preserved (Drexel and Preiss, 1995; M.R.W., unpublished data). The presence of both ancient and modern hydrothermal areas provides an opportunity to study the >200-million-year history of a terrestrial hydrothermal system.

The Paralana hot spring lies on the Paralana Fault Zone. Past and present hydrothermal activity is probably due to meteoric water (possibly augmented by Great Artesian Basin water) percolating down to hot rocks and then upwelling to the surface via faults (Blissett, 1971). The water appears to be heated by a combination of geothermal heating resulting from recent uplift along the fault line and the decay of radioactive minerals. Maximum water temperatures at the spring have been recorded as 62.2°C (Mawson, 1927), 60°C (Grant, 1938), and 63°C (this study). Gas that bubbles up from the sandy bottom of the pool is composed of 88.1% N_2, 11.9% CO_2, and trace helium and hydrogen. The spring also releases radon from the fission of radium, with higher levels in the gas (29,000 Bq/L) than in the water (2,000–5,800 Bq/L) (Grant, 1938; Beverley Environmental Impact Statement, 1998).

We report here the bacterial composition of the Paralana hot spring, as ascertained by culture-independent 16S SSU rRNA analysis. This approach provides a better representation of the bacteria present than do traditional culturing methodologies, which are estimated to only reveal <1–5% of microbial species present in the environment (Hugenholtz and Pace, 1996). Our results indicate that the Paralana hot spring supports a diverse community comprising representatives of nine bacterial divisions.

MATERIALS AND METHODS

Sample collection and DNA extraction

Temperature at sampling sites was measured with a portable digital thermometer (Thermoscan TP200); pH was recorded using pH test strips (range 0–14; Sigma). Nine sediment and/or mat samples (Table 1) were collected from the Paralana Hot Spring, which is located at 30°10′35″S, 139°26′26″E in the northeastern region of the Flinders Ranges, South Australia. Samples were stored at −20°C until processed for nucleic acid extraction. DNA was extracted using the FastPrep bead beating procedure (Bio101), using a modification of a previously described method (Yeates and Gillings, 1998). The sample (400 mg) was aseptically transferred to a 2-mL Multimix FastDNA tube containing a lysing matrix. To this was added 122 μL of MT buffer (Bio101; 1% sodium dodecyl sulfate, 0.5% Teepol, and PVP40 with EDTA and Tris) and 780 μL of 0.1 *M* sodium phosphate, pH 8. Cells were disrupted by processing in a FastPrep instrument (30 s at 5.5 m/s). Following centrifugation in a microcentrifuge (15,000 *g* for 5 min), 600 μL of DNA-containing supernatant was transferred to a fresh sterile tube. A protein precipitation solution (150 μL of 3 *M* potassium acetate and 4% glacial acetic acid) was added, and the sample was vortex-mixed and left to stand for 5 min. It was then recentrifuged, and the supernatant was collected as before. DNA binding matrix (600 μL of glassmilk from Bio101, diluted 1:5 with 6 *M* guanidine isothiocyanate) was added to the supernatant prior to rotating for 5 min. Following another centrifugation, the DNA-glassmilk pellet was resuspended in 800 μL of Wash solution (70% ethanol and 0.1 *M* sodium acetate) and rotated for 5 min. After centrifuging the mixture, the pellet was air-dried, resuspended in 200 μL of TE buffer (10 m*M* Tris-Cl and 1 m*M* EDTA, pH 8), and allowed to stand for 10 min. Following a final pelleting step, the DNA-containing supernatant (180 μL) was collected. The amount and quality of the DNA extracted were assessed by electrophoresis through a 0.8% agarose gel. A DNA extraction control, in which 400 μL of sterile water was used instead

Table 1. Sample Descriptions

Sample[1]	*Description*	*Temperature (°C)*
A	Brown biofilm + sediment	60–63
B	Sediment 2–3 cm under A	60–63
C	Black mat + sediment	48
D	Gray sediment (3–4 cm down) under blue-green mat	49
E	Diaphanous biofilms and slimy mats + sediment	60
F	White filamentous encrustation on sticks (on pool bottom) + sediment	60
G	White biofilm on green mat	59
H	Floating green and green-yellow mat	53
I	Benthic green-yellow and emerald green soft mat forming sheets and pinnacles	48

[1]All samples were ~pH 7. Samples A–H were obtained from the source pool; sample I was from the main pool.

of sample, was processed as above to test for laboratory contamination.

16S rRNA polymerase chain reaction (PCR) and recombinant libraries

Community 16S rRNA genes were PCR-amplified from each DNA sample with Bacteria domain-specific PCR primers PB36 (5′-AGR GTT TGA TCM TGG CTC AG-3′) and PB38 (5′-GKT ACC TTG TTA CGA CTT-3′) (Saul *et al.,* 1993). PB36 and PB38 bind to, respectively, positions 8–27 and 1,492–1,509 of the 16S rRNA gene [*Escherichia coli* gene numbering (Blattner *et al.,* 1997)]. Sample DNA (~100 ng) was amplified using the following cycling conditions: 94°C for 3 min (1 cycle); 94°C for 45 s, 55°C for 30 s, 72°C for 90 s (25 cycles); 72°C for 7 min (1 cycle). PCR products were purified using a High Pure PCR Product Purification Kit (Roche). A library of the purified 16S rRNA genes was prepared for each Paralana site using the pGEM-T Easy Vector System (Promega) and subsequent transformation into Max Efficiency DH5α competent *E. coli* cells (Invitrogen). For each library (A-I), 95 recombinant (i.e., white) *E. coli* colonies were selected and grown overnight (37°C) in 150 μL of Luria broth containing 100 μg/mL ampicillin. Cultured cells were lysed (94°C for 5 min), and debris was pelleted by centrifugation (5 min). Recombinant plasmid 16S rRNA gene inserts were reamplified from 2 μL of the lysate (supernatant) with pGEM-T vector-specific PCR primers (PGEMF, 5′-GCC GCG GGA ATT CGA TT-3′; PGEMR, 5′-CGA ATT CAC TAG TGA TT-3′). Amplified products were screened by restriction fragment length polymorphism (RFLP) with the restriction enzyme *Hae*III (recognition site GG ↓ CC; 37°C overnight) to determine the diversity of 16S rRNA genes in each library. The resulting *Hae*III restriction fragments were size-fractionated by 6% polyacrylamide gel electrophoresis. Comparative analysis of 16S rRNA RFLP patterns (ribotypes) was performed using GelCompar *II* computer software (version 2.5, Applied Mathematics). Restriction fragments <60 bp were excluded from this analysis.

Sequencing

DNA sequencing reactions were performed with the ABI Prism BigDye Terminator kit and analyzed with an ABI PRISM 377 DNA Sequencer. pGEM vector-specific (see above) and bacterial 16S rRNA internal PCR primers (16S.1, 5′-ACT CCT ACG GGA GGC AGC AG-3′; 16S.3, 5′-GGA TTA GAT ACC CKG GTA GTC C; 16S.5, 5′-GCT CGT TGC GGG ACT TAA CC-3′) were used for sequencing. All sequences were compared with GenBank database entries using both BLAST (National Center for Biotechnology Information; Altschul *et al.,* 1990) and FASTA (University of Virginia; Pearson and Lipman, 1988) alignment tools.

RESULTS

Description of the Paralana hot spring

The Paralana system consists of two interconnected freshwater pools. A small, irregularly shaped pool (designated the source pool, ~1 × 2 m) forms where the source water emerges through alluvial sand and gravel (Fig. 1, upper

panel). The water then flows around a large rock and through sediment to form a larger pool (main pool, ~10 × 5 m; Fig. 1, lower panel); Paralana Creek drains this pool at the end opposite the source. The source pool has a neutral pH, and temperatures measured ranged from 48°C to 63°C. In addition, gas was observed bubbling up through sediment at the source, presumably containing the radon gas described by Grant (1938). The presence of a complex microbial community was suggested by the numerous colored benthic and floating mats and biofilms in both pools (Fig. 2). A description of the samples taken for 16S rRNA analysis is given in Table 1.

16S rRNA genes from Paralana hot spring samples A–I

PCR amplification of community 16S rRNA genes from biomass DNA with Bacteria-specific primers produced the expected amplification product (i.e., ~1.5 kb in size). An additional, fainter, 1.7-kb PCR product was present in samples A and E. Although this product may represent an artifact, larger 16S rRNA genes have been found in some Gram-positive, thermophilic bacteria, including *Desulfotomaculum australicum*, an organism isolated from artesian water in Australia (Love *et al.*, 1993). No PCR product was observed in the DNA extraction control. Thus, 16S rRNA libraries (named for the respective samples A–I) were constructed, and cloned 16S rDNA inserts were screened using *Hae*III RFLP. Approximately 180 different RFLP patterns (ribotypes) were observed in 793 recombinants (Table 2). An example of the ribotypes obtained is shown in Fig. 3. Most sample libraries contained a relatively high diversity of ribotypes. This is highlighted by the Shannon–Weaver diversity index [H' (Shannon and Weaver, 1963)] values of >2.5 obtained for all samples except F and I ($H' = 0.57$ and 1.73, respectively). The range of possible H' values for the Paralana libraries varies from 0 (i.e., only one ribotype per sample) to ~4.4 (i.e., the number of ribotypes = number of recombinants analyzed).

FIG. 1. The Paralana hot spring. Upper panel: The smaller source pool, from which the spring water emerges. **Lower panel:** The larger main pool forms from water flowing around and under the large boulder seen in both panels (left side of upper panel and right side of lower panel). Paralana Creek is to the left of the main pool. The size of both pools has been observed to vary over time.

The 16S rRNA recombinants selected for sequencing represented both the more dominant ribotypes in each library, which occurred at a frequency of ~10% or greater, and a selection of less frequent ribotypes. Only one 16S rRNA sequence, represented by recombinant E12H, may have been chimeric (i.e., composed of regions from two or more different 16S rRNA genes) as determined by the CHECK_CHIMERA program at the Ribosomal Database Project (http://rdp.cme.msu.edu/html; Maidak *et al.*, 2001). The taxonomic identity of the sequences, based on BLAST and FASTA database searches of GenBank, is given in Table 3; also shown is their percent representation within the nine samples. The vast majority of Paralana 16S rRNA genes displayed >90% sequence homology (i.e., identity) to database sequences; half of these were ≥95% homologous.

Sequence-based description of bacterial communities

The 16S rRNA sequences identified in the Paralana hot spring indicated the presence of representatives from nine bacterial divisions (Fig. 4 and Table 3). Seven of these are confirmed divisions, and two are candidates. Two sequences were of an uncertain affiliation. All samples except G contained at least one dominant sequence; sample G displayed a much more even distribution of sequence types.

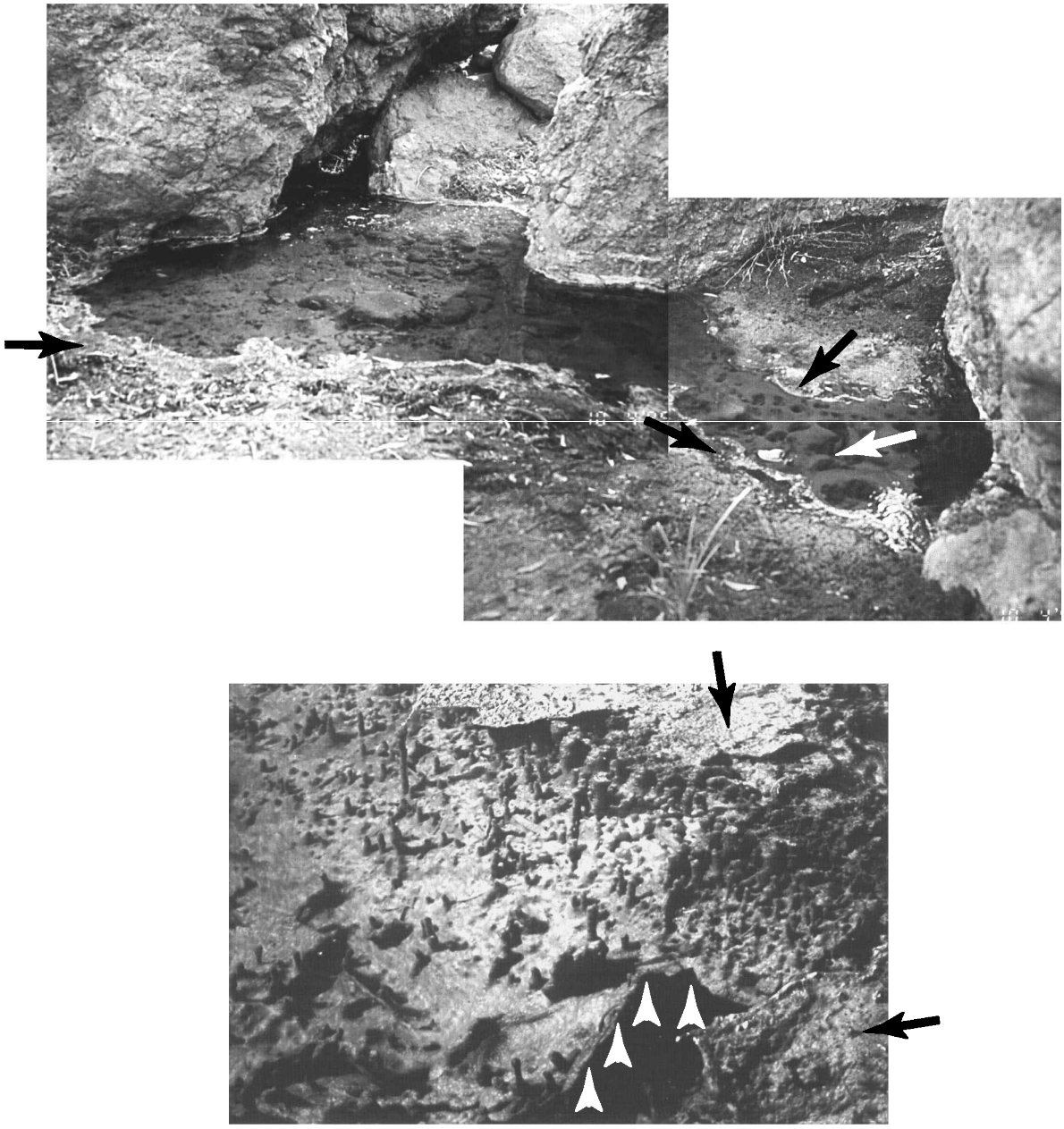

FIG. 2. Macroscopic evidence of microbial communities in the Paralana hot spring. The source and main pools contained a number of mats and biofilms. For example, floating green mats were present along the edge of the source pool (the montage in the **upper panel;** black arrows), and a dark benthic mat or biofilm covered some of the bottom (white arrow). Samples A–H were collected within 1 m of each other, with A and B near the rock on the far right of the pool. **Lower panel:** The bottom of the main pool was almost completely covered by a soft (unmineralized) green-yellow and emerald green mat that formed large sheets and pinnacles. Sample I was collected from this mat. Fragments of a similarly colored mat floating on the pool surface (black arrows) appeared to be derived from the benthic mat; the white arrowheads indicate a section of the benthic mat in the process of being dislodged.

Samples A and B (60–63°C). Located at the source, these sites were the hottest sampled. They differed in vertical, but not horizontal, position. Sample B, a coarse, sandy sediment with some small, brown biomaterial, was taken 2–3 cm below sample A, a benthic brown biofilm that coated a finer sandy sediment and leaves. The 16S rRNA libraries A and B displayed a wide, yet generally similar range of division-level diversity that encompassed six and five bacterial divisions, respectively (Table 3). The affiliation of two sequences was uncertain. Sample A was dominated

Table 2. Ribotype Screening Results

16S rRNA library	*Number of recombinants screened*	*Number of ribotypes observed*	*H′*
A	95	33	2.98
B	86	33	2.85
C	91	33	2.53
D	76	51	3.66
E	84	60	3.85
F	82	7	0.57
G	93	36	3.32
H	94	30	2.58
I	92	18	1.73

H′ = Shannon–Weaver diversity index (Shannon and Weaver, 1963).

by sequences with ≥96% homology to members of the *Nitrospira* and candidate OP8 divisions. The dominant organism at site A is a clearly defined member of the candidate OP8 division (Table 3). The representative recombinant isolate sequenced (A3D) was 98% homologous with clone OPB95 from Obsidian Pool (temperature 74°C) in Yellowstone (Hugenholtz *et al.*, 1998a). The sequence of the other dominant member of sample A (represented by Paralana recombinant A8H) shares 96% identity with *Nitrospira moscoviensis.* This bacterium is a nitrite-reducing chemolithotroph that was first isolated from a corroded iron pipe in a Moscow municipal heating system (Ehrich *et al.*, 1995).

Three ribotypes predominated in sample B. Their sequences matched with the following divisions (Table 3): (1) green sulfur bacteria—Paralana recombinant B5H, 98% homology with uncultured clone SM1H02, identified within travertine depositional facies at Angel Terrace, in Yellowstone (G.T. Bonheyo *et al.*, 2001, unpublished GenBank entry); (2) Low G+C Gram-positive bacteria—recombinant B12C, 91% homology with OS Type K from Octopus Spring, also at Yellowstone [temperature = 50–55°C (Weller *et al.*, 1992)]; and (3) unassigned division—recombinant B5D, 93% homology with environmental clone GR-WP33-30 (Radeva and Selenska-Pobell, 1999), which may belong to the δ-Proteobacteria

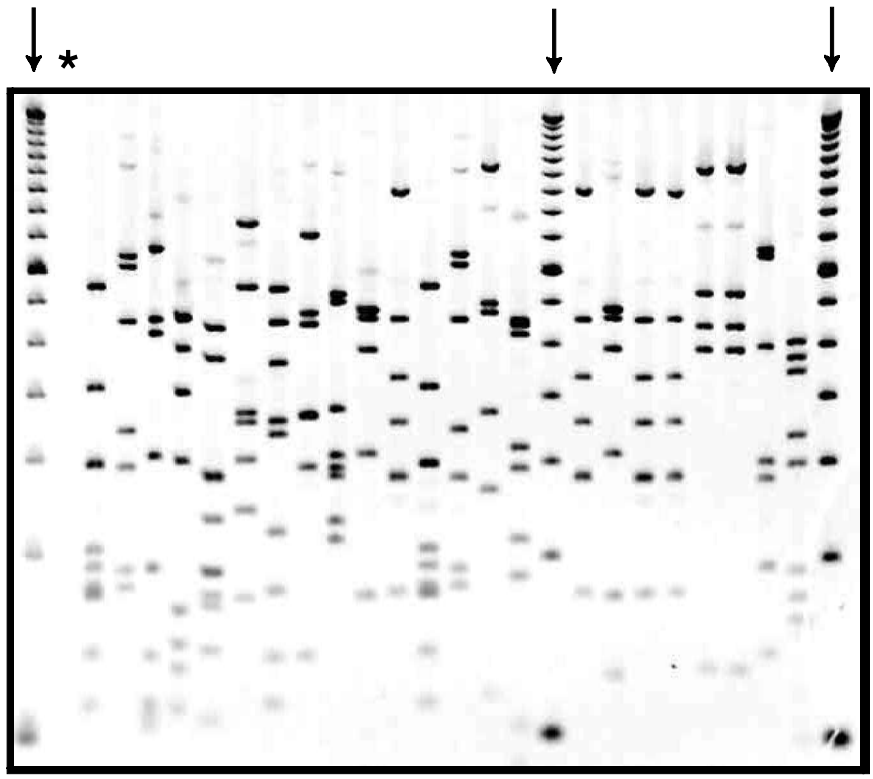

FIG. 3. An example of the different ribotypes obtained from the Paralana hot spring. The 16S rRNA genes were PCR-amplified from sample C recombinants and digested with *Hae*III. The resulting DNA fragments were separated by electrophoresis in a 6% polyacrylamide gel. A negative image of the gel is shown here. Fragment migration was from top to bottom. Lanes containing molecular weight standards (arrows) and the negative PCR water control (*) are indicated.

subdivision. Both samples A and B contained small numbers of sequences displaying homology (84–99%) with members of *Nitrospira,* the green non-sulfur bacteria, and the β- and δ-Proteobacteria (Table 3). Furthermore, thermophilic representatives (*Bacillus* spp.) of the Low G+C Gram-positive division that grow vigorously at 60°C have been cultured from sample A in preliminary experiments (data not shown).

Sample C (48°C). Although this sample was visible as a black mat lying upon sediment prior to collection, vortex-mixing prior to DNA extraction revealed soil-like material intermingled with green and brown biofilm. Sequence analysis indicated that this sample was dominated by three different cyanobacterial populations (sequences), representing 58% of 91 recombinants examined. Two of the cyanobacterial populations were dominant (Table 3). Recombinant I1G was homologous (99%) to *Oscillatoria amphigranulata,* a filamentous organism originally isolated from a New Zealand hot spring (Garcia-Pichel and Castenholz, 1990). The other dominant sequence (recombinant C2F) was 90–92% homologous to 16S rRNA genes from members of a number of different cyanobacterial orders, including Oscillatoriales, Chroococcales, and Nostocales. Maximum likelihood phylogenetic analysis performed using the software program PAUP* (Swofford, 2002) suggested that C2F is related to either an Oscillatoriales sequence or to *Synechococcus lividus* (Chroococcales). *S. lividus* is a major component of the Octopus Spring cyanobacterial mat (Weller *et al.,* 1992; Ward *et al.,* 1998). The presence of Oscillatoriales and *Lyngbya* (the other filamentous cyanobacterial population identified in this sample) was also demonstrated by microscopic examination of sample C wet mounts (data not shown). Two other sample C ribotypes sequenced matched with members of the β-Proteobacteria subdivision (recombinants F2D and I9H, Table 3).

Sample D (49°C). The community profile was similar to that of sample C, although only one cyanobacterial sequence type dominated. This was represented, as in sample C, by recombinant C2F. Another recombinant examined (E12H) displayed sequence homology with a member of the OP12 candidate division [clone OPB54 (Hugenholtz *et al.,* 1998a)], which, like OP8, does not contain cultured representatives. Sample D had a very high diversity of ribotypes ($H' = 3.66$, Table 2), with 51 different types identified in 76 recombinants screened.

Sample E (60°C). This sample contained a delicate, thin brown biofilm and slimy mats. Although its temperature was substantially different to that of sample D, there were only minor differences noted in rRNA sequence types (Table 3). Specifically, this related to one occurrence each of sequences matching with *N. moscoviensis* and clone OPB95 of the OP8 candidate division. Sample E displayed further relatedness with sample D: a high level of ribotype diversity, with 60 types present in 84 recombinants ($H' = 3.85$, Table 2).

Sample F (60°C). This sample consisted of a white filamentous encrustation on sticks at the bottom of the source pool (~20 cm deep), and a brown, stringy material evident after vortex-mixing. Some sand and silt were also collected. Sample F was overwhelmingly dominated by one ribotype (72 of 82 recombinants analyzed; sample H' value of 0.57), identified as a β-proteobacterium (Table 3). The representative recombinant, F2D, had a high level of homology (99%) with *Hydrogenophilus thermoluteolus,* a thermophilic, chemolithotrophic hydrogen oxidizer (Hayashi *et al.,* 1999). *H. thermoluteolus* sequences were also identified in samples A, C, and G. This and one other β-proteobacterial 16S rRNA sequence (recombinant I9H) were the most widespread, but generally minor, constituents of the other eight hot spring samples (Table 3).

Sample G (59°C). This sample was mechanically strong, being difficult to disrupt by vortex-mixing. Once disrupted, substantial amounts of green, brown, and rust-colored diaphanous biofilms were observed. Thirty-six ribotypes were seen amongst 93 recombinants, indicating a high level of 16S rRNA sequence diversity ($H' = 3.32$). Unlike the other samples, no single sequence type dominated. The sequences identified accounted for between 1% and 9% of the total number of recombinants analyzed, and represented seven confirmed and candidate divisions. Sequences with ≥95% database similarity (Table 3) represented Cyanobacteria [recombinant H11A, 97% homology with *Fischerella muscicola* (Turner *et al.,* 1999)], green sulfur bacteria (recombinant B5H, 98% with an uncultured bacterium), β-Proteobacteria (recombinant F2D, 99% with *H. thermoluteolus*), *Nitrospira* [recombinant

TABLE 3. DISTRIBUTION AND TAXONOMIC AFFILIATIONS OF 16S rRNA SEQUENCES IDENTIFIED IN PARALANA HOT SPRING SAMPLES A–I

Recombinant sequenced	*Bases sequenced*	*Bacterial division*	*Closest database relative*	*% homology*[1]	*GenBank accession number*	*% occurrence of sequence type in each hot spring sample*[2]								
						A (60–63°C)	*B (60–63°C)*	*C (48°C)*	*D (49°C)*	*E (60°C)*	*F (60°C)*	*G (59°C)*	*H (53°C)*	*I (48°C)*
F4F	1,345	CFB group	*Tuber borchii* symbiont (probably Cytophagales; uncultured)	84	AF070444						1			
G4F	1,363	CFB group	Clone SB-5 (probably Cytophagales; uncultured)	90	AF029041							5		
C2F	1,361	Cyanobacteria	Representatives of various orders	90–92	e.g., L04709			**33**	**16**	**14**			5	7
H9E	955	Cyanobacteria	*Lyngbya* spp. (Oscillatoriales)	91	AJ000714			8	1	1			**17**	**21**
H11A	1,321	Cyanobacteria	*F. muscicola* (Stigonematales)[3]	**97**	AF132788							8	**18**	
I1G	1,378	Cyanobacteria	*O. amphigranulata* (Oscillatoriales)[4]	**99**	AF317504			**16**	1	2			**23**	**51**
G10C	1,371	Green non-sulfur bacterium	Clone OPB34 (uncultured)	85	AF027044	1	2					4		
G11D	1,324	Green non-sulfur bacterium	Clone OPB65 (uncultured)	92	AF027036	4	3					3		
B5H	1,326	Green sulfur bacterium	SM1H02 (uncultured)	**98**	AF445702	3	**12**					4		

B12C	1,376	Low G+C Gram-positive	OS type K (uncultured)	91	L04711	2	**16**							
A8H	1,453	Nitrospira	*N. moscoviensis*	**96**	X82558	**16**	3			1				
G5C	1,437	Nitrospira	*T. islandicus*	**95**	X96726	1	2					6		
A3D	1,403	OP8	Clone OPB95 (uncultured)	**98**	AF027060	**20**				1				
E12H	1,505	OP12	Clone OPB54 (uncultured)	**95–97**[5]	AF027087				3	2		1		1
F2D	1,436	β-Proteobacteria	*H. thermoluteolus*	**99**	AB009828	1		1			**88**	8		
I9H	1,426	β-Proteobacteria	SBR1021 (uncultured)	94	AF204250	2	1	3	3	2			2	5
B5E	1,452	δ-Proteobacteria	*Desulfuromonas* spp.	84	AF357914	2	2						2	
A3F	1,278	Uncertain affiliation	SHA-114 (uncultured)	90	AJ290978	6						9		
B5D	1,363	Uncertain affiliation (δ-Proteobacteria?)	GR-WP33-30 (uncultured)	93	AJ296569		**9**							

[1]We have considered sequences displaying ≥95% homology (bold) as providing a good measure of taxonomic affiliation, whilst those <95% give a more approximate indication.

[2]Dominant sequence types in a sample are shaded; percentages for each sample do not add up to 100% because not all 16S rRNA recombinants were sequenced.

[3]Also known as *Mastigocladus laminosus*.

[4]Only an incomplete (953-bp) *O. amphigranulata* sequence exists in GenBank. However, the almost perfect homology (99%) over this region gives us confidence in the identification.

[5]Recombinant E12H was ~95–97% homologous over most of the 16S rRNA sequence, except for a small region (~100 bp) near the 5′ end that displayed no homology with clone OPB54.

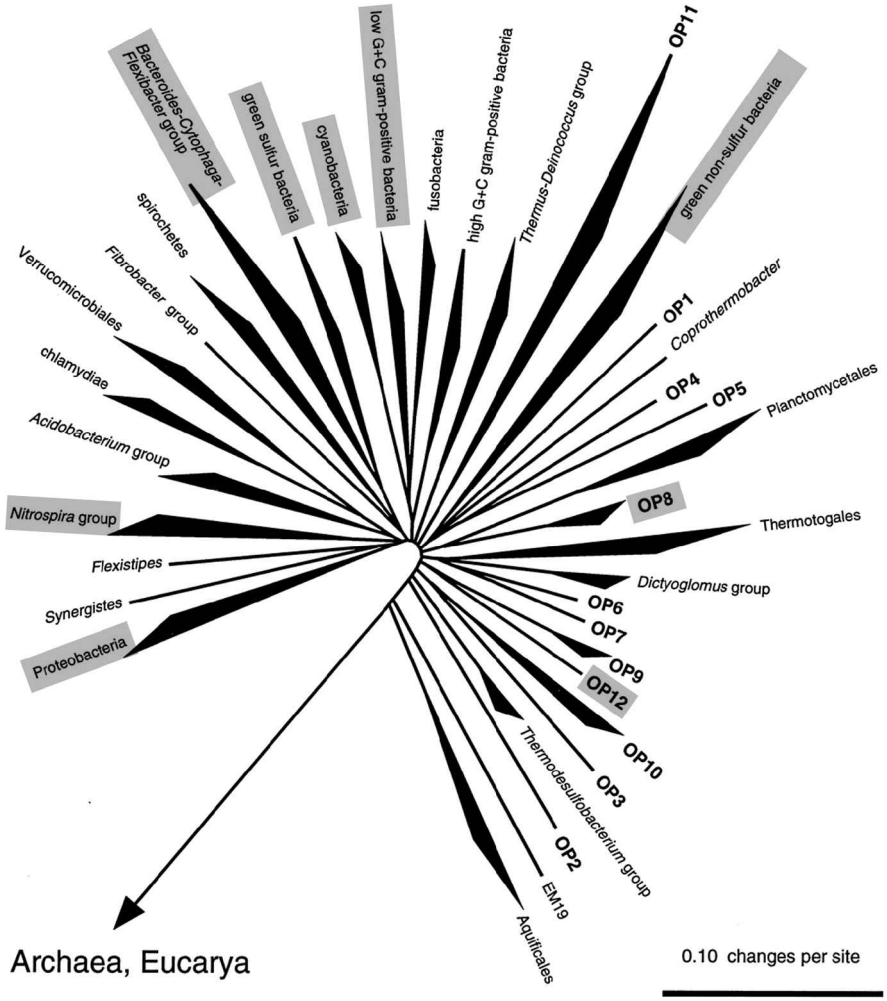

FIG. 4. Division-level distribution of the Paralana 16S rRNA sequences in the domain Bacteria. Divisions represented in the Paralana hot spring are indicated by shading on the 16S rRNA-based evolutionary distance tree of Hugenholtz *et al.* (1998a). Reprinted with permission from Hugenholtz *et al.* (1998a). Scale bar indicates 0.1 changes per nucleotide.

G5C, 95% with *Thermodesulfovibrio icelandicus,* an Icelandic hot spring isolate (Sonne-Hansen and Ahring, 1999)], and OP12 clone OPB54 (95–97% homology). Lower sequence homologies were observed with members of the Cytophaga–Flexibacter–Bacteroides (CFB) group, the δ-Proteobacteria, green non-sulfur bacteria, another Cyanobacteria, and an isolate of uncertain affiliation (Table 3).

Sample H (53°C) and sample I (48°C). When examined *in situ,* sampling sites H (a green-yellow/green floating mat in the source pool) and I (green-yellow and emerald green benthic mat in the main pool) appeared to be predominantly cyanobacterial. After vortex-mixing, sample H also contained small amounts of reddish-brown biofilm. Sample I had some rust-colored "slime." Cyanobacterial sequences dominated both samples, with their respective ribotypes representing 63% and 79% of recombinants analyzed. Four cyanobacterial sequences were identified in sample H, with three dominating (Table 3). The most prevalent, as in sample C, was *O. amphigranulata* (recombinant I1G). The other dominant sequences belonged to *F. muscicola* (recombinant H11A), which is a heterocystous branching member of the Stigonematales, and *Lyngbya* spp. [recombinant H9E (U. Nuebel, 1997, unpublished GenBank entry)]. Two minor ribotype sequences were homologous with recombinant C2F (Table 3) and an uncultured β-proteobacterium [clone SBR1021 (Crocetti *et al.,* 2000)] identified in an enhanced biological phosphorus removal process

(Paralana recombinant I9H). Database matches suggested that this latter recombinant may be a member of the purple non-sulfur bacteria (*Rhodocyclus* spp.).

The community profile of sample I was very similar to that of H, except that *F. muscicola* sequences were not identified, and an OP12 representative was present (Table 3). Over half of the ribotypes identified in sample I were *O. amphigranulata* (recombinant I1G). The other dominant sequence type present was *Lyngbya* spp., also identified in sample H (recombinant H9E). Based on its H' value of 1.73, sample I was the second least diverse Paralana sample analyzed.

DISCUSSION

We have conducted a qualitative, culture-independent 16S rRNA survey of the bacterial composition of the Paralana hot spring in order to characterize the microbial biota. There are no previous reports of comprehensive DNA- or culture-based studies of an Australian hot spring, and no such description of a radon hot spring anywhere in the world. Other researchers have concentrated on the hot springs of Yellowstone National Park in the United States and, in particular, Octopus Spring and Obsidian Pool (for example, Hugenholtz *et al.,* 1998a; Ferris *et al.,* 2001). As a result, these two springs are the best characterized in terms of community composition. A small number of other hot spring systems in the United States, Indonesia, Japan, and Iceland have been studied using molecular DNA techniques (Yamamoto *et al.,* 1998; Skirnisdottir *et al.,* 2000; Baker *et al.,* 2001). We sampled multiple sites (nine) representing different niches and growth types (mats, biofilms, and sediment) to ensure that we obtained a comprehensive description of the bacterial diversity supported by the Paralana spring. In general, other studies have only examined one or two samples, and often these only represent a single, visually dominant microbial population (e.g., filaments, streamers). We used 16S rRNA gene analysis because, despite its acknowledged limitations (von Wintzingerode *et al.,* 1997), its common use in recent years has revealed the breadth of diversity within terrestrial microbial biota (Hugenholtz *et al.,* 1998b). Identification of bacteria using 16S rRNA sequence database matches generally provides a good correlation with phylogenetic placement between the division and genus levels. However, the taxonomic affiliation of the Paralana bacterial sequences remains presumptive until confirmed by a detailed phylogenetic analysis.

Paralana supports a considerable diversity of Bacteria, with representatives of nine divisions identified. These include Cyanobacteria, β- and δ-Proteobacteria, the CFB group (probably representatives of the Cytophagales), Low G+C Gram-positives, Nitrospira (including both *Nitrospira* and *Thermodesulfovibrio*), green non-sulfur bacteria, green sulfur bacteria, and the candidate OP8 and OP12 divisions. Based on 16S rRNA techniques, only Obsidian Pool has a higher level of bacterial division level diversity, with 26 divisions (including 12 candidate divisions) (Hugenholtz *et al.,* 1998a). All nine Paralana divisions were identified in samples from the source pool into which the spring water emerges. Sample I, shown to contain Cyanobacteria, OP12, and β-Proteobacteria, was taken to identify the Bacteria present in the emerald green benthic mat covering most of the bottom surface of the main pool.

The presence of cyanobacteria in Paralana was not surprising, considering its temperature (48–63°C) and neutral pH. Both culture-dependent and -independent studies have indicated that cyanobacteria are common where temperatures are below their upper limit of ~74°C and pH values are above ~5 (Ward and Castenholz, 2000). Cyanobacteria displayed the largest intradivision diversity seen in Paralana, with four different 16S rRNA sequence types identified. Based on their high sequence homology values (97% and 99%, respectively), it is very likely that both *F. muscicola* and *O. amphigranulata* (or close relatives) are present in Paralana. It is also probable that *Lyngbya* spp. are present, as examples were identified in preliminary microscopic studies of the Paralana samples. Both *O. amphigranulata* and *F. muscicola* (previously known as *Mastigocladus laminosus*) are thermophiles that have been previously identified in hot springs environments in New Zealand and worldwide, respectively (Castenholz, 1976; Ward and Castenholz, 2000). The cyanobacterial communities in the Paralana hot spring occupy one of two distinct habitats. They form either a floating mat, or a submerged benthic mat or biofilm. Apart from the absence of *F. muscicola* in most of the benthic mats (sample G was the exception), both habitats were qualitatively the same with respect to cyanobacterial composition (the floating mat in

the main pool was not examined). Experiments aimed at obtaining a more quantitative indication of mat composition (for example, fluorescent *in situ* hybridization with 16S rRNA probes or flow cytometry) are required. The columnar/pinnacle-shaped structures in the benthic mat of the main pool were similar in appearance to structures containing *Phormidium* and *Synechococcus* cyanobacterial species in some Yellowstone hot springs (Walter *et al.,* 1976; Brock, 1978). The Yellowstone structures are mineralized by the deposition of silica (Walter *et al.,* 1972), but this is not the case with those in Paralana.

The majority of the Paralana 16S rRNA sequences show a high level of sequence similarity to those of Bacteria previously identified in hot springs by either culture- or DNA-based (culture-independent) techniques. For example, the closest database relatives of Paralana recombinants G10C, G11D, B5H, B12C, A3D, and E12H were found in Yellowstone National Park hot springs (Weller *et al.,* 1992; Hugenholtz *et al.,* 1998a; G.T. Bonheyo *et al.,* 2001, unpublished GenBank entry). Paralana supports other phototrophic microbes in addition to Cyanobacteria, viz., members of the green sulfur and green non-sulfur bacterial divisions. Proteobacteria were ubiquitous in the Paralana hot spring samples, reflecting the large size and diversity of this group (Garrity *et al.,* 2002). One 16S rRNA gene with a sequence homology of 99% with the β-proteobacterium *H. thermoluteolus* indicates that this species (or a close relative) is present in Paralana. *H. thermoluteolus,* originally isolated from soil around a Japanese hot spring, is an aerobic, facultatively chemolithoautotrophic hydrogen oxidizer that grows optimally at ~50–52°C and pH 7 (Hayashi *et al.,* 1999)—conditions that are found in Paralana. The other β-proteobacterial sequence we identified was not homologous to a hot spring-derived 16S rRNA gene sequence. Instead, it was identified in a mesophilic environment (22 ± 2°C): a sequence batch reactor used by Crocetti *et al.* (2000) for studying enhanced biological phosphorus removal from wastewater. The closest pure-cultured bacterial relative of the Paralana sequence is *Sterolibacterium denitrificans,* a cholesterol-oxidizing denitrifier (S.E. Tarlera, 2001, unpublished GenBank entry), which appears to be a member of the purple non-sulfur Rhodocyclus group.

The identification of a 16S rRNA sequence closely related (96% homology) to *N. moscoviensis,* an obligate chemolithoautotroph that oxidizes nitrite (NO_2^-), is of interest. Although originally isolated from an iron pipe in a Russian heating system [temperature unknown (Ehrich *et al.,* 1995)], its optimal growth temperature is 39°C (range 33–40°C). Until our study, *N. moscoviensis* only appears to have been associated with mesophilic environments, such as a fluidized bed reactor [30°C (Schramm *et al.,* 1998)], activated sludge (Burrell *et al.,* 1999), and freshwater aquaria [~26°C (Hovanec *et al.,* 1998)]. It has also been identified in Australian freshwater caves at a temperature (19°C) close to that considered psychrotrophic (Holmes *et al.,* 2001). The division Nitrospira was also represented, predominantly in Paralana sample G, by a member of the *Thermodesulfovibrio* subdivision, with *T. islandicus* being the closest database relative. This sulfate-reducing thermophile (optimal growth temperature of 65°C; range 45–70°C) was originally isolated from a microbial mat in an Icelandic hot spring (Sonne-Hansen and Ahring, 1999).

The identification of OP8 and OP12 sequences appears to be the first report of these recently described candidate divisions in Australia. This finding thereby considerably extends their known geographic distribution. OP8 and OP12 members have only been detected in culture-independent DNA-based studies. Apart from their original description in Obsidian Pool (Hugenholtz *et al.,* 1998a), 16S rRNA sequences of OP8 members have previously been identified in a number of other aquatic environments. These include a hydrocarbon- and chlorinated solvent-contaminated aquifer in the United States (Dojka *et al.,* 1998), the Cariaco basin water column [1,310 m deep (Madrid *et al.,* 2001)], mobile deltaic mud in a Papua New Guinean gulf (Todorov *et al.,* 2000), subterranean geothermal water in Iceland (Marteinsson *et al.,* 2001), and a shallow hydrothermal vent in Greece (Sievert *et al.,* 2000). The respective OP8 sequences from these studies shared between 85% and 92% homology with the original Obsidian Pool sequences. In contrast, OP12 sequences have previously only been identified in Obsidian Pool (Hugenholtz *et al.,* 1998a). The dominance of an OP8 sequence in Paralana sample A could provide an opportunity for a more comprehensive study of OP8 organisms. Furthermore, this sample may be the ideal starting material for developing culture strategies for a candidate division with no isolated representatives. Interestingly, no OP8 sequences were iden-

tified in sample B. This was an unexpected observation considering its close physical proximity and similar temperature to sample A. One possible explanation is that one of the minor sample B ribotypes we did not sequence represented a related OP8 species.

The Paralana water contains high levels of radon, measured at between ~2,000 and 5,800 Bq/L (Grant, 1938; Beverley Environmental Impact Statement, 1998). The value for the gas was ~29,000 Bq/L (Grant, 1938), which is $\sim 2 \times 10^6$ times greater than the average background level for outdoor air; it is also $\sim 2 \times 10^5$ fold above the "indoor air action level" at which it is recommended that remedial mitigation measures be taken (U.S. Environmental Protection Agency, 2001a,b). Whilst radon has been detected in some Yellowstone hot springs, this was not in the context of microbial communities, and radon levels were much lower than those present at Paralana (Clark and Turekian, 1990). With one exception (recombinant B5D, see below), none of the Paralana 16S rRNA sequences represents Bacteria previously identified in ionizing radiation environments. In particular, it was somewhat surprising that there was no evidence in Paralana of the extremely radiation-resistant *Deinococcus* genus, thermophilic members of which have been cultured from a hot spring in Italy (Ferreira *et al.*, 1997). The 16S rRNA sequence of Paralana recombinant B5D, only seen in sample B, was 93% homologous to a sequence originally identified in an ionizing radiation environment—that of clone GR-WP33–30, detected in the anaerobic drain waters of uranium waste piles (Radeva and Selenska-Pobell, 1999). Sequence database searches suggest that GR-WP33–30 may be a δ-proteobacterium (data not shown). Members of this bacterial subdivision have also been identified in uranium environments by Chang *et al.* (2001). Some members of *Bacillus* are resistant to ionizing radiation (for example, Kristensen and Christensen, 1981). However, the specific *Bacillus* spp. identified thus far in preliminary Paralana culture studies have not been reported to be radiation-resistant.

CONCLUSION

The culture-independent analysis of the Paralana hot spring bacterial community reported here is part of a program to characterize the microbial composition of this radon-rich hydrothermal site. We are currently conducting a similar study of the archaeal population. The majority of the bacterial representatives identified were those expected in a hot spring environment; however, they are not those expected in an ionizing radiation environment. Targeted molecular studies using PCR with primers specific for radiation-resistant microbes should determine whether known radiophilic extremophiles (for example, *Deinococcus* spp.) are present. The absence of studies on the effect of radon on microbes makes it difficult to ascertain if the Paralana radon levels are sufficient to cause cellular damage.

Knowledge of the Paralana 16S rRNA sequences permits certain *in situ* experiments to be conducted. An example is analysis of the distribution and structure of the Paralana microbial communities at the microscopic scale by fluorescent *in situ* hybridization. This technique could also be used to reveal the interactions and morphology of individual microbial cells, information that is relevant to the analysis of the ancient Mt. Painter hydrothermal sites. Finally, fluorescent *in situ* hybridization can be applied to experiments aimed at determining the microbial makeup of the columnar structures in the larger Paralana pool. Analysis of these is particularly significant if fossilized examples are identified in the Mt. Painter deposits.

Other potential studies cannot be performed *in situ*, but will require the availability of pure microbial cultures. An example is determination of the lipid composition of the Paralana microbes. Our identification of the Bacteria present in Paralana will enhance the chances of a successful microbial isolation by allowing a more rational, directed approach to culturing. Lipid profiles will be particularly useful for relating any lipid biomarkers found in the ancient Mt. Painter hydrothermal sites with the relevant extant Bacteria. Furthermore, the signature (i.e., diagnostic) lipids of the Paralana microbes could potentially be used for determining the composition of the microbial community (Jahnke *et al.*, 2001), thereby complementing molecular DNA studies of the hot spring.

Our results indicate that a diverse community of Bacteria is supported by the Paralana hot spring. Whilst similar findings have been reported previously, the presence of radon makes Paralana unique and argues for its suitability as an analogue for ionizing radiation environments, which may have been common on the early Earth

and Mars. It is clear that Paralana will add to the rapidly growing knowledge base of modern and ancient hydrothermal systems on Earth.

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ABBREVIATIONS

CFB, Cytophaga–Flexibacter–Bacteroides; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; rRNA, ribosomal RNA; SSU, small 16S subunit.

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