Development of fluorescent adjacent hybridization probes and their application in real-time PCR for the simultaneous detection and identification of *Fervidobacterium* and *Caloramator*

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A stretch of nucleotides consisting of a conserved region and an adjacent upstream variable region in the 16S rDNA of members of domain *Bacteria* was identified as a suitable target site for developing a real-time PCR adjacent hybridization assay. A single universal fluorogenic cyanin 5-labelled probe, CY5 1046+, targeting the conserved region, and two FITC-labelled probes, Calo and Fervi, targeting the variable region were designed and synthesized for the identification and differentiation of the thermophilic anaerobes *Caloramator* and *Fervidobacterium*. The simultaneous hybridization of probes CY5 1046+ and Fervi to the 16S rDNA target sites of *Fervidobacterium* species during PCR amplification resulted in an increase in fluorescence emission that was monitored continuously in a LightCycler. The differences in the temperature of disassociation of the hybridization probes (T_m) due to compositional variation in the nucleotide bases of the probe and target sequences enabled *Fervidobacterium islandicum* DSM 5733^T to be differentiated from *Fervidobacterium gondwanense* ACM 5017^T and *Fervidobacterium nodosum* ATCC 35602^T. The simultaneous hybridization of probes CY5 1046+ and Calo to the 16S rDNA target sequence of *Caloramator indicus* ACM 3982^T during PCR amplification also resulted in an increase in fluorescence emission. However, as the target sequence of *C. indicus* ACM 3982^T is identical to those of the other three *Caloramator* species, *Caloramator fervidus* ATCC 43204^T (formerly *Clostridium fervidus*), *Caloramator proteoclasticus* DSM 10124^T and *Caloramator coolhaasii* DSM 12679^T, further species discrimination on the basis of T_m will not be possible, making probe Calo a useful genus-specific probe. The devised method was subsequently used to detect and identify *Fervidobacterium* and *Caloramator* isolates from our previously uncharacterized culture collection and from enrichment cultures. The assay is cheap and flexible, as only a battery of inexpensive FITC probes for differentiating other members of domain *Bacteria* needs to be synthesized and DNA templates prepared by a simple lyse-boil method, in addition to purified DNA, can also be used.

Keywords: Great Artesian Basin of Australia, thermophilic anaerobes, 16S rDNA, real-time PCR, fluorescent adjacent hybridization probes

INTRODUCTION

The advent of molecular biology techniques such as PCR and automated sequencing of 16S rRNA genes has revolutionized our understanding of microbial diversity, with evidence suggesting that 90–99% of microbes from any given environment remain uncultured (Amann *et al.*, 1995). Natural and man-made environments including volcanic ecosystems, subsurface environments such as oilfields and aquifers, thermal spas and compost ecosystems (Reysenbach *et al.*, 1994; Harmsen *et al.*, 1997; Ravot *et al.*, 1995; Andrews & Patel, 1996; Chrisostomos *et al.*, 1996) have an enormous wealth of thermophilic microbes that has remained untapped. Micro-organisms from such environments are of great interest as they not only represent ancient lineages in the tree of life (Winker & Woese, 1991) but also possess biotechnologically important thermostable enzymes (Wynter *et al.*, 1995, 1996). The challenge now is to culture these microbes in the laboratory. It is evident that the current enrichment and isolation techniques are biased towards fast-growing microbial populations that potentially out-compete novel groups such as the slow-growing populations (Dunbar *et al.*, 1997). Initiating enrichment cultures under a wide variety of growth parameters should be an important procedure for discovering novel groups of thermophilic microbes, but this has been largely overlooked because of technical difficulties associated with handling the large numbers of cultures generated from such a process. Consequently, simple, specific and rapid detection and identification methods for species differentiation from enrichment cultures need to be developed. A powerful method, termed whole-cell hybridization, which relies on the use of extensive 16S rRNA sequence database resources for devising probes, can be used for such studies but is time-consuming to perform (Amann *et al.*, 1995). The application of real-time PCR for identifying microbes is an alternative complementary method to whole-cell hybridization but has not attracted a great deal of attention. During real-time PCR, an increase in fluorescence due to the hybridization of fluorescently labelled DNA probes to complementary targets in DNA amplicons is measured. We have recently reported the use of real-time PCR assays targeting the rRNA genes for the simultaneous detection, identification, quantification and differentiation of members of *Leptospira* and *Leptonema* (Woo *et al.*, 1997, 1998a, b) using a LightCycler (Idaho Technology, Idaho Falls, ID, USA). The LightCycler is an air thermal cycler with a built-in fluorimeter and has the ability to generate rapid thermal transitions resulting from a circulating air vortex (Wittwer *et al.*, 1997). We report in this paper the identification of a 16S rDNA target region suitable for the development of fluorescent adjacent hybridization probes and its application in the real-time detection of the thermoanaerobic genera *Fervidobacterium* and *Caloramator* in pure and enrichment cultures.

METHODS

Source of cultures and DNA extraction. Table 1 lists the pure cultures and enrichment cultures and their sources. Type cultures were purchased from the DSMZ, the ATCC and the Australian Collection of Microorganisms (ACM). Thermophilic cultures, isolated from the Great Artesian Basin of Australia and maintained at -80°C at the Microbial Technology Culture Collection (MTCC), Griffith University, were resuscitated (Patel *et al.*, 1991; Andrews & Patel, 1996; Ravot *et al.*, 1995; Wynter *et al.*, 1995, 1996). Water, sediment and microbial mat samples used for initiating enrichment cultures were collected from two bores of the Great Artesian Basin, registration numbers 17263 (Terrick Terrick) and 132, and their run-off channels as described previously (Denman *et al.*, 1991; Patel *et al.*, 1991; Andrews & Patel, 1996). All cultures were grown in anaerobic Trypticase/yeast extract/glucose (TYEG) medium (Patel *et al.*, 1985a, b) for up to 48 h at between 55 and 70°C as indicated (Table 1).

Crude DNA was extracted from 10 ml cultures by washing and resuspending centrifuged cell pellets in 10 mM Tris/HCl (pH 8.0) containing 0.75 mg each of achromopeptidase and lysozyme ml^{-1} , followed by an incubation at 37°C for 20 min. The samples were then heated for 15 min at 95°C and debris was removed by centrifugation. Cell lysis during the procedure was determined by examining aliquots from different steps and time intervals under a phase-contrast microscope. The hexadecyltrimethyl ammonium bromide (CTAB) method was used to prepare pure DNA (Wilson, 1987).

Design of primers and adjacent hybridization probes. Representative 16S rRNA gene sequences of 2000 members of the various phyla of the domain *Bacteria* were extracted from the GenBank database, release 125 (Benson *et al.*, 2000), and from the Ribosomal Database Project (RDP) version 8.1 (Maidak *et al.*, 2001) and aligned as described previously (Andrews & Patel, 1996). Primers and probes against useful target regions were determined and their usefulness in real-time PCR assays was checked by using a set of criteria and guidelines recommended by Idaho Technology as described previously (Woo *et al.*, 1997, 1998a, b). The most specific and ideal probe candidates for identifying members of the genera *Caloramator* and *Fervidobacterium* were confirmed by BLAST analysis and from T_m calculations. The melting temperatures (T_m) of the probes were estimated on the basis of thermodynamic data of base pairing by using a website-based calculator (<http://alces.med.umn.edu/rawtm.html>). The propensity of the probes to self-anneal and to anneal to the PCR amplification primers was predicted by the Oligo Selection program (Hillier & Green, 1991). The probe and primer target nucleotide positions are reported using the *Escherichia coli* numbering scheme (Winker & Woese, 1991). The probes and primers were synthesized by Genset Pacific (formerly Pacific Oligos) Pty Ltd, Lismore, Australia.

Rapid-cycle PCR and continuous monitoring of product. The monitoring of fluorescence emission during PCR was performed in a LightCycler, which is an air thermal cycler with a built-in fluorimeter. A 10- μl PCR contained 0.5 μM primers F1 and R5, 2 ng purified genomic DNA (or less for DNA template prepared by the boiling method), 0.2 mM dNTPs, 3 mM MgCl_2 , 50 mM Tris/HCl (pH 8.3), 0.25 mg BSA ml^{-1} (Sigma A2153), 0.4 U *Taq* DNA polymerase (Promega), 0.2 μM cyanin 5 (Cy5)-labelled universal probe

Table 1. Thermophilic, anaerobic cultures/enrichments used in this study

All cultures were grown in pre-reduced anaerobic TYEG medium as described in Methods. Type cultures were incubated at 65 °C. Unidentified isolates were incubated at the temperatures indicated in parentheses for up to 24 h. Enrichment cultures were incubated for 48 h at 60 °C and examined microscopically for growth and cell morphology. The probe T_m values were determined from at least three independent assays. Type cultures used as negative controls included *Thermoanaerobacter thermohydrosulfuricus* ATCC 35947^T, *Dictyoglomus thermophilum* DSM 567^T and *E. coli* K12. RN, Registered number of the bore; ND, not determined; NG, no growth.

Strain designation/morphology	Sample type and source/reference	Probe used		Probe T_m (°C)
		Fervi	Calo	
Type cultures				
Genus <i>Fervidobacterium</i>				
<i>F. nodosum</i> ATCC 35602 ^T	Patel <i>et al.</i> (1985b)	+	—	55.8 ± 1.7
<i>F. gondwanense</i> ACM 5017 ^T	Andrews & Patel (1996)	+	—	55.5 ± 1.7
<i>F. islandicum</i> DSM 5733 ^T	Huber <i>et al.</i> (1990)	+	—	61.7 ± 0.5
Genus <i>Caloramator</i>				
<i>C. indicus</i> ACM 3982 ^T	Chrisostomos <i>et al.</i> (1996)	—	+	66 ± 2.0
Unidentified cultures				
MTCC AB11B (65 °C)	RN 4164, Orange mats, 66 °C	—	—	—
MTCC AB17 (65 °C)	RN 3034, Orange mats, 65 °C	—	—	—
MTCC AB18 (70 °C)	RN 3034, Below source, 70 °C	+	—	54.4 ± 0.5
MTCC AB19 (65 °C)	RN 3034, 64 °C	+	—	54.4 ± 1.0
MTCC AB20 (55 °C)	RN 3034, Green mats, 53 °C	+	—	54.4 ± 0.1
MTCC AB23 (50 °C)	RN 5142, Thick mats, 47 °C	—	—	—
MTCC AB31 (70 °C)	RN 94, Yellow mats, 70 °C	—	—	—
MTCC SG58 (55 °C)	RN 37, Microbial mat, 54 °C	+	—	55
MTCC SG59 (55 °C)	RN 37, Floating mat, 52 °C	—	ND	—
MTC SG60 (55 °C)	RN 37, Sediment, 52 °C	ND	—	—
MTCC SG64 (60 °C)	RN 127, Sediment, 59 °C	ND	+	67.1
MTCC 75-2 (65 °C)	RN 132, Sample B	ND	—	—
MTCC SG76 (65 °C)	RN 132, Sample C	—	+	66 ± 1.0
MTCC SG80 (65 °C)	RN 89, Yellow/green mats	+	+	54.5 and 67.1
Enrichment cultures from RN 17263				
Small rods	Bore source, water sample A, 88 °C	—	—	—
Rods	Middle of run-off channel, sediment sample D, 75 °C	—	—	—
Rods	Side of run-off channel, sediment sample C, 66 °C	—	—	—
Rods with/without spheroids	Close to source, grey mat, 75 °C	+	—	61.2 ± 0.7
Rods, filaments	Side of run-off channel, red mat, 65 °C	—	—	—
Rods, filaments	Run-off channel, green mat, 57 °C	—	—	—
No growth	Run-off channel, brown mat, 52 °C	NG	NG	NG
Enrichment culture from RN 132				
Rods	Sediment sample, 60 °C	—	+	65.6

CY5 1046+ and 0.16 µM FITC-labelled specific probe. It was necessary to lower the concentration of the FITC-labelled probes from the 0.2 µM recommended by Idaho Technology so as to bring the initial fluorescence reading within the scale (6–10) of the LightCycler. Reactions were loaded into glass capillary tubes and snap-sealed using plastic caps (part 1720; Idaho Technology). The PCR cycle was as follows: initial denaturation at 94 °C for 15–30 s followed by 30–45 cycles of denaturation at 94 °C for between 0 s and combined annealing at between 50 or 55 °C for 20–30 s and extension at 72 °C for 30 s. The temperature transition rate was set at 20 °C s⁻¹. Acquisitions of fluorescence signals were carried out at the end of every annealing step for 100 ms from up to 24 samples. Temperature curves

for the release of hybridized fluorogenic probes were obtained immediately after PCR, by increasing the temperature linearly from 45 to 94 °C at a rate of 0.2 °C s⁻¹ over a 2 min period with signal acquisition mode set to continuous. A PC and software accompanying the LightCycler provided an interface for the operation and for analysis of the results. Cycle-to-cycle fluorescence emission readings were plotted on the computer screen for continuous monitoring of PCR products. At the end of each run, experimental data were saved to text files that could be analysed with the software provided or imported into a spreadsheet program for normalization or presentation. The effect of sample-to-sample variation was minimized by normalization of raw fluorescence data. This was achieved by subtracting the

minimum value from all data points, dividing the result by the maximum value and multiplying by 100. Replicate samples with different DNA concentrations prepared by different DNA extraction methods were used and variation in the T_m from run to run was observed in some cases. Therefore, the T_m values are reported as being within a range rather than an absolute value. The generation of PCR amplicons was verified by removing the reaction mixtures from the capillary tubes and electrophoresis on a 1.5% (w/v) agarose in Tris/acetate/EDTA buffer. A λ phage DNA *Hind*III digest (Promega) was used as a size marker.

RESULTS AND DISCUSSION

Design of PCR primers and adjacent hybridization probes

A stretch of nucleotides from positions 1026 to 1076 was identified as a suitable target region for the development of fluorescent hybridization probes. Within this stretch, the nucleotides from position 1046 to 1076 were conserved across most members of domain *Bacteria* and the upstream region, from position 1026 to 1044, was variable and hence suitable for the development of FITC probes for genus and/or species differentiation (Fig. 1). A universal Cy5-labelled probe CY5 1046+ (5'-Cy5-AGGTGTC-ATGGTIGTCGTCAGCTCGTGT-PO₄-3'), capable of binding to the conserved region, and two FITC-labelled probes, Fervi (5'-CCGCAAGGTAGGGA-GCCAGCA-FITC-3') and Calo (5'-GCCCTTGGG-GGAACGGTGAGA-FITC-3'), respectively specific for binding to the variable nucleotide regions for members of the genera *Fervidobacterium* and *Caloramator*, were developed. Results from BLAST analysis

against the GenBank database, release 125, indicated that the probe CY5 1046+ was specific to most members of domain *Bacteria* and that probes Fervi and Calo were respectively specific to members of *Fervidobacterium* and *Caloramator*.

A forward primer, F1 (5'-CTCCTACGGGAGG-CAGCAG-3', positions 339–357), and a reverse primer, R5 (5'-GGGGTTGCGCTCGTTG-3', positions 1115–1100), which straddled the fluorophore probe hybridization site between positions 1026 and 1076, were chosen as PCR amplification primers. Primers F1 and R5 bind to conserved regions of the 16S rDNA of members of domain *Bacteria* and produce an amplicon of approximately 776 bp. The theoretical T_m values of 0.5 μ M of primers F1 and R5 when reconstituted in 50 mM monovalent cations were respectively calculated to be 64 and 56 °C. The theoretical T_m values of probes Fervi and Calo under the same conditions were respectively calculated to be 60 and 67 °C. Therefore, the T_m values of the fluorogenic probes were higher than those of the amplification primers, enabling the probes to hybridize to the target regions of the amplicons during the annealing/extension segment of each PCR cycle, resulting in an increase in Cy5 fluorescence emission (F2) with a concomitant decrease in fluorescein emission (F1) and measured as a ratio of F2:F1.

Real-time detection assay with pure cultures

An increase in fluorescence was observed with DNA preparations (CTAB-purified DNA or crude DNA prepared by the simple lyse-boil method) for *Calora-*

Fig. 1. (a) Nucleotide stretch from positions 1026 to 1076 within the 16S rRNA gene suitable for the development of adjacent fluorescent hybridization probes for the detection and identification of thermoanaerobic members of the genera *Fervidobacterium* and *Caloramator* using real-time PCR. The primer pair F1 (positions 339–357, →) and R5 (positions 1115–1100, ←) produces amplicons of approximately 770 nt. The amplicon straddles the target (positions 1046–1076) for the universal, degenerate, Cy5-labelled oligonucleotide probe CY5 1046+ and an upstream genus/species target (positions 1026–1044) for FITC-labelled oligonucleotide probes, the two primers being separated from each other by a single nucleotide, C at position 1045. During hybridization, the 3'-FITC probe serves as a donor in fluorescence resonance energy transfer (FRET) to the adjacent 5'-labelled Cy5 acceptor probe, resulting in the emission of fluorescence, which is measured in a LightCycler. The labelling of the 3' ends with FITC for the FITC probes and the blocking of the 3' end by phosphorylation for the Cy5 probe stops extension during PCR. Dashes indicate alignment gaps introduced for other members of domain *Bacteria* during probe design. (b) A schematic representation of (a) showing primer-binding sites (arrows), probe-binding sites (bars) and the single nucleotide separating the two probes (indicated by the up arrow). Positions in (a) and (b) are numbered in accordance with the scheme proposed for the *E. coli* 16S rRNA (Winker & Woese, 1991).

Fig. 2. (a) Increases in fluorescence during specific hybridization of the probes Calo and CY5 1046+ to the target sites in the 16S rDNA of *Caloramator* strain MTCC SG75-2 during PCR, as measured in a LightCycler. Purified DNA was prepared using CTAB (□) and crude DNA was prepared by the lyse-boil method (○). *E. coli* DNA (△) and *F. nodosum* DNA (×) prepared by the lyse-boil method were used as negative controls. (b) Melting curves, generated from the dissociation of the fluorescent probes from *C. indicus* ACM 3982^T and pure cultures of *Caloramator* strains MTCC 75-2, MTCC SG80 and MTCC SG64, show a T_m of 67.8 °C.

mator strain MTCC SG75-2 (Fig. 2a) and *Caloramator indicus* ACM 3982^T, but not with the negative controls *E. coli* K12 and *Fervidobacterium nodosum* ATCC 35602^T, in the presence of probes Calo and CY5 1046+ (Fig. 2a). Similar results were obtained with DNA preparations of *F. nodosum* ATCC 35602^T, *Fervidobacterium gondwanense* ACM 5017^T and *Fervidobacterium islandicum* DSM 5733^T, but not with the negative controls *E. coli* K12, *Dictyoglomus thermo-*

philum ATCC 35947^T and *Thermoanaerobacter thermohydrosulfuricus* DSM 567^T, in the presence of probes Fervi and CY5 1046+. The initiation of increase in fluorescence was DNA concentration dependent and could be detected as early as cycle 10 with higher concentrations of DNA (e.g. 6 ng) and at cycle 29 with low DNA concentrations (e.g. 6 fg) (Spanevello, 2001). A 776 bp PCR product was always amplified from all DNA preparations, including those from negative-control cultures, and observed on agarose gels. This was expected and is a result of amplification from the binding of universal bacteria-specific primers to all template DNA. However, an increase in fluorescence was not observed from the negative controls, since the PCR product possessed the binding site for the universal probe CY5 1046+ but lacked the hybridization site for specific FITC probes, and this is in line with our expectations.

Immediately following the completion of real-time PCR, the PCR products were melted by a continuous gradual and uniform increase in temperature from 50 to 95 °C, resulting in the disassociation of the fluorescent probe, from which the probe T_m was calculated. The T_m for *Caloramator* strain MTCC SG75-2 and *C. indicus* ACM 3982^T was determined to be 66 ± 2 °C (Fig. 2b). Similar results can be expected for the other members of the genus *Caloramator*, which currently includes *Caloramator fervidus* ATCC 43204^T, previously known as *Clostridium fervidus* (Patel *et al.*, 1987), *Caloramator proteoclasticus* DSM 10124^T (Tarlera *et al.*, 1997) and *Caloramator coolhaasii* DSM 12679^T (Plugge *et al.*, 2000), as their target sequences are identical to that of *C. indicus* ACM 3982^T (Fig. 1). Probe-to-target sequence mismatch can assist in distinguishing between species, as mismatches can produce a significant difference in the T_m of melting curves. It was therefore expected that the T_m values for all three *Fervidobacterium* species would be different, as the Fervi probe and *F. islandicum* DSM 5733^T target sequence were identical but there was one nucleotide mismatch with *F. nodosum* ATCC 35602^T and two nucleotide mismatches with *F. gondwanense* ACM 5017^T. The T_m for *F. islandicum* DSM 5733^T was determined to be 61.7 ± 0.5 °C and was substantially higher than the values of 55.8 ± 1.7 °C for *F. nudism* ATCC 35602^T and *F. gondwanense* ACM 5017^T. This inconsistency between the experimental data and expected result may be due to errors in the sequences of *Fervidobacterium* species, but could also be due to the unpredicted binding properties of fluorescent hybridization probes in real-time PCR assay.

Detection and identification of *Caloramator* and *Fervidobacterium* strains from the MTCC

The real-time PCR assay was used successfully to identify *Caloramator* and *Fervidobacterium* species from 14 randomly chosen cultures isolated previously from the Great Artesian Basin of Australia and stored with the Griffith University MTCC (Table 1). Of these, two strains (MTCC SG64 and MTCC SG76) were

identified as *Caloramator*, four (MTCC AB18, MTCC AB19, MTCC AB20 and MTCC SG58) as members of the *F. nodosum*–*F. gondwanense* group and a further strain (MTCC SG80) was found to be a mixed culture composed of members of *Caloramator* and the *F. nodosum*–*F. gondwanense* group. Re-examination of the mixed culture MTCC SG80 under a phase-contrast microscope indicated that only some cells had the typical terminal protuberances, termed spheroids, characteristic of *Fervidobacterium*, whereas others were non-spheroid-bearing, morphologically larger, atypical rods. The fine distinction between the two morphotypes could have been missed easily during culture purification procedures of the isolate. We believe that the method reported here could be useful for routine or random purity checks of strains in culture collections, as pure cultures have sometimes been reported to be mixed (Erbezniak *et al.*, 1997). The remaining seven strains were not identifiable as *Caloramator* and *Fervidobacterium* and were deemed to represent novel species.

TYEG enrichment cultures and their identification

In our hands, TYEG medium was found to support the growth of a range of known and unidentified thermophilic anaerobe taxa, including members of *Caloramator* and *Fervidobacterium*, that had previously been cultured using a variety of different media (Wynter *et al.*, 1996; Huber *et al.*, 1990; Patel *et al.*, 1985b, 1991). Consequently, TYEG medium was used for initiating enrichments from eight samples collected from the Great Artesian Basin of Australia. Seven of the eight enrichments were positive for growth and grew rapidly (< 48 h) at 60 °C. Six of the positive enrichments consisted of rods and/or filaments, whereas the seventh enrichment initiated from grey mats consisted of rods with terminal spheroids, a morphological trait of *Fervidobacterium* species (Patel *et al.*, 1985b, 1991) (Table 1). The failure of one of the enrichments to develop is perhaps a reflection of the inability of cells from the low-temperature habitat (52 °C) to grow at a higher temperature (60 °C) and/or the inadequacy of TYEG to support growth.

The real-time PCR assay was used to determine the identity of the seven enrichment cultures. The enrichment culture initiated from the sediment of bore RN 132 was identified as a *Caloramator* species and the enrichment culture initiated from the grey mat of bore RN 17263 was identified as an *F. islandicum*-like strain (Table 1). Interestingly, the pure culture MTCC SG75, identified as a strain of *Caloramator*, had been isolated from the sediment of bore RN 132 some 3 years ago and was again detected in the enrichment culture in our present studies. This indicates the usefulness of the technique for studies on pure and enrichment cultures. The remaining five enrichment cultures not positively identified as *Caloramator* or *Fervidobacterium* were deemed to represent novel strains.

Advantages of the method

16S rDNA is widely used in microbial systematics and taxonomy and over 20000 sequences are available in databases (Maidak *et al.*, 2001; Benson *et al.*, 2000). We have taken advantage of the availability of these databases and have identified a moderately conserved region and an adjacent upstream variable nucleotide region in the 16S rDNA for use in a simple and rapid real-time PCR assay. The probe CY5 1046+, which is more expensive to synthesize, was designed by introducing dI at positions 1051 and 1061 to increase its degeneracy, so that it could act as a universal 16S rDNA probe and bind to all members of domain *Bacteria*, while the FITC probes, which are less expensive to synthesize, would target the adjacent upstream variable region to give the required genus- and/or species-distinguishing specificity. Consequently, by simply replacing the FITC probes that target approximately position 1026 and ensuring that the theoretical T_m of the new probes is 3–10 °C higher than the amplification primers, it will be possible to detect and identify members of domain *Bacteria*. We have recently synthesized FITC-labelled specific probes for the pathogens *Campylobacter* and *Arco-bacter* species and used these successfully for their identification in conjunction with probe CY5 1046+ (M. Abu-Hallaweh and B. K. C. Patel, unpublished). Therefore, we believe that this simple approach will not only save costs for probe synthesis but will also save considerable time, as it will be unnecessary to apply the time-consuming and complex rules of probe design every time a FITC probe is developed.

We have developed two specific probes for identifying members of the genera *Caloramator* and *Fervidobacterium* and tested them experimentally on pure cultures and enrichment cultures using the real-time PCR assay. The results suggest that a vast majority of the cultures from the Griffith University MTCC collection and from the enrichments are not members of *Caloramator* or *Fervidobacterium*, but may be other thermoanaerobes such as *Thermoanaerobacter* or novel taxa. It will therefore be necessary to develop additional complementing probes for known thermophilic anaerobic taxa. Once this is achieved, it will be possible to identify novel microbes rapidly from culture collections and/or enrichment cultures. The real-time PCR has the further advantage that it lends itself to quantification. We are currently developing the technique further for quantifying *Fervidobacterium* and *Caloramator* directly in environmental sample DNA. This will provide direct evidence of their presence, numbers and diversity in different habitats.

A bottleneck for molecular methods is the time-consuming step of preparing pure DNA, which is generally regarded as necessary for PCR. However, in the real-time PCR assay reported here, crude DNA was also used, prepared by a simple lyse-boil and centrifuge step, with no adverse outcomes. A similar

crude DNA extraction procedure for real-time PCR detection of *Leptospira* species has been reported by us previously (Woo *et al.*, 1997).

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