

## Hydrogen utilising bacteria from the forestomach of eastern grey (*Macropus giganteus*) and red (*Macropus rufus*) kangaroos

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**Abstract.** Reductive acetogenesis is an alternative to methanogenesis for removing hydrogen produced during enteric fermentation. In Australia, kangaroos have evolved an enlarged forestomach analogous to the rumen of sheep and cattle. However, unlike sheep and cattle, kangaroos produce very little methane from enteric fermentation. From samples of gut contents from five eastern grey and three red kangaroos, we were not able to detect methanogens using a PCR protocol, but did detect the formyltetrahydrofolate synthetase (FTHFS) gene (likely to be used for reductive acetogenesis) in all animals. Isolations to recover acetogens resulted in two different classes of hydrogen consuming bacteria being isolated. The first class consisted of acetogens that possessed the FTHFS gene, which except for *Clostridium glycolicum*, were not closely related to any previously cultured bacteria. The second class were not acetogens but consisted of enterobacteria (*Escherichia coli* and *Shigella*) that did not possess FTHFS genes but did utilise hydrogen and produce acetate. Enumeration of the acetogens containing the FTHFS gene by real-time PCR indicated that bacteria of the taxa designated YE257 were common to all the kangaroos whereas YE266/YE273 were only detected in eastern grey kangaroos. When present, both species occurred at densities above  $\times 10^6$  cell equivalents per mL. *C. glycolicum* was not detected in the kangaroos and, unlike YE257 and YE266/273, is unlikely to play a major role in reductive acetogenesis in the foregut of kangaroos.

### Introduction

Methane is a major greenhouse gas contributing heavily to global warming. From enteric methane generated during ruminal fermentation, cattle and sheep contribute 50.5% of Australia's total methane emissions (59.3 million tonnes of carbon dioxide equivalent annually) (NGGIC 2007) and 10.3% of the nation's total greenhouse gas emissions.

The anaerobic fermentation of plant material, as occurs in the rumen of sheep and cattle and the forestomach and/or hindgut of other herbivores, results in the generation of excess hydrogen. It is necessary to remove this hydrogen or it will inhibit further fermentation through the inability to re-oxidise essential enzyme cofactors, such as NADH (Wolin *et al.* 1997). The generation of methane is one mechanism for removing the excess hydrogen. Methane is generated in the rumen by methanogenic archaea that utilise hydrogen to reduce carbon dioxide, and is a significant electron sink in the rumen ecosystem. However, there are other mechanisms for removing excess hydrogen that have been suggested as alternatives to methanogenesis, including sulfate, nitrate, fumarate reduction and reductive acetogenesis (Mackie and Bryant 1994; Nollet *et al.* 1997; Joblin 1999; Fonty *et al.* 2007). Reductive acetogenesis appears the more likely of these alternatives to be able to displace methanogenesis as it does not depend on the availability of substrates such as sulfate or fumarate. Reductive acetogenesis combines hydrogen with carbon dioxide to form acetate, which is then utilised by the host animal as a source of energy. Reductive acetogenesis is undertaken by a broad range of genetically diverse bacteria (Mackie and Bryant 1994; Joblin 1999) and replaces methanogenesis as the dominant mechanism for hydrogen

removal, with considerable energetic advantages, in many anaerobic ecosystems (Breznak and Switzer 1986; Fievez *et al.* 2001). For example, wood-eating termites produce sufficient acetate from reductive acetogenesis to account for 33% of their total energy requirement (Breznak and Switzer 1986). Also, ostriches have evolved an enlarged hindgut for the fermentation of plant material, and have a similar feed passage rate to other herbivores and rates of plant cell wall degradation comparable with ruminants. Reductive acetogenesis often out-competes methanogenesis in the ostrich hindgut and represents 25% of total acetate production, accounting for 7% of an adult birds maintenance energy requirements (Fievez *et al.* 2001).

Kangaroos and wallabies, like ruminants, have a pre-gastric fermentation of plant material in an enlarged forestomach (Hume 1982). In ruminants methane represents around 10% of digestible energy intake (Dellow *et al.* 1988), but most often methane from the macropod marsupials is considerably lower. Kempton *et al.* (1976) reported a complete lack of methane emissions from eastern grey kangaroos (*Macropus giganteus*) foregut but a small amount of methane was formed in the hindgut. von Engelhardt *et al.* (1978) reported methane emission from the tammar wallaby (*Macropus eugenii*) but this was still low (1–2% of digestible energy intake) compared with ruminants. A further study by Dellow *et al.* (1988), where four species of macropods were studied, supports the latter but may also point to a difference in methane production between macropod species, particularly between the larger grazing kangaroos and smaller browsing wallaby species. As a percentage of total gas emitted, two wallaby species, the red-

necked wallaby (*Macropus rufogriseus*) and swamp wallaby (*Wallabia bicolor*) emitted gases containing between 5.4 and 9.8% methane, whereas the eastern grey kangaroo (*M. giganteus*) and eastern wallaroo (*Macropus robustus robustus*) only emitted between 0.5 and 1.8% methane. Interestingly, the swamp wallaby, whilst emitting methane also emitted considerable hydrogen (9.8–11%), which the other species did not.

The objectives of the current study were to (i) determine the presence or absence of reductive acetogens and methanogens in forestomach contents of two species of large grazing kangaroos, that is, five eastern grey and three red kangaroos; (ii) isolate and characterise reductive acetogens and other hydrogen utilising bacteria; and (iii) enumerate populations of the isolated reductive acetogens in kangaroo forestomach contents using real-time PCR.

## Materials and methods

### Sample collection, bacterial isolation and phenotype characterisation

Samples were collected under the Queensland Parks and Wildlife Scientific Purposes Permit number W0/001397/00/SAA. Samples of foregut contents (approximately 15–25 g) were collected from kangaroos that had been grazing predominantly curly Mitchell grass (*Astrelba lappacea*) pastures on Croxdale Research Station (DPI&F, Western Queensland). Samples were taken from the tubiform region of the forestomach (Hume 1982) and stored frozen with cryoprotectant as previously reported (Ouwerkerk *et al.* 2005). Initial work was performed on samples from eight kangaroos but samples from four more kangaroos were included towards the end of the work.

Anaerobic technique, isolation and culture followed standard anaerobic procedures (Hungate 1969; Holdeman *et al.* 1977). Isolation of hydrogenotrophic bacteria was based on methods and RF30 medium reported by Joblin *et al.* (1990). Samples (0.5 mL) of foregut contents were thawed, mixed with 4.5 mL of dilution solution (Ogimoto and Imai 1981), serially diluted at  $\times 10^{-2}$  increments to  $\times 10^{-8}$  and the head-space of each tube was pressurised to 206.8 kPa with hydrogen. Cultures were incubated with shaking at 39°C for up to 5 days and the head-space pressure measured to determine removal of gases. The highest dilution tubes showing marked reductions in gas pressure were selected, 0.5 mL aliquots removed, again serially diluted, and the dilutions were cultured using the roll-tube method of Hungate (1969), as previously described (Klieve *et al.* 1998). The head-space of each tube was pressurised to 206.8 kPa with hydrogen and cultures were incubated with shaking at 39°C for up to 5 days. Individual colonies were selected, placed into dilution solution and the above process repeated until it was deemed by visual inspection that a single colony type had been isolated. Isolates were maintained by passaging in RF30 broth culture with the head-space pressurised with hydrogen. Storage was by the method of Teather (1982).

Phenotypic characteristics recorded were colony and cellular morphology, sporulation, motility and Gram reaction, as determined by staining (Holdeman *et al.* 1977). Microscopy was with an Olympus BH microscope (Olympus, Tokyo, Japan) at  $\times 400$  and  $\times 1000$ .

Volatile fatty acid (VFA) production following growth of isolates in broth was measured as previously described by Ouwerkerk and Klieve (2001).

### DNA extraction, PCR amplification and restriction enzyme digestion

Methods for DNA extraction, PCR amplification of 16S rRNA, *stx* genes, restriction enzyme digestion with *MspI*, *CfoI* and *HaeIII* (Roche, Mannheim, Germany) and analysis by manual assignment to groups based on all three digestion patterns have been reported previously (Karch and Meyer 1989; Ouwerkerk and Klieve 2001; Ouwerkerk *et al.* 2002; Burow *et al.* 2005).

Detection of methanogens in samples of foregut contents was via a PCR assay directed at archaea. Archaeal PCR primers were ARCH46F, 5'-YTA AGC CAT GCR AGT-3', and ARCH1100R, 5'-YGG GTC TCG CGC GTT RCC-3' (Barns *et al.* 1994; Ovreås *et al.* 1997), and were from conserved sequences unique to the archaeal 16S rRNA gene. Detection of reductive acetogens was via a PCR assay directed at conserved sequences in the formyltetrahydrofolate synthetase (FTHFS) gene. FTHFS is a functional gene in the reductive acetogenesis pathway. The primers used were FTHFS-F, 5'-TTY ACW GGH GAY TTC CAT GC-3' and FTHFS-R, 5'-GTA TTG DGT YTT RGC CAT ACA-3' (Leaphart and Lovell 2001).

### Enumeration of kangaroo reductive acetogen populations by real-time Taq nuclease assay

The development and application of real-time Taq nuclease assays (TNA) based on 16S rRNA gene sequence data, for enumeration of *Clostridium glycolicum* YE255, kangaroo reductive acetogen YE257 and the kangaroo reductive acetogen group YE266/YE273 were based on those developed previously for *Megasphaera elsdenii*, *Butyrivibrio fibrisolvens*, *Streptococcus bovis* and *Ruminococcus bromii* (Ouwerkerk *et al.* 2002; Klieve *et al.* 2003, 2007). The development followed the same steps in terms of DNA extraction, PCR amplification, sequencing and sequence analysis of 16S rRNA genes, PCR calibration and verification of specificity. Primers and probes were selected from 16S rRNA gene sequence data using Primer Express Version 2 software (Applied Biosystems, Foster City, CA, USA) to fit the specifications for a minor groove binder (MGB) probe moiety and the RealMasterMix probe mix (Eppendorf, Hamburg, Germany). Identified primers and probe were checked for specificity using the programs Probe Match version 2.1 (Maidak *et al.* 2000) against the Ribosomal Database Project, and the BLAST program (Altschul *et al.* 1990) at the National Centre for Biotechnology Information (NCBI) site against the GenBank database. The 5'→3' sequences of the selected primers and probe are presented in Table 1. The probes were labelled with 6-carboxyfluorescein phosphoramidite (6'FAM) as the 5' reporter dye and included a non-fluorescent quencher attached to the 3' MGB moiety.

Real-time TNA were run on a Rotor-Gene RG-3000 (Corbett Research, Mortlake, NSW, Australia) under the following conditions – 1 cycle at 94°C for 1 min; then 45 cycles at 94°C for 10 s, 64°C for 30 s; and a final cycle of 30°C for 1 min.

Quantitative standards containing known numbers of each of the three reductive acetogens were prepared by directly

**Table 1. Primers and probes used to enumerate reductive acetogens by real-time *Taq* nuclease assay**

| Species of reductive acetogen       | Primer or probe        | 5'→3' base sequence                        |
|-------------------------------------|------------------------|--|
| <i>Clostridium glycolicum</i> YE255 | Forward primer YE255F1 | AAC CTG CCT CAT<br>ACA CAT GGA TAA         |
|                                     | Reverse primer YE255R2 | AGC TTT GAT AAG AAA<br>TAC ATG TGA ATC TCT |
|                                     | Probe YE255P2          | ACC GAA AGG TAT<br>GCT AAT A               |
| Isolate YE257                       | Forward primer YE257F2 | CCG GTC TGT AAT GAG<br>ACC TTT TCT T       |
|                                     | Reverse primer YE257R2 | CAC GAG CTG ACG<br>ACA ACC AT              |
|                                     | Probe YE257P2          | CAG AGG AGA CAG GTG GT                     |
| Isolates YE266 and YE273            | Forward primer YE266F2 | TCC CTC TGA CAG ACT<br>CTT AAT CGA         |
|                                     | Reverse primer YE266R2 | CGA CAC GAG CTG<br>ACG ACA AC              |
|                                     | Probe YE257P2          | CAG AGG AGA CAG<br>GTG GT                  |

enumerating pure cultures of the respective bacterium. Cells were directly enumerated through triplicate counts using a Petroff-Hauser Bacteria Counter (Arthur H. Thomas Co., Philadelphia, PA, USA), as per the manufacturer's instructions, at a magnification of 400× with an Olympus BH-2 microscope. Cells were diluted in TE buffer (10 mM Tris, 1 mM EDTA) or pelleted rumen fluid resuspended in TE buffer, to  $10^{10}$  cells mL<sup>-1</sup> and a log dilution series prepared down to  $10^2$  cells mL<sup>-1</sup>. Genomic DNA was isolated from 1 mL of these dilutions via bead beating (Yu and Forster 2005) and the DNA resuspended in 500 µL sterile water.

The specificity of the *Taq* nuclease assay was evaluated using genomic DNA isolated from the following bacterial isolates: *Bacteroides fragilis* 683, *Butyrivibrio fibrisolvens* strains AR12, AR27, AR73, ATCC 19171 and YE44, *Clostridium butyricum* strains YE12 and YE15, *C. glycolicum* YE255, *Escherichia coli* K13 (ATCC 15766), *E. coli* YE261, *Shigella flexneri* YE254, *Eubacterium ruminantium* AR2, *Eubacterium cellulosolvens* YE257, *Eubacterium rectale* YE131, *Fusobacterium necrophorum* AR4, *Lactobacillus* sp. YE07, YE08, and YE16, *Prevotella ruminicola* subsp. *brevis* AR20, *P. ruminicola* AR29, *Prevotella* sp. YE139, *Ruminococcus flavefaciens* AR45, *Ruminococcus productus* YE168, *Ruminococcus albus* AR67, *Selenomonas ruminantium* AR55, *Streptococcus bovis* AR25, SB15, YE01, and 2B, *Streptococcus intermedius* AR36, and *Megasphaera elsdenii* YE34. In addition, genomic DNA isolated from both bovine and ovine rumen contents was used to evaluate assay specificity.

#### Clone library preparation

In an attempt to identify the predominant hydrogen utilising bacteria in mixed cultures, well grown cultures that were actively utilising hydrogen from two different grey kangaroos were centrifuged, DNA extracted from the harvested cells, the

16S rRNA genes PCR amplified and clone libraries constructed using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Plasmid DNA containing a copy of the original product was extracted from clones using the QIAprep spin miniprep kit (Qiagen, Doncaster, Vic., Australia), the 16S rRNA gene insert re-amplified by PCR and digested with restriction enzymes (as above) to identify redundant clones. DNA sequence was obtained and analysed for representatives of each unique cloned gene.

#### DNA sequencing and sequence analysis

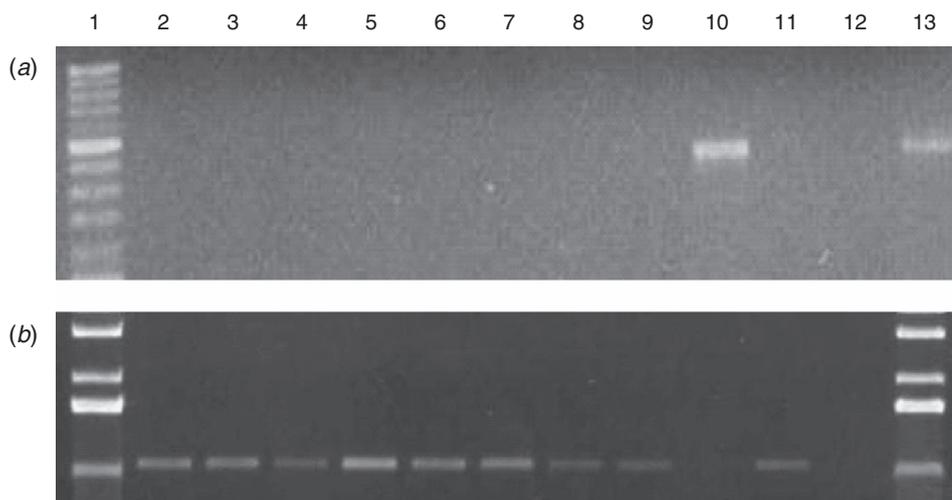
DNA sequencing and sequence analysis followed the same protocols as previously described (Klieve *et al.* 2007). Sequencing of the PCR amplified 16S rRNA gene was performed using the ABI prism dye terminator cycle sequencing ready reaction kit version 3.1 with amplitaq DNA polymerase FS and a model 373A DNA sequencing system (PE Applied Biosystems, Foster City, CA, USA) at the Griffith University Sequencing Facility (Brisbane, Qld, Australia). All procedures were performed according to the manufacturer's protocols. The primers used for sequencing the 16S rRNA gene were 27F, 530F, 1525R and 907R (Stackebrandt and Charfreitag 1990; Lane 1991). Sequence fragments were assembled using Sequencher Ver 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). Assembled 16S rRNA gene sequences were compared with GenBank, EMBL, and DDBJ non-redundant nucleotide databases using the gapped BLAST database search program (Altschul *et al.* 1990) at the National Centre for Biotechnology Information (NCBI). Similar 16S rRNA gene sequences were included in the phylogenetic analysis and the sequences aligned using ClustalW (Ludwig *et al.* 2004). The alignments were optimised manually and phylogenetic analyses carried out using PAUP\*, Version 4.0b8 (Swofford 1998). Trees were generated using maximum parsimony (P), maximum likelihood and distance matrix (D) analyses. Before constructing the trees a series of likelihood (L) ratio tests were performed using Modeltest Version 3.7 (Posada and Crandall 1998) to determine the most suitable nucleotide substitution model to use for the L and D analyses. The model selected was a general time reversible model (GTR) with estimates of invariant sites (I) and among site heterogeneity (G) (summarised as GTR+I+G) for the 1290 bases of 16S rRNA gene sequence used to produce the trees. Bootstrap resampling (P, 1000 replicates; D, 1000 replicates; L, 100 replicates) was used to assess the branch support of all inferred trees.

Nucleotide sequences have been deposited in the GenBank database under the accession numbers FJ966225 to FJ966228 inclusive.

## Results

#### Presence of archaeal 16S rRNA genes and FTHFS genes in kangaroo forestomach contents

Results of PCR assays to detect either archaeal 16S rRNA genes or FTHFS genes in eight eastern grey and red kangaroos (and one sheep as a positive control for the presence of methanogens) are presented in Fig. 1.



**Fig. 1.** PCR of DNA from kangaroo foregut contents to detect (a) archaea and (b) the FTHFS gene. Lanes (a) 1 and (b) 1 and 13, DNA size markers; (a, b) lanes 2–9, kangaroo forestomach content DNA from grey 1, red 2, grey 3, red 4, red 5, grey 6, grey 7 and grey 8, respectively; (a) lane 10, positive control (DNA from *Sulfolobus acidocaldarius*); lanes (a) 13 and (b) 11, sheep rumen; and lanes (a) 11 and (b) 12, negative control (no DNA).

Samples of total DNA from the foregut contents of eight kangaroos were positive for bacteria (data not shown), negative for archaea and positive for the FTHFS gene. Archaea were detected in the sheep sample but were not detected in the kangaroo samples.

#### *Isolation of hydrogen utilising bacteria, phenotypic, biochemical and genetic characteristics*

A total of 28 hydrogen consuming bacteria were isolated from the forestomach of eastern grey and red kangaroos. Key characteristics are presented in Table 2.

These isolates may represent up to eight species of bacteria. A large number of bacteria isolated were closely related to *E. coli* or *Shigella* spp. These bacteria reduced the gas pressure in Hungate tubes by between 30 and 50%, produced small amounts of acetic acid (3–4 mM) but did not possess an FTHFS gene. Given the relationship of these bacteria to known pathogenic bacteria and particularly those that have Shiga toxin genes (*stx* genes), two of the bacteria (YE254 and YE261) were investigated, by PCR, for the presence of these genes. Both were negative.

Three or four of the species isolated appear to be reductive acetogens, removing hydrogen and carbon dioxide, producing

**Table 2.** Phenotypic characteristics and DNA sequence similarity of the 16S rRNA gene to that of their nearest relatives (BLAST analysis) of hydrogen utilising bacterial isolates from kangaroo forestomach contents

Ac, acetic acid; FTHFS, formyltetrahydrofolate synthetase; I-Bu, iso-butyric acid; I-Val, iso-valeric acid; Pr, propionic acid

| No. of isolates (representative isolate) and host <sup>A</sup> | Morphology  | Reduction in gas pressure (initially 220.6–248.2 kPa) (%) | FTHFS gene PCR | Fermentation acids (mM)          | Closest match using BLAST                   | DNA identity (%) (no. of base pairs) |
|--|---|---|----------------|----------------------------------|---|--------------------------------------|
| 1 (YE254), grey  | Short motile rods                                   | 30  | –              | Ac (3)                           | <i>Shigella boydii</i>                      | 99 (1475)                            |
| 1 (YE255), grey  | Long pleomorphic motile rods with subterminal spore | 100 <sup>B</sup>  | +              | Ac (36), I-Bu (0.6), I-Val (1.2) | <i>Clostridium glycolicum</i>               | 99 (1460)                            |
| 4 (YE257), red   | Long non-motile rods in chains                      | 80–100  | +              | Ac (15)                          | Isolate YE59 (Ouwerkerk <i>et al.</i> 2005) | 92 (1406)                            |
| 5 (YE261), grey  | Cocco-bacillus, non-motile                          | 40–50   | –              | Ac (4), Pr (0.2)                 | <i>Escherichia coli</i> O157:H7             | 99 (1505)                            |
| 2 (YE266), grey  | Long thin non-motile rods                           | 100   | +              | Ac (24)                          | Isolate YE59                                | 91 (1486)                            |
| 5 (YE268), grey  | Short motile rods                                   | 30  | –              | Ac (3.5)                         | As for YE254 <sup>C</sup>                   | –                                    |
| 5 (YE273), grey  | Long non-motile rods                                | 100   | +              | Ac (20.5)                        | Isolate YE59                                | 91 (1483)                            |
| 5 (YE278), grey  | Short motile rods                                   | 30  | –              | Ac (3)                           | As for YE254 <sup>C</sup>                   | –                                    |

<sup>A</sup>Groups of isolates from grey kangaroos were from four different animals (YE254 and 255 from one animal, YE266 and 268 from another, YE261 from another and YE273 and 278 from a fourth).

<sup>B</sup>100% indicates that at the end of bacterial growth the gas phase of the culture tube was under a vacuum, i.e. below atmospheric pressure.

<sup>C</sup>These isolates had restriction fragment length polymorphism digestion patterns identical to YE254 when digested with three different restriction enzymes and were deemed to be of the same species (Ouwerkerk and Klieve 2001).

acetate and containing the FTHFS gene. Of these acetogens only one was genetically closely related to a previously cultivated species, in this case *C. glycolicum* (99% similarity in 16S rRNA gene sequence over 1460 bp). The other three species (represented by YE257, YE266 and YE273) were only distantly related to a named cultured bacterium, *Ruminococcus gnavus* with 91% similarity across 1482 bp of the 16S rRNA gene. These isolates were also related to another unknown species isolated from a grey kangaroo, YE59, which itself has no close culturable relatives (Ouwerkerk *et al.* 2005). From 16S rRNA gene sequence analysis, YE257 had 96% similarity over 1481 bp to YE266 and YE273 but was clearly distinct from them. Also, YE266 and YE273 shared 99% similarity over 1486 bp and are probably the same species. The three different tree building algorithms used on the 16S rRNA gene sequence data for the isolates and other culturable reductive acetogens were largely congruent and Fig. 2 contains the maximum likelihood (L) tree with branch support above 80% marked on the nodes for the three analyses. All species of kangaroo reductive acetogens consumed hydrogen and carbon dioxide vigorously, reducing the pressure in Hungate tubes from 220.6–248.2 kPa to a vacuum after 16 h incubation. The three novel species (YE257, YE266 and YE273) appear to be homoacetogenic, as the only volatile fatty acid produced during culture was acetic acid (averaging ~20 mM culture fluid). The concentration of acetic acid produced by the reductive acetogens was 5–8-fold higher than produced by the Enterobacterial isolates. *C. glycolicum* YE255 was quite different again, producing more acetic acid than the other species (36 mM) and also quantities of the branched chain fatty acids iso-butyric and iso-valeric acids.

Isolation of hydrogen utilising bacteria in pure culture was not always successful. These mixed cultures, with only two to three dominant species remaining, were characterised using clone libraries to elucidate the predominant hydrogen utilising species present. The species identified through this process are presented in Table 3. Although the number of clones examined was limited (17), over 50% were enterobacteria (*E. coli* and *S. flexneri*) and 30% represented the sulfur reducing bacterium *Desulfovibrio desulfuricans*. Attempts to isolate the latter bacterium were not successful.

#### Enumeration of acetogens in kangaroo forestomach contents

No non-specific amplification was observed when assaying the panel of rumen bacterial isolates. Genetically, YE266 and YE273 were very closely related and could not be differentiated from each other with separate real-time PCR assays, thus the same assay enumerates both isolates.

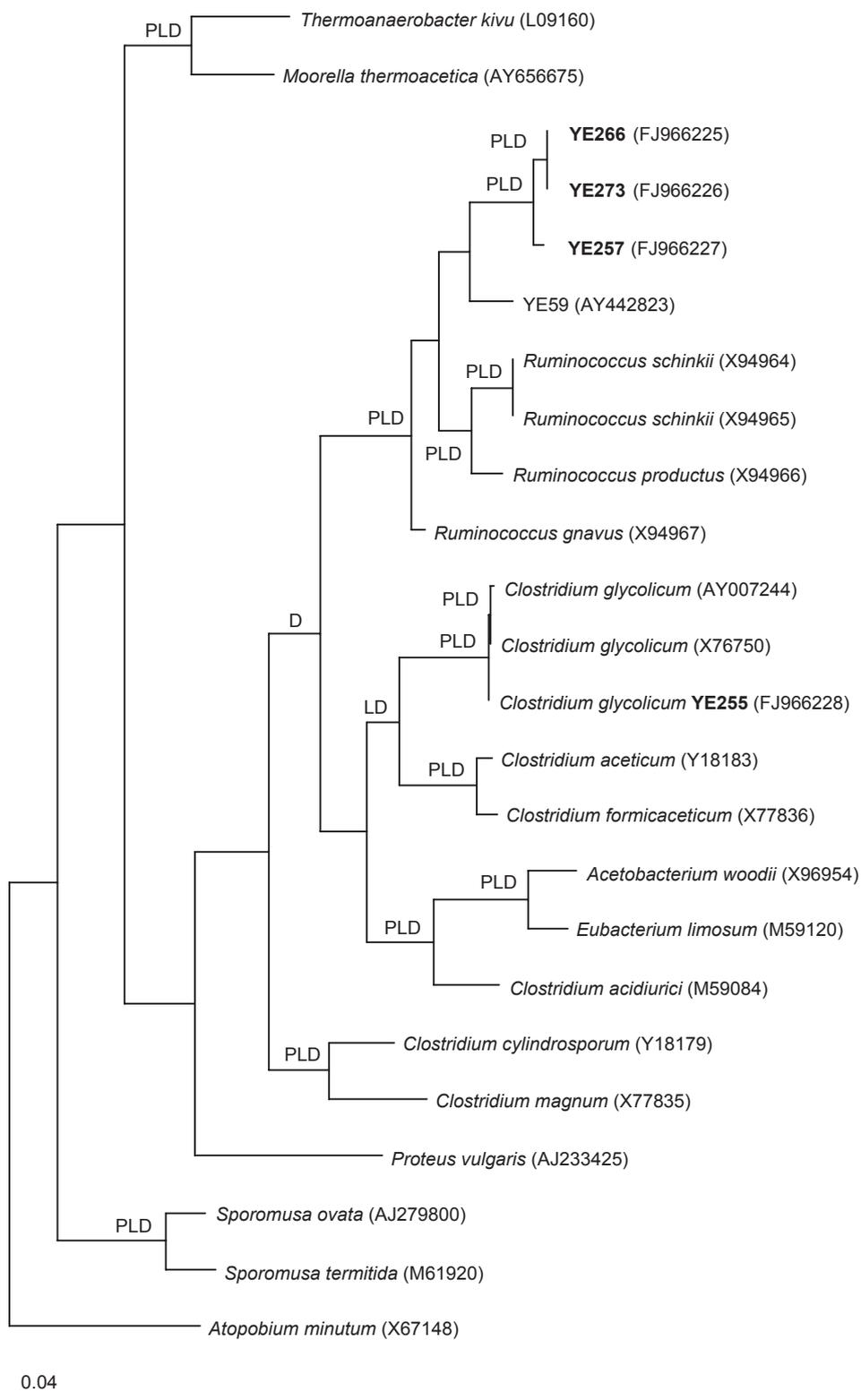
The assays were used to enumerate populations of each bacterium in twelve kangaroo foregut samples (Fig. 3). Amongst the kangaroos, strains YE266 and YE273 were common to the grey kangaroos but below detectable levels in all of the red kangaroos, while YE257 was common to nearly all the kangaroos. Grey kangaroo GB 10 was negative for all the acetogens and *C. glycolicum* YE255 was below detection limits in all of the kangaroos. YE257 and YE266/273, when present, were present in the kangaroos at appreciable population

densities, generally between  $\times 10^7$  and  $\times 10^8$  cells per gram of contents.

#### Discussion

The primer set used in the initial PCR studies was designed to detect archaea *per se* and at the time this work was done covered all of the known methanogens from ruminants. In the eight kangaroos investigated using this assay, methanogen populations were below detectable limits although the DNA was amplifiable (positive results in both a general 16S rRNA gene and an FTHFS gene PCR). This finding is in keeping with earlier work, with eastern grey kangaroos, that demonstrated that they produced little if any methane from foregut fermentation (Kempton *et al.* 1976; Dellow *et al.* 1988). This is not to suggest that there are no methanogens in kangaroos, as methanogens have been isolated (Evans *et al.* 2008) and enumerated from foregut contents of tammar wallabies (Evans *et al.* 2009). In tammar wallabies, methanogens were present at densities of  $\times 10^5$ – $10^6$  cells per gram of contents, which is very low compared with ruminants, where methanogens account for between 0.5 and 3% of total microbes (or  $\sim \times 10^8$ – $10^9$  cells per gram of contents) (Mackie *et al.* 2002). If the difference in emissions reported by Dellow *et al.* (1988) were due to lower population densities in larger kangaroos then populations could be below the PCR detection threshold in eastern grey and red kangaroos. In contrast to the paucity of methanogens, at least those detectable with the primer set used, the fact that DNA from foregut contents of the kangaroos was positive for the presence of FTHFS genes suggests that reductive acetogenesis may play a role in hydrogen removal in kangaroos, as it does in the gut of termites and ostriches (Breznak and Switzer 1986; Fievez *et al.* 2001).

Isolation of reductive acetogens resulted in two distinct groups of species, reductive acetogens and enterobacteria. Recently, similar enterobacteria have been isolated from rumen contents, when acetogens had been targeted for isolation, and been shown to be fumarate reducing bacteria (Fonty *et al.* 2007). Although this remains to be established for the enterobacteria from macropods it would appear distinctly possible that these bacteria perform a similar role. Another aspect of hydrogen utilisation by enterobacteria in gastrointestinal environments is in the context of reducing nitrite concentrations in the rumen. Sar *et al.* (2005) reported that addition of *E. coli* W3110 to rumen contents *in vitro* reduced methanogenesis. They concluded that this was due to the ability of this strain to reduce nitrite accumulation and nitrite toxicity when nitrate was used as a means of reducing methanogenesis. However, *E. coli* and *Shigella* may have a more direct involvement in removing hydrogen through the reduction of fumarate and production of acetate and succinate. Although the isolates obtained in this study were closely related to pathogenic enterobacteria and specifically Shiga toxin producing strains, *stx* genes were not detected in a limited sample of the isolates from kangaroos. A recent study has indicated that *stx* negative strains of *E. coli* O157:H7 are commonly isolated from the gut of cattle, wild bird excreta and various environmental sources (Wetzel and LeJeune 2007).



**Fig. 2.** A maximum likelihood (L) tree constructed from 1290 bases of 16S rRNA gene sequence indicating the positions of the bacterial isolates that contained FTHFS genes to related species and other known acetogenic bacteria. Bootstrap values for parsimony (P, 1000 replicates), distance (D, 1000 replicates) and likelihood (L, 100 replicates) analyses are indicated with a corresponding letter if they exceeded 80% support. *Atopobium minutum* was used as the out-group. The bar represents a sequence divergence of 4%. GenBank database accession numbers for the microorganisms are indicated.

**Table 3. DNA sequence similarity of the 16S rRNA gene to that of their nearest relatives (BLAST analysis) of hydrogen utilising bacteria in two mixed cultures from kangaroo forestomach contents**  
RFLP, restriction fragment length polymorphism

| 16S rRNA gene RFLP group <sup>A,B</sup> | No. of identical clones | Closest match using BLAST          | DNA similarity (%) (no. of base pairs) |
|---|-------------------------|------------------------------------|--|
| <i>Mixed culture 1</i>                  |                         |                                    |  |
| A                                       | 4                       | <i>Escherichia coli</i>            | 99 (1426)                              |
| B                                       | 4                       | <i>Desulfovibrio desulfuricans</i> | 99 (1386)                              |
| C                                       | 1                       | <i>Enterococcus avium</i>          | 99 (1455)                              |
| <i>Mixed culture 2</i>                  |                         |                                    |  |
| B                                       | 2                       | <i>Desulfovibrio desulfuricans</i> | 99 (1401)                              |
| D                                       | 1                       | <i>Bacteroides distasonis</i>      | 97 (1244)                              |
| E                                       | 5                       | <i>Shigella flexneri</i>           | 99 (1444)                              |

<sup>A</sup>Five different RFLP profiles for clones from the two mixed cultures were observed (data not shown).

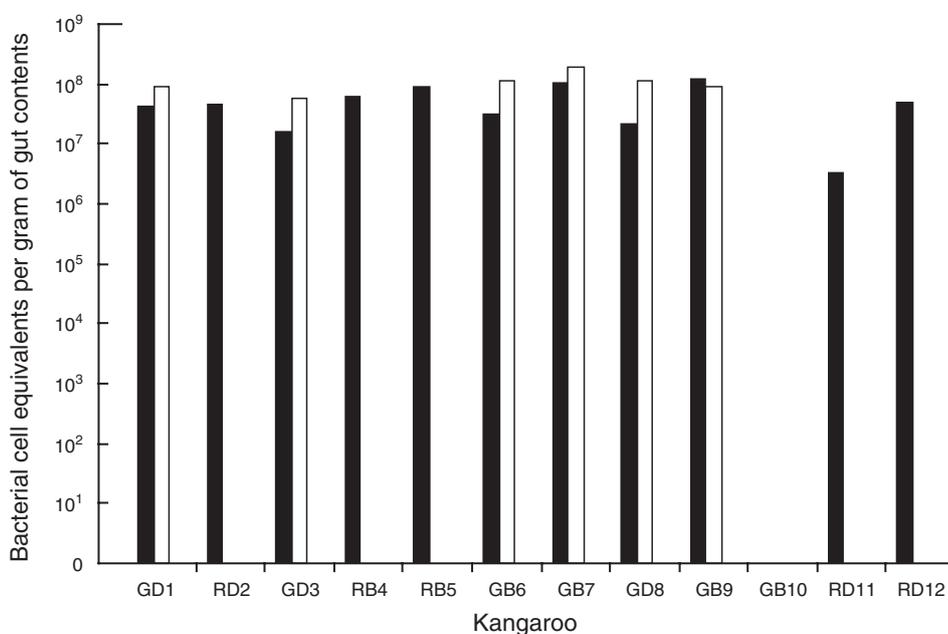
<sup>B</sup>As defined by Ouwerkerk and Klieve (2001).

Of the reductive acetogens that have been isolated, only one, *C. glycolicum* YE255 was closely related to other culturable or known reductive acetogens that have been assigned to a taxon. The other two to three species (represented by YE257, YE266/YE273) were distantly related (91% DNA similarity) to a validly described species, *Ruminococcus gnavus*. All of these isolates readily utilise hydrogen and carbon dioxide to the extent that they are able to reduce gas pressure within sealed containers from a strong positive pressure (248 kPa) to below atmospheric pressure, thus creating a vacuum, following incubation for as little as 16 h. All of these bacteria also produced acetate. Unlike the novel reductive acetogens (YE257 and YE266/YE273), *C. glycolicum* YE255 produced more acetate and also

branched chain fatty acids during growth. The branched chain fatty acids and additional acetate are indicative of amino acid fermentation by this species and thus *C. glycolicum* YE255 would be a non-homoacetogen and not have an obligate requirement for hydrogen to be able to grow and survive. The novel strains on the other hand only produced acetate, suggesting that they are homoacetogens. This, however, will require further clarification.

The current collection of reductive acetogens is limited and there are likely to be many more taxa present than we can currently obtain in pure culture. However, characterisation of two mixed cultures that readily utilised hydrogen also identified the presence of a third class of hydrogen utilising bacteria, *Desulfovibrio desulfuricans*, a well known sulfur reducing bacterium. This species should be readily culturable (Holdeman *et al.* 1977), but despite specifically targeting the isolation of this species from the mixed cultures, we were unable to do so. Another observation from these cultures was once again the prevalence of the enterobacterial strains.

Real-time PCR assays were used to enumerate populations of the reductive acetogens that were isolated from kangaroo forestomach contents to determine how widespread their occurrence was and whether their populations were of a density that could be regarded as significant. *C. glycolicum* YE255 was undetectable in any of the kangaroos despite the fact that it had been isolated from them, indicating that this species is unlikely to play an important role in hydrogen consumption *in vivo*. This species is likely to be present at very low population densities but prefers culture conditions more than the other acetogens, therefore creating a positive culture bias *in vitro*. The other reductive acetogens, when present in the forestomach were always present in reasonably dense populations ( $10^6$ – $10^8$  per mL), suggesting that they may



**Fig. 3.** Population densities of cultured kangaroo reductive acetogens in 12 kangaroos. G, grey; R, red. Reductive acetogens YE257 (■); YE266 and/or YE273 (□).

play a role in the overall hydrogen economy within the kangaroo foregut. However, there does appear to be a host-animal influence on which of these species is able to colonise the forestomach. YE266/273 was only detected in eastern grey kangaroos whereas YE257 was detected in all of the kangaroos suggesting that the latter is probably less fastidious and is not influenced by host-animal specificity. In the eastern grey kangaroo, both YE266/273 and YE257 were present in about equal numbers and at similar density to YE257 in red kangaroos, which may indicate that direct competition between these species of reductive acetogens is not a factor in the exclusion of YE266/273 from red kangaroos.

In the future we shall be constructing clone libraries of FTHFS genes from kangaroo forestomach contents to enable a more comprehensive view of the diversity of FTHFS containing bacteria that are likely to be reductive acetogens in these animals to be obtained. Whether the enterobacterial species are required as companion species for other hydrogen utilisers, as well as being responsible for a more limited utilisation themselves, is also worthy of further study.

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