

ORIGINAL ARTICLE

Investigation of the microbial metabolism of carbon dioxide and hydrogen in the kangaroo foregut by stable isotope probing

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Kangaroos ferment forage material in an enlarged forestomach analogous to the rumen, but in contrast to ruminants, they produce little or no methane. The objective of this study was to identify the dominant organisms and pathways involved in hydrogenotrophy in the kangaroo forestomach, with the broader aim of understanding how these processes are able to predominate over methanogenesis. Stable isotope analysis of fermentation end products and RNA stable isotope probing (RNA-SIP) were used to investigate the organisms and biochemical pathways involved in the metabolism of hydrogen and carbon dioxide in the kangaroo forestomach. Our results clearly demonstrate that the activity of bacterial reductive acetogens is a key factor in the reduced methane output of kangaroos. In *in vitro* fermentations, the microbial community of the kangaroo foregut produced very little methane, but produced a significantly greater proportion of acetate derived from carbon dioxide than the microbial community of the bovine rumen. A bacterial operational taxonomic unit closely related to the known reductive acetogen *Blautia coccooides* was found to be associated with carbon dioxide and hydrogen metabolism in the kangaroo foregut. Other bacterial taxa including members of the genera *Prevotella*, *Oscillibacter* and *Streptococcus* that have not previously been reported as containing hydrogenotrophic organisms were also significantly associated with metabolism of hydrogen and carbon dioxide in the kangaroo forestomach.

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Introduction

The production of methane by domestic ruminants is well recognised as an important component of anthropogenic greenhouse gas emissions, contributing ~17–25% of the global methane budget (Lassey, 2007; Conrad, 2009). Methane is generated by ruminants as a by-product of the enteric fermentation of forage material in the anaerobic conditions of the foregut. The hydrogen-dependent reduction of CO₂ to methane by hydrogenotrophic methanogenic

archaea is essential for normal function of the rumen microbial ecosystem, as accumulation of excess hydrogen in the system inhibits fermentation by preventing regeneration of NAD⁺. This leads in turn to reduced microbial production and forage digestion (Wolin *et al.*, 1997).

Although hydrogenotrophy is predominately carried out by methanogens in the rumen, alternative pathways apparently exist in other comparable gut ecosystems. In Australia, kangaroos and wallabies graze on the same native pastures as cattle, and digest forage via microbial fermentation in an enlarged foregut analogous to the rumen, yet methane production by kangaroos is minimal or non-existent (Kempton *et al.*, 1976; Englehardt *et al.*, 1978; Dellow *et al.*, 1988). Ouwerkerk *et al.* (2009) were unable to detect the presence of methanogenic archaea in the foreguts of kangaroos they

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surveyed, while Klieve *et al.* (2012) have recently demonstrated that the presence of methanogens in the kangaroo foregut is variable, with archaea apparently absent from many individuals.

The reasons why methanogenesis predominates in ruminants while alternative pathways for hydrogenotrophy apparently predominate in kangaroos are not well understood. It has been suggested (Attwood and McSweeney, 2008) that the hydrogen and CO₂ produced by fermentation in the kangaroo foregut is utilised predominately by acetogenic bacteria, which reduce carbon dioxide to acetate via the acetyl-CoA pathway (Drake *et al.*, 2008). Ouwerkerk *et al.* (2009) isolated several novel bacterial strains belonging to the family Lachnospiraceae from the foregut of kangaroos, which utilise hydrogen and produce acetate in *in vitro* cultures. Quantitative PCR assays confirmed that these bacteria are present in high numbers in the kangaroo foregut. The presence of a distinct acetogen population has also been observed in the forestomach of another macropod species (*Macropus eugenii*), which differs from the native acetogen population of the bovine rumen (Gagen *et al.*, 2010).

If acetogenesis does predominate in the kangaroo foregut, the acetate produced could be absorbed by the host animal, thus supplying a significant proportion of the host's energy needs (Henning and Hird, 1970; Sheppach, 1994). Reductive acetogenesis is therefore a pathway with the potential to reduce or completely eliminate methane production by the gut ecosystem, while simultaneously offering a significant energetic advantage to the host animal. If the nature of the interactions that allow acetogenesis to predominate in some enteric fermentation systems could be understood, it may be possible to promote reductive acetogenesis in the rumen ecosystem, thereby reducing livestock methane emissions while simultaneously improving the efficiency of feed utilisation.

The objective of this study was to investigate CO₂ and hydrogen metabolism in the kangaroo foregut so that the mechanisms that allow kangaroos to ferment plant material without producing large quantities of methane could be better understood. This was done by using stable isotope tracing to track the biological fate of CO₂ in the kangaroo foregut and the bovine rumen and by using RNA stable isotope probing (RNA-SIP) techniques to identify bacteria in the kangaroo foregut that are capable of hydrogen-dependent CO₂ metabolism.

Materials and methods

Collection of gut samples

See Supplementary Materials 1 and 2 for full details of sample collection procedures.

Samples of the liquid portion of the forestomach contents of three wild male Eastern Grey kangaroos (*M. giganteus*) were collected in the field near

Milmerran, Queensland. Samples of rumen fluid from four *Bos taurus* cattle that had been fed a diet consisting primarily of whole barley grain for 110 days were collected immediately after slaughter. Samples of rumen fluid of grass-fed cattle were collected from three rumen-fistulated *B. taurus* × *B. indicus* cattle housed at the Centre for Advanced Animal Studies at Gatton, Queensland. In all cases, liquid digesta was mixed with sterile anaerobic glycerol to give a final glycerol concentration of 30–40% and frozen on dry ice. During collection of all samples, a portion of liquid digesta was also collected into separate bottles that did not contain glycerol. These bottles were transported on ice and frozen at the earliest opportunity. This digesta was used for preparation of media for the incubation experiment.

Media

Anaerobic media containing 33% (*v/v*) of the appropriate digesta type (kangaroo, grain-fed rumen or grass-fed rumen) were prepared in the manner described previously (Klieve *et al.*, 1989). The media was supplemented with buffered mineral salts, bicarbonate and trace elements at concentrations designed to approximately simulate the natural conditions of the bovine rumen (Erdman, 1988) and kangaroo forestomach (Beal, 1984; Dellow *et al.*, 1988) (see Supplementary Material 3 for full details of media composition). All media were prepared under an anaerobic gas mix consisting of 95% CO₂ and 5% H₂. Aliquots (100 ml) of the media were dispensed into 500-ml bottles, with the headspace filled with 95% CO₂/5% H₂, which were sealed and autoclaved.

Minimal carbon anaerobic salts medium (MCAS, Supplementary Material 3) was designed in-house to simulate the major ion concentrations found in the kangaroo forestomach (Beal, 1984; Dellow *et al.*, 1988). Bicarbonate medium for isotope labelling incubations was identical to MCAS, except that it contained 100 mM of stable carbon isotope labelled (¹³C) NaHCO₃ with the NaCl concentration adjusted to give the same sodium concentration as MCAS (Supplementary Material 3).

Isotope tracing to track the biological fate of CO₂ in simulated bovine and kangaroo foregut fermentations

The gut content samples collected as described above were thawed, then 50-ml aliquots were centrifuged to pellet the microbial biomass and solid digesta particles. The supernatant containing glycerol was discarded, then the pellets were washed twice by centrifuging and resuspending in MCAS medium. The pellet was resuspended in 50 ml MCAS medium and added to a 500-ml Wheaton bottle containing 100 ml of the appropriate digesta-based medium (kangaroo, grain-fed rumen or grass-fed rumen). All washing and inoculation steps were performed in an atmosphere of 95% CO₂/5% H₂ in an anaerobic chamber (Coy, Grass

Lake, MI, USA). After inoculation, each 500-ml bottle was supplemented with 1.0 ml of 538 mM solution of ^{13}C -labelled NaHCO_3 (99 atom % ^{13}C , Sigma-Aldrich, St Louis, MO, USA), which had been flushed with nitrogen to remove dissolved oxygen, to produce a final concentration of 105 mM bicarbonate that consisted of 5% ^{13}C -labelled bicarbonate (equivalent to $\delta^{13}\text{C}_{\text{VPDB}}$ 3,683‰). Control incubations were identical but were supplemented with 1.0 ml of 538 mM unlabelled (^{12}C) NaHCO_3 solution. After addition of NaHCO_3 , the bottles were sealed and incubated at 39 °C.

Five-millilitre samples of the fluid portion and gas headspace were taken immediately after inoculation (time 0 samples), and at 3 h, 6 h, 24 h, 48 h and 7 days post inoculation. Samples were taken by piercing the butyl rubber stopper of the culture bottle with a 21-gauge needle with a syringe attached. Gas samples were transferred to evacuated 4.5-ml glass vials (Labco, Lampeter, UK). Fluid samples to be used for analysis of volatile fatty acids (VFAs) were transferred to sterile polypropylene vials to which 20 μl of saturated HgCl_2 solution was added and mixed to stop microbial activity. These samples were stored frozen at -20 °C prior to further processing. On the day of extraction, the frozen samples were thawed, and the internal standard 3-methylvaleric acid (3 mV) was added to give a final concentration of 4.0 mM. VFAs were extracted and purified from the thawed fluid samples by vacuum distillation (Vreman *et al.*, 1978).

Gas chromatography-isotope ratio mass spectrometry

Gas chromatography combustion isotope ratio mass spectrometry (GC-c-IRMS) was used to determine the concentrations and carbon stable isotope ratios ($\delta^{13}\text{C}_{\text{VPDB}}$) of acetate, propionate, butyrate and valerate in the fluid portion of the gut fermentations. For the gas headspace samples, both the concentration and carbon stable isotope ratio of methane was determined. For carbon dioxide, only the carbon stable isotope ratio was determined as the very high initial concentration of 95% carbon dioxide prevented accurate measurement of changes in concentration of carbon dioxide in the headspace. Details of the GC-c-IRMS procedures used are provided in Supplementary Material 4.

Variability between sample categories in both concentration and carbon isotope ratios of methane and VFAs were tested for significance using repeated measures analysis of variance (RM-ANOVA). All data analysis was conducted in GenStat version 14.2.0.6286 (VSN International, Hemphstead, UK).

Incubation of gut samples for RNA stable isotope labelling

An RNA-SIP experiment was conducted separately from the isotope-tracing experiment. This

experiment was performed under enrichment conditions designed to encourage growth of hydrogenotrophic organisms. A sample from a single female Eastern Grey kangaroo (*Macropus giganteus*) was incubated at 39 °C under an equimolar mixture of 206 kPa H_2/CO_2 for 3 days to enrich the sample for micro-organisms that were capable of utilising H_2/CO_2 for growth. One hundred millilitres of fluid was then centrifuged to pellet the biomass within the sample. This pellet was washed twice in MCAS. The final washed pellet was resuspended in 30 ml MCAS.

Aliquots (1 ml) of the washed and resuspended pre-incubated foregut content were used to inoculate 20 ml Hungate tubes containing 10 ml ^{13}C -bicarbonate (99 atom % ^{13}C) medium under an atmosphere of nitrogen gas. The tubes were then pressurised with $^{13}\text{CO}_2$ gas (99 atom % ^{13}C , Sigma-Aldrich) to 103 kPa above atmospheric pressure. H_2 gas was then added to a final pressure of 206 kPa to give a total of 0.2 mmol each of $^{13}\text{CO}_2$ and H_2 in the headspace. Unlabelled control incubations were identical except that the medium contained unlabelled (^{12}C) bicarbonate, and the tubes were pressurised with $^{12}\text{CO}_2$ and H_2 . Additional controls were also set up which contained ^{13}C -labelled bicarbonate medium and $^{13}\text{CO}_2$, but no hydrogen. These controls were used to identify organisms that were able to assimilate the ^{13}C -label without hydrogen present.

The tubes were incubated at 39 °C, with replicate samples removed at 3 h, 16 h and 7 days post inoculation. Cells were recovered from the medium by centrifugation, then immediately flash-frozen in liquid nitrogen.

RNA extraction

Frozen cell pellets were resuspended and thawed in 10 mM Tris, 1 mM EDTA buffer (pH 8.0) containing 15 mg ml⁻¹ lysozyme and incubated for 10 min at room temperature. Total nucleic acid was then extracted and precipitated according to Yu and Forster's (2005) protocol. The total nucleic acid pellets were resuspended in nuclease-free water and processed with the RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions to remove contaminating DNA and residual protein. RNA was eluted in nuclease-free water and quantified using the RiboGreen RNA quantification kit (Life Technologies, Carlsbad, CA, USA).

Isopycnic centrifugation and fractionation

^{13}C -labelled RNA was resolved from unlabelled RNA by isopycnic centrifugation in caesium trifluoroacetate (CsTFA) using a modified version of the protocol of Whiteley *et al.* (2007) (see Supplementary Material 5) for detailed protocol). A total of 600 ng of RNA was loaded per gradient, then each gradient was fractionated after

ultracentrifugation. Each ultracentrifuge run included a control gradient containing an equimolar mixture of ^{13}C and ^{12}C RNA from *Escherichia coli* (Supplementary Material 6). The RNA was recovered from each gradient fraction by precipitation in isopropanol, using glycogen as a carrier and resuspended in 30- μl nuclease-free water.

Quantification of CsTFA gradient fractions and reverse transcription

The quantity of RNA in each gradient fraction was measured using Ribogreen according to the manufacturer's recommendations. For each gradient, the three fractions closest to the 'heavy' and 'light' RNA peaks were identified by comparison to the *E. coli* control gradient from the same ultracentrifuge run and selected for further processing. For each fraction, 200 pg of RNA was reverse-transcribed using random hexamers and Thermoscript reverse transcriptase (Life Technologies) according to the manufacturer's instructions. Preliminary comparisons of the bacterial communities represented in the 'heavy' and 'light' fractions of CsTFA gradients were performed using denaturing gradient gel electrophoresis (Supplementary Material 7). The results of these denaturing gradient gel electrophoresis comparisons were used to determine the incubation time that resulted in differential incorporation of isotope label by the primary utilisers of $\text{CO}_2/\text{HCO}_3^-$, rather than 'downstream' incorporation of labelled metabolites by all the bacteria present in the sample (Supplementary Material 7).

Amplicon pyrosequencing

The cDNA produced by reverse transcription of the three 'heavy' and three 'light' CsTFA fractions obtained from samples incubated for 16 h was pooled to produce one 'heavy' and one 'light' cDNA solution for each gradient. This cDNA was then used as template for the production of 16S rRNA gene amplicons for pyrosequencing (see Supplementary Material 8 for detailed PCR protocol). Sequence data were processed, clustered into operational taxonomic units at the 97% identity level and classified as described previously (Eigeland *et al.*, 2012; Gulino *et al.*, 2013). All data processing was performed in QIIME 1.5.0 (Caporaso *et al.*, 2010).

Identification of ^{13}C -labelled operational taxonomic units

Operational taxonomic units (OTUs) representing organisms that incorporated the ^{13}C -label from bicarbonate/ CO_2 in the presence of hydrogen were identified using a χ^2 analysis (Sokal and Rohlf, 1969) to identify the OTUs whose distribution between 'heavy' and 'light' gradient fractions was significantly affected by the presence of ^{13}C -label in the media used for incubation. OTUs that were

significantly ($P < 0.001$) associated with CO_2/H_2 utilisation were identified by comparison to the Greengenes (DeSantis *et al.*, 2006) and Genbank (Benson *et al.*, 2011) databases. Full details of the statistical procedure used are given in Supplementary Material 9.

Mining kangaroo foregut and bovine rumen data sets

To obtain estimates of the prevalence of bacteria associated with H_2 and CO_2 metabolism in the kangaroo forestomach and bovine rumen the 16S rRNA sequences of OTUs that were identified as representatives of H_2 - and CO_2 -utilising organisms in the *in vitro* SIP experiment were used to query a database of bacterial 16S rRNA gene sequences that was generated in a separate study ((Gulino *et al.*, 2013) MG-RAST project IDs 3340 and 3374). This database includes bacterial 16S rRNA gene sequences obtained from the rumen of grass-fed cattle, grain-fed cattle, grey kangaroos (*M. giganteus*), red kangaroos (*M. rufus*) and wallaroos (*M. robustus*).

Results

Isotope tracing to compare the fates of carbon dioxide/bicarbonate in the bovine rumen and kangaroo forestomach

The *in vitro* fermentations performed with kangaroo forestomach and bovine rumen contents showed very clear differences between kangaroos and cattle in the pathways of CO_2 metabolism. In bovine rumen fermentations, both grain-fed and grass-fed, methane was detectable in the headspace 3 hours after inoculation, and continued to accumulate for the following 7 days of the experiment (Figures 1a and 2a). In contrast, kangaroo fermentations produced a measurable quantity of methane only after 7 days incubation, and this quantity was significantly ($P < 0.01$) lower than in rumen fermentations (Figures 1a and 2a).

Analysis of the carbon isotope ratios of CO_2 and methane in the headspace gases of the *in vitro* fermentations spiked with ^{13}C -labelled bicarbonate showed that the bicarbonate in solution rapidly established equilibrium with CO_2 in the headspace. The $\delta^{13}\text{C}$ value of CO_2 rose rapidly during the first 6 h of the experiment, then remained relatively stable (Figure 1c). The methane produced by the rumen bacterial community in the presence of labelled (^{13}C) bicarbonate was strongly labelled for both grass-fed and grain-fed fermentations, indicating that it was derived from $\text{CO}_2/\text{HCO}_3^-$ (Figure 1b). In kangaroo fermentations however, the small quantity of methane that was produced remained relatively unlabelled, indicating that it was derived from substrates other than CO_2 (Figure 1b).

Clear differences between the kangaroo forestomach and bovine rumen were also observed in fermentations using unlabelled (^{12}C) bicarbonate.

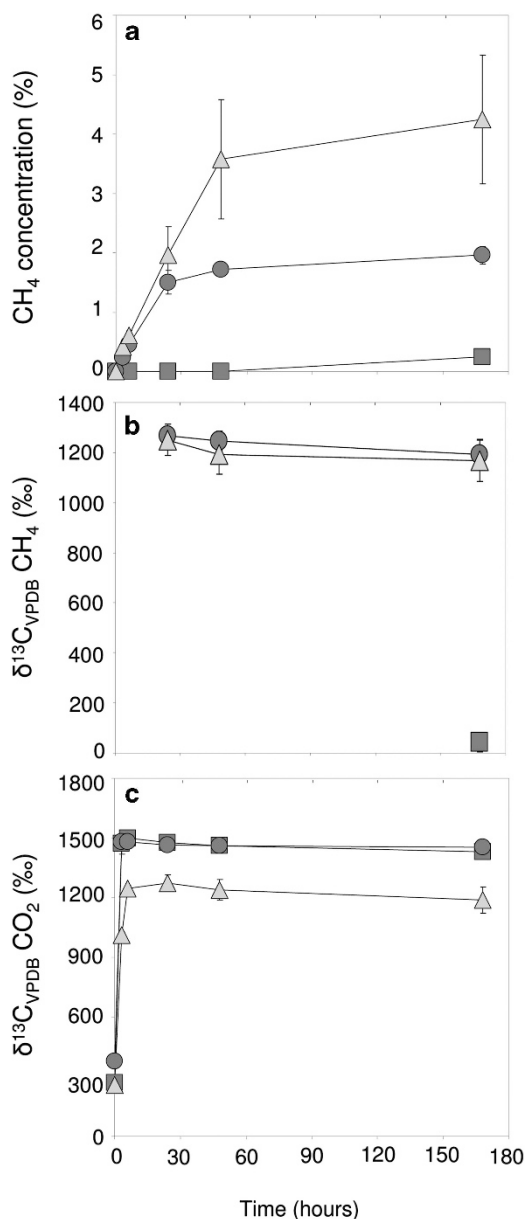


Figure 1 Mean (\pm s.e.) concentration (a) and $\delta^{13}\text{C}_{\text{VDPB}}$ (b) of methane and $\delta^{13}\text{C}_{\text{VDPB}}$ of CO_2 (c) in headspace gas of *in vitro* gut content fermentations spiked with $\text{H}^{13}\text{CO}_3^-$. Fermentations performed using kangaroo forestomach content produced significantly less methane than fermentations performed with bovine rumen fluid, and the small quantity of methane that was produced remained relatively unlabelled, indicating that it was largely not derived from CO_2 . \blacktriangle Bovine rumen fluid (grain-fed) ($n=4$). \bullet Bovine rumen fluid (grass-fed) ($n=4$). \blacksquare Kangaroo forestomach content ($n=3$). Early time points for $\delta^{13}\text{C}$ of methane are missing because carbon isotope ratios could not be determined until sufficient methane had accumulated to allow measurement.

The methane produced by bovine rumen fermentations consistently had a $\delta^{13}\text{C}$ value 40–50‰ lower than the $\delta^{13}\text{C}$ of the CO_2 in the headspace (Figure 2). The small quantity of methane produced in kangaroo fermentations however had a $\delta^{13}\text{C}$ value only 2–8‰ lower than the CO_2 in the headspace (Figure 2).

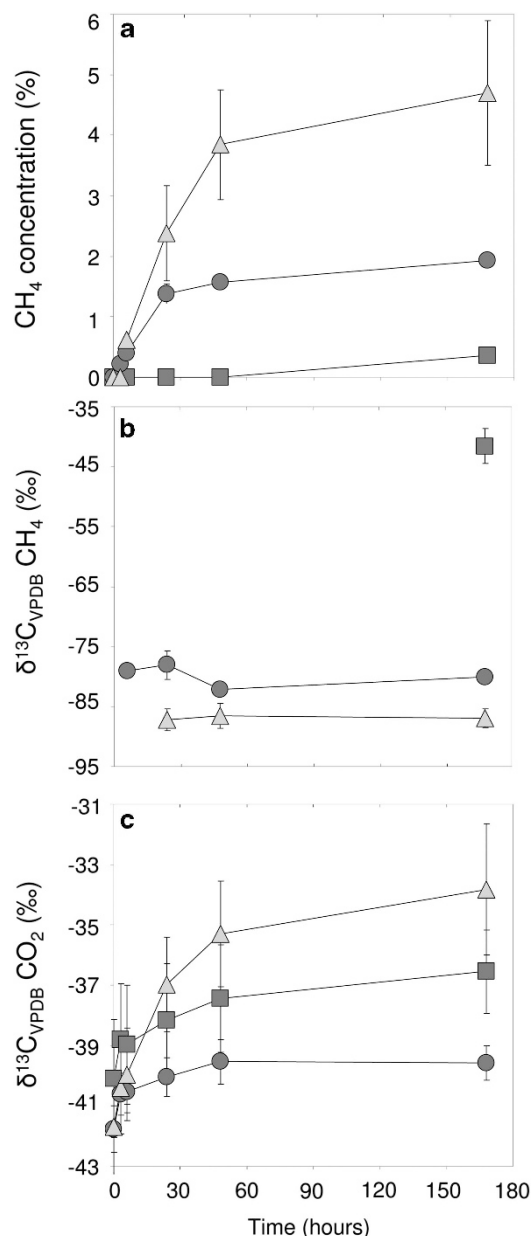


Figure 2 Mean (\pm s.e.) concentration (a) and $\delta^{13}\text{C}_{\text{VDPB}}$ (b) of methane and $\delta^{13}\text{C}_{\text{VDPB}}$ of CO_2 (c) in headspace gas of *in vitro* gut content fermentations spiked with unlabelled (^{12}C) HCO_3^- . Both grain-fed and grass-fed bovine rumen fermentations produced methane that was strongly depleted in ^{13}C relative to CO_2 , consistent with H_2/CO_2 -dependent methanogenesis. Fermentations performed with kangaroo forestomach content produced significantly less methane than bovine rumen fluid fermentations, and the small quantity of methane that was produced was not depleted in ^{13}C relative to CO_2 , indicating that it was produced by a pathway other than H_2/CO_2 -dependent methanogenesis. \blacktriangle Bovine rumen fluid (grain-fed) ($n=4$). \bullet Bovine rumen fluid (grass-fed) ($n=3$). \blacksquare Kangaroo forestomach content ($n=3$). Early time points for $\delta^{13}\text{C}$ of methane are missing because carbon isotope ratios could not be determined until sufficient methane had accumulated to allow measurement.

Analysis of the stable carbon isotope ratios of the VFAs produced in the *in vitro* fermentations clearly showed that conversion of CO_2 to acetate is much more active in the kangaroo foregut than in the

bovine rumen. Kangaroo foregut fermentations spiked with ^{13}C -labelled bicarbonate produced strongly labelled acetate, whereas bovine rumen content fermentations produced only slightly labelled acetate (Figure 3b). Repeated measures ANOVA showed that this observed difference in the extent of acetate labelling between kangaroos and cattle was significant ($P < 0.05$). Production of ^{13}C -labelled propionate occurred in all three systems (Figure 3d), and the extent of labelling did not differ significantly between sample types.

RNA-SIP to identify bacteria associated with metabolism of carbon dioxide/bicarbonate and hydrogen.

Figure 4 illustrates the distribution of OTUs significantly associated with hydrogen/ CO_2 utilisation between the 'heavy' and 'light' fractions of CsTFA gradients derived from samples incubated in the presence of either $^{13}\text{CO}_2/\text{H}^{13}\text{CO}_3^-$ or $^{12}\text{CO}_2/\text{H}^{12}\text{CO}_3^-$. Although many OTUs were detected in both the 'heavy' and 'light' gradient fractions, χ^2 analysis allowed the identification of those OTUs for which the distribution of RNA between the 'heavy' and 'light' fractions changed significantly when $^{13}\text{CO}_2/\text{H}^{13}\text{CO}_3^-$ was present.

Bacterial OTUs that were identified by RNA-SIP and χ^2 analysis as CO_2/H_2 metabolisers under the conditions used in the *in vitro* isotope labelling incubations are presented in Table 1. Sequences representing each OTU have been submitted to the Genbank database under accession numbers (KC632738–KC633091). Raw sequence reads have been submitted to the NCBI Sequence Read Archive under accession numbers (SRP033591, SRS512720, SRS512721, SRS512722, SRS512723, SRS513566 and SRS513567).

Among the OTUs identified as CO_2/H_2 metabolisers in kangaroos one is of particular interest (OTU 236, Table 1) because of its very close 16S rRNA gene sequence similarity to the known reductive acetogen *Blautia coccooides* (Kamlage *et al.*, 1997; Liu *et al.*, 2008). Comparison to our database obtained by 16S rRNA gene amplicon pyrosequencing of the forestomach contents of kangaroos and the rumens of cattle showed that sequences sharing $\geq 97\%$ 16S rRNA gene similarity to this OTU were present in all macropods sampled (22 individual animals) and represented an average of 0.30% ($\pm 0.21\%$) of total 16S reads. In the rumen of cattle however, sequences sharing $\geq 97\%$ 16S rRNA gene similarity to this OTU were only present in 40% of animals sampled (9 of 22 individual animals), where

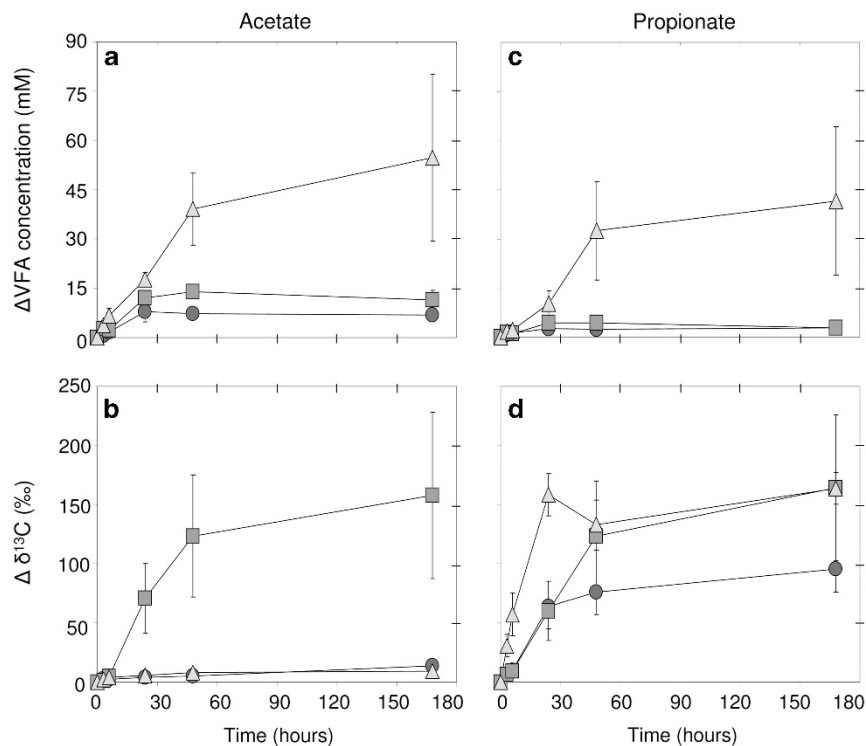


Figure 3 Mean (\pm s.e.) changes relative to time 0 in concentration and $\delta^{13}\text{C}_{\text{VDPB}}$ of acetate (a, b) and propionate (c, d) in *in vitro* gut content fermentations spiked with $\text{H}^{13}\text{CO}_3^-$. Fermentations performed with kangaroo forestomach content resulted in significantly more incorporation of ^{13}C label than fermentations performed with bovine rumen fluid, indicating that reductive acetogenesis contributes to a larger proportion of the acetate pool in kangaroos than it does in cattle. Additional data for butyrate and valerate are presented in Supplementary Figure S4. \blacktriangle Bovine rumen fluid (grain-fed) ($n = 4$). \bullet Bovine rumen fluid (grass-fed) ($n = 3$). \blacksquare Kangaroo forestomach content ($n = 3$).

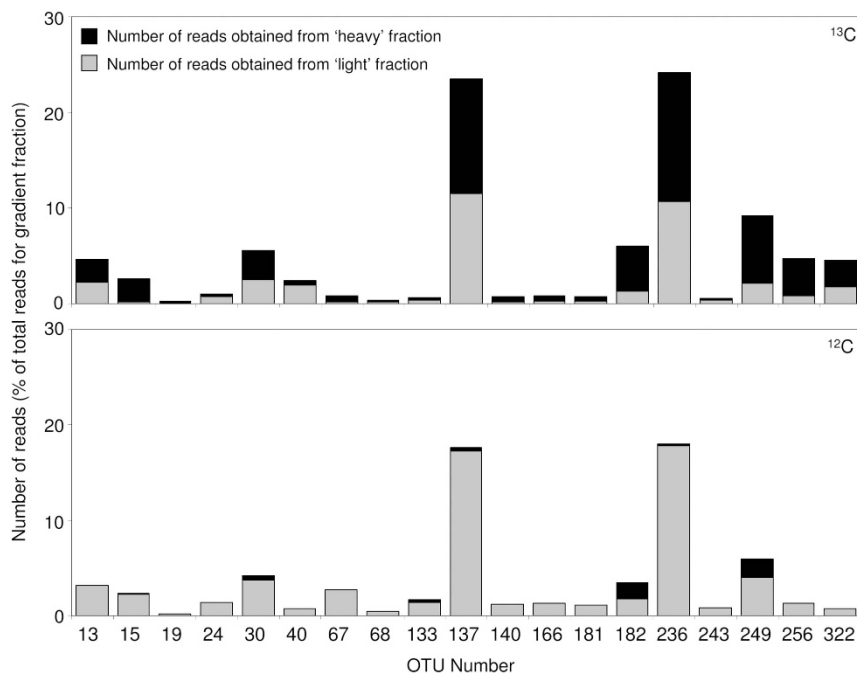


Figure 4 Number of 16S rRNA amplicon reads per OTU obtained from 'heavy' and 'light' fractions of CsTFA gradients. Only OTUs for which a statistically significant labelling effect was observed are shown. The CsTFA gradients were loaded with RNA extracted from kangaroo forestomach contents that had been incubated for 16 h in the presence of hydrogen and either labelled (^{13}C , upper figure) or unlabelled (^{12}C , lower figure) carbon dioxide and bicarbonate. Number of reads for each OTU are expressed as a percentage of the total number of reads obtained for each gradient fraction to normalise for variation in total read number between samples.

they represented an average of 0.05% ($\pm 0.11\%$) of total 16S reads.

Discussion

While the isotope tracer experiments were conducted under conditions designed to approximately simulate *in situ* conditions in the bovine rumen and kangaroo forestomach, there were technical and logistical limitations that prevented all conditions from being mimicked exactly. The collection of kangaroo forestomach material from remote sites necessitated cold storage and preservation of samples in glycerol during transportation. This glycerol was removed by centrifugation and washing of sample biomass prior to inoculation of the incubations. Previous work in our laboratory has demonstrated that a diverse range of viable bacteria and archaea can be recovered from gut samples stored frozen in glycerol solution (Ouwkerk *et al.*, 2005); however, it is likely that the viability of some organisms, especially fungi and protozoans, with potentially important ecological roles would have been adversely affected by cold storage. The temperature of incubation may have also affected the results to some extent. The typical temperature of the kangaroo forestomach is slightly lower than that of the bovine rumen (36–37°C versus 39°C). For the sake of consistency and to avoid introducing additional sources of variation between the kangaroo and bovine systems, all of the incubations for

both the chemical isotope-tracing experiment and the RNA-SIP experiment were conducted at 39°C. Despite these limitations, very clear and significant differences in formation of fermentation end products were observed between the kangaroo forestomach and bovine rumen systems.

Reductive acetogenesis is more active in the kangaroo forestomach than in the bovine rumen

The significantly reduced production of methane in kangaroo forestomach incubations supports previous studies that found that kangaroos produce little or no methane in comparison to ruminants (Kempton *et al.*, 1976; Englehardt *et al.*, 1978; Dellow *et al.*, 1988). This reduced methane output and the fact that the small quantity of methane produced in the kangaroo forestomach fermentations remained relatively unlabelled in the presence of $^{13}\text{CO}_2/\text{H}^{13}\text{CO}_3^-$ indicate that methanogenesis is not only less active in the foregut of kangaroos than in the rumen of cattle but also occurs by a fundamentally different pathway. It is clear from these observations that methane is not a major sink for CO_2 in the foregut of eastern grey kangaroos.

The results of the isotope analysis of the VFAs produced in the three fermentation systems in the presence of $^{13}\text{CO}_2/\text{H}^{13}\text{CO}_3^-$ clearly indicate that the conversion of CO_2 to acetate via the reductive acetogenesis pathway is markedly more active in the kangaroo forestomach than in the bovine rumen. When considered in combination with the results of

Table 1 Bacterial OTUs that were identified by RNA-SIP as CO₂/H₂ metabolisers in *in vitro* incubations of kangaroo forestomach content

OTU	P-value (χ^2 test)	Nearest cultured relative (<i>GreenGenes</i>) (accession number) (with source where available)	% identity to nearest cultured relative
13	<10 ⁻¹⁵	<i>Clostridium clostridioforme</i> str. 136069/2010 (HM008264.1) (human blood culture)	100
15	<10 ⁻¹⁵	<i>Streptococcus sanguinis</i> str. TTE17 (GU561372.1) (human mouth)	99
19	7.50 × 10 ⁻⁸	<i>Clostridium aldenense</i> str. RMA 9741 (DQ279736.1) (human clinical culture)	99
24	8.99 × 10 ⁻¹⁵	<i>Prevotella salivae</i> str. EPSA11; JCM 12084 (NR_024816.1) (human oral cavity)	93
30	<10 ⁻¹⁵	<i>Clostridium lactatifermentans</i> str. G17 (NR_025651.1) (chicken caeca)	93
40	1.47 × 10 ⁻⁶	<i>Streptococcus suis</i> SC84 (CP003736) (human meningitis)	97
67	<10 ⁻¹⁵	<i>Oscillibacter valericigenes</i> strain Sjm18–20 (NR_041340) (Valerate producing bacterium)	93
68	1.30 × 10 ⁻⁶	<i>Streptococcus rattii</i> str. ATCC19645 (AJ420201.1)	98
133	3.35 × 10 ⁻⁴	<i>Paracoccus</i> sp. str. CCGE3098 (seedborne endophyte)	100
137	<10 ⁻¹⁵	<i>Enterococcus durans</i> str. IMAU60172 (FJ917726.1) (fermented dairy products)	100
140	<10 ⁻¹⁵	<i>Gemmiger formicilis</i> str. ATCC 27749 × 2–56 (GU562446.1) (chicken ceaca)	95
166	<10 ⁻¹⁵	<i>Gemmiger formicilis</i> str. ATCC 27749 × 2–56 (GU562446.1) (chicken ceaca)	94
181	<10 ⁻¹⁵	<i>Streptococcus</i> sp. YE54 (AY442818.1) (Kangaroo forestomach)	97
182	<10 ⁻¹⁵	<i>Streptococcus mitis</i> str. MG3 (GU561382.1) (human oral cavity)	100
236	<10 ⁻¹⁵	<i>Blautia coccoides</i> str. JCM 1395 (AB571656.1)	100
243	8.71 × 10 ⁻⁶	<i>Barnesiella intestinihominis</i> str. JCM 15079 (AB547647.1)	88
249	<10 ⁻¹⁵	<i>Escherichia</i> sp. str. II_B13 (HM028651.1) (duck hatchery air)	100
256	<10 ⁻¹⁵	<i>Streptococcus caballi</i> str. 151 (EF364098.1) (horse hindgut)	98
322	<10 ⁻¹⁵	<i>Parasporobacterium paucivorans</i> (NR_025390.1) (freshwater sediment)	89

Abbreviation: OTU, operational taxonomic unit.

the gas headspace analysis, these results very strongly suggest that reductive acetogenesis is an important sink for carbon dioxide and hydrogen in the kangaroo foregut.

Analysis of the fractionation of carbon isotopes of methane produced in fermentations using unlabelled (¹²C) bicarbonate provided further evidence for a difference in the dominant methanogenic pathways occurring in the foregut of kangaroos. The strong isotope fractionation observed between headspace CO₂ and methane in the bovine rumen fermentations (Figure 2) was consistent with isotopic fractionation that occurs during reduction of CO₂ to methane (Krzycki *et al.*, 1987; Botz *et al.*, 1996). In contrast, relatively little difference was observed in the $\delta^{13}\text{C}$ values of methane and CO₂ in the kangaroo fermentations (Figure 2). When the carbon isotope ratios of acetate and methane in the kangaroo fermentations are compared however, the observed difference of -17 to -21‰ is more consistent with what would be expected for acetoclastic methanogenesis (Krzycki *et al.*, 1987; Conrad *et al.*, 2002; Penning *et al.*, 2006).

The incorporation of ¹³C label into propionate in all three systems (Figure 3) was unexpected. The most likely explanation for this production of labelled propionate is that ¹³C-labelled CO₂ was incorporated during the conversion of pyruvate to oxaloacetate, which is subsequently converted to propionate via malate, fumarate and succinate. The fact that the extent to which this process occurred did not differ significantly between the three systems suggests that it is a less important factor than reductive acetogenesis in explaining the reduced methane output of kangaroos relative to ruminants.

Identification of CO₂/H₂ utilising bacteria by RNA-SIP
RNA-SIP combined with 16S amplicon pyrosequencing and statistical comparison of the sequences obtained from 'heavy' and 'light' gradient fractions proved to be an effective approach for the identification of organisms involved in CO₂/H₂ metabolism in the kangaroo forestomach. This approach allowed OTUs corresponding to labelled RNA to be distinguished from the background 'smear' of RNA, which results in the generation of PCR product from all CsTFA gradient fractions. As has been the case with many other SIP studies (for example, Neufeld *et al.*, 2007; DeRito and Madsen, 2009; Bertram *et al.*, 2013), in this study it was necessary to conduct the incubations under enrichment conditions that were not entirely representative of *in situ* conditions in order to achieve sufficient amounts of isotope labelling to allow separation of labelled and unlabelled biomolecules. In order to verify the presence and abundance of the organisms identified as CO₂/H₂ utilisers in the SIP experiment in live kangaroos, the 16S rRNA sequences of OTUs identified were used to query a database of bacterial 16S rRNA gene sequences that had been generated in a previous study (Gulino *et al.*, 2013).

The identification of several putative CO₂/H₂-utilising bacteria, including the known reductive acetogen *Blautia coccoides*, and the confirmation that these bacteria occur in large numbers in at least three large kangaroo species (Figure 5) represent important steps towards a more complete understanding of the bacterial community involved in hydrogenotrophy in the kangaroo forestomach. The association of *Blautia* spp. with H₂/CO₂ utilisation in the kangaroo forestomach supports

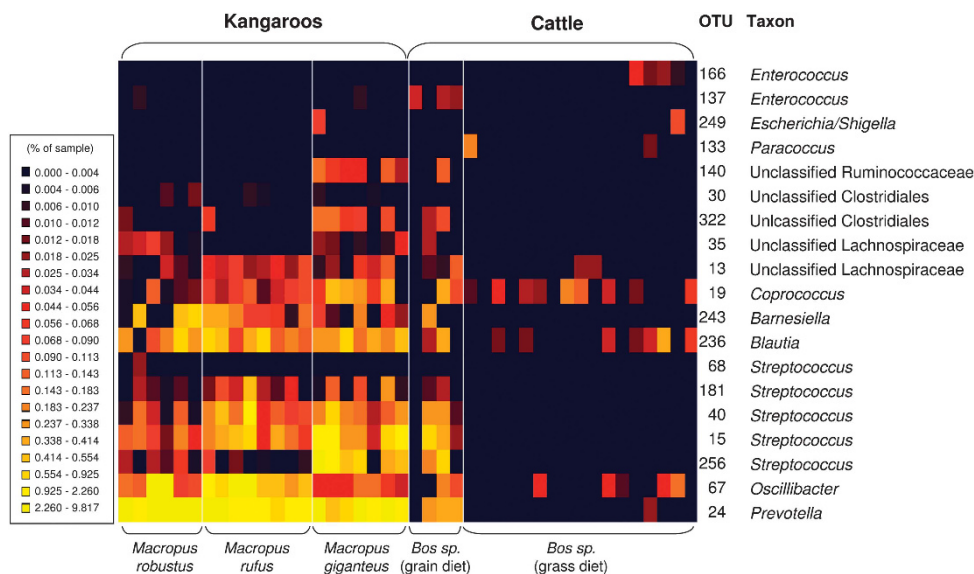


Figure 5 Heatmap representing the relative abundances in kangaroos and cattle of bacterial OTUs identified as H_2/CO_2 utilisers in the kangaroo foregut. For each sample, the colours represent the percentage of all bacterial 16S rRNA gene sequences obtained in 16S amplicon pyrosequencing surveys that share $\geq 97\%$ identity to representative sequences of H_2/CO_2 utilising OTUs identified in the RNA-SIP experiment.

the hypothesis that reductive acetogens have an important role as hydrogenotrophs in macropods (Attwood and McSweeney, 2008; Ouwkerk *et al.*, 2009). Gagen *et al.* (2010) detected sequences of the Acetyl-CoA synthase gene (which is specific to the reductive acetogenesis pathway) in the forestomach of the Tammar wallaby (*Macropus eugenii*) that shared $>99\%$ similarity to the draft genome of *Blautia hydrogenotrophica*. It is interesting to note that the RNA-SIP experiment did not detect any other known reductive acetogens, despite the fact that Gagen *et al.* (2010) and Ouwkerk *et al.* (2009) have demonstrated through both culture-based and culture-independent means that a considerable diversity of reductive acetogens exists in the macropod forestomach. It is possible that not all of these acetogens are metabolically active *in situ*, or that the incubation conditions used in the SIP experiment did not favour their activity. It is also possible that some of the other phylogenetic groups identified by RNA-SIP as H_2/CO_2 utilisers contain previously unrecognised acetogens or other hydrogenotrophs.

Some OTUs identified by RNA-SIP were of particular interest because of their relatively high abundance in the forestomach of kangaroos. Foremost among these is OTU 24 (Table 1), which shared 98% 16S rRNA gene sequence similarity with a *Prevotella sp.* that was present in all individuals of all species of kangaroos previously surveyed, and comprised up to 9.8% of all 16S sequence reads in the forestomach of some animals (Figure 5). Comparison of the 16S rRNA gene sequences of this OTU to the Greengenes (DeSantis *et al.*, 2006) and GenBank (Benson *et al.*, 2011) databases indicated that its closest cultured relative was a *Prevotella sp.* cultured from the human oral cavity (Table 1), while its closest uncultured relative

was a *Prevotella* sequence recovered from a metagenomic survey of the foregut of the Tammar wallaby (Pope *et al.*, 2010). Unfortunately, it is difficult to speculate about the role of this OTU purely on the basis of its relatively distant phylogenetic relationships to other organisms. Members of the genus *Prevotella* have diverse functions in a variety of gut ecosystems, including the human colon and the bovine rumen, but have not been previously reported as being involved in the metabolism of H_2 and CO_2 .

OTUs classified within the genera *Streptococcus* and *Oscillibacter* were also identified as H_2/CO_2 utilisers (Table 1), and 16S rRNA gene sequences of these OTUs were detected in high numbers in previously surveyed kangaroos (Figure 5). These genera have not been previously reported as containing hydrogenotrophic organisms, and again, it is difficult to speculate about the ecological roles of these OTUs in the kangaroo forestomach on the basis of their phylogenetic relationships. Work is currently focussed on isolating these bacteria in pure culture so that their potential roles as hydrogenotrophs can be investigated further.

Comparison of the 16S rRNA gene sequences of CO_2/H_2 -utilising bacteria obtained from the RNA-SIP experiment to 16S rRNA gene sequences of the bacterial communities present in the bovine rumen led to an unexpected observation. Sequences closely related to many of the bacteria identified as being involved in CO_2/H_2 metabolism in the kangaroo forestomach were also present in the rumen of cattle that had been fed a grain-based diet, but were absent or only present in much lower numbers in the rumen of cattle fed a grass-based diet (Figure 5). The reason for this apparent similarity in the composition of the CO_2/H_2 -utilising bacterial community in the kangaroo forestomach and the

rumen of grain-fed cattle is not yet clear. It is known that cattle raised on a grain-based diet produce less methane than cattle on a grass-based diet (Harper *et al.*, 1999; Doreau *et al.*, 2011). It is possible that the reduced methane production observed in grain-fed cattle and kangaroos is related to the presence of hydrogenotrophic bacteria that provide an alternative hydrogen sink to methanogenesis.

While it is now clear that reductive acetogenesis is more active in the kangaroo forestomach than the bovine rumen, the channelling of CO₂ and hydrogen into acetate production does not entirely explain the drastically reduced methane output of kangaroos. Our data also provides some evidence that acetoclastic methanogens are active in the kangaroo forestomach, so it is possible, in principle at least, that methane production could still occur via the acetoclastic pathway. While some acetate was apparently converted to methane in our *in vitro* fermentations, it not clear how much acetate would be diverted into this pathway *in situ*. Acetoclastic methanogenesis has a major role in carbon cycling in many anaerobic systems such as wetland and marine sediments (Conrad *et al.*, 2002). In gut ecosystems however the continual export of acetate limits its recycling through the microbial community. While it is known that the epithelial cells of the mammalian gut are efficient scavengers of acetate and other VFAs (Pennington, 1952; Henning and Hird, 1970; Dijkstra, 1994), there are currently little experimental data available to indicate whether or not the host might be able to outcompete acetoclastic methanogens in rates of acetate uptake. Further studies using isotopically labelled acetate tracers could shed light on these questions.

Strains closely related to at least some of the bacteria involved in reductive acetogenesis in the kangaroo forestomach (for example, *Blautia coccooides*) were found to be already present in the rumens of some but not all cattle (Figure 5). The ecological factors that allow these organisms to proliferate in kangaroos and remain competitive against hydrogenotrophic methanogens while carrying out the thermodynamically less favourable process of reductive acetogenesis are not well understood. It appears that the growth of methanogens is suppressed in the kangaroo foregut ecosystem, as evidenced by the significantly lower quantities of methane produced in the kangaroo forestomach fermentations relative to the bovine rumen fermentations, and the drastically different structure of archaeal communities observed in the kangaroo forestomach by Klieve *et al.* (2012). The mechanisms of this suppression are currently unknown, and future work should focus on investigating the possibility of both host-mediated and intermicrobial mechanisms of methanogen suppression in kangaroos. If the factors underlying the suppression of methanogens and the proliferation of reductive acetogens in the kangaroo forestomach can be elucidated, it may be possible to design strategies

to mitigate ruminant methane production by manipulating these factors in the rumen.

Conclusions

The data presented here provide strong evidence that reductive acetogenesis is an important sink for carbon dioxide and hydrogen in the kangaroo foregut. The dominance of reductive acetogenesis as a pathway for hydrogen removal in the kangaroo foregut explains the relatively low production of methane by kangaroos, and suggests that kangaroos are able to utilise feed energy more efficiently than ruminants. *Blautia* spp. appear to have a key role as reductive acetogens in the kangaroo forestomach, whereas the role of other bacteria including *Prevotella*, *Oscillibacter* and *Streptococcus* spp. as hydrogenotrophs is less clear.

Conflict of Interest

The authors declare no conflict of interest.

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