Rumen Bacterial Diversity With and Without Mulga (*Acacia aneura*) Tannins

J.J. Plumb[1], L.L. Blackall[1] and A.V. Klieve[2*]

Abstract

Feral goats are able to survive in many semi-arid areas of Australia. Under drought conditions, the only form of available feed is often mulga, which has a very high content of condensed tannins (5–24% dry weight). While feral goats apparently thrive on this diet, sheep do very poorly and lose liveweight rapidly. It has been shown that the transfer of rumen contents from feral goats to sheep can significantly improve mulga digestion, suggesting that the ruminal microflora of feral goats may contain tannin tolerant or degrading bacteria. To identify likely communities or associations of bacteria that may undertake this task, a comparative study of the bacterial ecology of the rumens of feral goats fed mulga and sheep fed either mulga or grass was undertaken. This study used the culture independent techniques of generation of 16S rDNA clone libraries and fluorescence in situ hybridisation (FISH) probing. From the clone libraries, bacteria were mainly (>90%) within the divisions *Cytophaga-Flexibacter-Bacteroides* (CFB) and low mol% G+C Gram positive bacteria (LGCGPB). In animals fed mulga, the CFB predominated (goat – 82% CFB and 11% LGCGPB; sheep – 78% CFB and 21% LGCGPB) whereas in sheep fed grass, the LGCGPB predominated (25% CFB vs 74% LGCGPB). In all clone libraries, few bacterial species were closely related to previously cultured bacteria, making it difficult to assign phenotypic traits. FISH probing of mulga fed –rumen (feral goats and sheep) or –fermentor samples demonstrated a predominance of CFB and gamma proteobacteria. This first molecular ecological study of tannin associated microbial communities suggested that bacteria from these two groups may be either more tolerant to tannins or able to degrade tannins. Further work will be required to elucidate the important members of these groups and to obtain them in culture.

DOCUMENTATION of the use of mulga (*Acacia aneura*) as a source of feed for sheep in southwest Queensland dates back to the late 1800s. During this time, mulga has enabled the sheep industry to survive and prosper in the area to the point where the region is responsible for more than 40% of the state's wool production. Mulga-based pasture associations extend over 150 million hectares of the continent, including also South Australia, Western Australia and New South Wales where its presence is also of value to the wool industry.

[1] The Department of Microbiology, The University of Queensland, Brisbane, Qld, 4072
[2] Sheep and Wool Institute, Animal Research Institute, Queensland Department of Primary industries, Locked Mail Bag No. 4, Moorooka, Qld, 4105
* Corresponding author

The high frequency of drought in these regions requires that livestock rely heavily on mulga for survival. Supplementation of stock with nitrogen, phosphorous and sulphur is necessary to overcome the nutrient deficiencies induced by the high concentration of condensed tannins (CTs: 5–24% dry weight) in the mulga leaf. The action of CTs, now widely recognised as a plant chemical defence against herbivory, is one of reducing plant protein digestibility. In high concentrations (greater than 3 g/kg) tannins may act as anti-feedants because they:

1. complex with food protein;
2. bind microbial enzymes, reducing fermentation and degradation of fibrous tissue;
3. bind digestive enzymes in general, reducing their activity; and
4. have an astringent taste.

KEYWORDS: Rumen, Mulga, Condensed tannins, Bacterial diversity

The failure of these compounds to be broken down during passage through the digestive tract results in a reduction of the nutritional value of mulga.

While sheep do very poorly and lose liveweight rapidly when fed a diet comprising predominantly mulga, without supplementation, feral goats apparently thrive on this diet. It has been demonstrated that both feral goat rumen fluid and a fermentor-enriched consortium of micro-organisms from the feral goat rumen improve the nutritional value of mulga when administered to mulga-fed sheep (Miller et al. 1995, 1997) Therefore, it appears likely that micro-organisms present in the feral goat rumen are able to tolerate the presence of high levels of mulga tannins and also digest this harsh fodder. Inoculation with pure cultures of *Streptococcus caprinus*, a bacterium isolated from feral goat rumen fluid (Brooker et al. 1994) and capable of disrupting tannic acid-protein complexes in vitro, does not enhance protein digestion, suggesting that a consortium of micro-organisms may be involved in enhancing digestion (Miller et al. 1996). On this basis and assuming that at least some, and maybe the majority, of organisms in the consortium are not culturable, a comparative study of the bacterial ecology of the rumen of feral goats fed mulga and sheep fed either mulga or grass was undertaken. This study used the culture independent techniques of generation or construction of 16S rDNA clone libraries and fluorescent in situ hybridisation (FISH) probing.

Materials and Methods

Construction of clone libraries

Rumen samples were collected from a feral goat browsing on mulga (Charleville region, Western Queensland), a sheep being fed mulga in a feeding trial (Charleville) and grass fed sheep (Brisbane). Samples were stored frozen, with glycerol, until required for DNA extraction.

Community DNA was extracted by standard methods (Maniatis et al. 1982) and purified by electrophoresing through a 0.8% low melting point agarose gel. From the purified DNA, 16S rDNA was amplified by PCR using primers to conserved regions. PCR products were purified again using low melting point agarose gel electrophoresis.

16S rRNA gene libraries were constructed either by ligating purified 16S rDNA amplicons into the plasmid vector and transforming into competent cells using the TA Cloning Kit (Invitrogen) or constructed in a like manner using the pGem-T plasmid vector (Promega) and competent cells (Stratagene). Clones with full sized inserts were detected by either: 1) plasmid extractions, alkaline lysis followed by PEG precipitation; or 2) direct lysis PCR using the plasmid specific primers. With the latter method, clones containing 16S rDNA inserts were determined based on the size of the PCR products obtained.

The DNA sequence of cloned 16S rDNA inserts were determined using automated sequencing and the data phylogenetically analysed using methods from Blackall et al. 1994.

Fluorescent in situ hybridisation (FISH)

Rumen samples were collected from a feral goat browsing on mulga (Charleville), a sheep being fed mulga in a feeding trial (Charleville) and grass fed sheep (Brisbane). Samples were fixed according to previously described methods, using both ethanol and ethanol/paraformaldehyde as cell fixatives (Wagner et al. 1994). Samples were then stored at −20 °C until used for whole cell probing.

The probes used for FISH probing of rRNA in the microbial cells in fixed rumen samples were: EUB338 (domain Bacteria), ALF1b (alpha proteobacteria), BET42a (beta proteobacteria), GAM42a (gamma proteobacteria), CF319a (*Cytophaga-Flavobacterium*), BAC303 (*Bacteroides-Prevotella*) and HGC69a (high mol% G+C Gram positive bacteria). These probes were chosen as they cover some of the major lines of descent within the domain Bacteria. The BAC303 probe was also chosen due to the abundance of clones in the CFB phylum, observed previously (see results). Oligonucleotides were synthesised with a 5'-C6-TFA aminolinker and labelled with tetramethylrhodamine-5-isothiocyanate and 5(6)-carboxyfluorescein-N-hydroxysuccinimide-ester (Amann et al. 1990).

Hybridisation of fixed rumen samples was performed as previously described (Erhart et al. 1997). Probed samples were mounted with Citifluor and visualised using a Nikon Microphot-FXA microscope with filter blocks V-2B, B-2A and G-2A and a Leitz Wetzlar NPL FLUOTAR 100x fluorescence oil immersion objective. Cell counts were taken by viewing at least 20 different fields and counting usually between 1500 and 2000 cells.

Results

Clone libraries

Evolutionary distance trees constructed to compare clone sequences with representatives from the domain *Bacteria*, revealed the presence of bacteria belonging to several different phyla. In the feral goat

library, 82% (69/84) of clone sequences were affiliated with the *Cytophaga-Flexibacter-Bacteroides* (CFB) phylum. Sequences affiliated with the low mol% G+C Gram positive bacteria (LGCGPB) comprised 11% (9/84) of the feral goat library, while the remaining 7% (6/84) were not affiliated with any phylum in the domain *Bacteria*. The vast majority of the clone sequences in the mulga-fed sheep and the grass-fed sheep belonged to two phyla, the CFB and the LGCGPB. In the grass-fed sheep library, members of the LGCGPB phylum comprised the majority of phylotypes (74.2%), whereas in the mulga-fed sheep library, 78% of clones were in the CFB phylum. Two out of 193 sequences analysed did not fall within these two phyla. The clone sequences affiliated with the CFB phylum were generally positioned in 4 different places associated with the bacteroides subgroup. Cluster I formed an outlying part of the subgroup. Cluster II is more closely affiliated with the true *Bacteroides* such as *Bacteroides fragilis*. Cluster III and cluster IV are more closely affiliated with species of *Prevotella* (Figure 1).

Novel bacterial diversity was assessed by comparing clone sequences with reference sequence data. Using >97% sequence similarity as an arbitrary criterion for defining a 'species' (Bond et al. 1995), only four clones from the feral goat library were determined to be closely related to previously described rumen species [*Prevotella ruminicola* (3 clones) and *Selenomonas ruminantium*].

Figure 1 is presented as an example of the genetic diversity of clones from the feral goat library in the CFB phylum and how closely these relate to described bacterial species.

In the grass and mulga fed sheep libraries, about 22% (42/193) of clones were determined to be affiliated with reference sequences. Only 5 of these 42 clones were affiliated to previously described rumen bacteria (*Ruminococcus bromii* and *Butyrivibrio fibrisolvens*). The majority of clones in these libraries were dissimilar to reference sequences with 49.7% (96/193) ranging between 90% and 97% similarity and the remaining 28.5% (55/193) less than 90% similar.

Fluorescent in situ hybridisation (FISH)

Greater than 75% of DAPI stained cells was detected using the EUB338 probe. In the three rumen samples, numbers of bacteria within the CFB phylum were higher than for any of the other bacterial groups detected by the probes employed. Almost half of all bacterial cells in the grass-fed sheep sample that hybridised with EUB338 also hybridised with BAC303 and, when combined with data for the CF319a probe, 76.4% of EUB338 probing cells were detected as being members of the CFB phylum. In the feral goat sample, 52.7% of bacteria were detected as being members of the CFB phylum. In all samples, bacteria belonging to different subclasses of the proteobacteria were also detected with alpha and gamma subclasses generally more numerous than the beta proteobacteria. Members of the high mol% G+C Gram positive bacteria phylum were not detected in significant numbers in any of the samples studied. At the time the study was undertaken, a probe was not available for bacteria characterised as low mol% G+C Gram positive bacteria.

Discussion.

The research undertaken to compare mulga-degrading communities in feral goats and sheep indicates that bacteria belonging to the CFB phylum and gamma subclass of the proteobacteria are likely to be important in the digestion of mulga and therefore could be the main reservoir of tannin tolerance or possibly degradation, in feral goats. According to FISH probing, members of these two phyla comprise the majority of bacteria present in the feral goat rumen. However, bacteria belonging to these phyla are also numerically dominant in rumen bacterial populations of grass-fed sheep. It is not known whether these species of CFB are identical to the CFB members in the feral goat rumen, nor whether they possess similar phenotypes in relation to mulga tannin tolerance or digestion. Lower numbers of gamma proteobacteria were present in the rumen of grass-fed sheep compared with the feral goat rumen, indicating a possible role for this group in mulga digestion. As many of the clone sequences retrieved from the ecosystems studied represent novel species, it is difficult to infer phenotypic traits to these bacteria. Of interest, however, is a report of the resistance to the negative effects of condensed tannins by a species of *Prevotella* (Jones et al. 1994) which may indicate an ability of closely related species within the CFB phylum to tolerate tannin compounds.

While this first molecular ecological study of tannin associated microbial communities in the rumen suggests that bacteria from these two groups may be either more tolerant to tannins or able to degrade tannins, further work will be required to elucidate the important members of these groups and their role in tannin metabolism.

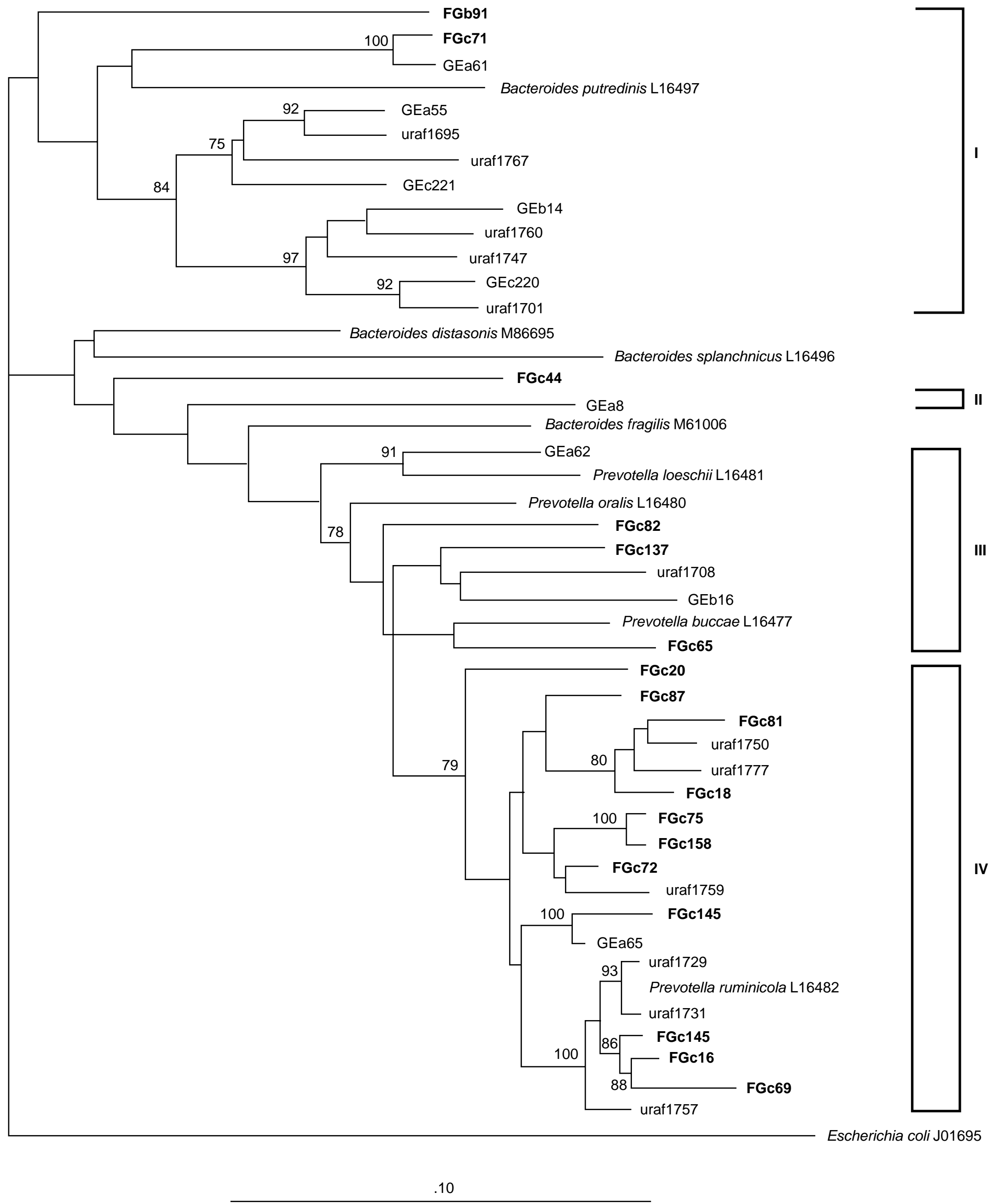

Figure 1. Evolutionary distance tree based on partial 16S rDNA sequence data (361 nucleotides) showing the phylogenetic position of representative clones within the CFB phylum. Clones derived from feral goat rumen contents are in bold type and have a FG designation. Designates beginning GE or uraf are other clonal sequences.

As future directions, we suggest the following may help further elucidate important tannin resistant or degrading bacteria:

(a) the design of new probes for members of the CFB phylum and other novel species which may be used to help clarify their role;
(b) a focused effort to isolate in culture CFB species present in the feral goat rumen that are currently uncultivable so that phenotype and tannin resistance/degradation can be investigated; and
(c) FISH probing of fixed sectioned mulga leaf retrieved from the rumen to identify attached bacteria.

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Characterisation of Tannin-Resistant Bacteria from the Rumen Fluid of Feral Goats and Camels with Restriction Analysis of Amplified 16S rDNA

A.S. Tjakradidjaja[1], J.D. Brooker[1] and C.D.K. Bottema[1]

Abstract

Acacia sp. and *Caliandra callothyrsus* contain tannin at concentration of 8–11% DM. These legumes can only be digested by ruminants that naturally adapt to feed with high tannin content. This capability is due to the presence of tannin-resistant bacteria in their rumen, such as *Streptococcus caprinus* and *Selenomonas ruminantium* K2, which were isolated from the rumen fluid of feral goats browsing Acacia. Other tannin-resistant bacteria also exist in the rumen. These bacteria have been isolated recently from the rumen fluid of feral goats and camels in enrichment experiments using tannic acid or tannin extracts from Acacia and Caliandra leaves as limiting substrates. These tannin-resistant bacteria were grouped morphologically into Gram-positive streptococci (6 isolates), Gram-positive cocci/rods (3 isolates), Gram-negative coccus (1 isolate), Gram-negative curved rods (6 isolates), and Gram-negative slender rods (4 isolates). These isolates have been identified by physiological and biochemical tests, as well as API test, and the possible genera are *Streptococcus* sp., *Leuconostoc* sp. or *Lactobacillus* sp., *Megasphaera* sp. or *Prevotella* sp., *Selenomonas* sp., *Butyrivibrio* sp. or *Clostridium* sp. Confirmation of these genera is still necessary, for example, by the use of a molecular approach. The present study was conducted to characterise tannin-resistant bacteria using 16S rDNA restriction fragment length polymorphism (RFLP) analysis. This method was capable of identifying isolates that belong to *Streptococcus* sp. and *Selenomonas* sp. The other two isolates appeared to be *Lactobacillus* sp. and *Butyrivibrio* sp. However, restriction analysis of amplified 16S rDNA did not characterise Gram-negative coccus to be the same genus as identified phenotypically. This bacterium could be characterised by sequencing its amplified 16S rDNA as *Escherichia coli*. This method also confirmed the identification of the other two isolates to be *Lactobacillus* sp. and *Butyrivibrio* sp. This study indicated that restriction analysis of amplified 16S rDNA followed by sequencing of 16S rDNA are useful for characterisation of tannin-resistant bacteria. This characterisation is important to study the role of tannin-resistant bacteria to digest legume leaves that contained tannin at high concentration.

Acacia sp. and *Caliandra callothyrsus* contain tannin at concentration of 8–11% dry matter (DM) which limits their utilisation as animal feeds (Soebarinoto 1986; Elliott and McMeniman 1987). However, ruminants that naturally adapt to feed with high tannin content were able to digest those legumes. This capability is due to the presence of tannin-resistant bacteria in their rumen.

Two tannin-resistant bacteria, *Streptococcus caprinus* and *Selenomonas ruminantium* K2, had been isolated from the rumen fluid of feral goats browsing *Acacia* sp. (Brooker et al. 1994; Skene and Brooker 1995). However, these species are not the only bacteria tolerating tannin. Other tannin-resistant bacteria also exist in the rumen. Twenty bacteria that are resistant to tannin have been isolated recently from the rumen fluid of feral goats and camels in enrichment experiments. In these experiments, tannic acid or tannin extracts from Acacia and calliandra were used as limiting substrates (Tjakradidjaja et al. 1997; unpublished data).

These isolates were grouped based on their morphology and identified phenotypically with

[1] Department of Animal Science, The University of Adelaide, Glen Osmond, Adelaide SA 5064 Australia

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physiological tests, biochemical reactions and API tests (Table 1). However, the metabolic identification techniques were not sufficient to distinguish the organisms that were similar (Johnson 1985). This conventional approach should be integrated with molecular approach, i.e. studying nucleic acids of bacteria to confirm the identification of bacteria (Johnson 1985; Staley 1996; Tiedje and Zhou 1996).

An example of molecular techniques for bacterial identification is amplification of DNA using polymerase chain reaction (PCR) which is usually employed together with other methods, such as restriction fragment length polymorphism (RFLP), random amplified polymorphism DNA (RAPD) or PCR ribotyping (Randles et al. 1996; Momol et al. 1997). In the present study, tannin-resistant bacteria were characterised with RFLP analysis of PCR-amplified 16S rDNA.

Materials and Methods

Bacteria

All isolates were characterised in this experiment (Table 1). To confirm this identification, several ruminal and non-ruminal bacteria were also included as references. These bacteria were *Streptococcus* (S) *bovis* WJ-1, *S. caprinus* 2.2, *S. gallolyticus* (*S. bovis* biotype I), *Selenomonas* (Sel.) *ruminantium* HD4, *Sel. ruminantium* K2, *Butyrivibrio* (B.) *fibrisolvens* E14, *Prevotella* (P.) *ruminicola*, *Ruminococcus* (R.) *albus*, *Clostridium* (C.) *perfringens*, *Lactobacillus* (L.) *plantarum*, *Megasphaera* (M.) *elsdeni*, *Enterococcus* (Ent.) *faecalis*, *Bacillus* (Bac.) *fragilis* and *Escherichia* (E.) *coli* ED8299.

DNA extraction and amplification

Extraction of DNA was carried out by the method of Ausubel et al. (1989). The purified DNAs of tannin-resistant and reference bacteria were used as templates in PCR. The 16S rDNA genes were amplified with PCR using two universal ribosomal DNA primers: fD1 (5'GAA TTC GTC GAC AGA GTT TGA TCC TGG CTC AG3') and rP2 (5'AAG CTT GGA TCC ACG GCT ACC TTG TTA CGA CTT3').

The PCR mixture contained : 5 μL 10x PCR buffer (Gibco BRL), 0.4 mM dNTP consisting of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), 30 pmol of each primer, 1.5 mM $MgCl_2$ (Gibco BRL), 2.5 unit Taq polymerase enzyme (Gibco BRL), 50 ng purified DNA and sterilised water to make up 50 μL. The amplification of DNA was performed using a Perkin Elmer Cetus DNA Thermal Cycler (Norwalk, Conn.). The reaction was

Table 1. Tannin-resistant bacteria isolated from the rumen fluid of feral goats and camels.

Group number	Isolates	Tannin sources in enrichment experiments	Bacterial groups based on Gram-staining morphology	Possible genera as identified phenotypically
I	K1T – goat	Tannic acid	Gram-positive streptococci	*Streptococcus* sp.
	C1T – camel			
	C13A – camel	Acacia		
	G13C – goat	Caliandra		
	G23C – goat			
	C13C – camel			
II	G33A – goat	Acacia	Gram-positive cocci/rods	*Leuconostoc* sp. or *Lactobacillus* sp.
	C23A – camel			
	G43C – goat	Calliandra		
III	C43C – camel	Calliandra	Gram-negative coccus	*Megasphaera* sp. or *Prevotella* sp.
IV	K2T – goat	Tannic acid	Gram-negative curved rods	*Selenomonas* sp.
	C2T – camel			
	G13A – goat	Acacia		
	G33C – goat	Calliandra		
	C23C – camel			
	C53C – camel			
V	G23A – goat	Acacia	Gram-negative slender rods	*Butyrivibrio* sp. or *Clostridium* sp.
	G53C – goat	Calliandra		
	G63C – goat			
	C33C – camel			