16S rDNA sequencing of *Ruminococcus albus* and *Ruminococcus flavefaciens*: design of a signature probe and its application in adult sheep

Denis O. Krause,¹ Brian P. Dalrymple,¹ Wendy J. Smith,¹
Roderick I. Mackie² and Christopher S. McSweeney¹

Author for correspondence: Denis O. Krause. Tel: +61 7 3214 2723. Fax: +61 7 3214 2881.
e-mail: denis.krause@tag.csiro.au

¹ CSIRO Division of Tropical Agriculture, Long Pocket Laboratories, Private bag # 3, Indooroopilly 4068, Australia

² Department of Animal Sciences, University of Illinois, Urbana, USA

The ruminococci are an important group of fibrolytic bacteria inhabiting the rumen. Seventeen strains of presumptively identified *Ruminococcus* were evaluated by a combination of nearly complete and partial 16S rDNA sequence that identified all strains as either *Ruminococcus albus* or *Ruminococcus flavefaciens*. All sequences fell into cluster IV of the clostridia, while other species of ruminococci (eg. *Ruminococcus obeum*, *Ruminococcus gnavus*, *Ruminococcus lactaris*) fall into cluster XIVa of the clostridia. *Ruminococcus* cluster IV sequences were used to design a 16S rRNA oligonucleotide probe to assess the relative abundance of target populations in a stable ruminal environment. A stable population (animals fed eight times per day) was established in sheep so that statistically robust comparisons could be made in the absence of variation due to diurnal rumen fluctuations. The steady state populations were sampled six times over a 24 d period and direct microscopic counts (DC), total culturable counts (TCC), and total cellulolytic counts (CEL) were determined. DC and culturable data (TCC and CEL) were compared with relative abundance estimates of *Ruminococcus* IV and *Fibrobacter succinogenes*. A combination of the *Ruminococcus* and *F. succinogenes* probes accounted for 4.0% of the bacterial population and cellulolytic bacteria (measured by most-probable numbers) were 5.2% of the total culturable count. These data suggest that a major portion of the *Ruminococcus* and *Fibrobacter* diversity has been cultured and is represented by available sequences. Steady state populations were measured over several days in three sheep and an estimate of variation in DC, TCC, CEL and 16S-based data were obtained. These variance estimates could be used to determine the theoretical sample sizes required to obtain statistically significant differences under different experimental conditions.

Keywords: *Ruminococcus*, 16S rDNA, phylogeny, statistics, rumen

INTRODUCTION

Fibre (lignocellulose) digestion is an anaerobic process in animals and usually takes place in the rumen or hind-gut (Mackie, 1997). Several genera of bacteria are

involved in fibre degradation and it is primarily carried out by *Butyrivibrio*, *Fibrobacter* and *Ruminococcus* spp. *Fibrobacter* and *Ruminococcus* spp. are the most fibrolytic, possibly related to their adherent ability, while *Butyrivibrio fibrisolvens* is a free-floating organism (Weimer, 1996). Phenotypic and phylogenetic analysis of *Fibrobacter* (Amann *et al.*, 1992; Lin *et al.*, 1994; Lin & Stahl, 1995; Montgomery *et al.*, 1988; Montgomery & Macy, 1982) and *Butyrivibrio* (Forster *et al.*, 1996, 1997) is extensive but the diversity and

Abbreviations: CEL, cellulolytic counts; DC, direct microscopic counts; TCC, total culturable counts.

The GenBank accession numbers for the sequences reported in this paper are shown in the Methods section '16S rDNA sequencing'.

phylogenetic relationships among cellulolytic ruminococci have not been well studied.

Ruminococcus flavefaciens was first isolated by Sijpesteijn (1951), had a yellow pigment when grown on cellulose, grew in chains on cellobiose, and was weakly Gram-positive. *Ruminococcus albus* was first isolated by Hungate (1957), but it does not produce a yellow pigment, forms diplococci on cellobiose and is also weakly Gram-positive. The carbohydrate-fermentation and end-product profiles of *R. flavefaciens* and *R. albus* are almost identical; however, only *R. flavefaciens* produces succinate as one of its major fermentation end products (Hespell *et al.*, 1997).

16S rRNA analysis has provided a framework for evaluating microbial communities without the need for prior cultivation (Amann *et al.*, 1995; Pace, 1997). These studies have provided significant insights into a variety of microbial ecosystems (Pace, 1997), but can only be conducted if probes for target organisms are available. *Fibrobacter* and *Ruminococcus* spp. are considered the most important fibrolytic bacteria inhabiting the rumen, and oligonucleotide probes (16S rRNA-based) have been designed for *Fibrobacter* spp., allowing for a more complete assessment of their ecology (Amann *et al.*, 1992; Lin *et al.*, 1994; Lin & Stahl, 1995). Oligonucleotide probes designed to the ruminococci tend to be strain specific and do not circumscribe organisms at the species or genus level (Odenyo *et al.*, 1994a, b). *R. albus* and *R. flavefaciens* are major contributors to ruminal fibre degradation and a signature probe that covers these species is essential to further our understanding of their role in rumen ecology.

Estimating population sizes in the rumen is difficult because microbial populations fluctuate dramatically during the day (Hungate, 1966; Leedle *et al.*, 1982). In addition, traditional cultivation techniques (Dehority *et al.*, 1989) or methods based on Northern blotting of 16S rRNA are variable (Raskin *et al.*, 1997). However, a systematic understanding of the size and extent of variation in a microbial ecosystem is critical for efficient experimental design. In ruminal ecology, estimates of variation have been made in relation to culturable count data (Dehority *et al.*, 1989) and an analysis of experimental error associated with various aspects of hybridization studies with rRNA have been obtained (Raskin *et al.*, 1997). However, there are no published data that evaluate the variation obtained with Northern-blot analysis of 16S rRNA obtained from ruminal studies.

In this paper, 16S rDNA RFLP and 16S rDNA sequence analyses were conducted to assess the phylogenetic diversity of *R. albus* and *R. flavefaciens*, and sequence information was used to design a 16S rRNA-based oligonucleotide probe. This probe was used to estimate the proportion of *R. albus* and *R. flavefaciens* in the rumen of adult sheep in a statistically robust manner, and comparisons were made with direct microscopic counts (DC), total culturable counts (TCC) and, total cellulolytic counts (CEL). Variation could be statistically

defined and theoretical estimates of samples sizes required to make statistically significant comparisons were calculated.

METHODS

Isolation and origin of *Ruminococcus* strains. Tipperary Sanctuary in the Northern Territory of Australia maintains a collection of exotic ruminants, including addax (*Addax nasomaculatus*), for research purposes. Ruminal contents from an adult addax were obtained by stomach tubing and digesta were deposited into a glass jar. Immediately after collection, 1 ml digesta was injected into a Hungate tube containing either basal medium (see below), 25 mg cellulose discs (see below) and 25 mg dewaxed cotton thread, or basal medium and 50 mg rhodes grass (*Chloris gayana*). The Hungate tubes were transported to Brisbane, Australia, and the cultures grown to stationary phase at 39 °C. The enrichments on cellulose discs and cotton thread or rhodes grass were maintained by transfer of 200 µl culture every 96 h using an 18-gauge needle, while the enrichment on rhodes grass was transferred every 72 h.

Isolation of cellulolytic bacteria from the addax enrichment was performed according to the roll-tube method of Hungate (1969), but with some modifications. Cellulose roll tubes contained 2% (w/v) ball-milled filter paper (Whatman no. 1) and 0.1% (w/v) cellobiose in basal medium. Enrichments (cellulose discs plus cotton thread or rhodes grass) were serially diluted in roll tubes and incubated for 5–7 d at 39 °C. Isolates were checked microscopically for purity and for the ability to grow on cellulose. Unfortunately we were not able to maintain R1-addax (R, rhodes grass enrichment) and C14-addax (C, cellulose enrichment) as actively growing cultures but were able to retain a DNA sample for sequence analysis.

Fifteen additional bacterial isolates from several geographic regions were analysed (Table 1). A total of ten isolates were from Australia (AR strains, LP-9155, Y1 and the two addax strains), one from Scotland (SY3), one from England (RF1Ba) and the remainder were from North America (Ra8, FD-1, B199, B146 and R13e2). Most of the isolates came from the rumen of cattle and sheep but one (R13e2) was from a reindeer.

Composition of media. The composition of the basal medium was (per litre): 2.5 g yeast extract, 150 ml clarified rumen fluid, 150 ml mineral-1 solution [3 g $K_2HPO_4 \cdot 3H_2O$ (100 ml)⁻¹], 150 ml mineral-2 solution (contains per 100 ml: 3 g KH_2PO_4 , 6 g $(NH_4)_2SO_4$, 6 g NaCl, 1.23 g $MgSO_4 \cdot 7H_2O$, 1.58 g $CaCl_2 \cdot 2H_2O$), 2 ml trace mineral salts solution (contains per 100 ml: 0.5 mg $ZnSO_4 \cdot 7H_2O$, 0.15 mg $MnCl_2 \cdot 4H_2O$, 1.5 mg H_3BO_3 , 1.0 mg $CoCl_2 \cdot 6H_2O$, 0.05 mg $CaCl_2 \cdot 2H_2O$, 0.1 mg $NiCl_2 \cdot 6H_2O$, 0.15 mg $Na_2MoO_4 \cdot 2H_2O$, 7.5 mg $FeCl_2 \cdot 4H_2O$), 3.2 mmol acetic acid, 1.4 mmol propionic acid, 1.1 mmol butyric acid, 1.1 mmol isobutyric acid, 0.31 mmol methylbutyric acid, 0.31 mmol valeric acid, 0.31 mmol isovaleric acid, 2.3 mmol phenylacetic acid, 1 g L-cysteine HCl and 0.01% resazurin. Media were prepared anaerobically according to the methods of Hungate (1950) as modified by Bryant (1972). The medium for total culturable bacteria was similar to the basal medium but with some modifications (Mackie & Wilkens, 1988). Total cellulolytics were determined by the three-tube most-probable number method (Dehority *et al.*, 1989) with basal medium and cellulose discs as the carbon source. The anaerobic gas was a 95% CO_2 :5% H_2 mix, 4 mg Na_2CO_3 ml⁻¹ was included as a medium buffer (pH 6.7). Cellulose discs (Whatman no. 1) were prepared by

Table 1. Source of *Ruminococcus* strains used in this study

Strain	Source/host	Source
<i>R. albus</i> strains		
B199	Cow	Marvin Bryant*
AR67	Sheep	Tom Bauchop†
Ra8	Cow	Marvin Bryant*
SY3	Sheep	Colin Stewart‡
<i>R. flavefaciens</i> strains		
FD-1	Tablet containing ruminal organisms	Marvin Bryant*
LP-9155	Cattle	Chris McSweeney§
R13e2	Reindeer	Burk Dehority
RF1Ba	Sheep	Colin Orpin¶
AR72	Sheep	Tom Bauchop†
AR71	Sheep	Tom Bauchop†
Y1	Sheep	Geoff Gordon**
AR69	Sheep	Tom Bauchop†
AR46	Sheep	Tom Bauchop†
AR47	Sheep	Tom Bauchop†
B146	Cow	Marvin Bryant*
R1-addax	Addax	This study
C14-addax	Addax	This study

* University of Illinois, Urbana, USA.

† University of New England, Armidale, Australia.

‡ Rowett Research Institute, Aberdeen, UK.

§ CSIRO Tropical Agriculture, Indooroopilly, Australia.

|| Ohio State University, Columbus, USA.

¶ AFRC Institute of Animal Physiology and Genetics Research, Babraham, UK.

** CSIRO Animal Production, Prospect, Australia.

incubation (with stirring) at room temperature with phosphoric acid (1M) for at least 1 h. Discs were then rinsed several times with distilled water before use.

Direct microscopic counts (DC). A 1 g ruminal sample was diluted 10-fold with anaerobic diluent (Mackie & Wilkens, 1988) and vortexed for 1 min. The diluted sample was placed in a counting chamber and cell numbers were converted to cells per ml by multiplying by 2×10^8 .

16S rDNA amplification. The 16S rDNA was amplified from a 1/100 dilution of stationary phase culture with universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGYTACCTTGTACGACT-3'). PCR reactions contained (per 20 µl) 2 µl 10× PCR buffer, 0.2 µl $MgCl_2$ (250 mM), 0.4 µl dNTPs (10 mM), 10 pmol each primer, 1 U *Taq* polymerase (Promega) and 0.5 µl 1/100 dilution of culture. Cycling conditions were one cycle of 94 °C for 5 min, 60 °C for 1 min and 72 °C for 90 sec, then 31 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 90 sec. The final cycle was 94 °C for 1 min, 50 °C for 1 min and 72 °C for 8 min.

Restriction digests. Approximately 100 ng 16S rDNA PCR product was digested with restriction enzymes for at least 2 h according to the manufacturer's instructions. Fifteen microlitres of the restricted product was electrophoresed on a 1% (0.5× TBE) agarose gel.

16S rDNA sequencing. Nearly complete 16S rDNA sequence data were obtained for (GenBank accession numbers are shown in parentheses) *R. flavefaciens* AR46 (AF104837), *R. flavefaciens* R1-addax (AF104835), *R. flavefaciens* C14-addax (AF104834), *R. flavefaciens* Y1 (AF104846), *R. flavefaciens* AR72 (AF104841), *R. flavefaciens* FD-1 (AF104844), *R. albus* Ra8 (AF104833) and *R. albus* AR67 (AF104839). Approximately the first 462 bp from the 5' end were sequenced for the remaining strains (GenBank accession numbers are shown in parentheses); *R. flavefaciens* B146 (AF104842), *R. flavefaciens* AR47 (AF104838), *R. flavefaciens* AR69 (AF104840), *R. flavefaciens* R13e2 (AF104847), *R. flavefaciens* LP-9155 (AF104836), *R. albus* B199 (AF104843) and *R. albus* SY3 (AF104845).

Concentrations of DNA after PCR amplification were measured by image analysis (Kodak BioMax 1D) of ethidium-bromide-stained agarose gels and approximately 25 ng of each product was included in a 20 µl sequencing reaction. Sequences were obtained in both orientations from at least two separate PCR products using universal primers (Ash *et al.*, 1993). All reactions were carried out with the ABI Prism cycle sequencing kit (Applied Biosystems) and gels were run on an ABI automated sequencer (Applied Biosystems).

Phylogenetic analysis. Restriction fragments were sized and coded as the presence (1) or absence (0) of unique bands.

These binary-coded characteristics were analysed by the Dollop procedure (Farris, 1977) using the PHYLIP computer program (Felsenstein, 1993).

Sequence data were aligned with CLUSTAL W (Thompson *et al.*, 1994) and the following alignment strategy was followed for complete and partial sequences. (i) Nearly complete sequences of ruminococci obtained in this study were aligned with nearly complete sequences of highly similar (> 90% similarity) organisms (not ruminococci) found in the Ribosomal Database Project (Maidak *et al.*, 1997). (ii) Sequences were manually adjusted with reference to secondary structure providing a robust template alignment. (iii) Partial sequences were added to the template alignment iteratively using the profile alignment procedure available on CLUSTAL W (Thompson *et al.*, 1994).

Phylogenetic analysis was by the distance method of Jukes & Cantor (1969) and tree topology was inferred by the neighbour-joining algorithm (Nei & Li, 1979) with TREECON software (Van de Peer & De Wachter, 1994). All sequence-based trees were analysed by bootstrapping of 1000 trees. The phylogenetic analysis was checked by using the maximum-likelihood method available on PHYLIP (Felsenstein, 1993).

Design of 16S rRNA oligonucleotide probes. A signature probe for *R. albus* and *R. flavefaciens* was designed and is designated with a 'IV' (S-G-Rum IV-0132-a-A-17; 5'-TGKT-AATACCYCATAAY-3') to differentiate it from cluster XIVa ruminococci (Collins *et al.*, 1994). The wash temperature was determined experimentally as 34 °C with 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate). The probe sequences were checked for specificity with the CHECK-PROBE procedure on the Ribosomal Database Project (Maidak *et al.*, 1997) and assessed empirically by hybridization to target ruminococci as well as a diverse set of ruminal bacteria (see Table 2).

DNA extraction. A 1 ml sample from an *in vitro* culture was centrifuged (10000 g) for 5 min, the supernatant removed and the pellet resuspended in 1 ml 100 mM Tris/HCl (pH 8), 10 mM EDTA, 0.2 ml 10% SDS and 0.1 ml proteinase K (10 mg ml⁻¹), and incubated at 55 °C for 2 h. After the sample was divided in two (approx. 0.65 ml each), 0.12 ml 5M NaCl and 0.065 ml (10%) hexadecyltrimethylammonium bromide (CTAB) were added to each tube prior to incubation at 65 °C for 10 min. Each sample was extracted with 0.7 ml chloroform/isoamyl alcohol (24:1) and centrifuged (10000 g) for 15 min. The supernatant was removed and mixed with 0.225 ml 5M ammonium acetate and 0.55 ml 100% 2-propanol, incubated on ice for 1 h and centrifuged (10000 g) for 15 min and the supernatant aspirated. The DNA pellet was washed with cold 70% ethanol, dried under vacuum and resuspended in TE buffer (0.1 ml) containing 5 µg RNase ml⁻¹ (Boehringer Mannheim). The DNA suspension was loaded onto a Wizard DNA clean up column (Promega) and the eluted DNA (50 µl) was used in PCR reactions.

RNA extraction. A 1 ml sample of ruminal digesta was taken with a P1000 pipette with the tip cut off so that sufficient plant material was included in the sample. The RNA was extracted by 5 min of bead-beating according to the procedure of Stahl *et al.* (1988).

Probe hybridization protocol. Oligonucleotide labelling with digoxigenin and hybridizations were carried out as previously described (Krause & Russell, 1996). The RNA concentration was measured spectrophotometrically and adjusted to 100 ng µl⁻¹. Three volumes 2% glutaraldehyde were added to the RNA and allowed to denature for 10 min at 25 °C. Before application to positively charged nylon membranes (Boeh-

ringer Mannheim) a volume equaling 1 µg per slot was diluted with 0.0002% bromophenol blue and 1 µg polyadenylic acid ml⁻¹. One microgramme of RNA was applied to nylon membranes, and the membranes were baked at 120 °C for 30 min. The membranes were prehybridized with hybridization solution [25% formamide, 5 × SSC, 50 mM Na₂HPO₄, 2% blocking reagent (Boehringer Mannheim), 2% SDS, 0.1% N-lauroylsarcosine] for at least 2 h. Digoxigenin (Boehringer Mannheim) labelled probe (10 ng ml⁻¹) was added to the hybridization solution and allowed to hybridize overnight. The membranes were then washed at the appropriate stringency for each probe with 1 × SSC. Membranes were subsequently processed according to the manufacturer's instructions (Boehringer Mannheim). Total bacterial 16S rRNA was determined by hybridization with a universal eubacterial probe S-D-Bact-0338-a-A-18 (Amann *et al.*, 1990).

Animal experiments. Animal experiments had the following objectives. (1) To establish a steady-state population in the rumen so that baseline statistical variation in microbial populations could be assessed using traditional and 16S rRNA based methods. This was accomplished by feeding sheep eight equal-sized meals within a 24 h period using automated feeders. (2) A common rumen sample would be used for estimation of the relative abundance of cellulolytic bacteria using traditional and 16S rRNA based methods. (3) Variance analysis could be conducted on data to determine sample sizes required to obtain statistically significant differences when 16S rRNA slot-blotting is used to study ruminal populations.

Three adult sheep were fistulated (McSweeney, 1989) and housed in metabolism crates. Eight-times-per-day automated feeders were used to provide feed to animals on a continuous basis during a 24 h period. Sheep were fed a rhodes grass diet augmented with 50 of a protein supplement. The sheep had free access to water 24 h per day. Ruminal samples were taken 1 h after the 10 am feeding. All animal experimental procedures were approved and monitored by an institutional animal ethics committee.

Statistical analysis. All statistical analyses were done with Statistica 5.0 software. The experimental unit consisted of three animals for each point. Data were analysed using analysis of variance procedures and treatment means were compared using orthogonal comparisons. Bacterial count data did not have homogeneous variances and were transformed using a log transformation procedure. Determination of sample sizes was according to Snedecor & Cochran (1980). The following formula was used:

$$n = (Z_{\alpha} + Z_{\beta})^2 \sigma^2_D / \delta^2$$

where n is the number of samples required, Z is the standard normal deviate, α and β are the probabilities that the standard normal deviate will lie within the specified normal distribution, σ is the standard deviation determined by experimentation and is critical in determining the sample size; if the variation increased, so does the number of samples required, and δ is the power of the test: the probability of showing a true difference between treatments of a specified size (usually 80 or 90%).

RESULTS

Analyses based on 16S rDNA RFLP analysis

Enterobacterial repetitive intergenic consensus, repetitive extragenic palindromic, and 16S–23S rDNA spacer

Fig. 1. Diagrammatic representation of 16S rDNA RFLPs from ruminococci analysed in this study. Enzymes used were *AluI* (a) and *DdeI* (b). Representatives of each cluster type are given with particular reference to ruminal or gut isolates.

polymorphisms have previously indicated that the *Ruminococcus* isolates used in this work are genetically distinct (Krause *et al.*, 1999). Restriction enzymes were selected that would differentiate between strains of ruminococci and were not selected with phylogenetic analysis in mind. *AluI*, *DdeI*, *HincI*, *HinfI*, *HpaII*, *MboI*, *MseI* and *Sau3AI* were initially assessed but *AluI* and *DdeI* were the most useful in differentiating strains. *DdeI* fragments were particularly effective in differentiating between *R. albus* and *R. flavefaciens*, and *AluI* was useful in separating closely related strains. Restriction enzymes were used to estimate a preliminary phylogeny (Fig. 1) and *R. flavefaciens* and *R. albus* were grouped separately. All strains except AR71 and RF1Ba were sequenced.

16S rDNA RFLP analysis was useful in determining culture purity prior to sequence analysis. FD-1, AR71, AR72, AR46, R13e2 and Y1 were contaminated and 16S rDNA RFLP gave bands adding up to more than 1500 bp. These strains were streaked on cellobiose agarose plates, then well-separated colonies were selected and transferred to cellulose medium to check for ability to degrade cellulose.

Several methods of phylogenetic analysis using restric-

Fig. 2. Cluster analysis derived from 16S rDNA RFLPs of *Ruminococcus* strains digested with *AluI* and *DdeI*.

tion-character data were used but all gave widely different tree topologies. However, the Dollop procedure (Farris, 1977) produced a tree most similar to that derived from sequence analysis (Fig. 2). Dollop analysis of restriction fragments (Fig. 1) separated *R. albus* and *R. flavefaciens* into two clusters. In the *R.*

Fig. 3. Phylogenetic relationships among *Ruminococcus* sequences in this study. Nearly complete sequences are in bold. Bootstrap values are expressed as a percentage of 1000 random trees. The scale represents 0.1 mutations per site and is represented as a percentage.

flavefaciens cluster, two major groups could be defined; strains AR71, AR72, R13e2, LP-9155 and RF1Ba in one group, and R1-addax, C14-addax, AR69, B146, AR46, Y1 and FD-1 in another group. This second cluster could be subdivided into two further groups, the largest of which included the Addax isolates. Based on this preliminary analysis, only strains that were obviously different were sequenced.

16S rDNA sequence analysis

Nearly complete sequence data were obtained for strains AR67, Ra8, FD-1, AR72, Y1, C14-addax, R1-addax and AR46. Partial sequence of the first 462 bp from the 5' end was obtained for strains SY3, B199, LP-9155, R13e2, AR69, AR47 and B146, but RF1Ba and AR71 were not sequenced. Similarity analysis placed all strains within *R. albus* or *R. flavefaciens* and the closest relative was *Ruminococcus callidus* (Fig. 3). Among *R. albus* strains, sequence similarity (462 bp) was at least 87.4%, and 89.8% among *R. flavefaciens*. *R. callidus* was more closely related to *R. flavefaciens* (mean similarity, 88.7%) than to *R. albus* (mean similarity, 83.9%). *Ruminococcus bromii* is a non-cellulolytic *Ruminococcus* sp. that is closely related to *R. flavefaciens* and *R. albus*, with a mean sequence similarity of 79.3%. Phylogenies were estimated using the neighbour-joining method and bootstrap analysis tested the robustness of tree nodes (Fig. 3). The phylogeny was relatively robust (bootstrap values between 90 and 95) but several nodes were notably weak: FD-1 and LP-9155, and Y1 and AR72.

16S rRNA oligonucleotide probes

Oligonucleotide probes could be designed that were highly specific for cluster IV *Ruminococcus* spp. (referred to as *Ruminococcus* IV). The 16S rRNA oligonucleotide probe designed (S-G-Rum IV-0132-a-A-

17) was highly specific for *R. albus* and *R. flavefaciens*, had little identity with cluster XIVa ruminococci, and did not result in significant hybridization to a group of phylogenetically diverse ruminal and non-ruminal bacteria (Table 2).

Experiments with adult sheep

Total numbers of bacteria in the rumen were measured as the DC [1.6×10^{10} (g digesta) $^{-1}$] or total culturable counts [TCC; 9.6×10^9 (g digesta) $^{-1}$], and did not vary significantly ($P < 0.05$) on a daily basis (Table 3). Total cellulolytic (CEL) most-probable numbers averaged 0.5×10^9 (g digesta) $^{-1}$ (Table 3). *Ruminococcus* IV (S-G-Rum IV-0132-a-A-17) made up 1.6% of the population and *F. succinogenes* (S-S-F.succ-0650-a-A-20) 1.8% (Table 3). Together, *Ruminococcus* IV plus *F. succinogenes* made up 4.0% of the eubacterial population. CEL were 3.1% of the DC and different ($P > 0.1$) from *Ruminococcus* IV plus *F. succinogenes* (4.0%), and *Ruminococcus* IV plus *F. succinogenes* was different ($P < 0.1$) when CEL were expressed as a proportion of the TCC (5.2%).

Base-line variance (σ^2) for a number of parameters was determined by analysing sheep ruminal samples on six separate days (spread over a 24 d period) from three sheep. The data over the 24 d period varied (expressed as cv) as little as 14.5% for the TCC, and as much as 50.5% for CEL/TCC (Table 4). The cvs for 16S rRNA based analyses were fairly large and were approximately 40%. This variance was used to determine how many samples would be required to detect a 20% difference between treatments at the 95% confidence interval and for most parameters was four samples. In a hypothetical experiment with 50% more variation (*ad libitum* feeding), generally only one more sample is required if the same statistical criteria are applied (Table 4).

Table 2. Hybridization of 16S rRNA probes to a phylogenetically diverse group of bacteria

Bacterial strain	S-D-Bact- 0338-a-A-18	S-G-Rum IV-0132-a-A-17
<i>R. flavefaciens</i> AR71	+	+
<i>R. flavefaciens</i> AR72	+	+
<i>R. flavefaciens</i> R13e2	+	+
<i>R. flavefaciens</i> LP-9155	+	+
<i>R. flavefaciens</i> RF1Ba	+	+
<i>R. flavefaciens</i> R1-addax	+	+
<i>R. flavefaciens</i> C14-addax	+	+
<i>R. flavefaciens</i> AR69	+	+
<i>R. flavefaciens</i> B146	+	+
<i>R. flavefaciens</i> AR46	+	+
<i>R. flavefaciens</i> AR47	+	+
<i>R. flavefaciens</i> Y1	+	+
<i>R. flavefaciens</i> FD-1	+	+
<i>R. albus</i> B199	+	+
<i>R. albus</i> Ra8	+	+
<i>R. albus</i> AR67	+	+
<i>R. albus</i> SY3	+	+
<i>R. callidus</i> ATCC 27760	+	—
<i>R. bromii</i> ATCC 27255	+	—
<i>B. fibrisolvens</i> OB156	+	—
<i>B. fibrisolvens</i> H17c	+	—
<i>Escherichia coli</i>	+	—
<i>Eubacterium cellulosolvens</i> 5494	+	—
<i>Eubacterium ruminantium</i> GA195	+	—
<i>F. succinogenes</i> S85	+	—
<i>Lactobacillus vitulinus</i> B26	+	—
<i>Megasphaera elsdenii</i>	+	—
<i>Prevotella ruminicola</i> 23	+	—
<i>Prevotella ruminicola</i> GA33	+	—
<i>Ruminobacter amylophilus</i> 70	+	—
<i>Selenomonas ruminantium</i> HD4	+	—
<i>Streptococcus bovis</i> JB1	+	—
<i>Streptococcus bovis</i> K11	+	—
<i>Succinomonas amylolytica</i> B ₂ 4	+	—
<i>Succinovibrio dextrinosolvens</i> 22B	+	—
<i>Treponema bryantii</i> B ₂ 5	+	—

Table 3. Cellulolytic bacterial populations in adult sheep determined by cultivation or 16S rRNA oligonucleotide probes

The following comparisons are significantly different ($P < 0.05$): DC vs TCC, CEL/DC vs CEL/TCC. Comparison between *Ruminococcus* IV + *F. succinogenes* and CEL/TCC is also different ($P < 0.1$). The sample size was six for all determinations.

Item	Microscopic and culturable counts [$\times 10^{-9}$ (g digesta) ⁻¹]			Proportion CEL (%)		16S rRNA oligonucleotide probes (%) [*]		
	DC	TCC	CEL	CEL/DC	CEL/TCC	<i>Ruminococcus</i> IV	<i>F. succinogenes</i>	<i>Ruminococcus</i> IV + <i>F. succinogenes</i>
Mean	16.3	9.6	0.5	3.1	5.2	2.2	1.8	4.0
SD	3.9	1.4	0.2	1.5	2.6	0.7	0.7	1.0

^{*}Relative abundance of *Ruminococcus* IV (S-G-Rum IV-0132-a-A-17) and *F. succinogenes* (S-S-F.succ-0650-a-A-20) expressed as a proportion of eubacteria (S-D-Bact-0338-a-A-18).

Table 4. Sample size required to determine statistically significant differences between treatments

Raw data used to calculate means is shown in Table 3. All abbreviations are the same as Table 3.

Item	Sampling day						Variation analysis*			
							This study		Hypothetical study with 50% more variation	
	1	3	5	10	16	24	CV	n	CV	n
DC [$\times 10^{-9}$ (g digesta) $^{-1}$]	16	20	13	17	21	11	23.8	4	35.6	5
TCC [$\times 10^{-9}$ (g digesta) $^{-1}$]	8.9	12.2	9.7	9.6	8.9	8.2	14.5	3	21.8	4
CEL [$\times 10^{-9}$ (g digesta) $^{-1}$]	0.8	0.4	0.3	0.7	0.2	0.5	46.6	4	69.9	5
CEL/DC (%)	5.0	2.2	2.2	4.1	1.1	4.3	49.1	4	73.7	5
CEL/TCC (%)	9.0	3.5	2.9	7.3	2.6	5.7	50.5	4	75.7	5
<i>Ruminococcus</i> IV (%)	2.9	2.9	1.8	2.5	1.3	1.7	42.3	4	63.4	5
<i>F. succinogenes</i> (%)	2.3	1.8	2.7	1.1	1.9	0.8	40.4	4	60.7	5
<i>Ruminococcus</i> IV + <i>F. succinogenes</i> (%)	5.2	4.7	4.5	3.6	3.2	2.5	25.9	4	38.8	5

* Sample size required to detect a 20% difference with 95% confidence.

DISCUSSION

Phenotypically, the genus *Ruminococcus* includes several isolates from gastrointestinal environments (Hespell *et al.*, 1997). In the rumen *R. albus* and *R. flavefaciens* are usually the predominant species, while *R. bromii*, *R. callidus*, *Ruminococcus torques*, *Ruminococcus gnavus*, *Ruminococcus lactaris* and *Ruminococcus obeum* are usually isolated from the hind-gut of non-ruminants (Bryant, 1986). Previous studies (Rainey & Janssen, 1995; Willems & Collins, 1995) have indicated that the ruminococci are polyphyletic and form two main groups within the clostridia (Collins *et al.*, 1994): cluster IV represented by *R. flavefaciens* but also including *R. albus*, *R. bromii*, and *R. callidus* and cluster XIVa encompassing *R. torques*, *R. gnavus*, *R. lactaris* and *R. obeum*. However, these studies (Rainey & Janssen, 1995; Willems & Collins, 1995) only sequenced one strain of each of *R. albus* and *R. flavefaciens*.

The genus *Ruminococcus* falls within the *Peptococcaceae*, which is a family of anaerobic Gram-positive cocci comprising five genera; *Peptococcus*, *Peptostreptococcus*, *Ruminococcus*, *Sarcina* and *Coprococcus* (Ezaki *et al.*, 1994). *Ruminococcus* is phenotypically distinguishable from other *Peptococcaceae* by the presence of meso-diaminopimelic acid at position three in the peptidoglycan (Ezaki *et al.*, 1994), but additional informative phenotypic characteristics appear to be lacking. All strains sequenced in this study were ruminal isolates and 16S rDNA sequence analysis placed these organisms into cluster IV of the clostridia (Collins *et al.*, 1994). *R. callidus* was originally isolated from the hind-gut of a human (Holdeman & Moore, 1974), and is most closely related to *R. flavefaciens*. *R. bromii* is a non-cellulolytic, starch-fermenting *Ruminococcus* that has

been isolated from the rumen and hind-gut (Bryant, 1986).

It is interesting to note that the hind-gut isolates are predominantly in cluster XIVa (Collins *et al.*, 1994) and suggest an ecological and phylogenetic division within ruminococci isolated from the gastrointestinal tract. In the case of *Fibrobacter*, all strains had similar phenotypes but *F. succinogenes* could be subdivided on the basis of 16S rDNA sequence data and ecological niche (*F. succinogenes*, rumen; *F. intestinalis*, hind-gut). This apparent phylogenetic and ecological distinction between ruminococci is a basis for further work to define consensus phenotypes that could be used as a basis for reclassification of currently described *Ruminococcus* species.

The monophyletic nature of *R. albus* and *R. flavefaciens* made it possible to design an oligonucleotide probe that covered all strains but excluded the closely related species *R. bromii* and *R. callidus*. This oligonucleotide probe also excluded all cluster XIVa *Ruminococcus* spp. An important aspect of oligonucleotide design is that it provides a hierarchical approach for comparing culturable and uncultured diversity. Oligonucleotide probes can be used to determine the relative abundance of a particular species (Lin *et al.*, 1994; Lin & Stahl, 1995) or groups of organisms at higher levels of hierarchical complexity such as kingdoms or domains (Fry *et al.*, 1997). If a set of probes with increasing levels of hierarchical complexity are used on the same sample, an estimate of undescribed diversity can be obtained (Lin *et al.*, 1994; Lin & Stahl, 1995). Ruminal bacteria enriched on cellulose medium should be composed mainly of *Ruminococcus* and *Fibrobacter* as these are the major cellulolytic species [*Butyrivibrio* is xylanolytic (Hespell *et al.*, 1997; Hungate, 1966)] and relative

abundances determined with 16S rDNA probes should provide some evidence of the extent of diversity circumscribed by the probes.

Published literature (see reviews by Dehority & Orpin, 1988; Hungate, 1966) indicates that the TCC in the rumen is usually 12–80% of the direct count. These studies demonstrated that a large proportion of ruminal diversity could be cultured, unlike that in many other environments where only 0.1–10% is culturable (Head *et al.*, 1998; Pace, 1997). Values obtained were: $1.6 \times 10^{10} \text{ ml}^{-1}$ for the DC, $9.6 \times 10^9 \text{ ml}^{-1}$ for the TCC and $5 \times 10^8 \text{ ml}^{-1}$ for the CEL count. The TCC was 58.9% of the DC and the CEL count was 3.1% of the DC or 5.2% of the TCC (Table 3). Although the medium used for the enumeration of the TCC cultivates most of the important ruminal bacteria, there are some groups that are not enriched. Notable exceptions are: (1) methanogens, which can make up 3–5% of the population and require a hydrogen head-space for growth (Lin *et al.*, 1996), (2) slow-growing obligate amino-acid fermenting bacteria that are approximately 1% of the population (Chen & Russell, 1989; Yang & Russell, 1993), and (3) protozoa. These considerations have important implications when 16S rRNA oligonucleotide probe data are interpreted (see below).

Even though traditional methods for enumerating micro-organisms suffer from cultivation bias, they provide a useful framework within which to compare 16S rRNA based data. 16S rRNA analysis with the *Ruminococcus* IV probe indicated that their relative abundance was 2.2%, and probing with the *F. succinogenes* probe gave a relative abundance of 1.8% (Fig. 3). The values for *F. succinogenes* are similar to those reported in the literature using 16S rRNA analysis (Lin *et al.*, 1994), and cultivation-based numbers for *Ruminococcus* and *Fibrobacter* are between 2 and 10%, depending on the diet (Dehority & Orpin, 1988; Hespell *et al.*, 1997; Stewart & Flint, 1989). Therefore, within the context of the published literature, the relative abundance of the ruminococci estimated with the *Ruminococcus* IV probe is reasonable, but the probe is unlikely to cover all the natural diversity represented by *R. albus* and *R. flavefaciens*.

Ruminococcus IV + *F. succinogenes* was 4% of the TCC, but significantly different ($P < 0.1$) from the total CEL (5.2% of TCC). This difference was 23% and several factors may have resulted in this discrepancy including the following. (1) Oligonucleotide probes for the *F. succinogenes* and *Ruminococcus* in this study have been designed from sequence obtained from cultured bacteria. Possible uncultured bacteria may not be covered by the probe sequences. A clone library of the addax consortia revealed *Ruminococcus* sequences that were not homologous to sequences of cultured bacteria (unpublished data). However, the *Ruminococcus* IV probe would have hybridized with these isolates. (2) The cellulolytic count probably contains non-cellulolytic bacteria. It is well known that some ruminal bacteria can feed on breakdown products (cellodextrins) of

cellulose (Russell, 1985) resulting in an artificially high CEL count. (3) The TCC does not include all ruminal micro-organisms but the universal probe used to normalize the blots would include these organisms. Even though there are obvious flaws with the comparisons made, what is apparent is that the probes employed cover a significant amount of the cultured diversity of ruminal cellulolytic bacteria. The *Ruminococcus* IV probe in particular is a valuable tool for use in ecological studies and provides relative abundance values that are in accordance with present knowledge of the ecology of the rumen.

Molecular ecological analyses of microbial ecosystems is a new and emerging field, and over the last 10 years there has been a large emphasis on the development of new and improved techniques (Stackebrandt & Goebel, 1994; Suzuki & Giovannoni, 1996; Van De Peer *et al.*, 1994; Wagner *et al.*, 1994; Zheng *et al.*, 1996). To date, the vast majority of studies which employ these techniques have been descriptive and little quantitative and statistical rigour has been applied. In contrast, macroecological studies utilize statistical methods extensively to interpret processes and populations (Link & Sauer, 1998). One of the most fundamental aspects of experimental design is an understanding of the amount of variation inherent in the ecosystem studied and the techniques used. An estimate of the variation with 16S rRNA determined populations has not to our knowledge been conducted on ruminal samples. However, Raskin *et al.* (1997) have made a systematic study of the variation associated with 16S rRNA slot blotting.

Baseline variation was determined by obtaining cultivation and 16S rRNA based data on steady-state ruminal populations. The cv for 16S rRNA based data was as high as 42.3% and in most cases was similar to that obtained with cultivation techniques (Table 4). This variance is used to calculate sample sizes required to obtain statistical differences. In this study, four samples are required to detect a 20% difference with statistical significance (95% confidence interval), but this increased to approximately five samples when sheep were fed *ad libitum* [hypothetical example; 50% increase in variance estimated from literature (Leedle *et al.*, 1982)] (Table 4). These data provide a basis for the design of rumen ecological experiments for other investigators. If, for example, an investigator wishes to detect a 10% decline in a rumen bacterial population using 16S rRNA probes, the variance associated with the technique determined in this study could be used to calculate the sample size: six samples to obtain a significant difference at the 95% confidence interval.

From our data, and the examples given above it appears that four to six samples would be adequate for most purposes, irrespective of whether the animals are fed *ad libitum* or not. The likely reason is that variation associated with slot-blot analysis of rRNA is high, and instead of taking more samples, effort should be put into decreasing the error associated with blotting. Raskin *et al.* (1997) systematically assessed this issue and suggested

that the majority of the variation is due to the stringency washes and membrane properties.

In conclusion, this study has shown that *Ruminococcus* within cluster IV of the clostridia are monophyletic and the ruminal species can be placed into either *R. albus* or *R. flavefaciens*. An important feature of this work is that an oligonucleotide probe for cluster IV ruminococci was designed and tested, and that a significant proportion of the *Ruminococcus* diversity is covered by the probe. It is, however, clear that there is likely to be undescribed *Ruminococcus* diversity not covered by the *Ruminococcus* IV probe. The *Ruminococcus* IV and *F. succinogenes* probes were used to describe the variation in steady-state ruminal populations and the number of samples required to obtain statistically significant differences between treatments could be calculated.

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