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Isolation of previously uncultured rumen bacteria by dilution to extinction using a new liquid culture medium

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ABSTRACT

A new anaerobic medium that mimics the salts composition of rumen fluid was used in conjunction with a dilution method of liquid culture to isolate fermentative bacteria from the rumen of a grass-fed sheep. The aim was to inoculate a large number of culture tubes each with a mean of <1 culturable cell, which should maximize the number of cultures that develop from a single bacterium. This minimizes the effort that has to be put into purifying the resultant cultures. Of 1000 tubes, 139 were growth positive. Of the 93 that were able to be subcultured, 54 (58%) appeared to be pure cultures. The phylogenetic placements of these 54 cultures, together with another 6 cultures obtained from a preliminary study, were determined. Using a criterion of <93% 16S rRNA gene sequence identity to a previously named bacterium as a proxy for defining a new genus, 27 (45%) of the 60 cultures belonged to 14 potentially novel genera. Many of these had 16S rRNA genes that shared >97% sequence identity to genes of uncultured bacteria detected in various gastrointestinal environments. This strategy has therefore allowed us to cultivate many novel rumen bacteria, opening the way to overcoming the lack of cultures of many of the groups detected using cultivation-independent methods.

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1. Introduction

The rumen contains a mixed community of microorganisms that ferments the feed ingested to products that are more readily utilized by the host animals. Instead of directly degrading plant material in the digestive tract, microbial degradation of the feed in the rumen, a foregut modification, results in the formation of volatile fatty acids that are absorbed across the rumen wall for use by the ruminant host (Hungate, 1966). In addition, microbial protein that is formed in the rumen is an important source of dietary nitrogen for the host animal. This complex interaction with the rumen microbes allows ruminants to feed on plant material rich in cellulose and hemicelluloses, which are poorly used by the animal itself, and so exploit a food source especially abundant in natural grasslands. Humans have domesticated some of these animals, and exploit the microbially mediated conversion of low value plant material to yield ruminant products like meat, wool, milk, and leather.

The microbial community that carries out the fermentation is made up of bacteria, archaea, fungi, and protozoa. Much of the primary attack on the plant material ingested by the animal is mediated by bacteria. The majority of these bacteria belong to only four phyla: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Spirochaetes* (Firkins and Yu, 2006). Krause and Russell (1996) surmised that the species diversity of rumen bacteria was much higher than the 22 species described as being dominant when they reviewed the literature in 1996. Surveys of the 16S rRNA genes of bacteria in the rumen of different animals have since revealed a vast diversity of bacterial genera and species that have not been characterized, largely because there are no cultured representatives (e.g., Brulc et al., 2009; Edwards et al., 2004; Firkins and Yu, 2006; Kocherginskaya et al., 2001; Koike et al., 2003; Larue et al., 2005; Nelson et al., 2003; Ozutsumi et al., 2005; Ramsak et al., 2000; Sundset et al., 2007; Tajima et al., 1999, 2007; Yang et al., 2010a,b). The roles of most of these bacteria in the fermentation that occurs in the rumen remains unknown, and so we still lack a complete understanding of this economically and socially important microbial system. Foundation researchers developed media and methods for cultivating rumen bacteria, many of which were reviewed by McSweeney et al. (2005). A wide range of rumen bacteria has been isolated using these and other methods (Stewart et al., 1997), but, until recently, the lack of simple tools for classifying the isolates limited identification of phylogenetically novel isolations among the collections obtained. The advent of routine gene sequencing technologies and the availability of large public databases for comparative analysis have allowed rapid identification of new bacterial isolates on the basis of their 16S rRNA gene sequences (Clarridge, 2004).

One of the difficulties in studying the rumen bacterial community is the sensitivity to oxygen that the majority of the species exhibit. Methods have been developed that allow the isolation and manipulation of pure cultures of rumen bacteria, using closed vessels under

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oxygen-free gas mixes, and artificial media that provide the growth requirements of the bacteria (Plugge, 2005; Sonnenwirth, 1972). These processes require some degree of skill and experience, and anyone who has isolated pure cultures of anaerobes appreciates the much slower progress and greater effort required compared to isolating most aerobes. Since the diversity of rumen bacteria is large, isolation of large numbers of cultures is especially daunting. Separation of cells of the different species that might grow, so that pure cultures can be derived, is not straight forward, since plating on gel surfaces is more difficult when working under anoxic conditions, although not impossible. If liquid cultures are used instead of solidified media, the species in a mixed inoculum that is best able to grow on that medium will dominate the culture, and will be repeatedly isolated, at the expense of species that are not pre-adapted to rapid growth in the microbiological medium.

In this study, we attempted to solve two of the problems facing microbiologists trying to isolate anaerobes from systems such as the rumen. Firstly, we developed a clear growth medium that chemically mimics the rumen milieu. Secondly, we used this medium in conjunction with a cultivation strategy that circumvented some of the problems of pure cultivation isolation of anaerobes, by attempting to avoid the enrichment of mixed cultures.

2. Materials and methods

2.1. Use of animals

The use of animals was approved by the AgResearch Grasslands Animal Ethics Committee and complied with the AgResearch Ltd. Code of Ethical Conduct for the Use of Animals in Research, Testing and Teaching, as prescribed in the Animal Welfare Act of 1999 and its amendments.

2.2. Medium preparation

A bicarbonate-buffered mineral medium supplemented with vitamins was developed and used to culture rumen bacteria. The mineral salts solution contained (in 950 ml of distilled water) 1.4 g of KH_2PO_4, 0.6 g of $(NH_4)_2SO_4$, 1.5 g of KCl, 1 ml of trace element solution SL10 (Widdel et al., 1983), 1 ml of selenite/tungstate solution (Tschech and Pfennig, 1984), and 0.4 ml of a 0.1% (w/v) resazurin solution. The mineral salts solution was mixed and then boiled under O_2-free 100% CO_2, before being cooled in an ice bath while it was bubbled with 100% CO_2. Once the mineral salts solution was cool, 4.2 g of $NaHCO_3$ and 0.5 g of L-cysteine·HCl·H_2O were added per litre. The cooled and supplemented mineral salts solution was then dispensed into Hungate tubes (16 mm dia., 125 mm long; Bellco Glass, Vineland, NJ, USA), while being gassed with 100% CO_2, at 9.5 ml of medium per tube. The tubes were closed with black butyl rubber seals and perforated plastic caps. This gave an initial headspace of 100% CO_2 in these tubes. These tubes were sterilized by autoclaving for 20 min at 121 °C. The tubes containing the medium, which was designated RM02, were stored in the dark for at least 24 h before use. When required, 0.5 ml of GenRFV (see section 2.3) was added per 9.5 ml of RM02. The final pH was 6.5.

2.3. Preparation of rumen fluid and substrates

Rumen contents were collected from two ruminally fistulated cows that had been fed pasture hay for 48 h after being on a rye-grass clover pasture. The cows were dry female Friesian crosses, 8–10 years old, and weighed about 570 kg each. Feed was withheld from the animal after 4 pm and rumen contents collected the next day at 9:30 am. The material was filtered through a double layer of cotton cheesecloth with a mesh size of approx. 1 mm (Stockinette; Cirtex Industries Ltd., Thames, New Zealand), and then fine particulate material was removed from the liquid fraction by centrifugation at 10,000 *g* for 20 min. The supernatant (rumen fluid) was stored frozen at −20 °C. The rumen fluid was thawed before use, and any new precipitates that formed were removed by centrifugation at 12,000 *g* for 15 min. The supernatant was retained and bubbled for 10 min with 100% N_2 gas, before inactivating viruses by autoclaving for 15 min in partly filled serum vials closed with blue butyl rubber stoppers and aluminium closures (20 mm diameter; Bellco) under 100% nitrogen. The anoxic conditions are to limit the formation of unwanted oxidation products during autoclaving. The autoclaved rumen fluid was then stirred under air, and 1.63 g of $MgCl_2 \cdot 6H_2O$ and 1.18 g of $CaCl_2 \cdot 2H_2O$ added per 100 ml. This formed a heavy precipitate, which was removed by centrifuging at 30,000 *g* and 4 °C for 60 min. The supernatant is the clarified rumen fluid. To each 100 ml of clarified rumen fluid were added: 0.34 g of D-glucose, 0.34 g of D-cellobiose, 0.30 g of D-xylose, 0.30 g of L-arabinose, 0.88 ml of Na L-lactate syrup (50% in water), 2 g of casamino acids, 2 g of Bacto-peptone and 2 g of yeast extract. This was well mixed and then bubbled with N_2 gas for 15 min, before being transferred through a 0.22 μm pore size Millex GP sterile filter (Millipore Corp., Bedford, MA, USA) via a sterile syringe and needle into a sterile N_2-flushed serum vial sealed with a blue butyl rubber stopper and an aluminium closure (Bellco). Two millilitres of Vitamin 10 concentrate (see section 2.4) was then added per 100 ml of preparation by syringe and needle. This clarified rumen fluid containing substrates and vitamins was designated GenRFV.

2.4. Vitamin 10 concentrate

Vitamin 10 concentrate contained 1000 ml of distilled water, 40 mg of 4-aminobenzoate, 10 mg of d-(+)-biotin, 100 mg of nicotinic acid, 50 mg of hemicalcium D-(+)-pantothenate, 150 mg of pyridoxamine hydrochloride, 100 mg of thiamine chloride hydrochloride, 50 mg of cyanocobalamin, 30 mg of D,L-6,8-thioctic acid, 30 mg of riboflavin and 10 mg of folic acid. After preparation, the solution was well mixed and then bubbled with N_2 gas for 15 min, before being transferred through a 0.22-μm pore size Millex GP sterile filter (Millipore) using a sterile syringe and needle to a sterile sealed N_2-flushed serum vial (Bellco).

2.5. Rumen samples for cultivation

All samples were collected from the same ruminally fistulated wether sheep (Romney cross, 9 years old, 80 kg). This animal was kept at the AgResearch Grasslands Campus, and allowed to feed *ad libitum* on a rye-grass clover pasture. Samples of total rumen contents were collected via the fistula on 4 different occasions. All sampling was conducted at 9.30 to 10.30 am, within 1 h of the animal being taken from the pasture. Samples were collected in 450-ml glass containers with screw-top sealable metal lids. The containers were gassed with CO_2 prior to use, and filled to the top with sample, before being capped and brought to the laboratory for use within 30 min of collection.

2.6. Cultivation of bacteria

Forty grams of rumen contents from the sheep were blended in 360 ml of RM02 under anoxic conditions using a Waring blender (Waring Products Inc., Torrington, CT, USA) with a glass chamber and tight-fitting rubber lid on the HI setting for four bursts of 20 s each, with an interval of 30 s between each burst. The glass vessel was gassed with CO_2 prior to use and during subsequent manipulations. Using a 16-gauge hypodermic needle and a sterile plastic syringe, 1 ml was transferred from the blender into a 150 ml serum bottle sealed with a blue butyl rubber stopper and an aluminium closure (Bellco) and containing 99 ml of RM02 supplemented with 5% (vol/vol) of GenRFV under a gas phase of 100% CO_2. Further serial dilutions were

then made, using sterile syringes and needles, to serum vials containing RM02 supplemented with 5% (vol/vol) GenRFV, to produce suspensions with different amount of rumen contents. The last three or four dilutions were used to inoculate 50 to 250 tubes of culture media, each with 0.1 ml of inoculum, in Hungate tubes containing 9.5 ml of RM02 supplemented with 0.5 ml of GenRFV. These tubes were incubated at 39 °C in the dark for at least 14 days. This entire procedure, from blending to incubation, was performed three times, each on a different day and using freshly prepared suspensions from the same sheep, and are designated experiments 2, 3, and 4.

An initial investigation, experiment 1, to test the effects of blending time, was performed in essentially the same way, except that five replicates each were inoculated from 13 serial 10-fold dilution steps. This was performed on samples that had been blended for different lengths of time (20 to 120 s, in 20 s steps).

Most-probable-number (MPN) counts were calculated (Hurley and Roscoe, 1983) from the tubes in which bacteria grew, which was estimated by the appearance of turbidity and by examination of the cultures by phase contrast microscopy and after Gram staining with the Burke method (Doetsch, 1981). The significance of differences between MPNs was calculated as described by Cochran (1950).

2.7. DNA extraction using a Chelex resin

Cells were harvested from 2 ml of growing culture by centrifugation at 14,000 *g* for 5 min. The supernatant was removed and 500 µl of UV-treated water were added. The pellet was resuspended, and then harvested again by centrifugation at 14,000 *g* for 5 min. DNA was extracted from a suspension of the pellet in 200 µl of 6% (w/v) Chelex 100 (InstaGene Matrix; Bio-Rad Laboratories Inc., Hercules, CA, USA) by heating at 56 °C for 30 min, then mixing by vortexing for 10 s, followed by boiling at 100 °C for 8 min and again mixing by vortexing for 10 s. Cell debris was removed from the suspension by centrifugation at 12,000 *g* for 3 min. The supernatant was diluted 1/10 in UV-treated water, and then 1 µl of that was used as the template for PCR. UV-treated water was prepared by filtering distilled water through a 0.2-µm pore size sterile filter, autoclaving the water, then irradiating 2-ml aliquots in 2-ml polypropylene tubes with UV light (254 nm, 6 W) at a distance of 100 mm for 4 h.

2.8. PCR amplification of 16S rRNA genes

Each reaction contained 5 µl of DNA polymerase Taq buffer (Roche Diagnostics NZ Ltd., Auckland, New Zealand), 2.5 mM $MgCl_2$ (Roche), 5 pmol of 27f primer (5′-GAGTTTGATCMTGGCTCAG-3′; modified from Lane (1991)), 5 pmol of 1492r primer (5′-GGYTACCTTGTTACGACTT-3′; Lane (1991)), 0.2 mM of each dNTP (Roche), 1 U of Taq DNA polymerase (Roche), and template DNA as described above, and made up to 50 µl with UV-treated water.

PCR was carried out using a Px2 thermal cycler (Thermo Electron Corp., Milford, MA, USA). After an initial denaturation step at 94 °C for 4 min, amplification occurred during 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1.5 min. Following these 35 cycles, there was a final extension step at 72 °C for 10 min. PCR products were purified using the Wizard Gel and PCR Clean-up System (Promega Corp., Madison, WI, USA), according to the manufacturer's instructions.

All DNA sequencing reactions were conducted by the Allan Wilson Centre Genome sequencing service. This service included fluorescent labelling of products using the BigDye Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit, subsequent removal of unincorporated fluorescent dideoxy NTPs by cleanup and precipitation of products, and capillary separation on an ABI3730 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA). Results were returned as ABI tracefiles. Each amplified 16S rRNA gene product was sequenced using the primers 27f (see previous discussion), 514r, 926f, 1100r, and 1492r (Lane, 1991). Sequencing data were manually checked and assembled using ContigExpress within the VectorNTI suite (Invitrogen, Carlsbad, CA, USA).

2.9. Phylogenetic analyses

The 16S rRNA gene sequences were compared with those in the GenBank database (Benson et al., 2008) using the BLASTN application (Altschul et al., 1990) to obtain an approximate phylogenetic placement by comparison to genes from classified organisms, to identify reference sequences from cultured isolates for phylogenetic tree-building, and to identify highly similar 16S rRNA genes found in culture-independent surveys. A more detailed phylogenetic analysis was carried out using the ARB software package (Ludwig et al., 2004). 16S rRNA gene sequences were aligned against references in the ARB SILVA database (SSURef version 89.1, released February 2007 [Pruesse et al., 2007]), with additional sequences added from GenBank (Benson et al., 2008). An evolutionary distance dendrogram was constructed from *Escherichia coli* positions 1500 to 41,500 (Pruesse et al., 2007) with the bacterial position variability filter provided, using the Olsen substitution model and neighbor-joining tree-building algorithm. The reliability of branching patterns was assessed separately by neighbour joining and parsimony analysis (DNAPARS, 100 bootstrap replications). 16S rRNA gene sequences of the isolates were deposited in GenBank under the accession numbers GU324357 to GU324416.

2.10. Microscopy

Agarose-coated slides were prepared and a small amount of culture added as described by Pfennig and Wagener (1986). Phase contrast photomicrographs were taken using a DM2500 microscope (Leica Microsystems, Wetzlar, Germany) with a 100× oil phase contrast objective, and the images captured digitally using Leica Application Suite software (Leica).

2.11. Calcium and magnesium determinations

Calcium and magnesium concentrations in three samples of 9.5 ml of RM02 supplemented with 0.5 ml of GenRFV were determined, each in duplicate, using inductively coupled plasma emission spectrometry (Varian Vista CCD Simultaneous Axial ICP-OES; Varian Inc., Walnut Creek, CA, USA) by the procedures described by Lee (1983).

Table 1
Concentrations of ions and total CO_2 in rumen fluid and the concentrations in medium RM02 supplemented with GenRFV.

Component	Concentration (mmol per litre)	
	In rumen fluid[a]	In RM02 + GenRFV[b]
Ca^{2+}	<1–11	3
Mg^{2+}	<1–6	4
K^+	18–93	30
Na^+	57–156	50
Cl^-	10–44	36
PO_4^{3-}	1.6–42	10
"S"[c]	1.2–2	5
NH_3/NH_4^+	1–30	10
Total CO_2	22–36	50

[a] Data from Durand and Kawashima (1980), Emanuele and Staples (1994), Evans and Davis 1966, Geishauser and Gitzel (1996), Johnson and Aubrey Jones (1989), and von Engelhardt and Hauffe (1975).
[b] Values for Ca^{2+} and Mg^{2+} by measurement; others by calculation.
[c] Calculated from total sulfur-containing compounds or from elemental analyses.

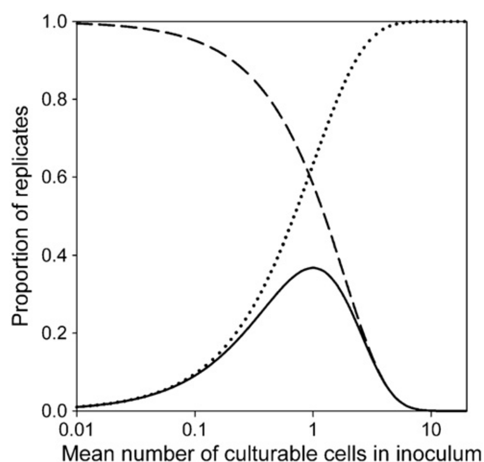

Fig. 1. The effect of inoculum size on the number of cultures derived from single cells. The proportion of replicates that grow, among all replicates inoculated, increases as the mean number of culturable cells in the inoculum increases (dotted line). The proportion of replicates that will be inoculated with a single culturable cell (solid line) is maximal when the mean inoculum size is one viable cell. The proportion of replicates that grow and are derived from a single culturable cell increases with decreasing inoculum size (dashed line). The calculations were made as described by Button et al. (1993).

3. Results and discussion

3.1. Development of growth medium

Many cultured rumen bacteria have a requirement for components in the rumen fluid, and it is probable that some of the uncultured bacteria in the rumen have similar requirements. However, rumen fluid also contains large amounts of suspended and colloidal material that often results in a cloudy growth medium when it is added as a supplement. Since growth of bacteria in liquid culture is most easily detected as an increase in the turbidity of the medium as the number of microbial cells increases, background turbidity due to the medium itself will make detection more difficult. This will be complicated even more if the concentrations of readily available energy sources in the medium are to be kept low to mimic the steady state concentrations in the rumen (Takahashi and Nakamura, 1969; Wright and Hungate, 1967), with low expected culture densities as a result.

A new medium was devised to reflect the concentrations of inorganic components of rumen fluid. The composition of rumen fluid reported in six different studies was used as a guide to develop a balanced salt solution that resulted in elemental concentrations within the reported ranges (Table 1). Substrates and other additions were made to the sterilized salts solution from separately sterilized stock solutions. This was done to minimize the formation of products in the Maillard reaction, which can inhibit the growth of rumen bacteria (Marounek et al., 1995).

Rumen fluid was clarified by precipitation of colloidal and other materials with calcium and magnesium salts. The clarified rumen fluid was then added to the growth medium. The resultant growth medium was clear and not strongly coloured. The final concentrations of calcium and magnesium in the fully supplemented medium were analyzed by plasma emission spectrometry to be 2.99 mM and 4.02 mM, respectively.

3.2. Estimation of MPN of rumen heterotrophs

In a preliminary trial (experiment 1), we found that the MPN counts were not significantly different for different blending times of 20 to 120 s ($P = 0.17$ to 1.0 for pairwise comparisons of MPNs). The tubes in this experiment were assessed for growth each day for 2 weeks, and the MPN counts did not increase any further after 7 days. We selected a blending protocol of 4 bursts of 20 s, with an interval of 30 s between each burst, for our further experiments.

We diluted fresh rumen contents on three occasions (experiments 2, 3, and 4), and used these to inoculate 50 to 250 tubes at each of 3 or 4 different inoculum levels. These were incubated for 14 days. In all three experiments, fewer cultures were positive for growth with an increasing dilution of the inocula, and <2% of the cultures inoculated with the terminal dilution were growth positive. This allowed calculation of MPNs (with lower and upper confidence limits) of 1.3×10^9 (6.3×10^8, 2.5×10^9), 3.3×10^9 (2.5×10^9, 4.4×10^9), and 3.6×10^9 (2.9×10^9, 4.4×10^9) culturable cells per g of fresh rumen contents for experiments 2, 3, and 4, respectively.

3.3. Cultivation of rumen bacteria

By diluting an inoculum to introduce a mean of ≤1 culturable cell into a tube (or other culture vessel), a significant number of cultures can be obtained that are derived from single culturable cells (Button et al., 1993). The inoculum may contain unculturable cells, but after growth of the culturable cells, these will essentially be of no consequence. Subsequent subculture will eliminate these. Some mixed cultures may also be obtained, where one microorganism will grow because of the activity of another (or both may be mutually dependent on each other), or where more than one cell initiates growth independently.

The proportion of all inoculated tubes that develop cultures derived from single cells is greatest when the mean inoculum size is 1, but at this inoculum size, only ~58% of all positive cultures will be derived from single cells (Fig. 1). If the mean inoculum size is decreased, the number of positive cultures (i.e. receiving ≥1 culturable cell) and the number of pure cultures (i.e. receiving exactly 1 culturable cell) decreases, but the proportion of positives that are

Table 2
Cultures obtained by inoculating 1000 tubes containing RM02 and GenRFV with different dilutions of fresh rumen contents (experiments 3 and 4).

Stage	Inoculum (g of fresh rumen contents)				Totals
	1.0×10^{-10}	2.5×10^{-11}	6.3×10^{-11}	1.6×10^{-12}	
Estimated culturable cells per tube[a]	0.35	0.09	0.022	0.0055	
Total tubes inoculated	375	250	250	125	1000
Tubes inoculated in expts 3/4	125/250	125/125	125/125	125/0	500/500
Total growth positive tubes	103	29	6	1	139
Unable to be subcultured	34	10	2	0	46
Mixed cultures	31	7	1	0	39
Actual “pure” cultures	38	12	3	1	54
Expected pure cultures[b]	87.3	27.2	5.9	1.0	121.4

[a] Calculated from the MPN (mean of both experiments).
[b] The expected number of pure cultures, i.e., derived from a single culturable cell in the inoculum, was calculated as from probability theory governing the dilution of culturable cells (Button et al., 1993).

potentially pure cultures, because they have arisen from only one culturable cell, increases. If the mean inoculum size is too large, too few cultures will be derived from a single culturable cell, while if it is too small, too few cultures will be obtained (Fig. 1). We therefore aimed for inocula containing an expected mean of 0.05 to 0.5 viable cell per culture tube.

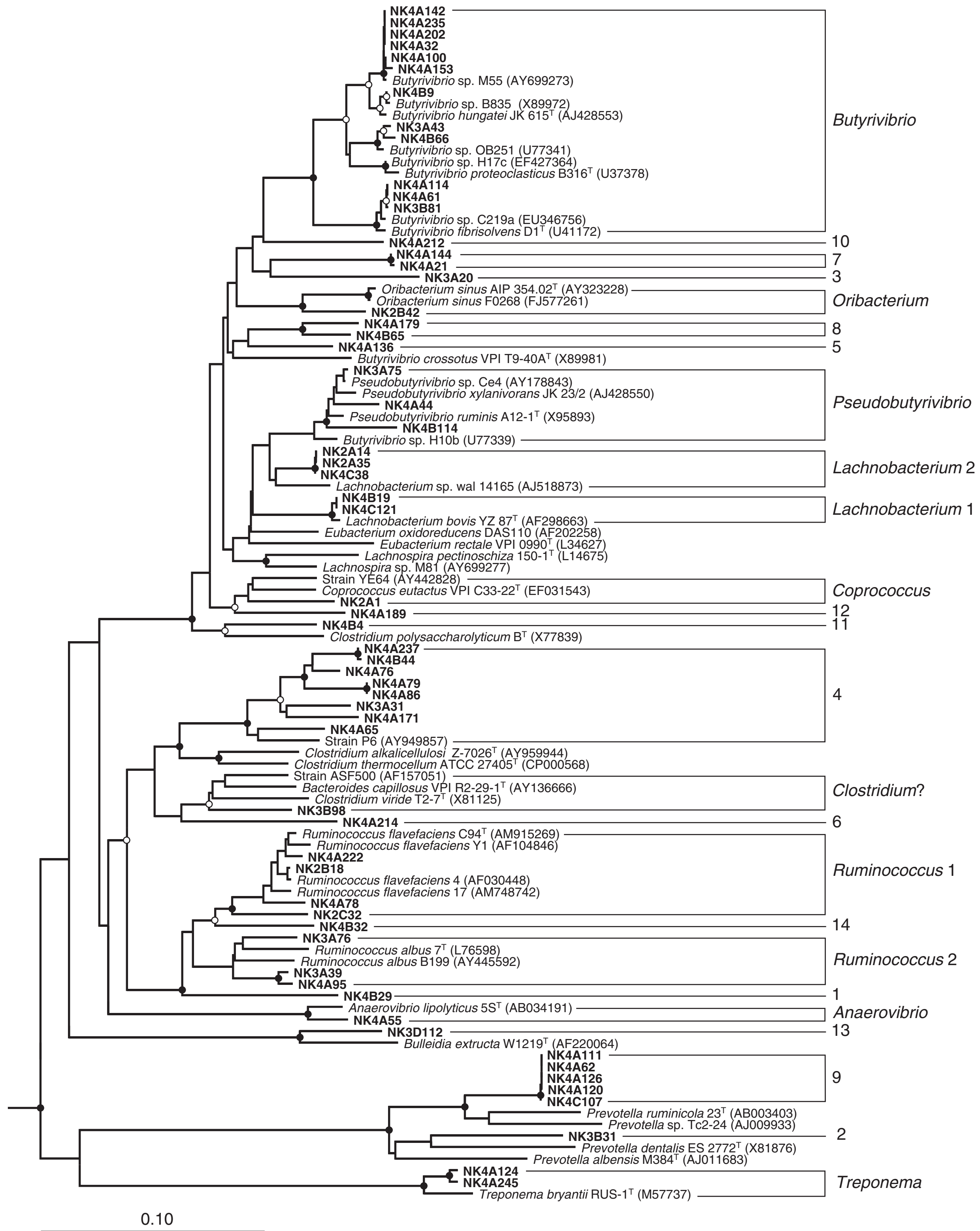

Fig. 2. Phylogenetic dendrogram illustrating the relationships of 16S rRNA gene sequences derived from the cultures obtained in this study (in bold font) and those of selected reference sequences. The putative genus-level designations are shown to the right of the dendrogram. GenBank accession numbers are given after each reference sequence designation. The superscript ^{T} indicates a type strain. The frequency with which nodes were recovered in a parsimony-based bootstrap analysis is indicated by symbols: ○, at nodes recovered in ≥85% of trees generated from bootstrapped data sets; ●, at nodes recovered in ≥99% of trees. Nodes recovered in <85% of trees have no symbol. The 16S rRNA gene sequences of *Methanobrevibacter gottschalkii* strains HO^T (GenBank accession number U55238) and PG (U55239), *Methanobrevibacter* sp. strain SM9 (AJ009958), and *Methanosphaera stadtmanae* strain MCB-3^T (CP000102) were used to root the dendrogram (not shown). Scale bar = 0.1 nucleotide substitution per nucleotide position.

Of the 1000 tubes inoculated in experiments 3 and 4, 139 cultures grew to visible turbidity (Table 2). Based on the probability theory governing the dilution of viable cells (Button et al., 1993), 121 of these could be expected to be derived from a single culturable cell, and the remaining 18 (i.e., 13%) to be derived from an inoculum containing >1 culturable cell. Forty-six (33%) of the 139 cultures did not grow following subculture. Thirty-nine of the remaining 93 cultures were mixed cultures of more than one bacterium, since they were either clearly impure when observed by microscopy or yielded mixed 16S rRNA gene sequences. This left a total of 54 isolates that seemed to be pure cultures. The proportion of mixed cultures (39 of 93 cultures, or 42%) was much higher than the 13% expected. The inoculum sizes were calculated from the number of culturable cells. This is less than the total cell number in the samples, which was estimated at 1.7×10^{10} cells/g of fresh rumen contents (I. M. van Scheepstal and P. H. Janssen, unpublished data). When multiple cells are inoculated into the same culture, the activity of some species may aid in the initiation of growth of other cells when those latter cells would not have grown if inoculated alone. This may account for the larger than expected number of mixed cultures. We have not proven that the 54 "pure" cultures are truly axenic, but we have obtained stable cultures that could be subcultured reproducibly, and that are dominated by a single morphological form and that yield unambiguous 16S rRNA gene sequences, as expected for pure cultures and cultures dominated by one strain. Not all of the cultures can be expected to grow easily on agar plates or in roll tubes, and so we sought first to identify those cultures that were potentially novel species.

There was a considerable morphological diversity among the cultures. Among the morphological forms obtained were cocci, a great variety of curved and straight rods, and tightly coiled thin cells, reminiscent of spirochetes. The cells of some cultures occurred singly, while others formed aggregates of different kinds. The cells of one culture, NK4B19, seemed to form a long cable-like matrix with which cells were associated. This morphological variation suggested an underlying phylogenetic diversity.

3.4. Identification of cultures

The 54 cultures obtained in experiments 3 and 4, summarized in Table 2, together with 6 cultures obtained the same way in experiment 2, were identified by comparative analysis of partial 16S rRNA gene sequences. The cultures fell into three of the bacterial phyla: *Firmicutes* (87%), *Bacteroidetes* (10%), and *Spirochaetes* (3%). Nearly all of the cultures that were in the phylum *Firmicutes* were affiliated with the order *Clostridiales*, with the exception of one isolate that was a member of the order *Erysipelotrichales*. In some rumens, members of the *Firmicutes* made up >80% of bacteria present (Firkins and Yu, 2006; Brulc et al., 2009). We did not determine how representative of the original samples our collection is. Instead, we concentrated on the phylogenetic novelty of the isolates we cultured.

Based on the phylogenetic placement of the cultures in coherent clusters (Fig. 2), and the sequence differences between the 16S rRNA genes of the cultures and their closest relatives (Fig. 3), we grouped the 60 cultures into 23 genus-level groups (Table 3). If the similarity of the 16S rRNA gene sequence of an isolate was >93% to that of the type strain of a recognized species, we assigned the isolate to the appropriate genus. Some authors suggest that 16S rRNA gene sequence similarities of <95 or even <98% indicate that organisms are likely to be from different genera (Clarridge, 2004; Everett et al., 1999), but we preferred to use a more conservative threshold. In fact, detailed characterization is required to see if the cultures truly differ sufficiently from described bacteria to be considered representatives of novel genera, and the definition of the genus-level groups in this study should therefore be considered to be tentative.

The process of placing cultures into genus-level groups highlighted inconsistencies in the current classification system. For example, the closest named relatives of cultures NK3B98, NKA214, and NK4B4, and of the eight cultures that grouped with strain P6, were members of the clostridial clusters III and XVIa, which include members of the genus *Clostridium* (Collins et al., 1994). However, these *Clostridium* spp. are phylogenetically distinct from those members of the genus *Clostridium*

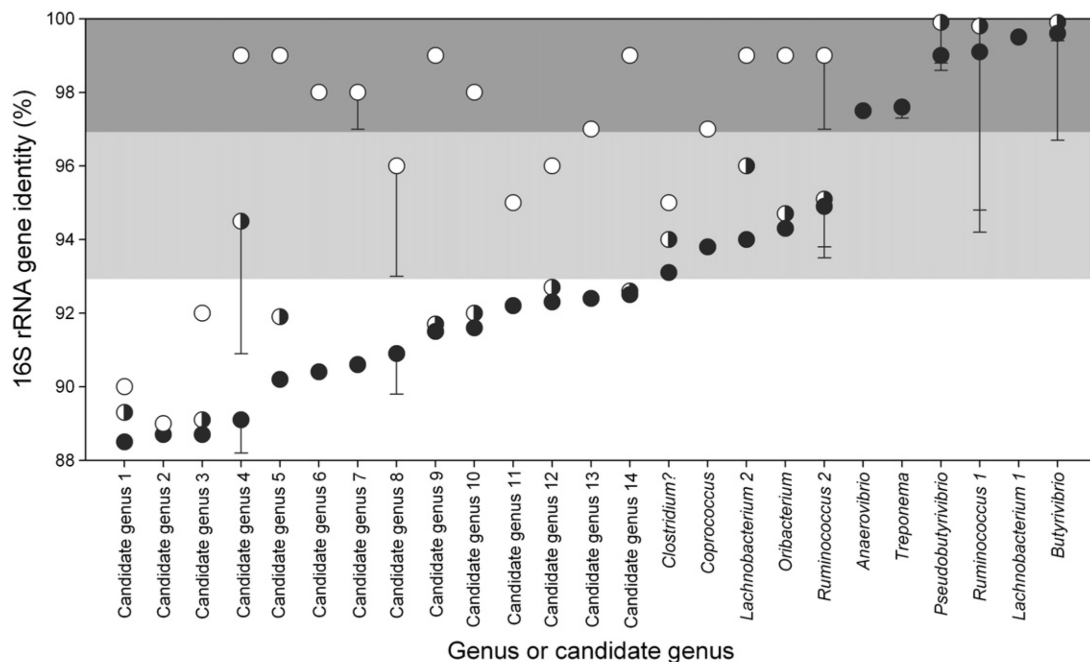

Fig. 3. The similarity of 16S rRNA gene sequences from cultures obtained in this study to the most similar homologs from type strains (●), from other cultured isolates (◑), and from culture-independent surveys of 16S rRNA genes where no type strains with >97% identity were identified (○). The thin vertical bars indicate the range when there were multiple isolates in a genus-level group. Where the bars overlap, the upper horizontal cap indicates the end of the bar associated with the upper symbol. The genus-level groups are those designated in Table 3 and shown in Fig. 2. The dark grey shading indicates sequence similarities >97%, indicative of possible membership of the same species (Stackebrandt and Goebel, 1994). The lighter grey shading indicates sequence similarities of 93–97%, our estimate of membership of the same genus but probably different species (see text).

Table 3
Taxonomic assignment of new cultures, with closest cultured relatives, identified by comparative analysis of 16S rRNA gene sequences. Sequence similarities were calculated from aligned gene sequences in ARB (Ludwig et al., 2004).

Cultures	Genus assignment	Closest cultured isolate and type strain[a] (GenBank accession)	Similarity (%)
NK4B29	Candidate genus 1	*Ruminococcus albus* B199 (AY445592)	89.3
		Ruminococcus albus 7[T] (L76598)	88.5
NK3B31	Candidate genus 2	*Prevotella dentalis* ES 2772[T] (X81876)	88.7
NK3A20	Candidate genus 3	*Butyrivibrio* sp. H17c (EF427364)	89.1
		Butyrivibrio proteoclasticus B316[T] (U37378)	88.7
NK3A31, NK4A65, NK4A76, NK4A79, NK4A86, NK4A171, NK4A237, NK4B44	Candidate genus 4	Strain P6 (AY949857)	90.9–94.5
		Clostridium alkalicellulosi Z-7026[T] (AY959944)	88.2–89.1
NK4A136	Candidate genus 5	*Lachnospira pectinoshiza* M81 (AY699277)	91.9
		Lachnospira pectinoshiza 150-1[T] (L14675)	90.2
NK4A214	Candidate genus 6	*Bacteroides capillosus* VPI R2-29–1[T] (AY136666)[b]	90.4
NK4A21, NK4A144	Candidate genus 7	*Butyrivibrio hungatei* JK 615[T] (AJ428553)	90.5–90.6
NK4A179, NK4B65	Candidate genus 8	*Eubacterium rectale* VPI 0990[T] (L34627)	89.8–90.9
NK4A62, NK4A111, NK4A120, NK4A126, NK4C107	Candidate genus 9	*Prevotella ruminicola* Tc2-24 (AJ009933)	91.6–91.7
		Prevotella ruminicola 23[T] (AB003403)	91.4–91.5
NK4A212	Candidate genus 10	*Butyrivibrio* sp. H17c (EF427364)	92.0
		Butyrivibrio proteoclasticus B316[T] (U37378)	91.6
NK4B4	Candidate genus 11	*Clostridium polysaccharolyticum* B[T] (X77839)	92.2
NK4A189	Candidate genus 12	Strain YE64 (AY442828)	92.7
		Coprococcus eutactus VPI C33-22[T] (EF031543)	92.3
NK3D112	Candidate genus 13	*Bulleidia extructa* W1219[T] (AF220064)	92.4
NK4B32	Candidate genus 14	*Ruminococcus flavefaciens* 17 (AM748742)	92.6
		Ruminococcus flavefaciens C94[T] (AM915269)	92.5
NK3B98	*Clostridium?*	Strain ASF500 (AF157051)	94.0
		Clostridium viride T2-7[T] (X81125)	93.1
NK2A1	*Coprococcus*	*Coprococcus eutactus* VPI C33-22[T] (EF031543)	93.8
NK2A14, NK2A35, NK4C38	*Lachnobacterium* 2	*Lachnobacterium* sp. wal 14165 (AJ518873)	95.9–96.0
		Lachnobacterium bovis YZ 87[T] (AF298663)	93.9–94.0
NK2B42	*Oribacterium*	*Oribacterium sinus* F0268 (FJ577261)	94.7
		Oribacterium sinus AIP 354.02[T] (AY323228)	94.3
NK3A39, NK3A76, NK4A95	*Ruminococcus* 2	*Ruminococcus albus* B199 (AY445592)	93.8–95.1
		Ruminococcus albus 7[T] (L76598)	93.5–94.9
NK3A43, NK4B66	*Butyrivibrio*	*Butyrivibrio* sp. OB251 (U77341)	99.4–99.5
		Butyrivibrio proteoclasticus B316[T] (U37378)	96.7–96.8
NK4A55	*Anaerovibrio*	*Anaerovibrio lipolyticus* 5S[T] (AB034191)	97.5
NK4A124, NK4A245	*Treponema*	*Treponema bryantii* RUS-1[T] (M57737)	97.3–97.6
NK3A75, NK4A44, NK4B114	*Pseudobutyrivibrio*	*Pseudobutyrivibrio ruminis* Ce4 (AY178843)	98.6–99.9
		Pseudobutyrivibrio ruminis A12-1[T] (X95893)	98.8–99.0
NK2B18, NK2C32, NK4A78, NK4A222	*Ruminococcus* 1	*Ruminococcus flavefaciens* 4 (AF030448)	94.8–99.8
		Ruminococcus flavefaciens C94[T] (AM915269)	94.2–99.1
NK4B19, NK4C121	*Lachnobacterium* 1	*Lachnobacterium bovis* YZ 87[T] (AF298663)	99.5
NK3B81, NK4A61, NK4A114	*Butyrivibrio*	*Butyrivibrio* sp. C219a (EU346756)	99.5
		Butyrivibrio fibrosolvens D1[T] (U41172)	99.0
NK4A32, NK4A100, NK4A142, NK4A153, NK4A202, NK4A235	*Butyrivibrio*	*Butyrivibrio* sp. M55 (AY699273)	99.5–99.8
		Butyrivibrio hungatei JK 615[T] (AJ428553)	98.3–98.6
NK4B9	*Butyrivibrio*	*Butyrivibrio* sp. B835 (X89972)	99.9
		Butyrivibrio hungatei JK 615[T] (AJ428553)	99.6

[a] Type strains are designated [T].
[b] *Bacteroides capillosus* is not a members of the phylum *Bacteroidetes*, but a member of the phylum *Firmicutes* (see Fig. 2).

that are closely related to the type species, *C. butyricum*. The new cultures, the existing species currently classified as members of the genus *Clostridium* that fall in these two clusters, and the misclassified *Bacteroides capillosus*, could be classified into new genera. Similarly, the clusters of *Prevotella* spp. and *Ruminococcus* spp. have sufficient phylogenetic depth that they may encompass several genera each, with 16S rRNA gene sequence similarities of <93% between cultures classified in the same genus (Krause et al., 1999; Willems and Collins, 1995).

3.5. Relationships with cultured bacteria

Of the 60 new cultures, 19 had no cultured relatives at all with 16S rRNA gene sequence similarities of >93%, suggesting they belonged to genera that have not yet been named and described (Table 3, Fig. 3). An additional eight cultures, in candidate genus 4, had one cultured relative that has no standing in taxonomy, and even then, this isolate, strain P6, had only 91 to 95% 16S rRNA gene sequence similarity to these eight cultures (Table 3, Figs. 2 and 3). In total, 27 of the 60 cultures fell into 14 novel candidate genus-level groups. Thus, 45% of cultures belonged to potentially novel genera, and, of the 23 genus-level groups found, nearly two-thirds (61%) were potentially new. If 16S rRNA gene sequence similarities of <96% (Everett et al., 1999) or higher (Clarridge, 2004) were used to indicate potential affiliations to different genera, more cultures could be classified as belonging to potentially novel genera. For example, at a 96% threshold to define genera, an additional five potentially novel genera could be defined, represented by nine cultures.

A further 11 cultures had 16S rRNA gene sequences sufficiently different from those of validly named species (<97%) to allow us to suggest that they are likely to be members of novel species (Table 3, Figs. 2 and 3). Stackebrandt and Goebel (1994) suggested that bacteria with 16S rRNA gene sequences of <97% identity are likely to belong to different species, based on the correlation of 16S rRNA gene sequence divergence and whole-genome similarities based on DNA–DNA hybridization. Other authors have suggested other threshold values, up to 99%, as the cutoff between species (Clarridge, 2004).

The remaining 22 of the 60 cultures had 16S rRNA gene sequences that shared >97% gene sequence similarity with 16S rRNA genes from validly described named species of bacteria (Table 3, Figs. 2 and 3).

These were members of the genera *Anaerovibrio, Butyrivibrio*, *Lachnobacterium, Pseudobutyrivibrio*, *Ruminococcus*, and *Treponema*. Some of these cultures may represent new species, but the degree of 16S rRNA gene sequence similarity (>97%) means that they may also be members of currently described species. How many of these 22 cultures truly represent new species can only be determined using other genetic and phenotypic characteristics, and would require detailed comparison to the type strains of related species.

3.6. Relationships with molecularly detected bacteria

No close matches (i.e., no identities >93%) to the 16S rRNA genes of cultures in the candidate genera 1, 2, and 3 were found in GenBank (Fig. 3). These three cultures seem to represent new, as-yet un-named genera and appear to have no close relatives detected in culture-independent analyses of microbial communities. The cultivation of such bacteria suggests a hidden phylogenetic diversity in the rumen hitherto undetected by molecular ecology or cultivation-based approaches.

Twenty of the cultures, which fell into eight of the 14 novel candidate genera, were close relatives of bacteria detected in surveys of 16S rRNA genes in a number of different intestinal systems. The 16S rRNA genes of these 20 cultures shared >97% sequence identity with genes detected directly in gut environments, even though the differences to the closest cultured and named relatives were much greater (Fig. 3). Close relatives of these were detected in many different gastrointestinal environments (Brulc et al., 2009; Ley et al., 2008; Nelson et al., 2003; Ozutsumi et al., 2005; Sundset et al., 2007; Tajima et al., 1999, 2007; Yang et al., 2010a,b). These cultures are therefore representative of molecularly detected bacteria that are known to occur in gastrointestinal environments, particularly the rumen, but for which no isolates have been reported previously.

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