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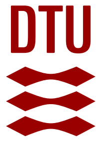

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Anaerobe

ENVIRONMENTAL MICROBIOLOGY

Rumen Bacterial Community Transition During Adaptation to High-grain Diet

Kiyoshi Tajima[1], Shozo Arai[2], Koretsugu Ogata[1], Takafumi Nagamine[1], Hiroki Matsui[1], Mutsumi Nakamura[1], Rustam I. Aminov[3]* and Yoshimi Benno[1,4]

[1]STAFF-Institute, Rumen Microbiology Research Team, 446-1, Kamiyokoba, Tsukuba, Ibaraki 305-0854, Japan; [2]National Institute of Animal Health, Department of Pathology and Physiology, Kannondai, Tsukuba, Ibaraki 305-0856, Japan; [3]Department of Animal Sciences, University of Illinois, Urbana, IL 61801, U.S.A; [4]Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-0198, Japan

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Key Words: Metabolic acidosis, 16S rDNA library, phylogenetic analysis, community structure

Transitional changes of the ruminal bacterial community structure in cows during the switch from roughage to high-grain diet were monitored by PCR amplification and sequencing of 16S rDNA clone libraries. In total, one hundred fifty 16S rDNA sequences of almost full-length (1.4 kb) were analysed from three libraries prepared from the rumen fluid on day 0, 3, and 28 of switch to high-grain diet. In the first library (day 0, hay diet) of 51clones, 90.2% of sequences were belonging to the low G+C Gram-positive bacteria (LGCGPB) phylum, with the minor inclusion of the *Cytophaga-Flavobacter-Bacteroides* (CFB; 3.9%), *Proteobacteria* (3.9%) and high G+C Gram-positive bacteria (HGCGPB; 2.0%) phyla-related sequences. Six LGCGPB sequences were clustered with the well-known cellulolytics of the rumen, *Ruminococcus flavefaciens* and *R. albus*. In the second library (day 3 of high-grain diet) of 58 clones, the LGCGPB-related sequences still dominated (72.4%), albeit being represented by other species than in the first library. In particular, this library was enriched by representatives of *Selenomonas-Succiniclasticum-Megasphaera* group IX (17.2%), lactobacilli-(6.9%) and *Butyrivibrio fibrisolvens* lineage 3-related (8.6%) sequences. Other phyla were represented by CFB (22.4%) and HGCGPB (3.4%). In the third library (day 28 of high-grain diet) of 41 clones, 95% of sequences fell into the LGCGPB phylum. About half of them (46%) were clustered within the *Selenomonas-Succiniclasticum-Megasphaera* group in *Clostridium* cluster IX. No HGCGPB-related sequences were detected and CFB was represented by only single clone. No *Streptococcus bovis*-related sequences were detected in any of the three clone libraries.

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*Address correspondence to: Dr R.I. Aminov, Department of Animal Sciences, University of Illinois, 1207 W Gregory Dr., Urbana, IL 61801, U.S.A. E-mail: aminov@uiuc.edu

Introduction

The dependence of the rumen bacterial community structure on an animal's diet is a well-documented

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fact [1]. Most profound changes in the community structure occur during the weaning period and, in adult animals, during the dietary changes such as the switch from roughage to high-grain diet or transfer from hay/concentrate to pasture feeding. The switch to elevated carbohydrate levels in the diet, which is used to improve the productivity of cattle, often induces acidosis, with pH decline and a large increase in lactic acid levels in the rumen. Previous studies on the subject have focused on identifying lactate-producing and lactate-utilizing bacteria with the aim of selective inhibition of the former to alleviate clinical symptoms ranging from loss of appetite to death. The change of the community structure under acidosis conditions and during the stepwise adaptation to high-concentrate diets has been established in several studies [2–5]. Variations in the microbial population structure can be detected at different levels of acidosis, ranging from the acute to mild acidosis states [6]. In summary, these and other cultivation-based studies have demonstrated that *Streptococcus bovis,* which easily ferments starch and produces lactate, is a major culprit in lactic acidosis. Lactate-utilizing bacteria, *Selenomonas ruminantium* and *Megasphaera elsdenii*, which are able to tolerate low pH conditions, have been shown to be effective in preventing acid accumulation in the rumen [3,4]. However, the methodology of characterization of transient microflora during the development of acidosis has been essentially cultivation-based and, therefore, could have missed some important contributors to the process. Recent molecular analyses of rumen bacterial communities have demonstrated the presence of major groups of yet-to-be-cultivated rumen bacteria [7,8]. These were essentially static snapshots of the system and no functional role could be assigned to the uncultivated groups. In this work, the same molecular technology was applied to the dynamic system undergoing transition from one steady-state phase to another. That is, the succession of predominant rumen bacterial phylogroups during the switch from roughage to high-grain diets.

Materials and Methods

Sampling

Samples were obtained from eight fistulated dry Holstein cows (with the average body weight 560 ± 15 kg) kept at the National Institute of Animal Health (Tsukuba, Japan). Before the experiment, animals were maintained on a basal diet consisting of 3.5 kg hay, 1 kg hay-cube and 1.5 kg concentrate fed twice a day. The composition of the concentrate was: 24% of ground wheat, 20% of corn, 20% of wheat bran, 10% of soybean meal, 10% of linseed meal, 6% of gluten feed, 5% of rice bran, 4% of calcium carbonate, 0.5% of dicalcium phosphate and 0.5% of microelements and vitamins. On day 0, five animals were switched to a high grain diet for 4 weeks. This regimen consisted of two feedings, in the morning (0.5 kg of hay, 2.4 kg of concentrate, and 3.6 kg of barley) and evening (2.4 kg of concentrate and 3.6 kg of barley). The remaining three control animals continued on the basal diet. On the first day of the high-grain diet feeding, the representative rumen content samples were obtained anaerobically via fistula at 0, 1, 3, 6, and 9 h. Afterwards, the samples were taken once on day 2, 3, 5, 7, 10, 14, 21, and 28, before the morning feeding (e.g. 16 h after last feeding). The samples were squeezed though two layers of cheesecloth and immediately used for DNA extraction. Libraries 1, 2 and 3 were prepared from the rumen fluid samples collected on day 0, 3, and 28, respectively.

Chemical assay of rumen fluid

The pH of the rumen fluid was measured immediately upon collection with a pH meter (model PHL-10, DDK Co., Japan). Rumen fluid samples (2 ml) for determination of total and individual volatile fatty acids (VFA) (except lactate) were mixed with 1 ml of 3N sulfuric acid solution containing 12% m-phosphoric acid and centrifuged at 3000 rpm for 15 min. Supernatants were frozen at −20°C for later analysis. Concentration of VFA was determined by gas chromatography (model GC-4BM, Shimadzu, Japan) using crotonic acid as an internal standard [9,10]. The column temperature was set at 125°C and that of the injector and detector at 200°C. Nitrogen was used as a carrier gas, with flow rate of 40 ml/min. Rumen fluid samples for determination of lactic acid concentration were mixed with trichloroacetic acid to a final concentration of 10% and centrifuged at 3000 rpm for 15 min. Supernatants were frozen at −20°C for later analysis. Lactate concentration was assayed enzymatically [11,12] with the D-/L-lactate F-kit (Roche Diagnostics, Germany) according to the manufacturer's protocol. Total concentration of lactate in Table 1 is the sum of concentration of both isomers.

DNA extraction

To minimize animal-to-animal variations, the aliquots of rumen fluid from five animals were mixed before DNA extraction. Total DNA from rumen fluid was extracted as described by Whitford *et al.* [7] using a mini Bead-Beater (Biospec Products, Bartlesville, USA) for cell lysis. 500 μL of rumen fluid was mixed

Table 1. Characteristics of rumen fluid during transition from hay to high-grain diet

Sample	Ruminal pH		Total lactate (mM)		Total VFA (mM)		Percent of ruminal volatile fatty acid/ total VFA: Acetate (%)		Propionate (%)		Butyrate (%)	
	Ctrl[a]	HGD[b]	Ctrl	HGD	Ctrl	HGD	Ctrl	HGD	Ctrl	HGD	Ctrl	HGD
0h	6.9±0.5[c]	7.0±0.3	0.9±0.1	0.8±0.3	83.0±0.5	85.8±0.3	67.8±1.6	69.1±1.6	16.4±0.8	16.5±1.2	10.8±2.2	9.8±0.9
1h	6.7±0.3	6.5±0.3	1.3±0.3	1.3±0.3	109.2±0.3	107.3±0.3	66.4±0.7	65.8±2.2	18.0±0.8	18.3±1.6	10.7±1.3	11.6±0.8
3h	6.4±0.2	6.2±0.6	1.3±0.4	1.2±0.5	107.1±0.2	113.4±0.6	66.2±1.2	64.2±1.5	17.6±0.2	18.6±1.1	11.2±1.1	12.9±1.1
6h	6.7±0.4	6.0±0.3*	1.2±0.1	1.1±0.5	94.0±0.4	109.9±0.3*	66.7±1.0	64.1±1.7*	16.8±0.6	18.7±1.6	11.9±1.0	13.3±1.2
9h	6.2±0.3	5.5±0.2**	1.2±0.1	1.2±0.4	109.5±0.3	124.9±0.2	65.5±0.8	60.3±1.7**	18.1±0.7	20.1±1.7	11.7±0.8	16.0±1.6**
1d	6.9±0.5	6.1±0.4	1.2±0.3	2.3±2.9	89.4±0.5	106.2±0.4*	67.9±1.8	59.1±3.3**	16.2±0.5	17.9±1.8	11.0±2.8	18.2±3.1*
2d	6.9±0.6	6.1±0.8	1.4±0.2	1.8±0.2*	90.5±0.6	83.1±0.8	67.4±2.1	61.7±10.6	16.9±1.2	18.8±4.2	10.9±2.3	14.5±9.2
3d	7.2±0.3	6.4±0.4*	1.0±0.9	2.0±1.1	83.6±0.3	91.9±0.4	67.7±1.9	57.0±7.0*	16.1±0.3	21.9±4.9	10.6±1.3	13.8±5.2
5d	7.1±0.1	6.8±0.4	1.3±0.6	2.4±0.7	82.4±0.1	84.7±0.4	69.0±2.7	56.0±6.2*	15.9±0.3	24.3±7.8	10.5±0.9	11.6±5.3
7d	7.6±0.4	6.4±0.8	0.6±0.0	3.1±2.2	77.5±0.4	91.2±0.8	65.6±6.1	55.7±6.5	18.5±2.8	24.8±9.5	10.1±1.2	12.2±4.5
10d	7.2±0.3	6.5±0.6	1.0±0.1	2.2±1.3	69.4±0.3	87.1±0.6	67.1±4.6	49.3±4.4**	16.4±1.3	35.6±6.9	10.4±0.8	7.4±3.3
14d	7.5±0.2	7.0±0.2*	1.3±0.3	2.2±0.4*	63.7±0.2	66.7±0.2	68.9±1.0	51.3±7.4**	15.6±0.6	31.8±9.6	10.4±1.0	8.9±4.1
21d	7.3±0.1	6.7±0.7	1.2±0.1	2.6±1.8	67.7±0.1	71.5±0.7	68.8±1.6	56.0±8.0*	15.3±0.6	24.0±10.3	11.1±1.9	10.3±3.9
28d	7.1±0.1	7.0±0.5	1.2±0.3	2.1±1.2	77.9±0.4	61.8±0.5	67.6±0.5	53.9±7.8	8.9±11.1	27.2±9.0	11.8±1.6	10.0±3.5

[a] Ctrl, control group.
[b] HGD, high-grain diet group.
[c] Values are means ± S.D.
*$P < 0.05$.
**$P < 0.01$.

with an equal volume of TE buffered phenol (pH 8.0) in a 2 mL tube with 0.5 g of glass beads. After adding 20 µl of 20% SDS, the tubes were shaken three times for 2 min with 3 min intervals on ice. Then the tubes were centrifuged at 15000 rpm for 5 min. The supernatant was transferred to a fresh tube, extracted with buffered phenol and isopropanol precipitated. Nucleic acids were dissolved in TE buffer (pH 8.0) and treated with DNase-free RNase. Samples were re-extracted with buffered phenol, ethanol precipitated and dissolved in TE buffer.

PCR, cloning and sequencing

Protocols for PCR amplification of 16S rDNA molecules and for cloning and sequencing of amplified products were described earlier [8]. In brief, primers used for PCR amplification of 16S rDNA were 27f and 1544r [13]. PCR was performed with the ExTaq kit (TAKARA SHUZO, Japan) according to the manufacturer's directions. To avoid the preferential amplification effect of the standard number of cycles [14], the number of cycles was reduced to 10. The PCR program comprised 3 min of initial denaturation at 95°C, then ten cycles of 30 s at 95°C, 30 s at 60°C, 90 s at 72°C, followed by the final 7 min extension at 72°C. PCR products were directly cloned into the TA Cloning Kit (Invitrogen, USA) and the recombinant colonies were randomly picked up. The recombinant plasmids were extracted using the alkaline lysis miniprep method [15]. Cycle sequencing was performed with the ThermoSequenase kit (Amersham, Berkshire, UK). The sequencing reaction products were read on a LI-COR M4000L automated DNA sequencer (LI-COR, USA). Sequences were read on both strands.

Sequence analysis

Sequence assemblage was done using the Auto Assembler™ program v.1.4 (Applied Biosystems, USA). Then sequences were tested for chimera formation with on-line CHIMERA_CHECK v.2.7 program provided by the Ribosomal Database Project (http://www.cme.msu.edu/rdp). All reference sequences were obtained from the GenBank database. Homology search against database entries was done using on-line BLAST search [16]. Sequences were aligned using the multiple sequence alignment program CLUSTAL W ver 1.74 [17]. Gaps and positions with ambiguities were excluded from the phylogenetic analysis. Phylogenetic analysis was performed using the neighbor-joining method [18]. Statistical significance of branching was verified by bootstrap analysis [19] involving the construction and analysis of 1000 trees

from the bootstrapped data sets. Operational taxonomic units (OTU) were defined as having more than 97% sequence similarity. This value discriminates between bacterial species previously defined on the basis of DNA-DNA reassociation values [20].

Nucleotide sequence accession numbers

Nucleotide sequences have been deposited in the GenBank database under the accession numbers AB034001–AB034150.

Results

pH and total and individual VFA concentrations of rumen fluid

The shift from roughage to high-grain diet was accompanied by a number of changes in the chemical composition of the rumen fluid. In general, the average value of pH was lower in the grain-fed group, although this was statistically significant only for four measurements (Table 1). Broad sample variations among experimental and control animals precluded more accurate conclusions. Lactate concentration was generally low in both diet groups and the statistically significant increase of lactate concentration on high-grain diet was detected only in two samples (Table 1). Concentration of total VFAs in the rumen fluid tended to be higher on the grain diet, although extensive animal-to-animal variation proscribed statistical significance (Table 1). Beginning from day 0 (6 h sample), the proportion of acetate among total VFAs was consistently lower in the high-grain diet group in comparison with control (Table 1). On the contrary, the propionate ratio in total VFAs was generally higher on the high-grain diet. The proportion of butyrate in the beginning of experiment significantly increased, with peak on day 1, then stabilized approximately at the control levels on day 5 and further (Table 1).

Similarity with database sequences

In total, one hundred fifty clones were analysed from three libraries prepared from the rumen fluid on day 0, 3 and 28 of the experiment. Fifty-one clones were analysed from library 1 (hay diet before the switch to high-grain diet). DNA similarity analysis showed that twenty-one of them could be assigned to the OTUs represented in databases (Table 2). However, only a single clone (4C0d-18) had the similarity with a cultured isolate, *Selenomonas ruminantium*, the other twenty clones were related to sequences retrieved in other *in vitro* experiments (Table 2). The remaining thirty sequences in this library had a similarity level with the database sequences of less than 97%.

Fifty-eight clones were analyzed in the second library (day 3 on high-grain diet). This library had more matches (in the similarity range of 97 to 100%) with cultivated rumen isolates than the other two libraries (Table 3). These OTUs included eighteen sequences and were defined as belonging to *Prevotella ruminicola* (four clones: 4C3d-1, 4C3d-15, 4C3d-18, and 6C3d-16), *P. bryantii* (three clones: 3C3d-16, 5C3d-4, and 5C3d-8), *Lactobacillus ruminis* (three clones: 3C3d-2, 4C3d-19, and 5C3d-14), *Megashaera elsdenii* (three clones: 3C3d-7, 3C3d-18, and 5C3d-9), *Butyrivibrio fibrisolvens* (three clones: 3C3d-17, 4C3d-7, and 4C3d-11), and *Bifidobacterium thermophilum* (two clones: 3C3d-20 and 4C3d-16). The other twelve clones in the same similarity range gave database matches with uncultured sequence entries (Table 3). The remaining twenty-eight sequences in this library had the similarity with database sequences of less than 97% (Table 3).

In library 3 (day 28 from the switch to high grain diet), from forty-one clones analysed, the only sequence similar to the cultivated rumen bacteria was 2C28d-3, with close match to *S. ruminantium* (Table 4). Two other clones (4C28d-15 and 4C28d-23) gave hits in the same similarity range with *in vitro*-retrieved rumen bacterial sequences (Table 4). The remaining thirty eight sequences had less than 97% of similarity with database entries.

Phylogenetic analysis

Succession of main bacterial phyla during adaptation to high-grain diet was evaluated by phylogenetic analyses of the three libraries. In the first library (day 0, hay diet) about 90.2% of clones were belonging to the low G+C Gram-positive bacterial (LGCGPB) phylum, with the minor inclusion of the *Cytophaga-Flavobacter-Bacteroides* (CFB) (3.9%), proteobacteria (3.9%) and high G+C Gram-positive bacterial (HGCGPB) (2.0%) phyla-related sequences (Figure 1). Among LGCGPB, six sequences (4C0d-7, 4Cd0-11, 3C0d-5, 4C0d0-16, 3C0d-15, and 4C0d-6) were clustered with the well-known cellulolytics of the rumen, *Ruminococcus flavefaciens* and *R. albus* (Figure 1). For the majority of LGCGPB sequences, however, the situation resembles our previous study [8], where a few sequences were clustered with known cultivated rumen isolates.

In the second library (day 3 on high-grain diet), the LGCGPB-related sequences still dominated (72.4%), albeit being represented by other species than in the first library (Figure 2). In particular, this library was

Table 2. Similarity values of 16S rDNA sequences from library of day 0

Clone	Phylum	Nearest relative	Similarity (%)
3C0d-1	LGCGPB[a]	**URB[b] 12-74 (16S rDNA clone)**	**99.1**
3C0d-2	HGCGPB[c]	*Atopobium parvulus*	93.0
3C0d-3	LGCGPB	URB RFN10 (16S rDNA clone)	91.9
3C0d-4	LGCGPB	**URB RF35(16S rDNA clone)**	**99.6**
3C0d-5	LGCGPB	*Ruminococcus flavefaciens*	96.6
3C0d-6	LGCGPB	**URB JW13 (16S rDNA clone)**	**97.5**
3C0d-7	LGCGPB	URB RF21 (16S rDNA clone)	91.9
3C0d-8	LGCGPB	*Termitobacter aceticus*	92.4
3C0d-9	LGCGPB	**URB 12-116 (16S rDNA clone)**	**98.4**
3C0d-10	LGCGPB	URB RF35 (16S rDNA clone)	96.2
3C0d-11	LGCGPB	URB RC15 (16S rDNA clone)	91.7
3C0d-12	LGCGPB	**URB RFN8 (16S rDNA clone)**	**98.5**
3C0d-14	LGCGPB	**URB RC6 (16S rDNA clone)**	**97.7**
3C0d-15	LGCGPB	*Ruminococcus albus*	95.4
3C0d-16	LGCGPB	**URB RF35 (16S rDNA clone)**	**97.5**
3C0d-17	LGCGPB	URB RF21 (16S rDNA clone)	91.5
3C0d-19	LGCGPB	**URB RFN7 (16S rDNA clone)**	**97.3**
3C0d-20	LGCGPB	**URB RFN65 (16S rDNA clone)**	**98.1**
4C0d-2	Proteobacteria	NA[d]	
4C0d-3	Proteobacteria	NA	
4C0d-5	LGCGPB	**URB JW13 (16S rDNA clone)**	**98.5**
4C0d-6	LGCGPB	*Ruminococcus albus*	94.8
4C0d-7	LGCGPB	**URB RCP6 (16S rDNA clone)**	**97.5**
4C0d-8	CFB[e]	NA	
4C0d-9	LGCGPB	**URB RFN71 (16S rDNA clone)**	**98.2**
4C0d-10	LGCGPB	**URB RC30 (16S rDNA clone)**	**98.8**
4C0d-11	LGCGPB	**URB RCP6(16S rDNA clone)**	**99.6**
4C0d-12	LGCGPB	*Eubacterium ruminantium*	96.0
4C0d-13	LGCGPB	*Ruminococcus callidus*	90.9
4C0d-14	LGCGPB	URB RFN8 (16S rDNA clone)	90.6
4C0d-15	LGCGPB	URB RC5 (16S rDNA clone)	92.3
4C0d-16	LGCGPB	*Ruminococcus albus*	95.8
4C0d-17	LGCGPB	*Clostridium cellulolyticum*	90.0
4C0d-18	LGCGPB	***Selenomonas ruminantium***	**98.9**
4C0d-19	LGCGPB	*Clostridium* sp.	92.5
5C0d-2	LGCGPB	URB RF21 (16S rDNA clone)	91.5
5C0d-3	LGCGPB	URB RF21 (16S rDNA clone)	91.3
5C0d-4	LGCGPB	URB RC31 (16S rDNA clone)	96.8
5C0d-6	LGCGPB	URB RCP6 (16S rDNA clone)	91.0
5C0d-7	LGCGPB	URB RCP6 (16S rDNA clone)	91.0
5C0d-8	LGCGPB	**URB RF35 (16S rDNA clone)**	**98.1**
5C0d-9	LGCGPB	**URB RFN7(16S rDNA clone)**	**99.8**
5C0d-10	LGCGPB	**URB JW13 (16S rDNA clone)**	**97.2**
5C0d-11	CFB	**URB 30-12 (16S rDNA clone)**	**99.4**
5C0d-12	LGCGPB	**URB 12-124 (16S rDNA clone)**	**98.4**
5C0d-13	LGCGPB	*Eubacterium ruminantium*	93.8
5C0d-14	LGCGPB	URB RFN8 (16S rDNA clone)	96.7
5C0d-16	LGCGPB	Uncultured eubacterium WCHB1-54	92.6
5C0d-19	LGCGPB	**URB 12-74 (16S rDNA clone)**	**98.9**
5C0d-23	LGCGPB	*Clostridium* sp.	91.7
5C0d-24	LGCGPB	*Mitsuokella multiacida*	94.7

[a]LGCGPB, low G+C Gram-positive bacteria.
[b]URB, unidentified rumen bacterium.
[c]HGCGPB, high G+C Gram-positive bacteria.
[d]NA, not available.
[e]CFB, *Cytophaga-Flavobacter-Bacteroides* group.
Database sequences with >97% similarity are shown in bold.

enriched by lactobacilli- (6.9%) and *Butyrivibrio fibrisolvens* lineage 3-related (8.6%) sequences. Conversely to the first library, no ruminococci-related sequences were detected in this library and it was enriched by sequences related to the *Selenomonas-Succiniclasticum-Megasphaera* group (clones 5C3d-10, 3C3d-5, 3C3d-10, 3C3d-9, 4C3d-9, 6C3d-34, 6C3d-30, 3C3d-7, 3C3d-18, and 5C3d-9). No sequences were affiliated with *S. bovis*. The HGCGPB phylum was represented by two sequences (3C3d-20 and 4C3d-16) clustered with *Bifidobacterium lactis*. There was also the considerable increase in the number of CFBs (22.4%), which were mainly represented by ruminal prevotellas.

In the third library (day 28 on high-grain diet), the LGCGPB phylum was represented by 95% of clones.

Table 3. Similarity values of 16S rDNA sequences from library of day 3

Clone	Phylum	Nearest relative	Similarity (%)
3C3d-1	CFB[a]	**URB[b] RCP19 (16S rDNA clone)**	**99.5**
3C3d-2	LGCGPB[c]	***Lactobacillius ruminus***	**99.5**
3C3d-3	LGCGPB	*Ruminococcus bromii*	94.5
3C3d-5	LGCGPB	*Selenomonas ruminantium*	96.1
3C3d-6	LGCGPB	*Eubacterium* sp. C2	93.2
3C3d-7	LGCGPB	***Megasphaera elsdenii***	**99.3**
3C3d-8	LGCGPB	*Eubacterium rectale*	95.1
3C3d-9	LGCGPB	*Selenomonas ruminantium*	95.9
3C3d-10	LGCGPB	*Selenomonas ruminantium*	96.0
3C3d-12	LGCGPB	*Lactobacillus pontis*	93.3
3C3d-13	LGCGPB	**URB RFN71 (16S rDNA clone)**	**99.8**
3C3d-14	LGCGPB	**URB RC21 (16S rDNA clone)**	**99.8**
3C3d-15	LGCGPB	*Eubacterium cellulosolvens*	91.3
3C3d-16	CFB	***Prevotella bryantii***	**99.6**
3C3d-17	LGCGPB	***Butyrivibrio fibrisolvens***	**98.2**
3C3d-18	LGCGPB	***Megasphaera elsdenii***	**98.8**
3C3d-19	LGCGPB	*Butyrivibrio fibrisolvens*	95.3
3C3d-20	HGCGPB[d]	***Bifidobacterium thermophilum***	**98.1**
4C3d-1	CFB	***Prevotella ruminicola***	**99.7**
4C3d-2	LGCGPB	**URB 12-116 (16S rDNA clone)**	**98.4**
4C3d-3	HGCGPB	*Atopobium parvulus*	94.9
4C3d-5	CFB	URB JW31 (16S rDNA clone)	95.1
4C3d-6	CFB	*Prevotella bivia*	92.4
4C3d-7	LGCGPB	***Butyrivibrio fibrisolvens***	**98.6**
4C3d-8	LGCGPB	*Eubacterium cellulosolvens*	92.1
4C3d-9	LGCGPB	**URB RFN69 (16S rDNA clone)**	**97.8**
4C3d-10	CFB	*Prevotella brevis*	95.8
4C3d-11	LGCGPB	***Butyrivibrio fibrisolvens***	**98.6**
4C3d-12	LGCGPB	*Eubacterium* sp. C2	93.2
4C3d-14	LGCGPB	*Eubacterium rectale*	95.0
4C3d-15	CFB	***Prevotella ruminicola***	**98.8**
4C3d-16	HGCGPB	***Bifidobacterium thermophilum***	**98.0**
4C3d-17	LGCGPB	*Eubacterium rectale*	95.1
4C3d-18	CFB	***Prevotella ruminicola***	**99.7**
4C3d-19	LGCGPB	***Lactobacillus ruminus***	**99.5**
4C3d-20	CFB	**URB RFN6 (16S rDNA clone)**	**98.0**
5C3d-4	CFB	***Prevotella bryantii***	**99.8**
5C3d-5	LGCGPB	*Butyrivibrio fibrisolvens*	92.0
5C3d-8	CFB	***Prevotella bryantii***	**99.7**
5C3d-9	LGCGPB	***Megasphaeroa elsdenii***	**99.6**
5C3d-10	LGCGPB	*Selenomonas ruminantium*	96.1
5C3d-11	LGCGPB	**URB RC21 (16S rDNA clone)**	**99.8**
5C3d-13	LGCGPB	**URB 12-116 (16S rDNA clone)**	**98.3**
5C3d-14	LGCGPB	***Lactobacillus ruminus***	**99.5**
5C3d-16	LGCGPB	*Butyrivibrio fibrisolvens*	92.2
5C3d-17	LGCGPB	URB 12-124 (16S rDNA clone)	92.5
5C3d-20	LGCGPB	*Butyrivibrio fibrisolvens*	92.0
6C3d-10	LGCGPB	*Rumincoccous bromii*	96.1
6C3d-11	LGCGPB	**URB 12-116 (16S rDNA clone)**	**98.6**
6C3d-12	LGCGPB	*Rumincoccous bromii*	96.2
6C3d-13	LGCGPB	*Rumincoccous* sp.	90.4
6C3d-16	CFB	***Prevotella ruminicola***	**97.5**
6C3d-25	CFB	**URB RC24 (16S rDNA clone)**	**97.0**
6C3d-27	LGCGPB	*Rumincoccous bromii*	96.2
6C3d-30	LGCGPB	**URB RF4 (16S rDNA clone)**	**97.2**
6C3d-31	LGCGPB	URB RF26 (16S rDNA clone)	95.8
6C3d-33	LGCGPB	*Rumincoccous bromii*	96.2
6C3d-34	LGCGPB	**URB RR4 (16S rDNA clone)**	**98.4**

[a]CFB, *Cytophaga-Flavobacter-Bacteriodes*
[b]URB, unidentified rumen bacterium.
[c]LGCGPB, low G+C Gram-positive bacteria.
[d]HGCGPB, high G+C Gram-positive bacteria.
Database sequences with >97% similarity are shown in bold.

About half of them (46%) clustered within the *Selenomonas-Succiniclasticum-Megasphaera* group (Figure 3). No HGCGPB-related sequences were detected and CFBs were represented by the only clone, 4Cd28-23 (Figure 3).

Discussion

On the macro level, the chemical composition of the rumen fluid during the switch from hay to high-grain diet can be considered as the development of

Table 4. Similarity values of 16S rDNA sequences from library of day 28

Clone	Phylum	Nearest relative	Similarity (%)
2C28d-1	LGCGPB[a]	URB[b] RF22 (16S rDNA clone)	92.0
2C28d-3	LGCGPB	***Selenomonas ruminantium***	**99.3**
2C28d-4	LGCGPB	URB RF22 (16S rDNA clone)	93.0
2C28d-5	LGCGPB	URB RF22 (16S rDNA clone)	92.2
2C28d-6	LGCGPB	URB RF22 (16S rDNA clone)	92.1
2C28d-7	LGCGPB	URB RF22 (16S rDNA clone)	93.0
2C28d-8	LGCGPB	*Dialister pneumosintes*	94.8
2C28d-9	CVG[c]	NA[d]	
2C28-11	LGCGPB	*Mitsuokella multiacida*	94.7
2C28-12	LGCGPB	URB RF22 (16S rDNA clone)	92.2
3C28d-2	LGCGPB	*Mitsuokella multiacida*	95.7
3C28d-3	LGCGPB	*Mitsuokella multiacida*	96.5
3C28d-4	LGCGPB	*Eubacterium sp. C2*	92.6
3C28d-5	LGCGPB	*Eubacterium cellulosolvens*	90.3
3C28d-6	LGCGPB	*Butyrivibrio fibrisolvens*	93.2
3C28d-7	LGCGPB	*Mitsuokella multiacida*	95.7
3C28d-8	LGCGPB	URB RFN45 (16S rDNA clone)	94.5
3C28d-9	LGCGPB	*Mitsuokella multiacida*	95.8
3C28d-10	LGCGPB	URB RFN45 (16S rDNA clone)	94.9
3C28d-11	LGCGPB	*Mitsuokella multiacida*	95.9
4C28d-2	LGCGPB	*Mitsuokella multiacida*	95.9
4C28d-3	LGCGPB	*Mitsuokella multiacida*	96.6
4C28d-4	LGCGPB	URB RFN7 (16S rDNA clone)	93.7
4C28d-5	LGCGPB	*Mitsuokella multiacida*	96.6
4C28d-6	LGCGPB	*Mitsuokella multiacida*	96.3
4C28d-7	LGCGPB	*Dialister pneumosintes*	94.1
4C28d-8	LGCGPB	Uncultured bacterium A11	96.7
4C28d-9	LGCGPB	*Eubacterium cellulosolvens*	95.0
4C28d-10	LGCGPB	*Dialister pneumosintes*	94.4
4C28d-12	LGCGPB	*Clostridium thermocellum*	91.4
4C28d-13	LGCGPB	*Eubacterium cellulosolvens*	90.2
4C28d-14	LGCGPB	*Mitsuokella multiacida*	95.1
4C28d-15	LGCGPB	**URB RFN42 (16S rDNA clone)**	**97.7**
4C28d-16	LGCGPB	URB RNF45 (16S rDNA clone)	95.1
4C28d-17	LGCGPB	Uncultured bacterium A54	90.9
4C28d-18	LGCGPB	URB RF30 (16S rDNA clone)	94.0
4C28d-19	LGCGPB	*Ruminococcus bromii*	94.9
4C28d-20	LGCGPB	*Fusobacterium sulci*	92.2
4C28d-22	LGCGPB	*Butyrivibrio fibrisolvens*	93.2
4C28d-23	CFB[e]	**URB 12-109 (16S rDNA clone)**	**99.5**
4C28d-24	LGCGPB	*Eubacterium cellulosolvens*	94.9

[a]LGCGPB, low G+C Gram-positive bacteria.
[b]URB, unidentified rumen bacterium.
[c]CVG, *Chlamydiales-Verrucomicrobia* group.
[d]NA, not available.
[e]CFB, *Cytophaga-Flavobacter-Bacteroides* group.
Database sequences with >97% similarity are shown in bold.

subacute acidosis [6,21]. When the ruminal pH is higher than 5.5 on a high-grain diet, lactic acid does not accumulate in the rumen [4]. In the present study, the rumen pH was maintained within the range of 5.5 to 7.0 and the concentration of lactate in the rumen fluid was low. The 'breakpoint' was contemplated to be at day 3 of the experiment. At this time, consistent changes in the proportion of propionate (increased) and acetate (decreased) in total VFA of grain-fed cows were established. Total VFA concentration also followed this trend, being established at the higher level than control. Clinical symptoms in animals (decreased food intake) were diagnosed as metabolic acidosis. Upon reaching the second steady-state phase (day 28) the animals recovered.

In accordance with these macro trends, PCR-retrieved 16S rDNA libraries were made from the rumen fluid of day 0, 3, and 28. Unlike our previous homology searches [8], the number of hits (in the range of 97–100%) with database entries was substantially higher (Tables 2, 3 and 4). However, the majority of these hits, especially in libraries 1 and 3, were with *in vitro*-retrieved sequences. This reiterates again the necessity of new isolation works based on specific sequence signatures of new phylogroups in the rumen. Library 2, which describes the system in the transient state, had a surprisingly high number of hits with cultivated rumen isolates in comparison with libraries 1 and 3 (31% vs ~2%). Apparently, this phase is dominated by bacteria which are more

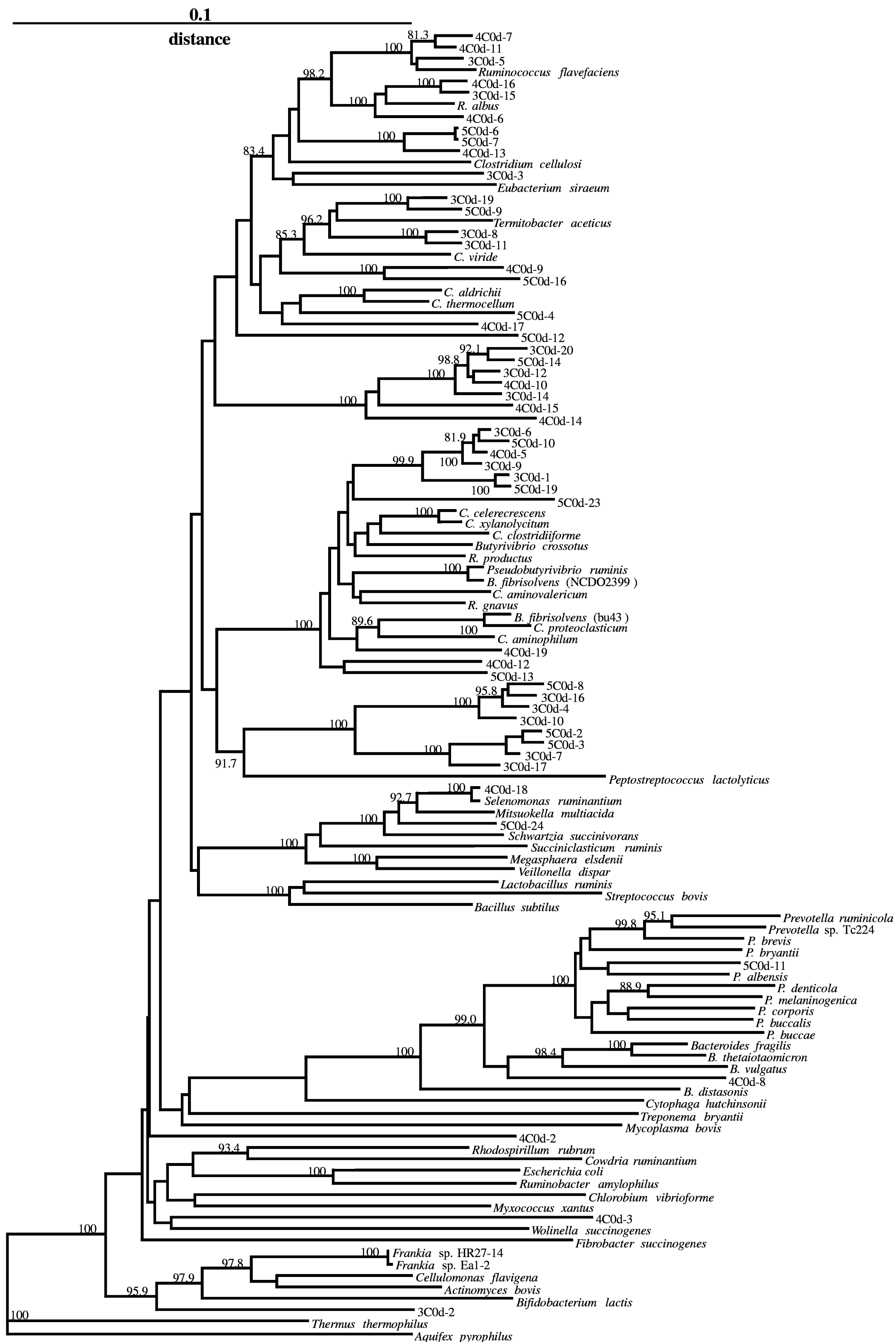

Figure 1. Phylogenetic tree of 16S rDNA sequences from library of day 0. The *Aquifex pyrophilus* sequence is used as the outgroup for rooting the tree. Numbers above each node are confidence levels (%) generated from 1000 bootstrap trees.

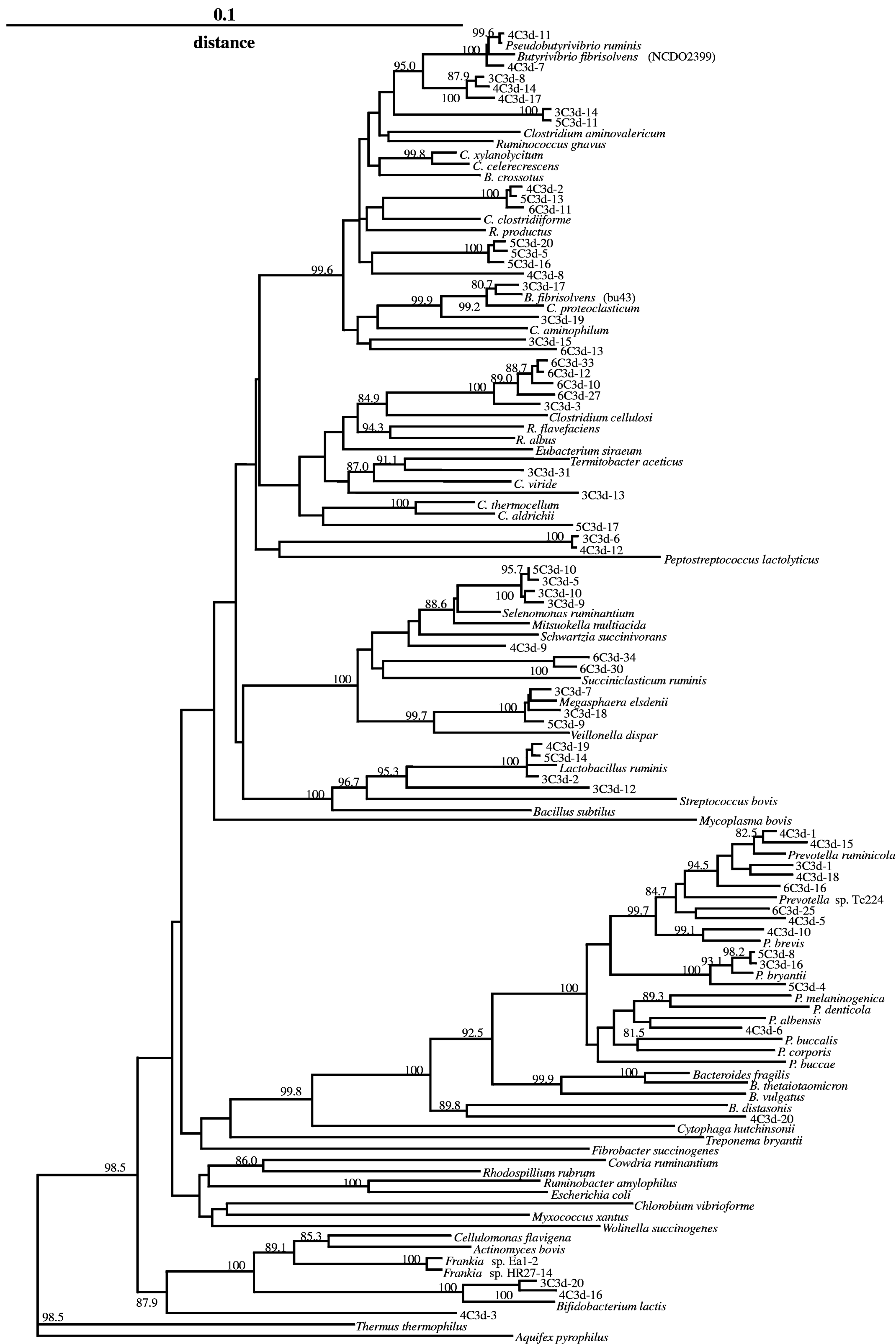

Figure 2. Phylogenetic tree of 16S rDNA sequences from library of day 3. Other definitions are as in Figure 1.

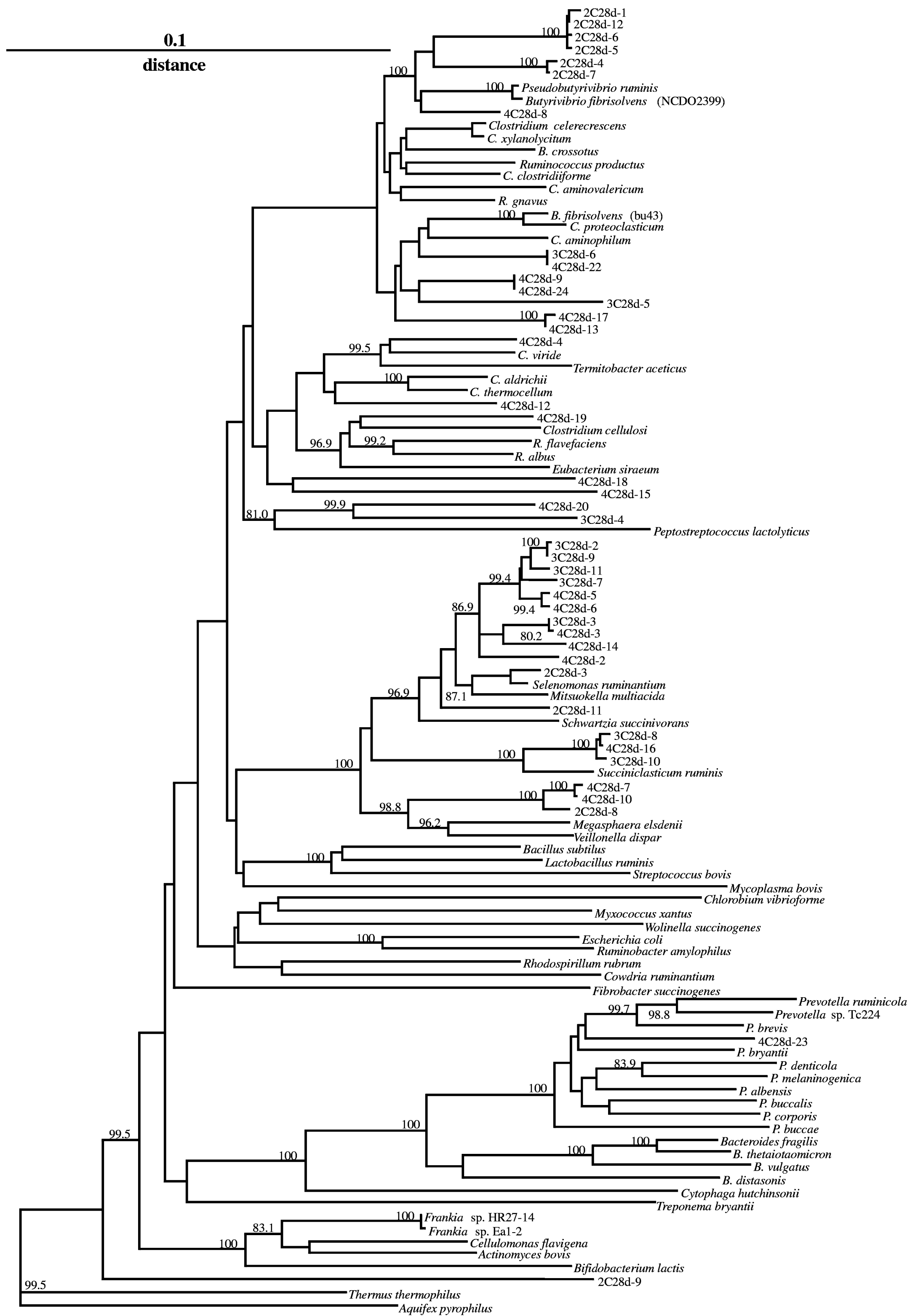

Figure 3. Phylogenetic tree of 16S rDNA sequences from library of day 28. Other definitions are as in Figure 1.

tolerant of high substrate concentrations commonly used in laboratory media and the growth of which is relatively independent from specific symbiotic or nutritional interactions with other bacteria (or archaea/eukarya). Another question is why this phase is dominated by such bacteria. Libraries 1 and 3 were obtained from the steady-state phases of the system. Under these conditions, predominant

bacterial components of the system form highly specialized or symbiotic relations such as syntrophy with other bacteria or archaea/eukarya. Obviously, reproducing such complex interactions under laboratory conditions is a very challenging task, especially when not all components of this dynamic equilibrium are known. It is because of this that the majority of sequences in libraries 1 and 3 are not represented by cultivated relatives. When the system is disturbed (library 2), it becomes dominated by 'self-sufficient' bacteria, which need little or none of the integrated network components supplied by other bacteria and/or archaea/eukarya. Certainly, the fast growth in pure cultures under laboratory conditions favors such bacteria and because of it, they are well represented among cultivated rumen isolates.

Similarly to our previous observations [8], the majority of sequences in all three libraries are located within the LGCGPB phylum (90.2%, 72.4%, and 98.0% in libraries 1, 2, and 3, respectively). This numerical prevalence suggests the functional importance of the phylum representatives under conditions of high-fiber and high-starch diets as well as in transition between the two. In library 1 (roughage diet), this phylum was represented by sequences clustered around the known cellulolytics such as ruminococci. Not surprisingly, no sequences were related to *S. bovis* and only two sequences were residing within *Selenomonas-Succiniclasticum-Megasphaera*, the group of known lactate utilizers. The functionality of other major phylogroups cannot be accessed this time because of absence of cultivated isolates. However, they do have high level of similarity to sequences retrieved in earlier experiments [7,8]. This would allow the design of specific primers and probes for isolation of representatives from these phylogroups. In addition, subtractive analysis against the high-grain diet libraries would be helpful in establishing the functionality of these phylogroups in the plant cell wall degradation processes.

In the second library (three days after the switch to high-grain diet), the ruminococci-related sequences disappeared and other LGCGPB representatives started to dominate. In particular, these are the sequences related to a lactic acid-producing bacterium, *L. ruminis,* and to lactate-utilizing bacteria such as *S. ruminantium, M. elsdenii* and *B. fibrisolvens.* Although this confirms some earlier observations, which have been based on cultivation methodology [6, 22], no clones clustering with the *S. bovis* sequence could be detected on this grain-rich diet.

In library 3 (28th day of high-grain diet), the representatives of LGCGPB were also predominant and the tendencies, which were observed in library 2, were more evident. In particular, approximately half of the LGCGPB-related sequences were affiliated with *Selenomonas-Succiniclasticum-Megasphaera* group, a considerable increase from the previous 26%. This cluster has been previously described as *Clostridium* group IX [23]. Representatives of this group such as *M. multiacida* and *S. ruminantium* are phylogenetically close [24] and utilize a similarly wide range of substrates including lactate. Numerical prevalence of this lactate-utilizing group may explain why the residual lactate concentration in the rumen fluid during the experiment was very low: apparently, most of it was metabolized by this group of bacteria. *S. bovis* has been shown to be one of the predominant bacteria under rumen acidosis conditions [3,4] but, similarly to library 2, no related sequences were retrieved in this clone library. Ninety-nine sequences were analysed in two libraries from high-grain diet and the total resolution is close to one percent. Although dominated by cultivation-based criteria, the real proportion of this bacterium may be much lower, less than one percent from the total bacterial diversity of the rumen. In addition, the main metabolic product of this bacterium, lactate, was detected at very low concentrations throughout the experiment. Thus, two factors may have contributed to the observed low lactate concentration: the low numbers of *S. bovis* and the elevated numbers of lactate-utilizers belonging to the *Selenomonas-Succiniclasticum-Megasphaera* group. As expected, no ruminococci-related sequences of cellulose-degraders were observed in this library.

Sequences from other phyla were less numerous and included representatives from the CFB, *Proteobacteria,* and HGCGP phyla. The relative scarcity of CFBs in *in vitro* libraries is in contrast to the earlier observations made with cultivation technique [24]. This may be explained by the relative ease of propagation of these bacteria under laboratory conditions and their subsequent overrepresentation in enumeration works. We have also found that CFBs are dominant in cultivation-based analyses of the rumen bacterial diversity (unpublished data), while *in vitro* analysis of the same samples indicate LGCGPB as dominant inhabitants of the rumen [8]. The higher proportion of CFBs (22.4%) in the transient phase (library 2) also suggest that these bacteria represent the 'self-sufficient' part of the system, discussed earlier which easily overgrows others during the substrate abundance in the rumen and which is quite tolerant to high-substrate laboratory media. The only two sequences of *Proteobacteria* (both from library 1) were not affiliated with any known ruminal proteobacteria. Representatives of HGCGPB were transient (detected only in library 2) and were affiliated with a known lactic acid producer, *B. lactis* (Figure 2).

In conclusion, analysis of bacterial succession with PCR-derived libraries confirmed some earlier

cultivation-based observations such as disappearance of ruminococci and prevalence of lactate-producing and utilizing bacteria during acidosis. However, the range of bacterial diversity involved in the process also includes species with no cultivated relatives. Despite the increased number of hits with previously published sequence data from the rumen, the majority of clones in this work is still represented by unique sequences. This dictates the necessity of further sequencing efforts to cover the broader range of molecular diversity. Design and application of probes and primers based on major molecular phylotypes would be helpful for intentional isolation with the aim of their functional characterization and determining their contribution to the development of acidosis.

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