APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2003, p. 6808–6815 Vol. 69, No. 11
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.11.6808–6815.2003

Ecology of Uncultivated *Oscillospira* Species in the Rumen of Cattle, Sheep, and Reindeer as Assessed by Microscopy and Molecular Approaches

Roderick I. Mackie,[1]* Rustam I. Aminov,[1]† Wenping Hu,[1] Athol V. Klieve,[2] Diane Ouwerkerk,[2] Monica A. Sundset,[3] and Yoichi Kamagata[4]

Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801[1]; Queensland Beef Industry Institute, Queensland Department of Primary Industries, Moorooka, Queensland 4105, Australia[2]; Department of Arctic Biology and Institute of Medical Biology, University of Tromsø, N-9037 Tromsø, Norway[3]; and National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305-8566, Japan[4]

Received 24 March 2003/Accepted 1 September 2003

The ecology of the uncultured, but large and morphologically conspicuous, rumen bacterium *Oscillospira* spp. was studied. *Oscillospira*-specific 16S rRNA gene sequences were detected in North American domestic cattle, sheep from Australia and Japan, and Norwegian reindeer. Phylogenetic analysis of the sequences obtained allowed definition of three operational taxonomic units within the *Oscillospira* clade. Consistent with this genetic diversity, we observed atypical smaller morphotypes by using an *Oscillospira*-specific fluorescence in situ hybridization probe. Despite the visual disappearance of typical large *Oscillospira* morphotypes, the presence of *Oscillospira* spp. was still detected by *Oscillospira*-specific PCR in the rumen of cattle and sheep. These observations suggest the broad presence of *Oscillospira* species in various rumen ecosystems with the level, and most likely the morphological form, dependent on diet. An ecological analysis based on enumeration of the morphologically conspicuous, large-septate form confirms that the highest counts are associated with the feeding of fresh forage diets to cattle and sheep and in two different subspecies of reindeer investigated.

Morphologically unique, large bacteria of the genus *Oscillospira*, which have not been grown in pure culture yet, can be routinely detected microscopically in the rumen contents of cattle and sheep (11, 18). A number of *Oscillospira* spp. are responsive to the advent of green pastures and fluctuate seasonally (13, 17) (see also Table 1). The only species of *Oscillospira* described in *Bergey's Manual of Systematic Bacteriology* (8) is *Oscillospira guilliermondii*, and this entry refers to the original morphological description by Chatton and Perard in 1913. Warner (34) proposed that at least two strains or species be included in the *Oscillospira* group based on cell diameter and the tendency to form spores as the main characteristic differences. This implies that there are different morphological forms or species related to different diets or in different gut ecosystems.

Detection and identification of microbial populations are the most basic prerequisites for microbial ecology studies. Over the last decade, several molecular techniques have been developed that when applied to the rumen microbiota have revealed enormous microbial diversity not recognized previously because of limitations and biases inherent in the cultivation approach (24, 30–32, 37). The cultivation-independent approach provides technology for detection and monitoring of microorganisms such as *Oscillospira*, for which growth requirements are unknown and undetermined. The only requirement for the development of molecular detection techniques is the availability of a marker molecule. Fortunately, several 16S rRNA gene sequences have recently been retrieved from this large bacterium isolated by flow cytometric sorting (39), and this enabled the design of various molecular probes for detection and monitoring. We designed and validate here PCR and PCR-denaturing gradient gel electrophoresis (DGGE) procedures for the detection of *Oscillospira* spp. and used these techniques to determine the occurrence of this bacterium in different ruminants and during diet shifts in cattle and sheep, as well as to estimate the genetic diversity of this unique group of bacteria.

MATERIALS AND METHODS

Sample collection. Rumen samples were obtained from three species of ruminants in three different geographic regions. Whole rumen fluid was obtained from two rumen-cannulated Hereford steers maintained at the Beef Research Farm, Department of Animal Sciences, University of Illinois at Urbana—Champaign. In winter, steers were kept indoors and fed medium-quality grass-legume-hay ad libitum. During the remainder of the year, steers were allowed to graze green Timothy (*Phleum pratense*) pasture. Representative rumen content samples were collected through the rumen cannula by using a scoop, filtered through two layers of cheesecloth, placed on ice, and transported to laboratory. The numbers of *Oscillospira* in the filtered rumen fluid sample were determined by direct count under a phase-contrast microscope by using a hemocytometer chamber. *Oscillospira* organisms were identified by their large size and distinct morphology (11, 18). This sample set supplied DNA for PCR and PCR-DGGE analysis.

Rumen samples were collected from four healthy semidomesticated female adult reindeer (*Rangifer tarandus tarandus*) feeding on natural winter pasture dominated by lichens in northern Norway (28, 29). This sample set supplied DNA for PCR-DGGE analysis. A further sample collection of whole rumen contents for *Oscillospira* counts was obtained from free-ranging male reindeer calves on fresh coastal natural summer pasture ($n = 3$) and winter pastures ($n = 5$) in Northern Norway and from male reindeer calves fed pelleted reindeer feed

* Corresponding author. Mailing address: Department of Animal Sciences, 1207 W. Gregory Dr., University of Illinois at Urbana-Champaign, Urbana, IL 61801. Phone: (217) 244-2526. Fax: (217) 333-8804. E-mail: r-mackie@uiuc.edu.

† Present address: Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, United Kingdom.

(RF-80 with a chemical composition of 10.3% crude protein [CP], 8.2% water-soluble carbohydrates, 15.9% cellulose, and 27.9% hemicellulose) (25) in summer ($n = 5$) and winter ($n = 5$) maintained at the Department of Arctic Biology, University of Tromsø. Rumen samples were also obtained from adult female reindeer ($n = 5$) on natural autumn pasture (10 September 2001) and from adult female Svalbaard reindeer (*Rangifer tarandus platyrhynchus*) ($n = 5$) on natural autumn pasture (29 April to 5 May 2001). Reindeer were sacrificed, the gastrointestinal tracts were removed immediately, and samples of whole rumen content were stored in 70% ethanol at 4°C until counted.

Rumen samples were collected from adult cannulated sheep housed in indoor pens at Queensland Department of Primary Industries, Brisbane, Australia, in a balanced crossover design experiment with four sheep per group in two 26-day periods. Sheep were fed lucerne pellets to standardize rumen microbial populations prior to introduction of the experimental regimen. Sheep in gronp 1 were fed fresh-cut Kikuyu (*Pennisetum clandestinum*) grass for 26 days before being switched to a diet containing cracked barley grain and chaffed hay plus 1% urea–$(NH_4)_2SO_4$. After the diet crossover, the grain was increased stepwise from 10 to 30 to 50 to 70% of the daily feed intake at 4-day intervals. Animals were kept on the 70% grain diet for last 14 days of the period. Sheep in group 2 were initially fed the grain diet increased stepwise at 4-day intervals from 0 to 10 to 30 to 50 to 70% levels before being switched to the fresh-cut Kikuyu grass diet for the last 26 days after the diet crossover. Rumen samples were collected by using a sampling tube, and a 4-ml aliquot added to 16 ml of formal-saline preservative for counting. *Oscillospira* organisms in rumen fluid samples were enumerated by using a counting chamber as described above for cattle (11, 18).

DNA extraction. Total genomic DNA from 200-mg samples of rumen content from cattle, reindeer, and sheep was isolated by using the Ultraclean Soil DNA isolation kit (catalog no. 12800-100; MoBio Laboratories, Solana Beach, Calif.). The amount of DNA extracted was 5 to 10 μg/200 mg of wet sample. The same procedure was employed for extraction of DNA from soil and pasture samples.

Hybridization probe and PCR primers. A PCR primer set, OSCI-FW (5′-AGGAGTTTTCGGACAACGG) and OSCI-RV (5′-ATTCAAGGGGTACCGTCTTC), was designed based on retrieval of 16S rRNA gene sequences from *Oscillospira* organisms (39). A hybridization probe for fluorescent in situ hybridization (FISH) (5′-CCGCACCTAGTATTGATC) was as described earlier (39). A universal bacterial set of primers, 27f and 1525r (14), was used in control amplifications of total DNAs from rumen contents to verify the quality of DNA templates before amplification with the *Oscillospira*-specific primers. The specificity of *Oscillospira*-specific probe and primers was initially verified by using the GenBank (2) and RDPII (16) databases. At least two mismatches were allowed with nontarget sequences. All primers and probes were synthesized commercially (OPERON, Alameda, Calif.).

PCR and DGGE. PCR amplifications were performed with a GeneAmp PCR system 2400 thermocycler (Perkin-Elmer, Norwalk, Conn.). A typical PCR mixture contained 125 ng of genomic DNA, 25 pmol of each primer, 1× ExTaq reaction buffer (PanVera Corp., Madison, Wis.), 100 μM concentrations of each deoxynucleoside triphosphate, and 1.0 U of ExTaq DNA polymerase (PanVera Corp.), adjusted to a total volume of 50 μl. PCR cycling was done with initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 45 s, 59°C for 45 s, and 72°C for 90 s, and with a final extension step at 72°C for 7 min. The PCR products were separated by gel electrophoresis in 3% (wt/vol) NuSieve agarose (FMC Bioproducts, Rockland, Maine) and visualized with ethidium bromide. For PCR-DGGE, the forward primer had a GC-clamp attached at the 5′ end (19). The optimized PCR protocol initially consisted of eight touchdown cycles, which were performed while decreasing the annealing temperature from 66 to 59°C (1°C/cycle), followed by 22 additional cycles as described above. Electrophoresis was performed at 60°C and 150 V for 2 h, followed by 200 V for 1 h, by using the D-Gene System (Bio-Rad Laboratories, Richmond, Calif.). After the run, gels were rinsed in double-distilled H_2O, fixed in a solution of 10% ethanol and 0.5% acetic acid, and silver stained. Gel images were captured and digitized by using a Bio-Rad GS-710 calibrated imaging densitometer connected to a G3 Macintosh computer with Diversity Database fingerprinting software.

Cloning and sequencing. DNA bands were excised from DGGE gels, crushed, and equilibrated in Tris-EDTA buffer at room temperature overnight. Eluted DNA solution (1 μl) was used for reamplification by using the same set of *Oscillospira*-specific primers but without the GC-clamp. PCR products were directly cloned by using the pGEM-T Easy Vector System II kit (Promega Corp., Madison, Wis.) according to the manufacturer's protocol. DNA sequence analysis of inserts in recombinant plasmids was performed on both strands at the University of Illinois Biotechnology Center. Online similarity search was performed by using the basic local alignment search tool (BLAST) family of programs in GenBank (15). The GenBank accession numbers for the eight 409-bp *Oscillospira* seqnences generated here are AY244475 to AY244482.

FISH. The FISH procedure essentially followed the method described by Amann (1) with our modifications (39). Rumen fluid and pure culture sample preparations were hybridized in 8 μl of the hybridization solution (Sigma, St. Louis, Mo.) containing 1 μl of probe (28 ng) at 48°C for 2 h. After hybridization, the slides were washed in hybridization buffer for 20 min at 48°C, rinsed with distilled water, and air dried. Slides were mounted by using the antifade mounting medium (SlowFade Antifade Kit; Molecular Probes, Eugene, Oreg.). In preliminary experiments, the slides were viewed with a Nikon epifluorescence EFD-3 microscope equipped with a suitable filter set (Nikon). Sequences of phylogenetically close, but nontarget, bacterial species exhibited at least two mismatches with the probe sequence and produced no FISH signal demonstrating high probe specificity. For subsequent confocal microscopy, a Fluoview FV300 laser-scanning biological microscope (version 3.00; Olympus, New York, N.Y.) was used. Transmission electron micrographs (TEMs) were obtained from rumen content of reindeer by using the methods described by Olsen and Mathiesen (20).

RESULTS AND DISCUSSION

PCR detection. The sensitivity of light microscopic detection of *Oscillospira* in rumen fluid may be low, and one of the objectives of the present study was to develop a more sensitive PCR detection technique allowing detection at levels beyond the sensitivity of the light microscopic without relying on the specific morphology of cells for enumeration. For this purpose, we designed a set of PCR primers based on *Oscillospira* 16S ribosomal DNA (rDNA) sequence generated from flow cytometric sorting of large cells (39). Since there was no positive culture available for the specificity test, the specificity of the primer set was checked against the GenBank and RDPII databases and by amplification and sequencing of PCR products from total ruminal DNA. For all of the rumen samples with conspicuous *Oscillospira* morphotypes surveyed by the PCR detection assay, amplification yielded the expected 409-bp product. This finding was confirmed by cloning and sequencing the PCR amplicon. No amplification was observed when phylogenetically related ruminal bacterial species (*Sporobacter termitidis*, *Clostridium perfringens* 3624A, *Ruminococcus albus* 7, and *Ruminococcus flavefaciens* FD-1) were used as negative controls in verification experiments. In addition, sequences amplified with this primer set from total ruminal DNA templates formed a phylogenetically coherent group with the sequences isolated earlier by the cell-sorting technique (39) (Fig. 2).

Diet change in cattle. Although not quantified, the *Oscillospira*-specific PCR signals from the rumen fluid of cattle feeding on Timothy pasture were more prominent than those of animals kept indoors. Dilutions of equal amounts of total rumen genomic DNA from the two diets, used in amplifications with the OSCI primer set, demonstrated that the DNA from the pasture-fed animals still produced PCR signal at a 10^{-2} dilution, whereas samples from animals kept indoors showed no amplification at a 10^{-1}dilution. Longitudinal microscopic detection and enumeration of *Oscillospira* and PCR detection performed on two rumen-cannulated steers kept at the University Research Farm showed that distinct *Oscillospira* morphotypes were consistently observed in all samples from both animals when fed on green pasture. However, two weeks after the transition from pasture fed to indoor fed, no typical *Oscillospira* morphotypes could be detected microscopically. These morphotypes reappeared again 18 days after the advent of pasture feeding. It is interesting that more than 2 weeks were needed for the reappearance of *Oscillospira* but quickly

TABLE 1. Ecological analysis of *O. guilliermondii*-like organisms as determined by light microscopic counts

Herbivore	Gut compartment	No. (10^6/g or ml)	Morphological description and/or diet	Geographic location	Source or reference
Guinea pig	Cecum		Original description	France	8
Rabbit	Cecum		See *Bergey's Manual*	France	8
Sheep	Rumen		Gram-negative, multicellular rods, 5 by 8- to 50-μm, motile, large circular spores (3.5-μm diam); most frequently formed on lush pasture	Australia	18
Sheep	Rumen	0.0003–0.047	Counts were highest on clover, low on grass; no *Oscillospira* in pen-fed sheep supplemented with grain plus protein; responds to the advent of green pasture and fluctuates seasonally	Australia	17
Sheep	Rumen	0.003–19.50	Varies with season and individual; 50% wheat plus 50% lucerne chaff diet; required ca. 12 days for reappearance after starvation	Australia	33
Sheep	Rumen	0.34	50% wheat plus 50% lucerne chaff	Australia	34
		0.09	50% lucerne plus 50% grain	Australia	34
		0.10	50% wheat plus 50% lucerne; interval feeding	Australia	35
		20	Wheat chaff to appetite	Australia	36
		0.89	Grazing animals	Australia	36
Water buffalo	Rumen	0.67	Grass, wheat straw plus concentrate supplement	India	24
Zebu cattle	Rumen	0.044	Grass, wheat straw plus concentrate supplement	India	24
Water buffalo	Rumen	4.7	Grass, wheat straw plus concentrate	India	13
Sheep	Rumen	2.0	Pasture	Orkney, Scotland	22
		25	Seaweed	Orkney, Scotland	22
Cattle and sheep	Rumen		Attach to cuticular surface of white clover, lucerne, and rye grass; none on leaves prior to ingestion	New Zealand	3
Reindeer	Rumen	7.0	Grasses and sedges (summer)	Svalbard, Norway	21
		0.8	Mosses and fibrous plants (winter)	Svalbard, Norway	21
Reindeer	Rumen	1–24	Natural pasture (autumn)	Svalbard, Norway	This study
		1–68	Natural pasture (winter)	Svalbard, Norway	This study
Reindeer	Rumen	20–50	Natural pasture (summer)	Northern Norway	This study
		7–50	Natural pasture (autumn)	Northern Norway	This study
		0.4–24	Natural pasture (winter)	Northern Norway	This study
		0.4–150	Pelleted reindeer feed (summer)	Northern Norway	This study
		0.3–20	Pelleted reindeer feed (winter)	Northern Norway	This study
Sheep	Rumen	0.5–3.8	Kikuyu pasture	Australia	This study
		2.1–2.3	10% grain inclusion	Australia	This study
		2.1	30% grain inclusion	Australia	This study
		0.8–0.9	50% grain inclusion	Australia	This study
		0.06–0.0005	70% grain inclusion	Australia	This study
Cattle	Rumen	0.25–0.68	Grass pasture (summer)	Illinois	This study
			Indoor feeding (winter); hay plus grain—no visible form	Illinois	This study

reached the level characteristic of pasture-fed animals (1.2×10^5) within just 1 week after the first detection. However, all samples produced the specific 409-bp PCR amplicon, irrespective of diet or the presence or absence of the specific *Oscillospira* morphotype evaluated microscopically. To exclude the possibility of *Oscillospira* entering the rumen with feed, total DNA from the pasture grass and soil samples was isolated and subjected to amplification with the OSCI primer set, with negative results. Clarke (3) also reported that *Oscillospira* was not found on leaves prior to ingestion based on examination of material by light microscopy.

Enumeration of *Oscillospira* in reindeer. An ecological analysis (Table 1) confirmed that the high counts of the large morphologically conspicuous form of *Oscillospira*, termed *O. guilliermondii*, were recorded in Orkney sheep consuming seaweed diets (2.5×10^7 per g of ingesta) and in Svalbard reindeer on summer pasture (7×10^6 per g of ingesta) (20, 21). The results from the present study show that consistently high counts were obtained from the rumen of reindeer in northern Norway and Svalbard (1×10^7 to 5×10^7 per g of ingesta).

Diet shift from grass to grain diets and vice versa in sheep. In order to evaluate the effect of diet change from lush forage to grain-containing diets, a changeover experiment was performed with sheep. Microscopic counts showed that on the lucerne pellet standard diet numbers of *Oscillospira* were 1.3×10^5 to 1.7×10^5 per g of ingesta. However, after the changeover *Oscillospira* numbers decreased on the 50% grain diet. After the animals were on a 70% grain diet for 10 days, the numbers of *Oscillospira* decreased to 50 per g of ingesta. For group 2, the numbers of *Oscillospira* decreased to 5×10^5 for animals on the 70% cracked barley diet. However, 8 days after the shift to fresh Kikuyu grass, the numbers of *Oscillospira* had increased to 1.1×10^5 to 1.9×10^5 per g of ingesta. Importantly, all samples produced the specific 409-bp PCR amplicon for *Oscillospira* regardless of diet (data not shown).

PCR-DGGE and phylogeny. In order to access the diversity of *Oscillospira* in different ruminant species, the *Oscillospira*-specific primers were used in conjunction with PCR-DGGE (Fig. 1). This analysis demonstrated the presence of at least seven different phylotypes in these animals based on gel band-

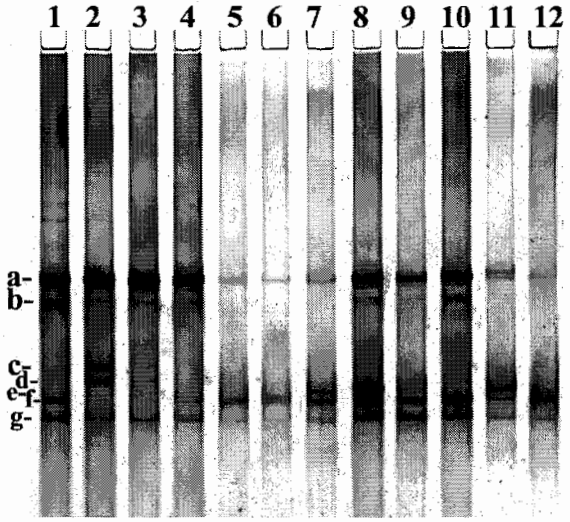

FIG. 1. DGGE analysis PCR-amplified 16S rRNA fragment of *Oscillospira* with the OSCI primers with an attached GC-clamp. The figure shows the DGGE separation pattern of PCR fragments obtained from Norwegian reindeer (lanes 1 to 4), cattle (lanes 5 and 6), and sheep (lanes 7 to 12). The arrows indicate the representatives of DNA bands, which were excised and subjected to cloning and sequencing.

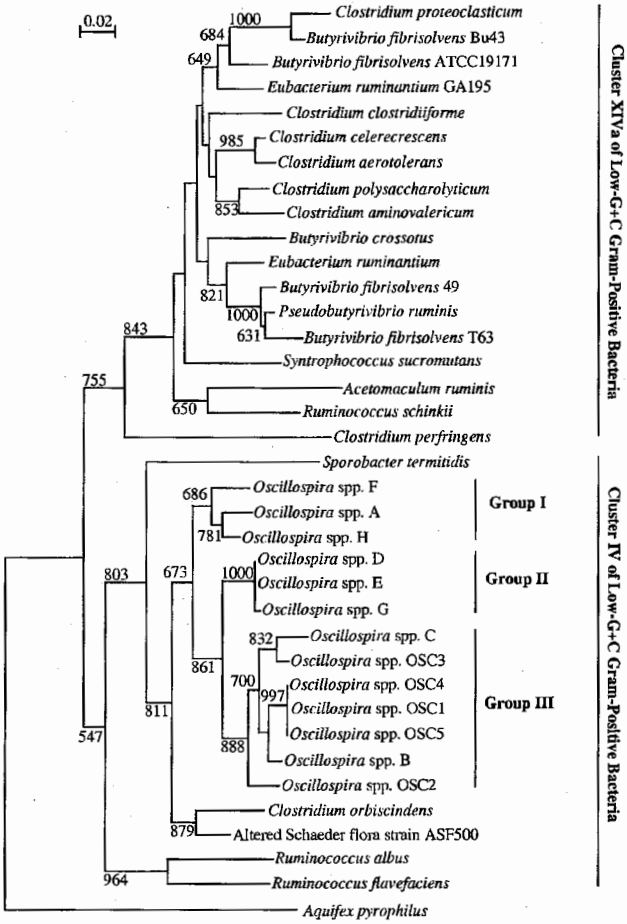

FIG. 2. Phylogenetic placement of partial 16S rDNA sequences, amplified with the *Oscillospira*-specific primer set OSCI, within the main rumen bacterial phyla. Previously cloned *Oscillospira* sequences are labeled OSC1 through OSC5. The sequences generated in this work are labeled A through H. The *Aquifex pyrophilus* sequence is used as the outgroup for rooting the tree. Numbers above each node are confidence levels generated from 1,000 bootstrap trees (5). The scale bar is in fixed nucleotide substitutions per sequence position.

ing patterns, with the most diverse group detected in the Norwegian reindeer (six phylotypes), with four phylotypes in the Australian sheep and three phylotypes in U.S. domestic cattle. Phylotypes a, f, and g were universally detected in all animal species, whereas phylotypes c and d were unique to reindeer, they were detected only in two animals. Phylotype e was encountered only in sheep from Australia. Eight DGGE bands were cloned, sequenced, and incorporated into the DNA similarity analysis, together with the five previously cloned sequences obtained from sheep in Japan (39). This analysis suggests the existence of at least three subgroups (possibly species) of *Oscillospira* in the rumen. In the subsequent phylogenetic analysis, these 13 cloned sequences were incorporated into the rumen bacterial phylogenetic tree (Fig. 2). All *Oscillospira* sequences formed a tight phylogenetic group within the cluster IV of the low-G+C gram-positive bacterial (LGCGPB) phylum with 78% bootstrap support. The nearest cultivated neighbors of the *Oscillospira* group were the human colonic isolate *Clostridium orbiscindens* (91% 16S rDNA similarity) (26, 38) and the ASF500 strain (*Clostridium* sp.) component of the murine altered Schaedler flora (92% similarity) (4). The sequence of *Quinella ovalis*, another large, morphologically conspicuous, rumen bacterium (12), was located within the cluster IX of the LGCGPB phylum. Despite the phylogenetic clustering in cluster IV of the LGCGPB phylum, the sequences of *Oscillospira* are quite heterogeneous and are represented by at least three groups, with the first group being represented by clones A, F, and H, the second group being represented by clones D, E, and G, and the third, most numerous group, being represented by clones OSC1 to OSC5 and B and C (Fig. 2). It is noteworthy that the sequences of *Oscillospira* cells, which were cloned after cell sorting (39), form a separate cluster with close DNA similarity values, suggesting possible large morphotype selection during the cell size sorting procedure (Fig. 2). With the cell sorting procedure, other less morphologically prominent or smaller forms may have escaped detection, collection, and subsequent 16S rRNA gene analysis. However, in PCR-generated libraries, these sequences were successfully retrieved and analyzed, confirming the existence of several *Oscillospira* species in the rumen. The sequence data are limited to an analysis of the 409-bp 16S rRNA gene and do not support any host species-specific affiliation of *Oscillospira*. For example, the genetically coherent group II is represented by sequences from American cattle, Australian sheep, and Norwegian reindeer (Fig. 2).

Thus, we failed to detect any host species-specific association of these operational taxonomic units, suggesting the widespread presence of the same operational taxonomic units in geographically distant and diverse ruminant species consuming a variety of diets. Although a limited number of *Oscillospira* sequences were obtained and analyzed in the

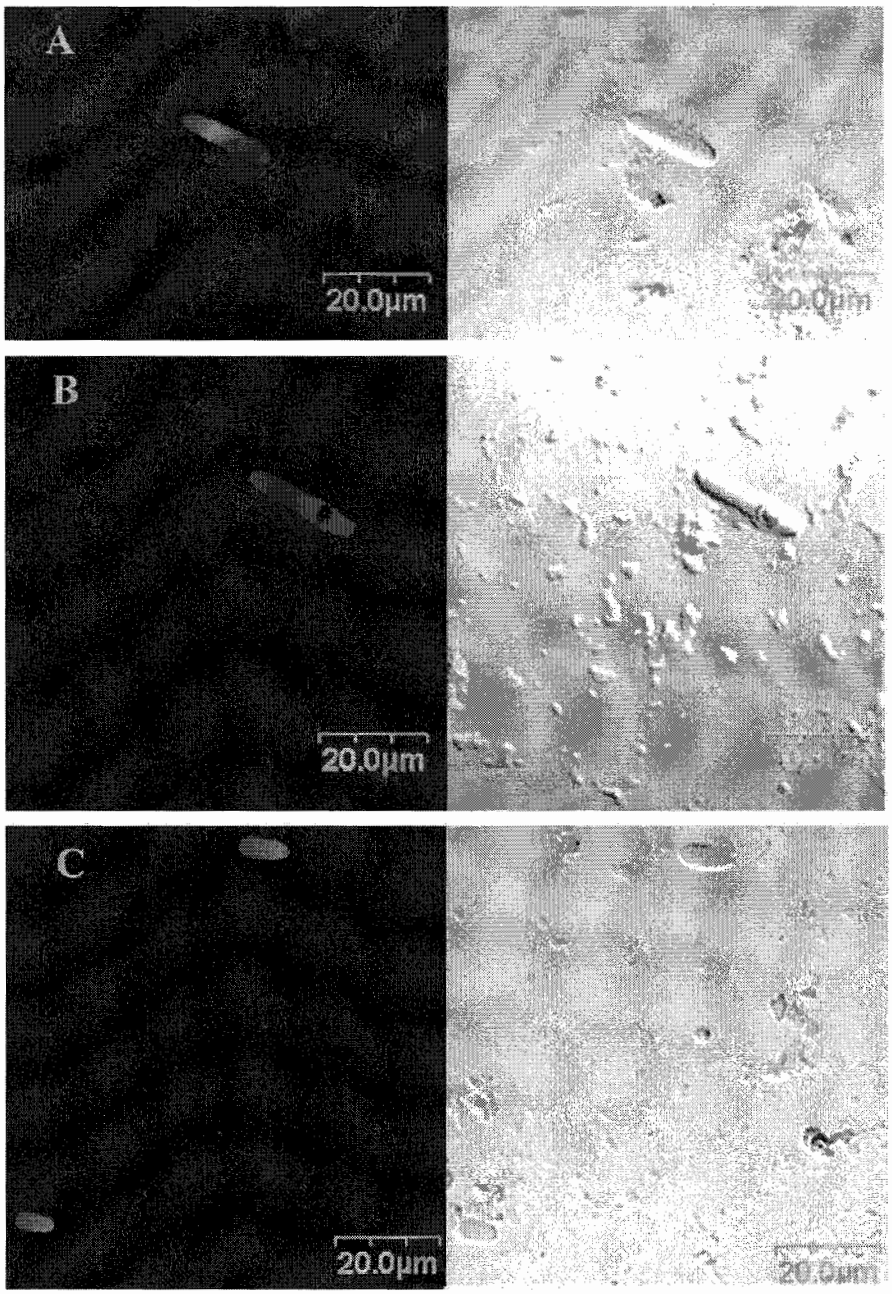

FIG. 3. Whole-cell FISH analysis of rumen fluid from cattle in Illinois. The cells were hybridized with Cy3-labeled oligonucleotide probe specific for *Oscillospira*. Images were obtained by confocal laser-scanning microscopy, with differential interference contrast (left) and fluorescence (right) images displayed for each identical field, showing the morphologically identified forms of *Oscillospira*. Panel A demonstrates characteristic morphology of *Oscillospira*, i.e., large, septate rod. Note the lack of probe penetration and staining of the spore (B) and the smaller, atypical septate form (C) that nevertheless gives a positive FISH signal. Scale bar, 20 μm.

present preliminary study, more representative analyses may uncover broader diversity and the presence of these fascinating organisms in other ruminants as well as in the cecum and colon of a wide range of herbivores, including humans. In fact, the nearest cultivated neighbors of the *Oscillospira* group included the human colonic isolate *C. orbiscindens* capable of cleaving the flavonoid C-ring (26, 38) and the AFS 500 (*Clostridium* sp.) component of the murine altered Schaedler flora. Obviously, higher-scoring BLAST hits were observed with uncultivated representatives of the rumen, pig, human, chicken, and rodent gastrointestinal microbiota. *Sporobacter termitidis*, a bacterial species isolated from the termite gut (10) that grows exclusively on a limited range of methoxylated aromatic compounds with ring cleavage, has a similarity of 88.1%. In order to perform this analysis adequately, we require full-length 16S rRNA gene sequence or

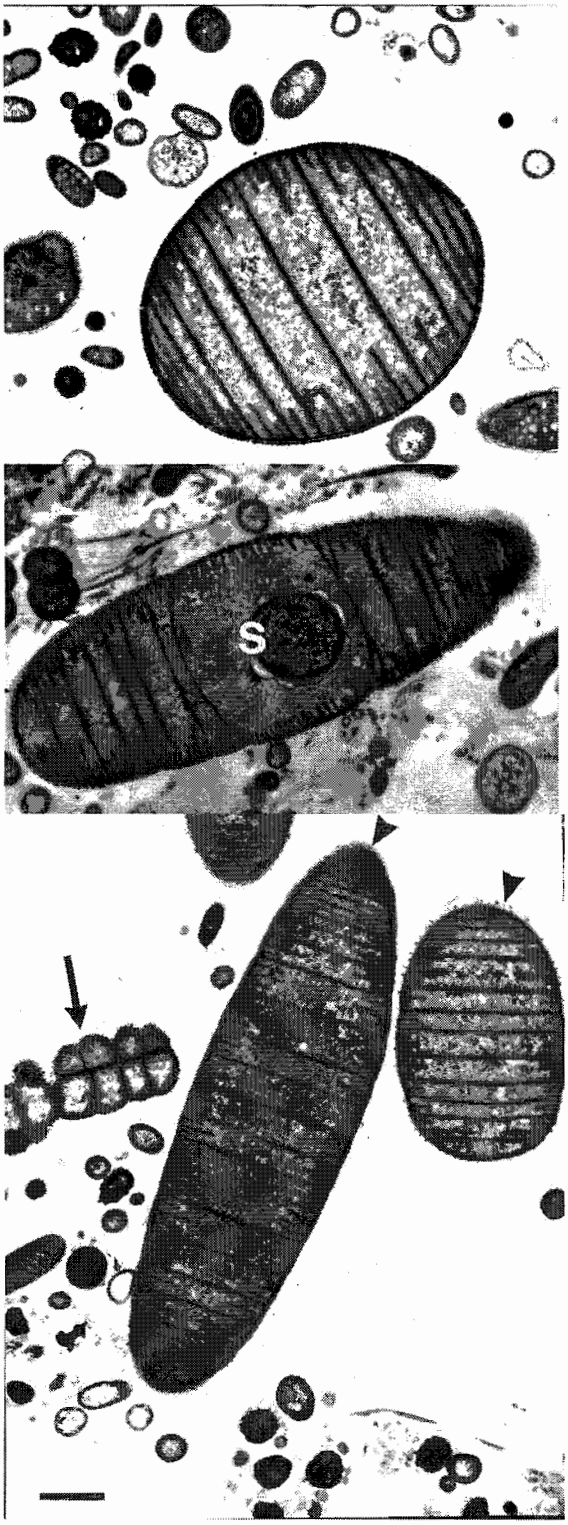

FIG. 4. TEMs of *O. guilliermondii* obtained from rumen content of Norwegian reindeer documenting an atypical, smaller, oval form (length, 5 μm) with septation (top panel); a morphologically conspicuous septate rod with round central spore (s) (middle panel); and both a morphologically conspicuous large, septate form (ca. 10 μm) and a smaller oval form (4 μm) (bottom panel). The two arrowheads indicate the long and oval forms, respectively. Animals, diet, and sampling of reindeer were as described by Olsen and Mathiesen (20). Scale bar, 1 μm.

another molecule other than SSU rDNA, which may be too highly conserved, to provide sufficient resolution of speciation and biogeographical principles initiated in the present study (23, 27).

Cell morphology and electron microscopy. Rumen samples were examined in an attempt to determine whether there were other unidentified morphological forms of *Oscillospira* in the rumen. As shown in Fig. 3, bacteria with the characteristic morphology of *Oscillospira* were observed by FISH, although these bacteria differed significantly in size (ranging from 40 to 50 μm to 5 to 8 μm in length). Some cells contain an intracellular circular region that binds probe poorly and are presumably spores (Fig. 3B). Specific treatment may be needed for FISH probe penetration of spores (6). Sporulation events during the life cycle of this bacterium have been well documented (9, 34). Using this probe, we were also able to detect another ruminal morphotype in cattle, which is unusual for *Oscillospira* (Fig. 3C). No obvious *Oscillospira*-specific septations were present, and the general morphology differed from the more typical multicellular morphotype shown in Fig. 3A and B. The ultrastructure of characteristic *Oscillospira* cells has been described by Grain and Senaud (9), but we present here TEMs as further evidence of two distinct morphological forms: a large rod divided by multiple, closely spaced cross-walls (10 μm) (Fig. 4, bottom panel), and a small oval form divided by three to five cross walls (4 to 5 μm) (Fig. 4, top and bottom panels). Thus, in addition to genetic diversity, these observations add further evidence for the morphological heterogeneity of *Oscillospira* populations in the rumen and for the possibility of a life cycle that includes elongation by septation and a spore stage (Fig. 3B; Fig. 4, middle panel).

The rapid association or colonization of the cuticular surface of plant material by *Oscillospira* suggests that this might constitute a specific habitat for this bacterium (3). It is likely that nutrients or other chemoattractents are released during mastication of freshly ingested forage, attracting these and other motile bacteria to this material. However, with no pure culture available, these observations remain anecdotal and unproven. Nevertheless, the conspicuous morphology of this bacterium enabled enumeration based on direct microscopic counts of rumen contents during various dietary regimens such as the transfer of cattle from indoor housing to green pasture and during a diet crossover experiment in sheep. Microscopic count data showed the highest numbers of this specific morphotype were recorded during green pasture feeding and ranged from 2.5×10^5 to 6.8×10^5 per ml of rumen fluid from cattle (Table 1). Direct microscopic counts of *Oscillospira*-like bacteria in reindeer are high (generally 10^7 but as high as 1.5×10^8; Table 1) and are associated not only with a diet of fresh summer pasture but also with winter diets containing large quantities of lichens and subarctic plants, including graminoids, shrubs, and heathers (28, 29). Unusually high numbers of *Oscillospira* were reported in the rumen of sheep on the island of North Ronaldsey, Orkney, Scotland, fed seaweed (2.5×10^7) (28). Seaweed is a rich source of mannitol that can constitute up to 35% of kelp dry weight (7). Thus, the abundance of *Oscillospira* in these sheep was linked to utilization of this six-carbon sugar alcohol by Orpin et al. (22), who observed that in mixed culture *O. guilliermondii* survived for extended periods with mannitol as a carbon source. Similar in vitro cultivation experiments in

our laboratory, using centrifugation to concentrate *Oscillospira* as an inoculum and based on direct microscopic counts, did not demonstrate improved survival on mannitol compared to the same concentration of mixed carbohydrates (glucose-cellobiose-maltose-starch) generally added to ruminal, habitat-simulating media.

The advent of microbial molecular ecology techniques during the last decade has revolutionized the approaches used in detection, enumeration, and classification of previously uncultivated microorganisms. Our *Oscillospira*-specific PCR assay suggests that, despite the visual disappearance of this bacterium during indoor feeding on a mixed hay diet of cattle and on high levels of grain feeding in sheep, the specific signal was still detected in ruminal samples. This discrepancy may be either due to low sensitivity of microscopic examination or because of the presence of other *Oscillospira* species or forms without the conspicuous morphology. Indeed, during FISH analysis we encountered atypical forms, which hybridized at high stringency with the *Oscillospira* probe (Fig. 3). This was supported by the observation of small oval, septate forms 4 to 5 μm in length (Fig. 4) using transmission electron microscopy. We postulate that this atypical form is responsible for the PCR signal in the absence of typical *Oscillospira* morphotypes. At present, it is not clear whether this is another species of *Oscillospira* or if it is a different morphological form of the same bacterium that undergoes morphological changes during its life cycle induced by the diet shift. Therefore, only the large, morphologically conspicuous, visually obvious form is the one detected and counted by light microscopy. Since we detected a considerable level of genetic diversity within this group of bacteria based on cloning and sequencing of bands separated by PCR-DGGE, the most plausible explanation for the observed morphological heterogeneity could be the underlying genetic diversity of *Oscillospira* in the rumen. This idea is supported by the observation that the morphologically based flow cytometric cell sorting also selected the sequences that phylogenetically cluster within this group (Fig. 2) (39). It is likely that the genetically distant phylogenetic groups I and II may be represented by different, atypical forms of *Oscillospira* dominant on the mixed hay diet.

In conclusion, although *Oscillospira* has been widely observed for almost 90 years, it remains uncultured. Modern molecular microbial ecology techniques have enabled us to place this large, morphologically conspicuous bacterium with the group IV LGCGP bacterial group. Combined evidence from PCR, DGGE, and FISH analyses strongly supports genetic diversity and morphological heterogeneity with the possibility of three (sub)species and the possibility of a life cycle that includes elongation by septation, as well as a spore stage.

ACKNOWLEDGMENTS

We thank Andrea Turner, Luke Burrow, and the farm staff at the Animal Research Institute, Yerongpilly, Queensland, Australia, for technical assistance during the sheep trial partially funded by the Australian Research Council. We are grateful to Svein Disch Mathiesen and Pål Vegar Storeheier for their assistance in sampling the reindeer and to the Departments of Electron Microscopy and Morphology at the Faculty of Medicine, University of Tromsø, Tromsø, Norway, for assistance with the TEMs of reindeer *Oscillospira*.

The reindeer project was supported by the Reindeer Husbandry Research Fund, the University of Tromsø, and the Norwegian Research Council. Research at the University of Illinois was supported by the Agricultural Research Station, University of Illinois at Urbana-Champaign, and by USDA-NRI Competitive Grants Program 42.0 (award 99-35206-7950).

REFERENCES

1. **Amann, R. I.** 1995. In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes, p. 1–15. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.
2. **Benson, D. A., M. S. Boguski, D. J. Lipman, J. Ostell, B. F. Ouellette, B. A. Rapp, and D. L. Wheeler.**1999. GenBank. Nucleic Acids Res. **27:**12–17.
3. **Clarke, R. T. J.** 1979. Niche in pasture-fed ruminants for the large rumen bacteria *Oscillospira*, *Lampropedia*, and Quin's and Eadie's ovals. Appl. Environ. Microbiol. **37:**654–657.
4. **Dewhirst, F. E., M. S. Boguski, D. J. Lipman, J. Ostell, B. F. Oullette, B. A. Rapp, and D. L. Wheeler.** 1999. Phylogeny of the defined murine microbiota: altered Schaedler flora. Appl. Environ. Microbiol. **65:**3287–3292.
5. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
6. **Fischer, K., D. Hahn, W. Honerlage, and F. Schonholzer.** 1995. In situ detection of spores and vegetative cells of *Bacillus megaterium* in soil by whole cell hybridization. Syst. Appl. Microbiol. **18:**265–273.
7. **Gerard, V. A.** 1989. Seaweeds, p. 205–212. *In* O. Kitami and C. W. Hall (ed.), Biomass handbook. Gordon and Breach Scientific Publications, New York, N.Y.
8. **Gibson, T.** 1986. Genus *Oscillospira* Chatton and Perard 1913, p. 1207. *In* P. H. A Sneath et al. (*ed.*), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore, Md.
9. **Grain, J., and J. Senaud.** 1976. *Oscillospira guilliermondii*, a rumen bacteria: ultrastructural study of the trichome and of sporulation. J. Ultrastruct. Res. **55:**228–244.
10. **Grech-Mora, I., M. L. Fardeau, B. K. C. Patel, B. Ollivier, A. Rimbault, G. Prensier, J. L. Garcia, J. L., and E. Garnier-Sillam.** 1996. Isolation and characterization of *Sporobacter termitidis* gen. nov., sp. nov., from the digestive tract of the wood-feeding termite *Nasutitermes lujae*. Int. J. Syst. Bacteriol. **46:**512–518.
11. **Hungate, R. E.** 1966. The rumen bacteria, p. 8–90. *In* R. E. Hungate (ed.), The rumen and its microbes. Academic Press, Inc., New York, N.Y.
12. **Krumholz, L. R., M. P. Bryant, W. J. Brulla, J. L. Vicini, J. H. Clark, and D. A. Stahl.** 1993. Proposal of *Quinella ovalis* gen. nov., sp. nov., based on phylogenetic analysis. Int. J. Syst. Bacteriol. **43:**293–296.
13. **Kulkerni, V. D., H. C. Pant, G. S. Rai, and J. S. Rawat.** 1971. The seasonal variation in the concentration of protozoa and *Oscillospira guilliermondii* organisms in the rumen fluid of grazing buffalo. Indian Vet. J. **48:**137–142.
14. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Inc., New York, N.Y.
15. **Madden, T. L., R. L. Tatusov, and J. Zhang.** 1996. Application of network BLAST server. Methods Enzymol. **266:**131–141.
16. **Maidak, B. L., J. R. Cole, C. T. Parker, Jr., G. M. Garrity, N. Larsen, T. G. Li, B. Lilburm, M. J. McCaughey, G. J. Olsen, R. Overbeek, S. Pramanik, T. M. Schmidt, J. M. Tiedje, and C. R. Woese.** 1999. A new version of the RDP (ribosomal database project). Nucleic Acids Res. **27:**171–173.
17. **Moir, R. J.** 1951. The seasonal variation in the ruminal microorganisms of grazing sheep. Austr. J. Agric. Res. **2:**322.
18. **Moir, R. J., and M. J. Masson.** 1952. An illustrated scheme for the microscopic identification of the rumen microorganisms of sheep. J. Pathol. Bacteriol. **64:**343–350.
19. **Muyzer, G., E. C. De Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
20. **Olsen, M. A., and S. D. Mathiesen.** 1998. The bacterial population adherent to plant particles in the rumen of reindeer fed lichen, timothy hay or silage. Rangifer **18:**55–64.
21. **Orpin, C. G., S. D. Mathiesen, Y. Greenwood, and A. S. Blix.** 1985. Seasonal changes in the ruminal microflora of the high-arctic Svalbard reindeer (*Rangifer tarandus platyrhynchus*). Appl. Environ. Microbiol. **50:**144–151.
22. **Orpin, C. G., Y. Greenwood, F. J. Hall, and I. W. Paterson.** 1985. The rumen microbiology of seaweed digestion in Orkney sheep. J. Appl. Bacteriol. **59:**585–596.
23. **Palys, T., K. Nakamura, and F. M. Cohan.** 1997. Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. Int. J. Syst. Bacteriol. **47:**1145–1156.
24. **Pant, H. C.** 1966. A note on concentration of "*Oscillospira guilliermondii*-type" organisms in the rumen ingesta of buffalo and zebu cattle. Indian Vet. J. **43:**111–113.
25. **Sletten, H., and K. Hove.** 1990. Digestive studies with a feed developed for realimentation of starving reindeer. Rangifer **10:**31–38.
26. **Stackebrandt, E., I. Kramer, J. Swiderski, and H. Hippe.** 1999. Phylogenetic basis for a taxonomic dissection of the genus *Clostridium*. FEMS Immunol. Med. Microbiol. **24:**253–258.

27. **Staley, J. T.** 1999. Bacterial biodiversity: a time for place. ASM News **65:** 681–687.
28. **Storeheier, P. V., S. D. Mathiesen, N. J. C. Tyler, and M. A. Olsen.** 2002. Nutritive value of terricolous lichens for reindeer in winter. Lichenologist **34:**247–257.
29. **Storeheier, P. V., S. D. Mathiesen, N. J. C. Tyler, I. Schjelderup, and M. A. Olsen.** 2002. Utilization of nitrogen and mineral-rich vascular forage plants by reindeer in winter. J. Agric. Sci. **139:**1–10.
30. **Tajima, K., R. I. Aminov, T. Nagamine, K. Ogata, M. Nakamura, H. Matsui, and Y. Benno.** 1999. Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. FEMS Microbiol. Ecol. **29:**159–169.
31. **Tajima, K., S. Arai, K. Ogata, T. Nagamine, H. Matsui, M. Nakamura, R. I. Aminov, and Y. Benno.** 2000. Rumen bacterial community transition during adaptation to high-grain diet. Anaerobe **6:**273–284.
32. **Tajima, K., T. Nagamine, H. Matsui, M. Nakamura, and R. I. Aminov.** 2001. Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. FEMS Microbiol. Lett. **200:**67–72.
33. **Warner, A. C. I.** 1962. Enumeration of rumen microorganisms. J. Gen. Microbiol. **28:**129–146.
34. **Warner, A. C. I.** 1966. Diurnal changes in the concentrations of microorganisms in the rumens of sheep fed to appetite in pens or at pasture. J. Gen. Microbiol. **45:**243–251.
35. **Warner, A. C. I.** 1966. Periodic changes in the concentrations of microorganisms in the rumen of sheep fed limited diets once daily. J. Gen. Microbiol. **45:**213–245.
36. **Warner, A. C. I.** 1966. Diurnal changes in the concentrations of microorganisms in the rumen of a sheep fed limited ration every 3 h. J. Gen. Microbiol. **45:**237–242.
37. **Whitford, M. F., R. J. Foster, C. E. Beard, J. Gong, and R. M. Teather.** 1998. Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. Anaerobe **4:**153–163.
38. **Winter, J., M. R. Popoff, P. Grimont, and V. D. Boekkenheuser.** 1991. *Clostridium orbiscindens* sp. nov., a human intestinal bacterium capable of cleaving the flavonoid C-ring. Int. J. Syst. Bacteriol. **41:**355–357.
39. **Yanagita, K., A. Manome, X.-Y. Meng, T. Kanagawa, H. Tsuchida, R. Mackie, and Y. Kamagata.** 2003. Flow cytometric sorting, phylogenetic analysis and in situ detection of *Oscillospira guilliermondii*, a large morphologically conspicuous but uncultured ruminal bacterium. Int. J. Syst. Evol. Microbiol. **53:**1609–1614.