Flow cytometric sorting, phylogenetic analysis and *in situ* detection of *Oscillospira guillermundii*, a large, morphologically conspicuous but uncultured ruminal bacterium

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Flow cytometric sorting based on its large cell size enabled an enriched fraction of *Oscillospira guillermundii* cells to be obtained from the rumen contents of a sheep. Phylogenetic analysis based on cloned 16S rDNA sequences indicated that the bacterium is a member of the low-G + C Gram-positive bacterial cluster. *Sporobacter termitidis* and *Papillibacter cinnamivorans* were the most closely related known species, with sequence similarities of only 86.3–88.1 %. Fluorescently labelled 16S rRNA-targeted oligonucleotide probes specific for *Oscillospira* were designed and applied to the rumen sample from which the enriched fraction was obtained. The probes hybridized specifically with the large, morphologically conspicuous *Oscillospira* cells.

Oscillospira guillermundii is a large bacterium (3–6 × 10–40 µm), often observed in the rumen contents of sheep and cattle as well as the alimentary tract of other herbivorous animals. *Oscillospira* is characterized as a Gram-positive bacterium, with closely spaced transverse septa and endospores (Gibson, 1974, 1986; Grain & Senaud, 1976; Stewart *et al.*, 1997). Although this bacterium was first described almost a century ago (Chatton & Pérard, 1913), growth in pure culture has not been reported (Gibson, 1974, 1986; Stewart *et al.*, 1997), hence, little is known of its ecological role and physiological properties in the intestinal tract. Clarke (1979) found that *Oscillospira* and other large bacteria attached rapidly to the cuticular surface of clover and grass leaves in the rumen, suggesting that the cuticle of green leaves constitutes a specific niche for these bacteria. Warner (1966) investigated the relationship between grazing behaviour and changes in rumen microbial populations in sheep and found that the population of *Oscillospira* in the rumen fluctuated and the length of the trichome also

changed depending upon the amount of feed consumed. Kurihara *et al.* (1968) reported that total counts of *Oscillospira* in the sheep rumen tended to decrease in the presence of ciliates. Although the number of *Oscillospira* cells is relatively small compared with other bacterial cells, they may make a significant contribution to rumen fermentation because of their large biomass, roughly equivalent to that of ruminal ciliate protozoa (Clarke, 1979; Williams & Coleman, 1997).

To date, culture-based techniques have not enabled the phylogenetic relatedness of *Oscillospira* to known bacteria to be studied. Flow cytometry (FCM), a well-established technology in cell biology and medical microbiology, is now being applied to microbial ecology studies. FCM allows the rapid analysis of bacterial communities and enables single cells to be detected, quantified and sorted according to differences in size, DNA content or phylogenetic affiliation as assessed by fluorescently labelled rRNA-targeted oligonucleotide probes (Amann *et al.*, 1990; Button *et al.*, 1996; Davey & Kell, 1996; Jansson & Prosser, 1997; Wallner *et al.*, 1997). Very recently, Zoetendal *et al.* (2002) have detected and enumerated uncultured *Ruminococcus obeum*-like bacteria in human faecal samples by fluorescent *in situ* hybridization (FISH) and FCM using 16S rRNA-targeted probes. In this study, *Oscillospira* cells were sorted by FCM based on cell size and the 16S rDNA was amplified and

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Abbreviations: FCM, flow cytometry; FISH, fluorescent *in situ* hybridization.

The GenBank/EMBL/DDBJ accession numbers for the *O. guillermundii* 16S rDNA sequences OSC1–OSC5 are AB040495–AB040499.

sequenced. 16S rRNA-targeted oligonucleotide probes were designed to detect specifically this large, morphologically conspicuous but uncultured ruminal bacterium.

Rumen contents were obtained from a Corriedale sheep weighing approximately 50 kg. The sheep was fed twice daily at 9:00 am and 4:00 pm with 600 g timothy hay. In addition, the sheep received 150 g concentrate feed (Chikushi 5P) at 9:00 am. Rumen contents were collected through a ruminal cannula at 2:00 pm. The contents were centrifuged (200 g, 5 min) to remove large particles, consisting mainly of undigested fibres, and the supernatant was strained through a filter (mesh size, 35 µm) to remove suspended dietary particles. Bacterial cells in the filtrate were centrifuged (5000 g, 5 min) and the pellet was resuspended in 1 × PBS (130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2). The resultant suspension was used for FCM analysis and sorting.

FCM analysis and cell sorting were performed with a Coulter Epics Elite ESP instrument (Beckman Coulter) equipped with an argon ion laser (488 nm, 15 mW). The instrument was used to measure forward-angle light scatter (FS; related to size) and side-angle light scatter (SS; related to shape). Iso Flow (Beckman Coulter) was used as sheath fluid. The nozzle diameter was 100 µm. Sheath and sample pressures were kept constant and an analytical rate of approximately 200 events s⁻¹ was maintained by diluting rumen fluid sample. The data were collected as two-dimensional

histograms and analysis was done with the Coulter Epics Elite software (version 4.5).

The FCM histogram of the sheep rumen sample is shown in Fig. 1. FCM sorting resulted in three distinct fractions, indicated as gates A, B and C. Microscopic observation revealed that gate A contained a number of ciliate cells, gate B contained cell debris of ciliates and gate C contained *Oscillospira* cells. Transmission electron micrographs revealed that the cells in gate C showed a cross-wall structure or septum described previously as characteristic of *O. guillermondii* (Gibson, 1974; Grain & Senaud, 1976) (Fig. 2). The *Oscillospira* cells accounted for over 95 % of total cells in the gate C fraction. Based on the total organisms sorted, the *Oscillospira* cells accounted for less than 0.4 % of the total ciliate and bacterial cells in the rumen sample, an enrichment factor of greater than 230. We again sorted the original rumen sample and approximately 5000 *Oscillospira* cells were collected for further 16S rDNA cloning analysis and *in situ* hybridization experiments.

The PCR amplification mixture contained (per 50 µl reaction): 1 µl collected cells (approx. 100 *Oscillospira* cells), 1 × *Taq* polymerase buffer, 2.5 U *Taq* polymerase (Applied Biosystems), 10 µmol dNTPs and 25 pmol of each primer. The PCR primer set for the PCR was the Eubacteria primer set Eub 10F (5'-AGAGTTTGTATCCTGGCTCAG) and Eub 1500R (5'-GGTTACCTTGTTACGACTT) (Weisburg *et al.*, 1991). The PCR amplification conditions were as

Fig. 1. FCM histogram of the sheep rumen sample. FCM cell sorting was performed by measuring forward-angle light scatter (FS) and side-angle light scatter (SS). The compositions of gates A, B and C are described in the text.

Fig. 2. Transmission electron micrograph of an FCM-sorted *Oscillospira* cell. Cells sorted through FCM were fixed with 2.5% (w/v) glutaraldehyde overnight and then post-fixed in 1.5% (w/v) osmium tetroxide at 4 °C for 3 h. The fixed cells were stained with uranyl acetate for 1 h at room temperature, dehydrated and embedded in Spurr low-viscosity resin. Thin-sections of the cells were cut with an ultramicrotome (Reichert Ultracut N) and examined using a transmission electron microscope (Hitachi H-7000) operated at 75 kV.

follows: denaturation and DNA extraction at 95 °C for 9 min, followed by 35 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min. PCR products were purified with a MicroSpin S-400 HR column (Pharmacia Biotech). Cloning of the PCR product was carried out by using pT7 blue T-vector (Novagen), a DNA ligation kit (Takara Shuzo) and *Escherichia coli* DH5 α (Toyobo). Cloned 16S rDNAs were prepared from randomly selected recombinants and used as templates for DNA sequencing. Sequencing was conducted with a DNA sequencer (model 377; Applied Biosystems) using sequencing primers as described previously (Hiraishi *et al.*, 1994). The sequences were analysed with closely related sequences of reference organisms from the BLAST network service (Altschul *et al.*, 1990) and Ribosomal Database Project 2 (RDP2) (Maidak *et al.*, 1999). Sequence data were aligned with CLUSTAL W (Thompson *et al.*, 1994). A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) with the MEGA program package (Kumar *et al.*, 1993). Bootstrap resampling analysis (Felsenstein, 1985) for 100 replicates was performed to estimate the confidence of the tree topologies.

Twenty randomly chosen clones were partially sequenced. Sequencing revealed that all of the cloned sequences formed one cluster and, therefore, five clones (OSC1, OSC2, OSC3, OSC4 and OSC5) were chosen for full sequencing and analysis. These sequences were found to have more than 97.0 % similarity to each other. Phylogenetic analysis indicated these sequences to be affiliated with the low-G+C subclass of the Gram-positive bacteria and to be distantly related to *Sporobacter termitidis* and *Papillibacter cinnamivorans*, with 86.3–88.1 % sequence similarity (Fig. 3). *S. termitidis* is, as its name indicates, an anaerobic bacterium isolated from the paunch of the wood-feeding termite *Nasutitermes lujae*. *S. termitidis* is a chemo-organotroph that grows exclusively on a limited range of methoxylated aromatic compounds including 3,4,5-trimethoxybenzoate and 3,4,5-cinnamate with ring cleavage and produces acetate as a major end product, suggesting that this bacterium may contribute, in part, to the degradation of

lignocellulosic matter in the digestive tract of the termite (Grech-Mora *et al.*, 1996). *P. cinnamivorans* is a strictly anaerobic bacterium that has been isolated from anaerobic digester sludge and is capable of metabolizing several methoxycinnamates (Defnouv *et al.*, 2000).

Whether or not *Oscillospira* is capable of utilizing these substrates in the rumen ecosystem is unknown. To date, all attempts to grow the FCM-sorted *Oscillospira* cells anaerobically on various substrates have been unsuccessful. However, since *Oscillospira* was shown to be associated with the cuticular surface of clover and grass leaves in the rumen (Clarke, 1979), it is possible that they are involved in degradation of the plant cell wall. Indeed, the closest known ruminal species are *Ruminococcus albus* and *Ruminococcus flavefaciens*, prominent fibre-degrading bacteria (Stewart *et al.*, 1997). The other notable finding was that the *Oscillospira* sequences are closely affiliated with cloned sequences within clostridial cluster IV obtained from the caecum of broiler chickens (GenBank accession no. AF376230) and the rumen content of a cow (Tajima *et al.*, 2000), forming a coherent group with sequence similarities of 90.2–91.1 %. Interestingly, a cloned sequence from a human faecal sample also fell into this cluster (Suau *et al.*, 1999), suggesting that micro-organisms within this cluster are widespread in the alimentary tracts not only of herbivores but also of omnivores.

Two *Oscillospira*-specific probes, S-G-Osci-621-a-A-19 (OSC 621) and S-G-Osci-808-a-A-20 (OSC 808), described using standardized nomenclature according to Alm *et al.* (1996), were designed using the BLAST network service (Altschul *et al.*, 1990) and RDP2 (Maidak *et al.*, 1999) for FISH (Table 1). The probes were labelled with FITC for *in situ* hybridization. FITC-labelled bacterial domain probe EUB 338 (Amann *et al.*, 1990) and archaeal domain probe ARC 915 (Stahl & Amann, 1991) were used as controls. Rumen fluid was fixed with 4 % (w/v) paraformaldehyde/PBS (130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2) for 3 h at 4 °C, washed once with PBS and stored in PBS/ethanol (1:1, v/v) at –20 °C until use. The fixed cells were spotted onto a slide (Bokusui Brown) and allowed to

Fig. 3. Phylogenetic tree of cloned *O. guillermondii* 16S rDNA sequences OSC1, OSC2, OSC3, OSC4 and OSC5. The tree was constructed using the neighbour-joining method. Numbers at nodes show bootstrap values obtained from 100 resamplings. Bar, 0.02 substitutions per nucleotide. GenBank/DBJ/EMBL accession numbers are shown in parentheses.

Table 1. 16S rRNA-targeted oligonucleotide probe sequences used in this study

Standardized probe names were derived according to the nomenclature of Alm *et al.* (1996).

Probe	Standardized name	Sequence (5'-3')	Specificity	Target site (<i>E. coli</i> numbering)
EUB 338	S-D-Bact-0338-a-A-18	GCTGCCTCCCGTAGGAGT	Bacteria	338–355
ARC 915	S-D-Arch-0915-a-A-20	GTGCTCCCCGCGCAATTCCT	Archaea	915–934
OSC 621	S-G-Osci-621-a-A-19	GACCCGCAGTTTCAAATGC	<i>Oscillospira</i>	621–643
OSC 808	S-G-Osci-808-a-A-20	CCGCACACCTAGTATTGATC	<i>Oscillospira</i>	808–831

air-dry (Amann *et al.*, 1990). After dehydration in 50, 70 and 100 % ethanol (3 min each), 10 µl prewarmed hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, 0.01 % SDS, 20 % formamide, 25 ng probe) was spotted onto the fixed cells on the slide. The slide was incubated for 2 h at 46 °C in a 50 ml conical tube as an equilibrated chamber. After hybridization, the slide was washed with washing buffer (318 mM NaCl, 20 mM Tris/HCl, 0.01 % SDS) for 30 min at 48 °C. The slide was rinsed with distilled water, air-dried and mounted with Citifluor solution. Fluorescence was detected under an AX-80TR microscope (Olympus Optical) fitted for epifluorescence with a high-pressure mercury bulb and a fluorescence mirror unit for FITC. Hybridization signals were recorded with a C5810 colour chilled 3 CCD camera

(Hamamatsu Photonics) controlled by C5810 remote. Exposure times were 0.01 and 1 s for phase-contrast and epifluorescence microscopy, respectively.

Epifluorescence microscopy revealed that both probes hybridized specifically with *Oscillospira* cells in both the sorted fraction and the original rumen sample (Fig. 4). Bacterial domain probe EUB 338 hybridized with most of the bacterial cells, including *Oscillospira* cells, under the same conditions, whereas the archaeal domain probe ARC 915 did not hybridize with *Oscillospira* cells (data not shown).

Together with *Oscillospira*, *Quinella ovalis* and 'Magnarovum

Fig. 4. FISH detection of *Oscillospira* cells using *Oscillospira*-specific probe OSC 621. Phase-contrast (A, C) and fluorescent (B, D) micrographs of FCM-sorted *Oscillospira* cells (corresponding to the gate C fraction in Fig. 1) (A, B) and the original rumen sample before sorting (C, D).

eadii are also known as relatively large bacteria often seen in the rumen. The 16S rDNA sequence of *Q. ovalis* was determined and found to be affiliated with the *Selenomonas-Megasphaera-Sporomusa* cluster (Krumholz *et al.*, 1993). '*Magnovum eadii*' was characterized on the basis of cell wall analysis and biochemical tests (Orpin, 1976), although its phylogenetic position remains undetermined. Since pure cultures of these bacteria are not available, these studies were performed using enrichment culture or differential centrifugation to obtain cells. Considering the fact that a number of anaerobic protozoa and bacteria present in the rumen have not yet been cultured, molecular techniques such as FISH are powerful, culture-independent methods of monitoring their ecology. The FISH probes specific for *Oscillospira* designed and evaluated in this study could be used to evaluate changes in the population and morphology of this bacterium in relation to diet and other conditions.

In conclusion, FCM sorting and subsequent 16S rDNA cloning, sequence analysis and probe design specific for *Oscillospira* were successful in determining the phylogenetic position of this morphologically distinct but uncultured ruminal bacterium.

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