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Naturally Occurring DNA Transfer System Associated with Membrane Vesicles in Cellulolytic *Ruminococcus* spp. of Ruminal Origin

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A genetic transformation system with similarities to those reported for gram-negative bacteria was found to be associated with membrane vesicles of the ruminal cellulolytic genus *Ruminococcus*. Double-stranded DNA was recovered from the subcellular particulate fraction of all the cellulolytic ruminococci examined. Electron microscopy revealed that the only particles present resembled membrane vesicles. The likelihood that the DNA was associated with membrane vesicles (also known to contain cellulosomes) was further supported by the adherence of the particles associated with the subcellular DNA to cellulose powder added to culture filtrates. The particle-associated DNA comprised a population of linear molecules ranging in size from <20 kb to 49 kb (*Ruminococcus* sp. strain YE73) and from 23 kb to 90 kb (*Ruminococcus albus* AR67). Particle-associated DNA from *R. albus* AR67 represented DNA derived from genomic DNA of the host bacterium having an almost identical HindIII digestion pattern and an identical 16S rRNA gene. Paradoxically, particle-associated DNA was refractory to digestion with EcoRI, while the genomic DNA was susceptible to extensive digestion, suggesting that there is differential restriction modification of genomic DNA and DNA exported from the cell. Transformation using the vesicle-containing fraction of culture supernatant of *Ruminococcus* sp. strain YE71 was able to restore the ability to degrade crystalline cellulose to two mutants that were otherwise unable to do so. The ability was heritable and transferred to subsequent generations. It appears that membrane-associated transformation plays a role in lateral gene transfer in complex microbial ecosystems, such as the rumen.

The rumen ecosystem comprises a complex of dense microbial communities of bacteria, archaea, protozoans, fungi, and bacteriophages (18). The fermentation effected by this complex microbiota is responsible for the conversion of plant feedstuffs to compounds that can be utilized by the animal. Hence, the fermentations and interactions of the microbes are central to ruminant digestion and nutrition. Of considerable interest are the exchange of genetic material between ruminal bacteria and the mechanisms that may enable this process to occur. While extrachromosomal elements, such as plasmids, transposons, and bacteriophages, are well known from ruminal bacteria, few examples of genetic transfer have been documented, and such transfers are largely inferred from the acquisition of antibiotic resistance genes (21). In addition to the transfer of extrachromosomal genetic elements, the presence of transformation systems, in which the chromosomal DNA of the host bacterium is "broken up" and exported from the cell, is known for a variety of bacterial species but has not been reported for ruminal bacteria. Naturally occurring transformation systems are associated with the release of DNA-containing particles from the cell and comprise two main types, phage-like systems based on particles with a viral appearance (4, 7, 25) and cell wall/membrane-based structures that slough off from the cell surface that are variously referred to as vesicles, transformasomes, or blebs (3, 8, 9, 15).

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The current work elucidated a transformation system associated with membrane vesicles of the ruminal cellulolytic genus *Ruminococcus*, a very important genus in the fermentation of plant material in the rumen due to its ability to digest crystalline cellulose, the most stable plant structural polysaccharide (2, 6).

MATERIALS AND METHODS

Bacteria and culture conditions. *Ruminococcus albus* AR67, *Ruminococcus flavefaciens* AR6, AR45, AR69, and AR72, *Butyrivibrio fibrisolvens* AR5, and the bacterial culture conditions and media used have been described previously (12). *Ruminococcus* sp. isolates YE71, YE73, YE74, YE75, YE76, YE78, YE82, and YE83 were isolated from ovine rumen contents in the current work with crystalline cellulose (Whatman no. 1 filter paper or powdered cellulose [Sigma]) as the sole source of carbon for growth.

Cellulolytic bacteria were maintained in cellulose disk (CD) broth, which comprised two or three disks of filter paper (Whatman no. 1) cut with a paper hole punch in 5 ml of rumen fluid (RF)-based broth (12), in which the filter paper was the sole source of carbon for growth. Noncellulolytic bacteria and Cel$^-$ mutants of strain YE71 were maintained on RF medium with cellobiose as the sole carbon source. Cultures were incubated at 39°C.

Concentration of subcellular particles. Cellulolytic bacteria used as sources of subcellular particles were maintained in CD broth but were transferred to RF-cellobiose broth (10 or 100 ml) and grown for 1 to 2 days, depending on the rate of growth of the strain. Subcellular particles were harvested from the culture supernatant, separated from bacterial cells, and concentrated by ultrafiltration (0.45-μm and 0.2-μm Durapore membrane filters [Millipore Corporation, Bedford, MA]) and differential centrifugation, as previously reported for the isolation and concentration of bacteriophage particles (12).

Electron microscopy. Samples of concentrated particles were examined by transmission electron microscopy. Negative staining was performed on butvar-coated grids with either 2% phosphotungstic acid at pH 6.5 or 3% ammonium molybdate at pH 5.1. Samples were examined using a Philips CM10 transmission electron microscope (Philips, Einhoven, The Netherlands).

Isolation and characterization of nucleic acid. Nucleic acid was isolated and purified from concentrated samples by using standard methods (19). The conditions used for digestion of isolated nucleic acids by DNase I and RNase A (Roche, Mannheim, Germany) and with restriction endonucleases HindIII and EcoRI and for digestion of PCR products with CfoI, MspI, and HaeIII were the conditions specified by the manufacturer (Roche).

The routine electrophoretic techniques were the techniques described by Maniatis et al. (19). DNA size was determined by comparison with a HindIII digest of phage λ or a 100-bp DNA size marker (Roche). Pulsed-field gel electrophoresis (PFGE) was performed as previously described (14), except that the machine used was a contour-clamped homogeneous electric field (Bio-Rad, Hercules, CA). The DNA size marker was a phage λ concatemer (Bio-Rad).

PCR amplification, sequencing, and sequence analysis of 16S rRNA genes. The 16S rRNA genes were enzymatically amplified from genomic DNA or subcellular particles (26) using PCR methods previously described by Ouwerkerk and Klieve (24). One nanogram of target DNA was used, and the standard PCR assay consisted of 30 cycles.

Sequencing was performed with an ABI Prism dye terminator cycle sequencing Ready Reaction kit with Amplitaq DNA polymerase FS and a model 373A DNA sequencing system (PE Applied Biosystems Inc., Foster City, CA) by following the manufacturer's protocols. The primers used for sequencing were 27F, 530F, 926F, 1114F, 342R, 787R, 1100R, and 1525R (16). Sequencing reactions were performed at the Griffith University DNA Sequencing Facility (School of Biomolecular and Biomedical Science, Griffith University, Queensland, Australia). Sequence fragments were assembled using ContigExpress from the Vector NTI Suite 6 package (Informax, Frederick, MD) (www.informaxinc.com). The Gapped BLAST database search program (1) at the National Centre for Biotechnology Information was used to compare sequences.

The almost full-length 16S rRNA gene sequences were aligned with similar sequences using the ARB software package (17), and a phylogenetic tree was reconstructed using the neighbor-joining method, the Kimura correction, and an evaluation of 1,000 bootstrapped trees with the Phylo_win program (5).

Attachment of subcellular particles to cellulose powder. To ascertain whether the subcellular particles containing DNA were likely to be membrane vesicles, samples of a particle concentrate from *R. albus* AR67 were incubated with cellulose powder. In cellulolytic ruminococci, cellulosomes are also associated with membrane vesicles, which attach to cellulose (10) and therefore sediment with cellulose upon low-speed centrifugation. Whether DNA can be extracted from cellulose-sedimented vesicles can then be determined.

The subcellular particle fraction (not concentrated) from a 100-ml overnight culture of *R. albus* AR67 in RF-cellobiose broth was transferred to an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI), and 20- to 25-ml aliquots were placed in four sterile serum bottles, two of which contained 100 mg of cellulose powder. The serum bottles were sealed, removed from the anaerobic chamber, and incubated at 39°C for 2 h with mixing every 15 min. Suspensions were then transferred to centrifuge tubes and centrifuged at 300 × *g* for 5 min. The pellet was retained for extraction of DNA from the cellulose fraction. The supernatants were centrifuged again at 30,000 × *g* and 4°C for 2 h to pellet the remaining subcellular particles. DNA was extracted from material pelleted in both the low- and high-speed centrifugations.

Production of Cel$^-$ mutants. To demonstrate transformation, it is necessary to have genetically distinct strains with observable phenotypes that are selectable, such as antibiotic resistance. As libraries of genetically altered ruminococci are not available to our knowledge, mutants of strain YE71 that were not capable of disrupting crystalline cellulase were created.

From an overnight culture (RF-cellobiose broth), 0.1 ml of *Ruminococcus* sp. strain YE71 was transferred to 5 ml RF-cellobiose broth containing 50 μl of a 0.1-mg ml^{-1} ethidium bromide (Bio-Rad) or acridine orange (Sigma-Aldrich, Castle Hill, New South Wales, Australia) solution. The cultures were incubated overnight at 39°C prior to spread plate inoculation of 10-μl portions onto either plain RF-cellobiose agar plates or plates that had been impregnated with ethidium bromide or acridine orange at the concentrations described above. The plates were incubated for a further 24 to 48 h, after which well-separated colonies were picked and duplicate plated on RF-cellobiose and RF-cellulose (powdered cellulose) agar plates. The plates were incubated for 7 to 10 days. Colonies that grew on RF-cellobiose but either did not grow or showed some growth but no zone of clearing on RF-cellulose were selected for further study. Wild-type YE71 was used as a positive control. The putative Cel$^-$ mutants were grown in RF-cellobiose broth overnight prior to replating on RF-cellulose agar plates and subculturing in CD broth to confirm the Cel$^-$ status. The cultures were incubated for 7 to 10 days.

DNA was extracted from Cel$^-$ mutants, and the 16S rRNA gene was PCR amplified and digested with restriction enzymes CfoI, MspI, and HaeIII for comparison with wild-type YE71 to confirm their authenticity.

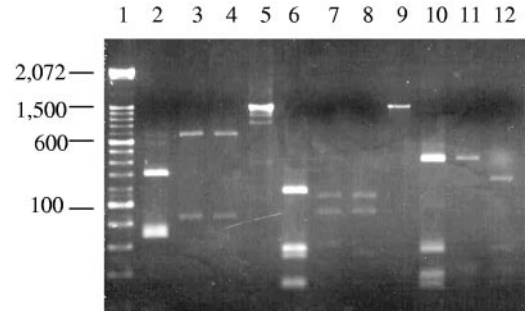

FIG. 1. Restriction endonuclease digests of the 16S rRNA genes from *Ruminococcus* sp. strain YE71 that were not cut (lane 5) or cut with CfoI (lane 2), HaeIII (lane 6), or MspI (lane 10); rRNA genes from *R. albus* AR67 that were not cut (lane 9) or cut with CfoI (lane 3), HaeIII (lane 7), or MspI (lane 11); and rRNA genes from *R. flavefaciens* AR45 that were cut with CfoI (lane 4), HaeIII (lane 8), or MspI (lane 12). Lane 1 contained the 100-bp ladder (Roche) molecular size marker.

Transformation experiments. A series of experiments were performed to determine whether membrane vesicles from YE71 (fresh or refrigerated for 7 days) and AR67 were capable of transducing Cel$^-$ mutants (YE71-2 and YE71-11) and *B. fibrisolvens* AR5 (a hemicellulolytic bacterium) to a Cel$^+$ phenotype (able to degrade crystalline cellulose). The basic experiment involved harvesting membrane vesicles (see above) from a 100-ml RF-cellobiose broth culture of either wild-type YE71 or AR67. The vesicle fraction was resuspended in approximately 600 μl of dilution solution (23) and stored at 4°C until it was used.

Recipient Cel$^-$ bacteria were grown overnight in 5 ml RF-cellobiose broth (one preparation for each treatment) and concentrated to a volume of 200 μl by centrifugation at 1,000 × *g* for 10 min and resuspension in dilution solution. Under anaerobic conditions 200 μl of vesicle concentrate was added to each sample of concentrated recipient bacteria, and the mixture was incubated at 39°C for 2 h before it was added to 5 ml CD broth and incubated for up to 3 weeks. The negative control treatments were (i) recipient bacteria without added vesicles and (ii) vesicles added to a concentrate from sterile RF-cellobiose medium (treated as described above for the bacterial cultures). The positive controls were wild-type bacteria (YE71 or AR67 depending on the experiment) in CD broth and the Cel$^-$ mutants incubated in RF-cellobiose instead of CD broth (to ensure survival during the experimental procedure). Controls were prepared and incubated like the test organisms. CD broth media were observed for signs of cellulose degradation (disk swelling, thinning, release of fibers, disk disintegration) over a 3-week period.

RESULTS

***Ruminococcus* sp.** In order to expand the range of cellulolytic ruminococci available for study and the range of *R. albus* strains in particular, a number of cellulolytic bacteria were isolated in the current work from an in vitro fermentation of rumen contents, as previously reported for other fermentations (13, 20). The in vitro fermentor was inoculated with ovine rumen contents, and oaten chaff-lucerne (70:30) was used as the food source. Isolates YE71, YE73, YE74, YE75, YE76, YE78, YE82, and YE83 were highly cellulolytic cocci that grew predominately in pairs. PCR amplification of the 16S rRNA gene, digestion of the PCR product with CfoI, HaeIII, and MspI, and comparison of DNA fragment banding patterns on electrophoretic gels showed that all isolates were members of the same species but were also genetically distinct from *R. albus* AR67 and the *R. flavefaciens* strains (Fig. 1). The DNA sequence of the 16S rRNA gene of YE71 (GenBank accession no. AY367006) was most closely related to that of *R. albus* 7

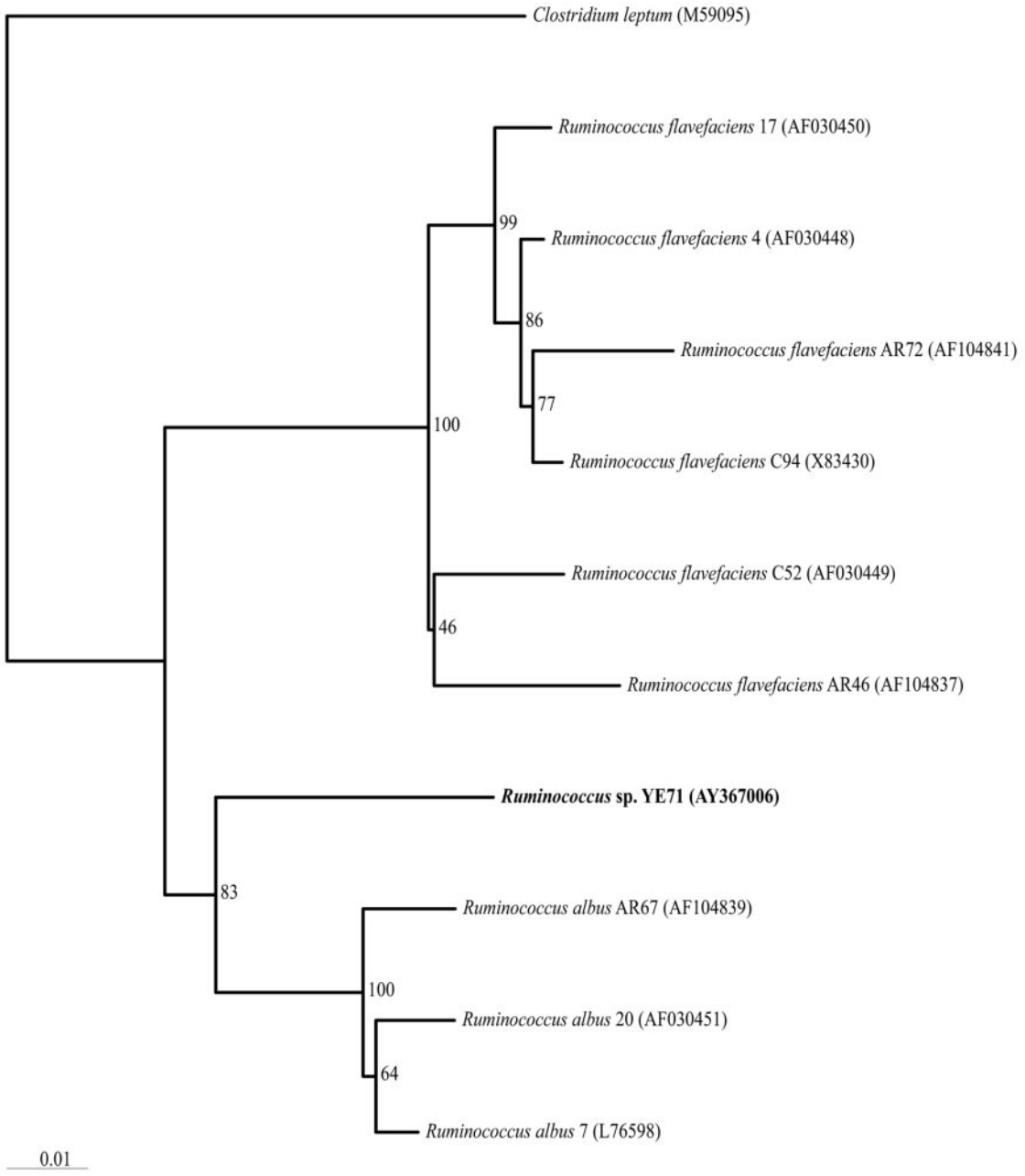

FIG. 2. Phylogenetic tree indicating the relationships of *Ruminococcus* sp. strain YE71 and related ruminococci. Bar = 1% sequence divergence. Database sequence accession numbers for known organisms are indicated in parentheses.

(93.2% binary similarity); however, the difference from related ruminococci is great enough that this organism could be regarded as a separate species (24) (Fig. 2).

DNA characteristics. Nucleic acid was recovered from the subcellular fraction of all the cellulolytic ruminococci examined. This nucleic acid was confirmed to be double-stranded DNA as it was completely digested by DNase I but not by RNase A. The DNA completely dissociated upon heating at 95°C. DNA profiles for selected ruminococci, as they appeared on a conventional agarose gel, are shown in Fig. 3a. The DNA recovered from different bacterial strains varied in appearance on gels. Generally, one main band was present at the limit of migration of linear DNA, just above the 23-kb DNA marker. Some strains (e.g., the YE strains) initially produced three bands, one at around the 23-kb marker, one faint band that migrated more slowly, and one band that migrated much faster, which was suggestive of plasmid DNA with supercoiled, circular, and linear forms present. However, the profiles of these strains tended to vary, and often just the single band at around 23 kb was present.

PFGE of DNA from subcellular particles from *R. albus* AR67 and *Ruminococcus* sp. strain YE73 (Fig. 3b) revealed

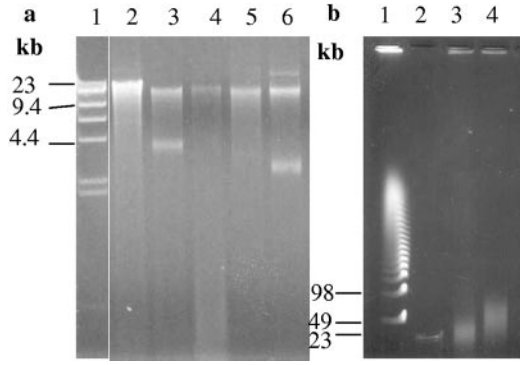

FIG. 3. DNA extracted from subcellular particles and electrophoresed on either a conventional agarose gel (a) or a PFGE gel (b). (a) Lane 1, HindIII digest of phage λ DNA size marker; lanes 2 to 6, *R. albus* AR67, *R. flavefaciens* AR69, AR72, and AR6, and *Ruminococcus* sp. strain YE73, respectively. (b) Lane 1, lambda ladder size marker; lane 2, HindIII digest of phage λ DNA size marker; lanes 3 and 4, DNA from *Ruminococcus* sp. strain YE73 and *R. albus* AR67, respectively.

that the DNA consisted of a population of linear DNA molecules that varied in length from < 20 kb to 49 kb for strain YE73 and from 23 kb to 90 kb for strain AR67. The average length of molecules was different in the two strains; the average length of the YE73 molecules was approximately 30 kb, and the average length of the AR67 molecules was approximately 50 kb. The presence of a small amount of DNA remaining in the well may have indicated the presence of some circularized molecules.

Restriction digestion of particle-associated DNA from *R. albus* AR67 with HindIII (Fig. 4) did not result in a specific banding profile that corresponded to the linear length of the DNA but instead resulted in a complex profile consisting of many bands. Digestion of genomic DNA from *R. albus* cells (Fig. 4) gave a profile that was almost identical to that obtained for the particle-associated DNA. The only discernible difference was that a couple of bands in the digest of the particle-

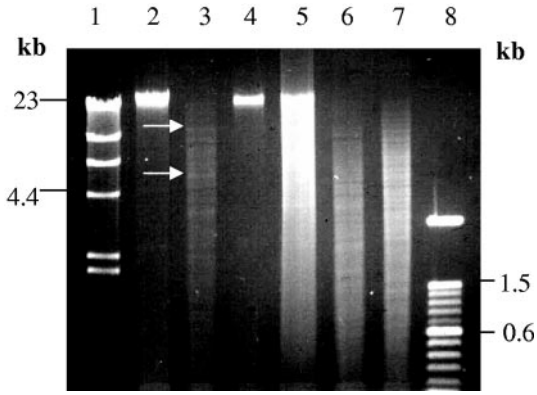

FIG. 4. Restriction digestion of chromosomal DNA extracted from whole cells of *R. albus* AR67 (lanes 5, 6, and 7, uncut and HindIII and EcoRI digests, respectively) and DNA from subcellular particles from *R. albus* AR67 (lanes 2, 3, and 4, uncut and HindIII and EcoRI digests, respectively). Lanes 1 and 8 contained DNA size markers. The arrows indicate DNA bands that appear to be overrepresented in the HindIII digest of the DNA from subcellular particles.

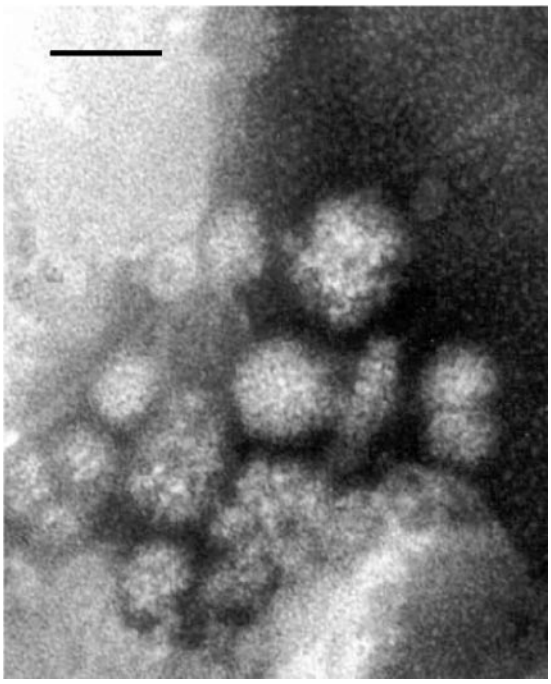

FIG. 5. Electron micrograph of membrane vesicle-like structures from *Ruminococcus* sp. strain YE71. Bar = 50 nm.

associated DNA appeared to be overrepresented compared to the genomic DNA digest (Fig. 4). Paradoxically, digestion of AR67 genomic DNA with EcoRI resulted in a complex profile, like that obtained by digestion with HindIII, but EcoRI did not digest particle-associated DNA (Fig. 4). Furthermore, PCR amplification of the 16S rRNA gene from particle-associated DNA from *R. albus* AR67 resulted in a PCR product with a restriction profile identical to that of the PCR product of the 16S rRNA gene amplified from AR67 genomic DNA.

Electron microscopy. No bacteriophage-like particles were found when concentrated samples of DNA-containing particles from *R. albus* AR67 and *Ruminococcus* sp. strain YE71 were observed by electron microscopy. The only common particles that might be expected to contain DNA resembled the membrane vesicle structures reported previously for *R. albus* F-40 (10). An electron micrograph is shown in Fig. 5.

Cellulose binding. DNA was extracted from the cellulose powder fraction following incubation with subcellular particles, and noticeably less DNA was present in the supernatant following incubation (Fig. 6), suggesting that the subcellular particle DNA was associated with the same structures (membrane vesicles) that were associated with the cellulosomes that had attached to the cellulose powder.

Cel$^-$ mutants. Acridine orange appeared to have little effect on the growth of *Ruminococcus* sp. strain YE71, and the overnight growth in the presence of this compound appeared to be similar to the growth of control cultures. Ethidium bromide, on the other hand, severely reduced the growth rates. Following incubation with acridine orange and ethidium bromide, a total of 75 colonies were selected and examined for the ability to use powdered cellulose. Five clones that had been incubated with ethidium bromide showed a reduced ability to grow on cellu-

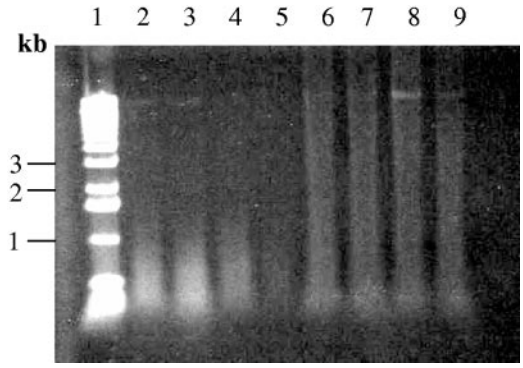

FIG. 6. DNA extracted from the cellulose powder fraction following incubation with subcellular particles from *R. albus* AR67 (lanes 2 and 3, cellulose powder added; lanes 4 and 5, no cellulose added) and DNA extracted from particles remaining in the supernatant following the removal of cellulose powder (lanes 6 and 7, cellulose powder added; lanes 8 and 9, no cellulose added). Lane 1 contained a 1-kb DNA size marker.

lose. Two of these clones (YE71-2 and YE71-11) could not grow on cellulose at all, and when they were inoculated into CD broth, no signs of growth or degradation of the cellulose disks were apparent after 3 weeks of incubation. DNA extraction, PCR amplification of the 16S rRNA gene, and restriction digestion of the PCR product produced banding patterns identical to that of wild-type strain YE71, whose pattern was distinct from those of the other ruminococci (Fig. 1).

Transformation. Subcellular particles from wild-type strain YE71 consistently (three trials) conferred the ability to degrade crystalline cellulose on bacterial mutants that were otherwise unable to do so. Acquisition of this property was quite slow, and the first signs of degradation of the cellulose disks (disk swelling, release of fibers, disk disintegration) did not appear until 7 to 10 days, but then they continued to develop for up to 3 weeks. CD broth media with either a mutant alone or subcellular particles alone showed no signs of degradation of the cellulose disks over the same period.

The acquisition of the ability to degrade crystalline cellulose by the mutants appeared to be heritable, as cells from both transduced mutants were sequentially subcultured twice in CD broth and the rate and extent of cellulose degradation increased so that there was complete disintegration of the disks within 7 days by the second subculture. The appearance of the transformed cells was not noticeably different from the appearance of the wild type.

Transformation of the hemicellulolytic ruminal bacterium *B. fibrisolvens* AR5 with YE71 vesicles was not successful.

DISCUSSION

The cellulolytic ruminococci of the rumen appear to possess a subcellular lateral gene transfer mechanism for transformation. Double-stranded DNA was isolated from particles in culture supernatants that were filterable and sedimentable by centrifugation. Commonly, the only nucleic acid-containing entities with these properties are bacteriophages (12), but no particles having typical phage morphology were observed by electron microscopy. The only "particles" that were observed were amorphous, mainly globular material of variable size that resembled membrane vesicles, as described by Kim et al. (10). The presence of these particles was not surprising as the bacteria under investigation were cellulolytic ruminococci, which are known to release cellulosome-containing vesicles. However, nucleic acid has not previously been associated with these particles. As cellulosome-containing membrane vesicles adhere to cellulose, the addition of cellulose powder to culture supernatant and transfer of a proportion of the extractable DNA from the fluid to the powder suggest that the DNA is associated with membrane vesicles.

DNA was present in culture filtrates from all of the cellulolytic ruminococci investigated, and therefore, this appears to be a common feature in this genus. The DNA, at least the DNA of *R. albus* AR67, appeared to be chromosomal DNA from the host bacterium itself. The majority of the DNA was linear and variable in size. The average size was different for different species of *Ruminococcus*, and based on the endonuclease digestion pattern the entire genome appeared to be present in the overall population of membrane vesicles. The presence of the bacterial 16S rRNA gene further supports this assertion. The amount of DNA associated with each particle and how it is packaged remain to be determined, but it appears that the DNA is processed and packaged for inclusion with the particles. First, the DNA was cut into relatively short linear pieces compared to the bacterial chromosome length. In the HindIII digestion profile there were a couple of DNA bands that appeared to be overrepresented in the particle-associated DNA compared to chromosomal DNA, and these bands may represent repetitive DNA sequences possibly used for packaging. The most intriguing aspect of the particle-associated DNA is its refractivity to digestion with EcoRI, even though the chromosomal DNA from the same bacterium was susceptible to digestion with this enzyme. Restriction modification systems are known for *R. albus* (22), but differential protection of DNA exported from the bacterial cell has not been reported. In addition to the particle-associated DNA reported here, it was previously noted that phage DNA from *R. albus* AR67 was not digested by EcoRI (11). The most plausible explanation for this is that only DNA exiting the bacterial cell is restriction modified, which implies that either the modification system or the chromosomal DNA is compartmentalized. The results also bring into question whether restriction endonucleases or just the restriction protection system is present in the bacterial cell. Considerably more research is required to answer these questions, but it does appear that DNA associated with membrane vesicles is specifically processed for export from the cell.

The presence of the 16S rRNA gene has been used as an indicator of generalized transducing phage systems in bacteria (26). This methodology is equally relevant to other subcellular particles that are filterable and sedimentable in a manner similar to the manner observed for phages. The particle-associated DNA that we describe here appears to be very similar to the "bleb-associated" or "vesicle-associated" transformation systems reported for *Haemophilus influenzae* (9), *Neisseria gonorrhoeae* (3), *Pseudomonas aeruginosa* (8), and *Escherichia coli* (15). These gram-negative bacteria all produce surface vesicles that slough off from the bacterial cell. The ruminococci are gram positive, and similar systems do not appear to have been reported previously for gram-positive bacteria. However, sim-

ilar to the gram-negative bacteria, the ruminococci do produce surface-associated vesicles that slough off from the cell (10). It appears that the surface vesicles of the ruminococci serve a dual purpose, degradation of plant fibrous material and lateral gene transfer.

While the membrane vesicle system of the ruminococci has many characteristics of a transformation system, further evidence is required to clarify the functionality. With genetically well-defined bacterial species, such as the gram-negative pathogens discussed above, functionality is often demonstrated by "rescuing" genetically mutated cell lines or by transfer of new traits, such as antibiotic resistance. Unfortunately, libraries of defined mutants of rumen bacteria in general and ruminococci in particular are not available for such studies. To overcome this limitation, we created mutants that were unable to grow on crystalline cellulose, which resulted in a phenotype that was easily identifiable because of the inability to degrade filter paper. Despite the fact that the mutants were crudely produced and undefined, we demonstrated that concentrated suspensions of vesicles from the wild-type bacterium were able to consistently "rescue" these mutants and enable them to degrade crystalline cellulose. The time necessary for the transformed mutants to show signs of cellulose utilization was long, indicating that the frequency of transformation was probably low, as would be expected. More importantly, the acquisition of cellulose degradation by the mutants was heritable, as indicated by sequential subculture in CD broth media of the rescued mutants. Further work, preferably with well-defined mutants, is required to more fully understand this system.

In conclusion, it appears that the cellulolytic ruminococci possess a transformation system associated with vesicles, giving this organelle at least a dual role in the biology of the ruminococci. This discovery removes the limitation of such systems to gram-negative bacteria and indicates that transformation plays a role in lateral gene transfer in complex microbial ecosystems, such as the rumen.

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