

Isolation and Presumptive Identification of Adherent Epithelial Bacteria ("Epimural" Bacteria) from the Ovine Rumen Wall

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One hundred sixty-one strains of adherent bacteria were isolated under anaerobic conditions from four sites on the rumen epithelial surface of sheep fed hay or a hay-grain ration. Before isolation of bacteria, rumen tissue was washed six times in an anaerobic dilution solution, and viable bacteria suspended in the washings were counted. Calculation indicated that unattached bacteria would have been removed from the tissue by this procedure, but a slow and progressive release of attached bacteria also occurred. Nevertheless, a wide range of characteristic morphological types remained associated with the epithelium as demonstrated by scanning electron microscopy. Most of these types were represented among the isolates. Characterization and presumptive identification of the isolates showed that 95.0% belonged to previously described genera of functionally significant rumen bacteria, including *Butyrivibrio* sp. (31.1%), *Bacteroides* sp. (22.4%), *Selenomonas ruminantium* (9.9%), *Succinivibrio dextrinosolvens* (8.7%), *Streptococcus bovis* (8.1%), *Propionibacterium* sp. (4.3%), *Treponema* sp. (3.1%), and *Eubacterium* sp., *Lachnospira multiparus*, and *Ruminococcus flavefaciens* (2.5% each). Eight isolates (5.0%) were not identified. *L. multiparus* was recovered only from hay-fed animals; all other genera were obtained from animals fed either ration. All *S. bovis* strains and two strains each of *Bacteroides* sp. and *Butyrivibrio* sp. were aerotolerant; all other strains were strictly anaerobic. Bacteria representing the gram-positive, facultatively anaerobic flora associated with rumen wall tissue (R. J. Wallace, K.-J. Cheng, D. Dinsdale, and E. R. Ørskov, *Nature* (London) **279**:424-426, 1979) were therefore not recovered by the techniques used; instead a different fraction of the adherent population was isolated. The term "epimural" is proposed to describe the flora associated with the rumen epithelium.

Extensive association of bacteria with the rumen epithelium in sheep was revealed in a scanning electron microscope (SEM) study by Bauchop et al. (4). It was shown that bacteria remained firmly attached to the epithelium during vigorous in vitro washing of rumen wall tissue. The morphological types and quantitative distribution of adherent bacteria at different wall sites varied fairly consistently among animals, and although some sites supported a mixed bacterial population, others were colonized by a single morphological type. This finding, together with the discovery of bacterial forms not previously described in rumen contents, suggested that a specific flora might exist on the rumen epithelial surface.

Subsequent studies of the adherent population from the rumen of cattle and sheep (29, 30, 35) have provided some support for this suggestion. Although the distribution of attached bac-

teria in cattle may vary with the ration fed (30), the adherent population contains consistently higher proportions of gram-positive (29), facultatively anaerobic (16), and ureolytic (16, 35) bacteria than are found in the luminal population. Indeed, the principal source of bacteria with these characteristics in rumen contents may be the adherent population itself (16); exfoliation of distal epithelial cells would release their associated bacteria from the rumen wall. Specific functions attributed to the adherent population include reductive metabolism of oxygen (16) and hydrolysis of urea (14, 17, 35) entering the rumen across the wall, and digestion of keratinized epithelial cells (29). The adherent population is considered to be both taxonomically and functionally distinct from the populations of bacteria associated with feed particles and rumen fluid (14, 16, 19, 35).

Support for the concept of taxonomic specific-

ity in the adherent population has been derived from the ultrastructural appearance of adherent cells seen by transmission electron microscopy in thin sections of rumen wall tissue (29), and the identification of some facultatively anaerobic components of the adherent population (16) as members of genera, such as *Micrococcus* and *Staphylococcus*, which are normally not significant components of the luminal flora (5, 23). The adherent population is numerous, however (35), and clearly comprises a much wider range of morphological types in situ than is represented by these genera (4, 29); consequently they may represent only a proportion of the taxonomic groups of rumen bacteria capable of adhering to rumen epithelium.

This paper reports the isolation, under anaerobic conditions, and presumptive identification of bacteria from the rumen epithelial surface of sheep.

MATERIALS AND METHODS

Animals. Six Dorset \times Columbia crossbred sheep, 12 to 15 months old, were used as a source of rumen tissue. Eight months before the experiments began the sheep were randomly segregated into two groups; one group was fed 100% brome-alfalfa hay and the other was fed a 62% chopped brome-alfalfa hay-10% grain ration (26). Salt licks and water were freely available to both groups. For some experiments rumen fluid was obtained from a fistulated Guernsey steer fed brome-alfalfa hay.

Rumen wall tissue. Sheep were stunned and killed by jugular vein severance. The abdomen was immediately opened and the esophagus and duodenum were ligated. The intact stomach was excised, placed in a sterile container and transported to the laboratory. Tissue handling procedures were begun within 10 min of death and were performed as rapidly as possible, aseptically, and under a stream of oxygen-free CO₂. Three pieces of rumen wall tissue (3.5 cm in diameter) were cut from each of four sites (Fig. 1) on the midline of each rumen using a sterile scalpel and template. These sites were the roof of the cranial rumen, the roof of the dorsal rumen, the floor of the caudodorsal blind sac, and the floor of the caudoventral blind sac. They were expected to support populations of adherent bacteria which varied consistently between animals in morphology and density (4, 30). Each tissue sample was washed sequentially up to 10 times in 100-ml volumes of sterile anaerobic dilution solution (ADS; 7) at room temperature; the suspended tissue was shaken manually through 30 excursions in each volume. From each washed sample, duplicate subsamples (0.95 cm in diameter) were cut with a sterile no. 5 corkborer. One subsample was prepared for SEM, and the other was used for bacterial isolation.

Anaerobic techniques. The cultural technique used most extensively in this work was that of Hungate (22), as modified by Bryant and Burkey (7); it was employed in conjunction with the V.P.I. Anaerobic Culture System (Bellco Glass Inc., Vineland, N.J.) described by Holdeman and Moore (21). Anaerobic

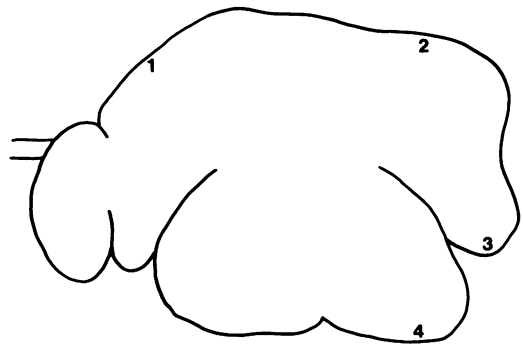


FIG. 1. Diagram of reticulorumen from left side showing tissue sampling sites. All sites on midline: (1) roof of cranial rumen, (2) roof of dorsal rumen, (3) floor of caudodorsal blind sac, (4) floor of caudoventral blind sac.

conditions were maintained by displacing all air in media and dilution fluids with CO₂ made oxygen-free by passage over heated copper. Resazurin (0.0001%) was added as an indicator of redox potential. Some phases of the work were carried out in an anaerobic chamber (model 75A, Germfree Laboratories Inc., Miami, Fla.) containing a 90% CO₂-10% H₂ atmosphere and an incubator similar to that described by Balish et al. (3). Sulfide and residual oxygen were removed from the atmosphere by circulating it sequentially over trays of activated charcoal (10 to 18 mesh; 2) and a palladium catalyst (Deoxo type D; Englehard Industries of Canada Ltd., Toronto, Ontario, Canada) contained in unheated catalyst trays (Coy Laboratory Products Inc., Ann Arbor, Mich.). Gases were obtained from Canadian Liquid Air Ltd., Montreal, Quebec, Canada; CO₂ was Coleman instrument grade (<20 ppm O₂), and H₂ was ultra-high purity grade (<2 ppm O₂).

Isolation of bacteria from rumen tissue. Adherent bacteria were isolated from washed rumen tissue samples, prepared as described above, using medium 10 (12). In some cases isolations were made on agar contained in roll tubes (25 by 142 mm; Bellco Glass Inc.). The epithelial surface of a piece of tissue, or of a papilla cut from it, was heavily smeared over a narrow area at the bottom of the tube, and the resulting inoculum was streaked over the remaining agar surface while the tube was rotated (21). In other cases isolations were made in the anaerobic chamber in petri plates containing prerduced agar medium from which cysteine hydrochloride and sodium sulfide (12) had been omitted. The tissue sample was homogenized manually in the chamber for 2 min in 0.5 ml of medium 10 lacking reducing agents and agar, using a sterile Potter-Elvehjem tissue grinder. The homogenate was then streaked on the agar surface in each of six plates, which were incubated without removal from the chamber. Roll tubes and plates were incubated at 39°C for 7 days. Colonies representative of different types observed microscopically were then transferred to tubes containing medium 10 lacking agar. These cultures were incubated at 39°C until growth was visible. Purity of the resulting isolates was established by restreaking the broth cultures on medium 10 contained in roll

tubes and reisolating typical colonies. Stock cultures of the isolates were maintained in carbohydrate agar maintenance medium (9) at -70°C . Working stocks were maintained in the same medium and transferred biweekly.

Authentic bacterial strains. Nine bacterial strains were used as controls in the characterization of isolates obtained from rumen wall epithelium. They were *Bacteroides ruminicola* subsp. *ruminicola* 23, *Butyrivibrio fibrisolvens* D1 and 49, *Eubacterium ruminantium* GA195, *Lachnospira multiparus* 40, *Megasphaera elsdenii* B159, *Ruminococcus flavefaciens* C94, and *Selenomonas ruminantium* GA192. All of the strains were obtained from M. P. Bryant, University of Illinois. Stock cultures of the bacteria were maintained as described above.

Characterization of isolates. To characterize the isolated strains, colony type, Gram reaction, morphology, motility, and flagellation were determined using cultures grown in medium 10 and the methods described by Bryant and Small (10). The capacity of the strains to grow in the presence of oxygen was tested in medium 10 modified by omission of Na_2CO_3 , cysteine hydrochloride, and sodium sulfide; the medium was prepared, inoculated, and incubated without anaerobic precautions although the tubes were gassed briefly with CO_2 at the time of inoculation since some strains probably required CO_2 for growth (23). This procedure did not result in complete reduction of resazurin contained in the medium. Biochemical tests were carried out by the method of Holdeman and Moore (21), using peptone-yeast extract basal medium supplemented with 5% clarified rumen fluid (CRF2; 8) and appropriate substrates added at recommended concentrations (21). Sufficient 5% clarified rumen fluid to meet all anticipated requirements was prepared in a single batch to avoid variability in composition (25). Cellulose hydrolysis was tested as described by Mann (27). Soluble products produced during fermentation of glucose, cellobiose, maltose (1% each), cellulose, or lactate (0.76%) in supplemented peptone-yeast extract basal medium were determined by the quantitative gas chromatographic procedure of Salanitro and Muirhead (33), using heptanoic acid as internal standard. In this procedure both short-chain volatile and nonvolatile acids are converted to the *n*-butyl esters and separated simultaneously. Residual glucose in fermentations was determined enzymatically using Sigma kit no. 510 (Sigma Chemical Co., St. Louis, Mo.), and residual maltose and cellobiose were determined by the anthrone procedure (31). Cellulose utilization was not quantitated. Culture pH was measured using a Radiometer PHM 64 pH meter equipped with a GK 2303C combination electrode (Bach-Simpson Ltd., London, Ontario, Canada). All tests were carried out at least in duplicate, and appropriate controls were included throughout. Isolates were identified by the method of Holdeman and Moore (21), Buchanan and Gibbons (11), Bryant (6), and Ziolecki et al. (36).

Enumeration of bacteria. Viable rumen bacteria were enumerated by the roll-tube method (24) using medium 10. Cultures were prepared in triplicate and incubated at 39°C for 7 days.

SEM. Samples of washed rumen tissue were fixed for 24 to 48 h at room temperature in glutaraldehyde

(3%) contained in 0.1 M cacodylic acid buffer (pH 7.4). When pure cultures of bacteria were to be examined, cultures in medium 10 lacking agar were filtered (pore size, $0.45\ \mu\text{m}$; Nuclepore Corp., Pleasanton, Calif.), and the filters were fixed for 10 min in the same solution. After fixation, tissue pieces and filters with adherent bacteria were dehydrated in a graded ethanol series (10 to 90% in 10% steps $\rightarrow 100\% \times 3$, with distilled water as the diluent). They were then infiltrated with amyl acetate (50% $\rightarrow 100\% \times 2$, with absolute ethanol as diluent) and critical point dried with liquid CO_2 in a Polaron E-3000 critical point dryer (Polaron Instruments Inc., Warrington, Pa.). The dried samples were mounted on aluminum stubs, coated with approximately 20 nm of gold in a Technico-Hummer shadow caster (Technics Inc., Alexandria, Va.), and examined in a Mark 2A Stereoscan SEM (Cambridge Scientific Instruments Ltd., Cambridge, England). Micrographs were recorded on Kodak 6057 film.

RESULTS

To determine the viability of rumen bacteria in ADS, and therefore the suitability of this solution for extensive washing of rumen tissue, duplicate portions of rumen fluid collected from a steer fed brome-alfalfa hay were diluted with ADS to provide suspensions (A and B) initially containing 10^4 and 10^8 colony-forming units per ml. Enumeration of viable bacteria at intervals during incubation of the suspensions at room temperature showed that the proportions of bacteria losing viability were 12.9 and 6.9% after 0.5 h, and 30.9 and 20.6% after 2 h, in suspensions A and B, respectively. On the assumption that adherent and luminal bacteria would behave similarly in ADS, and because adherent bacteria were not exposed to the solution for more than 0.75 h, it was concluded that ADS did represent a suitable washing medium.

To determine the number of washings required to remove the majority of nonadherent bacteria from rumen tissue, samples obtained from cranial rumen and dorsal rumen sites in three sheep were washed 10 times, and viable bacteria in the first, third, sixth, and tenth washes were enumerated. The rate of dilution of bacteria present after the first wash (Fig. 2) was substantially lower than the rate which would have occurred if 0.5 ml of solution adhered to the tissue after each wash and if all bacteria associated with the tissue were unattached. This shows that a proportion of residual adherent bacteria were removed during each wash; microscopic examination of these washing solutions showed that some of these bacteria were associated with detached epithelial cells. Calculation showed that unattached bacteria would not have exceeded one per ml of ADS after six washes. On the basis of these results, tissue to be used for SEM and bacterial isolations was washed six times.

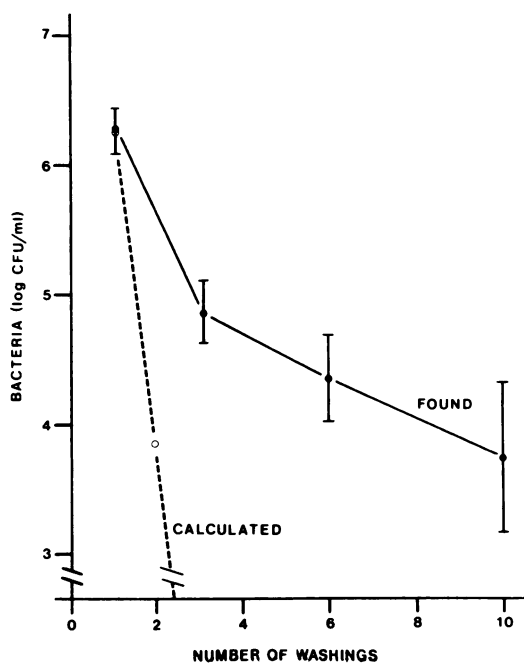


FIG. 2. Effect of washing of rumen tissue on removal of adherent and nonadherent bacteria. Symbols: ●, count of viable bacteria in washing medium. Bars indicate standard deviations from mean counts obtained in three experiments. ○, Calculated rate of dilution of bacteria (for assumptions made see text).

Washed tissue from all four rumen sites sampled supported a mixed population of adherent bacteria (Fig. 3). In some cases (Fig. 4A) the morphological types represented included cocci, straight and curved rods, and spirochetes, and in others (Fig. 4B) the epithelium supported several layers of adherent bacteria. Extensive exfoliation of the epithelium was observed in the cranial rumen site of a sheep fed the hay-concentrate ration (Fig. 4C); one or more epithelial cells had been completely removed from some areas of this sample, leaving cavities containing numerous bacteria of varied morphology (Fig. 4D). Many of these bacteria were clearly adhering one to another rather than to the underlying epithelium directly.

A total of 161 pure strains of adherent bacteria were isolated from roll-tube or petri plate cultures inoculated from washed rumen tissue. Of these isolates, 153 (95.0%) were presumptively assigned to 10 previously described genera of rumen bacteria (Table 1). These included *Butyrivibrio* sp. (31.1%), *Bacteroides* sp. (22.4%), *Selenomonas ruminantium* (9.9%), *Succinivibrio dextrinosolvens* (8.7%), *Streptococcus bovis* (8.1%), *Propionibacterium* sp. (4.3%), *Treponema* sp. (3.1%), and *Eubacterium* sp., *Lachno-*

spira multiparus, and *Ruminococcus flavefaciens* (2.5% each). Eight isolates (5.0%) could not be identified on the basis of the characters determined. Representative strains of all taxonomic groups were recovered from at least one site in the rumen of animals fed the hay or hay-grain rations, except *L. multiparus* which was recovered only from hay-fed animals. No distinction could be made between types of organisms initially cultured in roll tubes or on petri plates in the anaerobic chamber; similar types in similar proportions were obtained using both techniques. A high proportion of the characteristic morphological types of bacteria seen in situ by SEM examination of washed rumen epithelium were represented among the isolates, as judged by SEM observation of pure cultures. The majority of isolates (77.6%) were gram-negative, 16.8% were gram-positive, and the remainder (5.6%) were gram-variable. None formed spores. Most of the strains were obligate anaerobes, showing either no growth or barely visible growth within 1 week in the presence of oxygen. However, all of the isolates of group IX (Table 1), two of group III, and one of each of groups I and II showed heavy growth and completely reduced resazurin within 24 h under the same cultural conditions. These strains were aerotolerant.

The most numerous groups of isolates (group III, Table 1) were strains of *Butyrivibrio* sp., and collectively the second most numerous groups (groups I and II) comprised strains presumptively identified as *Bacteroides* sp. Group I (23 strains) showed only a weak capacity to ferment glucose, cellobiose, or maltose. In medium initially containing 1% glucose, the mean percentage of substrate fermented by these strains was 24.2 (standard deviation = 18.7). The major fermentation product produced was propionate, with smaller amounts of formate, acetate, *iso*-butyrate, *iso*-valerate, and lactate. Only two strains produced succinate. Strains of group I resembled several *Bacteroides* sp. isolated from clinical sources or the human intestine (21) but not previously described rumen strains of this genus. Strains of group II, however, showed characteristics typical of *Bacteroides ruminicola*. The mean percentage of glucose fermented was 71.5 (standard deviation = 13.1), and most strains actively fermented cellobiose and maltose. All of these strains produced acetate and succinate, and most produced formate and propionate as well.

Characteristics of the strains in groups XII and XIII (Table 1) did not resemble those of previously described bacteria, either from rumen contents (23) or the rumen wall (16). Cells of the

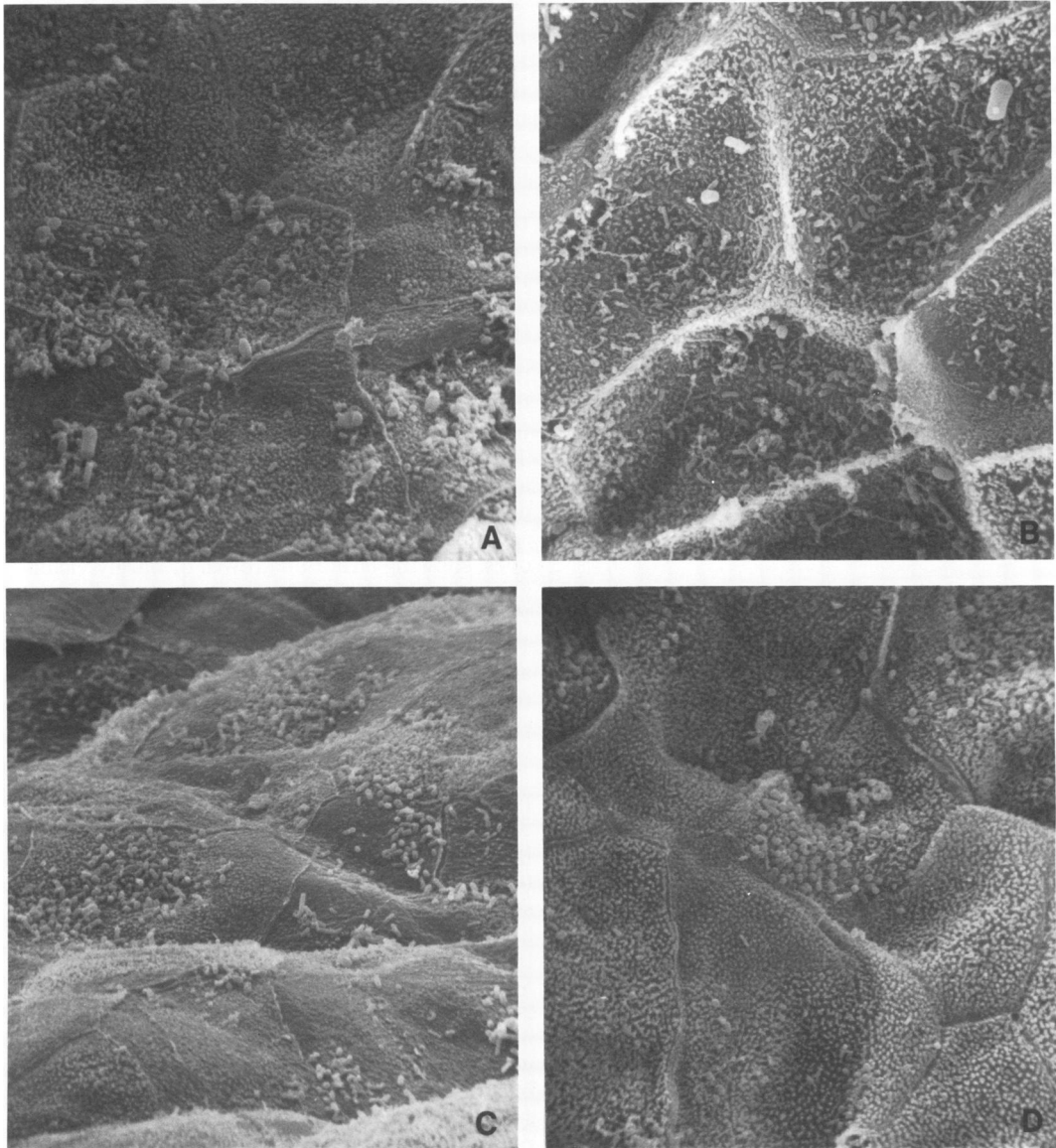


FIG. 3. Scanning electron micrographs of the epithelial surface of washed tissue from the rumen of sheep. (A) Cranial rumen, hay ration. (B) Dorsal rumen, hay-grain ration. (C) Caudodorsal blind sac, hay-grain ration. (D) Caudoventral blind sac, hay-grain ration. Magnification of all, $\times 2,200$.

five strains in group XII were gram-variable, spherical to elongated nonmotile cocci, arranged in pairs and clumps. All of the strains hydrolyzed starch but fermented starch weakly; no strain fermented glucose or maltose, hydrolyzed cellulose, or utilized lactate. One strain fermented cellobiose, producing acetate and succinate, with smaller amounts of formate and lactate, and reducing the culture pH from 6.9 to 5.2. The three strains in group XIII were isolated only

from the cranial rumen site in one sheep fed the hay-grain ration. The predominant morphology in this group was rod shaped, but cultures were highly pleomorphic, showing large ovoid forms on initial isolation as well. These forms decreased in size during continued subculturing of the strains on carbohydrate agar maintenance medium. Cells of all strains were gram-negative and motile by peritrichous flagella. In addition to flagella, extracellular fibrous material was

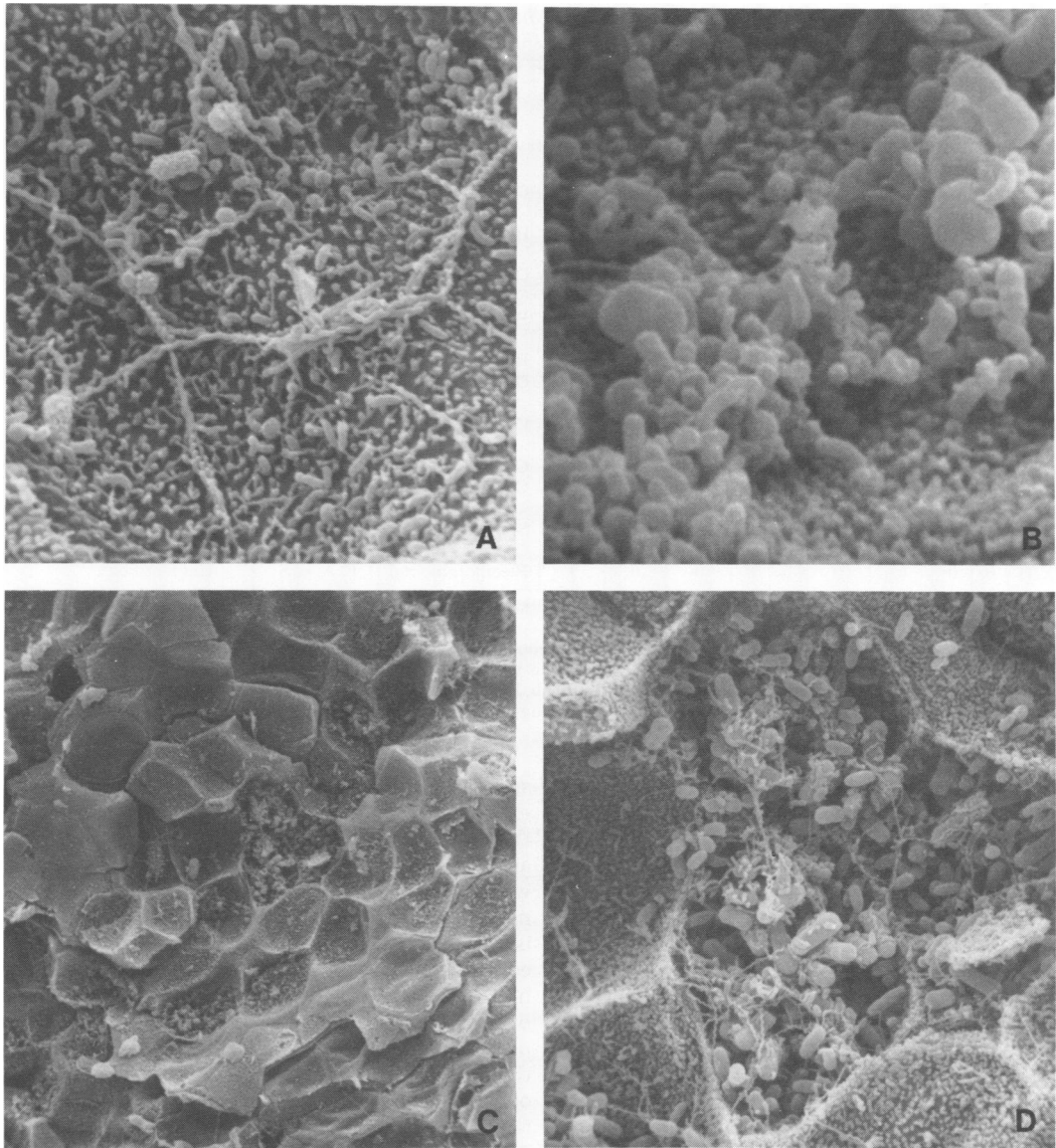


FIG. 4. Scanning electron micrographs of the epithelial surface of washed tissue from the rumen of sheep. (A) Cranial rumen, hay-grain ration, showing mixed population of attached bacteria. Magnification, $\times 5,500$. (B) Cranial rumen, hay ration, showing dense population of attached large cocci and smaller straight and curved rods. Magnification, $\times 11,000$. (C) Cranial rumen, hay-grain ration, showing exfoliation of epithelial cells. Magnification, $\times 550$. (D) Detail of (C) showing mixed bacterial population within cavity left by exfoliated epithelial cell. Magnification, $\times 2,200$.

observed in stained smears and SEM preparations. All of the isolates in this group hydrolyzed starch but not cellulose, and fermented maltose but not glucose or cellobiose. Soluble products of maltose fermentation were formate, acetate, and succinate; in addition one strain produced small amounts of propionate. Further work is

required to characterize the isolates in groups XII and XIII more fully.

Of all the isolates examined, 73.0, 61.3, and 65.0% fermented glucose, cellobiose, and maltose, respectively, causing a decrease in culture pH to 6.0 or less within 7 days. Starch was hydrolyzed by 23.3% of the isolates, but <1%

TABLE 1. Selected characteristics of 161 strains of adherent bacteria isolated from the epithelium in four sites in the rumens of sheep fed two rations

Group	No. of strains isolated from the following sites ^a								Gram reaction ^b	Morphology	Soluble products of carbohydrate fermentation ^{c,d}	Presumptive identification
	Hay ration				Hay-grain ration							
	CR	DR	CDBS	CVBS	CR	DR	CDBS	CVBS				
I	2	2	3	3	3	5	2	3	-	Coccoid rod	(f), (a), P, i-b, i-v	<i>Bacteroides</i> sp.
II	1	2	1	0	1	4	1	3	-	Rod	(f), A, (p), S	<i>Bacteroides ruminicola</i>
III	9	9	8	5	7	1	7	4	-	Curved rod	F, (a), B, L	<i>Butyrivibrio</i> sp.
IV	2	0	1	0	0	0	0	1	v	Coccoid rod	F, a, b, L, s	<i>Eubacterium</i> sp.
V	1	1	1	1	0	0	0	0	-	Filamentous rod	F, A, l	<i>Lachnospira multiparus</i>
VI	3	0	1	1	0	1	1	0	+	Rod	f, A, P, (i-v), s	<i>Propionibacterium</i> sp.
VII	1	0	2	0	1	0	0	0	+	Coccus	f, A, l, S	<i>Ruminococcus flavefaciens</i>
VIII	1	2	0	0	5	4	2	2	-	Crescentic rod	f, a, p, (L), s	<i>Selenomonas ruminantium</i>
IX	1	1	1	1	2	3	1	3	+	Coccus	f, L	<i>Streptococcus bovis</i>
X	0	0	1	4	2	2	2	3	-	Curved rod	F, A, (l), S	<i>Succinivibrio dextrinosolvens</i>
XI	0	0	0	1	1	0	1	2	-	Spirochete	F, A, (l), s	<i>Treponema</i> sp.
XII	0	1	0	0	0	2	1	1	v	Coccus	f, A, l, S	Unidentified
XIII	0	0	0	0	3	0	0	0	+	Pleomorphic rod	F, A, p, S	Unidentified

^a Abbreviations: CR, Cranial rumen; DR, dorsal rumen; CDBS, caudodorsal blind sac; CVBS, caudoventral blind sac.

^b v, Gram variable.

^c Substrate for group VII strains, cellulose; for group XII, cellobiose; for group XIII, maltose; for all other groups, glucose. Substrate concentration in each case except cellulose, 1%; cellulose was Whatman no. 1 paper (27).

^d Abbreviations: f, F, formate; a, A, acetate; p, P, propionate; b, B, *n*-butyrate; i-b, *iso*-butyrate; i-v, *iso*-valerate; l, L, lactate; s, S, succinate. Lower case, production of <1 mmol per 100 ml; upper case, production of ≥1 mmol per 100 ml; (), product not formed by all strains in group.

hydrolyzed cellulose or utilized lactate. Some strains of *Butyrivibrio* sp. and most strains of *Succinivibrio dextrinosolvens* adhered to the glass culture tube when grown in medium 10 lacking agar.

DISCUSSION

Before adherent bacteria could be isolated from rumen epithelium, it was necessary to wash rumen wall tissue to remove nonadherent luminal bacteria present through contact with rumen contents. Ideally, washing should remove all nonadherent but no adherent bacteria. Factors determining the optimum washing procedure include the area and extent of papillation of the epithelial surface, the number of nonadherent bacteria associated with the tissue, and the firmness of adhesion of attached bacteria. Tissue was washed once by Bauchop et al. (4) and twice by McCowan et al. (29) before fixation for SEM examination. We made a comparison between the actual rate of dilution of bacteria in the first solution in which pieces of fresh rumen tissue were washed and the rate which would have occurred in nine subsequent washings if 0.5 ml of solution adhered to the tissue after each wash and if all bacteria associated with the tissue were unattached. This showed that luminal bacteria would not have exceeded one per ml after

six washes and that adherent bacteria greatly exceeded unattached bacteria associated with the fresh tissue. A proportion of the remaining adherent bacteria were removed during each wash. Based on these results, we subjected rumen tissue to be used for SEM and bacterial isolations to six consecutive washes; this ensured complete removal of luminal bacteria but it also had the effect of removing a greater proportion of the adherent population than would be expected with more limited washing. Therefore, the mean density of bacterial cover seen by SEM on the epithelial surface appeared lower than that observed by other workers (4, 29, 30). Nevertheless, a wide range of characteristic morphological types of bacteria, representing virtually all of the types previously depicted, remained attached to the epithelium and were subsequently isolated.

The washing procedure used involved exposure of bacteria to the washing medium for periods up to 45 min. Cultural counts of aerobic bacteria are markedly affected by diluent composition (34), and diluents likely influence the viability of rumen anaerobes. The ADS of Bryant and Burkey (7), however, used here to wash rumen tissue, was shown not to cause substantial destruction of the mixed rumen microflora during periods of exposure up to 2 h.

The proportion of adherent bacteria which lost viability during the washing of rumen tissue was therefore probably small, but whether some taxonomic groups were more sensitive to ADS than others was not determined.

McCowan et al. (29) showed by electron microscopy that highly keratinized distal epithelial cells from the bovine rumen became colonized by bacteria, usually of one morphological type, which digested them. Penetration of the intercellular space underlying colonized cells by the same morphological type of bacteria was also noted. Subsequent exfoliation of these cells would be expected to leave cavities in the epithelium containing the colonizing bacterial form. In the present study, close examination of areas of epithelium undergoing exfoliation revealed the presence of cavities containing numerous bacteria of varied morphology rather than of a single type. Hofman (20) observed similar cavities, attributing their origin to bursting of cells in the surface epithelial layer. The subsequent work of McCowan et al. (29), however, and the appearance of the cavities by SEM (Fig. 4D), suggests that they result instead from the exfoliation of one or more distal epithelial cells. The mixed bacterial population within the cavities could then arise from pre-exfoliative penetration of the subcellular space or postexfoliative colonization by luminal bacteria or by adherent bacteria from adjacent epithelium.

Presumptive identification of bacterial isolates recovered from washed rumen epithelium showed that a high proportion belonged to genera and species common to rumen contents and of functional significance in the rumen fermentation (23). The majority of these isolates did not show the characteristics of gram positivity and facultative anaerobiosis previously attributed to the epithelial flora (16). A functionally significant component of the adherent population (35) was therefore not recovered by the techniques used here. However, the higher proportion of *Butyrivibrio* sp. found (31.1%) may be consistent with the presence on rumen epithelium of many bacteria with a typically gram-positive cell wall ultrastructure (29), since two strains of *Butyrivibrio fibrisolvens*, although producing a gram-negative reaction to the standard staining technique, were of the gram-positive morphological type (15). The notable absence of genera such as *Micrococcus* and *Staphylococcus* (16) from the isolates may be due either to their removal or death during washing or to their inability to form visible colonies under the anaerobic isolation conditions used; if these genera selectively colonize moribund epithelial cells (18), they could have remained attached to

such cells as those separated from the epithelium. A similar mechanism has been postulated to account for the more extensive removal of aerobic cocci than anaerobes during simple washing of human skin (28). Detached epithelial cells from rumen tissue were seen in washings, but their attached bacteria were not identified. Alternatively, these genera may be located more superficially, or adhere to epithelial cells less firmly, than the types of organisms isolated in this study. The results therefore indicate that through extensive washing of rumen epithelium we have isolated components of the adherent flora not previously recognized. Further work is required to determine the quantitative distribution of this predominantly anaerobic flora on the rumen wall, to establish its functional significance in the ecology of the rumen, and thus to assess the degree of taxonomic and functional specificity (4, 16) associated with the epithelial flora as a whole.

Bacterial attachment to solid surfaces in the rumen is now recognized to be a common phenomenon, involving attachment to feed particles (13) and ciliate protozoa (32) as well as to rumen epithelium. Although the mechanisms of adhesion of rumen bacteria to these objects are incompletely understood, the acidic polysaccharides existing in either pure or conjugated form in the bacterial slime coat have been implicated in the adhesion process (29). Other mechanisms may be involved in the attachment of some morphological types of bacteria to feed particles, however, since extracellular material appeared to be absent from bacilli intimately associated with the walls of coastal bermudagrass leaf cells (1). More than one mechanism may therefore be involved in the attachment of rumen bacteria to rumen epithelial cells. Moreover, the capacity of individual bacterial strains to associate with epithelium may be influenced by nutritional and environmental factors which remain to be elucidated.

An adequate descriptive term applicable to bacteria associated with the rumen epithelium, the tissue lining the rumen wall, is lacking. The term "adherent" used by several authors (16, 18, 19, 30) with its connotation of "stickiness," may be misleading because the mechanisms of attachment of bacteria to epithelial cells remain undefined and may vary. Accordingly we propose the term "epimural" (Gr. prep. epi upon; L. adj. muralis fr. murus wall) to describe the flora which is now known to be extensively and consistently associated with the rumen epithelium in domestic ruminants. The term could also be applied to bacteria associated with the gut mucosa in other animals.

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