APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1989, p. 3080–3084
0099-2240/89/123080-05$02.00/0

Vol. 55, No. 12

Degradation of Barley Straw, Ryegrass, and Alfalfa Cell Walls by *Clostridium longisporum* and *Ruminococcus albus*

V. H. VAREL,[1]* A. J. RICHARDSON,[2] AND C. S. STEWART[2]

Roman L. Hruska U.S. Meat Animal Research Center, Agricultural Research Service, U.S. Department of Agriculture, Clay Center, Nebraska 68933,[1] and Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, United Kingdom[2]

Received 31 July 1989/Accepted 25 September 1989

The recently isolated ruminal sporeforming cellulolytic anaerobe *Clostridium longisporum* B6405 was examined for its ability to degrade barley straw, nonlignified cell walls (mesophyll and epidermis) and lignified cell walls (fiber) of ryegrass, and alfalfa cell walls in comparison with strains of *Ruminococcus albus*. *R. albus* strains degraded 20 to 28% of the dry matter in barley straw in 10 days, while the clostridium degraded less than 2%. A combined inoculum of *R. albus* SY3 and strain B6405 was no more efficient than SY3 alone, and the presence of *Methanobacterium smithii* PS did not increase the amount of dry matter degraded. In contrast, with alfalfa cell walls as the substrate, the clostridium was twice as active (28% weight loss) as *R. albus* SY3 (15%). The percentages of dry matter degraded from ryegrass cell walls of mesophyll, epidermis, and fiber for the clostridium were 50, 47, and 32%, respectively, and for *R. albus* SY3 they were 77, 73, and 63%, respectively. Analyses of the predominant neutral sugars (arabinose, xylose, and glucose) in the plant residues after bacterial attack were consistent with the values for dry matter weight loss. Measurements of the amount of carbon appearing in the fermentation products indicated that *R. albus* SY3 degraded ryegrass mesophyll cell walls most rapidly, with epidermis and fiber cell walls being degraded at similar rates. Strain B6405 attacked alfalfa cell walls at a rate greater than that of any of the ryegrass substrates. These results indicate an unexpected degree of substrate specificity in the ability of *C. longisporum* to degrade plant cell wall material.

The cellulolytic bacterium *Clostridium longisporum* was first isolated from a bovine rumen by Hungate (12, 13). The first isolates were very actively cellulolytic, but they were eventually lost. Recently, Varel (21a) described the isolation of new strains of this organism and found that *C. longisporum* hydrolyzed preparations of alfalfa cell walls more extensively than did strains of other prominent plant cell wall-degrading rumen bacteria [*Ruminococcus albus*, *R. flavefaciens*, *Fibrobacter* (*Bacteroides*) *succinogenes*, and *Butyrivibrio fibrisolvens*] with which it was compared.

The distribution and organization of structural polymers in the cell walls of dicotyledonous plants, such as alfalfa and clover, differ from those in monocotyledons, including grasses (21). Such differences inevitably affect the way bacteria degrade these substrates. It has been shown, for example, that the pectinolytic bacterium *Lachnospira multiparus* macerates leaflets of clover upon incubation in vitro but has no comparable effect on ryegrass leaves (5). Since *C. longisporum* has been found in the rumens of animals fed either legume or grass forage, it was of obvious interest to measure the activity of a recent isolate against ryegrass cell walls and against a comparatively highly lignified forage, barley straw. We report here the activity of *C. longisporum* B6405 against alfalfa, barley straw, and ryegrass cell walls in comparison with the activity of strains of *R. albus* previously shown to be active in the degradation of straw (9, 15) and ryegrass (6). In this study, evidence for synergy between *C. longisporum* and *R. albus* in the degradation of straw was sought and the effect of the presence of a methanogen, *Methanobrevibacter smithii*, on plant cell wall degradative activity was examined.

* Corresponding author.

MATERIALS AND METHODS

Bacteria. *C. longisporum* B6505 was isolated as described by Varel (21a). *R. albus* SY3 was isolated as described by Wood et al. (22), and strains J1, J2, and J6 were isolated as described by Stewart and Duncan (19). *M. smithii* PS was from the Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany (as strain DSM 861).

Anaerobic methods and culture maintenance. The anaerobic methods used were similar to those of Bryant (4). Bacteria were routinely maintained on agar stabs (10 ml) of M2 (11), a habitat-simulating medium incorporating rumen fluid and three carbohydrates, cellobiose, glucose, and maltose, each at 0.2% (wt/vol). The medium was prepared and maintained under O_2-free CO_2 in screw-capped Hungate tubes (16 by 125 mm) fitted with butyl rubber septum stoppers (Bellco Glass, Inc., Vineland, N.J.). *M. smithii* PS was maintained on medium M2 without carbohydrates (medium M2-CH_2O) under an atmosphere of 80% H_2–20% CO_2.

Incubations with plant cell walls (see below) were in medium V1, which contained (per 100 ml) 15 ml of clarified rumen fluid which had been preincubated at 38°C for 5 days to remove readily fermentable substrates, 15 ml each of mineral solutions 1 and 2 from medium M2 of Hobson (11), 0.05 g each of Casitone (Difco Laboratories, Detroit, Mich.) and Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 0.1 g of yeast extract, 0.4 g of $NaHCO_3$, 0.1 g of cysteine hydrochloride, 0.1 mg of resazurin, and 55 ml of distilled water.

Incubations with plant cell walls. The main objective of the study was to compare the activity of *C. longisporum* with that of *R. albus* for each cell wall type. The scale of the incubations and the concentration of plant cell walls in the media were varied, because in some cases only limited quantities of plant cell walls were available.

Barley straw was the straw (without chemical pretreatment; particle size, 425 to 600 μm) described by Kolankaya

et al. (15). Straw (250 mg) was weighed into Wheaton bottles (100-ml volume) (Phase Separations Ltd., Clwyd, United Kingdom) before addition of 50 ml of medium V1 under O_2-free CO_2, sealing with butyl rubber stoppers retained with aluminum crimp seals, and autoclaving (121°C, 15 min).

Alfalfa cell walls were prepared as described by Varel (21a). Wheaton bottles, medium addition, and autoclaving were as for barley straw, except that the amount of starting material was 200 mg per bottle. Separated leaf mesophyll, epidermis, and fiber cell walls from ryegrass were prepared as described by Chesson et al. (6). The incubation conditions were those used for alfalfa, but the amount of mesophyll cells weighed into the bottles was reduced to 120 mg.

The experiment in which the various *R. albus* strains were compared involved addition of 50 mg of alfalfa cell walls or barley straw (425- to 600-μm particles) to Hungate tubes (16 by 125 mm) to which 9 ml of medium V1 was added before the tubes were sealed with septum stoppers and autoclaved as described above.

Inocula of the cellulolytic bacteria were grown on plant cell walls (or mixtures of cell walls) to condition the cells to growth on cellulosic substrates. *R. albus* SY3 and *C. longisporum* B6405 were grown separately in Wheaton bottles containing medium V1 with barley straw (250 mg) that had been ball milled for 48 h to a fine powder to aid pipetting. These cultures were incubated for 72 h at 38°C. Wheaton bottles were inoculated with 2.5 ml of the appropriate culture, except that when both strains SY3 and B6405 were added, the volume of inoculum added was 1.25 ml of each. *M. smithii* was pregrown in medium M2-CH_2O at 38°C for 72 h (optical density, 0.70), and 2.5 ml of the resulting culture was used as an inoculum when appropriate. Previous studies have shown that weight from inoculum dry matter is negligible (21a), and it was not considered in the dry matter loss value, nor was the weight from microbial growth. Corrections were made for solubilization of the substrate due to autoclaving.

Essentially the same procedure was used to prepare inocula with alfalfa cell walls and ryegrass as substrates, except that the inocula consisted of 5 ml of the relevant culture grown on a mixture of approximately equal proportions (100 mg) of alfalfa walls and ryegrass mesophyll, epidermis, and fiber cell walls. The inocula (0.5 ml) for comparison of *R. albus* strains were grown on a mixture (200 mg each) of ball-milled barley straw and alfalfa cell walls for 48 h. When appropriate, 1.0 ml of *M. smithii* PS grown as described above was also added.

After static incubation at 38°C for the desired period, cell wall residues were recovered by centrifugation (2,000 × *g*, 20 min), washed twice with distilled H_2O, and then lyophilized. When removal of culture supernatant samples was necessary, 1.0 ml of the liquid phase was withdrawn aseptically with a sterile plastic syringe fitted with a 23-gauge hypodermic needle.

Chemical analyses. Fermentation products were analyzed by the method of Schooley et al. (18) or by high-pressure liquid chromatographic methods published elsewhere (21a). H_2 gas values were normalized to 20°C and 1 atm (101.29 kPa). CO_2 was not measured and not included in the total carbon fermentation products presented (see Fig. 2). The products analyzed for SY3 were formate, acetate, and ethanol; for B6405, they were formate, acetate, butyrate, and ethanol. Neutral sugars in cell wall residues were analyzed by gas chromatography (1), following derivatization to alditol acetates (2). Cellulose was determined by the method of Updegraff (20), and uronic acid was measured by the method of Blumenkrantz and Asboe-Hansen (3). Soluble carbohydrates in culture supernatants were analyzed with anthrone for hexoses and with orcinol for pentoses (10).

TABLE 1. Compositions of barley straw and fiber from alfalfa and ryegrass before incubation with bacteria

Component	% Dry matter		
	Barley straw[a]	Alfalfa fiber	Ryegrass fiber[b]
Cellulose	33.0	35.7	40.0
Hemicellulose	28.1	13.3	45.0
Glucose	41.4	40.4	46.6
Arabinose	3.0	2.6	3.7
Xylose	20.9	10.7	25.0
Uronic acid	3.3	3.8	3.8
Total phenolic compounds		12.8	5.0
Lignin	14.9	15.9	5.0
Protein (N × 6.25)	3.3	7.7	1.7

[a] From Graham and Aman (8) and Graham et al. (9).
[b] From Chesson et al. (6) and Gordon et al. (7).

Statistics. All analyses were performed by analysis of variance and least-significant difference with the Statistical Analysis System with a factorial design (17). The main effects tested were substrate and bacteria strain.

RESULTS

Chemical analysis of substrates. The chemical compositions of barley straw, Italian ryegrass (var. Perma) fiber cells, and the alfalfa cell wall preparation are summarized in Table 1. The barley and alfalfa cell walls were comparable in cellulose and lignin content, while hemicellulose in barley was two times greater than in alfalfa. Ryegrass fiber had greater amounts of cellulose and hemicellulose but less lignin than the other substrates.

Incubations with barley straw. *R. albus* SY3 and *C. longisporum* B6405 were incubated with barley straw both alone and in combination. The incubations were performed both with and without *M. smithii* PS. Approximately 12% of the straw was solubilized by autoclaving; upon subsequent incubation, it was found that although *R. albus* SY3 degraded around 20% of the straw during 10 days of incubation, *C. longisporum* B6405 had no significant degradative activity (Fig. 1A). A combined inoculum of strains SY3 and B6405 was no more active than SY3 alone, and the presence of *M. smithii* PS did not increase degradative activity (Fig. 1A). Strain SY3 was much more active in production of H_2 than was strain B6405 (Table 2); in this respect, the mixed culture of these two strains was intermediate to the pure cultures (Fig. 1B). In the presence of *M. smithii* PS, H_2 was normally found only in trace amounts (data not shown), but it was slightly more evident in the mixed culture of SY3 and PS (Fig. 1B). Methane accumulated in cultures inoculated with *M. smithii* PS and was produced in greatest quantity in cultures that contained *R. albus* SY3 (Fig. 1C). Addition of *M. smithii* PS to *R. albus* SY3 or *C. longisporum* B6405 reduced the amounts of formate and ethanol but increased the concentration of acetate detected (data not shown). Strain B6405 produced small amounts of acetate, butyrate, formate, and ethanol, but when *M. smithii* PS was present in coculture with strain B6405, the amounts of individual products detected were too small to allow accurate quantification. Likewise, when the substrate was alfalfa cell walls and strain PS was added to either or both of the cellulolytic organisms, no increase in the amount of substrate degraded was observed.

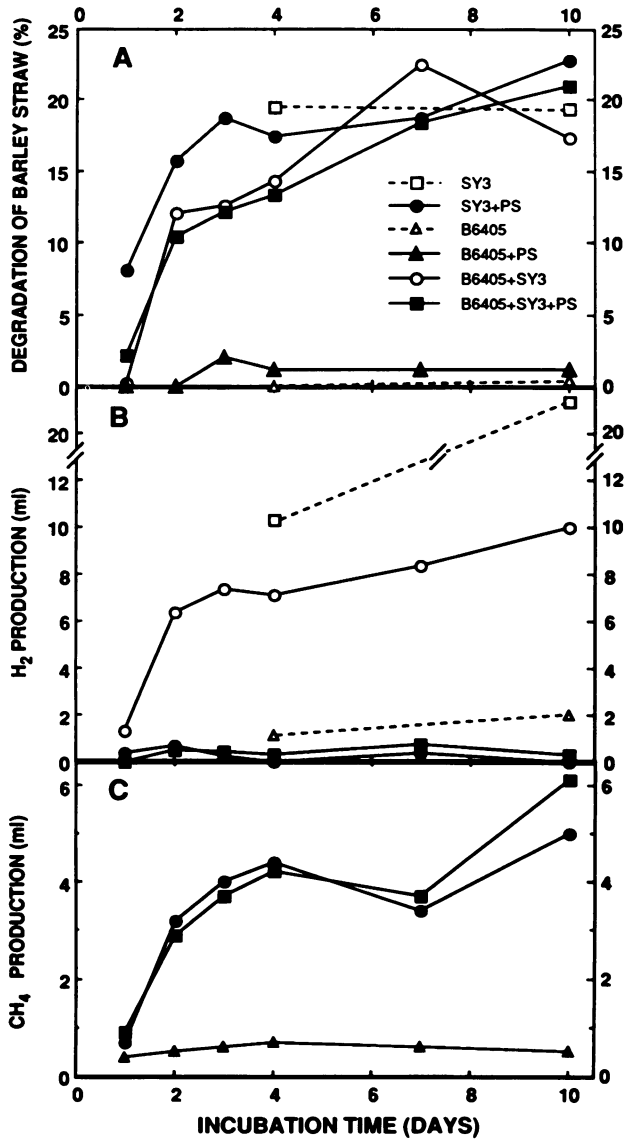

FIG. 1. Degradation and H_2 and CH_4 production (n = 3) from barley straw by *R. albus* SY3, *C. longisporum* B6405, and *M. smithii* PS.

Fermentation rates. The losses in weight of alfalfa and ryegrass cell walls that accompanied incubation with *R. albus* SY3 or *C. longisporum* B6405 for 75 h are shown in Table 2. Strain B6405 was about twice as effective against alfalfa as was strain SY3. In contrast, with the ryegrass fractions, *R. albus* SY3 consistently degraded more dry matter than did the clostridium. These results are supported by the appearance of carbon in the fermentation products (Fig. 2). *R. albus* always produced more H_2 than did the clostridium (Table 2). Analysis of the predominant neutral sugars (arabinose, xylose, and glucose) in the residues after bacterial attack revealed that *C. longisporum* removed all three of these sugars from alfalfa more extensively than did *R. albus*, but the opposite was true for the ryegrass fractions, with *R. albus* being the most effective.

Degradation of alfalfa and barley straw by strains of *R. albus*. Results from the initial experiments suggested that *R. albus* SY3 was more active against barley straw than against alfalfa cell walls. To confirm and extend this observation, SY3 and three other strains of *R. albus* were incubated with straw and alfalfa cell walls for 10 days. Measurements of the percent loss in weight of the plant material and H_2 production (Table 3) confirmed this difference in susceptibility, although the difference was greater with some strains than with others (e.g., contrast strain J2 with strain J6).

DISCUSSION

Results from this study demonstrate the different abilities of *R. albus* strains and *C. longisporum* to degrade barley straw, alfalfa cell walls, nonlignified cell walls of ryegrass (mesophyll and epidermis), and lignified ryegrass fiber cell walls. These substrates are expected to vary considerably in their contents of structural carbohydrates within the plant cell walls; thus, differences in attack by ruminal strains are expected.

C. longisporum solubilized the hemicellulose fraction of alfalfa more extensively than do other rumen isolates (21a); however, this organism did not grow in the presence of larchwood xylan, xylose, or arabinose. Osborne and Dehority (16) have recently shown that although *Bacteroides ruminicola* H2b is unable to utilize purified pectin, it can degrade and utilize forage pectin. Thus, isolation and characterization of rumen bacteria on purified substrates may be misleading with regard to their role and importance in overall rumen fermentation. Similarly, the data (Table 2) indicate that *C. longisporum* was able to degrade arabinose and xylose from cell walls of alfalfa and ryegrass.

It was surprising to see *C. longisporum* degrade barley

TABLE 2. Degradation of dry matter, arabinose, xylose, and glucose from plant fractions and H_2 production upon incubation with *R. albus* SY3 or *C. longisporum* B6405 for 75 h

Substrate fraction	Mean % degradation of component by indicated strain								H_2 production (ml)[b]	
	Dry matter[a]		Arabinose		Xylose		Glucose		SY3	B6405
	Sy3	B6405	SY3	B6405	SY3	B6405	SY3	B6405		
Alfalfa (fiber)	14.9[c]	28.3[d]	33.0	59.2	2.0	8.3	15.7	23.5	6.2[e]	4.5[f]
Ryegrass										
Fiber	63.1[c]	31.9[d]	82.8	26.5	86.7	26.5	89.0	42.2	21.0[e]	6.0[f]
Mesophyll	76.7[c]	50.0[d]	96.2	44.1	98.5	49.4	97.5	79.9	11.5[e]	5.5[f]
Epidermis	73.1[c]	47.1[d]	95.4	56.0	89.5	58.9	96.9	80.4	18.0[e]	8.6[f]

[a] Pooled standard error, 0.97. Means (n = 3) not having the same superscript differ ($P < 0.01$). The residues from triplicate tubes (duplicate for mesophyll) were pooled and analyzed for arabinose, xylose, and glucose.

[b] Pooled standard error, 0.12. Means (n = 3) not having the same superscript differ ($P < 0.01$).

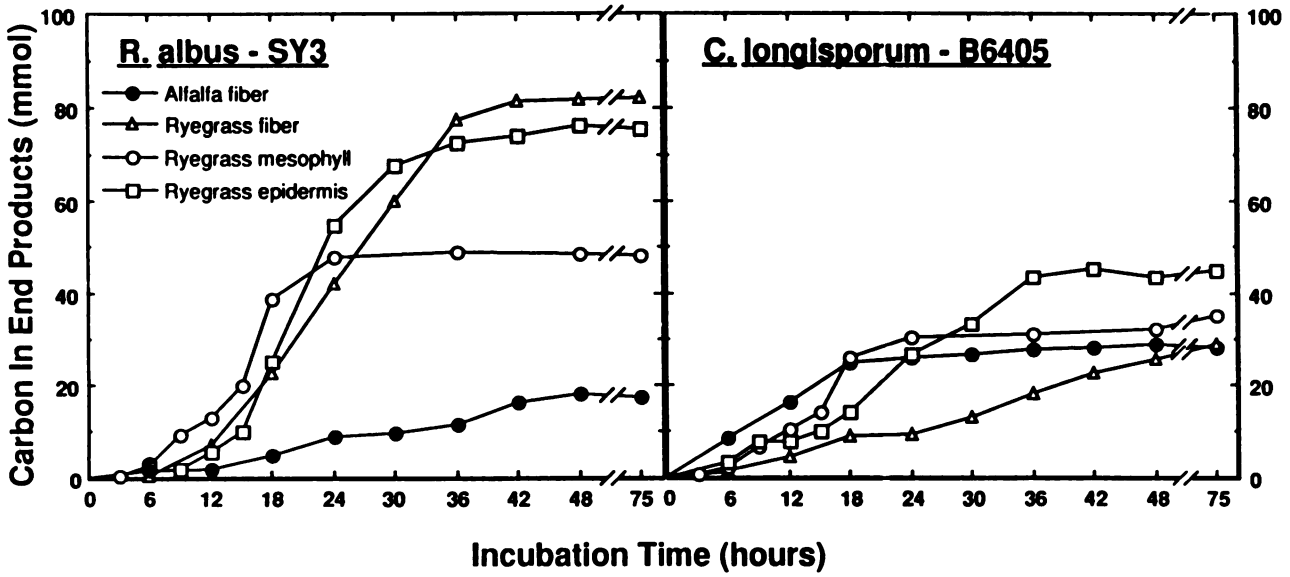

FIG. 2. Analyzed carbon fermentation products of strains SY3 and B6405 incubated with alfalfa fiber or ryegrass fiber, epidermis, or mesophyll cell walls.

straw and ryegrass so poorly in comparison with *R. albus* SY3, although Kock and Kistner (14) have demonstrated the limited ability of clostridia to attack native cellulose in grass. Barley straw and ryegrass have much higher hemicellulose contents than does alfalfa. Many clostridia have a very active pectinase, and it was initially thought that pectin is the substrate degraded in alfalfa by *C. longisporum*, which in turn would give the appearance of greater loss of dry matter. Our studies were done with alfalfa cell wall preparations which involved boiling ground alfalfa in neutral detergent for 1 h. Presumably, this would remove most of the pectin and eliminate it from playing a role in determining the amount of substrate solubilized. The results on degradation of the various substrates point out our limited knowledge about the cell wall matrix in forage plants, especially regarding how ruminal fibrolytic organisms attack this matrix. Not enough is known of the properties of the enzyme systems involved in the hydrolysis of plant cell wall polymers by *C. longisporum* to explain the substrate specificity of strain B6405 reported here. Characterization of these enzyme systems could be particularly informative.

When cultures of *R. albus* and *C. longisporum* were combined with barley straw as the substrate, no increase in solubilization of the substrate over that caused by *R. albus* by itself was observed. This suggests that *C. longisporum* attacks the same polysaccharides as *R. albus* does, yet with much less efficiency. *M. smithii* PS had no effect on the amount of substrate solubilized by either organism, which suggests that accumulation of H_2 is not inhibitory to the metabolism of either organism. Addition of *M. smithii* merely shifted fermentation products from less reduced ones, such as formate and ethanol, to more oxidized ones, such as acetate.

Our results (Table 2) confirmed those of Chesson et al. (6) and others in that nonlignified cell walls (mesophyll and epidermis) were more extensively degraded than more lignified fiber cell walls. While *R. albus* was superior to *C. longisporum* in degrading both substrates, little difference between the extents of mesophyll and epidermis cell wall degradation by *R. albus* or *C. longisporum* was observed. On the basis of the appearance of carbon in the fermentation products (Fig. 2), we conclude that *R. albus* SY3 degraded the mesophyll cell walls most rapidly, followed by a close similarity between the rates of epidermis and fiber cell wall degradation. The reason for the latter observation is unclear because the fiber cell walls are more lignified (6). However, the results obtained with *C. longisporum* (Fig. 2) point out that lignin alone is not indicative of the rate of fermentation because alfalfa cell walls (15% lignin) were fermented at a faster rate than ryegrass fiber (5% lignin). Epidermal cells may be partly protected by the presence of a cuticle (19).

TABLE 3. Degradation of dry matter and production of H_2 from alfalfa fiber and barley straw by strains of *R. albus* after 10 days

R. albus strain	Mean % degradation[a]		Mean H_2 production (ml)[b]	
	Alfalfa	Barley	Alfalfa	Barley
SY3	18.8^c	28.0^d	1.3^e	1.9^f
J1	20.5^c	23.0^d	0.6^e	1.0^f
J2	10.9^c	24.6^d	0.5^e	1.3^f
J6	22.9^c	28.4^d	2.0^e	2.4^f

[a] Pooled standard error, 0.41. Means (n = 4) in each row without a common superscript differ ($P < 0.01$).
[b] Pooled standard error, 0.01. Means (n = 4) in each row without a common superscript differ ($P < 0.01$).

ACKNOWLEDGMENTS

We thank Susan Anderson and Graham Calder for gas chromatography assistance, Peter Dewey for sugar and cell wall analyses, and Debbie Brown for typing the manuscript.

V.H.V. was the recipient of an OECD fellowship, and the work was conducted in the laboratory of C.S.S.

LITERATURE CITED

1. **Bacon, J., and A. H. Gordon.** 1980. The effect of various deacetylation procedures on the nylon bag digestibility of barley straw and of grass cell walls recovered from sheep faeces. J. Agric. Sci. 4:361–367.
2. **Blakeney, A. B., P. J. Harris, R. J. Henry, and B. A. Stone.** 1983. A simple and rapid preparation of alditol acetates for monosac-

charide analysis. Carbohyr. Res. **113**:291–299.
3. **Blumenkrantz, N., and G. Asboe-Hansen.** 1973. New method for quantitative determination of uronic acids. Anal. Biochem. **54**:484–489.
4. **Bryant, M. P.** 1972. Commentary on the Hungate technique for the culture of anaerobic bacteria. Am. J. Clin. Nutr. **25**: 1324–1328.
5. **Cheng, K.-J., D. Dinsdale, and C. S. Stewart.** 1979. Maceration of clover and grass leaves by *Lachnospira multiparus*. Appl. Environ. Microbiol. **38**:723–729.
6. **Chesson, A., C. S. Stewart, K. Dalgarno, and T. P. King.** 1986. Degradation of isolated grass mesophyll, epidermis and fibre cell walls in the rumen and by cellulolytic rumen bacteria in axenic culture. J. Appl. Bacteriol. **60**:327–336.
7. **Gordon, A. H., J. A. Lomax, K. Dalgarno, and A. Chesson.** 1985. Preparation and composition of mesophyll, epidermis and fiber cell walls from leaves of perennial ryegrass. (*Lolium perenne*) and Italian ryegrass (*Lolium multiflorum*). J. Sci. Food. Agric. **36**:509–519.
8. **Graham, H., and P. Aman.** 1984. A comparison between degradation *in vitro* and *in sacco* of constituents of untreated and ammonia treated barley straw. Anim. Feed Sci. Technol. **10**: 199–211.
9. **Graham, H., P. Aman, O. Theander, N. Kolankaya, and C. S. Stewart.** 1985. Influence of heat sterilization and ammoniation on straw composition and degradation by pure cultures of cellulolytic rumen bacteria. Anim. Feed Sci. Technol. **12**: 195–203.
10. **Herbert, D., P. J. Phipps, and R. E. Strange.** 1971. Chemical analysis of microbial cells, p. 209–344. *In* R. J. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 5B. Academic Press, Inc. (London), Ltd., London.
11. **Hobson, P. N.** 1969. Rumen bacteria, p. 117–132. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 3B. Academic Press, Inc. (London), Ltd., London.
12. **Hungate, R. E.** 1957. Microorganisms in the rumen of cattle fed a constant ration. Can. J. Microbiol. **3**:289–311.
13. **Hungate, R. E.** 1966. The rumen and its microbes, p. 43–49. Academic Press, Inc. (London), Ltd., London.
14. **Kock, S. G., and A. Kistner.** 1969. Extent of solubilization of α-cellulose and hemicellulose of low-protein teff hay by pure cultures of cellulolytic rumen bacteria. J. Gen. Microbiol. **55**:459–462.
15. **Kolankaya, N., C. S. Stewart, S. H. Duncan, K.-J. Cheng, and J. W. Costerton.** 1985. The effect of ammonia treatment on the solubilization of straw and the growth of cellulolytic rumen bacteria. J. Appl. Bacteriol. **58**:371–379.
16. **Osborne, J. M., and B. A. Dehority.** 1989. Synergism in degradation and utilization of intact forage cellulose, hemicellulose and pectin by three pure cultures of ruminal bacteria. Appl. Environ. Microbiol. **55**:2247–2250.
17. **SAS Institute.** 1986. SAS user's guide: statistics. SAS Institute, Inc., Cary, N.C.
18. **Schooley, D. L., F. M. Kubiak, and J. V. Evans.** 1985. Capillary gas chromatographic analysis of volatile and non-volatile organic acids from biological samples as the t-butyldimethylsilyl derivatives. J. Chromatogr. Sci. **23**:385–390.
19. **Stewart, C. S., and S. H. Duncan.** 1985. The effect of avoparcin on cellulolytic bacteria of the ovine rumen. J. Gen. Microbiol. **131**:427–435.
20. **Updegraff, D. M.** 1969. Semi-micro determination of cellulose in biological materials. Anal. Biochem. **32**:420–424.
21. **Van Soest, P. J.** 1987. Nutritional ecology of the ruminant. Comstock Publishing Associates, Ithaca, N.Y.

21a. **Varel, V. H.** 1989. Reisolation and characterization of *Clostridium longisporum*, a ruminal sporeforming cellulolytic anaerobe. Arch. Microbiol. **152**:209–214.

22. **Wood, T. M., C. A. Wilson, and C. S. Stewart.** 1982. Preparation of the cellulase from the cellulolytic anaerobic rumen bacterium *Ruminococcus albus* and its release from the bacterial cell wall. Biochem. J. **205**:129–137.