Characteristics of Saponin-Utilizing Bacteria from the Rumen of Cattle

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Evidence for the microbial degradation of plant saponins is limited to few investigations. Rothrock *et al.* (1955) have reported that the saponins from *Dioscorea* tubers can be cleaved into their component parts, diosgenin and the sugar moiety, by *Aspergillus terreus*. Among the bacteria, yeast, and molds examined, only strains of *Penicillium*, *Aspergillus*, and *Fusarium* were capable of hydrolyzing the *Dioscorea* saponin. Rubin *et al.* (1953) have isolated unidentified bacteria which grew in a medium with *Dioscorea composita* saponin as the sole source of carbon. Procedures for the isolation of legume saponins have been developed by Thompson *et al.* (1957) and these investigators have shown alfalfa saponins are plant glycosides with a triterpenoid sapogenin nucleus and a carbohydrate component.

Bloat symptoms have been produced in sheep by the oral and intravenous administration of water soluble alfalfa saponins (Lindahl *et al.*, 1957). Thus the possible relationship of bacterial degradation of legume saponins to bloat assumes particular interest. The proposal has been advanced that, in bloat, ruminal microorganisms might be responsible for an excess production of polysaccharide slime when cattle are on lush pastures such as clover and alfalfa (Hungate *et al.*, 1955), and when animals are maintained on feed-lot rations containing a high percentage of carbohydrate (Jacobson and Lindahl, 1955). The slime contributes to a stable froth formation in which the rumen fermentation gases are retained as numerous small gas bubbles in the ingesta. Changes in the ruminal microbial populations have been shown to occur with the onset of feed-lot bloat symptoms (Gutierrez *et al.*, 1959). This investigation has been directed at the isolation and the characteristics of rumen bacteria capable of degrading alfalfa saponins. A preliminary part of our findings has been published (Gutierrez *et al.*, 1958).

Experimental Methods

Ruminal fluids from six steers on a diet of freshly cut alfalfa (*Medicago sativa*) was used as inocula in the initial experiments. Samples of the rumen contents were removed by stomach tube and serially diluted into rumen fluid agar medium enriched with 0.5 per cent composite alfalfa saponins. A control series of tubes in which the saponin was omitted was also inoculated in parallel. Organisms able to decompose the substrate were detected by the larger numbers and colony size when compared to the nonsaponin control. The basal medium for the original isolation of the bacteria had the following percentage composition in tap water: NH_4Cl, 0.05; NaCl, 0.1; $MgSO_4$, 0.005; $CaCl_2$, 0.005; resazurin, 0.0001; strained rumen fluid, 20.0; and agar, 1.5. Cysteine hydrochloride, 0.04 per cent; $NaHCO_3$, 0.5 per cent; and alfalfa saponins, 0.5 per cent were autoclaved separately and added to the melted agar tubes at the time of inoculation. The autoclaved saponin solution showed no reducing materials when tested with Benedict's solution. Carbon dioxide was used to provide anaerobiosis. For the analysis of fatty acids and gases, the isolated strains were grown in liquid basal medium plus 0.5 per cent peptone and 0.25 per cent yeast extract (Difco)[1] and the rumen fluid omitted. Detection of acid production with brom-thymol-blue and visual observation of turbidity were the criteria employed for utilization of various carbohydrates in the latter medium. The shake culture and anaerobic techniques used were described by Hungate (1950).

Total volatile fatty acids were determined by steam distillation of the culture fluid and the distillate titrated with 0.02 N NaOH using nitrogen as the gas phase. The acids were separated on a chromatographic cellulose column and identified from their Duclaux constant (Carroll and Hungate, 1954). For the analysis of lactic and succinic acids the culture fluid residue remaining after steam distillation was extracted with ether for 24 hr, titrated with 0.02 N $Ba(OH)_2$ and the lactic acid quantitatively determined by the method of Friedmann *et al.* (1927). Succinic acid was precipitated from the ether extract with 5 volumes of ethanol and determined from the dry weight of the barium succinate. Formic acid was oxidized to CO_2 with $HgCl_2$ and determined from the weight of the insoluble calomel. Ethanol was separated by alkaline distillation of the culture fluid, oxidized to acetic acid with K_2CrO_4, and the amount estimated by Duclaux distillation. Fermentation gases were identified with a semimicro-modification of the Newcomber-Haldane gas analysis apparatus and the carbon dioxide dissolved in the medium was determined in an absorption train with 0.8 N NaOH.

[1] Difco Laboratories, Inc., Detroit, Michigan.

Results and Discussion

Isolation of butyric acid-producing rods. Many colonies developed in the rumen fluid agar tubes containing the alfalfa saponins as an energy source 24 hr after the inoculation with rumen contents, whereas the non-saponin control series of tubes showed very little growth. Microscopic examination of a large number of the colonies from the saponin series showed that small, gram negative, curved rods were the predominant organisms. Colonies were picked from the higher dilutions and inoculated into a second series of tubes

TABLE 1

Colony counts of presumptive saponin-digesting bacteria in animals on a green alfalfa diet

Steer No.	Millions per ml of Rumen Fluid
61	680
79	20
479	180
488	200
490	8
491	4

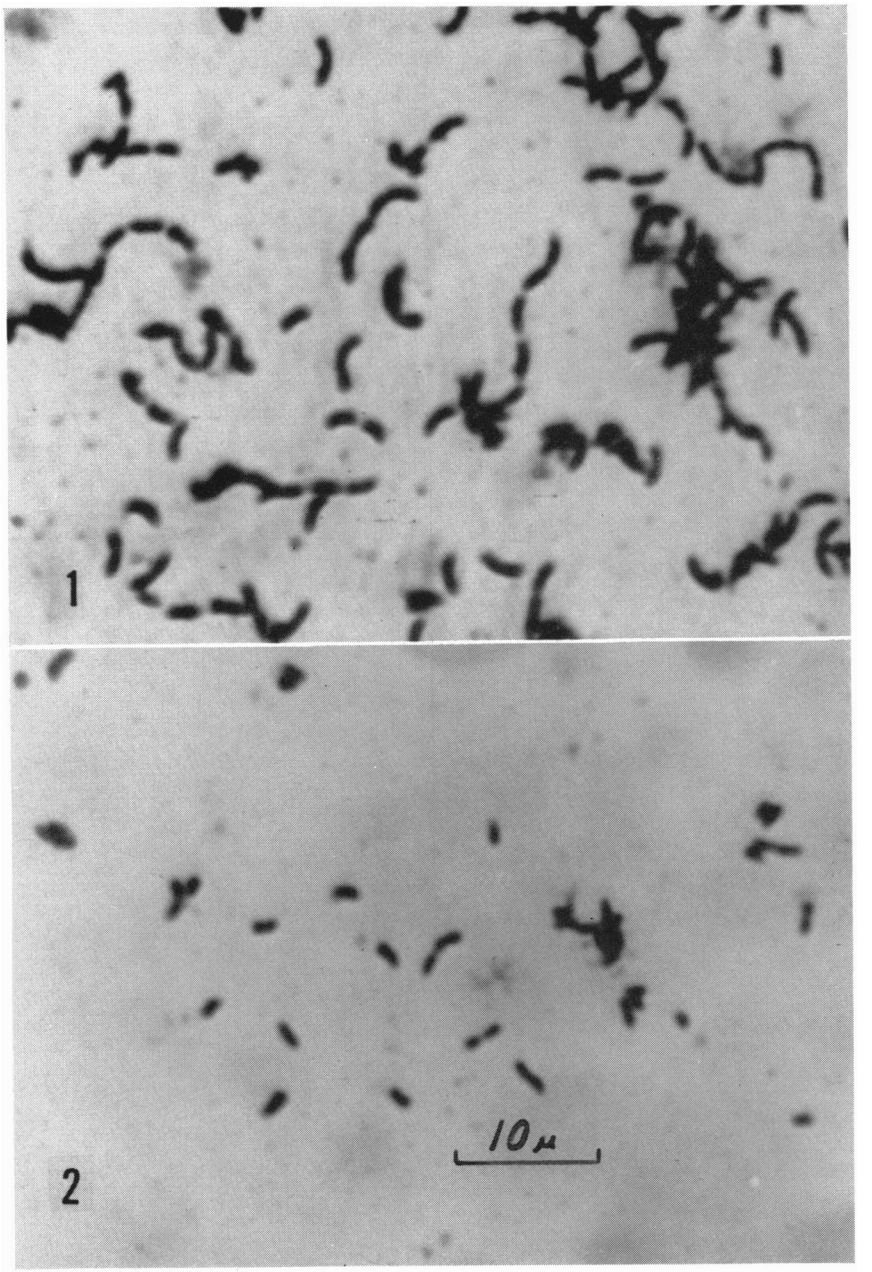

Figure 1. Gentian violet stain of strain 61-2 grown in yeast extract peptone plus saponin. Butyric acid-producing rods. Original magnification 1455 ×.

Figure 2. Gram stain of strain 55N grown in yeast extract peptone plus saponin. Succinic acid-producing rods. 1455 ×.

to obtain pure cultures. Sixteen strains of curved rods capable of attacking alfalfa saponins were isolated in this manner from the rumen contents of the six steers and three strains were selected for further study. A wide variation was encountered in the counts of bacteria able to grow at the expense of the saponin. Estimates made from the colonies appearing in the serial dilutions from the different animals on the fresh alfalfa diet gave a range of 680 to 4 million per ml (table 1). Saponin digesters were also isolated from a steer on an alfalfa hay diet with a count of 100,000 per ml.

Surface colonies on rumen fluid agar were grayish-white, smooth, entire, and up to 4 mm in diameter. Deep colonies were lens-shaped, and upon initial isolation the colonies had a mucoid appearance that was lost on subculture. In liquid medium supplemented with saponin, growth was flocculent with some of the sediment adhering to the bottom of the tube. The cells were gram negative, motile, curved rods, 0.8 μ × 2.0 to 4.0 μ long (figure 1). Some of the strains were smaller. Spores were not observed. The cells were usually in singles or pairs, and when grown on the yeast-peptone medium were larger than on the rumen fluid medium supplemented with the saponin. Smears of colonies showed the rods were rather evenly separated, suggesting that adhesive capsular material might be present. Large capsules such as those seen in some strains of rumen cocci were not present. The organism was nitrate negative, gelatin was not liquified, and indole was not produced. Hydrogen sulfide was produced, and the Voges-Proskauer reaction was negative. The following carbohydrates were fermented: lactose, maltose, galactose, D-xylose, cellobiose, sucrose, glucose, fructose, salicin, and arabinose. Starch was hydrolyzed. Growth did not occur with raffinose, esculin, or trehalose.

The culture medium used initially for the analysis of the metabolic end products from alfalfa saponins included 20 per cent rumen fluid and did not permit accurate analysis of the acids produced by the bacteria. Subsequent tests showed the organisms could grow well in yeast-extract peptone medium, and the identification of acids and gas was carried out in this medium with improved results. The fermentation vessels were 100-ml round bottom flasks equipped with inlet and outlet tubes to facilitate gas measurement. Fifty ml of autoclaved yeast-extract peptone broth was inoculated with 2 ml of a 24-hr liquid culture of a presumptive saponin-digesting strain. Carbon dioxide gas and 0.5 per cent sodium bicarbonate was the buffer system and 0.04 per cent cysteine hydrochloride was added as a reducing agent. Control flasks were inoculated and incubated, but received no saponin. Carbon dioxide, formic, acetic, butyric, and lactic acids were identified as fermentation products of alfalfa saponins from strain 61-2 (table 2). Traces of ethanol and propionic acid were also present. Large quantities of viscous slime were produced in the saponin flasks, whereas very little growth was evident in the control medium. The slime material was harvested by centrifugation, dried at 100 C, and weighed. Fermentation flasks were prepared to compare the fermentation products and slime production of saponin with that of glucose. The fermentation products from the latter substrate were the same; but, whereas approximately 50 per cent of saponin appeared as cellular and slime matter, only 10 per cent of the glucose was converted to slime by strain 61-2 (table 3). Acid hydrolysis of the slime produced significant amounts of copper-reducing compounds indicating its polysaccharide or other glycosidic nature. A carbon fermentation balance calculated for glucose showed approximately 61 per cent of the carbon was recovered in the products. The composite nature of the alfalfa saponins of unknown molecular weight prevented comparison of carbon balances with glucose.

The mechanism of breakdown of the alfalfa saponins by the bacterial strains remains to be determined, but one possibility is that the sugar component of the saponin is split off and utilized by the bacteria leaving the intact sapogenin in the medium. The sapogenin may contribute to the increased slime that was observed in the saponin bacterial cultures as compared to glucose cultures (table 3). The morphology, cultural traits, and fermentation products of this first group of saponin digesters resemble the atypical strains of *Butyrivibrio fibrisolvens* described by Bryant and Small (1956) and

TABLE 2

*Fermentation products of alfalfa saponins and glucose by strain 61-2**

Product	Alfalfa Saponins (200 mg)	Glucose (230 mg)
Carbon dioxide	1.90	2.20
Butyric acid	0.15	0.16
Acetic acid	0.23	0.13
Lactic acid	0.14	0.33
Formic acid	0.52	0.63

* In mmoles; the values for alfalfa saponins are taken from Gutierrez *et al.*, 1958.

TABLE 3

Cellular and slime harvests of strains 61-2 and 55N

Substrate	Medium	Cells and Slime*	
		61-2	55N
		mg	*mg*
200 mg saponin..	Yeast-extract peptone	85	65
200 mg saponin..	20% rumen fluid broth	119	105
1.0 g saponin....	20% rumen fluid broth	452	302
230 mg glucose..	Yeast-extract peptone	22	79

* Dry weight.

the strains probably should be placed in this species of rumen bacteria.

Isolation of succinic-acid producing rods. A second type of saponin-digesting organism was isolated from the rumen contents of five heifers which were fed a timothy hay ration. The numbers of colonies developing in the dilution series of agar shake tubes enriched with alfalfa saponins after inoculation ranged from 40,000 to 1,000,000 per ml; a parallel series of inoculated tubes without saponin showed fewer and smaller colonies. Twelve rod strains were isolated from the different animals and two representative strains were studied in sufficient detail for taxonomic evaluation.

In rumen fluid agar shake tubes supplemented with 0.5 per cent saponin, deep colonies were 0.5 to 2.0 mm, lenticular, with a grayish-white color; surface colonies were slightly larger, smooth, entire, and convex with a soft consistency. In yeast-extract peptone broth supplemented with saponin the cells were gram negative, nonmotile rods with rounded ends, 0.8 to 1.0 × 1.5 to 2.0 μ long (figure 2). When the broth was enriched with 0.5 per cent glucose, the cells were 2.0 to 4.0 μ long. Swollen cells and cells which stained unevenly were common. Spores were not observed. Examination of living cells with the phase microscope frequently revealed the rods evenly dispersed without movement, as would be expected with cultures capable of slime formation. The organism produced small amounts of H_2S, reduced nitrate to nitrite and was indole negative; the Voges-Proskauer reaction was negative and gelatin was not liquified. Growth occurred at 30, 37, and 45 C, but not at 20 and 50 C. Glucose, D-xylose, D-sorbitol, galactose, fructose, raffinose, salicin, sucrose, arabinose, trehalose, maltose, lactose, mannose, dextrin, inulin, and cellobiose were fermented. Starch was hydrolyzed. Esculin and glycerol were not attacked. Three other strains fermented identical carbohydrates. Growth in yeast extract-peptone broth supplemented with suitable carbohydrate showed an even turbidity with a ropy sediment. The strains grew well in rumen fluid starch feed-extract medium (Gutierrez, 1958) and, after initial isolation with the saponin enriched rumen fluid agar medium, the cultures were routinely transferred in the former medium. Growth of the rods in flasks provided with 0.5 per cent alfalfa saponins exhibited a slimy viscid property. In one experiment where the concentration of alfalfa saponin was increased to 1 per cent, the yeast extract-peptone broth was converted to a gelatinous slime after inoculation with a saponin-digesting strain.

TABLE 4

*Fermentation products of alfalfa saponins and glucose by strain 55N**

Product	Alfalfa Saponins (1 g)	Glucose (1 g)
Formic acid	0.56	0.86
Acetic acid	0.39	0.92
Succinic acid	0.50	2.14
Lactic acid	0.45	2.45

* Amounts are given in mmoles; 0.5 per cent substrate in 200 ml liquid medium.

The fermentation products from the breakdown of alfalfa saponins were analyzed in the same manner as for the study of the butyric acid rods. Inoculated flasks with no substrate served as controls. In flasks containing 200 ml yeast extract-peptone broth supplemented with 0.5 per cent alfalfa saponin, the following products were formed (in mmoles): formic acid, 0.56; acetic acid, 0.39; succinic acid, 0.50; and lactic acid, 0.45. No gas was produced in fermentation flasks provided with an initial gaseous phase composed of 5 per cent CO_2-95 per cent N_2. The analysis of the products of a second strain showed the same acids were formed.

By their morphology and end products, the succinic acid rods capable of degrading alfalfa saponins are related to *Bacteroides amylophilus* (Hamlin and Hungate, 1956), but their capacity of attacking a wide variety of carbohydrates prevents placing the strains in this species; *B. amylophilus* could utilize only maltose and starch. Our isolates have a closer relationship to the recently described *Bacteroides ruminicola* (Bryant *et al.*, 1958). Lactic acid was not reported as one of the end products of *B. ruminicola*, but this may be due to differences in the growth media employed in the two studies. The strains of saponin-digesting rods do not differ sufficiently from *B. ruminicola* to warrant separate species designation.

The genera *Butyrivibrio* and *Bacteroides* have been shown to be among the predominant groups of rumen bacteria (Bryant *et al.*, 1956; Bryant *et al.*, 1958), and the current findings indicate that strains of these two genera can attack legume saponins with a significant production of slime. In the case of the *Butyrivibrio* strains, the slime production is accompanied by gas evolution and illustrates how the biochemical activity of the rumen bacteria upon ingested plant compounds may alter the normal rumen fermentation. The increased slime gives rise to a stable frothy foam which traps the fermentation gases and interferes with the ruminant's gas eructation mechanism (Dougherty *et al.*, 1958). Hungate *et al.*, (1955) have observed an interesting correlation between foam production and the intensity of bloat symptoms of cattle on clover pasture. In cases of feed-lot bloat when animals were fed a high carbohydrate diet, slime producing *Streptococcus bovis* and *Peptostreptococcus elsdenii* increased in numbers in the rumen upon the onset of the bloat symptoms (Gutierrez *et al.*, 1959). The organisms responsible for slime production in the rumen belong to several genera, and the type of diet being fed plus the initial microflora present in the host undoubtedly influence, in

part, the groups of organisms which play a significant role. Further investigations are necessary before a complete evaluation can be made of the importance of slime production in the rumen as a contributing factor in the pathogenesis of bloat.

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SUMMARY

Gram negative, motile, curved rods able to attack soluble alfalfa saponins were isolated in large numbers from steers fed a freshly cut alfalfa diet. The rods produced CO_2, formic, acetic, butyric, and lactic acids plus traces of ethanol and propionic acid from saponin. The isolated strains are similar to atypical strains of *Butyrivibrio fibrisolvens*.

A second group of gram negative rods able to degrade alfalfa saponins was isolated from animals on a timothy hay ration, and the strains were studied for classification purposes. Analysis of the fermentation products from the breakdown of saponin showed formic, acetic, lactic, and succinic acids were formed. The strains were related to *Bacteroides ruminicola*.

Significant amounts of slime were produced from the degradation of saponin by strains of both genera. The production of slime from these plant compounds by the bacteria described in this paper is suggested as a significant factor in the bloat syndrome in animals on legume pastures.

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