CULTURE AND PHYSIOLOGY OF A STARCH-DIGESTING BACTERIUM 
(BACTEROIDES AMYLOPHILUS N. SP.) FROM THE BOVINE RUMEN 2

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Studies in this and other laboratories have disclosed a variety of starch-digesting rumen organisms, including Streptococcus bovis (Hungate et al., 1952), several cellulolytic strains (Hungate, 1950), and various non-cellulolytic bacteria (Gall et al., 1947; Gall and Huhtanen, 1951; Huhtanen and Gall, 1953a, b; Bryant and Burkley, 1953a, b; Jayko, 1953; McPherson, 1953; van der Wath, 1948) as well as protozoa of the genera Entodinium and Diplodinium (Berger, 1928) and Isotricha (Gutierrez, 1955, Sugden and Oxford, 1952). Starch digestion by most of the bacteria has been discovered after they have been isolated on other substrates. It seemed of interest to investigate whether use of starch in initial agar dilution series would disclose starch-digesting bacteria not previously encountered.

Starch digesters tentatively identified as S. bovis and as the weakly cellulolytic, butyric acid-producing rods (Hungate, 1950) were encountered and, in addition, rod shaped bacteria different from any previously described amylolytic organisms, and considered to be a new species, Bacteroides amylophilus.

MATERIALS AND METHODS

The culture and analytical methods were similar to those previously employed in this laboratory (Hungate, 1950). Rumen contents were serially diluted in a liquid basal medium with the percentage composition: K2HPO4, 0.05; KH2PO4, 0.05; NaCl, 0.1; (NH4)2SO4, 0.05; MgSO4, 0.01; CaCl2, 0.01; resazurin, 0.0001; cysteine hydrochloride, 0.07; and NaHCO3, 0.5. Each of the latter two ingredients was sterilized separately and added to the medium just before inoculation. Carbon dioxide was the gas phase.

To this basal medium was added one or more of the following additional nutrients: 0.5 per cent soluble starch or corn starch (S), 30 per cent rumen fluid (R), 1 per cent peptone (P), 0.5 per cent yeast extract (Y). A feed extract medium (F) was prepared by boiling 1 per cent alfalfa (Medicago sativa) hay and 0.5 per cent feed concentrate 3 in a solution containing the inorganic salts of the basal medium except that the NaHCO3 was omitted. The heated suspension was filtered while hot and the filtrate used to replace one-third of the basal medium. The concentration of corn starch added to this medium was reduced to 0.3 per cent.

In preliminary experiments, rumen contents were either diluted serially in the melted agar culture medium (R-S or F-R-S) or were diluted in liquid basal medium and then inoculated into R-S or F-R-S agar. The culture counts were about the same, using these two dilution procedures. Comparisons of various nutrients showed that counts on an agar medium containing feed extract plus corn starch (F-S) were as high as those when rumen fluid was also added.

Starch digestion could frequently be detected in young cultures in F-S or P-Y-S agar by a slight clearing of the medium around a colony. A more reliable index was lack of blue color around colonies flooded with Gram's iodine solution. Because the blue color in starch-containing areas rapidly disappeared due to reducing materials in the anaerobic medium, a considerable quantity of the iodine solution was added and the tube examined as quickly as possible. Organisms in subsurface starch-digesting colonies were still viable after addition of the iodine,

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3 The ingredients and their proportion in the concentrate meal were: corn gluten feed 40, hominy and corn meal 54, corn distillers dried grains 36, linseed oil meal 8, cane and corn sugar molasses 20.
though sufficient quantities to give a permanent blue color would undoubtedly have killed them.

RESULTS

Culture counts on starch medium. The total culture counts with the F-S medium are shown in table 1, together with the counts of Bacteroides amylophilus.

A large proportion of the colonies were starch digesters. S. bovis was conspicuous because of its large colonies. Amylolytic organisms resembling the "less actively cellulolytic rod" (Hungate, 1950) were also abundant. In the first experiment, amylolytic colonies different from either of these were observed after 24 hr of incubation. One strain (6-24) was isolated in pure culture and found to consist of gram negative non-motile rods somewhat variable in size and exhibiting a good deal of pleomorphism. The cells were often slightly tapered, with rounded ends. In some stained smears or in fresh mounts examined with the phase microscope, small intracellular granules could be seen.

The culture tubes of experiments 2 to 11 were examined for similar organisms. Starch-digesting colonies were picked, examined microscopically, and subcultured if the cell characteristics appeared similar to those of strain 6-24. Subsurface colonies were as much as 0.8 mm in diameter at 24 hr, lenticular with entire or irregular edges, white, and homogeneous in texture. Surface colonies were a little larger, white, translucent, convex, and possessed entire edges and smooth and glistening surface. Colony morphology was not sufficiently distinctive for identification until after a good deal of experience with several strains. Then identification was fairly accurate from colony morphology alone. Unfortunately, the distinguishing differences are so subtle that success is possible only after direct observation of many colonies.

In experiment 1, 17 per cent of the colonies contained starch digesters similar in cell morphology to strain 6-24. In experiments 4, 9, and 10 this type constituted 10, 15, and 5 per cent, respectively, of the total count. A pure culture was isolated from each of these experiments.

These starch digesters were not detected in the other experiments and, if present, must have occurred in numbers less than 10⁶/ml, the lowest dilution cultured.

The highest count (600 x 10⁶) of this type of starch digester was found with rumen contents from one of the Angus steers on a high grain ration, but a second animal on a similar diet did not show such high numbers. This indicates that occurrence of the organism is sporadic but that it may on occasion play an important role in the rumen.

Morphology. The three additional strains (7-20, 8-26, and 11-4) were morphologically quite similar to strain 6-24, with minor differences. Immediately after isolation, strain 7-20 showed plump oval rods with a few coccolid cells, and no visible granulation. Cells of strains 8-26 and 11-4 were quite pleomorphic in initial cultures and this was retained by strain 11-4 but soon lost by 8-26. Figure 1 illustrates the range

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>Source of Inoculum</th>
<th>Feed of Host Animal</th>
<th>Total Count</th>
<th>Count of Bacteroides amylophilus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fistulated steer no. 1</td>
<td>Grass-alfalfa hay</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Fistulated steer no. 1</td>
<td>Grass-alfalfa hay</td>
<td>6</td>
<td>&lt;1</td>
</tr>
<tr>
<td>3</td>
<td>Fistulated steer no. 1</td>
<td>Grass-alfalfa hay</td>
<td>190</td>
<td>&lt;1</td>
</tr>
<tr>
<td>4</td>
<td>Fistulated steer no. 1</td>
<td>Grass-alfalfa hay</td>
<td>63</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>Fistulated steer no. 2</td>
<td>Grass-alfalfa hay</td>
<td>63</td>
<td>&lt;1</td>
</tr>
<tr>
<td>6</td>
<td>Fistulated steer no. 2</td>
<td>Grass-alfalfa hay</td>
<td>190</td>
<td>&lt;1</td>
</tr>
<tr>
<td>7</td>
<td>Fistulated steer no. 2</td>
<td>Grass-alfalfa hay</td>
<td>190</td>
<td>&lt;1</td>
</tr>
<tr>
<td>8</td>
<td>Angus steer, no. 1</td>
<td>⅔ grain-⅓ hay</td>
<td>2000</td>
<td>&lt;1</td>
</tr>
<tr>
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<td>⅔ grain-⅓ hay</td>
<td>4000</td>
<td>600</td>
</tr>
<tr>
<td>10</td>
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<td>Hay-grain-silage</td>
<td>150</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>Producing milk cow no. 2</td>
<td>Hay-grain-silage</td>
<td>110</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>
of morphological variation encountered in these last two strains as observed with the phase microscope.

Except in strain 11-4 pleomorphism decreased after continued subculture in P-Y-S agar. Morphology still fluctuated to some extent and strain 8-26, which was examined microscopically over a longer period than the others, occasionally showed a few cells like the larger ones illustrated in figure 1. A photomicrograph of a stained smear of strain 8-26 showing the usual morphology of this strain, is reproduced in figure 2. Since the stain was crystal violet the internal structure of the cells does not show.

Transfer of 8-26 to a different medium occasionally increased pleomorphism but this result was not sufficiently consistent to permit analysis of the factors involved. Increasing age of the cultures correlated to some extent with increased size and pleomorphism.

A 12-hr culture of strain 8-26 showed coccoid to rod-shaped cells of uniform diameter and with little visible internal structure. At 24 hr the cells were longer, some swollen and some showing internal granular structure as illustrated in figure 1.

Like strain 6-24, some cells in the original cultures of 7-20 and 8-26 were gram positive and some contained internal gram positive granules. Most cells were entirely gram negative. Strain 11-4 was gram negative even in the original culture and all strains were gram negative after the first subculture. Cells from older cultures showed internal granules when stained with alkaline methylene blue. Motility was never detected and no spores were seen in any of the strains.

Figure 1. Drawing illustrating the various cell forms observed in a 26 hr culture of strain 11-4 in P-Y-S broth, first culture after isolation; living cells as seen with the phase microscope; magnification 1000 X.

Figure 2. Strain 8-26 in P-Y-S broth; stained with gentian violet; magnification 970 X. (a) 12-hr culture. (b) Same culture at 26 hr.
Physiological characteristics. Strains 7-20 and 8-26 grew at 35°C, better at 39°C and 45°C, and did not grow at 30°C and 55°C. Strain 8-26 grew best in P-Y-S broth at pH 6.8-7.8 and failed to grow at pH 6.0 and 8.8. The various acidities were obtained by adjusting the concentrations of bicarbonate and carbon dioxide.

In deep shake tubes containing F-S or P-Y-S agar no colonies developed in the upper oxidized region in which the resazurin was pink, but they developed in the lower anaerobic part, indicating obligate anaerobiosis.

In 7 days strain 11-4 liquefied 5 per cent gelatin added to P-Y-S broth but strain 8-26 showed no liquefaction in 14 days. Indole was not produced by three tested strains and two tested strains did not reduce nitrate. Hydrogen sulfide was not formed.

Baker and Nasr (1947), van der Wath and Myburgh (1941), and van der Wath (1948) reported that ruminal break-down of starch is associated with iodophilia of the responsible bacteria. When iodine was added to 12-hr agar cultures of strain 8-26 the small colonies showed no typical iodophilic reaction. They were white to light tan in color and surrounded by a small unstained zone of starch digestion.

In order to test more conclusively for iodophilia, raw unsterilized corn starch was added to a 9-hr culture in P-Y-S broth which had just begun to show growth. After further incubation for 2 hr many of the starch grains showed attached bacteria, as shown in figure 3, and the starch grains showed incipient disintegration. Even the cells attached to the starch grains were not iodophilic. It is doubtful that these organisms are responsible for the iodophilia often observed in rumen contents, though further studies such as those of Hobson and Mann (1955) on S. bovis might disclose other conditions under which intracellular polysaccharide would be stored.

Substrates fermented. All strains were tested, using P-Y broth plus the test material in 0.2 per cent concentration. Soluble substrates were sterilized by filtration. The concentration of NaHCO₃ was reduced to 0.1 per cent and 5 per cent CO₂ was employed to give a suitable pH but less buffering capacity. Tubes which showed growth, as indicated by turbidity, were checked for purity by microscopic examination and for acid production by adding brom thymol blue. Of the many substrates tested only starch and maltose supported growth and fermentation.

In order to test conclusively for glucose utilization it was added in 0.2 per cent concentration to one tube of P-Y-S broth and to a second was added 0.2 per cent maltose. Both tubes were inoculated and incubated. The starch in both tubes disappeared. After several days of additional incubation, the contents of the glucose culture showed a copious reduction of copper but none was detected in the maltose tube. The pH in the maltose tube was 5.7 whereas it was 6.4 with the glucose.

The ability of strain 8-26 to utilize hay was tested by inoculating P-Y broth tubes each containing 40 mg of ground alfalfa hay. A control P-Y-S broth tube showed abundant turbidity after incubation for 24 hr but the tubes containing hay showed no growth even after 9 days of incubation. From these experiments starch appears to be the chief energy-yielding substrate for growth of this organism. Occurrence of these bacteria in the hay-fed animals is somewhat surprising, though the starch in the hay may have been sufficient to support it in the numbers found.

Fermentation products. The products from fermentation of 300 mg of starch by strain 7-20 are shown in table 2. Peptone-yeast extract broth was the culture medium, and an inoculated flask without starch was analyzed as a control.

![Figure 3. Starch granules and attached bacteria 3 hr after addition of raw starch to a 9-hr culture of strain 8-26 in P-Y-S broth; phase contrast; 970 X.](http://jb.asm.org/)
No hydrogen, acetylmethylcarbinol, 2,3-butylen glycol, or glycerol could be detected.

Carbon dioxide (100 per cent) and 0.5 per cent NaHCO₃ were used in this experiment and the error involved in equilibrating the solution with gas was so large that the difference between initial and final CO₂ could not be measured accurately. Production of a fairly large quantity of succinic acid indicated that carbon dioxide might be fixed. It had been found in earlier experiments to be required for growth. In a quantitative analysis of the fermentation products of strain 8-26 the CO₂ was decreased to 5 per cent and the NaHCO₃ to 0.3 per cent and, as seen in table 3, some CO₂ was fixed.

The carbon recovery in these experiments was about 50 per cent based on the fermented starch. A test for polysaccharides yielding reducing sugars upon acid hydrolysis was negative.

Classification. The gram negative, non-spore forming, non-motile, anaerobic nature of the isolated strains suggests their inclusion in the genus Bacteroides. The intestinal habitat and the tendency toward pleomorphism are consistent with this assignment. According to the Bergey Manual the organisms key out as Bacteroides vesecus, but the carbohydrate fermentation pattern and the morphology are different. The strain differs from each of the other described species of Bacteroides in several characteristics, the most important being restriction to fermentation of maltose and starch. The strains are considered to belong to a new species, Bacteroides amylophilus n. sp., with the following description.

Cells rod-shaped, 0.9-1.6µ by 1.6-4.0µ, but often pleomorphic and exhibiting larger irregular shapes. Ends of cells rounded, sometimes tapered toward the rounded ends. Granular internal structure often visible in stained smears or in live cells examined with the phase microscope. Non-motile. None or slight capsule formation.

Colonies in deep agar lenticular, 0.8 mm in diameter, entire or irregular edges, white, soft butyrous in texture. Surface colonies larger, white, convex, translucent, with entire edges, smooth and glistening.

Gelatin may or may not be liquefied when added in 5 per cent concentration to peptone-


Obligately anaerobic, not growing in those portions of shake tubes in which resazurin is pink. Carbon dioxide required for growth and some fixed during fermentation. Growth at 35 C and 45 C but not at 30 C or 55 C. Optimum initial pH 6.8-7.0, range 6.5-7.8.

Substrates fermented: starch and maltose. Not fermented: arabinose, rhamnose, xylose, glucose, glucose-1-phosphate, fructose, galactose, mannose, lactose, sucrose, cellobiose, cellulose, raffinose, inulin, glycerol, mannitol, salicin, and esculin.

Fermentation products are acetic, succinic, and formic acids, and traces of ethanol. Lactic acid may be formed. Final pH in peptone-yeast extract broth is 5.5-5.7. The habitat is the rumen of cattle.

Cultures of this species were sent to the American Type Culture Collection but were reported to be non-viable when received. Broth cultures remain viable only two or three days and roll tube cultures only 3-4 days. Slant cultures were viable as long as 5 days. Unfortunately it has not been possible to maintain a stock culture because of obligations to the armed services of the senior author.
DISCUSSION

The pure cultures of starch-digesting bacteria previously isolated from the rumen differ from B. amylophilus in their morphology, fermentation products, or substrates fermented. The substrate specificity of B. amylophilus is the factor which has probably prevented its previous detection and isolation. The cellulose, glucose or cellulobiose usually used for isolation of rumen bacteria do not support its growth. McPheron (1953) used starch in the primary isolation medium but did not report on types other than amylolytic cocci. The RO-H₂ strains described by Huhtanen and Gall (1953a,b) are even more specific for starch than is B. amylophilus since they could not decompose maltose. They were minute motile rods forming acetic, propionic and butyric acid.

The action of B. amylophilus on starch is analogous to that of some of the rumen cellulolytic cocci on cellulose. In both instances the polysaccharide is split to a disaccharide which is used without any extracellular conversion to glucose.

Failure to demonstrate B. amylophilus in excess of 10⁴/ml in 7 out of 11 experiments suggests that it is not universally important in the rumen, but the high numbers in one experiment indicate that it occasionally plays an important role. It has a capacity for rapid proliferation; the growth rate in agar cultures is sufficient to give visible colonies after incubation for 11 hr. This is longer than the 6 hr needed for development of visible colonies of S. bovis, one of the most rapidly growing rumen bacteria, though the difference in growth rate is not quite as marked as these figures indicate, since the S. bovis colony includes much capsular material.

In the original culture experiments, there was some indication of an inverse relationship between numbers of S. bovis and B. amylophilus. This suggested an incompatibility between them, but tests with mixed pure cultures furnished no support for this hypothesis.

SUMMARY

A gram negative, non-motile, starch-digesting bacterium, Bacteroides amylophilus n. sp., occurs sporadically in the bovine rumen, occasionally in large numbers. Four strains were isolated from rumen samples obtained from a steer with a rumen fistula, an unfastulated steer, and a producing milk cow. The organism was not always present in these animals and was not found in three other animals which were examined, though no extensive search was made.

Plasmodism occurred to varying extents in the different strains. In large cells intracellular granules could be detected.

B. amylophilus is quite specific for starch, fermenting only starch and maltose of a large number of hexoses and disaccharides tested. It grows well on peptone-yeast extract medium if fermentable carbohydrate is provided. Principal identified fermentation products are acetic, formic, and succinic acids. A measurable quantity of carbon dioxide was fixed in one experiment. Traces of ethanol and lactic acid may be formed.

REFERENCES


Bryant, M. P. and Burkey, L. A. 1953a Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci., 36, 205–217.


