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Basal Medium for the Selective Enumeration of Rumen Bacteria Utilizing Specific Energy Sources[1]

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A 40% rumen fluid basal medium has been developed that without added substrate will support growth of about 10% or less of the total colony count obtained with 40% rumen fluid-glucose-cellobiose-starch-agar medium (RGCSA). The basal medium is prepared by anaerobic incubation of all ingredients in RGCSA medium except the carbohydrates, Na_2CO_3, and cysteine for 7 days at 38°C. After incubation, substrate(s), Na_2CO_3, and cysteine are added and the medium is tubed and sterilized as in normal medium preparation. When xylose was included with glucose, cellobiose, and starch as added carbohydrates in the incubated medium, colony counts were comparable to those obtained with RGCSA medium. The addition of specific carbohydrates or other substrates as energy sources to the basal medium suggested that the percentage of the bacterial population capable of utilizing these energy sources was influenced by the ration of the animal; however, considerable animal variation and day-to-day variation in a given animal was observed. Comparison of the population in animals fed either orchardgrass hay or 60% corn-40% orchardgrass (60-40) indicated little or no difference for the percentage of bacteria utilizing glucose, pectin, xylan, or mannitol. Increases in the percentages of xylose-, cellobiose-, glycerol-, and lactate-utilizing bacteria occurred with the orchardgrass hay ration, whereas the percentage of starch-digesting bacteria was increased significantly ($P < 0.01$) in the animals fed the 60-40 ration. A limited number of bacterial strains were isolated from the basal medium without added substrate, most of which were atypical with respect to the predominant rumen bacteria. Growth of these strains, even in complex media, was very slow and limited. Based on these data with isolated strains and colony counts obtained in roll tube medium containing only minerals, resazurin, agar, Na_2CO_3, and cysteine, the selective medium overestimated the percentage of bacteria able to use a specific energy source. This overestimate was 6 to 7% of the total culturable count.

Two procedures have been used to estimate the percentage of the total culturable rumen bacteria capable of fermenting specific substrates. The first involves random isolation of bacterial strains from nonselective medium and subsequent screening of these isolates for their ability to ferment the substrates under study (1, 3, 9, 11, 12). Aside from the obvious drawback of being very laborious and time consuming, some questions might be raised about the probability of picking colonies from a high dilution that are truly representative of the whole population. The second procedure would involve the use of a selective medium, in which a specific substrate would be the only added energy source. The latter method has been used quite successfully with cellulose as the sole added energy source, since only colonies surrounded by a clear zone in the cellulose agar medium are counted. In contrast, when soluble substrates are used, either all colonies must be counted or an arbitrary limit must be set on size of colony to be counted, assuming that only the larger colonies are growing on the added substrate. Considerable doubt exists as to whether the organisms are growing on the specific added energy source or on a component of the complex medium that is needed to meet the nutritional requirements of many species of rumen bacteria, and whether colony size is related to utilization of the added substrate. Bryant et al. (2) found that both of the above factors interfered in the estimation of lactate-fermenting species of rumen bacteria in a selective lactate medium.

Gilchrist and Kistner (5) and Kistner et al. (8) attempted to use selective-medium procedures to estimate the proportions of rumen bac-

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teria utilizing cellulose, starch, glucose, cellobiose, and lactate. However, they did not determine a simultaneous total viable count or report colony counts on their 30% rumen fluid basal medium without added substrates. Except for cellulose medium, incubation times used by these authors were extremely short: starch medium, 16 h; glucose and xylose media, 20 h; lactate medium, 96 h. With the lactate medium, they found that only 57% of the colonies counted were capable of utilizing lactate. Without a value for culturable bacterial numbers per gram of rumen contents, which can vary markedly with type and amount of ration, the percentage of bacteria utilizing specific substrates cannot be calculated, and shifts in the population would probably not be as evident solely on the basis of numbers obtained on the different selective media.

Selective-medium colony counts, using either xylan, pectin, or starch as the only substrate added to a 40% rumen fluid basal medium, were made by Grubb and Dehority (6). Counts were expressed as a percentage of the colony count obtained with a nonselective medium and for five replicate samples from the same sheep on the same ration; values for the xylan medium ranged from 70 to 113%; for pectin, 70 to 106%; and for starch, 71 to 136%. In a later study (7) it was found that 77.5% of the culturable rumen bacteria grew in the 40% rumen fluid basal medium without added substrate.

The present study was undertaken to develop a selective medium that could be used to estimate the percentage of the culturable rumen population capable of fermenting specific substrates. The basal medium, without an added energy source, should support little or no colony growth and, with the addition of the normal carbohydrate substrates (glucose, cellobiose, and starch), colony numbers should be comparable to those obtained in 40% rumen fluid-glucose-cellobiose-starch-agar (RGCSA) roll tubes.

MATERIALS AND METHODS

Three cross-bred wether sheep, weighing approximately 45 kg, were fed 800 g of chopped orchardgrass hay or 60% cracked corn and 40% chopped orchardgrass hay (60-40) daily. Both rations were supplemented daily with 20 g of a mineral-vitamin mix and 4.4 g of feed-grade urea. The animals were fed once daily at 9:00 a.m. and had free access to water. All sheep were surgically prepared with rumen fistulae, and composite samples of contents were collected from various locations within the rumen just before the daily feeding.

The anaerobic culture techniques, preparation of (RGCSA) roll tube medium and other media, and procedures for roll tubes were as previously described (7). Colonies were counted by using a binocular dissecting microscope after 7 days of incubation at 38°C. Rumen fluid for the preparation of media was obtained by suction from a fistulated steer just before the morning feeding of alfalfa hay. Unless designated otherwise, the supernatant obtained from centrifugation of whole rumen fluid at 1,000 × *g* for 10 min was used in all media.

The term "replicate experiments" indicates a separate sample of rumen contents, either from two animals on the same day or the same animal on different days. "Inoculum levels" refers to the rumen fluid dilution or grams of rumen contents added to the roll tube, i.e., 1×10^{-8}, 0.5×10^{-8}, or 1×10^{-9}. Actual colony counts were all converted to numbers per 10^{-8} g of rumen contents before calculation of mean values.

Methods used to study the bacterial strains isolated from the basal medium without substrate have been reported previously (4).

Statistical comparisons of the data were made with standard paired and nonpaired *t* tests.

RESULTS AND DISCUSSION

Since a 40% rumen fluid medium without added carbohydrates had been shown to support considerable colony growth (7), a series of experiments was set up with a non-rumen fluid basal medium. Rumen fluid was replaced by 0.2% Trypticase (BBL), 0.05% yeast extract (Difco), a 0.33% mixture of volatile fatty acids similar in composition to those in rumen fluid (3), and 0.0001% hemin. Without added carbohydrates, colony counts with the non-rumen fluid medium averaged 44% of the count obtained in RGCSA roll tubes. However, when carbohydrates were added to the non-rumen fluid medium, only 70% of the total colony numbers in RGCSA medium were obtained. This difference was significant ($P < 0.01$). Reducing the concentration of Trypticase, yeast extract, and volatile fatty acids by 75, 66, and 75%, respectively, significantly lowered colony count both with and without carbohydrates ($P < 0.05$). Deletion of all three of the above ingredients, along with carbohydrates, still allowed growth of colony numbers equal to about 15% of those obtained in the non-rumen fluid medium with carbohydrates. Based on these observations, further efforts to develop a non-rumen fluid selective medium were discontinued.

The only alternative possibility appeared to be that of using a 40% rumen fluid medium in which the available energy sources were removed. It was further reasoned that it might be possible to deplete the concentration of these energy sources by fermentation and still retain an adequate concentration of those compounds needed to meet the nutritional requirements of most species of rumen bacteria. Four different media, all without added carbohydrates, were

prepared as follows. (i) Forty percent rumen fluid-agar medium (RA) (RGCSA medium minus the carbohydrates). (ii) Incubated RA medium (IRA). All ingredients for RA medium except Na_2CO_3 and cysteine were placed in a round-bottom flask and gassed with CO_2 until reduced. The flask was sealed anaerobically with a rubber stopper (held in by wire) and placed in a 38°C incubator for 10 days. After incubation, the flask of medium was heated to 100°C for 5 min in the autoclave, opened anaerobically under CO_2, and placed in a 45°C water bath. Na_2CO_3 and cysteine were added, and the medium was tubed anaerobically in tubes (18 by 150 mm) and then sterilized in the tube. (iii) Forty percent clarified rumen fluid-agar medium (CRA), identical to RA except that the rumen fluid was centrifuged at 2,500 × *g* for 5 min, decanted through cheesecloth, and then centrifuged at 25,000 × *g* for 20 min. (iv) Rumen fluid (1,000 × *g* supernatant), which was incubated by itself, as described above, and after 10 days was used to prepare CRA medium. Colony counts obtained in these four media are shown in Table 1. Incubation of RA medium for 10 days before the addition of Na_2CO_3 and cysteine, tubing, and sterilizing significantly reduced colony counts. The magnitude of decrease was about 88%. In contrast, incubation of the 1,000 × *g* rumen fluid supernatant for 10 days, followed by clarification and inclusion in CRA medium, was not effective in reducing colony counts. An explanation for this difference is not obvious; however, inhibition of growth by fermentation products in the undiluted rumen fluid or small amounts of energy sources present in the agar are possibilities to be considered.

On the basis of these observations, several experiments were conducted to compare colony counts obtained from incubated and nonincubated 40% rumen fluid medium with and without added carbohydrates (Table 2). Incubation of RA medium (IRA) for 10 days reduced colony counts from 79.3% of the total count with RGCSA medium to 7.7%, similar to the results shown in Table 1. When glucose, cellobiose, and starch were added to the IRA medium (IRA + GCS), colony counts were increased to 87.4% of the RGCSA count. Although colony counts in IRA + GCS medium were significantly lower than in RGCSA medium, the above data suggested that the incubation procedure had considerably more potential for the development of a selective medium than any previously used media.

A study was conducted to determine what effect the length of the incubation period of IRA might have on colony counts in media without added carbohydrates. The results of the first experiment (Table 3) indicated that a marked decrease occurred in colony count during the first 3 days of incubation. This was followed by

TABLE 1. *Effect of incubating 40% rumen fluid-agar medium or rumen fluid for 10 days at 38°C upon colony counts in media without added substrate*

Medium[a]	Colony count/10^{-8} g of rumen contents[b]
40% RA	59.3 ± 8.6[c]
IRA	7.2 ± 0.8[d]
40% CRA	49.6 ± 7.1[c, e]
CRA prepared from incubated rumen fluid	43.6 ± 8.3[e]

[a] See text for definition of the various media.

[b] Mean and standard error of the mean. Each mean value was obtained from three replicate experiments with two inoculum levels and four roll tubes per level, or a total of 24 roll tubes.

[c-e] Means in the same column followed by different superscripts are significantly different at $P < 0.01$.

TABLE 2. *Effect of incubation upon colony counts in 40% rumen fluid medium with and without substrates*

Medium[a]	Colony count/10^{-8} g of rumen contents[b]	% of count in RGCSA medium
RA	37.1 ± 3.7[c]	79.3
RGCSA	46.8 ± 3.9[d]	100.0
IRA	3.6 ± 0.5[e]	7.7
IRA + GCS	40.9 ± 3.8[f]	87.4

[a] See text for definition of the various media.

[b] Mean and standard error of the mean. A total of 64 roll tubes are behind each mean value, i.e., eight replicates, two inoculum levels, and four tubes per level.

[c-f] Means in the same column followed by different superscripts are significantly different at $P < 0.01$.

TABLE 3. *Effect of incubation time upon colony counts in 40% rumen fluid medium without substrates*

Days of incubation at 38°C	Colony count/10^{-8} g of rumen contents[a]	
	Expt 1	Expt 2
0	49.0 ± 4.0[b]	60.2 ± 5.6[b]
3	16.5 ± 2.7[c]	
7	8.9 ± 1.8[d]	8.2 ± 1.0[c]
10	8.2 ± 1.5[d]	8.5 ± 1.4[c]
14	7.8 ± 1.7[d]	

[a] Mean and standard error of the mean. For each experiment there were four replicates, two inoculum levels, and four tubes per level for a total of 32 roll tubes behind each mean.

[b-d] Means in the same column with different superscripts are significantly different at $P < 0.01$.

a significant decrease ($P < 0.01$) between days 3 and 7 with no further change through day 14. Experiment 2 (Table 3) substantiated the observation that a 7-day incubation period was not different from a 10-day period. Because of the convenience of starting and ending the incubation on a week day, the 7-day incubation period was thus used in the remainder of our studies.

A rather extensive series of experiments was run in which colony counts in incubated and nonincubated media were compared. Media for these studies included a negative control without added carbohydrates, a positive control with added glucose, cellobiose, and starch, and selective media in which one of the following substrates was used: starch, xylose, xylan, glycerol, or lactate. Colony counts in the incubated selective medium were consistently lower than those in nonincubated medium, and in the case of glycerol and lactate were only 35 and 25%, respectively. However, when the positive controls containing added glucose, cellobiose, and starch were compared, the colony count in the incubated medium was lower in every experiment. When all 17 experiments were compared statistically, the difference was significant at $P < 0.01$.

Two conclusions could be readily drawn from these data. First, after incubation the medium became limiting or deficient in one or more factors that are essential to growth of certain species of rumen bacteria, and/or second, an energy source normally present in rumen fluid had been exhausted, and some rumen bacteria were present that could not utilize glucose, cellobiose, or starch. These possibilities were tested experimentally (Table 4). Either 5% rumen fluid, 0.017% yeast extract, or 0.02% xylose increased the counts in IRA + GCS to equal those in RGCSA medium. Addition of a volatile fatty acid mixture was without effect. On the basis of the xylose response, it appeared that the second hypothesis was involved; i.e., an energy source normally present in rumen fluid had been depleted and could not be replaced by glucose, cellobiose, or starch. Additional experiments were conducted to substantiate these results and evaluate the effect of adding xylose to regular RGCSA medium (Table 5). In general, addition of xylose to RGCSA medium was without significant effect, whereas the same response noted earlier was observed with IRA. Therefore, it was concluded that IRA contained all of the nutritional factors essential for the growth of the same spectrum of rumen bacterial species that are normally cultured in RGCSA medium. The possibility also exists that some bacteria were able to grow in RGCSA and not IRA, whereas other bacteria grew in IRA and not in RGCSA. Therefore, viable counts on the two media could be similar while the flora was different.

To evaluate the ability of the selective medium to measure differences in bacterial proportions between animals and rations, studies were conducted in which each of the carbohydrates used in the medium for total colony counts was individually added to the basal incubated medium. These data are shown in Ta-

TABLE 4. *Effect of various additions to incubated medium plus carbohydrates upon colony count*

Medium[a]	Additions to medium	Colony count/ 10^{-8} g of rumen contents[b]
RGCSA	None	105.1 ± 4.9[c]
IRA + GCS	None	87.7 ± 4.5[d]
IRA + GCS	5% Rumen fluid	105.5 ± 4.7[c]
IRA + GCS	VFA[e]	78.8 ± 1.7[d]
IRA + GCS	0.017% Yeast extract	109.3 ± 5.5[c]
IRA + GCS	Xylose[f]	116.8 ± 8.1[c]

[a] See text for medium abbreviations.

[b] Mean and standard error of the mean. Each mean represents a total of 32 roll tubes, i.e., four replicates, two inoculum levels per replicate, and four tubes per inoculum level.

[c,d] Means in the same column with different superscripts are significantly different at $P < 0.01$.

[e] 0.083% of volatile fatty acid (VFA) mixture (3).

[f] Total concentration of added carbohydrates was 0.1%, 0.02% each of glucose, cellobiose, and xylose, plus 0.04% starch.

TABLE 5. *Effect of including xylose as an energy source in incubated and nonincubated medium*

Medium[a]	Colony count/ 10^{-8} g of rumen contents[b]	
	Expt 1	Expt 2
RGCSA	174.9 ± 8.2[c]	109.0 ± 3.4[c, d]
RGCSA + xylose	178.4 ± 11.9[c]	116.0 ± 4.4[c, d]
IRA + GCS		96.6 ± 1.5[c, e]
IRA + GCS + xylose		113.2 ± 2.2[d]
RA		84.0 ± 2.7[e]
IRA		14.9 ± 1.5[f]

[a] See text for medium abbreviations. Total carbohydrate concentration in the media was 0.1%, i.e., 0.025% each of glucose and cellobiose plus 0.05% starch or 0.02% each of glucose, cellobiose, and xylose plus 0.04% starch.

[b] Mean and standard error of the mean. Each mean in experiment 1 is based on four replicates of four tubes each or 16 tubes. Experiment 2 consisted of three replicates, two inoculum levels per replicate, and four tubes per inoculum level or a total of 24 roll tubes for each mean.

[c-f] Means in this same column with different superscrpts are significantly different at $P < 0.01$.

ble 6, with the colony count on the selective medium reported as a percentage of the total count. For sheep 2, fed the 60-40 ration, total colony count and percentage of starch-utilizing bacteria varied considerably between the 2 consecutive sampling days. In contrast, the total count over 3 sampling days was fairly constant for sheep 3, whereas marked variation occurred in the counts on all of the selective media. A comparison of the mean values for the selective media between sheep 2 and 3, both fed the 60-40 ration, indicates quite large differences in the percentage of glucose- and xylose-fermenting bacteria. Daily variation was also evident in the counts obtained with samples of rumen contents for sheep 1, fed orchardgrass hay. The only obvious trends between the sheep fed the 60-40 ration and the hay-fed sheep were that the animal fed hay appeared to have a higher percentage of cellobiose-fermenting bacteria along with the lower percentage of starch digesters.

While the preceding experiment was being carried out, it was noted that colony size on the incubated medium was considerably smaller than normally found in RGCSA roll tubes with 0.1% total added carbohydrates. Two experiments of four replicates each were set up to compare colony counts with (i) 0.1% total carbohydrates in RGCSA medium plus xylose (RGCSXA) and (ii) IRA + GCS + xylose (IRA + GCSX) medium and 0.2% total carbohydrates in IRA + GCSX medium. Two inoculum levels and four roll tubes per level were used in each replicate. The 0.1% added carbohydrate level contained 0.02% each glucose, cellobiose, and xylose plus 0.04% starch. In the first experiment, the 0.2% level contained 0.04% each glucose, cellobiose, and xylose plus 0.08% starch, whereas 0.05% of each carbohydrate was used in experiment 2. No significant differences in colony count were noted in either experiment between the three media, and because of increased colony size, counting was much easier with 0.2% total carbohydrate in the incubated medium. On this basis, the concentration of added carbohydrate in incubated media was increased to 0.2% for all subsequent studies. The 0.05% level was used for each of the carbohydrates in IRA + GCSX medium.

The percentage of rumen bacteria utilizing starch, xylan, and pectin in two sheep, one fed the 60-40 ration and the other 100% orchardgrass, is presented in Table 7. Total count and the percentage of starch-digesting bacteria were significantly higher in the sheep fed the 60-40 ration ($P < 0.01$). The mean percentages for xylan- and pectin-utilizing bacteria were not different; however, daily variations tended to be greater for these two carbohydrates. In the concentrate-fed sheep, the percentage of starch-utilizing bacteria was significantly higher ($P < 0.05$) than percentages for xylan and pectin utilizers. No difference between these three groups was observed in the orchardgrass-fed animal. The data on the percentage of starch digesters and the data in Table 6 for these same two animals are similar, i.e., 83.3 and 95.8 for sheep 2 and 62.7 and 60.6 for sheep 1.

Table 8 presents the percentages of the total

TABLE 6. *Effect of animal, ration, and day upon the percentage of rumen bacteria utilizing the individual carbohydrates included in the medium for total colony counts*[a]

Sheep	Ration[b]	Date	Total colony count[c]	% of total colony count utilizing:				
				Glucose	Cellobiose	Starch	Xylose	None
2	60-40	10-21	67.9	42.2	53.8	75.2	45.0	10.0
		10-22	98.9	53.9	58.6	91.4	42.8	8.7
Mean ± SE[d]			83.4 ± 15.5	48.0 ± 5.8	56.2 ± 2.4	83.3 ± 8.1	43.9 ± 1.1	9.4 ± 0.6
3	60-40	10-28	47.6	71.9	68.4	88.7	77.2	11.0
		10-29	42.0	66.4	39.8	64.2	61.9	11.1
		11-1	56.8	100.8	60.4	80.4	42.9	15.1
Mean ± SE			48.8 ± 4.3	79.7 ± 10.7	56.2 ± 8.5	77.8 ± 7.2	60.7 ± 9.9	12.4 ± 1.4
1	OG	10-24	96.6	66.2	88.9	77.6	85.6	11.3
		10-25	94.4	66.8	62.4	58.8	56.8	7.8
		11-5	66.2	60.5	75.5	51.7	57.4	11.7
Mean ± SE			85.7 ± 9.8	64.5 ± 2.0	75.6 ± 7.6	62.7 ± 7.7	66.6 ± 9.5	10.2 ± 1.2

[a] Total count was determined in IRA + GCSX medium (0.02% each of glucose, cellobiose, and xylose plus 0.04% starch). Individual carbohydrates were added at 0.1%. Each daily mean is based on eight individual roll tubes, i.e., two inoculum levels, four tubes per level.

[b] 60-40, 60% corn-40% orchardgrass hay; OG, orchardgrass hay.

[c] Colony count per 10^{-8} g of rumen contents.

[d] SE, Standard error of the mean.

TABLE 7. *Percentage of the rumen bacterial population utilizing starch, xylan, and pectin in a sheep fed 60% corn-40% orchardgrass and a sheep fed orchardgrass*[a]

Sheep	Ration[b]	Date	Total colony count[c]	% of total colony count utilizing:		
				Starch	Xylan	Pectin
2	60-40	11-18	180.5	98.9	39.4	56.1
		11-19	129.2	92.0	62.7	81.0
		11-28	161.0	99.1	61.8	63.4
		11-29	123.8	93.4	78.0	40.9
Mean ± SE[d]			148.6 ± 13.4[e]	95.8 ± 1.8[e]	60.5 ± 7.9[e]	60.4 ± 8.3[e]
1	OG	11-14	62.2	54.0	85.0	69.6
		11-15	59.6	52.4	64.4	62.8
		12-2	43.9	59.6	52.5	56.2
		12-5	46.1	76.2	52.0	40.1
Mean ± SE			53.0 ± 4.6[f]	60.6 ± 5.4[f]	63.5 ± 7.7[e]	59.4 ± 4.4[e]

[a] Total colony count was determined in IRA + GCSX medium (0.05% each of glucose, cellobiose, starch, and xylose). Individual carbohydrates were added at 0.2%. Each daily mean is based on eight individual roll tubes, i.e., two inoculum levels, four tubes per level.

[b-d] See Table 6.

[e,f] Means in the same column followed by different superscripts are significantly different at $P < 0.01$.

TABLE 8. *Percentage of the rumen bacterial population utilizing lactate, glycerol, and mannitol in a sheep fed 60% corn-40% orchardgrass and a sheep fed orchardgrass*[a]

Sheep	Ration[b]	Date	Total colony count[c]	% of total colony count utilizing:		
				Lactate	Glycerol	Mannitol
2	60-40	12-16	85.1	38.0	53.7	44.0
		12-17	97.5	28.8	27.2	29.8
		12-30	162.5	28.4	28.6	49.6
		12-31	155.5	26.8	25.8	23.8
Mean ± SE[d]			125.2 ± 19.8[e]	30.5 ± 2.5[e]	33.8 ± 6.6[e]	36.8 ± 6.0[e]
1	OG	12-19	40.4	52.6	54.3	32.0
		12-20	48.2	47.8	58.6	35.1
		12-30	72.6	31.8	31.8	37.0
		12-31	71.4	24.5	28.2	24.4
Mean ± SE			58.2 ± 8.2[f]	39.2 ± 6.6[e]	43.2 ± 7.7[e]	32.1 ± 2.8[e]

[a-f] See Table 7.

population that could utilize lactate, glycerol, and mannitol in these same two sheep. Within each animal or ration, no significant differences were found in the percentages utilizing the three substrates, although the percentage of bacteria utilizing lactate and glycerol tended to be higher in the sheep fed the orchardgrass ration.

Data were compiled from all of the studies in which both total colony counts and colony counts on IRA medium had been determined. On the basis of 36 replicate experiments, each with two inoculum levels and four roll tubes per level, the basal incubated medium supported the growth of 9.6 ± 0.6% of the total culturable bacteria, with a range of 3.5 to 16.2%. Total colony counts varied from 24.6 to 259.5 per 10^{-8} g of rumen contents. A significant correlation, $r = 0.6$ ($P < 0.01$), was found between total and IRA colony counts. This was expected, since the percentage of colonies in IRA medium was fairly constant over the 10-fold increase in total count.

A question of major interest with respect to those colonies that developed in the IRA roll tubes was whether these same bacterial species would be present in all selective medium roll tubes. If so, the determined percentage of total rumen bacteria utilizing any specific substrate would be higher than the absolute percentage by that amount. To investigate this question, we tried to isolate bacteria from roll tubes of IRA medium. Although efforts were hampered by the extremely small size of the colonies, 10 cultures were eventually obtained. Five of the cultures grew quite rapidly in RGCA slants, showing visible growth in 24 h, whereas the remaining five required 48 to 72 h of incubation before growth could be observed. Observations with the phase microscope revealed that three

of the faster-growing cultures were mixed and consisted of two or three morphological types commonly found in the rumen. The remaining seven appeared to be pure cultures, and their morphology and physiological characteristics are shown in Table 9.

Strain 4 was presumptively identified as belonging to the genus *Butyrivibrio*; however, the characteristics of the six additional strains were quite atypical with respect to predominant rumen bacterial species. Strains 1, 3, and 6 showed limited growth only in the complete medium with 10% rumen fluid and glucose substrate; strain 7 was extremely limited in ability to grow in any of the broth media; and strains 9 and 14, which were quite similar, appeared to grow best in the medium without carbohydrate. However, this latter observation was confounded by the production of a black precipitate in these cultures, presumed to be ferrous sulfide.

A second approach to the question posed earlier, concerning the colonies growing in IRA roll tubes, was to determine colony count in a medium containing only minerals, resazurin, Na_2CO_3, cysteine, and agar. Four replicate experiments, each with two inoculum levels and four roll tubes per level, were conducted. All colonies that developed were extremely small; however, colony counts ranged from 4.4 to 11.1% of the total count, with a mean of 6.8 ± 0.7%. It is assumed that these organisms are capable of utilizing cysteine as an energy source.

Based on the characteristics of the bacterial strains isolated from IRA medium, it appears that most of these organisms would be present in all of the selective media. Although numbers were extremely low, 6 of 10 (60%) of the strains were atypical with respect to the predominant rumen bacteria, and their growth was quite slow and limited in all media. Since the mean percentage of colonies in IRA medium was 9.6% of the total count, 60% of this value, or 5.8% of the total count, might therefore be expected to grow in all the selective media. This value compares quite closely with the 6.8% of the total count that grew in a medium containing only minerals, resazurin, Na_2CO_3, and cysteine. If one wishes to determine the absolute percentage of the rumen population utilizing a specific substrate, the above data suggest that the values obtained on the present selective medium are probably about 6 to 7% high. Use of the percentages directly obtained in selective media should all be relative and quite applicable to estimating differences between animals, days, and rations.

The only valid comparisons of other work with the present data would appear to be those

TABLE 9. *Morphology and physiological characteristics of rumen bacteria isolated from the basal incubated medium without added substrate*

Characteristic	Strain[a]						
	1	3	4	6	7	9	14
Cells							
Morphology	Rod	Rod	Curved rod	Rod	Rod	Cocci in chains	Cocci in chains
Dimensions (μm)							
Width	0.8–1.0	1.0	0.4–0.5	0.8–1.0	0.4–0.5	0.5	0.5
Length	1.0–4.0	1.5–4.0	1.0–2.5	1.0–4.0	0.5–1.0		
Motility	−	+	+	−	+	−	−
Gram stain	−	−	−	−	−	− to ±	− to ±
H_2S production	+	+	−	+	+	+	+
Growth in slants (h)[b]	48	24	24	72	48	72	48
Growth in Trypticase-yeast extract medium	−	+	+	−	−	−	−
Growth in complete medium[c]	0.02 (48)[d]	0 (168)	1.31 (24)	0.01 (72)	0 (168)	0 (168)	0 (168)
Plus 10% rumen fluid	0.21 (96)	0.42 (24)	1.32 (24)	0.17 (72)	0.09 (60)	0.06 (120)	0.10 (108)
Minus carbohydrate	0.06 (72)	0.04 (132)	0 (168)	0.12 (132)	−[e]	−[e]	−[e]
Cellobiose substrate	0.02 (186)	0.09 (24)	1.27 (24)	0.03 (42)	0.12 (186)	0.07 (114)	0.07 (114)
Xylose substrate	0 (168)	0 (168)	0.43 (96)	0.02 (168)	0 (168)	0 (168)	0 (168)
Starch substrate	−[e]	0.16 (168)	0.52 (96)	0.02 (71)	0.08 (119)	0.09 (96)	0.08 (96)
Starch hydrolysis	−	−	−	−	−	−	−
Fermentation end products[f]	b	aFL	FBL	L	b	−	1

[a] All strains were obligate anaerobes.

[b] Hours required before visible growth was observed in RGCA slants.

[c] Defined medium plus acid hydrolysate of casein and 0.0002% hemin (10), containng 0.5% glucose as an energy source.

[d] Values are expressed as optical density at 600 nm. The figures in parentheses indicate hours required to reach maximum optical density.

[e] A black precipitate was produced which interfered with optical density measurements.

[f] Small letters, <1 meq/100 ml of medium; capital letters, >1 meq/100 ml of medium. B, Butyric and higher acids; A, acetic; F, formic; L, lactic.

results obtained by nonselective medium isolation and screening procedures. The percentages of bacterial strains hydrolyzing starch and producing acid from glucose, cellobiose, and xylose were estimated by Bryant and Burkey (1) for isolates from rumen contents of a cow fed several different rations. Values for isolates hydrolyzing starch were low (18%) when the animal was fed a straw ration, intermediate (32 to 43%) on the alfalfa or alfalfa-concentrate ration, and highest (56%) on the concentrate ration. The percentage of isolates hydrolyzing starch from a second cow fed the same alfalfa ration was considerably higher, 49.7 versus 33.1%. This confirms our observations on animal differences. All of their percentages for starch digesters were considerably lower than those found in the present study. In contrast, their percentages and ration differences were quite similar to our values for glucose-, cellobiose-, and xylose-utilizing bacteria.

Slyter and Putman (12) reported that 52 to 79% (mean, 65.5%) of the bacterial strains isolated on four different days from a steer fed low levels of pelleted roughage could utilize starch. This compares with our range of 51.1 to 77.6% and mean of 61.5% for seven replicates from a sheep fed a low level of orchardgrass hay (Tables 6 and 7). Using 6% as a blank medium correction, as discussed earlier, our value decreases to 55.5%. It is difficult to draw precise conclusions from these two studies because of animal and ration differences; however, the percentages of starch utilizers are in similar ranges.

In a later study, Slyter et al. (11) reported that 88.8 and 94.6% of the strains isolated from two steers fed unlimited amounts of a 90% corn ration could hydrolyze starch. By using a 90% wheat ration, values of 86.5 and 81.6% were found in two additional steers. These data give a mean of 87.9% for the four steers fed the 90% concentrate rations. This compares quite well with our mean of 87.0% for starch utilizers (Tables 6 and 7) in rumen contents from two sheep fed the 60% corn ration. Again correcting for the blank medium (minus 6%), our value decreases to 81%, which seems quite reasonable when comparing a 60% corn ration fed at a limited level to a 90% concentrate ration fed ad libitum.

No particular explanation can be offered for the necessity of also adding xylose as a substrate in incubated medium to obtain counts similar to those in RGCSA medium. Obviously certain strains might be present that can only utilize pentoses, which are removed during incubation; however, it appears just as plausible that another substrate is removed and that those strains can also utilize xylose but not the other hexoses. Further experiments with other carbohydrates, alcohols, or acids in place of xylose might help explain this observation.

The data presented indicate that a 7-day preincubation of 40% RA medium reduces the concentration of endogenous energy sources to a level suitable for use in a selective medium, without removing required nutritional factors. With this medium, considerable variation between days and animals was observed in the percentages of the total rumen population utilizing specific substrates, which in turn decreases the probability of detecting ration differences. Comprehensive studies on these sources of variation, possibly including time samples from the same animal within a day, are needed to determine whether such rapid shifts in bacterial proportions do occur in the rumen population.

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