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Differential Carbohydrate Media and Anaerobic Replica Plating Techniques in Delineating Carbohydrate-Utilizing Subgroups in Rumen Bacterial Populations

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A basal (BC) medium devoid of added carbohydrates, a complete (CC) medium containing nine carbohydrates, and a variety of differential (DC) media containing one or two carbohydrates were developed for enumerating rumen bacteria. The colony counts on the BC medium were 85 to 100% of those obtained on the CC medium. These colonies were pinpoint size (≤1 mm in diameter) but increased in size (2 to 5 mm in diameter) when carbohydrates were subsequently added. With the CC medium or other media tested, the colony counts were 20 to 50% higher on plates than on roll tubes and were about 35% of the direct cell counts. The lower colony counts on roll tubes were shown to result primarily from the loss of viability due to heat stress. The DC media were found by plating techniques to be suitable for differentiating mixed rumen bacterial populations into subgroups based upon carbohydrate utilization as shown by differences in subgroup profiles found within solid and liquid fractions of rumen contents, within rumen contents from animals fed high-forage and high-grain diets, and by correct colony formations by pure cultures of rumen bacteria on appropriate DC media. With simple modifications and use of an anaerobic glove box, replica plating methods and the CC and DC media were found to be a suitable means of rapidly determining the range of utilizable carbohydrate energy sources of rumen bacteria.

Nonselective isolation and enumeration of the predominant rumen bacteria has been accomplished by using a variety of rumen fluid-containing media (6, 8, 14, 19) or more defined media (11) in which the rumen fluid has been replaced by the addition of Trypticase, yeast extract, hemin, and volatile fatty acids. With the use of these media, it has been documented that the total rumen microbial population varies in number both with time after feeding (7, 8, 10, 30) and with different dietary regimens (8, 10, 17, 23, 25-27, 30). However, definite information is limited or lacking concerning the interactions among the various carbohydrate-utilizing subgroups of bacteria that occur with alterations in diet or degree of digestion of dietary components.

The idea for developing a series of selective or differential media is not new, although success in this area has been rather marginal with ruminal and gastrointestinal tract species of major ecological significance. To date, only minimally species-specific enrichment media are available for anaerobic rumen bacteria. These include the mannitol-containing medium for *Selenomonas* sp. (28; R. B. Hespell and M. P. Bryant, *in* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel, ed., *The Procaryotes—A Handbook on Habitats, Isolation and Identification of Bacteria*, in press) and the rutin-containing medium for *Butyrivibrio* sp. (13; Hespell and Bryant, in press). Recently, however, studies of Dehority and Grubb (15) used rumen fluid-based media to which a single carbohydrate energy source was added in an attempt to determine the percentage of the total anaerobic bacterial population capable of utilizing the added substrate. For several reasons, including use of plating or replica plating rather than roll tube techniques, we tried to develop a more reproducible rumen fluid-containing basal (BC) medium for separating mixed rumen bacteria into groups based upon growth on differential carbohydrate (DC) media.

MATERIALS AND METHODS

Bacteria. Pure cultures of anaerobic rumen bacteria were obtained from the culture collection of the Department of Dairy Science at the University of Illinois. The medium used for their maintenance was the RGCA slant medium of Bryant and Burkey (6) as modified by Bryant and Robinson (9).

Bacterial populations in rumen contents were obtained from a fistulated, nonlactating Holstein cow fed a diet consisting of 10 kg of alfalfa hay and 3.6 kg of concentrate (75% forage/25% grain as dry matter). The diet was fed in equal portions twice daily at 6 a.m.

and 4 p.m. Later, a Holstein steer fed corn and corn silage in a ratio of 85% grain/15% forage (as dry matter) was also used to obtain samples for comparative purposes. All samples of rumen contents were obtained 3 to 4 h after the morning feeding in the following manner. The contents in the rumen were first manually mixed to obtain a more homogeneous sample. Handfuls of rumen contents were removed and squeezed through two layers of cheesecloth, and the resultant liquid portion was collected in a flask to one-half the flask's volume. Whole rumen contents were added to completely fill the flask, which was then sealed with a one-way, check-valve stopper and immediately transported to the laboratory. Maximum time for collection and transport was 20 min. In the laboratory, the rumen contents were blended for 1 min in a Waring blender at maximum setting while being vigorously bubbled with O_2-free CO_2. The blended mixture was squeezed through four layers of cheesecloth, and the liquid was collected in a second CO_2-filled flask. The resulting rumen fluid either was used for incubated clarified rumen fluid preparation or was serially diluted in 10-fold amounts (each tube mixed 2 s) in S buffer (Table 1) and inoculated into tubes of medium for roll tube preparation or onto plates of medium.

Purification of medium constituents. Agar (225 g; Difco Laboratories, Detroit, Mich.) was washed three times in 3 liters of 60°C distilled water. After each wash, the agar-water suspension was centrifuged (5,000 × *g*, 20 min, 4°C), after which the supernatant liquid was decanted. The washed agar was spread into thin layers, frozen, broken up into small pieces, and lyophilized until dry.

The carbohydrates in various media were prepared as follows. (i) For cellulose, reagent-grade powdered cellulose (25 g; Whatman, Clifton, N.J.) was washed by filtration three times alternately with 100-ml portions of 0.2 N HCl and 0.2 N NaOH. The cellulose was then rinsed once with distilled water and dried (30°C). (ii) For pectin, citrus pectin (50 g; Sunkist, lot 3442) was purified by extraction twice with 500-ml portions of 70% (vol/vol) aqueous ethanol and dried (37°C; 22). (iii) For rutin, a commercial preparation of rutin (25 g; ICN, Irvine, Calif.) was purified by crystallization from absolute ethanol and recrystallization from hot water (16, 18). For xylan, reagent-grade larchwood xylan (25 g; Sigma Chemical Co., St. Louis, Mo.) was washed twice with 100-ml portions of 95% ethanol and rinsed with 100 ml of absolute ethanol before drying (30°C).

TABLE 1. *Composition of S buffer*[a]

Ingredient	% in buffer	Final concn (mM)
$CaCl_2 \cdot H_2O$	0.02	1.0
$MgSO_4$	0.025	2.0
KCl	0.06	8.0
NaCl	0.06	10.0
PIPES buffer[b]	0.15	5.0
Resazurin (0.1%)	0.20	0.01
R_1 salts[c]	0.10	
Distilled water	96.20	
Dithiothreitol (0.1 M)[d]	1.50	1.5
NaH_2PO_4 (0.5 M; pH 7.0)[d]	2.00	10.0

[a] Prepared under N_2; weight/volume basis; pH adjusted to 7.0 with KOH before autoclaving.

[b] Piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); pK_a 6.8.

[c] Prepared as described by Hespell and Canale-Parola (20) and Canale-Parola et al. (12).

[d] Filter-sterilized solutions, added after autoclaving.

Incubated clarified rumen fluid was prepared by a modification of the procedure of Dehority and Grubb (15). Flasks containing a magnetic stirring bar, plus 35 ml each of minerals one and two (modified from Bryant and Burkey [6]; see Table 2) and 300 ml of distilled water, were bubbled with O_2-free CO_2 for 15 min. Then 2 ml of 2.5% (wt/vol) L-cysteine hydrochloride was added to each flask, and bubbling with CO_2 was continued for another 5 min. The flasks were then stoppered with butyl rubber stoppers. (If incubation flasks are to be stored before use, it is suggested that autoclaving and aseptic techniques be used.) Fresh rumen contents (300 ml; prepared as described above) were added to each flask under CO_2 to yield a 40% (vol/vol) rumen fluid mixture. The vessels were closed with stoppers fitted with one-way (gravity-type) check valves. The pH of the mixture in each flask was then adjusted to 6.8 immediately and periodically throughout the incubation period (3 to 5 days, 39°C, rotary bath). All pH adjustments were made while the flasks were being flushed with CO_2 and using CO_2-equilibrated 1.0 M Na_2-CO_3. After completion of the incubation, the rumen fluid mixture was clarified by centrifugation (16,300 × *g*, 30 min, 4°C). The resultant supernatant fluid constituted the incubated clarified rumen fluid (ICRF) and was frozen until needed. To minimize possible between-batch differences, portions from two or three separately prepared batches of ICRF were thawed and combined in equal proportions for use in medium preparation.

Media. Throughout this study, sterile anaerobic techniques as outlined by Hungate (21) and modified by Bryant (5) were used. Common rumen bacteriological media used included medium 98-5 and medium 10 as described by Bryant and Robinson (8) and Caldwell and Bryant (11), respectively. The rutin medium was prepared as described by Cheng et al. (13), using ICRF and washed agar. The mannitol medium was prepared as described by Tiwari et al. (28), using washed agar.

The BC medium was designed such that it would supply the nutrients necessary for initiating growth of the predominant rumen bacteria. Complete carbohydrate (CC) medium consisted of the BC medium plus an array of carbohydrates (Table 2). DC media each contained the BC medium plus a single carbohydrate, except for the cellulose/cellobiose- and xylan/xylose-DC media, which contained these dual carbohydrates. For each medium, the carbohydrate(s) was added to give a final total carbohydrate concentration of 0.40% (wt/vol).

ICRF was added (40%, vol/vol) to the various media. However, as the preparation of ICRF itself resulted in a 40% (vol/vol) solution (see above), the actual concentration of rumen fluid in the finished CC and DC media was 16% (vol/vol).

Inoculation of media. Inoculation of roll tubes

TABLE 2. *Complete and differential carbohydrate media*[a]

Ingredient	% in medium
Carbohydrate[b]	0.45
Trypticase	0.20
Yeast extract	0.05
Mineral one[c]	4.0
Mineral two[c]	4.0
Hemin solution (0.01%)[d]	1.0
Volatile fatty acid solution[e]	1.0
Resazurin (0.1%)	0.1
Incubated clarified rumen fluid (40%, vol/vol)	40.0
Agar (washed)	2.0
Distilled water	47.0
Na_2S/L-cysteine hydrochloride solution (2.5%/2.5%)[f]	1.0
Na_2CO_3 solution (8%)[g]	5.0

[a] Prepared under CO_2; weight/volume unless otherwise specified; final pH 6.8.

[b] CC medium: cellulose, cellobiose, glucose, maltose, pectin, soluble starch, xylan, and xylose, 0.05% each, plus glycerin, 0.05% (vol/vol). DC media: cellulose + cellobiose, 0.2% each; glucose, 0.4%; glycerin, 0.4%; maltose, 0.4%; pectin, 0.4%; soluble starch, 0.4%; xylan + xylose, 0.2% each.

[c] Modified from Bryant and Burkey (6) as follows: (i) mineral one = K_2HPO_4, 0.6%; and (ii) mineral two = KH_2PO_4, 0.6%; $(NH_4)_2SO_4$, 0.6%; NaCl, 1.2%; $MgSO_4 \cdot 7H_2O$, 0.255%; $CaCl_2 \cdot 2H_2O$, 0.169%.

[d] 10 mg of hemin dissolved in 50 ml of ethanol plus 50 ml of 0.05 M NaOH.

[e] Acetic, 17 ml; propionic, 6 ml; butyric, 4 ml; *iso*-butyric, *n*-valeric, isovaleric, and DL-α-methylbutyric, 1 ml each, plus distilled water to 100 ml after adjusting the pH to 7.5 with NaOH.

[f] Prepared as a single, sterile (121°C, 10 min) solution and added after autoclaving.

[g] Prepared as a sterile (121°C, 15 min; equilibrated with CO_2, 30 min) solution and added after autoclaving.

was with 0.1 and 0.5 ml of appropriately diluted rumen contents. Four replicates per amount of inoculum were done. Immediately after inoculation each tube was rolled (30 to 40 s), using ice water applied to the tube with a sponge. The inoculated tubes were incubated (37°C, 5 days), after which the colonies observable with a Quebec colony counter were counted.

Agar plates were prepared and inoculated within an anaerobic glove box (temperature, 22 to 24°C; atmosphere, 95% Ar and 5% H_2). Disposable plastic petri dishes (60 by 15 mm) and all other plasticware were allowed to equilibrate within the glove box for at least 24 h before use to ensure removal of traces of surface-adhering O_2. The media were prepared as for roll tubes and, in the sealed flasks, were brought into the glove box. After pouring 12 to 15 ml per plate, we held the plates in the glove box for 24 to 48 h to remove excess surface moisture, after which we placed them into modified pressure cookers (see below) which were flushed with CO_2 and kept under a slight positive pressure while being stored within the glove box. Within the pressure cookers, the agar plates remained moist and reduced even after 1 month of storage.

Using an autopipette (P200, Gilson Pipetman), plates were inoculated in triplicate with 50-μl portions of diluted rumen contents, and the inoculum was dispersed with a sterile, bent glass rod. The range of dilutions used allowed for development of up to 200 colonies per plate, but only plates containing between 10 and 100 colonies were used for calculating the number of viable bacteria.

Direct cell counts. Using a Petroff-Hausser counting chamber, we made direct cell counts on the prepared rumen contents that were diluted in 10% (vol/vol) formaldehyde–0.85% (wt/vol) NaCl solution as described by Warner (29).

Replica plating. The classical methods of Lederberg and Lederberg (24) were used as the basis for replica plating. Pure cultures of rumen bacteria were spot inoculated in a grid pattern onto a plate (60 by 15 mm) of the CC medium, using a sterile loop or toothpick. Due to the variation in colony sizes, only 10 cultures were inoculated onto each master plate. These were incubated for 48 h at 37°C to allow for sufficient colony growth (3 to 5 mm in diameter) before use for replica plating.

Several preliminary experiments were conducted to determine the best transfer method. Although velveteen was used in the original technique (25), we used filter paper disks (Whatman no. 1, 4, or 50). The paper disk was attached to a wooden block or a flat-bottomed 50-ml flask which fit within the inner perimeter of the petri dishes. These "replicators" were wrapped in aluminum foil, autoclaved (121°C, 15 min), dried (30°C, 24 h), and then allowed to equilibrate for at least 48 h in the anaerobic glove box before use.

Incubation of plates. Two systems for incubating plates outside the anaerobic glove box were used. The first utilized commercially available 3.5-liter stainless-steel pressure cookers which were modified in the following manner. The automatic air vent was plugged, and the pressure regulator was removed and replaced with a stopcock valve situated between short lengths of butyl rubber tubing. One free end was then secured to the vent pipe. The lid gasket was reinforced by an internal support consisting of a length of wire passed through an equivalent length of butyl rubber tubing which fit snugly within the inner surface of the sealing gasket.

After the lids were secured, the pressure cookers were evacuated and flushed three times with CO_2 gas provided from an external tank via a gassing manifold situated within the glove box (3). To ensure no entry of air into these vessels during incubation outside the glove box, a positive pressure (1.4×10^4 Pa) was maintained via connection of the stopcock valve to an external gassing manifold set up in a walk-in incubator.

Although relatively easy to construct and inexpensive, the modified pressure cookers had shortcomings, including periodic replacement of gaskets and necessity of keeping them attached to a gassing manifold. For greater reliability, anaerobic incubation vessels approximately the same size as the pressure cookers were constructed after the model of Balch et al. (2). These vessels could accommodate about 21 (100 by 15 mm) or 64 (60 by 15 mm) petri dishes.

RESULTS

Colony counts on various media using roll tubes or plates. At least as many bacteria were cultured on the CC medium as on medium 98-5 or medium 10, regardless of whether plates or roll tubes were used (Table 3). The addition of xylan to medium 98-5 increased the number of bacteria cultured, as previously noted by Henning and Van der Walt (19). Regardless of the medium used, higher colony counts (20 to 50%) were observed on plates than on roll tubes. Deletion of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), yeast extract, hemin, and volatile fatty acids from the CC medium resulted in a decrease of about 19% of the total count. Deletion of only the carbohydrates from the CC medium (i.e., BC medium) supported the growth of slightly fewer bacteria than did the CC medium. The colonies on BC medium, however, rarely exceeded pinpoint size (≤1 mm in diameter). The number and size of the colonies growing on BC medium suggested that all nutrients necessary to initiate growth of most of the bacteria were present. The concentration of total carbohydrates in the BC medium was found to be quite low (about 100 μg/ml) and was derived primarily from the ICRF component.

TABLE 3. *Comparison of the total colony counts on roll tubes or plates of various media*

Medium	Expt no.	Colony count × 10^8/ml in rumen contents[a]	
		Roll tubes	Plates
CC	1	25.8 ± 3.9	33.0 ± 4.2
	2	30.0 ± 2.1	24.8 ± 1.6
	3	19.8 ± 1.4	24.4 ± 1.8
	4	24.0 ± 1.6	41.7 ± 4.2
	5	24.5 ± 2.0	33.4 ± 1.4
	$\bar{x}$	24.1 ± 2.0	33.2 ± 1.4
CC-Z[b]	1	15.1 ± 2.4	28.0 ± 5.1
	2	26.1 ± 1.4	23.1 ± 2.0
	3	19.3 ± 0.9	24.3 ± 1.7
	$\bar{x}$	20.2 ± 1.0	25.1 ± 1.9
BC	1	20.3 ± 3.2	30.9 ± 5.7
	2	26.1 ± 1.3	25.6 ± 0.6
	3	19.0 ± 1.0	23.1 ± 0.9
	$\bar{x}$	21.8 ± 1.2	26.5 ± 1.9
98-5[c]	1	22.2 ± 2.6	35.5 ± 3.0
	2	29.0 ± 2.2	24.6 ± 1.6
	3	18.9 ± 2.5	24.0 ± 1.9
	4	25.9 ± 5.6	49.9 ± 5.0
	5	24.6 ± 2.9	32.0 ± 2.4
	$\bar{x}$	24.1 ± 2.0	33.2 ± 1.4
98-5 + xylan (0.05%)	4	27.0 ± 5.0	46.5 ± 3.3
	5	28.4 ± 1.4	34.0 ± 1.9
	$\bar{x}$	27.7 ± 2.6	40.3 ± 1.9
Medium 10[c]	4	21.4 ± 1.7	41.1 ± 2.2
	5	22.4 ± 1.2	27.1 ± 1.9
	$\bar{x}$	21.9 ± 1.0	34.1 ± 1.4

[a] Data are means ± standard deviations of colony counts obtained on either eight roll tubes (four at two levels of inocula) or nine plates (three at three levels of inocula) for the respective media. Each experiment used different batches of media and freshly collected rumen contents.

[b] Trypticase, yeast extract, hemin, and volatile fatty acid solutions were deleted.

[c] Prepared according to Bryant and Robinson (8) and Caldwell and Bryant (11), respectively.

To test whether colony size was being limited by the low concentration of carbohydrate in the BC medium, plates of BC medium were inoculated with appropriate dilutions of mixed rumen bacteria. After 5 days of incubation, the colonies on the plates were counted. If the colonies were then overlaid with CC with 0.1% added carbohydrate medium, or replica plated onto CC or onto CC with 0.1% added carbohydrate medium plates, then over 90% of the pinpoint colonies on the original BC plates grew to a larger size (3 to 5 mm in diameter) after an additional 5 days of incubation. The majority of these newly enlarged colonies that were randomly examined exhibited a single type of cell morphology. Using microscopic observations, we tentatively identified these as belonging to the predominant types of rumen bacteria.

Colony counts on plates versus roll tubes. Since 20 to 50% higher colony counts were consistently obtained on plates than on roll tubes (Table 3), this aspect was further investigated. To examine whether potential heat/cold stresses associated with roll tube preparation might explain this difference, several experiments were done (Table 4). We found that regardless of the method of enumeration, heating at 47°C for as short a time as 0.5 min was detrimental, as 5 to 20% loss in viability of the total bacterial population resulted. The colony counts on roll tubes as well as the colony counts with the agar overlay method declined continuously with time of heating. The percentage of the initial numbers of viable bacteria remaining after exposure for 1 min or longer to 47°C was higher for the dilution buffer than for roll tubes or overlays. Although the same bacterial dilution tube was used as an inoculum, the initial counts (0 min) were always higher on plates than on roll tubes.

Comparison of direct and viable counts and the effect of blending. The blending of rumen contents for 1 min under CO_2 resulted in an increase of 29 and 38% in viable count with roll tubes and plates, respectively (Table 5).

TABLE 4. *Loss of bacterial viability in rumen contents subjected to 47°C heat exposures for various times during roll tube preparation and plating procedures*

Culture method[a]	Expt no.[b]	Initial colony count × 10^8/ml in rumen contents[c]	% of initial colony count with time of 47°C heat exposure (min)[d]					
			0	0.5	1	3	5	10
(i) Roll tubes	1	11.8 ± 3.8	100	87	76	59	55	45
	2	16.3 ± 3.5	100	95	78	70	53	22
	3	25.0 ± 4.9	100	85	78	59	57	33
	$\bar{x}$	17.7 ± 2.4	100	89	77	63	55	33
(ii) Plates: overlays	4	20.7 ± 3.1	100	85	67	54	15	14
	5	16.9 ± 2.6	100	80	72	38	25	11
	$\bar{x}$	18.8 ± 2.0	100	83	70	46	20	13
(iii) Plates: dilution buffer	2	19.9 ± 4.7	100	90	87	89	88	93
	3	30.0 ± 9.3	100	79	79	79	58	26
	4	24.4 ± 3.1	100	88	87	84	75	74
	5	16.9 ± 1.9	100	88	77	67	34	22
	$\bar{x}$	23.0 ± 2.8	100	86	83	80	64	54

[a] Rumen contents were appropriately diluted in S buffer and then either (i) inoculated into roll tubes containing 5 ml of 47°C molten 2% agar CC medium, which were then held at 47°C for intervals of 0 to 10 min before rolling; or (ii) inoculated within the anaerobic glove box into tubes containing 1-ml portions of 47°C molten 0.8% agar CC medium, which were then held at 47°C for the specified periods before overlaying onto plates of 2% agar CC medium; or (iii) dispensed into empty tubes in 0.5-ml portions, which were then held at 47°C for the timed periods, after which appropriate amounts were removed and spread onto plates of 2% agar CC medium. The completed roll tubes and plates were incubated for 5 days at 37°C under 100% CO_2, after which the colony counts were determined.

[b] The same animal was used for all experiments. The diet fed was changed, however, during the time over which the samples were collected. Diet for experiments 1 through 3: 70% alfalfa hay, 30% concentrate; diet for experiment 4: 70% alfalfa hay, 30% corn silage; diet for experiment 5: 100% alfalfa hay. All diets were fed at 1.2 times maintenance.

[c] Data are means ± standard deviations of the colony counts obtained for each experiment (eight roll tubes or nine plates each). $\bar{x}$, Grand mean of all experiments for each kind of heating.

[d] For each time point, the value is (mean colony count/initial colony count) × 100.

TABLE 5. *Enumerating efficiencies and the effect of 1-min blending on total bacterial counts of rumen contents*

Cultivation method	Parameter	Treatment of rumen contents		% Increase
		Not blended	Blended	
Roll tubes	Direct count[a]	78.6 ± 0.7	101.8 ± 0.9	26
	Viable count[b]	18.7 ± 2.19	26.4 ± 2.8	29
	Enumerating efficiency[c]	24%	26%	2
Plates	Direct count	90.5 ± 0.9	140.0 ± 0.7	35
	Viable count	31.5 ± 3.7	50.5 ± 2.8	38
	Enumerating efficiency	35%	36%	1

[a] Mean ± standard deviation of cell counts per milliliter of rumen contents × 10^8.

[b] Mean ± standard deviation of colony counts (on CC medium) per milliliter of rumen contents × 10^8. Each mean represents a total of 24 roll tubes (three experiments) or 18 plates (two experiments). Each of the total of five experiments was conducted separately.

[c] (Viable count/direct count) × 100.

Similar increases in direct cell count were also noted. Blending did not increase the enumerating efficiency of either plates or roll tubes.

Carbohydrate subgroup profiles in various fractions of rumen contents. Samples of rumen contents were collected (as described in Materials and Methods) and processed in several different ways (see footnote *a*, Table 6). The standard sample contained both the bacteria present in the fluid portion plus the bacteria that were associated either with forage particles or with aggregates of microflora that were dislodged or disrupted, respectively, by the blending treatment. Sample A contained the bacteria present in the fluid portion, and sample B contained those associated with the solid portions

TABLE 6. *Carbohydrate subgroup profiles of rumen bacterial populations within differently prepared rumen content samples*[a]

Differential carbohydrate medium	Colony count in rumen contents[b]				Arithmetic total of colony counts of A + B
	Standard sample	Sample A	Sample B	Sample C	
CC	24.6 ± 3.2	12.7 ± 1.2	13.0 ± 0.8	22.8 ± 1.8	25.7 ± 0.7
BC	21.6 ± 1.9	12.0 ± 2.0	7.5 ± 1.4	20.4 ± 2.7	19.5 ± 1.2
Cellulose-cellobiose	16.9 ± 3.0	8.1 ± 2.1	10.6 ± 0.7	17.0 ± 1.5	18.7 ± 1.1
Glucose	17.5 ± 2.4	11.8 ± 1.2	5.5 ± 1.6	15.2 ± 1.1	17.3 ± 1.0
Soluble starch	16.6 ± 2.2	11.5 ± 2.6	7.6 ± 1.8	16.2 ± 2.4	19.1 ± 1.6
Maltose	17.6 ± 1.8	7.1 ± 1.9	6.8 ± 1.8	15.5 ± 1.9	13.9 ± 1.3
Xylan-xylose	13.2 ± 3.2	6.6 ± 1.7	9.5 ± 1.3	12.2 ± 1.2	16.1 ± 1.1
Glycerol	4.4 ± 1.1	2.4 ± 1.0	2.8 ± 0.9	4.1 ± 1.2	5.2 ± 0.7
Casein[c]	1.5 ± 0.4	0.7 ± 0.2	0.7 ± 0.4	1.6 ± 0.5	1.4 ± 0.2

[a] The diet was 100% alfalfa hay, fed at maintenance level. Rumen contents were collected 3 h after the morning feeding.

[b] Data are means ± standard deviation of colony counts per milliliter of rumen contents × 10^8. Each mean represents a total of 18 plates (two separate experiments). For the standard sample, the bacterial suspension was prepared from rumen contents as described in Materials and Methods. For sample A, the liquid fraction was obtained by squeezing rumen contents through cheesecloth. This liquid was then blended (1 min) under CO_2 and used. For sample B, the solid fraction remaining from A was blended (1 min) under CO_2 with an amount of anaerobic dilution solution (Bryant and Burkey [6]) equal to the volume of the liquid fraction of A and then squeezed through cheesecloth, and the resultant fluid used. For sample C, the liquid of A plus the liquid of B, 50% (vol/vol each), was used.

[c] Only bacterial colonies with hydrolysis zones were counted.

of rumen contents. Sample C contained both of these populations by combining equal portions of the liquids from the preparation of samples A and B. With the CC medium, the colony counts obtained with "free" bacteria (sample A) and the presumably "attached" bacteria (sample B) were approximately 52 and 53%, respectively, of those obtained with the standard sample. Recombining the free and presumably attached bacterial populations (sample C) resulted in colony counts equivalent to those observed with the standard samples but less than the predicted value (arithmetic total of colony counts of samples A + B). With the BC medium, formation of pinpoint colonies always resulted.

The bacteria present in these various samples were also enumerated by using various DC media. In comparing the attached population (sample B) and the unattached population (sample A), threefold-higher colony counts were observed for the former than for the latter on the cellulose/cellobiose- and xylan/xylose-DC media (Table 6). The converse was found with the glucose-DC medium. Approximately equal colony counts for these two samples were observed when soluble starch-, maltose-, or glycerol-DC media were used. For a given sample, the colony counts on any single DC medium were variable and often less than 50% of the colony counts observed on the CC medium. The one exception to this was the unattached population (sample A) on the glucose-DC medium. Slightly more colonies formed here than on the CC medium. With the attached population (sample B), the colony numbers decreased successively with cellulose/cellobiose-, xylan/xylose-, and soluble starch-DC media.

Growth of known rumen bacterial species on DC media. To ascertain whether use of the array of DC media was a valid way to discriminate specific carbohydrate-utilizing subgroups, we examined the growth responses of strains of rumen bacteria with known substrate specificities by using the various DC media. To accomplish this, a replica plating technique (see Materials and Methods) was used to identically inoculate plates of each DC medium. To determine the extent of carryover carbohydrate contamination when the replica transfers were made, a two-dimensional replica plating experiment was devised. The first dimension was carried out as follows. Master plates of CC medium containing 10 pure cultures of rumen bacteria were prepared, and after incubation each master plate was replicated onto plates of six different DC media. After incubation to allow development of sufficient colony size, each of these primary plates was used as a secondary master plate. Each secondary master plate served to inoculate a second row of six plates. Five of these plates contained an added carbohydrate source different from that on the second master plate, and the order of these plates was randomized for inoculation. The sixth plate contained the same

carbohydrate as added to the particular secondary master plate, and served both as a control to indicate that complete transfer through the row of plates had taken place and as a measure of the extent of carbohydrate carryover.

If carryover of carbohydrates between plates was responsible for the development of colonies on the respective DC medium plates (thus giving false-positive results), then the observed arrays of colony formations on the second-dimension plates would be expected to be different from the arrays obtained in the first dimension. Both sets of data would then contain different arrays of false-positive results. Such false responses, however, were not found when 20 different species of rumen bacteria were examined in this manner. In the primary plates, only six colony formations out of 200 possible were not as expected. In the second plates, only 95 colony formations out of the 1,440 possible were not as expected. In addition, 25% of the secondary mismatches were identical to the mismatches in the first dimension. These results suggested that although some carbohydrate may be carried over, it was not a major factor affecting the carbohydrate growth substrate utilization responses exhibited by the rumen bacteria. This was further supported by the results of other experiments in which the colony formations on various DC media by pure cultures of rumen bacteria were examined in detail. We found that all cultures formed large colonies on the CC medium, but only pinpoint colonies were formed on the BC medium. This was true regardless of the location of the plates of these media in the array during the inoculation procedure.

Although within individual experiments the matching of observed results to expected results varied slightly, data from seven separate experiments indicated that the overall correlation of the known substrate specificities of the bacteria with the carbohydrates in the DC and the rutin and mannitol media was 99% positive.

Carbohydrate subgroup profiles of rumen bacteria in animals fed different diets. Subgroup profiles were examined by using the array of DC and the rutin and mannitol media inoculated with bacterial dilutions prepared from standard samples of rumen contents obtained from animals fed either a high-forage or a high-grain diet. The data (Table 7) indicated that both the direct cell count and the viable cell count (on CC medium) were more than twice as high in the rumen contents from the high-grain-fed animal than in the animal fed the high-forage diet. The plating efficiencies calculated from the CC medium counts and the direct cell counts were 26 and 29% for the high-forage and high-grain diets, respectively. The data obtained with the BC medium indicated that for both diet samples, the number of pinpoint colonies developing upon this medium was 90% or more of the colony counts observed on the CC medium.

The percentages of the total number of colonies developing on the DC media varied both within a given rumen content sample and between samples from animals fed the two diets (Table 7). Except for the cellulose/cellobiose- and the xylan/xylose-DC media, the numbers of colonies were consistently higher for rumen contents from the high-grain-fed animal than for

TABLE 7. *Comparison of the carbohydrate subgroup profiles of rumen bacterial populations from rumen contents of animals fed either a high-forage or a high-grain diet*[a]

Differential carbohydrate medium	High forage		High grain	
	Colony count[b]	% of total[c]	Colony count	% of total
Direct count	65.8 ± 10.1		136.3 ± 23.2	
CC	16.9 ± 2.1	100	39.4 ± 4.6	100
BC	15.2 ± 1.1	90	38.2 ± 1.0	97
Cellulose-cellobiose	10.4 ± 1.5	61	13.6 ± 2.9	35
Glucose	7.4 ± 2.3	44	33.8 ± 2.0	86
Glycerol	3.0 ± 2.3	18	23.5 ± 3.1	60
Maltose	7.7 ± 1.8	45	27.6 ± 0.8	70
Pectin	4.9 ± 0.6	29	19.7 ± 1.1	50
Soluble starch	10.8 ± 1.2	64	32.0 ± 4.9	81
Xylan-xylose	8.8 ± 0.9	52	18.5 ± 2.1	47
Mannitol	0.9 ± 0.3	5	19.9 ± 1.7	51
Rutin	1.8 ± 1.0	10	18.0 ± 1.0	46

[a] High-forage diet: 70% forage as alfalfa hay, 30% corn silage fed at maintenance level; high-grain diet: 85% grain as corn, 15% forage as corn silage, fed at 1.25× maintenance.

[b] Data are means ± standard deviation of colony counts per milliliter of rumen contents × 10^8. Each mean represents a total of 18 plates (two experiments).

[c] (Colony count on given DC medium/colony count on CC medium) × 100.

the high-forage-fed animal. This was true whether the data were considered on an absolute or on a percentage basis. About 61% of the high-forage bacterial population formed colonies on the cellulose/cellobiose-DC medium as opposed to only 35% of the high-grain bacterial population. On the other hand, there were far higher percentages of the bacterial population from the high-grain-fed animal capable of forming colonies on the DC media containing soluble carbohydrates such as glucose, maltose, or soluble starch than from the population in the high-forage-fed animal. Only 18% of the bacterial population in the rumen contents from the high-forage-fed animal grew on the glycerol-DC medium, compared with 60% of the population from the high-grain-fed animal.

Qualitative examinations of colonies having different morphologies were done by randomly picking colonies from each medium and observing them by phase-contrast microscopy. With the cellulose/cellobiose-DC medium, the colonies contained typical coccus- or rod-shaped bacteria resembling ruminococci, butyrivibrios, and bacteroides-type bacteria. In the high-grain diet samples, the majority of the cellulolytic colonies were butyrivibrios as indicated by clearing zones around of the colonies containing cells typical of butyrivibrio-type bacteria. On the various soluble carbohydrate DC media (glucose, maltose, and soluble starch) was a random mix of colony morphologies which represented the bacterial cell types of the predominant rumen bacteria. Lachnospira-type as well as bacteroides-type bacteria predominated on the pectin-DC medium, especially with the high-grain diet samples.

The mannitol-containing medium supported the growth of almost exclusively selenomonad isolates. However, a small percentage of the colonies formed on this medium from the high-grain diet samples were black and consisted of large cocci in singles and pairs. Since we found that some strains of *Megasphaera elsdenii* grew on mannitol, these isolates were also presumed to be *Megasphaera* species. On the rutin-containing medium, the colonies from the high-forage rumen bacterial population were butyrivibrios and bacteroides-type organisms, whereas colonies from the high-grain diet samples were about equally distributed between butyrivibrio- and selenomonad-type isolates. Deposition of the yellow quercetin precipitate was associated with greater than 90% of the colonies growing on the rutin medium.

DISCUSSION

The first major attempt to develop selective or differential media for enumerating rumen bacteria was that by Dehority and Grubb (15). However, in preliminary experiments we found that their medium and its method of preparation had certain drawbacks. Heating of their medium after preincubation resulted in lysis of many of the bacterial cells and a variable release of intracellular products (proteins, nucleic acids, etc.). In addition, the finished medium was quite opaque, making it unsuitable for accurately detecting pinpoint colonies (especially in roll tubes) or faint zones of cellulose or protein hydrolysis surrounding some colonies. We also found that preincubation of the rumen fluid medium without pH adjustment did not lead to a reduction in the level of endogenous carbohydrates (data not shown). Rather, the total carbohydrate level increased, presumably due to release of carbohydrate components from the agar matrix. We believe that our methodology and the resultant array of media used in our studies overcome these drawbacks. While we were completing our experimentation and preparing this paper, Allison et al. (1) published a modification of the Dehority and Grubb procedures and media which were used in enumerating bacterial populations from pig cecal and colon contents. Their modifications would also appear to overcome the first two drawbacks, but not the last one. Albeit improved, the Allison et al. (1) procedure could result in loss of heat-labile components in the rumen fluid which undergoes a double heat treatment, but definitive evidence on this aspect is lacking.

The CC medium was found to be capable of supporting the growth of the predominant rumen bacteria (Table 3). At least as many colonies grew on the CC medium as on other media. The colonies on the BC medium appeared to be limited by the amount of available carbohydrates provided by the ICRF component. This conclusion was supported by total carbohydrate analysis of the ICRF and by the observation that the colonies on the BC medium grew to only pinpoint size, but more than 90% of these colonies increased in size when carbohydrates were subsequently made available. These results are consistent with those reported by Dehority and Grubb (15), who estimated that about 6% of the total number of colonies would grow only as pinpoint colonies on all their selective carbohydrate media. These authors also found that the number of pinpoint colonies on their basal IRA medium was 9 to 12% of the number on their complete GCSX/IRA medium. However, on our own basal (BC) medium, the pinpoint colony counts were 85% or more of the large-size colony counts on the complete (CC) medium (Tables 6 and 7). The basis for these differences in colony

counts on the basal medium is not known.

The consistent observation of higher colony counts on plates than on roll tubes was likely the result of certain differences between the two methods. Specifically, with roll tubes but not plates, the inoculation exposes the bacteria within the inoculum to molten agar and to subsequent rapid cooling. The data (Table 4) indicate that exposure to 47°C results in the loss of viability of some bacteria, and that heat stress in media containing agar (whether at 0.8 or 2.0%, wt/vol) constitutes a more serious heat stress to the bacteria than does heat stress in dilution buffer devoid of agar. There also appears to be no further loss in viability due to the secondary cold stress involved in roll tube preparation, since similar trends in loss of viability occurred in both roll tubes and agar overlays onto plates. Based upon our findings, if roll tubes are to be used, we suggest that the roll tube media be maintained at the lowest temperature possible and that after inoculation the roll tubes be rolled immediately to minimize heat stress and loss of viability.

Another explanation for why plate colony counts were found to be consistently higher than roll tube counts may stem from the fact that surface colonies, particularly pinpoint-sized ones, are more readily observed on a flat surface and hence more readily counted. Using plates rather than roll tubes avoids the necessity of looking through the medium to detect colonies. This aspect is accentuated with roll tubes if the medium is somewhat opaque, as with cellulose or xylan substrate, or if it contains coagulated materials or feed particles. The CC medium used in this study is quite translucent and allows for easy detection of all colony sizes and areas of substrate hydrolysis (e.g., cellulose) that may surround colonies.

Some significant effects of blending (1 min) rumen contents were also found. This treatment resulted in substantial (30% or more) increases in both direct and viable counts of rumen bacteria. The observation that the enumerating efficiencies for both roll tubes and plates remained the same before and after blending suggests that blending, under the conditions specified, is not a major detriment to the viability of the bacterial population. This does not rule out the possibility that some bacterial lysis may occur during blending. The data further suggest that blending may serve to dislodge many bacteria from feed particles or to break up clumps of microflora to increase the number of bacteria that appear free in suspension. Since some major differences were found in the groups of bacteria associated with the liquid and solid portions of rumen contents (Table 6), the deletion of blending during enumeration of rumen bacteria may lead to serious underestimates of both the numbers and types of bacteria present in a given sample.

The data from the specific DC media which reflect the carbohydrate subgroup profiles of the various samples and fractions of rumen contents can only be interpreted to indicate trends. Further work is in progress to evaluate the variation within subgroup profiles with respect to time after feeding and diet fed the animal. However, the present data for our animal fed alfalfa hay indicate that the carbohydrate subgroup profiles for the liquid (sample A) and the solid (sample B) fractions of rumen contents were as expected. The free or unattached bacteria were predominantly soluble carbohydrate utilizers (glucose, soluble starch, and, to a lesser extent, maltose). The attached bacteria, on the other hand, were predominantly those capable of utilizing the substrates in the cellulose/cellobiose- and the xylan/xylose-DC media.

In an attempt to differentiate the truly cellulolytic bacteria from the noncellulolytic ones capable of using cellobiose, the colonies with hydrolysis zones were enumerated. However, zone detection proved difficult since the thickness (8 mm) of the plate medium was too great, and hence the zones were too diffuse. Preliminary experiments indicate that thinner plates (3 to 4 mm) can be used to facilitate hydrolysis zone detection. An additional way to enhance the degree of hydrolysis may be to separate these two carbohydrates and thereby enumerate the true cellulolytic bacteria in the sample by using a cellulose-DC medium. With the xylan/xylose-DC medium, counting the number of colonies with hydrolysis zones was not as difficult. The even opaqueness of the medium was easily cleared by the growth of some of the colonies. The number of colonies detected with zones was quite variable, however, and did not correlate well with the total number of colonies present (therefore the numbers are not reported). These observations were consistent with those of Henning and Van der Walt (19) working with xylan-degrading bacteria from the sheep rumen. In conjunction with their evidence, we believe that the xylan/xylose-DC medium yields a reasonably accurate measure of the number of xylanolytic bacteria in the rumen.

Hydrolysis zones surrounding colonies were also detected with the soluble starch-DC medium if the plates were flooded with Grams iodine solution. Such enumeration could be done only if incubation was for 3 days or less. After 3 days the zones from separate colonies had merged, making accurate enumeration difficult. Shorter incubation times allowed zone detection around only the fast-growing starch-hydrolyzing

bacteria. The 5-day incubation period used in the present experiments was necessary to permit development for counting all colonies present.

Replica plating was used with pure cultures of rumen bacteria with known substrate specificities. The data from these experiments confirm that the DC media can be used as a tool to determine the catabolic capabilities of rumen bacteria with respect to carbohydrate utilization. The 90% positive correlation within experiments suggested that this technique can be used in quick screening procedures for identifying new isolates or mutants in genetic experiments with rumen bacteria. An important implication arose from these replica plating data. Most of the bacterial species tested were isolated 10 to 20 years ago and subsequently maintained in laboratory culture. Yet most species exhibited the same carbohydrate substrate specificities as when they were first isolated and characterized. The data imply that the general patterns of carbohydrate utilization among rumen bacterial species are fairly stable genetic characteristics.

The DC media were used to compare carbohydrate subgroup profiles in rumen contents of a high-forage-fed animal and a high-grain-fed animal. These diets were chosen as extremes in order to exaggerate possible differences in carbohydrate subgroup composition. As expected from the total cell counts observed in previous studies (8, 10), both the total number of colonies (CC medium) and direct cell counts were found to be much higher in the high-grain-fed animal than in the high-forage-fed animal at 3 h postfeeding (Table 7). The resident rumen population on the high-forage diet reflected the higher proportion of cellulosic materials and the lower amount of readily digestible feedstuffs, since the cellulolytic and xylanolytic bacteria were the predominant groups. However, the colonies on the soluble starch-DC medium were numerically greater than either of these groups within a given diet and reflected the general capacity of many rumen bacteria either to ferment or hydrolyze starch or to utilize its breakdown product, maltose. With the high-grain diet, the rumen bacterial population was predominantly soluble carbohydrate utilizers: the colonies on glucose, soluble starch, and maltose media were 86, 81, and 70% of the total colony count, respectively. Far fewer bacteria (35%) were to develop colonies on the cellulose/cellobiose-DC medium. Although the animals, diets, and sampling times were different, our results are consistent with the data obtained for total cell counts and by characterization of randomly picked isolates in previous studies (7, 8, 10, 17, 26, 27).

The replica plating data with pure cultures indicated that some strains of *Megasphaera* and *Selenomonas* were able to use glycerol for growth. Since these bacteria are known to be among the predominant microflora in animals fed high-grain diets (7, 11), it was not unexpected that the percentage of the populations utilizing glycerol increased from 18% on the high-forage diet to 60% on the high-grain diet. The same trend was observed for utilization of pectin, which is a substrate used by *Bacteroides ruminicola* as well as by butyrivibrios, succinivibrios, and lachnospiras (4, 11, 14, 23). However, these two trends were not observed by Dehority and Grubb (15) with sheep fed orchardgrass or orchardgrass plus cracked corn. The number of colonies on the mannitol-containing medium was only 5% of the total number of colonies in the high-forage rumen sample, yet 51% of the total colonies in the high-grain sample grew on the mannitol medium. By random picking of colonies, these were found to contain bacteria of morphologies representative of selenomonads (as expected), although some colonies contained cells typical of butyrivibrios and megasphaeras. Lastly, the rutin medium supported about five times as many colonies (primarily butyrivibrios) from the high-grain diet samples than from the high-forage samples.

In summary, the complete (CC) medium was found to be totally satisfactory for accurately enumerating rumen bacteria. Whether used with roll tubes or with plates, the CC medium gave colony counts equal to previously found with established media (Table 3). The CC medium was found to support the growth of at least 20 major species of rumen bacteria. The replica plating data obtained with colony formations by pure cultures on various media together with differences in subgroup profiles observed between the solid and liquid fractions of rumen content samples (Table 6), or between rumen contents samples of animals fed different diets (Table 7), clearly indicate that the differential media can be used to separate the mixed rumen population into catabolic subgroups on the basis of carbohydrate utilization. Plates were consistently found to be superior to roll tubes, yielding consistently higher colony counts (Tables 3, 4, and 5). In addition, we found that with appropriate modifications, the replica plating techniques commonly used with aerobic bacteria can be used with anaerobic rumen bacteria. Finally, the media and methodologies used in our studies need not be restricted to use with rumen contents. With modifications, these techniques could be applied to studies on anaerobic bacteria in other portions of the gastrointestinal tract (e.g., cecum, large intestine), in other animals (e.g., horses, pigs, raccoons, squirrels, humans, fish), or in anaerobic sediments.

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