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Nutritional Features of the Intestinal Anaerobe *Ruminococcus bromii*

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Of six strains of *Ruminococcus bromii* studied, five grew in a minimal chemically defined medium containing minerals, NH_4^+ as nitrogen source, sulfide or sulfate as sulfur source, fructose as energy and carbon source, isobutyrate or 2-methylbutyrate and carbonic acid-bicarbonate as additional carbon sources, and the vitamins biotin, riboflavin, pyridoxine, vitamin B_{12} (replaced by L-methionine), pantethine, and tetrahydrofolate. The strains also could utilize cysteine or thiosulfate but not methionine; and strain Z3 failed to use dithiothreitol, thioglycolate, sulfite, or β-mercaptoethanol as sole sources of sulfur. Mixtures of amino acids, peptides (Casitone), urea, nitrate, asparagine, or glutamine failed to replace NH_4^+ as N source. Three strains isolated from Americans were identical in nutritional features, whereas one from a Japanese and one from a South African native differed slightly in having requirements for fewer vitamins. One strain from the cecum of a sow grew well in a rumen fluid-supplemented medium but not in the various chemically defined media plus Casitone. The nutritional features suggest that the environment which selects *R. bromii* contains relatively little amino acid nitrogen and a relatively large amount of NH_4^+-N and indicate that these bacteria must depend upon other bacteria such as those that produce NH_4^+ from urea or protein and those that produce branched-chain volatile acids to grow.

Detailed information on the nutritional characteristics of representative, important microbial species of a given ecosystem is essential to an adequate qualitative understanding of the ecology, taxonomy, and metabolism of the species and gives important clues to the chemical nature of the environment which has selected the species. Nutritional information may also be valuable in the development of specific selective cultural media for use in isolation, enumeration, and genetic studies of given species.

Although a large amount of nutritional information is available concerning anaerobic species of the rumen ecosystem (4, 6, 16), nutritional studies on the major anaerobic species of the intestinal tract has only just begun (12, 17). In fact, the species of anaerobes of greatest importance in ecosystems, such as the large bowel, are only beginning to be understood (14).

Ruminococcus bromii is of particular ecological interest, because it is among the most numerous species in human and swine feces, yet it was not detected until workers initiated studies using special anaerobic techniques and media developed originally for study of rumen anaerobes (13, 14; see also group 10 of Eller et al. [9] and Jennifer Gossling, Ph.D. thesis, West Virginia Univ., Morgantown, 1973). This organism was probably not found by other workers because of failure to use adequate anaerobic techniques or to include an energy source used by *R. bromii* in the isolation medium. It does not utilize amino acids, and usually not glucose, as an energy source but ferments starch and maltose and, usually, fructose. It was also considered possible that it required growth factors present in rumen fluid or fecal extract but of limited occurrence in some other more commonly used crude nutrient sources (13).

In the present study, we describe chemically defined and minimal media and indicate some nitrogen and sulfur sources utilized for growth of *R. bromii*.

MATERIALS AND METHODS

Except for *R. bromii* strain Z3, which was isolated by Eller et al. (9), the strains studied, Virginia Polytechnic Institute (VPI) 0851, VPI 6833, VPI J3-2A, and VPI S6B-47, were obtained through the courtesy of the Anaerobe Laboratory, Virginia Polytechnic Institute. Most of the work was conducted with strain Z3.

The anaerobic culture methods used were those described by Hungate (11) with modifications (3).

Inocula for the experimental media were prepared as follows. A stab culture, grown on rumen fluid-glucose-cellobiose-starch agar slants (6) and stored in the refrigerator for 1 day to 2 weeks, was stab inoculated into fresh slants. After 18 to 24 h of incubation at 37 C, the fresh culture was transferred into tubes containing 5 ml of the basal defined medium shown in Table 1 supplemented with 40% (vol/vol) clarified rumen fluid (8), which was found to contain other essential factors for growth. After 18 to 24 h of growth, one 4-mm platinum loop of this culture (about 0.01 ml) was inoculated into duplicate tubes (13 by 100 mm) containing 5 ml of experimental medium. In an experiment done to determine sulfur sources, media were used without an added reducing agent such as cysteine or sulfide, inoculum was 0.1 ml per tube so that reducing materials generated by the inoculum culture reduced the medium, and serial transfers of the cultures were made in the experimental media to dilute out possible sulfur sources carried over with the inoculum.

Growth was estimated as optical density at 600 nm with a Bausch and Lomb spectronic-20 colorimeter. Each value given is a mean for duplicate tubes of medium. Culture purity was checked periodically by wet-mount observations with the phase-contrast microscope.

The medium listed in Table 1 was used for all experimental media with variations as indicated. The media and all solutions were prepared as previously described (3, 5, 6). Heat labile compounds, including urea, pantethine, and tetrahydrofolic acid, were filter-sterilized, aseptically tubed with CO_2 gas phase in 5-ml amounts, and stored in the refrigerator. They were added to previously autoclaved media before it was tubed.

TABLE 1. *Composition of the basal medium for studies on the nutrition of Ruminococcus bromii*

Components	Percentages	
Fructose	0.5	(wt/vol)
Mineral solution 3[a]	5.0	(vol/vol)
Hemin solution	0.0002	(wt/vol)
Resazurin	0.0001	(wt/vol)
VFA solution[b]	0.3	(vol/vol)
B-vitamin solution[c]	0.5	(vol/vol)
$FeSO_4 \cdot 7H_2O$	0.0004	(wt/vol)
$(NH_4)_2SO_4$ (120 mM)	5.0	(vol/vol)
Casitone (Difco, vitamin-free)	0.2	(wt/vol)
Cysteine hydrochloride-water (2.5% solution)	2.0	(vol/vol)
Sodium carbonate (8% solution)	5.0	(vol/vol)
CO_2 gas phase (pH 6.7)		

[a] Mineral solution 3 contained per liter: 18 g of KH_2PO_4; 18 g of NaCl; 0.53 g of $CaCl_2 \cdot 2H_2O$; 0.4 g of $MgCl_2 \cdot 6H_2O$; 0.2 g of $MnCl_2 \cdot 4H_2O$; and 0.02 g of $CoCl_2 \cdot 6H_2O$.

[b] Volatile fatty acid solution contained 36 ml of acetic acid, 14.8 ml of propionic acid, 10.6 ml of butyric acid, 1.8 ml of isobutyric acid, and 2.0 ml each of *n*-valeric acid, DL-2-methylbutyric acid, and isovaleric acid.

[c] B-vitamin solution contained per 100 ml: 20 mg each of thiamine hydrochloride, calcium-D-pantothenate, nicotinamide, riboflavin, and pyridoxine hydrochloride; 1 mg of *p*-aminobenzoic acid, 0.25 mg each of biotin and folic acid, and 0.1 mg of vitamin B_{12}.

RESULTS

Five of the six strains studied grew well in a chemically defined medium containing fructose, minerals, CO_2-bicarbonate, cysteine, biotin, riboflavin, pyridoxine, vitamin B_{12}, tetrahydrofolate, pantethine, isobutyric acid, and methionine (Table 2). Strains Z3, 6833, and S6B47 were identical in all growth requirements studied and all of the B-vitamins or related factors indicated above were essential. Other experiments showed that the vitamin B_{12} requirement of these three strains is replaced by methionine, as in *Bacteroides fragilis* (17) and many other bacteria (12), and that the tetrahydrofolate and pantethine requirements were not replaced by folate and pantothenate, respectively. Strain J3-2A differed from these three strains in that pantethine (or pantothenic acid) and pyridoxine were not required and the vitamin B_{12} requirement was not replaced by methionine. Strain A2-6 differed in that neither pantethine (or pantothenic acid), riboflavin, nor vitamin B_{12} was required. Strain 0851 grew well in the inoculum medium which contained rumen fluid but failed to grow in any of the chemically defined media (Table 2), or in media containing constituents shown in Table 1 plus pantethine and tetrahydrofolate. Further studies to determine the nature of the factor(s) in rumen fluid required by this strain were not done.

Other studies showed that isobutyric acid was essential for growth of four of the five strains which grew in defined media and was stimulatory to the other strain A2-6. The requirement was not replaced by a mixture of amino acids or Casitone as it is in some organisms (6). Further studies with Z3 (Fig. 1) showed that approximately 0.05 mM isobutyrate was required for optimal growth yield and that DL-2-methylbutyrate replaced the requirement for isobutyrate. These results also suggest that isovalerate can be used in place of the other acids; however, a two- or threefold larger amount was required. Studies with *Ruminococcus albus* (1) and *Bacteroides succinogenes* (20) showed that these bacteria have a branched-chain fatty acid requirement satisfied by either 2-methylbutyrate or isobutyrate, and that activity found in commercial isovalerate samples is often due to contamination with D-2-methylbutyrate.

TABLE 2. *Effect of vitamins and methionine on growth of six strains of R. bromii in a chemically defined medium*

Ingredient deleted[a]	Strain[b]				
	Z3	6833	S6B47	J3-2A	A2-6
None	0.75 (31)	0.69 (40)	0.81 (31)	0.75 (24)	0.50 (60)
Biotin	0.11 (96)	0.25 (94)	0.09 (18)	0.04 (30)	0
Pantethine	0	0	0	0.88 (20)	0.60 (50)
Tetrahydrofolate	0.04 (26)	0	0	0	0
B_{12} and methionine	0	0.01 (96)	0	0.05 (60)	0.69 (50)
B_{12}	0.90 (24)	0.74 (40)	0.82 (24)	0	0.81 (45)
Riboflavin	0.03 (24)	0	0	0	0.71 (50)
Pyridoxine	0.05 (68)	0	0	0.95 (24)	0.45 (73)

[a] The complete defined medium contained fructose, mineral 3, $(NH_4)_2SO_4$, $FeSO_4$, CO_2, carbonate, cysteine, biotin, riboflavin, vitamin B_{12}, and pyridoxine, as indicated in Table 1, and contained further additions of tetrahydrofolate (0.09 μg/ml), pantethine (0.5 μg/ml), 0.5 mM L-methionine, and a 0.05 mM concentration each of isovaleric, DL-2-methylbutyric, and isobutyric acids.

[b] Strain 0851 failed to grow in any of the defined media. Values expressed as optical density. Numbers in parentheses indicate hours required to reach maximal optical density on the second serial transfer.

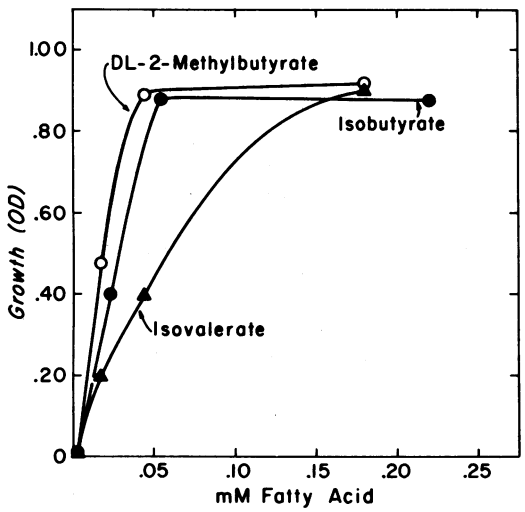

FIG. 1. *Growth response of strain Z3 R. bromii to different levels of branched-chain volatile fatty acids in a basal culture medium as given in Table 1 except that hemin and Casitone were deleted; pantethine, tetrahydrofolate, and methionine were added; and volatile acids were added as indicated.*

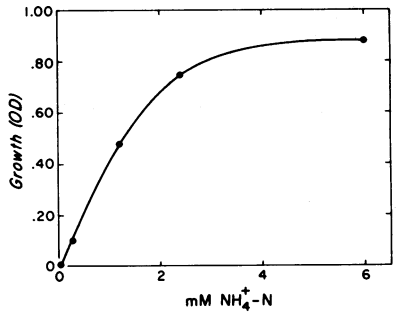

FIG. 2. *Growth response of strain Z3 R. bromii to NH_4^+-N in a low-nitrogen basal medium with a composition like that shown in Table 1, except that hemin and Casitone were deleted, pantethine and tetrahydrofolate were added, and $(NH_4)_2SO_4$ was added as indicated.*

Whether the present sample of isovalerate had similar contamination was not determined.

Results in Fig. 2 and Table 3 show that strain Z3 grew well with NH_4^+ as the nitrogen source and that free amino acids or peptides were not effectively utilized. The growth obtained when Casitone or Casamino Acids were added to an otherwise low-nitrogen basal medium (Table 3) was undoubtedly due to NH_4^+ contamination. Based on the growth response of *R. bromii* on these products compared to limiting levels of ammonia-N (Fig. 2), the NH_4^+ contamination would be equal to only about 3% or less of the total N present. Other results indicate that the other strains capable of growth in defined media have nitrogen requirements identical to those indicated above for strain Z3. In further experiments, all strains failed to grow in a medium containing a mixture of free L-amino acids (17) in similar proportions to those present in casein and added to give a final concentration of 41 mM amino acid-N, and growth yields in this medium with NH_4^+ added were essentially identical with or without the amino acid mixture. In an experiment involving strain Z3 only, neither urea, NO_3^-, NO_2^-, asparagine, nor glutamine-N were utilized as sole source of nitrogen in place of NH_4^+.

Results in Table 4 show that all of the strains capable of growth in defined media have similar sulfur requirements. They utilized sulfide, sul-

fate, thiosulfate, and cysteine as sole sulfur sources. Methionine, as indicated in the studies in which it replaced vitamin B_{12}, was utilized but did not serve as sole sulfur source. In another experiment with the same basal medium except that 1 mM dithiothreitol was added as a reducing agent, the above results with strain Z3 were confirmed, and it was further shown that sulfite, thioglycolate, β-mercaptoethanol, and dithiothreitol were not utilized as sole sources of sulfur. The inability of the organism to utilize sulfite seems peculiar in that sulfate is used. One can speculate that a transport system for sulfite is absent in *R. bromii*.

DISCUSSION

The results demonstrate that many strains of *R. bromii* can be grown in a relatively simple minimal chemically defined medium containing minerals, NH_4^+ as N source, sulfide or sulfate as sulfur source, fructose as energy and carbon source, isobutyrate or 2-methylbutyrate and carbonic acid-bicarbonate as additional carbon sources, and the vitamins biotin, riboflavin, pyridoxine, vitamin B_{12}, pantethine, and tetrahydrofolate.

The strains were selected for study on the basis of diversity of isolation source, and it is of interest that the three strains which were identical in nutritional features were all isolated from Americans (Z3 from a man in Ill., and 6833 and S6B47 from women in Va.). Strain J3-2A, isolated from a Japanese man, and A2-6, from a South African native in the bush, were somewhat different in their patterns of vitamin requirements. The one strain from a non-human source, i.e., strain 0851 from the cecum of a hog, failed to grow in the media not containing rumen fluid. Further studies to establish the nutrient requirements of this strain and others from non-human and human sources should be of interest.

TABLE 3. *Effect of ammonia, amino acids (Casamino Acids), and peptides (Casitone) as nitrogen sources on growth of strain Z3 R. bromii*

Additions of the basal[a]	Maximal growth[b]
None	0.02 (48)
6 mM NH_4^+-N	0.93 (41)
6 mM NH_4^+-N + 17 mM Casitone-N	0.97 (35)
17 mM Casitone-N	0.18 (30)
6 mM NH_4^+-N + 17 mM Casamino Acids-N	0.87 (35)
17 mM Casamino Acids-N	0.23 (30)

[a] As given in Table 1 plus 0.5 μg of pantethine per ml and 0.09 μg of tetrahydrofolate per ml with changes in $(NH_4)_2SO_4$ and Casitone as indicated above. The 17 mM Casitone-N or Casamino Acids-N was calculated on the assumption that these products contain about 12% nitrogen, i.e., 0.2% was added to the media.

[b] Values expressed as optical density. Numbers in parentheses indicate hours required to reach maximal growth.

The nutritional features of this bacterium, which is one of the most numerous intestinal organisms, are surprisingly similar to those of rumen organisms such as *R. albus*, an important cellulolytic bacterium. The latter organism usually requires 2-methylbutyrate or isobutyrate (1, 4). NH_4^+ is essential as the main nitrogen source, and nitrogen from materials such as amino acids, peptides, nitrate, and urea are not effectively used (4, 5). *R. albus* also usually requires biotin and pyridoxine, but differs from *R. bromii* in requiring *p*-aminobenzoic acid but usually not tetrahydrofolate (or folate) or pantethine (or pantothenic acid) (4). *R. albus*, of course, requires different energy sources such as cellulose or cellobiose. Another anaerobe which is perhaps of intestinal origin, *Fusobacterium symbiosum*, also requires pantethine (12, 15).

The fact that *R. bromii* very effectively utilizes NH_4^+ nitrogen but not nitrogen of amino acids or peptides suggests that NH_4^+ is the main nitrogen source available for its growth in the intestinal or cecal environment, i.e., there is probably little survival value in the ability to efficiently utilize amino acids. It is of interest that many rumen bacteria (6, 7) also lack the ability to effectively use exogenous amino acid nitrogen. *R. bromii* is probably dependent upon other bacteria in the tract which produce NH_4^+ from proteinaceous materials, and upon organisms such as the urease-forming *Peptostreptococcus productus* (18) which produce NH_4^+ from urea (19).

It probably also depends upon organisms such as *B. fragilis*, which produce the required branched-chain volatile fatty acids from branched-chain amino acids present in proteins. This is similar to the interaction in the rumen in which organisms such as *Bacteroides ruminicola* produce the branched-chain volatile acids required by organisms such as *R. albus* and *B. succinogenes* (2).

The ability of *R. bromii* to utilize sulfate as sulfur source is of interest. This is done via an assimilatory rather than a dissimilatory sulfate reducing process, as no sulfide can be detected in cultures grown in media containing sulfate (9, 13). There is little documentation of assimilatory types of sulfate reducing bacteria among major, strictly anaerobic saccharoclastic bacteria of the gastrointestinal tract. *Lachnospira multiparus* utilizes sulfate (10) but other

TABLE 4. *Effect of various sulfur sources on the growth response of strains of R. bromii in a sulfur-free basal medium*

Additions to basal[a]	Strain[b]				
	Z3	6833	S6B47	J3-2A	A2-6
1 mM Na_2S	1.0 (11)	0.91 (11)	1.0 (11)	0.89 (21)	1.07 (35)
1 mM Na_2SO_4	0.95 (13)	0.79 (25)	0.92 (20)	0.91 (20)	1.00 (20)
1 mM cysteine	0.82 (13)	0.72 (20)	0.75 (13)	0.99 (13)	0.88 (30)
1 mM thiosulfate	0.71 (43)	0.80 (20)	0.73 (35)	0.83 (25)	0.71 (10)
1 mM methionine	0	0	0	0	0
None	0	0	0	0	0

[a] The sulfur-free basal medium was as indicated in Table 1 but with Casitone, cysteine, and hemin deleted; with equimolar NH_4Cl-N and $FeCl_2$ replacing $(NH_4)_2SO_4$-N and $FeSO_4$; and with additions of pantethine and tetrahydrofolate.

[b] Results expressed as maximal optical density (and hours of incubation) after three serial 0.1-ml transfers.

rumen bacteria so far studied and *B. fragilis* do not (17). Most of these bacteria very effectively utilize sulfide, and sulfide-producing dissimilatory sulfate reducers are found in the rumen (M. R. Bennink and M. P. Bryant. Abstr. XII, Conf. Rumen Function., 1973). Further studies on sulfate-reducing bacteria of the bowel would be of interest. It is possible that addition of sulfate as sole sulfur source would have utility in the development of a selective medium for isolation of *R. bromii*.

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