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Nutritional Features of *Bacteroides fragilis* subsp. *fragilis*

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Studies of three reference strains of *Bacteroides fragilis* subsp. *fragilis* showed that they grow well in a minimal defined medium containing glucose, hemin, vitamin B_{12}, minerals, bicarbonate-carbon dioxide buffer, NH_4Cl, and sulfide. The vitamin B_{12} requirement of 0.1 ng/ml was replaced with 7.5 μg of methionine. Cysteine or sulfide was an excellent source of sulfur, thioglycolate was a poor source, and thiosulfate, methionine, β-mercaptoethanol, dithiothreitol, sulfate, or sulfite did not serve as sole sources of sulfur. Neither single amino acids, nitrate, urea, nor a complex mixture of L-amino acids or peptides effectively replaced ammonia as the nitrogen source. Comparative studies with a few strains of other subspecies of *B. fragilis* including *B. fragilis* subsp. *vulgatus*, *B. fragilis* subsp. *thetaiotaomicron*, and *B. fragilis* subsp. *distasonis* indicate that they exhibit similar growth responses in the minimal medium. A single strain of *B. fragilis* subsp. *ovatus* required other materials. The results indicate the great biosynthetic ability of these organisms and suggest that, in their ecological niche within the large intestine, many nutrients such as amino acids are in very low supply, whereas materials such as ammonia, heme, and vitamin B_{12}, or related compounds, must be available during much of the time.

One of the predominant organisms in the normal flora of the large intestine of man and other animals is the gram-negative, nonsporeforming, anaerobic, nonmotile rod, *Bacteroides fragilis* (7). Recent studies of more than 300 strains of *B. fragilis* indicate that there is a continuum of variants (8). Many intermediate strains do not conform to any of the combination of reactions described in the literature for this organism. However, analysis of certain sets of characteristics showed clusters of strains within the species and these clusters have been designated subspecies. The *Anaerobe Laboratory Manual* (8) lists five subspecies of *B. fragilis*: *B. fragilis* subsp. *fragilis*, *B. fragilis* subsp. *distasonis*, *B. fragilis* subsp. *ovatus*, *B. fragilis* subsp. *thetaiotaomicron*, and *B. fragilis* subsp. *vulgatus*. Additional study of these subspecies and strains, which fit none of them, is needed to determine whether their separation is adequate (10).

In a study of the growth requirements of *B. fragilis*, in which the source of the seven strains was not given and identification was not documented, Tamimi et al. (21) reported that they failed to grow in a defined medium containing 20 pure amino acids, vitamins, purines, pyrimidines, glucose, and trace elements. Hemin was not included in the study, and supplementation of the medium with sodium thioglycolate was necessary for good growth. Quinto (17, 18) and Quinto and Sebald (19) showed that three strains of *Ristella pseudoinsolita*, which is now considered synonymous with *B. fragilis* (1), had very simple nutrient requirements in that they could be grown in a medium containing only glucose, minerals, hemin, bicarbonate, carbon dioxide, and various amino acids, none of which was essential. Great discrepancies between the two studies are apparent. A nutritional study of well-characterized strains of the various subspecies was warranted, and information generated may be of value in relationship to their taxonomy and identification and may help generate information relative to the chemical environment and ecology of the intestinal tract which has selected them.

The present study was initiated to determine the minimal nutrient requirements of three strains of *B. fragilis* subsp. *fragilis*, one strain, NCTC 9343, being the proposed neotype of the species and genus. A comparison of the nutritional features of this subspecies to the other subspecies of *B. fragilis* was also investigated.

MATERIALS AND METHODS

The following strains of *B. fragilis* were supplied by the Anaerobe Laboratory, Virginia Polytechnic Institute (VPI), Blacksburg; *B. fragilis* subsp. *fragilis* NCTC 9343 (VPI 2553); VPI 2044 and 2552; *B. fragilis* subsp. *vulgatus* ATCC 8482 (VPI 4245) and VPI 0980-1; *B. fragilis* subsp. *thetaiotaomicron* NCTC

10582 (VPI 5482); *B. fragilis* subsp. *distasonis* ATCC 8503 (VPI 4243) and VPI 0434; *B. fragilis* subsp. *ovatus* VPI 0916. Strain H of *B. fragilis* subsp. *fragilis* (13) was obtained from Ronald Gibbons, Forsythe Dental Clinic, Boston. *B. ruminicola* subsp. *ruminicola* strain 23 and *B. amylophilus* strain H18 were from the culture collection of the Microbiology Division of this Department. Strain H18 was originally obtained from P. N. Hobson, Rowett Research Institute, Aberdeen, Scotland.

The anaerobic culture techniques used were similar to those described by Hungate (9) as modified by Bryant (2).

B. fragilis strains were maintained on rumen fluid-glucose-cellobiose-starch-agar slants as indicated by Bryant and Robinson (4). The inoculum medium, similar to that used in the study of *B. ruminicola* (15), contained the components shown in Table 1. It was prepared as previously described (15) and was tubed in 5-ml amounts (13 by 100 mm tubes). The various experimental media were modifications of the inoculum medium, and specific compositions are given for each experiment in the results section. All components except carbonate and cysteine were added before autoclaving the medium unless otherwise indicated.

Inocula for experimental media were prepared as follows. A stab culture, grown on rumen fluid-glucose-cellobiose-starch-agar slants and stored in a refrigerator for 1 day to 2 weeks, was stab-inoculated into a slant of the same medium. After overnight growth, this fresh culture was transferred by loop to the inoculum medium. After 10 to 24 h of growth, one 4-mm platinum loop of this culture (about 0.01 ml) was inoculated into each tube of experimental medium. Growth was recorded as optical density (OD) using a Spectronic 20 colorimeter (Bausch and Lomb) set at 600 nm. When serial transfers were to be made in experimental media to test for nutrient carry-over, the culture was allowed to proceed to OD 0.90 to 1.10 before it was transferred to a medium of the same kind. If a culture did not reach this value, it was transferred when growth approached the stationary phase.

Culture purity was checked periodically by observation of wet mounts, Gram strains, and occasionally by inoculation into tubes of Trypticase Soy Agar (BBL) with 0.5% glucose added, incubated under aerobic conditions. The medium will support the growth of the usual contaminant but not of the obligate anaerobes such as *B. fragilis*.

Most of the NH_4^+ was removed from a 5% vitamin-free Casitone solution by placing 100 ml in a Dow Hollow Fiber Beaker Dialyzer (Bio-Rad Laboratories). Hydrostatic pressure was adjusted to obtain a flow of 58 ml of distilled water per min through the fibers, and dialysis was continued for 30 min. The casein solution was then autoclaved at 15 psi for 15 min. The NH_4^+ content of the casein solution dropped from 5.37 mM to 1.06 mM, and the total nitrogen from 399 mM to 241 mM. It was added to experimental medium to give 30 mM total nitrogen (0.13 mM NH_4^+) of a low NH_4^+ peptide source. NH_4^+ was determined by combining the Conway microdiffusion technique as modified by Obrink (14) with a Nessler colorimetric method (22). Total nitrogen was determined by the method of Johnson (11).

TABLE 1. *Composition of the inoculum medium for studies on the nutrition of Bacteriodes fragilis subsp. fragilis*

Components	Percentages	
Glucose	0.5	(w/v)
Mineral solution 3[a]	5.0	(v/v)
Hemin solution	0.0001	(w/v)
Resazurin solution	0.0001	(w/v)
VFA solution[b]	0.45	(v/v)
B-vitamin solution[c]	0.5	(v/v)
$FeSO_4 \cdot 7H_2O$	0.0004	(w/v)
$(NH_4)_2SO_4$ (6 mM)	5.0	(v/v)
Casitone (Difco, vitamin-free)	0.2	(w/v)
Cysteine·HCl·H_2O (2.5% solution)	2.0	(v/v)
Sodium carbonate (8% solution)	5.0	(v/v)
CO_2 gas phase		

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

[b] Volatile fatty acid (VFA) solution contained 36 ml of acetic 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 thiamin-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}.

The amino acid mixture contained: 25 mg each of L-histidine-hydrochloride, L-tryptophan, glycine, and L-tyrosine; 50 mg each of L-arginine-hydrochloride, L-phenylalanine, L-methionine, L-threonine, and L-alanine; 75 mg each of L-lysine, L-serine, L-valine, L-isoleucine, L-proline, and L-aspartic acid; 100 mg of L-leucine; and 224 mg of L-glutamic acid. The mixture was dissolved in 50 ml of distilled water and added to medium at a concentration of 20% (v/v) (about 41 μmol of nitrogen per ml).

RESULTS

Deletions from inoculum medium. The effects of various deletions of ingredients from the inoculum medium on growth of three strains of *B. fragilis* subsp. *fragilis* were initially determined. Results for strain 9343, from experiments done on each strain and in which the results were similar, are shown in Fig. 1. These results from data collected after the first transfer from the inoculum medium, plus further data on growth of the three strains after four serial transfers on each of the media (data not shown), indicated that the volatile fatty acid mixture was not necessary and was sometimes slightly inhibitory (medium 3 versus 1). In further experiments, the mixture was deleted. B

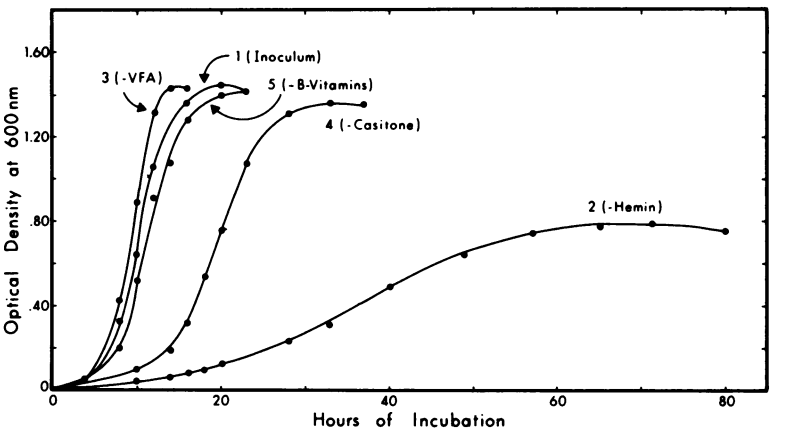

FIG. 1. *Growth of strain 9343 Bacteroides fragilis subsp. fragilis in the inoculum medium and in media minus certain ingredients of the inoculum medium. Medium 1 is the complete inoculum medium as given in Table 1, 2 is minus hemin, 3 is minus VFA, 4 is minus Casitone, and 5 is minus B vitamins. OD determinations represent the means of three 13 by 100 mm tubes read at 600 nm.*

vitamins were not essential (medium 5 versus 1) but were sometimes slightly stimulatory, especially to strain 2552. Hemin was not shown to be essential, even after four serial transfers, but growth without it was very poor (OD 0.45 to 0.70 in 94 to 100 h). Casitone was somewhat stimulatory, but the organisms grew very well without it (medium 4 versus 1). It is evident that these organisms grow well in a defined medium with ammonia serving as the main possible nitrogen source.

Methionine substitution for vitamin B_{12}. When the B-vitamin solution and Casitone were both deleted from the inoculum medium, no growth occurred. In an experiment involving single deletions of B vitamins from the inoculum medium without Casitone, vitamin B_{12} was the only vitamin shown to be essential. The minimum amount of B_{12} required for good growth (OD 1.10 in 20 h), as determined with strain 9343, was 0.075 to 0.10 ng/ml, whereas about 0.04 ng/ml was required for half-maximal growth. A further experiment showed that L-methionine would replace Casitone or vitamin B_{12} in the growth of these strains (Table 2), as it does for many other organisms. In the absence of B_{12}, the strains required about 7.4 μg of methionine per ml for optimal growth, whereas about 2.5 μg/ml resulted in about half-maximal growth.

White et al. (25) showed that other methyl donors such as betaine, choline, and dimethylacetothetin will replace the methionine requirement in *Pseudomonas denitrificans*. When the above compounds, *S*-adenosyl-methionine, or DL-homocysteine were added (0.15 mM) in place of methionine, they proved to be ineffective. Whether vitamin B_{12}-like factors will replace vitamin B_{12} was not studied.

TABLE 2. *Growth of strain 9343 of Bacteriodes fragilis subsp. fragilis in media with and without Casitone, methionine, vitamin B_{12}, and vitamin solution*

Additions to basal medium[a]	Growth[b] (OD)
None	0.01 (106)
B_{12}	1.22 (20)
Vitamin solution	1.19 (20)
Methionine	1.17 (32)
B_{12} + methionine	1.20 (24)
Casitone	1.34 (14)
B_{12} + Casitone	1.37 (14)

[a] The basal medium was the same as the inoculum medium given in Table 1 except that the Casitone, volatile fatty acid, and vitamin solutions were deleted. Additions were as indicated above and in concentrations as in the inoculum medium, except for methionine which was added at a concentration of 0.5 mM.

[b] Growth is indicated as the mean maximal OD of three tubes after three serial transfers. Numbers in parentheses indicate hours of incubation required to reach the maximal OD. Similar results were obtained with strains 2552 and 2044.

Nitrogen sources. Results in Fig. 2 show that NH_4^+ serves as the source of nitrogen and that growth-limiting concentrations are between about 0 and 5 to 6 mM.

The strains showed little ability to utilize the nitrogen of a complex mixture of free amino acids, although strain 2552 grew to a very limited extent (Table 3). These results also show that these strains have a much more limited ability to utilize the nitrogen of peptides (low-NH_4^+ Casitone) than does *B. ruminicola*,

which is well documented (15, 16) as an organism which very effectively utilizes oligopeptides or NH_4^+ but not free amino acids. *B. ruminicola* and *B. amylophilis* were included as controls to show that the media contained the correct ingredients. Note that the latter organism requires ammonia and utilizes no other nitrogen sources (4, 5). In confirmation of the relatively poor ability to use the nitrogen of peptides, growth equal to about 20% or less of that obtained with equivalent NH_4^+-nitrogen was obtained with strains 2044 and 9343 when gelatin, casein, or Trypticase (2 or 5 mM nitrogen level) was the nitrogen source. Strain 2552 grew slightly better, especially on Trypticase which allowed growth equal to about 50% of that of NH_4^+. Urea, nitrate, or single amino acids (those indicated in the mixture), added at a 2 mM level, were not utilized as nitrogen source.

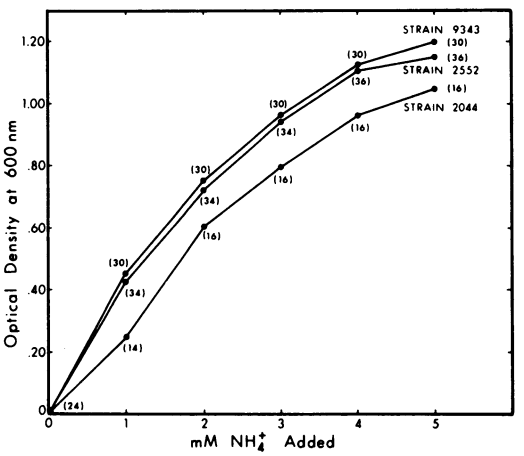

FIG. 2. *Growth response of strains of Bacteroides fragilis subsp. fragilis to limiting levels of ammonia. The basal medium used is the same as the inoculum medium (Table 1) except that Casitone, VFA solution, and ammonium sulfate solution were omitted. The values plotted are the means of three replicate tubes and the numbers in parentheses are the hours required to reach maximal OD.*

Sulfur sources. By replacing $(NH_4)_2SO_4$ and $FeSO_4$ in the inoculum medium with equimolar NH_4Cl and $FeCl_2$ and deleting the cysteine and Casitone, a sulfur-free basal medium was obtained. Dithiothreitol (Calbiochem) was added as a reducing agent, not used as a sulfur source, and various sulfur compounds were tested for their ability to serve as the sole source of sulfur. Cysteine and sulfide each were excellent as sole sources of sulfur (Table 4). Equimolar sodium thioglycolate allowed only about one-third the growth yield and required a much longer incubation time (two to three times) when compared to either cysteine or sulfide and methionine, β-mercaptoethanol, sulfite, or sulfate were not used. A significant amount of growth was noted with $Na_2S_2O_3$, but it was later learned that some thiosulfate is converted to sulfide in the presence of dithiothreitol (20). Therefore, $Na_2S_2O_3$ is probably not used as a sole sulfur source for these strains.

Results on the level of the sulfur requirement using the basal medium of Table 4 and cysteine as the sulfur source showed that the strains required about 0.8 mM sulfur for optimal growth yield and about 0.35 mM for half-maximal yield. These levels are about one-seventh of the nitrogen requirement (Fig. 2).

Other subspecies of B. fragilis. Strains of *B. fragilis* subsp. *vulgatus*, *B. fragilis* subsp. *thetaiotaomicron*, and one strain of *B. fragilis* subsp. *distasonis* exhibited the same growth pattern as *B. fragilis* subsp. *fragilis* (Table 5).

TABLE 3. *Comparison of growth with strains of Bacteroides fragilis, B. ruminicola strain 23, and B. amylophilus strain H18 in a basal medium to which various nitrogen sources were added*

Nitrogen source added[a]	*B. amylophilus*	*B. ruminicola*	*B. fragilis*		
			2552	2044	9343
None	0 (168)	0.01 (48)	0.05 (96)	0.02 (16)	0.02 (40)
Casitone + NH_4^+	0.72 (16)	1.22 (16)	1.15 (16)	1.05 (16)	1.10 (16)
NH_4^+	0.66 (16)	1.20 (24)	1.15 (16)	1.10 (16)	1.10 (16)
Low-NH_4^+ Casitone	0 (168)	1.30 (24)	0.70 (24)	0.37 (40)	0.72 (72)
Amino acid mixture	0 (168)	0 (168)	0.30 (168)	0 (168)	0 (168)

[a] The basal medium was similar to that given for the inoculum medium (Table 1) except that $(NH_4)_2SO_4$ and Casitone were deleted. The Casitone contributed approximately 26 mM nitrogen mainly as tryptic peptides of casein. The NH_4^+ was 6 mM (added as $(NH_4)_2SO_4$). The low-NH_4^+ Casitone was treated to remove most of the NH_4^+ by the Hollow fiber dialysis technique (see Materials and Methods) and was added to give 30 mM nitrogen. The amino acid mixture contained about 41 mM nitrogen. Methionine (0.5 mM) was added to media with no nitrogen or NH_4^+ only, because of the known requirement of *B. ruminicola*. Data represent the average of duplicate tubes; numbers in parentheses indicate hours of incubation required to reach the maximal OD.

They grew well in the minimal medium and showed the requirement for vitamin B_{12} which is replaced by methionine. However, strain 0434 of the *B. fragilis* subsp. *distasonis* has an obligate requirement for methionine and no requirement for B_{12}, while the strain of *B. fragilis* subsp. *ovatus* seems to require one or more amino acids and, possibly, some vitamins. This minimal medium apparently lacks some growth factor(s) stimulatory for strain H as growth was poor in all cases. In another experiment, it was shown that either cysteine or sulfide can also serve as the sole source of sulfur for the strains of *B. fragilis* subsp. *vulgatus*, *B. fragilis* subsp. *thetaiotaomicron*, and *B. fragilis* subsp. *distasonis*, except for strain 0434 which, as indicated above, also required methionine.

TABLE 4. *Growth responses of strain 9343 of Bacteroides fragilis subsp. fragilis in media with various filter-sterilized single sources of sulfur added*

Additions to basal[a]	Growth (OD)[b]
None	0
L-Cysteine	1.11 (24)
Na_2S	1.09 (24)
Sodium thioglycolate	0.35 (68)
$Na_2S_2O_3$	0.58 (62)
Na_2SO_3, Na_2SO_4 L-Methionine or β-Mercaptoethanol	0

[a] The basal medium is similar to the inoculum medium (Table 1) except that $(NH_4)_2SO_4$ and $FeSO_4$ are replaced with equimolar NH_4Cl and $FeCl_2$; Casitone, volatile fatty acid, and vitamin solution are omitted; vitamin B_{12} (0.5 ng/100 ml) and 1.0 mM dithiothreitol were added. The sulfur sources were filter sterilized with 0.45-μm filters (Millipore, type HA), and added in 1.0 mM final concentration after the medium was autoclaved and cooled.

[b] OD values represent the mean of three replicate tubes with the numbers in parentheses being the hours of incubation. Essentially identical results were obtained with strains 2552 and 2044.

DISCUSSION

The present study of *B. fragilis* shows that most strains have very simple nutritional requirements, in that a minimal chemically defined medium of NH_4^+, glucose, sulfide, vitamin B_{12}, carbon dioxide-bicarbonate buffer, hemin, and minerals supported excellent growth of most strains. Subspecies could not be differentiated on the basis of nutrient requirements.

The results are in general agreement with the work of Quinto (18) on strains which were not identified in regard to subspecies in that purines and pyrimidines were not required and no B vitamins were essential when a complete amino acid mixture was present. While Quinto reported hemin to be essential, in the present study it appeared to be only highly stimulatory as poor growth occurred through many serial transfers on medium without hemin. However, it is evident that the Casitone included in the medium contains a trace amount of hemin or factors that replace hemin for *B. fragilis*, as D. R. Caldwell (University of Wyoming) has shown that many of the strains of the present study fail to grow in similar media in which Casitone is replaced by cysteine unless hemin is included (manuscript in preparation).

It would be of interest to perform further

TABLE 5. *Comparison of growth between Bacteroides fragilis subsp. fragilis strain 9343 and other subspecies of B. fragilis in a vitamin and amino acid-free basal medium to which was added vitamin B_{12}, methionine, B vitamin solution, or Casitone*

Subspecies	Strain	Additions				
		None	B_{12}	Methionine	B vitamins	B vitamins plus Casitone
fragilis	9343	0 (139)	1.18 (22)	1.22 (24)	1.17 (24)	1.28 (12)
vulgatus	8482	0 (108)	1.20 (24)	1.17 (26)	1.15 (24)	1.36 (12)
vulgatus	0980-1	0 (100)	1.10 (20)	1.12 (24)	1.10 (20)	1.28 (12)
thetaiotaomicron	10582	0 (108)	1.12 (24)	1.15 (24)	1.04 (22)	1.31 (12)
distasonis	8503	0 (108)	1.10 (46)	1.11 (42)	1.05 (58)	1.14 (25)
distasonis	0434	0 (108)	0 (132)	1.18 (20)	0 (132)	1.36 (12)
ovatus	0916	0 (132)	0 (132)	0 (132)	0 (132)	1.18 (30)
fragilis	H	0 (95)	0.48 (56)	0.33 (66)	0.44 (83)	0.42 (56)

[a] The basal medium is similar to the inoculum medium given in Table 1, except that Casitone, volatile fatty acid, and vitamin solutions were deleted. Additions were as indicated above and in concentrations as in the inoculum medium, except that B_{12} and methionine were added at concentrations of 5 ng/100 ml and 0.5 mM, respectively. Growth is indicated as the mean maximal OD of three tubes after three serial transfers. Numbers in parentheses indicate hours of incubation required to reach the maximal OD.

studies on the specificity of the hemin requirement of *B. fragilis* and to compare this with *B. ruminicola*. This species utilizes various porphyrins, uroporphyrinogen, coproporphyrinogen, certain iron-free metalloporphyrins, hemes, and certain heme-proteins containing readily removable hemes, but not porphyrin biosynthesis intermediates preceding the tetrapyrole stage or related compounds such as uroporphyrin, chlorophyll, pheophytin, phycoerythrin, bilirubin, or pyrrole (6). As in *B. ruminicola* (25), all subspecies of *B. fragilis* utilize heme to produce a *b*-type cytochrome (C. A. Reddy and M. P. Bryant, 1967, Bacteriol. Proc. p. 40; Gail Herrstrom and M. P. Bryant, unpublished data, 1973).

The results show that strains of *B. fragilis* subsp. *fragilis* have poor ability to utilize organic nitrogen compounds such as amino acids and that NH_4^+ is the preferred nitrogen source. This suggests that this organism occupies a niche in the natural habitat in which there is little survival value in maintenance of systems (presumably transport systems, 16) for efficient utilization of preformed cell monomers such as amino acids and it has lost this ability. On this basis, it can be speculated that there is little organic nitrogen available for growth in its natural environment, the large intestine. On the other hand, one can speculate that NH_4^+, hemin, and vitamin B_{12} or related compounds are readily available in the large intestine during much of the time. NH_4^+ should be readily available, as approximately 20 to 25% of a person's daily urea excretion is normally recycled to the intestine from the blood and hydrolyzed to NH_4^+ by ureolytic bacteria. In humans this may release 3.0 to 4.0 g of NH_4^+-nitrogen daily in addition to that arising from other sources (24). NH_4^+ is known to be the main nitrogen source for rumen bacteria, and little free amino acids or peptides are available for microbial growth in the rumen during much of the time (5, 9).

The relatively simple nutrient requirements shown in the present study should be of considerable value in further studies on *B. fragilis*. This information could be used together with information on relative sensitivity to antibiotics, etc. (23), for development of truly selective methods of isolation and enumeration. The minimal medium has value in genetic studies and can be useful in systematic studies for separation of *B. fragilis* from many other species in the family *Bacteriodaceae*. For example, *B. ruminicola* and *B. succinogenes* would not grow in the medium because of requirements for B vitamins other than B_{12} (4), and preliminary studies show that *B. ochraceus*, strains of all subspecies of *B. melaninogenicus*, *B. biacutus*, *B. clostridiiformis*, *Fusobacterium fusiforme*, *F. nucleatum*, and *F. necrophorum* will not grow on the minimal medium because of requirements for more B vitamins or vitamin K (12), more complex nitrogen sources, or unknown reasons.

ACKNOWLEDGMENTS

M. R. Crabill maintained the strains utilized in this study and performed the experiment indicated in Table 3. The research was partially supported by grant 35-331, Department of Agriculture, and by the Agricultural Experiment Station of the University of Illinois.

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