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1,4-Naphthoquinone and Other Nutrient Requirements of *Succinivibrio dextrinosolvens*

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Three strains of *Succinivibrio dextrinosolvens* isolated from the rumen of cattle or sheep under diverse conditions grew well in a minimal medium containing glucose, minerals, cysteine, methionine, leucine, serine, ammonia, 1,4-naphthoquinone, *p*-aminobenzoic acid, and bicarbonate-carbonic acid buffer, pH 6.7. When menadione or vitamin K_5 was substituted for 1,4-naphthoquinone, the growth rate was somewhat depressed. Growth was poor with vitamin K_1 and phthiocol, and other related compounds were inactive. In the absence of ammonia, further addition of the amino acids aspartic acid, arginine, histidine, and tryptophan was necessary for good growth of type strain 24, but the other two strains grew well only in media containing ammonia. Strains C18 and 22B produced urease and grew well when ammonia replaced urea. When urea replaced ammonia, strain 24 grew poorly and urease activity could not be detected. Strain 24 required no B-vitamins, but the other two strains were stimulated by *p*-aminobenzoic acid. The methionine requirement was not replaced by vitamin B_{12}, betaine, or homocysteine. Cysteine was replaced by sulfide in strain 24 but less well in the other two strains. Very poor growth was obtained when sulfate replaced cysteine. The half-saturation constant for ammonia during growth of *S. dextrinosolvens* is more than 500 μM, a much higher value than that of many rumen bacteria.

Although much is known about the nutrition of many important species of rumen bacteria (4, 6, 7, 10), *Succinivibrio dextrinosolvens* (2, 8) has received little attention. It is usually isolated in large numbers only from the rumen of cattle or sheep fed relatively high starch diets or other diets containing large amounts of rapidly fermented carbohydrate (2, 8, 13) and, under some conditions, may be one of the major rumen bacteria fermenting dextrins and grass levan. Its fermentation products include mainly acetate and succinate, with a relatively large uptake of CO_2 (8, 13). It was the most numerous bacterium producing urease in the rumen of cattle fed a relatively high grain and corn silage diet (15).

In early studies with glucose media, *S. dextrinosolvens* was shown to require a high level of HCO_3^--CO_2 for good growth (5). In a basal medium containing glucose, minerals, enzymatic casein hydrolysate, B-vitamins, cysteine, and HCO_3^--CO_2, growth of a relatively large inoculum of one strain was highly stimulated by rumen fluid and ammonia, and factors in casein hydrolysate were essential (6). Acetate or a mixture of isobutyrate and valerate isomers had no effect on growth, but one or more B-vitamins was stimulatory (6). When NH_4^+, peptides, or a mixture of free amino acids was added as nitrogen source, little NH_4^+ was produced or used, and ^{14}C from free amino acids was very effectively incorporated into cellular material (7). Scardovi (13), working with another strain, found that a factor present in rumen fluid was necessary for good growth.

In the present study we describe minimal chemically defined culture media for growth of three strains of *S. dextrinosolvens* and show that the rumen fluid requirement is replaced by 1,4-naphthoquinone.

MATERIALS AND METHODS

The strains used were from the culture collection of the Microbiology Division, Department of Dairy Science, University of Illinois. Strain 24 (ATCC 19716) is the type strain of the species and was isolated from the rumen of a cow on a high-grain diet in Maryland (5, 8). Strain 22B was isolated from the rumen of a sheep fed dried grass cubes and hay in England (S. N. Wilson, J. Gen. Microbiol. 9:i–ii, 1953) and was provided by S. R. Elsden. Strain C18 was a more recent isolate from an Illinois cow fed mainly grain and corn silage

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TABLE 1. Composition of the basal medium for nutritional studies of *S. dextrinosolvens*

Component	%
Glucose	0.3 (wt/vol)
Mineral solution[a]	5.0 (vol/vol)
Trace mineral solution[b]	0.5 (vol/vol)
$FeSO_4$ (10 mM)	0.2 (vol/vol)
Resazurin (0.1% [wt/vol])	0.1 (vol/vol)
L-Cysteine-hydrochloride (2.5% [wt/vol])[c]	2.0 (vol/vol)
Na_2CO_3 solution (8% [wt/vol])[d]	5.0 (vol/vol)
CO_2 gas phase (pH 6.7)	

[a] The mineral solution contained (grams/liter) KH_2PO_4, 18; NaCl, 18; $CaCl_2 \cdot 2H_2O$, 0.53; $MgCl_2 \cdot 6H_2O$, 0.4; $MnCl_2 \cdot 4H_2O$, 0.2; and $CoCl_2 \cdot 6H_2O$, 0.02 in distilled water.

[b] Trace mineral solution contained 10 mg each of $ZnSO_4 \cdot 7H_2O$, H_3BO_3, Na_2SeO_3, $Na_2MoO_4 \cdot 2H_2O$, and $NiCl_2 \cdot 6H_2O$, 5 mg of $CuSO_4 \cdot 5H_2O$, and 2 mg of $Al(SO_{45})_2 \cdot 12H_2O$ made to 100 ml with distilled water.

[c] The cysteine solution was autoclaved separately, tubed, and stored with an N_2 gas phase (3).

[d] The Na_2CO_3 solution was autoclaved separately, aseptically equilibrated with CO_2, and stored with a CO_2 gas phase (3).

and was one of many urease-forming *S. dextrinosolvens* strains isolated (15).

Anaerobic methods and media. The anaerobic techniques were those of Hungate as modified by Bryant (3).

The composition of the basal medium is shown in Table 1. The medium, with additions, was autoclaved at 121°C for 15 min. The separately sterilized cysteine and Na_2CO_3 solutions were then added, and the medium was tubed in 5-ml amounts into rubber-stoppered culture tubes (13 by 100 mm) as previously described (3).

Some solutions added to the basal medium were as follows: 0.3% (vol/vol) volatile fatty acid (VFA) solution was added, containing 2.0 ml each of DL-2-methyl-*n*-butyric, *n*-valeric, isobutyric, and isovaleric acids adjusted to pH 7.0 with NaOH and made to 50 ml with distilled water. The B-vitamin solution contained 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 0.2 mg of cobalamin in 100 ml of distilled water, and 0.5% (vol/vol) was added to the medium. Five percent (vol/vol) of 120 mM $(NH_4)_2SO_4$ was added as a source of ammonia nitrogen. These and other additions to the basal medium are indicated in the respective experiments.

Strains were maintained in carbohydrate medium maintenance slants (6). For nutrition experiments, one loop (about 0.01 ml) from the inoculum medium into 5 ml of experimental media (triplicate tubes) was used. The inoculum medium was the basal medium with Casitone (0.2% [wt/vol]), VFA mixture, $(NH_4)_2SO_4$ solution, B-vitamins, and 1% of rumen fluid (6) added. In later experiments, the inoculum was from the simplest defined medium, relevant to the experiment, which allowed good growth.

Growth was measured by absorbance at 600 nm (A_{600}), using a Bausch and Lomb Spectronic 70 with triplicate culture tubes (13 by 100 mm). Cultures were usually serially transferred on a given medium three times before the final growth measurements were made. An absorbance value of 1.0 was equal to about 0.67 mg of cellular dry weight per ml of medium.

RESULTS

Naphthoquinone requirement. The basal medium with B-vitamins, Casitone (0.2% [wt/vol]), and 6 mM $(NH_4)_2SO_4$ added did not support growth through three transfers of any of the three strains. However, with the addition of 1% (vol/vol) of rumen fluid or certain vitamin K-like compounds growth was good. Results with these additions to the growth medium, using strain 24 (Fig. 1), indicated that 1,4-naphthoquinone supported the best growth. Somewhat slower growth rates were obtained with rumen fluid or menadione, and vitamin K_5 gave a long lag in growth followed by good growth. Phthiocol and vitamin K_1 supported poor growth after long lags, and other compounds tested did not support growth. Similar results were obtained with strains 22B and C18 except that vitamin K_5 supported somewhat faster growth than menadione did.

Strain 24 grew best with 2.6 μM or more 1,4-naphthoquinone added; below this level growth rates were lower. Below 0.26 μM, both growth rates and extent of growth were drastically reduced.

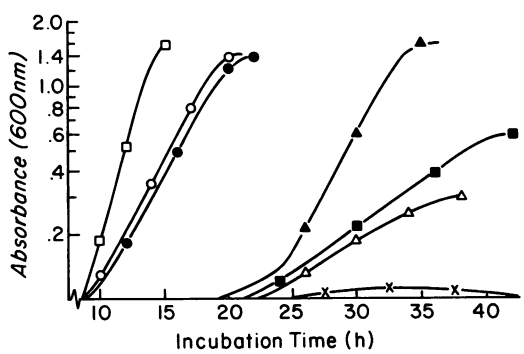

FIG. 1. The effect of compounds related to vitamin K in replacing the rumen fluid requirement for growth of *S. dextrinosolvens* strain 24 in the basal medium (Table 1) supplemented with Casitone (0.2% [wt/vol]), 6 mM $(NH_4)_2SO_4$, VFA mixture, and B-vitamins. Symbols: ○, rumen fluid (1% [vol/vol]); ●, 52 μM menadione; □, 52 μM 1,4-naphthoquinone; ■, 52 μM phthiocol; △, 52 μM vitamin K_1; ▲, 52 μM vitamin K_5; and ×, no additions or 52 μM naphthaline, hydroquinone, toluoquinone, or coenzyme Q. Points are the means of three tubes after three transfers on a given medium.

TABLE 2. The effect of certain amino acids on the growth of *S. dextrinosolvens* strain 24 in a medium[a] with and without ammonia

Additions[b]	Growth (A_{600})			
	Plus NH_3		Minus NH_3	
None	0.01	(100)[c]	0.02	(48)
ser, leu, arg, asp, his, trp	0.11	(36)	0.15	(20)
met, ser, leu, arg, asp, his, trp	1.90	(14)	1.90	(16)
met, ser, leu, arg, asp	1.86	(14)	1.60	(24)[d]
met, ser, leu	1.80	(14)	0.32	(48)
met, ser	1.60	(20)[d]	0.07	(40)
met, leu	1.67	(20)[d]	0.09	(32)
met	0.28	(72)	0.02	(48)

[a] The medium was that in Table 1 supplemented with the VFA mixture and 52 μM 1,4-naphthoquinone.

[b] Additions where indicated included 6 mM $(NH_4)_2SO_4$, 1.34 mM DL-methionine (met), 2.85 mM L-serine (ser), 3.04 mM L-leucine (leu), 0.95 mM L-arginine (arg), 2.25 mM L-aspartate (asp), 0.52 mM L-histidine (his), and 0.49 mM L-tryptophan (trp).

[c] Values in parentheses indicate hours to reach maximum absorbance.

[d] Cultures with abnormal growth as indicated in the text.

Nitrogen requirements. *S. dextrinosolvens* strain 24 was unable to grow in the basal medium supplemented with 52 μM 1,4-naphthoquinone, B-vitamins, and VFA mixture or in the same medium supplemented with 6 mM $(NH_4)_2SO_4$. However, growth in the latter medium was good if Casitone (0.2% [wt/vol]), Casamino Acids (0.2% [wt/vol]), or a complex mixture of amino acids was added. If $(NH_4)_2SO_4$ was deleted, growth in the medium with Casitone was somewhat depressed, but not in the medium with Casamino Acids or in the medium with a complex mixture of amino acids.

These results indicated that ammonia and cysteine alone would not satisfy the nitrogen requirements of strain 24 but that other amino acids were required. The results for media without ammonia added also suggested that free amino acids were better sources of nitrogen than peptides (Casitone) were.

The effect of various amino acid combinations on the growth of *S. dextrinosolvens* strain 24 in the presence and absence of ammonia is shown in Table 2. Methionine was necessary for good growth regardless of the other amino acids present; however, methionine, cysteine, and ammonia supported only feeble growth. If serine and leucine were also added, growth was excellent. If either serine or leucine was deleted from this medium, growth was good, but abnormal, and was characterized by large swollen cells, longer chains of cells, and by clumps of cells that were hard to disrupt. The methionine requirement could not be replaced by betaine, homocysteine, or vitamin B_{12}, and the VFA mixture had no effect on growth regardless of the number of amino acids present in the medium.

In the absence of ammonia further amino acid supplementation was necessary to obtain good growth of strain 24 as shown in Table 2 and in other experiments not shown. The medium with just those amino acids necessary for good growth when ammonia was present, i.e., methionine, serine, leucine, and cysteine, supported very poor growth in the absence of ammonia. Further addition of aspartate and arginine allowed much better growth, but the cultures were abnormal as indicated above. The further addition of histidine and tryptophan resulted in the best growth observed without ammonia, and the addition of other amino acids had little effect. In contrast to strain 24, strain 22B grew more slowly, and strain C18 grew very poorly without ammonia even when a complete mixture of amino acids was added to the medium; yet all three strains grew very well with only ammonia, methionine, cysteine, serine, and leucine as nitrogen sources.

The minimal amount of ammonia required to give near optimal growth of strain 24, with only methionine, cysteine, serine, and leucine as added amino acids, was about 2 mM (Table 3). With ammonia levels of 1 mM or less, the extent of growth was drastically lower, and the growth rate was lower.

Urea added to the medium in place of ammonia supported excellent growth of strains 22B and C18 but only poor growth of strain 24 (Table 4). Strong urease activity, indicated by ammonia production in the spot test of Wozny et al. (15), was obtained with strains 22B and C18 in the culture containing 25 mM urea, but no ammonia was detected with strain 24. Tests on the uninoculated medium and on the stock urea solution by

TABLE 3. The effect of levels of ammonia on the growth of *S. dextrinosolvens* strain 24 in a medium containing the amino acids necessary to give good growth when ammonia was in excess[a]

Ammonia (mM)	Growth (A_{600})	Growth rate[b] (A_{600})/h
0	0.06 (15)[c]	
0.5	0.42 (23)	0.17
1.0	0.73 (21)	0.27
2.0	1.60 (18)	0.42
3.0	1.70	0.44

[a] The medium was that in Table 1, with 52 μM 1,4-naphthoquinone, 1.34 mM DL-methionine, 3.04 mM L-leucine and 2.85 mM L-serine added.

[b] Growth rate during exponential growth.

[c] Hours to reach maximum absorbance.

TABLE 4. Effect of the addition of ammonia or urea on the growth of *S. dextrinosolvens* strains in a medium containing only those amino acids necessary to allow good growth when ammonia was in excess[a]

Addition	Growth (A_{600})		
	Strain 24	Strain 22B	Strain C18
None	0.07 (86)[b]	0.05 (66)	0.06 (32)
$(NH_4)SO_4$ (5 mM)	1.60 (18)	1.90 (16)	1.56 (14)
Urea (5 mM)[c]	0.82 (38)	1.75 (18)	1.52 (16)
Urea (25 mM)[c]	0.90 (32)	1.72 (16)	1.46 (16)

[a] The medium was that described in Table 1 supplemented with 52 μM 1,4-naphthoquinone, 50 ng of *p*-aminobenzoic acid per ml, 1.34 mM DL-methionine, 3.04 mM L-leucine, and 2.85 mM L-serine.

[b] Hours to reach maximum absorbance.

[c] Urea was filter sterilized.

the spot test or by the indophenol test (9) indicated no ammonia contamination.

B-vitamin requirements. Strain 24 grew very well when none of the B-vitamins was added to the basal medium supplemented with 1,4-naphthoquinone and various utilizable nitrogen sources (Tables 2 and 3), and none of the B-vitamins stimulated the growth of strain 24. However, although none of the B-vitamins was essential to the growth of the other two strains, *p*-aminobenzoic acid was stimulatory to their growth. In the absence of *p*-aminobenzoic acid, both strains 22B and C18 had reduced growth rates, and the extent of growth was greatly reduced in strain C18.

Sulfur requirements. Little or no growth was obtained in the basal medium supplemented with 1,4-naphthoquinone, methionine, serine, and leucine, with NH_4Cl and $FeCl_2$ replacing their sulfate salts and with 1 mM dithiothreitol replacing cysteine. The addition of cysteine to this medium allowed excellent growth of all three strains. The replacement of cysteine with 1 mM sulfide allowed excellent growth of strain 24, slower growth of strain 22B, and much less growth of strain C18. When cysteine was replaced with 1 mM Na_2SO_4, strain 24 grew with a greatly reduced growth rate, and strains 22B and C18 did not grow.

DISCUSSION

This is the first documentation of a naphthoquinone, menadione, or vitamin K requirement by a bacterium known to be important in the rumen. *Bacteroides levii*, previously thought to be a strain of *Bacteroides melaninogenicus*, has long been known to require menadione or 1,4-naphthoquinone (11) but was found in only small numbers in the rumen.

The amino acids required for good growth of *S. dextrinosolvens* are somewhat greater in number than those required by most known species of rumen bacteria. A few other species also require methionine and cysteine but not serine and leucine (4). When ammonia was deleted from the medium, strain 24 then required additional amino acids for good growth. These included arginine and aspartic acid and, to a lesser extent, histidine and tryptophan. The other two strains were stimulated by ammonia even in a medium containing many amino acids. That strain 24 grew well with mixtures of free amino acids fits well with previous results showing that it very effectively incorporated ^{14}C from a mixture of ^{14}C-amino acids as compared with many other rumen bacteria when grown on a medium containing free amino acids, peptides, and ammonia as possible nitrogen sources (7). The fact that ammonia was more stimulatory to growth in media containing mainly oligopeptides (Casitone) as a nitrogen source than in media containing many free amino acids suggests that strain 24 is not very effective in the utilization of oligopeptides as compared with species such as *Bacteroides ruminicola* (12). Previous results indicated that *S. dextrinosolvens* did not produce ammonia in a medium containing a large amount of amino acid and peptide nitrogen (1).

The present results show that strain 24 does not have a very good affinity for ammonia as the maximum growth rate with 0.5 mM ammonia in the medium was less than half of that in the same medium with 2.0 mM or more (Table 3). Thus, the half-saturation constant for ammonia for growth in media in which ammonia is required is in the order of 500 μM or more. Many species of rumen bacteria have half-saturation constants for ammonia of less than 50 μM (14).

The present results show that *S. dextrinosolvens* strain C18, shown earlier to contain urease (15), retained it, and strain 22B, not previously shown to have urease, also has the enzyme. This, along with the study of Wozny et al. (15), suggests that most strains of the species contain urease. However, although the type strain 24 of the species grew very slowly when urea replaced ammonia (Table 4), no ammonia was detected and no $^{14}CO_2$ was detected in a urease assay involving [^{14}C]urea (J. A. Patterson and R. B. Hespell, personal communication). Thus, the type strain either contains no urease or a greatly modified activity.

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