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Branched-Chain Amino Acid Fermentation by a Marine Spirochete: Strategy for Starvation Survival

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An anaerobic marine spirochete (strain MA-2) fermented glucose and formed ethanol, acetic acid, CO_2, and H_2 as end products. The organism required carbohydrates as growth substrates. Amino acids did not support the growth of strain MA-2. However, when the spirochete was grown in media containing branched-chain amino acids and glucose, significant quantities of 4- and 5-carbon branched-chain volatile fatty acids were formed in addition to products of glucose fermentation. Smaller quantities of branched-chain alcohols were also formed under these conditions. The spirochete converted L-valine, L-isoleucine, and L-leucine to isobutyric, 2-methylbutyric, and isovaleric acids, respectively. CO_2 formation accompanied each of these conversions. Spirochete MA-2 did not require branched-chain amino acids for growth, but these compounds could serve as sole sources of nitrogen for the organism. In addition, the survival of starving cells (no growth substrate available) of spirochete MA-2 was prolonged significantly when L-valine, L-isoleucine, and L-leucine were present in starvation media. Starving cells fermented these amino acids, forming adenosine 5'-triphosphate and branched-chain fatty acids. Our findings indicate that energy derived from amino acid fermentation allows the spirochete to survive periods of growth substrate starvation. Apparently, dissimilation of branched-chain amino acids can provide this bacterium with maintenance energy for cell functions not related to growth. In its natural environment spirochete MA-2 may catabolize branched-chain amino acids as a strategy for survival when growth substrates are not available.

Numerous bacteria from aquatic sediments, the rumen, the mammalian large intestine, and other anaerobic habitats produce significant quantities of branched-chain volatile fatty acids (2, 12, 18, 19, 23, 26, 27). These fatty acids have four or five carbon atoms and are released by the bacteria into the external environment. Bacteria that are known to form these products include *Megasphaera elsdenii* and various species of *Bacteroides*, *Eubacterium*, *Peptostreptococcus*, *Treponema*, and *Clostridium* (18, 26). Available evidence indicates that the branched-chain fatty acids are derived from branched-chain amino acids (2). Thus, isobutyric, 2-methylbutyric, and isovaleric acids are formed from L-valine, L-isoleucine, and L-leucine, respectively. It is not clear why bacteria produce these fatty acids, especially since at least some of the bacteria that produce them do not utilize valine, isoleucine, or leucine as sole carbon and energy sources for growth (2, 13). Few studies have dealt with the catabolism of branched-chain amino acids by pure cultures of anaerobic microorganisms. Thus, despite the widespread occurrence of branched-chain volatile fatty acid formation by anaerobic bacteria, the physiological significance of this microbial process is largely unknown.

Recently, we have isolated from marine intertidal mud an anaerobic spirochete (strain MA-2) able to catabolize branched-chain amino acids. As described in this article, we found that this spirochete required a fermentable carbohydrate for growth and that it did not utilize amino acids as growth substrates. However, spirochete MA-2 fermented L-valine, L-isoleucine, and L-leucine to form four- and five-carbon branched-chain fatty acids and alcohols as end products. The present studies were carried out primarily for the purpose of elucidating the significance of this fermentation process with regard to the overall physiology of spirochete MA-2 and, possibly, of other bacteria. Data presented in this paper indicate that branched-chain amino acid fermentation provides the spirochete with maintenance energy for growth-unrelated cell functions that allow cells to survive during periods of starvation. In an accompanying paper we describe ATP-yielding enzymatic pathways utilized by spirochete MA-2 for the fermentation of branched-chain amino acids (15).

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sented at the Annual Meeting of the American Society for Microbiology, Miami Beach, Florida, 11 through 16 May 1980 [abstr. no. I46].)

MATERIALS AND METHODS

Isolation of spirochete MA-2. The spirochete (strain MA-2) was isolated from black, viscous, sulfide-rich mud that contained decaying plant material. The mud was collected at Woods Hole, Mass., from a marshy area subject to regular tidal flooding from a seawater pond. This marshy area has been described previously (6).

The isolation procedure involved migration of the spirochetes through an agar medium as described by Canale-Parola (9), except that agar medium was placed in glass bottles instead of petri dishes. Narrow-necked glass bottles (60-ml capacity) were half filled with isolation medium (see below) and stoppered with cotton. Immediately after sterilization by autoclaving, the cotton stoppers were replaced with neoprene rubber stoppers, and the air atmosphere within was replaced with N_2. Then, the isolation medium was allowed to solidify at an angle of approximately 45°. After the medium solidified, a small cylindrical well was melted near the center of each agar slant by touching the agar medium with the hot tip of a sterile pipette. A small volume of marsh mud was placed into each well, and the bottles were incubated at 30°C. Spirochetes present in the mud inoculum multiplied and migrated through the agar medium forming a subsurface growth veil that usually was visible after 5 days of incubation (9). Cells from the outer edge of the growth veil in one of the enrichment cultures were used to obtain a pure culture of strain MA-2 by repeated serial dilutions through agar medium in tubes (N_2 atmosphere).

Media and growth conditions. Spirochete MA-2 was cultivated anaerobically in an atmosphere of N_2 by the technique of Hungate (20). Media were heated to the boiling point or until the resazurin indicator, when present, became colorless. Then, the media were distributed into 18- by 142-mm anaerobic culture tubes (Bellco Glass, Inc., Vineland, N.J.; 10 ml of medium per tube) while the tubes were being flushed with N_2. The culture tubes were sealed with neoprene stoppers and autoclaved. Cells were mass cultured in sealed Florence flasks containing media in an N_2 atmosphere. Cultures were grown routinely at 30°C.

The isolation medium used in the bottle slants contained 0.02 g of vitamin-free casein hydrolysate, 0.05 g of sodium thioglycolate, 10 mmol of Tris-hydrochloride (pH 7.2), 1 ml of growth factor solution, and 1 g of agar (Difco Laboratories, Detroit, Mich.) in 75 ml of seawater and 25 ml of distilled water. The pH of the medium was adjusted to 7.8 with KOH. Pure cultures of spirochete MA-2 were obtained by performing serial dilutions (as described above) in isolation medium supplemented with 0.1% (wt/vol, final concentration) cellobiose.

The growth factor solution used was described previously (16). However, coenzyme A and inositol were omitted, and the growth factor solution components were dissolved in half-strength artificial seawater (5). The artificial seawater consisted of 0.6 M NaCl, 0.1 M $MgSO_4 \cdot 7H_2O$, 0.02 M KCl, and 0.02 M $CaCl_2 \cdot 2H_2O$ (5). The pH of the growth factor solution was adjusted to 7.0 with KOH before sterilization by filtration.

The spirochete was grown in complex medium (MSM broth) that contained (per 50 ml of artificial seawater and 45 ml of distilled water): yeast extract (Difco), 0.4 g; peptone (Difco), 0.2 g; Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 0.2 g; D-glucose, 0.2 g; and L-cysteine-hydrochloride, 0.05 g. To this solution 5 ml of a M Tris-hydrochloride buffer solution was added. The pH of 50 mM Tris-hydrochloride solution (the final concentration of Tris-hydrochloride buffer in medium MSM) was 7.5 at 30°C. The final pH of medium MSM was 7.5. Strain MA-2 was also cultivated in defined marine medium (MMM broth) that contained (per 100 ml of basal medium): D-glucose, 0.2 g; L-asparagine, 0.25 g; L-cysteine-hydrochloride, 0.05 g; trace elements solution, 0.05 ml; resazurin (0.1% wt/vol), 0.1 ml; and growth factors solution, 1 ml. The basal medium (BM) contained 50 mM Tris-hydrochloride (pH 7.5 at 30°C), 0.33 mM K_2HPO_4, and 0.1 mM $FeSO_4 \cdot 7H_2O$ in half-strength artificial seawater (5). The final pH of medium MMM was 7.5. In some experiments MMM broth was supplemented with 0.1% (wt/vol, final concentration) each L-valine, L-isoleucine, and L-leucine (MMM-AA broth).

The trace elements solution contained (per 450 ml of distilled water): $MnCl_2 \cdot 4H_2O$, 875 mg; $ZnCl_2$, 125 mg; $CuCl_2 \cdot 2H_2O$, 125 mg; $NiCl_2 \cdot 6H_2O$, 125 mg; $CoCl_2 \cdot 6H_2O$, 125 mg; $NaMoO_4 \cdot 2H_2O$, 62 mg; and Na_2SeO_3, 62 mg.

Spirochete MA-2 was maintained in a marine glucose-Trypticase-yeast extract medium (MGTY) containing 0.2 g of D-glucose, 0.1 g of Trypticase, 0.1 g of yeast extract, 0.05 g of L-cysteine-hydrochloride, 0.1 ml of resazurin (0.1% wt/vol), 5 ml of 50 mM Tris-hydrochloride buffer (pH of 50 mM Tris-hydrochloride solution was 7.5 at 30°C), 75 ml of seawater, and 20 ml of distilled water. The final pH of this medium was 7.5. Agar (0.8 g/100 ml of medium) was added for solid media. Strain MA-2 was maintained by incubating stab cultures at 15°C, a temperature at which slow growth occurred. Cultures were transferred every 8 days.

The ability of strain MA-2 to utilize various carbon compounds for growth was investigated by inoculating growing cells into MMM broth to which potential substrates had been added in place of glucose. Each compound was sterilized separately and added to a final concentration of 0.2% (wt/vol), except for L-valine, L-isoleucine, L-leucine, L-proline, glycine, sodium succinate, and lithium lactate, which were added to a final concentration of 0.1% (wt/vol). Casein hydrolysate, casein, and sodium 2-ketoglutarate were added to final concentrations of 1.0, 0.4, and 0.3% (wt/vol), respectively. Ball-milled cellulose was added to a final concentration of 0.6% (wt/vol). Growth yields of the spirochete were determined after three transfers of the cells (2% [vol/vol] inoculum) into media containing the substrate being tested.

The ability of the spirochete to utilize various nitrogen sources for growth was determined by inoculating growing cells into MMM broth to which nitrogenous compounds had been added in place of asparagine and cysteine. In these media 0.1% (wt/vol) $Na_2S \cdot 9H_2O$ replaced cysteine as the reducing agent. Each com-

pound to be tested was added to a final concentration of 0.1% (wt/vol), except for L-asparagine and L-glutamic acid which were added at final concentrations of 0.25% (wt/vol). NH_4Cl was added at a final concentration of 1.0% (wt/vol). Growth yields of the spirochete were determined after four transfers of the cells (2% [vol/vol] inoculum) into media containing the nitrogen source being tested.

Growth measurements. Growth yields were determined turbidimetrically and by direct cell counts as described previously (16).

Preparation of cell suspensions. Cells grown in MSM broth were harvested by batch centrifugation at 5,000 × *g* at 5°C. The cells were washed twice and then suspended in 50 mM Tris-hydrochloride (pH 7.5 at 30°C) and 4 mM dithiothreitol solution in half-strength artificial seawater. The dithiothreitol was added after the solution had been boiled and quickly chilled. The solution was stored under N_2. Cell suspensions were kept under N_2 whenever possible.

Analysis of fermentation products. Quantitative analysis of fatty acids and branched-chain alcohols produced by growing cells and cell suspensions of spirochete MA-2 was carried out by gas-liquid chromatography in a Varian 3700 gas chromatograph (Varian Instrument Division, Palo Alto, Calif.) equipped with a flame ionization detector. Samples (1 ml) of spent growth medium were placed in screw-capped test tubes (16 by 125 mm). The growth medium in the tubes was acidified with 0.03 ml of 6 M H_2SO_4 and then extracted with 1 ml of diethyl ether by inverting the tube 50 times. The ether layer was removed and used for analysis. Nonvolatile fatty acids were prepared for gas-liquid chromatography as described by Holdeman et al. (18). Products were separated on a glass column (6 ft in length by 0.25 in. in outer diameter) packed with SP-1000/H_3PO_4 (10/1%) on 100/120 Chromosorb WAW (Supelco, Inc., Bellefonte, Pa.). Injector and detector temperatures were 200°C. Carrier gas (N_2) flow was 30 ml/min. Immediately after sample injection (7-μl samples), the oven temperature was increased from 100 to 130°C at a rate of 4°C/min. Amounts of fermentation products were estimated by measurement of areas under peaks as compared with standard curves prepared with known amounts of acids and alcohols. Isovaleric and 2-methylbutyric acids were resolved on a stainless steel column (6 ft by 0.125 in. [ca. 1.8 m by 0.313 cm]) packed with SP-1000/H_3PO_4 (0.15/0.15%) on 80/100 Carbopack C (Supelco). Separation was carried out isothermally at 130°C.

Gaseous fermentation products were determined by connecting sealed cultures (after growth had occurred) to a fermentation train (25). Acetoin, diacetyl, and 2,3-butanediol were determined by means of assays described previously (11). Ethanol and glucose were assayed enzymatically by using alcohol dehydrogenase and glucose oxidase, respectively (Ethyl Alcohol Reagent Set and STATZYME glucose reagent; Worthington Diagnostics, Freehold, N.J.). Ammonia was also determined enzymatically (Ammonia diagnostic kit; Sigma Chemical Co., St. Louis, Mo.). Uptake of L-[*U*-^{14}C]glucose by growing cells was determined as described previously (17).

Amino acid metabolism by cell suspensions. Reaction mixtures were incubated in double-sidearm Warburg vessels. At the end of the reaction period H_2SO_4 was added to terminate the reaction and release soluble CO_2. The flasks were opened 30 min after the reactions were terminated. Hyamine hydroxide present in the center well was added to 15 ml of scintillation fluid (Aquasol-2; New England Nuclear Corp., Boston, Mass.), and radioactivity was determined in a Beckman LS-100 liquid scintillation counter. Volatile fatty acids present in the reaction mixtures were analyzed by gas-liquid chromatography as described above. Samples were simultaneously collected and quantitated by means of a glass column (packed with SP-1000/H_3PO_4 [10/1%] as described above) that was fitted with a 10:1 glass microcollector/splitter (Varian, Inc.). The small side of the glass splitter was connected to a detector base fitted with a flame tip assembly and detector tower. The large side of the glass splitter was connected to the alternate detector base present in Varian 3700 gas chromatographs. This detector base was open and contained no flame tip assembly. Thus, 10% of the injected sample passed through a flame ionization detector and was quantitated, and 90% of the sample was emitted from the detector base. The emitted samples were collected on Whatman 3MM filter paper circles (2.3-cm diameter) that had been soaked in 0.1 N NaOH and stored at −25°C. Filter papers were placed in glass vials containing scintillation fluid, and radioactivity was determined. Specific activities of CO_2 released in the reactions were determined by assuming that CO_2 and branched-chain fatty acid were formed in equimolar amounts.

Survival experiments. Cells to be used in survival experiments were grown in MSM broth and harvested by centrifugation when the cultures reached the exponential growth phase. Equal quantities of cells were washed twice and finally suspended in liquid media from which glucose was omitted (either MMM broth or MMM-AA broth). NH_4Cl (1% [wt/vol] final concentration) replaced L-asparagine as a nitrogen source in these media. The cell suspensions were incubated at 25°C (N_2 atmosphere). The numbers of viable spirochetes present in starvation media were determined as follows. Starving cells serially diluted in MGTY broth were inoculated into tubes of MGTY agar. After 24 to 48 h of incubation at 30°C, the colonies that had developed in these agar medium deeps were counted. Numbers of viable spirochetes present in starvation media MMM and MMM-AA were determined by using agar medium deeps each containing from 25 to 100 colonies.

Microscopy. A Zeiss GFL phase-contrast microscope was used for light microscopy observations and for direct cell counts. Cells examined by electron microscopy were grown in MSM broth. Equipment and methods used for electron microscopy were described previously (11).

Chemicals. All chemicals used were of reagent grade. L-[*U*-^{14}C]glucose, L-[*U*-^{14}C]valine, L-[*U*-^{14}C]isoleucine, and L-[*U*-^{14}C]leucine were obtained from New England Nuclear Corp., Boston, Mass.

Other experimental procedures. Casein hydrolysis was determined as described by Holdeman et al. (18), except that cells were streaked on anaerobic slants of MSM agar (0.75 g of agar per 100 ml of medium) that was supplemented with 2.0% (wt/vol) casein.

RESULTS

Characterization of spirochete MA-2. Cells of strain MA-2 were helical in shape, had tight coils, and, for the most part, measured 0.4 by 10 to 15 μm. Electron microscopy of strain MA-2 revealed morphological features similar to those of other spirochetes (10). Two periplasmic fibrils were present per cell, one inserted near one end of the protoplasmic cylinder and the other inserted near the opposite end. An outer sheath surrounded both periplasmic fibrils and the protoplasmic cylinder. Spirochete MA-2 grew optimally in media containing between 200 and 300 mM NaCl. When NaCl was not added to growth media, the spirochete did not grow. NaCl could not be replaced by KCl. Thus, spirochete MA-2 had a specific requirement for relatively high concentrations of Na^+. On the basis of this requirement it was concluded that strain MA-2 is a marine bacterium (22).

Utilization of carbon compounds. Spirochete MA-2 grew to densities of 5.4×10^8 cells per ml in a chemically defined marine medium (MMM broth) that contained D-glucose as the sole energy source. The spirochete did not grow in MMM broth from which glucose was omitted. When glucose in MMM broth was replaced by D-fructose, D-galactose, cellobiose, maltose, sucrose, or D-xylose, growth yields were 3.5×10^8 cells per ml or greater. Cellulose did not support growth of strain MA-2. Amino acids added to the medium as acid-hydrolyzed casein did not serve as growth substrates for the spirochete. Furthermore, L-valine, L-isoleucine, L-leucine, glycine, and L-proline did not support growth of MA-2 cells when present either singly or in combinations in defined media. The spirochete also failed to utilize fumarate, 2-ketoglutarate, lactate, pyruvate, or succinate for growth, even when these compounds were present together with branched-chain amino acids in defined medium. These data indicate that spirochete MA-2 is a saccharolytic bacterium and that it lacks the ability to use compounds other than carbohydrates as growth substrates. In this respect it resembles other free-living anaerobic and facultatively anaerobic spirochetes that have been described previously (10, 14). Branched-chain amino acids, even when supplied in combination with other amino acids or organic acids, did not support growth of the spirochete.

Fermentation products. The major products of glucose fermentation by spirochete MA-2 growing in MMM broth were (per 100 μmol of glucose utilized): ethanol, 105.6 μmol; acetic acid, 66.3 μmol; H_2, 105.6 μmol; and CO_2, 164.0 μmol. A small amount (1 μmol) of lactic acid was also produced. The following compounds were assayed for, but not detected: succinate, fumarate, malate, oxaloacetate, formate, butyrate, propionate, valerate, acetoin, 2,3-butanediol, and diacetyl. The oxidation-reduction balance was 1.03. Carbon recovery, including glucose used for synthesis of cell material (10% of the total glucose utilized), was 95%, as determined by measuring L-[U-^{14}C]glucose (specific activity, 4,200 cpm/μmol) incorporation into growing cells. When the spirochete was grown in MMM broth supplemented with branched-chain amino acids (medium MMM-AA), or in a complex medium containing glucose, yeast extract, trypticase, and peptone (MSM broth), significant amounts of the branched-chain volatile fatty acids isobutyric, 2-methylbutyric, and isovaleric acids were formed together with products of glucose fermentation (Table 1). Smaller quantities of the branched-chain alcohols isobutanol and isoamyl alcohol were also produced under these conditions (Table 1). The branched-chain products accounted for a small but significant proportion of the total fermentation products. Furthermore, the branched-chain products were formed throughout the growth of spirochete MA-2. Trace quantities of branched-chain products were detected in MMM broth in which strain MA-2 cells had grown. These products were

TABLE 1. *Fermentation acids and alcohols produced by spirochete MA-2 growing cells*

Medium[a]	Growth yield (10^8 cells/ml)	Products (nmol/ml of growth medium)[b]				
		Isobutyric acid	2-Methylbutyric acid	Isovaleric acid	Isobutanol	Isoamyl alcohol
MSM	9.2	350	402	440	130	32
MMM	5.3	27	<10	12	24	<10
MMM-AA	5.0	143	256	203	64	64

[a] See the text for composition of media. MSM is a complex marine medium containing yeast extract, trypticase, and glucose; MMM is a defined marine medium containing glucose; MMM-AA is MMM containing L-valine, L-isoleucine, and L-leucine. Cultures were incubated until they reached the late exponential phase of growth.

[b] Nanomoles of acetic acid produced per milliliter of MSM, MMM, and MMM-AA, respectively, were 10,200, 6,200, and 5,300. Nanomoles of ethanol produced per milliliter of MSM, MMM, and MMM-AA, respectively, were 12,000, 4,600, and 5,400.

probably derived from amino acids that were synthesized from glucose by the spirochete during growth.

When cell suspensions of the spirochete were incubated with individual L-*U*-^{14}C-labeled branched-chain amino acids, radioactivity was detected in specific branched-chain fermentation products. These experiments showed that MA-2 cells converted L-leucine to isovaleric acid, L-isoleucine to 2-methylbutyric acid, and L-valine to isobutyric acid (Table 2). CO_2 formation accompanied each of these conversions.

Utilization of nitrogen sources. Spirochete MA-2 grew in chemically defined media containing branched-chain amino acids as sole sources of nitrogen. L-Valine, L-isoleucine, and L-leucine served as sole nitrogen sources for the spirochete when supplied singly or in combination. The spirochete fermented each amino acid and formed relatively large amounts of branched-chain end product (Table 3). The increased quantity of isobutanol formed when L-valine was present as the sole nitrogen source was particularly striking. L-Asparagine, L-glutamic acid, and ammonium chloride also served as sole nitrogen sources for spirochete MA-2. When any one of these compounds was supplied to cells in combination with L-valine, L-isoleucine, and L-leucine, the amount of branched-chain product formed was reduced by more than 50%. This indicates that additional nitrogen sources partially inhibit branched-chain amino acid fermentation by strain MA-2. Free ammonia was not excreted by cells growing in defined media containing branched-chain amino acids.

Spirochete MA-2 hydrolyzed casein. When cells were grown in MMM broth containing casein, but not free branched-chain amino acids or other nitrogenous compounds, significant quantities of branched-chain fermentation products were formed.

Effect of amino acids on cell survival and growth yields. The effect of branched-chain amino acids on survival of starved cell populations was examined to determine whether spirochete MA-2 derives maintenance energy from these compounds when growth substrates are not available. Numbers of viable cells surviving starvation conditions were determined in the presence and absence of L-valine, L-isoleucine, and L-leucine (Fig. 1). The data show that in the presence of exogenous amino acids, the viability of starving cells is markedly prolonged over a period of 54 h. The effect of amino acids was most pronounced after 27 h. At this time 10^8 cells per ml were viable in starvation medium supplemented with amino acids. In contrast, unsupplemented medium contained only 6×10^6 viable cells per ml. Significant amounts of branched-chain volatile fatty acids accumulated in the medium that contained amino acids (Fig. 2). This indicates that starving cells fermented L-valine, L-isoleucine, and L-leucine. Branched-chain alcohols were not detected in the starvation media. Furthermore, acetic acid was not formed by either population of cells.

TABLE 2. *Fermentation of L-U-^{14}C-amino acids by cell suspensions of MA-2*[a]

^{14}C-amino acid	Sp act of amino acid (cpm/nmol)	Sp act of products (cpm/nmol)			
		CO_2	Isobutyric acid	2-Methylbutyric acid	Isovaleric acid
L-Leucine	900	150	<10	<10	710
L-Isoleucine	850	144	<10	660	<10
L-Valine	760	153	660	<10	<10

[a] The following were added to each Warburg vessel: L-*U*-^{14}C-amino acid, 5 μmol; dithiothreitol, 12 μmol; Tris buffer (pH 7.5), 150 μmol; NaCl, 900 μmol; KCl, 30 μmol; $MgSO_4 \cdot 7H_2O$, 150 μmol; $CaCl_2 \cdot 2H_2O$, 30 μmol; and 10^{11} cells in a final volume of 3.0 ml. Center well contained 0.2 ml of 1.0 M hydroxide of Hyamine 10X. Incubation was for 5 h at 30°C under argon. Reactions were terminated by addition of 600 μmol of H_2SO_4.

TABLE 3. *Nitrogen source utilization by growing cells of spirochete MA-2*[a]

Nitrogenous compound(s) added[b]	Growth yield (10^8 cells/ml)	nmol of product/ml of growth medium				
		Isobutyric acid	2-Methylbutyric acid	Isovaleric acid	Isobutanol	Isoamyl alcohol
L-Valine, L-isoleucine, and L-leucine	6.3	530	910	1,650	60	35
L-Valine	4.8	559	18	51	253	ND[c]
L-Leucine	3.2	55	23	1,080	13	17
L-Isoleucine	0.8	19	431	29	ND	<10
None	0.1	<10	14	19	ND	ND

[a] Cells were grown in medium MMM from which L-asparagine and L-cysteine were omitted. $Na_2S \cdot 9H_2O$ replaced cysteine as the reducing agent (0.1 g/100 ml). Cultures were incubated until they reached the late exponential phase of growth.

[b] Nitrogenous compounds were added at 0.1 g per 100 ml of medium.

[c] ND, Not detected.

The effect of L-valine, L-isoleucine, and L-leucine on yields of cells growing in a medium containing a growth-limiting concentration of glucose was examined (Table 4). No significant difference in growth yields or growth rates was observed when cells were grown in defined media containing or not containing branched-chain amino acids.

DISCUSSION

The results described in this paper show that, in the absence of growth substrates, the survival of spirochete MA-2 cells is prolonged when exogenous branched-chain amino acids are available. Under these conditions starving cells ferment exogenous L-valine, L-isoleucine, and L-leucine, forming branched-chain fatty acids as final products. As reported in an accompanying paper (15) fermentation of branched-chain amino acids by strain MA-2 results in ATP generation via substrate-level phosphorylation. These observations indicate that spirochete MA-2 derives maintenance energy from branched-chain amino acid catabolism. Apparently amino acid catabolism is a survival strategy that allows spirochetes to persist in marine habitats that are temporarily devoid of growth substrates. It is possible that this mechanism of cellular maintenance also operates in other branched-chain amino acid-fermenting bacteria that, like spirochete MA-2, are unable to use valine, isoleucine, or leucine as growth substrates. Proteases produced by spirochetes probably increase the concentration of free branched-chain amino acids available in natural environments. Endocellular protein may also serve as a source of branched-chain amino acid.

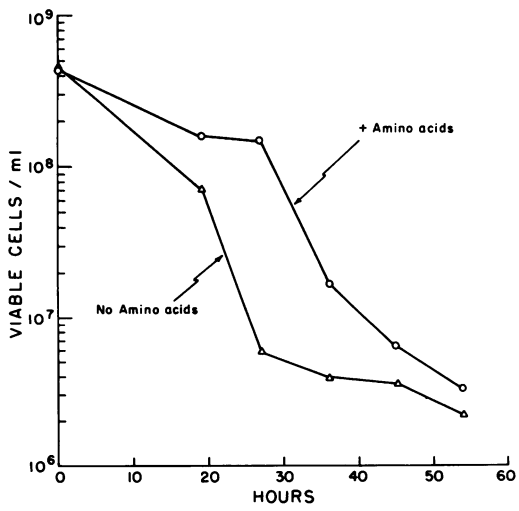

FIG. 1. *Survival of spirochete MA-2 cells suspended in starvation media (no growth substrate available) containing 0.1% (wt/vol, final concentration) each L-valine, L-isoleucine, and L-leucine or not containing amino acids. Viable cells per milliliter were measured as colony-forming units.*

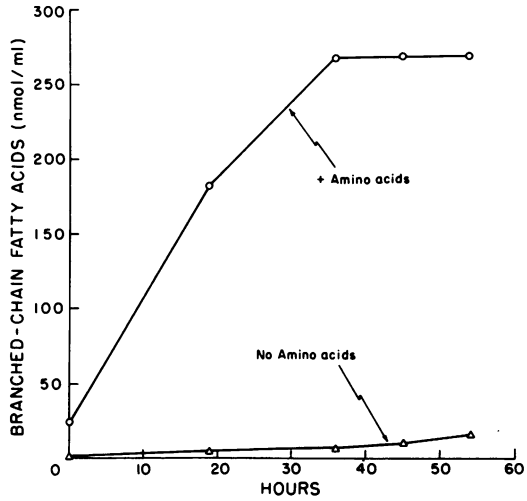

FIG. 2. *Effect of L-valine, L-isoleucine, and L-leucine (0.1% [wt/vol] final concentration of each amino acid) on formation of branched-chain fatty acids by MA-2 cells suspended in starvation media (no growth substrate available).*

Inasmuch as spirochete MA-2 obtains ATP from amino acids, it is likely that valine, isoleucine, and leucine provide this bacterium with energy for growth as well as for cell maintenance. The relative amounts of ATP that growing cells derive from glucose and branched-chain amino acids can be calculated since preliminary data indicate that strain MA-2 ferments glucose by pathways similar to those utilized by other anaerobic free-living spirochetes (10). Thus, it may be expected that cells obtain a net yield of two molecules of ATP from the metabolism of one molecule of glucose to pyruvate via the Embden-Meyerhof pathway. One additional molecule of ATP would be formed per molecule of acetic acid or branched-chain fatty acid produced (10, 15). On the basis of results of fermentation product analyses (see above and Table 4) it may be estimated that the ATP contributed by branched-chain amino acid catabolism would be 4 to 5% of the total ATP derived by dissimilatory activities.

In media containing a growth-limiting concentration of glucose, branched-chain fatty acids are produced from amino acids throughout the entire period of growth of spirochete MA-2, as well as after depletion of glucose. Thus, in view of the estimated ATP yield, it may be predicted that yields of cells grown in media containing a

TABLE 4. *Growth response of MA-2 cells to glucose and amino acids*

Addition to MMM[a]	Growth yield (10^8 cells/ml)		Population doubling time[b] (h)	nmol of branched-chain fatty acids/ml of medium[c]
	Mean[b]	Range		
No addition	No growth			0
Glucose	4.9	4.7–5.1	7.5	20 ± 0.0
L-Leucine, L-isoleucine, and L-valine	No growth			0
Glucose, L-leucine, L-isoleucine, and L-valine	5.0	4.8–5.1	7.5	558 ± 24.0

[a] In these experiments, medium MMM lacked glucose except when indicated. Glucose concentration was 5 μmol/ml of medium; this was a growth-limiting concentration. The concentration of each amino acid was 8 μmol/ml of medium.

[b] Average of triplicate determinations.

[c] Total amount of isovaleric, 2-methylbutyric, and isobutyric acids. Average of triplicate determinations ± standard deviation.

growth-limiting concentration of glucose would increase by approximately 4 to 5% in the presence of branched-chain amino acids. However, cell yields of spirochetes grown in defined media not containing amino acids varied by as much as 8% as determined by the methods we used (Table 4). Thus, under these experimental conditions, it was not possible to detect growth increases resulting from generation of ATP from amino acids.

Strain MA-2 cells growing in glucose-containing media assimilate carbon from L-*U*-^{14}C-labeled branched-chain amino acids present as medium components, but it is not known to what extent these amino acids are used for synthesis of cellular material. It is likely that the amino acids serve as substrates for protein synthesis or as precursors of branched long-chain fatty acids used to manufacture cellular lipids (21). However, cells may not be able to convert leucine, isoleucine, and valine into additional cellular components required for macromolecular synthesis and cell growth. Thus, failure of spirochete MA-2 to use these amino acids as growth substrates may be ascribed to biosynthetic deficiencies. In view of our finding that catabolism of branched-chain amino acids yields ATP, spirochete MA-2 would be expected to utilize leucine, isoleucine, and valine as energy sources for growth in the presence of other organic compounds that are able to satisfy cellular biosynthetic requirements. However, we found that branched-chain amino acids did not support the growth of strain MA-2 when they were present in media in combination with a variety of potential carbon sources such as other amino acids or organic acids.

The amounts of branched-chain volatile fatty acids produced by spirochete MA-2 are small as compared to the amounts of other fermentation products formed by this organism. Cells apparently possess some regulatory mechanism that allows only a limited amount of ATP to be generated via this metabolic route. Thus, another possible explanation for the failure of branched-chain amino acids to support growth of spirochete MA-2 is that cells are not able to generate sufficient ATP from these compounds to allow for significant increases in cell numbers.

We have shown that spirochete MA-2 forms branched-chain alcohols as well as fatty acids from branched-chain amino acids. The significance of branched-chain alcohol formation is not understood. Spirochete MA-2 cells may form these reduced products to maintain charge balance.

This paper constitutes the first documented report of amino acid fermentation by a free-living spirochete. Our studies indicate that many, but not all, marine free-living spirochetes ferment valine, isoleucine, and leucine. Six of 14 independently isolated strains of marine spirochetes formed significant quantities of branched-chain volatile fatty acids when grown in complex media. All six isolates were obligate anaerobes, and none was able to utilize branched-chain amino acids as growth substrates. Three obligately anaerobic strains, including *Spirochaeta litoralis* (strain R1), and five facultatively anaerobic strains of spirochetes did not form significant amounts of isobutyric, 2-methylbutyric, or isovaleric acids when they were grown anaerobically.

In addition to providing spirochetes with nitrogen for growth and a source of maintenance energy for cell survival, branched-chain amino acid catabolism may be important in nutritional interactions that occur between spirochetes and other anaerobic bacteria present in marine environments. It has been shown that rumen bacteria which ferment branched-chain amino acids interact with cellulose-degrading bacteria that require isobutyric, 2-methylbutyric, and isovaleric acids as essential growth factors (7, 8, 24). Rumen cellulolytic bacteria use branched-chain volatile fatty acids to synthesize long-chain branched fatty acids and branched-chain amino acids (1, 3, 4). Amino acid-catabolizing bacteria

derive a competitive advantage by associating with cellulolytic bacteria because they obtain soluble sugars for growth from degraded cellulose (8). It is possible that marine spirochetes are involved in similar microbial interactions. Spirochetes occur in high numbers in viscous, sulfide-rich marsh sediments that contain large amounts of decaying plant material (28). There are indications that spirochetes associate and interact with cellulose-degrading bacteria in these anaerobic marine environments inasmuch as spirochetes are frequently observed in anaerobic enrichment cultures for marine cellulolytic bacteria that are inoculated with marsh sediments. Since known spirochetes do not ferment cellulose, it is likely that spirochetes present in these enrichments utilize soluble sugars released from cellulose by cellulolytic bacteria for growth. Currently we are attempting to isolate a branched-chain fatty acid-requiring cellulolytic bacterium from anaerobic marine sediments. This organism would be useful in studies aimed at determining the nature of possible interactions between marine spirochetes and anaerobic cellulolytic bacteria.

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