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A Gram-Negative Anaerobic Bacterium That Utilizes *O*-Methyl Substituents of Aromatic Acids

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A novel strain of gram-negative anaerobic rods which utilized *O*-methyl substituents of monoaromatic acids as a sole organic source of carbon was isolated from municipal sewage sludge. Energy for growth seemed to be generated by an acetate formation pathway. The growth yield in defined medium was 7.9 g (dry weight) of cells per mol of ferulate utilized. This isolate and other *O*-demethylating anaerobes may play a role in the turnover of acetate and the metabolism of highly methoxylated lignaceous materials in anaerobic environments.

Aromatic compounds with *O*-methyl substituents are widespread in nature; they are major components of lignin and are formed metabolically from tyrosine and phenylalanine in plants and animals. Acetate is a key intermediate in anaerobic environments (11), and recent work shows that one of the possible routes of acetate formation is through anaerobic O-demethylation of aromatic acids or phenols (1, 9). Bache and Pfennig (1) reported that isolates of *Acetobacterium woodii* (2) from sewage sludge or freshwater sediments can produce acetate with *O*-methyl groups as the sole organic carbon source. Carbon balance data suggested that acetate is formed from the *O*-methyl carbon and carbon dioxide. Other recently isolated obligate anaerobes are also able to mediate this O-demethylation reaction. These bacteria are gram-positive rods, except for the novel gram-negative coccus isolated from the rumen by L. R. Krumholtz and M. P. Bryant (Abstr. Third Int. Symp. Microb. Ecol. K-17, p. 56, 1983; personal communication). In this paper, we present the characteristics of a new isolate, an anaerobic O-demethylating gram-negative rod, distinct from those previously described.

Isolation of the O-demethylating anaerobe was accomplished in four phases by stringent anaerobic methods (8, 12). First, enrichment cultures were established that metabolized 1.5 mM ferulate (4-hydroxy-3-methoxycinnamate) to methane and carbon dioxide as previously described (5). The methanogenic activity in enrichments was inhibited subsequently by the addition of 2-bromoethanesulfonic acid, an analog of the essential coenzyme M in methane formation (15). After a 2-bromoethanesulfonic acid-inhibited enrichment culture had completely metabolized the organic carbon source, the ferulate, nitrogen, and phosphorus sources were replenished. The initial enrichment cultures were thus closed systems subjected to 4 to 8 cycles of discontinuous feeding over a period of 6 to 8 months. Second, the O-demethylating population was selectively enriched by several successive subcultures with inocula diluted 10^{-5} to 10^{-7} in fresh defined minimal medium formulated as previously described (4) with ferulate as the sole organic carbon source. Third, isolated colonies were obtained in Hungate roll tubes containing ferulate defined minimal medium solidified with 2% agar. Fourth, the strain was further purified by three successive transfers of isolated single colonies on a nonselective complex medium, Schaedler agar (BBL Microbiology Systems, Cockeysville, Md.). Several isolates of the same type were obtained from different enrichments. One of these, TH-001, was retained for further work.

Cellular morphology was established by phase-contrast microscopy. Wet mounts (Fig. 1) showed that cells were rods 0.6 μm in diameter and 3.3 ± 0.9 μm long (± standard deviation). Occasional motile cells were seen. There were no spores. In old cultures, many cells had a rounded spheroplast form. Cells from actively growing cultures consistently stained gram negative, with no Gram stain variability observed. Thin sections examined by electron microscopy had a cell wall morphology consistent with the ultrastructure of a gram-negative eubacterium.

A number of growth parameters of TH-001 were examined to better characterize the isolate. The strain required strict anaerobic conditions for growth. Stock cultures of TH-001 were transferred monthly and maintained on 1.5% agarose slants of ferulate FC medium. The composition of this defined medium was the same as that of defined minimal medium except that $FeCl_2 \cdot 4H_2O$ was decreased to 0.0018 g/liter, and the reducing agent was changed to L-cysteine (0.035%). Surface colonies on FC agar medium (1.5% agarose) containing D-glucose (0.1%) as the carbon source were 0.5 to 1 mm in diameter after 4 to 7 days. Colonies were transparent to whitish, circular, and flat with an entire margin. Older and larger colonies were mucoid, while small colonies had a firmer consistency. In 0.8 and 1.0% agarose agar shake tubes (13), colonies were 2 mm in diameter, lens-shaped, and white. No growth in FC broth or agarose took place without the addition of ferulate or other utilizable organic carbon sources. In minimal broth, the doubling time at 37°C was approximately 7 h with ferulate and 3 h with glucose. For both substrates, growth took place at 25, 37, and 42°C but not at 48°C. Growth was also observed in FC broth with D-fructose (5.5 mM) or glycerol (13.5 mM) but not with pyruvate (4 mM) or DL-lactate (5 mM). Sulfate and nitrate were not reduced.

No growth occurred with ferulate analogs that lack *O*-methyl substituents, such as caffeate (3,4-dihydroxycinnamate) or cinnamate, each tested at 2 mM. No growth occurred with other aromatic compounds lacking *O*-methyl groups. Compounds tested were phloroglucinol, pyrogallol, and phenylalanine (each tested at 2 mM). No growth was observed with H_2-CO_2 (70:30). No growth occurred with several C_1 compounds including methanol (5 and 50 mM), formate (5

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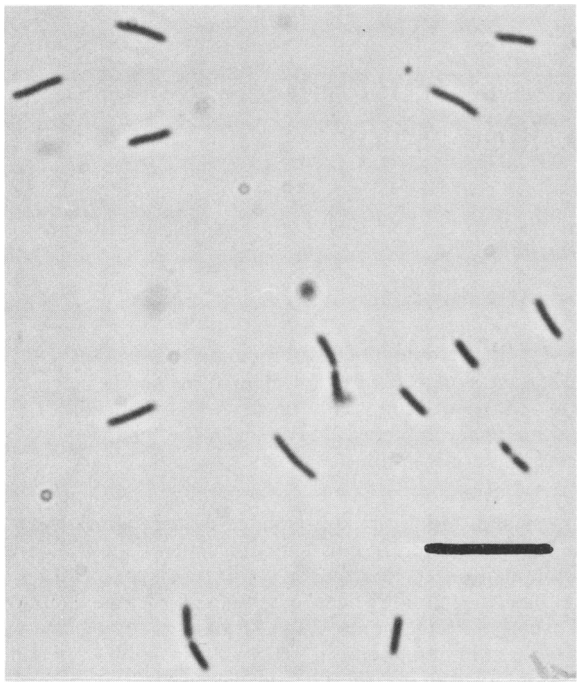
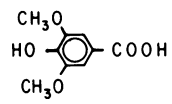
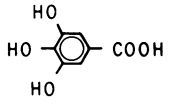
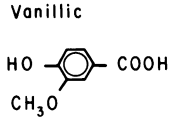
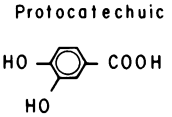
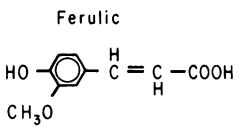
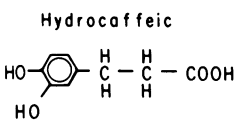


FIG. 1. Suspension of TH-001 cells grown in ferulate defined medium. A culture actively growing in ferulate defined FC medium was centrifuged at 10,400 × *g* for 15 min, and the cell pellet was resuspended in a small volume of fresh anaerobic medium. Bar, 10 μm.

and 50 mM), or formate combined with fumarate (45 mM and 25 mM).

The metabolism of methoxy compounds and of glucose was examined by gas chromatography, high-pressure liquid chromatography, and UV spectroscopy to identify and quantitate fermentation substrates and end products. The culture fluid was analyzed for volatile fatty acids and methylated derivatives of nonvolatile acids by the sample preparation methods of Holdeman and Moore (7) and by packed column gas chromatography. The 6-ft (183-cm) glass column was packed with 10% SP-100–1% H_3PO_4 on 100/120 mesh chromosorb W AW (Supelco, Inc., Bellefonte, Pa.) and operated isothermally at 150°C with the injection port and flame ionization detector at 250 and 300°C, respectively. The helium carrier gas flow rate was 30 ml/min. Analysis for aromatic acids was by high-pressure liquid chromatography with a Spherisorb C18 bonded reversed phase column run isocratically with a moving phase composed of (vol/vol) 23.5% methanol, 4.7% acetonitrile, and 71.8% of an aqueous 5 mM formic acid solution as described by Kaiser and Hanselmann (9). The same high-pressure liquid chromatography column was run isocratically with 0.01 N perchloric acid to confirm and quantitate the presence of acetate and to rule out the presence of formate (3). Acetate was the only fermentation product detected when cells were grown in glucose FC medium or glucose FC medium supplemented with 1% peptone and 1% yeast extract. This strongly suggests that TH-001 ferments glucose via a homoacetate pathway.

TABLE 1. Metabolism of O-methylated aromatic acids[a]

Substrate acid	Products	
	Aromatic acid	Acetic acid
Syringic 	Gallic 	+
Vanillic 	Protocatechuic 	+
Ferulic 	Hydrocaffeic 	+

[a] No growth occurred with caffeate, cinnamate, phloroglucinol, pyrogallol, phenylalanine, methanol, or formate.

TABLE 2. Acetate yields after growth on O-methylated compounds

Substrate	Concn (mM)	Amt of acetate found (mM)[a]	Molar ratio (amt of acetate found per *O*-methyl group)
Syringate	1.7	0.89 ± 0.08	0.26
Vanillate	2.0	0.69 ± 0.07	0.34
Ferulate	1.7	0.29 ± 0.05	0.17

[a] Values are means ± standard deviations.

O-Methylated aromatic acids served as a sole organic carbon source in the defined FC medium (Table 1). The end products were identified as acetate and hydroxylated derivatives of the respective aromatic acids. The amounts of end products formed were determined in triplicate cultures grown on each substrate. Substrates were converted stoichiometrically to their demethoxylated derivatives, whereas the amount of acetate produced varied with different substrates (Table 2). Strain TH-001 also reduced the unsaturated side chain of ferulate, forming hydrocaffeate as the hydroxylated aromatic end product. This reductive capability has been observed in anaerobes that utilize *O*-methyl groups (16; Krumholtz and Bryant, Abstr. Third Int. Symp. Microb. Ecol., 1983) but is also present in other anaerobes (14). Syringate with two methoxyl substituents gave the highest acetate yield, while ferulate gave the lowest. The amount of acetate formed per *O*-methyl group was lower for ferulate than for the other two aromatic compounds. This is not

TABLE 3. Molar growth yields in defined medium[a]

Substrate	Concn (mM)	Amt of substrate used (μmol)	Amt of cells (dry wt) formed (mg)	Growth yield (g/mol of substrate utilized)
Ferulate	0.7	69.6	0.55	7.9
Glucose	5.6	56	1.82	32.5

[a] The ferulate experiment was performed with duplicate 100-ml cultures in stoppered serum bottles; the glucose experiment was performed with 10-ml cultures in Hungate tubes.

TABLE 4. Antimicrobial susceptibility of TH-001

Target	Antibiotic	Medium[a]	MIC (μg/ml)
Peptidoglycan synthesis	Ampicillin	1	0.92
	D-Cycloserine	1	18.0
Protein synthesis	Streptomycin	2	11.0
	Chloramphenicol	3	9.0
RNA polymerase	Rifampicin	2	0.0095
DNA synthesis	Nalidixic acid	2	95.0

[a] Media used were: 1, FC with 1.7 mM ferulate as the organic carbon source; 2, Schaedler broth; 3, FC with 0.1% glucose.

surprising since the reduction of the side chain double bond also can generate metabolic energy (16).

A variety of media modifications were tested in an attempt to counteract the tendency of cells to lyse readily when growing in batch culture. Good cell survival was attained with growth in the medium of Tschech and Pfenning (16) modified by the addition of cysteine (0.035%) and resazurin (0.001 g/liter) and a decrease in KH_2PO_4 (0.07 g/liter). This defined medium containing either ferulate or glucose as the sole organic carbon source was used to determine molar growth yields (Table 3). The concentration of ferulate was measured by its UV A_{320} in media supernatants appropriately diluted in 0.01 N HCl; glucose concentration was measured enzymatically (Sigma Chemical Co., St. Louis, Mo.). A standard curve was obtained for TH-001 grown with 0.5% glucose relating A_{620} to the dry weight of cells washed with deionized water and dried to a constant weight at 85°C. Three concentrations of each substrate were tested to establish appropriate growth-limiting substrate levels for the growth yield experiments. Cell dry weight was determined from the absorbance of duplicate cultures measured after substrate utilization was completed. After the absorbance values of control cultures incubated in the medium without any added carbon source were subtracted, the corresponding dry weights were obtained from the standard curve. The molar growth yields were 7.9 g (dry weight) of cells per mol of ferulate metabolized and 32.5 g (dry weight) of cells per mol of glucose.

An antimicrobial susceptibility profile was obtained for various antibiotics by using broth dilution tests (10) to establish the MICs for growth inhibition (Table 4). These data indicate that TH-001 is not like archaebacteria (6), since it is sensitive to antibiotics that affect the synthesis of cell wall peptidoglycan.

Strain TH-001 is an anaerobic gram-negative rod that ferments glucose or *O*-methyl substituents of aromatic acids to form acetate and saturates the double bond in the side chain of ferulate. It is the first reported gram-negative rod capable of these metabolic transformations and is a novel isolate that can be differentiated from *A. woodii*, a gram-positive demethoxylating anaerobe, and other O-demethylating anaerobic bacteria on the basis of the Gram reaction, the cellular morphology, the range of substrates utilized, and the temperature range compatible with growth. Strain TH-001 has also been scrutinized at the Virginia Polytechnic Institute Anaerobe Laboratory, Blacksburg (personal communication, L. V. Holdeman) for a range of identifying characteristics (7), including a characterization of its polyacrylamide gel electrophoresis profile of soluble proteins. This analysis has confirmed that TH-001 is quite different from *A. woodii* and other known anaerobes. This strain, along with other recently reported organisms (16; Krumholtz and Bryant, personal communication), may play a key role in the little-understood turnover of acetate and the catabolism of lignaceous compounds in anaerobic environments.

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