Clostridium hydroxybenzoicum sp. nov., an Amino Acid-Utilizing, Hydroxybenzoate-Decarboxylating Bacterium Isolated from Methanogenic Freshwater Pond Sediment

XIAOMING ZHANG,^{1†} LINDA MANDELCO,² AND JUERGEN WIEGEL^{1*}

Department of Microbiology and Center for Biological Resource Recovery, University of Georgia, Athens, Georgia 30602¹ and Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801²

Clostridium hydroxybenzoicum JW/Z-1^T (= ATCC 51151 = DSM 7310) (T = type strain), isolated from freshwater pond sediment, is a nonmotile, gram type-positive, spore-forming, amino acid-utilizing, anaerobic rod. This bacterium produces two inducible enzymes that catalyze the decarboxylation of *para*-hydroxybenzoates. The phenols produced are not utilized. *C. hydroxybenzoicum* requires yeast extract for growth. Sugars are not utilized. Sodium ions and acetic acid stimulate growth. The optimal temperature and optimal pH for growth are 33 to 34°C and 7.2 to 8.2, respectively. The DNA base composition of the type strain is 35.5 mol% guanine plus cytosine, whereas the DNA base compositions of the type strains of *Clostridium sticklandii* and *Clostridium aminovalericum* are 33 and 33.5 mol% guanine plus cytosine, respectively, as determined by a chemical method. 16S rRNA sequence analysis groups strain JW/Z-1^T most closely with *Clostridium purinolyticum* and *Clostridium acidurici* (10.6 and 11 inferred changes per 100 bases, respectively). However, *C. hydroxybenzoicum* does not utilize uric acid, hypoxanthine, xanthine, adenine, or guanine. The cell wall type is A1α (L-Lys direct).

Various aromatic compounds, either of natural origin or of anthropogenic origin, can be transformed and mineralized in various anaerobic environments (4, 12, 14–16, 41, 43). Hydroxybenzoate is one of the key aromatic intermediates in the anaerobic degradation of the biopolymer lignin, its hydrolytic products, and similar aromatic compounds. In a previous study of the anaerobic degradation of 2,4-dichlorophenol, we found that mineralization of 2,4-dichlorophenol to methane and carbon dioxide in a freshwater sediment sample required the sequential action of at least five different bacteria (45). During additional studies, a phenol-degrading enrichment culture was established, from which *Clostridium* sp. strain JW/Z-1^T (T = type strain) was isolated (44). This bacterium decarboxylates 4-hydroxybenzoate and 3,4-dihydroxybenzoate to phenol and catechol, respectively. In this paper we describe additional properties of this organism and propose that strain JW/Z-1^T be the first member of the new species, *Clostridium hydroxybenzoicum*.

MATERIALS AND METHODS

Organisms and media. *C. hydroxybenzoicum* JW/Z-1^T was isolated from a freshwater sediment sample in Athens, Ga. (44). *Clostridium aminovalericum* ATCC 13725^T and *Clostridium sticklandii* ATCC 12662^T were obtained from The American Type Culture Collection, Rockville, Md.

ATCC medium 1626 (1) was used to grow *C. aminovalericum*. *C. sticklandii* and *C. hydroxybenzoicum* were grown in mineral medium (44) supplemented with 1% yeast extract, 0.1% glycine, 0.1% L-arginine, and 0.1% L-lysine or in other media (see below).

To test whether the growth of *C. hydroxybenzoicum* was stimulated in the presence of 10 mM adenine, hypoxanthine, uric acid, guanine, or xanthine, mineral basal medium contain-

ing 0.3% yeast extract and 0.1% glycine was used. Peptone-yeast extract medium supplemented with 20 mmol each of glycine and L-lysine per liter was used to grow cells for the lipid fatty acid analysis (gas chromatography of fatty acid methyl esters [Microbe Inotech Laboratories, Inc., St. Louis, Mo.]).

Tests involving cell suspensions. *C. hydroxybenzoicum* was grown in mineral medium supplemented with 1.5% yeast extract and 10 mM each of glycine, L-arginine, and L-lysine. Cells were harvested anaerobically by centrifugation. The resulting cell pellet was suspended in prerduced mineral medium (pH 7.0) without yeast extract or any other carbon and energy source to an optical density at 600 nm of 1.0 to 1.3. Incubation was started after 10 mmol of glycine, L-arginine, or L-lysine or combinations of these amino acids per liter was added to the suspensions. Each preparation was incubated for 22 h at 30°C, during which time the optical density decreased by 10% because of lysis of some cells.

Tests involving growing cultures. Gelatin hydrolysis, indole production, and sulfide production were tested as described by Smibert and Krieg (32).

Fermentation products. Volatile fatty acids were determined by gas chromatography as described previously (44). Acetic acid was determined enzymatically by using reagent kit 148-261 obtained from Boehringer Mannheim GmbH, Mannheim, Germany.

Amino acid determination. Amino acids were analyzed as dansyl amino acid derivatives by using the methods of Kaneda et al. (19) and Bayer et al. (3). Dansylation was performed by the method of Tapuhi et al. (34). A 6-μl portion of a sample was added to 400 μl of 100 mM NaHCO₃. In cell suspension tests, 4 mmol of valine per liter was added to the NaHCO₃ solution as the internal standard. A 200-μl aliquot of a 5-dimethylaminonaphthalene-1-sulfonyl chloride solution (6 mg/ml of acetonitrile) was added to start the reaction. The sample was incubated at 60°C for 10 min in the dark, vortexed for 2 min, and then centrifuged for 10 min. The supernatant from the sample was stored at –20°C until it was analyzed. A 50- to 100-μl portion of the supernatant was injected into a C18 high-performance liquid chromatography column. The

* Corresponding author. Phone: (706) 542-2651. Fax: (706) 542-2674. Electronic mail address: jwiegel@uga.cc.uga.edu.

† Present address: EPA Environmental Research Laboratory, 960 College Station Road, Athens, GA 30605-2720.

two running solvents used were 100% methanol (solvent A) and 30% methanol–70% 100 mM Tris-HCl buffer (pH 7.75) (solvent B). A linear gradient was made by increasing the proportion of solvent B in a solvent A-solvent B mixture from 5 to 25% at a rate of 0.5%/min and then increasing the proportion of solvent B at a rate of 2%/min until the proportion of solvent B was 95%. The flow rate of the mobile phase was 1.0 ml/min. The wavelength used for detection was 254 nm.

Electron microscopy. Transmission electron microscopy was performed with a model JEM-100 CXII electron microscope (JEOL, Tokyo, Japan). Samples for ultrathin sectioning were prepared as described previously (20, 33), and uranyl acetate and lead citrate were used for poststaining (13).

Cell wall analysis. Cell walls were prepared and peptidoglycan structure was determined by using the methods described by Schleifer and Kandler (30). Amino acids and peptides were separated and characterized by thin-layer chromatography on cellulose sheets.

Sequence analysis of 16S rRNA and data analysis. Total RNA was isolated from *C. hydroxybenzoicum* JW/Z-1^T by using a customary procedure (22, 38, 39). A combination of dideoxynucleotide chain termination and reverse transcriptase (5, 21, 29) was used. The synthesized DNA strands were labeled with thio-³⁵S-labeled dATP (5). A standard set of primers specific for (eu)bacterial 16S rRNAs was used (36). The sequences were aligned by using sequence editor ae2 (C. R. Woese, University of Illinois) and a representative collection of (eu)bacterial 16S rRNAs (26, 28). Corrected pairwise distances (expressed as the estimated numbers of changes per 100 nucleotides) were computed from percentages of similarity (1,286 nucleotides were compared) by using the Jukes-Cantor correction (18) as modified by G. J. Olsen (36) to accommodate the actual nucleotide ratios. Dendrograms were constructed from evolutionary distance matrices by using the algorithm of De Soete (9).

RESULTS

Many morphological and physiological properties of strain JW/Z-1^T, including its characteristic decarboxylation of *para*-hydroxybenzoate and analogous compounds, have been described previously (42, 44). A further description of the characteristics of this organism is given below, mainly to provide a basis for comparison with similar organisms.

Cell wall structure and type. *C. hydroxybenzoicum* cells, rods with pointed ends, exhibited the typical cell wall structure of gram-positive organisms (Fig. 1). The cell wall type was A1 α (L-Lys direct) (36a).

Lipid fatty acid composition. The following fatty acid methyl esters were found: 12:0 (2.1%), 14:0 (18.4%), 16:1 *cis*-7 (2.2%), 16:1 *cis*-9 (10.7%), 16:0 (9.0%), 18:1 *cis*-9 (6.3%), and 18:0 (7.4%). The following aldehydes were found: 16:0 (4.4%) and 18:0 (3.2%). And the following dimethyl acetals were found: 16:0 (15.4%), 16:1 *cis*-9 (2.8%), 18:0 (8.4%), 18:1 *cis*-9 (2.3%), and 18:1 *cis*-11 (2.1%).

Biochemical reactions. Resuspended cells from exponentially growing cultures that were tested by using Virginia Polytechnic Institute manual procedures and methods described by Smibert and Krieg (32) were negative for catalase activity, reduction of nitrate to nitrite, the lipase reaction, gelatin liquefaction, hydrolysis of starch, glycogen, or esculin, lecithinase activity, and acid production from glycogen, cellobiose, maltose, fructose, arabinose, glucose, mannose, ribose, xylose, trehalose, and sorbitol; the cells were positive for indole and sulfide formation.

Utilization of amino acids and nitrogenous compounds.

More than 60% of the free amino acids glycine, arginine, and lysine present in 1% yeast extract-containing medium were utilized during batch culture of *C. hydroxybenzoicum*. Additions of glycine, L-arginine, and L-lysine, singly and in combinations, to the yeast extract-containing medium had different effects on growth (Table 1) and on the production of volatile fatty acids in growing cultures (Table 2) and concentrated cell suspensions (Table 3). The decreases in aspartate, glutamate, valine, methionine, isoleucine, leucine, and phenylalanine contents observed after growth in the yeast extract-containing medium were not affected significantly by the addition of the amino acids arginine, glycine, and lysine (data not shown). Addition of arginine plus glycine increased the final optical density by more than twofold. When lysine was added to the yeast extract-containing medium, alanine was utilized; and when arginine or glycine or both were added, the alanine and proline contents increased. Also, the serine content increased in cultures supplemented with arginine. Because *C. hydroxybenzoicum* is close to pyrinolytic organisms on the 16S rRNA tree, we tested the utilization of uric acid, hypoxanthine, xanthine, guanine, and adenine. However, no significant stimulation of growth was observed when the individual compounds were added at a concentration of 10 mM to the growth medium (44) without additional amino acids.

Influence of sodium and potassium ions and acetic acid on growth. Up to a concentration of 200 mM, sodium chloride stimulated and potassium chloride inhibited the growth of *C. hydroxybenzoicum* when these compounds were added to medium containing 0.1 mM sodium ions and 100 mM potassium ions (Fig. 2). Monensin inhibited the growth of *C. hydroxybenzoicum* at a concentration of 1.5 μ M (1 ppm) or higher (other antibiotics were not tested). Addition of 10 mmol of sodium acetate per liter or 10 mmol of potassium acetate per liter to medium that contained about 80 mM sodium ions and 20 mM potassium ions before the addition resulted in a 45% increase in growth (Fig. 3A). In contrast, addition of 10 mmol of sodium chloride per liter of medium or 10 mmol of potassium chloride per liter of medium led to only a slight stimulation of growth (Fig. 3A). However, addition of 100 mmol of sodium acetate per liter of medium, 100 mmol of potassium acetate per liter of medium, 100 mmol of sodium chloride per liter of medium, or 100 mmol of potassium chloride per liter of medium had similar stimulatory effects on growth (Fig. 3B).

DNA and RNA analysis. The evolutionary relationships of *C. hydroxybenzoicum* to other clostridia and other species closely related to clostridia as determined by a 16S rRNA sequence analysis are shown in Fig. 4 and Table 4. The three organisms that were most closely related to *C. hydroxybenzoicum* were *Clostridium purinolyticum*, *Clostridium acidurici*, and *Clostridium sordellii* (10.6, 11.0, and 15.9 inferred changes per 100 bases, respectively).

As determined by the chemical method of Whitman et al. (37), the DNA base compositions of *C. hydroxybenzoicum* and the type strains of the amino acid-utilizing species *C. sticklandii* and *C. aminovalericum* were 35.5, 33.0, and 35.5 mol% G+C, respectively.

DISCUSSION

Comparison of *C. hydroxybenzoicum* with similar clostridia.

(i) **Amino acid utilization.** Addition of glycine or glycine plus arginine stimulated the growth of *C. hydroxybenzoicum* (Table 1), indicating that glycine (as electron acceptor) and arginine (as electron donor) can function as a pair for a Stickland-like reaction (25). In contrast to the results obtained with other clostridia which use the energy-producing arginine deaminase

FIG. 1. Transmission electron micrographs of *C. hydroxybenzoicum* cells. Bars = 0.2 μ m. (A) Resolution of the cell wall with an undulating surface layer (thick arrow) and the cytoplasmic membrane (thin arrow) of a cell apparently in the horizontal plane. (B) Typical cell of *C. hydroxybenzoicum*. (C) Last stage of cell division. Note the pointed ends of the cells.

pathway (8, 24, 31), adding arginine alone to the yeast extract-containing medium did not stimulate the growth of *C. hydroxybenzoicum*; however, arginine almost completely disappeared from the culture, and about 60% of the arginine was recovered

as ornithine (Table 1). The addition of arginine resulted in decreased growth (Tables 1 and 2), suggesting that uptake of arginine is an ATP-requiring process (35). On the basis of the growth obtained, the utilization of glycine and arginine, and

TABLE 1. Growth and amino acid utilization in medium with and without added amino acids

Addition(s)	Growth (%) ^a	Concn of amino acids utilized (mM) ^b				
		Ala	Arg	Gly	Lys	Orn
None	100	0.7	0.7	3.2	0.1	0
L-Arg (6 mmol/liter)	95	+0.5 ^c	7.0	3.1	0.1	+4.1
Gly (13 mmol/liter)	115	+3.6	0.8	12.1	0.1	0
L-Lys (7 mmol/liter)	161	4.6	0.8	3.1	9.1	0
L-Arg (6 mmol/liter) + Gly (13 mmol/liter)	215	+4.5	7.0	13.3	0.1	0
L-Arg (6 mmol/liter) + L-Lys (7 mmol/liter)	144	0.1	7.0	3.1	6.7	+4.1
Gly (13 mmol/liter) + L-Lys (7 mmol/liter)	204	1.5	0.8	6.5	9.1	0

^a A growth value of 100% was equivalent to an optical density of 0.213 at 600 nm. The growth medium contained 1% yeast extract.

^b Before the L-arginine, glycine, and L-lysine additions were made, the yeast extract-containing medium also contained the following concentrations of free amino acids: Ala, 10 mM; Arg, 1.2 mM; Gly, 3.3 mM; Lys, 2.2 mM; and Orn, 0.3 mM. The experimental values were corrected for these zero-time values. The proteins and peptides in the yeast extract-containing medium were not hydrolyzed before the concentrations of the amino acids in the yeast extract-containing medium or the supernatant of a grown culture were determined.

^c A plus sign indicates that the amino acid was produced.

the products formed, ornithine seems to act as an electron donor and energy source in *C. hydroxybenzoicum*, as it does in other amino acid-utilizing clostridia (8). The addition of lysine alone slightly stimulated growth, and butyrate was the major product (Tables 2 and 3). Cell suspensions of *C. hydroxybenzoicum* formed more butyrate than acetate when lysine was added, which indicated that butyrate was produced from acetate or acetyl moieties during lysine catabolism (2). However, a cell suspension supplemented with L-lysine and L-arginine did not form more volatile acids than the control (Table 3). This inhibition might have been due in part to the presence of a cotransport system for lysine and arginine (8).

From the data described above it appears that *C. hydroxybenzoicum* is an organism that has the ability to utilize amino acids like other peptidolytic organisms by using Stickland-like reactions.

The fatty acid profile of *C. hydroxybenzoicum* falls into the group of Elsdén et al. (11) which contains fatty acids that have mainly odd numbers of carbon atoms, 14:0 acids as the major fatty acids, and no significant amounts of iso and anteiso acids. *C. hydroxybenzoicum* formed also as main acids the unsaturated acids 16:1 *cis*-9 and 18:1 *cis*-9 and the saturated acids 16:0 and 18:0. Thus, the *C. hydroxybenzoicum* pattern does not really match any of the previously described fatty acid patterns. However, the fatty acid patterns of anaerobic gram-positive organisms are apparently very sensitive to variations in physiological state and growth conditions; thus, a quantitative comparison with previously published values and values from other libraries is questionable.

Although the characteristic property of *C. hydroxybenzoicum* is the transformation of hydroxybenzoate, which is an intermediate in aromatic amino acid utilization in many peptidolytic

clostridia, this bacterium does not metabolize aromatic amino acids and does not produce *p*-hydroxyphenyl acids from tyrosine; thus, *C. hydroxybenzoicum* also differs in this respect from physiologically similar peptidolytic and amino acid-utilizing clostridia, including *Clostridium sporogenes*, *Clostridium botulinum* (types A, B, F, and G), *Clostridium subterminale*, and *C. sticklandii* (10). When *C. hydroxybenzoicum* was compared with the groups suggested by Mead (23), it fit loosely with the organisms in group IIA; δ -aminovalerate is not produced, and arginine and glycine are utilized. This group contains *C. botulinum* and *Clostridium histolyticum*, but both of these organisms differ from *C. hydroxybenzoicum* in many important properties (Table 5).

The inhibitory effect of monensin on growth and the amino acid utilization combined with the stimulation of *C. hydroxybenzoicum* growth by sodium ions in sodium-deficient medium (Fig. 2) suggests that *C. hydroxybenzoicum* utilizes a sodium-proton antiport and a sodium-amino acid symport(s) (7, 28, 40).

(ii) **G+C content and 16S rRNA sequence analysis.** *C. hydroxybenzoicum* has a DNA base composition of 35.5 mol% G+C (44), which is similar to the DNA base compositions of several other mesophilic clostridia, including *C. sticklandii* (31 mol% G+C as determined by the thermal denaturation method; 33 mol% G+C as determined by a chemical method [this study]), *C. aminovalericum* (33 mol% G+C as determined by the thermal denaturation method; 35.5 mol% G+C as determined by a chemical method [this study]), *Clostridium acetium* (33 mol% G+C as determined by the thermal denaturation method), *Clostridium formicoaceticum* (34 mol% G+C as determined by the thermal denaturation method), and *Clostridium propionicum* (35 mol%) (6). *C. acetium* and *C. formicoaceticum* are glycolytic homoacetogens; *C. propionicum*

TABLE 2. Growth and volatile fatty acid production in culture medium with and without added amino acids

Addition(s)	Growth (%) ^a	Concn of volatile fatty acids produced (mM)					
		Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate
None	100	2.4	0	0.2	2.1	0.2	0.03
Arg (10 mM)	66	2.4	0	0.1	1.2	0.1	0.03
Gly (10 mM)	216	13.0	2.0	0.6	2.2	1.1	0.03
Lys (10 mM)	128	3.1	1.1	0.2	12.7	0.4	0.05
Arg (10 mM) + Gly (10 mM)	469	16.8	1.5	0.4	1.5	0.7	0.04
Arg (10 mM) + Lys (10 mM)	93	3.1	1.5	0.2	17.6	0.4	0.05
Gly (10 mM) + Lys (10 mM)	375	15.3	1.8	0.6	7.7	1.1	0.05

^a A growth value of 100% was equivalent to an optical density of 0.092 at 600 nm. The mineral medium was supplemented with 0.38% yeast extract, 0.86% NaCl, and 20 times the vitamin concentration described previously (36).

TABLE 3. Utilization of three amino acids and production of volatile fatty acids by a *C. hydroxybenzoicum* cell suspension

Addition(s) ^a	Concn of amino acids utilized (mM)					Concn of volatile fatty acids produced (mM)				
	Ala	Arg	Gly	Lys	Orn	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate
None	0				0	0.7	0	0	0.5	Tr
Arg (10 mM)	0	9.9			+7.5 ^b	0.9	0	0	0.4	Tr
Gly (11 mM)	0		3.5		0	5.4	0	0.14	0.3	0.19
Lys (12 mM)	0			7.5	0	7.6	0.7	0.08	11.8	Tr
Arg (10 mM) + Gly (10 mM)	+1.5	10.0	10.0		+5.3	10.6	0	0.17	0.5	0.17
Arg (10 mM) + Lys (10 mM)	0	9.7			+6.5 ^c	0.7	0	0	0.6	Tr
Gly (11 mM) + Lys (10 mM)	0		4.0	1.7	0	6.9	0	0.11	3.6	0.31

^a The organism was grown on yeast extract-containing medium supplemented with 10 mM glycine, L-arginine, and L-lysine and resuspended in mineral medium lacking any carbon and energy sources. The optical density at 600 nm of the cell suspension was about 1.3. After the additions, the cell suspensions were incubated at 30°C for 22 h before the analysis was performed.

^b A plus sign indicates that the amino acid was produced.

^c Since the retention times and coefficient constants of lysine and ornithine are very similar, this value may be less accurate than the other values.

produces propionate as a major fermentation product. The DNA G+C content of *C. aminovalericum* is very similar to that of *C. hydroxybenzoicum*, but other properties of these two organisms, such as fermentation products, the ability to decarboxylate hydroxybenzoate, the ability to utilize substrates, and the 16S rRNA sequences, are significantly different. Thus, all of these species with similar G+C contents are physiologically and phylogenetically different from *C. hydroxybenzoicum*.

On the basis of 16S rRNA sequence analysis data, *C. hydroxybenzoicum* is much more closely related to the purinolytic clostridia, *C. purinolyticum* and *C. acidurici*, than to the typical amino acid utilizers that have similar G+C contents (*C. sticklandii* and *C. aminovalericum*) and to other peptidolytic clostridia (27). However, since growth of *C. hydroxybenzoicum* is not stimulated by the addition of 10 mM adenine, hypoxanthine, uric acid, guanine, or xanthine to the medium, *C. purinolyticum* and *C. acidurici* differ physiologically from *C. hydroxybenzoicum*.

Thus, although the genus *Clostridium* is very heterogeneous

and the 16S rRNA sequence of *C. hydroxybenzoicum* places it outside the group of species around *C. butyricum* (the type species), which represents clostridia sensu stricto, we place this organism tentatively in a new *Clostridium* species, *C. hydroxybenzoicum*, until more closely related organisms are identified and rules for reorganization of the genus have been formulated.

Description of *Clostridium hydroxybenzoicum* sp. nov. *Clostridium hydroxybenzoicum* (hy. drox. y. ben. zo' i. cum. Gr. n. hydro, water; Gr. n. oxys, acid; N. L. n. hydroxy, water acid; Ger. and Fr. n. benzoin, frankincense of Java; Ger. n. benzoessäure, resin obtained from the tree *Styrax benzoin*; N. L. adj. hydroxybenzoicum, pertaining to the organic acid hydroxybenzoic acid, referring to the characteristic feature of this organism, the reversible decarboxylation of 4-hydroxybenzoate and 3,4-dihydroxybenzoate) (42, 44). Two differently induced decarboxylases are responsible for the reversible decarboxylation of 4-hydroxybenzoate and the decarboxylation of 3,4-dihydroxybenzoate. The use, however, of the decarboxylation products,

FIG. 2. Effect of sodium ions on the growth of *C. hydroxybenzoicum*. Mineral medium was supplemented with 0.2% yeast extract, 0.2% glycine, 0.2% arginine, and 10 times the vitamin concentration used previously (44). The medium contained 0.1 mM sodium and 100 mM potassium after inoculation. NaCl (A) or KCl (B) was added to the sodium-deficient medium described above at a concentration of 10 mM (●), 20 mM (□), 50 mM (■), 100 mM (△), or 200 mM (▲). ○, control (no NaCl or KCl added).

FIG. 3. Effect of acetic acid on the growth of *C. hydroxybenzoicum*. Mineral medium was supplemented with 1% yeast extract, 20 times the vitamin concentration used previously (44), 80 mM sodium, and 20 mM potassium ions. (A) Compounds added at a concentration of 10 mmol per liter of medium. (B) Compounds added at a concentration of 100 mmol per liter of medium. Symbols: ■, sodium acetate; ●, sodium chloride; □, potassium acetate; △, potassium chloride; ○, control.

FIG. 4. Phylogenetic tree for *C. hydroxybenzoicum* and related organisms based on 16S rRNA sequences (see Materials and Methods and Table 4). Bar = 10% inferred changes per 100 nucleotides. *E. tenue*, *Eubacterium tenue*; *M. elsdenii*, *Megasphaera elsdenii*; *B. subtilis*, *Bacillus subtilis*. *B. subtilis* was used as an outgroup to root the tree.

e.g., phenol and catechol, as a carbon or energy source by *C. hydroxybenzoicum* was not observed.

C. hydroxybenzoicum cells are strictly anaerobic, slightly curved rods that are 0.35 to 0.76 μm in diameter and 2.5 to 5.1 μm long, depending on the growth conditions and the growth stage. The organism is nonmotile, and no flagella have been

observed in negatively stained cells by use of an electron microscope. Sporulating cells elongate to between 5.0 and 8.0 μm long and produce terminal swollen sporangia (drumstick type). Spores are round to slightly oval (diameter, 0.8 to 1.1 μm). The Gram reaction of the cells from the early to late exponential growth phase is negative. However, lipopolysaccharides are not detected, indicating that the organism is gram positive. The cell wall type is A1 α (L-Lys direct).

The pH supporting growth ranges from 6.1 to 10, and the optimal pH is 7.2 to 8.2. The temperature range for growth is 12 to 41°C, and the optimal temperature is 33 to 34°C.

C. hydroxybenzoicum requires yeast extract for growth. Acetic acid and sodium ions at concentrations of up to 10 and 200 mM, respectively, stimulate growth, whereas 5% NaCl completely inhibits growth. Neither $\text{CO}_2\text{-HCO}_3^-$ nor sulfide is required for growth; addition of nitrate, sulfate, or thiosulfate has no effect on growth. During growth in mineral medium containing 1.5% yeast extract and 200 mM acetate, 5 mM butyrate, 4 mM isovalerate, 1 mM propionate, 1 mM isobutyrate, 0.2 mM isopentanol, and traces of valerate are formed, but H_2 is not formed. Addition of 0.1% each of glycine, arginine, and lysine to yeast extract-containing medium stimulates growth three- to fivefold. Carbohydrates are not fermented; there is no significant acid formation from carbohydrates by resting cells. Addition of H_2 plus CO_2 , ethanol, propanol, isobutanol, propionic acid, butyric acid, isobutyric acid, valeric acid, pyruvic acid, lactic acid, uric acid, adenine, hypoxanthine, guanine, or xanthine does not stimulate growth.

Tests for catalase activity, reduction of nitrate to nitrite, lipase activity, gelatin liquefaction, hydrolysis of starch, glycogen, or esculin, and lecithinase activity are negative; tests for indole and sulfide formation are positive.

The DNA base composition is 35.5 mol% G+C (as determined by the chemical method).

So far, *C. hydroxybenzoicum* has been enriched and isolated only from methanogenic sediment from a freshwater pond in Athens, Ga.

TABLE 4. Evolutionary distances between *C. hydroxybenzoicum* and organisms that are members of the clostridial subphylum

Organism	Evolutionary distance																
	<i>C. hydroxybenzoicum</i>	<i>C. purinolyticum</i>	<i>C. acidurici</i>	<i>C. sordellii</i>	<i>Eubacterium tenue</i>	<i>C. lituseburense</i>	<i>C. manganotii</i>	<i>C. sticklandii</i>	<i>C. aminovalericum</i>	<i>C. oroticum</i>	<i>C. scatologenes</i>	<i>C. pasteurianum</i>	<i>C. butyricum</i>	<i>C. barkeri</i>	<i>Megasphaera elsdenii</i>	<i>C. quercicolum</i>	<i>C. thermoaceticum</i>
<i>C. purinolyticum</i>	10.9																
<i>C. acidurici</i>	11.6	6.2															
<i>C. sordellii</i>	16.0	12.9	13.4														
<i>Eubacterium tenue</i>	15.8	13.0	13.4	1.0													
<i>C. lituseburense</i>	17.3	13.4	14.3	3.7	4.0												
<i>C. manganotii</i>	17.1	15.4	14.7	6.3	6.5	7.1											
<i>C. sticklandii</i>	18.0	15.9	16.3	10.8	10.8	12.7	13.5										
<i>C. aminovalericum</i>	16.8	15.6	15.6	15.1	15.9	15.4	16.6	17.4									
<i>C. oroticum</i>	17.8	17.3	16.5	15.0	15.6	16.3	16.5	18.1	8.5								
<i>C. scatologenes</i>	17.4	15.5	16.0	15.4	15.5	16.3	16.8	17.2	18.0	17.6							
<i>C. pasteurianum</i>	16.1	14.2	14.9	16.5	16.8	17.8	18.0	17.6	18.0	17.9	6.0						
<i>C. butyricum</i>	17.1	15.1	15.8	17.1	17.5	17.0	16.9	18.4	18.0	17.3	8.6	8.0					
<i>C. barkeri</i>	16.7	14.9	14.3	18.2	17.9	18.8	18.2	20.0	19.5	20.5	18.3	17.4	19.1				
<i>Megasphaera elsdenii</i>	22.8	20.1	22.8	22.9	23.4	22.6	25.3	22.4	22.2	22.6	23.1	22.2	23.8	23.9			
<i>C. quercicolum</i>	20.7	18.2	20.0	20.9	21.0	21.1	20.9	23.1	21.7	22.6	22.4	21.2	23.0	21.2	13.2		
<i>C. thermoaceticum</i>	17.6	16.4	16.6	17.6	17.5	18.3	18.2	18.4	19.4	19.5	18.1	17.7	19.2	18.0	17.6	16.4	
<i>Bacillus subtilis</i>	19.3	16.5	16.2	18.1	17.9	18.6	18.7	19.6	19.5	19.8	17.3	16.0	18.0	17.4	21.3	18.0	15.7

TABLE 5. Characteristics that differentiate *C. hydroxybenzoicum* from related bacteria^a

Characteristic	<i>C. hydroxybenzoicum</i>	<i>C. aminowaltericum</i>	<i>C. hisiolyticum</i>	<i>C. purinolyticum</i>	<i>C. scatologenes</i>	<i>C. sticklandii</i>	<i>C. thermoaceticum</i>
G+C content (mol %)	35.5	35.5	NR ^b	29	27	33	54
Decarboxylation of:							
4-OHB	+	—	—	—	—	—	+
3,4-OHB	+	—	—	—	+	—	+
Fermentation of:							
Glucose	—	+	+	—	+	—	+
Glycine	+	NR	+	+	NR	+	NR
Arginine	+	NR	+	NR	+	+	NR
Lysine	+	NR	—	NR	NR	+	NR
Gelatin hydrolysis	—	—	+	—	—	—	—
Gram stain reaction	—	+	+	+	+	+	+
Motility	—	+	+	+	+	+	±
Cell size (µm)	0.35–0.67 × 2.5–5.1	0.56–0.71 × 1.9–5.1	0.5–0.9 × 9.2–13	1.1–1.6 × 2.7–6.9	0.5–1.6 × 3.1–21.2	0.3–0.5 × 1.3–3.8	0.4 × 2.8
Cell shape	Slightly curved rods	Straight rods	Straight rods	Straight rods	Straight rods	Straight rods	Straight rods
Spore shape	Round	Round	Oval	Round	Oval	Oval	Round
Spore position	Terminal	Terminal	Central to subterminal	Terminal	Terminal to subterminal	Subterminal	Terminal
Cell wall contains	L-Lys direct	meso-DAP ^c	meso-DAP, glutamine, alanine	meso-DAP	meso-DAP	NR	L-L-DAP, glycine ^d
Optimum temp (°C)	33–34	NR	37	36	33–37	30–37	55–60
H ₂ production	—	+	+	—	+	+	+
Nitrate reduction	—	—	—	—	±	—	+
Indole production	+	—	—	—	—	—	NR

^a Most data for *C. aminovalericum*, *C. hisiolyticum*, *C. purinolyticum*, *C. scatologenes*, *C. sticklandii*, and *C. thermoaceticum* are from reference 6; the exceptions are the data for decarboxylation of 4-hydroxybenzoate (4-OHB) and 3,4-dihydroxybenzoate (3,4-OHB) and the G+C content data (as determined by the chemical method) for *C. aminowaltericum* and *C. sticklandii*, which were determined in this study.

^b NR, not reported.

^c meso-DAP, meso-diaminopimelic acid.

^d L-L-DAP, L-L-diaminopimelic acid.

Type strain. Strain JW/Z-1 is the type strain of *C. hydroxybenzoicum* ATCC 51151 = DSM 7310. The G+C content of this strain is 35.5 mol%, and the accession number of its 16S rRNA is L11305. The properties of the type strain are the same as those described above for the species.

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REFERENCES

- American Type Culture Collection. 1989. Catalogue of bacteria and bacteriophages, 17th ed. American Type Culture Collection, Rockville, Md.
- Barker, H. A. 1981. Amino acid degradation by anaerobic bacteria. *Annu. Rev. Biochem.* **50**:23–40.
- Bayer, E., E. Grom, B. Kaltenecker, and R. Uhmman. 1976. Separation of amino acids by high performance liquid chromatography. *Anal. Chem.* **48**:1106–1109.
- Berry, D. F., A. J. Francis, and J. M. Bollag. 1987. Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. *Microbiol. Rev.* **51**:43–49.
- Biggin, M. D., T. J. Gibson, and G. F. Hing. 1983. Buffer gradient gels and ³⁵S label as an aid to rapid DNA sequence determination. *Proc. Natl. Acad. Sci. USA* **80**:3936–3965.
- Cato, E. P., W. L. George, and S. M. Finegold. 1986. *Clostridium* Prazmowski 1880, 23^{AL}, p. 1141–1200. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 2. The Williams & Wilkins Co., Baltimore.
- Chen, G., and J. B. Russell. 1989. Sodium-dependent transport of branched-chain amino acids by a monensin-sensitive ruminal *Peptostreptococcus*. *Appl. Environ. Microbiol.* **55**:2658–2663.
- Cunin, R., N. Glansdorff, A. Pierard, and V. Stalon. 1986. Biosynthesis and metabolism of arginine in bacteria. *Microbiol. Rev.* **50**:314–352.
- De Soeto, G. 1983. A least square algorithm for fitting additive trees to proximity data. *Psychometrika* **48**:621–626.
- Elsden, S. R., and M. G. Hilton. 1979. Amino acid utilization patterns in clostridial taxonomy. *Arch. Microbiol.* **123**:137–141.
- Elsden, S. R., M. G. Hilton, K. R. Parsley, and R. Self. 1980. The lipid fatty acids of proteolytic clostridia. *J. Gen. Microbiol.* **118**:115–123.
- Evans, W. C., and G. Fuchs. 1988. Anaerobic degradation of aromatic compounds. *Annu. Rev. Microbiol.* **42**:289–317.
- Frasca, J. M., and V. R. Parks. 1965. A routine technique for double-staining ultrathin sections using uranyl and lead salts. *J. Cell Biol.* **25**:157–161.
- Genthner, B. R. S., G. T. Townsend, and P. J. Chapman. 1990. Effect of fluorinated analogues of phenol and hydroxybenzoates on the anaerobic transformation of phenol to benzoate. *Biodegradation* **1**:65–74.
- Hagblom, M. 1990. Mechanisms of bacterial degradation and transformation of chlorinated compounds. *J. Basic Microbiol.* **30**:115–141.
- Hale, D. D., W. Reineke, and J. Wiegel. Chlorophenol degradation. In G. R. Chaudry (ed.), *Biological degradation and bioremediation technologies of toxic chemicals*, in press. Timber Press, Portland, Ore.
- Hippe, H., J. R. Andreesen, and G. Gottschalk. The genus *Clostridium*—nonmedical, p. 1800–1866. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), *The prokaryotes*. Springer-Verlag, New York.
- Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21–132. In H. N. Munro (ed.), *Mammalian protein metabolism*. Academic Press, New York.
- Kaneda, N., M. Sato, and K. Yagi. 1982. Analysis of dansyl amino acids by reversed-phase high-performance liquid chromatography. *Anal. Biochem.* **127**:49–54.
- Kellenberger, E., A. Ryter, and J. Sechaud. 1958. Electron microscope study of DNA-containing plasma. II. Vegetative and mature phage DNA as compared with normal bacterial nucleosides in different physiological states. *J. Biophys. Biochem. Cytol.* **4**:671–678.
- Lane, D. J., B. Pace, G. J. Olsen, and D. A. Stahl. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. USA* **82**:6955–6959.
- Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* **3**:208–218.
- Mead, G. C. 1971. The amino acid fermenting clostridia. *J. Gen. Microbiol.* **67**:47–56.
- Mitruka, B. M., and R. N. Costilow. 1967. Arginine and ornithine catabolism by *Clostridium botulinum*. *J. Bacteriol.* **93**:295–301.
- Nisman, B. 1954. The Stickland reaction. *Bacteriol. Rev.* **18**:16–42.
- Olsen, G. J., R. Overbeek, N. Larsen, T. L. Marsh, M. J. McCaughey, M. A. Maciukenas, W. M. Kuan, T. J. Macke, and C. R. Woese. 1992. The ribosomal database project. *Nucleic Acids Res.* **20**(Suppl.):2199–2200.
- Paster, B. J., J. B. Russell, C. M. J. Yang, J. M. Chow, C. R. Woese, and R. Tanner. 1993. Phylogeny of ammonia-producing ruminal bacteria *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, and *Clostridium aminophilum* sp. nov. *Int. J. Syst. Bacteriol.* **43**:107–110.
- Russell, J. B. 1987. A proposed mechanism of monensin action in inhibiting ruminal bacterial growth: effects on ion flux and protonmotive force. *J. Anim. Sci.* **64**:1519–1525.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
- Schleifer, K. H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* **36**:407–477.
- Schmidt, G. C., M. A. Logan, and A. A. Tytell. 1952. The degradation of arginine by *Clostridium perfringens* (BP6K). *J. Biol. Chem.* **198**:771–783.
- Smibert, R. M., and N. R. Krieg. 1981. General characterization, p. 409–443. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), *Manual of methods for general bacteriology*. American Society for Microbiology, Washington, D.C.
- Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**:31–43.
- Tapuhi, Y., D. E. Schmidt, W. Lindner, and B. L. Karger. 1981. Dansylation of amino acids for high-performance liquid chromatography analysis. *Anal. Biochem.* **115**:123–129.
- Van Kessel, J. S., and J. B. Russell. 1992. Energetics of arginine and lysine transport by whole cells and membrane vesicles of strain SR, a monensin-sensitive ruminal bacterium. *Appl. Environ. Microbiol.* **58**:969–975.
- Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. vanEtten, J. Maniloff, and C. R. Woese. 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. *J. Bacteriol.* **171**:6455–6467.
- Weiss, N. Personal communication.
- Whitman, W. B., S. Jersong, S. Sohn, D. S. Caras, and U. Premachandran. 1986. Isolation and characterization of 22 mesophilic methanococci. *Syst. Appl. Microbiol.* **7**:235–240.
- Woese, C. R., R. Gutell, R. Gupta, and H. F. Noller. 1983. Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. *Microbiol. Rev.* **47**:621–669.
- Woese, C. R., M. Sogin, D. A. Stahl, B. J. Lewis, and L. Bonen. 1976. A comparison of the 16S ribosomal RNAs from mesophilic and thermophilic bacilli. *J. Mol. Evol.* **7**:197–213.
- Yamato, I., and Y. Anraku. 1990. Mechanism of Na⁺/proline symport in *Escherichia coli*: reappraisal of the effect of cation binding to the Na⁺/proline symport carrier. *J. Membr. Biol.* **114**:143–151.

41. Young, L. Y. 1984. Anaerobic degradation of aromatic compounds, p. 487–523. In D. E. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, New York.
42. Zhang, X., R. L. Gherna, and J. Wiegel. 1992. Decarboxylation of hydroxybenzoate by strictly anaerobic bacteria in methanogenic sediments and pure cultures, p. 498–504. In DEHEMA, Proceedings of the International Symposium on Soil Decontamination Using Biological Processes, Karlsruhe.
43. Zhang, X., T. V. Morgan, and J. Wiegel. 1990. Conversion of ^{13}C -1 phenol to ^{13}C -4 benzoate, an intermediate in the anaerobic degradation of chlorophenols. FEMS Lett. **67**:63–66.
44. Zhang, X., and J. Wiegel. 1990. Isolation and partial characterization of a *Clostridium* species transforming *para*-hydroxybenzoate and 3,4-dihydroxybenzoate and producing phenols as the final transformation products. Microb. Ecol. **20**:103–121.
45. Zhang, X., and J. Wiegel. 1990. Sequential anaerobic degradation of 2,4-dichlorophenol in freshwater sediments. Appl. Environ. Microbiol. **56**:1119–1127.