

## Isolation and Characterization of *Desulfitobacterium dehalogenans* gen. nov., sp. nov., an Anaerobic Bacterium Which Reductively Dechlorinates Chlorophenolic Compounds

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**An organism that is able to reductively *ortho*-dechlorinate 2,4-dichlorophenol and 3-chloro-4-hydroxyphenylacetate (3-Cl-4-OHPA) was isolated from a methanogenic lake sediment. This organism, an anaerobic, motile, Gram-type-positive, rod-shaped bacterium, grew in the presence of 0.1% yeast extract when pyruvate, lactate, formate, or hydrogen was used as the electron donor for reductive dehalogenation of 3-Cl-4-OHPA. Sulfite, thiosulfate, and sulfur were reduced to sulfide, nitrate was reduced to nitrite, and fumarate was reduced to succinate. Dissimilatory reduction of sulfate could not be demonstrated, and no adenylylsulfate reductase was detected with an immunoassay. The organism fermented two pyruvate molecules to one lactate molecule, one acetate molecule, and one carbon dioxide molecule. The pH and temperature optima for both growth and dechlorination of 3-Cl-4-OHPA were 7.5 and 38°C, respectively. The doubling time under these conditions was approximately 3.5 h. On the basis of the results of a 16S rRNA analysis and the inability of the organism to use sulfate as an electron acceptor, strain JW/IU-DC1 is described as the type strain of the new taxon *Desulfitobacterium dehalogenans* gen. nov., sp. nov.**

Although there have been many reports of reductive dehalogenation of chloroaromatic compounds in sediments and enrichment cultures (25), only two obligate anaerobes have been isolated in pure culture, the Gram-type-negative sulfate-reducing bacterium *Desulfomonile tiedjei* DCB-1 (10, 28) and a clostridium-like strain, DCB-2 (22).

In this paper we describe the isolation and characterization of an anaerobic Gram-type-positive organism, *Desulfitobacterium dehalogenans* gen. nov., sp. nov., that is capable of reductive *ortho*-dechlorination of chloroaromatic compounds such as 2,4-dichlorophenol (2,4-DCP) and 3-chloro-4-hydroxyphenylacetate (3-Cl-4-OHPA).

### MATERIALS AND METHODS

**Origin of enrichment culture and organism.** The enrichment culture used in this study was obtained from a freshwater sediment collected from a pond located in a wooded area of the Sandy Creek Nature Park, Athens, Ga. (7, 19). *Desulfitobacterium dehalogenans* JW/IU-DC1<sup>T</sup> (T = type strain) was isolated from the enrichment culture as described below.

**Enrichment, isolation, and cultivation of the dechlorinating organism.** Using oxygen-free nitrogen gas, we prepared media by the Hungate technique (21) and sterilized media by autoclaving them at 121°C for 40 min. Unless indicated otherwise, cultures were grown anaerobically at 37°C in Hungate tubes (borosilicate glass; 16 by 125 mm; Belco Glass, Inc., Vineland, N.J.) containing 5 to 10 ml of medium.

The enrichment culture was grown in a mineral medium described previously (39), except that our medium contained 2 mM KH<sub>2</sub>PO<sub>4</sub> and 40 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS). Unless indicated otherwise below, the enrichment medium was supplemented with yeast extract (0.2 to 1%) as a carbon source. The pH was kept

within the range from 8.0 to 8.4 by adjusting it with anaerobic sterile 2 N NaOH. The medium used for further enrichment and isolation was prepared by using the spent medium from a coculture of *Clostridium* sp. strain JW/IU-YU1 and a facultative anaerobic coccus (JW/IU-DC1), both of which were isolated previously from the primary stable enrichment culture. The spent medium was obtained by growing the coculture in the mineral medium described above supplemented with 0.5 or 0.1% yeast extract and 0.1% (wt/vol) glucose. After centrifugation of the cultures (which were kept in Hungate tubes), transfer of the supernatant into new sterile, anaerobic tubes, autoclaving for 40 min at 121°C, and adjustment of the pH to 8.2 with anaerobic, sterile 2 N NaOH, the supernatant was supplemented with 15 mM 3-Cl-4-OHPA. Final isolation was accomplished by repeatedly isolating single colonies growing in agar (0.6 and 0.9%, wt/vol) shake cultures, using the enrichment medium described above supplemented with 2 mM pyruvate, 2 mM formate, and 1 mM 3-Cl-4-OHPA. The mineral medium (base medium) used for cultivation of the purified culture was the same as the enrichment medium (see above) except that it contained 40 mM KH<sub>2</sub>PO<sub>4</sub> and no TAPS. The pH was adjusted to 7.5 with anaerobic sterile 2 N NaOH after the medium was autoclaved at 121°C for 40 min. Unless indicated otherwise, the medium was supplemented with 0.1% yeast extract, 20 mM pyruvate, and 10 mM 3-Cl-4-OHPA (purified-culture medium).

**Light microscopy and electron microscopy.** Phase-contrast light microscopy (model PM-10AD; Olympus Optical Co., Ltd., Tokyo, Japan) was used for routine examinations and for taking photomicrographs of agar-coated slides. Electron microscopy and preparation of cells for ultrathin sectioning were carried out as described previously (13, 17, 29) by using uranyl acetate and lead citrate for poststaining. Cells were negatively stained as described by Valentine et al. (30) and Beuscher et al. (3) by using 2% uranyl acetate.

**Gram stain reaction and Gram type.** Gram staining was performed by a modified Hucker method (12), using an Enhanced Gram Stain Kit (Carr Scarborough Microbiologicals, Inc., Decatur, Ga.). The Gram type (34) was determined

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by the polymyxin B-lipopolysaccharide assay as described previously (36).

**Substrate utilization and electron donors for dehalogenation.** Tubes containing the base medium described above were supplemented with 0.1% yeast extract and filter-sterilized substrates at final concentrations of 0.2% (wt/vol) for the sugars and 20 mM for all of the other organic substrates. Control tubes contained no added carbon source. Cultures grown on 20 mM pyruvate at 37°C and pH 7.5 were used as inocula (2% vol/vol). The use of potential electron donors for reductive dehalogenation was assayed in cultures containing 10 mM 3-Cl-4-OHPA. Tubes containing hydrogen as the electron donor were incubated with shaking at 150 rpm (New Brunswick Scientific Co., Inc., Edison, N.J.). A culture grown on medium containing 20 mM pyruvate plus 10 mM 3-Cl-4-OHPA was used as the inoculum. Growth was monitored by determining the increase in optical density at 600 nm at 37°C and pH 7.5. Assays for reductive dechlorination (see below) were performed in duplicate. The maximum incubation time was 11 days.

**Electron acceptors.** The use of different electron acceptors was studied with cultures grown in base medium supplemented with 0.1% yeast extract and various test compounds, using 20 mM pyruvate as the carbon source at 37°C and pH 7.5. All assays were carried out in duplicate. Two uninoculated tubes were used as controls. Chlorophenolic compounds and products of the reduction of these compounds were quantified by high-performance liquid chromatography (HPLC) (Rabbit HP System; Rainin Instrument Co.), using a C<sub>18</sub> reversed-phase column as described by Kohring et al. (19). The test for dissimilatory reduction of nitrate (5 mM) and fumarate (10 mM) was performed in Hungate tubes containing purified-culture medium. Concentrations of nitrate and nitrite were measured spectrophotometrically as described by Hanson and Philips (14). Fumarate and succinate were quantified by gas chromatography, using a model 5880A instrument (Hewlett-Packard, Avondale, Pa.) and a Chromosorb WAW 10% SP-1000 glass column (Supelco, Inc., Bellefonte, Pa.), and were measured with a flame ionization detector as described previously (35). Purified-culture medium lacking Na<sub>2</sub>S and supplemented with 0.1% FeSO<sub>4</sub> was used to assay for dissimilatory reduction of sulfate, sulfite, thiosulfate, and sulfur. Tubes were supplemented with 5 and 10 mM Na<sub>2</sub>SO<sub>4</sub>, 2 mM Na<sub>2</sub>SO<sub>3</sub>, 5 and 20 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, and sulfur powder (about 0.2% added with a spatula in an anaerobic chamber). All tubes were pressurized with hydrogen (20 lb/in<sup>2</sup>). Adenylylsulfate reductase was detected with an immunoassay test kit for the detection of sulfate-reducing bacteria (Conoco Specialty Products, Houston, Tex.).

**pH and temperature ranges.** The pH and temperature ranges for growth and dechlorination were determined by growing the dechlorinating organism in Hungate tubes containing 10 ml of purified-culture medium. The medium used to determine the pH range contained 25 mM potassium phosphate and 25 mM TAPS as buffers rather than 40 mM potassium phosphate. The pH was kept constant by frequently adjusting it with sterile anaerobic 2 N NaOH. The pH range was determined at 37°C. The temperature range was determined at pH 7.5 by using a temperature gradient incubator (Scientific Industries, Inc., Bohemia, N.Y.) set at temperatures ranging from 1.6 to 53°C with a shaking speed of 15 spm.

**Sporulation.** To induce spore formation, *Desulfitobacterium dehalogenans* was grown under various conditions, including in liquid or agar (0.6%, wt/vol)-solidified purified-culture medium. In a coculture with *Clostridium* sp. strain JW/IU-YU1 and facultatively anaerobic coccus strain JW/IU-YU2,

*Desulfitobacterium dehalogenans* was grown in 0.5% yeast extract medium supplemented with 0.4 mM 2,4-DCP at 37°C and pH 8.0. Cultures were analyzed for spore formation by phase-contrast light microscopy and by testing for heat resistance (i.e., growth after treatment at 87°C for 30 min).

**Fermentation products.** H<sub>2</sub> and CO<sub>2</sub> were determined by gas chromatography (Varian, Walnut Creek, Calif., instrument equipped with a Porapak Q 80/100-mesh column [Supelco] and a thermal conductivity detector). The temperatures of the injection port and the column were 160 and 120°C, respectively. The carrier gas was nitrogen, and the flow rate was 30 ml/min. Volatile fatty acids and alcohols, as well as nonvolatile fatty acids, were analyzed as described previously (35) by using a model 5880A gas chromatograph (Hewlett-Packard) equipped with a Chromosorb WAW 10% SP-1000 glass column (Supelco) and a flame ionization detector. Formic acid contents were determined by monitoring NAD reduction at 340 nm in the presence of NAD-dependent formate dehydrogenase obtained from yeast cells (Boehringer Mannheim GmbH, Mannheim, Germany). Acetic acid contents were determined enzymatically by using an acetic acid reagent kit (catalog no. 148-261; Boehringer Mannheim GmbH).

**Determination of G+C content.** DNA was isolated as described by Ausubel et al. (2) by using CsCl gradient ultracentrifugation. The guanine-plus-cytosine (G+C) content was determined after enzymatic digestion and HPLC separation of nucleosides as described by Whitman et al. (32) and Mesbah et al. (24).

**16S rRNA isolation, sequencing method, and data analysis.** 16S rRNA was isolated from strain JW/IU-DC1<sup>T</sup> by previously described procedures (23, 38). The dideoxynucleotide chain termination method (4, 27), adapted for direct rRNA sequencing using reverse transcriptase (20), was employed. Synthesized strands were labeled by including [ $\alpha$ -<sup>35</sup>S]thio-dATP (4). A standard set of primers (usually eight) specific for (eu)bacterial 16S rRNAs (4, 20, 31) was routinely used. All sequences were >90% complete. The sequences were aligned by using sequence editor ae2 (C. R. Woese) with the sequences of a representative collection of (eu)bacterial 16S rRNAs (26, 37). Corrected pairwise distances (expressed as estimated numbers of changes per 100 nucleotides) were computed from percentages of similarity by using the Jukes-Cantor correction (16) as modified by G. J. Olsen (described in reference 31) to accommodate the actual nucleotide ratios. Dendograms were constructed from evolutionary distance matrices by using the algorithm of De Soete (9).

**Sensitivity to oxygen.** Sensitivity of bacterial growth and dechlorination of 3-Cl-4-OHPA to oxygen was determined in 160-ml serum bottles containing 10 ml of purified-culture medium (which contained no reducing solution) under a nitrogen atmosphere in the presence of 0, 0.5, 1, 2, 5, and 9% air. The medium in each tube was inoculated with 0.25 ml of a culture of strain JW/IU-DC1<sup>T</sup> in the exponential growth phase. The bottles were incubated in a shaking incubator (New Brunswick Scientific Co.) at 37°C and 150 rpm. The maximum incubation time was 15 days.

**Effect of sodium chloride on growth and dechlorination.** Sensitivity of growth and dechlorination to NaCl was tested by transferring portions (2%, vol/vol) of an exponentially growing culture into fresh purified-culture medium containing 0, 0.5, 1, 1.5, 2, and 2.5 M NaCl.

**Nucleotide sequence accession number.** The GenBank accession number for the 16S rRNA sequence of strain JW/IU-DC1<sup>T</sup> is L28946.

TABLE 1. Evolutionary distances between *Desulfotobacterium dehalogenans* and related organisms<sup>a</sup>

Organism	Evolutionary distance																								
	<i>Desulfotobacterium dehalogenans</i> JW/IU-DC1 <sup>T</sup>	<i>Desulfotomaculum orientis</i>	<i>Desulfotomaculum ruminis</i>	<i>Desulfotomaculum nigrificans</i>	<i>Heliobacterium chlorum</i>	<i>Sporomusa paucivorans</i>	<i>Megasphaera elsdenii</i>	<i>Desulfotomaculum australicum</i>	<i>Desulfotomaculum thermobenzoicum</i>	<i>Acetogenium kivui</i>	<i>Clostridium thermoaceticum</i>	<i>Clostridium thermosaccharolyticum</i>	<i>Clostridium fervidus</i>	<i>Clostridium butyricum</i>	<i>Clostridium pasteurianum</i>	<i>Clostridium perfringens</i>	<i>Clostridium kluyveri</i>	<i>Clostridium barkeri</i>	<i>Clostridium acidurici</i>	<i>Clostridium lituseburense</i>	<i>Clostridium aminovalericum</i>	<i>Clostridium symbiosum</i>	<i>Clostridium thermocellum</i>	<i>Clostridium thermolacticum</i>	
<i>Desulfotomaculum orientis</i>	9.2																								
<i>Desulfotomaculum ruminis</i>	14.6	16.9																							
<i>Desulfotomaculum nigrificans</i>	15.0	17.4	7.8																						
<i>Heliobacterium chlorum</i>	14.7	16.9	16.1	15.7																					
<i>Sporomusa paucivorans</i>	19.0	18.1	21.9	19.9	20.5																				
<i>Megasphaera elsdenii</i>	18.6	19.2	20.7	18.9	22.5	15.5																			
<i>Desulfotomaculum australicum</i>	15.4	16.1	14.5	13.6	17.3	21.2	21.1																		
<i>Desulfotomaculum thermobenzoicum</i>	14.8	16.4	14.1	12.3	17.2	19.6	20.4	4.7																	
<i>Acetogenium kivui</i>	17.7	18.0	18.4	17.4	18.2	20.3	20.3	12.2	12.2																
<i>Clostridium thermoaceticum</i>	15.4	16.4	16.4	14.3	17.4	19.0	18.4	11.2	10.6	10.8															
<i>Clostridium thermosaccharolyticum</i>	20.8	21.6	19.0	18.2	19.7	23.6	19.5	15.9	15.5	12.8	12.8														
<i>Clostridium fervidus</i>	18.2	19.5	19.2	18.0	17.3	22.4	21.5	16.8	16.0	14.6	14.3	15.5													
<i>Clostridium butyricum</i>	20.1	21.1	20.9	19.7	20.8	23.6	25.2	21.8	21.4	21.6	18.8	19.4	15.4												
<i>Clostridium pasteurianum</i>	21.2	20.4	22.1	20.7	19.6	22.3	24.6	22.2	22.9	20.8	18.2	19.8	14.7	6.9											
<i>Clostridium perfringens</i>	18.9	18.6	21.5	20.5	19.7	25.2	25.2	20.3	20.8	19.6	18.9	18.2	15.4	6.5	8.6										
<i>Clostridium kluyveri</i>	21.5	21.1	20.3	21.1	20.4	23.8	24.9	21.8	20.7	20.4	18.0	17.7	15.0	11.2	9.2	10.9									
<i>Clostridium barkeri</i>	21.8	20.4	20.8	21.5	23.3	24.5	25.1	20.7	20.1	21.5	18.9	19.2	21.4	19.8	19.0	18.2	17.0								
<i>Clostridium acidurici</i>	18.2	20.4	19.2	18.6	20.8	24.6	24.8	18.1	18.0	19.0	15.7	17.7	16.9	14.6	15.4	15.1	15.6	14.0							
<i>Clostridium lituseburense</i>	18.6	20.8	19.3	19.3	20.9	24.5	23.2	19.0	19.6	19.2	16.6	19.8	18.1	15.0	16.5	14.7	17.6	18.5	13.3						
<i>Clostridium aminovalericum</i>	18.4	20.4	19.4	18.5	21.6	22.6	21.9	21.2	20.1	20.3	18.1	21.0	16.1	16.4	16.8	17.3	18.2	20.4	15.5	15.7					
<i>Clostridium symbiosum</i>	20.8	21.8	22.2	21.2	20.8	23.8	22.5	22.4	21.9	21.7	19.9	22.2	20.3	18.0	19.0	17.3	20.5	22.3	18.9	16.3	9.2				
<i>Clostridium thermocellum</i>	17.0	17.8	18.8	17.3	18.8	19.7	18.5	17.2	16.3	17.0	15.0	16.8	14.5	16.5	17.4	16.4	17.4	17.2	17.3	17.6	15.5	18.4			
<i>Clostridium thermolacticum</i>	18.0	18.6	19.5	19.0	19.5	21.2	18.8	17.7	16.7	16.9	15.5	18.1	15.0	20.5	20.4	20.5	19.2	19.7	18.5	19.9	14.8	17.3	7.8		
<i>Bacillus subtilis</i>	16.1	16.4	18.5	17.4	19.4	19.7	22.8	17.6	16.1	18.2	15.1	18.9	17.4	18.1	17.8	17.6	18.4	18.1	16.8	18.4	19.8	20.5	19.4	22.1	

<sup>a</sup> Only those positions having known bases in all sequences were used in the analysis. *Bacillus subtilis* was used as an unweighted outgroup.

## RESULTS AND DISCUSSION

**Isolation of dechlorinating strain JW/IU-DC1<sup>T</sup>.** The starting enrichment culture (7) grown in the presence of 1% (wt/vol) yeast extract contained approximately 10<sup>6</sup> to 10<sup>7</sup> dehalogenat-

ing cells per ml of culture, as determined by serial dilution (1:5). Changing the chlorinating compound from 1 mM 2,4-DCP (higher concentrations were inhibitory) to 15 mM 3-Cl-4-OHPA and using spent medium from a coculture of the yeast extract-utilizing organism *Clostridium* sp. strain JW/IU-YU1

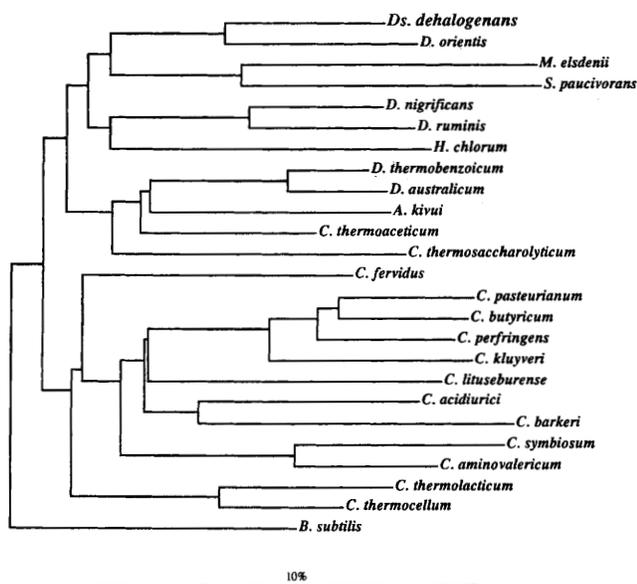


FIG. 1. Unrooted phylogenetic tree for *Desulfotomaculum dehalogenans* JW/IU-DC1<sup>T</sup> and related organisms based on 16S rRNA sequences (see Materials and Methods and Table 1). Bar = 10 inferred changes per 100 nucleotides. Abbreviations: A., *Acetogenium*; B., *Bacillus*; C., *Clostridium*; D., *Desulfotomaculum*; Ds., *Desulfotomaculum*; H., *Heliobacterium*; M., *Megasphaera*; S., *Sporomusa*. *Bacillus subtilis* was used as an arbitrary outgroup organism.

and a facultatively anaerobic coccus (JW/IU-YU2), both of which were isolated from the enrichment culture, increased the concentration of the dechlorinating organisms approximately 100-fold. The culture consisted mainly of two different organisms. A pure culture of the dechlorinating organism was finally obtained by isolating single colonies in soft agar shake cultures containing 0.6 and 0.9% (wt/vol) agar (21). Higher agar concentrations were inhibitory for the dechlorinating culture but not for the contaminating organism, an unidentified clostridium-like organism. A convenient routine check for the purity of this culture was the lack of growth on and in 1.2% (wt/vol) agar-solidified medium containing 20 mM pyruvate

and 0.3% yeast extract, a medium that many organisms grow well on.

**Phylogeny.** The 16S rRNA analysis of strain JW/IU-DC1<sup>T</sup> placed it in the *Desulfotomaculum*-*Clostridium* subphylum (Table 1; Fig. 1). *Desulfotomaculum orientis* is the most closely related species (phylogenetic distance, 9.2%). The new isolate is clearly separated from all groups of *Clostridium* species for which sequences are currently available, including the species regarded as *Clostridium sensu stricto*, closely related to the type species, *Clostridium butyricum*. Like the members of the genus *Clostridium*, the currently known species that are assigned to the genus *Desulfotomaculum* do not form a uniform and defined group around the type species, *Desulfotomaculum nigrificans*. We expect that in the near future both of these genera will be subdivided into different genera. In particular, *Desulfotomaculum orientis* is about 17% different from the type species, *Desulfotomaculum nigrificans*, and thus should belong to a different genus. Additional data (i.e., additional sequences of similar organisms) will be required to decide whether *Desulfotomaculum dehalogenans* and *Desulfotomaculum orientis* belong to the same genus. Many phenotypic properties of *Desulfotomaculum dehalogenans* (see below) and the species belonging to the genus *Desulfotomaculum* are similar (Table 2). However, on the basis of the results of the 16S rRNA analysis and the inability of the organism to carry out dissimilatory sulfate reduction but using a variety of electron acceptors, including elemental sulfur, we concluded that the strain which we isolated does not belong to any previously validly published genus and placed it, therefore, in a new genus and species, *Desulfotomaculum dehalogenans*.

**Description of the genus *Desulfotomaculum* gen. nov.** *Desulfotomaculum* (De.sul.fi.to.bac.te'ri.um. L. pref. de, from, off, away; L. n. sulfur, the element sulfur; L. n. sulfite, alteration of sulfate, referring to a less oxygenated sulfur anion; L. masc. n. bacter, rod; *Desulfotomaculum*, rod-shaped organism that reduces sulfite). The description of this genus is the same as that of *Desulfotomaculum dehalogenans*, the type and so far only species in the genus.

**Description of *Desulfotomaculum dehalogenans* sp. nov.** *Desulfotomaculum dehalogenans* (de.ha.lo'ge.nans. L. pref. de, off, away; Gr. n. hals, salt, sea; F. n. halogen, referring to the group VII elements; L. pres. part. dehalogenans, dehalogenating, split off halogens, referring to the characteristic property

TABLE 2. Selected properties of *Desulfotomaculum dehalogenans* and *Desulfotomaculum* species<sup>a</sup>

Species	Cell morphology	Morphology and location of spores	Growth with pyruvate without electron acceptors	Autotrophic growth	Electron acceptors			G+C content of DNA (mol%)	Oxidation of organic substrates
					Sulfate	Nitrate	Sulfur		
<i>Desulfotomaculum dehalogenans</i>	Straight or curved rods	Usually not present	+	-	-	+	+	45	Incomplete
<i>Desulfotomaculum orientis</i>	Straight or curved rods <sup>c</sup>	Slightly oval, terminal to subterminal <sup>c</sup>	- <sup>d</sup>	+ <sup>e</sup>	+ <sup>d</sup>	- <sup>d</sup>	- <sup>f</sup>	45 <sup>b,c</sup>	Incomplete <sup>c</sup>
<i>Desulfotomaculum nigrificans</i>	Rods <sup>c</sup>	Oval, central <sup>c</sup>	+ <sup>d</sup>	+ <sup>e,g</sup>	+ <sup>d</sup>	- <sup>d</sup>	- <sup>f</sup>	45 <sup>b,c</sup>	Incomplete <sup>c</sup>
<i>Desulfotomaculum ruminis</i>	Rods <sup>c</sup>	Oval, central <sup>c</sup>	+ <sup>d</sup>	+ <sup>e,g</sup>	+ <sup>d</sup>	- <sup>d</sup>	- <sup>f</sup>	49 <sup>b,c</sup>	Incomplete <sup>c</sup>

<sup>a</sup> *Desulfotomaculum dehalogenans* and the *Desulfotomaculum* species (5, 18) use lactate, pyruvate, H<sub>2</sub>, and formate but not acetate as electron donors for reduction of 3-Cl-4-OHPA and sulfate, respectively.

<sup>b</sup> The values for *Desulfotomaculum* species are different from the values in references 5 and 6.

<sup>c</sup> Data from reference 33.

<sup>d</sup> Data from reference 5.

<sup>e</sup> Data from reference 18.

<sup>f</sup> Data from reference 15.

<sup>g</sup> Determined in the presence of 1 mmol of acetate per liter.

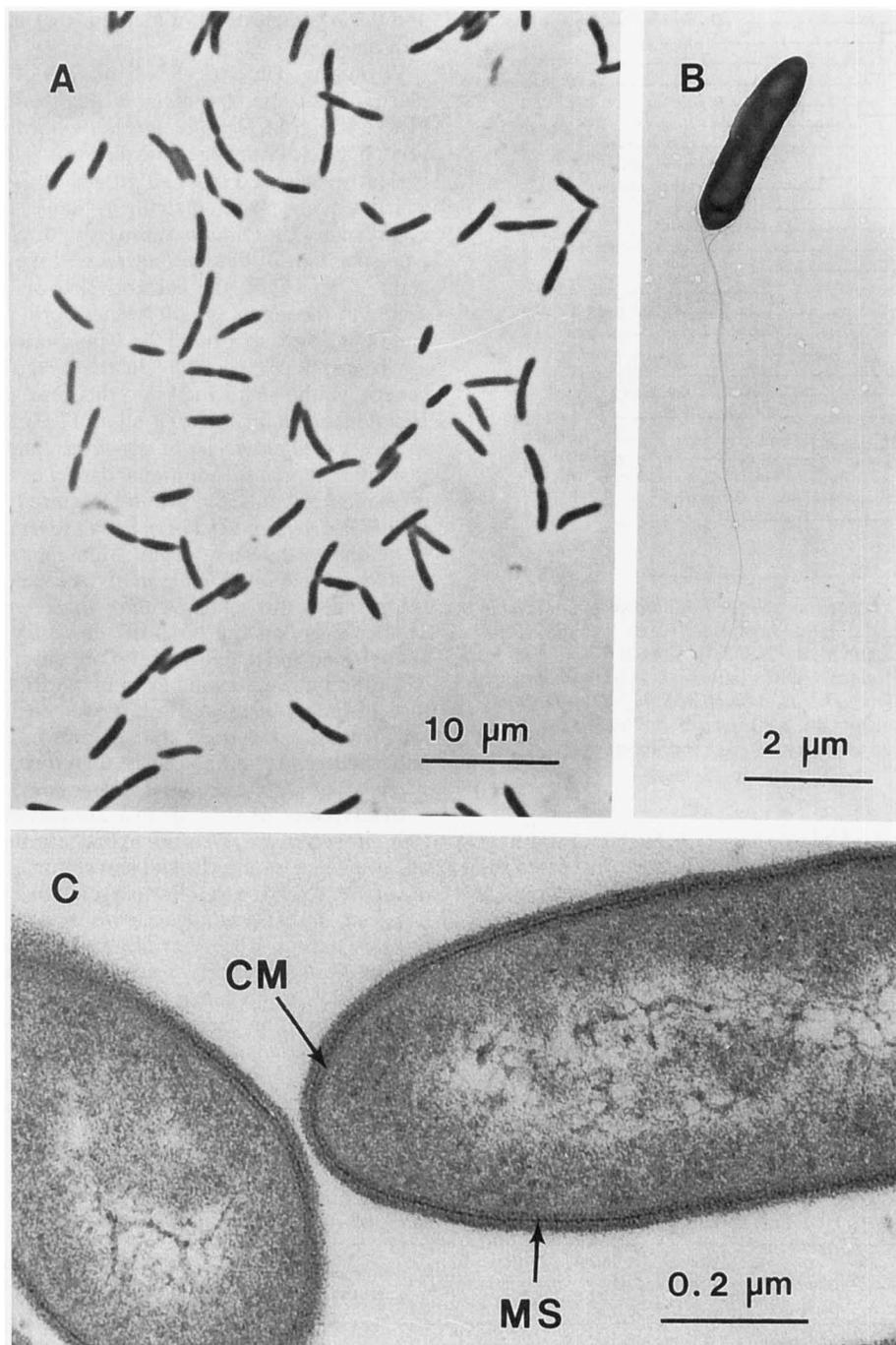


FIG. 2. Phase-contrast light micrograph (A) and electron micrographs of a negatively stained cell (B) and an ultrathin section (CM, cytoplasmic membrane; MS, murein sacculus) (C) of *Desulfitobacterium dehalogenans* in the middle exponential phase. *Desulfitobacterium dehalogenans* was grown in purified-culture medium at 37°C and pH 7.5.

of the organism to dehalogenate various chlorophenolic compounds).

**Colony morphology.** The organism does not grow in or on the surfaces of solid 1 to 1.5% agar media containing 0.1% yeast extract, 20 mM pyruvate, and 1 to 10 mM 3-Cl-4-OHPA. Colonies in 0.7 to 0.8% agar shake tubes are translucent, white, and spherical to slightly irregular and have diameters of 2 to 3 mm.

**Morphology of vegetative cells.** Exponential-phase cells that

are grown in liquid medium are slightly curved rods that are 0.5 to 0.7  $\mu\text{m}$  in diameter and 2.5 to 4  $\mu\text{m}$  long (Fig. 2A and B). The cells become more pleiomorphic (i.e., thicker and shorter cells appear) in the early stationary growth phase.

**Gram stain reaction and Gram type.** The Gram stain reaction is positive, regardless of the growth phase. Furthermore, tests for formation of the lipopolysaccharide-polymyxin B complex give no indications for the presence of lipopolysaccharide (36). Thus, the organism is a Gram-type-positive (34)

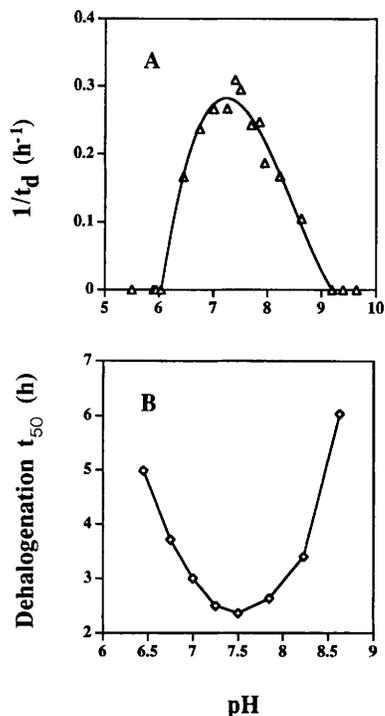


FIG. 3. Effects of pH on the doubling time ( $t_d$ ) of JW/IU-DC1<sup>T</sup> (A) and the observed time for 50% dehalogenation ( $t_{50}$ ) of 10 mM 3-Cl-4-OHPA (B). *Desulfitobacterium dehalogenans* was grown at 37°C in modified purified-culture medium (See Materials and Methods).

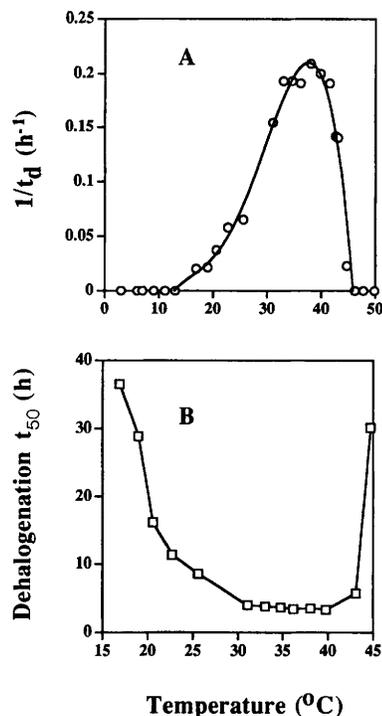


FIG. 4. Effects of temperature on the doubling time ( $t_d$ ) of JW/IU-DC1<sup>T</sup> (A) and the observed time for 50% dehalogenation ( $t_{50}$ ) of 10 mM 3-Cl-4-OHPA (B). *Desulfitobacterium dehalogenans* was grown in purified-culture medium at pH 7.5.

bacterium. This conclusion is supported by the fine structure of the cell wall, as revealed in photomicrographs of ultrathin sections (Fig. 2C), and also by the data from 16S rRNA analysis, which indicate that the organism belongs to the Gram-type-positive *Desulfotomaculum-Clostridium* subphylum (Fig. 1).

**Sporulation.** Although heat stability (25 min, 85°C) of the dechlorinating activity in the original sediment-free enrichment culture has been observed repeatedly, no spores were observed with the purified organism grown in liquid or solidified media (see Materials and Methods). Cells in the late stationary growth phase do not survive heat treatment at 87°C for 30 min.

**Motility and flagellation.** Most exponentially growing cells in purified-culture medium are motile, whereas cells in the later growth stages exhibit mainly tumbling motion. Each cell has one to four flagella (Fig. 2B).

**Growth properties.** (i) **Effects of pH and temperature on growth.** The pH range for growth and dechlorination of 3-Cl-4-OHPA is pH 6 to 9 (as determined at 37°C), and the optimum pH is 7.5 (Fig. 3). The shortest doubling time is about 3.5 h at pH 7.5 and 37°C. A similar pH profile with an optimum at about pH 7.5 has been observed for dechlorination of 2,4-DCP (data not shown). The temperature range for growth and dechlorination of 3-Cl-4-OHPA is 13 to 45°C, and the optimum temperature is around 38°C (Fig. 4).

(ii) **Effect of oxygen.** We determined the sensitivity of strain JW/IU-DC1<sup>T</sup> to oxygen by measuring growth and dechlorination of 3-Cl-4-OHPA in 160-ml serum bottles containing 10 ml of medium under a nitrogen gas phase containing different concentrations of air. Growth and dechlorination do not occur under aerobic conditions and when the concentration of air in

the gas phase is 5% or more. However, dechlorination does occur under a nitrogen atmosphere containing 2% air, indicating that the organism is resistant to microaerophilic conditions. Determining whether the organism can use O<sub>2</sub> as an electron acceptor will require further research. It has been shown recently that under special conditions some sulfate reducers, which have been assumed to be strictly anaerobic bacteria, not only can survive exposure to air (1) but also can utilize O<sub>2</sub> (8, 11).

(iii) **Effect of sodium chloride.** Growth and complete dechlorination of 10 mM 3-Cl-4-OHPA occur in the presence of 0.5 M NaCl, but growth and dechlorination do not occur in the presence of an concentration of 1 M or more. This observation is consistent with the previous observation that the enrichment culture is not active when it is transferred to marine sediments unless the sediment is diluted at least 2:1 with water, thus reducing the salt concentration (7).

(iv) **Substrates and fermentation products.** The only defined carbon source found which is utilized in the presence of 0.1% yeast extract under anaerobic conditions is pyruvate. Fermentation of 22.2 mol of pyruvate results in the formation of 10 mol of lactate, 9.2 mol of acetate, and 11.2 mol of carbon dioxide. We conclude that pyruvate is fermented according to the following equation:  $2CH_3COCOOH + H_2O \rightarrow CH_3CH(OH)COOH + CH_3COOH + CO_2$ . If the organism is grown on pyruvate in the presence of 3-Cl-4-OHPA or fumarate as an electron acceptor, equimolar amounts of 4-hydroxyphenylacetate and succinate, respectively, are produced instead of lactate. Substrates which do not support growth under anaerobic conditions in the presence of 0.1% yeast extract include arabinose, cellobiose, galactose, glucose, fructose, lactose, maltose, mannose, mannitol, raffinose, rhamnose, ribose,

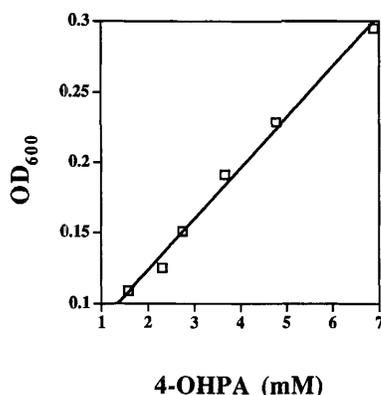


FIG. 5. Correlation between dechlorination of 3-Cl-4-OHPA (measured by the formation of the product 4-hydroxyphenylacetate [4-OHPA]) and growth of JW/IU-DC1<sup>T</sup> (optical density at 600 nm [OD<sub>600</sub>]). *Desulfotobacterium dehalogenans* was grown in purified-culture medium at 38°C and pH 7.5 by using pyruvate as the major carbon source and 3-Cl-4-OHPA as the major electron acceptor.

sucrose, xylose, lactate, ethanol, methanol, butyrate, i-butyrate, propionate, i-valerate, acetate, and formate.

(v) **Electron donors.** In the presence of 0.1% yeast extract and 10 mM 3-Cl-4-OHPA as an electron acceptor, pyruvate, lactate, formate, and hydrogen are used as electron donors for reductive dechlorination and, consequently, growth: the doubling times are 3.5, 6.2, 11.4, and 15.5 h, respectively. No growth occurs in the absence of 3-Cl-4-OHPA with lactate, formate, and hydrogen. Growth on pyruvate in the absence of 3-Cl-4-OHPA starts after a lag period of more than a day. However, strain JW/IU-DC1<sup>T</sup> cultures grown on 20 mM pyruvate in the presence of 10 mM 3-Cl-4-OHPA reach the stationary phase within 24 h. Growth in purified-culture medium is proportional to the amount of 3-Cl-4-OHPA converted to 4-hydroxyphenylacetate (Fig. 5). This indicates that *Desulfotobacterium dehalogenans* obtains energy through reductive dehalogenation. A thorough investigation to quantify the contribution of dehalogenation to growth yield is under way. The presence of cellobiose, glucose, fructose, mannitol, sucrose, i-butyrate, propionate, i-valerate, or acetate has no measurable beneficial effect on dechlorination or growth.

(vi) **Electron acceptors.** In addition to chlorophenolic compounds, such as 3-Cl-4-OHPA and 2,4-DCP, the following inorganic and organic compounds are used as electron acceptors in the presence of 0.1% yeast extract and 20 mM pyruvate: sulfite (2 mM), thiosulfate (20 mM), and sulfur (5%, wt/vol), which are reduced to sulfide; nitrate (5 mM) (reduced to nitrite); and fumarate (10 mM) (reduced to succinate). The test for dissimilatory sulfate reduction, however, is negative. Moreover, no adenylylsulfate reductase is detected by an immunoassay (see Materials and Methods), thus excluding the possibility that the organism is a sulfate-reducing bacterium. Besides 3-Cl-4-OHPA, cultures of JW/IU-DC1<sup>T</sup> grown in purified-culture medium reductively *ortho*-dechlorinate a variety of chlorophenols, including the dichlorophenols 2,3-dichlorophenol, 2,4-dichlorophenol, and 2,6-dichlorophenol, the trichlorophenols 2,3,4-trichlorophenol, 2,3,6-trichlorophenol, and 2,4,6-trichlorophenol, the tetrachlorophenols 2,3,4,6-tetrachlorophenol, 2,3,4,5-tetrachlorophenol, and 2,3,5,6-tetrachlorophenol, and pentachlorophenol. The following chlorophenols are not dehalogenated: 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,5-dichlorophenol, 3,4-dichlorophenol, 3,5-dichlorophenol, and 3,4,5-trichlorophenol.

**G+C content.** The G+C content of the genomic DNA is 45% mol% (as determined by the chemical method). Although this value is similar to values found for *Desulfotomaculum* species (Table 2), other properties justify placing this organism in a separate genus.

**Type strain.** Strain JW/IU-DC1 is the type strain of *Desulfotobacterium dehalogenans*; this strain has been deposited in the Deutsche Sammlung von Mikroorganismen as strain DSM 9161. The properties of this strain are the same as the properties described above for the species since it is the only strain that has been isolated so far.

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