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Isolation and Characterization of a Novel Bacterium Growing via Reductive Dehalogenation of 2-Chlorophenol

JAMES R. COLE,[1,2] AMY L. CASCARELLI,[2] WILLIAM W. MOHN,[2]† AND JAMES M. TIEDJE[1,2]*

Departments of Crop and Soil Sciences[1] and of Microbiology,[2] Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824

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A bacterium capable of anaerobic growth via reductive dehalogenation of 2-chlorophenol was isolated from a culture enriched from sediment taken from a small stream near Lansing, Mich. The organism, designated strain 2CP-1, is a gram-negative rod ca. 3 by 0.5 μm in size and is a catalase-negative, oxidase-negative, facultative anaerobe that forms small red colonies in anaerobic media. The organism grew in reduced anaerobic mineral medium supplemented with 2-chlorophenol, acetate, and vitamins, producing phenol as a product. It did not grow when either 2-chlorophenol or acetate was omitted. The growth yield was about 3 g of protein per mol of 2-chlorophenol dechlorinated, and the doubling time was 3.7 days. Only the ortho position was dehalogenated, and additional chlorines at other positions decreased or blocked ortho dechlorination. The organism also grew with fumarate as its electron acceptor. Dechlorination activity is inducible, since cultures grown in fumarate containing medium with 2-chlorophenol rapidly dechlorinated additional 2-chlorophenol, while cultures grown in the same medium without 2-chlorophenol did not. Analysis of the organism's 16S rRNA sequence revealed that it is a member of the delta proteobacteria, more closely related to the myxobacteria than to the sulfidogenic bacteria.

Bacteria present in many anaerobic environments have been shown capable of dehalogenating a variety of haloaromatic compounds. Although some of these natural reactions are probably fortuitous or cometabolic, aromatic dehalogenation reactions are generally energetically favorable (6). However, very few bacteria have been isolated that are able to grow through reductive dehalogenation reactions (13, 16, 19, 21). The best studied of these is *Desulfomonile tiedjei* DCB-1 (4, 19). *D. tiedjei* is a strictly anaerobic gram-negative sulfate-reducing bacterium, a member of the delta subdivision of the proteobacteria. *D. tiedjei* obtains energy for growth from reductive dehalogenation of 3-chlorobenzoate (5). *D. tiedjei* uses 3-chlorobenzoate as a respiratory electron acceptor, producing a membrane potential during dehalogenation (15).

Anaerobic enrichment cultures active on various chlorinated phenols have been obtained by a number of workers (27; for reviews, see references 8 and 16). One isolate, a gram-positive anaerobic spore-forming bacterium, is capable of removing chlorine from the *ortho* and *meta* positions of several chlorophenols (13). However, this organism has not been shown to benefit from the dechlorination process. Another gram-positive isolate, *Desulfitobacterium dehalogenans*, ortho-dechlorinates a wide range of chlorophenols and related compounds and appears to benefit from the dechlorination reaction when grown with pyruvate and yeast extract (22). Additionally, *D. tiedjei* DCB-1 can dechlorinate the *meta* position of polychlorinated chlorophenols but has not been shown to obtain energy from chlorophenol dechlorination (14).

We have isolated a gram-negative rod capable of growth in a defined medium on 2-chlorophenol and acetate (and vitamins). This organism dechlorinates *ortho*-chlorophenol, producing phenol as a product. Phylogenetic analysis of the organism's 16S rRNA sequence indicates that it is a member of the delta proteobacteria, as is *D. tiedjei*, but that the new isolate is more closely related to the myxobacteria than to *D. tiedjei* or the other sulfidogens.

* Corresponding author. Phone: (517) 353-9021. Fax: (517) 353-2917. Electronic mail address: 21394jmt@msu.edu.

† Present address: Department of Microbiology, University of British Columbia, Vancouver, Canada V6T 1Z3.

MATERIALS AND METHODS

Media and growth conditions. The basal medium was modified from the medium of Widdel and Pfennig (24) and consisted of the following (grams per liter): NaCl, 1; $MgCl_2$, 0.5; KH_2PO_4, 0.2; NH_4Cl, 0.3; KCl, 0.3; and $CaCl_2$, 0.015. In addition, it contained 1 ml of a trace element solution per liter (24), 1 ml of Na_2SeO_3–Na_2WO_4 solution per liter (3), and 10 mg of resazurin per liter. These components were boiled under N_2 and cooled to room temperature under N_2-CO_2 (95:5). Na_2S and $NaHCO_3$ were added to final concentrations of 1 and 30 mM, respectively, and the pH of the medium was adjusted to 7.5 by varying the CO_2 concentration in the headspace. The medium was dispensed into N_2-CO_2-flushed vessels and sterilized by autoclaving. The sterile medium was amended with the anaerobic vitamin mix of Wolin et al. (26), with addition of thiamine, 1,4-naphthoquinone, nicotinamide, hemin, and lipoic acid at concentrations of 50, 200, 500, 50, and 50 μg/liter, respectively (4). Other components were added from sterile anaerobic stocks.

The enrichment medium consisted of the basal medium amended with 2-chlorophenol, formate, acetate, and 2-bromoethane sulfonic acid (BESA) to final concentrations of 0.25, 10, 1, and 1 mM, respectively. Colonies were isolated in deep agarose shake cultures containing 10 ml of enrichment medium solidified with 1% low-gelling-temperature agarose (Boehringer Mannheim).

To test for aerobic growth, basal medium was prepared as described above for basal anaerobic medium but without Na_2S. After inoculation, cultures were amended with 5 ml of air (added by syringe) per anaerobic culture tube containing 10 ml of medium, giving approximately one-third the normal O_2 partial pressure. Aerobic agarose roll tubes contained 15 ml of

basal medium containing 0.1% yeast extract solidified with 1.5% low-gelling-temperature agarose rolled on the side of anoxic culture tubes, with 1.5 ml of air added to the headspace.

Growth rates and antibiotic sensitivity. Growth rates were measured by the increase in optical density for cultures grown with fumarate plus acetate and yeast extract plus O_2 and by the rate of increase in phenol product for cultures grown with 2-chlorophenol plus acetate because of the low cell density. Antibiotics were added from filter-sterilized anaerobic stock solutions to triplicate basal medium cultures amended with 1 mM acetate and 5 mM fumarate. Growth was defined as the appearance of visual turbidity and was confirmed by microscopic observation.

Analysis. Phenol and chlorophenols were analyzed by reverse-phase high-performance liquid chromatography (HPLC) with a Hibar RT C_{18} column (E. Merck), with a flow rate of 1.5 ml/min of 66:33:0.1 H_2O-CH_3CN-H_3PO_4 and a UV detector set to 218 nm. Products were verified by comparison with authentic standards. In addition, the assignment of phenol as the product was confirmed by comparing the peak $A_{218/230}$ ratio with that of authentic phenol.

Acetate, fumarate, succinate, and other volatile fatty acids were analyzed by ion-exclusion HPLC (20).

To measure protein yield, cultures were harvested by centrifugation, washed, and subjected to protein analysis by the method of Lowry after alkaline hydrolysis (9).

Substrate use. Triplicate 20-ml cultures of basal anaerobic medium were amended from sterile anaerobic stock solutions with potential growth substrates and inoculated with a 1% transfer from a culture grown on enrichment medium. Total cell counts in the inoculum (ca. 2.4×10^6 cells per ml) and in the test cultures after 44 days were determined by direct microscopic examination. Samples were concentrated by centrifugation and resuspended in 1/10th the initial volume of 0.1 M HCl, and cell counts were determined in a microscopic counting chamber. A 10-fold increase in total cell counts was considered evidence of growth.

To test the ability of growing cells to dehalogenate test substrates, cultures (20 ml) of basal anaerobic medium were amended with 10 mM formate, 1 mM acetate, and 250 μM 2-chlorophenol. Cultures were inoculated with a 1% transfer from an actively dehalogenating culture and periodically monitored for disappearance of 2-chlorophenol. After the initial 250 μM 2-chlorophenol had been transformed, replicate cultures were reamended with the halogenated substrate to be tested (ca. 100 μM). The cultures were monitored for remaining substrate by HPLC at days 5 and 34.

To test for dechlorination in washed cell suspensions, cultures were grown in the basal medium amended with 5 mM fumarate, 1 mM acetate, and 250 μM 2-chlorophenol. Cultures were reamended once with 250 μM 2-chlorophenol. After transformation of the 2-chlorophenol, cells were concentrated by centrifugation and resuspended in an anaerobic buffer consisting of the basal medium salts, 10 mM formate, 1 mM acetate, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.5]), 1 mM cysteine, and 1 mM titanium(III) citrate. Portions of the cell suspension were anaerobically transferred to duplicate 10-ml serum bottles containing the test substrate at a final concentration of ca. 100 μM. Suspensions were incubated at 25°C, sampled at intervals, and assayed by HPLC for disappearance of substrate and appearance of potential products.

16S rRNA sequencing and analysis. DNA was prepared by a method shown to be effective with diverse bacteria (23) from a 20-ml culture grown on the enrichment medium. The major portion of the 16S rRNA gene was amplified by the PCR method with two primers specific to conserved rRNA regions around bases 68 and 1400 (numbering according to the *Escherichia coli* 16S rRNA sequence [2]). The PCR mixtures contained (in 100 μl) 0.1% of the culture DNA, 0.2 mM (each) deoxynucleoside triphosphate, 0.8 mM base 68 primer and 0.08 mM base 1400 primer, 1× *Taq* DNA polymerase buffer (Promega), and 0.2 μl of *Taq* DNA polymerase (Promega). The mixture was heated to 94°C for 3 min and then cycled for 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. The PCR product was either gel purified by electrophoresis through a 1.2% agarose gel and extracted with GeneClean (Bio 101) and directly sequenced, or portions (10 μl) were transferred to new PCR mixes, as described above, but with only a single primer at 0.8 mM to allow single-stranded DNA synthesis. The volume of the single-stranded PCR mixtures was increased to 2 ml with Tris-EDTA buffer and purified from unreacted primers and nucleotides with a Centricon 30 microconcentrator (Amicon). Both double-stranded and single-stranded PCR DNA preparations were brought to a final volume of ca. 40 μl of Tris-EDTA, and 4 μl was used in each sequencing reaction mixture. Sequencing primers corresponding to conserved regions of rRNA genes were end labeled with ^{32}P (18) and used with the Sequenase sequencing kit (U.S. Biochemicals) to sequence the prepared PCR products.

The resulting sequence was analyzed with the programs and database of the Ribosomal Database Project (10). After initial placement with the program similarity rank, the sequence was hand aligned with a selection of representative rRNA sequences and a maximum likelihood phylogenetic tree was generated with the program fastDNAml.

RESULTS

Enrichment and isolation. Stream sediment from near Lansing, Mich., was placed in a 150-ml serum bottle with 50 ml of enrichment medium. This primary enrichment was incubated at 25°C and monitored at intervals by HPLC for disappearance of 2-chlorophenol. The 2-chlorophenol had disappeared by 4 months with the concomitant appearance of phenol. The enrichment was reamended with 2-chlorophenol to 100 μM. After about a month, the added 2-chlorophenol had almost totally disappeared and another 100 μM was added. After another month, the added 2-chlorophenol had almost totally disappeared and a 10% transfer was made into fresh medium containing 250 μM 2-chlorophenol. This transfer was monitored and reamended with 2-chlorophenol to 250 μM as depleted until a total of about 1 mM 2-chlorophenol had been transformed. The transfer process was repeated two more times. Microscopic examination of the third transfer revealed that the enrichment consisted primarily of short fat rods and long thin rods with smaller numbers of cells with several other morphologies.

The third enrichment transfer was serially diluted 1:10 into deep agarose shake cultures of enrichment medium. After about 4 weeks, cream-colored colonies became visible. After 6 weeks, small red colonies appeared. At 8 weeks, colonies were removed under an N_2-CO_2 atmosphere to anaerobic culture tubes containing fresh enrichment medium. The cream-colored colonies yielded cultures matching the short rods in the enrichment culture. Four of six of the tubes containing the small red colonies showed disappearance of 2-chlorophenol and accumulation of phenol. These cultures all contained only long thin rods (ca. 0.5 by 3 μm) similar to those in the enrichment culture. One isolate was chosen for further study. The culture was transferred several times and subjected to colony purification as described above two more times for a

total of three rounds. The culture appeared pure by microscopic observation and by other criteria described below.

A number of substrates were tested for their ability to support anaerobic growth of the dehalogenating isolate. The substrates that did not support growth were pyruvate (10 mM), lactate (10 mM) plus sulfate (5 mM), acetate (10 mM) plus sulfate (5 mM), glucose (1 mg/ml), Casamino Acids (1 mg/ml), citrate (10 mM), and glycerol (1 mg/ml). Of the substrates tested, only fumarate (1 mg/ml) and yeast extract (1 mg/ml) supported growth, although growth was initially poor on both of these substrates. No contamination was detected by microscopic examination of the cultures that grew on these substrates. Analysis of the fumarate culture media by HPLC showed a decrease in fumarate and accumulation of succinate. Acetate (1 mM) plus fumarate gave better growth than fumarate alone. Microscopic observation of cells from cultures grown anaerobically with fumarate showed that they tended to be longer than those from 2-chlorophenol-grown cultures (about 8 μm versus 3 μm), including some filaments >50 μm in length. Additionally, fumarate-grown cultures tended to form visible red clumps or aggregates of cells.

We also tested the organism for the ability to grow aerobically. Medium amended with 1 mg of yeast extract per ml in an approximately one-third partial O_2 pressure atmosphere supported much denser growth than yeast extract-amended anoxic cultures. The isolate also grew in similar aerobic cultures amended with acetate instead of yeast extract, although growth was much slower. Cells grown with yeast extract and air appeared morphologically similar to 2-chlorophenol-grown cells. Attempts to grow the organism under similar conditions but without $NaHCO_3$ and CO_2 failed, as did attempts to grow the organism in open vessels, possibly indicating a requirement for CO_2.

To further establish purity, colonies were picked from deep agar anaerobic shake tubes containing the basal medium amended with fumarate and in microaerophilic roll tubes containing yeast extract and air. After about 2 weeks, red colonies were visible in the fumarate shake tubes and were transferred to basal medium containing fumarate. The culture became visibly turbid in about 2 weeks. A 1% transfer of this fumarate-grown culture was transferred to the basal media with 2-chlorophenol, acetate, formate, and BESA. After 1 week, phenol was detected in the culture supernatant. Colonies became visible in the microaerophilic roll tube cultures after about 2 weeks. These colonies, embedded in agar, were red, as were the fumarate- and 2-chlorophenol-grown colonies in deep agar shakes. However, colonies on the agar surface appeared white, not red. Microscopically, both colonies contained cells with similar morphologies, thin rods resembling the 2-chlorophenol-grown cells. Colonies were transferred either to 25-ml culture tubes with 10 ml of anoxic basal medium with yeast extract (0.1%) and air (5 ml) or to enrichment medium (with 2-chlorophenol). The 2-chlorophenol-containing cultures accumulated phenol within 2 weeks. Similarly, transfers of the microaerophilic cultures to anaerobic media with 2-chlorophenol accumulated phenol within 1 week. Since isolation on these three different media produced cells with uniform morphological and physiological features, we consider the culture pure.

Characterization. The organism is a gram-negative, catalase-negative, and oxidase-negative facultative anaerobe. Doubling times on 2-chlorophenol plus acetate, fumarate plus acetate, and yeast extract aerobic cultures were 3.7, 2.5, and 0.8 days, respectively. Growth was inhibited by 50 μg of kanamycin per ml, 50 μg of chloramphenicol per ml, and 250 μg of rifampin per ml. Growth was not inhibited by nalidixic acid (200 μg/ml), streptomycin (250 μg/ml), or rifampin (50 μg/ml). The morphologies of the pure culture grown anaerobically with fumarate and aerobically with yeast extract are shown in Fig. 1. Cells grown on the acetate, 2-chlorophenol, and vitamin medium are rods (ca. 3 by 0.5 μm) very similar to those grown aerobically on yeast extract.

A total of 1,350 contiguous bases of the 16S rRNA gene of the isolate were determined, with 80% determined in both directions. An examination of signature sequences (1, 25) indicated that the new isolate contained the signature sequence 5′-CCUGACGCAGCRACGCCG-3′ (corresponding to *E. coli* positions 385 to 402), which is specific to members of the delta subdivision of the proteobacteria. The sequence was compared with other known delta proteobacterial sequences. The isolate shared the most 16S sequence similarity with the myxobacteria to the exclusion of the other delta proteobacteria and maps to a deep branch internal to the myxobacteria (see Fig. 3).

Evaluation of growth by reductive dechlorination. Three sets of agarose dilution tubes were prepared with vitamins, but without BESA or formate. One set contained both acetate (1 mM) and 2-chlorophenol (250 μM), while the two other sets contained either acetate or 2-chlorophenol. After about 6 weeks, colonies became visible in the set with both acetate and 2-chlorophenol, but colonies were not visible in the other two sets even after 12 weeks. Furthermore, we have sustained growth through four serial transfers with a 1% inoculum on the acetate–2-chlorophenol–defined vitamin medium.

Cell yield from growth by dechlorination was measured in two separate experiments. In the first experiment, replicate cultures were grown in serum bottles in the basal medium (100 ml) amended with vitamins, 1 mM acetate, and with or without about 250 μM 2-chlorophenol. Bottles with 2-chlorophenol were reamended with 2-chlorophenol as depleted. After growth, cultures were harvested and analyzed for substrate transformation and protein production (Table 1). In this experiment, 0.98 ± 0.01 phenol molecule was produced per chlorophenol molecule metabolized and 2.8 ± 0.1 chlorophenol molecules were transformed per acetate molecule metabolized. After we had determined in preliminary experiments that 2,6-dichlorophenol was a substrate for dechlorination and supported cell growth, we repeated the experiment with both 2-chlorophenol and 2,6-dichlorophenol. The protein yield per mol of 2,6-dichlorophenol was about twice the yield per mole of 2-chlorophenol, proportional to the amount of chlorine removed (Table 1). Since not all cultures began to grow at the same time, it is important to note that the growth yields remained similar regardless of the extent of growth.

To evaluate electron donors for growth by reductive dechlorination, triplicate cultures were grown in anaerobic culture tubes containing 20 ml of basal medium amended with vitamins, 2-chlorophenol (250 μM), and either BESA (1 mM), acetate (1 mM), and formate (10 mM) (enrichment medium); acetate (1 mM) alone; formate (10 mM) alone; BESA (1 mM) alone; or no added electron donor. Cultures were inoculated with a 2% transfer from a culture with all three potential electron donors. Cultures were monitored by HPLC, and more 2-chlorophenol was added as depleted. After 17 days, the cultures with all three potential electron donors averaged 1,053 ± 75 pmol of 2-chlorophenol dehalogenated per ml, while those with acetate alone averaged 1,024 ± 131 pmol/ml, those with formate alone averaged 700 ± 133 pmol/ml, those with BESA alone averaged 298 ± 21 pmol/ml, and those with no added donor averaged 259 ± 67 pmol/ml.

Characterization of dehalogenation. In a preliminary experiment, we determined that dechlorination occurred in medium amended with 2-chlorophenol, acetate, and low concentrations

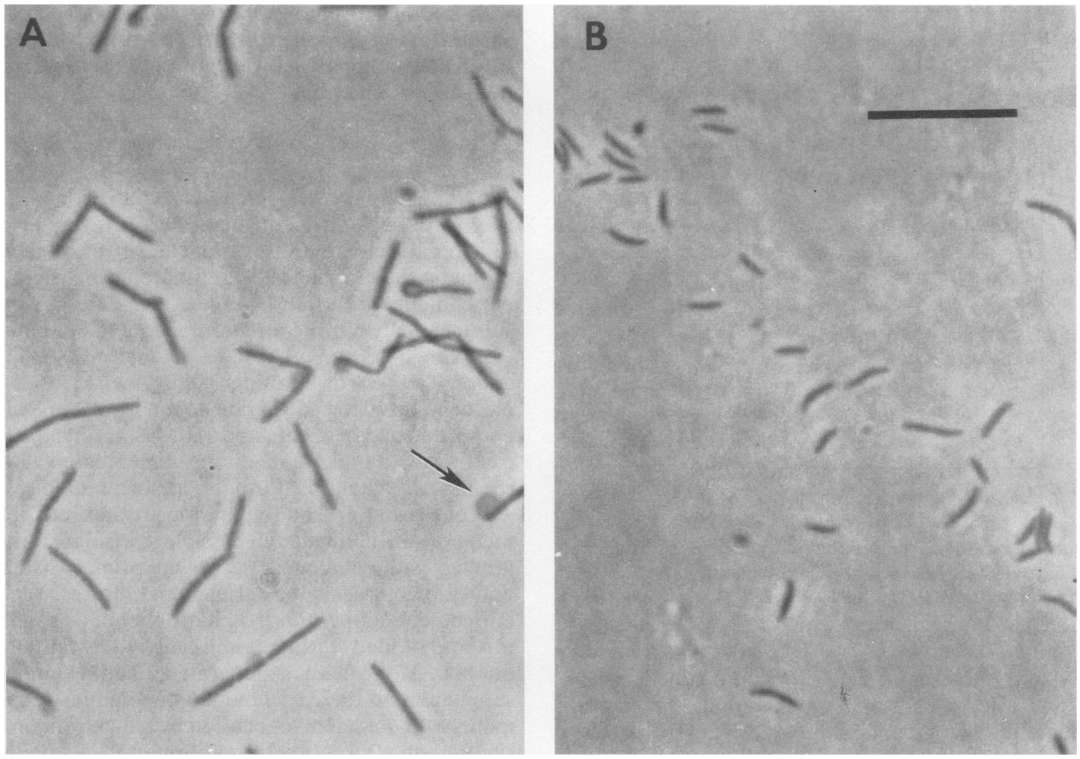

FIG. 1. Micrograph of 2CP-1 grown anaerobically in medium with fumarate (A) or aerobically in medium with yeast extract (B). Arrow points to bleb-like structure often found in older cultures (A). Bar, 10 μm.

(2 mM) of fumarate (although cultures amended with 2-chlorophenol and 10 mM fumarate did not show dechlorination of 2-chlorophenol). To test if the dechlorination activity was inducible, we inoculated two sets of triplicate fumarate plus acetate cultures with cells grown on fumarate plus acetate without 2-chlorophenol and amended one set of cultures with 250 μM 2-chlorophenol. We monitored the cultures containing 2-chlorophenol and reamended the cultures with additional 2-chlorophenol as depleted.

When the 2-chlorophenol-amended cultures had dehalogenated about 500 μM 2-chlorophenol, we amended both sets of cultures with 100 μM 2-chlorophenol. The cultures previously exposed to 2-chlorophenol rapidly dehalogenated the additional 2-chlorophenol, while the previously unexposed cultures

TABLE 1. Protein yield for 2CP-1 cultures grown on acetate and *ortho*-chlorophenols

Substrate[a]	Substrate consumed (pmol/ml)	Phenol product (pmol/ml)	Acetate consumed (pmol/ml)	Protein (pg/ml)	Substrate/acetate ratio	Protein/phenol (g/mol)[b]
Expt 1						
2-CP (a)	896	878	320	2,790	2.8	2.6
2-CP (b)	515	500	180	2,060	2.9	3.2
None[c]			−37 (65)	483 (80)		
Expt 2						
2-CP (a)		524		1,230		2.2
2-CP (b)		1,100		3,660		3.2
2-CP (c)		552		1,030		1.7
2,6-diCP (a)		326		2,960		8.8
2,6-diCP (b)		528		4,290		8.0
2,6-diCP (c)		476		3,210		6.6
None[c]				84 (2)		
Average[d]						
2-CP						2.6 (0.6)
2,6-diCP						7.8 (1.1)

[a] 2-CP, 2-chlorophenol; 2,6-diCP, 2,6-dichlorophenol. Data for each replicate culture are indicated by the letters a, b, and c in parentheses.
[b] Protein yield calculated after subtracting protein measured in control cultures.
[c] Average of triplicate control cultures with sample standard deviation in parentheses.
[d] Average of data from the two experiments.

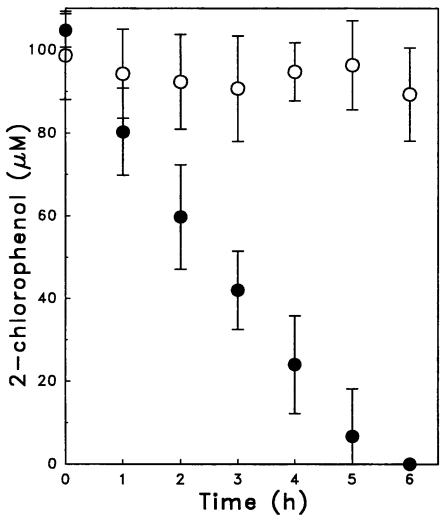

FIG. 2. Dehalogenation of 2-chlorophenol by cultures grown in the presence (●) or absence (○) of 2-chlorophenol. Datum points are the average of triplicate cultures.

showed little or no decrease in 2-chlorophenol concentration (Fig. 2). At 6 h after 2-chlorophenol addition, the protein concentrations of the two sets of cultures were nearly identical (10.2 ± 2.9 versus 7.8 ± 0.4 μg of protein per ml for the preinduced and uninduced cultures, respectively). Since this strain grows very slowly, the cell mass should have changed little over the 6-h experiment. With the protein content measured at the 6-h time point and the rate of 2-chlorophenol decrease during the first 4 h, the calculated dehalogenation rate was 2.0 mmol (g of total protein)$^{-1}$ h^{-1}.

We evaluated the substrate range for dehalogenation by adding to actively dehalogenating cultures potential substrates at a final concentration of ca. 100 μM. After 34 days, the following test substrates were not significantly transformed: 3- and 4-monochlorophenols; 2-, 3-, and 4-monobromophenols; 2-, 3-, and 4-monofluorophenols; 2-, 3-, and 4-monoiodophenols; 2,5-dichlorophenol; 2-chlorophenoxyacetic acid; and 2,4-dichlorophenoxyacetic acid. In contrast, 2-chlorophenol had virtually disappeared from parallel cultures within 5 days. Dehalogenation of various chlorophenols was also tested in induced cell suspensions (Table 2). This cell suspension assay, with a concentrated cell suspension, does not require the substrate to support cell growth. Indeed, one compound, 2,5-dichlorophenol, was dechlorinated by resting cells but did not support growth.

TABLE 2. Dehalogenation of chlorophenols by induced cell suspensions

Substrate[a]	Activity[b]	Product(s)[c]
2-Cl	++	Phenol
3-Cl	−	−
4-Cl	−	−
2,3-diCl	−	−
2,4-diCl	−	−
2,5-diCl	+	3-Cl
2,6-diCl	++	2-Cl, phenol
PCP	−	−

[a] Substrates (100 μM) were phenol derivatives substituted at the indicated positions. 2-Cl, 2-chlorophenol; 2,3-diCl, 2,3-dichlorophenol; PCP, pentachlorophenol.

[b] ++, dechlorination virtually complete after 4 h; +, dechlorination detected at 24 h; −, no dechlorination detected at 24 h.

[c] Detected products were phenol or phenol substituted at indicated positions. −, no product detected.

DISCUSSION

Although many halogenated aromatic compounds can serve as growth substrates for anaerobic enrichment cultures, very few organisms that are capable of transforming the aryl aromatic substrate have been isolated from these cultures. The enrichments are often complex; complete degradation of the substrate may require an interdependent community food web (7). The best-studied isolate, *D. tiedjei* DCB-1, can obtain energy for growth by using 3-chlorobenzoate as a respiratory electron acceptor (16), dehalogenating the chlorobenzoate, and producing benzoate and HCl (as products). This organism can grow in a mineral medium supplemented with only 3-chlorobenzoate, vitamins, formate, and acetate (5).

We reasoned that, by limiting other possible modes of metabolism, it might be possible to isolate other organisms growing with dehalogenation as the primary oxidant or at least limit the complexity of enrichment cultures. We supplied our enrichment cultures with 2-chlorophenol as an electron acceptor and added formate and acetate as potential electron donors. Along with a general defined vitamin mixture, we supplemented the enrichment with vitamins required for optimal growth and dehalogenation by a 3-chlorobenzoate-dechlorinating anaerobe (4). In addition, we added BESA to block both methanogenesis and syntrophic fermentation of the expected phenol product.

The enrichment obtained appeared morphologically simple after the first few transfers, with only two dominant morphotypes—short fat rods and longer thin rods. Both morphologies were present in our colony isolations. Only the longer thin rods metabolized 2-chlorophenol, so we speculate that the short fat rods may have been growing acetogenically, transforming the formate and CO_2 in the media into acetate. Because the 2-chlorophenol-dechlorinating isolate grew very slowly and only to relatively low densities, we took several steps to ensure that the isolate was pure, including colony isolation on additional media and transfer back into 2-chlorophenol-dechlorinating conditions.

The new isolate appears to gain energy from the dechlorination reaction. It grew for four 1% serial transfers into an anaerobic mineral medium amended with only vitamins, acetate, and 2-chlorophenol, producing phenol. The organism formed colonies on this medium solidified with agarose, but it did not form colonies when either acetate or 2-chlorophenol was omitted. In addition, the protein yield was proportional to the amount of chlorine removed and was about twice as high on 2,6-dichlorophenol as on 2-chlorophenol. Also, the stoichiometry of chlorine removed to acetate consumed (2.8) is in good agreement with the theoretical maximal value of four electron pairs produced per acetate oxidized to $2CO_2$, with the remaining reducing equivalents going to cell mass. Although there is no direct evidence, it is probable that, like *D. tiedjei*, 2CP-1 gains energy by using the chlorinated substrate as a respiratory electron acceptor.

In addition to the ability of the organism to grow by this unusual reaction, other evidence suggests that chlorophenol dechlorination is not a fortuitous or cometabolic reaction but instead is a reaction that has evolved for the use of *ortho*-chlorophenols or close natural analogs. The range of preferred substrates for dehalogenation by this isolate appears extremely limited, an unexpected feature for a fortuitous reaction. In

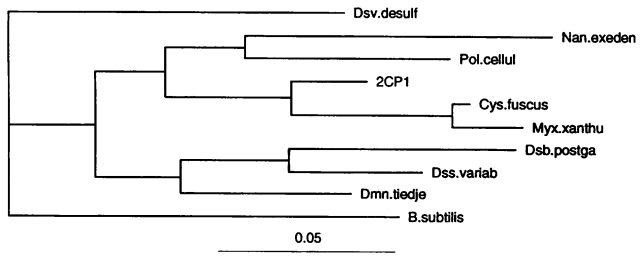

FIG. 3. Phylogenetic tree based on the 16S rRNA sequences of the 2-chlorophenol isolate and representative bacteria. Sequences used for comparison (GenBank accession numbers follow in parentheses) were *Desulfobacter postgatei* (Dsb.postga) (M26633), *Desulfosarcina variabilis* (Dss.variab) (M26632), *D. tiedjei* (Dmn.tiedje) (M26635), *Myxococcus xanthus* (Myx.xanthu) (M34114), *Cystobacter fuscus* (Cys.fuscus) (M94276), *Polyangium cellulosum* (Pol.cellul) (M94282), *Nannocystis exedens* (Nan.exeden) (M94279), *Desulfovibrio desulfuricans* (Dsv.desulf) (M34113), and *Bacillus subtilis* (M10606). Bar = 0.05 estimated substitution per sequence position.

addition, dehalogenation activity is not constitutive in this isolate but is induced by the presence of 2-chlorophenol, again implying specific recognition of substrate.

Comparison of the partial 16S sequence of the isolate with a database of 16S sequences indicated that the isolate is a member of the delta subdivision of proteobacteria, as is the 3-chlorobenzoate-dechlorinating bacterium *D. tiedjei*. The 16S sequence of the 2-chlorophenol isolate, however, does not place it among the sulfate-reducing bacteria but instead maps it to a deep branch in the myxobacteria (Fig. 3). On the basis of phylogenetic (and other) criteria, the myxobacteria have been divided into two suborders, the families *Cystobacterineae* (*Myxococcus*, *Stigmatella*, and *Cystobacter* genera) and *Sorangineae* (*Sorangium* and *Nannocystis* genera) (12). The phylogenetic placement of 2CP-1 corresponds to a deep branching in the family *Cystobacterineae*, with no near relatives. Such a deeply branching organism may hold useful information for the study of myxobacterial evolution. Although we have not observed gliding motility or fruiting body formation, the bleb-like structures, red pigmentation, and aerobic growth are all consistent with myxobacterial characteristics.

In addition to the myxobacteria and sulfate-reducing bacteria, the delta subdivision contains members capable of using a variety of substrates as physiological electron acceptors, including oxygen, nitrate, sulfate, sulfur, fumarate, and iron (11, 17, 24). It may not be surprising then, given the energetic benefit, that some members of this group are capable of utilizing halogenated compounds as physiological electron acceptors.

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