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Characterization of *Desulfitobacterium chlororespirans* sp. nov., Which Grows by Coupling the Oxidation of Lactate to the Reductive Dechlorination of 3-Chloro-4-Hydroxybenzoate

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Strain Co23, an anaerobic spore-forming microorganism, was enriched and isolated from a compost soil on the basis of its ability to grow with 2,3-dichlorophenol (DCP) as its electron acceptor. *ortho* chlorines were removed from polysubstituted phenols but not from monohalophenols. Growth by chlororespiration was indicated by a growth yield of 3.24 g of cells per mol of reducing equivalents (as 2[H]) from lactate oxidation to acetate in the presence of 3-chloro-4-hydroxybenzoate but no growth in the absence of the halogenated electron acceptor. Other indicators of chlororespiration were the fraction of electrons from the electron donor used for dechlorination (0.67) and the H_2 threshold concentration of <1.0 ppm. Additional electron donors utilized for reductive dehalogenation were pyruvate, formate, butyrate, crotonate, and H_2. Pyruvate supported homoacetogenic growth in the absence of an electron acceptor. Strain Co23 also used sulfite, thiosulfate, and sulfur as electron acceptors for growth, but it did not use sulfate, nitrate or fumarate. The temperature optimum for growth was 37°C; however, the rates of dechlorination were optimum at 45°C and activity persisted to temperatures as high as 55°C. The 16S rRNA sequence was determined, and strain Co23 was found to be related to *Desulfitobacterium dehalogenans* JW/IU DC1 and *Desulfitobacterium* strain PCE1, with sequence similarities of 97.2 and 96.8%, respectively. The phylogenetic and physiological properties exhibited by strain Co23 place it into a new species designated *Desulfitobacterium chlororespirans*.

Chlorophenolic compounds have been of environmental concern because of their extensive use in several industries and their toxicity. This has led to considerable efforts to study the degradation of these compounds under aerobic and strictly anaerobic conditions (22, 36). Pentachlorophenol (PCP), a widely used wood preservative, is degraded under methanogenic conditions via a series of reductive dechlorinations (26, 33, 34, 37, 39) and under aerobic conditions by several pure bacterial cultures via hydroquinone intermediates (32, 42, 46, 56). Other polychlorinated phenols and monochlorophenols are also degraded under anaerobic and aerobic conditions (1, 10, 17, 19, 23, 25, 28, 30, 40, 46, 57).

Anaerobic dechlorination has been widely observed in nature, although few pure cultures capable of coupling growth to this process via halorespiration have been isolated (4, 12, 18, 24, 35). Halorespiration refers to the ability of a microorganism to utilize the considerable energy released during reductive dehalogenation as the terminal process in a respiratory electron transport chain. Free-energy changes ($\Delta G^{\circ\prime}$) available from reductive dechlorination have been reported in the range of −140 to −160 kJ per reaction for chlorinated aromatic compounds (13) and −130 to −170 kJ per reaction for chlorinated aliphatic compounds (14). *Desulfomonile tiedjei* DCB-1 uses 3-chlorobenzoate (3-CBA) as a terminal electron acceptor and was the first organism capable of growing in this manner to be isolated (44). The anaerobic myxobacterium strains 2CP-1, 2CP-2, 2CP-C, and 2CP-3 all have been shown to grow at the expense of acetate oxidation and 2-chlorophenol (2-CP) dechlorination (4, 5). Recently, *Desulfitobacterium dehalogenans* JW/IU DC1, a gram-positive microorganism with the ability to remove *ortho*-substituted chlorines from polychlorinated phenols, was described (51). Direct evidence, however, for growth associated with halorespiration was not presented for this strain, although cell yields increased in the presence of a chlorinated substrate. The related *Desulfitobacterium* strain PCE1 was shown to grow by halorespiration on chlorophenols and tetrachloroethene (PCE) (18). PCE also serves as an electron acceptor for growth of two new genera, "*Dehalobacter restrictus*" and *Dehalospirillum multivorans* (24, 43).

Previously, we described microcosms and enrichment cultures with the ability to dechlorinate 2,3-dichlorophenol (2,3-DCP) to 3-CP and subsequently to phenol and methane (41). Here we describe the isolation of strain Co23, a spore-forming relative of *D. dehalogenans* with the ability to grow with lactate as an electron donor and several haloaromatic compounds as electron acceptors.

MATERIALS AND METHODS

Growth conditions. Cultures were grown in 160-ml serum bottles with 100 ml of boiled degassed medium or in 30-ml anaerobic culture tubes with 20 ml of medium and closed with butyl rubber stoppers. The mineral salts medium (47) consisted of 2 mM potassium phosphate buffer (pH 7.2 to 7.5) and (per liter) $CaCl_2 \cdot 2H_2O$, 0.015 g; $MgCl_2 \cdot 6H_2O$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.007 g; and Na_2SO_4, 0.005 g. A trace metals solution was added to give the following final concentrations (per liter): $MnCl_2 \cdot 4H_2O$, 5 mg; H_3BO_3, 0.5 mg; $ZnCl_2$, 0.5 mg; $CoCl_2 \cdot 6H_2O$, 0.5 mg; $NiSO_4 \cdot 6H_2O$, 0.5 mg; $CuCl_2 \cdot 2H_2O$, 0.3 mg; and $NaMoO_4 \cdot 2H_2O$, 0.1 mg. Selenium and tungsten were added to the following final concentrations (per liter): Na_2SeO_3, 0.003 mg, and Na_2WO_4, 0.008 mg. NH_4Cl was added to 8 mM. $NaHCO_3$ (10 mM) was added to buffer the medium, with a N_2 to CO_2 ratio of 95:5 added in the headspace. A vitamin solution (55) was provided after cooling. The cultures were incubated at 30°C unless indicated otherwise.

The solid medium used for culturing strain Co23 was identical to the basal medium with the following additions: 15 g of Bacto-Agar per liter; 10 mM

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pyruvate, 1.0 g of yeast extract per liter, and 10 mM 3-chloro-4-hydroxybenzoate (3Cl-4-HBA). R2A agar medium (Difco) was also used to grow strain Co23. Plates were incubated in an anaerobic glove box under a 97% N_2–3% H_2 atmosphere.

Liquid cultures of strain Co23 were grown on the mineral medium amended with 20 mM lactate and 1 mM 3Cl-4-HBA. After dechlorination had occurred, two 1.0% transfers were made to 20-ml volumes of medium, one containing lactate and 3Cl-4-HBA and one containing lactate only. Additional amendments of 3Cl-4-HBA (2 mM each time) were made after its dechlorination to 4-hydroxybenzoate (4-HBA), until the lactate had been depleted. To ensure continuous growth, 3Cl-4-HBA was never allowed to be completely removed. Growth was monitored by measuring the A_{600} in the anaerobic culture tubes. The cell yield was determined by dry-weight measurements from 100 ml of culture after the cells had consumed 20 mM lactate. The cells were concentrated by centrifugation in Corex glass tubes to a volume of 5 ml, which was subsequently filtered through a preweighed 0.2-μm-pore-size filter. The filters were dried for 2 h at 104°C, allowed to cool in a desiccator, and weighed until a constant mass was recorded.

Determination of electron donors and acceptors. The ability of strain Co23 to reductively dechlorinate 2,3-DCP with different electron donors was determined by using duplicate 20-ml culture tubes incubated at 30°C. Acetate, formate, butyrate, succinate, propionate, fumarate, lactate, pyruvate, crotonate, and H_2 were tested as potential electron donors. The volatile fatty acids (VFAs) and dicarboxylic acids were added to concentrations of 0.5 or 2.0 mM. H_2 (18 μmol or 6.6% [vol/vol]) was added to the headspace of the duplicate culture tubes. 2,3-DCP (100 μM) or 1 mM 3Cl-4-HBA was added as the electron acceptor. A 0.5% inoculum was added to each tube from a butyrate-, formate-, and 2,3-DCP-grown culture. The disappearance of 2,3-DCP and the appearance of 3-CP were monitored by high-pressure liquid chromatography (HPLC). 2,3-DCP was added three times at concentrations of 100 μM to those cultures exhibiting dechlorination activity. The appearance of 4-HBA was monitored in cultures receiving 3Cl-4-HBA as an electron acceptor. VFA utilization was monitored by HPLC, and H_2 use was determined by gas chromatography. Dechlorination and depletion of the electron donor constituted a positive test for growth.

The range of electron acceptors used by strain Co23 was determined with the same growth conditions as for the electron donor determination but with lactate (5 mM) or butyrate (5 mM) serving as the electron donor. The halogenated electron acceptors tested were 100 μM 2,3-DCP, 2,4-DCP, 2,5-DCP, 2,6-DCP, 2-CP, 3-CP, 4-CP, 3Cl-4-HBA, 3-chlorobenzoate, *o*-fluorophenol, *o*-bromophenol, *o*-iodophenol, 2,3,5-TCP, 2,4,6-TCP, 2,4,6-tribromophenol, pentachlorophenol (PCP), 3-chloroanisaldehyde, 3-chloro-L-tyrosine, and 3-chloro-4-hydroxyphenylacetate. Nitrate, sulfate, sulfite, thiosulfate, and fumarate were also tested at 5 mM. Sulfur was tested as an electron acceptor by addition of 0.1 ml of a powdered S^0 slurry to one set of tubes. The tubes were inoculated with a 0.1 to 0.5% inoculum from cultures grown on butyrate and 2,3-DCP or lactate and 3Cl-4-HBA. The concentrations of halogenated substrates were monitored by HPLC, and further substrate was added once depleted. Nitrate and fumarate concentrations were measured by HPLC. The use of sulfur compounds was determined by measuring growth, as indicated by A_{600}, compared with a lactate-only control culture. Lactate and butyrate consumption was monitored by HPLC to verify physiological activity.

Pasteurization and microscopy. To test for the presence of heat-resistant spores, fresh anaerobic medium containing lactate and 3Cl-4-HBA was heated to 80°C. A 1.0% inoculum from a 1-week-old Co23 culture was added to duplicate bottles, which were immediately placed back in an 80°C water bath for 10 min. The cultures were removed from the water bath, placed in the 30°C incubator, and monitored for dechlorination activity and consumption of lactate.

Microscopic observations were made by suspending portions of colonies and centrifuged suspensions of broth cultures in 10 μl of 5 mM phosphate buffer on agarose-coated slides. The coverslips were sealed with a molten mixture of 50% paraffin and 50% petrolatum. The cells were observed under oil immersion with a 60× phase-contrast objective lens. Gram staining was done by the modified Hucker method (11). Photographs were taken by using the automatic exposure system of the Leitz Orthoplan 2 microscope with TMAX 100 film.

Effect of temperature on growth and dechlorination. Cultures of Co23 were inoculated into anaerobic culture tubes and incubated at temperatures ranging from 15 to 75°C with 20 mM pyruvate and 1 mM 3Cl-4-HBA. Growth was monitored by measuring the A_{600} in the anaerobic culture tubes. To determine the temperature stability of the dechlorination activity in the absence of growth, Co23 was grown at 37°C on 20 mM pyruvate–1 mM 3Cl-4-HBA until it entered the stationary phase, after about 48 h. The resting-cell suspension was aliquoted (10-ml portions) into separate culture tubes, which were amended with 4 mM 3Cl-4-HBA and 10 mM pyruvate and incubated at temperatures ranging from 20 to 65°C. The rate of dechlorination in these cultures was monitored over the next 3 days by HPLC analysis.

16S rRNA gene isolation, sequencing, and analysis. Cells of Co23 were grown in 100 ml of medium containing lactate and 3Cl-4-HBA. Total DNA was isolated from Co23 by a method shown to work on diverse bacteria (52). The primers used to amplify near-full-length 16S rRNA gene sequences (5′AGAGTTTGATCCTGGCTCAG3′ and 5′AAGGAGGTGATCCAGCC3′) were modified from the primers FD1 and RD1 of Weisberg et al. (53) by removing the linker sequences. The PCR mixture consisted of 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 μM each primer, 1× *Taq* polymerase buffer, 0.75 U of *Taq* polymerase (Promega, Madison, Wis.), and 0.1 μg of DNA in a volume of 30 μl. Amplification was carried out with a GeneAmp PCR system 9600 thermocycler (Perkin Elmer, Norwalk, Conn.) with a program consisting of an initial denaturation at 92°C for 2 min 10 s followed by 30 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 2 min 10 s, and concluding with an elongation cycle at 72°C for 6 min 10 s.

The resulting PCR product was purified by gel electrophoresis through a 1% agarose gel and recovered with Gene Clean purification resin as recommended by the manufacturer (Bio 101, La Jolla, Calif.). The purified PCR product was cloned in vector pCRII with a TA cloning kit (InVitrogen, San Diego, Calif.). Plasmid DNA containing the 16S rRNA gene insert was isolated from one clone with a plasmid mini kit (Qiagen, Chatsworth, Calif.).

The DNA sequence of the insert was determined by automated fluorescent dye terminator sequencing with an ABI Catalyst 800 laboratory robot and ABI 373A Sequencer (Applied Biosystems, Foster City, Calif.). The primers used corresponded to conserved regions of the 16S sequence (54). Approximately 95% of the insert sequence was determined in both directions.

Initial phylogenetic placement was obtained with the Ribosomal Database Project (RDP) (27) electronic mail server program SIMILARITY_RANK. Related sequences and a preliminary alignment were obtained with the RDP programs SUBALIGNMENT and ALIGN_SEQUENCE. The alignment was completed with the sequence editor GDE obtained from the RDP. A maximum-likelihood phylogenetic tree was created with fastDNAml (38), using a weighting mask to include only unambiguously aligned positions with all other program options at their default values. This analysis was repeated on 100 bootstrap samples to obtain confidence estimates on the branching order (15). The program consense from PHYLIP (16) was used to determine the number of times that each group shown in the final tree was monophyletic in the bootstrap analysis. The final tree was then produced with the program TreeTool from the RDP.

The 16S rRNA sequence of *Clostridium* sp. strain DMC (EMBL accession number X86690) was obtained from the European Bioinformatics Institute web server (www.ebi.ac.uk). The following sequences were obtained from the RDP (GenBank accession numbers in parentheses): *Desulfotomaculum orientis* Singapore I (M34417), *D. dehalogenans* JW/IU-DC1 (L28946), *Peptococcus niger* (X55797), *Heliobacterium chlorum* (M11212), *Desulfotomaculum nigrificans* (X62176), *Desulfotomaculum ruminis* DL (M34418), *Bacillus subtilis* 168 (K00637 M10606 X00007), and *Escherichia coli* (J01695).

Chemical analyses. CPs, DCPs, and aromatic dechlorination products were analyzed on a Hewlett-Packard 1050 HPLC system with a Chemstation analysis package. The eluent was phosphoric acid (0.1%) plus methanol pumped at 1.5 ml/min, using a gradient from 48 to 55% methanol. A Hibar RP-18 (10 μm) column was used. Peaks were quantified at 218 nm on a UV multiwavelength detector. Samples (1 ml) from the cultures were made basic with 10 μl of 2 N NaOH, centrifuged for 6 min in a microcentrifuge, and filtered through Acrodisc LC13 polyvinylidene difluoride 0.45-μm-pore-size filters prior to HPLC analysis.

Nitrate and nitrite were analyzed by HPLC on a Whatman Partisil 10 SAX column in a Shimadzu HPLC system. The eluent was 50 mM phosphoric acid buffer (pH 3.0) pumped at a rate of 1 ml/min. The A_{210} was used for detection.

VFAs were analyzed with the Shimadzu HPLC system with a Bio-Rad Aminex HPX-87H ion exclusion column heated to 60°C and with 0.005 N H_2SO_4 as the eluent (21). Previously filtered samples were acidified to 0.25 N H_2SO_4 by adding 100 μl of 2.5 N H_2SO_4 to 900 μl of sample. Eluent was pumped at 0.6 ml/min, and VFAs were detected at 210 nm with a UV detector.

The headspace of the cultures was analyzed for H_2 and CO_2 with a Carle gas chromatograph equipped with a 1.83-m Porapak Q column and a thermal conductivity detector. The headspace pressure was normalized to atmospheric by venting with a needle before removing 0.3 ml of gas for injection into the gas chromatograph. Threshold concentrations of H_2 were measured with a gas chromatograph equipped with a reduction gas detector from Trace Analytical, Menlo Park, Calif.

Chemicals. CPs, DCPs, 3Cl-4-HBA, and 3-chloro-4-hydroxyphenylacetate were obtained from Aldrich Chemical Co. 4-HBA and 3-chloro-L-tyrosine were purchased from Sigma Chemical Co. 3-Chloroanisaldehyde was provided by Roberta Fulthorpe, University of Toronto.

Nucleotide sequence accession number. The DNA sequence described above was deposited as GenBank accession number U68528.

RESULTS

Isolation of *D. chlororespirans* Co23. Enrichment cultures inoculated from microcosms of compost soil (41) that exhibited complete dechlorination of 2,3-DCP were fed with 2,3-DCP and acetate and accumulated 3-CP. 2,3-DCP repeatedly added to this enrichment culture was completely dechlorinated in the *ortho* position. This activity was retained after transfer to new enrichment medium. Anaerobic agar shake cultures inoculated with the secondary enrichments yielded isolated colonies. Several colonies that were transferred to anaerobic broth

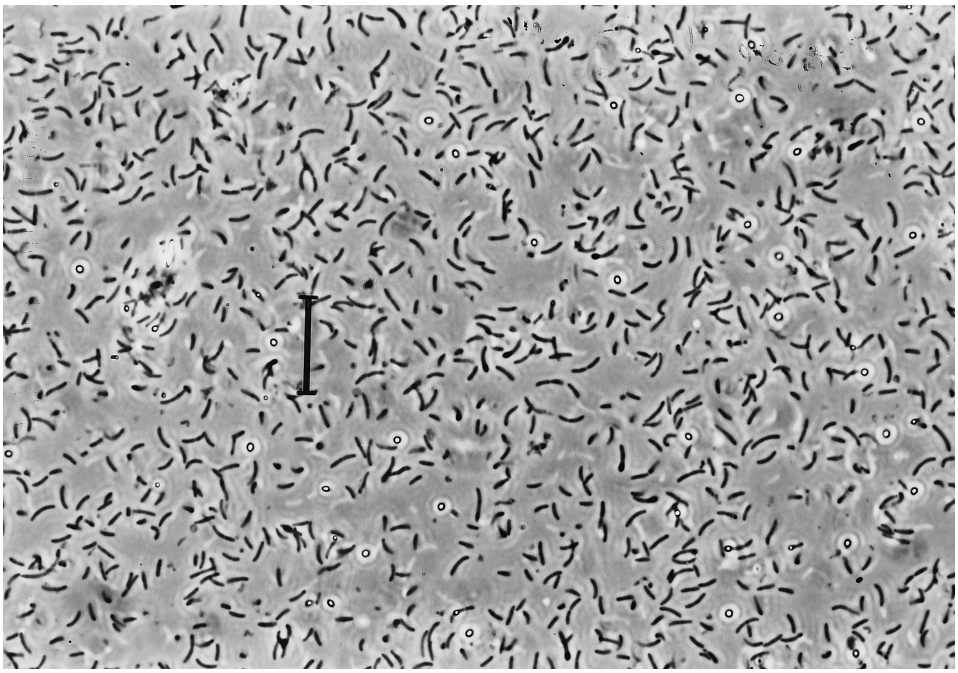

FIG. 1. Phase-contrast photomicrograph of strain Co23. Vegetative cells and the presence of spores and terminal swelling in cells developing spores are shown. Bar, 13 μm.

medium containing butyrate and formate dechlorinated 2,3-DCP. A survey of electron donors of one of the cultures showed that acetate no longer supported dechlorination of 2,3-DCP. Since 3-CP was inhibitory to growth and dechlorination activity at concentrations above 1 mM, additional chlorinated substrates which would not yield inhibitory products and therefore result in higher cell yields were evaluated. 3Cl-4-HBA was shown to be the optimum electron acceptor for growing the culture, since the product of dechlorination, 4-HBA, was not inhibitory at concentrations below 20 mM. The culture was then purified by dilution of dechlorination activity to extinction in medium containing 3Cl-4-HBA, butyrate, and formate. The purity of the resulting culture, designated strain Co23 (for compost soil origin), was confirmed by streaking culture fluid onto agar medium. Only one colony type was observed on both 3Cl-4-HBA and R2A agar media. Representative isolated colonies were transferred back into broth culture and shown to dechlorinate 3Cl-4-HBA.

The isolate is a motile, gram-negative, curved bacillus 3 to 5 μm long by 0.5 to 1 μm wide (Fig. 1). Terminal spores were present in some cells. To evaluate whether the refractal bodies (Fig. 1) were heat-resistant spores, duplicate cultures of Co23 were pasteurized. After exposure to 80°C, dechlorination and growth resumed after 1 week of incubation, confirming the ability of strain Co23 to survive the heat treatment.

Evidence for halorespiration. The ability of strain Co23 to couple reductive dechlorination to growth was determined by measuring increases in cell density in the presence and absence of 3Cl-4-HBA. The cell density increased in lactate-fed cultures only when 3Cl-4-HBA was added (Fig. 2). Stoichiometric generation of 4-HBA and acetate from the 3Cl-4-HBA and lactate, respectively, was observed. Lactate was not oxidized in the absence of an electron acceptor. The pH was periodically adjusted with 2 N KOH to 7.2 during growth because dechlorination activity was inhibited when the pH dropped to 6.2.

Additional evidence of halorespiration was provided in measurements of the hydrogen threshold and the fraction of electrons from the electron donor (fe) being used to reduce 3Cl-4-HBA. Hydrogen was consumed within a week to a threshold level of less than 1 ppm in duplicate cultures with excess 3Cl-4-HBA present in the medium. The fe was determined by plotting the number of micromoles of reducing equivalents (expressed as 2[H]) liberated in the oxidation of lactate to acetate against the number of micromoles of 4-HBA generated in the culture (Fig. 3). The slope of the resulting regression line is equivalent to the fe, which was determined to be 0.67. Values of 0.69 were calculated with cultures that used butyrate and formate as electron donors.

The mass balance of substrate use and cell yield when grown on lactate plus 3Cl-4-HBA is shown in Table 1. The cell yield

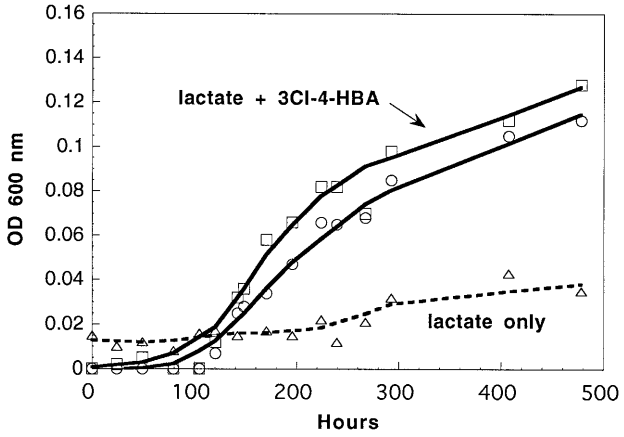

FIG. 2. Growth of strain Co23 as indicated by an increase in turbidity (OD_{600}). Duplicate cultures with lactate and 3-Cl-4-HBA are compared with a control culture containing lactate alone.

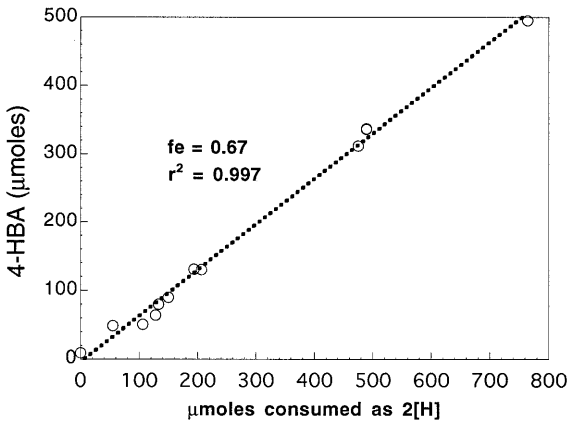

FIG. 3. Graphical determination of fe, the fraction of electrons from the electron donor accounted for by electron acceptor reduction, as indicated by the slope of the regression line. Electron pairs from lactate are plotted as reducing equivalents (2[H]) generated in the incomplete oxidation to acetate.

expressed as reducing equivalents (as 2[H]) used from growth on lactate was 3.2 g of cell dry weight per mol of reducing equivalents (as 2[H]), and the yield expressed on the basis of 3Cl-4-HBA dechlorinated was 6.90 g of cell dry weight per mol of 3Cl-4-HBA. With butyrate and formate as electron donors, the yield was 1.69 g of cells per mol of reducing equivalents (as 2[H]).

Range of electron donors and acceptors used. Strain Co23 was capable of using a wide range of electron donors for reductive dechlorination (Table 2). Growth was indicated by the measured consumption of both electron donor and acceptor over three successive feedings. All the organic acid electron donors except formate were incompletely oxidized to acetate. Pyruvate also supported growth in the absence of an electron acceptor and was fermented stoichiometrically to acetate.

The growth of strain Co23 on lactate and several different electron acceptors is shown in Fig. 4. No increase in turbidity occurred with lactate alone or with lactate plus sulfate. Sulfite, thiosulfate, and sulfur all supported growth as electron acceptors, but sulfate, nitrate, and fumarate did not. Several halogenated phenols supported the growth of strain Co23 (Table 3). *ortho*-substituted chlorines were removed from polychlorinated phenols added as an electron acceptor with two or more halogen substituents, with the exception of 2,4-DCP, 2,5-DCP, 2,3,5-DCP, and PCP. 2,4,6-Tribromophenol was dehalogenated to 4-bromophenol. Monosubstituted halogenated phenols were not utilized by strain Co23. 3Cl-4-HBA and 3-chloro-4-hydroxyphenylacetate were the only monochlorinated substrates used as electron acceptors.

Growth and dechlorination rates. Growth rates were measured with pyruvate as the sole carbon and energy source. Strain Co23 exhibited typical exponential growth with a doubling time (t_d) of 15.4 h when grown on pyruvate at 30°C (Fig. 5). Exponential growth did not occur with 3-Cl-4-HBA-fed cells, since concentrations of the electron acceptor in excess of 3 mM inhibited growth and it was difficult to maintain sufficient 3Cl-4-HBA in the growth medium (data not shown). Growth was observed from 15 to 37°C, with the optimal growth rate, expressed as μ_{max} (ln $2/t_d$), equal to 0.12 h^{-1} at 37°C (Fig. 6). No growth was observed above 45°C.

Dechlorination rates were determined for resting cells incubated at temperatures from 15 to 65°C. Measurable rates of dechlorination occurred at temperatures as high as 55°C, with the maximal rate observed at 45°C (Fig. 6). Complete dechlorination of 4 mM 3Cl-4-HBA occurred within 23 h at temperatures ranging from 25 to 45°C (Fig. 6). Although initial dechlorination rates at 50°C were significant, no further activity was observed after 11 h of incubation (data not shown). At 30°C, resting cells dechlorinated 3Cl-4-HBA at a rate of 3.44 ± 1.09 mmol h^{-1} g of cell dry $weight^{-1}$.

Phylogeny of strain Co23. Comparison of available 16S rRNA sequences with that of strain Co23 indicated that strain Co23 is a member of the *Desulfotomaculum* group of the gram-positive bacteria (RDP release version 5). Pairwise similarity values calculated between Co23 and the available near complete 16S sequences from the *Desulfotomaculum* group produced similarity values ranging from 82.2% identity with *P. niger* to 97.2 and 96.8% identity with *D. dehalogenans* JW/IU DC1 and *Desulfitobacterium* strain PCE1, respectively (Table 4). Partial 16S sequences are available for several other closely related bacteria. These sequences were included in the construction of a maximum-likelihood phylogenetic tree (Fig. 7). Bootstrap analysis of the maximum-likelihood phylogenetic tree confirmed the close relationship between strain Co23, *D. dehalogenans*, and strain PCE1.

DISCUSSION

The most distinctive feature of strain Co23 is that it was able to couple growth to dechlorination. The term "halorespiration" has been suggested for those organisms that are capable of utilizing the considerable energy released from reductive dehalogenation for oxidative phosphorylation. The $\Delta G^{\circ\prime}$ for reductively dechlorinating 2,3-DCP to 3-CP with H_2 is −144.3 kJ/mol of H_2 consumed (13). Since the conversion of lactate to acetate, CO_2, and H_2 has a $\Delta G^{\circ\prime}$ of −2.1 kJ per mol of H_2 formed and the conversion of butyrate to two acetate, H^+, and $2H_2$ has a $\Delta G^{\circ\prime}$ of +24.1 kJ per mol of H_2 formed, the coupling of these oxidations to the reduction of 2,3-DCP to 3-CP leads to a strongly exergonic reaction (49). Strain Co23 is one of only a few microorganisms described to grow by this process. The anaerobic myxobacterium strains 2CP-1, 2CP-2, 2CP-C, and

TABLE 1. Mass balance of electron donors and 3Cl-4-HBA consumed and yield of cells

Culture	Amt (mmol)			Cell mass, mg (mmol)	Yield (g/mol of substrate)		fs[a]
	2[H] from substrate equivalent[b]	4-HBA	Acetate		2[H] equivalents[b]	3Cl-4-HBA	
Lactate	2.89[c]	1.27	1.32	9.4 (0.084)[d]	3.24 (0.22)[c]	6.90 (1.11)[c]	0.285
Formate and butyrate	1.65	1.16	0.61	2.8 (0.025)[c]	1.69	2.42	0.15

[a] fs, fraction of electrons from electron donor incorporated into biomass.
[b] Reducing equivalents from electron donor oxidation are expressed as 2[H] equivalents.
[c] Two replicates were used for determination of millimoles of substrate, and three replicates were used to calculate yields shown as mean and 95% confidence interval in parentheses.
[d] Millimole equivalent of the cell mass based on a formula weight of 113, assuming a biomass composition of $C_5H_7O_2N$.

TABLE 2. Electron donors tested for use by strain Co23 in a minimal medium[a]

Substrate (mol)	Growth[b]		Product (mol)
	3Cl-4-HBA	2,3-DCP	
Acetate (1)	−	−	ND[c]
Formate (1)	+[d]	+[e]	None
Succinate (1)	−	−	ND
Propionate (1)	−	−	ND
Butyrate (1)	+	+	Acetate (2)
Crotonate	ND	+	Acetate
H_2	+[d]	+[d]	None
Fumarate (1)	ND	−	ND
Pyruvate (1)	+[f]	ND	Acetate (1)
Lactate (1)	+	ND	Acetate (1)

[a] 2,3-DCP and 3-Cl-4-HBA served as electron acceptors. Cultures were grown at 30°C.
[b] A positive score indicated growth, which was monitored by measuring the depletion of electron donor and acceptor.
[c] ND, not determined.
[d] Growth occurred only if acetate was present as a source of carbon.
[e] Formate utilization observed when butyrate was also added.
[f] Pyruvate supported growth both fermentatively and in the presence of an electron acceptor which was reduced.

TABLE 3. Electron acceptors tested with strain Co23[a]

Electron acceptor[b]	Growth[c]
2-CP	−
3-CP	−
4-CP	−
2-Fluorophenol	−
2-Bromophenol	−
2-Iodophenol	−
2,3-DCP (3-CP)	+
2,4-DCP	−
2,5-DCP	−
2,6-DCP (2-CP)	+
2,4,6-TCP (4-CP)	+
2,4,6-Tribromophenol (4-BrP)	+
2,3,5-TCP	−
PCP	−
3Cl-4-HBA (4-HBA)	+
3-Chlorobenzoate	−
3-Cl-4-OH-phenylacetate (4-OH-phenylacetate)	+
3-Chloro-L-tyrosine	−
3-Chloroanisaldehyde	−
Others	
Sulfate	−
Sulfite	+
Thiosulfate	+
Sulfur	+
Nitrate	−
Fumarate	−

[a] Growth was determined by measuring the depletion of the electron donor and electron acceptor as well as observing visual increase in culture turbidity. Lactate or butyrate was used as the electron donor. Cultures were incubated at 30°C.
[b] Dechlorination products are given in parentheses.
[c] +, support of growth; −, no growth.

2CP-3 were the first microorganisms described that are able to couple acetate oxidation to the reductive dechlorination of 2-CP (4, 5). *Desulfitobacterium* strain PCE1 also was shown to grow by lactate oxidation coupled to the reduction of 3-chloro-4-hydroxyphenylacetate (18). Some evidence suggests that *D. dehalogenans* JW/IU DC1 may also grow via halorespiration; however, yeast extract was always present in the medium, and growth in the absence of this additive at the expense of reductive dechlorination has not yet been established (51).

The evidence for the ability of strain Co23 to grow with halogenated substrates as electron acceptors is as follows. Lactate was shown to support considerable growth with 3Cl-4-HBA as an electron acceptor, but no growth occurred when only lactate was present. Similar results occurred when butyrate and formate were substituted for lactate. The reducing equivalents from the partial oxidation of lactate to acetate are partitioned to 3Cl-4-HBA dechlorination and to biomass. The fe was determined to be 0.67, implying that 67% of the available electrons from lactate are used for reductive dechlorination, which is consistent with the partitioning expected in respiratory processes (7). From the cell yield data, it is possible to calculate a value for fs, the fraction of electrons from lactate going to energy for cell synthesis (Table 1). Hence, fs + fe equals 0.96, which is close to the theoretical optimum of 1.0. Using both the fe and fs values, the following equation accounts for the mass balance of lactate oxidation, 3Cl-4-HBA reduction, and cell yield: 0.25 lactate + 0.064 H_2O + 0.355 3Cl-4-HBA + 0.0145 NH_3 → 0.214 acetate + 0.25 CO_2 + 0.0145 $C_5H_7O_2N$ + 0.355 4-HBA + 0.355 HCl.

Additional evidence for halorespiration is provided by the measured H_2 concentration in cultures incubated for 1 week. Cord-Ruwisch et al. (6) have shown that the steady-state con-

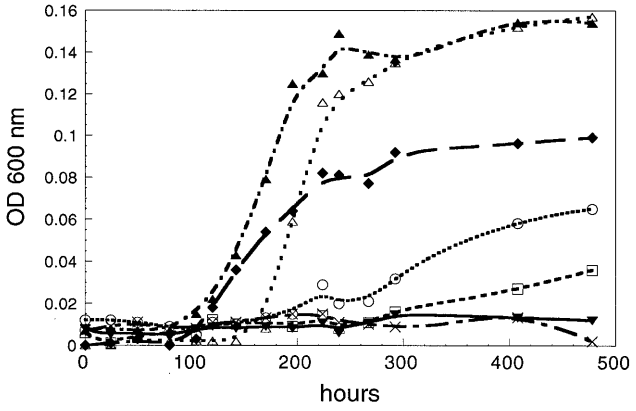

FIG. 4. Growth of strain Co23 on different electron acceptors and lactate as measured by increases in turbidity (OD_{600}). Symbols: □, sulfur; △, sulfite; ▲, thiosulfate; ×, sulfate; ○, 3-chloro-4-hydroxyphenylacetate; ◆, 3Cl-4-HBA; ▼, lactate. Data are means of duplicate cultures.

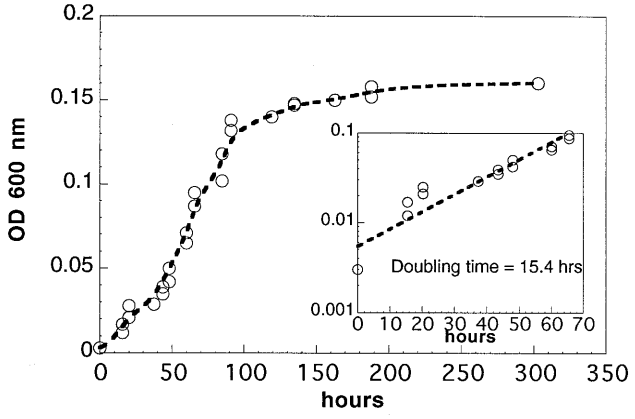

FIG. 5. Growth of strain Co23 on pyruvate at 30°C. Data are the means of triplicate cultures.

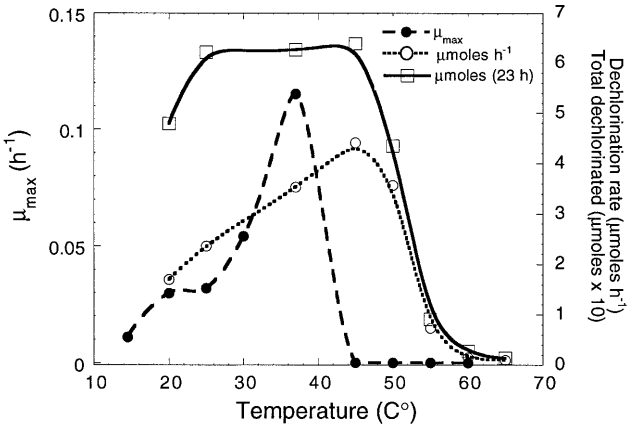

FIG. 6. Temperature dependence of growth of and dechlorination by strain Co23. Growth rates were determined by taking the mean of the μ_{max} for duplicate cultures. Dechlorination rates were determined with resting cells grown on pyruvate at 37°C and incubated at various temperatures with 3Cl-4-HBA and pyruvate. Also shown is the amount of 3Cl-4-HBA dechlorinated over 23 h.

centration of hydrogen in a culture is dependent largely on the available energy associated with the reduction of the terminal electron acceptor. The amount of energy released for oxidation and reduction reactions is inversely correlated with residual H_2 concentrations for a culture mediating those reactions. Our data showed an H_2 threshold of less than 1 ppm, which is in good agreement with the value expected if halorespiration were the process responsible for H_2 consumption (5). Other halorespiring strains were shown to lower H_2 concentrations to 0.7 ppm and to less than 0.5 ppm for *Desulfomonile tiedjei* DCB-1 (9) and 2CP strains (5), respectively.

The 16S rRNA sequence of strain Co23 places it close to *D. dehalogenans* JW/IU DC1 and *Desulfitobacterium* strain PCE1 (Table 4). In addition, secondary-structure features of the Co23 sequence are consistent with those of *D. dehalogenans*. This would at least place Co23 within the *Desulfitobacterium* genus, but the rRNA sequence differences and key phenotypic differences suggest that Co23 is a different species from *D. dehalogenans*. Recently, it has been shown that strains at 16S rRNA evolutionary distances of 2.5 to 3.0 (97 to 97.5% sequence identity) do not share 70% DNA-DNA homology and therefore would be considered members of different species (45). The 16S rRNA sequence of strain Co23 is 97.2% similar to *D. dehalogenans* and 97% similar to strain PCE1. Bootstrap analysis showed that *D. dehalogenans* and PCE1 always grouped together and that all three strains form a coherent phylogentic branch. The key features of these strains

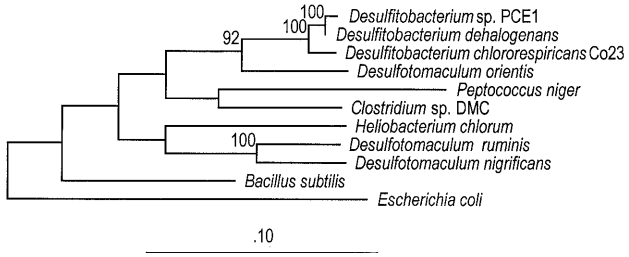

FIG. 7. Maximum-likelihood phylogenetic tree. Numbers at internal nodes are the percentage of 100 bootstrap samples in which the group to the right of the node was monophyletic. The scale is the expected number of substitutions per position.

and another similar strain, *D. hafniense* DCB-2, are compared in Table 5. The physiological differences of strain Co23 from the described species, *D. dehalogenans*, are the inability of strain Co23 to use nitrate and fumarate as an electron acceptor and its ability to form heat-resistant spores and to undergo homoacetogenic growth on pyruvate, all important physiological and taxonomic traits. Although strain Co23, *D. dehalogenans*, and *Desulfitobacterium* strain PCE1 use similar chlorinated substrates, a few of these halogenated compounds are different for the different strains. Strain Co23 is unable to use PCE or 2-CP, and both *D. dehalogenans* and *Desulfitobacterium* strain PCE1 have been shown to dechlorinate these compounds (18, 50). A phylogenetic comparison with *D. hafniense* DCB-2 is not possible since the 16S rRNA sequence of this organism is not available; however, this strain shares some physiological characteristics with Co23 that are not shared with *D. dehalogenans* (3). For example, *D. hafniense* DCB-2 was reported to grow homoacetogenically on pyruvate and produced visible spores (3, 31). Although many features of comparison have not yet been measured for strain DCB-2, it is unique in its ability to dechlorinate 3,5-DCP to 3-CP.

Although strain Co23 stains gram negative, the *Desulfotomaculum* group does not yield a reliable Gram stain (2). Utkin et al. (51) and Gerritse et al. (18) demonstrated a gram-positive cell wall for *D. dehalogenans* and *Desulfitobacterium* strain PCE, respectively, by transmission electron microscopy. Since strain Co23 is phylogenetically closely related to *D. dehalogenans* and strain PCE1, it is likely to have the same type of cell wall structure. Neither *D. dehalogenans* nor DCB-2 has yet been shown to grow via halorespiration, since both were always cultured with considerable amounts of yeast extract. The ability of *Desulfitobacterium* strain PCE1 to grow via chlororespiration indicates that this might be a common feature of the *Desulfitobacterium* genus.

TABLE 4. Pairwise similarity values for strain Co23 and selected bacterial species[a]

Species	% Similarity to:							
	D. dehalogenans	*Desulfitobacterium* strain PCE1	*D. chlororespirans* Co23	*Peptococcus niger*	*Heliobacterium chlorum*	*Desulfotomaculum nigrificans*	*Bacillus subtilis*	*Escherichia coli*
D. dehalogenans	100							
Desulfitobacterium strain PCE1	99.5	100						
D. chlororespirans Co23	97.3	96.8	100					
Peptococcus niger	83.3	83	83	100				
Heliobacterium chlorum	87.9	87.3	87	83.3	100			
Desulfotomaculum nigrificans	85.8	85.6	85.4	83.7	85.6	100		
Bacillus subtilis	84.3	84.2	84.1	81.7	83.5	83.8	100	
Escherichia coli	77.4	77.3	77.3	77.3	78.3	77.4	77.8	100

[a] Similarity values were calculated for 1,384 aligned residues. Species are as in Fig. 7.

TABLE 5. Comparison of strain Co23 with three other dehalogenating strains

Trait	Value for:			
	Co23	*D. dehalogenans* JW/IU DC1[a]	*Desulfitobacterium* strain PCE1[b]	*D. hafniense* DCB-2[c]
Gram stain	−[d]	+	+	−
Spores	+	−	−	+
Motility	+	+	+	+
Halorespiration[e]	+	ND[f]	+	+
3Cl-4-HBA dechlorination rate (mmol h^{-1} g of $cells^{-1}$)	3.44[g]	0.04[g]	ND	ND
Ready growth on solid medium[h]	+	−	+	+
Pyruvate fermentation product(s)	Acetate	Acetate, lactate	Acetate	Acetate
Electron donors for dechlorination				
Lactate	+	+	+	−
Pyruvate	+	+	+	+
H_2 + acetate	+	+	−	ND
Formate	+	+	+	−
Butyrate	+	−	+	−
Yeast extract	ND	+	+	+
Electron acceptors				
2-CP	−	±	+	−
2,4-DCP	−	+	ND	+
3,5-DCP	−	−	ND	+
PCE	−	−[i]	+	ND
Nitrate	−	+	−	+
Fumarate	−	+	+	−

[a] Data from reference 50.
[b] Data from reference 18.
[c] Data from reference 3.
[d] Strains gram negative, as is frequently observed for the gram-positive *Desulfotomaculum* group (2).
[e] Growth observed with lactate as the electron donor and a halogenated phenol as an electron acceptor, with no addition of yeast extract as a supplemental carbon and energy source.
[f] ND, not determined.
[g] Dehalogenation rate by resting cells of 3Cl-4-HBA is shown on a cell dry-weight basis. Rate data for *D. dehalogenans* are from reference 50.
[h] Colonies are easily propagated on anaerobic plate media with no special modifications to the medium or incubation conditions.
[i] *D. dehalogenans* was reported by Gerritse et al. (18) to dechlorinate PCE when grown on pyruvate and yeast extract.

The specificity that strain Co23 exhibits for removing *ortho*-substituted halogens from polyhalogenated phenols and the coupling to respiratory growth suggest that this capability has evolved for some considerable period. One hypothesis is that natural halogenated compounds are ubiquitous and abundant enough to select for and support novel anaerobic microbial populations. Fungi are a possible natural source of halogenated aromatic compounds. Recently, a white rot fungus was reported to generate concentrations of up to 75 mg of chlorinated anisyl metabolites, e.g., 3-chloroanisaldehyde, per kg of leaf litter (8). Bromophenolic compounds are produced by marine hemichordates and have been used to enrich for an anaerobic debrominating microorganism (48). Several other naturally occurring chlorophenolic compounds that could potentially serve as substrates for halorespiring microorganisms are discussed by Gribble (20).

Strain Co23 has some features desired for bioremediation, such as relatively rapid growth, compared with previously isolated aryl halorespirers. Since growth is selected by the chlorinated substrate, the process is easier to manage than if it were mediated by cometabolism. Strain Co23 has also been shown to dechlorinate a broad range of halogenated substrates in cell-free assays, generally being able to remove most *ortho*-substituted chlorines from PCP down to DCP (29). The temperature stability of the dechlorination activity may also be exploited by designing treatment systems that operate at higher temperatures. For example, at 45°C, the dechlorination activity would be stable, uncoupled from growth, and more rapid than at 37°C. Also, the spore-forming capability should provide for long-term survival of the inoculum in a habitat.

Strain Co23 is a good candidate for basic studies of the mechanism of halorespiration because it has higher growth rates and cell yields than *Desulfomonile tiedje* DCB-1 and the facultatively anaerobic myxobacterium 2CP-1 (4). Since it is a gram-positive microorganism, it is an important model system for comparison with the dechlorination process in gram-negative bacteria.

Description of *D. chlororespirans* sp. nov. *D. chlororespirans* (chlor.o.resp.i'.rans M. L. part. adj.; *chloro* referring to the group VII element chlorine; fr. L *respirare* to blow, breathe; *chlororespirans*, breathing chlorine, referring to the characteristic of coupling oxidation of electron donors to reductive removal of chlorines from various chlorophenolic compounds via a respiratory process used for obtaining energy for growth). Cells are slightly curved motile rods 3 to 5 μm long and 0.5 to 0.7 μm wide. Terminally located spores appear in late growth, and cultures are resistant to heat treatment at 80°C. Cells stain gram negative, but phylogenetically this organism is within the gram-positive *Desulfotomaculum* group. Growth is obligately anaerobic, and sulfite, thiosulfate, sulfur and *ortho*-substituted polychlorophenolic compounds are used as electron acceptors. 3-Chloro-4-hydroxybenzoate is the best substrate for halorespiration. Pyruvate, lactate, formate, butyrate, and H_2 are used as electron donors. Cells grow by partial oxidation of carbon substrates to acetate coupled to the reductive dechlorination of *ortho*-substituted chlorophenolic compounds. Growth of cells on pyruvate alone is homoacetogenic. The optimal growth temperature is 37°C. The pH range for growth is 6.8 to 7.5. Colonies grown on R2A agar medium are white, round, and smooth with a diameter of 1 to 2 mm after 1 week of growth.

The type strain is Co23. It has been deposited in the American Type Culture Collection. This organism was isolated from a Michigan residential compost soil.

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ADDENDUM

Following the revision of the manuscript, the 16S rRNA sequence of *D. hafniense* DCB-2 (X94975) was released by European Bioinformatics Institute. This sequence is 98.5% identical to that of *D. chlororespirans.*

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