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Resolution of Culture Clostridium bifermentans DPH-1 into Two Populations, a Clostridium sp. and Tetrachloroethene-Dechlorinating Desulfitobacterium hafniense Strain JH1

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Clostridium bifermentans strain DPH-1 reportedly dechlorinates tetrachloroethene (PCE) to cis-1,2-dichloroethene (cis-DCE) (4, 7, 14, 16, 17). These organisms belong to the delta/epsilon subdivisions of the Proteobacteria and the Firmicutes. Among the Firmicutes, all PCE-dechlorinating strains belong to the genera Dehalobacter and Desulfitobacterium, with one exception, the spore-forming Clostridium bifermentans strain DPH-1 (2). Spore-forming PCE dechlorinators may play relevant roles for initiating dechlorination following exposure to unfavorable conditions during physical-chemical remediation, including thermal treatment of PCE/TCE source zones. In this study, we report the resolution of the DPH-1 culture into two populations, a nondechlorinating Clostridium bifermentans strain and a nonsporulating, PCE-dechlorinating Desulfitobacterium hafniense strain, designated JH1.

Resolution of the DPH-1 culture into two populations. The DPH-1 culture was maintained in 160-ml (nominal capacity) serum bottles containing 100 ml anoxic, reduced, bicarbonate-buffered (30 mM) mineral salts medium (16) amended with acetate (5 mM), citrate (5 mM), yeast extract (2 g/liter), and PCE (240 μM, aqueous concentration). All cultures received 3% (vol/vol) inocula and were incubated at 24°C without agitation in the dark. Under these conditions, the DPH-1 culture reduced PCE to stoichiometric amounts of cis-DCE with the intermediate formation of TCE in 10 to 20 days, and this activity was stable upon repeated transfers. When culture fluid was spread on Luria Bertani (LB) agar plates, uniform colonies formed within 1 week of incubation inside an anoxic chamber (95% nitrogen-5% hydrogen, vol/vol). Unexpectedly, when cells from isolated colonies were transferred to liquid medium, PCE dechlorination activity was not recovered during a 6-month incubation period, although visible growth occurred within 1 day (Fig. 1).

In order to isolate the organism responsible for PCE dechlorination, two sequential dilution-to-extinction series were performed with 20-ml (nominal capacity) vials containing 9 ml of mineral salts medium amended with acetate (5 mM), H2 (10% headspace volume), and PCE (2.5 μl) dissolved in hexadecane (47.5 μl) to yield an initial aqueous-phase PCE concentration of approximately 460 μM (9). Dechlorination of PCE to cis-DCE occurred in the 10−10-dilution vial, which served as the source for the second dilution-to-extinction series. Dechlorination activity occurred in the 10−9-dilution vial, but when aliquots from this culture were transferred to LB agar plates, no colonies formed. Microscopic analysis corroborated the presence of two distinct organisms in the DPH-1 culture. Slender rods were observed in the 10−9-dilution vial, whereas the dominant organism in the original DPH-1 culture was a short, thick rod.

Phylogenetic analysis confirmed the presence of two populations in the DPH-1 culture. Genomic DNA was extracted from the nondechlorinating isolate obtained following clonal purification on agar plates, and 16S rRNA genes were PCR amplified using bacterial primers 8F and 1525R as described previously (13). The 16S rRNA gene amplicons were cloned, and four cloned fragments were sequenced (13). The 16S rRNA gene consensus gene sequence that was 97.3% similar to the reported clostridium bifermentans strain DPH-1 16S rRNA gene sequence (GenBank accession number Y18787.1). Alignment of GenBank accession numbers Y1878782.1, AY587781.1, EF052864.1, AY167932.1, DQ978211.1, DQ218319.1, AY587793.1, AY167941.1, AY39099, AY39100, AY39101, and EF052865.1) demonstrated that the DPH-1 sequence included a 30-bp repeat from position 1068 to 1097 (Escherichia coli numbering). The alignment revealed six additional mismatches be-
tween the reported DPH-1 16S rRNA gene sequence and the nondechlorinating *Clostridium* isolate, which likely represent sequencing errors and/or sequence variability (1, 15). Excluding the 30-bp repeat, the reported strain DPH-1 sequence and the sequence of the nondechlorinating isolate share 99.5% identity. Genomic DNA was extracted from the dechlorinating pure culture obtained following serial dilutions, and the 16S rRNA gene was amplified, cloned, and sequenced (13). The 16S rRNA gene sequence of the dechlorinating isolate was 99.6% similar (1.387 bp analyzed) to the 16S rRNA gene of *Desulfitobacterium hafniense* strain Y51 (GenBank accession number AP008230.1), a known PCE–to–cis-DCE dechlorinating bacterium (18). PCR with *Desulfitobacterium* 16S rRNA gene-targeted primers (8) yielded an amplicon diagnostic for *Desulfitobacterium* spp. with template DNA from the dechlorinating DPH-1 culture (data not shown). Therefore, we propose that the organism responsible for PCE dechlorination in the DPH-1 culture is a *Desulfitobacterium hafniense* strain, which was designated strain JH1.

**Physiological characterization of strain JH1**. Electron acceptor utilization was tested with 60-ml (nominal capacity) serum bottles containing 30 ml anoxic, reduced, bicarbonate-buffered mineral salts medium amended with 5 mM pyruvate, which supported fermentative growth and served as an electron donor and carbon source. The cultures were amended with undiluted chloroethanes, chloroethenes, chloromethanes, chloropropanes, or 2-chlorotoluene using a gas-tight Hamilton syringe (1800 series; Hamilton, Reno, NV) to yield final aqueous concentrations ranging from 100 to 250 μM. Chlorinated aliphatic compounds and 2-chlorotoluene were analyzed by gas chromatography as described previously (6). Hexachlorobenzene was added using a Hamilton syringe from a methanolic stock to give an aqueous concentration of 0.09 μM and analyzed by liquid-liquid extraction in hexane, followed by gas chromatographic separation and detection using an electron capture detector. 3-Chloro-4-hydroxybenzoate, nitrate, sulfate, and sulfite were added from anoxic, sterile, aqueous stock solutions by using plastic syringes to give final concentrations of 1 to 2 mM. 3-Chloro-4-hydroxybenzoate was analyzed as described previously (10), and inorganic anions were analyzed with a Dionex ICS-3000 ion chromatograph equipped with an AS14 4-mm column (Dionex, Sunnyvale, CA). Soluble Fe(III) [as Fe(III) citrate] and poorly crystalline Fe(III) oxide were prepared as described previously (16) and added at 5 mM (nominal) concentrations. Fe(II), total iron, ammonia, and sulfide concentrations were determined colorimetrically (3, 12).

Cultures of strain JH1 completely reduced PCE to cis-DCE (Fig. 2), TCE to cis-DCE, nitrate to ammonium, sulfite to sulfide, soluble Fe(III) to Fe(II), and poorly crystalline Fe(III) oxide to Fe(II). Cultures amended with 1,1,2,2-tetrachloroethane formed 1,1,2-trichloroethane (22% mol/mol), cis-DCE (57%), and trans-DCE (21%). Under the conditions tested, strain JH1 did not reduce 1,1,2-trichloroethane, 1,1-dichloroethane, 1,2-DCA, cis-DCE, trans-DCE, vinyl chloride, carbon tetrachloride, chloroform, dichloromethane, 1,2,3-trichloro-propane, 1,2-dichloropropane, 2-chlorotoluene, hexachlorobenzene, 3-chloro-4-hydroxybenzoate, or sulfate.

To test electron donor utilization, culture vessels were amended with 100 μM PCE and inocula (3%, vol/vol) from a culture that had consumed all pyruvate. Once PCE dechlorination ceased due to electron donor limitation, cultures were amended with potential electron donors, including acetate (5 mM), ethanol (170 μM), formate (5 mM), or H₂ (10% head-space volume). Strain JH1 used formate, ethanol, and H₂ as electron donors, but acetate did not support reductive dechlorination under the conditions tested. Some *Desulfitobacterium* spp. have been reported to form spores (20), but repeated efforts to recover activity from stationary-phase strain JH1 cultures exposed to temperatures ranging from 60 to 80°C for 10 min (5) were not successful.

**Identification of the PCE reductive dehalogenase gene**. The PCE reductive dehalogenase genes pceC (AJ277528) and pceA (AP008230.1) have been identified in the DPH-1 culture (11) and *Desulfitobacterium hafniense* strain Y51 (19), respectively. To amplify the pceC gene reported to occur in the DPH-1 culture, primers (pceCF, 5'-CGGTCATCAGAAATATCGG) and pceCR, 5'-GCTGAAGTATTATAAAGTT) were designed based on published degenerate primers (11). Genomic DNA from the mixed DPH-1 culture, strain JH1, and the *Clostridium* isolate served as templates in separate PCRs; however, none of the assays yielded the expected 81-bp amplicon over the ranges of annealing temperatures (32.6 to 41.6°C) and MgCl₂ concentrations (2.5 to 4.0 mM) and the number of PCR cycles (30 to 40) tested. To amplify the pceA gene reported to occur in strain Y51, primers

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**FIG. 1.** Growth in liquid medium inoculated with isolated colonies from LB agar plates. Optical density (OD; circles) and PCE concentration (squares) in live cultures (closed symbols) and cell-free controls (open symbols) were monitored. Data were averaged from duplicate cultures. The average variability between data points was 27%.

**FIG. 2.** Dechlorination of PCE (filled squares) to cis-DCE (open squares) with the intermediate formation of TCE (triangles) by strain JH1. Data were averaged from duplicate cultures. The average variability between data points was 35.0%.
(pceAF, 5'-CGGACATCGTGCTGCTGG; and pceAR, 5'-CTTGTCGGAGCAGTTT) were designed based on the degenerate primers reported previously (19). PCRs were carried out as described previously (13), but at an annealing temperature of 46.5°C. Amplicons of the expected size (1,000 bp) were obtained with genomic DNA from the DPH-1 culture and strain JH1, whereas genomic DNA from the Clostridium isolate did not yield a visible amplification product in ethidium bromide-stained agarose gels. The amplicons were purified (Qiagen QIAquick PCR purification kit; Germantown, MD) and sequenced using primers pceAF and pceAR. The sequence of the 935-bp fragment exactly matched the reported Y51 pceA gene sequence (19).

Desulfitobacterium hafniense strain JH1 shares many physiological properties with strain Y51 but, in contrast to strain Y51, dechlorinated 1,1,2-trichloroethane, whereas strain JH1 failed to reduce sulfate whereas strain Y51 reportedly reduced sulfate (18).

Coenrichment of PCE dechlorinators with Clostridium spp. is not unprecedented. For example, Sung et al. (17) reported a coenrichment consisting of the PCE dechlorinator Desulfuromonas michiganensis strain BB1 and Clostridium sphenoides. Hence, unexplored, possibly symbiotic nutritional interactions between Clostridium spp. and dechlorinators may exist. Understanding the interactions between dechlorinators and nondechlorinating populations is relevant for successful bioremediation, emphasizing the need for detailed studies of the ecology of bacteria capable of respiratory reductive dechlorination (i.e., dechlororespiration).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of strain JH1 and the nondechlorinating Clostridium isolate have been deposited in GenBank under accession numbers EU523374 and EU526032, respectively.

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