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A Freshwater Anaerobe Coupling Acetate Oxidation to Tetrachloroethylene Dehalogenation

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Strain TT4B has been isolated from anaerobic sediments known to be contaminated with a variety of organic solvents. It is a gram-negative, rod-shaped bacterium and grew anaerobically with acetate as the electron donor and tetrachloroethylene as the electron acceptor in a mineral medium. *cis*-Dichloroethylene was the halogenated product. This strain did not grow fermentatively and used only acetate or pyruvate as electron donors. Tetrachloroethylene and trichloroethylene were used as electron acceptors, as were ferric nitriloacetate and fumarate. Nitrogen and sulfur oxyanions were not able to substitute as the electron acceptor for this organism. Modest growth occurred in a two-phase system with 1 ml of hexadecane containing 50 to 200 mM tetrachloroethylene (aqueous concentrations, 25 to 100 μM) and 10 ml of anaerobic mineral solution with Na_2S as the reducing agent. Growth was completely inhibited at tetrachloroethylene levels above 100 μM.

Tetrachloroethylene (PCE) is one of the most widely used industrial solvents, with an annual U.S. production of 550 million pounds (*Federal Register*, 1985). Current and past use of PCE has caused widespread contamination (30), resulting in a clear requirement for biological or chemical treatment technologies.

Although this solvent is not degraded by aerobic bacteria (5), it is metabolized by anaerobic microorganisms through a reductive dechlorination process (40). There have been a number of reports describing the anaerobic dehalogenation of PCE, with ethylene as the product (8, 10, 16, 28). In each of these cases, the degradation of PCE is generally thought to proceed through trichloroethylene, one of the three dichloroethylene isomers, and finally through vinyl chloride to ethylene. Electron donors for this process include methanol (12, 16), hydrogen (28), and in some cases more complex substrates including wetland sediment (2), benzoate (33), and sucrose (7). There has been only one report of PCE dechlorination coupled to acetate oxidation (6), with trichloroethylene as the product.

Dehalospirillum multivorans has recently been isolated and grows by coupling the oxidation of hydrogen to the reduction of PCE, producing *cis*-dichloroethylene (32). Two other highly enriched cultures have also been reported to carry out similar reactions (19, 28), with one recently reported to convert PCE all the way to ethylene (28). In this paper, we report on the isolation and characterization of a unique pure culture coupling the oxidation of acetate to the reductive dechlorination of PCE, with *cis*-dichloroethylene as the product.

In addition, several organisms carry out the dehalogenation of PCE to trichloroethylene after having been grown on other compounds. These include two *Methanosarcina* spp. and *Desulfomonile tiedjei* (9, 13).

MATERIALS AND METHODS

Anaerobic methods and media. Anaerobic sediments were collected from a depth of 10 cm in a small stream in eastern Massachusetts. The stream is contaminated with industrial solvents including trichloroethylene and toluene at dissolved concentrations of 77 nM and 2.0 μM, respectively (22).

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A mineral medium was used with a 4:1 N_2-CO_2 gas phase and 0.35% sodium bicarbonate, similar to that previously described (23), except that it lacked both rumen fluid and casitone. The anaerobic techniques used were those of Hungate (20) as modified by Balch and Wolfe (1). The media were reduced with $Na_2S \cdot 9H_2O$ (final concentration, 0.025%) except when ferric iron was used as the electron acceptor. When organic solvents were added to the medium, stock solutions were generally dissolved in hexadecane under N_2-CO_2, sealed with a Teflon-coated butyl rubber serum stopper, and autoclaved. The hexadecane stocks were then added by syringe (1 ml per 10 ml of medium). All media containing organic solvents were sealed in serum tubes or bottles with a Teflon-coated serum stopper. Ferric nitriloacetate stock solution (neutralized with $NaHCO_3$) was filter sterilized and added to the growth medium to a final concentration of 10 to 25 mM. The Fe(II) concentration was determined as previously described (27). Hydrophilic sulfur was prepared as described previously (3) and added as a colloidal solution, and polysulfide was added from a stock solution (42).

All incubations were static at room temperature (21 to 24°C) unless otherwise indicated.

Pure cultures were isolated from enrichments grown on acetate as previously described (17). Pure cultures were obtained from roll tubes, incubated with the stopper down. The solid medium contained 5 mM sodium acetate and 200 mM PCE dissolved in 1 ml of hexadecane.

Nutrition and growth parameters. The nutrition and growth parameters were determined by monitoring growth by measuring the optical density (600 nm) in mineral medium. Because of the difficulties of monitoring optical density in a two-phase hexadecane-water system, growth with PCE as the electron acceptor was monitored by measuring either acetate consumption if it was the substrate or the conversion of PCE to *cis*-1,2-dichloroethylene for other electron donors.

Both the temperature and pH range for acetate consumption were tested in mineral medium with 6 mM acetate and 1 ml of hexadecane (containing 200 mM PCE). Acetate was monitored over time in all cases. The pH of the medium was adjusted with anoxic 1 M HCl or 1 M NaOH after it had been boiled and cooled under 4:1 N_2-CO_2 to obtain the desired pH within a range of 6.15 to 7.8. The pH was redetermined after autoclaving.

The optimal PCE concentration was determined in the same manner at pH 6.8, except that the concentration of PCE in the stock hexadecane was varied. Later experiments, including those in which the fermentation balance was determined and those used to determine the optimal concentration of pyruvate or fumarate for growth, used the mineral medium with 0.7% sodium bicarbonate to obtain a more desirable initial pH of 7.2. The fermentation balance experiment was set up with controls lacking an electron donor as well as controls lacking an electron acceptor.

Analytical methods. Growth on organic acids was determined by measuring changes in the optical density at 600 nm (18-mm serum tubes) in a Bausch and Lomb Spectronic 20 spectrophotometer. Generation times were calculated from the linear portion of a semilog plot of growth. The acetate concentration was determined by gas chromatography of acidified culture fluids (37). First-order acetate utilization rates were determined from the slope of the linear portion of a semilog plot of acetate utilization versus time.

Chlorinated ethylenes were determined by one of several procedures, all involving gas chromatography. For quantitative analysis, 0.5 to 1.0 ml of the culture headspace was removed and injected into a Carlo Erba HRGC 5300

mega series gas chromatograph (Fisons Instruments, Valencia, Calif.) equipped with a 30-m Restek RTX-5 capillary column (film thickness, 5 μm). Quantitative analysis of chlorinated ethylenes was also performed by injecting 1 μl of the hexadecane phase directly into a Hewlett-Packard 5890 Series II gas chromatograph equipped with a Carbograph VOC column (Alltech Associates, Inc., Deerfield, Ill.). Variability among triplicates was generally 5 to 10% of the total.

Nonvolatile organic acids were determined by high-pressure liquid chromatography (9). Proteins were determined by centrifugation of cells from the culture followed by treatment with 1 N NaOH at 90°C for 10 min. The protein levels were then determined by the bicinchoninic acid assay. Variability among triplicates was less than 20% and generally was 5 to 10%. Chloride was determined by ion chromatography (Dionex Corp., Sunnyvale, Calif.) with an AS-4A column. With this system, increases in chloride levels were quantifiable.

Electron microscopy. Exponential-phase cells were prepared for thin sectioning as described by Murray et al. (29). Electron micrographs were obtained with a Zeis EM-10 electron microscope.

Amplification, cloning, and sequencing of 16S rDNA. DNA was extracted from frozen cell pellets by the guanidine thiocyanate/diatom method for DNA isolation (4). 16S rDNA was selectively amplified by PCR amplification with bacterium-specific primers (S-D-Bact11aS-17 and S-D-Bact1512aA-16) (25). Reaction mixtures were in a total volume of 50 μl and contained 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM deoxynucleoside triphosphates, 0.5 U of *Taq* DNA polymerase (Pharmacia Biotech Inc., Piscataway, N.J.), DNA template at 0.04 to 0.4 ng/ml, and 10 pmol each of the two primers. Thermal cycling was carried out with a thermal cycler (Idaho Technology) as follows: an initial denaturation at 94°C for 30 s, followed by 30 cycles of 92°C for 15 s, 50°C for 15 s, and 72°C for 30 s. The amplified products were ligated directly into the cloning vector, pCRII (Invitrogen), with the original TA cloning kit (Invitrogen Corp., San Diego, Calif.).

Nucleotide sequences were determined by the dideoxynucleotide method (3). This involved cycle sequencing of purified plasmid preparations (Qiagen, Inc., Chatsworth, Calif.) with a Sequitherm sequencing kit (Epicentre Technologies, Madison, Wis.) and an infrared automated DNA sequencer (Li-Cor, Inc., Lincoln, Neb.) under conditions recommended by the manufacturers. Labeled M13 universal forward and reverse dye-labeled sequencing primers (31) and four additional oligonucleotide primers (24) were used to determine nearly complete 16S RNA sequences.

Sequence analysis. 16S rRNA sequences were aligned on the basis of conserved features of primary and secondary structures (38, 43). On the basis of this alignment, phylogenetic trees were constructed by using the ARB system and associated database (36). The sequences for other organisms represented in Fig. 5 were obtained from the ARB database (36). Trees were constructed by evolutionary-distance, maximum-parsimony, and maximum-likelihood methods (14). The phylogenetic tree in Fig. 5 was constructed by the maximum-likelihood method on the basis of inferred evolutionary distance and the correction of Jukes and Cantor to infer evolutionary distance (21). In addition to the evolutionary-distance method, relationships were examined by using the DNABOOT program of PHYLIP (Phylogeny Inference Package) (14); this program implements the bootstrap method of placing confidence limits on phylogenies by using parsimony.

Nucleotide sequence accession number. The GenBank accession number of the sequence described here is 134771.

RESULTS

Isolation of strain TT4B. Sediments were inoculated into the mineral medium described above, which contained 0.25% Bacto-Agar and 61 μM PCE with no hexadecane phase. PCE was added from a saturated stock solution. Agar was added as an energy source and formed a sloppy suspension. During the incubation period, the headspace was monitored for the disappearance of PCE. PCE was completely converted to *cis*-dichloroethylene over the course of the incubation. This product was identified by its retention time during gas chromatography and mass spectroscopy. A typical time course for the appearance of dehalogenated products is shown in Fig. 1. Over the course of the incubation, the agar was not liquefied. After repeated 10% transfers with agar and PCE (approximately every 2 weeks over a 3-month period), the enrichment culture was transferred into media with a variety of potential electron donors (concentrations tested in parentheses). Only glucose (0.5 mM) or acetate (1 mM), not hydrogen (50%), methanol, formate, butyrate (1 mM each), or toluene (50 μmol/liter) supported the dehalogenation activity. Since dehalogenation was not observed with many of the above compounds, it seemed unlikely that electron donor was carried over from the original inoculum. No abiotic dehalogenation was observed. The enrichment culture was then repeatedly transferred with acetate and PCE. Although growth was not detectable by turbidometric analysis at this PCE concentration, the culture was consistently transferable. A 1-ml hexadecane phase containing 200 mM PCE was subsequently used as a source of PCE, and transformation of the PCE to *cis*-dichloroethylene was observed. The enrichment culture was again repeatedly transferred, with slight turbidity and dispersion of the oil phase occurring as a function of growth. This enrichment culture was eventually diluted into roll tubes. These were incubated for 3 months with 10 mM acetate and 1 ml of hexadecane containing 200 mM PCE. Isolates were picked from the 10^{-4} dilution of the enrichment. Several of these isolates grew with acetate and PCE, showing slight turbidity. Their morphologies were identical, and they did not grow without PCE. Strain TT4B was used for further study.

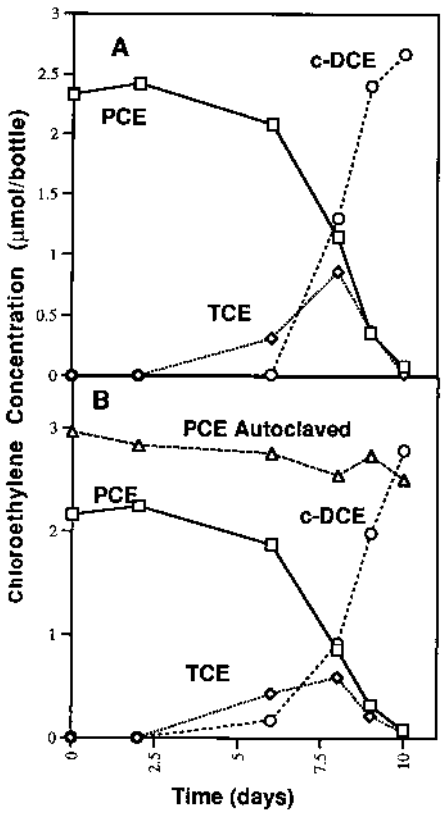

FIG. 1. Plot of the time course of the depletion of PCE and the accumulation of trichloroethylene (TCE) and *cis*-dichloroethylene (c-DCE) in enrichment cultures incubated with 0.25% agar (A) or 5 mM acetate (B) as the electron donor. Enrichment cultures were incubated in 40-ml serum bottles with 20 ml of a mineral medium.

Energy sources and fermentation products. Strain TT4B grows well in a mineral medium with either acetate or pyruvate as the electron donor and PCE, trichloroethylene, or fumarate as the electron acceptor. These results are based on turbidity in comparison with control cultures incubated without one component, either donor or acceptor, as well as on the protein determination (Table 1). This organism also uses ferric nitriloacetate as an electron acceptor, reducing 10 mM Fe(III) to Fe(II) from a 2% inoculum over a period of 30 to 40 days. Ferric nitriloacetate did not allow for significant growth yields as judged by turbidity. The influence of either pyruvate or

TABLE 1. Fermentation balances, cell protein yield and generation times of TT4B while growing on acetate

Electron acceptor and initial concn	Amt of acetate used (mM)[a]	Amt of electron acceptor used (mM)	Concn (mM) of products		Electron recovery (%)[b]	Protein yield (g/mol of acetate)[c]	Generation time (days)
			Succinate	Chloride[d]			
Fumarate (20 mM)	6.8	17.5	16.2		66	5.84	4
PCE							
10 mM[e]	4.6	9.9		17.9	98	0.60	2–4
20 mM[e]	9.4	17.4		32.6	88	0.71	3–4

[a] Corrected for acetate utilized in culture incubated without electron acceptor (0.54 mM).
[b] Includes electrons recovered in cell biomass assuming that cells are 50% protein with a composition of $C_{4.86}H_{8.85}O_{2.41}N_1$ and with 7.4% ash. The variability among triplicates was less than 20% and generally 5 to 10% of the total for all of the analyses in this table.
[c] Corrected for protein observed in culture incubated without electron acceptor.
[d] Both trichloroethylene and *cis*-dichloroethylene are recovered as products. See Table 2 for these data.
[e] Initial concentration if all of the solvent was in the aqueous phase.

fumarate concentration on the growth rate of this organism was determined. Although the relative concentration of fumarate has little influence on the growth rate, an increase in the pyruvate concentration from 10 to 80 mM, with an equivalent fumarate concentration, increased the growth rate from 0.17 to 0.61 day^{-1}. Growth rates with 20 and 40 mM pyruvate were 0.36 and 0.51 day^{-1}, respectively. Air-exposed and oxidized cultures (resazurin pink) would not grow or reduce PCE during incubation.

Compounds tested which were not used as electron donors for the reductive dechlorination of PCE include mannitol, fructose, glucose (2 mM each), glycerol, citrate, lactate, butyrate, ethanol (5 mM each), methanol, crotonate (10 mM each), formate (20 mM), hydrogen (210 μmol/10 ml of culture), Casamino Acids, tryptone (0.1% each), toluene, benzene (100 mM each in hexadecane), benzoate (1 mM), and phenol (0.5 mM). The following compounds were not dechlorinated in the presence of acetate (controls lacking acetate): 1,1,2,2-tetrachloroethane, 1,1,1,2-tetrachloroethane, 1,4-dichlorobenzene, carbon tetrachloride, and chloroform (all added as a 100 mM solution in hexadecane). Compounds not acting as respiratory electron acceptors for growth with acetate include nitrate, sulfate (20 mM each), polysulfide (10 mM), sulfur, and oxygen.

Strain TT4B would not grow on any of the substrates tested alone, including H_2-CO_2 (4:1 at 1.5 atm [152 kPa]), glucose, fructose, xylose (2 mM each), methanol, ethanol, lactate (10 mM each), formate (20 mM), Casamino Acids (0.1%), and yeast extract (0.1 and 0.5%). A reaction stoichiometry for strain TT4B growing with acetate as the electron donor and PCE or fumarate as the electron acceptor is presented (Table 1). In all cases, significant growth yields were obtained with similar generation times. Another product with a similar HPLC retention time to malate was observed in fumarate-grown cultures. If fumarate was partially converted to malate, this would account for the small discrepancy between the amount of fumarate used and the amount of succinate produced (Table 1). The products of PCE dehalogenation were quantitated; however, the recovery of volatile products was lower than expected (68 to 97%) for active cultures, most probably because of losses during the 30- to 40-day incubation period (Table 2).

Growth optimization. An attempt was made to improve the growth rate through the addition of yeast extract, clarified rumen fluid, Casitone, or 1,4-naphthoquinone to the growth medium. We observed that none of the amendments had a positive effect on growth as measured by acetate disappearance (Fig. 2A), including 1,4-naphthoquinone (50 μM) (data not shown) and that both yeast extract and Casitone caused significant inhibition. It could be argued that Casitone and yeast extract were either acting as alternative electron donors or as fermentative substrates. However, chloride production curves (resulting from the dechlorination of PCE) indicate a similar inhibitory phenomenon (Fig. 2B). Additional growth as a result of the addition of any of the amendments was not observed, and no fermentative growth was observed with either yeast extract or Casamino Acids alone at 0.1% each.

Acetate utilization rates with PCE as the electron acceptor indicated an initial pH range for growth of 6.5 to 7.4 with an optimum around 7.4. We did observe a significant decrease (0.2 pH unit) during the incubation at an initial pH of 6.8. No growth occurred at an initial pH of 6.2 or 7.8. The influence of the concentration of PCE on the acetate utilization rate was also determined (Fig. 3). The optimum concentration of PCE in hexadecane was 100 mM; below this concentration, it appeared to be limiting, and above, it appeared to inhibit acetate utilization. At 400 mM and above, no acetate utilization was observed. We have calculated the aqueous concentration of PCE in a hexadecane-water system (34) on the basis of both the oil/water partition coefficient (2,090) and Henry's law constant for PCE (27.5). With 1 ml of hexadecane and 10 ml of aqueous phase, the fraction of PCE in the medium is 0.47%, giving a dissolved concentration of 47 μM with 100 mM PCE in the hexadecane phase. Under these conditions, the fraction of PCE in the headspace is 0.7%. Growth as measured by acetate utilization occurred at 21 and 31°C but not at 16 or 35°C. No detectable differences in the growth rate could be determined for the growth at 21 and 31°C.

Structure. Cells were rod shaped (0.6 by 1.0 to 1.7 μm) with rounded ends. No spores have been observed. Electron micro-

TABLE 2. Concentration of chlorinated ethylenes observed in fermentation balance cultures

Electron Donor (Initial concn)	Initial PCE concn (mM)[a]	Concn of products (mM) in hexadecane[b]			Chloroethylene recovery (%)
		PCE	Trichloroethylene	Dichloroethylene	
Pyruvate					
4 mM	99	47	8.6	40	97
8 mM	210	102	14	60	84
Acetate					
5 mM	99	0	0	67	68
10 mM	210	36	1.2	112	71
None	99	107	0	0	108

[a] Concentration in the hexadecane phase as measured directly by gas chromatography.
[b] 98.8% of PCE is present in the hexadecane phase. Cultures were sacrificed, and the hexadecane was sampled after an incubation period of 25 to 35 days.

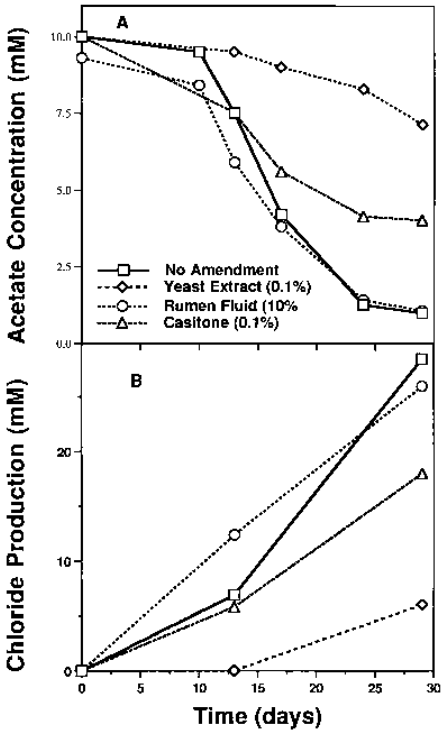

FIG. 2. Plots showing a time course of acetate consumption (A) and chloride production (B) during growth on acetate and PCE. Curves show the influence of different amendments to the growth medium. The variability between duplicates was less than 15% and generally was between 5 and 10%.

graphs revealed a typical gram-negative cell envelope (Fig. 4) with some narrow elongated internal inclusions adjacent to the cytoplasmic membrane.

16S rRNA sequence analysis. A similarity matrix determining the relationships among TT4B and other species from the delta subdivision of the *Proteobacteria* has been constructed. *Escherichia coli* was also included in our analysis to root the phylogenetic tree. The most similar organism to strain TT4B was *Desulfuromonas acetexigens* (96.9%), with three species of *Pelobacter* (*P. acetylenicus*, *P. venetianus*, and *P. carbinolicus*) all having slightly lower similarity values (94.4 to 95.3). Stackebrandt and Goebel (35) suggested that 16S sequence similarities of 97% or lower correlate to DNA-DNA reassociation values of less than 70%, and DNA-DNA reassociation values of 60 to 70% have been used as a recommended standard for defining a new species (41). On this basis, the isolate described here is a distinct species. The phylogenetic tree (Fig. 5) shows a statistically significant Bootstrap value [100] for the cluster containing *D. acetexigens* and strain TT4B. This group, several strains of *Pelobacter*, and all of the desulfuromonads form a stable cluster (100 Bootstraps) distinctly separated from the other groups of the delta subdivision of the *Proteobacteria*, in all analyses applying the different treeing methods.

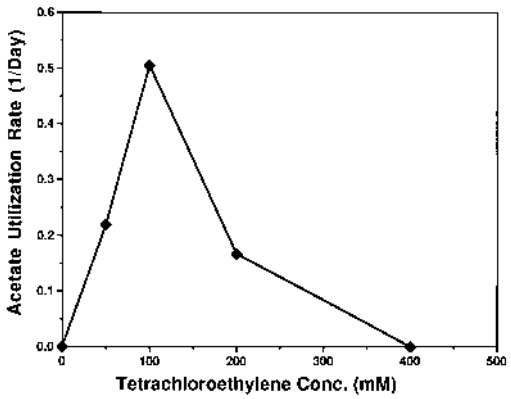

FIG. 3. Plot of acetate utilization rate versus initial PCE concentration in the hexadecane phase for cultures of TT4B growing with acetate and PCE. The variability between duplicates was less than 20%.

DISCUSSION

Although the complete degradation of PCE to ethylene and HCl has been shown to occur with mixed cultures and sediments (8, 10, 16, 28), no pure cultures which can carry out this reaction have been isolated. Several pure cultures which can carry out the partial dehalogenation, as described in this paper, have been described. These include *Dehalospirillum multivorans* (32), which couples the oxidation of H_2 to the reduction of PCE to *cis*-dichloroethylene, and "*Dehalobacter restrictus*" (18), which carries out a similar reaction. Neither of these previously described organisms will grow with acetate as an electron donor, and they differ from strain TT4B in this respect. Our physiological studies show that strain TT4B is most closely related to organisms in the genera *Desulfuromonas* and *Geobacter* on the basis of its ability to oxidize acetate. It differs from *Desulfuromonas* species in that it cannot utilize sulfur as electron acceptor. It also differs from organisms in the genus *Pelobacter* in that it will grow with acetate and use respiratory electron acceptors. It also differs from both of these genera in its ability to utilize PCE and trichloroethylene as electron acceptors as well as in the phylogenetic position. We have tested *Geobacter metallireducens* but have been unsuccessful in obtaining growth or dehalogenation by this organism with PCE and acetate.

The taxonomy of the Fe(III)-reducing bacteria has been recently reviewed by Lonergan et al. (26). Two clusters have been described, and strain TT4B fits within the "*Desulfuromonas*" cluster (Fig. 5), which includes species of *Desulfuromonas*, *Pelobacter*, and *Desulfuromusa*. Organisms within this cluster have the ability to reduce S^0 and/or Fe(III) (26), and several are capable of acetate oxidation. While *Pelobacter* and *Desulfuromonas* species have a common ancestor (11), they have different biochemical and physiological properties. Strain TT4B has several features in common with its closest known relative, *Desulfuromonas acetexigens* (15). Both are mesophilic and have a limited range of growth substrates, of which growth with acetate and fumarate is a common feature. *Desulfuromonas acetexigens* has a slightly higher pH optimum of 7.6 to 7.8 and also a slightly higher temperature optimum of 30 to 35°C. The strain described here has a statistically significant Bootstrap value [100] in the phylogenetic branch with *Desulfuromonas acetexigens*, confirming its relation to this species.

Because of the relatively low aqueous solubility of PCE (0.150 g/liter [34]) and its relative toxicity, the growth of microorganisms in an aqueous system with PCE as electron acceptor is limited. We have used a two-phase hexadecane-water system, which allows the chlorinated solvent concentration to be maintained at a low but steady level and functions to keep the concentration of chlorinated solvent products at similarly low levels. Previous isolates have been obtained in a similar manner (18, 32). This two-phase system represents an ideal model of the behavior of PCE in aquatic ecosystems. In the natural environment, PCE and other solvents interact in the

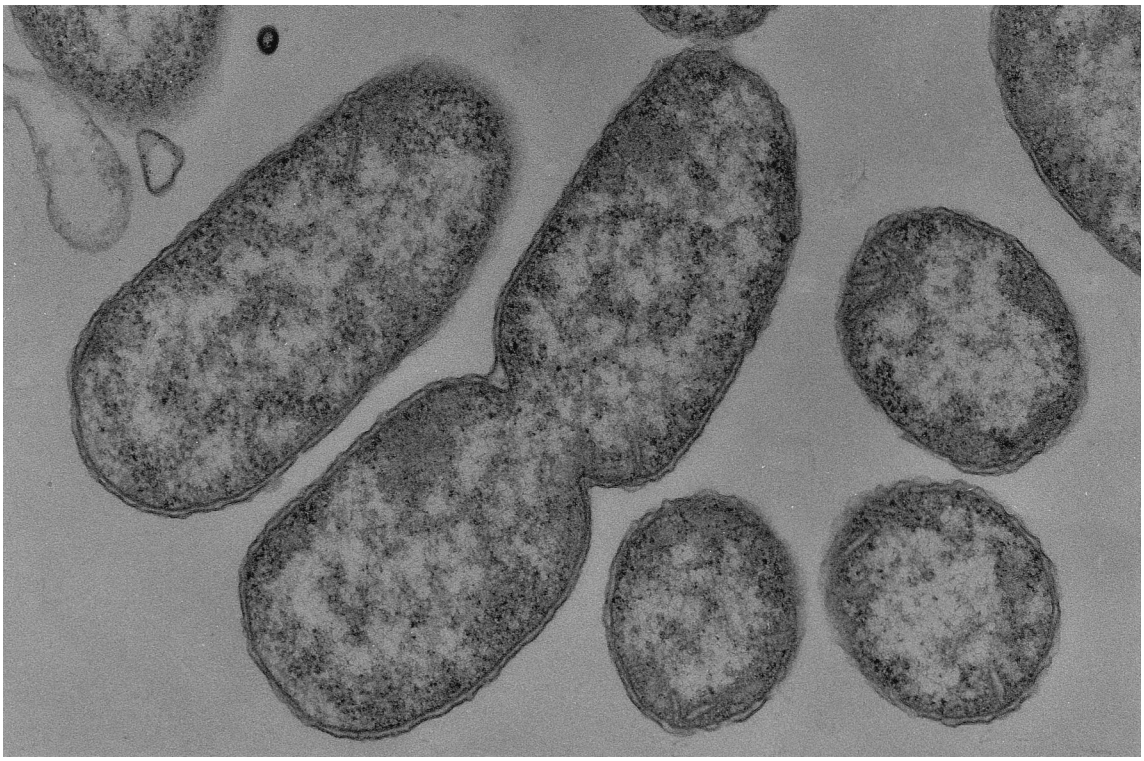

FIG. 4. Transmission electron micrograph of strain TT4B. Note what appear to be membrane-bound inclusions adjacent to the cytoplasmic membrane in cells. Total magnification, ×57,800.

same way with hydrophobic organic material as they do in the laboratory with hexadecane. The equilibrium is determined largely by the hydrophobicity of the contaminant and the fraction of organic matter in the system (34).

The growth of strain TT4B is dependent on the availability of PCE or fumarate as an electron acceptor. Growth yields are typically dependent on the change in Gibbs free energy of the corresponding reaction. Equations for the reactions carried out by strain TT4B are as follows (redox potential of chlorinated compounds taken from reference 39):

$$\text{Acetate} + 2\text{PCE} + 4\text{H}_2\text{O} \rightarrow 2\text{HCO}_3^- + 2\text{DCE} + 4\text{HCl} + 2\text{H}^+ \ (\Delta G^{\circ\prime} = -653 \text{ kJ/mol})$$

$$\text{Acetate} + 4 \text{ fumarate} + 4\text{H}_2\text{O} \rightarrow 2\text{HCO}_3^- + 4 \text{ succinate} + 2\text{H}^+ \ (\Delta G^{\circ\prime} = -247 \text{ kJ/mol})$$

The free energy obtained from the reduction of PCE is considerably greater than that obtained during the reduction of fumarate, yet the cell yield is lower (Table 1). This suggests

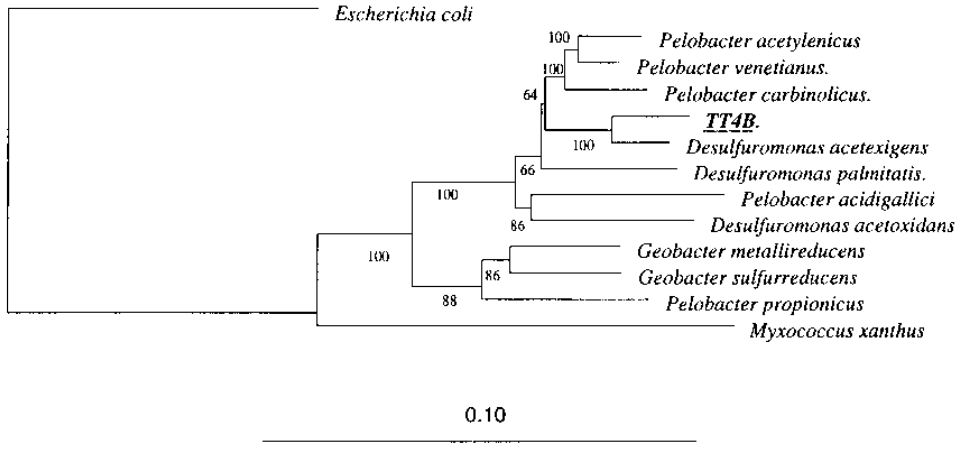

FIG. 5. Phylogenetic tree of TT4B and other representatives of the delta subclass of the *Proteobacteria* based on 16S rRNA sequence comparisons. The tree was constructed from a distance matrix by using the maximum-likelihood method and the correction of Jukes and Cantor (21). The scale bar indicates 0.01 estimated substitution per sequence. The DNABOOT program of PHYLIP (Phylogeny Inference Package) was used to calculate bootstrap values [100], placing confidence limits on phylogenies by using parsimony.

that either an energy input is needed during the metabolism of PCE or some part of the process is uncoupled from energy conservation. The latter hypothesis has been suggested to explain the low cell yield obtained during the growth of *Dehalospirillum multivorans* with PCE (32). Protein yields for growth of *Dehalospirillum multivorans* on H_2 and PCE were 1.4 g/mol of Cl^- released. Strain TT4B yielded 0.15 g of protein per mol of Cl^- while growing with acetate.

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