

Effects of Chloromethanes on Growth of and Deletion of the *pce* Gene Cluster in Dehalorespiring *Desulfitobacterium hafniense* Strain Y51

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The dehalorespiring *Desulfitobacterium hafniense* strain Y51 efficiently dechlorinates tetrachloroethene (PCE) to *cis*-1,2-dichloroethene (*cis*-DCE) via trichloroethene by PceA reductive dehalogenase encoded by the *pceA* gene. In a previous study, we found that the significant growth inhibition of strain Y51 occurred in the presence of commercial *cis*-DCE. In this study, it turned out that the growth inhibition was caused by chloroform (CF) contamination of *cis*-DCE. Interestingly, CF did not affect the growth of PCE-nondechlorinating SD (small deletion) and LD (large deletion) variants, where the former fails to transcribe the *pceABC* genes caused by a deletion of the promoter and the latter lost the entire *pceABCT* gene cluster. Therefore, PCE-nondechlorinating variants, mostly LD variant, became predominant, and dechlorination activity was significantly reduced in the presence of CF. Moreover, such a growth inhibitory effect was also observed in the presence of carbon tetrachloride at 1 μ M, but not carbon dichloride even at 1 mM.

Chlorinated organic solvents, such as tetrachloroethene (PCE) and trichloroethene (TCE), have been recognized worldwide as some of the most serious environmental pollutants. Because highly chlorinated chemicals are typically not degraded through oxygenation by aerobic bacteria, anaerobic dehalorespiring bacteria have received increased attention in the last decade. Dehalorespiration is an efficient dechlorination mechanism in which halogenated compounds can be used as the final electron acceptor. This reductive dechlorination is coupled with energy-yielding phosphorylation. Dehalorespiring bacteria are believed to play an important role in the degradation of chlorinated environmental pollutants. Since *Desulfomonile tiedjei* DCB-1, capable of the reductive dechlorination of chlorobenzoates, was first reported (5, 16), a number and variety of dehalorespiring bacteria have been isolated to date (4, 10, 11, 13, 17, 18, 22). *Desulfitobacterium hafniense* strain Y51 is one of these bacteria that dechlorinates PCE to *cis*-1,2-dichloroethene (*cis*-DCE) via TCE (19).

We previously purified and characterized the reductive PCE dehalogenase PceA and cloned the corresponding *pceA* gene from *Desulfitobacterium hafniense* strain Y51 (GenBank accession no. AY706985) (20). Subsequently, the rest of the *pce* genes, such as *pceB*, *pceC*, and *pceT*, were cloned (GenBank accession no. AY706985) (7, 20). The *pceABCT* gene cluster was situated between the two nearly identical IS elements termed *ISDesp1* and *ISDesp2*, which belong to the *IS256* family. Thus, the set of these genes could form a composite transposon (8). We isolated two different PCE-nondechlorinating variants that emerged during repeated subculturing of strain Y51 and named them SD (small deletion) and LD (large deletion) (8). Sequencing analysis revealed that the SD variant lost the *ISDesp1* element (ca. 1.6-kb DNA region upstream of

pceA), which includes the -35 promoter sequence of the *pceA* gene, so that the SD variant failed to transcribe the *pceA* gene. On the other hand, the LD variant lost ca. 6.5 kb including the *pceABCT* genes. This deletion took place by homologous recombination between *ISDesp1* and *ISDesp2* (8). Moreover, we found a significant growth inhibition when strain Y51 was cultured in the presence of 5 mM *cis*-DCE (7). After an extended lag time, the cells grew normally. This growth inhibition was observed to a lesser extent when the cells were grown with 5 mM PCE, but not with 5 mM TCE (7). In this study, we happened to find that the *cis*-DCE used in the previous study contained significant amounts of chloroform (CF). These findings led us to investigate how CF affects the growth of strain Y51 and the stability of the *pce* genes. We now report CF inhibits the growth of strain Y51, but not SD and LD variants, so that these deletion variants become predominant in the presence of CF. These findings should be important to the bioremediation and natural attenuation in the cocontaminated site by chloroethenes and chloromethanes.

MATERIALS AND METHODS

Chemicals. Chloroethenes and chloromethanes were purchased from the following companies: PCE, Ishizu Seiyaku (Osaka, Japan); TCE, Katayama Chemical (Osaka, Japan); *cis*-DCE, Tokyo Kasei Kogyo (Tokyo, Japan); all chloromethanes, GL Sciences (Tokyo, Japan). Quantitative analyses of contaminants in PCE, TCE, and *cis*-DCE were done at the Shimadzu Analytical and Measuring Center. CF contaminated the chloroethenes as follows: 0.62% (mol/mol) in *cis*-DCE, 0.00026% (mol/mol) in TCE, and 0.0017% (mol/mol) in PCE.

Strain and cultivation. *Desulfitobacterium* sp. strain Y51 was identified as *Desulfitobacterium hafniense* strain Y51 in a previous study (15). The SD and LD variants were isolated and characterized in a previous study (8). The wild-type (wt) strain Y51 and its SD and LD variants were anaerobically grown at 30°C in minimum medium containing yeast extract, pyruvate, and fumarate (MMYPPF medium) (45.9 mM K₂HPO₄, 8.8 mM KH₂PO₄, 1.7 mM sodium citrate, 0.4 mM MgSO₄ · 7H₂O, yeast extract [2.0 g/liter], 68.2 mM sodium pyruvate, 5.0 mM sodium fumarate, and 4.0 μ M resazurin sodium salt at pH 7.2) with or without chloroethenes or chloromethanes (19). Chloroethenes and chloromethanes dissolved in *N,N*-dimethylformamide were added to the culture medium after inoculation. The growth of strain Y51 and its variants was measured by their

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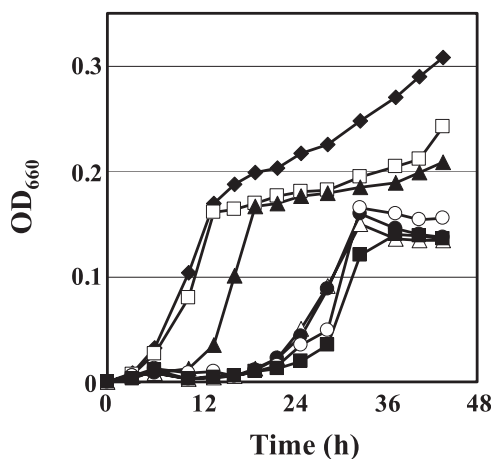


FIG. 1. Effect of CF on the growth of wt strain Y51. Strain Y51 was cultivated in MMYPF medium alone (\blacklozenge) or supplemented with 0.01 μM CF (\square), 0.1 μM CF (\blacktriangle), 1 μM CF (\triangle), 10 μM CF (\bullet), 100 μM CF (\circ), or 1,000 μM CF (\blacksquare). OD_{660} , optical density at 660 nm.

optical density at 660 nm. Growth was measured at least three times independently.

Production of biogenic *cis*-DCE. *cis*-DCE was produced biologically from TCE using a culture of strain Y51. Strain Y51 was grown in 1,000 ml MMYPF medium and harvested at the stationary phase by centrifugation at $4,000 \times g$ for 15 min. The cells were suspended in 160 ml MMYPF medium, which is a variant of MMYPF, lacking fumarate, and TCE was added at 6 mM. The complete conversion of TCE to biogenic *cis*-DCE was confirmed by gas chromatography as previously described (19). Samples of the liquid phase were extracted by a sterile syringe equipped with a 0.45- μm filter and added to fresh MMYPF cultures of strain Y51 to achieve appropriate *cis*-DCE concentrations.

Stability of the *pceA* and *tnpA* genes. The cells cultivated in MMYPF medium with or without CF were harvested at the stationary phase, and the genomic DNA was isolated according to the established procedures (21). The genomic DNA (500 ng) digested with EcoRI was subjected to the 0.8% agarose gel electrophoresis. Hybridization with digoxigenin (DIG)-labeled (Roche, Penzberg, Germany) *pceA* and *tnpA* as DNA probes was done to detect the presence or absence of the *pceA* and *tnpA* genes. This experiment was done in duplicate.

Detection of the *pceA* deletion variants. Strain Y51 was inoculated into MMYPF medium with or without chloromethanes and anaerobically cultivated until the cells reached the stationary phase. The cells were then spread onto the MMYPF agar medium. The agar (Sigma Aldrich Japan, Tokyo, Japan) was added to the MMYPF medium at 1.5%. Two hundred colonies were subjected to colony hybridization using the DIG-labeled *pceA* gene as a probe to count the *pceA* deletion variants. The frequency of loss of the *pceA* gene (as a percentage) was calculated as the ratio of the number of *pceA*-deleted colonies to the total number of colonies. This experiment was done three times independently.

PCE dehalogenase activity. The crude cell extracts were prepared from Y51 cells grown to stationary phase as previously described (19). PCE dehalogenase activity was measured in the following mixture (1.0 ml in a 5-ml serum vial): cell extracts (200 μg protein/ml), 2.5 mM sodium pyruvate, 0.2 mM NADH, 0.2 mM NADPH, 10 mM flavin adenine dinucleotide, 0.4 mM FeSO_4 , 3 mM methyl viologen, 25 mM imidazole, 5 mM dithiothreitol, and 10% glycerol at pH 7.5. The cell extract (200 μg protein/ml) of *Clostridium saccharoperbutylacetonicum* ATCC 13564 containing hydrogenase was added to reduce the methyl viologen. The vials were sealed with butyl rubber stoppers and crimped. PCE was added to get a final concentration of 600 μM . The reaction mixtures were incubated at 30°C with gentle shaking. The chloroethenes generated by the PCE dechlorination reaction were quantified by gas chromatography (19).

Immunoblotting of PCE dehalogenase. The cell extracts of strain Y51 (5 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The PCE reductive dehalogenase (PceA) was detected by immunoblotting using the anti-PceA antiserum as described previously (20).

RT-PCR of the *pce* transcripts. Cells of wt Y51 and SD variant cultivated in MMYPF medium were harvested at the mid-logarithmic phase. Total RNA from these cells was isolated by TRIzol reagent (GIBCO-BRL) according to the manufacturer's protocol. The resultant RNA was treated with DNase I for 1 h at

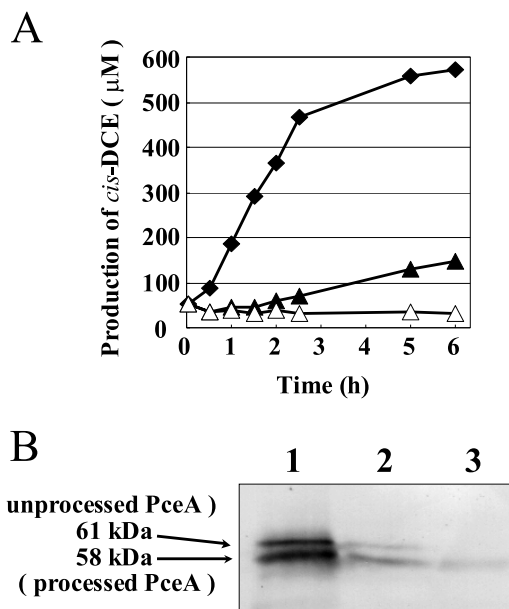


FIG. 2. (A) PCE dechlorination of cell extracts from wt strain Y51 after growth in MMYPF medium alone (\blacklozenge) or supplemented with 0.1 μM CF (\blacktriangle) or 1 μM CF (\triangle). (B) Immunoblot analysis using the anti-PceA antiserum of cell extracts from wt strain Y51. Lanes 1 to 3 depict strain Y51 grown in MMYPF medium, MMYPF with 0.1 μM CF, and MMYPF with 1 μM CF, respectively.

37°C and used as a template RNA for the reverse transcriptase PCR (RT-PCR) using the SuperScript III One Step RT-PCR synthesis system with Platinum *Taq* (Invitrogen) according to the manufacturer's protocol. For amplification of the intergenic regions between *pceA* and *pceB*, *pceB* and *pceC*, *pceC* and *pceT*, and *pceA* and *pceC* (see Fig. 4A), four sets of forward and reverse primers were chosen as follows: primer 1 (5'-TGGCATCTTGATTCCAACCG-3') and primer 2 (5'-AATCGCAGGAATAAGTGCCG-3'), primer 3 (5'-CGGCACTTATTCC TGCGATT-3') and primer 4 (5'-GCTCCTTGATTGCAAGACCT-3'), primer 5 (5'-GGTGACTATGGGACATGGGA-3') and primer 6 (5'-TAACCGGTACT GTTGCATCC-3'), and primer 1 and primer 4, respectively.

Northern blot analysis of the *pce* transcripts. Total RNA (20 μg) was electrophoresed through a 1.0% agarose gel with 15% (vol/vol) formaldehyde and transferred to a Hybond N membrane (Amersham Biosciences, Buckinghamshire, United Kingdom). Hybridization with *pceA* and *pceT* labeled with DIG as DNA probes and subsequent detection with the nitroblue tetrazolium-bromochloroindolylphosphate (Roche, Penzberg, Germany) were done according to the manufacturer's instructions.

RESULTS

Comparison of inhibitory effect between commercial and biogenic *cis*-DCE on the growth of *D. hafniense* strain Y51. In the previous study, 5 mM commercial *cis*-DCE inhibited the growth of strain Y51. However, it turned out that the commercial *cis*-DCE is contaminated by CF at 0.62% (mol/mol). Therefore, to investigate whether the commercial *cis*-DCE is intrinsically inhibitory, we compared the inhibitory effect between commercial and biogenic *cis*-DCE on the growth of strain Y51 (data not shown). The addition of commercial *cis*-DCE at 20 μM did not affect the growth of strain Y51. However, a prolonged lag period (ca. 24 h) was observed at 200 μM commercial *cis*-DCE, where the CF concentration was 1.2 μM in the culture medium. On the other hand, 200 μM biogenic *cis*-DCE did not affect growth. This result indicates that *cis*-DCE is not intrinsically inhibitory for the growth of strain Y51.

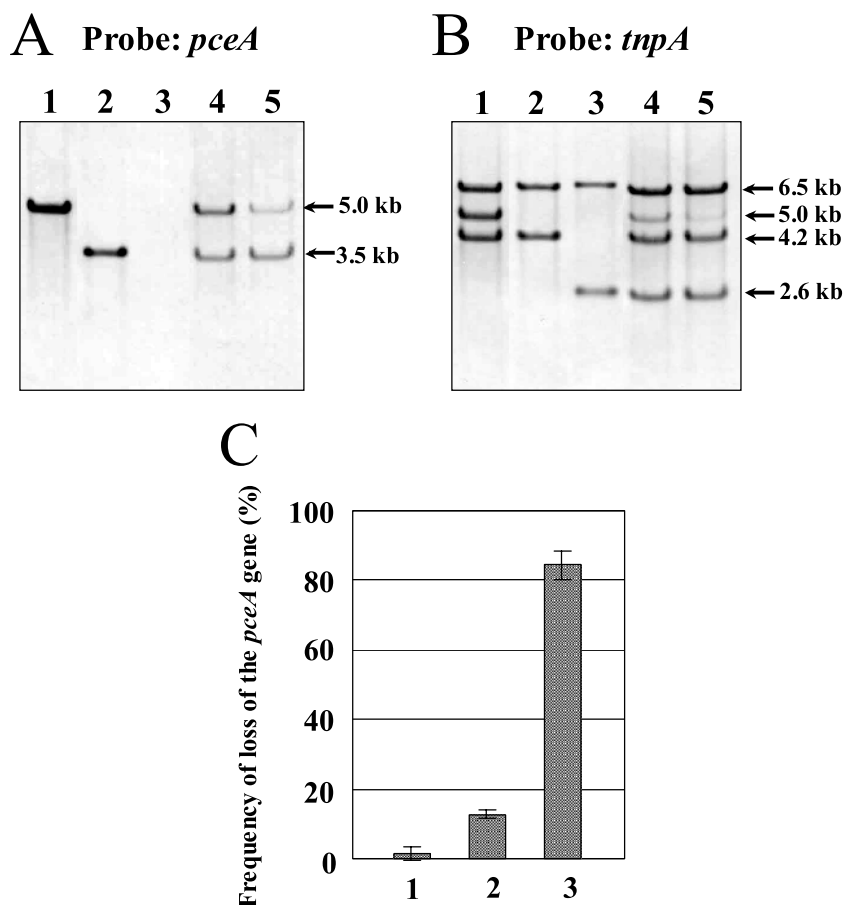


FIG. 3. (A) Southern blot analysis of the genomic DNA from strain Y51 grown with or without CF using *pceA* as a probe. Genomic DNA was digested by EcoRI. Strains and culture conditions for the lanes are as follows: lane 1, wt Y51, MMYPF medium; lane 2, SD variant, MMYPF; lane 3, LD variant, MMYPF; lane 4, wt Y51, MMYPF with CF at 0.1 μM ; lane 5, wt Y51, MMYPF with CF at 1 μM . (B) Southern blot analysis of the genomic DNA from strain Y51 grown with or without CF using *tnpA* as a probe. Genomic DNA was digested by EcoRI. Strains and culture conditions were the same as in panel A. (C) Effect of CF on the frequency of loss of the *pceA* gene in wt strain Y51. Two hundred colonies were subjected to colony hybridization using *pceA* as a probe. Culture conditions for the bars are as follows: 1, MMYPF medium; 2, MMYPF with CF at 0.1 μM ; 3, MMYPF with CF at 1 μM .

Inhibitory effect of CF on the growth of strain Y51. We investigated the effect of CF on the growth of strain Y51 (Fig. 1). Strain Y51 was inoculated into MMYPF medium, and CF was added at various concentrations. No significant growth delay was observed at 0.01 μM . However, a growth lag of ca. 10 h was observed at 0.1 μM CF. The growth lag was extended for ca. 24 h at 1, 10, 100 and 1,000 μM , and the cells grew normally after a prolonged lag phase in these cases.

PCE dehalogenase activity of the cells grown with CF. Because CF significantly affected the growth of strain Y51, we determined PCE dehalogenation activity when the cells were grown in MMYPF medium with or without CF (Fig. 2A). The cell extracts were subjected to a dechlorination assay. The cell extracts from strain Y51 grown without CF exhibited a significant dechlorination activity toward PCE. On the other hand, the cell extracts from strain Y51 grown with 0.1 μM CF showed reduced PCE dechlorination. The cell extract from strain Y51 grown with 1 μM CF had no PCE-dechlorinating ability. These results agreed with the results of the immunoblot analysis using the anti-PceA antiserum (Fig. 2B). The PceA protein signal

became significantly weaker in the cells grown with 0.1 μM CF and was dramatically weaker with 1 μM CF.

High frequency of emergence of *pceA* deletion variants in the presence of CF. Because the PCE dehalogenase activity of cells grown with CF was significantly reduced, we investigated how CF affects the *pce* genes. Southern blot analysis, using *pceA* as a probe, was done for the genomic DNA from strain Y51 cultivated in MMYPF medium with or without CF (Fig. 3A). The 5.0-kb and 3.5-kb EcoRI fragments originating from wt Y51 and SD variant were detected, respectively, and the signal was not detected from the LD variant. On the other hand, in wt Y51 grown with 0.1 and 1 μM CF, the intensity of the 5.0-kb band became faint, and the thin 3.5-kb band appeared. These results suggested that CF promotes the high frequency of emergence of the *pceA* deletion variant and slight emergence of SD variant at less than 1 μM . In addition, we used the *tnpA* gene as a probe to investigate the emergence of the LD variant, which had lost the *pce* genes (Fig. 3B). The genome analysis revealed that three *tnpA* genes are present in strain Y51 (15); three EcoRI fragments of 6.5, 5.0, and 4.2 kb were detected from wt Y51, in which the 6.5-kb EcoRI

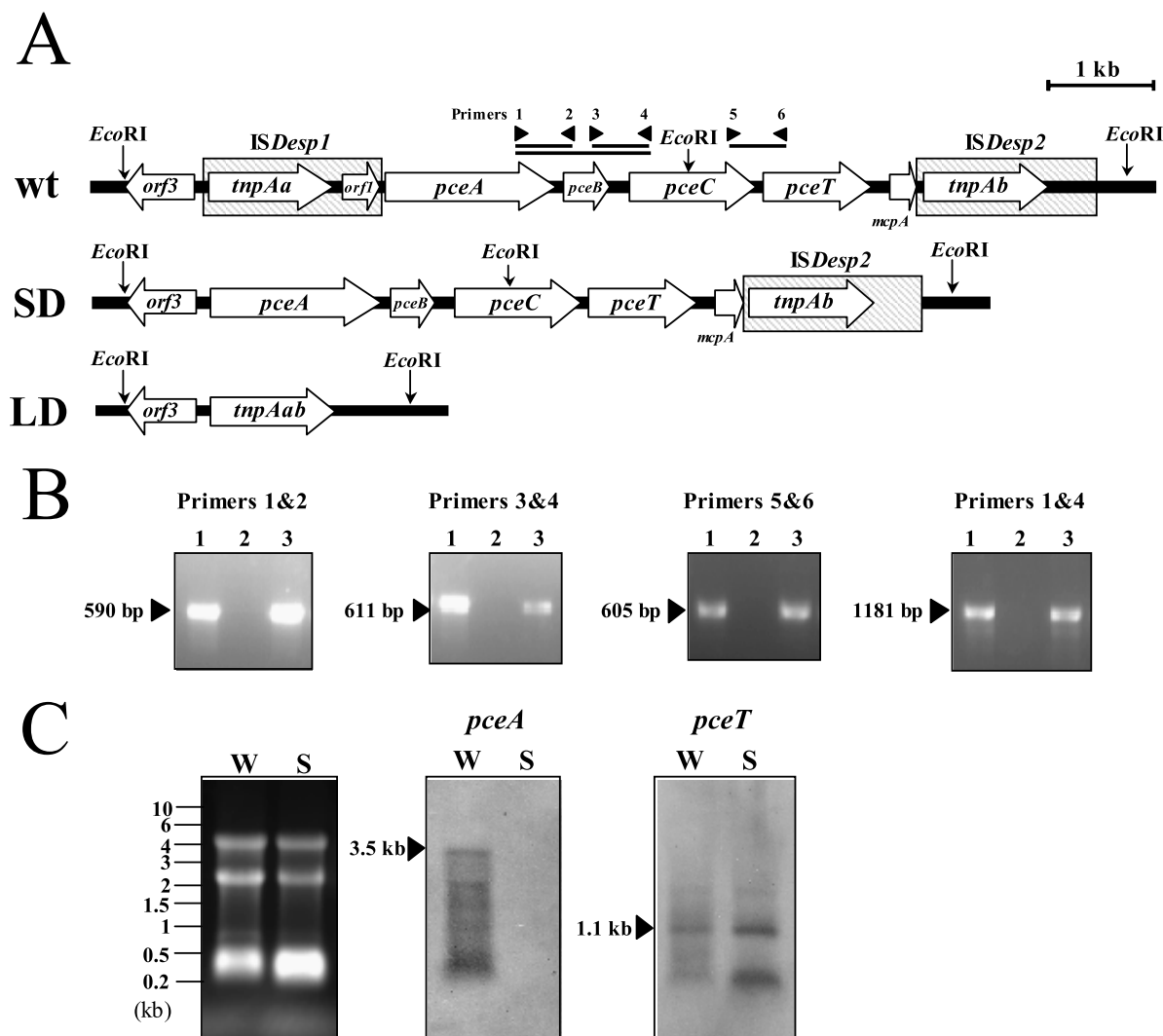


FIG. 4. (A) Gene organization around the *pce* gene cluster of wt strain Y51 and SD and LD variants. Primers for RT-PCR used in this study are depicted as arrowheads. The *EcoRI* sites are shown by vertical arrows. The mRNA transcripts detected by RT-PCR are shown as solid bars. (B) RT-PCR analyses for total RNA from wt strain Y51. RT-PCR was done targeting intergenic regions between *pceA* and *pceB* (primers 1 and 2), *pceB* and *pceC* (primers 3 and 4), *pceC* and *pceT* (primers 5 and 6), and *pceA* and *pceC* (primers 1 and 4), respectively. Lane 1, genomic DNA as the template; lane 2, RNA as the template, no RT; lane 3, RNA as the template, with RT. The sizes of RT products are indicated to the left of the blots. (C) Northern blot analyses using *pceA* and *pceT* as the probes for wt strain Y51 (W) and SD variant (S). Total RNAs from wt Y51 and SD variant were electrophoresed with RNA size markers (leftmost blot).

fragment is derived from *tnpA* located far from the *pce* gene cluster, the 5.0-kb fragment contains *tnpAa*, and the 4.2-kb fragment contains *tnpAb*. Because the SD variant lost *ISDesp1*, including the *tnpAa* gene, the 5.0-kb band disappeared (Fig. 4A). From the LD variant, the 2.6-kb band was detected, which contains a hybrid *tnpAab* gene generated by homologous recombination between *tnpAa* and *tnpAb* (8). The 6.5-kb band was detected in both SD and LD variants. In the cells grown with 0.1 and 1 μ M CF, the intensity of the 5.0-kb band (in wt Y51) became faint and the 2.6-kb band (in the LD variant) appeared. These results indicate that CF promotes the high frequency of emergence of the LD variant.

We also confirmed the *pceA* deletion variant by colony hybridization using *pceA* as a probe (Fig. 3C). Even after growth in the presence of 1 μ M CF, 84% \pm 4% of the cells lost *pceA*.

In addition, we confirmed that the 200 μ M biogenic *cis*-DCE did not affect the frequency of emergence of the *pceA* deletion variants (data not shown).

Effects of CF on the growth of SD and LD variants. We were interested in investigating the reason why the cells grew normally after an extended lag phase when wt strain Y51 was inoculated with CF. For this purpose, we investigated the growth of SD and LD variants in the presence or absence of CF. The SD and LD variants grew normally in the MMYPF medium using fumarate as an electron acceptor (data not shown). Surprisingly, no significant growth inhibition of these nondechlorinating variants was observed in the presence of CF at concentrations of 0.01, 0.1, 1, 10, 100, and 1,000 μ M (data not shown). Thus, such SD and LD variants seem to be selectively enriched in the presence of CF.

Effects of CD and CT on the growth of the strain and deletion of the *pceA* gene. We next investigated whether the negative effects caused by CF are also true for other chloromethanes, such as carbon dichloride (CD) and carbon tetrachloride (CT). First, we confirmed that strain Y51 could not dechlorinate CT and CD as well as it could dechlorinate CF (data not shown). The addition of CD at 1,000 μM did not affect the growth of wt Y51 and SD and LD variants (data not shown). However, the addition of CT caused a significant growth inhibition of wt Y51 even at 0.1 μM . The prolonged lag period of the growth of wt Y51 was observed with the increased concentrations of CT up to 100 μM as follows: 0.1 μM , ca. 6 h; 1 μM , ca. 21 h; 10 μM , ca. 24 h; and 100 μM , ca. 90 h. On the other hand, the growth of the SD and LD variants was not affected by CT up to 1 μM ; however, at 10 μM and 100 μM , an extension of the lag phase was observed as follows: 10 μM , ca. 6 h; and 100 μM , ca. 79 h. At 1,000 μM CT, the growth of wt Y51 and SD and LD variants was completely inhibited. Thus, CT exhibited growth inhibition not only for wt Y51 but also for the SD and LD variants, but to a much lesser extent for the latter two.

Subsequently, we investigated the effects of CT and CD on the stability of the *pceA* gene. Colony hybridization using *pceA* as a probe demonstrated that CT promoted enrichment of the LD variant, as did CF. The growth of strain Y51 with 1 μM CT resulted in the generation of $94\% \pm 6\%$ *pceA* deletion variants. However, CD did not generate such deletion variants even at 1,000 μM .

Transcriptional analyses of the *pce* gene cluster. We were interested in investigating the reason why growth inhibition was different for the wt strain Y51 and the SD and LD variants. Since the difference in the genotype between wt Y51 and the deletion variants is the regions around the *pceABCT* gene cluster, it is likely that the difference in the sensitivities to CF and CT between wt Y51 and its deletion variants is due to the difference in the expression of *pceABCT* genes. Therefore, RT-PCR and Northern blot analyses were done to investigate the transcription of the *pce* gene cluster (Fig. 4). RNA isolated from the cells grown with pyruvate (as an electron donor) and fumarate (as an electron acceptor) revealed that PceA is significantly produced in the absence of chloroethenes. RT-PCR toward the total RNA from wt Y51 with respective primer pairs designed to amplify the intergenic regions between *pceA* and *pceB* (using primers 1 and 2), *pceB* and *pceC* (primers 3 and 4), *pceC* and *pceT* (primers 5 and 6), and *pceA* and *pceC* (primers 1 and 4) yielded products with the expected sizes (Fig. 4B). Northern blot analyses revealed that ca. 3.5-kb mRNA was detected, indicating that the *pceA*, *pceB*, and *pceC* genes were transcribed in a polycistronic manner in wt Y51 (Fig. 4C). Also, ca. 1.1-kb mRNA revealed that *pceT* was transcribed in a monocistronic manner (Fig. 4C). On the other hand, in the SD variant, *pceA*, *pceB*, and *pceC* mRNA were not detected, but the *pceT* mRNA was transcribed as in wt Y51. These results indicated that *pceA*, *pceB*, and *pceC* were cotranscribed and that *pceT* was transcribed alone. However, the RT-PCR result (using primers 5 and 6) indicated that the *pceT* gene is also transcribed along with *pceABC* to some extent. Thus, the growth inhibition of wt strain Y51 by CF or CT seems to be caused by the interaction of PceA, PceB, or PceC with CF or CT.

DISCUSSION

In a previous study, the stability of *pceA* in *D. hafniense* strain Y51 was investigated after serial subculturing with or without chloroethenes (7). Initially, we found that the growth of strain Y51 was inhibited and more than 80% of the cells in a stationary phase lost the *pceA* gene after a single subculture in the presence of 5 mM *cis*-DCE. Afterward it turned out that the *cis*-DCE used in the previous study contained 0.62% (mol/mol) CF, which results in the contamination of 31 μM CF in the culture medium containing 5 mM *cis*-DCE. This concentration of CF is more than enough to inhibit the growth of wt strain Y51, but not SD and LD variants. We also confirmed that biogenic *cis*-DCE did not cause such growth inhibition. These results indicate that *cis*-DCE does not affect the growth of wt Y51 and enrichment of LD variant. PCE used in the previous study also stimulated the appearance of the LD variant at 5 mM but to a lesser extent than does commercial *cis*-DCE. This is simply because the addition of 5 mM PCE results in the contamination of 0.085 μM CF; this amount is much lower than that in 5 mM *cis*-DCE (31 μM CF). On the other hand, 5 mM TCE results in 0.013 μM CF, so that no significant growth inhibition or enrichment of LD variant occurred at this concentration. In the present study, we found that CF significantly affected dehalogenation activity and prevalence of *pce* gene deletion mutants at a concentration of 0.1 μM , but not at 0.01 μM . This is in perfect agreement with the effects observed for commercial chloroethene preparations.

CT also caused a growth delay in wt strain Y51. Unlike CF and CT, no negative effects were observed with CD. Therefore, the effects of chloromethanes on the growth inhibition of wt strain Y51 seem to be strictly dependent on the chlorine substitution on the methane structure. CT seems to have an additional toxic effect different from CF because the growth of SD and LD variants were also inhibited by CT at higher concentrations. Although wt Y51 did not dechlorinate these chloromethanes, *Desulfotobacterium hafniense* TCE1 did, forming a small amount of CD (9). Because strain TCE1 dechlorinates PCE to *cis*-DCE by PCE dehalogenase, which exhibits a 99.8% similarity to that of strain Y51, strain TCE1 may have other enzymes responsible for the reductive dechlorination of chlorinated methanes.

In the previous study, it was found that the LD variant was generated 6.5 times more frequently than the SD variant when wt strain Y51 was subcultured in MMYPF medium (8). This is comparable to the high emergence of LD variant when wt strain Y51 was cultured in the presence of CF and CT. These SD and LD variants were desensitized to CF even at 1,000 μM . The *pceA*, *pceB*, and *pceC* genes failed to be expressed in the SD variant because of the deletion of the promoter, and the same genes were completely deleted in the LD variant. In this context, the difference in the growth inhibition by CF between wt Y51 and the SD and LD variants seems to be caused by the presence or absence of three proteins encoded by *pceA*, *pceB*, and *pceC*. Among these proteins, PceA is a reductive dehalogenase, PceB is believed to be a membrane anchor protein associated with PceA, and PceC is an unknown protein similar to NosR that is involved in nitrous oxide (N_2O) respiration (3, 23). Our hypothesis is that one of these three proteins may interact with CF or CT to inhibit the growth of the wt strain.

Since SD and LD variants do not produce these proteins, CF does not inhibit the growth of SD and LD variants. Our efforts are currently focusing on which protein, if any, interacts with CF and what the mechanism is.

Maymo-Gatell et al. found that the commercially synthesized *cis*-DCE contained approximately 0.4% (mol/mol) CF, which was inhibitory for chloroethene utilization by *Dehalococcoides ethenogenes* strain 195 (12). They biologically produced *cis*-DCE from PCE using a *Desulfotobacterium* culture. Such a biogenic *cis*-DCE was readily utilized by strain 195 (12). Chloromethanes were reported to be inhibitory to a PCE dehalogenase of *Sulfurospirillum multivorans* (14). A 50% inhibition of the PCE dehalogenase reaction was observed at the following concentrations: 50 μ M for CD, 25 μ M for CF, and 100 μ M for CT. In the chloroethene-dechlorinating consortium, it was also reported that CF inhibited the dechlorination reaction, accumulating vinyl chloride (6). The complete inhibition of dechlorination of vinyl chloride by CF was observed at 2.5 μ M. Furthermore, CF is reported to be a potent inhibitor of methanogenesis (1, 2). However, the biochemical and genetic bases of these inhibitory effects by chloroethenes remain to be elucidated. In this study, we suggested for the first time that the growth inhibition by CF and CT could take place with interaction between a certain protein(s) (PceA, PceB, or PceC) and CF or CT in the halo-respiring *D. hafniense* strain Y51.

The results obtained in this study should be important from the viewpoints of toxicology and bioremediation including natural attenuation. It should be also important to investigate how CF affects various halo-respiring anaerobes in the environment because CF and CT are widely used and known as environmental contaminants all over the world. We are currently investigating how such anaerobes dechlorinate when chloromethanes and chloroethenes coexist in the environment.

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