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Biochemical and molecular characterization of a tetrachloroethene dechlorinating *Desulfitobacterium* sp. strain Y51: a review

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Abstract A strict anaerobic bacterium, *Desulfitobacterium* sp. strain Y51, is capable of very efficiently dechlorinating tetrachloroethene (PCE) via trichloroethene (TCE) to *cis*-1,2-dichloroethene (*cis*-DCE) at concentrations as high as 960 μM and as low as 0.06 μM. Dechlorination was highly susceptible to air oxidation and to potential alternative electron acceptors, such as nitrite, nitrate or sulfite. The PCE reductive dehalogenase (encoded by the *pceA* gene and abbreviated as PceA dehalogenase) of strain Y51 was purified and characterized. The purified enzyme catalyzed the reductive dechlorination of PCE to *cis*-DCE at a specific activity of 113.6 nmol min^{-1} mg $protein^{-1}$. The apparent K_m values for PCE and TCE were 105.7 and 535.3 μM, respectively. In addition to PCE and TCE, the enzyme exhibited dechlorination activity for various chlorinated ethanes such as hexachloroethane, pentachloroethane, 1,1,1,2-tetrachloroethane and 1,1,2,2-tetrachloroethane. An 8.4-kb DNA fragment cloned from the Y51 genome revealed eight open reading frames, including the *pceAB* genes. Immunoblot analysis revealed that PceA dehalogenase is localized in the periplasm of Y51 cells. Production of PceA dehalogenase was induced upon addition of TCE. Significant growth inhibition of strain Y51 was observed in the presence of *cis*-DCE, More interestingly, the *pce* gene cluster was deleted with high frequency when the cells were grown with *cis*-DCE.

Keywords *Desulfitobacterium* · Halorespiration · Tetrachloroethene · Dehalogenase · *pce* Genes

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Introduction

Halogenated aliphatic compounds, as well as halogenated aromatic compounds, are serious environmental pollutants. Recently, a number of studies have described reductive dechlorination during initial attack on halogenated hydrocarbons by some bacterial species. Such bacteria can grow by anaerobic respiration, a process that has been referred to as halorespiration or dehalorespiration [7, 13, 15, 26, 36, 45]. Tetrachloroethene (also referred to perchloroethylene; PCE) is a chlorinated hydrocarbon that has been widely applied in dry cleaning and other industries. Therefore, it has become an abundant pollutant of soil, groundwater, and atmosphere. Since highly chlorinated compounds are barely oxidized, degradation of these compounds has to be initiated by dechlorination by anoxic bacteria.

Some pure cultures have recently been reported to catalyze the reductive dechlorination of PCE to *cis*-1,2-dichloroethene (*cis*-DCE). These organisms belong to species of *Dehalospirillum* [23, 27, 33], *Desulfomonile* [6, 9], *Desulfitobacterium* [10, 11, 24, 38], *Dehalobacter* [14, 19], and *Clostridium* [4, 5]. Some strains belonging to *Dehalococcoides* spp. are able to convert PCE to ethene sequentially [18, 20–22, 46]. He et al. [12] recently identified a *Dehalococcoides* strain that uses DCEs and vinyl chloride (VC) but not PCE or trichloroethene (TCE) as metabolic electron acceptors.

PCE dehalogenases have been purified, and their genes cloned, from several bacteria [17–19, 27, 31, 35, 39]. The PCE dehalogenase *pceA* genes were found to be linked with the *pceB* genes coding for small hydrophobic proteins containing two or three transmembrane helices [18, 30, 37, 44], and PceB was assumed to act as a membrane anchor protein to link the dehalogenase to the respiratory chain. The presence of similar *pce* genes among different strains strongly indicates that these genes have a mechanism of transfer among these strictly anaerobic bacteria.

Unlike other *Desulfitobacterium* strains [3, 8, 32, 41–43], *Desulfitobacterium* sp. strain Y51, isolated in our laboratory, does not use chloroaromatic compounds, but exhibits a strong dechlorinating activity for PCE, converting it to *cis*-DCE. Because strain Y51 is a typical PCE-respiring bacterium, we describe here the nature of this organism, focusing on the biochemical and genetic basis of PCE dechlorination.

Growth characteristics and PCE dechlorination

Strain Y51 was originally isolated from soil in Fukuoka, Japan [38]. It grew anaerobically in MMYPF medium (minimal medium containing 0.2% yeast extract, 50 mM pyruvate and 50 mM fumarate) in the absence or presence of chloroethenes. The cell density (optical density at 660 nm, OD_{660}) reached 0.9 after 24 h (Fig. 1). Pyruvate was a good carbon source and also served as an electron donor. The addition of 50 mM fumarate greatly enhanced cell density, indicating that fumarate served as a good electron acceptor, i.e., fumarate respiration occurred [16]. Lactate, glycine and alanine support the growth of strain Y51, but succinate, citrate, malate, acetate, glutamate, alanine, methanol, ethanol, glucose, fructose, lactose and maltose do not. When pyruvate (50 mM) was the electron donor, strain Y51 grew to OD_{660} 0.05–0.1 with either nitrate, sulfite or sulfate (50 mM each) as an electron acceptor. No growth was observed with nitrite. In the presence of nitrate (20 mM), dechlorination was significantly inhibited. Nitrate may lead to a change in electron flow, and could be the final electron acceptor instead of PCE. This would explain why these compounds significantly inhibit dechlorination of PCE. Sulfite is known to bind to cobalamin upon formation of sulfito-cobalamin [27, 28, 40]. It was previously pointed out that inhibition of PCE reductive dechlorination by sulfite is due to the binding of this inhibitor to the cobalt of a corrinoid, suggesting that the PCE dehalogenase of strain Y51 also contains a corrinoid as a cofactor. On the other hand, sulfate did not inhibit dechlorination of PCE. Thus, the growth and PCE dechlorination ability of strain Y51 are highly dependent on the availability of external electron donors and electron acceptors. Oxygen completely inhibited the growth and dechlorination activity of strain Y51.

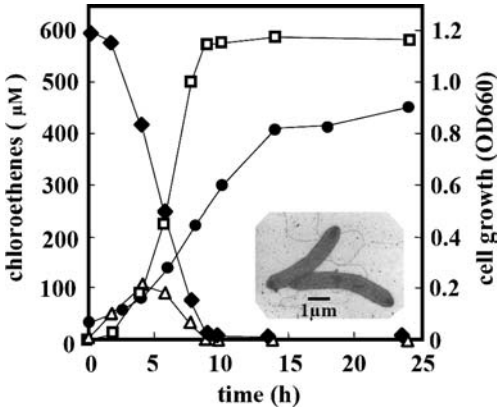

Fig. 1 Growth and tetrachloroethene (perchloroethylene; PCE) dechlorination of *Desulfitobacterium* sp. strain Y51 (*inset*). *Filled circles*, Cell growth (OD_{660}); *filled diamonds*, PCE; *open triangles*, trichloroethene (TCE); *open squares*, *cis*-1,2-dichloroethene (*cis*-DCE)

Dechlorination of chlorinated compounds

Examination of the PCE dechlorination rates by resting cells showed that maximum dechlorination (around 70 nmol min^{-1} mg cell $protein^{-1}$) was observed between 400 μM and 600 μM PCE. The Y51 resting cells were also incubated with various chlorinated alkenes, chlorinated alkanes and chlorinated aromatic compounds at a concentration of 100 μM. Y51 exhibited a wide range of dechlorination capacity towards these compounds (Table 1). Other than PCE and TCE, hexachloroethane, pentachloroethane, tetrachloroethanes, and 1,1,1,2,2,3,3-heptachloropropane were

Table 1 Substrate range of dechlorination by *Desulfitobacterium* sp. strain Y51. About 100 μM of each substrate was used. The resting cells used corresponded to a cell protein concentration of 0.26 mg mL^{-1}. No dechlorination was observed from *cis*-DCE; trans-DCE; 1,1-DCE; hexachloro-1,3-butanediene; 1,1,1-trichloroethane; 1,1,2-trichloroethane; 1,1,2,3-tetrachloropropane; 2-, 3-, 4-chlorobenzenes or 2-, 3-, 4-chlorophenyls

Substrate	Dechlorination rate (nmol min^{-1} mg cell $protein^{-1}$)	End product
Halogenated alkenes		
PCE	205.1	*cis*-1,2-DCE
TCE	303.3	*cis*-1,2-DCE
Halogenated alkanes		
Hexachloroethane	10.9	*cis*-1,2-DCE
Pentachloroethane	83.3	*cis*-1,2-DCE
1,1,1,2-Tetrachloroethane	96.2	1,1-DCE
1,1,2,2-Tetrachloroethane	32.1	*cis*-1,2-DCE
1,1,1,2,2,3,3-Heptachloropropane	19.2	Pentachloropropene

PCE Tetrachloroethene (perchloroethylene); *TCE* trichloroethene; *cis*-DCE *cis*-1,2-dichloroethene

dechlorinated. From 1,1,2,2-tetrachloroethane, pentachloroethane and hexachloroethane, *cis*-DCE was produced. From 1,1,1,2-tetrachloroethane, 1,1-DCE was the major product. From 1,1,1,2,2,3,3-heptachloropropane, pentachloropropene (M+, *m*/*z* 212; base peak, *m*/*z* 179) was detected by GC–MS analysis.

Induction of PceA dehalogenase activity

We examined the PceA dehalogenase activities for these cell extracts using 0.6 mM PCE as the substrate. The enzyme activity was 4.7 ± 0.4 nmol min^{-1} mg $protein^{-1}$ in cell grown in MMYP medium (control). Addition of PCE, TCE and fumarate resulted in enhanced PceA dehalogenase activities as follows: 44.3 ± 3.9 nmol min^{-1} mg $protein^{-1}$ for PCE; 130.9 ± 10.1 nmol min^{-1} mg $protein^{-1}$ for TCE; 13.8 ± 4.3 nmol min^{-1} mg $protein^{-1}$ for fumarate. On the other hand, addition of *cis*-DCE reduced the enzyme activity to 1.0 ± 0.3 nmol min^{-1} mg $protein^{-1}$. Enhanced or decreased enzyme production was also demonstrated by Western blot analysis using anti-PceA antibody (Fig. 2).

Purification and some properties of PceA dehalogenase

PceA dehalogenase was purified 2.5-fold in three chromatographic steps with a yield of 0.2%. The specific activity of the purified PceA dehalogenase toward PCE was 113.6 nmol min^{-1} mg $protein^{-1}$ at 37°C and pH 7.5. The enzyme was highly oxygen-sensitive and lost approximately 50% of its activity during incubation and stirring for 330 min at 4°C in the presence of air (Fig. 3). The highest dechlorination activity was observed at 37°C, but the activity was very low at 42°C. The pH optimum was between 7.0 and 7.5; at pH 6.0 or 9.5, dehalogenase activity was extremely low.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified PceA dehalogenase revealed a single protein band with an estimated molecular mass of approximately 58 kDa. Using the purified enzyme, the kinetic parameters were determined for various substrates. The assay mixture contained 4.6 μg enzyme and various concentrations (100–1,000 μM) of substrates. The apparent K_m values for PCE and TCE were determined to be 105.7 ± 24.8 μM and 535.3 ± 47.8 μM, respectively. The corresponding V_{max} values were 164.4 ± 27.2 nmol min^{-1} mg $protein^{-1}$ for PCE and 811.3 ± 39.0 nmol min^{-1} mg $protein^{-1}$ for TCE. Besides PCE and TCE, the same enzyme was also capable of dehalogenating various chloroethanes. Hexachloroethane was dehalogenated to *cis*-DCE via PCE and TCE. Pentachloroethane was dehalogenated to *cis*-DCE via TCE. 1,1,2,2-Tetrachloroethane was dehalogenated to *cis*-DCE. 1,1,1,2-Tetrachloroethane was dehalogenated to 1,1-DCE. These results indicate that at least two halogen atoms are reductively removed from the polychloroethanes to form chloroethenes. The K_m (μM) and V_{max} (nmol min^{-1} mg^{-1} $protein^{-1}$) values for these compounds were as follows: hexachloroethene, K_m 125.7 ± 14.6 and V_{max} 148.7 ± 10.4; pentachloroethane, K_m 619.9 ± 37.6 and V_{max} 876.2 ± 43, 4; 1,1,2,2-tetrachloroethane, K_m 336.5 ± 35.3 and V_{max} 42.4 ± 7.2; 1,1,1,2-tetrachloroethane, K_m 785.0 ± 71.4 and V_{max} 772.9 ± 59.7. The K_m and V_{max} values for hexachloroethane were comparable to those for PCE. Thus, the PceA dehalogenase of strain Y51 exhibited a broad range of dechlorination capability, not only for PCE and TCE, but also for various highly chlorinated ethanes.

Corrinoid enzymes are known to be inactivated by alkyl halides such as propyl iodide, and to be reactivated by light illumination. Also, sulfite is known to bind and form sulfito-cobalamin. In the presence of propyliodide

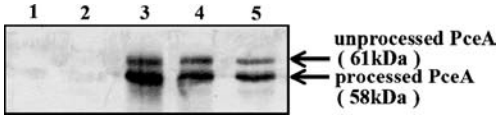

Fig. 2 Effect of various chloroethenes on production of PceA dehalogenase in *Desulfitobacterium* sp. Y51. Each chloroethene was added to the culture medium at a concentration of 0.6 mM. Western blot analyses using an anti-PceA-antibody was performed on crude extracts. *Lanes*: *1* No addition (control); *2cis*-DCE; *3* TCE; *4* PCE; *5* fumarate

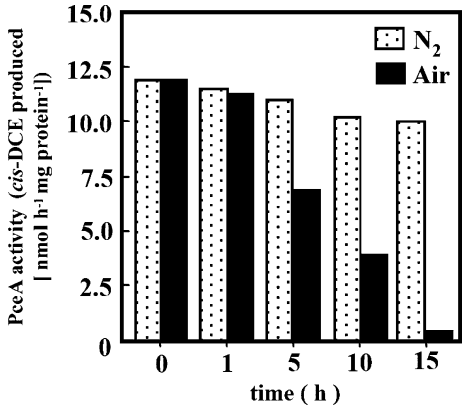

Fig. 3 Inactivation of PceA dehalogenase activity by exposure to air. Enzyme activity was measured for PCE dechlorination to *cis*-DCE after 1, 5, 10 and 15 h with gentle stirring at 4°C

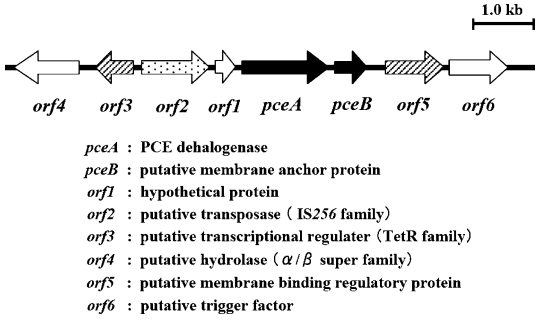

Fig. 4 Physical map of the 8.4-kb region surrounding the *pceAB* genes of *Desulfitobacterium* sp. strain Y51

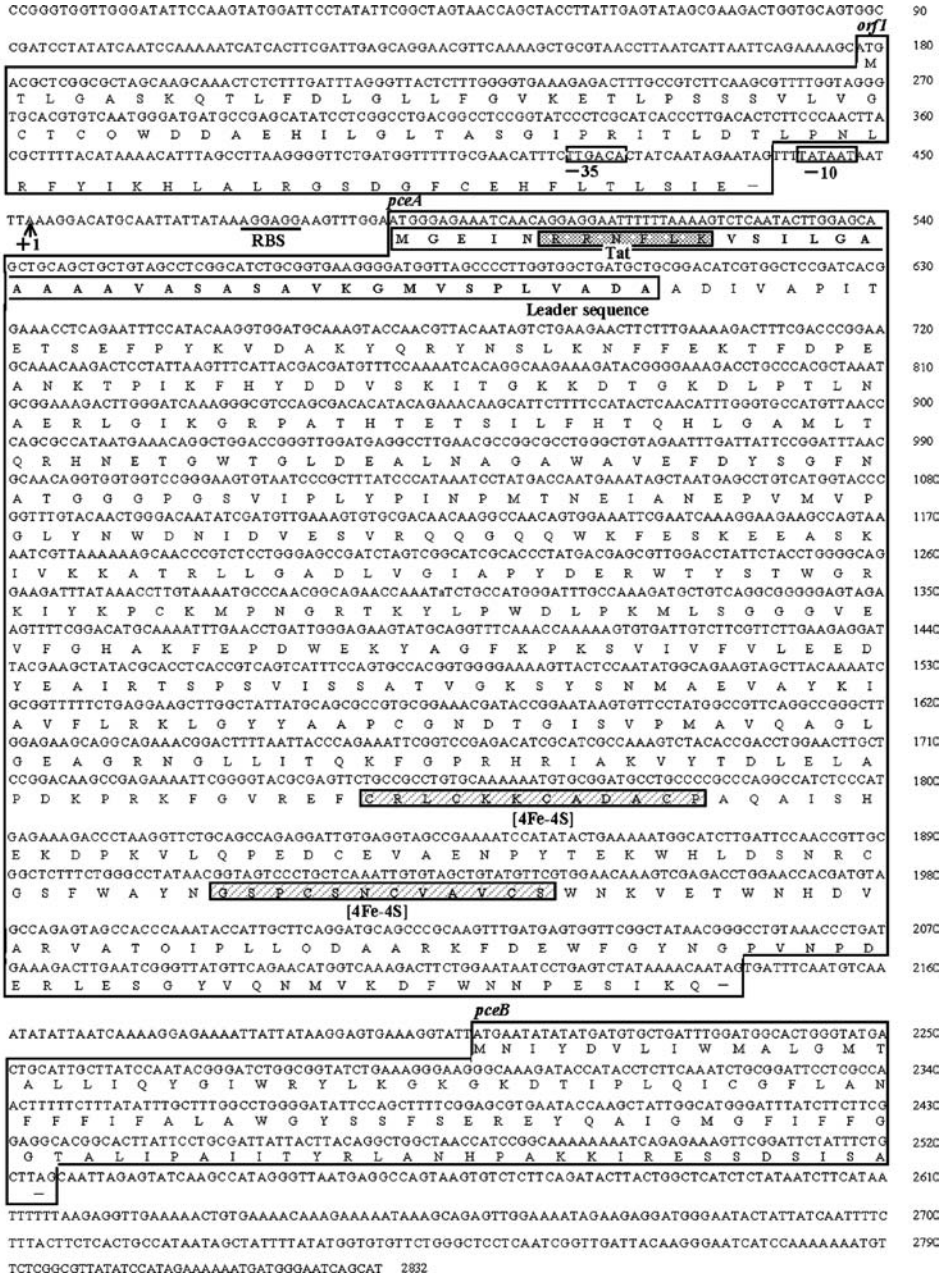

Fig. 5 Nucleotide sequence of 2,832 bp DNA including *orf1*, *pceA* and *pceB* of *Desulfitobacterium* sp. strain Y51 with the corresponding deduced amino acid sequence. The transcription start site of *pceA* is indicated by an *arrow* at position +1. The consensus promoter sequences identical to the *Escherichia coli* σ^{70} −35 and −10 regions are *boxed*; the ribosome binding site (RBS) is *underlined*; the leader sequence of PceA is *boxed*; the Tat signal sequence is highlighted in a *dark box*, and two possible [4Fe–4S] binding clusters are highlighted in *hatched boxes*

(25 mM), PceA dehalogenase activity decreased to 13%. When the mixture was then exposed to light, the activity was restored to 59% after 6 h and 90% after 24 h. The addition of 20 mM sulfite resulted in inhibition of the dehalogenase activity to 53%. These results suggest that a corrinoid is involved in the PceA dehalogenase of strain Y51.

Characterization of the *pceAB* gene

The N-terminal amino acid sequence of the purified PceA dehalogenase of strain Y51 was determined to be ADIVAPITETSEFPYKVDAK. The enzyme was digested with lysylendopeptidase, and the amino acid (aa) sequences of the six resulting peptides were determined. For PCR amplification using Y51 genomic DNA as the template, a pair of degenerate oligonucleotides was used to obtain a 1.0-kb PCR product. Using this 1.0 kb-DNA as a probe, a *Sma*I/ *Sph*I DNA fragment of 2.8-kb was obtained. Further gene walking allowed us to obtain an 8.4-kb DNA. This 8,354-bp nucleotide sequence revealed eight open reading frames (ORFs) (Fig. 4; GenBank accession nos. AB070709 and AY706985). The *pceA* gene codes for a protein containing the N-terminus and all six internal peptides of the purified PceA dehalogenase (Fig. 5). The deduced aa sequence of this ORF started 39 aa upstream of the N-terminus of PceA dehalogenase isolated from strain Y51. In this region the Tat (twin arginine translocation) consensus sequence RRXFXK [1,

2, 37] was detected. The Tat signal sequence is considered to be involved in the translocation of the enzyme into or across the cytoplasmic membrane. The molecular masses of the deduced 551-aa protein (unprocessed) and of the processed 512-aa protein were calculated to be 61,283 and 57,444 Da, respectively.

Consensus sequences similar to the binding motifs for two [4Fe–4S] ferredoxin-like clusters were present towards the C-terminal region of the PceA: CXXCXXCXXXCP (aa 420–431) and GXXCXXCXXXCS (aa 475–486). The aa sequence identity between the PceA of strain Y51 and the PceA of *D. multivorans* [30], the CprA of *D. dehalogenans* [44] and the TceA of *D. ethenogenes* 195 [18] were as low as 28.0, 26.3 and 23.9%, respectively. These values are surprisingly low when considering the similar function of these enzymes. With the exception of the signal sequence and the binding sequence of the [4Fe–4S] cluster, no significant similarities were found between these reductive dehalogenases. However, the aa sequence of PceA dehalogenase of strain Y51 is almost identical to the recently sequenced PceAs of *Dehalobacter restrictus* ([19], accession no. AJ439607), *Desulfitobacterium* sp. strain PCE-S (accession no. AY216592) and *D. hafniens* strain TCE1 (accession no. AJ439608). Only one aa difference was found between Y51-PceA and TCE1-PceA.

Downstream of *pceA*, one ORF (*pceB*, 315 bp) was detected (Fig. 4). The 105-aa protein encoded by this gene has a calculated molecular mass of 11,843 Da. Three hydrophobic regions in PceB were detected by a hydropathic plot, indicating the presence of three membrane-spanning helices. The PceB of *D. multivorans* [30] possess two, and the CprB of *D. dehalogenans* [44] and TceB of *D. ethenogenes* [18] possess three membrane-spanning helices. It is suggested that these proteins act as a membrane anchor for the reductive dehalogenases. However, no significant similarities in aa sequence between the PceB of strain Y51 and the above mentioned PceB, CprB and TceB were found. On the other hand, the aa sequence of Y51-PceB was almost identical with those of *D. restrictus* (99.0%), *Desulfitobacterium* sp. PCE-S (98.1%) and *D. hafniens* TCE1 (100%).

Sequence analysis of other ORFs

Four ORFs (*orf1*, *orf2*, *orf3* and *orf4*) were present upstream of the *pceA* gene (Fig. 4). The 86 aa-encoding *orf1* just upstream of *pceA* showed no significant sequence similarities to genes in the databases. *Orf2*, encoding 379 aa, was similar to the IS256 family transposase (accession no. AB032203) with 39% identity, while *orf3*, encoding 379 aa, showed high similarity with the TetR-family transcriptional regulator (31% identity, accession no. AAS41874). The consensus-binding motif of the TetR-family transcriptional regulator was found upstream of the *pceA* gene (Fig. 5). *Orf4*, encoding 308 aa, was similar to a α/β super family hydrolase (35% identity, accession no. Q97MJ4). There are two ORFs downstream of the *pceAB* genes: *orf5*, just downstream of *pceB* and encoding 366 aa, was similar to a membrane-binding transcriptional regulator (29% identity, accession no. AJ001308); *orf6*, consisting of 316 aa, was considered to be a trigger factor (20% identity, accession no. AE017012).

Expression of the *pceA* gene in *Escherichia coli*

Following induction with 1.0 mM IPTG of *E. coli* carrying pET32b-pceA, a PceA dehalogenase tagged with Trx, S, and $(His)_6$, with a molecular mass of approximately 77 kDa was detected in the insoluble fraction of cell extracts [39]. However, dehalogenase activity for PCE could not be detected in cell-free extracts of recombinant *E. coli*. Denaturation followed by refolding of the PceA dehalogenase fusion protein failed to recover dehalogenase activity. We used the solubilized fusion protein to raise an antibody.

Localization of PceA dehalogenase

Immunoblotting with the anti-PceA-antibody demonstrated that a protein with a molecular mass of 58 kDa, corresponding to mature PceA, was present in the periplasmic fraction (Fig. 6). Proteins with molecular masses of 61 and 58 kDa, which were considered to be the unprocessed and processed PceAs, respectively, were detected in the cytoplasmic fraction. In the inner and outer membranes, no proteins cross-reacting with the anti-PceA-antibody were detected. These results demonstrated that the mature PceA is located in the periplasm of strain Y51 cells. Thus, using the polyclonal antibody raised against PceA, it was clearly demonstrated that the mature PceA dehalogenase is localized in the periplasm, and that the unprocessed enzyme is localized in the cytoplasm. These findings are in agreement with the features of the aa sequence deduced from the *pceA* gene, where a hydrophobic stretch of 39 N-terminal amino acids (containing the consensus sequence RRXFXK) could act as a signal peptide. The remaining 512 aa can be exported into the periplasm via the Tat pathway [1, 2, 37]. The PCE dehalogenase of the Gram-negative bacterium *D. multivorans* was purified from the soluble fraction, which suggests that the enzyme is localized in the cytoplasm [29]. In contrast, the

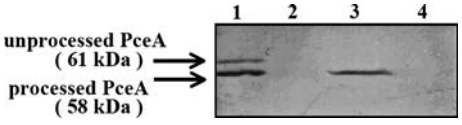

Fig. 6 Localization of the PceA dehalogenase in cells of *Desulfitobacterium* sp. strain Y51. Western blot analysis was performed using anti-PceA-antibody. Lanes: *1* Cytoplasmic fraction, *2* inner membrane fraction, *3* periplasmic fraction, *4* outer membrane fraction. Unprocessed 61-kDa and processed 58-kDa PceA proteins are indicated

PCE dehalogenases of *Desulfitobacterium* sp. strain PCE-S [25], *D. restrictus* [19, 34] and *D. ethenogenes* [17], were purified from the membrane fraction and were present as membrane-associated forms.

Effect of chloroethenes on growth of strain Y51 and deletion of the *pceA* gene

We investigated the effects of various chloroethenes on the growth of strain Y51. Addition of chloroethenes (at 5 mM each) to MMYPF medium (pyruvate and fumarate were used at 68 and 5 mM, respectively, in this experiment) affected the growth of strain Y51 to a greater or lesser extent. Addition of TCE resulted in the enhanced growth of strain Y51 after a slightly longer lag phase compared with the control (MMYPF medium without chloroethenes) (Fig. 7). On the other hand, addition of *cis*-DCE resulted in significant growth inhibition of strain Y51. Addition of PCE also inhibited growth of strain Y51, but to a lesser extent than *cis*-DCE. Growth reached 0.16 with *cis*-DCE and 0.26 with PCE with prolonged incubation (51 h, not shown). The *pceA* gene was found to be unstable, with ca. 0.2% of cells grown in MMYPF medium overnight losing the *pceA* gene upon repeated subculture. Interestingly, *cis*-DCE stimulated high frequency deletion of *pceA*. In the presence of 5 mM *cis*-DCE, 90% of cells lost the *pceA* gene after a single subculture, and 100% after five subcultures (Fig. 8). Addition of PCE also resulted in the deletion of *pceA*, but to a lesser extent. In this case, 20% of cells lost the *pceA* gene after a single subculture, and ca. 80% of cells lost the gene after five subcultures. In contrast to *cis*-DCE, TCE seemed to stabilize the *pceA* gene, so that, even after five subcultures with 5 mM TCE, no *pceA* deletion was observed (Fig. 8). Thus, TCE and *cis*-DCE have opposite effects in terms of the growth of strain Y51 and the stability of the *pce* genes. This was also true for PceA dehalogenase production (Fig. 2).

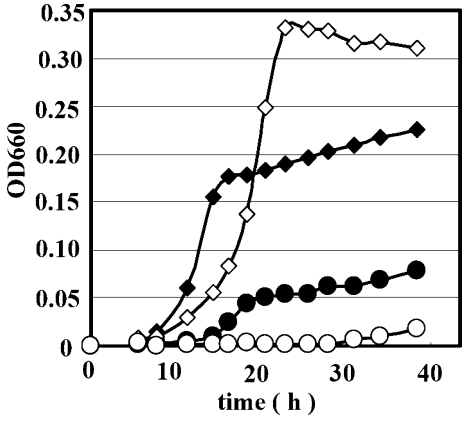

Fig. 7 Effect of various chloroethenes on the growth of *Desulfitobacterium* sp. strain Y51. About 5 mM each chloroethene was added to MMYPF medium. *Filled diamonds*, MMYPF; *filled circles*, MMYPF + PCE; *open diamonds*, MMYPF + TCE; *open circles*, MMYPF + *cis*-DCE

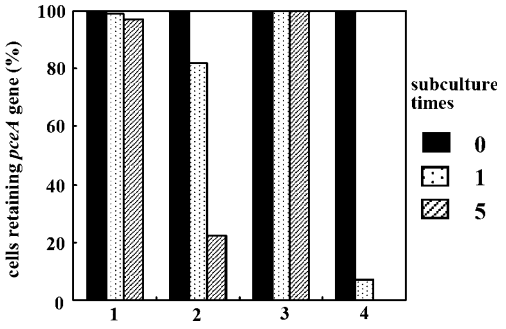

Fig. 8 Effect of PCE, TCE and *cis*-DCE on deletion of the *pceA* gene in *Desulfitobacterium* sp. strain Y51. At stationary phase following one or five subcultures, 100 colonies were examined for the presence or absence of the *pceA* gene by colony hybridization. Culture conditions: *1* MMYPF; *2* MMYPF + PCE; *3* MMYPF + TCE; *4* MMYPF + *cis*-DCE

Conclusions and perspectives

Halorespiring bacteria are generating increasing interest, because they are believed to play an important role in the degradation of chlorinated environmental pollutants. The ability to perform halorespiration appears to be widespread in nature. These strains include a variety of anoxic bacteria, among which the genus *Desulfitobacterium* has become recognized as a major group of halorespiring bacteria. Unlike other halorespiring *Desulfitobacterium* strains reported to date, *Desulfitobacterium* sp. strain Y51 cannot attack haloaromatic compounds, but it exhibited a strong capacity for dechlorination of chloroethenes and chloroalkanes, converting most of these compounds to *cis*-DCE. In this study, we characterized the biochemical and genetic basis of dechlorination in this particular anaerobe in some detail, but many items remain to be elucidated in order to further understand halorespiration in this strain, including the regulatory mechanism of the *pce* genes, the electron transfer system in halorespiration relevant to fumarate respiration, and dynamic genetic events of *pce* genes such as highly frequent deletion and gene transfer. Development of a host-vector system is also urgently required. Because the entire genome sequence of strain Y51 has recently become available (H. Nonaka et al. MS submitted), our efforts are now being focused on the above points.

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