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Note

Cloning of a Novel Dehalogenase from Environmental DNA

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Cloning of *pceA*, the gene of tetrachloroethene (PCE)-reductive dehalogenase, was undertaken from environmental DNA. Two genes were amplified using PCR primer deduced from *pceA*. Functional expression of these genes was unsuccessful in *Escherichia coli*, but PceA1 synthesized *in vitro* was enzymatically active. In recombinant *E. coli*, PceA1 formed a complex with host DnaK and caused filamentous growth.

Key words: dehalogenase; metagenome; tetrachloroethene; tetrachloroethylene; DnaK

Owing to their durability under harsh chemical and heat conditions, halogenated organic compounds are used industrially, *e.g.*, in the dry cleaning and washing of semiconductor. Once released into the environment, however, these compounds penetrate soils, accumulate in ground water, and often cause long-range pollution. Although physical and chemical methods (adsorption, oxidation, *etc.*) are used in the treatment of soils and of ground water contaminated with halogenated compounds, the application of microbial cleaning capacity in bioremediation is expected to prove a mild, safe technique. From environmental DNA, we attempted to clone the gene(s) of the enzyme involved in the detoxification of halogenated organic compounds, especially tetrachloroethylene (PCE). Several reports have appeared dealing with PCE reductive dehalogenase (PceA), the enzyme that dehalogenates PCE. We prepared environmental DNA from soil-containing groundwater samples collected in 50 domestic industrial zones. For DNA extraction, the ISOIL for Beads Beating Kit (Nippon Gene, Toyama) was used. Synthetic primers (5′-GAYAARCCNATHGAYTTYGG-3′ and 5′-CCRTANCCNARNGCRTCRTC-3′) were designed, referring to amino acid (nucleotide) sequences relatively conserved in several known *pceA* genes (accession nos. AF022812, AB194706, AY216592, and AY013367). Using these primers and the Ex-Taq system (Takara Bio, Shiga), gene fragments were amplified by PCR. Only one sample among the 50 environmental DNA preparations yielded an amplified fragment. Subsequent subcloning with a TOPO TA Cloning Kit (Invitrogen, California, USA) revealed that this PCR product contained two putative *pceA* genes (*pceA1* and *pceA2*). The full-length genes were obtained by Annealing Control Primer™ technology, using DNA Walking SpeedUp Premix Kit 2 (Seegene, Seoul, Korea). Amplification of each full-length *pce* gene was confirmed by a single PCR, using the environmental DNA template. The amino acid sequences were deduced from the genes: PceA1 was composed of 500 residues (56 kDa), and PceA2 of 493 residues (55 kDa). A homology search with the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) showed that both enzymes were highly homologous to PceA of *Sulfurospirillum* (*Dehalospirillum*) *multivorans*. As shown in Fig. 1, the homology levels were 93% (PceA1) and 63% (PceA2). At the N-terminus, each enzyme possessed a putative signal sequence containing the twin-arginine motif (RRXFXK) as a Sec-independent membrane translocation system. On the other hand, two iron-sulfur cluster binding motifs (CXXCXXCXXXCP and CXX[$_{2-12}$]CXXCXXXCP) are present at each C-terminus.[1,2] In common with *pceA* of *S. multivorans*[3] and *Desulfitobacterium* sp. strain Y51,[4] *pceB*, the gene encoding the putative membrane anchor protein of PceA, was located downstream of the *pceA* genes.

Next, the *pceA1* and *pceA2* genes deleted in the putative signal sequences were subcloned individually in pQE60 (Qiagen, Hilden, Germany) and introduced into *E. coli* BL-21. By electroporation,[5] six ampicillin-resistant transformants were obtained, and each of them was found to retain the intact insert and vector. Expression was however detected only in two clones by western blot analysis using anti-his-tag antibody, and the

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Abbreviations: PCE, tetrachloroethene; PceA, PCE reductive dehalogenase; GFAT, L-glutamine-D-fructose-6-phosphate aminotransferase

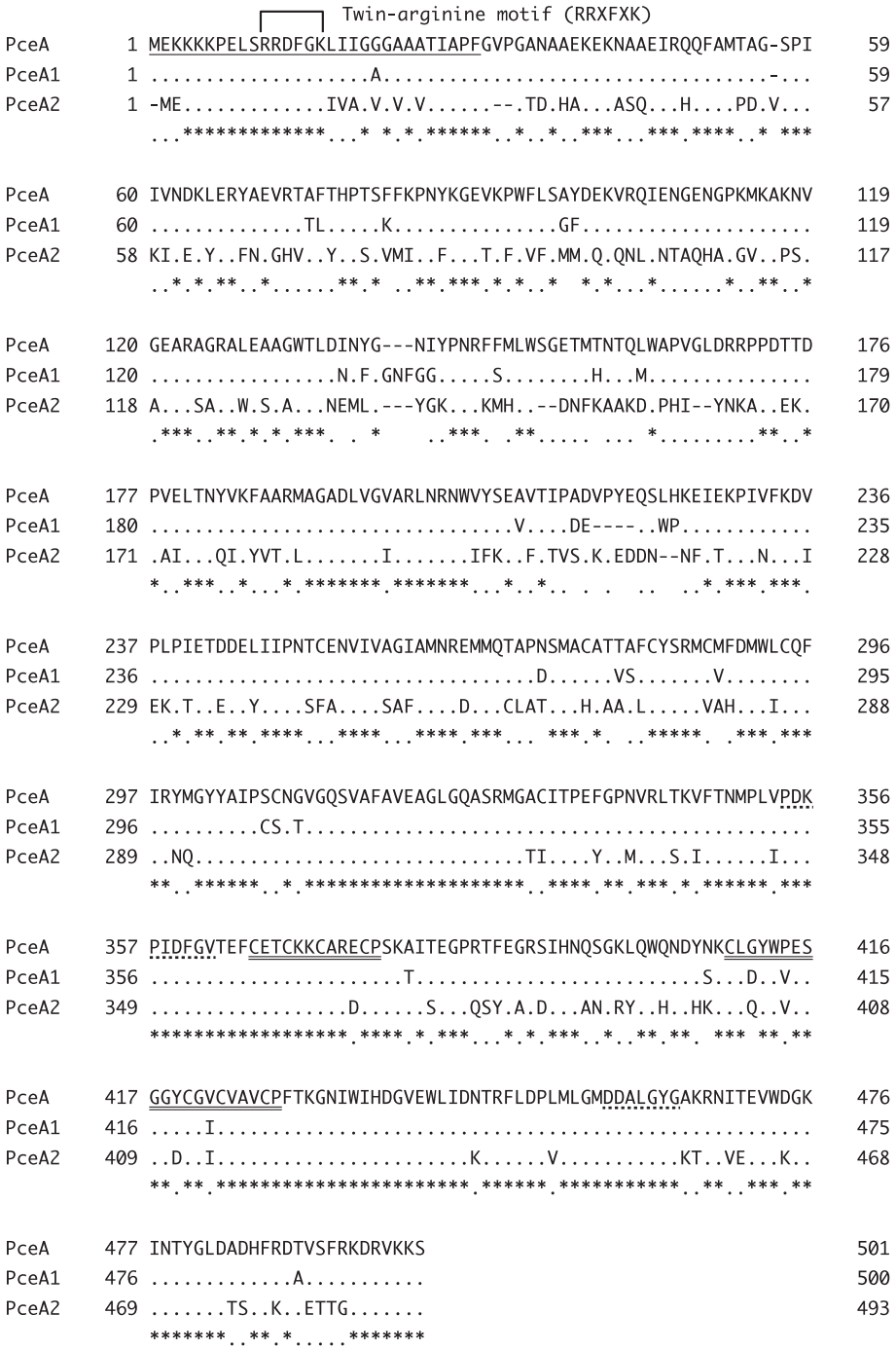

Fig. 1. Sequence Alignment of PCE Reductive Dehalogenases.

The chromosomal DNA sequence database was searched using BLASTP search algorithms. The amino acid sequence data were analyzed using GENETYX computer software (Software Development, Tokyo). The homologous amino acid residues found in all three enzymes (asterisks) and conserved substitutions (dots) are indicated. The deduced signal sequence containing the twin-arginine motif (RRXFXK) is underlined. Two possible iron-sulfur cluster binding motifs, CXXCXXCXXXCP and CXX[$_{2-12}$]CXXCXXXCP, are double-underlined. The broken underlined amino acid sequences are conserved in a few known *pceA* genes. Based on these sequences, synthetic primers were designed for cloning of the PCE reductive dehalogenase genes. The alignments were from the DDBJ/GenBank/EMBL accession numbers in parentheses, as follows: PceA of *Sulfurospirillum* (*Dehalospirillum*) *multivorans* (AAC60788); PceA1 (AB537448); PceA2 (AB537449).

expressed enzyme protein formed an insoluble inclusion body. The growth of the host cells, and hence yield of the expressed product, decreased on IPTG treatment. In spite of various experiments using vector pBAD, pCold and different host strains, no PceA activities were detectable in the transformant *E. coli* lysates.

In general, reductive dehalogenases are highly sensitive to oxygen and refractory to purification, owing to high content of cystein residue. Indeed the PceA1 and PceA2 molecules had 15 and 12 cystein residues respectively. Anaerobic cultures of *E. coli* and of yeast cells, however, did not yield PceA1 activities.

In consideration of these difficulties, PceA1 was synthesized *in vitro* using the WakoPURE system (Wako Pure Chemical Industries, Osaka).[6] After replacement of air phase with N_2 gas, the reaction mixture was incubated at 37 °C for 1 h. The resuling enzyme, the concentration of which was below the detection limit on SDS–PAGE, was assayed by the method of Neumann *et al.*[7] In brief, the synthetic enzyme was mixed with

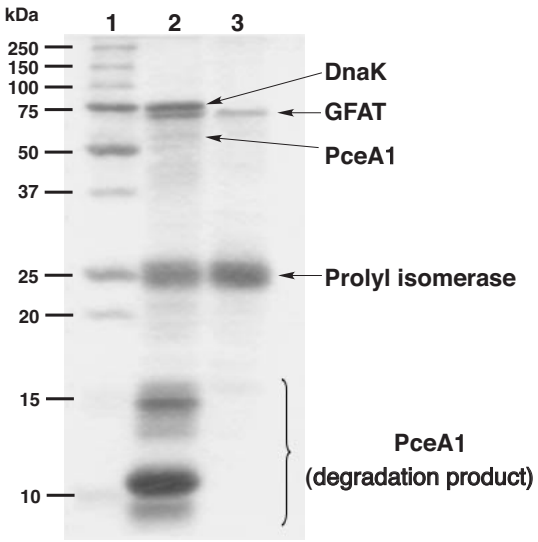

Fig. 2. Direct Interaction between His-Tag Fused PceA and DnaK *in Vivo* (His-tagged protein pull-down assay).

A culture of *E. coli* BL21 harboring recombinant plasmid (pCold harboring *pceA1*) or mock plasmid (pCold) was induced with 1 mM IPTG for 24 h at 15 °C, and the His tag-fused recombinant PceA1 or vector control was extracted, using BugBuster™ protein extraction reagent (Novagen, Madison, WI) containing 1.0 mM PMSF and 0.001 mM pepstatin. BugBuster™ extract was loaded on a Ni-NTA Superflow Cartridge (Qiagen, Hilden, Germany), and the His tag-fused PceA1 that stuck to the cartrige (lane 2) and the control (lane 3) were recovered with 250 mM imidazole. The eluates were subjected to 15% SDS–PAGE and stained with CBB R-250 dye. The proteins in the gel were transferred to a PVDF membrane, and the bands were cut off and analyzed with a PE Applied Biosystems model 491 Procise protein sequencer. GFAT stands for L-glutamine-D-fructose-6-phosphate aminotransferase.

PCE in Tris-pyruvate buffer, pH 7.3, containing methyl viologen, titanium citrate, DTT, and BSA, followed by incubation at 30 °C. Care was taken to minimize exposure to oxygen. After incubation for 1–4 h, the mixture was heated at 65 °C for 15 min, and the headspace gas, containing evaporated PCE and TCE, was subjected to GC/MS analysis. The protein synthesized *in vitro* dehalogenated PCE into trichloroethylene (TCE), at a rate of 2.30 µmole/h ml of reaction mixture. Except when determined immediately after synthesis, dehalogenase activity was undemonstrable, indicating high oxygensensitivity.

As mentioned above, the PceA of *S. multivorans* (*D. multivorans*) has 93% homology with PceA1, and the recombinant protein expressed in *E. coli* is similarly deficient in dehalogenase activity.[3] On the other hand, PceA did not exhibit toxicity against *E. coli*, and the amount of the expressed product was not small. The toxicity of PceA1 was unrelated to dehalogenase activity and its signal peptide.

A search for the host protein interacting with PceA1 was performed, using pull-down assay.[8] The PceA1 molecule formed a complex with the DnaK (Hsp70)[9] protein (Fig. 2). Through interaction with DnaK, a molecular chaperon involved in DNA replication, PceA1 probably shows toxicity against host *E. coli*. In accordance with the molecular interaction, *E. coli* cells harboring the *pceA1* plasmid underwent filament formation (Fig. 3), one of the properties of *dnaK* mutants.[10] Like *dnaK* strains,[10] the *pceA* transformant grew slowly, and easily lost the pPceA plasmid.

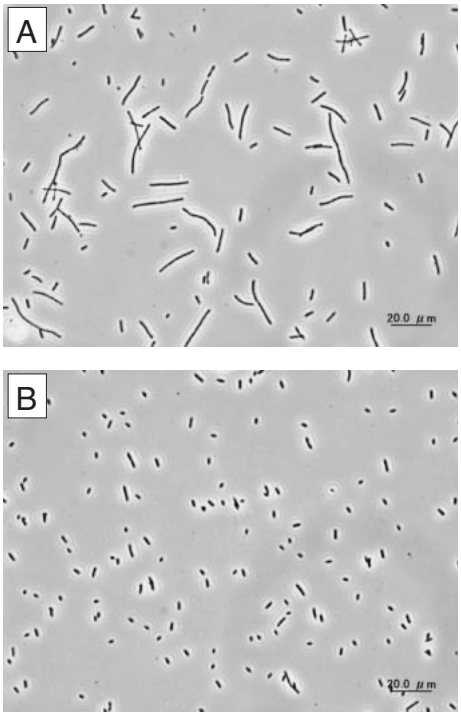

Fig. 3. Filamentation of the *E. coli pceA* Transformant.

Cells of strain XL1-Blue transformed with pPceA (pBAD bearing the *pceA* insert) (A) or pBAD (the insert-free control, B) were grown at 37 °C for 6 h in the presence of 0.2% L-arabinose, and were subjected to phase-contrast microscopy.

In spite of analysis of the enzymes and genes involved in dehalogenative respiration, efficient expression of PceA is still difficult, and establishment of gene manipulation system is urgently required. Furthermore, most dehalogenative bacteria are refractory to pure culture, and the development of a culture system is also essential. The PCR primers used in this study, designed for the cloning of *pceA*, are probably applicable in searching for new allied genes or dehalogenative bacteria, and in the monitoring of microbes in bioremediation.

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