Full Paper

Aerobic degradation of *cis*-dichloroethene by the marine bacterium *Marinobacter salsuginis* strain 5N-3

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An aerobic bacterium, designated strain 5N-3 (NBRC 113055), that degrades *cis*-dichloroethene (*c*DCE) was isolated from a sea sediment in Japan. Strain 5N-3 was able to degrade a certain amount of *c*DCE in the presence of pyruvate without the action of inducers. In the presence of inducers, such as phenol and benzene, the strain completely removed *c*DCE. By the application of 16S ribosomal RNA (16S rRNA) gene sequencing and average nucleotide identity analyses, the strain 5N-3 was identified as *Marinobacter salsuginis*. On the other hand, identified species of *Marinobacter* are not known to degrade *c*DCE at all. A draft genome sequence analysis of the strain 5N-3 suggested that the *dmp*-homologous operon (operon for phenol degradation) may be contributing to the aerobic degradation of *c*DCE. This is the first report on an aerobic marine bacterium that has been found to degrade *c*DCE.

Key Words: aerobic degradation; *c*DCE; *cis*-dichloroethene; marine bacterium; *Marinobacter*

Introduction

Chloroethylenes (perchloroethene (PCE), trichloroethene (TCE), *cis*-1,2-dichloroethene (*c*DCE), *trans*-1,2-dichloroethene (*t*DCE), 1,1-dichloroethene and vinyl chloride (VCM)) are common environmental contaminants. PCE and TCE have been used as dry cleaning solvents and metal degreasing agents around the world. Lesser chlorinated ethenes, such as *c*DCE and VCM, are formed by the reductive dechlorination of PCE, TCE, and

chlorinated ethanes. *c*DCE and VCM exhibit properties of low biodegradability and high toxicity. It is generally agreed that the presence of microorganisms capable of degrading chloroethenes is one of the important factors that can completely remove these compounds from their environments.

Many aerobic/anaerobic chloroethene-degrading bacteria have been isolated from terrestrial environments. It has been reported that, under aerobic conditions, many bacteria isolated from terrestrial environments, such as soils and rivers, are capable of degrading TCE, DCEs and VCM by co-metabolic oxidation (Mattes et al., 2010). On the other hand, a few species of *Mycobacterium*, *Pseudomonas*, *Nocardioides*, *Ochrobactrum*, and *Ralstonia* are known as VCM-assimilating bacteria (Mattes et al., 2010), and *Polaromonas* sp. strain JS666 is the only known *c*DCE-assimilating bacterium isolated from terrestrial environments (Coleman et al., 2002).

A few reports are available on chloroethene-degrading anaerobic bacteria found in marine environments. Reductive-dehalogenase-homologous (*rdhA*) genes were detected from the anaerobic marine subsurface sediments in the Pacific Ocean and their sequences were found to be similar to those of known anaerobic dehalorespiring bacteria, such as *Dehalococcoides* (Futagami et al., 2009). Additionally, Futagami reported that the enrichments of samples sourced from the subseafloor sediments of the Nankai Trough degraded TCE (Futagami et al., 2009, 2013). These reports suggested that anaerobic dehalorespiring bacteria inhabit marine, as well as terrestrial, environments. On the other hand, chloroethene-degrading aerobic bacteria have never been isolated from sea samples. In the current study, we report a *c*DCE-degrading aerobic bacterium belonging to the genus *Marinobacter* that was isolated from a sea sediment.

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Fig. 1. The rate of *c*DCE degradation by the isolates and several marine bacteria in liquid culture.

Each bacterium was cultivated in SNv medium consisting of 1,000 mg/l of pyruvate and 20 mg/l of *c*DCE at 25°C.

Each value is the average $\pm$ standard deviation (error bars) based on three independent experiments.

Materials and Methods

Enrichment and isolation of chloroethene-degrading bacteria.

Sea sediments were collected from the Kawasaki bay in Japan in February, 2011. To enrich and isolate aerobic bacteria degrading TCE, *c*DCE and VCM, 10 mg of the sediment was inoculated into 10 ml of modified SN medium (Waterbury et al., 1986) with a tenfold increase in vitamin concentration (SNv medium) supplemented with 20 mg/l of chloroethene in a 100-ml vial sealed with Teflon-coated butyl rubber stoppers and aluminum caps, and cultivated at 25°C under conditions of shaking at 150 rpm. Cultures that decreased the levels of chloroethenes were further subcultured in 1 ml of SNv medium supplemented with 5 mg/l of chloroethenes and various energy and carbon sources. As supplemental carbon sources, a sugar mix (consisting of 100 mg/l of glucose, arabinose, fructose and fucose) and 100–500 mg/l of yeast extract, glucose, arabinose, fructose, fucose or pyruvate were used. To isolate *c*DCE-degrading bacteria, the streak plate method was used to obtain single, isolated bacterial colonies on the plates of SNv agar containing 1,000 mg/l of pyruvate. To estimate their *c*DCE-degrading activities, each isolate was cultured separately in SNv medium supplemented with 5 mg/l of *c*DCE and 500 mg/l of pyruvate. Cell growth was measured by turbidity at 660 nm.

Bacterial strains, culture conditions, and chemicals.

Strains 5N-1, 5N-3 and 5N-13 were isolated in the study and strain 5N-3 was submitted to the Biological Resource Center, NITE (illustrated as NBRC) under the number NBRC 113055. *Marinobacter salsuginis* NBRC 109114^T,

Marinobacter adhaerens DSM 23420^T, *Marinobacter flavimaris* DSM 16070, *Marinobacter hydrocarbonoclasticus* DSM 8798^T, *Marinobacter koreensis* NBRC 106396^T, *Marinobacter sedimentalis* DSM 15400, *Arthrobacter pascens* NBRC 12139, *Ferrimonas marina* NBRC 102583^T, *Ferrimonas kyonanensis* NBRC 101286 and *Oceanobacter kriegii* NBRC 15467 were procured from the NBRC and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) culture collections. Modified SNv medium was used as the basal medium in this study. Cultures of the strains were maintained by routine subculturing on SNv medium supplemented with 1,000 mg/l of pyruvate, or on Marine Agar 2216 (MA; Becton Dickinson), at 25°C. 200 mg/l of *c*DCE and TCE in water, 700 mg/l of *c*DCE in DMSO and 1,000 mg/l of VCM in ethanol were used as stock solutions.

Measurement of TCE, *c*DCE and VCM by gas chromatography.

Concentrations of TCE, *c*DCE and VCM were measured by applying the technique of headspace gas chromatography with flame ionization detection (GC-FID) using an Agilent 6890N GC (Agilent, Palo Alto, CA, USA) equipped with a GC sampler 80 (Agilent) and a DB-624 capillary column (30 m-by-0.32 mm, J&W Scientific, Folsom, CA, USA). The headspace gas (250 μ l) was injected. The temperature of the oven was held for 5 min at 35°C, then increased to 165°C at a rate of 30°C/min and held constant at 165°C for 1 min (total runtime: 10.3 min). The injection and detection temperatures were 200°C and 250°C, respectively. Except for the enrichment and isola-

Fig. 2. The rate of *c*DCE degradation by strain 5N-3 in liquid culture.

Initial *c*DCE concentration was 20 mg/l and the cultures were incubated at 150 rpm at 25°C. Cell-free bottles with 4 ml of SN medium were used as negative controls. Cells were grown on a medium containing 1,000 mg/l of pyruvate, 1,000 mg/l of succinate, 200 mg/l of pristane, 200 mg/l of phenol, 1,000 mg/l of pyruvate and 200 mg/l of benzene in 4 ml of SN medium and also in 4 ml of Marine Broth. Each value is the average $\pm$ standard deviation (error bars) based on three independent experiments.

tion procedures, all measurements were conducted using triplicate culture systems.

Degradation activity by resting cells. Cells grown for 4 days on plates of SNv agar containing 1,000 mg/l of pyruvate were washed thrice with artificial seawater and then suspended in it. The optical density (OD₆₀₀) was adjusted to 10. *c*DCE (10 mg/l) was added into 1 ml of the cell suspension in a vial and the concentration of *c*DCE in the gas phase of the vial was monitored with a GC-FID.

Sequence analysis. The 16S rRNA gene sequence of the isolate was amplified by conducting a polymerase chain reaction (PCR) using the universal primer pair (Fukunaga et al., 2006). The draft genome was sequenced using a combined strategy of shotgun sequencing with 454 GS-FLX Titanium (Roche Diagnostics, Branford, CT, SA) and paired-end sequencing with MiSeq (Illumina, Inc., San Diego, CA, USA). The data obtained from these analyses were assembled using Newbler version 2.6. The assembled contig was submitted to the RAST (Rapid Annotations using Subsystems Technology) server (<https://rast.nmpdr.org/>) for automatic annotation. Average nucleotide identity (ANI) values based on the genome sequences were calculated as a substitute for DNA-DNA hybridization (Goris et al., 2007) using JSpecies (Richter and Rossello-Mora, 2009). The GenBank/EMBL/DDBJ accession numbers for the draft genomes of strains 5N-3 and NBRC 109114^T are BGZH01000001-BGZH01000010 and BGZI01000001-BGZI01000084, respectively.

Results and Discussion

Enrichment, isolation, and phylogenetic analysis of the *c*DCE-degrading bacterium

To obtain enrichment cultures with aerobic chloroethene-degrading activities, the sea sediment samples were inoculated in SNv media supplemented with TCE, *c*DCE and VCM along with various energy and carbon sources under aerobic conditions. Consequently, *c*DCE-degrading enrichment was obtained, but TCE- and VCM-decreases were not observed. Therefore, we focused on a *c*DCE-degrading enrichment sample designated 5N that was cultivated in SNv medium containing 5 mg/l of *c*DCE and 500 mg/l of pyruvate. The 5N enrichment was monitored constantly and a decrease in the concentration of *c*DCE was observed within 2 weeks (data not shown). Pure strains designated 5N-1, 5N-3, 5N-6 and 5N-13 were isolated from the 5N enrichment sample and similarities in their 16S rRNA gene sequences were found to match with *Azoarcus buckelii* (94.2%), *Marinobacter salsuginis* (99.9%), *Marinobacter hydrocarbonoclasticus* (100%) and *Thalassospira profundimar* (99.3%), respectively. Among the 4 strains, only strain 5N-3 could degrade *c*DCE. Strain 5N-3 grew on 200–1,000 mg/l of pyruvate, 1,000 mg/l of succinate, 200 mg/l of phenol, 200 mg/l of hexadecane or 200 mg/l of pristane as the sole carbon source and also in Marine Broth 2216, but *c*DCE, benzene or toluene were not used for the sole substrates for its growth. Strain 5N-3 grew well on Marine Broth rather than on the other substrates (data not shown).

Fig. 3. The rate of *c*DCE degradation activity by resting cells of strain 5N-3.

Initial concentration of *c*DCE was 7 mg/l and the cultures were incubated at 150 rpm at 25°C. Autoclaved cells were used as a control. Headspace of anaerobic vials was replaced with 100% nitrogen. Each value is the average $\pm$ standard deviation (error bars) based on three independent experiments.

Table 1. Differences in amino acid sequences of Dmp between strains 5N-3 and NBRC 109114.

DmpR	Positive regulator of dmp operon	99%
DmpK	Phenol hydroxylase, assembly protein	98%
DmpL	Phenol hydroxylase, P1 oxygenase component	100%
DmpM	Phenol hydroxylase, P2 regulatory component	100%
DmpN	Phenol hydroxylase, P3 oxygenase component	100%
DmpO	Phenol hydroxylase, P4 oxygenase component	100%
DmpP	Phenol hydroxylase, FAD- and [2Fe-2S]-containing reductase component	99%
DmpQ	ferredoxin-like protein	96%
DmpB	Catechol 2,3-dioxygenase	99%
DmpD	2-hydroxymuconic semialdehyde hydrolase	100%
DmpH	4-oxalocrotonate decarboxylase	37%
DmpF	Acetaldehyde dehydrogenase	40%
DmpG	4-hydroxy-2-oxovalerate aldolase	99%
DmpH	4-oxalocrotonate decarboxylase	100%
DmpI	4-oxalocrotonate tautomerase	100%

*c*DCE-degradation activity of strain 5N-3 in liquid culture

The time courses of *c*DCE degradation in the control and cultures of the isolates along with some *Marinobacter* species and other marine bacteria are shown in Fig. 1. All strains of the bacteria grew in SNv medium containing 500 mg/l of pyruvate and 5 mg/l of *c*DCE, but only strain 5N-3 clearly demonstrated approximately 60% of *c*DCE degradation. The demonstration of the phenotypic characteristic of *c*DCE degradation in *Marinobacter* is strain-specific and not species-specific or genus-specific. The rate of *c*DCE degradation by strain 5N-3 under liquid culture conditions in the presence of inducers and carbon sources is shown in Fig. 2. Strain 5N-3 could decrease *c*DCE by 50% in the medium containing 1,000 mg/l of pyruvate as the sole source of carbon and energy. On the other hand, strain 5N-3 was found to grow on 1,000 mg/l of succinate and 200 mg/l of pristane, as well as in Marine Broth, but these cultures demonstrated a low degradation of *c*DCE. Strain 5N-3 grew well on Marine Broth

rather than on 1,000 mg/l of pyruvate, but the *c*DCE degradation rate was more rapid on pyruvate than on Marine Broth. *c*DCE degradation activity may depend on the kind of substrate but not on the cell concentration. When 200 mg/l of phenol was used as a carbon and energy source, the strain removed *c*DCE completely. Additionally, when 200 mg/l of benzene was added as an inducer, strain 5N-3 completely removed *c*DCE from the culture supplemented with 1,000 mg/l of pyruvate. The highest capacity for degradation of *c*DCE by strain 5N-3 was observed under the conditions in which inducers such as benzene and phenol were present. Meanwhile, the strain could clearly degrade *c*DCE even in the absence of inducers. It is not clear why the culture system with pyruvate demonstrated a better *c*DCE-degradation activity than those with succinate, pristane, or Marine Broth.

Activity of *c*DCE-degradation by resting cells of strain 5N-3

It has been reported that aerobic bacteria degrading

*c*DCE in terrestrial environments use oxidizing enzymes such as monooxygenase or dioxygenase to oxidize chloroethenes (Mattes et al., 2010). To confirm the activity of oxidizing enzymes in strain 5N-3, the ability of resting cells to degrade *c*DCE was tested (Fig. 3). The heat-killed cells and resting cells under anaerobic conditions did not exhibit *c*DCE degradation, while the resting cells under aerobic conditions degraded more than 60% of *c*DCE within 29 hours. Because terrestrial *c*DCE degradation has been reported to require inducers (Ensign et al., 1992), the ability of the resting cells to degrade *c*DCE suggested that the strain 5N-3 might be degrading *c*DCE using oxidizing enzymes similar to the other *c*DCE-degrading aerobic bacteria and constantly expresses the required genes, unlike the other aerobic bacteria that are known to degrade *c*DCE.

Genomic analysis

The draft genome sequencing of strain 5N-3 and *M. salsuginis* NBRC 109114^T was performed. The genomes of both the strains, 5N-3 and NBRC 109114^T, consisted of chromosomes that were 4.7 Mbp in size. Their ANIb value was found to be 99.9% and this result led to the conclusion that they are the same species. The two strains commonly possessed more than 96% of the predicted protein-coding genes, such that strain 5N-3 and NBRC 109114^T have 63 and 79 unique predicted protein-coding genes, respectively. Both the strains also have the operon homologous to *dmp* operon (operon for phenol degradation) that codes for phenol hydroxylase, catechol 2,3-dioxygenase, ferredoxin-like protein, putative 5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase oxidoreductase protein, 2-hydroxymuconic-semialdehyde hydrolase, 4-hydroxy-2-oxovalerate aldolase, 4-oxalocrotonate decarboxylase, and 4-oxalocrotonate tautomerase (Table 1). The strain 5N-3 demonstrated a low capacity for *c*DCE degradation when grown on pyruvate without an inducing substrate and a high degradation capacity when grown on media containing phenol. It is suggested that the strain 5N-3 uses phenol hydroxylase for *c*DCE degradation like many terrestrial bacteria that degrade *c*DCE using aromatic hydrocarbon monooxygenase. On the other hand, it is not clear why *M. salsuginis* NBRC 109114^T could not degrade *c*DCE in the presence of pyruvate. There are reports that the expression of the *dmp* operon is regulated by the activator and sensor protein DmpR (Shingler et al., 1993). DmpR consists of four domains as follows: domain A is a sensor for inducing substrates like phenol, domain B is a linker between domain A and C, domain C is a transcriptional activator, and domain D is the DNA binding region. The amino acid sequence of DmpR in both the strains showed 100% similarity in domain B, domain C and domain D and a difference of 4 residues was observed in domain A. The difference in the amino acid residues in domain A of DmpR may be responsible for the expression of the *dmp*-homologous operon and *c*DCE degradation. Or the difference in the amino acid sequence of other proteins in the *dmp* operon in both the strains (Table 1) may be concerned with *c*DCE degradation activity. Alternatively, the hypothetical proteins predicted to be present only in the genome of the strain 5N-3 may be involved in the degradation of *c*DCE. Further analyses are necessary

to elucidate the mechanism of *c*DCE degradation in strain 5N-3.

Conclusion

First, a marine *c*DCE-degrading strain 5N-3 was isolated from a sea sediment and identified to be *M. salsuginis*. The strain could degrade *c*DCE with pyruvate, as well as with phenol and benzene as inducers. While many terrestrial bacteria need inducing substrates, such as phenol or toluene, to degrade chlorinated ethylenes (Mattes et al., 2010), this strain could degrade *c*DCE without the addition of such inducing substrates. Additionally, the genome analyses inferred that strain 5N-3 uses phenol hydroxylase for the degradation of *c*DCE, but we could not decipher its unique mechanism in the genome. These findings suggest that the natural decomposition of *c*DCE may occur in non-polluted marine environments. Further research on bacterial degradation of chlorinated ethylenes in the marine environment may provide new evidence of microbial functions in the marine ecosystem.

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