論 文 の 内 容 の 要 旨

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論文題目

Anaerobic benzene degradation pathway of *Azoarcus* sp. DN11

（*Azoarcus* sp. DN11 株の嫌気的条件におけるベンゼン分解経路の解明）

Introduction

Benzene is a common soil and groundwater pollutant, which causes a major global health concern because of its carcinogenicity. It has long been known that benzene can be degraded by bacteria under aerobic conditions using oxygen-dependent pathways containing mono- or dioxygenases to attack initially and hydroxylate benzene ring to form catechol. The development of anoxic zones in hydrocarbon-contaminated subsurface sites renders the elucidation of its associated metabolic pathways highly relevant for the groundwater bioremediation technique. Three initiating pathways have been proposed for putative anaerobic benzene activation: (a) methylation to toluene, (b) carboxylation to benzoate and (c) hydroxylation to phenol (Figure 1). (Vogt *et al., Microb. Biotechnol.,* 4:10-724, 2007). However, the genes involved in the initial benzene activation have not yet been identified.

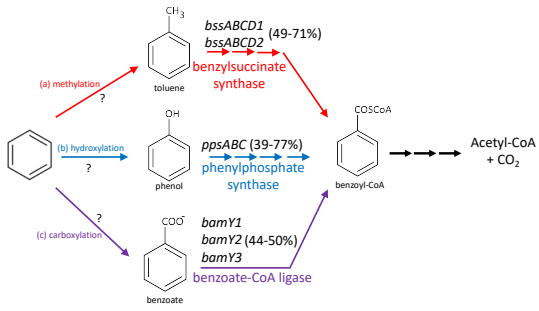


Figure 1. Proposed pathways for the anaerobic benzene degradation. Putative genes encoding enzymes for anaerobic degradation of toluene, phenol and benzoate were identified within the DN11 genome. Percentage values represent amino acid homology with that of *Geobacter metallireducens*.

Azoarcus sp. DN11, a denitrifying bacterium, was isolated from gasoline-contaminated groundwater in Kumamoto, Japan (Kasai *et al.*, *Appl. Environ. Microbiol.*, 72:3586-3592, 2006). Furthermore, DN11 has been shown to be capable of degrading benzene, toluene, benzoate, but not phenol, under both aerobic and anaerobic conditions (Kasai *et al.*, *Environ. Sci. Technol.*, 41:6222-6227, 2007). These studies highlight the potential of DN11 for aromatic hydrocarbon bioremediation.

The aim of this study was to elucidate the detailed mechanism of anaerobic benzene degradation pathway of *Azoarcus* sp. DN11.

Genome sequence-based prediction of *Azoarcus* sp. DN11 genes related to benzene degradation

The whole genome sequencing of *Azoarcus* sp. DN11 was performed using Illumina technology to identify its genetic characteristics with focus on the aromatic degradation capability of DN11. The genes and degradation pathways for aromatic pollutants were investigated using the NCBI and KEGG databases. Analysis of DN11 whole genome sequence genomic data identified putative genes encoding enzymes for anaerobic degradation of toluene, phenol and benzoate, which suggests that DN11 may employ

all the three pathways to degrade benzene anaerobically (Figure 1). On the other hand, aerobic benzene degradation pathways containing putative monooxygenases and dioxygenases were also found in the genome of DN11. The complete genome sequence of DN11 was published in the Microbiology Resource Announcements (Devanadera *et al.*, *Microbiol. Resour. Announc.*, 8:11, 2019).

GC-MS analysis of metabolites produced during anaerobic benzene degradation by DN11

Metabolic intermediates produced by DN11 during anaerobic benzene degradation were analyzed by GC-MS for incorporation of [^{13}C] to shed light on the possible route of degradation by the strain. Media preparations and laboratory manipulations were conducted under strict anoxic conditions for anaerobic experiments. DN11 cells were precultured in CGY medium (Watanabe *et al.*, *Appl. Environ. Microbiol.* 65:2813-2819,1999) under aerobic conditions and then inoculated into BSM anaerobic medium (Kasai *et al.*, *Environ. Microbiol.*, 7:806-818, 2005) supplemented with 15 µM [^{13}C] benzene (carbon source) and 6 mM nitrate (electron acceptor). During active benzene degradation, metabolites was extracted from the culture medium using diethyl ether as described by Ulrich *et al.* (*Environ. Sci. Technol.,* 39:6681-6691, 2005). GC-MS analysis showed the presence of [^{13}C] phenol as an intermediate of anaerobic [^{13}C] benzene degradation by DN11 (Figure 2A). Mass spectral evidence also supported the production of [^{13}C] phenol from [^{13}C] benzene during anaerobic benzene degradation by DN11 (Figure 2B). On the other hand, [^{13}C] toluene or [^{13}C] benzoate was not detected (data not shown). These findings suggest that phenol is an intermediate in the anaerobic benzene metabolism of DN11 and that the biodegradation proceeds via the hydroxylation pathway (pathway b in Figure 1).

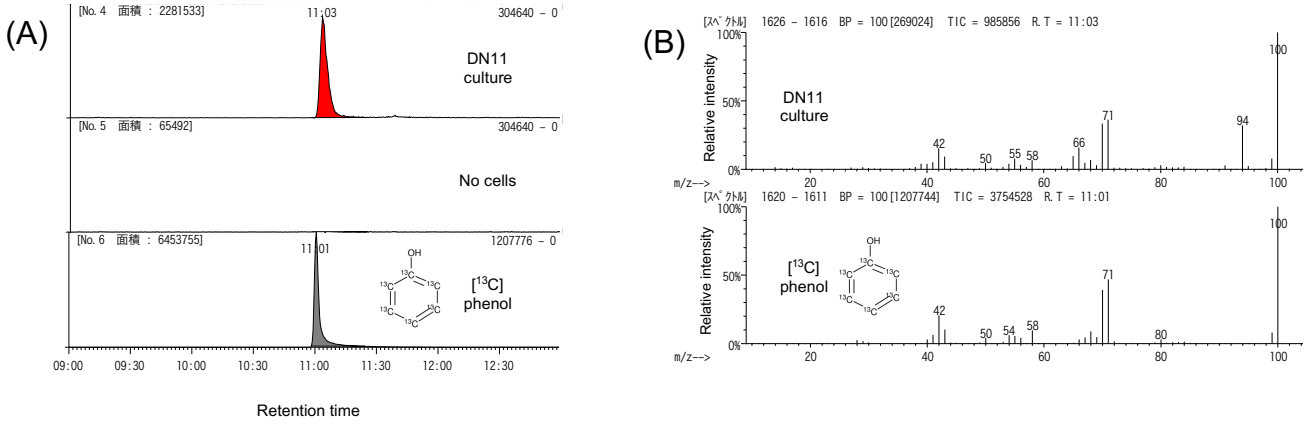


Figure 2. [^{13}C] Phenol peak (A) in benzene-degrading DN11 culture after 14 days of incubation and the mass spectral fragmentation pattern as compared with the [^{13}C] phenol standard (B).

Using ^{18}O-labelled water, oxygen incorporated into benzene to produce phenol can be shown to come from water. The GC-MS analysis for the detection of ^{18}O-labelled phenol produced during anaerobic benzene degradation by DN11 was performed using diethyl ether extracts from benzene-degrading cultures in 10.5 mL BSM with 42.95% ^{18}O-labelled water. The mass spectrum of the phenol peak in benzene-degrading DN11 cultures with ^{18}O-labelled water showed no *m/z* 96 peak which represents the ^{18}O-phenol.

The result suggested that the oxygen incorporated into benzene to produce phenol under anaerobic degradation by DN11 was not derived from water.

Transcriptomic analysis of the genes related to benzene degradation pathway of DN11 using RNA-Sequencing

In order to obtain a clearer picture of the anaerobic benzene degradation pathway of DN11, comparison of the whole transcriptome of DN11 cells grown on benzene and benzoate as its sole substrates using RNA-Sequencing was performed. High quality total RNA was extracted from DN11 cells during active anaerobic benzene and benzoate degradation. After depletion of rRNA using Ribo-Zero kit (Epicentre) from the samples, cDNA extraction and library preparation were performed using Illumina TruSeq^{TM} RNA sample preparation kit v2. Sequencing data were generated on MiSeq system (Illumina) with MiSeq Reagent Kit v3 following the manufacturer's protocol. The raw RNA-Seq reads were analyzed using CLC Genomics Workbench software (Version 7.5.2, CLC Bio). High quality RNA-Seq reads were used to analyze the gene expression with the RNA-Seq analysis tool of CLC. RPKM of genes were calculated to compare the levels of genes expressed in benzene- and benzoate-grown cells. The results of the whole transcriptomic analysis of the genes related to benzene degradation pathway of DN11 revealed the simultaneous transcription of putative aerobic benzene oxidation genes and putative anaerobic phenol degradation genes. The genes in the phenol degradation pathway of DN11 showed higher RPKM values in benzene-degrading cultures than those in benzoate-degrading cultures (Figure 3). Although some of the genes involved in the putative toluene pathway showed higher RPKM in benzene- than benzoate-grown cells, the RPKM values were not as high as those of the genes in the phenol pathway. The putative genes for initial benzoate degradation were also not significantly expressed in benzene cultures when compared to benzoate cultures. Although anaerobic conditions and manipulations were employed during experiments on the benzene degradation of DN11, the higher expression of putative benzene monooxygenase genes (*dmpKLMNOP* encoding for phenol hydroxylase and *tmoABCDEF* encoding for toluene monooxygenase) (Figure 4) were observed in benzene-degrading cultures than in benzoate-degrading cultures.

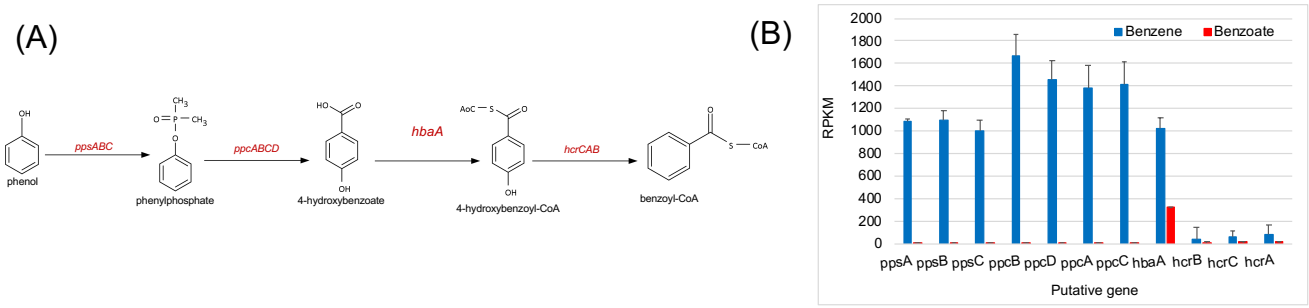
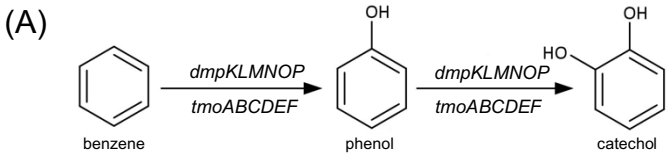


Figure 3. The putative genes in the anaerobic phenol pathway of DN11 (A) and its RPKM values (B) in DN11 cells grown on benzene and benzoate.



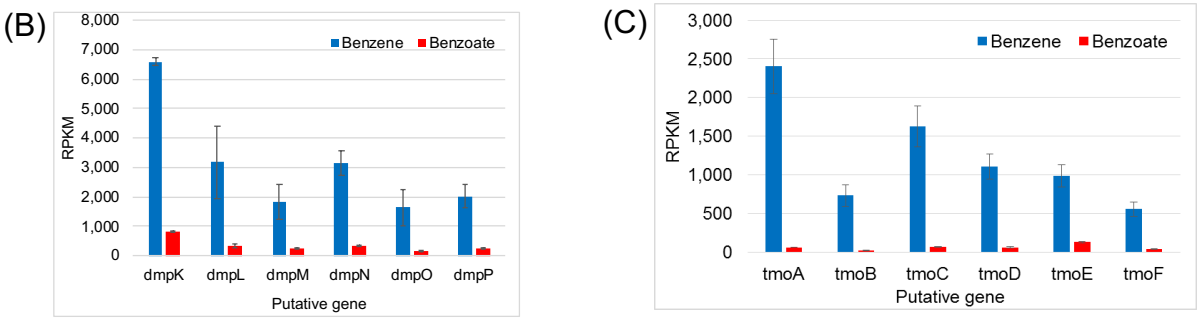


Figure 4. The putative genes in the benzene monooxygenase pathway of DN11 (A) and their RPKM values (B: gene *dmpKLMNOP* encoding for phenol hydroxylase; C: *tmoABCDEF* encoding for toluene monooxygenase) in DN11 cells grown on benzene and benzoate.

It was suggested that intracellular oxygen can be produced by *Candidatus* M. oxyfera and γ-proteobacterium strain HdN1 from nitrite by employing the yet unknown nitric oxide dismutase (Nod) (Ettwig *et al.*, *Nature*, 464:543-548, 2010; Ettwig *et al.*, *Front. Microbiol.,* 3:273, 2012). The oxygen produced is then used for oxidation pathways involving oxygenases. It is hypothesized that DN11 employs similar mechanism to activate benzene monooxygenase and dioxygenase genes during anaerobic benzene degradation. The phenol produced from the aerobic degradation of benzene activates the anaerobic phenol degradation pathway. Similar to the continuous culture studied by Atashgahi *et al.* (*Sci. Rep.,* 8:4490, 2018), no nitric oxide dismutase genes were found in the genome of DN11. This suggests a novel pathway for the production of oxygen from nitrate during anaerobic benzene degradation of DN11.

Summary and future prospects

Through genome-wide analysis, this study has confirmed the ability of DN11 to degrade benzene and other aromatic compounds under both aerobic and anaerobic conditions. Isotope labelling-based analysis has shown the production of phenol as a key metabolite in the anaerobic benzene degradation of DN11. However, oxygen incorporated into benzene was not derived from water. Moreover, whole transcriptomic analysis of benzene-degrading nitrate-reducing DN11 cultures has revealed the simultaneous expression of aerobic benzene and anaerobic phenol degradative genes even though strict anaerobic conditions were employed. An intra-aerobic denitrification pathway facilitated by an uncharacterized nitric oxide dismutase to produce oxygen from nitrite has been proposed recently (Ettwig *et al.*, *Nature*, 464:543-548, 2010). DN11 may have employed this pathway to degrade benzene anaerobically. Incubations with ^{18}O-labelled nitrate will be performed to determine the participation of nitrate in the oxygen production during anaerobic benzene degradation of DN11. Further studies are needed to characterize the putative nitric oxide dismutase to clearly describe its function and confirm the novel mechanism of oxygen production in oxygen-deficient environments. In general, this study has provided information of the possibility of the novel pathway of anaerobic benzene degradation in pure culture. The underlying genetics and biochemistry of biodegradation process would aid in the determination of the potential applicability of microbes in bioremediation strategies.