

博士論文

Anaerobic benzene degradation pathway of *Azoarcus* sp. DN11

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

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論文の内容の要旨

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論文題目

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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.

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

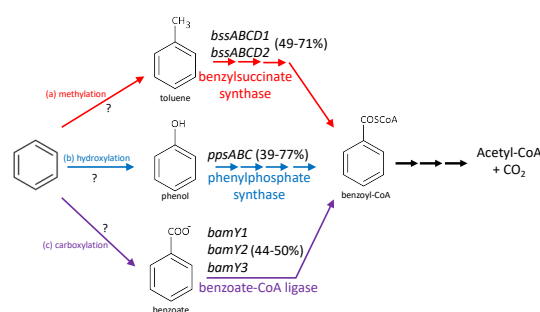


Figure 1. Proposed pathways for the anaerobic benzene degradation. Putative genes encoding enzymes for anaerobic degradation of toluene, phenol and benzoate were identified within the DN11 genome. Percentage values represent amino acid homology with that of *Geobacter metallireducens*.

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 were 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).

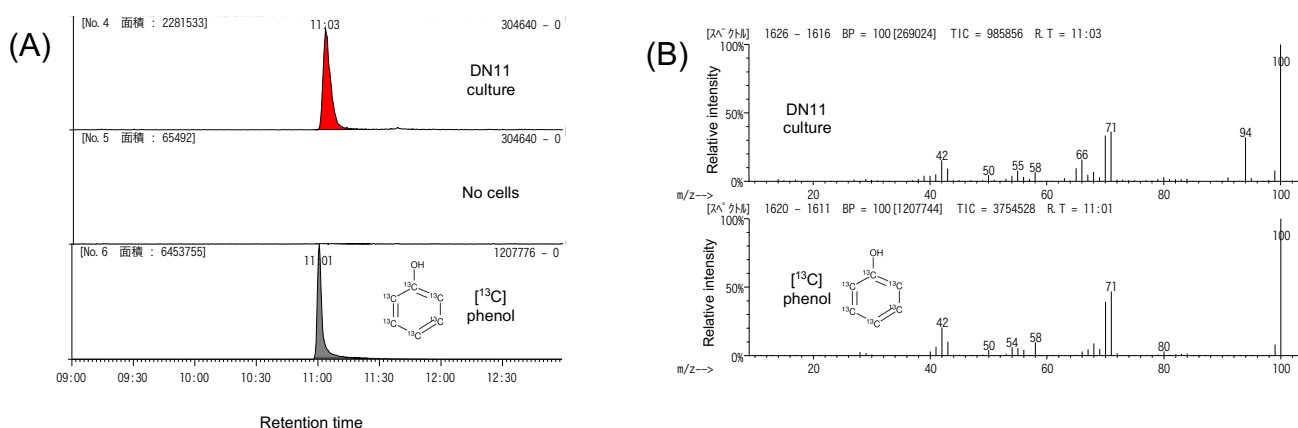


Figure 2. [^{13}C] Phenol peak (A) in benzene-degrading DN11 culture after 14 days of incubation and the mass spectral fragmentation pattern as compared with the [^{13}C] phenol standard (B).

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™ 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.

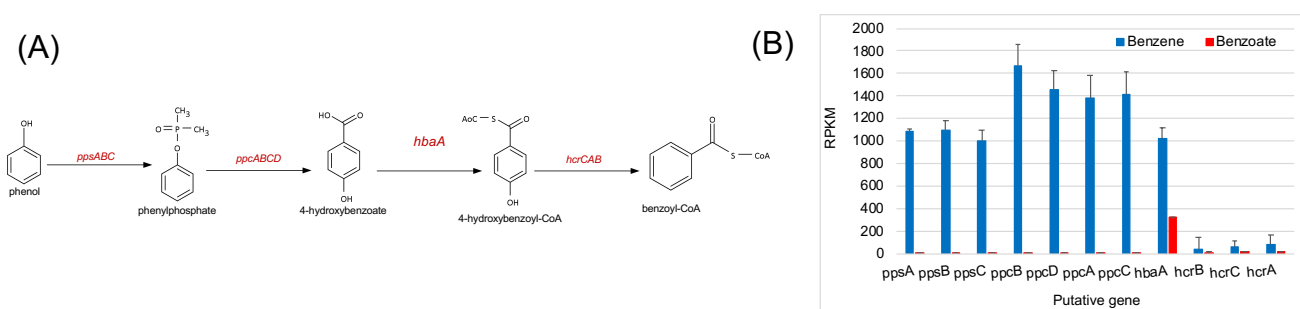
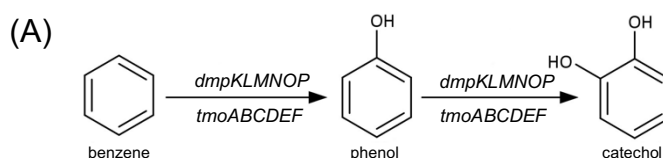


Figure 3. The putative genes in the anaerobic phenol pathway of DN11 (A) and its RPKM values (B) in DN11 cells grown on benzene and benzoate.



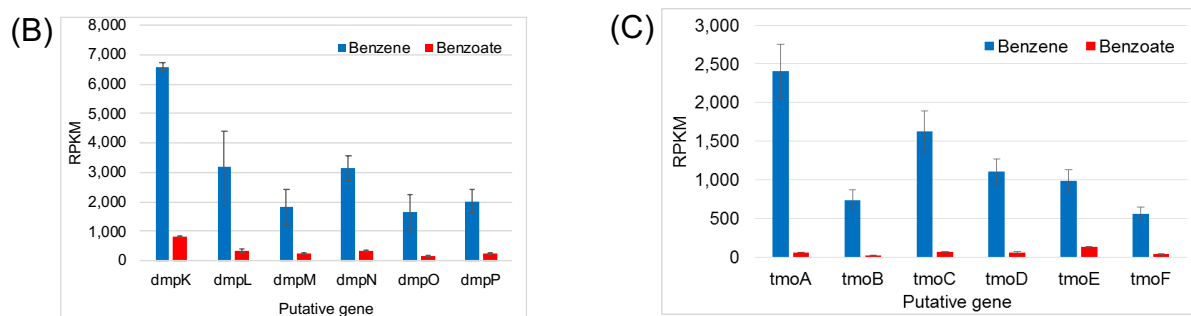


Figure 4. The putative genes in the benzene monooxygenase pathway of DN11 (A) and their RPKM values (B: gene *dmpKLMNOP* encoding for phenol hydroxylase; C: *tmoABCDEF* encoding for toluene monooxygenase) in DN11 cells grown on benzene and benzoate.

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.

List of abbreviations

^{13}C	carbon-13 isotope
^{14}C	carbon-14 isotope
^{18}O	oxygen-18 isotope
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSM	Basal salt medium
BTEX	benzene toluene ethylbenzene xylene
Cld	chlorite dismutase
CoA	Coenzyme A
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
et al.	and others
g	gram
GC	guanine-cytosine
GC-MS	gas chromatography-mass spectrophotometry
h	hour
HPLC	High Performance Liquid Chromatography
KAAS	KEGG Automatic Annotation Server
KEGG	Kyoto Encyclopedia of Genes and Genomes
km	kilometer
KO	KEGG orthology
L	liter
M	molar
m/z	mass/charge
Mb	Megabase
MiGAP	Microbial Genome Annotation Pipeline
min	minute
mM	millimolar
MP	mate-pair
MRA	Microbiology Resource Announcements
NADH	β -Nicotinamide adenine dinucleotide
Nod	Nitric oxide dismutase
OD	optical density
PE	paired-end

PGAP	Prokaryotic Genome Annotation Pipeline
pH	negative logarithm of the hydrogen ion concentration
ppm	parts per million
RNA	ribonucleic acid
RNA-Seq	RNA-Sequencing
RNase	ribonuclease
RPKM	Reads Per Kilobase of transcript, per Million mapped reads
rpm	revolutions per minute
RT	retention time
SIP	stable isotope probing
sp.	Species
U.S.	United States of America
v/v	volume per volume
w/v	weight per volume
WGS	Whole genome sequencing
xg	relative centrifugal force
μ	micro

Chapter 1

Introduction

1.1 Groundwater contamination and the implication of the present study

Groundwater contamination of aromatic compounds is a very serious environmental problem that we face in recent years. Petroleum hydrocarbons from crude oil and refined oil products such as gasoline and diesel are very toxic to all living things and can be lethal to humans. According to the U.S. Energy Information Administration website (<https://www.eia.gov/>), the total world petroleum consumption in 2012 reached a record-breaking of approximately 88.9 million barrels per day. The widespread use of petroleum products has greatly contributed to environmental pollution because of its disposal and accidental crude oil spills. The 2018 Sanchi oil tanker collision and explosion accident in East China Sea resulted in the loss of 32 lives as well as the release of an estimated 136,000 tons of condensate creating four separate oil slicks which covered a total area of 100 square km. It is considered to be the most environmentally damaging oil spill in the 21st century (Chen *et al.*, 2020). The worldwide occurrence of hydrocarbon contamination requires remediation due to its potential threat to human health and negative effects on the environment. When such contamination occurs, a group of hydrocarbons called BTEX (benzene, toluene, ethylbenzene and xylene) is of concern because of their mobility and toxicity (Figure 1.1).

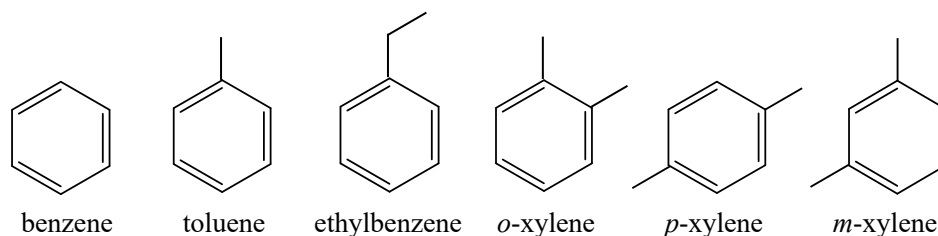


Figure 1.1. Chemical structures of BTEX compounds.

BTEX are naturally occurring compounds in crude oil and can be introduced into the environment via spills, vehicle emissions or industrial effluents similar to the Sanchi collision

accident. It can also be dissolved in water and may be found in surface and groundwater at contaminated sites. Benzene is the most carcinogenic among the BTEX due to its haematotoxicity and high volatility which makes exposure through inhalation possible. Prolong exposure to low levels of benzene (10 ppm) can increase the risk of leukemia and bone marrow depression (Thornton, 1995). The xenobiotic property and high chemical stability of benzene render its persistence subsurface environment, thus, undergoes very slow degradation process (Canzano *et al.*, 2014). Therefore, the removal of pollution or contaminants such as benzene has gained much attention in recent years.

Physical, chemical and biological methods to alleviate BTEX contamination from groundwater can be employed (Kofoed & Rollins, 2007). The method of remediation depends on the chemical nature of the contaminant. However, bioremediation is a promising remediation technology that has received a great deal of attention worldwide recently. *In situ* bioremediation utilizes naturally occurring or introduced organisms such as bacteria, plants, animals or fungi to biodegrade the contaminants of concern. This technique is also minimally invasive to the site to be treated as it provides the treatment in place without removal of soil or groundwater and transport of contaminants (Vidali, 2001). The natural approach of bioremediation produces minimal waste products and is relatively cost-effective compared to other remediation treatments which makes it a very attractive solution for waste treatment (Bonaventura & Johnson, 1997).

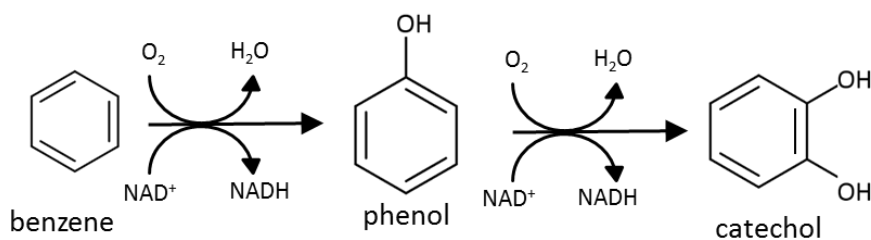
The most important factor of bioremediation is the microorganism that will degrade a particular environmental hazard. A tremendous variety of microorganisms have been isolated and used for remediation of hydrocarbons because of their diverse metabolic capabilities to degrade xenobiotics (Sandrin & Maier, 2003). Some bacteria are known to utilize hydrocarbon as their sole energy source (Yakimov *et al.*, 2007). However, the knowledge on the anaerobic metabolism of aromatic compounds especially benzene and its degradation pathway by bacterial isolates is still limited for the at least the past decade. Therefore, the studies on physiology and metabolic characteristics of hydrocarbon-degrading bacteria have environmental significance as it provides a

better understanding on mechanisms of microbial biodegradation that could be helpful on its application on the actual contaminated sites.

1.2 Aerobic benzene biodegradation

Indigenous microorganisms have been known to degrade benzene in the presence of molecular oxygen (Fritsche & Hofrichter, 2008). Microbial metabolic pathways for the aerobic degradation of benzene usually contain monooxygenase or dioxygenase to attack initially and hydroxylate benzene ring to form a catechol derivative as shown in Figure 1.2 (Gibson & Parales, 2000).

Monooxygenase reaction



Dioxygenase reaction

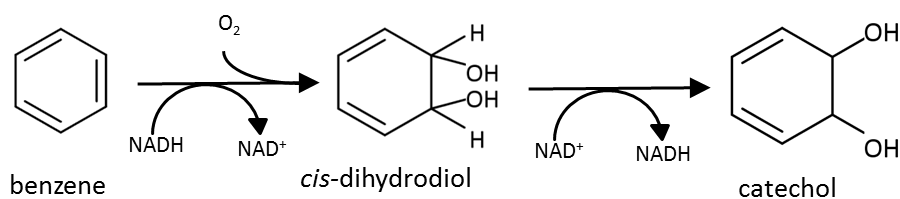


Figure 1.2. Aerobic benzene degradation pathways follow monooxygenase or dioxygenase systems.

The bacterial monooxygenase system consists of soluble di-iron monooxygenase which incorporates one oxygen atom from the oxygen as the initial hydroxylation reaction. This group can be divided into four groups based on the structure, biochemistry and genetic information: (1) methane monooxygenase (sMMOs), (2) Amo alkene monooxygenases, (3) phenol hydroxylases and (4) four component alkene/aromatic monooxygenases. Regarding the aerobic benzene

degradation, the phenol hydroxylases and the four component alkene/aromatic monooxygenases are involved in the degradation process (Leahy *et al.*, 2003). Most of these enzymes have fairly broad substrate specificity ranging from aromatic compounds and can catalyze the oxygen-dependent hydroxylation of benzene to form phenol as an intermediate.

The dioxygenase system incorporates both oxygen atoms from an oxygen molecule to the benzene ring to give the intermediate *cis*-dihydrodiol. These enzymes can be found extensively in different sites in nature and participate in both anabolic and catabolic metabolisms (Jindrová *et al.*, 2002). Aerobic degradation of aromatic compounds is an important process that are catalyzed by dioxygenases. Dioxygenase enzyme has a broad substrate specificity and can catalyze reactions with a wide range of substrates such as toluene, naphthalene, chlorobenzene and fluorobenzene (Geary *et al.*, 1990).

1.3 Anaerobic benzene degradation

Benzene have been considered persistent in anoxic environments for a long time. It was only until the last two decades that many aromatic compounds including benzene was discovered to be biodegradable under various electron-accepting conditions (Foght, 2008). Studies on anaerobic benzene metabolism are highly relevant because hydrocarbon-contaminated groundwater sites are often under oxygen-limiting conditions due to low solubility and rapid microbial consumption of oxygen. Moreover, benzene is considered to be the most difficult to remediate among the BTEX compounds because of the stability of its ring structure. Although studies on microcosms and laboratory enrichment cultures capable of degrading benzene under anaerobic conditions have been increasing recently, the number is still low compared to literature describing cultures with the ability to degrade other hydrocarbons such as toluene or xylene. Furthermore, isolation of pure cultures poses a big challenge due to very slow biodegradation rate of benzene and slow growth of available microbial consortia. Because of this, the anaerobic benzene degradation pathway has not been clearly elucidated.

1.3.1 Anaerobic benzene-degrading microorganisms

Several researches have shown that some microcosms and microbial enrichments can degrade benzene and fully oxidize to carbon dioxide in the complete absence of oxygen under iron-reducing, sulfate-reducing, methanogenic and denitrifying conditions (Abu Laban *et al.*, 2009; Chakraborty & Coates, 2005; Lovley *et al.*, 1996; Ulrich & Edwards, 2003; Vogt *et al.*, 2011). Increasing attention has been paid to the isolation and characterization of microorganisms with the potential application of degrading environmental contaminants such as BTEX.

Pure isolates with anaerobic benzene-degrading abilities have been characterized (Chakraborty & Coates, 2005; Holmes *et al.*, 2011; Zhang *et al.*, 2013). Furthermore, the genes responsible for the initial benzene activation under anoxic conditions present a biochemical novelty. *Dechloromonas* strain RCB and JJ are two pure cultures capable of degrading benzene anaerobically under denitrifying conditions (J. B. Coates *et al.*, 2001). Strain RCB was isolated from river sediment using chlorobenzoate as electron donor and chlorate as electron acceptor. Strain JJ was isolated from lake sediment using a humic substance and nitrate as electron donor and acceptor, respectively. Both strains are phylogenetically closely related and belong to the newly described *Dechloromonas* genus in the Betaproteobacteria (Achenbach *et al.*, 2001; J. B. Coates *et al.*, 2001). Strain RCB can also degrade toluene, ethylbenzene and all the three isomers of xylene under nitrate-reducing conditions. Furthermore, strain RCB also possess the ability of degrading benzene using perchlorate and chlorate as electron acceptors (Chakraborty *et al.*, 2005).

The hyperthermophilic archaeon *Ferroglobus placidus* which can grow at 85°C has been shown to oxidize a variety of aromatic compounds including benzene, benzoate, phenol, 4-hydroxybenzoate, and benzaldehyde using Fe (III) as sole electron acceptor (Tor & Lovley, 2001). *F. placidus* was isolated in a hydrothermal vent in Vulcano island in Italy where aromatic compounds such as benzene were detected (Hafenbradl *et al.*, 1996). It is the first and only isolated hyperthermophile Archaea that can oxidize aromatic compounds under anoxic conditions. This provides insights into the metabolic profiles of microorganisms in extreme environments and may play an important part in the carbon cycle of such environments (Holmes *et al.*, 2012).

The abundance of *Geobacter* species associated with aromatic compound degradation under Fe(III)-reducing conditions have been described in several studies (Anderson *et al.*, 1998; Botton & Parsons, 2007; Butler *et al.*, 2010; Tobler *et al.*, 2007). It is, therefore, considered that *Geobacter* species are major players in anaerobic degradation under Fe(III)-reducing conditions (Kunapuli *et al.*, 2007). Recently, *G. metallireducens*, a strictly anaerobic bacterium, was shown to be capable of oxidizing benzene under iron-reducing conditions (Zhang *et al.*, 2012). It was isolated from an iron-reducing zones of a petroleum-contaminated aquifer in Minnesota. In addition to benzene, *G. metallireducens* can also grow benzoate, toluene and acetate as electron donors. Tremblay *et al.* (2012) have established a genetic system for *G. metallireducens* to evaluate the metabolic mechanisms and pathways for benzene degradation.

Weelink *et al.* (2008) reported the isolation of a chlorate-reducing benzene-degrading culture *Alicyclophilus denitrificans* from a stable enrichment culture obtained from a wastewater treatment soil samples. Although it is a denitrifying bacterium, *A. denitrificans* is not capable of growing on benzene with nitrate. A unique feature of this bacterium among other benzene degraders is its ability to dismutate chlorite to oxygen and chloride under anaerobic conditions. The release of oxygen during chlorite reduction activates the aerobic pathways of benzene degradation. Therefore, *A. denitrificans* cannot be considered as a true anaerobic benzene degrading microorganism (Vogt *et al.*, 2011; Weelink *et al.*, 2010).

1.3.2 *Azoarcus* sp. DN11

Azoarcus species are members of nitrogen-fixing *Betaproteobacteria* known to degrade many aromatic compounds mainly toluene, ethylbenzene and xylenes (Ball *et al.*, 1996; Evans *et al.*, 1991; Fries *et al.*, 1994; López Barragán *et al.*, 2004; Rabus & Widdel, 1995). Species in this genus are usually found in contaminated groundwater and biodegradation zones (Zhou *et al.*, 1995). Kasai *et al.*, (2006) isolated *Azoarcus* sp. DN11, a denitrifying bacterium, from gasoline-contaminated groundwater in Kumamoto, Japan using an RNA-based stable isotope probing (SIP) and ¹⁴C-labelled benzene techniques. This bacterium can degrade benzene, benzoate, toluene and

xylene under both aerobic and anaerobic conditions (Kasai *et al.*, 2007). However, it could not grow on phenol as the sole carbon source under anaerobic conditions. DN11 was also tested for its bioaugmentation potential for benzene-contaminated groundwater and results showed that the initial 19.8 μM benzene concentration from the groundwater sample was depleted with nitrate consumption after 50 days of incubation. This proves the bioremediation potential of DN11 in removal of benzene and other environmental contaminants from polluted groundwater.

1.3.3 Anaerobic benzene degradation pathway

Up to now, literature regarding the initial benzene activation in the complete absence of oxygen is still fairly lacking mainly due to the difficulty in isolating pure cultures and slow biodegradation of benzene. Isotope-based labelling technique for the detection of ^{13}C - or ^{14}C -labelled metabolites during anaerobic benzene degradation is a common method used to elucidate the reaction mechanism. Three anaerobic benzene-degradation pathways have been proposed based on the metabolites identified in benzene-degrading cultures, namely, methylation to toluene, hydroxylation to phenol, and carboxylation to benzoate (Figure 1.3) (Vogt *et al.*, 2011). Toluene, phenol, and benzoate are further metabolized to a common intermediate, benzoyl-coenzyme A (CoA).

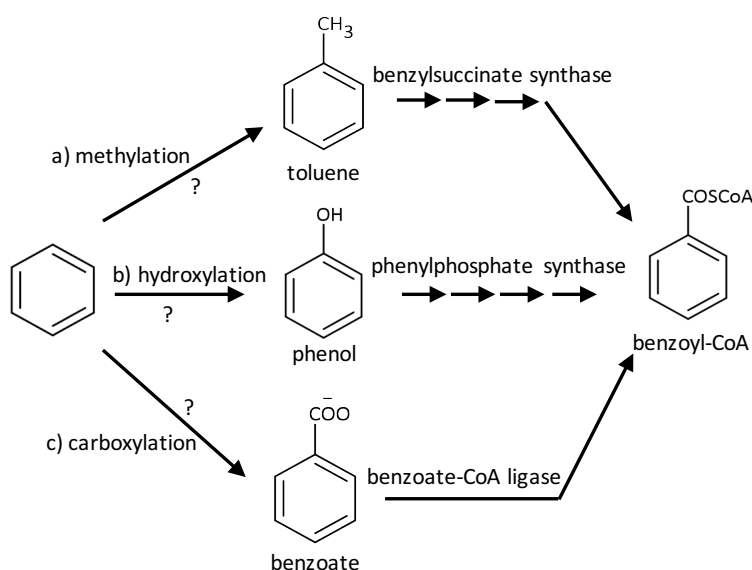


Figure 1.3. Three proposed pathways for anaerobic benzene degradation: a) methylation to toluene; b) hydroxylation to phenol; and c) carboxylation to benzoate. The three pathways further lead to benzoyl-CoA as central metabolite (modified from Vogt *et al.*, 2011).

1.3.3.1 Benzene methylation

Methylation of benzene involves substitution by strong electrophiles derived from S-adenosylmethionine as in Friedel-Crafts alkylation (Coates *et al.*, 2002). In bone marrow, methylation of benzene to toluene have been described mediated by S-adenosyl-methione-dependent alkylation (Flesher & Myers, 1991). Toluene is then converted to benzylsuccinate by addition of methyl group to fumarate by a radical enzyme benzylsuccinate synthase (Figure 1.4A). The detection of toluene as an intermediate in anaerobic benzene degradation was first observed by Ulrich *et al.* (2005) in a nitrate-reducing and methanogenic enrichment culture. ^{13}C -labelled toluene was detected by GC-MS when the culture was incubated with ^{13}C -labelled benzene. However, no pure cultures have been isolated that follows the methylation pathway in degrading benzene anaerobically, thus, warranting further research to confirm that benzene can actually be converted to toluene via methylation under anaerobic conditions.

1.3.3.2 Benzene hydroxylation

Benzene hydroxylation is another alternative pathway for anaerobic benzene degradation involving the incorporation of hydroxyl ions into the benzene ring forming phenol. Anaerobic phenol degradation is initiated by phenylphosphate synthase to convert phenol to phenylphosphate (Figure 1.4B). Vogel and Grbic-Galic (1986) have shown that benzene can be degraded under methanogenic conditions with the detection of phenol as intermediate. ^{18}O -labelled water was used to demonstrate that oxygen incorporated into the benzene ring was derived from water to form phenol. Another study demonstrated the production of phenol from benzene under anaerobic conditions with use of ^{13}C -labelled benzene and detection of ^{13}C -intermediates with GC-MS (Caldwell & Suflita, 2000). *Geobacter metallireducens* is a strictly anaerobic bacterium capable of oxidizing benzene via the phenol pathway under iron-reducing conditions (Zhang *et al.*, 2013). ^{13}C -labelled phenol was detected in benzene degrading *G. metallireducens* culture under anaerobic

conditions. Moreover, ^{18}O -labelled benzene was detected when the culture was incubated with ^{18}O -labelled water. The putative genes involved in the anaerobic phenol degradation in *G. metallireducens* were also differentially expressed during anaerobic benzene degradation as demonstrated using whole-genome microarray hybridization experiment. The researchers proposed that the genes Gmet 0231 and Gmet 0232 of *G. metallireducens* could be encoding for enzymes for hydroxylating benzene to phenol (Zhang *et al.*, 2014).

Kunapuli *et al.* (2008) suggested that phenol can be abiotically formed from benzene under iron- and sulfate-reducing conditions when samples were exposed to air during sampling. It is hypothesized that hydroxyl radicals play an important role in benzene ring hydroxylation, thus, posing difficulty in the interpretation of phenol formation from benzene during metabolite analysis. Although Chakraborty and Coates (2005) suggested that *Dechloromonas* strain RCB employs the hydroxylation pathway in degrading benzene under denitrifying conditions, the researchers could not identify the origin of the oxygen incorporated into benzene. Moreover, the strain RCB genome does not contain the known putative genes for the anaerobic benzene degradation but instead contains genes in the aerobic pathways for aromatic degradation (Salinero *et al.*, 2009). The intra-aerobic production of oxygen from the dismutation of nitrite (a known nitrate reduction intermediate) catalyzed by the putative nitric oxide dismutase has been recently demonstrated by Ettwig *et al.* (2012). Strain RCB may encode this enzyme activating the aerobic benzene pathways even under denitrifying conditions allowing oxygen-dependent benzene degradation (Vogt *et al.*, 2011; Weelink *et al.*, 2010). Additionally, a continuous culture has been shown to activate the both benzene monooxygenase and the carboxylation pathways in degrading benzene under nitrate-reducing conditions (Atashgahi *et al.*, 2018). Oxygen production was observed during benzene degradation suggesting the participation of the putative nitric oxide dismutase (Nod). However, Nod-encoding genes were not identified in the transcriptome of the culture.

1.3.3.3 Benzene carboxylation

Benzoate is a well-studied aromatic compound that is converted to benzoyl-CoA by

benzoate-CoA ligase enzyme (Figure 1.4C) (Carmona *et al.*, 2009). The formation of benzoate from the carboxylation of benzene has been reported under sulfate-reducing (Abu Laban *et al.*, 2009; Caldwell & Suflita, 2000), nitrate-reducing (Caldwell & Suflita, 2000; Kunapuli *et al.*, 2008) and methanogenic (Caldwell & Suflita, 2000; Ulrich *et al.*, 2005a) enrichment cultures. The hyperthermophilic archaeon *Ferroglobus placidus* has been shown to oxidize benzene to CO₂ via benzoate pathway with Fe (III) as e⁻ acceptor. However, due to lack of available genetic manipulation system for hyperthermophiles, the initial carboxylase gene responsible for initial benzene activation of *F. placidus* have not yet been discovered (Holmes *et al.*, 2011). Based on the research done by Abu Laban *et al.* (2010) on the combined metagenomics and metaproteomics analysis of the iron-reducing enrichment culture, a protein homologous to benzoate-CoA ligase was expressed during benzene degradation. A putative anerobic benzene carboxylase (Abc) was suggested to catalyze the benzene activation in the enrichment culture. However, further research on the enzyme activity of the putative benzene carboxylase is needed to determine the legitimacy of this enzyme.

1.4 Objectives of this study

The initial benzene activation via addition of a functional group is a crucial step in the degradation of benzene under anaerobic conditions. Several initial activation steps have been proposed for the benzene degradation. Bioremediation strategies for contaminated environmental sites will greatly depend on the mechanisms of anaerobic degradation by bacteria as it may assist in the design of the bioremediation methods. Therefore, understanding the genetics and biochemistry involved in the anaerobic benzene degradation is a prerequisite for application of the microorganism as bioremediation agent to the actual environmental sites. The current work aims to elucidate the mechanism for the anaerobic benzene degradation of *Azoarcus* sp. DN11 by detection of metabolites produced during the degradation and whole transcriptomic analysis of benzene-degrading DN11 cultures.

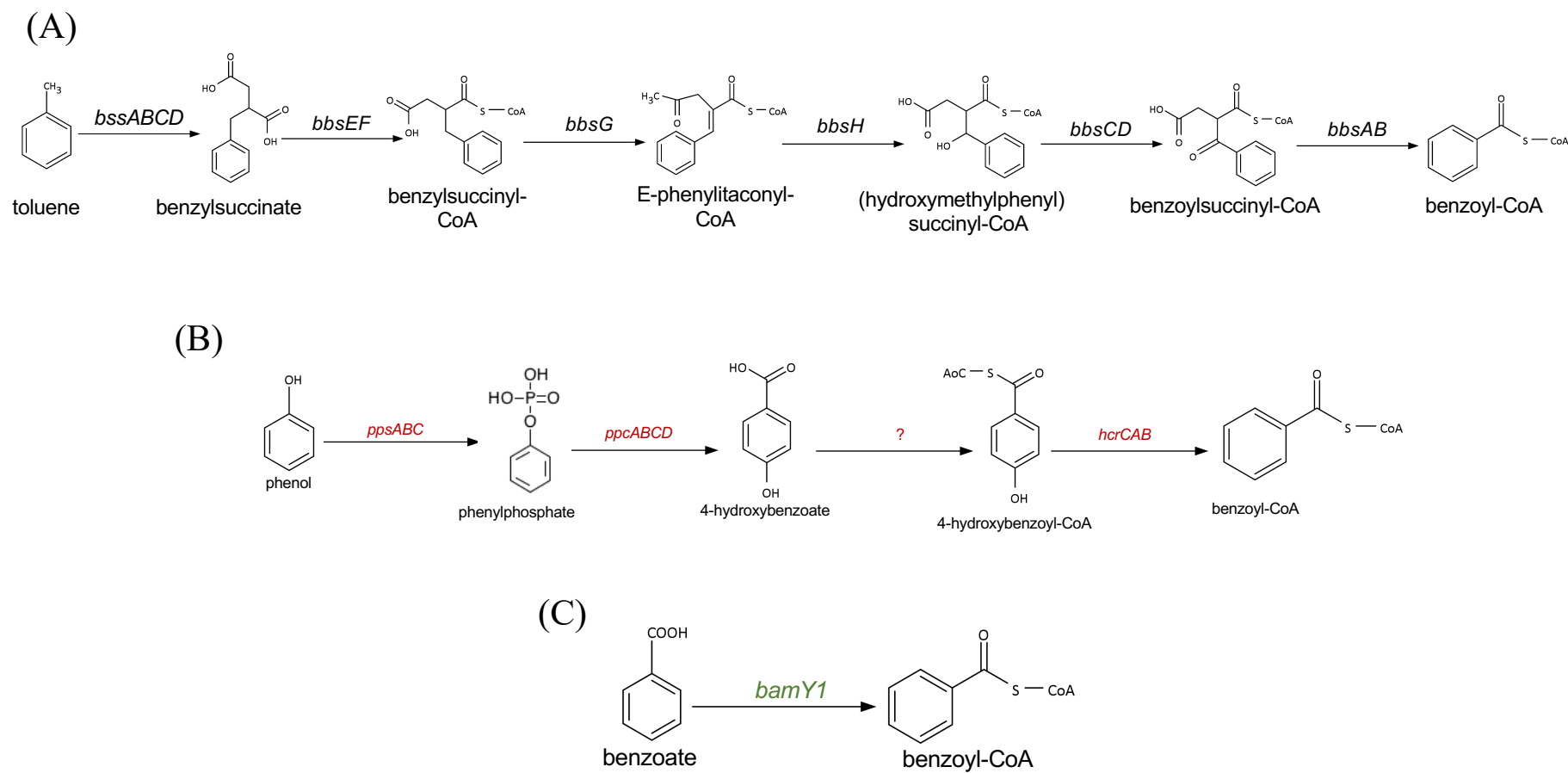


Figure 1.4. Anaerobic degradation pathways for toluene (A), phenol (B) and benzoate (C). The three pathways lead to a common aromatic intermediate, benzoyl-CoA.

Chapter 2

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

2.1 Introduction

This chapter describes the completion of the genome sequence of *Azoarcus* sp. DN11. The draft genome sequence of DN11 has been determined previously, but meaningful downstream analyses can be achieved from a genome sequence free of gaps and fragmentation from draft assemblies. The determination of the complete genome sequence of DN11 provided the basis for the analysis of the catabolic potential of DN11 with focus on the putative degradative genes involved in the benzene metabolism. Genomic analysis of the whole genome sequence of DN11 using KAAS and BLAST searching within the genome of DN11 were performed to construct putative pathways for aerobic and anaerobic benzene degradation. The complete genome sequence of DN11 also served as the reference for the transcriptomic studies described in the later chapter.

2.2 Materials and Methods

2.2.1 Source of bacteria

Azoarcus sp. DN11 was originally isolated from gasoline-contaminated groundwater in Kumamoto, Japan (Kasai *et al.*, 2006) and has been described previously (Kasai *et al.*, 2007). DN11 strain was kindly provided by Dr. Yuki Kasai of Chuo University, Tokyo, Japan. DN11 cells were kept preserved in 40% (v/v) glycerol at -80°C.

2.2.2 Cultivation of DN11 and genomic DNA extraction

Cells of DN11 were revived from frozen glycerol stock by streaking directly onto CGY (Watanabe *et al.*, 1998) agar plates supplemented with 30 µg/mL Polymyxin B and incubating for 3 days at 30°C under aerobic conditions. DN11 colonies were aseptically picked and grown in 5 mL CGY liquid medium overnight at 30°C on a horizontal test tube shaker set at 300 strokes/min.

DN11 cells were harvested for DNA extraction by centrifugation at 13,000 rpm for 2 min at room temperature. DNA were isolated using DNA isolation kit (Promega) following the manufacturer's protocol.

CGY medium

Bacto Casamino Acid	0.05%
Glycerol	0.05%
Yeast extract	0.01%
Agar	1.6%

The medium was autoclaved for 20 min at 121°C.

30 mg/mL Polymyxin B

Polymyxin B sulfate	150 mg
MilliQ water	5 mL

The solution was filter-sterilized through a nitrocellulose membrane (0.22 µm pore size, Millex®GP, Merck Millipore Ltd.) and stored at -20°C.

2.2.3 Whole genome sequencing of *Azoarcus* sp. DN11

The whole genome sequencing of *Azoarcus* sp. DN11 was performed as a collaborative effort with the other laboratory members to complete the previously obtained draft genome sequence.

For library preparation, TruSeq DNA PCR-free and Nextera mate-pair library preparation kits (Illumina) were used to generate a 1,300-bp paired-end (PE) PCR-free library and a 580-bp mate-pair (MP) library with an initial fragment size of 7,100 bp. Sequencing was performed by loading the libraries into a MiSeq 600-cycle V3 chemistry sequencing cartridge (Illumina), and 300 bp of each library end was sequenced with a MiSeq instrument (Illumina). All kits were used following the manufacturer's instructions. Newbler 2.8 (Roche) was used for genome assembly (default parameters, except for overlapMinMatchIdentity of 98 and allContigThresh of 0), with 1 million PE (260 Mb) and 0.67 million MP (120 Mb) reads that were quality trimmed using ShortReadManager 0.995 (score_threshold, 30; length_threshold, 21; and 21-mers occurring more than twice regarded as valid) (Yoshiyuki Ohtsubo *et al.*, 2012). In silico gap closing of the obtained scaffolds (67 in total) was done manually with AceFileViewer 1.5 and GenoFinisher 2.1 (default settings) (Yoshiyuki Ohtsubo *et al.*, 2012) using the MP data, resulting in the complete resolution of repeat-induced gaps and unambiguous assembly of a single replicon. This sequence was checked for errors derived from the gap closing step with FinishChecker (an accessory of GenoFinisher) (Y. Ohtsubo *et al.*, 2014). The gene prediction and annotation was performed using Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova *et al.*, 2016) and Microbial Genome Annotation Pipeline (MiGAP) (Sugawara *et al.*, 2009), and annotated sequence files were first compared using GenomeMatcher 2.203 (Yoshiyuki Ohtsubo *et al.*, 2008) to manually correct start/stop codon inconsistencies and then merged into a single annotated sequence file

2.2.4 Data availability and genome announcement

The complete genome sequence of *Azoarcus* sp. Strain DN11 was deposited at DDBJ/ENA/GenBank under the accession number CP021731. Raw sequencing data was deposited in the SRA under the accession number PRJNA388763.

Genome announcement has been made in the journal of Microbiology Resource Announcements (MRA) (Devanadera *et al.*, 2019).

2.2.5 *In silico* analysis of benzene metabolic pathways of DN11

In order to infer the metabolic repertoire of DN11, the genomic data of DN11 was mapped onto the metabolic networks derived from KEGG Automatic Annotation Server (KAAS) (<http://www.genome.jp/tools/kaas/>) (Moriya *et al.*, 2007). Genes data set “for Prokaryotes” option was selected as organism list in KEGG in addition to manual selection of relevant strains on aromatic degradation. The assignment method was run with “BBH (bi-directional best hit)” method in KEGG database. Using KEGG annotation, each gene acquired a KEGG Orthology (KO) number representing a node in a certain reference metabolic pathway in KEGG. The generated KO list was used to map the metabolic pathways of DN11 using KEGG mapper (<http://www.genome.jp/15eg/mapper.html>) (Ogata *et al.*, 1998).

To validate the KEGG mapping results, characterized genes and enzymes involved in benzene degradation from known aromatic degraders were used as queries within the genome of DN11 to identify putative homologs. Searches for homologs were performed through BLAST searching using CLC Genomics Workbench (version 7.5.2, Qiagen Bioinformatics) with default parameters. Comparative analysis based on pairwise sequence alignment was performed to calculate the identity percentage between two protein sequences using CLC software’s default parameters. The metabolic analysis of the genome sequence of DN11 was focused on the aromatic degradation of under aerobic and anaerobic conditions. The information of genes and proteins obtained from the mentioned sources was utilized to construct putative pathways for aromatic degradation of DN11 with focused on benzene metabolism.

2.3 Results and Discussion

2.3.1 Whole genome sequencing results

The whole genome of *Azoarcus* sp. DN11 was sequenced using MiSeq system. A total length of 4,956,835 bp long was obtained with a sequencing depth of 77× and GC content of 66.3%. The coding DNA sequences (CDSs) obtained were 4,593 with 4 copies of rRNA operons and 57 tRNA genes. Circular representations of complete genome of DN11 is shown in Figure 2.1.

2.3.2 Genomic analysis of genes involved in benzene degradation pathways

Genome sequence-based prediction was performed to identify the putative genes and proteins in the benzene degradation pathways of DN11. Figure 2.2 shows the overview of the pathways for the aromatic compound degradation as predicted by the KEGG mapper based on the whole genome of DN11. Figure 2.3 shows the generated KEGG map of the pathways for the benzoate degradation of DN11. Several pathways for the degradation of aromatic compounds were identified as indicated by the KEGG maps.

DEGRADATION OF AROMATIC COMPOUNDS

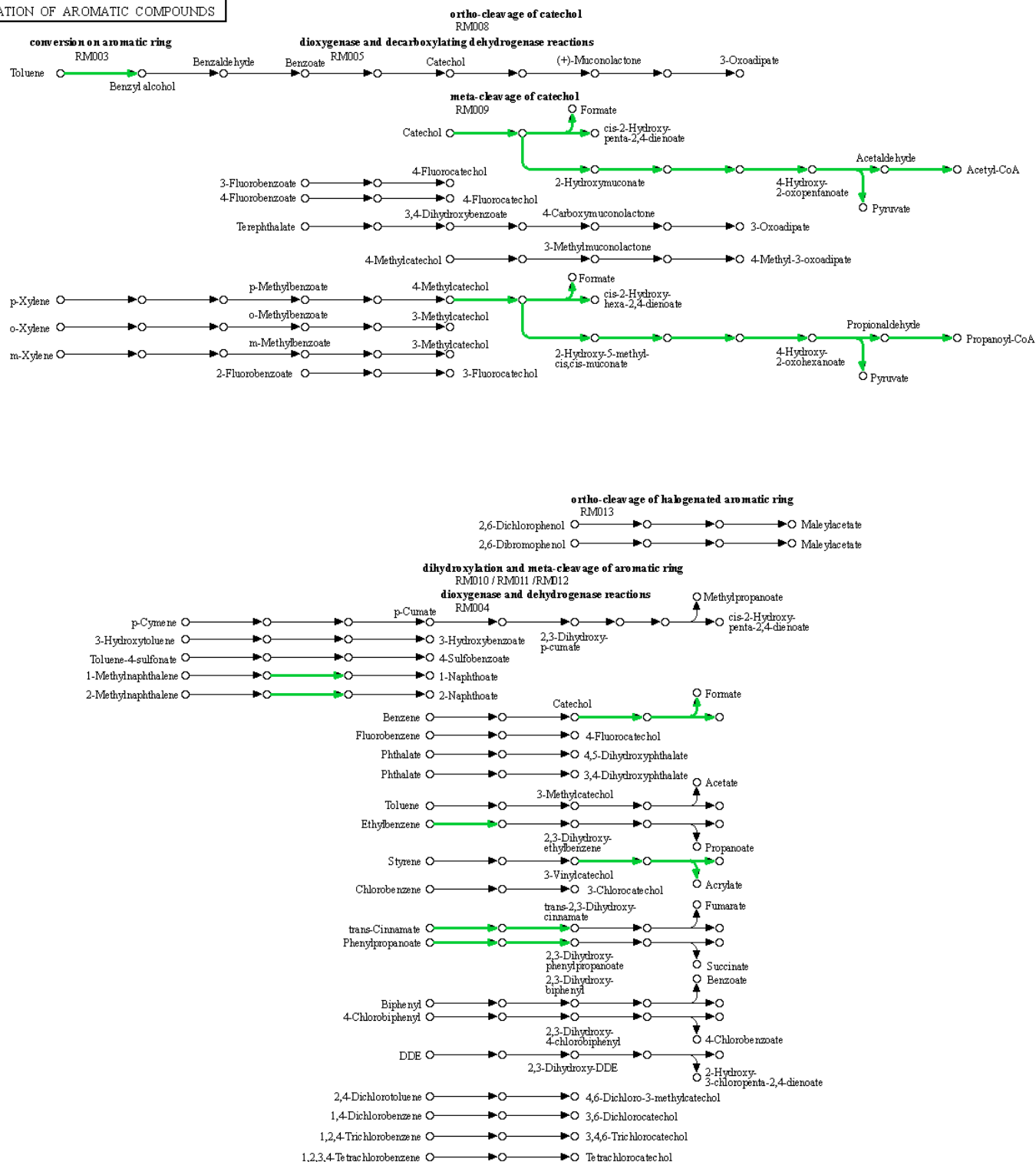


Figure 2.2. Overview of the pathways for the aromatic compound degradation of DN11 as predicted by the KEGG mapper. Green arrow indicates that the pathway exists in DN11 genome.

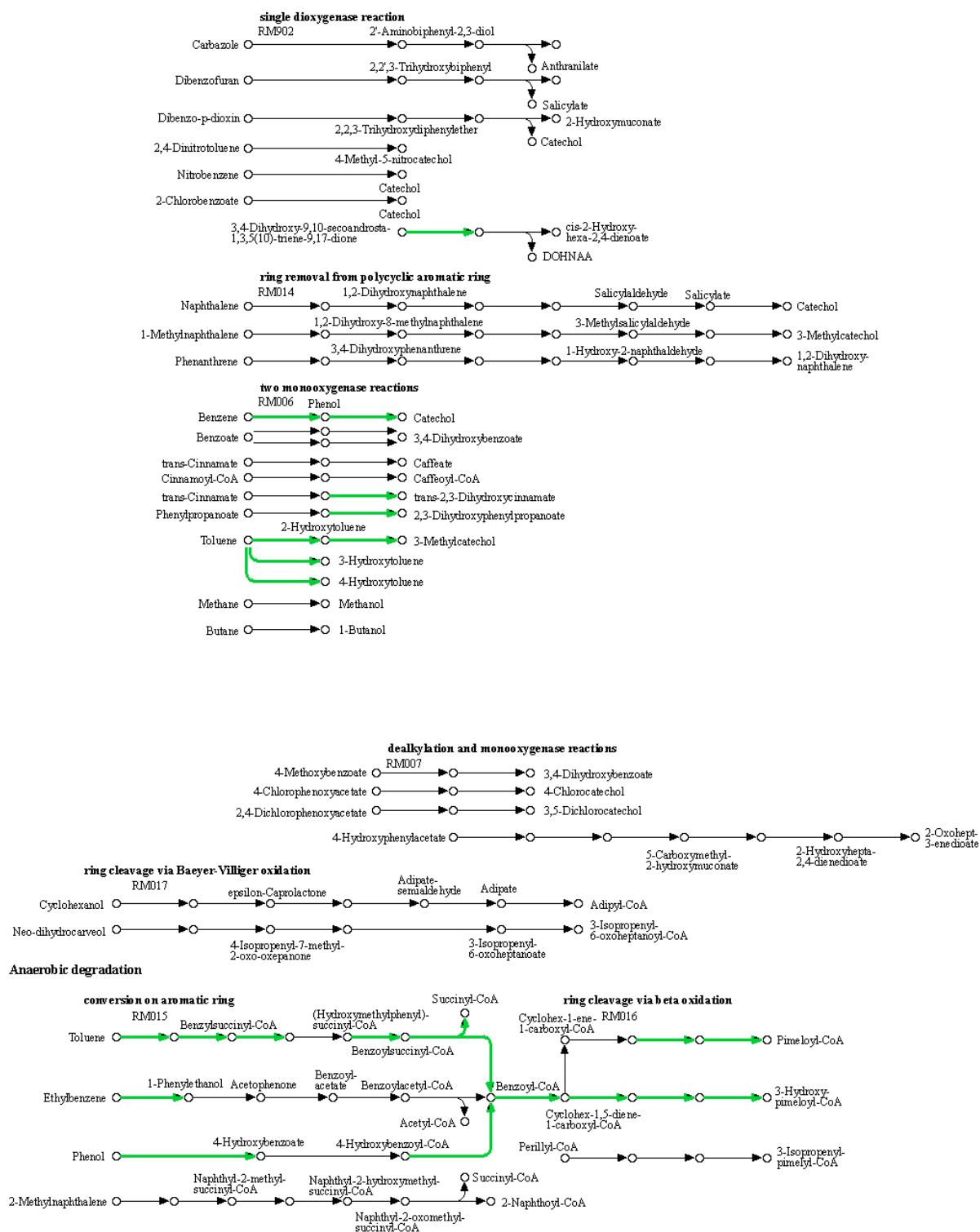


Figure 2.2. *Continued.*

2.3.2.1 Aerobic benzene degradation pathways

Two pathways, either through monooxygenase or dioxygenase reactions, can be utilized by microorganism for the aerobic benzene degradation. Representative proteins from known benzene degraders were obtained and used as queries to identify putative genes in DN11 genome that correspond to a specific reaction in a pathway. Putative pathways were constructed for monooxygenase and dioxygenase enzyme systems for the degradation of benzene. Tables 2.1 and 2.2 show the percentage identities of the putative proteins involved in the benzene monooxygenase degradation, and Table 2.3 shows the percentage identities for the dioxygenase enzymes. The putative aerobic benzene degradation pathways involving monooxygenase and dioxygenase systems were constructed as shown on Figure 2.4. The presence of both benzene monooxygenase and dioxygenase pathways suggests that DN11 may be able to degrade benzene aerobically using either of the two mechanisms. It should be noted, however, that dioxygenase enzyme has a broad substrate specificity and can catalyze reactions with a wide range of substrates such as toluene, naphthalene, chlorobenzene and fluorobenzene (Geary *et al.*, 1990).

2.3.2.2 Anaerobic benzene degradation pathways

The putative anaerobic pathways of DN11 for the degradation of toluene, phenol and benzoate were constructed based on the metabolic analysis of the whole genome sequence and the proposed pathways described by Abu Laban *et al.* (2009) and Zhang *et al.* (2013). Two gene clusters for the anaerobic toluene degradation were identified within the genome of DN11. Tables 2.4 and 2.5 show the comparison of the proteins involved in the two putative toluene degradation pathways of DN11 with that of *Geobacter metallireducens*. The two gene clusters for anaerobic toluene degradation are found in two different regions of the genome. Tables 2.6 and 2.7 show comparison of the proteins involved in the phenol and benzoate pathway, respectively, to that of the various representative organisms. The proposed pathways for the anaerobic biodegradation of benzene by *Azoarcus* sp. DN11 are shown in Figure 2.5. Two sets of gene clusters were found to be involved

in the anaerobic toluene degradation of DN11, one for phenol pathway and three putative enzymes were identified to be involved in the anaerobic benzoate degradation of DN11. These findings indicated the possibility that DN11 may use all three pathways, namely, methylation, carboxylation, and hydroxylation pathway to degrade benzene under anaerobic conditions.

Table 2.1. Comparison of the proteins involved in the putative four component type monooxygenase enzyme system (four component alkene/aromatic monooxygenase group) of DN11 responsible for benzene degradation with that of *Pseudomonas mendocina* KR1.

Putative gene number	Protein as annotated	Queries used for protein comparison				
		Gene	Organism	Protein	% Identity	Reference
10195	toluene monooxygenase	<i>tmoA</i>	<i>Pseudomonas mendocina</i> KR1	toluene-4-monooxygenase system protein A	69.78	Yen <i>et al.</i> (1991)
10200	toluene monooxygenase	<i>tmoB</i>		toluene-4-monooxygenase system protein B	44.94	
10205	2Fe-2S ferredoxin	<i>tmoC</i>		toluene monooxygenase system ferredoxin subunit	55.75	
10210	monooxygenase	<i>tmoD</i>		toluene monooxygenase system protein D	56.60	
10215	toluene monooxygenase	<i>tmoE</i>		toluene monooxygenase system protein E	55.15	
10220	oxidoreductase	<i>tmoF</i>		toluene monooxygenase electron transfer component	38.60	

Table 2.2. Comparison of the proteins involved in the putative monooxygenase enzyme system (phenol hydroxylase group) of DN11 responsible for benzene degradation with that of *Pseudomonas* sp. strain CF600.

Putative gene number	Protein as annotated	Queries used for protein comparison				
		Gene	Organism	Protein	% Identity	Reference
10250	phenol hydroxylase	<i>dmpK</i>	<i>Pseudomonas</i> sp. strain CF600	phenol hydroxylase P0 protein	31.91	Shingler <i>et al.</i> (1992)
10255	phenol hydroxylase	<i>dmpL</i>		phenol hydroxylase P1 protein	51.49	
10260	phenol hydroxylase	<i>dmpM</i>		phenol hydroxylase P2 protein	45.05	
10265	phenol 2-monooxygenase	<i>dmpN</i>		phenol hydroxylase P3 protein	59.62	
10270	phenol hydroxylase	<i>dmpO</i>		phenol hydroxylase P4 protein	46.67	
10275	phenol hydroxylase	<i>dmpP</i>		phenol hydroxylase P5 protein	60.11	

Table 2.3. Comparison of the proteins involved in the putative dioxygenase enzyme system of DN11 responsible for benzene degradation with that of *Pseudomonas putida* ML2.

Putative gene number	Protein as annotated	Queries used for protein comparison				
		Gene	Organism	Protein	% Identity	Reference
21455	FAD-dependent oxidoreductase	<i>bedA</i>	<i>Pseudomonas putida</i> ML2	Benzene 1,2-dioxygenase system ferredoxin--NAD(+) reductase subunit	29	Tan <i>et al.</i> (1993)
21460	bifunctional 3-phenylpropionate/cinnamic acid dioxygenase ferredoxin subunit	<i>bedB</i>		Benzene 1,2-dioxygenase system ferredoxin subunit	57	
21470	benzene 1,2-dioxygenase	<i>bedC2</i>		Benzene 1,2-dioxygenase subunit beta	49	
21475	aromatic ring-hydroxylating dioxygenase subunit alpha	<i>bedC1</i>		Benzene 1,2-dioxygenase subunit alpha	61	
21450	cis-2,3-dihydrobiphenyl-2,3-diol dehydrogenase	<i>bnzE</i>		Cis-1,2-dihydrobenzene-1,2-diol dehydrogenase	55	

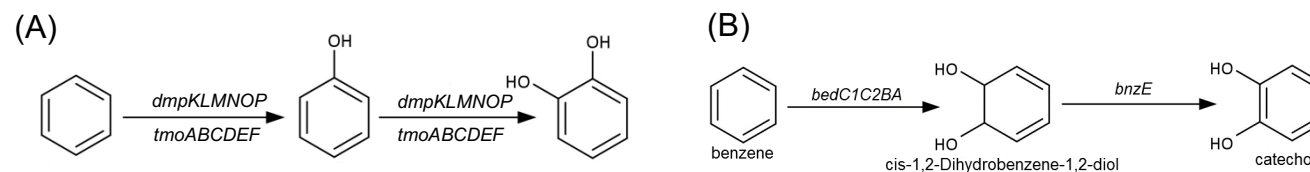


Figure 2.4. Putative pathways for aerobic benzene degradation of *Azoarcus* sp. DN11 involving monooxygenase (A) and dioxygenase (B) enzyme systems.

Table 2.4. Comparison of the proteins involved in the 1st putative toluene degradation pathway of DN11 with that of *Geobacter metallireducens*.

Putative Gene number	Protein as annotated	Queries used for protein comparison				
		Gene	Organism	Protein	% Identity	Reference
07535	benzylsuccinate synthase subunit alpha	<i>bssA</i>	<i>Geobacter metallireducens</i>	(R)-benzylsuccinate synthase, alpha subunit	71	Aklujkar <i>et al.</i> (2009)
07530	benzylsuccinate synthase subunit beta	<i>bssB</i>		(R)-benzylsuccinate synthase, beta subunit	53	
07540	benzylsuccinate synthase subunit gamma	<i>bssC</i>		(R)-benzylsuccinate synthase, gamma subunit	58	
07545	[benzylsuccinate synthase]-activating enzyme	<i>bssD</i>		(R)-benzylsuccinate synthase, delta subunit	49	
07440	benzylsuccinate CoA-transferase	<i>bbsE</i>		succinyl:(R)-benzylsuccinate coenzyme A transferase subunit	64	
07445	succinyl-CoA--benzylsuccinate CoA-transferase	<i>bbsF</i>		succinyl:(R)-benzylsuccinate coenzyme A transferase subunit	68	
07455	benzylsuccinyl-CoA dehydrogenase	<i>bbsG</i>		(R)-benzylsuccinyl-CoA dehydrogenase	70	
07460	enoyl-CoA hydratase	<i>bbsH</i>		(E)-2-benzylidenesuccinyl-CoA hydratase	73	
07430	2-[hydroxy(phenyl)methyl]-succinyl-CoA DH	<i>bbsC</i>		2-[hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase subunit	36	
07435	beta-ketoacyl-ACP reductase	<i>bbsD</i>		2-[hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase subunit	71	
07420	benzoylsuccinyl-CoA thiolase	<i>bbsA</i>		benzoylsuccinyl-CoA thiolase subunit	68	
07425	propanoyl-CoA acyltransferase	<i>bbsB</i>		benzoylsuccinyl-CoA thiolase subunit	76	

Table 2.5. Comparison of the proteins involved in the 2nd putative toluene degradation pathway of DN11 with that of *Geobacter metallireducens*.

Putative gene number	Protein as annotated	Queries used for protein comparison				
		Gene	Organism	Protein	% Identity	Reference
21890	benzylsuccinate synthase subunit alpha	<i>bssA</i>	<i>Geobacter metallireducens</i>	(R)-benzylsuccinate synthase, alpha subunit	71	Aklujkar <i>et al.</i> (2009)
21895	benzylsuccinate synthase subunit beta	<i>bssB</i>		(R)-benzylsuccinate synthase, beta subunit	53	
21885	benzylsuccinate synthase subunit gamma	<i>bssC</i>		(R)-benzylsuccinate synthase, gamma subunit	57	
21880	[benzylsuccinate synthase]-activating enzyme	<i>bssD</i>		(R)-benzylsuccinate synthase, delta subunit	49	
21975	benzylsuccinate CoA-transferase	<i>bbsE</i>		succinyl:(R)-benzylsuccinate coenzyme A transferase subunit	62	
21970	succinyl-CoA--benzylsuccinate CoA-transferase	<i>bbsF</i>		succinyl:(R)-benzylsuccinate coenzyme A transferase subunit	68	
21960	acyl-CoA dehydrogenase	<i>bbsG</i>		(R)-benzylsuccinyl-CoA dehydrogenase	70	
21955	enoyl-CoA hydratase	<i>bbsH</i>		(E)-2-benzylidenesuccinyl-CoA hydratase	75	
21985	2-[hydroxy(phenyl)methyl]-succinyl-CoA DH	<i>bbsC</i>		2-[hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase subunit	37	
21980	NAD(P)-dependent oxidoreductase	<i>bbsD</i>		2-[hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase subunit	69	
21995	benzoylsuccinyl-CoA thiolase	<i>bbsA</i>		benzoylsuccinyl-CoA thiolase subunit	69	
21990	propanoyl-CoA acyltransferase	<i>bbsB</i>		benzoylsuccinyl-CoA thiolase subunit	77	

Table 2.6. Comparison of the proteins involved in the putative phenol degradation pathway of DN11 with that of various microorganisms.

Putative gene number	Protein as annotated	Queries used for protein comparison				
		Gene	Organism	Protein	% Identity	Reference
14585	PEP-utilizing enzyme, mobile region	<i>ppsA</i>	<i>Geobacter metallireducens</i>	phenylphosphate synthase, alpha subunit	77	Aklujkar <i>et al.</i> (2009)
14590	phenylphosphate synthase subunit beta	<i>ppsB</i>		phenylphosphate synthase, beta subunit	69	
14595	protein stimulating phenylphosphate synthetase activity	<i>ppsC</i>		phenylphosphate synthase, gamma subunit	39	
14610	phenylphosphate carboxylase subunit alpha	<i>ppcA</i>	<i>Azoarcus</i> sp. strain EbN1	Alpha subunit of phenylphosphate carboxylase	95	Rabus <i>et al.</i> (2005)
14600	phenylphosphate carboxylase subunit beta	<i>ppcB</i>		Beta subunit of phenylphosphate carboxylase	95	
14615	phenylphosphate carboxylase subunit gamma	<i>ppcC</i>		Phenylphosphate carboxylase, gamma subunit	93	
14605	phenylphosphate carboxylase subunit delta	<i>ppcD</i>		Phenylphosphate carboxylase, delta subunit	67	
15675	4-hydroxybenzoate--CoA ligase2	<i>hbaA</i>	<i>Thauera aromatica</i>	4-hydroxybenzoate--CoA ligase	45	Breese and Fuchs (1998)
02105	4-hydroxybenzoyl-CoA reductase subunit beta	<i>hcrB</i>		4-hydroxybenzoyl-CoA reductase flavoprotein subunit	48	
02115	4-hydroxybenzoyl-CoA reductase subunit gamma	<i>hcrC</i>		4-hydroxybenzoyl-CoA reductase iron-sulfur cluster-binding subunit, putative	72	
02110	4-hydroxybenzoyl-CoA reductase subunit alpha	<i>hcrA</i>		4-hydroxybenzoyl-CoA reductase subunit	59	

Table 2.7. Comparison of the proteins involved in the putative benzoate degradation pathway of DN11 with that of various microorganisms.

Putative gene number	Protein as annotated	Queries used for protein comparison				
		Gene	Organism	Protein	% Identity	Reference
12090	4-hydroxybenzoate--CoA ligase	<i>bamY</i>	<i>Geobacter metallireducens</i>	benzoate--CoA ligase	50	Aklujkar <i>et al.</i> (2009)
22715	4-hydroxybenzoate--CoA ligase				49	
21860	benzoate-CoA ligase family protein				44	

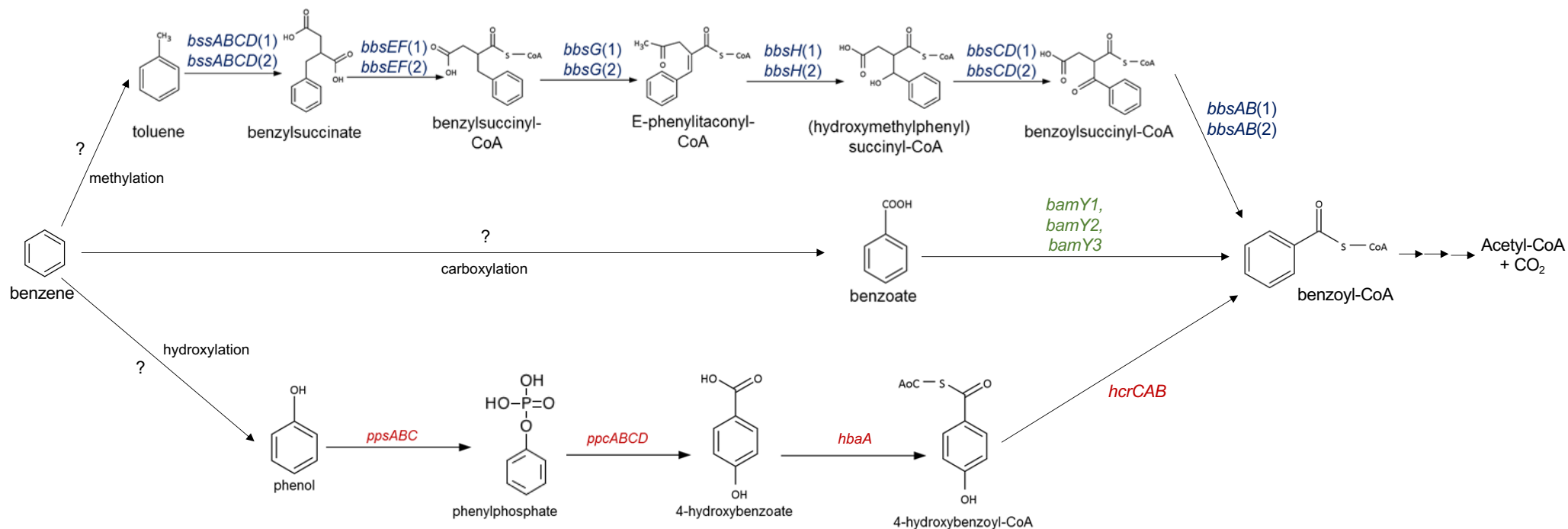


Figure 2.5. Proposed pathways for the anaerobic biodegradation of benzene by *Azoarcus* sp. DN11. Two sets of putative gene clusters were found to be involved in the methylation of benzene to toluene. Three putative genes encoding for enzymes involved in the degradation of benzoate to benzoyl-CoA were found in the proposed carboxylation pathway.

2.3.2.3 Nitrate metabolism

Based on the generated KEGG map, DN11 genome contained the complete genes for the nitrate metabolism (Figure 2.6). Moreover, the complete genes in the nitrate metabolism pathway were predicted from the genome of DN11 based on comparison with the known nitrate-reducing microorganisms (Table 2.7 and Figure 2.7). The proposed pathway for the growth of DN11 under nitrate-reducing conditions agrees with the experimental evidence that DN11 is a denitrifying bacterium capable of degrading benzene under nitrate-reducing conditions (Kasai *et al.*, 2007).

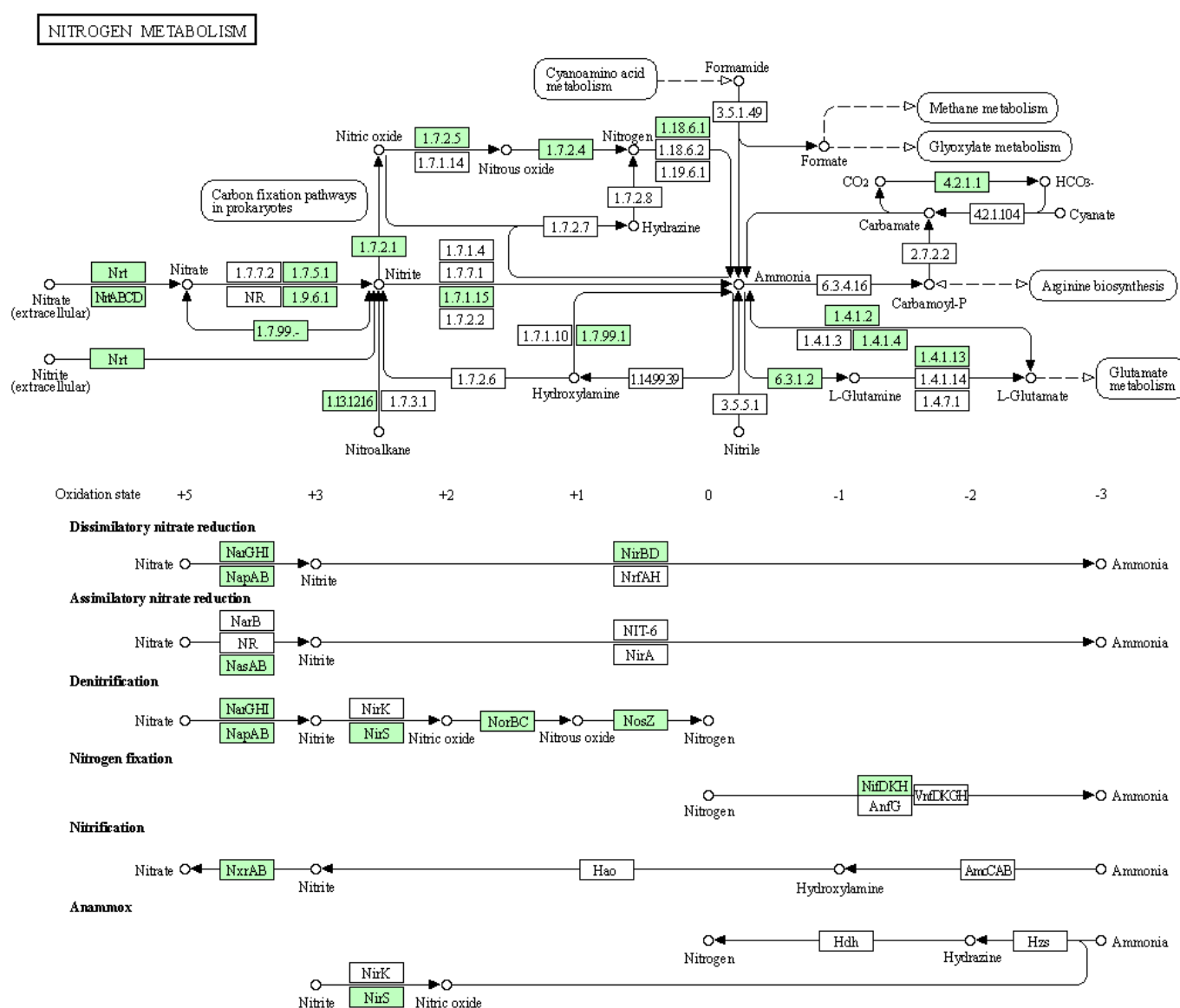


Figure 2.6. Overview of the pathways for the nitrogen metabolism of DN11 as predicted by the KEGG mapper. Orthologs of enzymes highlighted in green were found in DN11 genome.

Table 2.8. Comparison of the proteins involved in the denitrification pathway of DN11 with that of various microorganisms.

Putative gene number	Protein as annotated	Queries used for protein comparison				
		Gene	Organism	Protein	% Identity	Reference
07270	nitrate reductase subunit beta	<i>narH</i>	<i>Escherichia coli</i> (strain K12)	Respiratory nitrate reductase 1 beta chain	63	Blasco <i>et al.</i> (1990)
07275	nitrate reductase subunit alpha	<i>narG</i>		Respiratory nitrate reductase 1 alpha chain	58	
07260	respiratory nitrate reductase subunit gamma	<i>narI</i>		Respiratory nitrate reductase 1 gamma chain	47	
17520	nitrite reductase	<i>nirS</i>	<i>Alcaligenes eutrophus</i>	Cytochrome cd1 nitrite reductase	69	Rees <i>et al.</i> (1997)
08895	nitric-oxide reductase large subunit	<i>norB</i>	<i>Pseudomonas stutzeri</i> (<i>Pseudomonas perfectomarina</i>)	Nitric oxide reductase subunit B	70	(Heiss <i>et al.</i> (1989)
07240	nitrous-oxide reductase	<i>nosZ</i>	Paracoccus denitrificans	Nitrous-oxide reductase	46	Hoeren <i>et al.</i> (1993)
08290	nitrous-oxide reductase	<i>nosZ</i>		Nitrous-oxide reductase	45	

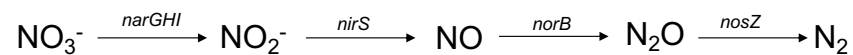


Figure 2.7. Proposed pathway for the nitrate reduction in DN11.

Chapter 3

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

3.1 Introduction

The three proposed pathways for the anaerobic benzene degradation (Figure 1.3) produces three unique intermediate metabolites (phenol, toluene and benzoate) which are often used as the targets for the identification of pathways employed by an organism. A common and useful technique used to investigate the reaction mechanism of anaerobic benzene degradation is the detection of intermediate metabolites produced under benzene-degrading conditions. The isotope-based detection and identification of intermediate metabolites is aided by the use of gas chromatography-mass spectrophotometry (GC-MS) as an analytical technique. [^{13}C]-labelled benzene and [^{18}O] labeled water was used to label the intermediate metabolite that was produced during the anaerobic degradation by DN11. The application of isotopic labeling and GC-MS to elucidate the based on the labeled degradation products provided insights into the possible mechanism of benzene degradation by DN11.

3.2 Materials and Methods

3.2.1 Pre-cultivation of DN11

Cells of DN11 were revived from glycerol stock by streaking directly onto CGY (Watanabe *et al.*, 1998) agar plates supplemented with 30 µg/mL Polymyxin B and incubating for 3 days at 30°C under aerobic conditions. DN11 colonies were aseptically picked and grown in 5 mL CGY liquid medium overnight at 30°C on a horizontal test tube shaker set at 300 strokes/min. The cells were subcultured into a baffled flask with 100 mL CGY liquid medium incubated overnight at 30°C on an orbital shaker (120 rpm).

CGY medium

Bacto Casamino Acid	0.05%
Glycerol	0.05%
Yeast extract	0.01%
Agar	1.6%

The medium was autoclaved for 20 min at 121°C.

30 mg/mL Polymyxin B

Polymyxin B sulfate	150 mg
MilliQ water	5 mL

The solution was filter-sterilized through a nitrocellulose membrane (0.22 µm pore size, Millex®GP, Merck Millipore Ltd.) and stored at -20°C.

3.2.2 Preparation of anaerobic medium

Anaerobic cultivation of DN11 was performed in modified basal salt medium (BSM) as described by Kasai *et al.* (2005).

Preparation of stock solutions

Trace element solution

HCl (25%)	10 ml
FeCl ₂ · 4H ₂ O	1.5 g
CoCl ₂ · 6H ₂ O	190 mg
MnCl ₂ · 4H ₂ O	100 mg
ZnCl	70 mg
H ₃ BO	62 mg
Na ₂ MoO ₄ · 2H ₂ O	36 g
NiCl ₂ · 6H ₂ O	24 mg
CuCl ₂ · 2H ₂ O	17 mg

MilliQ water	990 mL
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FeCl₂ was first dissolved in HCl before diluting in MilliQ water. The remaining salts were added, and the final volume was adjusted to 1 L. The solution was autoclaved for 20 min at 121°C and stored at 4°C.

Selenite-tungstate solution

NaOH	0.4 g
Na ₂ SeO ₃ • 5H ₂ O	6 mg
Na ₂ WO ₄ • 2H ₂ O	8 mg
MilliQ water	1 L

The solution was autoclaved for 20 min at 121°C and stored at 4°C.

Vitamin solution

D(+) Biotin	2.5 mg
Nicotine acid	25 mg
Thiamine-HCl	12.5 mg
<i>p</i> -Aminobenzoic acid	12.5 mg
Calcium D(+)-pantothenate	6.5 mg
Pyridoxine dihydrochloride	62.5 mg
MilliQ water	100 mL

The solution was filter-sterilized through a nitrocellulose membrane (0.22 µm pore size, Millex® GP, Merck Millipore Ltd.) and stored at 4°C.

Vitamin B₁₂ solution

Cyanocobalamine	5 mg
MilliQ water	100 mL

The solution was filter-sterilized through a nitrocellulose membrane (0.22 µm pore size, Millex® GP, Merck Millipore Ltd.) and stored at 4°C.

68 mM CaCl₂ • 2H₂O

CaCl ₂ • 2H ₂ O	10 g
MilliQ water	1 L

The solution was autoclaved for 20 min at 121°C and stored at 4°C.

200 mM MgSO₄ • 6H₂O solution

MgSO ₄ • 6H ₂ O	40.6 g
MilliQ water	1 L

The solution was autoclaved for 20 min at 121°C and stored at 4°C.

0.1% Resazurin solution

Resazurin sodium salt	0.01 g
MilliQ water	10 mL

The solution was filter sterilized through a nitrocellulose membrane (0.22 µm pore size, Millex® GP, Merck Millipore Ltd.) and stored at 4°C.

Preparation of Basal salt medium

Salt solution

KH ₂ PO ₄	0.52 g
K ₂ HPO ₄	1.06 g

NH ₄ Cl	0.2 g
NaNO ₃	0.5 g
Trace element solution	1 mL
Vitamin solution	1 mL
Vitamin B ₁₂ solution	1 mL
Selenite-tungsten solution	1 mL
68 mM CaCl ₂ · 2H ₂ O solution	10 mL
200 mM MgSO ₄ · 6H ₂ O solution	10 mL
0.1% Resazurin solution	0.5 mL
MilliQ water	1 L

Salts were dissolved in MilliQ water and 10 mL of the solution were dispensed to 20 mL flat bottom headspace vial (Agilent Technologies). To remove dissolved oxygen in the solution, the solution was sparged with N₂ gas for 20 min and the headspace of the vial for 1 min. Prior to sparging, nitrogen gas was passed through a heated copper column to remove contaminating oxygen. The vials were fitted with ETFE-coated butyl rubber septum and aluminum cap and were autoclaved for 20 min at 121°C. 0.1 mL CaCl₂ and 0.01 mL MgSO₄ solutions were added to the vials after autoclavation and under N₂/H₂ (96%:4%) gas mix atmosphere. This resulted to a total volume of 10.2 mL BSM in a headspace vial.

3.2.3 Preparation of inoculum

To prepare the inoculum for anaerobic experiments, DN11 cells aerobically grown in 100 mL preculture (Section 3.2.2) were collected during exponential growth through centrifugation at 7000×g for 5 min. The resulting cell pellet was washed with sterile 15 mL carbon-free BSM three times to remove contaminating CGY medium. DN11 cells were then resuspended in sterile BSM to a final optical density of approximately 0.5 at 600 nm. The cell suspension was transferred to an anaerobic vial sealed with ETFE-coated butyl rubber septum and aluminum cap. Prior to use, the headspace of the vial containing the cell suspension was subjected to gas exchange using nitrogen gas to remove oxygen gas.

3.2.4 Incubation with [¹³C] benzene

To determine the first intermediate formed in anaerobic benzene metabolism, benzene labeled with stable isotope [¹³C] was used as tracer in benzene degrading DN11 cultures. All manipulations were performed under strict anaerobic conditions inside an anaerobic glovebox (Coy Laboratory Products Inc., Michigan, USA) under N₂/H₂ (96%:4%) gas mix atmosphere. The anaerobicity of the chamber was maintained by performing routine mix gas exchange with a heated palladium catalyst. Incubations were performed in a 20 mL headspace vial containing sterile 10.2

mL BSM supplemented with 6 mM NaNO₃ (electron acceptor) and 0.00005% resazurin (redox indicator). The medium was inoculated with 0.3 mL DN11 inoculum (as prepared in Section 3.2.4) resulting to a final volume of 10.5 mL. Labelled [¹³C] benzene (¹³C₆, 99% purity; Cambridge Isotope Laboratories, Andover, MA) was added to the vial to a final concentration of 15 µM using a syringe. The same samples were prepared as negative control but without inoculation of DN11. The vial was sealed with butyl rubber septum and aluminum cap and incubated under anaerobic conditions at 30°C. Samples for cell enumeration by crystal violet staining and GC-MS analysis of headspace benzene concentration and metabolite detection were taken at a specific time interval over the course of degradation. The headspace benzene peak area ratio was calculated using the formula:

$$\text{Benzene peak area ratio} = \frac{\text{DN11 benzene peak area}}{\text{Negative control benzene peak area}}$$

The identification of the intermediate metabolites of anaerobic benzene degradation by DN11 were based on their molecular mass ions and comparison with authentic standards. Labelled [¹³C] toluene (¹³C₆, 99% purity), [¹³C] benzoate (¹³C₆, 99% purity) and [¹³C] phenol (¹³C₆, 99% purity) as authentic standards were purchased from Cambridge Isotope Laboratories, Andover, MA.

3.2.5 Incubation with [¹⁸O] H₂O

To determine the oxygen source in the production of metabolites during anaerobic benzene degradation by DN11, biodegradation test in isotopically-labelled water was set-up. Labelled water (water-¹⁸O, 97 atom %¹⁸O) was obtained from Sigma-Aldrich, UK. The procedure described in Section 3.2.5 was used with the following modifications. The incubation was performed in an anaerobic medium with 42.95% H₂¹⁸O supplementation. One headspace vial containing 4.51 mL H¹⁸O₂, 5.69 mL BSM with 6 mM NaNO₃ and 0.00005% resazurin, 0.3 mL DN11 inoculum (as prepared in Section 3.2.4), and 15 µM unlabeled benzene was prepared. The sample was taken after 14 days of incubation under anaerobic conditions at 30°C for metabolite extraction and GC-MS analysis.

3.2.6 Extraction of metabolites

Radioisotope-labeled metabolic intermediates produced during anaerobic benzene degradation by DN11 was extracted from the benzene-degrading cultures by the use of diethyl ether (Kanto Chemical Co., Inc.) following the method described by Ulrich *et al.* (2005) with modifications. Briefly, three samples were obtained for each sampling point. Each sample was immediately transferred to a separate acid-washed, screw-capped test tubes. The samples were acidified to $\text{pH} \leq 2$ by addition of 2 M HCl. The extraction of metabolites was performed by adding 5 mL diethyl ether to the tubes. The sample was extracted three times with diethyl ether for a total of 15 mL extracts from one sample. The extracts from the three samples were pooled together with the combined total of 45 mL extracts and was dried over anhydrous sodium sulfate. The extracts were concentrated to approximately 1 mL under nitrogen gas stream on ice. The concentrated extracts were subjected to GC-MS analysis to determine the metabolites produced during anaerobic benzene degradation.

3.2.7 Analytical techniques

GC-MS analyses were carried out on an Agilent GC 6890 gas chromatograph (Agilent Technologies, USA) connected to JEOL JMS-K9 mass spectrophotometer (JEOL, USA) with splitless injection. The GC-MS was equipped with Agilent J&W HP-1 column (60 m \times 0.320 mm ID \times 1.00 μm film; Agilent Technologies, USA). Inlet temperature was maintained at 230°C and injections were performed in splitless mode. Helium was used as the carrier gas at a flow rate of 1 mL/min.

For headspace benzene analysis, 50 μL of the headspace was injected into the injection port of the GC-MS with the initial oven temperature of 50°C held for 5 min and then was raised to 200°C at a rate of 10°C/min.

For detection of metabolites, 1 μ L of the concentrated ether extract was injected to the GC-MS with the following oven temperature gradient: 50°C held for 1 min and then was raised to 200°C at a rate of 10°C/min.

HPLC analysis for benzoate concentration was performed using Hitachi L-2000 equipped with Pegasil-octadecyl-silica (ODS) column (4.6 by 250 mm; Senshu Kagaku). The mobile phase used was 2% acetic acid and methanol with program for the gradient system shown on Table 3.1. The flow rate was set to 1 mL/min, the column temperature was kept at 40°C, and the detection was done at 254 nm. The injection volume of the sample was 30 μ L.

Table 3.1 Gradient system for HPLC analysis of benzoate concentration.

Time (min)	2% Acetic acid (% vol)	Methanol (% vol)
0.0	85	15
22.5	60	40
25.0	0	100
40.0	0	100
50.0	85	15

3.3 Results and Discussion

3.3.1 Incubation with [^{13}C] benzene

3.3.1.1 Anaerobic [^{13}C] benzene degradation by *Azoarcus* sp. DN11

The utilization of [^{13}C] benzene as electron donor and nitrate as electron acceptor by DN11 under anoxic conditions was monitored and confirmed using GC-MS. [^{13}C] benzene was degraded by DN11 under anaerobic conditions as shown by the decreasing benzene peak area ratio of DN11 samples to negative control with gradual increase in cell concentration over time (Figure 3.1).

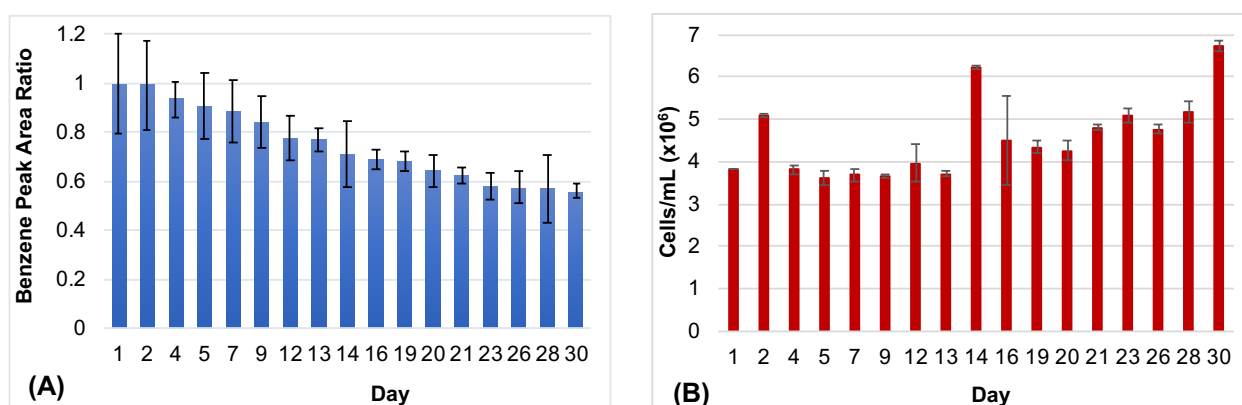


Figure 3.1. Ratio of headspace [^{13}C] benzene peak area of DN11-supplemented samples to those of negative control (A) and cell concentration of DN11 (B) over time.

3.3.1.2 Detection of phenol as intermediate

The GC peak of the authentic [^{13}C] toluene standard was eluted at retention time of 7.38 min using selected ion mode at m/z 98. No [^{13}C] toluene peaks were detected for DN11 samples and negative control at 7.38 min (Figures 3.2 and 3.3, respectively). The results suggest that toluene was not produced as an intermediate in the anaerobic benzene degradation of DN11. Similar results were found for all the DN11 extracts and negative control from different sampling points.

The retention time of the authentic [^{13}C] benzoate standard was detected at 14.13 min using selected ion mode at m/z 128. GC peaks at retention time of 14.23 to 14.28 were detected for both DN11 samples and negative control at m/z 128 (Figures 3.4 and 3.5, respectively). However, the same peaks were detected for both the DN11 samples and negative control (Figures 6 and 7). The

results suggest that benzoate was not produced as an intermediate in the anaerobic benzene degradation of DN11.

[¹³C] phenol GC peaks were detected for both DN11 extracts and negative control as shown by its comparison to the authentic [¹³C] phenol peak eluted at retention time of 11 min using *m/z* 100 (Figures 3.6 and 3.7). However, the phenol peaks for DN11 extracts were much larger in terms of height and peak area than that of the negative control.

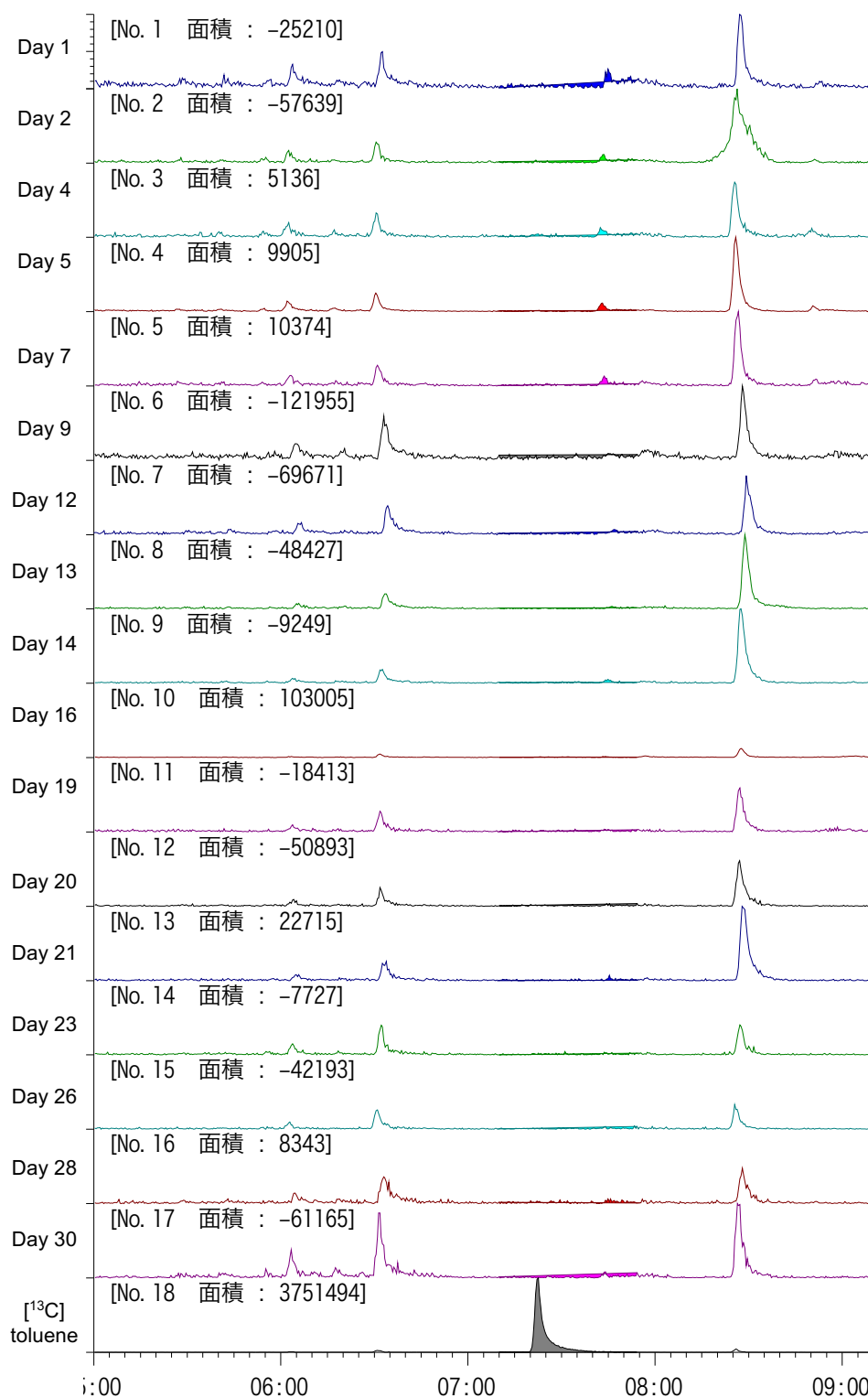


Figure 3.2. GC peak of authentic [¹³C] toluene standard (15 μM) and DN11 extracts at *m/z* 98. The retention time of authentic [¹³C] toluene standard is at 7.63 min.

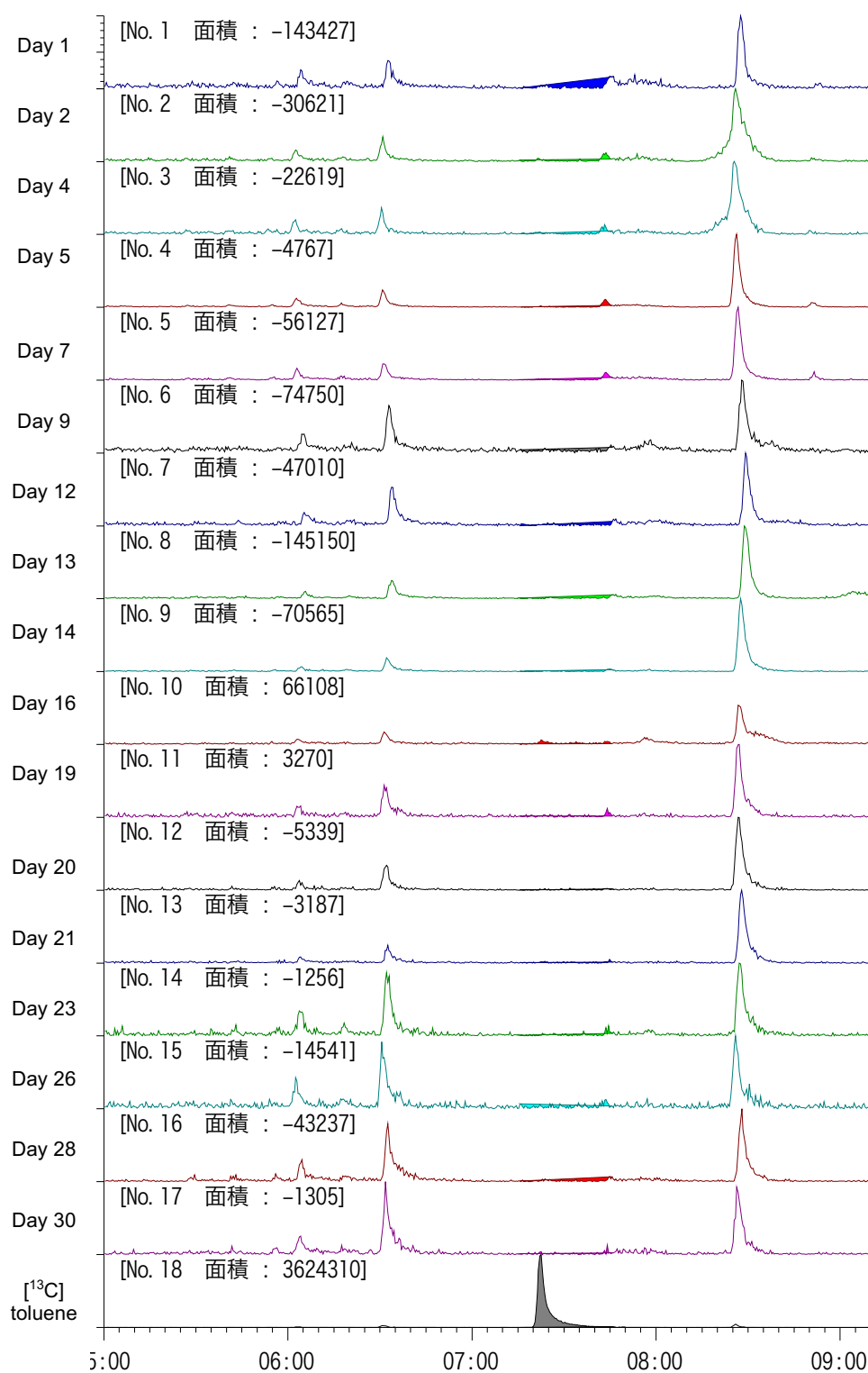


Figure 3.3. GC peak of authentic [¹³C] toluene standard (15 mM) and negative control at m/z 98. The retention time of authentic [¹³C] toluene standard is at 7.63 min.

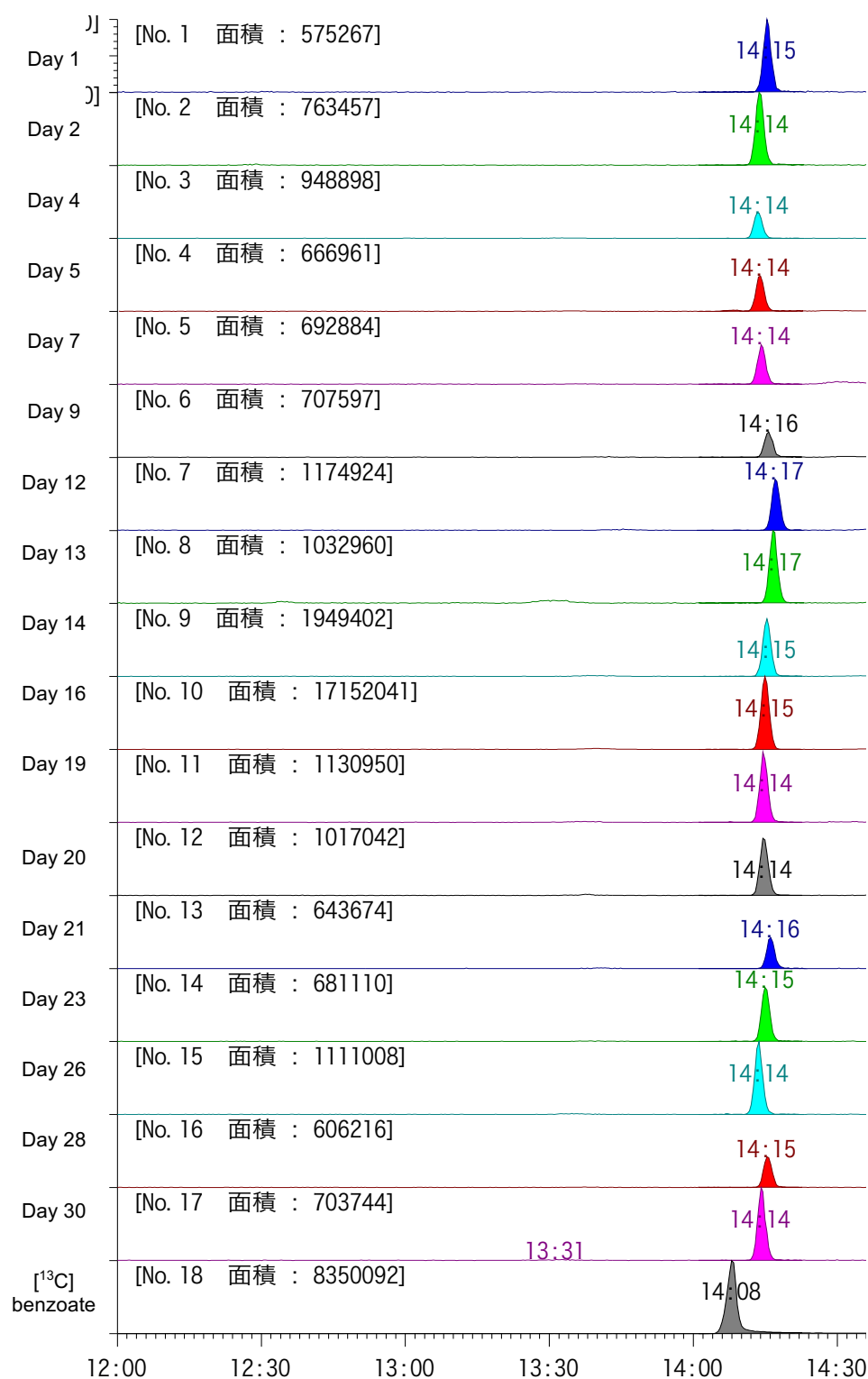


Figure 3.4. GC peak of authentic [¹³C] benzoate standard (50 mM) and DN11 extracts at *m/z* 128. The retention time of authentic [¹³C] benzoate standard is at 14.13 min.

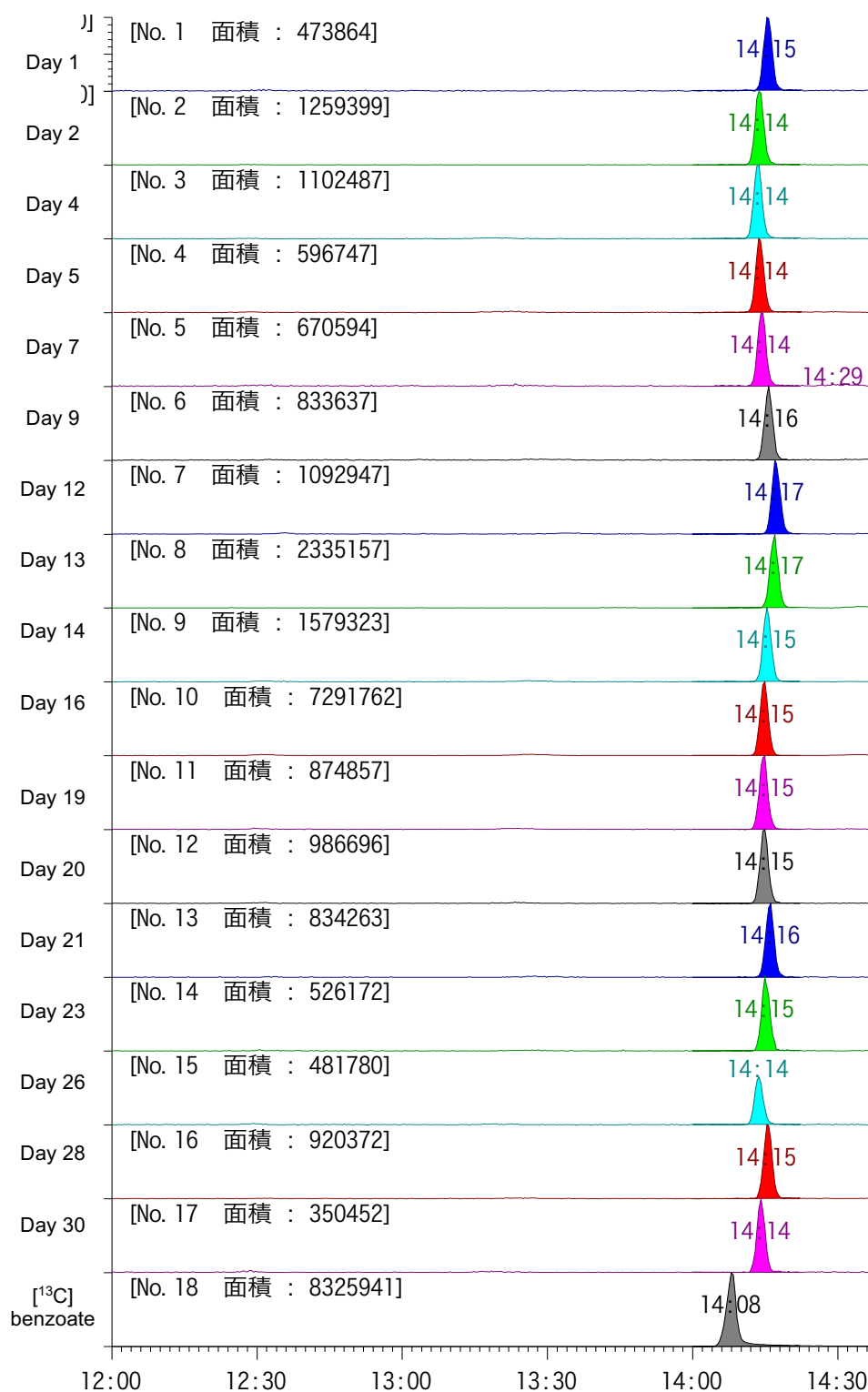


Figure 3.5. GC peak of authentic [¹³C] benzoate standard (50 mM) and negative control at m/z 128. The retention time of authentic [¹³C] benzoate standard is at 14.13 min.

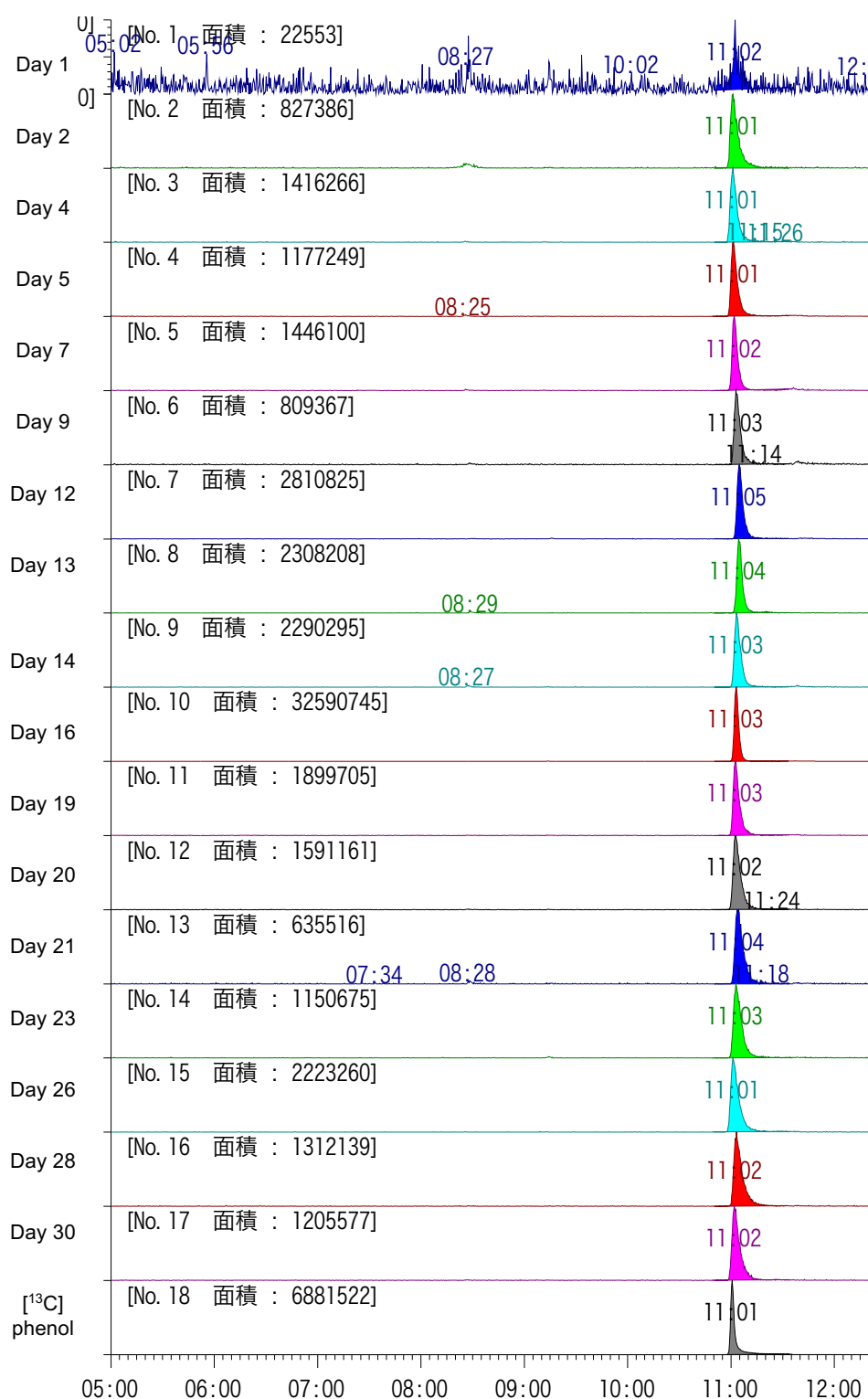


Figure 3.6. GC peak of authentic [¹³C] phenol standard (50 mM) and DN11 extracts at m/z 100. The retention time of authentic [¹³C] phenol standard is at 11 min.

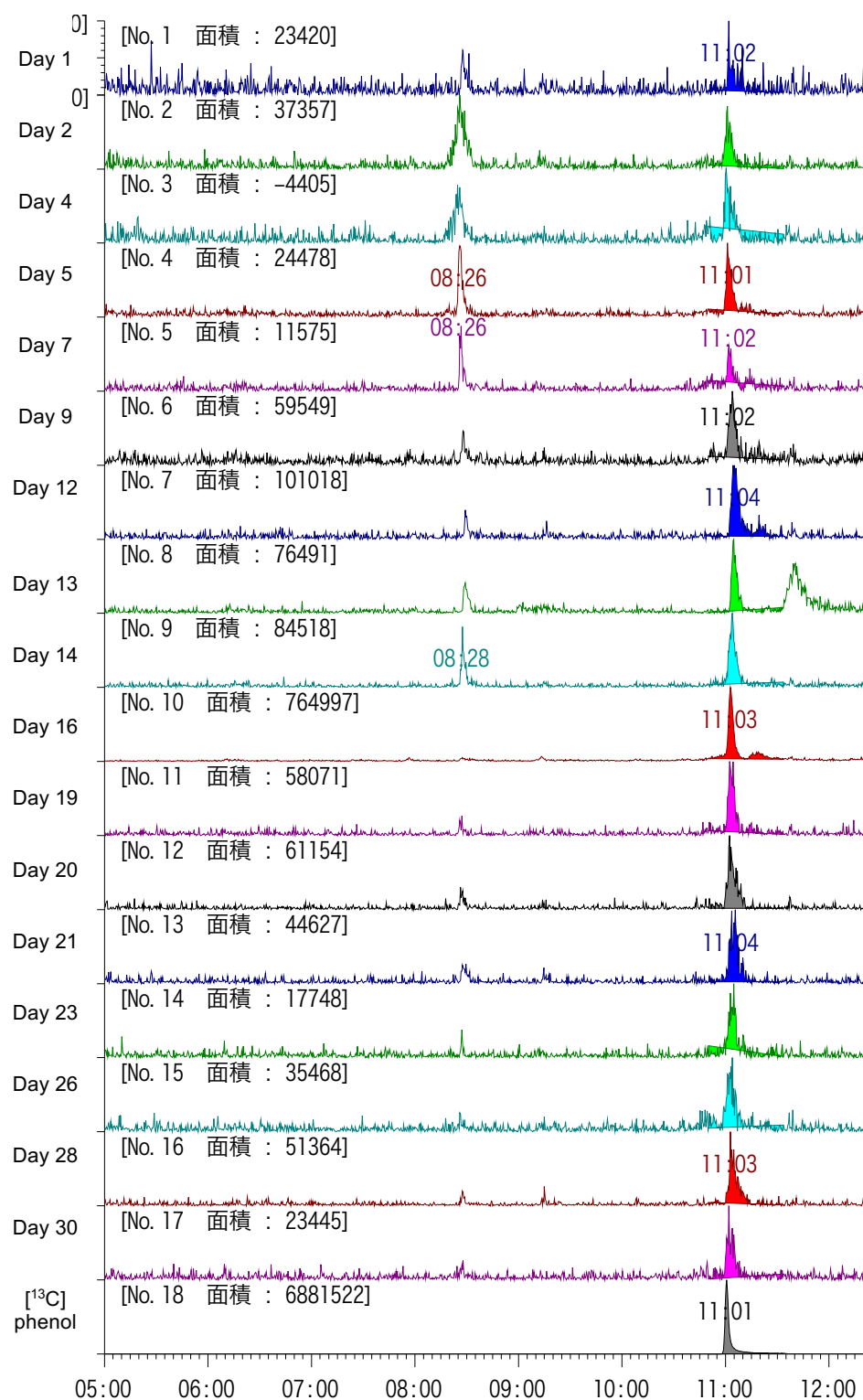


Figure 3.7. GC peak of authentic ^{13}C phenol standard (50 mM) and negative control at m/z 100. The retention time of authentic ^{13}C phenol standard is at 11 min.

The mass spectrum of an authentic [^{13}C] phenol standard consists primarily of fragments at m/z 18, 28, 42, 58, 71 and 100. The mass spectrum at the retention time of [^{13}C] phenol standard for DN11 samples was similar to that of the authentic [^{13}C] phenol standard. On the other hand, the mass spectrum of negative control extracts was different from that of the [^{13}C] phenol standard (Figure 3.8). These data confirm that phenol is a metabolite of anaerobic benzene degradation in DN11 cultures and that the degradation proceeds via the hydroxylation pathway.

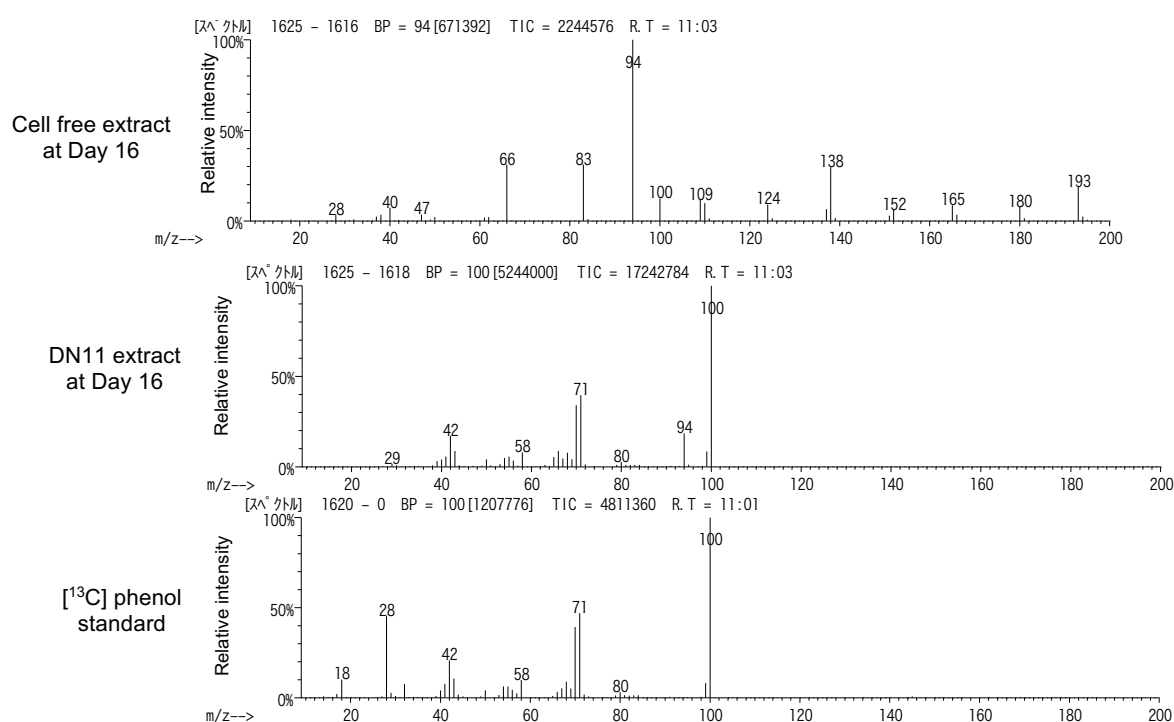


Figure 3.8. Mass spectra of authentic [^{13}C] phenol standard (50 μM), DN11 extract and negative control at their respective retention times using m/z 1100.

3.3.2 Incubation with [^{18}O] 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 unlabeled water and 42.95% ^{18}O -labelled water. The phenol formed in benzene-degrading cultures were extracted using diethyl ether after 14 days of incubation under anaerobic conditions. Figure 3.9 shows that the phenol peaks was detected in benzene-degrading DN11 cultures in unlabeled water and ^{18}O -labelled water as compared with the authentic phenol standard peak at RT 10.58 min.

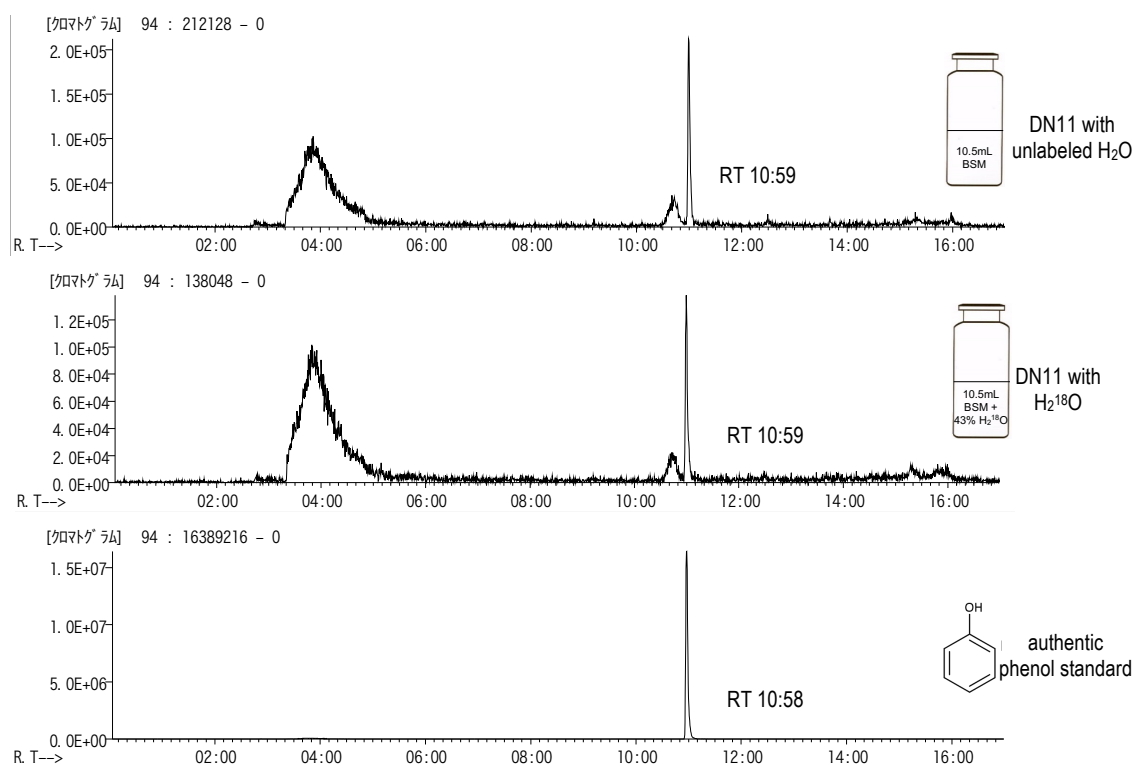


Figure 3.9. GC phenol peak detected in benzene-degrading DN11 culture with unlabeled water and 42.95% ^{18}O -water incubated for 14 days. Authentic phenol standard peak was eluted at RT 10:58.

The mass spectrum of the extract from DN11 sample supplemented with ^{18}O -water did not show a m/z 96 peak which represents the ^{18}O -phenol. On the other hand, the mass spectrum of the phenol peak from DN11 culture in unlabeled water showed no +2 peak (Figure 3.10). These results suggest that water did not take part in the production of phenol from benzene under anaerobic conditions. Hence, the oxygen atoms originated elsewhere.

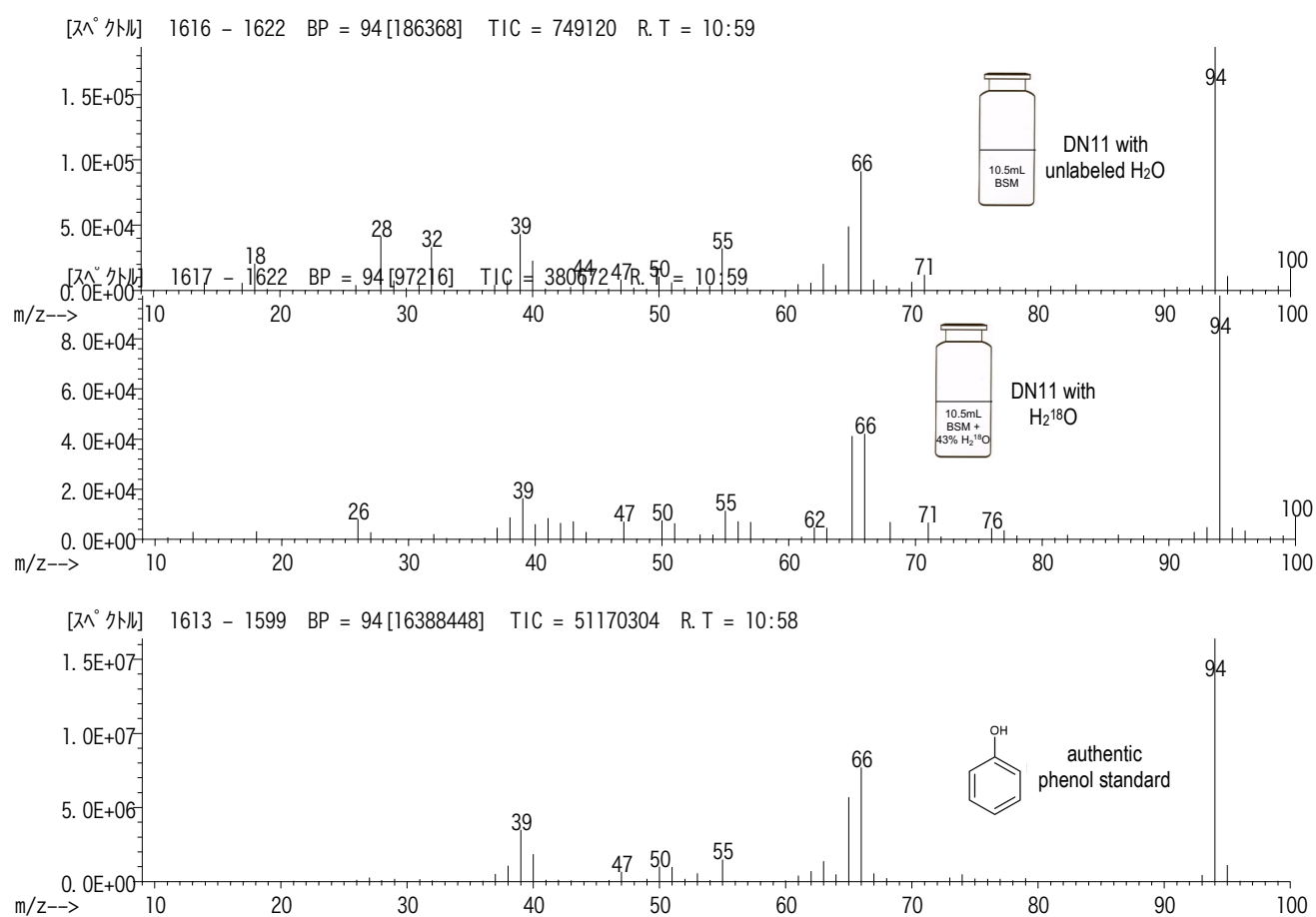


Figure 3.10. Mass spectra of GC phenol peaks of extracts from DN11 cultures in 43% ^{18}O -water and unlabeled water at m/z 94. Mass spectrum of authentic phenol standard was used as a comparison.

3.3.3 Anaerobic benzene degradation in the absence of nitrate

DN11 cells were incubated in anaerobic medium containing 15 μM benzene but without supplementation of nitrate (electron acceptor) as oxygen contamination during benzene degradation was suspected. Figure 3.11 shows the benzene peak area ratio of DN11 to the negative control at different sampling points during anaerobic incubation in nitrate-free medium. Very little change was observed in the benzene peak area ratio through time indicating that little or no degradation occurred in the culture. Moreover, a benzene peak ratio of close to 1 was observed in all the samples from different time points and the values are relatively close to each other. This result suggests that the anaerobic benzene degradation of DN11 was dependent on the presence of nitrate, thus, confirming that the degradation occurred during incubation with benzene was, in fact, under anaerobic conditions.

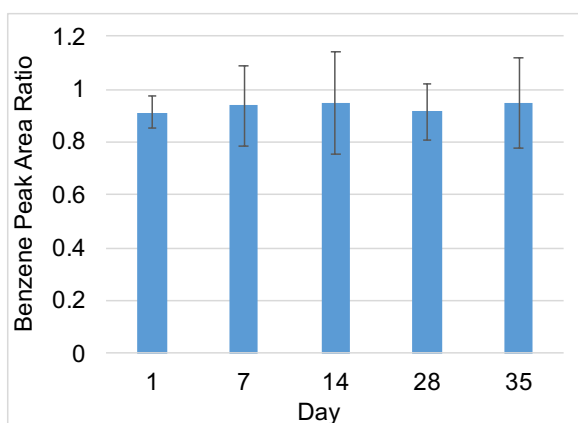


Figure 3.11. Benzene peak area ratio of DN11 to the negative control at different sampling points during anaerobic incubation in nitrate-free medium.

3.3.4 Discussion

In order to analyze the metabolites produced during anaerobic benzene degradation by DN11, GC-MS analysis of ether extracts from benzene-degrading cultures was performed. During anaerobic benzene degradation, samples for ether extraction were taken periodically (Figure 3.1). The metabolic intermediates of benzene biodegradation by DN11 were identified from their molecular mass ions (m/z) and by comparing their mass spectra and retention times with that of authentic standards. The current results suggest that phenol is involved in the anaerobic degradation of benzene by DN11 and that water does not take part in the formation of phenol. Therefore, oxygen atoms incorporated in the production of phenol is derived elsewhere. It has been identified that phenol was produced during transformation of benzene under anaerobic conditions in microbial consortium under various electron acceptors (Botton & Parsons, 2007; Caldwell & Suflita, 2000; Grbic-Galic & Vogel, 1987; Ulrich *et al.*, 2005b; Vogel & Grbic-Galic, 1986). Moreover, the formation of phenol from benzene-degrading pure cultures under anaerobic conditions was shown in nitrate-reducing *Dechloromonas* strain RCB under nitrate-reducing conditions and iron-reducing *Geobacter metallireducens* (Chakraborty and Coates 2005; Zhang *et al.* 2013, respectively). The detection of phenol, but not toluene or benzoate, in benzene-degrading DN11 cultures indicates that the degradation proceed via the hydroxylation pathway.

It has been reported that iron- and sulfate-reducing cultures exhibited abiotic formation of phenol from autoxidation of benzene due to contact with air during sampling and analysis (Abu Laban *et al.*, 2009; Kunapuli *et al.*, 2008). In the current study, extraction of metabolites was also performed in anoxic culture medium without inoculation of DN11. Although small peaks were found to elute at m/z 100 and at a similar retention time with the [^{13}C] phenol standard (Figure 10), these were generally more than 90% smaller in terms of peak area than the [^{13}C] phenol peaks found in samples with DN11 (Figure 9). These peaks were too small to be considered as product of autoxidation of benzene to produce phenol. The mass spectral profile of these peaks did not correspond to that of the [^{13}C] phenol. These results demonstrate that abiotic formation of phenol from benzene does not occur in benzene-degrading, nitrate-reducing DN11 cultures. Moreover, no

other nitrate-reducing cultures have been shown to demonstrate the abiotic production of hydroxylated aromatic compounds from benzene.

GC-MS analysis for the detection of ^{18}O -phenol in benzene-degrading DN11 cultures supplemented with ^{18}O -labelled water was performed to provide evidence on the origin of oxygen in the production of phenol from benzene. Vogel and Grbic-Galic (1986) first reported the incorporation of oxygen from water into benzene under anaerobic conditions. In benzene-degrading *Geobacter metallireducens* cultures supplemented with [^{18}O] water, [^{18}O] labelled phenol was detected suggesting that the oxygen molecule was derived from water (Zhang *et al.*, 2013). Based on the results of the current study, ^{18}O -labelled phenol was not detected in DN11 culture enriched with 42.95% ^{18}O -labelled water which suggests that water was not involved in the formation of phenol from benzene. Due to the possible oxygen contamination, degradation test was performed in nitrate-free culture medium. Little or no degradation was observed in nitrate-free medium suggesting that the anaerobic benzene degradation of DN11 was dependent on the presence of nitrate, thus, confirming that the degradation occurred during incubation with benzene was, in fact, under anaerobic conditions. It has been reported that the adsorption of benzene into the butyl rubber septums occurred during incubation resulting to loss of small amounts of benzene (Chakraborty & Coates, 2005; J. B. Coates *et al.*, 2001; J. D. Coates *et al.*, 1996; Grbic-Galic & Vogel, 1987). Although complete elimination of oxygen from the medium is nearly impossible, the effect of oxygen contamination in the current setup was very minimal.

Similarly, the formed phenol from the anaerobic degradation of benzene in *Dechloromonas* strain RCB cultures supplemented with 50% H_2^{18}O and nitrate as electron acceptor produced only small amounts of ^{18}O -phenol which suggests that water is not the source of hydroxyl group for the formation of phenol (Chakraborty & Coates, 2005). After sequencing, it was found out that *Dechloromonas* strain RCB does not possess genes for degradation of aromatic compounds (Salinero *et al.*, 2009). Therefore, the mechanism for the anaerobic benzene degradation of *Dechloromonas* strain RCB has yet to be clarified. On the contrary, genes encoding enzymes responsible for the anaerobic degradation of toluene, benzoate and phenol were found within the

genome of DN11 (Section 2.3.2.2). The question still arises as to the source of oxygen in the phenol production from benzene by DN11. An intra-aerobic pathway of oxygen production from nitrate reduction by employing the putative nitric oxide dismutase (*nod*) has been shown in *Candidatus M. oxyfera* and γ -proteobacterium strain HdN1 (Ettwig *et al.* 2010, 2012, respectively). This intracellular production of oxygen may explain the absence of ^{18}O -labelled phenol in DN11 cultures with ^{18}O -labelled water. DN11 may employ this pathway producing oxygen under nitrate-reducing conditions to activate oxygenic mechanism of benzene degradation.

Chapter 4

RNA-Sequencing analysis of genes expressed during anaerobic benzene degradation by DN11

4.1 Introduction

Recent technological developments in NGS technologies have contributed to a plethora of studies on gene expression analysis. The new RNA-Sequencing (RNA-Seq) technology has been widely used in recent years for the comprehensive profiling of gene expression and understanding gene regulation and signaling networks. RNA-Seq is low cost and highly reproducible compared to microarray technology. However, bioinformatics software packages available is still improving and no gold standard method has been established for analysis of RNA-Seq data. This chapter reports on the whole transcriptomic analysis of genes involved in the benzene degradation using RNA-Seq. The RNA-Seq reads were mapped to the complete genome sequence of DN11 described in Chapter 2. Profiles of gene expression by DN11 were used to analyze the genes upregulated during benzene degradation and clarify the benzene pathway utilized by DN11.

4.2 Materials and methods

4.2.1 Sample collection

DN11 cells during active degradation of benzene or benzoate under anaerobic conditions were collected. Cells were grown on 15 μ M benzene or 1 mM benzoate as sole carbon source under anaerobic conditions following the incubation method described in Section 3.2.1 – 3.2.4 (but without using labeled compounds). The benzene and benzoate concentrations during incubation were monitored at specific time points to determine the appropriate sampling time for cell collection. Cell samples (with biological and technical replicates) were harvested inside the anaerobic chamber using a single-well vacuum filtration Millipore assembly (Merck Millipore Ltd) consisting of vacuum filtering flask attached to a glass microanalysis filter holder. A sterile hydrophilic polycarbonate membrane filter (0.2 μ m pore size, 25 mm diameter, Isopore, Merck Millipore Ltd) were clamped into the filtration assembly. During active degradation of the carbon source, DN11 liquid cultures were immediately applied to the membrane and were pulled through the filter by the vacuum. The membrane filters were transferred to a 50 mL centrifuge tube and were then washed with 1 mL BSM to dislodge the cells from the membrane into the medium. To stabilize and protect mRNA from degradation, 2 mL RNeasy Protect Bacteria Reagent (Qiagen) was immediately mixed with cell suspension following the protocol recommended by the manufacturer. The reagent was removed by centrifugation for 10 min at 5000 \times g and the resulting RNeasy Protect-treated cell pellet were stored at -80°C prior to RNA extraction. The general overview of the RNA-Seq workflow is shown in Figure 4.1.

4.2.2 RNA isolation and purification

The total RNA from DN11 cells grown on benzene and benzoate under anaerobic conditions were extracted using the RNeasy Mini Kit (Qiagen) per manufacturer's recommendation. To remove contaminating DNA, the extracted RNA samples were treated with RQ1 RNase-free DNase (Promega) following the manufacturer's instructions. The DNase-treated RNA samples

were purified using RNeasy Mini Kit. The RNA quality and quantity were initially assessed using DU 800 Spectrophotometer (Beckman Coulter). For more accurate assessment, the integrity and concentration of each RNA sample were evaluated with Qubit RNA HS assay kit using Qubit RNA HS assay kit (Life Technologies) on Qubit 4.0 Fluorometer and RNA 6000 Nano Kit (Agilent Technologies, Inc) on Agilent 2100 Bioanalyzer, respectively, following the manufacturer's protocol.

4.2.2 Ribosomal RNA (rRNA) depletion

For mRNA isolation, Ribo-Zero rRNA Removal Kit (Illumina) was used to deplete rRNA from the total RNA samples according to manufacturer's instructions. The resulting mRNA sample was resuspended in 18 µL Elute, Prime, Fragment Mix (as supplied in the TruSeq RNA sample prep kit). The integrity and concentration of the purified mRNA samples were evaluated on Qubit 4.0 Fluorometer using Qubit RNA HS assay kit (Life Technologies) and Agilent 2100 Bioanalyzer using RNA 6000 Pico Kit (Agilent Technologies, Inc), respectively, following the manufacturer's protocol.

4.2.3 RNA-Seq library construction and sequencing

The purified mRNA was fragmented and converted to cDNA library with TruSeq RNA sample Prep Kit v2 (Illumina) following the Low Sample Protocol recommended by the vendor. Briefly, the RNA was eluted, fragmented and primed for 8 minutes and the first strand cDNA was prepared using random hexamers and reverse transcriptase. After second strand cDNA synthesis, end repair, addition of single A base, adaptor ligation, DNA fragments were enriched followed by solid-phase PCR amplification to produce the sequencing library. The Kapa Library Quantification kit was used to quantify the size of the library on a qPCR (7300 Real Time PCR system) following the protocol described by the manufacturer. The quality of the library was assessed on Agilent 2100 Bioanalyzer using Agilent DNA High Sensitivity Kit (Agilent Technologies, Inc). Sequencing of pooled cDNA libraries were conducted on MiSeq instrument (Illumina) using MiSeq Reagent Kit v3 (paired-end, 150 cycle) (Illumina) following the manufacturer's protocol.

4.2.4 RNA-Seq data analysis

The RNA-Seq reads were preprocessed and analyzed using the RNA-Seq module of CLC Genomics Workbench (version 7.5.2, Qiagen Bioinformatics). Preprocessing of raw sequencing data was accomplished by quality trimming of poor quality terminal nucleotides and short reads, and removal of RNA adapters using the default parameters. Quality reports were generated after preprocessing to evaluate the preprocessed raw data.

Sequence reads were mapped onto the coding sequences (CDS) of *Azoarcus* sp. DN11 genome using default settings. Mapping reports were generated after the alignment process. RPKM (reads per kilobase per million) values of the putative genes related to benzene degradation from the cells grown on benzene or benzoate were compared to analyze the gene expression levels. All downstream analyses were based on high quality data. Figure 4.2 shows the RNA-Seq analysis workflow used in investigating the anaerobic benzene mechanism of DN11.

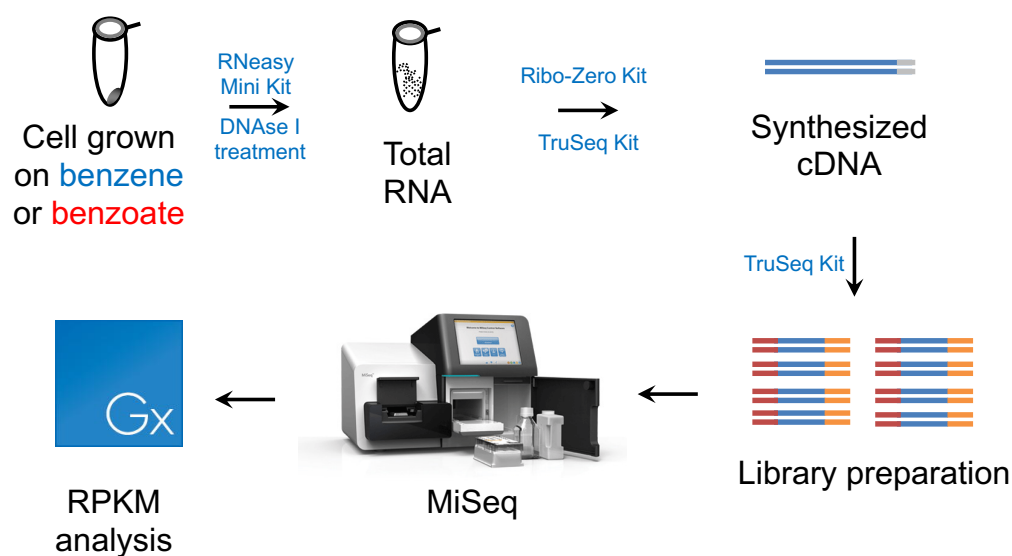


Figure 4.1. Overview of the RNA-Seq workflow used in this study.

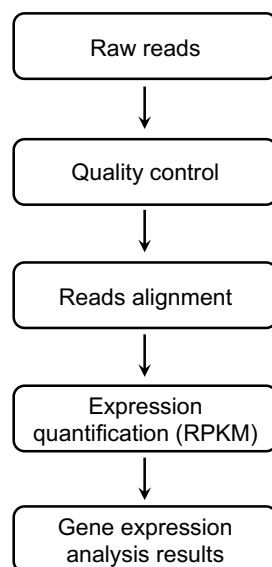


Figure 4.2. RNA-Seq analysis workflow. CLC Genomics Workbench was used as the software for bioinformatics analysis.

4.3 Results and discussion

4.3.1 Sequencing results

The sequencing and mapping results of RNA-Seq data are summarized in Table 4.1. Overall, the alignment mapping percentage of all the samples were greater than 85% indicating a good alignment rate. A global comparison of the calculated RPKM of the genes between biological replicates showed a high correlation of samples grown on benzene and benzoate indicating that the results are reproducible (Pearson correlation coefficient of $r=0.98$ and $r=0.99$, respectively) (Figure 4.3). These findings attest to the fine quality of the sequencing results. Hence, the transcriptome data were deemed suitable for subsequent downstream analysis.

Table 4.1. Sequencing and mapping results of RNA-Seq of benzene- and benzoate-grown cells. The number in the library name indicates technical replicate number.

Substrate	Biological Replicate	Library Name	Total Number of Raw Reads	Mapped reads	% Alignment to DN11 genome sequence
Benzene	1	B1	5,429,490	4,628,234	85.24
		B2	5,207,298	4,572,960	87.82
	2	B3	6,732,042	6,075,066	90.24
		B4	3,888,666	3,714,472	95.52
Benzoate	1	T1	4,446,212	4,073,502	91.62
		T2	2,378,910	2,027,340	85.22
	2	T3	4,376,694	3,875,014	88.54
		T4	5,066,728	4,442,332	87.68

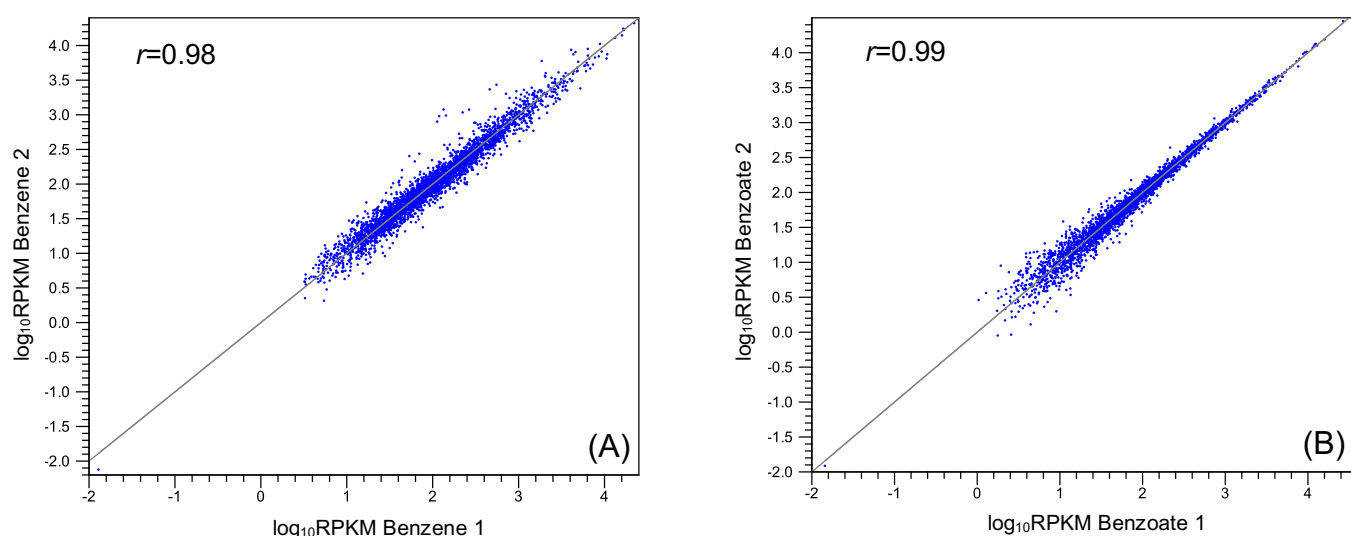


Figure 4.3. Scatter plots of log₁₀RPKM values of the genes between biological replicates within each benzene- and benzoate-grown cells libraries showing the Pearson's correlation coefficient. (A) benzene-grown cells library. (B) Benzoate-grown cells library. Each dot represents the RPKM value of a specific gene.

4.3.2 Expression of genes associated with anaerobic benzene degradation pathways

The whole transcriptome analysis using RNA-Seq revealed that the genes in the putative phenol pathway of DN11 showed higher RPKM values in benzene-degrading cultures versus their RPKM in benzoate-degrading cultures (Figure 4.4). Although some of the genes involved in the putative toluene pathway showed higher RPKM in benzene- than benzoate-grown cells (Figure 4.5), the RPKM values were not as high as those of the genes in the phenol pathway. As mentioned above, the putative genes for initial benzoate degradation were also not significantly expressed in benzene cultures when compared to benzoate cultures (Figure 4.6). These RNA-Seq analysis results together with the GC-MS detection of phenol as intermediate in benzene-degrading cultures (Chapter 3 Figure 3.6) provide evidence that the phenol hydroxylation pathway is important for anaerobic benzene degradation of DN11.

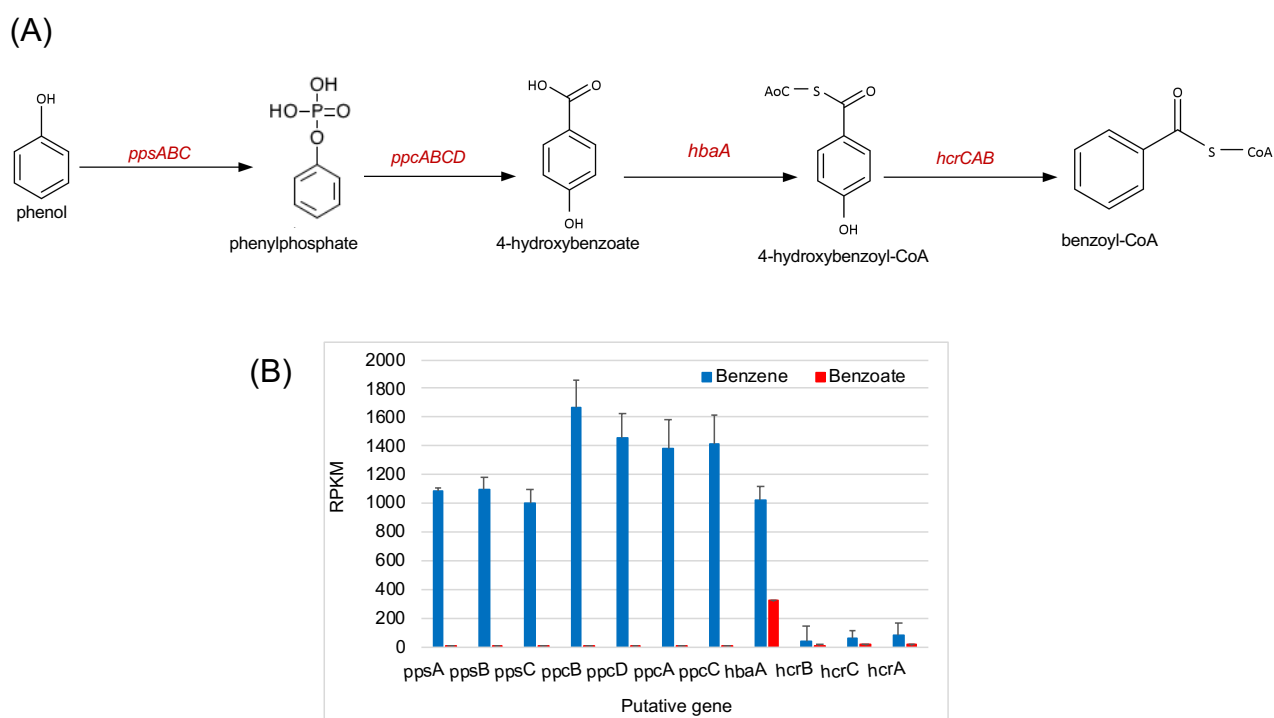


Figure 4.4. The genes in the putative anaerobic phenol pathway of DN11 (A) and its RPKM values (B) in DN11 cells grown on benzene and benzoate.

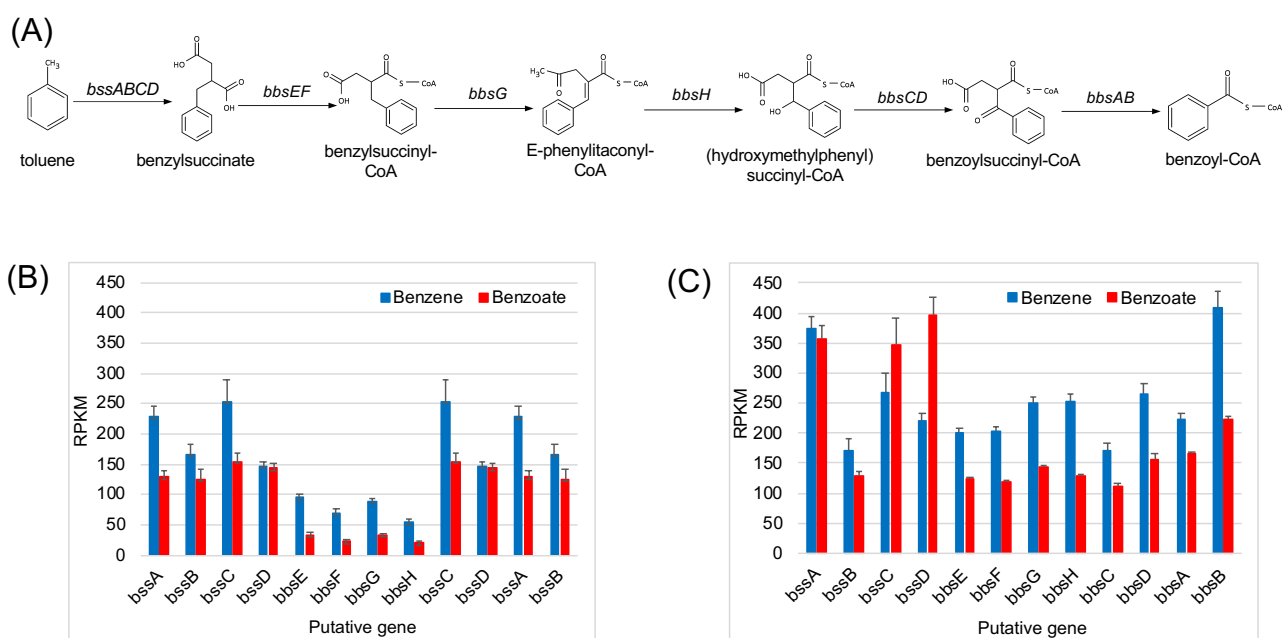


Figure 4.5. The putative genes in the anaerobic toluene pathways of DN11 (A) and their RPKM values (B: 1st gene cluster; C: 2nd gene cluster) in DN11 cells grown on benzene and benzoate. Two putative gene clusters for anaerobic toluene pathway were found in DN11.

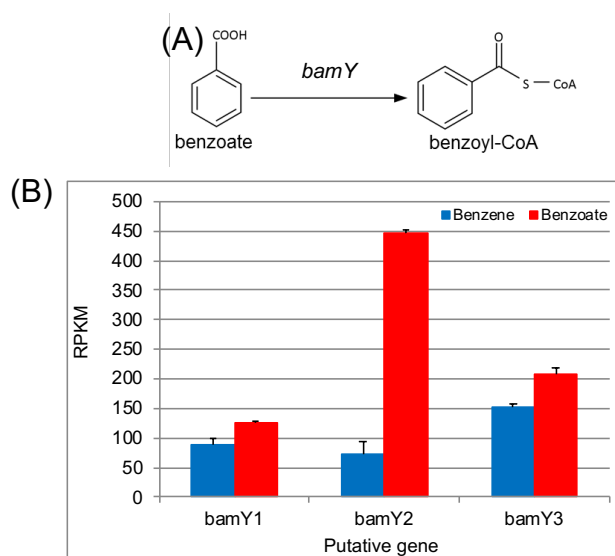


Figure 4.6. The putative genes in the putative anaerobic benzoate pathway of DN11 (A) and their RPKM values (B) in DN11 cells grown on benzene and benzoate. Three putative genes for anaerobic benzoate pathway were found in DN11.

4.3.3 Expression of genes associated with aerobic benzene degradation pathways

Although anaerobic conditions and manipulations were employed during experiments on the benzene degradation of DN11, higher expression of putative benzene monooxygenase genes (*dmpKLMNOP* encoding for phenol hydroxylase and *tmoABCDEF* encoding for toluene monooxygenase) in the putative monooxygenase benzene pathways were observed in benzene-degrading cultures than in benzoate-degrading cultures (Figure 4.7). The expression levels of benzene dioxygenase genes (*bedC1C2BA* encoding for benzene 1,2-dioxygenase and *bnzE* encoding for cis-1,2-dihydrobenzene-1,2-diol dehydrogenase) (Figure 4.8) were higher in benzene-degrading cultures than in benzoate-degrading cultures. However, the expression of benzene monooxygenase genes was much higher than that of the benzene dioxygenase genes which suggest that benzene dioxygenases have little participation in the benzene degradation by DN11. It is also suggested that benzene dioxygenase was not the main enzyme used by DN11 to degrade benzene which could be due to the wide range specificity of benzene dioxygenase to other aromatic compounds (Geary *et al.*, 1990).

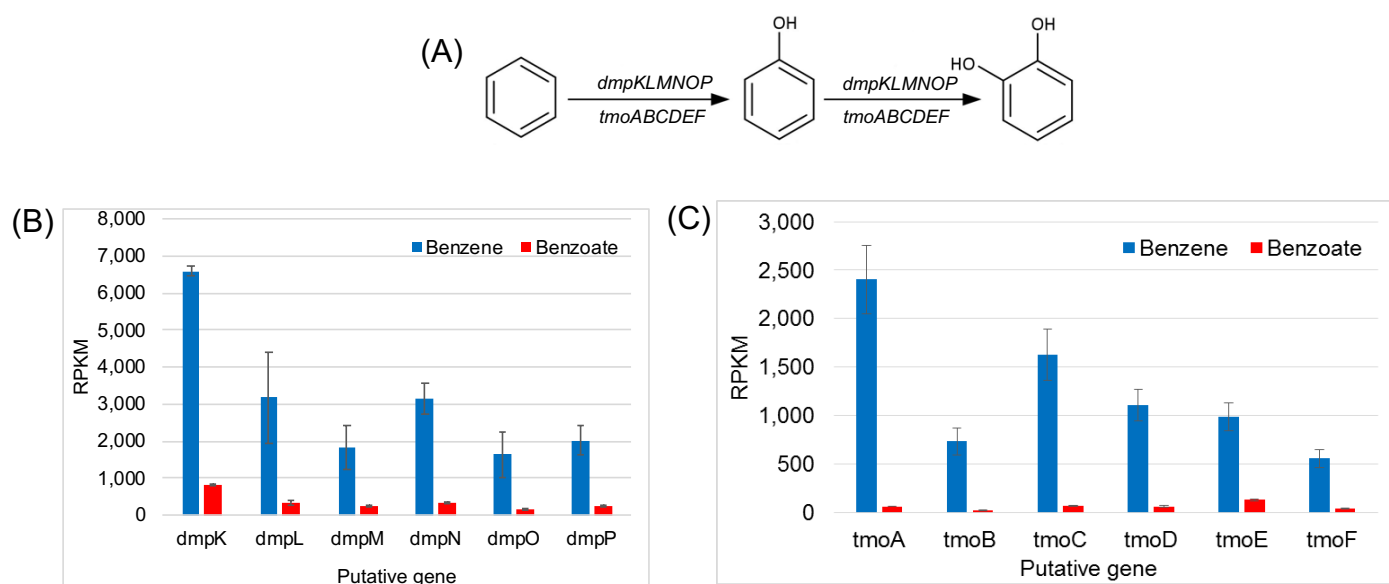


Figure 4.7. The putative genes in the putative monooxygenase benzene pathway of DN11 (A) and their RPKM values (B: gene *dmpKLMNOP* encoding for phenol hydroxylase; C: *tmoABCDEF* encoding for toluene monooxygenase) in DN11 cells grown on benzene and benzoate.

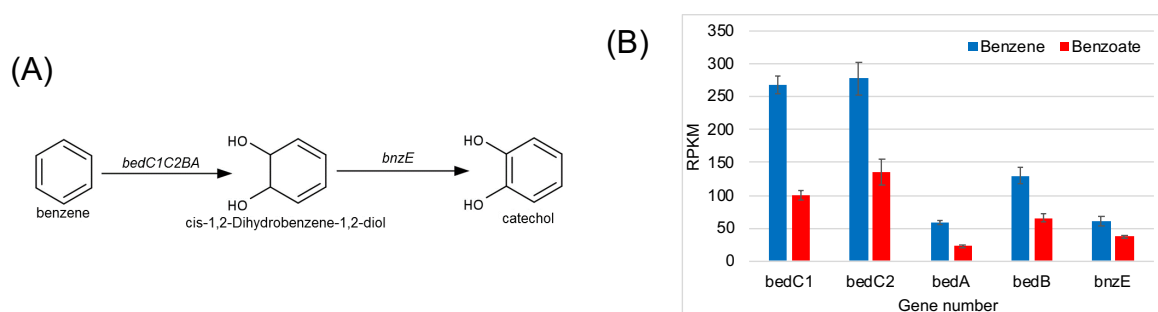


Figure 4.8. The putative genes in the benzene dioxygenase pathway of DN11 (A) and their RPKM values in DN11 cells grown on benzene and benzoate.

4.3.4 Expression of genes associated with nitrate metabolism

The expression of denitrification genes (nitrate, nitrite, nitric oxide and nitrous oxide reductase genes) was detected in both DN11 cells grown in benzene or benzoate (Figure 4.9). The expression of the nitrate reductase genes (*narGHI*) was notably higher among the other genes in benzene-degrading cultures than benzoate-degrading cultures.

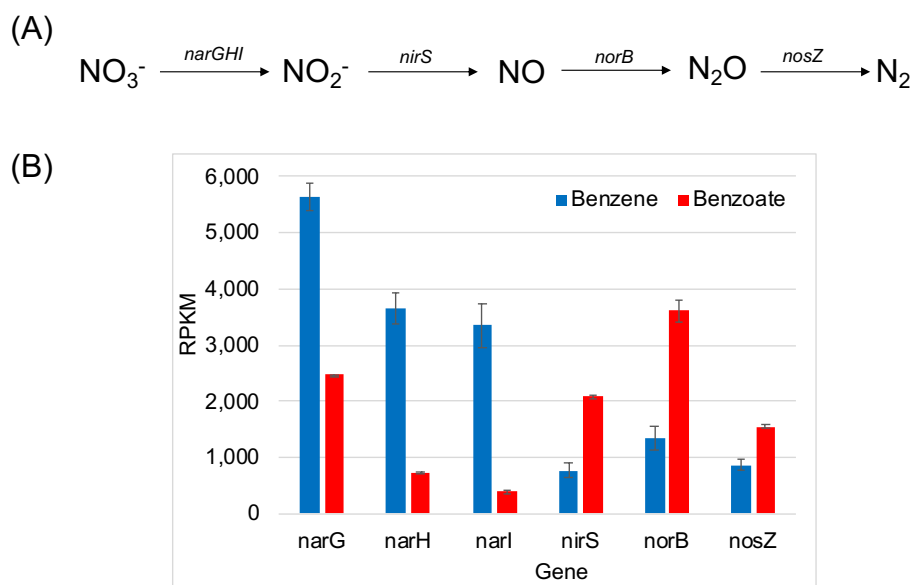


Figure 4.9. The putative genes in the denitrification pathway of DN11 (A) and their RPKM in DN11 cells grown on benzene and benzoate.

4.3.5 Discussion

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. 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 aerobic benzene and anaerobic phenol degradative genes. The genes in the putative phenol pathway of DN11 showed higher RPKM values in benzene-degrading cultures versus their RPKM in benzoate-degrading cultures (Figure 4.4). Although anaerobic conditions and manipulations were employed during experiments on the benzene degradation of DN11 and incubations were performed under nitrate-reducing conditions, higher expression of putative benzene monooxygenase genes (*dmpKLMNOP* encoding for phenol hydroxylase and *tmoABCDEF* encoding for toluene monooxygenase) (Figure 4.7). The higher expression of monooxygenase genes than that of the dioxygenase gene suggest that monooxygenase reaction system was the main enzymes used by DN11 to degrade benzene. Geary *et al.* (1990) suggested that benzene dioxygenase was not the main enzyme used by DN11 to degrade benzene which could be due to the wide range specificity of benzene dioxygenase to other aromatic compounds.

A similar finding has been reported from the transcriptome of a benzene-degrading nitrate-reducing continuous culture by Atashgahi *et al.* (2018) where benzene monooxygenase genes and a putative anaerobic benzene carboxylase genes were highly transcribed during benzene degradation. Oxygen formation from nitrate was detected in the culture during anaerobic benzene degradation. It was suggested intracellular oxygen was produced by dismutation of nitrate to form oxygen and nitrogen molecules catalyzed by the putative nitric oxide dismutase (Nod) enzyme. The oxygen produced is then used to activate the oxidation pathways involving oxygenases. This “intra-aerobic”

pathway of oxygen production from nitrite (a known nitrate reduction intermediate) was first described in methane-oxidizing *Candidatus M. oxyfera* and alkane-degrading γ -proteobacterium strain HdN1 by employing nitric oxide dismutase (Nod) (Ettwig *et al.* 2010; 2012, respectively). In a similar fashion, the “intra-aerobic” pathway for benzene degradation was observed in chlorate-reducing *Alicyclophilus denitrificans* (Weelink *et al.*, 2008). Oxygen was produced by from chlorate which was catalyzed by chlorite dismutase (Cld). It was also suggested that *Dechloromonas* strain RCB genome may encode genes for the putative nitric oxide dismutase because the strain was capable of anaerobic benzene degradation even without possessing the known putative genes for the anaerobic benzene degradation

It is hypothesized that DN11 employs similar mechanism to activate benzene monooxygenase in degrading benzene under anoxic conditions. The phenol produced from the aerobic degradation of benzene activates the anaerobic phenol degradation pathway (Figure 4.10). The question still arises as to the source of oxygen in the phenol production from benzene by DN11. Although no direct experimental data was obtained for the oxygen production from nitrate in benzene-degrading DN11 cells, evidences from the isotope labeling experiment and transcriptomic data agree that oxygen activated the aerobic benzene pathways of DN11 during benzene degradation under denitrifying conditions. Notably, the anaerobic benzene degradation did not occur when DN11 was incubated in nitrate-free medium suggesting that nitrate was required in order for the degradation to proceed (Chapter 3 Figure 3.11). This also confirmed that the degradation occurred during incubation with benzene was, in fact, under anaerobic conditions and that the effect of oxygen contamination was very minimal.

Similar to the continuous culture studied by Atashgahi *et al.* (2018), no homologous nitric oxide dismutase genes were found in the genome of DN11. The full characterization of the nitric oxide dismutase has not yet been done which makes it difficult for the identification of homologous genes in the genome of DN11. Furthermore, the actual enzymatic function of Nod has not yet been conducted in detail. Nevertheless, a recent study has shown the abundant distribution of putative *nod* gene was found in wastewater treatment plants, hydrocarbon-contaminated sites and oil

reservoirs suggesting that nitric oxide dismutation could occur in these ecosystems (Hu *et al.*, 2019). Furthermore, the expression of denitrification genes was observed in DN11 cells during anaerobic benzene degradation with nitrate reductase genes (*narGHI*) showing the highest transcription values (Figure 4.9). This suggests a yet unknown or novel pathway for the production of oxygen from nitrate during anaerobic benzene degradation of DN11. Detection of [¹⁸O]-labeled phenol in DN11 cells incubated with [¹⁸O]-labeled nitrate or nitrite would provide experimental data that supports the production of oxygen from nitrate or nitrite during benzene degradation under denitrifying conditions. Acquisition of experimental evidence to support the oxygen production from nitrate under anoxic conditions in DN11 would provide insights into the anaerobic benzene degradation pathway which is an important process in bioremediation in wastewater-treatment sites. A better understanding of the anaerobic degradation mechanism would aid in the design and optimization of bioremediation strategies for removal of various environmental contaminants.

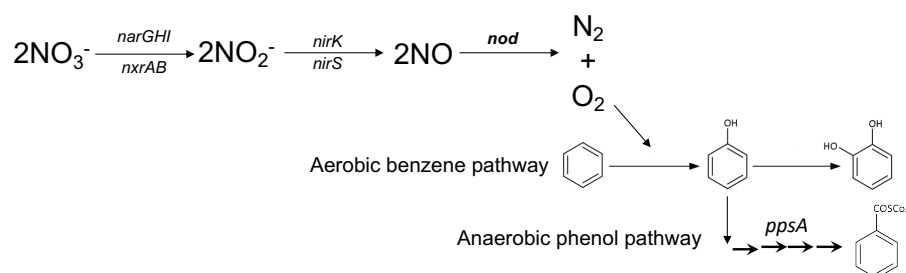


Figure 4.10. Anaerobic benzene degradation pathway involving production of oxygen from nitrate. Oxygen produced from nitrate activates the oxygenic degradation pathways of benzene. The phenol produced as an intermediate of the aerobic benzene pathway is degraded simultaneously through the anaerobic phenol degradation pathway.

Chapter 5

Summary and future prospects

The data presented in this study provide insights into the possible anaerobic benzene degradation mechanism of *Azoarcus* sp. DN11.

The whole genome sequence of *Azoarcus* sp. DN11 was successfully completed and the ability of its to degrade benzene and other aromatic compounds under both aerobic and anaerobic conditions was predicted through genome-wide analysis. Putative genes in the benzene monooxygenase and dioxygenase pathways as well putative genes in the anaerobic phenol, toluene and benzoate were identified in the DN11 genome. These results also highlight the great potential of DN11 for application in bioremediation.

The detection of [^{13}C]-labeled phenol as metabolite in the anaerobic benzene degradation of DN11 as shown by the results on the isotope labelling-based analysis suggest that the hydroxylation reaction was involved in the degradation of benzene under anoxic conditions. However, benzene-degrading DN11 cultures incubated with [^{18}O]-labeled water did not produce [^{13}C]-labeled phenol suggesting that the oxygen incorporated into benzene was not derived from water.

The whole transcriptomic analysis of benzene-degrading nitrate-reducing DN11 cultures has revealed the simultaneous expression of aerobic benzene and anaerobic phenol degradative genes even under strict anaerobic conditions. An intra-aerobic denitrification pathway facilitated by an uncharacterized nitric oxide dismutase to produce oxygen from nitrite has been proposed recently (Ettwig *et al.*, 2010; 2012). DN11 may have employed this pathway to produce oxygen from nitrate which, in turn, activated the oxygenic pathways of benzene degradation. Incubations with [^{18}O]-labelled nitrate and subsequent detection of with [^{18}O]-labelled phenol will support the evidence on the production of oxygen from nitrate during anaerobic benzene degradation by DN11. I have recently optimized the nitrite concentration for the anaerobic benzene degradation by DN11 under nitrite-reducing conditions. In the future, [^{18}O]-labelled nitrate will be used to confirm if DN11 can

produce oxygen from nitrite which can activate the aerobic benzene degradation pathway under anaerobic conditions.

There is a need for more studies regarding the characterization of the putative nitric oxide dismutase to clearly describe its function and confirm the novel mechanism of oxygen production in oxygen-deficient environments. This discovery of novel genes involved in anaerobic benzene degradation would serve a great biochemical novelty in the bioremediation of benzene. Deletion of genes that were highly expressed during anaerobic benzene degradation in DN11 will provide additional evidence to support the possible activation of aerobic benzene degradation pathways under nitrate-reducing conditions. The underlying genetics and biochemistry of biodegradation process is needed before application of the bacteria on the contaminated sites. Finally, the bioremediation potential of DN11 will be evaluated in a large-scale setup before actual application to contaminated sites. Experiments will be performed under aerobic conditions to determine the optimum conditions for preculturing DN11 cells before application to the actual sites. Different media composition and concentration will be tested for the most efficient degradation of benzene by DN11.

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Acknowledgments

First of all, I would like to thank my sensei, **Dr. Hideaki Nojiri**, for accepting me as a student in his laboratory and for his knowledge and guidance all throughout my research. I thank **Dr. Chiho Suzuki-Minakuchi** for her hands-on guidance on my experiments and all the experimental techniques she has imparted. I also have also received useful suggestions and valuable advices during my progress reports from **Dr. Kazunori Okada**. This research would not be possible if it weren't for these brilliant professors that I was lucky enough to work with.

I would also like to give appreciation to all the **previous and present members of the Laboratory of Environmental Biochemistry** for making my daily laboratory life smooth and worthwhile.

Lastly, I am forever grateful for my family for the support and unconditional love. None of this research could have been completed without help of these people.