Doctoral Dissertation

Characterization of dichlorobenzenes degrading consortia obtained from a contaminated site

（汚染現場から得たジクロロベンゼン分解コンソーシアムの解析）

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Abstract

Chlorinated benzenes (CBs) are important industrial intermediates and solvents (Jim A.Field., 2008). CBs are used as solvents, fumigants, and intermediates in the production of pesticides. They have caused an increasing concern over the fate and persistence of such halogenated compounds in the environment. Their widespread use has resulted in broad distribution of these compounds in the environment. U.S. Environmental Protection Agency reported contaminant level of 0.6 mg l−1 for 1,2-dichlorobenzene (1,2-DCB or O-DCB) in drinking water. However, the widespread use of CBs cause serious health hazard to human being and pollution problems to the environment, therefore, the research about contamination by CBs has become a new issue. And bacteria that are able to use these compounds as sole source of carbon and energy have been isolated from polluted environments (Sander *et al.*, 1991; Spiess *et al.*, 1995). Bioremediation refers to the use of microorganisms to degrade contaminants that pose environmental and human risks. Bioremediation processes typically involve the actions of many different microbes acting in parallel or sequence to complete the degradation process (Sylvia *et al*., 2005).

Previous studies have shown that the degrading bacteria added into the soil usually failed in competing with the indigenous microorganisms (JG Streeter, 1994). Therefore, it is necessary to find degrading microorganisms that can survive the competition with the indigenous microorganisms. The aim of this study was to find microorganisms that perform ability to degrade dichlorobenzenes (DCBs) in actual contaminated soil and ground water. Stable consortia were created by enrichment culture from the actual contaminated sites contaminated by original chlorinated organic compound including benzene, 1,2-DCB and 1,4-dichlorobenzene (1,4-DCB or P-DCB). The enrichment culture's environment will support the growth of a

selected microorganism, while inhibiting the growth of others. Enrichment may be a good way to select the competitive microorganisms which could degrade DCB. Here four bacterial consortia (consortium 1, 2, 3, 4) which were enriched with 40 mg/l of 1,2-DCB and two consortia which were enriched with 40 mg/l 1,3-DCB (1,3-DCB or M-DCB) and 1,4-DCB were evaluated. Each of the combination of 1,2-DCB, 1,3-DCB and 1,4-DCB was added as sole carbon and energy source in the enrichment cultures.

In the consortia, some evidence of dominant bacteria existing in consortia have been confirmed by DGGE and 16S rRNA gene cloning library results. Normally, the dominant bacteria which could be competitive after many generations of enrichment are possibly substrate degraders (Massol Deya *et al*., 1977; Springael *et al*., 2002). So, there is high possibility that the dominant stable coexisting bacteria after many generations of enrichment in consortium 1, 2, 3, and 4 could possibly be dominant 1,2-DCB degraders. And bacteria belonged to *Acidovorax* sp. was dominant at 9^{th} generation but disappeared after 15^{th} generation of 1,2-DCB degrading consortium 3 and 4 possibly also possessed the 1,2-DCB degradation ability. It presents always two genera of strains coexist even after many generations of each 1,2-DCB degrading consortium by cloning library method. DCB(s) degraders were successfully isolated from consortia. They were close to *Acidovorax soli* BL21, *Acidovorax defluvii* BSB411, *Ralstonia pickettii* 12J, *Variovorax soli* NBRC106424 and *Pandoraea thiooxydans* ATSB16, respectively, which was the first research to report that bacteria belong to *Variovorax* sp. and *Pandoraea* sp. possess the DCBs degradation ability. Based on the degradation characterizations results, this is the first research to report that bacteria could degrade maximum of 1,2-DCB around 160 mg/l. Specific growth rates of isolates were calculated to indicate that DCB inhibited cells growth because of the decrease of specific growth rate under high concentrations. *Acidovorax* sp. sk40 and *Acidovorax* sp. could degrade CB and 1,2-DCB. *Variovorax* sp. and *Pandoraea* sp. could degrade CB, 1,2-DCB, 1,3-DCB, and 1,4-DCB. *Ralstonia* sp. sk41 and *Ralstonia* sp. could degrade CB, 1,2-DCB, and 1,4-DCB. DCBs degrading strains released stoichiometric chloride ion in DCB degradation process.

It is interesting to find that always two stable bacteria coexisted in consortium 1, 2, and 3. By

analysis of specific growth rates of these bacteria, it indicated that the specific growth rates of strain sk40 and sk41 were the same at 40 mg/l, so that both bacteria could coexist in the consortium. However, if the concentration was changed, the balance was destroyed. the specific growth rates of strains in stable consortium 2, 3 and 4 were also investigated. It showed that both strains possess the same specific growth rate at each concentration of 1,2-DCB and it may be a good evidence on the reason for coexistence of these two bacteria in consortium 2. Strains possess the different specific growth rate and the specific growth rate at 40 mg/l of 1,2-DCB. This may be a good reason for the disappearance of strain after many generations of enrichment. These coexisting results were also confirmed by mathematics calculation based on cells growth models. These results provide a good understanding of the stable coexistence exhibited by the two 1,2-DCB degrading bacteria in consortia.

Aromatic-ring-hydroxylating dioxygenase (ARHDO) gene core region of α-subunit was discovered from isolates. ARHDOs are key enzymes in the aerobic bacterial metabolism of aromatic compounds. ARHDOs catalyze the first step in the metabolism of aromatic hydrocarbons under aerobic conditions by incorporation of molecular oxygen (Paul M. Taylor *et al*., 2002). The prototype catalytic reaction is to add two hydroxyl groups to vicinal carbons, thereby destroying the aromatic system and yielding dihydrodiol compounds of cis stereochemistry (Butler *et al*., 1997; Boyd and Sheldrake, 1998). ARHDOs are so important that the function in the degradation of aromatic pollutants (Silke Kahl and Bernd Hofer, 2003) which describes the development and validation of a system for the rapid isolation and characterization of specific ARHDO activities. Isolated strains possess the DCB(s) degradation ability. Each isolated strain have functional DCB dioxygenases enzyme system which were separated to two groups. The functional gene found from strains were close to *tecA* gene which has been reported that found from chlorobenzene degrading bacteria and *bphA* gene which was found from biphenol degrading bacteria. And this is the first report that bacteria possess *bphA* gene also can degrade DCB. *tecA* and *bphA* may be taken from outside in bacterial evolution. Not only α-subunit determine the degradation of DCB isomers. Or the substrate transporters on plasmids determine the DCB degradation ability by different isolates.

The other DCB isomers degrading consortium (1,3-DCB degrading consortium and 1,4-DCB degrading consortium), and the other isolations and genetic experiment were also carried out in this study. To date, there have been no reports regarding the mechanism governing stable coexistence, as stable substrate-degrading enrichments are considered complex cultures affected by complex factors (Haruta et al. 2002). The mechanism of stable coexistence of these two degraders will be our future study. Moreover, to the best of our knowledge, this is the first report to construct the stable enrichment coexisted with two degrading strains during the degradation of 1,2-DCB. The isolated strains which possess robust 1,2-DCB-degrading activity, show good potential for future use in bioremediation.

Chapter 1

General introduction

Soil and groundwater contamination by hazardous organic (carbon-based) chemicals of industrial origin was identified in the US as a serious problem beginning in the 1970s. Small amounts of organic chlorinated liquid solvents in particular can cause extensive contaminate to sub-surface drinking water resources because they are considered toxic at very low concentrations (Janick F. Artiola *et al.*, 2006). Halogenated aromatic compounds constitute one of the largest groups of environmental pollutants as a result of their widespread use. Chlorobenzenes (CBs) are extensively used in chemical synthesis of pesticides, pharmaceuticals and dyes (M. Ziagova *et al.*, 2007). They represent a group of chemicals that are widely used as industrial solvents and degreasers, and are commonly encountered in the subsurface near industrial areas where they have been manufactured or used (Dominguez, Rosa F., *et al.*, 2008). Their widespread use has resulted in broad distribution of these compounds in the environment. The ubiquitous distribution of chlorinated aromatic compounds in the biosphere has caused public concern over the possible effects on the quality of life. CBs are lipophilic compounds, chemically stable in nature, resistant to photodegradation and can cause liver and renal cancer in mammals (Banni, Sebastiano, *et al.*, 1999). Over 90% of contaminated sites identified and listed by US Environmental Protection Agency (USEPA) contain toxic chlorinated solvents like trichloroethenes, benzene, and dichlorobenzenes. Fig. 1-1 shows the image of the CBs contaminated soil and ground water site.

CBs include monochlorobenzene (MCB), dichlorobenzene (DCB) isomers (1,2-DCB; 1,3-DCB; and 1,4-DCB), TCBs, TeCBs, PCB, and HCB. These compounds have low USA federal drinking water standards (i.e., maximum contaminant levels [MCLs] ranging from 0.075 mg/l for 1,4-DCB to 0.6 mg l/l for 1,2-DCB) and relatively high water solubility, ranging from 75 mg/l for 1,4-DCB to 500 mg/l for MCB (Dominguez, Rosa F., *et al.*, 2008). Among these toxic chlorinated solvents, dichlorobenzenes (DCBs) are very important chlorinated chemical solvents. All DCBs are artificial synthesized but not natural compounds. 1,2-DCB is a colorless

to pale yellow liquid used to make herbicides; 1,3-DCB is a colorless liquid used to make herbicides, insecticides, medicine, and dyes; 1,4-DCB is a colorless to white solid with a strong, pungent odor which is used as a pesticide and a deodorant (Haigler, *et al.*, 1988; de Bont, J. A., *et al.*,1986; Schraa, Gosse, *et al.*, 1986; Spain, Jim C., and Shirley F. Nishino., 1987). Table 1-1 shows the physical characterization and toxicity of DCB isomers (Haigler, *et al.*, 1988; de Bont, J. A., *et al.*,1986).

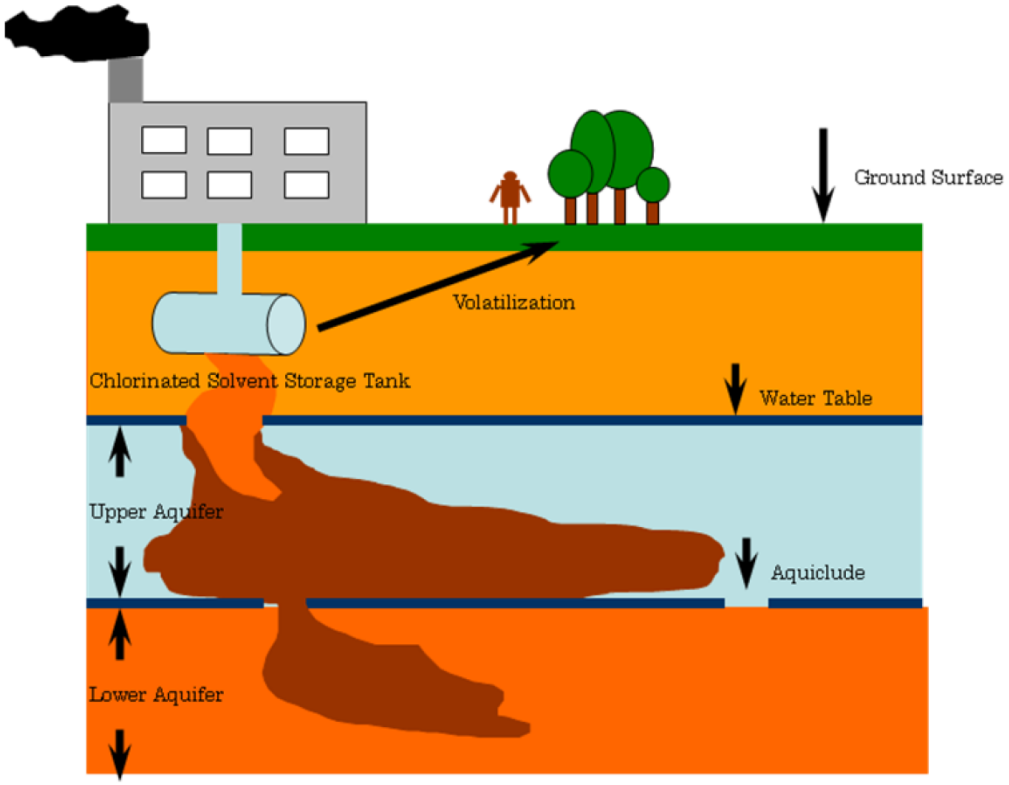


Fig. 1-1 Image of contaminated soil site and underground water

It is said that exposure to DCBs mostly occurs from breathing indoor air or workplace air. Exposure to high level of 1,2- or 1,4-dichlorobenzene may be very irritating to the eyes and nose, cause difficult breathing, and an upset stomach. Extremely high exposures to 1,4-DCB can result in dizziness, headaches, and liver problems. 1,2-, 1,3-, and 1,4-DCB have been identified in at least 281, 175, and 330, respectively, of the 1,662 National Priorities List sites identified by the Environmental Protection Agency (EPA, 2006). U.S. EPA promulgated a Maximum Contaminant Level of 0.6 mg l^{-1} for 1,2-DCB in drinking water (USEPA, office of water, 1995).

When exposed to air, it slowly changes from a solid to a vapor. Most people can smell 1,4-dichlorobenzene in the air at very low levels (USEPA, 2006).

Table 1-1 Characters of DCB isomers

Contents	**1,2-DCB**	**1,3-DCB**	**1,4-DCB**
Melting Point	-16.7°C	-24.8°C	52.8～53.5°C
Boiling Point	180.3°C	173°C	173～174°C
Density (25°C)	1.3007 g/cm^3	1.2884 g/cm^3	1.25～1.46 g/cm^3
Vapor Pressure	0.196 kPa	0.18 kPa	0.17 kPa
Water Solubility	155.8 mg/l	125 mg/l	60 mg/l
Solubility in organic solvents	Miscible	Miscible	Miscible
Henry's Law Constant (25°C)	193 Pa・m^3/mol	192 Pa・m^3/mol	262 Pa・m^3/mol
Flash Point (closed cup)	66°C	63°C	66°C
Flammability Limits	Upper 9.2% Lower 2.2%	Upper 7.8% Lower 1.8%	Upper 5.9% Lower 1.7%
Acute Intraperitoncal Toxicity	LD_{50} (rat): 840 mg/kg LD_{50} (mouse): 1228 mg/kg	LD_{50} (rat): 500 mg/kg LD_{50} (mouse): 1470 mg/kg	LD_{50} (rat): 500 mg/kg LD_{50} (mouse): 2950 mg/kg

Halogenated aromatic compounds are one of the largest group of environmental pollutants, among them, CBs are used for chemical synthesis pesticides, pharmaceuticals, dyes and so on. Now, the extensive use, toxicity, persistence and accumulation of these kinds of chlorinated benzenes have been a serious environmental problem (Aelion *et al.*, 1987). Actually, applying to the bioremediation by degrading microorganisms in the contaminated site, it is necessary to consider the competition by other microorganisms (indigenous microorganisms). So, because of the competition with the indigenous microorganisms, it often happens that there is no microoganism which has a strong degradation ability left during biodegradation processes. Some species disappeared by competition during biodegradation processes (Springael *et al.*, 2002). Therefore, it is necessary to find a DCB isomers degrading microorganism having the ability to overcome competition from indigenous microorganisms.

Thus, stable consortium should be created by enrichment culture that was used 1,2-DCB as a substrate to provide carbon and energy source. To analyze the degradation characterization of major microorganisms in the consortium, microorganisms were taken from the consortium in this study, the isolation was aimed at finding a microorganism that can exhibit high degrading performance in actual contaminated soil. The genetic analysis is helpful to understand the reason of stable coexistence of bacterial strains in the degrading consortium. In the further study, decomposition experiments under simulated contaminated soil will be conducted.

Chapter 2

Construction and characterization of dichlorobenzene isomers degrading consortia

DCB isomers are chemically stable in environment, and it is very difficult to remove this kind of aromatic organic compound from environment. Recently, DCB isomers are known to be degraded under appropriate conditions in the laboratory by bacteria isolated from soil and water (Reineke and Knackmuss 1984; Yadav *et al*., 1995; Mars *et al*., 1997; Kiernicka *et al*., 1999; Adebusoye *et al*., 2007; Baptista *et al*., 2008; Field and Sierra-Alvarez 2008). Biodegradation has become an useful strategy to remove DCBs polluted soil or groundwater (de Bont *et al.*, 1986). This suggests that biodegradation might be an effective method for the removal of DCBs accidentally released into the environment (Spain, J.C., 1987; Holliger, C *et al.*, 1992).

Bioremediation refers to the use of microorganisms to degrade contaminants that pose environmental and human risks. Bioremediation processes typically involve the actions of many different microbes acting in parallel or sequence to complete the degradation process (Sylvia *et al*., 2005). In various natural and engineered environments, many species of microorganisms stably coexist by interacting with each other and effectively exert various functions. Particularly in engineered environments, achieving the functional and structural stability of microbial communities for long term is considered to be an important issue (Briones *et al.*, 2003; Canstein *et al.*, 2001). There is a report describing the construction of a stable co-culture consisting of two bacterial strains in mutualistic or commensalistic association (Dolhopf *et al.*, 2003) and there is another report noting that the addition of a third microorganism alters the relationships between two microorganisms (Kerr *et al.*, 2002). In addition, one previous study described the relationship between two degrading strains present in a stable degrading enrichment capable of extensive growth on chlorinated aromatic compounds (Adebusoye *et al*., 2007). Meanwhile, a separate study reported two strains that were isolated from phenol-degrading enrichment and

maintained stable phenol degradation abilities more than 3 years (Thomas *et al*., 2002). These findings show that it is possible for two or more degrading strains to coexist in a stable enrichment when cultivated in the presence of a sole carbon and energy source. However, the mechanisms underlying such a stable coexistence within degrading enrichment have yet to be elucidated.

In this study, DCB degrading consortia were constructed to analyze the biodiversity in stable degrading consortia. Enrichment may be a good way to select the competitive microorganisms which could degrade DCB. Here four consortia (consortium 1, 2, 3, 4) which were enriched with 40 mg/l of 1,2-DCB and two consortia which were enriched with 40 mg/l 1,3-DCB and 1,4-DCB were evaluated. The main objectives of this chapter are to investigate the consortia which are showing stable coexistence in enrichment process even after many transferred generations, and to elucidate its' microbial diversity.

Chapter 3

Isolation, identification and characterization of DCBs degrading bacteria

Biodegradation of halogenated aromatic compounds has been the subject of previous reviews (Stefan Beli *et al*., 1999). The isolation of 1,2-, 1,3-, and 1,4-DCB degrading bacteria have been studied by many researchers. Previous studies characterized a strain of *Acidovorax avenae* capable of biodegrading CB, 1,2-DCB, 1,3-DCB, and 1,4-DCB (Monferran *et al*. 2005), and a strain of *R. pickettii* L2 capable of biodegrading CB, 1,3-DCB, and 1,4-DCB, but not 1,2-DCB (Zhang *et al*. 2011), indicating that certain bacterial strains within the same group are able to biodegrade certain DCBs, but not all of the isomers (Haigler *et al*., 1988; Schraa *et al*., 1986; Field and Sierra, 2008). The previously reported bacteria which have an ability to degrade DCB isomers are listed in Table 3-1. Most of these isolates belong to *Proteobacteria* group, while *Staphylococcus xylosus* and *Bacillus cereus* belong to *Firmicutes* group.

Biodegradation pathways of CB, 1,2-DCB, 1,3-DCB, and 1,4-DCB have been thoroughly studied. DCB isomers are provided to bacteria as a sole carbon and energy source, and DCB isomers are degraded to dihydrodiol, dichlorocatechol, dichloro-cis-muconic acid, and the other intermediate (Haigler *et al*., 1988).

Analysis of the kinetic properties of the *Alcaligenes* sp. strain R3 indicated that the maximum specific growth rate is high, with values of 0.175 h^{-1} (Spiess *et al*., 1995). The specific growth rates at 0.1 mM (14.6 mg/l) of 1,2-DCB were estimated to 0.196 and 0.145 h^{-1} for *Pseudomonas* sp. and *Staphylococcus xylosus* (Ziagova *et al*., 2007), respectively.

Table 3-1 Summary of DCBs degraders

Aerobic bacterial strains capable of growing on DCBs as a sole source of carbon		
Bacterial strain	Congener	References
Burkholderia sp. strain PS12	1,2-DCB	Sander et al.(1991)
Burkholderia sp. strain PS14	1,2-DCB	Sander et al.(1991) Rapp Timmis(1999)
Pseudomonas sp. strain GJ60	1,2-DCB	Oldenhuis et al.(1989)
Pseudomonas sp. strain JS100	1,2-DCB	Haigler et al.(1988)
Pseudomonas sp. strain P5	1,2-DCB	Van Der Meer et al.(1987)
Staphylococcus xylosus	1,2-DCB	A. Mrozik et al.(2002)
Acidovorax avenae	1,2-DCB	Monferran et al.(2005)
Bacillus cereus strain DL-1	1,2-DCB	Huihui Liu et al.(2011)
Alcaligenes sp. strain OBB65	1,3-DCB	Debont et al.(1986)
Burkholderia sp. strain PS12	1,3-DCB	Sander et al.(1991)
Burkholderia sp. strain PS14	1,3-DCB	Sander et al.(1991)Rapp Timmis(1999)
Pseudomonas sp. strain P51	1,3-DCB	Van Der Meer et al.(1987)
Bacillu cereus PF-11	1,3-DCB	L, Wang et al.(2003)
Acidovorax avenae	1,3-DCB	Monferran et al.(2005)
Ralstonia pickettii L2	1,3-DCB	Li Li Zhang et al.(2011)
Alcaligenes sp. R3	1,4-DCB	Oltmanns et al.(1988)
Burkholderia sp. PS12	1,4-DCB	Sander et al.(1991)
Burkholderia sp. PS14	1,4-DCB	Sander et al.(1991)Rapp Timmis(1999)
Hydrid strain WR1323	1,4-DCB	Oltmanns et al.(1988)
Pseudomonas aeruginosa RHO1	1,4-DCB	Oltmanns et al.(1988)Brunsbach(1994)
Pseudomonas sp. B1	1,4-DCB	Oltmanns et al.(1988)
Pseudomonas sp. JS150	1,4-DCB	Haigler et al.(1992)
Pseudomonas sp. JS6	1,4-DCB	Spain and Nishino(1987)
Pseudomonas sp. P51	1,4-DCB	Van Der Meer et al.(1987)
Alcaligenes sp. strain A175	1,4-DCB	Schraa et al.(1986)
Unidentified strain 1474	1,4-DCB	Nishino et al.(1994)
Xanthobacter flavus 14p1	1,4-DCB	Sommer an Gorisch(1997)
Rhodococcus phenolicus	1,4-DCB	Rehfuss and Urban (2005)
Acidovorax avenae	1,4-DCB	Monferran et al.(2005)
Ralstonia pickettii L2	1,4-DCB	Li Li Zhang et al.(2011)

Mostly, DCB could be biodegraded completely, but a report showed some bacteria could not degrade DCB completely. The percentage of 1,2-DCB consumption is ranging between 78 and 51% for *Pseudomonas* sp. and between 45 and 22% for *Staphylococcus xylosus* (Ziagova *et al*., 2007), respectively depending on its initial concentration.

Comparing cell growth rates of the two bacterial species is evident that *Pseudomonas* sp. is more tolerant to high 1,2-DCB concentrations, since it presents higher values comparing to *Staphylococcus xylosus*. This is an important indication for the degrading efficiency of each strain, as it is closely associated to the size of bacterial population (higher number of available degrading enzymes). Moreover, these results are in agreement with the general finding that Gram-negative bacteria are more resistant to toxic pollutants, compared with Gram-positive bacteria, possibly due to the lack of protection by the outer membrane (Robertson *et al*., 1996; Ziagova *et al*., 2007). But there was little report to show the maximum degradation capacity of DCBs.

There was little research about the degradation rate of DCBs by bacteria. A report about *A. avenae* didn't describe the degradation rate of DCBs but showed that 0.136 mM 1,2-DCB (approximately 20 mg/l) could be completely degraded within 45 h (Monferran *et al*., 2005). Most report researched the metabolic degradation of DCB, but a report showed the possibility of cometabolic degradation of 1,2-DCB by *Pseudomonas* sp. and *Staphylococcus xylosus* with glucose (Ziagova *et al*., 2007). Bacterial metabolism of halogenated aromatic compounds has been the subject of recent reviews (Zeyaullah *et al*., 2009; Reineke *et al*., 1984). Biodegradation pathways of chlorobenzoic acids (Reineke *et al*., 1980; Reineke *et al*., 1982; Reineke *et al*., 1984;), chlorophenols (Evans *et al*., 1971; Tiedje *et al*., 1969), chlorobenzene (Reineke *et al*., 1984), 1,2-DCB (Haigler *et al*., 1988), 1,3-DCB (de Bont *et al*., 1986) ,1,4-DCB (Spain Jim C. *et al*., 1987) have been thoroughly studied in pure cultures of bacteria. The most common catabolic pathways involve conversion of the parent molecules to chlorocatechols (Evans W. C. *et al*., 1971; Reineke W. *et al*., 1984) or dichlorocatechols (Reineke W. *et al*., 1984; de Bont J. A. *et al*., 1986; Haigler *et al*., 1988; Oltmanns *et al*., 1988; Spiess Elke *et al*., 1995). The initial attack on dichlorobenzene could be at the 1 or 2 position to form 4-chlorophenol or

2,5-dichlorophenol. A second adjacent hydroxylation would produce the ring fission substrate 4-chlorocatechol or 3,6-dichlorocatechol (JIM C. SPAIN *et al*., 1987; Schraa Gosse *et al*., 1986). Alternatively, attacking at the 1,2- or 2,3- position would lead to the formation of either 4-chlorocatechol or 3,6-dichlorocatechol. Dechlorination did not occur until after the ring opening (Schraa Gosse *et al*., 1986).

Chloride was eliminated by lactonization of chloromuconic acids and subsequent reactions to yield P-ketoadipic acid (Reineke W. *et al*., 1984). During growth with DCB in pure culture, stoichiometric amounts of chloride were released (Schraa Gosse *et al*., 1986; JIM C. SPAIN *et al*., 1987; de Bont J. A *et al*., 1986; Haigler *et al*., 1988)

In this study, four 1,2-DCB degrading consortia were successfully constructed in chapter 2. In the consortia, some evidence of dominant bacteria existing in consortia have been confirmed by DGGE and 16S rRNA gene cloning library results. Normally, the dominant bacteria which could be competitive after many generations of enrichment are possibly substrate degraders (Massol Deya *et al*., 1977; Springael *et al*., 2002). So, there is high possibility that the dominant stable coexisting bacteria after many generations of enrichment in consortium 1, 2, 3, and 4 could possibly be dominant 1,2-DCB degraders. And bacteria belonged to *Acidovorax* sp. was dominant at 9^{th} generation but disappeared after 15^{th} generation of 1,2-DCB degrading consortium 3 and 4 possibly also possessed the 1,2-DCB degradation ability. The results in chapter 2 gave us a motivation to isolate and identify the degrading strains from 1,2-DCB degrading consortia. Additionally, characterizations of DCB degradation by isolates would also be analyzed as a first report in this part.

Chapter 4

Activity of degrading bacteria in the stable DCB degrading consortium

In various natural and engineered environments, many species of microorganisms stably coexist by interacting with each other and effectively exert various functions (Hoiling, C. S. *et al*., 1997). Particularly in engineered environments, achieving the functional and structural stability of microbial communities for long term is considered to be an important issue (Briones *et al.*, 2003; Canstein *et al.*, 2001). But it is very difficult to clarify the mechanism of the stable coexistence of many species of microorganisms in the consortium (Freilich, S. *et al*., 2011). In addition, there were some reports describing the construction of a stable co-culture. A stable consortium was constructed consisting of two bacterial strains in mutualistic or commensalistic association (Dolhopf *et al.*, 2003). A cellulose-degrading defined mixed culture consisting of five bacterial strains were analyzed (Souichiro Kato *et al*., 2005). Methane production by biological pretreatment using a constructed microbial consortium has been researched (Zhang, Q. *et al*., 2011). However, there is other report noting that the addition of a third microorganism alters the relationships between two microorganisms (Kerr *et al.*, 2002). Competition and the coexistence of species in a mixed podocarp stand were talked by Richard P. Duncan (1991). Coexistence of species competing for some special shared resources was researched by Robert Armstrong (1976).

It is said that two functionally similar bacterial strains can coexist in aerobic granules. The compact structure of aerobic granules was discovered to provide spatial separation for stable coexistence of bacteria with similar functions (He-Long Jiang *et al.*, 2007). This may be the reason for stable coexistence of microorganisms in consortium. Most reports were talking about that one substrate degrading microorganism coexisting with the other microorganisms who supported the substrate degrading microorganism but no degradation ability. While there was little report about that two or more than two dominant substrate-degrading bacteria coexist in the consortium after many generations of enrichment.

In this study, stable consortia were constructed successfully in chapter 2. By isolation and identification study in chapter 3, six 1,2-DCB degrading strains were successfully isolated (*Acidovorax* sp. sk40 and *Ralstonia* sp. sk41 were isolated from consortium 1; *Variovorax* sp. skdcb1 and *Pandoraea* sp. sk44 were isolated from consortium 2; *Acidovorax* sp. skno3 and *Ralstonia* sp. skdcb11 were isolated from consortium 3 and 4). For the six isolates, the relation between the 1,2-DCB concentration and the growth rate was obtained by the Haldane's and newly modified Haldane's model. It indicated that there were always two stable dominant 1,2-DCB degrading bacteria in consortium 1 and 2 even after many generations of enrichment. However, many previous studies reported that some species disappeared by competition during biodegradation process because of bacterial competition in consortium (Springael *et al*., 2002).

This chapter aims to assess the stable coexistence of two strains in consortium 1 and 2 with the similar 1,2-DCB degradation pattern in consortia. 1,2-DCB degradation kinetics were analyzed to compare the difference of degradation kinetics of each strain. Specific growth rates (μ) were analyzed in chapter 3. And it was calculated to describe the existence of strains in this chapter. By investigation of 1,2-DCB degradation kinetics especially specific growth rate of these strains, it indicated a good understanding of coexistence of two strains coexisting in consortia. However, the dominant bacteria in consortium 3 and 4 were disappeared after many generations of enrichment. This phenomenon was also described by a good evidence by the 1,2-DCB degradation kinetics of each strain in consortia. This is the first research to report that two substrate-degrading bacteria could be constructed and stable after many transferred generations.

Chapter5

Functional genes of DCBs found in isolates from 1,2-DCB degrading consortium

A family of aromatic-ring-hydroxylating dioxygenases (ARHDOs) is key enzyme in the aerobic bacterial metabolism of aromatic compounds. ARHDOs catalyze the first step in the metabolism of aromatic hydrocarbons under aerobic conditions by incorporation of molecular oxygen (Paul M. Taylor *et al*., 2002). The prototype catalytic reaction is to add two hydroxyl groups to vicinal carbons, thereby destroying the aromatic system and yielding dihydrodiol compounds of cis stereochemistry (Butler *et al*., 1997; Boyd and Sheldrake, 1998). ARHDOs are so important that the function in the degradation of aromatic pollutants (Silke Kahl and Bernd Hofer, 2003) which describes the development and validation of a system for the rapid isolation and characterization of specific ARHDO activities.

ARHDOs incorporate two atoms of dioxygen (O_2) into their substrates in the dihydroxylation reaction. The product is *cis*-1,2-dihydroxycyclohexadiene, which is subsequently converted to benzene glycol by a *cis*-diol dehydrogenase.

A large family of multicomponent mononuclear (non-heme) iron oxygenases has been identified. Bacterial ARHDOs constitute two different functional parts: hydroxylase components and electron transfer components. Hydroxylase components are either $(\alpha\beta)_n$ or $(\alpha)_n$ oligomers. Two prosthetic groups, a Rieske-type [Fe_2S_2] center and a mononuclear iron, are associated with the α-subunit in the $(\alpha\beta)_n$-type enzymes. Electron transfer components are composed of ferredoxin oxidoreductase and Rieske-type [Fe_2S_2] ferredoxin. In benzoate and toluate 1,2-dioxygenase systems, a single protein containing reductase and Rieske-type ferredoxin domains transfers the electrons from NADH to the hydroxylase component. In the phthalate 4,5-dioxygenase system, phthalate dioxygenase reductase (PDR) has the same function. The most extensively studied ARHDOs enzyme systems have been isolated from *Pseudomnas* species. Gibson and co-workers (1971) were the first to show that a dioxygenase system of *Pseudomonas putida* oxidized benzene to generate cis-benzene dihydrodiol. Several other

ARHDOs have since been isolated and characterized from other strains of *Pseudoinonus*: pyrazon dioxygenase (Sauber *et al*., 1977); naphthalene dioxygenase (Ensley *et al*., 1982; Takizawa *et al*., 1994; Yang *et al*., 1994); toluene dioxygenase (Subramanian *et al*., 1979); benzoate 1,2-dioxygenase (Yamaguchi and Fujisawa, 1978, 1980, 1982); phthalate dioxygenase (Batie *et al*., 1987); 4-chlorophenylacetate 3,4-dioxygenase (Markus *et al*., 1986); 2-halobenzoate 1,2-dioxygenase (Fetzner *et al*., 1992); ortho-halobenzoate 1 ,2-dioxygenase (Romanov and Hausinger, 1994; Haak *et al*., 1995); benzene dioxygenase (Axcell and Geary, 1975; Irie *et al*., 1987); and biphenyl dioxygenase (Haddock *et al*., 1993; Erickson and Mondello, 1993). The multicomponent dioxygenases system have been classified into five groups (IA, IB, IIA, IIB and III) based on the number and some characteristic features of the compounds (Butler & Mason, 1997; Silke Kahl and Bernd Hofer, 2003) (Table 5-1). The terminal oxygenase proteins of this class consist of an iron-sulfur protein with a Rieske-type [2Fe-2S] cluster and a mononuclear iron, and the electron transport chain may consist of either one or two separate proteins (Jeong W. N. *et al*., 2001). This classification of ARHDOs is widely accepted since it explained systematically the non-heme iron-containing ring-hydroxylating oxygenases very well (Jeong W. N. *et al*., 2001). But, in recent years, with much more identification and characterization of many other oxygenases, it became clear that this system will not be able to classify all of these oxygenases (Jeong W. N. *et al*., 2001). A new classification based on the amino acid sequence of the terminal oxygenase component that has catalytic activity and reflects the phylogenetic affiliation showed in Fig. 5-1.

By this new classification, the Rieske-type [2Fe-2S] cluster binding site sequence is generally expressed as Cys-X1-His-X15-17-Cys-X2-His, but detailed information showed that group 1 had sixteen or eighteen amino acids between the first His and the second Cys residue, while groups 2, 3, 4 had seventeen amino acids. And the two His residues of the Fe^{2+} binding site are separated by three to five amino acids. Again, detailed information showed that the two residues are separated by three to four amino acids for group 1, four for groups 2, 3, and four to five for group 4 (Fig. 5-1 and 5-2).

Research about the core region of ARHDOs which could encode the catalytic site of the large

subunit of chlorobenzene dioxygenase had been reported (Monferra´n *et al*., 2005). The core region was detected by PCR amplification around 700 bp shown in agarose and confirmed by DNA sequencing. The consensus primers which were used to amplify the core region had been studied (Kahl and Hofer, 2003).

Genetic analyses of TecA DCB dioxygenase found from *Burkholderia* sp. strain PS12 had been studied (Stefan Beil *et al*., 1998). This enzyme catalyzed the first step in the mineralization of DCB. Components of TecA were constructed as TecA1, A2, A3, and A4 which could determine the α-subunit, β-subunit, ferredoxin, and ferredoxin reductase, respectively (Fig. 5-3). The consensus primers were designed to amplify *tecA1* gene which could encode the α-subunit of TecA DCB dioxygenase.

In this study, six DCB(s) degrading strains were successfully isolated from consortia. It indicated that all of these DCB(s) degraders possess the DCB dioxygenase to degrade DCB. The consensus primers for amplification of core region of α-subunit aromatic-ring-hydroxylating dioxygenase were used to try to amplify the important core region genes. Then, based on the sequences of core region, the taxonomy of genes encoding α-subunit of DCB dioxygenase were determined. There were some reports to research that bacteria possess TecA dioxygenase can degrade DCB. Additionally, the consensus primers for amplification of α-subunit TecA dioxygenase gene (*tecA1*) were used to amplify the α-subunit of DCB dioxygenase genes. Whether or not all of the isolated DCB degrading strains possess the same TecA dioxygenase system will be my main study in this part.

Chapter 6

General conclusions and future prospects

Soil contamination by DCB has been found in places such as pesticide factory sites in Japan, there are many cases where low concentration DCB is widely distributed, and there is no effective purification method. In recent years, the research has been performed in the field of in-situ bioremediation. It is considered that bioremediation which is a natural process utilizing natural microorganism is friendly to environment. Moreover, because it can be carried out on site without causing a major disruption nor a large amount of energy, it needs a lower cost than other treatment process.

Therefore, bioremediation is expected as a fundamental restoration technique. In this study, samples were obtained from polluted site contaminated mainly by 1,2-DCB. Experiments were conducted to investigate the 1,2-DCB degradation ability of the enriched consortia. It aims to isolate and identify DCB degraders from consortia; to analyze the degradation characterization of major degraders in the consortia to find DCB degrading bacteria that can exhibit high degrading performance in actual contaminated soil; to examine the DCB degrading enzyme which is helpful to understand the degradation mechanism of DCB by bacteria.