Characterization of Novel Desulfurizing Bacteria as Biocatalysts Applicable to Deep Desulfurization of Light Gas Oils

新規脱硫細菌の機能解析と軽油の超深度脱硫への応用

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Preface

Fossil fuels such as petroleum and coal are essential energy sources for human life. In fossil fuels, light gas oil (LGO) as a middle distillate of crude oil is used for diesel engine vehicles. However, LGO contains large amounts of organosulfur compounds, and combustion of LGO generates sulfur oxides. In addition, the resulting sulfur oxides poison catalysts used for removal of the nitrogen oxides and the particulate matter in diesel engine vehicles, causing serious environment problems such as acid rain and air pollution.

Today, LGO is treated by hydrodesulfurization using metallic catalysts in the presence of hydrogen gas under extremely high temperature and pressure. However, current hydrodesulfurization faces difficulties in removing complicated organosulfur compounds. In contrast, biodesulfurization using microorganisms as biocatalysts is a promising technology to remove organosulfur compounds in LGO under mild conditions, and has attracted attention.

In this thesis, with the objective to achieve deep desulfurization of LGO using biodesulfurization, the author isolated and characterized novel desulfurizing bacteria and found that these bacteria are promising biocatalysts applicable to deep desulfurization of LGOs. I hope that the studies in this thesis will provide useful information for not only the development of biodesulfurization process, but also the studies in the fields of microbiology, biotechnology, and applied chemistry.

Toshiki Furuya

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Chapter 1

Review:
Biodesulfurization of Light Gas Oil

1.1. Introduction

Fossil fuels such as petroleum and coal are essential energy sources for human life. In fossil fuels, light gas oil (LGO) as a middle distillate of crude oil is used for diesel engine vehicles. However, LGO contains large amounts of organosulfur compounds, and combustion of LGO generates sulfur oxides. In addition, the resulting sulfur oxides poison catalysts used for removal of the nitrogen oxides and the particulate matter in diesel engine vehicles, causing serious environment problems such as acid rain and air pollution. Thus, regulations for the sulfur level in diesel oil have become increasingly strict as shown in Table 1.1 [1]. It is planned to reduce the sulfur level to 50 ppm S (mg sulfur per liter oil) by 2005 in the European Union and to 15 ppm S by 2006 in the USA. Similarly, in Japan, the regulations have been enforced to reduce the sulfur level to 50 ppm S by the end of 2004.

Today, LGO is treated by hydrodesulfurization using metallic catalysts in the presence of hydrogen gas under extremely high temperature and pressure. However, current hydrodesulfurization faces difficulties in removing complicated organosulfur compounds. In contrast, biodesulfurization using microorganisms as biocatalysts is a promising technology to remove organosulfur compounds in LGO under mild

Table 1.1. Regulations for sulfur levels in diesel oil.

Year	Country	Current sulfur level (ppm)	Target sulfur level (ppm)
1998	Taiwan	5000	500
2000	Thailand	5000	500
	Korea	2000	500
2004	Japan	500	50
2005	European Union	350	50
2006	United States	500	15

conditions, and has attracted attention ([1-4] for review).

In this chapter, the author describes an overview of the biodesulfurization of LGO. The author explained two desulfurization methods, i.e., hydrodesulfurization and biodesulfurization, and demonstrated that biodesulfurization is a promising technology to achieve deep desulfurization of LGO. Based on the review in this chapter, the author clarified the objective of this thesis.

1.2. Sulfur compounds in light gas oil

Petroleum is predominantly composed of hydrocarbons. After carbon and hydrogen, sulfur is the third most abundant element in petroleum [5]. Sulfur levels in crude oil range from 500 to 50000 ppm S, depending on the source. Sulfur levels in LGO as a middle distillate of crude oil often exceed 5000 ppm S. LGO contains various organosulfur compounds, such as thiols, sulfides, disulfides, thiophenes, benzothiophenes (BTHs), dibenzothiophenes (DBTs), naphthothiophenes (NTHs), and benzonaphthothiophenes (BNTHs) as shown in Fig. 1.1. Particularly, heterocyclic sulfur compounds such as BTHs and DBTs are contained much in LGO [2].

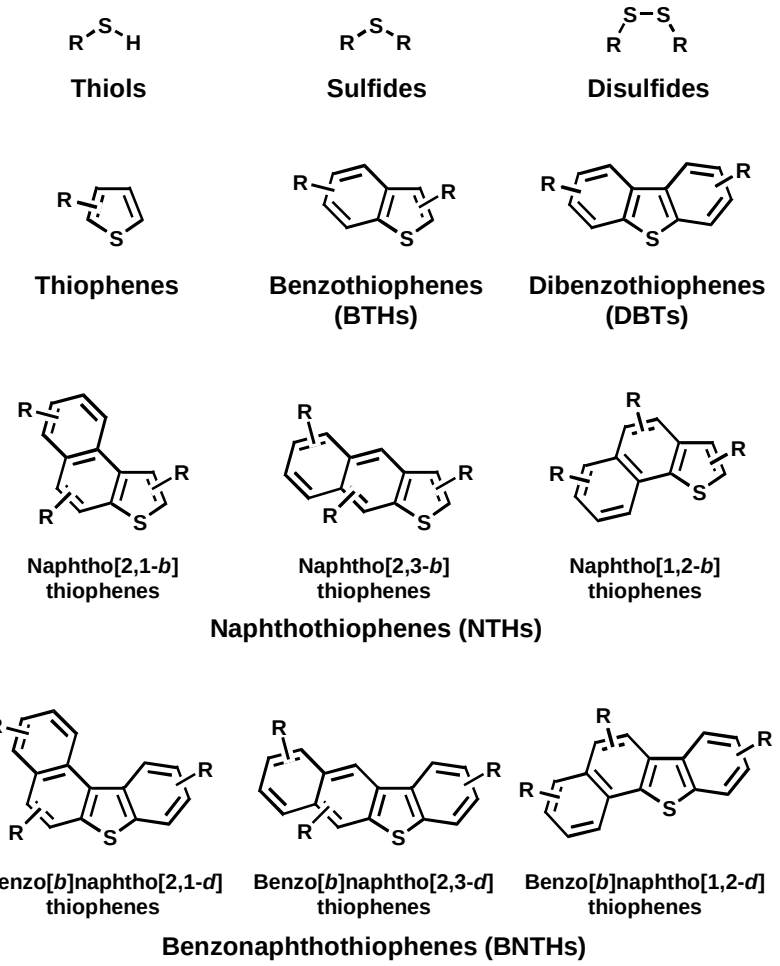


Fig 1.1. Organosulfur compounds in LGO. R, Alkyl substitution.

1.3. Deep desulfurization of light gas oil

1.3.1. Hydrodesulfurization

Today, LGO is treated by hydrodesulfurization (HDS) using metallic catalysts in the presence of hydrogen gas under extremely high temperature and pressure [6]. Generally sulfided $Co/Mo/Al_2O_3$ or $Ni/Mo/Al_2O_3$ is used as a metallic catalyst, and $Co/Mo/Al_2O_3$ exhibits higher desulfurizing activity toward LGO than $Ni/Mo/Al_2O_3$. The typical reaction temperature and pressure are 300 to 350˚C and 50 to 100 atm, respectively. The sulfur atom in the molecule of a sulfur compound is hydrogenated

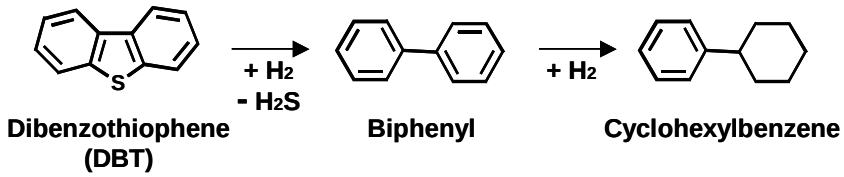


Fig 1.2. The pathway of DBT desulfurization by $Co/Mo/Al_2O_3$.

by the catalyst in the presence of hydrogen gas, and the resulting H_2S is removed from LGO.

DBT is a representative sulfur compound detected in LGO. The pathway of DBT desulfurization by $Co/Mo/Al_2O_3$ is shown in Fig. 1.2. DBT is desulfurized to biphenyl as the major product with a small amount of cyclohexylbenzene. HDS is effective in desulfurizing various sulfur compounds, but the reactivity is dependent on the local environment of the sulfur atom in the molecule and the overall structure of the molecule. HDS can desulfurize simple sulfur compounds such as thiols, sulfides, disulfides, and heterocyclic sulfur compounds with no or small alkyl substitutions. However, heterocyclic sulfur compounds with alkyl substitutions adjacent to the sulfur atom such as 4,6-dimethyl-DBT is recalcitrant to HDS. This is because alkyl substitutions at the 4- and 6-positions of DBT prevent the catalyst from interacting with the sulfur atom due to steric hindrance as shown in Fig 1.3.

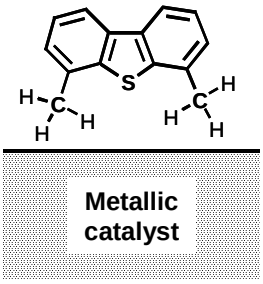
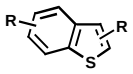
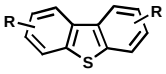
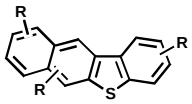
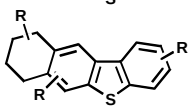


Fig 1.3. Steric hindrance by alkyl substitutions for metallic catalyst.

Table 1.2. Sulfur compounds detected in hydrodesulfurized LGO [a] [7].

Sulfur compound	Molecular structure	Sulfur content (%)[b]
BTHs		1.1
DBTs		92.7
BNTHs		0.9
BHNTHs		5.4

[a] The hydrodesulfurized LGO contained 400 ppm S.
[b] Percent based on total sulfur content in the LGO are shown.

Sulfur compounds detected in hydrodesulfurized LGO are shown in Table 1.2 [7]. In the hydrodesulfurized LGO containing 400 ppm S, the remaining sulfur compounds more than 90% are alkylated DBTs. A small amount of BTHs, BNTHs, and benzotetrahydronaphthothiophenes (BHNTHs) with alkyl substitutions also remain after HDS. NTH is an asymmetric structural isomer of DBT, and it is difficult to distinguish NTHs from DBTs based on their mass spectra. However, it is considered that NTHs are also contained in LGO following HDS although NTHs seem to be more susceptible to HDS than DBTs. These findings indicate that current HDS faces difficulties in removing complicated heterocyclic sulfur compounds such as alkylated DBTs.

1.3.2. Biodesulfurization

As regulations for the sulfur level in diesel oil become stricter, more of the HDS-recalcitrant sulfur compounds must be removed from LGO. However, their

removal by HDS will require more intense conditions leading to higher operating cost, energy consumption, and CO_2 emission. Moreover, intense conditions cause hydrogenation of aromatic and alkene (olefin) compounds, reducing octane value of LGO.

In contrast, biodesulfurization using microorganisms as biocatalysts is a promising technology to remove these recalcitrant organosulfur compounds in LGO under mild conditions [1-4]. Generally biocatalysts can selectively accept even complicated molecules as substrates. To date, many microorganisms possessing degrading abilities toward heterocyclic sulfur compounds have been isolated as described below. The reaction proceeds at ambient temperature and pressure. In addition, aerobic microorganisms use oxygen gas in the air for the reaction instead of expensive hydrogen gas used for HDS. Therefore, biodesulfurization has the advantages of low operating cost, energy consumption, and CO_2 emission, and has attracted attention from the

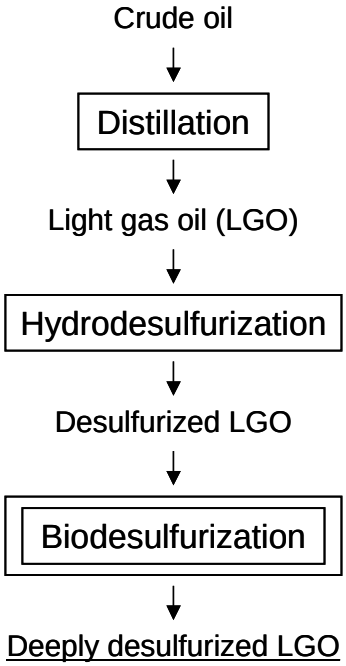


Fig 1.4. Biodesulfurization as a technology complementary to current HDS.

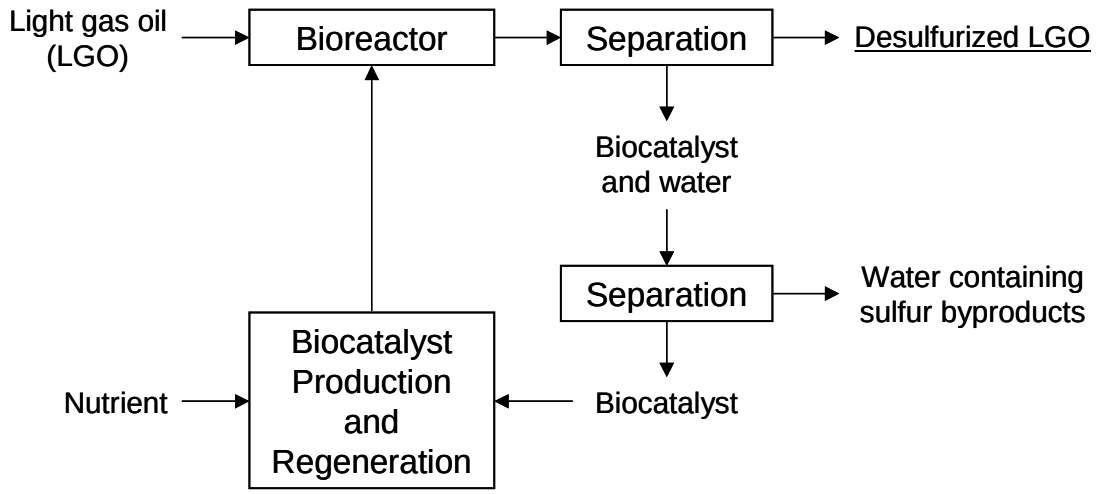


Fig 1.5. Proposed biodesulfurization process.

viewpoint of a technology complementary to current HDS, particularly after HDS, to achieve deep desulfurization of LGO as shown in Fig. 1.4.

Proposed biodesulfurization process is shown in Fig. 1.5 [3]. Cells possessing desulfurizing ability toward LGO are produced as biocatalysts through cultivation, and the resulting biocatalysts are incubated with LGO in oil-water two-phase bioreactor. In the reactor, sulfur compounds in LGO are oxidized to water-soluble sulfur byproducts by the biocatalysts. After the reaction, desulfurized LGO is recovered from the oil-water emulsion in separation unit. Then, the cells are separated from the water containing sulfur byproducts, and are regenerated.

1.4. Biodesulfurization of DBT

1.4.1. Three types of DBT-degrading microorganisms

Many microorganisms possessing DBT-degrading abilities have been isolated to date. These DBT-degrading microorganisms are mainly classified into three types according to their DBT degradation pathways [1].

DBT-degrading microorganisms of type 1 utilize DBT as the sole source of carbon and energy. Thus, these microorganisms degrade DBT through a pathway with the cleavage of carbon-carbon bonds, often referred to as the Kodama pathway, as shown in Fig. 1.6 [8, 9]. DBT is first oxidized at the 1,2-positions by dioxygenase, followed by *meta*-cleavage of the dioxygenated aromatic ring. Some DBT-degrading bacteria of this type, mainly *Pseudomonas* strains [8], have been isolated. However, in this pathway, the sulfur atom is not removed from DBT during the degradation. On the other hand, although degraded DBT metabolites such as 3-hydroxy-2-formylbenzothiophene are water-soluble and removed from oil, the carbon skeleton of aromatic

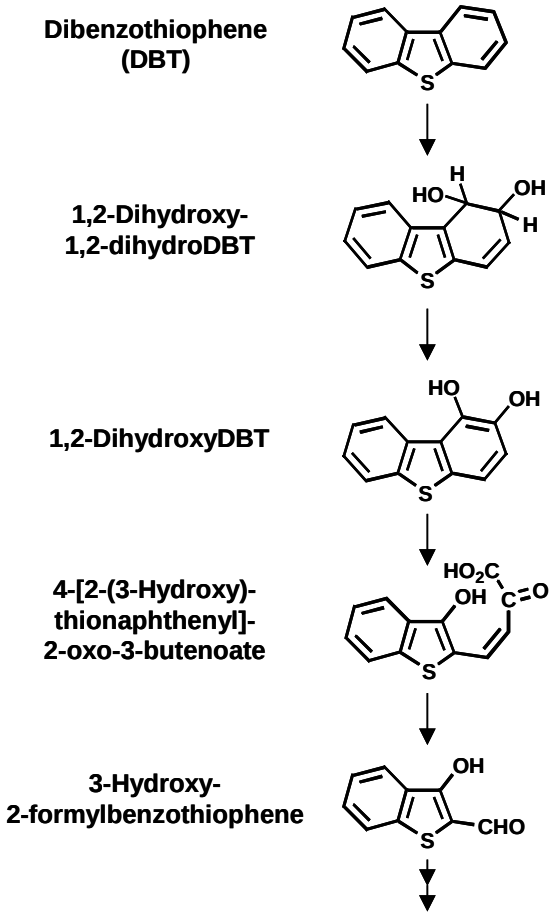


Fig 1.6. The DBT degradation pathway with the cleavage of carbon-carbon bonds.

compounds without sulfur in addition to DBT may be destroyed by this type of microorganisms, leading to a loss of energy content of fuel.

DBT-degrading microorganisms of type 2 utilize DBT as the sole source of sulfur, carbon, and energy. Thus, these microorganisms degrade DBT through a pathway with the cleavage of carbon-sulfur bonds in addition to carbon-carbon bonds as shown in Fig. 1.7 [10, 11]. DBT is first oxidized to DBT sulfone via DBT sulfoxide. DBT sulfone is then oxidized by angular dioxygenase, leading to cleavage of the thiophene ring. The reaction is followed by *meta*-cleavage of the dioxygenated aromatic ring in

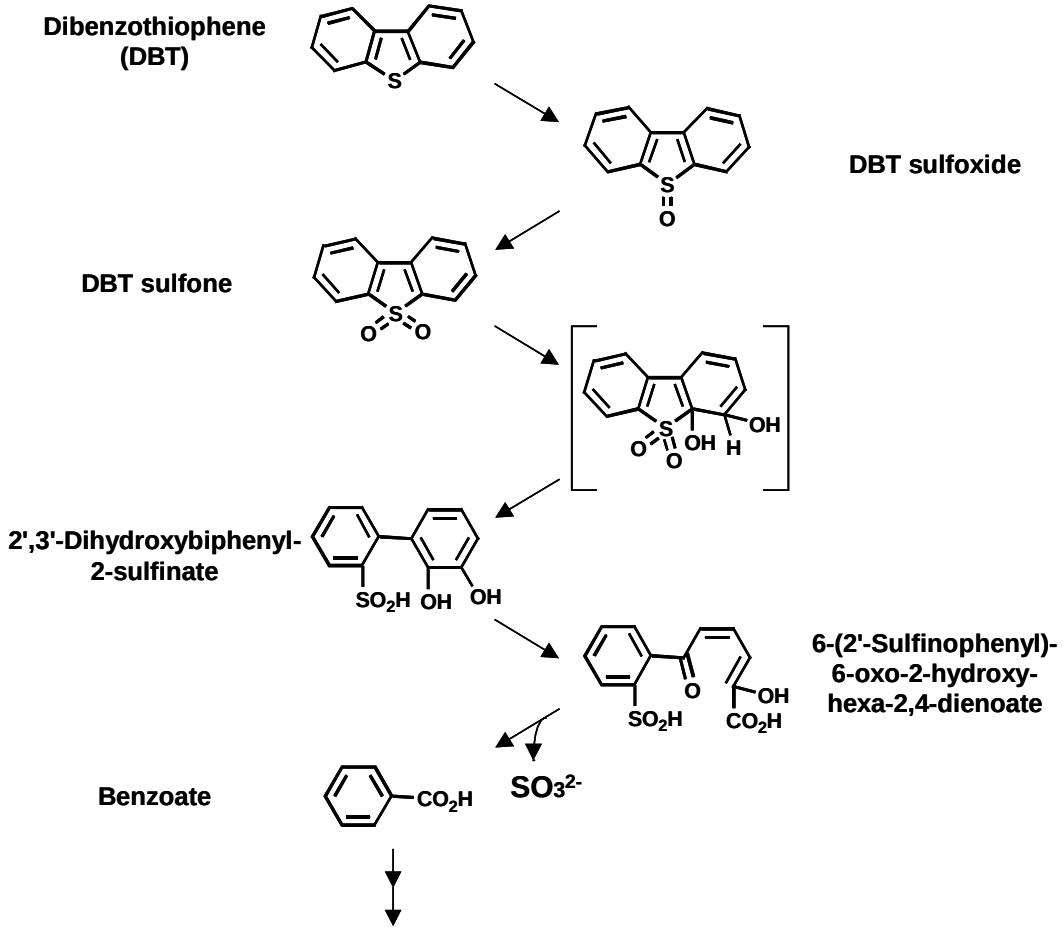


Fig 1.7. The DBT degradation pathway with the cleavage of carbon-carbon and carbon-sulfur bonds.

2',3'-dihydroxybiphenyl-2-sulfinate. Finally, DBT is completely mineralized with the release of the sulfur atom as sulfite. Only one DBT-degrading bacterium of this type, *Brevibacterium* sp. DO [10], has been isolated. However, in this pathway, the carbon skeleton of DBT is destroyed with reducing energy content of the molecule. In addition, DBTs with alkyl substitutions at the 4- and 6-positions such as 4,6-dimethyl-DBT may not be degraded since the alkyl substitutions at the 4- and 6-positions disturb the angular dioxygenation.

DBT-degrading microorganisms of type 3 utilize DBT as the sole source of sulfur. Thus, these microorganisms degrade DBT through a pathway with the selective cleavage of carbon-sulfur bonds as shown in Fig. 1.8 [12-14]. This pathway, often referred to as the 4S pathway, has been extensively studied so far using *Rhodococcus* sp. IGTS8, which was discovered by Kilbane J. J [15-17]. DBT is first oxidized to DBT sulfone ($DBTO_2$) via DBT sulfoxide (DBTO) by DBT monooxygenase. $DBTO_2$ is then converted to 2'-hydroxybiphenyl-2-sulfinate (HBPSi) by $DBTO_2$ monooxygenase, leading to cleavage of the thiophene ring. HBPSi is finally desulfurized to 2-hydroxybiphenyl (2-HBP) with the release of the sulfur atom as sulfite by HBPSi desulfinase. In addition to *Rhodococcus* sp. IGTS8 [12-17], many bacteria of this type have been isolated, for example, *Rhodococcus* sp. ECRD-1 [18], *R. erythropolis* D-1 [19], *R. erythropolis* H-2 [20], *R. erythropolis* KA2-5-1 [21], *Rhodococcus* sp. SY1, formerly identified as *Corynebacterium* sp. [22], *Mycobacterium* sp. G3 [23], *Gordonia nitida* CYKS1 [24], and *Paenibacillus* sp. A11-2 [25]. This pathway is practical since the sulfur atom is selectively removed without reducing energy content of the molecule and the removal of the sulfur atom is not affected much by the alkyl substitutions on the molecule.

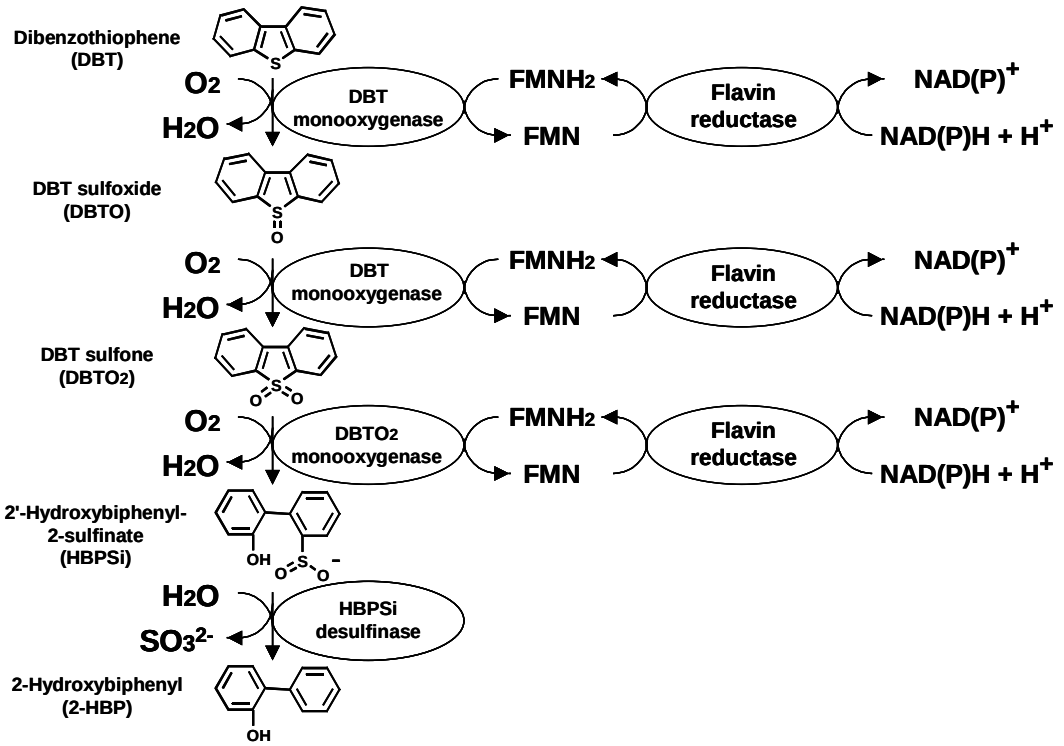


Fig 1.8. The DBT degradation pathway with the selective cleavage of carbon-sulfur bonds.

In addition to three types of DBT-degrading microorganisms, it was reported that some sulfate-reducing microorganisms [26] and sulfur-oxidizing microorganisms [27] possessed DBT-degrading abilities.

1.4.2. DBT desulfurization with the selective cleavage of carbon-sulfur bonds

The sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds is practical, and has been extensively studied so far. *Rhodococcus* sp. IGTS8 is a representative strain possessing the ability to desulfurize DBT through this pathway [12-17]. The genes involved in DBT desulfurization were cloned from *Rhodococcus* sp. IGTS8 and characterized [28-31]. The DBT-desulfurizing phenotype is endowed by a 4-kb gene located on a 120-kb linear plasmid. The genes constitute

a single operon consisting of the three genes, *dszA*, *dszB*, and *dszC*, which are responsible for DBT desulfurization through the sulfur-specific degradation pathway. That is, DBT monooxygenase as the gene product of *dszC* first oxidizes DBT to $DBTO_2$ via DBTO, $DBTO_2$ monooxygenase as the gene product of *dszA* then converts $DBTO_2$ to HBPSi, leading to cleavage of the thiophene ring, and HBPSi desulfinase as the gene product of *dszB* finally desulfurizes HBPSi to 2-HBP with the release of the sulfur atom as sulfite (Fig. 1.8). These three enzymes were purified from *Rhodococcus* strains [32, 33, 34], and particularly DszC and DszA from *R. erythropolis* D-1 [35, 36] and DszB from *R. erythropolis* KA2-5-1 [37] were well characterized. DszC is a 180 kDa homotetramer with a monomeric molecular mass of 45 kDa, DszA is a 100 kDa homodimer with a monomeric molecular mass of 50 kDa, and DszB is a 40 kDa monomer. The reaction catalyzed by DszB is a rate-limiting step in the pathway since it is the slowest of the reactions. The turnover rate of DszB for HBPSi desulfination is only 1 to 7 per min [32, 34, 37].

In addition to these three enzymes, flavin reductase has been found to be essential in combination with two flavin-dependent monooxygenases DszC and DszA [32, 35, 36, 38, 39]. Flavin reductase uses NAD(P)H to catalyze the reduction of FMN and the resulting reductant $FMNH_2$ is used to activate oxygen by the terminal monooxygenases. The gene encoding flavin reductase (*dszD*) was also cloned from *Rhodococcus* sp. IGTS8 [32] and characterized. The *dszD* gene is located on the genome far from the *dszABC* genes located on the plasmid in *Rhodococcus* sp. IGTS8. DszD was purified from *Rhodococcus* sp. IGTS8 [32] and *R. erythropolis* D-1 [40] and characterized. DszD is a 86 kDa homotetramer with a monomeric molecular mass of 22 kDa.

There are only a few reports on application of these DBT-desulfurizing bacteria to

real fossil fuels including diesel oil. *Rhodococcus erythropolis* KA2-5-1 [21], *Rhodococcus* sp. ECRD-1 [41-43], and *Gordonia nitida* CYKS1 [24] led to 30-90% reductions of sulfur content in diesel oils although components of sulfur in diesel oils and reaction conditions were variable. It was also reported that recombinant *Rhodococcus* strains carrying multiple copies of DBT-desulfurization genes exhibited improved desulfurizing ability toward diesel oils [44, 45].

1.4.3. Thermophilic DBT desulfurization

HDS is performed under extremely high-temperature conditions (300-350˚C). Thus, high-temperature LGO following HDS is cooled by exchanging heat with HDS-untreated LGO, seawater, and air, before next processes. It is considered that biodesulfurization is effective as a technology complementary to current HDS, particularly after HDS (Fig. 1.4). Taking account of this, thermophilic DBT-desulfurizing microorganisms may be more practical than mesophilic ones. If biodesulfurization could be performed at high temperatures, it would be unnecessary to cool HDS-treated LGO to ambient temperatures. In addition, contamination by undesirable microorganisms would be avoided at high temperatures. Moreover, enzymes derived from thermophilic microorganisms generally tend to exhibit higher activity and stability than those from mesophilic ones.

A thermophilic DBT-desulfurizing bacterium *Paenibacillus* sp. A11-2 [25] has been isolated to date. This bacterium could desulfurize DBT at around 50˚C through the sulfur-specific degradation pathway (Fig. 1.8). In addition, the DBT-desulfurization genes (*tdsABC*) equivalent to *dszABC* were cloned and characterized [46]. The flavin reductase gene (*tdsD*) was also cloned and

characterized [47]. However, there are no reports on thermophilic DBT-desulfurizing microorganisms except this strain.

1.5. Biodesulfurization of BTH

Some microorganisms possessing BTH-desulfurizing abilities have been isolated to date, for example, *Gordonia* sp. 213E [48], *Rhodococcus* sp. T09 [49], and *Paenibacillus* sp. A11-2 [50]. These bacteria desulfurize BTH through sulfur-specific degradation pathways with the selective cleavage of carbon-sulfur bonds analogous to

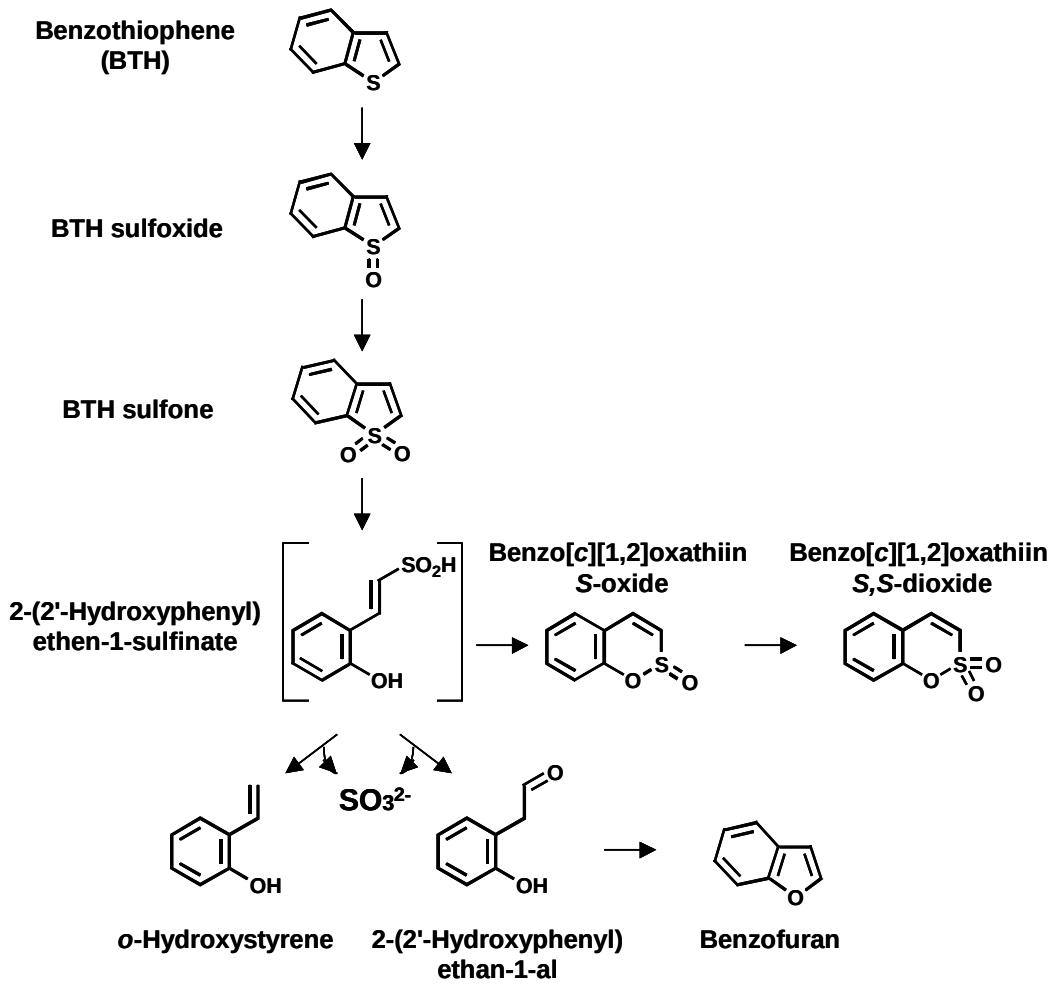


Fig 1.9. The BTH degradation pathway with the selective cleavage of carbon-sulfur bonds.

the pathway for DBT desulfurization (Fig. 1.8) as shown in Fig. 1.9. *Gordonia* sp. 213E and *Rhodococcus* sp. T09, which were isolated for its ability to grow in a medium with NTH as the sole source of sulfur, could desulfurize BTH but not DBT. In contrast, *Paenibacillus* sp. A11-2 could also desulfurize DBT in addition to BTH since this bacterium was isolated for its ability to grow in a medium with DBT as the sole source of sulfur. BTH is first oxidized to BTH sulfone via BTH sulfoxide. BTH sulfone is then converted to 2-(2'-hydroxyphenyl)ethen-1-sulfinate, leading to benzo[*c*][1,2]oxathiin *S*-oxide and benzo[*c*][1,2]oxathiin *S,S*-dioxide through dehydration. 2-(2'-hydroxyphenyl)ethen-1-sulfinate is finally desulfurized to 2-(2'-hydroxyphenyl)ethan-1-al, leading to benzofuran through dehydration, by *Gordonia* sp. 213E and *Rhodococcus* sp. T09, which could desulfurize BTH but not DBT. On the other hand, 2-(2'-hydroxyphenyl)ethen-1-sulfinate is finally desulfurized to *o*-hydroxystyrene by *Paenibacillus* sp. A11-2, which could desulfurize both BTH and DBT. This pathway is practical since the sulfur atom is selectively removed without reducing energy content of the molecule.

1.6. Biodesulfurization of NTH

Only one microorganism possessing naphtho[2,1-*b*]thiophene (NTH)-degrading ability, *Pseudomonas* sp. W1 [51], has been isolated to date. This bacterium utilizes NTH as the sole source of carbon and energy, and degrades NTH through a pathway with the cleavage of carbon-carbon bonds analogous to the pathway for DBT degradation (Fig. 1.6) as shown in Fig. 1.10. NTH is first oxidized at the 6,7- or 8,9-positions by dioxygenase, followed by *meta*-cleavage of the dioxygenated aromatic ring. However, in this pathway, the sulfur atom is not removed from NTH during the

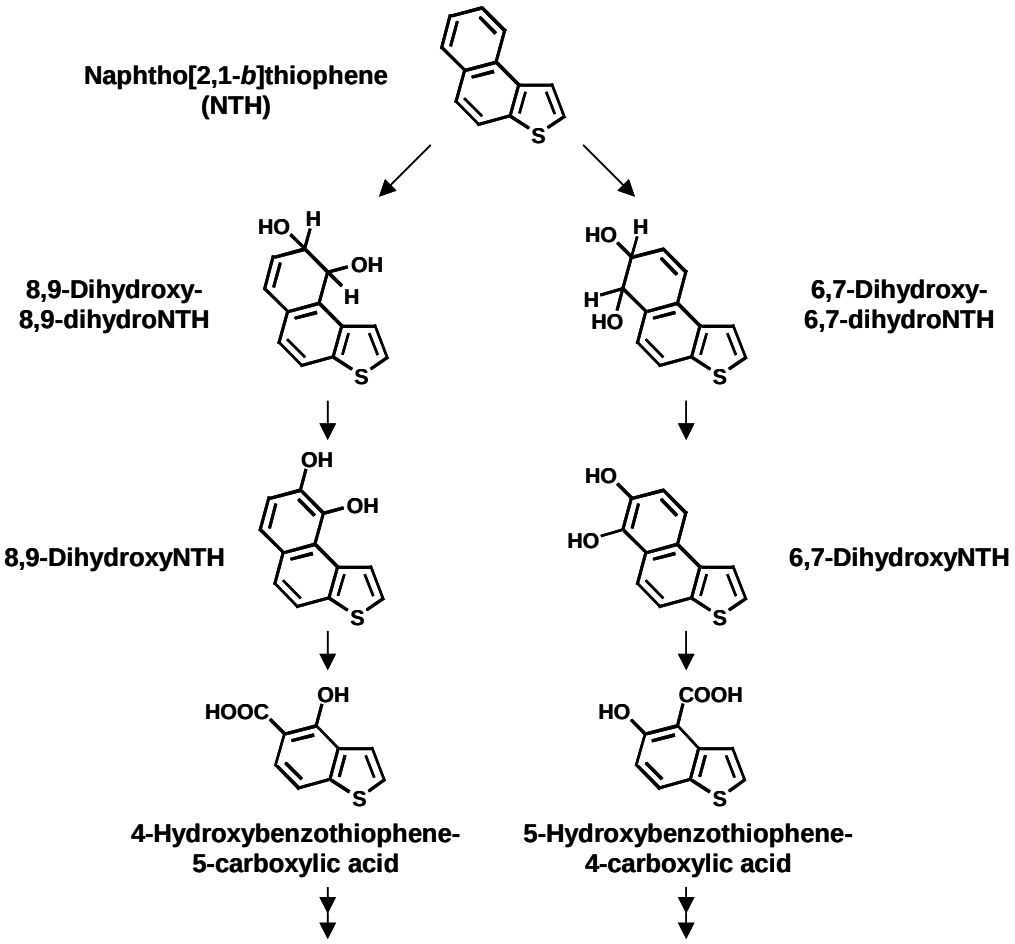


Fig 1.10. The NTH degradation pathway with the cleavage of carbon-carbon bonds.

degradation, and the carbon skeleton of NTH is destroyed with reducing energy content of the molecule.

There are no reports on microorganisms possessing the ability to desulfurize NTH through the sulfur-specific degradation pathway.

1.7. Objective of this thesis

Current HDS faces difficulties in removing complicated heterocyclic sulfur compounds. As regulations for the sulfur level in diesel oil become stricter, more of

the HDS-recalcitrant sulfur compounds must be removed from LGO. However, their removal by HDS will require more intense conditions leading to higher operating cost, energy consumption, and CO_2 emission. In contrast, biodesulfurization has the advantages of low operating cost, energy consumption, and CO_2 emission, and has attracted attention from the viewpoint of a technology complementary to current HDS, particularly after HDS, to achieve deep desulfurization of LGO. Sulfur components in LGO following HDS are confirmed to be largely DBTs and a small amount of BTHs, BNTHs, and BHNTHs. Thus, these compounds are targeted for biodesulfurization.

In this thesis, with the objective to achieve deep desulfurization of LGO using biodesulfurization, the author isolated two types of novel desulfurizing bacteria as biocatalysts applicable to deep desulfurization of LGO and characterized them.

First type is novel thermophilic DBT-desulfurizing bacteria, *Bacillus subtilis* WU-S2B and *Mycobacterium phlei* WU-F1, which are a clearly different type of bacteria from the thermophilic strain previously reported. The author examined the desulfurizing abilities of WU-S2B and WU-F1 and found that these bacteria possessed high desulfurizing abilities toward DBT and its derivatives over a wide temperature range up to 50˚C. In addition, it was found that both growing and resting cells of WU-F1 could efficiently desulfurize HDS-treated LGOs over a wide temperature range up to 50˚C. Moreover, all the genes involved in DBT desulfurization were cloned from these bacteria and characterized.

Second type is a novel NTH-desulfurizing bacterium, *Rhodococcus* sp. WU-K2R, which is the first isolate possessing the ability to selectively desulfurize NTH through sulfur-specific degradation pathways. The author examined the desulfurizing ability of WU-K2R and found that this bacterium could preferentially desulfurize asymmetric

heterocyclic sulfur compounds such as NTH and BTH through sulfur-specific degradation pathways with the selective cleavage of carbon-sulfur bonds.

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Chapter 2

Materials and Methods

2.1. Introduction

In this chapter, the author describes the materials and methods generally used for the studies in this thesis. Methods of cultivation and resting cell reaction were concisely explained. Recombinant DNA techniques were described according to the publication by Sambrook et al. [1] or manufacturer's protocols. Analytical methods to determine organosulfur compounds were also explained. Further specific and detailed explanations were described in Materials and methods in each chapter.

2.2. Bacterial strains and plasmids

2.2.1. Bacterial strains

The bacterial strains used in the present study are listed in Table 2.1. *Bacillus subtilis* WU-S2B, *Mycobacterium phlei* WU-F1, and *Rhodococcus* sp. WU-K2R were newly isolated in the present study. These bacterial strains were stored in micro-tubes containing 10% (v/v) glycerol at −80˚C.

2.2.2. Phage and plasmids

The phage and plasmids used in the present study are listed in Table 2.2.

Table 2.1. Bacterial strains used in the present study.

Strain	Relevant properties	Source or reference
Bacillus subtilis WU-S2B	Thermophilic DBT[a]-desulfurizing bacterium	Chapter 3
Mycobacterium phlei WU-F1	Thermophilic DBT[a]-desulfurizing bacterium	Chapter 3
Rhodococcus sp. WU-K2R	NTH[a]-desulfurizing bacterium	Chapter 5
Escherichia coli		
XL1-Blue MRA (P2)	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 gyrA96 relA1 lac* (P2 lysogen)	Stratagene
JM109	*recA1 endA1 gyrA96 thi-1 hsdR17*($r_k^-m_k^+$) *e14*$^-$*(mcrA*$^-$*) supE44 relA1 Δ(lac-proAB)*/F' [*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*]	Takara Bio
DH5α	F$^-$ *ø80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17*($r_k^-m_k^+$) *phoA supE44 λ*$^-$ *thi-1 gyrA96 relA1*	Takara Bio
BL21 (DE3)	F$^-$ *dcm ompT hsdS*($r_B^-m_B^-$)*gal λ* (DE3)	Novagen

[a] DBT, dibenzothiophene; NTH, naphthothiophene.

Table 2.2. Phage and plasmids used in the present study.

Phage and plasmids	Relevant properties	Source
Phage		
Lambda DASH II	Multicloning site vector	Stratagene
Plasmids		
pGEM-T	Multicloning site vector; Ap^r, *lac* promoter	Promega
pUC19	Multicloning site vector; Ap^r, *lac* promoter	Takara Bio
pUC118	Multicloning site vector; Ap^r, *lac* promoter	Takara Bio
pSTV28	Multicloning site vector; Cm^r, *lac* promoter	Takara Bio
pKK223-3	Multicloning site vector; Ap^r, *tac* promoter	Amersham
pET21-a	Multicloning site vector; Ap^r, T7 promoter	Novagen

2.3. Cultivation

2.3.1. Cultivation of *Bacillus subtilis* WU-S2B

Cultivation of *B. subtilis* WU-S2B was performed using A-2 medium shown in

Table 2.3. Each composition of metal solution and vitamin mixture is shown in Table 2.4 and Table 2.5, respectively. A-2 medium was supplemented with dibenzothiophene (DBT) or another organosulfur compound as the sole source of sulfur. Unless otherwise indicated, cultivation was performed at 50˚C with reciprocal shaking at 240 strokes per min in test tubes (18 by 180 mm) containing 5 ml of A-2 medium with DBT or another organosulfur compound.

Table 2.3. Composition of A-2 medium[a].

Ingredients	
Glucose	5.0 g
NH_4Cl	2.0 g
KH_2PO_4	6.3 g
K_2HPO_4	8.0 g
$MgCl_2 \cdot 6H_2O$	0.2 g
Metal solution[b]	2.0 ml
Vitamin mixture[c]	1.0 ml
Distilled water	to 1,000 ml

[a] The pH was adjusted to 7.0 with 2 M NaOH.
[b] Composition of metal solution is shown in Table 2.4.
[c] Composition of vitamin mixture is shown in Table 2.5.

Table 2.4. Composition of metal solution.

Ingredients	
NaCl	1.0 g
$CaCl_2$	2.0 g
$MnCl_2 \cdot 4H_2O$	0.5 g
$FeCl_2 \cdot 4H_2O$	0.5 g
$CuCl_2$	0.05 g
$ZnCl_2$	0.5 g
$Na_2MoO_4 \cdot 2H_2O$	0.1 g
$Na_2WO_4 \cdot 2H_2O$	0.05 g
10 M HCl	10 ml
Distilled water	to 1,000 ml

Table 2.5. Composition of vitamin mixture.

Ingredients	
NaCl	1.0 g
Calcium pantothenate	400 mg
Inositol	200 mg
Niacin	400 mg
Pyridoxine hydrochloride	400 mg
p-Aminobenzoic acid	200 mg
Cyanocobalamin	0.5 mg
Distilled water	to 1,000 ml

2.3.2. Cultivation of *Mycobacterium phlei* WU-F1

Cultivation of *M. phlei* WU-F1 was performed using AF medium shown in Table 2.6. AF medium was supplemented with DBT or another organosulfur compound as the sole source of sulfur. Unless otherwise indicated, cultivation was performed at 50˚C with reciprocal shaking at 240 strokes per min in test tubes (18 by 180 mm)

Table 2.6. Composition of AF medium[a].

Ingredients	
Glucose	5.0 g
NH_4Cl	1.0 g
KH_2PO_4	1.0 g
K_2HPO_4	8.0 g
$MgCl_2 \cdot 6H_2O$	0.2 g
Metal solution[b]	10.0 ml
Vitamin mixture[c]	1.0 ml
Distilled water	to 1,000 ml

[a] The pH was adjusted to 7.5 with 2 M NaOH.
[b] Composition of metal solution is shown in Table 2.4.
[c] Composition of vitamin mixture is shown in Table 2.5.

containing 5 ml of AF medium with DBT or another organosulfur compound.

2.3.3. Cultivation of *Rhodococcus* sp. WU-K2R

Cultivation of *Rhodococcus* sp. WU-K2R was performed using MG medium shown in Table 2.7. MG medium was supplemented with naphthothiophene (NTH) or another organosulfur compound as the sole source of sulfur. Unless otherwise indicated, cultivation was performed at 30˚C with reciprocal shaking at 240 strokes per min in test tubes (18 by 180 mm) containing 5 ml of MG medium with NTH or another organosulfur compound.

Table 2.7. Composition of MG medium[a].

Ingredients	
Glucose	5.0 g
NH_4Cl	1.0 g
KH_2PO_4	2.0 g
K_2HPO_4	4.0 g
$MgCl_2 \cdot 6H_2O$	0.2 g
Metal solution[b]	10.0 ml
Vitamin mixture[c]	1.0 ml
Distilled water	to 1,000 ml

[a] The pH was adjusted to 7.0 with 2 M NaOH.
[b] Composition of metal solution is shown in Table 2.4.
[c] Composition of vitamin mixture is shown in Table 2.5.

2.3.4. Cultivation of *Escherichia coli* strains

Cultivation of *E. coli* strains was mainly performed using Luria-Bertani (LB) medium shown in Table 2.8. LB medium was supplemented with appropriate antibiotics when they were required. Unless otherwise indicated, cultivation was performed at 37˚C with reciprocal shaking at 240 strokes per min in test tubes (18 by

Table 2.8. Composition of LB medium[a].

Ingredients	
Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Distilled water	to 1,000 ml

[a] The pH was adjusted to 7.0 with 2 M NaOH.

180 mm) containing 5 ml of LB medium with appropriate antibiotics. For LB medium agar plate, 1.5% (v/v) agar was added to the medium. On the other hand, NZY medium agar plate (Table 2.9) was used for cultivation of *E. coli* XL1-Blue MRA (P2) at 37˚C.

Table 2.9. Composition of NZY medium[a].

Ingredients	
NaCl	5.0 g
$MgSO_4 \cdot 7H_2O$	2.0 g
Yeast extract	5.0 g
NZ amine	10.0 g
Distilled water	to 1,000 ml

[a] The pH was adjusted to 7.5 with 2 M NaOH.

2.4. Resting cell reaction

Cultivation was performed in 500-ml flasks containing 200 ml of a medium. Cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4˚C, washed twice with a buffer, and suspended in the same buffer. The optical density at 660 nm (OD_{660})

of cell suspension was appropriately adjusted. A substrate such as DBT and NTH was added to test tubes containing cell suspension. Unless otherwise indicated, resting cell reactions were performed at 50˚C with reciprocal shaking at 180 strokes per min. For desulfurization of light gas oil, 50 ml of light gas oil was added to 100-ml flasks containing 5 ml of cell suspension. Resting cell reactions were performed at 45˚C with reciprocal shaking at 180 strokes per min.

2.5. Enzyme assays for flavin reductase activity

Flavin reductase activity was determined at 50˚C by monitoring decrease in absorbance at 340 nm due to oxidation of NAD(P)H. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.3 mM NAD(P)H, 0.01 mM FMN, and enzyme in total volume of 1 ml. One unit of activity is defined as the amount of enzyme necessary to oxidize 1 μmol of NAD(P)H per min ($\varepsilon_{340} = 6.22 \times 10^3\ M^{-1} \cdot cm^{-1}$).

2.6. Protein determination

Protein concentration was determined by using a protein assay kit (Bio-Rad, CA, USA) with a bovine serum albumin as the standard according to manufacturer's protocols.

2.7. DNA extraction

For preparation of total DNAs, WU-S2B and WU-F1 were cultivated as described in Chapter 2.3. In addition, the cells of WU-F1 were incubated at 37˚C with 50 μg/ml D-cycloserine and 100 μg/ml lysozyme, harvested by centrifugation at 6,000 × *g* for 15

min at 4˚C, and then used as the source of total DNA [2]. Total DNA was prepared with a QIAGEN genomic-tip (QIAGEN, Tokyo, Japan) according to manufacturer's protocols.

Phage DNA and plasmid DNA were prepared with a QIAGEN lambda kit (QIAGEN) and a GFX micro plasmid prep kit (Amersham Biosciences, NJ, USA), respectively, according to manufacturer's protocols.

2.8. Purification of DNA fragments

DNA fragments were purified with a GFX PCR DNA and gel band purification kit (Amersham Biosciences) according to manufacturer's protocols.

2.9. Transformation of *Escherichia coli*

E. coli strains were transformed with plasmid DNAs by electroporation (Gene Pulser; Bio-Rad). Competent cells of *E. coli* (50 μl) prepared for electroporation and 1 μl of plasmid were added to an ice-cold cuvette with a 0.2-cm gap (Bio-Rad). The cells were pulsed at 2 kV and 25 μF with the resistance set at 400 Ω, and were immediately diluted with 1 ml of SOC medium composed of 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose. Transformed cells were incubated at 37˚C for 1 h, spread onto LB medium agar plates (Table 2.8) with appropriate antibiotics, and incubated at 37˚C until colonies developed.

On the other hand, lambda DASH II phage DNA was packaged into phage *in vitro* with a Gigapack III Gold packaging kit (Stratagene, CA, USA) according to

manufacturer's protocols. Phage was propagated in *E. coli* XL1-Blue MRA (P2). Transformed cells were spread onto NZY medium agar plates (Table 2.9), and incubated at 37˚C until plaques developed.

2.10. PCR amplification

PCR amplification was performed using thermal cycler (iCycler; Bio-Rad). Reaction mixture contained PCR buffer composed of Tris-HCl, KCl, and $MgCl_2$, dNTP, DNA template, PCR primers, and DNA polymerase such as *Taq*, *Ex Taq*, and *Pyrobest* (Takara Bio, Tokyo, Japan). Each concentration in reaction mixture was determined according to manufacturer's protocols corresponding to each DNA polymerase. The reaction mixture was incubated at 95˚C for 5 min, and then subjected to 30 cycles of amplification (1 min at 95˚C for denaturation, 1 min at a temperature appropriate to PCR primers for annealing, and 1min per 1-kb DNA template at 72˚C for extension), followed by incubation at 72˚C for 10 min.

2.11. Southern hybridization

DNA probes were labeled with DIG (digoxigenin) DNA Labeling Kit (Roche, Basel, Switzerland) according to manufacturer's protocols. A DNA library on agar plates was transferred to Hybond-N+ nylon membranes (Amersham Biosciences). The membranes were hybridized with labeled DNA probes at 68˚C in hybridization buffer composed of 5 × SSC, 1% (w/v) Blocking reagent (Roche), 0.1% (w/v) *N*-lauroylsarcosine, and 0.02% (w/v) SDS. The hybridized membranes were washed twice at 25˚C in 2 × SSC with 0.1% (w/v) SDS, and then washed twice at 68˚C in 0.1 ×

SSC with 0.1% (w/v) SDS. DIG DNA probes hybridizing with target DNA interact with Anti-DIG-AP (alkaline phosphatase) conjugate (Roche), which catalyzes the reaction of 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium salt (NBT) (Roche) to blue dye precipitate.

2.12. DNA sequencing and sequence analysis

Samples for DNA sequencing were prepared with DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences). Reaction mixture contained 8 μl of Premix, 11.5 μl of DNA template, and 0.5 μl of 10 pmol/μl primer. The reaction mixture was incubated at 95˚C for 1 min, and then subjected to 30 cycles of amplification (20 sec at 95˚C, 15 sec at 50˚C, and 1 min at 60˚C), followed by incubation at 60˚C for 1 min. The resulting fragments were purified with AutoSeq G-50 (Amersham Biosciences) according to manufacturer's protocols. DNA sequencing was performed with an ABI PRISM 310 genetic analyzer (Applied Biosystems, CA, USA). Nucleotide sequence was determined by complete sequencing of both strands, with multiple sequencing of some regions.

Sequence analysis was performed with a Genetyx-Mac version 10.1 (SDC, Tokyo, Japan). Homology search of sequence was performed with the FASTA program of the DNA Data Bank of Japan (DDBJ). Phylogenetic tree based on sequence homology was constructed with the ClustalW program of DDBJ.

2.13. Analytical methods

2.13.1. Measurement of cell growth

Cell growth was measured turbidimetrically at 660 nm by using spectrophotometer (UV-1200; Shimadzu, Kyoto, Japan).

2.13.2. Measurement of aromatic compounds

Aromatic compounds were determined by using high-performance liquid chromatography (HPLC) (type LC-10A; Shimadzu) equipped with a Puresil C_{18} column (Waters, MA, USA). The mobile phase was acetonitrile-water (1:1, v/v) or acetonitrile-tetrahydrofuran-water (1:1:3, v/v/v) and the flow rate was 1.0 ml/min. The culture broth and reaction mixture were acidified to pH 2.0 with 6 M HCl and extracted with ethyl acetate. The extract was filtered through a 0.20-μm-pore-size polytetrafluoroethylene (PTFE) membrane filter (Advantec Toyo, Tokyo, Japan) for HPLC analysis. Aromatic compounds were detected spectrophotometrically at 254 nm, and the amounts of them were calculated from standard calibration curves.

The molecular structures of metabolites produced through desulfurization of hetrocyclic sulfur compounds were analyzed by using gas chromatography-mass spectrometry (GC-MS) (type 5890II; Hewlett-Packard, Ontario, Canada) equipped with a 30-m type HP-5 column (Hewlett-Packard). The carrier gas was helium and the flow rate was 1.0 ml/min. The injection temperature was maintained at 280˚C. The oven temperature was programmed to start at 40˚C, which was held for 3 min, and increased to a final temperature of 280˚C at a rate of 10˚C/min. The combined extracts were dried over anhydrous sodium sulfate, evaporated, and redissolved in ethyl acetate for GC-MS analysis.

2.13.3. Measurement of light gas oils

To determine components in light gas oils, the culture broth and reaction mixture were centrifuged at 24,000 × *g* for 1 h at 20˚C, and each oil phase was collected as the upper fraction. The oil phase was analyzed by using gas chromatography with an atomic emission detector (GC-AED) (type 5890II/5921A; Hewlett-Packard) to determine sulfur and carbon components, and by using ANTEK 7000V (Antek, TX, USA) to determine total sulfur concentration [3].

2.14. Chemicals

DBT was purchased from Tokyo Kasei (Tokyo, Japan). NTH and light gas oils were kindly supplied by the laboratory of the Japan Cooperation Center, Petroleum (Shizuoka, Japan). Tryptone, yeast extract, and agar were purchased from BD (MD, USA). All other reagents were of analytical grade and commercially available.

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Chapter 3

Thermophilic Biodesulfurization of Dibenzothiophene and Its Derivatives by *Bacillus subtilis* WU-S2B and *Mycobacterium phlei* WU-F1

3.1. Introduction

Light gas oil (LGO) for diesel engine vehicles contains large amounts of organosulfur compounds and combustion of LGO generates sulfur oxides, causing serious environment problems such as acid rain and air pollution. Today, LGO is treated by hydrodesulfurization (HDS) using metallic catalysts in the presence of hydrogen gas under extremely high temperature and pressure, but heterocyclic sulfur compounds cannot be completely removed. Dibenzothiophene (DBT) is generally recognized as a model compound for recalcitrant hetrocyclic sulfur compounds since various types of DBT derivatives are detected in LGO following HDS. In contrast, biodesulfurization using DBT-desulfurizing microorganisms as biocatalysts is a promising technology to remove organosulfur compounds in LGO under mild conditions, and has attracted attention from the viewpoint of a technology complementary to current HDS to achieve deep desulfurization of LGO (Chapter 1) [1].

Many mesophilic DBT-desulfurizing microorganisms have been isolated as desulfurizing biocatalysts to date [1], and particularly *Rhodococcus* sp. IGTS8 has been extensively studied so far [2]. These bacteria desulfurize DBT through a

sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds (Chapter 1.4) [3]. That is, DBT is first oxidized to DBT sulfone ($DBTO_2$) via DBT sulfoxide by DBT monooxygenase [4]. $DBTO_2$ is then converted to 2'-hydroxybiphenyl-2-sulfinate (HBPSi) by $DBTO_2$ monooxygenase [5], leading to cleavage of the thiophene ring. HBPSi is finally desulfurized to 2-hydroxybiphenyl (2-HBP) with the release of the sulfur atom as sulfite by HBPSi desulfinase [6]. On the other hand, thermophilic DBT-desulfurizing microorganisms may be more practical than mesophilic ones for application of biodesulfurization to high-temperature LGO immediately after HDS (Chapter 1.4.3). If biodesulfurization could be performed at high temperatures, it would be unnecessary to cool HDS-treated LGO to ambient temperatures. It was reported that a sulfur-oxidizing microorganism *Sulfolobus acidocaldarius* could oxidize the sulfur atom in DBT to sulfate at 70˚C [7], and that *Paenibacillus* sp. A11-2 could desulfurize DBT at around 50˚C through the sulfur-specific degradation pathway [8]. However, there are no reports on thermophilic DBT-desulfurizing microorganisms except these strains.

In this chapter, the author describes the thermophilic desulfurization of DBT and its derivatives by the newly isolated bacteria, *Bacillus subtilis* WU-S2B and *Mycobacterium phlei* WU-F1. The author examined the desulfurizing abilities of these bacteria and found that WU-S2B and WU-F1 possessed high desulfurizing abilities toward DBT and its derivatives over a wide temperature range up to 50˚C.

3.2. Materials and methods

3.2.1. Cultivation of *B. subtilis* WU-S2B and *M. phlei* WU-F1

Cultivation of *B. subtilis* WU-S2B and *M. phlei* WU-F1 was performed using A-2

and AF media (Chapter 2.3), respectively. The media were supplemented with 0.54 mM (100 ppm) DBT or one of its derivatives, i.e., 2,8-dimethyl-DBT, 4,6-dimethyl-DBT, and 3,4-benzo-DBT (see Fig. 3.3), as the sole source of sulfur. To suspend DBT and its derivatives in the media, 9.1% (v/v) *n*-tridecane (0.5 ml *n*-tridecane to 5 ml medium) and 17% (v/v) *n*-tridecane (1 ml *n*-tridecane to 5 ml medium) were added to A-2 and AF media, respectively. Cultivation was performed at 50˚C with reciprocal shaking at 240 strokes per min in test tubes (18 by 180 mm) containing 5 ml of A-2 or AF medium with DBT or one of its derivatives and *n*-tridecane. Single-colony isolation was performed on Luria-Bertani (LB) medium agar plates (Chapter 2.3.4).

3.2.2. Resting cell reaction

Cultivation was performed at 45˚C in 500-ml flasks containing 200 ml of A-2 or AF medium with 0.27 mM DBT and 0.50% (v/v) *n*-tridecane over 69 h for WU-S2B and 45 h for WU-F1. Cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4˚C, washed twice with 0.1 M potassium phosphate buffer (pH 7.0 for WU-S2B and pH 7.6 for WU-F1), and suspended in the same buffer. The optical density at 660 nm (OD_{660}) of cell suspension was adjusted to 40 for WU-S2B and 50 for WU-F1. Reaction mixture contained 0.6 ml of the cell suspension, 0.81 mM (150 ppm) DBT or one of its derivatives, and 90 µl *n*-tridecane for WU-S2B and 9 µl *n*-tridecane for WU-F1. Resting cell reactions were performed in L-formed test tubes with reciprocal shaking at 140 strokes per min for WU-S2B and 180 strokes per min for WU-F1.

3.2.3. Analytical methods

Cell growth was measured turbidimetrically at 660 nm (Chapter 2.13.1). DBT, its derivatives, and 2-HBP were determined by using high-performance liquid chromatography (HPLC) (Chapter 2.13.2). The molecular structures of metabolites produced through desulfurization of DBT and its derivatives were analyzed by using gas chromatography-mass spectrometry (GC-MS) (Chapter 2.13.2).

3.2.4. Chemicals

DBT, 2-HBP, and 2,8-dimethyl-DBT were purchased from Tokyo Kasei (Tokyo, Japan). $DBTO_2$ and 3,4-benzo-DBT (benzo[*b*]naphtho[2,1-*d*]thiophene) were purchased from Aldrich (WI, USA). 4,6-Dimethyl-DBT was kindly supplied by the laboratory of the Japan Cooperation Center, Petroleum (Shizuoka, Japan). All other reagents were of analytical grade and commercially available.

3.3. Results

3.3.1. Identification of thermophilic DBT-desulfurizing bacteria, WU-S2B and WU-F1

To isolate thermophilic DBT-desulfurizing microorganisms, approximately 1000 samples of soil, waste water, and oil sludge in Japan were collected as sources of microorganisms. A small amount of each sample was suspended in distilled water at an appropriate concentration, and 0.2 ml of this suspension was inoculated into a test tube containing 5 ml of A-2 or AF medium with DBT and cultivated at 50˚C for 3 to 5 days. Aliquots of some turbid cultures were then transferred into fresh medium. After 5 subcultivations, the culture broth was appropriately diluted with distilled water

and spread onto LB medium agar plates. After cultivation at 50˚C for 3 to 5 days, colonies formed on the plates were again inoculated into liquid A-2 or AF medium with DBT. By means of repeating cultivation in liquid A-2 or AF medium and single-colony isolation on LB medium agar plates, strains showing stable growth at 50˚C in the media with DBT as the sole source of sulfur were selected. Among the strains, two bacteria, WU-S2B for A-2 medium and WU-F1 for AF medium, were chosen for further studies.

WU-S2B (Fig. 3.1A) was a rod-shaped bacterium with the dimensions of 0.6 to 0.8 μm by 2.5 to 4.0 μm. This strain was Gram-positive, catalase-positive, and oxidase-negative and formed spores. Further taxonomical identification of WU-S2B was performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany), and a partial sequence of the 16S ribosomal DNA of WU-S2B was found to have 99.8% identity to that of the type strain of *B. subtilis*. From these results, WU-S2B was identified as *B. subtilis*.

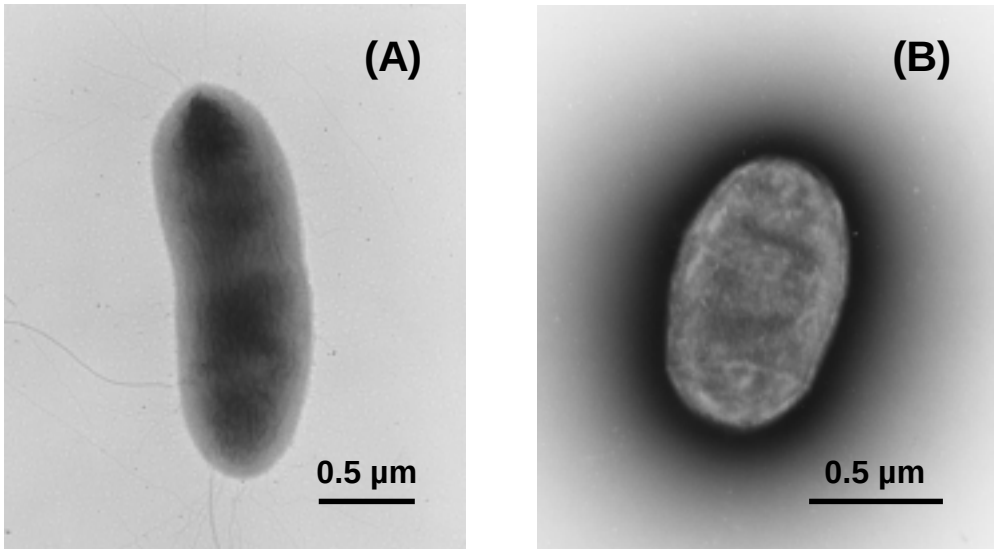

Fig. 3.1. Electron micrographs of a *B. subtilis* WU-S2B cell (A) and a *M. phlei* WU-F1 cell (B). Scale bar, 0.5 μm.

WU-F1 (Fig. 3.1B) was a rod-shaped bacterium with dimensions of 0.5 to 1.0 μm by 1.5 to 2.0 μm. This strain was Gram-positive, catalase-positive, and oxidase-negative and did not form spores. Further taxonomical identification of WU-F1 was performed by DSMZ. The fatty acid pattern of WU-F1 was typical of the genus *Mycobacterium*, and the mycolic acid pattern of this strain was similar to that of the type strain of *M. phlei*. In addition, a partial sequence of the 16S ribosomal DNA of WU-F1 was found to have 100% identity to that of the type strain of *M. phlei*. From these results, WU-F1 was identified as *M. phlei*.

3.3.2. Growth characteristics on DBT

B. subtilis WU-S2B and *M. phlei* WU-F1 grew at 50˚C in the media with DBT as the sole source of sulfur and produced one predominant metabolite which showed the same retention time as authentic 2-HBP by HPLC analysis. In addition, since by GC-MS analysis the mass spectrum of this metabolite corresponded with that of authentic 2-HBP (data not shown), it was identified as 2-HBP. WU-S2B and WU-F1 also utilized $DBTO_2$ instead of DBT as the sole source of sulfur and produced 2-HBP (data not shown). Therefore, it was concluded that WU-S2B and WU-F1 desulfurized DBT through the sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds as previously reported for other DBT-desulfurizing bacteria [3]. The time courses of DBT desulfurization at 50˚C by growing cells of WU-S2B and WU-F1 are shown in Fig. 3.2. WU-S2B degraded 0.54 mM DBT within 5 days to produce 2-HBP (Fig. 3.2A) and WU-F1 degraded 0.54 mM DBT within 3 days (Fig. 3.2B). On the other hand, it was confirmed that these strains did not utilize DBT as the sole source of carbon nor as the sole source of carbon and sulfur (data not shown). It

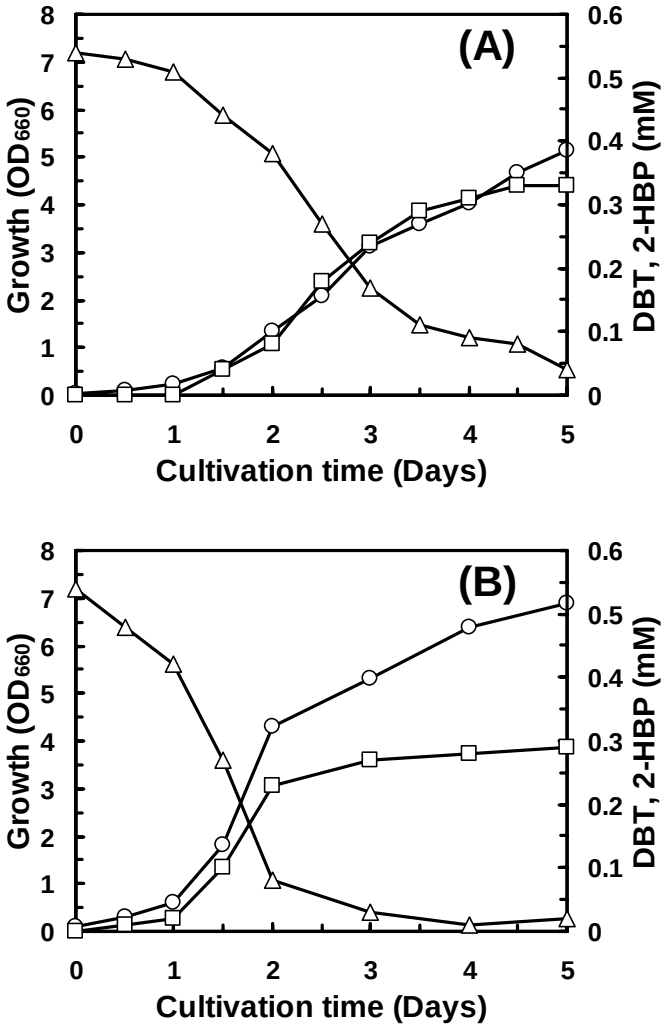

Fig. 3.2. Time courses of DBT desulfurization at 50˚C by growing cells of *B. subtilis* WU-S2B (A) and *M. phlei* WU-F1 (B). WU-S2B and WU-F1 were cultivated in A-2 and AF media, respectively, with 0.54 mM DBT as the sole source of sulfur. Symbols: ○, growth; △, DBT; □, 2-HBP.

was also confirmed that these strains did not utilize 2-HBP as the sole source of carbon (data not shown).

3.3.3. Desulfurization of DBT derivatives

Various types of DBT derivatives are detected in LGO following HDS [1]. Although commercially available DBT derivatives are limited, 2,8-dimethyl-DBT,

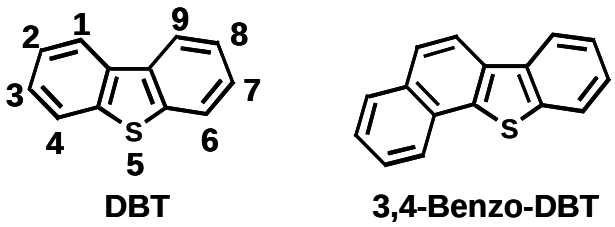

Fig. 3.3. Molecular structures of DBT with ring numbering and 3,4-benzo-DBT.

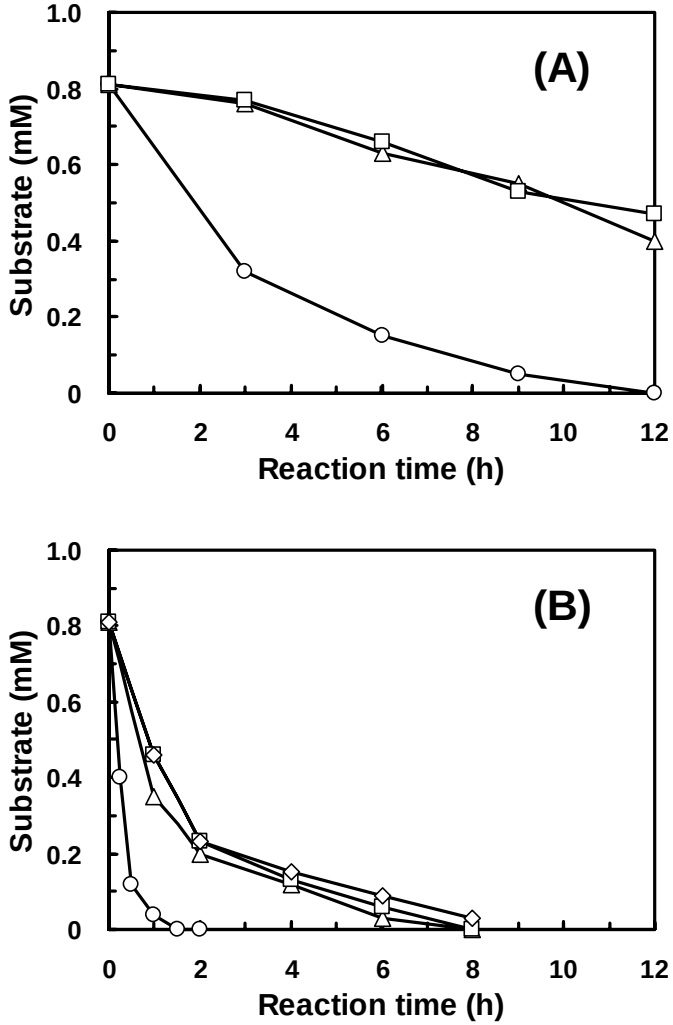

Fig. 3.4. Time courses of degradation of DBT derivatives at 50˚C by resting cells of *B. subtilis* WU-S2B (A) and *M. phlei* WU-F1 (B). The reaction mixture contained 0.6 ml of the cell suspension, 0.81 mM DBT or one of its derivatives, and *n*-tridecane. Symbols: ○ , DBT; △ , 2,8-dimethyl-DBT; □ , 4,6-dimethyl-DBT; ◇ , 3,4-benzo-DBT.

4,6-dimethyl-DBT, and 3,4-benzo-DBT (Fig. 3.3) were tested. *B. subtilis* WU-S2B and *M. phlei* WU-F1 grew at 50˚C in the media with 2,8-dimethyl-DBT, 4,6-dimethyl-DBT, or 3,4-benzo-DBT as the sole source of sulfur (data not shown). Then, the time courses of degradation of DBT derivatives at 50˚C by resting cells of WU-S2B and WU-F1 were examined and shown in Fig. 3.4. WU-S2B degraded 0.81 mM DBT within 12 h, and also degraded approximately 50% of 2,8-dimethyl-DBT and 4,6-dimethyl-DBT (Fig. 3.4A). In addition, it was confirmed that WU-S2B exhibited desulfurizing activity toward 3,4-benzo-DBT (data not shown). On the other hand, WU-F1 degraded 0.81 mM DBT within 90 min, and also degraded 0.81 mM of 2,8-dimethyl-DBT, 4,6-dimethyl-DBT, and 3,4-benzo-DBT within 8 h (Fig. 3.4B). By GC-MS analysis, the mass spectra of the metabolites produced through desulfurization of 2,8-dimethyl-DBT, 4,6-dimethyl-DBT, and 3,4-benzo-DBT revealed that these metabolites were the compounds including a monohydroxybiphenyl structure, corresponding to 2-HBP produced through DBT desulfurization (data not shown).

3.3.4. Effects of temperature on DBT desulfurization

To investigate the potential for thermophilic biodesulfurization, the effects of temperature on DBT-desulfurizing activities of *B. subtilis* WU-S2B and *M. phlei* WU-F1 were examined using resting cells of these strains. As shown in Fig. 3.5A, WU-S2B and WU-F1 exhibited DBT-desulfurizing activities over a wide temperature range up to 50˚C. DBT-desulfurizing activity of WU-F1 was much higher than that of WU-S2B. Resting cells of WU-F1 exhibited high DBT-degrading activity over a wide temperature range of 20 to 52˚C and at the highest level at 50˚C. On the other hand, the resting cells produced 2-HBP at the highest level at 45˚C. Temperature

dependence of DBT-desulfurizing activity of WU-F1 was compared with those of the mesophilic DBT-desulfurizing bacterium *Rhodococcus* sp. IGTS8 and the thermophilic DBT-desulfurizing bacterium *Paenibacillus* sp. A11-2 [8]. As shown in Fig. 3.5B, WU-F1 was found to exhibit DBT-desulfurizing activity over a wide temperature range

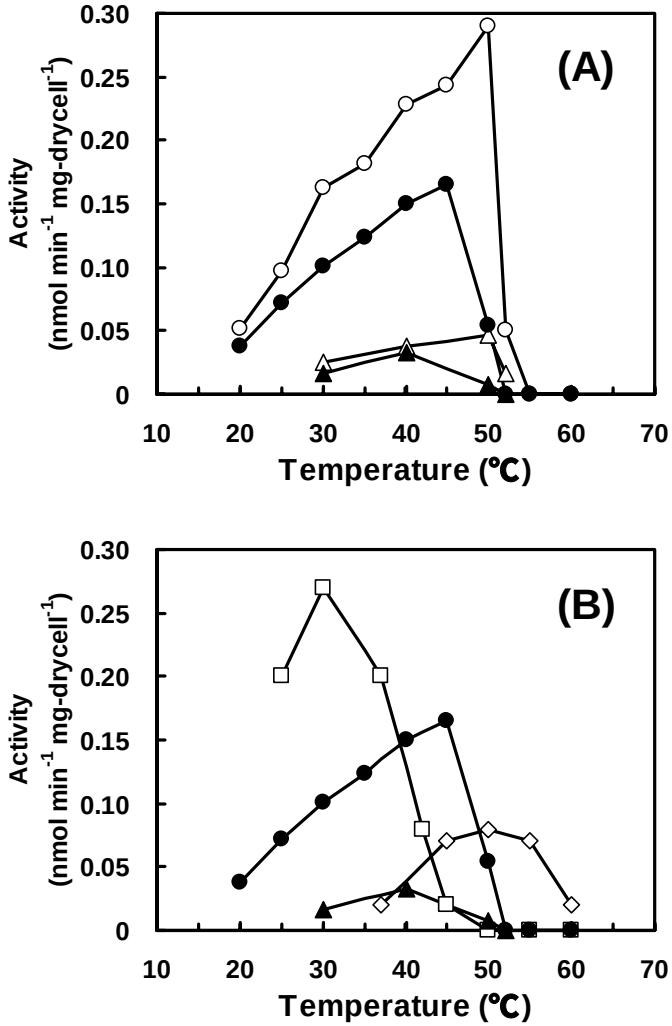

Fig. 3.5. Effects of temperature on DBT-desulfurizing activities of *B. subtilis* WU-S2B and *M. phlei* WU-F1 (A) and comparison of them with those of other bacteria (B). In A, the reaction mixture was the same as outlined in the legend to Fig. 3.4. The resting cell reaction was performed at various temperatures over 6 h for WU-S2B and 1 h for WU-F1. DBT-degrading activities (△for WU-S2B and ○ for WU-F1) and 2-HBP-producing activities (▲ for WU-S2B and ● for WU-F1) are shown. In B, 2-HBP-producing activities of *Rhodococcus* sp. IGTS8 (□) and *Paenibacillus* sp. A11-2 (◇) were taken from [8].

covering mesophilic *Rhodococcus* sp. IGTS8 and thermophilic *Paenibacillus* sp. A11-2. Particularly, WU-F1 exhibited much higher DBT-desulfurizing activity at around 45˚C than the other bacteria.

3.4. Discussion

In this chapter, the author described the thermophilic desulfurization of DBT and its derivatives by the newly isolated bacteria, *B. subtilis* WU-S2B and *M. phlei* WU-F1. Both *Bacillus* and *Mycobacterium* are gram-positive, but these genera are entirely distinct phylogenetically from each other. Most of mesophilic DBT-desulfurizing bacteria reported are coryneform bacteria such as *Rhodococcus* strains [1], and only one thermophilic DBT-desulfurizing bacterium reported is a *Paenibacillus* strain [8], which is closely related to the genus *Bacillus*. *M. phlei* WU-F1 is the first isolate as a thermophilic coryneform bacterium possessing DBT-desulfurizing ability. It is interesting to note that most of DBT-desulfurizing microorganisms are Gram-positive bacteria, particularly coryneform and bacillus-type bacteria.

WU-S2B and WU-F1 desulfurized DBT through the sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds as previously reported for the other DBT-desulfurizing bacteria (Chapter 1.4) [3]. This pathway is practical since the sulfur atom is selectively removed without reducing energy content of the molecule and the removal of the sulfur atom is not affected much by the alkyl substitutions on the molecule. Through the experiments, WU-S2B and WU-F1 efficiently desulfurized alkylated DBTs such as 2,8-dimethyl-DBT and 4,6-dimethyl-DBT (Fig. 3.4). As for *R. erythropolis* H-2, the initial reaction rate for 4,6-dimethyl-DBT was approximately 50% of that for 2,8-dimethyl-DBT [9]. On the other hand, as for WU-S2B and WU-F1, the

initial reaction rate for 4,6-dimethyl-DBT was almost the same as that for 2,8-dimethyl-DBT (Fig. 3.4), indicating that the substrate specificities of WU-S2B and WU-F1 were different from that of *R. erythropolis* H-2.

WU-S2B and WU-F1 exhibited DBT-desulfurizing activities over a wide temperature range up to 50˚C (Fig. 3.5A). Particularly, WU-F1 exhibited much higher DBT-desulfurizing activity than the other DBT-desulfurizing bacteria (Fig. 3.5B). The cell surfaces of *Mycobacterium* strains possessing mycolic acids with many carbon atoms are more hydrophobic than those of the other bacteria lacking mycolic acids [10], and this property might be advantageous for *M. phlei* WU-F1 to take up hydrophobic compounds such as DBT and its derivatives. As previously reported, the thermophilic bacterium *Paenibacillus* sp. A11-2 exhibited high DBT-desulfurizing activity over a temperature range of 45 to 55˚C, but the activity decreased at around 30˚C [8]. In contrast, WU-F1 exhibited high DBT-desulfurizing activity even at 30˚C (Fig. 3.5).

In conclusion, the author confirmed that the newly isolated bacteria *B. subtilis* WU-S2B and *M. phlei* WU-F1 possessed high desulfurizing abilities toward DBT and its derivatives over a wide temperature range up to 50˚C. Particularly, WU-F1 exhibited much higher DBT-desulfurizing activity than any other DBT-desulfurizing bacteria previously reported. These results suggest that WU-F1 may be a promising biocatalyst for practical biodesulfurization.

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Chapter 4

Thermophilic Biodesulfurization of Naphthothiophene and 2-Ethylnaphthothiophene by *Mycobacterium phlei* WU-F1

4.1. Introduction

Dibenzothiophene (DBT) is generally recognized as a model compound for recalcitrant hetrocyclic sulfur compounds since many types of DBT derivatives are detected in light gas oil (LGO) following hydrodesulfurization (HDS) [1]. In Chapter 3, the author described that the newly isolated bacteria *Bacillus subtilis* WU-S2B and *Mycobacterium phlei* WU-F1 possessed high desulfurizing abilities toward DBT and its derivatives over a wide temperature range up to 50˚C. Moreover, it has recently become apparent that in addition to DBT derivatives, naphtho[2,1-*b*]thiophene (NTH) derivatives (see Fig. 4.3) are also detected in LGO following HDS although NTH derivatives are minor components in comparison with DBT derivatives (unpublished data). Therefore, NTH, which is an asymmetric structural isomer of DBT, may also be a model target compound for deep desulfurization (Chapter 1) [2].

Although many DBT-desulfurizing microorganisms have been isolated as desulfurizing biocatalysts to date [1], there are no data related to NTH desulfurization by the bacteria which can desulfurize DBT and/or benzothiophene [3-5]. There is only one report on NTH degradation by *Pseudomonas* sp. W1 (Chapter 1.6) [2]. However, this bacterium utilized NTH as the sole source of carbon and energy with reducing

energy content of the molecule, and the sulfur atom was not removed from NTH during the degradation.

In this chapter, the author describes the thermophilic desulfurization of NTH and 2-ethyl-NTH by the DBT-desulfurizing bacterium *M. phlei* WU-F1 (Chapter 3). The author examined the desulfurizing ability of this bacterium and found that WU-F1 could efficiently desulfurize asymmetric organosulfur compounds, NTH and 2-ethyl-NTH, in addition to symmetric DBT derivatives through a sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds under high-temperature conditions. This is the first report describing the biodesulfurization of NTH and 2-ethyl-NTH through the sulfur-specific degradation pathway.

4.2. Materials and methods

4.2.1. Cultivation of *M. phlei* WU-F1

Cultivation of *M. phlei* WU-F1 was performed using AF medium (Chapter 2.3.2). The medium was supplemented with 0.27 mM (corresponding to 50 ppm NTH) of each heterocyclic sulfur compound, i.e., NTH, 2-ethyl-NTH, DBT, and 4,6-dimethyl-DBT, as the sole source of sulfur. To suspend heterocyclic sulfur compounds in the medium, 1% (v/v) *n*-tridecane was added. Cultivation was performed at 50˚C with reciprocal shaking at 240 strokes per min in test tubes (18 by 180 mm) containing 5 ml of the medium with each heterocyclic sulfur compound and *n*-tridecane.

4.2.2. Resting cell reaction

Cultivation was performed at 45˚C in 500-ml flasks containing 200 ml of the medium with 0.27 mM DBT or 0.1 g/l Na_2SO_4 as the sole source of sulfur and 0.50%

(v/v) *n*-tridecane for 45 h. Cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4˚C, washed twice with 0.1 M potassium phosphate buffer (pH 7.6), and suspended in the same buffer. The optical density at 660 nm (OD_{660}) of cell suspension was adjusted to 50. DBT or one of its derivatives and 9 µl *n*-tridecane were added to L-formed test tubes containing 0.6 ml of the cell suspension to a final concentration of 0.81 mM (150 ppm) DBT and its derivatives. Resting cell reactions were performed at 50˚C with reciprocal shaking at 180 strokes per min.

4.2.3. Analytical methods

Cell growth was measured turbidimetrically at 660 nm (Chapter 2.13.1). Heterocyclic sulfur compounds were determined by using high-performance liquid chromatography (HPLC) (Chapter 2.13.2). The molecular structures of metabolites produced through 2-ethyl-NTH desulfurization were analyzed by using gas chromatography-mass spectrometry (GC-MS) (Chapter 2.13.2).

4.2.4. Chemicals

DBT was purchased from Tokyo Kasei (Tokyo, Japan). NTH, 2-ethyl-NTH, and 4,6-dimethyl-DBT were kindly supplied by the laboratory of the Japan Cooperation Center, Petroleum (Shizuoka, Japan). All other reagents were of analytical grade and commercially available.

4.3. Results

4.3.1. Desulfurization of NTH and 2-ethyl-NTH

M. phlei WU-F1 grew at 50˚C in AF medium with NTH or 2-ethyl-NTH as the

sole source of sulfur. An optical density at 660 nm indicating growth of WU-F1 with NTH reached 1.0 for 5 days and that with 2-ethyl-NTH reached 3.0 for 2 days. Growing cells of WU-F1 degraded 39% of 0.27 mM NTH within 5 days. In addition, the growing cells degraded 64% of 0.27 mM 2-ethyl-NTH within 2 days. These results indicated that WU-F1 exhibited a faster growth rate and higher degrading activity for 2-ethyl-NTH than for NTH. Moreover, the growth rate and the degrading activity of WU-F1 for 2-ethyl-NTH were comparable to those for DBT and 4,6-dimethyl-DBT (data not shown).

The time course of degradation of heterocyclic sulfur compounds at 50˚C by resting cells of WU-F1 was examined. As shown in Fig. 4.1, resting cells of WU-F1 grown with DBT degraded 67 and 83% of 0.81 mM NTH and 2-ethyl-NTH, respectively, within 8 h. These results indicated that the desulfurizing enzymes

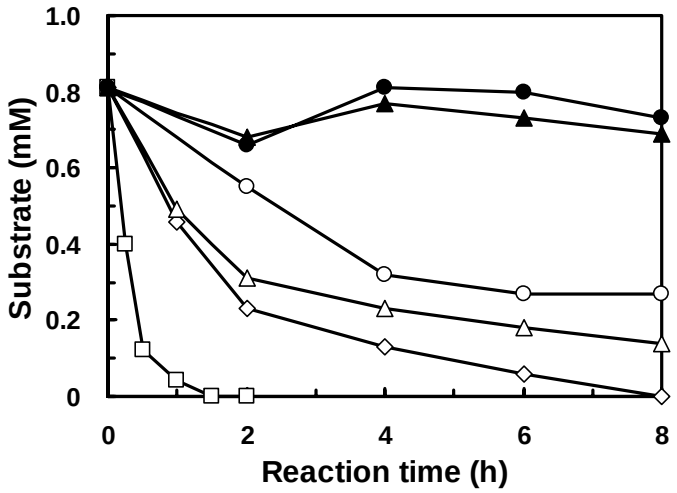

Fig. 4.1. Time course of degradation of heterocyclic sulfur compounds at 50˚C by resting cells of *M. phlei* WU-F1. The resting cells were prepared through cultivation in AF medium with DBT (○, △, □, ◇) or Na_2SO_4 (●, ▲) as the sole source of sulfur. The reaction mixture contained 0.6 ml of the cell suspension (OD_{660} = 50), 0.81 mM of each heterocyclic sulfur compound, and 9 μl *n*-tridecane. Symbols: ○ and ●, NTH; △ and ▲, 2-ethyl-NTH; □, DBT; ◇, 4,6-dimethyl-DBT.

produced through the cultivation with DBT also catalyzed the desulfurization of both NTH and 2-ethyl-NTH. In contrast, the resting cells grown with Na_2SO_4 instead of DBT as the sole source of sulfur degraded neither NTH nor 2-ethyl-NTH, indicating that the enzymes necessary for the desulfurization of NTH and 2-ethyl-NTH were not produced through the cultivation with Na_2SO_4. In resting cell reaction, the degrading activity for 2-ethyl-NTH was lower than those for DBT and 4,6-dimethyl-DBT.

4.3.2. 2-Ethyl-NTH-desulfurizing pathway

M. phlei WU-F1 was cultivated to the end of exponential growth phase in AF medium with 2-ethyl-NTH as the sole source of sulfur, and the culture broth was extracted with ethyl acetate. The resulting extract containing metabolites produced

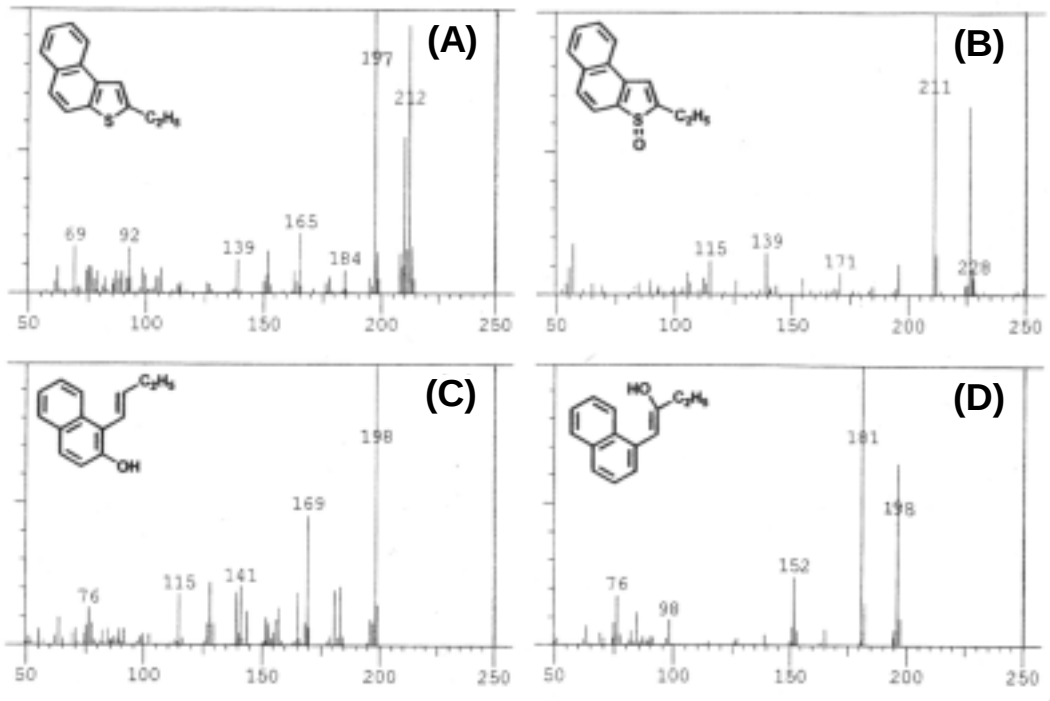

Fig. 4.2. GC-MS analysis of desulfurized 2-ethyl-NTH metabolites produced by *M. phlei* WU-F1. (A), 2-Ethyl-NTH; (B), 2-ethyl-NTH sulfoxide; (C), 1-(2'-hydroxynaphthyl)-1-butene; (D), 1-naphthyl-2-hydroxy-1-butene.

through 2-ethyl-NTH desulfurization was analyzed by GC-MS. As shown in Fig. 4.2, three compounds were detected as metabolites, in addition to 2-ethyl-NTH (M^+, m/z = 212) (Fig. 4.2A). Since standard compounds were commercially unavailable, the molecular structures of metabolites were deduced from these mass spectra. One metabolite was assigned to 2-ethyl-NTH sulfoxide (M^+, m/z = 228) (Fig. 4.2B). The high-abundance fragment ion at m/z = 211 corresponds to loss of the oxygen atom from the molecular ion. The other metabolites (Fig. 4.2C,D) were considered to include no sulfur atom in their molecular structures. One was assigned to 1-(2'-hydroxynaphthyl)-1-butene (M^+, m/z = 198) (Fig. 4.2C). The fragment ion at m/z = 169 corresponds to

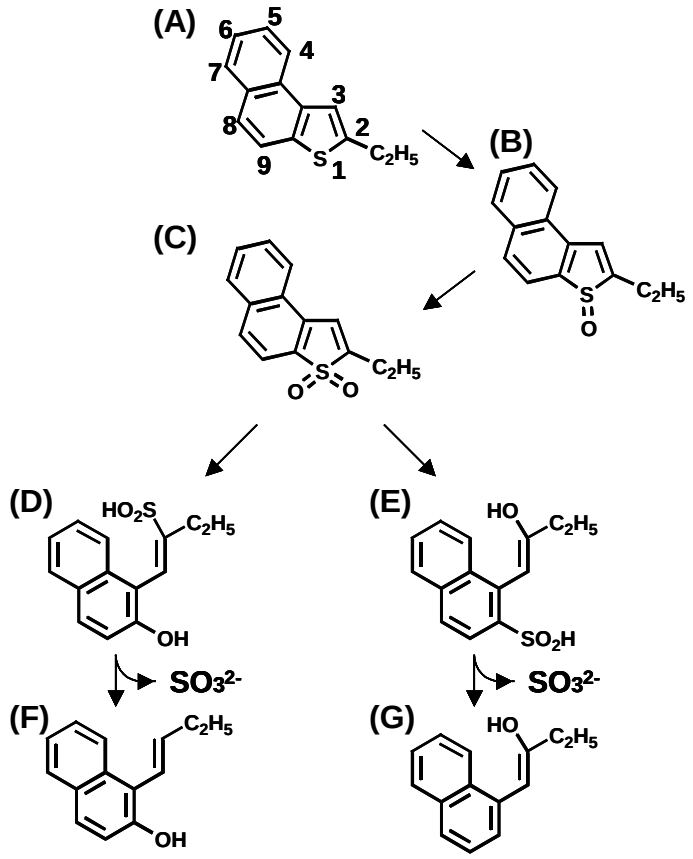

Fig. 4.3. Proposed pathway of 2-ethyl-NTH desulfurization by *M. phlei* WU-F1. Compounds (C), (D), and (E) were not identified and are indicated as postulated metabolites. Ring numbering of NTH is also shown.

loss of the ethyl group from the molecular ion, and the fragment ion at m/z = 141 corresponds to further loss of the vinyl group. The other was assigned to 1-naphthyl-2-hydroxy-1-butene (M^+, m/z = 198) (Fig. 4.2D). The high-abundance fragment ion at m/z = 181 might be due to loss of the hydroxyl group from the molecular ion, and the fragment ion at m/z = 152 might be due to further loss of the ethyl group.

On the other hand, desulfurized 2-ethyl-NTH metabolites were not detected when WU-K2R was cultivated in AF medium with Na_2SO_4 instead of NTH as the sole source of sulfur (data not shown). Based on the deduced structures of these metabolites and DBT-desulfurizing pathway previously reported [3, 4], the pathway for 2-ethyl-NTH desulfurization by WU-F1 was proposed as shown in Fig. 4.3. It was concluded that WU-F1 desulfurized 2-ethyl-NTH through the sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds.

4.4. Discussion

In this chapter, the author described the thermophilic desulfurization of NTH and 2-ethyl-NTH by the DBT-desulfurizing bacterium *M. phlei* WU-F1. This is the first report describing the biodesulfurization of NTH and 2-ethyl-NTH through the sulfur-specific degradation pathway (Fig. 4.3). This pathway is practical since the sulfur atom is selectively removed without reducing energy content of the molecules and the removal of the sulfur atom is not affected much by the alkyl substitutions on the molecules. WU-F1, which possessed high desulfurizing ability toward DBT and its derivatives under high-temperature conditions (Chapter 3), could also desulfurize NTH and 2-ethyl-NTH efficiently at 50˚C (Fig. 4.1). From the viewpoint of practical

applications (Chapter 1.4.3), biodesulfurization at around 50˚C of the HDS-treated LGO containing various types of DBT and NTH derivatives is advantageous since it would be unnecessary to cool high-temperature LGO immediately after HDS to ambient temperatures.

It is interesting to note that WU-F1 exhibited a faster growth rate and higher degrading activity for 2-ethyl-NTH than for NTH (Fig. 4.1). In comparison with NTH, ethyl group at the 2-position of 2-ethyl-NTH might increase the affinity of substrate recognition by the desulfurizing enzymes or substrate uptake by the cells, leading to a faster growth rate and higher degrading activity for 2-ethyl-NTH than for NTH. This property might be practical since ethyl group at the 2-position of 2-ethyl-NTH seems to prevent metallic catalysts from interacting with the sulfur atom due to steric hindrance through HDS.

We confirmed that WU-F1 produced only one metabolite with no sulfur, 2-hydroxybiphenyl from a symmetric DBT. In addition, WU-F1 also produced only one metabolite with no sulfur from an asymmetric 3,4-benzo-DBT (Chapter 3) as reported for *R. erythropolis* H-2 [6]. In contrast, it is interesting to note that WU-F1 produced two metabolites with no sulfur (Fig. 4.3F and 4.3G) from an asymmetric 2-ethyl-NTH, probably due to the distinct recognition of two types of carbon-sulfur bonds in the postulated metabolite, 2-ethyl-NTH sulfone (Fig. 4.3C), by the desulfurizing enzyme(s). However, no pronounced metabolites after NTH desulfurization were detected by GC-MS analysis (data not shown). Although the author does not have a clear explanation for this, it might be due to the lability of desulfurized NTH metabolites. However, since WU-F1 utilized NTH as the sole source of sulfur but not as the sole source of carbon (data not shown), it is presumed

that NTH was also desulfurized through the sulfur-specific degradation pathway.

In conclusion, the author confirmed that *M. phlei* WU-F1 could efficiently desulfurize asymmetric organosulfur compounds, NTH and 2-ethyl-NTH, in addition to symmetric DBT derivatives through the sulfur-specific degradation pathway under high-temperature conditions. These results suggest that WU-F1 may be a promising desulfurizing biocatalyst possessing a broad substrate specificity toward organosulfur compounds.

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Chapter 5

Biodesulfurization of Naphthothiophene and Benzothiophene by *Rhodococcus* sp. WU-K2R

5.1. Introduction

It has recently become apparent that in addition to dibenzothiophene (DBT) derivatives, naphtho[2,1-*b*]thiophene (NTH, see Fig. 5.4B) derivatives are also detected in light gas oil (LGO) following hydrodesulfurization (HDS) although NTH derivatives are minor components in comparison with DBT derivatives (unpublished data). Therefore, NTH, which is an asymmetric structural isomer of DBT, may also be a model target compound for deep desulfurization (Chapter 1) [1].

In Chapter 4, the author described that the thermophilic DBT-desulfurizing bacterium *M. phlei* WU-F1 could also desulfurize NTH and 2-ethyl-NTH through a sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds. This pathway is practical since the sulfur atom is selectively removed without reducing energy content of the molecules and the removal of the sulfur atom is not affected much by the alkyl substitutions on the molecules. However, there are no other reports on microorganisms possessing NTH-desulfurizing ability although the author found the sulfur-specific degradation pathway for NTH and 2-ethyl-NTH in *M. phlei* WU-F1.

In this chapter, the author describes the desulfurization of NTH and benzothiophene (BTH, see Fig. 5.4A) by the newly isolated bacterium, *Rhodococcus* sp.

WU-K2R. The author examined the desulfurizing ability of this bacterium and found that WU-K2R could preferentially desulfurize asymmetric heterocyclic sulfur compounds such as NTH and BTH through sulfur-specific degradation pathways with the selective cleavage of carbon-sulfur bonds.

5.2. Materials and methods

5.2.1. Cultivation of *Rhodococcus* sp. WU-K2R

Cultivation of *Rhodococcus* sp. WU-K2R was performed using MG medium (Chapter 2.3.3). The medium was supplemented with 0.27 mM (50 ppm) NTH or each heterocyclic sulfur compound, i.e., NTH sulfone ($NTHO_2$), BTH, 3-methyl-BTH, 5-methyl-BTH, DBT, DBT sulfone ($DBTO_2$), and 4,6-dimethyl-DBT, as the sole source of sulfur. To suspend heterocyclic sulfur compounds in the medium, 1% (v/v) ethanol or *N,N*-dimethylformamide was added. Cultivation was performed at 30˚C with reciprocal shaking at 240 strokes per min in test tubes (18 by 180 mm) containing 5 ml of the medium with each heterocyclic sulfur compound and ethanol or *N,N*-dimethylformamide. Single-colony isolation was performed on Luria-Bertani (LB) medium agar plates (Chapter 2.3.4).

5.2.2. Analytical methods

Cell growth was measured turbidimetrically at 660 nm (Chapter 2.13.1). Heterocyclic sulfur compounds were determined by using high-performance liquid chromatography (HPLC) (Chapter 2.13.2). The molecular structures of metabolites produced through desulfurization of NTH and BTH were analyzed by using gas chromatography-mass spectrometry (GC-MS) (Chapter 2.13.2).

5.2.3. Chemicals

BTH and DBT were purchased from Tokyo Kasei (Tokyo, Japan). 3-Methyl-BTH and 5-methyl-BTH were purchased from Lancaster (NH, USA). $DBTO_2$ was purchased from Aldrich (WI, USA). Benzofuran (BFU) was purchased from Wako (Osaka, Japan). NTH, $NTHO_2$, 4,6-dimethyl-DBT, and naphtho[2,1-*b*]furan (NFU) were kindly supplied by the laboratory of the Japan Cooperation Center, Petroleum (Shizuoka, Japan). The purity of these heterocyclic compounds was more than 95% except $NTHO_2$. The purity of $NTHO_2$ was slightly low (90%), but no sulfur compound except $NTHO_2$ was detected in the reagent. All other reagents were of analytical grade and commercially available.

5.3. Results

5.3.1. Identification of NTH-desulfurizing bacterium, WU-K2R

To isolate NTH-desulfurizing microorganisms, approximately 1000 samples of soil, waste water, and oil sludge in Japan were collected as sources of microorganisms. A small amount of each sample was suspended in distilled water at an appropriate concentration, and 0.2 ml of this suspension was inoculated into a test tube containing 5 ml of MG medium with NTH and cultivated at 30˚C for 3 to 5 days. Aliquots of some turbid cultures were then transferred into fresh medium. After 5 subcultivations, the culture broth was appropriately diluted with distilled water and spread onto LB medium agar plates. After cultivation at 30˚C for 3 to 5 days, colonies formed on the plates were again inoculated into liquid MG medium with NTH. By means of repeating cultivation in liquid MG medium and single-colony isolation on LB medium agar

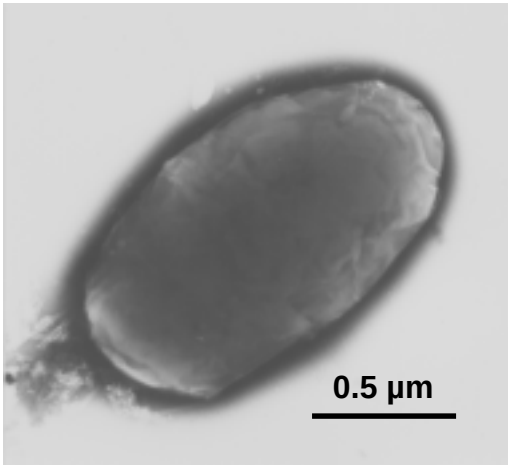

Fig. 5.1. Electron micrograph of a *Rhodococcus* sp.WU-K2R cell. Scale bar, 0.5 µm.

plates, strains showing stable growth in the medium with NTH as the sole source of sulfur were selected. Among the strains, one bacterium, WU-K2R, was chosen for further studies.

WU-K2R (Fig. 5.1) was a rod-shaped bacterium with the dimensions of 0.5 to 1.0 µm by 1.5 to 2.0 µm. This strain was Gram-positive, catalase-positive, and oxidase-negative and did not form spores. Further taxonomical identification of WU-K2R was performed by the National Collection of Industrial and Marine Bacteria Japan Ltd. (Shizuoka, Japan), and the 16S ribosomal DNA sequence of WU-K2R was found to have 99.9% identity to those of *Rhodococcus* sp. DSM43943 (accession number X80616), *Rhodococcus opacus* 1CP (accession number Y11893), and *Rhodococcus koreensis* (accession number AF124343). From these results, WU-K2R was tentatively identified as *Rhodococcus* sp.

5.3.2. Growth characteristics on NTH

Rhodococcus sp. WU-K2R grew in MG medium with NTH as the sole source of

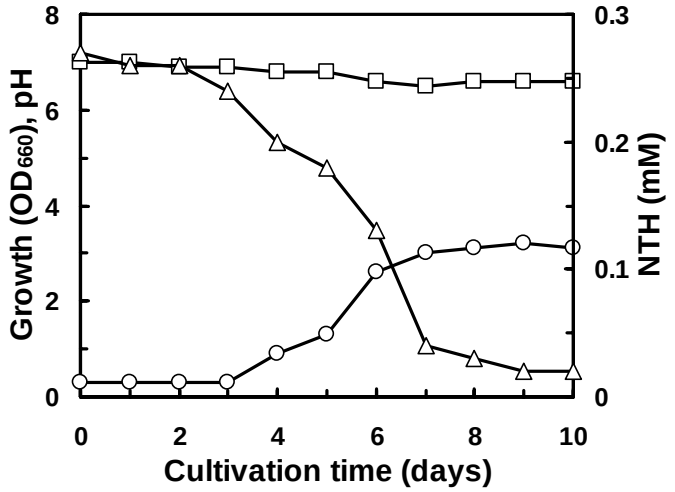

Fig. 5.2. Time course of NTH degradation by growing cells of *Rhodococcus* sp. WU-K2R. WU-K2R was cultivated in MG with 0.27 mM NTH as the sole source of sulfur and 1% (v/v) *N,N*-dimethylformamide. Symbols: ○, growth; □, pH; △, NTH.

sulfur. The time course of NTH degradation by growing cells of *Rhodococcus* sp. WU-K2R is shown in Fig. 5.2, and WU-K2R degraded 0.27 mM NTH within 7 days. On the other hand, it was confirmed that WU-K2R did not utilize NTH as the sole source of carbon nor as the sole source of carbon and sulfur (data not shown). It was also confirmed that WU-K2R did not grow with NFU (see Fig. 5.4B) as the sole source of carbon (data not shown); WU-K2R did not grow even in MG medium including 5 g of glucose per liter with Na_2SO_4 and 0.25 g of NFU per liter, indicating that this compound was toxic to this strain for growth.

5.3.3. Desulfurization of heterocyclic sulfur compounds

The degradation of heterocyclic sulfur compounds by growing cells of *Rhodococcus* sp. WU-K2R was examined. As shown in Table 5.1, WU-K2R showed the growth in MG medium with $NTHO_2$, as well as NTH, as the sole source of sulfur and degraded 97% of 0.27 mM $NTHO_2$ within 5 days. In addition, WU-K2R grew

TABLE 5.1. Degradation of heterocyclic sulfur compounds by growing cells of *Rhodococcus* sp. WU-K2R[a].

Sulfur source	Growth (OD_{660})[b]	Degradation (%)
No substrate	0.0	
NTH	4.3	80
$NTHO_2$	2.3	97
BTH	2.0	57
3-Methyl-BTH	0.3	ND[c]
5-Methyl-BTH	1.0	ND[c]
DBT	0.1	0
$DBTO_2$	0.1	0
4,6-Dimethyl-DBT	0.1	0

[a] WU-K2R was cultivated in MG medium with 0.27 mM of each heterocyclic sulfur compound as the sole source of sulfur and 1% (v/v) ethanol.
[b] The OD_{660}s of 0.1 were due to insoluble DBT, $DBTO_2$, or 4,6-dimethyl-DBT itself in the medium but were not due to the growth of WU-K2R; no growth of WU-K2R was confirmed by microscopic observation.
[c] ND, not determined.

with BTH, 3-methyl-BTH, and 5-methyl-BTH. Since BTH, 3-methyl-BTH, and 5-methyl-BTH are volatile, test tubes were tightly sealed to restrict admission of air. Under these conditions, growth occurred with BTH only and none was observed with the methylated BTHs (data not shown). However, WU-K2R grew with the methylated BTHs when silicone caps which freely admitted air were used (Table 5.1). When NTH and BTH were simultaneously provided for WU-K2R, this strain degraded 9.0 and 59% of each substrate at 0.27 mM, respectively, within 5 days. On the other hand, WU-K2R did not utilize DBT, $DBTO_2$, or 4,6-dimethyl-DBT as the sole source of sulfur.

5.3.4. NTH- and BTH-desulfurizing pathways

Rhodococcus sp. WU-K2R was cultivated to the end of exponential growth phase in MG medium with NTH or BTH as the sole source of sulfur, and each culture broth was extracted with ethyl acetate. First, the extract containing metabolites produced through NTH desulfurization was analyzed by GC-MS. As shown in Fig. 5.3, three compounds as metabolites were detected in addition to NTH (M^+, m/z = 184) (Fig. 5.3A). One metabolite was identified as NTH sulfone ($NTHO_2$, M^+, m/z = 216) (Fig. 5.3B) since its GC-MS spectrum was identical to that of authentic $NTHO_2$. The other metabolites were considered to include no sulfur atom in their molecular structures. One was identified as 2'-hydroxynaphthylethene (HNE, M^+, m/z =170) (Fig. 5.3C). The fragment ion at m/z = 141 corresponds to loss of the vinyl group from the molecular ion. The other was identified as naphtho[2,1-*b*]furan (NFU, M^+, m/z =168) (Fig. 5.3D) since its GC-MS spectrum was identical to that of authentic NFU.

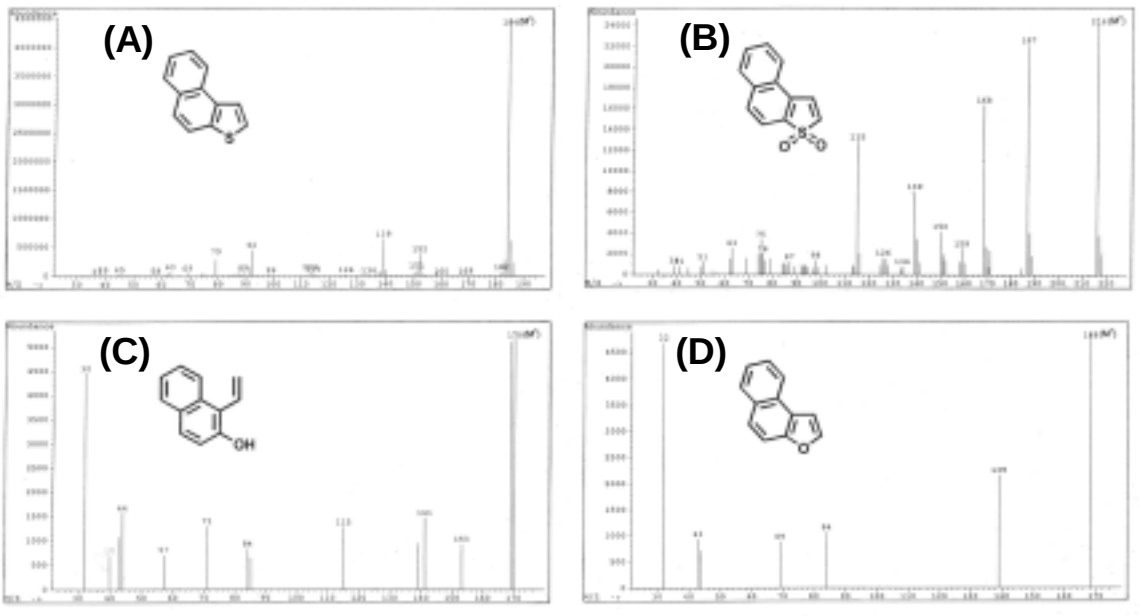

Fig. 5.3. GC-MS analysis of desulfurized NTH metabolites produced by *Rhodococcus* sp. WU-K2R. (A), NTH; (B), $NTHO_2$; (C), HNE; (D), NFU.

TABLE 5.2. GC-MS analysis of desulfurized BTH metabolites produced by *Rhodococcus* sp. WU-K2R.

Metabolites	Fragment ions (m/z)
BTH	134 (M^+), 89, 67, 63
$BTHO_2$	137, 109, 166 (M^+), 63, 76, 118, 89
BcOTO	118, 89, 166 (M^+), 63
$BcOTO_2$	89, 118, 182 (M^+), 63
o-Hydroxystyrene	91, 120 (M^+),69, 66
HPEal	107, 136 (M^+), 77, 51, 89, 63
BFU	118 (M^+), 89, 63

The extract containing metabolites produced through BTH desulfurization was then analyzed by GC-MS. As shown in Table 5.2, six compounds as metabolites were detected in addition to BTH (M^+, m/z = 134). These six metabolites were identified as BTH sulfone ($BTHO_2$, M^+, m/z = 166), benzo[*c*][1,2]oxathiin *S*-oxide (BcOTO, M^+, m/z = 166), benzo[*c*][1,2]oxathiin *S,S*-dioxide ($BcOTO_2$, M^+, m/z = 182), *o*-hydroxystyrene (M^+, m/z = 120), 2-(2'-hydroxyphenyl)ethan-1-al (HPEal, M^+, m/z = 136), and benzofuran (BFU, M^+, m/z = 118) since GC-MS spectra of these metabolites were identical to those reported by other researchers who analyzed desulfurized BTH metabolites in detail [2, 3].

On the other hand, desulfurized NTH and BTH metabolites were not detected when WU-K2R was cultivated in MG medium with Na_2SO_4 instead of NTH or BTH as the sole source of sulfur (data not shown). Based on the deduced structures of these metabolites and BTH-desulfurizing pathways previously reported [2, 3], the pathways for NTH and BTH desulfurization by WU-K2R were proposed as shown in Fig. 5.4. It was concluded that WU-K2R desulfurized NTH and BTH through the sulfur-specific degradation pathways with the selective cleavage of carbon-sulfur bonds.

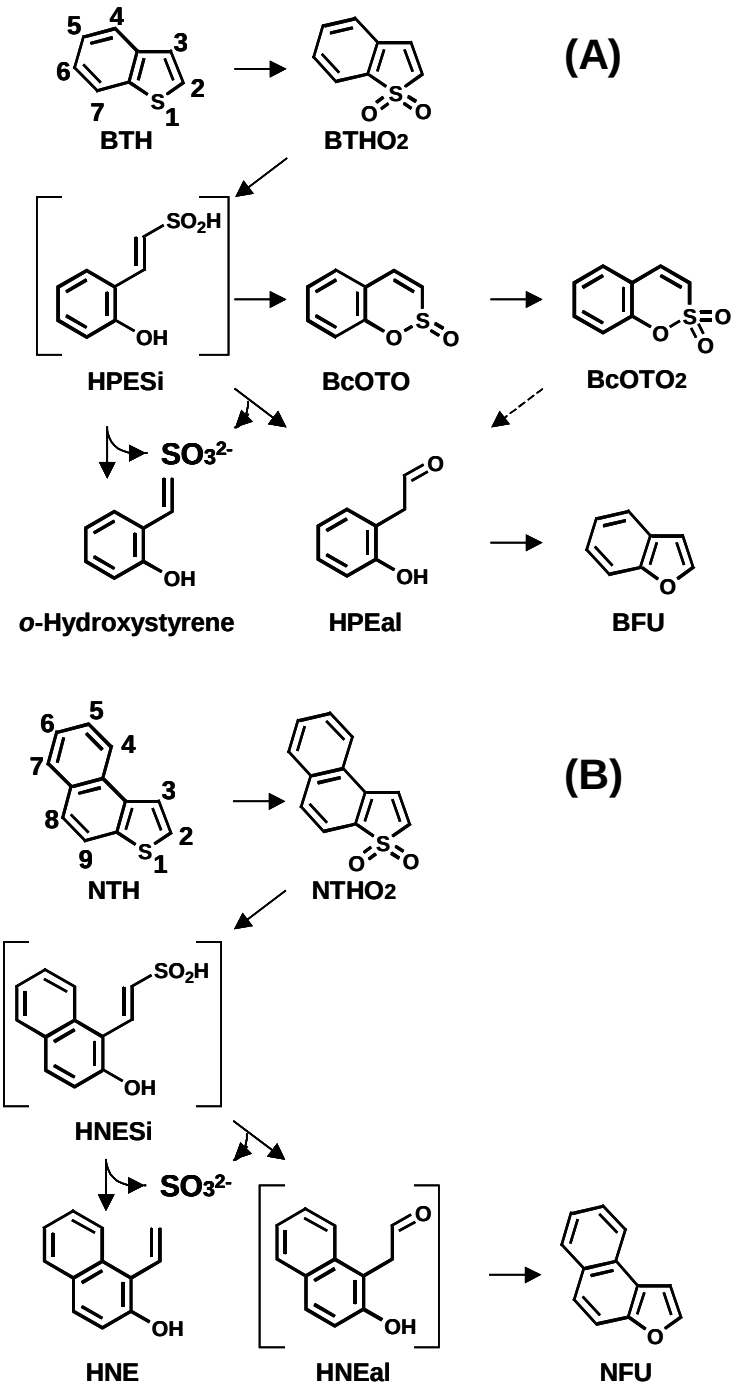

Fig. 5.4. Proposed pathways of BTH (A) and NTH (B) desulfurization by *Rhodococcus* sp. WU-K2R. Compounds in brackets, that is, HPESi in A, and 2-(2'-hydroxynaphthyl)ethen-1-sulfinate (HNESi) and 2-(2'-hydroxynaphthyl)-ethan-1-al (HNEal) in B were not identified and are indicated as postulated metabolites. Ring numbering of BTH and NTH are also shown. The dotted arrow in A indicates that BFU might be produced via HPEal from $BcOTO_2$ (see text).

5.4. Discussion

In addition to symmetric DBT derivatives, asymmetric heterocyclic sulfur compounds such as NTH and BTH derivatives are also detected in LGO following HDS. Some microorganisms possessing BTH-desulfurizing abilities have been isolated, for example, *Gordonia* sp. 213E [2], *Rhodococcus* sp. T09 [4], and *Paenibacillus* sp. A11-2 [3]. These bacteria desulfurize BTH through sulfur-specific degradation pathways (Chapter 1.5). *Gordonia* sp. 213E and *Rhodococcus* sp. T09 could desulfurize BTH but not DBT. In contrast, *Paenibacillus* sp. A11-2 could also desulfurize DBT in addition to BTH since this bacterium was isolated for its ability to grow in a medium with DBT as the sole source of sulfur. It is interesting to note that DBT-desulfurizing bacterium *R. erythropolis* KA2-5-1 could desulfurize alkylated BTH but not BTH [5]. However, there are no reports on microorganisms possessing NTH-desulfurizing ability through a sulfur-specific degradation pathway except *M. phlei* WU-F1 (Chapter 4). *M. phlei* WU-F1, which possessed high desulfurizing ability toward DBT and its derivatives under high-temperature conditions, could also desulfurize NTH and 2-ethyl-NTH through the sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds. However, as described below, the NTH desulfurizing pathways of *M. phlei* WU-F1 and *Rhodococcus* sp. WU-K2R (shown in this chapter) are considered to be different.

In this chapter, the author described the desulfurization of NTH and BTH by the newly isolated bacterium, *Rhodococcus* sp. WU-K2R. The 16S ribosomal DNA sequences of WU-K2R and, surprisingly, *Rhodococcus* sp. T09 (desulfurizing BTH [4]) were found to have 99.9% identity to that of *Rhodococcus* sp. DSM43943 (accession number X80616), but *Rhodococcus* sp. T09 was confirmed to show no

NTH-desulfurizing activity in a medium with NTH as the sole source of sulfur, in which WU-K2R showed the activity (data not shown). Therefore, *Rhodococcus* sp. WU-K2R is concluded to be a different type of desulfurizing bacterium from *Rhodococcus* sp. T09.

As shown in Table 5.1, *Rhodococcus* sp. WU-K2R degraded 80% of 0.27 mM NTH within 5 days, and WU-K2R showed much higher NTH-desulfurizing activity than *M. phlei* WU-F1 since WU-F1 degraded only 39% of 0.27 mM NTH within 5 days (Chapter 4). WU-K2R exhibited a faster growth rate and higher NTH-degrading activity in the presence of ethanol (Table 5.1) than in the presence of *N,N*-dimethylformamide (Fig. 5.2). This might be related to the fact that WU-K2R could utilize ethanol but not *N,N*-dimethylformamide as the sole source of carbon (data not shown). WU-K2R also degraded $NTHO_2$, suggesting that this strain desulfurized NTH via $NTHO_2$, as shown in Fig. 5.4B. In addition, WU-K2R grew with BTH, 3-methyl-BTH, and 5-methyl-BTH when silicone caps which freely admitted air were used (Table 5.1), but with tightly sealed caps restricting admission of air, growth occurred with BTH only and none was observed for the methylated BTHs (data not shown). These results suggest that WU-K2R might require more O_2 to utilize the methylated BTHs.

On the other hand, WU-K2R did not utilize DBT, $DBTO_2$, or 4,6-dimethyl-DBT as the sole source of sulfur (Table 5.1). Therefore, WU-K2R is considered to preferentially desulfurize asymmetric heterocyclic sulfur compounds such as NTH and BTH rather than symmetric DBT derivatives.

BTH-desulfurizing bacteria reported by other researchers desulfurized BTH through the sulfur-specific degradation pathways [2-4], in which BTH was first oxidized

to $BTHO_2$ via BTH sulfoxide. $BTHO_2$ was then converted to 2-(2'-hydroxyphenyl)ethen-1-sulfinate (HPESi), leading to BcOTO and $BcOTO_2$ through dehydration. HPESi was finally desulfurized to HPEal, leading to BFU through dehydration, by *Gordonia* sp. 213E [2] and *Rhodococcus* sp. T09 [4], which could desulfurize BTH but not DBT. On the other hand, HPESi was finally desulfurized to *o*-hydroxystyrene by *Paenibacillus* sp. A11-2 [3], which could desulfurize both BTH and DBT. In the present study, it was confirmed that *Rhodococcus* sp. WU-K2R desulfurized BTH through the sulfur-specific degradation pathway, as shown in Fig. 5.4A. In addition, since both BFU and *o*-hydroxystyrene were detected as metabolites with no sulfur (Table 5.2), WU-K2R is considered to possess two types of carbon-sulfur bond cleavage reactions toward HPESi. Moreover, BFU might be produced via HPEal from $BcOTO_2$, as previously reported in the case of DBT desulfurization, in which $DBTO_2$ monooxygenase also recognizes dibenz[*c,e*][1,2]oxathiin *S,S*-dioxide as a substrate to produce 2,2'-dihydroxybiphenyl [6]. Similarly, since metabolites with no sulfur from NTH were identified as NFU and HNE, it was concluded that WU-K2R desulfurized NTH through the same pathway (or quite similar one) as that for BTH desulfurization, as shown in Fig 5.4B. On the other hand, it was suggested that *M. phlei* WU-F1 produced only the compounds corresponding to HNE and not those corresponding to NFU as metabolites with no sulfur from 2-ethyl-NTH (Chapter 4). Therefore, the NTH-desulfurizing pathway of WU-K2R is considered to be different from that of *M. phlei* WU-F1. In the NTH-desulfurizing pathway of WU-K2R, desulfurized NTH metabolites corresponding to desulfurized BTH metabolites such as BcOTO, $BcOTO_2$, and HPEal were not detected by GC-MS analysis, and the total amount of desulfurized NTH metabolites

detected was much lower than that of degraded NTH (data not shown). Although the author does not have a clear explanation for this, it might be due to the lability of desulfurized NTH metabolites, as suggested in Chapter 4, or the existence of an additional degradation pathway(s) for NTH [7]. However, since desulfurized NTH metabolites were not detected when WU-K2R was cultivated in MG medium with Na_2SO_4 instead of NTH as the sole source of sulfur (data not shown), these metabolites (Fig. 5.3) were derived from NTH, indicating that the pathway shown in Fig. 5.4B clearly contributes to NTH desulfurization by WU-K2R.

In conclusion, the author confirmed that the newly isolated bacterium *Rhodococcus* sp. WU-K2R could preferentially desulfurize asymmetric heterocyclic sulfur compounds such as NTH and BTH through the sulfur-specific degradation pathways with the selective cleavage of carbon-sulfur bonds. These results suggest that WU-K2R may be a promising biocatalyst for practical biodesulfurization complementary to microorganisms possessing desulfurizing ability toward symmetric DBT derivatives.

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Chapter 6

Cloning and Functional Analysis of the Dibenzothiophene-Desulfurization Genes from *Bacillus subtilis* WU-S2B and *Mycobacterium phlei* WU-F1

6.1. Introduction

Biodesulfurization is a promising technology to remove recalcitrant organosulfur compounds such as dibenzothiophene (DBT) and its derivatives more selectively under much milder conditions than hydrodesulfurization using metallic catalysts (Chapter 1) [1]. Thus, many mesophilic DBT-desulfurizing microorganisms have been isolated as desulfurizing biocatalysts to date [1]. Particularly, *Rhodococcus* sp. IGTS8 has been extensively studied so far, and the genes involved in DBT desulfurization (*dszABC*) were cloned and characterized [2-4]. The *dszABC* genes constitute a single operon consisting of the three genes, *dszA*, *dszB*, and *dszC*, which are responsible for DBT desulfurization through a sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds (Chapter 1.4) [2, 3]. That is, DBT monooxygenase as the gene product of *dszC* first oxidizes DBT to DBT sulfone ($DBTO_2$) via DBT sulfoxide [5], $DBTO_2$ monooxygenase as the gene product of *dszA* then converts $DBTO_2$ to 2'-hydroxybiphenyl-2-sulfinate (HBPSi), leading to cleavage of the thiophene ring [6], and HBPSi desulfinase as the gene product of *dszB* finally desulfurizes HBPSi to 2-hydroxybiphenyl (2-HBP) with the release of the sulfur atom

as sulfite [7].

In addition, from the viewpoint of practical applications (Chapter 1.4.3), a thermophilic DBT-desulfurizing bacterium *Paenibacillus* sp. A11-2 [8] has been isolated and the DBT-desulfurization genes (*tdsABC*) equivalent to *dszABC* were cloned and characterized [9]. Moreover, in Chapter 3, the author and collaborators isolated thermophilic DBT-desulfurizing bacteria, *Bacillus subtilis* WU-S2B and *Mycobacterium phlei* WU-F1, and confirmed that these bacteria possessed high DBT-desulfurizing abilities over a wide temperature range up to 50˚C.

In this chapter, the author describes the cloning and functional analysis of the DBT-desulfurization genes from *B. subtilis* WU-S2B and *M. phlei* WU-F1. By nucleotide sequence analysis, it was surprisingly found that the nucleotide sequences of the DBT-desulfurization genes from WU-S2B and WU-F1 are completely the same as each other. The genes constitute a single operon consisting of the three genes designated *bdsA*, *bdsB*, and *bdsC* for bacterial desulfurization. Although the nucleotide sequence of *bdsABC* shows homology to those of *dszABC* and *tdsABC*, it was confirmed that *bdsA* was independently located away from the *dszA* cluster and *tdsA* by phylogenetic analysis. In addition, heterologous expression analysis in *Escherichia coli* indicated that the abilities of WU-S2B and WU-F1 to desulfurize DBT over a wide temperature range were endowed by the thermophilic DBT-desulfurization genes, *bdsABC*.

6.2. Materials and methods

6.2.1. Chemicals

DBT and 2-HBP were purchased from Tokyo Kasei (Tokyo, Japan). $DBTO_2$ was

purchased from Aldrich (WI, USA). HBPSi was kindly supplied by the laboratory of the Japan Cooperation Center, Petroleum (Shizuoka, Japan). All other reagents were of analytical grade and commercially available.

6.2.2. Bacterial strains, phage, plasmids, and cultivation

B. subtilis WU-S2B and *M. phlei* WU-F1 were used as the source of total DNAs for cloning of DBT-desulfurization genes. For preparation of total DNAs, WU-S2B and WU-F1 were cultivated at 50˚C in test tubes (18 by 180 mm) containing 5 ml of A-2 and AF media (Chapter 2.3), respectively, with 0.54 mM DBT, followed by incubation at 37˚C with 50 μg/ml D-cycloserine and 100 μg/ml lysozyme for WU-F1 (Chapter 2.7) [10]. *E. coli* XL1-Blue MRA (P2) and lambda DASH II phage vector (Stratagene, CA, USA) were used for cloning of DBT-desulfurization genes. *E. coli* JM109 and a cloning vector pGEM-T (Promega, WI, USA) were used for cloning of PCR products. *E. coli* JM109 and a cloning vector pUC19 (Takara Bio, Tokyo, Japan) were used for subcloning of DBT-desulfurization genes from recombinant phage vectors. *E. coli* JM109 and an expression vector pKK223-3 (Amersham Biosciences, NJ, USA) were used for subcloning and expression studies. *E. coli* strains were cultivated in Luria-Bertani (LB) medium (Chapter 2.3.4) supplemented with 50 μg/ml ampicillin. For heterologus expression analysis, the recombinant strains of *E. coli* were cultivated at 37˚C in test tubes (18 by 180 mm) containing 5 ml of LB medium with ampicillin and 0.27 mM DBT, $DBTO_2$, or HBPSi.

6.2.3. Recombinant DNA techniques

Recombinant DNA techniques were performed as described by Sambrook et al.

[11] or according to manufacturer's protocols (Chapter 2). Total DNAs from WU-S2B and WU-F1 were prepared with a QIAGEN genomic-tip (QIAGEN, Tokyo, Japan). Phage DNA and plasmid DNA were prepared with a QIAGEN lambda kit (QIAGEN) and a GFX micro plasmid prep kit (Amersham Biosciences), respectively. DNA fragments were purified with a GFX PCR DNA and gel band purification kit (Amersham Biosciences). DNA sequencing was performed with an ABI PRISM 310 genetic analyzer (Applied Biosystems, CA, USA). Nucleotide sequence was determined by complete sequencing of both strands, with multiple sequencing of some regions. Sequence analysis was performed with a Genetyx-Mac version 10.1 (SDC, Tokyo, Japan). Homology search of sequence was performed with the FASTA program of the DNA Data Bank of Japan (DDBJ). Phylogenetic tree based on sequence homology was constructed with the ClustalW program of DDBJ.

6.2.4. Construction of total DNA library

Total DNA of WU-S2B or WU-F1 was partially digested with *Sau*3AI and dephosphorylated with bacterial alkaline phosphatase. The DNA fragments were ligated to lambda DASH II phage DNA previously digested with *Bam*HI. The recombinant phage DNA was packaged into phage *in vitro* with a Gigapack III Gold packaging kit (Stratagene). The resulting recombinant phage was propagated in *E. coli* XL1-Blue MRA (P2). Transformed cells were spread onto NZY medium agar plates (Chapter 2.3.4), incubated at 37˚C, and then used as total DNA library of WU-S2B or WU-F1.

6.2.5. Screening of total DNA library by plaque hybridization

The total DNA library of WU-S2B or WU-F1 was screened by plaque hybridization with a DNA probe. The DNA probe was prepared by PCR amplification (Chapter 2.10) with the total DNA of WU-S2B or WU-F1 as the template using primers based on the conserved regions in deduced amino acid sequences of the DBT-desulfurization genes, *dszABC* and *tdsABC* from *Rhodococcus* sp. IGTS8 [4] and *Paenibacillus* sp. A11-2 [9], respectively. The PCR products were labeled with DIG (digoxigenin) DNA Labeling Kit (Roche, Basel, Switzerland). The total DNA library of WU-S2B or WU-F1 on NZY medium agar plates was transferred to Hybond-N+ nylon membranes (Amersham Biosciences), and the membranes were hybridized with the DNA probe (Chapter 2.11).

6.2.6. Construction of expression plasmids

A 3.4-kb fragment containing backward region of *bdsA* and complete *bdsB* and *bdsC* was prepared from pU1Nh, which included *bdsABC* in pUC19 as described later, by the digestion with *Bss*SI and *Hin*dIII. In addition, a 0.3-kb fragment containing forward region of *bdsA* was amplified by PCR (Chapter 2.10) from pU1Nh using two oligonucleotide primers 5'-GCCAAGCTTTGGTCTCCGGTAACTGATCCC-3' (the *Hin*dIII restriction site is underlined) and 5'-GACCACATTCCACGAGATCCGGC-3' (the *Bss*SI restriction site is underlined), and amplified DNA fragments were digested with *Hin*dIII and *Bss*SI. The 3.4-kb fragment and the 0.3-kb fragment were inserted into pKK223-3 previously digested with *Hin*dIII to locate the *bdsABC* genes under control of *tac* promoter. The resulting recombinant plasmid (pKBDS) was amplified in *E. coli* JM109 cells, and the insert was sequenced to ensure that the correct

construction had been obtained. The pKBDS construct was then introduced into *E. coli* JM109 for expression of the *bdsABC* genes. For heterologus expression analysis in *E. coli*, the deletion plasmids, pKBDSA including *bdsA*, pKBDSB including *bdsB*, and pKBDSC including *bdsC* were constructed from pKBDS with restriction endonuclease digestion and self-ligation.

6.2.7. Resting cell reaction

Recombinant *E. coli* cells were cultivated at 37˚C in 500-ml flasks containing 100 ml of LB medium with ampicillin for 16 h. The cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4˚C, washed twice with 100 mM potassium phosphate buffer (pH 7.0), and suspended in the same buffer. The optical density at 660 nm (OD_{660}) of cell suspension was adjusted to 20. Fifty microliters of a 10 g/l DBT stock solution in *n*-tridecane was added to 10 ml screw-cap test tubes containing 1 ml of the cell suspension to a final concentration of 0.27 mM DBT. Resting cell reactions were performed with inverted shaking at 50 rpm for 1 h.

6.2.8. Analytical methods

DBT and the metabolites produced through DBT desulfurization were determined by using high-performance liquid chromatography (HPLC) (Chapter 2.13.2).

6.2.9. Nucleotide sequence accession number

The nucleotide sequence discussed in this paper is available from GeneBank under Accession No. AB076745 as the DBT-desulfurization genes of *B. subtilis* WU-S2B, *bdsABC*. The nucleotide sequence of the DBT-desulfurization genes of WU-F1 is

completely the same as that of WU-S2B, as described later.

6.3. Results

6.3.1. Cloning of DBT-desulfurization genes from *B. subtilis* WU-S2B

The DBT-desulfurization genes, *dszABC* and *tdsABC,* have already cloned from

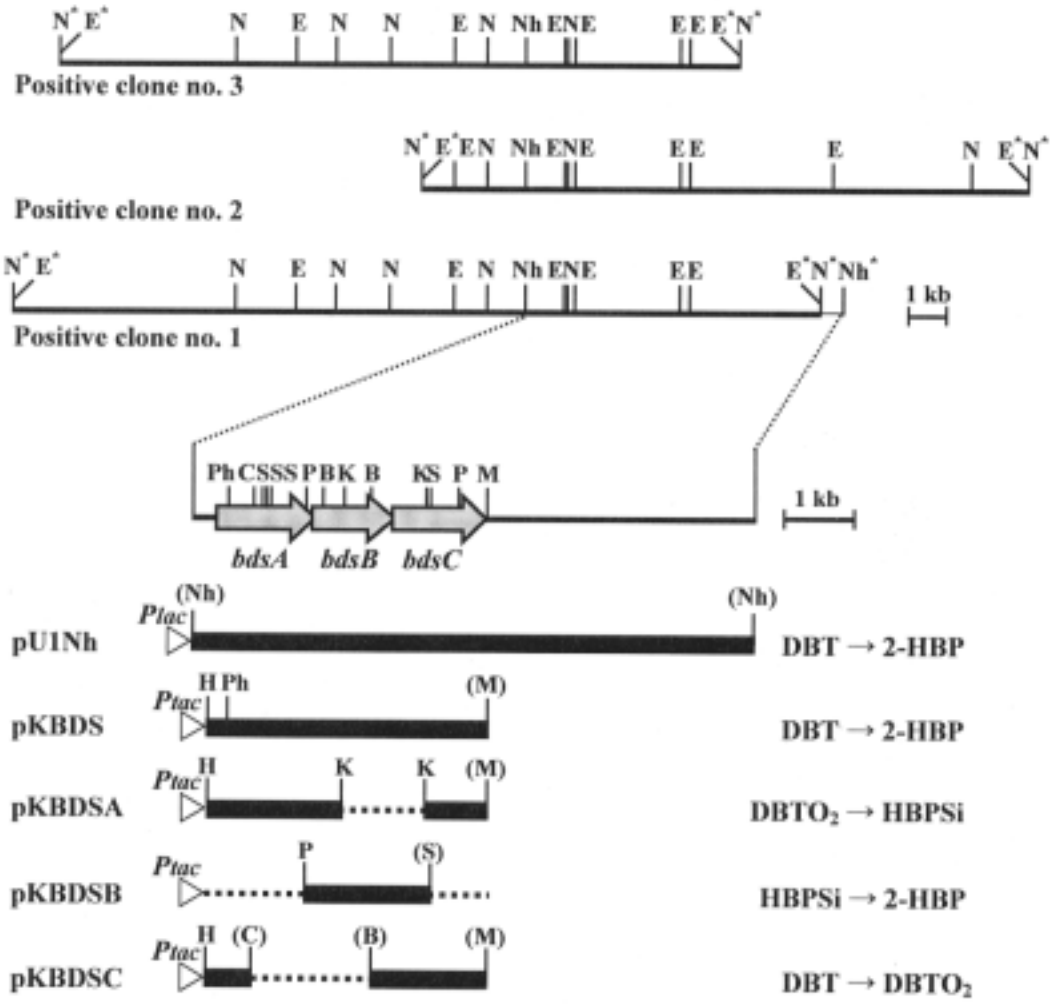

Fig. 6.1. Restriction map of three positive clones and subclones for the *bdsABC* genes from *B. subtilis* WU-S2B. The restriction enzyme sites derived from the phage vector are shown with asterisk and the sites lost by blunting are shown in parenthesis. B, *Bpu*1102I; C, *Cpo*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; N, *Not*I; Nh, *Nhe*I; P, *Pst*I; Ph, *Psh*AI; S, *Sac*II. *Plac* and *Ptac* (triangle) show the directions of *lac* and *tac* promoter in pUC19 and pKK223-3, respectively.

mesophilic *Rhodococcus* sp. IGTS8 [4] and thermophilic *Paenibacillus* sp. A11-2 [9], respectively. The conserved regions in the deduced amino acid sequences of *dszABC* and *tdsABC* were searched and identified as AEARNFG between 429 and 447 nucleotide from the initiation codon of *dszA* and GFDRFWR between 99 and 116 nucleotide from the termination codon of *dszC*. Based on these amino acid sequences, two degenerated primers 5'-GCIGARGCIMGIAAYTTYGG-3' and 5'-CGTIGCGCCA IAAGCGGTC-3' (I, inosine) were designed, and used for PCR amplification with the total DNA of *B. subtilis* WU-S2B as the template. The resulting 3.2-kb amplified DNA fragments were inserted into pGEM-T, and the insert was sequenced. Since the deduced amino acid sequence of this PCR product showed approximately 60% homology to those of *dszABC* and *tdsABC*, strongly suggesting that the amplified region was a part of DBT-desulfurization genes of WU-S2B. Then, based on the nucleotide sequence of the amplified region, two oligonucleotide primers 5'-GCATGAC ATCCGATACG-3' and 5'-TAGTTTGGGTGGGTTCC-3' were designed, and used for second PCR amplification with the total DNA of WU-S2B as the template. The resulting PCR product was labeled with digoxigenin and used as the DNA probe.

The DNA library of WU-S2B was constructed as described in Materials and methods and contained approximately 8,000 plaques. By plaque hybridization with the DNA probe, three positive plaques were obtained and selected as candidates for cells carrying DBT-desulfurization genes from WU-S2B. As shown in Fig. 6.1, restriction endonuclease digestion and Southern hybridization analyses revealed that all the inserts from three positive clones included the same region of the total DNA of WU-S2B and that a 7.1-kb *Nhe*I fragment of positive clone no. 1 included the entire region of the DBT-desulfurization genes as described later. Then, the 7.1-kb *Nhe*I fragment was

subcloned into pUC19 previously digested with *Xba*I to construct pU1Nh.

6.3.2. Characterization of the nucleotide sequence of *bdsABC*

The nucleotide sequence of the hybridizing region and its adjacency in pU1Nh was determined, and three open reading frames (ORFs) were identified to be aligned in the same direction. As shown in Fig. 6.2, the nucleotide sequences of the ORFs show significant homology to those of the *dsz* operon from *Rhodococcus* sp. IGTS8 [4] and the *tds* operon from *Paenibacillus* sp. A11-2 [9]. Thus, each ORF homologous to *dszA*, *dszB*, and *dszC* of IGTS8 was designated *bdsA*, *bdsB*, and *bdsC*, respectively, for bacterial desulfurization. The overall nucleotide sequence of the *bds* operon, *bdsABC*, shares 61.0% identity with that of *dszABC* from *Rhodococcus* sp. IGTS8 and 57.8% with that of *tdsABC* from *Paenibacillus* sp. A11-2. In the *bds* operon, the tail of *bdsA* shows a 4-bp overlap with the head of *bdsB*, as previously reported in the cases of the *dsz* and *tds* operons [4, 9]. In addition, *bdsB* also shows a 4-bp overlap with *bdsC* although the overlap does not exist in the *dsz* and *tds* operons. On the other hand, it was confirmed that no ORFs existed close to the *bdsABC* genes, at least in the upstream region of 3.3 kb and the downstream region of 0.4 kb, except two ORFs in the upstream region showing low homology to unknown hypothetical proteins (data not shown).

The first ORF, *bdsA*, encodes a protein of 453 amino acid residues (Fig. 6.2A). The deduced amino acid sequence of *bdsA* shares 78.8% and 64.4% identity with those of the $DBTO_2$ monooxygenases, DszA and TdsA from *Rhodococcus* sp. IGTS8 and *Paenibacillus* sp. A11-2, respectively. In addition, BdsA was found to show appreciable homology to several flavin-dependent monooxygenases. For example, BdsA shares 41.5%, 41.2%, and 32.2% identity with SnaA, pristinamycin II_A synthase

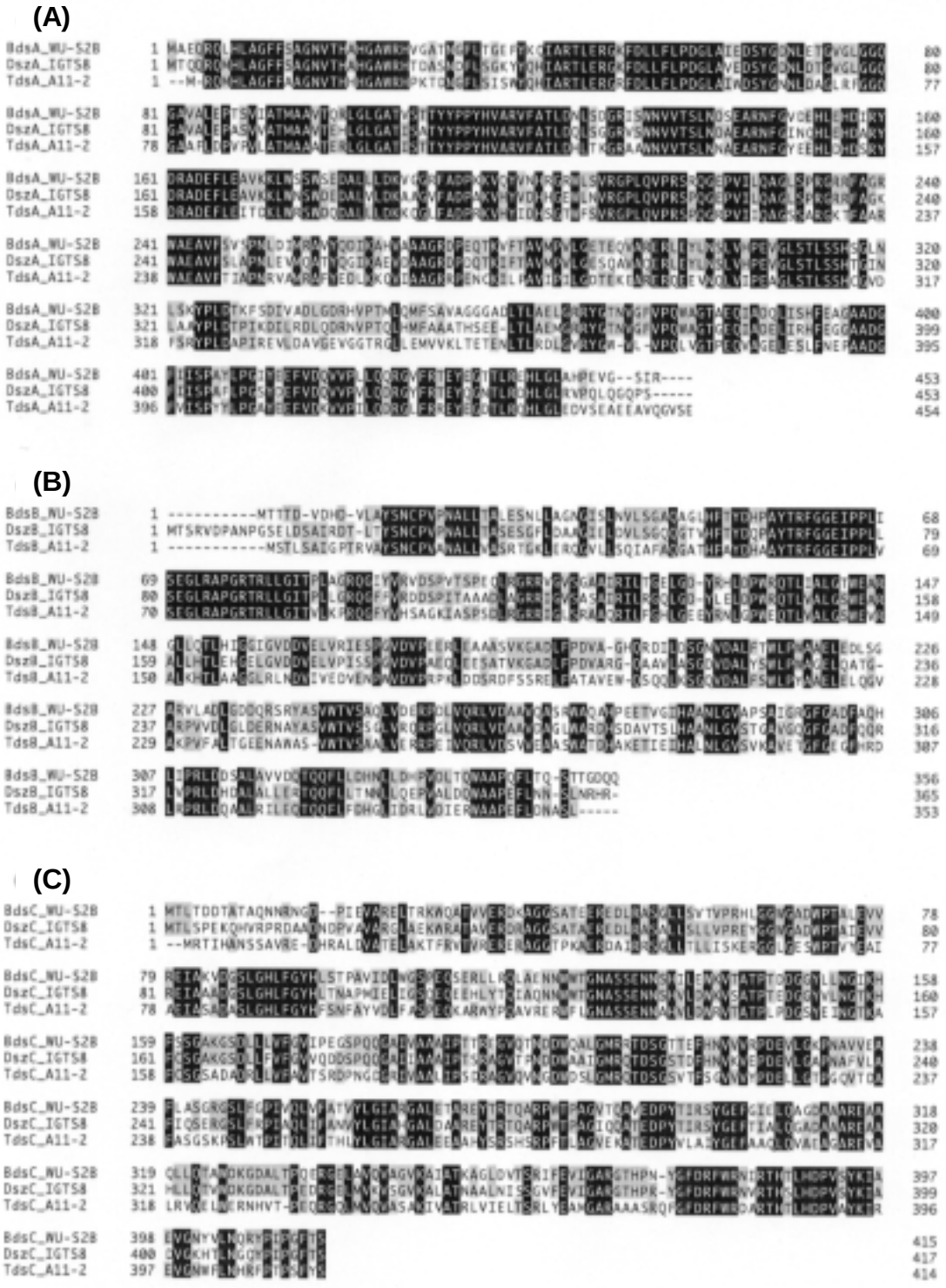

Fig. 6.2. Multiple sequence alignment of the DBT-desulfurizing enzymes. Black shading indicates identical residues in all the members. Gray shading indicates identical residues in two members. The Bds enzymes are from *B. subtilis* WU-S2B (accession number AB076745). The Dsz enzymes are from *Rhodococcus* sp. IGTS8 (L37363). The Tds enzymes are from *Paenibacillus* sp. A11-2 (AB033997). (A), $DBTO_2$ monooxygenases BdsA, DszA, and TdsA; (B), HBPSi desulfinases BdsB, DszB, and TdsB; (C), DBT monooxygenases BdsC, DszC, and TdsC.

of *Strepromyces pristinaespiralis* [12], NtaA, nitrilotriacetate monooxygenase of *Chelatobacter heintzii* [13], and EmoA, EDTA monooxygenase of a EDTA-degrading bacterium BNC1 [14], respectively. The sequence alignment of BdsA and its equivalents, DszA and TdsA, is shown in Fig. 6.2A. BdsA shows high homology in its N-terminal region of approximately 250 amino acid residues and its C-terminal region of approximately 50 amino acid residues to the equivalents. As for flavin-dependent monooxygenases, BdsA shares some conserved regions with them, but shows no homology in its N-terminal region of approximately 100 amino acid residues to them such as SnaA, NtaA, and EmoA (data not shown). In addition, as shown in Fig. 6.3A, phylogenetic analysis indicated that the $DBTO_2$ monooxygenases formed a cluster in

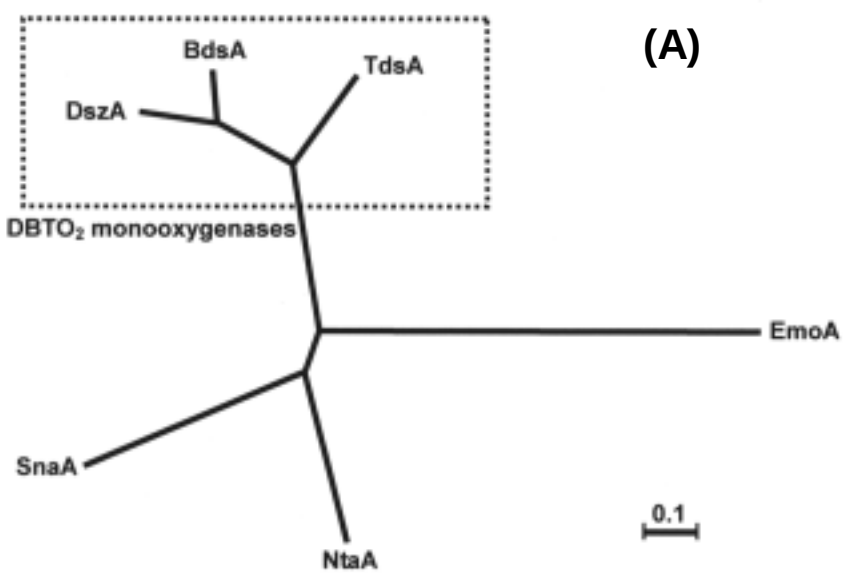

Fig. 6.3A. Phylogenetic tree of the flavin-dependent monooxygenases related to BdsA. Scale bar, 0.1 substitution per site. BdsA, $DBTO_2$ monooxygenase of *B. subtilis* WU-S2B (accession number AB076745); DszA, $DBTO_2$ monooxygenase of *Rhodococcus* sp. IGTS8 (L37363); TdsA, $DBTO_2$ monooxygenase of *Paenibacillus* sp. A11-2 (AB033997); SnaA, pristinamycin II_A synthase subunit A of *Strepromyces pristinaespiralis* (U21215); NtaA, nitrilotriacetate monooxygenase of *Chelatobacter heintzii* (U39411), EmoA, EDTA monooxygenase of a EDTA-degrading bacterium BNC1 (AF176664).

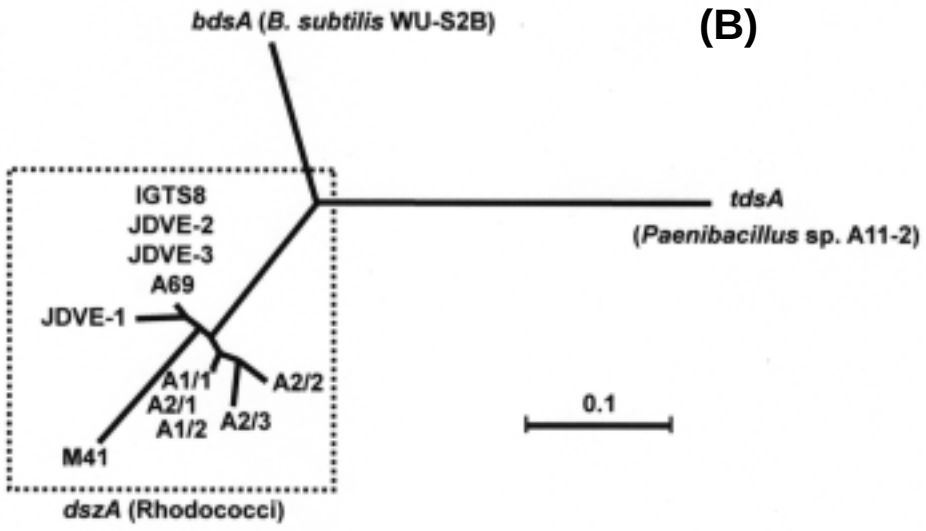

Fig. 6.3B. Phylogenetic tree of the $DBTO_2$ monooxygenase genes equivalent to *bdsA*. Scale bar, 0.1 substitution per site. *bdsA*, gene encoding $DBTO_2$ monooxygenase of *Bacillus subtilis* WU-S2B (accession number AB076745); *tdsA*, gene encoding $DBTO_2$ monooxygenase of *Paenibacillus* sp. A11-2 (AB033997); IGTS8, gene encoding $DBTO_2$ monooxygenase of *Rhodococcus* sp. IGTS8 (L37363); A1/1, *dszA*-like gene of uncultured *Rhodococcus* sp. A1/1 (AF322033); A1/2, *dszA*-like gene of uncultured *Rhodococcus* sp. A1/2 (AF322034); A2/1, *dszA*-like gene of uncultured *Rhodococcus* sp. A2/1 (AF322036); A2/2, *dszA*-like gene of uncultured *Rhodococcus* sp. A2/2 (AF322035); A2/3, *dszA*-like gene of uncultured *Rhodococcus* sp. A2/3 (AF322037); A69, *dszA*-like gene of *Rhodococcus* sp. A69 (AF322041); JDVE-1, *dszA*-like gene of uncultured *Rhodococcus* sp. JDVE-1 (AF322038); JDVE-2, *dszA*-like gene of uncultured *Rhodococcus* sp. JDVE-2 (AF322039); JDVE-3, *dszA*-like gene of uncultured *Rhodococcus* sp. JDVE-3 (AF322040); M41, *dszA*-like gene of *Rhodococcus* sp. M41 (AF322042).

the flavin-dependent monooxygenases. However, as shown in Fig. 6.3B, it was confirmed that *bdsA* was independently located away from the *dszA* cluster [15] and *tdsA*.

The second ORF, *bdsB* encodes a protein of 356 amino acid residues (Fig. 6.2B). The deduced amino acid sequence of *bdsB* shares 68.1% and 53.1% identity with those of the HBPSi desulfinases, DszB and TdsB, respectively. In addition, BdsB was found to show low homology (around 30% identity) to substrate binding proteins responsible for sulfonate or sulfate ester utilization such as SsuA, AsfC, and AtsR [16].

The last ORF, *bdsC*, encodes a protein of 415 amino acid residues (Fig. 6.2C).

The deduced amino acid sequence of *bdsC* shares 72.9% and 51.2% identity with those of the DBT monooxygenases, DszC and TdsC, respectively. In addition, BdsC was found to show low homology to several acyl-CoA dehydrogenases (data not shown).

6.3.3. Cloning of DBT-desulfurization genes from *M. phlei* WU-F1

DBT-desulfurization genes were also cloned from *M. phlei* WU-F1 by the similar strategy to that for *B. subtilis* WU-S2B. Two positive plaques were obtained from the DNA library of WU-F1 containing approximately 4,000 plaques, and both the inserts from two positive clones included the entire region of the DBT-desulfurization genes (data not shown). The nucleotide sequence of the DBT-desulfurization genes from WU-F1 was determined, and found to be completely the same as that of WU-S2B. Thus, the DBT-desulfurization genes from *M. phlei* WU-F1 also designated *bdsABC*. In addition, it was confirmed that the nucleotide sequence adjacent to the *bdsABC* genes of WU-F1 was also completely the same as that of WU-S2B, at least in the upstream region of 3.3 kb and the downstream region of 0.4 kb. Any differences have not been found in nucleotide sequences adjacent to the *bdsABC* genes of WU-S2B and WU-F1 thus far.

6.3.4. Heterologous expression of *bdsABC* in *E. coli*

The recombinant *E. coli* cells carrying pU1Nh or pKBDS, which included *bdsABC* in pKK223-3, desulfurized DBT to 2-HBP (data not shown), indicating that the *bdsABC* genes were expressed in *E. coli*. Then, the contribution of each ORF, i.e., *bdsA*, *bdsB*, and *bdsC* to DBT desulfurization was examined. As shown in Fig. 6.1, the recombinant *E. coli* cells carrying pKBDSA, which included only *bdsA*, converted

$DBTO_2$ to HBPSi. The recombinant *E. coli* cells carrying pKBDSB, which included only *bdsB*, desulfurized HBPSi to 2-HBP, and the recombinant *E. coli* cells carrying pKBDSC, which included only *bdsC*, oxidized DBT to $DBTO_2$. These results indicated that the gene products of *bdsABC* were responsible for desulfurization of DBT to 2-HBP via $DBTO_2$ and HBPSi.

6.3.5. Effects of temperature on DBT desulfurization by recombinant *E. coli*

To investigate the potential of the *bdsABC* genes for thermophilic biodesulfurization, the effects of temperature on DBT-desulfurizing activity of the recombinant *E. coli* carrying pKBDS was examined using the resting cells. As shown in Fig. 6.4, the recombinant *E. coli* cells exhibited DBT-desulfurizing activitiy over a

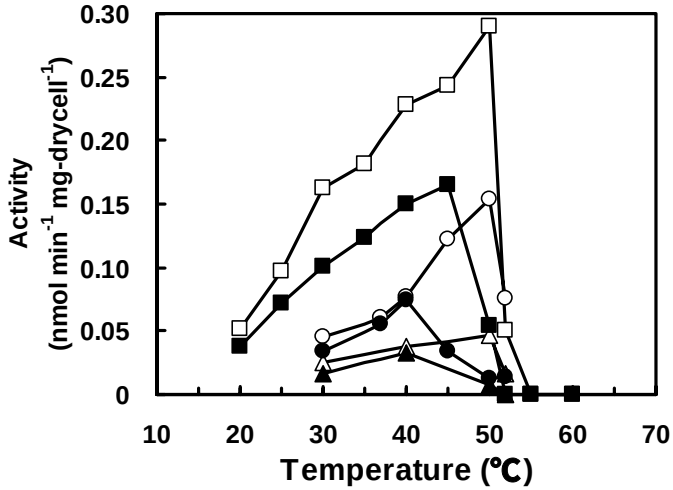

Fig. 6.4. Effects of temperature on DBT-desulfurizing activitiy of the recombinant *E. coli* carrying *bdsABC*. The reaction mixture contained 50 µl of a 10 g/l DBT stock solution in *n*-tridecane and 1 ml of the cell suspension in a 10 ml screw-cap test tube to give a final concentration of 0.27 mM DBT. Resting cell reactions were performed with inverted shaking at 50 rpm for 1 h. Symbols: ○ and ●, DBT-degrading and 2-HBP-producing activities of the recombinant *E. coli* carrying pKBDS, respectively; △and ▲, DBT-degrading and 2-HBP-producing activities of *B. subtilis* WU-S2B, respectively; □and ■, DBT-degrading and 2-HBP-producing activities of *M. phlei* WU-F1, respectively.

wide temperature range up to 52˚C. Resting cells of the recombinant *E. coli* exhibited high DBT-degrading activity over a wide temperature range of 30 to 52˚C and at the highest level at 50˚C. On the other hand, the resting cells produced 2-HBP at the highest level at 40˚C. In addition, the recombinant *E. coli* showed similarity in temperature dependence of DBT-desulfurizing activity to *B. subtilis* WU-S2B and *M. phlei* WU-F1.

6.4. Discussion

In this chapter, the author described the cloning and function analysis of the DBT-desulfurization genes from *B. subtilis* WU-S2B and *M. phlei* WU-F1. The DBT-desulfurization genes were cloned by plaque hybridization with the DNA probe based on the conserved regions in deduced amino acid sequences of the DBT-desulfurization genes, *dszABC* and *tdsABC* from *Rhodococcus* sp. IGTS8 [4] and *Paenibacillus* sp. A11-2 [9], respectively. By nucleotide sequence analysis, we surprisingly found that the nucleotide sequences of the DBT-desulfurization genes from WU-S2B and WU-F1 are completely the same as each other. The genes constitute a single operon consisting of the three genes, *bdsA*, *bdsB*, and *bdsC*, and heterologous expression analysis in *E. coli* indicated that the gene products of *bdsABC* were responsible for desulfurization of DBT to 2-HBP via $DBTO_2$ and HBPSi.

BdsA was found to show appreciable homology to several flavin-dependent monooxygenases (Fig. 6.3A), which require flavin reductase (NAD(P)H:FMN oxidorductase, E.C. 1.6.8.1) for their activities [17]. Flavin reductase uses NAD(P)H to catalyze the reduction of FMN and the resulting reductant $FMNH_2$ is used to activate oxygen by the terminal monooxygenases. The DBT monooxygenases, DszC and TdsC,

as well as the $DBTO_2$ monooxygenases, DszA and TdsA, require flavin reductase for their activities [18]. Although BdsC shows no appreciable homology to any flavin-dependent monooxygenases, this enzyme was found to share some conserved regions with 4-hydroxyphenylacetate 3-monooxygenases which are also flavin-dependent (data not shown) [17].

Phylogenetic analysis indicated that the $DBTO_2$ monooxygenases formed a cluster in the flavin-dependent monooxygenases (Fig. 6.3A). However, it was confirmed that *bdsA* was independently located away from the *dszA* cluster and *tdsA* (Fig. 6.3B). In addition, although the nucleotide sequence of *bdsABC* from thermophilic *B. subtilis* WU-S2B and *M. phlei* WU-F1 shows homology to that of *tdsABC* from thermophilic *Paenibacillus* sp. A11-2, there were appreciable differences between them (Fig. 6.2). As previously reported, *Paenibacillus* sp. A11-2 exhibited high DBT-desulfurizing activity over a temperature range of 45 to 55˚C, but the activity decreased at around 30˚C [8]. In contrast, WU-S2B and WU-F1 exhibited high DBT-desulfurizing activity even at 30˚C (Chapter 3). These differences in temperature dependence of DBT-desulfurizing activity might be due to the differences in nucleotide sequences of the DBT-desulfurization genes, *bdsABC* and *tdsABC*.

The nucleotide sequences of the DBT-desulfurization genes from WU-S2B and WU-F1 were completely the same as each other. These results suggest that the *bdsABC* genes might be transferred by horizontal transmission. In addition, the nucleotide sequence adjacent to the *bdsABC* genes of WU-F1 was also completely the same as that of WU-S2B, at least in the upstream region of 3.3 kb and the downstream region of 0.4 kb. These results indicate that the *bds* promoter is recognized in both the *Bacillus* and *Mycobacterium* strains.

The recombinant *E. coli* cells carrying *bdsABC* exhibited DBT-desulfurizing activity over a wide temperature range up to 52˚C (Fig. 6.4). In addition, the recombinant *E. coli* showed similarity in temperature dependence of DBT-desulfurizing activity to *B. subtilis* WU-S2B and *M. phlei* WU-F1, indicating that the abilities of WU-S2B and WU-F1 to desulfurize DBT over a wide temperature range were endowed by the thermophilic DBT-desulfurization genes, *bdsABC*. On the other hand, the gene products of *bdsA* and *bdsC* encoding flavin-dependent monooxygenases could couple with a natural flavin reductase(s) existing in *E. coli* since a heterologous flavin reductase gene was not introduced into the recombinant *E. coli* in the present study. The recombinant *E. coli* exhibited higher DBT-desulfurizing activity than WU-S2B, but lower activity than WU-F1 (Fig. 6.4). The differences in DBT-desulfurizing activities among WU-S2B, WU-F1, and the recombinant *E. coli* might be due to the differences in abilities to take up substrate, expression levels of *bdsABC*, and/or flavin reductase activities among them. These results imply that the ability of host cell is critical for high DBT-desulfurizing activity.

It has recently been reported that another thermophilic *M. phlei*, GTIS10, possessed high DBT-desulfurizing ability [19]. However, it is surprising to note that the DBT-desulfurization genes of *M. phlei* GTIS10 were identical in nucleotide sequence to the *dszABC* genes of *Rhodococcus* sp. IGTS8. It was confirmed using purified enzymes that the gene products of *bdsABC* exhibited the activity at higher temperatures and higher heat stability than those of *dszABC* (unpublished data). These results indicate that *M. phlei* WU-F1 possesses more suitable components for thermophilic biodesulfurization than *M. phlei* GTIS10. However, the author does not have a clear explanation for the fact that *M. phlei* GTIS10 exhibited DBT-desulfurizing

activity even at 57˚C.

In conclusion, the author confirmed that *B. subtilis* WU-S2B and *M. phlei* WU-F1 possessed the same DBT-desulfurization genes as each other, and that the abilities of WU-S2B and WU-F1 to desulfurize DBT over a wide temperature range were endowed by the thermophilic DBT-desulfurization genes, *bdsABC*. In addition, for practical biodesulfurization, the *bdsABC* genes may be useful genetic resources to improve desulfurizing biocatalysts by genetic engineering [20].

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Chapter 7

Cloning of the Flavin Reductase Gene Involved in DBT Desulfurization from *Bacillus subtilis* WU-S2B through Coexpression Screening Using Indigo Production as Selective Indication

7.1. Introduction

Many dibenzothiophene (DBT)-desulfurizing microorganisms have been isolated as desulfurizing biocatalysts to date [1]. Particularly, a mesophilic DBT-desulfurizing bacterium *Rhodococcus* sp. IGTS8 has been extensively studied so far, and the genes involved in DBT desulfurization (*dszABC*) were cloned and characterized [2-4]. Moreover, in Chapter 6, the author and collaborators cloned and characterized the DBT-desulfurization genes (*bdsABC*) of thermophilic DBT-desulfurizing bacteria, *Bacillus subtilis* WU-S2B and *Mycobacterium phlei* WU-F1.

These bacteria desulfurize DBT through a sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds as shown in Fig. 7.1 [2, 3]. In this pathway, DBT is oxidized to DBT sulfone via DBT sulfoxide by DBT monooxygenase (DszC/BdsC), DBT sulfone is converted to 2'-hydroxybiphenyl-2-sulfinate (HBPSi) by DBT sulfone monooxygenase (DszA/BdsA), and HBPSi is desulfurized to 2-hydroxybiphenyl (2-HBP) with the release of the sulfur atom as sulfite by HBPSi desulfinase (DszB/BdsB). In addition, flavin reductase (NAD(P)H:FMN

oxidorductase, E.C. 1.6.8.1) has been found to be essential in combination with two flavin-dependent monooxygenases, DszC/BdsC and DszA/BdsA. Flavin reductase uses NAD(P)H to catalyze the reduction of FMN and the resulting reductant $FMNH_2$ is used to activate oxygen by the terminal monooxygenases. Other members of the two-component monooxygenase class have also been reported. Luciferase from luminous bacteria is a typical two-component monooxygenase and has been thoroughly characterized [5]. Styrene monooxygenase [6], 4-hydroxyphenylacetate 3-monooxygenase [7], pyrrole-2-carboxylate monooxygenase [8], nitrilotriacetate monooxygenase [9], and EDTA monooxygenase [10] have been discovered in the degradation pathway of each substrate. In addition, monooxygenases of this type have been found to be involved in biosyntheses of antibiotics such as pristinamycin II_A and valanimycin [11, 12].

On the other hand, it is known that recombinant strains of *E. coli* expressing genes encoding various monooxygenases and dioxygenases can oxidize indole to blue pigment indigo [6, 13-17]. Recently, it has been reported that the DBT monooxygenase DszC from *Rhodococcus* sp. IGTS8 also catalyzed the conversion of indole to indigo in the presence of the flavin reductase (DszD) [18]. Thus, it occurred to us to apply this indigo production as selective indication for cloning a gene encoding flavin reductase from *B. subtilis* WU-S2B (Fig. 7.1). That is, if the recombinant *E. coli* carrying the DBT-desulfurization genes *bdsABC* acquires a gene encoding flavin reductase and these genes are coexpressed, indole which is provided from a natural metabolic pathway(s) in *E. coli* would be converted to indigo via indoxyl, resulting in the formation of a blue colony on a nutrient agar plate.

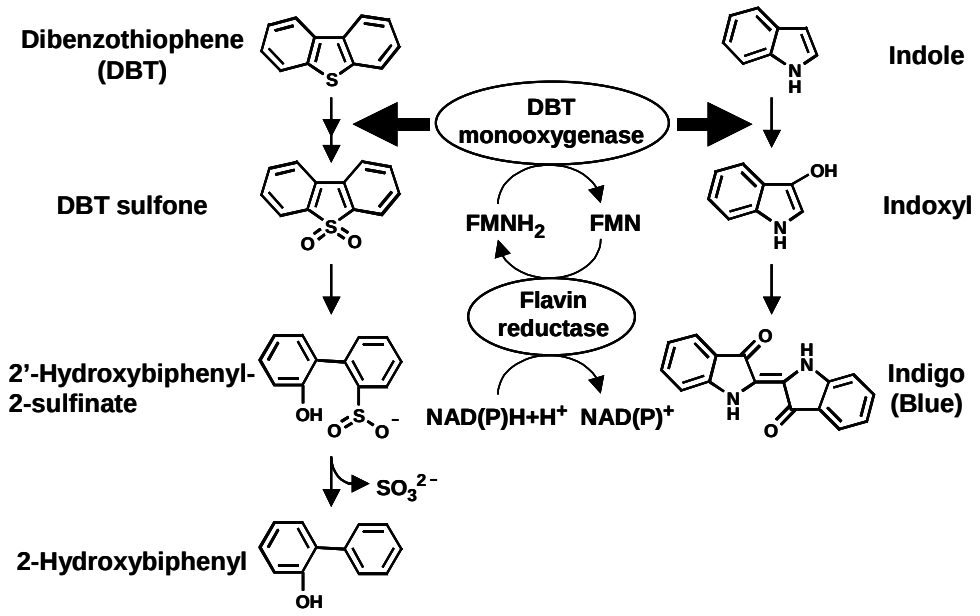

Fig. 7.1. Conversion of indole to indigo by DBT monooxygenase in the presence of flavin reductase. Indole is provided from a natural metabolic pathway(s) in *E. coli*. Indoxyl is spontaneously transformed to indigo [13].

In this chapter, the author describes the cloning of the flavin reductase gene involved in DBT desulfurization from *B. subtilis* WU-S2B through coexpression screening using indigo production as selective indication. Using this method, a gene encoding flavin reductase (*frb*) was cloned from *B. subtilis* WU-S2B. In addition, coexpression of *frb* with *bdsABC* was critical for high DBT-desulfurizing ability over a wide temperature range of 20 to 55˚C. Moreover, these results suggest that this coexpression screening using indigo production as selective indication may be widely applicable for cloning novel genes encoding either component of flavin reductase or flavin-dependent monooxygenase which efficiently couples with the other component in two-component monooxygenases.

7.2. Materials and methods

7.2.1. Chemicals

DBT was purchased from Tokyo Kasei (Tokyo, Japan). NADH and NADPH were purchased from Oriental Yeast (Tokyo, Japan). FMN was purchased from Wako Pure Chemicals (Osaka, Japan). All other reagents were of analytical grade and commercially available.

7.2.2. Bacterial strains, plasmids, and cultivation

B. subtilis WU-S2B was used as the source of total DNA for cloning of a flavin reductase gene. For preparation of total DNA, WU-S2B was cultivated at 50˚C in test tubes (18 by 180 mm) containing 5 ml of A-2 medium (Chapter 2.3.1) with 0.54 mM DBT. *E. coli* JM109 and an expression vector pSTV28 (Takara Bio, Tokyo, Japan) were used for cloning and expression studies. *E. coli* BL21 (DE3) and an expression vector pET21-a (Novagen, Darmstadt, Germany) were used for overexpression studies. *E. coli* strains were cultivated in Luria-Bertani (LB) medium (Chapter 2.3.4) supplemented with antibiotics (100 μg/ml ampicillin and/or 35 μg/ml chloramphenicol) when they were required. The recombinant plasmid pKBDS includes the DBT-desulfurization genes *bdsABC* from WU-S2B in pKK223-3 (Chapter 6). The nucleotide sequence of *bdsABC* is available from GeneBank under Accession No. AB076745.

7.2.3. Recombinant DNA techniques

Recombinant DNA techniques were performed as described by Sambrook et al. [19] or according to manufacturer's protocols (Chapter 2). Total DNA from WU-S2B

was prepared with a QIAGEN genomic-tip (QIAGEN, Tokyo, Japan). Plasmid DNA was prepared with a GFX micro plasmid prep kit (Amersham Biosciences, NJ, USA). DNA fragments were purified with a GFX PCR DNA and gel band purification kit (Amersham Biosciences). DNA sequencing was performed with an ABI PRISM 310 genetic analyzer (Applied Biosystems, CA, USA). Nucleotide sequence was determined by complete sequencing of both strands, with multiple sequencing of some regions. Sequence analysis was performed with a Genetyx-Mac version 10.1 (SDC, Tokyo, Japan). Homology search of sequence was performed with the FASTA program of the DNA Data Bank of Japan (DDBJ).

7.2.4. Cloning of a flavin reductase gene through coexpression screening

Total DNA of WU-S2B was partially digested with *Sau*3AI. After electophoresis, fragments of 2 to 4 kb in length were excised from agarose gel and purified with a GFX PCR DNA and gel band purification kit. The DNA fragments were ligated by using DNA ligation kit ver. 2 (Takara Bio) to pSTV28 previously digested with *Bam*HI and dephosphorylated with bacterial alkaline phosphatase. The resulting recombinant plasmid and pKBDS, which includes the DBT-desulfurization genes *bdsABC* from WU-S2B in pKK223-3 (Chapter 6), were simultaneously introduced into *E. coli* JM109. Transformed cells were spread onto LB medium agar plates with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol, incubated at 37˚C, and used as a DNA library of WU-S2B. Blue colonies due to indigo production on LB medium agar plates were selected as candidates for cells carrying a flavin reductase gene from WU-S2B.

7.2.5. Amplification of the flavin reductase gene

The flavin reductase gene (*frb*) was subcloned and expressed under control of T7 promoter. The gene was amplified by PCR (Chapter 2.10) from the recombinant plasmid (pSTVfrb) including *frb* in pSTV28, which was obtained through the coexpression screening as described above, using two oligonucleotide primers P1 (5'-TTCCAT**ATG**AAAGTATTAGTGCTCGCATTT-3' [the *Nde*I restriction site is underlined and the ATG initiation codon is indicated by boldface type]) and P2 (5'-CACAAGCTT**TTA**AAATGACTTCAGCACATG-3' [the *Hin*dIII restriction site is underlined and the TTA termination codon is indicated by boldface type]). Amplified DNA fragments were digested with *Nde*I and *Hin*dIII and inserted into pET21-a. The resulting recombinant plasmid (pETfrb) was amplified in *E. coli* JM109 cells, and the insert was sequenced to ensure that the correct construction had been obtained. The pETfrb construct was then introduced into *E. coli* BL21 (DE3) for expression of the *frb* gene.

7.2.6. Expression of *frb* in *E. coli*

Recombinant *E. coli* BL21 (DE3) carrying pETfrb was cultivated at 30˚C in 500-ml flasks containing 100 ml of LB medium with 100 μg/ml ampicillin. After cultivation for 6 h, 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium, and cultivation was continued for additional 6 h. The cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4˚C, washed twice with 50 mM potassium phosphate buffer (pH 7.0), and suspended in the same buffer with 1 mM dithiothreitol and 10% glycerol. Then, they were disrupted with an ultraoscillator at 20 kHz. The cell debris was removed by centrifugation at 16,000 × *g* for 30 min at

4˚C.

7.2.7. Enzyme assays for flavin reductase activity

Flavin reductase activity was determined at 50˚C by monitoring decrease in absorbance at 340 nm due to oxidation of NAD(P)H (Chapter 2.5). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.3 mM NAD(P)H, 0.01 mM FMN, and the cell-free extracts in total volume of 1 ml. One unit of activity is defined as the amount of enzyme necessary to oxidize 1 μmol of NAD(P)H per min ($\varepsilon_{340} = 6.22 \times 10^3\ M^{-1} \cdot cm^{-1}$).

7.2.8. Protein determination

Protein concentration was determined by using a protein assay kit (Bio-Rad, CA, USA) with a bovine serum albumin as the standard (Chapter 2.6).

7.2.9. Construction of a DNA cassette with *frb* and *bdsABC*

A 0.8-kb fragment including *frb* and its upstream region was amplified by PCR (Chapter 2.10) from pSTVfrb using two oligonucleotide primers P3 (5'-GACGCGTGA GCTCCGTTTTCACCTGT-3' [the *Mlu*I restriction site is underlined]) and P4 (5'-GAAGCTTGAGCTCATAAGCCGGGCTT-3' [the *Hin*dIII restriction site is underlined]). Amplified DNA fragments were digested with *Mlu*I and *Hin*dIII and inserted into pKBDS to locate the fragment at downstream region of the DBT-desulfurization genes *bdsABC* in the same direction. The resulting recombinant plasmid (pKBDSfrb) was amplified in *E. coli* JM109 cells, and the insert was sequenced to ensure that the correct construction had been obtained. The pKBDSfrb construct

was then introduced into *E. coli* JM109 for expression of the *bdsABC* genes and the *frb* gene.

7.2.10. Coexpression of *frb* with *bdsABC* in *E. coli*

Recombinant *E. coli* cells carrying pKBDSfrb or pKBDS were cultivated at 25˚C in 500-ml flasks containing 100 ml LB medium with 100 μg/ml ampicillin. After cultivation for 12 h, 0.2 mM IPTG was added to the medium, and cultivation was continued for additional 12 h. The cells were harvested and washed in the same way as described above, and suspended in 50 mM potassium phosphate buffer (pH 7.0). The optical density at 660 nm (OD_{660}) of cell suspension was adjusted to 20. Eleven microliters of a 10 g/l DBT stock solution in *n*-tridecane was added to 10 ml screw-cap test tubes containing 0.6 ml of the cell suspension to a final concentration of 0.5 mM DBT. Resting cell reactions were performed with inverted shaking at 50 rpm for 30 min. DBT and its desulfurized metabolite 2-HBP were determined by using high-performance liquid chromatography (HPLC) (Chapter 2.13.2).

7.2.11. Nucleotide sequence accession number

The nucleotide sequence discussed in this paper is available from GeneBank under Accession No. AB125067 as the flavin reductase gene of *B. subtilis* WU-S2B, *frb*.

7.3. Results

7.3.1. Cloning of a flavin reductase gene through coexpression screening

It has been reported that the DBT monooxygenase DszC from *Rhodococcus* sp. IGTS8 catalyzed the conversion of indole to indigo in the presence of the flavin

reductase DszD [18]. In addition, it was confirmed that the recombinant *E. coli* cells expressing both the DBT monooxygenase gene *bdsC* from *B. subtilis* WU-S2B and a flavin reductase gene *tdsD* from another thermophilic DBT-desulfurizing bacterium *Paenibacillus* sp. A11-2 [20] resulted in the formation of blue colonies on a LB agar plate (unpublished data). Thus, to clone a gene encoding flavin reductase from *B. subtilis* WU-S2B, indigo production by coexpression of a corresponding gene with *bdsC* in *E. coli* was used as a selection (Fig. 7.1). The DNA library of WU-S2B was constructed as described in Materials and methods, and contained approximately 5,000 colonies. Among them, two colonies showing clearly blue color were selected as candidates for cells carrying a flavin reductase gene from WU-S2B. The two recombinant strains were cultivated in liquid LB medium for 18 h at 37˚C, and the resulting blue pigment was extracted with *N,N*-dimethylformamide from the pellets after centrifugation. By thin-layer chromatography (TLC) analysis [21], the components of the pigment were confirmed to be largely indigo (blue) and a small amount of indirubin (red) (data not shown).

The nucleotide sequences of the inserts in pSTV28 from the two positive clones were determined and found to be the same as each other. The insert in the recombinant plasmid designated pSTVfrb was approximately 1.4 kb in size and contained one complete open reading frame (ORF) and one partial ORF. The complete ORF encodes a protein of 174 amino acid residues with a molecular weight of 19,663. As shown in Fig. 7.2, the deduced amino acid sequence of the ORF shares 61% identity with that of a nitroreductase (YwrO) of *B. amyloliquefaciens* [22], which could catalyze the reduction of FMN in addition to nitro compounds such as 5-aziridinyl-2,4-dinitrobenzamide. Thus, the ORF was designated *frb* for flavin reductase of *B. subtilis* WU-S2B since this

Fig. 7.2. Multiple sequence alignment of the flavin reductases related to Frb. Black shading indicates identical residues in all the members. Gray shading indicates identical residues in two members. Bs_Frb, flavin reductase of *B. subtilis* WU-S2B (accession number AB125067); Ba_YwrO, nitroreductase of *B. amyloliquefaciens* (AF373598); Bs_YwrO, hypothetical protein of *B. subtilis* (Z99122).

gene product Frb was confirmed to exhibit flavin reductase activity, as described later. The deduced amino acid sequence of *frb* also shares 65% identity with that of a hypothetical protein (YwrO) of *B. subtilis* [23], which was identified by the genome sequencing project. On the other hand, the deduced amino acid sequence of the partial ORF, which was located downstream of *frb* in the opposite direction, shares 81% identity with that of a hypothetical transporter protein of *Ralstonia solanacearum* (Accession No. AL646086) [24] and shows low homology to those of unknown hypothetical proteins (around 40% identity).

7.3.2. Expression of *frb* in *E. coli*

The flavin reductase gene *frb* was subcloned to exclude the partial ORF and expressed under control of T7 promoter. The gene was amplified by PCR from pSTVfrb obtained as described above, and was inserted into pET21-a. The resulting recombinant plasmid, pETfrb, was introduced into *E. coli* BL21 (DE3). Using cell-free extracts of *E. coli* BL21 (DE3) carrying pETfrb, it was confirmed that this gene

Table 7.1. Expression of the flavin reductase gene *frb* in *E. coli*.

Strain	Specific activity (U/mg)[a]	
	NADH	NADPH
E. coli (pETfrb)	9.58	12.70
E. coli (pET21-a)	0.57	0.76
B. subtilis WU-S2B	0.39	0.19

[a] Flavin reductase activity was determined at 50˚C with NAD(P)H as electron donors and FMN as an electron acceptor.

product Frb exhibited flavin reductase activity as shown in Table 7.1. When *frb* was overexpressed in the presence of IPTG, the specific activity in cell-free extracts of the recombinant *E. coli* was about 25-fold higher for NADH than that of wild type strain, *B. subtilis* WU-S2B. In addition, Frb used NADPH as well as NADH to catalyze the reduction of FMN. On the other hand, it was confirmed that *E. coli* BL21 (DE3) carrying only *frb* showed no productivity of indigo (data not shown).

7.3.3. Coexpression of *frb* with *bdsABC* in *E. coli*

A DNA cassette with *frb* and the DBT-desulfurization genes *bdsABC* was constructed as described in Materials and methods, and *frb* was coexpressed with *bdsABC* in *E. coli*. As shown in Fig. 7.3, resting cells of *E. coli* JM109 (pKBDSfrb) carrying both *frb* and *bdsABC* exhibited high DBT-desulfurizing activity over a wide temperature range of 20 to 55˚C. The DBT-desulfurizing activity of *E. coli* JM109 (pKBDSfrb) was the highest at 40˚C and about 27-fold higher than that of *E. coli* JM109 (pKBDS) carrying only *bdsABC*. These results indicated that coexpression of

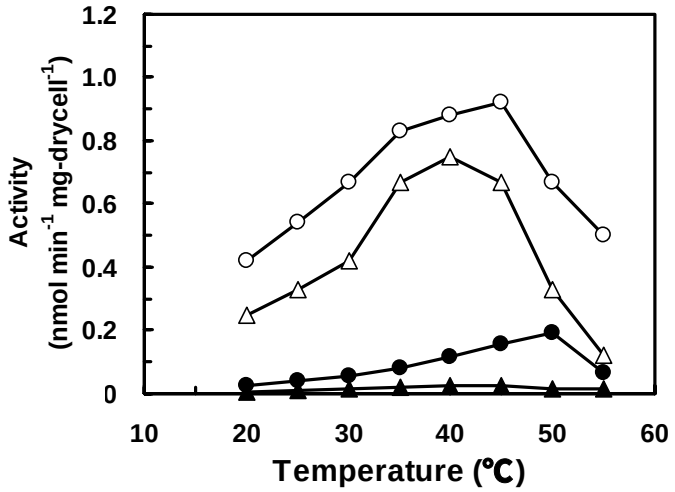

Fig. 7.3. Coexpression of the flavin reductase gene *frb* with the DBT-desulfurization genes *bdsABC* in *E. coli*. The reaction mixture contained 11 μl of a 10 g/l DBT stock solution in *n*-tridecane and 0.6 ml of the cell suspension in a 10 ml screw-cap test tube to give a final concentration of 0.5 mM DBT. Resting cell reactions were performed with inverted shaking at 50 rpm for 30 min. Symbols: ○ and △, DBT-degrading and 2-HBP-producing activities of the recombinant *E. coli* carrying both *frb* and *bdsABC,* respectively; ● and ▲, DBT-degrading and 2-HBP-producing activities of the recombinant *E. coli* carrying only *bdsABC,* respectively.

frb with *bdsABC* was critical for high DBT-desulfurizing ability over a wide temperature range.

7.4. Discussion

In this chapter, the author described the cloning of the flavin reductase gene (*frb*) involved in DBT desulfurization from *B. subtilis* WU-S2B through the coexpression screening using indigo production as selective indication. It was confirmed that the recombinant *E. coli* cells expressing the DBT monooxygenase gene *bdsC* from *B. subtilis* WU-S2B possessed the ability to oxidize indole to blue pigment indigo in the presence of the flavin reductase TdsD from another thermophilic DBT-desulfurizing bacterium *Paenibacillus* sp. A11-2 [20]. Thus, to clone the gene encoding flavin

reductase from WU-S2B, indigo production by coexpression of the corresponding gene with *bdsC* in *E. coli* was used as a selection (Fig. 7.1). Using this method, the corresponding gene *frb* was obtained from the recombinant strain forming a blue colony due to indigo production on a LB agar plate, and it was confirmed that this gene product Frb exhibited flavin reductase activity (Table 7.1). In addition, coexpression of *frb* with the DBT-desulfurization genes *bdsABC* from *B. subtilis* WU-S2B was critical for high DBT-desulfurizing ability over a wide temperature range of 20 to 55˚C (Fig. 7.3). The DBT-desulfurizing activity of *E. coli* JM109 carrying both *frb* and *bdsABC* was about 27-fold higher at 40˚C than that of *E. coli* JM109 carrying only *bdsABC*. The low DBT-desulfurizing activity of *E. coli* JM109 carrying only *bdsABC* was attributed to a natural flavin reductase(s) existing in *E. coli*.

Frb shares 61% identity in amino acid sequence with the nitroreductase YwrO of *Bacillus amyloliquefaciens*, which can catalyze the reduction of FMN in addition to nitro compounds such as 5-aziridinyl-2,4-dinitrobenzamide (Fig. 7.2). It was reported that the nitroreductase used both NADH and NADPH to catalyze the reduction of FMN [22]. In the present study, it was found that Frb also used both NADH and NADPH. On the other hand, Frb shows the highest homology in amino acid sequence to the hypothetical protein YwrO in *B. subtilis* (Fig. 7.2), which was identified by the genome sequencing project [23]. It is interesting to note that *frb* shares only 65% identity with the hypothetical protein although both of the two proteins were derived from the bacteria belonging to the same species *B. subtilis*.

It has been reported that recombinant strains of *E. coli* expressing genes encoding various monooxygenases and dioxygenases could oxidize indole to indigo. Dioxygenases which activate oxygen with mononuclear iron center such as naphthalene

dioxygenase [13], monooxygenases with binuclear iron center such as toluene, phenol, and dimethyl sulfide monooxygenases [14-16], and cytochrome P450s [17] have been found to catalyze the conversion of indole to indigo. Styrene monooxygenase [6] as a two-component monooxygenase also catalyzes this reaction. In addition, it has been recently reported that direct evolution of a flavoprotein oxygenase, 2-hydroxybiphenyl 3-monooxygenase, resulted in an enzyme variant that catalyzes the conversion of indole to indigo [21] although the wild-type enzyme does not catalyze this reaction. There seems to be no relationship between the reaction mechanism of oxygenases and their ability to catalyze the conversion of indole to indigo. In addition, through the present study, it has been confirmed that the DBT monooxygenase BdsC from *B. subtilis* WU-S2B also catalyzed the conversion of indole to indigo in the presence of the flavin reductase (Fig. 7.1). It is interesting to note that BdsC oxidizing the sulfur atom in DBT molecule possesses the ability to oxidize the carbon atom in indole molecule. There seems to be no strict relationship between the substrate specificity of oxygenases and their ability to catalyze the conversion of indole to indigo although aromatic oxygenases tend to catalyze this reaction. Thus, this indigo production provides a simple method as selective indication for cloning various oxygenase genes.

Two-component monooxygenases consisting of flavin-dependent monooxygenase and flavin reductase are involved in attractive biotranfomations [5-12]. The genes encoding the two components of the enzymes are often located very close to each other, but sometimes very far [7]. The *dszD* gene encoding flavin reductase involved in DBT desulfurization from *Rhodococcus* sp. IGTS8 is located on the genome far from the *dszA* and *dszC* genes encoding flavin-dependent monooxygenases, which are located on the plasmid [4]. If a flavin reductase gene is located very far from a flavin-dependent

monooxygenase gene, it is impossible to clone the genes encoding the two components of the enzyme simultaneously. Since it was confirmed that no flavin reductase gene existed close to the *bdsABC* genes of *B. subtilis* WU-S2B, at least in the upstream region of 3.3 kb and the downstream region of 0.4 kb (Chapter 6), in the present study the coexpression screening using indigo production as selective indication was utilized to clone the *frb* gene encoding flavin reductase.

In conclusion, the author confirmed that coexpression of the flavin reductase gene *frb*, which was cloned using this method, with the DBT-desulfurization genes *bdsABC* was critical for high DBT-desulfurizing ability over a wide temperature range. On the other hand, it has been reported that a flavin reductase from *Vibrio harveyi* was coupled with DBT and DBT sulfone monooxygenases, DszC and DszA, from *Rhodococcus* strains [25, 26] and that a flavin reductase from *Vibrio fischeri* was coupled with a EDTA monooxygenase from a bacterial strain DSM 9103 [10] and a isobutylamine *N*-hydroxylase from *Streptomyces viridifaciens* MG456-hF10 [12]. These findings indicate that flavin reductases are exchangeable for each other to stimulate a flavin-dependent monooxygenase. Therefore, the coexpression screening in the present study may be widely applicable for cloning endogenous or heterologous genes encoding novel flavin reductases which efficiently couple with a flavin-dependent monooxygenase. Reversely, novel flavin-dependent monooxygenases whose activities cannot be detected *in vitro* due to low or no flavin reductase activity might be obtained through the coexpression screening with a flavin reductase gene using indigo production as selective indication.

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Chapter 8

Cloning of the Flavin Reductase Gene Involved in DBT Desulfurization from *Mycobacterium phlei* WU-F1 and Purification and Characterization of the Cloned Enzyme

8.1. Introduction

Many dibenzothiophene (DBT)-desulfurizing microorganisms have been isolated as desulfurizing biocatalysts to date [1]. These bacteria desulfurize DBT through a sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds (Chapter 1.4) [2, 3]. The conversion is initiated by consecutive sulfur atom-specific oxidations by two monooxygenases, and flavin reductase (NAD(P)H:FMN oxidorductase, E.C. 1.6.8.1) is essential in combination with these flavin-dependent monooxygenases. Flavin reductase uses NAD(P)H to catalyze the reduction of FMN and the resulting reductant $FMNH_2$ is used to activate oxygen by the terminal monooxygenases.

In Chapter 6, the author and collaborators cloned and characterized the DBT-desulfurization genes (*bdsABC*) of thermophilic DBT-desulfurizing bacteria, *Bacillus subtilis* WU-S2B and *Mycobacterium phlei* WU-F1. Moreover, in Chapter 7, the gene encoding flavin reductase (*frb*) was cloned from *B. subtilis* WU-S2B through coexpression screening using indigo production as selective indication.

In this chapter, the author describes the cloning of the flavin reductase gene

involved in DBT desulfurization from *M. phlei* WU-F1 and purification and characterization of the cloned enzyme. A gene encoding flavin reductase (*frm*) was cloned from *M. phlei* WU-F1. In addition, the author purified this gene product, Frm, from the recombinant *E. coli* and characterized, and found that Frm exhibited high flavin reductase activity over a wide temperature range. The enzyme also exhibited high stability under high-temperature and even acidic and alkaline conditions. Moreover, coexpression of *frm* with *bdsABC* was critical for high DBT-desulfurizing ability over a wide temperature range. This is the first report describing the characterization of *Mycobacterium* flavin reductase.

8.2. Materials and methods

8.2.1. Chemicals

DBT was purchased from Tokyo Kasei (Tokyo, Japan). NADH and NADPH were purchased from Oriental Yeast (Tokyo, Japan). FMN was purchased from Wako Pure Chemicals (Osaka, Japan). All other reagents were of analytical grade and commercially available.

8.2.2. Bacterial strains, plasmids, and cultivation

M. phlei WU-F1 was used as the source of total DNA for cloning of a flavin reductase gene. For preparation of total DNA, WU-F1 was cultivated at 50˚C in test tubes (18 by 180 mm) containing 5 ml of AF medium (Chapter 2.3.2) with 0.54 mM DBT, followed by incubation at 37˚C with 50 μg/ml D-cycloserine and 100 μg/ml lysozyme (Chapter 2.5) [4]. *E. coli* DH5α and an expression vector pUC118 (Takara Bio, Tokyo, Japan) were used for cloning and expression studies. *E. coli* BL21 (DE3)

and an expression vector pET21-a (Novagen, Darmstadt, Germany) were used for overexpression studies. *E. coli* DH5α and an expression vector pSTV28 (Takara Bio, Tokyo, Japan) were used for coexpression studies. *E. coli* strains were cultivated in Luria-Bertani (LB) medium (Chapter 2.3.4) supplemented with antibiotics (100 μg/ml ampicillin and/or 35 μg/ml chloramphenicol) when they were required. The recombinant plasmid pKBDS includes the DBT-desulfurization genes *bdsABC* from WU-S2B in pKK223-3 (Chapter 6). The nucleotide sequence of *bdsABC* is available from GeneBank under Accession No. AB076745.

8.2.3. Recombinant DNA techniques

Recombinant DNA techniques were performed as described by Sambrook et al. [5] or according to manufacturer's protocols (Chapter 2). Total DNA from WU-S2B was prepared with a QIAGEN genomic-tip (QIAGEN, Tokyo, Japan). Plasmid DNA was prepared with a GFX micro plasmid prep kit (Amersham Biosciences, NJ, USA). DNA fragments were purified with a GFX PCR DNA and gel band purification kit (Amersham Biosciences). DNA sequencing was performed with an ABI PRISM 310 genetic analyzer (Applied Biosystems, CA, USA). Nucleotide sequence was determined by complete sequencing of both strands, with multiple sequencing of some regions. Sequence analysis was performed with a Genetyx-Mac version 10.1 (SDC, Tokyo, Japan). Homology search of sequence was performed with the FASTA program of the DNA Data Bank of Japan (DDBJ).

8.2.4. Construction of DNA library

Total DNA of WU-F1 was digested with *Pst*I. After electophoresis, fragments of

about 1.5 kb in length were excised from agarose gel and purified with a GFX PCR DNA and gel band purification kit. The DNA fragments were ligated by using DNA ligation kit ver. 2 (Takara Bio) to pUC118 previously digested with *Pst*I and dephosphorylated with bacterial alkaline phosphatase. The resulting recombinant plasmid was introduced into *E. coli* DH5α. Transformed cells were spread onto LB medium agar plates with 100 μg/ml ampicillin, incubated at 37˚C, and used as a DNA library of WU-F1.

8.2.5. Screening of DNA library by colony hybridization

The DNA library of WU-F1 was screened by colony hybridization with a DNA probe. The DNA probe was prepared by PCR amplification (Chapter 2.10) with the total DNA of WU-F1 as the template using primers based on the N-terminal and internal amino acid sequences of a flavin reductase partially purified from WU-F1. The PCR products were labeled with DIG (digoxigenin) DNA Labeling Kit (Roche, Basel, Switzerland). The total DNA library of WU-F1 on LB medium agar plates was transferred to Hybond-N+ nylon membranes (Amersham Biosciences), and the membranes were hybridized with the DNA probe (Chapter 2.11).

8.2.6. Amplification of the flavin reductase gene

The flavin reductase gene (*frm*) was subcloned and expressed under control of T7 promoter. The gene was amplified by PCR (Chapter 2.10) from the recombinant plasmid (pUCfrm) including *frm* in pUC118, which was obtained through colony hybridization as described above, using two oligonucleotide primers P1 (5'-TTCCAT**A TG**AGCGCAATCGATTTGAGCCCC-3' [the *Nde*I restriction site is underlined and the

ATG initiation codon is indicated by boldface type]) and P2 (5'-CACAAGCTT**TCA**GG CGGTGCCGAGGCGGCG-3' [the *Hin*dIII restriction site is underlined and the TCA termination codon is indicated by boldface type]). Amplified DNA fragments were digested with *Nde*I and *Hin*dIII, and inserted into pET21-a. The resulting recombinant plasmid (pETfrm) was amplified in *E. coli* DH5α cells, and the insert was sequenced to ensure that the correct construction had been obtained. The pETfrm construct was then introduced into *E. coli* BL21 (DE3) for expression of the *frm* gene.

8.2.7. Overxpression of *frm* and purification of the cloned enzyme

Recombinant *E. coli* BL21 (DE3) carrying pETfrm was cultivated at 25˚C in 500-ml flasks containing 100 ml of LB medium with 100 μg/ml ampicillin. After cultivation for 12 h, 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium, and cultivation was continued for additional 12 h. The cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4˚C, washed twice with 50 mM potassium phosphate buffer (pH 7.0), and suspended in the same buffer with 1 mM dithiothreitol and 10% glycerol. Then, they were disrupted with an ultraoscillator at 20 kHz. The cell debris was removed by centrifugation at 16,000 × *g* for 30 min at 4˚C.

The gene product, Frm, was purified from the cell-free extracts as described below. All purification steps were performed with an ÄKTA explorer (Amersham Biosciences) at 4˚C.

(i) Step 1: Anion-exchange chromatography. The cell-free extracts were applied to a HiLoad 26/10 Q-Sepharose HP column (2.6 by 10 cm) (Amersham Biosciences) which had been equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The

column was washed well with the same buffer, and the bound proteins were eluted with a linear gradient of 0 to 0.5 M KCl in the Tris-HCl buffer at a flow rate of 120 ml/h. The active fractions were combined and concentrated by ultrafiltration with a membrane filter YM-10 (Millipore, MA, USA).

(ii) Step 2: Gel permeation chromatography. The enzyme solution obtained in step 1 was loaded on a HiLoad 16/60 Superdex 75 pg column (1.6 by 60 cm) (Amersham Biosciences) which had been equilibrated with 50 mM Tris-HCl buffer (pH 8.0) with 0.15 M NaCl. The proteins were eluted with the same buffer at a flow rate of 30 ml/h, and the active fractions were combined.

(iii) Step 3: Affinity chromatography. The enzyme solution obtained in step 2 was dialyzed against 1.5 M potassium phosphate buffer (pH 7.6) and the dialyzed enzyme solution was applied to an FMN agarose column (1.6 by 5 cm) (Sigma, MO, USA) which had been equilibrated with the same buffer. The column was washed well with the same buffer, and the bound proteins were eluted with a linear gradient of 1.5 to 0.6 M potassium phosphate in the buffer at a flow rate of 60 ml/h. The active fractions were combined and used as the purified enzyme.

8.2.8. Enzyme assays for flavin reductase activity

Flavin reductase activity was determined at 50˚C by monitoring decrease in absorbance at 340 nm due to oxidation of NADH (Chapter 2.5). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.3 mM NADH, 0.01 mM FMN, and enzyme in total volume of 1 ml. One unit of activity is defined as the amount of enzyme necessary to oxidize 1 μmol of NADH per min ($\varepsilon_{340} = 6.22 \times 10^3\ M^{-1} \cdot cm^{-1}$).

8.2.9. Protein determination

Protein concentration was determined by using a Protein assay kit (Bio-Rad, CA, USA) with a bovine serum albumin as the standard (Chapter 2.6).

8.2.10. Molecular mass determination

The native molecular mass was estimated by gel permeation chromatography with a Superdex 75 column. The column was equilibrated with 50 mM Tris-HCl buffer (pH 8.0) with 0.15 M NaCl, and the proteins were eluted with the same buffer at a flow rate of 30 ml/h. The subunit molecular mass was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% polyacrylamide gels. The gels were stained with 0.25% Coomassie brilliant blue G-250 dissolved in 50% methanol-10% acetic acid, and then destained with 30% methanol-10% acetic acid.

8.2.11. Coexpression of *frm* with *bdsABC* in *E. coli*

A fragment containing *frm* was prepared from pETfrm by the digestion with *Xba*I and *Hin*dIII and inserted into pSTV28. The resulting recombinant plasmid (pSTVfrm) was amplified in *E. coli* DH5α cells, and the insert was sequenced to ensure that the correct construction had been obtained. Then, pSTVfrm and pKBDS, which includes the DBT-desulfurization genes *bdsABC* from WU-F1 in pKK223-3 (Chapter 6), were simultaneously introduced into *E. coli* DH5α.

Recombinant *E. coli* cells carrying pSTVfrm and pKBDS were cultivated at 25˚C in 500-ml flasks containing 100 ml LB medium with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol. After cultivation for 12 h, 0.2 mM IPTG was added to the medium, and cultivation was continued for additional 12 h. The cells were harvested and

washed in the same way as described above, and suspended in 50 mM potassium phosphate buffer (pH 7.0). The optical density at 660 nm (OD_{660}) of cell suspension was adjusted to 20. Eleven microliters of a 10 g/l DBT stock solution in *n*-tridecane was added to 10 ml screw-cap test tubes containing 0.6 ml of the cell suspension to a final concentration of 0.5 mM DBT. Resting cell reactions were performed with inverted shaking at 50 rpm for 30 min. DBT and its desulfurized metabolite 2-HBP were determined by using high-performance liquid chromatography (HPLC) (Chapter 2.13.2).

8.2.12. Nucleotide sequence accession number

The nucleotide sequence discussed in this paper is available from GeneBank under Accession No. AB125068 as the flavin reductase gene of *M. phlei* WU-F1, *frm*.

8.3. Results

8.3.1. Determination of N-terminal and internal amino acid sequences

Flavin reductase was purified 2400-fold from the wild type strain, *M. phlei* WU-F1, through the six chromatography steps, and SDS-PAGE of the purified preparation indicated the presence of one major band and several minor bands (data not shown). The N-terminal and internal amino acid sequences of the protein showing the major band were determined by using a protein sequencing system (type G1005A; Hewlett-Packard, Ontario, Canada), and were found to be SAIDLSPSTLREAFGFPSD GV and LRDLPALGISVLGEAHDVAART, respectively. The amino acid sequences showed approximately 70% homology to those of hypothetical flavin reductases of *M. tuberculosis* [6] and *M. leprae* [7], suggesting that the protein showing the major band

was a flavin reductase.

8.3.2. Cloning of a flavin reductase gene from *M. phlei* WU-F1

Based on the determined amino acid sequences, two degenerated primers 5'-GCIT TYGGICAYTTYCC-3' and 5'-GCIACRTCRTGIGCYTC-3' (I, inosine; Y, C+T; R, A+G) were designed, and used for PCR amplification with the total DNA of *M. phlei* WU-F1 as the template. The resulting 0.2-kb amplified DNA fragments were inserted into pGEM-T, and the insert was sequenced. The deduced amino acid sequence of this PCR product showed approximately 80% homology to those of hypothetical flavin reductases of *M. tuberculosis* [6] and *M. leprae* [7], and also showed appreciable homology to those of several flavin reductases in two-component monooxygenases [8]. Then, based on the nucleotide sequence of the amplified region, two oligonucleotide primers 5'-GGCGTGATCGCCATCGCC-3' and 5'-GAGCACACTGATCCCCAG-3' were designed, and used for second PCR amplification with the total DNA of WU-F1 as the template. The resulting PCR product was labeled with digoxigenin and used as the DNA probe.

The DNA library of WU-S2B was constructed as described in Materials and methods and contained approximately 1,200 plaques. By colony hybridization with the DNA probe, three positive colonies were obtained and selected as candidates for cells carrying a flavin reductase gene from WU-F1.

The nucleotide sequences of the inserts in pUC118 from the three positive clones were determined and found to be the same as each other. The insert in the recombinant plasmid designated pUCfrm was approximately 1.4 kb in size and contained one complete open reading frame (ORF). The ORF encodes a protein of 162 amino acid

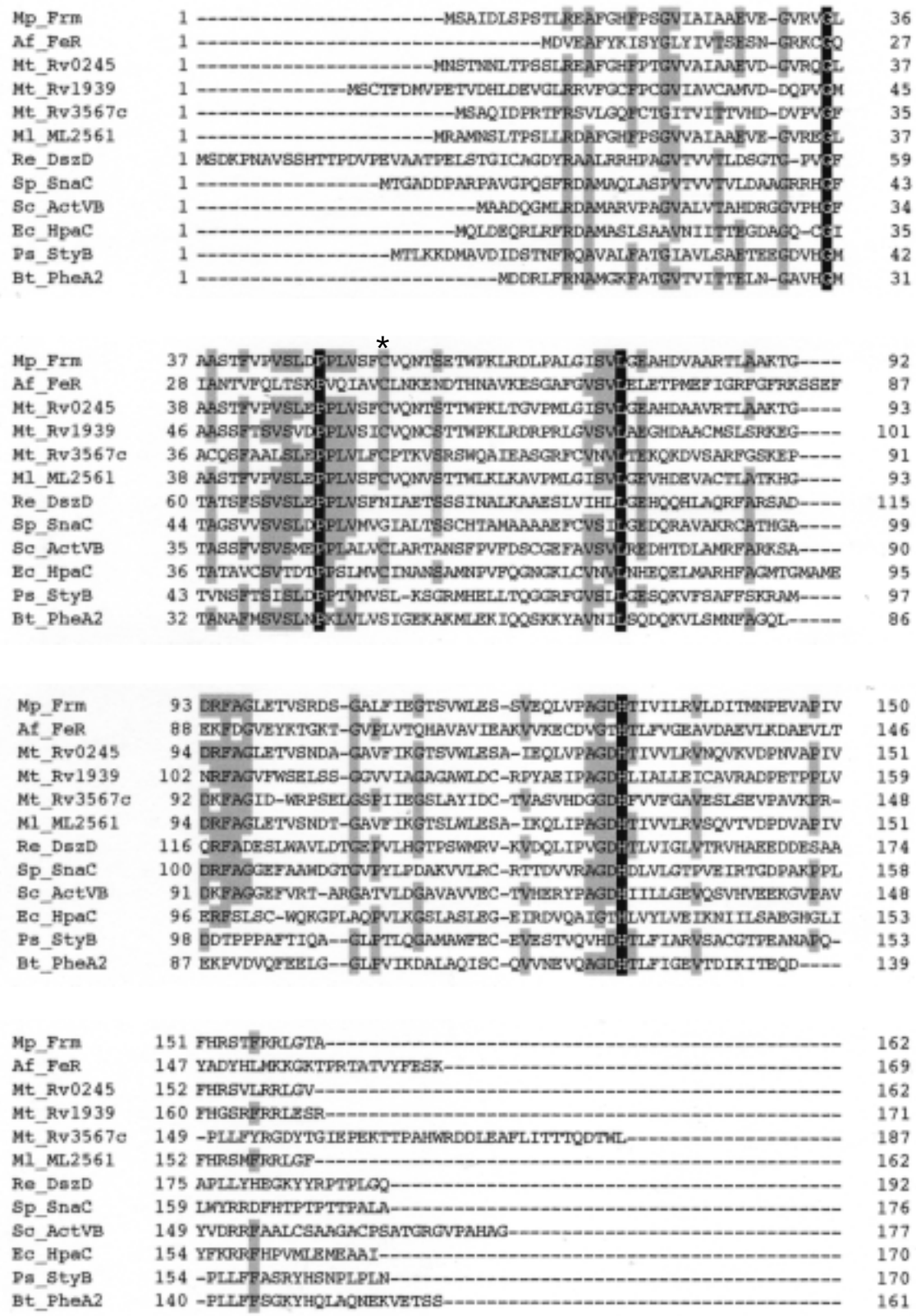
Mp_Frm
Af_FeR
Mt_Rv0245
Mt_Rv1939
Mt_Rv3567c
Ml_ML2561
Re_DszD
Sp_SnaC
Sc_ActVB
Ec_HpaC
Ps_StyB
Bt_PheA2

Fig. 8.1. Multiple sequence alignment of the flavin reductases related to Frm. Black shading indicates identical residues in all the members. Gray shading indicates identical residues in more than seven members. Asterisk indicates the position of the cysteine residue of Frm. Mp_Frm, flavin reductase involved in DBT desulfurization from *M. phlei* WU-F1 (accession number AB125068); Af_FeR, ferric reductase of *Archaeoglobus fulgidus* (AE001047); Mt_Rv0245, hypothetical flavin reductase of *M. tuberculosis* (AL021929); Mt_Rv1939, hypothetical flavin reductase of *M. tuberculosis* (Z84498); Mt_Rv3567c, hypothetical flavin reductase of *M. tuberculosis* (Z92774); Ml_ML2561, hypothetical flavin reductase of *M. leplae* (AL583926); Re_DszD, flavin reductase involved in DBT desulfurization from *Rhodococcus erythropolis* (AB051429); Sp_SnaC, flavin reductase involved in pristinamycin II_A biosynthesis from *Strepromyces pristinaespiralis* (U21216); Sc_ActVB, flavin reductase involved in actinorhodin biosynthesis from *S. coelicolor* (X63449); Ec_HpaC, flavin reductase component of 4-hydroxyphenylacetate 3-monooxygenase from *E. coli* (Z29081); Ps_StyB, flavin reductase component of styrene monooxygenase from *Pseudomonas* sp. (AJ000330); Bt_PheA, flavin reductase component of phenol hydroxylase from *Bacillus thermoleovorans* (AF140605).

residues with a molecular weight of 17,177. As shown in Fig. 8.1, the deduced amino acid sequence of the ORF shares 79% identity with that of a hypothetical flavin reductase (Rv0245) of *M. tuberculosis* [6] and 75% with that of a hypothetical flavin reductase (ML2561) of *M. leprae* [7], which were identified by the genome sequencing projects. In addition, the deduced amino acid sequence of the ORF shows appreciable homology to those of several flavin reductases in two-component monooxygenases (around 30% identity) [8], and was found to share some conserved regions with those of these flavin reductases (Fig. 8.1). Thus, the ORF was designated *frm* for flavin reductase of *M. phlei* WU-F1 since this gene product Frm was confirmed to exhibit flavin reductase activity, as described later.

8.3.3. Overxpression of *frm* and purification of the cloned enzyme

Expression of the flavin reductase gene *frm* by *E. coli* DH5α carrying pUCfrm, which was obtained as described above, was examined, but no expression was observed.

There is a possibility that the ribosome-binding site from *M. phlei* WU-F1 might not be effective in *E. coli*. Thus, *frm* was expressed under control of T7 promoter. The gene was amplified by PCR from pUCfrm, and was inserted into pET21-a. The resulting recombinant plasmid, pETfrm, was introduced into *E. coli* BL21 (DE3). Using cell-free extracts of *E. coli* BL21 (DE3) carrying pETfrm, it was confirmed that this gene product Frm exhibited flavin reductase activity. When *frm* was overexpressed in the presence of IPTG, the specific activity in cell-free extracts of the recombinant strain (127 U/mg) was 794-fold higher than that of the wild type strain, *M. phlei* WU-F1 (0.16 U/mg).

By using Q-Sepharose, Superdex 75, and FMN-agarose column chromatography, Frm was purified to homogeneity from cell-free extracts of *E. coli* BL21 (DE3) carrying pETfrm as shown in Table 8.1 and Fig. 8.2. Frm used NADH as an electron donor to catalyze the reduction of FMN, but did not use NADPH (data not shown). The native molecular mass of the enzyme was found to be 34 kDa by gel permeation chromatography. In addition, the subunit molecular mass of the enzyme was found to be 17 kDa by SDS-PAGE (Fig. 8.2). This value is in good agreement with the

Table 8.1. Purification of the flavin reductase Frm from the recombinamt *E. coli* [a].

Purification step	Protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Cell-free extracts	380	48,200	127	1	1 0 0
Q-Sepharose	61.3	18,100	295	2.3	37.5
Superdex 75	24.0	13,000	542	4.3	27.0
FMN-agarose	10.1	8,680	856	6.7	18.0

[a] Flavin reductase activity was determined at 50˚C with NADH as an electron donor and FMN as an electron acceptor.

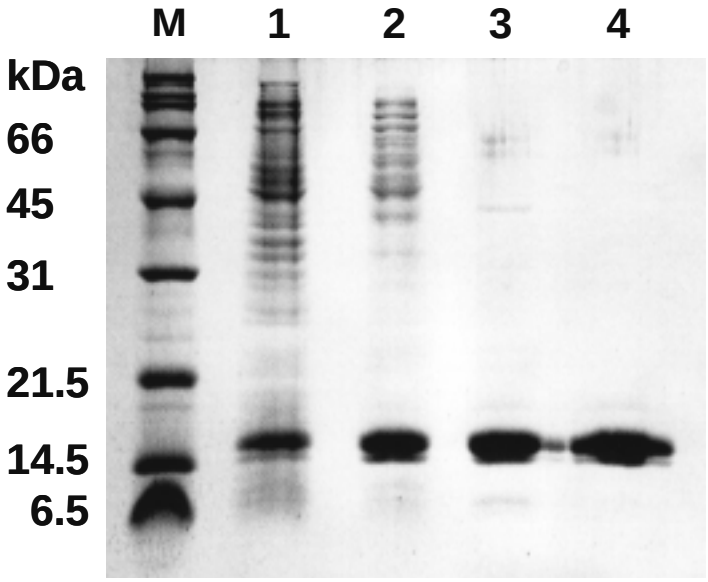

Fig. 8.2. SDS-PAGE of the flavin reductase Frm from the recombinamt *E. coli*. Lane M, marker proteins; lane 1, cell-free extracts; lane 2, Q-Sepharose; lane 3, Superdex 75; lane 4, FMN-agarose.

molecular mass predicted from the nucleotide sequence as described above. These results indicated that Frm was a 34 kDa homodimeric enzyme with a monomeric molecular mass of 17 kDa. It was also confirmed that the enzyme purified from the recombinant strain exhibited the same molecular masses as the enzyme partially purified from the wild type strain (data not shown). On the other hand, the purified Frm did not contain any tightly bound chromophore such as flavin since it had no absorption peak between 200 and 800 nm.

8.3.4. Effects of temperature and pH on the activity and stability of Frm

The effects of temperature and pH on the activity and stability of Frm were examined as shown in Fig. 8.3 and Fig. 8.4. When the enzyme activity was determined at various temperatures and pHs, the optimum temperature and pH were found to be 50˚C (Fig. 8.3A) and around 7.0 (Fig. 8.4A), respectively. To evaluate the

temperature stability of the enzyme, Frm was incubated at various temperatures for 30 min. The enzyme exhibited high stability under high-temperature conditions and retained more than 80% of the activity even at 60˚C (Fig. 8.3B). In addition, to evaluate the pH stability of the enzyme, Frm was incubated at various pHs for 24 h. The enzyme exhibited high stability even under acidic and alkaline conditions (Fig. 8.4B).

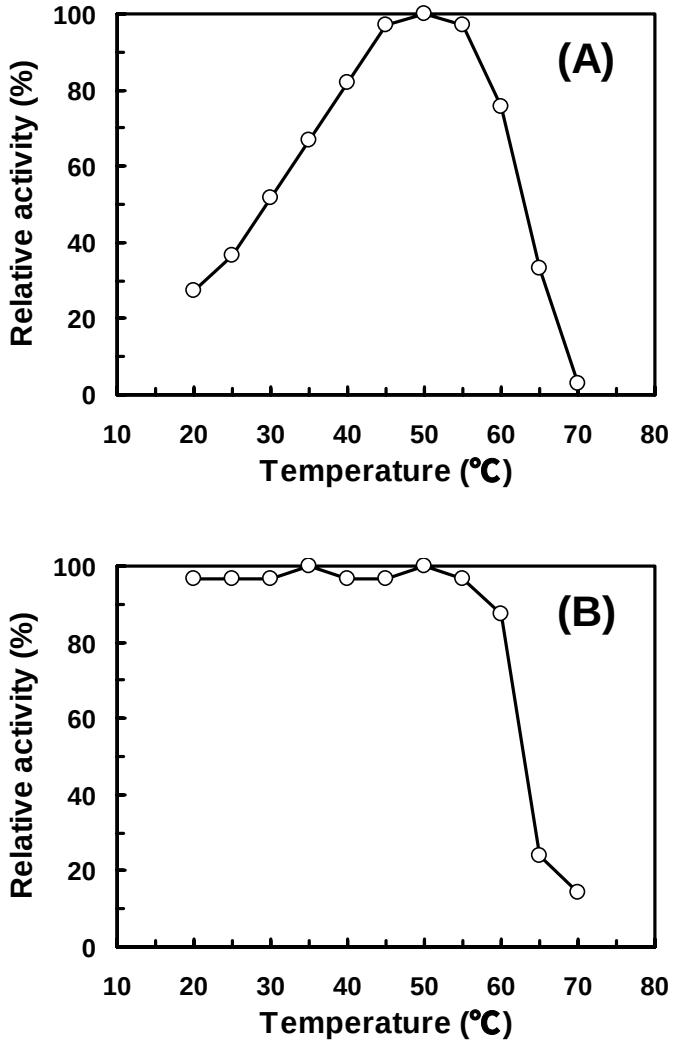

Fig. 8.3. Effects of temperature on the activity (A) and stability (B) of Frm. For determination of the stability, the enzyme was incubated at various temperatures for 30 min, and then the activity was measured as described in Materials and methods.

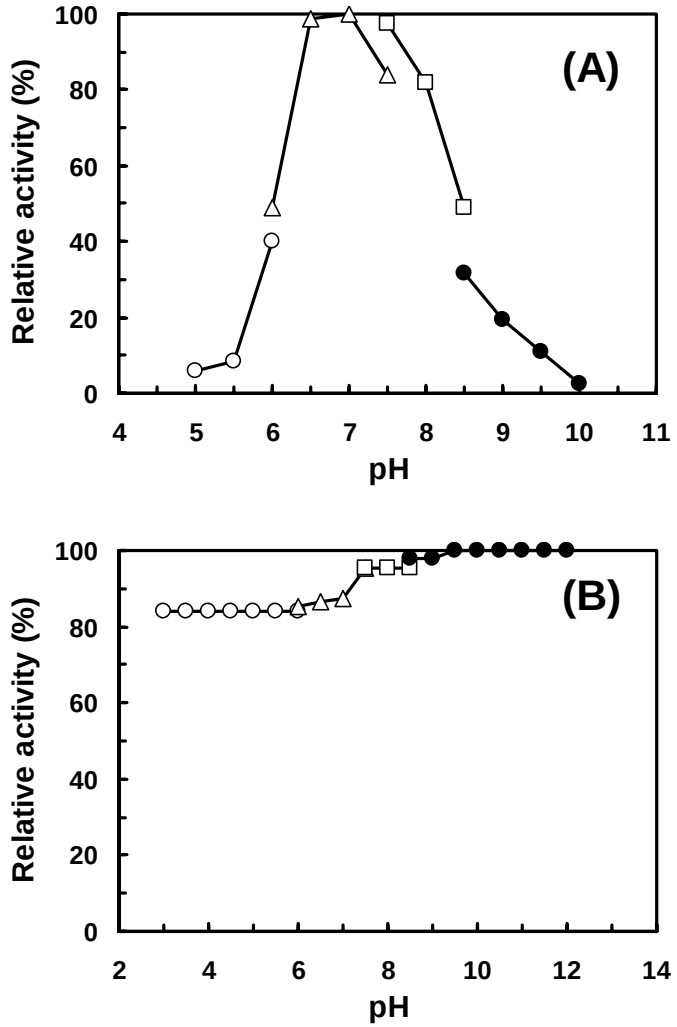

Fig. 8.4. Effects of pH on the activity (A) and stability (B) of Frm. For determination of the stability, the enzyme was incubated at various pHs for 24 h, and then the activity was measured as described in Materials and methods. Symbols: ○, citrate-phosphate buffer; △, potassium-phosphate buffer; □, Tris-HCl buffer; ●, glycine-NaOH buffer.

8.3.5. Effects of various compounds on the activity of Frm

The effects of various compounds on the activity of Frm were examined as shown in Table 8.2. All the metal ions tested did not activate Frm. Chelating reagents, i.e., EDTA and bipyridyl, did not inhibit the activity. These results suggested that metal ions were not required for the activity. Thiol reagents, i.e., 5,5'-dithio-bis-(2-nitorobenzoic acid) and *N*-ethylmaleimide, as well as Cu^{2+} and Ag^{+} strongly inhibited

Table 8.2. Effects of various compounds on the activity of Frm [a].

Compound	Relative activity (%)
None	100
5,5'-Dithio-bis(2-nitrobenzoic acid)	31.7
N-Ethylmaleimide	60.6
$CuCl_2$	0
$AgNO_3$	0
$CaCl_2$	93.5
$MgCl_2$	91.3
$MnCl_2$	90.4
$CoCl_2$	90.4
EDTA	97.8
2,2'-Bipyridyl	80.4

[a] Flavin reductase activity was determined as described in Materials and methods. All compounds were added to the reaction mixture at a 1 mM concentration except 5,5'-dithio-bis(2-nitrobenzoic acid) (0.1 mM).

the activity, suggesting that cysteine residue was involved in the activity.

8.3.6. Coexpression of *frm* with *bdsABC* in *E. coli*

pSTVfrm including *frm* in pSTV28 and pKBDS including the DBT-desulfurization genes *bdsABC* from *M. phlei* WU-F1 were simultaneously introduced into *E. coli* DH5α as described in Materials and methods, and *frm* was coexpressed with *bdsABC*. As shown in Fig. 8.5, resting cells of *E. coli* DH5α (pSTVfrm and pKBDS) carrying both *frm* and *bdsABC* exhibited high DBT-degrading activity over a wide temperature range of 20 to 55˚C. The DBT-degrading activity of *E. coli* DH5α (pSTVfrm and pKBDS) was the highest at 50˚C and about 7-fold higher than that of *E. coli* DH5α (pKBDS) carrying only *bdsABC*. These results indicated that coexpression of *frm* with *bdsABC* was critical for high DBT-desulfurizing ability over a wide

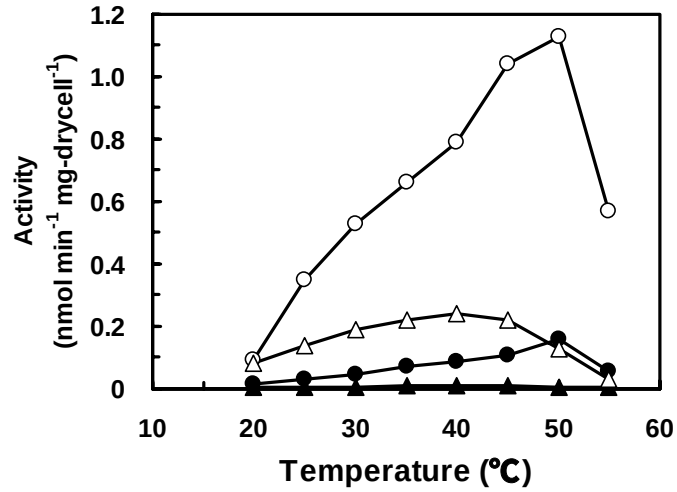

Fig. 8.5. Coexpression of the flavin reductase gene *frm* with the DBT-desulfurization genes *bdsABC* in *E. coli*. The reaction mixture contained 11 μl of a 10 g/l DBT stock solution in *n*-tridecane and 0.6 ml of the cell suspension in a 10 ml screw-cap test tube to give a final concentration of 0.5 mM DBT. Resting cell reactions were performed with inverted shaking at 50 rpm for 30 min. Symbols: ○ and △, DBT-degrading and 2-HBP-producing activities of the recombinant *E. coli* carrying both *frm* and *bdsABC,* respectively; ● and ▲, DBT-degrading and 2-HBP-producing activities of the recombinant *E. coli* carrying only *bdsABC,* respectively.

temperature range.

8.4. Discussion

Flavin reductases are the enzymes that catalyze the reduction of flavin such as FMN and FAD with NAD(P)H as electron donors. In two-component monooxygenase consisting of flavin reductase and flavin-dependent monooxygenase, the resulting free reduced flavin is used to activate oxygen by the terminal monooxygenase [8]. Luciferase from luminous bacteria is a typical two-component monooxygenase and has been thoroughly characterized [8]. Other members of the two-component monooxygenase class have also been reported. 4-Hydroxyphenylacetate 3-monooxygenase [9], styrene monooxygenase [10], phenol hydroxylase [11],

pyrrole-2-carboxylate monooxygenase [12], nitrilotriacetate monooxygenase [13], and EDTA monooxygenase [14] have been discovered in the degradation pathway of each substrate. In addition, monooxygenases of this type have been found to be involved in biosyntheses of antibiotics such as pristinamycin II_A and valanimycin [15-17].

In this chapter, the author described the cloning of the flavin reductase gene (*frm*) involved in DBT desulfurization from *M. phlei* WU-F1 and purification and characterization of the cloned enzyme. This bacterium desulfurizes DBT through the sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds (Chapter 1.4) [2, 3]. The conversion is initiated by consecutive sulfur atom-specific oxidations by two monooxygenases, and flavin reductase is essential in combination with these flavin-dependent monooxygenases. This is the first report describing the characterization of *Mycobacterium* flavin reductase.

The flavin reductase gene, *frm*, was cloned by colony hybridization with the DNA probe based on the N-terminal and internal amino acid sequences partially purified from WU-F1. The deduced amino acid sequence of *frm* shows appreciable homology to those of several flavin reductases in two-component monooxygenases such as HpaC [9], StyB [10], PheA2 [11], and SnaC [15] (around 30% identity), and shares some conserved regions with those of these flavin reductases (Fig. 8.1). Frm was also found to share some conserved regions with the ferric reductase of hyperthermophlic archaeon *Archaeoglobus fulgidus*, FeR [18, 19]. FeR catalyzed the reduction of Fe^{3+}-EDTA with NAD(P)H as electron donors. This enzyme required either FMN or FAD as a catalytic intermediate for Fe^{3+}-EDTA reduction. This enzyme also catalyzed the reduction of FMN and FAD with NAD(P)H as electron donors in the absence of Fe^{3+}-EDTA. FeR from a recombinant *E. coli* was recently crystallized and its structure

was solved [20]. His126 of FeR, which is conserved in all the homologues shown in Fig. 8.1, was found to interact with $NADP^+$, suggesting that His130 of Frm was involved in $NADP^+$ binding.

On the other hand, Frm contained only one cysteine residue, Cys54 (Fig. 8.1). Since the activity of Frm was strongly inhibited by thiol reagents (Table 8.2), Cys54 seemed to be involved in the activity. In addition, Cys45 of FeR, which corresponds to Cys54 of Frm, was found to interact with FMN, suggesting that Cys54 of Frm was involved in FMN binding.

Frm shows homology to three *M. tuberculosis* hypothetical flavin reductases (Rv0245, Rv1939, and Rv3567c) [6] and one *M. leprae* hypothetical flavin reductase (ML2561) [7], which were identified by the genome sequencing projects, and shares some conserved regions with these hypothetical flavin reductases (Fig. 8.1). Frm shares 53% identity with Rv1939. Rv1939 is located in the operon of Rv1936 to Rv1941, and Rv1936 shows low homology to hypothetical luciferases, suggesting that the products of Rv1939 and Rv1936 might function as a two-component monooxygenase. Frm also shares 33% identity with Rv3567c. Rv3567c is located in the operon of Rv3567c to Rv3570c, and Rv3570c shows low homology to flavin-dependent monooxygenases such as DBT monooxygenases, suggesting that the products of Rv3567c and Rv3570c might function as a two-component monooxygenase. In addtion, Rv3568c and Rv3569c in the operon share 82 and 75% identity with 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) and 2-hydroxy-6-oxo-phenylhexa-2,4-dinoate hydrolase (BphD), respectively, of *Rhodococcus* sp. RHA1 [21]. These results suggest that this operon might be related to degradation of aromatic compounds. In contrast, there seemed to be no genes encoding flavin-dependent enzymes close to

Rv0245 of *M. tuberculosis* and ML2561 of *M. leprae*. Thus, although the true physiological functions of these hypothetical flavin reductases are unclear, there is a possibility that they might be effective in activating certain biological functions.

Flavin reductase was purified 2400-fold from the wild type strain, *M. phlei* WU-F1. However, several minor bands were detected on SDS-PAGE, probably due to low concentration of the enzyme in cells of the wild type strain. Thus, the enzyme was purified to homogeneity from the recombinant *E. coli* expressing the flavin reductase gene, *frm*, at 794-fold higher level than the wild type strain (Table 8.1 and Fig. 8.2). The purified Frm was found to be a 34 kDa homodimeric enzyme with a monomeric molecular mass of 17 kDa. Frm exhibited high flavin reductase activity over a wide temperature range (Fig. 8.3A). Particularly, the activity reached 856 U/mg at 50˚C, which is at the highest level of the activity among all the flavin reductases previously reported. In addition, the enzyme exhibited high stability under high-temperature conditions and retained more than 80% of the activity even at 60˚C (Fig. 8.3B). It is interesting to note that the enzyme also exhibited high stability even under acidic and alkaline conditions (Fig. 8.4B).

Coexpression of *frm* with the DBT-desulfurization genes *bdsABC* was critical for high DBT-desulfurizing ability over a wide temperature range of 20 to 55˚C (Fig. 8.5). The DBT-degrading activity of *E. coli* DH5α carrying both *frm* and *bdsABC* was about 7-fold higher at 50˚C than that of *E. coli* DH5α carrying only *bdsABC*. In chapter 7, the gene encoding flavin reductase, *frb*, was cloned from *B. subtilis* WU-S2B, and coexpression of *frb* with *bdsABC* was also critical for high DBT-degrading ability over a wide temperature range. However, it is interesting to note that the deduced amino acid sequence of *frm* shows no appreciable homology to that of *frb*, indicating that these

enzymes belong to distinct flavin reductase families.

On the other hand, in chapter 6, it was confirmed that the nucleotide sequences of the DBT-desulfurization genes *bdsABC* from WU-F1 and WU-S2B were completely the same as each other, and that no flavin reductase gene existed close to the *bdsABC* genes, at least in the upstream region of 3.3 kb and the downstream region of 0.4 kb. These results suggest that the *bdsABC* genes and the flavin reductase genes might have evolved independently. That is, there is a possibility that the *bdsABC* genes might be transferred by horizontal transmission to *M. phlei* WU-F1 and *B. subtilis* WU-S2B, which possess the *frm* gene and the *frb* gene, respectively, on their genomes.

In conclusion, the author confirmed that Frm, which is the first flavin reductase purified from *Mycobacterium* strains, exhibited high flavin reductase activity over a wide temperature range. The enzyme also exhibited high stability under high-temperature and even acidic and alkaline conditions. In addition, coexpression of the flavin reductase gene *frm* with the DBT-desulfurization genes *bdsABC* was critical for high DBT-desulfurizing ability over a wide temperature range. These results suggest that Frm possesses useful properties for practical applications.

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Chapter 9

Thermophilic Biodesulfurization of Hydrodesulfurized Light Gas Oils by *Mycobacterium phlei* WU-F1

9.1. Introduction

Light gas oil (LGO) for diesel engine vehicles contains large amounts of organosulfur compounds and combustion of LGO generates sulfur oxides, causing serious environment problems such as acid rain and air pollution. Thus, regulations for the sulfur level in diesel oil have become increasingly strict [1], and it is planned to reduce the sulfur level to 50 ppm S (mg sulfur per liter oil) by 2005 in the European Union and to 15 ppm S by 2006 in the USA. Similarly, in Japan, the regulations have been enforced to reduce the sulfur level to 50 ppm S by the end of 2004. Today, LGO is treated by hydrodesulfurization (HDS) using metallic catalysts in the presence of hydrogen gas under extremely high temperature and pressure, but heterocyclic sulfur compounds such as dibenzothiophene (DBT) derivatives cannot be completely removed. Their removal by HDS will require more intense conditions leading to higher operating cost, energy consumption, and CO_2 emission. In contrast, biodesulfurization using DBT-desulfurizing microorganisms as biocatalysts is a promising technology to remove organosulfur compounds in LGO under mild conditions, and has attracted attention from the viewpoint of a technology complementary to current HDS to achieve deep desulfurization of LGO (Chapter 1) [1].

Many mesophilic DBT-desulfurizing microorganisms have been isolated as desulfurizing biocatalysts to date [1]. These bacteria desulfurize DBT through a sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds (Chapter 1.4) [2]. In addition, from the viewpoint of practical applications (Chapter 1.4.3), a thermophilic DBT-desulfurizing bacterium *Paenibacillus* sp. A11-2 [3] has been isolated. Moreover, in Chapter 3, the author and collaborators isolated thermophilic DBT-desulfurizing bacteria, *Bacillus subtilis* WU-S2B and *Mycobacterium phlei* WU-F1, and confirmed that these bacteria possessed high DBT-desulfurizing abilities over a wide temperature range up to 50˚C. Particularly, *M. phlei* WU-F1 also possessed high desulfurizing ability toward 4,6-dimethyl-DBT, a representative heterocyclic sulfur compound detected in LGO following HDS (Chapter 3).

There are many reports focusing on the desulfurization of model compounds such as DBT [1]. However, there are only a few reports on application to real fossil fuels including diesel oil, mainly using *Rhodococcus* strains. Mesophilic *Rhodococcus erythropolis* KA2-5-1 [4], *Rhodococcus* sp. ECRD-1 [5-7], and *Gordonia nitida* CYKS1 [8, 9] led to 30-90% reductions of sulfur content in diesel oils at 25-30˚C although components of sulfur in diesel oils and reaction conditions were variable. It was also reported that recombinant *Rhodococcus* strains carrying multiple copies of DBT-desulfurization genes exhibited improved desulfurizing ability toward diesel oils [10, 11]. As for thermophilic biodesulfurization of diesel oil, there is only one report on growing cells of *Paenibacillus* sp. A11-2 which desulfurized a diesel oil from 800 to 720 ppm S at 50˚C [3]. However, the reduction of sulfur content was only 10% and did not reach a practical level.

In this chapter, the author describes the thermophilic desulfurization of

hydrodesulfurized LGOs by the DBT-desulfurizing bacterium *M. phlei* WU-F1. The author examined the desulfurizing ability of this bacterium and found that both growing and resting cells of WU-F1 could efficiently desulfurize HDS-treated LGOs over a wide temperature range up to 50˚C.

9.2. Materials and methods

9.2.1. Cultivation of *M. phlei* WU-F1

Cultivation of *M. phlei* WU-F1 was performed using AF medium (Chapter 2.3.2). The medium was supplemented with 0.54 mM (100 ppm) DBT as the sole source of sulfur. To suspend DBT in the medium, 1% (v/v) *n*-tridecane was added. Cultivation was performed at 50˚C with reciprocal shaking at 240 strokes per min in test tubes (18 by 180 mm) containing 5 ml of the medium with DBT and *n*-tridecane. For LGO desulfurization tests using growing cells, 1% (v/v) of WU-F1 cells grown with DBT was inoculated into 500-ml flasks containing 100 ml of the medium with 2% (v/v) of LGO instead of DBT as the sole source of sulfur and was cultivated for 7 days.

9.2.2. Resting cell reaction

Cultivation was performed at 45˚C in 500-ml flasks containing 200 ml of the medium with 0.27 mM DBT as the sole source of sulfur and 0.50% (v/v) *n*-tridecane for 45 h. Cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4˚C, washed twice with 0.1 M potassium phosphate buffer (pH 7.6), and suspended in the same buffer. The optical density at 660 nm (OD_{660}) of cell suspension was adjusted to 50. Five milliliters of LGO was added to 100-ml flasks containing 5 ml of the cell suspension. Resting cell reactions were performed at 45˚C with reciprocal shaking at

180 strokes per min for 24 h. In addition, the LGO after biodesulfurization for 24 h was collected by centrifugation at 24,000 × *g* for 1 h at 20˚C, and the resting cell reaction was repeated with the collected LGO by changing the used cells for the fresh ones. For control reactions, a cell suspension autoclaved at 121˚C for 5 min was added to the reaction mixture.

9.2.3. Analytical methods

Cell growth was measured turbidimetrically at 660 nm (Chapter 2.13.1). To determine components in LGO, the culture broth and reaction mixture were centrifuged at 24,000 × *g* for 1 h at 20˚C, and each oil phase was collected as the upper fraction. The oil phase was analyzed by using gas chromatography with an atomic emission detector (GC-AED) (Chapter 2.13.3) to determine sulfur and carbon components, and by using ANTEK 7000V (Chapter 2.13.3) to determine total sulfur concentration.

9.2.4. Chemicals

Three types of hydrodesulfurized LGOs, B-LGO, F-LGO, and X-LGO, were tested in the present study. These LGOs were derived from distinct places of origin and produced through distinct processes of HDS. B-LGO contains 390 ppm S and is commercially used for diesel engine vehicles. F-LGO contains 120 ppm S and is used as deeply hydrodesulfurized LGO. X-LGO is a test sample produced through intensive HDS beyond the limit of the refinery plant and contains 34 ppm S. These LGOs were kindly supplied by the laboratory of the Japan Cooperation Center, Petroleum (Shizuoka, Japan). DBT was purchased from Tokyo Kasei (Tokyo, Japan). All other reagents were of analytical grade and commercially available.

9.3. Results

9.3.1. Desulfurization by growing cells

To investigate the potential for thermophilic biodesulfurization of hydrodesulfurized LGO, the effects of temperature on LGO-desulfurizing activity of *M. phlei* WU-F1 were examined using growing cells. WU-F1 grew in AF medium with F-LGO as the sole source of sulfur (data not shown). In addition, as shown in Fig. 9.1, growing cells of WU-F1 exhibited high desulfurizing activity toward LGO over a wide temperature range of 30 to 50˚C, and the sulfur content in F-LGO decreased from 120 to 42-48 ppm S over 7 days. In contrast, no reduction of sulfur content was detected for the uninoculated samples after treatment under the same conditions. Moreover, at 45˚C the growing cells desulfurized B-LGO from 390 to 100 ppm S and X-LGO from 34 to 13 ppm S, as shown in Table 9.1. Biodesulfurization using WU-F1 resulted in

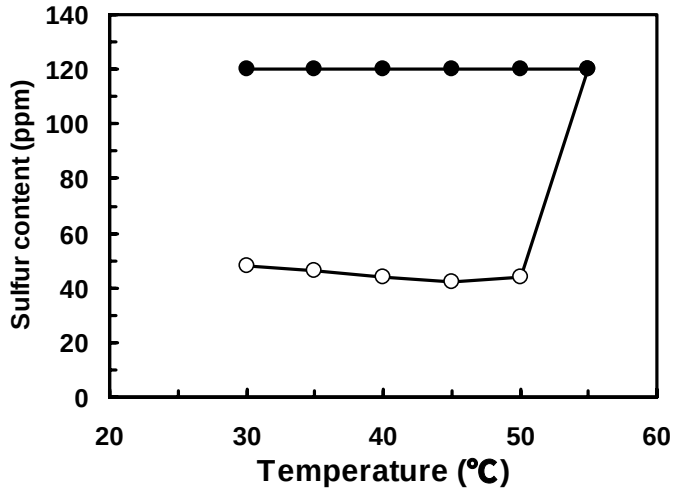

Fig. 9.1. Effects of temperature on LGO-desulfurizing activity of *M. phlei* WU-F1. WU-F1 was cultivated in AF medium with 2% (v/v) of F-LGO (120 ppm S) as the sole source of sulfur at various temperatures for 7 days. Symbols: ○, sulfur content after biodesulfurization; ●, sulfur content for uninoculated samples after treatment under the same conditions as inoculated ones.

Table 9.1. Desulfurization of hydrodesulfurized LGOs at 45˚C by growing cells of *M. phlei* WU-F1[a].

Oil	Growth (OD_{660})	Initial sulfur (ppm)	Final sulfur (ppm)	Desulfurization (%)
B-LGO	2.6	390	100	74
F-LGO	2.4	120	42	65
X-LGO	0.7	34	13	62

[a] WU-F1 was cultivated at 45˚C in AF medium with 2% (v/v) of each LGO as the sole source of sulfur for 7 days.

around 60-70% reduction of sulfur content of all three types of hydrodesulfurized LGOs. When these LGOs were recovered after biodesulfurization and provided for WU-F1 again as the sole source of sulfur, no growth was detected (data not shown). These results indicated that one round of cultivation was enough to remove sulfur compounds to the limit from each LGO.

9.3.2. Desulfurization by resting cells

Since growing cells of WU-F1 exhibited desulfurizing activity toward LGO, resting cell reaction was also examined. As shown in Fig. 9.2, when resting cells of WU-F1 were incubated at 45˚C with F-LGO in the reaction mixture containing 50% (v/v) oil for 24 h, the sulfur content in F-LGO decreased from 120 to 86 ppm S. Since no further reduction of sulfur content occurred after 24 h, the resting cell reaction was repeated by changing the used cells for the fresh ones every 24 h. As shown in Fig. 9.2, the resting cells desulfurized F-LGO from 120 to 50 ppm S through ten consecutive reactions. In contrast, no reduction of sulfur content was detected for the autoclaved cell suspension after treatment under the same conditions (data not shown), indicating that the reduction of sulfur content in Fig. 9.2 was not due to abiotic means

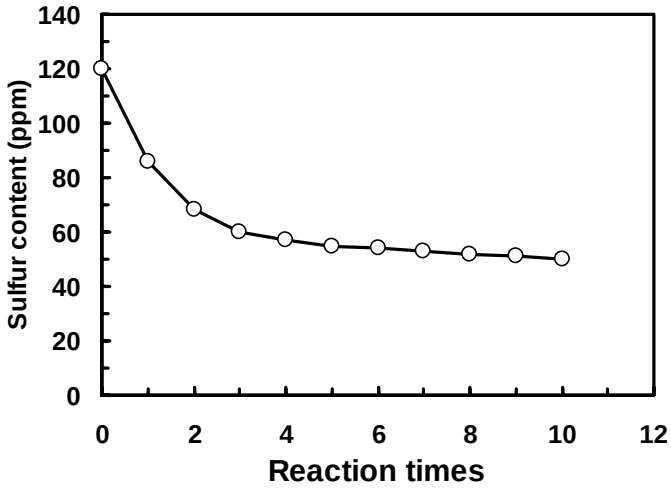

Fig. 9.2. Desulfurization of hydrodesulfurized LGO at 45˚C by resting cells of *M. phlei* WU-F1. The reaction mixture contained 5 ml of the cell suspension (OD_{660} = 50) and 5 ml of F-LGO (120 ppm S). Resting cell reaction was performed for 24 h and repeated by changing the used cells for fresh ones every 24 h.

such as adsorption of sulfur compounds to the cells. GC-AED analysis revealed that the peaks of alkylated DBTs including 4-methyl-DBT, 4,6-dimethyl-DBT, and 3,4,6-trimethyl-DBT significantly decreased after biodesulfurization (Fig. 9.3C,D). In addition, the resting cells desulfurized B-LGO from 390 to 292 ppm S through one and to 100 ppm S through six consecutive reactions (Fig. 9.3A,B). Similarly, the resting cells desulfurized X-LGO from 34 to 23 ppm S through one and to 15 ppm S through six consecutive reactions (Fig. 9.3E,F). On the other hand, it was confirmed by GC-AED analysis that carbon components in three types of hydrodesulfurized LGOs were almost the same before and after biodesulfurization (data not shown), indicating that the energy loss in these LGOs through biodesulfurization was little.

9.4. Discussion

In this chapter, the author described the thermophilic desulfurization of

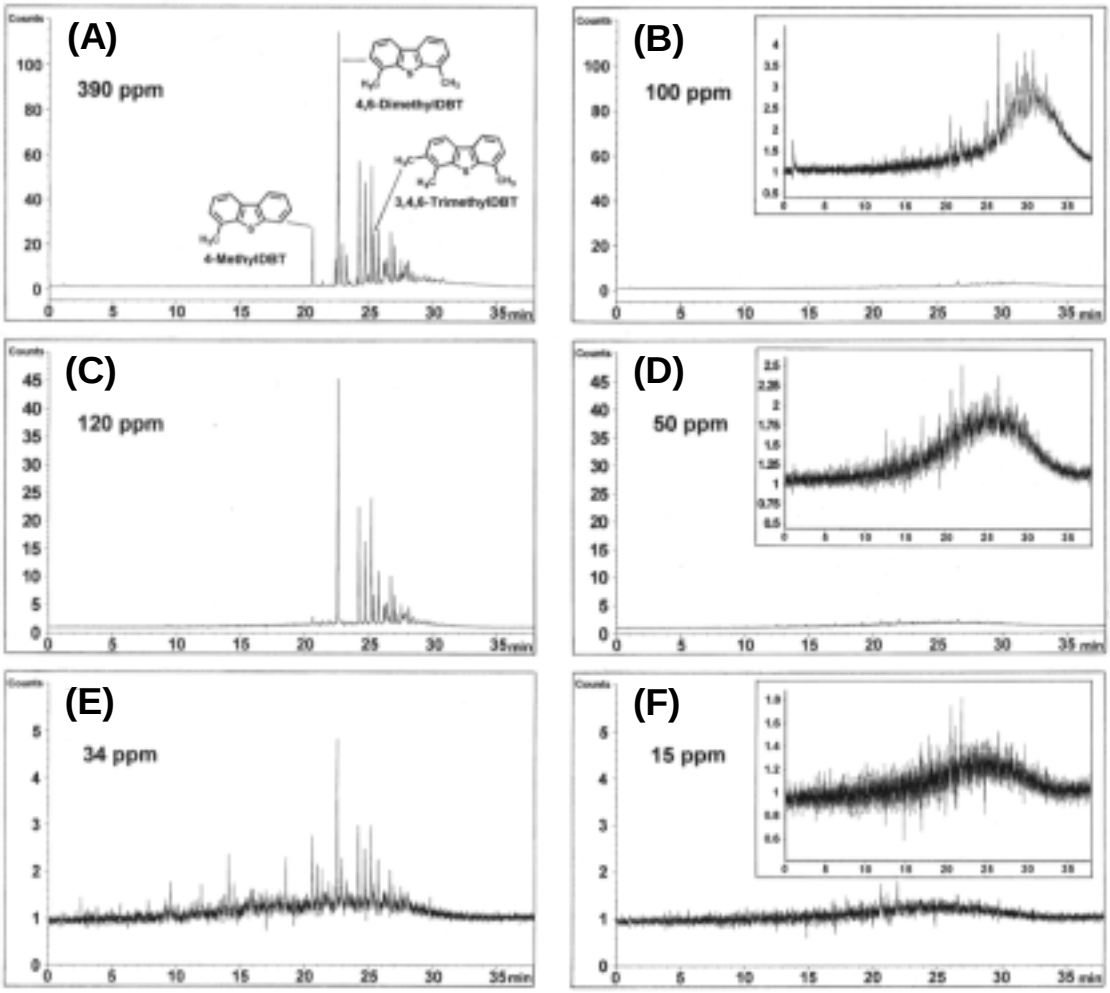

Fig. 9.3. GC-AED chromatograms of sulfur components in hydrodesulfurized LGOs before and after biodesulfurization at 45˚C by resting cells of *M. phlei* WU-F1. B-LGO (A), F-LGO (C), and X-LGO (E) were desulfurized by the resting cells, and GC-AED chromatograms after biodesulfurization are shown in B, D, and F, respectively. The sulfur content in each LGO is also shown. Inset; magnified GC-AED chromatograms after biodesulfurization.

hydrodesulfurized LGOs by the DBT-desulfurizing bacterium *M. phlei* WU-F1. Both growing and resting cells of WU-F1 could efficiently desulfurize HDS-treated LGOs over a wide temperature range up to 50˚C. The cell surfaces of *Mycobacterium* strains possessing mycolic acids with many carbon atoms are more hydrophobic than those of the other coryneform bacteria including *Rhodococcus* strains [12], and this property might be advantageous for *M. phlei* WU-F1 to take up hydrophobic compounds such as

DBT derivatives. Through the experiments, it was confirmed that WU-F1 could take up alkylated DBTs such as 4,6-diethyl-, 4,6-dipropyl-, and even 4,6-dibutyl-DBT (data not shown). It has been reported that a mesophilic DBT-desulfurizing bacterium *Mycobacterium* sp. G3 could also take up these alkylated DBTs and efficiently desulfurize a diesel oil from 116 to 48 ppm S [13]. In addition, it has recently been reported that another thermophilic *M. phlei*, GTIS10, possessed high DBT-desulfurizing ability [14]. These results indicate that *Mycobacterium* strains may be promising candidates for practical biodesulfurization of diesel oil besides the *Rhodococcus* strains that have been extensively studied so far.

WU-F1 grew in the medium with LGO as the sole source of sulfur, and the growing cells exhibited high desulfurizing activity toward LGO over a wide temperature range of 30 to 50˚C (Fig. 9.1). These results indicate that WU-F1 may have useful practical applications for biodesulfurization process since this property simplifies the temperature control of the process to treat high-temperature LGO immediately after HDS. On the other hand, much more sulfur was removed from B-LGO than from F-LGO, as shown in Table 9.1. It was also confirmed that much more glucose was consumed in the medium with B-LGO than with F-LGO (data not shown), indicating that sulfur was the limiting nutrient in the medium. However, a little more growth was observed for B-LGO compared with F-LGO. Although we do not have a clear explanation for this, it might be due to the differences in the toxicity to WU-F1 of these LGOs. In addition, at 50˚C, WU-F1 showed no growth with B-LGO (data not shown) although this strain sufficiently grew with F-LGO (Fig. 9.1), suggesting that B-LGO might be more toxic to this bacterium for growth than F-LGO.

Three types of hydrodesulfurized LGOs exhibited similar sulfur compound

composition to each other in GC-AED chromatograms and included alkylated DBTs such as 4,6-dimethyl-DBT regardless of the degree of HDS (Fig. 9.3A,C,E). This is because alkyl substitutions at the 4- and 6-positions of DBT prevent metallic catalysts from interacting with the sulfur atom due to steric hindrance (Chapter 1.3.1). On the other hand, after biodesulfurization of these LGOs, the peaks of alkylated DBTs including 4-methyl-DBT, 4,6-dimethyl-DBT, and 3,4,6-trimethyl-DBT significantly decreased (Fig. 9.3B,D,F), indicating that WU-F1 can selectively desulfurize alkylated DBTs regardless of the initial sulfur levels in the LGOs. Biodesulfurization using WU-F1 resulted in around 60-70% reduction of sulfur content of all three types of hydrodesulfurized LGOs (Table 9.1, Fig. 9.3). It is interesting to note that the reduction of sulfur content was almost constant as a function of percentage of the initial sulfur level. In contrast, the peaks in retention times at around 25-35 min still remained (Fig. 9.3B,D,F, insets). These peaks were presumed to be bulky compounds such as DBT derivatives with large alkyl and aromatic substitutions. There is a possibility that the desulfurizing enzymes have no activity toward these bulky compounds and/or that the cells have no permeability toward them since both growing and resting cells of WU-F1 failed to remove these compounds.

In conclusion, the author confirmed that both growing and resting cells of WU-F1 could efficiently desulfurize HDS-treated LGOs over a wide temperature range up to 50˚C. Particularly, WU-F1 could also desulfurize the intensively HDS-treated LGO (X-LGO) from 34 to 13-15 ppm S (Table 9.1, Fig. 9.3E,F), and to our knowledge this is the first report describing the biodesulfurization of a low-sulfur LGO with less than 50 ppm S. These results suggest that biodesulfurization using WU-F1 might be applicable to a ‘no sulfur’ specification of less than 15 ppm S, which we will be faced

with in the near future.

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Chapter 10

Summary and Conclusions

In this thesis, the author described the characterization of novel desulfurizing bacteria as biocatalysts applicable to deep desulfurization of light gas oils (LGOs). The summary of this thesis is as follows.

In Chapter 1, the author described an overview of the biodesulfurization of LGO. The author explained two desulfurization methods, i.e., hydrodesulfurization (HDS) and biodesulfurization, and demonstrated that biodesulfurization is a promising technology to achieve deep desulfurization of LGO. Based on the review, the author clarified the objective of this thesis.

In Chapter 2, the author described the materials and methods generally used for the studies in this thesis.

In Chapter 3, the author described the thermophilic desulfurization of dibenzothiophene (DBT) and its derivatives by *Bacillus subtilis* WU-S2B and *Mycobacterium phlei* WU-F1. Novel thermophilic DBT-desulfurizing bacteria, *B. subtilis* WU-S2B and *M. phlei* WU-F1, were isolated, which were a clearly different type of bacteria from the thermophilic strain previously reported. The author examined the desulfurizing abilities of these bacteria and found that WU-S2B and WU-F1 possessed high desulfurizing abilities toward DBT and its derivatives over a wide temperature range up to 50˚C. Particularly, WU-F1 exhibited much higher

DBT-desulfurizing activity than any other DBT-desulfurizing bacteria previously reported.

In Chapter 4, the author described the thermophilic desulfurization of naphthothiophene (NTH) and 2-ethyl-NTH by the DBT-desulfurizing bacterium *M. phlei* WU-F1. The author examined the desulfurizing ability of this bacterium and found that WU-F1 could efficiently desulfurize asymmetric organosulfur compounds, NTH and 2-ethyl-NTH, in addition to symmetric DBT derivatives through the sulfur-specific degradation pathway with the selective cleavage of carbon-sulfur bonds under high-temperature conditions. This is the first report describing the biodesulfurization of NTH and 2-ethyl-NTH through the sulfur-specific degradation pathway.

In Chapter 5, the author describes the desulfurization of NTH and benzothiophene (BTH) by *Rhodococcus* sp. WU-K2R. Novel NTH-desulfurizing bacterium, *Rhodococcus* sp. WU-K2R, was isolated, which is the first isolate possessing the ability to selectively desulfurize NTH through sulfur-specific degradation pathways. The author examined the desulfurizing ability of this bacterium and found that WU-K2R could preferentially desulfurize asymmetric heterocyclic sulfur compounds such as NTH and BTH through the sulfur-specific degradation pathways with the selective cleavage of carbon-sulfur bonds. These results suggest that WU-K2R may be a promising biocatalyst for practical biodesulfurization complementary to microorganisms possessing desulfurizing ability toward symmetric DBT derivatives.

In Chapter 6, the author described the cloning and functional analysis of the DBT-desulfurization genes from *B. subtilis* WU-S2B and *M. phlei* WU-F1. By nucleotide sequence analysis, it was found that the nucleotide sequences of the

DBT-desulfurization genes from WU-S2B and WU-F1 are completely the same as each other. The genes constitute a single operon consisting of the three genes designated *bdsA*, *bdsB*, and *bdsC*. Although the nucleotide sequence of *bdsABC* shows homology to those of *dszABC* from *Rhodococcus* sp. IGTS8 and *tdsABC* from *Paenibacillus* sp. A11-2, it was confirmed that *bdsA* was independently located away from the *dszA* cluster and *tdsA* by phylogenetic analysis. In addition, heterologous expression analysis in *Escherichia coli* indicated that the abilities of WU-S2B and WU-F1 to desulfurize DBT over a wide temperature range were endowed by the thermophilic DBT-desulfurization genes, *bdsABC*. Moreover, for practical biodesulfurization, the *bdsABC* genes may be useful genetic resources to improve desulfurizing biocatalysts by genetic engineering.

In Chapter 7, the author described the cloning of the flavin reductase gene involved in DBT desulfurization from *B. subtilis* WU-S2B through coexpression screening using indigo production as selective indication. The gene encoding flavin reductase, *frb*, was cloned from *B. subtilis* WU-S2B through coexpression screening using indigo production as selective indication. In addition, coexpression of *frb* with *bdsABC* was critical for high DBT-desulfurizing ability over a wide temperature range of 20 to 55˚C. Moreover, these results suggest that this coexpression screening may be widely applicable for cloning novel genes encoding either component of flavin reductase or flavin-dependent monooxygenase which efficiently couples with the other component in two-component monooxygenases.

In Chapter 8, the author described the cloning of the flavin reductase gene involved in DBT desulfurization from *M. phlei* WU-F1 and purification and characterization of the cloned enzyme. The gene encoding flavin reductase, *frm*, was

cloned from *M. phlei* WU-F1. In addition, the author purified this gene product, Frm, from the recombinant *E. coli* and characterized, and found that Frm exhibited high flavin reductase activity over a wide temperature range. The enzyme also exhibited high stability under high-temperature and even acidic and alkaline conditions. Moreover, coexpression of *frm* with *bdsABC* was critical for high DBT-desulfurizing ability over a wide temperature range of 20 to 55˚C. This is the first report describing the characterization of *Mycobacterium* flavin reductase.

In Chapter 9, the author described the thermophilic desulfurization of hydrodesulfurized LGOs by the DBT-desulfurizing bacterium *M. phlei* WU-F1. The author examined the desulfurizing ability of this bacterium and found that both growing and resting cells of WU-F1 could efficiently desulfurize HDS-treated LGOs over a wide temperature range up to 50˚C. Particularly, WU-F1 could also desulfurize the intensively HDS-treated LGO of 34 ppm S to 13-15 ppm S, and to our knowledge this is the first report describing the biodesulfurization of a low-sulfur LGO with less than 50 ppm S. These results suggest that biodesulfurization using WU-F1 might be applicable to a 'no sulfur' specification of less than 15 ppm S, which we will be faced with in the near future.

For commercial application of biodesulfurization, however, improvement of desulfurizing biocatalysts is essential, mainly in three areas, i.e., activity, longevity, and selectivity [1].

First, the activity over 20 nmol S/min/mg-drycell is required for commercialization [2], which is two or three orders of magnitude higher than the activity of native cell biocatalysts. The low activity is mainly attributed to the low

concentration of desulfurizing enzymes in the native cells. Multiplication of the copy number of desulfurization genes and replacement of the promoter by genetic engineering will be effective in enhancing the concentration [3, 4]. In addition, the activity of desulfurizing enzymes for sulfur compounds and the permeability of hosts for them affect on the activity. Moreover, conditions for biocatalyst production, biocatalyst regeneration, and desulfurization in bioreactor substantially affect on the activity.

Second, the longevity over 400 hours is required for commercialization [5], which is one or two orders of magnitude higher than the longevity of native cell biocatalysts. The low longevity is partly attributed to the low tolerance of the native hosts for the oil. It will be important to screen proper hosts possessing high tolerance for the oil [6, 7]. In addition, conditions for biocatalyst production, biocatalyst regeneration, and desulfurization in bioreactor substantially affect on the longevity.

Third, since the selectivity of native cell biocatalysts for sulfur compounds is too high, the range of substrates desulfurized by the biocatalysts is narrow [2, 5]. The reduction of sulfur content in LGO over 90% is required for commercialization. There is a possibility that the native desulfurizing enzymes have no activity for bulky sulfur compounds and that the native hosts have no permeability for them. Genetic engineering such as directed evolution and gene shuffling will be effective in broadening substrate range of the enzymes [8, 9]. It may also be important to screen proper hosts possessing high permeability for bulky sulfur compounds [10].

In conclusion, in this thesis, with the objective to achieve deep desulfurization of LGO using biodesulfurization, the author isolated and characterized novel desulfurizing

bacteria and found that these bacteria are promising biocatalysts applicable to deep desulfurization of LGOs. These bacteria and the cloned genes may be useful resources to improve desulfurizing biocatalysts for commercial application of biodesulfurization. The outcome of the studies in this thesis will provide a lot of important and useful information for not only the development of biodesulfurization process, but also the studies in the fields of microbiology, biotechnology, and applied chemistry.

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研 究 概 要

ディーゼル車の燃料として使用されている軽油は、硫黄分を除去するために金属触媒を用いた水素化脱硫によって処理されているが、アルキルジベンゾチオフェン類などの有機硫黄化合物は脱硫が困難であるため軽油中に残存している。これらの難脱硫性化合物は燃焼により硫黄酸化物を生成し、さらに窒素酸化物や粒子状物質の生成を促すことから酸性雨や大気汚染の原因となっている。したがって、軽油中の硫黄分をさらに低減することが求められている。

微生物を生体触媒として利用するバイオ脱硫は、これらの難脱硫性化合物を除去するための有効な手段の 1 つと考えられる。すなわち、金属触媒は多種多様な有機硫黄化合物を脱硫可能であるが、アルキルジベンゾチオフェン類などの脱硫はそのアルキル基が立体障害となるために困難である。これに対して、生体触媒は基質に対する特異性が高く、これらの難脱硫性化合物を選択的に脱硫することが可能と考えられる。さらに、生体触媒は常温常圧条件下での反応を可能とすることから環境負荷を低減することも可能である。したがって、アルキルジベンゾチオフェン類などを脱硫可能な微生物を探索し、取得した微生物の脱硫機能を解明して、軽油の超深度脱硫への応用を図ることは、生物化学の分野に有用な基礎的知見をもたらすばかりでなくバイオプロセスの実用へ向けた応用的側面からも極めて重要である。

本論文では、新規脱硫微生物の取得および機能解析と軽油の超深度脱硫への応用を目的として研究を行い、その成果をまとめた。実際のプロセスを想定して高温条件下でジベンゾチオフェンを脱硫可能な微生物を探索し、新規な好熱性ジベンゾチオフェン脱硫細菌 2 株を取得することに成功した。水素化脱硫処理後の軽油中にはアルキルジベンゾチオフェン類以外にもナフトチオフェン類が残存しているが、ナフトチオフェンを特異的に脱硫可能な細菌を初めて取得することにも成功した。また、2 株の好熱性ジベンゾチオフェン脱硫細菌から脱硫に関わる全遺伝子をクローニングし、塩基配列の解析および大腸菌における発現試験を通して当該細菌の脱硫機能を遺伝子レベルで解明した。さらに、当該細菌を軽油の脱硫に実際に応用し、その有効性を評価した。

本論文は 10 章より構成されている。

第 1 章では、微生物を生体触媒として利用するバイオ脱硫について概説した。微生物の脱硫機能についてまとめ、さらにバイオ脱硫を金属触媒を用いた水素化脱硫と比較し、その予想される有効性について述べた。これらを背景として、本研究の意義と目的を明らかにした。

第 2 章では、本研究で用いた主な実験方法について説明した。すなわち、各種細

菌の培養方法、有機硫黄化合物および軽油の脱硫方法、遺伝子工学的手法、ならびに脱硫代謝産物の分析方法などについて述べた。

第 3 章では、新規な好熱性ジベンゾチオフェン（DBT）脱硫微生物を探索し、取得した細菌 2 株の機能を解析した。高温条件下で行われている金属触媒を用いた水素化脱硫を補完する技術としてバイオ脱硫を適用する場合、水素化脱硫後の高温状態にある軽油を処理することが可能であれば冷却に要するコストを削減できるため有利である。そこで 50℃の高温条件下で DBT を唯一の硫黄源として増殖可能な微生物を探索し、新規な好熱性 DBT 脱硫細菌 *Bacillus subtilis* WU-S2B および *Mycobacterium phlei* WU-F1 の 2 株を取得することに成功した。これらの細菌は炭素、硫黄原子間の結合を選択的に切断して DBT 分子から硫黄原子のみを除去する代謝経路で DBT を脱硫することを明らかにした。また DBT 脱硫の温度依存性について検討し、両株とも 50℃の高温域を含む広範な温度条件下で DBT を効率的に脱硫した。さらに DBT 誘導体に対する脱硫活性についても検討し、50℃の高温条件下で DBT のみならず水素化脱硫では除去が困難な 4,6-ジメチル DBT などのアルキル DBT 類も効率的に脱硫可能なことを明らかにした。とくに、WU-F1 は 0.8 mM の DBT および 4,6-ジメチル DBT をそれぞれ 90 分、8 時間で完全に分解した。

第 4 章では、*M. phlei* WU-F1 を用いてナフトチオフェン（NTH）および 2-エチル NTH の脱硫について検討した。水素化脱硫処理後の軽油中にはアルキル DBT 類以外にも微量ではあるが NTH 類が残存しており、超深度脱硫を実現するためにはこれらの難脱硫性化合物も除去できることが望ましい。そこで NTH 誘導体に対する脱硫活性について検討した結果、WU-F1 は 50℃の高温条件下で 0.8 mM の NTH および 2-エチル NTH を 8 時間でそれぞれ 67%、83%分解し、これらの有機硫黄化合物も効率的に脱硫可能なことを明らかにした。

第 5 章では、新規な NTH 脱硫微生物を探索し、取得した細菌の機能を解析した。NTH を唯一の硫黄源として増殖可能な微生物を探索し、NTH を特異的に脱硫可能な細菌 *Rhodococcus* sp. WU-K2R を初めて取得することに成功した。NTH 脱硫代謝産物を同定して代謝経路を推定し、WU-K2R は炭素、硫黄原子間の結合を選択的に切断する経路で NTH を脱硫することを明らかにした。さらに有機硫黄化合物に対する脱硫活性について検討した結果、WU-K2R は対称構造の DBT を脱硫しないが NTH やベンゾチオフェンなどの非対称構造の有機硫黄化合物を効率的に脱硫し、DBT 脱硫細菌とは異なる新規な性質を有することを明らかにした。

第 6 章では、*B. subtilis* WU-S2B および *M. phlei* WU-F1 から DBT 脱硫遺伝子をクローニングし、その機能を解析した。プラークハイブリダイゼーション法により、WU-S2B および WU-F1 から DBT 脱硫遺伝子を取得することに成功した。塩基配列を解析した結果、WU-S2B と WU-F1 は細菌の属種が異なり系統的に離れているにも関わらず DBT 脱硫遺伝子の塩基配列は同一であることを明らかにし、これを

bdsABC と命名した。*bdsABC* は既報の常温性 DBT 脱硫細菌 *Rhodococcus* sp. IGTS8 の DBT 脱硫遺伝子（*dszABC*）と 61.0%の相同性を示した。さらに、大腸菌における発現試験を行った結果、*bdsABC* を保持する組換え大腸菌は原株 WU-S2B および WU-F1 と同様に 50℃の高温域を含む広範な温度条件下で DBT を効率的に脱硫し、WU-S2B および WU-F1 の広範な温度条件下で脱硫可能な性質は *bdsABC* に依存していることを明らかにした。このように、当該細菌の優位性を遺伝子レベルで明らかにした。

第 7 章では、*B. subtilis* WU-S2B から DBT 脱硫に関わるフラビンレダクターゼ遺伝子をクローニングし、その機能を解析した。DBT 脱硫代謝経路において DBT の酸化反応を触媒するモノオキシゲナーゼの活性発現には、酸化反応に必要な還元型フラビンを供給するフラビンレダクターゼが不可欠である。そこで、インドール酸化活性を指標にした共発現スクリーニング法により、WU-S2B からフラビンレダクターゼ遺伝子 *frb* を取得することに成功した。*frb* の発現産物は高温条件下で高いフラビンレダクターゼ活性を示した。さらに、大腸菌内で *frb* を DBT 脱硫遺伝子 *bdsABC* と共発現させたところ、*bdsABC* のみを保持する大腸菌（大腸菌保有のフラビンレダクターゼにより活性を発現）と比較して 40℃における脱硫活性が 27 倍に向上した。また、当該遺伝子をクローニングするために考案した共発現スクリーニング法は、種々のフラビンレダクターゼ遺伝子やモノオキシゲナーゼ遺伝子のクローニングに広く応用可能なことを示した。

第 8 章では、*M. phlei* WU-F1 から DBT 脱硫に関わるフラビンレダクターゼ遺伝子をクローニングし、その機能を解析した。コロニーハイブリダイゼーション法により、WU-F1 からフラビンレダクターゼ遺伝子 *frm* を取得することに成功した。塩基配列を解析し、*frm* は前述の *frb* と推定アミノ酸配列でほとんど相同性を示さず、異なるファミリーに属する酵素遺伝子と考えられた。さらに、大腸菌内で *frm* を DBT 脱硫遺伝子 *bdsABC* と共発現させたところ、*bdsABC* のみを保持する大腸菌と比較して 40℃における脱硫活性が 28 倍に向上した。また、大腸菌高発現株から当該酵素 Frm を精製し、その酵素的諸性質を解明した。Frm は 60℃の高温域を含む広範な温度条件下で高いフラビンレダクターゼ活性を示し、60℃、30 分の熱処理後も 80%以上の活性を維持していた。

第 9 章では、*M. phlei* WU-F1 を用いて水素化脱硫処理された軽油の脱硫について実際に検討した。WU-F1 の菌体を 45℃の高温条件下、50%の軽油存在下で水素化脱硫処理された軽油に作用させたところ、120 ppm の硫黄分を含む市販の軽油を 50 ppm に、さらに 34 ppm の硫黄分を含む試験用の低硫黄濃度軽油を 15 ppm にまで脱硫した。バイオ脱硫処理した軽油をガスクロマトグラフィー－原子発光検出器で分析し、4,6-ジメチル DBT などのアルキル DBT 類に相当するピークが消失していることを確認した。このように、当該細菌は高温条件下で DBT のみならず実際の軽

油も効率的に脱硫可能であり、優れた脱硫生体触媒であることを明らかにした。

第 10 章では、本研究を総括した。本研究によって得られた知見をもとに微生物を生体触媒として利用したバイオ脱硫の有効性を評価し、実用化へ向けてさらに解決すべき点について述べた。