Characterization of Novel Enzymes Converting Aromatics and Their Application to Production of Valuable Aromatics

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Preface

Aromatics are mainly derived from coal and petroleum, and converted into valuable compounds used as intermediates of medicines, agrochemicals, and industrial chemicals. Recently, industrial organic syntheses of valuable compounds are carried out under the conditions at high pressure and high temperature which gives much negative impact on the earth environment. However, in the organic syntheses, a large amount of unnecessary by-products are also formed, and a process for separating by-products from main products as valuable compounds is then required.

In contrast, the bioconversion of aromatics using enzymes as biocatalysts has been focused as practical alternatives to the conventional organic syntheses because it provides high selectivity toward substrates and environmentally benign conditions. Selective removal of unnecessary components from aromatics and the synthesis of valuable compounds from aromatics using novel enzymes are thus expected to be significant model processes for the selective and ecological conversion of aromatics.

In this thesis, to develop a novel method of selectively converting aromatics under environmentally benign conditions, the author studied the molecular characterization of novel enzymes converting aromatics, including their purification, characterization, gene-cloning, and over-expression. Especially, the author examined biodesulfurization of aromatic sulfur compounds and enzymatic regioselective carboxylation of aromatics. I hope that the studies in this thesis will provide meaningful information for not only development of novel bioconversion process of aromatics, but also the studies in the fields of microbiology, biotechnology, and applied chemistry.

Yuichiro Iwasaki

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1.1. Introduction

Aromatics are mainly derived from coal and petroleum, and converted into valuable compounds used as intermediates of medicines, agrochemicals, and industrial chemicals. Recently, industrial organic syntheses of valuable compounds are carried out under the conditions at high pressure and high temperature which gives much negative impact on the earth environment. However, in the organic syntheses, a large amount of unnecessary by-products are also formed, and a process for separating by-products from main products as valuable compounds is then required.

In contrast, the bioconversion of aromatics using enzymes as biocatalysts has been focused as practical alternatives to the conventional organic syntheses because it provides high selectivity toward substrates and environmentally benign conditions. Selective removal of unnecessary components from aromatics and the synthesis of valuable compounds from aromatics using novel enzymes are thus expected to be significant model processes for the selective and ecological conversion of aromatics.

In this chapter, the author describes an overview of the bioconversion of aromatics. The author explained two conversion methods of aromatics, i.e., nonbiological conversion and bioconversion, and demonstrated that the bioconversion is a significant model process for the selective and ecological conversion of aromatics. Based on the review in this chapter, the author clarifies the objective of this thesis.

1.2. Aromatics in the chemical industry

Aromatics are hydrocarbons with benzene rings, and so called because of their distinctive perfumed smell. Almost all aromatics are mainly derived from coal and petroleum. The main substances in this group such as benzene, toluene, and xylene (Fig. 1.1.) are used as starting materials for consumer products. Everyday items made with aromatics can be found in our daily life. They include a diverse range of products such as medicines, agrochemicals, cosmetics, computers, paints, vehicle components, cooking utensils, household fabrics, carpets, and sports equipment.

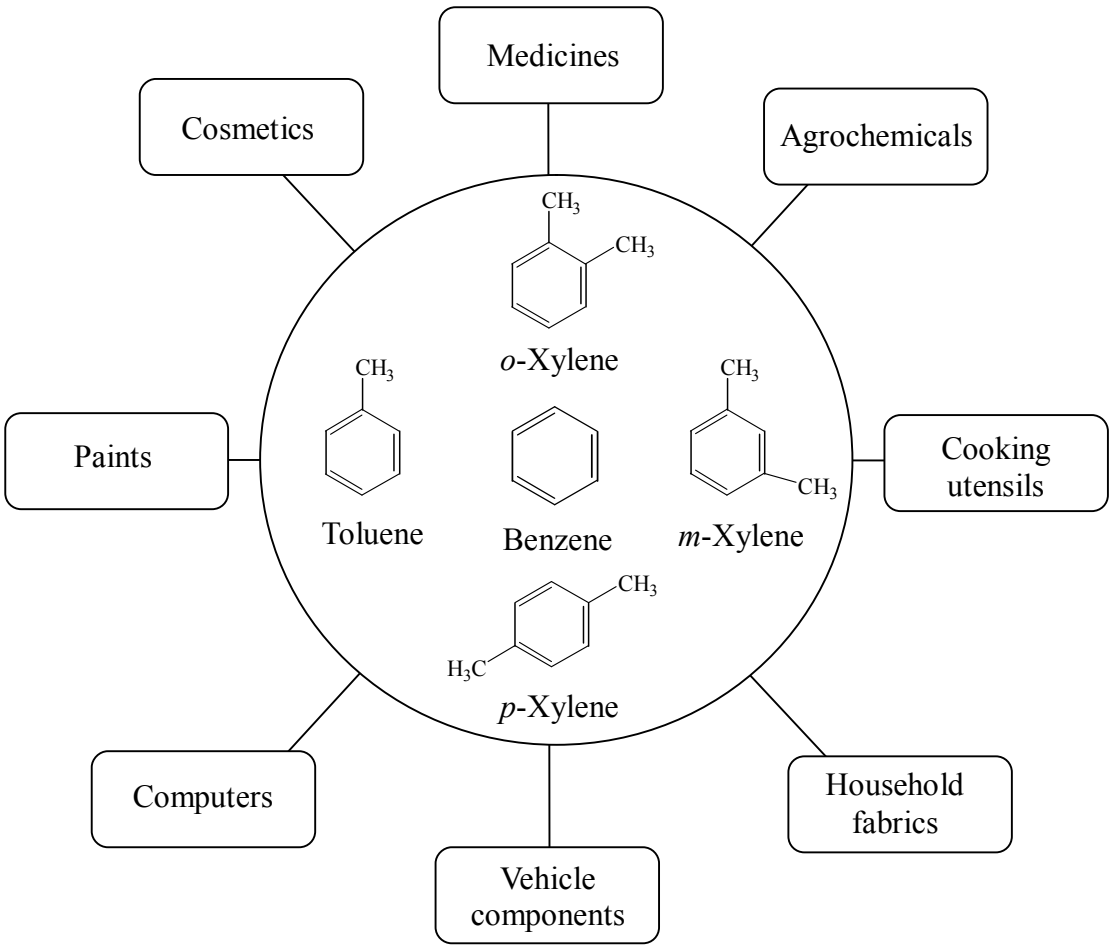


Fig. 1.1. Main substances in aromatics for the chemical industry.

On the other hands, developments in science and technology using aromatics over the last two decades have increased soil and water pollutions. Phenols and polycyclic aromatic hydrocarbons (PAHs) are commonly occurring industrial pollutants and are often found as co-contaminants in the environment. PAHs are widely distributed in the environment [1] and are found in numerous natural and industrial sources, including tars and creosote. PAHs are toxic to a wide variety of organisms and particular PAHs have been shown to be carcinogenic [2]. Phenols are also common industrial pollutants of soil and groundwater and have been found in soils underlying chemical storage depots, manufactured gas plants, soils at former creosote, and wood preserving plants [3]. Phenols have relatively high water solubility (more than 84 g/l) and are widely known to be toxic to a range of organisms.

In addition to industrial sources, aromatics also have generated from natural sources. For example, a study of the concentrations of phenols present in the capillary water of the soil of a high-altitude spruce forest showed that the total concentration of phenols was only 2 μg/l. These monomers, which included vanillic acid, 4-hydroxybenzoic acid, and cinnamic acid were only 1% of the total phenols, which consisted of mostly polymerized aromatics [4].

1.3. Bioconversion of various aromatics

Because of the widespread occurrence of aromatics, microorganisms able to use aromatics as a carbon and energy source can be found in many environments. This ability of microorganisms to degrade aromatics has many practical applications. Examples include the biological treatment of aromatics-containing industrial wastewater

[5], the bioremediation of sites polluted with aromatics such as PAHs [1], and the desulfurization of aromatic sulfur compounds [6].

On the other hand, the bioconversions of aromatics have also been studied for production of valuable compounds, such as di- and trihydroxy aromatic compounds that have many applications as shown by worldwide production of catechol, resorcinol, and hydroquinone at a level of 110,000 tons/year [7]. Although some of these compounds cannot be easily synthesized industrially and the industrial organic syntheses are often lengthy and require expensive starting materials [9], genetic-engineered toluene-*o*-xylene monooxygenase from *Pseudomonas* sp. enables to synthesize valuable hydroxylated benzenes from inexpensive materials, such as benzene, toluene, phenol, cresol, catechol, hydroquinone, and resorcinol [9] (Fig. 1.2). In this way, the bioconversion of aromatics can provide a more cost-effective, more selective approach, and more environmentally benign conditions than conventional organic syntheses.

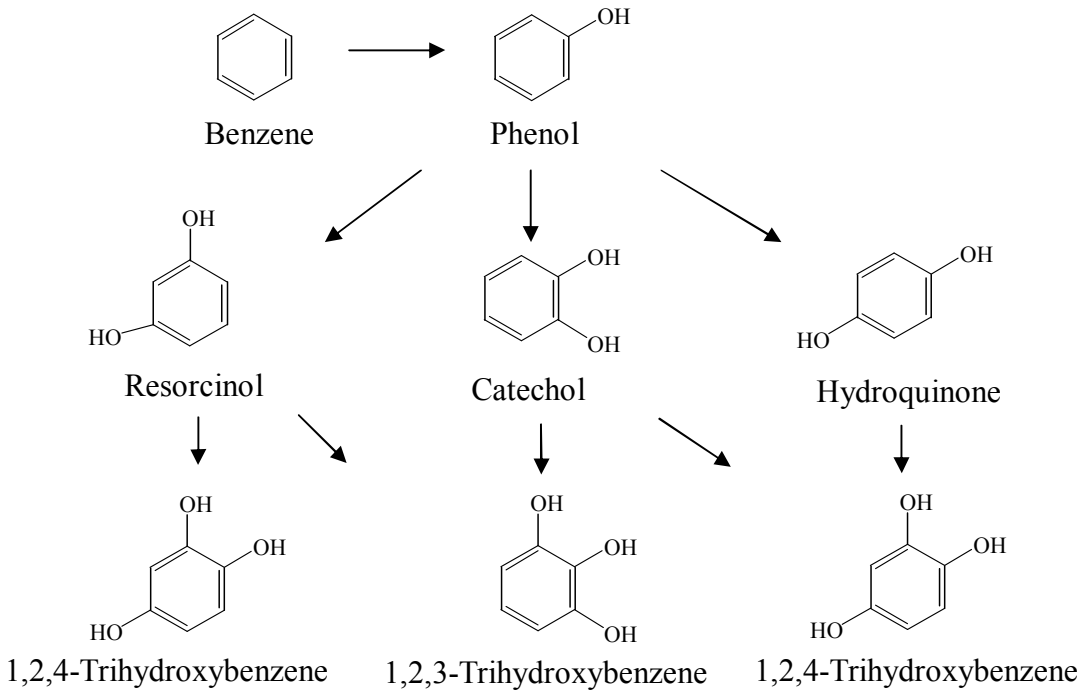


Fig. 1.2. Enzymatic oxidation of benzene into several aromatics using genetic-engineered toluene-*o*-xylene monooxygenase from *Pseudomonas* sp.

1.4. Desulfurization of aromatic sulfur compounds

1.4.1. Aromatic 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 [10]. Sulfur levels in crude oil range from 500 to 50000 ppm S, depending on the source. Sulfur levels in light gas oil (LGO) as a middle distillate of crude oil often exceed 5000 ppm S. LGO contains various aromatic sulfur compounds, such as thiols, sulfides, disulfides, thiophenes, benzothiophenes (BTHs), dibenzothiophenes (DBTs), naphthothiophenes (NTHs), and benzonaphthothiophenes (BNTHs) as shown in Fig. 1.3. Particularly, heterocyclic sulfur compounds such as BTHs and DBTs are contained much in LGO [11].

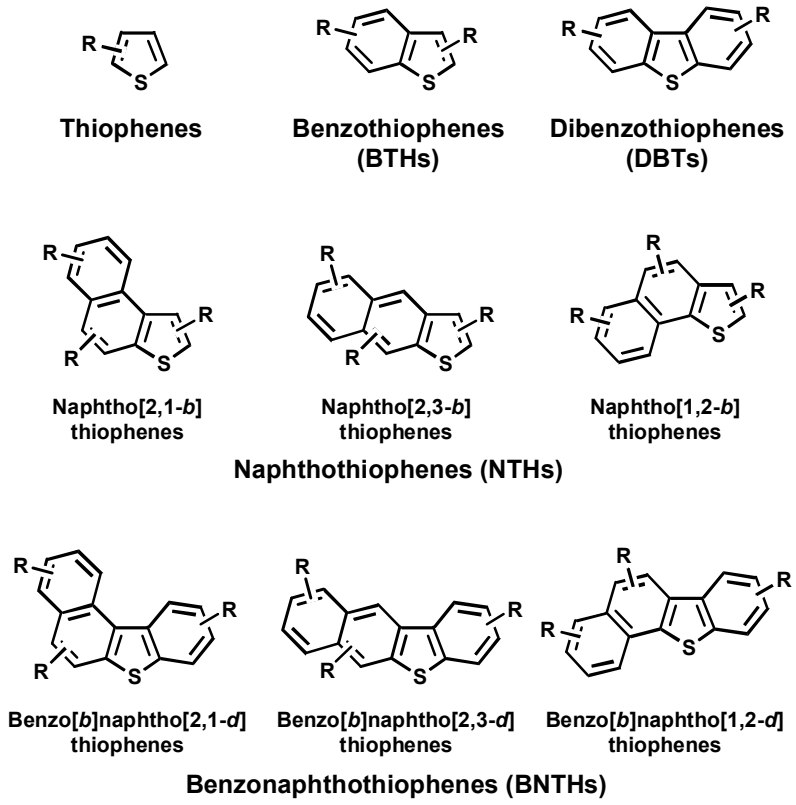


Fig. 1.3. Organosulfur compounds in LGO. R, Alkyl substitution.

1.4.2. Hydrodesulfurization

Today, LGO is treated by hydrodesulfurization (HDS) using metallic catalysts in the presence of hydrogen gas under extremely high temperature and pressure [12]. Generally sulfide $Co/Mo/Al_2O_3$ 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 by the catalyst in the presence of hydrogen gas, and the resulting H_2S is removed from LGO.

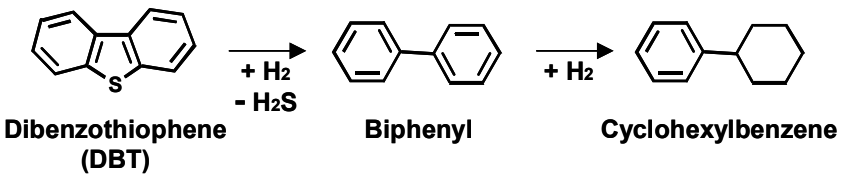


Fig. 1.4. The pathway of DBT desulfurization by $Co/Mo/Al_2O_3$.

DBT is a representative aromatic sulfur compound detected in LGO. The pathway of DBT desulfurization by $Co/Mo/Al_2O_3$ is shown in Fig. 1.4. 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. 1.5.

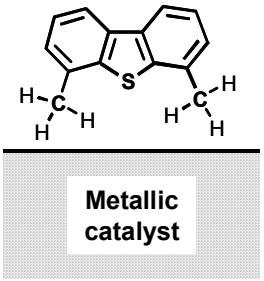
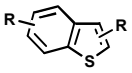
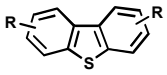
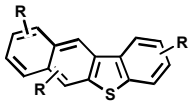
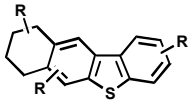


Fig. 1.5. Steric hindrance by alkyl substitutions for metallic catalyst.

Table 1.1. Sulfur compounds detected in hydrodesulfurized LGO [a]

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.

Aromatic sulfur compounds detected in hydrodesulfurized LGO are shown in Table 1.1 [13]. 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. These findings indicate that current HDS faces difficulties in

removing complicated heterocyclic sulfur compounds such as alkylated DBTs.

1.4.3. Biodesulfurization of dibenzothiophene (DBT)

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 [6].

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 [14-19]. The genes involved in DBT desulfurization were cloned from *Rhodococcus* sp. IGTS8 and characterized [20-23]. 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. 1.6). These three enzymes were purified from *Rhodococcus* strains [24-26], and particularly DszC and DszA from *R. erythropolis* D-1 [27, 28] and DszB from *R. erythropolis* KA2-5-1 [29] 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 [24, 26, 29].

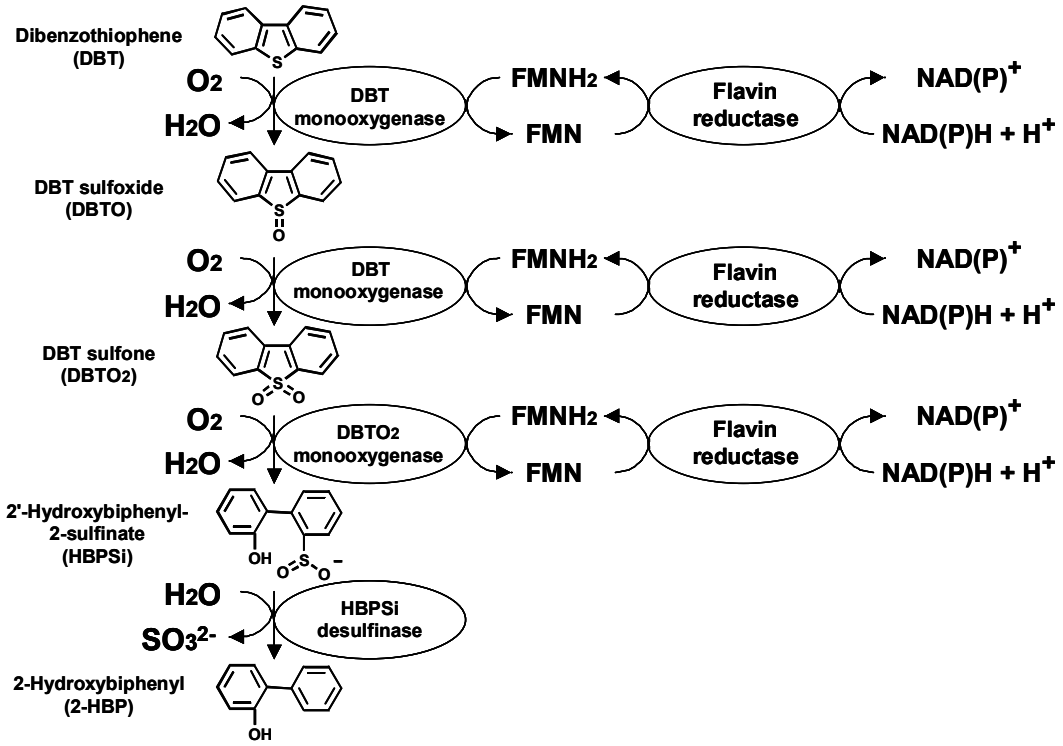


Fig. 1.6. The DBT degradation pathway with the selective cleavage of carbon-sulfur bonds.

In addition to these three enzymes, flavin reductase has been found to be essential in combination with two flavin-dependent monooxygenases DszC and DszA [24, 27, 28, 30, 31]. 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 [24] 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 [24] and *R. erythropolis* D-1 [32] 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 [33], *Rhodococcus* sp. ECRD-1 [34-36], and *Gordonia nitida* CYKS1 [37] 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 [38, 39].

The pathway shown in Fig. 1.6, often referred to as the 4S pathway, has been extensively studied so far using *Rhodococcus* sp. IGTS8, which was discovered by Kilbane [17-19]. In addition to *Rhodococcus* sp. IGTS8, many bacteria of this type have been isolated, for example, *Rhodococcus* sp. ECRD-1 [40], *R. erythropolis* D-1 [41], *R. erythropolis* H-2 [42], *R. erythropolis* KA2-5-1 [33], *Rhodococcus* sp. SY1, formerly identified as *Corynebacterium* sp. [43], *Mycobacterium* sp. G3 [44], *Gordonia nitida* CYKS1 [37], and *Paenibacillus* sp. A11-2 [45]. 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. It was also reported that some sulfate-reducing microorganisms [46] and sulfur-oxidizing microorganisms [47] possessed DBT-degrading abilities.

As regulations for the sulfur level in diesel oil become stricter, in near future the amount of HDS-recalcitrant aromatic sulfur compounds to be removed from LGO must become greater than that of today. However, the 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, the biodesulfurization using microorganisms as biocatalysts is a promising technology to remove sulfur atom from recalcitrant aromatic sulfur compounds in LGO under mild conditions [6, 11, 48, 49]. 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 above. The biodesulfurization using microorganisms as biocatalysts proceeds at ambient temperature and pressure. Therefore, the 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 as shown in Fig. 1.7.

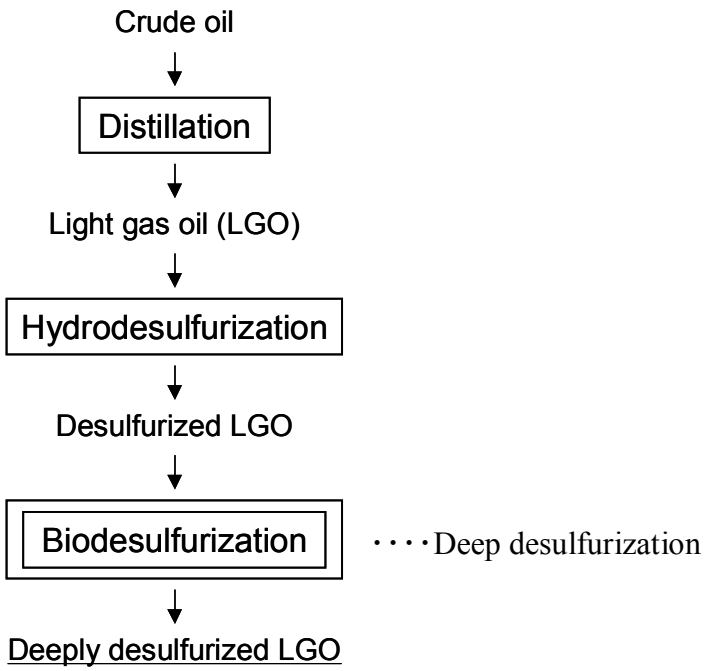


Fig. 1.7. Biodesulfurization as a technology complementary to current HDS.

Proposed biodesulfurization process is shown in Fig. 1.8 [48]. 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 by-products 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.

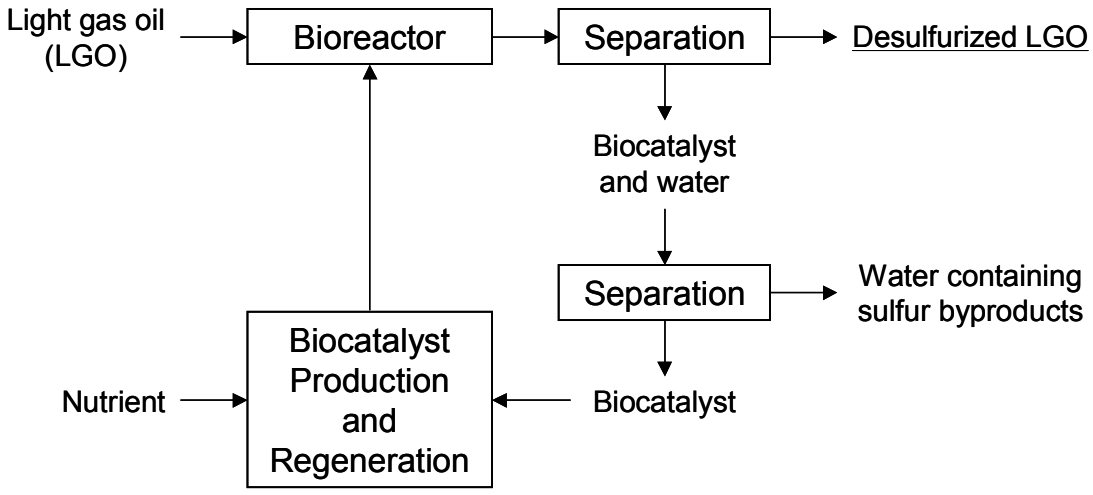


Fig. 1.8. Proposed biodesulfurization process.

1.4.4. 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. 1.7). 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 [45] has been isolated to date. This bacterium could desulfurize DBT at around 50˚C through the sulfur-specific degradation pathway (Fig. 1.6). In addition, the DBT-desulfurization genes (*tdsABC*) equivalent to *dszABC* were cloned and characterized [50]. The flavin reductase gene (*tdsD*) was also cloned and characterized [51]. However, reduction of the sulfur content in diesel oil was only 10% by the reaction with growing cells of *Paenibacillus* sp. A11-2, and did not reach a practical level [45].

1.5. Carboxylation of aromatics to produce valuable chemicals

1.5.1. Aromatic carboxylic acids in the chemical industry

Among several aromatics, aromatic carboxylic acids and their derivatives are widely used as anti-phlogistic, anti-rheumatic, anti-pyretic, and anaesthetic drugs to treat many diseases (Fig. 1.9) [52]. Non-steroid anti-phlogistic drugs (NSAD) are effective in principle for checking the metabolism of arachidonic acid (the precursor of prostaglandins), which causes increased sensibility of pain and temperature receptors.

A better known group of anti-phlogistic drugs are derivatives of salicylic acid. Pure salicylic acid as well as benzoic acid are used only externally as irritative and

keratolytic agents. The derivatives of salicylic acid such as acetylsalicylic acid, sodium salicylate, salicylamide, and γ-resorcylic acid are used as anti-phlogistic, analgesic, anti-rheumatic, anti-pyretic drugs, and intermediates of medicines, agrochemicals, and industrial chemicals [53].

Aromatic carboxylic acid derivatives are also in the group of anaesthetic drugs having influence on the nerve endings. The derivatives of benzoic acid such as cocaine, hexylcaine and others are applied only in topical anaesthesia in stomatology, laryngology, and ophthalmology. The derivatives of 4-hydroxybenzoic acid such as cyclomethycaine and paretoxycaine as well as derivatives of acetylsalicylic acid, e.g. edan have similar applications. The derivatives of 4-aminobenzoic acid such as procaine and tetracaine, being less toxic, are also used as a nerve block and spinal anaesthesia [54].

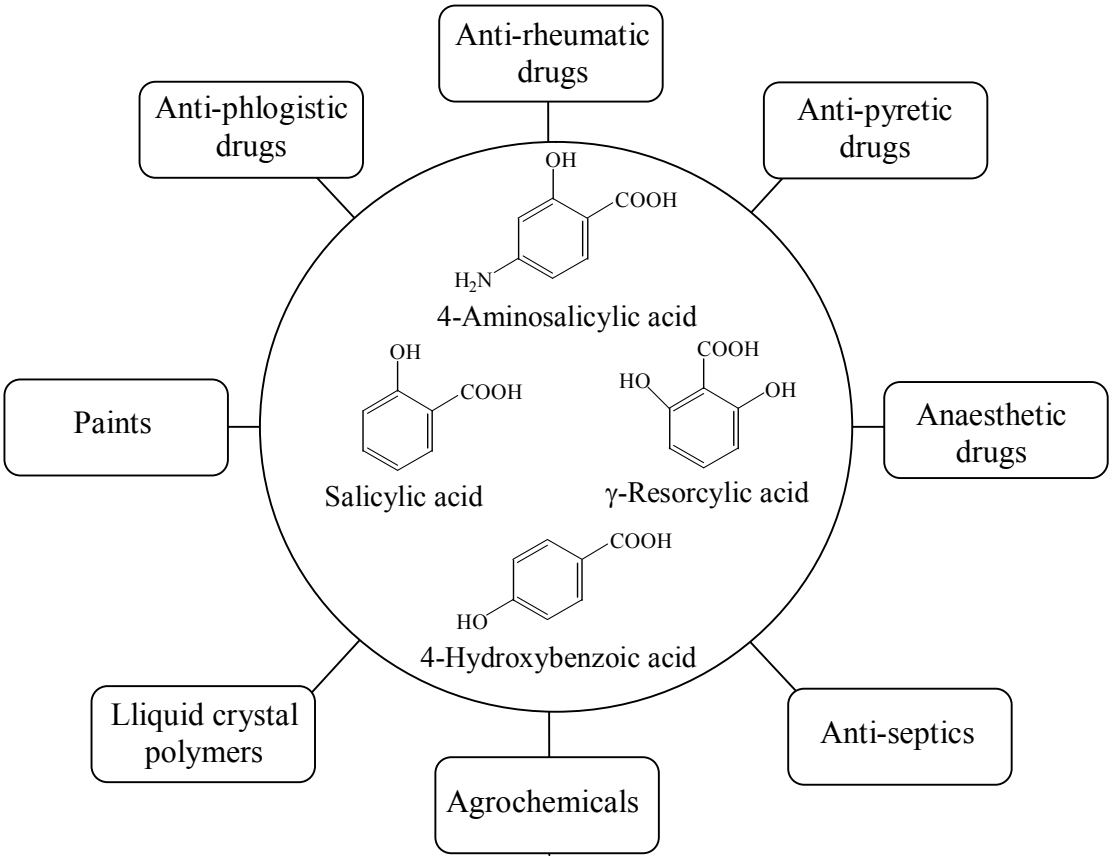


Fig. 1.9. Aromatic carboxylic acids for the chemical industry.

1.5.2. Kolbe-Schmitt reaction

The Kolbe-Schmitt reaction is known to be one of the typical and efficient methods to use carbon dioxide in organic reactions [55]. The reaction has been used for over 100 years for production of aromatic carboxylic acids used for intermediates of medicines, agrochemicals, industrial chemicals, paints, and preservatives of foods, cosmetics etc.

Many improvements of the Kolbe-Schmitt reaction have been reported for long periods of time. The initial work was performed already about one and a half centuries ago by Kolbe [55]. Salicylic acid was obtained by heating mixture of phenol and sodium in the presence of carbon dioxide at atmospheric pressure, as shown in Fig. 1.10.

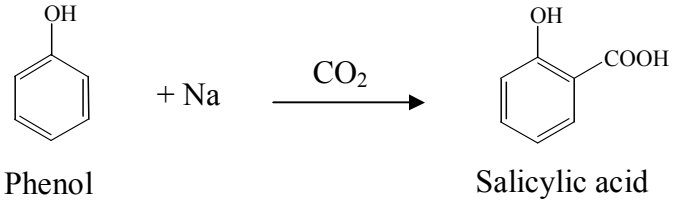
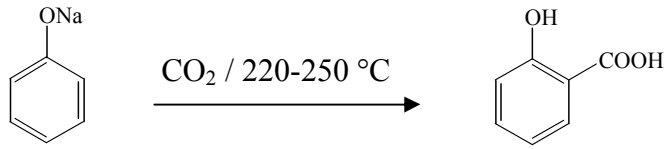


Fig. 1.10. Kolbe synthesis.

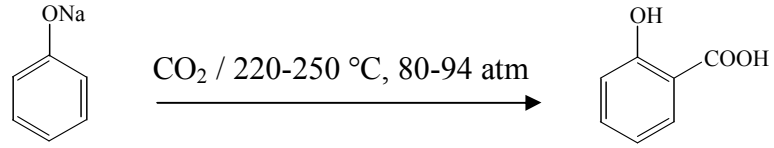
Although there was a larger request for salicylic acid, this synthesis was not easily scaled out. Also, the yields on a laboratory level changed remarkably under seemingly similar reaction conditions. This was a result of volatilization of the reactant phenol and the consumption of sodium by reactions to species other than the salicylic acid anion, e.g., sodium phenoxide and sodium carbonate. For these reasons the solid hygroscopic sodium phenoxide, prepared from evaporated solutions of phenol and sodium hydroxide, was later used directly as a starting reagent by Kolbe [55], as shown in Fig. 1.11.



Phenol Salicylic acid

Fig. 1.11. Kolbe synthesis.

The dried sodium phenoxide was heated in an iron retort to 180 °C and carbon dioxide passed slowly over the hot salt. About half of the phenol distilled under these conditions so that the yield was never larger than 50%. Schmitt modified the Kolbe procedure by applying pressure which resulted in greatly improved yields [55]. Dry sodium phenoxide is heated with carbon dioxide at 120-130 °C for several hours at a pressure of about 80 to 94 bar. No phenol is lost, and almost a quantitative yield of salicylic acid is obtained. This now so-called Kolbe-Schmitt reaction (Fig. 1.12) is still the standard method for the production of a wide variety of aromatic carboxylic acids.



Phenol Salicylic acid

Fig. 1.12. Kolbe-Schmitt reaction.

The reaction is performed with alkali metal phenoxide and CO_2 at high pressure and high temperatures, and is greatly affected by metal ions of phenoxides [55]. For example, the carboxylation of sodium phenoxide mainly yields salicylic acid (Fig. 1.12), while potassium phenoxide yields a mixture of salicylic acid and 4-hydroxybenzoic acid, as shown in Fig. 1.13.

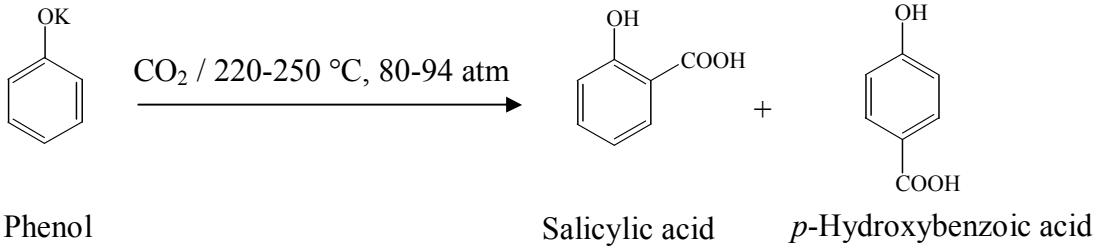


Fig. 1.13. Kolbe-Schmitt reaction.

Recent works on the Kolbe-Schmitt reaction are concerned with the effects of alkali and alkaline earth metals on the Kolbe-Schmitt reaction [56] and with achieving carboxylation with high regioselectivity [57]. However, regioselective carboxylation of aromatics with high yields and no by-products cannot be easily in such production, and a process for separation of by-products is then required [56] (Table 1.2).

Table 1.2 Carboxylation of various alkali and alkaline earth metal phenoxides with carbon dioxide (Data from Rahim *et al*., 2002 [56])

Metal	Reaction conditions			Recovered phenol (%)	Yield (%)[a]				
	CO_2 (MPa)	Temp. (°C)	Time (h)		SA	*p*HBA	4HIPA	2HIPA	Total[b]
Mg	5.0	150	1.0	96.0	4.0	0	0	0	4.0
Mg	5.0	260	5.0	14.7	74.0	3.0	0	8.0	93.0
Ca	5.0	150	1.0	70.0	26.7	2.8	0	0	29.5
Ca	5.0	200	1.0	54.4	42.7	2.8	trace	trace	45.5
Ba	5.0	150	1.0	20.0	76.0	3.0	0.6	0	80.2
Ba	5.0	260	5.0	16.0	64.8	2.0	5.5	11.0	99.8
Rb	5.0	150	1.0	6.5	39.8	51.5	1.8	0	94.9
Rb	5.0	200	1.0	1.4	37.0	51.6	7.8	1.8	107.8
Cs	5.0	150	1.0	29.5	20.7	48.0	1.6	0.	71.9
Cs	5.0	260	1.0	6.0	26.5	19.0	35.0	12.5	140.5

a) Determined by HPLC. An unidentified peak was observed in run nos. 17 (ca. 1.0%) and 18 (ca. 3.9%) of retention time at 8.5 min. SA, salicylic acid; *p*HBA, 4-hydroxybenzoic acid; 4HIPA, 4-hydroxyisophthalic acid; 2HIPA, 2-hydroxyisophthalic acid.

b) Total yield of the carboxylation based on the amount of phenol used.

1.5.3. Enzymatic carboxylation of aromatics

Several enzymes catalyzing carboxylation of aromatics to form aromatic carboxylic acids have been found in microorganisms. Carboxylation and decarboxylation of aromatics are central metabolic processes of microorganisms and some of them are catalyzed by a broad spectrum of aromatics decarboxylases. Aromatics decarboxylases are generally oxidative and depend on metal ions and cofactors such as thiamin pyrophosphate, puridoxal-5'-phosphate, and biotin with or without ATP [58]. On the other hand, nonoxidative aromatics decarboxylases do not use either molecular O_2 or cofactors for decarboxylation activities [59-71], and some of them have the unique ability that reversibly catalyzing the regioselective carboxylation [60-62, 64, 66, 70]. One of the properties of these reversible and nonoxidative aromatics decarboxylases is that their reactions are generally sensitive to O_2, and both decarboxylation and carboxylation activities are decreased in the presence of O_2 [60-62, 70].

Recently, reversible and nonoxidative vanillic acid decarboxylase (Vdc) having the advantage that they are insensitive to O_2 and need no cofactors for their activities has been found from *Streptomyces* sp. D7 [64]. Because the enzymatic regioselective carboxylation of guaiacol into vanillic acid is performed by only mixing Vdc, guaiacol, and CO_2 source in aqueous phase (Fig. 1.14), such enzymatic carboxylation is considered to be easily applicable to practical production of aromatic carboxylic acids.

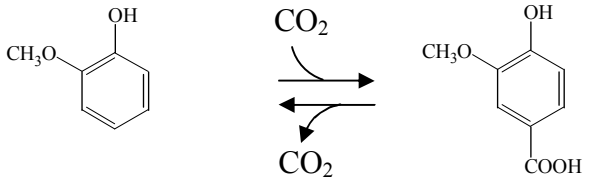


Fig. 1.14. Enzymatic reversible conversion of vanillic acid and guaiacol using vanillic acid decarboxylase from *Streptomyces* sp. D7.

1.6. Objective of this thesis

Conventional conversion process of aromatics faces difficulties in selective removing unnecessary components from complicated aromatics and in selective production of valuable compounds such as aromatic carboxylic acids. In addition, conditions for such process at high pressure and high temperature lead to negative impact on the environment. In contrast, the bioconversion of aromatics using enzymes as biocatalysts has been focused because its advantage of providing high selectivity and more environmentally benign conditions compared with those for the conventional organic syntheses. The removal of unnecessary components from aromatics and the synthesis of valuable compounds from aromatics using novel enzymes are thus expected to be a significant model process for the selective and ecological conversion of aromatics.

In this thesis, to develop a novel method of selectively converting aromatics under environmentally benign conditions, the author studied the molecular characterization of novel enzymes converting aromatics, including their purification, characterization, gene-cloning, and over-expression. Especially, the author examined biodesulfurization of aromatic sulfur compounds and enzymatic regioselective carboxylation of aromatics.

First, the author describes the cloning of the DBT-desulfurization genes from the thermophilic DBT-desulfurizing bacterium *M*ycobacterium *phlei* WU-F1 and the enhancement of DBT-desulfurizing activity by genetic engineering of *M. phlei* WU-F1. The author cloned the DBT-desulfurization genes from WU-F1 and found that the nucleotide sequences of the genes were completely the same to those of *Bacillus subtilis* WU-S2B. Moreover, the author constructed a *Mycobacterium-E. coli* shuttle vectors applicable to genetic engineering of *M. phlei* WU-F1, and succeeded in enhancing the

DBT-desulfurizing activity of *M. phlei* WU-F1 by the increase of copy numbers of genes related to DBT-desulfurization under high temperature at 45ºC.

Second, the author describes the molecular characterization of novel reversible aromatics decarboxylases from *Rhizobium radiobacter* WU-0108 and *Trichosporon moniliiforme* WU-0401, which are the first enzymes catalyzing the carboxylation of resorcinol to form γ-resorcylic acid and phenol to form salicylic acid, respectively.

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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 are concisely described. Recombinant DNA techniques are basically described according to the publication by Sambrook et al. [1] or manufacturer's protocols. Analytical methods to determine aromatics are also described. Further specific and detailed explanations for experimental methods will be described in the section of Materials and methods in each chapter.

2.2. Chemicals

Dibenzothiophene, γ-resorcylic acid, resorcinol, salicylic acid, and phenol were purchased from Tokyo Kasei (Tokyo, Japan). Tryptone, yeast extract, and agar were purchased from BD (MD, USA). All other reagents were of analytical grade and commercially available.

2.3. Bacterial and yeast strains and plasmids

2.3.1. Bacterial and yeast strains

The bacterial and yeast strains used in the present study are listed in Table 2.1.

Rhizobium radiobacter WU-0108 and *Trichosporon moniliiforme* WU-0401 were newly isolated in the present study. These bacterial and yeast strains were stored in micro-tubes containing 10% (v/v) glycerol at −80˚C.

2.3.2. Phage and plasmids

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

Table 2.1. Bacterial and yeast strains used in the present study.

Strain	Relevant properties	Source or reference
Mycobacterium phlei WU-F1	Thermophilic DBT[a]-desulfurizing bacterium	Chapter 3
Rhizobium radiobacter WU-0108	γ-Resorcylic acid degrading bacterium	Chapter 5
Trichosporon moniliiforme WU-0401	Salicylic acid degrading yeast	Chapter 7
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
BL21 (DE3)	F$^-$ *dcm ompT hsdS*($r_B^- m_B^-$)*gal λ* (DE3)	Novagen

[a] DBT, dibenzothiophene.

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		
pUC19	Multicloning site vector; Ap^r, *lac* promoter	Takara Bio
pUC118	Multicloning site vector; Ap^r, *lac* promoter	Takara Bio
pET21-a	Multicloning site vector; Ap^r, T7 promoter	Novagen

Ap^r, ampicillin resistance gene; *lac* promoter, lactose operon promoter.

2.4. Cultivation

2.4.1. Cultivation of *Mycobacterium phlei* WU-F1

Cultivation of *M. phlei* WU-F1 was carried out using AF medium shown in Table 2.3. AF medium was supplemented with DBT as the sole source of sulfur. Unless otherwise indicated, cultivation was carried out at 45˚C with reciprocal shaking at 240 strokes per min in test tubes (18 by 180 mm) containing 5 ml of AF medium with DBT. For preparation of AF agar plates, 15 g/l of agar was added to AF medium.

Table 2.3. 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.

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

2.4.2. Cultivation of *Rhizobium radiobacter* WU-0108

Cultivation of *R. radiobacter* WU-0108 was carried out using RA medium shown in Table 2.6. Unless otherwise indicated, cultivation was carried out at 30˚C with reciprocal shaking at 120 strokes per min in test tubes (18 by 180 mm) containing 5 ml of RA medium. For preparation of RA agar plates, 15 g/l of agar was added to RA medium.

Table 2.6. Composition of RA medium[a].

Ingredients	
γ-Resorcylic acid	0.76 g
NaH_2PO_4	0.5 g
Na_2HPO_4	1.0 g
$NaNO_3$	0.5 g
$(NH_4)_2SO_4$	0.5 g
$CaCl_2 \cdot 2H_2O$	0.025 g
$MgSO_4 \cdot 6H_2O$	0.5 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 1 M NaOH.
[b] Composition of metal solution is shown in Table 2.4.
[c] Composition of vitamin mixture is shown in Table 2.5.

2.4.3. Cultivation of *Trichosporon moniliiforme* WU-0401

Cultivation of *T. moniliiforme* WU-0401 was carried out using SA medium shown in Table 2.7. Unless otherwise indicated, cultivation was carried out at 30˚C with reciprocal shaking at 120 strokes per min in test tubes (18 by 180 mm) containing 5 ml of SA medium. For preparation of SA agar plates, 15 g/l of agar was added to SA medium.

Table 2.7. Composition of SA medium[a].

Ingredients	
Sodium salicylate	0.8 g
KH_2PO_4	2.5 g
K_2HPO_4	0.5 g
$NaNO_3$	0.5 g
$(NH_4)_2SO_4$	0.5 g
$MgSO_4 \cdot 6H_2O$	0.5 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 6.0 with 2 M HCl.
[b] Composition of metal solution is shown in Table 2.4.
[c] Composition of vitamin mixture is shown in Table 2.5.

2.4.4. Cultivation of *Escherichia coli* strains

Cultivation of *E. coli* strains was mainly carried out using Luria-Bertani (LB) medium shown in Table 2.8. Appropriate antibiotics were supplemented to LB medium, if when they were necessary. Unless otherwise indicated, cultivation was carried out at 30˚C with reciprocal shaking at 240 strokes per min in test tubes (18 by 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 2.8. 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.

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.

2.5. Resting cell reaction

Cultivation of each strains (WU-F1, WU-0108, WU-0401, and *E. coli*) was carried out in 500-ml flasks containing 200 ml of an appropriate medium (Chapter 2.3). Cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4˚C, washed twice with an appropriate buffer, and suspended in the same buffer. The optical density at 660 nm (OD_{660}) or 600 nm (OD_{600}) of cell suspension was appropriately adjusted. A substrate such as DBT, γ-resorcylic acid, resorcinol, salicylic acid, and phenol was added to test tubes containing cell suspension. Unless otherwise indicated, resting cell reactions were carried out with reciprocal shaking at 180 strokes per min.

2.6. Enzyme assays for reversible decarboxylase activities

Reversible decarboxylase activities were determined by monitoring increase in amounts of products due to carboxylation or decarboxylation of aromatics. One unit (U) of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of product per minute.

2.7. 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.

2.8. DNA extraction

For preparation of total DNAs or cDNAs, strains (WU-F1, WU-0108, and WU-0401) 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 or cDNA was prepared with a QIAGEN genomic-tip (QIAGEN, Tokyo, Japan) or Sepasol-RNA I Super (Nacalai tesque, Kyoto, 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.

2.9. 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.

2.10. 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. Each 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.

2.11. 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*, and *Ex Taq* (Takara Bio, Tokyo, Japan). Each concentration of ingredients in the 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.

Rapid amplification of 5' and 3'cDNA ends were generated by using a BD SMART^{TM} RACE cDNA Amplification Kit (Takara, Shiga, Japan) according to the manufacturer's protocols.

2.12. 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.

2.13. 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. On the other hand, CUGA sequencing, a method for DNA sequencing using RNA polymerase, was carried out by NIPPON GENE (Tokyo, Japan) to sequence hairpin regions.

Sequence analysis was carried out with a Genetyx-Mac version 10.1 (SDC, Tokyo, Japan). Homology search of sequence was carried out 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.

2.14. Analytical methods

2.14.1. Measurement of cell growth

Cell growth was measured turbidimetrically at 660 nm for bacteria, and 600 nm for *Trichosporon* spp. by using spectrophotometer (UV-1200; Shimadzu, Kyoto, Japan).

2.14.2. 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 methanol-50 mM K_2HPO_4-KH_2PO_4 buffer (pH 8.0) (25:75, v/v) and the flow rate was 0.8 ml/min. The culture broth and reaction mixture were acidified to pH 2.0 with 6 M HCl and filtered using a 0.2-μm polytetrafluoroethylene (PTFE) membrane filter (Advantec Toyo, Tokyo, Japan) for HPLC analysis. Aromatics were detected spectrophotometrically at 254 nm, and the amounts of them were calculated from standard calibration curves.

2.15. Gibbs assay

Phenolic compounds such as phenol and salicylic acid in the reaction mixtures were visually detected by Gibbs assay, which was carried out by the addition of 30 μl of 1.0 M $NaHCO_3$ solution and 20 μl of 5.0 mM Gibbs reagent dissolved in ethanol solution into 50 μl reaction mixtures. Gibbs assay was observed to produce a blue mixture with phenol, light blue with salicylate or blown with catechol, as shown in Fig. 2.1.

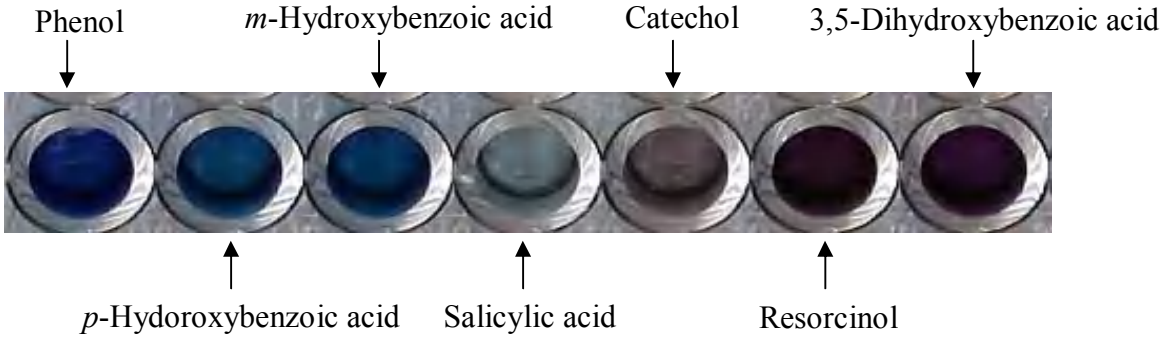

Fig. 2.1. Gibbs assay of several phenolic compounds.
Each phenolic compounds was prepared at 5 mM.

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Chapter 3

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

3.1. Introduction

Biodesulfurization is a promising technology to remove sulfur atom from recalcitrant aromatic sulfur compounds such as dibenzothiophene (DBT) and its derivatives more selectively under more benign 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 1.4.3) [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] was isolated and the DBT-desulfurization genes (*tdsABC*) equivalent to *dszABC* were cloned and characterized [9]. Moreover, thermophilic DBT-desulfurizing bacteria, *Bacillus subtilis* WU-S2B and *Mycobacterium phlei* WU-F1, were isolated 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 *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 desulfurization of DBT.

3.2. Materials and methods

3.2.1. Chemicals

DBT and 2-HBP were purchased from Tokyo Kasei (Tokyo, Japan). All the other chemicals used were commercially available and of chemically pure grade.

3.2.2. Bacterial strains, phage, plasmids, and cultivation

M. phlei WU-F1 was used as the source of total DNAs for cloning of DBT-desulfurization genes. For preparation of total DNAs, WU-F1 was cultivated at 45˚C in test tubes (18 by 180 mm) containing 5 ml of AF media (Chapter 2.4.1) 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 2.8) [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 pUC19 (Takara Bio, Tokyo, Japan) were used for subcloning of DBT-desulfurization genes from recombinant phage vectors. *E. coli* strains were cultivated in Luria-Bertani (LB) medium (Chapter 2.4.4) supplemented with 50 μg ampicillin/ml.

3.2.3. Recombinant DNA techniques

Recombinant DNA techniques were carried out as described by Sambrook et al. [11] or according to manufacturer's protocols (Chapter 2). Total DNAs from 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 carried out 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 carried out with a Genetyx-Mac version 10.1 (SDC, Tokyo, Japan). Homology search of sequence was carried out with the FASTA program of the DNA Data Bank of Japan (DDBJ).

3.2.4. Construction of total DNA library

Total DNA of 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 2.4.4), incubated at 37˚C, and then used as total DNA library of WU-F1.

3.2.5. Screening of total DNA library by plaque hybridization

The total DNA library of WU-F1 was screened by plaque hybridization with a DNA probe. The DNA probe was prepared by PCR amplification (Chapter 2.11) with the total DNA of 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-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 2.12).

3.2.6. 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.

3.3. Results

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

The DBT-desulfurization genes, *dszABC* and *tdsABC,* were already cloned from 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 *M. phlei* WU-F1 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-F1. 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-F1 as the template. The resulting PCR product was labeled with digoxigenin and used as the DNA probe.

The DNA library of WU-F1 was constructed (Chapter 3.2.4) and contained approximately 4,000 plaques. By plaque hybridization with the DNA probe, two positive plaques were obtained and selected as candidates for cells carrying DBT-desulfurization genes from WU-F1. As shown in Fig. 6.1, restriction endonuclease digestion and Southern hybridization analyses revealed that all the inserts from two positive clones included the same region of the total DNA of WU-F1 and that a 7.1-kb *Nhe*I fragment of positive clone no. 2 included the entire region of the

DBT-desulfurization genes. 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, at least in the upstream region of 3.3 kb and the downstream region of 0.8 kb. Thus, the DBT-desulfurization genes from *M. phlei* WU-F1 also designated *bdsABC*. No difference was found in nucleotide sequences adjacent to the *bdsABC* genes of WU-S2B and WU-F1 thus far.

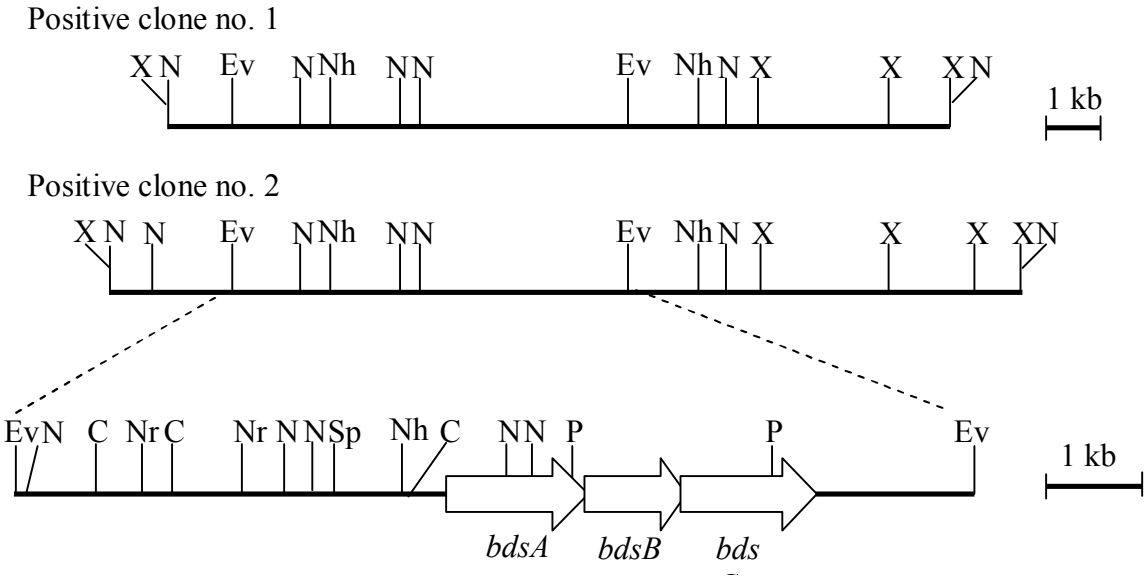

Fig. 6.1. Restriction map of three positive clones and subclones for the *bdsABC* genes from *M. phlei* WU-F1. The restriction enzyme sites derived from the phage vector are shown with asterisk and the sites lost by blunting are shown in parenthesis. C, *Cla*I; Ev, *Eco*RV; N, *Not*I; Nh, *Nhe*I; Nr, *Nru*I; P, *Pst*I; Sp, *Sph*I; X, *Xba*I.

As for DBT-desulfurizing bacterium *Paenibacillus* sp. A11-2, two open reading frames (ORFs) on both sides of DBT-desulfurization genes (*tdsABC*) encoded a putative transposase and were similar to a bacterial insertion sequence, suggesting these ORFs might constitute a transposable. Since the nucleotide sequence of the DBT-desulfurization genes from WU-F1 was the same as that of WU-S2B, it is considered that *bdsABC* might constitute a transposable unit. However, ORFs

encoding putative transposase were not located on both sides of *bdsABC*, the upstream region of 3.3 kb and the downstream region of 0.8 kb.

3.4. Discussion

In this chapter, the author described the cloning of the DBT-desulfurization gene from *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, 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, *bdsA*, *bdsB*, and *bdsC*.

The nucleotide sequences of the DBT-desulfurization genes from WU-S2B and WU-F1 were completely the same for each other. These results suggest the possibility 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 0.8 kb. These results indicate that the *bds* promoter is recognized in both the *Bacillus* and *Mycobacterium* strains.

Recently, it was reported that another thermophilic *M. phlei* GTIS10 possessed high DBT-desulfurizing ability [12]. However, it is interestingly 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 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 situation that *M. phlei* GTIS10 exhibited DBT-desulfurizing activity even at 57˚C.

In conclusion, the author confirmed that *M. phlei* WU-F1 and *B. subtilis* WU-S2B possessed the same DBT-desulfurization genes as each other, and that the abilities of WU-F1 and WU-S2B 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 must be useful as genetic resources to improve desulfurizing biocatalysts by genetic engineering [13].

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Chapter 4

Enhancement of DBT-desulfurizing Activity of *Mycobacterium phlei* WU-F1 by Genetic Engineering

4.1. Introduction

Fossil fuel contains a large amount of organosulfur compounds, and combustion of fossil fuel generates sulfur oxides, leading to harmful effects on the environment such as acid rain and air pollution [1]. Today, for reduction of sulfur contents, petroleum is treated by hydrodesulfurization (HDS) using metallic catalysts through the refinery process. However, heterocyclic sulfur compounds such as dibenzothiophene (DBT) and alkylated DBTs are recalcitrant against conventional HDS process. Therefore, the biodesulfurization (BDS) using microbial catalysts capable of desulfurizing HDS-resistant sulfur compounds, such as alkylated DBTs, has been focused since BDS would reduce the impact toward the environment with respect to energy consumption and CO_2 evolution [1].

Several mesophilic microorganisms capable of desulfurizing DBT, a model aromatic sulfur compound recognized as a target for deeper desulfurization, through carbon-sulfur bond-targeted reactions have been isolated, and almost all of them are coryne-form bacteria, such as *Rhodococcus* sp. IGTS8 [2], *R. erythropolis* KA2-5-1 [3], *R. erythropolis* D-1 [4], *R. erythropolis* ECRD-1 [5] and *Gordonia* sp. CYKS1 [6]. On the other hand, thermophilic DBT-desulfurizing microorganisms might be more advantageous than mesophilic ones for application to a BDS process following HDS

since it would be unnecessary to cool the HDS-treated oil to ambient temperature and avoid the contamination of other microorganisms. *Paenibacillus* sp. A11-2 was previously isolated as a thermophilic bacterium [7, 8]. However, reduction of the sulfur content in diesel oil was only 10% by the reaction with growing cells of *Paenibacillus* sp. A11-2, and did not reach a practical level [7]. On the other hand, three thermophilic DBT-desulfurizing bacteria, *Bacillus subtilis* WU-S2B [9], *M. phlei* WU-F1 [10], and *M. phlei* WU-0103 [11] were isolated. Among them, *M. phlei* WU-F1 has been well characterized and found to desulfurize DBT over the temperature range from 30 to 52ºC [10, 12], and its DBT-desulfurization genes related to DBT-desulfurization pathway have been determined (Chapter 1.4.3) [13, 14]. Since reduction of the sulfur content in HDS-treated light gas oil is around 60-70% by growing cells of *M. phlei* WU-F1 [15], *M. phlei* WU-F1 is desirable and practical strain for thermophilic BDS process. On the other hand, as other thermophilic desulfurizing bacteria, *M. phlei* GTIS10 showing the desulfurizing activity at 50ºC [16] and *Mycobacterium* sp. X7B desulfurizing DBT at 25-45ºC [17] have been also reported.

Genetic engineering systems for the desulfurizing microorganisms might be useful for enhancement of the biocatalytic activity and development of practical BDS process. Several mesophilic microorganisms such as *Rhodococcus* sp. [18], *Pseudomonas* sp. [19], *Escherichia coli* [20] were used as hosts for expression of DBT-desulfurization genes derived from *Rhodococcus*, and enhancement of DBT-desulfurizing activities were achieved. However, no recombinant thermophilic microorganisms overexpressing the DBT-desulfurization genes was reported so far. In addition, no shuttle vector applicable to transformation of thermophilic *Mycobacteria* such as *M. phlei* WU-F1 was reported so far.

In this chapter, the author describes the enhancement of DBT-desulfurizing activity by genetic engineering of the thermophilic DBT-desulfurizing bacterium *M. phlei* WU-F1. The *Mycobacterium-E. coli* shuttle vector designed as pUALS was constructed to generate the recombinant *M. phlei* WU-F1 with increased copy numbers of genes related to DBT-desulfurization. The DBT-desulfurizing activity of the recombinant *M. phlei* WU-F1 through the resting-cell reaction at 45°C was approximately 2-fold higher than that of the parental strain. Therefore, this is the first report describing the enhancement of DBT-desulfurizing activity of thermophilic bacteria by genetic engineering.

4.2. Materials and methods

4.2.1. Chemicals

DBT and 2-HBP were purchased from Tokyo Kasei (Tokyo, Japan). All the other chemicals used were commercially available and of chemically pure grade.

4.2.2. Bacterial strains, plasmids, and cultivation

M. phlei WU-F1 [10] was used as the host strain, and the sources of *bdsABC* [13] and *frm* [14]. *M. phlei* WU-F1 was cultivated as described previously (Chapter 2.4.1). *E. coli* JM109 and pUC19 (Takara Shuzo, Kyoto, Japan), pMSC1 (NCIMB, Shizuoka, Japan) and enzymes for recombinant DNA techniques (Nippon Gene, Toyama, Japan) were purchased. The plasmid pHP45Ω is an *E. coli* vector plasmid carrying a streptomycin-resistance gene derived from the Ω interposon [21], and was obtained from National Institute of Genetics (Shizuoka, Japan) and used for construction of a shuttle vector. pMSC1 is a *Mycobacterium-E. coli* shuttle cosmid vector carrying the

putative replication genes derived from the cryptic plasmid in *M. fortutitum*, pAL5000, and kanamycin-resistance gene and ampicillin-resistance gene [22]. *E. coli* strains were grown in either Luria-Bertani (LB) medium or SOC medium [23]. The *E. coli* and *M. phlei* WU-F1 transformants were selected on LB agar plates containing 30 μg ml^{-1} streptomycin.

4.2.3. Recombinant DNA techniques

The techniques for DNA manipulations for cloning of the desulfurization genes from *M. phlei* WU-F1 were basically the same as described previously [13, 23].

4.2.4. Construction of a *Mycobacterium-Escherichia coli* shuttle vector

Three plasmids described above, pUC19, pHP45Ω, and pMSC1, were used for construction of the *Mycobacterium-E. coli* shuttle vector, as shown in Fig. 4.1. In brief, pUC19 was digested with *Sph*I and *Hin*dIII, and ligated with the *Sph*I and *Hin*dIII digested-fragment including the streptomycin resistance gene from pHP45Ω. The *Ssp*I and *Ema*I digested-fragment including the ampicillin resistance gene was removed from the resulting plasmid to construct pUCmsph-A. And then, pUCmsph-A was digested with *Sma*I, and combined with the *Eco*RV and *Hpa*I digested-fragment including the putative replication genes from pMSC1 to construct the *Mycobacterium-E. coli* shuttle vector, designated as pUALS.

4.2.5. Construction of the recombinant plasmids

The recombinant plasmids were constructed, as shown in Fig. 4.1. pUALS was digested with *Sph*I, and ligated with *Nde*I and *Hin*dIII digested-fragment (5.5 kb)

including *bdsABC* of *M. phlei* WU-F1, as described previously (Chapter 3.3.1). And then, the resulting plasmid was digested with *Hin*dIII, and ligated with *Nru*I digested-fragment (0.8 kb) including *frm* [14] encoding a flavin reductase to construct pUALSABCD.

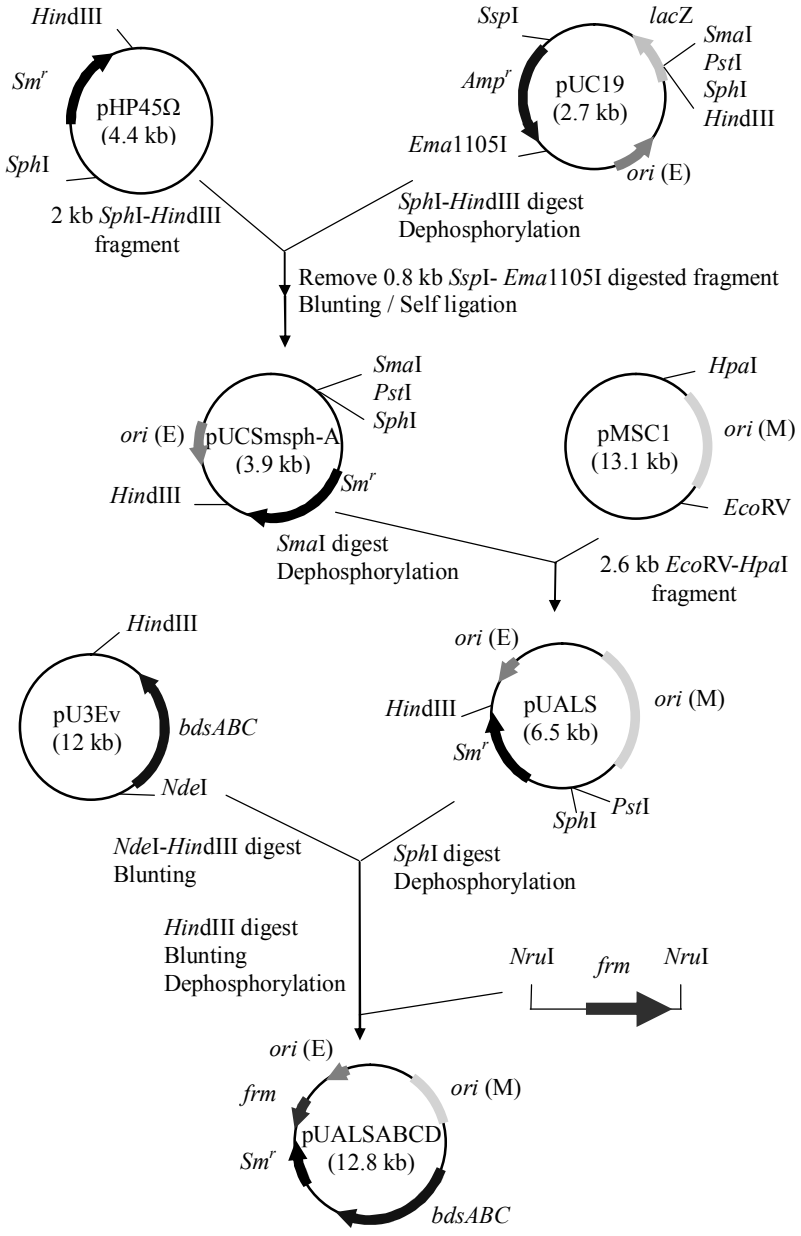

Fig. 4.1. Construction of a *Mycobacteirum-E.coli* shuttle vector pUALS and recombinant plasmid pUALSABCD.

Abbreviation: *Amp*^{r}, ampicillin resistance gene; *Sm*^{r}, streptomycin resistance gene; *ori*(E), *E. coli* orign of replication; *ori*(M), *Mycobacterium* sp. orign of replication; *lacZ*, β-galactosidase gene. Arrows indicate genes and the direction of transcription.

4.2.6. Transformation of *M. phlei* WU-F1 by electroporation

M. phlei WU-F1 cells were grown in 100 ml LB at 45ºC with shaking, and harvested at optical density at 660 nm (OD_{660}) of 0.5-1.0. The cells were washed three times with ice-cold 10% glycerol, and then concentrated by 100-fold. These cells were stored in small portions of 200 μl at -80ºC. A portion of the cells was mixed with each recombinant plasmid, pUALS or pUALSABCD, in a 2 mm-gap electrocuvette (Bio-Rad Japan, Tokyo, Japan), and the mixture was supplied at 12.5 kV/cm electric pulse by a Gene Pulser II (Bio-Rad Japan, Tokyo, Japan) connected to a pulse controller (25 μF capacitor, 400 Ω external resistance). The pulse-given mixture was diluted immediately with 0.4 ml SOC medium, and incubated at 37ºC for 3 h with shaking. Then, the culture was appropriately diluted and spread on LB plates containing 30 μg ml^{-1} streptomycin, and cultivation was done at 30ºC for 4 days. A colony-forming unit (CFU) was defined as the colony number of transformants, which appeared on the plates, per μg of plasmid DNA.

4.2.7. Resting cell reaction

Recombinant *M. phlei* WU-F1 harboring each recombinant plasmid, pUALS or pUALSABCD, were cultivated at 45ºC for 4 days in AF medium (Chapter 2.4.1) containing 30 μg ml^{-1} streptomycin and DBT (0.54 mM, in *n*-tridecane), and then diluted 40-fold with AF medium containing 30 μg ml^{-1} streptomycin and DBT (0.27 mM, in *n*-tridecane) followed by shaking at 45ºC till late log phase (OD_{660} 4.5-5.5). Cells were harvested at 4ºC by centrifugation at 10,000 g for 15 min, washed twice and resuspended in 0.1 M potassium phosphate buffer (pH 7.6) to give an OD_{660} of 40. Nine microliters of substrate solution, DBT (0.54 mM, in *n*-tridecane), was added to 0.6

ml of the cell suspension in a test tube. The reaction was carried out at 45ºC for 15 min with reciprocal shaking at 190 strokes per minute. After the reaction, the bacterial suspension was acidified by adding 9 µl of 6 M-HCl and extracted with 1.5 ml of ethylacetate. The extract filtered using a 0.2-µm PTFE membrane filter (Advantec Toyo, Tokyo, Japan) and then analyzed by gas-chromatography (GC-2010, Shimadzu, Kyoto, Japan) equipped with a 30 m × 0.25 mm Film Thickness 0.25 µm type DB-5 column (J&W Scientific, CA) to measure concentrations of DBT and 2-hydroxybiphenyl (2-HBP).

4.3. Results

4.3.1. Transformation of *M. phlei* WU-F1 with plasmid pUALS

M. phlei WU-F1 itself as a host strain showed potentially resistance toward kanamycin and ampicillin, but not streptomycin resistance. Therefore, the streptomycin resistance gene derived from the Ω interposon was used as a marker for selection of transformants. The *Mycobacterium-E. coli* shuttle vector, pUALS, harboring the streptomycin resistance gene, *E. coli* orign of replication, and *Mycobacterium* sp. orign of replication was constructed and introduced into *M. phlei* WU-F1 cells by electroporation. The transformation frequency of *M. phlei* WU-F1 with pUALS was 40 CFU on AF agar plate incubated at 45ºC.

Since pUALS is a low-copy plasmid in *M. phlei* WU-F1 but not in *E. coli*, the presence of the recombinant plasmid was confirmed by electroduction between transformants and *E. coli* [25]. Although the bands of plasmids pUALS and pUALSABCD were not directly detected on agarose-gel stained by ethidium bromide as for the total DNA of transformants WUF1/pUALS and WU-F1/pUALSABCD, the use

of PCR with adequate primers and/or electroduction between the transformants and *E. coli* enabled the detection of plasmids pUALS and pUALSABCD (details not shown).

4.3.2. Desulfurization of DBT by the recombinant strains derived from *M. phlei* WU-F1

M. phlei WU-F1 and the transformants harboring pUALSABCD (WU-F1/pUALSABCD) and pUALS (WU-F1/pUALS) were cultivated for appropriate intervals to show the same growth phases and the highest DBT-desulfurizing activities for each strain among the cultivation times examined by resting cell reactions of each strain at 45ºC. The DBT-desulfurizing activity of WU-F1/pUALS was almost the same as that of WU-F1 as a host strain. On the other hand, the DBT-desulfurizing activity of WU-F1/pUALSABCD was 0.67 nmol/min/mg-dry-cell-weight, corresponding to approximately 2-fold higher than that of WU-F1/pUALS, as a control, and WU-F1 (Fig. 4.2).

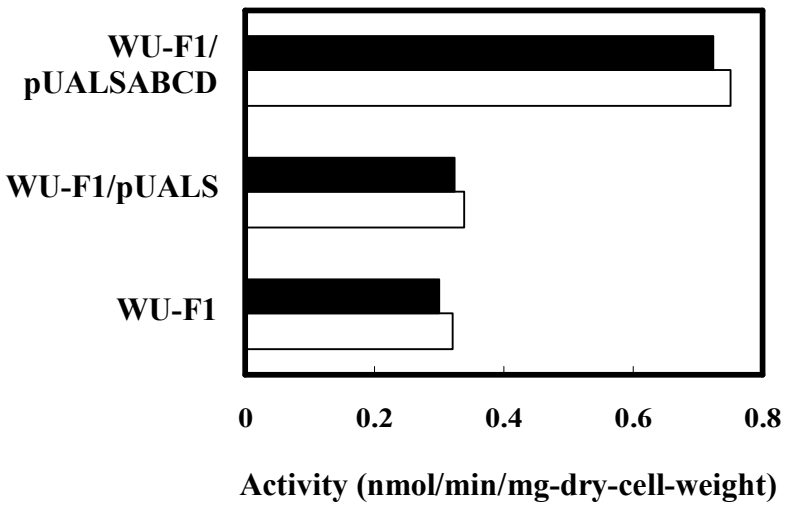

Fig. 4.2. DBT-degrading and 2-HBP-producing activities of the recombinant strains derived from *M. phlei* WU-F1. Closed bars, 2-HBP-producing activities; open bars, DBT-degrading activities.

4.4. Discussion

In this chapter, the author described the enhancement of DBT-desulfurizing activity by genetic engineering of the thermophilic DBT-desulfurizing bacterium *M. phlei* WU-F1. The transformation frequency of *M. phlei* WU-F1 with pUALS by electroporation is lower than those of previously reported in [24] describing transformation of various mycobacterial species with a pAL5000-based *Mycobacterium-E. coli* shuttle vector, although transformation of *M. phlei* was not reported in [24] and the marker gene used in this study is different from the one in [24]. Moreover, several pAL5000-based *Mycobacterium-E. coli* shuttle vectors were used for analysis of the gene function and gene regulation responsible for mycobacterial diseases [24, 25], and were only used for transformation of mesophilic bacteria at 30-37ºC. Therefore, in this study, pAL5000-based pUALS was newly constructed for a thermophilic bacterium *M. phlei* WU-F1, and the transformants harboring pUALS showed the growth at even 45ºC. Therefore, the author considers that the mycobacterial replicon of pAL5000 was functionable at least up to 45ºC.

On the other hand, it is well known that pAL5000-based *Mycobacterium-E. coli* shuttle vectors show generally low copy numbers in *Mycobacteria* but not in *E. coli* [24]. Therefore, the presences of the recombinant plasmids were confirmed by electroduction [25].

The DBT-desulfurizing activity of WU-F1/pUALSABCD was 0.67 nmol/min/mg-dry-cell-weight, corresponding to approximately 2-fold higher than that of WU-F1/pUALS, as a control, and WU-F1. Therefore, these results clearly indicate that gene dosage effect on DBT-desulfurizing activity by the increase of copy numbers of genes related to DBT-desulfurization in *M. phlei* WU-F1 as a host. Similar results

describing enhancement of DBT-desulfurizing activity by genetic engineering was reported by Hirasawa et al. [18], who described that the DBT-desulfurizing activity of the recombinant strain, a mesophilic bacterium *Rhodococcus erythropolis* KA2-5-1 harboring one DBT-desulfurizing gene cluster and one flavin reductase gene on *Rhodococcus-E. coli* shuttle vector, was approximately 2-fold higher than that of the parental strain [18].

Even under the conditions of various temperature up to 50ºC, *M. phlei* WU-F1 shows higher DBT-desulfurizing activity than other strains reported so far [10], and desulfurizes asymmetric organosulfur compounds, NTH and 2-ethylNTH, in addition to symmetric DBT derivatives by the resting cell reaction as described previously [12]. Therefore, *M. phlei* WU-F1 efficiently desulfurized HDS-treated light gas oil over a temperature range up to 50ºC [15]. These results suggest that WU-F1 might be a promising desulfurizing biocatalyst possessing a broad substrate specificity toward aromatic sulfur compounds and a host strain to enhance the biodesulfurization abilities of thermophilic desulfurization.

In conclusion, the author constructed a *Mycobacterium-E. coli* shuttle vectors applicable for genetic engineering of *M. phlei* WU-F1, and succeeded in enhancing the DBT-desulfurizing activity of *M. phlei* WU-F1 by genetic engineering under high temperature at 45ºC. Although the shuttle vectors such as pUALS and pUALSABCD are low-copy plasmids in *M. phlei* WU-F1 and promoter regions of *bdsABC* and *frm* are native ones, gene dosage effects were confirmed as for DBT-desulfurization. These results suggest the possibility to enhance the biodesulfurization abilities of thermophilic desulfurization of *M. phlei* WU-F1 by genetic engineering for a desirable and practical thermophilic BDS process.

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Chapter 5

Cloning and Functional Analysis of the Reversible γ-Resorcylic Acid Decarboxylase Gene (*rdc*) from *Rhizobium radiobacter* WU-0108

5.1. Introduction

In microbial degradation-pathway for aromatics, enzymes catalyzing oxidative and nonoxidative decarboxylation of aromatics have been reported [1-9]. Although several enzymes of nonoxidative decarboxylation have been purified [3-7], among these nonoxidative decarboxylases, 4-hydroxybenzoate decarboxylase and 3,4-hydroxybenzoate decarboxylase from *Clostridium hydroxybenzoicum* JW/Z-1T [3,4], pyrrole-2-carboxylate decarboxylase from *Bacillus megaterium* PYR2910 [5], and indole-3-carboxylate decarboxylase from *Arthrobacter nicotianae* FI1612 [6] have been found to catalyze reversible reactions, in other words carboxylation of organic compounds. As for these enzymes, only the gene encoding 4-hydroxybenzoate decarboxylase from *C. hydroxybenzoicum* JW/Z-1 was already cloned and characterized [10]. One of the properties of these nonoxidative decarboxylases is that their reactions were generally sensitive to O_2, and both of the activities of decarboxylation and carboxylation were found to decrease as for 4-hydroxybenzoate decarboxylase, 3,4-hydroxybenzoate decarboxylase, and indole-3-carboxylate decarboxylase in the presence of O_2 [3, 6]. Pyrrole-2-carboxylate decarboxylase purified from *B. megaterium* PYR2910 showed stable activities in the presence of O_2 although addition

of dithiothreitol was necessary [6]. On the other hand, some O_2-insensitive, nonoxidative aromatics decarboxylases were purified, but no activity catalyzing reversible reaction has been reported [7, 8, 11, 12].

γ-Resorcylic acid (γ-RA, 2,6-dihydroxybenzoic acid) is used as an intermediate of medicine, herbicide, and industrial chemicals. Although γ-RA is produced industrially by the carboxylation toward resorcinol (RE, 1,3-dihydroxybenzene) under high temperature and high pressure, large amount of β-resorcylic acid (2,4-dihydroxybenzoic acid) is also generated as a by-product, and a process for separating by-product is then required [13].

In this chapter, the author describes the molecular characterization of the reversible and nonoxidative γ-RA decarboxylase from *Rhizobium radiobacter* WU-0108, including its purification, characterization, and gene-cloning. For selective synthesis of γ-RA from RE, microorganisms possessing a novel enzyme catalyzing regioselective carboxylation of RE to form γ-RA was screened from 50 soil samples. The nonoxidative γ-RA decarboxylase (Rdc) that reversibly catalyzes the regioselective carboxylation of RE to form γ-RA (Fig. 5.1) was found from *R. radiobacter* WU-0108. The gene (*rdc*) encoding Rdc was cloned and heterologously expressed in *Escherichia coli* cells. Therefore, this is the first report describing the enzyme that catalyzes the regioselective carboxylation of RE to form γ-RA.

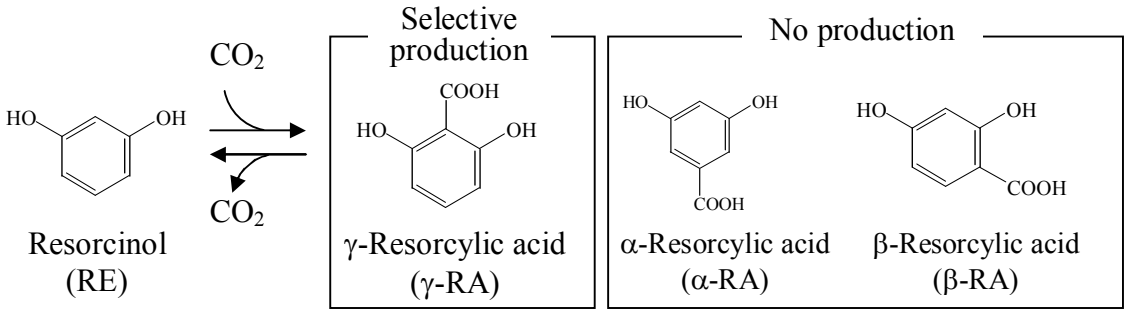

Fig. 5.1. Enzymatic reversible conversion of γ-resorcylic acid and resorcinol.

5.2. Materials and methods

5.2.1. Chemicals

α-Resorcylic acid (α-RA), β-RA, γ-RA, 2,3-dihydroxybenzoic acid, RE, and catechol were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All the other chemicals used were commercially available and of chemically pure grade.

5.2.2. Bacterial strains, plasmids, and cultivation

R. radiobacter WU-0108 was used as the source of total DNAs for cloning of the gene (*rdc*) encoding the reversible and nonoxidative γ-RA decarboxylase (Rdc). For preparation of total DNAs, WU-0108 was cultivated at 30˚C in test tubes (18 by 180 mm) containing 5 ml of RA media (Chapter 2.4.2). *E. coli* JM109 and a cloning vector pUC118 (Takara Bio, Tokyo, Japan) were used for construction of a partial DNA library of WU-0108. *E. coli* JM109 was cultivated in Luria-Bertani (LB) medium (Chapter 2.4.4) supplemented with 50 μg ampicillin/ml. *E. coli* BL21 (DE3) and pET21-d were used for over-expression of *rdc*. Recombinant *E. coli* BL21 (DE3) carrying a recombinant plasmid derived from pET21-d was cultivated at 30°C in 500 ml Erlenmeyer-flasks containing 100 ml LB medium supplemented with 100 μg ampicillin/ml and 0.1 mM isopropyl-β-D-thiogalactopyranoside with reciprocal shaking at 120 strokes/min.

5.2.3. Resting cell reaction

R. radiobacter WU-0108 was cultivated for 48 h at 30°C in 500 ml Erlenmeyer flasks containing 100 ml RA medium with reciprocal shaking at 120 strokes/min (Chapter 2.4.2). After cultivation, the cells were harvested by centrifugation at 10,000

× *g* for 20 min at 4°C, washed twice with 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.0), and suspended with 10 ml of the same buffer. Then, 50 µl of 0.5 M RE solution was added to 1 ml of the cell suspension containing 150 mM $NaHCO_3$ (pH 9.6) in a 2-ml microcentrifuge tube. The reaction was allowed to proceed for 24 h at 30°C, 50 µl of 12 M HCl was added to stop the reaction. The mixture was then centrifuged at 6,000 × g for 10 min at 4°C and the supernatant filtered using a 0.2-µm PTFE membrane. The amount of substrates and reaction products were measured by HPLC according to Chapter 2.14.2..

5.2.4. Preparation and purification of reaction product from resorcinol

For preparation of the reaction product from RE, 220 mg of RE and 520 mg $NaHCO_3$ were added to 80 ml of a resting cell suspension (OD_{660} 20) in 500 ml Erlenmeyer flasks. The mixture was incubated with shaking for 24 h at 30°C, and then extracted with 200 ml ethylacetate. The resulting extract was concentrated on a rotary evaporation equipment and applied to a silica column chromatograph packed with Wakogel C-200 (Wako Pure Chemical, Osaka, Japan). The elution was carried out with methanol-chloroform-ammonia (5 : 13 : 0.8, v/v/v). The fraction including product was concentrated by rotary evaporation, and then lyophilized and subjected to NMR analysis as described previously [15].

5.2.5. NMR analysis

^{13}C-NMR, ^{1}H-NMR, and heteronuclear multiple bond coherence spectra were obtained using a JEOL JNM-LA 500 spectrometer (JEOL, Tokyo) operated at 150 MHz (^{13}C-NMR) and 600 MHz (others) with sodium 2, 2-dimethyl-2-silapentane-5-sulfonate

(DSS) as an internal standard.

5.2.6. Preparation of cell-free extract of *R. radiobacter* WU-0108

As the basal buffer, 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 8.0) was used. *R. radiobacter* WU-0108 was cultivated for 48 h at 30°C in 500 ml Erlenmeyer flasks containing 100 ml RA medium with reciprocal shaking at 120 strokes/min. The cells in 800 ml of culture broth were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C, washed twice with the basal buffer (pH 8.0), and suspended in 10 ml of the basal buffer. The suspended cells were disrupted with an ultraoscillator (Sonifier 450; Branson, CT, USA) at 20 kHz for 20 min at 4°C. The cell debris was removed by centrifugation at 16,000 × *g* for 30 min at 4°C. The resulting supernatant was used as the cell-free extracts to further purification through the following 3 steps by the AKTA system (Amersham Biosciences, NJ, USA).

5.2.7. Purification of a novel γ-resorcylic acid decarboxylase (Rdc)

All purification procedures were carried out at 4°C or on ice. The cell-free extracts were applied to a Toyopearl-DEAE 650M column (Tohso, Tokyo, Japan) equilibrated with the basal buffer and washed with 90 ml of the same buffer. Elution was carried out with a continuous linear gradient of 0 to 1.0 M NaCl in 150 ml of the basal buffer at a flow rate of 2 ml/min. The active fractions (0.1 to 0.17 M NaCl) showing carboxylation of RE to form γ-RA were collected and dialyzed for 18 h against the basal buffer supplemented with 1.0 M $(NH_4)_2SO_4$ for applying to a butyl FF column of hydrophobic interaction chromatography. The dialysate was applied to a HiPrep 16/10 Butyl Fast Flow column (Amersham Biosciences) equilibrated with the basal

buffer supplemented with 1.0 M $(NH_4)_2SO_4$, and washed with 70 ml of the same buffer. Elution was carried out with a continuous linear gradient of 1.0 to 0 M $(NH_4)_2SO_4$ in the 140 ml of basal buffer at a flow rate of 2 ml/min. The active fractions (0.84 to 0.71 M $(NH_4)_2SO_4$) were collected and combined. The combined solution was applied to a HiLoad 16/10 Phenyl Sepharose High Performance column (Amersham Biosciences) equilibrated with the basal buffer supplemented with 0.5 M $(NH_4)_2SO_4$, and washed with 70 ml of the same buffer. Elution was carried out with a continuous linear gradient of 0.5 to 0 M $(NH_4)_2SO_4$ in 140ml of the basal buffer at a flow rate of 2 ml/min. The active fractions (0.46 to 0.40 M $(NH_4)_2SO_4$) were collected, dialyzed against the basal buffer, and then concentrated by ultrafiltration.

5.2.8. Enzyme assays

For measurement of decarboxylase activity, the standard reaction conditions were as follows: the mixture contained 50 μg of protein and 3 mM γ-RA in 100 mM K_2HPO_4-KH_2PO_4 buffer (pH7.0) to a final volume of 1 ml in a 1.5-ml microcentrifuge tube. The reaction was started by the addition of γ-RA, and incubated with shaking at 40°C for 30 min. For measurement of carboxylase activity, the standard reaction conditions were as follows: the mixture contained 10 μg of protein, 30 mM RE, and 1 M $KHCO_3$ in 50 mM K_2HPO_4-KH_2PO_4 buffer (pH7.0) to a final volume of 1 ml in the 1.5-ml microcentrifuge tube. The reaction was started by the addition of RE, and incubated with shaking at 40°C for 30 min. Both of the reactions, decarboxylation and carboxylation, were stopped by addition of 10 μl of 12 M HCl. When other substrates were used instead of γ-RA or RE, other assay conditions were the same as those of standard conditions if otherwise indicated. When reactions were carried out in the

absent of O_2, 10 ml screw cap test tube was used, and the head space of the test tube was substituted with CO_2 or N_2 gas. Other variations of the assay conditions are indicated in the figure legends and tables. The amounts of γ-RA, RE, and other substrates were determined by using HPLC (Chapter 2.14.2). One unit (U) of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of product per minute.

5.2.9. Protein analysis

The molecular mass was determined by the AKTA system (Amersham Biosciences) with a Superdex 200 HR 10/30 column at a flow rate of 0.5 ml/min by using 20 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.2) as the eluent. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out using 12% polyacrylamide by the method of Laemmli [16]. The N-terminal amino acid sequence of the reversible γ-RA decarboxylase was determined by Takara Bio Inc. (Shiga, Japan). Protein concentrations were measured by the method of Bradford [17] by using the Coomassie Protein Assay Kit (Pieace Co., IL, USA) with bovine serum albumin as the standard.

5.2.10. Recombinant DNA techniques

Recombinant DNA techniques were basically carried out as described in Chapter 2, and by Sambrook et al. [18] or according to the previous report [19].

5.2.11. Construction of DNA library

Total DNA of *R. radiobacter* WU-0108 was digested with *Sal*I. After

electrophoresis, fragments of 3-4 kb in length were excised from agarose gel and purified. The DNA fragments were ligated into pUC118 and then introduced to *E. coli* JM109 to construct a partial DNA library of *R. radiobacter* WU-0108.

5.2.12. Screening of DNA library by colony hybridization

For preparation of a probe, PCR was carried out with total DNA of *R. radiobacter* WU-0108 and two primers as follows, 5'-ATT GCT GGA CGG AAC TG-3' as the sense primer and 5'-AGC TTC ACC CAG GCA TT-3' as the anti-sense primer. The amplified fragment was then labeled by the digoxigenin-11-dUTP random prime method, using a DIG DNA labeling kit (Roche Diagnostics, Tokyo, Japan). The labeled fragment was used as a probe (designated as RDC-DIG probe) to screen the partial DNA library of *R. radiobacter* WU-0108 by colony hybridization. The library was cultivated on LB agar plate containing 50 μl of ampicillin/ml, and the colonies were transferred to Hybond-N+ nylon membranes (Amersham Biosciences). The membranes were probed with RDC-DIG at 68°C in 5 × SSC, 1%(w/v) Blocking reagent, 0.1%(w/v) N-lauroylsarcosine and 0.02%(w/v) SDS, and washed twice at 25°C in 2 × SSC and at 68°C in 0.1 × SSC.

5.2.13. Amplification of *rdc*

The gene was amplified by PCR from the recombinant plasmid (pC2) including *rdc* in pUC118, which was obtained through the colony hybridization as described above, using two oligonucleotide primers as follows, 5'-AAC TCC ATG GAA GGC AAG GTC-3' [the *Nco*I restriction site is underlined] as the sense primer and 5'-CAT GTC GAC CGA ACG GCT GCA-3' [the *Sal*I restriction site is underlined] as the

anti-sense primer.

5.2.14. Expression of *rdc* in *E. coli*

Amplified DNA fragments were digested with *Nco*I and *Sal*I, separated by agarose gel electrophoresis, inserted into pET21-d (Novagen, WI, USA). The resulting recombinant plasmid (pENS10) was amplified in *E. coli* JM109 cells, and the insert was sequenced to ensure that the correct construction had been obtained. The plasmid pENS10 was then introduced into *E. coli* BL21 (DE3) for expression of *rdc*. Recombinant *E. coli* BL21 (DE3) carrying pENS10 was cultivated at 30°C in 500 ml Erlenmeyer-flasks containing 100 ml LB medium supplemented with 100 μg ampicillin/ml and 0.1 mM isopropyl-β-D-thiogalactopyranoside with reciprocal shaking at 120 strokes/min. After cultivation for 18 h, cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C, and then cell-free extract was prepared (Chapter 5.2.6). Site-directed mutants were created using the Gene Tailor Site-Directed Mutagenesis System (Invitrogen, CA, USA).

5.2.15. Nucleotide sequence accession number

The nucleotide sequence discussed in this paper is available from DDBJ under accession number AB185333 as the reversible γ-RA decarboxylase gene of *R. radiobacter* WU-0108, *rdc*.

5.3. Results

5.3.1. Identification of γ-resorcylic acid degrading bacteria, WU-0108

Small amounts of 50 soil samples collected from all over the Kanto area of Japan were suspended in 1 ml distilled water and centrifuged at 1500 × *g* for 5 min at 4°C to remove solid components. The resulting supernatant was appropriately diluted with distilled water, and aliquots of 50 µl were inoculated into test tubes containing 5 ml of RA liquid medium and cultivated at 30°C for 6 to 8 days. Aliquots of 50 µl of some turbid cultures showing optical density at 660 nm (OD_{660}) more than 0.5 were then transferred into 5 ml of fresh RA liquid medium. After four subcultivations, to confirm whether RE was accumulated, the culture filtrate was analyzed by TLC. Among the 40 turbid cultures, TLC analysis revealed that RE was accumulated in four cultures from independent soil samples. These cultures were appropriately diluted with distilled water and spread onto RA agar plates. After cultivation at 30°C for 3 to 5 days, each single colony formed on the plates was isolated and inoculated again into liquid RA medium. By repeated cultivation in liquid RA medium and single-colony isolation on RA agar plates, strains showing stable growth more than OD_{660} 1.0 and the accumulation of RE in liquid RA medium were selected. Among the strains, one bacterium designated WU-0108 was selected for further studies.

Strain WU-0108 was a rod-shaped bacterium with dimensions of 0.8 µm by 1.5 to 2.0 µm. This strain was gram-negative, motile, catalase positive, and oxidase positive, and did not form spores. Further taxonomical identification of strain WU-0108 was carried out by the National Collection of Industrial and Marine Bacteria Japan Ltd. (Shizuoka, Japan), and the 16S ribosomal DNA sequence of WU-0108 was found to have 99.7% identity to that of *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*). From these results, WU-0108 was identified as *R. radiobacter* (Fig. 5.2). Since WU-0108 was determined to belong to *R. radiobacter*, it was confirmed whether

the carboxylation activity of RE would be detected on several *Rhizobium* strains, *i.e. R. radiobacter* IFO 13532T, *R. radiobacter* IFO 12607, and *R. radiobacter* IFO 12664. Since all of three strains could grow in RA medium, resting cells of these three strains were prepared and subjected to the tests of carboxylation of RE. Since the carboxylation activity of RE to form γ-RA was observed for all these strains, such an activity might be universally existed in *R. radiobacter* (data not shown).

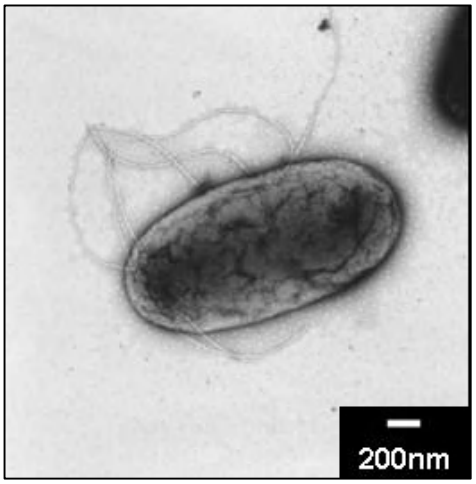

Fig. 5.2. Electron micrographs of a *R. radiobacter* WU-0108 cell. Scale bar, 200 nm.

5.3.2. Carboxylation of resorcinol to form γ-resorcylic acid by resting cell reaction

The synthesis of γ-RA from RE by the resting cells of strain WU-0108 was investigated. The TLC analysis revealed that the resting cells of strain WU-0108 produced from RE remarkably only one product which shows Rf value of 0.316 on TLC identical to that of authentic γ-RA. The product was extracted from the reaction mixture, purified by silica gel column chromatography, and subjected to NMR analysis. Thirty mg of product was obtained from 220 mg of RE in the reaction mixture, with the molar conversion yield of 9.7% (mol/mol). The two-dimensional HMBC spectrum

indicated that the product isolated was identified as γ-RA (Fig. 5.3). The resting cells of strain WU-0108 cultivated in LB medium did not show the both activities of RE-carboxylation and γ-RA-decarboxylation. On the other hand, the resting cells of strain WU-0108 cultivated in LB medium supplemented with γ-RA slightly showed the both activities (data not shown), suggesting that the enzyme was an inducible protein by γ-RA.

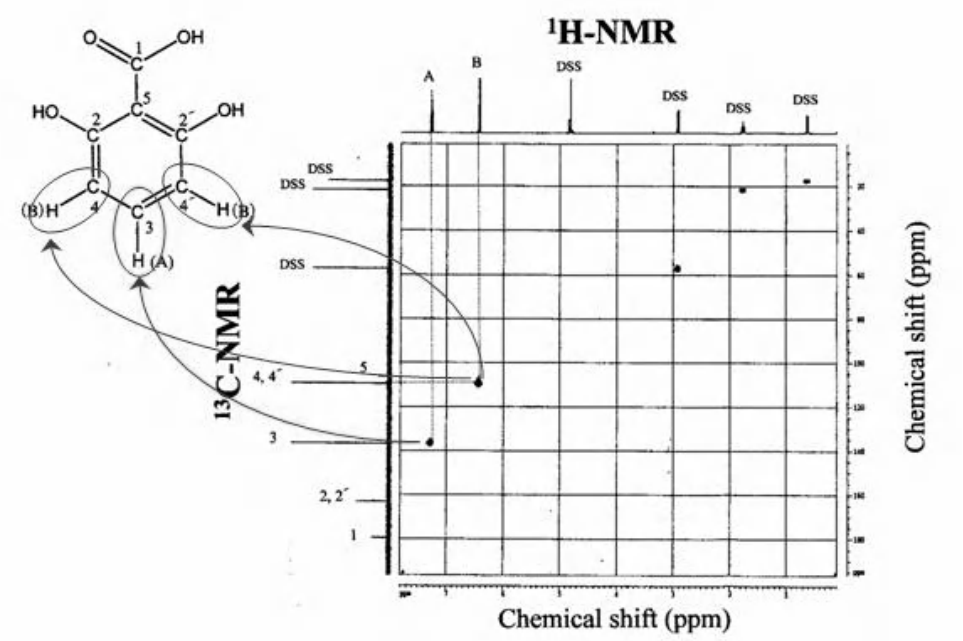

Fig. 5.3. ^{1}H-^{13}C shift correlation NMR spectrum of the product from resorcinol.

5.3.3. Purification of a novel γ-resorcylic acid decarboxylase (Rdc)

Based on the carboxylase activity converting RE to γ-RA, the RE-carboxylation enzyme was purified to homogeneity from cell extracts of *R. radiobacter* WU-0108 (Chapter 5.2.7). Purification steps are shown in Table 5.1. The RE-carboxylation enzyme was purified 14.2-fold with a yield of 14%, and the specific activity of the purified enzyme for carboxylation of RE to γ-RA was 1.57 U/mg. The purified enzyme produced a 34 kDa single band on the gel by SDS-PAGE (data not shown). The native molecular mass of the enzyme was found to be 130 kDa by gel filtration

(data not shown), suggesting that the enzyme has a homotetrameric structure.

Table 5.1 Purification of the reversible γ-RA decarboxylase from *Rhizobium radiobacter* WU-0108

Step	Total Protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
Cell-free extract	144	0.11	16.0	1.00	100
DEAE-Toyoperl	25.2	0.58	14.7	5.28	92
Butyl FF	6.6	0.87	5.7	7.83	36
Phenyl Sepharose HP	1.4	1.57	2.2	14.2	14

[a] U, One unit (Unit) was defined as the amount of enzyme that synthesizes one μmol of γ-RA per minute from RE.

5.3.4. Properties on decarboxylation of Rdc

The purified enzyme also catalyzed the decarboxylation of γ-RA to RE, indicating that this enzyme is a reversible γ-RA decarboxylase. Therefore, properties as for the decarboxylation activity of this enzyme were examined. The optimal pH and optimal temperature for decarboxylation activity were 7.0 and 60°C, respectively (Fig. 5.4).

The enzyme was stable up to 40°C and retained 60% of its activity after the treatment of heating at 50°C for 30 min. The optimal concentration of γ-RA for enzyme activity was 10 mM (data not shown). The effects of various metal ions and chemical reagents are shown in Table 5.2. The enzyme activity was inhibited by $AgNO_3$ (100% inhibition) and $HgCl_2$ (79%), and especially diethyl pyrocarbonate (DEPC, 34%) as a histidine residue-specific inhibitor. Other metal ions tested and sulfhydryl group inhibitor, such as *p*-chloromercuribenzoic acid, *N*-ethylmaleimide, and iodoacetamide, had no inhibition on enzyme activity. The enzyme activity was activated by $MgCl_2$ (32% activation). Additions of EDTA, pyridoxal 5'-phosphate,

avidin, biotin, NADH, and NADPH to reaction mixture had no or slight effect on the enzyme activity. Hydroxylamine, which is known as an inhibitor of pyridoxal 5'-phosphate-dependent decarboxylases, also had no effect. From these results, this enzyme was found to be a nonoxidative decarboxylase that requires no cofactors such as pyridoxal 5'-phosphate, NADH, and NADPH.

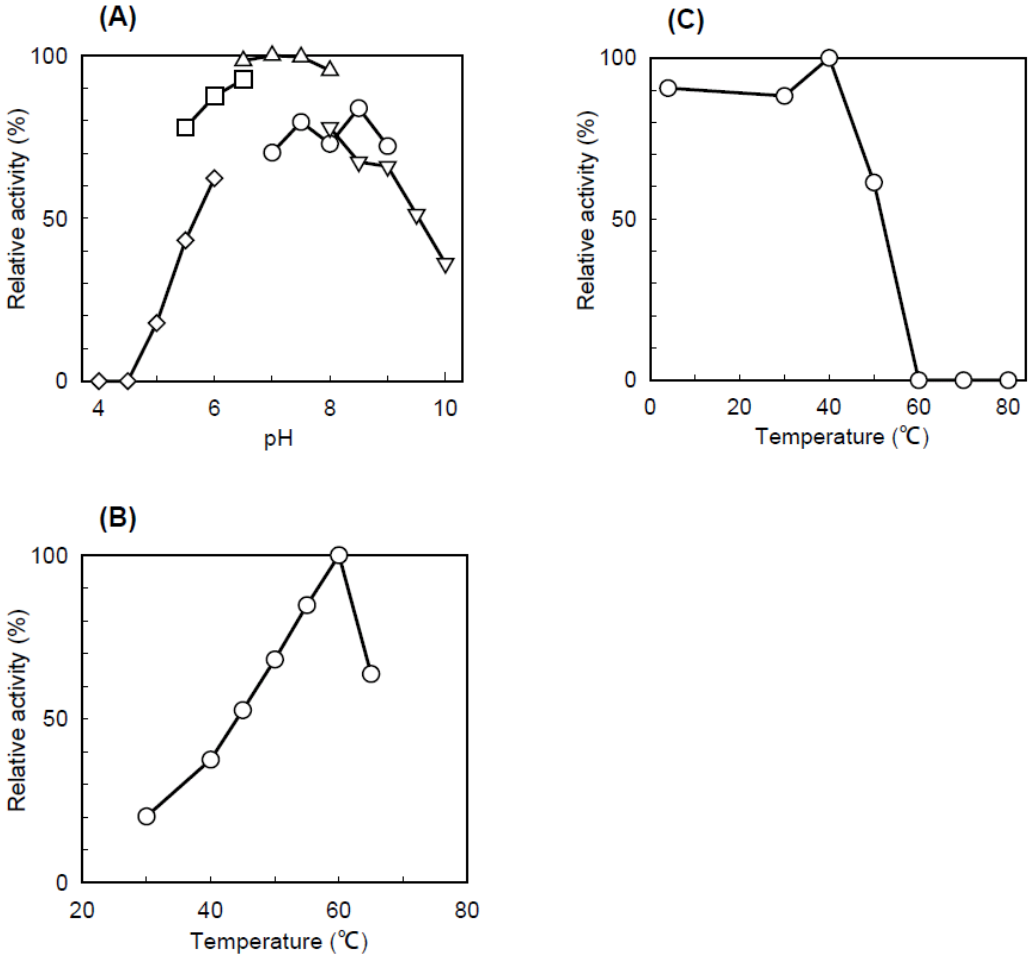

Fig. 5.4. Effects of pH and temperature on decarboxylation activity. (A) Effect of pH on activity. Assays for measurement of activity were done under the standard condition (Chapter 5.2.8) with modification of buffer. The following buffers (100 mM) were used: ◇; Citrate-NaOH pH 4.0 - 6.0, □; Mes-NaOH pH 5.5 - 6.5, △; K_2HPO_4-KH_2PO_4 pH 6.5 - 8.0, ○; Tris-HCl pH 7.0 - 9.0, ▽; H_3BO_3-NaOH pH 8.0 - 10.0. (B) Effect of temperature on activity. Assays for measurement of activity were done under the standard condition. (C) Effect of temperature on stability. After the purified enzyme was preincubated at the indicated temperature for 30 min, the remaining activity was measured under the standard condition. The remaining activity was expressed relative to the activity, taken as 100%, when reaction mixture was not preincubated.

Table 5.2 Effect of various compounds on carboxylation and decarboxylation activity of the reversible γ-RA decarboxylase (Rdc)

Compound	Relative activity (%)	
	Decarboxylation	Carboxylation
None	100	100
NaCl	97	100
KCl	100	100
$MgCl_2$	132	106
$MnCl_2$	104	109
$CaCl_2$	98	99
$HgCl_2$	21	94
$ZnCl_2$	86	89
$CuCl_2$	87	86
$AgNO_3$	0	12
EDTA	99	100
N-Ethylmaleimide	99	104
Iodoacetamide	103	100
p-Chloromercuribenzoic acid	92	90
Diethyl pyrocarbonate	66	62
Pyridoxal 5'-phosphate	99	98
Avidin	98	97
Biotin	99	97
NADH	99	99
NADPH	102	99
Hydroxylamine	101	93
$NaBH_4$	98	89

The carboxylation and decarboxylation reactions were done under the standard conditions supplemented with the compounds tested at 1 mM except for hydroxylamine (20 mM), NaBH4 (10 mM), avidin (0.2 U/ml), NADH (2.5 U/ml), and NADPH (2.5 U/ml).

The purified enzyme catalyzed the decarboxylation of γ-RA to stoichiometric amounts of RE with a specific activity of 1.36 U/mg. The *K*m, *V*max and *k*cat values derived from Lineweaver-Burk plots with the enzyme at 50°C and pH 7.0 were 0.035 mM, 3.69 mM/min and 2.8×10^3 min^{-1}, respectively, for γ-RA. The enzyme also catalyzed the decarboxylation of 2,3-dihydroxybenzoic acid to stoichiometric amounts of catechol with a specific activity of 0.95 U/mg. The *K*m, *V*max and *k*cat values derived from Lineweaver-Burk plots with the enzyme at 50°C and pH 7.0 were 0.035 mM, 0.70 mM/min and 5.2×10^2 min^{-1}, respectively, for 2,3-dihydroxybenzoic acid. The enzyme did not catalyzed the decarboxylation of salicylic acid (2-hydroxybenzoic acid), 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 2,3,4-trihydroxybenzoic acid, 3,4,5-trihydroxybenzoic acid, 4-aminobenzoic acid, *o*-hydroxyphenylacetic acid, and vanillic acid (4-hydroxy-3-methoxybenzoic acid). These results indicated that the substrate recognition of the purified enzyme seems to depend strictly on dihydroxybenzoic acid with three neighboring groups in the active center of the enzyme.

5.3.5. Properties on carboxylation of Rdc

The best CO_2 source was bicarbonates with $KHCO_3$ leading to 7.67 mM γ-RA from 25 mM RE, followed by NH_4CO_3 (93% relative activity), $NaHCO_3$ (85%), K_2CO_3 (4.4%) and Na_2CO_3 (0.40%). The carboxylation activity showed a substrate saturation dependence from HCO_3 - with optimal HCO_3 - concentration above 2.5 M (Fig. 5.5). The optimal substrate concentration of RE and optimal temperature for enzyme activity were 45 mM and 45°C, respectively (Fig. 5.5). The enzyme was stable up to 40°C and retained 60% of its activity after the treatment of heating at 50°C for 30 min.

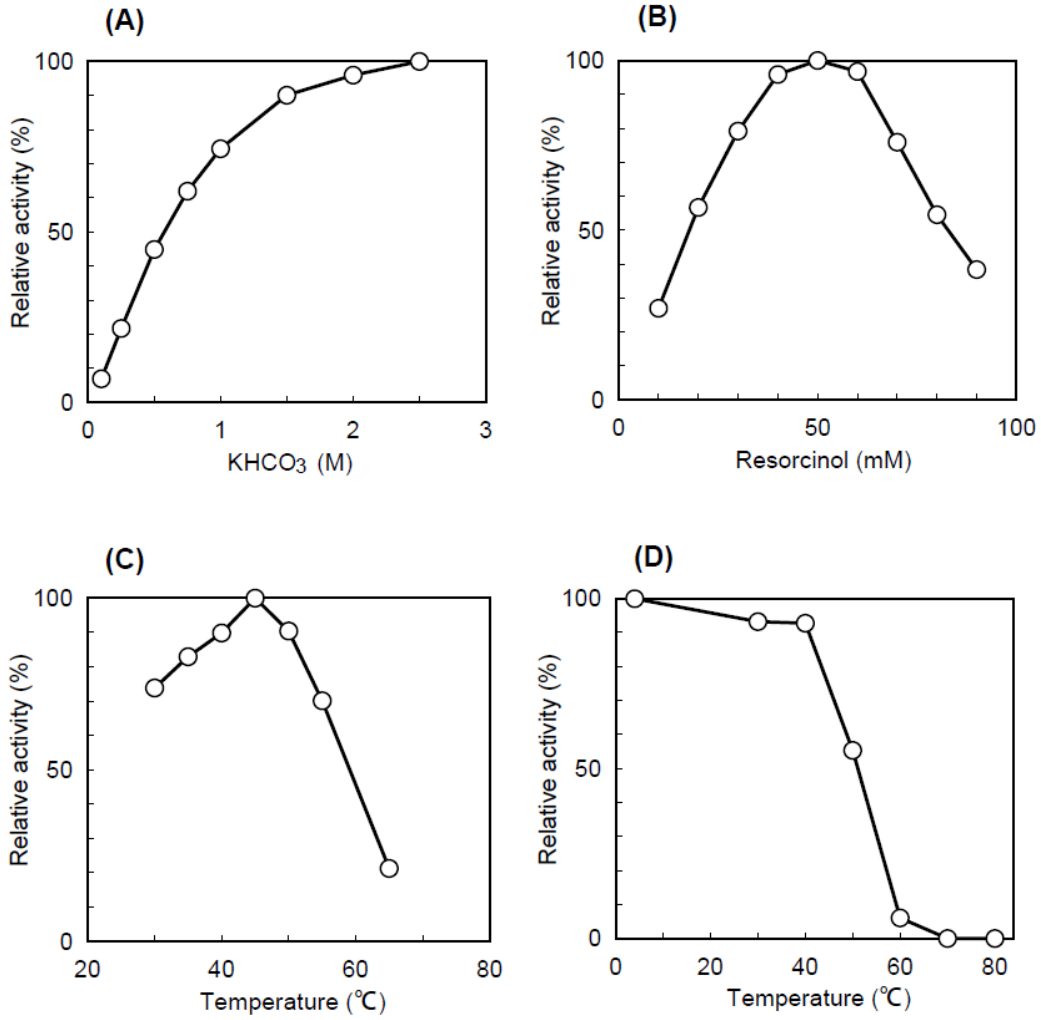

Fig. 5.5. Effects of substrate concentration and temperature on carboxylation activity. (A) Effect of $KHCO_3$ concentration on activity. Assays for measurement of activity were done under the standard condition (Chapter 5.2.8) with modification by the addition of different amounts of $KHCO_3$. (B) Effect of RE concentration on activity. Assays for measurement of activity were done under the standard condition with modification by the addition of different amounts of RE. (C) Effect of temperature on activity. Assays for measurement of activity were done under the standard condition. (D) Effect of temperature on stability. After the purified enzyme was preincubated at the indicated temperature for 30 min, the remaining activity was measured under the standard condition. The remaining activity was expressed relative to the activity, taken as 100%, when reaction mixture was not preincubated.

The effects of various metal ions and chemical reagents are shown in Table 5.2. The RE-carboxylation activity was inhibited by $AgNO_3$ (88% inhibition) and DEPC (38%). Addition of other metal ions tested and sulfhydryl group inhibitor to reaction

mixture, such as *p*-chloromercuribenzoic acid, *N*-ethylmaleimide, and iodoacetamide, had no or slight effect on enzyme activity.

The purified reversible γ-RA decarboxylase catalyzed the regioselective carboxylation of RE to stoichiometric amounts of γ-RA with a specific activity of 2.56 U/mg. The *K*m, *V*max and *k*cat values derived from Lineweaver-Burk plots with the enzyme at 50°C and pH 7.0 were 7.1 mM, 1.62 mM/min and 6.0×10^3 min^{-1}, respectively, for RE. The enzyme also catalyzed the regioselective carboxylation of catechol into stoichiometric amounts of 2,3-dihydroxybenzoic acid with a specific activity of 0.56 U/mg, not 3,4-dihydroxybenzoic acid. The enzyme did not catalyzed the carboxylation of phenol, hydroquinone, and 1,2,3-benzenetriol.

5.3.6. Effects of O_2 on Rdc

All of the already-known reversible decarboxylases were sensitive to O_2, and required the addition of reducing agents and/or anaerobic condition during the handling [3-6]. Since the enzyme activities of the purified Rdc were stable during the purification under the conditions in the presence of air containing O_2, there was some possibility that Rdc would be insensitive to O_2 for the reactions. Therefore, the effects of O_2 on the carboxylation activity were examined. As shown in Fig. 5.6, none of the effect on the activity was observed under the conditions substituted with N_2 or CO_2 atmosphere, compared with the standard condition in a shaking reaction without exchange of the gas in the head space, in other words without removal of O_2. This result indicates that the purified Rdc is insensitive to O_2 for reactions.

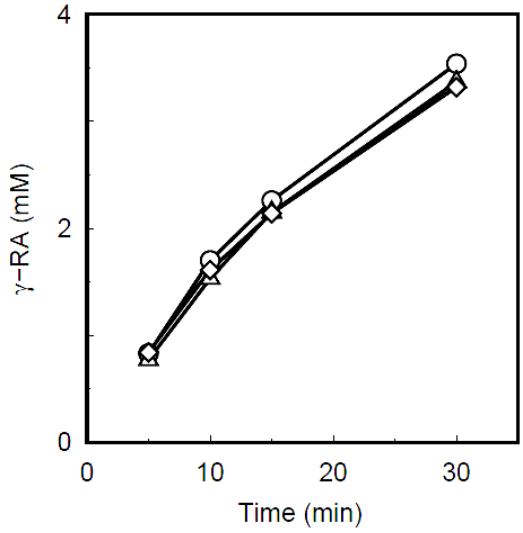

Fig. 5.6. Effects of oxygen on carboxylation activity of Rdc. Assays for measurement of activity were carried out under the standard condition (Chapter 5.2.8) modification by the exchange of the gas in the head space. Symbols: ○; CO_2, △; N_2, ◇; Air.

5.3.7. Cloning of *rdc* from *R. radiobacter* WU-0108

The first 22 residues of the N-terminal amino acid sequence of the purified γ-RA decarboxylase enzyme was determined to be Met-Gln-Gly-Lys-Val-Ala-Leu-Glu-Glu-His-Phe-Ala-Ile-Pro-Glu-Thr-Leu-Gln-Asp-Ser-Ala-Gly. The 22 amino acid sequence showed 100% identity to Atu2529 (NP533200), conserved hypothetical protein, of *Agrobacterium tumefaciens* C58 and 71% to the first 14 residues of 2,3-dihydroxybenzoic acid decarboxylase (DHBD, EC 4.1.1.46, AR224196 and P80402) of *Aspergillus niger* [9, 20, 21] and *A. oryzae* [22]. Based on the N-terminal amino acid sequence of the reversible γ-RA decarboxylase purified and nucleotide sequence of genome DNA data of *Agrobacterium tumefaciens* C58, primers were synthesized as follows, 5'-ATT GCT GGA CGG AAC TG-3' as the sense primer and 5'-AGC TTC ACC CAG GCA TT-3' as the anti-sense primer. PCR was carried out with these primers and total DNA of *R. radiobacter* WU-0108 as template. An approximately 600 bp fragment was amplified and cloned into pGEM-T easy vector. The amplified fragment was then labeled and used as a probe (designated as RDC-DIG

probe) to screen the partial DNA library of *R. radiobacter* WU-0108 by colony hybridization.

When total DNAs of *R. radiobacter* WU-0108, digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I and *Xba*I, were analyzed by Southern hybridization using the RDC-DIG probe, a *Pst*I fragment of about 8 kb, a *Sal*I fragment of about 3.5 kb, and *Bam*HI, *Eco*RI, *Hin*dIII and *Xba*I fragments of more than 20 kb in size each, were detected as hybridizable bands. The *Sal*I fragments of total DNA of *R. radiobacter* WU-0108 were used to construct a partial DNA library, which contained approximately 400 colonies. By colony hybridization using the RDC-DIG probe and the partial DNA library, two positive clones were obtained.

5.3.8. Nucleotide sequence analysis of *rdc*

Both of the insert fragments in plasmids of the positive clones designated pC1 and pC2 were approximately 3.4 kb in size and contained the same DNA region: one partial open reading frame (ORF1) and two complete ORFs (ORF2 and ORF3). These three ORFs were located in the same direction. ORF2 encodes a protein of 327 amino acid residues with a molecular weight of 37,430. The molecular size and the N-terminal amino acid sequence of the protein encoded by ORF2 were identical to those of the reversible γ-RA decarboxylase purified from *R. radiobacter* WU-0108. Moreover, since this gene product was conformed to exhibit reversible γ-RA decarboxylase activity as described later, ORF2 was designated *rdc* for reversible γ-RA decarboxylase of *R. radiobacter* WU-0108. The nucleotide sequence and deduced amino acid sequence of *rdc* show 92% and 96% identities to those of conserved hypothetical protein Atu2529(NP533200) of *A. tumefaciens* C58, respectively. The

deduced amino acid sequence of *rdc* shows 30% and 42 % identities to the DHBD (AR224196) of *A. niger* [9, 20, 21] and a 5- carboxyvanillate decarboxylase (LigW, AB033664) involved in the catabolism of the lignin-related biphenyl by *S. paucimobilis* SYK-6 [8], respectively. Moreover, deduced amino acid sequence of *rdc* shows 32-44 % identities to the hypothetical proteins such as P00305171 (32%), NP_442548 (35%), NP_879425 (43%), NP_886820 (44%), and NP_882627 (44%). On the other hand, as to the primary structure of reversible and nonoxidative aromatic decarboxylase, only 4-hydroxybenzoate decarboxylase (Ohb1, AAD50377) from *C. hydroxybenzoicum* JW/Z-1T was reported [3], but the deduced amino acid sequence of *rdc* shows no homology to that of Ohb1.

The deduced amino acid sequence of the partial ORF1, which was located at upstream of *rdc*, shows 96% identity to that of a putative oxidoreductase, Atu2530 (NP533201), of *A. tumefaciens* C58. The deduced amino acid sequence of the other complete ORF3, which was located downstream of *rdc*, shows 92% identity to that of a putative maleylacetate reductase, Atu2528 (NP533199), of *A. tumefaciens* C58 and shows low homology to those of putative maleylacetate reductase of other microorganisms (around 40% identity). The locus of these two ORFs in the insert fragment of the positive clones, pC1 and pC2, were identical to those of genome DNA data of *A. tumefaciens* C58

5.3.9. Overexpression of *rdc* in *E. coli*

The *rdc* gene was amplified by PCR and subcloning in pET21(+) to generate plasmid pENS10 (Chapter 5.2.14). The *rdc* gene was expressed in *E. coli* BL21(DE3) harboring plasmid pENS10 with the aid of its T7 promoter. SDS-PAGE analysis of

crude cell extracts from *E. coli* BL21(DE3) harboring pENS10 revealed the presence of single band of 34 kDa that did not appear in control extracts from BL21(DE3) harboring pET21d(+) (data not shown). RE-carboxylation activity of the cell-free extract of *E. coli* BL21(DE3) harboring pENS10 was 0.393 U/mg, indicating that the high level-expression of *rdc* was successfully done since the activity was 3.6 times higher than that of original strain *R. radiobacter* WU-0108.

5.3.10. Determination of active site residues of Rdc

Since both decarboxylation and carboxylation activities of Rdc were inhibited by DEPC as a histidine residue-specific inhibitor (Table 5.2), it was presumed that a histidine residue in Rdc might be one of the active site residues. Based on the alignment among nonoxidative decarboxylases, it was found that two histidine residues at position 164 and 218 might be the center of this enzyme (Figs. 5.7 and 5.8). Therefore, His-164 and His-218 were replaced with glutamine by site-directed mutagenesis. The resultant mutant proteins H164Q and H218Q were over-

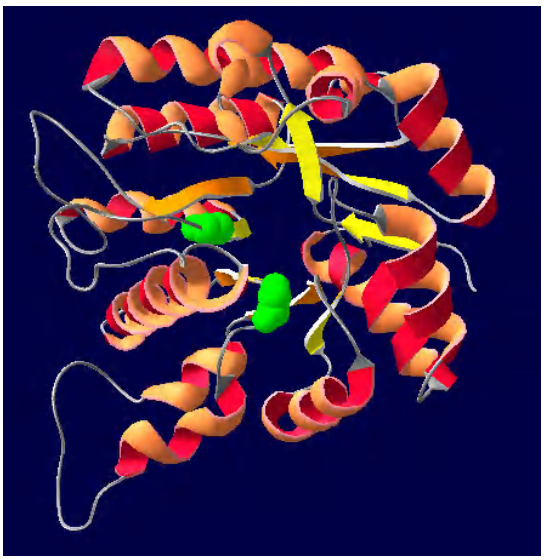

Fig. 5.7. 3D modeling analysis of Rdc by Deep view/Swiss-Pdb Viewer. Green regions indicate two histidine residues at position 164 and 218.

expressed as the soluble proteins and showed neither decarboxylation nor carboxylation activities, indicating that both of His-164 and His-218 of Rdc are essential for the catalytic activities of decarboxylase and carboxylase (Fig. 5.8).

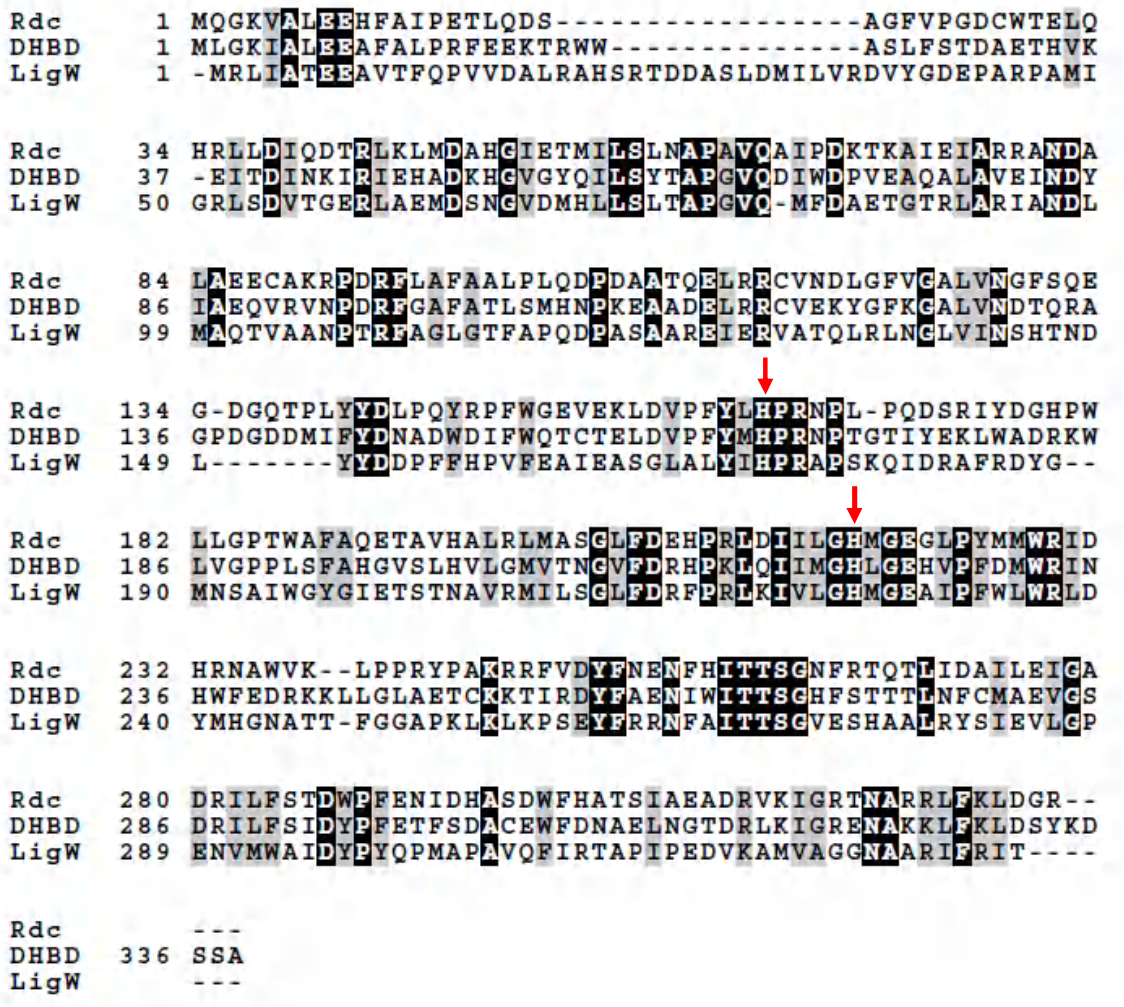

Fig. 5.8. Multiple sequence alignment of nonoxidative decarboxylases. Black shading indicates identical residues in all the members. Gray shading indicates identical residues in two members. Rdc, γ-resorcylic acid decarboxylase from *R. radiobacter* WU-0108 (accession number Q60FX6); DHBD, 2,3-dihydroxybenzoic acid decarboxylase from *A. niger* (accession number Q2USF4); LigW, 5-carboxyvanillate decarboxylase from *S. paucimobilis* SYK-6 (accession number Q8RJ47). Arrows indicate two histidine residues at position 164 and 218.

5.4. Discussion

With the objective to isolate and characterize a novel enzyme forming γ-RA (2,6-dihydroxybenzoic acid) from RE (1,3-dihydroxybenzene), one bacterial strain *Rhizobium radiobacter* WU-0108 was newly isolated from soil for its ability to utilize γ-RA as the sole source. Reversible conversion of γ-RA and RE was observed in whole cell suspensions and cell-free extracts of *R. radiobacter* WU-0108. The enzyme catalyzing the reversible conversion of γ-RA and RE from *R. radiobacter* WU-0108, reversible γ-RA decarboxylase (Rdc), was purified and characterized. As to the reversible and nonoxidative decarboxylase, 4-hydroxybenzoate decarboxylase and 3,4-hydroxybenzoate decarboxylase from *C. hydroxybenzoicum* JW/Z-1T [3, 4], pyrrole-2-carboxylate decarboxylase from *B. megaterium* PYR2910 [5], and indole-3-carboxylate decarboxylase from *A. nicotianae* FI1612 [6] have been previously reported. These already-known reversible decarboxylase were sensitive to O_2, but Rdc from *R. radiobacter* WU-0108 was insensitive to O_2. Moreover, under both conditions in the presence and absence of O_2, the carboxylation activities of Rdc were not affected, suggesting that the handling of the strain WU-0108 and the enzyme would be easy for industrial production of γ-RA. On the other hand, the substrate specificity of Rdc was strict as similar to the other already-known decarboxylases, and Rdc converted RE to γ-RA without by-product.

The gene, *rdc*, encoding the reversible γ-RA decarboxylase Rdc from *R. radiobacter* WU-0108 was cloned and expressed in *E. coli*. The locus of ORFs in the flanking region of *rdc* was identical to that of Atu2529, showing 92% identity to *rdc*, in *A. tumefaciens* C58. In the flanking region of Atu2529 in *A. tumefaciens* C58, the genes encoding putative benzoate transport protein and hydroxylquinol 1,2-dioxygenase

exist at upstream of Atu2530. The *rdc* gene and the ORFs in flanking region might be concerned with the degradation of aromatics. In the cultivation with rich medium such as LB medium, *R. radiobacter* WU-0108 showed the reversible reaction of RE and γ-RA with the addition of γ-RA to medium, but not without γ-RA. Moreover, several other strains of *R. radiobacter* (formerly *A. tumefaciens*) also showed both activities for RE-carboxylation and γ-RA-decarboxylation. These results indicated that the ability for reversible reaction of RE and γ-RA would be commonly conserved in the species of *R. radiobacter* for the degradation of aromatics. Although over-expression of the *rdc* gene in *E. coli* was detected on SDS-PAGE, the carboxylation activity of cell-free extracts of recombinant *E. coli* was 3.6 times higher than that of *R. radiobacter* WU-0108. This low enhanced rate of activity in *E. coli* would be due to high level expression of *rdc* with induction by aromatics such as γ-RA in *R. radiobacter* WU-0108.

The genes encoding the nonoxidative aromatics decarboxylase were cloned and characterized [8, 10, 23-29]. Based on the CDD (Conserved Domain Database) and COG (Clusters of Orthologous Groups) search, Rdc from *R. radiobacter* WU-0108 and the orthologous proteins such as LigW and DHBD show homologies to predicted metal-dependent hydrolase of the TIM-barrel fold protein (COG2159) and amidohydrolase (pfam04909). The reversible and nonoxidative 4-dihydroxynbenzoate decarboxylase (Ohb1) from *C. hydroxybenzoicum* belongs to 3-octaprenyl-4-hydroxybenzoate carboxy-lyase (UbiD) family (pfam01977) and contains the conserved sequence EGP[F/Y][G/V][D/E]XXGXY of the UbiD family [30]. The nonoxidative decarboxylase 4,5-dihydoxyphthalate decarboxylase, Pht5 from *Pseudomonas putida* [27], PhtC from *Arthrobacter keyseri* [28], and PhtD from

Pseudomonas testosterone [29], belong to TauA family (COG0715). The other nonoxidative aromatic acid decarboxylase such as ferulic acid decarboxylase Fdc from *Bacillus pumilus* [23], *p*- coumaric acid decarboxylase PdcC from *Lactobacillus plantarum* [24], phenolic acid decarboxylase from *B. subtilis* (BSPAD, AF017117), *B. pumilus* (BPPAD, AJ278683), *L. plantarum* (LPPAD, U63827), *Pediococcus pentosaceus* (PPPAD, AJ276891), and *Bacillus* sp. BP-7 (PadA, CAD37333) [25, 26] belong to PA decarbox family (pfam05870). Rdc from *R. radiobacter* WU-0108 and the orthologous proteins such as LigW and DHBD show no homology to the decarboxylases of the UbiD, aldolase or PA decarbox family. Moreover, they contain no similar sequence to EGP[F/Y][G/V][D/E]XXGXY and their molecular mass of subunit are approximately 30 kDa with different to approximately 20 kDa and 55 kDa of other nonoxidative decarboxylases. The subunit-molecular mass of the already-known reversible decarboxylase such as Ohb1, pyrrole-2-carboxylate decarboxylase, and indole-3-carboxylate decarboxylase were approximately 55 kDa. These facts suggest that Rdc would be novel reversible nonoxidative decarboxylase.

It was reported that the presence of histidine, tryptophan and cysteine residues at the active site of DHBD from *A. niger* [20]. Moreover, it was reported that the active site of DHBD was cysteine residue of LLGAETCK in the positions of 244 to 252 aa [21]. Although Rdc from *R. radiobacter* WU-0108 has a similar sequence of LAEECA in the positions of 84 to 94 aa, the position of these residues are different and Rdc was not affected by sulfhydryl-inhibitor. On the other hand, Rdc was affected by DEPC as a histidine-inhibitor. The multiple alignment of Rdc and the orthologous proteins including hypothetical proteins indicated that two histidine residues were conserved but not any cysteine residues. The site-direct mutagenesis of Rdc suggests

that the conserved two histidine residues at positions of 164 and 218 are concerned to both activities of RE-carboxylation and γ-RA-decarboxylation as active site residues. The already-known reversible decarboxylase such as Ohb1, pyrrole-2-carboxylate decarboxylase, and indole-3-carboxylate decarboxylase were affected by sulfhydryl-inhibitors and sensitive to O_2. Therefore, the O_2-insensitivity of Rdc must be concerned to the situation that two histidine residues but not cysteine residue are active sites of Rdc.

In conclusion, the reversible and nonoxidative γ-RA decarboxylase (Rdc) that catalyzes the regioselective carboxylation of resorcinol to form γ-resorcylic acid was found in *Rhizobium radiobacter* WU-0108. The author succeeded in the molecular characterization of this novel enzyme, including its purification, characterization, and gene-cloning.

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Chapter 6

Application of Recombinant *Escherichia coli* Cells Overexpressing *rdc* to γ-Resorcylic Acid Production

6.1. Introduction

γ-Resorcylic acid (2,6-dihydroxybenzoic acid, γ-RA, see Fig. 5.1) is used as an intermediate for the production of medicines, herbicides, and industrial chemicals. γ-RA is produced industrially from resorcinol (1,3-dihydroxybenzene, RE, see Fig. 5.1) by organic synthesis, according to the Kolbe-Schmitt reaction, a carboxylation reaction at high pressure and high temperature [1]. However, in such production, a large amount of β-RA (2,4-dihydroxybenzoic acid) is also generated as a by-product, and a process for separating β- and γ- RAs is then required [2].

Several decarboxylases reversibly catalyzing the carboxylation of phenolic and cyclic organic compounds have been reported [3-6]. The author and collaborators found a γ-RA decarboxylase (Rdc) from *Rhizobium radiobacter* WU-0108, which reversibly catalyzes the regioselective carboxylation of RE leading to the synthesis of γ-RA without by-products (Fig. 5.1), and described the molecular characterization of this novel enzyme Rdc, including its purification, characterization and gene cloning (Chapter 5). Rdc has some unique properties; for example, although decarboxylases reversibly catalyzing carboxylation show decreased activities in the presence of O_2 and require cofactors, such as NAD(P)H and pyridoxal 5'-phosphate [3-6], Rdc shows a constant activity regardless of the presence or absence of O_2 and requires no cofactor

(Chapter 5.3.6), suggesting the effectiveness of handling Rdc for practical γ-RA production from RE. Recently, other γ-RA decarboxylases reversibly catalyzing carboxylation with properties similar to those of Rdc reported by other researchers [7, 8].

In this chapter, the author describes the enzymatic production of γ-RA using recombinant *Escherichia coli* cells expressing the gene (*rdc*) encoding Rdc with the aim of developing a novel method of selectively producing γ-RA from RE under environmentally benign conditions compared with the conditions needed for the Kolbe-Schmitt reaction. A recombinant *E. coli* expressing *rdc* converted 20 mM resorcinol to 8.8 mM γ-resorcylic acid with a 44% (mol/mol) yield at 30°C for 7 h.

6.2. Materials and methods

6.2.1. Chemicals

Authentic γ-RA and RE (Tokyo Kasei Kogyo) used in this study were purchased. All the other chemicals used were commercially available and of chemically pure grade.

6.2.2. Cultivation of recombinant *E. coli* overexpressing *rdc*

E. coli BL21 (DE3)/pENS10 cells (Chapter 5.2.14) were cultivated at 30°C in 500 ml Erlenmeyer flasks containing 200 ml Luria-Bertani (LB) medium (Chapter 2.4.4) supplemented with 100 μg ampicillin/ml and 0.2 mM IPTG as an inducer of the T7 promoter with reciprocal shaking at 120 strokes/min.

6.2.3. Resting cell reaction

The cells cultivated were harvested by centrifugation at 6,000 × g for 10 min at 4°C, and washed twice with 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.0). Unless otherwise indicated, the cultivation was carried out for 18 h. The washed cells were used for the resting cell reaction for γ-RA production from RE. The standard reaction conditions were as follows. A reaction mixture containing *E. coli* BL21 (DE3)/pENS10 cells (26 g dry-cells/l), 5 mM RE, and 150 mM $KHCO_3$ in 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.0) in 1 ml in a 1.5-ml microcentrifuge tube was incubated at 30°C with reciprocal shaking at 120 strokes/min. Unless otherwise indicated, the incubation was performed for 16 h. The reaction was stopped by adding 10 μl 12 M HCl, the mixture was then centrifuged at 6,000 × g for 10 min at 4°C and the supernatant filtered using a 0.2-μm PTFE membrane.

6.2.4. Analytical methods

Cell growth was measured turbidimetrically at 660 nm. γ-RA and RE in the supernatant were determined by HPLC, as described previously (Chapter 2.14.2).

6.3. Results

6.3.1. Reaction conditions of γ-resorcylic acid production

To optimize the conditions for the resting cell reaction using the recombinant *E. coli* cells, the author examined the reaction conditions by changing the standard temperature, $KHCO_3$ concentration, and reaction time described above. The optimal conditions were: 30°C, a $KHCO_3$ at 3 M (saturated concentration), and a reaction time of 16 h. As shown in Fig. 6.1, 8.8 mM γ-RA was produced from 20 mM RE with a

maximum yield of 44% (mol/mol). On the other hand, 26 mM γ-RA of maximum concentration was produced from 70 mM RE. The initial RE concentration was therefore fixed at 20 mM as the optimal concentration.

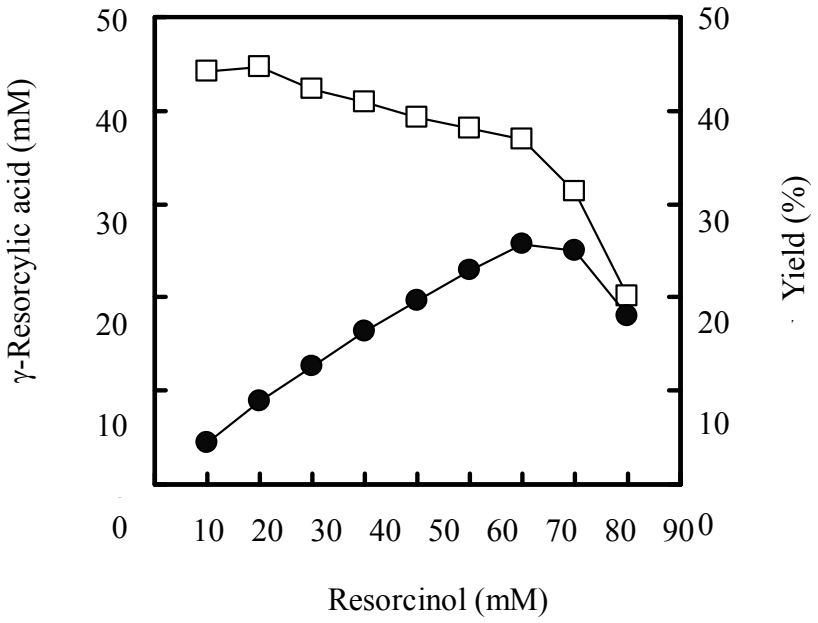

Fig. 6.1. Effects of initial RE concentration on γ-RA production by resting cell reaction using *E. coli* BL21 (DE3)/pENS10 cells (26 g dry-cells/l). Experiments were carried out under standard conditions at various RE concentrations. Symbols**;** closed circle, γ-RA; open square, γ-RA yield based on the amount of RE supplied.

6.3.2. Biosynthesis of γ-resorcylic acid by resting cell reaction

The time course of γ-RA production by the resting cell reaction using the recombinant *E. coli* cells is shown in Fig. 6.2. The concentration of γ-RA reached its maximum, 8.8 mM, at 7 h and thereafter, remained constant. Under these conditions, γ-RA was the only product in the reaction mixture. These results indicate that recombinant *E. coli* cells expressing *rdc* are applicable as efficient and convenient

biocatalysts to the selective production of γ-RA from RE.

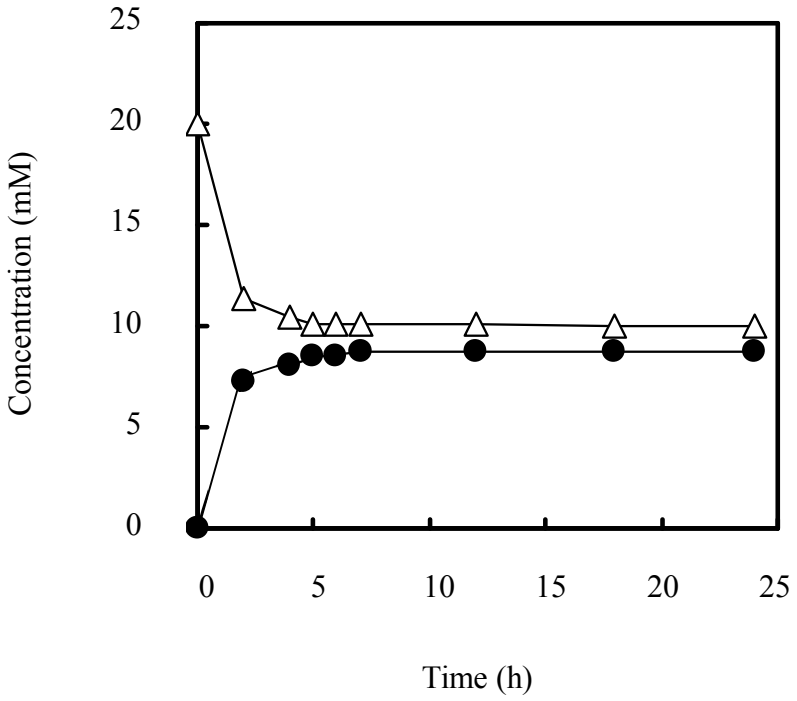

Fig. 6.2. γ-RA production by resting cell reaction using recombinant *E. coli* BL21 (DE3)/pENS10 under optimal conditions. Symbols**;** closed circle, γ-RA; open triangle, RE.

6.4. Discussion

In this chapter, the author described the enzymatic production of γ-RA using recombinant *E. coli* cells expressing *rdc* with the aim of developing a novel method of selectively producing γ-RA from RE under environmentally benign conditions compared with the conditions needed for the Kolbe-Schmitt reaction. A recombinant *E. coli* expressing *rdc* converted 20 mM resorcinol to 8.8 mM γ-resorcylic acid with a 44% (mol/mol) yield at 30°C for 7 h.

After 7 h, the recombinant *E. coli* cells were separated from the reaction mixture

by centrifugation at 6,000 × g for 30 min at 4°C and were recyclable at least five times for use as biocatalysts for the selective production of γ-RA from RE: in each reaction, 8.8 mM γ-RA was produced from 20 mM RE over 7h. Because the recombinant *E. coli* cells maintained a stable Rdc activity for a long time (more than 24 h), this stable Rdc activity is favorable for practical γ-RA production.

The author also examined whether the resting cell reaction using recombinant *E. coli* cells is applicable to the carboxylation of other phenolic compounds. By performing the 16-h reaction under the same conditions described in Fig. 6.1 and 6.2, the author confirmed the production of 4-aminosalicylic acid from 20 mM 3-aminophenol with a yield of 7.8% and that of 2,3-dihydroxybenzoic acid from 20 mM catechol with a yield of 8.5%.

In conclusion, the author showed that enzymatic γ-RA production can be achieved using *E. coli* cells expressing *rdc* as biocatalysts under environmentally benign conditions compared with the conditions for the conventional organic synthesis, such as that using the Kolbe-Schmitt reaction. Therefore, the author considers that the method described here is a significant model process for the selective and ecological carboxylation of phenolic compounds.

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Chapter 7

Cloning and Functional Analysis of the Reversible Salicylic Acid Decarboxylase Gene (*sdc*) from *Trichosporon moniliiforme* WU-0401

7.1. Introduction

Aromatic carboxylic acids such as salicylic acid, 4-hydroxybenzoic acid, 4-aminosalicylic acid, and γ-resorcylic acid are used as intermediates of medicines, herbicides, and industrial chemicals. Today, these compounds are produced industrially from aromatics by the organic synthesis, according to the Kolbe-Schmitt reaction, a carboxylation reaction at high pressure and high temperature [1]. However, in such production, a large amount of by-products are also generated, and a process for separating by-products is then required [1].

In contrast, the enzymatic carboxylation of aromatics has been focused because selective and ecological carboxylation of phenolic compounds might be possible under environmentally benign conditions compared with those for the Kolbe-Schmitt reaction. Several enzymes catalyzing carboxylation of aromatics into aromatic carboxylic acids have been found in biological metabolisms [4-16]. However, in spite of the fact that salicylic acid is the well-known aromatic carboxylic acid used for a precursor of acetylsalicylic acid and that it is widely used as a nonsteroidal anti-inflammatory and known to be the analgesic aspirin [2], there has been no report on the enzyme catalyzing

the carboxylation of phenol to form salicylic acid.

Carboxylation and decarboxylation of aromatics are central metabolic processes of microorganisms and some of them are catalyzed by a broad spectrum of aromatics decarboxylases. Aromatics decarboxylases are generally oxidative and depend on metal ions and cofactors such as thiamin pyrophosphate, pyridoxal-5'-phosphate, and biotin with or without ATP [3]. On the other hand, nonoxidative aromatics decarboxylases do not use either molecular O_2 or cofactors for decarboxylation activities [4-16], and some of them have the unique ability of reversibly catalyzing the regioselective carboxylation [5-7, 9, 11, 15]. One of the properties of these reversible and nonoxidative aromatics decarboxylases is that their reactions are generally sensitive to O_2, and both decarboxylation and carboxylation activities are decreased in the presence of O_2 [5-7, 15]. However, in chapters 5 and 6, reversible and nonoxidative γ-resorcylic acid decarboxylase (Rdc) from *R. radiobacter* WU-0108 shows a constant activity regardless of the presence or absence of O_2 and requires no cofactors for its activities. Because the enzymatic regioselective carboxylation of resorcinol to form γ-resorcylic acid is carried out by only mixing Rdc, resorcinol, and CO_2 source in aqueous phase, the enzymatic carboxylation using reversible and nonoxidative decarboxylases is considered to be easily applicable to practical production of aromatic carboxylic acids.

In this chapter, the author describes the molecular characterization of the reversible and nonoxidative salicylic acid decarboxylase from *Trichosporon moniliiforme* WU-0401, including its purification, characterization, and gene-cloning. For selective synthesis of salicylic acid from phenol, microorganisms possessing a novel enzyme catalyzing regioselective carboxylation of phenol to form salicylic acid were

screened from 3000 soil samples. The nonoxidative salicylic acid decarboxylase (Sdc) that reversibly catalyzes the regioselective carboxylation of phenol to form salicylic acid (Fig. 7.1) was found from *T. moniliiforme* WU-0401. The gene (*sdc*) encoding Sdc was cloned and heterologously expressed in *Escherichia coli* cells. Therefore, this is the first report describing the enzyme that catalyzes the regioselective carboxylation of phenol to form salicylic acid.

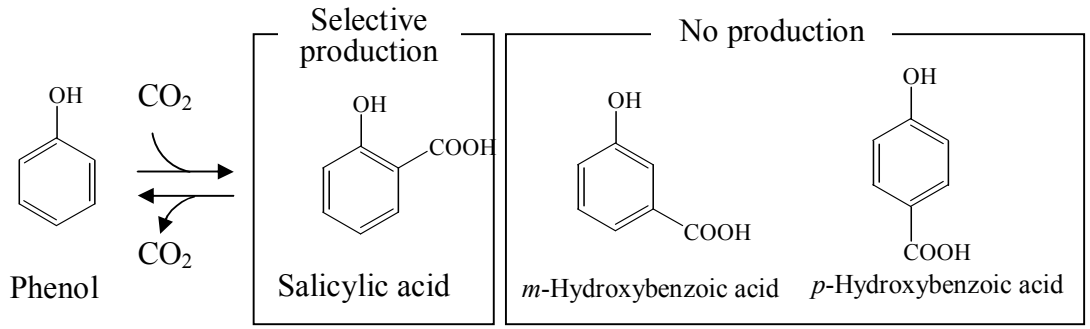

Fig. 7.1. Enzymatic reversible conversion of salicylic acid and phenol.

7.2. Materials and methods

7.2.1. Chemicals

Salicylic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, catechol, and phenol were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All the other chemicals used were commercially available and of chemically pure grade.

7.2.2. Bacterial strains, plasmids, and cultivation

T. moniliiforme WU-0401 was used as the source of total DNAs for cloning of the gene (*sdc*) encoding the reversible and nonoxidative salicylic acid decarboxylase (Sdc). For preparation of total DNAs, WU-0401 was cultivated at 30˚C in test tubes (18 by 180

mm) containing 5 ml of SA media (Chapter 2.4.3). *E. coli* JM109 was cultivated in Luria-Bertani (LB) medium (Chapter 2.4.4) supplemented with 50 μg ampicillin/ml. *E. coli* BL21 (DE3) and pET21-d were used for over-expression of *sdc*. Recombinant *E. coli* BL21 (DE3) carrying a recombinant plasmid derived from pET21-d was cultivated at 30°C in 500 ml Erlenmeyer-flasks containing 100 ml LB medium supplemented with 100 μg ampicillin/ml and 0.1 mM isopropyl-β-D-thiogalactopyranoside with reciprocal shaking at 120 strokes/min.

7.2.3. Resting cell reaction

T. moniliiforme WU-0401 was cultivated for 48 h at 30°C in 500 ml Erlenmeyer flasks containing 100 ml SA medium with reciprocal shaking at 120 strokes/min (Chapter 2.4.3). After cultivation, cells were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C, washed twice with 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.0), and suspended with 10 ml of the same buffer. Then, 50 μl of 0.5 M phenol solution was added to 1 ml of the cell suspension containing 150 mM $KHCO_3$ (pH 9.6) in a 2-ml microcentrifuge tube. The reaction was allowed to proceed for 24 h at 30°C, and 50 μl of 12 M HCl was added to stop the reaction. The mixture was then centrifuged at 6,000 × g for 10 min at 4°C and the supernatant filtered using a 0.2-μm PTFE membrane. The amount of substrates and reaction products were measured by HPLC according to Chapter 2.14.2.

7.2.4. Preparation and purification of reaction product from phenol

For preparation of the reaction products from phenol, 376 mg of phenol was added to 100 ml of a resting cell suspension (OD_{600} 40) in 500 ml Erlenmeyer flasks.

The mixture was incubated with shaking for 24 h at 30°C, and then extracted with 200 ml of ethylacetate. The resulting extract was concentrated on a rotary evaporation equipment and applied to a silica column chromatograph packed with Wakogel C-200 (Wako Pure Chemical, Osaka, Japan). The elution was carried out with methanol-chloroform-ammonia (5 : 13 : 0.8, v/v/v). The fraction including product was concentrated by rotary evaporation, and then lyophilized and subjected to NMR analysis as described previously [17].

7.2.5. NMR analysis

^{13}C-NMR, ^{1}H-NMR, and heteronuclear multiple bond coherence spectra were obtained using a JEOL JNM-LA 500 spectrometer (JEOL, Tokyo) operated at 150 MHz (13C-NMR) and 600 MHz (others) with sodium 2, 2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard.

7.2.6. Preparation of cell-free extract of *T. moniliiforme* WU-0401

As for the basal buffer, 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.0) was used. *T. moniliiforme* WU-0401 was cultivated for 48 h at 30°C in 500-ml Erlenmeyer flasks containing 200 ml of SA medium with reciprocal shaking at 120 strokes/min. The cells in the 200-ml culture broth were harvested by centrifugation at 10,000 × g for 20 min at 4°C, then washed twice with the basal buffer (pH 7.0). The washed cells were frozen in liquid N_2 and ground into a fine powder using a mortar and pestle. The powdered cells were suspended in 10 ml of the basal buffer (pH 7.0). Cell debris were removed by centrifugation at 16,000 × g for 30 min at 4°C. The resulting supernatant was used as the cell-free extract. The resulting supernatant was used as

the cell-free extracts to further purification through the following 4 steps by the AKTA system (Amersham Biosciences, NJ, USA).

7.2.7. Purification of a novel salicylic acid decarboxylase (Sdc)

All purification procedures were carried out at 4°C or on ice. The cell-free extracts were applied to a Toyopearl-DEAE 650S column (Tohso, Tokyo, Japan) equilibrated with 25 mM Tris-HCl buffer (pH 8.0) and washed with 90 ml of the same buffer. Elution was carried out with a continuous linear gradient of 0 to 1.0 M KCl in 150 ml of the same buffer at a flow rate of 2 ml/min. The active fractions (0.14 to 0.15 M KCl) showing decarboxylation of salicylic acid to form phenol were collected, and applied to a Q-sepharose FF column (GE Healthcare, Tokyo, Japan) equilibrated with 25 mM Tris-HCl buffer (pH 8.0) and washed with 90 ml of the same buffer. Elution was carried out with a continuous linear gradient of 0 to 1.0 M KCl in 150 ml of the same buffer at a flow rate of 2 ml/min. The active fractions (0.22 to 0.26 M KCl) were concentrated by ultrafiltration, and applied to a Superdex 200 HR 10/30 column (GE Healthcare, Tokyo, Japan) equilibrated with 25 mM Tris-HCl buffer (pH 8.0) and washed with 90 ml of the same buffer. Elution was carried out with same buffer at a flow rate of 0.5 ml/min. The active fractions (fractions containing proteins with the molecular mass of approximately 140 kDa) were collected for applying to a Bio-Scale CHT-1 column (GE Healthcare, Tokyo, Japan) equilibrated with 10 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.0) and washed with 90 ml of the same buffer. Elution was carried out with a continuous linear gradient of 0 to 500 mM K_2HPO_4-KH_2PO_4 in 150 ml of the same buffer at a flow rate of 2 ml/min. The active fractions (0.09 to 0.10 mM K_2HPO_4-KH_2PO_4) were collected, and then concentrated by ultrafiltration.

7.2.8. Enzyme assays

For measurement of decarboxylase activity, the standard reaction conditions were as follows: the mixture contained 10 μg of protein and 30 mM salicylic acid in 100 mM MES-NaOH buffer (pH5.5) to a final volume of 1 ml in a 1.5-ml microcentrifuge tube. The reaction was started by the addition of salicylic acid, and the mixture was incubated with shaking at 40°C for 1 h. For measurement of carboxylase activity, the standard reaction conditions were as follows: the mixture contained 100 μg of protein, 20 mM phenol, and 2.5 M $KHCO_3$ in 100 mM K_2HPO_4-KH_2PO_4 buffer (pH7.0) to a final volume of 1 ml in a 1.5-ml microcentrifuge tube. The reaction was started by the addition of phenol, and the mixture was incubated with shaking at 30°C for 1 h. Both of the reactions, decarboxylation and carboxylation, were stopped by heating at 60°C for 1h. When other substrates were used instead of salicylic acid or phenol, other assay conditions were the same as those of standard conditions if otherwise indicated. When reactions were done in the absent of O_2, 15 ml screw cap test tube was used, and the head space of the test tube was substituted with Ar gas. Other variations of the assay conditions are indicated in the figure legends and tables. The amounts of salicylic acid, phenol, and other substrates were determined by using HPLC according to Chapter 2.14.2. One unit (U) of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of product per minute.

7.2.9. Protein analysis

The molecular mass was determined by the AKTA system (GE Healthcare, Tokyo, Japan) with a Superdex 200 HR 10/30 column at a flow rate of 0.5 ml/min by using 25

mM Tris-HCl buffer (pH 8.0) as the eluent. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out using 12% polyacrylamide by the method of Laemmli [18]. The partial amino acid sequence of the reversible salicylic acid decarboxylase was determined by Shimadzu Biotech (Ibaraki, Japan). Protein concentrations were measured by the method of Bradford [19] by using the Coomassie Protein Assay Kit (Pieace Co., IL, USA) with bovine serum albumin as the standard.

7.2.10. Recombinant DNA techniques

Recombinant DNA techniques were basically carried out as described in Chapter 2, and by Sambrook et al. [20] or according to the previous report [21].

7.2.11. Reverse transcriptase-polymerase chain reaction (RT-PCR)

According to manufacturer's protocol, total RNA was isolated from powdered cells of *T. moniliiforme* WU-0401 (Chapter 7.2.6), using Sepasol-RNA I Super (Nacalai tesque, Kyoto, Japan) followed by Cloned DNase I (Takara, Shiga, Japan) treatment with Cloned RNase Inhibitor (Takara, Shiga, Japan).

Approximately 0.1 μg of total RNA from *T. moniliiforme* WU-0401 was used as templates for reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR was carried out using ReverTra-Plus-^{TM} kit (TOYOBO, Osaka, Japan) according to manufacturer's protocol and a library of cDNAs was constructed. The primers used for RT-PCR were designed from the partial amino acid sequences of Sdc as follows: 5'-CTC TAC ATC GCC CCC AA-3' as the sense primer and 5'-GTA GAT GGT GTA GCC GAT GCC-3' as the anti-sense primer. Amplified fragment of approximately 100

bp was sequenced using a DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Tokyo, Japan) and a ABI Prism310 (Applied Biosystems, CA, USA).

7.2.12. 5' and 3' RACE

The primers used for 5' and 3' RACE were designed from the amplified fragments thorough RT-PCR as follows: 5'- GAG ATT CTC AAC CCG TGC GGC AA -3' as the sense primer and 5'- TTG CCG CAC GGG TTG AGA ATC TC -3' as the anti-sense primer. Total RNA from *T. moniliiforme* WU-0401 was converted into mRNA using a Poly(A)$^+$ Isolation Kit (NIPPON GENE, Tokyo, Japan) according to manufacturer's protocol, and mRNA generated was used as templates for 5' and 3' RACE. Rapid amplification of 5' and 3'cDNA ends were generated by using a BD SMART^{TM} RACE cDNA Amplification Kit (Takara, Shiga, Japan) according to manufacturer's protocol. Two amplified fragments containing a complete open reading frame (ORF1), approximately 400 bp from 5' RACE and 1000 bp from 3' RACE, were sequenced.

7.2.13. Expression of *sdc* in *E. coli*

The gene (*sdc*) encoding reversible salicylic acid decarboxylase of *T. moniliiforme* WU-0401 was subcloned and expressed under control of T7 promoter. Based on the nucleotide sequence of ORF1 encoding the salicylic acid decarboxylase (details are described later), the primers used for amplification of *sdc* were designed as follows: 5'-TTA AAA CAC ATC CAT CCA TAT GCG-3' [the *Nde*I restriction site is underlined] as the sense primer and 5'-TTC ATT ACT AAG CTT CTA AGC CTC CGA G-3' [the *Hind*III restriction site is underlined] as the anti-sense primer. The gene *sdc*

was amplified by PCR from the cDNA of *T. moniliiforme* WU-0401. Amplified DNA fragments were digested with *Nde*I and *Hind*III, separated by agarose gel electrophoresis, inserted into pET21-d (Novagen, WI, USA). The resulting recombinant plasmid (pSDC) was amplified in *E. coli* JM109 cells, and the insert was sequenced to ensure that the correct construction had been obtained. The plasmid pSDC was then introduced into *E. coli* BL21 (DE3) for expression of the *sdc* gene. Recombinant *E. coli* BL21 (DE3) carrying pSDC was cultivated at 30°C in 500 ml Erlenmeyer-flasks containing 100 ml LB medium supplemented with 100 μg ampicillin/ml and 0.1 mM isopropyl-β-D-thiogalactopyranoside with reciprocal shaking at 120 strokes/min. After cultivation for 18 h, cells were harvested by centrifugation at 6,000 × g for 10 min at 4°C, and then cell-free extract was prepared (Chapter 5.2.6).

7.2.14. Gibbs assay

Phenolic compounds such as phenol and salicylic acid in the reaction mixtures were visually detected by Gibbs assay, which was carried out by the addition of 30 μl of 1.0 M $NaHCO_3$ solution and 20 μl of 5.0 mM Gibbs reagent dissolved in ethanol solution into 50 μl reaction mixtures. Gibbs assay was observed to produce a blue mixture with phenol, light blue with salicylate or blown with catechol, as shown in Fig. 2.1.

7.3. Results

7.3.1. Identification of salicylic acid degrading yeast, WU-0401

Small amounts of 3000 soil samples collected from many areas of Japan were

suspended in 1 ml distilled water and centrifuged at 1500 × g for 5 min at 4°C to remove solid components. The resulting supernatant was appropriately diluted with distilled water, and aliquots of 50 µl were inoculated into test tubes containing 5 ml of SA liquid medium and cultivated at 30°C for 6 to 8 days. Aliquots of 50 µl of some turbid cultures showing the value more than 0.5 of optical density at 660 nm (OD_{660}) were then transferred into 5 ml of fresh SA liquid medium. After four subcultivations, to confirm whether phenol was accumulated, the culture filtrate was analyzed by Gibbs assay and HPLC. Among the 1000 turbid cultures, Gibbs assay (Chapters 2.15 and 7.2.14) revealed that phenol was accumulated in two cultures from independent soil samples. The culture was appropriately diluted with distilled water and spread onto agar plates of SA medium. After cultivation at 30°C for 3 to 5 days, each single colony formed on the plates was isolated and inoculated again into liquid SA medium. By repeated cultivation in liquid SA medium and single-colony isolation on SA medium agar plates, strains showing stable growth more than OD_{660} 1.0 and accumulation of phenol in liquid SA medium were selected. Among the strains, two microorganisms designated strains WU-0401 and WU-0501were selected for further studies. Through the comparative experiments, strain WU-0401 showed a higher degradation yield for salicylic acid than strain WU-0501 (details not shown). Therefore, strain WU-0401 was mainly used in this study.

Strain WU-0401 cells were observed as budding yeast cells forming wet colonies on YM medium agar plates. This strain formed true hyphae and arthroconidia but not pseudohyphae and sexual reproductive organs. Further taxonomical identification of strain WU-0401 was performed by Techno Suruga Co., Ltd. (Shizuoka, Japan), and the 28S ribosomal DNA sequence of strain WU-0401 was found to show 100% identity to

that of *Trichosporon moniliiforme*. From these results, strain WU-0401 was identified as *T. moniliiforme* (Fig. 7.2). Similarly, strain WU-0501 was also identified as *T. moniliiforme*. Since both of the strains WU-0401 and WU-0501 were identified as *T. moniliiforme*, it was confirmed whether salicylic acid degradation activity would be detected in strains closely related to the species of *T. moniliiforme*, namely, *T. moniliiforme* NBRC 1527, *T. cutaneum* NBRC 1198^{T}, and *T. asteroides* NBRC 0173. Because all three strains could grow in SA medium, resting cells of these three strains were prepared to examine whether phenol would be produced through the degradation of salicylic acid. Activities of salicylic acid degradation and phenol production were detected in all the three strains (data not shown). Therefore, these results indicate that the metabolic pathway for salicylic acid degradation via phenol might be widely distributed in strains closely related to the species of *T. moniliiforme*.

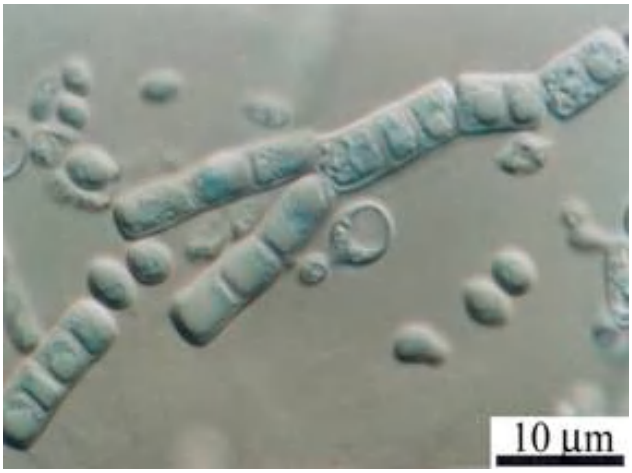

Fig. 7.2. Electron micrographs of a *T. moniliiforme* WU-0401 cells. Scale bar, 10 μm.

7.3.2. Carboxylation of phenol to form salicylic acid by resting cell reaction

The TLC analysis revealed that only one product which shows Rf value of 0.074 on TLC identical to that of authentic salicylic acid was detected by a resting cell

reaction of strain WU-0401 mixing with phenol and $KHCO_3$. The product was extracted from the reaction mixture, purified by silica gel column chromatography, and subjected to NMR analysis. Ten mg product was obtained from 376 mg phenol in the reaction mixture, with the molar conversion yield of 1.8% (mol/mol). The two-dimensional HMBC spectrum indicated that the product isolated was identified as salicylic acid (Fig. 7.3). Resting cells of strain WU-0401 cultivated in LB medium did not show degradation and synthesis of salicylic acid. On the other hand, a resting cell of strain WU-0401 cultivated in LB medium supplemented with 20 mM salicylic acid showed either reaction (data not shown). These results suggested that enzymes responsible for degradation and synthesis of salicylic acid might be inducible. Salicylic acid synthesis from phenol with $KHCO_3$ was detected when a cell-free extract of strain WU-0401 was used instead of a resting cell of strain WU-0401. Moreover, the three strains closely related to the species of *T. moniliiforme* also showed salicylic acid synthesis from phenol with $KHCO_3$ (data not shown).

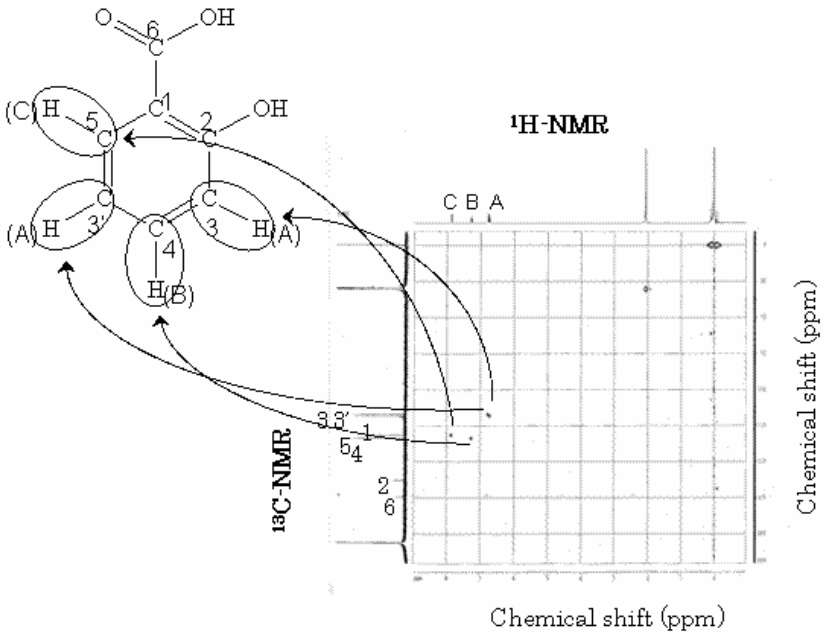

Fig. 7.3. ^{1}H-^{13}C shift correlation NMR spectrum of the product from phenol.

7.3.3. Purification of a novel salicylic acid decarboxylase (Sdc)

Based on the decarboxylase activity converting salicylic acid into phenol, the salicylic acid decarboxylase was purified to homogeneity from cell-free extracts of *T. moniliiforme* WU-0401 (Chapter 7.2.7). The salicylic acid decarboxylase was purified 20.8-fold with a yield of 5.5%, and the specific activity of the purified enzyme for salicylic acid decarboxylation into phenol was 0.47 U/mg (Table 7.1). The purified enzyme produced a 40 kDa single band on the gel by SDS-PAGE (data not shown). The native molecular mass of the enzyme was found to be 140 kDa by gel filtration (data not shown), suggesting that the enzyme has a homotetrameric structure.

Table 7.1 Purification of the reversible salicylic acid decarboxylase from *T. moniliiforme* WU-0401

Step	Total protein (mg)	Specific activity (U/mg)	Total activity (U)[a]	Purification (fold)	Yield (%)
Cell-free extract	18.9	0.023	0.43	1.00	100
TOYOPEARL DEAE	1.79	0.069	0.12	3.05	29
Q-Sepharose FF	0.80	0.13	0.11	5.89	25
Superdex 200	0.35	0.23	0.082	10.4	19
Bio-scale CHT-1	0.050	0.47	0.023	20.8	5.5

[a] U, One unit (Unit) was defined as the amount of enzyme that synthesizes one μmol of phenol per minute from phenol.

7.3.4. Properties on decarboxylation of Sdc

The optimal pH and optimal temperature for decarboxylation activity were 5.5 and 40°C, respectively (Fig. 7.4). The enzyme was stable up to 40°C and retained 40% of its activity after the treatment of heating at 50°C for 1 h. The effects of various metal ions and chemical reagents were investigated (Table 7.2). The enzyme activity was inhibited by $AgNO_3$ (100% inhibition), $HgCl_2$ (100%), *p*-chloromercuribenzoic

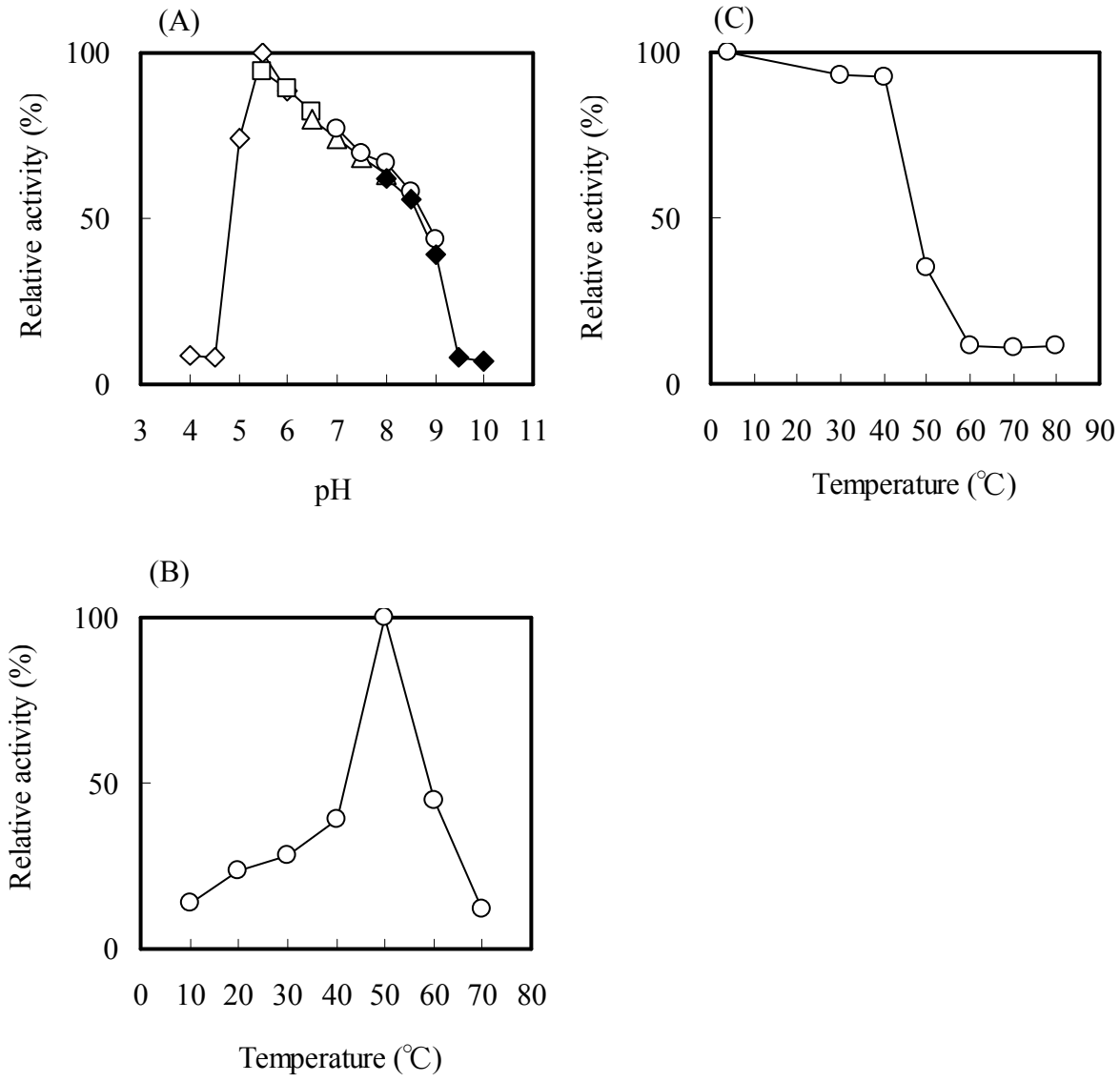

Fig. 7.4. Effects of pH and temperature on decarboxylation activity. (A) Effect of pH on activity. Assays for measurement of activity were done under the standard condition as described in Materials and Methods with modification of buffer. The following buffers (100 mM) were used: ◇; Citrate-NaOH pH 4.0 - 6.0, □; Mes-NaOH pH 5.5 - 6.5, △; K_2HPO_4-KH_2PO_4 pH 6.5 - 8.0, ○; Tris-HCl pH 7.0 - 9.0, ◆; H_3BO_3-NaOH pH 8.0 - 10.0. (B) Effect of temperature on activity. Assays for measurement of activity were done under the standard condition. (C) Effect of temperature on stability. After the purified enzyme was preincubated at the indicated temperature for 1 h, the remaining activity was measured under the standard condition. The remaining activity was expressed relative to the activity, taken as 100%, when reaction mixture was not preincubated.

Table 7.2 Effects of various compounds on carboxylation and decarboxylation activity of the reversible salicylic acid decarboxylase

Compound	Relative activity (%)	
	Decarboxylation	Carboxylation
None	100	100
NaCl	97	80
KCl	92	81
$MgCl_2$	106	90
$MnCl_2$	105	83
$CaCl_2$	84	60
$HgCl_2$	0	6
$ZnCl_2$	111	84
$CuCl_2$	86	30
$FeCl_2$	93	149
$NiCl_2$	54	15
$AgNO_3$	0	40
EDTA	95	104
N-Ethylmaleimide	94	72
Iodoacetamide	97	82
p-Chloromercuribenzoic acid	18	51
Diethyl pyrocarbonate	79	63
Pyridoxal 5'-phosphate	88	93
Avidin	95	103
Biotin	91	82
NADH	90	86
NADPH	83	82
Hydroxylamine	91	77
NaBH4	78	65

The carboxylation and decarboxylation reactions were done under the standard conditions supplemented with the compounds tested at 1 mM except for hydroxylamine (20 mM), NaBH4 (10 mM), avidin (0.2 U/ml), NADH (2.5 U/ml), and NADPH (2.5 U/ml).

acid (82%) as a sulfhydryl group inhibitor, $NiCl_2$ (46%), and diethyl pyrocarbonate (21%) as a histidine residue-specific inhibitor. Other metal ions tested and sulfhydryl group inhibitor, such as N-ethylmaleimide and iodoacetamide, had no inhibition on enzyme activity. Additions of EDTA, pyridoxal 5'-phosphate, avidin, biotin, NADH, and NADPH to reaction mixture had no or slight effect on the enzyme activity.

Hydroxylamine, which is known as an inhibitor of pyridoxal 5'-phosphate-dependent decarboxylases, also had no effect. From these results, this enzyme was found to be a nonoxidative decarboxylase that requires no cofactors such as pyridoxal 5'-phosphate, NADH, and NADPH.

The purified enzyme catalyzed the decarboxylation of salicylic acid into stoichiometric amounts of phenol with a specific activity of 0.47 U/mg. The K_m, V_{max} and k_{cat} values derived from Lineweaver-Burk plots with the enzyme at 40°C and pH 5.5 were 1.08 mM, 1.22×10^{-2} mM/min and 2.03×10 min^{-1}, respectively, for salicylic acid. The enzyme also catalyzed the decarboxylation of β-resorcylic acid (2,4-dihydroxybenzoic acid) into resorcinol (1,3-dihydroxybenzene), γ-resorcylic acid (2,6-dihydroxybenzoic acid) into resorcinol, and 4-aminosalicylic acid into 3-aminophenol. The enzyme Sdc did not catalyzed the decarboxylation of 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, Protocatechuic acid (3,4-dihydroxybenzoic acid), 2,3-dihydroxybenzoic acid, α-resorcylic acid (3,5-dihydroxybenzoic acid), 3-methylsalicylic acid, 4-methylsalicylic acid, and vanillic acid (4-hydroxy-3-methoxybenzoic acid). These results indicated that the substrate recognition of the purified Sdc for decarboxylation seems to depend strictly on hydroxybenzoic acid with two neighboring hydroxyl and carboxyl groups in the active center of the enzyme.

7.3.5. Properties on carboxylation of Sdc

The purified Sdc also catalyzed the carboxylation of phenol into salicylic acid, indicating that this enzyme is a reversible salicylic acid decarboxylase. Therefore, properties as for the carboxylation activity of Sdc were examined. The carboxylation

activity showed a substrate saturation dependence from HCO_3^- with optimal HCO_3^- concentration above 2.5 M (Fig. 7.5). The optimal substrate concentration of phenol and optimal temperature for enzyme activity were 30 mM and 30°C, respectively (Fig. 7.5). Sdc was stable up to 30°C and retained 60% of its activity after the treatment of heating at 40°C for 1 h.

The effects of various metal ions and chemical reagents on Sdc activities are investigated (Table 7.2). The phenol-carboxylation activity was inhibited by $HgCl_2$ (94% inhibition), $NiCl_2$ (85%), $CuCl_2$ (70%), $AgNO_3$ (60%), *p*-chloromercuribenzoic acid (49%) as a sulfhydryl group inhibitor, $CaCl_2$ (40%), diethyl pyrocarbonate (37%) as a histidine residue-specific inhibitor, and $NaBH_4$ (35%). Addition of other metal ions tested and sulfhydryl group inhibitor to reaction mixture, such as N-ethylmaleimide and iodoacetamide, had no or slight effect on enzyme activity. The Sdc activity was activated by $FeCl_2$ (49% activation).

The purified Sdc catalyzed the regioselective carboxylation of phenol into stoichiometric amounts of salicylic acid. The K_m, V_{max} and k_{cat} values derived from Lineweaver-Burk plots with the enzyme at 30°C were 1.23×10^{2} mM, 1.23×10^{-2} mM/min and 6.64×10 min^{-1}, respectively, for phenol. Sdc also catalyzed the carboxylation of resorcinol into β- and γ- resorcylic acid, and 3-aminophenol into 4-aminosalicylic acid. Sdc did not catalyze the carboxylation of catechol, and cresol.

7.3.6. Effects of O_2 on Sdc

Almost all of the already-known reversible aromatics decarboxylases were sensitive to O_2, and required the addition of reducing agents and/or anaerobic condition

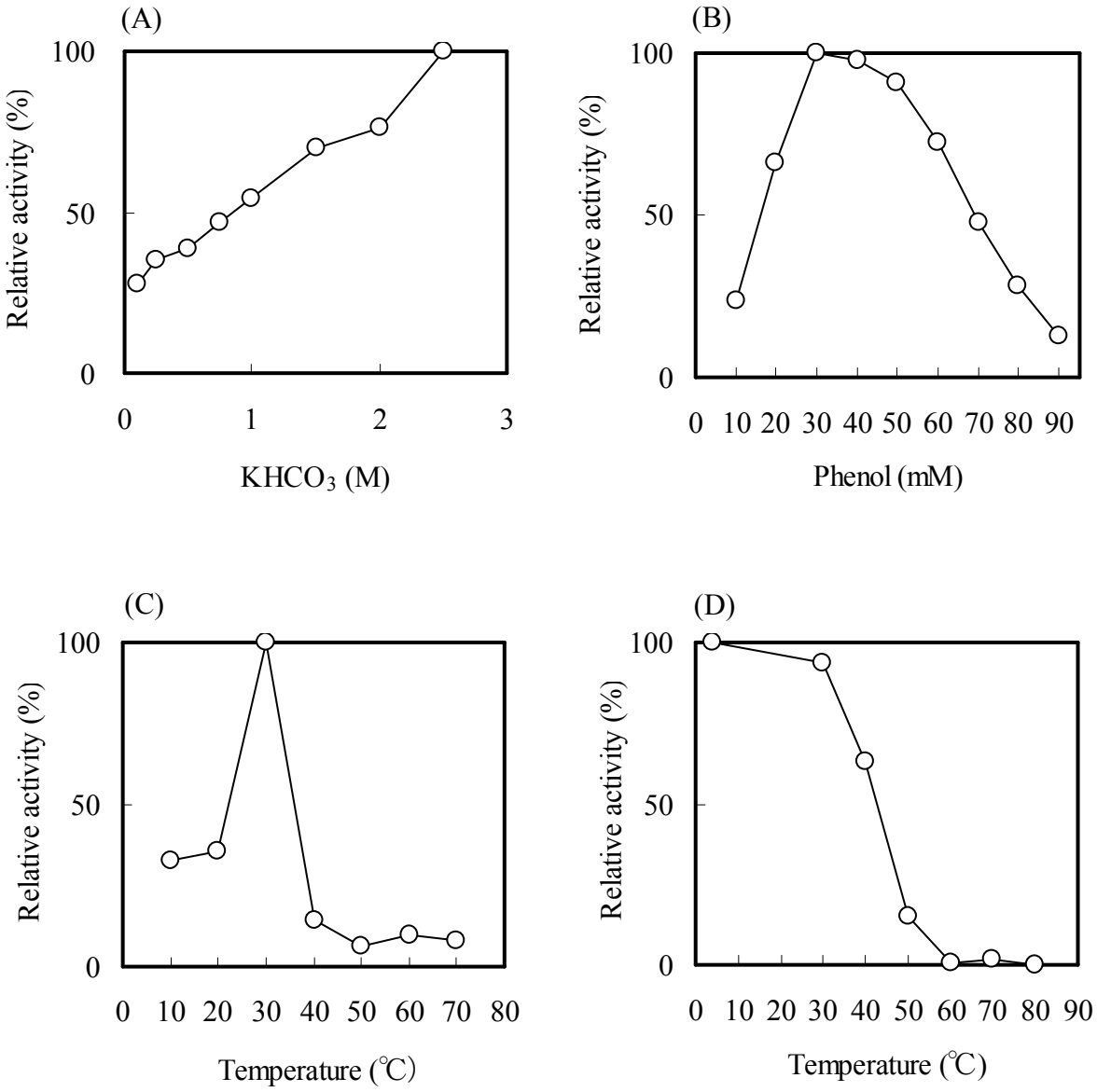

Fig. 7.5. Effects of substrate concentration and temperature on carboxylation activity. (A) Effect of $KHCO_3$ concentration on activity. Assays for measurement of activity were done under the standard condition as described in Materials and Methods with modification by the addition of different amounts of $KHCO_3$. (B) Effect of phenol concentration on activity. Assays for measurement of activity were done under the standard condition with modification by the addition of different amounts of phenol. (C) Effect of temperature on activity. Assays for measurement of activity were done under the standard condition. (D) Effect of temperature on stability. After the purified enzyme was preincubated at the indicated temperature for 1 h, the remaining activity was measured under the standard condition. The remaining activity was expressed relative to the activity, taken as 100%, when reaction mixture was not preincubated.

during the handling [5-7, 15]. Because the enzyme activities of the purified Sdc were stable during the purification under the conditions in the presence of air containing O_2, there was some possibility that the reversible salicylic acid decarboxylase would be insensitive to O_2 for the reactions. Therefore, we examined the effects of O_2 on the carboxylation activity (Fig. 7.6). None of the effect on the activity was observed under the conditions substituted with Ar atmosphere, compared with the standard condition in a shaking reaction without exchange of the gas in the head space, in other words without removal of O_2. These results indicate that the purified Sdc is insensitive to O_2 for reactions.

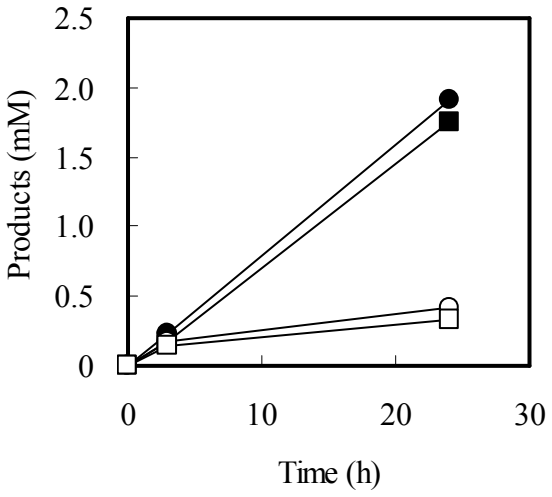

Fig. 7.6. Effects of oxygen on decarboxylation and carboxylation activities of Sdc. Assays for measurement of activity were done under the standard condition (Chapter 7.2.8) with modification by the exchange of the gas in the head space. Symbols: ○; Carboxylation of phenol in Air, △; Carboxylation of phenol in Ar, ●; Decarboxylation of salicylic acid in Air, ▲; Decarboxylation of salicylic acid in Ar.

7.3.7. Cloning of *sdc* from *T. moniliiforme* WU-0401

Two partial amino acid sequences, 10 and 11 residues, of the purified Sdc were determined to be Val-Lys-Ala-Glu-Leu-Tyr-Ile-Ala-
Pro-Asn and Val-Gly-Ile-Gly-Tyr-Thr-Ile-Tyr-Leu-Ile-Tyr, respectively.

Based on these amino acid sequences and codon usage of *T. moniliiforme*, primers used for RT-PCR were synthesized (Chapter 7.2.11). RT-PCR was performed with the primers and cDNA *T. moniliiforme* WU-0401 as template. Based on the nucleotide sequence of an approximately 100 bp amplified fragment thorough RT-PCR, primers used for 5' and 3' RACE were synthesized (Chapter 7.2.12). 5' and 3' RACE were carried out with the primers and cDNA of *T. moniliiforme* WU-0401 as template. Amplified fragments, approximately 400 bp from 5' RACE and 1000 bp from 3' RACE, were sequenced. A complete 1035 bp open reading frame (ORF1) was contained in these fragments.

7.3.8. Nucleotide sequence analysis of *sdc*

The molecular size and the partial amino acid sequence of the protein encoded by ORF1 were identical to those of Sdc purified from *T. moniliiforme* WU-0401. Moreover, since this gene product was conformed to exhibit reversible salicylic acid decarboxylase activity as described later, ORF1 was designated *sdc* encoding the reversible salicylic acid decarboxylase (Sdc) of *T. moniliiforme* WU-0401. The deduced amino acid sequence of *sdc* shows 50% and 40 % identities to the 2,3-dihydroxybenzoic acid decarboxylase (DHBD, AP007151) of *Aspergillus oryzae* [22] and a γ-resorcylic acid decarboxylase (Rdc, AB185333) of *R. radiobacter* WU-0108 (Chapter 5), respectively. On the other hand, as to the primary structure of

reversible and nonoxidative aromatic decarboxylase, 4-hydroxybenzoate decarboxylase (Ohb1, AAD50377) from *C. hydroxybenzoicum* JW/Z-1T was also reported [5, 7], but the deduced amino acid sequence of *sdc* shows no homology to that of Ohb1. Since both decarboxylation and carboxylation activities of Sdc were inhibited by *p*-chloromercuribenzoic acid and diethyl pyrocarbonate as a sulfhydryl group and histidine residue-specific inhibitor (Table 7.2), it was presumed that a cysteine and histidine residue in Sdc might be one of the active site residues. Based on the alignment among aromatics decarboxylases, it was found that one cysteine residue at position 120 and two histidine residues at positions 169 and 224 might be the center of this enzyme (Figs. 7.7 and 7.8).

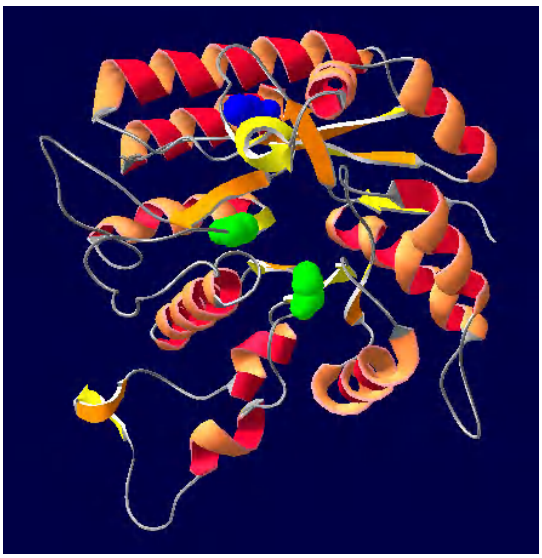

Fig. 7.7. 3D modeling analysis of Sdc by Deep view/Swiss-Pdb Viewer. Green regions indicate two histidine residues at position 169 and 224. Blue regions indicate one cysteine residues at position 120.

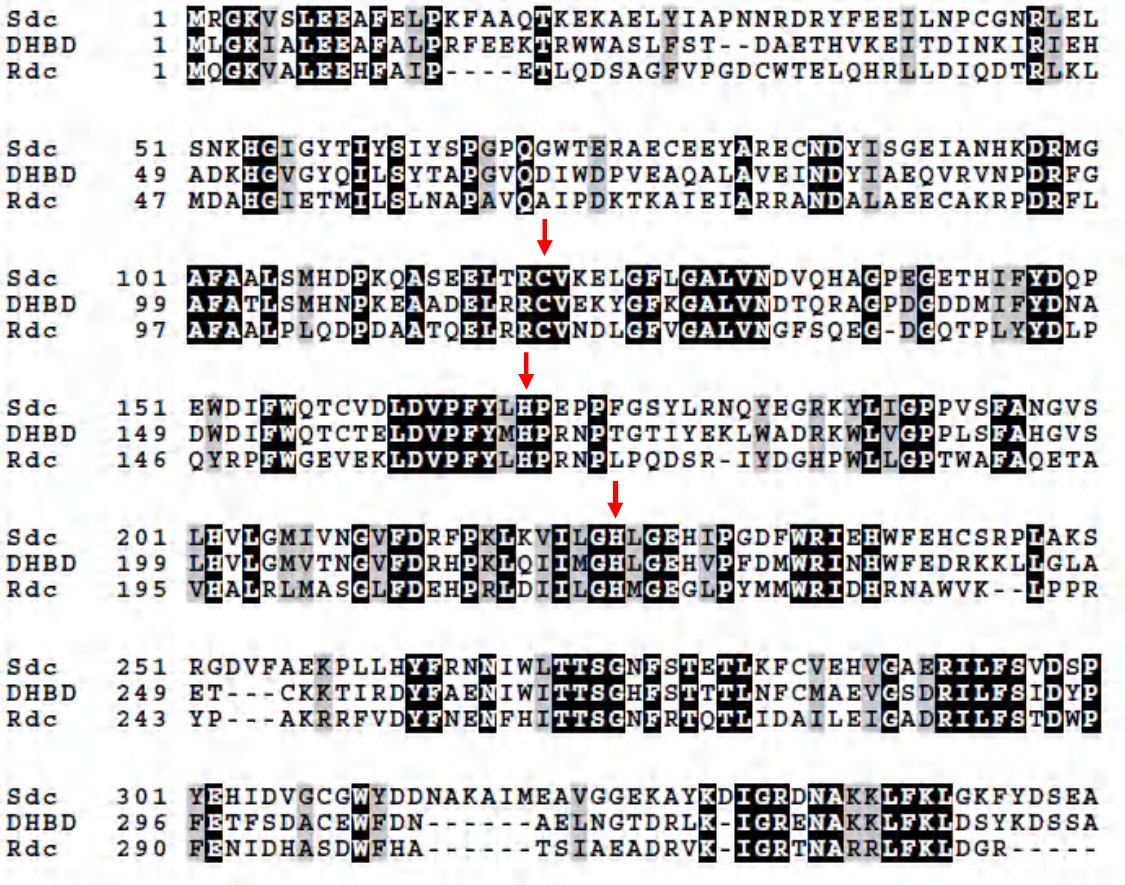

Fig. 7.8. Multiple sequence alignment of nonoxidative decarboxylases. Black shading indicates identical residues in all the members. Gray shading indicates identical residues in two members. Sdc, salicylic acid decarboxylase from *T. moniliiforme* WU-0401 (this study); Rdc, γ-resorcylic acid decarboxylase from *R. radiobacter* WU-0108 (accession number Q60FX6); DHBD, 2,3-dihydroxybenzoic acid decarboxylase from *A. oryzae* (accession number Q2USF4). Arrows indicate one cysteine residue at position 120 and two histidine residues at position 169 and 224.

7.3.9. Overexpression of *sdc* in *E. coli*

The *sdc* gene was amplified by PCR and subcloning in pET21(+) to generate plasmid pSDC (Chapter 7.2.13). The *sdc* gene was expressed in *E. coli* BL21(DE3) harboring plasmid pSDC with the aid of its T7 promoter. SDS-PAGE analysis of a crude cell extract from *E. coli* BL21(DE3) harboring pSDC revealed the presence of single band of 40 kDa that did not appear in control extracts from BL21(DE3) harboring pET21d(+) (data not shown). Phenol-carboxylation activity of a cell free extract of *E.*

coli BL21(DE3) harboring pSDC was 0.08 U/mg, indicating that the high level-expression of *sdc* was successfully done since the activity was 4 times higher than that of original strain *T. moniliiforme* WU-0401.

7.4. Discussion

With the objective to isolate and characterize a novel enzyme forming salicylic acid from phenol, yeast strains *T. moniliiforme* WU-0401 and WU-0501 were newly isolated from soil for its ability to utilize salicylic acid as the sole carbon source. Especially, we detected reversible conversion activities of salicylic acid and phenol in whole cell suspensions and a cell-free extract of *T. moniliiforme* WU-0401 cells. The novel enzyme catalyzing the regioselective carboxylation of phenol into salicylic acid, reversible salicylic acid decarboxylase (Sdc) from *T. moniliiforme* WU-0401, was purified and characterized. As to the reversible and nonoxidative decarboxylase, 4-hydroxybenzoate decarboxylase and 3,4-hydroxybenzoate decarboxylase from *C. hydroxybenzoicum* JW/Z-1T [5, 6], vanillic acid decarboxylase (Vdc) from *Streptomyces* sp. D7 [9], pyrrole-2-carboxylate decarboxylase from *B. megaterium* PYR2910 [11], indole-3-carboxylate decarboxylase from *A. nicotianae* FI1612 [15], and γ-resorcylic acid decarboxylase from *Rhizobium radiobacter* WU-0108 (Chapter 5), from *Rhizobium* sp. MTP-10005, and from *Agrobacterium tumefaciens* IAM12408 [23, 24] have been previously reported. These already-known reversible decarboxylase without γ-resorcylic acid decarboxylase [23, 24] and vanillic acid decarboxylase [9] were sensitive to O_2, but Sdc from *T. moniliiforme* WU-0401 was insensitive to O_2. On the other hand, the substrate specificity of Sdc was strict as similar to the other

already-known decarboxylases, and Sdc converted phenol into salicylic acid without the generation of 4-hydroxybenzoic acid. Moreover, under both conditions in the presence and absence of O_2, the carboxylation activities of Sdc were not affected, suggesting that the handling of the recombinant *E. coli* cells expressing *sdc* and the enzyme would be readily applicable to practical salicylic acid production.

Because strains WU-0401 and WU-0501 were determined to belong to *T. moniliiforme*, we confirmed whether the synthesis and degradation of salicylate would be detected for several closely related to species of *T. moniliiforme*, i.e. *T. moniliiforme* NBRC 1527, *T. cutaneum* NBRC 1198^T, and *T. asteroides* NBRC 0173. Because all of three strains could grow in SA medium, resting cells of these three strains were prepared and subjected to synthesis and degradation of salicylate. The activity of degradation or synthesis of salicylate was observed for all these 3 strains (data not shown). Therefore, salicylic acid decarboxylase reversibly catalyzing the carboxylation of phenol might be widely distributed in strains closely related to the species of *T. moniliiforme*.

In the cultivation with rich medium such as LB medium, *T. moniliiforme* WU-0401 showed the reversible reaction of phenol and salicylic acid with the addition of salicylic acid to medium, but not without salicylic acid. Although over-expression of the *sdc* gene in *E. coli* was detected on SDS-PAGE, the carboxylation activity of cell-free extract of recombinant *E. coli* was 5 times higher than that of *T. moniliiforme* WU-0401. This low enhanced rate of activity in *E. coli* would be due to high level expression of the *sdc* gene with induction by salicylic acid in *T. moniliiforme* WU-0401.

Several genes encoding the nonoxidative aromatics decarboxylase were thus far cloned and characterized [4, 7-10, 12-14, 16]. Based on the CDD (Conserved Domain Database) and COG (Clusters of Orthologous Groups) search, Sdc from *T. moniliiforme*

WU-0401, the orthologous proteins such as DHBD, and Rdc show homologies to the predicted metal-dependent hydrolase of the TIM-barrel fold protein (COG2159) or amidohydrolase (pfam04909). The reversible and nonoxidative 4-dihydroxynbenzoate decarboxylase (Ohb1) from *C. hydroxybenzoicum*, vanillic acid decarboxylase (Vdc) from *Streptomyces* sp. D7 belongs to 3-octaprenyl-4-hydroxybenzoate carboxy-lyase (UbiD) family (pfam01977) and contains the conserved sequence EGP[F/Y][G/V][D/E]XXGXY of the UbiD family [25] (Fig. 7.9). The nonoxidative 4,5-dihydoxyphthalate decarboxylase (Pht5) from *Pseudomonas putida* [10], and PhtD from *Comamonas testosteroni* [8] belong to TauA family (COG0715). The other nonoxidative aromatics decarboxylase such as ferulic acid decarboxylase (Fdc) from *Bacillus pumilus* [16], *p*-coumaric acid decarboxylase (PdcC) from *Lactobacillus plantarum* [4], and phenolic acid decarboxylase (PadA) *Bacillus* sp. BP-7 [13] belong to PA decarbox family (pfam05870). Sdc from *T. moniliiforme* WU-0401 and the orthologous proteins such as DHBD and Rdc show no homology to the decarboxylases of the UbiD, TauA, and PA decarbox family. Moreover, they contain no similar sequence to EGP[F/Y][G/V][D/E]XXGXY and their molecular mass of subunit are approximately 35 kDa with different to approximately 20 kDa and 55 kDa of other nonoxidative aromatics decarboxylases. The subunit-molecular mass of the already-known reversible decarboxylase such as Ohb1, pyrrole-2-carboxylate decarboxylase, and indole-3-carboxylate decarboxylase were approximately 55 kDa. These results suggest that Sdc would be novel reversible and nonoxidative aromatics decarboxylase.

It was reported that active-site peptide of DHBD from *A. oryzae* is LLGLAETCK in the positions of 244-252 aa [14]. Moreover, it was reported that two histidine

residues at positions of 164 and 218 of Rdc from *R. radiobacter* WU-0108 are related to both activities of resorcinol-carboxylation and γ-resorcylic acid-decarboxylation as active site residues (Chapter 5.3.10). Since both decarboxylation and carboxylation activities of Sdc were inhibited by *p*-chloromercuribenzoic acid and diethyl pyrocarbonate as a sulfhydryl group and histidine residue-specific inhibitor (Table 7.2),

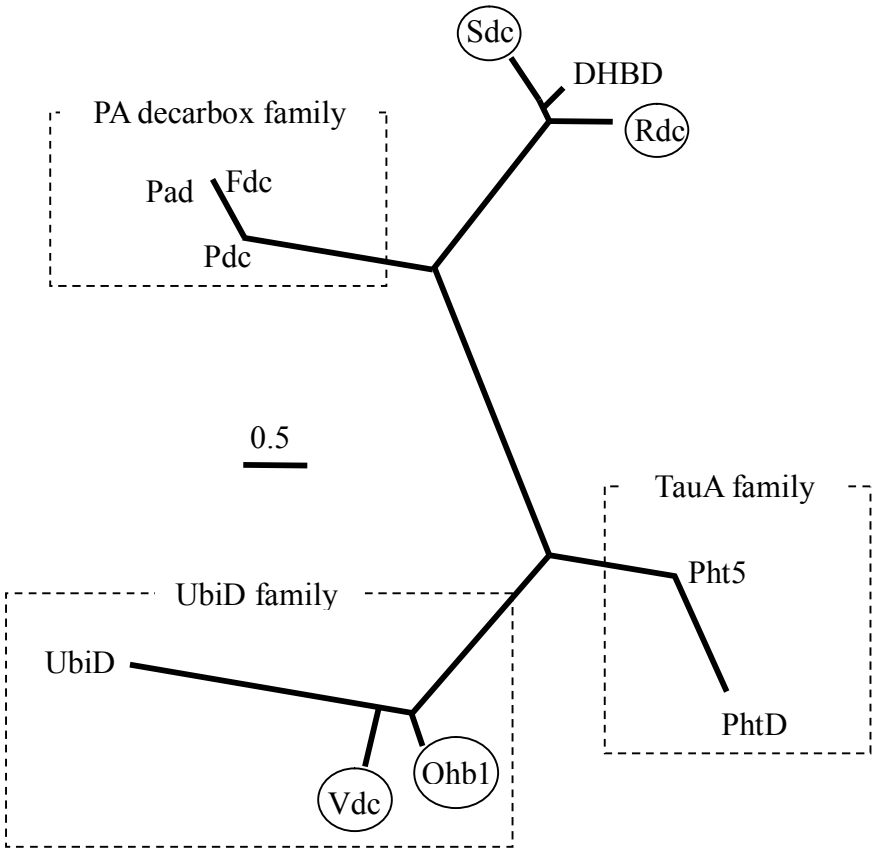

Fig. 7.9. Phylogenetic tree of the nonoxidative aromatics decarboxylases related to Sdc. Scale bar, 0.5 substitution per site. The decarboxylases that reversibly catalyze the carboxylation are circled. Sdc, salicylic acid decarboxylase from *T. moniliiforme* WU-0401; Rdc, γ-resorcylic acid decarboxylase from *R. radiobacter* WU-0108 (AB185333); DHBD, 2,3-dihydroxybenzoic acid decarboxylase of *A. oryzae* (AP007151); Ohb1, 4-dihydroxynbenzoate decarboxylase from *C. hydroxybenzoicum* (AAD50377); Vdc, vanillic acid decarboxylase from *Streptomyces* sp. D7 (AF134589); UbiD, 3-octaprenyl-4-hydroxybenzoate carboxy-lyase (Polyprenyl *p*-hydroxybenzoate decarboxylase) from *E. coli* (AE014075); Pht5, 4,5-dihydoxyphthalate decarboxylase from *P. putida* (BAA02513); PhtD, 4,5-dihydoxyphthalate decarboxylase from *C. testosteroni* (Q59727); Fdc, ferulic acid decarboxylase from *B. pumilus* (Q45361); PdcC, *p*-coumeric acid decarboxylase from *L. plantarum* (AAC45282); PadA, phenolic acid decarboxylase from *Bacillus* sp. BP-7 (CAD37333).

it was presumed that a cysteine and histidine residue in Sdc might be one of the active site residues. Based on the alignment among aromatics decarboxylases, it was found that one cysteine residue at position 120 and two histidine residues at position 169 and 224 might be the center of Sdc (Figs. 7.7 and 7.8), indicating that Cys-120, His-169, and His-224 of Sdc might be essential for the catalytic activities of decarboxylase and carboxylase (Fig. 7.8).

In conclusion, the reversible and nonoxidative salicylic acid decarboxylase (Sdc) that catalyzing the regioselective carboxylation of phenol to form salicylic acid was found in *T. moniliiforme* WU-0401. Therefore, the author succeeded in the molecular characterization of this novel enzyme, including its purification, characterization, and gene-cloning.

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Chapter 8

Application of Recombinant *Escherichia coli* Cells Overexpressing *sdc* to Salicylic Acid Production

8.1. Introduction

Salicylic acid is a precursor of acetylsalicylic acid, which is widely used as a nonsteroidal anti-inflammatory and is known to be the analgesic aspirin [1]. Salicylic acid is produced industrially from phenol (Chapter 1.5.2) by organic synthesis, according to the Kolbe-Schmitt reaction, a carboxylation reaction at high pressure and high temperature [2]. However, in such production, a large amount of 4-hydroxybenzoic acid is also generated as a by-product, and a process for separating 4-hydroxybenzoic acid is then required [2].

Several decarboxylases reversibly catalyzing the carboxylation of phenolic and cyclic organic compounds have been reported [3-6]. As shown in Chapter 7, the author and collaborators found a salicylic acid decarboxylase (Sdc) from *Trichosporon moniliiforme* WU-0401, which reversibly catalyzes the regioselective carboxylation of phenol leading to the synthesis of salicylic acid without by-products (Fig. 7.1), and described the molecular characterization of this novel enzyme Sdc, including its purification, characterization, and gene cloning (Chapter 7). Sdc has some unique properties; for example, although decarboxylases reversibly catalyzing carboxylation show decreased activities in the presence of O_2 and require cofactors, such as NAD(P)H and pyridoxal 5'-phosphate [3-6], Sdc shows a constant activity regardless of the

presence or absence of O_2 and requires no cofactor (Chapter 7.3.6), suggesting the effectiveness of handling Sdc for practical salicylic acid production from phenol.

In this chapter, the author describes the enzymatic production of salicylic acid using recombinant *Escherichia coli* cells expressing the gene (*sdc*) encoding Sdc with the objective of developing a novel method of selectively producing salicylic acid from phenol under environmentally benign conditions compared with those needed for the Kolbe-Schmitt reaction. A recombinant *E. coli* expressing *sdc* converted 40 mM phenol to 10.6 mM salicylic acid with a 27% (mol/mol) yield at 30°C for 9 h.

8.2. Materials and methods

8.2.1. Chemicals

Authentic salicylic acid and phenol (Tokyo Kasei Kogyo) used in this study were purchased. All the other chemicals used were commercially available and of chemically pure grade.

8.2.2. Cultivation of recombinant *E. coli* overexpressing *rdc*

E. coli BL21 (DE3)/pSDC cells (Chapter 7.2.13) were cultivated at 30°C in 500 ml Erlenmeyer flasks containing 200 ml Luria-Bertani (LB) medium (Chapter 2.4.4) supplemented with 100 μg ampicillin/ml and 0.2 mM IPTG as an inducer of the T7 promoter with reciprocal shaking at 120 strokes/min.

8.2.3. Resting cell reaction

The cells cultivated were harvested by centrifugation at 6,000 × g for 10 min at

4°C, and washed twice with 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.0). Unless otherwise indicated, the cultivation was carried out for 18 h. The washed cells were used for the resting cell reaction for salicylic acid production from phenol. The standard reaction conditions were as follows. A reaction mixture containing *E. coli* BL21 (DE3)/pSDC cells (26 g dry-cells/l), 5 mM phenol, and 150 mM $KHCO_3$ in 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.0) in 1 ml in a 1.5-ml microcentrifuge tube was incubated at 30°C with reciprocal shaking at 120 strokes/min. Unless otherwise indicated, the incubation was performed for 16 h. The reaction was stopped by adding 10 μl 12 M HCl, the mixture was then centrifuged at 6,000 × g for 10 min at 4°C and the supernatant filtered using a 0.2-μm PTFE membrane.

8.2.4. Analytical methods

Cell growth was measured turbidimetrically at 660 nm. Salicylic acid and phenol in the supernatant were determined by HPLC, as described previously (Chapter 2.14.2).

8.3. Results

8.3.1. Reaction conditions of salicylic acid production

To optimize the conditions for the resting cell reaction using the recombinant *E. coli* cells, the author examined the reaction conditions by changing the standard temperature, $KHCO_3$ concentration, and reaction time described above. The optimal conditions were: 30°C, a $KHCO_3$ at 3 M (saturated concentration), and a reaction time of 16 h. As shown in Fig. 8.1, 4 mM salicylic acid was produced from 20 mM phenol

with a maximum yield of 20% (mol/mol). On the other hand, 6.4 mM salicylic acid of maximum concentration was produced from 40 mM phenol. Since the recombinant *E. coli* cells after 9-h reaction at 20 mM phenol were not recyclable for use as biocatalysts, the initial phenol concentration was therefore fixed at 40 mM as the optimal concentration.

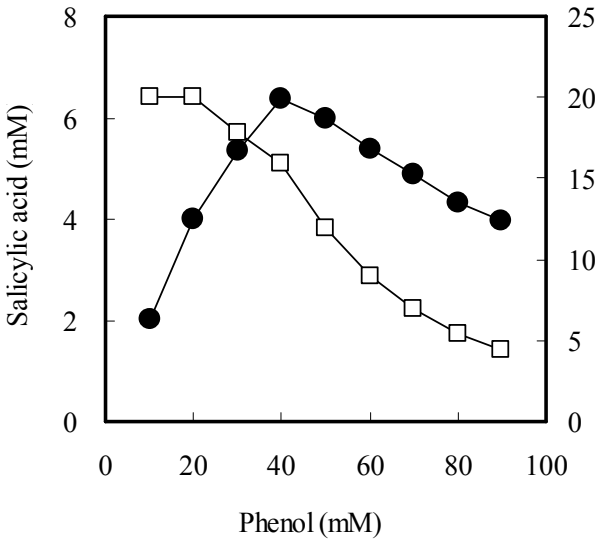

Fig. 8.1. Effects of initial phenol concentration on salicylic acid production by resting cell reaction using *E. coli* BL21 (DE3)/pSDC cells (26 g dry-cells/l). Experiments were performed under standard conditions at various phenol concentrations. Symbols**:** closed circle, salicylic acid; open square, salicylic acid yield based on the amount of phenol supplied.

8.3.2. Biosynthesis of salicylic acid by resting cell reaction

The time course of salicylic acid production by the resting cell reaction using the recombinant *E. coli* cells is shown in Fig. 8.2. The concentration of salicylic acid reached its maximum, 10.6 mM, at 9 h and thereafter, remained constant. Under these conditions, salicylic acid was the only product in the reaction mixture. These results indicate that recombinant *E. coli* cells expressing *sdc* must be applicable as efficient and convenient biocatalysts to the selective production of salicylic acid from phenol.

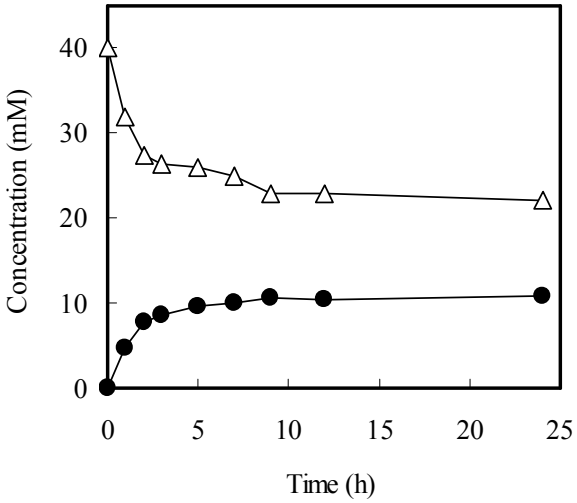

Fig. 8.2. Salicylic acid production by resting cell reaction using recombinant *E. coli* BL21 (DE3)/pSDC under optimal conditions. Symbols**:** closed circle, salicylic acid ; open triangle, phenol.

8.4. Discussion

In this chapter, the author described the enzymatic production of salicylic acid using recombinant *E. coli* cells expressing *sdc* with the aim of developing a novel method of selectively producing salicylic acid from phenol under environmentally benign conditions compared with the conditions needed for the Kolbe-Schmitt reaction. A recombinant *E. coli* expressing *sdc* converted 40 mM resorcinol to 10.6 mM salicylic acid with a 27% (mol/mol) yield at 30°C for 9 h.

Although the recombinant *E. coli* BL21 (DE3)/pENS10 cells were recyclable at least five times for use as biocatalysts for the selective production of γ-resorcylic acid from resorcinol (Chapter 6), the recombinant *E. coli* BL21 (DE3)/pSDC cells after 9-h reaction at 20 mM phenol were not recyclable for use as biocatalysts. On the other hand, the recombinant *E. coli* cells maintained a stable Sdc activity for a long time (more than 24 h), and such a stable Sdc activity is favorable for practical salicylic acid production.

In conclusion, the author showed that enzymatic salicylic acid production can be achieved using *E. coli* cells expressing *sdc* as biocatalysts under environmentally benign conditions compared with those for the conventional organic synthesis, such as that using the Kolbe-Schmitt reaction. Therefore, the author considers that the method described here is a significant model process for the selective and ecological production by carboxylation of phenol to form salicylic acid.

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Chapter 9

Summary and Conclusions

In this thesis, the author described the characterization of novel enzymes converting aromatics as biocatalysts applicable to the selective and ecological conversion of aromatics. The summary of this thesis is as follows.

In Chapter 1, the author described an overview of the bioconversion of aromatics. The author explained two conversion methods of aromatics, i.e., nonbiological conversion and bioconversion, and demonstrated that bioconversion is a significant model process for the selective and ecological conversion of aromatics. Based on the review in this chapter, the author clarifies 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 describes the cloning and functional analysis of the DBT-desulfurization genes from *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 desulfurization of DBT.

In Chapter 4, the author describes the enhancement of DBT-desulfurizing activity by genetic engineering of the thermophilic DBT-desulfurizing bacterium *M. phlei* WU-F1. The *Mycobacterium-E. coli* shuttle vector designed as pUALS was constructed to generate the recombinant *M. phlei* WU-F1 with increased copy numbers

of genes related to DBT-desulfurization. The DBT-desulfurizing activity of the recombinant *M. phlei* WU-F1 through the resting-cell reaction at 45°C was approximately 2-fold higher than that of the parental strain. Therefore, this is the first report describing the enhancement of DBT-desulfurizing activity of thermophilic bacteria by genetic engineering.

In Chapter 5, the author describes the molecular characterization of the reversible and nonoxidative γ-RA decarboxylase from *Rhizobium radiobacter* WU-0108, including its purification, characterization, and gene-cloning. For selective synthesis of γ-RA from RE, microorganisms possessing a novel enzyme catalyzing regioselective carboxylation of RE to form γ-RA was screened from 50 soil samples. The nonoxidative γ-RA decarboxylase (Rdc) that reversibly catalyzes the regioselective carboxylation of RE to form γ-RA was found from *R. radiobacter* WU-0108. The gene (*rdc*) encoding Rdc was cloned and heterologously expressed in *Escherichia coli* cells. Therefore, this is the first report describing the enzyme that catalyzes the regioselective carboxylation of RE to form γ-RA.

In Chapter 6, the author described the enzymatic production of γ-RA using recombinant *E. coli* cells expressing *rdc* with the aim of developing a novel method of selectively producing γ-RA from RE under environmentally benign conditions compared with the conditions needed for the Kolbe-Schmitt reaction. A recombinant *E. coli* expressing *rdc* converted 20 mM resorcinol to 8.8 mM γ-resorcylic acid with a 44% (mol/mol) yield at 30°C for 7 h.

In Chapter 7, the author describes the molecular characterization of the reversible and nonoxidative salicylic acid decarboxylase from *Trichosporon moniliiforme* WU-0401, including its purification, characterization, and gene-cloning. For selective

synthesis of salicylic acid from phenol, microorganisms possessing a novel enzyme catalyzing regioselective carboxylation of phenol to form salicylic acid were screened from 3000 soil samples. The nonoxidative salicylic acid decarboxylase (Sdc) that reversibly catalyzes the regioselective carboxylation of phenol to form salicylic acid was found from *T. moniliiforme* WU-0401. The gene (*sdc*) encoding Sdc was cloned and heterologously expressed in *Escherichia coli* cells. Therefore, this is the first report describing the enzyme that catalyzes the regioselective carboxylation of phenol to form salicylic acid.

In Chapter 8, the author described the enzymatic production of salicylic acid using recombinant *E. coli* cells expressing *sdc* with the objective of developing a novel method of selectively producing salicylic acid from phenol under environmentally benign conditions compared with the conditions needed for the Kolbe-Schmitt reaction. A recombinant *E. coli* expressing *sdc* converted 40 mM phenol to 10.6 mM salicylic acid with a 27% (mol/mol) yield at 30°C for 9 h.

For commercial application of bioconversion of aromatics, improvement of biocatalysts and conversion process is essential.

As for the biodesulfurization of DBT, the activity over 20 nmol S/min/mg-drycell is required for commercialization [1, 2], which is approximately 20 times higher than the recombinant cell biocatalysts constructed in this study. The low activity is mainly attributed to the low concentration of desulfurizing enzymes in the 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.

As for the enzymatic carboxylation of phenols, first, the conversion yield over 70% is required for commercialization [5-7], which is two or three times higher than the yield in this study using recombinant cell biocatalysts. The low yield is mainly attributed to the reversible conversion of products and starting materials by reversible decarboxylases. The reversible conversion of products and starting materials was apparently limiting the maximum yield of products. The resin-based products removal will be effective in increase the yield [8]. The products removal from reaction mixture will prevent the reversible decarboxylases from reversible conversion of products to form starting materials. Second, the longevity is required for commercialization, which is partly attributed to the tolerance of the hosts for the phenols used as starting materials. For example, in this study, toxicity of phenols toward the recombinant cell biocatalysts was apparently limiting the maximum concentration of products and the recycle use of biocatalysts. It will be important to screen proper hosts possessing high tolerance for the phenols [9].

In conclusion, in this thesis, to develop a novel method of selectively converting aromatics under environmentally benign conditions, the author studied the molecular characterization of novel enzymes converting aromatics, including their purification, characterization, gene-cloning, and over-expression. These studies have resulted in application to biodesulfurization of aromatic sulfur compounds and to enzymatic regioselective carboxylation of aromatics. The microorganisms, enzymes, and genes

found through the studies in this thesis may be useful resources to improve biocatalysts for commercial application to bioconversion of aromatics. The outcome of the studies in this thesis will provide meaningful information for the bioconversion process of aromatics, but also the studies in the fields of microbiology, biotechnology, and applied chemistry.

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Yuichiro Iwasaki

研 究 概 要

分子内にベンゼン環を含む有機化合物は芳香族化合物と総称されており、石炭や石油などの化石燃料中に多く含まれている。芳香族化合物から得られる各種の誘導体は、化学製品あるいは医薬品の原料として重要であり、その合成（化学的変換）には有機化学的方法が用いられることが多い。しかし、従来の有機合成プロセスでは高温高圧条件下での反応が利用されることが多く、目的物質への選択的な変換が困難であるため廃熱や不要な副生成物の発生といった環境への負荷が大きな問題となっている。

一方、酵素や微生物を生体触媒として利用した芳香族化合物の変換は、常温常圧条件下で目的物質への選択的な変換が可能であることから、環境調和型の新規な方法として重要視されている。すなわち、従来の有機合成プロセスに代替可能な方法として、酵素による芳香族化合物の変換により新規な有用物質の生産方法を構築することは極めて重要である。また、芳香族化合物の混合物から不要な成分を酵素を利用して選択的に除去する方法を構築することも極めて重要である。これらの実現によって環境調和型かつ効率的な芳香族化合物変換プロセスの新展開が期待できる。

本論文では、環境調和型な芳香族化合物の微生物変換プロセスの構築を目的とした研究を行い、その成果をまとめた。すなわち、新規な芳香族化合物変換酵素を発見し諸性質を明らかにするとともに、遺伝子クローニングおよび機能解析を行った。また、遺伝子工学的手法により各種遺伝子を高発現させた微生物細胞を生体触媒として利用することによって新規な微生物変換プロセスを構築した。具体的には、石油中には多くの芳香族硫黄化合物とくにジベンゾチオフェン（DBT）類が含まれており、エネルギーとして燃焼することで硫黄酸化物を生成するため、酸性雨や大気汚染の原因となっている。そこで、DBT 脱硫活性の向上を目的として、好熱性脱硫細菌由来の DBT 脱硫酵素遺伝子をクローニングするとともに、好熱性脱硫細菌の細胞内における当該遺伝子のコピー数を増大させた組換え体を作製し DBT 脱硫能力を評価した。一方、γ - レゾルシン酸およびサリチル酸は、染料、香料、医薬品原料など多方面で利用されているが、従来の有機合成プロセスでは高温高圧を必要とする Kolbe-Schmitt 法によって生産されており、環境への負荷が大きい。そこで、γ - レゾルシン酸とサリチル酸についての新規な代謝経路を有する微生物を取得するとともに、それぞれに対応する新規な可逆的脱炭酸酵素の存在を明らかにした。さらに、これらの酵素をコードする遺伝子について大腸菌における高発現を可能として、当該酵素の新規性を遺伝子レベルで解明した。また、当該酵素遺伝子を高発現させた組換え大腸菌を利用し、レゾルシノールからのγ - レゾルシン酸とフェノールからのサリチル酸の選択的生産に応用した。

本論文は 9 章より構成されている。

第 1 章では、芳香族化合物の微生物変換について概説した。芳香族化合物の微生物変換と芳香族化合物変換酵素の報告例についてまとめ、芳香族化合物に関する微生物変換を従来の有機合成法や金属触媒を用いた反応と比較し、その特徴について述べた。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、各種微生物の培養方法、芳香族化合物の微生物変換に関する方法、酵素の分析方法、遺伝子工学的手法、ならびに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 DBT 脱硫細菌 *Mycobacterium phlei* WU-F1 由来の DBT 脱硫酵素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、*M. phlei* WU-F1 から DBT 脱硫酵素遺伝子をクローニングした。さらに、CUGA シーケンス法により当該遺伝子の上流領域約 3 kb および下流領域約 1 kb の塩基

配列を決定した。DBT 脱硫に関わる DNA の塩基配列を解析し、*Bacillus subtilis* WU-S2B 由来の DBT 脱硫酵素遺伝子（*bdsABC*）と比較したところ、両株は属種が異なり系統的に離れているにも関わらず、塩基配列は同一であった。以上の結果は、脱硫遺伝子群 *bdsABC* が進化系統学的に遠距離に位置する菌種にまで水平伝達された可能性を示唆した。

第 4 章では、*M. phlei* WU-F1 の遺伝子組換えによる DBT 脱硫能力の強化について検討した。*Mycobacterium phlei-Escherichia coli* シャトルベクターを作製し、WU-F1 の宿主－ベクター系を構築した。*bdsABC* と DBT の酸化反応に関与するフラビンレダクターゼ遺伝子（*frm*）をシャトルベクターに連結し、*M. phlei* WU-F1 の細胞内に導入することにより、当該遺伝子のコピー数を増大させた組換え体 *M. phlei* WU-F1/pUALSABCD を作製した。さらに、*M. phlei* WU-F1/pUALSABCD を用いた休止菌体反応による DBT 脱硫を検討し、野生株の *M. phlei* WU-F1 と比較して 45℃における DBT 脱硫活性が約 2 倍（0.67 nmol/min/mg-dry-cell-weight）に向上したことを示した。

第 5 章では、レゾルシノールからγ－レゾルシン酸を合成する能力を示す新規酵素を発見し、その解析を目的として、*Rhizobium radiobacter* WU-0108 由来の可逆的γ－レゾルシン酸脱炭酸酵素（Rdc）をコードする遺伝子（*rdc*）をクローニングし機能を解析した。γ－レゾルシン酸を唯一の炭素源として増殖可能な微生物として *R. radiobacter* WU-0108 を取得した。WU-0108 の休止菌体はレゾルシノールをカルボキシル化して、選択的にγ－レゾルシン酸を合成する能力を示した。当該反応に関与する酵素として Rdc を精製し、酵素的諸性質を検討した。Rdc は、従来の脱炭酸酵素とは異なり補酵素や ATP 等を必要とせず、酸素存在下でも反応が進行する実用性に優れた能力を有することを明らかにした。精製酵素の N 末端アミノ酸配列を基に作成したプローブを用いて、WU-0108 の部分 DNA ライブラリーから目的の遺伝子（*rdc*）をクローニングした。*rdc* は、327 個のアミノ酸残基から成る 37.4 kDa のタンパク質をコードしており、従来の脱炭酸酵素とは相同性が低く、新規な酵素であることをアミノ酸配列から明らかにした。さらに、*rdc* への部位特異的変異導入により、Rdc においては 164 番目と 218 番目の His 残基が活性に関与していることを明らかにした。

第 6 章では、*rdc* を高発現させた組換え大腸菌細胞を利用したγ－レゾルシン酸生産について検討した。pET ベクターを利用して *rdc* を高発現させた組換え大腸菌 *E. coli* BL21 (DE3)/pENS10 を作製した。無細胞抽出液を用いてレゾルシノールのカルボキシル化活性を検討したところ、組換え大腸菌 *E. coli* BL21 (DE3)/pENS10 について比活性は 0.93 U/mg で、原株 WU-0108 のそれと比較して約 3.6 倍のカルボキシル化活性を示した。さらに、*E. coli* BL21 (DE3)/pENS10 の休止菌体反応を利用したレゾルシノールの位置選択的カルボキシル化反応を最適化した。反応は、*E. coli* BL21 (DE3)/pENS10 の細胞懸濁液にレゾルシノールと炭酸水素カリウムを混合し、30℃で 7 時間振とうすることによって行い、8.8 mM のγ－レゾルシン酸を 20 mM のレゾルシノールから最大収率 44%で生産することに成功した。

第 7 章では、フェノールからサリチル酸を合成する能力を示す新規酵素を発見し、その解析を目的として、*Trichosporon moniliiforme* WU-0401 由来の可逆的サリチル酸脱炭酸酵素（Sdc）をコードする遺伝子（*sdc*）をクローニングし機能を解析した。サリチル酸を唯一の炭素源として増殖可能な微生物として *T. moniliiforme* WU-0401 を取得した。WU-0401 の休止菌体はフェノールをカルボキシル化して、選択的にサリチル酸を合成する能力を示した。当該反応に関与する酵素として Sdc を精製し、酵素的諸性質を検討した。Sdc は、Rdc と同様に、補酵素や ATP 等を必要とせず、酸素存在下でも反応が進行する実用性に優れた能力を有することを明らかにした。精製酵素の内部アミノ酸配列を基に作成したプライマーを用いて、逆転写 PCR と 5`-RACE および 3`-RACE 法を利用して目的の遺伝子（*sdc*）をクローニングした。*sdc* は、349 個のアミノ酸残基から成る

39.7 kDa のタンパク質をコードしており、従来の脱炭酸酵素とは相同性が低く、新規な酵素であることをアミノ酸配列から明らかにした。

第 8 章では、*sdc* を高発現させた組換え大腸菌細胞を利用したサリチル酸生産について検討した。pET ベクターを利用して *sdc* を高発現させた組換え大腸菌 *E. coli* BL21 (DE3)/pSDC を作製した。無細胞抽出液を用いてフェノールのカルボキシル化活性を検討したところ、組換え大腸菌 *E. coli* BL21 (DE3)/pSDC について比活性は 2.03 U/mg で、原株 WU-0401 のそれと比較して約 5 倍のカルボキシル化活性を示した。さらに、*E. coli* BL21 (DE3)/pSDC の休止菌体反応を利用したフェノールの位置選択的カルボキシル化反応を最適化した。反応は、*E. coli* BL21 (DE3)/pSDC の細胞懸濁液にフェノールと炭酸水素カリウムを混合し、30℃で 9 時間振とうすることによって行い、10.6 mM のサリチル酸を 40 mM のフェノールから最大収率 27%で生産することに成功した。

第 9 章では、本研究を総括した。本研究成果により得られた新規な芳香族化合物変換酵素（BdsABC、Rdc、Sdc）を利用した各種芳香族化合物に対する微生物変換能力を評価し、実用化へ向けてさらに解決すべき点などについて述べた。

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