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Characterization and crystal structures of carbazole degrading genes (CarBaBb and CarC) from *Sphingomonas* sp. KA1

List of abbrevations

APS	:	ammonium peroxodisulfate
CAR	:	carbazole
DHBP	:	2,3-dihydroxybiphenyl
DW	:	distilled water
Da	:	Dalton
EDTA	:	ethylenediaminetetracetic acid
FPLC	:	fast protein liqiud chromatography
Fig.	:	figure
HCl	:	hydrochloric acid
HMW	:	high molecular weight
HODA	:	2-hydroxy-6-oxohexa-2,4-dienoates
IPTG	:	isopropyl-β-D-thiogalactoside
K_{cat}	:	turnover number
K_m	:	Michaelis constant
LB medium	:	Luria-Bertani medium
LMW	:	low molecular weight
MCS	:	multi-cloning site
MES	:	2-(*N*-morpholino) ethanesulfonic acid
MOPS	:	3-(*N*-morpholino) propanesulfonic acid
MWCO	:	molecular weight cut-off
M_r	:	relative molecular weight
O.D.	:	optical density
PAGE	:	polyacrylamide gel electrophoresis
PAHs	:	polycyclic aromatic hydrocarbons
PCR	:	polymerase chain reaction
SDS	:	sodium dodecyl sulfate
TE	:	tris-acetate / EDTA
TEMED	:	*N,N,N',N'*-tetramethylenediamide
Tris	:	tris (hydroxymethyl) aminomethane
V_0	:	initial rate
Vmax	:	maximum rate
conc.	:	concentration
g	:	gram(s)
hr	:	hour(s)
ht	:	histidine-tagged
min	:	minute(a)
rpm	:	rounds per minute
w/v	:	weight / volume

Chapter 1

Introduction

The widespread of dioxin-like substances produced through usage of herbicides and coke ovens have severe impact on the environment. Dioxin-like substance are known to be highly toxic to all organism. Furthermore, due to their stability, they reside in the environment for long periods. The half life for 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) in soil and riverbed sediments was reported to be from 1 to 10 years [(1)]. Carbazole, a compound produced from impurities of fossil fuel, share the same plane structure of dioxin and characterized as a recalcitrant chemical. It was also reported to have substantial carcinogen effects[(2)]. Recently, bacteria that can grow on carbazole as sole carbon and nitrogen source have been isolated from the soil, other than carbazole, they also posses the ability to degrade dioxin-like chemicals[(3,15)].

Due to its widespread in the environment in low concentrations, dioxins are difficult to be processed by physical and chemical methods. Bioremediation is a method of utilizing microbacteria ability to degrade pollutants that reside in the environment. In comparison to physical and chemical methods, bioremediation provides an economical way to clean the environment from recalcitrant pollutants while leaving behind small impact on the environment. Bioaugmentation, subset of bioremediation involves introducing new bacteria to polluted area to improve the biodegradation of pollutants. Method like this promotes the isolation of new bacteria with high activity for biodegrading recalcitrant pollutants.

Catabolisms of aromatic compounds in aerobic microbes start with introducing hydroxy groups to the benzene ring. This reaction is conducted by the enzyme called dioxygenase. Degradation pathways of naphthalene and biphenyl, members of polycylic aromatic hydrocarbons (PAHs) family, are very well studied[(4)]. The pathways starts with the production of *cis*-1,2-dihydro-1,2-dihydroxynaphthalene and *cis*-2,3-dihydro-2,3-dihydroxybiphenyl from naphthalene and biphenyl respectively. This type of dioxygenation is called angular dioxygenation. The angular dioxygenation also detected in dibenzo-*p*-dioxin (DD)、fluorene (FN) and diphenyl ether (DE) degradation pathways[(15)]. Products of angular dioxygenation are unstable and ring cleavage easily occur producing cathecol derivatives. In the carbazole pathway, 2 oxygen molecule are added to carbazole through angular dioxygenation to produce a diol derivative which is later converted to 2'-aminobiphenyl-2,3-diol through spontaneous ring cleavage[(3,4)] (Fig 1-1).

After the addition of hydroxyl groups to aromatic compounds, these compounds were cleaved in 2 known ways; intradiol and extradiol dioxygenation. Intradiol dioxygenation is catalyzed by intradiol dioxygenases which typically have non-heme Fe(III) at their active site. Intradiol dioxygenases cleaves rings at the C-C bond between the vicinal hydroxyl groups. This type of cleavage is also known as *ortho*-cleavage. The latter type of dioxygenation is catalyzed by extradiol dioxygenases which possess non-heme Fe(II) at their active site. Extradiol dioxygenases catalyzes ring cleavage at the C-C bond adjacent to the vicinal hydroxyl groups. This is also known as *meta*-cleavage[(4)].

Extradiol dioxygenase have been classified into 2 families based on substrate specificity, monocyclic and bicyclic substrates by Harayama and Rekik[(5)]. Eltis et al. initially classified extradiol dioxygenase based on phylogenetical analyses. Two families were suggested where type I enzymes showed conserved consensus and type II enzymes have low mutual sequence identity[(6)]. Extradiol dioxygenase have also been classified into 3 classes on the basis of sequence alignments as proposed by Spence et al[(7)]. Class I of extradiol dioxygenase comprises of single-domain enzymes such as BphC2 and BphC3 from *Rhodococcus globerulus* P6. Class II has a two-domain enzyme in which the C-terminal domain binds Fe(II) and is catalytically active. An example of class II enzyme is BphC from *Pseudomonas* strain LB400. Class III enzyme has a two-domain enzyme in which the N-terminal domain contains conserved histidine residues such as MhpB from *E.coli*. Spence et al. also proposed the evolutionary relationship among the three classes. From the conserved histidine residues observed from enzymes of the 3 classes, the duplication of class I gene followed by the mutation and loss of function of N-terminal domain is thought to give class II. Class III could be explained by duplication of class I gene with mutation and loss of function of C-terminal domain.

Structures of several extradiol enzymes were determined by X-ray crystallography. Fe(II) ligands of BphC from *Pseudomonas* strain LB400 were determined as 2 histidine residues and a glutamate residue (His-146, His-210 and Glu-260) located in the C-terminal domain[(8)]. His-12, His-61 and Glu-242 act as Fe(II) ligands in LigB from Sphingomonas paucimobilis SYK-6, a class III enzyme. Also revealed was a unique βαββ stucture motif in class II enzymes. This motif is repeated once in each C-terminal domain and N-terminal domain. Structure of LigB did not reveal any structural motif. This example shows that class II and class III enzymes are structurally different despite the evolutionionary relationship hypothesis. However, the orientation of the 3 residues which hold the Fe(II) ion are almost similar to BphC and LigB.

The *car* genes of *Pseudomonas resinovorans* CA10 have been studied most extensively among other carbazole degrading bacteria[(9)]. Extradiol dioxygenase of strain CA10 is a class III extradiol enzyme designated CarBaBb coded by *carBa* and *carBb* genes. Although most extradiol dioxygenase are homo-multimeric, CarBaBb is made of two proteins like LigAB enzyme. CarBaBb is made of CarBb, a larger subunit which carries the catalytic site and CarBa, a smaller subunit which function remains unknown. Although the catalytic site of this enzyme is on the larger subunit, activity was only detected when both proteins were expressed. CarBaBb showed strong meta-cleavage activity towards biphenyl-type substrates but showed weak activity towards catechol-type substrates[(10)]. The *carBa* and *carBb* genes were also found in *car* genes of *Sphingomonas* sp. KA1. Significant differences between car_{CA10} and car_{KA1} gene cluster have been shown but the identity of $CarBaBb_{CA10}$ and $CarBaBb_{KA1}$ is 40% (Fig. 1-2). However, the crystal structure of CarBaBb enzyme has not been determined.

CarC hydrolase follows CarBaBb enzyme in the carbazole degradation pathway from both strains CA10 and KA1[9]. It was proposed that this enzyme played an important role in substrate speceficity for chlorinated aromatic compounds. $CarC_{CA10}$ and $CarC_{KA1}$ identity is shown to be 57% (Fig. 1-3)[11]. Phylogenetic studies of this hydrolase indicate that it belongs to family of proteins with α/β folds. Hernaez et al. indicated that hydrolases can be divided into several groups that correspond well enzymes' preferred substrates[12]. From this phylogenetic grouping, most hydrolases involved in the degradation of monocyclic and bicyclic aromatic compounds are in group I and III (Fig. 1-4)[13]. The substrate specificity of CarC was similar to that hydrolases classified into group I, but CarC has closer phylogenetic relation with group III. This shows that CarC hydrolases have intermediate characteristics and might be classified into a new group other than group III.

Recently, more bacteria have been isolated from not only soil samples, but from marine samples as well. Some are reported to have the abilities to degrade a wide range of recalcitrant compounds. Studies like there would lead to discoveries of novel pathways and enzymes. However, without the understanding of mechanism of enzymes related to degradation of recalcitrant pathways, it is difficult to develop a technology for enhancing biodegradation of xenobiotics. Studies on enzymes includes characterization, biochemical analysis and structure determination. Hence, in this study we conducted characterization and biochemical analysis on two enzymes belong to the degradation pathways of carbazole from *Sphingomonas* sp. KA1. The enzymes reported are *meta*-cleavage enzyme CarBaBb and hydrolase CarC.

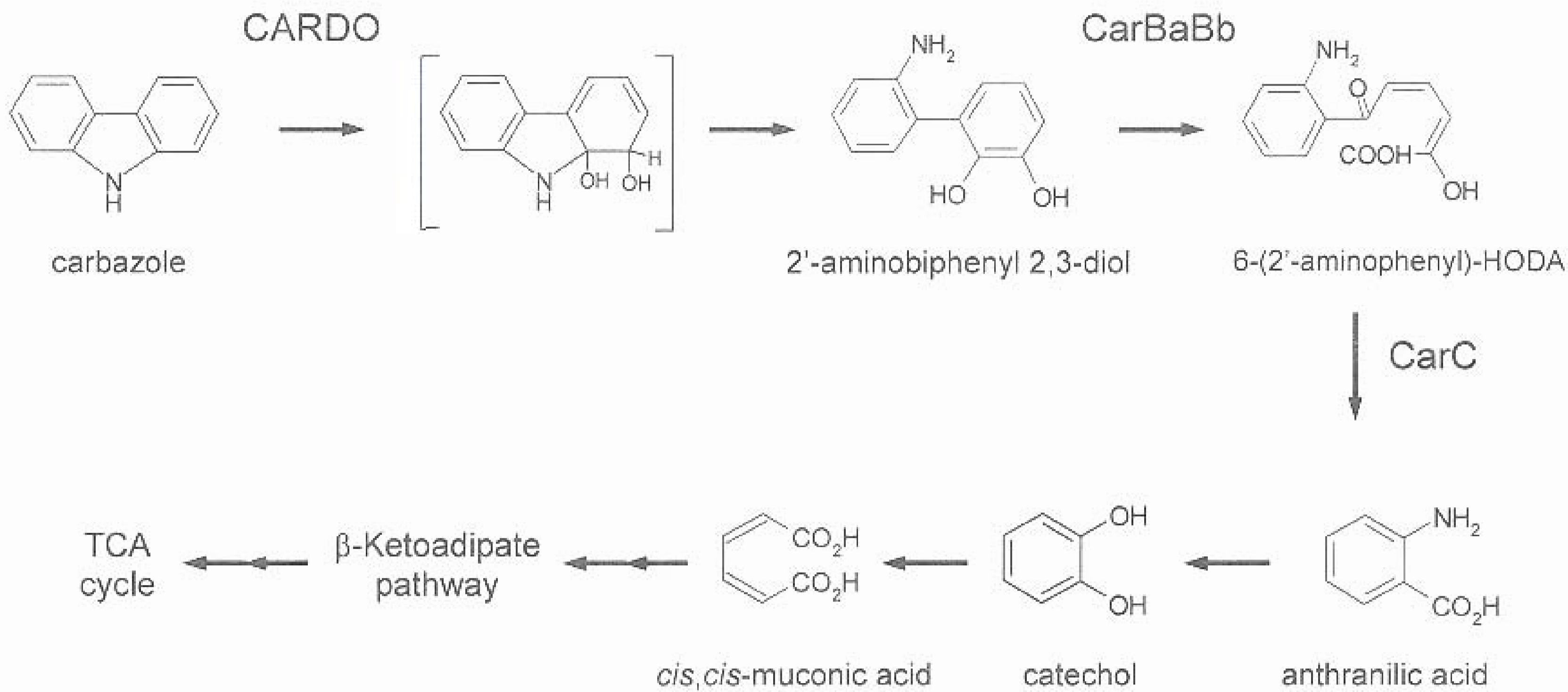

Fig. 1-1. Carbazole degradation pathway for *Pseudomonas resinovorans* CA10

Bacteria	Substrates	Enzymes	Class
Rhodococcus sp. P6	Biphenyl	BphC2P6	I
Rhodococcus sp. RHA1	Ethylbenzene	EtbC	II
Pseudomonas sp. KKS102	Biphenyl	BphC	II
Burkholderia cepacia LB400	Biphenyl	BphC	II
Pseudomonas fluorescens IP01	Cumene	CumC	II
Pseudomonas putida mt-2	Xylene	XylE	II
Pseudomonas resinovorans CA10	Carbazole	CarBaBb	III
Sphingomonas sp. KA1	Carbazole	CarBaBb	III
Rhodococcus sp. DFA3	Dibenzofuran	FlnD1D2	III
Sphingomonas paucimobils SYK-6	Protocatechuate	LigAB	III

Table 1-1. List of bacteria, extradiol dioxygenases, substrates and their classes

```
CarBa_CA10      ---MARYEVDRLIQDMSKKEGLIGRVIDTPSDVFEEYGLTPPERTALLEGTPQALASIGV 57
CarBa_KA1       MGTTASYPVNRLMQELFTNPGNVELFRADREALYERYGLSSAQRAALDEGGFGALTAVGL 60
                    * * *:**:*:: .: * :  .    .  ::*.***:..:*:** **   **:::*:

CarBa_CA10      HPILQMHYLMYKNPEMATHVSIKDYSDMLKGGA 90
CarBa_KA1       HPVLQMHHFMLTNPMAPDFVSVKAYRKMVDRNG 93
                **:****::* .**  .  .**:* * .*:. ..


CarBb_CA10      MGKIVAAGGTSHILMSPKGCEESAARVVNGIAELGRRLKEARPDVLVIITSDHMFNINLS 60
CarBb_KA1       MAEIVGVFATSHVLFGSPDGDAQALRVVEGMNEIGRRVRLLRPDLLIVIGSDHLFNITTR 60
                *.:**.. .***:*:.. . : .* ***:*: *:***::  ***:*::* ***:***.

CarBb_CA10      MQPRFVVGIADSYTPMGDMDIPRDLVPGSREVGRAIALQADEDGFDLCQAEEYSLDHGIM 120
CarBb_KA1       LQPPFTVGVSDTFTPLGDMDIEQRPFAGNRAFAESLCARA-ADRFDLAQGEELRPDHGVM 119
                :** *.**::*::**:***** :  ..*.* ...::. :*  * ***.*.**   ***:*

CarBb_CA10      IPILFMG-MKEIPVVPVIVNINTDPIPSARRCVALAESIRQAIEKRTPDGCRVAVVGAGG 179
CarBb_KA1       VPLMFIDPDGRIPVVPIYVNANMTPPPTAARAAQLGDIVAEAVGLDLPSHLRVVVVATGG 179
                :*::*:.   .*****: ** *   * *:* *.. *.: : :*:    *.  **.**.:**

CarBb_CA10      LSHWLCVPRHGEVSEKFDHMVMDELVRGNAEKLVAMGNEAIIDQGGNAGVEILTWIMAAV 239
CarBb_KA1       LSHWINIPGHGEVNAEFDRRVIAAFQSGDMRWLRAIDTETLLKNAGNGGLEIVNWVMAAA 239
                ****: :* ****. :**: *:  :  *: . * *:..*:::.:.**.*:**:.*:***.

CarBb_CA10      ASEASSGEKVFYEAMTQWFTGIGGMEFHVK 269
CarBb_KA1       TLPGRRAEKIYYEPMPQWMTGMGGIAIV-- 267
                :  .  .**::**.*.**:**:**: : :
```

'*' indicates positions which have a single, fully conserved residue

':' indicates that conserved substitutions have been observed

'.' indicates that semi-conserved substitutions have been observed

Fig. 1-2. ClustalW alignments between $CarBaBb_{CA10}$ and $CarBaBb_{KA1}$

```
CarC_CA10       MLNKAEQISEKSESAYVERFVNAGGVETRYLEAGKGQPVILIHGGGAGAESEGNWRNVIP 60
CarC_KA1        ----------MVLAATAGRSVTVRGMETYYHEQGSGDVVVLVHGGGAGADSMGNWRGVMP 50
                             :* . * *.. *:** * * *.*: *:*:*******:* ****.*:*

CarC_CA10       ILARHYRVIAMDMLGFGKTAKPD--IEYTQDRRIRHLHDFIKAMNFDGKVSIVGNSMGGA 118
CarC_KA1        VLADRYRVIAVDMLGFGRTAKPADPFVFSQAARTDHLAGFLDALGLS-NVALVGNSMGGA 109
                :** :*****:******:****   : ::*  *  ** .*:.*:.:. :*::********

CarC_CA10       TGLGVSVLHSELVNALVLMGSAGLVVEIHEDLRPIINYDFTREGMVHLVKALTNDGFKID 178
CarC_KA1        SALGVAVERPGLVRKLVLMGSAGLVSKIDPALEPVLGYDFTREGMIRLVRALTTDNFQID 169
                :.***:* :. **. ********** :*.  *.*::.********::**:***.*.*:**

CarC_CA10       DAMINSRYTYATDEATRKAYVATMQWIREQGGLFYDPEFIRKVPVPTLVVHGKDDKVVPV 238
CarC_KA1        DAMIDYRYALSVDPETRRAYSATMQWIRDQGGLYYEDDYIRRITAPTLIVNGKLDKVVPL 229
                ****: **: :.*  **:** *******:****:*: ::**::..***:*:** *****:

CarC_CA10       ETAYKFLDLIDDSWGYIIPHCGHWAMIEHPEDFANATLSFLSRRADITRAAA 290
CarC_KA1        ANAYKFLELIGPSWGYIMPDCGHWAMIEHPVDFARTTAAFIEAAQ------- 274
                 .*****:**. *****:*.********** ***.:* :*:.
```

'*' indicates positions which have a single, fully conserved residue

':' indicates that conserved substitutions have been observed

'.' indicates that semi-conserved substitutions have been observed

Fig. 1-3. ClustalW alignments between $CarC_{CA10}$ and $CarC_{KA1}$

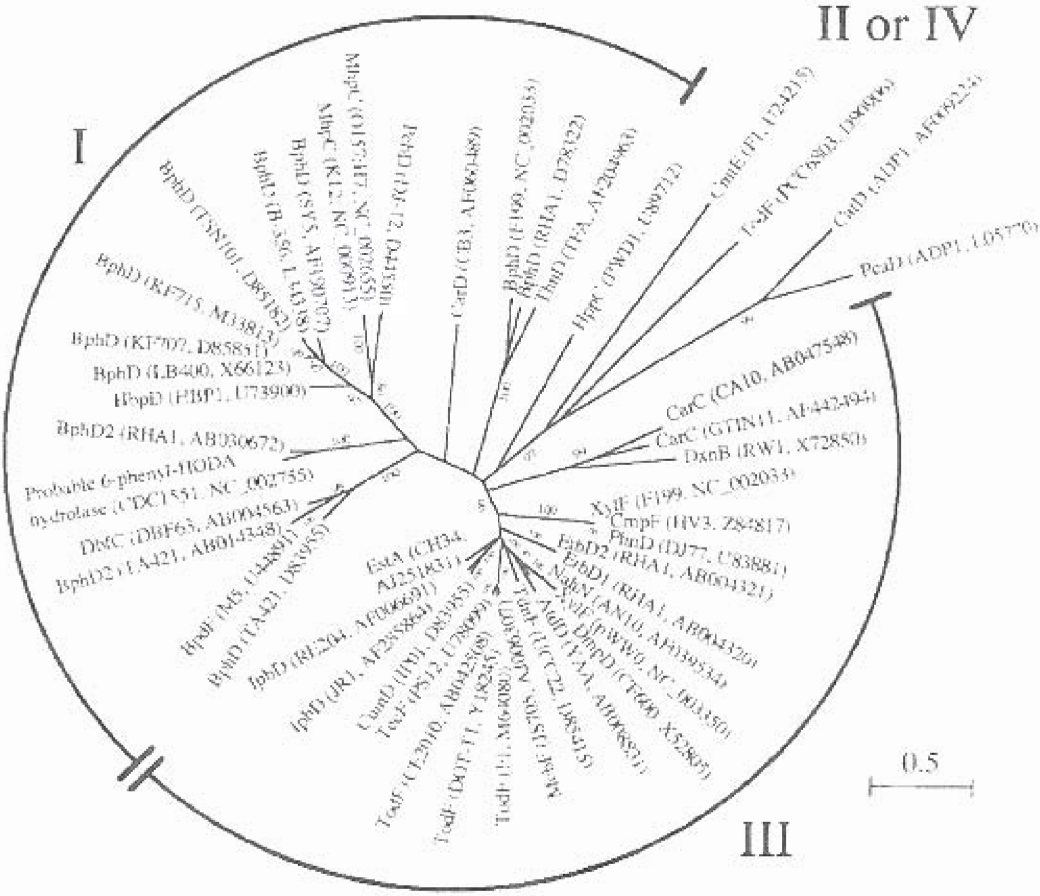

Fig. 1-4. Phylogenetic tree of meta-cleavage compound hydrolases[13]

Chapter 2

Determination of growth conditions for expression of 2'-aminobiphenyl-2,3-diol 1,2-dioxygenase (CarBaBb) from *Sphingomonas* sp. KA1

2.1 Introduction

Among class III extradiol dioxygenases reported, most of them are single-subunit proteins. Still very few two-subunit extradiol dioxygenases were reported. Two-subunit meta-cleavage enzymes are considered to be unique as both subunits are needed to be active enzymes. It is also intriguing as how two-subunit extradiol dioxygenases related in the evolution of the extradiol dioxygenase family.

CarBaBb from *Pseudomonas resinovorans* CA10 was extensively studied. High yields of $CarBaBb_{CA10}$ were achieved from *E.coli* expression and biochemical and structural studies were possible. Although $CarBaBb_{KA1}$ has amino acid identity to $CarBaBb_{CA10}$, $CarBaBb_{KA1}$ expression levels were low in *E.coli* hosts making studies difficult. In this chapter, several experiments were conducted to determine the optimal expression condition for $CarBaBb_{KA1}$ in *E.coli* host.

2.2 Materials and Methods

2.2.1 Media and reagents

Composition and preparation of medium and buffers used in this study are shown in table 2-1.

2.2.2 Chemicals

Chemicals used for buffers used in this study such as nuclease and protease tested tris(hydroxymethyl)aminomethane and sodium chloride are products of Nacalai tesque, inc (Kyoto, Japan). Glycerol and imidazole are from Kanto Chemical Co., Inc. All chemicals were of analytical or molecular biology grade and of the highest purity available.

2.2.3 Enzymes and Kits

Plasmid High Purity Extraction kit (Roche) was used for plasmid maintanence according to manufacturer's protocol.

2.2.4 Bacterial strains, plasmids

Bacterial strains and plasmids used for this study are shown in table 2-2.

2.2.5 Molecular genetic methods

2.2.5.1 Preparation of plasmid DNA

Plasmids used in this study were periodically prepared using either *E.coli* strain DH5α or JM109. Plasmids were extracted from *E.coli* cells using High Purity Extraction kit (Roche). Purity and concentration of plasmids were inspected using UV/vis spectrophotometer (GE Healthcare). Extracted plasmids were stored in dH_2O or TE buffer at -20 °C until use.

2.2.5.2 Preparation of competent *E.coli*

Preparation of competent *E.coli* was performed following method described by Hanahan (1989). *E.coli* strain was streaked directly from glycerol stock onto the surface of an ϕb medium plate and incubated at 37 °C for 16 hr. A colony was inoculated into 5 ml of ϕb medium and grown at 37 °C until $O.D._{550}$ reaches 0.3. Cells were transferred into 200 ml of ϕb medium and shake at 18 °C until $O.D._{550}$ became 0.48. The culture cell was kept on ice for 5 minutes and centrifuged at 3000 rpm at 4 °C for 5 min. The cell pellet was washed thoroughly with 20 ml of TfbI solution and kept on ice for 15 min. Next, the washed cell pellet was resuspended with 2 ml of TfbII solution and dispensed into aliquots (100 µl in 1.5 ml tubes). The aliquots were snap-freezed in dry ice and stored at -80 °C until use.

2.2.5.3 Transformation of *E.coli* with plasmid DNA

Plasmid DNA was transformed into chemically competent *E.coli* using standard method (Sambrook et al., 1989). Plasmid DNA or ligated products were mixed with 100 µl of competent cells and the tube was kept on ice for 30 minutes. Then the mixture was subjected to heat shock at 42 °C for exactly 45 seconds and immediately chilled on ice for 2 min. 500 µl of SOC medium were added to the tube and incubated at 37 °C for at least 30 min. The mixture was spreaded onto 2xYT plates containing appropriate antibiotics. The plate was incubated at 37 °C for 18 hr until colonies appear.

2xYT medium (per liter)	
Bacto-tryptone	16 g
Bacto-yeast extract	10 g
NaCl	5 g
Bacto-agar (if needed)	15 g

ϕa medium (per liter)	
Bacto-tryptone	5 g
Bacto-yeast extract	20 g
$MgSO_4 \cdot 7H_2O$	5 g
Bacto-agar	15 g

LB broth (per liter)	
Bacto-tryptone	16 g
Bacto-yeast extract	10 g
NaCl	5 g
Bacto-agar (if needed)	15 g

SOC medium (per 100 ml)	
Bacto-tryptone	2 g
Bacto-yeast extract	0.5 g
NaCl	0.05 g
250 mM KCl	1 ml
1 M Glucose*	2 ml
0.5 M $MgCl_2$*	2 ml

* Added after autoclave

Table 2-1 Media compositions.

Strains or plasmids	Characteristics	Sources or references
Bacterial strains		
Escherichia coli DH5α	F^-, Φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(r_K^-, m_K^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Takara shuzo
Escherichia coli JM109	*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(r_K^- m_K^+), e14$^-$ (*mcrA*$^-$), *supE*44, *relA*1, Δ(*lac-proAB*)/F' [*traD*36, *proAB*$^+$, *lac* I^q, *lacZ*ΔM15]	Takara shuzo
Escherichia coli BL21(DE3)	F^-, *ompT*, *hsdS*$_B$ ($r_B^- m_B^-$), *gal*, *dcm*, (DE3)	Novagen
Escherichia coli HB101	*supE*44, Δ(*mcrC-mrr*), *recA*13, *ara*-14, *proA*2, *lacY*1, *galK*2, *rpsL*20, *xyl*-5, *mtl*-1, *leuB*6, *thi*-1	Takara shuzo
Plasmids		
pET-26b	Km^r, pBR322 origin, *lacI*, T7 promoter	Novagen
pUCA525	Ap^r, pUC119 with both 0.3-kb *Sph*I-*Bgl*II insert from pTCARBaF and *Sal*I-*Bgl*II insert from pTCARBbR; carries *carBaBb*	Iwata et al., 2003
pUCA526	Ap^r, pUCA525 with 1.1-kb *Sph*I-*Sal*I insert from pUCA525; carries tandemly duplicated *carBaBb*	Iwata et al., 2003
pBKA101	Ap^r, pBluescript II SK(-) with 3.8-kb of *Ecor*RI insert of strain KA1 DNA	Inoue et al., 2003

Table 2-2. Bacterial strains and plasmids.

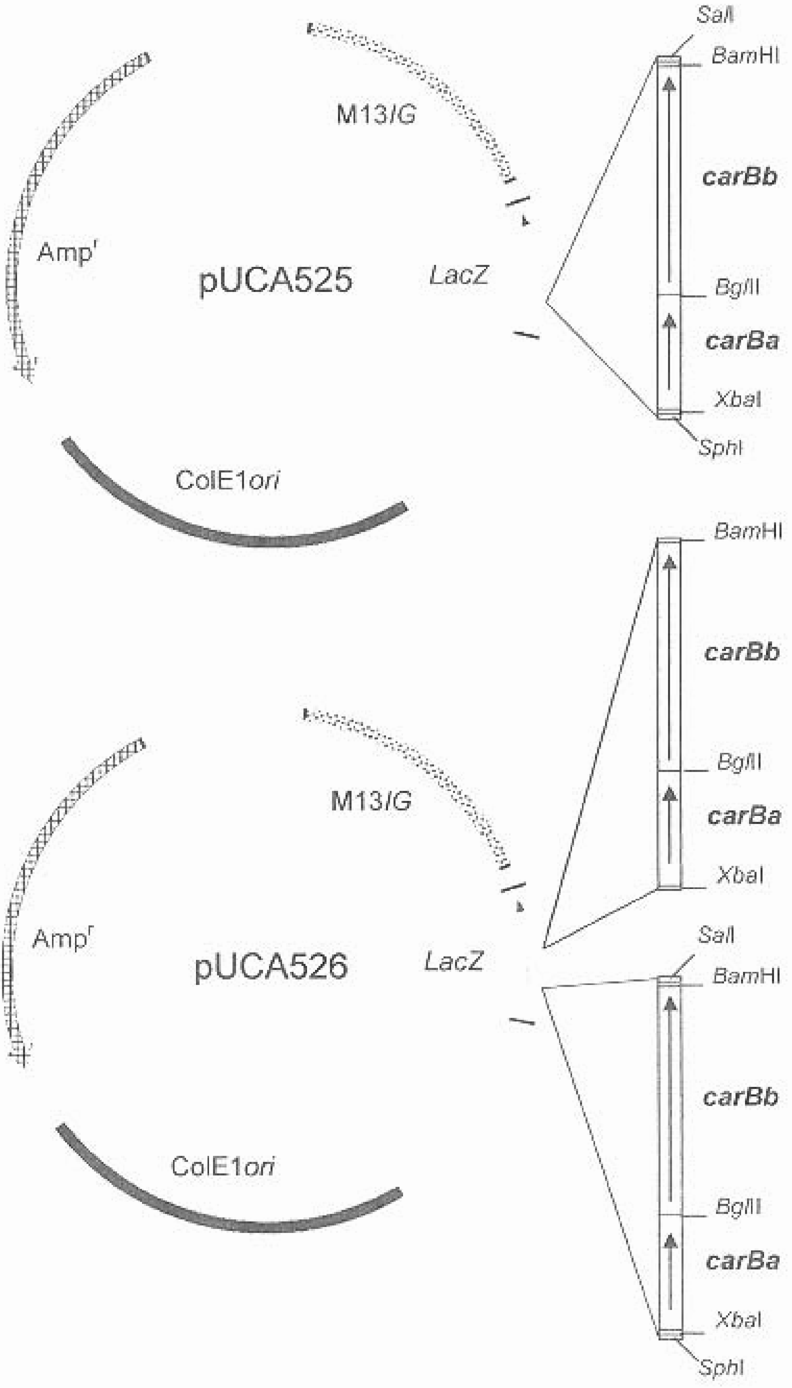

Fig. 2-1. Plasmid construction of pUCA525 and pUCA526.

2.2.6 Optimizing growth and expression conditions for ht-CarBaBb

Expressions with double tandem gene insert

Plasmid pUCA525 and pUCA526 are similar except the later has a pair of genes coding ht-CarBaBb protein which was inserted tandemly. To assess the expression level of both plasmids, each plasmid was transformed into *E.coli* BL21(DE3) and grown in 2xYT medium. Extract of cells were taken and were analyzed using 15% SDS-PAGE gel.

Optimizing growth and expression condition for CarBaBb

To obtain maximum yield of CarBaBb protein, expression conditions need to be optimized. Several known factors affecting protein expression such as growth medium, growth temperature, IPTG induction time, expression host were tested to find the best expression for this protein.

Growth medium

To test the most suitable growth medium for CarBaBb expression, 3 medium LB broth, 2xYT medium, Buiyon medium were tested. Plasmid pUCA526 was transformed into *E.coli* BL21(DE3) and grown at 37 ˚C for 12 hours using selected medium containing 100 µg amphicillin. Two ml of cells were centrifuged and sonificated (Branson Digital Sonifier 250) using 20 mM Tris-HCl pH 7.5 as buffer. Crushed cells were centrifuged at 15000 rpm, 15 min (Tomy MX-301) to separate insoluble fractions. Expression levels of each fraction were analyzed using 15% SDS-PAGE gel.

Growth temperature

Optimal growth temperature were tested with 30 ˚C, 37 ˚C and 40 ˚C. Plasmid pUCA526 transformed *E.coli* BL21(DE3) were pre-cultured in 5 ml 2xYT medium at 37 ˚C for 3 hours before inoculated into 200 ml flasks of 2xYT at different temperatures. Cells were grown for 12 hours before analysis with 15% SDS-PAGE gel.

IPTG induction time

IPTG induction timing to produce most proteins were tested by addition of IPTG (final concentration 1 mM) at different growth stages, $O.D._{600}$ 0, 0.5 and 1.7. Samples were taken periodically after application of IPTG and were analyzed by SDS-PAGE.

Expression host

Several *E.coli* strains were used to obtain maximum protein expression. E.coli strains tested were DH5α, JM109, HB101 and BL21(DE3). Each strain were transformed with pUCA526 and were grown with 2xYT medium. Each strain were sampled to be analyzed with SDS-PAGE.

Erlenmeyer buffled flasks and Sakaguchi flasks

2 types of flasks were used for growing expression host in this study, the 500 ml Erlenmeyer buffled flask and 500 ml Sakaguchi flask . 200 ml 2xYT medium were filled in each flask with appropriate antibiotic. Erlenmeyer buffled flasks were shaken in a circular motion while Sakaguchi flasks were shaken in a reciprocal motion. Flasks were shaken at 37 °C.

2.2.7 Preparation of crude extract samples (small-scale)

Crude extract samples were prepared by sampling 2 ml of bacteria and centrifuged in 2 ml tubes at 5000 rpm (TOMY MX-301) 4 °C for 10 min. Medium were discarded and cell pellets were resuspended with 1 ml of ice cooled 20mM Tris-HCl pH 7.5 10% glycerol buffer. Cells were later sonicated with microtip (0.5 seconds pulses of 30 seconds at 30% output) with the tube submerged in ice. Sonicatications were repeated 3 times with 2 min of chilling on ice in between. Sonicated samples were then centrifuged at 15000 rpm 4 °C for 30 min to separate soluble and insoluble fractions. Soluble fractions were moved to fresh tubes and insoluble pellets were resuspended with 1 ml of the same buffer.

2.2.8 SDS-PAGE

SDS-PAGE analysis were performed according to Laemmli method using Bio-Rad Mini PROTEAN-3 apparatus. Chemicals and compositions of gels used in this study are shown in table 2-3. LMW kit E standards (Amersham Biosciences) ranging from 14.4 – 97.0 kDa were used as protein standards. SimplyBlue Safe stain (Invitrogen) was used for staining of gels according to supplied protocols.

Chemicals:

Acrylamide/Bisacryamide mixture (19:1) (Bio-Rad Lab.)

10% Ammonium peroxodisulfate (APS)

4x Tris-HCl / SDS (pH 6.8); Concentration gel buffer

4x Tris-HCl / SDS (pH 8.8); Separating gel buffer

N,N,N',N'-Tetramethylenediamine (TEMED)

2x SDS sample buffer

4x Tris-HCl / SDS buffer (pH 6.8)	25 ml
Glycerol	20 ml
SDS	4 g
2-Mercaptoethanol	2 ml
Bromophenol blue	1 mg

Mixture is filled up to 100 ml with DW

10x SDS / electrophoresis buffer

Tris	15.1 g
Glycine	72.0 g
SDS	5.0 g

Mixture is fillep up to 500 ml with DW

	Separation gel			Concentration gel
	10%	12%	15%	
30% (w/v) Acrylamide/Bisacryamide	1.66 ml	2.00 ml	2.50 ml	o.22 ml
DW	2.08 ml	1.75 ml	1.25 ml	1.02 ml
4x Tris-Hcl / SDS	1.25 ml			0.42 ml
10% APS	17 µl			8 µl
TEMED	3 µl			2 µl

Table 2-3. Composition for SDS-PAGE mini gels.

2.3 Results

Expression with double tandem gene inserts

Expression level difference was tested between pUCA525 carrying 1 copy of *carBaBb* genes and pUCA526 carrying 2 copies of *carBaBb* genes. Strong bands of approximately 30 kDa appeared. This band was not observed in the cells harboring pUC119 which served as a negative control. This relative molecular weight corresponded well to CarBb subunit deduced amino acid sequence of . CarBa subunit was not observed due to the its small molecular weight and the poor resolution of 15% glycine gel at low molecular weight regions.Cells harboring pUCA526 expressed more CarBaBb protein. CarBaBb protein expression increased with IPTG induction time length for both plasmids.

Growth temperature

Expression level for growth temperature 30 °C, 37 °C and 40 °C was examined. CarBaBb was expressed the most when cells were grown at 37 °C. Cells grown at 30 °C showed moderate expression while 40 °C showed almost no expression of CarBaBb protein.

IPTG induction time

Cells that were induced during exponential growth phase (O.D. 0.5) produce more CarBaBb with time. The production of CarBaBb was maximum at approximately 16 hours after IPTG induction. Cells that were induced during stationary phase (O.D. 1.7) showed similar expression pattern, increasing with time. In comparison, cells grown with IPTG added during stationary phase produce more CarBaBb than.cells induced during exponential phase.

Expression hosts

All strain tested showed no significant difference in expression level for CarBaBb. Among the hosts tested, strain BL21(DE3) produced the least CarBaBb in the insoluble fraction. This proved that BL21(DE3) has better compatibility with expressing CarBaBb proteins.

Erlenmeyer buffled flasks and Sakaguchi flasks

Both flasks showed contant increase of CarBaBb proteins with growth time. But, cells grown in Sakaguchi flasks expressed more proteins. The mass expression of CarBaBb protein is due to the better aeration from the to-and-fro shaking of Sakaguchi flasks comparing to the conventional rotation shaking of Erlenmeyer buffled flasks.