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Cloning and Expression of *meta*-Cleavage Enzyme (CarB) of Carbazole Degradation Pathway from *Pseudomonas stutzeri*

Ariane Leites Larentis[1,2*], **Tito Lívio Moitinho Alves**[1] **and Orlando Bonifácio Martins**[2]
[1]Laboratório de Bioprocessos; Programa de Engenharia Química - COPPE; Centro de Tecnologia; Universidade Federal do Rio de Janeiro; Cidade Universitária; Ilha do Fundão; C. P. 68502; 21945-970; ariane@peq.coppe.ufrj.br; Rio de Janeiro -RJ -Brasil. [2]Laboratório de Biologia Molecular; Instituto de Bioquímica Médica; Centro de Ciências da Saúde; Universidade Federal do Rio de Janeiro; Cidade Universitária; Ilha do Fundão; 21941-590; Rio de Janeiro -RJ -Brasil

ABSTRACT

In this work, the 1082bp PCR product corresponding to carBaBb genes that encode the heterotetrameric enzyme 2'-aminobiphenyl-2,3-diol 1,2-dioxygenase (CarB), involved in the Pseudomonas stutzeri *ATCC 31258 carbazole degradation pathway, was cloned using the site-specific recombination system. Recombinant clones were confirmed by PCR, restriction enzyme digestion and sequencing. CarB dioxygenase was expressed in high levels and in active form in* Escherichia coli *BL21-SI using the His-tagged expression vector pDEST™17 and salt induction for 4h.*

Key words: Carbazole, 2'-aminobiphenyl-2,3-diol dioxygenase, HOADA, heterologous gene expression, petroleum denitrogenation, biodegradation

INTRODUCTION

Carbazole and its dibenzopyrrole derivatives are recalcitrant heterocyclic aromatic compounds and potentially pollutants, commonly found in petroleum and other fossil fuels (Benedik et al., 1998). The presence of these nitrogenated compounds, of which carbazole is one of the major species, is characteristic of Brazilian oils and deleterious to the refining process. The coupling of nitrogenated compounds biodegradation pathways to the expensive hydrotreating processes used for N and S removal (HDN and HDS, respectively) is a strategy of current great biotechnological interest. Due to the high selectivity and mild conditions of most bioprocesses, they could be envisaged as alternative to hydrotreating processes that employ high temperature and pressure, as well as alter other petroleum constituents (Benedik et al., 1998). Some carbazole-degrader bacteria, as *Pseudomonas* sp., able to grow in carbazole as sole carbon and nitrogen source, have been described in the literature. The general carbazole degradation pathway to anthranilic acid involves two different dioxygenases (carbazole and 2'-aminobiphenyl-2,3-diol) and a hydrolase, whose genes are present in an 6kb operon into a megaplasmid found in those bacteria (Sato et al. , 1997[a,b]).

The second pathway enzyme, 2'-aminobiphenyl-2,3-diol 1,2-dioxygenase (CarB), is an extradiol dioxygenase that catalyzes the *meta*-cleavage of the cathecolic ring of the compound 2'-aminobiphenyl-2,3-diol to produce 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoic acid (HOADA). This enzyme is encoded by both *carBa*

* Author for correspondence

and *carBb* genes and is probably an $\alpha_2\beta_2$-heterotetramer. The catalytic site is composed by a ferrous ion present in a conserved LigB-domain in the subunit CarBb (Iwata et al., 2003). CarBa did not present significant homologies to other nucleotide or amino acid sequences (Sato et al., 1997[a]). The schematic *meta*-cleavage reaction is presented in Fig. 1.

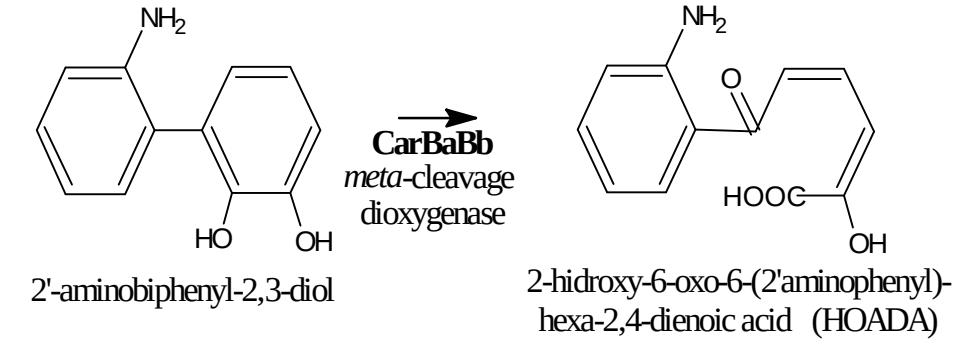

Figure 1 - *Meta*-cleavage catalyzed by CarB (2'-aminobiphenyl-2,3-diol 1,2-dioxygenase). 2'-aminobiphenyl-2,3-diol is the product of carbazole conversion by carbazole dioxygenase (CarA).

Recently, the *meta*-cleavage enzymes from *Pseudomonas* sp. CA10 and LD2 were cloned and expressed (Sato et al., 1997[a], Iwata et al., 2003; Riddle et al., 2003). The recombinant DNA technology permits to enhance protein concentration and to control the expression levels, which is indispensable for obtaining high concentrations of biocatalysts, proteins with biological activities and with high economic values for biotechnological applications. The use of enzymes that can tolerate extreme conditions of temperature, pressure, solvents and pH is strategic for bioremediation and biorefining processes. However, no homologue carbazole biodegradation enzymes were found in thermophilic microorganisms deposited in GenBank (Larentis et al., 2002). A promising approach is the expression of these mesophilic enzymes into extremophilic hosts. For instance, the two-component enzyme CarBaBb from *Sphingomonas* sp. GTIN11 was expressed in the thermophile *Thermus thermophilus* (Park et al., 2004). Nevertheless, the expression of heterologous genes in thermophiles is incipient, and more studies are required to improve the expression levels.

In this work, the 2'-aminobiphenyl-2,3-diol 1,2-dioxygenase from *P. stutzeri* was cloned using the site-specific recombination system and expressed in high levels in *E. coli*. This is a versatile cloning approach because it could provide high efficiency and fidelity cloning, independent of vector function or host (Hartley et al., 2000). Thus, it could be visualized as a cloning system for expression in thermophiles.

MATERIALS AND METHODS

P. stutzeri total DNA extraction

Genes encoding 2'-aminobiphenyl-2,3-diol 1,2-dioxygenase were obtained from *P. stutzeri* ATTC 31258 (Hisatsuka and Sato, 1994) total DNA. The genomic DNA was extracted with Wizard®SV Genomic DNA Purification System (Promega).

PCR

CarBa and *carBb* genes were amplified by PCR with *P. stutzeri* genomic DNA as template. Primers were made as described for *P. resinovorans* CA10 deposited in GenBank (Sato et al., 1997[a,b]) and adding CACC sequence on direct primer 5', before start codon for entry vector ligation. PCR was performed using a PTC100 thermal cycler (MJ Research) with Platinum® *Pfx* DNA Polymerase (Invitrogen). Salts (KCl and $MgCl_2$), dNTPs and primers concentrations, as well

as the cycling conditions, were used as described in Shepherd and Lloyd-Jones (1998). PCR product was analyzed by 1% agarose gel electrophoresis and eluted with ultrapure water from 1% low melt agarose gel with phenol/chloroform in the final concentration of 10ng/μL to clone into TOPO/GATEWAY™ system (Invitrogen).

Cloning of genes encoding CarB

The 1082bp PCR product of *CarBaBb* genes was ligated to the kanamycin-resistant 2580bp Entry Vector pENTR/D-TOPO® (Invitrogen), through the overhang sequence GTGG ligation to primer complementary sequence CACC by the action of topoisomerase (Heyman et al., 1999). This vector presents the 100bp- *att*L1 and *att*L2 recombination sites to produce T7 promoter His-tagged Expression Vector by the action of LR Clonase (Invitrogen) in the reaction with 125bp- *att*R1 and *att*R2 sites presented in the 6354bp plasmid pDEST™17 (Invitrogen), that presents ampicillin resistance (Hartley et al., 2000). His-tagged recombinant proteins are used for purification in Ni columns. The cloning strategy employed for *carBaBb* using topoisomerase and site-specific recombination is shown in Fig. 2.

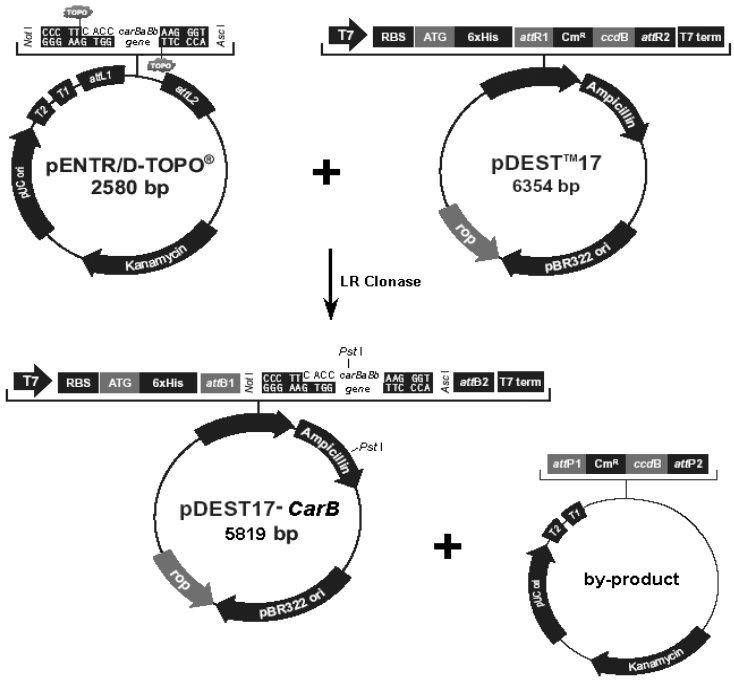

Figure 2 - Cloning strategy employed for *carBaBb* using topoisomerase and site-specific recombination.

Plasmid DNA was prepared from *E. coli* cells by alkaline lysis (Sambrook et al., 1989). The cloning confirmations were made by PCR (conditions described in previous section), plasmid restriction with endonucleases *Not*I (GC▾GGCCGC) for Entry Vector ligated to *CarBaBb* and *Pst*I (CTGCA▾G) for Expression Vector, and by sequencing. Restriction endonucleases were used according to the manufacturer's instructions (Amersham Biosciences). Construction of plasmids was analyzed by agarose gel electrophoresis. DNA sequencing was performed using Cycle Sequency Big Dye Terminators in ABI 377 DNA Sequencer (Applied Biosystems).

***E. coli* transformation**

E. coli DH10B was used as host for plasmid DNA cloning and sequencing. *E. coli* BL21-SI was used for salt-inducible protein expression under the control of T7 promoter and protease deficient for minimizing heterologous protein degradation (Bhandari and Gowrishankar, 1997). Cells were previously submitted to ultrapure water washes (Dower et al., 1988) and stored at -70°C. Hosts

transformations were made by electroporation by a 5ms electric discharge with 1.80kV, 25μF and 200Ω using Gene Pulser® II (Bio-Rad) and selection with the appropriate antibiotic medium plates, standing overnight at 37°C.

Cells cultivations

E. coli DH10B was cultivated at 37°C in LB (NaCl 1%, bactotryptone 1% and yeast extract 0.5%) and BL21-SI was grown in the same medium lacking NaCl (LBON) with the appropriate antibiotics (kanamycin or ampicillin at final concentrations of 50 or 100μg/mL, respectively).

CarB expression

E. coli BL21-SI harboring recombinant His-tagged Expression Vector was cultivated in 10mL LBON medium at 37°C until reached the absorbance 0.8 at 600nm. Then, CarB expression was induced with 0.3M NaCl and incubated for 4h. 1mL samples (before salt induction and with 4h) were harvested and the pellets were stored at -20°C.

Preparation of cell extract

The pellets from 1mL samples were resuspended in GET (glucose 50mM, EDTA 10mM and Tris-HCl 25mM), sonicated and submitted to total protein concentration measurement by Bradford method (Bradford, 1976), with bovine serum albumin as standard.

SDS-PAGE

18% SDS-Polyacrilamide Gel Electrophoresis was performed with 20μg of cell extract in a Bio-Rad apparatus. Gel was stained with Coomassie brilliant blue R-250.

Determination of activity

CarB activity for the 2,3-dihydroxybiphenyl (analogous to 2'-aminobiphenyl-2,3-diol) were analyzed by spraying the *E. coli* BL21-SI colonies with recombinant plasmids on plates and visually observing the formation of the yellow metabolite 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) (Sato et al., 1997[a]; Riddle et al., 2003; Park et al., 2004).

RESULTS AND DISCUSSION

A 1082bp gene was amplified by PCR using a proofreading DNA polimerase, corresponding to the expected size for *carBaBb* (Fig. 3). This PCR product was cloned into pENTR/D-TOPO® vector and the selected kanamycin-resistant colonies were analyzed by PCR and restriction enzyme digestion. From 12 selected colonies, 8 were confirmed as positive for *carBaBb* gene product ligation.

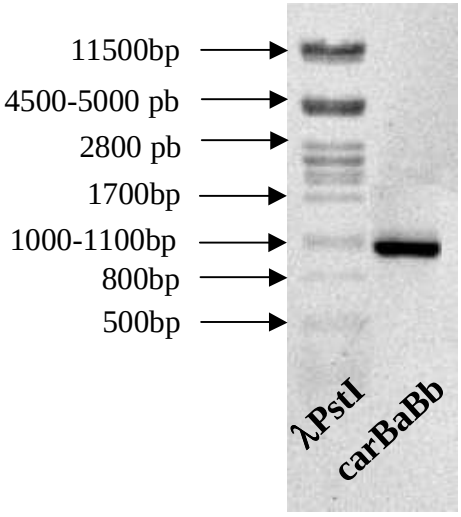

Figure 3 - Agarose gel (1%) of PCR *carBaBb* amplification product (~1100bp confirmed with λ*Pst*I size ladder).

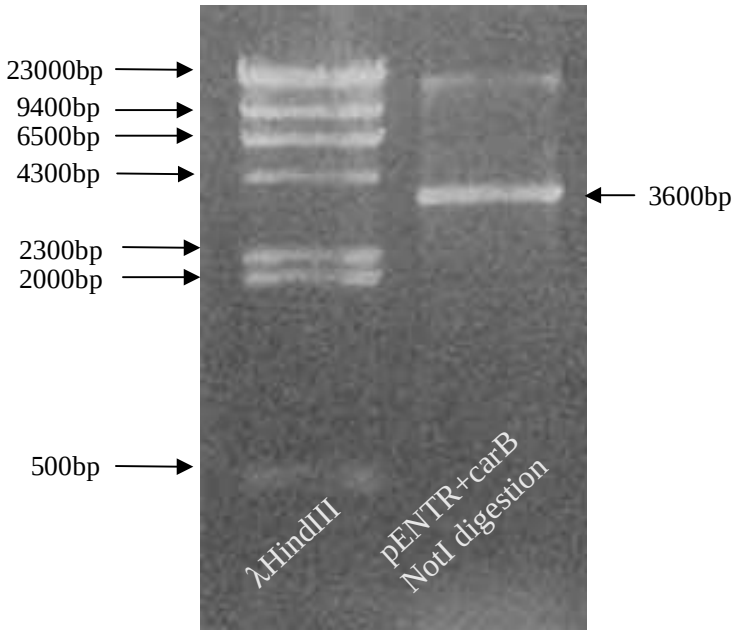

Figure 4 - 1% agarose gel with ~3600bp corresponding to restriction of positive pENTR/D-TOPO® + *carBaBb* clones with *Not*I (size confirmed with λ*Hind*III DNA ladder).

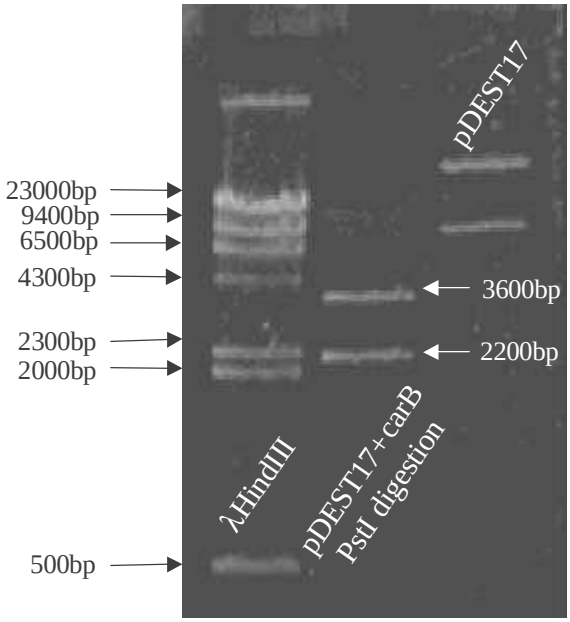

Figure 5 - 1% agarose gel with *Pst*I restriction of constructed CarB expression plasmid (two bands of 3.6kb and 2.2kb confirmed with λ*Hind*III DNA size ladder).

These positive clones were confirmed by the 1082bp band amplification by PCR and also by plasmid linearization with *Not*I in the position 673 of pENTR/D-TOPO® vector, producing a 3600bp fragment in agarose gel (Fig. 4).

A confirmed plasmid pENTR/D-TOPO® ligated to *carBaBb* gene was chosen for sequencing and submission to recombinant reaction and production of expression vector. The sequencing confirmed the *carBaBb* gene. Ampicillin resistance selected the transformant colonies and 12 of them were analyzed by PCR and *Pst*I restriction (the enzyme site is presented in position 3116 of pDEST™17 and 181 of *carBaBb* gene). *Pst*I restriction analysis confirmed the production of two bands: 3600bp and 2200bp for all selected clones, as well as the confirmation of the 1082bp amplification by PCR. These results confirmed the high specificity of recombinases reaction, as 100% of analyzed colonies were positive, as indicated in the literature (Hartley et al., 2000). The *Pst*I restriction for the 5819bp pDEST17+c*arB* plasmid is shown in Fig. 5.

The proteins were expressed by salt induction for 4h. SDS-PAGE showed two bands of 29 and 10kDa, corresponding to CarBb and CarBa expected protein sizes, respectively (Fig. 6). Expression results showed the enrichment of the target protein in the cell extract and tests on plates confirmed the enzymatic activity. Expression levels obtained for site-specific recombination system were similar to those obtained for well-known pUC cloning system (Iwata et al., 2003). Therefore, the site-specific recombination system was confirmed to be a simple and rapid two-step fidelity cloning process for proteins overexpression, instead of the use of traditional restriction endonucleases and ligase cloning. It also takes advantage of the versatility of the recombination system to transfer the cloned gene into different vectors and hosts, as extremophiles, which is very interesting for biotechnological applications.

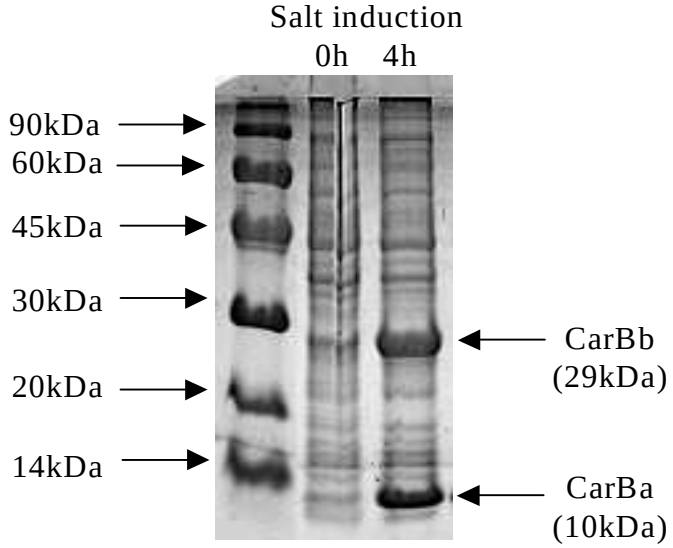

Figure 6 - SDS-PAGE of CarBa and CarBb obtained by BL21-SI cell extract samples without and with 4h of salt induction.

Further steps would include the use of cloning system to obtain all carbazole degradation pathway enzymes and to improve enzymatic expression by scaling-up the growth of recombinant cells, allowing applications in nitrogen biodegradation.

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RESUMO

Carbazol e seus derivados são compostos nitrogenados aromáticos, presentes comumente em petróleo e potencialmente poluentes. A rota de biodegradação de carbazol a ácido antranílico em Pseudomonas sp. é composta por três enzimas responsáveis, respectivamente, pelas reações de dioxigenação angular, meta-clivagem e hidrólise. A segunda enzima da rota, 2'-aminobifenil-2,3-diol 1,2-dioxigenase (CarB), codificada por dois genes (carBa e carBb), é um heterotetrâmero com atividade catalítica na quebra do anel catecol do susbtrato na posição meta. Neste trabalho, foi clonado o produto de PCR de 1082pb correspondente aos genes carBaBb da bactéria degradadora de carbazol Pseudomonas stutzeri ATCC 31258. A estratégia de clonagem empregada foi a de recombinação sítio-específica e a construção dos plasmídeos foi confirmada por PCR, digestão com enzima de restrição e seqüenciamento. A enzima ativa foi expressa em altas concentrações em vetor pDEST™17 com cauda de histidina e promotor T7 em Escherichia coli BL21-SI com indução por NaCl durante 4h.

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