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Research Article

Characterization of a Naphthalene Catabolic Gene Cluster and Heterologous Expression of Naphthalene Dioxygenase Genes from *Rhodococcus ruber* OA1

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Abstract

Background and Objective: Naphthalene is a Polycyclic Aromatic Hydrocarbon (PAH) pollutant which is toxic and widespread in the environment. *Rhodococcus ruber* OA1 is a gram-positive bacterium which can utilize naphthalene as the sole carbon and energy source for growth, but no molecular biological research has been conducted on its naphthalene catabolic gene cluster. The objective of this study was to characterize the naphthalene catabolic gene cluster and naphthalene dioxygenase (NDO) from *R. ruber* OA1. **Methodology:** The gene cluster for naphthalene degradation from *R. ruber* OA1 was identified by *in situ* hybridization and sequence analysis. Then the genes *narAa* and *narAb* encoding the large and small subunit of naphthalene dioxygenase, as part of the cluster were sub-cloned and heterologously expressed to confirm their functions. **Results:** *In situ* hybridization and sequence analysis revealed a 43,754 bp fragment in the cloned plasmid, including the gene cluster *narAaAbBC* encoding the proteins responsible for converting naphthalene to salicylaldehyde. After the *narAa* and *narAb* genes were sub-cloned and expressed, the enzymatic activity was assayed, which revealed that *narAa* and *narAb* were functional in *Escherichia coli* BL21 (DE3). Sub-cloning and heterologous expression of the *rub1* gene revealed that the *rub1* gene product was not indispensable for naphthalene degradation. **Conclusion:** A gene cluster for naphthalene biodegradation was identified in *R. ruber* OA1 for the first time. The *narAa* and *narAb* genes were sub-cloned and heterologously expressed to confirm their functions.

Key words: Biodegradation, naphthalene, *Rhodococcus ruber*, gene cluster, naphthalene dioxygenase

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Naphthalene, a model compound of Polycyclic Aromatic Hydrocarbons (PAHs), is ubiquitous environmental pollutant, generated primarily during the incomplete combustion of organic materials[1]. Due to its toxicity, various techniques have been investigated to eliminate the pollutant, of which microbial decontamination is the most effective and eco-friendly way[2].

Up to date, numerous naphthalene degrading bacterial species have been isolated and characterized[3-5]. The genetics of naphthalene metabolism have been studied in detail in Gram-negative *Pseudomonas* bacteria and diverse naphthalene catabolic genes and pathways were summarized in a number of excellent review articles[6-8]. Usually, the initial step in the aerobic metabolism of naphthalene involves the hydroxylation of an aromatic ring via naphthalene dioxygenase (NDO), resulting in the formation of cis-1,2-dihydroxy-1,2-dihydronaphthalene (cis-naphthalene dihydrodiol). A typical NDO is a multicomponent enzyme system consisting of an iron-sulfur flavoprotein ($ferredoxin_{NAP}$ reductase), $ferredoxin_{NAP}$ and terminal iron-sulfur-containing oxygenase (ISP_{NAP}) which has an $\alpha_2\beta_2$ subunit composition, with each α subunit containing a Rieske-type [2Fe-2S] center[9,10]. The a subunit of NDO, more conservative than other components and decisive for substrate specifity, has been used to generate probes targeting the specific genes[11].

Compared to Gram-negative bacteria, only limited information on naphthalene degradation in Gram-positive bacteria is available[12]. Research on Gram-positive bacteria, especially the genus *Rhodococcus*, showed that they possess extremely wide catabolic versatility, which makes them suitable for application in remediation of PAH contaminated environments[13-15]. The genes organization analysis of three *Rhodococcus* strains has shown that the genes in *nar* region are not arranged into a single operon and a number of genes which were shown to be essential for naphthalene catabolism in Gram-negative bacteria are not located in the region[7,14,16]. It means that Gram-positive strains are likely to possess distinct naphthalene-degradation mechanisms and the genetic basis for naphthalene degradation in Gram-positive strains requires further research.

Rhodococcus ruber strain OA1 (CCTCC AB2015073) is an aromatic compound degrading bacterium isolated from a pharmaceutical wastewater treatment plant in Zibo, China. It can utilize aromatic compound, such as naphthalene, salicylate and catechol as sole carbon and energy source for growth[17]. Although several Gram-positive bacteria have been investigated on naphthalene catabolic gene cluster[12,18], no molecular biological research has been conducted on naphthalene catabolic gene cluster in *R. ruber*. In the present study, genes involved in naphthalene metabolism were identified from *R. ruber* OA1 according to the methods of Kimura *et al.*[19] and Zhang *et al.*[20]. The objective of this research was to study the expression of genes encoding NDO in *R. ruber* OA1 and enzymatic activity. The research will enrich knowledge on the genetic basis of naphthalene biodegradation in Gram-positive bacteria.

MATERIALS AND METHODS

Bacterial plasmids and strains: *Rhodococcus ruber* strain OA1 (CCTCCAB2015073) was used in this study as a source of naphthalene catabolic gene cluster. CopyControl™ HTP Fosmid Library Production Kit and EPI300 *E. coli* plating strain were purchased from Epicentre (Madison, WI, USA). *E. coli* strain Trans1-T1 and *pEASY*-Blunt E1 Expression Kit were purchased from Transgen (Beijing, China).

Media and growth conditions: *Rhodococcus ruber* OA1 was cultivated in liquid Luria-Bertani (LB) medium when needed. EPI300 $T1^R$ plating strain was cultivated at 37°C in LB broth containing 10 mM $MgSO_4$ and 0.2% maltose. Recombinant proteins were overexpressed in *E. coli* strain Trans1-T1 and grown at 37°C, with shaking, in LB broth containing 100 g mL^{-1} ampicillin.

Construction of genomic fosmid library: A genomic library was constructed from strain *R. ruber* OA1 using the CopyControl™ Fosmid Library Production Kit with pCC2FOS™ Vector and the phage T1-resistant EPI300 *E. coli* plating strain. Firstly, approximately 40 kb genomic DNA was extracted from *R. ruber* OA1 cells, as described in Metagenomic DNA Isolation Kit For Water (E picentre, USA). The DNA fragments were then end-repaired, ligated into the fosmid vectors, packaged and plated on *E. coli* EPI300 $T1^R$ following kit instructions. The copycontrol fosmid clones were selected at 37°C on LB plates containing chloramphenicol (12.5 μg mL^{-1}). The titer of the packaged copycontrol fosmid clones was also determined and the genomic library were stored at -70°C.

***In situ* hybridization:** Based on the gene sequence of the large subunit of naphthalene dioxygenase (*narAa*) of the naphthalene-degrading strain *Rhodococcus* sp. DB11 (GenBank No. ADM94823.1), a pair of primers (NAR-F 5′-TACAGCAACACGGGAAGTCT-′3 and NAR-R 5′-TCACTGCGGTCCAGAACAAT-3′) was designed to amplify

the *narAa* gene of *R. ruber* OA1. Using the genomic DNA of *R. ruber* OA1 as the template, a digoxigenin (DIG)-labeled nucleotide probe targeting the large subunit gene of the naphthalene dioxygenase (DIG-NAR) was generated by PCR (Applied Biosystems, Singapore) using a PCR DIG Probe Synthesis Kit (Roche, USA).

Colonies from the OA1 genomic library were transferred to membranes (GE Healthcare, UK), followed by denaturation of colonies. Hybridizations of the genomic fosmid library with the DIG-NAR probe were carried out according to the manufacturer's instructions. Genes homologous to *narAa* were detected with DIG Nucleic Acid Detection Kit. Positive colonies were identified based on the location of the blue spots on the membranes and were then transferred to LB broth containing chloramphenicol (12.5 μg mL^{-1}).

Sequencing and bioinformatics analysis of the naphthalene degrading gene cluster: According to the results of *in situ* hybridization, positive colonies were screened and confirmed by PCR with the primers NAR-F and NAR-R and sequenced by Sanger sequencing. The confirmed positive clone was sequenced by next-generation high-throughput sequencing using an Illumina HiSeq 2000 sequencer[21]. The sequences were assembled with the SOAPdenovo program and annotation was performed using the KEGG and COG databases.

Cloning and heterologous expression of *narAaAb* and *rub1*: Based on the sequence of the *nar* gene cluster from *R. ruber* OA1, PCR primers were designed by Primer Premier 5. The primer set consisting of *narA-1*F 5'-ATGCTGAGCAACGAACTCC-3' and *narA-1*R 5'-TCACATGATCAGGGCGAGG-3' was used to amplify the putative NDO and the primer set of *rub1*-F 5'-ATGTCATCCCAGCTCTCCCGTTAC-3' and *rub1*-R 5'-CTAATGAAGATTCTGATCGCGACG-3' was used to amplify the putative rubredoxin from *R. ruber* OA1. Plasmid DNA extracted from the positive clone was used as the template. TransStart FastPfu DNA polymerase was used according to the manufacturer's instructions (Trans Gen Biotech, China). The PCR products were purified with the EZ-10 Spin Column Gel Extraction Kit (Sangon, China) before being ligated into the *pEASY*-Blunt E1 vector at 37°C for 10 min. Recombinant plasmids were transferred to *E. coli* Trans1-T1 cells. Single colonies grown overnight on LB plates containing ampicillin (100 mg mL^{-1}) were inoculated into 5 mL LB broths (with ampicillin 100 mg mL^{-1}) and incubated overnight at 37°C.

Positive colonies of the correct orientation and size were identified by PCR and sequencing (Biosune, China). The recombinant plasmid was extracted and transferred to *E. coli* BL21 (DE3). 0.5 mL of the confirmed recombinant strain, grown overnight, was inoculated into 50 mL of LB broth containing ampicillin and shaken at 37°C to an optical density at 600 nm (OD_{600}) of approximately 0.5, at which point 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG) was added to induce the expression of the target gene and the cells were grown for an additional 4 h at 30°C. Cells were harvested by centrifugation (8,000× g, 20 min, 4°C), washed twice with PBS (PH 7.0) and stored at -20°C.

Preparation of cell extract: Cell extracts were prepared by resuspending cell pellets in 5 mL PBS (pH 7.0) and disrupted by ultrasonication in an ice-water bath for 10 min with pulsing on 5 sec and off 10 sec (40 W, SONICS, USA). The supernatant and precipitates were separated by centrifugation at 13,000× g for 20 min at 4°C. The obtained membrane-free supernatant was referred to as cell extracts and used as the enzyme solution for further assays. The supernatant protein and precipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide).

Enzyme assay: The catalytic activity of *narAaAb* depended on the presence of NADH in the reaction mixture. The NDO activity was estimated spectrophotometrically (Thermo, USA) by detecting the absorbance decrease of NADH at 340 nm[22]. One unit of enzyme activity was defined as the amount of enzyme required for the conversion of 1 mM of NADH min^{-1} at 37°C. Reaction mixtures (200 μL) contained 0.1 M PBS (pH 7.0), 2.5 mM NADH and 25 μL naphthalene (200 mg L^{-1}) in ddH_2O. To activate the enzyme, 0.1 mM $(NH_4)_2Fe(SO_4)_2$ was added to the reaction system. The reaction was initiated by the addition of an appropriate amount of the cell extract and incubated at 37°C for 20 min. Protein concentration was quantified using Bradford assays. In addition, the absorption of the mixture was recorded between 280 and 500 nm using a microplate spectrophotometer. Spectra were recorded at 0, 5, 10, 15 and 20 min following the addition of an appropriate amount of cell extract.

To check whether Rub1 was indispensable part of NDO or not, the specific activity of NarAaAb-Rub1 was estimated spectrophotometrically (Thermo, USA) by a decrease in the absorbance of NADH at 340 nm in 0.1 M PBS, pH 7.0, containing 2.5 mM NADH, 0.1 mM $(NH_4)_2Fe(SO_4)_2$ and 200 mg L^{-1} naphthalene. Assays were initiated by the addition of an appropriate amount of *narAaAb* and *rub1* crude enzyme.

Phylogenetic analyses: A phylogenetic tree was constructed based on the BLAST results of searches against the NCBI database using the NarAa and NarAb. Multiple alignments were obtained by ClustalX 1.83. Phylogenetic trees were constructed using the Neighbor-joining method in MEGA5.

Accession number: The nucleotide sequence of the *nar* gene cluster of *R. ruber* OA1 was submitted to GenBank under the accession number: KY072804.

RESULTS

Genomic library construction and screening: A genomic library was constructed and the titer of the packaged copycontrol fosmid clones was 5.0×10^4 CFU mL^{-1}. A 212 bp DIG-NAR probe was generated and the library was screened by *in situ* hybridization using the DIG-NAR probe. A positive clone was obtained containing a gene homologous to *narAa* gene.

Sequencing and bioinformatics analysis of the positive plasmid DNA: Plasmid DNA was extracted from the positive clone and sequencing and bioinformatic analysis revealed the presence of a 43,754 bp fragment in the plasmid, which included a gene cluster encoding the proteins involved in the conversion of naphthalene to salicylaldehyde (*nar* gene cluster) and a *mhp* gene cluster encoding the proteins for conversation of hydroxyphenylpropionic acid to acetyl-CoA. The sizes, locations and directions of the genes in the plasmid are shown in Fig. 1. The *nar* cluster was organized into the following manner: *fixA* encoding the putative flavoprotein, *rub1* encoding the putative rubredoxin, two regulator genes (*narR1* and *narR2*), *orf7* encoding a protein of unknown function, two genes encoding the large (*narAa*) and small (*narAb*) subunits of NDO, *orf1* encoding a protein of unknown function, a gene encoding the cis-naphthalene dihydrodiol dehydrogenase (*narB*) and a gene encoding hydratase-aldolase (*narC*). The *mhp* cluster was organized into the gene cluster *mhpDCRABFE*.

Notably, sequence analysis revealed the putative products of the *narAa* and *narAb* genes had only 26 and 25% amino acid identity with the corresponding subunits (accession number AAO64274 and AAO64275) from *P. putida* NCIB 9816-4, while they had 100% nucleotide identity to the corresponding genes from *Rhodococcus* sp. I24, which converts indene to *cis*-(1R,2S)-indandiol and keto-OH-indan[23]. It is worth noting that two components of the electron transfer chain, designated as *fixA* and *rub1*, were founded upstream of the two *narR* genes (Fig. 1).

Heterologous expression of *narAaAb* and *rub1* genes: A 1929 bp fragment containing the *narAa* and *narAb* genes was amplified from the positive plasmid DNA from *R. ruber* OA1. The 1,407 bp *narAa* gene encoded a protein of 468 amino acids, while the *narAb* gene encoded a protein of 172 amino acids. SDS-PAGE analysis was used to check the expression of NDO in *E. coli* BL21(DE3) containing *pEASY*-Blunt E1-narAaAb. Compared to the negative control of *E. coli* BL21(DE3)(*pEASY*-Blunt E1), two apparent protein bands of 55 kDa and 23 kDa were presented (Fig. 2), which were consistent with the size of the NDO α subunit and β subunits from *Rhodococcus* sp. NCIMB12038[24].

Interestingly, a *rub1* gene (28711-28971) located 2.9 kb upstream of *narAa* had 98% nucleotide identity to the *nidE* gene for rubredoxin from *Rhodococcus opacus* TKN14 (Accession No. AB206671). As a component of a typical NDO for converting naphthalene and various (di) methylnaphthalenes into their corresponding cis-dihydrodiols from TKN14, the rubredoxin was found to be indispensable for the ring dihydroxylation action of the naphthalene skeleton[25]. In order to ascertain whether the protein product of *rub1* from OA1 had similar function as rubredoxin, the 261 bp *rub1* gene was obtained by PCR. The recombinant plasmid transformed to *E. coli* BL21(DE3) was induced by IPTG, then SDS-PAGE revealed a protein band with an apparent molecular mass of 9 kDa.

Catalytic activity of NDO: The activity of *narAaAb* was estimated by a decrease in the absorbance of NADH at 340 nm. The specific activity of NDO in the cell extracts was found to be 20.29 U mg^{-1} of protein. Partial wavelength scanning of the reaction solution between 280 and 500 nm revealed a peak corresponding to the decreased absorption of NADH at 340 nm (Fig. 3a).

Fig. 1: Organization of naphthalene degradation gene cluster (KY072804) from *Rhodococcus ruber* strain OA1
Arrows indicate the sizes, locations and directions of the coding genes. Straight lines indicate the non-coding regions between the coding genes

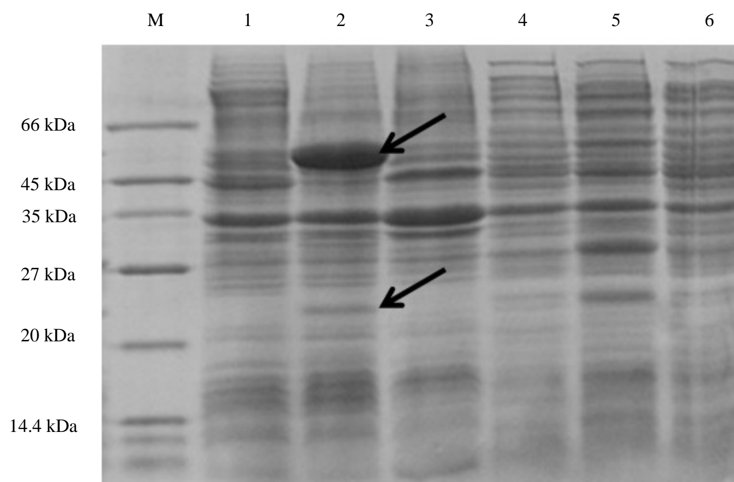

Fig. 2: SDS-PAGE analysis of the expressed proteins from *pEASY*-Blunt E1-*narAaAb*

M: protein molecular weight marker, Lane 1,4: The precipitated proteins and supernatant proteins of *E. coli* BL21 (DE3) (*pEASY*-Blunt E1) cells, respectively, Lane 2,5: The precipitated proteins and supernatant proteins of *E. coli* BL21 (ED3) (*pEASY*-Blunt E1-*narAaAb*) cells, respectively, which were induced with 0.5 mM IPTG, Lane 3,6: The precipitated proteins and supernatant proteins of non-induced *E. coli* BL21 (ED3) (*pEASY*-Blunt E1-*narAaAb*) cells, respectively. The arrows indicate the protein bands of 55 kDa and 23 kDa for α subunit and β subunit of NDO, respectively

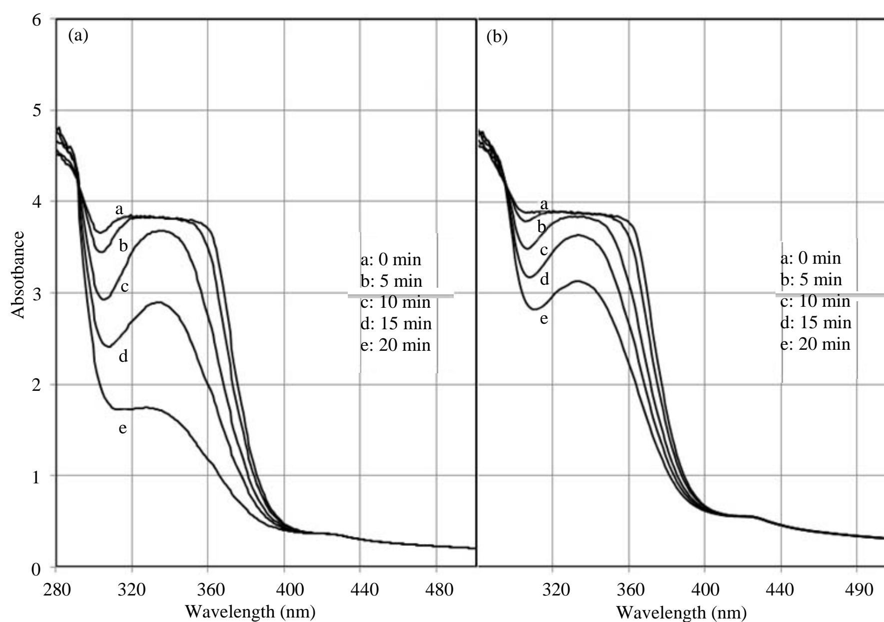

Fig. 3: Wavelength scanning of the reaction mixtures observed between 280 and 500 nm by microplate spectrophotometer, (a) Reaction mixtures contained 0.1 M PBS (pH 7.0), 0.1 mM $(NH_4)_2Fe(SO_4)_2$, 2.5 mM NADH and an appropriate amount of cell extract from *E. coli* BL21 (ED3) (*pEASY*-Blunt E1-*narAaAb*), (b) Reaction mixtures contained an appropriate amount of cell extract from *E. coli* BL21 (ED3) (*pEASY*-Blunt E1-*rub1*) besides the same component as a

The enzymatic activity assay of NarAaAb-Rub1 showed that its specific activity was 7.33 U mg^{-1} of protein, which was lower than with only NarAaAb. Partial wavelength scanning of reaction mixture showed a change in the spectra revealed a peak of decreasing absorption at 340 nm (Fig. 3b), which was different from that with only NarAaAb.

Phylogenetic analysis: The BLAST analysis against the NCBI database showed that the nucleotide sequence of putative NDO genes from *R. ruber* OA1 had high similarity (91-99% nucleotide identity) to *narAa* and *narAb* genes of large and small subunits of naphthalene 1,2-dioxygenase from other *Rhodococcus* spp.. The homologous amino acid

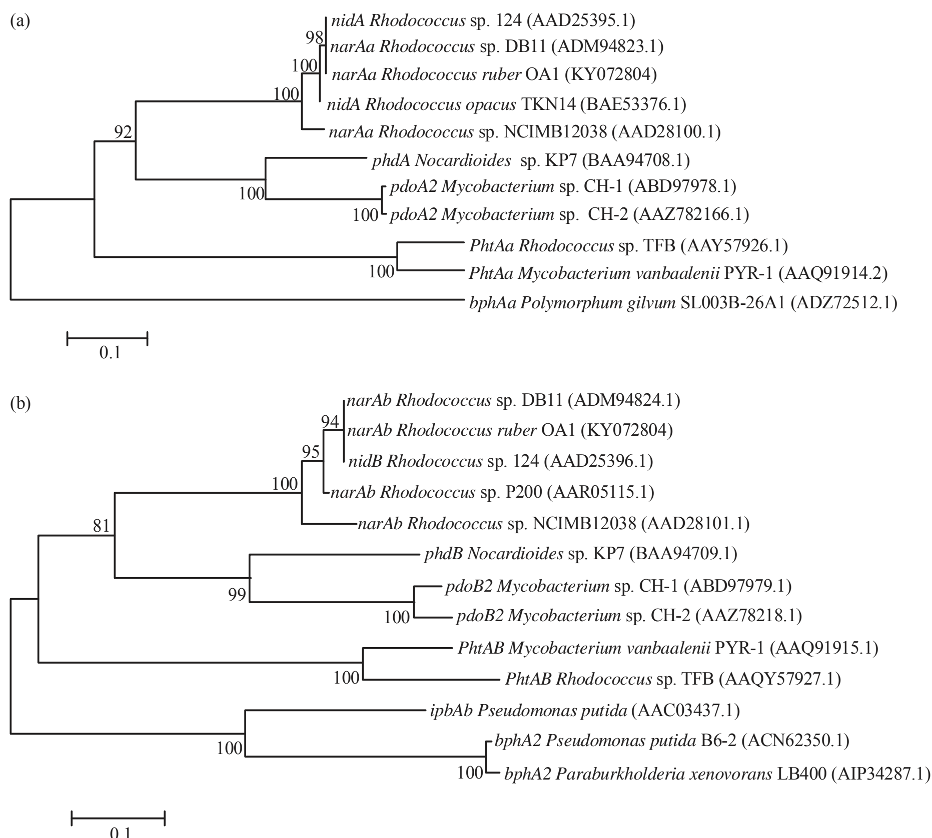

Fig. 4(a-b): Phylogenetic trees of *narAa* and *narAb* of *R. ruber* strain OA1 with related proteins, (a) Large (α) subunit of naphthalene 1,2-dioxygenase, *narAa*, (b) Small (β) subunit of naphthalene1,2-dioxygenase, *narAb*

The accession numbers of the sequences in GenBank are shown in parentheses. *BphAa* from *Polymorphum gilvum* SL003B-26A1 was used as outgroup for *narAa*. *IpbAb* from *Pseudomonas putida* and *bphA2* from *Pseudomonas putida* B6-2 and *Paraburkholderia xenovorans* LB400 were chosen as three outgroups for *narAb*

sequences were aligned and the resulting phylogenetic trees are shown in Fig. 4. The phylogenetic tree showed that NarAa of OA1 clustered with NarAa of *Rhodococcus* sp. DB11 and *Rhodococcus* sp. NCIMB12038 and NidA of *Rhodococcus* sp. I24 and *Rhodococcus opacus* TKN14 (Fig. 4a). Furthermore, NarAa of OA1 revealed close genetic distance with three other Gram-positive bacteria, including *Nocardioides* sp. KP7, *Mycobacterium* sp. CH-1 and *Mycobacterium* sp. CH-2. The *narAb* gene, located downstream of *narAa*, encoded a product with high sequence homology to the small (β) subunit of naphthalene 1,2-dioxygenase of *Rhodococcus* sp.. The phylogenetic tree using the *narAb* fragment and the published homologous amino acid sequences showed similar subgroups as *narAa* (Fig. 4b).

DISCUSSION

In this study, a new naphthalene catabolic gene cluster *nar* from *R. ruber* OA1 was identified by screening a genomic library and by further functional determination. The organization of the genes encoding naphthalene degrading enzymes in strain OA1 showed low similarity (25-38% amino acid identity) to those in Gram-negative bacteria, while it was consistent to some degree with those in other Gram-positive bacteria[12,26]. The characteristic naphthalene catabolic genes from *Rhodococcus* may help to reveal the unique naphthalene degradation mechanism which is different from that in *Pseudomonas*. Therefore, further research on the genetic basis of PAH degradation for Gram-positive degrader is required.

Gene organization comparison of gene region indicated that the *nar* region from strain OA1 showed a high similarity to those present in *Rhodococcus* and *Gordonia* (Fig. 5). The genes encoding NDO large and small subunits (*narAaAb/nidAB*), cis-naphthalene dihydrodiol dehydrogenase (*narB/nidC*) and putative aldolase (*narC/nidD*) were present in the analyzed strains. The *narC* gene shared high homology with the putative aldolase gene from the analyzed strains except strain NCIMB12038. The *narR1* and *narR2* genes from strain OA1, NCIMB12038 and *Gordonia* CC-NAPH129-6

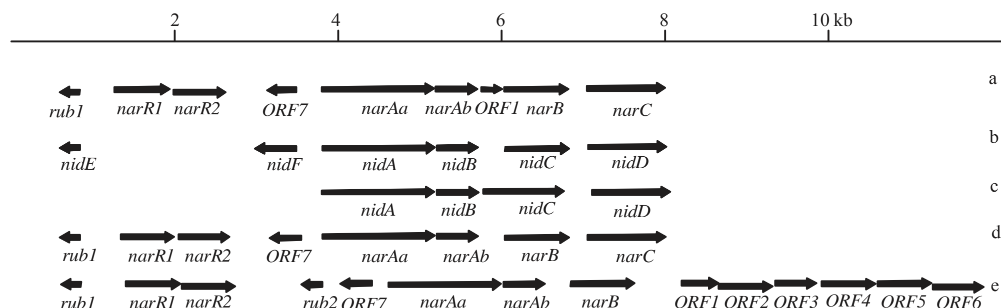

Fig. 5: Organization of the sequenced genes aligned to related naphthalene degradation gene clusters

Arrows indicate the whole genes included in transcription units, a: *Rhodococcus ruber* OA1 (GenBank accession number KY072804), b: *Rhodococcus opacus* TKN14 (GenBank accession number AB206671.1), c: *Rhodococcus* sp. I24 (GenBank accession number AF121905.1), d: *Gordonia* sp. CC-NAPH129-6 (GenBank accession number GQ848233.3), e: *Rhodococcus* sp. NCIMB12038 (GenBank accession number AF082663.3)

showed high homology to putative naphthalene degradation regulator proteins that belong to GntR-like regulator protein family[27]. A *rub1* gene was found upstream of the two subunits of NDO except *Rhodococcus* sp. I24. The putative protein encoded by *rub1* showed approximately 30% sequence identity to a putative rubredoxin that is a key component of multiple-degradative-enzyme systems[28,29]. A two components electron transport chain consisting of rubredoxin and rubredoxin reductase has been previously reported for alkane hydroxylase in *Pseudomonas* and *Rhodococcus*[29,30]. The putative proteins encoded by *rub1* and *rub2* in *R. opacus* R7 exhibited a high level of similarity to rubredoxin, which is involved in electron transfer[12]. Comparative analysis showed that the *rub2* gene from strain NCIMB12038 had no identity to genes in any other strain analyzed. Since no reductase or ferredoxin components for the typical NDO were found in the strains analyzed, it seems possible that rubredoxin might work with ISP_{NAP} as part of the dioxygenation system instead of ferredoxin and an unknown host-derived reductase may function as a ferredoxin reductase to transfer electrons from NAD(P)H to ISP_{NAP} with rubredoxin[25]. However, how the rubredoxin is involved in electron transfer in the dioxygenation system has not yet been identified.

Around the *nar* region, a *fixA* gene was found upstream of the *rub1* gene (Fig. 1). The BLAST analysis of the deduced amino acid sequence of *fixA* showed high identity with the electron transfer flavoprotein of *Rhodococcus* spp.. Previous studies have shown that the reductase component of NDO from *Pseudomonas* sp. strain NCIB 9816 was an iron-sulfur flavoprotein that transfers electrons from NAD(P)H to $ferredoxin_{NAP}$[31]. This implies that *fixA* of *R. ruber* OA1 might function like an electron transfer flavoprotein, but the real function of *fixA* in *R. ruber* OA1 needs to be further investigated.

Besides, the *narAaAb* and *rub1* genes from *R. ruber* OA1 were heterologously expressed in *E. coli* BL21 (DE3). Based on enzymatic activity, it was speculated that the NDO encoded by *narAaAb* was necessary for converting naphthalene to cis-naphthalene dihydrodiol, while the rubredoxin encoded by *rub1* of *R. ruber* OA1 was not an essential component of the NDO. The results indicated that the catalytic mechanism involved in naphthalene degradation in strain OA1 might be different from that described in *R. opacus* TKN14.

CONCLUSION

In conclusion, an *nar* gene cluster consisting of genes involved in conversion of naphthalene to salicylaldehyde was characterized in *R. ruber* OA1. The *narAa* and *narAb* genes encoding putative NDO were sub-cloned and expressed in *E. coli* BL21(DE3) and the specific activity was 20.29 U mg^{-1} of protein. Further enzymatic experiments of NarAaAb-Rub1 confirmed NarAa and NarAb as the functional components of NDO in *R. ruber* OA1.

SIGNIFICANCE STATEMENT

This study describes the identification and organization of naphthalene catabolic genes that can be beneficial for revealing the genetic basis of naphthalene biodegradation in *R. ruber* OA1. The cloning and expression of genes encoding NDO will have a broad applications value in remediating environmental PAHs pollutions.

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