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Overexpression, purification and characterization of a new salicylate hydroxylase from naphthalene-degrading *Pseudomonas* sp. strain ND6

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KEYWORDS
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Summary

A new salicylate hydroxylase from naphthalene-degrading *Pseudomonas* sp. strain ND6, NahU, has been identified. The *nahU* is an isofunctional gene of the classic salicylate hydroxylase gene, *nahG*, and situated outside the transcriptional unit forming the naphthalene degradation lower pathway. Both genes, *nahU* and *nahG* of *Pseudomonas* sp. ND6, have been cloned and overexpressed in *Escherichia coli* BL21(DE3). NahU contains 429 amino acid residues and NahG contains 434 amino acid residues. SDS-PAGE analysis showed that both NahG and NahU are about 47 kDa. Both enzymes exhibit broad substrate specificities and metabolize salicylate, sulfosalicylate, aspirin, methylsalicylate, chlorosalicylate and 3,5-dinitrosalicylate. The comparison of the K_m and V_{max} values for NahG and NahU demonstrated that NahU possesses a higher binding ability to salicylate and cofactors and catalytic efficiency.

Introduction

The bacterial oxidation of naphthalene has been extensively investigated especially in *Pseudomonas* strains (Yen and Serdar, 1988), such as the archetype *Pseudomonas putida* G7, possessing the naphthalene catabolic plasmid NAH7 (Dunn and Gunsalus, 1973; Yen and Gunsalus, 1982), *P. putida* NCIB 9816-4, possessing the NAH plasmid pDTG1 (Serdar and Gibson, 1989; Simon et al., 1993; Dennis and Zylstra, 2004), and *P. stutzeri* AN10 possessing the naphthalene-dissimilatory genes in chromosome (Bosch et al., 1999a, b, 2000). Their naphthalene- degrading genes are organized in two operons: the upper pathway operon (*nah* operon) coding for the enzymes involved in the conversion of naphthalene to salicylate (*nahAaAbAcAdBFCED*), the lower pathway operon (*sal* operon) coding for the conversion of salicylate to tricarboxylic acid cycle intermediates through the *meta*-cleavage

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pathway enzymes (*nahGTHINLOMKJ*). The regulatory gene codes a single protein, NahR, acts as a positive regulator for both promoters, and salicylate functions as inducer (Schell and Wender, 1986).

The *nahG* gene, the gene most proximal to the naphthalene degradation lower pathway, codes for salicylate hydroxylase, a flavoprotein, converts salicylate to catechol (via decarboxylative hydroxylation). Mechanistic-kinetic properties of the salicylate hydroxylase have been studied extensively (Einarsdottir et al., 1988; Suzuki and Katagiri, 1981; Wang and Tu, 1984; Wang et al., 1984). Briefly, the enzyme binds salicylate and an external reductant (NADH or NADPH) in a random order pattern to form a reduced enzyme-substrate complex, and then a molecular oxygen binds to the complex for production of catechol, CO_2, and H_2O. Up to now, the *nahG* genes in several naphthalene-degrading strains have been cloned and sequenced, such as *P. putida* G7 (You et al., 1991), *P. putida* S-1 (Suzuki et al., 1996), *P. putida* KF715 (Lee et al., 1996), *P. stutzeri* AN10 (salicylate hydroxylase gene *nahG* and its isofunctional gene *nahW*, Bosch et al., 1999a, b, 2000), *Sphingomonas* sp. AJ1 (GenBank accession no. AB000564), *P. putida* NCIB 9816 (GenBank accession no. X83926), *P. putida* NCIB9816-4 (Dennis and Zylstra, 2004), *P. fluorescens* (GenBank accession no. AY048764), *P. fluorescens* NKNS3 (GenBank accession no. AY429511) and *P. fluorescens* A88 (GenBank accession no. AY43938).

Pseudomonas sp. strain ND6 is a naphthalene-degrading bacterium isolated from industrial wastewater in Tianjin, China, and its naphthalene-degrading genes have been located on plasmid pND6-1 (Zhang et al., 2000). The complete nucleotide sequence and annotation of pND6-1 have been reported (Li et al., 2004). There were two pairs of duplicate naphthalene catabolic genes in pND6-1 sequence. The *nahU* (ND016) and *nahV* (ND013) are isofunctional genes of *nahG* (ND091) and *nahF* (ND058), respectively.

In this study, the two isofunctional salicylate hydroxylase genes, *nahU* and *nahG*, from *Pseudomonas* sp. ND6 were cloned and overexpressed in *Escherichia coli*, their corresponding enzymes were purified and characterized.

Materials and methods

Bacterial strains, plasmids and culture conditions

Pseudomonas sp. strain ND6 was aerobically cultured in LB medium or minimal medium containing 0.2% naphthalene (W/V) at 30 °C (Zhang et al., 2000). *Escherichia coli* DH5α and BL21 (DE3) were used as hosts for cloning and expression of the both salicylate hydroxylase genes of pND6-1. When necessary, ampicillin was supplemented to medium at 100 μg ml^{-1}. The cloning and expression plasmid pET21b(+) were purchased from TaKaRa (China) and Novagen (USA), respectively. Naphthalene-degrading bacteria were isolated by serial batch enrichments in mineral medium containing 0.9 g l^{-1} KH_2PO_4, 6.5 g l^{-1} $Na_2HPO_4 \cdot 12H_2O$, 0.4 g l^{-1} $(NH_4)_2SO_4$, 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$, and 2 g l^{-1} naphthalene as sole source of carbon (Zhang et al., 2000). The naphthalene-degrading strains D1–D8 were isolated from industrial wastewater of Tianjin Dagang Oil Field, China. The naphthalene-degrading strains J1–J6 were isolated from activated sludge of Tianjin Jizhuangzi Wastewater Treatment Facility.

PCR and cloning of *nahU* and *nahG*

To clone the salicylate hydroxylase genes *nahG* and *nahU*, two pairs of primers were designed as follows: G-f, 5′-GGAGACATCATATGAAAAACAATAA-ACCTGGCTTG-3′, G-r, 5′-CCAA*TCTCGA*GCCCTTGAC-GTAGCACACCC-3′; U-f, 5′-GGAGACATCATATGCAAA-ATTCTACTTC TGCTCTGAA-3′, U-r, 5′-CCAAT*CTC-GA*GGGCCGCTTGCGCGC-3′ (the *Nde*I site is underlined and the *Xho*I site is italicized). PCR was performed with pND6-1 DNA as the template. pND6-1 was isolated according to reported procedures (Li et al., 2004). The amplified fragments were individually digested with *Nde*I and *Xho*I, and ligated to similarly digested pET21b(+), respectively. In the two recombined plasmids, *nahG* or *nahU* genes are under the control of T7 promoter and processes C-terminal six-His tag. The plasmids were transformed into *E. coli* DH5α and subsequently transformed into *E. coli* BL21 (DE3). The inserts of the two clones were sequenced by Sangon (Shanghai, China) to ensure that no mutation had been incorporated during the PCR. Restriction endonuclease digestions and ligations with T4 DNA ligase were done in accordance with the manufacturer's instructions. *E. coli* strains were transformed by standard procedures (Sambrook et al., 1989). PCR fragment recovery kit, restriction endonucleases and T4 DNA ligase were obtained from TaKaRa (China)

Overexpression and identification of NahU and NahG

Precultures were prepared by inocubating individual colony into 10 ml of LB medium supplemented with ampicillin (100 μg ml^{-1}) and shaken at

37 °C for 10 h. Then, the culture was diluted 100-fold in 500 ml of the same medium and shaken at 37 °C to an OD_{600} of 0.6. Protein expression was induced by adding IPTG to 10 μM. The culture was shaken overnight at 20 °C.

Preparation of cell extracts

The cells were harvested by centrifugation at 4000*g* for 10 min at 4 °C, washed with a 33 mM potassium phosphate buffer (pH 7.0), resuspended in the same buffer, and ruptured by sonication at 400 watt for 60 cycles of 5 s with 15 s intervals, after which cell debris was removed by centrifugation at 10,000*g* for 30 min at 4 °C. The clear supernatant was kept on ice and used for enzyme purification.

Purification of NahU and NahG

NahU and NahG were purified as His-tagged proteins by Ni-NTA His Bind® Resin (Novagen, USA). All subsequent procedures were carried out below 10 °C. The cell extracts were passed two times through the column to ensure maximum binding. After washing twice with 6 ml wash buffer (33 mM potassium phosphate, pH 7.0, 300 mM NaCl and 40 mM imidazole), the target protein was eluted with elution buffer (33 mM potassium phosphate, pH 7.0, 300 mM NaCl and 80 mM imidazole). The molecular weights were determined by SDS-PAGE performing on a discontinuous gel according to the standard procedures (Sambrook et al., 1989).

Enzyme assays

Salicylate hydroxylase activities were measured following NADH-oxidation activity as a decrease in the absorbance at 340 nm, using a UV–VIS Spectrophotometer Cary 100 (VARAN, USA). The reaction mixture was composed of 150 μM sodium salicylate (or derivatives), 120 μM NADH (Sangon, China), 10 μM FAD (Sigma, USA) and 33 mM potassium phosphate buffer (pH 7.0) in a volume of 500 μl. One unit of enzyme activity represented the oxidation of 1 μM of NADH per min measured at 25 °C. Each experiment was repeated five times, and the representative data were statistically analyzed. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976). Specific activities are expressed as units per milligram of protein. Kinetics parameters of NahG and NahU were determined by varying the concentration of salicylate, NADH or FAD in the presence of constant concentrations of other substrates. The K_m and V_{max} values were calculated by double-reciprocal plots.

Dot-blot hybridization

nahU and *nahG* DNA generated by PCR were used as hybridizing probes. The probes were labeled with ^{32}P-α-dCTP by using a random primed DNA labeling kit (BioDev, China). Bacterial total DNA of naphthalene-degradation strains were isolated and hybridized with *nahG* and *nahU* probes by a standard method (Ausubel et al., 1989).

Results and discussion

Sequence comparison between NahU and the other ten salicylate hydroxylases

The complete nucleotide sequences of the 102 kb naphthalene catabolic plasmid pND6-1 have been determined previously (Li et al., 2004), showing two isofunctional salicylate hydroxylase genes, *nahG* and *nahU*. The novel salicylate hydroxylase gene, *nahU*, was situated outside of the lower pathway operon containing the classic salicylate hydroxylase gene *nahG*. The neucleotide sequence comparison of *nahU* (1290bp) and *nahG* (1305bp) showed that the two genes possess 66.51% identity. The *nahU* gene encodes a polypeptide of 429 amino acid residues with a calculated molecular mass of 46804. The *nahG* gene encodes a polypeptide of 434 amino acid residues with a calculated molecular mass of 47480. NahU and NahW only exhibit 19.44% sequence identity. The identities of NahU and the other nine salicylate hydroxylases are between 23.43% and 65.98%. All these salicylate hydroxylases revealed the well-conserved amino terminal FAD-binding site (GxGxxG) (Eggink et al., 1990; Wierenga et al., 1986) except the *nahW*. A dendrogram comparing the NahU of *Pseudomonas* sp. ND6 and their homologs is shown in Fig. 1.

Catalytic activities of NahU and NahG

Salicylate hydroxylase activities were obtained with extracts of induced cells. Both NahU and NahG exhibited broad substrate specificities and metabolized salicylate, sulfosalicylate, aspirin, 3-methylsalicylate, 5-methylsalicylate, 5-chlorosalicylate and 3,5-dinitrosalicylate. However, the relative rates by which the substituted analogs

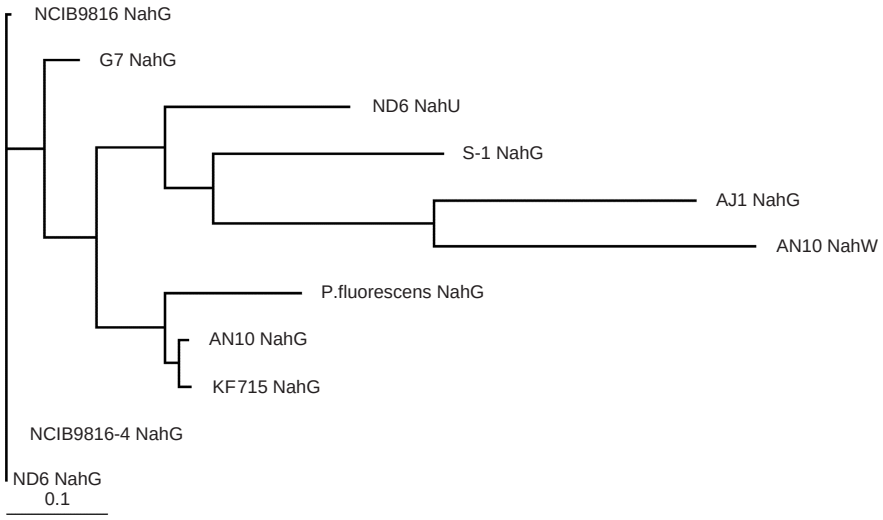

Figure 1. The dendrogram of similarities between 11 bacterial salicylate hydroxylases. The bar indicates 10% estimated sequence divergence. The following salicylate hydroxylase sequences were used: *P. stutzeri* AN10 NahW (GenBank accession no. AF039534), *Sphingomonas* sp. AJ NahG (AB000564), *P. stutzeri* AN10 NahG (AF039534), *P. putida* G7 NahG (M60055), *P. putida* KF715 NahG (S80995), *P. putida* NCIB9816 NahG (X83926), *P. putida* NCIB9816-4 NahG (AF491307), *Pseudomonas* sp. ND6 NahG (AY208917), *P. fluorescens* NahG (AY048764), *P. putida* S-1 NahG (D67098), *Pseudomonas* sp.ND6 NahU (AY208917).

were transformed differed considerably (Table 1). To most substrates in this experiment, NahU revealed more efficient, except sulfosalicylate and 5-chlorosalicylate. Whereas 3,5-dinitrosalicylate was transformed by NahG at a rate lower than that of salicylate, it could not be transformed by NahU. Sulfosalicylate was transformed at a high rate by NahG, but it was transformed at a lower rate of 7% by NahG. Both NahG and NahU seem to be a very efficient catalyst for the transformation of aspirin and show higher activities of methylsalicylate than chlorosalicylate.

Table 1. Salicylate hydroxylase activities of NahU and NahG from *Pseudomonas* sp. ND6

Substrate	Salicylate hydroxylase	
	NahG	NahU
Salicylate	3.17 (100)	23.44 (100)
Sulfosalicylate	4.24 (134)	1.53 (7)
Aspirin	4.12 (130)	25.64 (109)
3-Methylsalicylate	4.04 (128)	10.59 (45)
5-Methylsalicylate	3.91 (123)	18.79 (80)
5-Chlorosalicylate	3.63 (115)	5.25 (22)
3,5-Dinitrosalicylate	1.27 (40)	0 (0)

Salicylate hydroxylase activities were obtained with extracts of induced cells. Activities are measured in micromoles of NADH min^{-1} mg^{-1} protein. Numbers in parenthesis are relative activities (%).

Purification and kinetic analysis of NahU and NahG

The *nahU* and *nahG* genes of *Pseudomonas* sp. ND6 were ligated into the downstream of the T7 promoter in pET21b(+) and expressed in *E. coli* BL21(DE3). By cultivation aerobically of *E. coli* BL21 (DE3) in LB medium at 37 °C with IPTG as the inducer, NahU and NahG were overexpressed mainly in the form of inclusion bodies. When the cultures were incubated at 20 °C soluble NahU and NahG were produced. The reason why a lower temperature favors the native state is related to a number of factors, including a decrease in the driving force for protein self- association, a slower rate of protein synthesis, changes in the folding kinetics of the polypeptide chain (Georgiou and Valaxt, 1996).

The purified NahU and NahG both exhibited a single band of about 47 kDa on SDS-PAGE (Fig. 2). According to the molecular masses, both salicylate hydroxylases of *Pseudomonas* sp. starin ND6, NahG and NahU, belong, like NahG of *P. putida* G7 (You et al., 1990) and *Pseudomonas putida* S-1 (Suzuki et al., 1996), to the subgroup of low-molecular-mass flavincontaining monooxygenases, which are approximately 45 kDa in size (Harayama et al., 1992). The kinetic parameters for the NahU and

NahG were determined by double-reciprocal plots and were shown in Table 2. Enzyme kinetic assay revealed that the K_m and V_{max} of NahU are both higher than that of NahG (Table 2). These results may suggest that either the ability of binding salicylate and cofactors or the catalytic efficiencies of NahU are higher than that of NahG. Experiments to identify promoter regions and to clarify the regulatory mechanism of the two salicylate hydroxylase genes of *Pseodomonas* sp. ND6 are now underway.

Hybridization of nahU and nahG with the total DNA of naphthalene-degrading bacteria

Dot-blot hybridization was conducted to show the presence of *nahU* and *nahG* homologs in different naphthalene-degrading bacteria. Naphthalene-degrading strains J1–J6 were isolated from activated sludge of Tianjin Jizhuangzi Wastewater Treatment Facility, China. Naphthalene-degrading strains D1–D8 were isolated from industrial wastewater of Tianjin Dagang Oil Field, China. The experimental results showed that *nahG* gene presents in all naphthalene-degrading strains (D1–D8 and J1–J6), while *nahU* gene only presents in naphthalene-degrading strains D1–D8 (Fig. 3). This indicated that although *nahU*-like genes are prevalent, they are not necessarily present in all naphthalene-degrading bacteria. The presence of *nahU*-like genes was probably related to the environmental conditions where bacteria grew. Repeated degradation genes in bacteria possess important ecological significance. It has been suggested that the presence of two salicylate hydroxylases was advantageous to the host (Bosch et al., 1999a, b). Standard gene regulatory mechanisms allow cells to adjust their metabolism to the range of conditions most frequently encountered. When extreme conditions cannot be accommodated by gene regulatory mechanism, selection is imposed for increasing the copy number of a gene or a set of linked genes that can improve growth (Roth et al., 1996).

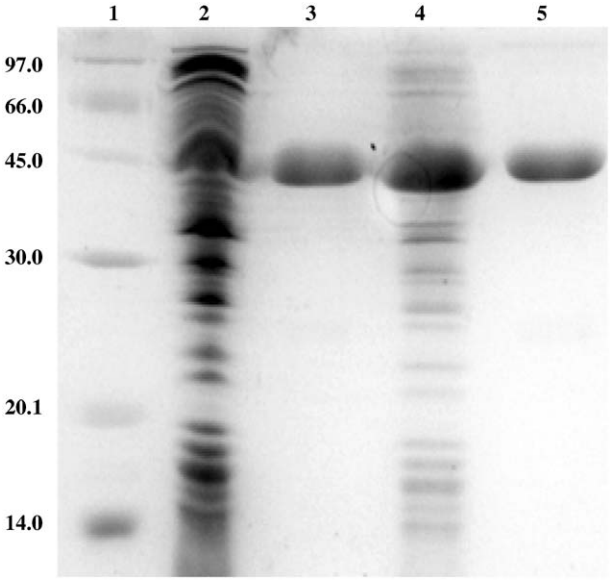

Figure 2. SDS-PAGE of NahG and NahU from *Pseudomonas* sp. ND6. Lane 1, protein molecular weight markers (kDa); lane 2, crude cell extract of NahG; lane 3, purified NahG; lane 4, crude cell extract of NahU; lane 5, purified NahU.

Conclusions

Our data suggest that there are two isofunctional salicylate hydroxylase, NahG and NahU, in *Pseudomonas* sp. ND6. We have cloned and expressed *nahU* and *nahG* genes in *E. coli* and the gene products were purified by His-tag affinity chromatography. Enzyme kinetic assays revealed that the K_m and V_{max} of NahU are both higher than those of NahG. NahU exhibited a broad range of substrate specificity and metabolized salicylate, sulfosalicylate, aspirin, methylsalicylate, chlorosalicylate and 3,5-dinitrosalicylate. The dot-blot analysis showed that although *nahU*-like genes are prevalent, they are not necessarily present in all naphthalene-degrading bacteria. The presence of *nahU*-like genes was probably related to the environmental conditions where bacteria grew.

Table 2. Kinetic parameters of salicylate hydroxylases NahG and NahU from *Pseudomonas* sp. ND6

Enzyme	K_m (μM)			V_{max} (μmol min^{-1} mg^{-1})
	Salicylate	NADH	FAD	
NahG	0.50	23.26	0.37	11.68
NahU	4.94	79.74	1.98	271.00

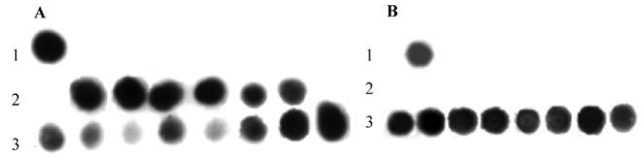

Figure 3. Dot-blot hybridization of *nahG* and *nahU* from *Pseudomonas* sp. ND6 to the total DNAs of naphthalene-degrading strains. (A) Dot-blot hybridization using *nahG* as probe. Line 1, positive control DNA. Line 2, strains J1–J6 (from the left to the right). Line 3, strains D1–D8 (from the left to the right); (B) Dot-blot hybridization using *nahU* as probe. Line 1, positive control DNA. Line 2, strains J1–J6. Line 3, strains D1–D8.

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