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Cloning, heterologous expression, and functional characterization of the nicotinate dehydrogenase gene from *Pseudomonas putida* KT2440

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Abstract 6-Hydroxynicotinate can be used for the production of drugs, pesticides and intermediate chemicals. Some *Pseudomonas* species were reported to be able to convert nicotinic acid to 6-hydroxynicotinate by nicotinate dehydrogenase. So far, previous reports on NaDH in *Pseudomonas* genus were confused and contradictory each other. Recently, Ashraf et al. reported an NaDH gene cloned from *Eubacterium barkeri* and suggested some deducted NaDH genes from other nine bacteria. But they did not demonstrate the activity of recombinant NaDH and did not mention NaDH gene in *Pseudomonas*. In this study we cloned the gene of NaDH, *ndhSL*, from *Pseudomonas putida* KT2440. NdhSL in *P. putida* KT2440 is composed of two subunits. The small subunit contains [2Fe2S] iron sulfur domain, while the large subunit contains domains of molybdenum cofactor and cytochrome *c*. Expression of recombinant *ndhSL* in *P. entomophila* L48, which lacks the ability to produce 6-hydroxynicotinate, enabled the resting cell and cell extract of engineering *P. entomophila* L48 to hydroxylate nicotinate. Gene knockout and recovery studies further confirmed the *ndhSL* function.

Keywords Nicotinate · 6-Hydroxynicotinate · Nicotinate dehydrogenase (NaDH) · *ndhSL* · *Pseudomonas putida* KT2440

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Introduction

Nicotinate (also known as nicotinic acid, vitamin B3) is an precursor of NAD(P), which is essential for the growth of plants, animals and microbes (Alhapel et al. 2006). In bacteria (including both aerobic and anaerobic), nicotinate metabolism starts with hydroxylation of C_6 on the pyridine ring, resulting in 6-hydroxynicotinate (Ensign and Rittenberg 1964; Harary 1957a, b; Hughes 1955), which is subsequently converted into 1,4,5,6- tetrahydro -6-oxonicotinate (THON) (Holcenberg and Tsai 1969; Pastan et al. 1964; Tsai et al. 1966) or 2,6-dihydroxynicotinate (Harary 1957a, b). In some aerobes, e.g., *Pseudomonads* sp., 6-hydroxynicotinate may be oxidized to 2, 5-dihydroxypyridine (Hughes 1955).

Since the nineties of last century, researches on the conversion of aromatic rings or heterocyclic rings by microbes increased significantly in order to aid the production of drugs, pesticides and intermediate chemicals (Berry et al. 1987; Nakano et al. 1999; Yanisch-Perron et al. 1985). Because 6-

hydroxynicotinate can be used in the production of chloronicotiny pesticides as an intermediate (Hurh et al. 1994; Hurh and Nagasawa 1994; Yoshida and Nagasawa 2000), studies on the production of 6-hydroxynicotinate using microbes gradually immerged. In 1985, a Swiss company acquired a strain of *Pseudomonas putida* (NCIB 10521) and two strains of *Achromobacter xylosoxidans* (DSM2402 and DSM 2783) that can convert nicotinate to 6-hydroxynicotinate (Andreesen and Fetzner 2002). Thereafter, several new bacterial strains, including *Achromobacter xylosoxidans*LK1 (Nagel and Andreesen 1989), *P. fluorescens* TN5 (Hurh et al. 1994; Hurh and Nagasawa 1994), and *Serratia marcescens* (Hurh et al. 1994) IFO12648 were also reported to be able to hydroxylate nicotinic acid. Recently, we have reported that resting cells of *P. putida* NA-1 (Lu et al. 2005) and *Comamonas testosterone* JA1 (Yuan et al. 2005) can efficiently convert nicotinate yielding large quantity of 6-hydroxynicotinate. Because of the potential application of 6-hydroxynicotinate in industries, studies on the property of NaDH are necessary.

In bacteria, hydroxylation of nicotinate is catalyzed by nicotinate hydroxylase (Hunt et al. 1958; Hunt 1959). Oxygen tracing studies have demonstrated that the O in the hydroxyl group comes from H_2O not O_2 (Hunt et al. 1958; Hirschberg and Ensign 1971a, b, 1972), thus the enzyme was renamed nicotinate dehydrogenase (Amano et al. 2007; Andreesen and Fetzner 2002; Nagel and Andreesen 1989). Although studies on the isolation and property of nicotinate dehydrogenase started 30 years ago, little progress was made because the enzyme is multi-subunit membrane protein. Until 2006, Ashraf et al. reported a clone of one dehydrogenase genes screened from gene library of anaerobic bacterium *E. barkeri* (Alhapel et al. 2006). The enzyme is composed of four subunits, flavoprotein, [2Fe2S] cluster and two Mo(V) chains which were, respectively encoded by transcriptional-coupled genes *ndh*F, *ndh*S, *ndh*L and *ndh*M. However, it is not clear whether Ashraf et al. produced active recombinant enzyme in engineering bacterium. Although they suggested after GenBank sequence search that the other nine species, SAR86 clade γ-proteobacterium, *Bradyrhizobium* sp. BTAi1, *Bradyrhizobium japonicum* USDA110, *Burkholderia xenovorans* LB400, *Rhodopseudomonas palustris* HaA2, *Polaromonas* sp. JS666, *Eubacterium barkeri, Sllicibacter pomeroyi* DSS-3, *Magnetospirillum magneticum* AMB-1 and *Mehizobium loti* MAFF303099, also have similar NaDHs, they did not mention NaDH gene in nicotinate converting *Pseudomonas* species. To study the property of NaDH in *Pseudomonas*, we have cloned and functionally expressed NaDH gene from whole genome-sequenced model strain *P. putida* KT2440.

Materials and methods

Plasmids, bacteria and culture

Bacterial strains and plasmids used in this study were listed in Table 1. *P. putida* KT2440 and *P. entomophila* L48 were cultured in LB containing 50 mM nicotinic acid (Hunt 1959) at 30°C overnight with shaking. *Escherichia coli* were cultured in LB at 37°C overnight with shaking. Antibiotics used were ampicillin (100 μg ml^{-1}), kanamycin (30 μg ml^{-1}), and tetracycline (12.5 μg ml^{-1}) (Caponi and Migliorini 1999). Solid culture media contained 2% (wt/v) of agar in LB.

PCR

Genomic DNA was isolated from *P. putida* KT2440 according to ref (Davis et al. 1980). Plasmid DNA was prepared from *E. coli* with alkaline lysis method (Caponi and Migliorini 1999). Agarose gel electrophoresis, restriction digestion, alkaline phosphatase treatment and ligation were conducted as described (Caponi and Migliorini 1999). DNA gel extraction was performed according to (Tautz and Renz 1983). DNA primers (Table 2) were purchased from Sangon Bio Co., Ltd (Shanghai, China). PCR was performed as described by (Sambrook et al. 1989; Caponi and Migliorini 1999). Annealing temperature was 60°C. PCR product was cloned in pMD18-T vector. DNA was sequenced by Sangon Bio Co., Ltd (Shanghai, China). *E. coli* and *Pseudomonas* sp. competent cells were prepared according to (Dower et al. 1988) and (Iwasaki et al. 1994), respectively.

Construction of pJB866H vector

Oligonucleotide Hislink-S and Hislink-F were annealed into 6HIS + TGA linker (*Hin*dIII-*Spe*I-6his-TGA-*Eco*RI) according to Anearning Scheme (Stemmer et al. 1995) and cloned in pUC18, yielding

Table 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics[a]	Reference(s)
Strains		
P. putida		
KT2440	Wild type strain	Nelson et al. (2002)
KT2440Δ*ndhSL*	*ndhSL* gene knockout strain.KT2440Δ*ndhSL*::*kan*	This work
KT2440Δ*ndhSL* [pJB866H::*ndhSL*]	KT2440Δ*ndhSL*-containing the pJB866H::*ndhSL*	This work
P. entomophila		
L48	Wild type strain	Vodovar et al. (2006)
E.coli		
DH10B	Cloning host. F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lac*X74 *deoR recA1 endA1 ara*Δ*139*Δ(*ara leu*) *7,697 galU galK* λ^- *rpsL nupG*λ^- *tonA*	Novagen
BL21(DE3)	Expression host. F^-*dcm ompT hsdS*$_B$(r_B^- m_B^-) *gal* λ(DE3) (pLysS Cm^r)	Novagen
HB101	Helper strain containing the plasmid pRK2013. F^- Δ(*gpt-proA*)*62 leuB6 glnV ara-14 galK2 lacY1* Δ(*mcr-mrr*) *rpsL20* (Str^r) *xyl-5 mtl-1 recA13*	Wenzel et al. (2005), Quenee et al. (2005)
Plasmid		
pUC18	Cloning vector. MCS, rep (pMB1) Ap^r. 2.7 kb	Boyer and Roulland-Dussoix (1969)
pMD18-T	TA cloning plasmid for sequence. Ap^r. 2.7 kb	TaKaRa
pRSETB	Expression vector. Ap^r. 2.9 kb	Invitrogen
pUC18H	Derivative of pUC18 in which a his_6 + TGA linker was inserted to *Hin*dIII/*Eco*RI site. Ap^r. 2.7 kb	This work
pJB866	RK2 expression vector containing the Pm promoter and the gene encoding the regulatory protein XylS. Tc^r. 8.3 kb	Blatny et al. (1997a, b)
pJB866H	Derivative of pJB866 in which a his_6 + TGA linker was inserted to *Hin*dIII/*Eco*RI site. Tc^r. 8.3 kb	This work
pRSETB::*ndhSL*	pRSETB containing the *ndhSL* gene *from P. putida* KT2440. Ap^r. 7.0 kb	This work
pJB866H::*ndhSL*	pJB866H containing the *ndhSL* gene *from P. putida* KT2440. Tc^r. 12.4 kb	This work
pRK2013	Helper plasmid for conjugation. Km^r.	Wenzel et al. (2005), Quenee et al. (2005)
pACYC177	Vector containing the *kan* gene. Km^r. 3.9 kb	Wenzel et al. (2005)
pEX100Tlink	Gene replacement vector. Ap^r. 6.2 kb	Quenee et al. (2005)
pDSL	pEX100Tlink containing 5′ and 3′ flanking sequence of *ndhSL*::*kan*. Km^r. 6.9 kb	This work

[a] For antibiotic resistances: *Ap*^{r} ampicillin, *Tc*^{r} tetracycline, *Km*^{r} kanamycin, *Str*^{r} streptomycin, *Cm*^{r} chloramphenicol

pUC18H. Subsequently, pUC18H was double digested with *Hin*dIII/*Eco*RI, and the insert was cloned into pJB866, yielding a plasmid pJB866H.

Construction of *ndhSL* recombinant

ndhSL was amplified with primers KTSLS and KTSLF. The amplicon was digested with *Nco*I and *Hin*dIII, inserted at *Afl*III and *Hin*dIII sites of pJB866H vector. The C-terminus of the foreign gene was fused with 6 × HIS on the vector, which was then used to transform *E. coli* DH10B. A DH10B [pJB866H::*ndhSL*] recombinant was thus obtained.

ndhSL induced expression

The recombinant plasmid [pJB866H::*ndhSL*] was introduced into *P. entomophila* L48. The transformants were then cultured at 30°C with shaking until $OD_{600} = 0.1$ (about 2 h) and induced with *m*-toluic

Table 2 PCR primers used in this study[a]

Primer	Sequence (5′–3′)	Restriction site
KTSLS	5′-GGG**CCATGG**GATGCAAACAACCATCTCCCTG-3′	*Bam*HI
KTSLF	5′-CCC**AAGCTT**TCAGTGGCTGCCAGGGTTG-3′	*Hin*dIII
Hislink-S	5′-**AGCTTACTAGT**CACCACCACCACCACCACTGA**G**-3′	*Hin*dIII, *Spe*I, *Eco*RI
Hislink-F	5′**-AATTC**TCAGTGGTGGTGGTGGTGGTG**ACTAGTA**-3′	*Eco*RI, *Spe*I, *Hin*dIII
U-SL-S	5′-GGG**GAGCTC**GTTGCACCAGAGGAGTCGCGAG-3′	*Sac*I
U-SF	5′-GGG**GGTACC**GCTCATCTCTGGAGGTTGCTTGC-3′	*Kpn*I
D-SL-S	5′-GGG**CCATGG**CAAGGTCACTGATGAAGGTCAGC-3′	*Nco*I
D-SL-F	5′-GGG**TCTAGA**CAAGACGATCGAGCAGGTCAAC-3′	*Xba*I.
Kan-S	5′-GGG**GGTACC**AAAGCCACGTTGTGTCTCAAAATC-3′	*Kpn*I
Kan-F	5′-GGG**CCATGG**TTAGAAAAACTCATCGAGCATC-3′	*Nco*I

[a] All primers were purchased from Sangon Bio Co., Ltd (Shanghai, China). Restriction sites are indicated in bold

acid (final concentration 2 mg ml^{-1}) for 12 h at 30°C with shaking. Bacteria were harvested by centrifugation, washed twice with 20 mM phosphate buffer pH 7.0, suspended in the washing buffer containing PMSF (10 μM), and disrupted with ultrasound (400 W, 5–10 min) until clear. Cell debris was discarded after high-speed centrifugation.

SDS–PAGE

SDS-PAGE was performed according to (Laemmli 1970), with the concentrations of polyacrylamide being 12.5 and 5% (w/v) in separation and condensation gels, respectively. The protein staining solution contained Coomassie blue R-250 (0.1%, w/v) in 10% (w/v) acetic acid and 40% (w/v) ethanol. Destaining solution was H_2O/ethanol/acetic acid (50:40:10).

Western blotting

Proteins were separated with SDS–PAGE, blotted for 70 min onto PVDF membrane (MILLIPORE Immobilon-P) at 0.9 mA cm^{-2}. The blotting buffer contained 25 mM Tris, 190 mM glycine in 20% (v/v) aqueous methanol. His Tag monoclonal antibody (NOVAGEN) was used for Western blotting analysis.

NaDH activity determination

Nicotinate hydroxylation reaction was performed in 1.5 ml tube in 0.5 ml reaction mixture (Yuan et al. 2005) containing 20 mM buffer (pH 7.0), 5 mM nicotinate and an appropriate amount of bacterial cells or cellular extract. The reaction was allowed to take place for 2 h at 30°C with shaking. When cellular extract was tested for nicotinate hydroxylation, PMS (50 μM) was added to the reaction mixture to serve as the electron acceptor (Nagel and Andreesen 1990). At the end of the reaction, the cells or enzyme was inactivated by heating (100°C for 5 min) and removed by centrifugation.

The production of 6-hydroxynicotinate was analyzed using Agilent 1100 (USA) HPLC and quantitated with external standard. The column was ZORBAX ODS (4.6 mm i.d. × 250 mm, 5 μm). The liquid phase was methanol and water (50:50, v/v, pH 3.0) and the velocity was 1 ml min^{-1}. The detection wavelength was 260 nm and the detector was Agilent G1314A UV. The reaction mixture was diluted to an appropriate concentration for assay.

One unit of NaDH activity was defined as the enzyme amount needed to produce 1 μmol of 6-hydroxynicotinate in 1 min at the test conditions (Lu et al. 2005; Yuan et al. 2005).

Gene knockout and recover

Two 1 kb segments, located at the upstream and downstream of the *ndhSL* gene of *P. pudia* KT2440, respectively, were amplified using primers U-SL-S, U-SL-F, D-SL-S and D-SL-F. A 1 kb segment of kan^r was amplified from pACYC177 using primers Kan^r-S and Kan^r-L. The upstream segment of *ndhSL* was digested with *Sac*I and *Kpn*I. The downstream segment of *ndhSL* was digested with *Nco*I and *Xba*I. The kan^r segment was digested with *Kpn*I and *Nco*I. The three amplicons were then ligated to pEX100Tlink vector that had been digested with *Sac*I and *Xba*I, with the

kan^r segment ligated in between the two *ndhSL* segments. This resulted in a recombinant plasmid pDSL, which contained a *sac*B reverse selection maker and *kan*^{r}. The plasmid was used to transform *E. coli* DH10B, resulting in *E. coli* DH10B[pDSL].

An *ndhSL* gene knockout strain, *P. putida* KT2440Δ*ndhSL,* was prepared as described (Lu et al. 2005). Briefly, with the help of pRK2013 plasmid in *E. coli* HB101, the *ndhSL* gene was replaced by *kan*^{r} from pDSL after triple conjugation among *E. coli* DH10B[pDSL], *E. coli* HB101 and *P. putida* KT2440. The gene knockout was verified with PCR. A recovery strain, *P. putida* KT2440Δ*ndh*SL[pJB866H::*ndhSL*] was obtained by electric transformation of *P. putida* KT2440Δ*ndhSL* with [pJB866H::*ndhSL*].

Results

Cloning of *ndhSL*

Since NaDH exists in *E. barkeri* and possibly exists in nine other strains according to the report by Ashraf et al. (Alhapel et al. 2006), we searched the genome of *P. putida* KT2440 and identified a gene cluster possibly encoding NaDH, named *ndhSL*. This gene cluster has two genes, *ndhS* and *ndhL*, encoding NdhS and NdhL (Fig. 1), respectively. The initiation codon of *ndhS* is located at position 4,449,926 nt of *P. putida* KT2440 genome. The coding region ORF is 474 bp, coding for 158 amino acid residues with an estimated molecular weight of 16,685 Da. The other gene, *ndh*L is located between nt 4,450,396 and 4,453,959 of *P. putida* KT2440 genome, with a size of 3,564 bp, coding 1,188 amino acid residues. The estimated molecular weight is 127,874 Da. NdhSL was found to exhibit higher similarity (identities of 63.3–64.5%) with NaDHs from *B. japonicum* USDA110, *R. palustris* HaA2 and *Polaromonas* sp. JS666 containing a cytochrome *c* binding domain, however it shows low similarity (identities of 9–11.8%) with the NaDHs from *E. barkeri*, *S. pomeroyi* DSS-3, *M. magneticum* AMB-1 and *M. loti* MAFF303099 containing a flavin-binging domain. The PCR amplicon using primers KTSLS and KTSLF derived from *ndhSL* was identical to the genomic sequence (GenBank accession: EU604833).

Expression of recombinant *ndhSL*

Since *ndhSL* cannot be expressed in *E. coli* BL21 (DE3) (data not shown), we attempted to express it in *P. entomophila* L48, which has no nicotinate hydroxylation ability. *ndhSL* was inserted behind *Pm* promoter in plasmid pJB866H and obtained [pJB866H::*ndh*SL], which was used to transform *P. entomophila* L48, resulting in recombinant *P. entomophila* L48 [pJB866H::*ndhSL*]. After induction, the expression of recombinant NdhSL was examined with anti-His following SDS–PAGE. An expected 130 kDa protein was detected in cell extract of L48 [pJB866H::*ndhSL*]. Cells transformed with empty-vector or control sample did not show the same protein (Fig. 2).

Functional characterization of recombinant NaDH

The NaDH activity in resting cells of recombinant L48 [pJB866H::*ndhSL*] and cell extract was determined with HPLC. HPLC analysis confirmed the presence of a product which was subsequently identified as 6-hydroxynicotinate using LC-MS and NMR (data not shown). The extract from control strain and non-induced culture had no NaDH activity (Fig. 3). The enzymatic activities were 1.89 U mg^{-1} dry weight and 1.18 U mg^{-1} protein for resting cells of recombinant L48 [pJB866H::*ndhSL*] and its cell-free extract, respectively.

Knockout of *ndhSL*

To further confirm that *ndhSL* encodes NaDH in *P. putida* KT2440, we performed gene knockout study.

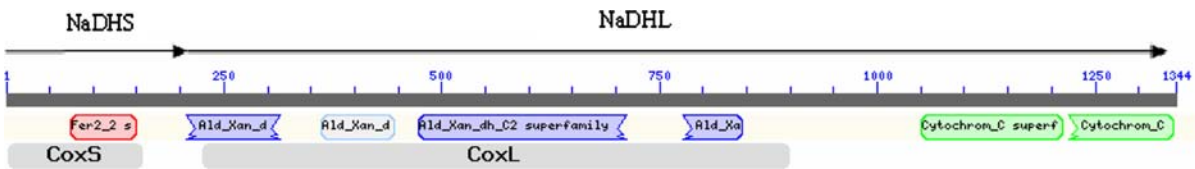

Fig. 1 Gene cluster of NdhSL indicated by comparing to known sequences of NaDH in the Swiss protein Data Library

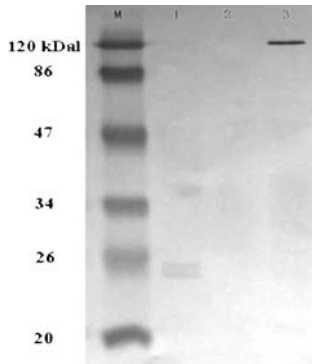

Fig. 2 Western blotting detection of NdhSL in recombinant *P. entomophila* L48. Detection of predicted NaDH subunits in Western blotting of cell-free extract separated by SDS–PAGE. The distinctive band indicated the *ndhL* coding protein with a C-terminal his_6-tag. *Lane 1*, *m*-toluic acid-induced empty-vector-containing *P. entomophila* L48 [pJB866H] extract. *Lane 2*, non-induced *ndhSL*-containing *P. entomophila* L48 [pJB866H::*ndhSL*] extract. *Lane 3*, *m*-toluic acid-induced *ndhSL*-containing *P. entomophila* L48 [pJB866H::*ndhSL*] cell extract

A strain, *P. putida* KT2440Δ*ndhSL* in which *ndhSL* was deleted, was generated. An examination of NaDH activity in the *ndhSL* knockout strain indicated that this strain has lost its ability to hydroxylate nicotinate. In contrast, a recovery strain, *P. putida* KT2440Δ*ndhSL*[pJB866H::*ndh*SL] which was generated by transformation of *P. putida* KT2440Δ*ndhSL* with pJB866H::*ndh*SL plasmid, regained its ability to hydroxylate nicotinate. The enzymatic activities were 1.91 U mg^{-1} dry weight and 1.23 U mg^{-1} protein in the recovered strain. These data further demonstrated that *ndhSL* encodes NaDH in *P. putida* KT2440.

Amino acid sequence similarity

Since the genomes of *P. putida* W619, *P. putida* F1, *P. putida* GB-1 and *P. fluorescens* PfO-1 were completely sequenced, we analyzed amino acid sequence similarities of NaDHs among these strains and species. Results (Table 3) showed that NaDHs shared 90% identity of amino acid sequences among strains of *P. putida*, while the NdhSL in *P. fluorescens* PfO-1 shared 32% identity of amino acid residues with *P. putita*. In *P. putita*, all NaDHs contained [2Fe–2S], molybdopterin and cytochrome *c* domains, but not FAD. Both molybdopterin and cytochrome *c* were in the same subunit. In *P. fluorescens* PfO-1, a species of the same genus, the three structural domains are separated in three subunits. This NaDH structural arrangement is very different from that in *P. putida*, but is similar to other cytochrome *c* containing NaDH (Alhapel et al. 2006).

Discussion

Since nicotinate is an important element in biological functions, extensive study has been carried on its biosynthesis and related enzymes' application. During the screening of microorganisms that can hydroxylate nicotinate to 6-hydroxynicotinate, we found *P. putida* KT2440 is able to perform the reaction with high efficiency.

By performing a protein–protein BLAST search with the amino acid sequences of Ndh proteins from *E. barkeri* and other 9 bacteria against the genome

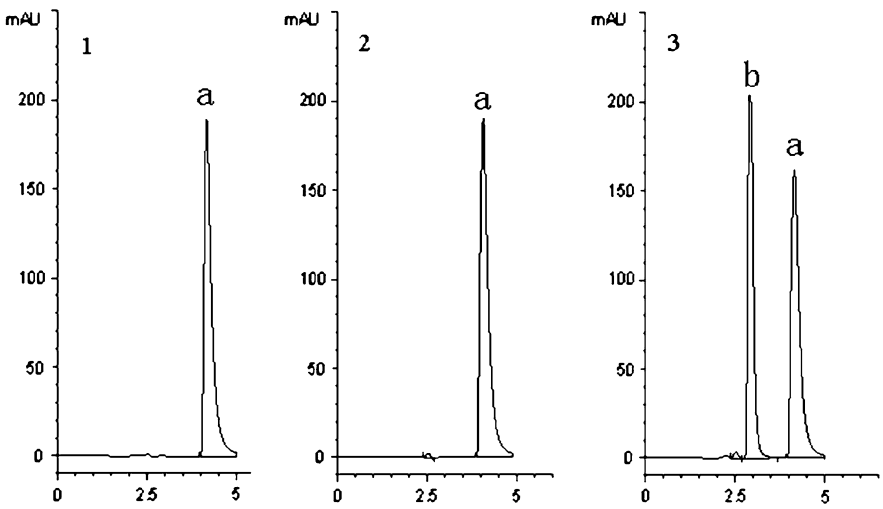

Fig. 3 HPLC detection of transformation of nicotinate by recombinant *P. entomophila* L48. *1 m*-toluic acid-induced empty-vector-containing *P. entomophila* L48 [pJB866H]. *2* Non-induced *ndhSL*-containing *P. entomophila* L48 [pJB866H::*ndhSL*]. *3 m*-toluic acid-induced *ndhSL*-containing *P. entomophila* L48 [pJB866H::*ndhSL*]. **a** Nicotinate, **b** 6-hydroxynicotinate

Table 3 Predicted gene clusters associated with NaDH from *Pseudomonas* strains

Strains	Corresponding subunit/domain of predicted NaDH						Identity (% in aa overlap)
	2 × [2Fe–2S]		Molybdopterin		Cytochrome *c*		
	Product size[a]	Coding sequence[b]	Product size	Coding sequence	Product size	Coding sequence	
P. putida W619	155	2,381,320–2381787c	1,187	2,377,741–2381304c	C-terminal fusing to molybdopterin		90(1220aa)
P. putida F1	157	2,166,125–2166598c	1,187	2,162,565–2166128c	C-terminal fusing to molybdopterin		98(1320aa)
P. putida GB-1	157	3,980,945–3,981,418	1,187	3,981,415–3,984,978	C-terminal fusing to molybdopterin		95(1287aa)
P. fluorescens PfO-1	151	2,828,122–2828577c	749	2,825,873–2828122c	450	2,824,503–2825855c	32(450aa)

[a] For Numbers of amino acid

[b] For position of start codon-position of stop codon in genome

database of *P. putida KT2440*, we identified a gene cluster, *ndhSL* which coding for NaDH. Expression of the recombinant *ndhSL* in *P. entomophila* L48, which lacks the ability to produce 6-hydroxynicotinate, enabled the resting cell and cell extract of the engineered strain to hydroxylate nicotinate. *P. putida KT2440 ndhSL* gene knocked out strain abolished the transformation activity. This, unequivocally, demonstrated that *ndhSL* is the nicotinate dehydrogenase.

During the preparation of this manuscript, José Jiménez et al. published similar work (Jiménez et al. 2008), they identified a nicotinate metabolism gene cluster, with nicotinate dehydrogenase as a crucial gene in it.

Detail comparison between their work and us showed that we are working on the same genes. The *ndhSL* was named *nicAB* in their study. They obtained functional nicotinate dehygrogenase gene in pBBR1MCS-5 constitutive gene expression vector, whereas we used pJB866, in which the genes cloned were under the tight regulation and gene expression initiates upon induced with *m*-toluic acid. And, we both could not express the genes in *E. coli*. José Jiménez et al. elucidated the whole pathway, while we focused on the nicotinate dehygrogenase. Overall, our results complemented PNAS result.

The hydroxylation of nicotinate dehygrogenase, albeit extensive work has been done so far, remains an unresolved question. In our case, nicotinate dehygrogenase from *P. putida KT2440* could not hydroxylate 3-cyanopyridine, yet enzymes of *P. fluorescens* R2f, the engineered strain José Jiménez et al. used, origin have the nicotinate and 3-cyanopyridine hydroxylation function, though the 3-cyanopyridine hydroxylation efficiency is relatively low (5% activity as nicotinate hydroxylase). It was already reported that the NaDH purified from *P. fluorescens* TN5 by Hurh and Nagasawa (1994) can hydroxylate both nicotinate and also 3-cyanopyridine. Therefore, it is possible that the host strain *P. fluorescens* R2f itself has the ability to hydroxylate 3-cyanopyridine. Further work to find enzymes that can hydroxylate one or both substrates are under way in our lab.

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