Biodegradation (2010) 21:593–602
DOI 10.1007/s10532-010-9327-2

ORIGINAL PAPER

Cloning, expression and functional analysis of nicotinate dehydrogenase gene cluster from *Comamonas testosteroni* JA1 that can hydroxylate 3-cyanopyridine

Yao Yang · Ting Chen · Pengjuan Ma · Guangdong Shang · Yijun Dai · Sheng Yuan

Received: 7 November 2009 / Accepted: 13 January 2010 / Published online: 2 February 2010

Abstract A nicotinate dehydrogenase (NaDH) gene cluster was cloned from *Comamonas testosteroni* JA1. The enzyme, termed $NaDH_{JA1}$, is composed of 21, 82, and 46 kDa subunits, respectivley containing [2Fe2S], Mo(V) and cytochrome *c* domains. The recombinant $NaDH_{JA1}$ can catalyze the hydroxylation of nicotinate and 3-cyanopyridine. $NaDH_{JA1}$ protein exhibits 52.8% identity to the amino acid sequence of $NaDH_{KT2440}$ from *P. putida* KT2440. Sequence alignment analysis showed that the [2Fe2S] domain in $NaDH_{JA1}$ had a type II [2Fe-2S] motif and a type I [2Fe-2S] motif, while the same domain in $NaDH_{KT2440}$ had only a type II [2Fe-2S] motif. $NaDH_{KT2440}$ had an additional hypoxanthine dehydrogenase motif that $NaDH_{JA1}$ does not have. When the small unit of $NaDH_{JA1}$ was replaced by the small subunit from $NaDH_{KT2440}$, the hybrid protein was able to catalyze the hydroxylation of nicotinate, but lost the ability to catalyze hydroxylation of 3-cyanopyridine. In contrast, after replacement of the small subunit of $NaDH_{KT2440}$ with the small subunit from $NaDH_{JA1}$, the resulting hybrid protein $NaDH_{JAS+KTL}$ acquired the ability to hydroxylate 3-cyanopyridine. The subunits swap results indicate the [2Fe2S] motif determines the 3-cyanopyridine hydroxylation ability, which is evidently different from the previous belief that the Mo motif determines substrate specificity.

Keywords *Comamonas testosteroni* · Nicotinate dehydrogenase (NaDH) · Nicotinic acid · 6-Hydroxynicotinic acid · 3-Cyanopyridine · 3-Cyano-6-hydrxoypyridine

Introduction

Nicotinate (also known as nicotinic acid, vitamin B3) is an important component of nicotinamide adenine dinucleotide phosphate {NAD(p)} that plays an essential role in animals, plants and microbes (Alhapel et al. 2006). It has been reported that the pathway of nicotinate metabolism varies among different species of microbes (including aerobic and anaerobic). However, the first step of nicotinate metabolism is invariably the production of 6-hydroxynicotinic acid by hydroxylation of C_6 on the pyridine ring (Ensign and Rittenberg 1964; Harary 1957; Hughes 1955; Hunt et al. 1958). Nicotinate dehydrogenase (NaDH) is the enzyme that catalyze this first step in bacteria (Andreesen and Fetzner 2002).

Since the 1980s research on the conversion of aromatic rings or heterocyclic rings by microbes has increased significantly in order to aid in the

Y. Yang · T. Chen · P. Ma · G. Shang · Y. Dai · S. Yuan (✉)
Jiangsu Engineering and Technology Research Center for Industrialization of Microbial Resources, Jiangsu Key Laboratory for Biodiversity and Biotechnology, College of Life Science, Nanjing Normal University, 1Wenyuan Rd, Nanjing 210046, People's Rebuplic of China
e-mail: yuansheng@njnu.edu.cn; yuansheng2008@126.com

production of drugs, pesticides and intermediate chemicals (Berry et al. 1987; Yanisch-Perron et al. 1985). Because 6-hydroxynicotinate and 3-cyano-6-hydroxypyridine can be used in the production of chloronicotiny pesticides as an intermediate (Hurh et al. 1994a, b; Yasuda et al. 1995), studies on the production using microbes gradually immerged (Fig. 1). 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. 1994a, b), and *Serratia marcescens* IFO12648 (Hurh et al. 1994a) were also reported to be able to hydroxylate nicotinate. *Comamonas testosteroni* MCI2848 (Yasuda et al. 1995) was reported to be able to hydroxylate 3-cyanopyridine to 3-cyano-6-hydroxypyridine. Recently, we have reported that resting cells of *P. putida* NA-1 (Lu et al. 2005) and *C. testosteroni* JA1 (Yuan et al. 2005) can efficiently convert nicotinate yielding large quantity of 6-hydroxynicotinate, and *C. testosterone* JA1 can also hydroxylate 3-cyanopyridine to 3-cyano-6-hydroxypyridine.

Isolation and purification of NaDH began in 1950s. However, reports about the size and cofactor components of NaDH did not agree with each other. Behrman and Stanier (1957) described the isolation and purification of an NaDH from *P. putida* N-9, where the enzyme existed in the insoluble membrane fraction, interacting with cytochrome to exert its function (Andreesen and Fetzner 2002). In 1958, Hunt reported the initial purification of an NaDH from *P. fluorescens* KB1 and showed that the enzyme

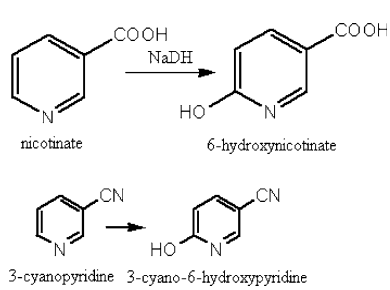

Fig. 1 The hydroxylation of nicotinate and 3-cyanopyridine

also existed in membrane as an insoluble component. Ultraviolet spectrometry study demonstrated that flavin and cytochrome were the cofactors of this NaDH (Hunt 1959). Later on in 1973, Jones demonstrated by ultraviolet spectrometry that the NaDH isolated from *P. ovalis* might contain heme instead of flavin as the cofactor (Jones 1973; Jones and Hughes 1972).

In 1994, Hurth and Nagasawa purified an NaDH from *P. fluorescens* TN5 and demonstrated that the NaDH was a membrane protein of 80 kDa. SDS–PAGE analysis indicated that the NaDH from *P. fluorescens* TN5 was composed of a single chain (Hurh et al. 1994b). In1989, Nagel reported that an NaDH from *Bacillus niacini* was 300 kDa. SDS–PAGE analysis showed that the enzyme had three bands, i. e., 85, 34 and 20 kDa, presumably three subunits. Ultraviolet spectrophotometry analysis suggested that the enzyme had a flavoprotein and iron-sulfur center (Nagel and Andreesen 1989). In 2006, Ashraf et al. reported for the first time the NaDH-encoding genes in *Eubacterium barkeri* (Alhapel et al. 2006) and showed that the enzyme was encoded by a cluster of four transcriptional-coupled genes, *ndh*F, *ndh*S, *ndh*L and *ndh*M respectively for the 33, 23, 50 and 37 kDa subunits. These four functional subunits were respectively a flavoprotein, a iron-sulfur cluster and two Mo(V) chains. In 2008, we have reported the *ndh*SL gene cluster in *P. putida* KT2440 encoding an NaDH of 140 kDa, composed of 16- and 128-kDa subunits. The enzyme has a [2Fe2S] domain, two Mo(V) domains and a cytochrome *c* domain. These findings were consistent with reports that NaDH has a cytochrome cofactor in *P. putida* N-9 and *P. fluorescens* KB1 (Andreesen and Fetzner 2002; Hunt 1959), but inconsistent with the reports that NaDH has heme as the cofactor (Jones 1973; Jones and Hughes 1972). The *P. putida* NaDH was also different from the one in *P. fluorescens* TN5 where the enzyme has a 80 kDa chain. Therefore, to determine the structural characteristics of NaDH, it is necessary to determine the NaDH from more bacterial sources.

The NaDH isolated from *P. fluorescens* TN5 by Hurth and Nagasawa was also capable of hydroxylating 3-cyanpyridine (Hurh et al. 1994b). However the recombinant NaDH we have expressed from *P. putida* KT2440 can only hydroxylate nicotinate, but not 3-cyanopyridine. Therefore, we speculate that

there might be 3-cyanopyridine hydroxylase. We have previously reported that *C. testosteroni* JA1 is capable of hydroxylating nicotinate and 3-cyanopyridine (Yang et al. 2009). Therefore, this strain is a good model of study (Yuan et al. 2005). Here, we report the cloning and functional expression of an NaDH from *C. testosteroni* JA1, hoping to determine whether 3-cyanopyridine hydroxylation is also catalyzed by NaDH, whether this NaDH has special structure, or if there could be an additional enzyme that catalyze 3-cyanopyridine hydroxylation.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids were shown in Table 1. *C. testosteroni* JA1 and *P. entomophila* L48 were shaken in LB media containing 50 mM nicotinate at 30°C overnight. *E. coli* was shaken in LB at 37°C overnight. Antibiotics ampicillin (100 μg ml^{-1}), kanamycin 30 μg ml^{-1}, and tetracycline (12.5 μg ml^{-1}) were used. Solid culture was done with the above media plus 2% (w/v) agar.

DNA manipulation

Genomic DNA of *C. testosteroni* JA1 was extracted as described in Molecular Cloning (Davis et al. 1980). Recombinant plasmids were extracted with alkline lysis method as described by Sambrook et al. (1989). Gel electrophoresis, DNA restriction digestion, alkline phosphatase treatment and ligation were performed with conventional method (Sambrook et al.1989). Recovery of DNA from gel was done as described by Tautz and Renz (1983). Primers (Table 2) were synthesized by Shanghai Biotech (Shanghai, China). Conventional PCR was performed according to Sambrook et al. (1989). Annealing temperature was 60°C. The PCR product was inserted to pMD18-T (TaKaRa biotech, Dalian, China). Sequencing was done by Shanghai Biotech. Competent cells (*E. coli* and *Pseudomonas sp.*) were prepared according to Dower et al. (1988) and Iwasaki et al. (1994), respectively.

Induced expression of recombinants

The expression vector [pJB866H:: *ndhSLM*] was extracted from recombinant bacterium DH10B [pJB866H:: *ndhSLM*], then introduced by electroporation

Table 1 Strains and plasmids used in this work

Strain or plasmid	Genotype or relevant characteristics[a]	References
P. entomophila		
L48	Wild-type strain	Vodovar et al. (2006), Yang et al. (2009)
P. putida		
KT2440	Wild type strain	Nelson et al. (2002)
C. testosteroni		
JA1	Wild-type strain	Yuan et al. (2005)
E.coli		
DH10B	Cloning host. F^- *mcrA*Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX*74 *deoR recA1 endA1 ara*Δ*139*Δ(*ara leu*)*7697 galU galK* λ^-*rpsL nupG*λ^- *tonA*	Novagen
Plasmid		
pMD18-T	TA cloning plasmid for sequence. Ap^r.2.7 kb	TaKaRa
pJB866H	Derivative of pJB866 in which a his_6 + TGA linker was inserted to *Hind*III/*Eco*RI site. Tc^r. 8.3 kb	Blatny et al. (1997a, b), Yang et al. (2009)
pNKTS	An insertion [*ndhS* from *P. putida* KT2440 + *ndhLM* from *C. testosteroni* JA1] into plasmid pJB866H,Tc^r, 12.4 kb	This work
pNJAS	An insertion [*ndhS* from *C. testosteroni* JA1 + *ndhLM* from *P. putida* KT2440] into plasmid pJB866H,Tc^r, 12.8 kb	This work

Antibiotic resistances are indicated as follows: Ap^r, ampicillin; Tc^r, tetracycline

Table 2 PCR primers used in this study

Primer	Sequence (5′–3′)	Restriction site
JA-1	5′-CTGYGGSCTSGGCGAATGYGG-3′	
JA-2	5′-CCRTARCARCCGGCDGCYTC-3′	
JA-3	5′-GGGCAGGTCTGGACCGGCTCGCAGAAC-3′	
JA-4	5′-GGGCTTCAGCGGCACGGAGCAGGAG-3′	
JA-5	5′-GGGGATGGTGTTGCTGGCAATGGTGG-3′	
JA-6	5′-GGGGACGAGCTGTATGTGGACTTTGC-3′	
JA-7	5′-GGGGACAAGAGCGGCGTCACCTCCGTG-3′	
JA-8	5′-GGGCACGTTGCCGTGCACCTGATGAC-3′	
JA-9	5′-GGGCTGGACCAAGCAGGATCTGGTC-3′	
JA-10	5′-CCCGAGGCCGTCTTGTCCGTCAGAG-3′	
JA-SLM-S	5′-GGGATGAAATTCACTCTGAACGGCCAG-3′	
JA-SLM-F	5′-CCCATGCGCCTGGGTCTGCTTGCGC-3′	
JA-SLM$_1$-S	5′-GGG**CTCGAGGGTCTCGC**ATGAAATTCACTCTGAACGGCCAG-3′	*Xho*I, *Bsa*I
JA-SLM$_1$-F	5′-GGGCCTTGC**ACACGT**CGTGAGGGTTC-3′	*Afl*III
JA-SLM$_2$-S	5′-GGGCTCACG**ACGTGT**GCAAGGATATTG-3′	*Afl*III
JA-SLM$_2$-F	5′-CCC**GCGGCCGCAAGCTT**ATGCGCCTGGGTCTGCTTGCGC-3′	*Not*I, *Hind*III
KT-S–S	5′-GGG**GAGCTCCCATGGC**TATGCAAACAACCATCTCCCTGC-3′	*Sac*I, *Nco*I
KT-S–F	5′-CCC**AAGCTTTCTAGA**TCATGGCTTTCTCCGGGTCTCG-3′	*Hind*III, *Xba*I
JA-LM-S	5′-GGG**GAGCTCTCTAGA**ATGTCTAGAATGAAATTCTTCACCAAAGAC-3′	*Sac*I, *Xba*I
JA-LM-F	5′-CCC**AAGCTT**ATGCGCCTGGGTCTGCTTGCG-3′	*Hind*III
JA-S-S	5′-GGG**GAGCTCGGTCTCGC**ATGAAATTCACTCTGAACGGCCAG-3′	*Sac*I, *Bsa*I
JA-S-F	5′-CCC**AAGCTTTCTAGA**TCAGGCAGCCTTTGCGGTTGTGG-3′	*Hind*III, *Xba*I
KT-La-S	5′-GGG**GAGCTCTCTAGA**ATGTCTAGAATGAACCACTCACAACAGGTGC-3′	*Sac*I, *Xba*I
KT-La-F	5′-CCC**AAGCTT**GTGGCAA**GCATGC**CCAATGCG-3′	*Hind*III, *Sph*I
KT-Lb-S	5′-GGG**GAGCTCG GCATGC**TTGCCACCGCCTTGC-3′	*Sac*I, *Sph*I
KT-Lb-F	5′-CCC**AAGCTT**GTGGCTGCCAGGGTTGGCGC-3′	*Hind*III

All primers were purchased form Sangon Bio Co., Ltd (Shanghai, China). Restriction sites are in bold

to *P. entomophila* L48. The resulting recombinant *P. entomophila* L48 [pJB866H:: *ndhSLM*] was cultured at 30°C for 2 h (OD 600 nm = 0.1), then induced with m-toluic acid (2 mg/ml) for 12 h with shaking. The cells were harvested by centrifugation, resuspended in 20 mM PBS (pH 7.0). After addition of PMSF (final concentration 10 μM), the cells were disrupted by sonication (400 W 5–10 min) until the sample became clear. Debris was removed by high speed centrifugation. The supernatant was saved for further analysis (Yang et al. 2009).

SDS–PAGE

SDS–PAGE was performed according to Laemmli (1970). The concentrations of the separation gel and focusing gel were 12.5% and 5% (w/v). Protein staining solution was Coomassie blue R-250 [0.1% (w/v) in 10% (w/v) acetic acid and 40% (w/v) ethanol. The washing solution was made up of water, ethanol and acetic acid (50:40:10).

Western blotting

Protein samples were separated with SDS–PAGE, blotted (for 90 min) onto PVDF membrane (MILLIPORE Immobilon-P) with blotting buffer (25 mM Tris, 190 mM glycine in 20% (v/v) aqueous methanol). The electric filed was 0.9 mA cm^{-2}. The antibody was His·Tag Monoclonal Antibody (NOVAGEN).

NaDH activity assay

The hydroxylation of nicotinate or 3-cyanopyridine was performed in 1.5 ml centrifuge tubes. Each reaction (0.5 ml) contained 20 mM phosphate buffer (pH 7.0), 5 mM nicotinate or 5 mM 3-cyanopyridine, cell extract. The reaction mix was shaken (200 rpm) at 30°C for 2 h. When cell extract was used, 50 μM PMS was added as the electron acceptor (32). At the conclusion of the reaction, enzyme was inactivated by heating (100°C for 5 min), centrifuged to remove precipitate and supernatant was saved for analysis.

Product was analyzed with high efficient liquid chromatography (Agilent 1100, USA). The detector was Agilent G1314A UV. The column was ZORBAX ODS (4.6 mm i.d. × 250 mm, 5 μm). For detection of 6-hydroxy-nicotinamide, the mobile phase was methanol and water (50:50, pH 3.0) at a velocity 1 ml/min. The detection wave length was 260 nm. For the detection of 6-hydroxy-3-cyanopyridine, the mobile phase was methanol and water at 30:70 (V:V, pH 3.0), the speed was 1 ml/min and the wave length was 230 nm. The products were diluted appropriately and quantified with external reference method (Yuan et al. 2005).

Under the above condition, the amount of enzyme that produces 1 μmol of hydroxylated product in 1 min is 1 Unit (U). The specific enzyme activity was defined as the amount of enzyme activities contained in 1 mg dry cell or protein.

Results

The cloning of *ndh*

Based on the conserved amino acid sequences of the known NaDH from *P. putida* KT2440 and three other strains, degenerated primers JA-1 and JA-2 were designed (Fig. 2). Using the genomic DNA from *C. testosteroni* JA1 as template, a 1.5-kb segment of of $NaDH_{JA1}$ was amplified (not shown). The multiple reverse PCR was employed to amplify the flanking regions: (1) Genomic DNA from *C. testosteroni* JA1 was digested with *Nco* I. The recovered fragment was used as the template to amplify the upstream 0.3 kb and downstream 0.9 kb with primers JA-3, JA-5, JA-4 and JA-6. Alignment analysis indicated the initiation sequence was included in upstream fragment.

USDA110 -------^{134}NLCRCG^{140}-------^{842}GESASVP^{849}-------

HaA2 ------- ^{158}NLCRCG^{164}-------^{896}GESASVP^{903}-------

JS666 ------- ^{134}NLCRCG^{140}-------^{880}GESASVP^{887}-------

KT2440 ------- ^{133}NLCRCG^{139}-------^{862}GESASVP^{869}-------

Fig. 2 Alignment of conserved NaDH amino acid sequences from four sources (ClustalV programs). Abbreviation: USDA110—NaDH from *Bradyrhizobium japonicum*, USDA110; HaA2—NaDH from *Rhodopseudomonas palustris* HaA2; JS666—NaDH from *Polaromonas sp*.JS666; KT2440—NaDH from *P. putida* KT2440

(2) *Sal* I digested genomic DNA was used to amplify further downstream sequence 1.4 kb with primers JA-7 and JA-8. (3) *C. testosteroni* JA1 genomic DNA was digested with *Pst* I and recovered fragment was used to amplify a 1.5 kb fragment with primers JA-9 and JA-10. Alignment analysis showed the sequence contained the stop codon. Finally, the entire sequence encoding $NaDH_{JA1}$ was amplified with primers JA-SLM-S and JA-SLM-F, cloned with TA cloning vector pMD18-T*ndhSLM*, and sequenced (Miyamoto et al. 2002).

The entire sequence encoding $NaDH_{JA1}$ was termed *ndh* cluster, which was 4.5 kb in size (GenBank accession: EU418449). The operon contained three genes termed *ndh*S, *ndh*L and *ndh*M, respectively encoding the NaDH*S*, NaDH*L* and NaDH*M* chains. The coding sequence of *ndh*S had a start codon at nucleotide (nt) 203 and a stop codon TGA at nt 902, encodes a peptide of 203 amino acid residues, 20,981 Da. The coding sequence of *ndh*L was 2295 bp, starting at nt 904 and ending at nt 3198, encoding a peptide chain of 765 amino acid residues, 82,389 Da. There were 2 bp between the initiation codon of *ndh*L and the termination codon of *ndh*S. The coding sequence of *ndh*M started at nt 3222 and ending at nt 4517, encoding a peptide chain of 432 amino acid residues, 46,042 Da. There were 24 bp between the initiation codon of *ndh*M and the termination codon of *ndh*L.

The similarity of NaDH amino acid sequences from different sources were analyzed with Mega 3.1. $NaDH_{JA1}$ amino acid sequence shows 52.8–54.6% identities with those containing cytochrome *c* in bacteria (e.g., *B. japonicum* USDA110, *R. palustris* HaA2, *Polaromonas sp*.JS666 and *P. putida* KT2440), while it exhibits 8.9–10.3% identities with

NaDHs containing FAD (e. g., *E. barkeri, S. pomeroyi* DSS-3, *M. magneticum* AMB-1and *M. loti* MAFF303099).

Expression of recombinant *ndhSLM*

The *ndh* cluster was amplified in two fragments (primers JA-SLM_1-S and JA-SLM_1-F for the first fragment and primers JA-SLM_2-S and JA-SLM_2-F for the second fragment). After purification, the two fragments were digested with restricting pairs *Bsa*I/*Afl*III and *Afl*III/*Hind*III, and then linked to pJB866H that was digested with *Afl*III, *Hind*III, obtained pJB866H*ndhSLM* which possesses a His·tag in the downstrean of *ndhSLM*. The plasmid was then introduced to *P. entomophila* L48, resulting in *P. entomophila* L48[pJB866H::*ndhSLM*]. Using ant-His·tag antibody, western blot analysis explored that a new expression band appeared at the expected size (around 46 kDa) of NaDHM in *P. entomophila* L48[pJB866H::*ndhSLM*] compared to *P. entomophila* L48 (Fig. 3), indicating that *ndhSLM* could sucesfully express in *P. entomophila* L48[pJB866H::*ndhSLM*].

The recombinant expression $NaDH_{JA1}$ from *P. entomophila* L48[pJB866H::*ndhSLM*] was able to convert nicotinate into 6-hydroxynicotinic acid, while the control cell extract was unable to convert nicotinate, suggesting that $NaDH_{JA1}$ has NaDH activity (Table 3). In addition, in the conversion solution containing 3-cyanopyridine instead of nicotinate, 6-hydroxy-3-cyanopyridine could be detected (Table 3), indicating $NaDH_{JA1}$ was also able to hydroxylate 3-cyanopyridine.

Analysis of functional domain of $NaDH_{JA1}$ compared to $NaDH_{KT2440}$

Similarity analysis explored that $NaDH_{JA1}$ protein showed 52.8% identity to the amino acid sequence of the $NaDH_{KT2440}$. The functional domain composition and arrangement are identical in the two enzymes. The amino acid sequence similarities between the two enzymes were 95/176 (53% identity), 406/755 (53% identity) and 193/427 (45% identity) for the [2Fe2S] domain, Mo domain, and cytochrome *c* domain respectively. The three functional domains of $NaDH_{JA1}$ were independently located in the three subunits NaDHS, NaDHL, NaDHM, while in $NaDH_{KT2440}$, [2Fe2S] domain was in NaDHS subunit, and the NaDHL is a single chain composed of Mo domain and cytochrome *c* domain.

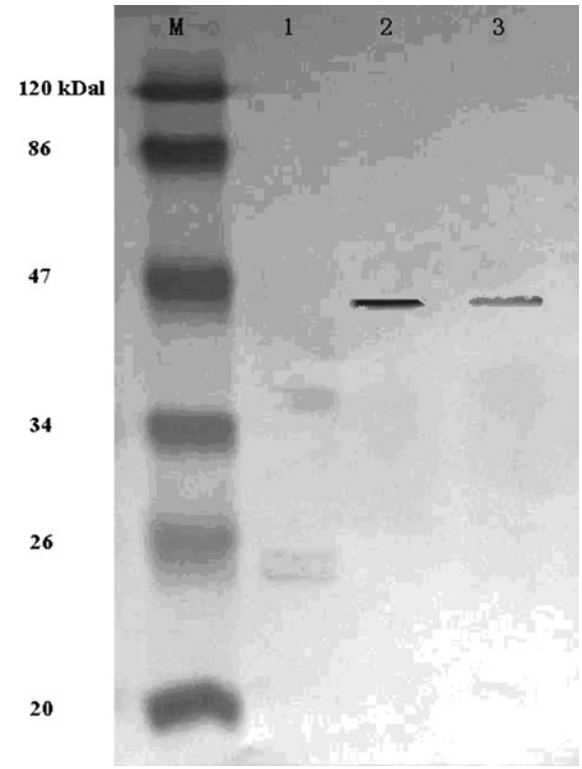

Fig. 3 Western blot analysis of of *ndh*M expression in *P. entomophila* L48. M—Protein Marker; 1—extract of noneinduced *P.entomophila* L48[pJB866H::*ndhSLM*]; 2—total protein of induced *P.entomophila* L48[pJB866H::*ndhSLM*]; 3—cell extract from induced *P.entomophila* L48[pJB866H::*ndhSLM*]

Table 3 NaDH activity in extract of cells expressing recombinant *ndhSLM* ($n = 9$)

Substrate	Crude extract Sp activity (U/mg of protein)	
	Nicotinate	3-Cyanopyridine
Plasmid control	<0.01	<0.01
None induced	<0.01	<0.01
Induced	2.07 ± 0.02	1.72 ± 0.03

We further analyzed differences in functional domains of $NaDH_{JA1}$ and $NaDH_{KT2440}$ (Fig. 4). The compositions of cytochrome *c* domain were the same in $NaDH_{KT2440}$ and $NaDH_{JA1}$. However, there were differences in the [2Fe-2S] center. In $NaDH_{KT2440}$, the [2Fe-2S] cluster consisted of type II motif (Doolittle 1995; Grether-Beck et al.1994). In

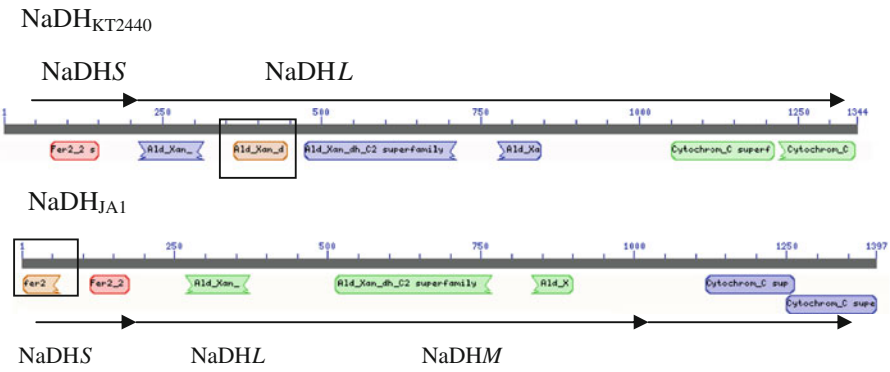

Fig. 4 Alignment of amino acid sequences from NaDH$_{JA1}$ and NaDH$_{KT2440}$. Two frames shows two functional domain differences between the NaDH$_{JA1}$ and NaDH$_{KT2440}$: (1) NaDH$_{JA1}$ contained type I [2Fe-2S] (residues 3–81); (2) NaDH$_{KT2440}$ had an aldehyde oxidase and xanthine dehydrogenase a/b hammerhead domain (residues 361–447)

contrast, the NaDH$_{JA1}$ contained both type II (residues 102–177) and type I [2Fe-2S] (residues 3–81) motifs (Doolittle 1995; Grether-Beck et al.1994). In addition, there were also differences in the molybdenum domain. NaDH$_{KT2440}$ had an aldehyde oxidase and xanthine dehydrogenase a/b hammerhead domain (residues 361–447) that NaDH$_{JA1}$ did not (Doolittle 1995; Grether-Beck et al.1994).

Hybrid recombination of NaDH$_{KT2440}$ and NaDH$_{JA1}$

In order to interrogate whether the substrate specificity was determined by the [2Fe-2S] domain or by the Mo domain, we performed a hybrid recombination experiment where the *ndhS* was swapped between NaDH$_{KT2440}$ and NaDH$_{JA1}$.

Using DNA from *P. putida* KT2440 as the template, *ndh*S$_{KT2440}$ was amplified with primers KT-S–S and KT-S–F. Similarly, using DNA from *C. testosteroni* JA1 as the template, *ndh*LM$_{JA1}$ was amplified with primers JA-LM-S and JA-LM-F. The two fragments were digested with two pairs of restriction enzymes (*Nco*I/*Xba*I and *Xba*I/*Hind*III) and inserted into pJB866H vector double digested with *Afl*III and *Hind*III, resulting in plasmid pNKKTS. Reversely, *ndh*S$_{JA1}$ was amplified with primer pair JA-S–S/JA-S–F, *ndh*L$_{KT2440a}$ and *ndh*L$_{KT2440b}$ were amplified with primer pairs KT-La-S/KT-La-F and KT-Lb-S/KT-Lb-F. The three fragments were double digested with *Bsa*I/*Xba*I, *Xba*I/*Sph*I, *Sph*I/*Hind*III respectively and inserted to vector pJB866H. The hybrid recombinant plasmid pNJAS was constructed as such. Using pNKTS and pNJAS to transform *P.entomophila* L48, recombinant cells *P. entomophila* L48[pNJAS] and *P. entomophila* L48[pNKTS] were obtained. The expressed recombinant proteins were respectively termed NaDH$_{JAS+KTL}$ and NaDH$_{KTS+JALM}$. The recombinant cell extracts were used to catalyze hydroxylation of nicotinate and 3-cyanopyridine. Results were shown in Table 4. The domain-swapped recombinant proteins were both able to catalyze hydroxylation of nicotinate, suggesting the domain-swapped recombinant proteins were folded correctly and were functional. This result also suggested that the differences in NaDHS subunits from *P. putida* KT2440 and *C. testosteroni* JA1 did not affect the hydroxylation function. However, proteins containing the NaDH*S* subunit from *P. putida* KT2440 (NaDH$_{KT2440}$ and NaDH$_{KTS+JALM}$) were unable to catalyze hydroxylation of 3-cyanopyridine. In contrast, proteins containing the NaDH*S* subunit from *testosteroni* JA1 (NaDH$_{JA1}$ and NaDH$_{JAS+KTL}$) were. These results suggest that the NaDH*S* subunit determines 3-canopyridine hydroxylation activity. Fig. 5 showed that HPLC profiles of the domain swap experiment.

Table 4 Conversion of nicotinate and 3-cyanopyridine by NaDH$_{KTS+JALM}$ and NaDH$_{JAS+KTL}$ ($n = 9$)

	Nicotinate	3-Cyanopyridine
NaDH$_{KT2440}$	1.89 ± 0.02	<0.01
NaDH$_{JA1}$	2.07 ± 0.03	1.72 ± 0.02
NaDH$_{KTS+JALM}$	1.92 ± 0.04	<0.01
NaDH$_{JAS+KTL}$	2.02 ± 0.02	1.56 ± 0.03

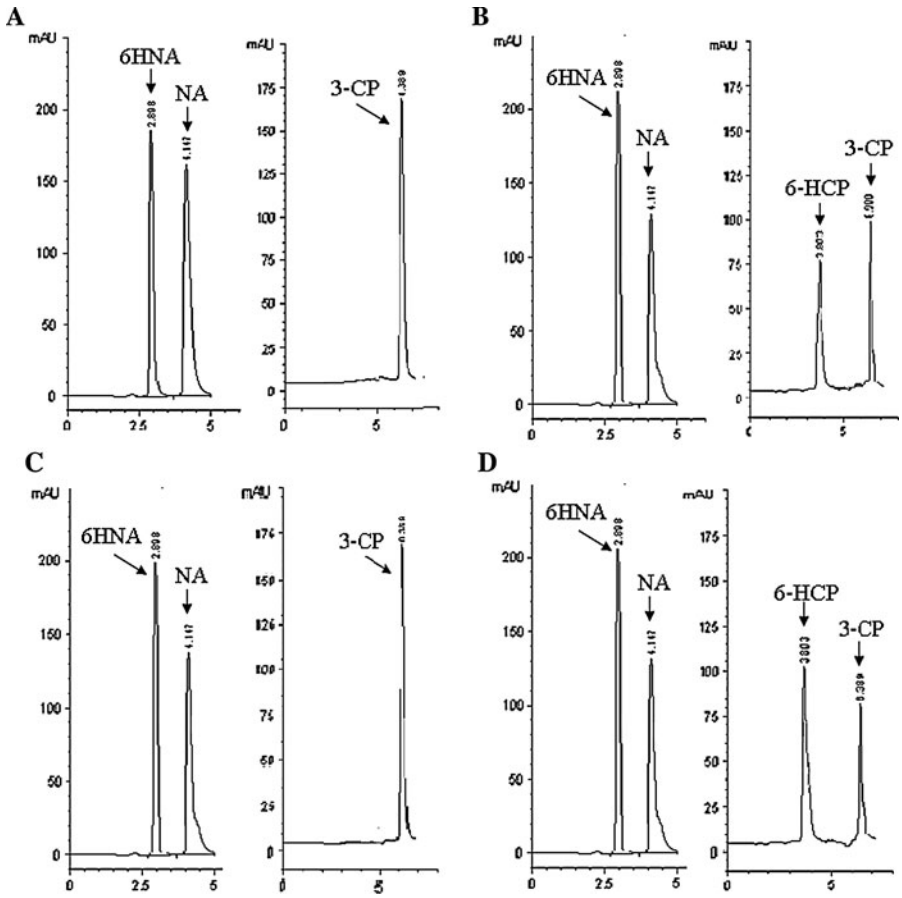

Fig. 5 HPLC profiles of *ndhS* swapped recombinant proteins. **a** Before swap, $NaDH_{KT2440}$ could catalyze the hydroxylation of nicotinate, but not 3-cyanopyridine. **b** Before swap, $NaDH_{JA1}$ could catalyze the hydroxylation of both nicotinate and 3-cyanoridine. **c** After swap, $NaDH_{KTS+JALM}$ could catalyze the hydroxylation of nicotinate, but not 3-cyanopyridine. **d** After swap, $NaDH_{JAS+KTL}$ could catalyze the hydroxylation of both nicotinate and 3-cyanoridine

Discussion

We have cloned and expressed recombinant *ndhSLM* cluster from *C. testosteroni* JA1. The recombinant protein ($NaDH_{JA1}$) was able to catalyze hydroxylation of nicotinate, indicating the cloned sequence encodes NaDH. The recombinant $NaDH_{JA1}$ was also able to catalyze the conversion of 3-cyanopyridine to 6-hydroxy-3-cyanopyridine, indicating $NaDH_{JA1}$ was also able to catalyze the hydroxylation of 3-cyanopyridine. Therefore, NaDH $_{JA1}$ of *C. testosteroni* JA1 was apparently different from that of *P. putida* KT2440 where the NaDH was only capable of converting nicotinate (Yang et al. 2009). Thus, we demonstrated that the hydroxylation of nicotinate and 3-cyanopyridine was catalyzed by the same enzyme in *C. testosteroni* JA1 and there is no additional 3-cyanopyridine hydroxylase.

$NaDH_{JA1}$ encoded by *ndhSLM* gene cluster in *C. testosteroni* JA1 was 149 kDa, composed of 21, 82 and 46 kDa subunits, respectively containing [2Fe-2S], Mo(V) and cytochrome *c* domains. Similar to $NaDH_{KT2440}$, NaDH $_{JA1}$ was distinguished apparently from those NaDHs containing FAD such as $NaDH_{barkeri}$ (Alhapel et al. 2006), by having a cytochrome *c* domain. The Mo(V) domains in $NaDH_{barkeri}$ was found to exist in both NaDHL and NaDHM subunit(Alhapel et al. 2006), while the Mo(V) domain and cytochrome *c* domain in $NaDH_{KT2440}$ was found to co-exist in a subunit NaDHL (Jiménez et al. 2008; Yang et al. 2009). In contrast, here we found that the Mo(V) and cytochrome *c* domains in $NaDH_{JA1}$ were respectively in two separate subunits NaDHL and NaDHM. Thus, NaDHs may have 2, 3 or 4 subunits and different functional domains may exist separately or together in different subunits, varying with different species, which is believed to be the results of evolutionary (Doolittle 1995).

In 1994, Hurth and Nagasawa et al. purified one NaDH from *P. fluorescens* TN5, demonstrated that the NaDH also catalyzes hydroxylation of 3-cyanopyridine (Hurh et al. 1994b). In 2008, Jiménez et al.

reported the cloning of $NaDH_{KT2440}$ from *P. putida* KT2440 and the recombinant expression of $NaDH_{KT2440}$ in *P. fluorescens* R2f. They showed their recombinant cell extract was able to catalyze nicotinate hydroxylation. The extract was also able to catalyze weakly 3-cyanopyridine hydroxylation, accounting for 4% of the hydroxylation activities (Jiménez et al. 2008). However, *P. fluorescens* was even reported to be able to catalyze 3-cyanopyridine hydroxylation (Hurh et al. 1994b), so the interference of endogenous enzyme from the host cell could not be excluded. We have also cloned $NaDH_{KT2440}$ from *P. putida* KT2440 but expressed it in *P. entomophila* L48 that has no endogenous 3-cyanopyridine hydroxylation activity. Our recombinant $NaDH_{KT2440}$ showed nicotinate hydroxylation activity but no 3-canoppridinde hydroxylation activity (Yang et al. 2009), which is consistent with the result that *P. putida* KT2440 could only hydroxylate nicotinate in our previous study (Yang et al. 2009). Therefore in the current study, we chose to analyze *C. testosteroni* JA1 which is capable of hydroxylating both nicotinate and 3-cyanopyridine to explore whether NaDH can catalyze the hydroxylation of both nicotinate and 3-cyanopyridine, or whether there is an independent enzyme for 3-cyanopridine hydroxylation. Results demonstrated the recombinant $NaDH_{JA1}$ can hydroxylate both substrates. Analysis of sequence similarity showed that $NaDH_{KT2440}$ shares 63–69.1% identities with those NaDH containing cytochrome *c* domains, but only exhibits 52.8–54.6% identities with $NaDH_{JA1}$. It is therefore possible that the NaDHs of less similar sequences lead to wider substrate specificity of $NaDH_{JA1}$. The current study showed that the major sequence differences between $NaDH_{JA1}$ and $NaDH_{KT2440}$ lay in the [2Fe-2S] and Mo(V) domains. $NaDH_{KT2440}$ has only one type II [2Fe-2S] motif, while $NaDH_{JA1}$ has one type II [2Fe-2S] and a type I [2Fe-2S] motifs. The Mo(V) domain in $NaDH_{KT2440}$ also includes a hypoxanthine dehydrogenase motif which $NaDH_{JA1}$ does not have. If the small subunit of $NaDH_{JA1}$ is replaced by the small subunit from $NaDH_{KT2440}$, the hybrid protein $NaDH_{KTS+JALM}$ was able to catalyze nicotinate hydroxylation, but lost the ability to catalyze 3-cyanopyridine hydroxylation. Conversely, if the small subunit of $NaDH_{KT2440}$ was replaced by the one from $NaDH_{JA1}$, the hybrid $NaDH_{JAS+KTL}$ acquired the ability to catalyze 3-cyanopyidine hydroxlation. These results indicate that the [2Fe2S] domain determines the 3-cyanopyridine hydroxylation ability. Studies of XO family of molybdenum proteins have shown that molybdenum domain is the activity center of quinoline 2-oxidoreductase. There is a substrate canal in the center interact with the substrate, while [2Fe2S] motif is implicated in electron transduction (Bonin et al. 2004). There is also report that the Mo domain in the activity center of aldehyde oxidoreductase is involved in substrate binding, while the [2Fe2S] domain is responsible for the formation of a stable clamp structure together with Mo domain, which did not prove whether [2Fe2S] domain participates in substrate specificity determination (Kisker et al. 1997). In the future, further study is required to determine what key residues in [2Fe2S] domain of $NaDH_{JA1}$ are responsible for specific binding of 3-cyanopyridine.

Acknowledgments We thank Isabelle Vallet-Gely, Bruno Lemaitre laboratory, FRANCE, and He Jian, Nanjing Agriculture University, Department of Life Science, for kindly providing *Pseudomonas putida* L48 and *Pseudomonas putida* KT2440, respectively. This work was supported by the Key Fundamental Research Program of Jiangsu Higher Education Institution of China (06KJA21016), the Natural Science Foundation of Jiangsu Higher Education Institution of China (04KJB180071).

References

Alhapel A, Darley DJ, Wagener N, Eckel E, Elsner N, Pierik AJ (2006) Molecular and functional analysis of nicotinate catabolism in *Eubacterium barkeri*. Proc Natl Acad Sci USA 103:12341–12346

Andreesen JR, Fetzner S (2002) The molybdenum-containing hydroxylases of nicotinate, isonicotinate, and nicotine. Met Ions Biol Syst 39:405–430

Behrman EJ, Stanier RY (1957) The bacterial oxidation of nicotinic acid. J Biol Chem 228:923–945

Berry DF, Francis AJ, Bollag JM (1987) Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. Microbiol Rev 51:43–59

Blatny JM, Brautaset T, Winther-Larsen HC, Haugan K, Valla S (1997a) Construction and use of a versatile set of broad-host-range cloning and expression vectors based on the RK2 replicon. Appl Environ Microbiol 63:370–379

Blatny JM, Brautaset T, Winther-Larsen HC, Karunakaran P, Valla S (1997b) Improved broad-host-range RK2 vectors useful for high and low regulated gene expression levels in gram-negative bacteria. Plasmid 38:35–51

Bonin I, Martins BM, Purvanov V, Fetzner S, Huber R, Dobbek H (2004) Active site geometry and substrate recognition of the molybdenum hydroxylase quinoline 2-oxidoreductase. Structure 12:1425–1435

Davis RW, Botstein D, Roth JR (1980) A manual for genetic engineering: advanced bacterial genetics. Cold Spring Harbor, NY

Doolittle RF (1995) The multiplicity of domains in proteins. Annu Rev Biochem 64:287–314

Dower WJ, Miller JF, Ragsdale CW (1988) High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res 16:6127–6145

Ensign JC, Rittenberg SC (1964) The pathway of nicotinic acid oxidation by a *Bacillus* species. J Biol Chem 239:2285–2291

Grether-Beck S, Igloi GL, Pust S, Schilz E, Decker K, Brandsch R (1994) Structural analysis and molybdenum-dependent expression of the pAO1-encoded nicotine dehydrogenase genes of *Arthrobacter nicotinovorans*. Mol Microbiol 13:929–936

Harary I (1957) Bacterial fermantation of nicotinic acid. II. Anaerobic reversible hydroxylation of nicotinic acid to 6-hydroxynicotinic acid. J Biol Chem 227:823–831

Hughes DE (1955) 6-Hydroxynicotinic acid as an intermediate in the oxidation of nicotinic acid by *Pseudomonas fluorescens*. Biochem J 60:303–310

Hunt AL (1959) Purification of the nicotinic acid hydroxylase system of *Pseudomonas fluorescens* KB1. Biochem J 72:1–7

Hunt AL, Hughes DE, Lowenstein JM (1958) The hydroxylation of nicotinic acid by *Pseudomonas fluorescens*. Biochem J 69:170–173

Hurh B, Ohshima M, Yamane T, Nagasawa T (1994a) Microbial production of 6-hydroxynicotinic acid, an important building block for the synthesis of modern insecticides. J Ferment Bioeng 77:382–385

Hurh B, Yamane T, Nagasawa T (1994b) Purification and characterization of nicotinic acid dehydrogenase from *Pseudomonas fluoescens* TN5. Ferment Bioeng 78:19–26

Iwasaki K, Uchiyama H, Yagi O, Kurabayashi T, Ishizuka K, Takamura Y (1994) Transformation of *Pseudomonas putida* by electroporation. Biosci Biotechnol Biochem 58:851–854

Jiménez JI, Canales A, Jiménez-Barbero J, Ginalski K, Rychlewski L, García JL, Díaz E (2008) Deciphering the genetic determinants for aerobic nicotinic acid degradation: the *nic cluster* from *Pseudomonas putida* KT2440. Proc Natl Acad Sci USA 105(32):11329–11334

Jones MV (1973) Cytochrome *c* linked nicotinic acid hydroxylase in *Pseudomonas ovalis* Chester. FEBS Lett 32:321–324

Jones MV, Hughes DE (1972) The oxidation of nicotinic acid by *Pseudomonas ovalis* Chester. The terminal oxidase. Biochem J 129:755–761

Kisker C, Schindelin H, Rees DC (1997) Molybdenum-cofactor-containing enzymes: structure and mechanism. Annu Rev Biochem 66:233–267

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lu WH, Wang X, Xu L, Dai YJ, Yuan S (2005) Induction of nicotinic acid hydroxylase activity of *Pseudomonas putida* NA-1 and optimization of transformation conditions. Acta Microbiologica Sinica 45:6–9

Miyamoto Y, Johdo O, Nagamatsu Y, Yoshimoto A (2002) Cloning and characterization of a glycosyltransferase gene involved in the biosynthesis of anthracycline antibiotic beta-rhodomycin from *Streptomyces violaceus*. FEMS Microbiol Lett 206:163–168

Nagel M, Andreesen JR (1989) Molybdenum-dependent degradation of nicotinic acid by *Bacillus* sp. DSM 2923. FEMS Microbiol Lett 59:147–152

Nelson KE, Weinel C, Paulsen IT, Dodson RJ, Hilbert H, Martins dos Santos VA, Fouts DE, Gill SR, Pop M, Holmes M, Brinkac L, Beanan M, DeBoy RT, Daugherty S, Kolonay J, Madupu R, Nelson W, White O, Peterson J, Khouri H, Hance I, Chris Lee P, Holtzapple E, Scanlan D, Tran K, Moazzez A, Utterback T, Rizzo M, Lee K, Kosack D, Moestl D, Wedler H, Lauber J, Stjepandic D, Hoheisel J, Straetz M, Heim S, Kiewitz C, Eisen JA, Timmis KN, Dusterhoft A, Tummler B, Fraser CM (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ Microbiol 4:799–808

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, vol 2. Cold Spring Harbor Laboratory Press, NY

Tautz D, Renz M (1983) An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. Anal Biochem 132:14–19

Vodovar N, Vallenet D, Cruveiller S, Rouy Z, Barbe V, Acosta C, Cattolico L, Jubin C, Lajus A, Segurens B, Vacherie B, Wincker P, Weissenbach J, Lemaitre B, Medigue C, Boccard F (2006) Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium *Pseudomonas entomophila*. Nat Biotechnol 24:673–679

Yang Y, Yuan S, Chen T, Ma PJ, Shang GD, Dai YJ (2009) Cloning, heterologous expression, and functional characterization of the nicotinate dehydrogenase gene from *Pseudomonas putida* KT2440. Biodegradation 20:541–549

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119

Yasuda M, Sakamoto T, Sashida R, Ueda M, Morimoto Y, Nagasawa T (1995) Microbial hydroxylation of 3-cyanopyridine to 3-cyano-6-hydroxypyridine. Biosci Biotechnol Biochem 59:572–575

Yuan S, Yang Y, Sun J, Dai YJ (2005) A combined technology of growing culture hydroxylation of nicotinic acid and resting cells hydroxylation of 3-cyanopyridine by *Comamonas testosterone* JA1. Eng Life Sci 5:369–374