International Biodeterioration & Biodegradation 63 (2009) 988–992

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

PanB is involved in nicotine metabolism in *Pseudomonas putida*

Hailei Wei [a,b,1], Liping Lei [c,1], Shuo Liu [d], Zhenyuan Xia [c], Xingzhong Liu [b,*], Peigui Liu [a,**]

[a] Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China
[b] Key Laboratory of Systematic Mycology and Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Datun Rd., Chaoyang District, Beijing 100101, China
[c] Yunnan Tobacco Research Institute, Yuxi, China
[d] Affiliated High School of Beijing Jiaotong University, Beijing, China

ARTICLE INFO

Article history:
Received 23 November 2008
Received in revised form
19 January 2009
Accepted 9 February 2009
Available online 17 July 2009

Keywords:
Biodegradation
Nicotine
Pseudomonas putida
Ketopantoate hydroxymethyltransferase

ABSTRACT

A nicotine-sensitive mutant was generated from the nicotine-degrading bacterium, *Pseudomonas putida* strain J5, by mini-Tn5 transposon mutagenesis. This mutant was unable to grow with nicotine as the sole carbon source but could grow with glucose. Sequence analysis showed that the Tn5 transposon inserted at the site of the ketopantoate hydroxymethyltransferase gene (*panB*), which had 54% identity to PanB in *Escherichia coli* K-12. In-frame deletion of the *panB* gene abolished the nicotine-degrading ability of strain J5, while complementation with *panB* from *P. putida* J5 and *E. coli* K-12 restored the degrading activity of the mutant to the wild-type level. These results suggest that ketopantoate hydroxymethyltransferase is a crucial enzyme in nicotine metabolism in *P. putida* J5.

1. Introduction

According to the World Health Organization, 1.1 billion people smoke tobacco cigarettes worldwide, consuming about 6000 billion cigarettes every year. The main alkaloid synthesized by the tobacco plant is nicotine, which can constitute as much as 3% of tobacco leaf dry mass (Armstrong et al., 1998). When the cigarette smoke is inhaled, 90% of the nicotine is quickly absorbed by the lungs (Zhang et al., 2003). Statistics from the American Lung Association indicate that about 350,000 Americans die prematurely each year from lung cancer, emphysema, coronary heart disease, and other diseases caused by cigarette smoking. In addition to harming individuals who smoke, cigarettes and other tobacco products can damage the environment because the tobacco-manufacturing process generates many toxic chemicals as waste, and chief among these is nicotine. When the nicotine content exceeds 500 mg kg^{-1} dry weight, the non-recyclable, powdery waste is classified as "toxic and hazardous" by European Union Regulations (Civilini et al., 1997).

Some microorganisms are able to degrade nicotine and play an important role in tobacco-manufacturing by altering the nicotine content of toxic and hazardous waste. In particular, species of the genera *Arthrobacter* and *Pseudomonas* are able to extract energy by metabolizing nicotine (Hochstein and Rittenberg, 1958; Thacker et al., 1978; Schenk et al., 1998; Ruan et al., 2005). The nicotine metabolic pathway following the pyridine route encoded on the megaplasmid pA01 was well elucidated in *Arthrobacter nicotinovorans* (Baitsch et al., 2001; Igloi and Brandsch, 2003). However, the molecular mechanisms of nicotine degradation and metabolism in *Pseudomonas* are less well documented, though putative intermediates were identified, such as pseudoxynicotine, methylamine, 3-succinoylpyridine, and 6-hydroxy-3-succinoylpyridine (Thacker et al., 1978; Wang et al., 2007).

Pseudomonas putida J5 (Wei et al., 2008), isolated from the tobacco rhizosphere, is an efficient nicotine-degrading strain. In this study, the *P. putida* J5 mutants produced by Tn5 mutagenesis were screened for the inability to metabolize nicotine, and a ketopantoate hydroxymethyltransferase gene was cloned and its function in nicotine metabolism was determined.

2. Materials and methods

2.1. Strains, plasmids, and growth conditions

Characteristics of strains and plasmids are listed in Table 1. *P. putida* was grown in Luria–Bertani (LB) medium or M9 (Sambrook and Russell, 1998) medium at 28 °C, whereas *Escherichia coli* was grown at

* Corresponding author. Tel.: +86 10 64807512; fax: +86 10 64807505.
** Corresponding author. Tel.: +86 0871 5223125.
E-mail addresses: liuxz@im.ac.cn (X. Liu), pgliu@kib.ac.cn (P. Liu).
[1] Hailei Wei and Liping Lei contributed equally to this work.

0964-8305/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibiod.2009.02.010

Table 1
Strains and plasmids used in this study.

Strains or plasmids	Characteristics	Source
Pseudomonas putida strains		
J5	Ap^r; wild type	Wei et al., 2008
J5ΔB	Ap^r; *panB* deletion mutant	This study
J5ΔB-B	Ap^r; J5ΔB containing pCMS2-B	This study
J5ΔB-K12B	Ap^r; J5ΔB containing pK12-B	This study
Escherichia coli strains		
K-12	Δ(*lac pro*) *rif nalA ara argE*(Am)	Sambrook and Russell, 1998
DH5α	F^- *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*Δ (*argF-lacZYA*)*I169Φ80lacZ ΔM15*	Sambrook and Russell, 1998
DH5α (λpir)	*thi pro hsdR* $hsdM^+$ *recA* RP4-2-Tc::Mu-Km::Tn*7 λpir*	Miller and Mekalanos, 1988
HB101	Δ*(gpt-proA)62 gln V44 recA13*	Sambrook and Russell, 1998
Plasmids		
pUTKm	Ap^r; Km^r; delivery plasmid for Tn5; R6K replicon	Herrero et al., 1990
pBluescript II SK+	Ap^r; ColE1 origin	Stratagene
pBBR1MCS-2	Km^r; *mob, rep, lacZα*$^+$	Kovach et al., 1995
pSR47S	Km^r; *oriT sacB*	Andrews et al., 1998
pRK600	Cm^r; ColE1 replicon with RK2 transfer region, helper plasmid	Finan et al., 1986
pBS-M10	Ap^r; pBluescript containing 4.5 kb *Pst*I fragment	This study
pBSΔBR	Ap^r; pBluescript containing 720 bp *Eag*I–*Sac*I fragment	This study
pBSΔB	Ap^r; pBSΔBR containing 1.3 kb *Sal*I–*Eag*I fragment from pBS-M10	This study
p47SΔB	Km^r; pSR47S containing 2.1 kb *Sal*I–*Sac*I fragment from pBSΔB	This study
pCMS2-B	Km^r; pBBR1MCS-2 containing 1.5 kb *Eco*RI–*Hin*dIII *panB* fragment from *P. putida* J5	This study
pK12-B	Km^r; pBBR1MCS-2 containing 1.0 kb *Eco*RI–*Hin*dIII *panB* fragment from *E. coli* K-12	This study

Ap^r, Km^r, and Cm^r indicate resistance to ampicillin, kanamycin, and chloromycetin, respectively.

37 °C in LB medium. For plasmid propagation and selection of transformants, media were supplemented with antibiotics at appropriate concentrations as follows: 100 μg ml^{-1} ampicillin, 50 μg ml^{-1} kanamycin, and 20 μg ml^{-1} chloramphenicol.

2.2. DNA manipulations and sequencing

Chromosomal DNA of bacteria was extracted by the CTAB method (Del Sal et al., 1988). Plasmid DNA extractions and other molecular assays were performed according to standard procedures (Sambrook and Russell, 1998). Nucleotide sequencing was performed by Invitrogen Co. Ltd., China. Nucleotide and deduced amino acid sequences were analyzed with the on-line BLAST search engine in GenBank.

2.3. Tn5 transposon mutagenesis

Tn5 mutagenesis of *P. putida* J5 was carried out by triparental mating as described by Revelles et al. (2005). *P. putida* J5, *E. coli* DH5α (λpir) containing the donor plasmid pUTKm, and *E. coli* HB101 containing the helper plasmid pRK600 were grown overnight in LB medium on a shaker at 200 rpm. A suspension containing 1 ml from each of these three cultures was mixed and then centrifuged. The cells were harvested by discarding the supernatant, washed once with autoclaved distilled water, and finally suspended in 0.2 ml of sterilized water. The cell suspensions were spotted on one LB plate lacking antibiotics. After incubation at 30 °C for 6 h, cells were scraped off and resuspended in 1 ml of autoclaved distilled water. Serial dilutions were spread onto fresh M9 plates containing 1.0 g l^{-1} glucose, 50 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin. After incubation at 30 °C for 48 h, individual colonies were picked out as Tn5 insertional conjugants and inoculated onto M9 media with either 1.0 g l^{-1} nicotine or 1.0 g l^{-1} glucose as the sole carbon source. The mutants, which could not grow on nicotine media but could grow on the glucose media, were selected and streaked to yield single colonies.

2.4. Southern hybridization and cloning of the Tn5 insertion site

To determine the copy number of Tn5 transposon in mutant strain, total DNA was digested with *Eco*RI, *Pst*I, *Bam*HI, *Hin*dIII, and *Sal*I; separated by electrophoresis on 0.8% (w/v) agarose gel and transferred onto nylon membrane (Hybond-N+; Amersham, GE Healthcare, Piscataway, NJ, USA). A 600 bp fragment of Tn5 transposon containing the kanamycin resistance gene was amplified with a set of primers Km421 (5′-ATG TCG GGC AAT CAG GTG CG-3′) and Km1016 (5′-GGC AAG ATC CTG GTA TCG GTC-3′). The PCR product was then used to prepare a digoxigenin-labeled probe using the PCR DIG Probe Synthesis Kit (Roche). Hybridization and detection were conducted according to the protocol for the DIG High Primer DNA Labeling and Detection Starter Kit I (Roche). The insertion site of the transposon was cloned into pBluescript by the shot-gun strategy with appropriate restriction endonuclease according to the result of Southern blotting. The positive clone harboring flanking sequences of Tn5 transposon, resistant to kanamycin, was obtained and sequenced by two primers (Tn5-39: 5′-CGG CAT AGG CGG CCA GAT C-3′ and Tn5-1571: 5′-CCG CAC TTG TGT ATA AGA GT-3′) located at the two ends of the mini-Tn5. This made it possible to read outside the transposon and within the chromosomal insert.

2.5. Construction of a panB in-frame deletion-mutant strain

To create a *panB* gene deletion allele, two fragments flanking *panB* gene were generated by endonuclease digestion and PCR. A 1.3 kb fragment was created by digestion with *Sal*I and *Eag*I from pBS-M10, and the other 0.7 kb fragment was amplified with primers M10-318 (5′-CGT TGA TCC CGG CCG ACC TG-3′), which introduced *Eag*I site (underlined), and M10-393 (5′-ACC ACG AGC TCA GCA CGC TG-3′), which introduced the *Sac*I site (underlined). The standard PCR reaction involved 5 min at 94 °C; followed by 35 cycles of 40 s at 94 °C, 40 s at 58 °C, and 1 min at 72 °C; and finally 10 min at 72 °C. After being digested with relevant restriction enzymes, the two fragments were inserted into pBluescript to create pBSΔB. An approximately 2.0 kb *Sac*I–*Sal*I fragment, including the *panB* gene with 220 bp deletion, was lifted and ligated

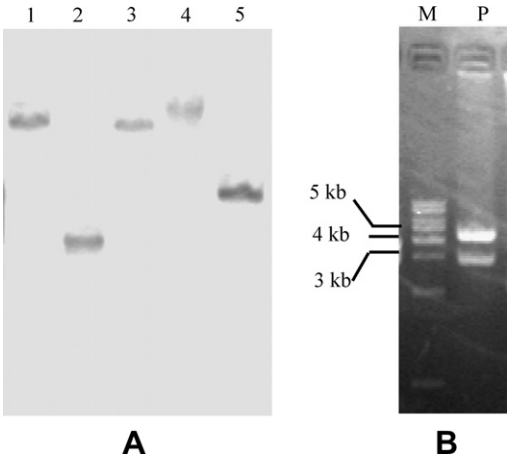

Fig. 1. Southern hybridization analysis of *Pseudomonas putida* J5-M10 (A) and restriction enzyme digestion analysis of pBS-M10 with *Pst*I (B). In (A), 1, 2, 3, 4, 5 represent the genomic DNA of J5-M10 after digestion with *Eco*RI, *Pst*I, *Bam*HI, *Hin*dIII, or *Sal*I, respectively. In (B), M, KB marker; P, pBS-M10 digested with *Pst*I.

into pSR47S. The last suicide plasmid p47SΔB was used in a two-step strategy to introduce the shortened *panB* locus into the chromosome of *P. putida* J5 according to previous methods (Wei and Zhang, 2006). Primers PanB-F (5′-CGC GCT ACA CCA ATG CCG TG-3′) and PanB-R (5′-ATG GCC GCT GTG CAG GTT GC-3′) were used to confirm a double crossover event.

To complement the *panB* mutant, a 1.5-kb fragment containing the putative upstream promoter and coding region of the *panB* gene was amplified from *P. putida* J5 with primers PanB-F and PanB-R. Primers K12B-F (5′-GGG AAG CTT GCT ATC GAA CCC G-3′) and K12B-R (5′-GGC GAA TTC GCT GAC GCA GC-3′) were also used to amplify the *panB* gene from *E. coli* K-12 for complementing the *panB* mutant. The amplified intact *panB* genes were inserted into the shuttle vector pBBR2MCS, and the resulting plasmids were introduced into strain J5ΔB by triparental mating (Wei and Zhang, 2006).

2.6. Detection of nicotine concentration

P. putida J5 and its derivatives were cultured to stationary phase in LB medium, and 0.1 ml of the cell suspension was inoculated into 100 ml of M9 media containing 1.0 g l^{-1} nicotine and incubated at 30 °C. Uninoculated sterile medium was used as a blank control. To determine cell density, the absorbance (600 nm) of 3 ml of culture was determined with a spectrophotometer at 2 h intervals. The cell suspensions were then centrifuged, and the nicotine concentration of the supernatant was determined by high-pressure liquid chromatography (HPLC) (Lei et al., 2007).

3. Results

3.1. Isolation of a P. putida J5 mutant unable to metabolize nicotine

The genes involved in metabolism of nicotine were characterized by mutagenesis with the transposon Tn5. Following this procedure, a mini-Tn5 mutagenesis library of *P. putida* J5 was constructed, and one mutant J5-M10 that failed to grow in the M9 medium with 1.0 g l^{-1} nicotine as the sole carbon source was screened from 1820 transformants.

3.2. Cloning of the adjacent DNA to a Tn5 insertion

Southern hybridization analysis showed that only one band was detected from the genomic DNA of J5-M10 that was respectively digested with *Eco*RI, *Pst*I, *Bam*HI, *Hin*dIII, and *Sal*I (Fig. 1A). This suggested that J5-M10 had a single copy insertion of Tn5 in its genome. Genomic sequences flanking the insertion site of J5-M10 were cloned by the shot-gun strategy with *Pst*I according to the result of Southern blotting. The resulting plasmid (pBS-M10) contained about 4.4 kb foreign fragment including 1.6 kb of the mini-Tn5 plus 2.8 kb of chromosomal DNA (Fig. 1B). Sequence analysis revealed that the Tn5 cassette was inserted into a putative open reading frame of about 801 bp and encoding a polypeptide of 266 amino acids with a molecular mass of 27.8 kDa. The deduced amino acid sequence showed 54% identity to ketopantoate hydroxymethyltransferase (PanB) of *E. coli* K-12 (Fig. 2), in which PanB initiates the first reaction of pantothenate biosynthesis (Jones et al., 1993). The insertion occurred at the site of 185 amino acids of PanB. The nucleotide sequence of the *P. putida* J5 *panB* gene has been deposited in the GenBank database under accession no. EU037098.

3.3. Effect of the panB gene on nicotine-degrading ability

Plasmid p47SΔB was used in a two-step strategy to introduce the deleted *panB* locus into the chromosome of *P. putida* J5 according to previous methods (Wei and Zhang, 2006). The deletion mutant was confirmed by PCR with primers PanB-F and PanB-R (Fig. 3). A 1.5 kb fragment was amplified from wild-type strain J5 while only a 1.3 kb fragment was amplified from strain J5ΔB, which indicated that a sequence of the *panB* gene containing about 200 bp had been deleted from strain J5ΔB.

Under optimal conditions, strain J5 could fully degrade 1.0 g l^{-1} nicotine for about 14 h but no nicotine could be degraded after deletion of the *panB* gene (Fig. 4). The ability to degrade nicotine was restored by complementation with the *panB* gene, whether that gene was from *P. putida* J5 or *E. coli* K-12. These results

```
J5-PanB    MPEVTLTTLHGRKAKGEKITMLTCYDATFAKVASQAGVDVLLVGDSLGMVLQGHDSTLPVCVKRGNEGAL    70
K12-PanB   MKPTTISLLQKYKQEKKRFATITAYDYSFAKLFADEGLNVMLVGDSLGMTVQGHDSTLPVAVRRGAPNCL    70
Consensus  m   t   l   k         t yd  fak     g  v lvgdslgm  qghdstlpv v rg    l

J5-PanB    ILADLPFMAHATAEQAFANCATLMQAGAHMIKLEGAAWLAETIRLLAERGVPVCAHMGLTPQTVNVLGGY    140
K12-PanB   LLADLLFMTYATPEQAFENAATVMRAGANMVKIEGGEWLVETVQMLTERAVPVCGHLGLTPQSVNIFGGY    140
Consensus   ladl fm  at eqaf n at m aga m k eg  wl et   l er vpvc h gltpq vn  ggy

J5-PanB    KVQGRQEAQARQMRADAIALEQAGAAMLLLECVPSELAAEITQAVSIPVIGIGAGSATDGQVLVLHDMLG    210
K12-PanB   KVQGRGDEAGDQLLSDAILALEAAGAQLLVLECVPVELAKRITEALAIPVIGIGAGNVTDGQILVMHDAFG    210
Consensus  kvqgr      q   da ale aga  l lecvp ela  it a  ipvigigag  tdgq lv hd  g

J5-PanB    LSLSGRVPKFVKNFMVGQPDIHSALVAYVTAVRDVSFPASEHGFS                            255
K12-PanB   ITG.GHIPKFAKNFLAETGDIRAAVRQYMAEVESGVYPGEEHSFH                            254
Consensus      g  pkf knf     di  a   y   v     p  eh f
```

Fig. 2. Protein sequence alignments of PanB from *Pseudomonas putida* J5 and *Escherichia coli* K-12. Identical amino acids are indicated by the gray background.

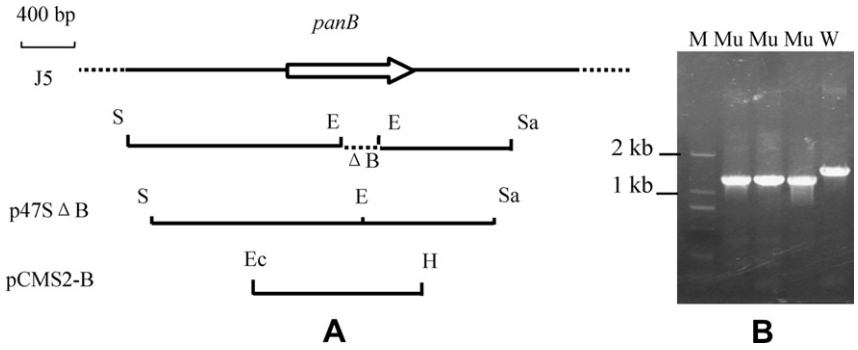

Fig. 3. Genetic analysis of the *panB* gene (A) and deletion analysis by PCR with primers PanB-F and PanB-R (B). Hollow arrows represent the location and orientation of the genes in the J5 chromosome of *Pseudomonas putida*. Suicide plasmid p47SΔB containing the deleted *panB* gene was constructed by ligating the *Sal*I–*Eag*I and *Eag*I–*Sac*I fragments. Plasmid pCMS2-B contained the intact *panB* gene via PCR with primers PanB-F and PanB-R. Restriction enzyme abbreviations: S, *Sal*I; E, *Eag*I; Sa, *Sac*I. M, 1 KB marker; Mu, J5ΔB; W, *P. putida* J5.

indicated that ketopantoate hydroxymethyltransferase took part in nicotine metabolism in *P. putida* J5.

4. Discussion

In the tobacco rhizosphere, many bacteria, and especially species of *Pseudomonas* and *Arthrobacter*, have adapted to using nicotine as a source of nutrients and have thereby developed biochemical pathways to decompose this organic heterocyclic compound. The best-elucidated metabolic pathway of nicotine degradation following the pyridine route is the one encoded on the megaplasmid pAO1of *A. nicotinovorans*. All of the six degrading enzymes and their genes have been isolated and cloned, in which the heterotrimeric molybdenum enzyme nicotine dehydrogenase (NDH) catalyzes the first nicotine-degrading reaction, hydroxylation of the pyridine ring to 6-hydroxynicotine (Brandsch, 2006; Freudenberg et al., 1988). Next, the pyrrolidine ring is oxidized by the flavoprotein 6-hydroxy-L-nicotine oxidase (Schenk et al., 1998). The 6-hydroxy-methylmyosmine formed in this reaction spontaneously converts into 6-hydroxypseudooxynicotine by the addition of water to the double bond (Brandsch, 2006). A second hydroxylation at C2 of 6-hydroxypseudooxynicotine, a step catalyzed by ketone dehydrogenase (KDH), yields 2,6-dihydroxypseudooxynicotine. And then the 2,6-dihydroxypseudooxynicotine hydrolase (PONH) cleaves 2,6-dihydroxypseudooxynicotine into 2,6-dihydoxypyridine and γ-N-methylaminobutyrate (Sachelaru et al., 2006). A further hydroxylation by 2,6-dihydroxypyridine hydroxylase (DHPH) gives trihydroxypyridine that spontaneously forms nicotine blue with the appearance of the pigment in the growth medium (Baitsch et al., 2001). Same metabolic pathway of nicotine degradation was identified in *Nocardioides* sp. JS614 (Ganas et al., 2008). Previous researches have documented that nicotine metabolism in *Pseudomonas* might follow the pyrrolidine pathway via methylmyosmine, pseudooxynicotine, 3-succinoylpyridine, 2,5-dihydroxypyridine (Kaiser et al., 1996; Thacker et al., 1978; Wang et al., 2007). The ring of 2,5-dihydroxypyridine was then cleaved to yield maleamic acid, and hydrolytic deamination of maleamic acid generated maleic acid (Behrman and Stanier, 1957). It was proposed that ring cleavage occurred between carbons 5 and 6, and pyruvic acid was present among the end products (Kaiser et al., 1996; Schräder et al., 2002). Basic insights into steps and intermediates in nicotine catabolism have been gained almost 50 years ago, whereas the enzymes which catalyze various steps in this pathway have been poorly characterized. Only recently, the gene for 6-hydroxy-3-succinoylpyridine hydroxylase catalyzing 6-hydroxy-3-succinoylpyridine (HSP) directly to 2,5-dihydroxypyridine was cloned from *P. putida* S16 (Tang et al., 2008). In addition, no other progress was made on molecular and biochemical work on nicotine metabolism by Pseudomonads.

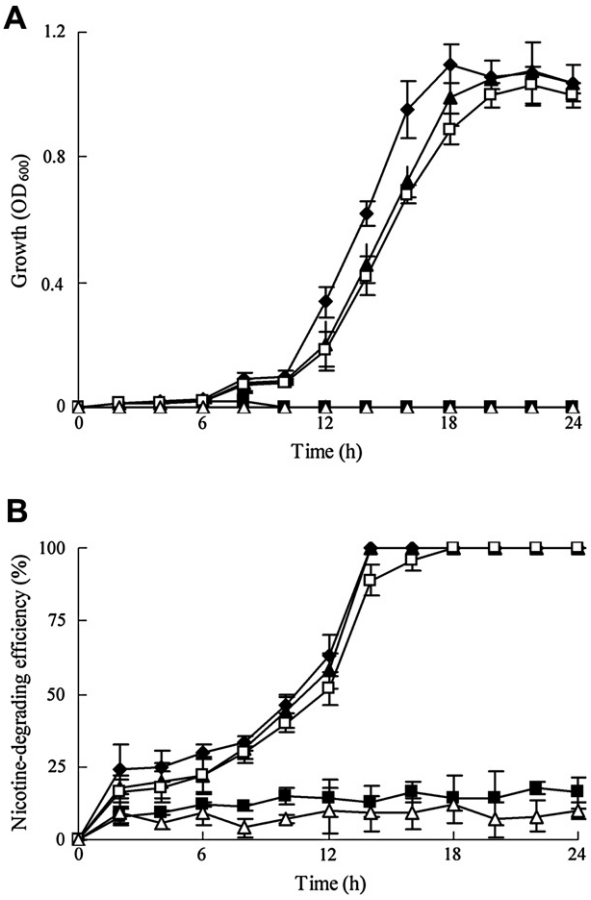

Fig. 4. Growth curve (A) and nicotine-degrading efficiency (B) of *P. putida* J5-M10 and its derivatives. All strains were grown in M9 (1 g l^{-1} nicotine as the sole carbon source), and growth (OD600) and nicotine-degrading efficiency were monitored every 2 h. Nicotine-degrading efficiency was calculated according to previous study (Lei et al., 2007). Strain J5 (◆), J5ΔB (■), J5ΔB-B (▲), J5ΔB-K12B (□), and blank control (△).

The vitamin pantothenate, a vital and central metabolic compound in all organisms, is the precursor of the predominant acyl group carriers of coenzyme A (CoA) and acyl carrier protein (Jackowski, 1996). In *E. coli*, the specific biosynthesis pathway of pantothenate consists of only four steps (Geerlof et al., 1999; Vallari and Rock, 1985), in which ketopantoate hydroxymethyltransferase initiates the first reaction by using ketoisovalerate to generate ketopantoate (Cronan et al., 1982; Jones et al., 1993). It is well known that pyruvic acid is the direct precursor of ketoisovalerate in many bacteria such as *Escherichia*, *Salmonella*, and *Pseudomonas*

(Jackowski, 1996; Rubio and Downs, 2002; Shimizu et al., 1988). Just as mentioned above, strains of *Pseudomonas* degraded nicotine via pyrrolidine pathway and 2,5-dihydroxypyridine, finally leading to the formation of pyruvic acid (Schräder et al., 2002). Given the known reaction mechanism and general chemical considerations, it was proposed that nicotine, when used as the carbon source by *P. putida* J5, was catabolized and transformed into simple carbohydrate as pyruvic acid and then participated in the synthesis of vitamin pantothenate and CoA, which supplied the energy for the normal functioning. In this study, deletion of the *panB* gene in *P. putida* J5 abolished the nicotine-degrading ability and the mutation phenotypes could be restored by complementation with *panB* genes from either *P. putida* J5 or *E. coli* K-12. These results suggest that ketopantoate hydroxymethyltransferase is a crucial enzyme in nicotine catabolism and pantothenate synthesis in *P. putida* J5.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (30460007, 30760011) and the Science and Technology Program of the Yunnan Tobacco Co. Ltd (04A17).

References

Andrews, H.L., Vogel, J.P., Isberg, R.R., 1998. Identification of linked *Legionella pneumophila* genes essential for intracellular growth and evasion of the endocytic pathway. Infection and Immunity 66, 950–958.

Armstrong, D.W., Wang, X., Ercal, N., 1998. Enantiomeric composition of nicotine in smokeless tobacco, medicinal products, and commercial reagents. Chirality 10, 587–591.

Baitsch, D., Sandu, C., Brandsch, R., 2001. A gene cluster on pAO1 of *Arthrobacter nicotinovorans* involved in the degradation of the plant alkaloid nicotine: cloning, purification and characterization of 2,6-dihydroxypyridine 3-hydroxylase. Journal of Bacteriology 183, 5262–5267.

Behrman, E.J., Stanier, R.Y., 1957. The bacterial oxidation of nicotinic acid. Journal of Biological Chemistry 228, 923–945.

Brandsch, R., 2006. Microbiology and biochemistry of nicotine degradation. Applied Microbiology and Biotechnology 69, 493–498.

Civilini, M., Domenis, C., Sebastianutto, N., de Bertoldi, M., 1997. Nicotine decontamination of tobacco agro-industrial waste and its degradation by microorganisms. Waste Management & Research 15, 349–358.

Cronan Jr., J.E., Little, K.J., Jackowski, S., 1982. Genetic and biochemical analyses of pantothenate biosynthesis in *Escherichia coli* and *Salmonella typhimurium*. Journal of Bacteriology 149, 916–922.

Del Sal, G., Manfioletti, G., Schneider, C., 1988. A one-tube plasmid DNA mini-preparation suitable for sequencing. Nucleic Acids Research 16, 9878.

Finan, T.M., Kunkel, B., de Vos, G.F., Signer, E.R., 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. Journal of Bacteriology 167, 66–72.

Freudenberg, W., König, K., Andreesen, J.R., 1988. Nicotine dehydrogenase from Arthrobacter oxidans: a molybdenum containing hydroxylase. FEMS Microbiology Letters 52, 13–18.

Ganas, P., Sachelaru, P., Mihasan, M., Igloi, G.L., Brandsch, R., 2008. Two closely related pathways of nicotine catabolism in *Arthrobacter nicotinovorans* and *Nocardioides* sp. strain JS614. Archives of Microbiology 189, 511–517.

Geerlof, A., Lewendon, A., Shaw, W.V., 1999. Purification and characterization of phosphopantetheine adenylyltransferase from *Escherichia coli*. Journal of Biological Chemistry 274, 27105–27111.

Herrero, M., de Lorenzo, V., Timmis, K.N., 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. Journal of Bacteriology 172, 6557–6567.

Hochstein, L.I., Rittenberg, S.C., 1958. The bacterial oxidation of nicotine: I. Nicotine oxidation by cell-free preparations. Journal of Biological Chemistry 234, 151–156.

Igloi, G.L., Brandsch, R., 2003. Sequence of the 165-kilobase catabolic plasmid pAO1 from *Arthrobacter nicotinovorans* and identification of a pAO1-dependent nicotine uptake system. Journal of Bacteriology 185, 1976–1986.

Jackowski, S., 1996. Biosynthesis of pantothenic acid and coenzyme A. In: Neidhardt, F.C. (Ed.), *Escherichia coli* and Salmonella: Cellular and Molecular Biology, second ed., vol. 1. ASM Press, Washington, D.C., pp. 687–694.

Jones, C.E., Brook, J.M., Buck, D., Abell, C., Smith, A.G., 1993. Cloning and sequencing of the *Escherichia coli panB* gene, which encodes ketopantoate hydroxymethyltransferase, and overexpression of the enzyme. Journal of Bacteriology 175, 2125–2130.

Kaiser, J.P., Feng, Y.C., Bollag, J.M., 1996. Microbial metabolism of pyridine, quinoline, acridine, and their derivatives under aerobic and anaerobic conditions. Microbiology and Molecular Biology Reviews 60, 483–498.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., Peterson, K.M., 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166, 175–176.

Lei, L.P., Xia, Z.Y., Wang, Y., Wei, H.L., Liu, X.Z., 2007. Isolation and characterization of nicotine-degrading bacterial strain L1. Chinese Journal of Agricultural Biotechnology 4, 721–722.

Miller, V.L., Mekalanos, J.J., 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. Journal of Bacteriology 170, 2575–2583.

Revelles, O., Espinosa-Urgel, M., Fuhrer, T., Sauer, U., Ramos, J.L., 2005. Multiple and interconnected pathways for *L*-lysine catabolism in *Pseudomonas putida* KT2440. Journal of Bacteriology 187, 7500–7510.

Ruan, A.D., Min, H., Peng, X.H., Huang, Z., 2005. Isolation and characterization of *Pseudomonas* sp. strain HF-1, capable of degrading nicotine. Research in Microbiology 156, 700–706.

Rubio, A., Downs, D.M., 2002. Elevated levels of ketopantoate hydroxymethyltransferase (PanB) lead to a physiologically significant coenzyme A elevation in *Salmonella enterica* serovar Typhimurium. Journal of Bacteriology 184, 2827–2832.

Sachelaru, P., Schiltz, E., Brandsch, R., 2006. A functional *mobA* gene for molybdopterin cytosine dinucleotide cofactor biosynthesis is required for activity and holoenzyme assembly of the heterotrimeric nicotine dehydrogenase of *Arthrobacter nicotinovorans*. Applied and Environmental Microbiology 72, 5126–5131.

Sambrook, J., Russell, D.W., 1998. Molecular Cloning: a Laboratory Manual, third ed. Cold Spring Harbor Laboratory Press.

Schenk, S., Hoelz, A., Kraus, B., Decker, K., 1998. Gene structures and properties of enzymes of the plasmid-encoded nicotine catabolism of *Arthrobacter nicotinovorans*. Journal of Molecular Biology 284, 1323–1339.

Schräder, T., Thiemer, B., Andreesen, J.R., 2002. A molybdenum-containing dehydrogenase catalyzing an unusual 2-hydroxylation of nicotinic acid. Applied Microbiology and Biotechnology 58, 612–617.

Shimizu, S., Kataoka, M., Chung, M.C., Yamada, H., 1988. Ketopantoic acid reductase of *Pseudomonas maltophilia* 845. Purification, characterization, and role in pantothenate biosynthesis. Journal of Biological Chemistry 24, 12077–12084.

Tang, H.Z., Wang, S.N., Ma, L.Y., Meng, X.Z., Deng, Z.X., Zhang, D.K., Ma, C.Q., Xu, P., 2008. A novel gene encoding 6-hydroxy-3-succinoylpyridine hydroxylase in nicotine degradation by *Pseudomonas putida* strain S16. Applied and Environmental Microbiology 74, 1567–1574.

Thacker, R., Rbrvig, O., Kahlon, P., Gunsalus, I.C., 1978. NIC, a conjugative nicotine–nicotinate degradative plasmid in *Pseudomonas convexa*. Journal of Bacteriology 135, 289–290.

Vallari, D.S., Rock, C.O., 1985. Isolation and characterization of *Escherichia coli* pantothenate permease (*panF*) mutants. Journal of Bacteriology 164, 136–142.

Wang, S.N., Liu, Z., Tang, H.Z., Meng, J., Xu, P., 2007. Characterization of environmentally friendly nicotine degradation by *Pseudomonas putida* biotype A strain S16. Microbiology 153, 1556–1565.

Wei, H.L., Lei, L.P., Xia, Z.Y., Liu, S., Liu, P.G., Liu, X.Z., 2008. Characterisation of a novel aerobic nicotine-biodegrading strain of *Pseudomonas putida*. Annals of Microbiology 58, 41–45.

Wei, H.L., Zhang, L.Q., 2006. Quorum-sensing system influences root colonization and biological control ability in *Pseudomonas fluorescens* 2P24. Antonie van Leeuwenhoek 89, 267–280.

Zhang, Y.D., Luo, C.R., Wang, H.L., Lu, G.F., 2003. Advances in microbial degradation of nicotine and its application. Tobacco Science and Technology 12, 3–7.