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Characterization of 2,5-dihydroxypyridine dioxygenases from *Sinorhizobium* sp. L1

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The *Sinorhizobium* sp. L1 bacteria utilize 3-hydroxypyridine and nicotinic acid by different pathways. The biosynthesis of the 2,5-dihydroxypyridine 5,6-dioxygenase in *Sinorhizobium* sp. L1 is induced by 3-hydroxypyridine as well as nicotinic acid, however, the distinct isoforms of the enzyme are produced depending on the applied inducer. Both isoforms of the enzyme have been purified and characterized. A 10.4 kb DNA fragment has been cloned from *Sinorhizobium* sp. L1 by using data of the *de novo* sequencing of the purified enzymes. According to the nucleotide sequence analysis, the cloned fragment encodes a part of the degradation pathway of nicotinic acid. The *orf9* gene has been cloned and expressed in *Escherichia coli* cells. The cells produce an active 2,5-dihydroxypyridine 5,6-dioxygenase.

Key words: *Sinorhizobium* sp., 2,5-dihydroxypyridine, 2,5-dihydroxypyridine 5,6-dioxygenase, 3-hydroxypyridine, nicotinic acid

INTRODUCTION

The pyridine ring is the basic structure of widely distributed compounds such as dyes, industrial solvents, herbicides, and pesticides. The pyridine ring is a major constituent of natural plant alkaloids and coenzymes such as nicotinamides. Pyridine compounds can be used by various microorganisms as a carbon, nitrogen and energy source via special catabolic pathways (Fetzner, 2000; Kaiser et al., 1996).

Catabolism and initial hydroxylation steps of monocarboxylated pyridines such as 2-carboxypyridine (picolinic acid) (Kiener et al., 1993; Siegmund et al., 1990; Tate, Ensing, 1974), 3-carboxypyridine (nicotinic acid) (Nagel, Andreesen, 1989; Nakano et al., 1999; Ueda, Sashida, 1998) and 4-carboxypyridine (isonicotinic acid) (Kretzer et al., 1993; Singh, Shukla, 1986) have been studied in detail. Nicotinate dehydrogenases, catalyzing the hydroxylation reactions, were purified from *Bacillus niacini* (Nagel, Andreesen, 1990), *Pseudomonas fluorescens* TN5 (Hurh et al., 1994), *Eubacterium barkeri* (previously *Clostridium barkeri*) (Gladyshev et al., 1996), *Ralstonia*/*Burkholderia* strain DSM 6920 (Schräder et al., 2002) and *Pseudomonas putida* KT2440 (Jimenez et al., 2008). Isonicotinate dehydrogenase

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was purified from *Mycobacterium* sp. INA1 (Kretzer et al., 1993) and picolinic acid 6-hydroxylase was purified and characterized from *Arthrobacter picolinophilus* (Tate, Ensing, 1974).

2,5-Dihydroxypyridine is an intermediate metabolite, a formation of which is observed during aerobic degradation of some pyridine compounds including nicotinic acid and nicotine (Fetzner, 2000; Kaiser et al., 1996; Jimenez et al., 2008). The oxidative cleavage of 2,5-dihydroxypyridine catalyzed by 2,5-dihydroxypyridine 5,6-dioxygenase is an important catabolic step starting a maleamic pathway (Jimenez et al., 2008).

Unfortunately, there are no data about characterized enzyme or / and genes involved in the transformation of 3-hydroxypyridine by microorganisms. Recently we have isolated the *Sinorhizobium* sp. L1 bacteria capable to utilize both 3-hydroxypyridine and nicotinic acid as a single carbon and energy source (Karvelis, Meškys, 2004). In this study, we are demonstrating that the biosynthesis of 2,5-dihydroxypyridine 5,6-dioxygenase in *Sinorhizobium* sp. L1 is induced by 3-hydroxypyridine as well as nicotinic acid, however, the different isoforms of the enzyme are produced depending on the applied inducer. We have been able to identify and characterize a gene cluster encoding a part of the nicotinic acid degradation pathway in *Sinorhizobium* sp. L1. In addition, a recombinant 2,5-dihydroxypyridine 5,6-dioxygenase has been expressed *Escherichia coli*.

MATERIALS AND METHODS

Chemicals. Nicotinic, 6-hydroxynicotinic acids and 3-hydroxypyridine were obtained from Merck. 2,5-Dihydroxypyridine was synthesized according to (Berhman, Pitt, 1958).

Bacterial strains. *Sinorhizobium* sp. L1, capable to degrade 3-hydroxypyridine and pyridine-3-carboxylic acid was described previously (Karvelis, Meškys, 2004). *Escherichia coli* DH5α (ϕ80dlacZΔM15 Δ(lacZY-argF) U169, deoR, recA1, endA1, hsdR17($r_K^- m_K^+$), dup E44, thi-1, gyrA96, relA1) was obtained from Pharmacia, *E. coli* BL21 F^-, *omp*T, *gal*, *hsd*S_B ($r_B^- m_B^-$), *dcm*, *lon* (DE3) from Avidis.

Bacterial vectors. pUC19/PstI, pUC19/SalI, pUC19/HindIII, pUC19, and pTZ57R/T were obtained from Fermentas Thermofisher; pET19 was obtained from Invitrogen.

Media and growth conditions: *Sinorhizobium* sp. L1 cells were grown in the EFA medium, (g/l): K_2HPO_4 – 8.8, $(NH_4)_2SO_4$ – 1.0, NaCl – 2.35, yeast extract – 0.5, $MgSO_4 \times 7H_2O$ – 0.18, salt solution – 10 ml/l; salt solution (g/l): $CaCl_2 \times 2H_2O$ – 2.0, $MnSO_4 \times 4H_2O$ – 1.0, $FeSO_4 \times 7H_2O$ – 0.6, $NaMoO_4 \times 2H_2O$ – 0.5, all components were dissolved in 0.1 N HCl and added to EFA1 medium before cultivation. Nutrient Broth and Nutrient Agar (NA) (Oxoid) were used for cultivation of *E. coli* cells. All media and solutions were autoclaved at 1 atm for 30 min. *E. coli* was cultivated at 37 °C, *Sinorhizobium* sp. L1 was cultivated at 30 °C. In liquid medium, bacteria were cultivated with aeration and shaking at 180 rpm.

DNA isolation and manipulation. Total DNA from *Sinorhizobium* sp. L1was isolated by method proposed by Woo et al. (18). Plasmid DNA from *E. coli* was isolated by alkaline lysis method (Maniatis et al., 1982). Restriction endonucleases, phosphotase (FastAP), T4 DNA ligase were purchased from Fermentas Thermofisher and used as recommended. DNA for sequencing was purified using ZYMO Plasmid MiniPREP (Zymo Research). *E. coli* cells were prepared for electroporation by the method of Sharma & Schimke (Sharma, Schimke, 1996). Electroporation into 100 μl of the cells was performed using electroporator 2510 (Eppendorf, Germany) with electrical conditions selected as 2.0 kV/cm and impulse duration of 5.0–5.6 ms. Transformed *E. coli* bacteria were incubated at 37 °C for 1 hour, thereafter cells were spread on solid NA with ampicillin.

DNA sequencing and analysis. Primers for nicotinic acid degradation operon genes were designed using *de novo* sequences of peptides. The sequencing was performed at Macrogen (South Korea). Vector NTI 9.0 (Gorelenkov et al., 2001) was used for DNA and protein analysis. The nearest homologues for phylogenetic analysis of proteins were picked by using the BLAST family programs (NCBI) (Altschul et al., 1990).

Resting cells reactions. *Sinorhizobium* sp. L1 cells were pre-grown in 20 mL of EFA medium, containing the appropriate pyridine compound at 30 °C overnight with aeration. Cells were transferred into the 50 mL of the same media, and

the cultivation continued for next 24–48 hours. The cells were harvested by centrifugation at 10 000 *g* for 20 minutes and washed twice with 50 mM potassium phosphate buffer (pH 7.2). The washed cells were suspended in the 1 mL of the same buffer, and the 0.1–0.2 mM of substrate was added. Cells were resuspended and immediately harvested by centrifugation. The supernatant was transferred to the quartz cuvette, and the primary spectrum was read over a range of 200 to 400 nm. Later, the supernatant and the harvested cells were transferred to the same reaction cell and further incubated. The spectra were recorded in appropriate time intervals after centrifugation of cells, using Helios γ spectrophotometer (Thermo).

Preparation of cell-free extracts. The cells grown in the liquid media were harvested by centrifugation and washed twice with 20 mM potassium phosphate buffer (pH 7.0), then resuspended in the same buffer, and disrupted by sonication at 22 kHz. The cells debris was removed by centrifugation (12 000 *g* for 20 min).

Enzyme assays. Activity of 2,5-dihydroxypyridine 5,6-dioxygenase was analyzed as described previously (Gauthier, Rittenberg, 1971). One unit of activity was defined as the amount of enzyme necessary to oxidize 1 μmol of 2,5-dihydroxypyridine per min. Activity of nicotinate dehydrogenase was analyzed as described previously (Jimenez et al., 2008). One unit of activity was defined as the amount of enzyme necessary to oxidize 1 μmol of nicotinate per min. Other enzymes were detected as described previously (Jimenez et al., 2008; Gauthier, Rittenberg, 1971).

Protein *de novo* sequencing. The proteins were extracted from the polyacrylamide gel after separation. Samples were prepared for the mass spectrometry analysis according to the specifications of the Proteomics Centre at the Institute of Biochemistry, Vilnius University. The mass spectra were analyzed using the GPS Explorer™ De Novo Explorer program.

RESULTS AND DISCUSSION

The *Sinorhizobium* sp. L1 bacteria were isolated previously as 3-hydroxypyridine-degrading organism (Karvelis, Meškys, 2004). The analysis of substrate specificity showed that this microorganism could utilize various compounds as a substrate (Table 1). Moreover, the different combinations of catabolic enzymes were induced in the presence of the appropriate carbon sources (Table 1). The utilization of 3-hydroxypyridine was always accompanied by formation of green pigment which turned brown. A more detailed analysis of utilization of nicotinic acid showed it was degraded by the formation of 2,5-dihydroxypyridine. The detected enzymatic activities supported this conclusion (Table 1). However, the growth in the presence of nicotinic acid was not accompanied by the pigment formation, notwithstanding that the metabolic pathway proceeded via biosynthesis of 2,5-dihydroxypyridine. Hence, it was

Table 1. The detected enzymes in the *Sinorhizobium* sp. L1 cell-free extracts. The bacteria were grown in mineral media containing an appropriate carbon source (final concentration 0.2%). (+) – the activity was detected, (–) – no activity was detected, (±) – a very weak activity

Enzyme activity	Carbon source					
	Succinic acid	Nicotinamide	6-Hydroxynicotinic acid	Nicotinic acid	3-Hydroxypyridine	3-Hydroxymethylpyridine
Nicotinamide deaminase	–	+	–	–	–	–
Nicotinate dehydrogenase	–	+	+	+	–	+
6-Hydroxynicotinate 3-monooxygenase	–	+	+	+	–	±
2,5-Dihydroxypyridine 5,6-dioxygenase	+	+	+	+	+	+
Maleamic acid deaminase	+	+	+	+	+	+

proposed that 3-hydroxypyridine and nicotinic acid induced the different catabolic pathways. The presence of different regulation systems was confirmed by investigation of resting *Sinorhizobium* sp. L1 cells. The cells pre-grown in the media containing 3-hydroxypyridine could degrade 3-hydroxypyridine and 2,5-dihydroxypyridine only. No bioconversion was observed with the nicotinic acid. Vice versa, the cells pre-grown in the presence of the nicotinic acid were able to degrade the nicotinic and 2,5-dihydroxypyridine, but 3-hydroxypyridine was not attacked.

The analysis of the cell-free extract showed that 2,5-dihydroxypyridine 5,6-dioxygenase is induced both in the presence of 3-hydroxypyridine and nicotinic acid. To elucidate whether the same or the distinct forms of dioxygenase were produced, the 2,5-dihydroxypyridine 5,6-dioxygenases were purified from differently induced biomass of *Sinorhizobium* sp. L1. The results are presented in Tables 2 and 3.

The SDS-PAGE analysis showed that the purified proteins were almost homogeneous (Fig. 1, lanes 2 and 3), but differed in molecular masses of subunits. The 3-hydroxypyridine-induced cells of *Sinorhizobium* sp. L1 produced the 2,5-dihydroxypyridine 5,6-dioxygenase consisting of 39.8 kDa subunits (isoform A) (Fig. 1, lane 3). The molecular mass of the subunit of the 2,5-dihydroxypyridine 5,6-dioxygenase from the nicotinate-induced cells was determined to be 38.3 kDa (isoform B) (Fig. 1, lane 2).

The molecular masses of the native enzymes determined by gel-filtration were 230 ± 2 kDa and 214 ± 2 kDa, respectively. The purified enzymes are similar by molecular masses to 2,5-dihydroxypyridine 5,6-dioxygenase from *Pseudomonas putida* N-9, KT2440 and S16 (Jimenez et al., 2008; Gauthier, Rittenberg, 1971; Tang et al., 2008).

Both isoforms showed similar catalytic properties. Both enzymes needed oxygen and Fe(II) for the full activity. The optimal pH for the oxidation of 2,5-dihydroxypyridine by the isoform A and B was 7.5–7.75 and 7.25–7.5, accordingly. The K_m values for 2,5-dihydroxypyridine at pH 7.2 were 175 and 150 μM for the isoform A and B, respectively. Both dioxygenases were inhibited by 2,5-dihydroxypyridine when the substrate concentration was higher than 0.8 mM. The enzymes were sensitive to chelators of metal ions. However, the enzymes were different in the specific activity 10-fold.

The purified enzymes were analyzed by the *de novo* sequencing. Three peptides identified

Table 2. Purification of 2,5-dihydroxypyridine 5,6-dioxygenase from the nicotinate-induced cells of *Sinorhizobium* sp. L1

Step	Total activity, U	Total protein, mg	Specific activity, U/mg	Purification, -fold	Yield, %
Cell-free extract	5 400	500	11	1	100
Ultracentrifugation	3 950	340	11.6	1.05	73.1
30–40% ammonium sulphate	2 280	115	19.8	1.8	44.2
GigaCapQ	1 760	19	92.6	8.4	32.6
Resource 15PHE	430	1.4	307	27.9	8
Resource 15Q	320	0.64	500	45.5	5.9

Table 3. Purification of 2,5-dihydroxypyridine 5,6-dioxygenase from the 3-hydroxypyridine-induced cells of *Sinorhizobium* sp. L1

Step	Total activity, U	Total protein, mg	Specific activity, U/mg	Purification, -fold	Yield, %
Cell-free extract	385	192.5	2	1	100
30–40% ammonium sulphate	210	97	2.2	1.1	54.5
GigaCapQ	180	23	7.8	3.9	46.7
Resource 15PHE	27	1.1	24.5	12.25	7
Resource 15Q	19	0.32	59.4	29.7	4.9

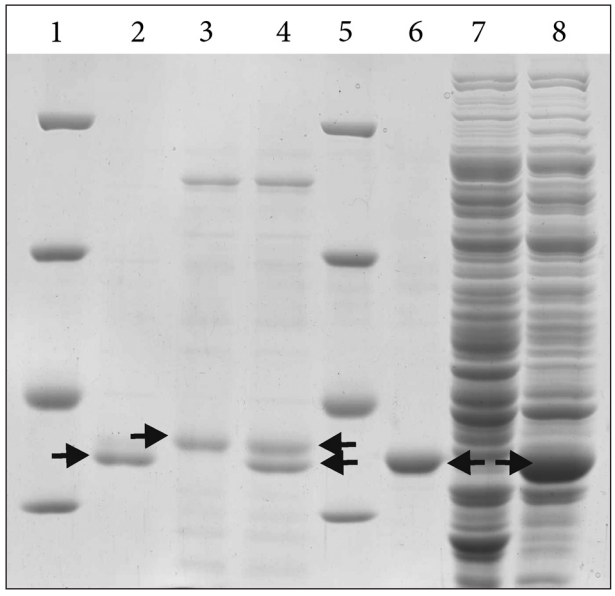

Fig. 1. The SDS-PAGE analysis of 2,5-dihydroxypyridine 5,6-dioxygenases from *Sinorhizobium* sp. L1. 1) and 5) protein ladder (from top to bottom 116, 66.2, 45 and 35 kDa); 2) the purified 2,5-dihydroxypyridine 5,6-dioxygenase from 3-hydroxypyridine-induced cells of *Sinorhizobium* sp. L1; 3) the purified 2,5-dihydroxypyridine 5,6-dioxygenase from nicotinate-induced cells of *Sinorhizobium* sp. L1; 4) the mixture of samples 2 and 3; 6) the purified recombinant 2,5-dihydroxypyridine 5,6-dioxygenase from cells of *E. coli* BL21(DE3); 7) the control, cell-free extract of *E. coli* BL21(DE3); 8) the cell-free extract of *E. coli* BL21(DE3) / pET19orf9. 2,5-Dihydroxypyridine 5,6-dioxygenases are marked by small arrows

in the 2,5-dihydroxypyridine 5,6-dioxygenase A had the following sequences IITAVEPPEIIAR, MMPYPELR, and PYPELR. In addition, IDYADAFIAAGKR, VITAVEPAPLIGR, WDHGEPAAF, and VVVISKGDER peptides were identified in the isoform B. The BLAST analysis showed that three of them of the isoform B were homologous to the hypothetical protein (YP_002978554) from *Rhizobium leguminosarum bv. trifolii* WSM1325. The corresponding degenerated oligonucleotides based on this protein encoding gene sequence as well as *de novo* sequencing data were synthesized. The 685 bp DNA fragment was amplified using the developed oligonucleotides and total DNA from *Sinorhizobium* sp. L1. The pF1R4A plasmid containing this fragment was constructed and the DNA sequence of the cloned insert was determined. The BLAST analysis showed that the amplified DNA was most homologous to genes of 2,5-dihydroxypyridine 5,6-dioxygenases from various microorganisms. The full gene, encoding 2,5-dihydroxypyridine 5,6-dioxygenase was identified by screening the gene library of *Sinorhizobium* sp. L1 using the oligonucleotides adapted to the determined sequence of the 685 bp DNA fragment. One clone harboring the pL1Pst25 plasmid containing a 10.3-kb DNA insert and resulting in a positive PCR reaction was selected for further analysis.

Sequence analysis of the cloned DNA fragment from pL1Pst25 revealed eleven putative ORFs (Fig. 2). The Shine-Dalgarno sequences were de-

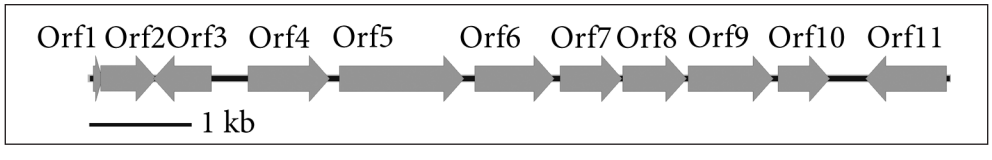

Fig. 2. Graphical representation of the DNA fragment (10351 bp) from *Sinorhizobium* sp. L1 cloned in the pL1Pst25 plasmid

Table 4. Summary of ORFs identified by significant homology in pL1Pst25 plasmid

ORF	Length, (a.a.)	Nearest homolog	GenBank Accession No.	E value
Orf1	35	carbon-monoxide dehydrogenase, *Rhodopseudomonas palustris* HaA2	ABD07809	6E-06
Orf2	224	hypothetical protein CTS44_14338 *Comamonas testosteroni* S44	EFI60974	9E-81
Orf3	233	GntR family transcriptional regulator *Comamonas testosteroni* S44	EFI60973	6E-32
Orf4	329	hypothetical periplasmic protein TorT *Marinomonas* sp. MED121	EAQ65112	3E-86
Orf5	503	sugar ABC transporter ATP-binding protein *Marinomonas* sp. MED121	EAQ65113	3E-172
Orf6	290	Ribose / xylose / arabinose / galactoside ABC-type transport systems, permease component *Marinomonas* sp. MED121	EAQ65114	6E-90
Orf7	249	maleate *cis-trans* isomerase *Pseudomonas putida* S16	ADN26549	2E-148
Orf8	257	hydrolase (deformylase), alpha / beta fold family *Octadecabacter antarcticus* 238	EDY88475	7E-137
Orf9	343	2,5-dihydroxypyridine 5,6-dioxygenase *Pelagibacterium halotolerans* B2	AEQ53770	0.0
Orf10	208	N-carbamoylsarcosine amidase *Pelagibacterium halotolerans* B2	AEQ53771	7E-110
Orf11	327	putative formaldehyde dehydrogenase, glutathione-independent *Dermacoccus* sp. Ellin185	EFP57883	2E-104

tected in the upstream regions of deformylase (GAGAGGGCGAG***ATG***) and 2,5-dihydroxypyridine 5,6-dioxygenase genes (GAGAGGAAATAGCGCG***ATG***).

Four of OFRs (Orf6, Orf7, Orf8, Orf9) shared the significant sequence homology with enzymes participating in the biodegradation of nicotinic acid (Jimenez et al., 2008). The predicted functions of ORFs from pL1Pst25 are presented in Table 4. The comparison of gene organization in pL1Pst25 plasmid and the similar gene clusters from other bacteria showed that the genes encoding the degradation of 2,5-dihydroxypyridine from *Sinorhizobium* sp. L1 differed from the well characterized *nic* operon from *Pseudomonas putida* KT2440 (Jimenez et al., 2008). Hence, the transport system proteins were rarely present in the *nic* related gene clusters, but these clusters usually contained 6-hydroxynicotinate 3-monooxygenase gene located near the core genes consisting of maleate isomerase, *N*-formylmaleamate deformylase, 2,5-dihydroxypyridine dioxygenase and maleamate amidase encoding sequences.

To clarify a function of the *orf9* gene it was cloned into pET19 expression vector and the protein synthesis was induced in *E. coli* BL21 (DE3) cells (Fig. 1, lane 8) using standard molecular techniques. Most of protein was produced in the inclusion bodies, but part of the recombinant 2,5-dihydroxypyridine dioxygenase remained soluble. The soluble protein was purified to homogeneity (Fig. 1, lane 7) and showed an expected activity in the presence of 2,5-dihydroxypyridine. Moreover, the SDS-PAGE analysis of the purified recombinant 2,5-dihydroxypyridine 5,6-dioxygenase led to conclusion that the protein was the isoform B (Fig. 1, lane 6). This observation strengthens the conclusion that the cloned DNA fragment encodes the part of the nicotinic acid degradation operon in *Sinorhizobium* sp. L1.

CONCLUSION

Sinorhizobium sp. L1 cells utilized 3-hydroxypyridine and nicotinic acid via formation of 2,5-dihydroxypyridine, which was subsequently

oxidized by different 2,5-dihydroxypyridine 5,6-dioxygenase isoforms, depending on the metabolic pathway. For the first time the gene encoding 2,5-dihydroxypyridine 5,6-dioxygenase from *Sinorhizobium* spp. was expressed in *E. coli* BL21 (DE3) cells and the active recombinant enzyme was purified.

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SINORHIZOBIUM SP. L1 2,5-DIHIDROKSIPIRIDINO 5,6-DIOKSIGENAZIŲ CHARAKTERIZAVIMAS

Santrauka

Nustatyta, kad 3-hidroksipiridino ir nikotino rūgšties skaidymas *Sinorhizobium* sp. L1 ląstelėse vyksta skirtingais keliais. Pastebėta, kad *Sinorhizobium* sp. L1 ląstelėse 3-hidroksipiridinas ir nikotino rūgštis indukuoja skirtingas 2,5-dihidroksipiridino 5,6-dioksigenazės izoformas. Abi fermento izoformos buvo išgrynintos ir charakterizuotos. Panaudojus baltymų *de novo* sekvenavimo metodą, buvo klonuotas 10,4 kb DNR fragmentas iš *Sinorhizobium* sp. L1. Nukleotidų sekos analizė rodo, kad klonuotas fragmentas koduoja dalį nikotino rūgšties degradacijos kelio. Klonavus *orf9* geną *Escherichia coli* ląstelėse, buvo stebėta aktyvios rekombinantinės 2,5-dihidroksipiridino 5,6-dioksigenazės raiška.

Raktažodžiai: *Sinorhizobium* sp., 2,5-dihidroksipiridinas, 2,5-dihidroksipiridino 5,6-dioksigenazė, 3-hidroksipiridinas, nikotino rūgštis