SHORT REPORT
doi: 10.2306/scienceasia1513-1874.2005.31.307

ScienceAsia 31 (2005): **307-311**

Vitamin B-6 Degradation by Pyridoxamine-Pyruvate Transaminase and Pyridoxine 4-Oxidase from *Ochrobactrum anthropi* and *Enterobacter cloacae*-like Bacteria

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Received 13 Jun 2004
Accepted 23 Mar 2005

ABSTRACT: Two different pathways, pathway I and II, for vitamin B-6 degradation are known in bacteria. Pyridoxamine-pyruvate transaminase and pyridoxine 4-oxidase, respectively, use pyridoxamine and pyridoxine as a substrate in pathway I of vitamin B-6 degradation. Among 500 bacterial isolates, four isolates, T2, T4, T5, and T6, were identified as the fast growing bacterial isolates using pyridoxine as a carbon source. The activities of pyridoxine 4-oxidase and pyridoxamine-pyruvate transaminase were determined by analyzing the reaction product, pyridoxal, by isocratic reverse-phase HPLC. Only two isolates, T2 and T6, showed pyridoxine 4-oxidase activities of 4.08 and 9.38 Unit/mg (pmol/min/mg protein), respectively. The activity of pyridoxamine-pyruvate transaminase was 6.31 Unit/mg in T2 isolate and 6.11 Unit/mg in T6 isolate. Some biochemical characteristics and nucleotide sequences (500 bases) of 16S rDNA suggested that T2 and T6 were closely related to *Ochrobactrum anthropi* and *Enterobacter cloacae*, respectively. This is the first report of pyridoxamine-pyruvate transaminase and pyridoxine 4-oxidase, enzymes in vitamin B-6 degradation pathway in *O. anthropi* and *E. cloacae*-like bacteria.

KEYWORDS: pyridoxine 4-oxidase, pyridoxamine-pyruvate transaminase, vitamin B-6 degradation pathway, vitamin B-6.

INTRODUCTION

Vitamin B-6 is a water–soluble vitamin which consists of six natural forms: pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their 5'-phosphate esters, which are pyridoxine 5-phosphate (PNP), pyridoxal 5-phosphate (PLP), and pyridoxamine 5'-phosphate (PMP), respectively. Two degradation pathways of vitamin B-6 in bacteria have been reported.[1] In the first pathway (or pathway I), both PM and PN were degraded through 8 sequential steps to succinic semialdehyde, ammonia, and carbon dioxide. This pathway was found in *Pseudomonas* sp. MA-1[1] and *Microbacterium luteolum*.[2] In the second pathway (or pathway II), only PN was degraded in 5 subsequent steps to 2-hydroxymethyl succinic semialdehyde, acetic acid, ammonia, and carbon dioxide. *Arthrobacter* sp. was found to degrade vitamin B-6 via the second pathway.[3]

Both PM-pyruvate transaminase and PN 4-oxidase were in the pathway I of vitamin B-6 degradation. Pyridoxamine-pyruvate transaminase (EC 2.6.1.30) that catalyzed the reaction of PM and pyruvate to PL and L-alanine, was purified and characterized from *Pseudomonas* sp.[4-5] Unlike most transaminases, PM-pyruvate transaminase of *Pseudomonas* sp. required no coenzyme for its activity. The optimum pH for its reaction was 9.2. The K_m value for PM was 13 μM and that for PL was 12 μM. PM-pyruvate transaminase was a tetrameric protein that had a molecular mass of 153 kDa, as determined by equilibrium sedimentation. The partial amino acid sequence around the pyridoxal-binding site of this enzyme was different from that of the corresponding peptides that found from other pyridoxal phosphate-dependent enzymes.[4–7] Although, purification, characterization, and partial amino acid sequence determination of the PM-pyruvate transaminase of this bacteria has been reported, the nucleotide sequence of the gene encoding the enzyme and the protein structure are still unknown.

Pyridoxine 4-oxidase, which catalyzes the reaction of PN to PL requires FAD as a coenzyme. The nucleotide sequence of this enzyme from *M. luteolum* is known.[2] It belongs to the GMC oxidoreductase family.[2] Recently, the gene encoding pyridoxine 4-oxidase was also found in a nitrogen-fixing symbiotic bacterium, *Mesorhizobium loti*.[8] It was unexpectedly found that the PN 4-oxidase

of *M. loti* played different ecological roles. For this reason, identification of PN 4-oxidase in the other bacteria is of interest, since it will help improve the understanding of the role of enzymes in vitamin B-6 degradation pathway in the environment.

Here we report bacterial isolation using medium with PN as a sole carbon source. Bacteria that showed PM-pyruvate transaminase and PN 4-oxidase activities were identified by some of their physiological and biochemical characteristics, including their16S rDNA gene sequences.

Materials and Methods

Materials Pyridoxine hydrochloride, pyridoxamine dihydrochloride, pyridoxal hydrochloride and flavin adenine dinucleotide (FAD) were obtained from Sigma. API 20 E and NE kits were purchased from Biomerieux (Lyon, France). Sodium pyruvate was obtained from Himedia. All other chemicals were of analytical grade.

Isolation and Culture Medium All microorganisms were isolated from soil from a depth of approximately 10 cm from various sites in Khon Kaen University. Soil samples (1 g) were suspended in 5 ml distilled water. The soil suspension was diluted to 1:10, 1:50, and 1:100 with distilled water. Twenty – five microliters of each dilution was spread onto a separate PN agar plate[2] (0.2% PN, Edinburgh minimum medium (EMM) without potassium hydrogen phthalate or glucose, and 1.5% (w/v) agar) and incubated at 30°C for 2-3 days. After 4-6 times of transferring single colonies onto agar medium that contained 0.5% PN, single colonies with differences in size, color, and shape were grown on the same agar medium. Those colonies varied in size, shape, and color were selected.

Measurement of Vitamin B-6 Compounds Vitamin B-6 compounds were measured by the isocratic reversed-phase HPLC method described by Yagi *et al.*[9] with slight modification. A 0.6 mm ID X 15 cm Shim-pack CLC-ODS column was packed with 5 μm particles of reverse phase material (Shimadzu, Tokyo, Japan). Flow rate of the column buffer was 1.0 ml/min. Samples (50 μl) were injected with an autosampler injector (Shimadzu, Tokyo, Japan). Data were collected with a C-R7A chromatopac (Shimadzu, Tokyo, Japan). Column effluent was monitored with a Shimadzu RF-10A spectrofluorometer detector with a 12 μl flow cell. Retention times of standard PM, PL, and PN were 5.0, 10.3, and 14.3 min, respectively.

Growth Conditions Each isolate was aerobically grown in LB medium (5 ml) at 30°C for 12 h. Cell pellets were aseptically harvested and transferred to 50 ml PN liquid medium. The culture was allowed to grow under the same condition as described and growth was followed by OD_{660}.

Enzyme Assays Cells were grown at 30°C for 14 h in PN liquid medium. The cells (1g) were harvested and resuspended into 5 ml sonication buffer, pH 8.0 (20 mM potassium phosphate containing 20 μM FAD and 1 mM phenylmethylsulfonyl fluoride). The suspension was sonicated at 4-15°C for 12 min with a BRANSON 450 ultrasonic sonifier (Branson, Danbury, USA). Cell extract was obtained by centrifugation at a 5000 x g for 10 min at 4°C.

The assays of PM-pyruvate transaminase and PN 4-oxidase were done as described by Wada and Snell[4] and Kaneda *et al.*[2] with slight modification. Each enzyme was assayed by measuring the increase of the reaction product, PL. The reaction mixture (0.4 ml) of PN 4-oxidase contained 0.1 M Tris-acetate buffer (pH 7.0), 5 mM PN, and the crude enzyme. The pyridoxamine-pyruvate transaminase reaction mixture (0.5 ml) consisted of 50 mM Tris-HCl buffer (pH 8.5), 1 mM PM, 1 mM sodium pyruvate, 1 mM EDTA, and the crude enzyme. The reaction mixture was incubated at 30°C. Enzyme reactions were terminated at 30 min intervals (30, 60, 90 and 120 min reactions) by addition of 9 M H_2SO_4 (66 μl), and the mixture was centrifuged. The supernatant was diluted to 100 times with distilled water to a final volume of 1 ml before mixing with 0.33 ml of 69-72% perchloric acid followed by filtering through a Minisart-RC filter (Sartorius, Goettingen, Germany). The filtrate (50 μl) was applied to HPLC. One unit of each enzyme was defined as the amount of the enzyme required to produce 1 pmole of pyridoxal per min at 30°C.

Protein Assay Protein concentration was determined by the dye-binding method.[10] Increase of absorbance at 595 nm was compared with the standard, bovine serum albumin.

Bacterial Identification Pure colonies were streaked on Luria-Bertani (LB) agar. Initial investigations were based on the conventional methods: Gram staining, growth on Mac Conkey agar, catalase and oxidase activities, motility, fermentation/oxidation of glucose, and triple sugar iron (TSI) test. In addition, other biochemical tests were done by using the API 20 E and 20 NE kits. The manufacturers' instructions were followed precisely for the inoculation and incubation of the strip. The results were analyzed by using Analytical Profile Index (APILAB Plus version 3.3.3 software). Cell morphologies were determined under light microscopy (1000 X).

16S rDNA Sequencing Genomic DNAs were prepared by using standard methods.[11] The 16S ribosomal DNA (rDNA) genes of bacterial samples were amplified under standard conditions using the universal 16S rDNA primers; forward primer (fD1: 5'-AGAGTTTGATYMTGGCTCAG-3') and reverse primer (rP2: 5'-ACGGCTACCTTGTTACGACTT-3')[12] (Bioservice Unit, NSTDA, Bangkok, Thailand). PCR products were examined by electrophoresis on a 1 % agarose gel. DNA was stained with ethidium bromide and visualized under UV light. Purified DNA fragments of 1,500 bp from PCR were sequenced by the Bioservice Unit, NSTDA, Thailand. Sequences of approximate 500 bp of DNA were compared with those available in the GenBank, EMBL and the DDBJ databases using the gapped BLASTN 2.2.7 program through the National Center for Biotechnology Information Server (http://www.ncbi.nlm.nih.gov).[13]

Results

Isolation Bacteria were isolated from 5 soil samples. Approximately 500 isolates were grown on the selective medium having PN as a sole carbon source for 7 days. Among 500 isolates, 12 isolated bacteria were picked according to their differences in colony size, morphology, and color. All isolates showed slow growth in PN liquid medium. According to their growth rates (Fig. 1), the bacteria were divided into two groups. Two isolates, T4 and T5, grew faster than the others. T4 and T5 isolates reached the late log phase within 8 h with their OD_{600} at 0.08. While the other isolates entered the late log phase with the OD_{600} at approximately 0.06 at 14-18 h. T2 showed good growth with PN utilization, too, with OD_{600} nearly 0.08 but at 18 h. T6 showed diauxic growth during 14 to 47 h and maximum growth at 90 h with OD_{600} at approximately 0.12. From the growth rates of these isolates, four isolated bacteria (T2, T4, T5, and T6) were selected.

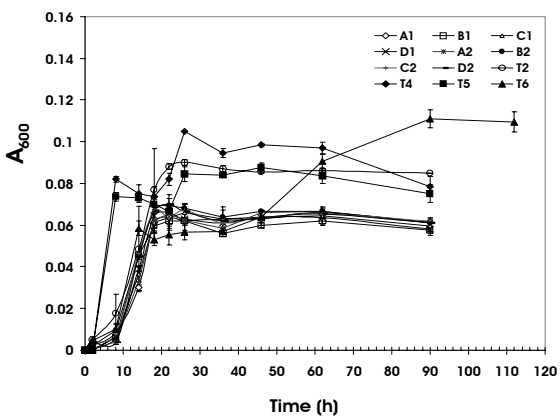

Fig 1. Growth rates of the 12 isolated bacteria in PN liquid medium incubated at 30 °C.

Identification of PL and Enzyme Activities Analyses of pyridoxine 4-oxidase and PM-pyruvate transaminase activities were done every 30 min for 2 h (Fig. 2). The data showed that only T2 and T6 isolates could produce PL in the PN 4-oxidase reaction mixtures. Maximum activities of the PN 4-oxidases of the T2 and T6 isolates were obtained at 30 and 60 min, respectively, after which the enzyme activities were decreased (Fig. 2a). The product was eluted at the same retention time (10.3 min) as that of the standard PL by isocratic HPLC. In addition, when the standard PL was used as the internal standard, it was coeluted with the reaction product. These results indicated that the reaction product was PL. The maximum PN 4-oxidase activities of the T2 and T6 isolates were 4.08 and 9.38 Units/mg protein, respectively. The activity of PM - pyruvate transaminase was also determined by

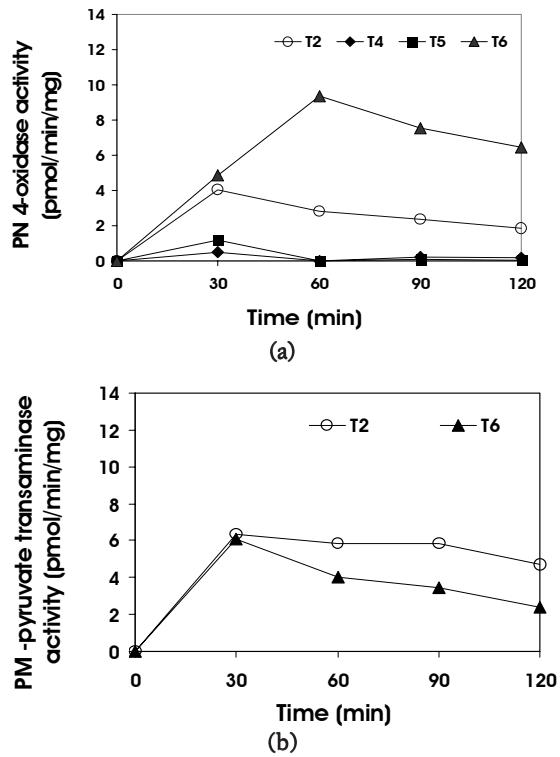

Fig 2. Enzyme activities of isolated bacteria grown in PN liquid medium at 30°C for 24 h. **a)** PN 4-oxidase activities of 4 bacteria (T2, T4, T5, and T6). Each reaction mixture contained an equal volume of crude enzyme with 20 μM FAD, 0.1 M Tris-acetate (pH 7.0), and 5 mM PN. **b)** PM-pyruvate transaminase activities of 2 bacteria (T2 and T6). Each reaction mixture contained an equal volume of crude enzyme with 20 μM FAD, 50 mM Tris-HCl (pH 8.5), 1 mM PM, 1 mM sodium pyruvate, and 1 mM EDTA. The amount of PL was detected by HPLC as described in the methods.

measuring PL in T2 and T6 isolates. Maximum activities of PM-pyruvate transaminase of both T2 and T6 isolates were at 30 min, after which the enzyme activities were decreased (Fig. 2b). The results showed the maximum activities of the enzymes from T2 and T6 were 6.31 and 6.11 Unit/mg protein, respectively.

Bacteria Identification The results of Gram staining showed that both T2 and T6 isolates were Gram-negative bacilli. Six biochemical characteristics of T2 and T6 are shown in Table 1. The biochemical characteristics together with those from the API 20E and NE system (Table 2) analyzed with the APILAB software, showed that T2 was 99.9% closed to *Ochrobactrum anthropi*. The identification of T6 showed doubtful results that it was 59.2% related to *Enterobacter cloacae*, and 40.7% related to *Enterobacter sakazakii*.

Table 1. Some biochemical characteristics of T2 and T6.

Biochemical Test	T2	T6
MacConkey agar	White	White
Catalase test	Positive	Positive
Oxidase test	Positive	Positive
Motility test	Negative	Negative
Oxidation	Positive	Positive
Fermentation	Negative	Positive
TSI test	K/K	A/A
	No gas	Gas, no H_2S

From approximately 500 base sequences of 16S rDNA of T2 and T6, the T2 16s rDNA was 96% identical to that of *Ochrobactrum anthropi* and the T6 16s rDNA was 95% identical to that of *Enterobacter cloacae*.

Discussion

Degradation of vitamin B-6 occurs by two pathways. Pathway I is found in bacteria which are able to use PM or PN as a sole carbon source. PM and PN are substrates of pyridoxamine–pyruvate transaminase and pyridoxine 4-oxidase, respectively. The product of both enzymes is PL. In this study, we isolated bacteria which could use PN as a sole carbon source. Four of twelve isolates showed high growth rates in PN medium compared with the others, but only two of those showed pyridoxine 4-oxidase activities. The result of identification of both bacteria showed that T2 was closely related to *O. anthropi* and T6 was closely related to *E. cloacae*. Activities of both pyridoxine 4-oxidase and pyridoxamine-pyruvate transaminase from T2 and T6 were quite low when compared to the other strains that have been reported (*Pseudomonas* sp. and *M. luteolum*).[2, 4-5] Probably components in the medium were not optimal for both new strains. After changing some ingredients in the media, both strains showed higher activities of both enzymes, which could be detected by spectrophotometer (data not shown).

The results in Fig. 2a showed that the PN 4-oxidase

Table 2. Biochemical results of T2 and T6 by API 20 NE and E, respectively.

API 20 NE Tests	Results of T2 isolate	API 20 E Tests	Results of T6 isolate
Reduction of nitrates to nitrites	Positive	β-Galactosidase	Positive
Reduction of nitrates to nitrogen	Negative	Arginine dihydrolase	Positive
Indole production	Negative	Lysine decarboxylase	Negative
Acidification of glucose	Negative	Ornithine decarboxylase	Positive
Arginine dihydrolase	Negative	Citrate utilization	Positive
Urease	Positive	H_2S production	Negative
Hydrolysis (β-galactosidase)	Negative	Urease	Negative
Hydrolysis (protease)	Negative	Tryptophane deaminase	Negative
β-galactosidase	Negative	Indole production	Negative
Assimilation of		Acetoin production	Positive
- glucose	Positive	Gelatinase	Negative
- arabinose	Positive	Fermentation/Oxidation of	
- mannose	Positive	- glucose	Positive
- mannitol	Negative	- mannitol	Positive
- N-acetyl-glucosamine	Positive	- inositol	Positive
- maltose	Positive	- sorbitol	Positive
- gluconate	Negative	- rhamnose	Positive
- caprate	Negative	- sucrose	Positive
- adipate	Negative	- melibiose	Positive
- malate	Positive	- amygdalin	Positive
- citrate	Negative	- arabinose	Positive
- phenyl-acetate	Negative		

activities of T2 and T6 were increased to the maximum at 30 and 60 min, respectively. After that, the enzyme activities were decreased. The PM-pyruvate transaminase activities of both isolates also showed maximum activity at 30 min and then declined, as shown in Fig. 2b. We hypothesized that the product of both PN 4-oxidase and PM-pyruvate transaminase, PL, was used as a substrate of other enzymes contained in the crude enzyme. One of the enzymes in vitamin B-6 pathway that can catalyze the reduction of PL and NAD+ to 4-pyridoxolactone and NADH, is pyridoxal dehydrogenase.[14] The detection of 4-pyridoxolactone produced by T2 and T6 (data not shown), thus supported our idea that the decrease of PN 4-oxidase activity was due to decreasing PL, which was catalyzed by other enzymes in the crude enzyme.

To the best of our knowledge, this is the first report on the capabilities of *O. anthropi* and *E. cloacae*–like strains in vitamin B-6 degradation. The Gram-negative bacterium, *O. anthropi*, is grouped in the denitrifying bacteria that play a role in transforming nitrogen compounds to gaseous nitrogen compounds (N_2O, NO, and N_2) in the environment. Moreover, they were reported to be able to degrade xenobiotic compounds such as atrazine, urea-formaldehyde and chlorophenols.[15] The enteric bacterium, *E. cloacae*, was able to reduce the nitro group of many different nitroaromatic compounds, a large number of which have toxic, mutagenic, and carcinogenic properties. They are strategically situated to play a key role in metabolism of foreign compounds to which a host was exposed.[16-17]

In general, all organisms can transform the vitamin B-6 they intake into coenzyme forms in their metabolic pathways. But, some bacteria can also use vitamin B-6 as a sole carbon source. The ability of the PM-pyruvate transaminases and PN 4-oxidases in T2 and T6 to degrade other substances should be further studied, especially in characterization of the enzymes and the biochemical roles.

Acknowledgements

The authors wish to thank Assoc. Prof. Dr. Somdei Kanokmedhakul, Dept. of Chemistry, for IR spectrum of 4-pyridoxolactone. Thanks are also to Assist. Prof. Dr. Nipa Milintawisamai and Assist. Prof. Dr. Paweena Pongdontri for their valuable advice. This work was granted by Research and Development Institute (RDI), Khon Kaen University.

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