

A new *Arthrobacter* strain utilizing 4-hydroxypyridine

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For the first time, a Gram-positive microorganism capable of degrading 4-hydroxypyridine was isolated by enrichment techniques from soil. The isolate IN13 utilizes also 2-hydroxypyridine as a sole carbon, nitrogen and energy source. During the degradation of 2-hydroxypyridine a soluble blue pigment was accumulated in the culture. The 16S rRNA-based phylogeny analysis has indicated that the IN13 isolate belongs to the genus *Arthrobacter* and with *Arthrobacter* sp. A4Z24, *Arthrobacter* sp. A1-1, and several uncultured strains forms a separate group. The biodegradation of 4-hydroxypyridine and 2-hydroxypyridine was investigated. Biodegradation studies with washed cells confirmed 4-hydroxypyridine and 2-hydroxypyridine consumption. IN13 growing in a medium with 2-hydroxypyridine tends to lose its 4-hydroxypyridine degrading activity at a significant rate.

Key words: 4-hydroxypyridine, 2-hydroxypyridine, biodegradation, *Arthrobacter*

INTRODUCTION

Aromatic *N*-heterocycles represent a significant class of industrial pollutants, however, their biotransformation pathways are not well understood. Pyridine is widely used as a solvent and an intermediate in the production of piperidine, agricultural chemicals, drugs, dyes, rubber products and in many other applications. The pyridine ring is a major constituent of natural plant alkaloids, pyridoxyl derivatives, and coenzymes such as nicotinamides. Microorganisms have multiple ways of degrading pyridine and its derivatives. The biodegradation under aerobic conditions has been studied intensively [1, 2]. Microbial utilisation of hydroxypyridines is initiated by the hydroxylation of the ring, yielding di- and trihydroxylated intermediates and followed by ring fission [3–10]. During the transformation of 2-hydroxypyridine a blue pigment usually appears in the culture medium. It has been suggested that the blue pigment is probably a result of 2,3,6-trihydroxypyridine oxidation [6].

4-hydroxypyridine-degrading bacteria are uncommon, and only Houghton and Cain [3] and Watson et al. [5] reported that one strain of *Achromobacter* sp. and one strain of *Agrobacterium* sp. could transform 4-hydroxypyridine into 3,4-dihydroxypyridine. Both 4-hydroxypyridine-degrading organisms belong to Gram-negative bacteria, and they are unable to transform 2- or 3-hydroxypyridine. Studies with $H_2^{18}O$ demonstrated that in the presence of NADP or an artificial dye (methylene blue), the oxygen used for the oxidation of 4-hydroxypyridine to 3,4-dihydroxypyridine by the enzyme 4-hydroxypyri-

dine-3-hydroxylase was derived from water [5]. Later Watson et al. [11] reported that cell extracts of an *Agrobacterium* sp. transformed 3,4-dihydroxypyridine into 3-formiminopyruvic acid, pyruvic acid, two molecules of formic acid, and ammonium. 3,4-dihydroxypyridine is also an intermediate of mimosine (β -*N*-(3-hydroxy-4-pyridone)- α -aminopropionic acid) degradation in *Rhizobium* [12, 13].

The metabolism of hydroxylated pyridines under anaerobic conditions has been studied only scarcely. Kaiser and Bollag [14] investigated the degradation of monohydroxypyridines under denitrifying and sulfate-reducing conditions by a mixed culture obtained from sewage sludge. 3-Hydroxypyridine was metabolized after a lag period of two months, whereas 2- and 4-hydroxypyridines were not transformed.

The aim of the present study was to isolate and characterize a new 4-hydroxypyridine-degrading bacterium.

MATERIALS AND METHODS

Chemicals. Pyridine, 4-hydroxypyridine, 2-, 3-, 4-carboxypyridine, pyridine-*N*-oxide were obtained from Merck. 2-Hydroxypyridine and 2-methylpyridine were from Sigma-Aldrich.

Bacterial strains. Strain IN13 was isolated throughout this work.

Media: EFA (g/l): K_2HPO_4 10.0, KH_2PO_4 4.0, yeast extract 0.5, $(NH_4)_2SO_4$ 1.0, 2-hydroxypyridine 2.0, $MgSO_4 \times 7H_2O$ 0.2, salt solution 10 ml/l., pH 7.2; Salt solution (g/l): $CaCl_2 \times 2H_2O$ 2.0, $MnSO_4 \times 4H_2O$ 1.0, $FeSO_4 \times 7H_2O$ 0.5, all components were dissolved in 0.1 N HCl and added into EFA medium after sterilization. Kozar

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mineral medium (g/l): NaCl 5.0, $\text{NH}_4\text{H}_2\text{PO}_4$ 1.0, K_2HPO_4 1.0, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 0.4, pH 7.2 with KOH; Nutrient agar medium (g/l): 28.0.

Isolation of 4-hydroxypyridine-degrading bacteria.

The enrichment culture technique using 4-hydroxypyridine as a sole carbon source in the Kozier mineral medium was used to isolate 4-hydroxypyridine-degrading organisms. Samples of polluted soils were cultivated aerobically at 30 °C in a mineral medium containing 4-hydroxypyridine for at least 48 hours. After cultivation the aliquots were diluted and spread on the Kozier agar plates containing 0.2% of 4-hydroxypyridine. The fastest-growing colonies were picked and purified by streaking repeatedly on the nutrient agar medium. On evidence of growth on 4-hydroxypyridine, bacteria were isolated by streak plating on solidified Kozier medium and solidified Kozier medium containing 0.2 % 4-hydroxypyridine

Phylogenetic analysis of rRNA-encoding genes. Genomic DNA was isolated according to [15]. The 16S rRNA encoding gene was amplified using two universal primers: w001 (5'-agagtttgatcmtggctc-3') and w002 (5'-gntacctgtgtacgactt-3') [16]. The PCR-amplified DNA fragment was purified with a DNA extraction kit and cloned using the InsT/Aclone™ PCR Product Cloning Kit (Fermentas MBI, Lithuania). The cloned 16S ribosomal DNA was sequenced at the Sequencing Centre (Institute of Biotechnology, Lithuania). Web-based similarity searches were performed using the Ribosomal Database Project-II Release 9 (<http://rdp.cme.msu.edu/>). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 [17].

Biodegradation studies. All liquid cultures were incubated at 30 °C with shaking at 150 rpm unless otherwise noted. The strain IN13 was grown in 1-l flasks containing 50 ml of EFA medium supplemented with 0.2% 4-hydroxypyridine or 2-hydroxypyridine. Cells were harvested after 24 h by centrifugation (5 min, 6000 g) and washed twice in 0.9% NaCl prior to be used for biodegradation studies. Biodegradation studies were performed in 50 mM potassium phosphate buffer, pH 7.2, containing 10 mM of 4-hydroxypyridine or 2-hydroxypyridine. The cells were suspended in this solution and the suspension was aerobically incubated at 20 °C. The mixtures without incubation served as a control. Following centrifugation to remove the cells, 4-hydroxypyridine or 2-hydroxypyridine consumption was estimated after 10 min by recording absorbance from 200 nm to 350 nm.

Preparation of cell free extract and SDS-PAGE analysis. Bacteria were grown in appropriate media, harvested, washed with 0.9% NaCl, resuspended in 50 mM Tris-HCl buffer, pH 8.0, and disrupted by sonication. Cell debris was removed by centrifugation at 10.000 g for 45 min. The resulting cell-free extracts were analysed by SDS-PAGE using slab gels (5% stacking gel and 14% resolving gel) at pH 8.3. Gels were stained for proteins with Coomassie brilliant blue R-250.

Nucleotide accession numbers. The 16S rRNA encoding DNA sequence for strain IN13 has been submitted to EMBL Nucleotide Sequence Database (accession number AM236151).

RESULTS AND DISCUSSION

Isolation and characterisation of 4-hydroxypyridine-degrading bacteria. From enrichments inoculated with different soil samples (using the procedure described in Materials and Methods), several bacterial isolates were obtained, which grew in a mineral medium supplemented with 4-hydroxypyridine. The isolate, designated as IN13, was selected for further studies. A 1.5 kb DNA fragment encoding 16S rRNA was amplified using specific primers and a genomic DNA isolated from the IN13 strain. The PCR fragment was cloned into pTZ57R/T vector and sequenced. Analysis of nucleotide sequence of 16S rDNA from the IN13 strain and a comparison with the data in GeneBank showed this microorganism to be related to *Arthrobacter* species (a group of Gram-positive and GC-rich bacteria) (Fig 1.) and with *Arthrobacter* sp. A4Z24, *Arthrobacter* sp. A1-1 and several uncultured strains to form a separate branch of the phylogenetic tree.

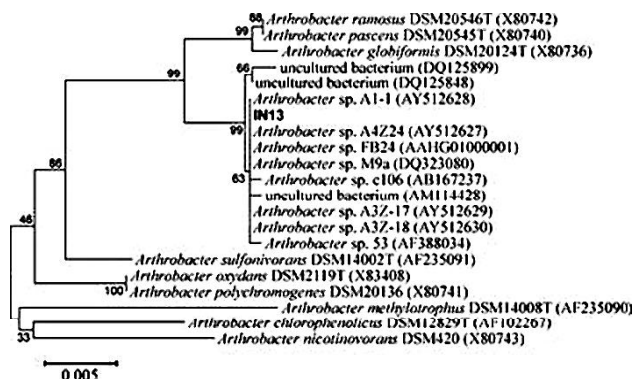


Fig. 1. Phylogenetic tree of IN13 isolate based on 16S rRNA gene sequences. The 16S sequences of *Arthrobacter* spp. strains obtained from the GenBank database were aligned as the reference. GenBank accession numbers are listed parenthetically. Analysis was based on the neighbour-joining method [17]. Differences among sequences are indicated by the sums of the horizontal lengths; the scale bar is in fixed nucleotide substitutions per sequence position. Numbers indicate bootstrap values in 100 trials

Cultivation and biodegradation studies

Various pyridine derivatives were tested as substrates for *Arthrobacter* sp. IN13. The growth both on solid and in liquid media was analysed. The strain was found utilize 4-hydroxypyridine or 2-hydroxypyridine as a sole carbon and energy source in the medium. The other pyridine derivatives, such as 3-hydroxypyridine, 2-, 3-, 4-carboxypyridine, 2-methylpyridine, pyridine-*N*-oxide, and pyridine were not catabolized. It should be noted that bacteria utilizing a single pyridine derivative were described in a

large number of studies [1-10], but IN13 strain is unique in utilizing 4-hydroxypyridine and 2-hydroxypyridine. The biotransformation of 2-hydroxypyridine by *Arthrobacter* sp. IN13 is accompanied by accumulation of a blue pigment, maybe caused by spontaneous oxidation of 2,3,6-trihydroxypyridine intermediate [1, 2]. A bright blue pigment was produced during the early log phase. Subsequently the medium changed to purple and finally to brown, after which there was no apparent change in colour. The series of pigments were produced only when 2-hydroxypyridine was present in the media. Colour intensity was proportional to the amount of 2-hydroxypyridine added to the media. Production of pigments during the biodegradation of 2-hydroxypyridine has been reported with other *Arthrobacter* sp. Ensign and Rittenberg [18] observed production of a blue pigment during 2-hydroxypyridine degradation by *Arthrobacter crystallopoietes*. In liquid cultures this blue pigment was transitory, and the medium ultimately turned orange-brown in colour. A blue pigment with ultraviolet and visible spectra, identical to the pigment produced by *A. crystallopoietes* has been reported during degradation of 2-hydroxypyridine by *Arthrobacter pyridinolis*, *Arthrobacter* sp. PY21 and other microorganisms [7, 8, 19]. No pigment was observed when *Arthrobacter* sp. IN13 grew in media containing 4-hydroxypyridine.

Biodegradation studies with washed cell suspensions were carried out as described in Materials and Methods after 24 h of cultivation in an appropriate medium. Data are shown in Figs. 2-4. Oxidation of 4-hydroxypyridine by the resting cells of *Arthrobacter* sp. IN13 is inducible in cells grown in the presence of 4-hydroxypyridine. When cells were grown in either chemically defined or in complex media in the absence of 4-hydroxypyridine, no degradation of 4-hydroxypyridine by whole cells was observed. However, both 2-hydroxypyridine and 4-hydroxypyridine are actively consumed by 2-hydroxypyridine-grown cells (Figs. 3 and 4, curves 1, 2). 4-hydroxypyridine-grown cells also consumed 2-hydroxypyridine, but very slowly (Fig. 4, curves 3, 4). Moreover, it has been found that IN13 tends to lose 4-hydroxypyridine degrading activity at a significant rate after a prolonged growth in a medium with 2-hydroxypyridine (data not shown). All these data suggest that there are differences among the pathways of 4-hydroxypyridine and 2-hydroxypyridine degradation, but 2-hydroxypyridine can induce utilization of 4-hydroxypyridine, or the enzymes participating in the catabolism of 2-hydroxypyridine can transform 4-hydroxypyridine non-specifically under *in vitro* conditions using the rest cells. The second alternative is indirectly supported by SDS-PAGE analysis of cell-free extracts (Fig. 5). Several different proteins are inducible during cultivation of *Arthrobacter* sp. IN13 in a growth medium supplemented with 2-hydroxypyridine and 4-hydroxypyridine (Fig. 5). If 2-hydroxypyridine could induce the synthesis of proteins participating in the catabolism of 4-hydroxypyridine, the patterns of inducible protein in SDS-PAGE should be identical or more similar.

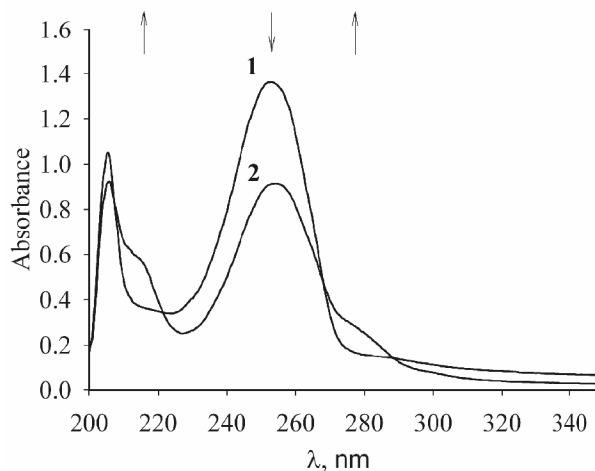


Fig. 2. Degradation of 4-hydroxypyridine by washed cells of *Arthrobacter* sp. IN13 grown on 4-hydroxypyridine. Biodegradation studies were performed in 50 mM potassium phosphate buffer, pH 7.2, containing 10 mM of 4-hydroxypyridine. Cells were suspended in this solution and the suspension was aerobically incubated at 20 °C. Following centrifugation to remove cells, the consumption of 4-hydroxypyridine was estimated recording absorbance in the 200–350 nm range. Arrows mark decrease or increase in absorbance. 1 – after 0 min; 2 – after 10 min

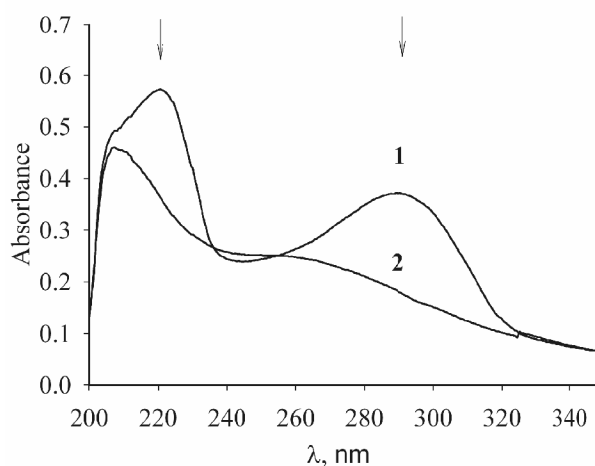


Fig. 3. Degradation of 2-hydroxypyridine by washed cells of *Arthrobacter* sp. IN13 grown on 2-hydroxypyridine. Biodegradation studies were performed in 50 mM potassium phosphate buffer, pH 7.2, containing 10 mM of 2-hydroxypyridine. Cells were suspended in this solution and the suspension was aerobically incubated at 20 °C. Following centrifugation to remove the cells, the consumption of 2-hydroxypyridine was estimated recording absorbance in the 200–350 nm range. Arrows mark decrease or increase in absorbance. 1 – after 0 minutes; 2 – after 10 min

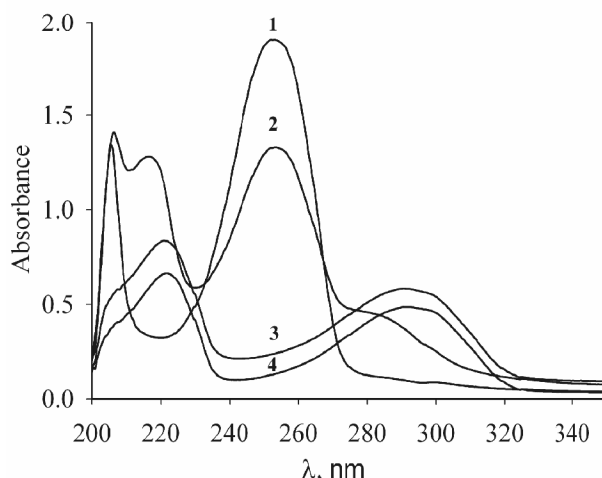


Fig. 4. 1, 2 – degradation of 4-hydroxypyridine by washed cells of *Arthrobacter* sp. IN13 grown on 2-hydroxypyridine. 3, 4 – degradation of 2-hydroxypyridine by washed cells of *Arthrobacter* sp. IN13 grown on 4-hydroxypyridine. Biodegradation studies were performed in 50 mM potassium phosphate buffer, pH 7.2, containing 10 mM of appropriate substrate. The cells were suspended in this solution and the suspension was aerobically incubated at 20 °C. Following centrifugation to remove the cells, the consumption of substrate was estimated recording absorbance in the 200–350 nm range. 1, 3 – after 0 min; 2, 4 – after 10 min

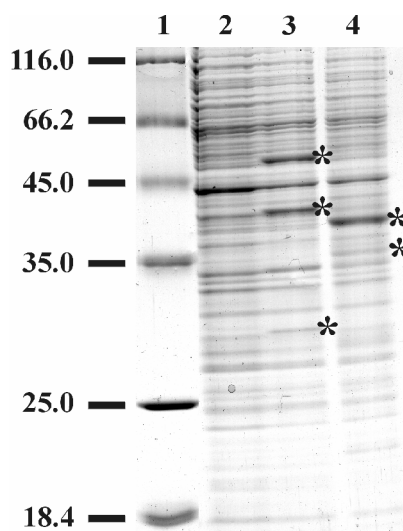


Fig. 5. 14% SDS-PAGE of cell-free extracts from *Arthrobacter* sp. IN13. 1 – protein molecular mass marker (kDa); 2 – cells grown on succinate; 3 – cells grown on 4-hydroxypyridine; 4 – cells grown on 2-hydroxypyridine. Inducible proteins are marked by asterisks on the right side of the lanes

CONCLUSIONS

The isolate *Arthrobacter* sp. IN13 is the first characterized 4-hydroxypyridine degrading Gram-positive bacterium. It appears to be unique among the reported 4-hydroxypyridine degraders since previously isolated microorganisms were unable to utilize 2-hydroxypyridine.

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NAUJAS *ARTHROBACTER* GENTIES MIKROORGANIZMAS, SKAIDANTIS 4-HIDROKSIPIRIDINĄ

Santrauka

Pirmą kartą išskirtas gram-teigiamas bakterinis kamienas, sugėbantis aerobinėmis sąlygomis skaidyti 4-hidroksipiridiną ir naudoti jį kaip vienintelį anglies, azoto ir energijos šaltinį. Remian-

tis 16S rRNR geno nukleotidų sekos analize nustatyta, kad IN13 bakterinis kamienas priklauso *Arthrobacter* genčiai ir su keliais nekultivuojamais kamienais, taip pat *Arthrobacter* sp. A4Z24, *Arthrobacter* sp. A1-1 ir kitais formuoja atskirą filogenetinio medžio šaką. Nustatyta, kad *Arthrobacter* sp. IN13 kaip vienintelį anglies, azoto ir energijos šaltinį gali pasisavinti ir 2-hidroksipiridiną. Iki šiol literatūroje nebuvo duomenų, kad vienas bakterinis kamienas galėtų transformuoti abu šiuos junginius. Be to, buvo pastebėta, kad IN13 bakterinis kamienas,

kultivuojamas su 2-hidroksipiridinu, yra linkęs prarasti sugebėjimą naudoti 4-hidroksipiridiną kaip vienintelį anglies, azoto ir energijos šaltinį. Nustatyta, kad intaktinės ląstelės *in vitro* efektyviai skaido 4-hidroksipiridiną ir 2-hidroksipiridiną; be to, *Arthrobacter* sp. IN13 ląstelės, užaugintos terpėje su 2-hidroksipiridinu, *in vitro* transformuoja ir 4-hidroksipiridiną, o ląstelės, užaugintos su 4-hidroksipiridinu, 2-hidroksipiridiną skaido pastebimai silpniau.