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# Isolation and characterization of novel pyridine dicarboxylic acid-degrading microorganisms

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Five novel microorganisms capable of pyridine dicarboxylic acids degradation were isolated from a soil. Microorganisms utilizing pyridine-2,3-dicarboxylic acid as a sole carbon source were identified as *Rhodococcus* sp. 23C1, *Mycobacterium frederiksbergense* 23ON and *Cupriavidus campinensis* 23K8. This is the first report describing the representatives of these genus capable of degrading this compound. A pyridine-2,3-dicarboxylic acid dehydrogenase (quinolinate dehydrogenase) activity was detected in *Rhodococcus* sp. 23C1 and *Mycobacterium frederiksbergense* 23ON. The enzyme was partially purified from *Rhodococcus* sp. 23C1. Based on detection of nicotinic acid hydroxylase, 6-hydroxynicotinic acid hydroxylase and 2,5-dihydroxypyridine dioxygenase activities in the cell-free extract, a novel pathway of degradation of pyridine-2,3-dicarboxylic acid proceeding via formation of nicotinic acid was proposed for *Cupriavidus campinensis* 23K8. A bacterial isolate aerobically degrading pyridine-2,6-dicarboxylic acid was identified as *Achromobacter* sp. JS18. A novel pathway of pyridine-2,6-dicarboxylic acid degradation with 3-hydroxypicolinic acid as an intermediate was proposed for this bacteria. A pyridine-3,5-dicarboxylic acid-degrading bacterial isolate 35KP identified as *Xanthobacter* sp. was characterized for the first time. A phenazine methosulphate-dependent pyridine-3,5-dicarboxylate dehydrogenase activity was detected in the cell-free extract of *Xanthobacter* sp. 35KP.

**Keywords:** pyridine-2,3-dicarboxylic acid, pyridine-2,6-dicarboxylic acid, pyridine-3,5-dicarboxylic acid, pyridine-3,5-dicarboxylate dehydrogenase, biodegradation

## INTRODUCTION

In the past decades, biocatalysis has emerged as an important tool in the industrial synthesis of bulk chemicals, pharmaceutical and agrochemical intermediates, active pharmaceuticals, and food ingredients. Selective hydroxylation of aromatic compounds is among the most challenging chemical reactions in synthetic chemistry and has gained steadily increasing attention during recent years, particularly because of the use of hydroxylated aromatics as precursors for pharmaceuticals [1–3]. The chemo- and regioselective hydroxylation of the pyridine ring has few analogues in non-enzymatic chemistry, and biocatalysis represents a potential new and mild synthetic route to substituted pyridinols, many of which are potential drugs or agrochemicals. Microbial hydroxylation of pyridines has been studied extensively [4–8]. Various *N*-heterocyclic compounds and their derivatives are converted into useful chemicals by microbial or enzymatic oxidation [9–16]. The microbial conversions of pyridine carboxylic acids also open ways for synthesis of industrially relevant hydroxylated derivatives. For example, 6-hydroxynicotinic acid is an important material in the synthesis of imidacloprid, a potential insecticide, and modified nucleotides [17–20], and 2-hydroxynicotinic acid is the starting compound for the synthesis of 2-chloronicotinic acid [21]. Catabolism and initial hydroxylation steps of monocarboxylated pyridines such as 2-carboxypyridine (picolinic acid) [22–25], 3-carboxypyridine (nicotinic acid) [26–28], and 4-carboxypyridine (isonicotinic acid) [29, 30] have been studied in detail. Nicotinate dehydrogenases, catalyzing the hydroxylation reactions, were purified from *Bacillus niacini* [31], *Pseudomonas fluorescens* TN5 [32], *Eubacterium barkeri* (previously *Clostridium barkeri*) [33], *Ralstonia*/*Burkholderia* strain DSM 6920 [34] and *Pseudomonas putida* KT2440 [35]. Isonicotinate dehydrogenase was purified from *Mycobacterium* sp. INA1 [29] and picolinic acid 6-hydroxylase was purified and characterized from *Arthrobacter picolinophilus* [22].

The microbial conversion of pyridine dicarboxylic acids has been less studied. A putative degradation pathway of

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pyridine-2,6-dicarboxylic acid (dipicolinic acid) via 3-hydroxydipicolinic acid [36, 37], the regioselective hydroxylation of pyridine-2,3-dicarboxylic acid (quinolinic acid), pyridine-2,4-dicarboxylic acid (lutidinic acid) and pyridine-2,5-dicarboxylic acid (isocinchomeronic acid) using microbial cells has been reported [38, 39]. 2,3-, 2,5-, 2,6 and 3,4-dicarboxypyridine were oxidized by phthalic acid-degrading microorganisms [40, 41]. Fermentation of pyridine-2,6-dicarboxylic acid by the consortium of strictly anaerobic microorganisms has been also observed [42]. However, microorganisms capable to use or convert pyridine-3,5-dicarboxylic acid (dinicotinic acid) have not been isolated yet.

The present work describes the isolation and characterization of novel pyridine dicarboxylic acids-degrading bacteria including the first identified microorganism able to degrade pyridine-3,5-dicarboxylic acid. The isolated microorganisms show a good potential to be applicable as biocatalysts as well as an interesting source for novel pyridine ring attacking oxygenases.

## EXPERIMENTAL

### Chemicals

Chemicals were purchased from Sigma-Aldrich and Fluka (Buchs, Switzerland) and were of the highest purity available. Nutrient agar and yeast extract were purchased from Oxoid (Hampshire, UK). 2,5-Dihydroxypyridine was synthesized according to [43]. DEAE FF Sepharose and Phenyl-Sepharose 6 FF were obtained from GE Healthcare (Helsinki, Finland).

### Isolation of pyridine dicarboxylic acid utilizing microorganism

Soils and the enrichment culture technique were used to isolate pyridine dicarboxylic acid-degrading microorganisms. Samples of soils (5 g) were suspended in 20 ml of the mineral medium (KT medium (per litre of distilled water): 5.0 g NaCl, 1.0 g $NH_4H_2PO_4$, 1.0 g $K_2HPO_4$, 0.4 g $MgSO_4 \cdot 7H_2O$, pH 7.2 with KOH) containing an appropriate acid (0.05%) and cultivated aerobically at 30 °C for 1–3 weeks. After cultivation the aliquots were diluted and spread on the agar plates containing the KT medium supplemented with 0.05% of an appropriate acid and cultivated aerobically at 30 °C for 2–7 days. The largest colonies were selected and purified by streaking repeatedly on the Nutrient agar medium, EFA medium ((per litre of distilled water): 10.0 g $K_2HPO_4$, 4.0 g $KH_2PO_4$, 0.5 g yeast extract, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, salt solution 10 ml/l, pH 7.2; salt solution (per litre of 0.1 N HCl): 2.0 g $CaCl_2 \cdot 2H_2O$, 1.0 g $MnSO_4 \cdot 4H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$ was added into EFA medium after sterilization) and the KT medium supplemented with 0.05% of an appropriate acid. Pyridine-2,3-dicarboxylic, pyridine-2,5-dicarboxylic, pyridine-2,6-dicarboxylic, pyridine-3,4-dicarboxylic, and pyridine-3,5-dicarboxylic acid were used for the isolation of degrading microorganisms.

### Preparation of bacterial cells for whole-cell bioconversion experiments

The isolate 35KP was cultivated aerobically in 20 ml EFA medium supplemented with pyridine-3,5-dicarboxylic acid (0.05%) at 30 °C for 7 days. The cells were aseptically collected (3000 × *g*, 15 min), suspended into 200 ml KT medium supplemented with pyridine-3,5-dicarboxylic acid (0.05%) and cultivated aerobically at 30 °C for 24 hours. The cells were aseptically collected (3000 × *g*, 15 min) and washed twice with 0.9% NaCl. The isolates 23ON and 23K8 were cultivated aerobically in 200 ml EFA medium supplemented with pyridine-2,3-dicarboxylic acid (0.05%) at 30 °C for 5 days. Then pyridine-2,3-dicarboxylic acid was aseptically added (final concentration 0.05%) and the cells were additionally cultivated for 24 hours. The biomass was aseptically collected (3000 × *g*, 15 min) and washed twice with 0.9% NaCl. The isolate 23C1 was cultivated aerobically in 200 ml EFA medium supplemented with pyridine-2,3-dicarboxylic acid (0.05%) at 30 °C for 24 hours. Then the cells were aseptically collected (3000 × *g*, 15 min) and twice washed with 0.9% NaCl. The isolate JS18 was cultivated aerobically in 200 ml EFA medium supplemented with pyridine-2,6-dicarboxylic acid (0.05%) at 30 °C for 2 days. Then pyridine-2,6-dicarboxylic acid was aseptically added (final concentration 0.05%) and the cells were additionally cultivated at 30 °C for 24 hours. The biomass was aseptically collected (3000 × *g*, 15 min) and washed twice with 0.9% NaCl.

The same cultivation conditions were applied when other substrates were used as an inducer.

For all experiments, biomass (equivalent amount as from 10 ml of culture broth) was suspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.2) containing 0.1–1 mM of the appropriate substrate. The reactions were carried out at 20 °C.

### Taxonomic affiliation and phylogenetic analysis

DNA was extracted according to [44]. 16S rRNA encoding genes were amplified using universal primers w001 (5′-AGAGTTTGATCMTGGCTC-3′) and w002 (5′-GNTACCTTGTTACGACTT-3′) according to [45]. The PCR product was purified with a DNA purification kit and cloned into the pTZ57R/T plasmid (Thermo Fisher Scientific, Lithuania). The cloned 16S ribosomal DNA was sequenced at Macrogen (Netherlands). A phylogenetic tree was created by the neighbour-joining method [46]. The robustness of the tree was analyzed by 1000 bootstrap replications [47]. The evolutionary distances were computed using the maximum composite likelihood method [48] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA6 [49].

### Assays of enzymes

All reactions were carried out in triplicate at 30 °C. Quinolinate dehydrogenase activity was measured as published [38]

with modifications. The standard assay mixture contained 6 mM pyridine-2,3-dicarboxylic acid, 5 μM phenazine methosulfate (PMS), 50 μM dichlorophenol indophenol (DCPIP), 100 mM Tris-HCl buffer (pH 8.5) and enzyme solution in a total volume of 1 ml. One unit of enzyme was defined as the amount of enzyme catalyzing the reduction of 1 μmol of DCPIP ($\varepsilon_{600}$ = 16800 $M^{-1}$ $cm^{-1}$) per minute. The nicotinic acid 6-hydroxylase and 6-hydroxynicotinic acid 3-monooxygenase activities were analysed by measuring spectrophotometrically the reduction of $K_3[Fe(CN)_6]$ and oxidation of NADH, respectively [27, 50]. The standard assay mixture for nicotinic acid 6-hydroxylase contained 0.15 mM nicotinic acid, 50 mM potassium phosphate buffer (pH 7.2) and enzyme solution in a total volume of 1 ml. The reaction was started by the addition of 0.3 mM potassium hexacyanoferrate(II). The reduction of potassium hexacyanoferrate(II) ($\varepsilon_{417}$ = 5200 $M^{-1}$ $cm^{-1}$) was measured at 30 °C. One unit of activity was defined as the amount of enzyme necessary to reduce 1 μmol of hexacyanoferrate(II) per minute. The standard assay mixture for 6-hydroxynicotinic acid 3-monoxygenase contained 0.1 mM 6-hydroxynicotinic acid, 50 mM potassium phosphate buffer (pH 7.2) 10 μM FAD and enzyme solution in a total volume of 1 ml. The reaction was started by the addition of 0.2 mM NADH. The oxidation of NADH (ε = 6220 $M^{-1}$ $cm^{-1}$) was measured at 340 nm. One unit of activity was defined as the amount of enzyme necessary to oxidise 1 μmol of NADH per minute. Activity of 2,5-dihydroxypyridine dioxygenase was measured as described previously [51]. 1 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.2), 200 μM 2,5-dihydroxypyridine, 1 μM $FeSO_4$ and enzyme solution. The reaction was started by the addition of 2,5-dihydroxypyridine (ε = 5200 $M^{-1}$ $cm^{-1}$) and followed photometrically at 320 nm. One unit of activity was defined as the amount of enzyme necessary to oxidise 1 μmol of 2,5-dihydroxypyridine per minute. Picolinic acid dehydrogenase and 6-hydroxypicolinic acid hydroxylase were analysed as described previously [22, 52]. Pyridine-3,5-dicarboxylate dehydrogenase activity was analysed by measuring spectrophotometrically the reduction of DCPIP in the presence of PMS and substrate. The standard assay mixture contained 1.5 mM pyridine-3,5-dicarboxylic acid, 5 μM PMS, 50 μM DCPIP, 100 mM Tris-HCl buffer (pH 8.0) and enzyme solution in a total volume of 1 ml. One unit of enzyme was defined as the amount of enzyme catalyzing the reduction of 1 μmol of DCPIP ($\varepsilon_{600}$ = 16800 $M^{-1}$ $cm^{-1}$) per minute.

### Partial purification of pyridine-2,3-dicarboxylic acid dehydrogenase (quinolinate dehydrogenase)

All steps for the purification of quinolinate dehydrogenase were done at 4 °C and 10 mM Tris-HCl buffer (pH 8.5) (buffer A) was used unless otherwise specified. The cells of *Mycobacterium frederiksbergense* 23ON from 2 l of culture were washed with saline (0.9% NaCl), suspended in 12 ml of buffer A and then disrupted using an ultrasonic oscillator (22 kHz for 10 min). The cell debris was removed by centrifugation at 10000 *g* for 30 min. The cell-free extract was loaded on a DEAE FF column equilibrated with buffer A. The enzyme activity was eluted with a linear gradient of 0–1 M NaCl in buffer A. The active fractions were combined and concentrated by ultrafiltration (30 kDa cutoff). Solid NaCl was added to the final concentration of 2 M and the sample was loaded on a Phenyl FF column equilibrated with buffer A containing 2 M NaCl. The enzyme activity was eluted by decreasing of the concentration of NaCl. The active fractions were combined and concentrated by ultrafiltration (30 kDa cutoff).

## RESULTS AND DISCUSSION

### Isolation of pyridine dicarboxylic acid-degrading microorganisms

Through enrichment culture using pyridine dicarboxylic acids, three microorganisms (the isolates 23C1, 23ON, and 23K8) degrading pyridine-2,3-dicarboxylic acid, one microorganism (the isolate JS18) degrading pyridine-2,6-dicarboxylic acid and one microorganism (the isolate 35KP) degrading pyridine-3,5-dicarboxylic acid were isolated. All attempts to screen bacteria when pyridine-2,5-dicarboxylic or pyridine-3,4-dicarboxylic acid was used as the sole carbon source were unsuccessful. The ability of the isolated strains to grow on *N*-heterocyclic compounds as a source of carbon is presented in Table 1. All isolates were capable of using two or three different pyridine mono- or dicarboxylic acids. In addition to the pyridine acids, 2-, 3- and 4-hydroxypyridine were also tested as growth substrates, but none of the strains grew using these compounds, as measured by turbidity of the culture. All strains grew on succinate, which was used as a control compound in whole-cell reactions. A violet pigment was produced by the isolate 35KP in the presence of pyridine-3,5-dicarboxylic acid but not 3-carboxypyridine or other carbon sources.

Table 1. **Growth of isolated microorganisms on various carboxylated pyridines. + Moderate growth, ++ good growth, – no growth. Results were recorded after growth in mineral medium supplemented with 0.05% of carbon source at 30 °C for 4 days**

| Substrate | Bacterial strain | | | | |
|---|---|---|---|---|---|
| | 23K8 | 23C1 | 23ON | JS18 | 35KP[a] |
| Pyridine-2,3-dicarboxylic acid | + | ++ | ++ | – | – |
| Pyridine-2,6-dicarboxylic acid | + | + | + | ++ | – |
| Pyridine-2,5-dicarboxylic acid | – | – | – | – | – |
| Pyridine-3,4-dicarboxylic acid | – | – | – | – | – |
| Pyridine-3,5-dicarboxylic acid | – | – | – | – | ++[b] |
| 2-Carboxypyridine | – | – | – | ++ | – |
| 3-Carboxypyridine | ++ | – | – | + | ++ |
| 4-Carboxypyridine | – | – | – | – | – |

[a] After 7 days.

[b] A violet pigment.

**Identification of isolates**

The nucleotide sequences of the 16S rDNA of each isolate were determined by sequencing of the cloned DNR fragments obtained by PCR amplification. The constructed phylogenetic trees are presented in Figs. 1, 2. 23C1, 23ON, and 23K8 showed the highest 16S rDNA sequence similarity toward *Rhodococcus wratislaviensis*/*opacus* group (Fig. 1a), *Mycobacterium frederiksbergense* (Fig. 1b), and *Cupriavidus campinensis* (Fig. 1c), respectively. The similarity rank analysis showed that the pyridine-3,5-dicarboxylic acid-degrading microorganism 35KP is closely related to members of the genus *Xanthobacter* (Fig. 2b). Interestingly, the strain JS18 was found to have two types of 16S rDNA sequence, which showed 9 bp sequence differences (99.3% nucleotide identity). The possibility of contamination was eliminated because an identical result was obtained from new cultures that had been checked and confirmed to be uncontaminated. The possibility of PCR amplification error was also eliminated since identical results were obtained when PCR was repeated. Accordingly, it was concluded that the strain JS18 shows heterogeneity in 16S rDNA sequences between rRNA gene clusters. The two types of the 16S rDNA sequence were designated as type 1 and type 2 and used for phylogenetic analysis (Fig. 2a). Both 16S rDNA sequences showed the highest similarity toward the *Achromobacter insolitus*/*piechaudii*/*spanius* group. More than one copy of rRNA operons, which code for both the small-subunit and large-subunit rRNA, are often found in prokaryotes. It is generally assumed that all rRNA operons within a single cell are almost identical. A notable exception is the extremely halophilic archaeal genus *Haloarcula*, most species of which are known to harbour highly divergent rRNA operons that differ at ~5% of the nucleotide positions in the small-subunit rRNA gene [53].

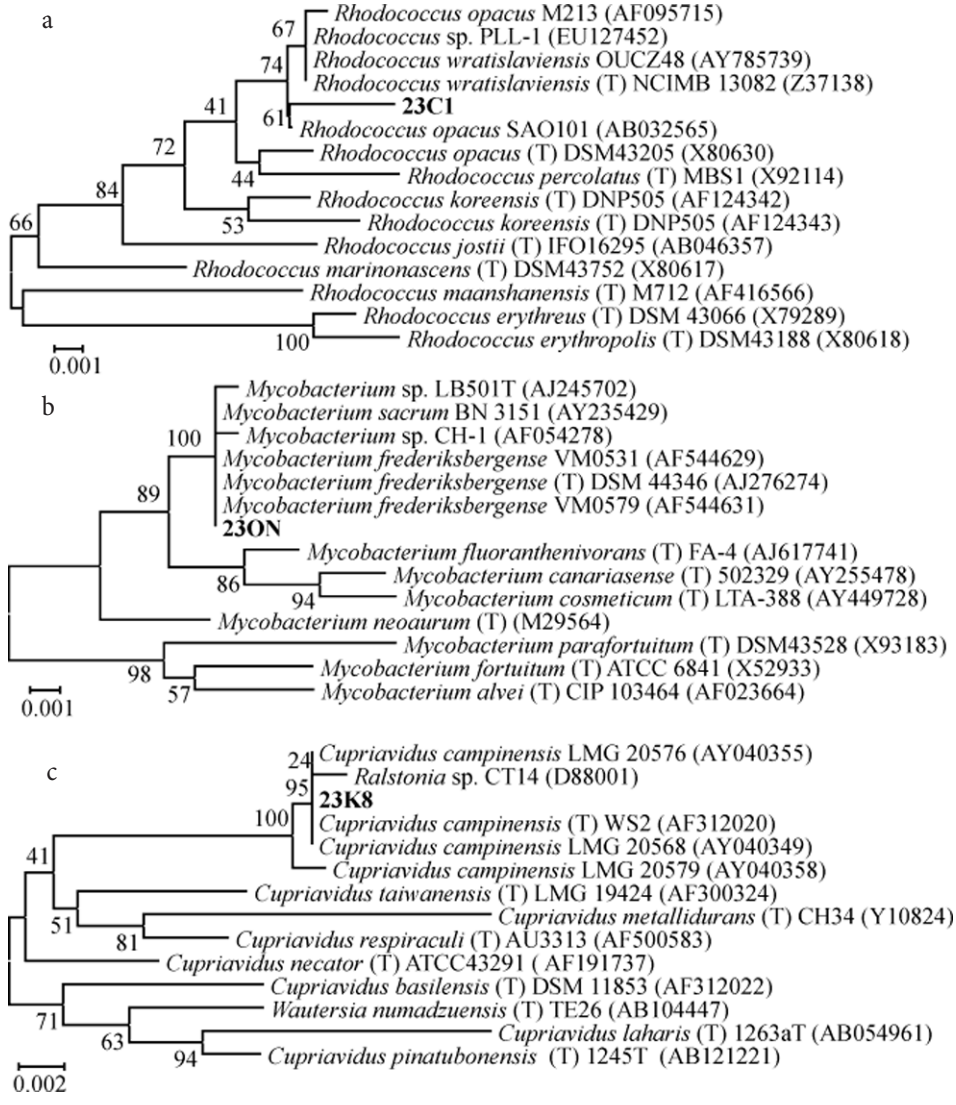


**Fig. 1.** Phylogenetic tree illustrating the relationship among partial 16S rDNA sequences of pyridine-2,3-dicarboxylic acid-degrading bacteria. A phylogenetic tree was created as described in *Materials and methods*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bars represent the number of base substitutions per site. T is type strain. Accession numbers are given in parentheses

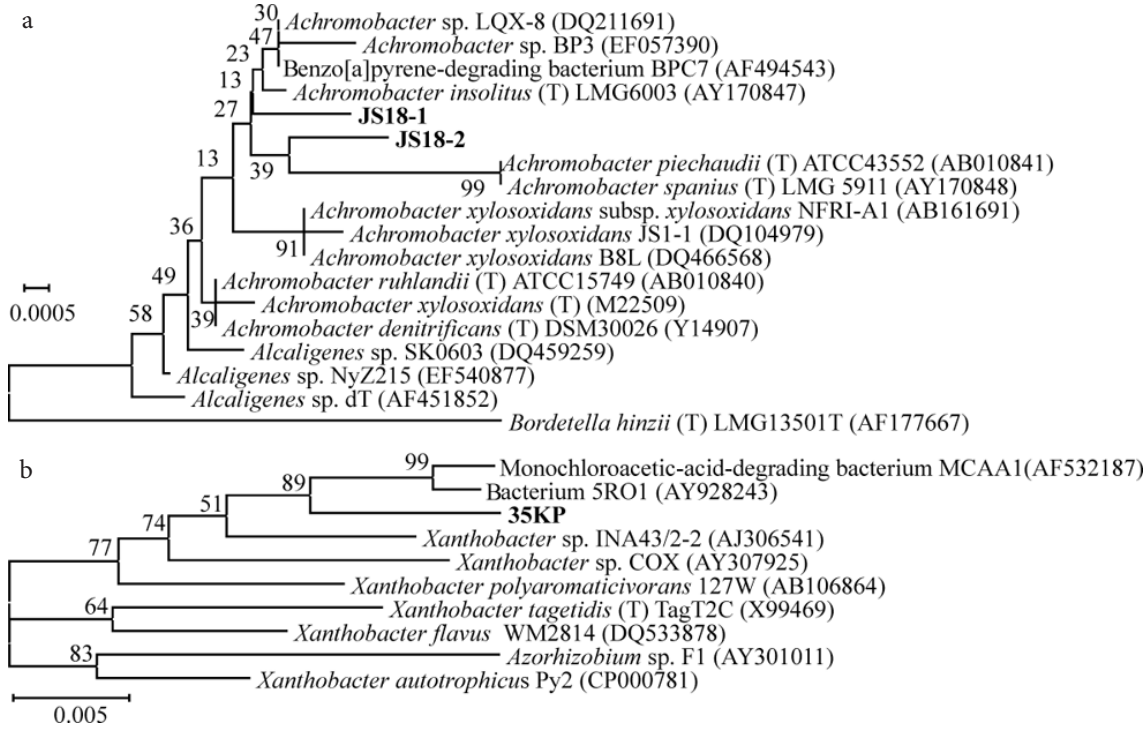


**Fig. 2.** Phylogenetic tree of the pyridine-2,6-dicarboxylic acid-degrading strain JS18 (a) and the pyridine-3,5-dicarboxylic acid-degrading strain 35KP (b). Other descriptions are as in Fig. 1

The existence of heterogeneous 16S rDNA sequences within a single organism has been reported also in *Camphylobacter helveticus*, *Clostridium paradoxum*, *Rhodococcus koreensis*, *Anaerospora hongkongensis* and *Aeromonas* spp. [54–56]. The 16S rRNA heterogeneity shown by the strain JS18 supports the possibility that inter-operon variability within a single strain may exist in more cases than known previously.

Based on phylogenetic analysis, the assignment of the isolates as *Rhodococcus* sp. 23C1, *Mycobacterium frederiksbergense* 23ON, *Cupriavidus campinensis* 23K8, *Xantobacter* sp. 35KP, and *Achromobacter* sp. JS18 was proposed. To our knowledge, the isolates 23C1 and 23ON are the first gram-positive bacteria known to be able of growing with pyridine-2,3-dicarboxylic acid as the sole source of carbon and energy. In addition, this is the first report of the degradation of pyridine-3,5-dicarboxylic acid by the bacteria.

### Conversion of pyridine-2,3-dicarboxylic acids by whole cells and cell-free extracts

The substrate specificities of three pyridine-2,3-dicarboxylic acid-degrading bacteria were examined by the whole-cells reaction. All of isolates pre-grown in the presence of quinolinic acid consumed this substrate. Uninduced cells did not catalyze any conversion. Quinolinic acid-induced cells of both *Mycobacterium frederiksbergense* 23ON and *Rhodococcus* sp. strain 23C1 did not use 6-hydroxynicotinic, 6-hydroxypicolinic, 2-hydroxynicotinic, or 3-hydroxypicolinic acid. Analysis of cell-free extracts of *M. frederiksbergense* strain 23ON and *Rhodococcus* sp. strain 23C1 showed the presence of quinolinate dehydrogenase. According to the literature, 6-hydroxypicolinic acid is an intermediate metabolite of pyridine-2,3-dicarboxylic acid degradation [39]; however, repeated attempts made to register oxidation of this intermediate by adding NADP(H), NAD(H), methylene blue, DCPIP, NBT, FAD, FMN, or their combination into the cell-free extracts both *M. frederiksbergense* 23ON and *Rhodococcus* sp. 23C1 bacteria met with no success. The same negative results were observed if 6-hydroxynicotinic acid was used as a substrate. Quinolinate dehydrogenase from *Rhodococcus* sp. 23C1 was partially purified and characterized. The overall purification of the enzyme was 21-fold with a yield of 35.4% (Table 2). The partially purified enzyme was not homogeneous according to SDS-PAGE (data not shown); however, a specific activity was about 20-times higher compared with the analogous enzyme from *Alcaligenes* sp. UK21 [38]. The activity of quinolinate dehydrogenase had maximum at pH 8.0 in 100 mM Tris-HCl buffer in the presence of phenazine methosulfate as in the case of the enzyme isolated from *Alcaligenes* sp. UK21 [38]. According to the collected data, the initial steps of degradation of pyridine-2,3-dicarboxylic acid by *Rhodococcus* sp. strain 23C1 and *M. frederiksbergense* strain 23ON may be analogous to the known pathway for the aerobic degradation of quinolinic acid found in the gram-negative bacterium *Alcaligenes* sp. strain UK21 [38]. The quinolinic acid dehydrogenase activity was detected in both microorganisms induced with pyridine-2,3-dicarboxylic acid. The enzyme partially purified from *Rhodococcus* sp. strain 23C1 is similar to the previously described one [38].

Table 2. **Partial purification of quinolinate dehydrogenase from *Rhodococcus wratislaviensis* strain 23C1. Data of a typical purification of the enzyme from 2 L of culture is presented**

| Step | Total protein, mg | Total activity, units | Specific activity, units/mg | Purification (-fold) | Yield, % |
|---|---|---|---|---|---|
| Cell-free extract | 120 | 3500 | 29.2 | 1 | 100 |
| DEAE FF Sepharose | 20.4 | 2232 | 109.4 | 3.7 | 63.8 |
| Phenyl FF Sepharose | 2 | 1238 | 619.1 | 21.2 | 35.4 |

Regarding *Wautersia campinensis* 23K8 bacteria, a different pattern of utilization of pyridine-2,3-dicarboxylic acid was observed. The changes in the UV/VIS spectrum during a typical biotransformation of pyridine-2,3-dicarboxylic acid by *Wautersia campinensis* 23K8 are illustrated in Fig. 3a. New absorption maxima were detected at 251 and 300 nm during the initial stages of bioconversion. A decrease in absorbtion in 200–330 nm region occurred after a prolonged incubation (Fig. 3a). The quinolinic acid-induced cells of *Wautersia campinensis* 23K8 did not convert both 3- neither 6-hydroxypicolinic nor 2-hydroxynicotinic acid; however, the cells efficiently consumed both 6-hydroxynicotinic (Fig. 3b) and nicotinic acid. Moreover, succinate- or nicotinic acid-induced cells were inactive against pyridine-2,3-dicarboxylic acid. Hence, the catabolism of quinolinic acid in *Wautersia campinensis* 23K8 was an inducible process. Activities of nicotinic acid dehydrogenase, nicotinic acid 6-monooxygenase, and 2,5-dihydroxypyridine dioxygenase were detected in pyridine-2,3-dicarboxylic acid-induced *Wautersia campinensis* 23K8 cells, but no quinolinic acid dehydrogenase activity was detected. A new pathway for the degradation of pyridine-2,3-dicarboxylic acid could be proposed for *C. campinensis* strain 23K8 on the basis of the whole-cell experiments and the enzymatic activities discovered in the cell-free extract. It is supposed that 6-hydroxynicotinic acid but not 6-hydroxypicolinic acid is an intermediate of pyridine-2,3-dicarboxylic acid degradation in the *C. campinensis* strain 23K8 (Fig. 4).

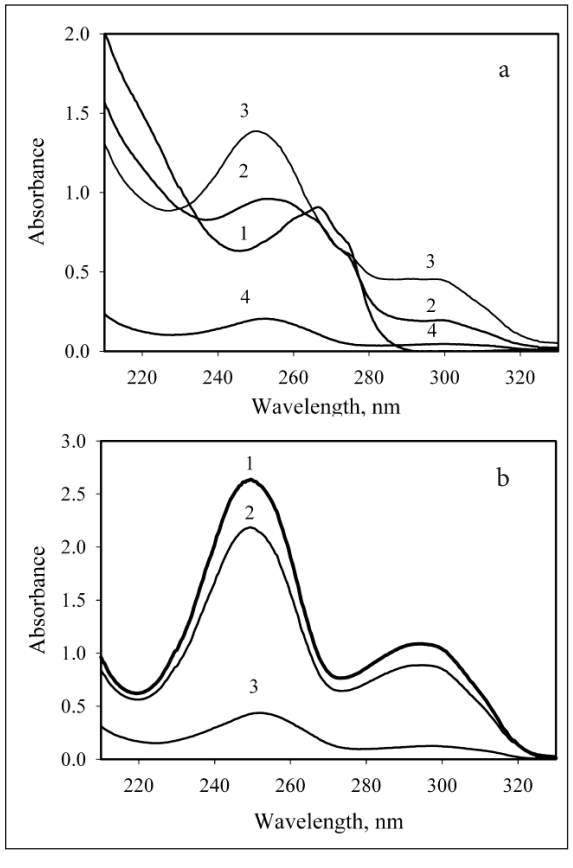


**Fig. 3.** Spectral changes during aerobic conversion of pyridine-2,3-dicarboxylic acid (a) and 6-hydroxynicotinic acid (b) by whole cells of *Cupriavidus campinensis* strain 23K8 ($A_{600\,nm} = 5$) in 50 mM potassium phosphate buffer (pH 7.2) at 20 °C. The initial substrate concentration was 0.2 mM. The reaction mixture after centrifugation (16000 × *g*, 1 min) was scanned at 0 min (1), 40 min (2), 60 min (3), and 16 hours (4) (a) and at 0 min (1), 40 min (2), and 16 hours (3) (b)

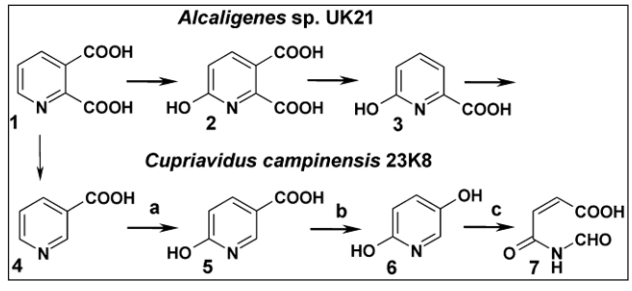


**Fig. 4.** Possible pathway of pyridine-2,3-dicarboxylic acid oxidation in *Cupriavidus campinensis* 23K8. The upper pathway found in *Alcaligenes* sp. UK21 is according to [38, 39]. Pyridine-2,3-dicarboxylic acid (1), 6-hydroxyquinolinic acid (2), 6-hydroxypicolinic acid (3), nicotinic acid (4), 6-hydroxynicotinic acid (5), 2,5-dihydroxypyridine (6) and *N*-formylmaleamic acid (7). Enzymatic activities detected in cell-free extracts of *Cupriavidus campinensis* 23K8 grown on pyridine-2,3-dicarboxylic acid: (a) nicotinic acid hydroxylase, (b) 6-hydroxynicotinic acid hydroxylase, and (c) 2,5-dihydroxypyridine dioxygenase

## Conversion of pyridine-2,6-dicarboxylic acid by *Achromobacter* sp. JS18

*Achromobacter* sp. JS18 cells pre-grown in the presence of pyridine-2,6-dicarboxylic acid consumed this substrate readily (Fig. 5a). Uninduced cells cultivated on acetate or succinate did not catalyze the conversion of pyridine-2,6-dicarboxylic acid. Consequently, the catabolism of pyridine-2,6-dicarboxylic acid in *Achromobacter* sp. JS18 cells is an inducible process. In addition, *Achromobacter* sp. JS18 cells, pre-cultivated in the presence of pyridine-2,6-dicarboxylic acid, were able also of converting picolinic and nicotinic acids (Fig. 5b, c) as well as 3-hydroxypicolinic acid (Fig. 6); however, the cells were not active towards 6-hydroxypicolinic acid. In addition, the *Achromobacter* sp. JS18 cells pre-grown in the medium containing picolinic acid also utilized 3-hydroxypicolinic acid. To clarify the pyridine-2,6-dicarboxylic acid degradation pathway in this strain,

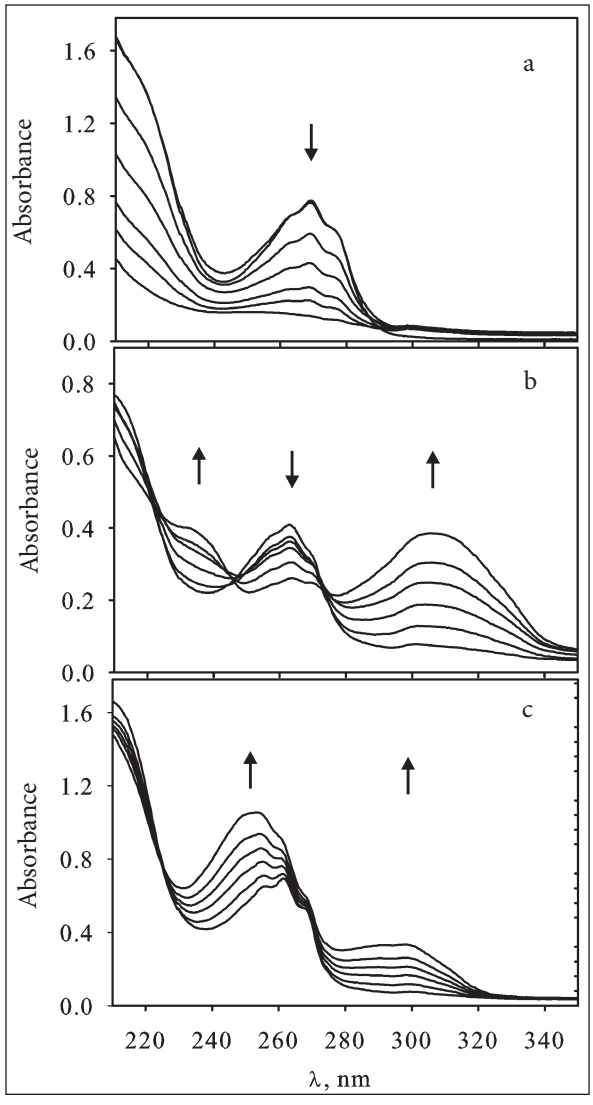


**Fig. 5.** Spectral changes during aerobic conversion of pyridine-2,6-dicarboxylic (a), picolinic (b) and nicotinic acid (c) by whole cells of *Achromobacter* sp. strain JS18 ($A_{600\ nm}$ = 5) in 50 mM potassium phosphate buffer (pH 7.2) at 20 °C. The initial concentration of the compound was 0.2 mM. The *Achromobacter* sp. cells were grown in the presence of pyridine-2,6-dicarboxylic acid. The reaction mixture after centrifugation (16000 × *g*, 1 min) was scanned at 0, 5, 15, 25, 35, 45, 60 min (a) or at 0, 10, 20, 30, 40, 55 min (b, c)

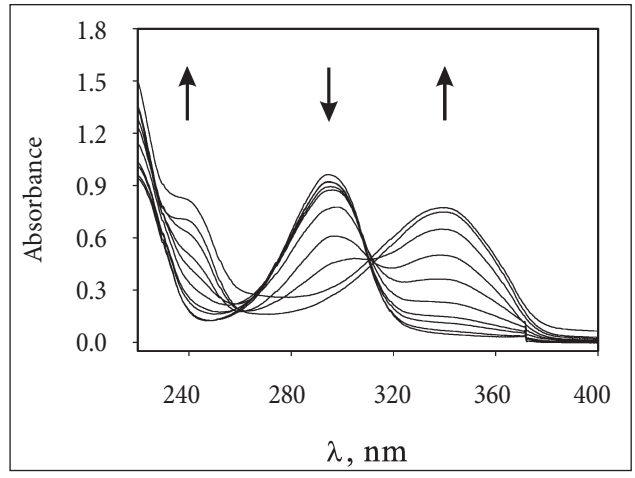


**Fig. 6.** Spectral changes during aerobic conversion of 3-hydroxypicolinic acid by whole cells of *Achromobacter* strain JS18 ($A_{600\ nm}$ = 5) in 50 mM potassium phosphate buffer (pH 7.2) at 20 °C. The initial substrate concentration was 0.2 mM. The *Achromobacter* sp. cells were grown in the presence of dipicolinic acid. The reaction mixture after centrifugation (16000 × *g*, 1 min) was scanned at 0, 5, 15, 25, 35, 45, 70, 100, 140, 200, 270 min

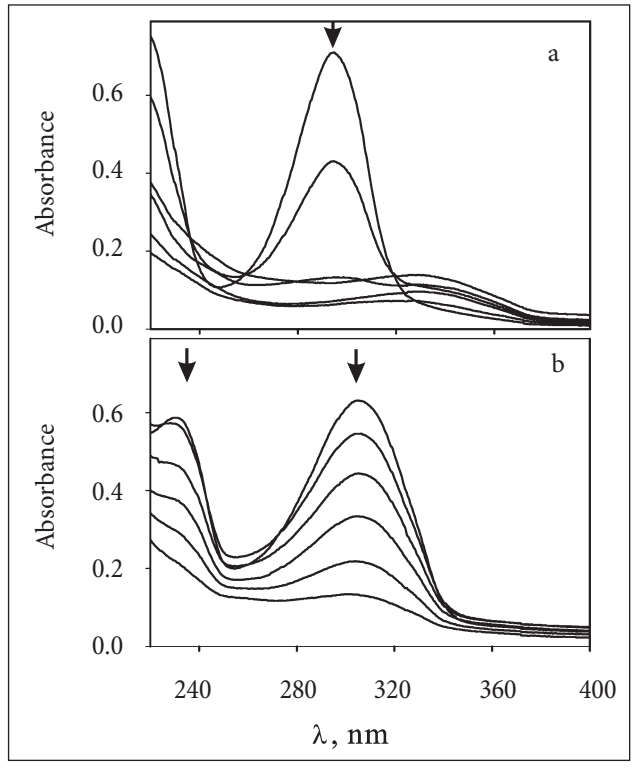


**Fig. 7.** Spectral changes during aerobic conversion of 3-hydroxypicolinic (a) and 6-hydroxypicolinic acid (b) by whole-cells of *Achromobacter* strain JS18 ($A_{600\ nm}$ = 5) in 50 mM potassium phosphate buffer (pH 7.2) at 20 °C. The initial substrate concentration was 0.2 mM. The *Achromobacter* sp. cells were grown in the presence of picolinic acid. The reaction mixtures after centrifugation (16000 × *g*, 1 min) was scanned at 0, 5, 15, 25, 35 and 45 min

the putative degradation enzymes were analyzed in the cell-free extract. The activity of nicotinate dehydrogenase (0.34 ± 0.07 U × $mg^{-1}$ of protein) and picolinate (6.85 ± 0.78 U × $mg^{-1}$ of protein) dehydrogenases as well as 2,5-dihydroxypyridine 5,6-dioxygenase (0.13 ± 0.02 U × $mg^{-1}$ of protein) was detected in pyridine-2,6-dicarboxylic acid-induced cells. A similar level of activities of these enzymes was observed both in picolinic and nicotinic acid-induced cells. However, only background activities (<0.01 U × $mg^{-1}$ of protein) were detected in the cell-free extracts of succinate- or acetate-cultivated *Achromobacter* sp. JS18 cells. It is supposed that the bioconversion of the picolinic and the nicotinic acids started by the hydroxylation at C6 [4, 5]. Since the *Achromobacter* sp. JS18 cells could use both 6-hydroxypicolinic and

6-hydroxynicotinic acids as a carbon source, these data pointed to the classic pathways of pyridine monocarboxylic acids metabolism [4, 5].

Pyridine-2,6-dicarboxylic acid, a typical constituent of the core of the bacterial endospore, is partially degraded by phthalate-utilizing bacteria isolated from marine sediments [40, 41, 57]. Hence, the marine strain CC9M partially oxidized pyridine-2,6-dicarboxylic acid to 2,3-dihydroxypicolinic acid [57]. It has been reported that a *Bacillus brevis* strain is able of utilizing pyridine-2,6-dicarboxylic acid and 2,3-dihydroxypyridine has been supposed as an intermediate [58]. According to the published data, dipicolinic acid is degraded by *Achromobacter* sp. via NAD(P)H-dependent hydroxylation at C3 (formation of 3-hydroxydipicolinic acid) and followed by a ring fission between the two neighbouring carbon atoms, C2 and C3 [36, 37]. In contrast to the previously described *Achromobacter* sp., NAD(P)H-dependent 2,6-dicarboxypyridine oxidation was not detected in the cell-free extract of *Achromobacter* sp. JS18 even if NAD(P)H was changed to other redox mediators such as methylene blue, nitroblue tetrazolium, DCPIP, PMS, FAD or FMN. However, it was found that 3-hydroxypicolinic might be an intermediate in the case of the *Achromobacter* sp. strain JS18 since the pyridine-2,6-dicarboxylic acid-induced cells started to convert 3-hydroxypicolinic acid in the whole cells experiment. The observed increase of absorbance in the 300 to 350 nm region resulting from the metabolism of 3-hydroxypicolinic acid is probably caused by the introduction of hydroxyl groups into the heterocyclic ring [41, 59, 60]. An increase of absorbance in the 260 nm region suggested that 3-hydroxypicolinic acid metabolism does not involve ring cleavage at the first stage. According to the whole-cell experiments and the enzymatic activities discovered in the cell-free extracts, a new pathway for the degradation of pyridine-2,6-dicarboxylic acid could be proposed for *Achromobacter* sp. JS18 (Fig. 8).

To evaluate a biocatalytic potential of *Achromobacter* sp. JS18, several compounds not supporting the growth were tested as putative substrates. Hence, the picolinate-induced *Achromobacter* sp. JS18 cells could transform 5-hydroxypicolinic acid. The final product was not identified, but it was proposed that the 5,6-dihydroxypicolinic acid was formed, since absorbance at 260 nm, specific for the carboxyl group, did not disappear; however, an absorbance near the 310 nm wavelength increased (data not shown). In addition, the *Achromobacter* sp. JS18 cells cultivated in the media containing nicotinic acid could hydroxylate pyridazine-3-carboxylic and 2-hydroxynicotinic acid, probably, at C6. The formed 2,6-dihydroxynicotinic acid easily autooxidated yielding a blue pigment. It was proposed that the quinonic dimer, in analogy with the product, obtained during the autooxidation of trihydroxypyridine, was formed [7].

### Conversion of pyridine-3,5-dicarboxylic acid in *Xanthobacter* sp. 35KP

The spectrophotometric analysis of pyridine-3,5-dicarboxylic acid conversion by *Xanthobacter* sp. 35KP cells showed that a new absorption maximum could be seen (Fig. 9). Nicotinic acid, 3-pyridinesulfonic acid, picolinic acid, 6-hydroxynicotinic acid, 2-hydroxynicotinic acid, 6-hydroxypicolinic acid, 3-hydroxypicolinic acid, isonicotinic acid as well as pyridine-3,4-dicarboxylic acid were inert as the substrates in the whole cell experiments. The *Xanthobacter* sp.

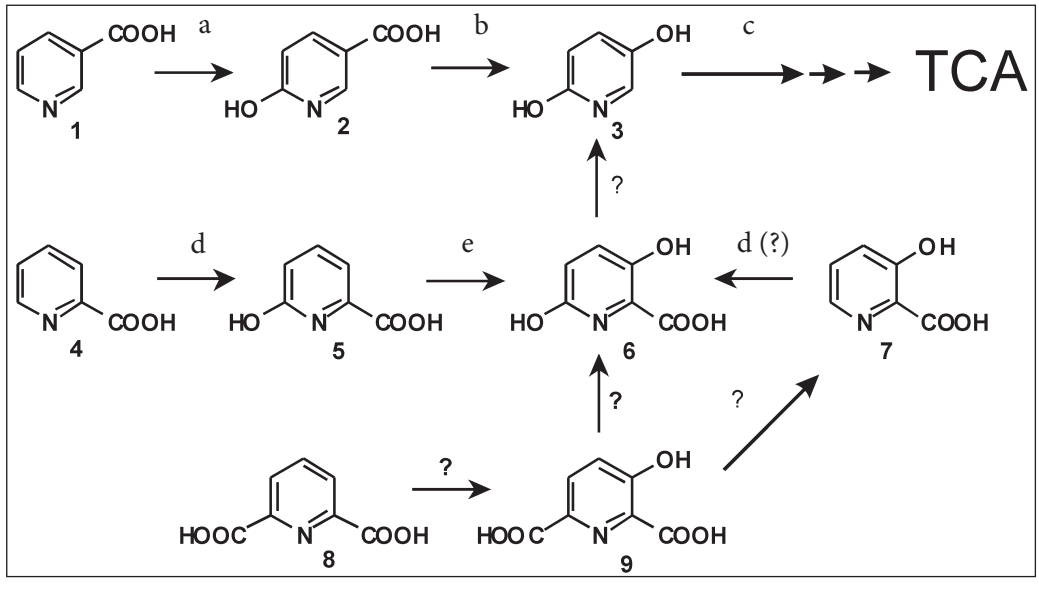


**Fig. 8.** The hypothetical pathway of degradation of nicotinic, picolinic and dipicolinic acids in *Achromobacter* sp. JS18: nicotinic acid (1), 6-hydroxynicotinic acid (2), 2,5-dihydroxypyridine (3), picolinic acid (4), 6-hydroxypicolinic acid (5), 3,6-dihydroxypicolinic acid (6), 3-hydroxypicolinic acid (7), dipicolinic acid (8), 3-hydroxydipicolinic acid (9). Enzymatic activity detected in cell-free extracts: nicotinic acid dehydrogenase (a), nicotinic acid 6-hydroxylase (b), 2,5-dihydroxypyridine 5,6-dioxygenase (c), picolinic acid dehydrogenase (d), 6-hydroxypicolinic acid 3-hydroxylase (e). ? is hypothetical reactions. TCA is tricarboxylic acid cycle

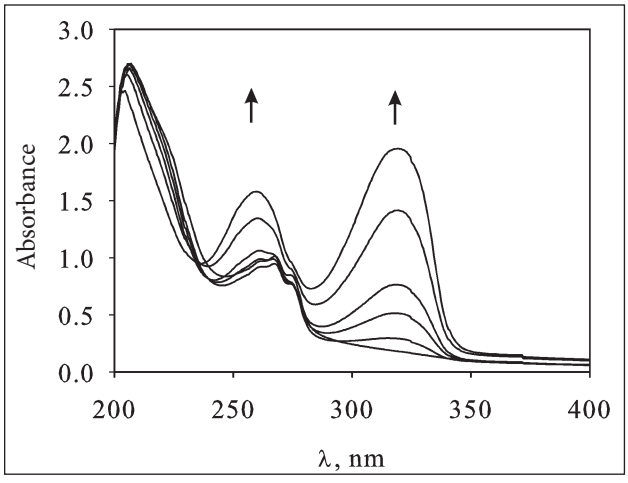


**Fig. 9.** Spectral changes during aerobic conversion of pyridine-3,5-dicarboxylic acid by whole cells of *Xanthobacter* sp. strain 35KP ($A_{600\ nm} = 5$) in 50 mM potassium phosphate buffer (pH 7.2) at 20 °C. The initial substrate concentration was 0.2 mM. The reaction mixture after centrifugation (16000 × *g*, 1 min) was scanned at 0, 10, 25, 35, 60, and 85 min

strain 35KP is the only strain described so far which can utilize pyridine-3,5-dicarboxylic acid as the sole source of carbon and energy. The metabolism of heterocyclic compounds very often involves ring hydroxylations, followed by ring cleavage [4]. Increased absorbance in the 320 nm region resulting from the conversion of dinicotinic acid by resting cells of *Xanthobacter* sp. strain 35KP is probably caused by the introduction of hydroxyl groups into the heterocyclic ring. Moreover, pyridine-3,5-dicarboxylic acid-dependent DCPIP reduction was observed in the cell-free extract of the strain 35KP (0.057 ± 0.006 U × $mg^{-1}$ of protein), but this activity was not detected in the succinate grown cells (<0.002 U × $mg^{-1}$ of protein). By analogy to degradation of other pyridine compounds hydroxylation at C2 was supposed. The next steps of the pathway, including those responsible for ring opening and characterization of pyridine-3,5-dicarboxylic acid hydroxylase, are currently under investigation.

## CONCLUSIONS

Five novel microorganisms capable of utilizing various pyridine dicarboxylic acids showed a wide substrate specificity and biocatalytic activity towards various pyridine compounds. Since hydroxylation of the pyridine ring has few analogues in non-enzymatic chemistry, the whole cells of pyridine dicarboxylic acid-degrading bacteria are very promising for chemical synthesis of hydroxylated pyridine carboxylic acids under mild conditions. Further studies have to be done to elucidate the entire catabolic pathways as well as to characterize the individual enzymes and to explore the biocatalytic potential of the isolated microorganisms in full.

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## NAUJŲ PIRIDINO DIKARBOKSIRŪGŠTIS SKAIDANČIŲ MIKROORGANIZMŲ IŠSKYRIMAS IR CHARAKTERIZAVIMAS

*S a n t r a u k a*

Hidroksilinti *N*-heterocikliniai junginiai – patrauklūs pirmtakai įvairių pramonei svarbių junginių sintezei, tačiau hidroksi grupės įvedimas į *N*-heterociklinį žiedą cheminiais metodais yra komplikuotas. Vienas iš šios problemos sprendimo būdų – panaudoti *N*-heterociklinius junginius, transformuojančius ar skaidančius mikroorganizmus.

Darbe aprašytos penkios naujos bakterijos, skaidančios piridino karboksirūgštis. Trys piridino-2,3-dikarboksirūgštį įsisavinantys mikroorganizmai identifikuoti kaip *Rhodococcus* sp. 23C1, *Mycobacterium frederiksbergense* 23ON ir *Cupriavidus campinensis* 23K8. Piridino-2,3-dikarboksirūgšties dehidrogenazė aptikta *Rhodococcus* sp. 23C1 ir *Mycobacterium frederiksbergense* 23ON ląstelėse, o fermentą iš *Rhodococcus* sp. 23C1 iš dalies pavyko išgryninti. Remiantis intaktinių ląstelių tyrimais bei identifikavus nikotino rūgšties ir 6-hidroksinikotino rūgšties hidroksilazių bei 2,5-dihidroksipiridino dioksigenazės aktyvumus neląsteliniuose ekstraktuose, buvo pasiūlytas naujas piridino-2,3-dikarboksirūgšties skaidymo kelias *Cupriavidus campinensis* 23K8 ląstelėse. Taip pat pastebėta, kad piridino-2,6-dikarboksirūgšties katabolizmas *Achromobacter* sp. JS18 ląstelėse vyksta iki šiol neaprašytu būdu. Pirmą kartą pavyko išskirti piridino-3,5-dikarboksirūgštį skaidantį mikroorganizmą – *Xanthobacter* sp. 35KP ir neląsteliame ekstrakte identifikuoti nuo fenazino metosulfato priklausomą piridino-3,5-dikarboksirūgšties dehidrogenazę.