Genetic diversity of 2-hydroxypyridine-degrading soil bacteria

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New bacterial strains capable of growth on 2-hydroxypyridine as a sole source of carbon and energy were isolated from contaminated soil. The genetic diversity of 26 2-hydroxypyridine-degrading bacteria was determined by restriction fragment length polymorphism (RFLP) of 16S rDNA. The isolates were distributed in 7 groups revealing a high level of microbial diversity. However, the analysis of enzymatic activities responsible for initial degradation of 2-hydroxypyridine showed a lower complexity. A correlation between catabolic pathways and genetic diversity is discussed.

Key words: 2-hydroxypyridine, degradation, 16S rDNA, RFLP

INTRODUCTION

N-heteroaromatics are produced by low-temperature carbonisation, coking, or gasification of fossil raw material and are the basic structure of widely distributed compounds such as dyes, industrial solvents, herbicides, and pesticides. Due to their relatively high water solubilities many heteroaromatic pollutants are readily transported to subsurface environments, resulting in contamination of groundwater. The biodegradation of pyridine derivatives under aerobic conditions has been studied intensively [for reviews see 1, 2]. Microbial utilisation of hydroxypyridines is initiated by hydroxylation of the ring yielding di- and trihydroxypyridine intermediates and followed by ring cleavage [3–7]. Certain members of the genera *Arthrobacter* and *Rhodococcus* are able to utilise 2-hydroxypyridine as a sole source of carbon and nitrogen [6–11]. During transformation of this pyridine derivative, a blue pigment, which is probably the result of 2,3,6-trihydroxypyridine condensation, appears in the culture medium [8, 9]. It is known that the first stage of enzymatic oxidation of 2-hydroxypyridine requires molecular oxygen and NADH and is stimulated by FMN or FAD [12]. Previously we have demonstrated that an FMN : NADH oxidoreductase (flavin reductase) is induced during catabolism of 2-hydroxypyridine in several *Arthrobacter* species [13]. This enzyme can be engaged by $FMNH_2$- or $FADH_2$-utilising monooxygenases as a supplier for the reductant used by the terminal oxygenases. Recently, evidence was presented for a cytochrome P450 to be involved in the initial oxidation of some heterocycles (morpholine, piperidine, and pyrrolidine) by *Mycobacterium* sp. strains [14–17]. In most cases bacterial P450-dependent monooxygenases are composed of three components and electrons are transferred from NAD(P)H via an FAD-containing reductase and a small iron-sulfur protein to the cytochrome P450 where catalysis of the monooxygenase reaction takes place [for a review see 18, 19]. Several P450-dependent monooxygenases are involved in the degradation of xenobiotics by *Rhodococcus* spp. [20], but to date there is not any report on P450-dependent monooxygenases

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involved in the biodegradation of pyridine derivatives.

In this communication we report the isolation of new 2-hydroxypyridine-degrading microorganisms and demonstrate their genetic and biochemical diversity. It is also shown that both flavin- and P450-utilising monooxygenases might be involved in initial steps of 2-hydroxypyridine utilization.

MATERIALS AND METHODS

Chemicals. 2-hydroxypyridine and pyridine were obtained from Merck. Lumichrome was from Sigma.

Bacterial strains. Strains PY11, PY21, PY22, KA1, KA2V2, KA2V3, KA3, and KA4 were described elsewhere [11, 13]. Bacterial strains RDDEP, PYX2, APP, P2G, GAZ1GAZ3, GAZ5, P3, PRH1, RD1, VM02, VSP4, VP3, VPW7, VP22, VP23, KA2 and VM22 were isolated through this work.

Media. Mineral medium KOZ [11] was used for enrichment cultures. TY broth and agar [11] were used as complete media. The routine culture medium (EFA) was as described [8]. All strains were grown at 30 °C.

Enrichment cultures and isolation. Enrichment cultures were prepared in a mineral medium (KOZ) containing 0.2% of 2-hydroxypyridine using soil samples from various contaminated sites. After several serial transfers, the cultures were streaked onto a solid mineral medium supplemented with 0.2% 2-hydroxypyridine. The best growing colonies were subsequently purified on EFA medium containing 0.2% 2-hydroxypyridine.

Characterisation of isolates. Standard microbiological tests and analysis were performed. Formation of a blue pigment was monitored as described previously [8]. DNA was extracted according to [21]. 16S rRNA encoding genes (1.5 kb fragments) were amplified using universal primers w001 (5'-AGAGTTTGATCMTGGCTC-3') and w002 (5'-GNTACCTTGTTACGACTT-3') [22]. The PCR products were purified with a DNA purification kit and cloned into pTZ57R/T plasmid (Fermentas MBI, Lithuania). Each cloned 16S ribosomal DNA was sequenced in both orientations by using ABI377 system.

Inhibition of 2-hydroxypyridine degradation. EFA medium, supplemented with 0.2% 2-hydroxypyridine and 140 μM lumichrome or 2 mM metyrapone, when necessary, was inoculated with a preculture of the test strain. Growth and pigment formation were monitored by following the absorbance at 600 and 650 nm, respectively.

Growth of cells and preparation of extracts for enzymatic assays. 1L flasks containing 200 ml of EFA medium supplemented with succinate or 2-hydroxypyridine were inoculated by overnight-grown cells and were aerobically incubated for an appropriate period. Biomass was collected by centrifugation (5000 g for 20 min) and washed with 0.9% NaCl. The cell paste was frozen at –20 °C until use. Cell-free extracts were prepared by suspending cell paste in 50 mM Tris-HCl buffer, pH 7.5. The cells were broken by ultrasonic treatment (22 kHz, 5 min) and cell debris was removed by centrifugation (5000 g for 30 min).

Enzyme assays. NADH-dependent flavin mononucleotide (FMN) reductase activity was measured as published previously [13].

RESULTS AND DISCUSSION

Strain isolation and characterisation. Several bacterial strains capable of using 2-hydroxypyridine were obtained from heavily contaminated soil. The isolates RDDEP, PYX2, APP, P2G, GAZ1, GAZ3, GAZ5, P3, PRH1, RD1, VM02, VSP4, VP3, VPW7, VP22, VP23, KA2 and VM22 were selected for this work. The strains were found to be gram-positive (except VSP4), pleomorphic rods. All strains utilised 2-hydroxypyridine as a sole carbon source, producing a blue pigment, therefore trihydroxypyridine intermediate can be expected [1, 2]. The RDDEP, PYX2, APP and GAZ5 strains also used pyridine as a sole substrate for growth. The RFLP analysis of 16S rDNA amplified from strains isolated previously and in this study showed that 2-hydroxypyridine-degrading microorganisms form seven distinct clusters, where group 1 consists of PY11, RDDEP, PYX2, APP and GAZ5 strains, group 2 consists of PY21, GAZ3, RD1, VM22 and KA2 strains, group 3 is the most abundant and includes PY22, P2G, P3, GAZ1, KA2V3, KA1, KA3, KA4, PRH1, VP3 and VP23 strains, group 4 contains two strains (KA2V2, VPW7) and three small groups 5, 6 and 7 each represented by one strain – VP22, VM02 and VSP4, respectively (Figure). It is notable that only strains from group 1 are capable to utilise pyridine as a sole carbon source. Partial sequences of the 16S rRNA encoding genes (approximately 500 bp from each end of amplified fragments) were determined at least for one representative of each group. The sequences of 16S rDNA from strains PY11, PY21 and PY22 have been determined previously [13]. According to the analysis of nucleotide sequences, strains from RFLP group 1 were related to *Rhodococcus pyridinovorans*, *R. coprophilus* or *R. rhodochrous* and the bacterium from group 7 to *Stenotrophomonas maltophilia*. Another strains were related to *Arthrobacter* spp.: group 2 showed high homology to *Arthrobacter oxydans* or *A. nicotianae*;

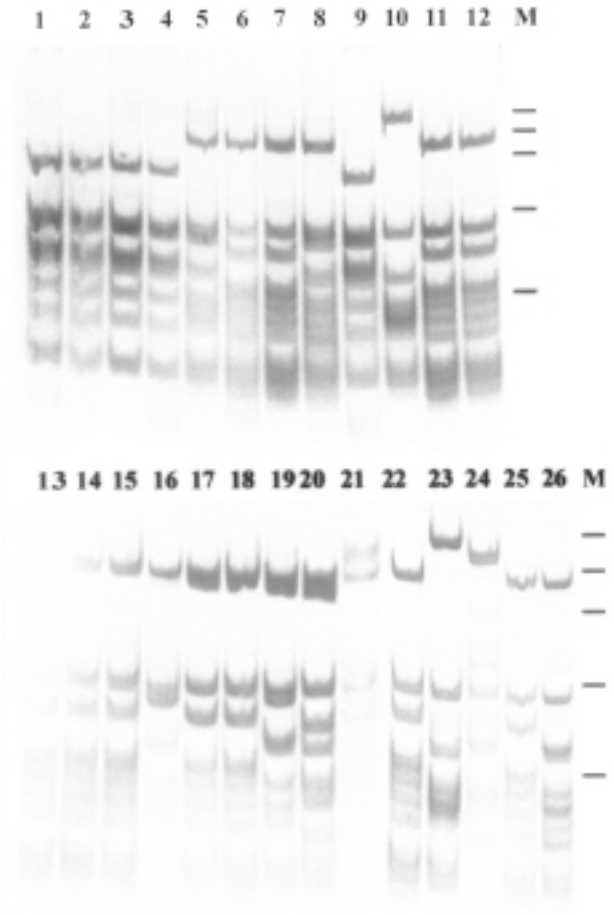

Figure. The RFPL analysis of the amplified 16S rDNAs from 2-hydroxypyridine-degrading bacterial strains. DNA was digested with *Msp*I and applied onto 8% PAAG. *1* – RDDEP, *2* – PYX2, *3* – PY11, *4* – APP, *5* – PY21, *6* – P2G, *7* – GAZ1, *8* – GAZ3, *9* – GAZ5, *10* – KA2V2, *11* – KA2V3, *12* – PY22, *13* – P3, *14* – KA4, *15* – KA3, *16* – KA2, *17* – KA1, *18* – PRH1, *19* – RD1, *20* – VM02, *21* – VSP4, *22* – VP3, *23* – VPW7, *24* – VP22, *25* – VP23, *26* – VM22. M – molecular mass marker, from top to bottom: 500, 400, 300, 200 and 100 bp

representatives from group 3 and 4 were similar to *Arthrobacter rhombi* and *Arthrobacter globiformis*, respectively; microorganisms from groups 5 and 6 belonged to unidentified arthrobacterial species. These results show quite a high genetic diversity of 2-hydroxypyridine-utilizing microorganisms. The next part of this study was asimed at elucidating whether the corresponding diversity exists at the enzymatic level.

Investigation of 2-hydroxypyridine-inducible enzymes. Some arthrobacterial strains are known to produce an inducible FR when cultivated in the presence of 2-hydroxypyridine [13]. In order to understand the mechanism of biodegradative pathway in new microorganisms, we were thus interested in giving evidences of the type of enzymes involved in the initial catabolic reactions. For this reason the bacterial strains were grown in EFA medium containing succinate (non-inducing conditions) or 2-hydroxypyridine (inducing conditions). Cell-free extracts were prepared and FR activity was assayed as described in Materials and Methods. It was found that: *i*) in all arthrobacterial strains tested (microorganisms from groups 2–6) the biosynthesis of FR was inducible in the presence of 2-hydroxypyridine and only traces of FR activity were observed if the cells had been grown in the presence of succinate; *ii*) 2-hydroxypyridine did not induce FR biosynthesis in the strains belonging to group 1. Additional evidences as to the role of flavine reductase in catabolic reactions were collected studying the influence of lumichrome on the pigment production. Lumichrome is known as a powerful inhibitor of FR [23]. It was found that all arthrobacterial strains isolated throughout this work did not produce the blue pigment, a side product of biodegradation, when grown in the presence of 2-hydroxypyridine and lumichrome, but the formation of pigment occurred when the cells were cultivated without the inhibitor. A similar strategy was applied to determine which class of oxygenases is responsible for 2-hydroxypyridine catabolism in microorganisms from group 1. In this case metyrapone, a known inhibitor of cytochrome P450-dependent monooxygenases [17], was used as a supplement of the cultivation medium. It was found that addition of metyrapone resulted in a significant inhibition of pigment production in all rhodococcal strains tested. All these data allow us to conclude that 2-hydroxypyridine degradation pathways are diverse and the type of monooxygenase involved in the initial steps of catabolism depends on bacterial species: flavine reductases are the main enzymes in *Arthrobacter* spp., but microorganisms from *Rhodococcus* group produce a cytochrome P450-related protein.

Thus, further progress in our understanding of bacterial 2-hydroxypyridine catabolism pathways will require a biochemical analysis of highly purified enzymes involved in oxidation reactions.

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References

1. Kaiser J-P, Feng Y, Bollag J-M. Microbiol Rev 1996; 60: 483–98.
2. Fetzner S. Appl Microbiol Biotechnol 1998; 49: 237–50.
3. Houghton C, Cain, RB. Biochem J 1972; 130: 879–93.
4. Cain RB, Houghton C, Wright KA. Biochem J 1974; 140: 293–300.
5. Watson GK, Houghton C, Cain RB. Biochem J 1974; 140: 265–76.
6. Gupta RC, Shukla OP. Ind J Biochem Biophys 1975; 12: 296–8.
7. Zefirov NS, Agapova SR, Terentiev PB et al. FEMS Microbiol Lett 1994; 118: 71–4.
8. Ensign JC, Rittenberg SC. Arch Microbiol 1963; 47: 137–53.
9. Kolenbrander PE, Lotong N, Ensign JC. Arch Microbiol 1976; 110: 239–45.
10. O'Loughlin EJ, Sims GK, Traina SJ. Biodegradation 1999; 10: 93–104.
11. Bondareva A, Marcinkevičienė L, Bachmatova I et al. Biologija 2000; 1: 22–4.
12. Kolenbrander PE, Weinberger M. J Bacteriol 1977; 132: 51–9.
13. Semėnaitė R, Duran R, Marcinkevičienė L et al. Biologija 2000; 2: 29–331.
14. Poupin P, Truffaut N, Combourieu B et al. Appl Environ Microbiol 1998; 64: 159–65.
15. Poupin P, Ducrocq V, Hallier-Soulier S et al. J Bacteriol 1999; 181: 3419–26.
16. Poupin P, Godon JJ, Zumstein E et al. Can J Microbiol 1999; 45: 209–16.
17. Schräder T, Schuffenhauer G, Sielaff B et al. Microbiology 2000; 146: 1091–8.
18. Degtyarenko KN. Protein Eng 1995; 8: 737–47.
19. Munro AW, Lindsay JG. Mol Microbiol 1996; 20: 1115–25.
20. Eltis LD, Karlson U, Timmis KN. Eur J Biochem 1993; 213: 211–6.
21. Woo TS, Cheng AF, Ling JM. BioTechniques 1992; 13: 696–7.
22. Godon JJ, Zumstein E, Dabert P et al. Appl Environ Microbiol 1997; 63: 2802–13.
23. Fieschi F, Niviere V, Frier C et al. J Biol Chem 1995; 270: 30392–400.

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GENETINĖ 2-HIDROKSIPIRIDINĄ METABOLIZUOJANČIŲ DIRVOŽEMIO BAKTERIJŲ ĮVAIROVĖ

S a n t r a u k a

Išskirti nauji bakteriniai kamienai, sugebantys metabolizuoti 2-hidroksipiridiną. Remiantis 16S rRNR genų sekomis ir jų restrikcinių fragmentų ilgio polimorfizmo tyrimais, nustatyta didelė 2-hidroksipiridiną metabolizuojančių mikroorganizmų įvairovė, o 26 tirti bakteriniai kamienai buvo suskirstyti į septynias grupes. Pastebėta, kad fermentų lygyje pradinių 2-hidroksipiridino katabolizmo kelių įvairovė yra daug mažesnė.