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ORIGINAL PAPER

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Regioselective hydroxylation of quinolinic acid, lutidinic acid and isocinchomeronic acid by resting cells of pyridine dicarboxylic acid-degrading microorganisms

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Abstract Microorganisms aerobically degrading quinolinic acid, lutidinic acid or isocinchomeronic acid were isolated and the microbial regioselective hydroxylation of these pyridine dicarboxylic acids was studied. *Alcaligenes* sp. UK21 cells converted quinolinic acid into 6-hydroxypicolinic acid, suggesting the involvement of two enzyme reactions catalyzing hydroxylation at position C6 and decarboxylation at position C3 of quinolinic acid. Resting cells of *Alcaligenes* sp. UK21 accumulated 94.9 mM 6-hydroxypicolinic acid (13.2 g l^{-1}), with a 96% molar conversion yield by 48 h incubation. *Rhizobium* sp. LA17 and *Hydrogenophaga* sp. IMA01 catalyzed the regioselective hydroxylation of lutidinic acid and isocinchomeronic acid into 6-hydroxylutidinic acid and 6-hydroxyisocinchomeronic acid, respectively. 6-Hydroxylutidinic acid accumulated up to 95.4 mM (17.5 g l^{-1}) by 24 h incubation in the resting cells reaction, using *Rhizobium* sp. LA17, with a 99% molar conversion yield. Resting cells of *Hydrogenophaga* sp. IMA01 produced 88.7 mM 6-hydroxyisocinchomeronic acid (16.2 g l^{-1}) by 24 h incubation, with a 81% molar conversion yield.

Introduction

Regioselectivity and high efficiency are characteristics of microbial hydroxylation reactions of *N*-heterocyclic aromatic compounds; and *N*-heterocyclic compounds with hydroxyl groups can be utilized for the synthesis of various pharmaceuticals and agrochemicals. For example, 6-hydroxynicotinic acid and 3-cyano-6-hydroxypyridine are important materials in the synthesis of imidacloprid, a potential insecticide (Kagabu et al. 1992; Moriya et al. 1992, 1993). Microbial hydroxylation at the carbon atom adjacent to nitrogen of pyridine monocarboxylic acids, such as nicotinic acid, picolinic acid and isonicotinic acid, has been studied extensively (Gupta and Shukla 1979; Hurh et al. 1994b; Kiener et al. 1993; Kretzer and Andreesen 1991; Kretzer et al. 1993; Nagel and Andreesen 1989; Nakano et al. 1999; Shukla et al. 1977; Siegmund et al. 1990; Singh and Shukla 1986; Tate and Ensign 1974; Tinschert et al. 1997; Ueda and Sashida 1998; Yasuda et al. 1995). We previously established the efficient conversion process of nicotinic acid and 3-cyanopyridine into 6-hydroxynicotinic acid and 3-cyano-6-hydroxypyridine, respectively, by bacteria.

In contrast, studies on microbial conversion of pyridine dicarboxylic acids are limited solely to the degradation pathway of dipicolinic acid (pyridine-2,6-dicarboxylic acid), which is found in *Bacillus* spores (Slieman and Nicholson 2001). A possible degradation pathway via 3-hydroxydipicolinic acid is reported (Arima and Kobayashi 1962; Kobayashi and Arima 1962). However, there has been no comprehensive report on microbial degradation of pyridine dicarboxylic acids (Fetzner 1998; Schwarz and Lingens1994). In the present study, we found that some microorganisms degrade quinolinic acid (pyridine-2,3-dicarboxylic acid), lutidinic acid (pyridine-2,4-dicarboxylic acid) and isocinchomeronic acid (pyridine-2,5-dicarboxylic acid). We therefore applied these microbial cells to the synthesis of 6-hydroxypicolinic acid, 6-hydroxylutidinic acid and 6-hydroxyisocinchomeronic acid.

Materials and methods

Chemicals

Quinolinic acid, lutidinic acid and isocinchomeronic acid (see Fig. 1) were obtained from Wako Pure Chemical Industries (Japan). Yeast extract was purchased from the Oriental Yeast Co. (Japan). All other chemicals used in this work were of analytical grade and commercially available.

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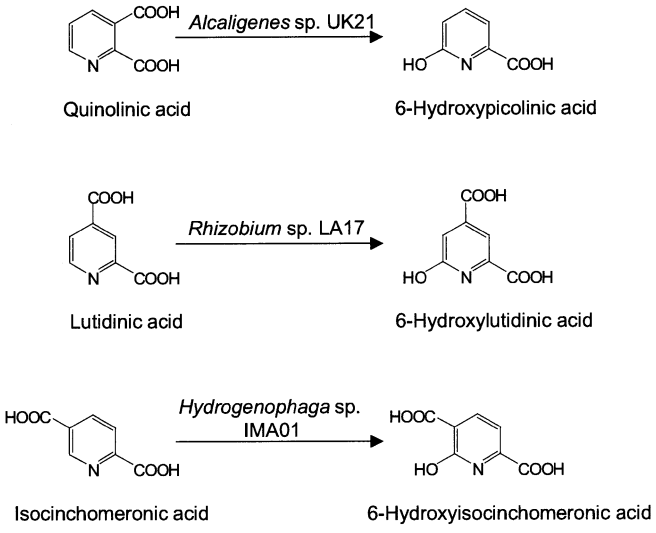

Fig. 1 Microbial aerobic degradation of quinolinic acid, lutidinic acid and isocinchomeronic acid

Medium and cultivation

For the isolation of quinolinic acid-, lutidinic acid- and isocinchomeronic acid-degrading microorganisms, conventional enrichment cultures were carried out aerobically at 28 °C in the following two media. Medium A contained 2.5 g pyridine dicarboxylic acid (either quinolinic acid, lutidinic acid or isocinchomeronic acid), 1 g KH_2PO_4, 3 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$ and 10 ml metal solution in 1 l tap water (pH 7.0). Medium B comprised medium A with 1% (w/v) NH_4Cl. Metal solution contained 400 mg $CaCl_2{\cdot}2H_2O$, 300 mg H_3BO_3, 40 mg $CuSO_4{\cdot}5H_2O$, 100 mg KI, 200 mg $FeSO_4{\cdot}7H_2O$, 400 mg $MnSO_4{\cdot}7H_2O$, 200 mg $Na_2MoO_4{\cdot}2H_2O$ and 10 ml conc. HCl in 1 l distilled water. Soils were used as the source to isolate pyridine dicarboxylic acid-degrading microorganisms.

For the preparation of resting cells, the following cultivation was carried out. The subculture was done at 28 °C for 1 day with reciprocal shaking in a test-tube containing 2 ml nutrient medium, consisting of 5 g peptone, 0.5 g yeast extract, 5 g meat extract and 2 g NaCl in 1 l tap water (pH 7.0). The subculture was transferred into a 500-ml shaking-flask containing 40 ml medium C; and the cultivation was done at 28 °C for 3 days. Medium C consisted of 2.5 g pyridine dicarboxylic acid, 1 g KH_2PO_4, 3 g K_2HPO_4, 1 g yeast extract, 0.5 g $MgSO_4{\cdot}7H_2O$ and 10 ml metal solution in 1 l tap water (pH 7.0).

The cell growth of *Alcaligenes* sp. UK21 was estimated turbidimetrically at 610 nm; and 0.650 mg dry cell weight ml^{-1} was equivalent to 1.0 unit of optical density at 610 nm. As for *Rhizobium* sp. LA17 and *Hydrogenophaga* sp. IMA01, 0.424 mg and 0.576 mg dry cell weight ml^{-1} were equivalent to 1.0 unit of optical density at 610 nm, respectively.

Identification of microorganisms

The identification of pyridine dicarboxylic acid-degrading microorganisms was carried out by the Deutsche Sammelung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Resting cells reaction

Cells harvested by centrifugation at 7,000 *g* at 4 °C for 20 min were suspended in 0.15 M NaCl and used as the resting cells. Cells from 30 ml culture broth were incubated with 50 µmol pyridine dicarboxylic acid and 250 µmol potassium phosphate buffer (pH 7.0) in a 2.0-ml reaction mixture. The reaction was carried out with reciprocal shaking (160 strokes min^{-1}) at 30 °C for 30 min and stopped by the addition of 2 ml methanol. The reaction product and remaining substrate were determined by HPLC on a Shimadzu LC-10AS and SPD-10A system with a Wakosil-II RS column (4.6×150 mm) purchased from Wako Pure Chemical Industries. A 96:4 (v/v) mixture of 10 mM potassium phosphate buffer (pH 2.5) and acetonitrile was used for elution. The flow rate was maintained at 1.0 ml min^{-1}; and the absorbance of eluates was monitored at 230 nm. Total activity was defined as the amount of hydroxylated product by resting cells per minute, derived from 1 ml culture broth.

Identification of reaction products

The reaction products from each pyridine dicarboxylic acid were isolated by a Dowex 1-X2 anion-exchange column and crystallized in distilled water. NMR spectrometries were analyzed with a Joel JNM-400 F17 NMR using DMSO-d_6. Mass spectrometry was analyzed with Quattoro-II (Micromass). 6-Hydroxypicolinic acid was a white needle crystalline solid [^{1}H-NMR (400 MHz, DMSO-d_6) gave: δ 3.46 (1H, s, OH), 6.57 (1H, d, J=9 Hz), 6.90 (1H, d, J=6.8 Hz), 7.48 (1H, dd, J=6.8 Hz) and 12.3 (1H, s, COOH); ^{13}C-NMR (100 MHz, DMSO-d_6) gave: δ 109.9, 123.4, 137.6, 140.0, 162.2 and 162.9; mass spectrometry gave m/z values of 139.9 ($M+H^+$, 100%) and 121.9 ($M–OH^+$, 5.7%)]. 6-Hydroxylutidinic acid was a white crystalline solid [^{1}H-NMR (400 MHz, DMSO-d_6) gave: δ 4.19 (1H, s, OH), 7.07 (1H, s), 7.33 (1H, s) and 12.3 (1H, s, COOH); ^{13}C-NMR (100 MHz, DMSO-d_6) gave: δ 109.0, 122.7, 139.8, 142.1, 162.8, 162.9 and 165.4; mass spectrometry gave m/z values of 181.8 ($M–H^-$, 100%) and 137.8 ($M–CO_2–H^-$, 5.2%)]. 6-Hydroxyisocinchomeronic acid was a yellowish-white crystalline solid [^{1}H-NMR (400 MHz, DMSO-d_6) gave: δ 3.48 (1H, s, OH), 7.19 (1H, s), 8.42 (1H, s) and 12.2 (1H, s, COOH); ^{13}C-NMR (100 MHz, DMSO-d_6) gave: δ 110.2, 120.4, 141.4, 145.7, 161.3, 164.4 and 164.6; mass spectrometry gave m/z values of 181.7 ($M–H^-$, 100%) and 137.7 ($M–CO_2–H^-$, 73.9%)].

Optimization of culture medium

To optimize the culture medium, the following carbon sources were added to medium C: glucose, lactose, sucrose, galactose, fructose, glycerol, sodium L-glutamate, sodium citrate and sodium fumarate. In order to optimize nitrogen sources in the culture medium, the following compounds were tested: yeast extract, corn steep liquor, casamino acids, NZ amine, malt extract, NH_4Cl, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$ and $NaNO_3$. Enzyme induction by the following compounds was examined: quinolinic acid, lutidinic acid, isocinchomeronic acid, nicotinic acid, 3-pyridinesulfonic acid, picolinic acid, 6-hydroxynicotinic acid, 2-hydroxynicotinic acid, 6-hydroxypicolinic acid, 3-hydroxypicolinic acid, isonicotinic acid, benzoic acid, salicylic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, *o*-phthalic acid, *m*-phthalic acid and *p*-phthalic acid. Cultivation was carried out at 28 °C for 3 days.

Results

Isolation of pyridine dicarboxylic acid-degrading microorganisms

Through enrichment culture using quinolinic acid, seven microorganisms degrading quinolinic acid were isolated. Among them, strain UK21 exhibited the highest activity and was identified as *Alcaligenes* sp. As for lutidinic acid-degrading microorganisms, six strains were isolated. Strain LA17 was selected as the most promising one to degrade lutidinic acid and was identified as *Rhizobium* sp.

The degradation of isocinchomeronic acid was found in only one isolated strain, *Hydrogenophaga* sp. IMA01.

Conversion of pyridine dicarboxylic acids by resting cells reaction

Resting cells reactions were carried out using quinolinic acid, lutidinic acid or isocinchomeronic acid as substrate; and the reaction products were identified as described in Materials and methods. The cells of *Alcaligenes* sp. UK21 converted quinolinic acid into 6-hydroxypicolinic acid. Therefore, *Alcaligenes* sp. UK21 catalyzed hydroxylation at position C6 and decarboxylation at position C3, resulting in the formation of 6-hydroxypicolinic acid (Fig. 1). The reaction product from lutidinic acid by the resting cells of *Rhizobium* sp. LA17 was 6-hydroxylutidinic acid. Isocinchomeronic acid was converted into 6-hydroxyisocinchomeronic acid by the cells of *Hydrogenophaga* sp. IMA01. Whereas quinolinic acid was hydroxylated and decarboxylated, lutidinic acid and isocinchomeronic acid were only hydroxylated at position C6. It might be plausible to conclude that the degradation of these pyridine dicarboxylic acids was initiated by the hydroxylation reaction at position C6.

Optimization of culture conditions

To increase the hydroxylation activity of pyridine dicarboxylic acid-degrading microorganisms, the culture media were optimized. The hydroxylation activities of quinolinic acid, lutidinic acid or isocinchomeronic acid were measured by resting cells reaction. The addition of 1% (w/v) galactose and 1% (w/v) yeast extract was effective on the growth of *Alcaligenes* sp. UK21, resulting in an enhancement of the formation of 6-hydroxypicolinic acid from quinolinic acid. The 6-hydroxypicolinic acid-producing activity of the bacterium was induced by quinolinic acid and 6-hydroxypicolinic acid. The reaction product, 6-hydroxypicolinic acid, was a more effective inducer (41.2 nmol min^{-1} mg^{-1} dry cell weight) than quinolinic acid (24.0 nmol min^{-1} mg^{-1} dry cell weight). The highest activity was observed after 60 h cultivation.

The culture conditions for *Rhizobium* sp. LA17 and *Hydrogenophaga* sp. IMA01 were also examined. The optimized medium for *Rhizobium* sp. LA17 comprised 4 g lutidinic acid, 1 g KH_2PO_4, 3 g K_2HPO_4, 15 g yeast extract, 0.5 g $MgSO_4 \cdot 7H_2O$ and 10 ml metal solution in 1 l tap water, pH 7.0. The lutidinic acid-producing activity of *Rhizobium* sp. LA17 reached a maximum after 60 h cultivation. The 6-hydroxyisocinchomeronic acid-producing activity of *Hydrogenophaga* sp. IMA01 was highest after 18 h cultivation in the following medium: 2 g isocinchomeronic acid, 1 g KH_2PO_4, 3 g K_2HPO_4, 5 g yeast extract, 5.0 g malt extract, 0.5 g $MgSO_4 \cdot 7H_2O$ and 10 ml metal solution in 1 l tap water, pH 7.0. The hydroxylation activities for lutidinic acid and isocinchomeronic acid were induced only in the presence of lutidinic acid and isocinchomeronic acid, respectively. Other pyridine-related compounds tested were inert as the inducer.

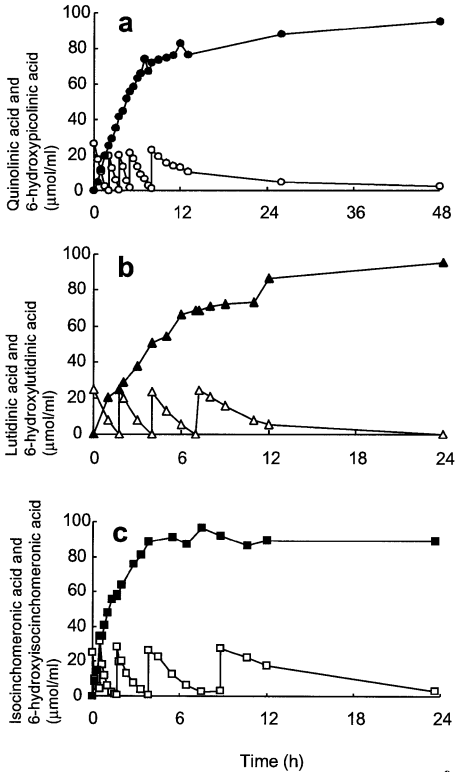

Fig. 2 Accumulation of 6-hydroxypicolinic acid (**a**), 6-hydroxylutidinic acid (**b**) and 6-hydroxyisocinchomeronic acid (**c**). The reaction mixture contained resting cells, 250 μmol each pyridine dicarboxylic acid and 1,250 μmol Tris-HCl (optimized pH) in 10 ml with shaking at 30 °C. Then, 100 mg (**a**), 254 mg (**b**) and 300 mg (**c**) resting cells (as dry weight) were added to the reaction mixture, respectively. *White circles* Quinolinic acid, *black circles* 6-hydroxypicolinic acid, *white triangles* lutidinic acid, *black triangles* 6-hydroxylutidinic acid, *white squares* isocinchomeronic acid, *black squares* 6-hydroxyisocinchomeronic acid

Synthesis of 6-hydroxypicolinic acid, 6-hydroxylutidinic acid and 6-hydroxyisocinchomeronic acid

For the synthesis of 6-hydroxypicolinic acid, 6-hydroxylutidinic acid and 6-hydroxyisocinchomeronic acid by the cells of pyridine dicarboxylic acid-degrading microorganisms, the resting cells reactions were optimized. The effects of pH, temperature and aeration were examined. With the resting cells of *Alcaligenes* sp. UK21, the highest amount of 6-hydroxypicolinic acid accumulated from quinolinic acid when the reaction was carried out in 75 mM Tris-HCl buffer (pH 7.5) at 35 °C with reciprocal shaking at 160 strokes min^{-1}. As shown in Fig. 2a, 94.9 mM 6-hydroxypicolinic acid (13.2 g l^{-1}) was produced from 105 mM quinolinic acid after 48 h, with a 91% molar conversion yield. Optimum conditions for

Table 1 Substrate specificity. Resting cells reactions using each strain were carried out under optimized conditions. The activity was calculated from the amount of product

Compound	Activity (nmol min^{-1} mg^{-1} dry cell weight)		
	Alcaligenes sp. UK21	*Rhizobium* sp. LA17	*Hydrogenophaga* sp. IMA01
Quinolinic acid	24.0	0	0
Lutidinic acid	0	14.8	0
Isocinchomeronic acid	0	0	43.7

the synthesis of 6-hydroxylutidinic acid and 6-hydroxyisocinchomeronic acid were also examined using *Rhizobium* sp. LA17 and *Hydrogenophaga* sp. IMA01, respectively. The cells of both strains showed their highest activity at 35 °C in 75 mM Tris-HCl buffer (pH 8.5) with reciprocal shaking at 160 strokes min^{-1}. Under optimized conditions, the resting cells of *Rhizobium* sp. LA17 accumulated up to 95.4 mM 6-hydroxylutidinic acid (17.5 g l^{-1}), with a 99% molar conversion yield (Fig. 2b). The resting cells of *Hydrogenophaga* sp. IMA01 accumulated 88.7 mM 6-hydroxyisocinchomeronic acid (16.2 g l^{-1}), with a 81% molar conversion yield (Fig. 2c).

Substrate specificity

The substrate specificities of three pyridine dicarboxylic acid-degrading bacteria were examined by the resting cells reaction (Table 1). The following heterocyclic and aromatic compounds were inert as the substrate: nicotinic acid, 3-pyridinesulfonic acid, picolinic acid, 6-hydroxynicotinic acid, 2-hydroxynicotinic acid, 6-hydroxypicolinic acid, 3-hydroxypicolinic acid, isonicotinic acid, benzoic acid, salicylic acid, pyridine-3,4-dicarboxylic acid, pyridine-3,5-dicarboxylic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, *o*-phthalic acid, *m*-phthalic acid and *p*-phthalic acid.

Discussion

We isolated the bacteria assimilating pyridine dicarboxylic acids, and characterized the initial reactions in each case. Pyridine dicarboxylic acid-degrading bacteria were applied to produce 6-hydroxypicolinic acid, 6-hydroxylutidinic acid and 6-hydroxyisocinchomeronic acid, which might be used for the synthesis of biologically active compounds. The resting cells of *Alcaligenes* sp. UK21, *Rhizobium* sp. LA17 and *Hydrogenophaga* sp. IMA01 accumulated 6-hydroxypicolinic acid, 6-hydroxylutidinic acid and 6-hydroxyisocinchomeronic acid, respectively, with high molar conversion yields. Further conversion of these reaction products was barely observed in the resting cells reaction. In our previous studies on the conversion of nicotinic acid into 6-hydroxynicotinic acid by *Pseudomonas fluorescens* and *Serratia marcescens*, nicotinic acid strongly inhibited the activity of 6-hydroxynicotinic acid 3-monooxygenase (Hurh et al. 1994a; Nagasawa et al. 1994). Therefore, the producing system of 6-hydroxynicotinic acid with a high conversion yield was established. The accumulation of the hydroxylated compounds in our present study suggests that enzymes degrading the accumulated compounds might be inhibited by quinolinic acid, lutidinic acid and isocinchomeronic acid, as in the case of nicotinic acid.

In the resting cells reaction, *Alcaligenes* sp. UK21 catalyzed decarboxylation at position C3 together with hydroxylation at position C6. We suspect that the hydroxylation and decarboxylation do not proceed at the same time. Probably, the intermediate of 6-hydroxypicolinic acid production from quinolinic acid is 6-hydroxyquinolinic acid, but not picolinic acid. When picolinic acid was incubated with the resting cells of *Alcaligenes* sp. UK21 cultivated in medium containing quinolinic acid as the inducer, the formation of 6-hydroxypicolinic acid was not observed at all. Thus, the first step of 6-hydroxypicolinic acid production from quinolinic acid is assumed to be the hydroxylation of quinolinic acid; and the resulting 6-hydroxyquinolinic acid is decarboxylated in a second step.

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