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The Metabolic Pathway of 4-Aminophenol in *Burkholderia* sp. Strain AK-5 Differs from That of Aniline and Aniline with C-4 Substituents

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4-Aminophenol has highly toxic and mutagenic effects and induces DNA cleavage in mouse and human lymphoma cells (12, 22). This compound is an intermediate in the degradation of hydroxyacetanilide (7) and azo dyes (19). However, little is known about the metabolism of 4-aminophenol by bacteria (1). 3-Nitrophenol-grown cells of *Ralstonia eutropha* JMP 134 convert nitrobenzene to hydroxylaminobenzene, 2-aminophenol, and 4-aminophenol (16). Hydroxylaminobenzene is transformed by 3-nitrophenol-grown cells of *Pseudomonas putida* 2NP8 to 1,4-benzenediol via 4-aminophenol (25). A number of reports indicate that 4-aminophenol might be a key intermediate in the biodegradation of nitrobenzenes and amines (7, 19, 25). Our aim was to elucidate a biodegradation pathway for 4-aminophenol by analyzing metabolites.

Here we report the isolation of a 4-aminophenol-assimilating bacterium and propose a metabolic pathway for 4-aminophenol. In addition, the characterization of a 1,2,4-trihydroxybenzene 1,2-dioxygenase from strain AK-5 is described.

**MATERIALS AND METHODS**

**Organism and growth conditions.** Strain AK-5 was enriched from rice field soil from the Hyogo Prefecture. The basal medium containing 4-aminophenol was prepared by methods described previously (3). Succinate-glucose medium was a modified basal medium containing 1.0% (wt/vol) sodium succinate, 1.0% (wt/vol) D-glucose, and 0.04% (wt/vol) NH₄NO₃ as the sole carbon and nitrogen sources instead of 4-aminophenol.

**Purification of 1,2,4-Trihydroxybenzene 1,2-Dioxygenase.** 1,2,4-Trihydroxybenzene 1,2-dioxygenase activity was assayed by the method of Latus et al. (10). The molar extinction coefficient of 4.44 × 10³ at 243 nm for maleylacetic acid was used (20). Protein concentrations were measured by the method of Lowry et al. (11).

Cells (25 g [wet weight]) of strain AK-5 were suspended in 20 mM Tris-HCl (pH 8.0) (buffer A). Cell extract (fraction 1) was prepared and treated with streptomycin sulfate (fraction 2) as described previously (3). Fraction 2 was fractionated with ammonium sulfate (32 to 50% saturation). After centrifugation (20,000 × g for 10 min), the pellet was dissolved in buffer A. The solution was dialyzed against buffer A (fraction 3, 90 ml). Fraction 3 was applied to a DE52 cellulose column (2.1 by 26 cm), and proteins were eluted with a linear gradient (0 to 0.4 M NaCl) at a flow rate of 40 ml h⁻¹. The active fractions were pooled (fraction 4; 60 ml). Fraction 4 was applied to a DEAE-Cellulofine A-800 column (2.0 by 15 cm), and proteins were eluted with a linear gradient (0 to 0.35 M) of NaCl at a flow rate of 30 ml h⁻¹. The active fractions were pooled (fraction 5; 30 ml). Fraction 5 was applied to a phenyl-Cellulofine column (1.6 by 7.5 cm), and proteins were eluted with a linear gradient (0.5 to 0 M) of (NH₄)₂SO₄ at a flow rate of 30 ml h⁻¹. The active fractions were pooled (fractions 6; 28 ml).

**Effect of various compounds on enzyme activity.** The effect of metal salts and chelating and sulfhydryl agents on enzyme activity with 1,2,4-trihydroxybenzene was monitored spectrophotometrically. Inhibition of the enzyme activity by substrate analogues was examined by incubating the enzyme (25 μg ml⁻¹) with each analogue (0.05 mM) in 3 ml of 100 mM sodium-potassium phosphate buffer (pH 7.5) at 24°C for 1 min. The enzyme reaction was started by adding 1,2,4-trihydroxybenzene.

**Production and isolation of metabolites.** The reaction mixture contained 84 ml of 100 mM sodium-potassium phosphate buffer (pH 7.5), 3.0 ml of cell suspension (0.57 mg [dry weight] of cells ml⁻¹), and 3 ml of 10 mM 4-aminophenol.

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TABLE 1. Purification of 1,2,4-trihydroxybenzene 1,2-dioxygenase from Burkholderia sp. strain AK-5

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Sp act (U·mg⁻¹)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (cell extract)</td>
<td>340,200</td>
<td>0.12</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2 (streptomycin sulfate)</td>
<td>240,700</td>
<td>0.09</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>3 (ammonium sulfate)</td>
<td>230,1400</td>
<td>0.16</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>4 (DE52)</td>
<td>80,150</td>
<td>0.53</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>5 (DEAE-Cellulofine A-800)</td>
<td>30,20</td>
<td>1.5</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>6 (Phenyl-Cellulofine)</td>
<td>6.0</td>
<td>5.5</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>7 (Cellulofine GCL-1000 sf)</td>
<td>3.0, 0.14</td>
<td>0.9</td>
<td></td>
<td></td>
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*Fractions 1 to 7 refer to the fractions obtained at the end of steps 1 to 7, respectively, of the purification procedure. See the text for details.

After incubation with shaking at 30°C for 5 min, cells were broken by ultrasonic disintegration. The solution was then adjusted to pH 3.0 with 3 N HCl, and precipitated proteins were removed by centrifugation at 20,000 g × 10 min. The supernatant was concentrated with an evaporator to 20 ml and then extracted with ethyl acetate. The upper layer was recovered and evaporated to dryness. The accumulated products reacted with 

**RESULTS AND DISCUSSION**

Identification of a 4-aminophenol- assimilating bacterium. Strain AK-5 is a motile rod of 0.8 to 1.2 by 2.4 to 3.6 μm with polar flagella. It is aerobic, gram negative, and non spore forming, and catalase and oxidase positive. It produces acid oxidatively from D-glucose, D-fructose, D-sorbitol, D-mannitol, lactose, maltose, and sucrose. It does not produce H2S, indole, or acetoin and does not hydrolyze gelatin. The nucleotide sequence of the 16S rRNA gene of strain AK-5 (1,520 bp; accession no. AB103080) was 97.9 and 98.1% identical to that of Burkholderia sp. strain NF100 (AB025790) and Burkholderia sp. strain S4.9 (AF247496), respectively (6, 8). Thus, strain AK-5 was identified as a species of *Burkholderia*.

Strain AK-5 grew well on 4-aminophenol as the sole carbon, nitrogen, and energy source (Fig. 1). 4-Aminophenol was rapidly degraded during the exponential phase. The consumption of 4-aminophenol correlated with an increase in cell density and protein content. High concentrations of 4-aminophenol (>18.0 mM) inhibited growth. Strain AK-5 grew well at pHs from 5 to 6.5 and poorly at pHs >6.5.

**Purification and properties of the purified dioxygenase.** The 1,2,4-trihydroxybenzene 1,2-dioxygenase from strain AK-5 was present in cell extracts of 4-aminophenol-grown cells but not in cell extracts of succinate-glucose-grown cells; therefore, the synthesis of the enzyme was inducible. The enzyme was purified 108-fold, with an overall yield of 0.9% (Table 1). The apparent molecular mass was determined to be 85 kDa by gel filtration, and the molecular mass was determined to be 81 kDa by SDS-PAGE, which indicated that the enzyme is a monomer.

After DE52 chromatography, the dioxygenase from strain AK-5 was stable for several weeks in buffer A containing 250 mM NaCl. The dioxygenase from *Burkholderia cepacia* AC1100 is also stable at a high salt concentration (5). The enzyme from strain AK-5 maintained more than 100% activity after a 10-min incubation at temperatures up to 50°C and showed maximal activity at pH 7.0. The enzyme had a high activity only for 1,2,4-trihydroxybenzene, with $K_m$ and $V_{max}$ values of 9.6 μM and 6.8 μmol·min⁻¹·mg of protein⁻¹, respectively. Such a remarkably narrow substrate specificity is shared with the dioxygenase from *Trichosporon cutaneum* (17).

Among the substrate analogues tested, 1,4-benzenediol, 1,4-methylcatechol, and 4-chlorocatechol decreased the enzyme activity to 10% and 25%, respectively. Among the metal salts tested, the enzyme was completely inhibited by 1 mM HgCl₂, 1 mM MgSO₄, and 1 mM AgNO₃. The addition of 1 mM α,α’-dipyridyl, EDTA, or phenanthroline, or Na₂S decreased the enzyme activity to 48, 49, 0, and 25%, respectively.

**TABLE 2. Mass spectra of the metabolites from 4-aminophenol and reaction product from 1,2,4-trihydroxybenzene**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fragments of the trimethylsilylated product (m/z, assignment, relative intensity [%])</th>
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<tbody>
<tr>
<td>II (1,4-benzenediol)</td>
<td>254 (M⁺, 92.3), 239 (M⁺−CH₂OH, 100), 147 ([CH₃]₂O−OSi(CH₃)₃)⁺, 20, 135 (M⁺−O−Si(CH₃)₃, 2.0, 35.5)</td>
</tr>
<tr>
<td>III (1,2,4-trihydroxybenzene)</td>
<td>342 (M⁺, 31.4), 327 (M⁺−CH₂OH, 2.4), 239 (M⁺−Si(CH₃)³, 100), 147 ([CH₃]₂O−OSi(CH₃)₃)⁺, 1.7, 73 (Si(CH₃)₃)⁺, 22.3)</td>
</tr>
<tr>
<td>IV (maleylactic acid)</td>
<td>374 (M⁺, 0.8), 359 (M⁺−CH₂OH, 9.9), 315 (M⁺−CH₂−CO₂, 2.1), 257 (M⁺−COOSi(CH₃)₃, 100), 241 (1.37), 219 (1.3) 197 (1.0) 147 ([CH₃]₂O−OSi(CH₃)₃)⁺, 2.2, 73 (Si(CH₃)₃)⁺, 43.3)</td>
</tr>
</tbody>
</table>
**Proposed pathway of 4-aminophenol metabolism.**

4-Aminophenol (0.20 mM) was degraded, with elimination of ammonia (0.17 mM), by 4-aminophenol-grown whole cells of strain AK-5. Metabolites were analyzed by GC and GC-MS. Tri-methylsilylated 4-aminophenol (M^+ = 253) had a GC retention time of 10.7 min. Major peaks at 8.6 and 12.1 min were also observed. The mass spectra (Table 2) and the GC retention times (R^t) of compounds II and III agreed with those of the trimethylsilylated authentic 1,4-benzenediol (R^t = 8.6 min) and 1,2,4-trihydroxybenzene (R^t = 12.1 min), respectively (Fig. 2). The enzymatic reaction product derived from 1,2,4-trihydroxybenzene showed an absorption peak at 243 nm. The mass spectrum of the trimethylsilylated reaction product (compound IV) is in agreement with that of maleylactic acid (Table 2) (15).

The oxygen uptake rates of 4-aminophenol-grown whole cells with 4-aminophenol, 1,4-benzenediol, and 1,2,4-trihydroxybenzene were 22, 10, and 12 μmol min^{-1} mg of protein^{-1}, respectively. In contrast, the oxygen uptake rates of succinate-glucose-grown whole cells with these compounds were less than 1 μmol min^{-1} mg of protein^{-1}. The oxygen uptake rates of 4-aminophenol- and succinate-glucose-grown whole cells with phenol, catechol, or 1,4-benzoquinone were less than 1 μmol min^{-1} mg of protein^{-1}. These results indicated that the enzymes responsible for 4-aminophenol, 1,4-benzenediol, and 1,2,4-trihydroxybenzene metabolism were induced in 4-aminophenol-grown cells.

Figure 2a shows the proposed metabolic pathway of 4-aminophenol in strain AK-5. 4-Aminophenol was converted to 1,2,4-trihydroxybenzene via 1,4-benzenediol; 1,2,4-trihydroxybenzene 1,2-dioxygenase catalyzed the conversion of 1,2,4-trihydroxybenzene to maleylactic acid. Presumably, the benzene ring of 4-aminophenol is subjected to two hydroxylation steps to yield 1,2,4-trihydroxybenzene. The proposed pathway differs from previously reported metabolic pathways for aniline and anilines with a methyl-, chloro-, sulfo-, or carboxy-functional-group substituent at the C-4 position (Fig. 2b) (2, 4, 13, 24). The initial reaction in the degradation of anilines is catalyzed by a dioxygenase and yields the corresponding 1-amin-2-hydrodiols as the first metabolites. Subsequent oxidation of 1-amin-2-hydrodiols leads to the formation of the catechols. The dioxygenation and dehydrogenation steps to form catechols in these species are similar irrespective of which functional group at the C-4 position of aniline is the electron donor or electron acceptor.

Hughes et al. (9) have reported that, in *Pseudomonas putida* strain TW3, 4-hydroxylaminobenzoate lyase converts 4-hydroxylaminobenzoate to protocatechuate, replacing the amino group by a hydroxyl group. Likewise, strain AK-5 could possibly require lyase activity in the initial step of 4-aminophenol metabolism, which we propose to be the direct conversion of 4-aminophenol to hydroquinone.

When the purified enzyme was added to a reaction mixture containing 1,2,4-trihydroxybenzene, the absorption peak at 243 nm, corresponding to maleylactic acid, and the absorption peak at 260 nm, arising from the auto-oxidation of 1,2,4-trihydroxybenzene (23), increased slowly. In contrast, the auto-oxidation product did not accumulate when cell extract and the (NH_4)_2SO_4 fraction were used in the assay. The cell extract might contain enzymes that inhibit the nonenzymatic reaction or that reduce the product. We are currently investigating enzymes involved in the transformation of the auto-oxidation product.

**REFERENCES**

2. Aoki, K., R. Shinike, and H. Nishira. 1983. Metabolism of aniline by Rhodo-
acetanilide) and other substituted acetanilides by a Penicillium species. An-
tonic Leeuwenhoek. 41:239–247.
14. Norwitz, G., and N. Keliiher. 1982. Spectrophotometric determination of aromatic amines by the diazotization-coupling technique with 8-amino-1-
hydroxynaphthalene-3,6-disulfonic acid and N-(1-naphthyl)ethylenediamine as the coupling agents. Anal. Chem. 54:807–808.
15. Rieble, S., D. K. Joshi, and M. H. Gold. 1994. Purification and characteri-
tization of a 1,2,4-trihydroxybenzene 1,2-dioxygenase from the basidiomycete Phanerochaete chrysosporium. J. Bacteriol. 176:4838–4844.
17. Sze, I. S. Y., and S. Dagley. 1984. Properties of salicylate hydroxylase and hydroxyquinol 1,2-dioxygenase purified from Trichosporon cutaneum. J. Bacteri-
el 159:353–359.
21. Werber, K., and M. Osborn. 1969. The reliability of molecular weight de-