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Microbial Transformation of Aniline Derivatives: Regioselective Biotransformation and Detoxification of 2-Phenylenediamine by *Bacillus cereus* Strain PDa-1

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A bacterial isolate, strain PDa-1, grew well on basal medium supplemented with 2-phenylenediamine, sucrose, and ammonium nitrate and completely transformed 2-phenylenediamine. The isolate was identified as *Bacillus cereus*. The product formed from 2-phenylenediamine was identified by EI-MS and NMR as 2-aminoacetanilide; whole cells converted 2-phenylenediamine to the product with a 76% molar yield. Whole cells also showed a broad substrate specificity toward 20 of 26 tested arylamines with substituent groups of various size and positions. Especially 2-aminobenzoic acid, 4-aminosalicylic acid, 5-aminosalicylic acid, and 2-aminofluorene were converted completely to the corresponding product with an aminoacetyl group. Cell extracts of strain PDa-1 had a high arylamine *N*-acetyltransferase activity. The partially purified enzyme converted 2-phenylenediamine to 2-aminoacetanilide. Strain PDa-1 constitutively expressed the enzyme in the absence of 2-phenylenediamine. Effects of 2-phenylenediamine and 2-aminoacetanilide on growth indicated that this enzyme probably plays a role in the detoxification of toxic arylamines in this strain.

[**Key words:** *Bacillus cereus*, 2-phenylenediamine, 2-aminoacetanilide, arylamine, arylamine *N*-acetyltransferase]

Aniline and its derivatives are major industrial raw materials and intermediates used for the production of dyes, plastics, pesticides and herbicides. The compounds are released into the environment both accidentally and through routine disposal by waste-water facilities. They can then be transformed to more toxic aromatic compounds by microbial, physical, and chemical processes (1). Bacteria not only mineralize anilines via the corresponding catechol derivatives (2, 3), but also transform them through acetylation (4) and *N*-oxidation (5).

Phenylenediamines and aniline are listed as harmful chemical substances in the Pollutants Release and Transfer Register (PRTR) system of the Ministry of Economy, Trade, and Industry in Japan (http://www.meti.go.jp/policy/chemical_management/law/). Phenylenediamines have highly toxic and mutagenic effects (6). These compounds are the main metabolites of a bactericidal substance or an azo-compound. For example, in a liquid culture, *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) converts the fungicide 2,6-dichloro-4-nitroaniline to 2,6-dichloro-*p*-phenylenediamine (7) and a methanogenic granular sludge converts the azo dye acid orange 52 to sulfanilic acid and *N,N*-dimethyl-1,4-phenylenediamine, which is then transformed to 1,4-phenylenediamine (8). To our knowledge the transformation of phenylenediamines by soil microorganisms has not yet been observed. We therefore attempted to isolate soil microorganisms that can mineralize or metabolize 2-, 3-, or 4-phenylenediamine and to reveal the metabolic fate of the phenylenediamines.

Here we report the isolation of a strain of *Bacillus cereus* able to transform 2-phenylenediamine and other arylamines. The enzyme involved in the transformation, arylamine *N*-acetyltransferase (NAT), was characterized, and the effect of various factors on growth and synthesis of the enzyme were studied.

MATERIALS AND METHODS

Bacterial strain and growth conditions Phenylenediamine-transforming bacteria were enriched from rice-field soil from the Hyogo Prefecture using basal medium containing 0.1% (w/v) phenylenediamine·2HCl with a 2-, 3-, or 4-substitution, 1.0% (w/v) sucrose, and 0.15% (w/v) NH_4NO_3 (phenylenediamine medium). Solutions of the components were prepared separately and mixed according to methods described previously (9). Briefly, solution A contained 2 g sucrose as an additional carbon source and 0.3 g NH_4NO_3 as an additional nitrogen source in 100 ml; the final pH was adjusted to pH 5.5 or 6.8. Solution B contained metal ions. Solution C contained 0.2 g 2-, 3-, or 4-phenylenediamine·2HCl and 0.6 g $Na_2HPO_4 \cdot 12H_2O$ in 70 ml; the final pH was adjusted to pH

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5.5 or 6.8.

Of 25 isolated phenylenediamine-transforming bacteria, strain PDa-1 grew well on 0.1% (w/v) 2-phenylenediamine medium (pH 6.8) and transformed this substrate almost completely. Strain PDa-1 was selected for further study and was maintained on 2-phenylenediamine medium (pH 6.8) supplemented with 1.0% (w/v) Polypepton (Wako Pure Chemicals, Osaka) instead of sucrose and NH_4NO_3. Whole cells (resting cells) and cells used for the purification of NAT were cultured in 110 ml of 2-phenylenediamine medium (pH 6.8) in a 500-ml flask for 18 h at 30°C with shaking.

Identification of strain PDa-1 Strain PDa-1 was identified according to morphological and biochemical characteristics, such as the Gram reaction, flagella type, catalase activity, oxidase activity, and the OF test using methods described previously (10), and by 16S rRNA gene sequence analysis. Total DNA of strain PDa-1 was prepared as reported previously (11). The 16S rRNA gene of strain PDa-1, corresponding to a region between positions 8 and 1542 in the gene of *Escherichia coli*, was amplified using a previously reported method (12) and sequenced using the Thermo Sequenase Primer Cycle sequencing kit with 7-deaza-dGTP (Amersharm Pharmacia Biotech, Buckinghamshire, UK) and a Shimadzu DSQ-2000L DNA analyzer (Shimadzu, Kyoto). The partial nucleotide sequence of the 16S rRNA gene of strain PDa-1 reported here was deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB247137.

Isolation of the transformed product of 2-phenylenediamine Whole cells of strain PDa-1 grown in 2-phenylenediamine medium were suspended to an OD_{660} of 30 (11.7 mg cells [dry wt.] ml^{-1}) in 100 ml of a reaction mixture containing 0.3 g KH_2PO_4, 1.0 g $Na_2HPO_4 \cdot 12H_2O$, 0.05 g NaCl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.05 mg each of $CaCl_2 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, and $ZnCl_2$, 0.091 g 2-phenylenediamine·2HCl (final concentration 5 mM), and deionized water at pH 7.5 and incubated with shaking at 30°C for 20 h. After centrifugation (20,000×*g* for 10 min), the supernatant was concentrated with an evaporator to 5 ml and then extracted with ethyl acetate. The upper layer was recovered and evaporated to dryness. The compound was purified by TLC on a silica gel $60F_{254}$ aluminum sheet (Merck, Darmstadt, Germany) using ethyl acetate as the solvent. The purified compound (8.8 mg) was analyzed by DI-MS, GC-MS, and NMR as described below.

Enzyme assay Arylamine *N*-acetyltransferase (NAT) activity was assayed by monitoring the increase of 2-aminoacetanilide from 2-phenylenediamine using HPLC as described below. The reaction mixture contained 220 µl of 50 mM sodium-potassium phosphate buffer (pH 7.1), 10 µl of 30 mM EDTA·2Na, 10 µl of 30 mM dithiothreitol, 10 µl of 5 mM 2-phenylenediamine, and 60 µl of enzyme solution. After 5 min pre-incubation at 30°C, the reaction was started by adding 20 µl of 10 mM acetyl-CoA; the mixture was incubated at 30°C for 1 h. The reaction was stopped by adding 30 µl of acetonitrile. One unit of NAT was defined as the amount of enzyme that catalyzed the formation of 1 µmol of the product per hour under the assay conditions. Protein concentrations were measured using the method of Lowry *et al*. (13).

Purification of NAT Cells (23.8 g [wet wt.]) of strain PDa-1 were harvested from 4.4 *l* of 2-phenylenediamine medium. The cells were suspended in 20 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA and 1 mM dithiothreitol (buffer A). Cell extract (fraction 1) was prepared and treated with streptomycin sulfate (fraction 2) to remove nucleic acids as described previously (9). Fraction 2 was fractionated with ammonium sulfate (50–80% saturation). After centrifugation (20,000×*g* for 10 min), the pelleted precipitate was dissolved in buffer A (fraction 3). After dialysis, the fraction was applied to a DE52 cellulose column (1.5×17.5 cm), and proteins were eluted with a linear gradient (0 to 0.4 M NaCl). The pooled active fractions (fraction 4, 27 ml) were dialyzed and applied to a POROS 50HQ column (0.46×10.0 cm; PerSeptive Biosystems, Cambridge, MA, USA) and proteins were eluted with a linear gradient (0 to 0.5 M) using a BioCAD Workstation system (PerSeptive Biosystems). $(NH_4)_2SO_4$ (final concentration 2.0 M) was added to the pooled active fractions (fraction 5, 30 ml), and the mixture was applied to a POROS 20PE column (0.46×1.0 cm; PerSeptive Biosystems); proteins were eluted with a linear gradient (2.0 to 0 M) of $(NH_4)_2SO_4$ using a BioCAD Workstation system. The pooled active fractions (fraction 6, 36 ml) were concentrated and loaded onto a Toyopearl HW-55F column (2.0×97 cm); proteins were eluted with buffer A containing 0.2 M NaCl. The enzyme purity was checked by SDS–PAGE (14).

Effects of various factors on growth and the transformation of 2-phenylenediamine Strain PDa-1 was pre-cultured in 7 ml of 2-phenylenediamine medium in a test tube at 30°C for 24 h with shaking. Six ml of the pre-culture was used to inoculate test medium in a 500-ml flask. All experiments were done in triplicate and average values are shown.

Effects of aeration Strain PDa-1 was cultured in a 500-ml flask containing 50, 70, or 110 ml of 2-phenylenediamine medium with shaking at 140 rpm until the late-exponential phase. Cells were harvested from each culture, and NAT activity was assayed.

Effects of carbon source Test media (110 ml) contained one of six sugars, one of five sugar alcohols, or one of nine organic acids instead of sucrose. Cultures were sampled every 4 h to measure growth, the remaining 2-phenlyenediamine, and the accumulated 2-aminoacetoanilide.

Effects of nitrogen source Test media (110 ml) contained sodium nitrate, ammonium chloride, urea, or one of 20 amino acids instead of ammonium nitrate. The culture was sampled every 4 h to measure growth, the remaining 2-phenlyenediamine, and the accumulated 2-aminoacetoanilide.

Effects of the concentration of 2-phenylenediamine and its product The test medium (7 ml in a test tube) contained 2-phenylenediamine or 2-aminoacetanilide at a concentration ranging between 0 to 15 mM. After 18 h of incubation, cultures were sampled to measure growth, the remaining 2-phenylenediamine, and 2-aminoacetoanilide.

Induction of NAT Strain PDa-1 was cultured in 2-phenylenediamine medium, the medium containing arylamine (2-aminobenzoic acid, 4-aminosalicylic acid, 5-aminosalicylic acid, or 2-aminofluorene) instead of 2-phenylenediamine, and the medium containing 1.0% (w/v) sucrose and 0.15% (w/v) NH_4NO_3 and lacking 2-phenylenediamine for 24 h at 30°C with shaking. Cells were harvested from the culture and NAT activity was assayed.

Substrate specificity Since we could not obtain enough purified NAT to examine the substrate specificity of the enzyme, whole cells of strain PDa-1 were used instead. Twenty-six arylamines (see Table 2) were tested in the same reaction mixture used for the identification of the product from 2-phenylenediamine. The remaining arylamine was measured spectrophotometrically using a diazo coupling reaction (15) and HPLC as described below. Each accumulated product was extracted with ethyl acetate and analyzed by MS as described below.

Products (5.5 and 6.5 mg) from 4-aminosalicylic acid and 5-aminosalicylic acid were also isolated from each resting cell reaction mixture (total volume 10 ml) using the procedures described above and analyzed by DI-MS, GC-MS, and NMR.

Analytical methods UV absorption spectra of reaction products were recorded with a Beckman DU 650 spectrophotometer. The dissolved oxygen in the culture was measured with a dissolved oxygen meter (model DO-25P·DKK-TOA Corporation, Tokyo); the initial dissolved oxygen concentration was 6.3 mg l^{-1}. Products of arylamines were analyzed with a Hitachi M-2500 mass spectrometer (direct inlet mode, DI) with electron-impact (EI) ionization (70 eV). ^{1}H- and ^{13}C-NMR spectra of the product from 2-phe-