Biodegradation of Carbazole by *Pseudomonas* spp. CA06 and CA10

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Two bacterial strains, CA06 and CA10, that assimilate carbazole (CAR) as the sole source of carbon and nitrogen were isolated from 202 farm soil samples and 4 activated sludge samples, and identified as *Pseudomonas* spp. Growth conditions for strains CA06 and CA10 on CAR were examined. Anthranilic acid (AN) and catechol (CAT) were identified as the main metabolites of CAR by high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). When strains CA06 and CA10 were cultivated in a medium containing 17 mM CAR, 1.4 mM AN, and 0.1 mM CAT were accumulated in the culture broth, but AN disappeared after 140 h of incubation. An initial oxidation product, 2'-aminobiphenyl-2,3-diol, and a *meta*-cleavage product, 2-hydroxy-6-oxo-6-(2'-aminophenyl)hexa-2,4-dienoic acid, were tentatively identified in the culture broth of CAR by GC-MS. When AN was used for a substrate in culture by these strains, CAT and a small amount of cis,cis-muconate was detected by HPLC. This conversion suggested the existence of an ortho-cleavage. The activities of the *meta*-cleavage enzymes for biphenyl-2,3-diol (the initial oxidation intermediate analog of 2'-aminobiphenyl-2,3-diol), 3-methylcatechol, and CAR were measured using the crude cell extracts of CAR- and AN-grown cells. The *meta*-cleavage enzymes of two strains for biphenyl-2,3-diol was induced during the growth on CAR, but not induced by AN. Based on these results, a CAR degradation pathway is proposed.

Serious contamination of ground water by hazardous chemicals is of environmental interest because of the importance of ground water as a natural resource. Several azaarenes, including carbazole, quinoline, and other nitrogen-containing aromatic carbons have been found in ground water.1) Nestler characterized the major compounds of a coal tar distillate, which included polynuclear aromatic hydrocarbons, nitrogen-containing aromatic compounds, and oxygen-containing and sulfur-containing aromatic compounds.2) CAR and quinoline are contained in coal tar and shale oils. They are well-known to be mutagenic, and toxic.3) Although CAR is known as an environmental pollutant and a recalcitrant molecule, it is useful as an industrial raw material for dyestuffs, medicines, and plastics. Four hundred tons of CAR was used in 1985 in Japan.

Bohonos *et al.* have reported the biodegradation of CAR and other azaarenes using six types of water samples as inocula.4) Fedorak *et al.* have described the method for analyzing 26 alkyl (C1–C5) CARs in Norman Wells crude oil and their degradation by a mixed microbial population.5) However, no degradation products nor pathways of CAR have yet been reported. In this report, we describe the biodegradation of CAR by a soil isolate, strain CA06, and an activated sludge isolate, strain CA10, capable of using CAR as the sole source of carbon, nitrogen, and energy. A degradation pathway of CAR by these strains is also proposed.

**Materials and Methods**

_Growth conditions._ The cells were grown in 500-ml flasks containing 100 ml of medium with agitation at 150 rpm on a rotary shaker at 30°C. An autoclaved mineral salts medium (minimal medium: MM) was added with the CAR solution in dimethyl sulfoxide (3%) to make the desired CAR concentrations.

Media and microorganism. The MM had the following composition (g/liter): Na₂HPO₄·12H₂O, 2.2; KH₂PO₄, 0.8; FeSO₄·7H₂O, 0.015; CaCl₂·2H₂O, 0.015; MgSO₄·7H₂O, 0.015; yeast extract, 0.025; and deionized water to make 1 liter. The initial pH was adjusted to 7.0. The culture medium was sterilized at 120°C for 15 min.

Strains CA06 and CA10 were isolated on the basis of the ability to grow on CAR as the sole source of carbon and nitrogen. Strain CA06 was isolated from soil by enrichment and serial transfer with CAR (0.1% [w/v]) as the growth substrate. Each of the soil samples was suspended in deionized water (1 g/10 ml), and filtered through filter paper (Advantec Toyo No. 2, Toyo Roshi Kaisha, Ltd., Japan). Then the filtrate was put on a cellulose acetate filter with a pore size of 0.45 μm (Toyo Roshi Kaisha, Ltd., Japan) to collect bacteria. The membrane filters were suspended in 100 ml of MM supplemented with 100 mg of CAR and the suspensions were cultivated at 30°C for 2 weeks.

Strain CA10 was isolated from the activated sludges by the same method as the soil samples. Each of the activated sludge samples was filtered though filter paper. One milliliter of the filtrate was used for the enrichment culture. One milliliter of the enrichment culture was transferred to the fresh MM with CAR after two weeks, and then repeated three times at weekly intervals. Subcultures were spread on the nutrient agar medium and, after incubation at 30°C, single colonies appeared on the nutrient agar medium and were transferred to 10 ml of MM in test tubes with CAR. Well-grown colonies were selected and used for further experiments. The bacterium purified from the culture broth was identified on the basis of the information in Bergey's Manual of Systematic Bacteriology.

Chemicals. CAR was purchased from Katayama Chemical Industries Co., Ltd., Osaka, Japan. Although this CAR was of the highest purity commercially available, four recrystallizations in toluene were done for further purification. AN and CAT were purchased from Kanto Chemicals Co., Inc., Tokyo, Japan. All other chemicals were obtained from Wako.
Measurement of growth of bacteria with aromatic compounds. Bacteria inoculated from an agar slant were incubated with 5mg of various aromatic compounds in 5ml of MM with 0.38% [w/v] of ammonium nitrate for aromatic compounds without nitrogen in a test tube (18mm x 165mm) for 1-2 weeks at 30°C. Due to the insolubility of many of the substrates, growth was assessed visually by comparison with growth on AN after 18-20h of incubation.

Isolation of metabolites. Growing cultures (0.1% CAR, 14-16h incubation) of strains CA06 and CA10 were centrifuged (10,000 × g, 10 min) to remove the residual substrate and the cells. The supernatants were extracted with ethyl acetate after acidification to pH 3 with 6N HCl. The ethyl acetate layer was dried with sodium sulfate anhydrous and concentrated by a rotary evaporator at below 40°C. The concentrated ethyl acetate layer was methylated with diazomethane and concentrated by passage of nitrogen gas for the injection into a gas chromatograph-mass spectrometer (model JMS DX303) with a capillary column (15m, 0.32mm diameter, Ultra-I). A high-performance liquid chromatograph-mass spectrometer (model Shimadzu QP1000S) was used for identification of the metabolites without methyl esterification.

Analytical methods. The CAR, AN, and CAT concentrations of the culture were assayed by a high-performance liquid chromatograph (model Shimadzu LC6A; Shimadzu, Kyoto, Japan). The culture medium was mixed with 1.33 volumes of acetonitrile, and vortexed for 5sec. It was clarified by centrifugation (2min at 10,000 × g) in an Eppendorf Microfuge tube and analyzed by HPLC. An Inertsil ODS2 column (GL Sciences, Tokyo, Japan) was used, with an acetonitrile-water-0.05% trifluoroacetic acid mobile phase, typically run at 20% to 60% acetonitrile gradient for 8 min or 20% isocratic conditions, to monitor major products at an absorbance of 230nm. The optical density of the cell suspensions was measured at 610nm.

Measurement of enzyme activities. The meta-cleavage enzyme activities for the oxidation of biphenyl-2,3-diol, 3-methylcatechol, and CAT were measured using the crude cell extracts of CAR- and AN-grown cells. Cells were grown in the MM with CAR (0.1% [w/v]) or AN (0.1% [w/v]) for 16-18h at 30°C, and harvested by centrifugation. Assays were done with a spectrophotometer (model Shimadzu UV-160A; Shimadzu, Kyoto, Japan) at 30°C. Protein was measured by an assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the standard.

Results and Discussion

Enrichment, isolation, and characterization of the CAR using microorganisms

Two hundred and two soil samples from farms in Fukuoka, Oita, and Ibaraki prefectures and 4 activated sludge samples from sewage disposal plants were used for the isolation of bacteria that grew on CAR. Enrichment cultures were started by mixing 2g each of 20 soil samples and each activated sewage sample with 100ml of MM supplemented with 0.1g of CAR as the substrate. After 2 weeks, used of the substrate became visible by the increasing turbidity of the enrichment cultures.

Only one strain, CA06, was isolated from the 202 soil samples, while strain CA10 was obtained from one of the 4 activated sludge samples. These two bacterial strains grew well on CAR as the sole source of carbon, energy, and nitrogen. The reason the bacteria were more easily screened from the sludge samples than from the soil samples the enrichment culture technique was that the former, being from industries, contained various chemicals suitable for use by the bacteria and that probably contributed to the formation of the enzymes responsible for the degradation of CAR. CAR assimilating or partially decomposing bacteria are probably more widely distributed in activated sludge than in soil.

Strains CA06 and CA10 were both Gram-negative, non-sporeulated, motile, rod-shaped bacteria. The strains gave positive results for catalase, oxidase, and cytochrome oxidase and grew under strictly aerobic conditions. The guanine-plus-cytosine content of bacterial DNAs of CA06 and CA10 were estimated to be 63.3% and 61.9%, respectively. The ubiquinones of the two strains showed Q9 patterns. Based on these data, strains CA06 and CA10 were identified as Pseudomonas spp.

Effects of dispersion of substrate on the rate of biodegradation

The dispersion of water-insoluble CAR with dimethyl sulfoxide enhanced mineralization of CAR by strains CA06 and CA10 (Fig. 1). Dispersion exhibited the rapid degradation of CAR without the consumption of dimethyl sulfoxide. The optimal concentration of dimethyl sulfoxide for the degradation of CAR was 3%. Subsequent studies were done with this concentration of dimethyl sulfoxide. The increasing surface area available for biodegradation seems to be effective in promoting the rate of CAR mineralization. Solubilization with a surfactant or by mechanical fragmentation of substrate has been reported to increase biodegradation of PCBs. Solubility is also important in the mineralization of CAR, since both the strains CA06 and CA10 showed poor growth upon the addition of CAR to the medium without dimethyl sulfoxide. The acclimated cultures of strains CA06 and CA10 showed similar CAR degradation patterns as shown in Fig. 2. CAR was completely degraded in 18h by the two strains.

Growth conditions for CAR biodegradation

Figure 3 shows the effects of CAR concentrations on the degradation of CAR and the formation of metabolites. At the concentrations of CAR from 5.7mm up to 17mm, both bacteria grew well on the substrate. At the concentration of CAR of 17mm, the consumption rate of substrate was slow compared with those of low substrate concentrations, and 1.4mm of AN and 0.1mm of CAT were accumulated in the culture broth.
Biodegradation of Carbazole by *Pseudomonas*

**Growth of bacteria with aromatic compounds**

The following compounds were used by CA06 and CA10 for growth: *p*-hydroxybenzoate, biphenyl-2,3-diol, AN, and gentisic acid. AN was used by the strains as a carbon and nitrogen source. Salicylic acid was used by CA10. *N*-Ethylcarbazole was weakly used by CA06. *N*-Methylcarbazole, naphthalene, fluorene, dibenzofuran, dibenzothiophene, biphenyl, 2-biphenylylamine, pyridine, aniline, isonicotinic acid, 4-pyridinol, and quinoline, were not used for the growth of either strain. A wide variety of aromatic compounds could not be decomposed by these strains. This suggested that the enzymes of strains CA06 and CA10 involved in the degradation of CAR have a similar range of substrate specificities. Although CAR was easily decomposed by both strains, *N*-ethylcarbazole was only weakly degraded by strain CA06 and *N*-methylcarbazole was not attacked by either strain. These results suggested the initial oxidation of degradation may occur at the nitrogen-binding carbon.

**Identification of CAR degradation products**

A culture shaken for 48 h (100 ml MM with 17 mM CAR) was analyzed by HPLC (Fig. 4). Major products (peaks 2 and 3) were detected at an absorbance of 230 nm by HPLC. Only one of the major metabolites (peak 2) showed identical HPLC chromatogram and GC-MS spectrum to those of authentic AN. Peak 3 was a metabolite with *m/z* = 220 (M+H) by HPLC-MS. In GC-MS, the fragment pattern of the minor metabolite (peak 1) agreed well with that of authentic CAT. When AN was used for a substrate (200 mg/100 ml MM with nitrogen source) in culture, CAT was obtained as the
Table 1. Minor Metabolites Produced from CAR by Strain CA10

<table>
<thead>
<tr>
<th>No.</th>
<th>Predicted name and structure</th>
<th>M⁺ (Methyl ester)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5-Oxo-5-(2'-aminophenyl)pent-2-enoic acid</td>
<td>219</td>
</tr>
<tr>
<td>2</td>
<td>6-Oxo-6-(2'-aminophenyl)hexa-4-enoic acid</td>
<td>233</td>
</tr>
<tr>
<td>3</td>
<td>2,6-Dioxo-6-(2'-aminophenyl)hexa-4-enoic acid</td>
<td>247</td>
</tr>
<tr>
<td>4</td>
<td>2-Hydroxy-6-oxo-6-(2'-aminophenyl)hexanoic acid</td>
<td>251</td>
</tr>
<tr>
<td>5</td>
<td>2-Hydroxy-6-oxo-6-(2'-aminophenyl)hexa-2,4-dienoic acid</td>
<td>261</td>
</tr>
<tr>
<td>6</td>
<td>2'-Aminobiphenyl-2,3-diol</td>
<td>229</td>
</tr>
</tbody>
</table>

* Metabolite numbers in Fig. 6.
† Metabolites were tentatively identified as diazomethane methyl ester derivatives.

and others were found (Table 1). The former had fragment ions at m/z 214 (M—CH₃), 198 (M—OCH₃), 186 (M—CH₂—CO), 158 (M—CH₃—CO—H₂CN), 130 (M—CH₃—CO—H₂CN—CO), and 77 (C₆H₅⁺). The latter had fragment ions at 246 (M—CH₃), 230 (M—OCH₃), and 146 (N-protonated 4-quinolinone which is a dominant fragment peak, and the rearrangement resembles that of n-butyl N-methylantranilic acid). The molecular formula of the product of m/z 261 was C₁₄H₁₄NO₄ (m/z 261.1002) by high-resolution MS. From these data and data on metabolites of biphenyl, they were tentatively identified as 2' -aminobiphenyl-2,3-diol (methylated m/z 229) and 2-hydroxy-6-oxo-6-(2'-aminophenyl)hexa-2,4-dienoic acid (methylated m/z 261).

Additionally, an oxopentenoate metabolite was identified on the basis of HPLC retention time by comparison with the sample produced by oxidative deamination of 2-allylglycine. These data suggest the involvement of a meta-cleavage step in the metabolic pathway of CAR to AN. This step resembles the pathway of biphenyl to benzoic acid.

**Enzyme activity in crude cell extracts**

When *Pseudomonas* sp. strains CA06 and CA10 were grown on CAR, the cultures turned to yellow. The analyses of metabolites also suggest that the degradation enzymes of CAR may contain a meta-cleavage enzyme of 2'-aminobiphenyl-2,3-diol. This compound is similar to the metabolites produced from the biphenyl substrates by the bacteria such as *Pseudomonas* that assimilate biphenyl and/or chlorinated biphenyls. Unfortunately, this compound is not commercially available, so the non-aminated analog biphenyl-2,3-diol was used instead. The enzyme activities of the catabolic meta-cleavage enzyme for biphenyl-2,3-diol, 3-methylcatechol, and CAT were measured with crude cell extracts of CAR- and AN-grown cells.

Table II shows the induction of meta-cleavage enzyme of biphenyl-2,3-diol in the two strains during growth on CAR as the substrate. The enzyme of strain CA10 showed weak activity for 3-methylcatechol. However, slight or no activity, and no development of yellow color caused by the
accumulation of meta-cleavage product was detectable when cells were grown on AN-culture.

The production of 2-hydroxy-6-oxo-6-(2'-aminophenyl)hexa-2,4-dienoic acid and the existence of a meta-cleavage enzyme of biphenyl-2,3-diol in crude cell extract demonstrated that strains CA06 and CA10 have a meta-cleavage pathway of CAR. In addition, the formation of CAT and cis,cis-muconate from AN also indicated the existence of an ortho-cleavage pathway. Only the major metabolites were identified as AN by HPLC and GC-MS, and the minor metabolites were tentatively identified as 2’-aminobiphenyl-2,3-diol (methylated \( m/z \) 229) and 2-hydroxy-6-oxo-6-(2’-aminophenyl)hexa-2,4-dienoic acid (methylated \( m/z \) 261). Strubel et al. reported that fluorene and dibenzo-furan were attacked via dioxygenation in the angular position.\(^{14,15}\) The initial oxidation product of CAR resembled that of dibenzo-furan. Identification of oxopentenoate by HPLC suggested the hydrolysis of 2-hydroxy-6-oxo-6-(2’-aminophenyl)hexa-2,4-dienoic acid.

Fig. 6. Total Ion Chromatogram (A) of a Methylated Acidic Ethyl Acetate Extract of Culture (100 ml of MM with 5.7 mm CAR) after 14 Hours of Incubation with Strain CA10.

The identity of each numbered metabolite is given in Table I. Peaks (*) were impurities of CAR. They were identified by a control experiment without inocula. Mass spectra of peak 5 (B) and peak 6 (C).
enzyme assays of crude cell extracts growth on AN and CAR. The conversion of AN to CAT and cis,cis-muconate by these strains suggested the existence of an ortho-cleavage. Based on these data, a proposed pathway of CAR is presented in Fig. 7.

One of the two strains was isolated from the 202 farm soil samples and the other was from the four activated sludge samples from sewage disposal plants. To elucidate the difference of distribution or frequency of CAR degrading bacteria in soil and activated sludge samples, we intend to investigate the genetic and the enzymatic aspects of the biodegradation of CAR by both strains. The isolated strain CA06 and CA10 assimilated CAR for growth factors. These strains may contribute to environmental cleaning of CAR, which is one of the most abundant industrially produced nitrogen-containing aromatic compounds.

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References