Selective and Continuous Degradation of Carbazole Contained in Petroleum Oil by Resting Cells of Sphingomonas sp. CDH-7

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Microbial degradation of carbazole (CA), a model of hard-removal heterocyclic nitrogen compounds contained in petroleum oil, was examined using Sphingomonas sp. CDH-7 isolated from a soil sample by screening for CA-assimilating microorganisms. CDH-7 used CA as a sole source of carbon and nitrogen, and metabolized CA to ammonia via antranilic acid as an intermediate product. When CDH-7 was cultivated in the medium containing CA at the concentration of 500 mg/l (2.99 mM), CA was completely degraded within 50 h. By the reaction with the resting cells of CDH-7, 500 mg/l of CA was completely degraded within 4 h, with 1.64 mM of ammonia accumulated in the reaction mixture. When CA was added at the concentration of 100 mg/l (0.599 mM) periodically to the reaction mixture ten times, 925 mg/l (5.54 mM) of CA was degraded within 48 h by the resting cells, and 4.50 mM of ammonia was accumulated in the reaction mixture with a 75.1% molar conversion yield based on total CA added. The resting cells could almost completely degrade CA in a two-liquid-phase system which consists of water and organic solvent, even in the presence of 20% (v/v) isooctane, n-hexane, cyclohexane, and kerosene as a model petroleum oil. In the presence of an organic solvent system such as 20% (v/v) p-xylene, toluene, and heptanol, however, CA degradation yields decreased.

Key words: biodegradation; carbozole; heterocyclic nitrogen compound; resting cell reaction; Sphingomonas sp.

Crude petroleum oil is a complex mixture of organic compounds including hydrocarbons and oxygen-containing, sulfur-containing, and nitrogen-containing organic compounds. Most of the nitrogen-containing organic compounds are removed from crude petroleum oil by the chemical and physical petroleum oil refinery processes currently used. However, it is very hard to remove heterocyclic nitrogen compounds such as carbozole (CA) by such processes using inorganic catalysts, and these compounds still remain in petroleum oil. When these nitrogen compounds are combusted, nitrogen oxides or other nitrogen oxidation products, NOx, are released into the atmosphere in addition to thermal NOx, which cause acid rain and air pollution. Moreover, the nitrogen-containing organic compounds such as CA poison the catalysts used in the refinery process for the petroleum oil. Therefore, it is necessary to establish a method for removing these nitrogen compounds from crude petroleum oil for global environmental protection. A bioprocess using bacteria degrading heterocyclic nitrogen compounds might be used since such a process enables selective degradation and proceeds under milder conditions than the chemical and physical processes, which need high-temperature and high-pressure conditions.

Several researchers have reported the microbial degradation of heterocyclic nitrogen compounds, and CA is often used as a model substrate for heterocyclic nitrogen compounds, since CA is used as an industrial raw material for the productions of dyes, medicines, and plastics, and is known as an environmental pollutant in ground water. Fedorak et al. reported the degradation of a wide range of 26-type alkylcarbazoles (C1 to C5) contained in Norman Wells crude oil by an oil-degrading mixed bacterial culture enriched by growth on CA. Ouchiya et al. identified some metabolites of CA and proposed a CA degradation pathway by Pseudomonas sp. CA 06 and CA 10, and Sato et al. reported the gene cloning and expression of CA degradation pathway enzymes. However, there have been no reports on the continuous degradation of CA by resting cells of CA-degrading microorganisms.

In this paper, we describe the degradation of CA by the resting cells of Sphingomonas sp. CDH-7, an newly isolated bacterium, using CA as a sole source of carbon and nitrogen. In addition, we describe the possibilities of continuous CA degradation, and CA degradation in a two-liquid-phase system which consists of water and petroleum oil.

Materials and Methods

Growth media and conditions. For the screening of CA-degrading microorganisms, a nitrogen-free synthetic medium (CDM) was used, containing (per liter of distilled water): 1.6 g Na2HPO4, 1.0 g KH2PO4, 0.5 g MgSO4·7H2O, 0.025 g CaCl2·2H2O, 2 ml metal solution, 1 ml vitamin mixture. The metal solution contained (per liter of distilled water): 1.5 g FeCl3·4H2O,
0.19 g CoCl₂·6H₂O, 0.1 g MnCl₂·4H₂O, 0.07 g ZnCl₂, 0.062 g H₃BO₃, 0.036 g Na₂MoO₄·2H₂O, 0.024 g NiCl₂·6H₂O, 0.017 g CuCl₂·2H₂O. Vitamin solution contained (per liter of distilled water): 400 mg Ca-pantothenate, 400 mg niacin, 400 mg pyridoxine-HCl, 200 mg inositol, 200 mg p-aminobenzoate, 50 mg cyanocobalamin. The initial pH of CDM was adjusted to 7.0, and per 1 liter of CDM 500 mg of CA was supplied as the sole source of carbon and nitrogen, and dispersed by the addition of n-hexadecane 0.5% (v/v). The medium was sterilized at 120°C for 20 min except the vitamin mixture, which was sterilized through a membrane filter with a pore size of 0.2 μm (Kurabo Co., Ltd., Osaka, Japan). The cells were cultivated at 30°C with reciprocal shaking at 120 rpm in a Teflon-sealed 100-ml vial containing 30 ml of CDM containing CA or a 500-ml Sakaguchi flask containing 100 ml of the medium. As the growth parameter of bacteria, optical density (O.D.) of the culture broth was measured at 660 nm by Shimadzu UV-1200 spectrophotometer (Shimadzu Co., Kyoto, Japan).

**Isolation of carbazole degrading microorganisms.** Approximately 350 samples from soil, wastewater, and petroleum oil sludge were collected, and 1.0 g of each sample was suspended in 10 ml of CDM. A portion of 100 μl of the suspension was added to 30 ml of the medium in a 100-ml vial, containing CA 1.0 g/l and n-hexadecane 0.5% (v/v). After 5 days of cultivation, 1 ml of the culture broth was transferred into fresh medium containing CA and n-hexadecane. The same enrichment subcultivations were done over four generations, and a well-grown subculture, showing O.D. more than 1.0, was spread on the nutrient agar medium with CA and cultivated at 30°C. The nutrient agar medium with CA contained (per liter of distilled water): 5.0 g CA, 5.0 g bacto-peptone, 2.5 g yeast extract, 2.5 g casamino acids, 2.5 g malt extract, 1 ml glycerol, 0.5 g MgSO₄·7H₂O, and 15 g agar. The initial pH was adjusted to 7.0. Grown colonies were isolated and purified by repeating single colony isolation. Each isolate was cultivated in CDM containing CA 500 mg/l and n-hexadecane 0.5% (v/v) at 30°C, and CA degradation was measured.

**Resting cell reaction.** Strain CDH-7 was cultivated in 500-ml flasks containing 100 ml of CDM with CA 500 mg/l and n-hexadecane 0.5% (v/v) for 35 h at 30°C. Cells were harvested by centrifugation at 10,000 × g for 10 min at 4°C, and washed twice with 50 mM K₂HPO₄-50 mM KH₂PO₄ buffer (50 mM potassium phosphate buffer, pH 7.0) and suspended in the same buffer. The cell suspension was cooled at 4°C and filtered through a 17G glass filter to remove the residual CA and n-hexadecane. The filtered cell suspension was centrifuged again and its cell density was adjusted to O.D. 3.3, equivalent to 2.2 mg/ml of resting cells as dry weight. The resting cell reaction was usually done in a 50-ml vial. The reaction mixture comprised of 10 ml of cell suspension and 1 mg or 5 mg of CA so that the CA concentration was adjusted 100 mg/l or 500 mg/l, respectively. Although 1 mg of CA was added to the reaction mixture as 100 μl of CA-ethanol solution (10 g/l), 5 mg of CA was added in a solid form with 100 μl of ethanol. n-Hexadecane was usually added to the reaction mixture at the concentration of 10% (v/v). The resting cell reaction was done at 30°C with reciprocal shaking at 120 rpm and stopped by acidification (below pH 2.0) by addition of 200 μl of 6 N HCl to the reaction mixture.

When dibenzofuran, fluorene, dibenzothiophene, biphenyl, naphthalene, or phenanthrene was used as a substrate, they were added to the reaction mixture to give 100 mg/l, as described above for CA: 100 μl of ethanol solution containing each compound (10 g/l).

**Analytical methods.** To detect and measure the amount of products, culture broth and resting cell reaction mixture were extracted with ethyl acetate containing dibenzofuran as an internal standard after acidification with 6 N HCl. The organic layer was filtered through a 0.2 μm PTFE membrane (Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and the concentrations of CA and anthranilic acid (AN) were measured by HPLC with a Shimadzu LC-10A system with a variable-wavelength detector (Shimadzu). A Puresil C18 column (Millipore, Tokyo, Japan) was used, with acetonitrile-water (50:50 v/v) as the mobile phase at the flow rate of 1.0 ml/min and temperature 40°C. The effluent was monitored at an absorbance of 230 nm. For the measurement of ammonia concentration, culture broth or resting cell reaction mixture was centrifuged at 12,000 × g for 10 min to remove the residual substrate and cells. The supernatant was filtered through the 0.2 μm membrane filter and used for the measurement by Ammonia Test Wako (Wako Pure Chemical Ind., Ltd., Osaka, Japan). For identification of the metabolites, the ethyl acetate fraction was concentrated by a rotary evaporator below 35°C, and analyzed by a gas chromatograph-mass spectrometer HP5971A (Hewlett-Packard) with a capillary column NB-1 (25 m × 0.32 mm; df=0.4 μm, GL Science, Co., Tokyo, Japan). Helium gas was used as a carrier. The column temperature was changed from 150°C to 250°C at the rate of 5°C/min. The temperature of injector and detector was maintained at 250°C and 280°C, respectively.

The concentrations of dibenzofuran, fluorene, dibenzothiophene, biphenyl, naphthalene, and phenanthrene were measured by HPLC as described above for measurement of CA.

**Results and Discussion**

**Isolation and characterization of CA degrading microorganisms**

Through the screening using CA as a sole source of nitrogen, five microorganisms growing on CA were isolated from the soil. The CA degradation by each isolate was examined under the conditions with initial CA concentration of 500 mg/l. Since CDH-7 showed the highest CA degradability of 100% in 5 days among the five isolates, it was used for further experiments. CDH-7 was a Gram-negative, non-sporulating, and rod-shaped bacterium, showed motility, and was also catalase-and
oxidase-positive. From these results, CDH-7 seemed to be a strain closely related to the genus Pseudomonas. However, as the membrane component, only sphingolipid-polsascharide was detected but not lipopolysaccharide. Further identification was done by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in detail, and the summarized results are shown in Table 1. From these results, CDH-7 was classified as Sphingomonas sp.

The ethyl acetate fraction prepared by extraction of the culture broth of CDH-7 was analyzed by GC-MS and HPLC. Anthranilic acid (AN) and 2'-aminobiphenyl-2,3-diol as a minor component were detected as metabolite products of CA degradation. Since AN has been reported to be the main metabolite of CA by several researchers, CDH-7 seems to have the same metabolizing pathway to produce AN during CA degradation. On the other hand, in the water-soluble fraction, through CA degradation much NH₃ was detected. These results indicate that CA was mineralized to NH₃ via AN as an intermediate product as shown in Fig. 1. Since AN is generally metabolized through either catechol or gentisic acid as an intermediate, NH₃ must have been released in these steps.

Use and mineralization of carbazole by Sphingomonas sp. CDH-7

<table>
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<tr>
<th>Properties of the strain</th>
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Symbols: +, positive; -, negative.

![Table 1. Identification of Strain CDH-7](image)

Fig. 1. Proposed CA Degradation Pathway by Sphingomonas sp. CDH-7.

The pathway is based on the reports of Ouchiyama and Sato. In the culture broth, we detected (II) and (IV) but not (III). Compounds: I, carbazole; II, 2'-aminobiphenyl-2,3-diol; III, 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoic acid; IV, anthranilic acid; V, catechol; VI, cis,cis-muconic acid.

The course of cultivation of CDH-7 and CA degradation is shown in Fig. 2. When CDH-7 was grown in the CDM containing 500 mg/l of CA and 0.5% (v/v) of n-hexadecane, CA was completely degraded after 50 h of cultivation. Strong CA degradation was observed after 20 h, and the growth of CDH-7, O.D.₆₆₀, increased in proportion to the CA degradation. AN was accumulated in the culture broth after 20-40 h transiently and then gradually disappeared. The presence of ammonia (NH₃) was slightly detected in the culture broth after 40-50 h of cultivation. CDH-7 could grow in the CDM without n-hexadecane, suggesting that CDH-7 has used CA as a sole source of carbon and nitrogen. However, the addition of n-hexadecane to the medium was favorable for CA degradation by growing cultures of CDH-7, due to increase of the growth rate and efficiency of CA degradation (data not shown). Since the solubilizations of CA by addition of dimethyl sulfoxide or a nonionic surfactant have been reported to increase the CA degradation, addition of n-hexadecane was considered to

![Fig. 2. Degradation and Mineralization of CA by CDH-7](image)
probably contribute to solubilize CA and increase the CA degradation by CDH-7.

Degradation of carbazole by resting cell reaction of CDH-7 in a two-liquid (oil/water) phase system

Considering the practical use of the resting cells for biodegradation of CA in crude petroleum oil, CA degradation might be examined in a two-liquid-phase system consisting of water and petroleum oil. Therefore, the CA degradation with the resting cells of CDH-7 was done in the presence of n-hexadecane as a model petroleum. The effects of concentration of n-hexadecane on CA degradation by resting cells were examined. The reaction was done for 30 min at the initial CA concentration of 100 mg/l, and the CA degradation ratio was measured. CA degradation was very low in the presence of n-hexadecane below 10% (v/v), probably due to the low dispersion efficiency of CA in the reaction mixture. The optimum n-hexadecane concentration was between 10% (v/v) and 30% (v/v) of the cell suspension, and the CA degradation ratio gradually decreased with more than 30% (v/v) n-hexadecane (data not shown) (probably because the cells of CDH-7 were damaged by the higher concentration of n-hexadecane.) Therefore, the resting cell reaction was done in the presence of 10% (v/v) n-hexadecane in further experiments.

The course of the resting cell reactions with CDH-7 at the initial CA concentrations of 100 mg/l and 500 mg/l are shown in Fig. 3(A) and (B), respectively. With the resting cells of CDH-7, 100 mg/l and 500 mg/l of CA were completely degraded within 30 min and 4 h, respectively. With the initial concentration of CA 100 mg/l, AN was not detected at all in the reaction mixture and the accumulation of NH₃ was observed throughout the reaction. On the other hand, with the initial concentration of CA 500 mg/l, AN accumulated transiently in the reaction mixture after 1 h as the CA degradation proceeded, and NH₃ was accumulated in accordance with decrease of CA and AN after 2 h. After 4 h, 1.64 mM of NH₃ was detected in the reaction mixture. These results indicate that CA was degraded completely by the resting cells of CDH-7. However, NH₃ accumulation was not equimolar to the CA degraded in the reaction mixture, suggesting that some CA metabolites such as NH₃ were assimilated as a nitrogen source for CDH-7.

Selective degradation of CA by the resting cells of CDH-7

To examine whether or not the resting cells of CDH-7 degrade organic compounds having similar carbon skeletons, the reactions toward dibenzofuran, fluorene, dibenzothiophene, and biphenyl were tested. However, the resting cells of CDH-7, cultivated in the medium containing CA as sole source of carbon and nitrogen as in Fig. 3, did not degrade these four organic compounds, and when CA, dibenzofuran, fluorene, dibenzothiophene, and biphenyl were added to the reaction mixture, only CA was degraded, as shown in Fig. 4. The reactions toward naphthalene and phenanthrene were also tried, however these compounds were not degraded by the resting cells of CDH-7 (data not shown). These results indicate that the resting cells of CDH-7 distinguished between CA and other organic compounds having similar carbon skeletons, and that selective degradation of CA was possible by the resting cell reaction using CDH-7. These properties of the CDH-7 resting cells might be suitable for selective degradation of CA in the petroleum oil since valuable organic compounds would have not been consumed or degraded. On the other hand, Sato et al.⁶ reported that Pseudomonas sp. CA-10 could degrade not only CA but also dibenzofuran.

**Fig. 3.** Degradation of CA by the Resting Cells of CDH-7 in the Presence of n-Hexadecane (10% Volume of the Reaction Mixture).

The cells were incubated in the reaction mixture containing CA at the initial concentration of 100 mg/l (A) or 500 mg/l (B). Symbols: ○, carbazole; ●, anthranilic acid; ▲, ammonia.

**Fig. 4.** Selective Degradation of CA with Dibenzofuran, Fluorene, Dibenzothiophene, or Biphenyl by the Resting Cells of CDH-7 in the Presence of n-Hexadecane (10% Volume of the Reaction Mixture).

(A) a, dibenzofuran; b, fluorene; c, dibenzothiophene; d, biphenyl. (B) Resting cell reaction by CDH-7. Each compound, 100 mg/l, was added to the reaction mixture. Symbols: ○, dibenzofuran; △, fluorene; ○, dibenzothiophene; ●, biphenyl; ●, CA.
and dibenzo-p-dioxin. Considering that CDH-7 probably metabolizes CA through the same pathway as to that of strain CA-10 as shown in Fig. 1, the differences in the degradation specificities might be due to the unique character of the CA-degrading enzymes of each strain. Shepherd et al.\textsuperscript{10} isolated a CA-degrading bacterium, \textit{Sphingomonas} sp. CD3, which mineralized only CA and did not use fluorene or dibenzothiophene. Such properties of CD3 are similar to those of CDH-7, although it is not clear whether or not CD3 could degrade CA selectively in the presence of other polyaromatic hydrocarbons.

**Continuous degradation of CA by the resting cells of CDH-7**

Since CA 500 mg/l added to the resting cell-reaction mixture was completely degraded within 4 h (Fig. 3(B)), the continuous CA degradation using the resting cells of CDH-7 was done. As shown in Fig. 5, 100 µl of CA-ethanol solution (10 g/l) was added periodically to the reaction mixture (10 ml) ten times so that the CA concentration was adjusted to 100 mg/l dropwise. Under these conditions, approximately 925 mg/l (5.54 mM) of CA was degraded in 48 h and 4.50 mM of NH\textsubscript{3} accumulated in the reaction mixture with a 75.1% molar conversion yield based on CA supplied (5.99 mM). We consider that higher accumulation of NH\textsubscript{3} in Fig. 5 was probably due to the increase of ethanol concentration by the periodical addition to the reaction mixture, which might have affected the cell membranes to promote the NH\textsubscript{3} release from the cells. After the reaction at 48 h, cell density (O.D.) was the same as that of initial time at 0 h. Therefore, it was confirmed that CDH-7 did not grow during the continuous degradation reaction. These results suggest the possibility of mineralization of CA to NH\textsubscript{3} using resting cells of CDH-7. However, CA added at the tenth time (48 h) was not completely degraded, and the accumulation of AN in the reaction mixture increased gradually after 25 h.

In the parallel experiments, we confirmed that CA degradation activity of the resting cells did not decrease even in the presence of 5.0 mM NH\textsubscript{3} or 2.0 mM AN. Therefore, NH\textsubscript{3} and AN seemed not to inhibit the CA degradation. However, we also confirmed that in the presence of ethanol at more than 5.0% (v/v) the CA degradation activity decreased (details not shown). Hence, we consider that the decrease in CA degradation activity after 36 h in Fig. 5 might have been explained by the following possibilities. One possibility is that ethanol accumulated in the reaction mixture during the continuous degradation damaged gradually the cells or the CA degrading enzymes. The other possibility is that consumption of cofactors such as NAD(P)H in the cells during the continuous degradation caused decrease of the activity of CA degrading enzymes. By the improvement of reaction conditions, for example, refreshing the reaction mixtures or reactivating the damaged cells, we presume that the resting cells might be available to further efficient CA degradation. On this point, further experiments are now in progress.

**CA degradation by resting cells in the presence of various organic solvents**

Removal of nitrogen from CA contained in petroleum oil requires that CA is degraded even in the presence of petroleum oil. Hence, CA degradation by the resting cells of CDH-7 in the presence of various organic solvents was tried. The 50 mM potassium phosphate buffer (pH 7.0) containing 4.4 mg/ml of cells as dry weight was used for the reaction. Since the log P values are generally related to the organic solvent tolerance of microorganisms,\textsuperscript{12,13} we examined CA degradation in the presence of several organic solvents with different log P values. As shown in Fig. 6, the resting cells of CDH-7 could degrade CA even in the presence of 20% (v/v) isooctane (log P=4.8), n-hexane (log P=3.9), cyclohexane (log P=3.4), p-xylene (log P=3.1), or kerosene. However, CA degradation decreased in the presence of 20% (v/v) p-xylene and toluene, and was lost almost completely in the presence of 20% (v/v) heptanol. The ratio of CA degradation seems to correlate

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\text{log } P & \text{Organic solvents} \\
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 & \text{Kerosene} (100\%) & \text{Kerosene} (50\%) & \text{Kerosene} (20\%) & 2.4 & 2.8 & 3.1 \\
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2.4 & \text{Heptanol} (20\%) & \text{Toluene} (20\%) & \text{p-Xylene} (20\%) & 3.4 & 3.9 & 4.8 \\
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3.4 & \text{Cyclohexane} (20\%) & \text{n-Hexane} (20\%) & \text{Isooctane} (20\%) & & & \\
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Fig. 5. Continuous Degradation of CA by the Resting Cells of CDH-7 in the Presence of n-Hexadecane (10% Volume of the Reaction Mixture).

The cells were incubated in the reaction mixture containing CA added periodically ten times at the initial concentration of 100 mg/l for 48 h. Symbols: ●, carbaolate; ■, antranilic acid; ▲, ammonia.

Fig. 6. Degradation of Carbazole by the Resting Cells of CDH-7 in the Presence of Various Organic Solvents.

The cells were incubated in the reaction mixture containing CA at the initial concentration of 100 mg/l for 1 h. Various organic solvents were added to the reaction mixture at the concentration of 20%, 50%, or 100% volume of the reaction mixture.
with the log $P$ values of the organic solvents. In the case of kerosene, a typical model of petroleum oil, cells of CDH-7 could degrade CA even at 100% (v/v) concentration (the same volume of the reaction mixture). These results support the possibility of the use of CDH-7 resting-cells in the degradation of CA contained in petroleum oil. Although CA degradation in the presence of 20% (v/v) toluene was 25.0% with the cell concentration of 4.4 mg/ml as dry weight, it increased up to 60.4% with the cell concentration of 11.0 mg/ml (data not shown). Therefore, it is possible to increase CA degradation activity in the presence of organic solvents with strong toxicity and low log $P$ values by increasing the cell concentration.

In this study, we have isolated Sphingomonas sp. CDH-7, which degrades CA as a sole source of carbon and nitrogen. Since the resting cells of this strain could degrade CA effectively and maintained their activity in the two-liquid-phase system consisting of water and organic (petroleum-model) solvents, we believe such a system must be available for the biodegradation of nitrogen-containing organic compounds remaining in petroleum oil.

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