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Biodegradation of carbazole by the seven *Pseudomonas* sp. strains and their denitrification potential

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ABSTRACT

Carbazole, one representative of non-alkaline nitrogen heterocyclic compounds, is widespread in the natural environment and harmful to human health. In this research, the seven bacterial strains using carbazole as their sole carbon, nitrogen and energy source were isolated from activated sludge of a coking wastewater treatment plant. All strains efficiently degraded 500 mg/L of carbazole in the medium within 36 h. Based on the DNA sequence and phylogenetic tree analysis, the seven strains were identified as the genera *Pseudomonas* with different evolutionary pathways. PCR analysis revealed that the seven isolates carried the *car* gene. Moreover, all of these strains could utilize and transform ammonium and nitrate efficiently, and the six strains except BC043 strain coded the nitrite reductase gene (*nirS*) and the nitrous oxide reductase (*nosZ*), that indicated their denitrification ability. All these strains may be useful in the bioremediation of environments contaminated by carbazole.

1. Introduction

Nitrogen heterocyclic compounds (NHCs), which are often generated from coking, petrochemical, and other related industries, are representative xenobiotics. Carbazole, quinoline, and pyridine are the typical NHCs in these industrial wastewaters. They are well-known to be toxic, teratogenic, and mutagenic pollutant [1,2].

Carbazole is a recalcitrant non-alkaline NHC. It is very difficult to be degraded by most of the microorganisms in environments. However, a few bacteria have evolved the necessary metabolic pathways and acquired the ability to degrade carbazole after a prolonged exposure to this pollutant [3]. These bacteria include *Pseudomonas resinovorans* CA10 [1], *Nocardioides aromaticivorans* IC177 [4], and *Sphingomonas* sp. KA1 [5]. For the first time, Ouchiyama et al. reported the degradation pathways and biodegradation products of carbazole by *P. resinovorans* CA10. This bacterial strain CA10 degraded carbazole to anthranilate and 2-hydroxypenta-2,4-dienoate through angular dioxygenation, *meta*-cleavage, and hydrolysis [1]. The enzyme carbazole 1,9-dioxygenase (CARDO), which participates in the angular dioxygenation and cleaves one of the two carbon–nitrogen bonds, is composed of terminal oxygenase (CarAa), ferredoxin (CarAc), and ferredoxin reductase (CarAd) [6]. CarAa is a unique type of oxygenase that shares low homology with other known dioxygenases based on the amino acid sequence homology and phylogenetic analysis [7,8].

It was also reported that during the biodegradation of carbazole, ammonium was produced as an inorganic product by the bacterial degrader [4,9], like the other degrader of NHCs [10]. However, little attention has been devoted to determine the further transformation of the NH_4^+–N by the same microbial degrader of carbazole. If the NH_4^+–N accumulated in the water, high concentration ammonium could not reach the discharge standard, even would cause serious environmental problems. Adding a new unit or modifying the treatment process to remove ammonia–nitrogen would increase the investment and operation cost. The bacterium with multiple functions could be regarded as an eligible solver.

In this study, the seven carbazole-degrading bacteria were isolated and phylogenetically analyzed. The biodegradation of carbazole by the seven strains was investigated, and the representative functional gene *carAa* was amplified from them. Their nitrification and denitrification potential was investigated using ammonium and nitrate brines. Furthermore, genes involved in the denitrifying process were detected, e.g. the nitrite reductase gene (*nirS*) and the nitrous oxide reductase gene (*nosZ*), that indicate the denitrification potential of these carbazole-degrading bacteria.

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2. Materials and methods

2.1. Chemicals

Carbazole was obtained from Sigma–Aldrich, Inc., USA. Dimethyl sulfoxide (DMSO) was purchased from Amresco, USA. NH_3–N, NO_2^-–N, and NO_3^-–N were received from the China Research Center of Certified Reference Materials. Tryptone and yeast extract were obtained from Oxoid Ltd., UK. Solvents for HPLC and GC/MS analysis were chromatographic grade. All other chemicals used in this study were analytical grade.

2.2. Media

Two kinds of basic media were used in the experiments. The Luria–Bertani (LB) medium [11] was used for bacteria enrichment and maintenance. The mineral salt medium (MSM), which does not contain N source as described by Bai et al. [12], was used as the basic ingredient for the bacterial degradation and transformation. The carbazole was dissolved in DMSO (30 g/L) and added to the MSM as the sole degradable carbon, nitrogen, and energy source in the biodegradation experiments. The MSM + NH_4Cl + glucose medium was used to determine the nitrification potential; and the MSM + KNO_3 + glucose medium was used to evaluate the denitrification potential of these carbazole-degrading strains. All media were sterilized at 121 °C for 20 min before use.

2.3. Bacteria cultivation and isolation

The seven carbazole-degrading bacteria that utilized carbazole as their sole sources of carbon, nitrogen, and energy were isolated from the activated sludge of the coking wastewater treatment plant of Shougang Group, Beijing, China. The strains were identified by 16S rRNA sequence analysis. Genomic DNA was extracted from each pure culture using a TIANamp Bacteria DNA Kit (Tiangen Biotech (Beijing) Co., Ltd.). The bacterial 16S rRNA gene sequence was amplified using the TaKaRa ExTaq hot-start polymerase (TaKaRa Bio., Japan) with the bacterial universal primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-TAC GGT TAC CTT GTT ACG ACT-3′) [13]. The PCR thermal program was at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min, and then kept at 4 °C. The sequences were analyzed by the ABI 3730*xl* DNA analyzer (Applied Biosystems, USA).

The sequences obtained from the seven strains were searched in the National Center for Biotechnology Information (NCBI) database by using the BLAST program. Selected sequences of the same genera were extracted from the GenBank database. Sequence analysis was performed by Bioedit software, and a phylogenetic tree was constructed using the neighbor-joining method in MEGA 4.0 [14].

2.4. Biodegradation of carbazole

A series of 250-ml Erlenmeyer flasks were used in the experiments. Each flask carried 100 ml of the MSM with a specific concentration of carbazole and the same initial amount of inoculated bacterium (OD_{602} = 0.1). In addition to above test flasks, the dead-cell control and negative control experiments were carried out at the same time. The dead-cell control flasks were inoculated with different species of heat-killed (autoclaved at 121 °C for 20 min) cells corresponding to the test flasks. The negative control flask had no inoculum. All flasks were sealed with sealfilm, shaken at 30 °C, 180 rpm, and sampled periodically. The samples were fully vortexed, centrifuged and extracted with isometric ethyl acetate for the carbazole analysis. All experiments including the negative controls were carried out in triplets.

2.5. PCR amplification and detection of dioxygenase in carbazole catabolism

In order to investigate the catabolic potential of the seven *Pseudomonas sp.* strains for carbazole, the total DNA extracted from each strain was analyzed for the presence of carbazole 1,9-dioxygenases (CARDO). The PCR amplification primer was designed from *Pseudomonas* sp. CA10 and *Pseudomonas* sp. OM1 as follows: sense: 5′-GCG AGC CGA AGA CAC TAA-3′, antisense: 5′-GCG TAG AAA TCC ACC ATA GC-3′. The PCR thermal program was set at 94 °C for 5 min followed by 30 cycles at 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min, and then kept at 4 °C. The TaKaRa Taq hot-start polymerase (TaKaRa Bio., Japan) was used for the PCR reaction. The sequence was analyzed by the same method as that used for 16S rRNA sequence analysis.

2.6. Nitrification and denitrification potential

A series of 500-ml Erlenmeyer flasks were used. Each flask was filled with 200 ml of the MSM + NH_4Cl + glucose or MSM + KNO_3 + glucose medium. The initial C/N ratio of the medium was kept at 25:1. All flasks were sealed with sealfilm, shaken at 30 °C, 180 rpm, and sampled periodically. The NH_4^+–N, NO_3^-–N, and NO_2^-–N were measured for the samples from the MSM + NH_4Cl + glucose medium; the NO_3^-–N and NO_2^-–N were measured for the samples from the MSM + KNO_3 + glucose medium.

For functional gene detection, two gene fragments encoding cytochrome cd1-containing nitrite reductase (*nirS*) and nitrous oxide reductase (*nosZ*), which participate in denitrification, were amplified from the total DNA of the seven carbazole-degrading strains using the following primers [15]:

nirS-F: 5′-CAC GG**Y** GT**B** CTG CGC AAG GGC GC-3′
nirS-R: 5′-CGC CAC GCG CGG **Y**TC **S**GG GTG GTA-3′
nosZ-F: 5′-CG**Y** TGT TC**M** TCG ACA GCC AG-3′
nosZ-R: 5′-CAT GTG CAG **N**GC **R**TG GCA GAA-3′

Bold-face letters denote degenerate positions. B, G + T + C; M, A + C; N, A + C + G + T; R, A + G; S, G + C; Y, C + T.

The PCR thermal program used was described in our previous study [16]. The TaKaRa Taq hot-start polymerase (TaKaRa Bio., Japan) was used for the PCR reaction. The PCR products were separated by 1% agarose gel electrophoresis and stained using SYBR Safe DNA gel stain (Molecular Probes, USA).

2.7. Plasmid isolation

In order to determine the location of the genes encoding carbazole degradation and the genes participating in denitrification, plasmid DNA was isolated from each strain by the modified alkaline lysis method [11]. Positive control test was synchronized with the whole plasmid extraction.

2.8. Analytical methods

Bacterial growth was monitored by OD_{602} using a UV–Vis spectrophotometer (Shimadzu UV-2401PC).

The carbazole concentrations were analyzed by a high performance liquid chromatography (HPLC) system (Shimadzu $LC10AD_{VP}$, $SPD10A_{VP}$ UV–Vis Detector; Rheodyne 7725i manual injector; Diamonsil C18 reverse-phase column, 250 mm × 4.6 mm, 5 μm). A methanol and water solution (9:1) was used as a mobile