Desulfurization of alkylated forms of both dibenzothiophene and benzothiophene by a single bacterial strain

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Abstract

Thirty-five bacterial strains capable of converting dibenzothiophene into 2-hydroxybiphenyl were isolated. Among them Rhodococcus erythropolis KA2-5-1 was chosen for further characterization because of its ability to retain high desulfurization activity stably. PCR cloning and DNA sequencing of a KA2-5-1 genomic DNA fragment showed that it was practically identical with dszABC genes from Rhodococcus sp. IGTS8, a representative carbon–sulfur-bond-targeted dibenzothiophene-degrading bacterium. KA2-5-1 desulfurized a variety of alkyl dibenzothiophenes through the specific cleavage of their C–S bonds. In addition, unexpectedly, KA2-5-1 also attacked alkyl benzothiophenes in a C–S-bond-targeted fashion. The purified monooxygenase, encoded by dszC of KA2-5-1, converted benzothiophene and dibenzothiophene into benzothiophene sulfone and dibenzothiophene sulfone, respectively, with the aid of an NADH-dependent oxidoreductase. This result raises the possibility that the same enzymatic step may be involved in desulfurization of alkylated forms of both dibenzothiophene and benzothiophene in KA2-5-1 cells. ß 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Desulfurization; Alkyl dibenzothiophene; Alkyl benzothiophene; Rhodococcus

1. Introduction

Heterocyclic sulfur compounds such as alkyl dibenzothiophenes and alkyl benzothiophenes are major sulfur components in the hydrodesulfurized oil fractions because they are highly recalcitrant to chemical catalysts. Several bacterial strains belonging to the genera Rhodococcus, Agrobacterium, Mycobacterium, and Paenibacillus can attack dibenzothiophene to produce 2-hydroxybiphenyl in a C–S-bond-targeted fashion [1]. Arthrobacter sp. ECRD-1 and Rhodococcus erythropolis H-2 can attack dibenzothiophene and even its alkyl forms to remove sulfur atoms [2,3]. Recently, a bacterium capable of desulfurizing benzothiophene in a C–S-bond-targeted fashion has also been reported [4]. However, there has not yet been a report of a single bacterium that can desulfurize both alkyl dibenzothiophenes and alkyl benzothiophenes. To construct a highly controllable biodesulfurization process, it is very attractive if any single bacterial strain can desulfurize both alkyl thiophenes. In the course of isolating bacteria that could maintain their desulfurization activities stably for a long time, we found that a R. erythropolis strain had the capability of desulfurizing dibenzothiophenes and benzothiophenes possessing alkyl group(s). It has also been demonstrated that the first step of alkyl benzothiophene desulfurization, sulfur oxygenation, can be catalyzed with the monooxygenase involved in the alkyl dibenzothiophene desulfurization by the bacterium R. erythropolis KA2-5-1.

2. Materials and methods

2.1. Microorganisms

Rhodococcus sp. IGTS8 (ATCC 53968), a desulfurizing bacterium cleaving the C–S bond of dibenzothiophene, was used as a control bacterium [5]. Escherichia coli JM109 was used as a cloning host.
2.2. Cultivation and resting cell reaction

The E. coli strain was grown in Luria–Bertani (LB) medium or modified M9 medium containing MgCl₂ instead of MgSO₄ at 37°C. R. erythropolis strains were grown in medium A [6]. The ability to desulfurize dibenzothiophenes and benzothiophenes was tested in the resting cell reaction system [7].

2.3. Chemical reagents

Dibenzothiophene, benzoquinone, 2,8-dimethyl dibenzothiophene, 2-hydroxybiphenyl, 3-methyl benzoquinone, 5-methyl benzoquinone, dibenzothiophene sulfone, dibenzothiophene sulfone and n-tetradecane were of analytical grade, commercially available. Sulfones of the benzothiophenes were synthesized by the procedure of Kropp et al. [8]. Other dibenzothiophene and benzothiophene derivatives were synthesized by and obtained from Nard Institute, Ltd. (Hyogo, Japan).

2.4. Gibb's assay and analysis of bacterial metabolites

Desulfurization activity was monitored using Gibb's reagent (2,6-dichloro (or dibromo) quinone-4-chloroimide) [7]. Dibenzothiophene and benzothiophene derivatives and their metabolites were analyzed by gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS) and gas chromatography with atomic emission detection (GC–AED) [9].

2.5. Cloning of dsz genes from Rhodococcus sp. IGTS8

PCR amplification of the DNA region (ca. 3.9 kb) covering from just upstream of a Shine–Dalgarno-like sequence of dszA to about 200 bp downstream of dszC was carried out with total DNA extracted from KA2-5-1 or IGTS8 and primers containing EcoRI recognition sequences. The PCR product of ca. 3.9 kb was digested with EcoRI and cloned in E. coli JM109 with the aid of vector pUC118.

2.6. Purification of DszC enzyme and enzyme assay

Purification of DszC enzyme from the frozen cells of KA2-5-1 was performed according to Ohshiro et al. [10]. The enzyme activity was determined by measuring the amount of dibenzothiophene or benzoquinone sulfone derived from the substrate dibenzothiophene or benzoquinone [10].

2.7. Western blotting

The cell extract of KA2-5-1 was electrophoresed in 12% (w/v) polyacrylamide gel in the presence of SDS and the proteins were electroblotted onto a polyvinylidene fluoride membrane. The proteins were detected with primary rabbit antiserum specific for IGTS8 Dsz enzymes and by the Immunoblotting ABC-POD(R) Kit (Wako Chemicals) according to the manufacturer's instruction.

3. Results and discussion

3.1. Isolation of R. erythropolis KA2-5-1

Enrichment culturing by incubating over 500 soil samples in medium A containing dibenzothiophene as a sole source of sulfur was repeated four times. More than half of the microbial cultures obtained were positive in Gibb's assay. GC analysis with the solvent extracts from these Gibb's assay-positive cultures showed a decrease in the amount of dibenzothiophene and the generation of a peak at 3.74 min, identical with the peak position of 2-hydroxybiphenyl. In the MS analysis, the product gave a signal at m/z 170 corresponding to the calculated molecular mass of 2-hydroxybiphenyl. Colony purification of desulfurizing bacteria from the Gibb's assay-positive cultures was carried out on agar plates containing medium A and 35 strains were isolated. In detailed morphological and physiological tests, 27 bacterial isolates showed properties close to those of Rhodococci. Among the other Gibb's assay-positive isolates identified were Enterobacter cloacae, Stenotrophomonas maltophilia, Corynebacterium aquaticum and Klebsiella planticola. It was found that the desulfurization activities of almost all of the bacterial isolates were markedly unstable during repetitive subculturing. To select stable desulfurizing bacterial strains, we made repeated subculturing of all the isolates in a rich liquid medium (LB medium). After 10 times of subculturing, only six strains still kept a high ability to convert dibenzothiophene into 2-hydroxybiphenyl. Among them, Rhodococcus sp. strain KA2-5-1 exhibited the highest desulfurizing activity and thus was chosen as the most suitable strain for further studies. Nucleotide sequence analysis of the 16S rRNA gene from this bacterium confirmed without doubt that this bacterium was R. erythropolis.

A 3.9-kb DNA fragment was amplified from KA2-5-1 DNA with specific primers which covered the entire region encoding the dszABC genes of IGTS8. Its restriction patterns were quite similar to those of the PCR product amplified from the corresponding region of IGTS8 DNA (data not shown). Nucleotide sequence analysis showed 99.9% homology between the sequences of the PCR-amplified DNA fragments derived from KA2-5-1 and IGTS8 [11]. The extreme homology in the dsz sequences between the two bacterial origins was further supported by Western blotting analysis with the antisera raised against the synthetic peptides designed on the basis of the partial amino acid sequences of IGTS8 Dsz enzymes.
3.2. Reaction of KA2-5-1 against dibenzothiophene and alkyl dibenzothiophenes

To test the desulfurizing activity of KA2-5-1 to alkyl dibenzothiophenes, it was grown in medium A containing dibenzothiophene as the sole sulfur source and the cell suspensions were incubated with various methyl and ethyl dibenzothiophenes dissolved in an equal volume of n-tetradecane. As shown in Table 1, the bacterial cells desulfurized all of the monomethyl, dimethyl, monoethyl, trimethyl, and tetramethyl dibenzothiophenes examined and the desulfurization efficiencies decreased in that order. GC–AED chromatograms of the degradation products showed that all of the alkyl dibenzothiophenes examined had lost their sulfur atoms (data not shown). In GC–MS analysis, the mass ions observed with the metabolites suggested that the alkyl dibenzothiophenes were desulfurized to corresponding monohydroxy alkyl biphenyls. These results indicate that KA2-5-1 can degrade various types of alkyl dibenzothiophenes as well as dibenzothiophene itself in a C–S-bond-targeted fashion.

3.3. KA2-5-1 can also desulfurize alkyl benzo thiophenes

It has been reported that IGTS8 can utilize dibenzothiophene but not benzo thiophene as the sole source of sulfur [12]. In contrast with this, Gordonia sp. strain 213E (NCIMB 40816) can desulfurize benzo thiophene, but is unable to grow in a mineral salts medium containing dibenzothiophene as the sole sulfur source [4]. The benzo thiophene-desulfurization-active 213E cells, which are grown in medium containing benzo thiophene as the sole sulfur source, cannot desulfurize dibenzothiophene. These reports suggest that the enzymatic system responsible for benzo thiophene desulfurization may be different from that for dibenzothiophene desulfurization. To examine the possible coexistence of benzo thiophene and dibenzothiophene desulfurization activities in the same bacterial cells, the utilization of benzo thiophene and its various derivatives by KA2-5-1 cells was tested. KA2-5-1 grew well in medium containing 3-methyl, 2-ethyl or 2,7-diethyl benzo thiophene as the sole sulfur source, suggesting that KA2-5-1 may transform some benzo thiophene derivatives to

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Desulfurization activity (mmol (kg dry cells)^{-1} h^{-1})</th>
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<tr>
<td>Dibenzothiophene</td>
<td>74.2</td>
</tr>
<tr>
<td>4-Methyl dibenzothiophene</td>
<td>44.4</td>
</tr>
<tr>
<td>4,6-Dimethyl dibenzothiophene</td>
<td>29.2</td>
</tr>
<tr>
<td>2,8-Dimethyl dibenzothiophene</td>
<td>27.4</td>
</tr>
<tr>
<td>2-Ethyl dibenzothiophene</td>
<td>29.5</td>
</tr>
<tr>
<td>3-Ethyl dibenzothiophene</td>
<td>26.0</td>
</tr>
<tr>
<td>3,4,6-Trimethyl dibenzothiophene</td>
<td>15.6</td>
</tr>
<tr>
<td>3,4,6,7-Tetramethyl dibenzothiophene</td>
<td>8.68</td>
</tr>
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Fig. 1. Enzymatic conversion of alkyl benzo thiophenes. Purified DszC enzyme (0.32 nmol) and 0.27 mM of the substrates were incubated in the reaction mixture. The enzymatic activities were determined as a function of sulfones produced from their respective substrates at the indicated times. Black circle, dibenzothiophene; half-tone circle, benzo thiophene; black triangle, 3-methyl benzo thiophene; half-tone triangle, 5-methyl-benzo thiophene.

Fig. 2. GC–MS chromatogram (A) of the metabolites of 3-methyl benzo thiophene and mass spectrum (B) of the final metabolite by R. erythropolis KA2-5-1. Peak 1, 2-isopropenylphenol; peak 2, 3-methyl benzo thiophene; peak 3, 2-hydroxybiphenyl; peak 4, 2-methyl 2-(2-hydroxyphenyl) ethene 1-sulfate (2MHPE sul fate). 2-Hydroxybiphen yl is derived from dibenzothiophene, which is a component of the culture media.
release sulphur which can be utilized for its growth. On the other hand, no significant growth was observed when benzothiophene, 2-methyl benzothiophene, 5-methyl benzothiophene, 7-methyl benzothiophene, 7-ethyl benzothiophene or 5,7-dimethyl benzothiophene was added in the medium as the sole sulphur source (Table 2). In conformity with the results obtained with the growing cell system, KA2-5-1 resting cells did not significantly attack benzothiophene and 5-methyl benzothiophene. However, the monooxygenase DszC from KA2-5-1 converted them to corresponding sulfoxides (Fig. 1). With the same enzyme system, other alkyl benzothiophenes such as 2-methyl, 3-methyl, 7-methyl, 2-ethyl, 7-ethyl, 5,7-dimethyl and 2,7-diethyl benzothiophene were also converted to corresponding sulfoxides. These results may be suggestive of the possible involvement of the same enzyme in the bacterial transformation of alkyl benzothiophenes and alkyl dibenzothiophenes.

Fig. 2A shows the GC–MS chromatogram of the metabolites of 3-methyl benzothiophene by KA2-5-1 cells. The main product was identified to be 2-isopropenylphenol by mass spectrum analysis (Fig. 2B). Besides 2-isopropenylphenol, a sulfur-containing peak was also detected. By the mass spectrum analysis of the peak at the retention time of 10.44 min, the component was identified as 2-methyl 2-(2’-hydroxyphenyl) ethene 1-sulphonate (2MHPE sulphonate). On the basis of the data on the analysis of the intermediates and the kinetics, the following pathway can be presumed for conversion of 3-methyl benzothiophene by KA2-5-1: 3-methyl benzothiophene → 3-methyl benzothiophene sulfoxide → 3-methyl benzothiophene sulphone → 2MHPE sulphonate → 2-isopropenylphenol. This pathway fulfills the steps of sequential sulfur oxygenation, thiophene ring cleavage and desulfination. It seems practically the same as the 4S pathway observed in the desulfurization of dibenzothiophene and 213E’s pathway proposed for the benzothiophene desulfurization in terms of the reaction mechanism.

To our knowledge, this is the first report of a microbe that can desulfurize both alkyl dibenzothiophenes and alkyl benzothiophenes. The use of the biocatalyst capable of attacking both forms of thiophenes should be convenient for deeper desulfurization of petroleum and its fractions.

Acknowledgements

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