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Desulfurization of Dibenzothiophene, Benzothiophene, and Other Thiophene Analogs by a Newly Isolated Bacterium, *Gordonia alkanivorans* Strain 1B

Luís Alves, Rita Salgueiro, Carla Rodrigues, Elsa Mesquita, José Matos, and Francisco M. Gírio*

Instituto Nacional de Engenharia, Tecnologia Inovacáo, Departamento de Biotecnologia, Estrada do Paço do Lumiar, 22, 1649-038 Lisboa, Portugal, E-mail: francisco.girio@ineti.pt

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Abstract

A novel bacterium, *Gordonia alkanivorans* strain 1B, was isolated from hydrocarbon-contaminated soil. Assessment of the biodegradation of distinct organic sulfur-compounds, such as dibenzothiophene (DBT), benzothiophene (BT), DBT sulfone, and alkylated tiophenic compounds, as the sole source of sulfur was investigated. *G. alkanivorans* strain 1B was able to remove selectively the sulfur from DBT while keeping intact the remaining carbon-carbon structure. Orthophenyl phenol (2-hydroxybiphenyl) was the only detected metabolic product. The bacterial desulfurization activity was repressed by sulfate. *G. alkanivorans* strain 1B consumed 310 µ*M* DBT after 120 h of cultivation, corresponding to a specific desulfurization rate of 1.03 µmol/(g of dry cells·h). When an equimolar mixture of DBT/BT was used as a source of sulfur in the growth medium, *G. alkanivorans* strain 1B assimilated both compounds in a sequential manner, with BT as the preferred source of sulfur. Only when BT concentration was decreased to a very low level was DBT utilized as the source of sulfur for bacterial growth. The specific desulfurization overall rates of BT and DBT obtained were 0.954 and 0.813 µmol/(g of dry cells·h), respectively. The newly isolated *G. alkanivorans* strain 1B has good potential for application in the biodesulfurization of fossil fuels.

Index Entries: Biodesulfurization; dibenzothiophene; benzothiophene; sulfur; *Gordonia alkanivorans*.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

The increasing utilization of fossil fuels owing to the demand of developed countries has resulted in sulfur oxide emission into the atmosphere being one of the main contributors to air pollution. These oxides are primarily responsible for acid rain owing to their reaction with water in the atmosphere, which results in the formation of sulfuric acid *(1)*. Additionally, sulfur oxides at low concentration in the atmosphere provoke irritation in the human respiratory system and damage plants *(2)*. This problem could be much more severe in the future; the estimated average sulfur content in the world's crude oil reserves is expected to increase 12% from 1990 to 2010 *(3)*.

Consequently, developed countries are imposing increasingly stringent restrictions on the maximum sulfur concentration present in fossil fuels in order to control the emission of sulfur *(4)*. In the European Union, petrochemical plants are mandatory in order to produce fuels of lower sulfur content by 2005 (maximum allowable level of 50 ppm) than the current content (maximum allowable level of 150 ppm). The current physicochemical process used in most refineries, so-called hydrodesulfurization, has technical limitations. It works inefficiently for removing the sulfur from organic aromatic compounds, such as benzothiophene (BT), dibenzothiophene (DBT), and their derivatives *(5)*. Consequently, hydrodesulfurization-refractory sulfur compounds from middle and heavy distillate range fuels pose a significant barrier to reaching very low sulfur levels *(6)*.

In the last two decades, an alternative/complementary biologic process for oil desulfurization involving bacteria with the ability to use either BT or DBT (model compounds) as the only source of sulfur was described *(7)*. However, most of the bacteria that metabolize DBT did not have the ability to degrade BT and vice versa *(8)*. In *Rhodococcus erythropolis* IGTS8, desulfurization of DBT occurs through the so-called 4S metabolic pathway, producing 2-hydroxybiphenyl (2-HBP) as an end product *(9,10)*. On the other hand, the desulfurization of BT by *Gordonia* sp. strain 213E was reported to yield 2-(2'-hydroxyphenyl)ethan 1-al *(11)*. Both metabolic pathways have the advantage of keeping the C-C structure of these sulfur-containing aromatic hydrocarbons intact, maintaining their combustion value. Other DBT-catabolic pathways reported in several other bacteria are not as attractive for industrial purposes because they either mineralize DBT to CO_2 *(12)* or partially destroy the DBT carbon structure *(13)*.

From a practical point of view, a bacterial strain that displays the ability to desulfurize both BT and DBT would be ideal. So far, this ability has been reported only in the bacteria *Paenibacillus* sp. strain A11-2 *(14)*, *Mycobacterium phlei* GTIS10 *(15)*, *Gordonia* strain CYKS1 *(16)*, *Nocardia* sp. strain CYKS2 *(17)*, and *Rhodococcus* spp. strain KT462 *(5)*. However, all of these studies reported only the single utilization of DBT or BT as the sole source of sulfur in the bacterial growth medium.

In this article, we describe the isolation and characterization of a novel strain, *Gordonia alkanivorans* 1B, toward the utilization of several inorganic and organic sources of sulfur for growth. We also discuss the kinetics of DBT and BT degradation by *G. alkanivorans* strain 1B during a mixed sulfur-source growth.

Materials and Methods

Chemicals

DBT (99%) was obtained from Acros Organics, DBT sulfone (97%) and BT (95%) were from Aldrich, 2-HBP was from Sigma, DMF was from Riedel-de Haën, and anthracene and ethyl acetate were from Merck. All other reagents were of the highest grade commercially available.

Culture Media and Growth Conditions

Sulfur-free mineral (SFM) culture medium contained 1.22 g of NH_4Cl, 2.5 g of KH_2PO_4, 2.5 g of $Na_2HPO_4{\cdot}2H_2O$, 0.17 g of $MgCl_2{\cdot}6H_2O$, and 1 L of milli-Q water. To the SFM medium was added 0.5 mL of a trace elements solution without sulfur containing 25 g/L of EDTA, 2.14 g/L of $ZnCl_2$, 2.5 g/L of $MnCl_2{\cdot}4H_2O$, 0.3 g/L of $CoCl_2{\cdot}6H_2O$, 0.2 g/L of $CuCl_2{\cdot}2H_2O$, 0.4 g/L of $NaMoO_4{\cdot}2H_2O$, 4.5 g/L of $CaCl_2{\cdot}2H_2O$, 2.9 g/L of $FeCl_3{\cdot}6H_2O$, 1.0 g/L of H_3BO_3, and 0.1 g/L of KI. For agar medium, 15 g/L of agar was added. Unless otherwise indicated, filter-sterilized glucose (10 g/L) was used as the only carbon source. DBT, BT, and the other hydrocarbons were dissolved in dimethylformamide (DMF) and added to the sterilized medium. All bacterial cultures were performed in shake flasks at 30°C, pH 6.9–7.0, with 150-rpm shaking.

Bacterial Isolation, Identification, and Maintenance

G. alkanivorans strain 1B was isolated from soil samples harvested from hydrocarbon-contaminated grounds at EXPO-98 area (Lisbon, Portugal). Soil samples (5 g) were suspended in sterilized SFM medium (100 mL) and supplemented with 5 g/L of yeast extract and 0.2 m*M* DBT. The slurry was filtered through sterilized filter paper (Whatman no. 42) to remove the major solids. Enrichment growth occurred in 1 wk to increase the number of bacterial cells and was used to seed a selective medium (SFM without yeast extract) containing DBT as the only S-source. From this growth medium, a serial dilution method was used for bacterial isolation. The isolated bacterial strain that showed a higher ability to grow in DBT was selected for further studies. After a classic microbiologic characterization based on colony morphology techniques, the isolate 1B was sent to Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany, for biochemical, physiologic, and chemotaxonomic properties and for a comparison of the first 500 bases among the 16S rDNA of strain 1B and other 16S rDNA from the DSMZ bacterial database. The bacterium

G. alkanivorans strain 1B was routinely maintained on agar SFM medium slants at 4°C in the dark and was transferred into fresh basal medium once a month. For long-term storage, stock cultures on liquid SFM were stored at –20°C medium containing 50% (w/v) glycerol.

Substrate Utilization

To check the ability of *G. alkanivorans* strain 1B to use several sulfur and carbon compounds, two independent sets of experiments of bacterial growth were performed. In the first set of experiments, *G. alkanivorans* strain 1B was cultured in SFM medium supplemented with different sulfur compounds (1 m*M*) using glucose (5 g/L) as the carbon source. In the second set of experiments, different carbon compounds (1 g/L) were used and DBT (200 µ*M*) served as the sole sulfur source.

Analytical Methods

Cell growth was monitored by measuring the optical density (OD) of culture at 600 nm. Dry cell weight (DCW) was determined using 0.22-µm cellulose acetate membranes after 18 h at 100°C. Five hundred microliters of the culture broth was acidified with 25 µL of 4 *M* HCl, and the organic metabolites were extracted with 2 mL of ethyl acetate during a 5-min vortex. After 1 h, 5 µL of the organic fraction was analyzed to detect and quantify DBT, BT, and other metabolic products using a gas chromatograph (Model CP 9001; Chrompack, Middelburg, The Netherlands) equipped with a flame ionization detector. Chromatography was accomplished over 40 min by using an oven temperature of 120°C for 5 min followed by a 4°C/min rise up to 250°C and held for 1 min at this temperature. The injector and detector temperatures were set to 250 and 335°C, respectively. The carrier gas used was nitrogen. In all gas chromatography (GC) measurements, a calibration curve between a known concentration of anthracene (standard) and different concentrations of 2-HBP, DBT, and BT was used. 2-HBP, DBT, and anthracene were separated using a Chromosorb WAW-DMCS column (80–100 mesh) with retention times of 8.2, 14.2, and 15.1 min, respectively. BT and anthracene were separated using a Chromosorb WHP column (100–120 mesh) with retention times of 4.2 and 20.2 min, respectively.

Results and Discussion

Taxonomic Characterization of Strain 1B

The screening of novel bacteria from soil samples contaminated with hydrocarbons allowed the isolation of several bacterial strains. Strain 1B was selected for further studies because it showed better growth when cultured using DBT as the only source of sulfur (data not shown). Strain 1B was an aerobic, Gram-positive, catalase-positive, oxidase-negative, and pink/orange-pigmented bacterium. The cells were shown to be short-

Table 1
Substrate Utilization by *G. alkanivorans* Strain 1B for Various Carbon Compounds[a]

Substrate	Growth (OD_{600})	Final pH
Control	0.23	6.83
Maltose	0.25	6.57
Arabinose	0.21	6.60
Galactose	0.21	6.67
Mannitol	1.08	6.60
Mannose	0.29	6.56
Glucose	1.88	6.37
Sucrose	1.37	6.64
2-HBP	0.26	6.95
Anthrone	0.57	6.92
Benzoate	0.21	6.94
n-Hexadecane	1.18	6.18
Naphthalene	0.23	6.85
Anthracene	0.28	6.93
Tetrahydrofuran	0.46	6.58
DBT sulfone	0.33	6.93

[a]The source of sulfur was DBT (0.2 m*M*). The data were obtained after 5 d of culture and are the average values obtained from triplicates, with an SD <5%. The control assay was carried out from a growth medium containing DBT as dual sources of carbon and sulfur.

branched hyphae, which disintegrated to rods and coccus-like elements when visualized by phase contrast microscopy. Nonmotile cells generally occurred in groups but sometimes singly. This morphologic appearance fitted the original description of *G. alkanivorans (18)*. Moreover, these data were later confirmed by the DSMZ German culture collection, showing that the chemical composition of the bacterial cell wall was based on the *meso* form of diaminopimelic acid and the nocardomycolic acid. Additional analysis of the mycolic acids by high-temperature GC showed that the strain synthesized a homologous series of mycolic acids ranging from C_{54} to C_{60}. The fatty acid pattern revealed unbranched, saturated, and unsaturated fatty acid plus tuberculostearic acid. A 100% sequence similarity of the first 500 bases of the 16S rDNA compared to the strain *G. alkanivorans* DSM 44369^T was also obtained.

Substrate Utilization

Considering the complex composition of fossil fuels in terms of sulfur compounds, it is advantageous that a bacterial strain can desulfurize most of those compounds. The ability of *G. alkanivorans* strain 1B to use several sources of carbon and sulfur was investigated. Table 1 presents the results of bacterial growth and the pH of the culture medium after 5 d of cultivation as a function of different sources of carbon. When DBT was used as the sole

Table 2
Substrate Utilization by *G. alkanivorans* Strain 1B for Various Sulfur Compounds[a]

Substrate	Growth (OD_{600})	Final pH
Control	0.27	6.80
DBT	1.18	6.66
DBT sulfone	1.64	6.66
BT	0.91	6.67
Elemental sulfur	4.61	4.35
2-Methylthiophene	1.53	6.70
2-Mercaptoethanol	1.76	5.33
Potassium sulfate	5.97	4.49
Sodium sulfide	6.13	5.63
Sodium sulfite	5.75	4.49

[a]The source of carbon was glucose (5 g/L). The data were obtained after 5 d of culture and are the average values obtained from triplicates, with an SD <5%. The control assay was carried out from a growth medium containing glucose as the source of carbon and with no source of sulfur added.

carbon and sulfur source (control assay), no significant bacterial growth was detected. Glucose, sucrose, *n*-hexadecane, and mannitol were in a decreasing order the most suitable sources of carbon to support bacterial growth with OD values of >1 unit. Strain 1B showed poor growth on tetrahydrofuran and anthrone, and for the other sources of carbon tested, including DBT sulfone and 2-HBP, no bacterial growth was observed.

Table 2 displays the effect of different sources of sulfur on *G. alkanivorans* 1B growth and the pH of the culture medium after 5 d of cultivation. In the control assay, in which no source of sulfur was added to the glucose mineral medium, no significant growth was obtained. Conversely, *G. alkanivorans* strain 1B showed vigorous growth (>4.5 OD units) on sulfate, sulfide, sulfite, and elemental sulfur and significant growth (>1 OD unit) on DBT, DBT sulfone, BT, 2-methylthiophene, and 2-mercaptoethanol. These results clearly demonstrated that *G. alkanivorans* strain 1B utilized different sources of sulfur-containing aromatic hydrocarbons, including alkylated thiophene, BTP, and DBT, suggesting its ability to decrease the sulfur content of fossil fuels.

Metabolic Pathway of DBT

In most biodesulfurization studies, DBT has been used as the representative molecule (model) of the thiophenic compounds present in the fossil fuels. When considering a practical application of fossil fuel desulfurization, it is important that the biocatalyst be able to use only DBT as the source of sulfur, to avoid weakening of the combustion value of fossil fuels. In this context, the metabolic pathway of DBT used by *G. alkanivorans* strain 1B was studied. Desulfurization of DBT by strain 1B was repressed in the

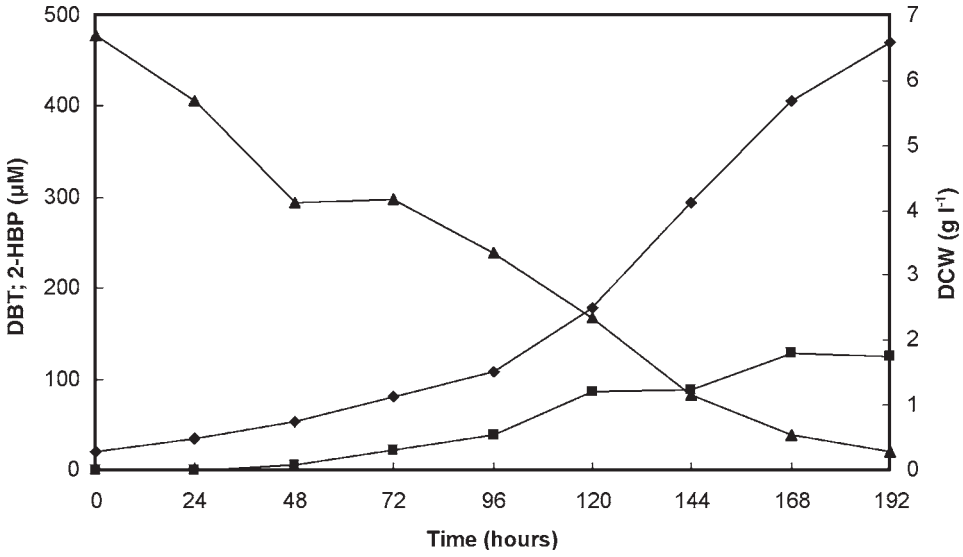

Fig. 1. Time-course of desulfurization of DBT by *G. alkanivorans* strain 1B. The bacterium was cultivated at 30°C, pH 7.0, in SFM medium with 10 g/L of glucose and 500 µ*M* DBT. (◆) DCW; (▲) DBT; (■) 2-HBP.

presence of sulfate in the culture medium (data not shown). This repression effect of desulfurization by sulfate has been described to be characteristic of the 4S metabolic pathway *(19)*. Further chromatographic analysis for DBT-derived metabolites revealed that 2-HBP was the only metabolite detected. Moreover, the bacterium was able to utilize DBT sulfone as the sole source of sulfur, with the end product still being 2-HBP (data not shown). Regarding the use of these thiophenic compounds as potential carbon sources for *G. alkanivorans* strain 1B, Table 1 shows that this bacterium was not able to use DBT, DBT sulfone, or 2-HBP as the sole source of carbon, which excludes the alternative van Afferden et al. *(12)* and Kodama et al. *(13)* pathways. Thus, desulfurization of DBT by *G. alkanivorans* strain 1B can be assumed to proceed as follows: DBT → DBT sulfone → 2-HBP + sulfate. Examination of the described bacterial DBT degradation pathways *(9,12,13)* reveals that the 4S pathway is the only known bacterial pathway where 2-HBP is produced as the end product of the metabolic pathway. Therefore, these results strongly suggest that *G. alkanivorans* strain 1B uses the 4S metabolic pathway for desulfurization of DBT. This proposal is in agreement with the proposed DBT metabolism for other strains of *Gordonia* spp. *(16,20)*.

Kinetics of Consumption of DBT by Strain 1B

Figure 1 displays the kinetics of the consumption of DBT by *G. alkanivorans* strain 1B. The consumption of DBT was observed after the first 24 h of culture, but the accumulation of 2-HBP in cultivation broth was observed only after 48 h, which might suggest the existence of a concentration-

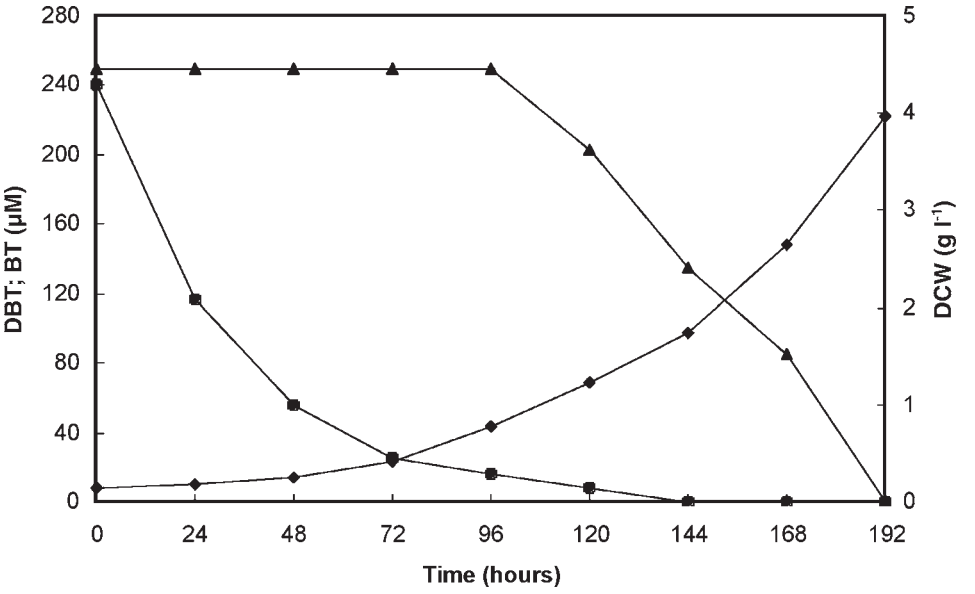

Fig. 2. Time-course of desulfurization of DBT and BT by *G. alkanivorans* strain 1B. Both hydrocarbons were added simultaneously to the culture medium in a concentration of 250 µ*M* each. (◆) DCW; (▲) DBT; (●) BT.

dependent efflux system. The concentration of DBT decreased from 478 to 168 µ*M* after 120 h of culture, corresponding to a specific desulfurization rate of 1.03 µmol of DBT/(g of dry cell·h). This rate is similar to that obtained with another *Gordonia* sp. strain, CYKS1 (0.917 µmol of DBT/[g of dry cell·h]), which reduced the concentration of DBT from 320 to 50 µ*M* after 120 h of bacterial cultivation *(16)*. The maximal extracellular concentration of 2-HBP was about 120 µ*M*, which is only 27% of the consumed DBT (450 µ*M*). This nonstoichiometric accumulation of 2-HBP had also been observed in *Bacillus subtilis* strain WU-S2B *(21)* and in *Nocardia* sp. strain CYKS2 *(17)*. This fact was probably owing to a transient accumulation of 2-HBP (up to a certain threshold) inside the cell during its formation. On the other hand, Wang and Krawiec *(22)* suggested that the difference might be owing to the volatile characteristics of 2-HBP.

Kinetics of Single and Mixed DBT/BT Consumption by Strain 1B

G. alkanivorans strain 1B utilizes both BT and DBT as a single source of sulfur, with DBT the preferred sulfur (Table 2). A *Nocardia* sp. strain (CYKS2) and a *Gordonia* sp. strain (CYKS1) that utilize DBT preferably to BT have also been reported *(16,17)*. Conversely, other bacterial strains have been described to show a faster metabolism of BT compared with DBT *(5,9)*. However, as far as we know, there are not reported data about the bacterial degradation kinetics of mixed DBT/BT sulfur sources.

Figure 2 shows the desulfurizing activity of *G. alkanivorans* strain 1B grown on glucose mineral medium containing DBT/BT as mixed source of

sulfur under equimolar concentrations. The preferred source of sulfur was BT, with DBT used only after 96 h, when the concentration of BT became residual. This suggests the existence of a nonspecific transport system able to uptake both thiophenic compounds, with the higher affinity for BT. For this mixed DBT/BT-containing growth medium, the specific desulfurization rates of BT and DBT were 0.954 and 0.813 μmol/(g of dry cell·h), respectively. One hundred percent of both BT and DBT in the culture medium was consumed after 144 and 192 h, respectively. The strains *Sinorhizobium* sp. KT55 *(23)* and *Rhodococcus* sp. WU-K2R growing on BT as the single source of sulfur only degraded 71 and 59%, respectively, of the initial concentration (100 and 270 μ*M*, respectively) after 5 d of culture *(8)*. The strain WU-K2R also preferentially degraded BT for a BT/naphthothiophene mixture.

It was previously described that BT cannot be desulfurized via the DBT-specific pathway and that DBT cannot be desulfurized through the BT-specific pathway for many bacterial species *(8,11)* despite the fact that their metabolic pathways apparently are rather similar *(24)*. This means that microbial fuel desulfurization technology might need to employ different bacterial systems in a complementary basis for enhancement of the desulfurization rate of the wide range of sulfur sources available in fuel oil. However, the ability of *G. alkanivorans* strain 1B to desulfurize DBT, BT, and other sulfur organic compounds suggests the usefulness of this strain for further genetic improvement of its desulfurization rate.

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