J Ind Microbiol Biotechnol (2007) 34:423–431
DOI 10.1007/s10295-007-0214-8

ORIGINAL PAPER

Comparative studies of phenotypic and genetic characteristics between two desulfurizing isolates of *Rhodococcus erythropolis* and the well-characterized *R. erythropolis* strain IGTS8

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Received: 12 October 2006 / Accepted: 4 February 2007 / Published online: 27 February 2007

Abstract Two *Rhodococcus erythropolis* isolates, named A66 and A69, together with the well-characterized *R. erythropolis* strain IGTS8 were compared biochemically and genetically. Both isolates, like strain IGTS8, desulfurized DBT to 2-hydroxybiphenyl (2-HBP), following the 4S pathway of desulfurization. Strain IGTS8 showed the highest (81.5%) desulfurization activity in a medium containing DBT at 30 °C. Strain A66 showed approximately the same desulfurization activity either when incubated at 30 °C or at 37 °C, while strain A69 showed an increase of desulfurization efficiency (up to 79%) when incubated at 37 °C. Strains A66 and A69 were also able to grow using various organosulfur or organonitrogen-compounds as the sole sulfur or nitrogen sources. The biological responses of A66, A69 and IGTS8 strains to a series of mutagens and environmental agents were evaluated, trying to mimic actual circumstances involved in exposure/handling of microorganisms during petroleum biorefining. The results showed that strains A69 and IGTS8 were much more resistant to UVC treatment than A66. The three desulfurization genes (*dszA*, *dszB* and *dszC*) present in strains A66 and A69 were partially characterized. They seem to be located on a plasmid, not only in the strain IGTS8, but also in A66 and A69. PCR amplification was observed using specific primers for *dsz* genes in all the strains tested; however, no amplification product was observed using primers for carbazole (*car*) or quinoline (*qor*) metabolisms. All this information contributes to broaden our knowledge concerning both the desulfurization of DBT and the degradation of organonitrogen compounds within the *R. erythropolis* species.

Keywords *Rhodococcus erythropolis* · Desulfurization · DBT · Denitrogenation · Petroleum biorefining · Genetic and phenotypic characterization

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Introduction

Fossil fuels contain large amounts of organosulfur and organonitrogen compounds and their presence can lead to the poisoning of catalysts used in the cracking of oil for commercial uses, and to environmental degradation. Whenever the fossil fuels are combusted, sulfur and/or nitrogen oxides are generated and released

to the atmosphere, contributing to acid rain and air pollution [17, 29]. Therefore, petroleum is treated by physical methods, but recalcitrant heterocyclic compounds such as dibenzothiophene (DBT) and their alkylated derivatives cannot be easily removed by the hydrodesulfurization process. Biotechnological upgrading of fossil fuels by microbial desulfurization and denitrogenation is of increasing interest. Both methods, based on naturally occurring bacteria that can remove organically bound sulfur or nitrogen from petroleum, offer the potential for a more selective and cost-effective method for lowering the sulfur/nitrogen content of petroleum products [6, 19]. DBT has been used for the last few decades as a polyaromatic sulfur model for the isolation and characterization of bacteria capable of transforming organosulfur compounds found in a variety of fossil fuels [17, 33], while carbazole has been used as a model for hard-removal heterocyclic nitrogen compounds contained in petroleum oil [13, 22].

A variety of DBT-desulfurizing bacteria have been reported [3, 5, 21, 27 among others]. The first and most extensively characterized is the patented strain *Rhodococcus erythropolis* IGTS8 [8]. This strain is able to extract sulfur from a variety of organosulfur compounds, including thiophenes, sulfides, mercaptans, sulfoxides and sulfones. In particular, IGTS8 can remove sulfur from DBT to yield 2-hydroxybiphenyl (2HBP) as the final product, meaning that there is no degradation of the carbon–carbon bonds in the DBT molecule [4, 11]. In contrast to DBT desulfurization, much less is known about bacteria capable of transforming organonitrogen compounds. Strains of *Ralstonia* sp., *Sphingomonas* sp., *Pseudomonas* sp., *Xanthomonas* sp., *Mycobacterium* sp. and, more recently, *Gordonia* sp. have been described as carbazole-degrading bacteria [13, 22, 27, 30].

Duarte et al. [5] studied the selective effects of sulfur-containing hydrocarbons, with respect to changes in bacterial community structure and selection of desulfurizing organisms and genes in soil. Two strains, denominated A66 and A69, were isolated from a polluted field soil contaminated with crude oil containing a high content of sulfur. Each strain was identified as *Rhodococcus* sp. and their ability to desulfurize DBT has been demonstrated. In the present study, since both isolates, as *R. erythropolis* IGTS8, display potential application in petroleum biorefining, they were characterized biochemically and genetically. We determined that both isolates are able to desulfurize DBT using the 4S pathway. The desulfurization abilities of strains A66 and A69 were compared at 30 and 37 °C with that presented by strain IGTS8, as well as the range of organosulfur and/or organonitrogen compounds that could serve as the sole sources of sulfur and/or nitrogen for strain growth. In addition, we screened for the biological responses of A66, A69 and IGTS8 strains to a series of mutagens and environmental agents. Using these agents, we were able to mimic the actual circumstances involved in the exposure/handling of microorganisms during petroleum biorefining, thus comparing cell sensitivities and mutabilities to chemical and environmental stresses in order to rank the most genetically stable strain. Finally, we also partially characterized the three desulfurization genes (*dszA*, *dszB* and *dszC*) present in strains A66 and A69. All this information contributes to broaden our knowledge concerning both the desulfurization of DBT and the degradation of organonitrogen compounds within the *R. erythropolis* species.

Materials and methods

Bacterial strains

The *Rhodococcus* sp. strains A66 and A69 were previously isolated from soil contaminated with crude oil [5] and were provided by PETROBRAS, a Brazilian oil company. The well-characterized desulfurizing strain *R. erythropolis* IGTS8 was a gift of Kilbane [11] and was used as a control in most experiments. Wild-type *E. coli* strain AB1157 was used as control for cell resistance and DNA repair/damage tolerance [2, 27]. *Pseudomonas* sp. IGTN9m [12] was used as positive control strain in the amplification reaction of the *carAa* and *qorL* genes.

Media and growth conditions

All strains were maintained in Luria-Bertani (LB–1% tryptone, 0.5% yeast extract, 0.5% NaCl) medium at 30 °C. The basal salts medium (BSM) was used to assay the strains for their ability to utilize DBT, carbazole or quinoline, as described before [27]. BSM contained (per liter) 0.2% glycerol, 4 g of $NaH_2PO_4 \cdot H_2O$, 4 g of $K_2HPO_4 \cdot 3H_2O$, 2 g of NH_4Cl, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.001 g of $CaCl_2 \cdot 2H_2O$, and 0.001 g of $FeCl_3 \cdot 6H_2O$. DBT ($0.2\ g\ l^{-1}$), carbazole ($1\ g\ l^{-1}$) or quinoline ($0.5\ g\ l^{-1}$) was added to a sterile medium. DBT was treated with germicidal ultraviolet C (UVC) radiation [14] or dissolved in 2,2,4,4,6,6,7-heptamethylnonane (HMN; 1:50, w/v) and then sterilized by filtration. Cultures (10^7 cells ml^{-1} washed three times in 50 mM potassium phosphate buffer—PBS, pH 7.0) were grown in BSM at 30 and 37 °C at 130 rpm in a shaking incubator for 15 days.

Analytical methods

After 15 days of incubation in BSM, the cultures broths were acidified to pH 2.0 with 2 N HCl and extracted twice with ethyl acetate (0.5 volume of ethyl acetate/volume of supernatant). To determine 2-HBP content and DBT, carbazole or quinoline concentration, the samples were analyzed using gas chromatography (GC model 5890 Hewlett-Packard) equipped with an SE-54 capillary column (30 m × 0.32 mm × 0.25 μm). The flow rate of the carrier gas (hydrogen) was 2 ml min^{-1}. The column temperature was programmed from 50 °C (held 1 min) to 220 °C with an increasing rate of 10 °C min^{-1}. The injector and detector temperatures were 200 and 220 °C, respectively. GC quantification of carbazole, quinoline, DBT and 2-HBP was performed by reference to standard curves with a series of dilutions of these pure compounds. The coefficients of variation observed with all of the above-mentioned compounds were lower than 5%. The molecular masses of the desulfurization products were deduced from their mass spectra. Gas chromatography–mass spectroscopy (GC–MS) analysis was performed using a SHIMAD ZU QP5000, equipped with an HP 5% capillary column (215 m × 0.20 mm × 0.25 μm). The column temperature was programmed from 50 °C (held for 3 min) to 220 °C with an increasing rate of 10 °C min^{-1}. The injector temperature was 200 °C and that of the interface was 200 °C. Mass spectrographs were compared with various libraries of mass spectrograph data prepared from known standard compounds.

Substrate range tests

To determine the range of organosulfur and/or organonitrogen compounds that could serve as the sole source of sulfur and/or nitrogen for growth of the strains tested, we used different compounds (0.1 g l^{-1} for organosulfur and 0.5 g l^{-1} for organonitrogen), which are presented in Table 2. The strains were cultivated in BSM with the various compounds for 10 days.

Treatment with chemical and physical agents

Cultures in the exponential growth phase (~2 × 10^8 CFU ml^{-1} in 10 ml LB) were centrifuged and resuspended in phosphate buffer and treated with increasing H_2O_2 concentrations (0.1 up to 10 M) or 4-Nitroquinoline oxide (30 μg per plate), as previously described [27]. Culture irradiation with UVC was performed with a GE 15-W lamp (G15T8, 254 nm) with increasing doses. After each dose, aliquots (100 μl) were taken, diluted and plated on solid LB. CFU were scored after 24–48 h and survival determined. UVC fluence was measured with a Latarjet dosimeter [27]. The results obtained were analyzed by ANOVA followed by the Student Newman Keuls multiple comparison test using the statistical program InStat version 3.01 (GraphPad Software, San Diego, CA, USA).

Table 1 DBT and DBT/HMN utilization by the *R. erythropolis* strains A66, A69 and IGTS8 incubated at different temperatures

Strains	DBT 30 °C	DBT 37 °C	DBT/HMN 30 °C	DBT/HMN 37 °C
A66	61.9[a]	56.8	45.0	45.0
A69	65.2	79.0	50.0	70.0
IGTS8	81.5	Nd	60.0	Nd

[a] The values correspond to the amount in percentage of substrate consumed after 15 days of incubation. DBT and DBT/HMN were added at 1 mM in BSM medium as the sole sulfur sources. Data are the mean results of the analysis of duplicate samples and the average standard for all data was 5% or less

Nd not determined

Mutagenesis

Spontaneous and induced (UVC) mutageneses were determined using the rifampicin resistance system [16, 27]. Aliquots (100 μl) from untreated (control) and UVC-treated cultures were plated in LB-rifampicin (100 μg ml^{-1}) and in LB to determine mutagenic frequency. Mutagenesis was expressed as the frequency of rifampicin resistant mutants (Rif^R) per 10^8 cells. The results obtained were analyzed by ANOVA, as described above.

Biochemical features

Tests for the utilization of substrates as a sole carbon source were performed with the API 50CH kit (Appareils et Procédés d'Identification, bioMérieux sa, Lyon, France), as described by the manufacturer. The API test galleries were incubated at 30 °C for 2 days.

DNA extraction and plasmid DNA isolation

Total DNA was extracted from strains using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA). DNA concentrations were determined spectrophotometrically using a GeneQuant apparatus (Amersham Pharmacia Biotech, New Jersey, USA). Plasmid DNA was isolated as previously described by Rosado and Seldin [25]. Agarose gel electrophoresis of total and plasmid DNAs was performed in 0.8% agarose gels in Tris–Borate–EDTA (TBE) buffer [26] at 25 V for 20 h at room temperature.

16S rRNA gene sequencing

PCR amplification of the 16S rRNA gene was performed using the primers 27FB 5′-AGAGTTTGA TCATGGCTC AG-3′ and 907RAB 5′-CCGTCAATT CMTTTGAGTTT-3′. The 50 µl reaction mix contained 1 µl of template DNA (50–100 ng), 200 mM Tris–HCl, pH 8.4, 50 mM KCl, 50 pmol of each primer, 0.2 mM of each deoxynucleoside triphosphate, 3 mM $MgCl_2$ and 5 U of Taq polymerase. The amplification conditions were as follows: 30 cycles at 94 °C (1 min 30 s), 50 °C (1 min 30 s) and 72 °C (1 min 30 s). A hot start (3 min at 94 °C) was applied to avoid initial mispriming and to enhance the specificity. A final extension step was run for 15 min at 72 °C and the reaction tubes were then cooled to 4 °C. The PCR product of 16S rDNA from the *Rhodococcus* strains was cloned using the pGEM-T Easy Vector according to the instructions of the manufacturer (Promega, Wisconsin, Madison, WI, USA). After the transformation of *E. coli* JM109-competent cells, clones were picked. Selected clones were then sequenced by using the dideoxynucleotide chain-termination method of a BigDye terminator cycle sequencing FS Ready Reaction kit (Applied Biosystems) and an ABI Prism 3100 automatic sequencer. Alignment was performed using the Clustal X [34], and the BLAST program was used to compare the 16S rRNA gene sequence obtained in this study with sequences deposited in the GenBank [1].

PCR amplification using *dsz*, *qor* and *car* primers

PCR amplification reactions for the *dsz* genes were performed as previously described [5] using the primers dszAf 5′-TCGATCAGTTGTCAGGGG-3′ and dszAr 3′-GGATGGACCAGACTGTTGAG-5′ for the *dszA* gene, dszBf 5′-ATCGAACTCGACGTCCTC AG-3′ and dszBr 3′-GGAACATCGACACCAGGA CT-5′ for the *dszB* gene, and dszCf 5′-CTGTTCGGAT ACCACCTCAC-3′ and dszCr 3′-ACGTTGTGGA AGTCCGTG-5′ for the *dszC* gene. *R.erythropolis* IGTS8 DNA was used as positive control. PCR products were sequenced as described above. The nucleotide sequence data reported have been deposited in the DDBJ/EMBL/GenBank databases. The amplification reaction of the *carAa* gene (involved in carbazole utilization) using the primers carAaf 5′-CTCTTGGCAA ACCATG TGCC-3′ and carAar 3′-CAGCCATCAG CCCGATTCAT-5′ was performed as described by Widada et al. [37]. The amplification reaction of the *qorL* gene (involved in quinoline utilization) was carried out with primers qorLf 5′-CGACCTGGTTT TGTCGGG-3′ and qorLr 3′-AGCGGCGCCTACA GAATCCGCA-5′ designed based on the sequences of this gene present in the Gen Bank. The PCR reaction was performed as follows: a hot start step at 96 °C for 1 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min 30 s and extension at 72 °C for 2 min. A last step of 10 min at 72 °C for the final extension was added. *Pseudomonas* sp. IGTN9m [12] was used as positive control for the amplification reactions of the *carAa* and *qorL* genes. Agarose gel electrophoresis of PCR products was performed in 1.4% agarose gels in TBE buffer at 70 V for 2 h at room temperature.

Southern blotting, probes and hybridization conditions

Southern hybridization was performed using the digoxigenin (DIG) DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany), as recommended by the supplier. Hybridization reactions were performed at 65 °C. DNA probes were obtained after PCR amplification of *Rhodococcus* sp. A69 chromosomal DNA with primers for the three *dsz* genes [5].

Results

Identification of strains A66 and A69

Strains A66 and A69 were previously isolated by Duarte et al. [5] and, at that time, presumptively identified as *Rhodococcus* sp. using a few morphological and biochemical properties. In this study, in an attempt to identify both strains at the species level, partial nucleotide sequences of their 16S rRNA genes were obtained (GenBank accession numbers: EF052850 for A66 and EF052854 for A69) and they showed more than 98% homology to *R. erythropolis*. Production of acid from different carbohydrates was further compared between the isolates and *R. erythropolis* IGTS8 using the API50CH kit. Strains A66, A69 and IGTS8 produced acid from glycerol, ribose, glucose, fructose, inositol, mannitol, sorbitol, esculin and D-arabitol. Acid was not produced from erythritol, D- and L-arabinose, D- and L-xylose, adonitol, β methyl-xyloside, galactose, mannose, L-sorbose, rhamnose, dulcitol, α methyl D-mannoside, α methyl D-glucoside, *N*-acetyl glucosamine, amygdaline, arbutine, salicine, cellobiose, lactose, maltose, melibiose, inuline, melezitose, raffinose, starch, glycogene, xylitol, β gentiobiose, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, L-arabitol, gluconate, 2 ceto and 5 ceto-gluconate in none of the strains tested. Only two carbohydrates were able to differentiate the three strains: A66 was not able to

produce acid from sucrose and IGTS8 was the only strain that showed a positive result from trehalose. From these results, strains A66 and A69 were considered members of the *R. erythropolis* species.

DBT desulfurization activity of *R. erythropolis* A66, A69 and IGTS8

The strains A66 and A69, like *R. erythropolis* IGTS8, desulfurized DBT to 2-HBP, following the 4S pathway of desulfurization. The end-product 2-HBP, present in the A66 and A69 culture supernatants, had been identified by GC-MS (Fig. 1), and its molecular mass was 170. Based on the GC analysis results, DBT utilization in BSM supplemented with 1 mM DBT or DBT/HMN as a sole sulfur source has been demonstrated (Table 1). DBT desulfurization rates were always higher in DBT than in DBT/HMN, and strain IGTS8 showed the highest (81.5%) desulfurization activity at 30 °C. The maximum specific desulfurization activity for strain IGTS8 has been observed at 30 °C [11]. Strain A66 showed approximately the same desulfurization activity either when incubated at 30 or at 37 °C. On the other hand, strain A69 showed an increase in desulfurization efficiency in BSM + DBT (65.2–79%) and in DBT/HMN (50–70%) when the incubation temperature was increased from 30 to 37 °C (Table 1).

Utilization of organic sulfur and nitrogen compounds

Strains A66 and A69 were cultivated in BSM with various organosulfur and organonitrogen substrates as the sole sources of sulfur or nitrogen in growth assays at 30 °C, and the results are listed in Table 2. The range of substrates utilized by both strains for growth is quite broad and the organosulfur compounds metabolized were very similar to the results reported for strain IGTS8 [8]. Strain A69 was able to grow using 2-phenylbenzothiazole, while strain A66 could not. Strain A69 also grew better than A66 in the presence of DBT-sulfone. Besides the sulfur compounds, strains A66 and A69 were also grown in media containing different nitrogen compounds, like carbazole and quinoline (Table 2).

Table 2 Availability of various sulfur and nitrogen compounds to *R. erythropolis* strains, A66 and A69

Organic sulfur or nitrogen compounds	Cell growth	
	A66	A69
Positive Control (BSM)	+++	+++
Dibenzothiophene (DBT)	++	++
DBT-Sulfone	+	++
Benzothiophene	+	+
2-Phenylbenzothiazole	–	+
2-Methyl-β-naphtothiazole	–	–
Thiazole	–	–
2-Methyl-dibenzothiophene	+	+
2-Methyl-thiophene	+	+
Carbazole	+	+
2-Epicatequine	–	–
7-Hydroxicoumarine	–	–
Quinoline	+	+
Imidazole	+	+
Butylpyrrolidine	+	+
Indole	+	+
2,6-Dichloroquinone-4-chloroimidine	–	–
Methylpropylpyrazine	–	–
Methylnaphtothiazole	–	–
Mercaptobenzothiazole	+	+
Catechol	–	–
Methylcatechol	–	–

Growth conditions: BSM medium (sulfur or nitrogen free), incubation during 10 days at 30 °C, 130 strokes/min. Concentration of the compounds 0.1 g l^{-1} (sulfur compounds) and 0.5 g l^{-1} (nitrogen compounds). Cell growth (+++) good; (++) regular; (+) scarce and (-) no growth

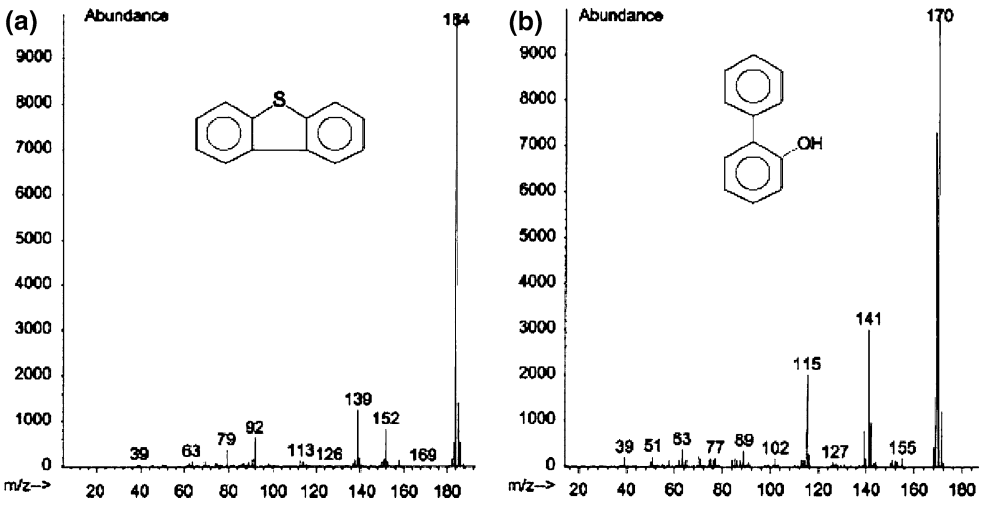

Fig. 1 GC–MS analysis of desulfurized DBT metabolites produced by *Rhodococcus erythropolis* strains A66 and A69. **a** DBT molecular mass, 184; **b** 2-HBP molecular mass, 170

The ability of strains A66 and A69 to metabolize carbazole or quinoline as nitrogen sources was further investigated by growing these strains in the presence of both compounds separately (6 mM carbazole and 3.87 mM quinoline), for 10 days at 30 and 37 °C, and then followed by GC analyses. Table 3 shows the results obtained using the two strains and also the ability of strain IGTS8 to grow using carbazole as a nitrogen source. Carbazole utilization was higher at 37 °C than at 30 °C for strains A66 and A69, showing an increase of nitrogen utilization from 19.3 to 28.5% and from 19.8 to 33.3%, respectively.

Physiological features of A66, A69 and IGTS8

Strains A66, 69 and IGTS8 were evaluated concerning their sensitivity to different physical and chemical agents, such as 4NQO, H_2O_2, and heat (52 °C for 2 h). All the strains tested were as sensitive as the wild-type *E. coli* strain AB1157, and displayed equivalent survival levels to these agents (data not shown). However, A69 and IGTS8 were much more resistant to UVC treatment than A66 and AB1157 strains ($P < 0.05$) (Fig. 2). AB1157 survivors were less than 0.02%, while A66, A69 and IGTS8 survivors were 0.07, 2.0 and 0.08%, respectively, after the UVC treatment (120 J m^{-2}). Considering survival levels at $LD_{1\%}$, the results point out that A66, IGTS8 and A69 strains are approximately 1.15, 1.6 and 2.0 times more resistant than AB1157 strain to UVC irradiation. Moreover, strain A69 was also more resistant to UVC than IGTS8 strain. Survival values observed for A69 at 80 and 120 J m^{-2} (Fig. 2) were statistically different from IGTS8 ($P < 0.05$).

In addition to the particular resistance against UVC toxicity, A66, A69 and IGTS8 were also extremely refractory to UVC-induced mutagenesis. While AB1157 strain had more than 100-fold increase in mutagenesis after UVC (30 J m^{-2}), in A66 and A69, UVC-mutagenic increments were not greater than 4-fold (Table 4).

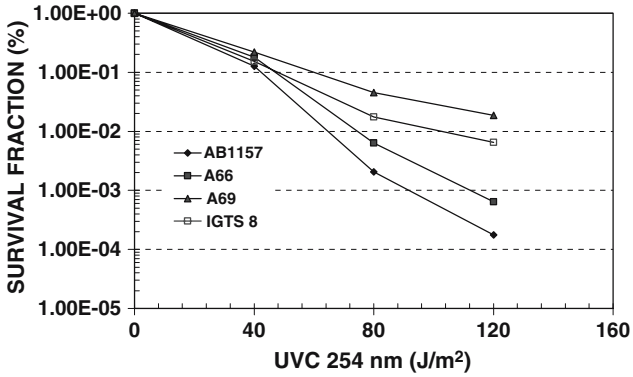

Fig. 2. Survival of *E. coli* AB1157, *Rhodococcus erythropolis* strains A66, A69 and IGTS8 strains after UVC treatment. Cultures in exponential growth phase were treated with increasing doses of UVC radiation (254 nm) as described in Materials and methods

Plasmid localization of *dsz* genes in strains A66 and A69

The *dsz* cluster (*dszA*, *dszB* and *dszC*) of *R. erythropolis* IGTS8 is located on a 120-kb linear plasmid [23]. In this study, plasmid DNA could be detected not only in IGTS8 but also in A66 and A69 strains. Using the *dszA*, *dszB* and *dszC* probes, the bands of the same size, corresponding to plasmid DNA, hybridized to the probes used in all the strains tested (data not shown).

PCR amplification using *dsz*, *car* and *qor* primers

The results obtained via PCR using the *dsz* primers designed by Duarte et al. [5] and DNA from

Table 3 Carbazole and quinoline utilization by the *R. erythropolis* strains A66, A69 and IGTS8 incubated at different temperatures

Strains	Carbazole 30 °C	Carbazole 37 °C	Quinoline 30 °C	Quinoline 37 °C
A66	19.3[a]	28.5	20.0	24.8
A69	19.8	33.3	29.9	30.2
IGTS8	25.0	Nd	0	Nd

[a] The values correspond to the amount in percentage of each substrate consumed after 15 days of incubation. Carbazole and quinoline were added at 6 mM and 3.87 mM, respectively, in BSM medium as the sole nitrogen sources. Data are the mean results of the analysis of duplicate samples and the average standard for all data was 5% or less

Nd not determined

Table 4 Spontaneous and UVC-induced mutagenesis in *E. coli* AB1157 compared to *Rhodococcus erythropolis* strains A66, A69 and IGTS8

AB1157	
0	2
30 J/m^2	263 (131)[a]
A69	
0	3
30 J/m^2	5 (1.6)
A66	
0	3
30 J/m^2	12 (4.0)
IGTS 8	
0	4
30 J/m^2	9 (2.25)

[a] The number in parenthesis is the fold increment over the spontaneous levels

R. erythropolis IGTS8, A66 and A69 showed PCR fragments of approximately the same size for *dszA* (560 bp), *dszB* (420 bp) and *dszC* (390 pb) for all strains tested (data not shown). PCR fragments were sequenced [GenBank accession numbers: A66–EF052851 (*dszA*), EF052852 (*dszB*) and EF052853 (*dszC*) and A69–EF052856 (*dszA*), EF052857 (*dszB*) and EF052858 (*dszC*)] and BLAST analysis confirmed their homology to *dszA*, *dszB* and *dszC* genes. No amplification product was found for *carA* or for *qorL* primers in IGTS8, A66 and A69, while both expected products were obtained using *Pseudomonas* sp. IGTN9m DNA.

Discussion

In this study, two strains isolated from an oil-contaminated soil by Duarte et al. [5] were identified as *R. erythropolis* after the nucleotide sequence analysis of part of their 16S rRNA genes. These strains (A66 and A69) showed DBT desulfurizing activity and, therefore, were compared with the well-known *R. erythropolis* strain IGTS8 [4, 8]. Phenotypic and genetic characteristics were used to broaden the understanding of biodesulfurization trait at the intra-species level. The three strains showed very similar fermentation pattern of 49 carbohydrates on the API 50 CH strip; however, acid production from sucrose (negative for A66) and trehalose (positive only for IGTS8) could be used to differentiate the strains tested.

All the strains showed the conversion of DBT to 2-HBP via the 4S pathway [21, 24]. However, specific desulfurization activity of IGTS8 was higher than that of the two other strains when they were tested in BSM supplemented with 1 mM DBT as a sole sulfur source at 30 °C. One explanation for the higher desulfurization activity presented by IGTS8 may be due either to the continuous passage of this culture in its laboratory of origin (Gas Technology Institute, Des Plaines, IL, USA) for the past 12 years under conditions where DBT served as the sole sulfur source for growth, or to differences in desulfurization phenotypes that exist within the same genus [36]. The first hypothesis has been suggested by Srinivasaraghavan et al. [32] to explain differences in DBT desulfurization efficiency between two strains of *Mycobacterium phlei*. When the desulfurization rates were determined in BSM + DBT at 37 °C, A69 showed an increase in the desulfurization activity, while A66 did not change its profile. As strains A66 and A69 can grow and desulfurize at 37 °C, this extends the differences observed between these strains belonging to the same species, and corroborates with the intra-species differences observed by Srinivasaraghavan et al. [32]. Various reports have demonstrated that the maximum desulfurization activity for *R. erythropolis* strain IGTS8 was observed at 30 °C [14, 15, 20]. Moreover, DBT dissolved in HMN has been also tested as a model oil system as proposed by Setti et al. [28]. The use of DBT dissolved in a hydrocarbon resulted in an increase of the desulfurization rate in *Nocardia* sp. [3]; however, in our case, all three strains showed a decrease in the desulfurization rates.

Strains A66 and A69 were cultivated in BSM with various organic sulfur and nitrogen compounds (Table 2). The strains grew on thiazole derivatives, sulfides and disulfides. Similarly to *Nocardia* sp. CYKS2 [3], but different from other strains previously reported [7, 21], it could utilize methylthiophene and benzothiophene. *R. erythropolis* IGTS8 has been previously reported to be unable to utilize thiphene and/or benzothiophene [14, 15, 20]. Besides the sulfur compounds, strains A66 and A69 were also grown in different nitrogen compounds. Both strains were able to grow with carbazole and with quinoline as the sole nitrogen sources. The selective removal of nitrogen from petroleum is a relatively neglected topic in comparison with sulfur removal. There is currently no biochemical pathway, or thermochemical process, for the selective removal of nitrogen from compounds typically present in petroleum [31]. Carbazole is a good model that is representative of nitrogen-containing compounds present in greatest abundance in many petroleum samples [18]. However, there is no known culture able to selectively cleave both C–N bonds in carbazole, leaving the rest of the molecule intact. More recently, a microorganism capable of selectively cleaving C–N bonds in quinoline and removing nitrogen from petroleum was isolated and partially characterized [12].

To better characterize the biological responses of strains IGTS8, A66 and A69 to a series of mutagens and environmental agents, we first evaluated their sensitivities to physical and chemical agents. Based on the results obtained, we can assume that A69 could be safely handled in outdoor environments, which are particularly exposed to high incidence of solar UV. It is important to emphasize that solar UV toxicity is primarily attributed to pyrimidine dimers, which are, incidentally, the main induced lesions in DNA after UVC irradiation [10]. These physiological characteristics prompt A69 to be a new potential alternative microorganism for biorefining purposes, given that it is resistant to environmental threats and is also endowed with high-fidelity DNA repair systems conferring genetic stability.

The results obtained via PCR using the *dsz* primers designed by Duarte et al. [5] and DNA from strains

IGTS8, A66 and A69 showed PCR fragments of the same size for *dszA* (560 bp), *dszB* (420 bp) and dszC (390 pb) for all strains tested, suggesting that the DNA sequences of the desulfurization genes are highly similar. Probes were also generated for *dszA*, *dszB* and *dszC* genes of the strain A69 by DIG-labeling the corresponding PCR products. In this study, plasmid DNA could be detected in all the strains tested, and using *dszA*, *dszB* and *dszC* as probes, the band corresponding to the 120 kb plasmid DNA known to encode for *dszABC* operon in *R. erythropolis* IGTS8 [23] was hybridized to the probes. The presence of same plasmid has already been demonstrated in *Mycobacterium* sp., which is phylogenetically closely related to *Rhodococcus* [9]. On the other hand, no amplification product was found for *carA* or *qorL* primers in all strains, although the expected product was obtained using *Pseudomonas* sp. IGTN9m DNA. In fact, no product was expected after PCR amplification using *qorL* and the IGTS8 DNA, as this strain was not able to utilize quinoline as a nitrogen source. In the cases where a PCR product was expected, it is possible that gene rearrangements occurred through evolutionary divergence in the natural environment and a different genetic organization of the *car* and *qor* gene clusters exists.

In the present study, the potential of the strains A66 and A69 to fulfill the major requirements needed for biorefining is demonstrated. Their inherent genetic stability should be considered very important, as well as the ability to grow using quite a broad range of organosulfur compounds, including 2-phenylbenzothiazole and DBT-sulfone. To be commercially useful, desulfurizing bacteria must be capable of degrading the sulfur content of oil, which contains a broad range of organic sulfur compounds [6]. Furthermore, these cultures can also cleave carbon–nitrogen bonds in aromatic organic compounds typically found in petroleum, such as carbazole and quinoline. These data broaden our knowledge of the *R. erythropolis* species, and new strains other than the well-characterized IGTS8 are now available as an alternative for the development of a commercially viable biorefining process. However, we still need a far better understanding of how these strains metabolize nitrogen compounds and also sulfur heterocyclic compounds through the Dsz pathway. A more in-depth view of the enzymology of the biorefining process is essential to overcome problems related to substrate acquisition, the supply of reducing equivalents and enzyme turnover rates for specific substrates [6]. The emergence of new in vitro tools for mutation and genetic rearrangement has enabled the limits of the biodesulfurization system to be extended, and directed evolution techniques have been applied to improve both the rate and extent of biodesulfurization by derivatives of *R. erythropolis* strain IGTS8 [35]. Application of these techniques, using new strains that are able to catalyze oxidative desulfurization and/or denitrogenation of a broad range of organic sulfur/nitrogen compounds, as those presented here, promises to improve both the range and robustness of these microbiologically catalyzed chemical transformations. However, we still have to consider that the commercial implementation of biorefining can be hampered by cost-effectiveness and also by the difficulty to integrate the bioprocess into the existing refining processes.

Acknowledgments This study was carried out as a part of the project of characterization of strains capable of desulfurization and denitrogenation, which was supported by PETROBRAS and FINEP (CTPETRO 00/2001).

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