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1 **Development of molecular driven screening for desulfurizing microorganisms targeting**
2 **the *dszB* desulfinase gene**

3

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12 Abstract

13 CONsensus DEgenerate Hybrid Oligonucleotide Primers (CODEHOP) were developed for the
14 detection of the *dszB* desulfinase gene (2'-hydroxybiphenyl-2-sulfinate desulfinase; EC
15 3.13.1.3) by polymerase chain reaction (PCR), which allow to reveal larger diversity than
16 traditional primers. The new developed primers were used as molecular monitoring tool to
17 drive a procedure for the isolation of desulfurizing microorganisms. The primers revealed a
18 large *dszB* gene diversity in environmental samples, particularly in diesel-contaminated soil
19 that served as inoculum for enrichment cultures. The isolation procedure using the
20 dibenzothiophene sulfone (DBTO₂) as sole sulfur source reduced drastically the *dszB* gene
21 diversity. A *dszB* gene closely related to that carried by *Gordonia* species was selected. The
22 desulfurization activity was confirmed by the production of desulfurized 2-hydroxybiphenyl
23 (2-HBP). Metagenomic 16S rRNA gene sequencing showed that the *Gordonia* genus was
24 represented at low abundance in the initial bacterial community. Such observation highlighted
25 that the culture medium and conditions represent the bottleneck for isolating novel
26 desulfurizing microorganisms. The new developed primers constitute useful tool for the
27 development of appropriate cultural-dependent procedures, including medium and culture
28 conditions, to access novel desulfurizing microorganisms useful for the petroleum industry.

29

30

31 Keywords: desulfurization, dibenzothiophene sulfone, 16S rRNA gene, *dszB* desulfinase
32 gene, *Gordonia*

33

34 1. Introduction

35 Sulfur is essential for living organisms since it is involved in enzyme catalytic sites,
36 protein structure, and respiratory chain. In the environment sulfur is found under various
37 forms, sulfate being the most readily available source of sulfur [1]. Organic compounds
38 containing sulfur atom constitute also potential sulfur sources such as sulfonate in which
39 sulfur is covalently bond to one carbon [2], and as sulfide in dibenzothiophene (DBT) where
40 the sulfur atom is bond to two carbons trapped in a heterocycle and surrounded by two
41 benzene cycles [3]. In these conformations the sulfur is difficult to access [4].
42 Microorganisms have developed several strategies to break C-S bond, depending on the
43 substrate, in order to access and assimilate the sulfur atom [5–9]. For DBT, three microbial
44 pathways that release the sulfur atom have been reported. In the Kodama pathway, the
45 benzene rings cleaved by oxygenases are then completely mineralized in carbon dioxide
46 (CO₂) releasing sulfate that serves as easily assimilable sulfur source [10,11]. In the Van
47 Afferden pathway, after an oxidative attack on the sulfur heteroatom, the DBT is completely
48 mineralized [12]. In the “4S” desulfurizing pathway, the sulfur atom is specifically extracted,
49 producing desulfurized 2-hydroxybiphenyl (2-HBP) and sulfur as sulfite, readily oxidized in
50 sulfate and consumed [13].

51 For the last three decades, DBT desulfurizing microorganisms have attracted the
52 scientific community because they present a potential for the development of petroleum
53 desulfurization processes [14–16]. Because of the relative low sulfur content in biomass, in
54 comparison to carbon and nitrogen, isolation of desulfurizing microorganism is challenging
55 [17,18]. The main difficulty for isolating desulfurizing microorganisms is that they are in
56 competition, for thiophenic compounds consumption, with hydrocarbon-degrading
57 microorganisms implementing the Kodama pathway [19]. It has been demonstrated that a
58 carbon to nitrogen ratio below five favours the Kodama pathway over the "4S" pathway [19].

59 Additionally, the sulfate released by the "4S" pathway is assimilated by non-desulfurizing
60 microorganisms [13,18]. Thus, the desulfurizing microorganisms are found in low abundance
61 (10 to 1000-fold less abundant than non-desulfurizing microorganisms) in microbial
62 communities even when DBT is the sole sulfur source [18,20].

63 The *Rhodococcus erythropolis* IGTS8 is considered as model microorganism for the
64 study of the "4S" desulfurizing pathway [21]. The "4S" desulfurizing pathway involves the
65 enzymes DszA, B, C, and D coded by the *dsz* operon [22]. The DszB, the desulfinase (2'-
66 hydroxybiphenyl-2-sulfinate desulfinase; EC 3.13.1.3) breaks the last C-S bond as final step
67 [18,23,24]. Because DszB has the narrowest substrate spectrum [25] it is a limited enzyme for
68 industrial application of the biodesulfurization process. However, DszB homologs have been
69 reported to desulfurize alkylated DBTs, BTs or to perform desulfurization in thermophilic
70 conditions [26–30], enlarging the substrate spectrum, which increases the potential for
71 biodesulfurization process. It is thus of paramount importance to develop innovative
72 approaches in order to discover novel DszB enzymes. The information on *dszB* diversity is
73 limited because most studies were based on the *dszB* gene sequence from *R. erythropolis*
74 IGTS8 [20,31]. Recent advances in molecular biology revealed novel *dszB* gene homologs
75 exhibiting 66 – 84% similarity to that of *R. erythropolis* IGTS8 [27,30,32], expanding
76 knowledge on *dszB* gene diversity. Such information is useful for the development of new
77 primers targeting a wide range of *dszB* genes, marker of desulfurizing potential, and for
78 constituting tools to monitor the presence of desulfurizing microorganisms in environmental
79 samples and enrichment cultures. The use of COnsensus DEgenerate Hybrid Oligonucleotide
80 Primers (CODEHOP) approach presents the advantage to reveal larger diversity of the target
81 genes [33]. The CODEHOP approach has been successfully used for revealing large diversity
82 of ring hydroxylating dioxygenase gene [34], but yet not employed for *dsz* genes.

83 This study aimed at developing molecular tools for monitoring the enrichment
84 procedure, at both taxonomic and functional levels, for the selection of bacteria containing
85 *dszB* genes. For this purpose enrichment cultures with DBT-sulfone (DBTO₂, the second
86 metabolite of “4S” desulfurizing pathway, avoiding the first step from DBT to DBTO₂, which
87 kinetic is limiting and energy consuming), as a sole sulfur source were performed with
88 samples from various environments. The taxonomic diversity shift in the prokaryotic
89 communities was followed through 16S rRNA gene sequencing. Polymerase Chain Reaction
90 (PCR), the novel CODEHOP based approach targeting *dszB* genes allowed the detection of
91 rare homologs in soil sample, compared to traditional primers. The enrichment cultures were
92 driven by detecting the presence of *dszB* gene with the newly developed PCR method
93 combined with the detection of DBTO₂ metabolite production, until bacterial isolates able to
94 desulfurize DBTO₂ were obtained. The present study will provide tools for accessing novel
95 desulfurizing microorganisms able to remove organosulfur from oil fractions without
96 degrading the carbon skeleton, requested as eco-technology for the petroleum industry.

97

98

99 **2. Material and Methods**

100 ***2.1. Source of microorganism and samples collection***

101 The strain *Rhodococcus erythropolis* IGTS8 was obtained from American Type
102 Culture Collection and used as positive control for metabolic activity and gene detection.
103 Various soils and sediments (30 ml) in contaminated areas were sampled in France, using
104 sampling sterile cores. Samples were maintained at 4°C before culture set up, and at -20°C for
105 molecular analysis.

106

107 *2.2.DNA extraction, PCR amplification, cloning and sequencing*

108 The DNA was extracted from environmental sample (250 mg) by using the DNeasy
109 PowerSoil kit following instructions of the provider (QIAGEN, Germany).

110 The V4-V5 region of 16S rRNA gene was amplified using 515F [35] and 928R [36]
111 primers (Table 1). The PCR mixture consisted on: 1 µl of extracted DNA, 9.5 µl of DEPC-
112 treated water, 1 µl of each primer at 20 µM, 12.5 µl AmpliTaq Gold 360 Master Mix 2X
113 (Thermo Fisher Scientific, USA). The 16S rRNA gene fragment amplification was performed
114 through 35 cycles of 94°C (45 s), 60°C (1 min) and 72 °C (1.5 min), with a hot start (94°C, 3
115 min) and a final extension step (72°C, 10 min). The 16S rRNA gene amplicon sequences were
116 performed by using the Illumina sequencing Genotoul platform (Toulouse, France).

117 Functional diversity was assessed by targeting *dszB* homologs using two sets of
118 primers: (i) the traditional primers, modified Sul2F [37] and BR [38], based on the *R.*
119 *erythropolis* IGTS8 sequence; and (ii) a new set of primers designed following the
120 CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) procedure [33] (Table
121 1). The steps for CODEHOP primers design are described in details in the Results and
122 Discussion section (Fig. 1). Briefly, primer design was based on conserved blocks of amino
123 acids (aa) within 352 aligned DszB amino acid sequences retrieved from the GenBank and
124 aligned with ClustalW (MEGA version 7.0). The j-CODEHOP design software, an integrated
125 tool into Base-by-Base [39], was used to design the primers. The length of the nondegenerate
126 5' clamp region was set to 15 to 18 base pairs (bp). The length of degenerated 3' region was
127 set to 2 to 3 amino acids. The specificity of CODEHOP primers was confirmed by *in silico*
128 analysis.

129 The reaction mixture contained 10.5 µl of DEPC-treated water, 0.5 µl of each primer
130 at 20 µM, 12.5 µl of AmpliTaq Gold 360 Master Mix 2X (Thermo Fisher Scientific, USA)

131 and 1 μ l of extracted DNA template. Amplification conditions with CODEHOP were as
132 follows: 35 cycles of 94°C (45 s), 58°C (45 s) and 72°C (1 min). A hot start (10 min at 94°C)
133 was applied to avoid mispriming. A final extension step was run for 10 min at 72°C and the
134 PCR tubes were stored at 4°C. PCR were performed in triplicate and products were pooled
135 before applications. The *dszB* amplicons obtained with both traditional primers or CODEHOP
136 were purified with the GFX PCR DNA and Gel Band Purification kit (GE Healthcare, USA)
137 and inserted into plasmid vector and cloned in *E. coli* using the PCR-TOPO Cloning kit
138 (Thermo Fisher Scientific, USA) following the instructions of the manufacturer. The size of
139 cloned amplicons was examined by migration on electrophoresis gel (1% agarose, 7.7 V.cm⁻¹,
140 25 min). Amplicons with expected size were Sanger sequenced at the Eurofins GATC Biotech
141 platform (Konstanz, Germany).

142

143 *2.3. Sequence and diversity analysis*

144 Illumina sequencing generated 32,990 reads, partitioned in two datasets corresponding
145 to the soil sample and the last enrichment culture DNAs. 16S rRNA gene sequence analyses
146 were performed using QIIME2 software (Quantitative Insights Into Microbial Ecology, [39]).
147 The datasets were demultiplexed and denoised, using dada2 package and truncating reverse
148 sequence to 237 bp. These cleaned sequences were then clustered in representative sequences,
149 in such a way to get 197 Amplicon Sequence Variants (ASVs). ASVs were classified
150 according to their taxonomical assignation at the sequence similarity threshold of 97% using
151 SILVA database (v132). Singletons, chloroplasts, mitochondria and unassigned at the domain
152 level were removed from datasets, resulting in 190 ASVs.

153 Regarding *dszB* amplified fragments, sequences were aligned with reference *dszB*
154 genes found in the NCBI database (accession numbers: AB033997, JQ657805, CP003347,

155 AB076745, KP202690, JF740062, AB070603, KT630580, EF026089, AY960127,
156 AY714057, AY278323, DQ444325, L37363, DQ062161, AY789136, AY714059, KJ021035,
157 AY678116, KC693733, EU364831, AY396519, AJ973325, CP035924) using the BioEdit
158 software (v7.5.2.0). Low quality reads were discarded (<300-bp length), then vector and
159 primers sequences were removed. The remaining reads were compared to the NCBI non-
160 redundant database using BLAST to ensure the top hit (sequence similarity) was a homolog
161 *dszB* gene. These reads were then sorted in cluster at 97% nucleotide sequence similarity, as
162 suggested by[40]. Diversity indexes were calculated using *vegan* and *diversity* packages on R.

163

164 *2.4.Enrichment and Isolation procedure*

165 Sulfur free medium (SFM) for enrichment cultures contained (l⁻¹ deionized water):
166 KH₂PO₄ 2.44 g, Na₂HPO₄ 5.44 g, NH₄Cl 1.22 g, MgCl₂·6H₂O 0.2 g, CaCl₂·2H₂O 0.001 g,
167 FeCl₃·6H₂O 0.001 g; 12.6 g/l of glucose were used as carbon source as used for growing
168 desulfurizing bacteria [41] and SFM was supplemented with 0.1% sulfur free trace element
169 solution SL-10 [42]. DBTO₂ dissolved in dimethylformamide at 0.1 M was added as sulfur
170 source at a final concentration of 300 μM. Enrichment procedure was performed through 6
171 successive transfers of 5-days cultures to 10 ml of fresh SFM medium, at 30°C at 150 rpm
172 [43].

173 After 6 sub-cultivations, the cultures were diluted with saline solution (0.9 %) from
174 10⁻⁷ to 10⁻³ factor, then spread onto SFM agar plate and incubated at 30°C. Separated colonies
175 were peaked and streaked onto LB agar medium plate to ensure their purity.

176

177 *2.5.Analytical methods*

178 Hydrophobic compounds were extracted by mixing culture with ethyl acetate (1:1 v/v)
179 containing pyrene as internal standard (1 mM) after shaking for 30 min. DBTO₂ metabolites
180 were detected in extracts by gas chromatography equipped with Flame Ionization Detector
181 (GC-FID, GC 6850 Agilent Technologies), HP-1 capillarity column (30 m*0.25 mm*0.25
182 μm). Helium (1 ml/min) was used as carrier gas and 1 μl of extract was injected with a split
183 ratio of 1/50. Column temperature varied from 200 to 240 °C, increasing by 5°C/min and
184 maintaining 240°C for 1 min. Flame Ionization Detector was settled at 290°C.

185

186 *2.6. Sequence data*

187 The sequence data generated in this study have been deposited in the EMBL sequence
188 database under the accession numbers MT900068-MT900251 for *dszB* gene sequences. The
189 complete dataset for 16S rRNA gene sequences was deposited in the NCBI Sequence Read
190 Archive (SRA) database (SUB7938384). It is available under the Bioproject ID
191 PRJNA658137.

192

193 **3. Results and Discussion**

194 *3.1. Consensus-degenerate hybrid oligonucleotide primers (CODEHOP) design targeting* 195 *desulfurizing microorganisms*

196 In order to detect a large diversity of *dszB* genes, a new primer set was designed
197 following CODEHOP procedure [33]. An alignment of DszB homolog protein sequences was
198 performed. Interestingly, although the ten known amino acid residues involved in the DszB
199 enzymatic activity were found to be conserved among the aligned sequences, two sequences
200 exhibited amino acid replacements. In the DszB sequence of *Gordonia* sp. RIPI (AJ973326),

201 the Arg70 was replaced by Cys, while in *Paenibacillus* sp. A11-2 (AB033997) the Gly183
202 was replaced by Trp. These amino acid residue replacements are not conservative and
203 therefore might affect the protein properties, conformation and activity as previously
204 demonstrated by site-directed mutagenesis of amino acid residues at position 63 and 65 for
205 the DszB of *R. erythropolis* KA2-5-1 [44]. The alignment identified 11 blocks of conserved
206 amino acids (Fig. 1). Looking for degenerated primers with a hybridization temperature
207 around 62°C, 3 blocks coded by 3 nucleotide sequences were obtained (Fig. 1). The block
208 *LGSWEAR* contains 2 residues, Leu152 and Trp155, which were found to be involved in
209 hydrophobic interaction with the substrate in *R. erythropolis* IGTS8 through hydrophobic
210 interaction with benzene cycles [45]. The corresponding consensus nucleotide sequence was
211 defined as forward primer (codF). The other two blocks, *TQQFLLDHQL* and *QQFLLDHQLL*
212 (Fig. 1), located near the carboxylic extremity of DszB homologs showing large overlap,
213 served to design the reverse primers. The corresponding consensus nucleotide sequences were
214 named respectively codR1 and codR2. The efficiency of the new designed primers to amplify
215 *dszB* fragments was verified using DNA template from *R. erythropolis* IGTS8, and a fuel
216 contaminated soil sample (RITA3). The *dszB* codF/R1 primer lead to a single amplification of
217 expected size (576 bp) using DNA from pure strain and soil samples, whereas codF/R2
218 primer lead to amplification of a fragment (579 bp) only from pure strain DNA. Cloning and
219 sequencing of PCR products obtained with codF/R1 primer from soil DNA showed 68 to 85%
220 of nucleotide identity with *dszB* gene of *Rhodococcus erythropolis* strain IGTS8, resulting in
221 the amplification of new *dszB* homologs. The *in silico* analysis confirmed the CODEHOP
222 primers specificity for *dszB* genes. Thus, this primers pair, allowing amplification from both
223 model strain and significantly different *dszB* homolog, was selected for further experiments.

224 In order to further validate the CODEHOP primers, the *dszB* gene diversity within the
225 microbial communities from a soil sample (RITA3) assessed by the CODEHOP (codF/R1)

226 primers was compared with that obtained by traditional (Sul2F/BR) primers. After cloning
227 and sequencing 189 sequences were obtained, among them 95 with traditional primers and 94
228 with the CODEHOP primers. 186 sequences were related to *dszB* homologs. The 3 remaining
229 sequences did not exhibit any similarity with known *dszB* homolog or sulfinase gene, at both
230 nucleotide and amino acid levels, indicating that they result from unspecific amplification.
231 The CODEHOP approach showed similar rate of specific amplification (98.5 %) to that of
232 traditional primer. However, the CODEHOP primers revealed greater diversity than the
233 traditional primers (Table 2). The 371 *dszB* sequences were dispatched in 11 variants (Fig. 2),
234 the three most dominant variants being affiliated to *Gordonia* (variant 02, 40%, and variant
235 07, 24%) and to *Rhodococcus* (variant 03, 20%). With the traditional primers, the dominant
236 variants were variant 07 (78%) and variant 03 (13%). Noteworthy, variant 03 and variant 07
237 were detected with both primers. Such result was in agreement with previous studies reporting
238 that CODEHOP approach revealed large diversity for various functional genes [34,46]. This
239 was as expected since CODEHOP primers were generated from conserved amino acid blocks
240 among known *dszB* homologs, while traditional primers were only based on the *Rhodococcus*
241 *erythropolis* IGTS8 *dszB* gene.

242 The DszB functional diversity was further examined by performing a phylogenetic
243 analysis. The phylogenetic analysis showed three major clusters (Fig. 3). Two clusters were
244 characterized by DszB sequences obtained with both sets of primers, and closely related to
245 sequences belonging to *Gordonia* and *Rhodococcus* species (Fig. 3, clusters 2 and 3). The
246 third cluster contained DszB sequences, obtained only with CODEHOP primers, closely
247 related to a DszB homolog called “ADRO2” (Fig. 3, cluster1). This homolog was identified
248 by metagenomics analysis from crude oil [37]. *In silico* analysis showed the absence of a
249 hybridization site on ADRO2 sequence corresponding to the forward traditional primer,
250 assuming amplification of this homolog would not, theoretically, be possible with traditional

251 primers. Moreover, the traditional forward primer hybridizes on the end of *dszA*, located
252 upstream *dszB* in currently known *dsz* operons. An operon organized differently will not yield
253 any PCR product with traditional primers. Hence this enhances the interest of using primers
254 designed from conserved amino acid blocks of DszB to detect and investigate *dszB* diversity
255 in environmental samples.

256

257 *3.2. Screening of biodesulfurization capacities in environmental samples and* 258 *enrichment cultures*

259 CODEHOP based screening was performed on different environmental samples
260 (Table 3) and *dszB* gene was detected in RITA3, GRAL and NOGR samples. These samples
261 were characterized by oil contamination, mainly diesel, due to anthropogenic activities, and
262 without cleaning actions since decades. It can be assumed that in these sites, readily
263 assimilable sulfur sources were consumed, and heteroaromatic sulfur compounds remained as
264 sole sulfur source, shaping local microbial communities at the benefice of specialized
265 microorganisms as desulfurizing bacteria in such particular ecotopes [20]. The *dszB* genes
266 were not detected in three environmental samples (PORT, MAHARIN and BERRE) with the
267 CODEHOP approach (Table 3), nor with the traditional (Sul2F/BR) primers. PORT and
268 MAHARIN samples were collected from a harbour sediment periodically dredged and a
269 creek-estuary mouth sediment, respectively. In these dynamic environments we hypothesise
270 that desulfurizing microorganisms are not abundant since pollutants such as marine fuel,
271 should have been removed or not-accumulated, along with heteroaromatic sulfur sources.
272 BERRE sediments, chronically contaminated by petrochemical wastes, are characterized by
273 hydrocarbon-degrading sulfate-reducing bacteria [47], creating unfavourable conditions for
274 the growth of aerobic desulfurizing microorganisms. Considering desulfurizing metabolism
275 requires easy access of sulfur sources [22,37,48,49] these three environments would not be

276 advantageous for aerobic microorganisms specialized in heteroaromatic compounds
277 desulfurization bearing any *dszB* gene homolog. Moreover, for many strains, *dsz* genes are
278 carried by plasmid [28,50], that can be lost in absence of adequate environmental selective
279 pressure.

280 In order to select active desulfurizing microorganisms, enrichment procedure was
281 performed using the environmental samples as inoculum. The enrichment was conducted in
282 presence of dibenzothiophene-sulfone (DBTO₂) as sole sulfur source. The desulfurization
283 potential of enrichment cultures was characterized by molecular and chemical approaches.
284 The detection of *dszB* genes by CODEHOP approach showed the same pattern observed in
285 environmental samples (Table 3). The *dszB* genes were detected in RITA3, GRAL and
286 NOGR enrichment cultures, showing that the initial presence of *dszB* gene in environmental
287 samples was maintained through enrichment procedure. In these *dszB*-positive enrichment
288 cultures the production of 2-hydroxybiphenyl (2-HBP) was detected (Table 3), assuming that
289 biodesulfurization activity occurred as previously described [41]. DBTO₂ was removed to a
290 higher extent compared to 2-HBP production, showing no-equimolar conversion as expected
291 through 4S pathway [51,52]. This could be explained by hydrocarbon degradation through
292 Kodama pathway [11,19]. Consistently, the genes involved in naphthalene degradation *nidA*
293 and *nahA* [53,54] were detected in the soil sample RITA3 and its corresponding enrichment
294 cultures (data not shown). Enrichment cultures from BERRE sample showed DBTO₂ removal
295 despite that we were unable to detect the presence of *dszB* gene, nor the production of 2-HBP
296 (Table 3). Such result supports the hypothesis that hydrocarbon-degrading microorganisms
297 were selected, instead of desulfurizing microorganisms. In this case biodegradation of DBTO₂
298 is performed via the Kodama pathway [19,20]. As shown in Table 3, DBTO₂ consumption
299 and 2-HBP production were not detected in enrichment cultures from PORT and MAHARIN
300 samples, which was in agreement with the fact that *dszB* genes were not detected.

301

302 **3.3.Reduction of functional and taxonomic diversity during enrichment culture**

303 The *dszB* diversity of RITA3 enrichment culture was characterized. This enrichment
304 culture was described by its desulfurization capacity (Table 3). Comparing the diversity
305 between the enrichment culture and the original soil, the enrichment culture selected the
306 variant 02 among the eleven variants identified in RITA3 soil sample (Fig. 2). Such result
307 indicated that the culture media and/or the culture condition were stringent, contributing to the
308 disappearance through enrichment of most DszB variants not yet described. Additionally, the
309 loss of these variants may be also explained by the inability of corresponding DszB enzymes
310 to metabolize DBTO₂ since the substrate specificity depends on amino acid composition as
311 demonstrated by site-directed mutagenesis [29,44,45]. Moreover, it has been shown that
312 desulfurizing activity is not just based on Dsz allozymes but also on cellular and extracellular
313 parameters [55–58], which might explain that *dsz* genes were mainly found in
314 *Mycobacterium*, *Rhodococcus* and *Gordonia* genera (Fig. 3). These genera are known to be
315 able to desulfurize DBTO₂ [38,59,60]. It is likely that these genera bring cellular and genetic
316 properties well adapted to desulfurization, including the ability to replicate and conserve *dsz*-
317 carrying plasmid as previously described [61]. Our enrichment conditions selected
318 microorganisms able to use DBTO₂ as sole sulfur source, carrying the *dszB* homolog
319 belonging to variant 02 (Fig. 2), associated with *Gordonia* species.

320 In order to further characterise the reduction of diversity during the enrichment
321 procedure, high throughput sequencing of 16S rRNA gene was performed to assess the
322 taxonomic diversity. After trimming and rarefaction, 14,677 sequences per sample were
323 obtained and clustered in Amplicon Sequence Variants (ASVs). As expected, the diversity

324 was higher in the initial soil community than in the enrichment culture (Table 2), confirming
325 that a stringent selection pressure was applied as observed for *dszB* diversity.

326 Soil bacterial community was mainly dominated by three genera (Fig. 4) including
327 sequences affiliated to two aerobic taxa *Pseudoxanthomonas* and *Chryseobacter* (36 and 13%
328 relative abundance, respectively) and strictly anaerobic *Paludibacter* (7%), which known
329 representatives were isolated from swamp [62–64]. The enrichment culture was dominated by
330 *Klebsiella* (49%) and strictly aerobic *Achromobacter* (8%) and *Xanthomonas* (4%), which are
331 usually found in environmental samples [65–67]. The abundance of the *Gordonia* genus,
332 which was associated to *dszB* variant 02 selected during the enrichment procedure, increased
333 slightly during the enrichment culture reaching 3% while it represented less than 1% in the
334 initial soil. The selected genera through enrichment revealed the presence of microorganisms
335 with the ability to degrade xenobiotic and aromatic compounds [65,67,68]. Among them,
336 *Achromobacter* sp., *Klebsiella* sp. and several *Gordonia* species were reported for their
337 capacity to perform biodesulfurization on heteroaromatic compounds such as DBTO₂ [68–70].
338 It is likely that the enrichment procedure with DBTO₂ was adapted for their selection. The
339 presence in high abundance of ASVs related to the *Enterobacteriaceae* family that have never
340 been reported exhibiting desulfurization capacities, suggested a co-selection, probably
341 behaving as cheaters by using sulfur produced by desulfurizing-microorganisms. Such
342 behaviour has been reported for *Enterobacter cloacae*, which was co-cultivated with *R.*
343 *erythropolis* IGTS8 in presence of DBTO₂ as sole sulfur source [18]. It has been
344 demonstrated that biodesulfurization performing microorganisms are able to export sulfide
345 out of the cell, making the sulfur available for non-specialists microorganisms that are 10 to
346 1,000 times more abundant than the desulfurizing microorganisms [13,20].

347

348 3.4. Isolation of desulfurizing strains

349 After the enrichment procedure, desulfurizing strains were isolated on SFM agar
350 plates. After 24h of incubation, large white colonies were observed. After 72h, little orange
351 colonies were observed in plates coming from 2-HBP producer cultures (Table 3).
352 Microscopic observations revealed that white colonies were yeasts while the orange colonies
353 were rod-shaped bacteria. Almost every orange colony owned *dszB* gene. Among the 210
354 isolates owning *dszB* gene, 45 were randomly tested for the biodesulfurization capacity, after
355 cultivation on SFM with DBTO₂ as sole sulfur source. The 11 isolates from RITA3 were able
356 to convert DBTO₂ into 2-HBP (Table 3). The sequence of the *dszB* genes from these isolates
357 shared 99 – 100% nucleotide sequence similarity with the *dszB* variant 02 (Fig. 2), affiliated
358 to *Gordonia* belonging strains, further confirmed by 16S rRNA gene analysis. Such
359 homogeneity in *dszB* gene sequences obtained from different samples confirmed the
360 stringency of the enrichment procedure, highlighting the “cultural bias” [71,72]. Since our
361 enrichment procedure showed large *dszB* gene diversity than that represented by the obtained
362 isolates, there is still the possibility to develop other culture media, such as changing carbon
363 sources and providing various sulfur sources, in order to isolate bacterial strains carrying
364 different *dszB* gene.

365

366 4. Conclusion

367 The enrichment procedure combined with molecular monitoring tools allowed the
368 selection of active microorganisms able to produce 2-HBP from DBTO₂ as sole sulfur source
369 in mineral medium. The enrichment culture selected bacterial genera usually found in
370 polycyclic aromatic hydrocarbons contaminated environment or able to degrade thiophenic
371 compounds through Kodama or 4S pathways. The CODEHOP primers developed in this

372 study, used as molecular tools to monitor the enrichment procedure, revealed a large *dszB*
373 gene diversity in the initial soils that was reduced during the enrichment procedure. However,
374 the final isolation step selected only one *dszB* gene variant affiliated to *Gordonia* highlighting
375 the necessity to develop culture media for the selection of other or even novel *dszB* genes. For
376 this purpose the CODEHOP primers represent innovative molecular tool to drive the
377 development of specific culture media. They provide the possibility to detect a wide range of
378 *dszB* genes in environmental samples and identify strains exhibiting *dszB* gene significantly
379 different from the model strain *Rhodococcus erythropolis* IGTS8. The capacity to access
380 novel desulfurizing microorganisms may allow the optimisation of biotechnology process for
381 the petroleum industry such as crude biodesulfurization.

382

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387 **Contributions**

388 ED, RD, CCL designed the study. ED performed experiments and analyzed the data. ED, RD,
389 CCL, LP wrote the manuscript. All authors read and approved the manuscript.

390 **Conflict of interest**

391 The authors declare that they have no conflict of interest.

392

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- 636
- 637

638 **Figure captions**

639 **Fig. 1** Sequence alignment of DszB homologs (352 amino acids) from desulfurizing bacterial
640 strains of *Gordonia* (AY678116, AY396519, KC693733, AJ973326, EU364831, EF026089),
641 *Agrobacterium* (AY960127), *Rhodococcus* (L37363, AY789136, AY278323),
642 *Mycobacterium* (AB070603), *Bacillus* (AB076745) and *Paenibacillus* (AB033997) species.
643 Shaded columns are identity nucleotides conserved in all sequences (blocks 1 to 11). In red,
644 consensus nucleotide sequence identified by CODEHOP procedure, and used to design
645 forward (codF) and reverse (codR) primers

646

647 **Fig. 2** Diversity of *dszB* gene in an environmental sample (RITA3 soil) assessed by
648 traditional and CODEHOP primers

649

650 **Fig. 3** Phylogenetic tree showing the affiliation of DszB deduced amino acid sequences (519
651 aa) from PCR amplified *dszB* genes fragments and known DszB homologs. Phylogenetic tree
652 was constructed with the neighbour-joining method. Bootstrap values (> 50%) for 1000
653 replicates are shown at the branch points. Colours indicate different clusters of DszB
654 homologs based on at least 97% nucleotide identity of coding sequence [36]. Square indicate
655 translated fragments amplified with traditional primers, and circle indicate fragments
656 amplified with CODEHOP primers. Sequence accession numbers are given in parentheses

657

658 **Fig. 4** Relative abundance of the dominant bacteria from the environmental sample (initial
659 soil community from RITA3 soil sample) and in the enrichment culture, at the genus level.
660 The number of ASVs per genus is indicated in parenthesis. Non-affiliated genera in
661 enrichment culture (representing 34% of relative abundance) belong to *Enterobacteriaceae*
662 family

663

664 **Table 1: Primers used in this study**

Targeted gene	Primers	Sequence 5'→3'	Reference
16S	515F	GTGTYCAGCMGCCGCGGTA	[35]
	928R	CCGYCAATTYMTTTRAGT	[36]
<i>dszB</i>	Sul2F	GCAACTGCACATGTTCGCCG	[37]
	BR	CTATCGGTGGCGATTGAGGC	[38]
	codF	CCTGGGCACCTGGGARGYNMG	This study
	codR1	GGTTGTGGTCGAGCAGGAAYTGYTGNGT	
	codR2	GCAGGTTGTGGTCGAGCARRAAYTGYTG	
<i>nidA</i>	PAH-RHD α GP F	CGGCGCCGACAAYYTYGTNGG	[53]
	PAH-RHD α GP R	GGGGAACACGGTGCCRTGDATRAA	
<i>nahA</i>	PAH-RHD α GN F	GAGATGCATACCACGTKGGTTGGA	
	PAH-RHD α GN R	AGCTGTTGTTCGGGAAGAYWGTGCMGTT	

665 Degenerated bases (IUPAC): Y = C or T, M = A or C, R = A or G, D = A, G or T, K = G or T, W = A or T, N = A, T, C or G

666

667 **Table 2: Alpha diversity indices of bacterial communities in the environmental sample**
 668 **(initial soil community from RITA3 soil sample) and in the enrichment culture**

Diversity index	Environmental sample			Enrichment culture	
	16S rRNA	<i>dszB</i> gene		16S rRNA	CODEHOP
		Traditional primers	CODEHOP		
Shannon	1.286	0.804	1.516	0.255	0
Richness	181	7	8	9	1
Pielou	0.619	0.287	0.505	0.368	-

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671 **Table 3: Genetic potential and desulfurization activity during the enrichment and**
 672 **isolation steps**

Environmental sample	Enrichment culture		Colonies		Isolated strain (n° positive/n° tested) *		
	<i>dszB</i> **	<i>dszB</i> **	DBTO ₂ *** removal	2-HBP*** production	<i>dszB</i> **	DBTO ₂ *** removal	2-HBP*** production
RITA3	+	+	+	+	66/96	11/11	11/11
BERRE	-	-	+	-	NT [§]	6/6	0/3
PORT	-	-	-	-	NT [§]	2/23	0/23
MAHARIN	-	-	-	-	NT [§]	2/23	0/23
GRAL1	+	+	+	+	31/37	NT [§]	1/1
GRAL2	+	+	+	+	22/24	NT [§]	1/1
GRAL3	+	+	+	+	34/48	NT [§]	1/1
NOGR1	+	+	+	+	26/30	NT [§]	1/1
NOGR2	+	+	+	+	10/21	NT [§]	1/1
NOGR3	+	+	+	+	21/31	NT [§]	NT [§]

673 *Number of strains showing positive DBTO₂ removal or 2-HBP production per number of strains tested. ***dszB* genes
 674 detected by PCR. ***DBTO₂ transformation and 2-HBP production determined by GC-FID. [§]NT: not tested

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AY678116: MAGRLSPGNPGSELDTGILDTLTYSNCPINALLTAWESGFLDAAGIELDILSGKQGTVHFTYDQPAYTRYGGEIPPLLSEGLRAPGRTRLLGITPILGRQGFFVGDSPITVAADLAGR 120
AY396519: MAGRLSPGNPGSELDTGILDTLTYSNCPINALLTAWESGFLDAAGIELDILSGKQGTVHFTYDQPAYTRYGGEIPPLLSEGLRAPGRTRLLGITPILGRQGFFVGDSPITVAADLAGR 120
KC693733: MAGRLSPGNPGSELDTGILDTLTYSNCPINALLTAWESGFLDAAGIELDILSGKQGTVHFTYDQPAYTRYGGEIPPLLSEGLRAPGRTRLLGITPILGRQGFFVGDSPITVAADLAGR 120
AJ973326: MAGRLSPGNPGSELDTGILDTLTYSNCPINALLTAWESGFLDAAGIELDILSGKQGTVHFTYDQPAYTCYGGGEIPPLLSEGLRAPGRTRLLGITPILGRQGFFVGDSPITVAADLAGR 120
EU364831: MAGRLSPGNPGSELDTGILDTLTYSNCPINALLTAWESGFLDAAGIELDILSGKQGTVHFTYDQPAYTRYGGEIPPLLSEGLRAPGRTRLLGITPILGRQGFFVGDSPITVAADLAGR 120
AY960127: MTSRVDPANPGSELDSAIRDTLTYSNCPVPNALLTASESGFLDAAGIELDVLSGQQGTVHFTYDQPAYTRFGGEIPPLLSEGLRAPGRTRLLGITPLLGRQGFFVRRDSDPITAAADLAGR 120
AY714057: MTSRVDPANPGSELDSAIRDTLTYSNCPVPNALLTASESGFLDAAGIELDVLSGQQGTVHFTYDQPAYTRFGGEIPPLLSEGLRAPGRTRLLGSTPLLGRQGFFVRRDSDPITAAADLAGR 120
L37363 : MTSRVDPANPGSELDSAIRDTLTYSNCPVPNALLTASESGFLDAAGIELDVLSGQQGTVHFTYDQPAYTRFGGEIPPLLSEGLRAPGRTRLLGITPLLGRQGFFVRRDSDPITAAADLAGR 120
AY789136: MTSRVDPANPGSELDSAIRDTLTYSNCPVPNALLTASESVFLDAAGIELDVLSGQQGTVHFTYDQPAYTRFGGEIPPLLSEGLRAPGRTRLLGITPLLGRQGFFVRRDSDPVTAAADLAGR 120
AY278323: MTSRVDPANPGSELDSAIRDTLTYSNCPVPNALLTASESGFLDAAGIELDVLSGQQGTVHFTYDQPAYTRFGGEIPPLLSEGLRAPGRTRLLGITPLLGRQGFFVRRDSDPITAAADLAGR 120
EF026089: MTGRVDTDTANSRLDGGHRDTLTFSNCPVPNALITASESGCLDAAGIELDVLSGKQGTVHFTYDHPAYTRFGGGIPPLLSEGLRAPGRTRLLGITPVLGRQGYFVRNDSPITSAADLAGR 120
AB070603: MT-----TTGID---RDILAYSNCVPNALLTALESNLLAGNGISLNVLSGAQAGLHFTYDHPAYTRFGGEIPPLISEGLRAPGRTRLLGITPLAGRQGIYVRADSPVTSPEQLRGR 120
AB076745: MT-----TTDVD---HDVLAYSNCVPNALLTALESNLLAGNGISLNVLSGAQAGLHFTYDHPAYTRFGGEIPPLISEGLRAPGRTRLLGITPLAGRQGIYVRVDSPVTSPEQLRGR 120
AB033997: -----MSTLSAIGPTRVAYSNCPVANALLVASRTGKLERQGVLLSQIAFAQGATHFAYDHAAYTRFGGEIPPLVSEGLRAPGRTRLLGITVLKPRQGFYVHSAGKIASPSDLRGR 120

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AY678116: RIGVSASAIRILRGELG-DYLQLDPWRQTLVALGSWEARALLHTLEHGELDVDDVELVPINSPGVDVPAEQLEDAATLKGADLFPDVAAGQAAVLDLDRGEVDALFSWLPWAAELEG-TGAR 240
AY396519: RIGVSASAIRILRGELG-DYLQLDPWRQTLVALGSWEARALLHTLEHGELDVDDVELVPINSPGVDVPAEQLEDAATLKGADLFPDVAAGQAAVLDLDRGEVDALFSWLPWAAELEG-TGAR 240
KC693733: RIGVSASAIRILRGELG-DYLQLDPWRQTLVALGSWEARALLHTLEHGELDVDDVELVPINSPGVDVPAEQLEDAATLKGADLFPDVAAGQAAVLDLDRGEVDALFSWLPWAAELEG-TGAR 240
AJ973326: RIGVSVSAIRILRGELG-DYLQLDPWRQTLVALGSWEARALLHTLEHGELDVDDVELVPINSPGVDVPAEQLEDAATLKGADLFPDVAAGQAAVLDLDRGEVDALFSWLPWAAELEG-TGAR 240
EU364831: RIGVSASAIRILRGELG-DYLQLDPWRQTLVALGSWEARALLHTLEHGELDVDDVELVPINSPGVDVPAEQLEDAATLKGADLFPDVAAGQAAVLDLDRGEVDALFSWLPWAAELEG-TGAR 240
AY960127: RIGVSASAIRILRGQLG-DYLELDPWRQTLVALGSWEARALLHTLEHGELGVDDVELVPISSPGVDVPAEQLEESATVKGADLFPDVARGQAAVLASGDVDALYSWLPWAGELQA-TGAR 240
AY714057: RIGVSASAIRILRGQLG-DYLELDPWRQTLVALGSWEARALLHTLEHGELGVDDVELVPISSPGVDVPAEQLEESATVKGADLFPDVARGQAAVLASGDVDALYSWLPWAGELQA-TGAR 240
L37363 : RIGVSASAIRILRGQLG-DYLELDPWRQTLVALGSWEARALLHTLEHGELGVDDVELVPISSPGVDVPAEQLEESATVKGADLFPDVARGQAAVLASGDVDALYSWLPWAGELQA-TGAR 240
AY789136: RIGVSASAIRILRGQLG-DYLELDPWRQTLVALGSWEARALLHTLEHGELGVDDVELVPISSPGVDVPAEQLEESATVKGADLFPDVARGQAAVLASGDVDALYSWLPWAGELQA-TGAR 240
AY278323: RIGVSASAIRILRGQLG-DYLELDPWRQTLVALGSWEARALLHTLEHGELGVDDVELVPISSPGVDVPAEQLEESATVKGADLFPDVARGQAAVLASGDVDALYSWLPWAGELQA-TGAR 240
EF026089: RIGVSASAIRILRGELG-DYLELDPWRQTLVALGSWEARALLHTLEHGSLDVDDVELDPINSPGVDVPAEQLEESATVKGADLFPDVAQGQAAVLNAGIDALYSWLPWAAELQA-TGAR 240
AB070603: RVGVSGAAIRILTGELG-DYRQLDPWRQTLIALGTWEARGLLQTLHIGGIGISDVELVRIESPGVDVPEERLEAAASVKGADLFPDVAHQSDILSSGNVDALFTWLPWAAELEDLSGAR 240
AB076745: RVGVSGAAIRILTGELG-DYRHLDPWRQTLIALGTWEARGLLQTLHIGGIGVDDVELVRIESPGVDVPEERLEAAASVKGADLFPDVAGHQRDILDSGNVDALFTWLPWAAELEDLSGAR 240
AB033997: RIGLSRAAQRILFGHLGEEYRNLGPWEQTLVALGSWEVRALKHTLAAGGLRLNDVIVEDVENPWVDVPRPKLDDSRDFSSRELFAVAWEWQSQQLKSGQVDALFSWLPYAAELELQGVAK 240

8 9 10 11 codR
AY678116: PVVDLGLDERNAYASVWTVSSELVDRPDLVQRLVDAVVDAGLWARDHGDAVTRLHAANLGVSPDAVGHGFGADFQQRLVPRLPDAVALLDR**TQQFLLSNQLL**QEPVALDQ 352
AY396519: PVVDLGLDERNAYASVWTVSSELVDRPDLVQRLVDAVVDAGLWARDHGDAVTRLHAANLGVSPDAVGHGFGADFQQRLVPRLPDAVALLDR**TQQFLLSNQLL**QEPVALDQ 352
KC693733: PVVDLGLDERNAYASVWTVSSELVDRPDLVQRLVDAVVDAGLWARDHGDAVTRLHAANLGVSPDAVGHGFGADFQQRLVPRLPDAVALLDR**TQQFLLSNQLL**QEPVALDQ 352
AJ973326: PVVDLGLDERNAYASVWTVSSELVDRPDLVQRLVDAVVDAGLWARDHGDAVTRLHAANLGVSPDAVGHGFGADFQQRLVPRLPDAVALLDR**TQQFLLSNQLL**QEPVALDQ 352
EU364831: PVVDLGLDERNAYASVWTVSSELVDRPDLVQRLVDAVVDAGLWARDHGDAVTRLHAANLGVSPDAVGHGFGADFQQRLVPRLPDAVALLDR**TQQFLLSNQLL**QEPVALDQ 352
AY960127: PVVDLGLDERNAYASVWTVSSGLVRQRPGLVQRLVDAAVDAGLWARDHSDAVTSLHAANLGVSTGAVGQGFADFQQRLVPRLDHDALALLER**TQQFLLTNNLL**QEPVALDQ 352
AY714057: PVVDLGLDERNAYASVWTVSSGLVRQRPGLVQRLVDAAVDAGLWARDHSDAVTSLHAANLGVSTGAVGQGFADFQQRLVPRLDHDALALLER**TQQFLLTNNLL**QEPVALDQ 352
L37363 : PVVDLGLDERNAYASVWTVSSGLVRQRPGLVQRLVDAAVDAGLWARDHSDAVTSLHAANLGVSTGAVGQGFADFQQRLVPRLDHDALALLER**TQQFLLTNNLL**QEPVALDQ 352
AY789136: PVVDLGLDERNAYASVWTVSSGLVRQRPGLVQRLVDAAVDAGLWARDHSDAVTSLHAANLGVSTGAVGQGFADFQQRLAPRLDHDALALLER**TQQFLLTNNLL**QEPVALDQ 352
AY278323: PVVDLGLDERNAYASVWTVSSGLVRQRPGLVQRLVDAAVDAGLWARDHSDAVTSLHAANLGVSTGAVGQGFADFQQRLVPRLDHDALALLER**TQQFLLTNNLL**QEPVALDQ 352
EF026089: PVVDLGLDERNAYGSVWTVSSGLVDRPDLVQRLVDAAVDAGLWARDHADAVTRLHAANLGVSTEAVGHGFGGLDFQHRLVPRLDLDRDALALLDR**TQQFLLSNDLL**HEPVALDQ 352
AB070603: VLADLGDDKRNRYASVWTVSAQLVDERPDQVQRLVDAAVQAGRWAQAHPEETVGIHAANLGVAPSAIGRFGADFQAHLIPTLDDSA LAVVDQ**TQQFLIDHNLL**DRPVDLTQ 352
AB076745: VLADLGDDQRSRYASVWTVSAQLVDERPDLVQRLVDAAVQASRWAQAHPEETVGIHAANLGVAPSAIGRFGADFQAHLIPRLDDSA LAVVDQ**TQQFLLDHNLL**DHPVDLTQ 352
AB033997: PVFALTGEE-NAWASVWTVSAAVVERRPEIVQRLVDSVVEAASWATDHAKETIEIHALNLGVSVKAVETGFGEGFHRDLRPRLDQAALRILEQ**TQQFLFDHGL**IDRLVDIER 352





