Isolation and characterization of *Bradyrhizobium* sp. SR1 degrading two β-triketone herbicides

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Abstract In this study, a bacterial strain able to use sulcotrione, a β-triketone herbicide, as sole source of carbon and energy was isolated from soil samples previously treated with this herbicide. Phylogenetic study based on16S rRNA gene sequence showed that the isolate has 100 % of similarity with several *Bradyrhizobium* and was accordingly designated as *Bradyrhizobium* sp. SR1. Plasmid profiling revealed the presence of a large plasmid (>50 kb) in SR1 not cured under nonselective conditions. Its transfer to *Escherichia coli* by electroporation failed to induce β-triketone degrading capacity, suggesting that degrading genes possibly located on this plasmid cannot be expressed in *E. coli* or that they are not plasmid borne. The evaluation of the SR1 ability to degrade various synthetic (mesotrione and tembotrione) and natural (leptospermone) triketones showed that this strain was also able to degrade mesotrione. Although SR1 was able to entirely dissipate both herbicides, degradation rate of sulcotrione was ten times higher than that of mesotrione, showing a greater affinity of degrading-enzyme system to sulcotrione. Degradation pathway of sulcotrione involved the formation of 2-chloro-4-mesylbenzoic acid (CMBA), previously identified in sulcotrione degradation, and of a new metabolite identified as hydroxy-sulcotrione. Mesotrione degradation pathway leads to the accumulation of 4-methylsulfonyl-2-nitrobenzoic acid (MNBA) and 2-amino-4 methylsulfonylbenzoic acid (AMBA), two well-known metabolites of this herbicide. Along with the dissipation of β-triketones, one could observe the decrease in 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibition, indicating that toxicity was due to parent molecules, and not to the formed metabolites. This is the first report of the isolation of bacterial strain able to transform two β-triketones.

Keywords β-Triketone · Sulcotrione · Mesotrione · Biodegradation · *Bradyrhizobium* sp. SR1

Introduction

The widespread use of herbicides over the last decades to control weeds and to ensure quality and high crop yields led to the contamination of the environment. Among popular plant protection products, β-triketones, which replaced atrazine banned in several European countries (Chaabane et al. 2008; Calvayrac et al. 2012), are used as selective pre- and postemergence herbicides for a wide range of broad-leaved and grass weeds in maize (Mitchell et al. 2001). Sulcotrione (2-[2-chloro-4-(methylsulfonyl)benzoyl]-1,3-cyclohexanedione) and mesotrione (2-[4-(methylsulfonyl)-2-nitrobenzoyl]-1,3-cyclohexanedione) (Fig. 1) released in 1993 and 2003, respectively, are chemically derived from leptospermone, a natural

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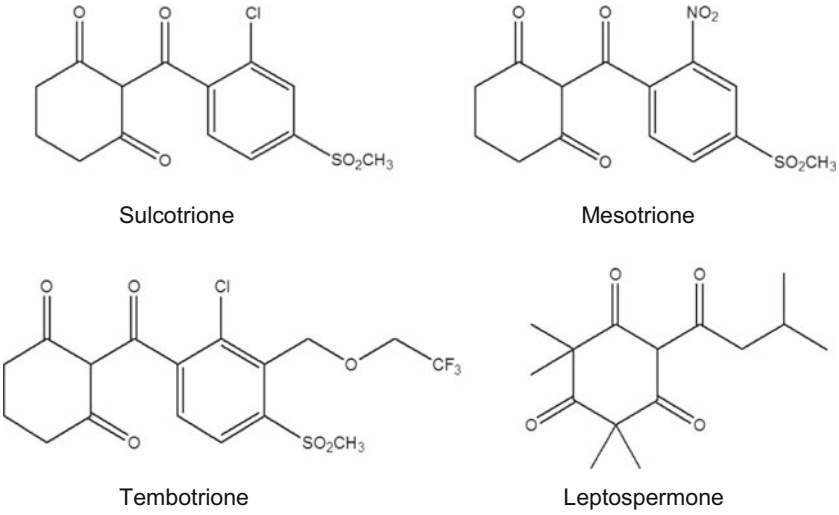

Fig. 1 Molecular structure of β-triketones herbicides

phytotoxin of the Californian bottlebrush plant, *Callistemon* spp. (Dayan et al. 2007). The target site for triketone herbicides was identified as the enzyme *p*-hydroxyphenylpyruvate dioxygenase (HPPD), which is found in a wide range of eukaryotic and prokaryotic organisms being involved in the catabolism of tyrosine. In plants, this enzyme catalyzes the conversion of *p*-hydroxyphenyl pyruvate (HPP) to homogentisate, which is the biosynthesis precursor of plastoquinone and α-tocopherol (Schulz et al. 1993; Lee et al. 1997; Meazza et al. 2002; Rocaboy et al. 2014). HPPD inhibition causes disruption of carotenoids biosynthesis and results in foliage bleaching of treated plants. All triketones are HPPD competitive inhibitors that form a tight complex with the enzyme by a mechanism of coordination to the active-site iron atom. Maize is known to exhibit natural tolerance to triketones using a nontarget mechanism based on rapid metabolic detoxification, involving a ring hydroxylation by the cytochrome P450 monooxygenase (P450), associated to a less sensitive form of the maize HPPD to triketones (Mitchell et al. 2001; Siehl et al. 2014).

Several works have detailed the environmental profile of triketone herbicides. Half-life (DT_{50}) in field varies between 16 and 122 days for sulcotrione and 6 and 34 days for mesotrione, depending on pH and organic matter content of soil (Rouchaud et al. 1998; Dyson et al. 2002; Chaabane et al. 2005; Crouzet et al. 2010). Although these herbicides are considered to have a favorable environmental and toxicological profiles (Shaner 2004), these molecules are of concern due to their potential toxicity for any living organisms harboring HPPD enzyme (Schulz et al. 1993; Wu et al. 2011; Joly et al. 2013). The toxicity of sulcotrione and mesotrione has been demonstrated on model organisms, such as *Tetrahymena pyriformis* and *Vibrio fischeri* (Microtox® bioassay). These two herbicides showed a toxicity level equal to or higher than atrazine (Bonnet et al. 2008; Joly et al. 2013). Recently, higher cytotoxicity and genotoxicity of sulcotrione mixture after photodegradation were shown in *Allium cepa* test as compared to the parent molecule (Goujon et al. 2014).

Microbial degradation is considered as the major dissipation process limiting the accumulation of pesticides in the environment (Anderson 1984; Walker and Welch 1991; Topp 2003; Arbeli and Fuentes 2007). For a range of pesticides, their repeated application was shown to lead to the selection of microbial populations able to use them as sources of nutrients and energy for their growth. This phenomenon is relying on the acquisition of degradation capacities through genetic mechanisms such as horizontal transfer, mutation, and genetic rearrangements (van der Meer et al. 1992). To date, only few papers reported the isolation and the characterization of bacterial strains able to degrade the two synthetic β-triketones. To our best knowledge, only one bacterial strain identified as *Pseudomonas* sp. 1OP has been found to be able to degrade sulcotrione (Calvayrac et al. 2012, 2014). Two *Bacillus* sp. strains isolated from agricultural soil and cloud water (Durand et al. 2006; Batisson et al. 2009) have been shown to completely and rapidly degrade mesotrione. A new mesotrione-degrading bacteria isolated from aquatic environment was identified as *Pantoea ananatis* (Pileggi et al. 2012). Contrary to the two *Bacillus* sp. strains, mesotrione was not a carbon source to *P. ananatis*, and glucose was required to start growth and degradation, suggesting a cometabolic process. Recently, *Escherichia coli* DH5-α was shown to possess a mechanism of adaptation to mesotrione involving glutathione-*S*-transferase (GST) enzyme (Olchanheski et al. 2014). Similarly to P450, GST enzymes have been commonly characterized in plants as nontarget based mechanisms for herbicide resistance (Ma et al. 2013). Then, *E. coli* DH5-α mesotrione degradation could be considered as an oxidative and nonspecific stress response. Until now, nothing is known about catabolic genes involved in sulcotrione and mesotrione degradation.

In this study, we report the isolation of a sulcotrione-degrading bacterial strain from an agricultural soil. Taxonomic and genetic characterization of the bacterial isolate was performed by 16S rRNA sequencing, plasmid profiling, and cloning. The capacity of the strain to degrade aerobically a range of synthetic (mesotrione, tembotrione) and natural (leptospermone) β-triketones was estimated. Metabolic pathways of sulcotrione and mesotrione were also investigated. The toxicity of these two herbicides and their related metabolites was estimated using HPPD inhibition test.

Materials and methods

Soil sampling and characteristics

Soil was collected from the surface layer (0–10 cm) of an experimental field located at the University of Perpignan, France. Soil samples were sieved (2 mm) and stored at 4 °C until use. The composition and characteristics of the soil was 13.9 % clay, 60.5 % silt, 25.6 % sand, 20 % soil humidity, 1.7 % organic matter, 0.9 % organic carbon, 0.98 $g.kg^{-1}$ total nitrogen, 15.5 meq 100 g^{-1} cation exchange capacity (CEC), 214 % Ca^{2+}/CEC and pH in water 8.1.

Chemicals and media

Analytical standards of sulcotrione (98.8 % purity) and 1,3-cyclohexanedione (CHD) (97.0 % purity) were purchased from Sigma-Aldrich (France). 2-Chloro-4-mesylbenzoic acid (CMBA) (95.0 % purity) was purchased from Acros Organics (France). Standards of mesotrione and tembotrione were purchased from Dr. Ehrenstorfer GmbH (Germany) as well as mesotrione metabolites 4-methylsulfonyl-2-nitrobenzoic acid (MNBA) and 2-amino-4-methylsulfonylbenzoic acid (AMBA). Leptospermone was kindly provided by F. E. Dayan (USDA, University of Mississippi, USA). Acetonitrile, methanol, and dichloromethane (HPLC quality) were purchased from Sigma-Aldrich and hydrochloric acid (38.0 %) from Prolabo (France).

β-Triketones solutions were prepared in water at initial concentration of 1 g L^{-1} (pH 9). They were then sterilized using a 0.2-μm filter, and finally added to mineral salts medium (MS) (Rousseaux et al. 2001) composed by: K_2HPO_4 (1.6 g L^{-1}), KH_2PO_4 (0.4 g L^{-1}), NaCl (0.1 g L^{-1}), $MgSO_4$ $7H_2O$ (0.2 g L^{-1}), $CaCl_2$ (0.02 g L^{-1}), $FeSO_4$, $7H_2O$ (5 mg L^{-1}), H_3BO_3 (2 mg L^{-1}), $MnSO_4$, H_2O (1.8 mg L^{-1}), $ZnSO_4$ (0.2 mg L^{-1}), $CuSO_4$ (0.1 mg L^{-1}), Na_2MoO_4 (0.25 mg L^{-1}), biotin (0.04 mg L^{-1}), and thiamine (0.1 mg L^{-1}). Each β-triketone herbicide or each metabolite was used as sole carbon source in liquid MS medium. Yeast extract mannitol medium (YM) containing (per liter) 2.5 g of yeast extract, 0.5 g of K_2HPO_4, 0.1 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCO_3$, and 10 g of mannitol (Vincent 1970) and TY medium composed by (per liter) 5 g tryptone, 3 g yeast extract, and 66 mg $CaCl_2$ were used as a rich medium. Luria–Bertani medium (LB) [tryptone (10 g L^{-1}), yeast extract (5 g L^{-1}), and NaCl (5 g L^{-1})] was used for *E. coli* plating.

Enrichment, isolation, and screening of microbial strains

Enrichment of sulcotrione-degrading bacteria was performed by adding 10 g of soil in an Erlenmeyer flask containing 90 mL of sterilized MS medium supplemented with sulcotrione at a final concentration of 35 mg L^{-1} (0.106 mM). The culture was incubated at 28 °C with continuous shaking at 150 rpm. After five enrichments, a serial dilution of the obtained bacterial consortium was done in MS liquid in order to reduce the culture microbial diversity. The 10^{-3}–10^{-8} dilutions were plated on both MS-sulcotrione and TY solid medium. Plates were incubated at 28 °C. Degrading capacity of dilution cultures was monitored. When about 50 % of the initial sulcotrione concentration was degraded, a new dilution series was started until three to four different growing colonies were obtained on TY plates. Each isolated colony was inoculated in liquid MS-sulcotrione to assess their degrading capacity. Regularly, 1-mL aliquot was sampled to monitor sulcotrione disappearance in the culture medium. Noninoculated cultures were used as control. A pure strain degrading the sulcotrione was isolated, and named SR1.

Analytical methodology

One-milliliter aliquots of liquid culture were analyzed by high-performance liquid chromatography (HPLC) Jasco apparatus equipped with a Phenomenex Luna C18 column (150×3.0 mm; 5 μm) and a diode array detector Jasco 875-UV set. The mobile phase was a mixture of water (AW) and acetonitrile (ACN) acidified by 0.1 % formic acid, delivered at a flow rate of 0.5 mL min^{-1}. A gradient method was used in order to improve molecules and metabolites separation: 0–4 min 70 % AW–30 % A-ACN, from 4 to 7 min 40 % AW–60 % A-ACN maintained for 15 min, then back to 70 % AW–30 % A-ACN.

Metabolites identification was accomplished using liquid chromatography–mass spectrometry (LC-MS) methodology. Primarily, Thermo Fisher Scientific LC-MS device, Accela HPLC coupled to a LCQ Fleet equipped with an electrospray ionization source and a 3D ion-trap analyzer was used. A full scan range in both positive and negative ion mode from *m/z* 60 to 300 in both positive was carried out, using the following gradient system: 90 %/10 % (AW/A-ACN) from 0 to 5 min, then 20 %/80 % (AW/A-ACN) maintained to 16 min. Confirmation of the nature of sulcotrione byproducts was carried out using an LC–high-resolution mass spectrometry

device: LC-HRMS Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher). Ions were generated using an electrospray ion source (ESI), and analysis was done in both positive and negative modes. The chromatographic system was equipped with a Luna PFP 2 (150×2.00 mm; 3 μm, Phenomenex). 0.1 % formic acid in Milli-Q water, and 0.1 % formic acid in ACN were used as mobile phase. The elution gradient were 3 % A-ACN (5 min), 3–95 % A-ACN(20 min), 95 % A-ACN (5 min), 95-3 % A-ACN (1 min), and 3 % A-ACN (5 min). Mass spectra analysis was effectuated using Xcalibur (version 2.0, Thermo Fisher) and MetAlign (version 041011, Arjen Lommen). For LC-MS analysis, aliquots were extracted from the bacterial culture medium as described by Calvayrac et al. (2012).

Purification was performed using a semi-preparative device (WATERS 1525 binary HPLC pump, Waters 248 DAD) equipped with a column ODS Uptisphere 5 μm. Mobile phase was a mixture of water and acetonitrile acidified by 0.1 % formic acid, delivered as follows: 20 % A-ACN (5 min), 95 % A-ACN until 22 min, then 20 % A-ACN until 35 min. The structure of sulcotrione metabolite was confirmed using 1H NMR (400 MHz, CDCl3).

Characterization of a sulcotrione-degrading isolate

Bacterial genomic DNA of SR1 strain was extracted by standard alkaline lysis method from one pure colony and used as template for PCR extraction reactions. The amplification reaction was carried out in 50 μl reaction volume containing 1.25 U of Go*Taq* DNA polymerase (Promega, USA) using 50 ng of DNA as template. 16S rRNA gene was amplified using universal primers 27f (5′-AGA GTT TGA TCH TGG CTC AG-3′) and 1492r (5′-TAC GGH TAC CTT GTT ACG ACT T-3′) (Gürtler and Stanisich 1996). PCR reactions were carried out in a thermocycler (Gradient Cycler, MJ Research, USA) under the following conditions: 5 min at 94 °C, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a 72 °C cycle for 15 min. The concentration of 16S rRNA amplicon was quantified using a spectrophotometer (Biophotometer, Eppendorf, Germany), and DNA quality was checked on 1 % agarose gel in 1× TBE buffer. The purity of the isolated bacteria was checked by 16S rRNA restriction fragment length polymorphism (RFLP). Restriction profiles were obtained by PCR products treatment using restriction enzymes *Alu*I and *Rsa*I (Fermentas, France). Separation of digestion products was effectuated on high-resolution 3 % agarose gel (MP Q-BIOgene, USA). DNA Molecular Weight Marker VIII (Roche Applied Science, France) was used to estimate the restriction fragments size.

16S rRNA amplicon was sequenced using the Sanger technology (Beckman Coulter Genomics). Sequence was deposited in GenBank under accession number KP683073. SR1 16S rRNA sequence was compared to closest sequences retrieved from GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) of the National Center for Biotechnology Information (NCBI). For phylogenetic analysis, multiple alignments were done with ClustalX program (Thompson et al. 1997), and neighbor-joining tree was calculated with the NJPlot software (Perrière and Gouy 1996).

Degradation of β-triketones herbicides by the isolated strain SR1

Capacity of SR1 to degrade β-triketone herbicides was estimated in resting cell experiments. SR1 was grown in YM incubated at 28 °C under agitation at 150 rpm. Bacterial culture was centrifuged at 4500*g* for 10 min at 4 °C. The pellet was then washed twice with MS medium and resuspended in 100 mL of MS containing herbicides at a concentration of 35 mg L^{-1} and an OD_{620} of 0.5 nm. Aliquots were regularly collected and frozen at −20 °C until analysis. Resting cell experiments were realized in triplicate. Noninoculated MS liquid controls supplemented either in β-triketone herbicide or in degradation product were maintained under the same conditions. As a negative control, similar experiments were carried out with four other *Bradyrhizobium* strains (*B. japonicum* USDA 110, *Bradyrhizobium sp.* LL13, *Bradyrhizobium* USDA 127, and *Bradyrhizobium* sp. G49) never exposed to sulcotrione.

SR1 strain plasmid curing assays

In order to establish a relationship between the degradation capacity and the presence of the plasmid, a curing experiment was carried out. The isolated strain was diluted (1/100 and 1/1000) and grown in YM medium without pesticide until the end of exponential growth phase. An aliquot was taken, and plasmid profile was done using Eckhardt method as previously described (Devers et al. 2007). Ten transfers (about 80 generations) were accomplished. Degradation capacity of the isolate was assessed after each transfer using resting cells experiment to monitor the evolution of sulcotrione-degrading ability.

SR1 strain plasmid isolation and transfer

SR1 strain was grown in YM liquid medium at 28 °C for 3 days with shaking at 150 rpm. Fifty milliliters of the grown culture was used to extract its plasmid using the Qiagen Plasmid midi kit (Qiagen, France). The plasmid was Tn5-labeled using the EZ-Tn5™<R6Kγ*ori*/KAN-2> insertion kit (Epicentre, USA) and introduced by electroporation in *E. coli* TransforMax™ EC100D™ pir^{+} cells according to the manufacturer's recommendations (Epicentre, USA). Transformants harboring SR1 plasmid was cultivated

overnight in LB medium containing Kanamycine (50 mg L^{-1}). SR1 Tn5-labeled plasmid was also introduced in electrocompetent cells of *Bradyrhizobium* sp. G49 by electroporation. Resting cell experiments were then carried out at 37 and 28 °C, respectively, for *E. coli* and *Bradyrhizobium* sp. G49 as previously described to assess sulcotrione degradation.

HPPD activity monitoring using colorimetric assay

Aliquots, regularly collected from SR1 resting cells, incubated with 35 mg L^{-1} of sulcotrione or mesotrione were analyzed with the colorimetric free cells bioassay developed by Rocaboy et al. (2014). Aliquots were firstly centrifuged (13, 500 rpm for 15 min) to remove SR1 cells from the supernatant. Each well of the 96-well microplate was filled with 12.5 or 25 μL of supernatant, and mixed with *E. coli* HPPD-induced cells and 1.2 mg mL^{-1} tyrosine. Triketone-positive controls were performed using 35 mg L^{-1} sulcotrione or mesotrione solution instead of resting cells supernatants. MS was added for controls. All tests were performed in triplicate. After 12 h of incubation, optical density of each well was measured at 405 nm.

Results

Isolation and characterization of sulcotrione-degrading bacteria

Enrichment culture was conducted using the soil of Perpignan that was previously exposed to sulcotrione (Chaabane et al. 2008) and shown to have a microflora able to degrade this compound with a half-life time of 8 days (Calvayrac et al. 2012). After five enrichment cycles, a mixed bacterial culture able to degrade sulcotrione was obtained (data not shown). After serial dilution of enrichment cultures and plating on both nutrient agar TY and MS agar containing sulcotrione as the sole carbon source, four pure isolates were obtained. The evaluation of their sulcotrione-degrading capacities revealed that only one isolate named SR1 was capable of efficiently degrade sulcotrione. The purity of SR1 strain was verified using RFLP fingerprinting of PCR-amplified 16S rDNA in both rich and MS medium (data not shown). This isolate was a Gram-negative, aerobic, motile, and rod-shaped bacterium.

SR1 16S rRNA sequence (1353 bp) showed 100 % of similarity with several *Bradyrhizobium* strains including *B. elkanii* strain SEMIA 6399, *B. japonicum* strain SEMIA 6154 and *Bradyrhizobium* sp. CMVU02. None of these isolates are known to degrade pollutants such as pesticides. Besides, resting cell experiments carried out with four *Bradyrhizobium* strains never previously exposed to sulcotrione showed that they were unable to degrade this herbicide (data not shown). Phylogenetic distances with other known triketones-degrading bacteria are presented in Fig. 2. Based on phylogenetic analysis, this isolate was named *Bradyrhizobium* sp. SR1. One could observe that the two known sulcotrione degraders belonged to the phylum of Proteobacteria in the α- and γ-Proteobacteria classes (*Bradyrhizobium* sp. SR1 and *Pseudomonas* sp. 1OP, respectively), the two mesotrione degraders belonged to the γ-Proteobacteria (*E. coli* DH5-α and *P. ananatis*) as well, and the two others belonged to the Firmicutes phylum (*Bacillus* sp. MES11 and *Bacillus* sp. 3B6).

Degradation of sulcotrione by *Bradyrhizobium sp.* SR1

Degradation kinetics of sulcotrione was established in resting cell experiments in MS medium supplemented with 35 mg L^{-1} of sulcotrione. One could observe that all the sulcotrione was completely degraded within 48 h in SR1 culture, while the concentration of sulcotrione remained stable in the control indicating the chemical stability of this molecule under our conditions (Fig. 5a, Supplementary data Fig. 1-A). Sulcotrione dissipation could be described by a first-order kinetic ($C=120e^{-0.075t}$, $R^2=0.913$, $n=3$) after a lag phase estimated to about 4 h. Half-life time was estimated to about 13 h (Supplementary data Fig. 1-A). During the degradation of sulcotrione by *Bradyrhizobium* sp. SR1, we searched for the two-known sulcotrione metabolites: CHD and CMBA. Under our experimental conditions, we did not detect CHD. However as previously reported for *Pseudomonas* sp. 1OP (Calvayrac et al. 2012), CMBA was identified using mass spectral in negative mode (ESI *m/z* 234) as the major metabolite. The apparition of this by-product is concomitant with the dissipation of sulcotrione with a molar recovery close to 90 % all during the time course of the herbicide degradation (Fig. 3a). Concomitantly with the appearance of CMBA (after 4 h of incubation), we observed the formation of another metabolite having a retention time different from those of CMBA and sulcotrione ones. This metabolite cannot be detected anymore when the sulcotrione was fully dissipated (Supplementary data Fig. 1-A). The LC-MS analysis corresponding to this peak (ESI *m/z* 343) allowed us to propose hydroxy-sulcotrione as an intermediate metabolite of sulcotrione that finally gives CMBA (Fig. 4). In order to confirm this hypothesis, the metabolite was purified by semipreparative chromatography from a large amount of SR1 resting cells prepared in MS-sulcotrione medium. Analysis of the metabolite in high-resolution LC-MS revealed the presence of a hydroxyl group on α-carbon of the carbonyl functional group. Additional proton NMR analysis of the purified metabolite clearly confirmed the absence of the hydroxyl group on the aromatic ring. Further resting cell experiments showed that SR1 was not able to degrade CMBA and CHD.

Resting cell experiments were also performed with higher concentrations of sulcotrione (100, 200, and 400 mg L^{-1}).

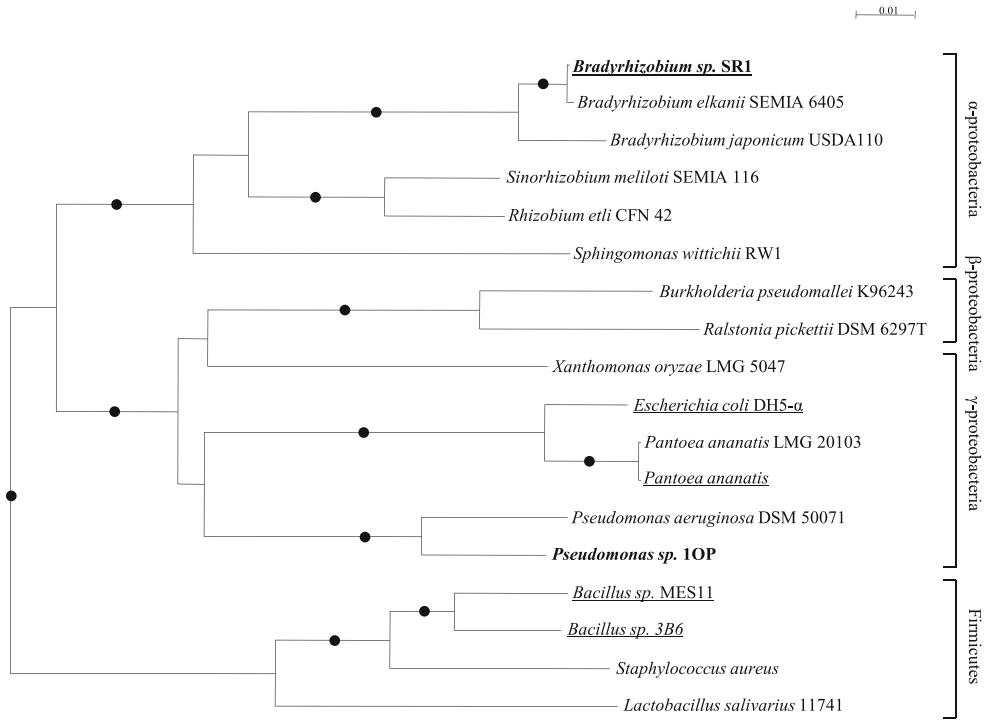

Fig. 2 Neighbor-joining phylogenetic analysis based on the multiple alignment of 16S rRNA sequences of *Bradyrhizobium* sp. SR1 with sulcotrione degrading strains (marked in *bold*), mesotrione degrading strains (*underlined*), and nondegrading strains. Nonavailable 16S rRNA sequences were replaced by their closest relative. Bootstrap values higher than 900 iterations out of 1000 are marked as *black circles*. Accession numbers of used strains are *Bradyrhizobium* sp. SR1 (KP683073), *Bradyrhizobium elkanii* strain SEMIA 6405 (FJ025109.1), *Bradyrhizobium japonicum* strain USDA 110 (AF363150.1), *Sinorhizobium meliloti* strain SEMIA 116 (FJ025128.1), *Rhizobium etli* CFN 42 (REU28916), *Sphingomonas wittichii* RW1 16S (NR_074268.1), *Burkholderia pseudomallei* (NR_074340.1), *Ralstonia pickettii* DSM 6297T (LN681565.1), *Xanthomonas oryzae* strain LMG 5047 (NR_026319.1), *Escherichia coli* DH5-α (KC768803.1), *Pantoea ananatis* strain CSA37 (KM091726.1), *Pantoea ananatis* LMG 20103 (NR_103927.1), *Pseudomonas aeruginosa* strain DSM 50071 (NR_026078.1), *Pseudomonas* sp. 1OP (JF303892.1), *Bacillus* sp. MES11(EU864320.1), *Bacillus macroides* (AF157696), *Staphylococcus aureus* strain ATCC (NR_115606.1), and *Lactobacillus salivarius* strain ATCC 11741(AF089108.2)

After 48 h of incubation, 66 and 48 mg L^{-1} of sulcotrione were degraded for an initial concentration of 100 and 200 mg L^{-1}, respectively, corresponding to a degradation rate of 66 and 24 %. For these two concentrations, sulcotrione biodegradation was completed after 5 days of incubation. However, at the highest concentration (400 mg L^{-1}), SR1 strain was not able to degrade anymore sulcotrione.

Degradation of other triketone herbicides by *Bradyrhizobium* sp. SR1

The ability of SR1 strain to degrade other synthetic (mesotrione and tembotrione) or natural triketones (leptospermone) was investigated in resting cell experiments showing that SR1 was only able to degrade mesotrione. However, the time required to get the full disappearance of 35 mg L^{-1} mesotrione (i.e., 20 days) was ten times longer than that required to dissipate the same quantity of sulcotrione (i.e., 2 days) under the same conditions. Kinetics of first order was also observed ($C = 102e^{-0.255t}$, $R^2 = 0.993$, $n = 3$) with a half-life time estimated to be about 3 days (Supplementary data Fig. 1-B).

Chromatographic analysis clearly showed that mesotrione biotransformation resulted in the generation of MNBA and AMBA, the two known mesotrione by-products (Durand et al. 2006). MNBA (ESI *m*/*z* 244) and AMBA (ESI *m*/*z* 214) appeared after about 24 h of incubation and then was accumulated in the medium up to 20 and 6 mg L^{-1}, respectively, by the end of the experiment. The cumulative profiles of metabolites during mesotrione transformation confirmed a quantitative transformation of mesotrione into MNBA and AMBA (Fig. 3b). Resting cell experiments showed that none of these two metabolites could be degraded by SR1.

Microbial toxicity of sulcotrione and mesotrione metabolites

In order to estimate the microbial toxicity of sulcotrione and mesotrione metabolites formed by *Bradyrhizobium* sp. SR1, HPPD inhibition capacity was assessed from the supernatants of resting cells using a colorimetric bioassay. The bioassay principle is that HPPD inhibitors decreased the ability of a recombinant *E. coli* clone to produce a melanine-like pigment resulting from tyrosine catabolism through HPP and

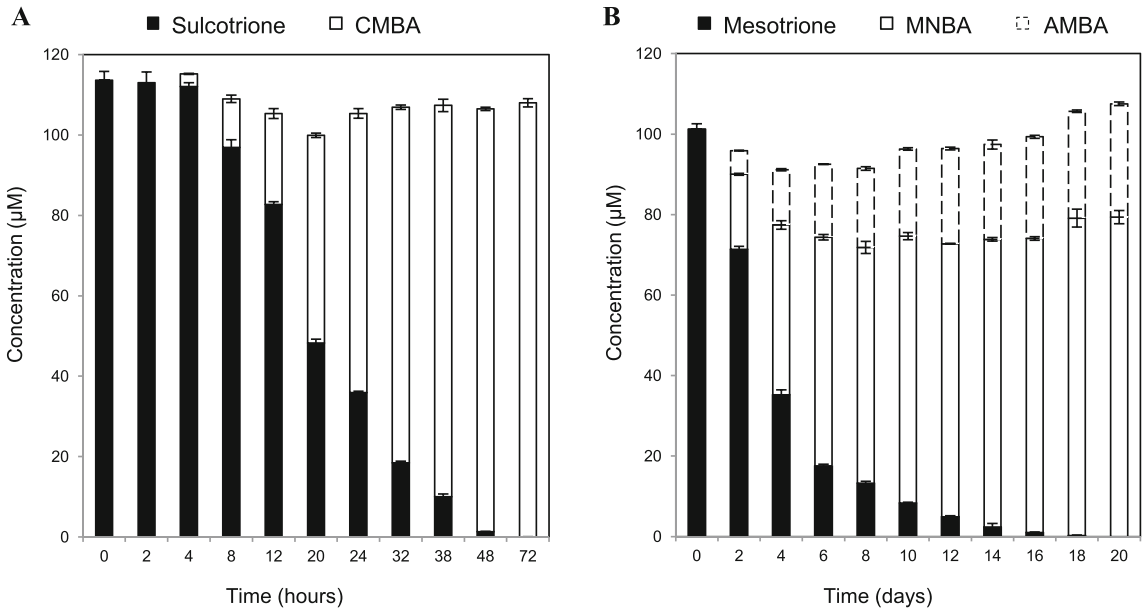

Fig. 3 Degradation kinetics of **a** sulcotrione and **b** mesotrione by *Bradyrhizobium* sp. SR1 in resting cell experiments. Cumulative formation of metabolites during sulcotrione and mesotrione degradation is represented. Standard deviations are indicated (n=3)

homogentisic acid. As expected, at T0, similar HPPD inhibition capacities were observed in SR1 culture and in the non-inoculated sulcotrione control (Fig. 5a). For 4 h, the HPPD inhibition capacity of the SR1 supernatant aliquots from sulcotrione resting cells was stable averaging 18±1.30 %. Thereafter, the HPPD inhibition decreased with time being negligible after 48 h of incubation. A significant linear regression (R^2=0.92) between the decrease in sulcotrione and HPPD inhibition was observed (Fig. 5b). Similarly, no HPPD inhibition was observed with SR1 supernatant aliquots from mesotrione resting cells after 20 days of incubation when no more mesotrione remained (data not shown).

Genetic localization of sulcotrione degradation in *Bradyrhizobium* sp. SR1

Attempts were done to identify the genetic localization of sulcotrione degradation in SR1. Keeping in mind that pesticide degradation by soil bacteria is often supported by large catabolic plasmids, this hypothesis was explored. Thus, a plasmid profile of the strain SR1 was performed and revealed the presence of a unique plasmid. Its size was estimated to be superior to 50 kb in comparison with a plasmid of known size (Fig. 6). Curing experiments were performed by cultivating SR1 isolate on a rich medium in the absence of selection pressure exerted by sulcotrione. After about 80 generations, the strain did neither lose its plasmid nor its capacity to degrade sulcotrione in resting cell experiments. In addition, the plasmid pSR1, labeled with Tn5, was successfully transferred to *E. coli* by electroporation (Fig. 6). However, transformed *E. coli* were not able to degrade sulcotrione in resting cell experiment. Furthermore, the insertion of the labeled plasmid into a nondegrading *B. japonicum* G49 was also assessed, but this transformation was unsuccessful.

Discussion

In this study, we report the isolation of SR1, a sulcotrione-degrading strain isolated from the soil of Perpignan, which was regularly exposed to this herbicide and known to host a microflora able to transform sulcotrione to CHD and CMBA (Chaabane et al. 2008). SR1 was able to use sulcotrione as the sole source of carbon and energy for its growth. 16S rRNA phylogeny analysis indicated that SR1 strain belongs to the α-Proteobacteria class, *Bradyrhizobium* genus and was named *Bradyrhizobium* sp. SR1. Interestingly, although this genus is mainly known for N-fixing symbiosis developed with leguminous plants, several studies have reported the involvement of various *Bradyrhizobium* strains in pesticide degradation

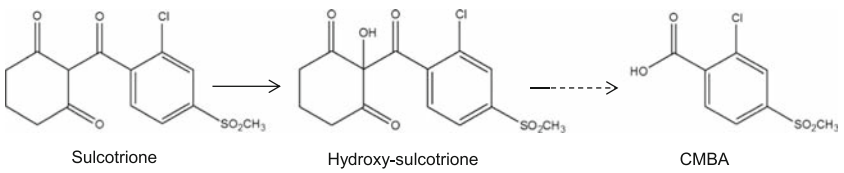

Fig. 4 Proposed scheme for the degradation of sulcotrione by *Bradyrhizobium* sp. SR1

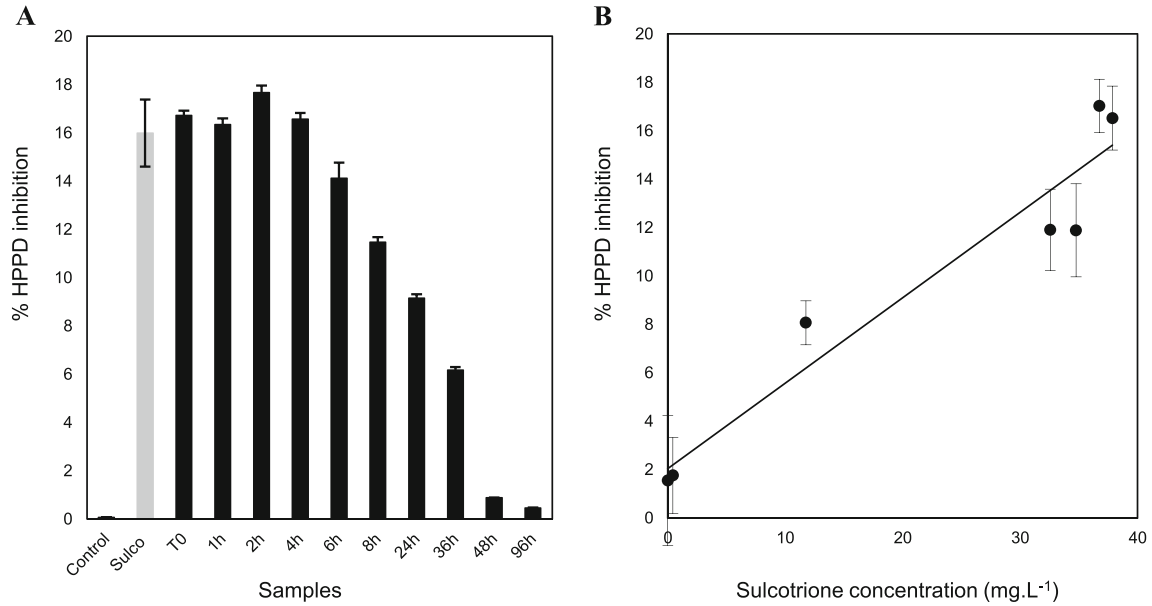

Fig. 5 **a** HPPD inhibitor capacity of SR1 resting cells incubated with 35 mg L^{-1} of sulcotrione and collected at different time (T0 to T96h) using colorimetric free cell bioassay. Control: negative control including SR1 resting cells incubated without sulcotrione. Sulco: noninoculated sulcotrione-positive control at a concentration of 35 mg L^{-1}. Standard deviations are indicated (n=3). **b** Linear regression between sulcotrione dissipation and HPPD inhibition

including herbicides such as 2,4-D (Kamagata et al. 1997; Huong et al. 2007), pentoxazone (Satsuma et al. 2000), simazine (Ozawa et al. 2004), and insecticide like methoxychlor (Satsuma et al. 2013).

Our work has demonstrated that SR1 was not only able to degrade sulcotrione but also mesotrione another herbicide belonging to the β-triketone family. To our best knowledge, this is the first report describing the isolation and the characterization of a bacterial strain able to degrade these two herbicides from triketone class. Up to now, only one bacterial isolate, *Pseudomonas* sp. 1OP, also isolated from the soil of Perpignan, was shown to degrade sulcotrione and to use it as sole carbon and energy sources under aerobic conditions (Calvayrac et al. 2012). One could observe that, as previously shown for *Pseudomonas* sp. 1OP by these authors, sulcotrione degradation with SR1 was leading to equimolar formation of CMBA metabolite. However, during the time course of sulcotrione degradation, we observed the transient accumulation of hydroxy-sulcotrione. This compound was previously detected as a metabolite resulting from abiotic degradation (i.e., photocatalysis treatment) of sulcotrione (Jovic et al. 2013). This observation led us to propose that SR1 degradation pathway is organized in two-step process: sulcotrione being first transformed to hydroxy-sulcotrione through oxidation, which is then transformed to CMBA and consecutively accumulated as a dead-end metabolite (Fig. 4). This is in accordance with Chaabane et al. (2008) reporting the accumulation of CMBA in the soil of Perpignan as a result of microbial transformation of sulcotrione. However, CHD the other degradation product of sulcotrione observed in the soil of Perpignan (Chaabane et al. 2008) was not detected in the culture media of strain SR1 neither by HPLC/UV nor by HPLC/mass spectrometry. Similar observation was done for *Pseudomonas* sp. 1OP (Calvayrac et al. 2012). As previously suggested, this discrepancy might be caused by abiotic transformation of CHD or to β-diketone oxidation catalyzed by enzymatic C–C bound breaking (Calvayrac et al. 2012). CMBA and CHD degrading tests have been carried out in resting cell experiments showing that SR1 was unable to degrade these two metabolites. SR1 was not able to degrade

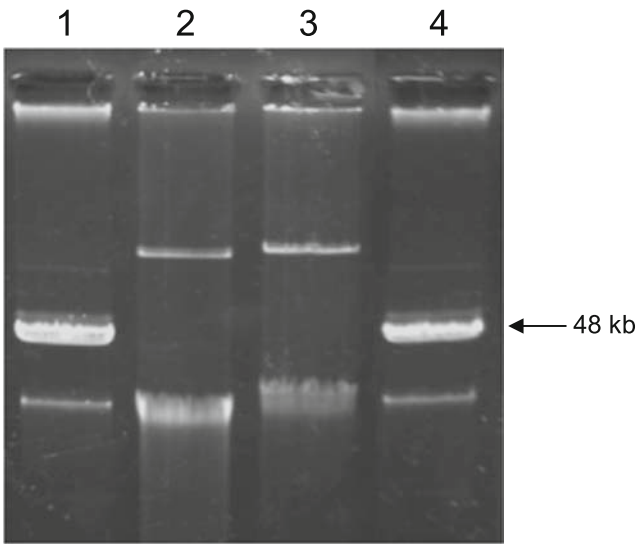

Fig. 6 Plasmid profiles from (*1* and *4*) *Sphingomonas* sp. SH., used as molecular weight marker, (*2*) *Bradyrhizobium* sp. SR1, and (*3*) *E. coli* pSR1::EZTn*5*. The size of the plasmid is indicated in kb

CHD probably because degradation pathway of this metabolite is inducible.

To date, several mesotrione-degrading bacteria have been isolated from different environments (i.e., water, soil, and clouds), but only a limited number of strains, all belonging to *Bacillus* genus, used this herbicide as a carbon source (Durand et al. 2006; Batisson et al. 2009; Pileggi et al. 2012). For *Bacillus* sp. 3B6, the major pathway of mesotrione degradation was initiated by a reduction of the nitro group of mesotrione resulting in the formation of several transformation products (M2, M3, and M4 as described in Supplementary data Fig. 2) leading to the formation of AMBA (Durand et al. 2010). A minor pathway leading almost directly to AMBA by an oxidative cleavage of mesotrione followed by a reduction of the nitro group was also observed. Although in the literature MNBA and AMBA have been described as the two main metabolites of mesotrione (Alferness and Wiebe 2002), MNBA was barely detected in *Bacillus* sp. 3B6 either because it was formed in an amount below its detection limit or because it was transformed since its formation in the medium (Durand et al. 2010). Similar mesotrione-degrading pathway was observed in *Bacillus* sp. MES11 (Batisson et al. 2009). According to our observations, the mesotrione degradation pathway of *Bradyrhizobium* sp. SR1 differed from that of *Bacilli* degraders, since (i) the three metabolites (M2, M3, and M4) resulting from the reduction of the nitro group of mesotrione were not detected during mesotrione degradation and (ii) both MNBA and AMBA were detected in the culture media. Additionally, resting cell experiments showed, that contrarily to *Bacilli* mesotrione-degrading strains, SR1 did not transform MNBA to AMBA. To explain this discrepancy, one could hypothesize, on the one hand, that SR1 strain harbors two distinct mesotrione-degradation pathways resulting in the formation of both MNBA and AMBA. On the other hand, mesotrione could be transformed into MNBA following the minor mesotrione-degradation pathway previously described in *Bacilli* strains, which is then transformed to AMBA (Supplementary data Fig. 2). Further studies are necessary to determine the pathway of degradation of mesotrione in strain SR1.

Although SR1 was able to degrade both sulcotrione and mesotrione, sulcotrione rate of degradation was ten times higher than that of mesotrione. This reveals that the selection driven by sulcotrione exposure led to the selection of SR1 harboring degrading enzymes highly specific for this herbicide. Among the other triketones tested, SR1 was only able to degrade mesotrione, which is having a chemical structure diverging from sulcotrione only by one substituent, $–NO_2$ instead of –Cl in position 2 of the aromatic group. Concerning tembotrione, the presence of 2,2,2-trifluoroethoxymethyl group in position 3 on the aromatic ring may contribute to the steric hindrance of the benzoyl 3-substituent on the enzymatic binding (Calvayrac et al. 2014) compromising its interaction with the catalytic site of SR1-degrading enzymes. On the contrary, the absence of the aromatic ring in the leptospermone may dramatically reduce its affinity for SR1 catabolic enzymes. A few number of pesticide-degrading strains are known to metabolize molecules belonging to the same family depending on the affinity of the degrading-enzyme to the pesticide (Karns et al. 1986; Strong et al. 2002; Karpouzas et al. 2005). As an example, biochemical studies carried out with *Mycobacterium brisbanense* strain JK1, a diuron-degrading bacterial isolate, showed that PuhA displayed the highest affinity for diuron, while this enzyme had lower affinities for other phenylureas such as chlorotoluron, isoproturon, linuron, and monolinuron (Khurana et al. 2009). It was suggested that the substrate specificity of PuhA was predominantly determined by steric factors, which could be the case for the enzymatic triketone degradation system of SR1.

The SR1 strain was not able to degrade sulcotrione at high concentrations. This might be explained by a possible dose-depending toxic effect of sulcotrione most likely resulting from the inhibition of a potential HPPD enzyme implicated in tyrosine metabolism of SR1. The presence of an *hppd* gene in *B. japonicum* USDA110 phylogenetically related to SR1 (Fig. 2) supports this hypothesis. Furthermore, the toxicity of triketone molecules and their related metabolites was estimated by monitoring HPPD inhibition. As expected, the obtained results confirmed that sulcotrione and mesotrione toxicity due to HPPD inhibition was mainly linked to parent molecule and revealed that the main metabolites displayed weak HPPD inhibitory capacities. These last observations are in contradiction with a previous study showing that CMBA metabolite displayed a higher toxicity than sulcotrione using Microtox assays with *V. fischeri* (Bonnet et al. 2008). Therefore, one could conclude that SR1-degrading activity is, on the one hand, beneficial to soil living organisms having sulcotrione-sensitive HPPD enzymes by removing the exposure to sulcotrione but, on the other hand, deleterious to soil organisms sensitive to sulcotrione metabolites, such as CMBA, which is accumulated during sulcotrione transformation. This ambivalent contribution of pesticide degraders was already reported for a range of pesticides that are transformed to toxic intermediates (i.e., phenylurea transformation to aniline derivatives, for review, see Hussain et al. 2015). For environmental safety, the ideal situation would be to have a synergetic cooperation between different microbial populations able to completely mineralize sulcotrione in soil.

Genes coding for degrading enzymes involved in different pesticide metabolisms, such as 2,4-D, isoproturon, and atrazine, are well known to form catabolic islands on plasmids spread all over the degrading community by horizontal gene transfer (Assinder and Williams 1990; Udikovic-Kolic et al. 2012; Gu et al. 2013). Recently, Calvayrac et al. (2014) showed that genetic system responsible for sulcotrione

degradation by *Pseudomonas* sp. 1OP was also plasmid borne. Several attempts were done in this work to determine if the degrading genes were localized on the large plasmid harbored by SR1. We showed that SR1 plasmid was not eliminated by curing experiment carried out on rich medium and that sulcotrione-degrading ability was maintained without pesticide selection pressure. The stability of this plasmid might be explained by the presence of essential genes for SR1 survival and growth. This is in agreement with previous studies showing that plasmids cannot be cured in rhizobia mainly because of the presence of genes essential for free-living growth (Finan et al. 1986). Although pSR1::Tn*5* was successfully transferred to *E. coli*, the transconjugant was not able to degrade sulcotrione. Based on this result, one could hypothesize that either catabolic genes are not plasmid borne or, for some reasons, genes located on SR1 plasmid are not expressed in the transconjugant.

Conclusion

We report the isolation and characterization of *Bradyrhizobium* sp. SR1 from an agricultural soil, which is able to biotransform two triketone herbicides. The dissipation of sulcotrione and mesotrione led to the accumulation of different known by-products, CMBA and MNBA/AMBA. Interestingly, hydroxysulcotrione was identified as a new metabolite, and a metabolic pathway was proposed. Further studies are required to identify and describe genes coding for enzymes involved in the transformation of these two triketones.

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Compliance with ethical standards All authors agreed to be listed and have approved of the manuscript, its content, and its submission to Environmental Science and Pollution Research. It has not been submitted or published elsewhere, whether partly or fully. All authors are in agreement with the ethical rules of ESPR.

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