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ORIGINAL ARTICLE

Isolation and characterization of indigenous 2,4-D herbicide degrading bacteria from an agricultural soil in proximity of Sauce Grande River, Argentina

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Abstract Our objective was to isolate and characterize indigenous bacteria able to use 2,4-D as a sole carbon (C) and energy source from an agricultural soil in the Sauce Grande River basin (Argentina). Culturable-dependant and molecular methods combined were used to identify and characterize putatively dominant indigenous degrading bacteria. Physiological traits, chloride release and biomass production showed the degradative capacity of the isolates obtained and high-performance liquid chromatography (HPLC) was used to corroborate the evidence. Degrading genes (*tfdA* and *tfdB*) were detected in all isolates, and their restriction fragment length polymorphisms (RFLP) were analyzed. Altogether, our results suggest that agricultural use of 2,4-D at recommended level leads to selection for a copiotrophic degrading population. The dominant genus able to metabolize 2,4-D in this soil was identified as *Cupriavidus* by 16S rRNA gene sequencing and the RFLP profiles of all isolates resembled that of *Cupriavidus necator* JMP134, the model organism for 2,4-D degradation. The strain EMA-G showed a remarkable performance in herbicide degradation (100 % removal in <1 day) in pure culture and is a favorite candidate for future biodegradation experiments.

Keywords 2,4-dichlorophenoxyacetic acid- degrading bacteria · *Cupriavidus* · *tfd* genes · Restriction fragment length polymorphism

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Introduction

Phenoxy herbicides have been produced and commonly applied at a large scale since the first half of the twentieth century. The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is extensively applied in agricultural fields worldwide to control dicotyledonous weeds in cereal crops and pastures, and to improve weed control obtained with glyphosate in no-till systems (Altieri and Pengue 2006; Roberts 1998). The herbicide is directly applied onto soil or sprayed over crops, and from there, often reaches superficial waters and sediments (Chinalia and Killham 2006). Later, animals and people may be exposed to 2,4-D through drinking water, which is worrisome as chlorophenoxyalkanoic acid herbicides (i.e., 2,4-D, 2,4-DB, MCPA and 2,4,5-T) are known endocrine-disrupting agents that have toxic effect to animals even at low concentrations (Pattanasupong et al. 2004)

Numerous work has been done to elucidate the physiological activity, mechanism of action, environmental fate of 2,4-D, and its potential for impacting soil microbial communities' structures and function (Baelum et al. 2008; Bouseba et al. 2009; Chinalia et al. 2007; Itoh et al. 2013). Yet fewer studies had dealt with the latest topic, particularly for low doses of herbicide (<5 mg kg^{-1} of active ingredient) simulating the agronomic use in the field (Bouseba et al. 2009; Ka et al. 1995; Merini et al. 2007; Vieuble-Gonod et al. 2005; Zabaloy et al. 2008, 2010).

2,4-D can be readily used as a carbon (C) and energy source by environmental microorganisms using at least two main different metabolic pathways, the α-ketoglutarate or the dehalogenase pathways (Ka et al. 1995; Kamagata et al. 1997; Kitagawa et al. 2002; Lerch et al. 2007; Muller et al. 2001; Vallaeys et al. 1996). The most studied catabolic pathway until

today follows the model encoded by plasmid pJP4 first isolated from *Cupriavidus necator* JMP134 (Lerch et al. 2007). *C. necator* JMP 134 harbors at least ten known genes involved in metabolism of 2,4-D; six of them are responsible for converting this compound to an intermediate of central metabolism (Chinalia et al. 2007). In the pathway initiated by the TfdA protein (a 2,4-D α-ketoglutarate dioxygenase encoded by *tfdA* gene) α–ketoglutarate is transformed into succinate and CO_2, and 2,4-D is converted into glyoxylate and 2,4-dichlorophenol (DCP). The next step in 2,4-D degradation is the transformation of DCP into 3,5-dichlorocatechol, which is catalyzed by a 2,4-dichlorophenol hydroxylase encoded in *tfdB* gene (Pérez-Pantoja et al. 2008). Most 2,4-D-degrading bacteria characterized to date are members of genera belonging to the β and γ subdivisions of the class *Proteobacteria* and were isolated from 2,4-D treated environments (Gonzalez et al. 2012; Kamagata et al. 1997; Lee et al. 2005). These β and γ subgroups carry 2,4-D degradation genes homologous to the canonical *tfdABCDEF* genes found in plasmid pJP4 of *C. necator* JMP134.

An understanding of the indigenous herbicide-degrading bacteria present in this agricultural soil is of the utmost importance, considering the high risk of herbicide contamination of the Sauce Grande River, a major water supply for the city of Bahía Blanca and the surrounding area (Orioli et al. 2008). Indigenous strains isolated in this work could be favorite candidates for bioaugmentation purposes for in situ cleanup strategies, as the survival and catabolic performance of exogenous introduced strains in bioremediation approaches is severely impaired by biotic and abiotic factors (Manzano et al. 2007; Marrón-Montiel et al. 2006). To provide de basis for testing these strains in future biodegradation studies, detailed knowledge on their biochemical and physiological properties and their genetic basis is required. The goal of this work was to isolate indigenous 2,4-D degraders from soil microcosms spiked with a low dose of 2,4-D, simulating agronomic use in the field. The putatively dominant population in microcosms was recovered by enrichment culture. Isolated degraders were identified by 16S rRNA gene sequencing, their degradation genes were characterized by restriction fragment length polymorphism (RFLP), and their 2,4-D degradation ability was confirmed by complementary methods.

Materials and methods

Soil sampling

The soil for this study was collected in an agricultural farm ("El Torino") in Saldungaray (38° 16′ S, 61° 41′ W) in the southern Pampas region, Buenos Aires, Argentina. The plot was selected because of its location in proximity of Sauce Grande River (< 100 m), where adequate herbicide management is critical to prevent water pollution. Soybean was cultivated for several years under no-till management, and pre-sowing weed control was done with 2,4-D and glyphosate until 2004. The last 2,4-D application occurred 29 months before the samples were taken. Since then, weed control was done with a mixture of fluorochloridone–cypermethrin–acetochlor for sunflower cultivation.

The soil is a sandy loam classified as Typic Argiudoll, with the following characteristics: pH (water) 6.6; total organic carbon, 20.1 g kg^{-1}, total nitrogen, 1.51 g kg^{-1} soil. Ten soil cores were randomly collected from the upper layer (0–10 cm) and then pooled to make a composite sample. Fresh, field-moist soil was sieved (< 5.6 mm) and kept at 4 °C until treatment within 2 weeks of sampling.

Chemicals

The herbicide 2,4-D (purity >98 %) was purchased from Fluka (Germany). All the other chemicals were of analytical reagent grade. The 2,4-D stock solution (25 mg ml^{-1}) was prepared by dissolving the necessary amount in methanol, filtered (<0. 22 μm) and stored at −20 °C.

Microcosms setup

Microcosms were prepared in 150-ml plastic flasks containing 40 g of soil, with caps loosely fitted to allow aeration. Before the experiment was initiated, soil microcosms were pre-incubated at their field moisture state for 7 days at 28 °C in the dark. One milliliter of a filter-sterilized aqueous solution of 2,4-D was spiked by dropping with pipette to give a final concentration of 5 mg kg^{-1} soil. Controls received only filter-sterilized distilled water. The dose of 2,4-D used here represents the concentration in soil after a single application of a commercial formulation (100 % isobutylic acid ester of 2,4-D) at the rate prescribed by the product label (0.4 l ha^{-1}), and expecting an interaction of herbicide and soil within the top 5 mm of the soil profile, for a soil with an apparent density of 1.3 ton m^{-3}. Further details on microcosms preparation can be found in a previous article (Zabaloy et al. 2010)

Enrichment cultures

Three replicate flasks per treatment were destructively sampled 1 month after treatment, and soil sub-samples (10 g dry wt. basis) were suspended in 0.85 % NaCl and used as the basis for a tenfold dilution series to estimate the most probable numbers of 2,4-D degrading bacteria ($MPN_{2,4\text{-}D}$) as described in Zabaloy et al. (2010). Enrichment cultures were prepared from the two most dilute positive tubes, using liquid mineral salts medium (MSM; K_2HPO_4 1.74 g l^{-1}, $NaHPO_4 \cdot 12H_2O$ 0.414 g l^{-1}, $(NH_4)SO_4$ 1.32 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.246 g l^{-1}, $Ca(NO_3)_2$ 24 mg l^{-1}, $Fe(NO_3)_3 \cdot 9H_2O$ 4.4 mg l^{-1}, $MnSO_4 \cdot$

H_2O 2 μM, $ZnSO_4 \cdot 7H_2O$ 2 μM, $CuSO_4 \cdot 7H_2O$ 2 μM, $NiSO_4$ 0.2 μM, $Co(NO_3)_2$ 0.2 μM, $NaMoO_4$ 0.2 μM, distilled water). The herbicide was added aseptically after sterilization, by diluting an appropriate volume of the stock solution (section 2.2). After two sequential transfers in liquid MSM supplemented with 100 mg l^{-1} of 2,4-D (Focht 1994), a loop full of each enrichment was streaked on solid MSM (2,4-D 250 mg l^{-1}). Positive isolates were streaked on nutritive agar (Laboratorios Britania, Argentina) and incubated 7 days at 28 °C to check for purity, and well-separated colonies were transferred to MSM (2,4-D 250 mg l^{-1}) agar slants. Glycerol stocks were stored at −20 °C. Morphological and physiological traits of the isolates were assessed (Holt 1994).

2,4-D degradation

Ten isolates were cultured in 10 ml of MSM+2,4-D 250 mg l^{-1} (1:50 inoculum: medium) in triplicate 50-ml Erlenmeyer flasks. Cultures were incubated 72 h at 28 °C. At the end of the incubation, 4-ml aliquots were centrifuged (10 min, 12,500 rpm), the supernatant was preserved and used for chloride release and spectrophotometrical analyses, and the cell pellet was stored at −20 °C for protein analysis. 2,4-D degradation was confirmed by UV scanning at 230–310 nm (Shimadzu 2100 UV–vis), chloride release (Focht 1994) and biomass protein quantitation by Bradford method (Bradford 1976).

Based on physiological differences (protein biomass and chloride release) that suggested slight differences in growth and degradation rates, respectively, the isolates EMA-G and EMA-K were selected for further characterization. Overnight-grown liquid cultures (OD_{550nm}=0.1) of the isolates were inoculated in 120 ml of MSM+2,4-D 250 mg l^{-1} (1:50) in triplicate Erlenmeyer flasks and incubated in a rotary shaker (28 °C, 90 rpm) for 72 h. Aliquots of 3-ml volume were drawn every 24 h, centrifuged (10 min, 12,500 rpm) to remove biomass and quantitate 2,4-D and 2,4-dichlorophenol (DCP) in the supernatant by HPLC. The analytical phase was performed at the Industrial and Biotechnological Microbiology Laboratory, University of Buenos Aires, Argentina. Supernatant was diluted 1:3 in methanol (HPLC grade), filtered (<0.45 μm) and injected in a HPLC Jasco module, equipped with detector UV-975 and hybrid column MS-C18 full end-capped (250×4.6 mm- 5 μm particles and 100-Å pore diameter, Waters), mobile phase methanol: phosphoric acid (pH 2.5) 65:35 (isocratic, 1 ml min^{-1}) and detection at 230 nm.

DNA extraction and PCR

Isolates and reference bacteria *Alcaligenes* sp. JD6 and *Burkholderia cepacia* TFD3 (courtesy of F. Martin-Laurent, INRA Dijon, France) were continuously grown in liquid MSM supplemented with 2,4-D as C source (250 mg l^{-1}) at 28 °C for 72 h. UltraClean Microbial DNA isolation kit (MoBio Laboratories, Carlsbad, USA) was used to extract DNA from 2-ml aliquots of bacteria cultures, following manufacturer's instructions.

Specific primers sets for the *tfdA* and *tfdB* genes previously described by Vallaeys et al. (1996) were used for selective amplification of the respective genes. *Burkholderia cepacia* TFD3 (Tonso et al. 1995) and *Alcaligenes* sp. JD6 (Vallaeys et al. 1996) were used as positive controls. Reaction mixtures (25 μl) for amplification of each gene contained 3.5 μl of nuclease-free water, 12.5 μl of 2× Green Go Taq Master Mix (Promega, Madison, WI, USA), 1.0 μl each of two primers (10 μM, forward and reverse), and 4 μl of DNA template. A touchdown program was carried out using a My Cycler thermal cycler (Bio-Rad, Hercules, CA, USA). A hot-start (3 min at 94 °C) was followed by 8 touchdown cycles (45 s at 94 °C; 30 s at 57-50 °C [decreasing 1 °C/cycle]; 45 s at 72 °C), 30 regular cycles (45 s at 94 °C; 30 s at 50 °C; 45 s at 72 °C), and a final extension step of 10 min at 72 °C. PCR products were revealed by electrophoresis in a 2 % agarose gel.

RFLP of *tfd* genes

The PCR products obtained with *tfdA* and *tfdB* primers were digested overnight with the restriction enzymes AluI and BsuRI (HaeIII) (Fermentas, Canada) according to the conditions described by the manufacturer. Restriction fragments (RF) were electrophoresed in either a 3.5 % (*tfdA*) or 2.5 % (*tfdB*) agarose gel (Metaphor, FMC Bioproducts, France) prepared in TBE buffer (60 V, 90–120 min). Gels were stained with ethidium bromide and analyzed using Kodak Digital Science Image Analysis Software version 3.0 (Eastman Kodak Company, NY).

Sequencing of 16S rRNA gene

Taxonomic identification of three isolates (EMA-G, EMA-K and EMA-N) was done following sequencing of 16S rRNA gene with universal primers by Macrogen Inc. (Seoul, Korea), and alignment with known sequences in GenBank (blast.ncbi.nlm.nih.gov/Blast.cgi). The strain EMA-N was sent for 16S rRNA sequencing as the microscopic observation showed larger bacilli than the other isolates. Nucleotide sequences for isolates EMA-G, EMA-K and EMA-N were assigned the following accession numbers: JX627313, JX627314, and JX627315.

Results

Physiological characterization

Ten pure isolates grew stably on MSM supplemented with 250 mg l^{-1} of 2,4-D. Morphological and physiological

characterization showed that all isolates were motile Gram-negative rods, formed non-pigmented colonies, tested negative for O-F (glucose) and gelatin hydrolysis, were positive for oxidase and catalase tests, and reduced NO_3^- to NO_2^-. They were able to use carboxylic acids (fumarate, succinate, acetate), amino acids (asparagine, glutamate), benzoate and fructose as growth substrates, while other sugars were not used (rhamnose, arabinose, cellobiose, xylose).

2,4-D degradation

All isolates grew with up to 250 mg l^{-1} herbicide as sole C source with complete primary degradation in less than 72 h. This was confirmed for all isolates by UV spectrophotometry, chloride release and biomass synthesis as determined by protein quantitation (Table 1). Herbicide utilization was associated with disappearance of the 283 nm-absorbance peak and almost stoichiometric release of chloride ions (Table 1). In addition, strains were further tested by HPLC and complete 2, 4-D degradation, with no DCP accumulation, was corroborated by this analytical approach (Fig. 1). The strain EMA-G showed better performance in 2,4-D degradation than EMA-K, as the former degraded 250 mg l^{-1} in less than 1 day, while the later initiated degradation (~5 % reduction in 2,4-D concentration) by that time.

Molecular analysis: presence and variability of *tfd*-genes

PCR amplification using the primers set specific for 2,4-D degrading genes *tfdA* (encoding a 2,4-D α-ketoglutarate dioxygenase) and *tfdB* (encoding a dichlorophenol hydroxylase) was done to elucidate the genetic basis for 2,4-D utilization in our isolates. With both *tfd*-specific primers, amplicons of the expected sizes for *tfdA* (370 bp) and *tfdB* (1.1 kb) were obtained from ten isolates and two reference strains (Fig. 2).

Table 2 shows the restriction patterns resulting from the digestion of *tfdA* and *tfdB* amplicons with two endonucleases (AluI and HaeIII). Restriction of *tfdA* amplicons from all isolates and *Alcaligenes* sp. JD6 with AluI and HaeIII originated two and three neat restriction fragments (RFs) (Pattern I), respectively. Restriction of *tfdA* amplicon of *B. cepacia* TFD3 with AluI produced three RFs (Pattern II) clearly distinct from the RFs of Pattern I (digestion with HaeIII was not determined). Pattern A, resulting from digestion of *tfdB* amplicons from all isolates and *Alcaligenes* sp. JD6 with AluI and HaeIII, showed two and four RF, respectively. Pattern B resulting from the restriction of *tfdB* amplicon of *B. cepacia* TFD3 showed four neat RFs with each endonuclease.

The almost full-length 16S rRNA gene sequence determined showed that the isolates were closely related to *Cupriavidus necator* (β-proteobacteria, Burkholderiales) with 99 % identity to *Cupriavidus necator* strain N-1 (Accession Nº NR 028766.1), type strain for the species.

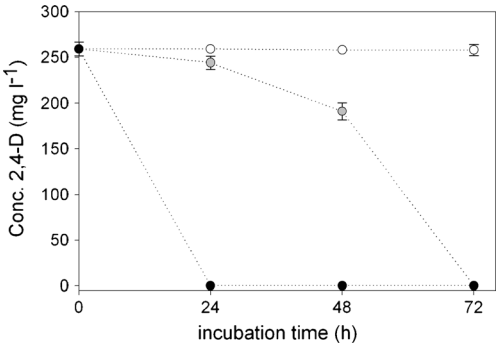

Fig. 1 Degradation of 2,4-D (250 mg l^{-1}) by selected isolates, measured by HPLC. Open circles: sterile control; filled circles: isolate G (black) and isolate K (gray). Values represent mean concentrations±S.E (n=3)

Table 1 Chloride release and biomass production of ten degrading isolates in MSM+2,4-D (250 mg l^{-1}≈1.1 mM)

Isolate	Released Cl^- (mM)	Protein (μg ml^{-1})[a]
EMA-A	1.1	2.5±0.1
EMA-E	1.1	2.5±0.2
EMA-F	1.1	1.1±0.3
EMA-F2	1.1	2.3±0.1
EMA-G	1.1	3.0±0.4
EMA-J	1.1	2.8±0.1
EMA-K	0.8	2.0±0.4
EMA-N	1.1	2.5±0.3
EMA-S	0.9	3.3±0.1
EMA-X	1.1	2.9±0.1

[a] Mean values±S.E (n=3)

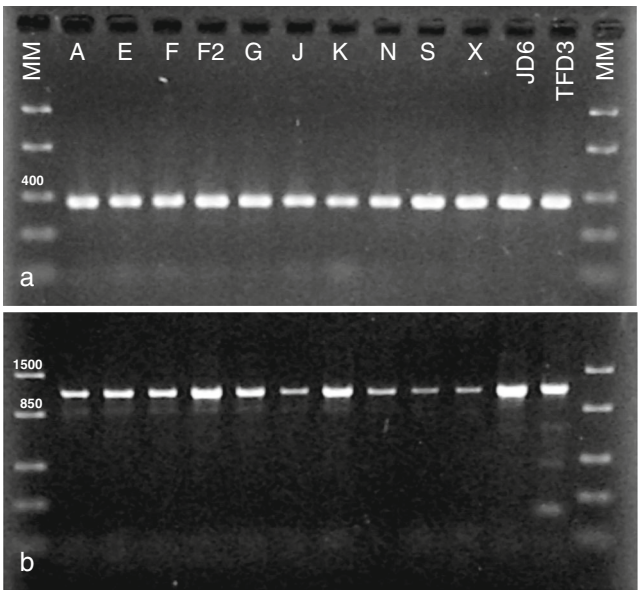

Fig. 2 PCR amplification of *tfdA* (**a**) and *tfdB* (**b**) genes from 10 isolates and two reference strains. Fragment sizes correspond to expected amplicon size for *tfdA* (370 pb) and *tfdB* (1.1 kb). MM=molecular size marker (50 bp-1.5 kb, Fermentas)

Table 2 Patterns observed after RFLP of *tfdA* and *tfdB* amplicons of ten isolates and two reference strains

Restriction Pattern	RF of *tfdA*[a](bp)		RF of *tfdB* (bp)	
	I	II	A	B
AluI	281	168	793	398
	90	119	322	307
		50		274
				138
BsuRI (HaeIII)	179 (12)	n.d	339 (1)	321 (6)
	115 (8)		262 (2)	268 (18)
	82 (8)		130 (2)	214 (14)
			58 (2)	89 (4)
				73 (8)

[a] Restriction fragment (RF) size in bp, corresponding to the average size of RFs of amplicons *tfdA* and *tfdB* obtained from ten isolates. In brackets, calculated differences with RFs for the canonical *tfd* genes digested with HaeIII as reported by Vallaeys et al. (1996). Italicized, estimated size of faint bands visualized in the gel

n.d not determined

Discussion

A previous study that evaluated the metabolic and degrading potential of this soil has shown it had a stable 2,4-D degrading population able to use the herbicide as C and energy source, which increased immediately after a low dose of 2,4-D and remained high for about 1 month after exposure had ceased (Zabaloy et al. 2010). In this study we isolated ten indigenous 2,4-D degrading bacteria belonging to the species *Cupriavidus necator* (formerly *Alcaligenes eutrophus, Ralstonia eutropha* and *Wautersia eutropha*) based on 16S rRNA gene sequence and physiological features in agreement with the genus description (Vandamme and Coenye 2004). This was consistent with our expectations, as the batch culture method used to enrich and isolate these bacteria is highly selective towards fast-growing copiotrophic microbes (Lee et al.2005; Macur et al. 2007). The fact that this taxonomic group was obtained from the most dilute positive MPN tubes may reflect its dominance in the 2,4-D degrading community in soil (Macur et al. 2007). In agreement, Cuadrado et al. (2010) reported that soils chronically exposed to phenoxy herbicides in the Humid Pampa in Argentina harbored a novel species of *Cupriavidus*, *Cupriavidus pampae*, able to degrade 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB).

Degradation performance was different between two strains, EMA-G and EMA-K, even though both were able to degrade a high concentration of 2,4-D (250 mg l^{-1}) in less than 72 h. The reasons for that difference are unclear. It could be argued that one of them was in better metabolic shape to cope with the "stressful" initial 2,4-D concentration, given that this phenoxy herbicide causes energy spilling due to metabolic uncoupling in the cells (Marrón-Montiel et al. 2006). The recovery after the transient lag in herbicide degradation in EMA-K could be explained either by consumption of by-products accumulated during early phase in the batch culture, or by the decrease in herbicide concentration at the end of the culture, with the consequent reduction in the toxic effect of 2,4-D. EMA-G degradation performance (lag and % herbicide removal) in medium containing 2,4-D was comparable to that of a *Pseudomonas cepacia* strain harboring plasmid pJP4 (Ka et al. 1994) and to a *Delftia* sp. strain (Gonzalez et al. 2012) isolated from an agricultural soil and a polluted river, respectively. The efficiency of our strain was even better than that of *C. necator* JMP134 grown on 2,4-D as sole source of C (250 mg l^{-1}) (Lerch et al. 2007). The lack of lag in 2,4-D degradation observed in EMA-G strain could make it a better competitor in comparison to EMA-K (e.g. in soil inoculations) as lag is important in determining fitness (Ka et al. 1994). Therefore, future applications of our degrading strains should evaluate not only the overall performance in 2,4-D degradation but also growth rates, lag in degradation and other parameters reflecting fitness.

Presence of *tfdA* and *tfdB* genes was confirmed by PCR. This allows inference of the metabolic pathway for 2,4-D degradation present in our isolates, even though DCP could not be detected in the HPLC analysis due to its lability. The enzyme TfdA of these isolates degrade 2,4-D by initial removal of the acetate side chain of 2,4-D to produce DCP and glyoxylate while oxidizing α-ketoglutarate to CO_2 and succinate. Enzyme TfdB subsequently catalyzes DCP oxidation to 3, 5-dichlorocatechol. This compound then undergoes *orto*-cleavage and subsequent oxidation steps that yield intermediates that will be assimilated through Krebs cycle (Pérez-Pantoja et al. 2008). Restriction patterns of *tfdA* and *tfdB* with HaeIII give insight into the genetic basis of 2,4-D degrading enzymes present in our isolates. The sizes of the RF's of Pattern I and A observed in all isolates and *Alcaligenes* sp. JD6 were in accordance with the reported sizes for canonical type I *tfdA* and type I *tfdB* genes present in self-transmissible plasmid pJP4 in *C. necator* JMP134 (Vallaeys et al. 1996). Follow-up studies will aim at isolating their degradative plasmids and determining *tfdA* and *tfdB* sequences in these isolates in order to assess the homology with the canonical type I *tfd* genes.

Future perspectives

The isolation of ten native strains of *Cupriavidus necator* able to rapidly degrade a high concentration of 2,4-D in pure culture, harboring *tfd* degrading genes comparable to those of self-transmissible plasmid pJP4 of *C. necator* JMP134 is promising. These strains could be used in future cell or plasmid-mediated bioaugmentation tests (once their plasmids are isolated and characterized) with better chances to survive in soil and sediments of the Sauce Grande River basin than introduced exogenous bacteria.

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