



Characterization of an isotroturon mineralizing bacterial culture enriched from a French agricultural soil

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ABSTRACT

The phenylurea herbicide isotroturon, 3-(4-isopropylphenyl)-1,1-dimethylurea (IPU), was found to be rapidly mineralized by a bacterial culture isolated from an agricultural soil regularly exposed to IPU. Molecular analysis of the bacterial culture by DNA fingerprinting, cloning and sequencing of the 16S rRNA genes revealed that it consisted of six different members among whom the dominant was related to *Sphingomonas* sp. Six bacterial strains belonging to genera *Ancylobacter*, *Pseudomonas*, *Stenotrophomonas*, *Methylobacterium*, *Variovorax* and *Agrobacterium* were isolated from the IPU-degrading culture. None of these were able to degrade IPU in pure culture and only the intact culture sustained the ability to mineralize IPU. The composition of the culture appeared stable suggesting that yet unknown interactions are involved in the IPU mineralization. IPU degradation involved the transitory accumulation of three known IPU metabolites 3-(4-isopropylphenyl)-1-methylurea, 3-(4-isopropylphenyl)-urea, and 4-isopropylaniline and their further degradation. Thus, it indicates a metabolic pathway initiated by two successive N-demethylations, followed by cleavage of the urea side chain. This culture did not degrade other structurally related phenylurea herbicides. The degrading activity of the bacterial culture was deeply influenced by the pH, being completely inhibited at pH 5.5 and optimal at pH 7.5.

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1. Introduction

The phenylurea herbicide isotroturon, 3-(4-isopropylphenyl)-1,1-dimethylurea (IPU), is among the most extensively used herbicides in conventional agriculture in Europe. It is applied for the prevention of the pre- and post-emergence weed developments in spring and winter wheat, barley and winter rye. It is relatively recalcitrant in the environment with 60% of the initial added amount remaining in the soil 3 months after its application (Nicholls et al., 1993). Due to its intensive and repeated usage as well as its persistence in the environment, IPU is frequently detected in the surface- and groundwater bodies at concentrations exceeding $0.1 \mu\text{g L}^{-1}$, the European Union drinking water limit (Muller et al., 2002). Ecotoxicological data suggest that IPU and several of its main metabolites are carcinogenic for animals and humans (Behera and Bhunya, 1990; Hoshiya et al., 1993) and harmful to aquatic invertebrates (Mansour et al., 1999), fresh water algae (Vallotton et al., 2009) and microbial communities (Widenfalk et al., 2008). So, there is a crucial interest in studying

the fate of IPU in the soil environment which acts as a recharge zone for underlying groundwater aquifers as well as nearby rivers, streams and lakes.

Microbial degradation is the primary mechanism for dissipation of IPU and related phenylurea herbicides in agricultural soils (Fournier et al., 1975; Cox et al., 1996). The degradation rate of pesticides is influenced by their bioavailability (Heitzer et al., 1992) and physico-chemical parameters such as pH and soil type (Aislabie and Lloydjones, 1995; Andrea et al., 2000). It is noteworthy that soil pH is among the most important factors controlling pesticide biodegradation and a positive correlation between soil pH and degradation rates has been described in several recent studies (Bending et al., 2003; El-Sebai et al., 2005; Rasmussen et al., 2005; Sun et al., 2009). Several studies have reported the adaptation of soil microflora to the enhanced IPU degradation in agricultural soils regularly exposed to this herbicide (Walker et al., 2001; Bending et al., 2003; El-Sebai et al., 2005). These observations have stimulated the research aimed at isolating and characterising microbial strains able to entirely mineralize IPU. *Sphingomonas* sp. SRS2 (Sørensen et al., 2001), *Arthrobacter* sp. N2 (Tixier et al., 2002), *Sphingomonas* sp. F35 (Bending et al., 2003), *Methylopila* sp. TES (El-Sebai et al., 2004), and *Sphingobium* strains YBL1, YBL2 and

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YBL3 (Sun et al., 2009) are the bacterial strains isolated and characterized for the degradation of IPU and other phenylurea herbicides. These bacterial isolates have different capabilities for degradation of IPU and other structurally related phenylurea herbicides.

Although described in details for *Sphingomonas* sp. SRS2, the IPU-metabolic pathway still remains to be elucidated for other isolates. Transient accumulation of MDIPU (3-(4-isopropylphenyl)-1-methylurea) following demethylation of the dimethylurea side chain of IPU has already been described as the initial step of degradation pathway (Sørensen et al., 2001; Sun et al., 2009). Some of the metabolites [MDIPU, DDIPU ((3-(4-isopropylphenyl)-urea) and 4-IA (4-isopropylaniline)] formed during the IPU degradation by *Sphingomonas* sp. SRS2 were identified and it was proposed that IPU metabolism is initiated by two successive N-demethylations, followed by the cleavage of the urea side chain and finally by the mineralization of the phenyl structure (Sørensen et al., 2001). Sørensen et al. (2003) also proposed different parallel degradation pathways for IPU by defined microorganisms in agricultural soils.

It should be noticed that most of the studies related to IPU degradation have been carried out with pure bacterial isolates from agricultural soils. Several studies have indicated that multi-member bacterial consortia are involved in the degradation of different pesticides (Udikovic-Kolic et al., 2008). One previous study reports the enhancement of IPU-degrading capability of *Sphingomonas* sp. SRS2 when grown in co-culture with strain SRS1, not able to degrade IPU (Sørensen et al., 2002). This observation suggests that bacterial consortia may also be involved in IPU degradation and that such synergistic interactions may have been overlooked previously. The current study was aimed at examining the complexity of an IPU-degrading bacterial community isolated from an agricultural soil treated regularly for over a decade with IPU. In this study, we have reported for the first time the characterization of an IPU-mineralizing bacterial culture enriched from a French agricultural soil. The initial steps of the IPU-metabolic pathway used by this bacterial culture were proposed based on the transient accumulation of three IPU metabolites during the mineralization process. The capability of this bacterial culture to degrade different phenylurea herbicides and the effect of pH on the IPU degradation kinetics were studied in liquid culture. Some of the bacterial members of this culture were isolated and tested for IPU degradation as well as for determining the diversity of the culture.

2. Materials and methods

2.1. Soil

Soil was sampled from an agricultural field located at “le Souich, France”. This field has annually been treated with IPU (1.8 kg ha^{-1}) for 10 years. Soil physico-chemical characteristics were: clay 17.2%, fine silt 29.7%, coarse silt 44.2%, fine sand 8.8%, coarse sand 1.2%, moisture content 25%, organic carbon 14.6 g kg^{-1} , organic nitrogen 1.9 g kg^{-1} , C/N ratio 7.8, Cation Exchange Capacity (CEC) $11.12 \text{ mmol } 100 \text{ g}^{-1}$, and pH (in water) 7.4 (El-Sebai et al., 2004). Surface soil samples (0–15 cm) were collected, sieved to 5 mm and stored at 4°C until used.

2.2. Herbicides

IPU (99.0% purity), diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea, 99.4% purity), linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea, 99.0% purity) and monolinuron (3-(4-dichlorophenyl)-1-methoxy-1-methylurea, 99.0% purity) were purchased from Riedel de Haen (Germany). Chlorotoluron (3-(3-chloro-p-tolyl)-1,1-dimethylurea, 99.7% purity) was obtained from Chem Ser-

vice (West Chester, USA). Analytical grade standards of the IPU metabolites MDIPU (99.4% purity), DDIPU (purity) and 4-IA (99.5% purity) were purchased from Dr. Ehrenstorfer GmbH (Augsberg, Germany). ^{14}C -ring-labelled IPU (specific activity 18 mCi mmol^{-1} ; 99% radiochemical purity) was purchased from International Isotopes (Munich, Germany).

2.3. Culture media

A mineral salt (MS) medium (Rousseaux et al., 2001) was used to estimate the capability of the bacterial culture to degrade IPU (provided as sole source of carbon and nitrogen) during enrichment cultures. For liquid culture studies, 50 mg L^{-1} of IPU was added, whereas, a solid IPU-MS medium was obtained by adding 15 mg L^{-1} agar (Biokar Diagnostics, France) and 2 mL L^{-1} of methanol dilution of IPU giving a final concentration of 500 mg L^{-1} . Isolation of the bacterial strains was done on Luria-Bertani medium (LB) [tryptone (10 g L^{-1}), yeast extract (5 g L^{-1}), NaCl (5 g L^{-1})] and MS medium supplemented either with 100 mg L^{-1} of casamino acids (Difco, Becton Dickinson, France) or with a defined mixture of different amino acids (Sørensen et al., 2001). A mineral salt (Knapp) buffer (Devers et al., 2004) was used to determine the capabilities of the bacterial culture to degrade different phenylurea herbicides. To assess the pH effect on degradation kinetics, pH was adjusted to targeted values by using 37% HCl and 3 M NaOH.

2.4. Enrichment cultures and isolation of bacterial strains

Selective enrichment cultures were established from soil slurries in order to isolate the IPU degrading bacteria. Briefly, 10 g of the soil of “le Souich” (dry weight equivalent) was added to 90 mL of IPU-MS medium and incubated on an orbital shaker (150 rpm) at 20°C . When about 50% of the initially added IPU was degraded, 10 mL of the soil slurry was transferred to 90 mL of fresh IPU-MS medium and incubated again under the same conditions. After the first four enrichments, 12 successive enrichment cycles were performed by transferring 100 μL of the IPU-degrading culture to 10 mL of the fresh IPU-MS medium. A bacterial culture having a stable community structure revealed by Amplified Ribosomal DNA Restriction Analysis (ARDRA) was obtained after performing about 16 enrichments. The bacterial culture was serially diluted and 100 μL aliquot of the fresh enrichment culture dilutions (10^{-3} – 10^{-6}) was plated on IPU-MS, MS supplemented with casamino acids, MS supplemented with amino acids and LB agar media. Different types of bacterial strains appearing on the media were purified by successive isolations on Petri plates and then preserved as stock in 25% sterile glycerol and kept frozen at -80°C for further experiments.

2.5. Determination of purity and of the ARDRA fingerprint of the bacterial culture

The composition of the bacterial culture was determined by amplifying the 16S and 18S rRNA genes by polymerase chain reactions (PCR), whereas, the fingerprint of the bacterial culture, revealing its dominant members, was assessed by ARDRA profiles at different times during the enrichment procedure.

2.5.1. 16S and 18S rRNA gene amplification

The cells of the bacterial culture were treated with proteinase K (Cheneby et al., 2004) before the PCR. Fungal 18S rRNA gene was amplified from the proteinase K extract of the bacterial culture by PCR using the primers nu-SSU-0817 (5'-TTAGCATGGAATAATTR AATAGGA) and nu-SSU-1536 (3'-ATTGCAATGCYCTATCCCCA) with the same PCR conditions as previously described by Edel-Hermann

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