

Isolation and Characterization of Diuron-degrading Bacteria from Lotic Surface Water

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Abstract

The bacterial community structure of a diuron-degrading enrichment culture from lotic surface water samples was analyzed and the diuron-degrading strains were selected using a series of techniques combining temporal temperature gradient gel electrophoresis (TTGE) of 16 S rDNA gene V1–V3 variable regions, isolation of strains on agar plates, colony hybridization methods, and biodegradation assays. The TTGE fingerprints revealed that diuron had a strong impact on bacterial community structure and highlighted both diuron-sensitive and diuron-adapted bacterial strains. Two bacterial strains, designated IB78 and IB93 and identified as belonging to *Pseudomonas* sp. and *Stenotrophomonas* sp., were isolated and shown to degrade diuron in pure resting cells in a first-order kinetic reaction during the first 24 h of incubation with no 3,4-DCA detected. The percentages of degradation varied from 25% to 60% for IB78 and 20% to 65% for IB93 and for a diuron concentration range from 20 mg/L to 2 mg/L, respectively. It is interesting to note that diuron was less degraded by single isolates than by mixed resting cells, thereby underlining a cumulative effect between these two strains. To the best of our knowledge, this is the first report of diuron-degrading strains isolated from lotic surface water.

Introduction

Pesticides applied in agricultural and urban areas constitute major sources of surface water and groundwater contamination that are currently causing serious environmental damage. Herbicides are able to reach

surface waters either directly by the herbicidal control of aquatic weeds or indirectly via the discharge of agricultural drainage water from treated land and via runoff after application on hard surfaces [23, 24, 32, 49].

Diuron, *N*-(3,4-dichlorophenyl)-*N*'*N*'-dimethyl-urea, is a phenylurea herbicide that has been widely used for the selective control of noncultivated areas, annual weeds, and field crops [46]. In France, the use of diuron has been restricted since 1997 (*Journal Officiel* dated 4/07/97) when it was detected in drinking water supplies. However, according to the French Environmental Institute [17], diuron is still detected in 34.6% of all river samples from the national basin system, and this rate changed only slightly between 1997 and 2002. Indeed, partly because of its low solubility in water (40 mg/L at 20°C), diuron is persistent in surface soil (Koc varying from 500 to 700) where it is prone to lateral movement via surface runoff and migration to both surface and groundwater [12, 31, 35]. Diuron can also disseminate by leaching after intensive rainfall, making it one of the main causes of groundwater pollution [21]. Diuron is slightly toxic to mammals and birds and moderately toxic to aquatic invertebrates [14, 27], but its main product of biodegradation, 3,4-dichloroaniline (3,4-DCA), exhibits a higher toxicity [44], even on natural bacterial communities. Hence, the European Commission (Directive 2000/60/CE) has designated diuron as a priority hazardous substance.

El Fantroussi *et al.* [7] have shown that the structure and metabolic potential of soil microbial communities are both clearly affected by the long-term application of urea herbicides, which promotes accelerated biodegradation. Microbial degradation is the primary vector of diuron dissipation from soil [13, 16], and numerous studies have underlined the diversity of the microorganisms involved [3, 5, 11, 19, 33, 36, 38, 39, 42, 43, 47, 48, 50, 52]. It has also been shown that if diuron penetrates aquatic

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ecosystems, it has an impact on the activity and diversity of bacterial communities [29, 41], and its degradation is mainly caused by the action of microorganisms.

There is therefore a serious need to assess the ability of microflora to acquire diuron adaptation or biodegradation capacities, especially in aquatic ecosystems, which are the final receptor environment for the molecule. Indeed, biodegradation could be a reliable and cost-effective technique for herbicide abatement. Although biodegradation in soils has been widely studied, there has been little research focusing on aquatic bacteria able to participate in remediation processes to eliminate or minimize diuron contamination of surface water and groundwater [2, 9]. The objective of this study was to isolate and characterize diuron-degrading bacteria from a water ecosystem, the Jauron River (Puy-de-Dôme, France) that has been sensitized to diuron exposure for more than 10 years (concentrations varying from 0.05 to 4.215 µg/L since 1997) [30].

Methods

Sampling Sites. Aquatic bacterial populations were sampled on 24 March 2003 at two sites of the Jauron river. Site Jau1, situated at the outlet of the watershed (total area: 11,975 ha) in an intensively farmed area, was used to represent agricultural pollution. Site Jau3, located 9 km upstream of site Jau1, collects water running out into an animal farming area that is subjected to relatively little pesticide treatment, and was considered as a nonpolluted reference point. The concentration of diuron at these sites varied from 0.06 to 2.6 µg/L between 2000 and 2004 [30], thus exceeding the authorized limit for herbicides in river water (0.1 µg/L). The sampling was realized before water exposure to diuron (and other herbicides used in the watershed basin) to ensure maximum diversity in the bacterial population at the beginning of the experiment.

Enrichment Cultures and Bacteria Isolation. The river water was filtered onto a 5-µm filter (SM type, Millipore) to conserve mainly the bacterial fraction. To estimate the capacity of the bacteria to degrade diuron, 10 mL of water (containing about 5.10^6 bacteria/mL) were added to 500 mL of a mineral salt solution (MS) [34] containing per liter of sterilized deionized water: 1.6 g K_2HPO_4 ; 0.4 g KH_2PO_4 ; 0.2 g $MgSO_4 \cdot 7 H_2O$; 0.1 g NaCl; 0.02 g $CaCl_2$; 1 mL of a salt stock solution (2 g/L H_3BO_3 ; 1.8 g/L $MnSO_4 \cdot H_2O$; 0.2 g/L $ZnSO_4 \cdot 7 H_2O$; 0.1 g/L $CuSO_4$; 0.25 g/L Na_2MoO_4 and 1 microcrystal of $CoNO_3$); 1 mL of a vitamin stock solution (5 mg/L thiamine-HCl; 2 mg/L biotin); and 1 mL of an $FeSO_4 \cdot 7 H_2O$ stock solution (5 g/L). The $FeSO_4$ and vitamin stock solutions were filter-sterilized. Diuron, added as source of

carbon (C), was dissolved in dimethyl sulfoxide (DMSO) to 0.3 g/mL and used as a stock solution to contaminate Erlenmeyer flasks. Pure diuron molecule powder was obtained from Rhône-Poulenc Agrochimie (France). Flasks with no added diuron but supplemented with DMSO were considered as negative controls. The diuron concentration in the enrichment medium was initially 25 mg/L and was gradually doubled at 2-week intervals in subsequent flasks, along a 56-day enrichment culture, to reach a maximum of 200 mg/L. The enrichment culture was realized by transferring 10 mL into 490 mL of fresh MS. The flasks were incubated at 28°C on an orbital shaker at 150 rpm in darkness to avoid photolysis of diuron. Bacterial growth was monitored at selected time points by DAPI staining (4',6'-diamidino-2-phenylindole), and the enrichment cultures were sampled weekly for molecular analyses and bacteria isolation. To isolate the strains able to degrade diuron, MS agar (MSA) plates (10 g/L agar) were spread with 0.1 mL of a 20-mg/mL diuron solution in acetone. Other nonseeded MSA plates were used as negative controls. The acetone was then air-evaporated. Tenfold serial dilutions of the weekly enrichment culture samples were spread onto the agar plates and incubated at 28°C in the dark for at least 8 days.

Deoxyribonucleic Acid (DNA) Extraction and Polymerase Chain Reaction (PCR) Amplification. Bacterial genomic DNA was extracted from 100 mL of bacterial cultures as described by Pesce *et al.* [29].

Total genomic DNA extracts were used as a template to PCR amplify V1 to V3 variable regions of 16S rDNA gene with the bacterium-specific forward primer GC-27f and the reverse primer 518r (MWG, Biotech; Table 1). The PCR products contained a GC-clamp of 40 bases added to the forward primer and had a total length of 566 bp (based on the reference strain *Escherichia coli* K-12). PCR reactions were performed in a total reaction volume of 50 µL containing: 200 µM of each deoxyribonucleotide triphosphate (dNTP), 0.5 µM of each primer, 1 × PCR buffer containing 1.5 mM $MgCl_2$, 1.25 U of Hot Star *Taq* DNA polymerase (Qiagen, Courtaboeuf, France), and 5 µL of genomic DNA. DNA amplification was carried out on a GeneAmp PCR system 2400 thermocycler (PerkinElmer) as follows: denaturation at 95°C for 15 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s per cycle, and a final extension step at 72°C for 10 min. For cloning (see below), amplification was performed with the 27f/518r primer pair under the same conditions. Amplification products were analyzed by electrophoresis on a 0.8% agarose gel (*w/v*) in 1 × Tris-borate-EDTA (TBE) containing 0.5 µg/mL ethidium bromide. DNA was quantified using the DNA quantitation kit fluorescence assay (Sigma-Aldrich, France) according to the manufacturer's instructions.

The entire 16 S rDNA of isolated bacteria was amplified using the 27f/1492r primers (Table 1). The program consisted of a denaturation at 95°C for 15 min followed by 30 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 45 s per cycle, and a final extension step for 10 min at 72°C.

Temporal Temperature Gradient gel Electrophoresis (TTGE). TTGE analyses were performed using a DCode system (Bio-Rad, France) on 300 ng of PCR products applied directly onto 7% (w/v) polyacrylamide gels containing 7 M urea, 1 × Tris–acetate–EDTA (TAE). Runs were performed in 1 × TAE at 67 V for 17 h with a temperature ranging from 66°C to 69.6°C and a ramp rate of 0.2°C per hour. After completion of electrophoresis, gels were stained for 1 h in the dark in a Gel Star nucleic acid gel stain bath (BMA) and photographed using a Versa Doc™ Imaging System (Bio-Rad) under UV transillumination.

Clone Library Construction of Single TTGE Bands and Sequencing. Each DNA band of interest was excised from the polyacrylamide gel and small gel pieces were placed into sterilized vials. The samples were subjected to passive diffusion (18 h at 37°C) after adding 200 µL of elution buffer (0.5 M ammonium acetate, 1 mM EDTA, pH 8). The supernatant containing DNA was then precipitated with ethanol (vol/2 vol) and incubated for at least 1 h at –20°C. After centrifugation (10,000×g, 4°C, 10 min), the DNA pellet was washed with 70% ethanol, vacuum-dried, and dissolved in 25 µL Tris–EDTA (TE) buffer (pH 8) overnight at 4°C. Five microliters of DNA were then used as template for a reamplification step using the GC27f/518r primers, as described above. Purity of the PCR reamplification product was checked by rerunning an aliquot on TTGE gel. If TTGE revealed more than just

the PCR-reamplified TTGE band, the target band was reexcised, reamplified, and controlled twice. The purified DNA band was then amplified with 27f/518r primers as described above (Table 1) before being cloned into a pGEM-T-Easy vector (Promega, Charbonnières, France) to construct clone libraries. At least four clones were reamplified and rerun on TTGE gel for comparison with the parent band. A minimum of two cloned fragments, which comigrated with the original bands, were bi-directionally sequenced (MWG-Biotech). Sequences were submitted to the National Center for Biotechnology Information (NCBI, BLASTn program, [1]) for species assignment and to the CHECK-CHIMERA program of the Ribosomal Database Project (RDP; [26]) to detect potential chimeric artifacts [20].

Entire 16S rDNA sequences of the three isolated strains (IB78, IB85, and IB93) were deposited in GenBank under Accession Nos: DQ983422, DQ983423, and DQ983424. Phylogenetic trees were built using the ARB software package [25].

Synthesis of Digoxigenin-labeled DNA Probe. Multiple alignments of partial 16S rDNA sequences performed on ClustalW software highlighted V1 to V3 variable regions and made it possible to design specific primers for each strain (Table 1). Probes were PCR-labeled with digoxigenin (PCR DIG labeling mix, Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. The PCR program was as follows: 95°C for 15 min, then 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s per cycle, and a final extension step at 72°C for 7 min.

Dot-blot and Colony Hybridization. First, to determine the specificity of the probes, the different recombinant pGEM-T plasmid harboring the partial 16S rDNA fragment were denatured at 95°C for 5 min and spotted onto

Table 1. Name and sequence of primers used in this study. The fragment length (bp) obtained after polymerase chain reaction (PCR) amplification with the forward (F) and reverse (R) primers was also indicated

Primer	Sequence (5'–3')	Fragment length (bp)	References
GC-clamp	CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGG		
27f	AGAGTTTGATCCTGGCTCAG	526	[51]
518r	ATTACCGCGGCTGCTGG		[28]
1492r	GGTTACCTTGTACGACTT	1484 (when coupled to the 27f primer)	[22]
78V3F	GTCTTCGGATTGTAAAGCAC	89	This study
78V3R	CGGTGCTTATTCTGTCCGG		This study
85V3F	GCCTTAGGGTTGTAAAGCTC	64	This study
85V3R	CGGGGCTTCTTCTCCGGA		This study
92V2F	GATTAGCTAATACCGCATAACG	79	This study
92V2R	AGACATCGGCCGCTCCTA		This study
92V3F	CCTTCGGGTTGTAAAGCAC	88	This study
92V3R	CGGTCCTTATTCTTCCGG		This study
93V2F	CTTACGCTAATACCGCATAACG	79	This study
93V2R	CGACATCGGCTCATTCAATCG		This study
93V3F	CCTTCGGGTTGTAAAGCCC	88	This study
93V3R	CGGTGCTTATTCTTTGGGTA		This study

positively charged nylon membranes (Hybond-N⁺, Amersham International). DIG-labeled control DNA (Roche Diagnostics) was used as positive control for the Dot-blot detection. The DNA was fixed by sandwiching the membrane between two pieces of Whatmann 3 MM paper and baking for 2 h in vacuum at 80°C. The membrane was then prehybridized in hybridized solution (5 × Sodium chloride-Sodium Citrate [SSC], 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulfate (SDS), 100 µg/mL salmon sperm, 50% formamide) for 2 h at 42°C, and the hybridized solution was discarded and replaced with fresh solution containing about 25 ng of denatured probe/mL, after which incubation was continued at 42°C overnight. The membrane was then washed twice (2 × SSC, 0.1% SDS) at room temperature for 5 min each time followed by two 15-min washes at 68°C in 0.1 × SSC, 0.1% SDS, and saturated in 1 × phosphate-buffered saline (PBS), 3% bovine serum albumin (BSA), 0.3% Tween 20 for 30 min at room temperature. Detection was performed by incubation for 30 min at room temperature with alkaline-phosphatase-conjugated anti-DIG antibodies diluted at 1/5000 in 1 × PBS, 0.1% Tween 20 and with NBT/BCIP substrate, as recommended by the manufacturer (Roche Diagnostics).

For bacteria isolation, colonies from the weekly samples of enrichment culture spread on MSA plates were streaked in duplicate onto Luria–Bertani agar (LB agar) plates and incubated at 28°C for 24 h. Colonies of one LB-agar plate were replicated by placing a nylon membrane aseptically on top of the bacteria. The membranes carrying the colony lifts were treated as described by Heikinheimo *et al.* [15] before being prehybridized, hybridized, and revealed, as described above.

Growth of Single Strains with Diuron and Degradation Capacity. Isolated bacteria were cultured in MS supplemented with diuron (20 mg/mL dissolved in DMSO) as main source of carbon for 23 days at 28°C in the dark on an orbital shaker. The diuron-degrading *Arthrobacter* sp. N2 [52] and the XL1 *E. coli* strains were used as positive and negative growth controls, respectively. To eliminate the possibility of DMSO serving as a main carbon source, control cultures amended with the same amount of DMSO, but no herbicide was included. Growth of pure culture was then monitored by measuring OD at 600 nm.

To determine diuron-degradation capacity, isolates were grown in Trypcase Soy (TS) broth medium (bioMérieux, Marcy l’Etoile, France) for 24 h at 28°C and centrifuged at 8000 × g for 15 min, and the resulting pellet was washed twice with mineral salt solution (MMK) [52]. Three and one half gram wet weight of cells (resting cells) were then resuspended in 100 mL of either 2 mg/L, 10 mg/L or 20 mg/L diuron solution (0.1% final DMSO) prepared from stock solutions of

diuron dissolved in DMSO, and were incubated at 28°C in the dark on a rotary shaker (200 rpm). The controls consisted of preparations incubated under the same conditions with diuron alone or with autoclave-killed bacteria (sterile control). Samples (5 mL) were regularly removed and centrifuged (15 min, 3000 × g). The supernatant (4 mL) was extracted with ethyl acetate (2 × 1 mL) and dried on MgSO₄. One hundred microliters of the combined crude extracts were then injected onto a high-performance liquid chromatography (HPLC) column. HPLC analyses were performed using a Waters 600E chromatograph fitted with a normal phase column (Nucleosil 3 µm Si 150 × 4.6 mm) at room temperature. Detection was performed using a Waters 486 UV detector set at 254 nm. The mobile phase used was ethyl acetate/cyclohexane 60/40 V/V and the flow rate was 0.7 mL/min. All the degradation test cultures were realized in duplicate. When degradation assays were performed with bacterial mix, 1.75 g wet weight (final biomass = 3.5 g) or 3.5 g wet weight of each strain were pooled.

Results

Enrichment of a Bacterial Culture that Degrade Diuron. Jauron river water samples taken from site Jau3 considered as nonpolluted reference point and from site Jau1 considered as representative of agricultural pollution were subcultured for 56 days in either control flasks or flasks containing increasing concentrations of diuron as main source of carbon for bacteria growth. In all flasks, bacteria were inoculated at about 10⁵ cells/mL (stained DAPI) and reached values ranging between 3.3 × 10⁶ and 1.0 × 10⁷ cells/mL over the 14-day incubation intervals. No difference in enumeration of total bacteria was observed between the control flasks and treated flasks.

To monitor bacterial population diversity over time and assess the impact of diuron on these communities, the V1–V3 16 S rDNA amplification products from weekly enrichment culture samples were analyzed by TTGE (Fig. 1). TTGE fingerprints showed that numerous faint bands were detected at day 0, suggesting a low density of each natural bacterial population. Shifts in band patterns were quickly observed (day 7) and revealed that the majority of day 0 faint bands disappeared and that some species became more prevalent in control and diuron-treated flasks. In samples from the reference site Jau3, there was no major difference between control and contaminated flasks over the first 14 days (Fig. 1B), whereas samples from the site Jau1 showed a different band pattern in diuron-treated flasks compared to controls, appearing as early as day 7 (Fig. 1A). After 21 days of culture, a second shift was observed in both control and treated flasks that highlighted some environmental condition-specific strains. Indeed, some bands

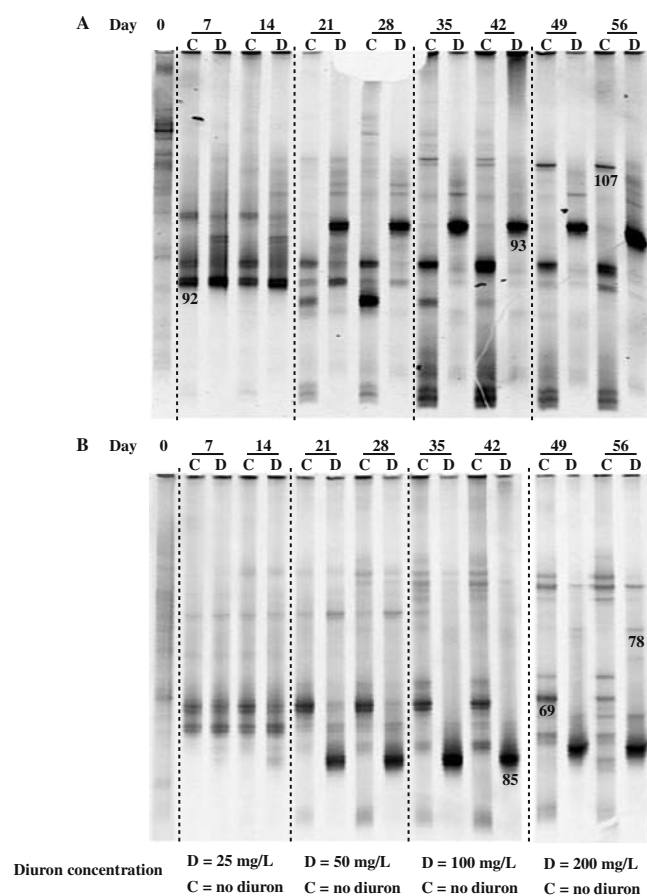


Figure 1. Study of bacterial community evolution by TTGE analysis of V1–V3 16S rDNA fragments in control (C; without diuron) and diuron-treated (D) enrichment cultures inoculated with bacteria from site Jau1 (A) and site Jau3 (B) from day 0 to day 56. Site Jau1 is representative of agricultural pollution, whereas site Jau3 was considered as a nonpolluted reference point. Numbers represent the bands of interest that were excised from the gel, purified, and sequenced.

were found only in diuron conditions and were completely absent in untreated Erlenmeyers. They persisted as preponderant bands in the corresponding diuron enrichment culture even with the highest concentration of the molecule. These specific patterns persisted until the end of the experiment (day 56; 200 mg/L). Furthermore, there was an increase in diversity in control flasks from the site Jau3 from day 35 to day 56, although the diuron-treated Erlenmeyer showed no major variation (Fig. 1B). This band pattern was not reproduced in the Jau1 samples (Fig. 1A).

Identification and Isolation of Diuron-degrading Strains. Dominant bands of interest (Fig. 1) corresponding to strains, which seemed to grow indifferently in the presence or absence of diuron (e.g., band 92, although with varying intensity), strains which were not able to develop or which disappeared in diuron-treated cultures (e.g., bands 107, 69), and strains that grew only in diuron-contaminated culture (e.g., bands, 78, 85, 93) were excised and purified. Before sequencing, and to confirm that the correct fragments were recovered, a control TTGE gel was run between parental bands and purified bands. The nearest neighbor and phylogenetic group of each sequence are listed in Table 2. The results indicated that the strains studied mainly belonged to Pseudomonadaceae and Rhizobiaceae families regardless of their sensitivity or their capacity to adapt to diuron exposure.

To isolate diuron-degrading bacterial strains, the enrichment cultures were plated on either diuron-supplemented or control MSA. There were no significant between-plate differences in cell density and colony morphology. Because it was impossible to distinguish bacteria between control and contaminated cultures, a colony hybridization method was used. With this in mind, sequences obtained from the different bands of

Table 2. Similarity to nearest neighbors and closest phylogenetic affiliation of TTGE band sequences detected in enrichment cultures based on BLASTn comparison to the GenBank database

Band	Similarity(%)	Nearest neighbor		
		Species	Accession number	Phylogenetic group
69	98	[<i>Agrobacterium tumefaciens</i>]	M11223	Rhizobiaceae, α proteobacteria
78	99	[<i>Pseudomonas mendocina</i>]	AJ006109	Pseudomonadaceae, γ proteobacteria
	99	{Uncultured gamma proteobacteria}	AY221609	Pseudomonadaceae, γ proteobacteria
	99	{ <i>Pseudomonas</i> sp.}	AM231055	Pseudomonadaceae, γ proteobacteria
85	98	[<i>Rhizobium</i> sp. SDW062]	AF345553	Rhizobiaceae, α proteobacteria
	99	{ <i>Agrobacterium tumefaciens</i> }	DQ468100	Rhizobiaceae, α proteobacteria
92	99	[<i>Ralstonia</i> sp. C1]	AY479983	Burkholderiaceae, β proteobacteria
	99	[Uncultured bacterium clone TM18]	AY838532	Uncultured bacterium
93	96	[Bacterium 7B9]	DQ298776	Uncultured bacterium
	96	[Uncultured bacterium clone negative-42]	AY527811	Uncultured bacterium
	99	{Uncultured bacterium clone rRNA020}	AY958793	Uncultured bacterium
	99	{ <i>Stenotrophomonas</i> sp. LXQ-11}	DQ256392	Pseudomonadaceae, γ proteobacteria
107	99	[<i>Pseudomonas nitroreducens</i> strain 0802]	AF494091	Pseudomonadaceae, γ proteobacteria

[]: alignment of V1 to V3 16S rDNA sequences; { } : alignment of entire 16S rDNA sequences

interest were aligned to design primers targeting variable regions of each strain (Table 1) and consequently generate probes. The cross-reactivity of the probes against other strains used in this study was tested and showed high specificity for each probe. Three colonies corresponding to bands 78, 85, and 93 (Fig. 1) were successfully isolated and were named strains IB78, IB85, and IB93, respectively. The 16S rDNA V1–V3 regions were reamplified to compare PCR products with parental bands and confirm that the colonies isolated were the colonies expected. Their entire 16S rDNA was then sequenced using the 27f/1492r primer couple (Table 1) and aligned using the BLASTn program. The results matched those obtained with the V1–V3 phylogenetic affiliation of these strains (Table 2) and confirmed that strains IB78, IB85, and IB93 are 99% similar to uncultured gamma *Proteobacteria*/*Pseudomonas* sp., *Agrobacterium tumefaciens*, and uncultured bacterium/*Stenotrophomonas* sp., respectively. ARB phylogenetic trees were also constructed (Fig. 2) that confirmed the BLASTn affiliation. Despite a series of attempts, we were unable to isolate the IB92 strain.

Capacity of the Isolates to Grow with and Degrade Diuron. Each isolate was first tested for its capacity to develop in MS medium supplemented with 20 mg/L of diuron. The resulting growth curves showed that the IB78 and IB93 isolates, but not the IB85 strain were able to grow in the presence of diuron (data not shown). The absence of turbidity in the MS supplemented only with DMSO provided additional suggestive evidence that the isolates grew at the expense of diuron.

We then investigated the capacity of each isolate to degrade 2 mg/L to 20 mg/L diuron in resting cells (3.5 g wet weight of cells in 100 mL MMK buffer). The kinetics of degradation for each strain are illustrated in Fig. 3. The IB78 isolate (Fig. 3A) presented a maximum percentage of diuron degradation of 25, 30, and 60% for 20, 10, and 2 mg/L diuron, respectively. In each case, diuron degradation occurred mainly during the first 24 h according to a first-order kinetic pattern, and then reached a plateau where degradation occurred only very slowly and weakly. Similar diuron degradation curves were observed for the IB93 isolate (Fig. 3B), which presented a maximum percentage of degradation of 20, 30, and 65% for 20, 10, and 2 mg/L of diuron, respectively. Diuron degradation by IB93 also took place during the first 24 h of incubation before reaching a plateau phase. However, another phenomenon was observed with the IB93 isolate, i.e., the diuron concentrations at the beginning of the experiments were lower than expected concentrations (1 mg/L instead of an expected 2 mg/L, 7.3 mg/L instead of 10 mg/L, and 17.3 instead of 20 mg/L). Such an effect has been observed previously in other experiments, where it was attributed to immediate biosorption. A rapid subsequent release of the herbicide was

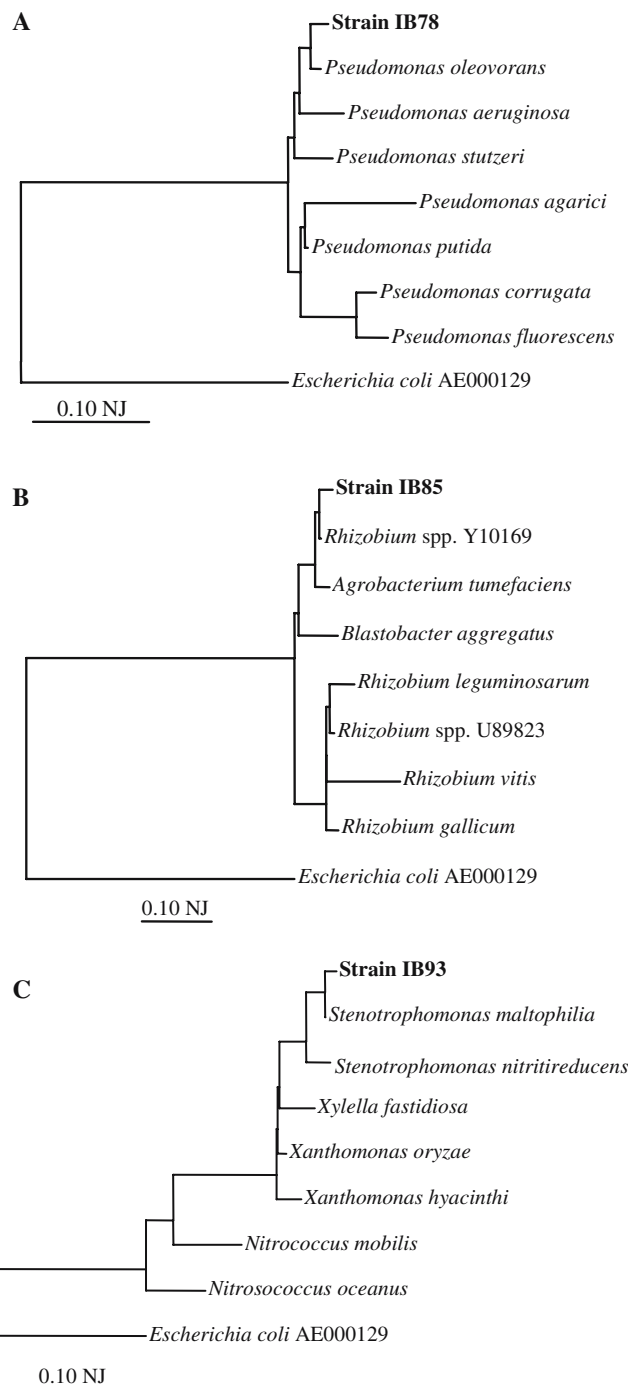


Figure 2. Phylogenetic trees based on the entire 16S rDNA sequence alignments. The trees were built using the neighbor-joining method with the use of the ARB software package [25]. A: IB78 isolate affiliation; B: IB85 isolate affiliation; C: IB93 isolate affiliation.

then observed and the diuron concentrations detected after 30 min of incubation corresponded almost to the theoretical values (1.7 and 9.3 mg/L instead of 2 and 10 mg/L, respectively). Controls containing the substrate alone or

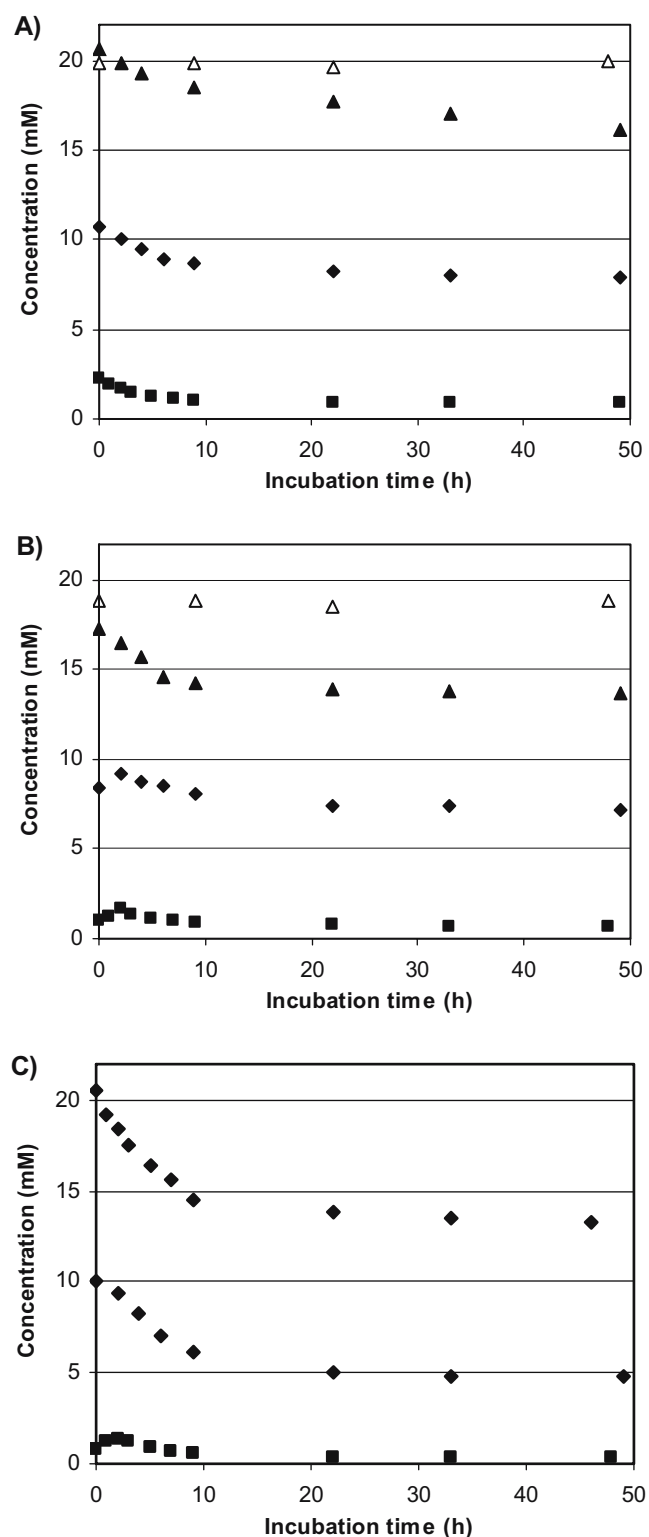


Figure 3. Degradation of diuron in resting cells (3.5 g wet weight of each strain in 100 mL MMK buffer). Assays were performed in the dark at 28°C with 2 mg/L (■), 10 mg/L (◆), and 20 mg/L (▲) of diuron. A: isolate IB78; B: isolate IB93; C: IB78 and IB93 combined. Δ: incubation of diuron with autoclave-killed bacteria (sterile control). Similar results were obtained in two independent experiments.

sterile controls incubated under the same conditions did not show any diuron disappearance (Fig. 3A and B). Based on these results, we can conclude that for each concentration tested, both the IB78 and the IB93 strains are able to degrade diuron with equivalent kinetics and at similar percentages, whereas after 5 days of incubation the IB85 isolate showed no diuron degradation (data not shown).

The capacity to degrade diuron was also tested with resting cells of mixed IB78 and IB93 isolates (3.5 g wet weight of each strain; Fig. 3C). The percentage of degradation reached 40, 50, and 85% (*versus* 20–25, 30, and 60–65% for strains tested independently) for 20, 10, and 2 mg/L, respectively, showing some concerted action of the two isolates. Similar to individual strains, degradation occurred during the first 24 h before the curve reached a plateau. In all cases, this plateau phase appeared not to be attributable to toxicity of a degradation product toward bacteria because neither 3,4-dichloroaniline nor any other degradation products could be detected by HPLC, and the bacterial counts on plates of viable bacteria remained stable until the end of the experiment (data not shown). The cumulative degradation was also confirmed when mix was realized with only 1.75 g wet weight of each strain because percentages of diuron degradation were at least equal to that observed with double biomass of individual strain (40% *versus* 30% for 10 mg/L diuron and 70% *versus* 60–65% for 2 mg/L diuron; data not shown).

Discussion

Enrichment Culture with Diuron. During enrichment culture, bacterial growth was observed in both diuron-treated and control flasks. The MS used by Rousseaux *et al.* [34] to isolate atrazine-degrading bacteria led to specific halos produced by colonies able to degrade atrazine on petri dishes and allowed a rapid selection of bacteria of interest. A similar light halo surrounding colonies has also been observed for diuron-degrading bacteria [52] and isoproturon-mineralizing bacteria [10]. However, in our study, there was no clear zone around the bacteria on the MSA spread with diuron, making it more difficult to isolate bacteria of interest. This could be caused by a loss of degradation capacity on solid media [8]. Furthermore, the isolation of only diuron-degrading bacteria was made more difficult by the riverine bacterial inocula, which would probably also transfer nutrient sources present in the mesoeutrophic-level water of the Jauron river [29]. The DMSO used to dilute the diuron molecule and the transfer of dead bacteria during the enrichment subculture stage may also bring additional nutrient sources for growth [3, 48]. On MSA plates, the bacterial background may be the result of carbon sources brought by impurities in the agar [3, 4, 8, 48] or by traces of the acetone used to introduce diuron on the surface agar plates.

Bacterial Response to Diuron Exposure. Contrary to spreading bacteria on agar plates, the TTGE approach allowed highlighting differences in bacterial populations of control and diuron-treated cultures. Indeed, we were able to reveal major shifts in dominant bacterial community with regard to natural populations (day 0) and between control and diuron-treated flasks (Fig. 1). As early as 7 days of enrichment culture, there was a drastic decrease of dominant bacteria community, probably as a result of the experimental adaptation phase and/or the unculturability of certain bacteria. Similar data were obtained by Pesce *et al.* [29] using a realistic diuron pollution level (10 µg/L) on bacteria sampled from the site Jau1 of the Jauron river at the same period of the year. They also showed that the strong impact of diuron on bacterial community diversity was only perceptible from the second week, after which only few variations were recorded in the treated microcosms. We obtained similar results because major shifts caused by high concentrations of diuron (25 to 200 mg/L) were also detected after the sixth day (seventh day and day 14 for the Jau1 and Jau3 samples, respectively; Fig. 1). This difference in lag before response to diuron could be attributable to a more rapid adaptation response from bacteria [6] from the site Jau1, which are more sensitized to this molecule than bacteria from the site Jau3. Indeed, bacteria from site Jau1 had regularly been exposed to diuron *in situ* in the Jauron river, at higher concentrations (0.06 to 2.6 µg/L) and for a longer period (two to 3 months/year) compared to bacteria from the site Jau3 (exposure to a maximum of 0.08 µg/L diuron 1 month/year) [30].

Identification and Isolation of Bacteria able to Degrade Diuron. TTGE bands of interest were sequenced and affiliated mainly to *Pseudomonadaceae* and *Rhizobiaceae* (Table 2). This phylogenetic affiliation was not surprising because it has previously been shown that *Pseudomonas* bacteria are ubiquitous in natural environments, generally grow rapidly in the presence of high concentrations of xenobiotics in enrichment culture and on agar plates, and are involved in the degradation of different phenylurea herbicides [4, 5, 7, 8, 33]. Nevertheless, one *Pseudomonas* species (band 107, Fig. 1A), although presenting close genetic affiliation to the diuron-degrading *Pseudomonas* sp. IB78 strain (99% similarity between bands 107 and 78), displayed widely different biodegradative capabilities because it was detected only in control flasks.

Only the strains IB78 (*Pseudomonas* sp.), IB85 (*Agrobacterium tumefaciens*), and IB93 (*Stenotrophomonas* sp.) could be successfully isolated. Strains showing 99% similarities with IB78 and IB93 isolates (Table 2)

have also been encountered in soils contaminated with metals and hydrocarbons ([18], accession No: AY221609) and in long-term pesticide-contaminated soils (Yang, unpublished data; accession No: DQ256392), respectively.

Pure-culture diuron degradation experiments in resting cells indicated that the strain IB85, although responsible for a strongly visible appearing band specific to treated flasks (Fig. 1B), was not able to degrade diuron. This result denotes that diuron had no toxic effect on this strain and suggests that the IB85 isolate may require a synergistic interaction with other bacteria to grow on the diuron molecule as sole C source. When isolates IB78 and IB93 were tested, we observed that within the first 24 h of incubation, these isolates responded to higher diuron concentrations by degrading the molecule more slowly and at a lower rate (Fig. 3). In all cases, this period was followed by a hitherto-unexplained plateau phase where only very low and slow additional degradation occurred.

Mixing IB78 and IB93 resting cells enhanced the degradation of diuron (Fig. 3). The concerted action of *Pseudomonas* sp. and *Stenotrophomonas maltophilia* has already been observed for degradation of 2,4-DNT [37], suggesting further evidence of their cumulative metabolic activities during the degradation of organic pollutants.

During these diuron degradation experiments, no 3,4-DCA was detected, which is in contrast with the majority of published studies that have reported an accumulation of 3,4-DCA as a final degradation product [3, 9, 40, 43–45, 47, 48, 52]. Conversely to El Fantroussi [8], 3,4-DCA was not detected even when incubation temperature was decreased to 17°C (data not shown). One potential explanation could be that isolates IB78 and IB93 were able to use an as yet undescribed degradation pathway that bypasses the formation of 3,4-DCA [5]. Another potential explanation could be that these two strains were able to degrade 3,4-DCA just as fast because no detectable accumulation level could be reached. This hypothesis is supported by preliminary results showing that isolate IB78 is able to degrade 3,4-DCA (5 mg/L) at about 50% within 24 h of incubation at 28°C (data not shown). It is also in concordance with the fact that a strain of *Pseudomonas* sp. has previously been shown to have a 3,4-DCA-degrading capacity [14].

Our laboratory is currently conducting research to identify the degrading gene involved in diuron biotransformation and the corresponding diuron degradation pathway.

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