Isolation from Agricultural Soil and Characterization of a Sphingomonas sp. Able To Mineralize the Phenylurea Herbicide Isoproturon

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A soil bacterium (designated strain SRS2) able to metabolize the phenylurea herbicide isoproturon, 3-(4-isopropylphenyl)-1,1-dimethylurea (IPU), was isolated from a previously IPU-treated agricultural soil. Based on a partial analysis of the 16S rRNA gene and the cellular fatty acids, the strain was identified as a Sphingomonas sp. within the α-subdivision of the proteobacteria. Strain SRS2 was able to mineralize IPU when provided as a source of carbon, nitrogen, and energy. Supplementing the medium with a mixture of amino acids considerably enhanced IPU mineralization. Mineralization of IPU was accompanied by transient accumulation of the metabolites 3-(4-isopropylphenyl)-1-methylurea, 3-(4-isopropylphenyl)-urea, and 4-isopropyl-aniline identified by high-performance liquid chromatography analysis, thus indicating a metabolic pathway initiated by two successive N-demethylations, followed by cleavage of the urea side chain and finally by mineralization of the phenyl structure. Strain SRS2 also transformed the dimethylurea-substituted herbicides diuron and chlorotoluuron, giving rise to as-yet-unidentified products. In addition, no degradation of the methoxy-methylurea-substituted herbicide linuron was observed. This report is the first characterization of a pure bacterial culture able to mineralize IPU.

The phenylurea herbicide isoproturon, 3-(4-isopropylphenyl)-1,1-dimethylurea (IPU), which is used for pre- and post-emergence control of annual grasses and broad-leaved weeds in wheat, rye, and barley crops, is among the most extensively used pesticides in conventional agriculture in Europe (34). Ecotoxicological data suggest that IPU and some of its metabolites are harmful to aquatic invertebrates (20), freshwater algae (25), and microbial activity (28). IPU is also suspected of being carcinogenic (2, 14). As a result of its widespread and repeated use, IPU is frequently detected in groundwater and surface waters in Europe in levels exceeding the European Commission drinking water limit of 0.1 μg l⁻¹ (23, 33, 34).

Degradation of IPU in agricultural soils occurs predominantly by microbiological processes (6, 22). Several studies have demonstrated a slow natural attenuation rate in various soils and subsurface environments with respect to mineralization of the phenyl structure (4, 15, 17, 18, 26, 35). The detection of IPU as an environmental pollutant and its apparently low mineralization potential has stimulated research aimed at isolating and characterizing microbial cultures able to mineralize IPU. Enrichment culture techniques have been used with varied success in attempts to isolate IPU-degrading microorganisms. In previous studies, slurries of mineral media and soils from different agricultural fields failed to degrade IPU (4, 19, 35). Enrichment on the IPU metabolite 3-(4-isopropylphenyl)-1-methylurea (MDIPU) as the sole source of carbon and energy recently yielded a mixed bacterial culture able to perform growth-linked mineralization of MDIPU and 4-isopropyl-aniline (41A) but with no degradation activity toward IPU (35). Several soil bacteria (7, 31) and soil fungi (3) are known to be able to catalyze transformation of the dimethylurea side chain of IPU, but there are no reports of microorganisms in pure culture able to mineralize the phenyl structure of IPU or any other phenylurea herbicides. Recently, El-Fantroussi (9) suggested that the lack of success in isolating pure cultures of phenylurea-mineralizing bacteria could be attributable to the involvement of consortia rather than single bacteria in the complete degradation.

In the present study we describe the isolation and characterization of an IPU-mineralizing Sphingomonas sp. (designated strain SRS2) from a British agricultural field that had previously been treated with IPU for several years. The study is the first to describe the isolation and characterization of a pure bacterial culture able to mineralize a phenylurea herbicide.

MATERIALS AND METHODS

Chemicals. Analytical-grade IPU (99.5% purity, 55-mg liter⁻¹ water solubility at 20°C), MDIPU (99.9% purity), 3-(4-isopropylphenyl)-urea (DDIPU) (98.3% purity), 4IA (99.5% purity), diuron (97.5% purity, 42-mg liter⁻¹ water solubility at 25°C), linuron (99.8% purity, 81-mg liter⁻¹ water solubility at 24°C), and chlorotoluuron (97.5% purity, 70-mg liter⁻¹ water solubility at 20°C) were purchased from Dr. Ehrenstorfer GmbH (Augsberg, Germany). Phenyl-U-¹⁴C]IPU (914 MBq mmol⁻¹, 97% radiochemical purity) (¹⁴C-IPU) was obtained from Amersham Life Science (Buckinghamshire, United Kingdom). Phenyl-U-¹³C]MDIPU (4.42 MBq mg⁻¹, 99% radiochemical purity) (¹³C-MDIPU) was purchased from the Institute of Isotopes (Budapest, Hungary). Phenyl-U-¹³C]4IA (773.3 MBq mmol⁻¹, >98% radiochemical purity) (¹³C-4IA) was purchased from International Isotope (Munich, Germany). The molecular structures of the compounds are presented in Fig. 1.

Enrichment and growth media. The mineral salt medium (MS) used for the enrichment and pure culture studies was modified from the HCMM2 medium.
Sterilized 100-ml properties and the sampling procedure have been described previously (35, 39).

Preceding IPU-treated agricultural fields located near Græse, Denmark, and near Wellesbourne (site E6, Deep Slade, United Kingdom). Details of the soil properties and the sampling procedure have been described previously (35, 39). Sterilized 100-ml flasks containing IPU (25 mg liter\(^{-1}\)) in 25 ml of MS were inoculated with 5 g of soil and sealed with airtight stoppers. The IPU had been added to the sterilized flasks from stock solutions in acetone (10 g liter\(^{-1}\)) and the solvent evaporated in a laminar flow bench before the addition of the liquid media. The flasks were placed in the dark at 20°C on an IKA Labortechnik KS 250 Basic Orbital Shaker (Staufen, Germany) at 100 rpm. Mineralization of the IPU was monitored by measuring the production of \(^{14}\)CO\(_2\) from added \(^{14}\)C-IPU as described below. Enrichment cultures showing mineralization of \(^{13}\)C-IPU were used to inoculate new flasks by transferring 0.1 ml to 49.9 ml of fresh IPU-containing MS medium. After more than 15 subcultures had been performed, a stable mixed bacterial culture was obtained. Since no IPU-degrading bacteria were obtained from the mixed culture by streaking samples onto various agar media, several successive dilutions (dilution ratio 1:10) were made to reduce the diversity within the mixed culture. 0.1 ml of each dilution were used to inoculate flasks with 49.9 ml of fresh IPU-containing (25 mg liter\(^{-1}\)) MS. \(^{14}\)CO\(_2\) production was measured for 30 days. Thereafter, the highest dilution able to mineralize \(^{13}\)C-IPU was diluted once again, and the procedure was repeated three times.

### Isolation, characterization, and identification.

To isolate pure cultures, aliquots (0.1 ml) were plated on different types of agar (NA, TSA, R2A, WA, and IPU agar). The plates were incubated for up to 1 month at 20°C. Colonies were removed and screened for their ability to degrade IPU in pure culture. After the successive dilutions of the enrichment culture, two strains of bacteria (designated SRS1 and SRS2) were isolated from plates with R2A. Stocks of both bacteria grown in R2B were maintained at ~80°C in 40% glycerol. Strain SRS2 was characterized and identified by Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, by analysis of the cellular fatty acids, partial sequencing of the 16S rRNA gene, and different physiological tests. Alignment of the partial 16S rRNA gene sequence was performed with sequence deposited in the GenBank database (National Center for Biotechnology Information) by using CLUSTAL W, version 1.8 (36). A neighbor-joining method (Neighbor-Joining/UPGMA, version 3.573c) from the PHYLIP software package (11) was used to estimate relatedness.

### Preparation of inoculum.

Prior to the pure culture degradation studies with the isolated strain SRS2, plate counts on R2A were correlated with optical density measurements (600 nm) in R2B. The strain was grown in 250-ml Erlenmeyer flasks containing 100 ml of R2B incubated on a platform shaker at 150 rpm (20°C). Cells were harvested in the late-exponential-growth phase by centrifugation (10 min, 3,500 × g, 20°C), washed twice in medium, and adjusted to a density of 5 × 10\(^8\) cells ml\(^{-1}\). Each flask was inoculated with washed cells suspended in 1 ml of mineral medium to provide a final density of 10\(^8\) cells ml\(^{-1}\).

### Degradation and mineralization.

All phenylurea and aniline compounds included in this study were added to sterilized flasks as previously described for IPU in the enrichment studies. The herbicides and their metabolites were measured by using a Hewlett-Packard Series 1050 HPLC System (16): 750-\(\mu\)l aliquots were filtered through a 0.45-\(\mu\)-m (pore-size) Tygon syringe filter (Scientific Resources, Eatontown, N.J.), and the last 25 \(\mu\)l was collected for analysis. Mineralization of \(^{14}\)C-labeled IPU, MDIPU, and 4IA was measured by trapping the evolved \(^{14}\)CO\(_2\) in an alkaline solution. Approximately 40,000 dpm of \(^{14}\)C-labeled compound and 1.25 mg (25 mg liter\(^{-1}\)) of unlabeled compound were added to each flask. Then, 49 ml of liquid medium was added, and the flasks were inoculated with 1 ml of the cell suspension. A 5-ml test tube holding 2 ml of 0.5 M NaOH was mounted in the flasks. Upon sampling, the alkaline solution was replaced with fresh solution and the used solution was mixed with 10 ml of Wallac OptiPhase HIsafe 3 scintillation cocktail (Turku, Finland) and counted on a Wallac 1409 liquid scintillation counter. The results were corrected for quenching and background radioactivity. Sterile or uninoculated controls were included in all experiments.

### RESULTS

**Mineralization of IPU in agricultural soils.** Different IPU mineralization patterns were observed in the two soils studied (Fig. 2) The IPU mineralization rates in slurries of the soil from Græse were constant, and only 6.0% ± 0.5% (\(n = 3\)) of the added \(^{14}\)C-IPU was metabolized to \(^{14}\)CO\(_2\) within 40 days. In contrast, rapid and accelerated mineralization of IPU was measured in the soil from Deep Slade. The initial degradation rate varied between replicates in the case of the latter soil, but by day 40, 40 to 53% of the \(^{14}\)C-IPU had been metabolized to \(^{14}\)CO\(_2\) in all flasks.

**Enrichment and isolation.** Aliquots of slurries of the soil from Deep Slade exhibiting the fastest mineralization were transferred to fresh IPU-containing MS medium. The mixed IPU-mineralizing cultures obtained were subcultured several times and then diluted serially. After three dilution cycles, aliquots of the highest dilution able to mineralize \(^{14}\)C-IPU were plated onto the R2A, WA, TSA, LB, NA, and IPU agars.
Growth of one colony type was seen on R2A and TSA after 2 days of incubation. Based on BIOLOG-GN (Biolog, Hayward, Calif.) profiles, these colonies were determined to be identical and were designated strain SRS1. Between days 5 and 6, another colony with a different morphology appeared on R2A but not on TSA. The strain was designated SRS2. No colonies were observed on WA, LB, NA, or IPU agar after 30 days at 20°C. Only strain SRS2 was able to degrade IPU and, to ensure purity, it was passed from IPU-containing MS to R2A three times.

Characterization and identification of strain SRS2. Strain SRS2 proved to be a gram-negative non-spore-forming rod with a width of 0.6 to 0.8 μm and a length of 1.5 to 2.5 μm. It is oxidase negative, catalase positive, aminopeptidase positive, and urease negative. It hydrolyzes esculin but not gelatin, DNA, or casein. It is negative in tests for indole production and denitrification. No growth was observed at 42°C after 7 days, and no growth was observed in BIOLOG-GN microplates. Growth on agar was restricted to R2A, where it forms reddish brown colonies within 6 to 7 days at 20°C. The degradative ability of strain SRS2 was very stable and was retained after several generations of nonselective growth on R2A. A whole-cell fatty acid profile revealed that the dominant fatty acids were 53.2% 18:1 (sum of 18:1ω7c, 18:1ω9t, and 18:1ω12t), 20.5% 16:1ω7c and/or 2OH(iso)15:0, 7.2% 2OH14:0, and 6.8% 16:0, which is typical for the genus *Sphingomonas* (1, 24). Upon comparison of a partial 16S rRNA gene sequence (425 bases) obtained from strain SRS2 with sequences from the GenBank Database, the highest degree of similarity (97%) was obtained with the 16S rRNA gene sequence of a dibenzo-p-dioxin-degrading *Sphingomonas* sp. strain RW1 (21, 41). Alignment of the partial 16S rRNA gene sequences revealed a close phylogenetic relationship to several *Sphingomonas* spp. (data not shown). The partial 16S rRNA gene sequences have been deposited in the GenBank Database under accession no. AJ251638.

Degradation and growth studies with *Sphingomonas* sp. strain SRS2. Strain SRS2 mineralized the phenyl structure of IPU slowly (Fig. 3A). Supplementing *Sphingomonas* sp. strain SRS2 with Casamino Acids significantly enhanced the degradation activity and resulted in mineralization of ca. 50% 14C-IPU to 14CO2 within 5 days (Fig. 3B). High-pressure liquid chromatography (HPLC) analysis revealed no IPU, metabolites, or unidentified peaks at the end of the experiment (data not shown). Although *Sphingomonas* sp. strain SRS2 was able to utilize IPU for growth (Fig. 4A), the growth was slow compared to cultures supplemented with amino acids (Fig. 4B and C). Growth of *Sphingomonas* sp. strain SRS2 was not supported on amino acids alone, as shown in controls of MS-CA, MS-A19, and MS-A2 without IPU (Fig. 4B and C and Table 1). Approximately 6.0 × 107 cells SRS2 were produced during degradation of 25 mg of IPU liter−1 (Table 1). Further studies revealed that SRS2 was able to mineralize IPU, MDIPU, and 4IA in MS medium containing L-methionine and glycine (MS-A2) (Fig. 5 and Table 1). HPLC analysis revealed transient accumulation of a main metabolite with the same retention time as MDIPU during the mineralization of IPU (Fig. 4). Trace amounts of metabolites with the same retention times as DDIPU and 4IA were also detected (data not shown). *Sphingomonas* sp. strain SRS2 also mineralized 14C-MDIPU and 14C-4IA (Fig. 5). The mineralization patterns for IPU and the two metabolites revealed that MDIPU was mineralized...
more slowly than IPU and 4IA, although the amount of $^{14}$C-labeled compound metabolized to $^{14}$CO$_2$ after 10 days was approximately the same for all three compounds (46 to 49%) (Fig. 5). No phenylurea or aniline compounds were detected at the end of the experiment. *Sphingomonas* sp. strain SRS2 was also able to utilize MDIPU, DDIPU, and 4IA for growth in mineral medium supplemented with L-methionine and glycine (MS-A2) (Table 1). Moreover, it was able to degrade diuron and chlorotoluron, both of which contain a dimethylurea side chain like IPU, giving rise to a reddish (chlorotoluron) or brown (diuron) coloration of the medium. No metabolites were detectable by HPLC after dissipation of the parent compound. The coloration of the medium made growth measurements by optical density impossible. No growth of strain SRS2 was observed by plate counts on R2A during degradation of diuron and chlorotoluron, however (Table 1). Linuron, a phe-
TABLE 1. Growth and degradation of phenylurea herbicides and IPU metabolites by 
Sphingomonas sp. strain SRS2 in 50 ml of MS medium with L-methionine and glycine 
(MS-A2) after incubation for 10 days

<table>
<thead>
<tr>
<th>Compound</th>
<th>Degradation</th>
<th>CFU ml⁻¹</th>
<th>OD₆₀₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPU</td>
<td>+</td>
<td>(6.1 ± 0.9) x 10⁷</td>
<td>0.026 ± 0.001</td>
</tr>
<tr>
<td>MDIPU</td>
<td>+</td>
<td>(5.8 ± 0.4) x 10⁷</td>
<td>0.022 ± 0.001</td>
</tr>
<tr>
<td>DDIPU</td>
<td>+</td>
<td>(5.9 ± 0.6) x 10⁷</td>
<td>0.022 ± 0.001</td>
</tr>
<tr>
<td>4IA</td>
<td>+</td>
<td>(5.9 ± 0.2) x 10⁷</td>
<td>0.025 ± 0.004</td>
</tr>
<tr>
<td>Diuron</td>
<td>+</td>
<td>(1.2 ± 0.8) x 10⁶</td>
<td>Colored⁺</td>
</tr>
<tr>
<td>Chlorotoluron</td>
<td>+</td>
<td>(1.2 ± 1.1) x 10⁶</td>
<td>Colored⁺</td>
</tr>
<tr>
<td>Linuron</td>
<td>-</td>
<td>(1.4 ± 1.1) x 10⁶</td>
<td>0.005 ± 0.001</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>(1.3 ± 1.0) x 10⁶</td>
<td>0.003 ± 0.001</td>
</tr>
</tbody>
</table>

‡ Each compound was provided at 25 mg liter⁻¹. Cell growth is given as the mean optical density at 600 nm (OD₆₀₀) of duplicates or triplicates (plating) with the standard deviation.

⁺ Degradation is based on HPLC results.

Plating on R2A medium.

Controls with MS-A2 and no addition of herbicides or metabolites.

See Results.

nylurea herbicide containing a methoxy-methyl side chain, was not degraded by 
Sphingomonas sp. strain SRS2, either. No degradation of any of the compounds included in this study was measured in controls without Sphingomonas sp. strain SRS2 (data not shown).

DISCUSSION

Inoculation of soils from two previously IPU-treated agricultural fields into liquid medium containing ¹⁴C-IPU revealed a substantial difference in the ability of the soil microorganisms to mineralize the labeled IPU to ¹⁴CO₂. Stable enrichment cultures able to rapidly mineralize IPU were obtained from the Græone of the soils (from Deep Slade). In contrast, the mineralization of IPU by Sphingomonas sp. strain SRS2, either. No attempts were made to homogenize the soils and the variation among replicate slurries of the Deep Slade soil may reflect a heterogeneous distribution of IPU-degrading bacteria, as recently demonstrated by Walker et al. (39) in a study of soil from Deep Slade.

A bacterial strain, designated SRS2, able to completely metabolize IPU to CO₂ and biomass was isolated from the Deep Slade soil. 16S rRNA gene sequencing and the characteristic cellular fatty acid composition strongly suggested that strain SRS2 belongs to the genus Sphingomonas. Strain SRS2 was phylogenetically related to several previously characterized Sphingomonas spp. able to degrade various aromatic and chloroaromatic compounds (1, 24, 41, 42). That the 16S rRNA gene sequence similarity obtained with the 16S rRNA gene sequence of Sphingomonas sp. strain RW1 (41) was no greater than 97% indicates that strain SRS2 is a member of a new species within Sphingomonas. Several previously characterized bacterial strains able to degrade xenobiotic aromatic compounds have recently been reclassified as members of the genus Sphingomonas, and it is becoming evident that Sphingomonas spp. are ubiquitous in the environment and possess broad catabolic capabilities (12, 40). Sphingomonas sp. strain SRS2 was unable to grow on rich media, thus indicating adaptation to oligotrophic conditions. Other Sphingomonas spp. have been isolated from oligotrophic environments such as river water and seawater (38, 41), bottled mineral water (8), and aquifer sediments (1, 12). This suggests that adaptation to oligotrophic conditions is a characteristic feature of several members of the genus Sphingomonas.

The transient accumulation of MDIPU indicates that the degradation of IPU by Sphingomonas sp. strain SRS2 is initiated by N-demethylation of the dimethylurea side chain (Fig. 6, step 1). MDIPU has previously been reported to be the main metabolite produced during the degradation of IPU in agricultural soils (4, 6, 13, 16, 19, 22). An alternative metabolic pathway involving initial hydroxylation of the isopropyl side chain resulting in 2-hydroxy-IPU [3-(4-(2-hydroxyisopropyl)-phenyl)-1,1-dimethylurea] has also been described in agricultural soils (19, 32). Measurements of both MDIPU and 2-hydroxy-IPU in soil porewater, surface runoff and a nearby creek after IPU treatment of an agricultural field revealed that both pathways are active in the environment (32). Trace amounts of the metabolites DDIPU and 4IA, which have previously been detected in agricultural soils (16, 19, 22, 35), were also detected during the mineralization of IPU by Sphingomonas sp. strain SRS2. Since strain SRS2 also degraded DDIPU and 4IA, we suggest that the metabolic pathway used by the strain comprises N-demethylation of MDIPU to DDIPU (Fig. 6, step 2), followed by cleavage of the urea side chain to 4IA (Fig. 6, step 3) and mineralization of 4IA to CO₂ and production of biomass (Fig. 5 and Table 1). Although microorganisms in agricultural soils are able to mineralize 4IA, the metabolite is rapidly bound, thereby reducing the extent of biodegradation (5, 29). Recently, we described a mixed bacterial culture from the Græe agricultural field that is able to mineralize MDIPU and 4IA (35) but not able to degrade DDIPU. A metabolic

FIG. 5. Mineralization of ¹⁴C-isoproturon (¹⁴C-IPU) (●) and the IPU metabolites ¹⁴C-MDIPU (■) and ¹⁴C-4IA (▲) in MS-A2 by Sphingomonas sp. strain SRS2. The initial concentration of each compound was 25 mg liter⁻¹. The data are mean values (n = 2). The bars indicate the standard deviation.
an Arthrobacter globiformis strain (designated D47) has recently been isolated from the Deep Slade agricultural field that is able to transform the phenylurea herbicides diuron, linuron, monolinuron, metoxuron, and IPU to their respective aniline derivatives (7). However, no degradation of the aniline metabolites was observed, and the transformation of IPU was slow, with 50% IPU remaining after 28 days at 20°C. A. globiformis D47 transformed IPU by a single step involving hydrolytic cleavage of the dimethylurea side chain to 4IA (37), in contrast to the metabolic pathway proposed for Sphingomonas sp. strain SRS2, which involves successive N-demethylation (Fig. 6). Supplementing A. globiformis D47 with glucose and NH₄Cl greatly enhanced the transformation of IPU (7), thus indicating a cometabolic process. Sphingomonas sp. strain SRS2 was able to mineralize IPU, MDIPU, DDIPU, and 4IA. Roberts et al. (31) isolated several bacteria from the Deep Slade agricultural field that are able to degrade IPU to MDIPU and DDIPU by two successive N-demethylation steps. However, the capacity to degrade DDIPU to 4IA (Fig. 6, step 3) and subsequent mineralization of the phenyl structure were not shown. The enrichment and pure culture studies described in that study were conducted in a medium supplemented with ethanol, which also suggests the involvement of cometabolic processes. Since only a few isolates able to degrade phenylurea herbicides have yet been described, knowledge of the enzymes involved in the degradation process is generally lacking. Some studies indicate specificity related to methoxy-methyl-substituted phenylurea herbicides (9, 10). An aryl acylamidase purified from Bacillus sphaericus ATCC 12123 isolated from agricultural soil had specificity related to methoxy-methyl-substituted phenylurea herbicides but no activity toward dimethyl-substituted phenylurea herbicides (10). El-Fantroussi (9) found a similar specificity for degradation of methoxy-methyl-substituted herbicides in a recent study of a bacterial consortium enriched from agricultural soil. In contrast to A. globiformis D47 and B. sphaericus ATCC 12123, further degradation of the aniline metabolites was observed with the consortium, thus suggesting mineralization of the methoxy-methyl-substituted herbicides. The nature of the metabolic pathways in the degradation of dimethylurea-substituted diuron and chlorotoluron by Sphingomonas sp. SRS2 remains to be elucidated and will be the subject of a future study.

The ability to enrich degraders able to mineralize IPU is related to the distribution of the involved metabolic pathways among members of the soil microbial community. Strains harboring the entire pathway could be able to proliferate from the mineralization of IPU, as observed with Sphingomonas sp. strain SRS2 isolated from the Deep Slade soil. In strains possessing only part of the metabolic pathway, in contrast, the mineralization rate might be limited by cometabolic or abiotic degradation steps, as previously proposed for the Græse soil (35). In conclusion, the Sphingomonas sp. isolated in the present study harbors the metabolic pathway for the mineralization of IPU. This involves two N-demethylation steps, followed by cleavage of the urea side chain leading to mineralization of the phenyl structure. The ability of the strain to transform diuron and chlorotoluron but not linuron suggests
specificity for dimethyl-substituted phenylurea herbicides, although this remains to be clarified.

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