

Growth in Coculture Stimulates Metabolism of the Phenylurea Herbicide Isoproturon by *Sphingomonas* sp. Strain SRS2

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Metabolism of the phenylurea herbicide isoproturon by *Sphingomonas* sp. strain SRS2 was significantly enhanced when the strain was grown in coculture with a soil bacterium (designated strain SRS1). Both members of this consortium were isolated from a highly enriched isoproturon-degrading culture derived from an agricultural soil previously treated regularly with the herbicide. Based on analysis of the 16S rRNA gene, strain SRS1 was assigned to the β -subdivision of the proteobacteria and probably represents a new genus. Strain SRS1 was unable to degrade either isoproturon or its known metabolites 3-(4-isopropylphenyl)-1-methylurea, 3-(4-isopropylphenyl)-urea, or 4-isopropyl-aniline. Pure culture studies indicate that *Sphingomonas* sp. SRS2 is auxotrophic and requires components supplied by association with other soil bacteria. A specific mixture of amino acids appeared to meet these requirements, and it was shown that methionine was essential for *Sphingomonas* sp. SRS2. This suggests that strain SRS1 supplies amino acids to *Sphingomonas* sp. SRS2, thereby leading to rapid metabolism of ¹⁴C-labeled isoproturon to ¹⁴CO₂ and corresponding growth of strain SRS2. Proliferation of strain SRS1 suggests that isoproturon metabolism by *Sphingomonas* sp. SRS2 provides unknown metabolites or cell debris that supports growth of strain SRS1. The role of strain SRS1 in the consortium was not ubiquitous among soil bacteria; however, the indigenous soil microflora and some strains from culture collections also stimulate isoproturon metabolism by *Sphingomonas* sp. strain SRS2 to a similar extent.

The phenylurea herbicide isoproturon [3-(4-isopropylphenyl)-1,1-dimethylurea] (IPU) is used extensively against annual grasses and broad-leaved weeds in cereal production across Europe, resulting in contamination of both surface water and groundwater (10, 32, 33). The natural attenuation of IPU, with respect to mineralization of the phenyl structure to CO₂, is either absent or very slow in groundwater aquifers (13, 19, 21, 22). The potential of surface soils to degrade IPU is thus of interest, since agricultural fields may function as groundwater recharge zones and hence determine the degree to which IPU degrades before leaching to the underlying aquifers. Degradation of IPU is predominantly microbial (5), but the herbicide may persist in soil for several years in a form that can be mobilized by rainfalls (14).

Attempts to isolate microorganisms in pure culture able to degrade phenylurea herbicides have often failed, possibly due to the involvement of bacterial consortia rather than single strains (9, 30, 34). El-Fantroussi (9) enriched a mixed bacterial culture able to degrade linuron and metobromuron from a previously linuron-treated orchard soil. None of the strains isolated were capable alone or in combination of degrading linuron or metobromuron. Analyzing the bacterial composition at various steps in the degradation, using denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA genes,

strongly suggested the involvement of a bacterial consortium in the degradation. Roberts et al. (30) and Sørensen and Aamand (34) also encountered difficulties in isolating pure cultures able to degrade phenylurea compounds from different phenylurea-degrading mixed bacterial cultures enriched from soil, thus indicating a lack of single strains able to proliferate through such degradation and hence supporting the involvement of bacterial consortia.

Recently we isolated *Sphingomonas* sp. strain SRS2 harboring the metabolic pathway for mineralization of ¹⁴C-ring-labeled IPU to ¹⁴CO₂ and biomass (35). In addition a second bacterium (designated strain SRS1) was isolated from the same highly enriched IPU-degrading culture, which was derived from an agricultural soil that had been regularly treated with IPU for more than 20 years. The aim of the present study is to describe the synergistic mineralization of IPU by this two-member consortium in batch experiments with liquid media or agricultural soil. This study shows that a bacterial strain with the metabolic capacity to degrade phenylurea herbicides may be stimulated by other soil bacteria providing growth factors or nutrients. Such synergistic interactions may be common in nature, possibly explaining the difficulties in isolating pure cultures of bacteria able to degrade phenylurea herbicides.

MATERIALS AND METHODS

Chemicals. Analytical-grade IPU (99.5% purity, 55-mg liter⁻¹ water solubility at 20°C) and reference standards for high-performance liquid chromatography (HPLC) analysis of 3-(4-isopropylphenyl)-1-methylurea (MDIPU), 3-(4-isopropylphenyl)-urea (DDIPU), and 4-isopropylaniline (4IA) were purchased from

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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).*

Bacterial strains. The IPU-degrading *Sphingomonas* sp. strain SRS2 and bacterial strain SRS1, both isolated from a mixed culture enriched from agricultural soil by providing IPU as the sole source of carbon and nitrogen, were studied in pure culture and in coculture. *Sphingomonas* sp. strain SRS2 is described by Sørensen et al. (35). Strain SRS1 was characterized by Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) by analysis of the cellular fatty acids, partial sequencing of the 16S rRNA gene, and various physiological tests. Alignment of the partial 16S rRNA gene sequence was performed with sequences deposited in the GenBank database (National Center for Biotechnology Information) using the BLAST algorithm (1). Additional soil bacteria studied in coculture with *Sphingomonas* sp. SRS2 were *Aeromonas hydrophila* DSM 30016, *Ralstonia eutropha* DSM 4058, *Bacillus subtilis* DSM 402, *Bacillus cereus* DSM 31, *Pseudomonas fluorescens* DSM 50148, *Pseudomonas fluorescens* DSM 50108, *Pseudomonas stutzeri* DSM 50227, *Pseudomonas aureofaciens* ATCC 13985, *Pseudomonas putida* ATCC 17514, *Pseudomonas* sp. strain ADP (24), *Pseudaminobacter* sp. strain C147 (37), *Sphingomonas herbicidovorans* DSM 11019, and a phenanthrene-degrading *Sphingomonas* sp. (designated strain 2phe528A) isolated from soil (16).

Media. A mineral salt (MS) medium without carbon and nitrogen sources (35) was used for the coculture studies. For the pure culture studies with *Sphingomonas* sp. strain SRS2, the MS medium was supplemented with 0.1 g of Casamino Acids (Difco, Detroit, Mich.) liter⁻¹ or 0.5 g of yeast extract (Difco) liter⁻¹. Additionally, four different MS-based specific amino acid mixtures were prepared containing 5.0 mg of each amino acid liter⁻¹. Amino acid mixture 1 contained L-glutamic acid, L-proline, L-lysine, L-valine, and L-leucine. Amino acid mixture 2 contained L-phenylalanine, L-isoleucine, L-aspartic acid, L-alanine, and L-threonine. Amino acid mixture 3 contained L-arginine, L-serine, L-histidine, glycine, and L-methionine. Amino acid mixture 4 contained L-cysteine, L-tryptophan, and L-tyrosine. All amino acids were purchased from ICN Biomedicals Inc. (Aurora, Ohio). R2A-based broth (R2B) for growth of *Sphingomonas* sp. SRS2 and soil bacterium SRS1 was prepared according to the method of Reasoner and Geldreich (28). All media were autoclaved (121°C, 20 min) before use.

Preparation of inocula. Strain SRS1 and *Sphingomonas* sp. SRS2 were stored in 15% glycerol at -80°C. Before each experiment the strains were thawed and grown individually in 250-ml Erlenmeyer flasks containing 100 ml of R2B. At 20°C, the flasks were placed on a platform shaker at 150 rpm. Each strain was harvested in the late-exponential growth phase by centrifuging (10 min, 3,500 × g, 20°C) and washed twice in sterile distilled water before inoculation. Unless otherwise stated, the densities of *Sphingomonas* sp. strain SRS2 and strain SRS1 were adjusted with MS media (liquid culture experiments) or distilled water (soil microcosm) to 5 × 10⁸ cells ml⁻¹ and 5 × 10⁶ cells ml⁻¹, respectively, in the inoculum. These cell suspensions were used to inoculate the liquid culture and the soil microcosms as described below. For both strains the adjustment of cell density was based on standard curves relating absorbance at 600 nm (*A*₆₀₀) to plate counts on R2A. Additional soil bacteria studied in coculture with strain SRS2 were thawed from storage at -80°C and grown on Nutrient Agar (Difco). Colonies were removed after 2 days of incubation at 20°C, washed twice in MS medium, and inoculated to a final density of approximately 10⁵ cells ml⁻¹.

Liquid culture experiments. For the mineralization experiments, an acetone stock solution containing both unlabeled and ¹⁴C-labeled IPU was added to sterilized airtight 100-ml flasks to give 1.0 mg of IPU and 40,000 dpm per flask. The acetone was evaporated in a laminar flow bench, and 49.0 ml (pure culture experiments) or 48.0 ml (coculture experiments) of liquid medium was added to each flask. After inoculation with 1.0 ml of cell suspension (pure culture experiments) or 1 ml of cell suspension of each strain (coculture experiments) to yield a final volume of 50.0 ml, the flasks were incubated in the dark at 20°C on an IKA Labortechnik KS 250 Basic Orbital Shaker (Staufen, Germany) at 100 rpm for up to 18 days. *Sphingomonas* sp. SRS2 was inoculated to give an initial density of 10⁷ cells ml⁻¹, and strain SRS1 and the other bacteria were inoculated to give an initial density of 10⁵ cells ml⁻¹ in the mineralization experiments. Mineralization of ¹⁴C-IPU was followed by trapping of the evolved ¹⁴CO₂ in an alkaline solution placed in a 5-ml test tube containing 2 ml of 0.5 M NaOH mounted within the flasks. Upon sampling, the NaOH was replaced with fresh alkaline solution. The samples were mixed with 10 ml of Wallac (Turku, Finland) OptiPhase HiSafe 3 scintillation cocktail and counted on a Wallac 1409 liquid scintillation counter. The counts were corrected for quenching and background radioactivity. Unless otherwise stated, the mineralization experiments were performed in triplicate, and uninoculated flasks served as controls for abiotic loss of ¹⁴C to the alkaline solution.

For the growth and degradation experiments an acetone stock solution con-

taining only unlabeled IPU was added to give 1.0 mg of IPU per flask. Media and bacteria were added as described for the mineralization experiments. The degradation and growth experiment cocultures of strain SRS1 and *Sphingomonas* sp. SRS2 were inoculated with 10⁴ cells of each strain ml⁻¹. IPU and the metabolites MDIPU, DDIPU, and 4IA were measured in the liquid media using a Hewlett Packard Series 1050 HPLC system as described by Juhler et al. (15). At regular intervals, 750-μl aliquots of the media were sampled from each flask and filtered through a 0.45-μm-pore-sized Titan syringe filter (Scientific Resources, Eatontown, N.J.) with the final 250 μl being collected for HPLC analysis. The flasks were also sampled for measurement of *A*₆₀₀ and for plating on R2A, where strain SRS1 and *Sphingomonas* sp. strain SRS2 were easily distinguished after 5 to 6 days of incubation at 20°C. To estimate the possibility of genes involved in the metabolism of IPU being transferred from *Sphingomonas* sp. strain SRS2 to strain SRS1, 10 randomly selected isolates of both strains grown on R2A were screened for degradation of IPU at each sampling point. The growth and degradation experiments were performed in triplicate, and uninoculated flasks and flasks without IPU were included as controls of abiotic degradation and growth in the MS medium.

Soil microcosm experiments. Surface soil was sampled from a previously IPU-treated agricultural field near Wellesbourne (site C6; Deep Slade, United Kingdom). Sampling and soil characteristics of site C6 are presented by Walker et al. (38). The soil from this site was selected based on its low potential for mineralization of IPU (3). Soil samples (wet weight, 10 g) were added to sterile 100-ml flasks. A parallel experiment was conducted with soil autoclaved twice for 60 min at 121°C at an interval of 24 h. Sterility was confirmed by plating on R2A and incubation at 20°C for one week. An acetone stock solution of unlabeled and ¹⁴C-labeled IPU was added to the soil, and the acetone was allowed to evaporate in a laminar flow bench before mixing the soil to give a final concentration of 20 mg of IPU kg of soil⁻¹ and approximately 340,000 dpm per flask. *Sphingomonas* sp. SRS2 was inoculated to give a density of 10⁷ cells g⁻¹, and strain SRS1 was inoculated to 10⁵ cells g⁻¹. Distilled water was used to adjust the moisture content of the soil to 15%. The soil microcosm experiment was performed in triplicate, and uninoculated flasks were included. Mineralization of ¹⁴C-IPU was followed by trapping the evolved ¹⁴CO₂ in an alkaline solution placed in a test tube mounted within each flask as described for the liquid culture experiments. The distribution of ¹⁴C into three different fractions were estimated after 70 days of incubation at 20°C. The fractions comprised mineralized ¹⁴C as ¹⁴CO₂, water-extractable ¹⁴C residues, and nonextractable ¹⁴C residues. The water-extractable fraction was estimated by adding 35.0 ml of 0.01 M CaCl₂ to each flask and incubating them on an orbital shaker at 200 rpm for 1 h, followed by centrifugation (20 min, 2,000 × g, 20°C) and measurement of the ¹⁴C present in the supernatant by liquid scintillation counting. Nonextractable ¹⁴C residues were estimated by combusting 0.1 g (dry weight) of the remaining soil in a Packard Oxidizer (model 507) under conditions of excess O₂ and capturing the ¹⁴CO₂ produced.

Data from the liquid culture and soil microcosm experiments were statistically analyzed using one way analysis of variance (ANOVA).

Nucleotide sequence accession number. The partial 16S rRNA gene sequences of strain SRS1 have been deposited in the GenBank database under accession no. AJ251639.

RESULTS

Characterization of strain SRS1. Strain SRS1 is a nonfluorescent yellow-pigmented gram-negative rod 0.8 to 0.9 μm wide and 1.8 to 3.5 μm long. It is oxidase positive, catalase positive, and urease negative. Strain SRS1 utilizes Tween 40, Tween 80, glucose, arabinose, sucrose, D-galactose, malate, phenylacetate, gluconate, glycerol, methyl pyruvate, monomethyl succinate, D-galacturonic acid, D-glucuronic acid, α- and β-hydroxybutyric acid, α-ketobutyric acid, D- and L-lactic acid, succinic acid, succinamic acid, glucuronamide, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, and L-threonine. It grows on R2A and 1/10-strength tryptic soy agar, forming slightly yellow to whitish brown colonies within 2 to 3 days at 20°C. The colonies were slimy and strongly adhesive to the agar surface. No growth was observed on nutrient agar (Difco) or Luria-Bertani agar (Difco). A whole-cell fatty acid profile revealed that the dominant fatty acids were 49.9%

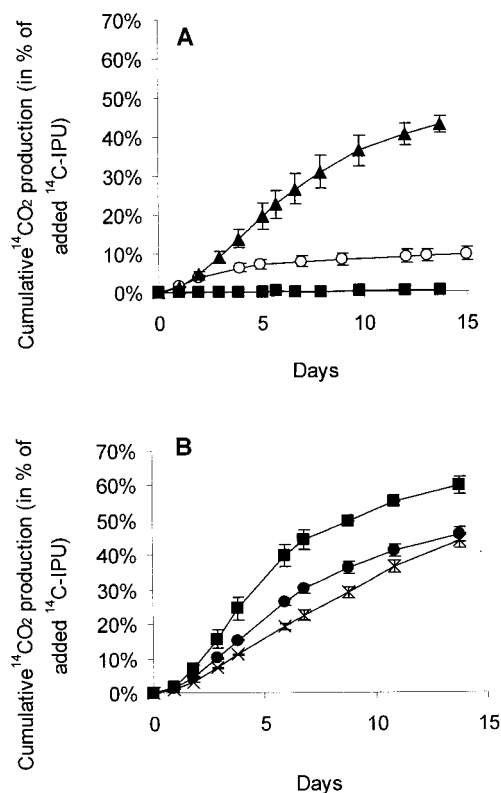


FIG. 1. Mineralization of ^{14}C -labeled isoproturon (^{14}C -IPU) in MS medium. (A) *Sphingomonas* sp. strain SRS2 in pure culture (○), strain SRS1 in pure culture (■), and *Sphingomonas* sp. strain SRS2 and strain SRS1 in coculture (▲). (B) *Sphingomonas* sp. strain SRS2 in coculture with *Pseudomonas* sp. ADP (■), *B. cereus* DSM 31 (●), and *B. subtilis* DSM 402 (×). The initial concentration of isoproturon was 20 mg liter $^{-1}$. The data are mean values ($n = 3$). The bars indicate the standard deviations.

16:1 ω 7c and/or 2OH(iso)15:0, 34.2% 16:0, 5.8% 18:1 (sum of 18:1 ω 7c, 18:1 ω 9c, and 18:1 ω 12t) and 3.2% cyclo-17:0. The fatty acid analysis points to the β -subdivision of the proteobacteria.

Upon comparison of a partial 16S rRNA gene sequence (470 bp) obtained from strain SRS1 with sequences from the GenBank database, the highest degree of similarity (95.7%, 463-bp overlap) was obtained with the 16S rRNA gene sequence of *Polaromonas vacuolata* isolated from Antarctica (12). Two 16S rRNA gene sequences obtained by Kaiser et al. (17) from agricultural soil (clone Wr0041) and by Lüdemann et al. (23) from paddy soil (clone oxSCC-37) showed a high degree of similarity to the 16S rRNA gene sequence of strain SRS1 (99.6%, 461-bp overlap and 98.9%, 462-bp overlap, respectively).

Mineralization of IPU by soil bacteria. Mineralization of ^{14}C -IPU was significantly enhanced by combining *Sphingomonas* sp. strain SRS2 with the soil bacterium SRS1 (ANOVA, $P < 0.05$), both of which were isolated from an IPU-degrading enrichment culture derived from agricultural soil (Fig. 1A). The percentage of ^{14}C -IPU metabolized to $^{14}\text{CO}_2$ within 14 days increased from $10.0\% \pm 2.0\%$ with *Sphingomonas* sp. SRS2 in pure culture to $43.1\% \pm 2.0\%$ when *Sphingomonas* sp. SRS2 was combined with strain SRS1 in coculture. Varying the

density of strain SRS1 (10^5 , 10^6 , 10^7 , and 10^8 ml $^{-1}$) in the coculture had no effect on the mineralization rate of ^{14}C -IPU (data not shown). No mineralization of ^{14}C -IPU was observed with strain SRS1 in pure culture. Additional studies with strain SRS1 in pure culture did not reveal any degradation of either IPU or the metabolites MDIPU, DDIPU, or 4IA (data not shown).

Combining *Sphingomonas* sp. strain SRS2 with a range of soil bacteria from culture collections revealed that three other strains also enhanced the mineralization of ^{14}C -IPU (Fig. 1B). No effect on the mineralization of ^{14}C -IPU by *Sphingomonas* sp. strain SRS2 was observed upon coculture with *A. hydrophila* DSM 30016, *R. eutropha* DSM 4058, *P. fluorescens* DSM 50148, *P. fluorescens* DSM 50108, *P. stutzeri* DSM 50227, *P. aureofaciens* ATCC 13985, *P. putida* ATCC 17514, *Pseudaminobacter* sp. C147, *S. herbicidovorans* DSM 11019 or *Sphingomonas* sp. strain 2phe528A (data not shown). The percentage of ^{14}C -IPU metabolized to $^{14}\text{CO}_2$ within 14 days was $59.9\% \pm 2.0\%$, $45.8\% \pm 2.0\%$, and $44.1\% \pm 3.0\%$ when *Sphingomonas* sp. strain SRS2 was cocultured with *Pseudomonas* sp. ADP, *B. cereus* DSM 31, and *B. subtilis* DSM 402, respectively. Neither IPU nor the metabolites MDIPU, DDIPU, or 4IA were detected in the media of these cocultures at the end of the experiment. None of the tested bacteria besides *Sphingomonas* sp. strain SRS2 mineralized ^{14}C -IPU to $^{14}\text{CO}_2$ in pure culture, and no degradation of IPU or formation of metabolites were detected after 15 days.

Mineralization of IPU by *Sphingomonas* sp. strain SRS2 in pure culture. Various supplements were examined for their effect on the mineralization of ^{14}C -IPU by *Sphingomonas* sp. strain SRS2 in pure culture. Within 8 days, $41.4\% \pm 4.1\%$ of the added ^{14}C -IPU was metabolized to $^{14}\text{CO}_2$ in R2B medium (Fig. 2A). Adding Casamino Acids or yeast extract to the MS medium, both of which are components of R2B medium, stimulated mineralization of ^{14}C -IPU by *Sphingomonas* sp. strain SRS2, resulting in $48.1\% \pm 1.5\%$ and $54.2\% \pm 4.3\%$ of the added ^{14}C -IPU being metabolized to $^{14}\text{CO}_2$ within 8 days (Fig. 2A). No significant difference was observed in the extent of ^{14}C -IPU mineralization to $^{14}\text{CO}_2$ after 8 days (ANOVA, $P > 0.05$); however, the lag phase was shortest with Casamino Acids, and the further studies thus focused on amino acids. Addition of various mixtures of amino acids to the MS medium revealed that L-arginine, L-serine, L-histidine, glycine, and L-methionine (amino acid mixture 3) increased the metabolism of ^{14}C -IPU to $^{14}\text{CO}_2$ (Fig. 2B) to a level not significantly different (ANOVA, $P > 0.05$) from the ^{14}C -IPU metabolism observed with Casamino Acids (Fig. 2A) or the isolated coculture (Fig. 1A). IPU metabolism by *Sphingomonas* sp. strain SRS2 was not enhanced by amino acid mixture 1, 2, or 4 (Fig. 2B). Exclusion of L-methionine and glycine from amino acid mixture 3 significantly (ANOVA, $P < 0.05$) decreased the initial mineralization rate of ^{14}C -IPU (data not shown). However, after 19 days the exclusion of L-arginine, L-serine, L-histidine, and glycine individually from amino acid mixture 3 did not significantly influence mineralization of ^{14}C -IPU (52.4% to 55.9% of ^{14}C -IPU metabolized to $^{14}\text{CO}_2$) (ANOVA, $P > 0.05$). In contrast, only $24.4\% \pm 0.2\%$ of ^{14}C -IPU was metabolized to $^{14}\text{CO}_2$ within this period when L-methionine was excluded from amino acid mixture 3.

Degradation of IPU by *Sphingomonas* sp. strain SRS2 in

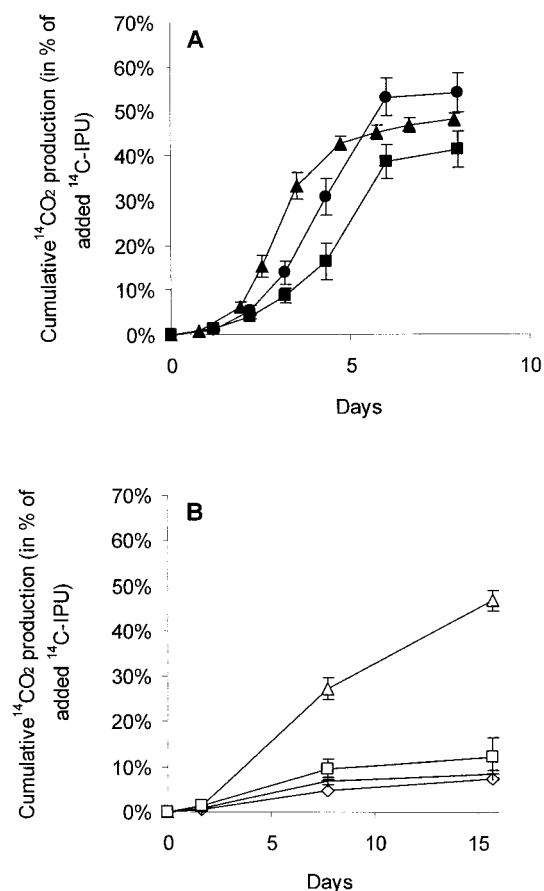


FIG. 2. Mineralization of ¹⁴C-labeled isoproturon (¹⁴C-IPU) by *Sphingomonas* sp. strain SRS2 in pure culture. (A) ¹⁴C-IPU mineralization in R2B (■) or in MS medium supplemented with 0.1 g of Casamino Acids (▲) liter⁻¹ or 0.5 g of yeast extract (●) liter⁻¹. (B) ¹⁴C-IPU mineralization in MS medium with amino acid mixture 1 (◇), amino acid mixture 2 (+), amino acid mixture 3 (Δ), or amino acid mixture 4 (□).

coculture with strain SRS1. Investigation of the dynamics of *Sphingomonas* sp. strain SRS2 and strain SRS1 growing in coculture during the metabolism of IPU revealed that degradation of IPU (20 mg liter⁻¹) was followed by growth to a maximum A_{600} of 0.03 measured at day 9 (Fig. 3A), corresponding to $6.1 \times 10^7 \pm 1.1 \times 10^7$ cells of *Sphingomonas* sp. SRS2 ml⁻¹ and $1.0 \pm 0.3 \times 10^7$ cells of strain SRS1 ml⁻¹ (Fig. 3B). Intermediate accumulation of the metabolite MDIPU was measured by HPLC, but after 8 days neither IPU nor the metabolite MDIPU, DDIPU, or 4IA was detected in the liquid culture (data not shown). No degradation was observed in the uninoculated controls. The A_{600} in controls without IPU was 0.01 at day 9, corresponding to $2.8 \times 10^6 \pm 1.6 \times 10^6$ cells of *Sphingomonas* sp. SRS2 ml⁻¹ and $4.9 \times 10^6 \pm 1.7 \times 10^6$ cells of strain SRS1 ml⁻¹ (Fig. 3B). Isolates of strain SRS2 randomly selected from R2A at different times during the incubation all had the ability to metabolize ¹⁴C-IPU to ¹⁴CO₂ when resuspended in liquid medium. None of the isolates of strain SRS1 tested had gained the ability to metabolize IPU during the experiment.

Mineralization of IPU in soil microcosms. The effect on the mineralization of ¹⁴C-IPU of introducing the coculture consisting of strain SRS1 and *Sphingomonas* sp. strain SRS2 or *Sphingomonas* sp. strain SRS2 in pure culture into agricultural soil was examined. After 70 days the indigenous soil microflora had mineralized $14.4\% \pm 1.4\%$ of the ¹⁴C-IPU (Fig. 4A). Introduction of *Sphingomonas* sp. strain SRS2 into this soil enhanced the initial rate of mineralization, and by the end of the experiment, a significantly higher fraction (ANOVA, $P < 0.05$) of the added ¹⁴C-IPU had been metabolized to ¹⁴CO₂ ($24.1\% \pm 2.3\%$) (Fig. 4A). The enhancement was initially slightly more pronounced when strain SRS1 was included in the inoculum. After 30 days, however, no significant difference was observed whether inoculating with the coculture or with *Sphingomonas* sp. SRS2 in pure culture (Fig. 4A). In contrast, only $0.3\% \pm 0.1\%$ of the added ¹⁴C-IPU was metabolized to ¹⁴CO₂ in sterilized soil after introduction of *Sphingomonas* sp. SRS2 alone, compared to $34.9\% \pm 7.9\%$ when added together with strain SRS1 (Fig. 4B). Table 1 shows the distribution of the initially added ¹⁴C after 70 days of incubation. Inoculation with strain SRS2 in pure culture as well as in coculture with strain SRS1 decreased the amount of extractable ¹⁴C.

DISCUSSION

In natural soils, bacteria are heterogeneously distributed and probably occur in multispecies rather than single-species communities. Close proximity within the community may facilitate metabolic cross-feeding between different species, which could be a prerequisite for the degradation of many organic pollutants introduced into the soil environment. The involvement of bacterial consortia derived from agricultural soils in the degradation of the extensively used phenoxyalkanoic-acid, triazine, and phenylurea herbicides has previously been described (8, 9, 20).

Pure cultures of bacteria and fungi isolated from agricultural soils treated with phenylurea herbicides have been shown to degrade the dimethylurea side chain of IPU, giving rise to various phenylurea or aniline metabolites (4, 6, 29). Recently we enriched a mixed bacterial culture from agricultural soil that utilizes IPU as its sole source of carbon and nitrogen during metabolism of ¹⁴C-IPU to ¹⁴CO₂ (35). After several successive dilutions the mixed culture was reduced to two different isolates. The first strain (designated SRS1) had no activity towards IPU or the metabolites MDIPU, DDIPU, or 4IA. 16S rRNA gene sequencing and fatty acid analysis suggest that strain SRS1 belongs to an unknown genus within the β-subdivision of the proteobacteria, phylogenetically related to several uncultured soil bacteria described by Kaiser et al. (17) and Lüdemann et al. (23). The second strain (designated SRS2), identified as a *Sphingomonas* sp. (35), was able to metabolize ¹⁴C-IPU to ¹⁴CO₂ and biomass although mineralization of the phenyl structure by this strain was slow in mineral medium. Growth in coculture with strain SRS1 stimulated the metabolism of ¹⁴C-IPU to ¹⁴CO₂, however, thus suggesting involvement of synergistic interactions between these two strains.

Sphingomonas spp. are known to possess broad metabolic capabilities (39), and their involvement in consortial degradation of various recalcitrant organic pollutants, such as biphenyl

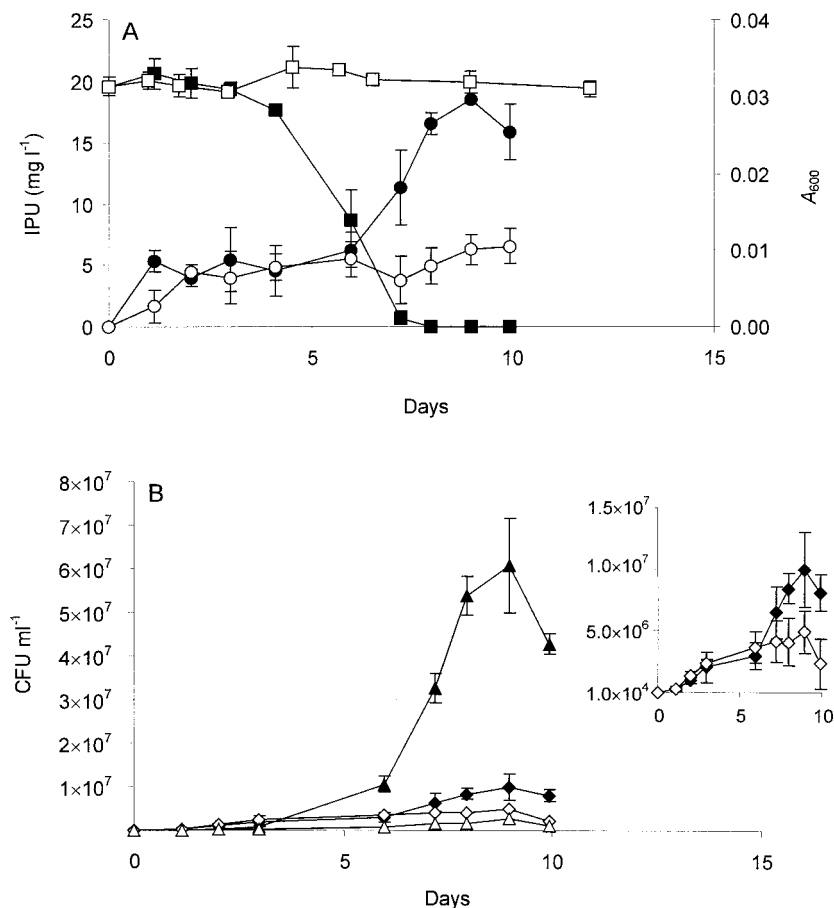


FIG. 3. Metabolism of isotreturon (IPU) by *Spingomonas* sp. strain SRS2 and strain SRS1 in coculture. (A) Degradation of IPU by the coculture (■) or in uninoculated controls (□) and growth of the coculture during metabolism of IPU (●) or in controls without IPU (○). (B) Growth of *Spingomonas* sp. SRS2 (▲) and strain SRS1 (◆) in the coculture during metabolism of IPU or in controls without IPU (△, ◇). The inset shows an enlargement of the growth data for strain SRS1. The data are mean values ($n = 3$). The bars indicate the standard deviation. Initial densities of both strains were 1.0×10^4 cells ml^{-1} .

(7), benzo[a]pyrene (18), and triclosan (11) has recently been demonstrated. The cooperative metabolic activities in bacterial consortia during degradation of organic pollutants generally involve two known mechanisms. The first is metabolic deficiencies, where the degrading bacteria are fastidious and depend on secondary strains providing various growth factors or nutrients (e.g., see references 11, 25, and 31). The second is associated metabolism, where cross-feeding with metabolites from the degradation pathway occurs within the consortium (e.g., see references 2, 8, 26, 27, and 36). Pelz et al. (27) revealed a network of metabolite cross-feeding within a four-member consortium that involved end products from one strain serving as a substrate for other strains, eventually leading to the mineralization of 4-chlorosalicylate. Hay et al. (11) isolated a *Spingomonas* sp. from a triclosan-degrading consortium derived from activated sludge. The strain harbored the metabolic pathway for metabolism of ¹⁴C-ring-labeled triclosan to ¹⁴CO₂, but the degradation depended on the presence of other bacteria or a complex medium, thus suggesting a requirement for growth factors or nutrients from its surrounding environment. Maymó-Gatell et al. (25) presented similar findings in a study of an anaerobic tetrachloroethene-degrading bacterium where

extensive degradation of tetrachloroethene required either cell components from other specific bacteria or a complex mixture of amino acids.

A specific mixture of amino acids was found to enhance the metabolism of ¹⁴C-IPU to ¹⁴CO₂ by *Spingomonas* sp. strain SRS2, and further studies revealed that removing L-methionine from the mixture significantly decreased the initial rate and extent of the IPU mineralization. This suggests that *Spingomonas* sp. strain SRS2 is auxotrophic, requiring components supplied by its surrounding environment. L-methionine appears to meet this requirement. The possibility that other growth factors or nutrients are supplied to *Spingomonas* sp. strain SRS2 by strain SRS1 cannot be excluded, however. Soil bacteria other than strain SRS1 also increased IPU mineralization when combined with *Spingomonas* sp. strain SRS2, although the majority of the tested strains had no stimulatory effect on the metabolism of IPU. This suggests that the role of strain SRS1 in the consortium is not ubiquitous among soil bacteria. Since no carbon, nitrogen, or energy sources other than IPU were added to the MS medium, most strains were probably inactive due to lack of appropriate substrates, thus entailing the possibility that other potential synergistic inter-

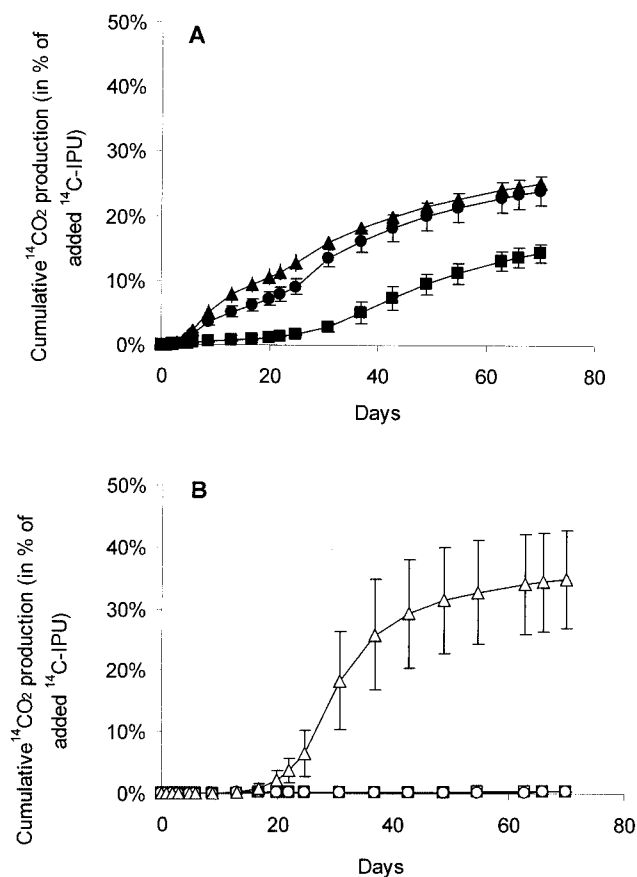


FIG. 4. Mineralization of ^{14}C -labeled isoproturon (^{14}C -IPU) in natural soil (A) or in sterilized soil (B) inoculated with *Sphingomonas* sp. strain SRS2 (●, ○) or the coculture consisting of *Sphingomonas* sp. strain SRS2 and strain SRS1 (▲, △) and in uninoculated controls (■, □). Initial concentration of isoproturon was 20 mg kg of soil $^{-1}$ (wet weight). The data are mean values ($n = 3$). The bars indicate the standard deviations.

actions may have been overlooked. Taken together, our findings strongly indicate that *Sphingomonas* sp. strain SRS2 has metabolic deficiencies but that these can be met by association with other bacteria.

Growth of the coculture of *Sphingomonas* sp. strain SRS2 and strain SRS1 during metabolism of IPU was observed as an

increase in the turbidity of the liquid culture. This was mainly related to the proliferation of *Sphingomonas* sp. strain SRS2, but minor growth of strain SRS1 was also detected. This is as would be expected, since strain SRS1 was selected during the enrichment procedure along with *Sphingomonas* sp. strain SRS2. The proliferation of strain SRS1 in the coculture suggests that it grows on unknown metabolites formed during the metabolism of IPU by *Sphingomonas* sp. strain SRS2. Alternatively, strain SRS1 may utilize compounds associated with debris from cells of *Sphingomonas* sp. strain SRS2 without any direct involvement in the metabolism of IPU.

Significant differences in the potential for degradation of IPU in soils sampled from different plots within the Deep Slade agricultural field have recently been reported (3, 38). The two-membered consortium characterized in this study was isolated from soil with rapid mineralization of ^{14}C -IPU sampled at the Deep Slade agricultural field (35). The Deep Slade soil used for the inoculation experiments was selected based on its low potential for mineralization of IPU (3). Introduction of *Sphingomonas* sp. strain SRS2 alone enhanced IPU mineralization in the soil (Fig. 4A). No significant difference in the mineralization of IPU was observed between inoculation with the consortium or *Sphingomonas* sp. strain SRS2 in pure culture, thus suggesting that strain SRS1 could be substituted for by indigenous soil microorganisms. Alternatively, a natural population of strain SRS1 is already present in the soil. Since approximately the same rate and level of IPU mineralization were observed among the replicate samples inoculated with *Sphingomonas* sp. strain SRS2, random events such as gene transfer between strain SRS2 and indigenous bacteria seem unlikely to explain the enhanced IPU mineralization. Surprisingly, no IPU mineralization was observed in sterilized soil inoculated with *Sphingomonas* sp. strain SRS2 unless strain SRS1 was included (Fig. 4B). This further supports the involvement of secondary strains in the metabolism of IPU by *Sphingomonas* sp. strain SRS2.

The amount of ^{14}C -IPU metabolized to $^{14}\text{CO}_2$ in the soil microcosms inoculated with the consortium was lower than expected from the liquid culture experiments. Walker et al. (38) showed that contrasting pH environments at different sites within the Deep Slade field influenced the degradation of IPU, which was significantly slower in soils having pHs of <7. The consortium was isolated from soil with a pH of 7.2, which might suggest that the pH of the soil used in the present study (pH

TABLE 1. Distribution of ^{14}C from ^{14}C -ring-labeled isoproturon (^{14}C -IPU) after 70 days in natural and autoclaved soil inoculated with *Sphingomonas* sp. strain SRS2 or *Sphingomonas* sp. strain SRS2 and strain SRS1 in coculture

Treatment	^{14}C recovery (%) ^a			
	Water-extractable ^{14}C residues	Nonextractable ^{14}C residues	$^{14}\text{CO}_2$	Total recovery
Natural soil				
<i>Sphingomonas</i> sp. SRS2	5.7 (± 1.5)	66.9 (± 4.8)	24.1 (± 2.3)	96.6 (± 3.7)
<i>Sphingomonas</i> sp. SRS2 + strain SRS1	4.0 (± 0.4)	67.8 (± 4.0)	25.2 (± 0.2)	97.0 (± 4.0)
No inoculation	12.7 (± 0.5)	74.4 (± 7.6)	14.4 (± 1.4)	101.6 (± 9.1)
Autoclaved soil				
<i>Sphingomonas</i> sp. SRS2	32.9 (± 1.6)	64.6 (± 0.7)	0.3 (± 0.1)	98.3 (± 1.5)
<i>Sphingomonas</i> sp. SRS2 + strain SRS1	8.7 (± 4.9)	54.0 (± 10.0)	34.9 (± 7.9)	97.6 (± 8.7)
No inoculation	41.1 (± 0.1)	56.1 (± 10.7)	0.4 (± 0.1)	97.6 (± 10.7)

^a Means and standard deviations (given in parentheses) of triplicates expressed as percentages of applied ^{14}C -IPU.

6.3) negatively effects *Sphingomonas* sp. strain SRS2, resulting in slower metabolism of ^{14}C -IPU to $^{14}\text{CO}_2$. However, the effect of soil pH on the success of inoculation with *Sphingomonas* sp. strain SRS2 remains to be elucidated and is currently the subject of a separate study. A lower level of availability of IPU or its metabolites in soil may also explain the lower amount of ^{14}C -IPU mineralized. We observed a large fraction of nonextractable ^{14}C residues following the mineralization of ^{14}C -IPU in the soil microcosms, suggesting a lower availability. Formation of nonextractable ^{14}C residues has also been observed in previous studies on the biodegradation of IPU in agricultural soils, and it has been suggested that this might be related to the sorption of aniline metabolites (3).

Our results demonstrate that synergistic interactions between different soil bacteria may be involved in the degradation of the phenylurea herbicide IPU in agricultural soil. This could explain the failure of several attempts to isolate microorganisms in pure culture able to mineralize or partially degrade phenylurea herbicides from mixed cultures capable of doing so. Unless the secondary strains in the consortia proliferate to some extent during metabolism, enrichment cultures will be unsuccessful despite the potential for rapid degradation that exists in the natural soil. In conclusion, we have isolated and characterized a two-member consortium from agricultural soil, consisting of the soil bacterium strain SRS1 and *Sphingomonas* sp. strain SRS2, able to rapidly metabolize ^{14}C -IPU to $^{14}\text{CO}_2$. Our results suggest that *Sphingomonas* sp. strain SRS2 is the actual degrading bacterium responsible for the metabolism of IPU, although strain SRS1 facilitates the mineralization by providing amino acids and/or other compounds. The minor proliferation of strain SRS1 in the consortium possibly indicates that it utilizes cell debris from *Sphingomonas* sp. strain SRS2 or unknown metabolites occurring during the metabolism of IPU. The reason for the selection of strain SRS1 during the enrichment procedure remains unclear, however.

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