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-isopropylaniline. 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 complicated by the mobility of IPU in soil (34) and the slow rate of degradation in groundwater (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 is 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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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 1 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 soil1 and approximately 340,000 dpm per flask. Sphingomonas sp. SR2 was inoculated to give a density of 107 cells ml-1 and strain SR1 was inoculated to give 108 cells ml-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 was 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). The nucleotide sequence accession numbers of strain SRS1 were deposited in the GenBank database under accession no. A251639.

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, mono-methyl 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. 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. 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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. herbicidivorans DSM 11019 or Sphingomonas sp. strain 2phe528A (data not shown). The percentage of $^{14}$C-IPU metabolized to $^{14}$CO$_2$ within 14 days was 59.9% ± 2.0%, 45.8% ± 2.0%, and 44.1% ± 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 $^{14}$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% ± 4.1% of the added $^{14}$C-IPU was metabolized to $^{14}$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% ± 1.5% and 54.2% ± 4.3% of the added $^{14}$C-IPU being metabolized to $^{14}$CO$_2$ within 8 days (Fig. 2A). No significant difference was observed in the extent of $^{14}$C-IPU mineralization to $^{14}$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 $^{14}$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 $^{14}$CO$_2$ (ANOVA, $P > 0.05$). In contrast, only 24.4% ± 0.2% of $^{14}$C-IPU was metabolized to $^{14}$CO$_2$ within this period when l-methionine was excluded from amino acid mixture 3.

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Mineralization of IPU in soil microcosms. The effect on the mineralization of $^{14}$C-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 $^{14}$C-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 $^{14}$C-IPU had been metabolized to $^{14}$CO$_2$ ($24.1\% \pm 2.3\%$) (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 $^{14}$C-IPU was metabolized to $^{14}$CO$_2$ 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 $^{14}$C 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 $^{14}$C.

**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 $^{14}$C-IPU to $^{14}$CO$_2$ (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 $^{14}$C-IPU to $^{14}$CO$_2$ 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 $^{14}$C-IPU to $^{14}$CO$_2$, 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
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 de-
ficiencies, where the degrading bacteria are fastidious and depend
on secondary strains providing various growth factors or nutri-
ents (e.g., see references 11, 25, and 31). The second is asso-
ciated 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 min-
eralization of 4-chlorosalicylate. Hay et al. (11) isolated a
Sphingomonas sp. from a triclosan-degrading consortium de-
formed 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 bac-
teria 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 Sphingomonas sp. strain
SRS2, and further studies revealed that removing L-methio-
nine from the mixture significantly decreased the initial rate
and extent of the IPU mineralization. This suggests that Sphin-
gomonas 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 Sphingomonas
sp. strain SRS2 by strain SRS1 cannot be excluded, however. Soil
bacteria other than strain SRS1 also increased IPU mineral-
ization when combined with Sphingomonas 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
ettailing the possibility that other potential synergistic inter-

FIG. 3. Metabolism of isoproturon (IPU) by Sphingomonas 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 Sphingomonas 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 × 10⁶ cells ml⁻¹.
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 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 $^{14}$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

### 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.

### 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Water-extractable $^{14}$C residues</th>
<th>Nonextractable $^{14}$C residues</th>
<th>$^{14}$CO$_2$</th>
<th>Total recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Natural soil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sphingomonas</em> sp. SRS2</td>
<td>5.7 (±1.5)</td>
<td>66.9 (±4.8)</td>
<td>24.1 (±2.3)</td>
<td>96.6 (±3.7)</td>
</tr>
<tr>
<td><em>Sphingomonas</em> sp. SRS2 + strain SRS1</td>
<td>4.0 (±0.4)</td>
<td>67.8 (±4.0)</td>
<td>25.2 (±0.2)</td>
<td>97.0 (±4.0)</td>
</tr>
<tr>
<td>No inoculation</td>
<td>12.7 (±0.5)</td>
<td>74.4 (±7.6)</td>
<td>14.4 (±1.4)</td>
<td>101.6 (±9.1)</td>
</tr>
<tr>
<td><strong>Autoclaved soil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sphingomonas</em> sp. SRS2</td>
<td>3.2 (±1.6)</td>
<td>64.6 (±0.7)</td>
<td>0.3 (±0.1)</td>
<td>98.3 (±1.5)</td>
</tr>
<tr>
<td><em>Sphingomonas</em> sp. SRS2 + strain SRS1</td>
<td>8.7 (±4.9)</td>
<td>54.0 (±10.0)</td>
<td>34.9 (±7.9)</td>
<td>97.6 (±8.7)</td>
</tr>
<tr>
<td>No inoculation</td>
<td>41.1 (±0.1)</td>
<td>56.1 (±10.7)</td>
<td>0.4 (±0.1)</td>
<td>97.6 (±10.7)</td>
</tr>
</tbody>
</table>

* 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 14C-IPU to 14CO2. 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 14C-IPU mineralized. We observed a large fraction of nonextractable 14C residues following the mineralization of 14C-IPU in the soil microcosms, suggesting a lower availability. Formation of nonextractable 14C 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 a two-member consortium from agricultural soil, consisting of the soil bacterium strain SRS1 and Sphingomonas sp. strain SRS2, able to rapidly metabolize 14C-IPU to 14CO2. 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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