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ORIGINAL PAPER

Isolation and characterization of an isoproturon mineralizing *Sphingomonas* sp. strain SH from a French agricultural soil

Sabir Hussain · Marion Devers-Lamrani · Najoi El Azhari · Fabrice Martin-Laurent

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Abstract The phenylurea herbicide isoproturon, 3-(4-isopropylphenyl)-1,1-dimethylurea (IPU), was found to be rapidly mineralized in an agricultural soil in France that had been periodically exposed to IPU. Enrichment cultures from samples of this soil isolated a bacterial strain able to mineralize IPU. 16S rRNA sequence analysis showed that this strain belonged to the phylogeny of the genus *Sphingomonas* (96% similarity with *Sphingomonas* sp. JEM-14, AB219361) and was designated *Sphingomonas* sp. strain SH. From this strain, a partial sequence of a 1,2-dioxygenase (*catA*) gene coding for an enzyme degrading catechol putatively formed during IPU mineralization was amplified. Phylogenetic analysis revealed that the *catA* sequence was related to *Sphingomonas* spp. and showed a lack of congruence between the *catA* and 16S rRNA based phylogenies, implying horizontal gene transfer of the *catA* gene cluster between soil microbiota. The IPU degrading ability of strain SH was strongly influenced by pH with maximum degradation taking place at pH 7.5. SH was only able to mineralize IPU and its known metabolites including 4-isopropylaniline and it could not degrade other structurally related phenylurea herbicides such as diuron, linuron, monolinuron and chlorotoluron or their aniline derivatives. These observations suggest that the catabolic abilities of the strain SH are highly specific to the metabolism of IPU.

Keywords Biodegradation · Isoproturon · Phenylurea · Catechol dioxygenase · pH

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Introduction

Much attention has been paid to the use of crop protection products in conventional agriculture in recent years owing to rising public concern about environmental contamination and their impact on human health. Phenylurea herbicides are among the most widely used class of crop protection products and are of particular significance as several have been recorded as contaminants of agricultural catchments and water resources in various parts of the world including Europe (Field et al. 1997; Stangroom et al. 1998; Thurman et al. 2000; Sorensen et al. 2003). They are used as selective weed killers in cereal cultivation and are moderately mobile in soil (Sorensen et al. 2003). In view of the poor mineralization rate of the phenyl ring and potential carcinogenic effects of phenylurea herbicides and their metabolites (Scassellati-Sforzolini et al. 1997; Tixier et al. 2001),

improved biodegradation of these compounds is of great importance.

Isoproturon (3-[4-isopropylphenyl]-1,1-dimethylurea), IPU, is one of the phenylurea herbicides used to prevent pre- and post-emergence weed development in winter cereals. IPU is used intensively, persists in soil, contaminates water resources and it is frequently detected in groundwater bodies at concentrations often exceeding the EU limit for drinking water (0.1 μg l^{-1}; Spliid and Koppen 1998; Muller et al. 2002). Although this compound has been banned in several countries or restricted to 1.2 kg ha^{-1} $year^{-1}$ since 2003 in several countries including France, it is still extensively used worldwide. Several studies have indicated the harmful effects of IPU and its metabolites on aquatic invertebrates, freshwater algae and microbial communities (Mansour et al. 1999; Widenfalk et al. 2008; Vallotton et al. 2009). IPU and its metabolites have also been suspected to be carcinogenic to human beings and animals (Behera and Bhunya 1990; Hoshiya et al. 1993). Given these harmful effects, it is important to eliminate or minimize IPU contamination in soil and water. Microbial biodegradation, which is the primary mechanism for dissipating IPU as well as other phenylurea herbicides from the soil (Fournier et al. 1975; Gaillardon and Sabar 1994; Pieuchot et al. 1996), could prove a reliable, cost-effective remediation technique for IPU abatement. Several studies have reported the adaptation of soil microflora to IPU degradation in response to repeated exposure to this herbicide over a long period on fields under cultivation (Bending et al. 2003; Sorensen and Aamand 2001; El-Sebai et al. 2005). The IPU degrading ability of soil microflora has been found to be influenced by various soil and environmental factors (Aislabie and Lloydjones 1995; Andrea et al. 2000; Bending et al. 2003; El-Sebai et al. 2007), among which pH is considered to be a key factor affecting biodegradation not only in isolated cultures (Bending et al. 2003; Hussain et al. 2009; Sun et al. 2009) but also in soils (Houot et al. 2000; El-Sebai et al. 2005).

The presence of IPU degradation potential has been reported in a variety of bacterial and fungal species isolated from soils of various regions throughout the world (Vroumsia et al. 1996; Khadrani et al. 1999; Castillo et al. 2001; Sorensen et al. 2001; Tixier et al. 2002; Bending et al. 2003; El-Sebai et al. 2004; Badawi et al. 2009; Hussain et al. 2009; Sun et al. 2009). Of all known IPU degrading isolates, only *Sphingomonas* sp. SRS2 (Sorensen et al. 2001), *Methylopila* sp. TES (El-Sebai et al. 2004) and *Sphingobium* sp. strains YBL1, YBL2, YBL3 (Sun et al. 2009) were found to mineralize IPU completely to CO_2 and biomass.

Although the complete IPU metabolic pathway still remains to be determined, the initial steps and processes involved have been described in recent studies (Sorensen et al. 2001, 2003; Hussain et al. 2009; Sun et al. 2009). Demethylation of the dimethylurea side chain of IPU resulting in transient accumulation of MDIPU (3-(4-isopropylphenyl)-1-methylurea) has been described as an initial, limiting step during IPU degradation (Sorensen et al. 2001; Sun et al. 2009; Hussain et al. 2009). Based on the accumulation of different metabolites, it has been suggested that the IPU metabolic pathway is initiated by two successive *N*-demethylations, followed by the cleavage of the urea side chain resulting in transient accumulation of 4-isopropylaniline and finally by the mineralization of the phenyl structure (Sorensen et al. 2001; Hussain et al. 2009). Various IPU metabolic pathways by defined microorganisms in agricultural soils were proposed by Sorensen et al. (2003). Recently, Sun et al. (2009) described the accumulation of catechol and the first metabolite after phenyl ring cleavage, *cis,cis*-muconic acid, during the mineralization of aniline by the IPU degrading *Sphingobium* sp. strain YBL2. A catechol degrading *catA* gene coding for 1,2-dioxygenase, catalyzing the opening of phenyl ring of catechol in many Gram positive and Gram negative bacteria, was also amplified by PCR from the IPU degrading strain YBL2. It was suggested that catechol, which is considered as a key intermediate during the phenyl ring cleavage of aromatic compounds, could also be a metabolite during IPU mineralization.

This study was carried out to isolate and characterize a bacterial population able to mineralize IPU by performing enrichment cultures from an agricultural soil of France that had adapted to IPU as a result of repeated exposure to IPU. Taxonomic and genetic characterization of the bacterial isolate was performed by cloning and sequencing 16S rRNA as well as catechol 1,2-dioxygenase gene. The ability of the bacterial strain to degrade IPU metabolites, various phenylurea herbicides and their aniline derivatives was also characterized. As pH is considered to be one

of the most important factors affecting the degrading capabilities of the soil microorganisms, its effect on IPU mineralization kinetics was also investigated.

Materials and methods

Soil sampling and characteristics

Nine soil samples (0–20 cm) were collected from an agricultural field periodically exposed to IPU located at the experimental farm of the French National Institute of Agronomical Research of Epoisses (Breteniere, France). The mean soil particle size distribution of the collected nine samples was clay 47.8%, fine silt 26%, coarse silt 18%, fine sand 3.5%, and coarse sand 4.7%. The physical and chemical characteristics of the soil samples are given in Table 1. The soil samples were sieved to 5 mm and stored at 4°C for less than 1 month until use.

Chemicals

Analytical grade IPU (99.0% purity), chlorotoluron (3-(3-chloro-*p*-tolyl)-1,1-dimethylurea, 99.7% purity), diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea, 99.4% purity), linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea, 99.0% purity) and monolinuron (3-(4-chlorophenyl)-1-methoxy-1-methylurea, 99.0% purity) were purchased from Riedel–de Haen (Germany). The IPU metabolites MDIPU (1-(4-isopropylphenyl)-3-methylurea, 99.0% purity), DDIPU (1-(4-isopropylphenyl) urea, 99.0% purity) and 4-IA (4-isopropylaniline, 99% purity) were purchased from Dr. Ehrenstorfer-Schafers (Augsburg, Germany). Aniline derivatives of various phenylurea herbicides [3-chloroaniline, 4-chloroaniline, 4-bromoaniline, 3-chloro-4-methylaniline, 3,4-dichloroaniline (3,4-DCA)] were purchased from Sigma–Aldrich (Germany). ^{14}C-ring-labeled IPU (specific activity 18 mCi mmol^{-1}; 99% radiochemical purity) was purchased from International Isotopes (Munchen, Germany).

IPU mineralization potential of soil

The IPU mineralization potential of the soil samples was determined by radiorespirometry using ^{14}C-ring-labeled isoproturon as previously described (El-Sebai et al. 2005). About 40 g equivalent dry weight of each soil was treated with 1.5 mg of IPU per kg of soil and 2 kBq of ^{14}C-ring-labeled IPU. The soil moisture was adjusted to 80% of its water holding capacity and incubated at 20°C in the dark for 90 days in closed respirometer jars (Soulas 1993).

Table 1 Physico-chemical and biological parameters of soil samples determined from nine different top soil samples (0–20 cm) collected at the experimental station of INRA of Epoisses samples (Breteniere, France)

Parameter	Maximum	Minimum	Mean	SD	CV (%)
Soil moisture (g 100 g^{-1})	26.9	23.5	24.8	1.26	5.07
Organic matter (g kg^{-1})	37.2	26.4	30.8	4.1	13.4
Organic carbon (g kg^{-1})	21.5	15.8	18.4	1.9	10.5
Total nitrogen (g kg^{-1})	1.87	1.65	1.71	0.07	4.02
C/N	11.3	8.9	10.2	0.9	8.8
pH	7.83	7.66	7.76	0.06	0.76
CEC (cmol^{+} kg^{-1})	22.3	21.0	21.59	0.38	1.77
Cultivable bacteria (log10 CFU g^{-1})	9.24	8.74	9.06	0.14	1.52
Microbial C biomass (mg kg^{-1})	374.9	295.9	336.1	27.8	8.3
A (% $^{14}CO_2$)	45.3	39.5	42.1	1.8	4.3
μm (% $^{14}CO_2$ day^{-1})	4.57	2.48	3.68	0.71	19.23
λ (days)	0.76	0.42	0.57	0.10	17.19

Physico-chemical parameters: equivalent humidity, organic matter content, organic carbon, total nitrogen, C/N ratio, pH and cation exchange capacity (*CEC*)

Biological parameters: number of cultivable bacteria, microbial C biomass, maximum percentage of mineralization (*A*), maximum mineralization rate (μm) and lag phase (λ)

$^{14}CO_2$ resulting from mineralization of ^{14}C-ring-labeled isoproturon was trapped in 0.2 M NaOH solution. The traps were changed regularly over the incubation period and analyzed for radioactivity content by liquid scintillation counting using ACSII scintillation fluid (Amersham).

Enrichment cultures and isolation of IPU degrading bacterial strain

In order to isolate IPU degrading bacterial strains, enrichment cultures were set up using a composite soil sample made of the nine samples. About 10 g of soil equivalent dry weight was suspended in 90 ml of mineral salt (MS) medium (Rousseaux et al. 2001) containing IPU as the sole source of carbon and nitrogen at a concentration of 50 mg l^{-1} for liquid medium (MS-IPU) and incubated at 20°C on an orbital shaker (150 rpm). Aliquots were taken regularly and the IPU remaining in the medium was quantified by HPLC as described previously (Hussain et al. 2009). When about 50% of the initially added IPU was degraded, 10 ml of this suspension was transferred to 90 ml of fresh MS medium and incubated under the same conditions. Twelve successive enrichment cycles were carried out in the same way and 1 ml aliquots of each enrichment culture were preserved at –20°C. After 12 enrichments, the culture was serially tenfold diluted and 100 µl of the 10^{-3} to 10^{-6} dilutions were inoculated onto solid MS medium containing 500 mg l^{-1} of isoproturon and nutrient agar (NA) medium (Difco, France). The plates were incubated at 20°C. Single colonies isolated from the plates were tested for IPU degradation in liquid MS-IPU media at 28°C under agitation (150 rpm). After a few days, the cells were harvested (3,220 g, 10 min) and the supernatant was analyzed to determine the amount of the remaining IPU using HPLC.

PCR amplification, cloning and sequencing of 16S rRNA and *catA* sequences of IPU mineralizing strain

Crude DNA was extracted from the frozen aliquots of the serial enrichment cultures using proteinase K and thermal shock treatment as previously described (Cheneby et al. 2004). Genomic DNA was extracted from the isolated IPU degrading bacterial strain collected at the late exponential stage of growth using QIAGEN Genomic DNA Isolation Kit (QIAGEN, France) according to the manufacturer's recommendations.

The 16S rRNA sequence was amplified using the universal primers 27f (5′-AGA GTT TGA TCH TGG CTC AG-3′) and 1492r (5′-TAC GGH TAC CTT GTT ACG ACT T-3′) (Gurtler and Stanisich 1996). The amplification reaction was carried out in a final 25 µl volume containing 2.5 µl of 10× Taq polymerase buffer, 200 µM of each dNTP, 1.5 mM of $MgCl_2$, 0.5 µM of each primer and 0.625 U of Taq polymerase. A volume of 2.5 µl of crude DNA or 25 ng of the genomic DNA was used as template for the PCR reactions. PCR was performed in a thermocycler (PTC 200 Gradient Cycler, MJ Research, Waltham, Mass) according to the following program: 1 cycle of 4 min at 94°C; 39 cycles of 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C and a final extension step at 72°C for 5 min. A *catA* gene fragment was amplified by PCR using *catA*r and *catA*f primers as recently described (Sun et al. 2009). The size of the 16S rRNA as well as *catA* amplicons was confirmed by separating on 1% agarose gel along with 1 kb size marker (Invitrogen, France).

The purified 16S rRNA amplicons were analyzed by restriction fragment analysis. The PCR products were digested using the restriction enzyme *Alu*I (Fermentas, France) and separated on 3% high resolution (MP Q-BIOgene, America) agarose gel along with the DNA molecular weight marker BVIII (Roche Applied Science, France).

For further analyses, 16S rRNA and *catA* PCR products were purified using the MinElute PCR Purification Kit according to manufacturer's recommendations (QIAGEN, France). The purified 16S rRNA and *catA* amplicons were cloned in pGEMT-EasyII vector according to the manufacturer's recommendations (Promega, Madison, WI, USA). The recombinant clones were screened by PCR using the SP6 and T7 universal primers and the following program: 94°C for 45 s, 35 cycles of 55°C for 45 s, 72°C for 90 s and a final extension step at 72°C for 7 min. Cloned 16S rRNA amplicon was sequenced by Cogenics (Meylan, France). The sequence was deposited in the GenBank database under the accession number HM191725. Cloned *catA* PCR product was sequenced using T7 primer with the CEQ8000 dye terminator cycle sequencing following the

manufacturer's recommendations (Beckman Coulter, France). The partial *catA* sequence was deposited in the GenBank database under the accession number HM191726.

Both 16S rRNA and *catA* sequences were compared to the known nucleotide sequences using BlastN (http://www.ncbi.nlm.nih.gov/BLAST). To construct phylogenetic trees based on 16S rRNA or *catA* sequences, multiple alignments were carried out using ClustalX (Thompson et al. 1997) and the data obtained was processed using NJ Plot using the neighbor joining method (Perrière and Gouy 1996).

Pairwise comparison of phylogenetic tree based on 16S rRNA with the one based on *catA* sequences was carried out using an applet developed by Nye et al. (2005).

Characterization of degrading capabilities of the isolated strain

The IPU mineralization kinetics of the isolated bacterial strain were determined by radiorespirometry using ^{14}C-ring-labeled IPU in a phosphate buffer defined as Knapp as previously described (Devers et al. 2004). The cells were harvested from MS-IPU medium at the late exponential phase, washed twice with Knapp buffer and resuspended to an optical density of 0.5 in Knapp buffer containing 50 mg l^{-1} of IPU and 2 kBq of ^{14}C-ring-labeled IPU. Cell suspensions ($n = 3$) were incubated for 3 days at 20°C on an orbital shaker (150 rpm) in closed sterile respirometer jars containing NaOH traps. The traps were changed regularly during the incubation period and analyzed for radioactivity content by liquid scintillation counting using ACSII scintillation fluid (Amersham). At the end of incubation, the amount of radioactivity remaining in the cell free medium as well as in the bacterial pellet was also analyzed by ACSII scintillation counting.

The ability of the isolated bacterial strain to degrade IPU, its known metabolites (i.e. MDIPU, DDIPU and 4-IA), various phenylurea herbicides (i.e. isoproturon, diuron, linuron, monolinuron and chlorotoluron) and their aniline derivatives (i.e. 4-IA, 3,4-DCA, 3-chloroaniline, 4-chloroaniline, 4-bromoaniline and 3-chloro-4-methylaniline) was estimated. The isolated strain was grown in MS medium, washed twice in Knapp buffer and then suspended in Knapp buffer containing 50 mg l^{-1} of the pesticide or metabolite, to obtain an optical density (600 nm) of 0.2. Three replicates were performed for each compound. The cells were incubated at 28°C under agitation (125 rpm) and aliquots from each suspension were taken regularly during the incubation period. The suspensions were centrifuged (3,220 × g, 10 min) and the supernatants were subjected to HPLC analysis in order to quantify the compound remaining in the suspension.

The impact of pH on IPU degradation kinetics was estimated. The pH of the Knapp buffer was adjusted to 5.5, 6.5, 7.5 or 8.5. At the end of incubation (3 days), cells were enumerated on Petri dishes. Cell suspensions were serially tenfold diluted and the 10^{-4} to 10^{-6} dilutions were plated on NA medium. The plates were incubated at 28°C and the CFU were enumerated after 10 days of incubation.

Modeling and statistical analysis of IPU degrading kinetics

To determine the kinetics parameters of the degradation of IPU, the degradation curves were fitted to a modified Gompertz model (Zwietering et al. 1990) using Sigma Plot 4.0. The model equation was: $y = A.\exp\{-\exp[1 + \mu m.\exp(1).(\lambda - t)/A]\}$ where y is the percentage of mineralization or degradation (%), t is the time (h), μm is the maximum mineralization or degradation rate (% h^{-1}), A is the maximum percentage of mineralization or degradation (%), and λ is the lag time (h). The parameters were validated using a Student's t test ($P < 0.005$).

In order to assess the impact of pH on IPU degradation, the kinetics parameters were subjected to a single-factor analysis of variance (one-way ANOVA) followed by a Fisher procedure ($n = 3$, $P < 0.001$) (Statview 4.55 software, Abacus Concept, Inc.). A similar approach was applied to assess the impact of pH on the estimation of the abundance of SH colonies.

Results

IPU mineralization kinetics in soil

This study was performed using nine soil samples collected from a field under rape seed/winter wheat/barley crop rotation. This field had been treated with

IPU (2 applications per year for 2 years of every 3 year rotation) for 10 years. The physical and chemical properties of the soil samples were found to be relatively homogenous with a low coefficient of variation (CV) (Table 1). The ability of the soil microflora to mineralize this herbicide estimated by radiorespirometry showed that all these soil samples were able to mineralize IPU under laboratory conditions (data not shown). Approximately 30–40% of the ^{14}C-ring-labeled IPU initially added was transformed to $^{14}CO_2$ over 2 weeks of incubation. The maximum IPU mineralization (A), estimated by fitting the modified Gompertz model to the IPU mineralization kinetics of the soil samples, averaged 42.1% $^{14}CO_2$ with a CV of 4.3% (Table 1). The other two kinetic parameters, the lag phase (λ) and the maximum mineralization rate (μm), averaged about 0.6 days and 3.68% $^{14}CO_2$ day^{-1}, respectively and were found to be moderately variable with a CV of 17.1% and 19.2%, respectively (Table 1). Determination of the remaining radioactivity in the soil samples indicated that the methanol extractable radioactivity was relatively low ranging from 5 to 8%, whereas, non-extractable radioactivity corresponding to bound residues varied from 40 to 50% of the initially added ^{14}C-ring-labeled IPU.

Enrichment and isolation of an IPU mineralizing bacterial strain

In order to characterize the IPU degrading populations responsible for the IPU mineralization observed in the field samples of Epoisses, the IPU mineralizing bacterial strain was isolated through enrichment cultures starting from a composite soil sample formed from the nine soil samples. This composite soil sample was transferred to fresh MS-IPU medium containing IPU as the sole source of carbon and nitrogen. Twelve successive enrichment cultures were carried out. During the enrichment procedure, the structure of the microbial communities in the soil slurries was evaluated by ARDRA fingerprinting. These analyses revealed a relatively complex microbial community of ten different bands (Fig. 1). During the early enrichments, the ARDRA profiles were simplified and very similar profiles were observed for the last four enrichments (9–12). In order to isolate pure IPU degrading bacteria, the IPU mineralizing bacterial culture obtained after 12 enrichments was serially diluted and inoculated on NA and MSA-IPU plates. After 5 days of incubation, different types of colonies appeared on NA media plates one of which was dominant (>80%). Similarly, the growth of different types of colonies was observed on MSA-IPU plates after about 15 days of incubation. The IPU degrading ability of the different types of colonies appearing on NA and MSA-IPU was tested in liquid MS-IPU medium. This showed that the dominant bacterial colony type present on the NA and MSA-IPU media plates was able to degrade IPU. On NA plates, the IPU degrading colonies were opaque, buff, smooth, circular and convex. On MSA-IPU media plates, the IPU degrading colonies formed a clear zone owing to the degradation of IPU crystals. The purity of the strain was ensured by passing it

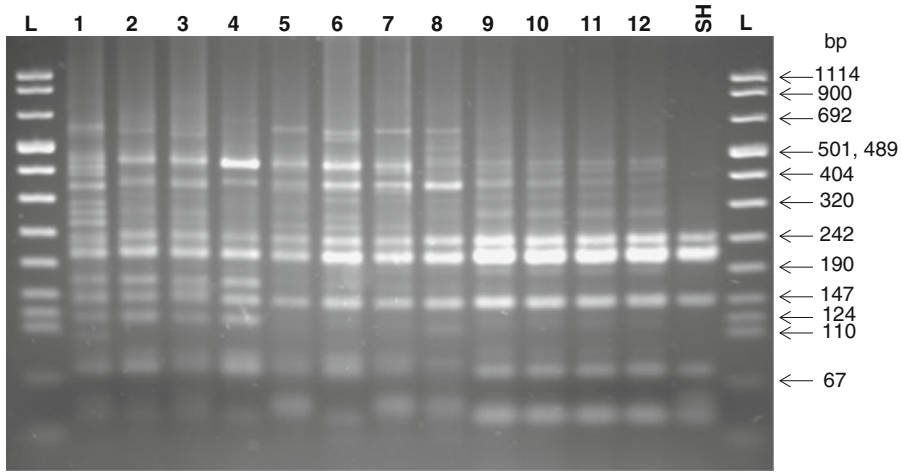

Fig. 1 ARDRA fingerprint produced from DNA samples extracted from enrichment culture aliquots. *Lanes 1–12* represent the ARDRA fingerprint of 12 consecutive enrichment cultures (from enrichment 1–12). *Lane SH* indicates the ARDRA fingerprint of the isolate *Sphingomonas* sp. SH and the *lane L* is the BVIII molecular weight marker (size indicated in base pairs)

twice from MS-IPU liquid to MSA-IPU plates and then testing its IPU degrading activity by HPLC.

Identification and phylogenetic characterization of IPU mineralizing strain

The 16S rRNA amplicon (1,447 bp) amplified from the isolated IPU degrading bacterial strain was sequenced and deposited in the GenBank database under accession number HM191725. The highest degree of similarity (96%) of this sequence was obtained with 16S rRNA gene sequence of *Sphingomonas* sp. JEM-14 (GenBank Ac. No. AB219361), an estrogen degrading bacterium. Given the phylogenetic relationship of this bacterial strain with several *Sphingomonas* spp. (Fig. 2), this isolate was named *Sphingomonas* sp. SH.

By performing a PCR assay targeting catechol 1,2-dioxygenase gene (Sun et al. 2009), a DNA fragment of about 417 bp was obtained (data not shown). The *catA* amplicon was sequenced and deposited in the GenBank database under the accession number HM191726. Homology searching using BLAST showed that the obtained gene fragment had 98% identity with the *catA* gene of *Sphingomonas* sp. KA1 (GenBank Ac. No. AB270530).

An *in silico* study was performed to establish the level of congruence between the corresponding *catA*

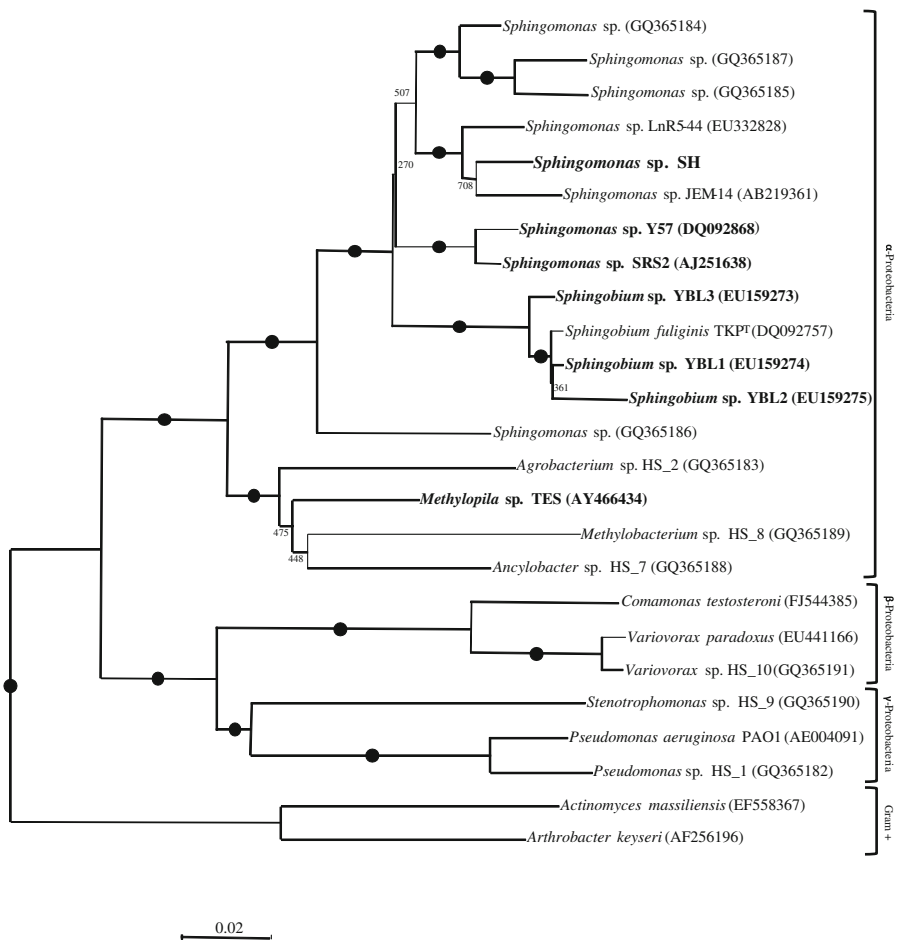

Fig. 2 Neighbor-joining phylogenetic analysis resulting from the multiple alignment of 16S rRNA gene sequences of *Sphingomonas* sp. SH with those of other IPU degrading strains found in GenBank database. IPU degrading bacterial isolates are in *bold*. GenBank accession numbers are given in *brackets*. Bootstrap values greater than 900‰ are marked as *black circles*. The phylogenetic distance is shown on a *scale bar*

and 16S rRNA sequence based phylogenies of 14 Proteobacteria including *Sphingomonas* sp. SH. The sequences were retrieved from the GenBank database by selecting known 16S rRNA and *catA* sequences identified in the same bacterial strain. The structure of the phylogenetic tree based on the alignment of 16S rRNA was consistent with the expected taxonomy exhibiting three clusters (i.e. 100% bootstrap) consisting of α-, β- and γ-Proteobacteria, respectively (Fig. 3a). Unlike 16S rRNA phylogeny, the *catA* based tree did not have these three significantly distinct clusters (Fig. 3b). However, *Sphingomonas* and *Sphingobium* spp. belonging to α-Proteobacteria were grouped together in the same cluster with 100% bootstrap value except for *Sphingomonas wittichii* RW1 which was found in another cluster (100% bootstrap value) of γ-Proteobacteria consisting of *Pseudomonas* spp. Similarly, the bacterial strains belonging to β-Proteobacteria were significantly grouped together in the 16S rRNA based phylogenetic tree. However, they were found to be spread and non-significantly (bootstrap <70%) grouped with α-Proteobacteria and γ-Proteobacteria in *catA* based phylogenetic tree. It can, therefore, be concluded that the 16S rRNA and *catA* based phylogenies were not congruent with each other because the same taxonomical structure was not found. This was also statistically confirmed by the pairwise comparison of *catA* and 16S rRNA phylogenies which revealed a probability of similarity of only 52.3%.

Degrading capabilities of *Sphingomonas* sp. SH

The degrading ability of the bacterial strain was estimated by HPLC measurements. IPU degradation kinetics by strain SH indicated that the complete degradation (>99%) of IPU occurred within 12 h of incubation (Fig. 4). Transient accumulation of a metabolite showing the same retention time as that of MDIPU was detected during the IPU degradation. Strain SH was also able to degrade the known metabolites of IPU (i.e. MDIPU, DDIPU and 4-IA).

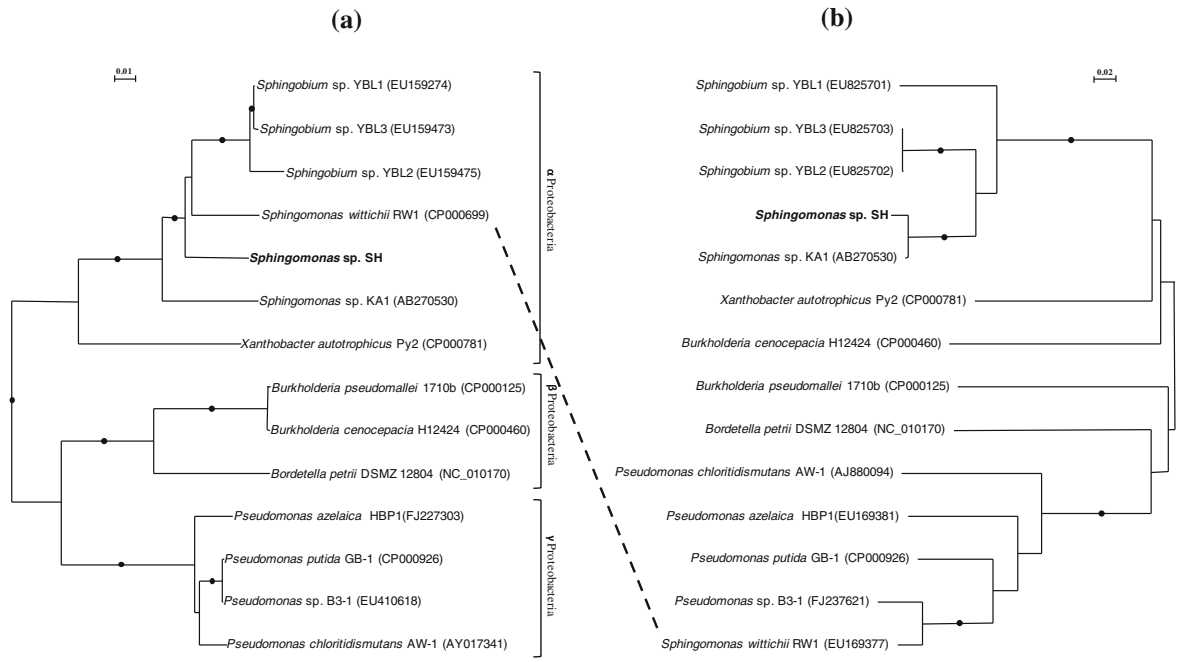

Fig. 3 Comparison of 16S rRNA and *catA* phylogenies of 14 bacterial strains. **a** Phylogenetic tree of 16S rRNA sequences resulting from the multiple alignment of 14 bacteria. The different clusters of different classes of bacteria are indicated. **b** Phylogenetic tree based on *catA* sequences of the 14 bacterial strains. The *broken line* between the two trees shows an example of non-congruence between the two phylogenies. GenBank accession numbers of the *catA* and 16S rRNA sequences are given in *brackets*. Bootstrap values greater than 900‰ are marked as *black circles*. The phylogenetic distance is shown on a *scale bar*

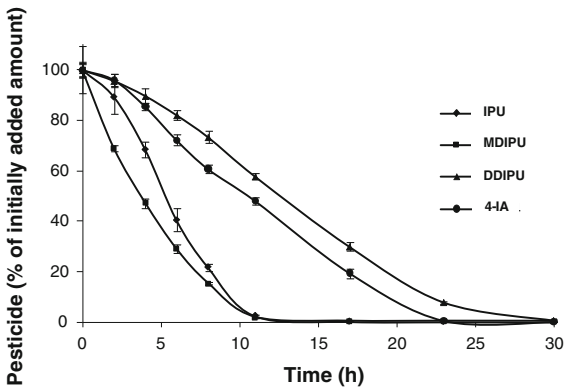

Fig. 4 Degradation kinetics of IPU, DMIPU, DDIPU and 4-IA by *Sphingomonas* sp. SH incubated in Knapp buffer at 28°C under 150 rpm agitation. *Error bars* indicate standard error ($n = 3$)

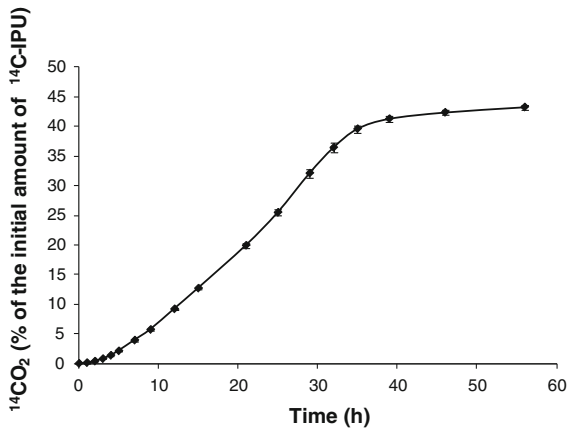

Fig. 5 Kinetics of ^{14}C-ring-labeled IPU mineralization by *Sphingomonas* sp. SH incubated at 28°C in Knapp buffer under agitation (150 rpm). *Error bars* indicate standard error ($n = 3$)

Complete degradation (>99%) of MDIPU, 4-IA and DDIPU occurred after 12, 23 and 30 h of incubation, respectively (Fig. 4.). The maximum degradation rate (μm [% h^{-1}]) calculated by modeling the degradation kinetics was found to be highest for IPU and MDIPU (μm = 14.2 and μm = 13.6, respectively) while the lowest rates were observed for DDIPU and 4-IA (μm = 5.4 and μm = 6.2, respectively).

Although *Sphingomonas* sp. SH was able to degrade IPU and its metabolites successfully, it could not degrade other phenylurea herbicides (diuron, linuron, monolinuron and chlorotoluron) even after 5 days of incubation (data not shown). Moreover, it was unable to degrade any of the aniline derivatives of other phenylurea herbicides i.e. 3,4-DCA, 3-chloroaniline, 4-chloroaniline, 4-bromoaniline and 3-chloro-4-methylaniline after 90 h of incubation.

Results of the radiorespirometric experiments indicated that over 40 h of incubation, the isolated bacterial strain had mineralized about 42% (±0.5%) of the ^{14}C IPU added initially (Fig. 5). Determination of the remaining radioactivity revealed that up to 39% (±2.4%) of the radioactivity added initially was detected in the bacterial pellet, suggesting that it was incorporated in the bacterial biomass, and only about 8% (±0.7%) remained in the culture media. The parameters of IPU mineralization kinetics deduced by modeling the mineralization data revealed a lag phase (λ) of 2 h and a maximum mineralization rate (μm) of 1.5% h^{-1}.

Effect of pH on IPU degradation kinetics

The effect of pH on the IPU degrading ability of the *Sphingomonas* sp. SH was estimated. The pH was shown to have a strong effect on the IPU degradation kinetics of the isolated bacterial strain in liquid culture (Fig. 6). *Sphingomonas* sp. SH had completely degraded IPU (>99%) after 44, 24, 18 and 36 h of incubation at pH values of 5.5, 6.5, 7.5 and 8.5, respectively. By fitting the modified Gompertz model to the degradation data, the highest μm (19.9) was observed at pH 7.5, while the lowest μm (3.5) was observed at pH 5.5 (Fig. 6a). Statistical analysis confirmed that μm was significantly higher at pH 7.5 than at other pH values ($P < 0.05$), while there was no significant difference between the μm at pH 5.5 and that at pH 8.5. However, μm for pH 6.5 (9.1) was significantly higher than for pH 5.5 and 8.5 but significantly lower than for pH 7.5 ($P < 0.05$). Thus, 7.5 was found to be the optimum pH value for the IPU degrading ability of *Sphingomonas* sp. SH and that the IPU degradation rate was significantly affected as the pH shifted towards more acidic or alkaline conditions.

In order to determine the effect of pH on the cultivability of *Sphingomonas* sp. SH, the colony forming units (CFU) of this strain were counted at different pH values at the end of the incubation period. Statistical analysis indicated that the abundance of the strain was significantly affected by the

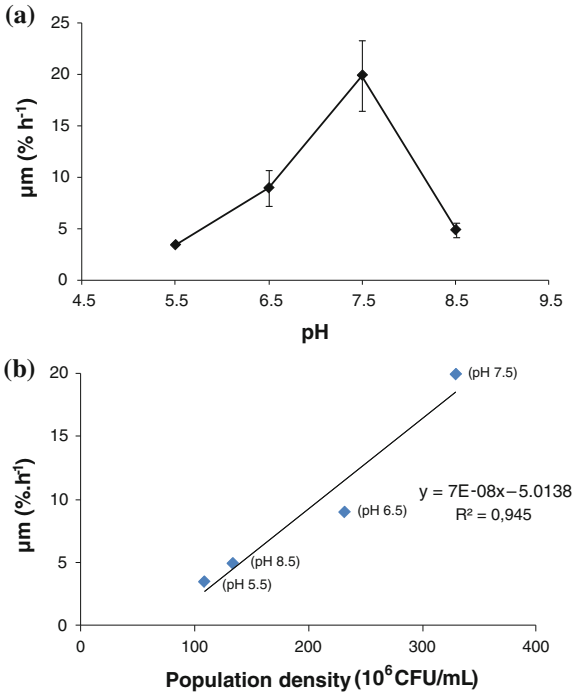

Fig. 6 **a**. Effect of pH on the maximum degradation rate (μm) of IPU by *Sphingomonas* sp. SH in liquid culture. *Error bars* indicate standard error ($n = 3$). **b**. Correlation between the maximum degradation rate (μm in % of IPU degraded per hour) and the number of *Sphingomonas* sp. SH colonies enumerated on nutrient agar medium (CFU ml^{-1}) after their incubation at different pH in Knapp buffer added with IPU

different pH values following the same pattern as that for the degradation. CFU was found to be significantly correlated ($\mu m = 7E\text{-}08$ cfu $ml^{-1} - 5.0183$, $R^2 = 0.945$) with the maximum degradation rate (μm) at different pH values (Fig. 6b).

Discussion

Accelerated degradation of IPU has already been reported in several soils repeatedly treated with this herbicide at various places in Europe including UK, Denmark and France (Sorensen et al. 2001; Bending et al. 2003; El-Sebai et al. 2005, 2007). Microbial degradation has been described as a primary mechanism responsible for IPU dissipation as well as other phenylurea herbicides from the soil (Fournier et al. 1975; Gaillardon and Sabar 1994; Pieuchot et al. 1996). In this study, it was observed that all nine soil samples collected from a field under rape seed/winter wheat/barley crop rotation, periodically exposed to IPU for 10 years, were able to mineralize about 40% of the ^{14}C-ring-labeled IPU initially added to $^{14}CO_2$ after only 31 days of incubation. These results revealed the IPU mineralizing ability of the experimental field in Epoisses which might result from the adaptation of the indigenous soil microflora to metabolize IPU in response to repeated exposure. The collected soil samples showed a relatively low variability in IPU mineralization ability which might be attributed to a low variation in the physical, chemical and microbial properties known to influence the pesticide biodegrading ability of the soil microflora (Bending et al. 2003; Briceno et al. 2007; El-Sebai et al. 2007; Vieuble-Gonod et al. 2009).

In order to characterize the bacterial populations responsible for IPU mineralization in the Epoisses field, enrichment cultures were initiated starting from a composite sample with IPU as sole source of carbon and nitrogen. Throughout the enrichment process, the evolution of the 16S rRNA genetic structure of the bacterial community was monitored by ARDRA fingerprinting. After successive enrichments, the complexity of the structure of the bacterial community was found to be simplified. It is more likely that the successive enrichments led to the disappearance of certain uncultivable bacterial strains and of the bacterial populations unrelated to IPU degradation leading to the dominance of the IPU degrading bacterial population. This observation is in accordance with other laboratory studies indicating that enrichment procedures using pesticides as a selection pressure induce significant changes in bacterial community composition (El-Fantroussi et al. 1999; Bending et al. 2003; El-Sebai et al. 2004).

Phylogenetic analysis based on 16S rRNA revealed that the strain SH showed up to 96% similarity with the 16S rRNA of *Sphingomonas* sp. JEM-14 and was clustered with several *Sphingomonas* strains. Within the *Sphingomonas* cluster that includes the strain SH, several bacterial isolates belonging to the Sphingomonadaceae family including *Sphingobium* (Sun et al. 2009) and *Sphingomonas* strains (Sorensen et al. 2001, 2002; Bending et al. 2003; this study), isolated from geographically distant soils (including China, Denmark, France and United Kingdom), have been shown to mineralize IPU. Interestingly, the linuron, another type of

phenylureas, was recently shown to be degraded by different strains belonging to genus *Variovorax*, like strain WDL1 (Breugelmans et al. 2007) and strain SRS16 (Sorensen et al. 2009) isolated from Belgium and Danish soils, respectively. These observations further reinforce the idea that members of this two genus, which are ubiquitous in the environment, harbor broad catabolic capabilities (White et al. 1996; Fredrickson et al. 1999; Sorensen et al. 2001), can rapidly adapt to phenylurea exposure and develop the ability to break it down as a source of carbon and nitrogen (Hussain et al. 2009).

Catechol, which is a key intermediate of the *β*-ketodipate pathway responsible for the degradation of many aromatic compounds (Alva and Peyton 2003; Nayak et al. 2009) and *cis,cis*-muconic acid, its aliphatic metabolite (Nayak et al. 2009), have also been detected during the mineralization of aniline by IPU degrading *Sphingobium* strains YBL1, YBL2 and YBL3 (Sun et al. 2009). In that study, it was reported that the *catA* gene coding for catechol 1,2-dioxygenase was detected in three IPU degrading *Sphingobium* strains thereby suggesting that phenyl ring of IPU might be degraded through *ortho*-cleavage of the catechol. These results show that the strain SH also harbors the *catA* gene. Phylogenetic analysis of the *catA* amplicon revealed that it shared about 98% homology with the *catA* of the *Sphingomonas* sp. KA1 (AB270530) and about 82% homology with that of the *Sphingobium* sp. YBL3 (Sun et al. 2009). In addition, the *catA* sequence of the strain SH was closely related to the *catA* genes present in various xenobiotic degrading members of the family *Sphingomonadaceae*. This may suggest that, for the strain SH, the mineralization of the IPU phenolic ring would have occurred through the formation of and degradation of catechol as an intermediate compound at some stage after 4-IA, as previously described (Sun et al. 2009). This finding may also be helpful in describing the later steps of IPU metabolism after 4-IA.

16S rRNA and *catA* phylogenies of 14 bacterial strains including *Sphingomonas* sp. SH were constructed and checked for congruence. The taxonomical structure of the selected 14 bacterial strains was reflected in 16S rRNA phylogeny but not in the *catA* phylogeny. In addition, pairwise comparison revealed a low probability (52.3%) further indicating that these two phylogenies are not congruent and suggesting that *catA* has been the subject of horizontal gene transfer, most likely as a result of the selection pressure exerted by the exposure of the soil microflora to contaminants including pesticides such as phenylurea herbicides.

The strain SH had the ability to mineralize about 40% of the added amount of ^{14}C IPU to $^{14}CO_2$ with almost the same amount incorporated into bacterial biomass. It is noteworthy that the amount of IPU evolved as $^{14}CO_2$ by the strain SH (42 ± 0.5%) was comparable to that of other IPU mineralizing bacterial strains like *Sphingomonas* sp. SRS2, *Methylopila* sp. TES and enriched bacterial culture confirming that strain SH mineralizes IPU as efficiently as previously isolated strains (Sorensen et al. 2001; El-Sebai et al. 2004; Hussain et al. 2009).

The transient accumulation of a metabolite with the same retention time as that of MDIPU during the IPU degradation by the strain SH and its further degradation suggests that IPU degradation might have been initiated by *N*-demethylation of the urea side chain as previously proposed (Sorensen et al. 2001; Hussain et al. 2009; Sun et al. 2009). Sorensen et al. (2001) and recently Hussain et al. (2009) have reported for *Sphingomonas* sp. SRS2 and a bacterial mixed culture, respectively, that IPU degradation was initiated by two successive *N*-demethylation producing MDIPU and DDIPU. This last metabolite was further transformed to 4-IA by the cleavage of the urea side chain and ultimately by mineralization of the phenyl ring. Based on the characterization of ability of the strain SH to degrade known IPU metabolites (i.e. MDIPU, DDIPU and 4-IA) and transient accumulation of a metabolite during the degradation kinetics of IPU, we hypothesize that strain SH transforms IPU through the demethylation mechanism and not through the hydroxylation of the isopropyl group or the hydrolysis of IPU which have been reported as two other routes of IPU degradation (Penning et al. 2010).

Although strain SH had the ability to degrade IPU and its known metabolites, it could not degrade any of the related phenylurea herbicides i.e. diuron, linuron, monolinuron and chlorotoluron or their aniline derivatives. This suggests that the degrading capabilities of SH are highly specific for IPU and its known metabolites. The enzymatic specificity of SH towards the IPU metabolic pathway was further highlighted by its ability to degrade only 4-IA (aniline derivative of IPU) and none of the aniline

derivatives of other phenylurea herbicides. Up to now different strains, able to degrade phenylurea herbicides among which IPU, have been isolated from geographically diverse regions of the world (Sorensen et al. 2001; Turnbull et al. 2001; Tixier et al. 2002; El-Sebai et al. 2004; Hussain et al. 2009; Sun et al. 2009). Among the known IPU degrading bacterial strains, *Arthrobacter globiformis* D47 (Turnbull et al. 2001) and *Arthrobacter sp. N2* (Tixier et al. 2002) are able to transform phenylurea herbicides to their corresponding aniline derivatives. However, *Sphingomonas* sp. SRS2 (Sorensen et al. 2001) as well as *Sphingobium* strains YBL2 and YBL3 (Sun et al. 2009) have been reported to mineralize IPU and to degrade closely related dimethyl substituted phenylurea herbicides such as diuron and chlorotoluron. In addition, more recently, *Sphingobium* sp. YBL1 (Sun et al. 2009) was shown to metabolize most of the phenylurea herbicides and their aniline derivatives. Unlike these strains, *Methylopila* sp. TES (El-Sebai et al. 2004) and recently, an enriched bacterial culture (Hussain et al. 2009) were found to mineralize only IPU without showing any degrading activity towards other structurally related phenylurea herbicides. *Sphingomonas* sp. SH has catabolic abilities similar to those of *Methylopila* sp. TES (El-Sebai et al. 2004) and the enriched bacterial culture (Hussain et al. 2009). It is possible that the strain SH as well as *Methylopila* sp. TES and the enriched bacterial culture possess a degrading ability specific for IPU.

pH is one of the most important factors which has been reported to have significant effects on microbial pesticide degradation not only in soils (El-Sebai et al. 2005, 2007; Bending et al. 2006) but also in isolated bacterial culture studies (Bending et al. 2003; Hussain et al. 2009; Sun et al. 2009). This study also highlights the effect of pH on the IPU degradation kinetics by the strain SH. The results indicate that the strain SH had the ability to completely degrade IPU between the pH range of 5.5–8.5 with an optimum activity at pH 7.5 ($\mu m = 19.9$) and reduced activity at pH 5.5 ($\mu m = 3.5$) and at pH 8.5 ($\mu m = 4.95$). These results are in accordance with similar patterns in pH regulation of IPU degradation kinetics by the bacterial species in liquid cultures (Bending et al. 2003; Hussain et al. 2009; Sun et al. 2009). They also confirmed studies reporting a significant correlation between the soil pH and the degradation of various pesticides (Houot et al. 2000; El-Sebai et al. 2005, 2007). Although the effect of pH on IPU degradation by soil microflora is well described by many previous studies (Bending et al. 2003; Hussain et al. 2009; Sun et al. 2009), the mechanisms responsible for this regulation have not been explained. However, the pH is known to affect the growth and survival of microbial populations (Russell and Dombrowski 1980; Sun et al. 1998; Rousk et al. 2009) which ultimately affects their activity. Enumeration of the SH population at the end of the incubation period for each pH value revealed that the maximum degradation rate (μm) was strongly correlated ($R^2 = 0.945$) with the number of SH bacteria counted at the end of incubation period (Fig. 6b). This suggests that pH might have an effect on the growth or survival of the SH bacterial population which might partly explain the variation in IPU degradation rate observed at different pH values. However, a more specific effect of pH on IPU degradation (abiotic and biotic IPU transformation, etc.) cannot be excluded and further research should be carried out to study the transcriptional and enzymatic regulation occurring in strain SH on exposure to IPU at different pH values.

In conclusion, the isolation of *Sphingomonas* sp. SH is the second report after *Methylopila* sp. TES (El-Sebai et al. 2004) of a soil borne bacterial isolate from agricultural soils in France, which is able to mineralize IPU and has degrading capabilities that are highly specific for metabolizing IPU. This study is possibly the first report showing the correlation between physiological effects and the IPU degradation capabilities of a bacterial isolate in response to variations in pH. It also suggests the involvement of *catA* in the lower degradation pathway of IPU. Further research is needed to elaborate the IPU metabolic pathway by identifying the genes coding for the enzymes involved in IPU mineralization either through metagenomic approaches or proteomic analysis.

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