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Isolation of an Isocarbophos-Degrading Strain of *Arthrobacter* sp. scl-2 and Identification of the Degradation Pathway

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Isocarbophos is a widely used organophosphorus insecticide that has caused environmental pollution in many areas. However, degradation of isocarbophos by pure cultures has not been extensively studied, and the degradation pathway has not been determined. In this paper, a highly effective isocarbophos-degrading strain, scl-2, was isolated from isocarbophos-polluted soil. The strain scl-2 was preliminarily identified as *Arthrobacter* sp. based on its morphological, physiological, and biochemical properties, as well as 16S rDNA analysis. The strain scl-2 could utilize isocarbophos as its sole source of carbon and phosphorus for growth. One hundred mg/l isocarbophos could be degraded to a nondetectable level in 18 h by scl-2 in cell culture, and isofenphos-methyl, profenofos, and phosmet could also be degraded. During the degradation of isocarbophos, the metabolites isopropyl salicylate, salicylate, and gentisate were detected and identified based on MS/MS analysis and their retention times in HPLC. Transformation of gentisate to pyruvate and fumarate *via* maleylpyruvate and fumarylpyruvate was detected by assaying for the activities of gentisate 1,2-dioxygenase (GDO) and maleylpyruvate isomerase. Therefore, we have identified the degradation pathway of isocarbophos in *Arthrobacter* sp. scl-2 for the first time. This study highlights an important potential use of the strain scl-2 for the cleanup of environmental contamination by isocarbophos and presents a mechanism of isocarbophos metabolism.

Keywords: Isocarbophos, biodegradation, *Arthrobacter* sp. scl-2, degradation pathway

Chemical pesticides are frequently applied in modern agricultural systems to ensure good harvests. However, the extensive use of chemical pesticides may easily lead to widespread environmental pollution. Isocarbophos (*O*-2-isopropoxycarbonylphenyl *O*-methyl phosphoramidothioate), a highly effective organophosphorus insecticide and acaricide, was originally synthesized in 1967 by the Bayer Company [21]. Isocarbophos is a potent acetylcholinesterase inhibitor and is widely used to control a variety of leaf-eating and soil insects such as aphids, spider mites, borers, and leaf rollers on rice, cotton, fruit, and other crops. Isocarbophos was introduced into China for agricultural use in 1981, and currently the annual production of pure isocarbophos in China is about 5,000 t [7]. The widespread application of isocarbophos has caused environmental pollution, and it was reported that in 2007 there was more than 9 mg/kg isocarbophos detected in Chinese celery. Isocarbophos is a moderately toxic compound, and the median lethal concentration (LC 50) of isocarbophos toward *Daphnia magna* was 13.9–353 mg/l after 48 h of static exposure [11]. Because of its toxicity and persistence, the remediation of isocarbophos pollutants has caused growing concern among environmental scientists.

Microorganisms play key roles in the detoxification of xenobiotics [15], and the use of microorganisms for bioremediation of isocarbophos-contaminated sites has received increasing attention as an efficient and cost-effective biotechnological approach. It was reported that isocarbophos could be degraded by immobilized mixed microorganisms, and the cyclic structure of isocarbophos was broken into simple inorganic compounds such as CO_2, H_2O, NH_3, H_2S, and H_3PO_4 [12]. The organphosphorus hydrolase OPHC2 was also reported to hydrolyze isocarbophos efficiently at 37°C in 50 mmol Tris–HCl buffer, pH. 8.0 [20]. However, studies on the biodegradation of isocarbophos have not been carried out extensively. To our knowledge, there have been no previous reports of microorganisms that are able to utilize isocarbophos as their sole carbon or phosphorus source for growth, and the detailed degradation pathway for isocarbophos remains unclear.

In the present study, the highly effective isocarbophos-degrading strain scl-2 was isolated. We found that this

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strain could use isocarbophos as its sole carbon and phosphorus source for growth. With the goal of elucidating a possible application in isocarbophos-contaminated environmental remediation, degradation of isocarbophos by scl-2 in liquid culture was studied, and the pathway by which scl-2 degrades isocarbophos was identified for the first time. This paper highlights an important potential use of pure culture microbial cells for the cleanup of organophosphorus-pesticide-contaminated environments, and also presents a mechanism for isocarbophos degradation.

Materials and Methods

Chemicals and Media

Isocarbophos (>99% purity) was purchased from The Pesticide Research Institute, Shanghai, China. Isopropyl salicylate (>99%) was purchased from Alfa Aesar Johnson Matthey company, and salicylic acid (>99.5% purity) was purchased from Wulian Chemical Plant, Shanghai, China. Gentisate (2,5-dihydroxybenzoic acid) (>98%), HPLC gradient grade petroleum ether, methanol, acetone, and ethyl acetate were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Antibiotics were purchased from Amresco. All other reagents used in this study were of analytical reagent grade.

Luria–Bertani (LB) medium, mineral salts medium (MSM) [10], and MPM (NaCl 1.0 g, $MgSO_4$ 0.02 g, NH_4SO_4 1.0 g, per liter) were used in this study. When necessary, isocarbophos dissolved in methanol was added to media at a final concentration of 100 mg/l.

Strain Isolation and Characterization

Isocarbophos-polluted soil samples were collected in Nantong, Jiangsu Province, China. The enrichment culture technique was used to isolate the isocarbophos-degrading strain [6]. Soil samples (5.0 g) were added to 100 ml of MSM with 100 mg/l isocarbophos and incubated at 30°C in a rotary shaker at 150 rpm. Dilutions of the sequential enrichment were plated onto MSM agar plates containing 100 mg/kg isocarbophos, and bacterial colonies grown on plates were tested for their isocarbophos-degrading capabilities. One strain, designated scl-2, which possessed the highest isocarbophos-degrading ability and could utilize isocarbophos as the sole carbon and phosphorus source, was purified and selected for further investigation.

The isolated strain was identified based on its morphological, physiological, and biochemical properties with reference to *Bergey's Manual of Determinative Bacteriology*, combined with 16S rDNA sequence analysis. Genomic DNA was extracted, and the 16S ribosomal RNA gene was amplified using the polymerase chain reaction (PCR) as described previously [10]. The nucleotide sequence coding for the 16S rRNA of strain scl-2 was sequenced by TaKaRa Biotechnology (Dalian) Co. Ltd. Alignment with different 16S rDNA sequences from GenBank was performed using Clustal X 1.8.3 [19] with default settings. Phylogenesis was analyzed with MEGA version 3.0 software, and distance was calculated using the Kimura 2-parameter distance model. Phylogenetic tree was built using the neighbor-joining method. Each dataset was bootstrapped 1,000 times.

Chemical Analysis

Isocarbophos and isopropyl salicylate in the cultures were extracted with an equal volume of dichloromethane [5]. The extracts were then dried over anhydrous Na_2SO_4 and evaporated under reduced pressure using a centrifugal evaporator at room temperature. Residual organic materials were redissolved in an equal volume of methanol [24]. The mobile phase was methanol:water (80:20, v:v), and the flow rate was 0.6 ml/min.

Salicylic acid and gentisate in the cultures were first acidified to pH 2.0 and then extracted with an equal volume of ethyl acetate. The extracts were then dried as described above and redissolved in an equal volume of the mobile phase. The mobile phase (adjusted to pH 2.0) was methanol:water (65:35, v:v), and the flow rate was 0.6 ml/min.

All samples were analyzed by high-performance liquid chromatography (Ettan LC; Amersham Bio-sciences, Uppsala, Sweden). The separation column used for HPLC (internal diameter, 4.6 mm; length, 25 cm) was filled with Kromasil 100-5C18. Isocarbophos, isopropyl salicylate, salicylic acid, and gentisate were detected by a UV-900 detector at 245 nm, 306 nm, 295 nm, and 320 nm respectively.

Degradation of Isocarbophos and Other Organophosphate Pesticides by scl-2 in Cell Culture

Cells of scl-2 were precultured in LB medium, harvested by centrifugation at 6,000 ×*g* for 5 min, and washed three times with sterilized water. For all experiments, cells were inoculated at a concentration of 10^8 cells/ml and samples were incubated at 30°C on a shaker at 150 rpm. MSM supplemented with 100 mg/l isocarbophos and other organophosphate pesticides such as methyl parathion, profenofos, isofenphos-methyl, phoxim, fenitrothion, or chlorpyrifos, was inoculated with scl-2 cells for degradation. The degradation efficiency was determined and estimated by the loss of substrate from the liquid culture. Medium inoculated with sterilized scl-2 cells was maintained according to the same conditions as the control. Remaining pesticides were measured by HPLC as described previously [4, 22].

Degradation of 100 mg/l isocarbophos in MPM versus scl-2 cell growth was carried out in order to determine whether scl-2 could utilize isocarbophos as its sole carbon and phosphorus source for growth. Cultures were regularly checked for bacterial growth and the degradation of isocarbophos. The cell density was monitored spectrophotometrically by measuring the absorbance at 600 nm using a Shimazdu UV–Vis recording spectrophotometer. All the treatments were replicated three times.

Identification of the Metabolites during Isocarbophos Degradation

For metabolite identification, scl-2 was inoculated into liquid MSM with 100 mg/l isocarbophos at 30°C. Four ml cultures were sampled from 1 h to 17 h, and 200 µl of 0.01 mol/l $FeCl_3$ was first added to verify whether there were metabolites containing phenol groups produced [13, 17]. $FeCl_3$ reacts with phenol groups, resulting in a colorimetric reaction that changes the medium to dark grey-blue. The concentration of the metabolites containing phenol groups was determined by spectrophotometer at 528 nm with $FeCl_3$ solution as the control. After 1–2 h of incubation, the culture medium was extracted with an equal volume of dichloromethane. The $FeCl_3$-culture sample with the highest absorbency at 528 nm was adjusted to pH 2.0 and extracted with an equal volume of ethyl acetate. Extracts from dichloromethane and ethyl acetate were analyzed respectively by Ettan LC with a mobile phase of methanol:water (80:20, v:v) and with a mobile phase (adjusted to pH 2.0) of methanol:water (65:35, v:v). The metabolites detected at 306 nm,

295 nm, and 320 nm were separated and identified by MS/MS, Finnigan TSQ Quantum Ultra AM (Thermal, U.S.A.). These metabolites were confirmed by the standard MS, ionized by electrospray with a positive or negative polarity, and scanned by normal mass range from 80 m/z to 300 m/z (mass-to-charge ratio). Characteristic fragment ions were detected in the second-order MS.

Enzymatic Gentisate Degradation Assay

Cell extracts of scl-2 were prepared by resuspending the bacterial pellets in ice-cold 100 mM phosphate-buffered saline (pH 7.5) and lysed by 3-s pulse sonication for 20 min. Cell debris was removed by centrifugation at 12,000 $\times g$ for 20 min at 4°C. Gentisate-degrading enzymes were assayed as described previously with modifications [8, 23]. Gentisate 1,2-dioxygenase (GDO) was assayed by measuring the increase in absorbance at 330 nm due to conversion of gentisate to maleylpyruvate. Maleylpyruvate isomerase was qualitatively monitored by measuring the change in absorbance at 330 nm due to maleylpyruvate disappearance in the presence of glutathione (GSH) [9].

Nucleotide Sequence Accession Number

The 16S rRNA gene sequence of *Arthrobacter* sp. scl-2 was deposited in the GenBank database under Accession No. EF626937.

RESULTS

Isolation and Characterization of the Isocarbophos-degrading Strain scl-2

Several bacterial strains were isolated from the isocarbophos-polluted soil samples by using isocarbophos as the sole carbon source. A strain designated as scl-2, which could degrade 100 mg/l isocarbophos in 18 h, was selected for further study. Results from biochemical tests showed that strain scl-2 is a Gram-positive, nonmotile, obligate aerobe with rod-shaped morphology during exponential growth and cocci morphology during the stationary phase. The strain scl-2 is oxidase and catalase positive, but is unable to hydrolyze starch, reduce nitrate, or grow in 10% NaCl. The phylogenetic tree of the 16S rDNA sequence is shown in Fig.1. The strain scl-2 was related to the *Arthrobacter* sp. lineage and closely clustered with two type strains, *A. nicotinovorans*^{T} and *A. histidinolovorans*^{T}, with sequence similarity scores of 99.8% and 99.2%, respectively. On the basis of morphological, physiological, and biochemical

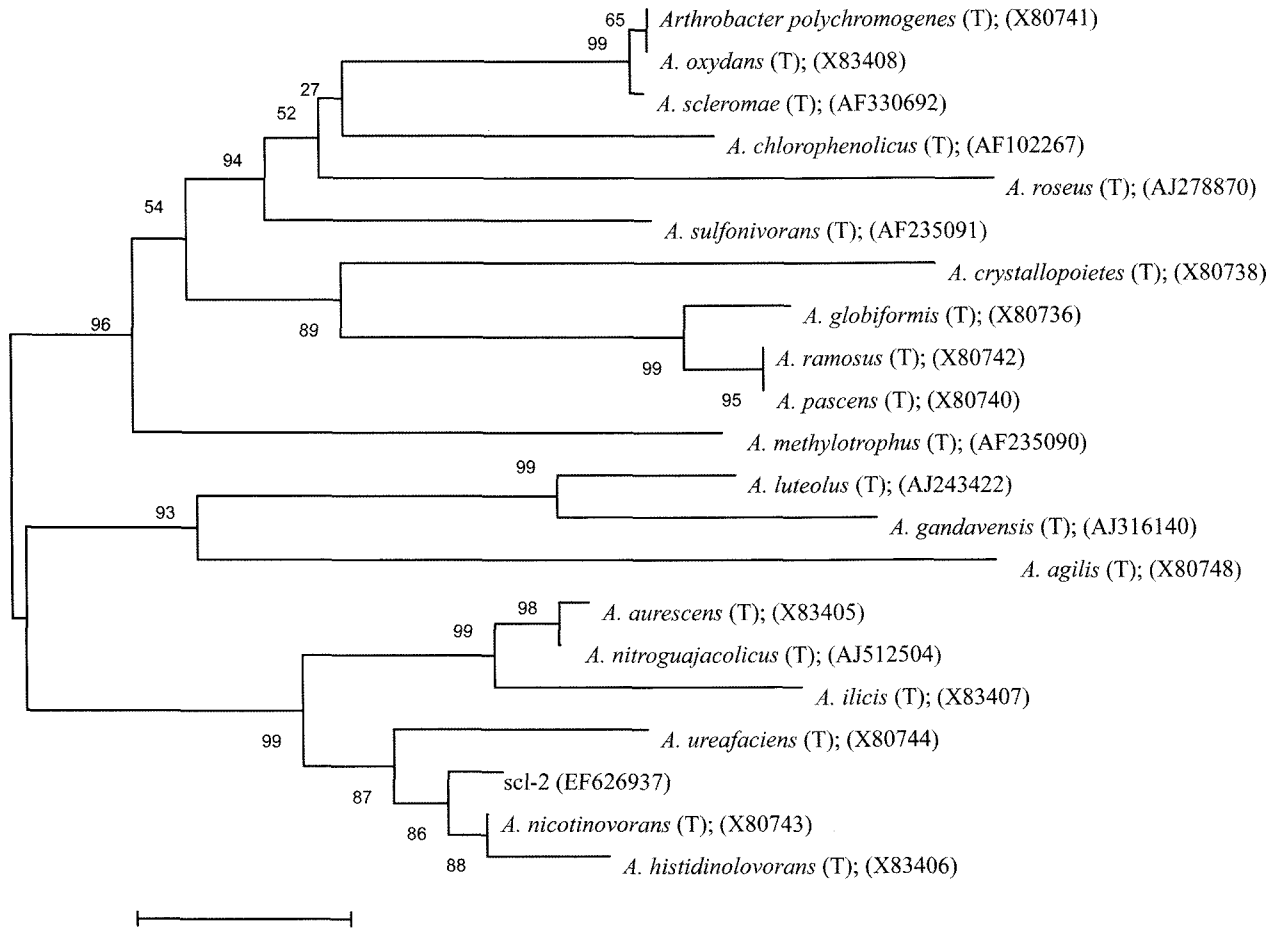

Fig.1. Phylogenetic analysis of strain scl-2 and related species by the neighbor-joining approach.
Bootstrap values obtained with 1,000 resamplings are indicated as percentages at all branches. The scale bars represent 0.005 substitutions per nucleotide position. The GenBank accession number for each microorganism is shown in parentheses after the species name.

properties, combined with 16S rDNA sequence analysis, the strain scl-2 was identified as *Arthrobacter* sp.

Degradation of Isocarbophos and Some Other Organophosphate Pesticides by scl-2

The strain scl-2 could utilize isocarbophos as its sole source of carbon and phosphorus for growth. The time course of isocarbophos degradation and scl-2 cell growth in MPM containing 100 mg/l isocarbophos is presented in Fig. 2. We observed that 100 mg/l isocarbophos was degraded to a nondetectable level after 18 h, and the cell density increased to 0.38 (OD_{600nm}). Studies on the degradation characteristics showed that the optimal temperature and initial pH were 35°C and 5.5–9, respectively. It was found that a high concentration of isocarbophos (>400 mg/l) was toxic to scl-2 cells and the degradation rate was greatly decreased. Alternative phosphorus, glucose, or organic nitrogen sources had nearly no effect on degradation of isocarbophos.

It was found that some other organophosphate pesticides such as profenofos, isofenphos-methyl, and phosmet could also be degraded by strain scl-2, whereas methyl parathion, phoxim, fenitrothion, and chlorpyrifos could not be degraded. The results showed that strain scl-2 could degrade a relatively wide range of substrates indicating significant potential for use in the bioremediation of complex organophosphate-pesticide-contaminated sites.

Identification of the Metabolites During Isocarbophos Metabolism

$FeCl_3$ was first used to verify whether there were any metabolites with phenol groups produced during isocarbophos degradation. During incubation, 4-ml culture samples were collected at periodic intervals. It was observed that, from hour 3 to hour 11, the color of the culture samples changed to purple black when 200 µl of $FeCl_3$ (original color is yellow) was added. The highest concentration of phenol-group-containing metabolites was detected at hour 5, and

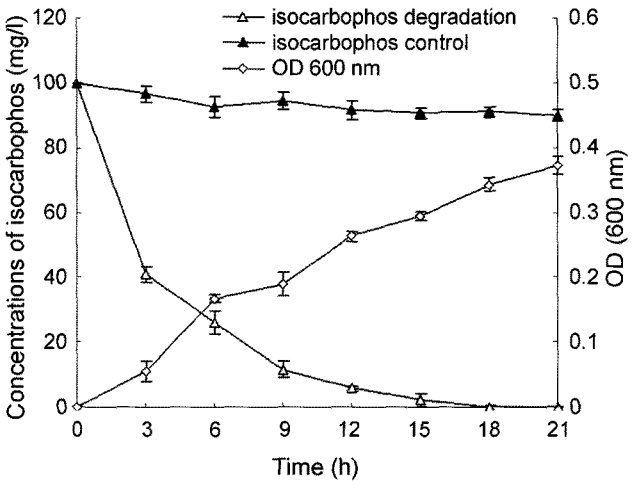

Fig. 2. Biodegradation of isocarbophos versus scl-2 cell growth in MPM supplemented with 100 mg/l isocarbophos at 30°C.
The data are represented as the means±standard deviation for triplicate incubations. When the error bar is not visible, it is within the data point.

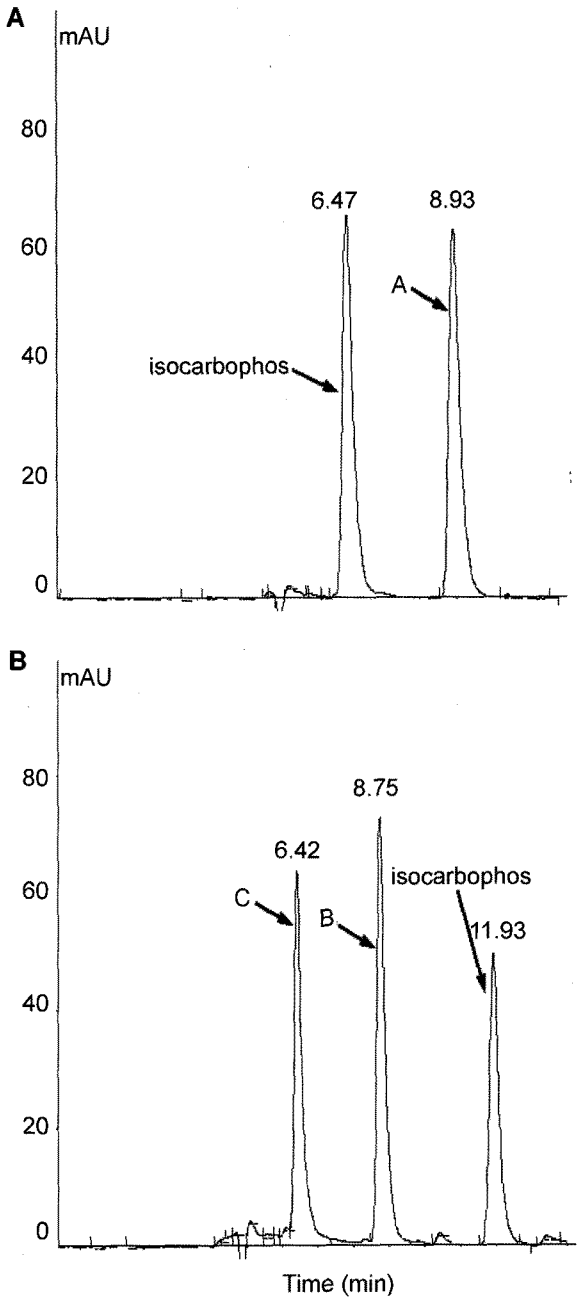

Fig. 3. Extracts obtained from dichloromethane (**A**) and ethyl acetate (**B**) were analyzed respectively by Ettan LC with a mobile phase of methanol:water (80:20, v:v) and with mobile phase (adjusted to pH 2.0) of methanol:water (65:35, v:v).
All these metabolites were detected by a UV-900 detector at 245 nm. Metabolites **A**, **B**, and **C** besides isocarbophos are marked with letters **A**, **B**, and **C** above their peaks.

the culture color returned to the original yellow after 11 h. These results indicate that the metabolites with phenol groups produced during degradation of isocarbophos were finally cleaved and transformed into other materials.

The metabolites of isocarbophos in the extracts of culture medium were isolated by Ettan LC (Fig. 3). Product *A* (R_t=8.93 min; λmax=306 nm) was detected in the culture medium extracted with dichloromethane, and product *B* (R_t=8.75 min; λmax=295 nm) and product *C* (R_t=6.42 min; λmax=320 nm) were detected in culture medium (adjusted to pH 2.0) extracted with ethyl acetate. Products *A*, *B*, and *C* were collected and identified by MS/MS.

In standard MS, prominent protonated molecular ions at *m/z* 137 $[M-H]^-$, *m/z* 153 $[M-H]^-$ and *m/z* 181 $[M+H]^+$ were found. The *m/z* of product *A* was 181 $[M+H]^+$, enabling the assignment of molecular ion (M^+) at *m/z*=180, the same as the standard material isopropyl salicylate. The characteristic fragment ion peaks of second order MS of product *A* at m/z=167, 139, 121, 93, 77, 69, 43, is shown in Fig. 4 (Product *A*). Product A had the same retention time (R_t= 8.93 min; λmax=306 nm) in HPLC as isopropyl salicylate. Therefore, product *A* was identified as isopropyl salicylate, which was derived as the result of cleaving the phosphodiester bond of isocarbophos.

The negative-ion chemical ionization of product *B* showed a prominent protonated molecular ion at *m/z*=137 $[M-H]^-$ and characteristic fragment ion peaks of second-order MS at *m/z*=65, 93 (Fig. 4, Product *B*). On the basis of these results and the retention time of product *B* (R_t=8.75 min; λmax=295 nm) in HPLC, product *B* was recognized as salicylate, which corresponds with the loss of isopropyl alcohol from isopropyl salicylate.

Product *C* showed a base peak at *m/z*=153 $(M-1)^-$, enabling the assignment of molecular ion (M^-) at *m/z*=154. The characteristic fragment ion peaks of second-order MS at *m/z*=109, 65 is shown in Fig. 4 (Product *C*). Product *C* was identified as gentisate according to the MS/MS and retention time (R_t=6.42 min; λmax=320 nm) in HPLC, which corresponds with the addition of OH^- to salicylate.

Isopropyl salicylate, salicylic acid, gentisate, and catechol were added to MSM for degradation by scl-2 cells, respectively. It was found that 100 mg/l isopropyl salicylate, salicylic acid, and gentisate could be degraded to a nondetectable level within 18 h. However, catechol could not be degraded by scl-2. These results further confirm that isopropyl salicylate, salicylic acid, and gentisate are metabolites produced during isocarbophos degradation, and indicate that in strain scl-2, isocarbophos was degraded *via* the gentisate pathway instead of the catechol pathway.

Enzymatic Gentisate Degradation Assay

When cell extracts were incubated with gentisate, the absorption spectrum changed rapidly from a peak with a λmax of 320 nm to 330 nm (Fig. 5), confirming that gentisate (λ_{max}=320 nm) was converted to maleylpyruvate (λ_{max}=330 nm) quickly [8]. Maleate could not be degraded by scl-2, indicating that maleylpyruvate was not degraded *via* the maleate pathway. It has been reported that maleylpyruvate will be converted to fumarylpyruvate by maleylpyruvate isomerase in the presence of glutathione, as observed by a change in the spectrum from a peak with a λ_{max} of 330 nm to 340 nm [24]. In our experiment, the spectrum changed from the peak with a λ_{max} of 330 nm to 308 nm when cell extracts were incubated with maleylpyruvate (Fig. 6). It

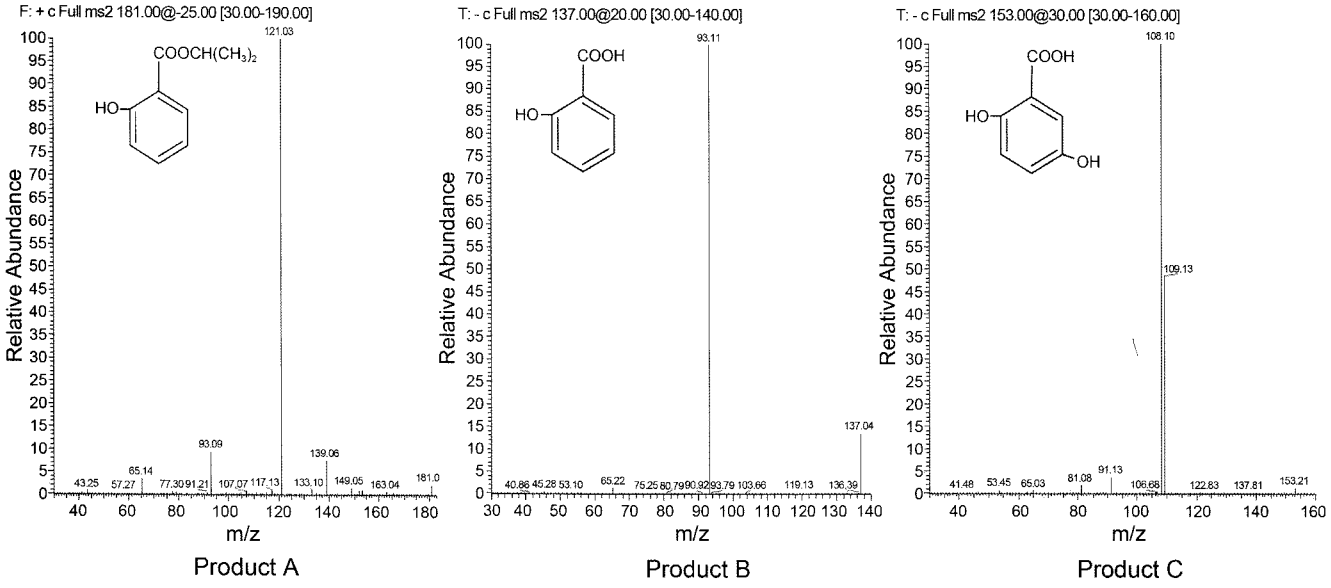

Fig. 4. The fragment ions of products **A**, **B**, and **C** in the second-order MS.
Products **A**, **B**, and **C** were identified as isopropyl salicylate, salicylic acid, and gentisate, respectively.

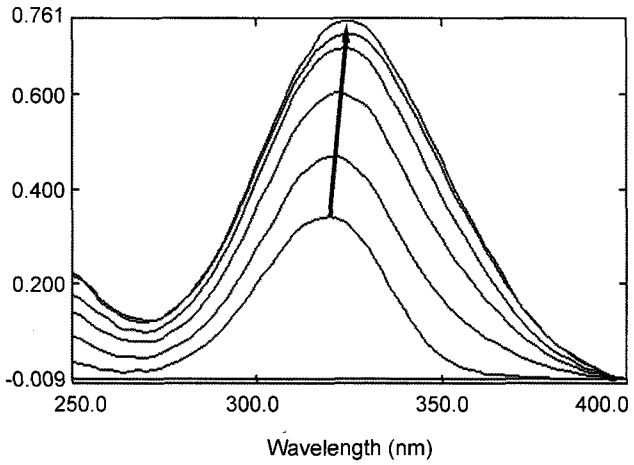

Fig. 5. Formation of maleylpyruvate from gentisate by cell extracts of scl-2 grown on LB medium.
Sample and reference cuvettes contained 100 mmol/l phosphate buffer (pH 7.4) and 600 μl of cell extracts containing 1.7 mg of protein in a 4-ml volume. Spectra were recorded before the addition of 0.32 μmol gentisate, and every 5 min thereafter.

was deduced that the isomerization reaction of maleylpyruvate to fumarylpyruvate was too fast to be seen, and the product with a λ_{max} of 308 nm was assumed to be the result of a spontaneous nonenzymatic reaction between the produced fumarylpyruvate and excess GSH, as was described by Lack [9].

Discussion

The extensive use of isocarbophos has caused serious environmental pollution, and cleanup of these pollutants

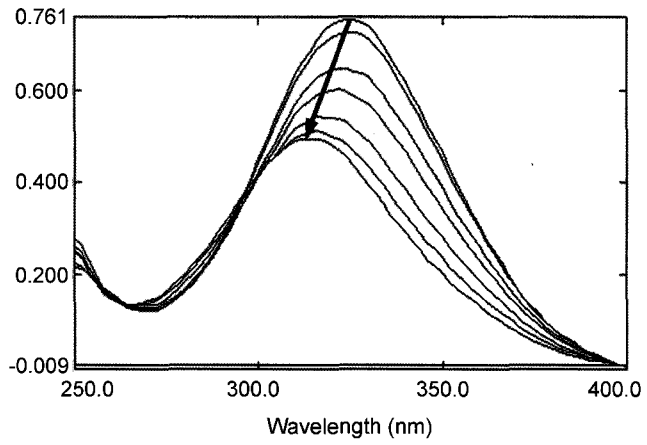

Fig. 6. Transformation of maleylpyruvate by cell extracts of scl-2.
Sample and reference cuvettes contained 100 mmol/l phosphate buffer (pH 7.4) and 600 μl of cell extracts containing 1.7 mg of protein in 4-ml volumes. The sample cuvette contained maleylpyruvate formed from gentisate by the action of cell extracts. Malylpyruvate had been previously transformed from 0.32 μmol gentisate by gentisate dioxygenase *in situ*. Spectra were recorded before the addition of glutathione and every 1 min thereafter.

by microorganisms is of great importance. In the present study, a highly effective isocarbophos-degrading strain, *Arthrobacter* sp. scl-2, which was capable of utilizing isocarbophos as its sole carbon and phosphorous source for growth, was isolated in our laboratory by the enrichment culture technique. We found that *Arthrobacter* sp. scl-2 could degrade a relatively wide range of substrates, and could also degrade some other organophosphorous pesticides such as profenofos, isofenphos-methyl, and phosmet. The phylogenetic *Arthrobacter* are metabolically versatile bacteria, and many *Arthrobacter* strains have been reported to degrade pollutants such as fluorene, 4-fluorophenol, atrazine, and so on [1, 2, 14]. However, this is the first report of an *Arthrobacter* strain that is capable of efficiently degrading isocarbophos. Our results also indicate that the genus of *Arthrobacter* has great ecological diversity and possesses significant potential for bioremediation of organophosphorous-pesticide-contaminated sites.

Even though several studies have focused on the biodegradation of isocarbophos, the degradation pathway of isocarbophos has not been studied extensively. In this paper, three metabolites that arise during isocarbophos degradation were identified and isolated by HPLC. These metabolites were identified as isopropyl salicylate, salicylate, and gentisate, respectively, based on the MS/MS analysis and their retention times in HPLC. Because isopropyl salicylate, salicylate, and gentisate could also be degraded by scl-2, a degradation pathway of isocarbophos in *Arthrobacter* sp. scl-2 is presented here (Fig. 7). The first step involved in the degradation pathway of isocarbophos was cleavage of the phosphodiester bond, producing the metabolite isopropyl salicylate. Then, isopropyl alcohol was lost from isopropyl salicylate to yield salicylate. It was reported that salicylate could be transformed *via* the catechol or gentisate pathway to produce catechol and gentisate, respectively [16, 24]. It was found that catechol could not be degraded by scl-2, whereas gentisate could be converted to fumarylpyruvate, indicating that scl-2 possesses salicylate 5-hydroxylase activities. Similarly to naphthalene degradation, isocarbophos was degraded *via* the salicylate and gentisate pathways [3]. The gentisate pathway was first reported by Tanaka *et al.* [18] and Lack [8], and numerous organic compounds have been reported to be transformed through this way. We determined that transformation of gentisate to maleylpyruvate was carried out by gentisate 1,2-dioxygenase (GDO) in strain scl-2. This was verified by a change of the absorption spectrum from a peak with a λ_{max} of 320 nm to 330 nm (Fig. 5). Maleylpyruvate was not degraded *via* the maleate pathway, and was found to be transformed to fumarylpyruvate, catalyzed by GSH-dependent maleylpyruvate isomerase. It was reported that fumarylpyruvate was hydrolyzed to pyruvate and fumarate by fumarylpyruvate hydrolase [23]. In strain scl-2, there were no metabolites detected during gentisate metabolism, so fumarylpyruvate was

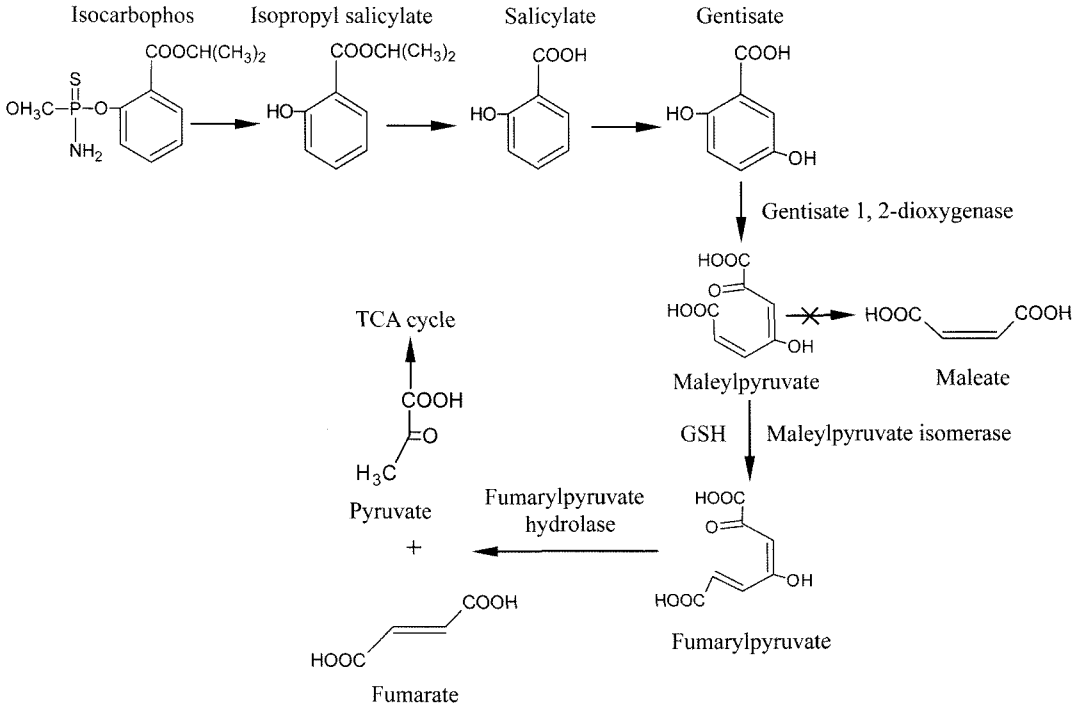

Fig. 7. Presumed degradation pathway of isocarbophos in *Arthrobacter* sp. scl-2.

presumed to be hydrolyzed to pyruvate and fumarate, and finally metabolized to CO_2 and H_2O *via* the TCA cycle. The work described here represents the first identification of the isocarbophos degradation pathway.

Acknowledgments

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