J. Pestic. Sci., 33(3), 266–270 (2008)
DOI: 10.1584/jpestics.G08-08

Note

Simultaneous biodegradation of chloro- and methylthio-*s*-triazines using charcoal enriched with a newly developed bacterial consortium

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(Received February 18, 2008; Accepted April 10, 2008)

A special type of charcoal, Charcoal A100, was enriched with a newly developed bacterial consortium using a perfusion method. The bacterial consortium consisted of a methylthio-*s*-triazine-degrading bacterium (*Rhodococcus* sp. FJ1117YT) and the chloro-*s*-triazine-degrading bacterial consortium CD7 (containing *Bradyrhizobium japonicam* CSB1, *Arthrobacter* sp. CD7w and *β-Proteobacteria* CDB21). Enriched charcoal was capable of degrading chloro-*s*-triazines (simazine and atrazine) and methylthio-*s*-triazines (simetryn and dimethametryn) simultaneously in sulfur-free medium. Almost complete degradation was observed after 4-day cultivation of chloro-*s*-triazines and 9-day cultivation of methylthio-*s*-triazines. These triazines were mineralized via their 2-hydroxy analogues.

Keywords: biodegradation, methylthio-*s*-triazines, chloro-*s*-triazines, bacterial consortium, Charcoal A100.

Introduction

The *s*-triazines are recognized as a major class of herbicides and are widely used in agriculture for controlling various weeds, and they have been detected in ground and surface water.[1–3] Among the removal technologies for such residual pesticides, bioremediation is considered to be the most cost-effective and safe technology; therefore, many studies concerning *s*-triazine-degrading bacteria have been reported.

In our study, bacterial consortium CD7[4] was obtained which can mineralize simazine, and a degrading bacterium *β-Proteobacteria* CDB21 was isolated from CD7 by Iwasaki *et al.*[5] It was considered that CD7 was more effective for bioremediation than strain CDB21 because strain CDB21 could not utilize simazine for its growth in mineral salt medium without cyanocobalamin, but CD7 could utilize simazine as sole carbon and nitrogen sources in mineral salt medium.[4,5] In addition, a special type of charcoal, Charcoal A100, enriched with CD7 was developed, and was placed under the subsoil of a golf course to degrade simazine. The simazine-degrading capability of the enriched charcoal was maintained for nearly 2 years.[4] Thus, Charcoal A100 enriched with CD7 is considered to be an effective material for bioremediation; however, CD7 and strain CDB21 could not degrade methylthio-*s*-triazines detected in river water,[3,6] a lake basin,[7] and river sediments,[8] while *Rhodococcus* sp. FJ1117YT[9] can transform methylthio-*s*-triazines to their hydroxyl analogues via sulfur oxidation, and accumulate hydroxy-*s*-triazines. It is considered that simultaneous degradation of chloro- and methylthio-*s*-triazines using a mixture of these bacteria is meaningful because river water[3,10] and river sediment[3] contamination with both triazines has been reported.

To take advantage of these useful properties of CD7 and Charcoal A100, this study describes the development of Charcoal A100 enriched with strain FJ1117YT and CD7, and demonstration of the simultaneous degradation of chloro- and methylthio-*s*-triazines using this novel material.

Materials and Methods

1. *Materials*

Simazine, atrazine, simetryn, and dimethametryn were purchased from Kanto Kagaku. Cyanuric acid and simazine-2-hydroxy were purchased from Dr. Ehrenstorfer GmbH. The sulfur-free mineral salt medium (MM) used in this study contained $Na_2HPO_4 \cdot 12H_2O$ 1.2 g/l and KH_2PO_4 0.5 g/l. Prior to autoclaving, the medium was supplemented with 10 ml/l of a sulfur-free solution of trace elements, which contained $MgCl_2 \cdot 6H_2O$ 2000 mg/l, $FeCl_2 \cdot H_2O$ 200 mg/l, $ZnCl_2$ 10 mg/l, $MnCl_2$ 5 mg/l, $CoCl_2$ 24 mg/l, $CuCl_2$ 5 mg/l, $NiCl_2 \cdot 6H_2O$ 5 mg/l, Na_2MoO_4 5 mg/l, H_3BO_4 30 mg/l, $Ca(OH)_2$ 50 mg/l, and EDTA 500 mg/l. Mineral salt medium containing sulfate (MM+S) was also used. MM+S contained $MgSO_4$ 20 mg/l and a solution of trace elements described by Yanze-Kontchou and Gschwind.[11] MM and MM+S were supplemented with *s*-triazines before autoclaving. Vitamin mixture was used as described by Fujii *et al.*[9]

2. *Analytical methods*

The concentration of *s*-triazines in the medium was determined using HPLC (Tosoh) using the following method. Separation of *s*-triazines was achieved at 40°C on an ODS column (Capcell Pak C18 UG120 3×250 mm, 5 μm particle size; Shiseido) with a mo-

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Published online July 14, 2008

bile phase containing a gradient mixture of acetonitrile and water (20%/80% 0 min–20%/80% 5 min, linear gradient–40%/60% 11 min, and linear gradient–20%/80% 15 min–20%/80% 20 min). The injection volume was 50 μl and the eluted compounds were detected by UV at 220 nm. Metabolites of s-triazines were determined using LC-ESI-MS. Separation was achieved using LC Alliance 2695 (Waters) system equipped with the same ODS column as above (40°C). A mixture of acetonitrile/0.2% acetic acid was used as the eluent (5%/95% 0 min, linear gradient–50%/50% 10 min–80%/20%10.1 min–80%/20% 15 min, and linear gradient–5%/95% 20 min). The injection volume was 10 μl. The ESI-MS system was Quattro micro API (Waters) with the flow rate of nebulization set at 100 l/h and the flow rate for desolvation gas (N_2) set at 500 l/h. The respective temperatures of source and desolvation were set at 100 and 350°C; and capillary voltage was 3.5 kV. Detection was performed by scanning between *m/z* 100 and *m/z* 300 under cone voltage of 35 V in positive ion mode. Cyanuric acid was detected at *m/z* 128 $[M-H]^-$ under cone voltage of 23 V in negative ion mode, and simazine-2-hydroxy was *m/z* 184 [M+H]+ under cone voltage of 35 V in positive ion mode.

3. *Enrichment of Charcoal A100 with strain FJ1117YT and CD7*

Charcoal A100 (Toyo Denka Kogyo) was used as the enrichment material. Enrichment of charcoal with a bacterial consortium was performed using a charcoal perfusion method, which was modified from the soil perfusion method described by Audus.[12] First, washed Charcoal A100 (7.5 g, dry weight) was packed in perfusion apparatus equipped with glass sintered filter, and then autoclaved. Subsequently, three pieces of stab cultures of CD7 on a MM agar plate containing 40 mg/l simazine were placed on the charcoal, and the charcoal layer was covered with glass fiber filter paper. Enrichment was performed in the dark at 25°C. MM (300 ml) containing 5 mg/l simazine was perfused with air lift using an air pump for 14 days. The concentration of simazine in the perfusion fluid was determined by HPLC, and the perfusion fluid was replaced twice. After 14 days, the glass fiber filter paper was replaced. A phosphate buffer suspension of strain FJ1117YT was placed on the filter paper. MM (300 ml) containing 5 mg/l simazine, 5 mg/l simetryn was perfused under similar conditions for 21 days, and the perfusion fluid was replaced once during the perfusion process. Lastly, MM (300 ml) containing 5 mg/l simetryn was used as perfusion fluid for 38 days, and was replaced once. The vitamin mixture was added twice. Total enrichment time was 73 days, and this material was used thereafter. Non-enriched Charcoal A100 was also perfused as a control under the same conditions.

4. *Preparation of DNA and analysis of 16S rRNA gene*

Adherent bacteria on the surface of Charcoal A100 enriched with strain FJ1117YT and CD7 were removed by sonication in phosphate buffer for 2 min. Charcoal A100 inhabited with strain FJ1117YT and CD7 was pulverized, and total DNA was extracted with Fast DNA Kit for soil (Q-Bio gene). Total DNA of strain FJ1117YT and each member of CD7 were extracted from suspensions of bacterial colonies grown on R2A agar (Difco) plates by a protocol for gram-positive bacteria of DNeasy Blood & Tissue Kit (QIAGEN). Nucleotide sequences of 16S rRNA genes of isolated bacteria were analyzed by direct sequencing of the PCR products described previously.[5]

5. *Denaturing gradient gel electrophoresis (DGGE) analysis*

Touchdown PCR was performed with a TP600 thermal cycler (Takara BIO) using GoTaq Green Master Mix (Promega). GM5F-gc-clamp and R534,[13] primers designed from a sequence of the variable V3 region of 16S rRNA, were used for PCR. DGGE analysis was performed using 6% polyacrylamide gel (ratio of acrylamide to bisacrylamide 37.5 : 1) in 1×TAE (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA) with a 30% to 60% denaturant gradient (100% denaturant containing 7 M urea and 40% formamide). Electrophoresis was performed at a constant voltage of 150 V and at a temperature of 60°C for 3.5 h by using the Dcode™ universal mutation detection system (Bio-Rad). After electrophoresis was completed, the gel was stained with SYBR gold (Invitrogen) for 20 min, rinsed, and the bands were visualized with a Molecular Imager Pharos FX plus system (Bio-Rad).

6. *Simultaneous degradation of chloro- and methylthio-s-triazines using Charcoal A100 enriched with strain FJ1117YT and CD7*

Charcoal A100 enriched with strain FJ1117YT and CD7 (0.4 g dry weight) was inoculated in MM (30 ml) containing 5 mg/l each of simazine, atrazine, simetryn, and dimethametryn or in MM+S containing 5 mg/l of each herbicide in 50 ml flasks. The flasks were shaken at 120 rpm at 25°C for 15 days. As controls, sterile Charcoal A100 and/or Charcoal A100 enriched with only CD7 were inoculated and shaken under the same conditions. The concentration of *s*-triazines in the medium was determined periodically by HPLC. Cyanuric acid, simazine-2-hydroxy, and hydroxy analogues of atrazine and dimethametryn in the medium after 15 days were measured by LC-ESI-MS.

Results and Discussion

1. *Enrichment of Charcoal A100 with a bacterial consortium*

The extent of enrichment of Charcoal A100 with strain FJ1117YT and CD7 was determined from the change in the concentration of simazine and simetryn in the perfusion fluid. In the first step of enrichment, CD7 was enriched in Charcoal A100. Concentration of simazine in the CD7-inoculated charcoal decreased faster than the control, and the degradation rate of simazine increased with every replacement of the perfusion fluid (data not shown). The charcoal was subsequently further enriched with strain FJ1117YT and perfused with MM containing simazine and simetryn. Simazine was degraded almost immediately (Fig. 1-A until Day21), in contrast to the slow degradation of simetryn (Fig. 1-B until Day21). The disappearance rate of

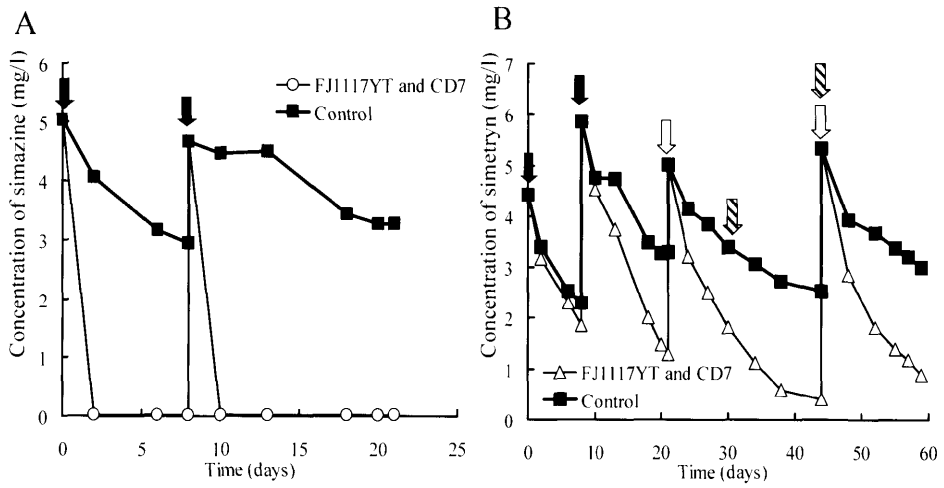

Fig. 1. Enrichment of strain FJ1117YT and CD7 in Charcoal A100 using a charcoal perfusion method. Changes in the concentrations of simazine (A) and simetryn (B) during enrichment are illustrated (both figures overlapped until 21 days). Charcoal A100 without enrichment was used as a control. In order to enrich CD7, MM containing 5 mg/l simazine was perfused for 14 days before these data. The arrows indicate as follows: replacement with MM containing simazine and simetryn (black), replacement with MM containing simetryn (white), and addition of vitamin mixture (striped).

simetryn was initialing almost the same as that of control, but increased after the second perfusion (Fig. 1-B on Day8). The decrease of pesticides in sterile Charcoal A100 was considered to occur because pesticides were adsorbed on Charcoal A100. In order to aid the growth of strain FJ1117YT,[9] vitamin mixture was added but had little effect on the enrichment of FJ1117YT. However, because the enrichment of CD7 was sufficient, MM containing simetryn without simazine was further perfused (Fig. 1-B after Day21), and the degradation rate of simetryn was further increased. This result indicates the enrichment of strain FJ1117YT in Charcoal A100.

2. *Detection of bacterial community in Charcoal A100 enriched with strain FJ1117YT and CD7*

Colony isolation from the bacterial consortium CD7 and subsequent analyses of 16S rRNA genes revealed that CD7 consisted of *Bradyrhizobium japonicam* CSB1, *Arthrobacter* sp. CD7w and strain CDB21. The presence of all members of CD7 and strain FJ1117YT enriched in Charcoal A100 were confirmed by PCR-DGGE analyses (Fig. 2).

3. *Simultaneous degradation of chloro- and methylthio-s-triazines, and detection of their metabolites*

Charcoal A100 enriched with strain FJ1117YT and CD7 was applied to simultaneous degradation of chloro- and methylthio-*s*-triazines. Simazine and atrazine were degraded to by 80–100% with Charcoal A100 enriched with both strain FJ1117YT and CD7 or CD7 alone after 9 days, and were completely degraded after 15 days (Fig. 3-A, B). On the other hand, simetryn and dimethametryn were degraded by over 80% with strain FJ1117YT and CD7 enriched charcoal in sulfur-free medium, but they were not degraded in the presence of sulfate (in MM+S) (Fig 3-C, D). In the sulfur-free medium with strain FJ1117YT and CD7 after 15 days, the concentrations of simazine-2-hydroxy and cyanuric acid were less than 0.1 mg/l and hydroxy analogues of atrazine and dimethametryn were not detected (data not shown). This result suggests that hydroxyl analogues of methylthio-*s*-triazines, which can not be metabolized by strain FJ1117YT, were mineralized by CD7 via *N*-ammelide analogue and cyanuric acid. These results also indicate that methylthio-*s*-triazines could be mineralized by the bacterial consortium that included strain FJ1117YT and CD7.

In conclusion, the respective degradation abilities of CD7 and strain FJ1117YT were successfully maintained in Charcoal

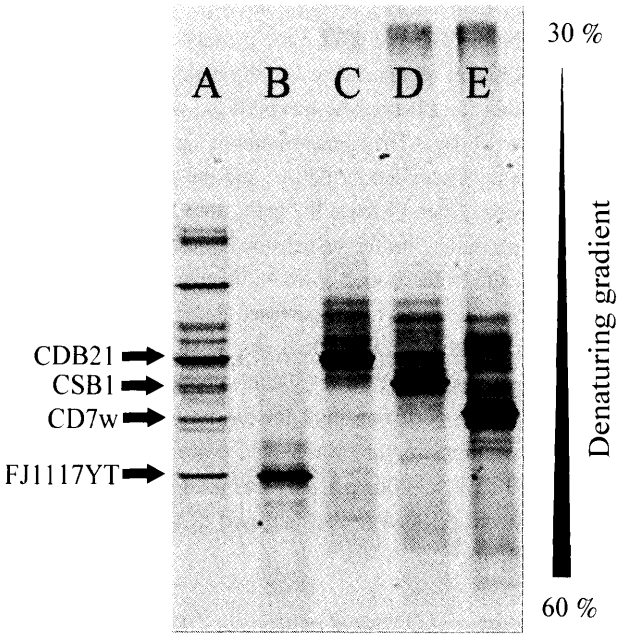

Fig. 2. PCR-DGGE band profiles of bacterial strains inhabiting Charcoal A100. Each lane represents DNA samples extracted from the following specimens: Charcoal A-100 enriched with FJ1117YT and CD7 (A); FJ1117YT (B); CDB21 (C); CSB1 (D); and CD7w (E).

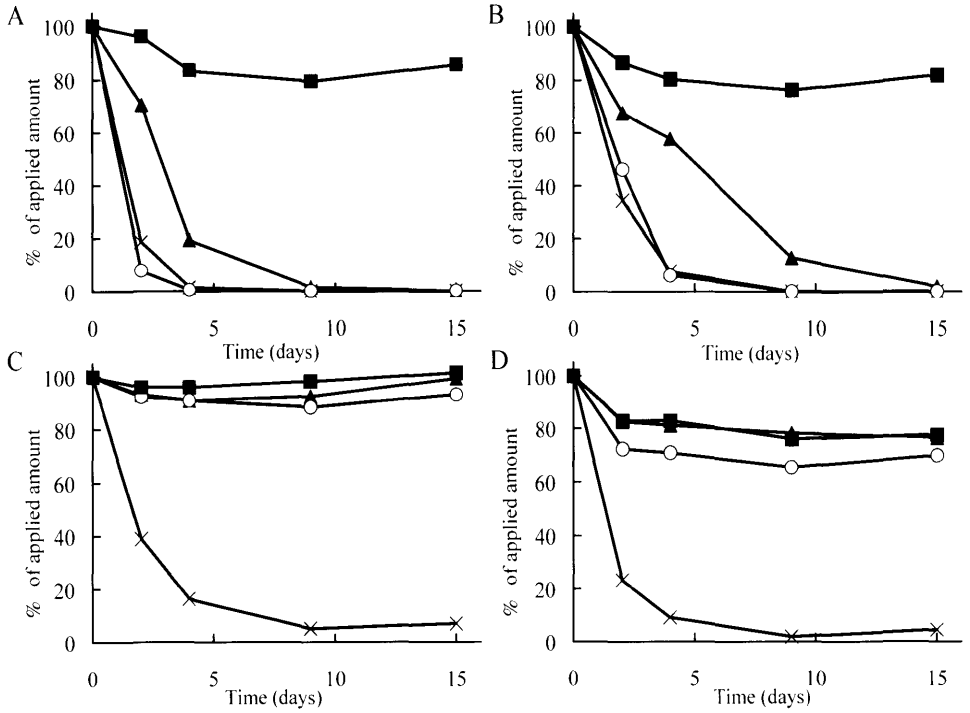

Fig. 3. Time course of simultaneous degradation of chloro- and methylthio-*s*-triazines with Charcoal A100 enriched with strain FJ1117YT and CD7. Degradation of simazine (A), atrazine (B), simetryn (C), and dimethametryn (D) by strain FJ1117YT and CD7 with (▲) or without (×) sulfate, with CD7 alone (○), and using non-enriched Charcoal A100 as a control (■) are shown.

A100. Chloro- and methylthio-*s*-triazines were degraded simultaneously and their metabolites were hardly detected. The expected metabolic pathways of chloro- and methylthio-*s*-triazines in the mixed culture are shown in Fig. 4. Degradation of methylthio-*s*-triazines by the bacterial consortium, strain FJ1117YT and CD7, was suppressed by the presence of sulfate (Fig. 3) as well as the culture of strain FJ1117YT reported previously.[9] However, Charcoal A100 enriched with strain FJ1117YT and CD7 could be a promising model to construct a multifunctional material enriched with bacterial consortium for in situ bioremediation. On the basis of this study, we will attempt to construct another charcoal material, which will include methylthio-*s*-triazines-degrading bacteria that are not suppressed by external sulfur sources.

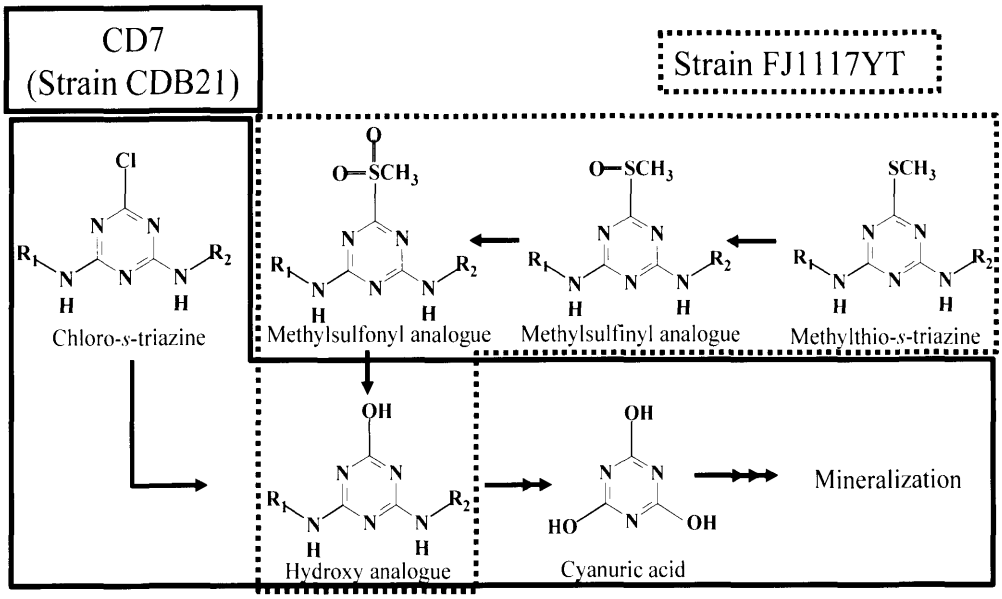

Fig. 4. The expected metabolic pathways of chloro- and methylthio-*s*-triazines degraded by CD7 (strain CDB21) and strain FJ1117YT. Simazine (R_1, R_2=C_2H_5) and atrazine [R_1=C_2H_5, R_2=$CH(CH_3)_2$] were selected as chloro-*s*-triazines, and simetryn (R_1, R_2=C_2H_5) and dimethametryn [R_1=C_2H_5, R_2=$CH(CH_3)CH(CH_3)_2$] were used as methylthio-*s*-triazines in this study.

Acknowledgments

This work was supported in part by a Grant-in-aid (Hazardous Chemicals) from the Ministry of Agriculture, Forestry, and Fisheries of Japan (HC-05-2441-1).

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