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Complete biodegradation of atrazine by a microbial community isolated from a naturally derived river ecosystem (microcosm)

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ABSTRACT

A microbial community, designated as AN4, capable of mineralizing the herbicide atrazine was isolated from a model river ecosystem (microcosm). The profile of degradation of atrazine by the AN4 community seemed to well reflect what occurred in the microcosm: rapid degradation of atrazine and transient accumulation of cyanuric acid, followed by relatively slow mineralization. The community comprised multiple phylogenetically distinct microbial strains, and the microbes were suspended and probably aggregated in the water phase of the microcosm. Denaturing gradient gel electrophoresis (DGGE) revealed that multiple bacterial strains exist in the AN4 community, and we successfully isolated two strains, which belonged to the genera *Nocardioides* and *Pedomicrobium*. *Nocardioides* sp. strain AN4-4 degraded atrazine to cyanuric acid and harbored the *trzN* and *atzC* genes encoding the *s*-triazine-degrading enzymes. This strain also degraded other chloro-substituted *s*-triazines like simazine and propazine, but it showed little degradability for simetryn (a methylthio-substituted *s*-triazine). Additionally, strain AN4-4 could grow on basal salt agar containing ethylamine or isopropylamine as the only carbon and nitrogen sources. Another strain, *Pedomicrobium* sp. strain AN4-9 could mineralize cyanuric acid alone. Therefore, we found that the coexistence of these two community members functionally serves to completely biodegrade atrazine.

1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) has been globally used as a herbicide from the 1950s to date to control a variety of upland weeds (Koskinen and Clay, 1997), primarily those growing during corn production. However, its herbicidal effect is often lost by repeated use over a span of several years (Zablotowicz et al., 2002). This indicates that atrazine-degrading microorganisms may accumulate in the soil. The atrazine-mineralizing *Pseudomonas* sp. strain ADP, which was isolated from a herbicide spill site, has been thoroughly investigated (de Souza et al., 1996; Boundy-Mills et al., 1997; Sadowsky et al., 1998; Martinez et al., 2001). On the basis of these investigations, researchers have elucidated the metabolic pathways, enzymes, and the corresponding genes involved in *s*-triazine herbicide degradation (Wackett et al., 2002; Shapir et al., 2007). The pathways for atrazine biodegradation by the ADP strain are almost identical and are globally distributed (Struthers et al., 1998; Topp et al., 2000b; Rousseaux et al., 2001; Strong et al., 2002; Piutti et al., 2003; Devers et al., 2007). The metabolic reactions involved are dechlorination by the AtzA or TrzN enzymes, and displacement of *N*-alkylamino groups by AtzB and AtzC, via the sequential metabolites hydroxyatrazine, *N*-isopropylammelide, and cyanuric acid. In the ADP strain, AtzDEF mineralizes the cyanuric acid. Some gram-negative bacteria have been reported to utilize cyanuric acid by the same enzymes (Devers et al., 2007; Iwasaki et al., 2007) or by TrzD (Karns, 1999; Rousseaux et al., 2001).

To date, atrazine-degrading microbes have been isolated mainly from soils enriched with such microbes. Compared to the soil environment, there has been limited information on atrazine-degrading microbes present in natural freshwater systems (López et al., 2005; Vargha et al., 2005). However, considering the transport of the herbicide by drainage or runoff, more detailed investigation is necessary to provide biological insight into the degradation of the herbicide and potential bioremediation applications. A previous study (Satsuma, 2006) reported that the atrazine in the water phase (1 mg L^{-1}) of the river microcosm degraded very rapidly and resulted in transient accumulation of cyanuric acid, followed by gradual mineralization. This metabolic feature strongly suggests that the atrazine added to the microcosm was mineralized by multiple microbial species or strains.

It is generally accepted that xenobiotic chemicals in natural environments are degraded by multiple microbial species (Alexander, 1981; Lappin et al., 1985; Mohapatra and Awasthi, 1997; Yanze-Kontchou and Gschwind, 1999). However, there are a few experimental proofs of isolated strains (Villarreal et al., 1991; de Souza et al., 1998a; Carvalho et al., 2002; Sørensen et al., 2002;

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Dejonghe et al., 2003). Previously, we screened four atrazine-mineralizing bacterial colonies from the river microcosm. Microscopic observation and denaturing gradient gel electrophoresis (DGGE) revealed that the atrazine-mineralizing bacterial colonies consist of multiple microbial strains. This paper elucidates an atrazine-mineralizing community, designated as AN4. We studied the community-level biodegradation of atrazine, and we report the phylogenetic classification and metabolic features of the isolated community members. Furthermore, to determine the potential degradation ability of the isolated strain AN4-4, we investigated the metabolic features of some *s*-triazines. To the best of our knowledge, this is the first characterized atrazine-degrading bacterial community isolated from a natural river environment in the absence of high concentrations of the herbicide or by repeated enrichment procedures.

2. Materials and methods

2.1. Chemicals

Atrazine, uniformly labeled with ^{14}C at the *s*-triazine ring ([^{14}C]atrazine), was synthesized and identified by the Institute of Isotopes Co. Ltd, Budapest, Hungary. Its specific radioactivity was 6.4 MBq mg^{-1}, and its radiochemical purity was greater than 98%. The following radioactive *s*-triazines were also uniformly labeled with ^{14}C at the *s*-triazine ring: [^{14}C]cyanuric acid (10.9 MBq mg^{-1}) purchased from Moravek Biochemicals Inc., Brea, CA, USA; [^{14}C]simazine (1.9 MBq mg^{-1}) purchased from Sigma Chemical Co., St. Louis, MO, USA; [^{14}C]simetryn (9.0 MBq mg^{-1}), which was synthesized and identified by Sigma Chemical, and supplied by Nippon Kayaku Co. Ltd., Tokyo, Japan; and [^{14}C]propazine (8.1 MBq mg^{-1}) and [^{14}C]ethylamine hydrochloride (25.4 MBq mg^{-1}) purchased from American Radiolabeled Chemicals Inc., St. Louis, MO, USA. Unlabeled atrazine and hydroxyatrazine were purchased from Kanto Kagaku Co. Inc., Tokyo, Japan. Hydroxysimazine and hydroxypropazine were purchased from Dr. Ehrenstorfer GmbH, Augsburg, Germany. Unlabeled cyanuric acid, ethylamine hydrochloride, and isopropylamine hydrochloride were obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan.

2.2. Media

The media used in this study are listed in Table 1. Nutrient agar or broth (Difco Laboratories, MI, USA) and R2A agar or broth (Wako) were prepared by dissolving an appropriate amount of the dehydrated media in Milli-Q water, followed by autoclaving at 121 °C. A sediment-extract (SE) medium was prepared as follows: the river sediment used for the microcosm was suspended in an approximately 10-fold volume of Milli-Q water, autoclaved for 20 min at 121 °C, further diluted with 10-fold Milli-Q water, and filtered aseptically. The basic salt (BS) medium comprised 3.0 mg $Na_2HPO_4{\cdot}12H_2O$, 1.2 mg KH_2PO_4, 2.5 mg $MgSO_4{\cdot}7H_2O$, 1.5 mg $CaCl_2{\cdot}2H_2O$, 62 μg H_3BO_3, 242 μg $Na_2MoO_4{\cdot}2H_2O$, 198 μg $MnCl_2{\cdot}6H_2O$, 54 μg $FeCl_3{\cdot}6H_2O$, 1.3 μg $CuSO_4{\cdot}5H_2O$, 1.4 μg $ZnSO_4{\cdot}7H_2O$, and 1.4 μg $CoSO_4{\cdot}7H_2O$ per liter of Milli-Q water. After autoclaving, the chemicals or test substances dissolved in Milli-Q water were added to each medium by filter sterilization.

Table 1
Media.

No.	Medium		Dissolved chemicals or test substances	
	Name	Dilution	Name	Concentration (mg L^{-1})
1	Nutrient agar	×100	Atrazine	1
2	Nutrient broth	×100	[^{14}C]atrazine	1
3	SE agar		Atrazine	2
4	SE broth		[^{14}C]atrazine	1
5	SE broth		Atrazine	1
6	SE broth		[^{14}C]atrazine	2
7	SE broth		[^{14}C]cyanuric acid	50
8	BS agar		Ethylamine	100
9	BS agar		Isopropylamine	100
10	R2A agar	×10	Atrazine	2
11	R2A broth	×10	Atrazine	2
12	R2A broth	×100	[^{14}C]atrazine	2
13	R2A broth	×100	[^{14}C]simazine	1.3
14	R2A broth	×100	[^{14}C]simetryn	2
15	R2A broth	×100	[^{14}C]propazine	1.3
16	R2A broth	×100	[^{14}C]cyanuric acid	2
17	R2A broth	×100	[^{14}C]ethylamine	0.7

2.3. Microcosm setup

The riverbed sediment and river water were collected in November from the Tonegawa River (35°58′N, 139°55′E) flowing through the southern part of Ibaraki Prefecture in Japan. The sampling location was surrounded by agricultural fields. The riverbed sediment was collected up to a depth of 10 cm. During collection, the sediment and river water were filtered through 2-mm and 0.2-mm sieves, respectively. The chemical properties of the river water and sediment have been described previously (Satsuma, 2006). In a 1-L culture flask (Culstir™), 800 mL of river water was gently added (up to a depth of approximately 10 cm) to the sediment; the amount of sediment was equivalent to 400 g on a fresh-weight basis (2.5 cm in depth). This flask (containing the microcosm) was then allowed to stand in the dark with gentle stirring (50 rev min^{-1}) for 3 d at 25 °C in order to stabilize the microflora (preincubation).

2.4. Monitoring of biodegradation in the microcosm

After preincubation of the microcosm, [^{14}C]atrazine was directly applied to its aqueous phase (1.0 mg L^{-1}). To collect the radioactive carbon dioxide ($^{14}CO_2$), duplicate 1-M NaOH traps (each 100 mL) were connected, and air was continuously introduced into the headspace of the flask containing the microcosm at approximately 50 mL min^{-1}. The microcosms were kept in the dark at 25 °C for 28 d with gentle stirring (50 rev min^{-1}). The radioactivity in the NaOH solution and water phase was measured every week. Additionally, an aliquot of the water phase of the microcosm was subjected to HPLC analysis in order to separate and quantify [^{14}C]atrazine and its degradation products.

2.5. Screening of atrazine-degrading bacteria

In the first tier, we screened atrazine-degrading bacteria, including those that may be responsible for partial transformation. An aliquot of the water phase of the microcosm was obtained at the rapid atrazine degradation stage; the aliquot was serially diluted with sterile water and inoculated on agar plates (Medium No. 1) by using the conventional pour-plate method, following which the plates were incubated for 14 d at 25 °C. We randomly picked 48 colonies from the agar (10–40 colonies per plate), and inoculated them into glass tubes containing broth medium with [^{14}C]atrazine (Medium No. 2). After cultivation for 14 d in the dark at 25 °C, a 10-μL aliquot of the medium from each tube was analyzed by TLC. Of the 48 colonies, bacteria in 47 colonies could degrade atrazine. In the next tier, we screened 46 colonies (excluding one colony showing weak degradability) for the ability to completely degrade atrazine (i.e., achieve mineralization). We transferred 10-μL aliquots of broth from each tube containing atrazine-degrading bacteria into glass tubes containing freshly prepared broth medium with [^{14}C]atrazine (Medium No. 2). To trap