J. Pestic. Sci., **31**(4), 420–423 (2006)
DOI: 10.1584/jpestics.G06-14

Note

Characterization of a *Nocardioides*-based, atrazine-mineralizing microbial colony isolated from Japanese riverbed sediment

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(Received May 11, 2006; Accepted July 20, 2006)

A multiple-member microbial colony (designated A14N) capable of mineralizing the herbicide atrazine has been isolated from Japanese riverbed sediment. We have determined the enzymes involved in the degradation route and the species composition of the colony. A14N converted atrazine to cyanuric acid *via* hydroxyatrazine and *N*-isopropylammelide as intermediates. Furthermore, A14N characteristically had the ability to cleave the triazine ring of cyanuric acid. A14N harbours genes coding atrazine-degrading enzymes, which are highly similar to those previously described: *trzN*, *atzB* and *atzC*. At least three phylogenetically different species, *Nocardioides* sp., *Mycobacterium* sp. and *Leptospira* sp. were present in A14N. Among these, *Nocardioides* sp. was closely related to the atrazine-degrading *Nocardioides* sp. strains previously isolated from agricultural soils. Thus, *Nocardioides* sp. in A14N could also be responsible for the degradation of atrazine to cyanuric acid.

Keywords: atrazine, degradation, mineralization, microbial colony, *Nocardioides*.

Introduction

Atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine) has been used as an herbicide globally scine the 1950s and is used to control a variety of upland weeds.[1] However, until the 1990s, its complete degradation route in the natural environment had remained unclear. In 1995, the *Pseudomonas* sp. strain ADP was reported as an atrazine-mineralizing bacterium by Mandelbaum *et al*.[2] A series of thorough investigations on strain ADP elucidated the metabolic pathways; six enzymes and their corresponding genes are involved in atrazine degradation.[3–10] Such intensive atrazine degraders have been consistently isolated by several researchers to date.[11–19] These atrazine-degrading microbes were isolated from soils enriched with such microbes by using relatively high atrazine concentrations (22–2500 ppm). These soils included a refluxed soil,[11] a contaminated soil[2,12] and soils repeatedly exposed to atrazine for several years.[15]

In the natural environment, the complete biodegradation of recalcitrant xenobiotics by an individual microbial strain is apparently rare. It is generally accepted that multiple microbial strains or species involved in the partial conversion of such chemicals would be common in nature, and these microorganisms play an important role in the synergistic mineralization of chemicals. Several multiple-member microbial consortia that are capable of the synergistic degradation of specific pesticides such as linuron,[20–23] isoproturon,[24] carbaryl[25] and simazine[26] have been isolated. An atrazine-mineralizing microbial consortium has also been reported.[27] In this consortium, degradation is initiated by *Clavibacter* sp. To date, we have isolated five microbial colonies capable of mineralizing atrazine using a simple model ecosystem consisting of riverbed sediment and its associated water. In this report, we will describe a colony designated as A14N with regard to its degrading enzymes and species composition. To our knowledge, this is the first report on an atrazine-mineralizing microbial colony in which a *Nocardioides* sp., a high-GC gram-positive bacterium, plays a major role. Furthermore, colony A14N includes a novel combination of bacterial strains which collectively mineralize atrazine.

Materials and Methods

1. Chemicals

Atrazine uniformly labelled with ^{14}C at the *s*-triazine ring ([^{14}C]atrazine) was used. This compound was synthesized and identified by the Institute of Isotopes Co., Ltd. Its specific radioactivity was 6.44 MBq/mg, and its radiochemical purity was greater than 98%. Cyanuric acid uniformly labelled with ^{14}C ([^{14}C]cyanuric acid) was purchased from Sigma Chemical Co., and used as a reference standard. Unlabelled atrazine and hydroxyatrazine (6-hydroxy-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine) were purchased from Kanto Kagaku Co., Ltd. *N*-isopropylammelide (4,6-dihydroxy-N^4-isopropy-1,3,5-triazine-2-amine), uniformly labelled with ^{14}C and detected as a microbial metabolite of atrazine from the other microbial colony, was identified by LC-MS/MS analysis (data not shown) and was tentatively used as a reference standard.

2. Sediment and water

Riverbed sediment and associated water were collected on 3 July 2002 from the Tonegawa River (35°58′N, 139°55′E) flowing through the southern part of Ibaraki prefecture in Japan. The surface layer of the riverbed sediment was collected from up to a depth of 10 cm. The sediment/water sample was immediately

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Published online September 7, 2006

subjected to microcosm preparation without storage.

3. Screening of atrazine-mineralizing colonies

Microbial colonies capable of degrading atrazine were screened as described in our previous studies.[28,29] Among them, atrazine-mineralizing colonies were then further screened by the following procedures. A 100-fold-diluted nutrient broth (Difco) containing 1 mg/l [^{14}C]atrazine was prepared. The liquid medium (2.5 ml each) was poured into a test tube in which a micro-test tube containing 1 M NaOH (1 ml) was mounted for collecting radioactive carbon dioxide ($^{14}CO_2$). The liquid media (10 μl each) in which the microbes were able to degrade atrazine were transferred to the new test tube as described above. After culturing in the dark for 3 weeks at 25°C, the trapped $^{14}CO_2$ in the micro-test tube was determined by liquid scintillation counting (LSC) analysis using an Aloka LSC-5100 liquid scintillation counter, after the addition of Packard ATOMLIGHT^{TM} scintillation cocktail. Compared with the non-inoculated control, colonies that generated significant amounts of $^{14}CO_2$ were selected.

4. Degradation test

The liquid medium (30 ml each, described in Section 3) in glass bottles inoculated with colony A14N was connected to an alkaline trap (1 M NaOH), and filter-sterilized air was introduced at a flow rate of 2 ml/min to collect $^{14}CO_2$. At appropriate intervals, atrazine degradation was monitored by HPLC,[29] and the amount of evolved $^{14}CO_2$ was determined. Intermediary metabolites were identified by TLC- and HPLC-co-chromatography and compared with the reference standards.

5. DNA extraction, PCR amplification and sequencing

The template DNA for PCR amplification of the genes of 16S rRNA and atrazine-degrading enzymes was extracted from bacterial cells by conventional methods such as phenolic extraction and isopropyl alcohol precipitation. The PCR primers used in this study and their corresponding annealing temperatures are listed in Table 1. The primers were synthesized by Kurabo Industries Ltd. or Rikaken Co., Ltd. PCR amplification was performed using a TP240 thermal cycler (Takara Bio). PCR for amplifying genes of 16S rRNA and atrazine-degrading enzymes was performed with an initial denaturation at 95°C for 3 min or 10 min when Takara EX Taq (Takara Bio) or Amplitaq Gold (Applied Biosystems) was used as DNA polymerase, respectively. Then, 32 cycles of denaturation (at 95°C for 30 s), annealing (at an appropriate temperature for 30 s) and extension (at 72°C for 1 min) were followed by a final extension at 72°C for 15 min. The PCR products were then cycle sequenced by Takara-Bio using the appropriate primers and the BigDye Terminator Ready Reaction Mix (Applied Biosystems), and sequences were obtained in both directions using an ABI Prism 337 DNA sequencer (Applied Biosystems). For PCR-DGGE targeting the V3 to V5 region of the bacterial 16S rRNA gene, we used the primers 341FGC with a GC clamp at the 5′ end, and 907R (Table 1). PCR amplification reactions were carried out using Takara EX Taq. Touchdown PCR was performed as follows. The initial denaturation was performed at 95°C for 3 min, and then denaturation was performed at 95°C for 1 min. The annealing temperature was initially set at 65°C and then decreased by 0.5°C per cycle for 1 min until 55°C. This was followed by primer extension at 72°C for 1 min. Next, 15 additional cycles were carried out at 55°C for 1 min (annealing), followed by extension at 72°C for 1 min. Finally, an extension step was carried out at 72°C for 15 min.

6. Denaturing gradient gel electrophoresis (DGGE)

The conditions for separation of the 16S rRNA gene fragments were as follows: a portion of PCR product was loaded onto a 6.5% (w/v) polyacrylamide gel with a denaturing gradient ranging from 45% to 65% (100% denaturant contained 7 M urea and 40% formamide) and run at 100 V for 5 hr at 60°C. After electrophoresis, the gels were stained with ethidium bromide for 20 min. For subsequent DNA re-amplification followed by the sequencing step, the central sections of selected DGGE bands were excised. Each gel fragment was soaked overnight at 4°C in a screw-cap tube (volume, 2 ml) containing sterilized water (50 μl). The exudates of DNA band samples were used as templates for subsequent PCR. The conditions for recovering the 16S rRNA gene were the same as for the original PCR although the forward primer 341F without the attached GC clamp was used. To confirm the existence of the species detected by DGGE, specific primers of corresponding species (Table 1) were designed based on the sequence information obtained from 16S rRNA gene fragments. Using the directly extracted DNA from colony A14N as a template, the remaining portion of their 16S rRNA genes was PCR-amplified using primer 9F or 1541R. With respect to *Mycobacterium* sp. and *Leptospira* sp., the forward and rear portions of each 16S rRNA gene were spliced to obtain a longer nucleotide sequence.

7. Phylogenetic analysis

A similarity search was performed by comparing the nucleotide sequence of each gene determined in this study with sequences deposited in the GenBank/DDBJ/EMBL database using the BLAST algorithm.[32]

Results and Discussion

1. Metabolism of atrazine and degrading genes

Using the total DNA extract from colony A14N, amplification of the genes coding atrazine-degrading enzymes, *trzN* and *atzBC*, was attempted using the primers described by Mulbry *et al*.[33] and de Souza *et al*.,[6] respectively. The expected PCR products of approximately 0.4 kb, 0.5 kb and 0.6 kb corresponding to *trzN*, *atzB* and *atzC*, respectively, were obtained. Each sequence was almost identical to that originally described and was found to have 100%, 98% and 99% similarity, respectively. Therefore, the genes could have originated common ancestors and could be widespread throughout the world.

We attempted to detect the genes *atzA*,[6] *atzDEF*[9,34] and *trzD*[35] by PCR, according to the conditions previously reported; however, the expected DNA fragments could not be obtained. Different primer pairs for *atzD* and *trzN* were also tested to detect

Table 1. PCR primers

Primer name	Target sequence	Nucleotide sequence from 5′ to 3′	Annealing temperature (°C)	Reference
genes from 16S ribosomal RNA				
341F	Universal for bacteria	CCT ACG GGA GGC AGC AG	61	30
341FGC	Universal for bacteria (with GC-clamp)	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG	65–55[a]	30
907R	Universal for bacteria	CCG TCA ATT CCT TTG AGT TT	61 or touchdown	31
9F	Universal for bacteria	GAG TTT GAT CCT GGC TCA G	56[b] or 50[c]	31
1541R	Universal for bacteria	AAG GAG GTG ATC CAG CC	55[d], 56[e] or 50[f]	31
NOC-F[g]	Specific for *Nocardioides* sp.	GCC ACC GGA AAC GGT GAT	55	This study
MYC-F[g]	Specific for *Mycobacterium* sp.	TTT CCT TCC TTG GGA TCC GTG	56	This study
MYC-R[h]	Specific for *Mycobacterium* sp.	CAC GGA TCC CAA GGA AGG AAA	56	This study
LEP-F[g]	Specific for *Leprospira* sp.	AGG TAT TAA CTC CTC TAG TAA	50	This study
LEP-R[h]	Specific for *Leprospira* sp.	TTA CTA GAG GAG TTA ATA CCT	50	This study
genes of atrazine-degrading enzymes				
C190-10	*trzN* (atrazine chlorohydrolase)	CAC CAG CACCTG TAC GAA GG	63	33
C190-11		GAT TCG AAC CAT TCC AAA CG		
atzBF	*atzB* (hydroxyatrazine	TCA CCG GGG ATG TCG CGG GC	69	6
atzBR	ethylaminohydrolase)	CTC TCC CGC ATG GCA TCG GG		
atzCF	*atzC* (*N*-isopropylammelide	GCT CAC ATG CAG GTA CTC CA	65	6
atzCR	isopropylaminohydrolase)	GTA CCA TAT CAC CGT TTG CCA		

[a] Touchdown PCR; see Materials and Methods, Section 5. [b] Combined with MYC-R. [c] Combined with LEP-R. [d] Combined with NOC-F. [e] Combined with MYC-F. [f] Combined with LEP-F. [g] Combined with 1541R. [h] Combined with 9F.

cyanuric acid-degrading genes, but the attempts were unsuccessful. Thus, the cyanuric acid-degrading enzymes occurring in A14N may be a novel type and have low similarity to those previously found. It appears reasonable to assume that there are variations in the enzymes catalyzing the degradation of relatively simple compounds such as cyanuric acid.

Figure 1 shows the dissipation of atrazine, the formation of intermediary metabolites in liquid media inoculated with colony A14N, and carbon dioxide generation. To date, the route of atrazine biodegradation has been well elucidated. The degradation route adopted by A14N and the enzymes described above were almost the same as those previously reported. Firstly, hydroxyatrazine was produced *via* oxidative dechlorination catalyzed by TrzN.[33] Next, hydroxyatrazine was converted to *N*-isopropylammelide through the release of the ethylamino group catalyzed by AtzB.[3] Finally, *N*-isopropylammelide was converted to cyanuric acid by the release of the isopropylamino group catalyzed by AtzC.[8] Cyanuric acid was further degraded *via* triazine ring cleavage to yield CO_2 and NH_3.

2. *Classification of members*

The DGGE results indicated that colony A14N consisted of at least three phylogenetically diverse microbial species. Sequence data containing more than 1000 bases of the 16S rRNA gene of each species were sequenced again for deposition in the GenBank/DDBJ/EMBL database (Table 2). As a result, the existence of three microbial species in A14N was confirmed. Among these, the sequence of *Nocardioides* sp. strain was completely identical

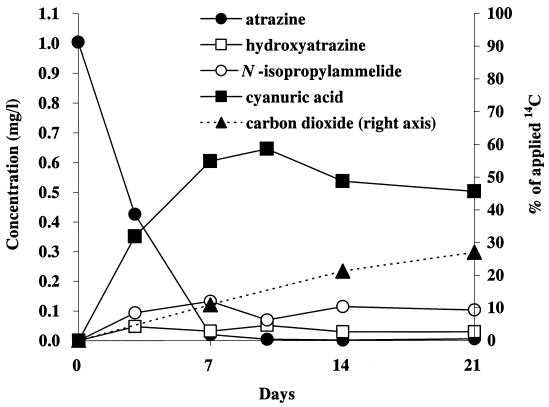

Fig. 1. Dissipation of atrazine and the formation of intermediary metabolites in 100-fold-diluted nutrient broth (left axis) and the generation of carbon dioxide (right axis). Data are the means of duplicate experiments.

Table 2. Phylogenetic classification of strains in colony A14N and most closely related strains

Strains in A14N	Accession No.	Most closely related strains in DDBJ database	Accession No.	Similarity (%)
Nocardioides sp. A14N	AB251600	*Nocardioides* sp. C157	AF253509	100
Mycobacterium sp. A14	NAB251601	*Mycobacterium duvalii*	U94745	98
Leptospira sp. A14	NAB251602	*Leptospira wolbachii*	AY631890	97

to those of the *Nocardioides* sp. strains C157 and SP12—atrazine-degrading microbial strains isolated from Canadian[16] and French[36] agricultural soils, respectively. Therefore, although colony A14N was suspended in the aqueous phase of the model ecosystem, it is likely that such a microbial consortium that is capable of mineralizing atrazine occurs also in terrestrial environments; however, the functional and ecological role of the other two strains remains unclear.

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