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Characterization of Novel Atrazine-Degrading *Klebsiella* sp. isolated from Thai Agricultural Soil

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***Abstract*—**Atrazine, a herbicide widely used in sugarcane and corn production, is a frequently detected groundwater contaminant. An atrazine-degrading bacterium, strain KB02, was obtained from long-term atrazine-treated sugarcane field soils in Kanchanaburi province of Thailand. Strain KB02 had a rod-to-coccus morphological cycle during growth. Sequence analysis of the PCR product indicated that the 16S rRNA gene in strain KB02 was ranging from 97-98% identical to the same region in *Klebsiella* sp. Based on biochemical, physiological analysis and 16S rDNA sequence analysis of one representative isolate, strain KB02, the isolates belong to the genus *Klebsiella* in the family Enterobacteriaceae. Interestingly that the various primers for *atzA*, *B* and *C* failed to amplify genomic DNA of strain KB02. Whereas the expected PCR product of *atzA*, *B* and *C* were obtained from the reference strain, *Arthrobacter* sp. strain KU001.

***Keywords*—**Atrazine; *atz* gene; Biodegradation; bioremediation ; Klebsiella

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I. Introduction

ATRAZINE (2-chloro-4-ethylamino-6-isopropylamino-striazine) is the herbicide that has been widely used in the world to control broadleaf weeds in the past 30 years. Due to its high mobility and long half life in soil, residues of both the parent compound and its derivatives have been detected in soil, surface, water and groundwater after year's application. Because of its widespread usage, atrazine concentrations in groundwater and surface water frequently exceed the 3-ppb health advisory level set by the U.S. Environmental Protection Agency [1]. The prevalence of atrazine in the environment has stimulated investi gations into the biodegradation of the class of compounds containing an *s*-triazine ring [2][3]. Many microorganisms were isolated and studied for their abilities in atrazine mineralization including members of genera *Pseudomonas, Acinetobacter, Agrobacterium, Arthrobacter*, *Rastonia and Norcardioides* [4][5][6][7]. Of all the studied bacteria, Pseudomonas sp. strain ADP might be the best-characterized atrazine mineralizing one [8]. These bacteria commonly initiate atrazine degradation by a hydrolytic dechlorination reaction. The genes encoding an atrazine chlorohydrolase (*atzA*) and the enzymes of two amidohydrolytic reactions (*atzB* and *atzC*), which together convert atrazine to the ring cleavage substrate cyanuric acid, have been cloned from *Pseudomonas* sp. strain ADP [9][10][11].

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This study aims to isolate and investigate atrazine-degrading bacteria from agricultural soil samples in Kanchanaburi province. Once atrazine-degrading bacteria were isolated, the physiological and biochemical properties were studied. Additionally, we sequenced their 16S rRNA genes and used the polymerase chain reaction (PCR) to amplify known atrazine-degrading genes comparison to previously studied atrazine degraders. Comparing the new isolates to previously characterized microorganisms will broaden our understanding of atrazine degraders and the varied gene combinations used.

II. Materials and Methods

A. Soil sampling

The bacteria described in this paper were isolated from soils obtained from Kanchanaburi province. All soils had been used to grow sugarcane and had been treated with atrazine for weed control according to normal farming practice for at least 20 years. The soil was stored without drying at 4°C before used for enrichment and isolation of atrazine-degrading bacteria.

B. Enrichment and isolation of atrazine-degrading bacteria

The isolation of native atrazine-degrading bacterial strains was performed by using the enrichment culture technique and dilution-plating and streaking on R-medium [12] containing atrazine. Enrichment preparation consisting of R-medium containing 25 mg of atrazine per liter as the sole nitrogen source were inoculated with soil (25%, wt/vol) and incubated aerobically with shaking at 30°C. Cultures were spreaded onto agar plates on R-medium with 500 mg/liter atrazine as the sole carbon and nitrogen source. This atrazine concentration is greater than the solubility limit, 33 mg/liter, and results in chalky suspension [8]. Colonies which developed cleared zones in the atrazine-containing R-medium agar were purified and routinely maintained on this medium.

C. Physiological and biochemical characterization

The isolates were characterized by conventional methods: Gram staining, oxidaes**,** catalaes, indole**,** methy red**,** citrate utilization**,** gelatin liquefaction**,** oxidation-fermentation, urease activities and motility. The morphology of bacterial colonies on TSA plates was observed.

D. Identification of bacterial strains by 16S rRNA gene sequence analysis

The genomic DNA of the strains was extracted and precipitated following the standard protocol for bacterial genomic DNA preparations [13]. The partial 16S rRNA genes were amplified by polymerase chain reaction (PCR) using the universal primers of 16S rRNA gene. The oligonucleotide primers used were 27F (5′- AGA GTT TGA TCM TGG CTC

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AG -3′) and 1492R (5′-TAC GGH TAC CTT GTT ACG ACT T-3′). The PCR mixture contained 1.5 U of *Taq* polymerase, 5 µl 10 x *Taq* buffer, 0.2 mM concentration of each deoxynucleoside triphosphate, 0.5 µM primers and 0.5 µl of DNA template. The PCR cycle parameters were as follows: 94°C for 4 min, 20 cycles of 94°C for 1 min, 50°C for 1.5 min and 72°C for 2 min. The resulting DNA sequences were compared with the 16S Rrna genes were available in GenBank nucleotide library by a BLAST search through the National Centre for Biotechnology Information (NCBI) Internet site (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were analyzed and concatenated by using DNASTAR (DNASTAR, Inc., Madison, Wis.) A multiple-sequence alignment was prepared by using CLUSTAL W [14], and phylogenetic trees were constructed by using the neighbor-joining method.

E. Detection of atrazine-degrading gene by PCR

Atrazine-degrading genes (*atz*) were detected in each bacterial community by PCR with gene-specific primers. Genomic DNA was prepared from single colonies resuspened in 100 µl Tris-EDTA buffer, heated at 95°C for 5 min and centrifuged briefly. The supernatant (5.0 µl) was used for PCR (25 µl final volume). For each bacterial community *atzA*, *B* and *C* sequences were amplified using the specific primer pairs listed in Table 1. PCR reactions were conducted using the conditions described above with optimal annealing temperature (see Table I for details).The PCR products (5 µl) were separated by electrophoresis on 1.0% agarose gel.

TABLE I
PRIMERS USED FOR PCR AMPLIFICATION OF ATRAZINE-DEGRADING GENES

Gene target	Primers	Sequences
*atz*A	atzA-F	5'-CCA TGT GAA CCA GAT CCT-3'
	atzA-R	5'-TGA AGC GTC CAC ATT ACC-3'
*atz*B	atzB-F	5'-CTT AAT GGC GTC GAT ATT CT-3'
	atzB-R	5'-AGG CGT TCG CAA TCG GCA AG-3'
*atz*C	atzC-F	5'-AGT CAG CGA AGG GCG TAG GTA TCA-3'
	atzC-R	5'-GAC AAA TCC GGG AGA CAC AAG GTT-3'

III. RESULTS

A. Isolation and Characterization of Strain KB02

A single colony of an atrazine-degrading bacterium was selected and isolated from sugarcane field soil by using the enrichment culture technique and restreaking three times on R-medium supplemented with atrazine. This isolates was subsequently designated as strain KB02. The results of gram stain indicated that strain KB02 is a gram-negative bacterium. Strain KB02 also appeared to show coccus morphology and cells occurred in singly or in pairs (Fig. 1). Isolates KB02 was large circular and produced white-pigmented colonies on TSA plates (Fig. 2).

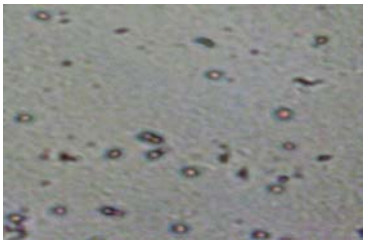


Fig. 1 Micrographs showing the cocci cells and gram stain of strain KB02

The biochemical properties of KB02 are shown in Table II. Analysis of these test indicated that the strain KB02 belong to the genus *Klebsiella*.

TABLE II
BIOCHEMICAL CHARACTERIZATION OF THE NOVEL BACTERIAL STRAINS KB02 ISOLATED FROM AGRICULTURAL SOIL COMPARED WITH *KLEBSIELLA* SP.

Biochemical test \ Strain	KB02	*Klebsiella* sp.
Gram	-	-
Oxidaes	-	-
Catalaes	+	+
Indole	-	-
Methy red	-	-
Citrate	+	+
Gelatin liquefaction	-	-
Oxidation-fermentation	+	+
Urease	+	+
Motility	+	+

B. Identification of atrazine-degrading bacteria

The near full-length 16S rRNA gene of isolates KB02 was sequenced and analyzed for bacterial identification. KB02 16S rRNA sequence showed high similarity of 97-98% with *Klebsiella* sp. The nucleotide sequences of the 16S rRNA gene from strain KB02 was deposited in GenBank as Accession No. HM989008. Therefore, this bacterium was named *Klebsiella* sp. strain KB02.

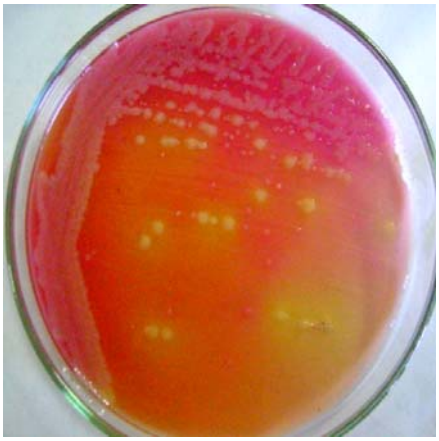


Fig. 2 Large circular and white-pigmented colonies of strain KB02 on TSA plates

C. Atrazine-degrading genes

Klebsiella sp. strain KB02 was analyzed for specific atrazine-degrading genes by PCR amplification using primers described in Table I. The expected PCR products of *atzA*, *B* and *C* genes were all obtained from the reference *Arthrobacter* sp. strain KU001. However, although conducted at various PCR conditions, these primers failed to amplify genomic DNA of *Klebsiella* sp. strain KB02 (data not shown).

IV. CONCLUSIONS

Atrazine is the most prevalent of the *s*-triazine formulations applied in the environment and is also less readily metabolized than certain other *s*-triazine compounds. A number of pure cultures have been found to metabolize cyanuric acid and ammeline as their sole source of nitrogen [2] but were unable

to use atrazine. In present study, we were successful in isolating a new atrazine-degrading bacteria from an agricultural soil that received yearly applications of the herbicide atrazine. This novel strain was characterized and identified, isolates was named *Klebsiella* sp. strain KB02. However, no amplification products were obtained from *Klebsiella* sp. KB02 using the primers of *atzA*, *atzB* and *atzC* genes, the well known reported genes responsible for the first two steps in atrazine degradation. This demonstrated the gene diversity of atrazine metabolism in different genera, more thorough molecular analysis will be necessary to elucidate the atrazine-degrading genes in this isolate.

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