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Phylogenetic Characterization of Atrazine-Degrading Bacteria Isolated from Agricultural Soil in Eastern Thailand

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***Abstract*—**In this study sugarcane field soils with a long history of atrazine application in Chachoengsao and Chonburi provinces have been explored for their potential of atrazine biodegradation. For the atrazine degrading bacteria isolation, the soils used in this study named ACS and ACB were inoculated in MS-medium containing atrazine. Six short rod and gram-negative bacterial isolates, which were able to use this herbicide as a sole source of nitrogen, were isolated and named as ACS1, ACB1, ACB3, ACB4, ACB5 and ACB6. From the 16S rDNA nucleotide sequence analysis, the isolated bacteria ACS1 and ACB4 were identified as *Rhizobium* sp. with 89.1-98.7% nucleotide identity, ACB1 and ACB5 were identified as *Stenotrophomonas* sp. with 91.0-92.8% nucleotide identity, whereas ACB3 and ACB6 were *Klebsiella* sp. with 97.4-97.8% nucleotide identity.

***Keywords*—**Atrazine-degrading bacteria, bioremediation, Thai isolate bacteria.

I. Introduction

ATRAZINE is a kind of selective inner-absorption conductive herbicide. It can be applied to corn, sorghum, sugarcane, tea gardens, orchards, Korean pine seedling nursery, and woodland to kill both annual and broadleaf weeds [1]. Atrazine residue in the soil and sprayed to cultivate the agricultural products can enter into the groundwater and surface water during irrigation of farmland [2], [3].

In states with high atrazine use, surface and ground water commonly have detectable levels of atrazine [4], [5]. Atrazine persists in soil and ground water and has the potential to leach into drinking water supplies [6]. Atrazine can also affect human and other animals directly because it can be concentrated by plants and transferred to the food chain [7]. The high incidence of atrazine contamination, along with an increasing concern about the toxicological properties of atrazine, has prompted researchers to seek bioremediation options for atrazine-polluted sites [8]. Many studies have shown that microorganisms in water and soil have the ability to degrade atrazine.

Some countries have been committed to isolate the atrazine-degrading strain since 1960s. Because of the diversity of biochemical characteristics and the strong ability to adapt to the environment, the bacteria play an important role in atrazine degradation. In this study, we isolated an atrazine degrading bacterium from soil in Eastern Thailand. Then we investigated the relevant phenotypic traits and phylogenetic relationships of their strain to determine its true taxonomic position.

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II. Materials and Methods

A. Isolation and Purification

The soil samples were collected from topsoil (0-10 cm) of a sugarcane field in Chachoengsao and Chonburi provinces of Thailand with long-term atrazine application. The soil was stored without drying at 4°C before used for enrichment and isolation of atrazine-degrading bacteria. The atrazine-degrading bacterial strains was isolated by dilution, plating and streaking on MS medium [9] supplemented with 500 µg/ml of atrazine. Colonies which developed cleared zones in the atrazine-containing MS medium agar were purified and routinely maintained on this medium.

B. Morphological Characterization

The isolates were characterized by conventional methods: Gram staining. The morphology of bacterial colonies on TSA plates was observed.

C. Amplification of Bacterial 16S rRNA Gene by PCR

The bacterial samples developed cleared zones in the atrazine-containing MS medium agar were selected. The genomic DNA of the strains was extracted and precipitated following the standard protocol for bacterial genomic DNA preparations [10]. 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 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.

D. Phylogenetic Analyses

A partial nucleotide sequence analysis (approximately 1,480 bases) of the 16S rRNA gene of Chachoengsao (ACS) and Chonburi (ACB) strains were determined by First BASE

Laboratories Sdn Bhd (Selangor Darul Ehsan, Malaysia) and used to perform a BLAST search [11] for related sequences. Sequences were analyzed and concatenated by using DNASTAR (DNASTAR, Inc., Madison, Wis.) A multiple-sequence alignment was prepared by using CLUSTAL W [12], and phylogenetic trees were constructed by using the neighbor-joining method.

III. Results

A. Isolation and Characterization of Atrazine-Degrading Strain

Atrazine-degrading bacteria were isolated by using the enrichment culture technique and restreaking three times on MS-medium supplemented with atrazine. Six bacterial strains, ACS1, ACB1, ACB3, ACB4, ACB5 and ACB6, were successfully identified from the sugarcane field soil sample. The results of gram stain indicated that these six isolates are gram-negative bacterium. All six isolates also appeared to show short rod morphology and cells occurred in singly or in pairs (Fig. 1). Isolates were large circular and produced white colonies on TSA plates (Fig. 2).

(a) (b) (c) (d) (e) (f)

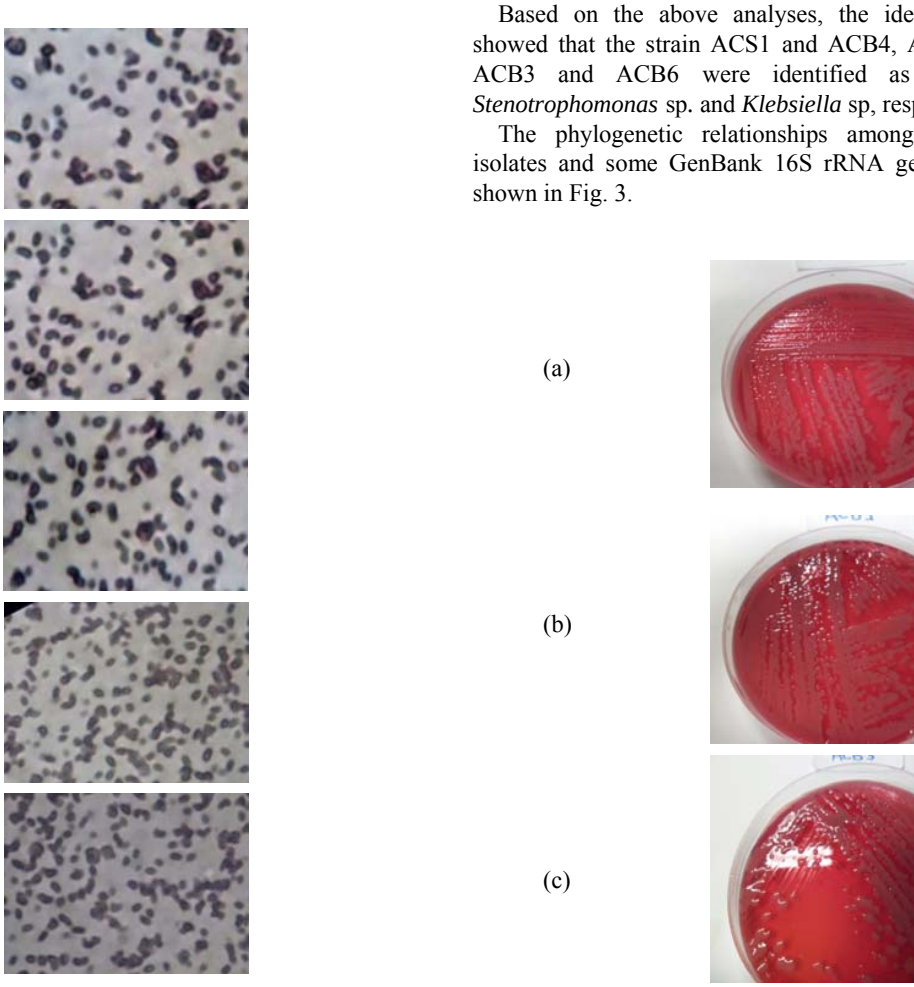

Fig. 1 Micrographs showing the short rod cells and gram stain of atrazine-degrading strains. ACS1 (a); ACB1 (b); ACB3 (c); ACB4 (d); ACB5 (e) and ACB6 (f).

B. Phylogenetic Relationships

A partial nucleotide sequence approximately 1,480 bp. of the 16S rRNA gene of ACS1, ACB1, ACB3, ACB4, ACB5 and ACB6 strains were sequenced and analyzed for bacterial identification.

The 16S rRNA gene sequence of ACS1 and ACB4 exhibited a 89-98% nucleotide identity with *Rhizobium* sp., and that of ACB1 and ACB5 showed a 91-92% 16S rRNA gene identity with *Stenotrophomonas* sp., whereas ACB3 and ACB6 16S rRNA sequences showed high similarity of 97-97% with *Klebsiella* sp.

Based on the above analyses, the identification results showed that the strain ACS1 and ACB4, ACB1 and ACB5, ACB3 and ACB6 were identified as *Rhizobium* sp., *Stenotrophomonas* sp. and *Klebsiella* sp, respectively.

The phylogenetic relationships among these six Thai isolates and some GenBank 16S rRNA gene sequences are shown in Fig. 3.

(a) (b) (c)

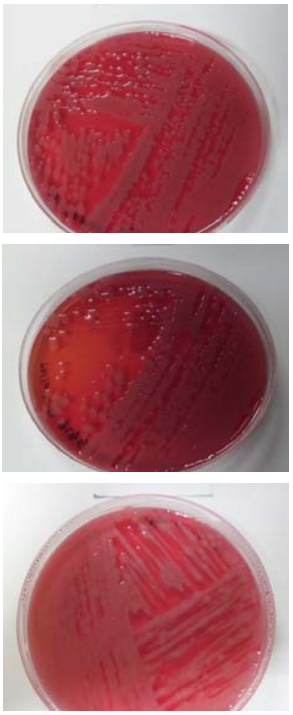

Fig. 2 Large circular and white-pigmented colonies of strain ACS1 (a); ACB1 (b); ACB3 (c); ACB4 (d); ACB5 (e) and ACB6 (f) on TSA plates

IV. Conclusions

In present study, we were successful in isolating several new atrazine-degrading microorganisms from an eastern Thai agricultural soil that received yearly applications of atrazine. Six short rod and gram-negative bacterial isolates, which were able to use this herbicide as a sole source of nitrogen were isolated. Based on 16S rDNA sequence analysis and phylogenetic relationships of atrazine-degrading Thai isolates, the isolates belong to the three genus including *Rhizobium*, *Stenotrophomonas* and *Klebsiella* in the Family Rhizobiaceae, Xanthomonadaceae and Enterobacteriaceae, respectively.

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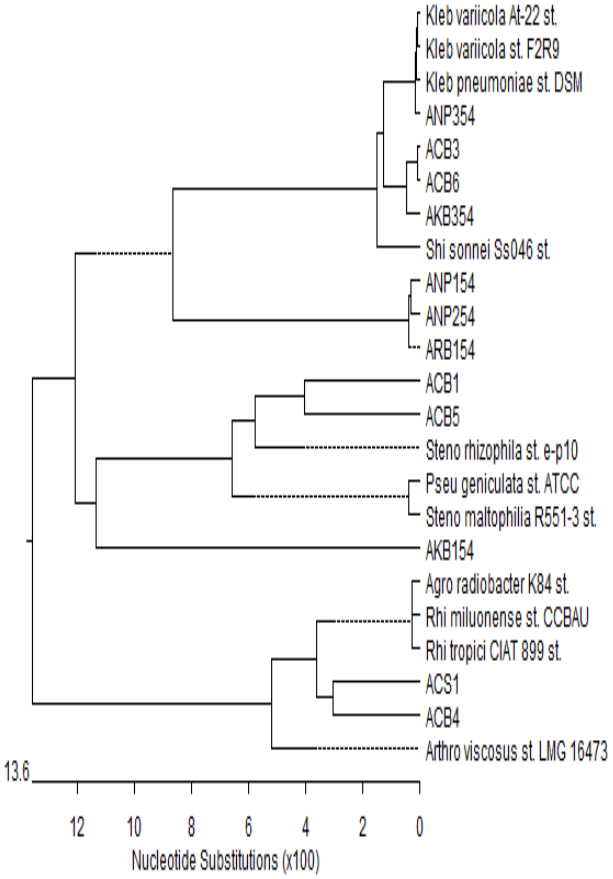

Fig. 3 Neighbor-joining tree based on the 16S rDNA sequences of the atrazine-degrading isolates (ACS1, ACB1, ACB3, ACB4, ACB5 and ACB6) with related 16S rDNA sequences found in GenBank database

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