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ORIGINAL PAPER

Biodegradation of atrazine by *Arthrobacter* sp. C3, isolated from the herbicide-contaminated corn field

H. Wang[1] · Y. Liu[1,2] · J. Li[1,2] · M. Lin[1] · X. Hu[1]

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Abstract The *s*-triazine herbicide, atrazine, has been well acknowledged as an important source causing contamination of soil, water, and sediment. Functional bacteria are one of the critical candidates for removing residual atrazine from contaminated environments. Here, seven bacterial strains showing atrazine-degrading ability were isolated from long-term atrazine-contaminated corn field and identified based on 16S rRNA gene sequencing. Among these bacterial isolates, a bacterium, later designated as *Arthrobacter* sp. C3, was found to be capable of completely degrading 25 mg/l atrazine. The high-performance liquid chromatography–mass spectrometry (HPLC–MS) analysis indicated that the atrazine was dechlorinated to hydroxyatrazine, a non-phytotoxic compound. The functional gene, *trz*N, which participates in the first step of atrazine degradation was successfully amplified and showed high similarity to the known *trz*N genes from different bacterial genera. Based on the HPLC–MS and the functional gene analysis, the functional bacterium C3 was speculated to degrade atrazine via dechlorination, which detoxified the herbicide. This study suggested a great potential of *Arthrobacter* sp. C3 to be used in indigenous bioremediation of atrazine in field.

H. Wang and Y. Liu have contributed equally to this work.

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✉ X. Hu
xkhu@yic.ac.cn

[1] Yantai Institute of Costal Zone Research, Chinese Academy of Sciences, Yantai 264003, China

[2] University of Chinese Academy of Science, Beijing 100049, China

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Introduction

Driven by the increasing use of herbicide, contamination caused by this kind of pesticide has received public and scientific attention. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine), an s-triazine herbicide, is extensively used in cropped soils to control weeds, especially during production of corn (Topp et al. 2000). For its high mobility and relatively long half-life, atrazine has been proved to easily contaminate different habitats, including soil, surface water, and ground water (Rousseaux et al. 2001). The residual of atrazine in environment not only could damage the following crops in the field and reduce populations of other organisms, but also has severe impacts on human health. The herbicide was reported as an endocrine-disrupting chemical, which could cause birth defects, reproductive tumors, and weight loss (Fernández et al. 2013). Thus, it is critical to eliminate residual atrazine from environment.

Bioremediation is the technique utilizing biological organisms to aid in removal of hazardous substances from a polluted area (Head et al. 2006; Louisa Wessels 2010). Microorganisms, especially bacteria, are key players in the process of bioremediation of most organic pollutants (Wang et al. 2009a, b). Atrazine could be biodegraded by either single functional bacterium or microbial consortia (Tortella et al. 2013; Yanze-Kontchou and Gschwind 1994). A number of bacterial strains affiliated into different genus, *Pseudomonas*, *Rhizobium*, *Acinetobacter*, *Arthrobacter*, and *Pseudaminobacter*, have been successfully isolated from agricultural soils, industrial wastewater, and other atrazine-polluted environment (Cai et al. 2003; Topp et al. 2000; Singh

et al. 2004; de Souza et al. 1998; Bouquard et al. 1997). For instance, Singh et al. (2004) isolated an *Acinetobacter* strain which could degrade atrazine as high as 250 ppm from a soil heavily contaminated with atrazine. The bacterium could utilize atrazine as a carbon, but not as a nitrogen source. On the other side, microbial consortium could be constructed by applying different functional isolates in the same process. Two bacterial isolates, *Klebsiella* sp. A1 and *Comamonas* sp., isolated from the sewage of a pesticide mill were combined as a microbial consortium for bioremediation of atrazine (Yang et al. 2010). A high atrazine-mineralizing efficiency was detected with 83.3 % mineralization of 5 g/l atrazine in 24 h. This should be attributed to the co-metabolic process of the two functional bacteria.

Even though dramatic improvements have been achieved, it is still necessary for isolating more efficient atrazine-degrading bacteria, given the large-scale use of atrazine and its toxicity. The aims of this study are (1) to isolate and identify atrazine-degrading bacteria and (2) to investigate the mechanisms of atrazine degradation and functional genes involved in the process.

Materials and methods

Sample collection

Soil samples were collected from a long-term (twice a year for at least 3 years) atrazine-sprayed corn field in east of China (N: 37.185926, E: 122.255335). Samples were collected in the 5–15 cm layer by removing surface soil. Samples used for isolating atrazine-degrading bacteria were kept in a cooler with insulation and ice packs, and transported to the laboratory in 2 h for further analysis.

Isolation and identification of atrazine-degrading bacterium

Immediately after samples were transferred to the laboratory, ten grams of soil samples and 25 mg/l (final concentration) of atrazine (Dr. Ehrenstorfer GmbH, Germany) were co-incubated in 100 ml mineral solution (7.01 mM K_2HPO_4, 2.94 mM KH_2PO_4, 0.81 mM $MgSO_4{\cdot}7H_2O$, 0.18 mM $CaCl_2$, 1.71 mM NaCl) for 7 days with shaking at 180 rpm at 30 °C. One ml of liquid culture was tenfold serially diluted to 10^{-7}, and 100 µl aliquots of each dilution was spread onto mineral agar which was prepared by adding 15 ‰ agar into mineral solution with 25 mg/l of atrazine. The plates were incubated at 30 °C for 48 h. Morphologically different colonies were streaked on mineral agar for isolation. Single colonies of each isolate were transferred to 10 ml of mineral solution to test the biodegrading capability. Yeast extract (1 ‰) was supplemented as carbon and nitrogen source. 1.8 ml of liquid culture was used for DNA extraction, while 1 ml of the residual was cryopreserved at −80 °C with 1 ml of 60 % glycerol. DNA was extracted from liquid cultures using the UltraClean microbial DNA isolation Kit (MoBio Laboratories, Carlsbad, CA). The 16S rRNA gene from each isolate was PCR amplified and sequenced using universal primers 27F and 1492R as described previously (Enticknap et al. 2006). 16S rRNA gene sequences from isolates were analyzed using the BLASTn tool at the National Center for Biotechnology Information (NCBI) website. Isolates were presumptively identified according to the identity of the closest cultured relative in the top BLAST hits.

Effect of exogenous carbon and nitrogen on the growth of the functional bacterium

Different carbon and nitrogen sources were added into the mineral solution mentioned above to assess the requirements for the growth of the functional bacterium. Treatments were as follows: mineral solution amended with (1) yeast extract (1 ‰, w/v) which could supply both carbon and nitrogen sources; (2) glucose (1 ‰, w/v) as carbon source; (3) NH_4NO_3 (1 ‰, w/v) as nitrogen source; and (4) no additional carbon and nitrogen sources (negative control). 25 mg/l of atrazine was added to all treatments and co-incubated for 48 h at 180 rpm, 30 °C. Treatments were performed in triplicate. Growth of the functional bacterium was determined by measuring the absorbance of the culture supernatant at 600 nm using a Unicam UV9100-visible spectrophotometer (Thermo Fisher Scientific, USA).

Degradation assay and mechanisms analysis

Exponentially growing functional bacterial strains were inoculated in mineral solution with 25 mg/l of atrazine and 1 ‰ yeast extract. Samples were collected after 3-day incubation at 30 °C. For the bacterial isolates later designated as C3, samples were collected for 72 h with a 6-h interval. Atrazine and its catabolite were extracted as follows. Aliquots (2 ml) of samples were extracted with 4 ml of chloroform for three times. The total of 12 ml organic phase was dried by volatilizing chloroform in vacuum and dissolved in 2 ml mixture of methyl cyanide and ddH_2O (1:1) for further analysis. High-performance liquid chromatography (HPLC) analyses were performed using an Agilent 1200 (USA), equipped with a Hypersil Gold C18 column (Thermo Fisher Scientific, USA, 4.6 × 150 mm). Eighty percent methyl cyanide and 20 % water were applied as the mobile phase at a flow rate of 1 ml/min using the absorbance wavelength of 230 nm. The biodegradation products of atrazine were detected by the LCQ Fleet mass spectrometer (Thermo Fisher Scientific, USA) coupled with HPLC.

Atrazine-degradation-related gene was detected by PCR amplification with primers specific for *atzA*, *atzD*, *trz*N, and *trz*D (de Souza et al. 1998; Mulbry et al. 2002; Rousseaux

Table 1 Atrazine-degrading bacterial isolates and their degradation efficiency

Isolates	Phylum	Closed cultured organism (GenBank accession no.)	Identity (%)	Degradation efficiency (%)
C1	Actinobacteria	*Streptomyces tricolor* HBUM174995 (FJ532400.1)	98	42.7
C2	Proteobacteria	*Burkholderia cenocepacia* MC0-3 (CP000960)	97	43.0
C3	Actinobacteria	*Arthrobacter* sp. SD41 (KC415036)	99	100
E1	Proteobacteria	*Variovorax* sp. S15 (HE662647.1)	98	40.4
E3	Proteobacteria	*Sphingobium* sp. YJ2 (HQ260908.1)	97	26.6
F1	Actinobacteria	*Rhodococcus opacus* (DQ474758.1)	98	45.6
F3	Actinobacteria	*Streptomyces* sp. NY05-11A (FJ546742.1)	97	39.6

et al. 2001; Topp et al. 2000). The PCR amplification was conducted in a 50 µl reaction system which includes 37.8 µl of sterilized distilled water, 5 µl of 10 × high-fidelity PCR buffer, 2 µl $MgSO_4$ (50 mM), 1 µl of a mix of dNTP (2.5 mM each), 1 µl of 100 µmM of different primers, 0.2 µl of Platinum® Taq DNA (Invitrogen Life Technologies, Carlsbad, CA, USA) and 2 µl of template DNA (20 ng/µl). The PCR amplification was conducted as follows. A 5 min initial denaturing period at 97 °C was followed by 29 cycles at 92 °C for 30 s, 60 °C (for *atzA* and *atzD*) and 55 °C (for *trz*N and *trz*D) for 2 min, 72 °C for 90 s, and a final extension of 72 °C for 30 min. PCR products were detected by electrophoresis using 1 % agarose gel. Amplicons were sequenced by Shanghai Life Technologies Biotechnology Co., Ltd. (Shanghai, China). Functional gene sequences were analyzed using the BLASTn tool at the NCBI website to aid in the selection of the closest reference sequences. Phylogenetic analyses were performed using the Mega 4 software package (Tamura et al. 2007), and sequences were aligned using the positional tree server with a data set containing the nearest relative matches. Trees were constructed using the neighbor-joining (Jukes-Cantor correction) algorithms. The robustness of the inferred tree topologies was evaluated after 1000 bootstrap replicates of the neighbor-joining data.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences and atrazine-degrading functional gene of bacterial isolates newly determined in this work have been deposited in GenBank under accession numbers KR265339 (strain C1), KR265340 (strain C2), KR181961 (strain C3), KR265341 (strain E1), KR265342 (strain E3), KR265343 (strain F1), KR265344 (strain F3), and KR263873 (*trz*N gene).

Results and discussion

Isolation and characterization of atrazine-degrading bacteria

A total of seven bacterial strains capable of degrading atrazine were isolated from atrazine-contaminated soil. 16S rRNA gene sequences indicated that these strains affiliated into different genera, including *Streptomyces*, *Burkholderia*, *Arthrobacter*, *Variovorax*, *Sphingobium*, and *Rhodococcus*. All these isolates showed high efficiency of degrading the herbicide, with values between 39.6 and 100 % (Table 1). A bacterial strain, designated as C3, which showed significant high atrazine-degrading capability, ca. 100 % degradation after 7-day enriching period, was chosen for further studies. After 48-h incubation on mineral agar plates, strain C3 forms circular, flat, and white colonies. Gram staining showed that C3 is a Gram-positive bacterial strain. 16S rRNA gene sequence comparisons indicated that strain C3 shows a high similarity (99 %) to the previously isolated bacterium *Arthrobacter* sp. SD41 (gene bank accession number, KC415036), which affiliated into the phylum Actinobacterium. Based on the phenotypic characteristics and phylogenetic analysis, strain C3 was identified as *Arthrobacter* sp.. The genus *Arthrobacter*, widely distributed in soil, has been proven to be capable of degrading various organic compounds. *A. crystallopoietes*, isolated from dichromate-contaminated soil, could efficiently degrade hexavalent chromium, Cr(VI), by producing a periplasmic chromate reductase (Camargo et al. 2004). A novel bacterial strain, designated as *Arthrobacter chlorophenolicus* sp. nov., could grow on high concentrations (up to 350 ppm) of 4-chlorophenol (4-CP) and showed great potential of bioremediation of 4-CP (Westerberg et al. 2000). Bacteria in this genus have also been found to be able to remediate *p*-nitrophenol (PNP), 2-methyl, 2-ethyl, 2-hydroxypyridine, phenol, and cresol isomer (Labana et al. 2005; Kar et al. 1997; O'Loughlin et al. 1999). Here, a bacterium affiliated into the genus *Arthrobacter* was isolated from atrazine-contaminated crop field. The strain showed a high atrazine-degrading efficiency which could degrade 25 mg/l atrazine in 6 h. Serial experiments with different concentrations of atrazine (5, 25, 100, 500 mg/l) were set up in this study to investigate the capability of atrazine degradation by strain C3. The results indicated that strain C3 could grow and degrade atrazine in medium with up to 500 mg/l atrazine (data not shown). Previous studies have revealed that functional bacteria could tolerate high concentration of atrazine. *A. nicotinovorans* HIM, a atrazine-

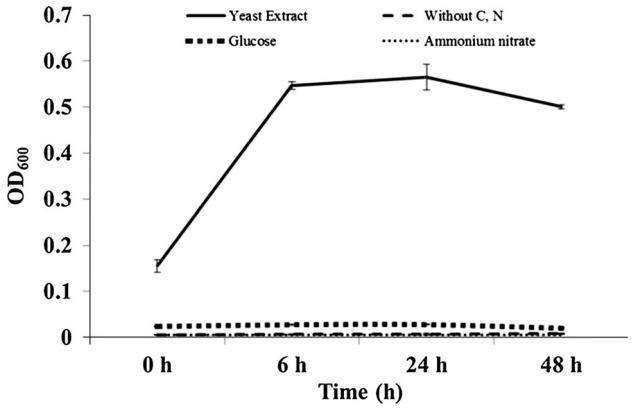

Fig. 1 Growth of the functional bacterium *Arthrobacter* sp. C3 in mineral solution amended with (*solid line*) yeast extract as both carbon and nitrogen sources; (*dash dot line*) glucose as carbon source; (*short dot line*) NH_4NO3 as nitrogen source; (*dash line*) no additional carbon and nitrogen sources

degrading bacterium isolated from New Zealand, could grow in medium with 1000 ppm atrazine (Aislabie et al. 2004). *A. aurescens* TC1 could grow in liquid medium with atrazine as the sole source of nitrogen, carbon, and energy, consuming up to 3000 mg of atrazine per liter (Strong et al. 2002). All these evidence indicated that the genus *Arthrobacter* had a potential to be used in high-concentration atrazine-contaminated field.

Nutrient effects on the growth of *Arthrobacter* sp. C3

Exogenous carbon and nitrogen sources are both essential for the growth of the functional bacterium *Arthrobacter* sp. C3. In the treatment (1), in which yeast extract was amended as both carbon and nitrogen sources, stationary phase could be attained in 6 h, while no obvious growth was observed till the end of the 48-h incubation period in all of the other three treatments (Fig. 1). The degradation efficiency was not measured for the treatment (2), (3), and (4) since no growth of the bacteria was detected.

Analysis of atrazine degradation and its catabolites

HPLC was applied to detect atrazine and its catabolites. Atrazine was detected in the growth medium with addition of functional bacterium *Arthrobacter* sp. C3 at the beginning of the degradation assay (0 h). An obvious single peak was measured at the retention time of 2.20 min (Fig. 2). After 6-h incubation, the peak could not be detected any more; instead, a novel peak with retention time of 3.75 min was observed (Fig. 2). These samples were also analyzed by mass spectrum to characterize the atrazine and its catabolites in the medium with inoculation of strain C3. The molecular mass and fragmentation pattern indicated that the catabolite encoded with the two peaks was conducted by loss of Cl with 35.5 mass units and acquisition of OH with 17 mass units (Fig. 3). The catabolite was consistent with the structure of the first-step catabolite, hydroxyatrazine.

Four critical functional genes, *atz*A, *atz*D, *trz*N, and *trz*D, involved in two key steps of bioremediation of atrazine were investigated to reveal the mechanism of atrazine degradation by strain C3. The gene *trz*N was successfully amplified, while all the other three genes were not detected.

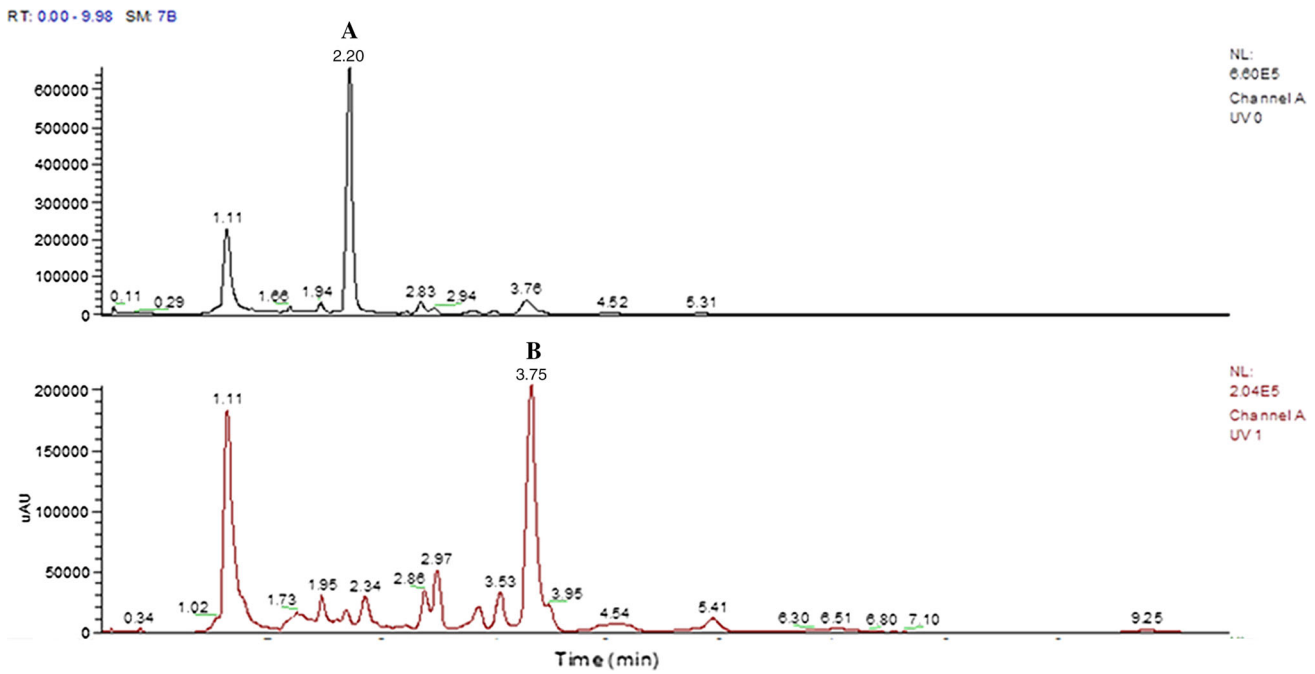

Fig. 2 Chromatograms of atrazine and its metabolic products after 0 and 6 h incubation with *Arthrobacter* sp. C3. The peak *A* indicated atrazine with a retention time of 2.20 min, while the peak *B* indicated a catabolite with a retention time of 3.75 min

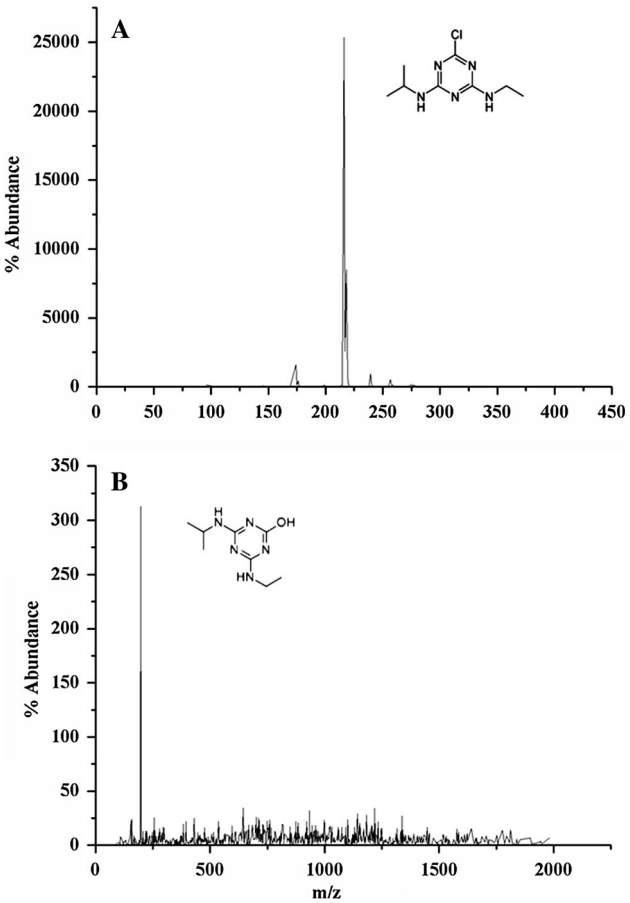

Fig. 3 Mass spectra of atrazine and its metabolic products obtained from culture extracts of strain C3 incubated in the presence of the herbicide after (**a**) 0-h incubation and (**b**) 6-h incubation. The mass spectrum of the transformation product is consistent with the hydroxytriazine structure (**b**)

PCR amplicon of *trz*N gene was then sequenced which resulted a 1312-bp sequence. Results of BLASTn indicated that the sequence has a high similarity (99 %) to the identified *Arthrobacter* sp. AD30 triazine hydrolase (*trz*N) gene. Phylogenetic analysis indicated that *trz*N gene of the newly isolated bacterial strain C3 grouped together with AD30 *trz*N gene, and had high similarities to all identified *trz*N gene from different microorganisms.

The pathway of atrazine degradation has been well characterized. Genes including *atz* group (*atz*A, *atz*B, *atz*C, *atz*D, *atz*E, *atz*F) and *trz* group (*trz*N, *trz*D, *trz*E, *trz*F) were acknowledged to be involved in the atrazine-catabolic pathway. *atz*A and *trz*N encoded with atrazine chlorohydrolase which catalyze atrazine to hydroxyatrazine in the first step of atrazine degradation. *atz*D and *trz*D were involved in the ring cleavage by expressing cyanuric acid amidohydrolase and catalyzing cyanuric acid to biuret. These were four key genes used for revealing the taxonomic and functional diversity of atrazine-degrading bacterial communities (Udiković-Kolić et al. 2010; Martinez et al. 2001; Li et al. 2008). These genes could also be used as gene indicators to distinguish different atrazine-degrading pathways conducted by the two different functional groups. Here, *atz*A, *atz*D, *trz*N, and *trz*D were applied to investigate the atrazine-degrading mechanism of functional bacterium *Arthrobacter* sp. C3. Among the four functional genes, only *trz*N was successfully amplified. The sequence of *trz*N amplicon was consistent with that previously found in atrazine-degrading bacteria *Arthrobacter*, *Pseudomonas*, and *Nocardioides* isolates (Sajjaphan et al. 2004; Mongodin et al. 2006; Yamazaki et al. 2008; Topp et al. 2000). By the expression of the enzyme trzN, strain C3 could successfully degrade atrazine to hydroxyatrazine, a chemical without phytotoxic activity. This was consistent with the degradation assay by HPLC–MS, in which only hydroxyatrazine was detected as the catabolite of atrazine degradation. Taken together, our results indicated that the strain *Arthrobacter* sp. C3 degraded atrazine by a hydrolytic dechlorination reaction, which is acknowledged to initiate atrazine degradation. The mineralization of atrazine could be completed by a single bacterial strain which has an integrated atrazine-metabolic pathway, or by a microbial consortium with different functional bacteria. Transformation into hydroxyatrazine by dechlorination was the key process for biodegradation and obligatory for complete mineralization of atrazine. Smith et al. (2005) found that the bacterial consortium including *Agrobacterium tumefaciens*, *Caulobacter crescentus*, *Pseudomonas putida*, *Sphingomonas yaniokuyae*, *Nocardia* sp., *Rhizobium* sp., *Flavobacterium oryzihabitans*, and *Variovorax paradoxus* could effectively mineralize atrazine. But the enrichment culture was destabilized by loss of *Nocardia* sp., which was responsible for dechlorination, and no longer able to degrade atrazine. Topp et al. (2000) also found that six out of their 14 atrazine-degrading isolates could not mineralize atrazine but produce a product coeluted with hydroxyatrzine. Here, the strain C3 could dechlorinate atrazine, the key process of detoxification. It was supposed that other functional bacteria would attend the residual process of mineralization of atrazine.

Conclusion

Overall, a functional bacterium C3, affiliated into the genus *Arthrobacter* in the phylum Actinobacteria, was successfully isolated in this study. The bacterium could dechlorinate atrazine to a non-phytotoxic compound, hydroxyatrzine. *trz*N gene the functional gene participating in the dechlorinating process of the first step of atrazine degradation was successfully amplified. Our results suggested that strain C3 could not mineralize the herbicide, but could detoxify atrazine to hydroxyatrzine. This study

opened an insight of bioremediation of atrazine by functional bacterium *Arthrobacter* sp. C3. The functional isolate *Arthrobacter* sp. C3 is believed to have a great potential to be used in bioremediation of atrazine in the field. Further studies will be conducted by applying the functional bacterium in indigenous bioremediation.

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