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# Isolation and characterization of atrazine-degrading *Arthrobacter* sp. AD26 and use of this strain in bioremediation of contaminated soil

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**Abstract**

A bacterial strain (AD26) capable of utilizing atrazine as a sole nitrogen source for growth was isolated from an industrial wastewater sample by enrichment culture. The 16S rRNA gene sequencing identified AD26 as an *Arthrobacter* sp. PCR assays indicated that AD26 contained atrazine-degrading genes *trzN* and *atzBC*. The *trzN* gene of AD26 only differs from the *trzN* of *Arthrobacter aurescens* TC1 by one base (A→T at 907) and one amino acid (Met→Leu at 303). The specific activity of *trzN* of AD26 in crude cell extract was 0.28 U/mg, which was 1.2 times that of TC1. This strain has shown faster growth and atrazine-degradation rates in atrazine-containing minimal media than two well characterized atrazine-degrading bacteria, *Pseudomonas* sp. ADP and *Arthrobacter aurescens* TC1. After incubating for 48 h at 30°C, the $OD_{600}$ of AD26 reached 2.6 compared with 1.33 of ADP. AD26 was capable of degrading 500 mg/L of atrazine in minimal medium at 95% in 72 h, while the degradative rates by TC1 and ADP were only 90% and 86%, respectively. A bioremediation trial of contaminated soil has indicated that AD26 can degrade as high as 98% of atrazine contained in soil (300 mg/kg) after incubating for 20 d at 26°C, nominating this strain as a good candidate for use in bioremediation programs.

**Key words**: atrazine; biodegradation; *Arthrobacter* sp. AD26; *trzN* gene; bioremediation

## Introduction

Atrazine is an *s*-triazine herbicide that has been used widely over the last 40 years for weed control in crops such as sorghum, maize, and sugarcane. This has resulted in the contamination of the environment. Atrazine has been detected in contaminated areas with concentrations as high as 1,100 μg/g in soil, 16 μg/L in surface water, and 1,500 μg/L in groundwater (Struthers *et al*., 1998). Since it has been implicated as an endocrine disrupting chemical (Singh *et al*., 2004), a rapid elimination of atrazine from the sites of application is crucial for a safe environment. For the remediation of atrazine-contaminated environments, an effective alternative to costly traditional physicochemical techniques is bioremediation (Wackett *et al*., 2002; Silva *et al*., 2004). Since 1985, a number of atrazine-degrading bacterial strains, belonging to diverse genera, have been isolated and characterized for research and bioremediation purposes. These include strains of *Agrobacterium* (Struthers *et al*., 1998), *Nocardia* (Giardi *et al*., 1985), *Rhodococcus* (Behki *et al*., 1993), *Pseudomonas* (Mandelbaum *et al*., 1995), *Rhizobium* (Bouquard *et al*., 1997), *Pseudaminobacter* (Topp *et al*., 2000), *Chelatobacter*, *Aminobacter*, *Stenotrophomonas* (Rousseaux *et al*., 2001), *Ralstonia* (Stamper *et al*., 2002), *Arthrobacter* (Strong *et al*., 2002), *Acinetobacter* (Singh *et al*., 2004), *Nocardioides* (Satsuma, 2006), *Alcaligens* (de Souza *et al*., 1998), and *Sinorhizobium* and *Polaromonas* (Devers *et al*., 2007).

*Pseudomonas* sp. ADP has become the best-studied atrazine-degrading bacterium so far in terms of the biochemistry and molecular biology of the atrazine degradation pathway (Wackett *et al.*, 2002). This strain can use atrazine as the sole source of nitrogen for growth. Atrazine mineralization occurs via six enzymatic steps, controlled by *atzA*, *atzB*, *atzC* genes, and *atzD*EF operon. The first three enzymes AtzA, AtzB, AtzC, catalyze the sequential hydrolytic reactions transforming atrazine to cyanuric acid. Cyanuric acid is then further hydrolyzed to $CO_2$ and $NH_3$ catalyzed by AtzD, AtzE, and AtzF. All six genes are harbored on catabolic plasmid pADP-1, which is completely sequenced (Martinez *et al*., 2001). *Pseudomonas* sp. ADP and the recombinant *Escherichia coli* expressing atrazine chlorohydrolase gene (*atzA*) from strain ADP are used for atrazine remediation in soil (Silva *et al*., 2004; Strong *et al*., 2000).

Another well studied atrazine-degrading bacterium is Gram-positive *Arthrobacter aurescens* TC1, which is able to grow with atrazine as the sole source of nitrogen, carbon, and energy, and metabolizes a wider range of *s*-triazine compounds, such as ametryn and prometryn (Strong *et al*., 2002; Sajjaphan *et al*., 2004). TC1 contains atrazine-degrading genes *trzN*, *atzB*, and *atzC* and degrades atrazine

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to cyanuric acid using the same pathway as *Pseudomonas* sp. strain ADP. The three degradation genes are situated on atrazine catabolic plasmid pTC1 (Mongodin *et al*., 2006). Being the first enzyme of atrazine catabolic pathway of TC1, triazine hydrolase TrzN transforms atrazine to hydroxyatrazine.

In this study, a bacterial strain that can degrade the elevated concentration of atrazine, *Arthrobacter* sp. AD26, was isolated from an industrial wastewater sample and characterized. The aim of this study was to describe the genetic organization and degradative characteristics of this strain and its use in bioremediation of atrazine-contaminated soil.

## 1 Materials and methods

### 1.1 Bacterial strains

Bacterial strain AD26 was isolated from an industrial wastewater sample of Jilin Pesticide Plant in northeast China by enrichment culture in this study. *Pseudomonas* sp. ADP (Mandelbaum *et al*., 1995) and *Arthrobacter aurescens* TC1 (Strong *et al*., 2002) were kindly provided by Dr. L. P. Wackett and Dr. M. J. Sadowsky, respectively. AD26 and TC1 can use atrazine, but not cyanuric acid, as the sole source of nitrogen and sucrose, or sodium citrate as the carbon source for growth. ADP can use atrazine or cyanuric acid as the sole source of nitrogen and sodium citrate, but not sucrose, as the carbon source for growth.

### 1.2 Growth media

Liquid atrazine medium (L) contained 0.9 g $KH_2PO_4$, 6.5 g $Na_2HPO_4{\cdot}12H_2O$, 0.2 g $MgSO_4{\cdot}7H_2O$, 3 g sucrose (for strains AD26 and TC1) or sodium citrate (for strain ADP) as the carbon source, and 0.5 g of atrazine as the sole nitrogen source. Atrazine was added from a 100 mg/ml stock solution (in methanol). The opaque solid medium contained the same mineral salts, carbon source, and atrazine as the liquid medium, and 2% agar.

### 1.3 Identification of strain AD26 by 16S rDNA sequence

The primer pair of 27F (5'-AGAGTTTGATCM TGGCTCAG-3') and 1492R (5'-CGGYTACCTTGTTAC GACTT-3') were used for amplification of 16S rDNA of AD26. The cloning, sequencing, and Blast analysis were conducted as described previously (Cai *et al*., 2003).

### 1.4 PCR assays of atrazine-degrading genes

The atrazine-degrading genes of strain AD26 were detected by PCR with the primers listed in Table 1. DNA amplifications were carried out in a Tpersonal thermocycler (Biometra, Germany) as follows: 5 min at 95°C, 35 cycles of 1 min at 94°C, 1 min at the optimal temperature for primer annealing, and 2 min at 72°C, plus an additional 10 min cycle at 72°C.

### 1.5 Atrazine measurements in liquid media and soils

The atrazine was extracted with 10 ml dichloromethane twice. The extract measurements in liquid media and soils were performed using a Hewlett-Packard 6890 gas chromatography system (Palo Alto, USA) equipped with a flame ionization detector and interfaced to an HP 7999A Chemstation (Strong *et al*., 2000).

### 1.6 Determination of TrzN activity in crude extract of strain AD26

The bacterial cells from 200 ml of AD26 culture were harvested by centrifugation, washed with 50 mmol/L potassium phosphate buffer (pH 8.0), and then resuspended in 20 ml of the same buffer with 20% glycerol. The cells were ruptured in an ice-water bath by sonification at an output of 400 W for 60 periods of 5 s with 15 s intervals. Subsequently, the cell debris was removed by centrifugation at 4°C for 30 min at 10,000 g. The clear supernatant (crude extract) was used for activity determination by monitoring the decrease in absorbance of ametryn for 10 min at 264 nm on a spectrophotometer (Shapir *et al*., 2005). One unit of enzyme was defined as the amount of enzyme that reduced 1 μmol of ametryn per minute at 25°C. Specific activity is expressed as units per milligram

**Table 1** PCR primers, annealing temperatures, and expected product sizes in the amplification of atrazine-degrading genes

| Gene | Primer | Sequence (5'→3') | Annealing (°C) | Size (kb) |
|---|---|---|---|---|
| *atzA* | *atzA*-F | TTGCGGTGCAGGTTTTTCGATG | 65 | 1.5 |
| | *atzA*-R | TGCAGCAACGGCGTCATTTC | | |
| *atzB* | *atzB*-BR1 | TCACCGGGGATGTCGCGGGC | 62 | 0.5[a] |
| | *atzB*-BR2 | CTCTCCCGCATGGCATCGGG | | |
| *atzC* | *atzC*-4BR1 | GCTCACATGCAGGTACTCCA | 46 | 0.6[a] |
| | *atzC*-3FFP | GTACCATATCACCGTTTGCCA | | |
| *atzD* | *atzD*-1 | GGAGACATCATATGTATCACATCGACGTTTTC | 60 | 1.1 |
| | *atzD*-2 | CCAATAAGCTTAGCGCGGGCAATGACTGCA | | |
| *atzE* | *atzE*-1 | TACGCGGTAAAGAATCTGTT | 50 | 1.0[a] |
| | *atzE*-2 | GGAGACCGGCTGAGTGAGA | | |
| *atzF* | *atzF*-1 | CGATCGGAAAAACGAACCTC | 55 | 0.9[a] |
| | *atzF*-2 | CGATCGCCCCATCTTCGAAC | | |
| *trzN* | *trzN*-1 | ATGATCCTGATCCGCGGACTGA | 54 | 1.37 |
| | *trzN*-2 | CTACAAGTTCTTGGGAATGAGTG | | |

[a] Amplification products are the internal regions of the genes.

of protein. Protein concentrations were determined by the Bradford assay using bovine serum albumin as a standard (Bradford, 1976).

### 1.7 Bioremediation of atrazine-contaminated soil

The bacterial cells from 20 ml of AD26, TC1, and ADP cultures were harvested by centrifugation, and washed with 0.9% NaCl and sterile water. Cells of AD26 and TC1 were resuspended in phosphate buffer (0.2 g/L $Na_2HPO_4 \cdot 12H_2O$, 0.1 g/L $KH_2PO_4$) containing 3 g/L sucrose, while ADP cells in the same buffer but containing 3 g/L sodium citrate. The cell densities in these suspensions were adjusted to $10^8$ cells/ml. 10 g of autoclaved soil, 3 mg of atrazine, and 1 ml of each bacterial suspension were mixed in sterile Petri dishes and incubated at 26°C for 20 d. Negative control contained no live cells. The soil was kept damp by spraying with sterilized water daily. Three dishes from each treatment were removed on day 0, 1, 2, 3, 5, 10, 15, and 20, and stored at –20°C.

### 1.8 GenBank accession numbers

The final sequences of 16S rDNA and triazine hydrolase gene *trzN* from strain AD26 were submitted to the GenBank under the accession numbers AF543695 and EU091479, respectively.

## 2 Results and discussion

### 2.1 Isolation and identification of atrazine-degrading strain AD26

Morphological and physiological characterization indicated that AD26 was a Gram-positive yellow-pigmented bacterium capable of utilizing both sucrose and sodium citrate as carbon sources and atrazine (not cyanuric acid) as a sole nitrogen source for growth. The 16S rRNA gene sequencing and Blast analysis showed that the identities of 16S rDNA of AD26 to thirteen other *Arthrobacter* strains are over 99%. These include *Arthrobacter* sp. AD12 (AY628690), *Arthrobacter* sp. AD2 (AY628691), *Arthrobacter* sp. ADH-2 (EF373977), *Arthrobacter* sp. pnp-3 (EF017808), *Arthrobacter* sp. AD3 (AY628689), *Arthrobacter* sp. ADX10 (EF488200), *A. ureafaciens* (X80744), *A. protophormiae* (AY577525), *Arthrobacter* sp. FR3 (AM113709), *Arthrobacter* sp. KI72 (AB262078), *A. keyseri* 12B (AF256196), *Arthrobacter* sp. PZC6 (EF028242), and *Arthrobacter* sp. WY (DQ656488). On the basis of 16S rRNA gene sequencing, AD26 was identified to be an *Arthrobacter* sp.

### 2.2 Detection of atrazine-degrading genes by PCR

Atrazine-degrading genes were amplified by PCR using the primers listed in Table 1. The results indicated that AD26 contained *trzN*, *atzB*, and *atzC* genes (Fig.1). Its composition of the atrazine-degrading genetic potential, therefore, seems to be similar to that of TC1. This is supported by the fact that AD26 cannot use cyanuric acid as the sole nitrogen source for growth. Recent reports proved that a number of bacteria that metabolize atrazine contain

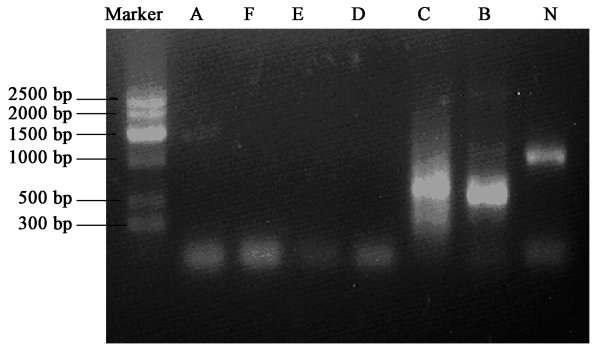


**Fig. 1** Agarose gel electrophoresis of PCR products of atrazine-degrading genes of strain AD26. A: *atzA*; F: *atzF*; E: *atzE*; D: *atzD*; C: *atzC*; B: *atzB*; and N: *trzN*.

*trzN-atzBC* genes. From an agricultural soil, Vibber *et al*. (2007) isolated six bacteria, which are all Gram-positive and group with microorganisms in the genera *Nocardioides* and *Arthrobacter*. These were capable of utilizing atrazine as the sole nitrogen source, whereas none of the isolates could utilize atrazine as the sole carbon and nitrogen source. One isolate contained *trzN-atzBC* and the other five contained *trzN-atzC*. Devers *et al*. (2007) recently characterized seven new atrazine-degrading bacteria and revealed for the first time the occurrence of *trzN-atzBC* gene composition in Gram-negative bacteria such as *Sinorhizobium* sp. and *Polaromonas* sp., Therefor, these results suggested that the *trzN-atzBC* gene composition may be widespread among Gram-positive and Gram-negative atrazine-degrading bacteria.

### 2.3 Sequence of *trzN* gene and activity of TrzN

Sequencing and analysis of the amplified *trzN* gene from AD26 revealed the 1,371 bp DNA fragment encoding a 456-amino acid protein (TrzN). It only differs from the *trzN* of TC1 by one base (A→T at 907), which results in one amino acid change (Met→Leu at 303). Enzyme assay indicated that the specific activity of TrzN of AD26 crude extract was 0.28 U/mg, which was 1.2 times that of TC1 (0.23 U/mg). It is inferred that the amino acid residue variation of AD26 TrzN at 303 was attributed to the increase of enzyme activity.

### 2.4 Biodegradation characteristics of AD26

The $OD_{600}$ of strain AD26 reached 2.6 after 48 h incubation at 30°C when atrazine (500 mg/L) was used as the sole nitrogen source. Under the same conditions, the $OD_{600}$ of strain ADP was only 1.33 (Fig.2). This indicated that AD26 possessed higher growth rate in comparison with ADP. The atrazine degradation rates of AD26, TC1, and ADP were compared and it showed a faster atrazine removal by AD26 than TC1 and ADP (Fig.3). After 36 h, nearly 81% of atrazine was removed by AD26 while only about 60% and 57% of atrazine were removed by TC1 and ADP, respectively. After 72 h, the atrazine remaining in AD26 culture was 5%, while there were still 10% and 14% atrazine remaining in TC1 and ADP cultures, respectively (Fig.3). After 36 h, the growth of AD26 successively reached the stationary phase and death phase, resulting in

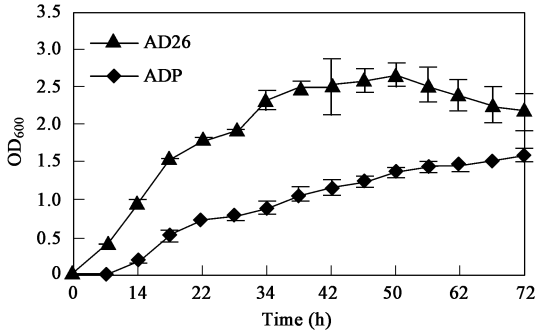


**Fig. 2** Growth curve of strains AD26 and ADP.

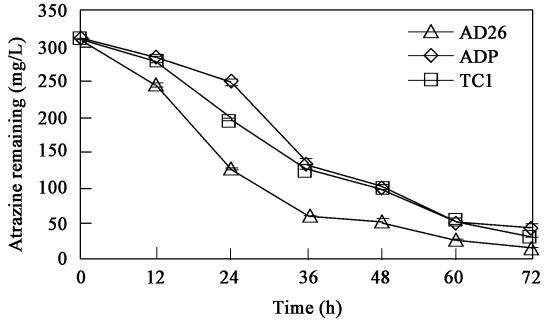


**Fig. 3** Curves of atrazine degradation of strains AD26, TC1, and ADP.

lower degradative rate. These results indicated that AD26 was a highly efficient atrazine-degrading bacterium and possessed great potential in bioremediation of polluted environments.

### 2.5 Bioremediation of atrazine-contaminated soil

Silva *et al*. (2004) and Strong *et al*. (2000) proved that bioaugmentation together with carbon source and phosphate biostimulation markedly increased atrazine degradation in soil by ADP and engineered *Escherichia coli* expressing an atrazine chlorohydrolase gene. To increase atrazine degradation in soil, AD26 and TC1 cells were resuspensed in sucrose (3 g/L)-phosphate (0.3 g/L) solution and were then added into the soil, while ADP cells were resuspensed in sodium citrate (3 g/L)-phosphate (0.3 g/L) solution and were then added into the soil. After incubation at 26°C for 20 d, 98.6% of atrazine was removed by AD26 from soil containing 300 mg/kg of atrazine. In comparison, 96.2% and 88.7% of atrazine were removed by TC1 and ADP, respectively, while only a 14% decline in atrazine was seen in the negative control (CK) without atrazine-degrading bacteria, probably as a result of chemical hydrolysis (Fig.4).

## 3 Conclusions

A highly efficient atrazine-degrading bacterial strain, *Arthrobacter* sp. AD26, was isolated from an industrial wastewater sample. The growth and atrazine-degradation rates of this strain in atrazine-containing minimal media were higher than those of the well studied atrazine-degrading bacteria, *Pseudomonas* sp. ADP, and *Arthrobacter aurescens* TC1.

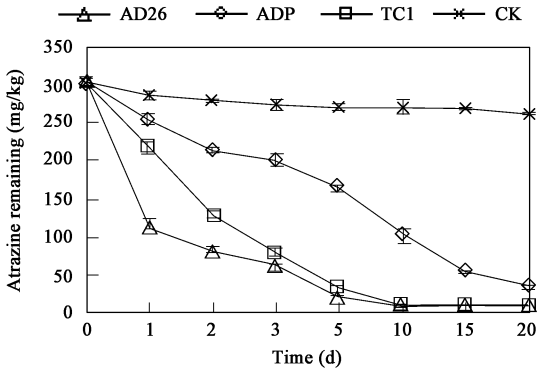


**Fig. 4** Degradation of atrazine in contaminated soil by strains AD26, TC1, and ADP.

*Arthrobacter* sp. AD26 contained atrazine-degrading genes *trzN* and *atzBC*. The *trzN* gene of AD26 only differs from the *trzN* gene of strain TC1 by one base and one amino acid. The specific activity of AD26 TrzN in crude extract was 0.28 U/mg, which was 1.2 times that of TC1.

Strain AD26 was shown to be capable of removing 98% atrazine in a contaminated soil containing the pollutant at 300 mg/kg after incubating at 26°C for 20 d, offering a great potential in the bioremediation of polluted environments.

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## References

Behki R, Topp E, Dick W, Germon P, 1993. Metabolism of the herbicide atrazine by *Rhodococcus* strains. *Appl Environ Microbiol*, 59(6): 1955–1959.

Bouquard C, Ouazzani J, Prome J, Michel-Briand Y, Plesiat P, 1997. Dechlorination of atrazine by a *Rhizobium* sp. isolate. *Appl Environ Microbiol*, 63(3): 862–866.

Bradford M M, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72(1-2): 248–254.

Cai B L, Han Y N, Liu B, Ren Y, Jiang C, 2003. Isolation and characterization of an atrazine-degrading bacterium from industrial wastewater in China. *Lett Appl Microbiol*, 36(5): 272–276.

de Souza M L, Seffernick J, Martinez B, Sadowsky M J, Wackett L P, 1998. The atrazine catabolism genes *atzABC* are widespread and highly conserved. *J Bacteriol*, 1998, 180(7): 1951–1954.

Devers M, Azhari N E, Kolic N U, Martin-Laurent F, 2007. Detection and organization of atrazine-degrading genetic potential of seventeen bacterial isolates belonging to divergent taxa indicate a recent common origin of their catabolic functions. *FEMS Microbiol Lett*, 273(1): 78–86.

Giardi M T, Giardina M C, Filacchioni G, 1985. Chemical and biological degradation of primary metabolism of atrazine by a *Nocardia* strain. *Agricul Biol Chem*, 49(6): 1551–1558.

Mandelbaum R T, Allan D L, Wackett L P, 1995. Isolation and characterization of a *Pseudomonas* sp. that mineralizes the *s*-triazine herbicide atrazine. *Appl Environ Microbiol*, 61(4):

1451–1457.

Martinez B, Tomkins J, Wackett L P, Wing R, Sadowsky M J, 2001. Complete nucleotide sequence and organization of the atrazine catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP. *J Bacteriol*, 183(19): 5684–5697.

Mongodin E F, Shapir N, Daugherty S C, DeBoy R T, Emerson J B, Shvartzbeyn A *et al*., 2006. Secrets of soil survival revealed by the genome sequence of *Arthrobacter aurescens* TC1. *PLoS Genetics*, 2(12): 2094–2106.

Rousseaux S, Hartmann A, Soulas G, 2001. Isolation and characterisation of new Gram-negative and Gram-positive atrazine degrading bacteria from different French soils. *FEMS Microbiol Ecol*, 36(2-3): 211–222.

Sajjaphan K, Shapir N, Wackett L P, Palmer M, Blackmon B, Tomkins J, Sadowsky M J, 2004. *Arthrobacter aurescens* TC1 atrazine catabolism genes *trzN*, *atzB*, and *atzC* are linked on a 160-kilobase region and are functional in *Escherichia coli*. *Appl Environ Microbiol*, 70(7): 4402–4407.

Satsuma K, 2006. Characterisation of new strains of atrazine-degrading *Nocardioides* sp. isolated from Japanese riverbed sediment using naturally derived river ecosystem. *Pest Manag Sci*, 62(4): 340–349.

Shapir N, Rosendahl C, Johnson G, Andreina M, Sadowsky M J, Wackett L P, 2005. Substrate specificity and colorimetric assay for recombinant *trzN* derived from *Arthrobacter aurescens* TC1. *Appl Environ Microbiol*, 71(5): 2214–2220.

Silva E, Fialho A M, Sa-Correia I, Burns R G, Shaw L J, 2004. Combined bioaugmentation and biostimulation to cleanup soil contaminated with high concentrations of atrazine. *Environ Sci Technol*, 38(2): 632–637.

Singh P, Suri C R, Cameotra S S, 2004. Isolation of a member of *Acinetobacter* species involved in atrazine degradation. *Biochem Biophys Res Commun*, 317(3): 697–702.

Stamper D M, Radosevich M, Hallberg K B, Traina S J, Tuovinen O H, 2002. *Ralstonia basilensis* M91-3, a denitrifying soil bacterium capable of using *s*-triazines as nitrogen sources. *Can J Microbiol*, 48(12): 1089–1098.

Strong L C, McTavish H, Sadowsky M J, Wackett L P, 2000. Field-scale remediation of atrazine-contaminated soil using recombinant *Escherichia coli* expressing atrazine chlorohydrolase. *Environ Microbiol*, 2(1): 91–98.

Strong L C, Rosendahl C, Johnson G, Sadowsky M J, Wackett L P, 2002. *Arthrobacter aurescens* TC1 metabolizes diverse *s*-triazine ring compounds. *Appl Environ Microbiol*, 68(12): 5973–5980.

Struthers J K, Jayachandran K, Moorman T B, 1998. Biodegradation of atrazine by *Agrobacterium* radiobacter J14a and use of this strain in bioremediation of contaminated soil. *Appl Environ Microbiol*, 64(9): 3368–3375.

Topp E, Zhu H, Nour S M, Houot S, Lewis M, Cuppels D, 2000. Characterization of an atrazine-degrading *Pseudaminobacter* sp. isolated from Canadian and French agricultural soils. *Appl Environ Microbiol*, 66(7): 2773–2782.

Wackett L P, Sadowsky M J, Martinez B, Shapir N, 2002. Biodegradation of atrazine and related *s*-triazine compounds: from enzymes to field studies. *Appl Microbiol Biotech*, 58(1): 39–45.

Vibber L L, Pressler M J, Colores G M, 2007. Isolation and characterization of novel atrazine-degrading microorganisms from an agricultural soil. *Appl Microbiol Biotechnol*, 75(4): 921–928.