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ORIGINAL ARTICLE

Growth of *Paenarthrobacter aurescens* strain TC1 on atrazine and isopropylamine during osmotic stress

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Abstract

Paenarthrobacter aurescens strain TC1 can use the herbicide atrazine and its degradation product isopropylamine as nutrients. Because osmotic stress can change the morphology of arthrobacters and decrease their metabolism of some carbon compounds, the effects of increasing NaCl concentrations on strain TC1 and its ability to utilize atrazine and isopropylamine were determined. Strain TC1 was cultured in minimal media with different NaCl concentrations and varying combinations of D-glucose, ammonium sulfate, atrazine, or isopropylamine. Growth was measured quantitatively as an increase in turbidity. Physiological effects were assessed using Biolog™ GP test plates and BD BBL Crystal GP or bioMérieux API 20E test systems. The effects of osmoprotective compounds were determined in liquid media and on agar plates. Strain TC1 formed multicellular myceloids and its growth rate slowed as the salt concentration increased, but the culture yields were similar up to 0.6 mol l^{-1} NaCl. The bacteria metabolized about half the carbon sources in Biolog™ GP test plates, but their use of some compounds and several hydrolytic activities decreased with high salt concentrations. However, strain TC1 grew well with atrazine and isopropylamine as the nitrogen source in media containing up to 0.6 mol l^{-1} NaCl. Growth in 0.8 mol l^{-1} NaCl was more limited but could be enhanced by glycine betaine, L-proline, and L-glutamate. *P. aurescens* strain TC1 can continue to use atrazine and isopropylamine as nutrients during osmotic stress and so may be particularly useful for remediation of contaminated soils with low water activity.

Keywords *Arthrobacter aurescens* · Atrazine · Bioremediation · Isopropylamine · Osmotic stress · *Paenarthrobacter aurescens*

Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a broadleaf and grass herbicide that has been commonly used in the USA, Europe, and Asia. Because of its toxic effects as an endocrine disrupter (Hayes et al. 2011), atrazine has now been banned in some developed countries. Whether it has additional effects on development, pregnancy, and cancer is still subject to analysis and debate (Boffetta et al. 2013; Goodman et al. 2014; Van Der Kraak et al. 2014). Because atrazine is chemically stable and highly mobile, it is often found in soils, surface waters, and ground waters long after its initial application (Jablonowski et al. 2011). A number of microorganisms have been identified which can degrade atrazine to less toxic metabolites and may be potentially useful for bioremediation of contaminated sites (Wackett et al. 2002; Ralebitso et al. 2002; Fan and Song 2014; Singh and Singh 2016). These include various Gram-positive bacteria (*Arthrobacter*, *Clavibacter*), Gram-negative bacteria (*Pseudomonas*, *Ralstonia, Shewanella*), and fungi (*Aspergillus*, *Penicillium*) that work individually or in combination (De Souza et al. 1998; Vibber et al. 2007; Satsuma 2009; Zhang et al. 2011; Zhang et al. 2012; Nousiainen et al. 2015; Wang et al. 2016; Ye et al. 2016).

Arthrobacter aurescens strain TC1 was one of the first atrazine-degrading bacteria to be identified and characterized in detail biochemically (Strong et al. 2002). This high G + C Gram-positive aerobe has now been reassigned to the genus *Paenarthrobacter* as *Paenarthrobacter aurescens* (Busse 2016). Strain TC1 uses the products of the genes *trzN*, *atzB*, and *atzC* to remove the chlorine residue and the two *N*-alkylamines from atrazine, leading the formation of cyanuric acid (Shapir et al. 2002; Shapir et al. 2005; Seffernick et al. 2007). Unlike some atrazine-degraders, *P. aurescens* strain

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TC1 does not metabolize cyanuric acid further, but it can use the two *N*-alkylamines (ethylamine and isopropylamine) as carbon, nitrogen, and energy sources (Strong et al. 2002). The genome of strain TC1 has been completely sequenced and consists of a circular chromosome and two large circular plasmids (Mongodin et al. 2006). The genes *trzN*, *atzB*, and *atzC* are located on the larger pTC1 plasmid. Degradation of the *N*-alkylamines can occur in three ways: (1) by copper amine oxidases, (2) by flavoprotein dehydrogenases, and (3) by the Ipu pathway, which involves reaction with glutamate to form γ-glutamyl-isopropylamide, oxidation, deamidation, and amine liberation (de Azevedo Wäsch et al. 2002). There are multiple genes for the first two groups of enzymes on the main chromosome and clusters of genes for the last pathway on the two plasmids (Shapir et al. 2007).

Like other arthrobacters, *P. aurescens* strain TC1 has genes that allow it to survive under a variety of environmental stresses (Mongodin et al. 2006) and so may be particularly suitable for bioremediation. Two key questions in the use of strain TC1 and other atrazine-degraders for bioremediation in situ are whether the genes needed for atrazine and *N*-alkylamine degradation will be expressed efficiently and whether the bacteria will grow under the conditions found at a contaminated site. Soils often vary in pH, salt composition, temperature, and saturation with water. The availability of water varies with the texture and composition of the soil and can be expressed in terms of soil moisture content, water potential, or water activity (Wildman 2016). Bastos and Magan (2009) found that the rates of cellular respiration and atrazine degradation in non-sterile soils varied with the water potential. Soils with reduced water potentials show limited mineralization of pesticides (Schroll et al. 2006) and decreased degradation of metolachlor (Rice et al. 2002) and mesotrione (Su et al. 2017). A decrease in water availability leads to osmotic stress on the microorganisms in the soil, which can result in a loss of intracellular water and the concentration of cellular contents (Brown 1976; Wood 2011). Osmotic stress can be imposed on bacteria in liquid cultures by the addition of salts like NaCl or sugars like sucrose (Sereno et al. 2001). NaCl concentrations of 0.6 mol l^{-1} (3.5% *w*/*v*) or 0.8 mol l^{-1} (4.7% *w*/*v*) reduce the water activity to 0.98 or 0.97, respectively, which is similar to that found in soils with water potentials of −2.78 or −4.19 MPa. Bacteria can adapt to osmotic stress and a decrease in water activity by the synthesis, uptake, and accumulation of a variety of osmotically compatible solutes (Galinski 1995; Kempf and Bremer 1998; Wood et al. 2001).

We have previously shown that imposition of osmotic stress on *Arthrobacter globiformis* strain ATCC 8010 by adding increasing concentrations of NaCl to a liquid medium leads to a reduction in the growth rate and the formation of clusters of branching cells called myceloids (Deutch and Perera 1992). Similar myceloids have been observed in response to vitamin- (Chan et al. 1973) or metal ion-deficiency (Germida and Casida 1980). The salt-induced myceloids of *A. globiformis* differ from normal cells in their physiology and responses to environmental stress (Malwane and Deutch 1999). They also show a reduced ability to degrade less common carbon sources as measured in BIOLOG™ carbon utilization test plates (Malwane and Deutch 1999). Of the 58 compounds that were used by normal cells, 33 showed a gradual or rapid decrease in the absorbance of the tetrazolium product as the salt concentration was increased. The effects of osmotic stress on the genera *Paenarthrobacter*, *Pseudoarthrobacter*, *Glutamicibacter*, *Paeniglutamicibacter*, and *Pseudoglutamicibacter* that were recently separated from the genus *Arthrobacter* sensu stricto (which includes *A. globiformis*) have not been described. In fact, salt tolerance was not used in the description of these new genera (Busse 2016). We hypothesized that *P. aurescens* strain TC1 might differ from *A. globiformis* in its response to osmotic stress and might not be able to degrade atrazine or *N*-alkylamines under conditions of reduced water activity. To answer these questions, we grew *P. aurescens* strain TC1 in the presence of increasing concentrations of NaCl. The data presented here show that strain TC1 can grow on atrazine and isopropylamine in the presence of relatively high salt concentrations and that growth can be enhanced by some compounds known to act as osmoprotectants. This bacterium thus may be particularly useful for bioremediation in soils with low water activity.

Materials and methods

Chemicals

Atrazine (technical USA/Domestic with surfactant) was provided by Syngenta Crop Protection, Inc. (Greensboro, NC, USA). It was added as a finely ground powder to agar media or as a 1% (*w*/*v*) stock solution in methanol to liquid media. Isopropylamine (> 99.5%) was obtained from Sigma Aldrich (St. Louis, MO, USA). All other chemicals and media components were obtained from Sigma Aldrich or Thermo Fisher Scientific (Waltham, MA, USA).

Bacteria and growth conditions

Paenarthrobacter aurescens strain TC1 was acquired from Dr. Michael Sadowsky at the University of Minnesota and stored at − 80 °C in 20% (*v*/*v*) glycerol. The ability of the bacteria to degrade atrazine was periodically confirmed by streaking them on agar plates with atrazine as the sole nitrogen source. Active stocks were grown at 30 °C on plates of tryptic soy broth agar containing 0.5% (*w*/*v*) yeast extract. The morphology of the bacteria was routinely checked by phase-contrast light microscopy with a Nikon Alphaphot microscope and a 100X oil immersion objective.

Liquid cultures were usually grown at 30 °C with aeration in 300 ml nephelometer flasks containing less than 1/10 volume of minimal R medium (Strong et al. 2002; Anon., American Type Culture Collection 2016) supplemented with 0.2% (*w*/*v*) D-glucose and 10 mmol l^{-1} $(NH_4)_2SO_4$. Culture turbidities were followed in a Klett-Summerson colorimeter with a red (660 nm) filter. The medium was modified by the addition of atrazine, isopropylamine, NaCl, or osmolytes as specified in each experiment. For some experiments, atrazine and isopropylamine were used at equivalent concentrations of 300 ppm (0.03%) since the degradation of one molecule of atrazine yields one molecule of isopropylamine. For other experiments, isopropylamine and ammonium sulfate were used at equivalent concentrations of 10 mmol l^{-1}. For liquid cultures containing atrazine, which were inherently turbid due to the insolubility of the substrate, the bacteria were grown in 5 ml of R medium in 25-ml flasks and then filtered through glass Pasteur Pipets containing a 1–2-cm plug of glass wool (Strong et al. 2002). The absorbance of the filtrates was measured in a Shimadzu UV 160U UV-visible spectrophotometer at 600 nm as an indicator of cell growth. Controls showed that this filtration step effectively removed the atrazine and let the bacteria through. For growth on agar plates, the R medium was solidified with 2% (*w*/*v*) Bacto-agar. To test the effect of potential osmolytes on the use of atrazine as a nitrogen source, the R medium agar contained 0.2% (*w*/*v*) D-glucose, 0.8 mol l^{-1} NaCl, and 0.03% atrazine. Some of the plates were spread with 100 μl of 0.1 mol l^{-1} glycine betaine, 0.1 mol l^{-1} L-proline, 0.1 mol l^{-1} choline, 0.1 mol l^{-1} trehalose, or 0.1 mol l^{-1} potassium L-glutamate prior to inoculation. For the determination of viable cell counts, bacterial cultures were serially diluted in 0.85% (*w*/*v*) NaCl and 100 μl aliquots spread on duplicate plates of tryptic soy broth agar containing 0.5% (*w*/*v*) yeast extract. Colonies were counted after incubation for 3 days at 30 °C. All growth and viability experiments were done at least three times.

Carbon source utilization and physiological experiments

Paenarthrobacter aurescens strain TC1 was grown in 10 ml of R medium with varying concentrations of NaCl, 0.2% (*w*/*v*) D-glucose and 10 mmol l^{-1} $(NH_4)_2SO_4$ at 30 °C with aeration to exponential phase in nephelometer flasks. The bacteria were harvested by centrifugation at 10,000×*g*, washed once with 0.85% (*w*/*v*) NaCl, and resuspended in Biolog™ inoculating fluid containing the same concentration of NaCl to give a concentration equivalent to 40 Klett Units with a red (660 nm) filter (OD_{600} = 0.6 or 25% transmittance at 600 nm). The wells of a standard Biolog™ GP test plate (Biolog, Inc., Hayward, CA, USA) were inoculated with 150 μl of bacteria and the plates incubated at 30 °C. The absorbance of each well at 595 nm was recorded after 3 days, and the value for the no carbon source control well subtracted from each of the other values. Absorbance values < 0.1 greater than the control were recorded as no use, values from 0.1 to 0.5 greater than the control were recorded as low use, and values > 0.5 greater than the control were recorded as high use. In similar experiments, exponential-phase cells grown in R medium with varying concentrations of NaCl, 0.2% (*w*/*v*) D-glucose, and 10 mmol l^{-1} $(NH_4)_2SO_4$ at 30 °C were harvested, washed, and resuspended in R medium with the same concentration of NaCl to give a concentration equivalent to 40 Klett Units. The cell suspensions were used to inoculate BD BBL Crystal GP identification test systems (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) or API 20E test systems (bioMérieux, Inc., Durham, NC, USA). The test systems were incubated at 30 °C for 2 or 3 days and scored as directed by the manufacturer. All physiological tests were done at least three times.

Results

Effects of osmotic stress on the growth and metabolism of *P. aurescens* strain TC1

Because the consequences of osmotic stress for *Paenarthrobacter aurescens* have not been previously described, we first assessed the effects of increased salt concentrations on the growth, morphology, and metabolism of *P. aurescens* strain TC1. The bacteria were grown in minimal R medium containing 0.2% (*w*/*v*) D-glucose as the carbon source, 10 mmol l^{-1} $(NH_4)_2SO_4$ as the nitrogen source, and increasing concentrations of NaCl (Fig. 1). As the salt concentration was raised, there was a somewhat longer lag phase and a gradual decrease in the growth rate. The yield of the cultures was similar with salt concentrations up to 0.6 mol l^{-1} NaCl but dropped with higher concentrations of NaCl (0.8 mol l^{-1}). We previously showed that *A. globiformis* strain ATCC 8010 could tolerate NaCl concentrations up to 1.2 mol l^{-1} when grown in the more enriched EYG medium containing yeast extract (Deutch and Perera 1992) and the same was true for *P. aurescens* strain TC1 (data not shown). In the absence of added salt, strain TC1 formed multicellular myceloids during the early stages of growth, grew as bent rods during exponential phase, and divided into shorter cocci during stationary phase like *A. globiformis*. In the presence of increasing concentrations of NaCl, the bacteria formed myceloids with short branches during both exponential phase and stationary phase and often aggregated into large clusters. The morphology of the myceloids of *P. aurescens* strain TC1 as seen by phase-contrast light microscopy was similar to that found with *A. globiformis* (Deutch and Perera 1992).

Because previous studies of *A. globiformis* strain ATCC 8010 indicated that osmotic stress altered its ability to utilize

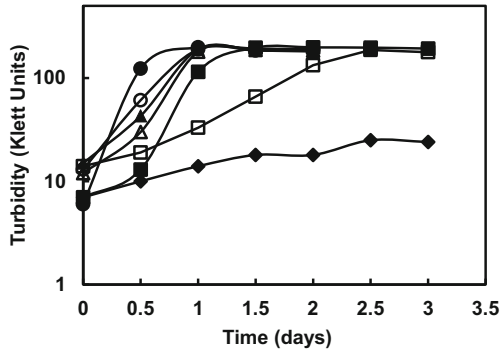

Fig. 1 Growth of *P. aurescens* strain TC1 at 30 °C with aeration in minimal R medium containing 0.2% (*w*/*v*) D-glucose, 10 mmol l^{-1} $(NH_4)_2SO_4$, and 0 NaCl (black circle), 0.1 mol l^{-1} NaCl (white circle), 0.2 mol l^{-1} NaCl (black triangle), 0.3 mol l^{-1} NaCl (white triangle), 0.4 mol l^{-1} NaCl (black square), 0.6 mol l^{-1} NaCl (white square), and 0.8 mol l^{-1} NaCl (black diamond). Turbidities were measured in Klett-Summerson colorimeter with a red (660 nm) filter

some potential carbon sources (Malwane and Deutch 1999), the effects of increasing concentrations of NaCl on the metabolism in *P. aurescens* strain TC1 were determined. The bacteria first were tested for carbon source utilization in Biolog™ GP2 plates containing 95 different compounds (Bochner and Savageau 1977; Bochner 1989) after growth in 0, 0.2 mol l^{-1}, or 0.4 mol l^{-1} NaCl. It was not possible to test the bacteria after growth in 0.6 mol l^{-1} NaCl because there was no color development in the wells after adding bacteria in inoculating fluid containing this higher salt concentration. The carbon compounds were divided into five groups: (1) those that were used at high efficiency at all salt concentrations (A_{595} of > 0.5 compared to the control well), (2) those that were used were low efficiency at all salt concentrations (A_{595} of 0.1 to 0.5 compared to the control well), (3) those that were used with decreasing efficiency as the salt concentration was increased, (4) those that were used with increasing efficiency as the salt concentration was increased, and (5) those that were not used at all (Table 1). *Paenarthrobacter aurescens* strain TC1 only used 48 of the 95 possible carbon sources (50%), which was less than was found with *A. globiformis* strain ATCC 8010 (58/95, 61%, Malwane and Deutch 1999). The metabolism of simple sugars, organic acids, and amino acids that directly enter the glycolytic pathway or the citric acid cycle including D-fructose, D-gluconate, D-glucose, D-mannose, acetic acid, 2-oxoglutarate (α-ketoglutarate), L-lactate, L-malate, succinate, L-glutamate, asparagine, and glycerol remained high after growth in the presence of 0.4 mol l^{-1} NaCl. The metabolism of some disaccharides (cellobiose, D-trehalose, sucrose) and amino acids (D-alanine, L-alanine) decreased as the salt concentration was increased. Interestingly, there were a few compounds including D-galacturonic acid, L-arabinose, and D-malic acid whose utilization was higher in 0.2 mol l^{-1} NaCl than in the absence of added salt. However, the use of some polymers (β-cyclodextrin, dextrin, mannan) did not occur. The reduction in the utilization of some carbon sources in response to osmotic stress and the inability of *P. aurescens* strain TC1 to degrade some polymers suggested there might be a problem in the utilization of atrazine or its products.

The carbon utilization studies were extended by examining the properties of *P. aurescens* strain TC1 in the BD BBL Crystal GP and in the bioMérieux API 20E test systems. Arthrobacters are commonly thought to be non-fermenters and to grow only by aerobic respiration (Morris 1960), so most of the wells in these test systems gave negative results. These systems, on the other hand, contain more tests for hydrolytic activity and so might be better predictors of the ability of the bacteria to degrade complex molecules like atrazine into their constituent parts. With the BD BBL Crystal GP system, hydrolysis of several glycosides (4-MU-β-D-glucoside, 4-MU-α-D-glucoside, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-α-D-maltoside) occurred in 0.2 or 0.4 mol l^{-1} NaCl but decreased in the presence of 0.6 mol l^{-1} NaCl. With the bioMérieux API 20E system, there was weak hydrolysis of ONPG, weak utilization of citrate, and weak fermentation of D-glucose and L-arabinose in the absence of NaCl and the intensity of these reactions decreased after growth in higher concentrations of salt. There was good hydrolysis of gelatin with bacteria grown in 0 or 0.2 mol l^{-1} NaCl but no breakdown of this polymer by bacteria grown in 0.4 or 0.6 mol l^{-1} NaCl. The decrease in hydrolytic activities in *P. aurescens* strain TC1 grown in high salt concentrations again suggested there might be a problem in atrazine utilization since this process requires the removal of the *N*-alkylamines from the ring structure.

Effects of osmotic stress on the growth of *P. aurescens* strain TC1 with atrazine and isopropylamine

Paenarthrobacter aurescens strain TC1 was originally isolated from a spill site in South Dakota USA after extracting the microorganisms from soil and directly plating them on minimal R medium agar plates containing 500 mg l^{-1} (500 ppm, 0.05%) of atrazine as the sole carbon and nitrogen source (Strong et al. 2002). To determine if strain TC1 can degrade atrazine and isopropylamine during osmotic stress, the bacteria first were streaked or spotted onto agar plates containing minimal R medium supplemented with different concentrations of NaCl, 0.2% (*w*/*v*) D-glucose as the primary carbon source, and 10 mmol l^{-1} $(NH_4)_2SO_4$, 10 mmol l^{-1} isopropylamine, or 300 ppm (0.03%) atrazine as the nitrogen source. After 7 days at 30 °C, streaks and colonies or spots were visible on all the plates at salt concentrations up to 0.6 mol l^{-1} NaCl.

To assess the growth of *P. aurescens* strain TC1 quantitatively, the bacteria were grown in three replicate 5-ml cultures in minimal R medium with 0.2% (*w*/*v*) D-glucose and 300 ppm (0.03%) atrazine or 300 ppm (0.03%) isopropylamine as the nitrogen source. After 7 days, the

Table 1 Utilization of carbon sources by *P. aurescens* strain TC1 in Biolog™ GP2 plates

Carbon compounds used with high efficiency	Carbon compounds used with low efficiency	Carbon compounds used with decreasing efficiency	Carbon compounds used with increasing efficiency	Carbon compounds not used
Tween 40	D-Galactose	Dextrin	L-Arabinose	α-Cyclodextrin
Tween 80	D-Psicose	N-Acetyl-D-Glucosamine	D-Galacturonic acid	β-Cyclodextrin
D-Fructose	D-Ribose	Arbutin	D-Malic Acid	Glycogen
D-Gluconic Acid	D-Sorbitol	D-Cellobiose		Inulin
D-Glucose	D-Xylose	Maltotriose		Mannan
D-Mannitol	*p*-Hydroxy-Phenyl-acetic Acid	Palatinose		N-Acetyl-β-D-Mannosamine
D-Mannose	Succinic Acid Mono-methyl Ester	Salacin		Amygdalin
Acetic Acid	Propionic Acid	Sucrose		D-Arabinol
β-Hydroxybutryic Acid	Pyruvic Acid	D-Trehalose		L-Fucose
α-Ketoglutaric Acid	N-Acetyl-L-Glutamic Acid	Turanose		Gentibiose
L-Lactic Acid	L-Alaninamide	D-Alanine		m-Inositol
L-Malic Acid	Glycyl-L-Glutamic Acid	L-Alanine		α-D-Lactose
Pyruvic Acid Methyl ester	L-Serine	L-Alanylglycine		Lactulose
Succinic acid				Maltose
L-Asparagine				D-Melezitose
L-Glutamic Acid				D-Melibiose
L-Pyroglutamic Acid				α-Methyl-D-Galactoside
Putrescine				β-Methyl-D-Galactoside
Glycerol				3-Methyl-Glucose
				α-Methyl-D-Glucoside
				β-Methyl-D-Glucoside
				α-Methyl-D-Mannoside
				D-Raffinose
				L-Rhamnose
-				Sedoheptulosan
				Stachyose
				D-Tagatose
				Xylitol
				α-Hydroxybutyric acid
				γ-Hydroxybutyric acid
				α-Ketovaleric Acid
				Lactamide
				D-Lactic Acid Methyl Ester
				Succinamic Acid
				2,3-Butandiol
				Adenosine
				2'-Deoxyadenosine
				Inosine
				Thymidine
				Uridine
				Adenosine-5'-Monophosphate
				Thymidine-5'-Monophosphate
				Uridine-5'-Monophosphate
				D-Fructose-6-Phosphate
				α-D-Glucose-1-Phosphate
				Glucose-6-Phosphate
				D-L-α-Glycerolphosphate

The test plates were inoculated with bacteria after growth in R medium containing 0, 0.2 mol l^{-1} NaCl, or 0.4 mol l^{-1} NaCl and resuspension in Biolog™ inoculation fluid containing the same salt concentration to give equivalent turbidities. The absorbance of each well at 595 nm was determined after 3 days at 30° C. "High efficiency" indicates an absorbance >0.5 greater than the control at all salt concentrations, "low efficiency" indicates an absorbance of 0.1-0.5 greater than the control at all salt concentrations, "decreasing efficiency" indicates that the absorbance declined as the salt concentration increased, "increased efficiency" indicates that the absorbance increased as the salt concentration increased, and "not used" indicates an absorbance <0.1 greater than the control (no substrate) well.

cultures were filtered through glass wool to remove the residual insoluble material (Strong et al. 2002) and the turbidity of the filtrate determined at 600 nm as a measure of cell growth. The final yield (A_{600}) in medium containing 10 mmol l^{-1} $(NH_4)_2SO_4$ was about 1.4 at all NaCl concentrations tested. Growth with atrazine and isopropylamine was less but persisted in NaCl concentrations as high as 0.6 mol l^{-1} (Fig. 2). Growth was barely detectable in 0.9 mol l^{-1} NaCl. The final turbidity (A_{600}) of about 0.3 with atrazine and isopropylamine in the absence of salt was similar to that seen by Strong et al. (2002) when 0.05% atrazine was added to R medium as the sole carbon and nitrogen source.

Because of the insolubility of the atrazine and the possible loss of bacteria (particularly the myceloids) during the filtration step, these experiments were difficult to do quantitatively. We therefore used isopropylamine as a substitute for atrazine in most of the remaining experiments and measured culture turbidities directly in nephelometer flasks with a Klett-Summerson colorimeter. Growth of *P. aurescens* strain TC1 with 20 mmol l^{-1} isopropylamine as the nitrogen source and 0.2% (*w*/*v*) D-glucose as the carbon source was slower than that shown in Fig. 1 with $(NH_4)_2SO_4$ but continued even in the presence of 0.8 mol l^{-1} NaCl (Fig. 3a). With 50 mmol l^{-1} isopropylamine as the sole carbon and nitrogen source, growth was even slower but again possible in the presence of 0.6 mol l^{-1} NaCl (Fig. 3b). Thus, *P. aurescens* strain TC1 can metabolize this compound even during osmotic stress. The appearance of the bacteria after growth in media containing isopropylamine in the absence of NaCl (normal bent rods) or in the presence of 0.2 to 0.8 mol l^{-1} NaCl (myceloids) as seen by-phase contrast microscopy was similar to that seen when ammonium sulfate was used as the nitrogen source.

Effects of osmolytes on the growth by *P. aurescens* strain TC1 with isopropylamine and atrazine

Many bacteria can adapt to osmotic stress through the cytoplasmic accumulation of small organic compounds called osmolytes by endogenous synthesis or by transport from the environment (Galinski 1995; Kempf and Bremer 1998; Wood et al. 2001). Species of *Arthrobacter* have been shown to accumulate trehalose and glycine betaine as osmoprotectants (Zevenhuizen 1992; Časaitė et al. 2006; Časaitė et al. 2011). Analysis of the genome of *P. aurescens* strain TC1 has indicated that it has the potential to form transporters for glycine betaine and L-proline as well as enzymes needed to convert choline to glycine betaine (Mongodin et al. 2006). To determine if the growth of strain TC1 during osmotic stress could be stimulated by compounds known to function as osmolytes, cultures were grown in minimal R medium containing 0.8 mol l^{-1} NaCl, 0.2% (*w*/*v*) D-glucose, 10 mmol l^{-1} $(NH_4)_2SO_4$, and 1 mmol l^{-1} concentrations of L-proline, glycine betaine, choline, L-glutamate, and trehalose. There was good stimulation of growth by glycine betaine and L-proline, some stimulation by L-glutamate, and little stimulation by choline or trehalose (Fig. 4a). To determine if these compounds might also affect the utilization of isopropylamine, *P. aurescens* strain TC1 was grown in R medium containing 0.8 mmol l^{-1} NaCl, 0.2% (*w*/*v*) D-glucose, 10 mmol l^{-1} isopropylamine as the nitrogen source, and 1 mmol l^{-1} concentrations of glycine betaine, L-proline, choline, L-glutamate, and trehalose. Again, there was good stimulation of growth by glycine betaine and L-proline, some stimulation by L-glutamate, and none by other compounds (Fig. 4b).

To determine if these osmoprotectants could also improve the growth of *P. aurescens* strain TC1 on atrazine, the bacteria were streaked or spotted on agar plates of R medium containing 0.8 mol l^{-1} NaCl, 0.2% (*w*/*v*) D-glucose, and 0.03% atrazine as the nitrogen source. Some of the plates had been previously spread with 100 μl of 0.1 mol l^{-1} glycine betaine, 0.1 mol l^{-1} L-proline, 0.1 mol l^{-1} choline, 0.1 mol l^{-1} trehalose, or 0.1 mol l^{-1} potassium L-glutamate. There was little growth on the unsupplemented plates, but growth was more obvious on the plates containing glycine betaine, L-proline, or L-glutamate. The other compounds had little effect.

In an attempt to quantify these results, *P. aurescens* strain TC1 also was grown overnight in liquid R medium and diluted 1/100 into 10 ml of liquid R medium containing 0.8 mol l^{-1} NaCl, 0.2% (*w*/*v*) D-glucose, 0.03% atrazine as the nitrogen source, and 0.1 mol l^{-1} glycine betaine, 0.1 mol l^{-1} L-proline, 0.1 mol l^{-1} choline, 0.1 mol l^{-1} trehalose, or 0.1 mol l^{-1} potassium L-glutamate. The turbidity and viable cell count in each culture were determined initially and again after 7 and 14 days. All of the cultures began with a relatively high turbidity [about 80 Klett $Units_{(600\ nm)}$] due to the insolubility of the atrazine and only showed a gradual increase over the next 2 weeks. Examination of the cultures by phase-contrast light microscopy indicated that there was growth of the bacteria in the cultures supplemented with L-proline, glycine betaine, or L-glutamate as shown by the formation of clusters of branching myceloid cells. These myceloids were similar to those seen in cultures with ammonium sulfate or isopropylamine as the nitrogen source. The viable cell counts remained relatively constant in the unsupplemented culture or in those supplemented with choline, trehalose, or L-glutamate for the first week and then began to decrease. The viable cell count for the cultures supplemented with glycine betaine increased about threefold in the cultures in the first week and then began to decrease. The

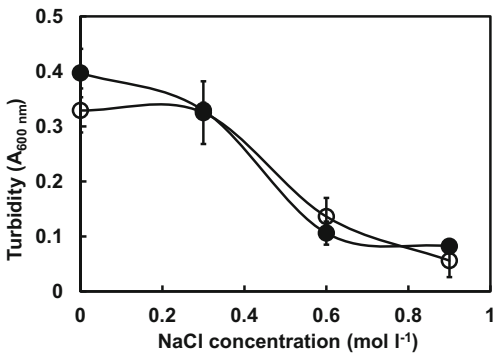

Fig. 2 Growth of *P. aurescens* strain TC1 at 30 °C with aeration in minimal R medium containing 0.2% (*w*/*v*) D-glucose, varying concentrations of NaCl, and 300 ppm atrazine (white circle) or 300 ppm isopropylamine (black circle) as the sole nitrogen source. After 7 days, three replicate samples were filtered through glass wool and the turbidities of the filtrates measured at 600 nm in a Shimadzu UV160U UV-visible spectrophotometer. The results show the means and standard deviations of the replicates at each salt concentration

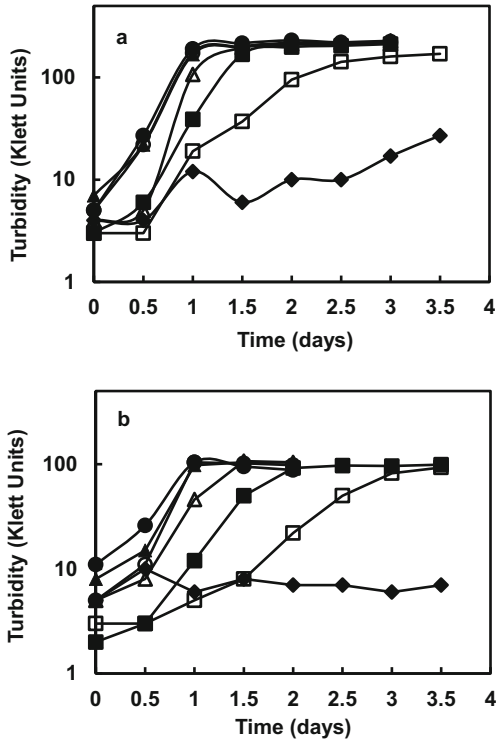

Fig. 3 **a** Shows the growth of *P. aurescens* strain TC1 at 30 °C with aeration in minimal R medium containing 0.2% (*w*/*v*) D-glucose, 20 mmol l^{-1} isopropylamine as the sole nitrogen source, and 0 NaCl (black circle), 0.1 mol l^{-1} NaCl (white circle), 0.2 mol l^{-1} NaCl (black triangle), 0.3 mol l^{-1} NaCl (white triangle), 0.4 mol l^{-1} NaCl (black square), 0.6 mol l^{-1} NaCl (white square), and 0.8 mol l^{-1} NaCl (black diamond). **b** Shows the growth of *P. aurescens* strain TC1 at 30 °C with aeration in R medium containing 50 mmol l^{-1} isopropylamine as the sole carbon and nitrogen source and 0 NaCl (black circle), 0.1 mol l^{-1} NaCl (white circle), 0.2 mol l^{-1} NaCl (black triangle), 0.3 mol l^{-1} NaCl (white triangle), 0.4 mol l^{-1} NaCl (black square), 0.6 mol l^{-1} NaCl (white square), and 0.8 mol l^{-1} NaCl (black diamond). Turbidities were measured in Klett-Summerson colorimeter with a red (660 nm) filter

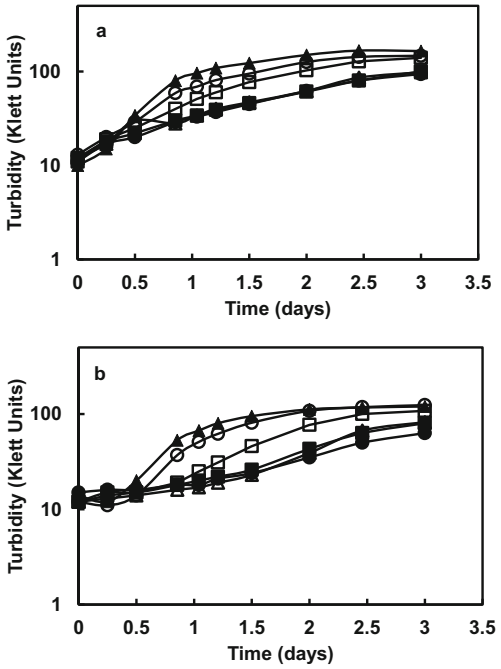

Fig. 4 **a** Shows the effects of osmolytes on the growth of *P. aurescens* strain TC1 at 30 °C with aeration in minimal R medium containing 0.8 mol l^{-1} NaCl, 0.2% (*w*/*v*) D-glucose, 10 mmol l^{-1} $(NH_4)_2SO_4$, and no added supplement (black circle), 1 mmol l^{-1} L-proline (white circle), 1 mmol l^{-1} glycine betaine (black triangle), 1 mmol l^{-1} choline (white triangle), 1 mmol l^{-1} trehalose (black square), or 1 mmol l^{-1} L-glutamate (white square). **b** Shows the effects of osmolytes on the growth of *P. aurescens* strain TC1 at 30 °C with aeration in minimal R medium containing 0.8 mol l^{-1} NaCl, 0.2% (*w*/*v*) D-glucose, 20 mmol l^{-1} isopropylamine as the sole nitrogen source, and no added supplement (black circle), 1 mmol l^{-1} L-proline (white circle), 1 mmol l^{-1} glycine betaine (black triangle), 1 mmol l^{-1} choline (white triangle), 1 mmol l^{-1} trehalose (black square), or 1 mmol l^{-1} L-glutamate (white square)

viable cell count in the culture supplemented with L-proline remained constant for 2 weeks. A major complication in these experiments was that the myceloids formed in media containing 0.8 mol l^{-1} NaCl still formed single colonies on an agar plate even though they were composed of multiple cellular units and tended to aggregate into clusters.

Discussion

Because of the stability and potential toxicity of atrazine, there has been a great deal of interest in the use of microorganisms for bioremediation of contaminated sites (Wackett et al. 2002; Ralebitso et al. 2002; Fan and Song 2014; Singh and Singh 2016). Among the factors that might limit degradation of atrazine are the soil moisture content (Issa and Wood 2005; Moreno et al. 2007; Bastos and Magan 2009), the pH (Weber 1993), and presence of other carbon and nitrogen sources (Assaf and Turco 1994; Gebendinger and Radosevich 1999; Zhang et al. 2014). We have focused on the issue of water availability and used increasing concentrations of NaCl as a way to reduce water activity. Our studies indicated that *P. aurescens* strain TC1 could grow in a minimal medium with ammonium as the nitrogen source at salt concentration up to 0.8 mol l^{-1}. NaCl concentrations of 0.6 mol l^{-1} (3.5%) or 0.8 mol l^{-1} (4.7%) reduce the water activity to 0.98 or 0.97, respectively. These low water activities limit the growth of many bacteria (Wildman 2016), and we have found that they can reduce the use of many potential carbon sources by arthrobacters (Malwane and Deutch 1999). *P. aurescens* strain TC1 used fewer compounds in the Biolog™ GP2 test plates than did *A. globiformis* strain ATCC 8010 and showed decreased hydrolytic activities in the BD BBL Crystal GP and API 20E test systems after growth in high concentrations of NaCl. However, use of atrazine and isopropylamine as a nitrogen source by *P. aurescens* strain TC1 still occurred in 0.6 mol l^{-1} or 0.8 mol l^{-1} NaCl. Thus, the answer to the general question of whether *P. aurescens* strain TC1 can continue to degrade these compounds during osmotic stress is yes.

Shapir et al. (1998) found that growth of the atrazine-degrading strain *Pseudomonas* sp. ADP in a saline atrazine medium decreased as the NaCl concentration increased. Growth was stimulated by prior exposure to 0.1% NaCl and by the presence of glycine betaine. Glycine betaine and other osmotically compatible solutes also have been found to stimulate microorganisms used in the treatment of anaerobic sludges (Vyrides and Stuckey 2017). We found that the use of isopropylamine and atrazine in the presence of 0.8 mol l^{-1} NaCl was stimulated by glycine betaine and L-proline. This suggests *P. aurescens* strain TC1 may be useful for bioremediation of atrazine-contaminated sites, particularly if the soil contains osmoprotective compounds from plant material or these are added as amendments. Strain TC1 is limited as an agent for bioremediation in that it cannot break down cyanuric acid. It thus may be helpful to combine *P. aurescens* strain TC1 with other microbes that can degrade this compound.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Human or animal participants No humans or animals were used in this project.

References

Anon., American Type Culture Collection, Manassas, VA, USA (2016) https://www.atcc.org/~/media/A4CF742B46514D579AAEB111AA55E7F9.ashx. Accessed 25 Aug 2016

Assaf NA, Turco RF (1994) Influence of carbon and nitrogen application on the mineralization of atrazine and its metabolites in soil. Pestic Sci 41:41–47

Bastos AC, Magan N (2009) *Trametes versicolor*: potential for atrazine bioremediation in calcareous clay soil, under low water availability conditions. Int Biodeterior Biodegradation 63:389–394

Bochner BR (1989) Sleuthing out bacterial identifies. Nature 339:157–128

Bochner BR, Savageau MA (1977) Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms. Appl Environ Microbiol 33:434–444

Boffetta P, Adami HO, Berry SC, Mandel JS (2013) Atrazine and cancer: a review of the epidemiological evidence. Eur J Cancer Prevent 22: 169–180

Brown AD (1976) Microbial water stress. Bacteriol Rev 40:803–846

Busse HJ (2016) Review of the taxonomy of the genus *Arthrobacter*, emendation of the genus *Arthrobacter sensu lato*, proposal to reclassify selected species of the genus *Arthrobacter* in the novel genera *Glutamicibacter* gen. nov., *Paeniglutamicibacter* gen. Nov., *Pseudoglutamibacter* gen. nov., *Paenarthrobacter* gen. nov., and *Pseudarthrobacter* gen. nov. and emended description of *Arthrobacter roseus*. Int J System Evol Microbiol 66:9–37

Časaitė V, Bružytė S, Meškys R (2006) Effect of glycine betaine on osmoadaptation of *Arthrobacter* strains. Biologija 4:46–48

Časaitė V, Povilonienė S, Mešklenė R, Rutkienė R, Meškys R (2011) Studies of dimethylglycine oxidase isoenzymes in *Arthrobacter globiformis* cells. Curr Microbiol 62:1267–1273

Chan ECS, Zyk B, Gomersall M (1973) Biotin deficiency in *Arthrobacter globiformis*: comparative cell ultrastructure and nonreplacement of the compound by structurally unrelated compounds. J Bacteriol 113: 394–402

de Azevedo Wäsch SI, van der Ploeg JR, Maire T, Lebreton A, Kiener A, Leisinger T (2002) Transformation of isopropylamine to L-alaninol by *Pseudomonas* sp. strain KIE171 involves *N*-glutamylated intermediates. Appl Environ Microbiol 68:2368–2374

De Souza ML, Newcombe D, Alvey S, Crowley DE, Hay A, Sadowsky MJ, Wackett LP (1998) Molecular basis of a bacterial consortium: interspecies catabolism of atrazine. Appl Environ Microbiol 64: 178–184

Deutch CE, Perera GS (1992) Myceloid cell formation in *Arthrobacter globiformis* during osmotic stress. J Appl Bacteriol 72:493–499

Fan X, Song F (2014) Bioremediation of atrazine: recent advances and promises. J Soils Sediments 14:1727–1737

Galinski EA (1995) Osmoadaptation in bacteria. Adv Microbial Physiol 37:273–328

Gebendinger N, Radosevich M (1999) Inhibition of atrazine degradation by cyanazine and exogenous nitrogen in bacterial isolate M91-3. Appl Microbiol Biotechnol 51:375–381

Germida JJ, Casida LE Jr (1980) Myceloid growth of *Arthrobacter globiformis* and other *Arthrobacter* species. J Bacteriol 144:1152–1158

Goodman M, Mandel JS, DeSesso JM, Scialli AR (2014) Atrazine and pregnancy outcomes: a systematic review of epidemiological evidence. Birth Defects Res B Dev Reprod Toxicol 101:215–236

Hayes TB, Anderson LL, Beasley VR, de Solla SR, Iguchi T, Ingraham H, Kestemont P, Kniewald J, Kniewald Z, Langlois VS, Luque EH, McCoy KA, Muñoz-de-Toro M, Oka T, Oliveira CA, Orton F, Ruby S, Suzawa M, Tavera-Mendoza LE, Trudeau VL, Victor-Costa AB, Willingham E (2011) Demasculinization and feminization of male gonads by atrazine: consistent effects across vertebrate classes. J Steroid Biochem Mol Biol 127:64–73

Issa S, Wood M (2005) Degradation of atrazine and isoproturon in surface and sub-surface soil materials undergoing different moisture and aeration conditions. Pest Manag Sci 61:126–132

Jablonowski ND, Schäffer A, Burauel P (2011) Still present after all these years: persistence plus potential toxicity raise questions about the use of atrazine. Environ Sci Pollut Res 18:328–331

Kempf B, Bremer E (1998) Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. Arch Microbiol 170:319–330

Malwane S, Deutch CE (1999) Adaptive characteristics of salt-induced myceloids of *Arthrobacter globiformis*. Antonie Van Leeuwenhoek 75:335–344

Mongodin EF, Shapir N, Daugherty SC, DeBoy RT, Emerson JB, Shvartzbeyn A, Radune D, Vamathevan J, Riggs F, Grinberg V, Khouri H, Wackett LP, Nelson KE, Sadowsky MJ (2006) Secrets of soil survival revealed by the genome sequence of *Arthrobacter aurescens* TC1. PLoS Genet 2:2094–2105

Moreno JL, Aliaga A, Navarro S, Hernández T, García C (2007) Effects of atrazine on microbial activity in semiarid soil. Appl Soil Ecol 35: 120–127

Morris JG (1960) Studies on the metabolism of *Arthrobacter globiformis*. J Gen Microbiol 22:564–582

Nousiainen AO, Björklöf K, Sagarkar S, Nielsen JL, Kapley A, Jorgensen KS (2015) Bioremediation strategies for removal of residual atrazine

in the boreal groundwater zone. Appl Microbiol Biotechnol 99: 10249–10259

Ralebitso TK, Senior E, van Verseveld HW (2002) Microbial aspects of atrazine degradation in natural environments. Biodegradation 13: 11–19

Rice PJ, Anderson TA, Coats JR (2002) Degradation and persistence of metolachlor in soil: effects of concentration, soil moisture, soil depth, and sterilization. Environ Toxicol Chem 21:2640–2648

Satsuma K (2009) Complete biodegradation of atrazine by a microbial community from a naturally derived river ecosystem (microcosm). Chemosphere 77:590–596

Schroll R, Becher HH, Dörfler U, Gayler S, Grundmann S, Hartmann HP, Ruoss J (2006) Quantifying the effect of soil moisture on the aerobic microbial mineralization of selected pesticides in different soils. Environ Sci Technol 40:3305–3312

Seffernick JL, Aleem A, Osborne JP, Johnson G, Sadowsky MJ, Wackett LP (2007) Hydroxyatrazine *N*-ethylaminohydrolase (AtzB): an amidohydrolase superfamily enzyme catalyzing deamination and dichlorination. J Bacteriol 189:6989–6997

Sereno AM, Hubinger MD, Comesaña JF, Correa A (2001) Prediction of water activity of osmotic solutions. J Food Eng 49:103–114

Shapir N, Mandelbaum RT, Gottlieb H (1998) Atrazine degradation in saline seawater by *Pseudomonas* sp. strain ADP. J Ind Microbiol Biotechnol 20:153–159

Shapir N, Osborne JP, Johnson G, Sadowsky MJ, Wackett LP (2002) Purification, substrate range, and metal center of AtzC: the *N*-isoprolyammelide aminohydrolase involved in bacterial atrazine metabolism. J Bacteriol 184:5376–5384

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

Shapir N, Mongodin EF, Sadowsky MJ, Daugherty SC, Nelson KE, Wackett LP (2007) Evolution of catabolic pathways: genomic insights into microbial *s*-triazine metabolism. J Bacteriol 189:674–682

Singh B, Singh K (2016) Microbial degradation of herbicides. Crit Rev Microbiol 42:245–261

Strong LC, Rosendahl C, Johnson G, Sadowsky MJ, Wackett LP (2002) *Arthrobacter aurescens* TC1 metabolizes diverse *s*-triazine ring compounds. Appl Environ Microbiol 68:5973–5980

Su W, Hao H, Wu R, Xu H, Xue F, Lu C (2017) Degradation of mesotrione affected by environmental conditions. Bull Environ Contam Toxicol 98:212–217

Van Der Kraak GJ, Hosmer AJ, Hanson ML, Kloas W, Solomon KR (2014) Effects of atrazine in fish, amphibians, and reptiles: an analysis based on quantitative weight of evidence. Crit Rev Toxicol 44(suppl 5):1–66

Vibber LL, Pressler MJ, Colores GM (2007) Isolation and characterization of novel atrazine-degrading microorganisms from an agricultural soil. Appl Microbiol Biotechnol 75:921–928

Vyrides I, Stuckey DC (2017) Compatible solute addition to biological systems treating waste/wastewater to counteract osmotic and other environmental stresses: a review. Crit Rev Biotechnol 37:865–879

Wackett LP, Sadowsky MJ, Martinez B, Shapir N (2002) Biodegradation of atrazine and related *s*-triazine compounds: from enzymes to field studies. Appl Microbiol Biotechnol 58:39–45

Wang H, Liu Y, Lin M, Hu X (2016) Biodegradation of atrazine by *Arthrobacter* sp. C3, isolated from the herbicide-contaminated corn field. Int J Environ Sci Technol 13:257–262

Weber JB (1993) Ionization and sorption of fomesafen and atrazine by soils and soil constituents. Pestic Sci 39:31–38

Wildman H (2016) Importance of water activity to soil microorganisms Poster available at https://www.researchgate.net/publication/305617763_Importance_of_water_activity_to_soil_microorganisms https://doi.org/10.13140/RG.2.1.3754.3924

Wood JM (2011) Bacterial osmoregulation: a paradigm for the study of cellular homeostasis. Annu Rev Microbiol 65:215–238

Wood JM, Bremer E, Csonka LN, Kraemer R, Poolman B, van der Heide T, Smith LT (2001) Osmosensing and osmoregulatory compatible solute accumulation by bacteria. Comp Biochem Physiol Part A 130:437–460

Ye JY, Zhang JB, Gao JG, Li HT, Liang D, Liu RM (2016) Isolation and characterization of atrazine-degrading strain *Shewanella* sp. YJY4 from cornfield soil. Lett Appl Microbiol 63:45–52

Zevenhuizen LP (1992) Levels of trehalose and glycogen in *Arthrobacter globiformis* under conditions of nutrient starvation and osmotic stress. Antonie Van Leeuwenhoek 61:61–68

Zhang Y, Jiang Z, Cao B, Hu M, Wang DX (2011) Metabolic ability and gene characteristics of *Arthrobacter* sp. strain DNS10, the sole atrazine-degrading strain in a consortium isolated from black soil. Int Biodeterior Biodegradation 65:1140–1144

Zhang Y, Cao B, Jiang Z, Dong X, Hu M, Wang Z (2012) Metabolic ability and individual characteristics of an atrazine-degrading consortium DNC5. J Hazard Materials 237-238:376–381

Zhang Q, Zhu L, Su J, Wang J, Xie H, Wang J, Wang F (2014) Impacts of nitrogen and phosphorus on atrazine-contaminated soil remediation and detoxification by *Arthrobacter* sp. strain HB-5. Environ Earth Sci 71:1465–1471