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Atrazine degradation in saline wastewater by *Pseudomonas* sp strain ADP

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Wastewater from atrazine manufacturing plants contains large amounts of residual atrazine and atrazine synthesis products, which must be removed before disposal. One of the obstacles to biological treatment of these wastewaters is their high salt content, eg, up to 4% NaCl (w/v). To enable biological treatment, bacteria capable of atrazine mineralization must be adapted to high-salinity conditions. A recently isolated atrazine-degrading bacterium, *Pseudomonas* sp strain ADP, originally isolated from contaminated soils was adapted to biodegradation of atrazine at salt concentrations relevant to atrazine manufacturing wastewater. The adaptation mechanism was based on the ability of the bacterium to produce trehalose as its main osmolyte. Trehalose accumulation was confirmed by natural-abundance ^{1}H NMR spectral analysis. The bacterium synthesized trehalose *de novo* in the cells, but could not utilize trehalose added to the growth medium. Interestingly, the bacterium could not produce glycine betaine (a common compatible solute), but addition of 1 mM of glycine betaine to the medium induced salt tolerance. Osmoregulated *Pseudomonas* sp strain ADP, feeding on citrate decreased the concentration of atrazine in non-sterile authentic wastewater from 25 ppm to below 1 ppm in less than 2 days. The results of our study suggest that salt-adapted *Pseudomonas* sp strain ADP can be used for atrazine degradation in salt-containing wastewater.

Keywords: atrazine; salt; industrial wastewater; wastewater treatment; *Pseudomonas*

Introduction

Over the last 40 years, the microbial degradation of *s*-triazines, especially atrazine (2-chloro, 4-ethylamino, 6-isopropylamino, *s*-triazine), has been intensively studied in various environments [6,29]. In early reports, only partial biodegradation of this compound was described [2,9,15]. To overcome its resistance to biodegradation, a physicochemical pre-treatment was required to enable complete mineralization [21]. Recently, several authors succeeded in the isolation of pure bacterial cultures which were able to mineralize atrazine completely [25,31,48].

S-triazines are widely used in various industries [18], with consequent problems of wastewater treatment. Wastewaters from the manufacture of *s*-triazine-containing pesticides, such as atrazine, simazine, propazine, and cyanazine, are especially problematic due to their high concentrations of residual chlorinated *s*-triazine compounds and other manufacturing by-products. Such wastewaters cannot be treated in regular municipal wastewater treatment plants, because of the prolonged persistence of atrazine residues [8]. The absence of microbial degraders and the unfavorable conditions for biodegradation have been found to be the major causes of the persistence of this herbicide [20], and its removal from drinking water appeared to be a difficult task. It had been suggested that the degradation of atrazine could be achieved under nitrogen-limited conditions. Subsequently, a significant number of pure cultures with atrazine-degrading potential have been reported, but these organisms failed to degrade atrazine in the wastewater even under nitrogen-limited conditions [7,18]. The failure of these bacteria was mainly attributed to high salinity [9] and to possible competition from nitrogen-fixing bacteria in the sludge [41]; these studies clearly revealed the importance of bacterial strains that could degrade atrazine under high salinity.

The salt concentration, that affects the osmotic strength of the environment, and the ionic composition are two physical factors that determine the ability of organisms to survive and proliferate in a given habitat [10]. The exposure of organisms to hyperosmolarity results in a decrease in their cytoplasmic water activities, and sudden plasmolysis results in the inhibition of a variety of physiological processes, ranging from nutrient uptake [35,36,43] to DNA replication [27]. Also, the synthesis of macromolecules is affected and this causes significant elevation of the cellular ATP level [28]. To grow in an osmotically stressful environment, a bacterial cell must establish and maintain its internal pressure above that of its surrounding medium [1], which it commonly achieves through the accumulation of osmotically active solutes (osmolytes, compatible solutes) in its cytosol [10,22,30,33]. Mostly, these osmolytes are low-molecular-weight organic compounds which exhibit the following characteristics: high solubility in water, a neutral net electric charge at a physiological pH, and a lack of *in vitro* toxicity toward enzymes at high but physiologically relevant concentrations [14,16,44]. The osmolytes fall into a few classes: amino acids (such as proline and glutamate), amino acids derivatives (such as glycine betaine and gamma-aminobutyric acid), peptides (such as *N*-acetylglutaminylglutamine amide), polyols (such as sugars, glycerol, and trehalose), methylamines, and urea

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[26,11,47]. Osmolytes such as glutamate or trehalose can be synthesized by microbes, whereas the others are transported in and accumulated from the medium [11]. A prominent example of the latter is the osmoregulated transport and intracellular accumulation of glycine betaine, which is known to protect a number of bacteria against osmotic stress [10,46]. The unusual dipeptide osmolyte, *N*-acetylglutaminylglutamine amide (NAGGN) has been found in only a few cases [38,39,40]. Smith *et al* [40] described the mechanism of NAGGN production and accumulation in *Rhizobium meliloti*. Trehalose, which is a common disaccharide, serves as an osmoprotectant in many different organisms, its two glucose molecules are linked by 1-1 α-glycoside [34,46], so that the sugar is nonreducing and has the unique quality of ensuring the long-term maintenance of membrane fluidity under conditions of dryness and desiccation [23,24]. The mechanism of trehalose production and accumulation in *Escherichia coli* has been well documented [3,4,17,19,42], but its role in *Pseudomonas* sp strain ADP is yet to be explored.

In the present study, we employed a potentially atrazine-mineralizing strain, *Pseudomonas* sp strain ADP and examined the influence of salt concentration on its growth, with a view to understanding the mechanism of its tolerance to high salinity. The complete degradative pathway of this bacterium had been proposed previously, and the characterization of a few genes involved in the earlier steps of atrazine degradation has been achieved recently [5,12,13,25]. The organic compounds accumulated by the organism during its adaptation to osmotic stress have been identified by means of NMR spectroscopy analysis. Finally, to simulate the natural conditions for bioremediation, this strain was introduced into salty industrial wastewater containing the herbicide, and its efficacy in degrading atrazine was studied in detail.

Materials and methods

Chemicals

Atrazine 98% was a gift from the Ciba-Geigy Corporation, Greensboro, NC, USA. All basic chemicals were of analytical grade and purchased from Merck, Darmstadt, Germany, except trehalose, glutamate and chelex-100, bought from Sigma Chemical Co (St Louis, MO, USA).

Microbial cultures

The atrazine-degrading bacterium, *Pseudomonas* sp strain ADP (*P*. ADP) used in the present study has been described previously [25]. Throughout these experiments, the cells were grown in 250 ml of atrazine medium at 35°C on an orbital shaker (125 rpm) according to Mandelbaum *et al* [24]. Cells were harvested after 24 h of incubation (late log phase) by centrifugation (6000 × *g*, 10 min), washed twice and resuspended in sterile saline solution (0.8% w/v).

Total bacterial counts

Total bacteria in the wastewater were counted using the dilution plate technique on nutrient agar (Difco Laboratories, Detroit, USA).

Determination of growth rates

Batch cultures: *P*. ADP was grown in 250-ml Erlenmeyer flasks containing 100 ml atrazine medium supplemented with various concentrations of salts (0.1–5% NaCl w/v). The media were inoculated with 10 μl of stationary-phase cells grown with 0.1% NaCl and incubated at 35°C on an orbital shaker (125 rpm). The growth of the cells was monitored at regular intervals by reading their absorbance at 600 nm. To measure adaptation of the cells to salinity, 1 ml of a late log culture grown in a medium containing 0.1 or 3% NaCl was inoculated into a 3% NaCl atrazine medium. Various osmolytes were added to the 3% NaCl medium to a final concentration of 1 mM, to study their effects on *P*. ADP growth rate. All batch culture experiments were carried out in triplicate.

Continuous culture: *P*. ADP was grown in a 2-L (1.5-L working volume) chemostat (Biof10, New Brunswick Scientific Co, Adison, NJ, USA). The dilution rate was 0.167 h^{-1} with double strength atrazine medium containing 0.1% NaCl (all media components were double strength, except for atrazine that was maintained at the original level of 100 ppm). Atrazine was the limiting growth factor and served as the sole nitrogen source. The culture was vigorously aerated and agitated at 300 rpm. The temperature of the chemostat was automatically controlled at 35°C, and the pH was automatically maintained at 7.3 by an automatic pH controller, model pH-40 (New Brunswick Scientific Co, Adison, NJ, USA), with sterile 0.5 M HCl solution. After the culture had reached a steady-state, the feeding solution in the reservoir was replaced with 3 or 5% NaCl in double-strength atrazine medium as previously described. Samples for bacterial counts and residual atrazine concentration determination were aseptically removed at selected time intervals. The *P*. ADP counts were made on plates containing atrazine solid medium by means of the clearing zones technique, as described by Mandelbaum *et al* [25].

Atrazine was analyzed by HPLC chromatography, with a HPLC Controller (SSI, PA, USA) fitted with an autosampler (Merck Hitachi L7200, Tokyo, Japan). Detection was made at 220 nm with a UV detector (Varian 9050, TX, USA). Separation was obtained on a C8 5-μm (250 × 4 mm) reverse-phase column (Merck, LiChrocart RP-8, Darmstadt, Germany) using a flow rate of 1 ml min^{-1}. The mobile phase was 70/30 (v/v) methanol/water with 50 mM ammonium acetate.

Sample preparation and NMR spectroscopy

Cultures were harvested at late log phase by centrifugation (6000 × *g*, 10 min). The cell pellet was suspended in 12 ml distilled water and sonicated for 4 min, under a 50% duty cycle, at 14 kHz frequency (Vibra Cell, Sonics and Materials Inc, Danbury, CT, USA). The broken cells were centrifuged for 10 min at 7000 × *g*, and the supernatant passed through a 0.5-ml Chelex-100 column for removal of metals, and freeze dried. Lyophilized samples were dissolved in 0.6 ml D_2O and ^1H NMR spectra were obtained at 300 MHz by using a Bruker DPX-300 spectrometer (Silberstreifen, Karlsruhe, Germany). The spectra were compared with those of authentic samples of trehalose and glutamine. For the quantitative determination of the osmolytes, 3 mg of sodium benzoate was added prior to the dissolution of the samples, as an external standard.

Atrazine degradation in authentic wastewater from a pesticide manufacturing plant

Industrial effluents were collected from a wastewater biological treatment facility in a herbicide factory. The physical and biological properties of the wastewater are summarized in Table 1.

Atrazine degradation was measured in 250-ml Erlenmeyer flasks containing 100 ml of industrial wastewater or saline (control) supplemented with 25 ppm atrazine. Sodium citrate was added to some of the treatments as a carbon source, to give a final concentration of 2% (w/v). Media were inoculated with either a non-adapted or 3% NaCl-adapted *P*. ADP suspension to give a final concentration of 5×10^7 cells ml^{-1}. Atrazine degradation by the wastewater indigenous microbial population was measured in non-inoculated wastewater solution supplemented with sodium citrate. The samples were incubated for 8 days at 35°C, under constant shaking (150 rpm). At regular intervals, samples (1 ml) from each treatment were removed, to evaluate atrazine degradation. Samples were filtered through a 0.45 μm polypropylene filter, and extracted with ethyl acetate/hexane (1/1 : v/v). The organic phase was collected and analyzed with a Varian Star 3400 cx, gas chromatograph (Varian, Harbor City, CA, USA) equipped with an autosampler (Varian 8200 cx). The GC was fitted with a temperature-programmable injector (Varian 1077), a thermionic specific detector, and a 30-m × 0.53-mm i.d., 1.5-μm film, DB-1 capillary column (J&W Scientific, Folson, CA, USA). The gas chromatograph was operated at an injector temperature of 260°C and a detector temperature of 300°C, and the column oven temperature was set at 195°C for 5.5 min after sample injection, and then raised by 20°C min^{-1} to 240°C. The carrier gas flow rate was 10 ml min^{-1} and the injection volume was 1 μl.

Results

Effect of osmotic stress on bacterial growth in batch culture

The length of the lag and the log phases was positively correlated with the increasing salinity of the growth medium (Figure 1). The lowest NaCl concentrations, 0.1, 1.0 and 2.0%, showed the shortest lag periods, 18, 20 and 30 h, respectively, followed by approximately 15 h of log phase. The 3.0 and 4.0% NaCl concentrations gave longer lag periods of 40 and 80 h, respectively, while the log phase

Table 1 Chemical and biological properties of the authentic industrial wastewater

pH	Bacterial count (cells ml^{-1})	Osmotic pressure (atm)	NaCl (meq L^{-1})	$N\text{-}NH_4$ (mg L^{-1})	$N\text{-}NO_3$ (mg L^{-1})	N-Total (mg L^{-1})	COD (mg L^{-1})
6.9	2×10^6	22	560	280.5	5.55	917.7	3450

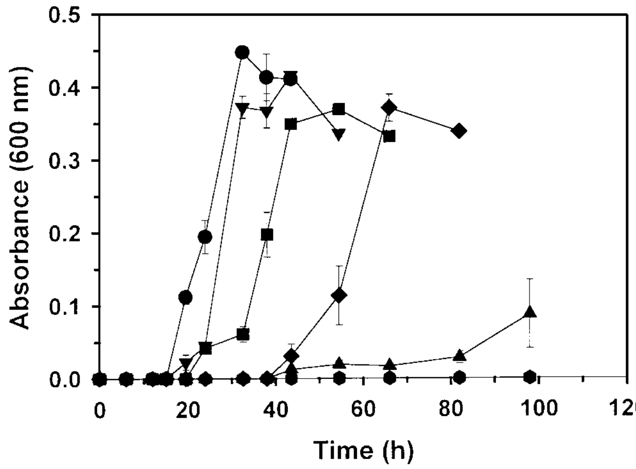

Figure 1 *P*. ADP growth rates on atrazine medium containing different NaCl (—●—) 0.1%; (—▼—) 1.0%; (—■—) 2.0%; (—◆—) 3.0%; (—▲—) 4.0%; (—⬢—) 5.0%. Bars represent the standard error.

of the former lasted for 25 h. *P*. ADP did not grow at all in 5.0% NaCl atrazine medium.

A clear adaptation effect was observed when cells pre-grown on 3% NaCl were transferred into 3% NaCl medium (Figure 2). The lag and the log phase of 3.0% NaCl-adapted bacteria were four and two times shorter, respectively, than those of non-adapted bacteria.

Effect of osmotic stress on bacterial growth in continuous culture

Complete degradation of atrazine (which was the sole nitrogen source and served as the limiting growth factor) indicated a physiologically healthy culture.

During the first 20–22 h (Figure 3a and b, respectively), a steady state with osmotic pressure of 4 atm (0.1% NaCl) was maintained, and atrazine was completely removed. The concentrations of bacteria were stable, at 1×10^8 cells ml^{-1} and 1×10^7 cells ml^{-1} (Figure 3a and b, respectively). When the NaCl concentration in the medium in the reservoir was raised to 3.0%, the osmotic pressure in the growth medium

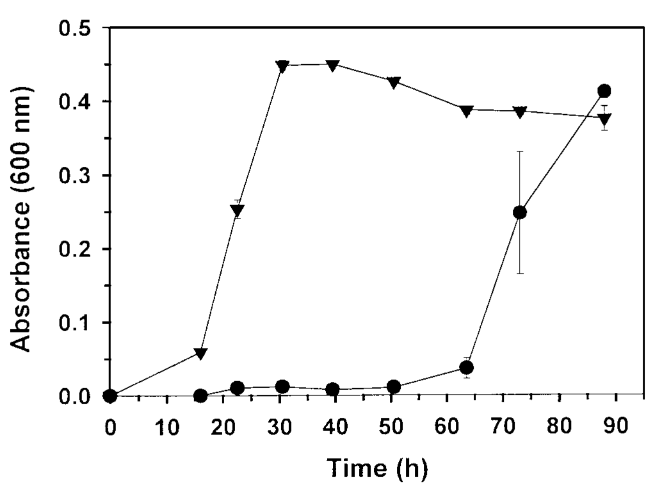

Figure 2 *P*. ADP growth rates on 3.0% NaCl atrazine medium with two types of inocula. (●) Inoculum previously grown on 0.1% NaCl; (▼) inoculum previously grown on 3.0% NaCl. Bars represent the standard error.

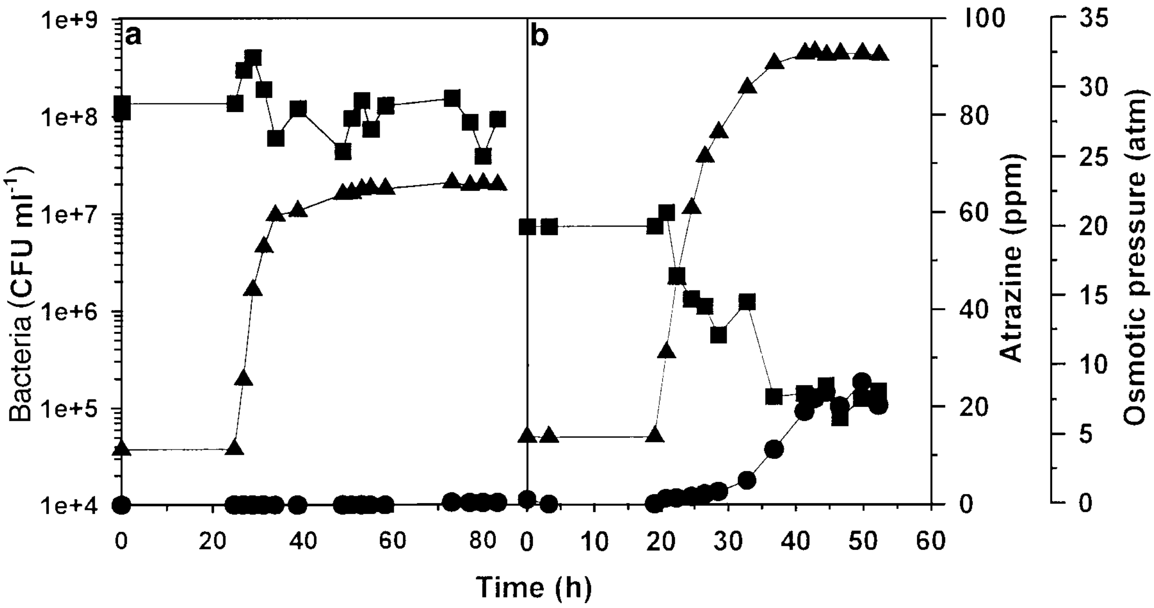

Figure 3 *P*. ADP growth and its atrazine degradation activity as affected by increasing medium salinity. (a) = 3% NaCl, (b) = 5% NaCl. (■) Bacterial concentration (CFU ml^{-1}); (▲) osmotic pressure (atm); (●) atrazine concentration (ppm).

increased from 4 to 22.5 atm in 20 h (Figure 3a). The bacteria resisted this modest osmotic pressure increase and their growth rate and the atrazine degradation rate did not change. When the reservoir medium NaCl concentration was raised to 5.0% NaCl, after 20 h the osmotic pressure increased from 4 to 32. 5 atm (Figure 3b). When the osmotic pressure rose above 20 atm, the bacterial concentration fell from 1×10^7 cells ml^{-1} to 1×10^5 cells ml^{-1}, because their growth rate was lower than the dilution rate. Atrazine residues started to appear and the atrazine concentration reached 20 ppm.

Salt tolerance mechanism

The growth rates of *P*. ADP in 0.1% NaCl and in 4.0% NaCl plus 1 mM glycine betaine were nearly identical (Figure 4). Interestingly the presence of glycine betaine in the medium reduced the lag period four-fold compared with the cells grown on 4.0% NaCl alone. Trehalose had only a minor effect on the growth rate in 4.0% NaCl. The lag period and the log phase growth rate showed the same behaviour with or without the addition of 1 mM of this osmolyte.

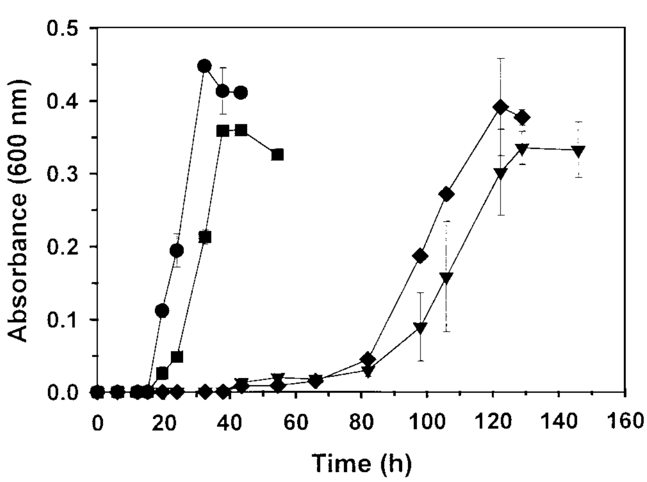

Figure 4 The effects of trehalose and glycine betaine addition on *P*. ADP growth rate. (●) Growth on 0.1% NaCl medium; (■) growth on 4.0% NaCl medium with the addition of 1 mM glycine betaine; (◆) growth on 4.0% NaCl medium with the addition of 1 mM trehalose; (▼) growth on 4.0% NaCl medium. Bars represent the standard error.

Natural-abundance ^{1}H NMR spectroscopy was used to identify the organic compounds within the cells of osmotically stressed culture of *P*. ADP (Figure 5). Accumulation of the disaccharide trehalose and a small amount of an unknown compound, suspected to be the unique dipeptide *N*-acetylglutaminylglutamine amide (NAGGN), were detected. The amounts of trehalose and NAGGN within late log phase cells, grown on 3% NaCl, were 82.2 mg g^{-1} protein and 25.4 mg g^{-1} protein, respectively.

Atrazine degradation by P. ADP in authentic wastewater

The chemical and biological properties of the industrial wastewater, collected from a herbicide manufacturing plant are summarized in Table 1. The wastewater osmotic pressure was 22 atm, and its pH was neutral (6.9); it contained high concentrations of COD and nitrogen compounds originating from the chemical herbicides synthesis process and from indigenous microbial activity in the wastewater. The bacterial count in the wastewater was 2×10^6 cells ml^{-1}.

Atrazine degradation rates in the authentic industrial wastewater by non-adapted and salt-adapted *P*. ADP, with or without the addition of sodium citrate as a carbon source, were measured (Figure 6). Addition of non-adapted bacteria, with or without citrate, did not significantly improve the atrazine degradation rate, compared with the degradation by the indigenous population alone. However, adapted bacteria reduced atrazine concentration from 25 to 3 ppm after 8 days. Addition of a carbon source further improved the degradation rate and complete degradation was observed after 4 days.

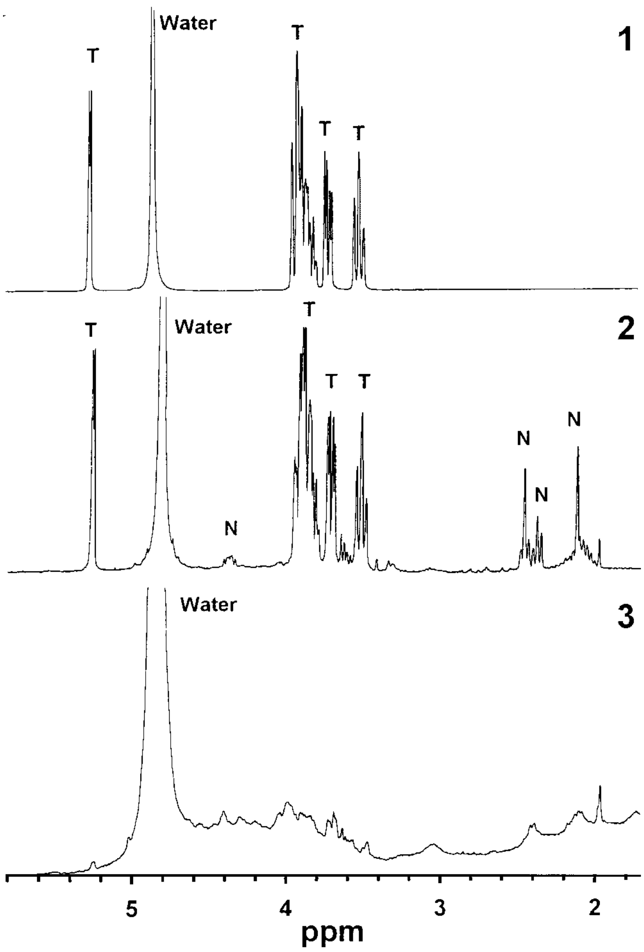

Figure 5 Natural-abundance ^{1}H NMR spectra of cell extracts from *P*. ADP. 1, Spectra of authentic trehalose; 2, spectra of extract of bacterial cells that were grown on 3.0% NaCl; 3, spectra of extract of bacterial cells that were grown on 0.1% NaCl; T, trehalose; N, NAGGN.

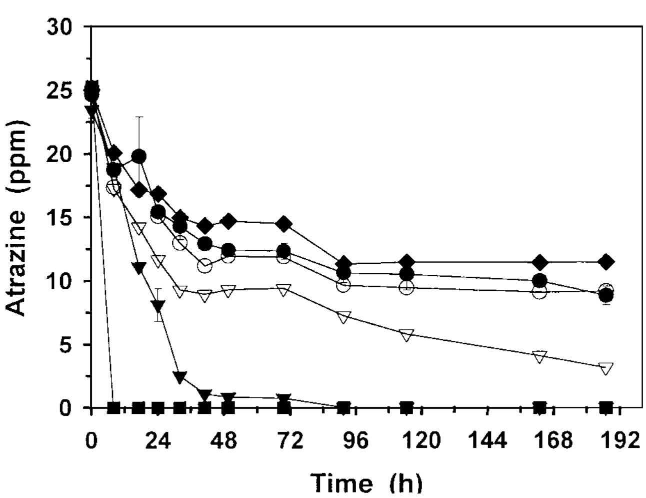

Figure 6 Atrazine degradation in authentic wastewater by non-adapted (●) and 3.0% NaCl adapted (▼) *P*. ADP, with or without the addition of citrate (solid and empty symbhols, respectively). (◆) Authentic wastewater without addition of bacteria; (■) saline water. Bars represent the standard error.

Discussion

Two of the major problems associated with atrazine degradation in wastewater from atrazine manufacturing plants are the scarcity of atrazine-mineralizing bacteria, and their inability to tolerate the high salt concentrations typical of atrazine-manufacturing wastewater [9]. In the present study, we used a recently isolated pure culture, capable of complete atrazine mineralization, and studied its ability to degrade atrazine under high salt concentrations, relevant to atrazine-manufacturing wastewater.

At supra-optimal NaCl concentrations, growth inhibition of *P*. ADP progressively increased with increasing salinity, as indicated by decreasing growth rates and increases in the time lag before growth started. However, the bacterium was able to adapt to salt stress after an initial inhibition period, and it is hypothesized that the bacterium activated its osmoregulation mechanism during the initial growth inhibition period. Also some temporary growth inhibition could be caused by the diversion of an increased proportion of the available fixed carbon and energy, to the synthesis of compatible solutes, rather than to maintenance and cell growth [46]. Osmoregulation in bacteria is not a new phenomenon and was reviewed by Csonka and Hanson [11], who described the initial adaptation period as a 'long-term or steady-state response'. When continuous cultures of *P*. ADP were progressively exposed to upshifts of salt content up to 22.5 atm osmotic pressure, their doubling time and atrazine degradation activity were not affected. These results indicate that under a gradual, slow increase in salt concentration, *P*. ADP has enough time to activate the osmoregulation mechanism to produce a compatible solute, in order to protect its cells from drying.

Using natural-abundance ^{1}H NMR spectral analysis we were able to identify the increase in content of two osmolyte types: trehalose, which was identified as the major compatible solute (Figure 5), and a much smaller amount (3.2 times less) of a different compatible solute, suspected to be the dipeptide, *N*-acetylglutaminylglutamine amide.

The addition of 1 mM trehalose to the growth medium did not relieve the growth inhibition, which suggests that trehalose is produced *de novo* in the cells and is not transported through the membranes of *P*. ADP.

However, the addition of 1 mM glycine betaine to the growth medium relieved the growth inhibition caused by high salinity, indicating that *P*. ADP possesses a glycine betaine uptake system. Osmotically regulated glycine betaine transport systems are widespread amongst halophilic and halotolerant eubacteria [14,37,45], and glycine betaine may be present in substantial quantities in hypersaline environments, where it is synthesized as a compatible solute by a wide range of phototrophic and cyanobacteria [32]. This is a common phenomenon that has been observed in several other bacterial species to which betaine also confers enhanced osmotic tolerance [11,14,40]. Nevertheless, *P*. ADP cells that were not exposed to glycine betaine did not produce it within the cells, as indicated by ^{1}H NMR spectral analysis (Figure 5).

Although the bacterium does not produce glycine betaine within the cells, there is a clear energetic benefit to be gained from the accumulation of external osmolytes, compared with their production within the cells [46].

Osmoregulated *P*. ADP bacteria were able to rapidly degrade atrazine in non-sterile authentic wastewater, in spite of the competition from the endogenous microbial population of the wastewater and the high salinity of the medium. Additionally, atrazine degradation proceeded in spite of the high content of nitrogen-containing compounds in the wastewater: nitrogen-rich conditions had previously been shown to inhibit *s*-triazine degradation in wastewater treatment [41]. Amendment of the wastewater with an additional carbon source in the form of sodium citrate greatly enhanced the degradation of atrazine. The high chemical oxygen demand (COD) of the wastewater could provide some carbon and energy sources for *P*. ADP, as indicated by the similar initial atrazine degradation rates in the citrate-amended and non-amended wastewater. However, after 24 h, the rate of degradation in the non-amended medium leveled, while the citrate-amended culture completely degraded atrazine. Apparently, there was no readily available carbon in the wastewater.

The results of the present study suggest that *P*. ADP can be used for atrazine degradation in salt-containing wastewater, but the benefit from the addition of an external carbon source (citrate) may be not worth the cost. Further studies of the ability of *P*. ADP to adapt to the utilization of carbon sources available in authentic wastewater are needed.

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