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Simultaneous degradation of atrazine and phenol by *Pseudomonas* sp. ADP: Effects of toxicity and adaptation

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ABSTRACT: The strain *Pseudomonas* sp. ADP is only able to degrade atrazine as sole nitrogen-source and therefore needs a carbon- and energy-source for growth. In addition to the typical C-source for *Pseudomonas*, Na_2succinate, the strain can also grow with phenol as carbon source. Thereby, phenol is oxidized to catechol by a multi-component phenol hydroxylase. Catechol is degraded via the *ortho*-pathway using the catechol-1,2-dioxygenase. It was possible to stimulate the strain in order to degrade very high concentrations of phenol (1000 mg/l) and atrazine (150 mg/l) simultaneously. With cyanuric acid, the major intermediate of atrazine degradation, as N-source both the growth rate and the phenol degradation rate were similar to those measured with ammonia as N-source. With atrazine as N-source growth rate and the phenol degradation rate were reduced to about 35% of those data obtained for cyanuric acid. This presents clear evidence that although the first three enzymes of the atrazine degradation pathway are constitutively present, either these enzymes or the uptake of atrazine are the bottleneck that diminishes the growth rate of *Pseudomonas* sp. ADP with atrazine as N-source. Whereas atrazine and cyanuric acid showed no significant toxic effect on the cells, phenol reduces growth and activates/induces typical membrane-adaptive responses known for the genus *Pseudomonas*. Therefore, *Pseudomonas* sp. ADP is an ideal bacterium to investigate the regulatory interactions between several catabolic genes and stress response mechanisms during the simultaneous degradation of toxic phenolic compounds and a xenobiotic N-source such as atrazine.

1 INTRODUCTION

Atrazine (2-chloro-4-(ethylamino)-6- (isopropylamino)-1,3,5-triazine) is a herbicide used for controlling broad-leaf and grassy weeds and is relatively persistent in soils (Hayes & Laws 1991). Atrazine and its metabolites have been detected in ground and surface waters at levels exceeding the Environmental Protection Agency's maximum contaminant level of 3 ppb (Hayes & Laws 1991).

Pseudomonas sp. ADP was the first isolated bacterium capable of degrading the herbicide atrazine (Mandelbaum et al. 1995, Wackett et al. 2002). Since then, most of the understanding of the genes and enzymes involved in atrazine degradation derives from studies using this strain, in which the first three enzymatic steps of atrazine degradation have been defined (de Souza et al. 1998). The genes *atzA*, *atzB*, and *atzC*, which encode the enzymes atrazine chlorohydrolase (AtzA), hydroxyatrazine ethylaminohydrolase (AtzB), and N-isopropylammelide isopropylaminohydrolase (AtzC), convert atrazine sequentially to cyanuric acid (de Souza et al. 1998). Cyanuric acid is catabolized by *Pseudomonas* sp. ADP to carbon dioxide and ammonia (de Souza et al. 1998). These first three genes have been localized on an approximately 100 kb plasmid, pADP-1 (de Souza et al. 1998). Recently, pADP-1 was completely sequenced and was shown to contain the genes for the complete catabolism of cyanuric acid to CO_2 and NH_3 as well namely, *atzD*, *atzE*, and *atzF* (30) as well. Structural and functional studies showed that the genes encoding the initial reactions of atrazine catabolism are not organized in an operon, but are dispersed and flanked by transposase copies.

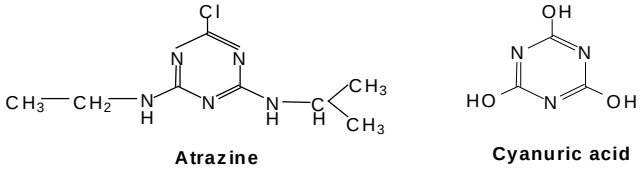

Figure 1. The investigated *s*-triazines.

However, the strain is only able to use atrazine as sole nitrogen-source and therefore needs an additional carbon- and energy source for growth. In the present, work we investigated the degradation of phenol in the presence of different N-sources including

the two *s*-triazines atrazine and cyanuric acid (Fig. 1). During our experiments we studied the effect of simultaneous degradation of those compounds as well as membrane-adaptive responses of the cells to the toxic compounds phenol and atrazine.

2 RESULTS

Pseudomonas sp. ADP can grow with phenol as sole carbon source. It cleaves catechol via the *ortho*-pathway using the catechol-1,2-dioxygenase, but was not able to degrade chlorophenols.

2.1 *Degradation of atrazine*

In order to adapt the ADP strain we selected atrazine concentrations of 100 or 150 mg/l atrazine, 2 g/l cyanuric acid and the phenol concentration of 500 mg/l which caused about 50 percent growth inhibition and the best physiological membrane adaptation reactions. Cyanuric acid is quite hydrophilic and therefore non-toxic and an easily degradable N-source. Although atrazine has a higher logP value than phenol (2.71 to 1.45) it is not that toxic to ADP as phenol (data not shown) and its complete degradation (cf. Fig. 2) was proved via HPLC-analysis (data not shown).

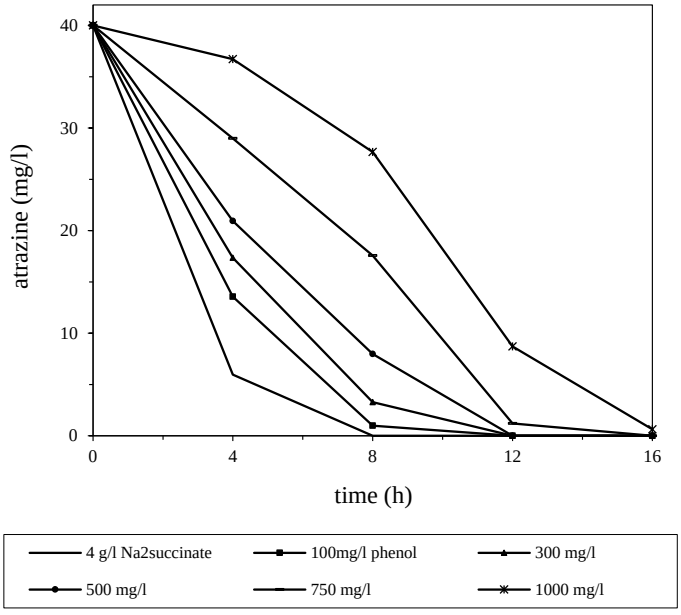

Figure 2. Degradation of atrazine by *Pseudomonas* sp. ADP with phenol as C-source. Due to the low water solubility of atrazine, the scale does not show the nominal concentration in the medium. Atrazine was applied in an amount of up to 150 mg/l by using a small reservoir made of an Eppendorf tube with a semi-permeable membrane. This guaranteed the constant presence of the maximum atrazine concentration which corresponds to the maximum solubility of this compound in water (30 mg/l). This application hindered a precipitation of atrazine in the medium that would have disturbed the measurement of the optical density (O.D.).

2.2 *Degradation of phenol*

Completely adapted, the strain was able to degrade phenol in amounts of 1000 mg/l totally within 8 hours with cyanuric acid as N-source (cf. Fig. 3). At the same time the growth rates μ were up to 0.32 h-1 which corresponds to a doubling time (tD) of about 2 hours. The degradation rates for phenol were up to 150 mg/h. These growth rates and the cell yields are comparable to those obtained with medium containing ammonia as N-source. Furthermore, there exists a correlation between an increased atrazine concentration and an increasing cell yield, although it takes the cells much longer to reach the same optical density.

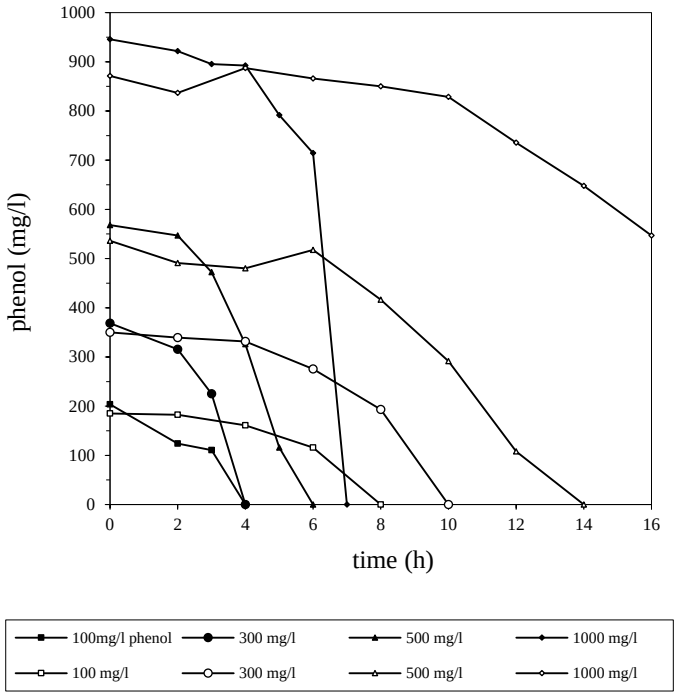

Figure 3. Comparison of the phenol degradation by *Pseudomonas* sp. ADP with cyanuric acid (■ closed symbols) or atrazine (□ open symbols) as sole N-source.

2.3 *Simultaneous degradation of atrazine and phenol*

With atrazine as N-source, phenol was completely degraded after about 30 hours, with decreased growth and degradation rates, respectively (μ: 0.12 h-1; tD: 5.75 h). It was possible to stimulate the strain to degrade very high concentrations of phenol and atrazine simultaneously and as shown in Fig. 4 at growth rates similar to those obtained in “normal” mineral medium only containing ammonia as N-source and salts supplemented with a standard vitamin solution.

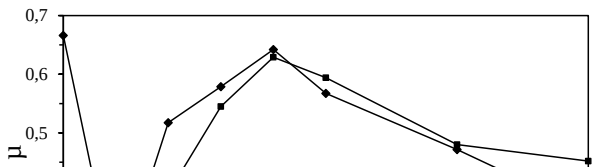

Figure 4. Comparison of the growth rates of *P*. sp. ADP growing on NH_4^+, cyanuric acid or atrazine as N-source. The "zero-points" correspond to the control cultures grown on Na_2-succinate as C-source.

2.4 *Physiological adaptation to atrazine and phenol*

As it is known that phenol as well as atrazine is a toxic compound, the adaptation of the strain was also investigated (Heipieper et al. 2003).

As it is shown in Fig. 5 the strain showed no increasing *trans/cis* ratio when cultivated on atrazine + Na_2Succinate, which confirms the assumption of atrazine not being very toxic. Nevertheless, an increasing degree of saturation of the membrane fatty acids was observed when atrazine was added as a toxin within the logarithmic growth phase.

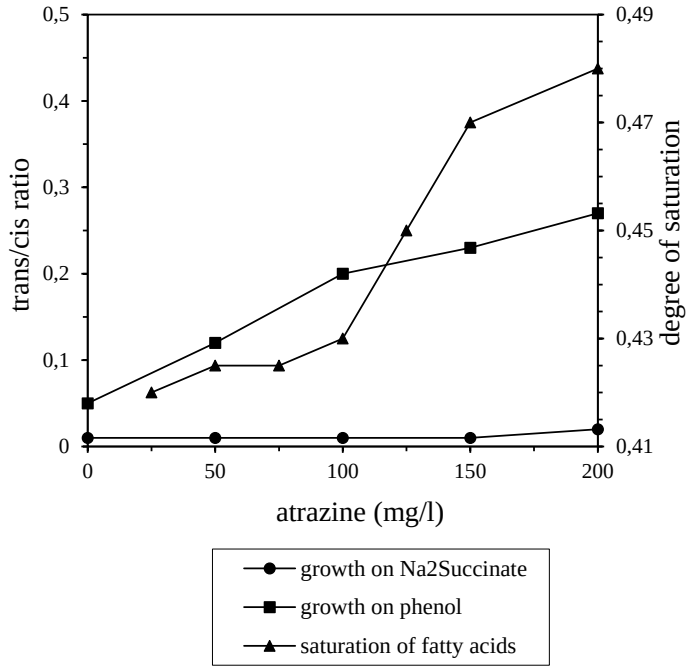

Figure 5. Effect of the atrazine concentration on changes in the membrane fatty acid composition of *Pseudomonas* sp. ADP.

Fig. 6 emphasizes the toxicity of phenol. The degree of saturation decreases significantly at 1000 and 1250 mg/l because the cells are no longer able to adapt to such high concentrations. As the *cis-trans* isomerase is in no need for energy or co-factors in order to function, the *trans/cis* ratio is still increasing at those concentrations.

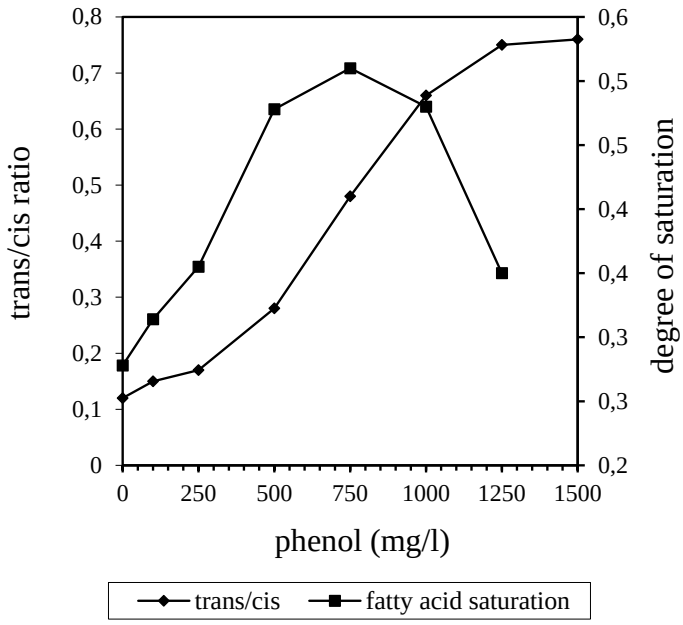

Figure 6. Effect of the phenol concentration on changes in the membrane fatty acid composition of *Pseudomonas* sp. ADP.

2.5 *Pseudomonas sp. strain ADP utilizes phenol via ortho-pathway of catechol degradation*

Phenol is usually degraded via the catechol degradation pathway. Two ways for catechol ring fission are existing: the *meta*- and *ortho*-pathway. The majority of bacteria use the *meta*-pathway of catechol degradation, especially if bacteria have multicomponent phenol hydroxylases like *Pseudomonas* sp. CF600 (Powlowski & Shingler 1990). However, there is also evidence that the multicomponent phenol hydroxylase and the *ortho*-pathway of catechol degradation can coexist (Ehrt et al. 1995). Both *meta*- and *ortho*-pathways are distinguishable by measuring characteristic enzymes, catechol-2,3-dioxygenase (C23O) for the *meta*-pathway, and catechol-1,2-dioxygenase (C12O) for the *ortho*-pathway. The activities of both enzymes were measured in ADP cells grown in phenol minimal media. Activity of C12O but no evidence of C23O could be detected in a crude extract of ADP cells. The activity of C12O in the crude extracts of logaritmically growing (6 hours) cells was 1.66 ± 0.24 μmol/mg min. This demonstrates that *Pseudomonas* sp. ADP uses the *ortho*-pathway of catechol degradation on phenol catabolism.

2.6 *The phenol degradation in ADP is down-regulated in rich medium*

Various reports have demonstrated that promoters of biodegradative operons are down-regulated in response to exponential growth in rich media irrespective of the presence of an effector, a phenomenon referred to as catabolite repression or exponential silencing (reviewed by Cases & de Lorenzo 2001). In order to study whether ADP's phenol degradation would also be repressed in rich medium, this strain was grown in LB medium in the presence of phenol and the activity of the *ortho*-pathway enzyme C12O was measured (data not shown). No activity of C12O could be detected when bacteria were grown in LB in the absence of phenol. In the presence of phenol, the C12O activity was only measurable when cells were entering the stationary phase and it increased during the stationary phase, demonstrating that expression of the phenol degradation pathway in *Pseudomonas* sp. ADP is also under physiological control.

3 CONCLUSIONS

It was possible to cultivate *Pseudomonas* sp. ADP with phenol as sole C- and energy source and simultaneously with atrazine or cyanuric acid as N-source. *Pseudomonas* sp. ADP is able to degrade both phenol and *s*-triazines at the same time.

With cyanuric acid as N-source both the growth rate and the phenol degradation rate are similar to those measured with ammonia as N-source (cf. Fig. 3, 4). With atrazine as N-source growth rate and the phenol degradation rate were reduced to about 35% of those data obtained with cyanuric acid. Due to the slight toxicity of atrazine the maximum growth rate occurred at a lower phenol concentration than with cyanuric acid or ammonia.

Although the first three enzymes of the atrazine degradation are constitutively present, they are the bottleneck that diminishes the growth rate of *Pseudomonas* sp. ADP with atrazine as N-source.

Pseudomonas sp. ADP is an ideal bacterium to investigate the degradation of phenolic compounds, the degradation of atrazine and the influence of stress adaptation mechanisms.

Additionally, the strain offers great opportunities to study the regulatory interactions between different degradation pathways and stress response mechanisms.

4 ACKNOWLEDGEMENTS

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