Nitrogen Control of Atrazine Utilization in *Pseudomonas* sp. Strain ADP

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Received 9 June 2003/Accepted 8 September 2003

**Pseudomonas** sp. strain ADP uses the herbicide atrazine as the sole nitrogen source. We have devised a simple atrazine degradation assay to determine the effect of other nitrogen sources on the atrazine degradation pathway. The atrazine degradation rate was greatly decreased in cells grown on nitrogen sources that support rapid growth of *Pseudomonas* sp. strain ADP compared to cells cultivated on growth-limiting nitrogen sources. The presence of atrazine in addition to the nitrogen sources did not stimulate degradation. High degradation rates obtained in the presence of ammonium plus the glutamine synthetase inhibitor MSX and also with an Nas\(^\text{−}\) mutant derivative grown on nitrate suggest that nitrogen regulation operates by sensing intracellular levels of some key nitrogen-containing metabolite. Nitrate amendment in soil microcosms resulted in decreased atrazine mineralization by the wild-type strain but not by the Nas\(^\text{−}\) mutant. This suggests that, although nitrogen repression of the atrazine catabolic pathway may have a strong impact on atrazine biodegradation in nitrogen-fertilized soils, the use of selected mutant variants may contribute to overcoming this limitation.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a herbicide of the s-triazine family used for broad-leaf weed control in both crop and noncrop lands. Its widespread use and high mobility in soil have led to its frequent detection in surface water and groundwater at concentrations exceeding the maximum levels allowed (21, 22, 30, 37). The high incidence of atrazine-contaminated water and the increasing concern about the toxicological and ecotoxicological properties of atrazine (3, 6, 16, 17) have boosted research directed toward bioremediation of atrazine-polluted sites.

A few laboratories have reported the isolation of bacteria with the ability to utilize atrazine, achieving in some cases the complete mineralization of the herbicide (see reference 29 and references therein). The best-characterized atrazine-mineralizing bacterial strain is *Pseudomonas* sp. strain ADP (23), which uses atrazine as the sole nitrogen source by means of a catabolic pathway comprising six enzymatic steps (25, 40). The complete degradative pathway is encoded in the 108-kbp conjugative catabolic plasmid pADP-1, which was recently sequenced (25). The *atzA*, *atzB*, and *atzC* genes, responsible for the conversion of atrazine to cyanuric acid, are harbored at three distant positions within a large (>40 kbp) unstable region in pADP-1. Loss of one or more of these genes is the cause of the frequent appearance of Atr\(^\text{−}\) (unable to utilize atrazine) mutants in nonselective medium (10). The genes involved in the s-triazine ring cleavage and ammonium release are clustered at a different location in pADP-1, to form the *atzDEF* operon (25). The *atzA*, *atzB*, and *atzC* genes have been shown to be widespread and plasmid borne in a number of independent isolates from different parts of the world (9, 10, 31, 39, 40).

The influence of nitrogen compounds on the efficiency of atrazine catabolism has been the focus of a number of studies, since most atrazine-degrading bacteria use it as a nitrogen source and agricultural soils are often rich in nitrogen due to routine fertilization. Nitrogen amendments have been shown to have a negative effect on atrazine biodegradation by indigenous populations in soils (1, 2, 4, 12). The effect of different sources on atrazine degradation has also been tested in pure cultures of degrading organisms, and both nitrogen-repressed and nonresponsive strains have been described (15, 28, 36). *Pseudomonas* sp. strain ADP has been shown to metabolize atrazine rapidly when previously grown on atrazine or glycine while degradation was significantly slower with cells grown on ammonium, nitrate, or urea (5, 19).

In this paper, we use a simple atrazine degradation assay in resting cell suspensions to characterize nitrogen control of the atrazine catabolic pathway in *Pseudomonas* sp. strain ADP. In addition, we determine the effect of nitrate amendment in soil on atrazine mineralization by *Pseudomonas* sp. strain ADP and describe a mutant that, by overriding nitrogen control, mineralizes atrazine efficiently in nitrate-amended soil.

**MATERIALS AND METHODS**

**Bacterial strains and growth media.** Minimal medium for *Pseudomonas* sp. strain ADP was previously described (24). Sodium succinate (1 g liter\(^{-1}\)) was always used as a carbon source. Nitrogen sources were added at a final concentration of 4 mM nitrogen, except for atrazine, which was supplied at the saturating concentration from a reservoir. Briefly, 0.15 ml of a 20-mg liter\(^{-1}\) atrazine solution in methanol was added to the cap of a microcentrifuge tube. The open side of the cap was topped with a piece of cellulose dialysis membrane (Sigma-Aldrich Chemie, Steinheim, Germany), which was sealed in place with the top ring of the same microcentrifuge tube. The sealed reservoir was added to the water in the medium and sterilized by autoclaving. This method provides a continuous supply of atrazine without the problems derived from its low water solubility (31 mg liter\(^{-1}\)). Minimal agar plates were prepared as described above.
but with the addition of 20 g of agar noble (Difco, Detroit, Mich.) liter⁻¹. Minimal atrazine agar plates included 0.4 g of atrazine liter⁻¹, added from a 20 g liter⁻¹ solution in methanol. Precipitation of atrazine crystals gives these plates a turbid appearance. Luria-Bertani (LB) agar was used as rich solid medium and was prepared by adding 0.4 g of atrazine liter⁻¹. Cultures were grown at 30°C, and shaking (~180 rpm) was used to aerate liquid cultures.

*Pseudomonas* sp. strain ADP (23) was used as a model atrazine-degrading bacterium. This strain is naturally resistant to ampicillin (40 mg/liter). Spontaneous Attr⁻ (deficient in atrazine utilization) mutants were isolated by taking advantage of the intrinsic instability of the atrazine-degradative phenotype (10).

Plasmid pADP-1 was isolated from an *Pseudomonas* sp. strain ADP grown on minimal medium containing 1 g of NH₄Cl liter⁻¹ and then subcultured in the same medium. After three serial dilutions, cells were plated on LB agar containing 0.4 g of crystallized atrazine liter⁻¹. Colonies not surrounded by clear halos were streaked on minimal agar plates containing cyanuric acid as the sole nitrogen source to test for the cyanuric acid utilization phenotype (Cya). Both Cya⁺ and Cya⁻ colonies were examined for the presence of atrazine catabolic genes by PCR and restriction analysis of the pADP-1 plasmid. Two derivatives, named MPO100 and MPO101, were chosen for further study. MPO100 is cured from pADP-1, as inferred from its Attr⁻ Cya⁻ phenotype, lack of amplification with specific atzA, atzB, and atzC primers (see reference 9 for primer sequences), absence of plasmid bands in plasmid preparations, and 100-fold-increased conjugation frequencies when compared with the recipient strain. MPO101 in Atz⁻ Cya⁺, and PCR and restriction analysis revealed that it harbors a deletion derivative of pADP-1 that lacks virtually the entire unstable region, including the atzA, atzB, and atzC genes and intervening sequences. A spontaneous Nas⁻ (deficient in nitrate assimilation) mutant derivative, named MPO102, was isolated based on nitrate resistance (14) by plating *Pseudomonas* sp. strain ADP on minimal medium containing KClO₃ (10 g liter⁻¹) and then scoring for slow growth on minimal agar plates containing nitrate as the sole nitrogen source. Spontaneous rifampin-resistant (Rifr) derivatives of these strains were isolated when required by mass plating on LB agar containing rifampin (20 mg liter⁻¹).

**Plasmid pADP-1 DNA preparation and manipulation.** Small-scale plasmid preparations from *Pseudomonas* sp. strain ADP were performed by a simple alkaline lysis method, followed by one phenol-chloroform (24:1, vol/vol) extraction and ethanol precipitation (32). Preparations displayed one single slow-migrating band when run on 0.8% agarose gels. Digestion with *EcoRV*, *HindIII* yielded band patterns consistent with those expected for pADP-1 according to the published sequence (25).

**Resting cell assay of atrazine degradation.** A simple resting cell assay was devised to measure the atrazine degradation of *Pseudomonas* sp. strain ADP based on a spectrophotometric atrazine chlorohydrolase assay described previously (7). Preinocula were grown to saturation on LB in the presence of 20 mg of ampicillin liter⁻¹ and 10 mg of rifampin liter⁻¹.

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The effect of ammonium was not due to the loss of the catabolic genes, since efficient atrazine degradation was evident when cultures were allowed to exhaust the ammonium by growing to stationary phase (data not shown). Several other nitrogen sources were tested with the same approach. The rate of atrazine degradation by cells grown on urea, proline, or nitrate was low regardless of the presence of atrazine in the growth medium (Fig. 1B to D). However, cyanuric acid, biuret, and serine failed to repress atrazine degradation both in the presence and in the absence of atrazine (Fig. 1E to G). Interestingly, nitrogen sources (ammonium, urea, and proline) that supported fast growth of Pseudomonas sp. strain ADP (doubling time, \( \frac{1}{2} \) h) repressed atrazine degradation while degradation was efficient with those nitrogen sources on which Pseudomonas sp. strain ADP exhibited slower growth (doubling time, >6 h), such as biuret, atrazine, or serine. Pseudomonas sp. strain ADP had similar doubling times (\( \frac{1}{2} \) h) when grown on nitrate and cyanuric acid, but degradation was observed with cyanuric acid-grown cells and not with nitrate-grown cells. However, this may relate to a specific positive effect of cyanuric acid on the degradative pathway (V. García-Gonzalez, unpublished data). Taken together, our results suggest that one or more steps in atrazine utilization are repressed under nitrogen-sufficient growth conditions and activated under nitrogen limitation. Activation does not appear to require atrazine or any of its metabolites (Fig. 1G). In addition, atrazine does not stimulate its own catabolism when a repressing nitrogen source is present (Fig. 1A to D).

Nitrogen incorporation to carbon backbones is required for repression of atrazine degradation. To test whether nitrogen control of atrazine degradation requires assimilation of the nitrogen source, we used MSX, an inhibitor of glutamine synthetase. Treatment of cells growing on ammonium with sublethal concentrations of MSX has been shown to provoke nitrogen starvation in several bacterial species by slowing down ammonium incorporation into carbon backbones and depleting the internal pools of glutamine (8, 34). A concentration of 0.5 mM MSX was chosen, since it had a severe effect on the growth rate but did not cause a complete inhibition of bacterial growth (Fig. 2A). Atrazine degradation by ammonium-grown cells was greatly stimulated when 0.5 mM MSX was also present in the growth medium, as assessed by resting cell assays (Fig. 2B), indicating that slow assimilation is sufficient to override ammonium-mediated repression of the pathway.

As an alternative means to test nitrogen signaling in Pseudomonas sp. strain ADP, a genetic approach was used. A Nas\(^{-}\) mutant (MPO102) was isolated that grows slowly on nitrate but exhibits a growth rate similar to that of the wild type on ammonium (Fig. 3A) and other nitrogen sources (data not
shown). Unlike the wild type, MPO102 degraded atrazine efficiently in resting cell assays when grown on nitrate alone or on nitrate and atrazine as nitrogen sources (compare Fig. 3B with 1C). These results strongly suggest that the signaling pathway responsible for nitrogen control of atrazine catabolism requires intracellular incorporation of nitrogen to carbon backbones. In addition, growth-limiting nitrogen assimilation is sufficient to induce atrazine degradation, regardless of the chemical nature of the nitrogen source tested.

Nitrate addition inhibits atrazine mineralization by Pseudomonas sp. strain ADP in soil. Nitrogen control of atrazine utilization may pose a limitation to degradation of the herbicide by Pseudomonas sp. strain ADP in nitrogen-rich agricultural soils. To address this issue, soil microcosm experiments were designed in which the rate of radiolabeled atrazine min-
eralization by *Pseudomonas* sp. strain ADP was determined in nonsterile low-nitrogen soil samples. In initial control experiments, the addition of wild-type atrazine-grown *Pseudomonas* sp. strain ADP to unamended soil greatly stimulated atrazine mineralization compared to a control to which heat-killed cells were added (Fig. 4A). Interestingly, a low level of atrazine mineralization was evident in the control vials, indicating that atrazine-mineralizing organisms were part of the natural flora in the soil. Atrazine mineralization by *Pseudomonas* sp. strain ADP was rapid, with more than 50% of the label released within the first 24 h after atrazine addition. Similar results were obtained with the MPO102 mutant (Fig. 4A). To determine the effect of nitrate on atrazine mineralization in soil, *Pseudomonas* sp. strain ADP cells were inoculated into nitrate-amended and unamended soil microcosms and allowed to acclimate to the soil environment for 6 days prior to the addition of atrazine (Fig. 4, B). Atrazine mineralization by *Pseudomonas* sp. strain ADP in the amended soil was decreased threefold compared to the unamended control, indicating that atrazine mineralization in soil is repressed by nitrate. Mineralization rates were lower than in the previous experiment, likely due to the decrease in the *Pseudomonas* sp. strain ADP population in the soil during the acclimation period (half-life, ~3.1 days).

**Atrazine mineralization by the MPO102 mutant is not repressed by added nitrate.** To test whether atrazine degradation by the MPO102 mutant is affected by the presence of nitrate in a soil environment, atrazine-grown MPO102 was also acclimated for 6 days in unamended and nitrate-amended soil microcosms. Radiolabeled atrazine was then added, and mineralization was monitored (Fig. 4C). The atrazine mineralization rate in the unamended soil was similar to that observed with wild-type *Pseudomonas* sp. strain ADP (compare unamended controls in Fig. 4B and C). Interestingly, there was no significant difference between mineralization rates in unamended and nitrate-amended soils when MPO102 was used. This difference between the wild-type and mutant strains is not due to increased survival of MPO102, since its half-life in nitrate-amended soil was slightly lower than that of the wild-type strain (2.3 days). These results indicate that atrazine degradation by MPO102 is not repressed by nitrate in the soil.

**DISCUSSION**

In this study, we have found that the presence of preferential nitrogen sources in the environment is detrimental to atrazine degradation by *Pseudomonas* sp. strain ADP. These results confirm and extend the observations of Bichat et al. (5). These authors noted that atrazine degradation by cells previously grown on ammonium, nitrate, or urea as the sole nitrogen source was slow compared to atrazine- or glycine-grown cells. Our data clearly show that certain nitrogen sources have an inhibitory effect on the catabolic pathway that is not prevented...
by the simultaneous presence of atrazine in the medium. This and the fact that efficient atrazine degradation can be achieved with compounds unrelated to the atrazine degradative pathway, i.e., serine (Fig. 1G) or glycine (5) as sole nitrogen sources indicate that neither atrazine nor any of its metabolites are required for induction of the pathway.

General nitrogen control is a well-characterized regulatory phenomenon in a number of gram-positive and gram-negative organisms (26). Our results suggest that atrazine degradation may be subjected to general nitrogen control resembling that described in enterobacteria: (i) regulation is likely to occur primarily at the gene expression level, since addition of ammonium to the assay buffer had no inhibitory effect on atrazine removal by atrazine-grown cells; (ii) atrazine catabolism is repressed in the presence of nitrogen sources that support fast growth of the strain, suggesting preferential nitrogen assimilation from these nitrogen sources; and (iii) nitrogen status is sensed from intracellular pools of metabolites, rather than from the nitrogen species present in the medium (11, 18, 33). Nitrogen control in the enterobacteria requires an alternative form of RNA polymerase holoenzyme that contains σ^54 (the product of rpoN) (26). Interestingly, an rpoN mutant of Pseudomonas putida KT2440 can grow normally on the atrazine degradation-repressing nitrogen sources ammonium, urea, and proline but fails to grow on the nonrepressing nitrogen sources serine and glycine (20). The only exception to this correlation is nitrate, which cannot be utilized as a nitrogen source by the P. putida rpoN mutant but represses atrazine degradation in Pseudomonas sp. strain ADP. However, comparison of the growth rates of the two organisms suggests that nitrate may be a growth-limiting nitrogen source for P. putida KT2440 but not for Pseudomonas sp. strain ADP. It is tempting to speculate that a conserved σ^54-dependent mechanism is responsible for general nitrogen control in the enterobacteria and the pseudomonads. In that regard, it is worth noting that genes homologous to the signal transduction and regulatory elements for general nitrogen control in the enterobacteria have been found in the sequenced genomes of Pseudomonas aeruginosa PAO1 and P. putida KT2440 (27, 35).

Many xenobiotic-degrading bacterial strains that perform well in laboratory media turn out to be poor degraders in a natural setting (42). One possible explanation for this phenomenon is the presence of environmental traits that have a negative impact on the expression of the degradative pathway. Our results indicate that nitrogen control of atrazine metabolism is functional under soil conditions and may therefore limit the potential of Pseudomonas sp. strain ADP for atrazine bioremediation in nitrogen-fertilized agricultural soils. On the other hand, the absence of nitrate repression in the MPO102 mutant and the fact that nitrate is the major nitrogen species in well- aerated soils (41) suggest that MPO102 may be a more suitable strain for bioremediation of atrazine-contaminated agricultural soils with a high nitrogen content.

ACKNOWLEDGMENTS

We thank Ines Canosa for critical review of the manuscript.

This work was supported by the European Union project QLK3-CT-1999-00041 and by a fellowship of the F.P.U. program of the Spanish Ministerio de Educacion y Cultura awarded to V.G.-G.

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