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DILIA RODRIGUEZ D'ALEMAN

ASUNTO	Radicación	08-68859
	Trámite	002
	Evento	001
	Actuación	519
	Oficio	
	Folios	003

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Patente de Invención	☐	Patente de Modelo de Utilidad	☐
Via Nacional	☐		
Via PCT (fase nacional)	☐	Cap. I ☐	Cap. II ☐

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 8 a 37	04/Julio/2008
Reivindicaciones:	Folios 38 a 40	04/Julio/2008
Dibujos:	Folios 41 a 49	04/Julio/2008
Prioridad:	CL 1982-2007	06/Julio/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Bacteria degradadora de herbicidas del tipo s-triazinas, un producto de bioremediación y un método de aplicación."

El problema planteado en esta solicitud consiste en suministrar métodos para la bioremediación de suelos contaminados con triazinas.

Para resolver este problema técnico la solicitud en estudio proporciona una cepa bacteriana capaz de degradar completamente o mineralizar contaminantes del tipo s-triazinas, como la simazina, que corresponde a la cepa Pseudomonas sp. MHP41, depositada bajo el número de acceso NRRL B-30908. Un producto para la bioremediación de ambientes contaminados con compuestos s-triazinas, en donde el producto comprende un inóculo bacteriano de dicha cepa Pseudomonas sp. MHP41.

Y un método para la bioremediación de un ambiente contaminado con s-triazinas, que utiliza dicho producto para la bioremediación.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12N1/00, C12S13/00, C12K17/14

4. No es una invención (Art 15 D 486 literales (b))

El objeto de la reivindicación 1 no se considera una invención, de acuerdo con el Art citado, ya que corresponde al aislamiento de un microorganismo existente en la naturaleza y que no ha sido modificado por el solicitante.

El objeto de las reivindicaciones 2 a 5 no se considera una invención, de acuerdo con el Art citado, ya que si bien se refiere a un producto, tanto la descripción como las reivindicaciones definen el producto como un inóculo de la bacteria, en el cual el solicitante no ha aportado a la composición ningún excipiente, por lo que el producto se reduce a el aislamiento de la bacteria de interés.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 6 a 12 no es patentable, de acuerdo con el Art 14 ya que dichas reivindicaciones se refieren a métodos que solo constituyen el dispersar en inóculo de *Pseudomonas* sp. MHP41 NRRL B 30908 en un ambiente contaminado. Además, el resultado del método corresponde a un proceso esencialmente biológico, ya que *Pseudomonas* sp. MHP41 NRRL B 30908 es un microorganismo **aislado**, sobre el cual el solicitante no realizó ninguna modificación y que tiene dentro de su actividad natural la degradación de s-triazinas

A pesar de que el capítulo reivindicatorio corresponde a materia no patentable, se aclara al solicitante que en el estado de la técnica se encontraron los siguiente documentos que anticipan la presente solicitud.

Ítem	Documento	Título	Fecha de Publicación*
D1	WO03025627	Optical biosensor with enhanced activity retention for detection of halogenated organic compounds	27/Marzo/2003
D2	Appl Environ Microbiol.; 70(4): 1907–1912	Simultaneous Degradation of Atrazine and Phenol by <i>Pseudomonas</i> sp. Strain ADP: Effects of Toxicity and Adaptation	Abril/2004

D1 divulga un producto de bioremediación de ambientes contaminados con s-triazina (Página 15 tabla 3 y figura 2), que incluye un inóculo de *Pseudomonas* sp. (Ver página 16 línea 15 a 19 y página 21 línea 19) y alginato de sodio (ver página 15 líneas 40 a 44), dicho inóculo puede estar o no liofilizado en un complejo protectorio estéril (página 13 línea 1 a 7).

http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=03025627A2&KC=A2&FT=D&ND=3&date=20030327&DB=EPODOC&locale=en_EP

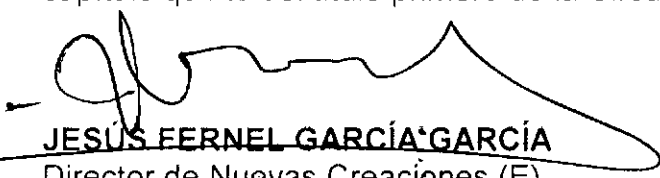
D2 divulga una cepa de *Pseudomonas* sp. ADP que es capaz de degradar atrazina como una única fuente de nitrógeno (resumen).

<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC383114/>

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante y presentarla por escrito

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JESÚS FERNEL GARCÍA GARCÍA
Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha

13 JUL 2012

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Simultaneous Degradation of Atrazine and Phenol by *Pseudomonas* sp. Strain ADP: Effects of Toxicity and Adaptation

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The strain *Pseudomonas* sp. strain ADP is able to degrade atrazine as a sole nitrogen source and therefore needs a single source for both carbon and energy for growth. In addition to the typical C source for *Pseudomonas*, Na₂-succinate, the strain can also grow with phenol as a carbon source. Phenol is oxidized to catechol by a multicomponent phenol hydroxylase. Catechol is degraded via the *ortho* pathway using catechol 1,2-dioxygenase. It was possible to stimulate the strain in order to degrade very high concentrations of phenol (1,000 mg/liter) and atrazine (150 mg/liter) simultaneously. With cyanuric acid, the major intermediate of atrazine degradation, as an N source, both the growth rate and the phenol degradation rate were similar to those measured with ammonia as an N source. With atrazine as an N source, the growth rate and the phenol degradation rate were reduced to ~35% of those 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 is the bottleneck that diminishes the growth rate of *Pseudomonas* sp. strain ADP with atrazine as an N source. Whereas atrazine and cyanuric acid showed no significant toxic effect on the cells, phenol reduces growth and activates or induces typical membrane-adaptive responses known for the genus *Pseudomonas*. Therefore *Pseudomonas* sp. strain ADP is an ideal bacterium for the investigation of the regulatory interactions among several catabolic genes and stress response mechanisms during the simultaneous degradation of toxic phenolic compounds and a xenobiotic N source such as atrazine.

Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine], a herbicide used for controlling broadleaf and grass weeds and is relatively persistent in soils (14). Atrazine herbicides have been detected in ground and surface water at levels exceeding the Environmental Protection Agency's maximum contaminant level of 3 ppb (14).

Pseudomonas sp. strain ADP was the first isolated bacterium capable of degrading the herbicide atrazine (22, 36). Since then, most of our understanding of the genes and enzymes involved in atrazine degradation has been derived from studies with this strain, in which the first three enzymatic steps of atrazine degradation have been defined (8). 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 (8). Cyanuric acid is further metabolized by *Pseudomonas* sp. strain ADP to carbon dioxide and ammonia (8). These first three genes have been located on a 10.1-kb plasmid, pADP-1 (8). Recently, the pADP-1 has been completely sequenced and was shown to contain the genes for the complete catabolism of cyanuric acid to CO₂ and NH₄ as well as *atzD*, *atzE*, and *atzF* (25). 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.

However, the strain is able to use atrazine and cyanuric acid as sole nitrogen sources and therefore needs an additional carbon and energy source for growth.

In this study, we investigated the degradation of phenol in the presence of different N sources, including the two s-triazines atrazine and cyanuric acid. Thus, we could demonstrate that the direct effects of the adaptation of the cells to phenol, as well as the degradation of atrazine to cyanuric acid, were the major bottleneck that limits effective degradation of atrazine. We also demonstrated that in the ADP strain, genes encoding phenol degradation are located on the chromosome. A multicomponent phenol hydroxylase converts phenol to catechol, which is further metabolized via the catechol degradation *ortho* pathway.

MATERIALS AND METHODS

Strains and chemicals. *Pseudomonas* sp. strain ADP was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ 21735) and has been described previously (22). The bacterial strains and plasmids used in this study are listed in Table 1. All chemicals were reagent grade and were obtained from commercial sources.

Culture conditions. *Pseudomonas* sp. strain ADP was cultivated in a mineral medium as described by Hartmans et al. (13). The N and C sources of the medium were modified for the specific purposes of the experiments. Adaptation of the cells to phenol was reached by growing the cells semicontinuously in batch cultures with increasing concentrations of phenol up to 1,000 mg/liter. The adapted cells were maintained on agar plates with 500 mg of phenol/liter as the sole C and energy source. Cells were grown in 50-ml shake cultures in a horizontally shaking water bath at 30°C. Growth was monitored by measuring the

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TABLE 1. Strains and plasmid used in this study

Strain or plasmid	Genetic characteristics	Reference
<i>Pseudomonas fluorescens</i> PC18	Phe ⁺ ; multicomponent phenol hydroxylase genes; <i>meta</i> pathway of catechol-degrading genes on chromosome	16
<i>P. putida</i> PaW85	Phe ⁺ ; <i>ortho</i> pathway of catechol degradation genes on chromosome	3
<i>P. putida</i> PaW340	Phe ⁺ Trp ⁺ Sm ^r ; <i>ortho</i> pathway genes on chromosome	11
<i>Pseudomonas</i> sp. strain ADP	Atr ^r Phe ⁺	23
<i>Pseudomonas</i> sp. strain ADP	Ap ^r ; <i>dmpKLMNOP</i> genes	2

medium (OD₆₀₀ 0.1). Atrazine was applied in amounts of 100 µg/ml. The cells were grown in an Erlenmeyer tube with a 100-ml volume of medium. The constant presence of the bacteria in the medium was determined by the maximum absorbance of the culture. The addition of atrazine hindered the precipitation of the cells, and the OD₆₀₀ would have affected measurement of the OD.

For the selection from overnight culture was transferred to 50 ml of fresh medium. The cells were grown exponentially for 2 to 4 h (until an OD₆₀₀ of 0.6 to 0.8). For all other biological experiments, all *Pseudomonas* strains were grown in 50 ml of LB medium (26) or M9 minimal medium (27). If needed, phenol was added to a final concentration of 250 mg/liter. *Pseudomonas putida* PaW340 was incubated in the presence of streptomycin at a concentration of 1,000 µg/ml and in the presence of tryptophan at a final concentration of 25 µg/ml.

Incubation with toxins. For measurements of the toxic effects of the investigated atrazine, cells were added to exponentially growing nonadapted cultures of *Pseudomonas* sp. strain ADP. The cultures were incubated in the presence of atrazine in 100 µl of water with 30°C. Then, the cells were incubated in 100 µl of 0.1 M sodium phosphate buffer (50 mM, pH 7.0) with 30°C for 2 h. The cells were then extracted.

Lipid extraction, transesterification, and fatty acid analysis. The lipids were extracted from the cells by ethanol-water as described by Bligh and Dyer (5). The fatty acid methyl esters (FAME) were prepared by incubation for 15 min at 95°C in 100 µl of methanol and 100 µl of acetyl chloride. The method of Morrison and Smith (28) was used for the analysis of the FAME.

Analysis of fatty acid composition by GC-MS. Analysis of FAME in hexane was performed using a quadrupole gas chromatography-mass spectrometry (GC-MS) system (HP6890 and HP5973, Hewlett-Packard, Palo Alto, Calif.) equipped with a split-splitless injector. A CP-Sil 88 capillary column (inside diameter 0.25 mm, length 30 m, 0.25-µm-thick film) (Chrompack, Middelburg, The Netherlands) was used for the separation of the FAME. The GC conditions were as follows: the oven temperature was held at 250°C. The split flow was 1.0 ml/min. The carrier gas was helium. The temperature program was 50°C for 1 min, 10°C/min to 250°C, 1 min, 20°C/min to 280°C. The MS conditions were as follows: the ionization energy at 70 eV. The peak areas were determined by using the chromatograms were used to determine the relative abundance of the fatty acids were identified by GC-MS and comparison with the data from the literature (from Sureda (Bellefonte, Pa.) and the analysis of the fatty acid composition of phenol was done using the cochromatographic method. The results were compared with 4-aminopyridine (4-aminopyridine-2,3-dicarboxylic acid) and 4-aminopyridine-2,3-dicarboxylic acid under alkaline conditions. The results were monitored spectrophotometrically at a wavelength of 254 nm.

Enzyme assays. Cells of strain ADP were grown overnight in M9 minimal medium containing 250 mg of phenol/liter. Then, the culture was diluted in fresh LB medium (250 mg of phenol/liter) or M9 minimal medium (250 mg of phenol/liter). To avoid any stationary-phase-induced effects, the bacteria culture was diluted with fresh medium to OD₆₀₀s of 0.1 to 0.2. Those starting culture in minimal medium and in LB or LB with phenol, respectively. The cell samples for enzyme assays were taken from cultures grown for 0, 8, 16, 24, and 32 h.

For the preparation of crude extracts, the collected cells were washed with 0.1 M KH₂PO₄ buffer (100 mM, pH 7.5), resuspended in the same buffer, and centrifuged at 10,000 × g for 5 min. The crude extracts were purified from cell debris and supernatant by centrifugation at 12,000 × g and 4°C for 30 min. The activities of catechol 1,2-dioxygenase (PheA (20)) and catechol 2,3-dioxygenase (PheB (21)) in crude extracts were monitored by examining the disappearance of

NADPH at 340 nm as described previously (4). For the measurement of endogenous oxidation of NADPH, the phenol substrate was omitted from the reaction mixture.

To detect catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) activities, the formation of the reaction products *cis-cis*-muconic acid and 2-hydroxymuconic semialdehyde was measured spectrophotometrically at 260 and 375 nm, respectively (10, 15). The protein concentration was measured according to the Bradford method (6).

DNA techniques. The method of Hansen and Olsen (12) was used for plasmid DNA preparation from *Pseudomonas* sp. strain ADP. The plasmid DNA was fractionated from the total DNA by a CsCl-ethidium bromide density gradient. Separation of genomic DNA in agarose gels and Southern blotting were carried out by the standard procedures described elsewhere (32). Digestion of DNA with restriction enzymes was performed according to the manufacturers' guidelines.

Two primer pairs were used to amplify the DNA region encoding the largest and most conserved subunit of the multicomponent phenol hydroxylase. The primers *dmpN*1 (5'-GCTGTCCTCACCCTCCG-3') and *dmpN*547 (5'-CCGCGGCCAGAACCACTTA-3') were designed to amplify the *dmpN* gene in *Pseudomonas* sp. strain CF600, and the second pair of primers used, *phe*149 (5'-CGATCGACGAGCTGCGCA-3') and *phe*212 (5'-GTTGTCAGCACTGATTCGAAGGACAA-3'), was the same as that designed by Watanabe et al. (37) based on the analysis of conserved sequences of the large subunit of the prevalent phenol hydroxylase in the environment.

Conjugation. *Pseudomonas* sp. strain ADP as a donor and *P. putida* PaW340 as a recipient were grown overnight in LB medium, and streptomycin was added to the growth medium of PaW340. Then, the cultures were diluted 1:20 (vol/vol) with fresh LB medium and cultivated for 3 h. The cultures were mixed in equal volumes, and 100 µl of the mixture was spotted onto LB medium plates overnight. The next morning, the bacteria were resuspended and washed with Na₂HPO₄-KH₂PO₄ buffer (100 mM, pH 7.5) and plated onto M9 minimal medium plates supplemented with 250 mg of phenol/liter as the only carbon source and with tryptophan. Streptomycin was added to selective plates for counterselection for growth of the donor cells.

RESULTS AND DISCUSSION

Growth of *Pseudomonas* sp. strain ADP with phenol as a sole C source and atrazine or cyanuric acid as a sole N source. *Pseudomonas* sp. strain ADP is able to degrade atrazine as a sole nitrogen source and therefore needs an additional carbon and energy source for growth. Besides the typical C source for *Pseudomonas*, Na₂-succinate, the strain can also grow with phenol as a carbon source. Although phenol showed toxic effects on the strain, it was possible to adapt strain ADP to high concentrations of the substance. For maintenance of the strain and for all further degradation experiments, phenol concentrations of 500 mg/liter were chosen, which caused ~50 percent growth inhibition and the strongest adaptive reactions.

Using the adapted cells, it was possible to degrade phenol as a carbon and energy source and atrazine as an N source simultaneously. To quantify the simultaneous degradation, it was decided to vary the phenol concentrations, whereas the initial

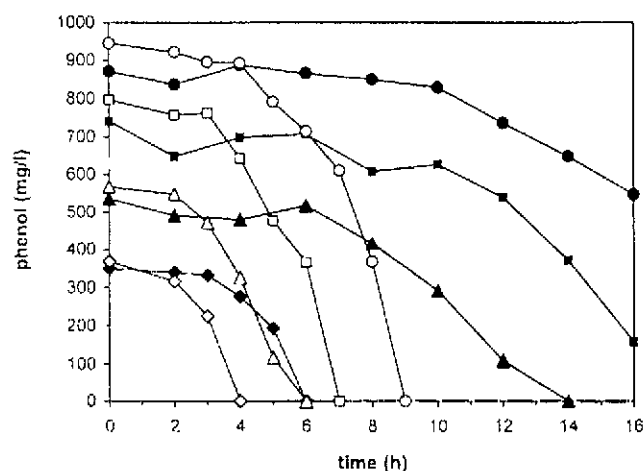


FIG. 1. Comparison of phenol degradation by adapted cells of *Pseudomonas* sp. strain ADP with 15 mM cyanuric acid (open symbols) and with 150 mg of atrazine/liter (closed symbols) as a sole N source at initial phenol concentrations of 300 (diamonds), 500 (triangles), 750 (squares), and 1,000 (circles) mg/liter.

concentrations of the tested N sources were kept stable. For this reason, we applied a nominal atrazine concentration of 150 mg/liter.

When atrazine was the N source, the strain was able to degrade initial amounts of up to 1,000 mg/liter. At higher concentrations, however, completely adapted cells were no longer able to degrade. Figure 1 shows the degradation of different phenol concentrations; atrazine degradation showed the same depletion curves (data not shown). The highest concentration of phenol was completely degraded after ~ 30 h. Thus, the growth rates were up to 0.12 h^{-1} , which corresponds to a doubling time of $\sim 5.75 \text{ h}$.

To compare these data with those for other s-triazines as N sources, the same series of experiments was conducted with the major intermediate of atrazine degradation, cyanuric acid (Fig. 1). In the presence of this N source, the strain degraded phenol completely: 1,000 mg/liter was completely degraded within 10 h. The growth rates were up to 0.32 h^{-1} , which corresponds to a doubling time of $\sim 2 \text{ h}$. The degradation rates for phenol were up to 150 mg/h.

Figure 2 summarizes the measured growth rates, depending on the N source and the initial phenol concentrations, respectively. In the presence of all investigated N sources, the cells showed a typical phenol-dependent course for the growth rates, with a maximum at a phenol concentration of 400 mg/liter. At higher concentrations, the toxic effects of phenol seem to reduce the growth rate of the cells. Comparing the N sources, the growth rates obtained for cyanuric acid were nearly the same as those measured for the N source typically used for this bacterium, ammonia. On the other hand, the growth rates measured for atrazine were only $\sim 35\%$ of those reached with the other two N sources and the same initial phenol concentrations (Fig. 2).

Contrary to this, cells grown with a constant initial phenol concentration of 500 mg/liter and various atrazine concentrations showed no significant differences in their growth rates. Thus, only a correlation between an increased atrazine con-

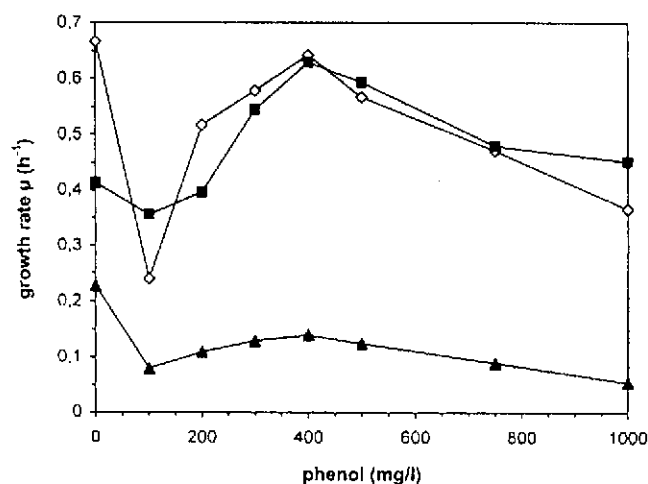


FIG. 2. Comparison of growth rates of *Pseudomonas* sp. strain ADP growing on NH_4^+ (■), cyanuric acid (◇), and atrazine (▲) as N sources. The data given for the concentration of 0 mg of phenol/liter correspond to the control cultures grown on Na_2 succinate as a C source.

centration and an increasing cell yield could be observed (data not shown).

These data showed that it was possible to cultivate *Pseudomonas* sp. strain ADP with phenol as a sole C and energy source and simultaneously with atrazine or cyanuric acid as an N source. To our knowledge, this is the first description of the simultaneous degradation of two hazardous compounds used by a single bacterium as the C and N source, respectively.

The differences between growth and phenol degradation rates obtained with cyanuric acid and with atrazine as N sources are difficult to explain. Atrazine had no major toxic effects on the cells, and in previous molecular biological studies, it had been shown that the first three enzymes involved in atrazine degradation to cyanuric acid are constitutively present in the cells (8). However, despite this information the degradation of atrazine to cyanuric acid seems to be the bottleneck that diminishes the growth rate of *Pseudomonas* sp. strain ADP with atrazine as an N source. One explanation for this obviously reduced efficiency in using atrazine might be the lower bioavailability of this substrate, which shows a much lower solubility than, e.g., cyanuric acid. Therefore, the reduced uptake of atrazine might be the factor causing the diminished growth rates with this compound.

Membrane adaptation of *Pseudomonas* sp. strain ADP to phenolic compounds and other forms of stress. The results mentioned above led to the conclusion that in addition to the regulation of the catabolic genes for either atrazine or phenol, toxicity might also directly influence the simultaneous degradation of atrazine and phenol. Therefore, we also investigated the toxic effects of the two compounds on strain ADP. For this reason, nonadapted cells grown in mineral medium with ammonia and succinate as the N and C source, respectively, were incubated with different concentrations of the compounds and growth, as well as membrane-adaptive responses, was observed.

Microorganisms are able to adapt to the presence of toxic organic compounds by using a whole cascade of adaptive

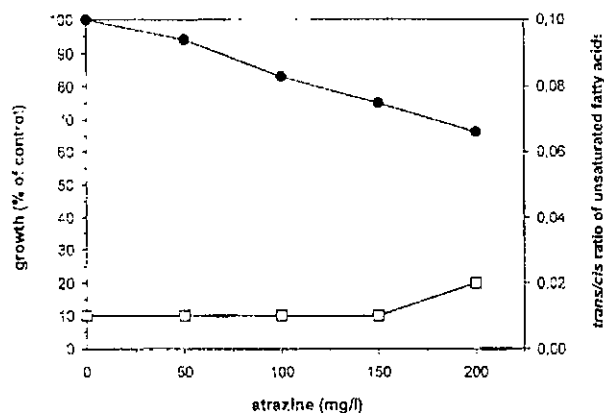


Fig. 3. Effect of atrazine on growth (●) and *trans/cis* ratio of unsaturated membrane fatty acids (□) of nonadapted cells of *Pseudomonas sp.* strain ADP.

growth (for a review see reference 31). Among the adaptive mechanisms changes in the fatty acid composition of the cell lipid are the most important reactions of bacteria to membrane active substances. One adaptive mechanism encountered by several *Pseudomonas* strains to grow in the presence of membrane-disrupting compounds is the isomerization of *cis*-unsaturated fatty acids to *trans*-unsaturated fatty acids (17, 35). This mechanism could also be found in *Pseudomonas sp.* strain ADP. The extent of the isomerization, usually expressed as the *trans/cis* ratio of unsaturated fatty acids, apparently correlates with the toxicity of organic compounds (18, 19). Additionally, a mutual dependence was found between the activation of this system and the induction or activation of other stress response mechanisms. Therefore, an increase in the *trans/cis* ratio can be used as an indicator of environmental stress (35).

Figure 3 shows the effects of atrazine on the growth and *trans/cis* ratio of strain ADP. Atrazine causes only a slight toxic effect on the bacteria. Even at concentrations above the water solubility limit (30 mg/liter) the growth of the cells was not inhibited. This is also reflected in the *trans/cis* ratio, which only a slight increase could be observed.

A similar reaction can be observed with phenol, as presented in Fig. 4. Although *Pseudomonas sp.* strain ADP is able

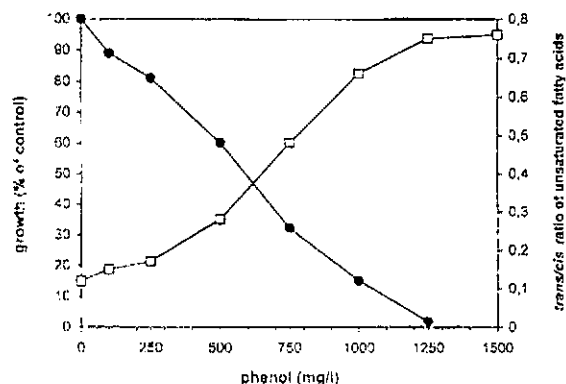


Fig. 4. Effect of phenol on growth (●) and *trans/cis* ratio of unsaturated membrane fatty acids (□) of nonadapted cells of *Pseudomonas sp.* strain ADP.

to degrade phenol, at the same time, this substrate evokes or causes a strong inhibition of cellular growth. In the presence of phenol, the *trans/cis* ratio of unsaturated fatty acids also showed a toxicity-dependent increase.

Increasing concentrations of atrazine had only minor inhibiting effects on the growth rate and cell adaptation of *Pseudomonas sp.* strain ADP. This can be explained by the very limited water solubility of the compound, which is only ~30 mg/liter under the experimental conditions. Very surprising was the observation that atrazine showed the slight increase in toxicity even at concentrations which were much higher than the solubility in water. This seems to be an indication of either an accumulation of the compound in the membranes of the cells or the presence of compounds produced by the cells which increase the solubility of atrazine. On the other hand, the simultaneous growth with phenol as a carbon source might provide the explanation for this increased toxicity of atrazine, because phenol also induces or activates the adaptive response mechanisms of the bacteria to this toxic compound. As toxic effects might indeed play a major role in the regulation of the catabolic genes during a simultaneous degradation of the two xenobiotic compounds, it was decided to characterize the phenol degradation in this strain.

Pseudomonas sp. strain ADP uses a multicomponent phenol hydroxylase for phenol oxidation. The first step of phenol degradation is determined by the phenol hydroxylase, which may contain only one subunit (monocomponent enzyme) or several subunits (multicomponent phenol hydroxylase). The multicomponent phenol hydroxylase of *Pseudomonas sp.* strain CF600 is well known and has six subunits, although only five seem to be needed for the production of this multicomponent enzyme (30). The genes that encode subunits of the multicomponent phenol hydroxylase of CF600 are located in a plasmid-encoded operon, the *dmp* operon (29). Many multicomponent phenol hydroxylase-encoding operons have a DNA sequence and organization similar to that of the *dmp* operon. On the other hand, some bacteria, such as *Pseudomonas sp.* strain EST1001 (20) and *Pseudomonas pickettii* strain PKO1 (21), use monocomponent phenol hydroxylases which do not show significant similarities.

Therefore, the first investigations of the molecular biology of phenol degradation in strain ADP were related to the description of the phenol hydroxylase the strain uses for phenol oxidation.

As the monocomponent phenol hydroxylase (PheA) of *Pseudomonas sp.* strain EST1001 and TbuD of *P. pickettii* PKO1 are measurable from crude cell extracts and use NADPH to add molecular oxygen to the aromatic ring, we made cell extracts of strain ADP and spectrophotometrically monitored the disappearance of NADPH at 340 nm. No phenol hydroxylase activity was measurable in the cell extracts (data not shown).

The multicomponent phenol hydroxylase is possibly involved in phenol oxidation. In order to prove this possibility, we used the PCR method to try to amplify a DNA segment of the ADP genome by using primers complementary to the gene of the largest and the most conserved subunit of the multicomponent phenol hydroxylase (37). The ~250-bp PCR product we obtained was sequenced, and the results indicated that ADP contains a sequence for the phenol hydroxylase large subunit

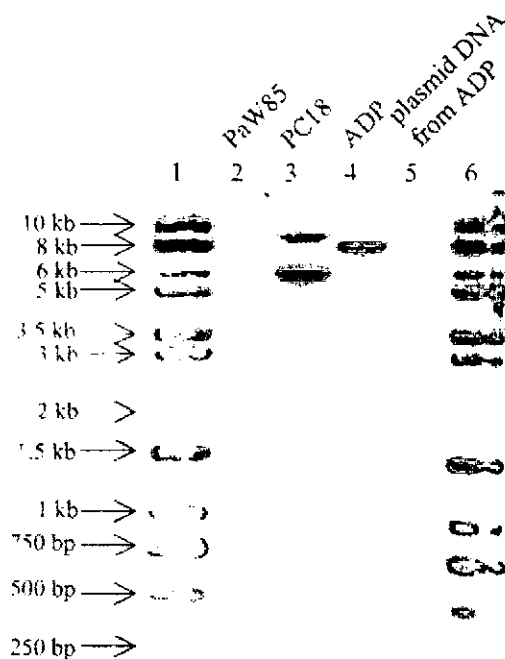


FIG. 5. Autoradiograph of Southern analysis of total EcoRI-digested DNA in *P. putida* PaW85 (lane 2), *Pseudomonas fluorescens* PC18 (lane 3), and the *Pseudomonas* sp. strain ADP (lane 4) and plasmid DNA of ADP digested with EcoRI (lane 5) hybridized with radiolabeled DNA specific for the *dmpN* gene of multicomponent phenol hydroxylase of *Escherichia coli* sp. strain CF600. The molecular sizes of the marker DNA (lanes 1 and 6) are given on the left.

of *AtzA* is very similar (94% identity [data not shown]) to that of *AtzA* from strain rN1 described by Watanabe et al. (37). The phenol hydroxylase in strain rN1 belongs, together with the other family hydroxylases, to group III of multicomponent phenol hydroxylases (37).

Localization of the phenol degradation genes in *Pseudomonas* sp. strain ADP. Bacteria can have their phenol-degrading genes located either on plasmids or on the chromosome (27). *Pseudomonas* sp. strain ADP contains two plasmids, pADP-1 and pADP-2 (8). pADP-1 was sequenced, and no genes exhibiting homology to the genes for phenol degradation could be found on the 709 kb plasmid (25). In order to test whether the phenol hydroxylase could be located on another plasmid, the 53 kb pADP-2, we conjugated ADP as a donor to the *Escherichia coli* strain PaW340 (which contains catechol degradation genes but lacks a phenol hydroxylase gene) (25). No *P. putida* PaW340 transconjugants were obtained on the selective plates. To specify the location of the phenol degradation genes in the ADP strain, Southern blot analysis of a total-DNA preparation of ADP was performed. A radiolabeled 537-bp PCR product, which was amplified from the *dmpA* gene of plasmid pVI261 by primers *dmp1* and *dmp537*, was used as a specific hybridization probe. The radiolabeled PCR product hybridized with the total DNA of ADP (Fig. 5, lane 4) and with the total DNA of PC18 (lane 3) as a positive control. No hybridization could be detected in the case of the plasmid DNA from ADP (lane 5) and the total DNA of PaW85 as a negative control (lane 2). This indicates that at

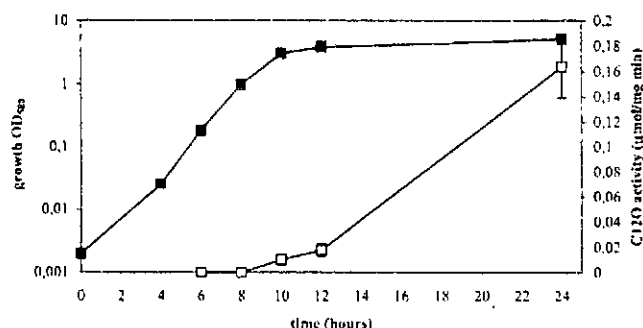


FIG. 6. Growth curve (■) and measurement of C12O activity (□) from cell extracts of *Pseudomonas* sp. strain ADP in LB medium supplemented with 250 mg of phenol/liter. Data (means $\pm$ standard deviations) from at least three independent experiments are presented.

least one subunit of the phenol hydroxylase gene in strain ADP is localized on the chromosome (Fig. 5).

Pseudomonas sp. strain ADP utilizes phenol via the *ortho* pathway of catechol degradation. Phenol is usually degraded via the catechol degradation pathway. There are two pathways for catechol ring fission, the *meta* and *ortho* pathways (34). The majority of bacteria use the *meta* pathway of catechol degradation, especially if the bacteria have multicomponent phenol hydroxylases, like *Pseudomonas* sp. strain CF600 (30, 34). However, there is also evidence that the multicomponent phenol hydroxylase and the *ortho* pathway of catechol degradation can coexist (9). Both *meta* and *ortho* pathways are distinguishable by measuring characteristic enzymes, C23O for the *meta* pathway and 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 logarithmically growing (6-h) cells was 1.66 ± 0.24 $\mu\text{mol/mg} \times \text{min}$. This demonstrates that *Pseudomonas* sp. strain ADP uses the *ortho* pathway of catechol degradation on phenol catabolism.

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 and de Lorenzo [7]). In order to study whether ADP phenol degradation would also be repressed in rich medium, the strain was grown in LB medium in the presence of phenol and the activity of the *ortho* pathway enzyme, C12O, was measured (Fig. 6). No C12O activity could be detected when the bacteria were grown in LB medium in the absence of phenol. In the presence of phenol, the C12O activity was measurable only when the cells were entering stationary phase, and it increased during stationary phase, demonstrating that expression of the phenol degradation pathway in *Pseudomonas* sp. strain ADP is also under physiological control.

The genes *atzA*, *atzB*, and *atzC*, which encode the enzymes of atrazine degradation to cyanuric acid, are constitutively expressed and are not regulated, either by induction of atrazine or by repression of other N sources (8, 25). However, the genes for the complete catabolism of cyanuric acid to CO_2 and NH_3 ,

atzE, and *atzB* (25), are regulated by RpoN (σ^{54}) and induced by cyanamic acid and N depletion (E. Santero, personal communication). As RpoN is also known to be involved in the regulation of many promoters that control phenol degradation pathways, there might be links between the regulations of both degradation pathways in *Pseudomonas* sp. strain ADP. Very recently, it was proven that the uptake of atrazine is repressed by non-renewable N sources, such as ammonia, urea, and nitrate (E. Santero, personal communication). Thus, the σ^{54} regulon for nitrogen metabolism, σ^{54} , seems to be responsible for this repression of atrazine uptake and degradation. On the other hand, it has also been shown that several catabolic genes for aromatic compounds are regulated by σ^{32} , or the main stress regulator, σ^{32} (σ^S) (29, 33).

With this background *Pseudomonas* sp. strain ADP is an ideal bacterium for the investigation of the regulatory interactions among several catabolic genes and stress response mechanisms during the simultaneous degradation of toxic phenolic compounds and a xenobiotic N source such as atrazine.

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