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EXPERIMENTAL ARTICLES

Characterization and In Situ Monitoring of Atrazine-Transforming Bacteria

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Abstract—The possible application of the in situ hybridization method to detect soil microflora able to transform the herbicide atrazine was investigated. Digoxigenin-labeled probes were shown to provide sufficient resolution when used either for hybridization on colonies or for in situ detection of bacteria in soil.

Key words: in situ hybridization, digoxigenin, atrazine, transformation, denitrification.

Atrazine, a chlorotriazine herbicide, is commonly used worldwide in cultures of corn, sorghum, pineapple, and sugar cane, as well as for treating orchards. This herbicide is effective against the most widespread and harmful of the weeds. However, its long aftereffect, exceeding one vegetation period, is a disadvantage limiting its applicability. The questions therefore arise of the effect of the herbicide on soil microflora and of the possibility of its microbial transformation. Since the 1960s, a number of studies on triazine decomposition have been performed in search of this information [1].

It is well known that a considerable part of the species constituting natural microbial communities are not culturable under laboratory conditions [2, 3]. Molecular methods based on the analysis of DNA or RNA sequences make it possible both to reveal and identify bacteria in natural environments, no matter culturable or not, and to concentrate on specific physiological or phylogenetic groups among diverse microorganisms. Research has demonstrated the applicability of in situ hybridization for the study of soil bacterial communities [4].

In the present work, the possible application of in situ hybridization to detect atrazine-transforming microflora in soil was investigated.

MATERIALS AND METHODS

The middle-podzolic, heavy-loamy soil (humus, 2.4%; pH of the salt extract, 8.2) of the Epoisse-Dijon parcel, France, was used for the investigation. Sterilization was performed by chloroform fumigation [5]. Atrazine was introduced into the soil in a concentration of 0.3 g/kg. The soil samples were incubated for two months. Radiorespirometry was used to monitor atrazine mineralization [6]. Atrazine with a ^{14}C label in the aliphatic chain was used (1776 MBq/mmol). The cell number of atrazine-transforming bacteria was estimated using a modified most-probable-number technique [7] on a Packard 1900TR-Tri-carb with the ACSII scintillation liquid (Amersham). The experiments were performed in triplicate.

In order to investigate the applicability of in situ hybridization to reveal atrazine-transforming microflora in soil, the strain *Pseudomonas* sp. ADP, obtained from the Soil and Water Institute (Israel), was used. The strain *Pseudomonas* sp. ADP was shown to be able to use atrazine as the sole nitrogen source, metabolizing it to carbon dioxide, ammonium, and chloride [8]. Sterile soil was inoculated with *Pseudomonas* sp. ADP in the concentrations of 10^4 and 10^6 CFU/g. The growth medium used to cultivate the microorganism contained (g/l) sodium citrate, 1.0; K_2HPO_4, 16.0; KH_2PO_4, 4.0; $MgSO_4 \cdot 7H_2O$, 2.0; NaCl, 1.0; $CaCl_2$, 0.2; $FeSO_4 \cdot 6H_2O$, 5.0; and 10 ml of a trace element solution of the following composition: $ZnSO_4 \cdot 7H_2O$, 0.4 g/l; $MgCl_2 \cdot 4H_2O$, 20 mg/l; H_3BO_3, 10 mg/l; $CoCl_2 \cdot 6H_2O$, 50 mg/l; $CuSO_4$, 0.2 g/l; $NiCl_2 \cdot 6H_2O$, 10 mg/l; and EDTA, 0.25 g/l. Atrazine was introduced into the medium in a concentration of 0.5 g/l. To estimate the ability of the strain to grow on nitrogen-containing pesticides, atrazine, propazine, prometrine, simazine, and diuron were introduced in 20.0 g/l concentrations. The strain was characterized on the basis of its morphological and physiological–biochemical characteristics [9].

The sequences complementary to the *atzA* gene, which is responsible for the first stage of atrazine degradation, were obtained using the PCR technique on a PCR Hybaid (Ceralabo) according to the procedure described in [11]. The following primers were used: 5'-CCATGTGAACCAGATCCT-3' and 5'-TGAAGCGTCCACATTACC-3'. The size of the amplified DNA probe was 0.5 kb. PCR was performed in a reaction mixture (50 μl) containing 5 μl 10× buffer (Appligene-Oncor, France), 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μM

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of each primer, 1.25 U *Taq*-polymerase (Appligene-Oncor, France), and 25 ng DNA. The reaction conditions were as follows: one cycle at 95°C for 4 min; five cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min; one cycle at 94°C for 1 min, 59, 58, 57, and 56°C for 1 min, and 72°C for 2 min; 26 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and one cycle at 72°C for 3 min. The DIG Oligonucleotide 3'-End Labeling Kit Boehringer Mannheim 98 was used for probe labeling.

Hybridization was performed both on colonies of bacteria isolated from soil and in situ, on filters and membranes after their incubation in soil. For this purpose, two paper disks moistened with 0.5 N NaOH were placed in a Petri dish, covered with membranes, and incubated for 7 min. Then, the membranes were washed twice for 2 min with 1 M Tris–HCl, pH 7.2, and afterwards with 0.5 M Tris–HCl and 1.5 M NaCl, pH 7.2, for 4 min. For further pronase treatment, a solution of 0.01 M Tris–HCl, 0.01 M EDTA, and 0.5% SDS, pH 8, was used; pronase (1 mg/ml) was added immediately before the treatment. The dishes were then closed, sealed with Parafilm, and incubated for 1 h at 37°C. The membranes were washed by shaking for 5 min in a solution of 0.3 M NaCl and 0.03 M Tris–sodium citrate and incubated for 1 h at 80°C.

For prehybridization, the membranes were immersed in the hybridization solution (0.6 M NaCl and 0.06 M Tris–sodium citrate, blocking agent, *N*-laurylsarcosine, and 20% SDS) in polyethylene bags and incubated in a water bath at 68°C for 2 h.

Hybridization was performed as follows. The probe was dissolved in the hybridization solution (2 μl per 10 ml). The membranes were incubated overnight at 68°C in shaking polyethylene bags with the probe. Washing was performed twice at room temperature in a solution of 0.3 M NaCl, 0.03 M Tris–sodium citrate, and 0.1% SDS. To eliminate the nonspecifically bound probe, the membranes were incubated in a water bath at 68°C in a solution of 0.015 M NaCl, 0.0015 M Tris–sodium citrate, and 0.1% SDS for 15 min.

Immunological determination was performed according to the standard procedure with an Anti-Dig-AP Fab antibody (Boehringer, Mannheim, Germany). CDP-Star™ was used as the chemiluminescent substrate.

RESULTS AND DISCUSSION

The morphological and physiological–biochemical characteristics of the atrazine-transforming strain *Pseudomonas* sp. ADP are presented in the table. Good growth of *Pseudomonas* sp. ADP was observed on agarized media with diuron and other triazine herbicides—atrazine, propazine, prometrine, and simazine—in concentrations of 20.0 g/l. Investigation of the dynamics of the cell number of the bacterium inoculated in soil showed that, during the first three weeks after inoculation of *Pseudomonas* sp. ADP, its concentration in the samples increased rapidly. After longer incubation, exceeding three weeks, the cell concentration in the soil decreased; the decrease was more rapid with a 10^6 CFU/g inoculum than with a 10^4 CFU/g inoculum (Fig. 1). After two months of incubation, atrazine mineralization for the 10^4 and 10^6 CFU/g inocula was 25 and 26%, respectively (Fig. 2).

Morphological and physiological–biochemical characteristics of *Pseudomonas* sp. ADP

Feature	*Pseudomonas* sp. ADP
Gram reaction	gram-negative
Cell shape	rods and coccobacilli
Motility in liquid medium	+
Yellow colony pigmentation	+
Oxidase	+
Catalase	+
Denitrification (to N_2)	+
Heterotrophic nitrification	+
N_2 fixation in vitro	–

Hybridization was performed during the third and the eighth weeks of soil incubation. Hybridization on colonies revealed a pronounced signal in both experimental setups; its intensity increased with incubation time. After a week of incubation, the signal spread all over the surface of contact with the membrane (Fig. 3a).

Replication of the filters incubated in soil to membranes and subsequent hybridization revealed interaction with the probe along the filter edge and partially in the center; this finding implies spatial redistribution of bacteria relative to the filter in soil (Fig. 3b).

Soil samples inoculated with 10^5 CFU/g were used for in situ hybridization. For this purpose, the nylon mem-

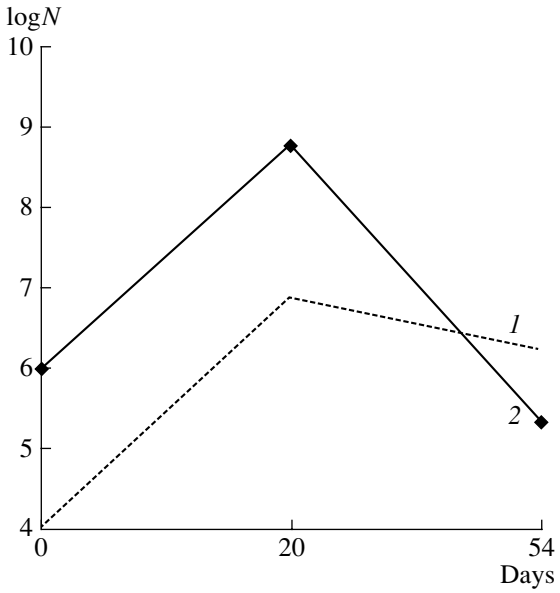

Fig. 1. Viable cell numbers of *Pseudomonas* sp. ADP in soil at different inoculation levels: (*1*) 10^4 and (*2*) 10^6 CFU/g.

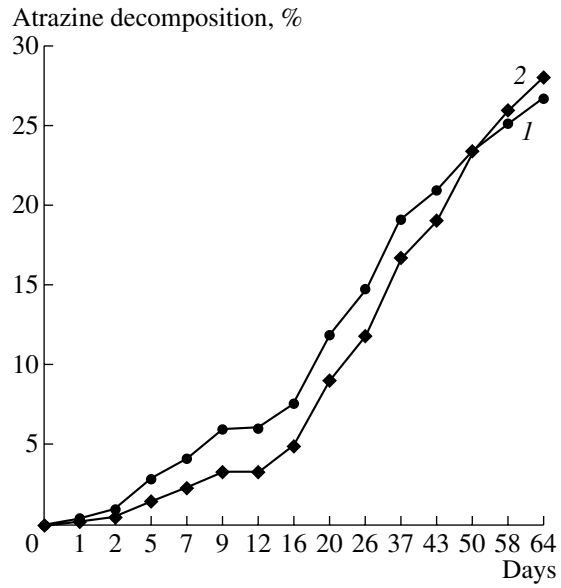

Fig. 2. Atrazine transformation in soil at different inoculation levels of *Pseudomonas* sp. ADP: (*1*) 10^4 and (*2*) 10^6 CFU/g.

branes were incubated directly in soil, withdrawn, and hybridized. The incubation time was two weeks. During the incubation period, progressive decomposition of up to 7% of atrazine was recorded. On the membranes incubated in situ in the soil, the signal was distributed uniformly on the surface, although it was less pronounced than in the case of hybridization with colonies (Fig. 4).

It is presently known that *Pseudomonas* sp. ADP has the genes *atzA*, *atzB*, and *atzC*, coding for the three sequential enzymes of the atrazine degradation pathway [8, 10]. At the first stage, AtzA catalyzes hydrolytic dechlorination of atrazine with the production of hydroxyatrazine, which is then transformed to *N*-isopropylammelide by AtzB. This last compound is transformed by AtzC. The proteins AtzA, AtzB, and AtzC belong to the aminohydrolase family together with urease, cytosine deaminase, and other enzymes. Data have been reported indicating that they diverged from a common ancestor and were converted to an atrazine-catabolism plasmid [10]. The genes *atzABC* were found to be highly conservative and widespread among atrazine-utilizing bacteria isolated from geographically remote sources [11]. The *Pseudomonas* sp. ADP genes of atrazine metabolism are more stable than those from other atrazine-utilizing bacteria. For instance, in *Pseudoaminobacter* sp., the *atzABC* genes do not always form a cluster on one plasmid and can be partially lost [12]. The *atzB* gene can be associated with a catabolite transposon and may be absent in some isolates [12].

We have studied the phenotypic characteristics of *Pseudomonas* sp. ADP and the applicability of the in situ hybridization technique to reveal atrazine-transforming bacteria in soil. The strain was found to be capable of denitrification with gas formation and of heterotrophic nitrification. Investigation of the dynamics of the cell number of this microorganism inoculated into soil demonstrated that, during the first three weeks after inoculation with *Pseudomonas* sp. ADP, a rapid increase in its cell number occurred and, afterwards, the cell con-

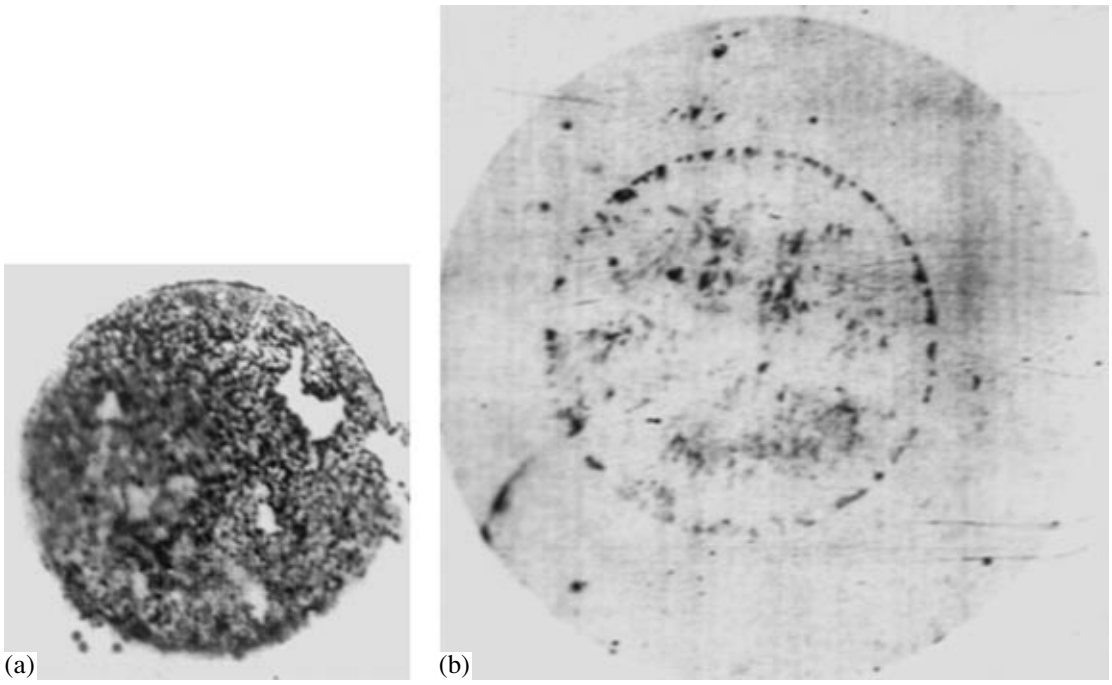

Fig. 3. Hybridization of bacterial DNA with a probe homologous to the *atzA* gene: (a) hybridization of colonies of *Pseudomonas* sp. ADP and (b) hybridization after incubation of filters in soil and subsequent replication to membranes.

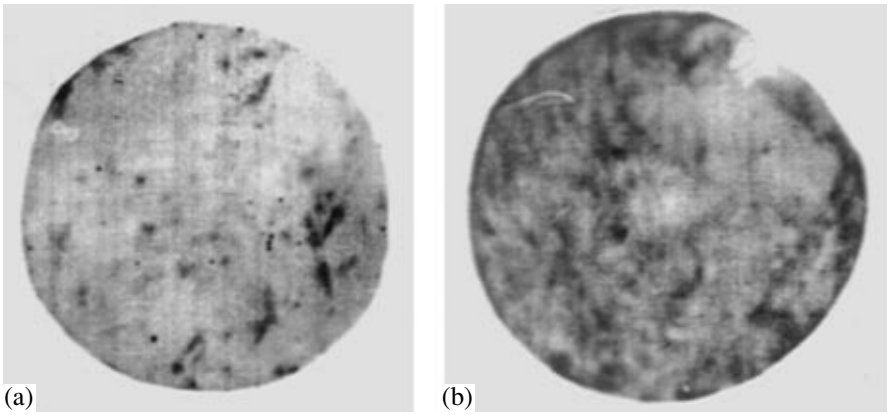

Fig. 4. In situ hybridization in soil with a probe homologous to the *atzA* gene: (a) control and (b) experiment with inoculation of *Pseudomonas* sp. ADP.

centration decreased. When 0.3 g/kg atrazine was introduced into the medium, up to 26% of the preparation was decomposed after two months; at initial stages of incubation, decomposition was more rapid with the 10^6 CFU/g inoculum.

Hybridization with a digoxigenin-labeled oligonucleotide probe complementary to the *atzA* gene sequence after replication of the membranes on the colonies revealed an intense signal, indicating good interaction with the probe. After the replication of the membranes on the filters incubated in soil, a pronounced signal was initially located along the edge of the filter paper disk and partially in the center, probably, in the area of atrazine application. Bacteria probably first attack the edge of a disk submerged in soil and afterwards colonize its entire surface. In situ hybridization on the membrane also revealed certain interaction with the probe, although the signal was less pronounced.

These results provide evidence of the applicability of the in situ hybridization method for the analysis of the distribution of xenobiotic-transforming bacteria in soil. Research in this area will be helpful for solving a number of important fundamental and applied problems.

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