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Degradation of 2,4,6-trinitrotoluene by Serratia marcescens

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A strain of Serratia marcescens, isolated from the soil of a contaminated site, degraded 2,4,6-trinitrotoluene (TNT) as the sole source of carbon and energy. At an initial concentration of 50 mg · L^{-1}, TNT was totally degraded in 48 h under aerobic conditions in a minimal salt medium. Reduction intermediates (4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene) were observed. The presence of a surfactant (Tween 80) is essential to facilitate rapid degradation.

Introduction

Natural nitroaromatic compounds are scarce. So far, only a few nitroaromatics have been isolated from natural sources. The antibiotic chloramphenicol is produced by several *Streptomyces* species (Pongs, 1979) and its bacterial degradation generates nitroaromatic acids and alcohols (Lingens *et al.*, 1966).

In contrast, nitroaromatics are of considerable industrial importance (Higson, 1992). They are used as explosives, dyes, polymer additives, pesticides and pharmaceuticals. They also serve as solvents or feedstocks in the preparation of aminoaromatic derivatives. Some of these compounds such as nitrobenzene or 2,4,6-trinitrotoluene are produced on a massive scale.

Many studies have shown that nitroaromatics and their degradation products are toxic or mutagenic to many life forms (Won *et al.*, 1976). These compounds are released in the environment through anthropogenic activities and have been detected in soils, waste-water and in air (Spain, 1995). In consequence, several nitroaromatics are listed as priority pollutants by several environmental protection agencies. Also, the most visible environmental problem caused by contamination with nitroaromatic compounds is the widespread contamination of soil by explosives (Spain, 1995). Biotreatment has been proposed for the remediation of nitroaromatics contaminated sites (Bennett, 1994; Roberts *et al.*, 1996) and the biodegradation of TNT has been the subject of many studies in recent years (Higson, 1992; Kaplan, 1992; Spain, 1995). We report here the fast degradation of TNT by a strain of *Serratia marcescens* isolated from a contaminated soil.

Materials and methods

Enrichment and isolation of microorganisms

Soil samples were collected near the waste-water stream (pink water) of a TNT-producing plant. This plant has produced TNT for about a century. The concentration of TNT in the soil samples was in the range 26–67 mg · kg^{-1} (dry weight).

Enrichment cultures were started with 20 g soil added to 200 mL minimal salt medium and incubated for 2 h at 22°C under constant shaking (150 rpm). For the first week enrichment period, aliquots (20 mL) of these soil suspensions were transferred to 200 mL minimal salt medium containing 50 mg TNT · L^{-1}. Then, during the following three-week enrichment period, the concentration of TNT was gradually increased to 100 mg · L^{-1}. Dilution and plating of the last subcultures on different solid media (Tryptic Soy Agar, Difco; Minimal salt agar with 50 mg TNT · L^{-1} and 55.5 mM of dextrose) provided colonies of different morphologies and growth rates. The minimal salt medium of pH 7.0, contained the following (in g · L^{-1} distilled water): $(NH_4)_2SO_4$ (2.38), $Na_2HPO_4 \cdot 7H_2O$ (2.68), KH_2PO_4 (1.36), $CaCl_2 \cdot 2H_2O$ (14.7), $MgSO_4 \cdot 7H_2O$ (0.246), NaCl (0.496), $FeSO_4 \Diamond 7H_2O$ (0.003), and 1.0 mL of oligoelement solution. The oligoelement solution

contained the following (in $g \cdot L^{-1}$ distilled water): $MnSO_4 \cdot H_2O$ (1.00), $Na_2MoO_4 \cdot 2H_2O$ (0.25), $Na_2B_2O_7 \cdot 10H_2O$ (0.2), $Co(NO_3)_2 \cdot 6H_2O$ (0.25), $CuCl_2 \cdot 2H_2O$ (0.25), $ZnCl_2$ (0.25).

Screening and degradation experiments

Initially, 17 isolates were screened for possible TNT-degrading activity. The bacterial growth and the biodegradation of TNT were monitored in 50 mL of a working medium containing 50 $mg \cdot L^{-1}$ of TNT as sole source of carbon and energy. Cultures were incubated at 22°C at 200 rpm on a rotary shaker. The concentration of residual TNT and the bacterial growth (colony forming units) were measured and evaluated after 12 and 17 days. With *Serratia marcescens*, the experiments were performed under the same conditions, but with more frequent monitoring and with an initial bacterial concentration adjusted to 1×10^7 cfu $\cdot$ mL^{-1}. The working medium of pH 6.8 contained the following (in $g \cdot L^{-1}$ distilled water: K_2HPO_4 (3.48), KH_2PO_4 (2.72), $MgSO_4 \cdot 7H_2O$ (0.05), $CaCl_2 \cdot 2H_2O$ (0.01), NH_4Cl (0.04), Tween 80 (1.0), benzyl alcohol (0.865). The oligoelement solution was identical to the one used in the minimal salt medium.

Isolate identification

Bacteria identifications were obtained with the Microscan procedure (Baxter Diagnostics Inc.). The *Serratia marcescens* strain described in this work has the characteristic red pigment (prodigiosin).

Analytical methods

TNT and metabolites were analyzed by high performance liquid chromatography (HPLC) using a monochromatic UV detector set at 254 nm or a photodiode array detector. The mobile phase was acetonitrile:water (40:60 V/V) with a gradient (7 minutes) to acetonitrile:water (70:30 V/V). Aliquots of 20 µL were injected onto a C-18 column (150 × 3.9 mm, 4 µ particles) at room temperature. The flow rate was 1 mL/min. Standard samples of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene were prepared by chemical or enzymatic reduction of TNT (Tatsumi *et al.*, 1981; Atkins and Wilson, 1986).

Results and discussion

Several bacterial strains, potentially able to use TNT as a sole source of carbon and energy, were isolated from the soil of a contaminated site following a one month enrichment period. Seventeen of these isolates were initially screened for their real TNT-degrading activity. Although most of these isolates were active, one consortium seemed more efficient for the degradation of nitroaromatics. After purification of this consortium, two bacteria were identified: *Serratia marcescens* and *Alcaligenes* sp. (Microscan procedure, Baxter). Degradation experiments with these two bacteria demonstrated that *Serratia marcescens* was by far the most effective TNT-degrader. The strain of this common soil and water, gram-negative bacteria produced the characteristic red pigment prodigiosin (Trias *et al.*, 1988).

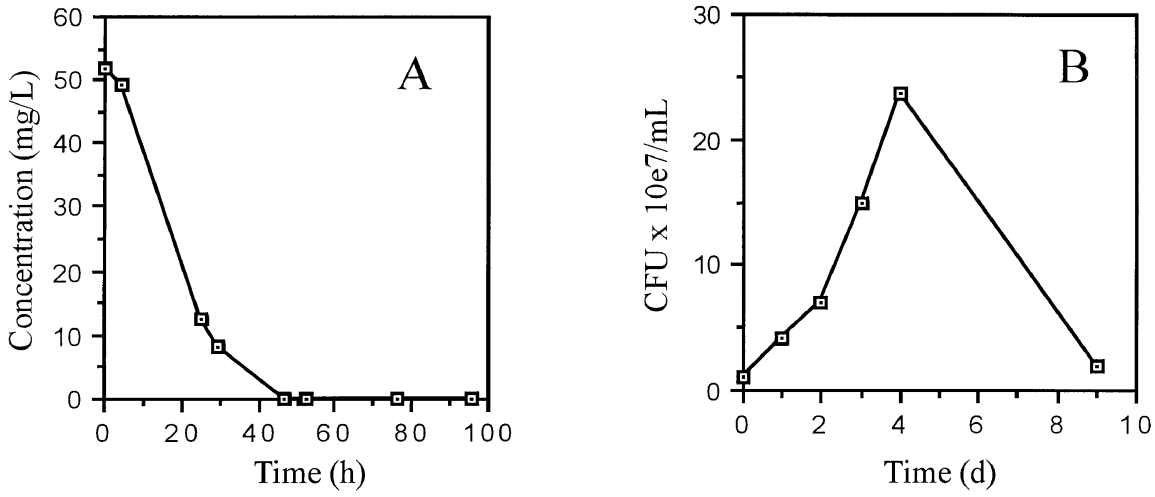

Figure 1 Biodegradation of TNT by Serratia marcescens in a minimum salt medium at pH 6.8. The initial bacterial concentraiton was adjusted to 1×10^7 cfu·mL^{-1} and the TNT concentration was measured by HPLC. A) Concentration of TNT. B) Growth of S. marcescens.

The transformation of TNT by *S. marcescens* was investigated in more details. Figure 1 presents a typical result. The initial concentration was set at 50 mg · L^{-1}. After 24 h, 70% of the TNT had disappeared from the culture medium and after 48 h the degradation was complete (Figure 1A). In the uninoculated control, the TNT concentration did not decrease, remaining at the original concentration of 50 mg · L^{-1}. Figure 1B shows the growth of *S. marcescens* during the biotransformation period. Maximal growth was observed after 48 h of incubation (48 to 96 h), by which time most of the TNT was transformed.

Two reduction metabolites, 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene were observed in the HPLC analysis. The sum of these metabolites accounted for less than one fifth of the initial sample (6–10 mg · L^{-1}). The amine concentration remained essentially constant for the rest of the experiment, indicating that these substrates cannot support growth. The bioreduction of the nitro groups on TNT has been reported by several workers but only a few recent studies have focused on metabolism of the aminonitrotoluenes (Alvarez *et al.*, 1996; Gilcrease and Murphy, 1995).

Degradation of TNT by bacteria has been observed under aerobic and anaerobic conditions. For instance, natural strains of *Pseudomonas* able to grow on TNT under aerobic conditions have been isolated (Boopathy *et al.*, 1994; Haïdour and Ramos, 1996). Interesting recent advances are the discoveries that pure cultures of *Clostridium* and *Desulfovibrio* species (anaerobes) can extensively degrade TNT (Costa *et al.*, 1996; Boopathy *et al.*, 1993). Also, the results of several studies indicate the ability of certain fungi to degrade TNT. Most notably, the white-rot basidiomycete *Phanerochaete chrysosporium* has been shown to be capable of degradation and even mineralization of TNT (Sublette *et al.*, 1992; Spiker *et al.*, 1992; Bumpus and Tatarko, 1994).

A series of experiments were conducted to determine the role of benzyl alcohol and Tween 80, two additives of the working medium. Benzyl alcohol is an enzyme inducer and could increase the rate of oxydo-reductase production. Figure 2A shows that the effect of benzyl alcohol on the transformation of TNT by *S. marcescens* is weak. Tween 80 (polysorbate 80) is a surfactant used to promote the release of enzymes from the microorganisms and the dispersion of TNT in the aqueous solution. Aromatic pollutants such as TNT are apolar, having both a low solubility in aqueous media and a high tendency for adsorption onto organic matter. Figure 2B shows that in the absence of polysorbate the transformation of TNT was very slow. Bioavailability enhancement of apolar pollutants by added surfactants or by biosurfactants produced by microorganisms has been shown to greatly improve the rate of biodegradation (Sabatini *et al.*, 1995).

In summary, we established (i) that the soil bacterium *S. marcescens* is able to degrade TNT rapidly and (ii) that the rate of this transformation is dependent on the presence of a surfactant. This study suggests that a versatile microorganism such as *S. marcescens* could play a role

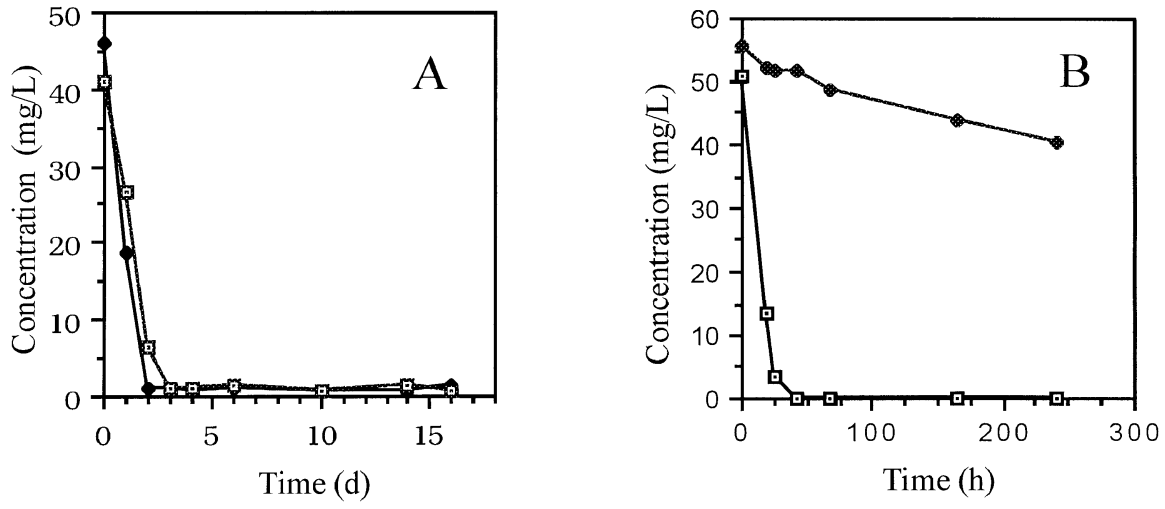

Figure 2 Effect of additives on the degradation of TNT by S. marcescens in a minimum salt medium at pH 6.8. The TNT concentration was measured by HPLC. A) With (—□—) and without (—●—) benzyl alcohol. B) With (—□—) and without (—●—) surfactant Tween 80.

in biological decontamination system. Further research efforts will be directed toward elucidating the TNT biodegradation pathway in this microorganism.

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