

Metabolism of 2,4,6-Trinitrotoluene by *Pseudomonas* sp. JLR11

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The main contaminant at many military sites is 2,4,6-trinitrotoluene (TNT). This explosive remains stable in the environment, and because it is toxic, there is an urgent need for remediation to clean up contaminated sites. *Pseudomonas* sp. JLR11 is a soil isolate able to use nitrate, TNT, or both as the N source under anaerobic conditions. At least two polypeptides were detected in cell-free extracts of TNT-grown cells that were not present in the cell-free extracts of nitrate-grown cells. Mass balances with unlabeled TNT revealed that about 85% of total N-TNT was incorporated as cell biomass, whereas only about 1% of [^{14}C]TNT was recovered as $^{14}\text{CO}_2$. Analyses of culture supernatants detected some pathway intermediates in the productive removal of nitro groups from TNT, such as 2,4,6-trinitrobenzaldehyde, 2-nitro-4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, and 4-hydroxybenzoic acid. This strain reduced a small fraction of the total TNT to monoaminodinitrotoluenes and diaminomononitrotoluenes, but these products accumulated with time and were not used by the strain as an N source. We discuss how the productive metabolism of TNT may occur.

Introduction

The worldwide annual production of 2,4,6-trinitrotoluene (TNT), the primary explosive used in the manufacture of munitions, is estimated at two million lb (1, 2). Manufacturing and decommissioning operations continue to generate large quantities of TNT as a waste product. Much of this waste has been deposited in soil and in unlined lagoons, having reached groundwaters through leaching (3, 4). The presence of multiple nitro groups on the aromatic ring of TNT limits its attack by oxygenases. Because of the electrophilic nature of the substituent, TNT readily oxidizes biological reductants, causing toxicity directly or through the formation of reactive products such as nitroarene radicals (5). TNT, which remains relatively stable in the environment, is toxic for many prokaryotes and eukaryotes and is mutagenic in *Salmonella typhimurium* (6–9). Because it is toxic, there is an urgent need for remediation to clean up contaminated sites.

Many studies have examined the feasibility of TNT remediation from waste streams and soils by conventional aerated microbial treatment processes including composts, activated sewage sludge, and soil slurry reactors. In these studies evidence for the reduction of nitro groups on the aromatic ring were found, with the final product frequently

being aminodinitrotoluenes (ADNT), diaminomononitrotoluenes (DANT), and azoxytetranitrotoluenes (10–17). In the presence of oxygen, the intermediate nitroso and hydroxylamino groups that form upon the reduction of nitro groups have a high propensity to react with each other to produce azoxydinitrotoluenes (19, 20) and with organic products in the surrounding milieu, which can result in a series of nitroaromatics covalently bound to humus material (2, 21).

A number of studies have found that mineralization of TNT under aerobic conditions can occur (22–28), although in most cases bacteria and fungi capable of using TNT as a source of nitrogen is the main outcome (12–14, 16, 17, 26, 30, 32, 33).

The limited TNT mineralization under aerobic conditions and the appearance of undesired TNT products have led to research on anaerobic processes as an alternative for TNT remediation (21). These processes have the potential advantages of rapid reduction at low redox potential and diminished polymerization reactions due to the absence of oxygen (2, 19).

Boopathy et al. (10) examined the degradation of TNT by a *Desulfovibrio* strain under different nutritional conditions and found that this organism used TNT as its sole source of nitrogen, forming toluene from TNT after 45 days of incubation. The catabolic steps in the process are unknown.

Several reports suggest that the ultimate product of TNT metabolism through anaerobic nitro-reductive routes is 2,4,6-triaminotoluene (TAT) (10, 18, 34–40). Preuss et al. (36) reported that TAT was unstable under their culture conditions, giving rise to unidentified decomposition products; however, Funk et al. (35) reported that a consortium of *Clostridium* strains and other anaerobes could apparently metabolize TAT via methylphloroglucinol and *p*-cresol, as these products transiently accumulated in culture supernatants.

In this study, we report the isolation of a pure culture of a *Pseudomonas* strain belonging to group I of rRNA, which utilizes TNT as an N-source under anaerobic conditions in the presence of glucose.

Experimental Section

Chemicals. TNT was obtained from Unión Española de Explosivos (Madrid, Spain) and was more than 99% pure. 2,6-Diamino-4-nitrotoluene (2,6DANT), 2,4-dinitrotoluene (2,4DNT), 2,6DNT, and 2,3DNT were from Aldrich Chemical (Madrid, Spain). Triaminotoluene was provided by Chem Services (West Chester, PA).

Synthesis of Chemicals. 4-Amino-2,6-dinitrotoluene (4ADNT) and 2,4-diamino-6-nitrotoluene (2,4DANT) were synthesized from TNT after equimolar reduction with $(\text{NH}_4)_2\text{S}$ under an N_2 atmosphere as described (19). 4-Hydroxylamino-2,6-dinitrotoluene (4OHADNT) was synthesized after equimolar reduction with zinc dust and ammonium chloride as described by Bauer et al. (41). The ^1H NMR data for 4ADNT and 2,4DANT were in agreement with those published by Nielsen et al. (42).

A total of 250 mg of [^{14}C]TNT (specific activity 11 mCi/mmol) was prepared from [ring-UL- ^{14}C]toluene basically as described by Michels and Gottschalk (43). The synthesized [^{14}C]TNT was extracted three times with ethyl acetate, and its purity was determined by GC. The [^{14}C]TNT was found to be more than 95% pure.

Organism, Culture Medium, and Growth Conditions. *Pseudomonas* sp. strain JLR11 was grown in N-free M8 minimal medium (19). When TNT, 2,4-DNT, and 2,6-DNT

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were used as a nitrogen source, they were supplied at 100 mg/L or as crystals in excess of its solubility in water. The volatile nitroaromatics *o*- and *m*-nitrotoluene were supplied through the vapor phase. Nitrate was used as a nitrogen source in some experiments at a concentration of 1–10 mM. Glucose (0.1 wt %/vol) was used as a C source. To achieve anaerobic conditions, the culture medium was flushed with N₂ to remove oxygen. Dissolved oxygen concentration throughout the assays was below 1 μ M, and the jars were kept under slightly positive pressure.

When bacterial cells were cultured in 100-mL conical flasks, the cultures were incubated at 30 °C and shaken at 200 strokes per min. When larger volumes were required, bacterial cells were grown in batch cultures in a 2-L bioreactor (Biostat B, Braun Biotech) at constant temperature (30 °C), pH (7.0 \pm 0.1), and stirring (600 \pm 2 rpm) throughout the assay. The bioreactor was periodically flushed with N₂ to maintain anaerobiosis during the assay.

Growth was measured by one of the following methods: (i) as the increase in turbidity of the culture at 660 nm, (ii) by counting colony forming units (cfu) per mL after spreading serial dilutions on LB medium plates (30), and (iii) as the increase in cellular protein. The protein content was measured by the Lowry method using bovine serum albumin as a standard (44).

Isolation of Metabolites. Culture supernatants were extracted twice with ethyl acetate at pH 7. The resulting aqueous solution was acidified to pH 2 with HCl and extracted again twice with ethyl acetate. The extracts were dried over anhydrous sodium sulfate, and the excess solvent was removed by evaporation under reduced pressure at 35 °C.

Solid-phase extraction (SPE) was also used to extract aromatic metabolites from culture medium, using a 30-mg OASIS cartridge as recommended by the manufacturer (Waters Incorporation, Madrid, Spain).

Analytical Methods. Products were analyzed by high-performance liquid chromatography (HPLC) on a Hewlett-Packard model 1050 chromatograph equipped with a diode array detector and a 5- μ m C18RP column (UltraCarb C30 Phenomenex 15 cm \times 4.6 mm).

The column was first washed with a mixture of acetonitrile (ACN) and a solution of 1% (vol/vol) acetic acid in water (2:8 [vol/vol]) for 2 min. Then a linear gradient was applied to reach 100% (vol/vol) ACN over 18 min. The flow was kept constant at 1 mL/min, and the detector was set at 230 and 254 nm to detect aromatic compounds.

Gas chromatography–mass spectrometry (GC–MS) analyses were done with an HP6890 GC–MS apparatus. The GC was equipped with a capillary 5% phenylmethyl silicone column (30 m \times 0.025 mm).

¹H nuclear magnetic resonance (¹H NMR) was performed with Bruker ARX400 (400 MHz) and Bruker AM300 (300 MHz) spectrometers on solutions prepared in deuterated acetone. Tetramethylsilane was used as an internal standard.

Other Methods. Glucose was enzymatically determined using a kit from Boehringer (Mannheim, Germany) (Catalog No. 716 251). Nitrite was determined by Snell and Snell's method (46). Ammonium was measured by the phenol–hypochlorite method of Solorzano (44). To determine nitrogen in the biomass, cells were harvested by centrifugation (5000g, 10 min) and dried at 50 °C to a constant weight. The dried material was digested by microwaves in the presence of H₂SO₄ and H₂O₂ (Maxidigest mx 4350 Prolabo). Nitrogen in the sample was determined according to Krom (47) by using an automatic nitrogen analyzer (Technicon autoanalyzer II system, Bran and Luebbe).

[¹⁴C]TNT in the culture medium and ¹⁴C associated to cell material were determined in samples prepared as follows: Cells growing in M8 minimal medium with (0.1 wt %/vol) glucose as the carbon source in the presence of TNT (50 000

cpm/mL) were harvested by centrifugation and washed in TNT-free culture medium. To determine the [¹⁴C]TNT remaining in the culture supernatants, we added 4 mL of scintillation liquid to 100 μ L of each different supernatant. ¹⁴C associated to the cell pellet was determined in washed cells suspended in about 200 μ L of water to which 4 mL of scintillation liquid was added. In all samples, ¹⁴C was counted in a Packard radiochemical counter.

Cell-Free Extracts and Electrophoretic Separation of Polypeptides. Cells were grown on minimal medium (200 mL) with glucose and nitrate or TNT as the N source until the late logarithmic phase. Cells were harvested as above, washed twice in 20 mM phosphate buffer (pH 7.0), and suspended in 2 mL of the same buffer. Then cells were disrupted in a French Press. The samples were centrifuged (12000g, 10 min), and the supernatant constituted the cell-free extract. Polypeptides were separated on sodium dodecyl sulfate–polyacrylamide gels according to Lammeli (48). Gels were stained with Coomassie blue.

Results

Isolation and Characterization of a Bacterial Strain That Utilizes TNT as an N Source under Anaerobic Conditions.

Water from a wastewater treatment plant in the city of Granada was diluted 100-fold in M8 minimal medium supplemented with TNT (100 mg/L) with and without glucose (1 g/L). Conical flasks were sealed with rubber stoppers and flushed with argon to remove the oxygen and then incubated at 30 °C with shaking for 2 weeks. Serial dilutions were spread on M8 minimal medium agar plates with 1 g/L glucose and TNT (100 mg/L) plus 1 mM NO₃[–] to favor growth. The plates were incubated at 30 °C in anaerobiosis jars, which were flushed three times with N₂ and then twice with argon to avoid nitrogen fixers. After 72 h, no colonies were found in enrichments without glucose, whereas a single type of colony appeared on plates from culture enrichments with glucose. A random colony, called JLR11, was chosen and identified as a Gram-negative facultative anaerobe belonging to the genus *Pseudomonas* biotype A on the basis of ApiNE 20 tests and phospholipid analysis (done by Microbial Identification Systems). *Pseudomonas* sp. JLR11 was able to use different nitroaromatic compounds as the sole N source (TNT, 2,6DNT, 2,4DNT, and 2NT) under aerobic and anoxic conditions but not 2,3DNT, 3NT, or 4NT. Moreover, it was unable to use monoaminodinitrotoluenes (2A,4,6DNT or 4A,2,6DNT) or diaminomononitrotoluenes (2,4DANT or 2,6DANT) as the sole N source. Inorganic nitrogen sources such as nitrate and nitrite can be used by the strain as the sole N source, either in aerobiosis or anaerobiosis. This strain was unable to grow under anaerobic conditions with glucose and NH₄⁺ as the sole nitrogen source. When the M8 minimal culture medium was supplemented simultaneously with an inorganic source of nitrogen (nitrate or nitrite) and TNT, both N sources were simultaneously used by this strain. None of the nitroaromatic compounds mentioned above were used as the sole C, N, and energy source. Indeed, growth on the above nitroaromatic compounds as the sole N source required sugars (glucose, sucrose, galactose), carboxylic acids (citrate, succinate, acetate), or glycerol as a C source to facilitate growth.

Cell-free extracts were prepared from *Pseudomonas* sp. JLR11 growing under anaerobic conditions on minimal medium with nitrate or TNT as an N source. Proteins were then separated in SDS–polyacrylamide gels as described in the Experimental Section. We found that at least four protein bands were present in the cell-free extracts from cells grown on TNT that were not present in NO₃[–]-grown cells (Figure 1). This suggests specific induction in *Pseudomonas* sp. JLR11 of proteins that are probably involved in TNT metabolism.

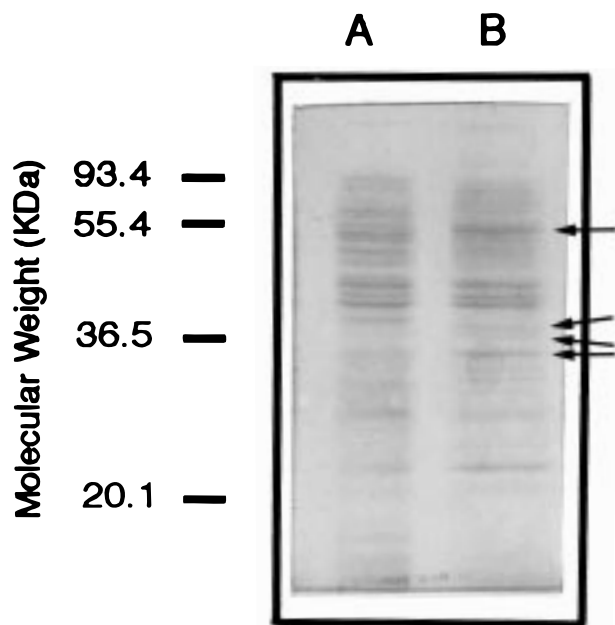


FIGURE 1. Separation of polypeptides from NO_3^- -grown (A) and TNT-grown (B) *Pseudomonas* sp. JLR11 in minimal medium with glucose as the sole C source. The arrows point out polypeptides made by TNT-grown cells.

In agreement with this observation was the appearance of a TNT-dependent NADH oxidase in the cell-free extracts prepared from cells grown on TNT (not shown).

Growth of *Pseudomonas* sp. JLR11 with TNT as the Sole N Source. To establish optimal growth conditions, bacterial yield and TNT consumption were determined in a 2-L bioreactor in batch cultures. We tested the effect of different pH values (from 5 to 9), temperatures (from 20 to 35 °C), and rates of stirring (from 200 to 1000 rpm). The culture medium was M8 minimal medium with 0.1% (wt/vol) glucose and 60 mg/L TNT. To determine the effect of pH, we kept temperature (30 °C) and the rate of stirring (600 rpm) constant. Similarly, to test the effect of temperature, we kept the rate of stirring (600 rpm) and pH (7.0) constant; when the rate of stirring was tested, pH (7.0) and temperature (30 °C) were kept constant. Optimal growth conditions in M8 minimal medium were found to be pH 7.0 ± 0.1 , 25–30 °C, and stirring at 400–1000 rpm; under these conditions more than 95% of the initial TNT concentration was removed during the first 7 days. Prolonged incubation for up to 20 days resulted in complete consumption of TNT. Standard assays were performed at pH 7.0, 30 °C, and 600 rpm. Figure 2 shows the growth of the strain with time, the consumption of TNT under these conditions, and the transitory accumulation of nitrite in the culture medium. Concomitant with the initial decrease in TNT concentration in the culture medium was NO_2^- accumulation to a maximum concentration of 270 μM (Figure 2) (between 50 and 200 μM in another six independent assays). Thereafter, nitrite concentration in the culture medium decreased (Figure 2) until it became negligible. Growth was monitored as the increase in cell protein (mg of cell protein/mL) and viable cells (cfu/mL). The number of cfu/mL and the bacterial protein content increased during the first 100–150 h, with a generation time of 3.7 h. After this, the cells entered the stationary phase, and although TNT concentration decreased further, growth was not significant. Glucose consumption was also determined; only 20% of the initial glucose concentration (0.1% wt/vol) was consumed, the rest remained in the culture medium.

The net increase in cell protein when the culture reached the stationary phase was 70 mg/L. Taking into account that

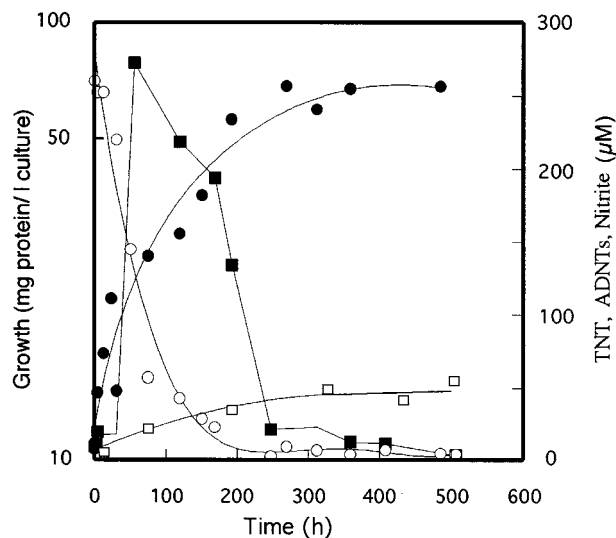
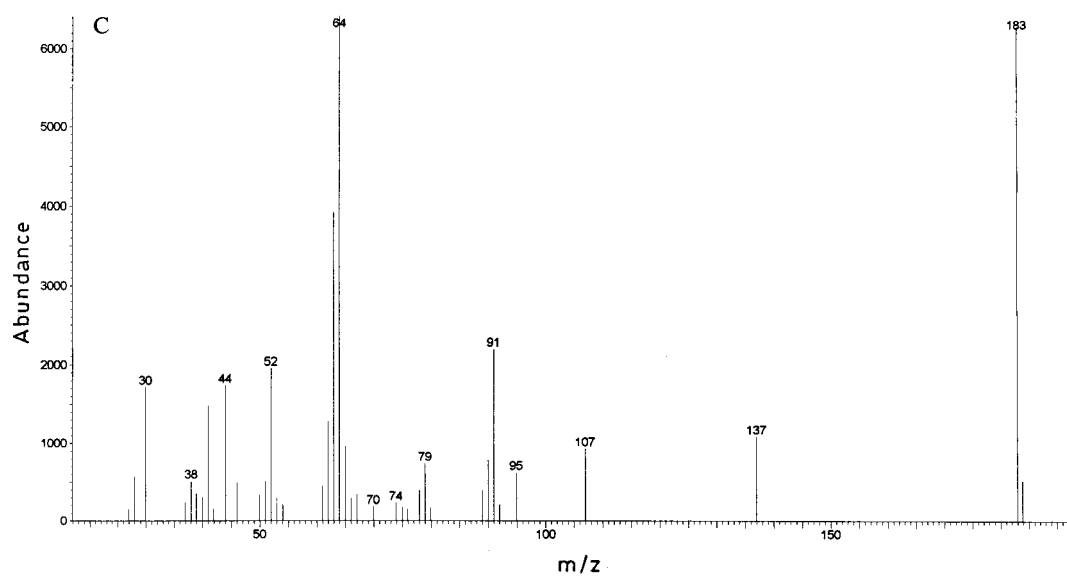
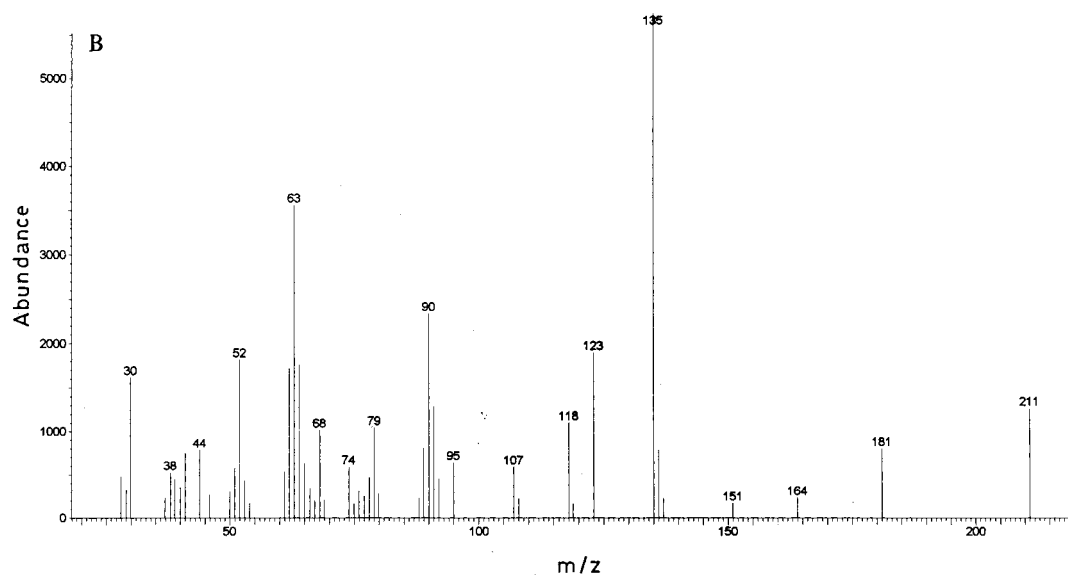
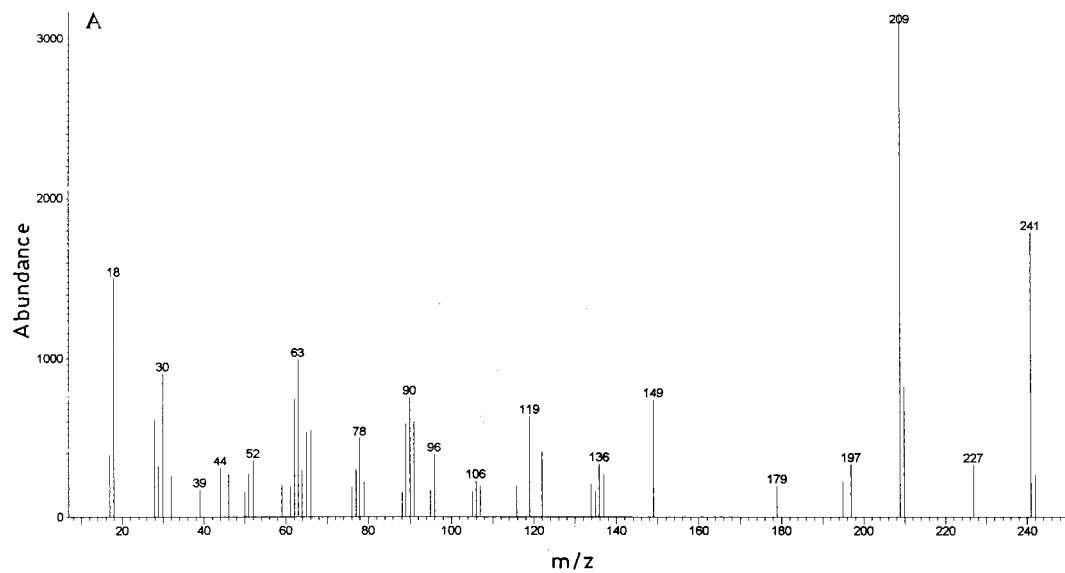


FIGURE 2. Growth of *Pseudomonas* sp. JLR11 at the expense of TNT and nitrite accumulation under anaerobiosis. *Pseudomonas* sp. JLR11 was grown in a batch culture with TNT as the sole nitrogen source and with glucose (1 g/L) as the carbon source. Changes with time in the concentration in culture supernatants of TNT (○), aminodinitrotoluenes (□), and nitrite (■) were determined. Growth was determined at the indicated times as the increase in cell protein (●).

the average N content of proteins is 15%, this means that 10.5 mg/LN from TNT was incorporated into the cell biomass. Given that the initial N content in the form of TNT was 12.4 mg, this represented about 85% of the initial N-TNT incorporated into the cell biomass. The same percentage was found when nitrogen was determined in dried cell biomass. The rest of the initial nitrogen (10–20% of the total) was found associated with aromatic compounds, mainly as reduced forms of TNT, which accumulated with time (Figure 2).

In other series of assays [^{14}C]TNT was used to determine whether the C skeleton of the TNT was mineralized or incorporated into cell material. These assays were carried out according to the following protocol: 80 mL of M8 minimal culture medium with 0.1% (wt/vol) glucose was placed in a 100-mL bioreactor with a CO_2 trap. To this suspension, we added [^{14}C]TNT to about 50 000 cpm/mL; once all the [^{14}C]TNT was dissolved, we added excess unlabeled TNT to oversaturation, a condition that favored the growth of *Pseudomonas* sp. JLR11. Thereafter oxygen was removed by flushing with N_2 , and about 10^5 cfu *Pseudomonas* sp. JLR11 per mL was added. The cultures were incubated at 30 °C with shaking, and samples were withdrawn periodically for the determination of $\text{Na}_2^{14}\text{CO}_3$ in the sodium hydroxide trap, remaining ^{14}C in the culture supernatant, and ^{14}C associated with trichloroacetic acid-precipitable cell material. In four independent assays, we found that about 1% of the total [^{14}C]TNT was released as $^{14}\text{CO}_2$, 45% appeared associated to cell material, and 15% remained in the culture supernatants as reduced forms of TNT. The rest of the ^{14}C was associated with volatile acids not trapped in NaOH, as also reported for *Clostridium* sp. (49). The nature of these volatile acids is currently under investigation.

Analysis of Metabolites in Culture Supernatants. The culture supernatants were analyzed periodically with HPLC. The chromatograms showed that as TNT decreased, some reduced forms of TNT accumulated, including 4A,2,6DNT and 2A,4,6DNT (not shown). The spectroscopic data of these compounds matched those reported before (42). The concentration of these metabolites increased with time, accounting for the transformation of about $10 \pm 2\%$ of the



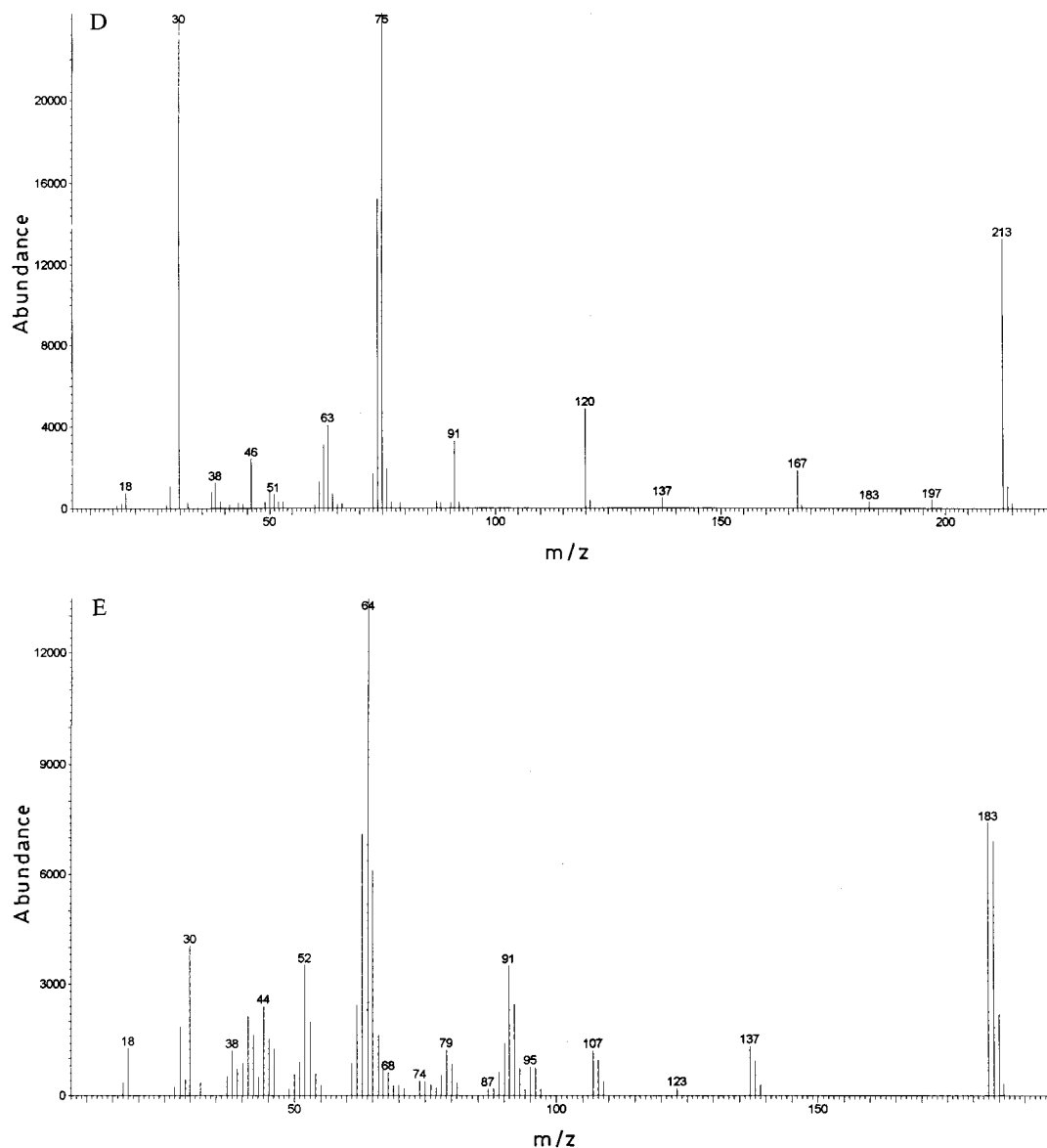


FIGURE 3. Mass spectra a of compound proposed to be 2,4,6-trinitrobenzaldehyde (A); 4-amino-2,6-dinitrobenzaldehyde (B); 3,5-dinitroaniline (C); 1,3,5-trinitrobenzene (D); and 2-nitro-4-hydroxybenzoic acid (E).

initial TNT. This suggests that they were dead-end products rather than pathway intermediates. Less than 1% of the initial TNT appeared as other reduced products, e.g., 4-hydroxyl-amino-2,6-dinitrotoluene, 2,4-diamino-6-nitrotoluene, and 2,6-diamino-4-nitrotoluene. No TAT was found. Other metabolites, most of them more polar than TNT, also accumulated. To identify them, culture supernatants were extracted with ethyl acetate or SPE; the products were purified by semipreparative HPLC and their UV-visible spectra; GC-MS fragmentation pattern and ^1H NMR spectrum were recorded. The following products were found:

Products 1 and 2. Their molecular masses were 240 and 211, and their mass spectra were consistent with the fragmentation pattern expected for 2,4,6-trinitrobenzaldehyde and 4-amino-2,6-dinitrobenzaldehyde (Figure 3, panels A and B).

Products 3 and 4. They had the same retention time, molecular mass, and mass fragmentation pattern as 3,5-dinitroaniline (Figure 3C) and 1,3,5-trinitrobenzene (Figure 3D), respectively. ^1H NMR data for 3,5-dinitroaniline isolated from the culture medium were identical to a pure standard (Table 1). The identity of these products suggested that the

TABLE 1. ^1H NMR Spectroscopic Data of TNT Metabolites^a

^1H NMR	product 3	product 5
H-2	7.88; d(2.02 Hz)	
H-3		7.62; d(2.05 Hz)
H-4	8.07; dd(2.02, 2.02 Hz)	
H-5		7.49; dd(2.05, 8.44 Hz)
H-6	7.88; d(2.02 Hz)	7.60; d(8.44 Hz)

^a Product 3 was identified as 3,5-dinitroaniline. Product 5 was identified as 2-nitro-4-hydroxybenzoic acid.

lateral $-\text{CH}_3$ group of TNT had been lost and that one of the nitro groups had been reduced.

Product 5. This metabolite had a molecular mass of 183. Its mass fragmentation pattern and ^1H NMR spectrum lead us to propose that it is 2-nitro-4-hydroxybenzoic acid (Figure 3E, Table 1).

Products 6 and 7. We also found metabolites without nitrogen in the aromatic ring whose mass spectrum and retention time in HPLC and UV-visible spectra were identical to those of 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid (data not shown).

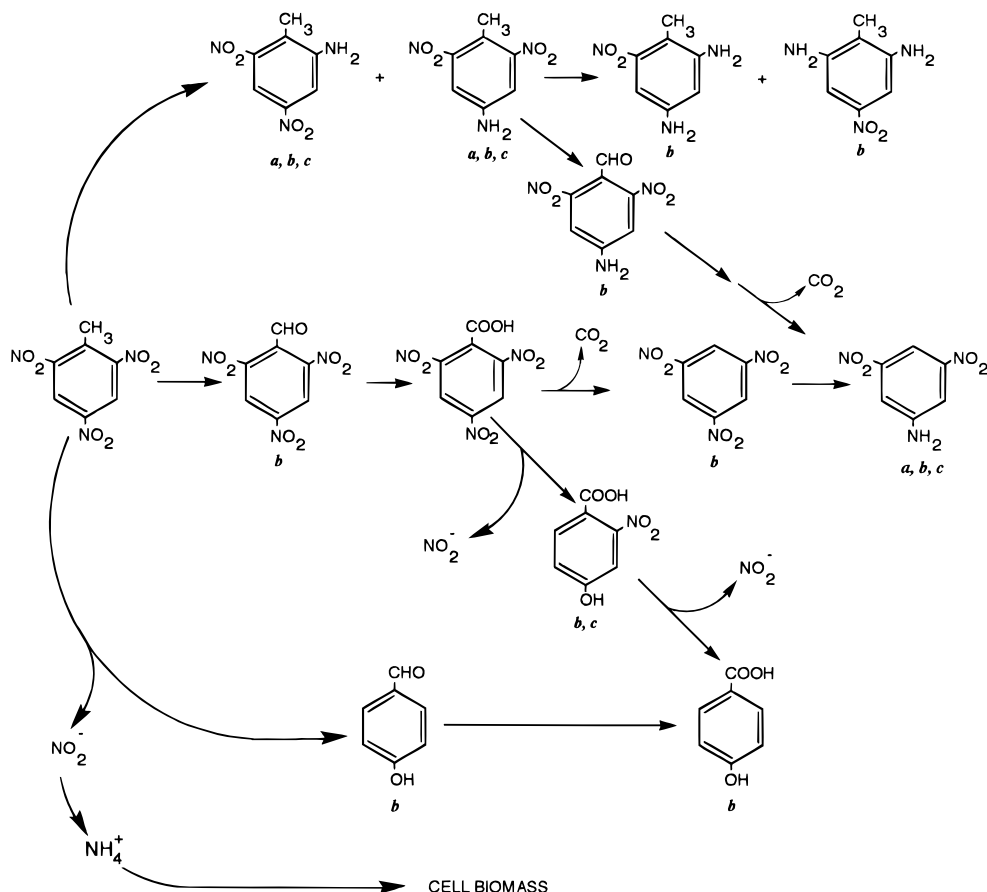


FIGURE 4. Proposed metabolic pathway for TNT degradation by *Pseudomonas* sp. JLR11. The products shown in the figure were identified by the following: a, UV-visible spectrum after HPLC separation; b, mass spectrum after GC; and c, ^1H NMR.

Discussion

This study describes the isolation of a *Pseudomonas* sp. strain called JLR11, which is able to grow under anaerobic conditions with TNT as the sole nitrogen source. This strain incorporated around 85% of N-TNT into N-organic nitrogen and is thus one of the most efficient TNT utilizers described to date, as most aerobic or anaerobic microbes that use TNT as an N source were not able to assimilate such a large proportion of N-TNT (50).

Pseudomonas sp. JLR11 could not grow with TNT as the sole C and N source and required a supplementary C source that facilitated the metabolism of TNT. The C/N ratio in TNT is 2.5, whereas in the microbial biomass the C/N ratio is between 7 and 10; therefore, extra C is required to achieve balanced N assimilation. Moreover, the TNT C skeleton is hardly metabolized (if at all) by this microorganism. In fact, the amount of $^{14}\text{CO}_2$ generated from $[^{14}\text{C}]\text{TNT}$ represented about 1% of the $[^{14}\text{C}]\text{TNT}$ supplied; this CO_2 may not represent true mineralization of TNT but rather the release of the $-\text{CH}_3$ lateral group upon its oxidation to the corresponding carboxylic acid and subsequent decarboxylation (See Figure 4 and below). It therefore also follows that the C source is probably needed not only to provide C skeletons for growth but also as the source of energy required to start the process of nitro group removal.

In some anaerobic microorganisms, TNT metabolism has been suggested to proceed via reduction to triaminotoluene. The subsequent fate of TAT is in most of the cases unknown, although methylphloroglucinol and *p*-cresol may be intermediates in the catabolic pathway (35, 40). We found that *Pseudomonas* sp. JLR11 reduced a small part of TNT (about 10–20% of total) to ADNT and trace amounts of 2,4-DANT and 2,6-DANT, which were not further metabolized and

seemed to constitute dead-end products. The unproductive reduction of TNT to MADNT and DAMNT indicates that this strain exhibits one or more (un)specific nitroreductase(s) like those described in other microorganisms (10–17). This represents a limitation in the productive use of TNT as an N source by *Pseudomonas* JLR11. In any case, it should be noted that the levels of ADNT and DANT are not comparable with the concentration detected in cultures of microorganisms that use the reductive pathway to TAT, which reduce most of the TNT to DANT (18). In the case of *Pseudomonas* sp. JLR11, the productive initial attack seems to involve nitrite release, as nitrite accumulated transiently with TNT consumption (Figure 2). This suggests that *Pseudomonas* sp. JLR11 uses a different initial pathway for TNT degradation. In the biotransformation of pyric acid by a *Rhodococcus* strain (2), in the biotransformation of TNT by a *Mycobacterium* strain (51), and in the utilization of TNT as an N source by *Pseudomonas* sp. clone A (19), a Meisenheimer complex may be the first intermediate product. The in vivo reaction leading from TNT to the TNT–Meisenheimer complex, which can occur in the absence of oxygen, requires that a hydride ion attack the aromatic nucleus and that once the non-aromatic complex is formed, aromaticity is achieved again upon nitrite release (2). Although the mechanism(s) of nitrite release from TNT by *Pseudomonas* sp. JLR11 is still unknown, it should be noted that a TNT-dependent NADH oxidase activity was detected in cell-free extracts of *Pseudomonas* sp. JLR11 grown on TNT and that specific polypeptides were present in cell-free extracts from TNT-grown cells that were not present in NO_3^- -grown cells.

Removal of all nitro groups in the TNT molecule probably does not occur through the same mechanism, as hydroxylated compounds at position 4 were found in aromatic compounds isolated from culture supernatants of *Pseudomonas* sp. JLR11

growing on TNT. We found two hydroxylated compounds lacking nitrogen, e.g., 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid, the key intermediate in the anaerobic metabolism of aromatic compounds (52). The conversion of TNT in these aromatics requires not only the removal of the nitro group but also the oxidation of the lateral methyl group of TNT to the corresponding aldehyde and carboxylic acid. Oxidation of the lateral alkyl chain of TNT may occur before removal of the nitro groups, as we found trace amounts of 4-amino-2,6-dinitrobenzaldehyde and 4-amino-2,6-dinitrobenzoic acid in culture supernatants. The latter compounds may be decarboxylated as release of CO₂ is supported by our assays with [¹⁴C]TNT and by our finding of 3,5-dinitroaniline and 1,3,5-trinitrobenzene in culture supernatants. 3,5-Dinitroaniline may also have been formed after decarboxylation of 1,3,5-trinitrobenzoic acid or 4-amino-2,6-dinitrobenzoic acid. We do not know the physiological role (if any) of this possible decarboxylation reaction, but decarboxylation of benzoic acid in anaerobiosis is related to ATP formation (53). The subsequent metabolism of the hydroxylated aromatic found in the culture supernatant is unknown at present.

Despite exhaustive efforts, we have not been able to identify more than seven compounds resulting from TNT metabolism by *Pseudomonas* sp. JLR11, and current efforts are being directed to the isolation of mutants that can provide further insight on TNT metabolism.

Acknowledgments

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Literature Cited

- Hartter, D. R. In *Toxicity of nitroaromatic compounds*; Hemisphere Publishing Corp.: New York, 1985; pp 1–14.
- Rieger, P.; Knackmuss, H. J. In *Biodegradation of nitroaromatic compounds*; Spain, J. C., Ed.; Plenum Publishing Co.: New York, 1995; pp 1–18.
- Pennington, J. C.; Patrick, W. H., Jr. *J. Environ. Qual.* **1990**, *19*, 559–567.
- Selim, H. M.; Xue, S. K.; Iskandar, I. K. *Soil Sci.* **1995**, *160*, 328–339.
- Mason, R. P.; Joseph, P. D. In *Toxicity of nitroaromatic compounds*; Hemisphere Publishing Co.: New York, 1985; pp 121–140.
- Spanggord, R. J.; Mortelmans, K. E.; Griffin, A. F.; Simmon, V. F. *Environ. Mutagen.* **1982**, *4*, 163–179.
- Styles, J. A.; Cross, M. F. *Cancer Lett.* **1983**, *20*, 103–108.
- Tan, E. L.; Ho, C. H.; Griest, W. H.; Tyndall, R. L. *J. Toxicol. Environ. Health* **1992**, *36*, 165–175.
- Won, W. D.; Disalvo, L. H.; N. J. *Appl. Environ. Microbiol.* **1976**, *31*, 576–580.
- Boopathy R.; Kulpa, C. F.; Wilsen, M. *Appl. Microbiol. Biotechnol.* **1993**, *39*, 270–275.
- Bradley, P. M.; Chapelle, F. H.; Landmeyer, J. E.; Schumacher, J. G. *Appl. Environ. Microbiol.* **1994**, *60*, 2170–2175.
- Funk S. B.; Pasti-Grisby, M. B.; Feliciano, E. C.; Crawford, D. L. In *Bioremediation of recalcitrant organics*; Hinchee, R. R., Hoepfel, R. E., Anderson, D. B., Eds.; Battelle: Columbus, OH, 1995; pp 329–250.
- Isbister, J. D.; Anspach, G. L.; Kitchens, J. F.; Doyle, R. C. *Microbiologica* **1984**, *7*, 47–73.
- Kaplan, D. L.; Kaplan, A. M. *Appl. Environ. Microbiol.* **1982**, *44*, 757–760.
- Won, W. D.; Heckly, R. J.; Glover, D. J.; Hoffsommer, J. C. *Appl. Microbiol.* **1974**, *27*, 513–516.
- Pennington, J. C.; Hayes, C. A.; Myers, K. M.; Ochman, M.; Gunnison, D.; Felt, D. R.; McCormick, E. F. *Chemosphere* **1995**, *30*, 429–438.
- Williams, R. T.; Ziegenfuss, P. S.; Sisk, W. E. *J. Ind. Microbiol.* **1992**, *9*, 137–144.
- Dann, G.; Lenke, H.; Reuss, M.; Knackmuss, H. J. *Environ. Sci. Technol.* **1998**, *32*, 1956–1963.
- Haïdour, A.; Ramos, J. L. *Environ. Sci. Technol.* **1996**, *30*, 2365–2370.
- Fiorella, P. P.; Spain, J. C. *Appl. Environ. Microbiol.* **1997**, *63*, 2007–2015.
- Lenke, H.; Warrelmann, J.; Dann, G.; Hund, K.; Sieglén, V.; Walter, U.; Knackmuss, H. J. *Environ. Sci. Technol.* **1998**, *32*, 1964–1971.
- Fernando, T. J.; Bumpus, J. A.; Aust, S. D. *Appl. Environ. Microbiol.* **1990**, *56*, 299–304.
- Scheibner, K.; Hofrichter, M.; Herre, A.; Michels, J.; Fritsche, W. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 452–457.
- Herre, A.; Michels, J.; Scheibner, K.; Fritsche, W. In *Fourth International In Situ and On Site Bioremediation Symposium*; Battelle Press: Columbus, OH, 1997; Vol. 2, pp 493–498.
- Widrig, D. L.; Boopathy, R.; Manning, J. F. *Environ. Toxicol. Chem.* **1997**, *16*, 1141–1148.
- Boopathy, R.; Manning, J.; Kulpa, C. F. *Water Environ. Res.* **1998**, *70*, 80–86.
- Ramos, J. L.; Haïdour, A.; Delgado, A.; Duque, E.; Fandila, M. D.; Gil, M.; Piñar, G. In *Biodegradation of nitroaromatic compounds*; Spain, J. C., Ed.; Plenum Press: New York, 1995; pp 53–68.
- Duque, E.; Haïdour, A.; Godoy, F.; Ramos, J. L. *J. Bacteriol.* **1993**, *175*, 2278–2283.
- Bell, B. A. In *Hazardous and Industrial Wastes*; Cole, C. A., Long, D. A., Eds.; Technomic Publishing: Lancaster, PA, 1984; pp 344–356.
- Carpenter, D. F.; McCormick, N. G.; Cornell, J. H.; Kaplan, A. *Appl. Environ. Microbiol.* **1978**, *35*, 949–954.
- Jones, A. M.; Grier, C. W.; Ampleman, G.; Thiboutot, S.; Lavigne, J.; Halawari, J. In *Bioremediation of recalcitrant organics*; Hinchee, R. E., Hoepfel, R. E., Anderson, D. B., Eds.; Battelle: Columbus, OH, 1995; pp 251–258.
- Nay, M. W.; Randali, C. W.; King, P. H. *J. Water Pollut. Control Fed.* **1974**, *46*, 485–497.
- Stahl, J. D.; Aust, S. D. *Biochem. Biophys. Res. Commun.* **1993**, *192*, 477–482.
- Ederer, M. M.; Lewis, T. A.; Crawford, R. L. *J. Ind. Microbiol. Biotechnol.* **1997**, *18*, 82–88.
- Funk, S. B.; Roberts, D. J.; Crawford, D. L.; Crawford, R. L. *Appl. Environ. Microbiol.* **1993**, *59*, 2171–2177.
- Preuss, A.; Rieger, P. G. In *Biodegradation of nitroaromatic compounds*; Spain, J. C., Ed.; Plenum Publishing Co.: New York, 1995; pp 69–86.
- Preuss, A.; Fimpel, J.; Dieckert, G. *Arch. Microbiol.* **1995**, *159*, 345–353.
- Regan, K. M.; Crawford, R. L. *Biotechnol. Lett.* **1994**, *16*, 1081–1086.
- Shin, C. Y.; Crawford, D. L. In *Bioaugmentation for site remediation*; Hinchee, R. E., Fredrickson, J., Alleman, B. C., Eds.; Battelle: Columbus, OH, 1995; pp 57–69.
- Lewis, T. A.; Ederer, M. M.; Crawford, R. L.; Crawford, D. L. *J. Ind. Microbiol. Biotechnol.* **1997**, *18*, 89–96.
- Bauer, H.; Rosenthal, S. M. *J. Am. Chem. Soc.* **1944**, *66*, 611–614.
- Nielsen, A. T.; Henry, R. A.; Norris, W. P.; Atkins, R. L.; Moore, D. W.; Leipe, A. H. *J. Org. Chem.* **1979**, *44*, 2449–2504.
- Michels, J.; Gottschalk, G. *Appl. Environ. Microbiol.* **1994**, *60*, 187–194.
- Lowry, D. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, J. R. *J. Biol. Chem.* **1951**, *193*, 265–275.
- Snell, F. D.; Snell, C. T. In *Colorimetric Methods of Analysis*; Van Nostrand Co.: New York, 1949; Vol. 3, pp 804–805.
- Solorzano, L. *Limnol. Oceanogr.* **1969**, *14*, 799–801.
- Krom, M. D. *Analyst* **1980**, *105*, 305–316.
- Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
- Crawford, R. L. In *Biodegradation of nitroaromatic compounds*; Spain, J. C., Ed.; Plenum Publishing Co.: New York, 1995; pp 87–98.
- Vanderloop, S. L.; Suidan, M. T.; Meteleb, M. A.; Maloney, S. W. In *Bioremediation of recalcitrant organics*; Hinchee, R. R., Hoepfel, R. E., Anderson, D. B., Eds.; Battelle: Columbus, OH, 1995; pp 225–230.
- Vorbeck, C.; Lenke, H.; Fischer, P.; Knackmuss, H. J. *J. Bacteriol.* **1994**, *176*, 932–934.
- Harwood, C. S.; Gibson, J. *J. Bacteriol.* **1997**, *179*, 301–309.
- Gallert, C.; Winter, J. *Appl. Microbiol. Biotechnol.* **1994**, *42*, 408–414.

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