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Biodegradation of 4-Nitrotoluene by *Pseudomonas* sp. Strain 4NT

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A strain of *Pseudomonas* spp. was isolated from nitrobenzene-contaminated soil on 4-nitrotoluene as the sole source of carbon, nitrogen, and energy. The organism also grew on 4-nitrobenzyl alcohol, 4-nitrobenzaldehyde, and 4-nitrobenzoate. 4-Nitrobenzoate and ammonia were detected in the culture fluid of glucose-grown cells after induction with 4-nitrotoluene. Washed suspensions of 4-nitrotoluene- or 4-nitrobenzoate-grown cells oxidized 4-nitrotoluene, 4-nitrobenzaldehyde, 4-nitrobenzyl alcohol, and protocatechuate. Extracts from induced cells contained 4-nitrobenzaldehyde dehydrogenase, 4-nitrobenzyl alcohol dehydrogenase, and protocatechuate 4,5-dioxygenase activities. Under anaerobic conditions, cell extracts converted 4-nitrobenzoate or 4-hydroxylaminobenzoate to protocatechuate. Conversion of 4-nitrobenzoate to protocatechuate required NADPH. These results indicate that 4-nitrotoluene was degraded by an initial oxidation of the methyl group to form 4-nitrobenzyl alcohol, which was converted to 4-nitrobenzoate via 4-nitrobenzaldehyde. The 4-nitrobenzoate was reduced to 4-hydroxylaminobenzoate, which was converted to protocatechuate. A protocatechuate 4,5-dioxygenase catalyzed *meta*-ring fission of the protocatechuate. The detection of 4-nitrobenzaldehyde and 4-nitrobenzyl alcohol dehydrogenase and 4-nitrotoluene oxygenase activities in 4-nitrobenzoate-grown cells suggests that 4-nitrobenzoate is an inducer of the 4-nitrotoluene degradative pathway.

Nitroaromatic compounds commonly occur as intermediates or by-products in the synthesis of solvents, dyes, and explosives. Their widespread use in industry has resulted in their emergence as environmental contaminants.

The bacterial degradation of polar nitroaromatic compounds such as the mononitrobenzoates (4, 5, 8, 12), the mononitrophenols (24, 32), and several of the dinitrophenol isomers (3, 11, 14) has been reported. The bacterial oxidation of a number of nonpolar nitroaromatic compounds has also been described. Several bacterial monooxygenase and dioxygenase enzymes that attack the aromatic ring of toluene have been shown to hydroxylate nitrobenzene (10). In addition, toluene dioxygenase can catalyze both mono- and dioxygenase attack on the mononitrotoluenes (19). In these instances, the nitro group was not removed and the compounds did not serve as growth substrates. Recently, the dioxygenase-mediated denitration of 2,4,5-trichloronitrobenzene (20) and 1,3-dinitrobenzene (7) has been suggested. This mechanism of denitration has been confirmed for 2,4-dinitrotoluene (26) and 1,3-dinitrobenzene (15) in *Pseudomonas* species.

The ability of oxygenases to attack 4-nitrotoluene (4-NT) has been reported previously. The TOL plasmid-encoded toluene monooxygenase can transform 4-NT to 4-nitrobenzyl alcohol and 4-nitrobenzaldehyde (6). In contrast, toluene dioxygenases from *Pseudomonas putida* F1 and *Pseudomonas* sp. strain JS150 oxidize 4-NT to 2-methyl-5-nitrophenol and 3-methyl-6-nitrocatechol (19). These oxygenases attack 4-NT at either the methyl group or the 2,3 position of the aromatic ring, and the nitro group remains intact. None of these reactions lead to further degradation of the molecule. Recently, bacterial strains able to degrade 4-NT were isolated, and the initial steps of the degradative pathway were described in a preliminary report (29). The present study reports the complete degradative pathway of a different bacterial isolate that uses 4-NT as a sole source of carbon, nitrogen, and energy.

MATERIALS AND METHODS

Isolation and growth of bacteria. A soil-groundwater composite sample from First Chemical Corp., Pascagoula, Miss., was used to inoculate 50 ml of nitrogen-free minimal salts (NMS) medium (3) supplemented with 25 mg of 4-NT and 200 mg of yeast extract per liter. The culture was incubated for 5 days at 30°C with constant shaking (200 rpm), and subsequent transfers (10%, vol/vol) were made to fresh NMS containing 50 mg of 4-NT per liter when the 4-NT disappeared. Samples of the culture fluid were transferred to agar (1.8%) plates of NMS medium with 4-NT supplied in the vapor phase via crystals placed on the lid of the inverted plates.

Strains were characterized by standard procedures (22) and with Biolog GN Microplates (Biolog, Inc., Hayward, Calif.). The 4-NT-degrading isolate was tested for growth on a number of aromatic compounds on NMS or minimal salts (27) agar plates. Auxanography (16) was used to screen the isolate for the ability to grow on nonvolatile substrates. Growth on the vapor phase of volatile substrates was tested with the compounds in small tubes placed on the lid of inverted agar plates or by incubating the agar plates in desiccators containing the volatile substrate.

Cultures were maintained on NMS agar plates with 4-NT supplied in the vapor phase as the sole source of carbon and nitrogen. For experiments with 4-NT-induced cells, cultures were incubated overnight in 1 liter of NMS supplemented with 400 mg of 4-NT. An additional 200 mg of 4-NT was added 3 h before cells were harvested. 4-Hydroxybenzoate- and 4-nitrobenzoate-grown cells were incubated overnight in 1 liter of NMS supplemented with 1.0 g of 4-hydroxybenzoate or 4-nitrobenzoate per liter. Media containing 4-hydroxybenzoate were supplemented with 1.5 g of $(NH_4)_2SO_4$. Prior to harvesting, 250 mg of the growth substrate was

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added and the cells were incubated for another 2 to 3 h. Cells were grown on glucose in 1.5 liters of NMS supplemented with 3.0 g of glucose and 1.5 g of $(NH_4)_2SO_4$.

Respirometry and enzyme assays. Cells were harvested by centrifugation, washed with 0.02 M potassium phosphate buffer (pH 7.0), and suspended in the same buffer. Oxygen uptake was measured polarographically at 25°C with a Clark-type oxygen electrode (25). Reaction mixtures contained 50 μM substrate, cells (0.15 to 0.3 mg of protein), and 0.02 M potassium phosphate buffer (pH 7.0) to a final volume of 1.8 ml.

Cell extracts for enzyme assays were prepared as previously described (25). NAD^+ reduction by benzaldehyde dehydrogenase and benzyl alcohol dehydrogenase was measured as described previously (31). The concentrations of the substrates and NAD^+ in the assays were 10^{-4} and 3×10^{-4} M, respectively. Spectrophotometric and polarographic measurements of protocatechuate 3,4-, 2,3-, and 4,5-dioxygenase were determined as described previously (1, 28, 30). Ring cleavage of protocatechuate was examined with cell extracts which were heat treated at 50°C for 5 min. Protein determinations were done by using the bicinchoninic acid method of Smith et al. (23).

Analytical methods. Samples of culture fluids were clarified by centrifugation and analyzed by high-performance liquid chromatography (HPLC). HPLC was performed on a μBondapak C_{18} column (10 μm; 3.9 by 300 mm; Waters Associates, Inc., Milford, Mass.) with trifluoroacetic acid (13.5 mM)-acetonitrile (40:60) as the mobile phase at a flow rate of 1.5 ml/min. 4-Hydroxylaminobenzoate and protocatechuate were separated with the acetonitrile–0.01 N sulfuric acid (10:90) mobile phase described by Groenewegen and de Bont (9). Compounds were detected by their UV A_{230} with an HP1040A diode array detector (Hewlett-Packard Corp., Palo Alto, Calif.) and identified by comparison with authentic standards. The identity of protocatechuate was confirmed by direct probe mass spectral analysis.

Nitrite release was determined as described by Smibert and Krieg (22). Ammonia release in whole cell experiments was measured by previously described methods (17). For experiments with cell extracts, ammonium was determined using test kit 170-B from Sigma to measure the oxidation of NADH in the presence of 2-ketoglutarate and glutamate dehydrogenase.

Accumulation of products. Products of 4-nitrobenzoate metabolism were accumulated anaerobically at room temperature in 50 ml of 0.02 M potassium phosphate buffer (pH 7.0). Buffers were sparged with argon prior to use, and the reaction was performed in an anaerobic chamber under an atmosphere of 10% H_2, 10% CO_2, and 80% N_2. The reaction mixture contained 1.4 ml of cell extract (39.8 mg of protein), 50 μM 4-nitrobenzoate, and 112 μM NADPH. The reaction mixture was analyzed by HPLC, and more 4-nitrobenzoate and NADPH were added as the 4-nitrobenzoate disappeared. When 800 μM 4-nitrobenzoate was converted, the reaction mixture was acidified to pH 3.0 with HCl. The precipitated protein was removed by centrifugation, and the supernatant fluid was extracted with ethyl acetate as described previously (10).

The stoichiometry of product formation from 4-hydroxylaminobenzoate by cell extracts was determined under similar anaerobic conditions. The reaction mixture contained 0.1 or 0.2 ml of cell extract and 500 μM 4-hydroxylaminobenzoate in 10 ml of 0.02 M potassium phosphate buffer. At appropriate times, 1 ml of the reaction mixture was transferred to microcentrifuge tubes on ice which contained 0.01 ml of 6 M HCl to terminate the reaction. Precipitated protein was removed by centrifugation, and the concentrations of substrate and products were determined by HPLC analysis.

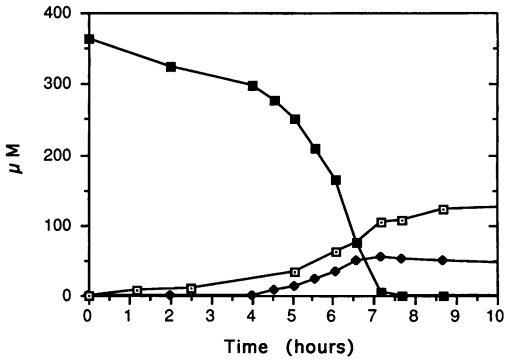

FIG. 1. Metabolism of 4-NT by glucose-grown cells of *Pseudomonas* sp. strain 4NT (A_{600} = 0.7). The metabolism of 4-NT (■) and the accumulation of 4-nitrobenzoate (◆) were monitored by HPLC. The accumulation of ammonia (⊡) was determined by the method of Parsons et al. (17).

Materials. 4-Hydroxylaminobenzoate was synthesized chemically as previously described (2), and its identity was confirmed by comparison with the previously published absorption and mass spectra (8). All other chemicals were of the highest purity commercially available.

RESULTS

Isolation and identification of bacteria. Biodegradation of 4-NT was detected in the mixed culture after 1 week of enrichment in the presence of 4-NT. A pure culture was obtained by repeated subculture on NMS agar plates with 4-NT supplied in the vapor phase as the sole source of carbon, nitrogen, and energy. The isolate was an oxidase- and catalase-positive, motile, gram-negative rod. The strain grew on *Pseudomonas* isolation agar (Difco) and was nonfluorescent on King's medium B (22). On the basis of these characteristics and the Biolog GN array, the strain was identified as a *Pseudomonas* sp. and designated strain 4NT. *Pseudomonas* sp. strain 4NT grew on 4-NT at concentrations approaching the solubility of 4-NT in water (400 mg/liter).

When glucose-grown cells were suspended in media containing 4-NT, a lag period of 4 h preceded rapid degradation of the substrate (Fig. 1). Degradation of 4-NT was accompanied by the accumulation of a small amount of 4-nitrobenzoate, which was identified by comparison of its HPLC retention time and UV absorbance with those of authentic standard. Ammonia but not nitrite was detected in the medium of cells degrading 4-NT (Fig. 1).

Pseudomonas sp. strain 4NT grew on 4-nitrobenzyl alcohol, 4-nitrobenzaldehyde, 4-nitrobenzoate, benzoate, benzyl alcohol, and 4-hydroxybenzoate (Table 1). These initial results suggested that 4-NT was mineralized by an initial oxidation of the methyl group to form 4-nitrobenzoate, which was further degraded.

Respirometry. Washed suspensions of 4-NT- and 4-nitrobenzoate-grown cells rapidly oxidized 4-nitrotoluene, 4-nitrobenzaldehyde, and 4-nitrobenzyl alcohol (Table 2). Lower rates of oxygen consumption were seen with protocatechuate. 4-Hydroxybenzoate-grown cells exhibited lower

TABLE 1. Growth characteristics of *Pseudomonas* sp. strain 4NT

Substrate	Growth	Substrate	Growth
4-NT	+	*m*-Toluate	−
4-Nitrobenzyl alcohol	+	*p*-Toluate	−
4-Nitrobenzaldehyde	+	3-Nitrotoluene	−
4-Nitrobenzoate	+	2-Nitrotoluene	−
Benzoate	+	*m*-Xylene	−
Benzyl alcohol	+	*p*-Xylene	−
Benzaldehyde	±	*o*-Xylene	−
4-Hydroxybenzoate	+	Pyridine	−
p-Cresol	−	*p*-Nitrophenol	−
4-Methylcatechol	−	4-Nitrocatechol	−
Benzene	−	4-Aminobenzoate	−
Toluene	−[a]	Protocatechuate	+

[a] Spontaneous mutants which grew on the test substrate appeared.

TABLE 2. Oxygen consumption by whole cells of *Pseudomonas* sp. strain 4NT

Substrate	Oxygen consumed (nmol/min/mg of protein) after growth with:			
	4-NT	4-Nitrobenzoate	4-Hydroxybenzoate	Glucose
4-NT	640	710	42	<10
4-Nitrobenzaldehyde	300	430	86	25
4-Nitrobenzyl alcohol	380	420	150	<10
Benzaldehyde	<10	120	110	<10
Benzyl alcohol	150	150	170	14
4-Hydroxybenzoate	<10	62	140	ND[a]
Protocatechuate	72	150	100	<10
4-Nitrobenzoate	<10	<10	<10	<10
4-Aminobenzoate	<10	<10	<10	<10
4-Methylcatechol	35	53	ND	ND
p-Cresol	27	40	<10	ND
4-Nitrocatechol	<10	<10	21	ND
4-Hydroxylaminobenzoate	<10	39	ND	ND

[a] ND, not determined.

rates of oxygen uptake with 4-NT, 4-nitrobenzyl alcohol, and 4-nitrobenzaldehyde than did 4-NT- and 4-nitrobenzoate-grown cells. Glucose-grown cells did not oxidize 4-NT or 4-nitrobenzyl alcohol but did exhibit low levels of 4-nitrobenzaldehyde oxidation.

Enzyme activities in cell extracts. Extracts prepared from 4-NT-, 4-nitrobenzoate-, and 4-hydroxybenzoate-grown cells exhibited benzaldehyde dehydrogenase activity with benzaldehyde and 4-nitrobenzaldehyde (Table 3). Benzyl alcohol dehydrogenase activity was either absent or present at very low levels in cell extracts subjected to ultracentrifugation (100,000 × *g*). Benzyl alcohol dehydrogenase activity toward benzyl alcohol and 4-nitrobenzyl alcohol was present in lysed suspensions of 4-NT-, 4-nitrobenzoate-, and 4-hydroxybenzoate-grown cells prior to ultracentrifugation; these are the values shown in Table 3.

Oxygen uptake by 4-NT- and 4-nitrobenzoate-grown cells was not stimulated by 4-nitrobenzoate. 4-Nitrobenzoate stimulated oxygen uptake with cell extracts of 4-NT-grown cells when NADPH was added. This finding suggested that 4-nitrobenzoate underwent an initial reduction to an oxidizable product similar to that which occurs during 4-nitrobenzoate degradation to protocatechuate by *Comamonas acidovorans* NBA-10 (8). The reaction was performed on a larger scale under anaerobic conditions as described in Materials and Methods, and the products were extracted. A compound with an HPLC retention time and absorption spectrum identical to those of protocatechuate was detected in the extract. The compound was purified by HPLC, and the identity was confirmed by direct probe mass spectral analysis. In a separate experiment, cell extracts of 4-NT-grown *Pseudomonas* sp. strain 4NT converted 4-hydroxylaminobenzoate to protocatechuate, which was identified on the basis of its HPLC retention time and absorption spectrum. Near stoichiometric amounts of protocatechuate (0.79 to 0.85 mol of protocatechuate per mol of 4-hydroxylaminobenzoate) and ammonia (0.56 to 0.82 mol of ammonia per mol of 4-hydroxylaminobenzoate) were formed anaerobically by cell extracts exposed to 4-hydroxylaminobenzoate.

Extracts of 4-NT-, 4-nitrobenzoate-, 4-hydroxybenzoate-, and glucose-grown cells converted protocatechuate to a yellow compound which appeared transiently in reaction mixtures. Protocatechuate 4,5-dioxygenase was present in cells grown on 4-nitrotoluene, 4-nitrobenzoate, 4-hydroxybenzoate, and glucose. Protocatechuate 4,5-dioxygenase activity was distinguished from protocatechuate 2,3- and 3,4-dioxygenase activities on the basis of the absorption spectrum of the ring fission product formed by heat-treated extracts. The ring fission product had a maximum absorbance in the visible range at 410 nm, which is consistent with α-hydroxy-γ-carboxymuconic semialdehyde (1). Protocatechuate 2,3-dioxygenase has been detected only in aerobic spore-forming bacteria (30), whereas protocatechuate 4,5-dioxygenase is induced in some nonfluorescent pseudomonads (1). The results suggest that protocatechuate is converted to α-hydroxy-γ-carboxymuconic semialdehyde by a protocatechuate 4,5-dioxygenase. In subsequent experiments (Table 3), protocatechuate dioxygenase activity was measured polarographically.

DISCUSSION

Two different mechanisms for the direct removal of nitro groups from nitroaromatic compounds by microorganisms have been described. This process can occur by either an oxidative (3, 7, 15, 24, 26, 33) or a reductive (13) route. Oxidation reactions that eliminate nitrite from nitroaromatic compounds are catalyzed by monooxygenase (24, 33) or dioxygenase (7, 15, 26) enzyme systems. A reductive elimination of nitrite from picric acid has recently been described for a *Rhodococcus* strain (13). In this pathway, one hydride ion is added to the aromatic ring of picric acid to form a Meisenheimer complex, which regains aromaticity with nitrite elimination to form 2,4-dinitrophenol.

An alternative mode of metabolism of aromatic nitro groups involves reduction by nitroreductase enzymes to form nitroso and hydroxylamino compounds, with eventual elimination of ammonia (4, 5, 8, 9, 12). Similar reductive pathways often result in the formation of aminoaromatic compounds as end products (21) which can accumulate to toxic levels. Recent evidence has shown that *C. acidovorans* NBA-10 reduces the nitro group of 4-nitrobenzoate via 4-nitrosobenzoate and 4-hydroxylaminobenzoate (9). In this pathway, the accumulation of aromatic amines is avoided by direct elimination of ammonia from 4-hydroxylaminobenzoate, with concomitant formation of protocatechuate (8, 9). A similar reductive pathway for the elimination of the nitro group from 2-nitrobenzoate without an interme-

TABLE 3. Enzyme activities in cell extracts of *Pseudomonas* sp. strain 4NT

Enzyme assayed and assay substrate	Sp act (nmol/min/mg of protein) after growth with:			
	4-Nitrotoluene	4-Nitrobenzoate	4-Hydroxybenzoate	Glucose
Benzaldehyde dehydrogenase				
Benzaldehyde	74	1,000	850	30
4-Nitrobenzaldehyde	98	1,800	410	77
Benzyl alcohol dehydrogenase				
Benzyl alcohol	71	58	75	<10
4-Nitrobenzyl alcohol	48	52	78	<10
Protocatechuate oxygenase	120	180	3,100	4,300

diate amino derivative was postulated for a *Flavobacterium* strain (12).

The presence of ammonia but not nitrite in cultures of *Pseudomonas* sp. strain 4NT exposed to 4-NT suggested that the nitro group was eliminated from the aromatic ring via a reductive pathway. The detection of 4-nitrobenzoate in culture fluids and the ability of the organism to grow on 4-nitrobenzyl alcohol, 4-nitrobenzaldehyde, and 4-nitrobenzoate (Table 1) suggested that 4-NT was oxidized to 4-nitrobenzoate prior to removal of the nitro group. Simultaneous adaptation studies and enzyme assays after growth on 4-NT confirm such a degradative pathway. This reaction sequence is similar to that of the TOL plasmid-mediated catabolism of toluene and has been suggested previously for the degradation of 4-NT by another *Pseudomonas* strain (29).

The oxidation of protocatechuate by intact cells and cell extracts of 4-NT-, 4-nitrobenzoate-, and glucose-grown cells suggested that 4-nitrobenzoate was converted to protocatechuate prior to ring cleavage. The ability of cell extracts to reduce 4-nitrobenzoate and 4-hydroxylaminobenzoate to protocatechuate implies that 4-hydroxylaminobenzoate is an intermediate in the conversion of 4-nitrobenzoate to protocatechuate as described for *C. acidovorans* (8, 9). Protocatechuate is oxidized by protocatechuate 4,5-dioxygenase in a reaction similar to that reported in some nonfluorescent pseudomonads (1). On the basis of these results, we propose the pathway shown in Fig. 2.

A previous study has shown that the TOL upper-pathway enzymes recognize 3- and 4-nitrotoluene as substrates but the XylR regulator protein does not recognize the nitrotoluenes as effectors, and so the pathway is not induced in pseudomonads harboring the TOL plasmid (6). The benzyl alcohol and benzaldehyde dehydrogenases of *Pseudomonas*

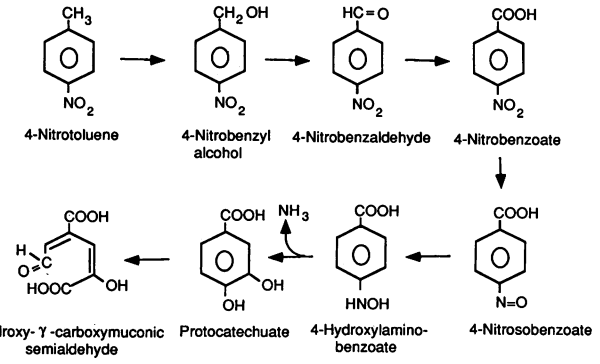

FIG. 2. Proposed pathway for the degradation of 4-NT by *Pseudomonas* sp. strain 4NT.

sp. strain 4NT act upon unsubstituted and 4-nitro-substituted substrates, and the strain grows well on benzyl alcohol and benzoate, suggesting that these enzymes are analogous to those of the TOL plasmid degradative pathway. *Pseudomonas* sp. strain 4NT grows poorly on toluene but gives rise to spontaneous mutants that grow well, which suggests that it may contain a TOL plasmid in which the regulatory protein recognizes nitrotoluene as an inducer. The relationship of the initial enzymes in the 4-NT degradative pathway to those of the TOL plasmid degradative pathway requires further study.

To our knowledge, this report and a very similar paper simultaneously submitted by Rhys-Williams et al. (18) are the first descriptions of a bacterial degradative pathway for any of the isomeric nitrotoluenes. The reductive elimination of the nitro group as ammonia from 4-NT differs from the previously reported dioxygenase-mediated removal of the nitro group of 2,4-dinitrotoluene by a *Pseudomonas* sp. (26). The results indicate that bacteria can employ both oxidative and reductive pathways for the removal of nitro groups from the nitrotoluenes and suggest strategies for the degradation of other nitroaromatic compounds.

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REFERENCES

1. **Arciero, D. M., A. M. Orville, and J. D. Lipscomb.** 1990. Protocatechuate 4,5-dioxygenase from *Pseudomonas testosteroni*. Methods Enzymol. **188**:89–95.
2. **Bauer, H., and S. M. Rosenthal.** 1944. 4-Hydroxylaminobenzene sulfonamide, its acetyl derivatives and diazotization reaction. J. Am. Chem. Soc. **66**:611–614.
3. **Bruhn, C., H. Lenke, and H.-J. Knackmuss.** 1987. Nitrosubstituted aromatic compounds as nitrogen source for bacteria. Appl. Environ. Microbiol. **53**:208–210.
4. **Cartwright, N. J., and R. B. Cain.** 1959. Bacterial degradation of the nitrobenzoic acids. Biochem. J. **71**:248–261.
5. **Cartwright, N. J., and R. B. Cain.** 1959. Bacterial degradation of the nitrobenzoic acids. 2. Reduction of the nitro group. Biochem. J. **73**:305–314.
6. **Delgado, A., M. G. Wubbolts, M.-A. Abril, and J. L. Ramos.** 1992. Nitroaromatics are substrates for the TOL plasmid upper-pathway enzymes. Appl. Environ. Microbiol. **58**:415–417.
7. **Dickel, O., and H.-J. Knackmuss.** 1991. Catabolism of 1,3-dinitrobenzene by *Rhodococcus* sp. QT-1. Arch. Microbiol. **157**:76–79.
8. **Groenewegen, P. E. J., P. Breeuwer, J. M. L. M. Van Helvoort, A. A. M. Langenhoff, F. P. De Vries, and J. A. M. de Bont.** 1992. Novel degradative pathway of 4-nitrobenzoate in *Comamonas acidovorans* NBA-10. J. Gen. Microbiol. **138**:1599–1605.

9. **Groenewegen, P. E. J., and J. A. M. de Bont.** 1992. Degradation of 4-nitrobenzoate via 4-hydroxylaminobenzoate and 3,4-dihydroxybenzoate in *Comamonas acidovorans* NBA-10. Arch. Microbiol. **158:**381–386.
10. **Haigler, B. E., and J. C. Spain.** 1991. Biotransformation of nitrobenzene by bacteria containing toluene degradative pathways. Appl. Environ. Microbiol. **57:**3156–3162.
11. **Jensen, H. L., and G. Lautrup-Larsen.** 1967. Microorganisms that decompose nitroaromatic compounds, with special reference to dinitro-orthocresol. Acta Agric. Scand. **17:**115–125.
12. **Ke, Y.-H., L. L. Gee, and N. N. Durham.** 1959. Mechanism involved in the metabolism of nitrophenyl-carboxylic acid compounds by microorganisms. J. Bacteriol. **77:**593–598.
13. **Lenke, H., and H.-J. Knackmuss.** 1992. Initial hydrogenation during catabolism of picric acid by *Rhodococcus erythropolis* HL 24-2. Appl. Environment. Microbiol. **58:**2933–2937.
14. **Lenke, H., D. H. Pieper, C. Bruhn, and H.-J. Knackmuss.** 1992. Degradation of 2,4-dinitrophenol by two *Rhodococcus erythropolis* strains, HL 24-1 and HL 24-2. Appl. Environment. Microbiol. **58:**2928–2932.
15. **Nishino, S. F., and J. C. Spain.** 1992. Initial steps in the bacterial degradation of 1,3-dinitrobenzene, abstr. Q-135, p. 358. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992.
16. **Parke, D., and L. N. Ornston.** 1984. Nutritional diversity of *Rhizobiaceae* revealed by auxanography. J. Gen. Microbiol. **130:**1743–1750.
17. **Parsons, T. R., Y. Maita, and C. M. Lalli (ed.).** 1984. A manual of chemical and biological methods for seawater analysis, p. 15–17. Pergamon Press, New York.
18. **Rhys-Williams, W., S. C. Taylor, and P. A. Williams.** A novel pathway for the catabolism of 4-nitrotoluene by Pseudomonas. J. Gen. Microbiol., in press.
19. **Robertson, J. B., J. C. Spain, J. D. Haddock, and D. T. Gibson.** 1992. Oxidation of nitrotoluenes by toluene dioxygenase: evidence for a monooxygenase reaction. Appl. Environ. Microbiol. **58:**2643–2648.
20. **Sander, P., R. M. Wittich, P. Fortnagel, H. Wilkes, and W. Francke.** 1991. Degradation of 1,2,4-trichloro- and 1,2,4,5-tetrachlorobenzene by *Pseudomonas* strains. Appl. Environ. Microbiol. **57:**1430–1440.
21. **Schackmann, A., and R. Muller.** 1991. Reduction of nitroaromatic compounds by different *Pseudomonas* species under aerobic conditions. Appl. Microbiol. Biotechnol. **34:**809–813.
22. **Smibert, R. M., and N. R. Krieg.** 1981. General characterization, p. 409–443. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
23. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.
24. **Spain, J. C., and D. T. Gibson.** 1991. Pathway for biodegradation of *p*-nitrophenol in a *Moraxella* sp. Appl. Environ. Microbiol. **57:**812–819.
25. **Spain, J. C., and S. F. Nishino.** 1987. Degradation of 1,4-dichlorobenzene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **53:**1010–1019.
26. **Spanggord, R. J., J. C. Spain, S. F. Nishino, and K. E. Mortelmans.** 1991. Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **57:**3200–3205.
27. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159–271.
28. **Whittaker, J. W., A. M. Orville, and J. D. Lipscomb.** 1990. Protocatechuate 3,4-dioxygenase from *Brevibacterium fuscum*. Methods Enzymol. **188:**82–88.
29. **Williams, W. R., S. C. Taylor, and P. A. Williams.** 1991. Microbial catabolism of 4-nitrotoluene. Presented at Pseudomonas 1991, a symposium sponsored by the Federation of European Microbiological Societies.
30. **Wolgel, S. A., and J. D. Lipscomb.** 1990. Protocatechuate 2,3-dioxygenase from *Bacillus macerans*. Methods Enzymol. **188:**95–101.
31. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. **124:**7–13.
32. **Zeyer, J., and P. C. Kearney.** 1984. Degradation of *o*-nitrophenol and *m*-nitrophenol by a *Pseudomonas putida*. J. Agric. Food Chem. **32:**238–241.
33. **Zeyer, J., and H. P. Kocher.** 1988. Purification and characterization of a bacterial nitrophenol oxygenase which converts *ortho*-nitrophenol to catechol and nitrite. J. Bacteriol. **170:**1789–1794.