A novel pathway for the catabolism of 4-nitrotoluene by *Pseudomonas*

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Eleven strains of *Pseudomonas* were isolated by selective enrichment on 4-nitrotoluene (4NT). They all utilized 4NT, 4-nitrobenzyl alcohol (4NBA) or 4-nitrobenzoate (4NBZate) as sole sources of carbon and nitrogen. One strain, TW3, was used for more detailed studies. 4NT-grown cells of TW3 take up O₂ when incubated in the presence of 4NBA, 4-nitrobenzaldehyde (4NBZ) and 4NBZate. HPLC analysis of culture supernatants showed that 4NBZ and 4NBZate were formed when 4NT-grown cells were incubated with 4NBA, whereas only 4NBZate was found when they were incubated with 4NBZ. Two dehydrogenases were detected in extracts of 4NT-grown cells. 4NBA dehydrogenase could be assayed by a dye-linked assay whereas 4NBZ dehydrogenase activity was linked to NAD⁺ reduction. No nitrite was detected in supernatants of 4NBZate-grown cells incubated with 4NBZate but the nitrogen appeared as ammonium. The only aromatic ring-cleavage dioxygenase that was induced during growth on the nitroaromatics was protocatechuate 3,4-dioxygenase. It is proposed that the pathway for 4NT catabolism proceeds via 4NBA, 4NBZ and 4NBZate and ultimately to protocatechuate with release of the nitro group as ammonium.

Introduction

Nitro-substituted aromatics are used in, and are the products of, a number of industrial processes. In particular, 2,4,6-trinitrotoluene (TNT) is a major product of the explosives industry and many sites are polluted with TNT itself and with mono- and dinitrotoluenes. However, by comparison with aromatic hydrocarbons and halogen-substituted aromatics there has been relatively little investigation of their microbial catabolism. Several reports of biotransformations of TNT and other nitrotoluenes by axenic cultures and mixed populations of microbes have been reported (McCormick *et al.*, 1976, 1978; Carpenter *et al.*, 1978; Davis *et al.*, 1981; Hallas & Alexander, 1983; Fernando *et al.*, 1990; Valli *et al.*, 1992) as well as cometabolic transformations by bacteria which catabolize unsubstituted toluene (Delgado *et al.*, 1992; Robertson *et al.*, 1992). In this paper we report a novel route for the complete catabolism of 4-nitrotoluene (4NT) in which the first transformations are analogous to the well characterized route for toluene catabolism in *Pseudomonas* strains with resident TOL plasmids (Assinder & Williams, 1990). In this pathway, the methyl

group is sequentially oxidized to an alcohol, aldehyde and carboxylic acid.

Methods

Bacterial cultures and their maintenance and growth. Eleven independent bacterial isolates (designated TW1–TW11) capable of growth on 4NT as sole source of carbon were isolated from soil samples by selective enrichment. All were classified as members of the genus *Pseudomonas* by the NCIMB, Torry Research Station, Aberdeen, UK; strain TW3 can produce fluorescent pigments and is probably *P. fluorescens* or *P. putida*. They were routinely maintained on slopes and plates of agar media containing minimal salts (Bauchop & Elsdon, 1960) with 5 mM-Na 4NBZate as sole carbon source. Growth on 4NT or 4NBA on solid surfaces was obtained using minimal salts agar with the carbon source supplied as solid in the lid of the inverted plate. Strain TW3 was used for all later experiments. Cells of TW3 were obtained from liquid cultures by inoculating into aerated 2 l vessels at 28 °C containing minimal salts medium with either dissolved 5 mM-Na 4NBZate or added solid 4NT or 4NBA; uninduced cells were routinely grown on 10 mM-acetate. Cells were harvested by centrifugation, washed in fresh Na/K phosphate buffer, pH 7.5, recentrifuged and stored at –20 °C until required. Cell-free extracts were prepared by sonication (Worsey & Williams, 1975).

Enzyme assays. The dye-linked 4NBA dehydrogenase was assayed in 3 ml made up of 50 µl 6.7 mM-dichlorophenolindophenol, 50 µl 20 mM-phenazine methosulphate and 2.7 ml 80 mM-Tris/HCl, pH 8.7 containing 3.0 mM-4NBA as substrate: the substrate was added previously to the Tris buffer as a 300 mM solution in dimethyl sulphoxide. The reaction was initiated by addition of 200 µl cell extract and the rate of disappearance of the blue colour was measured at

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Abbreviations: 4NBA, 4-nitrobenzyl alcohol; 4NBZ, 4-nitrobenzaldehyde; 4NBZate, 4-nitrobenzoate; 4NT, 4-nitrotoluene.

600 nm. Insignificant rates of change of absorbance were obtained with controls in which any one of the components was omitted; these included adding dimethyl sulphoxide without substrate to the buffer.

The activity of 4NBZ dehydrogenase was determined by measuring the increase in A_{340} due to NAD^+ reduction in a 3 ml assay containing 300 μl 50 mM- NAD^+ , 2.5 ml 400 μM -substrate dissolved in 80 mM-CAPS, pH 9.4. The substrate was added to the buffer as a 100 mM solution in dimethyl sulphoxide and the reaction was initiated by adding 200 μl cell extract. Controls with any one of the components omitted showed negligible rates of change in absorbance.

Ring cleavage dioxygenases were assayed by standard procedures: protocatechuate 3,4-dioxygenase (Fujisawa, 1970), protocatechuate 4,5-dioxygenase (Arciero *et al.*, 1990), catechol 2,3-dioxygenase (Sala-Trepat & Evans, 1971), catechol 1,2-dioxygenase (Murray *et al.*, 1972).

O₂ uptake experiments. Cells for respiration experiments were prepared either by growing in liquid medium on 5 mM-4NBZate or by growing on agar plates in the presence of 4NT. In the latter case cells were washed off plates into sterile minimal salts medium, centrifuged and washed twice more. Cells were suspended in 5 ml minimal salts medium and O_2 uptake was measured in a Clark oxygen electrode. Soluble substrates were present at a final concentration of 1 mM and sparingly soluble substrates were added as small quantities of solids (about 10 mg) into the chamber of the electrode. The endogenous uptake in the absence of substrate was measured before addition of substrate. Because of the variability of measuring uptake, only increases in the rate of uptake greater than threefold were taken as representing significant stimulation. The uptake of induced cells was compared with that of uninduced (10 mM-acetate-grown) cells.

Inorganic determinations. Nitrite was determined by a modification of the Griess-Ilosvay method according to Montgomery & Dymock (1961). Ammonium ions were measured with an ammonium ion probe (Model 8002-8, Kent Industrial Measurements) according to the manufacturer's instructions.

HPLC analysis. After removal of cells by filtration through 0.2 μm pore size GS filters (Millipore), the composition of supernatant fluids was determined by HPLC analysis on a Baker C18 reverse phase column running isocratically at a flow rate of 1 ml min⁻¹: the mobile phase consisted of a 60:40 (v/v) mixture of (a) 0.11% aqueous trifluoroacetic acid and (b) 60% acetonitrile containing 0.1% trifluoroacetic acid. The eluate was monitored at 254 nm by a PU4025 UV detector (Pye Unicam). The retention times for the relevant compounds differed somewhat on different occasions but authentic samples of the nitro compounds were always run at the beginning of a series of analyses. Typical retention times (min) were: 4NBA, 4.9; 4NBZate, 5.7; 4NBZ, 6.9; 4NT, 16.

Chemicals. All chemicals used were from commercially available sources (usually Sigma or Aldrich).

Results

Growth characteristics of 4NT⁺ isolates

All 11 isolates were tested for growth on solid and in liquid media on 23 different aromatic compounds as sole source of carbon. All grew on 4NT, 4NBA, 4NBZate, toluene, benzyl alcohol, benzoate, 4-hydroxybenzoate, 4-hydroxybenzaldehyde, *p*-cresol. No growth was detected on 4NBZ, 2-nitrotoluene, 3-nitrotoluene, 2,4-dinitrotoluene, 2,6-dinitrotoluene, 4-nitrocatechol, 4-amino-benzoate, *p*-xylene, *m*-xylene, naphthalene, 3-fluoro-catechol, 4-fluorobenzoate or toluidine. Strains TW4,

TW5, TW7 and TW8 grew on 3,4-dihydroxybenzaldehyde but the remainder did not.

During growth on the 4-nitro compounds only, cultures became progressively orange and supernatants developed a characteristic absorbance with a broad peak with two adjacent maxima at 430 and 466 nm. The causative compound(s) have not been identified. Because the colour continued to accumulate during growth and did not diminish in absorbance even during the late stages of growth, we have assumed that it is a by-product of metabolism rather than a direct metabolic intermediate.

Strains growing on the nitro-substituted substrates could use them as sole sources of nitrogen as well as carbon

O₂ uptake experiments

Cells of strain TW3 grown on 4NT showed significant (greater than threefold increase over the endogenous rate) increase in O_2 uptake in the presence of only 4NBA, 4NBZ, 4NBZate and protocatechuate (3,4-dihydroxybenzoate): there was no stimulation of uptake by 4-nitrocatechol, 4-aminobenzoate or 4-hydroxybenzoate. Toluene, 2,4-dinitrotoluene and 2,6-dinitrotoluene stimulated more modest increases (between two- and threefold), suggesting that there might be some non-specificity in the 4NT-metabolizing pathway, but 2- and 3-nitrotoluenes had no such effect. Uninduced acetate-grown cells showed no significant stimulation of respiration with any of the above compounds.

HPLC analysis of supernatant fractions

Cells of strain TW3 were grown for 24 h with either 4NT or 4NBA as the sole carbon source. Both cultures were then harvested by centrifugation, washed and resuspended in minimal medium containing either 4NT, 4NBA, 4NBZ or 4NBZate. Samples were taken at various times and were analysed for the presence of potential metabolites by HPLC. When incubated with 4NT, 4NBZate and smaller quantities of 4NBA could be detected in the medium (Fig. 1*a*) but no 4NBZ. In the case of incubation in the presence of 4NBA, both 4NBZ and 4NBZate appeared in the supernatants (Fig. 1*b*). After incubation with 4NBZ, only 4NBZate appeared; with 4NBZate, none of the other nitroaromatics were found in supernatants (data not shown).

In some incubations, particularly those in the presence of 4NBZate, an additional peak with a retention time of about 3.9 min appeared after 24 h. This was not 4-nitrocatechol which, by analogy with benzoate catabolism, is a possible metabolite of 4NBZate but is possibly the compound responsible for the orange colour which

Fig. 1. Quantitative appearance of metabolites during incubation of *Pseudomonas* strain TW3 with 4-nitroaromatics. (a) Incubation in the presence of excess 4NT. Cells grown with 4NT were inoculated into fresh medium containing sufficient solid 4NT to saturate the solution throughout the experiment. Accumulation of 4NBA (Δ) and 4NBZate ($\circ$). (b) Incubation in the presence of 4NBA. Cells grown with 4NT were incubated in fresh medium containing initially an excess of 4NBA. Accumulation of 4NBA (Δ), 4NBZate ($\circ$) and 4NBZ (∇). (c) HPLC trace showing separation of the authentic 4-nitroaromatics. Peaks: A, 4NT; B, 4NBZ; C, 4NBZate; D, 4NBA; E, 4-nitrocatechol.

Table 1. Substrate specificity of 4NBA dehydrogenase

Substrate	Relative activity*
4-Nitrobenzyl alcohol	100
2-Nitrobenzyl alcohol	15
3-Nitrobenzyl alcohol	30
4-Hydroxybenzyl alcohol	15
4-Methylbenzyl alcohol	190
Benzyl alcohol	130

* With respect to 4NBA (= 100).

appeared in the later supernatants since there was a qualitative relationship between the colour and the peak size.

Assay for 4NBA dehydrogenase

Attempts were made first to assay a 4NBA dehydrogenase linked to NAD^+ using the assay published for the TOL plasmid benzyl alcohol dehydrogenase (Worsey & Williams, 1975). No significant activity was detected in induced or uninduced cells under conditions where *P. putida* PaW1 (carrying the TOL plasmid pWW0) (Worsey & Williams, 1975) gave high induced activities which were active against both unsubstituted benzyl alcohol and 4NBA (data not shown).

However with an assay procedure, modified from Matsushita & Ameyama (1982), using dichlorophenol-indophenol (DCPIP) and phenazine methosulphate, it was possible to detect reduction of the DCPIP which was dependent upon the presence of 4NT-induced cell extract

and 4NBA. This reduction was also stimulated in the presence of other benzyl alcohols indicating that it has a broad specificity (Table 1).

To check that the activity was producing the expected product, an assay was allowed to proceed until all the DCPIP was utilized and a sample of the solution was analysed by HPLC. This clearly showed that 4NBZ had been formed (data not shown).

In the assay, the measured activity was proportional to the amount of cell extract added but depended upon the substrate in a hyperbolic manner with a K_m of about 1 mM. Activity was stable in extracts for several hours at 4 °C but heating to 60 °C for 10 min caused complete loss of activity. There was no strong evidence that the enzyme was membrane-bound. After using the procedure of Tassin *et al.* (1973) to prepare a membrane fraction, only about 30 % of the total activity was associated with the pellet fraction compared with the supernatant and its specific activity was only 10–15 % of that of the cell extract.

Assay for 4NBZ dehydrogenase

Using the assay conditions for benzaldehyde dehydrogenase from TOL plasmids (Worsey & Williams, 1975), NAD^+ -dependent activity against 4NBZ could be detected in cell extracts of 4NT-grown strain TW3. However, the glycine buffer used in the TOL assay caused problems with the assay at pH values above 9.9. CAPS proved to be a satisfactory alternative which caused no problems over its buffering range. At pH 9.4,

the assay was dependent upon the presence of substrate, NAD⁺ and cell extract. The activity was linear with regard to amount of cell extract added, hyperbolic with respect to substrate concentration but with a very low K_m in the order of 2 μ M. It was destroyed by heating, although it was somewhat more stable to heating than the alcohol dehydrogenase. It was active against 3-nitrobenzaldehyde (rate relative to 4NBZ 1.3) and benzaldehyde (rate relative to 4NBZ 0.5) but not against 2-nitrobenzaldehyde, 4-hydroxybenzaldehyde, or the 2,4- or 2,6-dinitrobenzaldehydes. NADP⁺ appeared to be only about 15% as effective as NAD⁺ as a cofactor.

To examine the product formed in the reaction, reactions with an excess of NAD⁺ over substrate were allowed to go to completion and the reaction analysed by HPLC. The results clearly showed the complete oxidation of 4NBZ to 4NBZate (data not shown).

Further metabolism of 4NBZate

To determine the fate of the nitrogen from the nitro group, cells were induced by growth on 4NBZate, centrifuged, washed and resuspended in minimal medium lacking any inorganic nitrogen but containing 5 mM-4NBZate. After incubating for 12 and 24 h, the supernatant was analysed for nitrite and ammonium. At both 12 and 24 h the nitrite concentration was only 2 μ M, whereas the ammonium concentration reached 5 mM after 12 h.

Extracts of cells grown on either 4NT, 4NBZate or acetate were assayed for aromatic ring cleavage enzymes to try to identify whether a route for conversion of an aromatic ring cleavage substrate to an aliphatic product could be identified. Specific activities of catechol 1,2-dioxygenase, catechol 2,3-dioxygenase and protocatechuate 4,5-dioxygenase were either very low or undetectable. However, protocatechuate 3,4-dioxygenase was induced by growth on both 4NT and 4NBZate: whereas acetate-grown cells contained only 24 mU (mg protein)⁻¹ min⁻¹, the activity for 4NT-grown cells was 230 mU (mg protein)⁻¹ min⁻¹ and for 4NBZate-grown cells, 850 mU (mg protein)⁻¹ min⁻¹.

A possible precursor of protocatechuate is 4-hydroxybenzoate. Extracts of 4NT-grown cells contained activities of 4-hydroxybenzoate hydroxylase which were higher [13 mU (mg protein)⁻¹ min⁻¹] than those found in acetate-grown cells (0.7) but were considerably lower than those found in 4-hydroxybenzoate-grown cells (200).

Discussion

Within the population of soil saprophytic bacteria, a number of strategies have evolved for the catabolism of substituted aromatic compounds. Toluene is metabolized

through a number of routes before the ring cleavage steps. In the TOD pathway, the ring is directly dihydroxylated producing 3-methyl catechol as the ring cleavage substrate (Zylstra & Gibson, 1989). An alternative route involves a monohydroxylation to produce *p*-cresol followed by the sequential oxidation of the methyl group to form 4-hydroxybenzoate, and protocatechuate is the ring cleavage substrate (Whited & Gibson, 1991). In the TOL plasmid-encoded pathway, the *xyl* gene products cause the sequential oxidation of the methyl group to produce benzoate, and the substrate for ring cleavage is unsubstituted catechol (Assinder & Williams, 1990).

We have shown that the initial steps in the catabolic pathway for 4-nitro-substituted toluene parallel the TOL plasmid pathway (Fig. 2). HPLC studies demonstrate that 4NBA, 4NBZ and 4NBZate all appear in the supernatants of induced cells and that the order in which they appear (hydrocarbon, alcohol, aldehyde and carboxylic acid) is the same as in the TOL pathway.

Both the range of growth substrates and stimulation of O₂ uptake are in agreement with the evidence from HPLC although on their own they would not serve as conclusive evidence for the pathway because of the possibilities of either (a) multiple overlapping pathways being present in the strains or (b) non-specificity of action of 4NT-induced enzymes. We have not been able to show growth on one of the postulated metabolites, 4NBZ, although its further metabolism to 4NBZate has been demonstrated by HPLC. This may be due either to toxicity or to the inability of the aldehyde to act as an inducer of the catabolic enzymes.

Finally we have demonstrated the presence in extracts of 4NT-grown cells of two dehydrogenase activities which convert 4NBA first to 4NBZ and then to 4NBZate,

Fig. 2. Initial steps in the proposed pathway for the catabolism of 4NT in strain TW3. The compounds are 4NT (I), 4NBA (II), 4NBZ (III), 4NBZate (IV), protocatechuate (V) and β -carboxy-*cis,cis*-muconate (VI).

and the products of both reactions have been detected by HPLC in cell extracts.

We originally thought that the genes and enzymes for conversion of 4NT to 4NBZate might bear some similarities to the TOL plasmid system for the unsubstituted and methyl-substituted toluenes, particularly since it has been demonstrated that the TOL system has activity against nitro-substituted toluenes and their metabolites (Delgado *et al.*, 1992). However, we have unsuccessfully probed Southern blots of genomic DNA from strain TW3 using TOL DNA probes carrying the genes for upper pathway enzymes. If there is DNA homology between the two systems it is at a level which cannot be detected by hybridization. The inability to cross-hybridize is matched by a major difference in the biochemistry of the two pathways at the level of the benzyl alcohol dehydrogenases. Whereas the TOL enzyme is linked to NAD⁺, we could detect no comparable activity in 4NT-induced TW3, but instead found an activity which required the dye couple dichlorophenolindophenol/phenazine methosulphate as electron acceptor. These differences suggests that any evolutionary relationship between the two systems is distant.

The second substituent of the aromatic ring is the nitro group. There are two main routes which have been described for the microbial metabolism of nitroaromatics. One involves direct removal as nitrite (Spain *et al.*, 1979; Zeyer & Kearney, 1984; Bruhn *et al.*, 1987), whereas the other involves its reduction through nitroso- and hydroxylamino-substituted metabolites (see Groenewegen *et al.*, 1992). In some cases the end-product is an amino-substituted aromatic although it is not certain whether the amino-compounds are ever direct metabolites of a complete catabolic pathway or are always either end-products or the result of side reactions. Recently a pathway for 4NBZate catabolism has been described in *Comomonas acidovorans* in which the nitro group is reduced through 4-nitrosobenzoate to 4-hydroxylaminobenzoate through the action of NAD(P)H-linked reductase(s) and the hydroxylamino group is then subsequently eliminated in a single novel step, the products of which are ammonium and protocatechuate (Groenewegen *et al.*, 1992; Groenewegen & de Bont, 1992). There is circumstantial evidence that TW3 also utilizes this route for the further metabolism of 4NBZate formed from 4NT: (a) no significant levels of nitrite could be detected in supernatants, whereas ammonium was released stoichiometrically; (b) 4-aminobenzoate was neither a growth substrate nor did it stimulate O₂ uptake in 4NT-induced cells; and (c) induced levels of protocatechuate 3,4-dioxygenase were present in 4NT-grown cells. In preliminary experiments we have also shown the presence in extracts of 4NT-grown cells of an

NADH-dependent activity using 4NBZate as substrate similar to the reductase described by Groenewegen *et al.* (1992) (data not shown).

The pathway described here for 4NT catabolism has also been independently demonstrated in a different isolate (Haigler & Spain, 1993).

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