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Degradation of 3-nitrophenol by *Pseudomonas putida* B2 occurs via 1,2,4-benzenetriol

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Abstract

Growth of *Pseudomonas putida* B2 in chemostat cultures on a mixture of 3-nitrophenol and glucose induced 3-nitrophenol and 1,2,4- benzenetriol-dependent oxygen uptake activities. Anaerobic incubations of cell suspensions with 3-nitrophenol resulted in complete conversion of the substrate to ammonia and 1,2,4-benzenetriol. This indicates that *P. putida* B2 degrades 3-nitrophenol via 1,2,4-benzenetriol, via a pathway involving a hydroxylaminolyase. Involvement of this pathway in nitroaromatic metabolism has previously only been found for degradation of 4-nitrobenzoate.

Reduction of 3 nitrophenol by cell-free extracts was strictly NADPH-dependent. Attempts to purify the enzymes responsible for 3-nitrophenol metabolism were unsuccessful, because their activities were extremely unstable. 3-Nitrophenol reductase was therefore characterized in cell-free extracts. The enzyme had a sharp pH optimum at pH 7 and a temperature optimum at 25°C. At 30°C, reductase activity was completely destroyed within one hour, while at 0°C, the activity in cell-free extracts was over 100-fold more stable. The K_m values for NADPH and 3-nitrophenol were estimated at 0.17 mM and below 2 μM, respectively. The substrate specificity of the reductase activity was very broad: all 17 nitroaromatics tested were reduced by cell-free extracts. However, neither intact cells nor cell-free extracts could convert a set of synthesized hydroxylaminoaromatic compounds to the corresponding catechols and ammonia. Apparently, the hydroxylaminolyase of *P. putida* B2 has a very narrow substrate specificity, indicating that this organism is not a suitable biocatalyst for the industrial production of catechols from nitroaromatics.

Introduction

Nitroaromatic compounds are used for the industrial production of explosives, dyes, pesticides and pharmaceuticals and have consequently entered the environment. Interest in the microbial degradation of these toxic compounds has led to the isolation of different microorganisms capable of degrading nitroaromatics.

Metabolism of nitroaromatics can occur via different mechanisms (Higson 1992; Marvin-Sikkema & De Bont 1994; Spain 1995). Mono- or dioxygenase activity on nitroaromatics or their initial reduction by a hydride ion (H^-) liberates the nitro group as nitrite and produces a mono- or dihydroxyaromatic compound (Jain et al. 1994; Spain & Gibson 1991; Zeyer & Kocher 1988; Spanggord et al. 1994; Lenke & Knackmuss 1992; Rieger & Knackmuss 1995). Degradation of nitroaromatics can also be initiated by reduction of the nitro group to an amine followed by deamination, yielding ammonia and the corresponding benzene derivative (Boopathy & Kulpa 1993; Boopathy et al. 1993).

Yet another pathway has been demonstrated in *Comamonas acidovorans* NBA–10 and two *Pseudomonas* species. In these organisms, metabolism of 4-nitrobenzoate is initiated by its partial reduc-

tion to 4-hydroxylaminobenzoate, which is subsequently converted to ammonia and the catechol 3,4-dihydroxybenzoate by a hydroxylaminolyase (Groenewegen et al. 1992a; Groenewegen & De Bont 1992; Haigler & Spain 1993; Rhys-Williams et al. 1993). Under anaerobic conditions, and in the presence of a suitable electron donor, *C. acidovorans* NBA–10 stoichiometrically converted 4- nitrobenzoate to these two products (Groenewegen & De Bont 1992).

Removal of toxic nitroaromatics from the environment is not the only incentive for studying the metabolism of these compounds. Catechols are useful starting compounds in the agrochemical, pharmaceutical and flavor industries. Chemical synthesis of catechols is notoriously difficult, as they are usually not stable under the reaction conditions employed (Gunstone 1960; March 1985; Olah et al. 1981). Biotechnological production of catechols using microorganisms with oxygenolytic activity (Van den Tweel 1988) suffers from the disadvantage that the required cofactor regeneration makes large-scale production expensive. Furthermore, oxygenolytic conversions have to be carried out in the presence of oxygen, which enhances autooxidation and polymerization of catechols. Anaerobic production of catechols from nitroaromatics may be a promising alternative.

Pseudomonas putida B2 (DSM 6376) was isolated from soil on 2- and 3-nitrophenol as the sole source of carbon and nitrogen (Zeyer & Kearney 1984). Degradation of 2-nitrophenol is initiated by a monooxygenase (Zeyer & Kearney 1984; Zeyer et al. 1986) which catalyses the conversion of 2-nitrophenol to catechol and nitrite with 1,2-benzoquinone as an intermediate (Zeyer & Kocher 1988). During growth on 3-nitrophenol stoichiometric concentrations of ammonia were released into the growth medium. Although the degradation pathway of 3-nitrophenol in *P. putida* B2 is unknown, cells grown on 3-nitrophenol were induced for substrate-dependent oxygen uptake with 3-nitrophenol and 1,2,4-benzenetriol as the substrates and not with 3-aminophenol and resorcinol (Zeyer and Kearney, 1984). This suggests that 3-nitrophenol is degraded via 1,2,4-benzenetriol via a similar pathway as was described for 4-nitrobenzoate degradation in *C. acidovorans* NBA–10. This implies that *P. putida* may be a suitable candidate for production of catechols under anaerobic conditions.

Goal of the study presented here was the characterization of the degradation pathway of 3-nitrophenol in *P. putida* B2 and the investigation of the possibilities for microbial catechol production with this organism.

Materials and methods

Organism and growth conditions

Pseudomonas putida B2 (DSM 6376) was obtained from the Deutsche Sammlung von Mikroorganismen. The organism was pregrown in mineral medium on glucose and was maintained at – 70°C in 1 ml aliquots after the addition of 20% glycerol to the culture.

Mineral medium without nitrogen contained per liter of demineralized water: 2.8 g KCl, 1.55 g K_2HPO_4, 0.85 g $NaH_2PO_4 \cdot H_2O$, 0.13 g K_2SO_4, 0.1 g $MgCl_2 \cdot 6H_2O$ and 1 ml of a trace elements solution, containing per liter demineralized water: 1.5 g $FeCl_2 \cdot 4H_2O$, 0.19 g $CoCl_2 \cdot 6H_2O$, 0.1 g $MnCl_2 \cdot 4H_2O$, 70 mg $ZnCl_2$, 36 mg $Na_2MoO_4 \cdot 2H_2O$, 24 mg $NiCl_2 \cdot 6H_2O$, 6 mg H_3BO_3 and 2 mg $CuCl_2 \cdot 2H_2O$. Mineral medium with nitrogen was as described above but contained 2 $g.l^{-1}$ $(NH_4)_2SO_4$ instead of KCl and K_2SO_4. After the addition of 3-nitrophenol the medium was adjusted to pH 7 and autoclaved at 120°C. Glucose was autoclaved separately at 120°C before addition to the medium.

Batch cultures for screening of carbon substrates contained 25 ml mineral medium with ammonium sulphate. Solid amino- and nitroaromatic compounds were added to a theoretical concentration of 1 mM. Other growth substrates were tested at 10 mM concentrations. The cultures were incubated at 30°C.

Chemostat cultures of *P. putida* B2 were grown in a 1 liter Applikon laboratory fermentor at 30°C, pH 6.5 and a dilution rate of 0.05 h^{-1}. The cultures were sparged with air and stirred at 800 rpm. The medium either contained 3 mM 3-nitrophenol, 3 mM glucose and 0.02% yeast extract or 3 mM glucose and 0.02% yeast extract as the growth-limiting substrates. For growth in the absence of 3-nitrophenol, mineral medium with nitrogen was used.

Measurement of substrate-dependent oxygen consumption

Respiration rates of cell suspensions were assayed polarographically at 30°C in a Biological Oxygen Monitor (Yellow Springs Instruments Inc.). Oxygen uptake measurements were performed with cells from chemostat cultures washed with 50 mM potassium phosphate buffer, pH 7. Substrates were dissolved in ethanol or demineralized water. Ethanol concentrations in the assay mixture did not exceed 0.2%, which neither influenced endogenous nor glucose- and

3-nitrophenol-dependent oxygen uptake rates. Calculations were based on an oxygen concentration of 236 μM in air-saturated water at 30°C. Results have been corrected for endogenous respiration rates and chemical oxidation of substrates.

Incubation of cell suspensions with 3-nitrophenol

For anaerobic experiments, washed cell suspensions from chemostat cultures grown on glucose and 3-nitrophenol were flushed with nitrogen. In some experiments a mixture of 10 mM potassium ferricyanide and 10 mM glucose or ethanol was included in the assay mixture. After the addition of 1 mM 3-nitrophenol, the assay mixtures were incubated in a shaking waterbath at 30°C. Samples (1 ml) were immediately mixed with 20 μl 50 mM $Na_2S_2O_4$ to prevent chemical oxidation of 1,2,4-benzenetriol and then centrifuged. Aerobic incubation of cell suspensions with 3- nitrophenol was performed as described above but in open bottles.

Incubation of hydroxylaminoaromatic compounds with intact cells and cell-free extracts

Solid hydroxylaminoaromatic compounds (theoretical concentration 1 mM) were added to 50 mM potassium phosphate buffer (pH 7), flushed with nitrogen and incubated in a shaking waterbath at 30°C. Washed cells from chemostat cultures grown on glucose and 3-nitrophenol were flushed with nitrogen and added to the assay mixture. Samples (1 ml) were mixed with 1 ml acetonitril to dissolve the substrates and then rapidly centrifuged.

Anaerobic incubations of hydroxylaminoaromatic compounds with cell-free extracts were performed as described above with the exception that the samples were not mixed with 1 ml acetonitril, but with 0.1 ml 6 N HCl to stop the reaction and deproteinize the samples before HPLC analysis. Cell-free extracts of *P. putida* B2 were prepared as described below.

Preparation of cell-free extracts

Cells from chemostat cultures grown on glucose and 3-nitrophenol were washed with 50 mM potassium phosphate buffer, pH 7 and disrupted on melting ice by sonication (5 min; 30% duty cycle; output control, 3) with a Branson Sonifier 250. Cell debris was removed by centrifugation at 48,000 x g for 60 min. The clear supernatant was used as the cell-free extract.

Enzyme assay for 3-nitrophenol reductase

The standard reaction mixture (1 ml) contained 25 mM potassium phosphate (pH 7), 0.1 mM 3-nitrophenol and 50 μl cell-free extract. NADPH (0.2 mM) was added to start the reaction. The decrease of A_{340} was recorded spectrophotometrically at 30°C and calculated 3-nitrophenol reductase activities were corrected for the low NADPH-oxidase activity of cell-free extracts. Specific enzyme activities were expressed in U.(mg protein)$^{-1}$. 1 U is defined as 1 μmol NADPH oxidized.min^{-1}.

Substrate specificity and inhibitors

Substrate specificity of nitroaromatic compound reduction by extracts was studied at pH 7 and 30°C. NADPH (0.2 mM) and NADH (0.2 mM) were tested as electron donors. Nitroaromatic and hydroxylaminoaromatic compounds were tested as electron acceptors at a concentration of 0.1 mM. Electron acceptors were dissolved in ethanol or demineralized water. Ethanol concentrations in the assay mixture were 1%, which inhibited the reductase activity with 3- nitrophenol by 25%. Relative activities with other substrates dissolved in ethanol were corrected for this inhibition.

The effect of potential reductase inhibitors was determined after 2 min preincubation in the assay mixture. Inhibitors tested included the sulfhydryl-binding agents *p*-chloromercurybenzoate (0.01 mM) and N-ethylmaleimide (0.01 and 0.1 mM), the metal-complexing agent EDTA (5 mM) and several metal ions (5 mM). Inhibition experiments were performed with cell-free extract prepared in 50 mM Tris-HCl, pH 7, to prevent precipitation of phosphate salts after the addition of metal ions.

Optimum temperature, pH optimum and kinetic constants

The temperature optimum of 3-nitrophenol reduction was studied at pH 7. The temperature in the cuvette was varied between 5 and 40°C. The pH optimum was determined at 30°C. Potassium acetate (pH 5 to 6), potassium phosphate (pH 6 to 8) and Tris-HCl (pH 8 to 9.5) were used as the assay buffer at a concentration of 25 mM. Kinetic constants were determined at pH 7 and 30°C. Values for K_m and V_{max} were calculated using non-linear regression following Michaelis-Menten kinetics.

Stability of 3-nitrophenol reductase activity

The thermostability of 3-nitrophenol reductase was tested by incubating cell-free extracts at 0 and 30°C. Enzyme activity was assayed at pH 7 and 30°C. The salt concentration in the assay mixture was increased by the addition of concentrated potassium phosphate, ammonium sulphate or potassium chloride solutions. The pH of all solutions added was 7.

Ammonium sulphate precipitation was performed by addition of solid ammonium sulphate to cell-free extract on ice. After precipitation of protein, the extracts were centrifuged for 20 min at 13,000 x g. The pellet was resuspended in 50 mM potassium phosphate buffer, pH 7, and reductase activity was measured in supernatant and resuspended pellet and also after combination of both. Both supernatant and resuspended pellet were desalted with a PD–10 column (Pharmacia) and reductase activity was again measured.

To stabilize enzyme activity the following additions to the extract buffer were tested: the protease inhibitors phenyl methylsulfonyl fluoride (1 mM) and EDTA (5 mM), the enzyme substrates 3-nitrophenol (1 mM), NADPH (1 mM) and $NADP^+$ (1 mM), the electron carriers reduced glutathion (0.1 mM), FMN (0.1 mM) and FAD (0.1 mM), the enzyme-solubilizing agents glycerol (10%), Tween–80 (0.5%) and Triton X–100 (0.5%) and the redox agents dithiothreitol (0.1 mM), cysteine (0.1 mM), sodium sulphite (1 mM) and sodium dithionite (1 mM). Stability of enzyme activity was also tested in 50 mM Tris-HCl, pH 7, as extract buffer.

Analytical procedures

Concentrations of aromatic compounds were measured with a Hewlett-Packard reversed-phase HPLC on a C_{18}- column (200 x 3 mm; Chromspher; Chrompack) using gradient elution in 0.01 N H_2SO_4 and acetonitril with a flow of 0.4 ml.min $^{-1}$. Detection was performed with a photodiode array detector (Hewlett Packard). 1,2,4-Benzenetriol was also analysed on a Chrompack CP 9000 GC and detected with a flame ionization detector. Water samples (0.5 μl) were injected on a CP Sil 8CB column (25 m x 0.32 mm; Chrompack). Temperatures of injector, column and detector were 220, 80 and 250°C, respectively. Split- and column flow were, respectively, 50 and 5.25 ml.min^{-1}. Analysis of catechols was also performed colorimetrically according to Friestad (1969).

Ammonia was analysed colorimetrically according to Weatherburn (1967) or enzymatically with the ammonia kit of Sigma Diagnostics. Protein concentrations were determined according to Bradford (1976).

Chemicals

4-Hydroxylaminophenylacetic acid was synthesized from 4-nitrophenylacetic acid by Dr. J.A. Jongejan (Delft University of Technology, The Netherlands). Other hydroxylaminoaromatic compounds were synthesized by partial chemical reduction of nitroaromatics and were over 95% pure (Syncom, The Netherlands). All other chemicals were of analytical grade and were obtained from commercial sources.

Results

Growth of Pseudomonas putida B2

P. putida B2 was originally isolated on 2- and 3-nitrophenol (Zeyer & Kearney 1984). To investigate whether other amino- and nitroaromatics could be used as carbon sources, growth on a number of these compounds was tested in aerobic batch cultures. Growth was observed on 1 mM concentrations of nitrobenzene, 2- and 4-nitrotoluene, 4-nitroanisole, 4-nitro–1-chlorobenzene, 4-nitrobenzonitrile, the methylesters of 4-nitrobenzoic acid and 4-aminobenzoic acid, aniline, 2- and 4-aminotoluene, 4-aminobenzonitrile and 2-aminobenzoic acid. Furthermore, *P. putida* B2 was able to grow on 10 mM concentrations of glucose, succinate, ethanol, 1-propanol and 1-butanol. Growth was not supported by 2-, 3- and 4-nitrobenzoic acid, 4-nitrophenylacetic acid, 2-aminophenol, 3- and 4-aminobenzoic acid, 4-aminoanisole, 4-amino–1-chlorobenzene, 4-aminophenylacetic acid, 2-propanol, 2-butanol, acetone, formaldehyde or phenol.

P. putida B2 biomass with a low endogenous oxygen uptake rate was routinely obtained from substrate-limited chemostat cultures grown on a mixture of 3 mM glucose, 3 mM 3-nitrophenol and 0.02% yeast extract in a mineral medium without an additional nitrogen source. These cultures contained 150 mg protein.l^{-1}. Glucose-limited chemostat cultures were grown under the same conditions, but without 3-nitrophenol and with ammonium sulphate as the nitrogen source. These cultures yielded 90 mg protein.l^{-1}.

Table 1. Substrate-dependent oxygen uptake by intact cells of *Pseudomonas putida* B2. Cells were grown on glucose or on a mixture of 3-nitrophenol and glucose. Substrate concentrations were 50 μM.

Substrate	Oxygen uptake rate (nmol O_2.(min.mg protein)$^{-1}$) after growth on:	
	glucose + 3-nitrophenol	glucose
3-nitrophenol	179	0
glucose	58	48
1,2,4-benzenetriol	16	0

Conversion of 3-nitrophenol by intact cells of P. putida B2

In order to characterize the degradation pathway of 3-nitrophenol in *P. putida* B2, oxygen uptake experiments and anaerobic incubations with 3-nitrophenol were performed. Cells grown on glucose and 3-nitrophenol showed substrate-dependent oxygen uptake with 3-nitrophenol, glucose and 1,2,4-benzenetriol (Table 1). Glucose-grown cells only revealed oxygen uptake with glucose and not with 3-nitrophenol and 1,2,4-benzenetriol (Table 1). Other possible intermediates in the degradation of 3-nitrophenol were also tested as substrates for oxygen uptake experiments. However, cells grown with or without 3-nitrophenol did not show oxygen uptake with pyrogallol, 3-aminophenol, 3- or 4-nitrocatechol, resorcinol or phenol.

Under anaerobic conditions, *P. putida* B2 cells pregrown on glucose and 3-nitrophenol stoichiometrically converted 1 mM 3-nitrophenol to 1,2,4-benzenetriol (Figure 1). Production of ammonia was also observed, although the end concentration was lower than the initial 3-nitrophenol concentration (Figure 1). Incubation with an initial 3-nitrophenol concentration of 10 mM completely inhibited this conversion (data not shown).

Due to its high oxygen sensitivity, detection of 1,2,4-benzenetriol posed problems. Its presence in anaerobic incubations with 3-nitrophenol was therefore verified by three analytical techniques. Firstly, HPLC analysis with a photodiode array detector yielded identical retention times and peak spectra with 1,2,4-benzenetriol standard solutions and samples of the anaerobic 3-nitrophenol incubations. Secondly, shifts in chromatograms and peak spectra after contact of standard solutions and incubation samples with air were also identical. These shifts in chromatograms and peak spectra are a result of the chemical oxidation of 1,2,4-benzenetriol to hydroxy–1,4-benzoquinone,

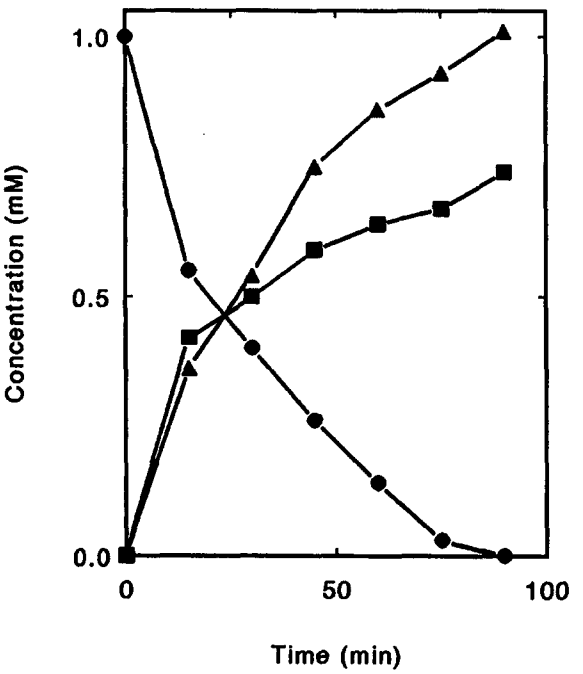

Figure 1. Anaerobic conversion of 3-nitrophenol by *Pseudomonas putida* B2. The protein concentration in the incubation mixture was 7.2 mg.ml^{-1}. ●: 3-nitrophenol; ▲: 1,2,4- benzenetriol; ■: ammonia.

which causes a colour change of the solutions from colourless to purple/red (Jain et al. 1994). This effect was reversible by the addition of sodium dithionite. Thirdly, gas chromatographic analysis of 1,2,4-benzenetriol standard solutions and incubation samples revealed the same retention time for the only peak present.

Initial rates of 3-nitrophenol degradation and 1,2,4-benzenetriol production under anaerobic conditions were both approximately 4 nmol.(min.mg protein)$^{-1}$ (Figure 1). As the first step in the degradation of 3-

nitrophenol in vivo is very likely to be an NAD(P)H-consuming reduction (see below), these low rates may well be due to a depletion of the NAD(P)H-pool in the cell. Under aerobic conditions, the NAD(P)H-yielding mineralization of 1,2,4-benzenetriol solves this problem (aerobic 3-nitrophenol degradation rates were approximately 40 nmol.(min.mg protein)$^{-1}$), but in the absence of oxygen 1,2,4-benzenetriol can not be further degraded (Jain et al. 1994; Rieble et al. 1994). Therefore, it was tested if a method for NAD(P)H-regeneration, previously found successful in the anaerobic production of a hydroxylated aromatic compound by bacterium NTB–1 (Groenewegen et al. 1992b), could also be applied here. Glucose or ethanol were added to the anaerobic incubations, while potassium ferricyanide was supplied as an artificial electron acceptor. However, anaerobic 1,2,4-benzenetriol production rates were not affected by these additions.

Specificity of the 3-nitrophenol-degrading system

The observed formation of 1,2,4-benzenetriol during metabolism of 3-nitrophenol in *P. putida* B2 suggests that a pathway similar to that in *C. acidovorans* NBA–10 is present in this organism. This implies that a hydroxylaminoaromatic is also intermediary produced during 3-nitrophenol metabolism. In order to investigate the specificity of the 3-nitrophenol-degrading enzyme system in *P. putida* B2, oxygen uptake experiments were performed with a set of synthesized hydroxylaminoaromatics (Meulenberg & De Bont 1995) and their corresponding nitroaromatic compounds as a substrate. *P. putida* B2, grown on 3-nitrophenol and glucose, only revealed oxygen uptake with hydroxylaminobenzene, 4-hydroxylaminoanisole and 2- and 4-hydroxylaminotoluene at relatively low rates of 26, 24, 18 and 14%, respectively (the oxygen uptake rate with 3-nitrophenol was 100%). Of the nitroaromatics tested, 4-nitro–1-chlorobenzene, methyl–4-nitrobenzoate, 4-nitrobenzonitrile and 4-nitroanisole showed oxygen uptake rates of 47, 46, 41 and 35%, respectively. The other compounds tested did not support substrate-dependent oxygen uptake.

Theoretically, absence of oxygen uptake might reflect incomplete conversion of the substrates to the corresponding catechols and ammonium by a hydroxylaminolyase. It was therefore tested if conversion of hydroxylaminoaromatics and nitroaromatics resulted in catechol production under aerobic or anaerobic conditions. However, none of the substrates but 3-nitrophenol were converted by intact cells of *P. putida* B2 and no significant production of either catechols or ammonia was found.

Reduction of 3-nitrophenol by cell-free extracts of P. putida B2

Cell-free extracts of *P. putida* B2 were able to reduce 3-nitrophenol with NADPH as an electron donor. Initial reaction rates were linearly proportional to the amount of cell-free extract added. NADH could not be used for the reduction of 3-nitrophenol. During conversion of 3-nitrophenol by cell-free extracts, equimolar amounts of ammonia were produced.

The activity of 3-nitrophenol reductase in cell-free extracts was very unstable. An increase in the ionic strength of the extract buffer with ammonium sulphate, potassium chloride or potassium phosphate, strongly and irreversibly inhibited the enzyme activity. Ammonium sulphate precipitation resulted in complete loss of enzyme activity. Desalting with a PD–10 column (Pharmacia) or ultrafiltration did not restore enzyme activity.

The activity of 3-nitrophenol reductase could not be stabilized by the addition of protease inhibitors (phenyl methylsulfonyl fluoride, EDTA), enzyme substrates (3-nitrophenol, NADPH, $NADP^+$), enzyme-solubilizing agents (glycerol, Tween–80 and Triton X–100), redox potential effectors (dithiothreitol, cysteine, sodium sulphite, sodium dithionite) and electron carriers (FMN, FAD, GSH) to the extract buffer. Also the use of another extract buffer did not result in an increased enzyme stability. For this reason, characterization of 3-nitrophenol reductase was carried out with cell-free extract.

Partial characterization of 3-nitrophenol reductase

The apparent Michaelis Menten constant of 3-nitrophenol reductase for NADPH was 0.17 mM. As a result of the very high affinity of the enzyme for 3-nitrophenol, the K_m for this substrate could not be determined accurately, but was estimated below 2 μM.

The enzyme had a sharp pH optimum at pH 7: only 20% of the optimum activity was found at pH 6 and 9. The temperature optimum of the reductase was found at 25°C, with approximately 30% of the optimum activity present at 5 and 35°C. When cell-free extract was stored at 30°C, the activity was destroyed within one hour, while on melting ice the enzyme was over 100-fold more stable. The stability at 30°C could not be

Table 2. Effects of inhibitors on 3-nitrophenol reductase activity in cell-free extracts of *Pseudomonas putida* B2. Results are given as percentages of enzyme activity without an inhibitor added (100% = 0.12 U.(mg protein)$^{-1}$).

Inhibitor	Concentration (mM)	Relative activity (%)
none	-	100
p-chloromercurybenzoate	0.01	0
N-ethylmaleimide	0.01	46
	0.1	7
EDTA	5	113
$CaCl_2$	5	23
$CoCl_2$	5	11
$MgCl_2$	5	47
$MnCl_2$	5	32
$MoCl_6$	5	57

increased by the protease inhibitors phenyl methylsulfonyl fluoride or EDTA.

Nitrophenol reductase was strongly inhibited by the sulfhydryl-binding agents *p*-chloromercurybenzoate and N-ethylmaleimide (Table 2). The complexing agent EDTA did not affect enzyme activity, but addition of different metal ions strongly inhibited enzyme activity.

The nitrophenol reductase activity in cell-free extract of *P. putida* B2 exhibited a broad substrate specificity. Except for 2-nitrobenzoic acid and 4-nitrocatechol, which were only slowly reduced, all other 15 nitroaromatic compounds tested were reduced at moderate or high rates (Table 3).

Hydroxylaminolyase activity in cell-free extracts of P. putida B2

The expected product of 3-nitrophenol reductase, 3-hydroxylaminophenol, cannot be purchased from commercial sources and, presumably, is unstable in aqueous solutions. Attempts to chemically synthesize this compound were unsuccessful. In an alternative approach to detect hydroxylaminolyase activity, it was tested if a range of available hydroxylaminoaromatic compounds (Meulenberg & De Bont 1995) could be converted by cell-free extracts. Redox-neutral conversion of these compounds to the corresponding catechols and ammonia was tested in anaerobic incubations with cell-free extract. During these incubations, none of the hydroxylaminoaromatics was converted and neither catechols nor ammonia were produced.

Table 3. Substrate specificity of 3-nitrophenol reductase in cell-free extracts of *Pseudomonas putida* B2. Results are given as percentages of enzyme activity with 3-nitrophenol (100% = 0.12 U.(mg protein)$^{-1}$). Substrate concentrations were 100 μM.

Substrate	Relative activity (%)
3-nitrophenol	100
2-nitrophenol	106
4-nitrophenol	33
4-nitrophenylalanine	39
4-nitrocatechol	6
3-nitrostyrene	94
nitrobenzene	122
2-nitrotoluene	31
4-nitrotoluene	131
4-nitroanisole	139
4-nitro–1-chlorobenzene	111
4-nitrobenzonitrile	92
methyl–4-nitrobenzoate	94
2-nitrobenzoic acid	10
3-nitrobenzoic acid	83
4-nitrobenzoic acid	67
4-nitrophenylacetic acid	83

Discussion

Growth of *P. putida* B2 on a mixture of 3-nitrophenol and glucose, induced the enzyme system for substrate-dependent oxygen uptake with 3-nitrophenol and 1,2,4-benzenetriol as substrates (Table 1). The oxygen uptake rates with 1,2,4-benzenetriol, although lower than those observed with 3-nitrophenol, were sufficient to convert all 3-nitrophenol via 1,2,4-benzenetriol in the chemostat. *P. putida* B2 did not reveal oxygen uptake with 3-aminophenol, 3- and 4-nitrocatechol, resorcinol and phenol. This indicates that a number of previously described degradation mechanisms of nitroaromatics, like reduction to aniline derivatives (Boopathy & Kulpa 1993; Rafii et al. 1991), monooxygenation to nitrocatechol or resorcinol (Jain et al. 1994; Spain & Gibson 1991; Zeyer & Kocher 1988) and initial attack by a hydride ion (H^-), yielding phenol (Lenke & Knackmuss 1992; Rieger & Knackmuss 1995), are unlikely to occur in *P. putida* B2 with 3-nitrophenol as the substrate.

During anaerobic incubation of cell suspensions with 3-nitrophenol, 1,2,4-benzenetriol was formed as an end product (Figure 1). We therefore propose that the pathway of 3-nitrophenol degradation in *P. putida* B2 is similar to the degradation of 4-nitrobenzoate in *C.*

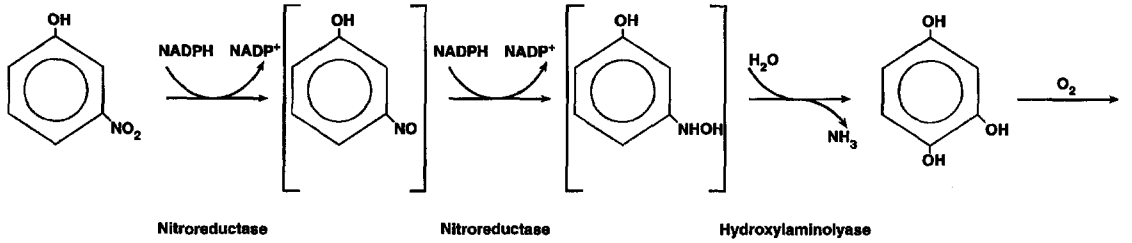

Figure 2. Hypothetical pathway of 3-nitrophenol degradation in *Pseudomonas putida* B2. Intermediates that were not identified are given between brackets.

acidovorans NBA–10 and two *Pseudomonas* species, involving a nitroaromatic reductase and a hydroxylaminolyase (Figure 2) (Groenewegen et al. 1992a; Groenewegen & De Bont 1992; Haigler & Spain 1993; Rhys-Williams et al. 1993). This makes *P. putida* B2 the first example of a hydroxylaminolyase-containing microorganism able to degrade a substrate other than 4-nitrobenzoate via this pathway.

Theoretically, lyase activity on 3-hydroxylaminophenol might also yield pyrogallol. However, the absence of oxygen uptake on this compound and the accumulation of 1,2,4-benzenetriol during anaerobic incubation with 3-nitrophenol argue against pyrogallol as an intermediate. Attempts to confirm the conversion of 3-hydroxylaminophenol to 1,2,4-benzenetriol by work at the level of in vitro enzyme activities have not been successful.

Intermediary production of 1,2,4-benzenetriol during degradation of nitroaromatics has previously been reported by Jain et al. (1994). These authors isolated a strain of *Arthrobacter* capable of degrading 4-nitrophenol via 1,2,4-benzenetriol, catalysed by a nitrite-releasing monooxygenase. Theoretically, dioxygenase-catalysed release of nitrite, as was described for biodegradation of 2,4-dinitrotoluene and for conversion of 3-nitrobenzoic acid by different *Pseudomonas* species (Nadeau & Spain 1995; Spanggord et al. 1991), would also yield 1,2,4-benzenetriol from 3-nitrophenol. However, as production of 1,2,4-benzenetriol in *P. putida* B2 was observed in the absence of oxygen and no nitrite but ammonia was produced, these enzyme activities can not explain the production of 1,2,4-benzenetriol in this organism.

Intact cells of *P. putida* B2 were unable to convert hydroxylaminoaromatic compounds to ammonia and the corresponding catechols. Likewise, cell-free extracts of *P. putida* B2 were unable to produce catechols from the hydroxylaminoaromatic compounds tested. We have made similar observations for other organisms degrading nitroaromatics via a hydroxylaminolyase. *Pseudomonas* 4NT (Haigler & Spain 1993) and other hydroxylaminolyase-containing microorganisms isolated on 4-nitrobenzoate (Meulenberg & De Bont 1995), were not able to convert any of the hydroxylaminoaromatic compounds tested. Apparently, the substrate specificity of the hydroxylaminolyases involved is extremely narrow. The conclusion must therefore be that, so far, anaerobic production of catechols has been found with two nitroaromatic compounds, 3-nitrophenol and 4-nitrobenzoate. Future research must yield the evidence if there are more opportunities for the anaerobic microbial production of catechols from nitroaromatics.

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