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Catabolism of 3-Nitrophenol by *Ralstonia eutropha* JMP 134

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***Ralstonia eutropha* JMP 134 utilizes 3-nitrophenol as the sole source of nitrogen, carbon, and energy. The entire catabolic pathway of 3-nitrophenol is chromosomally encoded. An initial NADPH-dependent reduction of 3-nitrophenol was found in cell extracts of strain JMP 134. By use of a partially purified 3-nitrophenol nitroreductase from 3-nitrophenol-grown cells, 3-hydroxylaminophenol was identified as the initial reduction product. Resting cells of *R. eutropha* JMP 134 metabolized 3-nitrophenol to *N*-acetylaminohydroquinone under anaerobic conditions. With cell extracts, 3-hydroxylaminophenol was converted into aminohydroquinone. This enzyme-mediated transformation corresponds to the acid-catalyzed Bamberger rearrangement. Enzymatic conversion of the analogous hydroxylaminobenzene yields a mixture of 2- and 4-aminophenol.**

In general, the presence of nitroaromatic compounds in nature is a major consequence of anthropogenic activities. Mononitroarenes such as nitrophenols are used in the manufacture of pesticides, pharmaceuticals, and dyes. 4-Nitrophenol occurs in soil as a breakdown product of widely used insecticides such as parathion or methyl parathion (27, 36).

Whereas the degradative pathways of 2-nitrophenol and 4-nitrophenol are well described, detailed information on the metabolism of 3-nitrophenol (3-NP) is lacking. *Pseudomonas putida* B2 was shown to convert 2-nitrophenol to nitrite and catechol by means of a nitrophenol monooxygenase (45). Catechol was further attacked by a catechol 1,2-dioxygenase and channeled into the *ortho*-cleavage pathway. In the case of 4-nitrophenol, a *Moraxella* sp. was described which is able to utilize 4-nitrophenol as its sole source of carbon (40). It was converted to hydroquinone with concomitant liberation of nitrite. *p*-Benzoquinone was proposed as the initial reaction product, which was subsequently reduced to hydroquinone. Hydroquinone is then degraded via γ-hydroxymuconic semialdehyde, maleylacetate, and β-ketoadipate (41). Recently, an alternative pathway for the degradation of 4-nitrophenol by an *Arthrobacter* sp. has been described (21). This catabolism is initiated by hydroxylation of the aromatic ring generating 4-nitrocatechol, which is further converted by a monooxygenase-catalyzed removal of the nitro group leading to 1,2,4-trihydroxybenzene (1,2,4-benzenetriol).

Although the liberation of ammonium instead of nitrite was observed during conversion of 3-NP, 3-aminophenol could not be identified as an intermediate (14, 44). Additionally, a 4-nitrophenol-degrading bacterium was shown to oxidize 3-NP to nitrohydroquinone as a dead-end metabolite when the cells were induced for 4-nitrophenol metabolism (31). In order to elucidate the degradative pathway of 3-NP, its metabolism was studied in more detail with *Ralstonia eutropha* (*Alcaligenes eutrophus*) JMP 134 (30), which can use 3-NP as its sole source of nitrogen, carbon, and energy (7).

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *R. eutropha* JMP 134, originally isolated as a 2,4-dichlorophenoxyacetate (2,4-D) degrader (30), was maintained on solid mineral media (11) with 2 mM 2,4-D. For the cultivation of the plasmid-free derivative strains JMP 222 and JMP 289 (10), nutrient broth agar plates with 100 μg of streptomycin per ml (JMP 222) and 25 μg of rifampin per ml (JMP 289) were used. For experiments with *R. eutropha* JMP 134, JMP 222, or JMP 289 in batch cultures, nitrogen-free mineral medium (24) containing 0.5 mM 3-NP and 10 mM succinate was used. Uninduced cells of *R. eutropha* JMP 134 were grown on ammonia (1 mM) and succinate (10 mM). For growth experiments with nitrobenzene as the sole source of nitrogen, succinate (10 mM) was used as the carbon and energy source, and nitrobenzene was supplied through the gas phase. For this purpose, the wide-bore cap of the Erlenmeyer flask was equipped with an insert vial (Ochs, Bovenden, Germany) that allowed nitrobenzene to evaporate into the gas phase of the flask. The incubation temperature was 30°C. Batch cultures in fluted Erlenmeyer flasks were incubated on a rotary shaker at 100 rpm. Solid media were prepared by the addition of 1.5% agar (Oxoid no. 1; London, United Kingdom).

In order to confirm 3-NP as the sole source of nitrogen, carbon, and energy, fluted Erlenmeyer flasks containing mineral medium (24) with or without 0.5 mM 3-NP were incubated at 30°C on a water bath shaker at 100 rpm. Growth was monitored spectrophotometrically by measurement of the turbidity at 546 nm. For the determination of the protein content of growing cells, 5 ml of the suspension had to be concentrated by centrifugation, and then the cells were resuspended to a final volume of 0.95 ml. Afterwards, the cells were disrupted by treatment with 0.05 ml of 10 M NaOH at 90°C for 10 min. Further treatment of the samples was performed by the method of Hartree (19).

Conjugation experiments. To transfer the plasmid pJP4 from strain JMP 134 into strain JMP 222, two different methods were used. During filter mating experiments, 0.1 ml of donor and 0.9 ml of recipient cultures, which were grown overnight at 30°C in 5 ml of nutrient broth, were mixed and incubated for 6 h at 30°C on a sterile nitrocellulose filter (1 cm^2), which was laid on a nutrient broth agar plate. After growth overnight, the cells were resuspended in 0.2 ml of 0.85% NaCl solution before plating of 0.1 ml onto agar plates (undiluted or diluted to 10^{-1} and 10^{-2}) containing 2 mM 2,4-D and 100 μg of streptomycin per ml as selection medium. As a control, the same procedure was done separately with unmixed donor and recipient cells. Additionally, they were diluted appropriately for plating onto nutrient broth agar plates to give cell counts. During liquid mating experiments, the conjugation mixture of donor and recipient cells was incubated in 5 ml of fresh nutrient broth with slow shaking (50 rpm). The culture was then centrifuged, washed, and resuspended in 0.2 ml of 0.85% NaCl solution before being plated onto the selection medium. Agar plates were incubated at 30°C for at least 3 days. Transconjugant strains were purified by subcultivation of single colonies on selection plates.

Experiments with resting cells. Cells of *R. eutropha* JMP 134 were grown with 0.5 mM 3-NP and 10 mM succinate. Fully induced 3-NP-grown cells were obtained by addition of 0.25 mM 3-NP 2 h before the cells were harvested by centrifugation. The cells were resuspended in phosphate buffer (50 mM [pH 7.4]) and incubated with 3-NP or nitrobenzene (0.5 mM) in a water bath shaker at 30°C. For resting cell experiments under anaerobic conditions, air was replaced by argon as described for the anaerobic experiments with cell extracts. The conversion of the substrates was monitored by high-performance liquid chromatography (HPLC) as described below.

Preparation of cell extracts. 3-NP-induced cells (see above) were resuspended in 50 mM phosphate buffer (pH 7.4) and disrupted by a French press (Amicon,

Silver Spring, Md.). Cell debris and membrane-bound proteins were removed by centrifugation at 100,000 × *g* for 30 min at 4°C (Beckman L8-70 ultracentrifuge; Beckman Instruments, Inc., Irvine, Calif.). Cell extracts were stored under an argon atmosphere on ice until used. The protein content of the cell extracts was determined by the method of Bradford (6).

Partial purification of a nitroreductase. The nitroreductase active on 3-NP was enriched at 4°C by use of a fast-performance liquid chromatography system (Pharmacia, Uppsala, Sweden). A cell extract (5.2 mg of protein [1.15 U]) prepared from *R. eutropha* JMP 134 was applied to a Sephacryl (dextran-bisacrylamide) column (S-200; bed volume, 190 ml; diameter, 16 mm; Pharmacia). The protein with the 3-NP-reducing activity was eluted between 104 and 118 ml of phosphate buffer (50 mM [pH 7.5]) at a flow rate of 1 ml/min. Fractions (2 ml each) were collected, and the activities of the 3-NP nitroreductase were determined as described above. Fractions containing the enzyme were pooled and applied to a Mono-Q column (HR 5/5; Pharmacia). The 3-NP nitroreductase was eluted after 14 ml of a 50-ml total gradient volume of 0 to 1,000 mM NaCl in phosphate buffer (50 mM [pH 7.5]) at a flow rate of 0.5 ml/min. Fractions (1 ml each) were collected and assayed for enzyme activity. The fraction with the highest activity (1 ml) was used for further experiments.

Enzyme assays. The activity of the 3-NP nitroreductase was measured spectrophotometrically by monitoring the decrease at 340 nm due to the oxidation of NADPH or NADH. After determination of the amount of 3-NP and NADPH which allows the highest initial reaction rate of the enzyme, the reaction mixtures that were used contained 0.2 μmol of the substrate, 0.2 μmol of NAD(P)H, 45.5 μmol of phosphate buffer (pH 7.4), and cell extract (50 to 200 μg of protein) in a final volume of 1 ml. Reactions were initiated by the addition of the substrate. The measurements under anaerobic conditions were conducted in 1-ml rubber-stoppered cuvettes flushed with argon. Specific activities of the 3-NP nitroreductase were calculated with a molar extinction coefficient (ε_{NADPH}) of 6,220 M^{-1} cm^{-1}. One unit is defined as a decrease of 1 μmol of NAD(P)H per min.

In order to analyze the initial enzymatic reaction of 3-NP, the partially purified 3-NP nitroreductase (0.2 ml, protein concentration of 0.02 mg/ml) or cell extracts of 3-NP-grown cells (0.2 ml, protein concentration of 0.28 mg/ml) were diluted with phosphate buffer (50 mM [pH 7.4]) to a final volume of 3.2 ml. The solutions were pipetted into serum bottles closed with gas-tight rubber septa. To reach anaerobic conditions, the solutions were evacuated and flushed with argon for at least three times before an overpressure of 0.5 bar was set with argon. Stock solutions of 3-NP and NADPH were flushed with argon before they were passed through the septa of the bottles by use of disposable syringes. After NADPH (final concentration, 0.75 mM) was added, the reaction was started with 3-NP (final concentration, 0.38 mM). The reaction mixture was incubated in a water bath shaker at 30°C. Samples drawn with syringes were directly analyzed by HPLC.

To measure the activity which converts hydroxylamino aromatics, cell extracts of 3-NP- or ammonia-grown cells of *R. eutropha* JMP 134 (final protein concentration, 0.07 mg/ml for the conversion of 3-hydroxylaminophenol and 0.38 mg/ml for the conversion of hydroxylaminobenzene) were diluted with phosphate buffer (50 mM [pH 7]). Extracts were placed in serum bottles and closed with gas-tight rubber septa. Stock solutions of 3-hydroxylaminophenol or hydroxylaminobenzene were gassed with argon prior to injection into the bottles. After starting of the reaction with 3-hydroxylaminophenol (initial concentration, about 0.8 mM, calculated from the amount of the chemical synthesized) or hydroxylaminobenzene (initial concentration, 0.4 mM), the reaction mixtures were incubated in a water bath shaker at 30°C. Samples were removed with syringes and directly analyzed by HPLC as described below.

Analytical methods. Ammonium ion concentration was determined by ion chromatography (IC; Dionex, Idstein, Germany) with a cation-exchange column (Ionpac CS10, 4 by 250 mm, with precolumn CG10; Dionex) using a conductivity detector with suppression technique. The flow rate of 0.03 M HCl as the mobile phase was 1 ml/min. The nitrite ion concentration was estimated by the photometric method of Griess-Ilosvay as modified by Shinn (26). The quantitative analysis of 3-NP, nitrobenzene, and metabolites was performed by ion-pair HPLC (Waters, Milford, Conn.) with a reversed-phase column (Lichrospher 100 RP8, 4.6 by 125 mm; Merck, Darmstadt, Germany). The liquid phase consisted of methanol containing hexane sulfonate (Pic B6; Waters) as solvent A and H_2O containing hexane sulfonate as solvent B. The conversion of 3-NP and nitrobenzene was measured with the following gradient system. The program started with 10% solvent A and 90% solvent B, changed continuously within 3.5 min to 40% solvent A and 60% solvent B, and remained at this composition for another 4.5 min. The ratio was then set back to 10% solvent A and 90% solvent B, remaining at this composition for at least 7 min to equilibrate the column for the next run. To quantify the enzymatic conversion of hydroxylaminobenzene, the mobile phase was 15% solvent A and 85% solvent B. To analyze the enzymatic turnover of 3-hydroxylaminophenol, the mobile phase was 10% solvent A and 90% solvent B. The flow rate of all mobile phases was 1 ml/min, and the detection was carried out at 210 nm. An HPLC system (Sykam, Gilching, Germany) equipped with a diode array detector (Philipps) was used with the mobile phase and an HPLC column as described above. Alternatively, 3-NP concentrations were measured spectrophotometrically (Beckman DU 20 spectrophotometer) at a wavelength of 392 nm after 0.02 ml of 10 M NaOH had been added to 1 ml of each sample (pH >12).

Isolation and identification of *N*-acetylaminohydroquinone. 3-NP-induced cells of *R. eutropha* JMP 134 were used to accumulate *N*-acetylaminohydroquinone. The cells were resuspended to a final volume of 50 ml in phosphate buffer (50 mM [pH 7.4]), and the cell suspension (optical density at 546 nm of 10) was poured into a serum bottle. After the air had been replaced by argon as described above, the anaerobic cell suspension was incubated with 0.5 mM portions of 3-NP in a water bath shaker at 30°C. Disappearance of 3-NP and formation of the metabolite were monitored by HPLC analysis. In order to obtain larger amounts of the metabolite, additional portions of 3-NP (0.4 mM) were added after 5 h and 24 h. After complete conversion of 3-NP (9.0 mg), the cells were removed by centrifugation. The cell-free culture fluid was acidified with HCl and extracted three times with equal volumes of ethyl acetate. The ethyl acetate extracts were combined, dried over $MgSO_4$, and evaporated to dryness. The colorless precipitate (9.7 mg) was used for nuclear magnetic resonance (NMR) and mass spectroscopic analyses. The ^{1}H-NMR spectrum was recorded on a Bruker AC 250 spectrometer (Rheinstetten, Germany; ^{1}H nominal frequency, 250.134 MHz) in d_6-dimethyl sulfoxide (DMSO) with tetramethylsilane as an internal standard. Capillary column gas chromatography-mass spectrum (GC/MS) analyses were performed on a Finnigan 4500 gas chromatograph (Finnigan, San Jose, Calif.) equipped with a fused silica column with both an electron-impact ionization (EI; 70 eV) and a chemical positive ionization detector (reagent gas methane; CI-CH_4).

Chemicals. Hydroxylaminobenzene was kindly supplied by Jim C. Spain, Armstrong Laboratory, Tyndall AFB, Fla. 3-Hydroxylaminophenol was synthesized from 3-NP prepared by reduction of 3-NP with zinc under neutral conditions by a modified procedure (3). Because of the sensitivity of the product to oxygen, all reaction steps were carried out under argon gas: 0.1 g of 3-NP and 0.12 g of NH_4Cl were dissolved in 20 ml of H_2O in a serum bottle, and the pH was adjusted to 6.8 with NaOH (2 M). Zn powder (0.1 g) was added, and the reaction mixture was evacuated and flushed with argon gas. The bottle was heated to 50°C in a water bath until the yellow color of 3-NP had disappeared (about 15 min). The reaction mixture was then immediately cooled with ice to prevent the formation of 3-aminophenol. Residual Zn powder was removed by filtration, and the aqueous solution was extracted three times with 20 ml each of oxygen-free ethyl acetate. The organic phases were combined and dried with Na_2SO_4, and the solvent was stripped off under an argon atmosphere. The product was characterized by spectroscopic methods. A ^{1}H-NMR spectrum was recorded on a Bruker AC 250 spectrometer (see above) in d_6-DMSO with tetramethylsilane as an internal standard. An MS was recorded on an MS 9/50 mass spectrometer (Kratos, Ramsey, N.J.) with EI at an electron energy of 70 eV.

Aminohydroquinone was prepared by the method of Kricheldorf and Thomson (22). 2,5-Dimethoxyaniline was dissolved in a mixture of aqueous HBr (40% [wt/wt]) and acetic acid and refluxed for several days. The reaction was monitored by HPLC. After complete conversion of 2,5-dimethoxyaniline, the acidic solution was reduced in volume under vacuum, and residual acid was removed by azeotropic evaporation with toluene. The resulting dark brown powder containing aminohydroquinone was stored at 4°C. Further purification of aminohydroquinone failed because of its instability in the presence of O_2. Solutions of the dark powder were used as a standard for aminohydroquinone.

RESULTS

Growth of *R. eutropha* JMP 134 and its derivatives on 3-NP. Bruhn et al. (7) reported that the 2,4-D-degrading *R. eutropha* JMP 134 can also utilize 3-NP as its sole source of carbon, energy, and nitrogen. In order to confirm this result, JMP 134 was inoculated into a mineral medium with 3-NP as its sole source of nitrogen, carbon, and energy. Because of its toxicity, the substrate could only be added in portions, so its concentration never exceeded 0.5 mM. With higher concentrations of 3-NP, the medium turned brown and growth ceased. As shown in Fig. 1, the consumption of 3-NP correlated with an increase in cell density as well as in protein content, whereas no growth was observed in the control. Ammonium was the only metabolite found in the medium.

R. eutropha JMP 222, a streptomycin-resistant mutant that was cured of the 2,4-D-degrading plasmid pJP4 (10), was unable to metabolize 3-NP. Supposing that at least the initial step of the catabolism of 3-NP was encoded on pJP4, a conjugation experiment with strain JMP 134 as the donor and strain JMP 222 as the recipient was carried out, and transconjugants were isolated by selection on streptomycin–2,4-D–agar plates. Although the plasmid pJP4 was transferred successfully (transfer rate, 4.7×10^{-2}), the transconjugant strain—designated as JMP-S3—was unable to convert 3-NP. Another authentic plasmid-free derivative of *R. eutropha* JMP 134, JMP 289 (10),

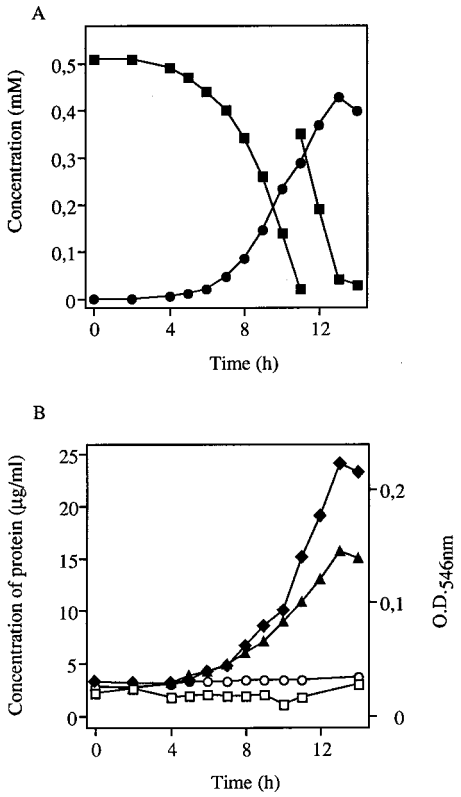


FIG. 1. Growth of *R. eutropha* JMP 134 with 3-NP as its sole source of nitrogen, carbon, and energy. 3-NP was added in portions so that the concentration never exceeded 0.5 mM in the culture fluid. The culture was incubated in a 500-ml fluted Erlenmeyer flask at 30°C on a rotary shaker. Disappearance of 3-NP (■) was measured spectrophotometrically, and formation of ammonia (●) was monitored by HPLC (A). Increase in cell density (▲ [control, ○]) was determined spectrophotometrically at 546 nm and as protein content of the culture fluid (◆ [control, □]), measured as biomass protein by a modification of the method of Hartree (19) (B). $O.D._{546\ nm}$, optical density at 546 nm.

however, utilized 3-NP as its sole nitrogen, carbon, and energy source. Therefore, the degradative pathway for 3-NP must be chromosomally encoded, and strain JMP 222 must be deficient in this sequence.

Characterization of the 3-NP nitroreductase. During growth of *R. eutropha* JMP 134 on 3-NP, ammonia was released into the medium, indicating a reduction of the nitro group. Cell extracts of both induced and uninduced cells of strain JMP 134 were tested for NADH- and NADPH-oxidizing activities with 3-NP as an electron acceptor. NADPH was shown to be the cosubstrate of a 3-NP nitroreductase (92 U/g of protein), and only 7% relative activity was found with NADH (6 U/g of protein). Ninety percent (83 U/g of protein) of the NADPH-oxidizing activity was also observed under anaerobic conditions, excluding the participation of oxygenase activities in the initial transformation of 3-NP. No measurable activity was detected in cell extracts of uninduced cells, demonstrating that 3-NP was not reduced in a nonspecific or nonenzymatic reaction.

Identification of 3-hydroxylaminophenol as a metabolite of 3-NP. In order to analyze the initial enzymatic reaction in the degradative pathway of 3-NP, the 3-NP nitroreductase was partially purified by a gel filtration chromatography step followed by an ion-exchange chromatography step. The enzyme fraction with the highest activity was incubated with 3-NP in the presence of NADPH. To prevent autoxidation of the reduction products of 3-NP, the experiment was carried out under anaerobic conditions. During conversion of 3-NP, only one oxygen-sensitive metabolite was formed in significant amounts as shown by HPLC analysis. The chromatographic properties and the UV-visible spectrum of the metabolite were clearly different from those of authentic 3-aminophenol.

The instability of the metabolite towards oxygen indicated that the enzyme presumably released a compound with a partially reduced nitro group as it exists in 3-hydroxylaminophenol. To confirm 3-hydroxylaminophenol as a metabolite, it was synthesized chemically. The dark oily material obtained by the chemical reduction of 3-NP contained only one major product, as shown by HPLC analysis. All attempts to purify the product by recrystallization failed. This corresponds to the observations by Entwistle et al. (13). The compound was identified by spectroscopic methods. The following ^{1}H-NMR resonances were observed: δ 6.15 ppm (d, 8.1 Hz, 4-H), δ 6.25 ppm (d, 8.1 Hz, 6-H), δ 6.32 ppm (broad singlet, 2-H) δ 6.90 ppm (t, 5-H). The low-field proton (5-H) is split into a triplet by two *ortho* couplings. No *meta* couplings appeared resolved in the DMSO spectrum. The EI-MS gave a small molecular ion peak (*m/z* 125) and a fragmentation ion (*m/z* 93, M^+·-NHOH). According to the chromatographic properties and the UV-visible spectra, the biologically obtained metabolite of 3-NP and the chemically synthesized compound proved to be identical.

During conversion of 3-NP by cell extracts of 3-NP-grown cells of *R. eutropha* JMP 134 in the presence of NADPH, only trace amounts of 3-hydroxylaminophenol were accumulated. In contrast to the experiment with the partially purified 3-NP nitroreductase, a different metabolite was observed, indicating further transformation of 3-hydroxylaminophenol. The same metabolite was observed when 3-hydroxylaminophenol was incubated with a cell extract of 3-NP-grown cells of *R. eutropha* JMP 134 under anaerobic conditions. No such turnover was catalyzed by a cell extract of ammonia-grown cells. By comparison with an authentic standard, 3-aminophenol was definitely excluded as a metabolite of 3-hydroxylaminophenol. The metabolite of 3-hydroxylaminophenol proved to be extremely unstable towards oxygen ($t_{1/2}$ = 5.7 min at pH 7.2 and room temperature), as shown by a red coloration which instantaneously appeared directly after exposure to air. Therefore, this metabolite could only be handled under anaerobic conditions.

Metabolism of nitrobenzene by *R. eutropha* JMP 134. In order to provoke the accumulation of metabolites that are indicative for the catabolic pathway of 3-NP, nitrobenzene as a structural analog was metabolized by 3-NP-grown cells of *R. eutropha* JMP 134. Noticeably, nitrobenzene did not serve as a growth substrate for *R. eutropha* JMP 134, but rapid development of a yellow color in the culture medium with nitrobenzene and succinate indicated its transformation. Only extracts grown with 3-NP plus succinate exhibited 3-NP nitroreductase activity against both 3-NP and nitrobenzene. Uninduced cells were inactive with both nitroaromatic compounds. The NADPH-dependent activity against nitrobenzene (86 U/g of protein) was comparable to that of 3-NP (92 U/g of protein). Resting cells as well as cell extracts (in the presence of NADPH) of *R. eutropha* JMP 134 pregrown with 3-NP and succinate readily converted nitrobenzene under anaerobic conditions into a mixture of hydroxylaminobenzene, 2-aminophenol, and 4-aminophenol. These products were identified by comparison of their chromatographic properties and UV-visible spectra with authentic compounds. The aminophenols were dead-end metabolites under both anaerobic and aerobic conditions.

Under anaerobic conditions, cell extracts of 3-NP-grown cells of *R. eutropha* JMP 134 converted hydroxylaminobenzene

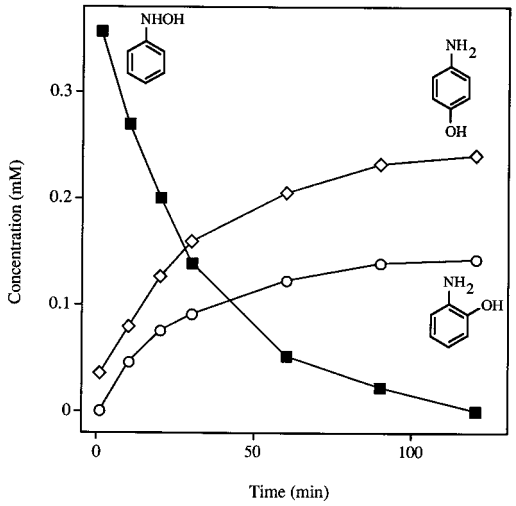


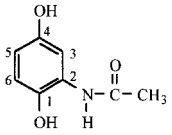
FIG. 2. Conversion of hydroxylaminobenzene by a cell extract of *R. eutropha* JMP 134. Hydroxylaminobenzene (0.4 mM) and the cell extract (soluble fraction, 0.38 mg of protein/ml) were incubated in phosphate buffer (50 mM [pH 7]) under an argon atmosphere at 30°C. The decrease in hydroxylaminobenzene and the formation of 2-aminophenol and 4-aminophenol were determined by HPLC.

(0.39 mM) to a mixture of 2-aminophenol (0.14 mM) and 4-aminophenol (0.24 mM) (Fig. 2). This enzymatic reaction required neither cofactors nor oxygen. In contrast, extracts from cells grown with ammonia and succinate did not catalyze this reaction.

Conversion of 3-NP by resting cells of *R. eutropha* JMP 134. Under aerobic conditions, 3-NP was shown to be metabolized with concomitant elimination of ammonia by 3-NP-grown resting cells of *R. eutropha* JMP 134. No other metabolites were detected by HPLC analysis. In contrast, under anaerobic conditions, the accumulation of an additional metabolite which proved to be more stable than the metabolite of 3-hydroxylaminophenol formed by a cell extract of strain JMP 134 was observed. Although the transformation rate of 3-NP under anaerobic conditions was about 20-fold slower than under aerobic conditions, the fact that 3-NP was initially attacked by a reductase indicated that the identity of the metabolite would help to elucidate the degradative pathway of 3-NP.

For further identification, the metabolite was isolated by extraction of the culture fluid with ethyl acetate after 3-NP had been completely consumed under anaerobic conditions. The isolated product was less sensitive to oxygen than that of the metabolite of 3-hydroxylaminophenol formed by a cell extract of strain JMP 134. The ^{1}H-NMR spectrum displayed a singlet at 2.13 ppm which is typical for an acetyl group (20). Three multiplets in the aryl proton region, integrating for one H each, showed another substituent having been introduced into the aryl ring. The coupling pattern clearly proved the 1,2,4 substitution of the aromatic ring (Table 1). Since the three aryl H resonances appeared at $\delta < 7.0$ ppm, the three substituents must all have positive mesomerism. From these data, an *N*-acetylaminohydroquinone structure was derived (Table 1). The NMR data were comparable to those of Gould et al. (16). GC/MS analysis of the metabolite definitely proves the proposed structure (Table 2). Elemental composition was established by the high-resolution data shown in Table 2. EI- and CI-MS data clearly demonstrated that an acetyl group has been introduced into the molecule, presumably at the end of the metabolic transformation.

TABLE 1. ^{1}H-NMR data of *N*-acetylaminohydroquinone (in d_6-DMSO)

	Chemical shifts δ [ppm] (relative intensity)	Splitting of signals	Coupling constants [Hz]
3-H	6.79 (1H)	d	$4J$ (3-H, 5-H) = 2.9
5-H	6.52 (1H)	dd	$3J$ (5-H, 6-H) = 8.7
			$4J$ (5-H, 3-H) = 2.9
6-H	6.73 (1H)	d	$3J$ (6-H, 5-H) = 8.7
$COCH_3$	2.13 (3H)	s	

Aminohydroquinone as a metabolite of 3-NP. Under anaerobic conditions, 3-NP, as well as 3-hydroxylaminophenol, was converted by cell extracts to a single metabolite. Compared with *N*-acetylaminohydroquinone (shoulder at 241 nm and λ_{max} at 298 nm), this metabolite showed the same retention volume (3.5 ml) but differed in its UV-visible absorption maxima (λ_{max} 218 and 291 nm) when analyzed by ion-pair HPLC. Whereas 3-NP is reduced by an NADPH-oxidizing enzyme, the further enzymatic transformation of 3-hydroxylaminophenol does not require any cofactors. Because the structural analog of 3-hydroxylaminophenol hydroxylaminobenzene was shown to be converted by an enzyme-mediated Bamberger rearrangement leading to a mixture of 2-aminophenol and 4-aminophenol, it was assumed that 3-hydroxylaminophenol would be rearranged to 3-aminocatechol, 4-aminocatechol, or aminohydroquinone. Because *N*-acetylaminohydroquinone was identified as a metabolite of 3-NP conversion by resting cells under anaerobic conditions, it was assumed that aminohydroquinone was the actual product formed from 3-NP reduction by cell extracts. Because of its extreme instability in the presence of oxygen ($t_{1/2}$ = 5.7 min [see above]), it could only be identified by comparison with a synthetic sample of aminohydroquinone (22). The chromatographic properties and absorption spectra of the synthetic product (retention volume of 3.5 ml, λ_{max} of 218 and 291 nm) and the metabolite in the reaction mixture were identical. Additionally, the absorption maxima corre-

TABLE 2. MS analysis of *N*-acetylaminohydroquinone

MS technique	*m/z* exptl value	*m/z*	*m/z* theoretical value	Fragment ion or elemental composition
EI (70 eV)		167		$M^{+\cdot}$
		149		$M^{+\cdot}$-H_2O
		167		$M^{+\cdot}$-CH_2=C=O
		124		M-CH_3CO^+
CI-CH_4[a]		168		MH^+
		167		$M^{+\cdot}$
		150		MH^+-H_2O
		149		$M^{+\cdot}$-H_2O
		126		MH^+-CH_2=C=O
		125		$M^{+\cdot}$-CH_2=C=O
High-resolution EI				
	167.0580		167.061	$C_8H_9NO_3$
	149.0475		149.051	$C_8H_7NO_2$
	125.0475		125.048	$C_6H_7NO_2$
	124.0397		124.040	$C_6H_6NO_2$

[a] With this extremely electron-rich chemical, a high percentage of charge exchange was observed in the CI mode.

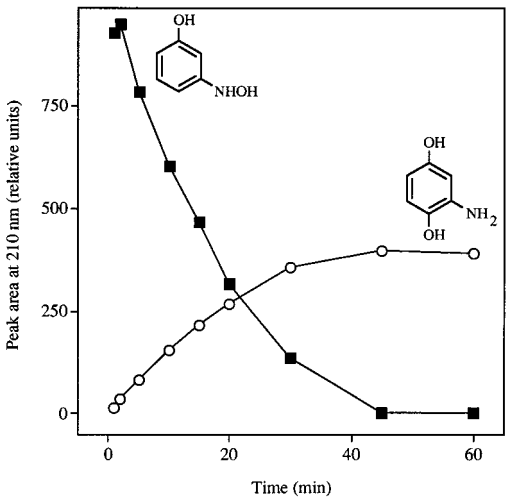


FIG. 3. Turnover of 3-hydroxylaminophenol by a cell extract of *R. eutropha* JMP 134 under anaerobic conditions. Soluble enzyme extract (0.07 mg of protein and chemically synthesized 3-hydroxylaminophenol (approximately 0.8 mM) were incubated in phosphate buffer (50 mM [pH 7]) under an argon atmosphere at 30°C. 3-Hydroxylaminophenol and aminohydroquinone were analyzed by HPLC.

sponded to the data λ_{max} of 221 and 290 nm described by Sunkel and Staude (42). Figure 3 shows the enzymatic conversion of 3-hydroxylaminophenol into aminohydroquinone. Because of the instability of both compounds, the enzymatic conversion of 3-hydroxylaminophenol into aminohydroquinone could only be performed under anaerobic conditions and could not be quantified because pure and authentic standards could not be obtained.

DISCUSSION

The 2,4-D-degrading *R. eutropha* strain JMP 134 was shown to utilize 3-NP as the sole source of nitrogen, carbon, and energy if nontoxic amounts of 3-NP were supplied. Higher concentrations of 3-NP (>0.5 mM) inhibited growth, and a brown coloration of the culture medium occurred, indicating accumulation and polymerization of O_2-sensitive metabolites. Zeyer and Kearney (44) likewise observed that cultures of *P. putida* B2 grew poorly with 3-NP (1 mM) and turned increasingly dark brown at a pH of 7 to 8. Better growth of this strain was observed at lower pH values.

The fact that the pJP4-containing transconjugant of JMP 222 (JMP-S3) did not metabolize 3-NP and that the plasmid-free derivative *R. eutropha* JMP 289 (10) harbors the ability to degrade 3-NP demonstrated that the catabolic pathway for the degradation of 3-NP is entirely chromosomally encoded and that the plasmid pJP4 does not function in the catabolic route. This result was confirmed by observation of 3-NP degradation with another transconjugant from which plasmid pJP4 was excluded (34). Therefore, the fact that *R. eutropha* JMP 222 did not transform 3-NP could only be explained as the result of one or several mutations in the regulatory genes or in the gene involved in the initial 3-NP catabolism.

Whereas 2-nitrophenol and 4-nitrophenol are initially attacked by monooxygenases followed by nitrite elimination (40, 45), the accumulation of ammonia into the medium of cultures of *R. eutropha* JMP 134 growing on 3-NP suggested an initial reductive attack of the nitro group. Similar observations were made with the 3-NP-degrading strain *P. putida* B2 (44). Interestingly, this strain metabolizes 3-NP along an initial reductive

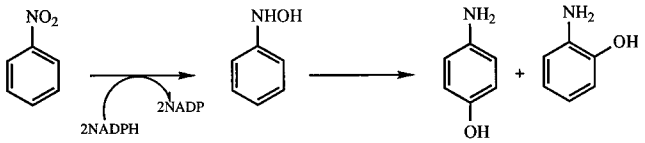


FIG. 4. Conversion of nitrobenzene into 2-aminophenol or 4-aminophenol.

sequence with 1,2,4-benzenetriol as an intermediate (25), whereas it denitrates 2-nitrophenol through an oxygenolytic mechanism.

Initial reduction of the nitro group of 3-NP by *R. eutropha* JMP 134 was indicated by the fact that 3-NP-induced cells exhibited high specific activities against 3-NP under both aerobic and anaerobic conditions. As observed for the nitrobenzene nitroreductase in *Pseudomonas pseudoalcaligenes* JS45 (28, 37), NADPH was clearly a better electron donor than NADH for the reduction of the nitro group of 3-NP.

3-Hydroxylaminophenol was definitely identified as the initial metabolite during conversion of 3-NP by the partially purified 3-NP nitroreductase. Partial reduction of the nitro group was also shown by Somerville et al. (37) with a purified nitrobenzene reductase which catalyzes the reduction of nitrobenzene to hydroxylaminobenzene at the expense of 2 mol of NADPH per mol of nitrobenzene. Since it occurred in the presence of oxygen, the authors suggested that this nitrobenzene nitroreductase is oxygen insensitive (type I enzyme).

The conversion of 4-hydroxylaminobenzoate to 3,4-dihydroxybenzoate has been identified as a key reaction in the degradative pathway of 4-nitrobenzoate (17) and 4-nitrotoluene (18, 32). This novel enzymatic reaction leads to a simultaneous elimination of ammonia and has recently been observed in the degradative pathway of 3-NP by *P. putida* B2 (25). The details of the mechanism are not yet clear. In contrast to this lyase reaction, which liberates ammonia, another crucial enzymatic reaction of a hydroxylamino compound was observed in the catabolism of nitroarenes where the nitrogen function is initially retained in the molecule. Nishino and Spain identified an enzyme which converts hydroxylaminobenzene to 2-aminophenol in the degradative pathway of nitrobenzene by *P. pseudoalcaligenes* JS45 (28). This intramolecular reaction is known as Bamberger rearrangement (2, 35, 38).

In contrast to the Bamberger rearrangement of hydroxylaminobenzene by *P. pseudoalcaligenes* JS45, cell extracts of *R. eutropha* JMP 134 were shown to convert hydroxylaminobenzene to a mixture of 2-aminophenol (37%) and 4-aminophenol (63%) (Fig. 2 and 4). Similar observations were reported by Corbett and Corbett for a *Rhodosporidium* sp. (8), in which 4-chloronitrobenzene had been transformed into 4-chloro-2-aminophenol and 4-aminophenol. The rearrangement into the *para* position leads to an exchange of the chlorine against a hydroxyl group.

In *R. eutropha* JMP 134, partial reduction of nitrobenzene and subsequent isomerization of the hydroxylaminobenzene yields 2- and 4-aminophenol as dead-end metabolites (Fig. 4). Correspondingly, 3-hydroxylaminophenol as a metabolite of 3-NP undergoes an enzyme-catalyzed rearrangement to aminohydroquinone (Fig. 5). Based on the observed transformation reactions, productive degradation of 3-NP must generate free aminohydroquinone. Whereas in cell extracts, aminohydroquinone was the end product of the anaerobic conversion, resting cells of *R. eutropha* JMP 134 additionally acetylated aminohydroquinone to *N*-acetylaminohydroquinone (Fig. 5). Acetylation of anilines has been demonstrated to be an important detoxification mechanism by microorganisms (5, 12, 43). A

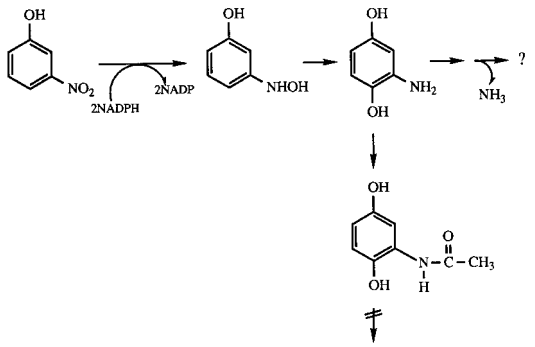


FIG. 5. Initial steps of the catabolic pathway of 3-nitrophenol by *R. eutropha* JMP 134.

corresponding mechanism was discussed by Gilcrease and Murphy for 4-*N*-acetylamino-2-amino-6-nitrotoluene as a conversion product of 2,4-diamino-6-nitrotoluene which was formed by the reduction of 2,4,6-trinitrotoluene by *Pseudomonas fluorescens* (15). Recently, a *Pseudomonas aeruginosa* strain was described which cometabolically transformed 2,4-dinitrotoluene by reduction of one or both nitro groups followed by an acetylation of the resulting amino group in the presence or absence of oxygen (29). In the case of *R. eutropha* JMP 134, the formation of *N*-acetylaminohydroquinone was observed only under anaerobic conditions. The transformation rate of 3-NP by resting cells under anaerobic conditions was considerably slower than that under aerobic conditions. This can be explained by the fact that sufficient amounts of reduction equivalents cannot be generated under anaerobic conditions. Thus, it is not clear whether *N*-acetylaminohydroquinone is a real metabolite or a detoxification product of 3-NP.

The identification of 3-hydroxylaminophenol as the metabolite of 3-NP is another example of how hydroxylamino aromatic compounds are key intermediates of the productive oxidative degradation of mononitroaromatic compounds. In contrast, Schackmann and Müller described a nitro group-reducing activity for 3-NP generating 3-aminophenol and 3-*N*-acetylaminophenol as dead-end metabolites. This complete reduction of the nitro group was carried out by resting cells of *Pseudomonas* sp. strain CBS3 and did not lead to a utilization of the corresponding amino compound (33). Up to now, complete reduction of the nitro group followed by a total and productive degradation of the corresponding amino compound has only been realized for the degradation of nitrobenzene (9) and 4-chloro-2-nitrophenol (4) by a coupled anaerobic-aerobic process. Characteristically, such a catabolic sequence has not yet been observed in a single organism. Therefore, oxidative assimilation of the carbon skeleton may be incompatible with complete reduction of the nitro group, which at least in non-defined cultures requires anaerobic conditions.

In contrast, simultaneous oxidative and reductive initial reactions were recently observed in the metabolism of 3-nitrotoluene. Resting cells of a *P. putida* strain, OU83, were shown to convert 3-nitrotoluene to 3-aminotoluene. Besides this, 3-NP was observed as a metabolite of 3-nitrotoluene along an oxidative pathway. Although the transformations of 3-nitrotoluene did not lead to a utilization of the compound, Ali-Sadat et al. described how 3-NP is further metabolized with release of nitrite into the medium (1). Therefore, up to now, two possibilities have been described for the elimination of the nitrogen function of 3-NP: one is the liberation of nitrite, and the other is the release of ammonia. Enzymatic reactions which resulted in an initial elimination of nitrite by oxidative as well as reductive reactions are well described (23, 39). In the degradation of 4-nitrobenzoate (17), 4-nitrotoluene (18, 32), and 3-NP by *P. putida* B2 (25), the elimination of ammonia occurred before ring cleavage, whereas 2-aminophenol as a key intermediate of nitrobenzene degradation is subject to a *meta* fission before the nitrogen function is liberated as ammonia (28). The mechanism of ammonia release in the degradative pathway of 3-NP by *R. eutropha* JMP 134 and the ring cleavage reaction are the subjects of current investigations.

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