A New 4-Nitrotoluene Degradation Pathway in a Mycobacterium Strain

TILMANN SPIESS,1,2 FRANK DESIERE,1† PETER FISCHER,3 JIM C. SPAIN,4 HANS-JOACHIM KNACKMUSS,1,2 AND HILTRUD LENKE1*†

Fraunhofer Institut für Grenzflächen- und Bioverfahrenstechnik,1 Institut für Mikrobiologie der Universität Stuttgart,2 and Institut für Organische Chemie der Universität Stuttgart,4 D-70569 Stuttgart, Germany, and Armstrong Laboratory/EQ-OL, Tyndall Air Force Base, Florida 32403-5323

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Mycobacterium sp. strain HL. 4-NT-1, isolated from a mixed soil sample from the Stuttgart area, utilized 4-nitrotoluene as the sole source of nitrogen, carbon, and energy. Under aerobic conditions, resting cells of the Mycobacterium strain metabolized 4-nitrotoluene with concomitant release of small amounts of ammonia; under anaerobic conditions, 4-nitrotoluene was completely converted to 6-amino-m-cresol. 4-Hydroxylysinotoluene was converted to 6-amino-m-cresol by cell extracts and thus could be confirmed as the initial metabolite in the degradation pathway. This enzymatic equivalent to the acid-catalyzed Bamberger rearrangement requires neither cofactors nor oxygen. In the same crucial enzymatic step, the homologous substrate hydroxylaminobenzene was rearranged to 6-amino-3-nitrotoluene. Abiotic oxidative dimerization of 6-amino-m-cresol, observed during growth of the Mycobacterium strain, yielded a yellow dihydrophenoxazinone. Another yellow metabolite (λ<sub>max</sub> 385 nm) was tentatively identified as 2-amino-5-methylunconic semialdehyde, formed from 6-amino-m-cresol by meta ring cleavage.

The production of nitroarenes, e.g., 2,4,6-trinitrotoluene (TNT) and 2,4,6-trinitrophenol (picric acid), as explosives during World War II has left both soil and groundwater extensively contaminated at the sites of former ammunition plants. Mononitrotoluenes and dinitrotoluenes, inevitable by-products in TNT production, are likewise found as contaminants in the environment of such plants.

Cometabolic attack on mononitrotoluenes by oxygenases has been described previously. Toluene dioxygenases from Pseudomonas putida F1 and Pseudomonas sp. strain JS 150 oxidatively attack the methyl group of 2- and 3-nitrotoluene (2-NT and 3-NT), yielding 2- and 3-nitrobenzylalcohol; with 4-nitrotoluene (4-NT), oxidation is directed towards the arene ring, leading to 2-methyl-5-nitrophenol and 3-methyl-6-nitrocatechol (22). The toluenone oxygenase encoded by the tol plasmid, however, oxidizes only the methyl group of 3-NT and 4-NT (22). Corresponding observations for the TOL-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase have been reported by Delgado et al. (7). Recent investigations have demonstrated reductive as well as oxidative initial attack during 3-NT metabolism in Pseudomonas putida OU83 (1). While 70% of the substrate was reduced to 3-aminotoluene, a minor part (30%) was converted, via 3-nitrobenzylalcohol, 3-nitrobenzaldehyde, and 3-nitrobenzoic acid, to 3-nitrophenol. The 3-nitrophenol was further metabolized with concomitant liberation of nitrite.

In contrast to the above-mentioned cometabolic transformations, all three mononitrotoluene isomers were shown to be biodegradable after adaptation of an activated-sludge system (26). Two different mechanisms were demonstrated for the productive degradation of 2-NT and 4-NT with single organisms. Oxidative release of nitrite was described with 2-NT (10). An et al. successfully demonstrated oxidative removal of the arene nitro group in vitro by a multicomponent enzyme system designated 2-NT-2,3-dioxygenase (2). Experiments with 18O<sub>2</sub> showed that both atoms of the oxygen molecule were added to the aromatic nucleus. The dihydrocyclohexadienediol thus formed spontaneously rearomatizes after elimination of nitrite, yielding 3-methylcatechol.

The isomeric 4-NT, in contrast, was oxidized to 4-nitrobenzoate, via 4-nitrobenzyalcohol and 4-nitrobenzaldehyde, prior to removal of the nitrogen function (9, 21). 4-Nitrobenzoate is reduced to 4-hydroxylaminobenzoate and subsequently converted, without participation of oxygen, to 3,4-dihydroxybenzoate and ammonia. This unusual catabolic pathway, with a hydroxylamino rather than an amino compound as key intermediate, was first described for the degradation of 4-nitrobenzoate by Comamonas acidovorans NBA 10 (8). We now report a new pathway for the degradation of 4-NT by a Mycobacterium strain, which was isolated by its ability to utilize 4-NT as the sole source of nitrogen in the presence of a readily degradable carbon source.

MATERIALS AND METHODS

Isolation, characterization, and growth of bacteria. A mixed soil sample from the Stuttgart, Germany, area was added to 50 ml of nitrogen-free mineral medium (13) and supplemented with 0.5 mM 4-NT as the nitrogen source and 10 mM succinate as the carbon source. Upon incubation of the suspension on a rotary shaker, bacterial growth and a slightly yellow coloration of the medium were observed. After 4-NT had disappeared from the medium, 5 ml of the suspension was transferred into fresh medium (50 ml). After 2 days of incubation, the samples were plated on agar plates (addition of 1.5% [wt/vol] agar no. 1; Oxoid Ltd., London, United Kingdom) with succinate (10 mM) in mineral medium. 4-NT was supplied to the plates via the gas phase in a desiccator containing crystals of 4-NT. After 7 days of incubation, single colonies were tested for their ability to utilize 4-NT as the sole source of nitrogen. One isolate, HL 4-NT-1, grew on sucinate-containing agar plates in a 4-NT atmosphere but not on control plates without 4-NT; it was characterized by the German Collection of Microorganisms as a Mycobacterium strain on the basis of its biochemical reactions, the presence of menaquinone, and the fatty acid composition of the cell envelope. The strain could be distinguished from the known mycobacterial species (11).
The strain was grown in batch culture with nitrogen-free mineral medium containing 0.5 mM 4-NT (or another nitroarene) and 10 mM succinate. The cultures were incubated at 30°C in fluted Erlenmeyer flasks on a rotary shaker (at 120 rpm). Growth was monitored by measuring the optical density at 546 nm. The cell debris and membrane-bound proteins were removed by centrifugation (10,000 × g for 30 min at 4°C). The protein content of the cell extracts was determined by the method of Bradford (6).

Preparation of cell extracts. Cells of Mycobacterium sp. strain HL 4-NT-1 were grown in nitrogen-free medium with 10 mM succinate and 0.5 mM 4-NT (induced cells) or 2 mM ammonia (uninduced cells). Fully induced cells were harvested by centrifugation at 12,000 × g for 15 min. The cell pellets were washed with phosphate buffer (pH 7.4) and resuspended in 50 mM phosphate buffer (pH 7.4) at a concentration of 0.1 to 0.2 mg of protein per ml. The reaction was started by adding the substrate, 6-AC (0.1 to 0.2 mM), NAD(P)H (0.2 to 0.3 mM), phosphate buffer (50 mM; pH 7.4), and cell extract (0.5 to 0.7 mg of protein per ml). The reaction was started by adding the substrate, 4-NT.

The hydroxylaminotoluene mutase activity was determined by monitoring the decrease in absorbance at 340 nm due to the conversion of NAD(P)H to NAD(P) by using well-established tests for nitroductases. The reaction mixtures contained 4-NT (0.1 to 0.15 mM), NAD(P)(H) (0.2 to 0.3 mM), phosphate buffer (50 mM; pH 7.4), and cell extract (0.5 to 0.7 mg of protein per ml). The reaction was started by adding the substrate, 4-NT.

Preparation of cell extracts. Cells of Mycobacterium sp. strain HL 4-NT-1 and cells of Pseudomonas aeruginosa 35 (12, 16) were harvested by centrifugation, suspended in 0.5 to 1 ml of 50 mM phosphate buffer (pH 7.4), and disrupted by four passages through a French pressure cell at 130 MPa (Amicon, Silver Spring, Md.). Cell debris and membrane-bound proteins were removed by centrifugation (100,000 × g for 35 min at 4°C). The protein content of the cell extracts was determined by the method of Bradford (6).

Enzyme assays. The 4-NT nitroreductase activity was determined spectrophotometrically by monitoring the decrease in absorbance at 340 nm due to the conversion of NAD(P)H to NAD(P) by using well-established tests for nitroductases (16, 24); the reaction mixtures contained 4-NT (0.1 to 0.15 mM), NAD(P)(H) (0.2 to 0.3 mM), phosphate buffer (50 mM; pH 7.4), and cell extract (0.5 to 0.7 mg of protein per ml). The reaction was started by adding the substrate, 4-NT.

The 4-hydroxylaminotoluene mutase activity was determined by monitoring the conversion of 4-hydroxytoluene to 6-AC (6-ac) by HPLC. The reaction mixture contained 0.2 mM 4-NT (dissolved with cell extract to a final concentration of 100,000 × g for 35 min at 4°C), Bechman Li-80 ultrafiltration, Beckman Instruments Inc., Irvine, Calif.). The protein content of the cell extracts was determined by the method of Bradford (6).

Analytical methods. The ammonium ion concentration was estimated by the photometric Berthelot method as modified by Parsons et al. (19). The nitrate ion concentration was monitored by the photometric method of Griess-Ilosvay as modified by Shinn (15). Concentrations of substrates and metabolites were monitored by reversed-phase or ion-pair HPLC (Waters, Milford, Conn.) with a reversed-phase column (Lichrospher 100 RP8, 4.6 by 125 mm; Merck, Darmstadt, Germany) and photometric detection at 230 nm. The flow rate was 1 ml/min. For monitoring the 4-NT concentration, the mobile phase was 50% acetonitrile–50% phosphate buffer (15 mM; pH 7.4). The concentrations of nitrobenzene, 2-amino-phenol, 6-AC, and product X were analyzed by HPLC with a mobile phase of 35% acetonitrile–65% phosphate buffer (15 mM; pH 7.4). The concentration of hydroxylaminobenzene was analyzed by ion-pair HPLC with 20% aqueous methanol containing hexane sulfonate (Pierce B6; Waters) as the mobile phase. 6-AC and product X were analyzed by ion-pair HPLC (mobile phase, 40% aqueous methanol with hexane sulfonate) or by reversed-phase HPLC (mobile phase, 5% methanol–95% of 0.2% H3PO4). An HPLC system (Sykam, Gilching, Germany), equipped with a diode array detector (Philips) was used for metabolite identification with the mobile phases and columns described above.

Isolation of the biologically obtained yellow coupling product. Mycobacterium sp. strain HL 4-NT-1 was cultivated in a batch culture (1,000 ml) with nitrogen-free mineral medium containing 0.6 × 10.5 mM 4-NT and 10 mM succinate. The culture flask was incubated at 30°C in a fluted Erlenmeyer flask on a rotary shaker (at 120 rpm). The disapperance of 4-NT and formation of the yellow metabolite were monitored by HPLC. After total consumption of 4-NT, the cells were removed by centrifugation, and the cell-free culture fluid was extracted twice with ethyl acetate. The ethyl acetate extracts were combined, dried with MgSO4, and evaporated to dryness. The yellow residue was purified by semipreparative HPLC on a reversed-phase column (Zorbax ODS, length, 250 mm; diameter, 21.2 mm [DuPont Chromatography, Wilmington, Del.]) with 75% phosphate buffer (15 mM; pH 7.4)–acetonitrile, 7.3 (vol/vol), as the mobile phase. Fractions containing the yellow oxidation product were concentrated; the resulting aqueous solution was extracted twice with ethyl acetate. The extracts were combined, dried with MgSO4, and evaporated to dryness, yielding brownish-yellow crystal line material, which was used for further experiments.

Spectroscopic methods. Coupled gas chromatography–mass spectrometry analyses were performed on a Hewlett-Packard 5890 (Palo Alto, Calif.) apparatus equipped with a fused-silica capillary column, in both electron impact (EI; 70 eV) and positive chemical ionization (CI) (reagent gas, methane) modes. 1H and 13C nuclear magnetic resonance (NMR) spectra of the yellow transformation product of 6-AC, obtained under both chemical and biological conditions, were obtained in CDCl3, solution, with tetramethylsilane (TMS) as the internal standard, on a Bruker (Rheinstetten, Germany) AC 250 spectrometer (nominal frequency, 250.134 MHz for 1H and 62.896 MHz for 13C).

Chemicals. 2-Amino-4,7-dimethyl-4,4a-dihydrophenoxazinone was synthesized as described by von Ahners et al. (29). Red crystals of the dihydrophenoxazine were obtained after recrystallization from toluene. 4-Hydroxylaminotoluene was prepared by reducing 4-NT with zinc under neutral conditions (5). Because of the sensitivity of the product toward oxygen, all reaction steps were carried out in an argon atmosphere: 4-NT (27.4 mg) and NH4Cl (120 mg) were dissolved in 10 ml of H2O and 10 ml of methanol in a serum bottle, the pH was adjusted to 6.8 with 2 M NaOH, Zn powder (0.1 g) was added, and the reaction mixture was evacuated and flushed with argon. The bottle was heated to 42°C on a water bath until the reaction was complete (14 h). Residual Zn powder was removed by filtration, and the filtrate was used for further experiments after dilution with phosphate buffer. A 5-methylpylicolic acid standard was obtained by incubation of 6-AC with a cell extract of P. pseudalcaligenes JS 45 (12). Hydroxylaminobenzene was kindly supplied by Shirley Nishino. All other chemicals were of the highest purity commercially available.

RESULTS

Isolation and characterization of a 4-NT-degrading bacterium. A bacterial strain, selected under nitrogen-limiting conditions, utilized 4-NT as the sole source of nitrogen in the presence of succinate as the carbon and energy source. The strain, designated HL 4-NT-1, was identified as a Mycobacte-rium strain (11). When it was grown with 4-NT and succinate, a yellow metabolite accumulated in the medium as 4-NT disappeared. Small amounts of ammonia transiently accumulated in the culture medium, which suggested that ammonia rather than nitrite was eliminated during 4-NT degradation. At 4-NT concentrations above 0.5 mM, the rate of conversion decreased and growth of the organism was inhibited. When 4-NT was supplied through the gas phase, Mycobacterium sp. strain HL 4-NT-1 was able to use it as the sole source of nitrogen, carbon, and energy in mineral medium on agar plates; no growth was observed on control agar plates. In batch cultures, the optical density at 546 nm increased from 0.15 to 0.72 in 69 h; in a control without 4-NT, the optical density increased only by 0.06. Interestingly, no yellow coloration appeared in the culture medium when 4-NT was the sole source of nitrogen, carbon, and energy.

Growth of Mycobacterium sp. strain HL 4-NT-1 with other substrates. Absolutely no growth of strain HL 4-NT-1 was observed when other nitroarenes were the sole source of nitrogen or carbon. The nitroarenes tested were nitrobenzene, 2-NT, 3-NT, 6-AC, 5-methyl-2-nitrophenol, 4-aminotoluene, or 4-nitrobenzoate. The strain also did not grow with protocatechue or 4-methylcatehol as the carbon and energy source.
Conversion of various substrates by resting cells of strain HL 4-NT-1. Resting cells of Mycobacterium sp. strain HL 4-NT-1 grown with 4-NT and succinate rapidly transformed 4-NT, nitrobenzene, and 6-AC under aerobic conditions. Transformation of nitrobenzene and 6-AC led to the accumulation of metabolites in the medium. 4-Aminotoluene, 4-nitrobenzoate, and 5-methyl-2-nitrophenol could not be detected as possible metabolites during transformation of 4-NT. Additionally, 4-Aminotoluene, 4-nitrobenzoate, and 5-methyl-2-nitrophenol were not transformed by resting cells under aerobic conditions. These initial results indicated that 4-NT is metabolized neither by initial oxidation of the methyl group to form 4-nitrobenzoate, as in the degradative pathway of 4-NT by Pseudomonas strains (9, 21), nor by complete reduction of the nitro group leading to 4-aminotoluene.

Metabolism of nitrobenzene. Because nitrobenzene, a structural analog of 4-NT, was also transformed by resting cells, its metabolism was investigated in detail. Resting cells, pregrown with 4-NT and succinate, readily converted nitrobenzene under aerobic conditions into 2-amino-phenol (17%) (retention volume, 2.2 ml; \( \lambda_{\text{max}} \) 231 and 286 nm), aniline (5%) (retention volume, 3.1 ml; \( \lambda_{\text{max}} \) 232 and 285 nm), and an unknown yellow compound (retention volume, 5.4 ml; \( \lambda_{\text{max}} \) 238 and 400 nm). 2-Aminophenol and aniline were identified from their chromatographic and UV characteristics by comparison with authentic material. In contrast, the formation of 4-aminotoluene was not observed with 4-NT as a substrate, indicating that the rate of nitro group reduction to the corresponding amino compound is negligible during 4-NT metabolism. 2-Aminophenol incubated in phosphate buffer under aerobic conditions without cells spontaneously converted to the same yellow compound that accumulated during nitrobenzene biotransformation. The results clearly indicate that the yellow compound is a nonbiological product of 2-amino-phenol.

Nitrobenzene is metabolized to 2-amino-phenol via hydroxylaminobenzene by P. pseudoalcaligenes JS 45 (16). To test whether a similar pathway is catalyzed by enzymes in Mycobacterium sp. strain HL 4-NT-1, a resting-cell experiment was carried out under anaerobic conditions with hydroxylaminobenzene as the substrate. Cells pregrown with 4-NT and succinate were added in an argon atmosphere to a hydroxylaminobenzene solution (nominal concentration, 0.55 mM). 2-Aminophenol was formed in stoichiometric amounts (Fig. 1). The results indicate clearly that hydroxylaminobenzene is completely converted to 2-amino-phenol under anaerobic conditions. In contrast to the nitrobenzene experiment under aerobic conditions, no yellow product was formed. The results indicate that the nonenzymatic conversion of 2-amino-phenol to the yellow product is an oxidative process. Transformation of hydroxylaminobenzene to 2-amino-phenol under anaerobic conditions was not catalyzed by uninduced cells pregrown with ammonia and succinate.

Identification of the yellow compound produced during conversion of 4-NT. Under aerobic conditions, 6-AC was readily converted by resting cells without accumulation of a yellow metabolite. When 6-AC was incubated without cells under aerobic conditions, however, a yellow product with the same chromatographic behavior and UV-visible spectrum as the yellow product accumulated during growth experiments with 4-NT was formed. For isolation of the yellow metabolite, the Mycobacterium strain was grown with 4-NT and succinate. When all the 4-NT disappeared from the medium, the yellow product was extracted with ethyl acetate. Purification by semipreparative HPLC yielded brownish-yellow crystals. When 6-AC was transformed chemically in the presence of air, as described by von Auwers et al. (29), red crystals were obtained after recrystallization from toluene. The two products had virtually identical chromatographic properties and UV-visible spectra (retention volume, 9.4 ml; \( \lambda_{\text{max}} \) 230 and 400 nm; additional flat maximum at 290 nm). The yellow products, obtained by chemical and biological transformation, were characterized by NMR. The NMR data showed that the chemically and biologically obtained products were identical. The chemical product was also analyzed by infrared spectroscopy and by high-resolution mass spectrometry to obtain the elemental composition (Table 1).

The peak for the molecular radical cation (M+; \( m/z \) 242) has 90% base peak intensity in the EI mass spectrum. In the CI (CH3) mass spectrum, \( m/z \) 243 is the base peak, because charge exchange with M+ predominates over protonation to the quasi-molecular ion (MH+, \( m/z \) 243) for the yellow metabolite. The spectrum indicates that the molecule must have an extremely conjugated aromatic structure. The extremely facile loss of a methyl radical from M+ (to \( m/z \) 227, 100%) indicates that there is a CH2 group at a quaternary position. The elemental composition C14H12N2O2 (Table 1), in combination with the 13C NMR data (Table 2), shows the yellow metabolite to be the product of oxidative dimerization of 6-AC (C6H8NO). The dihydrophenoxazinone structure derived from these results is given in Fig. 2. The compound 2-amino-4a,7-dimethyl-4a-dihydrophenoxazinone, has already been described in the literature (4) as the product of ferricyanide oxidation of 6-AC (C6H8NO).

The structural assignment is confirmed by the 1H NMR data, as well as by the very strong carbonyl band (1,695 cm\(^{-1}\)) and

<table>
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<th>( m/z ) (exp/calc)*</th>
<th>Relative intensities (% base peak)</th>
<th>Elemental composition</th>
<th>Fragment ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>242.105/242.105</td>
<td>85.3</td>
<td>C14H12N2O2</td>
<td>M+–H3C</td>
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<tr>
<td>227.081/227.082</td>
<td>100</td>
<td>C13H11N2O2</td>
<td>M+–HCO</td>
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<tr>
<td>213.104/213.103</td>
<td>13.7</td>
<td>C12H10N2O</td>
<td>M+–CHO</td>
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<tr>
<td>199.090/199.087</td>
<td>25.4</td>
<td>C12H9N2O</td>
<td>M+–CH2CO</td>
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<tr>
<td>173.072/173.071</td>
<td>30.6</td>
<td>C11H8N2O</td>
<td>M+–C3H4O</td>
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* exp, experimental; calc, calculated.
well-defined NH\textsubscript{2} stretching vibrations in the infrared spectrum.

**Conversion of 4-NT under anaerobic conditions.** Because both nitro- and hydroxylaminobenzene are converted to 2-aminophenol, a corresponding mechanism must be assumed for the conversion of 4-NT under anaerobic conditions. Indeed, 6-AC was identified as an intermediate of 4-NT degradation when resting cells, pregrown with 4-NT and succinate, were incubated in an argon atmosphere with a 4-NT solution (nominal concentration, 0.39 mM). Figure 3 shows that conversion of 4-NT to 6-AC is stoichiometric; the metabolite was identified unequivocally by comparison of its chromatographic properties and UV spectrum with those of authentic 6-AC (retention volume, 1.75 ml; \( \lambda_{\text{max}} \), 221, 234, and 289 nm). The yellow metabolite was produced more rapidly.

**4-Hydroxylaminotoluene as a metabolite of 4-NT.** To test whether conversion of 4-NT to 6-AC proceeds via 4-hydroxylaminotoluene, authentic 4-hydroxylaminotoluene was incubated under anaerobic conditions with extracts of cells grown with 4-NT and succinate. 4-Hydroxylaminotoluene substrate was completely converted to 6-AC; no 4-aminotoluene could be detected. The enzyme-catalyzed rearrangement of 4-hydroxylaminotoluene to 6-AC required neither cofactors nor oxygen. When the cell extract was heated to 100°C for 10 min, the enzyme activity was completely destroyed.

Extracts of cells grown with 4-NT and succinate were tested for the presence of enzymes that catalyzed 4-NT-dependent NADH and NADPH oxidation. This assay, which is well established in testing nitroreductases for degradation of nitroarenes (16, 24), did not reveal any detectable activity.

**Ring cleavage of 6-AC.** 6-AC accumulated as a dead-end product in the anaerobic conversion of 4-NT by resting cells of *Mycobacterium* sp. strain HL 4-NT-1; under aerobic conditions, 6-AC was readily converted by cells induced with 4-NT. With uninduced cells, grown with ammonia and succinate, 6-AC was transformed only after induction of the 4-NT-converting enzymes; the conversion stopped, however, after 170 min, as shown in Fig. 4. The same predominant metabolite accumulated in experiments with both induced and uninduced cells (retention volume, 1.75 ml; \( \lambda_{\text{max}} \), 221 and 270 nm). This metabolite, designated product X, was formed in larger amounts with uninduced than with induced resting cells (Fig. 4). When 6-AC was incubated without cells under the same conditions, 6-AC (0.48 mM) was oxidized to the dihydrophenoxazinone (0.19 mM) rather than to product X after 20 h.

Extracts from uninduced cells did not catalyze the conversion of 6-AC. Extracts of induced cells catalyzed the transient formation of a yellow intermediate (\( \lambda_{\text{max}} \), 385 nm) (Fig. 5), which was subsequently converted into another compound (\( \lambda_{\text{max}} \), 270 nm) (Fig. 5). In the presence of ferrous ions, the yellow metabolite was produced more rapidly.

When NAD was added to the reaction mixture at the time the yellow compound had reached maximum concentration, the disappearance of the yellow intermediate was much faster than without NAD. When NAD was added to the reaction mixture at the beginning, small amounts of the yellow intermediate appeared transiently; the final product (\( \lambda_{\text{max}} \), 270 nm) accumulated to a lesser extent. When the reaction mixture without NAD was analyzed by HPLC after complete conversion of 6-AC, it contained product X. Compound X was demonstrated by HPLC analysis to be identical to the 5-methylpi-
colinc acid obtained by conversion of 6-AC with cell extracts of *P. pseudoalcaligenes* JS 45 (12).

Extracts of induced cells did not catalyze the oxidation of either catechol or 4-methylcatechol; the ring fission enzyme activity was lower with 2-aminophenol than with 6-AC. When 6-AC was incubated with cell extracts in the presence of ferrous ion and NAD, 6-AC was converted with a specific activity of 0.045 mol/min/mg of protein as analyzed by HPLC. After complete conversion of 6-AC, 64% of the nitrogen was eliminated as ammonia. Without NAD, no release of ammonia could be detected.

DISCUSSION

Bacteria can utilize either oxidative or reductive reaction sequences for elimination of the nitro group from mononitroarenes. Oxidative elimination of nitrite from 2-nitrotoluene is catalyzed by a dioxygenase (2, 10). In contrast, hydroxylamino compounds have been established as crucial intermediates for productive nitroarene degradation in other microorganisms. In the degradative pathway of 4-NT in *Pseudomonas* strains, for example, the nitro group is reduced and eliminated as ammonia (9, 21). The substrate is initially oxidized to 4-nitrobenzoate and then reduced to 4-hydroxylaminobenzoate, which is converted, by a lyase reaction, to protocatechuate and ammonia. An analogous lyase reaction, converting 3-hydroxylaminophenol to 1,2,4-trihydroxybenzene, was recently postulated as a key reaction in the biodegradation of 3-nitrophenol by *Pseudomonas* sp. strain B2 (14). Enzymes in 3-nitrophenol-grown *Ralstonia eutropha* JMP 134, in contrast, catalyze the enzymatic transfer of the hydroxyl group of 3-hydroxylaminophenol from the nitrogen to the *ortho*-aryl position (24). An analogous reaction sequence has been established for nitrobenzene biodegradation by *P. pseudoalcaligenes* JS 45 (16). The reaction represents an enzymatic equivalent to the acid-catalyzed Bamberger rearrangement (3).

Whereas enrichments with 4-NT as the nitrogen, carbon, and energy source (9, 21) obviously led to the isolation of *Pseudomonas* strains with the above-described catabolic pathway of 4-NT, we now present an alternative pathway for the degradation of 4-NT by a *Mycobacterium* strain which was selected based on its ability to utilize 4-NT as the sole source of nitrogen. The degradative pathway of 4-NT (Fig. 6) involves reduction of the nitro to a hydroxylamino function without prior oxidation of the methyl group. The accumulation of 2-amino-4a,7-dimethyl-4,4a-dihydrophenoxazinone during aerobic growth of *Mycobacterium* sp. strain HL 4-NT-1 on 4-NT provides strong evidence that 6-AC is an intermediate in the pathway. Enzyme-catalyzed oxidative dimerization of o-aminophenols has been demonstrated to be a very effective strategy for the synthesis of phenoxazinones (4, 28); however, the oxidative coupling may also be effected chemically. With 4-NT as a substrate, the reaction sequence of oxidative dimerization is blocked at the 2-amino-4a,7-dimethyl-4,4a-dihydrophenoxazinone stage due to the methyl substituent in the angular (i.e., the 4a) position (4; this work). When 6-AC was kept in phosphate buffer, conversion to the dihydrophenoxazinone was almost quantitative even at concentrations below 1 mM. The aerobic incubation of 2-aminophenol in phosphate buffer also led to the formation of a yellow compound, which was identified as the compound that accumulated during transformation of nitrobenzene by resting cells of *Mycobacterium* sp. strain HL 4-NT-1. By analogy to 6-AC, 2-aminophenol is obviously subject to an oxidative dimerization.

When 4-NT was supplied to *Mycobacterium* sp. strain HL 4-NT-1 as the sole source of nitrogen, carbon, and energy, virtually no 2-amino-4a,7-dimethyl-4,4a-dihydrophenoxazinone was formed. During growth with 4-NT as the nitrogen source and succinate as the carbon and energy source, however, the dihydrophenoxazinone accumulated. This constitutes clear evidence that 6-AC, formed as an intermediate during the degradation of 4-NT, is not released into the medium when 4-NT is supplied as the sole source of nitrogen, carbon, and energy. Rather, it is degraded by the productive pathway presented in Fig. 6.

Conservation of two methyl groups in the dimeric product constitutes definitive proof that no methyl oxidation step is involved in the degradation of 4-NT by the *Mycobacterium*...
strain. Rather, degradation in this case proceeds via reduction of the nitro group to a hydroxylamino function. The 4-hydroxylaminotoluene thus formed is converted, in the next step, to 6-AC both by whole cells and by cell extracts. This pivotal reaction for the degradative pathway of 4-NT requires neither cofactors nor oxygen and is clearly analogous to the Bamberger-type rearrangements involved in the degradation of nitrobenzene (16) and 3-nitrophenol (24).

With hydroxylaminobenzene, the chemical rearrangement yields 4-aminophenol exclusively (25, 27). The 3-nitrophenol-degrading R. eutropha JMP 134 converts hydroxylaminobenzene to a mixture of 2- and 4-aminophenol (24). With the nitrobenzene-degrading P. pseudoalcaligenes JS 45 (16), in contrast, only 2-aminophenol is produced by the enzymatic rearrangement. The fact that the enzymes in Mycobacterium sp. strain HL 4-NT-1 convert nitrobenzene, via hydroxylaminobenzene, mainly to 2-aminophenol thus constitutes independent proof for the 4-NT degradation mechanism outlined in Fig. 6. The results also suggest that the enzyme in Mycobacterium sp. strain HL 4-NT-1 is more closely related to the analogous enzyme from P. pseudoalcaligenes JS 45 than to the one from R. eutropha JMP 134. Whereas P. pseudoalcaligenes JS 45 (16) can utilize 2-aminophenol as a growth substrate, Mycobacterium sp. strain HL 4-NT-1 does not. This explains the failure of the Mycobacterium strain to grow on nitrobenzene.

The fact that 6-AC is enzymatically converted in cell extracts under aerobic conditions to a yellow product indicates that the aromatic ring must be subject to enzymatic reaction and the mechanism of ammonia release are further degraded via an NAD-dependent step. Both this enzyme-mediated rearrangement catalyzed Bamberger-type rearrangement. To our knowledge, this represents the first such enzyme-mediated rearrangement in a gram-positive bacterium. Subsequent ring cleavage of 6-AC yields 2-amino-5-methylmuconic semialdehyde, which is recently purified and investigated in detail (12). It catalyzes the extradiol ring cleavage of 2-aminophenol as well as that of 6-AC; the 2-amino-5-methylmuconic semialdehyde thus formed is further converted, nonenzymatically, to 5-methylpicolinic acid. The results presented here clearly indicate that product X, formed from 6-AC by cell extracts or resting cells of the Mycobacterium strain, is in fact 5-methylpicolinic acid.

Accumulation of 5-methylpicolinic acid during transformation of 6-AC by induced cells indicates that 2-amino-5-methylmuconic semialdehyde must be formed faster than it is converted. The higher concentration of 5-methylpicolinic acid, accumulated during transformation of 6-AC by uninduced cells, seemed to inhibit 6-AC transformation. This observation may also explain the failure of the Mycobacterium strain to grow on 6-AC as the primary substrate.

The rapid, NAD-dependent disappearance of the meta ring fission product suggests that the 2-amino-5-methyl muconic semialdehyde was oxidized to the corresponding muconic acid. Ammonia was liberated during enzymatic conversion of 6-AC only in the presence of NAD, which indicates that ammonia must be eliminated after ring cleavage. This finding confirms that the aromatic ring of 6-AC is cleaved directly.

From our results, and by analogy to those with other microbial systems, the following pathway is proposed for the degradation of 4-NT by Mycobacterium sp. strain HL 4-NT-1 (Fig. 6): in the first step, 4-NT is partially reduced to 4-hydroxylaminotoluene, which is then converted to 6-AC by an enzyme-catalyzed Bamberger-type rearrangement. To our knowledge, this represents the first such enzyme-mediated rearrangement in a gram-positive bacterium. Subsequent ring cleavage of 6-AC yields 2-amino-5-methylmuconic semialdehyde, which is further degraded via an NAD-dependent step. Both this enzymatic reaction and the mechanism of ammonia release are currently being investigated in more depth.

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REFERENCES


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