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Oxidative Pathway for the Biodegradation of Nitrobenzene by *Comamonas* sp. Strain JS765

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Previous studies have shown that the biodegradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45 proceeds by the reduction of nitrobenzene through nitrosobenzene and hydroxylaminobenzene, followed by rearrangement to 2-aminophenol, which then undergoes *meta* ring cleavage. We report here the isolation of a *Comamonas* sp. that uses an oxidative pathway for the complete mineralization of nitrobenzene. The isolate, designated strain JS765, uses nitrobenzene as a sole source of carbon, nitrogen, and energy. Nitrobenzene-grown cells oxidized nitrobenzene, with the stoichiometric release of nitrite. Extracts of nitrobenzene-grown JS765 showed high levels of catechol 2,3-dioxygenase activity that were not abolished by heating the cell extracts to 60°C for 10 min. The ring cleavage product had an absorbance maximum at 375 nm, consistent with that of 2-hydroxymuconic semialdehyde. Both NAD-dependent dehydrogenase and NAD-independent hydrolase activities towards 2-hydroxymuconic semialdehyde were induced in extracts of nitrobenzene-grown cells. Catechol accumulated in the reaction mixture when cells preincubated with 3-chlorocatechol were incubated with nitrobenzene. Conversion of nitrobenzene to catechol by induced cells in the presence of 3-chlorocatechol and $^{18}O_2$ demonstrated the simultaneous incorporation of two atoms of oxygen, which indicated that the initial reaction was dioxygenation. The results indicate that the catabolic pathway involves an initial dioxygenase attack on nitrobenzene with the release of nitrite and formation of catechol, which is subsequently degraded by a *meta* cleavage pathway.

Recent work in several laboratories has revealed a variety of mechanisms that allow bacteria to use nitroaromatic compounds as growth substrates. Both oxidative and reductive mechanisms have been described for the initial reactions involved in the bacterial degradation of the mononitrophenols (33, 37), mononitrotoluenes (2, 12, 13, 26), 2,4-dinitrotoluene (34), and 4-nitrobenzoate (5, 6, 10).

We recently isolated and characterized *Pseudomonas pseudoalcaligenes* JS45, which utilizes nitrobenzene as the sole source of carbon, nitrogen, and energy (20). Strain JS45 degrades nitrobenzene via an initial reduction to hydroxylaminobenzene, at which the enzyme activity stops without further reduction to aniline (31). The hydroxylaminobenzene is rearranged to 2-aminophenol in an enzyme-catalyzed Bamberger-like reaction, which has not previously been reported in bacteria. 2-Aminophenol then undergoes *meta* ring cleavage with the subsequent release of ammonia. This reaction has not been reported in organisms for which 2-aminophenol was the physiological substrate. Because the pathway involves three unusual reactions, we surveyed several industrial sites contaminated with nitrobenzene, which included the four largest production facilities for nitrobenzene in the United States as well as associated industrial waste treatment plants, for the presence of bacteria able to degrade nitrobenzene. We hoped to answer the following questions regarding the distribution of nitrobenzene-degrading bacteria. The first was whether nitrobenzene-contaminated sites contain indigenous strains able to degrade nitrobenzene. The second objective was to determine whether the original nitrobenzene-degradative pathway that we described is universal. The partial reductive pathway was used by 154 of 155 isolates examined; however, an alternate, oxidative pathway was used for nitrobenzene mineralization by *Comamonas* sp. strain JS765. We report here the initial reactions involved in the oxidative pathway for nitrobenzene degradation.

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MATERIALS AND METHODS

Isolation and growth of bacteria. Groundwater (1 ml) and soil (1 g [wet weight]) from six sources (Table 1) were added to separate 250-ml flasks containing 125 ml of nitrogen-free minimal medium (BLK) (4). Nitrobenzene was provided in the vapor phase as the sole carbon and nitrogen source as described previously (20). Cultures were incubated at 30°C with shaking at 200 rpm in a Gyrotory shaker, and nitrobenzene concentrations in the BLK were monitored by high-performance liquid chromatography (HPLC) (20). Samples (10 ml) of the cultures were transferred to fresh BLK when concentrations of nitrobenzene in the culture medium decreased and the cultures became turbid. Nitrobenzene was added directly to the culture medium to a concentration of 1 mM in subsequent transfers. After 1 to 2 months of enrichment, samples of the cell suspensions were spread on agar plates with nitrobenzene provided in the vapor phase as the sole carbon and nitrogen source (20). Individual colonies were transferred into 5 ml of BLK containing 10 μM nitrobenzene as the carbon and nitrogen source. After 5 days of incubation at room temperature, nitrobenzene concentrations in the culture fluids were measured by HPLC. Cultures in which no nitrobenzene could be detected by HPLC were further screened for ammonia and nitrite release. Cultures were maintained on agar-solidified BLK with nitrobenzene provided as vapor.

Isolates were characterized by standard procedures (29), and gram-negative strains were characterized with GN microplates (Biolog, Inc., Hayward, Calif.). Fatty acid analysis was performed by the American Type Culture Collection. The ability to grow on other aromatic substrates was tested by auxanography (23). Volatile substrates were provided in the vapor phase. The ability to transform or cometabolize other aromatic substrates was tested with washed, nitrobenzene-grown cells. Substrates were added directly to BLK to a final concentration of 50 μM, and then the cultures were incubated at 30°C with shaking for 1 to 4 h.

Cells of strain JS765 were routinely grown in 2-liter shake flasks containing 1.25 liters of BLK supplemented with nitrobenzene (1 mM) and incubated with shaking (200 rpm) at 30°C. After 16 h, a second aliquot of nitrobenzene (100 μM) was added to the culture, and incubation was continued for 1 to 2 h until all of the nitrobenzene disappeared. Heavy inocula obtained by the addition of succinate (10 mM) or tryptic soy broth to the starter cultures yielded more-reliable growth. Cells were harvested by centrifugation and washed twice with fresh phosphate buffer (0.02 M, pH 7.0) before use in subsequent experiments. Uninduced cells were grown overnight in BLK supplemented with succinate (20 mM) and NH_4Cl (9.4 mM) or in tryptic soy broth.

TABLE 1. Selected characteristics of nitrobenzene-degrading strains

Isolate	Source of isolate	Gram reaction	Oxidase	Catalase	Release of:	
					NH_4^+	NO_2^-
JS45	Mississippi[a]	−	+	+	+	−
JS760	Mississippi[a]	−	+	+	+	−
JS761	Mississippi[a]	−	−	+	+	−
JS762	West Virginia[a]	+	−	+	+	−
JS763	Texas[a]	+	−	+	+	−
JS764	Mississippi[a]	+	+	+	+	−
JS765	New Jersey[b]	+ (−)[c]	+	+	−	+
JS766	Mississippi[b]	+	−	+	+	−
JS767	Mississippi[b]	−	+	+	+	−
JS768	Mississippi[b]	+	−	+	+	−
JS769	Florida[b]	−	−	+	+	−

[a] Samples were from manufacturing facilities.
[b] Samples were from waste treatment plants.
[c] Positive in fresh cultures, negative in older cultures.

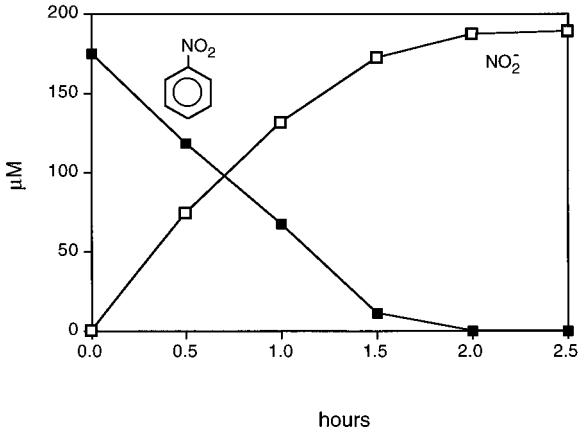

FIG. 1. Growth on nitrobenzene. Triplicate cultures were inoculated with nitrobenzene-grown cells. Stoichiometric release of nitrite accompanied the disappearance of nitrobenzene.

Preparation of cell extracts. Washed cells were broken by two passages through a French pressure cell at 20,000 lb/in^2. The exudate was divided and centrifuged for 60 min at 4°C: half at 100,000 × *g* and half at 26,000 × *g*. The pellets were discarded, and the supernatant fluids were stored on ice until used. Strain JS765 was resistant to breakage, but good yields were obtained when penicillin G (100 U/ml) was added to cultures 1 h before harvesting.

Enzyme assays. Catechol 1,2-dioxygenase (32), catechol 2,3-dioxygenase (32), nitrobenzene reductase, and 2-aminophenol 1,6-dioxygenase (20) were measured as described previously. 2-Hydroxymuconic semialdehyde (HMS) dehydrogenase activity was measured spectrophotometrically by monitoring the decrease in A_{375} of the catechol ring cleavage product. Reaction mixtures contained catechol (0.1 μmol), potassium phosphate (9.5 to 9.8 μmol, pH 7.5), and cell extract (0.1 to 0.3 mg of protein) in a final volume of 1 ml. HMS hydrolase activity was measured after the addition of NAD^+ (0.1 to 0.3 μmol) to the same reaction mixtures.

Inhibition of catechol-2,3-dioxygenase by 3-chlorocatechol and $^{18}O_2$ incorporation. Washed, nitrobenzene-grown cells were incubated with 3-chlorocatechol added in equal portions until oxygen uptake with 3-chlorocatechol and catechol was no longer stimulated by further additions of the substrate (800 μM added in 100 μM aliquots over a period of 45 min). Cells were harvested by centrifugation and suspended (3.7 mg of protein per ml) in 60 ml of fresh phosphate buffer (0.02 M, pH 7.0) containing 3-chlorocatechol (100 μM) and nitrobenzene (100 μM) under an atmosphere of $^{18}O_2$ (8%) and $^{16}O_2$ (9%) in N_2. After incubation for 30 min, the cells were removed by centrifugation, and the supernatants were extracted with equal volumes of ethyl acetate. The extracts were dried over anhydrous sodium sulfate, and the ethyl acetate was removed by flash evaporation at 30°C. The residue was dissolved in ethyl acetate and analyzed by gas chromatography-mass spectrometry (see below).

Analytical methods. HPLC analyses were performed on a Spherisorb C_8 column (Alltech, Deerfield, Ill.) with a gradient of acetonitrile and water as the mobile phase. Trifluoroacetic acid was added to both the acetonitrile and water (6.75 and 13.5 mM, respectively). The elution program began with 60% water and 40% acetonitrile, was changed to 40% water and 60% acetonitrile over 8 min, and was held at that ratio for 2 min. The flow rate was 1 ml/min. Compounds were monitored at UV A_{230} with an HP1040A diode array detector (Hewlett-Packard Co., Palo Alto, Calif.).

Gas chromatography-mass spectrometry analyses were performed on an HP5890 gas chromatograph equipped with a 30-m DB-5 fused silica capillary column and an HP5971 mass selective detector. Spectrophotometric analyses were performed on a Cary 3E UV-visible light spectrophotometer (Varian Associates, Sunnyvale, Calif.). Products of the reactions were identified by comparison with standards. Protein was measured by the bicinchoninic acid method (30). Nitrite (29) and ammonia (24) releases were measured by standard methods.

Chemicals. Hydroxylaminobenzene was prepared as previously described (20). Nitrobenzene, nitrosobenzene, 2-aminophenol, and catechol were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Catechol was purified by vacuum sublimation. 3-Chlorocatechol was obtained from Helix Biotech Corp., Richmond, British Columbia, Canada. [U-^{14}C]nitrobenzene (specific activity, 9.3 mCi/mmol; 96% radiochemical purity by HPLC) and NADase were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of the highest purity commercially available.

RESULTS

Isolation and identification of nitrobenzene-degrading bacteria. Strains that utilize nitrobenzene as a sole carbon, nitrogen, and energy source were isolated from all nitrobenzene-contaminated sites examined following a short enrichment period. Isolates were considered to degrade nitrobenzene if their presence in 5 ml of BLK containing nitrobenzene (10 μM) resulted in the disappearance of nitrobenzene within 5 days. Of 500 isolates tested, 155 degraded nitrobenzene. Many of these strains were gram positive, and although they grew well on solid surfaces in the presence of nitrobenzene vapor, they grew poorly in liquid cultures. The gram-negative isolates generally grew well in both liquid and solid cultures. Selected characteristics of representative nitrobenzene-degrading strains are shown in Table 1. *P. pseudoalcaligenes*, strain JS45, had been studied previously to determine the pathway of nitrobenzene catabolism (20) and was included here for comparison. The pathway involves the partial reduction of nitrobenzene through hydroxylaminobenzene and rearrangement to 2-aminophenol before ring cleavage and the release of ammonia. The nitrobenzene-degrading strains isolated in this study were examined to determine whether they release nitrogen from nitrobenzene as ammonia or nitrite. All the isolates released ammonia, except strain JS765, which released nitrite. The release of ammonia by the majority of the nitrobenzene-degrading strains was taken as evidence for the involvement of the reductive pathway analogous to that of *P. pseudoalcaligenes* JS45. In contrast, the release of nitrite suggested the presence of an oxidative pathway in strain JS765.

Strain JS765 is a motile, oxidase-positive, catalase-positive, denitrifying rod. It was gram positive in fresh cultures and gram variable in older cultures. Fatty acid analysis, however, gave a 0.843 similarity index to *Comamonas acidovorans*. Biolog GN plates gave a 0.673 similarity index to *Comamonas testosteroni*. On the basis of the above results, we designated the isolate *Comamonas* sp. strain JS765. Dense, washed suspensions of nitrobenzene-grown cells released stoichiometric amounts of nitrite during growth on nitrobenzene as the sole carbon, nitrogen, and energy source (Fig. 1). Cells provided with radiolabelled nitrobenzene released 37% of the initial radioactivity as $^{14}CO_2$ during growth on nitrobenzene for 2 h. Mercury-killed controls (12 μg of $HgCl_2$ per ml of culture medium) released neither CO_2 nor nitrite.

Utilization of other substrates. Strain JS45 was examined for growth on a variety of aromatic substrates by auxanography. Of those tested, only catechol and nitrobenzene served as growth substrates. No growth was detected on benzene; toluene; phe-

TABLE 2. Oxygen uptake by washed suspensions of selected nitrobenzene-degrading strains

Assay substrate[a]	O_2 uptake (μmol/min/mg of protein) by nitrobenzene (succinate)-grown cells[b]					
	JS45	JS760	JS763	JS764	JS765	JS768
Nitrobenzene	0.17 (0.01)	0.12 (<0.01)	0.06 (<0.01)	0.17 (0.04)	0.25 (0.06)	0.27 (0.06)
Hydroxylaminobenzene	0.47 (0.07)	0.33 (<0.01)	0.10 (<0.01)	0.28 (0.24)	0.01	0.50 (0.28)
2-Aminophenol	0.36 (0.11)	0.38 (<0.01)	0.08 (<0.01)	0.28 (0.29)	<0.01	0.45 (0.29)
Catechol[c]	0.04	0.04	0.04	0.03	0.91 (0.30)	0.05
2-Nitrophenol					<0.01	
3-Nitrophenol					0.11[d]	
4-Nitrophenol					0.18[d]	

[a] Substrate concentration, 100 μM.
[b] Oxygen uptake was measured polarographically (32). The values given are for nitrobenzene-grown cells, with values for succinate-grown cells in parentheses.
[c] There was no detectable activity for any culture with 3-nitrocatechol, 4-nitrocatechol, aniline, or phenol.
[d] Initial rate followed by a rapid decrease.

nol; 2-, 3-, and 4-nitrophenol; 2,4-, 2,5-, and 2,6-dinitrophenol; 1,2-, 1,3-, and 1,4-dinitrobenzene; 3- and 4-nitrocatechol; aniline; naphthalene; picric acid; 2-, 3-, and 4-nitrotoluene; 2,4- and 2,6-dinitrotoluene; 2,4,6-trinitrotoluene; or pyridine.

Preliminary experiments revealed that enzymes induced by growth on nitrobenzene have a broad substrate range and were able to transform many of the substrates that did not serve as growth substrates, including nitrophenols, nitrotoluenes, and naphthalene.

Respirometry. Washed suspensions of nitrobenzene-grown cells of strain JS45 and all the strains examined that released ammonia showed rapid oxygen uptake in the presence of hydroxylaminobenzene and 2-aminophenol, but not in the presence of catechol. This pattern of activity is consistent with the reductive catabolic pathway for nitrobenzene. In contrast, nitrobenzene-grown cells of strain JS765 rapidly oxidized nitrobenzene and catechol but not hydroxylaminobenzene and 2-aminophenol (Table 2). Oxygen consumption was 2.9 and 1.8 mol/mol of nitrobenzene and catechol, respectively. The difference of 1 mol between the two substrates suggested that 1 mol of oxygen is consumed in the production of catechol. This is the result that would be expected for a dioxygenation reaction. Two sequential monooxygenation reactions would require 2 mol of oxygen to produce 1 mol of catechol. Lack of stimulation of oxygen uptake by phenol, 2-nitrophenol, 3-nitrophenol, 4-nitrophenol, 3-nitrocatechol, and 4-nitrocatechol and the stoichiometry of oxygen consumption suggested that the initial reaction is dioxygenation at the 1,2 position, which would result in the formation of catechol and the release of nitrite. Uninduced cells of strain JS765 oxidized nitrobenzene and catechol at low constitutive rates.

Enzyme activities in cell extracts. Extracts of nitrobenzene-grown cells of JS765 catalyzed the oxygen-dependent disappearance of catechol and the transient appearance of a compound with a maximum value at A_{375}. This activity was only slightly reduced in extracts incubated at 60°C for 10 min. The yellow color was abolished upon acidification. These results are consistent with *meta* ring cleavage of catechol by catechol 2,3-dioxygenase to form HMS (9). Catechol 1,2-dioxygenase activity was not detected (Table 3).

A variety of catechols were assayed to determine the substrate specificity of the catechol 2,3-dioxygenase from *Comamonas* sp. strain JS765 (Table 4). The substrate range is similar to those reported for an *Azotobacter* sp. (27), *Rhodococcus rhodochrous* (28), *Pseudomonas picketii* (15), and *Pseudomonas arvilla* (17). The specific activity of catechol 2,3-dioxygenase in the preparation described in Table 4 is 10-fold lower than that shown in Table 3. Although there was considerable variation in specific activities among different enzyme preparations, in all cases the relative activities towards the various catechols remained the same.

Enzymes in freshly prepared extracts of nitrobenzene-grown cells catalyzed further metabolism of the yellow ring cleavage product, HMS, to colorless products. Activity was lost rapidly during storage of cell extracts, and no activity was detected in extracts that had been frozen.

HMS can be degraded via an NAD-dependent dehydrogenation reaction or by an NAD-independent hydrolase reaction. Either or both of these enzymes can be induced in other bacteria (27). Fresh crude extracts prepared from nitrobenzene-grown cells of JS765 appeared to contain both types of activities (Fig. 2). Catechol was converted rapidly to HMS, which subsequently disappeared slowly. The rate of disappearance of HMS was markedly enhanced by the addition of NAD (100 μM, final concentration). To determine whether the initial rate of removal of HMS was an NAD-dependent dehydrogenation reaction due to residual NAD in the crude cell extract or whether the activity was due to an NAD-independent hydrolase, residual NAD was removed from the reaction mixtures as follows: either cell extracts were dialyzed against two changes of phosphate buffer (0.02 M, pH 7.0) for 2 h before use, NADase (1 U/ml of cell extract) was preincubated at room temperature with cell extracts for 30 min prior to use in reaction mixtures, or an NADH generator (25) (6 mM glucose-6-phosphate and 5 U of glucose-6-phosphate dehydrogenase) was used in the reaction mixture. All three methods confirmed that the baseline activity was an NAD-independent hydrolase reaction but that the addition of NAD stimulated NAD-dependent dehydrogenation activity. To stimulate activity in the presence of the NADH-generating system, NAD was added to a final concentration of 200 μM.

Dioxygenation of nitrobenzene. The high constitutive rate of catechol oxidation in uninduced cells precluded the accumulation of catechol in cultures during induction and growth on nitrobenzene. However, the oxidation of catechol by catechol 2,3-dioxygenase in other systems is inhibited by 3-chlorocat-

TABLE 3. Activities in cell extracts

Enzyme assayed	Sp act (μmol/min/mg of protein) after growth on:		
	Nitrobenzene	Succinate	Tryptic soy broth
Catechol 1,2-dioxygenase	<0.01		
Catechol 2,3-dioxygenase	3.04	0.07	0.18
HMS hydrolase	0.05	0.01	<0.01
HMS dehydrogenase	0.08	0.02	<0.01

TABLE 4. Oxygen uptake by extracts of JS765

Assay substrate[a]	O_2 uptake (μmol/min/mg of protein) after growth on nitrobenzene[b]
Catechol	0.35
3-Nitrocatechol	<0.01
4-Nitrocatechol	<0.01
3-Methylcatechol	0.26
4-Methylcatechol	0.25
3-Chlorocatechol	0.27
Protocatechuate	<0.01

[a] Substrate concentration, 100 μM.
[b] For growth on succinate, the O_2 uptake with all substrates was <0.01 μmol/min/mg of protein.

echol (3, 14). When nitrobenzene-grown cells were preincubated with 3-chlorocatechol, catechol accumulated during subsequent incubations with 3-chlorocatechol and nitrobenzene (Fig. 3). The removal of 3-chlorocatechol by washing the cells prior to incubation with nitrobenzene restored the catechol 2,3-dioxygenase activity. However, catechol accumulated only in mixtures containing washed, nitrobenzene-grown cells preincubated with 3-chlorocatechol and then provided with nitrobenzene and 3-chlorocatechol. Catechol was not detected in mixtures without cells, with uninduced cells, or containing cells with nitrobenzene alone or cells with 3-chlorocatechol alone.

When the above experiment was repeated under an atmosphere that contained a mixture of N_2, $^{16}O_2$, and $^{18}O_2$, 41% of the catechol that accumulated was ^{18}O-labelled catechol with an *m/z* of 114. The remainder was unlabelled catechol with an *m/z* of 110. No catechol with an *m/z* of 112 was detected. The results confirm that the initial attack is a dioxygenation reaction rather than sequential monooxygenations. Nitrobenzene dioxygenase activity, however, was not detected in cell extracts.

DISCUSSION

All but one of the nitrobenzene-degrading organisms examined during the course of this study appeared to use the partial reductive pathway previously reported for nitrobenzene degradation (20). The partial reductive pathway for nitrobenzene degradation is greatly predominant, in both gram-positive and gram-negative bacteria, over a wide geographic distribution. The oxidative pathway was detected in only a single isolate. A possible explanation for this is that strain JS765 was isolated from an activated-carbon-based industrial waste treatment reactor, a well-aerated environment that can provide the additional oxygen requirements of the oxidative pathway. In contrast, the strains that use the pathway involving partial reduction were isolated from contaminated subsurface soil and groundwater in which oxygen is limiting.

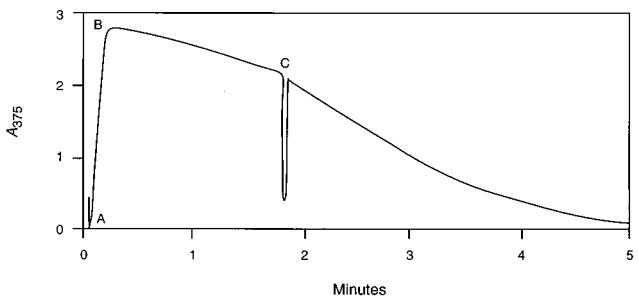

FIG. 2. Disappearance of HMS in mixtures containing cell extracts. (A) Catechol (100 μM) added to buffer containing cell extract, 6 mM glucose-6-phosphate, and 5 U of glucose-6-phosphate dehydrogenase; (B) maximum accumulation of HMS; (C) addition of NAD (200 μM).

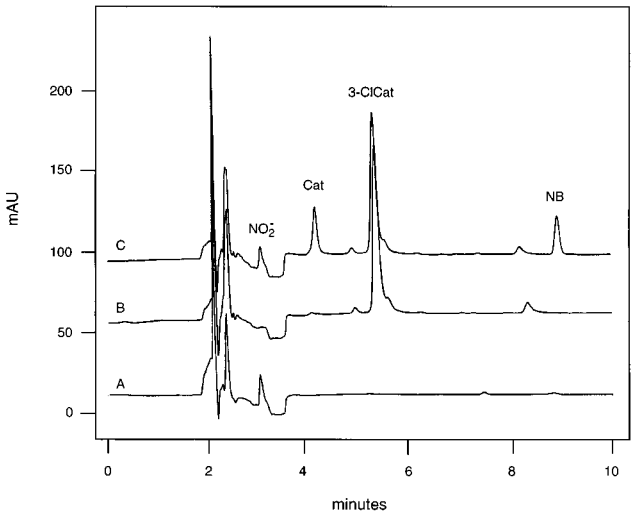

FIG. 3. Accumulation of catechol by catechol 2,3-dioxygenase-inactivated cells. Nitrobenzene (NB)-grown cells were preincubated with 3-chlorocatechol (3-ClCat) until oxygen stimulation was no longer detected upon the addition of catechol (Cat) or 3-chlorocatechol to cell mixtures. Cells were washed and suspended in buffer containing 3-chlorocatechol (100 μM) with the addition of nitrobenzene alone (no preincubation with 3-chlorocatechol) (A), 3-chlorocatechol alone (B), or nitrobenzene and 3-chlorocatechol (C).

The survey of nitrobenzene-contaminated sites and waste streams demonstrated that nitrobenzene-degrading organisms can be readily isolated from a variety of habitats. This may be contrasted with older studies (1, 16) in which nitrobenzene proved to be recalcitrant to removal. All of the isolates in this study came from sites with years of exposure to nitrobenzene, which suggests that chronic nitrobenzene contamination leads to the adaptation of the indigenous community to nitrobenzene. A similar conclusion was reached with respect to chlorobenzene contamination (21, 22). On the basis of our results, we believe that biodegradation of nitrobenzene contamination will not require inoculation with specific strains but can be initiated with the indigenous populations where conditions of chronic contamination exist.

Strain JS765 was unique in the release of nitrite during growth on nitrobenzene. Its ability to grow on catechol, as demonstrated by auxanography, and simultaneous adaptation studies together with its ability to release nitrite from nitrobenzene suggested the operation of an oxidative pathway. Two mechanisms for the oxidative removal of nitrite from the aromatic ring are known. The first mechanism described involves a monooxygenase attack that results in the replacement of the nitro group by a hydroxyl group (33). The monooxygenase-catalyzed reaction seems to require the presence of a hydroxyl group either *ortho* (36) or *para* (11, 33) to the nitro group. The second mechanism involves a dioxygenase attack that results in the simultaneous addition of two hydroxyl groups, one at the nitro-substituted position and the other at the adjacent position. The dioxygenase mechanism operates in pathways for biodegradation of 2,4-dinitrotoluene (34), 1,3-dinitrobenzene (8, 19) 3-nitrobenzoate (18), and 2-nitrotoluene (2, 13). The resulting dihydroxy intermediate undergoes a spontaneous rearrangement to form a catechol with the accompanying release of nitrite. The lack of oxidation of phenol, 3-nitrophenol, and

4-nitrophenol during simultaneous adaptation studies suggested a dioxygenation mechanism in strain JS765.

Although strain JS765 could grow on catechol, catechol-degrading enzymes were induced in nitrobenzene-grown cells, and cell extracts contained catechol 2,3-dioxygenase activity, the presence of catechol on the catabolic pathway could not be rigorously proven without the demonstration that nitrobenzene was converted to catechol. Inactivation of the catechol 2,3-dioxygenase with 3-chlorocatechol allowed the accumulation of catechol in cultures provided with nitrobenzene in the presence of 3-chlorocatechol, which demonstrated that catechol is a true pathway intermediate. The results of $^{18}O_2$ experiments clearly demonstrated that the initial attack is dioxygenation at the 1,2 position with the accompanying release of nitrite. The mechanism is analogous to those demonstrated for the nitroaromatic compounds cited above. It is remarkable that although the dioxygenase-catalyzed removal of the nitro group has only recently been discovered, it seems to be a widely used strategy for the degradation of nitroaromatic compounds. The genes that encode 2,4-dinitrotoluene dioxygenase from *Pseudomonas* sp. strain DNT are closely related to those that encode naphthalene dioxygenase in the pseudomonads (35). The relationships among the other dioxygenases remain to be elucidated.

The ease with which inactivation of the catechol 2,3-dioxygenase by 3-chlorocatechol was reversed in JS765 suggests that the mechanism is not an irreversible interaction at the active site, as reported for a catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 (3), but may be more similar to a reversible inactivation reported for another *P. putida* strain (14). However, inactivation of the catechol 2,3-dioxygenase in both of these cases was studied with purified and partially purified enzymes rather than with the whole cells as reported here.

The induction of both HMS hydrolase and dehydrogenase within a single organism has been reported for *Pseudomonas* sp. strain NCIB 9816 (7). Both activities are present in extracts prepared from cells of strain JS765 grown on nitrobenzene and are detectable at low levels in extracts of succinate-grown cells. The levels of activity reported here in the extracts of nitrobenzene-grown cells are low compared with the activity of catechol 2,3-dioxygenase but seem sufficient to support the rates of degradation of nitrobenzene indicated in Table 3. No effort was made to optimize the extraction or assay procedures for these enzymes, and as noted above, their activities were lost rapidly in cell extracts. When nitrobenzene-grown cells were incubated with catechol no HMS was detected in the reaction mixtures, which indicates that the levels of HMS-degrading activities are higher in intact cells.

We propose the pathway presented in Fig. 4 for the oxidative degradation of nitrobenzene by strain JS765 on the basis of the results presented above and by analogy with pathways for degradation of aromatic hydrocarbons and nitroaromatic compounds in other bacteria. Nitrobenzene dioxygenase attacks at the 1,2 position to yield a nitrohydrodiol, which spontaneously decomposes to catechol with the liberation of nitrite. Catechol 2,3-dioxygenase then catalyzes ring cleavage to form HMS. HMS dehydrogenase and hydrolase catalyze the initial steps in the further metabolism of the muconic semialdehyde.

The transformations of other nitroaromatic compounds by nitrobenzene-grown JS765 indicated that the dioxygenase enzyme induced during growth on nitrobenzene has a broad substrate range. In other bacteria able to degrade nitroaromatic compounds studied to date, both inducer specificity and substrate specificity have been high (12, 20, 34). Although the nitrobenzene degradation pathway in strain JS765 is induced only by exposure to nitrobenzene, the strain was isolated from a source which contained a mixture of many nitroaromatic compounds. An investigation is currently underway to determine the activities of strain JS765 in the presence of mixtures of nitroaromatic substrates.

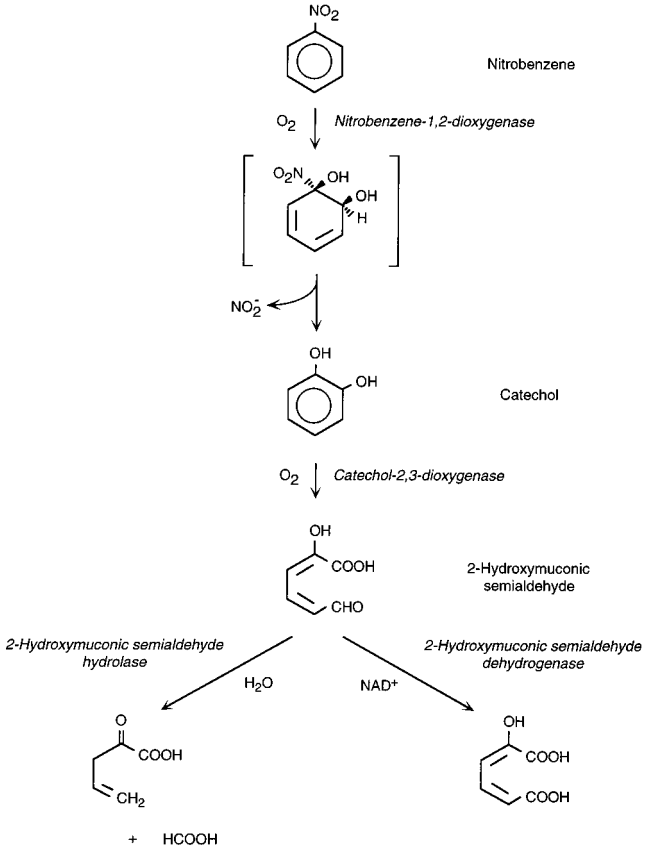

FIG. 4. Proposed pathway for oxidative degradation of nitrobenzene.

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