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Degradation of *o*-Nitrobenzoate via Anthranilic Acid (*o*-Aminobenzoate) by *Arthrobacter protophormiae*: A Plasmid-Encoded New Pathway

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An *Arthrobacter protophormiae* strain RKJ100, isolated by selective enrichment, was capable of utilizing *o*-nitrobenzoate (ONB$^+$) as the sole carbon, nitrogen, and energy source. The degradation of ONB proceeds through an oxygen insensitive reductive route as shown by the release of ammonia in the culture medium aerobically rather than nitrite ions. Thin-layer chromatography, gas chromatography, and gas chromatography-mass spectrometry of the intermediates have shown that ONB is degraded by a two-electron reduction of the nitro moiety, yielding *o*-hydroxylaminobenzoate and anthranilic acid. Quantitation of the intermediates, inhibition studies, and simultaneous induction studies have shown that anthranilic acid is produced as the terminal aromatic intermediate of a catabolic energy-yielding pathway and not as a side reaction taking place concurrently which is the first such report. A plasmid of approximately 65 kb was found to be responsible for harboring genes for ONB degradation in this strain. The same plasmid also encoded resistance to cobalt ions. © 2000 Academic Press

***Key Words: A. protophormiae; o*-nitrobenzoate degradation; new pathway; anthranilic acid; plasmid.**

Nitrobenzene, nitrophenols, nitrotoluenes, and nitrobenzoates are widely employed in the production of dyes, plastics, explosives, pharmaceuticals, polyurethane foams, elastomers and pesticides (1–3). Microorganisms either eliminate the nitro group as nitrite as in oxidative pathways (1–3, 5–7) or reduce the nitro group to an amino group as in reductive pathways (1–3, 8); aminoaromatic intermediates are then mineralized (9, 19) or accumulate as dead-end products which are highly cytotoxic and/or mutagenic (11–15). A third mechanism which involves an initial reduction of the ring and subsequent release of the nitro group from an intermediate Meisenheimer complex has now been elucidated (16, 17).

Reduction of the nitro group can be achieved by several different ways by nitroreductases. Oxygen-insensitive (type I) enzymes reduce the nitro group which results in the production of nitroso, hydroxylamino and amino derivatives as a consequence of a series of two-electron transfers (8, 18). This reaction is mainly found under anaerobic conditions and leads to the formation of a terminal aromatic amine which is highly reactive (19). The occurrence of hydroxylamino intermediates is well documented in various studies (1–3, 10, 20) but the nitroso intermediate is difficult to isolate in a productive catabolic pathway due to its highly reactive nature (1, 2, 19). The amino-substituted aromatic compound can also be produced as a dead end metabolite and in the presence of oxygen dark polymerization products can be formed (3, 21). On the other hand, oxygen sensitive (type II) enzymes yield a nitro anion radical as a result of one electron reduction of the nitro group (2, 22). The nitro anion radical may form superoxide after reacting with oxygen regenerating the parent nitro aromatic compound (2, 23). The reduction of the nitro group with elimination as ammonia has also been elucidated for the degradation of some nitro-substituted aromatic compounds. The degradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45 proceeds by the reduction of the nitro group to an amino-substituted phenol from which ring cleavage takes place (9). *Comamonas acidovorans* degrades *p*-nitrobenzoate by partial reduction of the nitro group to *p*-hydroxylaminobenzoate from which ammonia is released generating protocatechuate which undergoes ring fission (10, 24).

Anthranilic acid (*o*-aminobenzoate) has been implicated as an important intermediate in metabolic pathways of compounds containing an indole moiety by microorganisms (25–27) or as an intermediate in the biosynthesis of tryptophan (28–30). Earlier studies have shown that aminobenzoates are formed in the degradation of substituted nitrobenzoates as reductive

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side reactions which operate concurrently (3, 8, 31–34). Ke *et al.* found that a *Flavobacterium* sp., after growth on ONB, could not sequentially induce the enzymes to oxidize anthranilic acid (26). In all these studies anthranilic acid was not found to be an obligate intermediate in the direct energy-producing catabolic pathway but was postulated to be in a side reaction which operates concurrently. Bacteria transform anthranilic acid aerobically to catechol, salicylate or gentisate (32, 34–36). The pathway of anaerobic degradation of anthranilic acid is not very well understood but benzoate has been implicated in a study (37).

The present paper elucidates the oxygen-insensitive reductive catabolic pathway of ONB and provides evidence that anthranilic acid is not produced as a side reaction but is an obligate intermediate in a direct energy-yielding metabolic pathway by *Arthrobacter protophormiae* strain RKJ100. It was also demonstrated that the genes for the entire ONB degrading pathway are encoded on an approximately 65 kb plasmid in this strain which also encodes resistance to inorganic cobalt ions. To our knowledge, this is the first such conclusive and direct report.

MATERIALS AND METHODS

Isolation of strain RKJ100. Soil microorganism(s) capable of utilizing nitroaromatic compounds were enriched in the presence of *p*-nitrophenol (PNP) as sole carbon source. Soil samples from local agricultural fields which were sprayed with pesticides such as parathion or methyl parathion were used as the inoculum into minimal media (MM; 38). PNP (0.5 mM final concentration) was provided as the sole carbon and energy source and enrichment cultures were incubated at 30°C in a shaker at 200 rpm. Microbial growth was measured by the increase in optical density (OD) at 600 nm and the yellow color of PNP changed to colorless indicating its utilization by the microorganism(s). Following three subcultures, samples were plated out onto MM agar with PNP as sole carbon source. Following this rigorous exercise, one efficient PNP degrading microorganism was isolated and designated as strain RKJ100. This soil isolate was screened for growth on other nitroaromatic compounds and was also found to utilize ONB as sole source of carbon and energy.

Growth conditions and preparation of crude cell extracts. Minimal medium (MM) used and nitrite release assay were as described earlier (38). Ammonia was detected by measuring the oxidation of NADH in the presence of 2-oxoglutarate and L-glutamate dehydrogenase (Sigma Chemical Co., St. Louis, MO). RKJ100 was grown on ONB (0.5 mM final concentration) and succinate (10 mM) was also added to supplement the growth, wherever necessary. After overnight growth, cells were harvested, washed twice in ice cold potassium-phosphate buffer (pH 7.0) and resuspended in the same buffer (0.3 g cells/ml of buffer) at 4°C. For preparation of crude cell extracts lysozyme was added to a final concentration of 10 mg/ml and cells were incubated at 30°C for an hour. The cells were washed once more with the buffer to rinse off the lysozyme and lysed by two passages through a chilled French pressure cell at 20,000 lb/in^2. The supernatant (crude cell extract) was used for biotransformation studies.

Quantitation of intermediates. For quantitation of ONB depletion, RKJ100 was grown on ONB (0.5 mM) and samples were collected after every hour, centrifuged at 10,000 rpm for 10 min and the supernatants were monitored by scanning in the UV range from 200 to 300 nm in a Beckman DU 600 spectrophotometer. The decrease in the λ_{max} at 268 nm, which is the absorbance maximum of ONB at pH 7.0, was read and the residual ONB was calculated from a standard curve prepared using authentic ONB. Quantitation of total arylamines was achieved by the Bratton–Marshall procedure as mentioned by Rafii *et al.* (39) as well as by the colorimetric procedure outlined by Oren *et al.* (40). Nitroso- or hydroxylamino compounds were qualitatively observed by the colorimetric method of Harada and Omura as described earlier (21).

Plasmid curing. The derivatives incapable of utilizing ONB as sole carbon source were obtained spontaneously or by treatment with mitomycin C as described earlier (38), except that the concentration of mitomycin C was 5 μg/ml. Plasmid DNA isolations were carried out by all the methods cited in our previous communication (38), but the method of O'Sullivan and Klaenhammer (41) was found to be most appropriate. Purification of plasmid DNA by Cesium Chloride-Ethidium Bromide density gradients was carried out according to Sambrook *et al.* (42). Restriction endonuclease digestion with enzymes *Hin*dIII, *Pst*I, *Pvu*II was performed according to the instructions supplied by the manufacturer (Boehringer, Mannheim, Germany). The conditions for restriction endonuclease digestion and electrophoresis were as described by Jain *et al.* (43).

Heavy metal resistance of strain RKJ100 and its ONB$^-$ derivatives was determined by inoculating a loopful of overnight nutrient broth grown culture onto freshly prepared heavy metal plates as described by Bopp *et al.* (44).

Thin layer chromatography and gas chromatography. Thin layer chromatography (TLC) and gas chromatography (GC) of the intermediates in ONB degradation were carried out to elucidate the pathway. RKJ100 was grown in 500 ml of MM containing ONB or anthranilic acid as sole carbon source and samples (100 ml) were collected after every 2 h, centrifuged and extracted with ethyl acetate as mentioned earlier (38). These were evaporated to dryness and resuspended in 100–200 μl of methanol and analyzed. Different solvent systems for the separation of ONB intermediates consisted of: A, toluene:ethyl acetate:acetic acid (60:30:5); B, ethanol:ammonia: water (80:4:16) and C, n-butanol:glacial acetic acid:water (4:1:5). The compounds were detected under UV radiation, *o*-hydroxylaminobenzoate and anthranilic acid by their blue fluorescence and ONB by its quenching. Arylamines were also detected by spraying the TLC plates with Ehrlich's reagent which consisted of 1% *p*-dimethyaminobenzaldehyde in 200 ml of *n*-butanol:ethanol (30:70, v/v) in which 30 ml of conc. HCl was added (45, 46).

Gas chromatography was carried out as described earlier (38) except that the oven temp was kept at 250°C and injector and detector temp were at 280°C. Standard ONB, *o*-hydroxylaminobenzoate, anthranilic acid, protocatechuate, catechol, gentisate, 3-hydroxyanthranilic acid, 2,3-dihydroxybenzoate and salicylic acid (10 mg/ml in methanol) were also analyzed for comparison studies.

Gas chromatography-mass spectrometry (GC–MS). GC–MS analysis was carried out using a Shimadzu QP5000 GC–MS equipped with a quadrupole mass filter and DB-1 (100% dimethylpolysiloxane) capillary column (30 m $\times$ 0.25 mm), ionization of 70 eV, scan interval 1.5 s and mass range 40 to 500. The oven parameters were 150°C for 5 min with 10°C increase per minute to a final temperature of 300°C for 20 min; the injector temperature was kept at 250°C.

Synthesis of o-hydroxylaminobenzoate. *o*-Hydroxylaminobenzoate was synthesized as described by Bauer and Rosenthal (47), with minor modifications. ONB (1.7 g) and NH_4Cl (2 g) were dissolved in 35 ml dH_2O which contained 2 ml of 5 N NaOH. Zinc powder (1.5 g) was added over a period of 20–30 min with gentle stirring between 15–20°C. Zinc sludge was then filtered off and the filtrate was treated with 7.25 M HCl until the pH turned acidic and needle like crystals formed which were pale-yellow. These were vacuum filtered onto Whatmann No. 1 paper circles using Buchner funnel and allowed to dry; and then scrapped off and dissolved in methanol for analysis. Further purification of *o*-hydroxylaminobenzoate from the mixture was achieved by scrapping the spots from a preparative layer chro-

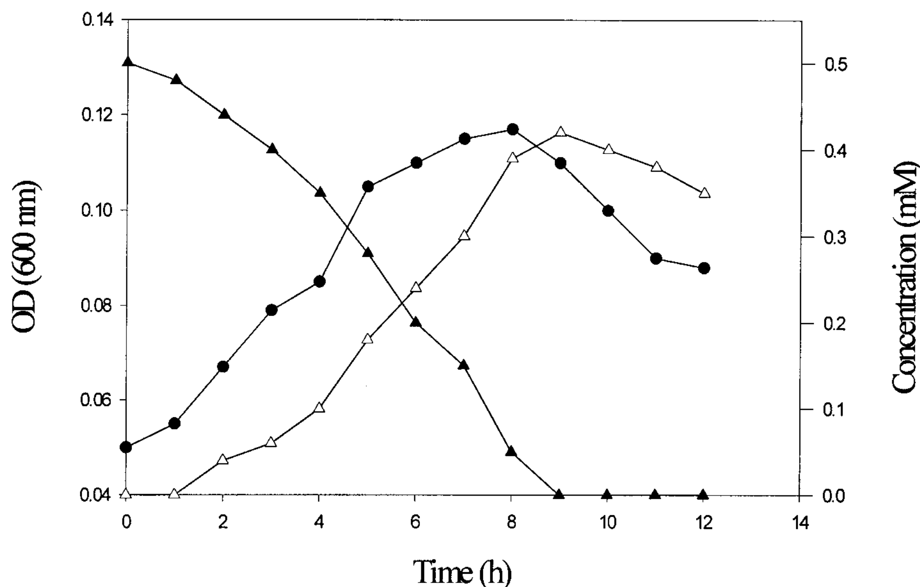

FIG. 1. Degradation of ONB by *A. protophormiae* strain RKJ100. ONB is metabolized with concomitant release of ammonia. The inoculum used was obtained from washed cells of RKJ100 grown overnight in ONB. ●, The culture optical density (OD); ▲, ONB concentration; △, ammonia concentration.

matographic plate 60 F_{254} (20 × 20 cm, thickness 2 mm, E. Merck, Germany) and the purity was checked by GC–MS.

Chemicals. ONB, anthranilic acid, protocatechuate, catechol, gentisate, 3-hydroxyanthranilic acid, 2,3-dihydroxybenzoate, salicylic acid, 2,2′-dipyridyl and *p*-dimethylaminobenzaldehyde (Ehlrich's reagent) were purchased from Sigma Chemical Co. (U.S.A.). 3,3′-(4,4′-Biphenylene) bis (2,5-diphenyl-2 H-tetrazolium chloride) was purchased from Aldrich Chemical Co. (USA). All other chemicals were of the highest purity available.

RESULTS AND DISCUSSION

Identification of RKJ100. Strain RKJ100 was identified on the basis of morphological and biochemical tests outlined in the Bergey's Manual of Systematic Bacteriology (48). Chemotaxonomical studies on the whole cells of strain RKJ100 for the identification of diaminopimelic acid and carbohydrates were carried out as described earlier (49). Strain RKJ100 was a gram positive, pleomorphic, oxidase negative, catalase positive, aerobic and motile organism. It utilized a variety of carbon and nitrogen sources, including adenine, D-fructose, citrate, pyruvate, meso-inositol, L-arabinose, xanthine, raffinose, hypoxanthine, D-xylose, sucrose, galactose, glucose and salicin. The microorganism was unable to utilize chitin, gelatin, starch, Tween 80, tyrosine, casein, guanine, testosterone, ethanol, sebacic acid, esculin and arbutin. Chemotaxonomy exhibited the presence of lysine as diagnostic diamino acid as well as alanine and glutamic acid. Glucose, xylose and ribose were present in the cell wall, mycolic acids were absent. Based on these morphological, biochemical and chemotaxonomical tests, strain RKJ100 was identified as *Arthrobacter protophormiae.*

Growth on ONB. Arthrobacter protophormiae RKJ100 was capable of utilizing ONB as sole source of carbon and energy. The microorganism also utilized ONB as a nitrogen source since exclusion of an additional nitrogen source (ammonium sulfate) in the MM did not affect its growth and ammonium ions were released rather than nitrite in the medium suggesting the involvement of reductive step(s) in the degradation of ONB. When strain RKJ100 was grown in the absence of another nitrogen source (ammonium sulfate), ammonium ions were released in the medium concomitant with the degradation of ONB. However, this was not stoichiometric indicating utilization of released ammonia as a source of nitrogen by the bacteria (Fig. 1). Strain RKJ100 was also capable of growth on anthranilic acid as sole carbon and energy source but failed to grow on salicylic acid, 3-hydroxyanthranilic acid and 2,3-dihydroxybenzoate which may be the probable intermediates in the pathway.

Identification of location of gene(s) for ONB degradation. It is well documented that many a times the degradative capabilities in bacteria are plasmid-encoded (38, 43, 50, 51). To determine if a plasmid(s) is present in strain RKJ100 that could be involved in ONB degradation, attempts were made to isolate plasmid(s) from the microorganism. A plasmid of app. 65 kb in size was found to be present using the method of O'Sullivan and Klaenhammer (41); *E. coli* V517 was used as standard plasmid size marker (Fig. 2). Purified DNA preparations by CsCl-EtBr gradient centrifugation from strain RKJ100 were digested with enzymes *Hin*dIII, *Pst*I, *Pvu*II to confirm the size of the plasmid (data not shown). Attempts were also made to obtain ONB^- derivatives (spontaneous and mitomycin C derived) to determine the role of plasmid(s) present in

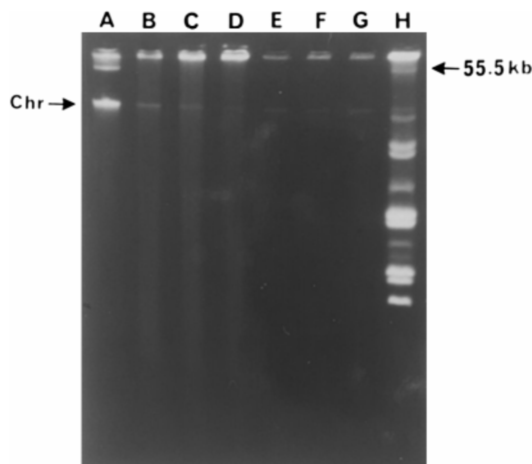

FIG. 2. Agarose gel electrophoresis of plasmid isolated from *A. protophormiae* strain RKJ100 and its cured derivatives. Lanes: A, strain RKJ100; B–D, spontaneous ONB$^-$ Cob^s derivatives; E–G, mitomycin C-derived ONB$^-$ Cob^s derivatives; H, *E. coli* V517 as standard plasmid size marker. Chr, chromosomal DNA.

RKJ100. The spontaneous loss of ONB$^-$ phenotype was app. 2% which increased to 10% when mitomycin C was present during growth. When such spontaneously or mitomycin C derived ONB$^-$ derivatives were checked for the presence of plasmid(s) by different methods (see Materials and Methods), the plasmid of approximately 65 kb in size was absent from them (Fig. 2). These ONB$^-$ derivatives were also checked for growth on anthranilic acid and they failed to grow. These results, therefore, suggested that the degradation of ONB is plasmid-encoded and that the genes for its degradation reside on a 65 kb plasmid. However, our attempts to transfer the purified plasmid in alternate hosts like *Pseudomonas putida* PaW340 (38) and in the cured plasmid-free derivatives of RKJ100 by triparental matings, chemical transformation, protoplast fusion or electroporation were unsuccessful.

It is often found that the resistance to heavy metals by bacteria are also plasmid-encoded (15, 44, 52). Strain RKJ100 and its ONB$^-$ derivatives (spontaneous and mitomycin C-derived) were tested for their resistance to different heavy metal salts such as zinc sulfate, sodium selenite, sodium selenate, cobaltous nitrate, sodium arsenite, sodium arsenate, mercuric chloride, ammonium molybdate etc. Based on these studies, it was concluded that the gene(s) for cobalt resistance is also encoded on the plasmid present in strain RKJ100 since this strain was resistant to cobalt whereas ONB$^-$ derivatives were sensitive.

Identification of intermediates in the pathway. Earlier reports indicate the presence of anthranilic acid in the pathway of ONB, but only as product of a side chain reaction; catechol was found to be the major metabolite of the oxidation of ONB (34, 45). To determine the ONB degradative pathway in strain RKJ100, TLC and GC studies were initially carried out on the extracted intermediates (see Materials and Methods). The TLC results using different solvent systems are shown in Table 1. ONB and anthranilic acid were detected in samples drawn from 2 h to 10 h intervals. Another blue fluorescing spot could be detected which corresponded well with that of *o*-hydroxylaminobenzoate synthesized in the laboratory. When TLC plates were sprayed with Ehlrich's reagent, an immediate yellow color was apparent in case of anthranilic acid and suspected *o*-hydroxylaminobenzoate spots indicative of amino compounds (45, 46). Similarly, strain RKJ100 was also grown on anthranilic acid as sole carbon source and supernatants were extracted and analyzed by TLC. Only anthranilic acid spots were apparent from 2 h to 12 h samples and after overnight growth no spots could be detected indicating the total utilization of anthranilic acid (data not shown). The samples processed for TLC were also analyzed for the presence of nitroso- or hydroxylamino compounds by a colorimetric test as reported earlier (21). The supernatants extracted after growth from 2 h to 10 h exhibited red color characteristic of nitroso- or hydroxylamino compounds, but samples drawn after overnight growth showed neither the

TABLE 1

Identification of Intermediates Formed in the Reductive Degradation of ONB by *Arthrobacter protophormiae* Strain RKJ100 by TLC and GC Analyses

	R_f values in different solvent systems used in TLC studies[a]						Retention time (min) in GC studies	
	A		B		C			
Compounds	(s)	(i)	(s)	(i)	(s)	(i)	(s)	(i)
o-Nitrobenzoate	0.48	0.48	0.48	0.48	0.71	0.72	2.880	2.875
o-Hydroxylaminobenzoate	0.56	0.57	0.56	0.57	0.87	0.87	3.820	3.802
Anthranilic acid	0.66	0.66	0.66	0.66	0.79	0.78	2.552	2.545

[a] Composition of the solvent systems are given in the text. R_f values calculated as distance of analyte from baseline/origin to distance traveled by solvent from baseline. s, standard compound used; i, putative intermediate identified.

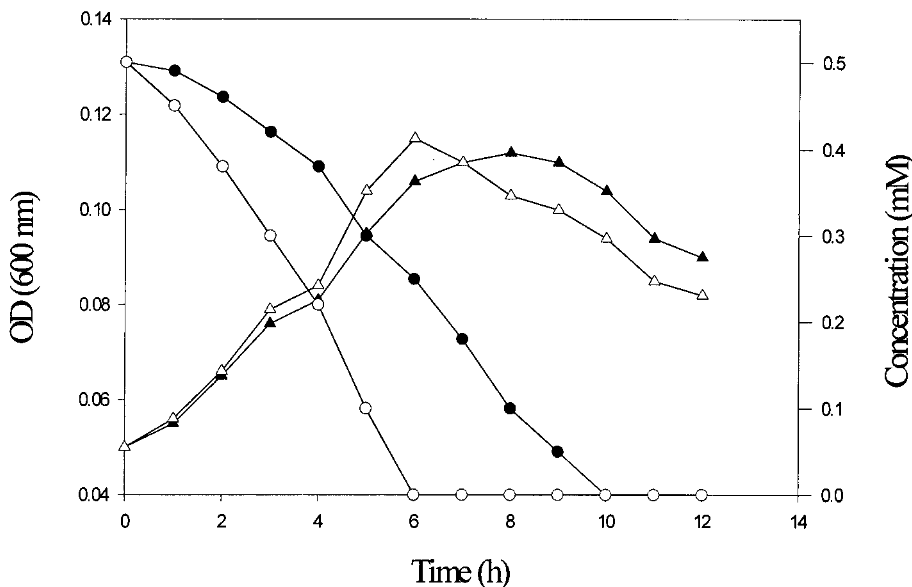

FIG. 3. Sequential and back induction studies of nitroreductase(s) in *A. protophormiae* strain RKJ100. △, The culture optical density (OD) which was induced with ONB and grown on anthranilic acid to exhibit sequential induction; ○, anthranilic acid depletion when the cells were induced with ONB; ▲, the culture optical density (OD) which was induced with anthranilic acid and grown on ONB to exhibit back induction; ●, ONB depletion when the cells were induced with anthranilic acid.

presence of any intermediates by TLC nor any color reaction was observed for nitroso- or hydroxylamino compounds again indicating their total degradation. The ONB^- derivatives of strain RKJ100 (spontaneous and mitomycin C derived) were also grown in ONB or anthranilic acid supplemented with succinate as additional carbon source and extractions were carried out after 6, 12, 24 and 48 h time intervals. No intermediates were detected in the cured derivatives even after 48 h of growth indicating the plasmid-encoded degradative capability in strain RKJ100.

To further confirm the presence of *o*-hydroxylaminobenzoate and anthranilic acid in the degradation pathway, GC studies were performed on the samples processed for TLC as described above. As shown in Table 1, the presence of ONB and anthranilic were indicated in samples drawn from 2 h to 10 h intervals based on the retention times of these compounds with that of the standards. A major peak at 3.80 min corresponded well with the synthesized *o*-hydroxylaminobenzoate. The presence of anthranilic acid and *o*-hydroxylaminobenzoate in the extracted samples was further confirmed in co-elution studies by addition of authentic anthranilic acid and *o*-hydroxylaminobenzoate in the above samples and analyzed by GC (data not shown) which proved the presence of these compounds in the biological extracts. The samples drawn after overnight growth did not show any of these compounds indicating their total degradation. To further ascertain whether anthranilic acid is converted into polar intermediate(s) such as catechol or protocatechuate, the ring cleavage enzyme was inhibited by incorporating 2,2′-dipyridyl in the growth medium, which chelates ferrous ions required for cleavage of the aromatic ring (4, 7, 53), along with ONB or anthranilic acid; succinate was added as an additional carbon source. The supernatants were extracted for intermediates after 6, 12, 24 and 48 h of growth and analyzed by TLC and GC studies. When ONB was used as substrate, *o*-hydroxylaminobenzoate and anthranilic acid accumulated even after 48 h of growth. However, in these studies there was no evidence of any other intermediates such as catechol or protocatechuate formed when ONB or anthranilic acid were used as substrates. Protocatechuate or catechol are typically important intermediates in the biodegradative pathways of nitrobenzoates (1–3, 10, 24, 32, 34). To exclude the possibility of any intracellular processing or permeability barriers of intermediates, the experiments described above were also carried out with crude cell extracts of RKJ100 grown on ONB and incubated with ONB or anthranilic acid. After 2 h, intermediates were extracted and analyzed by TLC and GC. The results were found comparable to that of the biotransformation experiments carried out with whole cells; i.e., no other intermediates were detected except *o*-hydroxylaminobenzoate and anthranilic acid which were utilized after further incubation. Thus, taken together, TLC and GC studies indicate that ONB is degraded via *o*-hydroxylaminobenzoate and anthranilic acid and the latter is the terminal aromatic intermediate from which ring-cleavage and ammonia release may take place thereafter.

Simultaneous adaptation studies. If anthranilic acid is not an obligate intermediate in the reductive

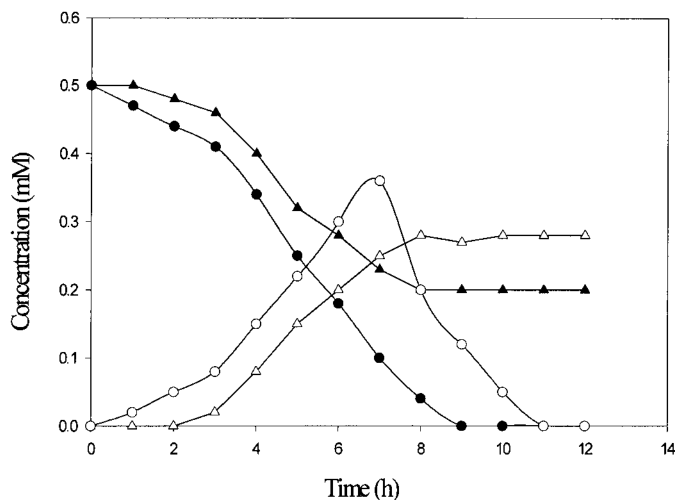

FIG. 4. Quantitation of ONB depletion and total arylamine formation by *A. protophormiae* strain RKJ100. ●, ONB depletion; ○, total arylamine formation; ▲, ONB depletion when ring cleavage was blocked by 2,2′-dipyridyl; △, total arylamine formation when ring cleavage was blocked by 2,2′-dipyridyl.

pathway of ONB, there should be an extended lag period to utilize anthranilic acid when cells are induced with ONB since enzymes to utilize anthranilic acid would not be synthesized (45). To test this hypothesis, two sets of experiments were carried out. In one set, the cells after being induced with ONB as sole carbon source were grown on anthranilic acid (to test for sequential induction) and in the other set, the cells were induced with anthranilic acid and grown on ONB as sole carbon source (to test for back induction). The depletion of ONB and anthranilic acid were monitored at hourly intervals. As shown in Fig. 3, ONB-induced cells grew on anthranilic acid and anthranilic acid-induced cells also grew on ONB without a lag period. Succinate grown cells always exhibited a lag of several hours before they could utilize either ONB or anthranilic acid as growth substrates indicating the inducible nature of enzymes. This shows that sequential and back induction of enzymes are very efficient in strain RKJ100. It could further be concluded that ONB-induced cells could utilize 0.5 mM of anthranilic acid in 6 h and anthranilic acid-induced cells could utilize 0.5 mM of ONB in 10 h thus proving our hypothesis of sequential and back induction in strain RKJ100. This was also checked by the extraction of intermediates and analyzed by TLC and GC; the results obtained were compatible to that of the spectrophotometeric quantitations; i.e., when RKJ100 was induced with ONB, it completely utilized anthranilic acid after 6 h (sequential induction) and anthranilic acid-induced cells could also completely utilize ONB in 10 h (back induction) (data not shown).

Quantitation of total arylamines. Since *o*-hydroxylaminobenzoate also reacted with the colorimetric tests for anthranilic acid, the combined concentrations of both the intermediates could be calculated by these procedures and are represented as total arylamines produced. These were measured in the deproteinized solution by diazotization and coupling with N-1-naphthylethylenediamine dihydrochloride according to the method of Rafii *et al.* (39) and also by the colorimetric method of Oren *et al.* (40). Although both methods yielded reproducible results, quantitations were more accurate by the diazotization method of Rafii *et*

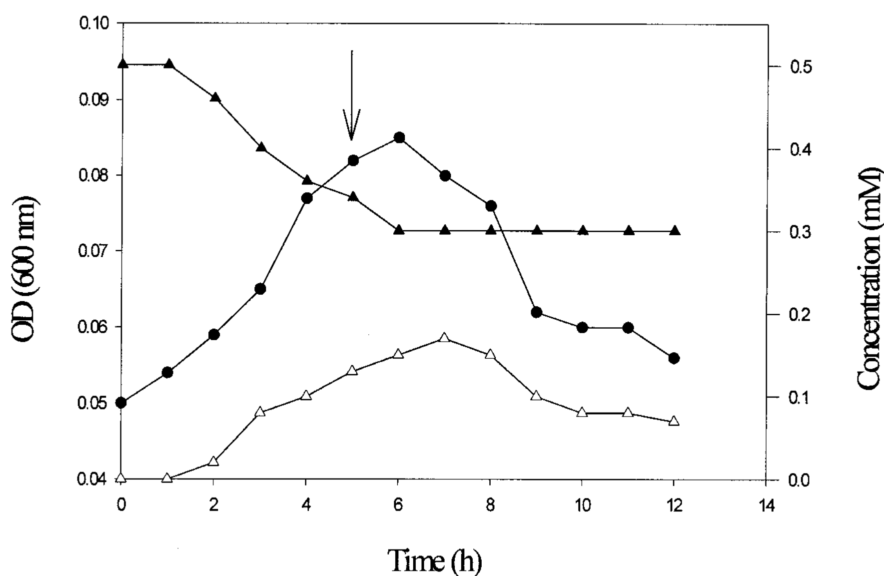

FIG. 5. Effect of sodium nitrite as nitroreductase inhibitor in *A. protophormiae* strain RKJ100. ●, culture optical density (OD); ▲, ONB depletion; △, total arylamine formation. The arrow indicates the time at which sodium nitrite was added in the medium.

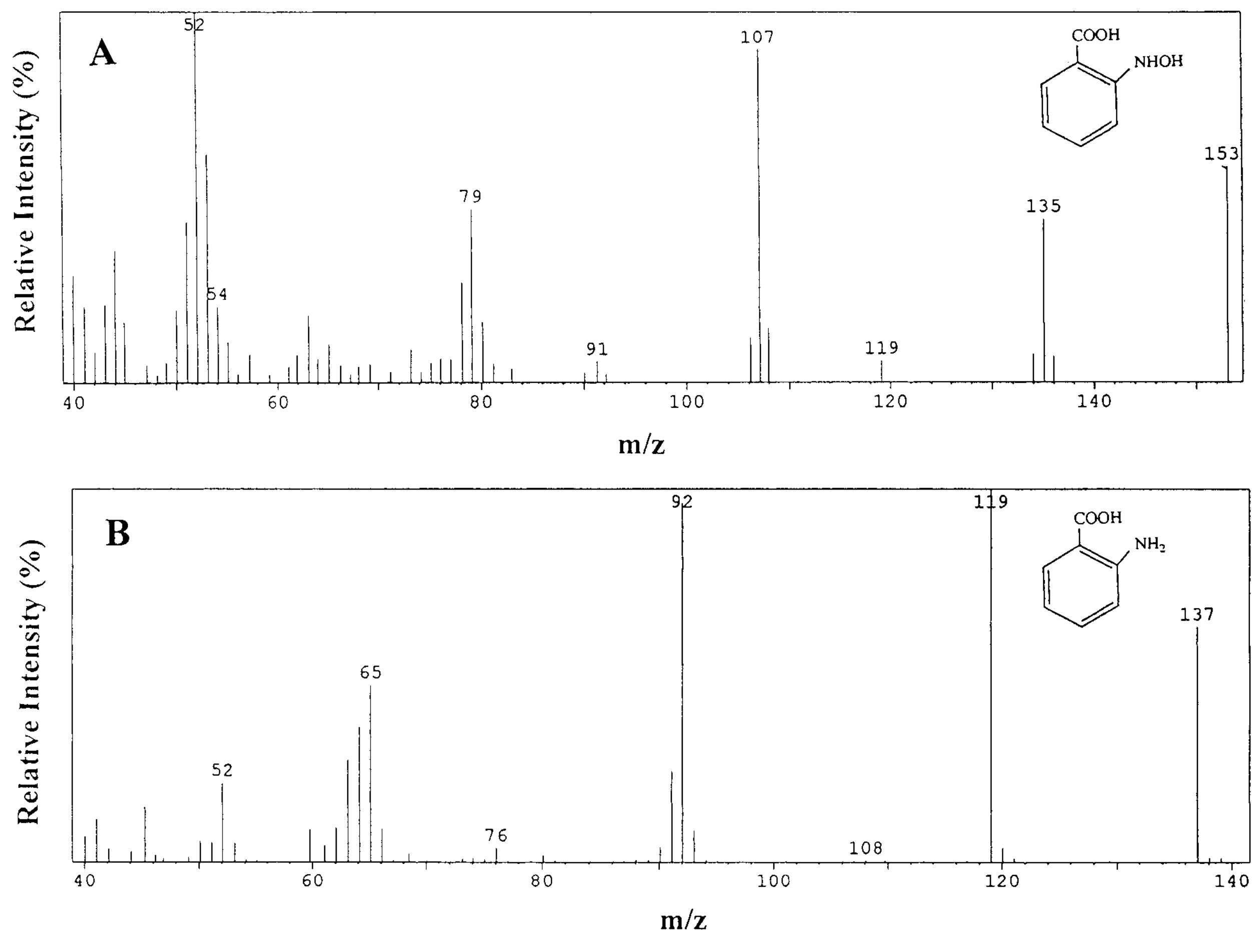

FIG. 6. (A) Mass spectrum of *o*-hydroxylaminobenzoate from cell extracts of *Arthrobacter protophormiae* strain RKJ100 following incubation with ONB. (B) Mass spectrum of anthranilic acid from cell extracts of *Arthrobacter protophormiae* strain RKJ100 following incubation with ONB.

al. (39) and are presented here. In one set of experiments RKJ100 was grown in the presence of ONB as sole carbon source, and in the other set 2,2′-dipyridyl was incorporated with ONB to block the ring cleavage of ONB (4, 7, 53) with succinate (10 mM) as additional carbon source. These results are shown in Fig. 4. Arylamines appeared in the growth medium after 2 h and peaked at 7 h. When the ring cleavage was blocked with 2,2′-dipyridyl, the degradation was slowed and arylamines appeared after 3 h and became constant after 8 h of growth. These observations were again checked by extracting intermediates from the supernatants after 3 h intervals and analyzed by TLC and GC. Based on these studies, it was concluded that in the absence of 2,2′-dipyridyl, *o*-hydroxylaminobenzoate and anthranilic acid were detected till 8 h of growth, but when 2,2′-dipyridyl was present in growth medium, *o*-hydroxylaminobenzoate and anthranilic acid could be detected even after 48 h of growth. This shows that *o*-hydroxylaminobenzoate and anthranilic acid are the major metabolites in the pathway of ONB and are not produced because of a side reaction operating concurrently as shown earlier for anthranilic acid and its isomers (3, 8, 21, 31–34, 39).

Effect of sodium nitrite as reductase(s) inhibitor. It is reported that nitrite acts as an inhibitor of nitroreductase and preferentially blocks the conversion of hydroxylamino compound into amino compounds (8). We performed another experiment in which strain RKJ100 was grown in the presence of ONB as sole carbon source and ONB depletion and arylamine formation were monitored after every hour of growth; around mid log-phase, 10 mM sodium nitrite was added in the medium. As shown in Fig. 5, after the addition of sodium nitrite, growth and ONB depletion ceased within an hour and the appearance of arylamines was not stoichiometric. This indicates that since the catabolic pathway of ONB involves anthranilic acid formation and the conversion of *o*-hydroxylaminobenzoate to anthranilic acid was blocked, the cells failed to grow further. When these experiments were repeated with glucose as an additional carbon source with ONB and nitrite added after 4 h of growth, the degradation of

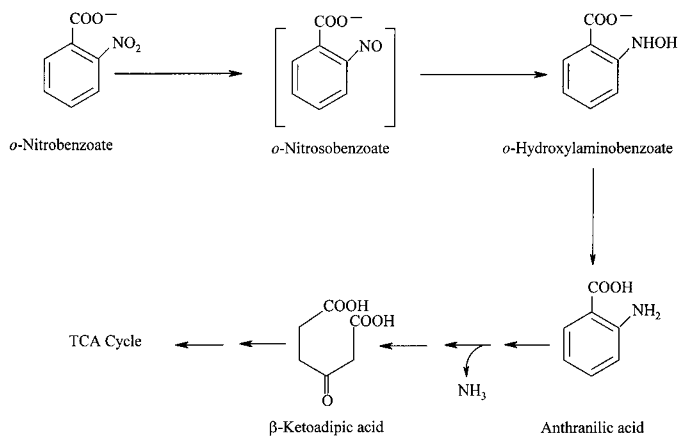

FIG. 7. Proposed initial pathway for the reductive degradation of ONB by *Arthrobacter protophormiae* strain RKJ100.

ONB ceased gradually but the microorganism could grow well (data not shown), confirming the earlier observation that nitrite inhibits nitroreductase(s) activity. This again clearly shows that anthranilic acid is the terminal aromatic intermediate in the aerobic reduction of ONB and is not a side reaction product.

GC–MS studies. The final confirmation of *o*-hydroxylaminobenzoate and anthranilic acid in the pathway of ONB degradation came from the GC–MS studies. Strain RKJ100 was grown on ONB as sole carbon source and the metabolites were extracted (see Materials and Methods). As shown in Fig. 6A, *o*-hydroxylaminobenzoate with a retention time of 11.16 min and a molecular ion [M^+] peak at *m/z* 153 corresponding to the molecular mass of *o*-hydroxylaminobenzoate and major fragmentation ions at *m/z* 135 (M^+ $-H_2O$), 107 (M^+ $-H_2O-CO$) and 79 (M^+ $-H_2O-CO-C_2H_2$) perfectly matched with the fragmentation pattern of synthesized *o*-hydroxylaminobenzoate. In addition, as shown in Fig. 6B, anthranilic acid with a retention time of 4.99 min and a molecular ion [M^+] peak at *m/z* 137 corresponding to the molecular mass of anthranilic acid and major fragmentation ions at *m/z* 119 (M^+ $-H_2O$), 108 (M^+ −CHO) and 92 (M^+ −COOH) was identical to the fragmentation pattern of authentic anthranilic acid.

Based on the quantitation and inhibition studies, and the chemical characterization of the isolated intermediates by TLC, GC and GC–MS studies, we propose a new pathway for the degradation of ONB via anthranilic acid which has been outlined in Fig. 7. It involves a two step reduction of ONB into *o*-hydroxylaminobenzoate and anthranilic acid. However, we were not able to isolate and characterize *o*-nitrosobenzoate which is the likely precursor of *o*-hydroxylaminobenzoate in the reductive pathway of ONB since nitroso intermediates are known to be highly reactive and defy direct isolation from *in vivo* studies (2, 19). Extracts from cells grown on ONB gave a strong positive purple color in the Rothera test (54) from anthranilic acid indicating subsequent formation of β-ketoadipate in the pathway.

Bacteria transform anthranilic acid aerobically to catechol, salicylate or gentisate (32, 34–36) from which ring cleavage takes place. However, we could not detect any other intermediate(s) from anthranilic acid in the catabolic reductive pathway in *Arthrobacter protophormiae* strain RKJ100 except β-ketoadipate. In this study, we show that anthranilic acid is a major intermediate in the reductive, plasmid-encoded degradation of ONB, however, the mechanism by which anthranilic acid is cleaved to form β-ketoadipate is the matter of future studies.

To our knowledge, this is the first direct and conclusive report which has shown anthranilic acid to be in a direct energy yielding catabolic reductive pathway of ONB and not as a side reaction product. Furthermore, this entire pathway is encoded on an approximately 65 kb plasmid in strain RKJ100. The bioremediation potential of this newly isolated *Arthrobacter protophormiae* is enormous since it utilizes ONB by the action of oxygen insensitive enzymes. In general, reduction of nitroaromatic compounds result in the production of substituted aminoaromatics as dead end products; which may even be more toxic than the parent compounds. We are currently cloning the gene(s) for ONB degradation using broad host-range cosmid vector(s) to gain an insight into the regulation of synthesis of ONB degradative enzymes at the molecular level.

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