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Anaerobic and Aerobic Degradation of Pyridine by a Newly Isolated Denitrifying Bacterium

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New denitrifying bacteria that could degrade pyridine under both aerobic and anaerobic conditions were isolated from industrial wastewater. The successful enrichment and isolation of these strains required selenite as a trace element. These isolates appeared to be closely related to *Azoarcus* species according to the results of 16S rRNA sequence analysis. An isolated strain, pF6, metabolized pyridine through the same pathway under both aerobic and anaerobic conditions. Since pyridine induced NAD-linked glutarate-dialdehyde dehydrogenase and isocitratase activities, it is likely that the mechanism of pyridine degradation in strain pF6 involves N–C-2 ring cleavage. Strain pF6 could degrade pyridine in the presence of nitrate, nitrite, and nitrous oxide as electron acceptors. In a batch culture with 6 mM nitrate, degradation of pyridine and denitrification were not sensitively affected by the redox potential, which gradually decreased from 150 to −200 mV. In a batch culture with the nitrate concentration higher than 6 mM, nitrite transiently accumulated during denitrification significantly inhibited cell growth and pyridine degradation. Growth yield on pyridine decreased slightly under denitrifying conditions from that under aerobic conditions. Furthermore, when the pyridine concentration used was above 12 mM, the specific growth rate under denitrifying conditions was higher than that under aerobic conditions. Considering these characteristics, a newly isolated denitrifying bacterium, strain pF6, has advantages over strictly aerobic bacteria in field applications.

Heterocyclic compounds are generated by many industries. Most of these chemicals are toxic to human health (39). Pyridine, one of the important *N*-heterocyclic compounds, occurs in the environment as a result of oil shale retorting, coal gasification, and pesticide use (40, 42). The presence of pyridine in the environment creates severe health hazards because pyridine is toxic and teratogenic.

The biodegradation of pyridine under aerobic conditions has been studied extensively (22, 38, 41). Aerobic bacteria capable of growing on pyridine have been isolated, and their metabolic pathways have been determined. However, most of the released pyridine is present in the anaerobic subsurface environments because of its water solubility (24). Accordingly, it is important to understand the biodegradation of pyridine under anaerobic conditions. The degradation of pyridine under anaerobic conditions is, however, less clear (20).

The anaerobic degradation of aromatic compounds under denitrifying (10, 11), methanogenic (46), sulfate-reducing (32), and ferric iron-reducing (27) conditions has been reported. Among several anaerobic metabolisms, denitrification is a facultative trait. In addition, nitrate is used as an electron acceptor in denitrification. Compared with other anaerobic electron acceptors, the use of nitrate in bioremediation can be advantageous in view of its water solubility, cost, and lack of toxicity. This high potential of denitrifying bacteria for biodegradation has been recognized, and therefore active research on the biodegradation of xenobiotics under denitrifying conditions is being conducted (4, 11, 12, 33).

In our laboratory, we have studied extensively the removal of pyridine in retort water by aerobic bacteria (22, 23, 35). To improve the efficiency of biodegradation in an oxygen-limited environment, we planned to use denitrifying bacteria. Here we describe the isolation of new bacteria that grow on pyridine as the sole carbon source under denitrifying conditions and the characterization of the isolated bacteria in the application aspects.

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MATERIALS AND METHODS

Enrichment and isolation. The basal medium used contained 0.90 g of K_2HPO_4, 0.54 g of KH_2PO_4, 0.1 g of KCl, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $CaCl_2 \cdot 2H_2O$, 1.5 g of $NaNO_3$, 1 ml of trace element solution, 1 ml of vitamin solution, and 1 ml of selenite-tungstate solution (45) per liter of deionized water. Individual salt and vitamin solutions were sterilized separately before being mixed into the basal medium. The medium was reduced with 0.1 ml of $Na_2S \cdot 9H_2O$ solution (10 g/liter; in a closed bottle) and 1 ml of resazurine solution (1 g/liter) was added as a redox indicator.

Enrichment cultures were initiated by inoculating the basal medium containing 0.5 g of pyridine per liter with 10% wastewater which was discharged from the industrial complexes in Taejon and Taegu, Korea. The cultures were grown in 125-ml serum bottles with butyl rubber stoppers and aluminum crimp seals. After the bottles were sealed, the 25-ml headspace of the serum bottles was filled with sterile N_2 or He gas (99.99%). The bottles were incubated at 30°C with intermittent shaking. After the complete disappearance of pyridine in the medium, 10 ml of the culture was transferred to 90 ml of fresh medium. After two successive transfers, 1 ml of the culture was transferred to 99 ml of fresh medium three times with successful enrichment. The bacteria were allowed to grow to confluency after each transfer.

Enrichment cultures were serially diluted into 0.95% saline solution and were spread onto plates containing nitrate-containing agar medium. The composition of nitrate agar medium per liter was 5 g of peptone, 3 g of beef extract, 1 g of KNO_3, and 15 g of Bacto Agar (Difco, Detroit, Mich.). The plates were incubated in an anaerobic jar (Difco) with a CO_2-H_2 gas-generating system and palladium catalysts (Difco). An anaerobic atmosphere was confirmed by the reduction of resazurine indicator strips. After 1 week of incubation at 30°C, single colonies were transferred into pyridine-containing minimal liquid medium in order to confirm their ability to metabolize pyridine under denitrifying conditions. For maintenance, the isolated strains were grown in nitrate agar medium or pyridine minimal agar medium.

Methods used for characterization of the isolated strain. The selected isolates were identified by primary tests such as Gram staining (3), morphology, motility, oxidase, catalase, and oxidation-fermentation tests (15). Two-day-old cells were used for the morphology and flagellar tests by transmission electron microscopy. The cells were negatively stained on a grid surface with 1% ammonium molybdate. After the grids were air dried, they were examined with a Hitachi H-800 electron microscope (Tokyo, Japan).

Sequence analysis of 16S rRNA. Extraction of genomic DNA and amplification of 16S rDNA were done as described previously (21). The PCR products were directly sequenced by using α-^{35}S-labeled dATP and a DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio). The generated 16S rRNA sequences were manually aligned with the corresponding sequences of representative strains of the β-subclass of *Proteobacteria* available from GenBank database. Evolutionary distances were calculated by the method of Jukes and Cantor (19). A phylogenetic tree was constructed by the neighbor-joining method (36).

Preparation of cell extracts and enzyme assays. For the preparation of a cell extract, 2.5 g (wet weight) of cells were suspended in 10 ml of 25 mM phosphate buffer (pH 7.0) containing 5% (wt/vol) glycerol and 2 mM $MgCl_2$. The cell suspension in a conical tube was maintained in an ice bath. Sonication was performed with a Vibra cell sonifier (Sonics and Materials, Danbury, Conn.); five 30-s bursts at 1-min intervals were used. The crude extract was centrifuged first at 10,000 × *g* for 30 min and then at 100,000 × *g* for 90 min. The clear supernatant obtained was used for measuring various enzyme activities. The protein content in the extract was estimated by the method described by Lowry et al. (28). Bovine serum albumin was used as a standard.

Isocitratase (EC 4.1.3.1) was determined as the semicarbazone of the product glyoxylate as described by Olson (30). Succinate-semialdehyde dehydrogenase, glutarate-dialdehyde dehydrogenase, and isocitrate dehydrogenase activities were determined by an increase in the optical density at 340 nm as a result of reduced coenzyme [NAD(P)H]. Amidase was measured by the formation of NH_3 from formamide in the reaction mixtures. The assay mixture (1 ml) contained 25 mM phosphate buffer (pH 7.5), 5% glycerol, 2 mM $MgCl_2$, 1 mM NAD(P), 1 mM substrate, and appropriate cell extracts. Reactions were started by the addition of carbon substrate. Enzyme activities were expressed as micromoles of the product formed per milligram of protein per minute.

Growth experiment. Cells were cultivated in the basal medium used in the enrichment cultures without vitamins. For the anaerobic culture, 1 ml of seed culture in the late log phase was inoculated into 125-ml serum bottles containing 100-ml portions of medium with 6 mM nitrate and 3 mM pyridine. After the serum bottles were sealed, they were flushed with N_2. The addition of sodium sulfide or vitamin C as a reducing agent had no significant effect compared with that of nitrogen flushing only. When nitrate was replaced by N_2O in some degradation experiments, the media were purged with N_2O and incubated with the headspace filled with N_2O. The bottles were incubated at 30°C and stirred with a magnetic bar. For the aerobic culture, the cells were cultivated in 250-ml Erlenmeyer flasks containing 50-ml portions of culture medium. The flasks were incubated in a gyratory shaker at 30°C. The medium used for anaerobic enrichment cultures without nitrate was used for the aerobic degradation of pyridine.

Analytical methods. Liquid samples for chemical analysis were taken with sterile N_2-flushed syringes. For analysis of metabolites and substrates, the samples were centrifuged and the supernatants were stored at 4°C until analyzed. Cell growth was monitored by measuring the optical density of the culture broth samples at 540 nm. The cell mass (dry weight) (DCW) was determined as described previously (23).

For detection of heterocyclic intermediates in pyridine degradation, the fermentation broth was separated from the cells by centrifugation and the supernatant was analyzed by UV spectra, high-performance liquid chromatography (HPLC), and gas chromatography-mass spectrometry (GC-MS). For the GC-MS analysis of the supernatant, the samples were thoroughly extracted with ethylacetate sequentially at pH 7, 3, and 10. The extracted organic layer was dried on sodium sulfate beads and then evaporated to a small volume. Before and after derivation with *N*-methyl, *N*-trimethylsilyltrifluoroacetamide (TSTFA), metabolites were analyzed with GC-MS (Fisons, Altrincham, England). A fused silica capillary column was coated with SE-30 cross-linked methylsilicone.

Pyridine concentration was measured by isocratic reverse-phase HPLC (23). Nitrate and nitrite concentrations in culture supernatant were determined by HPLC with a Vydeac 300IC anion-exchange column (Separation Group, Hesperia, Calif.) and a UV detector (254 nm). Phthalic acid (0.5 mM, pH 9.0 with triethylamine) was used as an eluent at a flow rate of 2 ml/min.

N_2O and N_2 concentrations were determined with a Shimazu model GC-14A gas chromatograph equipped with a thermal conductivity detector. Separation was carried out on a Porapak Q column (3 mm [inner diameter] by 2 m) at 50°C with He as a carrier gas at a flow rate of 40 ml/min. The total N_2O was determined from the N_2O measured in the headspace and Henry's constant at 30°C (13). The change of redox potential during incubation was measured with a redox-sensitive electrode (Ingold, Steinbach, Germany). Ammonia concentration was determined by monitoring the oxidation of NADH in the presence of α-ketoglutarate and L-glutamate dehydrogenase, using a test kit (Sigma, St. Louis, Mo.).

The specific growth rate (μ) and specific pyridine degradation rate (q_{pyr}) were calculated as described previously (23).

Nucleotide sequence accession number. The 16S rRNA sequence determined has been deposited in the EMBL database under accession no. U44853.

RESULTS

Enrichment and isolation. The enrichment of anaerobic bacteria that degrade pyridine with nitrate as an electron acceptor with industrial wastewater was attempted. Initially, a general enrichment medium for denitrifying bacteria was used (17). Although the enrichment was successfully initiated, we could not carry out further successive transfers with any bacterial source. For successful enrichment, the addition of selenite to the medium was necessary. After about 3 weeks of incubation, pyridine completely disappeared in an initial enrichment bottle containing 18 mM nitrate. However, when the initial concentration of nitrate was 18 mM, cell growth appeared to be severely inhibited. Therefore, we used 6 mM nitrate in the enrichment medium after the second transfer. The degradation rate of pyridine was accelerated during successive transfers. In the final stage of the enrichment cultures, the bacteria reached stationary phase within 2 days.

In order to isolate the pure cultures, the final transfer of the enrichment broth was serially diluted and spread onto the nitrate agar medium. After about 3 days of incubation in an anoxic atmosphere, colonies appeared in a nitrate agar medium. Each colony was inoculated into the pyridine minimal liquid medium, and its ability to metabolize pyridine under denitrifying conditions was successfully confirmed. When the inoculum size was 1%, all the isolates were fully grown within 3 days. Eight denitrifiers with similar colony morphology were isolated from different sources.

Morphological and physiological characteristics and evolutionary relationships. All the isolates were rod shaped and had similar sizes, with dimensions of 2.0 by 3.0 by 0.4 to 0.6 μm, after 2 days of incubation on the nitrate agar medium. An electron photomicrograph of a negatively stained preparation of the isolates showed that flagellation was evident and polar.

All the isolates were gram negative and motile. They had catalase and oxidase. With a commercially available API-NE kit (Analytab Products, Plainview, N.Y.), it was determined that the isolates were able to grow only on malate and the isolates gave negative results for all the other characteristics tested. Glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, caprate, adipate, citrate, and phenylacetate were not utilized. According to the API-NE test, we could not find any *Pseudomonas* species nor any non-*Enterobacteriaceae* with the characteristics of the strains isolated.

All the isolated strains could metabolize pyridine in the presence of oxygen. As expected from the enrichment cultures, they required selenite as an essential trace element in both the anaerobic and aerobic degradation of pyridine. They could degrade pyridine without vitamins under aerobic and anaerobic conditions.

The sequences of 16S rRNA from all the isolates were the same and were related to *Azoarcus* species (Fig. 1), which are various aromatic compound-degrading denitrifying bacteria (2, 12, 33). Similarities to 16S rRNA gene sequences of *Azoarcus tolulyticus* Td-15 and Td-17, and *Azoarcus evansii* KB740^T were 99.59, 99.25, and 100%, respectively.

Considering the cell growth rate and the pyridine degradation rate, strain pF6 was selected and further characterized. The temperature range at which strain pF6 could grow was between 20 and 40°C, with an optimum at 35°C. The optimum pH range of strain pF6 was found to be between 7.5 and 8.0.

Metabolic pathway of pyridine degradation. In order to detect heterocyclic intermediates, the supernatant collected dur-

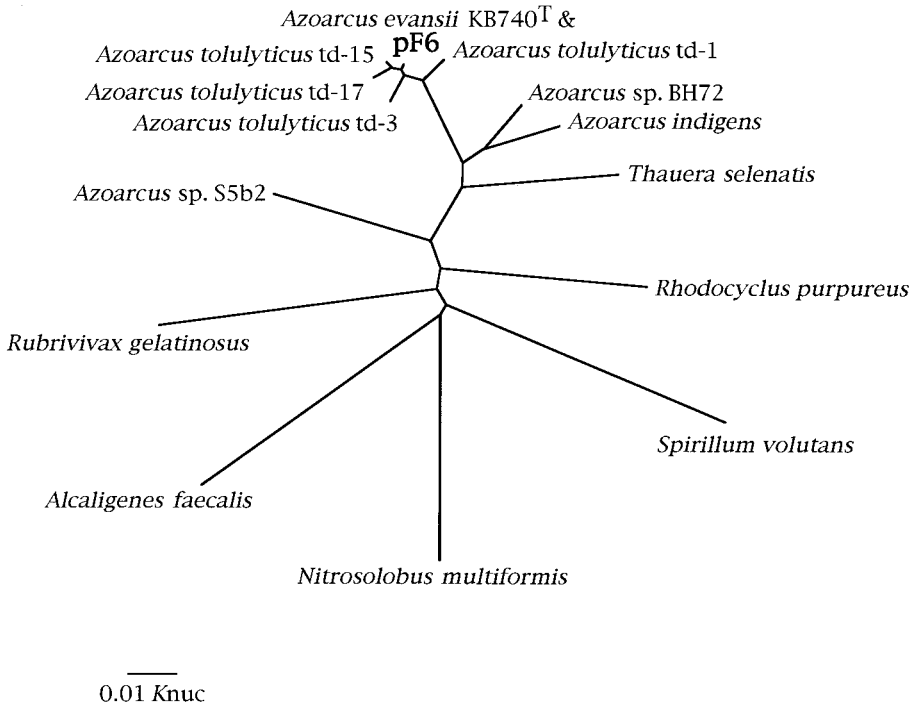

FIG. 1. Phylogenetic tree showing the position of a newly isolated denitrifying strain, pF6, within the representative taxa of the β-subclass of the *Proteobacteria* and known toluene-degrading denitrifiers. Scale bar represents 1 nucleotide substituent per 100 nucleotides (0.01 *K*nuc).

ing the cultivation was analyzed by HPLC and UV spectra. No new peak was found. Furthermore, no heterocyclic intermediates of pyridine were detected by GC-MS. Resuspended cells or extracts of cells grown with pyridine did not degrade 2-, 3-, or 4-hydroxypyridine, nor did they degrade 2,3-, 2,4-, or 2,6-dihydroxypyridine. Thus, it is apparent that hydroxyintermediates are not involved in pyridine metabolism by strain pF6.

Because pyridine was degraded under both anaerobic and aerobic conditions, the dependence of a metabolic pathway on the culture condition was tested by induction experiments. Chloramphenicol ranging from 50 to 250 mg/liter was used to inhibit de novo synthesis of protein. As shown in Fig. 2, pyridine was degraded without a lag period under each condition, indicating that pyridine was metabolized by the same pathway under both anaerobic and aerobic conditions. As the chloramphenicol concentration increased, the pyridine degradation rate decreased, probably because of the toxicity of chloramphenicol. When aerobically grown cells were transferred to anaerobic conditions, the pyridine degradation rate was slower. This might be because the denitrification system was not fully induced during a preincubation period.

Watson and Cain (44) and Sims et al. (41) were unable to demonstrate pyridine degradation using extracts of *Nocardia*, *Bacillus*, and *Micrococcus* species. In the present study, identical results were obtained with extracts of strain pF6. Despite our failure to detect heterocyclic intermediates and to demonstrate pyridine degradation by cell extracts, the following evidence for intermediates of pyridine metabolism was obtained. Two kinds of reductive pathways in pyridine degradation were known in aerobic bacteria isolated from soils (44). We tested the activities of some enzymes which are known to be involved in the important steps of pyridine metabolism, and the results were summarized in Table 1.

In anaerobic cultures, pyridine induced isocitratase and glutarate-dialdehyde dehydrogenase. Glutarate-dialdehyde dehydrogenase of strain pF6 was classified as EC 1.2.1.20 because NAD was a better coenzyme than NADP. These enzymes were also induced by pyridine under aerobic conditions. On the other hand, these enzyme activities were not detected in malate-grown cells under both anaerobic and aerobic conditions. Furthermore, succinate-semialdehyde dehydrogenase and formamidase were not induced by pyridine. These results indicate that the mechanism of pyridine degradation in strain pF6 is similar to that in the *Nocardia* species.

Anaerobic degradation of pyridine in a batch culture. The growth of cells, consumption of nitrate, and formation of nitrite, an intermediate, were monitored in an anaerobic batch culture (Fig. 3). Although N_2O is one of the general intermediates in the denitrification process (6, 8), it was not detected in this batch culture. Most anaerobes are inhibited at redox values higher than −100 mV (5). However, denitrification and degradation of pyridine by strain pF6 were not significantly affected by changes of the redox potential in the medium (Fig. 3A). Although the redox potential decreased linearly from 150 to −200 mV, μ and q_{pyr} were almost constant at 0.13 h^{-1} and 0.33 g of pyridine per g of cells (dry weight) (g_{DCW}) per h, respectively. During the assimilation of pyridine, ca. 80% of the nitrogen in the pyridine ring was released to the medium as ammonia (Fig. 3B).

Effects of electron acceptors on growth of cells and degradation of pyridine. In order to quantify the effects of electron acceptors on cell growth and the anaerobic degradation of pyridine, strain pF6 was cultivated in a minimal salts medium with 3 mM pyridine and various compounds as the sole electron acceptor. It could grow in the presence of nitrate, nitrite, or nitrous oxide but not in the presence of other anaerobic electron acceptors such as sulfate or selenate. In cultures with these electron acceptors, the growth rate, yield (amount of cell

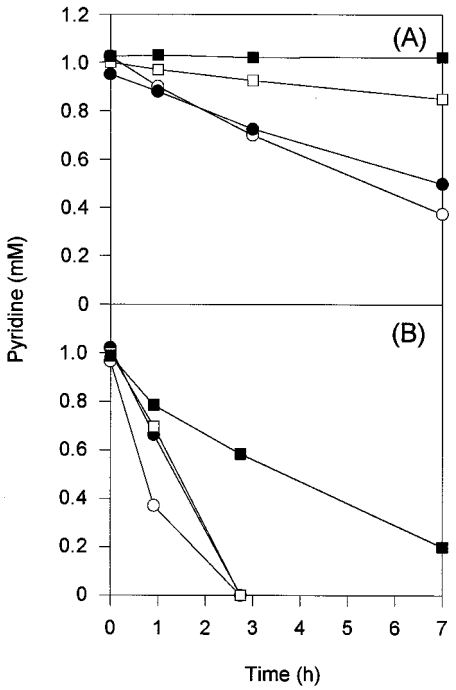

FIG. 2. Dependence of pyridine metabolism on aerobic and denitrifying conditions. Aerobically or anaerobically grown cells were washed with 25 mM phosphate buffer (pH 7.0) and preincubated for 12 h to induce an alternative respiratory system without pyridine. Aerobically grown cells were incubated in 50-ml serum bottles containing 50-ml portions of minimal medium with 6 mM nitrate. The serum bottles were flushed with N_2 gas and incubated at 30°C. Anaerobically grown cells were incubated in 250-ml Erlenmeyer flasks containing 50-ml portions of minimal medium. The flasks were incubated in a gyratory shaker at 30°C. After induction of the respiratory system, pyridine was added with electron acceptors (6 mM nitrate in anaerobic conditions) and various concentrations of chloramphenicol (in milligrams per liter) as follows: 50 (○), 100 (●), 150 (□), and 250 (■). Cell density used was 1.95 g/liter. (A) Aerobically grown cells were transferred to anaerobic conditions. (B) Anaerobically grown cells were transferred to aerobic conditions.

mass to amount of consumed pyridine [$Y_{X/\mathrm{pyr}}$] and amount of cell mass to amount of reduced electron acceptor [$Y_{X/\mathrm{E.A.}}$]), and molar ratio of pyridine to electron acceptors ($Y_{\mathrm{pyr/E.A.}}$) were calculated (Table 2).

To determine the optimal nitrate concentration for cell growth and pyridine degradation, nitrate ranging from 1.5 to 12 mM was used as an electron acceptor. At a nitrate concentration higher than 6 mM, cell growth was inhibited and $Y_{X/\mathrm{pyr}}$, $Y_{X/\mathrm{E.A.}}$, and $Y_{\mathrm{pyr/E.A.}}$ decreased significantly (Table 2). As shown in Fig. 4, strain pF6 grew in steps like diauxic growth at 9 mM nitrate. In the first growth phase, nitrate was completely reduced to nitrite. Before the second growth phase, the nitrite concentration decreased below 3 mM. At this time, we observed the disappearance of nitrite but did not observe cell growth and pyridine degradation. Thus, the accumulated nitrite appeared to inhibit cell growth. At 12 mM nitrate, cell growth was significantly inhibited and 8 mM nitrite accumulated. Although the nitrate concentration used significantly influenced cell growth, it had little influence on q_{pyr}. Accordingly, a nitrate concentration higher than 6 mM was unsuitable for the biodegradation of pyridine under denitrifying conditions. To confirm the inhibitory effect of nitrite accumulation, nitrite ranging from 3 to 12 mM was used as the sole electron acceptor. As summarized in Table 2, cell growth was inhibited at a higher concentration of nitrite. Furthermore, it was confirmed that a transient accumulation of nitrite in batch culture resulted from the lower nitrite uptake rate compared to the nitrate uptake rate.

When N_2O was used as an electron acceptor, the growth rate was approximately 50% of that at 6 mM nitrate. However, the

TABLE 1. Activities of some enzymes involved in pyridine metabolism[a]

Enzyme	Product measured	Sp act (μmol of product/min/mg of protein)[b]	
		Pyridine	Malate
Isocitrate dehydrogenase	NADPH	0.17	1.01
	NADH	<0.01	0.02
Isocitratase	Glyoxylate semicarbazone	0.24	0.02
Glutarate-dialdehyde dehydrogenase	NADPH	0.22 (0.31)	0.01
	NADH	2.61 (5.01)	0.03
Succinate-semialdehyde dehydrogease	NADPH	0.02 (0.02)	0.02
	NADH	<0.01 (0.03)	<0.01
Formamidase	NH_3	<0.01	<0.01

[a] Cells of strain pF6 were grown anaerobically under denitrifying conditions with either pyridine or malate as the sole carbon and electron source. Activities of enzymes in the cell extracts were measured.

[b] The numbers in parentheses represent the activity of enzyme extract prepared from cells grown aerobically.

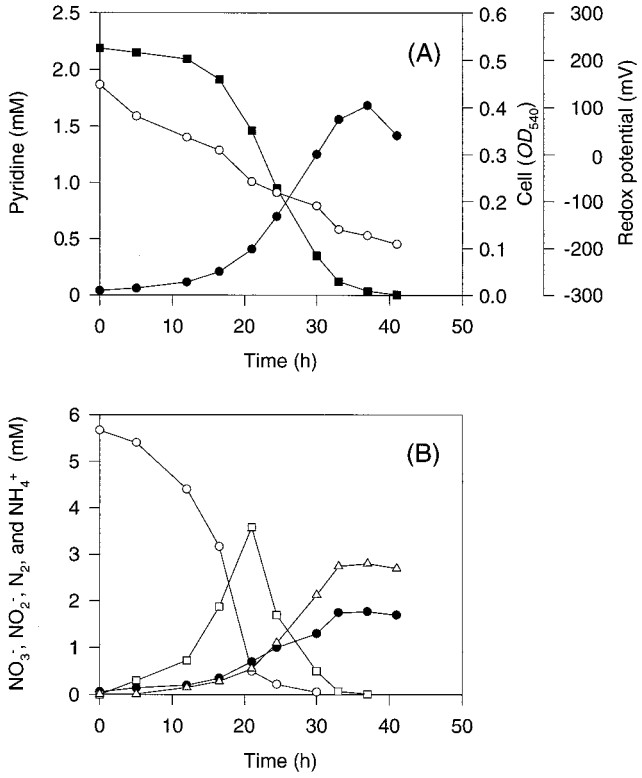

FIG. 3. Anaerobic culture of strain pF6 with pyridine. Experiments were performed at 30°C in 125-ml serum bottles with butyl rubber stoppers. Samples were taken by syringe at the times indicated. The headspace was filled with He. (A) Cell growth (●), pyridine concentration (■), and redox potential (○) are depicted. (B) Concentrations of nitrate (○), nitrite (□), N_2 (△), and ammonia (●) are depicted. OD_{540}, optical density at 540 nm.

TABLE 2. Quantitation of pyridine consumption and reduction of electron acceptors by strain pF6[a]

Condition and electron acceptor	Cell mass (DCW) formed (g/liter)	μ (h^{-1})	q_{pyr} ($g_{pyr}/g_{DCW}/h$)[b]	Reduction rate of electron acceptor (mmol/g_{DCW}/h)	$Y_{X/pyr}$ (g/mmol)[c]	$Y_{X/E.A.}$ (g/mmol)[d]	Pyr/E.A. (mmol/mmol)[e]
Aerobic condition							
Without electron acceptors	0.17	0.16	0.41		0.056	NA[f]	NA
Nitrate (9 mM)	0.16	0.15	0.44		0.053		
Nitrite							
3 mM	0.15	0.10	0.24		0.049		
9 mM	0.14	0.07	0.19		0.046		
Denitrifying condition							
Nitrate							
1.5 mM	0.03	0.09	0.32	7.53	0.035	0.016	0.49
3.0 mM	0.08	0.14	0.34	18.77	0.049	0.020	0.38
6.0 mM	0.12	0.11	0.38	24.10	0.052	0.019	0.41
9.0 mM	0.11	NA	NA	30.43	0.037	0.012	0.34
12.0 mM	0.03	0.10	0.35	32.90	0.021	NA	NA
Nitrite							
3.0 mM	0.04	0.08	0.13	3.27	0.046	0.012	0.26
6.0 mM	0.04	0.07	0.11	4.90	0.044	0.006	0.15
9.0 mM	0.05	0.04	0.12	4.30	0.042	0.005	0.11
12.0 mM	0.01	0.00	0.02	0.20	0.023	0.004	0.08
N_2O (21.0 mM[g])	0.16	0.05	0.12	17.30	0.058	0.010	0.17

[a] Strain pF6 was grown anaerobically in anoxic bottles containing 100-ml portions of medium with excess pyridine (3 mM) and various compounds as the sole electron acceptor. For comparison, aerobic experiments were carried out with 250-ml flasks containing 50-ml portions of medium. The preculture used to inoculate the test cultures was done in the presence of oxygen.
[b] g_{pyr}, gram of pyridine.
[c] Grams of cells (DCW) per millimole of pyridine consumed.
[d] Grams of cells (DCW) per millimole of electron acceptor reduced.
[e] Millimoles of pyridine consumed to millimoles of electron acceptor reduced.
[f] NA, not available.
[g] When the medium was purged with 100% N_2O, 21 mM N_2O was dissolved.

greatest cell mass was formed among the electron acceptors tested under denitrifying conditions because N_2O was highly soluble (21 mM in the test condition) and not toxic (10, 16).

Strain pF6 could metabolize pyridine in the presence of oxygen. q_{pyr} and μ were higher under aerobic conditions than those under anaerobic conditions (Table 2). In the presence of mixed electron acceptors, denitrifying bacteria prefer oxygen to other electron acceptors such as nitrate or nitrite (14, 25).

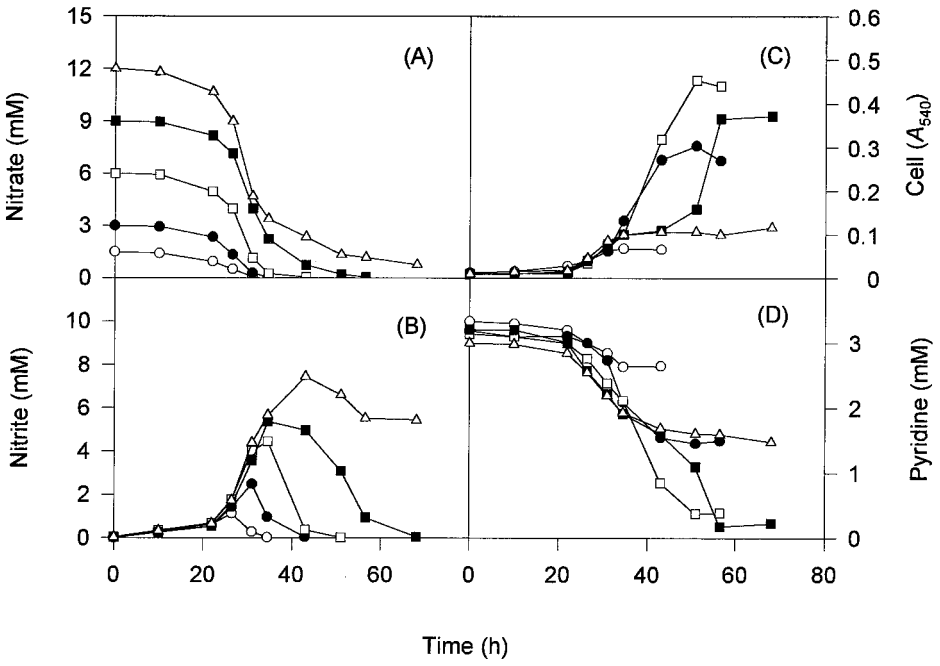

FIG. 4. Effects of initial nitrate concentration on cell growth and pyridine degradation. Initial nitrate concentrations used (in millimolar) were 1.5 (○), 3 (●), 6 (□), 9 (■), and 12 (△).

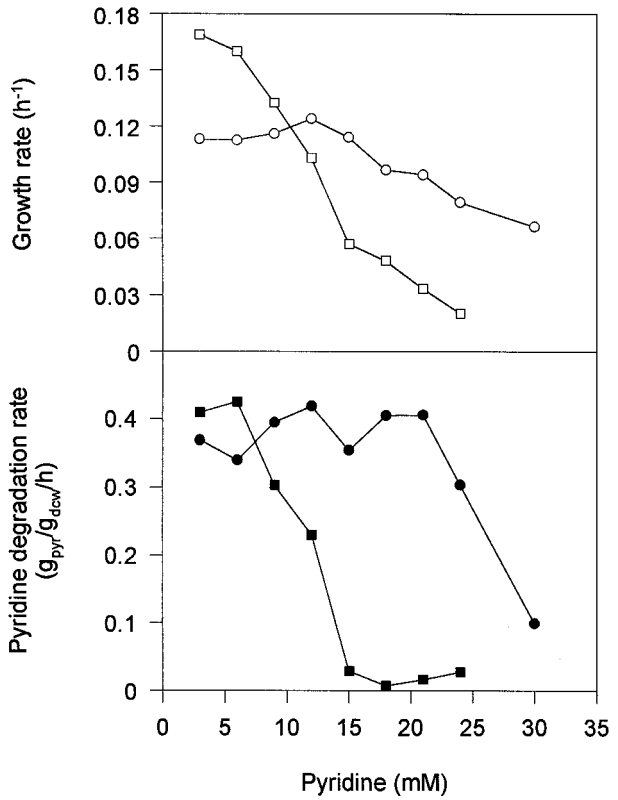

FIG. 5. Comparison of growth rate and pyridine degradation rate of strain pF6 incubated under aerobic and anaerobic conditions. (A) Growth rate for aerobic (□) and anaerobic (○) cultures. (B) Pyridine degradation rate for aerobic (■) and anaerobic (●) cultures. The initial concentration used ranged from 3 to 30 mM. g_{pyr}, grams of pyridine.

Similarly, nitrate had no effect on the growth of strain pF6 and the degradation of pyridine under aerobic conditions. However, in the presence of nitrite, aerobic growth and aerobic degradation of pyridine were severely inhibited. As 9 mM nitrite was added to the medium, μ decreased by 52%. In contrast to anaerobic conditions, the nitrite concentration did not decrease during aerobic incubation and the growth yield did not change.

Effects of pyridine concentration on cell growth and pyridine degradation. To investigate the effects of pyridine concentration on cell growth and pyridine degradation, the cells were cultivated in a medium containing various concentrations of pyridine (3 to 30 mM) and 6 mM nitrate under denitrifying conditions. For comparison, aerobic cultures were grown in a medium containing various concentrations of pyridine.

Under denitrifying conditions, a pyridine concentration of up to 15 mM had little influence on the pyridine degradation rate and growth rate of strain pF6. As the pyridine concentration was further increased to above 20 mM, μ decreased gradually (Fig. 5). A rapid decrease in q_{pyr} was observed above 20 mM pyridine. The inhibitory effects of pyridine on μ and q_{pyr} under aerobic conditions were more severe than those under denitrifying conditions. μ, which was higher than that under denitrifying conditions below 12 mM pyridine, significantly decreased above 12 mM pyridine and therefore became lower than that under denitrifying conditions. A rapid decrease in q_{pyr} was observed above 12 mM pyridine. Thus, it was found that the inhibitory effects of pyridine on cell growth and pyridine degradation were significantly influenced by the environment, though the same metabolic pathway seemed to be used in pyridine degradation under aerobic and anaerobic conditions.

DISCUSSION

The anaerobic biodegradation of pyridine by a new denitrifying bacterium was investigated in this study. As in the cultivation of strict anaerobic bacteria, such as sulfate-reducing bacteria (31), methanogens (18), and clostridia (47), the successful enrichment in this study was dependent on selenite. Certain enzymes in anaerobic metabolism such as formate dehydrogenase (9, 47) and hydrogenase (48) were known to contain selenium. However, the selenite requirement was not limited to the denitrifying condition, and the isolated strains required selenite in the aerobic degradation of pyridine. When the isolated strains were cultivated in the minimal salt media containing malate as the sole carbon source, selenium was required for growth. Therefore, the isolated strains may have essential selenoproteins that are not specifically involved in denitrification or pyridine degradation.

The agar shake dilution method was generally used for the isolation of denitrifying bacteria that degrade aromatic compounds (33, 43). However, a strict anaerobic condition was not necessary in this study. We successfully isolated these denitrifying bacteria from the culture plates incubated aerobically. In addition, a nitrate agar medium, instead of a minimal salt medium, was used as an isolation medium without any problem. The use of a complex medium allowed us to isolate the denitrifying bacteria rapidly from the enrichment cultures.

According to the physiological and phylogenetic characteristics of these isolates, all the new denitrifying bacteria appear to be closely related to each other and to be a new group of bacteria. Although some of their phenotypic and ecological characteristics were different from those of previously described strains of *Azoarcus* (34), the results of sequence analysis of 16S rRNA confirmed that these isolates were new strains of *A. evansii*. This finding coincided with recent phylogenetic findings suggesting that several isolated denitrifying strains that metabolize toxic aromatic compounds were members of this branch (2, 12, 33).

From the experiments on metabolic pathways, it was apparent that hydroxyintermediates were not involved in the pyridine metabolism of strain pF6. Pyridine was unlikely to be degraded by the hydroxylation pathway proposed by Zefirov et al. (49). Strain pF6 may degrade pyridine by one of two reductive pathways proposed by Watson and Cain (44). Succinate-semialdehyde dehydrogenase is involved in pyridine metabolism by *Bacillus*, *Brevibacterium*, and *Corynebacterium* species (37, 41, 44), whereas isocitratase and glutarate-dialdehyde dehydrogenase are involved in pyridine metabolism by *Nocardia* species (44). As shown in Fig. 6, one pathway for pyridine metabolism involves N–C-2 ring cleavage and subsequent deamination to glutarate dialdehyde. Glutarate dialdehyde is followed by successive oxidation to glutarate semialdehyde, glutarate, and acetyl coenzyme A. Acetyl coenzyme A may be assimilated via the glyoxylate cycle. Based on the observation that pyridine induced NAD-linked glutarate-dialdehyde dehydrogenase and isocitratase, it is likely that strain pF6 used this pathway for pyridine degradation. The other pathway proposed involves C-2–C-3 ring cleavage, accompanied by succinate-semialdehyde dehydrogenase and formamidase. However, since these enzymes were not induced by pyridine, it is unlikely that this pathway was used in strain pF6.

In a batch culture of strain pF6, 2.3 mM pyridine was completely degraded at the expense of 6 mM nitrate; therefore, the

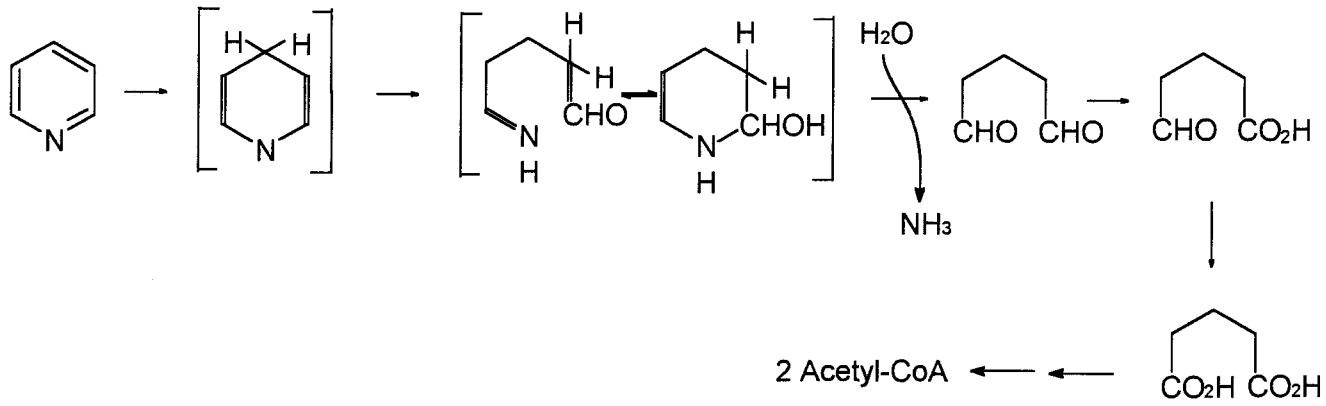

FIG. 6. Proposed pathway for pyridine metabolism (adopted from Watson and Cain with permission from the publisher [44]). Acetyl-CoA, acetyl coenzyme A.

molar ratio of pyridine to nitrate was 1:2.6. Considering the theoretical molar ratio of 1:4.4 for the complete oxidation of pyridine (26), 41% of the consumed pyridine may be assimilated into the biomass. The $Y_{X/\mathrm{pyr}}$ with 6 mM nitrate in denitrifying conditions decreased slightly (by 7%) from that in the presence of oxygen (Table 2). This small difference in $Y_{X/\mathrm{pyr}}$ was expected from the calculation of free energy change and the estimation of microbial yield in the reduction of nitrate or oxygen (29). The growth yield of strain pF6 is higher than those of other aerobic bacteria that degrade pyridine (22, 35). This could be advantageous for enhanced survival of applied microorganisms in bioremediation. In addition, the concentration of pyridine which strain pF6 can degrade completely is much higher than those of pyridine and pyridine derivatives in retort water (approximately 200 ppm). Accordingly, further dilution of retort water is unnecessary.

In liquid medium containing 18 mM nitrate, cell growth was severely inhibited by the toxic effect of the accumulated nitrite. The inhibitory effect of the accumulated nitrite on denitrification and cell growth has been observed in other denitrifying bacteria (1, 10). A transient accumulation of nitrite during batch culture was unavoidable because the rate of nitrate reduction was higher than that of nitrite reduction. Hence, the toxicity of accumulated nitrite can cause a problem in the application of denitrifying bacteria. The maximum concentration of nitrate which can be used without causing significant growth inhibition depended on the isolated strains. Thus, the optimal concentration of nitrate for maximum growth yield to electron acceptor ($Y_{X/\mathrm{E.A.}}$) should be determined for each strain.

Neither nitrate nor nitrite was used as an electron acceptor in aerobic conditions. However, nitrite was inhibitory to cell growth and the biodegradation of pyridine in aerobic conditions. When the denitrifying process was stopped by aeration or denitrifying environments were abruptly exposed to aerobic conditions, the accumulated nitrite could inhibit aerobic degradation of pyridine. Thus, in the simultaneous or sequential anaerobic and aerobic environments (7, 50), nitrite concentration should be regulated for efficient biodegradation.

In conclusion, a newly isolated denitrifying bacterium, strain pF6, has potential for use in the biodegradation of pyridine in the environment.

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