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Denitrification by a Soil Bacterium with Phthalate and Other Aromatic Compounds as Substrates

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A soil bacterium, *Pseudomonas* sp. strain P136, was isolated by selective enrichment for anaerobic utilization of *o*-phthalate through nitrate respiration. *o*-Phthalate, *m*-phthalate, *p*-phthalate, benzoate, cyclohex-1-ene-carboxylate, and cyclohex-3-ene-carboxylate were utilized by this strain under both aerobic and anaerobic conditions. *m*-Hydroxybenzoate and *p*-hydroxybenzoate were utilized only under anaerobic conditions. Protocatechuate and catechol were neither utilized nor detected as metabolic intermediates during the metabolism of these aromatic compounds under both aerobic and anaerobic conditions. Cells grown anaerobically on one of these aromatic compounds also utilized all other aromatic compounds as substrates for denitrification without a lag period. On the other hand, cells grown on succinate utilized aromatic compounds after a lag period. Anaerobic growth on these substrates was dependent on the presence of nitrate and accompanied by the production of molecular nitrogen. The reduction of nitrite to nitrous oxide and the reduction of nitrous oxide to molecular nitrogen were also supported by anaerobic utilization of these aromatic compounds in this strain. Aerobically grown cells showed a lag period in denitrification with all substrates tested. Cells grown anaerobically on aromatic compounds also consumed oxygen. No lag period was observed for oxygen consumption during the transition period from anaerobic to aerobic conditions. Cells grown aerobically on one of these aromatic compounds were also adapted to utilize other aromatic compounds as substrates for respiration. However, cells grown on succinate showed a lag period during respiration with aromatic compounds. Some other characteristic properties on metabolism and regulation of this strain are also discussed for their physiological aspects.

Denitrification by bacteria is generally regarded as being a result of anaerobic nitrate respiration (13, 17). Various carbon sources are utilized anaerobically as substrates for denitrification. However, the availability of aromatic compounds for this process has not been investigated as extensively.

Concerning investigations with pure bacterial culture, few strains have been known to be involved in denitrification with aromatic compounds (9). Although several strains of soil bacteria have been reported to utilize benzoate as a substrate for denitrification (19, 21, 22), these strains failed to utilize phthalate both under aerobic and anaerobic conditions. The anaerobic utilization of phthalate has only recently been reported (1). However, in this report, only denitrification with *o*-phthalate and benzoate has been described, and the effectiveness of other aromatic compounds as substrates for denitrification has not been studied in detail. Another bacterial strain has been reported to anaerobically utilize *o*-phthalate (6), but only positive growth on *o*-phthalate and nitrate has been briefly mentioned.

In any case, physiological aspects of denitrification with these substrates has received little consideration, and systematic investigation of denitrification with a series of aromatic compounds has been insufficient.

We report here the isolation of a new soil bacterium which can utilize *o*-phthalate and various other aromatic compounds anaerobically through nitrate respiration. The physiological characteristics of this strain for denitrification with phthalate and other aromatic compounds are also described.

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MATERIALS AND METHODS

Isolation of the organism. The organism was isolated from garden soil by selective enrichment culture. The basal medium contained (in grams per liter): KH_2PO_4, 1.0; $MgSO_4$, 0.2; $CaCl_2$, 0.02; K_2SO_4, 0.05; NaCl, 0.05; KNO_3, 5.0; NH_4Cl, 1.0; ferric citrate, 0.01; Na_2MoO_4, 0.005; H_3BO_3, 0.003; $MnCl_2$, 0.002; $ZnSO_4$, 0.0003; $CuSO_4$, 0.0002; $CoSO_4$, 0.00002; and carbon source, 2.0. The medium was adjusted to pH 7.4 with 1.0 N NaOH. Soil samples were inoculated into 10 ml of liquid medium in 20-ml test tubes. Selection was performed with *o*-phthalate as the sole carbon source. The test tubes were set in a desiccator and incubated at 30°C. Prior to incubation, the desiccator was evacuated and filled with pure nitrogen. Nitrogen was passed through an Oxy-Trap catalyst column (Gasukuro Kogyo Inc., Tokyo.) to remove residual oxygen (below 1 ppm) before use. The anaerobic condition was maintained by using the Gas-Pak system (Becton Dickinson Co.). Some cultures became turbid, and vigorous gas production was observed within 8 to 10 days. These cultures were enriched as described elsewhere (21, 22) and streaked on agar plates. The plates were incubated anaerobically as described above. The colonies isolated through the selection were streaked on nutrient agar plates (no. 2; Oxoid Co.) and incubated under aerobic conditions at 30°C. After repeated streakings, the isolated colonies were reinoculated into the basal medium to test anaerobic growth on aromatic compounds.

The isolated cultures were maintained on agar slants of the same medium.

Characterization of organism. Several taxonomic properties of the isolated organism were examined. The Gram stain, flagella stain, oxidase test, catalase test, and oxidation-fermentation test were performed by standard procedures (20). Growth tests on the temperature and pH of the medium were performed with basal medium containing *o*-phthalate and nitrate. The presence of plasmids was examined by the rapid agarose gel electrophoresis method (14).

Growth tests on carbon sources. To test the utilization of carbon sources, 0.1 ml of the liquid culture at full growth was inoculated into 10 ml of liquid medium containing an appropriate carbon source. Media were usually sterilized by autoclaving, except solutions of catechol and protocatechuate, which were sterilized by filtration. Anaerobic incubation was performed at 30°C as described above. For the aerobic growth test, the media were the same as for anaerobic growth except that nitrate was omitted and NH_4Cl was increased to 3 g/liter. Incubation was performed at 30°C on a reciprocal shaker. Growth was monitored by measuring the turbidity of the culture at 660 nm.

Unless otherwise described, two strains of *Pseudomonas aeruginosa* from the laboratory culture collection were used as reference strains in all control experiments. These strains have been characterized for the aerobic utilization of *o*-phthalate through protocatechuate.

Preparation of cell suspensions. Cell suspensions for the measurement of several biological activities were prepared from large-scale cultures. Large-scale cultivation under anaerobic condition was performed in 2-liter Erlenmeyer flasks filled with the basal medium containing an appropriate carbon source. These cultures were incubated under bubbling with pure nitrogen. Aerobic cultivation was performed in 1 liter of the medium for aerobic growth contained in a 5-liter Erlenmeyer flask on a rotary shaker. Cells were harvested by centrifugation at 10,000 × *g* for 20 min in the late log phase and washed with basal medium without carbon source or nitrate. The washed cells were resuspended in appropriate medium and used immediately.

Measurement of denitrification. Denitrification by cell suspensions was measured by gas chromatography (15, 18), with the following modifications. The reaction was carried out in 25-ml Thunberg tubes. Washed cells were resuspended in basal medium without a carbon source. The main compartment contained 5 ml of cell suspension, and the side arm contained 1 ml of the solution of carbon source. The final concentration of carbon source was adjusted to 2 g/liter in the standard condition.

The tubes were evacuated and filled with helium three times. Finally, the tubes were filled with helium containing 5% (vol/vol) neon as an internal standard (16). In some experiments with nitrite or nitrous oxide as a terminal electron acceptor, nitrate was omitted from the medium. Nitrite was added to the basal medium as $NaNO_2$ at 2 g/liter. Nitrous oxide was added as a 10% (vol/vol) mixture with helium in the gas phase. The tubes were incubated at 30°C on a reciprocal shaker. The reaction was started by mixing the contents of the main compartment and the side arm. At intervals, gas samples were withdrawn by a Shimadzu MGS-4 gas sampler and analyzed with a Shimadzu GC-3BT gas chromatograph. Gas analysis was performed by using the following columns at 50°C; molecular sieve 5A (3 mm by 1 m) for the separation of neon, nitrogen, and nitric oxide, and Porapak Q (3 mm by 1 m) for nitrous oxide. Helium was used as the carrier gas at a flow rate of 40 ml/min. Each gas component was determined by a Shimadzu Chromatopac E1A digital integrator.

Measurement of respiration. Washed cells were resuspended in basal medium without nitrate or a carbon source. Oxygen consumption by cell suspensions was measured by a Clark-type oxygen electrode at 30°C. Reactions were started by injection of the solution of carbon source into the reaction chamber. The output of the oxygen electrode was recorded by a Hitach recorder (model 056; Nissei Sangyo Co. Ltd.).

Other methods. The disappearance of aromatic compounds from the culture medium was monitored spectrophotometrically (22). The culture was centrifuged, and the resultant supernatant was diluted with 0.1 N HCl in methanol. The sample solution was scanned in the UV region at 250 to 300 nm. Phthalate concentration was estimated from a standard curve of absorbance at 274 nm. Catechol compounds in the culture medium were detected by Evans reagent (8). Nitrate was determined as nitrite after reduction with cadium metal (23).

Nitrite was determined colorimetrically by the diazo-coupling method (3).

Chemicals. Cyclohex-1-ene-carboxylic acid was prepared as described by Dutton and Evans (7). Cyclohexanecarboxylic acid, cyclohexane-1,2-dicarboxylic acid, and cyclohex-3-ene-carboxylic acid were from Tokyo Chemical Industry Co. Ltd. Neon, helium, and acetylene were from Suzuki Shokan Co. Ltd. All other chemicals were reagent grade.

RESULTS

Isolation and characterization of organism. A pure culture of the bacterium, strain P136, was isolated from garden soil by selective enrichment culture. Strain P136 was a gram-negative aerobic rod, 0.5 to 1.0 μm in diameter and 2.0 to 3.0 μm in length, motile with polar flagella, both oxidase and catalase positive, and nonsporeforming. Good growth was observed at pH 7 to 8, and no growth occurred below pH 5.5. Growth occurred at temperatures ranging from 25 to 35°C. Slow but significant growth was observed at 42°C. Growth was poor or negligible below 15°C and above 45°C.

Oxidation-fermentation tests were performed with arabinose, fructose, galactose, glucose, ribose, and xylose, and these sugars were found not to be fermented. Nitrate was reduced to nitrite, and further reduction of nitrite to molecular nitrogen was also evident.

Growth was poor on King A and King B mediun. No pigment formation was observed on these media. Colonies on nutrient agar were smooth, white, and opaque. Colonies on the defined medium under anaerobic conditions were pale white. No plasmid was detected in various cell preparations tested.

These characteristics suggested that strain P136 belonged to the genus *Pseudomonas*.

Growth tests on carbon sources. *Pseudomonas* sp. strain P136 utilized various aromatic compounds for aerobic and anaerobic growth (Table 1). Pimelate, adipate, succinate, and acetate also supported growth under aerobic and anaerobic conditions. *o*-Hydroxybenzoate, protocatechuate, catechol, phenol, cyclohexanecarboxylate, and cyclohexane-1, 2-dicarboxylate did not support either aerobic or anaerobic growth at any concentration tested.

Anaerobic growth on *o*-phthalate was accompanied by the disappearance of *o*-phthalate and nitrate from the culture medium. Nitrite was accumulated at the middle stage of growth and finally disappeared (Fig. 1). Anaerobic growth of *Pseudomonas* sp. strain P136 was dependent on the presence of nitrate or nitrite. Sulfate and thiosulfate failed to substitute as terminal electron acceptors. In either the light or the dark, no growth occurred on media lacking nitrate or nitrite under anaerobic conditions. Catechol compounds were not detected in the medium after either aerobic or anaerobic growth on *o*-phthalate.

Denitrification by cells grown anaerobically on aromatic compounds and other substrates. Cells grown anaerobically

TABLE 1. Growth of *Pseudomonas* sp. strain P136 on various substrates under aerobic and anaerobic conditions

Substrate	Growth[a]	
	Aerobic	Anaerobic
o-Phthalate	+	+
m-Phthalate	+	+
p-Phthalate	+	+
Benzoate	+	+
m-Hydroxybenzoate	−	+
p-Hydroxybenzoate	−	+
Cyclohex-1-ene-carboxylate	+	+
Cyclohex-3-ene-carboxylate	+	+

[a] +, Turbidity of the culture increased up to 1.6 to 2.0 within 48 h; −, turbidity of the culture did not exceed 0.2 after 48 h.

on *o*-phthalate and nitrate were capable of utilizing various other aromatic compounds as substrates for denitrification. Cells grown on other aromatic compounds also utilized the same range of substrates. Table 2 represents these results as the initial rate of production of molecular nitrogen with a series of substrates by various cell preparations. No lag period was observed in the production of molecular nitrogen with these substrates, and the addition of chloramphenicol (10 μg/ml) did not affect the onset of denitrification with these substrates.

Table 2 also represents the results with cells grown anaerobically on succinate or pimelate. No lag period was observed in the production of molecular nitrogen with succinate and pimelate by these cells. On the other hand, considerable lag periods were observed when the aromatic compounds were used as substrates for dentrification. The addition of chloramphenicol did not affect denitrification with succinate but completely inhibited the initiation of production of molecular nitrogen with aromatic compounds.

Experiments on denitrification with the compounds which did not support anaerobic growth were also performed. *o*-Hydroxybenzoate, protocatechuate, catechol, and cyclohexanecarboxylate were tested and also found to be unable to support denitrification by cells grown on *o*-phthalate. In any case, the production of nitric oxide or nitrous oxide was not observed in these experiments.

Denitrification by cells grown aerobically on aromatic compounds and other substrates. Experiments on denitrification by aerobically grown cells during the transition to anaerobic conditions were performed. Typical lag periods were observed in the production of molecular nitrogen with aromatic compounds by cells grown aerobically on *o*-phthalate. These

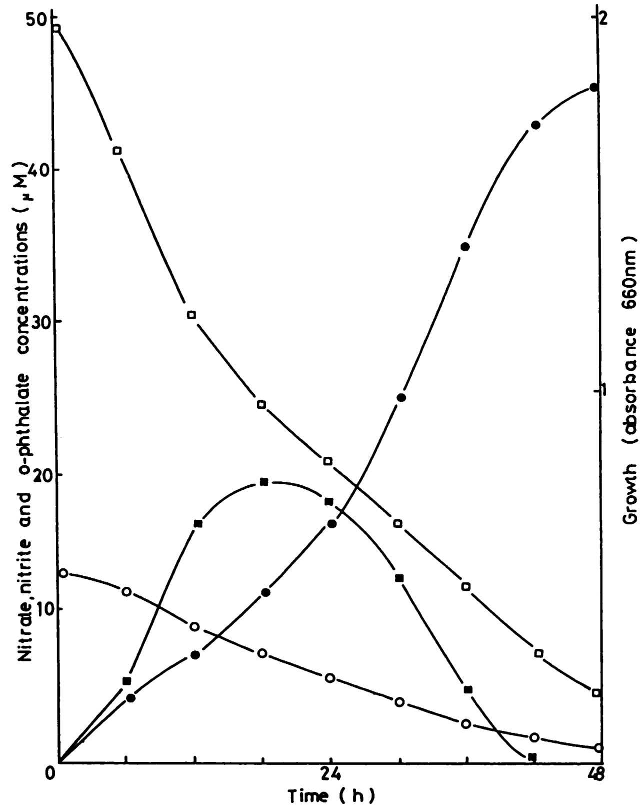

FIG. 1. Concomitant disappearance of *o*-phthalate and nitrate from the culture medium during anaerobic growth of *Pseudomonas* sp. strain P136. A 2-liter culture was anaerobically grown in 2-liter Erlenmeyer flask. Samples (10 ml) were aseptically removed at the times indicated. Concentrations of nitrate (□), nitrite (■), and *o*-phthalate (○) in samples were determined as described in Materials and Methods. Growth (●) was followed by measuring absorbance at 660 nm.

lag periods were observed in denitrification either with aromatic compounds or with other substrates. Transient production of nitrous oxide was observed in this process. Lag periods were also observed in experiments with cells grown on succinate. The addition of chloramphenicol completely inhibited the initiation of production of molecular nitrogen and nitrous oxide with all substrates tested. These results indicate that the lag periods in denitrification by these cells correspond to the time for the induction of enzymes

TABLE 2. Denitrification by *Pseudomonas* sp. strain P136 on various substrates

Substrate	Rate of N_2 production (μmol/mg [dry wt] per min) by cells grown on:							
	o-Phthalate	*m*-Phthalate	*p*-Phthalate	Benzoate	*m*-Hydroxy-benzoate	*p*-Hydroxy-benzoate	Pimelate	Succinate
o-Phythalate	0.37	0.36	0.36	0.34	0.29	0.30	0.01	0.01
m-Phthalate	0.35	0.39	0.37	0.32	0.31	0.31	0.005	0.01
p-Phthalate	0.38	0.36	0.40	0.29	0.26	0.29	0.02	0.003
Benzoate	0.28	0.30	0.29	0.33	0.31	0.32	0.01	0.02
m-Hydroxybenzoate	0.29	0.27	0.30	0.32	0.34	0.36	0.008	0.003
p-Hydroxybenzoate	0.30	0.32	0.30	0.33	0.35	0.37	0.005	0.007
Cyclohex-1-ene-carboxylate	0.34	0.33	0.33	0.36	0.37	0.36	0.007	0.006
Cyclohex-3-ene-carboxylate	0.31	0.29	0.30	0.31	0.27	0.27	0.004	0.003
Pimelate	0.27	0.30	0.29	0.26	0.30	0.31	0.32	0.19
Adipate	0.18	0.22	0.20	0.21	0.17	0.20	0.31	0.23
Succinate	0.20	0.19	0.20	0.18	0.19	0.19	0.28	0.24

required for denitrification rather than the time for the induction of enzymes to utilize aromatic compounds.

Denitrification with nitrite and nitrous oxide as terminal electron acceptors. The cells cultivated under anaerobic conditions on nitrate were also capable of utilizing nitrite and nitrous oxide as terminal electron acceptors. To investigate the relationship between the anaerobic metabolism of aromatic compounds and the source of electron acceptors, experiments were performed on denitrification with these electron acceptors. When cells were anaerobically grown on *o*-phthalate and nitrate, *o*-phthalate also supported the reduction of nitrite and nitrous oxide to molecular nitrogen. When the reduction of nitrous oxide was inhibited by the addition of 10% (vol/vol) acetylene in the gas phase (2), these cells produced nitrous oxide from nitrite. This process was also supported by anaerobic utilization of *o*-phthalate. Table 3 represents the initial rates of the production of molecular nitrogen and nitrous oxide by *o*-phthalate-grown cells and succinate-grown cells.

Oxygen consumption by cells grown under various conditions. The availability of aromatic compounds for aerobic respiration was investigated with various cell preparations by measuring oxygen consumption with the substrates. *o*-Phthalate, *m*-phthalate, *p*-phthalate, benzoate, and succinate were tested and proved to be able to support oxygen consumption by cells grown either aerobically or anaerobically on *o*-phthalate without lag periods. On the other hand, *m*-hydroxybenzoate and *p*-hydroxybenzoate did not support oxygen consumption by these cells. The addition of chloramphenicol did not affect oxygen consumption by these cells.

When cells were grown on succinate under both aerobic and anaerobic conditions, succinate supported oxygen consumption by these cells without lag periods. On the other hand, oxygen consumption with phthalate was initiated after lag periods in these cells. The addition of chloramphenicol did not affect oxygen consumption with succinate but completely inhibited the initiation of oxygen consumption with *o*-phthalate.

DISCUSSION

A new soil bacterium, *Pseudomonas* sp. strain P136, was isolated by selective enrichment for denitrification by anaerobic utilization of *o*-phthalate. This strain utilized *o*-phthalate and various other aromatic compounds anaerobically as substrates for growth and denitrification. In comparison with the bacterial strains reported for the anaerobic utilization of aromatic compounds, *Pseudomonas* sp. strain P136 showed quite different characteristics in its range of usable substrates. *m*-Phthalate, *p*-phthalate, cyclohex-1-ene-carboxylate, and cyclohex-3-ene-carboxylate were utilized by this strain. These compounds have not been reported to support anaerobic growth of and denitrification by the bacterial strains mentioned above. On the other hand, catechol, protocatechuate, and chclohexanecarboxylate were not utilized either aerobically or anaerobically by *Pseudomonas* sp. strain P136.

On the basis of the results from the studies on denitrification, it was suggested that the anaerobic utilization of aromatic compounds by strain P136 proceeds in a manner of simultaneous adaptation (Table 2). For example, *o*-phthalate-adapted cells were also capable of utilizing other aromatic compounds and cyclohexene-carboxylates without lag periods and de novo protein synthesis. Although *m*-hydroxybenzoate and *p*-hydroxybenzoate did not support the aerobic growth of this bacterium, these compounds were utilized as substrates for denitrification without lag periods by cells grown on other aromatic compounds. These results suggest that the aromatic compounds were utilized through the metabolic pathways under coordinate regulation in this strain. The occurrence of simultaneous adaptation in aerobic metabolism of cyclohexenecarboxylates and benzoate has been reported by Blakeley and Perish (4, 5). However, little is known about the occurrence of such regulation of the anaerobic metabolism of various aromatic compounds by denitrifying bacteria.

In the case of general aerobic metabolism of aromatic compounds, the absolute requirement for molecular oxygen and the direct incorporation of oxygen atoms from molecular oxygen into the substrates are well known (11). Although the concomitant disappearance of *o*-phthalate and nitrate (Fig. 1) suggests the cooperative metabolism of these substrates, experiments with nitrite and nitrous oxide showed no requirement for specific nitrogenous oxides for the anaerobic utilization of *o*-phthalate (Table 3). These results suggest that the possibility of direct interaction between the oxygen atoms of specific molecular species and aromatic compounds, such as in the case of aerobic metabolism of aromatic compounds, can be eliminated in the case of the anaerobic metabolism of aromatic compounds by this bacterium.

Williams and Evans described the anaerobic metabolism of benzoate by a *Moraxella* sp. through nitrate respiration and proposed a reductive metabolic pathway (22). In this pathway, benzoate is reduced to cyclohexanecarboxylate, followed by alicyclic ring cleavage. *Moraxella* sp. utilizes benzoate anaerobically through this pathway and aerobically via catechol, as in the case of general aerobic metabolism of benzoate. These pathways are induced alternatively depending on the culture conditions.

In contrast, *Pseudomonas* sp. strain P136 showed quite different properties. Cyclohexanecarboxylate was not utilized by this strain under any conditions tested. Experiments on oxygen consumption showed another different property of this strain. In the transition from anaerobic to aerobic conditions, no lag period for the induction of the alternative pathway was observed. These results suggest that the anaerobically grown cells possess the respiratory system to utilize molecular oxygen, and the cells grown by anaerobic utilization of *o*-phthalate were also capable of utilizing various other aromatic compounds as substrates for respiration without a lag time for adaptation.

In the case of photosynthetic bacteria, Hutber and Rib-

TABLE 3. Denitrification by *Pseudomonas* sp. strain P136 with nitrite and nitrous oxide as electron acceptors

Electron acceptor	Substrate	Rate of N_2 and N_2O production (μmol/mg [dry wt] per min) by cells grown on:			
		o-Phthalate		Succinate	
		N_2	N_2O	N_2	N_2O
Nitrite	*o*-Phthalate	0.39	ND[a]	0.02	ND
	Succinate	0.18	ND	0.20	ND
N_2O	*o*-Phthalate	0.45	—[b]	0.03	—
	Succinate	0.36	—	0.31	—
Nitrite + 10% C_2H_2	*o*-Phthalate	ND	0.45	ND	0.03
	Succinate	ND	0.23	ND	0.39

[a] ND, Not detected (<1 nmol/mg per min).
[b] —, Not done.

bons reported the immediate oxidation of cyclohexanecarboxylate and its derivatives by *Rhodopseudomonas palustris* grown on benzoate under anaerobic conditions in the presence of light (12). However, in this case, no growth or oxygen consumption was observed with benzoate under aerobic conditions. It is also widely accepted that, in the genus *Pseudomonas*, phthalate is usually metabolized via protocatechuate, and benzoate is metabolized via catechol, and these metabolic pathways are known to be regulated in independently from each other (10). On the other hand, *Pseudomonas* sp. strain P136 did not utilize either protocatechuate or catechol, and catechol compounds were not detected in the culture medium during growth on *o*-phthalate or benzoate under either aerobic or anaerobic conditions. These results indicate the characteristic physiological properties of *Pseudomonas* sp. strain P136, distinct from the other denitrifying bacteria which have been reported on the anaerobic utilization of aromatic compounds.

On the basis of the present study, we consider that this report represents the first systematic physiological investigation of denitrification with *o*-phthalate and a series of other aromatic compounds. Detailed biochemical studies on the anaerobic metabolism of aromatic compounds by this strain will be described in future publications.

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