APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1986, p. 1117–1122
0099-2240/86/111117-06$02.00/0

Vol. 52, No. 5

Anaerobic Oxidation of *p*-Cresol by a Denitrifying Bacterium

INGEBORG D. BOSSERT[1] AND L. Y. YOUNG[1,2]*

Department of Microbiology[1] and Department of Environmental Medicine,[2] New York University Medical Center, New York, New York 10016

Received 21 May 1986/Accepted 20 August 1986

Metabolism of *p*-cresol (pCr) under nitrate-reducing conditions is mediated by the denitrifying bacterial isolate PC-07. The methyl substituent of the substrate is oxidized anaerobically by whole-cell suspensions of PC-07 through a series of dehydrogenation and hydration reactions to yield *p*-hydroxybenzoate (pOHB) in stoichiometric proportions. The partially oxidized intermediates in the pathway *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde can also serve as substrates for pOHB formation. Nitrate is required as the external electron acceptor and is reduced to molecular N_2. Reduction of the nitrate is stoichiometric, with pCr serving as the electron donor. In addition, the molar relationship between the electron acceptor (NO_3^-) reduced to the electron donor oxidized decreased to approximately 2:3 and then to 1:3 when *p*-hydroxybenzyl alcohol or *p*-hydroxybenzaldehyde, respectively, served as substrates. The decreased ratios were to be expected when the partially oxidized intermediates served as substrates, because they provided correspondingly less reducing power for pOHB formation. The anaerobic oxidation of pCr by PC-07 demonstrates a mechanism whereby aromatic compounds can be transformed in anoxic environments.

The anaerobic biodegradation of benzoate and its analogs by nitrate-reducing bacteria has been well established (1, 6, 7, 20, 22–24, 26), and the metabolism of aromatic compounds in other reducing environments in which sulfate or carbonate serve as final electron acceptors has been reviewed (7, 21, 27). However, in most of these studies, the compounds under investigation were benzoate analogs that contain a carboxyl functional group attached to the aromatic ring. On the other hand, studies under anaerobic conditions on the biodegradation of phenolic compounds containing hydroxyl rather than carboxyl ring constituents have been limited. Biodegradation of phenolic analogs has been shown to occur in methanogenic consortia (4, 9, 28), with evidence indicating that demethylation may occur prior to ring fission (28). Field and laboratory studies have reported that cresols are biodegraded under nitrate-reducing conditions in soils and aquifers (10, 17, 22). Recently, cresol metabolism under sulfate-reducing conditions has also been observed (W. Smolenski and J. Suflita, Abstr. Annu. Meet. Soc. Environ. Toxicol. Chem., p. 21, 1985). In these reports and other studies on phenolic metabolism by nitrate reduction in enrichment cultures (2), the phenomenon generally has been examined under mixed culture conditions. That is, the role and activity of isolated species have not been clarified.

Recently, our laboratory has reported on the isolation of a two-membered bacterial coculture that utilizes *p*-cresol (pCr) as growth substrate under nitrate-reducing conditions (3a). One of the isolates, PC-07, oxidizes pCr to *p*-hydroxybenzoate (pOHB), which in turn is further metabolized by the second nitrate-reducing isolate PB-04. A closer examination of the pathway for the metabolism of the pCr methyl substituent to a carboxyl moiety under anaerobic conditions was of interest since O_2 is absent, thus precluding oxygenase activity and implicating a different nonoxygenase-mediated mechanism.

In the studies presented here we examine the pathway by which anaerobic oxidation of pCr takes place, as mediated by pure culture cell suspensions of the isolated bacterium PC-07. In addition, the role and requirement for nitrate during pCr oxidation was determined. These mechanisms may play an important role in the dissimilation of pCr and other phenolic compounds from anoxic environments.

MATERIALS AND METHODS

Media and cell culture. Strain PC-07, which metabolizes pCr under nitrate-reducing conditions, was grown in a nutrient medium (TYN) containing the following (per 1,000 ml of water): Trypticase soy broth (BBL Microbiological Systems, Cockneysville, Md.), 15.0 g; yeast extract (Sigma Chemical Co., St. Louis, Mo.), 5.0 g; and KNO_3, 3.0 g. The pH was adjusted to 7.1. Strict anaerobiosis (11) was maintained with oxygen-free argon (Linde Specialty Gases, Somerset, N.J.) during preparation, and 100-ml fractions were dispensed into argon-gassed serum bottles, which were subsequently sealed with butyl rubber stoppers (Bellco Glass, Inc., Vineland, N.J.). After autoclaving, filter-sterilized pCr (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added from an anaerobic stock solution to yield 300 mg/liter.

Incubation was at 30°C in the dark for 48 to 72 h, as required for turbid growth. For aerobic growth, PC-07 was inoculated into 125-ml Erlenmeyer flasks containing 25 ml of TYN without added nitrate and incubated at ambient temperature on a rotary shaker. Examination by phase-contrast microscopy, as well as uniform colony morphology on TYN agar plates, served as a check for culture purity. All chemicals serving as substrates were of 99% or higher purity (Aldrich; Fluka Chemical Corp., Hauppauge, N.Y.).

Experiments with washed cell suspensions. Cell cultures grown in TYN medium (100 ml) were harvested under argon gas by centrifugation at 6,800 × *g* for 10 min in capped polypropylene tubes. The cell pellets were washed with argon-gassed 40 mM phosphate buffer (pH 7.1), centrifuged again, and suspended in the anoxic phosphate buffer with

* Corresponding author.

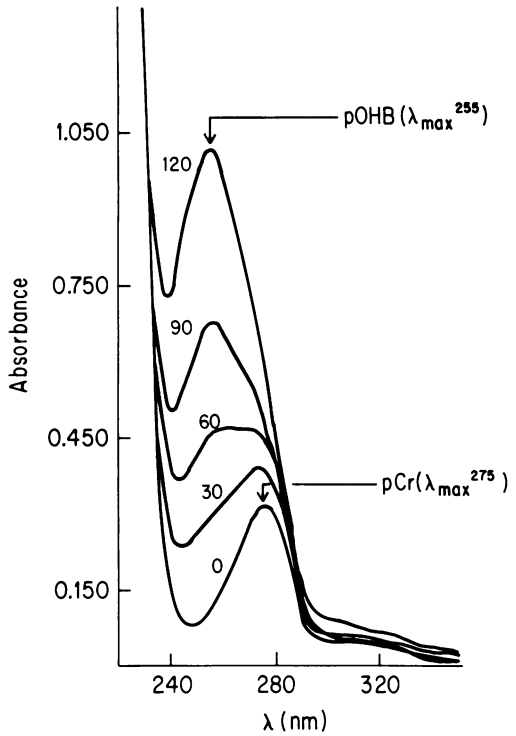

FIG. 1. UV spectra of pCr utilization by a PC-07 cell suspension. Anaerobically grown cells were suspended (0.42 mg of protein per ml) in phosphate buffer containing 15 mM KNO_3 and 3 mM pCr. The suspension was sampled at zero and at 30-min intervals, diluted 10-fold, and scanned from 350 to 190 nm. A distinct shift in absorbance at λ_{max} from 275 to 255 nm occurred with time as pCr was oxidized to pOHB.

added nitrate (5 to 15 mM KNO_3). The cell suspensions were transferred anaerobically with gassed syringes to sterile, argon-gassed, and stoppered Hungate tubes; for N_2 gas measurement, the cell suspensions were transferred to replicate 10-ml butyl rubber-stoppered serum bottles (Supelco, Inc., Bellefonte, Pa.). Substrate was added from filter-sterilized, anaerobic stock solutions to provide a 1 to 3 mM concentration. Incubation was at 25°C in the dark. Substrate and nitrate utilization, as well as product formation in the respective cell suspensions, were monitored at regular intervals by removing 0.1-ml samples from the incubation vessels with argon-gassed syringes. These were diluted 10-fold with 0.01 N HCl, passed through a 0.22-μm-pore-size filter, and analyzed by high-pressure liquid chromatography (HPLC). Formation of N_2 by nitrate reduction was determined by gas chromatography on isobaric samples of the headspace gas removed with a syringe (Pressure-Lok; Precision Sampling Corp., Baton Rouge, La.). The total amount of protein in the cell suspensions was determined by the method of Bradford (5) after the cells were treated with toluene to release the protein (18).

Analytical techniques. pCr and pOHB from filtered and diluted cell suspensions were quantified by HPLC (334 LC; Beckman Instruments, Inc., Fullerton, Calif.) with a methanol-water (60:40) mobile phase flowing through a Spherisorb octadecylsilane column (25 by 0.46 cm; Supelco) at 1 ml/min. A flowthrough UV detector measured the corresponding pCr and pOHB maxima at 275 and 255 nm, eluting at 5.5 and 3.0 min, respectively. The additional metabolites *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde were also quantified in this manner, with *p*-hydroxybenzyl alcohol eluting at 2.3 min (maximum absorbance [λ_{max}], 270 nm) and *p*-hydroxybenzaldehyde eluting at 3.9 min (λ_{max}, 260 nm). A linear standard curve served to quantitate the compounds. For a rapid, semiquantitative determination of substrate loss (including phenol, toluene, and *m*- and *o*-cresol) and pOHB formation, the supernatant from microfuged cell suspensions was diluted 10-fold with 0.01 N HCl and scanned from 350 to 190 nm with a UV spectrophotometer (Shimadzu UV-240; Shimadzu, Columbia, Md.).

Nitrate was quantified by HPLC as described by Braun and Gibson (6). N_2 from nitrate reduction was measured with a gas partitioner (Fisher Scientific Co., Springfield, N.J.) with thermal conductivity detection. The carrier flow rate was 30 ml of He per min through a molecular sieve column. To circumvent any correction for pressure fluctuations in the sealed incubation vessels as a result of N_2 production, the headspace gases were sampled and injected isobarically into the gas partition. N_2 solubility in the cell suspension was determined by the method of Umbreit (25) and included in subsequent calculations. A linear standard curve with a limit of detection of 5 μl of N_2 per 0.25-ml injection was used to quantitate N_2 in the gas samples.

RESULTS

Anaerobic oxidation of pCr to pOHB. UV scans of a washed cell suspension at timed intervals are illustrated in Fig. 1. A shift in absorbance from the characteristic λ_{max} of pCr at 275 nm to that of pOHB at 255 nm during incubation of PC-07 is shown. Mass spectral analyses have confirmed the transformation of pCr to pOHB (3a). Quantitation of the two compounds in the suspension by HPLC indicated that stoichiometric yields of pOHB were produced during the metabolism of pCr in these cell suspensions. These data (Fig. 2) show that for every 1 mol of pCr consumed 1 mol of pOHB was produced.

It should be noted that no metabolic intermediates were

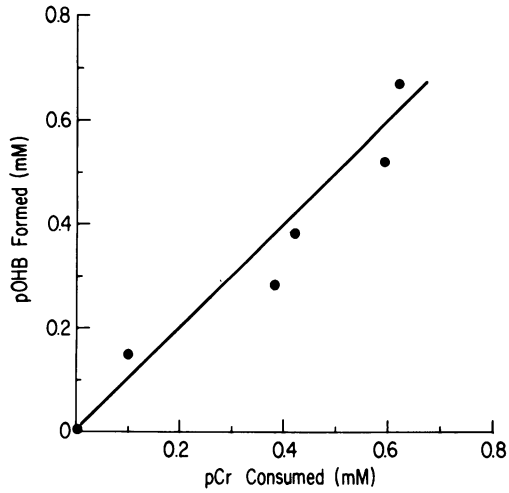

FIG. 2. Stoichiometry of pCr oxidation to pOHB by PC-07 cells under denitrifying conditions. Anaerobically grown cells were suspended (0.42 mg of protein per ml) in 15 mM KNO_3 with 3 mM pCr. Incubation was under argon at 25°C. Linear regression analysis of the data demonstrates a correlation coefficient of 0.968.

detected in the cell suspensions. This was most likely due to rapid metabolism by a larger cell mass. However, in growing cultures of PC-07, two transient intermediates of anaerobic pCr metabolism were detected by HPLC analysis. By comparing their respective elution times (2.3 and 3.9 min) and absorbance maxima (270 and 260 nm) with those of authentic compounds, the metabolites were identified as *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde, respectively. Based on their oxidation states, it is proposed that *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde are intermediates formed in sequence during pCr oxidation to pOHB under anaerobic conditions. This is supported by the transient formation first of *p*-hydroxybenzyl alcohol and then *p*-hydroxybenzaldehyde which is observed with respect to time during the shift in UV absorbance profiles of cell cultures metabolizing pCr (data not shown). Furthermore, when cell suspensions of PC-07 were incubated anaerobically in the presence of nitrate with either *p*-hydroxybenzyl alcohol or *p*-hydroxybenzaldehyde, these substrates were immediately oxidized to pOHB without a lag period.

No substrate metabolism was observed when cell suspensions were incubated anaerobically with structural analogs of pCr such as phenol, toluene, or *o*- or *m*-cresol. The type and position of ring substitution therefore suggest that the mechanism is specific for the anaerobic oxidation of the *para*-methyl group. However, 4-ethylphenol (pEP), an alkyl analog of pCr (4-methylphenol) was metabolized by PC-07. UV spectra of anaerobic cell suspensions incubated with either pEP or pCr demonstrate that pEP is oxidized by PC-07 at a rate which is substantially less than that for pCr. In addition, a slower rate of nitrate reduction reflected this decreased rate of metabolism (data not shown). A larger propyl substituent on the phenolic ring was not metabolized by PC-07 cells. It therefore appears that the size of the alkyl substituent affects the rate and ability of a substrate to be utilized.

Nitrate reduction during anaerobic pCr oxidation. Anaerobically grown cell suspensions demonstrated substrate metabolism under anaerobic conditions only when nitrate was available to serve as the electron acceptor (Fig. 3). No anaerobic metabolism occurred in the absence of nitrate. In addition, cell suspensions of aerobically grown cells incubated under nitrate-reducing conditions demonstrated no activity. This may be due to the absence of inducible nitrate reductases (19) that serve as a vehicle for electron transport under anaerobic conditions. Our attempts to provide an artificial electron acceptor such as phenazine methosulfate to the cell suspensions were unsuccessful due to toxicity to whole cells. Conversely, anaerobically grown cells were able to aerobically oxidize pCr in cell suspensions without nitrate. This suggests that O_2 alternately may serve as electron acceptor or that constitutive oxygenases are present.

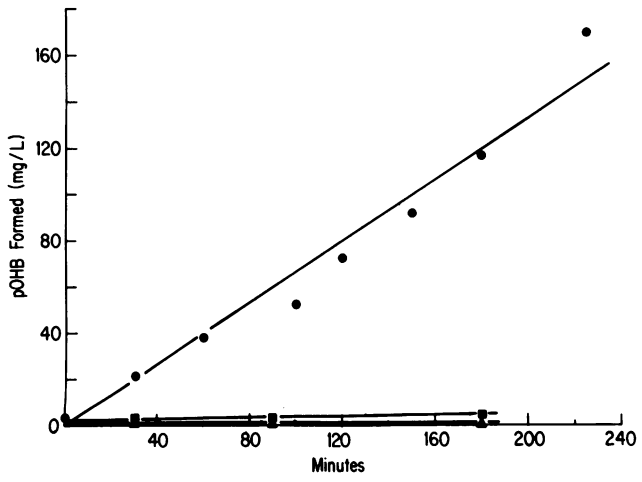

FIG. 3. Effect of aerobic growth and nitrate on anaerobic oxidation of pCr by PC-07 cells. Both aerobically (■) and anaerobically (●) grown cell suspensions (0.42 mg of protein per ml) were incubated under argon in phosphate buffer containing 3 mM pCr and 15 mM KNO_3. A suspension of anaerobically grown cells was incubated under identical conditions without KNO_3 (▲).

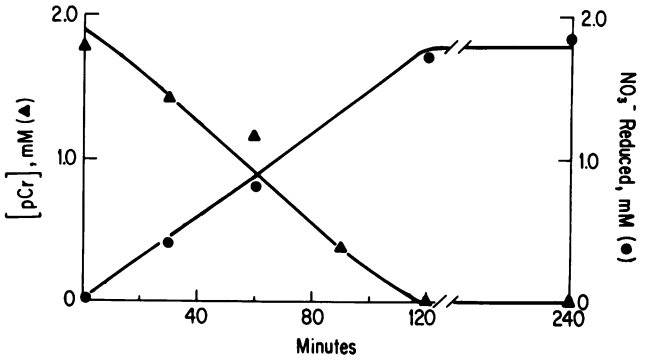

FIG. 4. Effect of electron donor (pCr) on nitrate reduction. A suspension (0.40 mg of protein per ml) of anaerobically grown PC-07 cells was prepared under argon in phosphate buffer containing NO_3^- in excess (15 mM) and 3 mM pCr. Incubation was at 25°C. pCr (▲) and nitrate (●) concentrations were measured at time zero and at 30, 60, 90, 120, and 240 min by HPLC, as described in the text.

The requirement for NO_3^- under anaerobic conditions is substantiated and supported by the results presented in Fig. 4. Nitrate was present in excess, and its reduction occurred only as long as pCr was available to serve as the electron donor. When pCr became limiting, nitrate reduction ceased.

The data presented in Fig. 5 further underscore the relationship between substrate oxidation and nitrate reduction by PC-07 in cell suspensions. On a molar basis, approximately equal amounts of NO_3^- were reduced when pCr served as the substrate for pOHB production. However, when the more oxidized *p*-hydroxybenzyl alcohol served as substrate, the molar ratio of NO_3^- used to pOHB formed was reduced to 2:3 and was further reduced to 1:3 when *p*-hydroxybenzaldehyde was the substrate. This demonstrates that each successively more oxidized metabolite in the reaction sequence generates proportionately and quantitatively less reducing power. Thus, nitrate serves as the terminal electron acceptor for the reducing equivalents generated at each step during pCr oxidation to pOHB via the intermediates *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde.

Nitrate is completely and quantitatively reduced to gaseous N_2. When PC-07 cell suspensions were incubated with 0.91 mM pCr, the amounts of pOHB formed (0.78 mM) and NO_3^- reduced (0.85 mM) demonstrated a 1:1.1 relationship, which approximates an expected 1:1.2 stoichiometry (3a). In addition, 0.44 mM N_2 (0.88 mM-N_2 nitrogen) was formed, which represents a 94% recovery of N_2 from the stoichiometric reduction of nitrate. Although nitrite and N_2O intermediates were detected and appeared transiently during

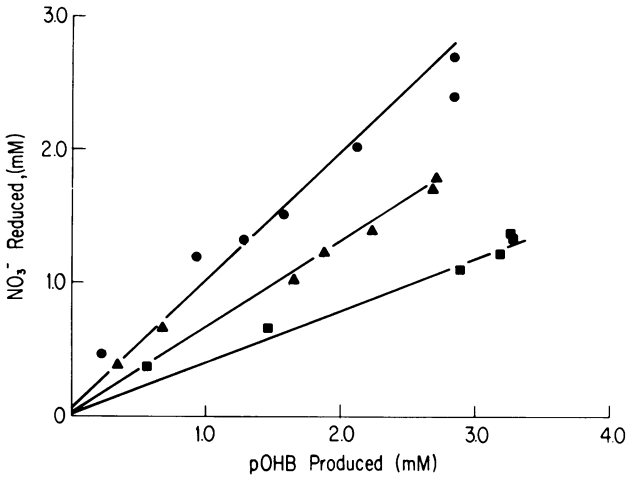

FIG. 5. Relationship of nitrate reduction to pOHB formation from pCr, *p*-hydroxybenzyl alcohol, or *p*-hydroxybenzaldehyde. Identical fractions of a cell suspension (0.62 mg of protein per ml) were incubated under argon in phosphate buffer containing 1.8 mM substrate and pCr (●), *p*-hydroxybenzyl alcohol (▲), or *p*-hydroxybenzaldehyde (■). Nitrate was added in excess (2.5 mM). Each incubation was sampled at time zero and then at 30-min intervals. Linear regression analysis of the data demonstrates a greater than 0.989 correlation coefficient for all substrates.

the course of incubation in growing cultures, they were not detected in the cell suspension assays, most likely because the high cell concentration produced a very rapid turnover which precluded their detection.

DISCUSSION

Results of recent studies from our laboratory have demonstrated that pCr is metabolized anaerobically as a sole carbon source by two bacterial isolates in coculture under nitrate-reducing conditions (3a). The individual activities of each member demonstrate a syntrophic mode for anaerobic pCr metabolism. One bacterial species (PC-07) exclusively oxidizes pCr to pOHB, yielding energy through electron transport but no net carbon for growth. The other bacterium (PB-04) is able to metabolize only the pOHB intermediate, thus providing growth carbon from ring fission for both members of the coculture.

Examination of the mechanisms of pCr metabolism under nitrate-reducing conditions by the isolated pure culture of PC-07 in the cell suspension studies presented here indicate that pCr metabolism proceeds via anaerobic oxidation, yielding pOHB, which is a known precursor for anaerobic ring fission (7, 23). It has been suggested previously (G. Bakker, Ph.D. thesis, Technical University, Delft, The Netherlands, 1977) that aromatic metabolism of phenols under denitrifying conditions occurs by hydrogenation and then ring cleavage without initial metabolism of the methyl group. These different mechanisms for cresol metabolism under nitrate-reducing conditions may be a consequence of different inoculum sources and physiological differences in the isolated microorganisms. In fact, unlike the isolated and mixed cultures described by Bakker (2), PC-07 is unable to utilize phenol.

Our results on the anaerobic oxidation of pCr by the PC-07 isolate are summarized in Fig. 6. The pathway illustrates a means whereby the methyl group of pCr is oxidized to a carboxyl moiety through a sequence of dehydrogenation and hydration reactions that are similar to a pathway observed in aerobic organisms (13). The *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde intermediates have been detected in sequence in growing cultures of PC-07 and can serve as substrates for oxidation to pOHB by whole-cell suspensions.

It is unlikely that oxidation of the methyl group is mediated by oxygenases under denitrifying conditions. Although Fabig et al. (8) have argued that traces of O_2 may be present in media used for metabolic studies under denitrifying conditions, the experimental protocol used here followed strictly anaerobic techniques (15) to exclude molecular oxygen, which was not the case in earlier studies. The oxidation state of the medium demonstrated a redox potential of less than 0 mV, as determined with a methylene blue redox indicator dye. Furthermore, the inability of aerobically grown cells to oxidize pCr anaerobically in the presence of nitrate underscores an anaerobic requirement for this denitrifying pathway.

Instead, the addition of oxygen to the molecule can occur through water by means of a methyl hydroxylase (12). The pCr is initially oxidized to the quinone methide structure (Fig. 6), a proposed, although undetected, intermediate for the initial incorporation of water-derived oxygen into the methyl carbon. This is based on a similar sequence of events for pCr oxidation by aerobic *Pseudomonas* strains (13, 14, 16; G. Whited and D. T. Gibson, personal communication). Isotopic ^{18}O studies have demonstrated that the incorporated methyl oxygen was derived from ^{18}O-labeled H_2O (12), with molecular O_2 serving only as an external electron acceptor and not as a reactant in the pathway. It was observed that cell-free activity was maintained under anaerobic conditions only if an artificial electron acceptor such as phenazine methosulfate was provided (14).

To our knowledge, this is the first report of such a pathway in pure culture under anaerobic, denitrifying conditions. A similar sequence recently has been observed in sulfate-reducing enrichment cultures (J. Suflita and W. Smolenski, personal communication). As reported here, nitrate is required as the terminal electron acceptor and is reduced to

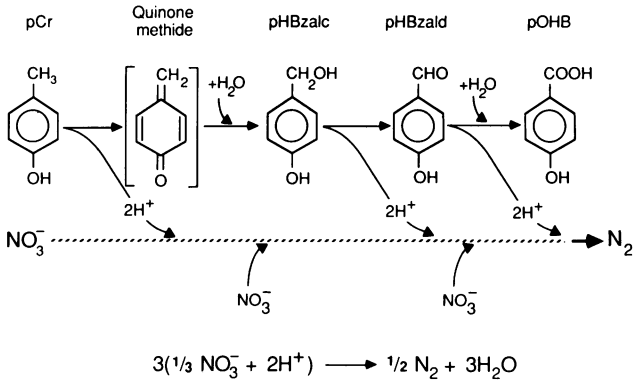

FIG. 6. Proposed model pathway for the anaerobic oxidation of pCr to pOHB by PC-07 under denitrifying conditions. Initial incorporation of water-derived oxygen occurs by a proposed methyl hydroxylase enzyme (13). Further oxidation can be mediated by alkyl dehydrogenases (3). Nitrate served as the terminal electron acceptor for each oxidation step, so that the reducing equivalents ($6H^+$) generated in the pathway served to reduce NO_3^- to N_2. Abbreviations: pHBzalc, *p*-hydroxybenzyl alcohol; pHBzald, *p*-hydroxybenzaldehyde.

N_2. Complete reduction to N_2 by PC-07 occurs even when nitrate is in excess. This may not always be the case; for instance, Braun and Gibson (6) reported that an aminobenzoate-degrading pseudomonad reduced nitrate to nitrite under anaerobic conditions, and further reduction of nitrite to N_2 occurred only when nitrate was exhausted from the medium.

As noted in the model presented in Fig. 6, each step in pCr oxidation can generate two reducing equivalents, producing a total of six electrons which serve to reduce nitrate to molecular N_2. Nitrate reduction is dependent on pCr to serve as the electron donor (Fig. 4). A reciprocal dependence of the electron donor to the electron acceptor has also been demonstrated (Fig. 3); that is, pCr is oxidized anaerobically only when nitrate is present.

The PC-07 isolate exhibits a limited substrate range. Thus far, results with pEP and *p*-propylphenol indicate that the size of the alkyl substituent affects its rate of metabolism. Structural analogs of pCr (e.g., phenol, toluene, and *o*- and *m*-cresol) were not metabolized by the anaerobic cell suspensions. The type and position of ring substitution therefore suggest that the mechanism is specific for anaerobic oxidation of the *para*-methyl group. Our observation of *m*-cresol biodegradation in other enrichment cultures (unpublished data), as well as studies reported for other analogs (2), suggest that more than one mechanism may operate under denitrifying conditions.

PC-07 can only partially metabolize pCr under nitrate-reducing conditions. After oxidation to pOHB, it lacks the metabolic capacity to cleave the aromatic nucleus and thus relies on subsequent ring fission by another nitrate-reducing microorganism. Metabolic energy, however, may be produced by electron transfer during oxidation of the methyl substituent. Perhaps such an energy-yielding strategy, which has been shown to take place in aerobic environments as well (13), provides a competitive advantage in environments with variable O_2 availability, such as polluted and eutrophic bodies of water, groundwater, or water-saturated soils. By conferring greater versatility, this pathway may have been evolutionarily conserved in such facultative aerobic microorganisms.

In summary, the results presented here demonstrate a mechanism by which a phenolic compound such as pCr is biodegraded in anaerobic, denitrifying environments. Anaerobic oxidation of the methyl group produces pOHB by way of *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde intermediates. Phenolic residues in the anaerobic environment may thus be detoxified and degraded in this manner.

ACKNOWLEDGMENTS

This study was supported in part by grant ECE-8118018 from the National Science Foundation and Public Health Service training grant AI07180 from the National Institutes of Health.

We thank K. B. Shipp for typing the manuscript.

LITERATURE CITED

1. **Aftring, P. R., B. E. Chalker, and B. F. Taylor.** 1981. Degradation of phthalic acids by denitrifying, mixed cultures of bacteria. Appl. Environ. Microbiol. **41**:1177–1183.
2. **Bakker, G.** 1977. Anaerobic degradation of aromatic compounds in the presence of nitrate. FEMS Microbiol. Lett. **1**:103–108.
3. **Bayly, R. C., and M. G. Barbour.** 1984. The degradation of aromatic compounds by the meta and gentisate pathways, p. 253–294. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.

3a. **Bossert, I. D., M. D. Rivera, and L. Y. Young.** 1986. *p*-Cresol biodegradation under denitrifying conditions: isolation of a bacterial coculture. FEMS Microbiol. Ecol. **38**:313–319.

4. **Boyd, S., and D. Shelton.** 1984. Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge. Appl. Environ. Microbiol. **47**:272–277.
5. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Braun, K., and D. T. Gibson.** 1984. Anaerobic degradation of 2-aminobenzoate (anthranilic acid) by denitrifying bacteria. Appl. Environ. Microbiol. **48**:102–107.
7. **Evans, W. C.** 1977. Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. Nature (London) **270**:17–22.
8. **Fabig, W., J. Ottow, and F. Mueller.** 1980. Failure of denitrifying bacteria to utilize benzoic acid under anaerobic conditions with nitrate as the only terminal electron acceptor. Eur. J. Appl. Microbiol. Biotechnol. **9**:133–135.
9. **Godsy, E., D. Goerlitz, and G. Ehrlich.** 1983. Methanogenesis of phenolic compounds by a bacterial consortium from a contaminated aquifer in St. Louis Park, Minnesota. Bull. Environ. Contam. Toxicol. **30**:261–268.
10. **Goerlitz, D. F., D. E. Troutman, E. M. Godsy, and B. J. Franks.** 1985. Migration of wood-preserving chemicals in contaminated groundwater in a sand aquifer at Pensacola, Florida. Environ. Sci. Technol. **19**:955–961.
11. **Healy, J. B., Jr., and L. Y. Young.** 1979. Anaerobic biodegradation of eleven aromatic compounds to methane. Appl. Environ. Microbiol. **38**:84–89.
12. **Hopper, D. J.** 1978. Incorporation of [^{18}O] water in the formation of p-hydroxybenzyl alcohol by the p-cresol methylhydroxylase from *Pseudomonas putida*. Biochem. J. **175**:345–347.
13. **Hopper, D. J., and D. G. Taylor.** 1975. Pathways for degradation of *m*-cresol and *p*-cresol by *Pseudomonas putida*. J. Bacteriol. **122**:1–6.
14. **Hopper, D. J., and D. G. Taylor.** 1977. The purification and properties of p-cresol (acceptor) oxidoreductase (hydroxylating), a flavocytochrome from *Pseudomonas putida*. Biochem. J. **167**:155–162.
15. **Hungate, R. E.** 1969. A roll tube method for cultivation of strict anaerobes, p. 117–132. *In* J. R. Norris and D. W. Ribbons (eds.), Methods in microbiology, vol. 3B. Academic Press, Inc., New York.
16. **Keat, M. J., and D. J. Hopper.** 1978. p-Cresol and 3,5-xylenol methylhydroxylases in *Pseudomonas putida* N.C.I.B. 9869. Biochem. J. **175**:649–658.
17. **Kuhn, E. P., P. J. Colberg, J. L. Schnoor, O. Wanner, A. J. Zehnder, and R. P. Schwarzenbach.** 1985. Microbial transformations of substituted benzenes during infiltration of river water to groundwater: laboratory column studies. Environ. Sci. Technol. **19**:961–968.
18. **Miller, J. H.** 1972. Assay of β-galactosidase, p. 353. *In* Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Payne, W. J., and W. L. Balderstrom.** 1978. Denitrification, p. 339–342. *In* D. Schlessinger (ed.), Microbiology—1978. American Society for Microbiology, Washington, D.C.
20. **Schennen, U., K. Braun, and H. J. Knackmuss.** 1985. Anaerobic degradation of 2-fluorobenzoate by benzoate-degrading, denitrifying bacteria. Appl. Environ. Microbiol. **161**:321–325.
21. **Sleat, R., and J. P. Robinson.** 1984. The bacteriology of anaerobic degradation of aromatic compounds. J. Appl. Bacteriol. **57**:381–394.
22. **Taylor, B. F.** 1983. Aerobic and anaerobic catabolism of vanillic acid and some other methoxy-aromatic compounds by *Pseudomonas* sp. strain PN-1. Appl. Environ. Microbiol. **46**:1286–1292.
23. **Taylor, B. F., W. L. Campbell, and I. Chinoy.** 1970. Anaerobic degradation of the benzene nucleus by a facultatively anaerobic microorganism. J. Bacteriol. **102**:430–437.

24. **Taylor, B. F., W. L. Hearn, and S. Pincus.** 1979. Metabolism of monofluoro- and monochlorobenzoates by a denitrifying bacterium. Arch. Microbiol. **122**:301–306.
25. **Umbreit, W. W.** 1972. Calibration of respirometers, p. 62. *In* W. Umbreit, R. Burris, and J. Stauffer (eds.), Manometric and biochemical techniques. Burgess Publishing Co., Minneapolis.
26. **Williams, R. J., and W. C. Evans.** 1975. The metabolism of benzoate by *Moraxella* species through anaerobic nitrate respiration. Biochem. J. **148**:1–10.
27. **Young, L. Y.** 1984. Anaerobic degradation of aromatic compounds, p. 487-523. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.
28. **Young, L. Y., and M. D. Rivera.** 1985. Methanogenic degradation of four phenolic compounds. Water Res. **19**:1325–1332.