

Biochemistry and Genetics of the Pathway  
for the Anaerobic Degradation of Aromatic Compounds  
by Eubacterium oxidoreducens.

by

John David Haddock

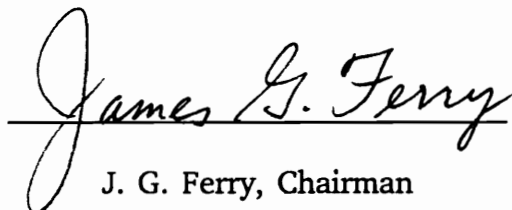
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Virginia Polytechnic Institute and State University  
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in

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APPROVED:


  
J. G. Ferry, Chairman



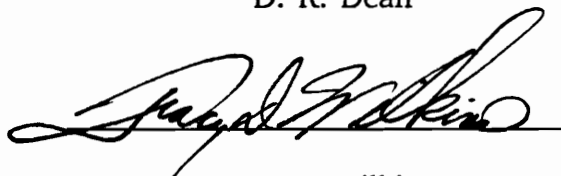
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BIOCHEMISTRY AND GENETICS OF THE PATHWAY  
FOR THE ANAEROBIC DEGRADATION OF AROMATIC COMPOUNDS BY  
EUBACTERIUM OXIDOREDUCENS

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ABSTRACT

The biochemical pathway for the anaerobic degradation of gallate, pyrogallol and phloroglucinol by Eubacterium oxidoreducens was investigated.

Phloroglucinol reductase was purified 90-fold, from the soluble fraction of cell extract, to electrophoretic homogeneity. The enzyme was an  $\alpha_2$  homodimer with a native  $M_r$  of 78,000, did not contain metals or cofactors and was specific for phloroglucinol and NADPH with a  $K_m$  of 800  $\mu$ M and 6.7  $\mu$ M respectively at pH 6.8. The  $K_m$  for phloroglucinol decreased with increasing pH. The enzyme catalyzed reaction was reversible and the equilibrium constant was 9.6.

Dihydroresorcinol was a competitive inhibitor of the reverse reaction ( $K_i = 756 \mu$ M). Dihydrophloroglucinol produced in cell extract with  $H_2$  as the reductant was identical to the compound produced by sodium borohydride reduction of phloroglucinol as shown by  $^1H$  NMR spectroscopy. The  $^{13}C$  NMR spectrum was consistent with the structural assignment of dihydrophloroglucinol. The mechanism of the proposed enzymatically catalyzed reaction is proposed to

involve transfer of a hydride equivalent from NADPH to the carbonyl carbon of the phloroglucinol dianion.

Mutant strains of *E. oxidoreducens* that showed no gallate decarboxylase or dihydrophloroglucinol hydrolase activity were isolated after mutagenesis with ethylmethane sulfonate and enrichment with ampicillin. The decarboxylase deficient mutants were unable to grow on gallate while pyrogallol and phloroglucinol supported growth. The hydrolase deficient mutants were unable to grow on any aromatic substrates and converted gallate to pyrogallol and dihydrophloroglucinol. The conversion of gallate to non-aromatic intermediates by cell extract of the wild-type strain was dependent on the presence of 1,2,3,5-benzenetetrol for the conversion of pyrogallol to phloroglucinol and on formate for the reduction of phloroglucinol to dihydrophloroglucinol. Transhydroxylase activity involved in the conversion of pyrogallol to phloroglucinol was induced by growth on aromatic substrates. The formate dehydrogenase was located in the soluble fraction of cell extract, and activity was protected from oxygen inactivation by sodium azide. The  $K_m$  for formate and NADP was 290  $\mu$ M and 140  $\mu$ M respectively at pH 7.5. The pH optimum for activity was 7.5 and maximum activity was observed at a temperature of 50°C.

## FOREWORD

This dissertation consists of ten sections. Section I is a general introduction to the subject of the degradation of aromatic compounds by anaerobic microorganisms. Section II is a comprehensive literature review. Sections III through VIII describe the experiments and results obtained in the laboratory. Section IX is a summary of the experimental results and presents the major conclusions. Section X is my curriculum vitae.

## DEDICATION

This dissertation is dedicated to the family: parents, Euclide Wayland and Joyce Haddock, Jr.; sister, Linda, her husband Jim Harrell, and daughter Katie; grandparents, E. W. and Emma Haddock, Sr., and Dessa Spung and Lawson Blanton, Sr.; Aunt Marcella and her husband Lou Kliman, and Uncle Lawson Blanton, Jr. and his wife, Ernestine.

## ACKNOWLEDGEMENTS

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## SECTION I. INTRODUCTION

Vast amounts of natural and synthetic aromatic compounds enter the environment continuously. Lignin and plant phenolics such as tannins and flavonoids are produced and released by plants. Aromatic hydrocarbons are released from natural oil seeps and through human exploitation of petroleum as an energy source. Derivatives of aromatic compounds are manufactured for use in agriculture, industry and in the home. Many of these compounds are toxic and are released into the environment through the application of pesticides or as wastes.

The microbial degradation of aromatic compounds to  $\text{CO}_2$  and  $\text{CH}_4$  plays a major role in the global carbon cycle and the removal of toxic compounds from the environment. In aerobic environments, oxygen is used as a terminal electron acceptor for the catabolism of aromatic compounds, but probably more importantly oxygen is utilized in the initial attack on the aromatic ring. Aerobic, aromatic degradation has been well studied and much is known concerning the biochemical pathways, enzymology and genetics of the microorganisms. In contrast, microbial degradation of aromatic compounds that enter important anaerobic habitats such as sediments, wastewater sludge digestors and the digestive tract of animals has not been investigated in detail. Strict anaerobes do not have the option to utilize molecular oxygen as a

reactant to overcome the resonance stabilization of the aromatic ring. Instead, anaerobes use reduction as a mechanism to prepare the ring for cleavage and further metabolism (1). Evidence for the validity of this hypothesis has been slowly accumulating since it was proposed (2), but the nature of the reactions involved remain unknown.

Eubacterium oxidoreducens is a gram positive, non-sporing rod that was isolated from the bovine rumen. The organism is a strict anaerobe with the ability to metabolize certain aromatic compounds in the absence of a terminal electron acceptor. Gallate, pyrogallol, phloroglucinol, and quercetin are degraded and support growth when either H<sub>2</sub> or formate are present. A pathway has been proposed for the degradation of these compounds by E. oxidoreducens (Fig. 1). Evidence for the pathway includes the demonstration of enzyme activities in cell extract and detection of the intermediates in cell extract or during growth. However, additional studies are needed in order to validate the pathway and to determine if any additional steps are involved.

The purpose of this study was: 1) investigate the reactions involved in the reductive pathway for the anaerobic degradation of aromatic compounds by E. oxidoreducens, with particular emphasis on the enzymology and mechanism of the reductive step, and; 2) determine the validity of the initial reactions of the proposed biochemical pathway. Towards these goals, phloroglucinol

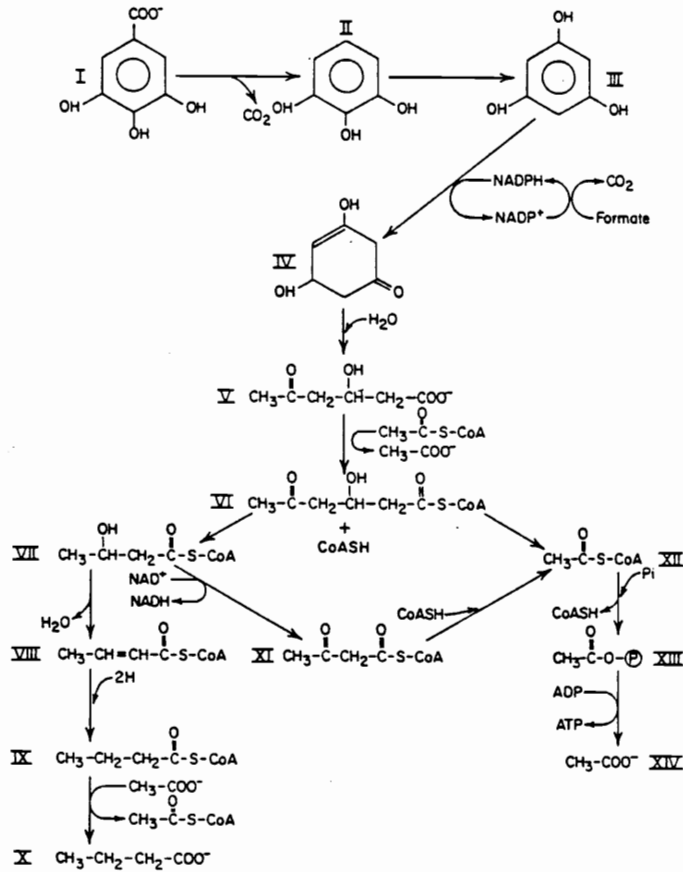


FIG. 1. Proposed pathway for the degradation of gallate by *Eubacterium oxidoreducens*. I, Gallate; II, pyrogallol; III, phloroglucinol; IV, dihydrophloroglucinol; V, 3-hydroxy-5-oxohexanoate; VI, 3-hydroxy-5-oxohexanoyl-CoA; VII, 3-hydroxybutyryl-CoA; VIII, crotonyl-CoA; IX, butyryl-CoA; X, butyrate; XI, acetoacetyl-CoA; XII, acetyl-CoA; XIII, acetyl phosphate; XIV, acetate. Krumholz et al., (4).



reductase was purified to homogeneity and characterized by measurements of its composition, reaction kinetics and thermodynamics. Formate dehydrogenase, which supplies electrons to phloroglucinol reductase, was characterized in cell extracts. Mutants were isolated and studied for consistency with the proposed pathway, and the requirements for the conversion of gallate to non-aromatic compounds by cell extract were determined.

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## SECTION II. LITERATURE REVIEW

### INTRODUCTION

Aromatic compounds constitute a class of organic molecules that are of major importance in the global storage and cycling of carbon. Petroleum, peat and coal deposits, lignin, plant phenolics and proteins contain a large aromatic fraction. Man's activities have increased the levels of natural and synthetic aromatic compounds released into the environment through the exploitation of natural resources and widespread application of pesticides. While microbial degradation or alteration of aromatic compounds by aerobic microorganisms is well known and major degradative pathways have been elucidated (eg. 30, 39, 49) anoxic degradative pathways are poorly understood. This lack of information is the result of difficulties encountered while working with anaerobic procedures, and because the anaerobic metabolism of aromatic compounds was previously thought to be a minor degradative route in nature. However, as early as 1934 Tarvin and Buswell (122) demonstrated that a variety of aromatic compounds were degraded under the strictly anaerobic conditions of methanogenic fermentations. Since the first demonstration of anaerobic aromatic degradation in pure cultures (105) an increasing number of bacteria of diverse physiological types have been shown to degrade a wide variety of aromatic compounds. These organisms generally fall into three physiological groups: i) photosynthetic bacteria, ii) anaerobic respirers,

including dissimilatory sulfate and nitrate reducers, and iii) non-respiring anaerobes. The latter group includes fermentative organisms, obligate proton-reducing acetogens and those involved in one-carbon metabolism. This review includes published studies concerning anaerobic degradation of monoaromatic compounds by mixed as well as pure cultures. The reader is also referred to the reviews of Evans (40), Sleat and Robinson (115), Young (144), Berry et al (16) and Evans and Fuchs (41). Scheline (108) reviewed anaerobic metabolism in the gastrointestinal tract from a pharmacological point of view and includes some information on reactions involving aromatic compounds. Anaerobic degradation of aromatic amino acids has been reviewed by Barker (12).

## I. PHOTOMETABOLISM

Although Tarvin and Buswell (122) first demonstrated the anaerobic degradation of several aromatic compounds to methane and CO<sub>2</sub> in mixed cultures derived from sewage sludge, little information was gained on the organisms or the biochemical pathways involved. The first demonstration of anaerobic aromatic degradation by a microorganism in pure culture was reported by Scher and Proctor (110). A Rhodopseudomonas sp. was isolated that could utilize benzoate as an organic electron donor. Several other strains

of Rhodopseudomonas spp. and Rhodospirillum rubrum grew anaerobically on benzoate, protocatechuate and catechol in the presence of light (105). They proposed a degradation pathway analogous to aerobic pathways in which oxygen is incorporated into the aromatic ring prior to ring cleavage. However, Dutton and Evans (33) proposed that R. palustris photo-metabolized aromatic compounds by a different route. They found that aerobic p-hydroxybenzoate degradation involved oxygenases, while addition of air to cells growing anaerobically on benzoate stopped benzoate degradation. Also, catechol, a key intermediate of the aerobic pathway was not degraded anaerobically. Hegeman (68) showed that enzymes involved in aerobic metabolism of p-hydroxybenzoate by R. palustris were induced by aerobic conditions and the presence of the substrate. In contrast, approximately 100 fold lower enzyme levels were found for cells grown anaerobically on this substrate. Evidence that anaerobic degradation involved a different mechanism was provided by radiotracer studies of Dutton and Evans (35), (36). They found that the aromatic ring was reduced before being cleaved, that oxygenases were absent and that anaerobic benzoate degradation was inducible. They proposed enzyme catalyzed reactions similar to those involved in the  $\beta$ -oxidation of fatty acids. Guyer and Hegeman (58) proposed a similar pathway based on evidence obtained with mutants of

R. palustris and radioisotopes. The failure of initial attempts to show benzoate degradation in cell extracts (36) was subsequently shown by Dutton and Evans, (34), (37) to involve the presence of fatty acids released during cell rupture, and that carboxyl groups were inhibitory. It was suggested that the inhibitory effects resulted from competition for cofactors involved in fatty acid metabolism. Coenzyme A involvement in the degradation of benzoate to pimelate was shown in R. palustris cell extract when it was discovered that benzoate conversion to benzoyl-CoA required ATP and  $Mg^{2+}$  (137). In addition, conversion of cyclohex-1-enecarboxylate, an intermediate of reductive benzoate degradation to pimelate, required NAD. Hutber and Ribbons (75) showed that the coenzyme A derivative of cyclohexane carboxylate was also formed by R. palustris and that the enzymes responsible for cyclohexanecarboxyl-CoA and benzoyl-CoA were induced when these compounds were growth substrates under anaerobic as well as aerobic conditions. All of the major enzyme activities necessary for fatty acid  $\beta$ -oxidation were identified in cell extracts. Harwood and Gibson (63) studied the uptake of benzoate by R. palustris and found that the intracellular formation of benzoyl-CoA was likely the first step involved in degradation and that it was responsible for maintaining a concentration gradient that allowed cells to take up benzoate from the medium at concentrations below 1  $\mu M$ . Geissler et al. (48) purified a soluble benzoate-

coenzyme A ligase from this organism. The enzyme required  $Mg^{2+}$ -ATP for activity and was insensitive to oxygen. Fluorobenzoates were also efficient substrates for the enzyme. Uptake of 4-hydroxybenzoate by this organism has also been studied, and apparently a different ligase enzyme is associated with this substrate (92). Harwood and Gibson (64) have shown that several different strains of *R. palustris* were able to grow anaerobically as well as aerobically on a wide range of substituted aromatic acids. Some compounds were degraded only under anaerobic but not aerobic conditions and vice versa. Experiments with mutants suggested the presence of two pathways of anaerobic metabolism that passed through benzoate or 4-hydroxybenzoate respectively.

## II. RESPIRATORY DEGRADATION

### A. Denitrification

Several aromatic compounds have been shown to be degraded anaerobically by denitrifying organisms that utilize nitrate as the terminal electron acceptor. These organisms are facultative anaerobes and often also have the ability to degrade the same aromatic compounds by aerobic pathways that are distinct from the anaerobic pathways.

Oshima (102) described two soil organisms resembling *Pseudomonas* spp. that were able to anaerobically degrade protocatechuate (3,4-dihydroxy-benzoic

acid) with nitrate in the medium. However, degradation occurred only when the two organisms were co-cultured. Taylor et al. (124) isolated Pseudomonas strain PN-1 from soil using p-hydroxybenzoate with nitrate as the terminal electron acceptor. This organism was also able to degrade benzoate anaerobically. Benzoate, p- and m-hydroxybenzoate and protocatechuate were degraded aerobically without nitrate. Aerobic degradation involved the enzyme protocatechuic acid-4,5-oxygenase which had a specific activity about 50-fold greater in extracts of aerobically compared to anaerobically grown cells. They concluded therefore, that the aerobic and anaerobic pathways of aromatic degradation were different. Taylor and Heeb (126) added support to this hypothesis using  $^{14}\text{C}$ -labeled benzoate. Protocatechuate and catechol, key intermediates of aerobic degradation pathways (49), failed to trap any radioactivity when Pseudomonas PN-1 was grown anaerobically on  $^{14}\text{C}$ -benzoate. Pseudomonas PN-1 was shown to anaerobically degrade several methoxylated aromatic compounds to the corresponding hydroxylated derivatives (123). Dehydroxylation then produced benzoic acid which was proposed as a central intermediate for the anaerobic degradation pathways of some aromatic compounds. Phenylpropane lignin derivatives were only degraded anaerobically, while most of the tested methoxylated aromatics that lacked a three-carbon side chain were demethylated both aerobically and anaerobically.

Blake and Hegeman (17) reclassified this organism as Alcaligenes xylosoxidans subspecies denitrificans PN1, and they found a small 17.4 kilobase plasmid that carried the genes necessary for anaerobic benzoate degradation in this organism. The plasmid was transmissible to strains of P. stutzeri and P. aeruginosa giving them the ability to grow anaerobically on benzoate. Williams and Evans (140, 141) described a Moraxella sp. that degraded benzoate, phloroglucinol (1,3,5-trihydroxybenzene) and several phenylpropane derivatives with nitrate as the terminal electron acceptor. Cyclohexane-carboxylate (CHCA), 2-hydroxy CHCA and adipic acid were identified as degradation products leading them to suggest a degradative pathway similar to the reductive pathway utilized by photosynthetic microorganisms (36). Phenol has also been used as a substrate for the isolation of denitrifiers. Bakker (6) isolated three gram-negative, flagellated rods on phenol and nitrate but growth was slow in pure culture. Enrichment cultures grew faster and were able to degrade a variety of aromatic compounds including benzoate, 3,4-dihydroxybenzoate, m- and p-hydroxybenzoate and o-, m-, and p-cresol. Catechol oxygenase levels were low and <sup>14</sup>C-phenol was used to show some incorporation of substrate into cell material (6). The list of aromatic compounds degraded by nitrate-respiring organisms includes the ubiquitous pollutants known as phthalates (1, 2). Afring et al. (1) described the anaerobic dissimilation of o-, m- and p-phthalate



by denitrifying mixed bacterial cultures. Afting and Taylor (2) described the isolation of a Bacillus sp. that required nitrate to anaerobically degrade o-phthalate to CO<sub>2</sub>. Benzoate was also degraded and was suggested to be an intermediate via decarboxylation of phthalate. Taylor and Ribbons (127) showed that o-phthalic acid was decarboxylated to benzoate anaerobically in the presence of nitrate. Nozawa and Maruyama (99) isolated a *Pseudomas* sp. that degraded o-, m- and p-phthalate as well as benzoate, cyclohex-1-ene-carboxylate and cyclohex-3-ene-carboxylate under denitrifying conditions.

Para-cresol (4-methylphenol) was anaerobically metabolized by a syntrophic association of two denitrifying organisms (19). Two gram-negative, facultative anaerobes were isolated from polluted river sediment. Isolate PC-07 oxidized p-cresol to p-hydroxybenzoate, which was oxidized by PB-04 to undetermined ring-fission products. Degradation by both organisms was nitrate dependent and was substrate specific since neither organism degraded the other aromatic compound. Para-hydroxybenzyl alcohol and p-hydroxybenzaldehyde were detected as intermediates of p-cresol degradation by PC-07 (20). Para-ethylphenol, but not p-propylphenol, toluene, or the ortho and meta isomers of cresol, was degraded by this organism. The aromatic hydrocarbons, m-xylene

(1,3-dimethylbenzene) and toluene were degraded primarily to CO<sub>2</sub> with nitrate reduction in anaerobic laboratory aquifer columns (148). Toluene, benzaldehyde, benzoate, m-toluyaldehyde, m-toluate, m-cresol, and p-hydroxybenzoate were degraded in columns previously adapted to m-xylene under denitrifying conditions (88).

Anaerobic degradation of aromatic compounds with ring substituents other than hydroxyl, methoxyl and methyl groups has also been shown for dissimilatory nitrate reducers. Braun and Gibson (23) isolated three strains of Pseudomonas spp. capable of anaerobic growth on anthranilate (2-amino-benzoate) with nitrate as the electron acceptor. Taylor et al. (125) showed that anaerobic cell suspensions of Pseudomonas PN-1 grown on p-hydroxybenzoate could metabolize o- and p-fluorobenzoate with release of the fluoride ion into the medium. However, this organism would not grow at the expense of m-fluorobenzoate. Schennen et al. (109) showed that the anthranilate-degrading Pseudomonas spp. described by Braun and Gibson (23) could grow on 2-fluorobenzoate and that an inducible enzyme, benzoyl-coenzyme A synthetase, was involved in the initial degradation reaction. Ziegler et al (149) showed the presence of a Mg<sup>+2</sup>-ATP-dependent aryl-CoA synthetase of broad substrate specificity in Pseudomonas strain KB 740 grown on benzoate under denitrifying conditions. Reduction of benzoyl-CoA was not

detected, however 2-aminobenzoyl-CoA reduction was detected in a partially purified preparation from extracts of cells grown anaerobically on 2-aminobenzoate. NADH as well as NADPH were reductants for the reaction.

## B. Sulfate Reduction

An anaerobic organism has been isolated that utilizes sulfate as the terminal electron acceptor and benzoate as the electron donor and carbon source (138). This organism, a new species of sulfate reducer, was named Desulfonema magnum and was found to utilize the aromatic compounds 4-hydroxybenzoate, hippurate, phenylacetate and 3-phenylpropionate, as well as C<sub>1</sub>-C<sub>10</sub> fatty acids, succinate and fumarate (139). The compounds 2-, 3-hydroxybenzoate and cyclohexane carboxylate were not utilized. Another sulfate-reducer, Desulfococcus niacini, which degrades nicotinate to CO<sub>2</sub> and ammonia, was isolated from marine sediments (78). It also degraded 3-phenylpropionate to CO<sub>2</sub> and to benzoate which was not further metabolized. Desulfobacterium indolicum and Desulfobacterium phenolicum were isolated from marine sediments after enrichment with indole and phenol, respectively (4, 5). D. indolicum degrades indole, anthranilic acid and quinoline to CO<sub>2</sub>, with sulfate as the electron acceptor. D. phenolicum also metabolizes indole and anthranilic acid as well as phenol, p-cresol, benzoate, 2- and 4-hydroxy-

benzoate, phenylalanine, phenylacetate and 4-hydroxyphenylacetate.

Desulfobacterium catecholicum was isolated from a catechol-degrading enrichment culture derived from an estuarine sediment (120). This strict anaerobe grew slowly with catechol, resorcinol, hydroquinone, benzoate, 4-hydroxybenzoate, protocatechuate, 2-aminobenzoate, phloroglucinol and pyrogallol as aromatic substrates and either sulfate or nitrate as electron acceptors. It also grew on 2-hydroxybenzoate with nitrate but not sulfate as the electron acceptor. Nitrate was reduced to ammonium.

### III. NON-RESPIRATORY DEGRADATION

Only a few anaerobes capable of the oxidization of aromatic compounds in the absence of light or exogenous electron acceptors have been isolated. Fermentative microorganisms often have slow growth rates and may be dependent on other organisms for the removal of fermentation end products. Organisms that degrade trihydroxylated aromatic compounds in pure culture have been described while those that degrade aromatic compounds with two or fewer hydroxyl groups have only been obtained in defined mixed cultures. A recent report has shown that certain facultative anaerobes belonging to the Enterobacteriaceae are able to slowly degrade ferulic acid while growing anaerobically in pure culture (54). These organisms appear to represent

exceptions to the generalization that only trihydroxylated aromatic compounds are cleaved by pure cultures under fermentative conditions. Pure cultures of anaerobes that metabolize only ring substituents of aromatic compounds without degrading the ring itself have been isolated. As members of microbial food webs these organisms play an important role in mineralization through the production of aromatic substrates necessary for the growth of anaerobes that can degrade the ring.

#### A. Pure Cultures

The first organism isolated in pure culture with the capacity to ferment an aromatic compound was a Clostridium sp. that anaerobically degraded the nitrogen-containing heterocyclic aromatic compounds nicotinic acid and nicotinamide (61). The isolate from river mud was able to ferment nicotinic acid to acetate, propionate, ammonia and CO<sub>2</sub>. The first step in the fermentation was a reversible hydroxylation resulting in the formation of 6-hydroxynicotinate (62). The organism was lost, however, before further studies could be made. Pastan et al. (103) re-isolated a similar organism that was subsequently named Clostridium barkeri (117). Tsai et al. (130) proposed a pathway for nicotinic acid degradation that involved partial ring reduction to

1,4,5,6-tetrahydro-6-oxonicotinic acid. The organism had high concentrations of a vitamin B<sub>12</sub> derivative subsequently shown to be involved in a rearrangement reaction after ring cleavage (89). Purification and characterization of the hydroxylase that catalyzed nicotinic acid oxidation to 6-hydroxynicotinic acid showed a requirement for NADP as the electron acceptor (71). Nicotinic acid hydroxylase activity in cell extract of C. barkeri was increased by growth on a medium supplemented with sodium selenite. Maximal activity occurred when the selenium concentration in the medium was 10<sup>-7</sup> M (77). Holcenberg and Tsai (72) partially purified the next enzyme in the pathway which catalyzed the reduction of 6-hydroxynicotinic acid to 6-oxo-1,4,5,6-tetrahydronicotinic acid. Since this enzyme required reduced ferredoxin for activity and would not substitute reduced NADP, it appeared that the oxidation and subsequent reduction reactions were not directly coupled through NADP(H).

The first organisms capable of fermenting a non-heterocyclic aromatic compound, phloroglucinol (1,3,5-trihydroxybenzene), were isolated from the rumen and were identified as strains of Streptococcus bovis and a Coprococcus sp. (129). The Coprococcus sp. produced two moles each of acetate and CO<sub>2</sub> per mole of phloroglucinol fermented (128). As with other anaerobes discussed so far, degradation involved ring reduction. Patel et al. (104) partially purified and characterized phloroglucinol reductase, which catalyzed the reduction of

phloroglucinol to dihydrophloroglucinol, with NADPH as the electron donor. Rhodopseudomonas gelatinosa (137) and Pelobacter acidigallici (107, 111) also have NADPH-dependent phloroglucinol reductase activity. P. acidigallici, isolated from salt and freshwater sediments, fermented gallate (3,4,5-trihydroxybenzoate), 2,4,6-trihydroxybenzene, pyrogallol (3,4,5-trihydroxybenzene) and phloroglucinol to acetate and CO<sub>2</sub> (111). This organism interacted with Acetobacterium woodii and Desulfobacter postgatei in defined co-culture as the second member of a food chain that mineralized 3,4,5-trimethoxybenzoate (82). A. woodii O-demethoxylated the substrate to gallate, which was fermented by P. acidigallici to acetate and CO<sub>2</sub>. The acetate was then oxidized to CO<sub>2</sub> by the sulfate reducer D. postgatei. Samain et al. (107) have proposed a unified pathway for degradation of trihydroxylated aromatics through: i) decarboxylation of the acidic constituents, if present; ii) rearrangement of hydroxyl groups to ring positions 1, 3 and 5 to form phloroglucinol; iii) ring reduction to dihydrophloroglucinol and; iv) degradation to acetate and CO<sub>2</sub>. Eubacterium oxidoreducens, a recent rumen isolate, ferments gallate, pyrogallol, phloroglucinol and crotonate to acetate, butyrate, and CO<sub>2</sub> (84). A unique requirement for growth on gallate was the addition of formate or H<sub>2</sub>, which were oxidized in approximate stoichiometric amounts with the aromatic substrate. NADP-dependent formate dehydrogenase (FDH) and

NADPH-dependent phloroglucinol reductase (PR) activities were detected in cell extracts and a scheme involving transfer of electrons from formate through NADP(H) to phloroglucinol was proposed (86). Dihydropchloroglucinol was the product of ring reduction. Krumholz et al (87) proposed a complete pathway for gallate degradation in this organism and demonstrated the presence of a number of intermediates and enzyme activities. Of significance was the demonstration of the ring cleaving enzyme, dihydropchloroglucinol hydrolase, and detection of 3-hydroxy-5-oxohexanoate as the product. Enzymes involved in  $\beta$ -oxidation and substrate level phosphorylation were also detected. All of the enzymes assayed appeared to be constitutive because crotonate grown cells had enzyme activities comparable to those in gallate plus formate grown cells. Pyrogallol-phloroglucinol isomerase, the second enzyme in the proposed pathway of gallate metabolism in *E. oxidoreducens* was partially purified and characterized (85). The enzyme had a native molecular weight of approximately 230,000 daltons and showed a single major band corresponding to 92,000 daltons on denaturing polyacrylamide gels. The enzyme contained iron, molybdenum and acid-labile sulfide and the reaction mechanism appeared to involve hydroxylation of pyrogallol to benzenetetrole followed by dehydroxylation to phloroglucinol.

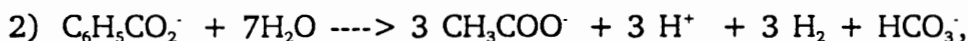


We have mass-cultured E. oxidoreducens in 10-L fermentors on 50 mM gallate plus 50 mM formate. A preliminary characterization of FDH in cell extracts (Section III) has shown a pH optimum of 7.5 and that maximum activity occurred at a temperature of 50°C. As with many formate dehydrogenases, enzyme activity in cell extract from E. oxidoreducens was extremely sensitive to air but was protected from inactivation by sodium azide. Protection by azide should facilitate the purification and study of this enzyme. FDH activity, as assayed by reduction of methyl viologen (MV), was recovered in the soluble fraction after centrifugation of cell extract at 105,500 x g for 1.5 hours in anaerobic phosphate buffer containing 20-percent sucrose. No significant activity was detected in the membrane fraction. The apparent  $K_m$  for formate was 290  $\mu\text{M}$  with MV as the electron acceptor and the apparent  $K_m$  for NADP was 140  $\mu\text{M}$  with formate as the electron donor. The apparent  $K_m$  for reduction of MV by formate was 1.4 mM. We have purified the phloroglucinol reductase from E. oxidoreducens to electrophoretic homogeneity (60). The enzyme is an  $\alpha_2$  homodimer with a native molecular weight of 78,000 daltons. No metals or cofactors were found, suggesting that the reaction mechanism may involve a direct hydride transfer from NADPH to phloroglucinol. Enzyme activity was insensitive to exposure to aerobic conditions and only NADPH and phloroglucinol were substrates for the enzyme.

Attempts to isolate an organism that fermented aromatic compounds with fewer than three hydroxyl groups attached to the ring were unsuccessful (42, 132, 133). An organism from phenylacetate enrichments of anaerobic digester sludge was isolated on succinate but required co-culture with a *Wolinella* sp. to degrade several aromatic compounds including mono- and dihydroxy-aromatics (11). Thermodynamic considerations offer an explanation for these results (42). Equations 1 and 2 show that the reactions for fermentation of benzene or benzoate to acetate and hydrogen are thermodynamically unfavorable under standard conditions (79).



$$\Delta G' = +72.2 \text{ kJ/mole.}$$



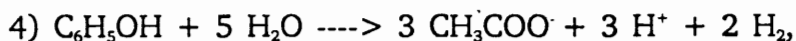
$$\Delta G' = +70.6 \text{ kJ/mole.}$$

However, Ferry and Wolfe (42) hypothesized that removal of benzoate fermentation products by methanogens allowed benzoate to be degraded with a favorable change in free energy. Equation 3 (132) shows that removal of hydrogen results in a favorably negative change in free energy.

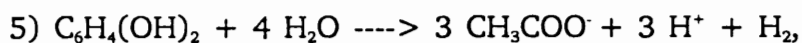


$$\Delta G' = -31.1 \text{ kJ/mole.}$$

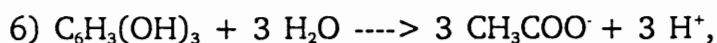
Equations 4-6 (79) show the effect that the number of ring hydroxyl groups has on the change in free energy for the fermentation.



$$\Delta G' = +6.55 \text{ kJ/mole.}$$



$$\Delta G' = -78.1 \text{ kJ/mole.}$$



$$\Delta G' = -158 \text{ kJ/mole.}$$

An increase in hydroxyl group number decreases the amount of hydrogen produced and increases the thermodynamic favorability of the reactions.

Hydroxyl groups may destabilize the aromatic nucleus by inducing the withdrawal of  $\pi$  electrons from the ring (121, 132). Theoretically, an organism capable of fermenting dihydroxy-aromatics such as catechol, resorcinol or hydroquinone should be able to grow in pure culture since the free energy change is negative (Eqn. 5). To date, however, only defined co-cultures of mono- and dihydroxy-aromatic degraders with hydrogen utilizing organisms have been described (11, 132). Equation 6 shows a relatively large negative  $G'$  for trihydroxybenzenes. As mentioned previously, organisms have been isolated in pure culture that can ferment trihydroxybenzenoids (84, 111, 129).

In addition to the influence of the number of ring hydroxyl groups on the thermodynamics of aromatic fermentation, their relative position on the ring also affects the degradation pathway (121, 132, 133). Since  $\beta$ -oxidation appears to be the mechanism by which aromatic ring carbon is metabolized anaerobically (40) hydroxyl groups at ring positions that would interfere with this process are usually removed, while those at positions where  $\beta$ -oxidation would occur are conserved (133). The reductive removal of hydroxyl groups may provide a small amount of energy for an aromatic degrader or an organism specializing in such a reaction, however a source of reducing equivalents would then be necessary (121). These authors have calculated that such a reaction would be thermodynamically feasible with electrons at the redox potential of the pyruvate/lactate couple, but not at the level of the fumarate/succinate couple. Rearrangement of ring hydroxyl groups to positions favoring  $\beta$ -oxidation occurs during the fermentation of gallic acid and pyrogallol by *P. acidigallici* (103, 117) and *E. oxidoreducens* (84).

## B. Syntrophic Cultures

The first benzoate-degrading organism described from a methanogenic consortium was obtained in co-culture with a hydrogen-utilizing *Desulfovibrio* sp. (96). The benzoate degrader, *Syntrophus buswellii* (95) also grew with the

methanogen Methanobacterium formicicum in the absence of sulfate. S. buswellii would not grow in pure culture on benzoate or on any other substrate tested. Because degradation of benzoate to acetate, CO<sub>2</sub> and hydrogen is thermodynamically unfavorable (42) removal of H<sub>2</sub> by the hydrogenotrophs, Desulfovibrio sp. or M. formicicum, was thought to be necessary for benzoate degradation by S. buswellii. Two additional syntrophic strains, P-2 and PA-1, have been isolated in co-cultures from phenylacetate or phenol degrading consortia (11). Wolinella succinogenes was used as the hydrogenotroph, which reduces fumarate to succinate (143). Strain P-2 in co-culture with W. succinogenes degraded benzoate, phenylacetate, hydrocinnamate, and phenol anaerobically. Strain PA-1 in co-culture with W. succinogenes degraded the same aromatics and also resorcinol (1,3-dihydroxybenzene), 2-aminobenzoate, ferulate (4-hydroxy-3-methoxycinnamate), gallate, pyrogallol, 4-aminophenol, phloroglucinol and catechol. Contrary to other described aromatic fermentors, which only degrade one or a few compounds, strains PA-1 and P-2 had an unusually broad substrate range for aromatic compounds. Shelton and Tiedje (112) isolated a syntroph that fermented benzoate in co-culture with a sulfate reducer and a methanogen. The benzoate degrader resembled S. buswellii and cooperated with other organisms in the degradation of 3-chlorobenzoate by a methanogenic consortium. An anaerobic microbial food web capable of

3-chlorobenzoate utilization was reconstituted from a pure culture of a dechlorinating organism and a defined co-culture of a benzoate fermentor and a hydrogen utilizing methanogen (32). One third of the hydrogen produced during benzoate fermentation to acetate and CO<sub>2</sub> was utilized by the dechlorinating organism for reductive dechlorination of 3-chlorobenzoate. The methanogen utilized the remainder of the hydrogen produced to reduce CO<sub>2</sub> to methane. Tschech and Schink (132) isolated two strictly anaerobic gram-positive, spore-forming, motile syntrophs from freshwater sediments. The isolates, which were placed in the genus Clostridium, degraded resorcinol and 2,4- and 2,6-dihydroxybenzoate in co-culture with Campylobacter spp., an association that did not involve interspecies hydrogen transfer. The nature of the dependence of degradation on the Campylobacter sp. could not be determined. However, additional isolates from marine sediments were obtained in association with sulfate reducers and methanogens. Benzoate, 2-hydroxybenzoate, and 3-hydroxybenzoate were syntrophically degraded by gram-negative anaerobes isolated in co-culture with D. vulgaris (133).

Syntrophic associations involving interspecies hydrogen transfer are not restricted to the degradation of aromatic compounds. Obligately syntrophic organisms have been isolated and described for the anaerobic degradation of propionate, butyrate and volatile fatty acids up to C-7 (18, 69, 91, 112).

As with many aromatic compounds, fermentation of fatty acids with the production of  $H_2$  as the electron sink is thermodynamically unfavorable.

Syntrophic association with hydrogenotrophs therefore lowers the  $H_2$  partial pressure sufficiently so that the reactions become favorable. Fatty acid-fermenting syntrophs probably utilize the  $\beta$ -oxidation pathway.

Syntrophomonas wolfei (90) has been shown to possess all of the necessary enzymes required for  $\beta$ -oxidation (142). Syntrophic organisms occupy an important niche in many anaerobic systems by completing the mineralization of less energetically favorable end products produced by fermentation of complex organic compounds. These organisms usually have slow growth rates in the laboratory so that aromatic compounds with two or fewer hydroxyl groups may not be degraded in an anaerobic habitat like the rumen which has a relatively short turnover time (84).

### C. Metabolism of ring methoxyl groups

Anaerobes have been isolated that remove the methyl group from aromatic compounds such as syringic acid (4-hydroxy-3,5-dimethoxybenzoate) and ferulic acid. O-demethylation results in the formation of hydroxylated aromatics (3) previously discussed as being important in determining subsequent degradation pathways. The methoxyl groups may be metabolized by dissimilation to acetate

through the folate-dependent Wood pathway (106). Bache and Pfennig (3) were the first to show anaerobic O-demethylation of aromatic compounds for strains of Acetobacterium woodii isolated from freshwater sediment and sewage sludge enrichments with vanillate (4-hydroxy-3-methoxybenzoate), syringate and 3,4,5-trimethoxycinnamate as substrates. Ten different methoxylated aromatics were stoichiometrically metabolized to their corresponding hydroxylated derivatives and acetate, however, the aromatic ring was not attacked. Growth yields of A. woodii on methoxylated aromatics were increased by reduction of the double bond in the acrylate side chain of caffeate and a corresponding decrease in acetate formation occurred (3, 131). Frazer and Young (46) isolated an organism that O-demethylated syringate, vanillate and ferulate. Radiotracer studies with vanillate showed that its O-methyl group carbon was converted to CO<sub>2</sub> and acetate in a 2:1 ratio (47). As with A. woodii, reduction of the double bond in the side chain of ferulate resulted in decreased acetate formation, suggesting the diversion of electrons of methoxyl group oxidation from acetate production to reduction of the acrylate side chain of ferulate. Reduction of the side chain, in place of acetate formation, may result in a greater overall negative free energy change (131). A facultative anaerobic Enterobacter sp. was isolated from ferulate enrichment cultures that transformed ferulate aerobically and anaerobically (52). Anaerobically,



O-demethylation, reductive dehydroxylation and reduction of the acrylate side chain were observed, but the aromatic ring itself was not attacked. The organism contained two plasmids that did not appear to be involved in coding for enzymes involved in ferulate metabolism (55). Clostridium pfennigii, isolated from steer rumen fluid, metabolized methoxylated aromatics to their hydroxylated derivatives and butyrate (83). In addition, pyruvate or carbon monoxide could be utilized as substrates. Mountfort and Asher (94) have isolated an anaerobe that metabolized the methoxyl group of several methoxylated aromatics but did not reduce the side chain double bond of phenylacrylate derivatives. Rather, butyrate and formate were produced, in addition to acetate. DeWeerd et al. (31) concluded that there was no relationship between the enzyme systems for O-demethylation of 3-methoxybenzoate and reductive dechlorination of 3-chlorobenzoate by DCB-1, an anaerobic dechlorinating organism.

#### IV. Methanogenic Fermentation of Aromatics

The first demonstration that aromatic compounds were degraded under anaerobic conditions was shown by Tarvin and Buswell (122) when it was shown that several aromatic compounds were completely converted to methane and CO<sub>2</sub> by anaerobic enrichment cultures. Clark and Fina (26) confirmed that

benzoic acid could be degraded to methane and CO<sub>2</sub> by anaerobic enrichments. However, the substitution of catechol and protocatechuate in place of benzoate stopped gas production, suggesting that these key intermediates of aerobic degradation pathways (49) were not involved. The low recovery of <sup>14</sup>CH<sub>4</sub> added as <sup>14</sup>C-bicarbonate, suggested that benzoate was the source of most of the methane carbon. Benzoate labeled at carbons 1 and 7 (ring and carboxyl carbons respectively), when added to rumen fluid or sewage sludge-derived enrichment cultures, converted the carboxyl carbon to CO<sub>2</sub> while the ring carbon was metabolized to methane (45). Nottingham and Hungate (98) also used ring labeled <sup>14</sup>C-benzoate to show anaerobic degradation of benzoate to methane and CO<sub>2</sub> by a sewage sludge-derived mixed culture. The fate of carbon-4 of benzoate in sewage sludge enrichments was shown by Fina et al. (44) to favor production of CO<sub>2</sub> over CH<sub>4</sub> by a factor of about 4.5 to 1. Propionate was detected as an intermediate with the carboxyl group containing the C-4 of benzoate. Acetate and butyrate were the only labeled volatile fatty acids detected when <sup>14</sup>C-benzoate was added to methanogenic enrichment cultures (113). Ferry and Wolfe (42) examined the microorganisms involved in methanogenic benzoate degradation. They showed that at least two populations of organisms were necessary for benzoate degradation. One group was involved in degradation of benzoate to H<sub>2</sub>, formate, acetate and CO<sub>2</sub>, which

were subsequently utilized as substrates by methanogenic bacteria. Based on the thermodynamics of the reactions involved, they concluded that a symbiotic relationship existed in which product removal by the methanogens was necessary for benzoate degradation to be energetically favorable. Grbic-Galic and Young (57) showed that the inhibition of methanogenesis with bromoethane sulfonic acid (BESA) still allowed aromatic degradation, but the concentration of metabolic intermediates increased. Studies on benzoate metabolism in lake sediment samples showed an almost immediate degradation of benzoate to methane, but that enrichment cultures required a long lag phase that was reduced by prior adaptation to fatty acid degradation (114). In other studies acetate shortened the lag phase for benzoate degradation presumably by stimulating methanogens, which remove end products of benzoate fermentation (26). Cyclohexane carboxylate (CHCA) and 2-hydroxy CHCA, intermediates of the proposed reductive pathway of anaerobic aromatic degradation (40), were degraded without a lag in the benzoate-degrading consortia described by Ferry and Wolfe (42) while cyclohex-1-ene-carboxylate, another proposed intermediate, did not support methanogenesis. However this compound was detected in extracts of sheep rumen liquor cultures that produced methane from benzoate (8). The absence of an uptake mechanism for some intermediate

compounds may preclude their use as exogenously supplied substrates. Keith et al. (80) found that benzoate-degrading sewage sludge enrichments utilized cyclohex-1-ene-carboxylate. Grbic-Galic and Young (57) proposed a reductive pathway that linked the degradation of benzoate and ferulate through common intermediates. Several fatty acids, including seven carbon mono- and dicarboxylic acids, as well as straight- and branched-chain volatile fatty acids, were detected in their benzoate and ferulate enrichment cultures. Although acetate-utilizing methanogens have not been isolated from any benzoate methanogenic enrichments, organisms with a morphology similar to acetate-degrading Methanothrix sp. have been observed (42, 114). Benzoate has served as an effective model compound for investigating anaerobic aromatic metabolism, but other aromatic compounds have also been shown to be degraded to  $\text{CH}_4$  and  $\text{CO}_2$ . Enrichment cultures derived from several different habitats were shown to degrade aromatic amino acids to  $\text{CH}_4$  and  $\text{CO}_2$  (10). Horowitz et al (73) compared the anaerobic aromatic biodegradation potential of lake sediment and sewage sludge. Various substituted benzoate and benzene derivatives were degraded under methanogenic conditions. However, analines were generally resistant to degradation. Indole, a potential intermediate of the anaerobic degradation of tryptophan (10) was degraded in methanogenic

cultures established with inoculum from an anaerobic activated carbon filter used to treat water containing indole, quinoline and methylquinoline (135). Healy et al. (67) showed that a methanogenic consortium degraded the aromatic lignin derivative ferulate by pathways that converged with proposed benzoate degradation pathways. Healy and Young (65) obtained enrichment cultures capable of producing  $\text{CH}_4$  and  $\text{CO}_2$  from catechol and phenol. Healy and Young (66) also found that eleven different aromatic compounds, several of which were lignin monomers, supported methanogenesis. There was a substantial lag phase in gas production after first exposure to the compounds, but upon subsequent feeding, adaptation of the microbial community shortened or eliminated the delay. Some cultures were simultaneously adapted to structurally similar compounds that had not been supplied as substrates originally. Dwyer et al. (38) studied the kinetics of methanogenic phenol degradation by a bacterial consortium immobilized in thin spaghetti-like strands of agar. Immobilization reduced the maximum degradation rate and apparent  $K_m$ , while providing protection from otherwise inhibitory substrate concentrations. Balba et al. (7) examined degradation of methoxybenzoates and protocatechuic acid (3,4-dihydroxybenzoate) in methanogenic consortia enriched with benzoate. O-demethylation occurred readily, but the resulting

phenolics required an adaptation period prior to degradation to methane. Knoll and Winter (81) found that the presence of high levels of  $H_2$  and  $CO_2$  in a methanogenic sewage sludge derived consortium reduced the rate of phenol degradation and that benzoate was formed from phenol and  $CO_2$ .

Decarboxylation of p-hydroxybenzoate to phenol and protocatechuic acid to catechol was observed. Methanogenesis from methoxylated aromatics involved fermentation of methoxyl groups to acetate by organisms similar to *A. woodii* (79). The resulting hydroxylated aromatic was further degraded to acetate and was not dependent on product removal by methanogens if three hydroxyl groups were present on the ring. If, however, catechol was formed, the ring was not degraded. The reason for the latter result was not clear since the calculated free energy change for conversion of catechol to methane and  $CO_2$  is favorable. Other investigators have found catechol to be degradable in methanogenic enrichments (7, 65, 66). Hydroquinone (1,4 dihydroxybenzene) and catechol (with hydroxyl groups para and ortho to one another, respectively) were degraded in methanogenic cultures, probably after dehydroxylation to phenol (121). In contrast, resorcinol and resorcyates with meta hydroxyl groups were not degraded by the enrichments. Tschech and Schink (132) found that enrichments supplied with resorcinol or resorcyate

isomers degraded these compounds to methane. Freshwater isolates were obtained from the enrichments in defined co-culture with a Campylobacter sp. that degraded 2,4- and 2,6-dihydroxybenzoate to acetate and butyrate. Hydrogen-utilizing methanogens or sulfate reducers could not replace Campylobacter and its role remained unexplained. Marine isolates degraded the two aromatics to acetate and  $H_2$  and required co-culture with methanogens to scavenge  $H_2$ . It was suggested that hydroxyl groups in positions meta to one another, as in resorcinol and resorcyates, are in favorable positions for degradation to acetate and butyrate, while ortho- and para-positioned dihydroxylated aromatics, such as catechol and hydroquinone, must be dehydroxylated to phenol prior to degradation. Reduction of the aromatic ring may not be necessary because the hydroxyl groups could stabilize a dione tautomer, which could then serve as a ring cleavage substrate (132). Young and Rivera (145) proposed phenol as the central intermediate for methanogenic metabolism of substituted phenols, since phenol was detected after BESA inhibition of methanogenesis of mixed anaerobic enrichment cultures that degraded p-cresol, hydroquinone and phloroglucinol.

Field and Lettinga (43) investigated the toxicity of gallotannic acid to methanogenesis during the anaerobic digestion of volatile fatty acids by

granulated sludge. Gallotannic acid, a polyester of gallic acid, severely decreased rates of methane production relative to unamended controls and those with additions of gallic acid and pyrogallol. However, gallotannic acid as well as gallic acid and pyrogallol were rapidly degraded by the sludge.

Aromatic compounds with ring substituents other than, or in addition to, carboxyl and hydroxyl groups have been shown to be susceptible to degradation in anaerobic habitats. Sewage sludge- and sediment-derived enrichment cultures reductively dehalogenated aromatic compounds (118). Complete mineralization to methane and  $\text{CO}_2$  occurred for some compounds which required complete dehalogenation before the aromatic ring could be attacked. A lag time in dehalogenation occurred for chloro-, bromo- and iodobenzoates in lake sediment upon initial exposure; however, the lag was eliminated or greatly reduced after acclimation to the substrate (74). Number, ring position and type of substituent groups all affected degradation. The kinetics of dechlorination of various chlorobenzoates in anaerobic lake sediments and methanogenic enrichment cultures was shown to follow Michaelis-Menten kinetics for the first chlorine atom. If, however, a second chlorine atom was present, its removal was competitively inhibited by the parent substrate. This effect was found for both 3,5-dichlorobenzoate and 4-amino-3,5-dichlorobenzoate (119). Boyd and Shelton (21) compared the degradation of isomers of mono- and dichloro-



phenols in acclimated and unacclimated sewage digester sludge. Cross-acclimation studies suggested that different populations of microorganisms were responsible for degradation of 2- and 3-chlorophenol. While both populations separately degraded 4-chlorophenol, a mixture of the two appeared to be responsible for 2,4- and 3,4-dichlorophenol degradation. Shelton and Tiedje (112) isolated several bacteria of different trophic levels from enrichment cultures growing on 3-chlorobenzoate. Degradation of the substrate apparently involved a symbiotic relationship between a dechlorinating organism, a benzoate degrader and hydrogen oxidizers. An organism capable of reductively dechlorinating 3-chlorobenzoate to benzoate was isolated and found to grow on a rumen fluid medium. An assessment of the anaerobic degradation of substituted phenols showed that ease of degradation was dependent on the type and position of the substituent group (22). Para-chlorophenol and o-cresol (2-methyl phenol) were the most resistant to degradation. Methoxylated phenols were demethylated to the corresponding hydroxylated phenol. Nitrophenols were also degraded. Pentachlorophenol, a widely used preservative and pesticide, was degraded to methane and CO<sub>2</sub> after sequential reductive removal of chlorine by two to three microbial populations. Pentabromophenol was similarly degraded (93). Gibson and Suflita (50) examined four anaerobic habitats for the ability to degrade benzoate, phenol and chlorinated aromatic

pesticides. While benzoate and phenol were degraded anaerobically at all sites, degradation of various chlorinated aromatics varied among the habitats. The presence of sulfate inhibited dechlorination. Smolenski and Suflita (116) found that o-, m-, and p-cresol degradation in anaerobic alluvial sand aquifer material was greater under sulfate reducing conditions than under methanogenic conditions. The aromatic hydrocarbons toluene and benzene were degraded in methanogenic consortia via ring hydroxylation as shown by incorporation of  $^{18}\text{O}$ -labeled water (134). A major route for toluene degradation in the cultures appeared to involve p-cresol and benzoic acid as intermediates while benzene degradation involved phenol as an intermediate (56).

## VI. ANAEROBIC LIGNIN DEGRADATION

Lignin is a heterogeneous high-molecular-weight aromatic polymer in which the basic phenylpropane subunits are randomly linked by intercarbon and ether bonds between aromatic nuclei and side chains (136). Lignin, the second most abundant terrestrially produced organic polymer (70) is the greatest source of the benzene ring in the environment (24). Lignin is resistant to microbial attack and until recently was not considered to be degraded under anaerobic conditions (59, 100, 146). Vanillic acid, a monoaromatic lignin derivative, and an aromatic dimer with an ether linkage were degraded in methanogenic lake

sediments, but high-molecular-weight synthetic lignin was not (147). This led Zeikus et al. (147) to conclude that the high-molecular-weight property was the major impediment to anaerobic lignin degradation. Dehydrodivanillin, a diaromatic lignin derivative, was shown to be degraded anaerobically by a mixed culture of rumen organisms (25) but contrary to most studies, little gas production was reported. Colberg and Young (27, 29) have shown an inverse correlation between increasing lignin molecular weight and its susceptibility to anaerobic degradation. Solubilized lignin fragments, "oligolignols", with a molecular weight of approximately 600 were shown to be partially degraded to volatile fatty acids, monoaromatic compounds, methane and CO<sub>2</sub> in anaerobic enrichment cultures (28). Inhibition of methanogenesis with bromoethane sulfonic acid (BESA) resulted in increased conversion of substrate and accumulation of intermediate degradation products relative to uninhibited cultures. Apparently, interspecies hydrogen transfer (76) of reducing equivalents to methanogens is not required for anaerobic depolymerization of lignin oligomers to mono-aromatic subunits. Recently, Benner et al. (14, 15) and Benner and Hodson (13) using sensitive radioisotope techniques, have shown slow but significant degradation of high-molecular-weight lignin derived from a variety of sources; degradation occurred in anaerobic freshwater and marine sediments. Coniferyl alcohol, an aromatic lignin precursor, was

completely degraded to  $\text{CO}_2$  and  $\text{CH}_4$ , with ferulic acid appearing as an intermediate (51). Ferulic acid was shown by Healy et al. (67) to be degraded methanogenically through a pathway proposed to merge with the benzoate degradation pathway proposed by Evans (40). Methanogenic degradation has been demonstrated for a large number of monoaromatic lignin derivatives (57, 66, 79). Limited attack on ring substituents of hydroxycinnamic acids in anaerobic enrichment cultures was shown by Nali et al. (97). Reactions involving reductive removal of p-hydroxyl groups and reduction of the side chain double bond were indicated by the degradation products. Ohmiya et al. (101) isolated an organism identified as Wolinella succinogenes that utilized ferulic acid as an electron acceptor when grown on a yeast extract medium. Disruption of methanogenesis by BESA resulted in the accumulation of a number of intermediate ferulate degradation products that were not detected in uninhibited cultures (53). Removal of methanogenesis as an electron sink for anaerobic aromatic degradation appeared to cause a shift from usual degradation pathways. Balba and Evans (9) showed that the  $\text{C}_3$ -phenyl compounds phenylpropionate and cinnamic acid were degraded without a lag to methane and  $\text{CO}_2$  by benzoate-adapted cultures, while phenylacetate degradation required an adaptation period. They suggested that  $\beta$ -oxidation of phenylpropionate yielded benzoate, which was readily degraded by the

benzoate-adapted microbial population, while  $\beta$ -oxidation of phenylacetate was blocked since the carbon beta to the carboxyl group was part of the aromatic ring.

## VII. CONCLUSIONS

Despite the fact that aromatic rings have increased stability from the negative resonance energy through delocalization of  $\pi$  electrons in the ring, numerous microorganisms utilize aromatic compounds as sources of carbon, energy and even electron acceptors. In aerobic environments aromatic compounds are more readily degraded by the involvement of oxygenase enzymes and with oxygen as the terminal electron acceptor. However in anaerobic environments microorganisms have evolved diverse strategies to unlock the potential of aromatics to support growth. Ring reduction, co-metabolism of substrates and interspecies hydrogen transfer result in the anaerobic metabolism of a wide range of substituted aromatic compounds. The oxidation level of ring carbons and the presence or absence of terminal electron acceptors appear to be major determining factors governing the mechanisms involved in degradation of aromatic compounds in anoxic habitats. Consequently, there is great metabolic diversity among anaerobes which is

highlighted by the fact that many of the described organisms are often new species.

The activity of anaerobes is important in carbon cycling in anoxic habitats and for the removal of toxic compounds released into the environment. The potential for exploitation of their specialized metabolic reactions has not been overlooked; however, the lack of basic research concerning the physiology, biochemistry and genetics of anaerobic aromatic degradation pathways currently limits their potential usefulness.

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### SECTION III. CHARACTERIZATION OF FORMATE DEHYDROGENASE IN CELL EXTRACT OF EUBACTERIUM OXIDOREDUCENS

#### SUMMARY

The formate dehydrogenase of E. oxidoreducens was characterized in cell extract prepared by French pressure cell lysis of cells grown on gallate plus formate. Enzyme activity was rapidly lost after exposure of cell extract to air while addition of sodium azide protected activity. The enzyme was located in the soluble fraction of cell extract after ultracentrifugation. The pH optimum was 7.5 and maximal activity occurred at 50°C. Methyl viologen and NADP were electron acceptors for formate oxidation with  $K_m$  values of 1.4 mM and 0.14 mM respectively. The  $K_m$  for formate was 0.29 mM.

#### INTRODUCTION

Formate dehydrogenase is an enzyme that has been found in a diverse group of microorganisms (3). The enzyme catalyzes the oxidation of formate and the reduction of a number of electron acceptors. In E. oxidoreducens formate or  $H_2$  are required for growth on gallate (6), and NADP is the electron acceptor for formate oxidation (7). It was postulated that formate oxidation to  $CO_2$  and phloroglucinol reduction to dihydrophloroglucinol were coupled through NADP(H) via catalysis of the reactions by formate dehydrogenase and

phloroglucinol reductase respectively (Fig. 1). The  $\Delta G'$  for the coupled reactions is -6.5 kcal/mol (Section V). Wolinella succinogenes is a rumen anaerobe with a membrane bound formate dehydrogenase that can be coupled to a membrane bound fumarate reductase via quinones and generates ATP by electron transport phosphorylation (5). The  $\Delta G'$  for the coupled reactions is -20.8 kcal/mol (Section V). The purpose of this study was to characterize the formate dehydrogenase in cell extract of E. oxidoreducens and to determine the cellular location of the enzyme in order to assess the potential that energy could be derived from formate oxidation coupled to phloroglucinol reduction in this organism.

## MATERIALS AND METHODS

Growth. E. oxidoreducens strain G-41 was obtained from L.R. Krumholz and maintained anaerobically on 1.0% agar slants of the previously described medium (6) with 20 mM each gallate and formate as substrates and omission of rumen fluid. Cell material was obtained by growth in 10 L fermentors (New Brunswick, NJ) in the same medium with 50 mM each gallate and formate. The fermentors were gassed continuously with  $N_2/CO_2$  (4/1) at 50 ml/min. The cells were harvested at the end of exponential growth with a continuous flow centrifuge (Cepa, model LE, New Brunswick, NJ) operated at 30,000 rpm. Cell paste was stored in liquid nitrogen until use.

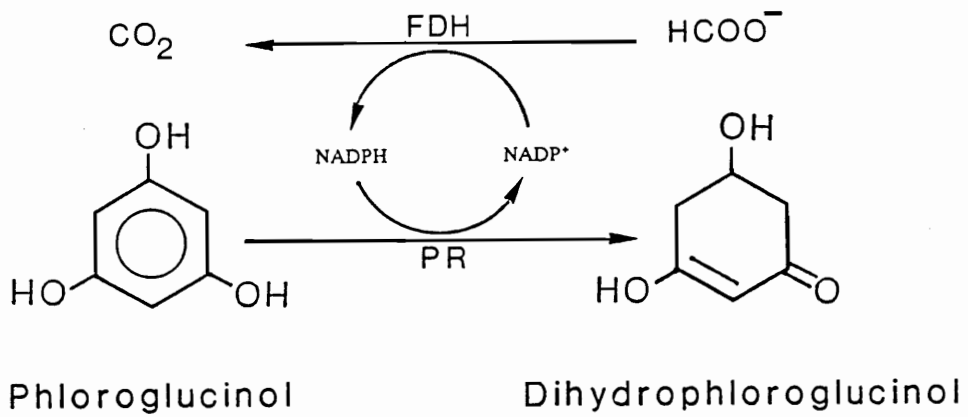


FIG. 1. Reaction scheme proposed for the coupling of formate oxidation to phloroglucinol reduction in *E. oxidoreducens*. Formate dehydrogenase (FDH), phloroglucinol reductase (PR). After Krumholz et al. (7).

All of the following procedures were done anaerobically under N<sub>2</sub> at 0-4°C unless noted otherwise.

Cell extract preparation. Thawed cell paste was resuspended in 2 volumes of 75 mM potassium phosphate buffer, pH 7.5, containing 2 mM 2-mercaptoethanol and 10 mM sodium azide. The cells were broken by two passages through a French pressure cell at 20,000 lb/in<sup>2</sup>. Approximately 50 µg of deoxyribonuclease I (Sigma) were added and the crude extract was centrifuged at 30,000 x g for 30 minutes. The supernatant was withdrawn with a 5.0 ml gas tight syringe and pelleted in liquid nitrogen for storage until use. Typical protein concentrations were about 20 mg/ml.

Ultracentrifugation. Sucrose solutions were prepared in 50 mM potassium phosphate buffer pH 7.5, containing 10 mM sodium azide. One ml of a 70% (w/v) sucrose solution was placed in the bottom of a 10 ml polycarbonate centrifuge tube and overlaid with 8 ml of a 20% sucrose solution followed by 0.92 ml of filtered cell extract. The tubes were sealed with gas-tight screw caps and centrifuged for 90 minutes at 4°C in a Beckman 50Ti rotor at an average relative centrifugal force of 105,500 x g. Fractions of 0.2 ml each were pumped from the bottom of the tube and collected in nitrogen purged serum vials. Fractions were assayed for formate dehydrogenase activity and protein concentration using the procedures described below.



Stability in air. Cell extract was prepared as described above with and without sodium azide in the breakage buffer. Extract was placed in 2 ml serum vials on ice and exposed to air with mild agitation. Samples (20  $\mu$ l) were periodically removed, placed in serum vials and purged with nitrogen prior to being assayed for formate dehydrogenase activity.

Enzyme assays. Formate dehydrogenase activity was determined on a dual beam spectrophotometer (Perkin Elmer model 552, Norwalk, CT) using serum-stoppered, glass cuvetts. The standard assay was performed in 0.5 ml of anaerobic buffer that consisted of 50 mM potassium phosphate and 2 mM 2-mercaptoethanol at 23-26°C. Methyl viologen (20 mM) or NADP (150  $\mu$ M) were electron acceptors and reduction was monitored spectrophotometrically at 603 nm ( $\epsilon = 11.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ ) and 340 nm ( $\epsilon = 6.22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ ) respectively. The sodium formate concentration was 20 mM and 7.5  $\mu$ g of cell extract protein were usually assayed. Protein was determined by the method of Bradford (2) with bovine serum albumin as the standard.

Temperature effects. Formate dehydrogenase activity was determined in the standard assay buffer, equilibrated to temperatures of 10, 20, 30, 40, 45, 50, 55, 60, 65, and 70°C with NADP (150  $\mu$ M) as the electron acceptor.

pH effects. Formate dehydrogenase activity was determined at room temperature with NADP as the electron acceptor. The standard assay buffer

with a pH of 6, 6.5, 7.0, 7.5, and 8.0, and 25 mM borate buffer of pH 8.0, 8.5, and 9.0 were used.

Kinetic constants.  $K_m$  and  $V_{max}$  values for formate, NADP and methyl viologen were determined using the standard assay for formate dehydrogenase and cell extract prepared without sodium azide. Values for formate were determined with 20 mM methyl viologen as the electron acceptor. Values for NADP and methyl viologen were determined with sodium formate (20 mM) as the electron donor.

## RESULTS

After ultracentrifugation, the peaks for formate dehydrogenase activity and protein were found in fractions collected near the top of the 20% sucrose layer (Fig. 2), indicating that the enzyme is soluble or is only loosely associated with the membrane. A cloudy region at the interface of the 20% and 70% sucrose layers indicated the presence of membranes. A protein peak for membrane associated proteins was found in this region.

Activity was sensitive to air with a 50% loss within 1.5 minutes after exposure. However, with 6-7 mM sodium azide in the cell extract, 87% of the initial activity remained after exposure to air for 34 minutes.

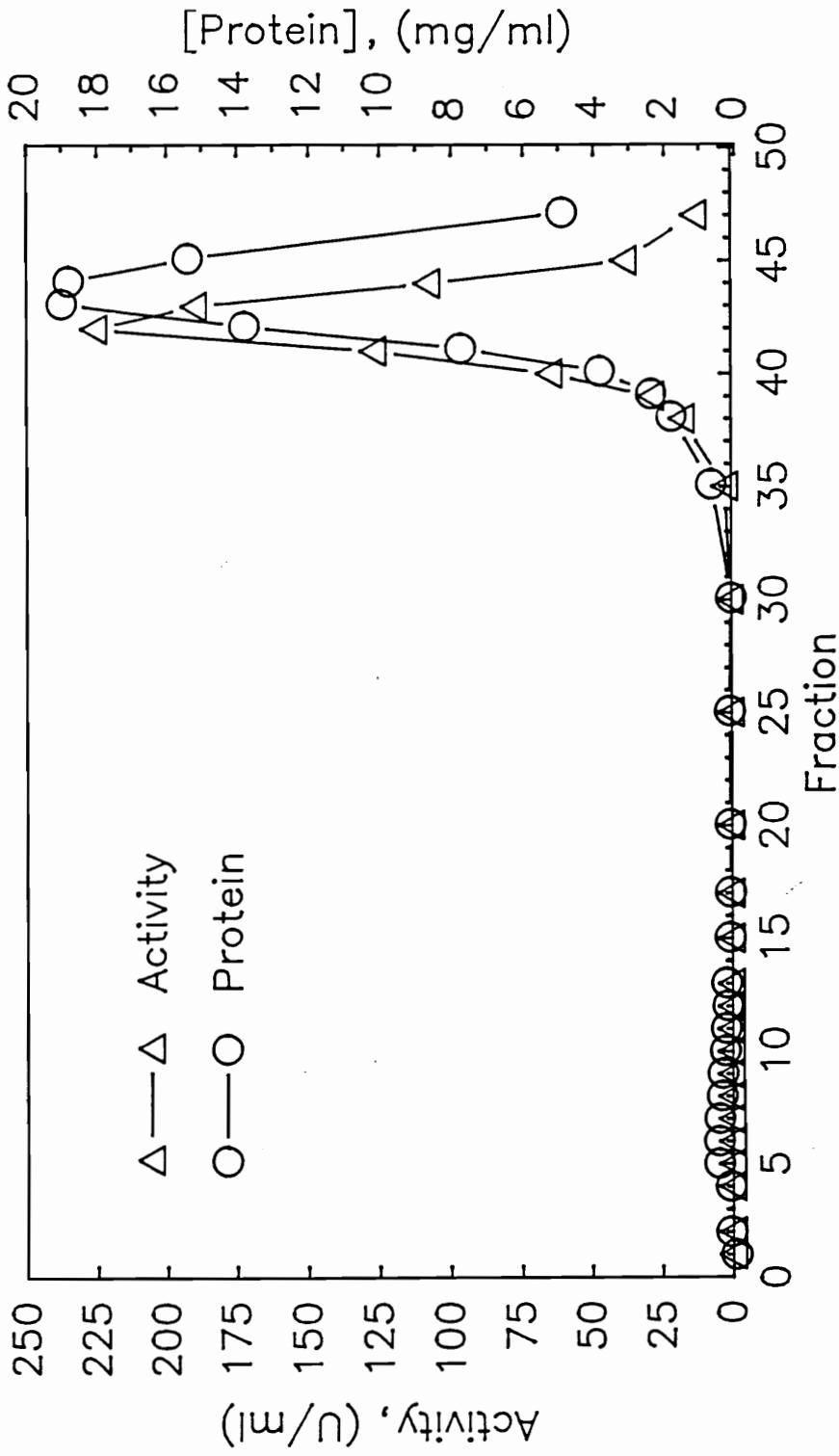


FIG. 2. Location of formate dehydrogenase activity and protein after ultracentrifugation of cell extract through 20% sucrose. Fractions 1-5, 70% sucrose layer; fractions 6-45, 20% sucrose layer.

Activity increased with increasing assay temperature and was maximal at 50 °C while no activity was detected at 70 °C (Fig. 3). The pH optimum for activity was 7.5 with NADP as the electron acceptor (Fig. 4). At pH 8.0, the activity in borate buffer was 38% of the activity found in phosphate buffer.

Values for the kinetic constants calculated by double reciprocal plots of activity versus substrate concentration are shown in Table 1.

## DISCUSSION

The extreme sensitivity to oxygen inactivation, protection by sodium azide and low  $K_m$  for formate for the formate dehydrogenase of E. oxidoreducens are properties typically associated with formate dehydrogenases of many obligately anaerobic bacteria (3). The enzyme from E. oxidoreducens is most similar to the formate dehydrogenase of Clostridium thermoaceticum (Table 2). Both oxidize formate and reduce NADP and methyl viologen and have similar  $K_m$  values for these substrates. The physiological role of this enzyme in C. thermoaceticum is the reduction of CO<sub>2</sub> or bicarbonate to formate (8). The C. thermoaceticum enzyme is the only one shown so far to carry out this thermodynamically unfavorable reaction (10, 11). The proposed function of formate dehydrogenase in E. oxidoreducens is formate oxidation to provide electrons for reduction of the aromatic ring (7), but it is not known if CO<sub>2</sub> can

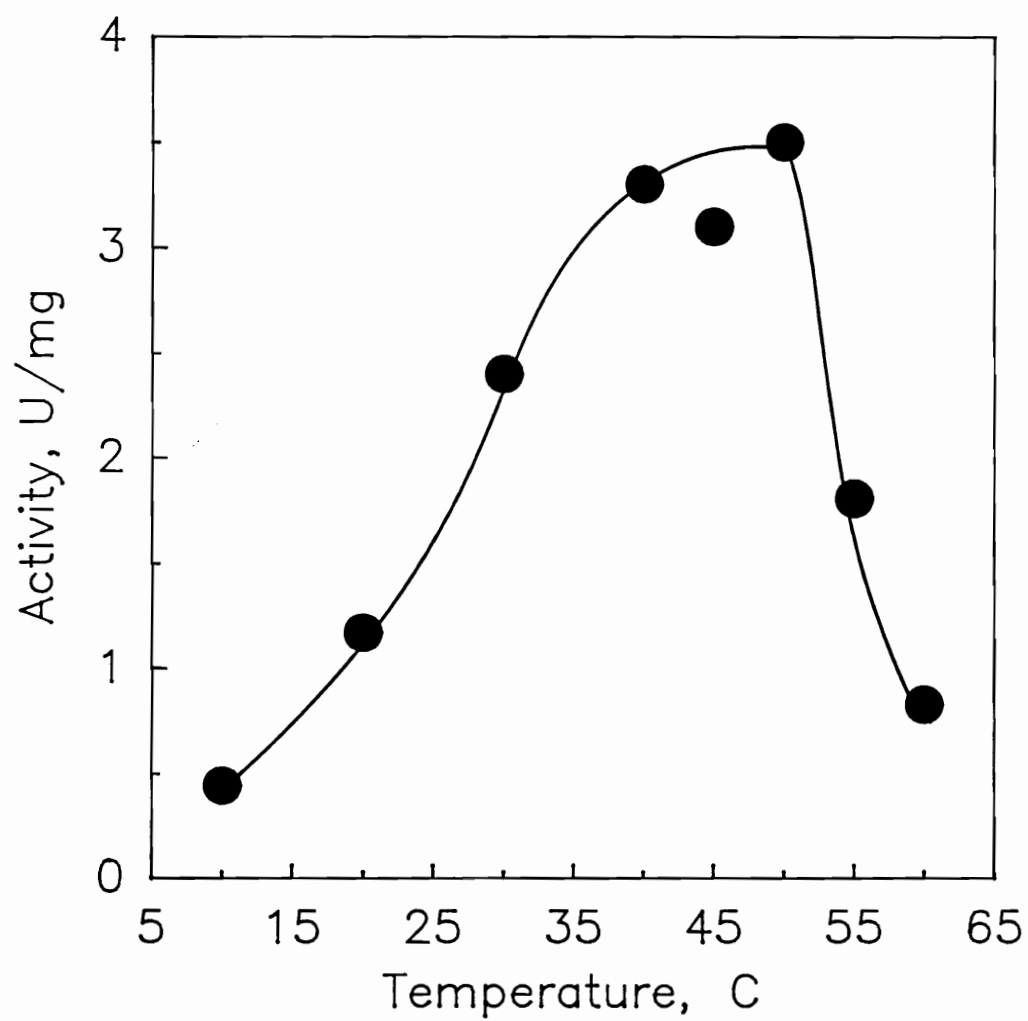


FIG. 3. Dependence of formate dehydrogenase activity on temperature.

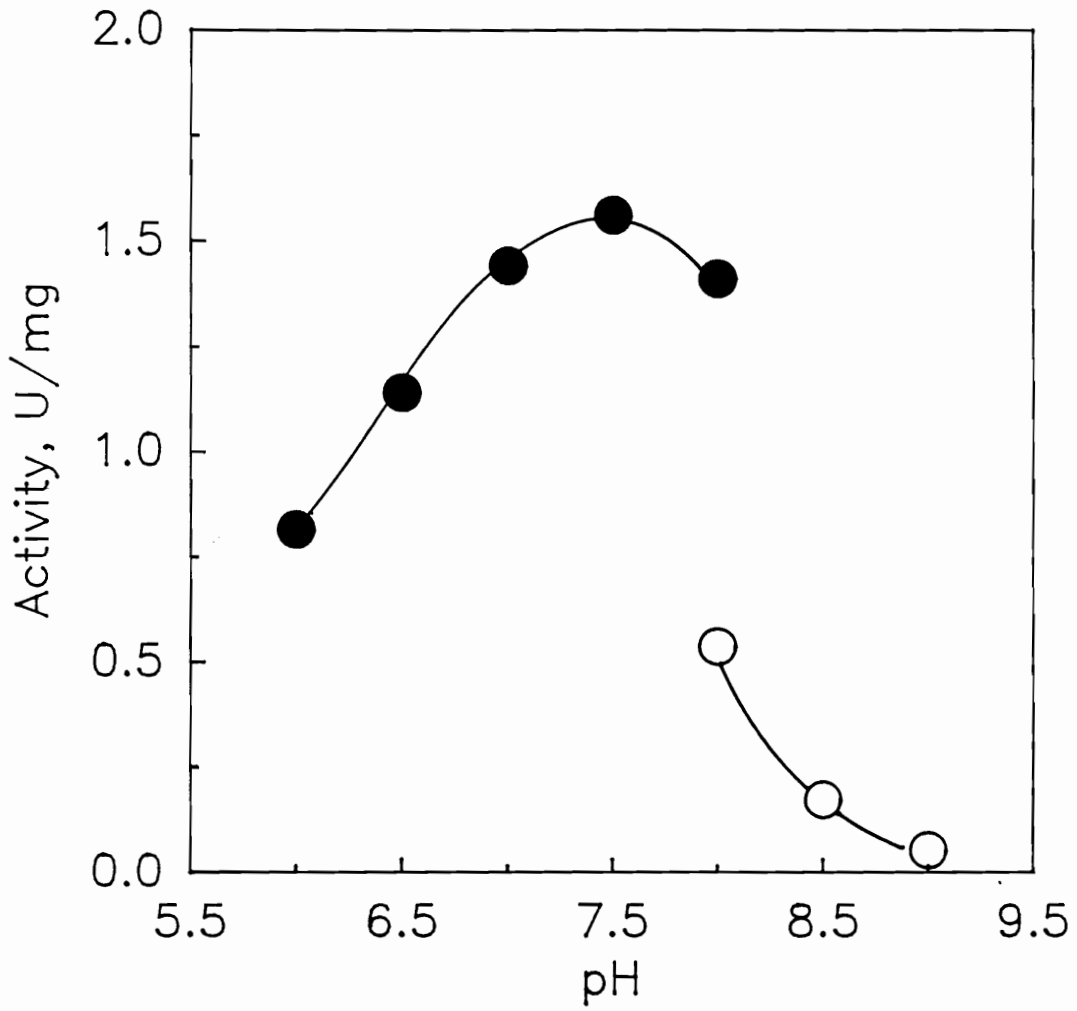


FIG. 4. Dependence of formate dehydrogenase activity on pH. ●, 50 mM potassium phosphate buffer; ○, 25 mM sodium borate buffer.

Table 1. Kinetic constants for formate dehydrogenase in cell extract of Eubacterium oxidoreducens.

Varied Substrate	Range of concentrations used (mM)	Electron Acceptor	Km (mM)	Vmax (U/mg) <sup>a</sup>
Formate	0.1-10	MV	0.29	1.4
NADP	0.014-0.16	NADP	0.14	3.9
MV <sup>b</sup>	0.19-38	MV	1.4	1.9

<sup>a</sup> Units/mg protein: 1 unit is equal to 1  $\mu$ mol acceptor reduced per minute.

<sup>b</sup> Methyl viologen.

Table 2. Some characteristics of formate dehydrogenase of Clostridium thermoaceticum<sup>a</sup>.

Substrate	K <sub>m</sub> <sup>b</sup> (mM)	pH Optimum	Temperature Optimum ( ° C)
Formate	0.227 <sup>c</sup> 0.083 <sup>d</sup>	7-9.5 <sup>c</sup>	85
NADP	0.109		
MV	2.35		

<sup>a</sup> Andreesen and Ljungdahl (10).

<sup>b</sup> 55 °C, pH 7.5.

<sup>c</sup> NADP as electron acceptor.

<sup>d</sup> Methyl viologen (MV) as electron acceptor.



be reduced to formate in this organism. If so, dihydrophloroglucinol should be able to supply the electrons for CO<sub>2</sub> or bicarbonate reduction since the reaction catalyzed by phloroglucinol reductase is reversible (Section IV).

The formate dehydrogenase K<sub>m</sub> for formate in E. oxidoreducens is 58 to 11 fold greater than the 0.005 to 0.026 mM formate concentrations reported for the bovine rumen (4), the habitat from which E. oxidoreducens was isolated. The organism may possess an efficient formate uptake system to provide higher intracellular concentrations or it may obtain additional reducing equivalents from H<sub>2</sub> in situ. The organism also grows on crotonate without a requirement for H<sub>2</sub> or formate. The K<sub>m</sub> for NADP is about 20-fold higher than the phloroglucinol reductase K<sub>m</sub> for NADPH (Section IV). The difference may be a mechanism to prevent over-reduction of the NADP pool when the formate concentration is high relative to the phloroglucinol concentration. Over-reduction of the NADP pool may explain the poor growth of E. oxidoreducens on crotonate and formate (7).

Formate dehydrogenase was not membrane bound when cell extract was fractionated by ultracentrifugation. The same results have been found for formate dehydrogenase from C. thermoaceticum (1). Association with the

membrane in vivo cannot be ruled out however. The formate dehydrogenase of W. succinogenes is involved in electron transport phosphorylation and is membrane bound with the site for formate oxidation oriented towards the periplasm (5). The electrons are passed, via a cytochrome mediated electron transport chain, to the inner membrane where fumarate is reduced to succinate by a membrane bound fumarate reductase. As a result, two protons are released into the periplasm and two are consumed in the cytoplasm. Electron transport from formate to fumarate involves a large free energy change due to a  $\Delta E' = 0.465$  V (calculated from ref. 10), and the ratio of ATP produced per 2 electrons transported is 1 (5). In E. oxidoreducens,  $\Delta E' = 0.141$  V for formate oxidation coupled to phloroglucinol reduction (Section V), phloroglucinol reductase was found in the soluble fraction (Section IV) and there is no net change in the hydrogen ion concentration on reduction since the proton consumed by reduction is balanced by the proton released from dihydrophloroglucinol which ionizes between pH 4.5 and 5.0 (Section VI). Therefore, the potential for generation of a proton gradient by E. oxidoreducens is significantly lower than that of W. succinogenes. The major function of formate dehydrogenase in E. oxidoreducens appears to be to supply electrons for reduction of the aromatic ring of phloroglucinol to dihydrophloroglucinol

which is the proposed ring cleavage substrate (7). Subsequent reactions may then generate ATP via substrate level phosphorylation.

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#### SECTION IV. PURIFICATION AND PROPERTIES OF PHLOROGLUCINOL REDUCTASE FROM EUBACTERIUM OXIDOREDUCENS G-41

##### SUMMARY

Phloroglucinol reductase was purified 90-fold to homogeneity from the anaerobic rumen organism Eubacterium oxidoreducens strain G-41. The enzyme is stable in the presence of air and is found in the soluble fraction after ultracentrifugation of cell extract. Ion-exchange, hydrophobic interaction, and affinity chromatography were used to purify the enzyme. The native  $M_r$  is 78,000 and the subunit  $M_r$  is 33,000 indicating an  $\alpha_2$  homodimer. The enzyme is specific for phloroglucinol and NADPH. The  $K_m$  and  $V_{max}$  are 600  $\mu M$  and 640  $\mu mol/min$  per mg (pH 7.2) for phloroglucinol, and 6.7  $\mu M$  and 550  $\mu mol/min$  per mg (pH 6.8) for NADPH; the  $K_m$  and  $V_{max}$  for the reverse direction are 290  $\mu M$  and 140  $\mu mol/min$  per mg (pH 7.2) for dihydrophloroglucinol, and 27  $\mu M$  and 220  $\mu mol/min$  per mg (pH 7.2) for NADP. The pH optimum is 7.8 in the forward direction and maximum activity occurs at 40°C. The pure enzyme is colorless in solution and flavins are absent. Analysis for Co, Mn, Mo, V, W, Se, Cu, Ni, Fe, and Zn indicated that these metals are not components of the phloroglucinol reductase. Cupric chloride, n-ethylmaleimide, and p-chloromercuribenzoate are potent inhibitors of enzyme activity. The properties of phloroglucinol reductase indicates that it functions

in the pathway of the anaerobic degradation of trihydroxybenzenes by catalyzing reduction of the aromatic nucleus prior to ring fission.

## INTRODUCTION

Aerobic microorganisms degrade many aromatic compounds with oxygenases which incorporate molecular oxygen into the aromatic molecule (6). In anaerobic habitats such as aquatic sediments, sludge digestors and the rumen, anaerobes also degrade a variety of aromatics. Though diverse in their physiology, all anaerobic microorganisms studied appear to utilize a pathway in which the aromatic ring is destabilized by reduction prior to cleavage (2, 4). Benzoic acid and anthranilic acid have been shown to require activation of the carboxyl group to Co-A derivatives prior to ring reduction (5, 7, 9, 21). However, in Eubacterium oxidoreducens and Pelobacter acidigallici gallic acid (3,4,5-trihydroxybenzoic acid) is decarboxylated and isomerized to phloroglucinol (1,3,5-trihydroxybenzene), the ring reduction substrate (11, 12, 16). E. oxidoreducens requires either formate or  $H_2$  as a co-substrate for growth on trihydroxylated aromatic compounds (10). The initial steps in the proposed catabolic pathway in this organism (11) are shown in Fig. 1. The second enzyme in the pathway which catalyzes the isomerization step has been

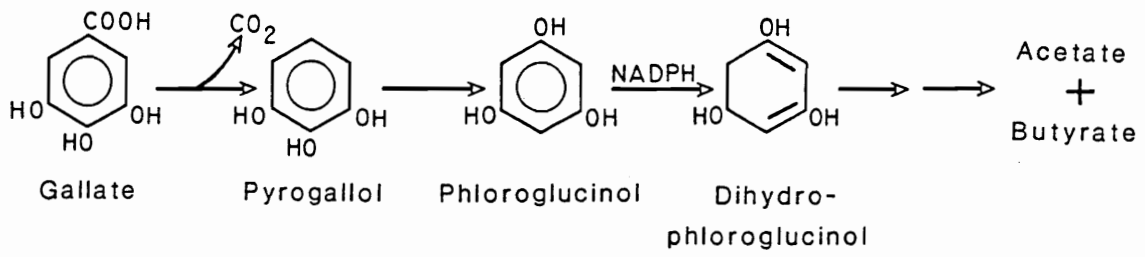


FIG. 1. Initial steps in the proposed pathway for the metabolism of gallic acid by *E. oxidoreducens* G-41. After Krumholz et al. (11).

partially purified (12). Here, we report the purification and characterization of phloroglucinol reductase, the next enzyme in the pathway proposed for the degradation of trihydroxylated aromatic compounds by E. oxidoreducens.

## MATERIALS AND METHODS

Cell material. Strict anaerobic techniques based on the methods of Hungate (8) and Balch and Wolfe (1) were used. E. oxidoreducens strain G-41 was obtained from L. R. Krumholz and maintained as described in 1% agar stabs with omission of rumen fluid (10). The gas phase was N<sub>2</sub>/CO<sub>2</sub> (4/1), the pH prior to autoclaving was 7.2, and the incubation temperature was 37°C. Cells were grown in 10-L batch cultures on 50 mM each sodium formate and gallic acid in the medium described above. Cultures were sparged with N<sub>2</sub>/CO<sub>2</sub>, 4/1, at 500 ml/min. During exponential growth the CO<sub>2</sub> flow rate was periodically decreased to compensate for the metabolic formation of CO<sub>2</sub>. Cells were harvested at the end of exponential growth with a continuous flow centrifuge (Cepa, Model LE, New Brunswick Scientific Co., Inc., New Brunswick, NJ) operated at approximately 30,000 rpm. Cell paste was stored in liquid N<sub>2</sub> until use.



Purification of phloroglucinol reductase. All procedures were done aerobically, and at 0-4°C unless otherwise noted. All buffers contained 20 mM potassium phosphate (pH 7.2) and 5% (w/v) glycerol unless indicated otherwise. Thawed cell paste (10 g wet weight) was suspended in 20 ml of buffer, and passed twice through a chilled French pressure cell at 20,000 lb/in<sup>2</sup>. Approximately 50  $\mu$ g of deoxyribonuclease I (Sigma) were added to the crude extract and incubated 10-15 minutes prior to centrifugation at 30,000 x g for 30 min. The supernatant solution was applied to a 2.5 x 11 cm DEAE-cellulose (Whatman DE-52) column equilibrated with buffer. Phloroglucinol reductase was eluted with a 300 ml, 0 to 0.25 M KCl linear gradient applied at 2.0 ml/min. The most active fractions were pooled, solid ammonium sulfate was added to 1 M final concentration, and the protein was applied to a 1 x 12 cm Phenyl Sepharose (Pharmacia) column equilibrated with buffer containing 1 M ammonium sulfate. The enzyme was eluted from the column at 0.5 ml/min with a 62.5 ml linear gradient decreasing from 1.0 to 0 M ammonium sulfate. The most active fractions were pooled and concentrated by ultrafiltration using a Centriprep YM-10 (Amicon) 10,000 Da cutoff membrane. The concentrated material was applied to a 1.3 x 5 cm column containing Reactive Red Agarose 120 type 1000 CL (Sigma) equilibrated with buffer containing 0.1 M KCl. The column was then washed with approximately 2 bed volumes of equilibration

buffer, and the enzyme was eluted with 10 ml of 1 mM NADPH in equilibration buffer applied at 0.2 ml/min. Active fractions were pooled, diluted with an equal volume of 5% (w/v) glycerol and applied to a 0.5 x 5 cm Mono-Q HR 5/5 anion exchange column (Pharmacia) equilibrated with buffer.

Phloroglucinol reductase was eluted with a 30 ml linear 0 to 0.35 M KCl gradient. The most active fractions containing the purified enzyme were pooled, and glycerol was added to a final concentration of 15% (w/v) before the enzyme was stored in liquid nitrogen.

Assay of phloroglucinol reductase. The standard assay for enzyme activity in the forward direction was done in 50 mM potassium phosphate buffer, pH 7.2, containing 100  $\mu$ M NADPH, 1 mM phloroglucinol dihydrate and indicated amounts of protein. Routine assays were done aerobically at 30°C with a Model 100-60 dual beam spectrophotometer (Hitachi, Ltd., Tokyo). The reaction was usually initiated by addition of the aromatic substrate and rates were determined by recording the oxidation of NADPH. The wide spectral band pass of the spectrophotometer lowered the extinction coefficient for NADPH from 6.22 to 4.58  $\text{mM}^{-1} \text{ cm}^{-1}$  which was determined with an NADPH standard (Sigma). Dihydrophloroglucinol production at 278 nm was recorded as a measure of enzyme activity in the indicated assays. An  $\epsilon_{278} = 24.3 \text{ mM}^{-1} \text{ cm}^{-1}$  was experimentally determined after reduction of phloroglucinol with

sodium borohydride (15). This value is lower than the one reported in Section VI due to the wide spectral band pass of the spectrophotometer. Unless otherwise noted 0.049  $\mu$ g of purified phloroglucinol reductase were assayed. Assays in the reverse direction were done at 30°C in 50 mM potassium phosphate buffer, pH 7.2, containing 3.5 mM dihydrophloroglucinol and 100  $\mu$ M NADP.

The substrate specificity of phloroglucinol reductase in the forward direction was determined with the standard assay and 0.49  $\mu$ g of the enzyme and substituting the test compound at 1.0 mM (menadione and orotic acid were 0.1 mM and 5-methoxyresorcinol was 0.27 mM) for phloroglucinol. Also, the same compounds were tested for their effects on activity in the forward direction with the standard assay and 0.049  $\mu$ g of enzyme and with phloroglucinol and the test compound at 1.0 mM each (except orotic acid and menadione at 0.1 mM). Anaerobic assays, where noted, were performed in stoppered N<sub>2</sub>-purged cuvettes and all reagents were made anaerobic by degassing under vacuum and flushing with N<sub>2</sub>. Gas tight syringes were used to make additions to the cuvettes. Aromatic stock solutions were always prepared and maintained under N<sub>2</sub>. Protein was determined by the method of Bradford (3) using Bio-Rad dye reagent (Bio-Rad Laboratories) and bovine serum albumin (Sigma) as the standard.

Electrophoresis. Polyacrylamide gel electrophoresis followed the method of Laemmli (14) with omission of sodium dodecylsulfate for native gels. An activity stain, based on the NADPH-dependent reduction of tetrazolium dye, was employed in native gels. After electrophoresis, gels were submerged for 15 min in 50 mM potassium phosphate buffer, pH 7.2. Dihydrophloroglucinol, NADP, phenazine ethosulfate, and p-iodonitrotetrazolium violet were added to a final concentration of 1.2 mM, 0.1 mM, 0.1 mM, and 1.0 mM respectively and the gels were incubated in the dark at 30°C for 1 hour. Phloroglucinol reductase activity was detected as a red band of reduced tetrazolium precipitate in the gel.

Isoelectric focusing. The procedure was done as recommended by the manufacturer on an LKB 2117 Multiphor II (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) unit using LKB Ampholine Pagplates, pH 3.5 - 10.0, maintained at 10°C. The standards that were used for gel calibration were: human carbonic anhydrase B, pI = 6.55; bovine carbonic anhydrase B, pI = 5.85;  $\beta$ -lactoglobulin A, pI = 5.20; soybean trypsin inhibitor, pI = 4.55; glucose oxidase, pI = 4.15; and amyloglucosidase, pI = 3.50.

Amino acid composition and N-terminal analyses. Both analyses were done by the Nucleic Acid Sequencing Facility, University of Virginia, Charlottesville, VA. The enzyme was dialyzed against distilled, deionized water

and lyophilized prior to being reduced and then alkylated with 4-vinylpyridine. Triplicate samples were hydrolyzed with vapor phase 6N HCl for 24 hours at 110°C. Amino acid standards (Pierce) were treated in tandem and used to correct for loss. The phenylisothiocyanate-derivatized amino acids were chromatographed using a Waters 840 LC System (Waters Associates, Milford, MA.) equipped with a Shandon 25 x 0.46 cm C<sub>18</sub> reverse phase, 3 micron column, and were eluted in a sodium acetate-acetonitrile gradient.

N-terminal amino acid sequence was determined with a model 470 A gas phase peptide sequencer (Applied Biosystems, Inc., Foster City, CA). The phenylthiohydantoin derivatives were identified with an on-line Applied Biosystems liquid chromatograph.

Metals. Neutron activation analysis for Co, Cu, Mn, Mo, Se, V, and W were done by General Activation Analysis, Inc., San Diego, CA. The enzyme was dialyzed against 0.1 mM EDTA and double distilled, deionized water with a YM-30 (Amicon) 30,000 Da cutoff membrane filter in a Centricon unit (Amicon). A 1.5 mg sample was irradiated in a TRIGA Mark I nuclear reactor for 30 seconds at a flux of  $2.5 \times 10^{12}$  neutrons/cm per sec and counted for short lived isotopes after a decay of one minute on a Ge(Li) detector coupled to a multi-channel gamma-ray spectrometer. A second irradiation was done for

30 minutes at a flux of  $1.8 \times 10^{12}$  neutrons/cm per sec with counting after one hour, one day, one week, and three weeks.

Fe, Ni, and Zn were determined with Models 180-70 and 180-80 atomic absorption spectrophotometers equipped with GA-3 graphite atomizers (Hitachi, Ltd., Tokyo), pyrolytically coated graphite furnace tubes, and Zeeman background correction. The enzyme was dialyzed against double distilled, deionized water and 136  $\mu\text{g}$  of protein were digested with concentrated ultrapure nitric acid (J.T. Baker) at  $160^\circ\text{C}$  for one hour prior to analysis.

Molecular weight. Native molecular weight was determined on a 1 x 35 cm Sephadex G-200 Superfine (Pharmacia) gel filtration column. The column was calibrated with chymotrypsinogen A ( $M_r = 25,000$ ), ovalbumin ( $M_r = 43,000$ ), bovine serum albumin ( $M_r = 67,000$ ), aldolase ( $M_r = 158,000$ ) and catalase ( $M_r = 232,000$ ). Purified enzyme, (20  $\mu\text{g}$ ) in 0.2 ml of a 5% (w/v) sucrose solution, was applied to the column which was developed with 100 mM Tris-HCl (pH 7.2) containing 0.2 M KCl and 0.1 mM phenylmethylsulfonyl fluoride applied at 0.02 ml/min.

Spectroscopy. The UV-visible absorption spectrum of purified phloroglucinol reductase was recorded on a Lambda-4B dual beam spectrophotometer (Perkin-Elmer, Norwalk, CT).

Chemicals. Chemicals of the highest available purity were purchased from Aldrich, Sigma, and Bio-Rad. 2,4,6-trihydroxy-benzoate and 5-methoxyresorcinol were purified by reverse phase high pressure liquid chromatography to remove contaminating phloroglucinol prior to use. All other chemicals were used without further purification. Dihydrophloroglucinol was synthesized by reduction of phloroglucinol with sodium borohydride and purified on a 2.5 x 40 cm Sephadex G-10 column (15).

## RESULTS

Enzyme purification. All phloroglucinol reductase activity was recovered in the soluble fraction after ultracentrifugation of cell extract in 20% (w/v) sucrose for 2 hours at 100,000 x g to pellet membranes (data not shown); therefore, the enzyme was purified from cell extract. Cell extract prepared anaerobically showed no loss of activity after exposure to air, therefore all purification steps were performed aerobically. Phloroglucinol reductase was purified 90-fold (Table 1) to homogeneity as indicated by a single protein band on denaturing gels (Fig. 2). Native gels also revealed a single protein band that corresponded with a band that was stained for activity with dihydrophloroglucinol and NADP as substrates.

Table 1. Purification of phloroglucinol reductase.

Step	Vol (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg)	Yield (%)	Fold purifi- cation
Cell extract	29.5	249	37.2	6.7	(100)	1
DEAE-cellulose	88.9	61	1.4	44	74	6.6
Phenyl Sepharose	42.6	64	1.4	44	37	6.6
Ultrafiltration	5.1	639	9.3	69	44	10.3
Red Agarose	4.3	707	1.3	544	41	80.7
Mono-Q	7.7	278	0.46	604	29	90.4

One unit is one  $\mu$ mole NADPH oxidized per minute.



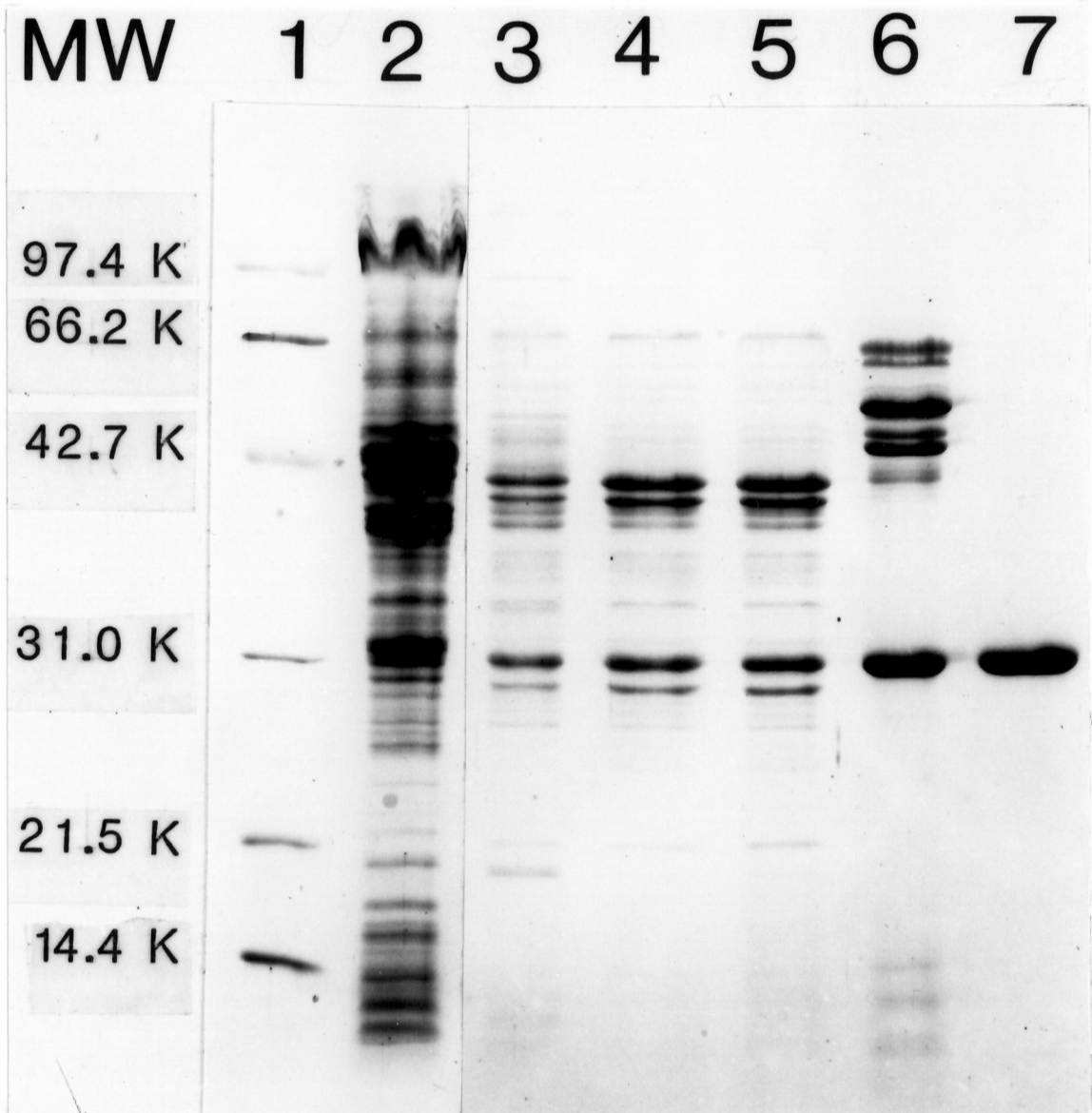


FIG. 2. Denaturing polyacrylamide gel electrophoretic analysis of each step during the purification of phloroglucinol reductase. Lane 1, molecular weight markers: phosphorylase b, bovine serum albumin, ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor, lysozyme. Lane 2, 113  $\mu$ g protein of cell extract. Lane 3, 28  $\mu$ g protein from the DEAE-cellulose column step. Lane 4, 29  $\mu$ g protein from the Phenyl Sepharose column step. Lane 5, 28  $\mu$ g protein from the ultrafiltration step. Lane 6, 10  $\mu$ g protein from the Red Agarose column step. Lane 7, 11  $\mu$ g protein from the Mono-Q column step. Samples were taken from pooled fractions after each step and subjected to electrophoresis on a 12% gel followed by staining with Coomassie Blue R-250 and destaining with 40% methanol/10% acetic acid.

Stability. Homogeneous phloroglucinol reductase had the same activity whether assayed under aerobic or anaerobic conditions. The enzyme was stable for several hours when kept on ice and retained full activity after being frozen with 15% (w/v) glycerol in liquid N<sub>2</sub>. When incubated at 30°C and pH 7.0, 82% of the initial activity was retained after 3.3 hours.

Physiochemical properties. Gel filtration chromatography of native phloroglucinol reductase gave an estimated  $M_r = 78,000$ , while denaturing gel electrophoresis showed one band with an estimated  $M_r = 33,000$ , suggesting that the native enzyme was purified as an  $\alpha_2$  homodimer. The discrepancy between the measured native molecular weight and that calculated from the subunit molecular weight is probably due to conformational differences among the protein standards and phloroglucinol reductase.

The amino acid composition is shown in Table 2. The reported values are the mean of triplicate determinations except for histidine and methionine, which have a  $n = 1$  due to losses during analysis. The pI was 4.4. The N-terminal amino acid sequence was Unknown-Val-Pro-Cys-Asn-Lys-Glu-Phe-Val-Unknown-Asn-Unknown-Phe-. The UV-visible absorption spectrum of the homogeneous enzyme showed a major peak at 276 nm (Fig. 3) with an extinction coefficient of  $97,600 \text{ M}^{-1} \text{ cm}^{-1}$  based on the native molecular weight.

Table 2. Amino acid composition of phloroglucinol reductase.

Amino Acid	Residues per molecule
Ala	95.6
Arg	23.4
Asp	84.3
Cys	20.1
Glu	76.5
Gly	119.5
His	14.8
Ile	31.4
Leu	35.6
Lys	22.3
Met	19.8
Pro	22.7
Phe	16.5
Ser	80.2
Thr	44.3
Tyr	32.4
Val	52.1

Results calculated per  $M_r = 78,000$ . Tryptophan was not determined.

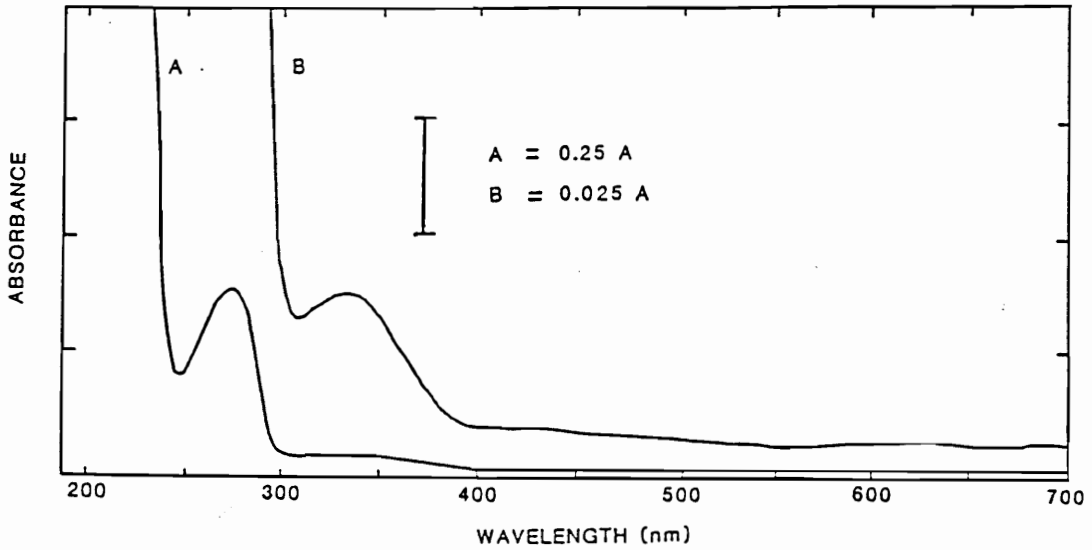


FIG. 3. UV-visible absorption spectrum of phloroglucinol reductase. 125  $\mu\text{g}$  enzyme in 170  $\mu\text{l}$  were diluted to 2 ml with buffer and concentrated by ultrafiltration through a Centricon YM-30 membrane filter (Amicon) to approximately 50  $\mu\text{l}$ . The dilution and concentration steps were performed two more times to remove remaining NADPH that was present during purification. The final volume was adjusted to 266  $\mu\text{l}$  with buffer and the spectrum was recorded in 1 cm quartz microcuvettes with buffer as a reference. The final protein concentration was 0.32 mg/ml.

The small peak at 335 nm was probably bound NADPH. The pure enzyme was colorless in solution at 2 mg/ml and the spectrum showed no absorbance in the visible region. No fluorescent material was released from the enzyme into solution after heat denaturation and precipitation with 7% trichloroacetic acid.

Based on the detection limits of neutron activation analysis, the levels of Co, Mn, Mo, V and W were less than 0.09 nmoles metal per nmole of native enzyme and the levels of Se and Cu were less than 0.3. Atomic absorption spectrophotometry showed that Fe, Ni and Zn were not components of the phloroglucinol reductase.

Temperature maximum and pH optimum. The maximum temperature for activity was 40°C (Fig. 4). The calculated activation energy for the reaction was 11,500 cal/mol. The pH optimum was 7.8 (Fig. 5).

Stoichiometry. The stoichiometry of the forward reaction catalyzed by phloroglucinol reductase was determined by comparing the rate of loss of NADPH at 20, 40, and 80  $\mu$ M with the rate of production of dihydrophloroglucinol, with 2 mM phloroglucinol as substrate. The ratio of the NADPH oxidization rate to the dihydrophloroglucinol production rate was 1.1.

Kinetics. Enzyme activity was reversible and obeyed Michaelis-Menten kinetics at low substrate concentrations. The apparent  $K_m$  and  $V_{max}$  values for phloroglucinol and NADPH in the forward direction and for

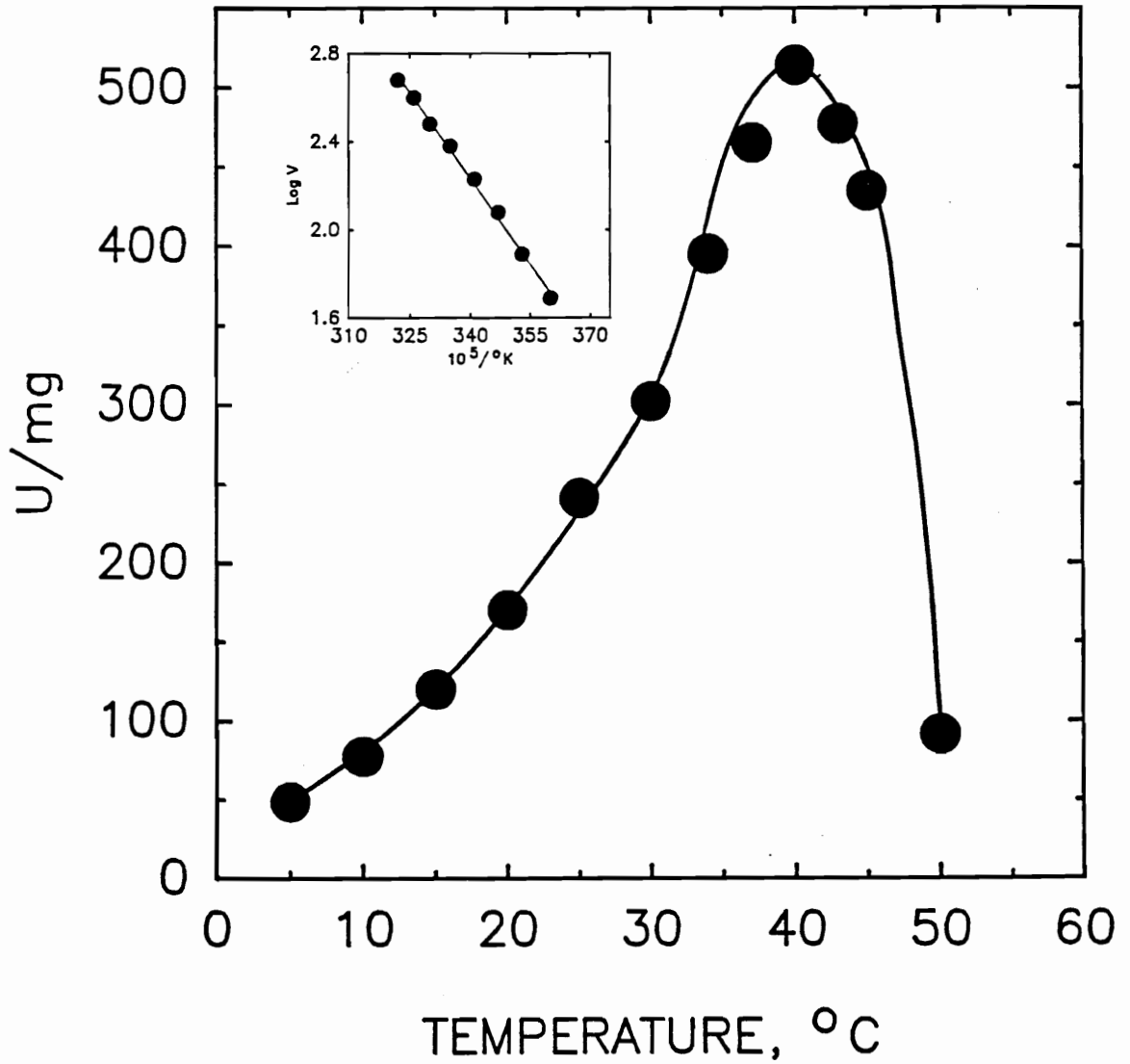


FIG. 4. Dependence of phloroglucinol reductase activity on temperature. The standard assay in the forward direction (see Materials and Methods) was used and 0.049 or 0.097  $\mu\text{g}$  of protein were equilibrated at the indicated temperature for 1 minute prior to initiation of the reaction. Inset, Arrhenius plot.

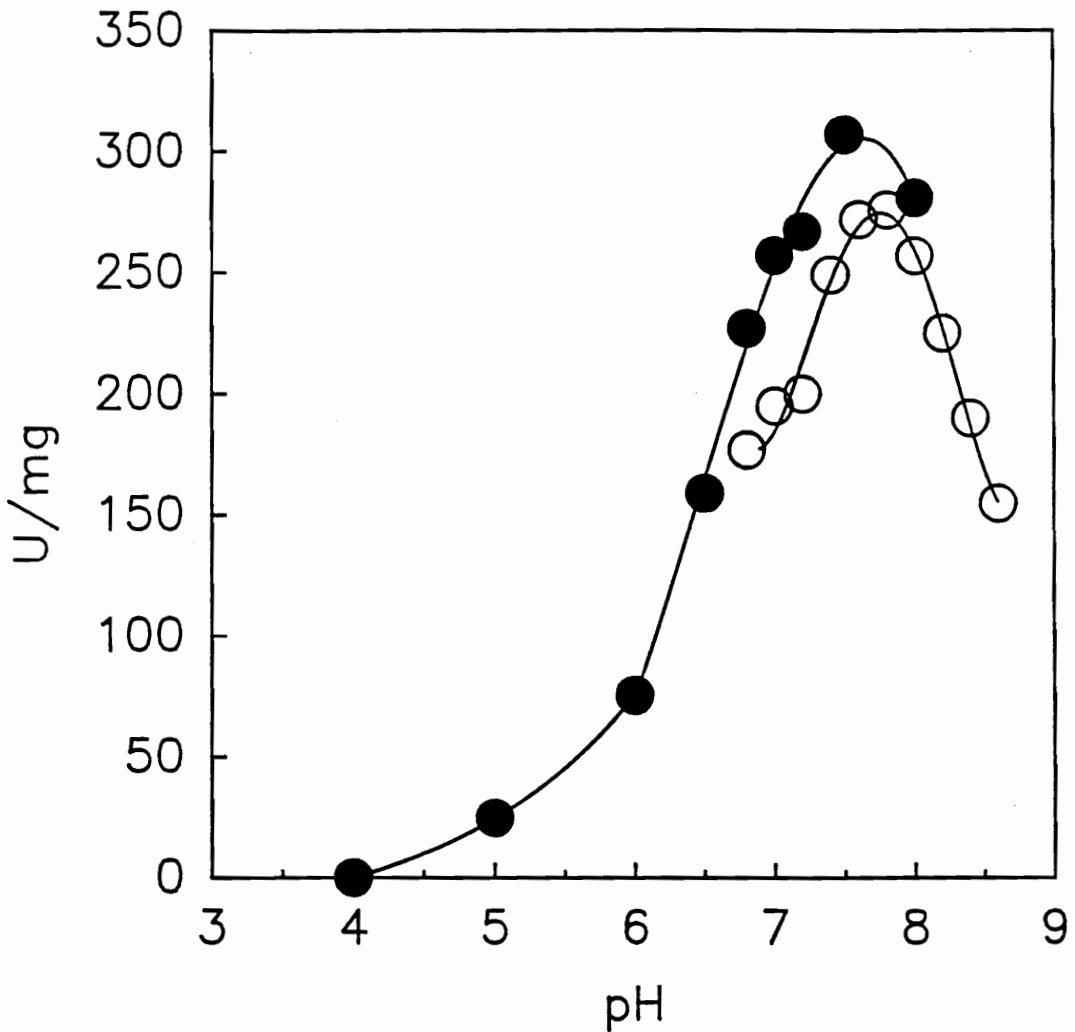


FIG. 5. Dependence of phloroglucinol reductase activity on pH. (●) 50 mM sodium acetate buffer, pH 4-5; 50 mM phosphate buffer, pH 6-8. (○) A combination of 3-[N-morpholino]propanesulfonic acid and its sodium salt (50 mM final concentration). The standard assay in the forward direction (see Materials and Methods) was used and 0.049 or 0.097  $\mu$ g of protein were assayed.

dihydrophloroglucinol and NADP in the reverse direction were determined by linear regression of reciprocal substrate concentration versus reciprocal reaction velocity (Table 3). Kinetic constants for NADPH were determined at pH 6.8 only owing to increased phloroglucinol absorbance at 350 nm and around 278 nm in alkaline solutions (19). Therefore, accurate measurements of NADPH oxidation or dihydrophloroglucinol formation at low  $\mu\text{M}$  NADPH concentrations were not possible above pH 6.8. The apparent  $K_m$  and  $V_{max}$  for phloroglucinol was dependent on pH (Fig. 6) indicating that either substrate or enzyme ionization or both were involved. At pH 7.6 and 8.0 enzyme activity was inhibited by high phloroglucinol concentrations and those data points were not included in calculations of the kinetic constants.

Enzyme specificity. Several substituted homocyclic and heterocyclic aromatic compounds were tested as substrates in the forward direction. None of the compounds listed in Table 4, except phloroglucinol, were substrates for the enzyme. Most showed less than 15% inhibition of activity when included in the assay with phloroglucinol as substrate (Table 4). Menadione, hydroquinone and 2,4,6-trihydroxybenzoate gave the greatest inhibition of activity. No activity was detected with phloroglucinol as substrate when 100  $\mu\text{M}$  NADH was substituted for NADPH as the reductant.



Table 3. Kinetic constants of phloroglucinol reductase.

Substrate	pH	K <sub>m</sub> ( $\mu$ M)	V <sub>max</sub> (U/mg)
(Forward Direction)			
Phloroglucinol <sup>c</sup>	6.8	800	510 <sup>a</sup>
	7.2	600	640 <sup>a</sup>
	7.6	530	910 <sup>a</sup>
	8.0	180	530 <sup>a</sup>
NADPH <sup>d</sup>	6.8	6.7	550 <sup>b</sup>
(Reverse Direction)			
Dihydrophloroglucinol <sup>e</sup>	7.2	290	140 <sup>a</sup>
NADP <sup>f</sup>	7.2	27	220 <sup>a</sup>

<sup>a</sup>One unit is 1  $\mu$ mole NADP(H) oxidized or reduced per min.

<sup>b</sup>One unit is 1  $\mu$ mole dihydrophloroglucinol produced per min.

<sup>c</sup>NADPH concentration = 100  $\mu$ M.

<sup>d</sup>Phloroglucinol concentration = 2.0 mM.

<sup>e</sup>NADP concentration = 100  $\mu$ M.

<sup>f</sup>Dihydrophloroglucinol concentration = 3.5 mM.

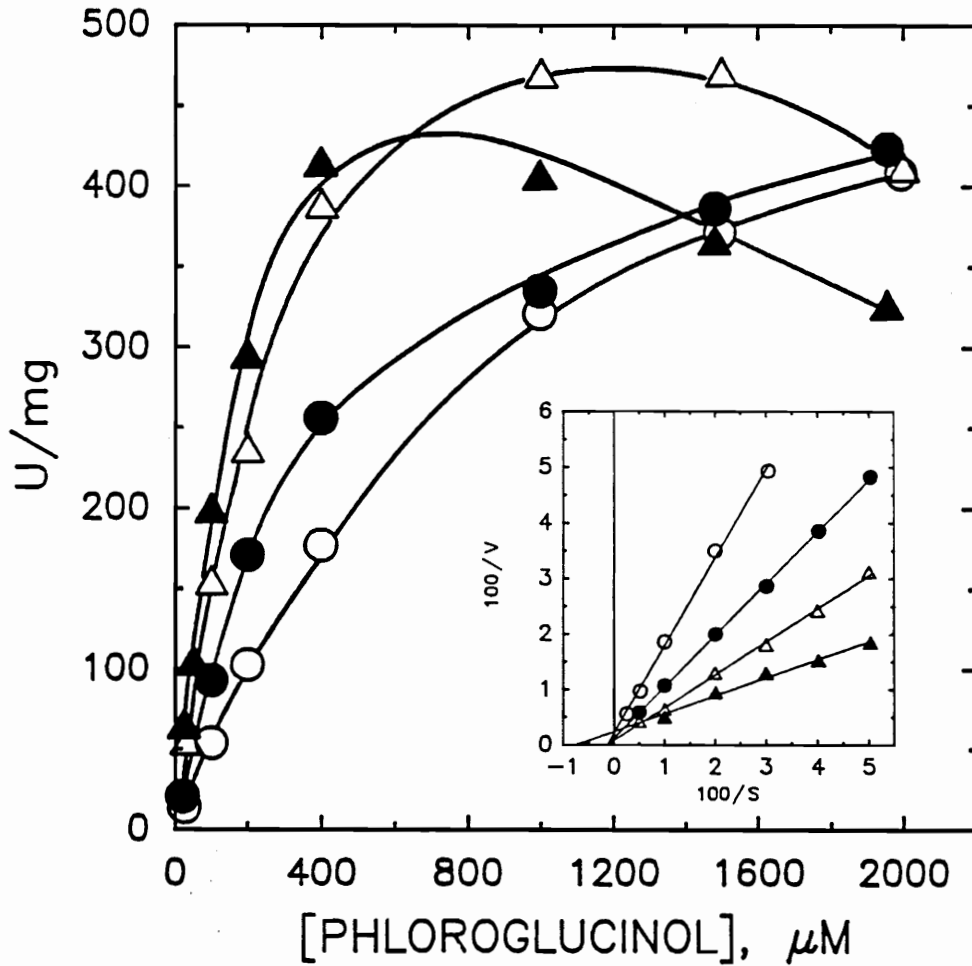


FIG. 6. Dependence of phloroglucinol reductase activity on phloroglucinol concentration. The standard assay in the forward direction (see Materials and Methods) was used except that the phloroglucinol concentration was varied and the reactions were carried out in 50 mM phosphate buffer at the indicated pH. (○), pH 6.8; (●), pH 7.2; (△), pH 7.6; (▲), pH 8.0. Inset, Lineweaver-Burke plots.

Table 4. Effects of aromatic compounds on the reduction of phloroglucinol by phloroglucinol reductase.

Substrate	Inhibition <sup>a</sup> (%)
Phloroglucinol <sup>b</sup>	(0)
Phenol	9
Catechol	14
Resorcinol <sup>b</sup>	5
Hydroquinone <sup>b</sup>	31
Pyrogallol <sup>b</sup>	5
1,2,4-Benzenetriol <sup>b</sup>	8
Orcinol <sup>b</sup>	0
5-Methoxyresorcinol <sup>b</sup>	9
3,5-Dihydroxybenzoate <sup>b</sup>	5
Gallate <sup>b</sup>	24
2,4,6-Trihydroxybenzoate <sup>b</sup>	49
3,5-Dimethoxyphenol	6
Benzoate	0
6-Hydroxynicotinate	12
Barbiturate	9
Orotate <sup>b,c</sup>	0
Menadione <sup>c</sup>	33

<sup>a</sup>Assays were performed in the presence of 1.0 mM phloroglucinol and unless otherwise noted, 1.0 mM aromatic compound.

<sup>b</sup>Assays performed under N<sub>2</sub>.

<sup>c</sup>Tested at 0.1 mM.

Inhibitors. The effects of potential inhibitors of enzyme activity are shown in Table 5. Of the sulfhydryl reagents tested, p-chloromercuribenzoate and n-ethylmaleimide were effective inhibitors whereas iodoacetate and iodoacetamide did not inhibit activity. Dithiothreitol had no effect on activity when included in the assay buffers at concentrations up to 20 mM.  $\text{Cu}^{2+}$  was a potent inhibitor in low concentrations. Diethylpyrocarbonate caused partial inhibition. In addition, 10 mM o-phenanthroline inhibited activity by 32% after 200 minutes, while 2.0 mM EDTA had no effect (data not shown).

## DISCUSSION

Although phloroglucinol reductase activity has been detected in several organisms (11, 15, 17, 20), purification of the enzyme has not been reported. Attempts to purify the enzyme from a Coprococcus sp. were unsuccessful, owing to instability of the enzyme under the conditions used (15). The purification scheme reported here produced good yields of enzyme with high specific activity. Affinity chromatography with Red Agarose was an especially useful step. Based on the recovery from cell extract, the phloroglucinol reductase from E. oxidoreducens appears to constitute greater than 1% of the soluble protein, consistent with its involvement in a major metabolic pathway (Fig. 1). Elution from the Phenyl Sepharose column required only a decreasing

Table 5. Inhibitors of phloroglucinol reductase.

Compound	Concentration (mM)	Incubation Time (min)	Inhibition (%)
p-Chloromercuribenzoate <sup>a</sup>	0.1	5	100
n-Ethylmaleimide <sup>a</sup>	1.0	60	94
Iodoacetate <sup>a</sup>	1.0	10	1
Iodoacetamide <sup>a</sup>	1.0	25	0
Diethylpyrocarbonate <sup>a</sup>	1.0	20	54
CuCl <sub>2</sub> <sup>b</sup>	0.1	0.17	100
CuCl <sub>2</sub> <sup>b</sup>	0.001	0.17	57
NiCl <sub>2</sub> <sup>b</sup>	0.1	0.17	10

<sup>a</sup>0.49  $\mu$ g of enzyme were incubated at 30°C in 0.05 ml of 50 mM phosphate buffer, pH 7.2 for the times indicated prior to analysis of .049  $\mu$ g for activity using the standard assay in the forward direction.

<sup>b</sup>0.049  $\mu$ g of enzyme were incubated at 30°C in 2.0 ml of assay buffer containing the metal ions and 100  $\mu$ M NADPH prior to initiation of the standard assay in the forward direction.

salt concentration suggesting that the enzyme was void of extremely hydrophobic domains. Analysis of the amino acid composition (13) revealed a low hydropathy index of -0.28, typical of soluble proteins. These results, and the presence of the enzyme in the soluble fraction after cell disruption, supports a cytoplasmic location in the cell; however, anchoring of the phloroglucinol reductase to the membrane by accessory proteins has not been ruled out. Formate dehydrogenase, which provides reducing equivalents for reduction of the aromatic ring, also appears to be located in the cytoplasm (Section II).

The enzyme in E. oxidoreducens has a native  $M_r = 78,000$  and apparently consists of two identical subunits of approximately 33,000. Patel et al. (15) reported a native  $M_r = 130,000$  for the partially purified enzyme from a Coprococcus sp. As reported here for the phloroglucinol reductase from E. oxidoreducens, both the apparent  $K_m$  and  $V_{max}$  for phloroglucinol are affected by pH. Patel et al. (15) reported  $K_m$  values of 30  $\mu M$  and 290  $\mu M$  (pH 7.4) for phloroglucinol and NADPH respectively for the partially purified enzyme from a Coprococcus sp. The enzyme in cell extract from P. acidigallici has a pH optimum of 7.2 and  $K_m$  values for phloroglucinol and NADPH of 760  $\mu M$  and 17  $\mu M$  respectively (10).

Phloroglucinol reductase is specific for NADPH in E. oxidoreducens, Coprococcus sp. (15), and P. acidigallici (16). The enzyme is specific for

phloroglucinol when purified from E. oxidoreducens, while other aromatic substrates were reported for the unpurified enzyme from the latter two organisms.

No evidence was obtained for the presence of intramolecular redox centers including flavins, iron-sulfur clusters, or potential redox active metals. These results imply a direct transfer of reducing equivalents from NADPH to phloroglucinol. Direct hydride transfer from NADPH to the keto tautomer of phloroglucinol is a testable hypothesis worthy of further investigation.

The possible involvement of sulfhydryl groups in maintaining a competent enzyme was implicated by the inhibition of activity with sulfhydryl agents; furthermore, the enzyme was rapidly inactivated with low amounts of  $\text{Cu}^{2+}$ . Cupric ions have been shown to oxidize neighboring cysteine residues with the formation of a disulfide bond (18). Phloroglucinol reductase has 20 cysteine residues per molecule which may account for its sensitivity to copper and other sulfhydryl reagents.

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## SECTION V: THERMODYNAMICS OF THE REACTION CATALYZED BY PHLOROGLUCINOL REDUCTASE OF EUBACTERIUM OXIDOREDUCENS

### SUMMARY

The equilibrium constant for the reversible reaction involved in the reduction of phloroglucinol by NADPH was determined by measurement of substrate and product concentrations after chemical equilibrium was established by addition of pure phloroglucinol reductase as a catalyst. The equilibrium constant was 9.5 and the calculated  $\Delta G'$  and  $\Delta E^\circ$  values were -1.35 kcal/mol and 0.0294 V respectively. With formate as the electron donor, the calculated  $K_{eq}$ ,  $\Delta G'$  and  $\Delta E^\circ$  values were 50,600, -6.52 kcal/mol and 0.141 V respectively. Therefore, the reduction of phloroglucinol is highly favorable with formate as a source of electrons.

### INTRODUCTION

The central hypothesis for microbial anaerobic aromatic degradation is that the ring must be reduced prior to cleavage (2). Even though a large number of aromatic compounds have been shown to be degraded by a diverse group of anaerobic microorganisms (1), no exceptions have been found for the requirement for ring reduction prior to ring cleavage. However, few studies have been conducted on the biochemistry of aromatic ring reduction and very

little is known about the enzymology and bioenergetics of the central step involved in anaerobic aromatic degradation. This study was carried out in order to describe the thermodynamics of phloroglucinol reduction by NADPH.

## MATERIALS AND METHODS

$K_{eq}$  determination. All reactions were performed aerobically at 30°C in 50 mM potassium phosphate buffer, pH 7.2, in a volume of 1.0 ml. The progress of the reactions were followed spectrophotometrically by measuring the decrease in absorbance at 340 nm and equilibrium was assumed to have been reached when there was no detectable change in absorbance after several minutes. Purified phloroglucinol reductase, 0.47  $\mu$ g, was added to initiate the reaction. The starting concentrations of phloroglucinol and NADPH were 1.0 mM and 0.05 mM respectively while dihydrophloroglucinol and NADP were not present initially when equilibrium was established from the forward direction. The starting concentrations for dihydrophloroglucinol and NADP were 0.1 mM each and no phloroglucinol or NADPH were present initially when equilibrium was established from the reverse direction. After equilibrium had been established the NADPH concentration was determined spectrophotometrically at 340 nm and the NADP concentration was calculated from the difference of the initial and final concentrations. Phloroglucinol and dihydrophloroglucinol were

determined by HPLC after acidification of the reaction mix to pH 2 with concentrated sulfuric acid.

HPLC. Dihydrophloroglucinol and phloroglucinol concentrations were determined by injecting 20  $\mu$ l per sample via a loop on to a 4 mm x 25 cm C<sub>18</sub> reverse phase column (Bio-Rad). The mobile phase was 5% methanol/95% 0.01N H<sub>2</sub>SO<sub>4</sub> pumped at a flow rate of 0.5 ml/min and the detector wavelength was 266 nm.

Phloroglucinol reductase purification. Cell extract was prepared as described (Section IV) except that the cell breakage buffer contained 20 mM potassium phosphate, pH 6.8, 50 mM KCl and 2 mM Na<sub>2</sub>EDTA. Cell extract was applied to a 2.6 cm x 15 cm DEAE-cellulose (Whatman DE-52) column equilibrated with the above described buffer with an EDTA concentration of 0.2 mM. Protein was eluted from the column with a 175 ml linear gradient from 0.1 to 0.35 M KCl with a flow rate of 2.0 ml/min. The most active fractions were pooled and applied to a 1.6 cm x 21 cm Reactive Red Agarose type 3000 CL (Sigma) column equilibrated with the DEAE-cellulose column equilibration buffer plus 150 mM KCl. Protein was eluted from the column with a 160 ml linear gradient from 150 mM to 2.5 M KCl at a flow rate of 0.8 ml/min. The most active fractions were pooled and concentrated by ultrafiltration using a Centriprep YM-30 (Amicon) 30,000 Da cut-off membrane. The retentate was

diluted to 15 ml with 20 ml potassium phosphate buffer, pH 6.8 and reconcentrated by ultrafiltration. The dilution and concentration procedure was repeated and the retentate was applied to a 1.2 cm x 4.6 cm column packed with Reactive Red Agarose type 1000 CL (Sigma) equilibrated with the DEAE-cellulose column equilibration buffer with 0.1 M KCl. Unbound protein was washed from the column with 10 ml of equilibration buffer and phloroglucinol reductase was eluted from the column with 10 ml of 1.0 mM NADPH at a flow rate of 0.2 ml/min and 1.0 ml fractions were collected. The four most active fractions showed a constant specific activity and were pooled, made to 15% (w/v) glycerol and stored frozen in liquid nitrogen.

## RESULTS AND DISCUSSION

The purified phloroglucinol reductase was homogeneous when electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel. The specific activity of the purified enzyme was  $655 \mu\text{mol}/\text{min}$  per mg which is slightly higher than that reported for the previously described method (Section IV). An advantage of the purification procedure described here over the previously described method is elimination of one column step.

The concentrations of phloroglucinol, NADPH, dihydrophloroglucinol and NADP were  $0.0488 \pm 0.0034$ ,  $0.00440 \pm 0.00056$ ,  $0.0436 \pm 0.0001$  and

0.0467  $\pm$  0.0014 (mean  $\pm$  standard deviation, n = 5) respectively after chemical equilibrium had been established from the forward direction and 0.0193  $\pm$  0.0003, 0.0217  $\pm$  0.0002, 0.0758  $\pm$  0.0004 and 0.0842  $\pm$  0.0004 (n = 3) respectively after chemical equilibrium had been established from the reverse direction.

The equilibrium constant for the reversible reaction catalyzed by phloroglucinol reductase was calculated using the equation:

$$[1] \quad K_{eq} = \frac{[\text{dihydrophloroglucinol}] \times [\text{NADP}]}{[\text{phloroglucinol}] \times [\text{NADPH}]}$$

The value obtained was 9.48 or 15.2 when equilibrium was established from the forward or reverse directions respectively. The former value was used in further calculations. The following equations were used for further calculations:

$$[2] \quad \Delta G' = -RT \ln K_{eq}$$

$$[3] \quad \Delta G' = -nF \Delta E'$$

$$[4] \quad \Delta E' = \frac{RT}{nF} \ln K_{eq}$$

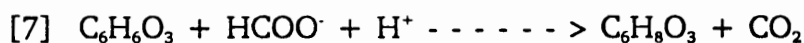
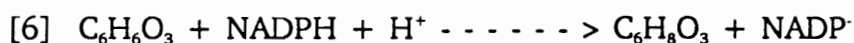
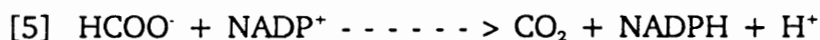
where R = 1.987 cal/mol per °K

$$T = 303 \text{ } ^\circ\text{K}$$

$$F = 23,063 \text{ cal/V per e}$$

$$n = e/\text{mol}$$

The calculated values for  $\Delta G'$  and  $\Delta E'$  are -1.35 kcal/mol and 0.0294 V for the reduction of phloroglucinol by NADPH. Formate dehydrogenase oxidizes formate to  $\text{CO}_2$  and reduces NADP to NADPH in *E. oxidoreducens* and supplies electrons for phloroglucinol reduction. Using values of  $E' = -0.432$  V for the  $\text{CO}_2$ /formate couple and  $E' = -0.320$  V for the NADP/NADPH couple (7),  $\Delta E' = 0.112$  V,  $\Delta G' = -5.17$  kcal/mol and  $K_{\text{eq}} = 5,330$  for the oxidation of formate to  $\text{CO}_2$  and reduction of NADP to NADPH. Coupling of formate oxidation with phloroglucinol reduction is described by the following reactions:



The overall  $G'$  for the reaction described by reaction [7] is the sum of the  $G'$  for the reactions described by [5] and [6] and the overall  $K_{\text{eq}}$  for [7] is the product of the  $K_{\text{eq}}$  of the two reactions. The calculated values for  $\Delta G'$  and  $K_{\text{eq}}$  for the overall reaction are -6.52 kcal/mol and 50,600 respectively.  $\Delta E'$  for the overall reaction is 0.141 V.

These values show that reduction of phloroglucinol with electrons from formate is energetically favorable but does not supply the -7.6 kcal/mol required for the synthesis of ATP under standard conditions (8). Oxidation of



formate is the most energetically favorable step and drives the reaction for phloroglucinol reduction strongly in the forward direction.

P. acidigallici (5, 6) and a Coprococcus sp. (4) also have NADPH-dependent phloroglucinol reductases. However, neither organism requires formate or hydrogen for growth on aromatic substrates, and the source of reducing equivalents has not been identified. Presumably the electrons are derived from  $\beta$ -oxidation of the ring cleavage product. P. acidigallici has a minimum doubling time of 2.0 h at 35°C and produces about 10 g dry weight cells per mole of gallate (6) while E. oxidoreducens has a minimum doubling time of 1.8 h at 39°C and produces 10 to 15 g dry weight of cells per mole of gallate (3). It appears that no advantage in growth rate is taken by E. oxidoreducens through the use of formate or H<sub>2</sub> for ring reduction. The data for the growth yields of E. oxidoreducens are too variable to determine if formate or H<sub>2</sub> increased cell yields (3). Some of the variability may be due to a rapid lysis of E. oxidoreducens following the end of the exponential phase of growth on gallate, which would result in an underestimate of cell yield, (unpublished observation).

Fumarate is an electron acceptor for formate oxidation in Wolinella succinogenes which results in the formation of ATP via generation of a membrane potential (8).  $E^{\circ} = 0.03$  V for the fumarate/succinate couple which

gives  $\Delta G' = -20.8$  kcal/mol for the transfer of 2 electrons from formate. This value is 3.2 times the standard free energy change for the transfer of electrons from formate to phloroglucinol. Formate dehydrogenase and fumarate reductase are also integral membrane proteins in W. succinogenes (8) while formate dehydrogenase and phloroglucinol reductase are located in the soluble fraction of E. oxidoreducens (Sections III and VI). Therefore, it seems unlikely that E. oxidoreducens generates ATP by electron transport phosphorylation during formate oxidation as has been shown for W. succinogenes.

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SECTION VI. DIHYDROPHLOROGLUCINOL: THE PRODUCT OF  
REDUCTION OF THE AROMATIC RING BY PHLOROGLUCINOL REDUCTASE  
OF EUBACTERIUM OXIDOREDUCENS.

SUMMARY

Selected structural, chemical and spectroscopic properties of dihydrophloroglucinol were investigated to provide information concerning the product of the reaction catalyzed by phloroglucinol reductase of E. oxidoreducens.  $^{13}\text{C}$  and  $^1\text{H}$  nuclear magnetic resonance (NMR) spectroscopy provided evidence that the compound exists as the enol tautomer in aqueous solution. UV-absorbance spectroscopy provided supporting evidence for the proposed structure which is related to that of dihydroresorcinol (1,3-cyclohexane dione). The molar absorbance value for dihydrophloroglucinol was  $26,900 \text{ M}^{-1} \text{ cm}^{-1}$ . Dihydroresorcinol was a competitive inhibitor with a  $K_i = 756 \mu\text{M}$  for the reverse reaction catalyzed by phloroglucinol reductase at pH 7.2 and  $30^\circ\text{C}$ .  $\text{H}_2$  served as the source of electrons for the reduction of phloroglucinol to dihydrophloroglucinol by cell extract of a mutant strain of E. oxidoreducens that lacked dihydrophloroglucinol hydrolase activity. Dihydrophloroglucinol produced by reduction of phloroglucinol by cell extract had UV-absorbance and  $^1\text{H}$  NMR spectra that were identical to the spectra of chemically reduced

phloroglucinol. A mechanism for the enzymatically catalyzed reaction is proposed.

## INTRODUCTION

Dihydrophloroglucinol has been proposed as an intermediate of the anaerobic degradation of phloroglucinol by a Rhodopseudomonas sp. (14), a Coprococcus sp. (10), Pelobacter acidigallici (11, 12) and E. oxidoreducens (5, 8). In neutral aqueous solution, dihydrophloroglucinol has a strong absorbance peak at 278 nm which has been used as evidence for its formation. However, the compound is not commercially available and details of its structural and chemical properties are not well known. This study was conducted to determine the structure and stability of dihydrophloroglucinol in order to assess possible mechanisms for its formation.

## MATERIALS AND METHODS

Chemical synthesis of dihydrophloroglucinol. Dihydrophloroglucinol was synthesized by sodium borohydride reduction of phloroglucinol (10). Fifty ml of distilled, deionized water (dd-H<sub>2</sub>O) was continuously sparged with O<sub>2</sub>-free N<sub>2</sub> and 3.24 g of phloroglucinol dihydrate (Chemical Dynamics) were added while stirring. After 15 minutes, 1.14 g of sodium borohydride were added and allowed to react 15 minutes while being stirred under N<sub>2</sub>. The pH was then

lowered to ~2 by drop-wise addition of concentrated HCl. The solution was then extracted with 3 x 100 ml ethylacetate. The ethylacetate was removed on a rotary vacuum evaporator and 15 ml dd-H<sub>2</sub>O were added to dissolve the residue. The solution was filtered through a 0.2  $\mu$ m filter and the water was removed by rotary vacuum evaporation. Five ml dd-H<sub>2</sub>O were added to dissolve the residue and 3 ml of the solution were removed for UV-absorbance spectroscopy and <sup>13</sup>C NMR spectroscopy. The water was removed from the remaining ~2 ml by vacuum evaporation and 10 ml D<sub>2</sub>O (100.0 atom %D, Aldrich Chemical Co.) were added and evaporated. Ten ml D<sub>2</sub>O were added and evaporated a second time and the residue was dissolved in ~6 ml D<sub>2</sub>O and the <sup>1</sup>H NMR spectrum was determined.

pH effects. Dihydrophloroglucinol was diluted to 0.0320 mM in dd-H<sub>2</sub>O at pH 0 to 4.8 (adjusted with HCl) and 4.4 to 6.9 in potassium phosphate buffer. The absorbance spectrum was scanned from 190 to 400 nm with the appropriate reference solution. The effects of acid and base on the absorbance spectrum of dihydrophloroglucinol in methanol were determined using a 0.032 mM solution to which 1 drop of 2 N NaOH was added to 100 ml of solution followed by 1 drop of 12 N HCl. Absorbance spectra were recorded before and after each addition. Resorcinol was treated by identical procedures for comparison. The molar absorbance value for dihydrophloroglucinol was

determined by sodium borohydride reduction of known concentrations of phloroglucinol with an excess of reductant. After completion of the reaction the absorbance was measured at 278 nm. Molar absorbance was calculated from the slope of the absorbance versus concentration plot for two independent determinations.

NMR spectroscopy.  $^{13}\text{C}$  NMR spectra were determined at ambient temperature in  $\text{H}_2\text{O}$  with a Bruker model WP 200 spectrometer with 3-(trimethylsilyl) tetradeutero sodium propionate (TSP) as an external reference.  $^1\text{H}$  NMR spectra were obtained at ambient temperature in  $\text{D}_2\text{O}$  with a Bruker model WP 270 spectrometer with TSP as an external reference.

Absorbance spectroscopy. Measurements were made on a Perkin Elmer model 552 dual beam instrument at room temperature using matched quartz cuvetts with a 1 cm pathlength.

Dihydrophloroglucinol synthesis by cell extract. Anoxic cell extract, 0.30 mg protein, of *E. oxidoreducens* mutant strain C21-21-7 (Section VII) was added to 10 ml of anoxic 100 mM potassium phosphate buffer, pH 7.2, that contained 16.2 mg of phloroglucinol dihydrate, 2 mg NADP and 2 mM 2-mercaptoethanol. Strain C21-21-7 is a mutant that lacks dihydrophloroglucinol hydrolase activity (Section VII, Table 2), and therefore is unable to metabolize dihydrophloroglucinol. The reaction was carried out at room temperature in a

stoppered, amber, 60 ml serum bottle. The headspace atmosphere was 100%  $\text{H}_2$  that had been passed through hot copper turnings and reduced palladium catalyst (BASF, Chemical Dynamics, S. Plainfield, NJ) before being introduced into the bottle. A constant flow of hydrogen was maintained by venting through a syringe needle inserted through the stopper. The reaction mixture was stirred with a teflon coated, magnetic stir bar. The progress of the reaction was determined by periodically diluting 10  $\mu\text{l}$  of the reaction mixture into 1.0 ml of 0.02N  $\text{H}_2\text{SO}_4$  and recording the UV absorbance spectrum. The reaction was terminated by the addition of 250  $\mu\text{l}$  of 4N HCl when the absorbance at 203 nm (phloroglucinol) had stopped decreasing and the absorbance at 256 nm (dihydrophloroglucinol) had stopped increasing. The pH, after addition of HCl, was 2.2. The reaction mixture was extracted with 3 x 50 ml ethylacetate. The ethylacetate was evaporated by rotary vacuum evaporation and the residue was dissolved in 20 ml  $\text{H}_2\text{O}$  and filtered through a 0.2  $\mu\text{m}$  syringe filter. The filtrate was rotary-vacuum evaporated to dryness and the residue was dissolved in 10 ml  $\text{D}_2\text{O}$ . The  $\text{D}_2\text{O}$  was evaporated and the residue was dissolved in 10 ml  $\text{D}_2\text{O}$  which was again removed by rotary vacuum evaporation. The residue was dissolved in 1.0 ml of  $\text{D}_2\text{O}$  and the  $^1\text{H}$  NMR spectrum was recorded.



Hydrogenase assay. The activity of hydrogenase in the mutant cell extract was determined spectrophotometrically at room temperature in 1.0 ml of 50 mM potassium phosphate buffer, pH 7.2, that contained 20 mM methyl viologen and 2 mM 2- mercaptoethanol under a 100% H<sub>2</sub> atmosphere. The increase in absorbance at 603 nm ( $\epsilon = 11.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ ) was measured after addition of 5  $\mu\text{l}$  of cell extract (7.5  $\mu\text{g}$  protein).

Dihydroresorcinol inhibition. Enzyme assays for phloroglucinol reductase activity in the reverse direction were performed aerobically at 30°C by recording the production of NADPH as described in Section IV. The initial concentration of NADP was 250  $\mu\text{M}$  and the dihydrophloroglucinol concentration was varied from 0.1 to 10.0 mM in the presence of various fixed concentrations of dihydroresorcinol (1,3 cyclohexane dione) (0 to 10 mM in a volume of 1.0 ml). The reaction was initiated by addition of 0.47  $\mu\text{g}$  of pure phloroglucinol reductase.

## RESULTS

The sodium borohydride reduction of phloroglucinol to dihydrophloroglucinol appeared to be complete. No phloroglucinol or unidentified peaks were detected when the product was analyzed by reverse phase HPLC. The product of the reaction was colorless if care was taken to

exclude all oxygen prior to addition of sodium borohydride.

Dihydrophloroglucinol was stable in air and aqueous preparations showed no change in absorbance for several months when neutralized with NaOH and frozen.

Several methods were attempted for the recovery of dihydrophloroglucinol produced by reduction of phloroglucinol by sodium borohydride. Gel filtration chromatography on a column of Sephadex G-10 (Patel et al., 1981) did not adequately separate dihydrophloroglucinol from the borate salts. Extraction of the acidified reaction solution into ethylether gave poor recoveries and the ether may have reacted with dihydrophloroglucinol. Extraction with ethylacetate gave a preparation with a low conductance indicating separation from the salts, however the extraction efficiency for dihydrophloroglucinol was only about 25%. Despite the low recovery, this method yielded the most homogeneous product and was used to isolate dihydrophloroglucinol for the spectroscopic analyses.

The absorbance maximum of dihydrophloroglucinol was dependent on the solvent pH. At low pH values in aqueous solution, the maximum occurred at 256 nm while at pH values above 5 the maximum was shifted to 278 nm (Fig. 1). The midpoint of the spectral shift occurred at a pH of 4.6 to 4.7. In methanol, the spectrum was nearly identical to that in water at pH 2.0

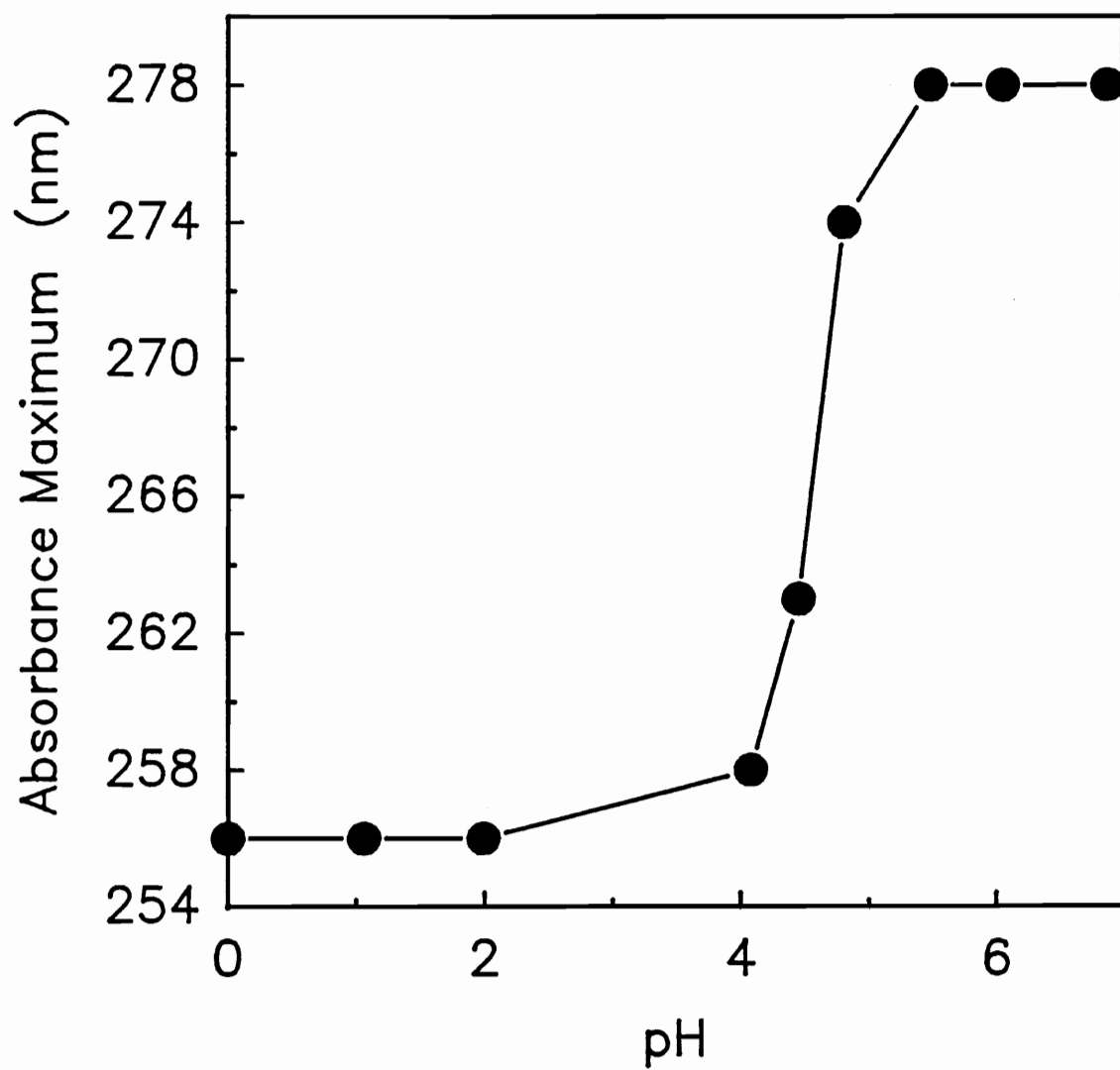


FIG. 1. The effect of pH on the absorbance maximum of dihydrophloroglucinol in aqueous solution.

with a maximum at 254 nm. Addition of base shifted the maximum to 278 nm, while addition of acid shifted the maximum back to 252 nm. However, in acidified methanol there was a slow decrease in absorbance at 254 nm indicating that decomposition was occurring. After approximately 24 hours the spectrum matched that of resorcinol, suggesting that the dihydrophloroglucinol had aromatized through the loss of one mole of water per mole of dihydrophloroglucinol.

With sodium borohydride added in excess, there was a linear relationship between the starting concentration of phloroglucinol and absorbance for dihydrophloroglucinol (Fig. 2). The calculated molar absorbance value at 278 nm was  $26,900 \text{ M}^{-1} \text{ cm}^{-1}$ . The starting pH of the phloroglucinol solution was about 4.5 but increased to approximately 9.2 after addition of the sodium borohydride. The wavelength maximum and molar absorbance value were unchanged when the pH of the dihydrophloroglucinol was adjusted to 7.2 in potassium phosphate buffer.

The  $^{13}\text{C}$  NMR spectra of dihydrophloroglucinol are shown in Fig. 3A and 3B. Peak assignments were aided by comparison with the spectrum of dihydroresorcinol (Fig. 4). Integration of the peak signals for the quantitative

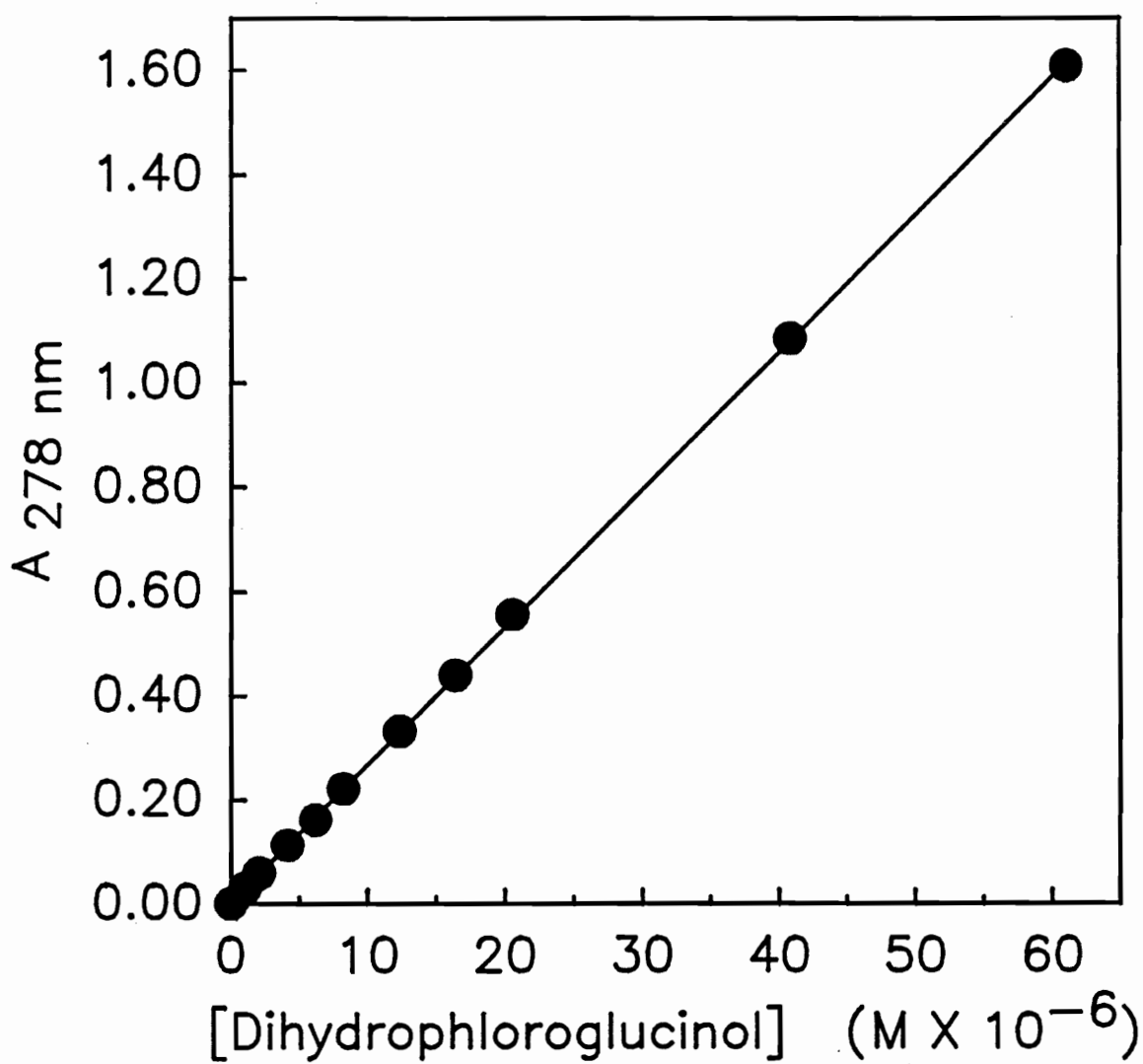


FIG. 2. Absorbance at 278 nm of dihydrophloroglucinol produced by reduction of various amounts of phloroglucinol with an excess of sodium borohydride.

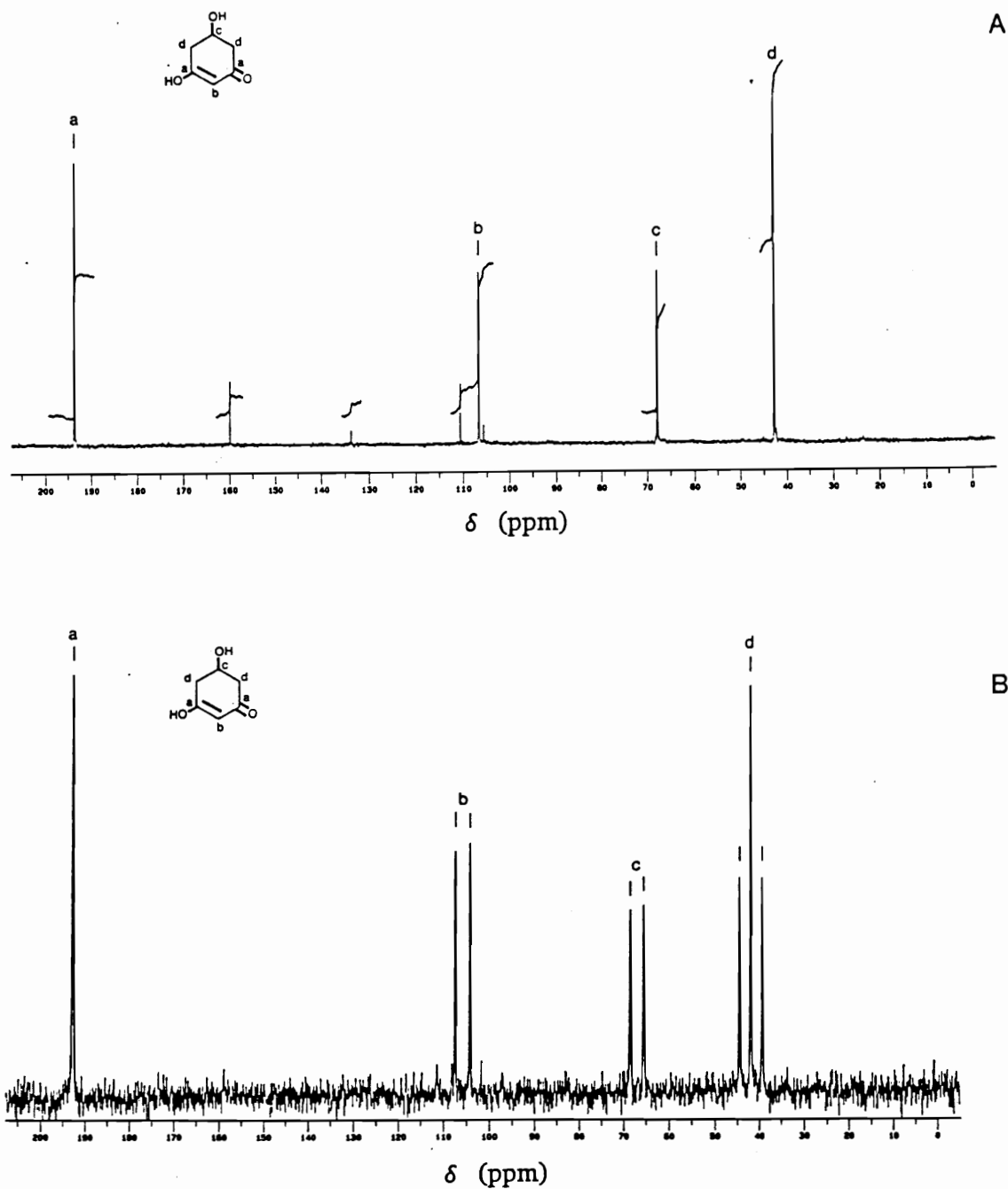


FIG. 3.  $^{13}\text{C}$  NMR spectra of dihydrophloroglucinol produced by reduction of phloroglucinol with sodium borohydride. The dihydrophloroglucinol concentration was 540 mM in  $\text{H}_2\text{O}$ , pH 2.2. A. Inverse gated spectrum with signal integration. B. Gated spectrum showing the C-H couplings.

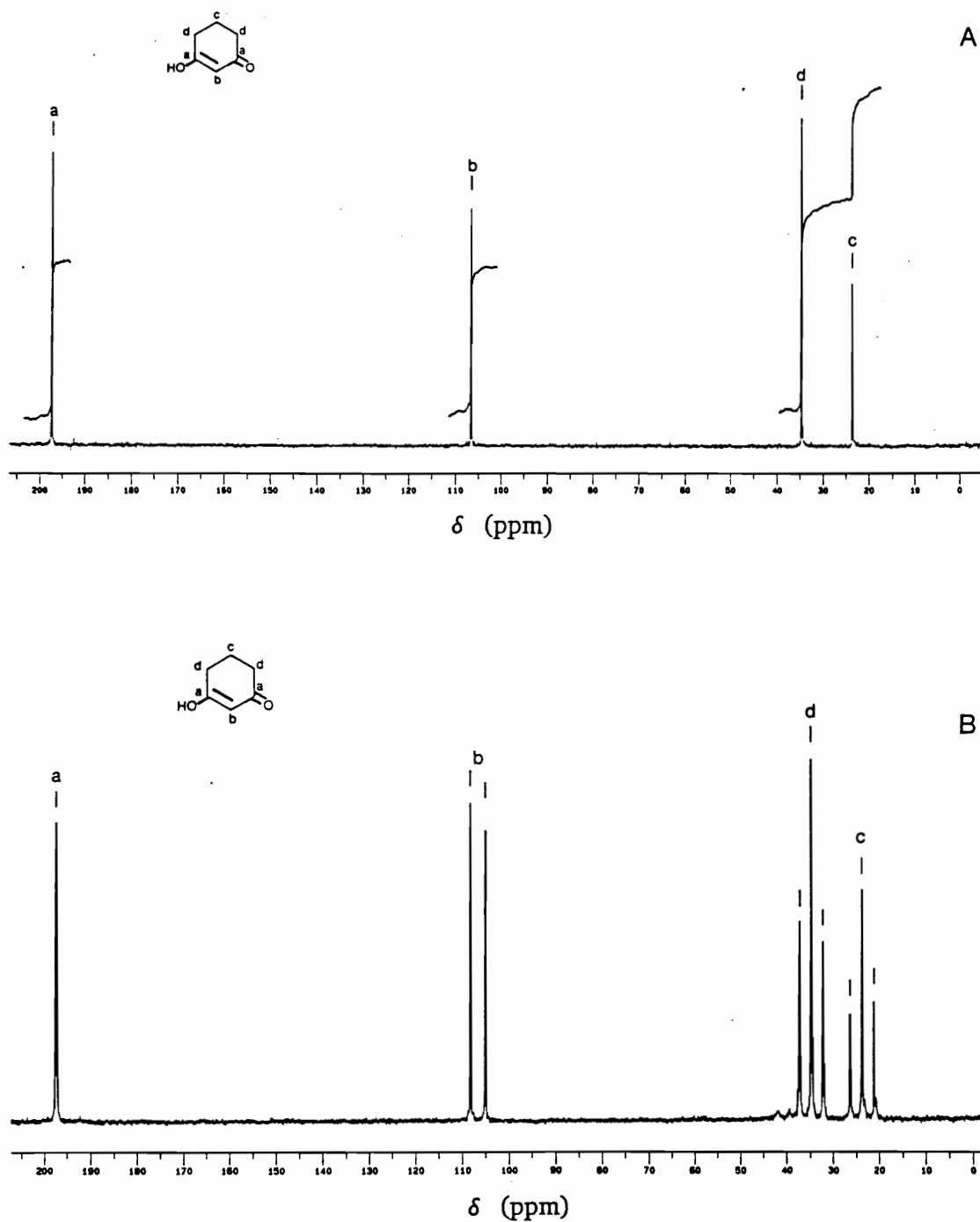


FIG. 4.  $^{13}\text{C}$  NMR spectra of dihydroresorcinol. The dihydroresorcinol concentration was  $\sim 750$  mM in  $\text{H}_2\text{O}$ , pH 2.7. A. Inverse gated spectrum with signal integration. B. Gated spectrum showing the C-H couplings.

carbon spectrum of dihydrophloroglucinol (Fig. 3A) showed approximately 2 carbons each for the carbonyl and methylene carbons and one each for the vinyl and alcoholic carbons. These results are confirmed by the spectrum in which the C-H couplings are revealed (Fig. 3B). The carbonyl carbons are not coupled to protons and have one peak, while the vinyl and alcoholic carbons have doublets which are indicative of coupling to one proton each. The methylene carbon has a triplet due to being coupled to two protons. Shifts for the carbonyl, vinyl and two of the methylene carbons of dihydroresorcinol are similar to those of dihydrophloroglucinol (Fig. 4A). An additional methylene peak is present at  $\delta = 23.6$  and the signal corresponding to the alcoholic carbon is not present. The C-H coupled spectrum of dihydroresorcinol supports these assignments (Fig. 4B).

The  $H^1$  NMR spectrum for dihydrophloroglucinol appears to show an  $A_2B_2X$  system (Fig. 5). The proton on the carbon with the hydroxyl group has a shift at  $\delta = 4.3$  and should occupy an equatorial position as shown by the coupling constants to the two neighboring equatorial methylene protons,  $J_{ax} = 4.082$  HZ, and to the two neighboring methylene axial protons,  $J_{bx} = 5.992$  HZ. The coupling constant would be expected to be in a range of 8-10 HZ for coupling between axial protons (13). The peaks at shifts of  $\delta \sim 2.65$  and  $\delta \sim 2.40$  correspond to the equatorial and axial methylene protons respectively,



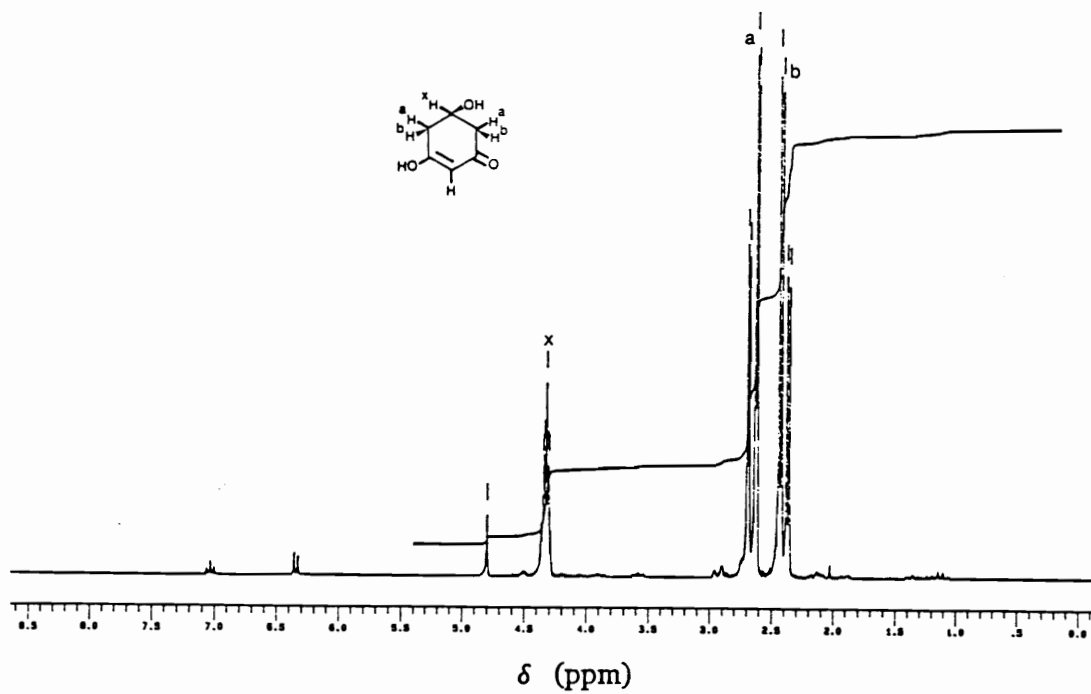


FIG. 5.  $^1\text{H}$  NMR spectrum of dihydrophloroglucinol produced by the reduction of phloroglucinol with sodium borohydride. The dihydrophloroglucinol concentration was 450 mM in  $\text{D}_2\text{O}$ , pD 2.2 (uncorrected).

which are coupled with a  $J_{ab} = 17.34$  HZ. No other proton signals are seen due to exchange with the solvent.

The hydrogenase activity of the mutant cell extract was  $36.6 \pm 1.07$  ( $X \pm SD$ )  $\mu\text{mol}/\text{min}$  per mg with NADP as the electron acceptor while dihydrophloroglucinol hydrolase activity was essentially zero (Section VII). Production of dihydrophloroglucinol by the cell extract was complete after 170 min of incubation as shown by the UV absorption spectrum and the  $^1\text{H}$  NMR spectrum which showed no peaks with shifts characteristic of aromatic protons (Fig. 6). The chemical shifts and proton-proton coupling patterns are identical to those found for the chemically synthesized compound (Fig. 5).

Dihydroresorcinol showed a competitive inhibition pattern for the oxidation of dihydrophloroglucinol by phloroglucinol reductase (Fig. 7). The calculated  $K_i$  was  $756 \mu\text{M}$  which is 2.6-fold higher than the  $K_m$  for dihydrophloroglucinol (Section VI).

## DISCUSSION

Dihydrophloroglucinol prepared by reduction of phloroglucinol with sodium borohydride has provided a source of this compound for biochemical studies. However, there is some confusion in the literature concerning its structure and reactivity. Fray (3) was the first to report a reaction between

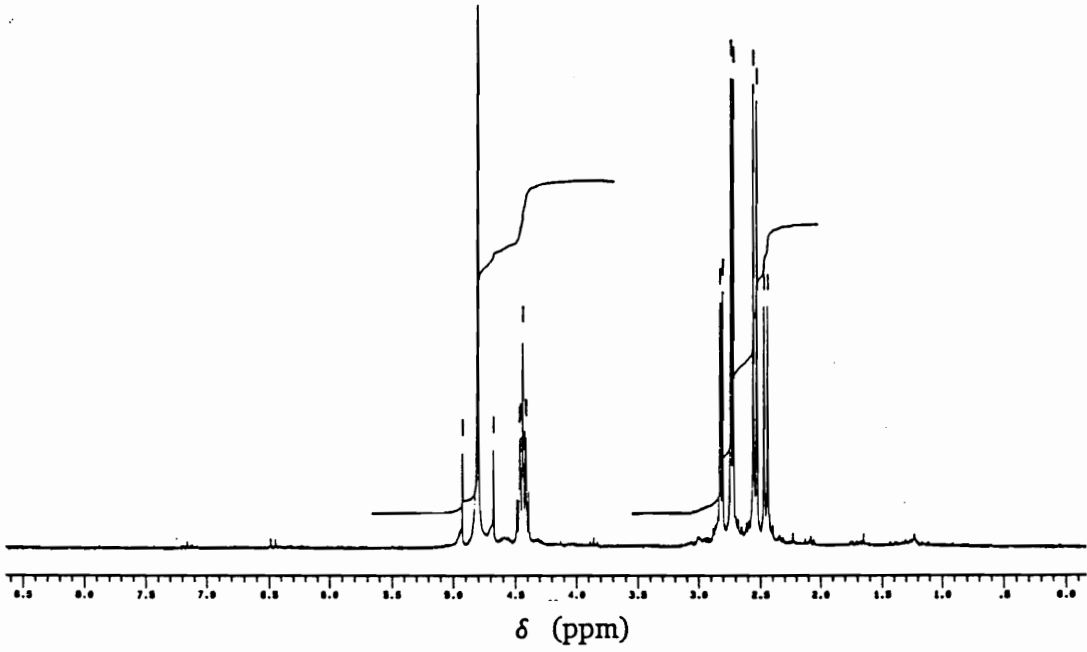


FIG. 6.  $^1\text{H}$  NMR spectrum of dihydrophloroglucinol produced by the reduction of phloroglucinol with  $\text{H}_2$  using cell extract from a mutant strain of *E. oxidoreducens* that lacks dihydrophloroglucinol hydrolase activity. The dihydrophloroglucinol concentration was 67 mM in  $\text{D}_2\text{O}$ , pD 2.2 (uncorrected).

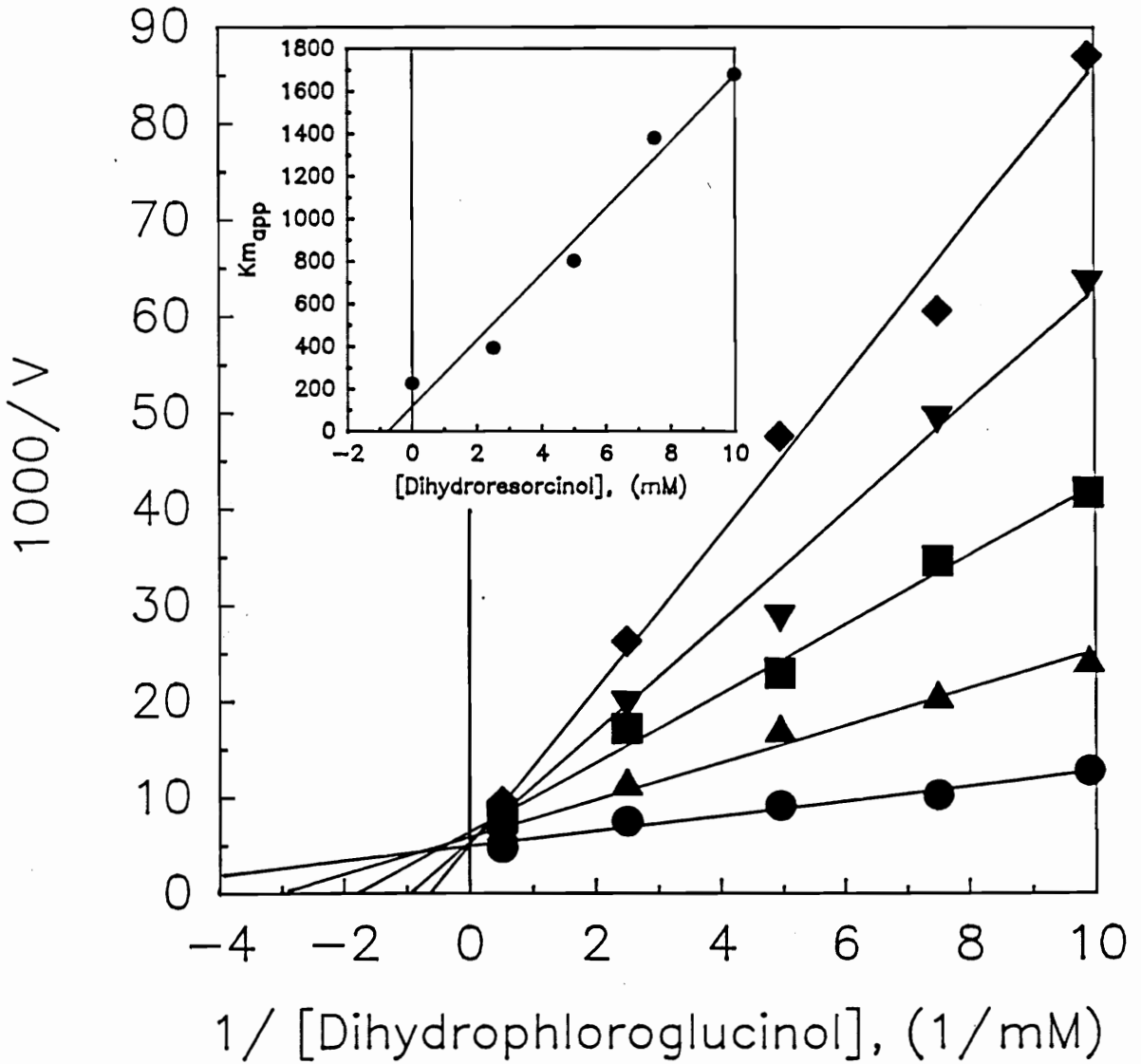


FIG. 7. Lineweaver-Burke plots of dihydrophloroglucinol oxidation by phloroglucinol reductase in the presence of various fixed concentrations of dihydroresorcinol. The dihydroresorcinol concentrations were 0 mM, ( $\bullet$ ); 2.5 mM ( $\blacktriangle$ ); 5.0 mM ( $\blacksquare$ ); 7.5 mM ( $\blacktriangledown$ ); and 10 mM ( $\blacklozenge$ ).  $V$  is  $\mu\text{mol NADPH}$  produced/min per mg protein. Inset, replot of  $K_{m_{app}}$  versus the concentration of dihydroresorcinol.

phloroglucinol and sodium borohydride. However, he isolated the reaction product after extensive reflux with benzene and resorcinol was recovered. Jamieson et al. (7) isolated a degradation product of phloroglucinol from the culture medium of a Pseudomonas sp. Mass spectral analysis gave a spectrum that resembled resorcinol which was considered to be derived from the dehydration of dihydrophloroglucinol. It is unclear if dehydration occurred during extraction and isolation or during the spectral analysis. Patel et al. (10) failed to isolate dihydrophloroglucinol using the method of Jamieson et al. (7), but did recover resorcinol. Krumholz et al. (8) stated that chemically synthesized dihydrophloroglucinol was identified by GC-MS but the data were not shown.

The results of this study show that dihydrophloroglucinol is the product of sodium borohydride reduction of phloroglucinol. The product is relatively stable in mildly acidic and neutral aqueous solutions but dehydrates to resorcinol in acidified methanol. The dihydrodiol produced during aerobic toluene degradation by Pseudomonas putida strain 39/D, also aromatizes via an acid catalyzed dehydration to form o-cresol (4). Dehydration of dihydrophloroglucinol offers an explanation for the repeated isolation of resorcinol in previous studies. Previous studies have also relied on absorbance at 278 nm as evidence for the presence of dihydrophloroglucinol. However, the

ultraviolet absorption spectrum of this compound is almost identical to that of dihydroresorcinol. Dihydrophloroglucinol shows an absorbance shift from lower to higher wavelengths with increasing pH in the interval of 4 to 5.

Dihydroresorcinol shows a similar deprotonation induced shift (13).

Dihydroresorcinol is commercially available and provides a model for the structure of dihydrophloroglucinol which can be described as 5-hydroxy-1,3-cyclohexane dione. Dihydrophloroglucinol, produced by sodium borohydride reduction, was shown to be a substrate for the reverse reaction catalyzed by phloroglucinol reductase (Section IV) and the  $^1\text{H}$  NMR spectra of enzymatically and chemically synthesized dihydrophloroglucinol were the same. Therefore both methods result in production of the same compound. Competitive inhibition by dihydroresorcinol in the reverse direction for the enzyme catalyzed oxidation of dihydrophloroglucinol to phloroglucinol probably is a result of the structural similarity of the two compounds. The additional hydroxyl group in the meta position of dihydrophloroglucinol apparently enhances binding and is probably the position in which oxidation occurs. The reaction is analogous to the oxidation of alcohols by alcohol dehydrogenases.

Phloroglucinol also ionizes in aqueous solutions and the dissociation constants for the first and second ionizations are 8.42 and 8.76 (1).

Phloroglucinol and its mono-dissociated anion show  $^1\text{H}$  NMR spectra

characteristic of aromatic compounds, while the dianion of phloroglucinol exists in the keto form (6). In the procedure described here for the synthesis of dihydrophloroglucinol, the pH of the phloroglucinol solution is ~4.5 initially, but increased to ~9.2 upon addition of sodium borohydride. When the phloroglucinol solution was buffered at pH 7.2 with 150 mM potassium phosphate, very little dihydrophloroglucinol was formed (data not shown). Addition of sodium borohydride to unbuffered aqueous solutions may increase the pH by consuming protons via reduction to hydrogen gas. This suggests that the keto form of the phloroglucinol dianion in solution is reduced by sodium borohydride in unbuffered aqueous solutions. Sodium borohydride reduction of ketones is a well-known organic reaction (9, Chapter 19). These observations and the observations that the phloroglucinol reductase  $K_m$  for phloroglucinol decreases with increasing pH while  $V_{max}$  increases with increasing pH (Section IV) suggest that the mechanism of the enzymatically catalyzed reaction involves hydride transfer from NADPH to the carbonyl carbon of the phloroglucinol dianion. Fig. 8 shows a hypothetical scheme that is consistent with these points. At physiological pH, the enzyme may induce formation of the keto form of the dianion by accepting three protons from the hydroxyl groups and donating one to form a methylene carbon. Cysteine with a  $pK_a$  of 8.37 for the sulfhydryl group and histidine with a  $pK_a$  of 6.04 for the

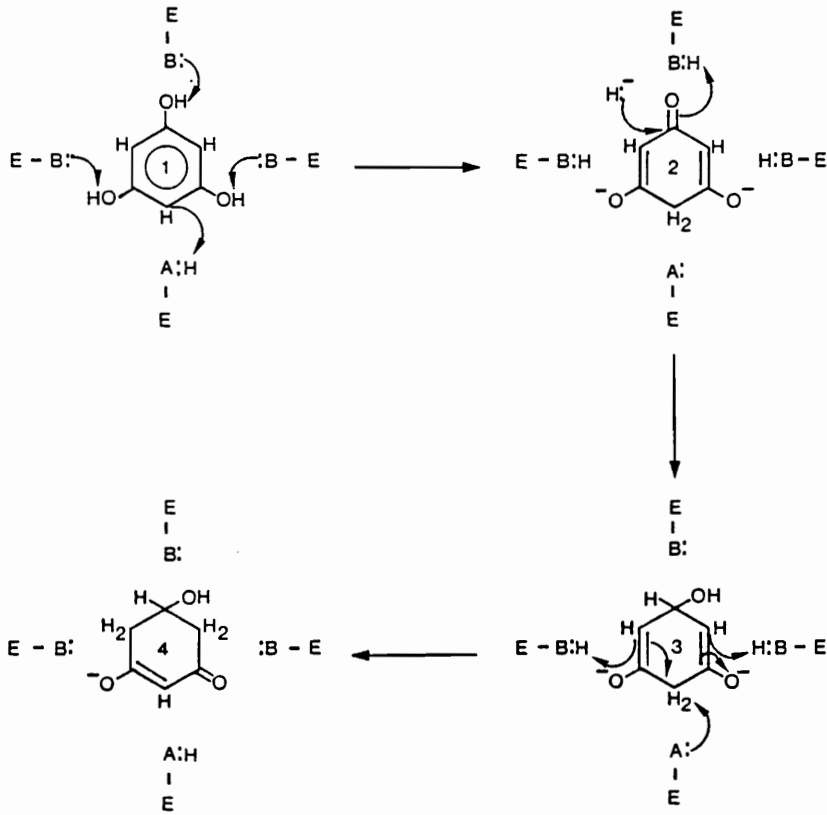


FIG. 8. Hypothetical mechanism for the reduction of phloroglucinol to dihydrophloroglucinol by phloroglucinol reductase. 1, Phloroglucinol; 2, dianion of the keto form; 3, hypothetical reduced intermediate; 4, dihydrophloroglucinol;  $E-B:$  and  $E-A:H$  basic and acidic amino acid groups respectively at the enzyme active site;  $H:-$ , hydride ion equivalent.



imidazole group (2) could serve as proton acceptors or donors for phloroglucinol ionization. Phloroglucinol reductase activity was shown to be very sensitive to certain sulfhydryl reagents such as p-chloromercuribenzoate, copper ions and n-ethylmaleimide (Section IV). Also, diethylpyrocarbonate, which reacts preferentially with histidyl residues, inhibited enzyme activity (Section IV). The amino acid compositional analysis showed that both of these amino acids were present in the enzyme (Section IV). A consequence of the proposed mechanism is a shift of two hydroxyl protons of phloroglucinol to the methylene carbons of dihydrophloroglucinol, which suggest a possible experimental test of the mechanism using isotopically labeled substrates in conjunction with NMR spectroscopy. Deuterated  $\text{NADP}^2\text{H}$  as reductant should result in deuterated dihydrophloroglucinol with a deuterium atom located on the alcoholic carbon which does not exchange with the solvent; therefore the  $^1\text{H}$  NMR spectrum of the product should not show the distinctive coupling pattern observed for dihydrophloroglucinol produced from the reduction of phloroglucinol with sodium borohydride (Fig. 5) and cell extract (Fig. 6). In addition, experiments may be devised in which the  $^1\text{H}$  NMR spectrum of the phloroglucinol dianion (13) could be observed upon binding of the substrate to the enzyme.

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SECTION VII. GENETIC ANALYSIS OF THE PATHWAY FOR ANAEROBIC  
AROMATIC DEGRADATION IN EUBACTERIUM OXIDOREDUCENS.

SUMMARY

A method for the production and isolation of mutant strains of Eubacterium oxidoreducens that are defective in the pathway for anaerobic, aromatic degradation was developed. Five mutant strains were isolated and characterized. Two strains could not grow on gallate as a substrate but could grow on pyrogallol and phloroglucinol. These strains lacked gallate decarboxylase activity and were unable to metabolize gallate during growth on crotonate. Two other strains could not grow on any aromatic substrates and lacked dihydrophloroglucinol hydrolase activity. These strains metabolized gallate to dihydrophloroglucinol and pyrogallol which were excreted into the growth medium during growth on crotonate. The activities of gallate decarboxylase, phloroglucinol reductase, dihydrophloroglucinol hydrolase and formate dehydrogenase were constitutively expressed in the wild-type strain during growth on crotonate but the transhydroxylase activity responsible for the conversion of pyrogallol to phloroglucinol was induced by growth on gallate. The conversion of pyrogallol to phloroglucinol by cell extract of E. oxidoreducens was dependent on the presence of 1,2,3,5-benzenetetrol or dimethyl sulfoxide (DMSO). DMSO was involved in the conversion of

pyrogallol to 1,2,3,5-benzenetetrol which catalyzed the conversion of pyrogallol to phloroglucinol.

## INTRODUCTION

The fate of aromatic compounds in the environment is of interest because they constitute a major fraction of the global organic carbon pool which is resistant to degradation (4). The exploitation of natural resources, industrial manufacturing processes and modern agricultural practices that rely on pesticide application have increased the levels of toxic aromatic compounds entering the environment. There is also renewed interest in the use of microorganisms for the conversion of waste biomass, such as lignin, into useful products. In relation to public health and nutrition, anaerobic metabolism in the human gut of ingested mutagenic plant phenolics such as quercetin may result in altered toxicological properties. Microbial degradation of aromatic compounds by aerobes involves oxygenase enzymes that hydroxylate and cleave the aromatic nucleus through the addition of molecular oxygen (4, 5). The biochemistry and genetics of the aerobic pathways have been extensively investigated in recent years. Degradation of aromatic compounds in the absence of oxygen has also been well documented (1, 7) and is thought to involve reduction of the aromatic nucleus as a general mechanism utilized by anaerobes that degrade

aromatic compounds (6). However, few detailed biochemical or genetic studies have been undertaken to investigate the validity of the hypothesized pathways for anaerobic aromatic degradation. Eubacterium oxidoreducens is a strict anaerobe that grows fermentatively on quercetin and the trihydroxylated aromatic monomers, gallate, pyrogallol, and phloroglucinol (15). This rumen isolate is one of only a few species obtained in pure culture that utilizes aromatic compounds as growth substrates in the absence of exogenously supplied electron acceptors (15, 20, 23). However, E. oxidoreducens is unique among these microorganisms because it requires formate or H<sub>2</sub> to supply electrons for the reductive attack on the aromatic ring. A pathway has been proposed for aromatic metabolism by this organism (16, 17). The initial transformations of the proposed pathway are shown in Fig. 1A. The pyrogallol-phloroglucinol isomerase has been partially purified and characterized (17). Brune and Schink (3) have investigated this reaction in Pelobacter acidigallici and suggest that it proceeds via transhydroxylation between the number four position of 1,2,3,5-benzenetetrol and the number five position of pyrogallol to form phloroglucinol and regenerate 1,2,3,5-benzenetetrol (Fig. 1B). Dimethyl sulfoxide (DMSO) will substitute for 1,2,3,5-benzenetetrol in the conversion of phloroglucinol to pyrogallol (3, 17) and may act as an electron acceptor for the

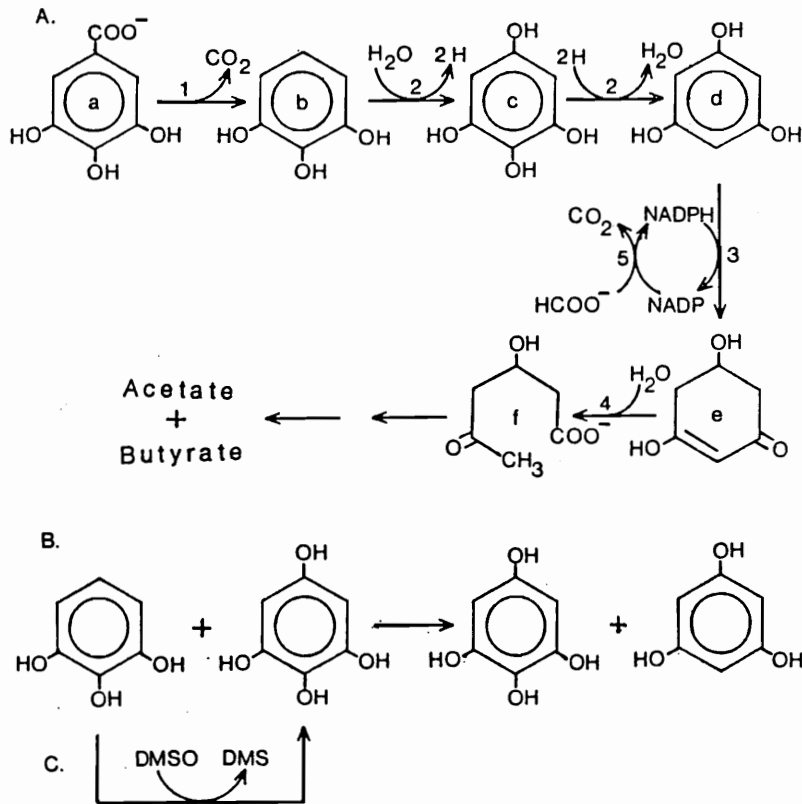


FIG. 1. Reactions that have been proposed for the initial steps for the anaerobic degradation of gallate. A, Proposed degradation pathway in *Eubacterium oxidoreducens*: a, gallate; b, pyrogallol; c, 1,2,3,5-benzenetetrol; d, phloroglucinol; e, dihydrophloroglucinol; f, 3-hydroxy-5-oxo-hexanoate; 1, gallate decarboxylase; 2, pyrogallol-phloroglucinol isomerase; 3, phloroglucinol reductase; 4, dihydrophloroglucinol hydrolase; 5, formate dehydrogenase. After Krumholz et al. (17) and Krumholz and Bryant (18). B, Reaction proposed to be catalyzed by a transhydroxylase which converts pyrogallol to phloroglucinol during gallate degradation in *Pelobacter acidigallici*. After Brune and Schink (3). C, Formation of 1,2,3,5-benzenetetrol from pyrogallol in the presence of DMSO (dimethyl sulfoxide). DMS (dimethyl sulfide). After (3, 18).

hydroxylation of pyrogallol with the formation of 1,2,3,5-benzenetetrol (Fig. 1C). Phloroglucinol reductase has been studied as a partially pure preparation from a Coprococcus sp. (19) and in cell extract from Pelobacter acidigallici (21). Phloroglucinol reductase from E. oxidoreducens has been purified to homogeneity and characterized (10). The enzyme is specific for NADPH in these organisms.

In order to validate and further characterize the proposed pathway for aromatic degradation in E. oxidoreducens and to provide strains for use in genetic studies, a method was developed for the chemical mutagenesis and isolation of strains with defects in particular biochemical steps of the pathway. This report describes the method and the characteristics of five strains that are unable to grow on gallic acid. Evidence is presented in support of the transhydroxylase reaction for pyrogallol conversion to phloroglucinol in this organism.

## MATERIALS AND METHODS

Organism and growth conditions. All procedures were performed anaerobically unless otherwise noted. The wild type strain of E. oxidoreducens G-41 was obtained from L. Krumholz and maintained on 1.5% agar slants in Balch tubes on the previously described basal medium (15) with 100 mM



crotonate as the growth substrate, 71.4 mM sodium bicarbonate and omission of rumen fluid. The gas phase was  $N_2/CO_2$ , 4/1, the pH prior to autoclaving was 7.2-7.4 and the incubation temperature was 37°C. Plating of the organism was done on approximately 20 ml of 1.5% agar media in an anaerobic chamber (Coy Products, Ann Arbor, MI) with an atmosphere of  $N_2$  and 5-10%  $H_2$  with sufficient  $CO_2$  to maintain the medium pH at about 7.8. Plates were inoculated with 0.1 ml of an appropriate dilution and spread with a bent glass rod. Inoculated plates were incubated in glass anaerobe jars (BBL, BioQuest) flushed with  $N_2/CO_2$ , 4/1 and 2 ml of 2.5%  $Na_2S \cdot 9H_2O$  were injected through a septum into an open petri plate in the jar prior to incubation at 37°C. Plates were examined for growth after 3-5 days. Cells for cell extract production were grown in 0.5 L nephelometer flasks containing 0.25 L of the basal medium and 100 mM crotonate or 100 mM crotonate plus 10 mM each gallate and formate. In addition, the wild type strain was grown in a 10 L fermentor on 50 mM each gallate and formate as previously described (10). Isolated mutant strains were tested for the ability to grow in 10 ml of liquid media on 20 mM each gallate plus formate, 5 or 10 mM each pyrogallol plus formate, or 10 mM each phloroglucinol plus formate. The strains were grown on 10 ml of 100 mM crotonate plus 15 mM each gallate plus formate to test for accumulation of intermediates. Mutant strains were maintained on agar slants of the basal

medium with 100 mM crotonate as substrate. Isolated strains were stored at -70°C in the basal medium amended with 25% glycerol.

Cell extract preparation. Cells were harvested from 250 ml cultures at the end of logarithmic growth by centrifugation at 7000 x g for 30 minutes, washed with 37 ml of anaerobic buffer containing 50 mM potassium phosphate and 2 mM 2-mercaptoethanol and recentrifuged at 12,100 x g for 15 minutes. The pellet was resuspended in 5 ml of the wash buffer, loaded into a French pressure cell inside a glove bag, and broken as previously described (10). The crude cell extract was collected in an anaerobic centrifuge tube and centrifuged at 30,000 x g for 30 min prior to storage of the supernatant in liquid nitrogen. The protein concentration of the extracts ranged from 2.2 to 4.0 mg/ml.

Enzyme assays. All assays were performed anaerobically at 37°C under N<sub>2</sub>, except phloroglucinol reductase assays which were done aerobically. The buffer used to prepare cell extract was also used for all enzyme assays. Spectrophotometric assays were performed on a Perkin Elmer model 552 instrument. The reaction volume was 1.0 - 1.1 ml unless otherwise noted.

Formate dehydrogenase activity was measured spectrophotometrically by following the appearance of NADPH at 340 nm ( $\epsilon = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$ ). The initial concentrations of sodium formate and NADP were 2.0 and 0.2 mM respectively and 10  $\mu\text{l}$  of cell extract were added to initiate the reaction.

Gallate decarboxylase activity was measured spectrophotometrically by following the disappearance of sodium gallate at 259 nm (16). The initial gallate concentration was 0.1 mM and a molar absorbance value of 9,000 was determined and used for rate calculations. Five to 20  $\mu$ l of cell extract were added to initiate the assay.

The standard assay for the determination of transhydroxylase activity (conversion of pyrogallol to phloroglucinol) was done by following the disappearance of pyrogallol in assay buffer containing 10% (v/v) DMSO (17). The reaction was initiated by addition of 100  $\mu$ l of cell extract to a 2.0 ml serum vial containing 1.0 ml of assay buffer and 5.5 mM pyrogallol. Samples (50  $\mu$ l) were removed every 5 min to 10 min for 30 min to 1 hour, and placed on ice in an aerobic glass test tube containing 5  $\mu$ l of 3N HCl. Four ml of molybdate reagent (21) were added and incubated 1 hour at room temperature prior to spectrophotometric measurement of absorbance at 352 nm. This reagent is specific for pyrogallol because it reacts with vicinal hydroxyl groups. A standard curve was prepared to convert absorbance values to pyrogallol concentration. In some experiments, 1,2,3,5-benzenetetrol replaced DMSO in the assay buffer, and pyrogallol, phloroglucinol and 1,2,3,5-benzenetetrol were quantitated by HPLC. Cell extract, 0.1 to 0.2 ml, was incubated at 37°C in serum vials containing 2.5 ml of anaerobic buffer with 5 mM pyrogallol and

either 10% (v/v) DMSO or 1.0 mM 1,2,3,5-benzenetetrol. Samples were periodically injected into anaerobic vials, acidified with concentrated  $\text{H}_2\text{SO}_4$  to a final concentration of 1% (v/v) and frozen. Just prior to analysis the samples were thawed and centrifuged at  $16,000 \times g$  to remove precipitated protein and 20  $\mu\text{l}$  of the supernatant were analyzed by HPLC.

Phloroglucinol reductase activity was determined as previously described (10) using a molar absorbance value of  $6,220 \text{ M}^{-1} \text{ cm}^{-1}$  for NADPH. One  $\mu\text{l}$  of cell extract was assayed and phloroglucinol was added to initiate the reaction.

Dihydrophloroglucinol hydrolase activity was determined spectrophotometrically by measuring the disappearance of dihydrophloroglucinol at 305 nm. A molar absorbance of  $640.2 \text{ M}^{-1} \text{ cm}^{-1}$  was determined and used for rate calculations. Either 25 or 50  $\mu\text{l}$  of cell extract were added to initiate the reaction.

EMS killing curve. Tubes of cells growing on 10 ml of 60 mM crotonate medium at a culture absorbance of about 0.5 (520 nm, 1.5 cm path length) were injected with ethylmethane sulfonate (EMS) to a final concentration of 1.5% (v/v). The tubes were gently rocked at  $37^\circ\text{C}$  and periodically sampled for plating on crotonate medium. The plates were incubated for 5 days and the colonies were counted.

Ampicillin inhibition. Susceptibility to growth inhibition by ampicillin was determined by inoculating 10 ml of medium containing 100 mM crotonate or 20 mM each gallate plus formate and containing 0, 5, 50, 100 or 250  $\mu\text{g/ml}$  of filter sterilized ampicillin. The tubes were incubated statically at 37°C and growth was followed by measuring the culture absorbance as described above.

Mutagenesis and mutant isolation. Two tubes holding 10 ml each of medium containing 100 mM crotonate were inoculated with 1 ml of an overnight grown culture of *E. oxidoreducens* and incubated without shaking until the culture absorbance equaled 0.5. Ethylmethane sulfonate was added to a final concentration of 1.5% (v/v) and the tubes were gently rocked at 37°C for fifteen minutes. The cells were then taken into a glove bag and filtered through a sterile 0.22  $\mu\text{m}$  membrane filter, resuspended in 10 ml of dilution blank medium (13) and refiltered. The washing and filtration procedure was repeated and the cells were resuspended in 5 ml of the basal medium containing no growth substrates. Two ml each of the resuspended cells were inoculated into 2 tubes containing 10 ml each of crotonate medium and incubated until the culture absorbance doubled to allow for growth recovery and segregation of the chromosomes. The cultures were pooled, filtered, and washed 2 x as described above and finally resuspended in 5 ml of the no-substrate medium. One ml each of the resuspended cells were inoculated into

4 x 10 ml of gallate plus formate medium (20 mM each) and incubated for one doubling of the culture absorbance. Filter sterilized ampicillin was added to a final concentration of 100  $\mu$ g/ml and the tubes were incubated for 6 hours. The cultures were pooled, filtered, washed 2 x and resuspended as described above and 2 ml each of the resuspended cells were inoculated into 2 x 10 ml of crotonate medium and incubated until the culture absorbance was 0.5. The cells were then diluted and spread on plates containing 100 mM crotonate medium.

Screening and isolation of mutants. After four days incubation at 37°C in anaerobe jars the plates were taken into a glove bag. Fifty individual colonies per plate were picked with sterile tooth picks and stabbed in a grid pattern into agar medium containing 20 mM each gallate plus formate. All plates were then incubated for three days after which the gallate plus formate plates were examined for growth. Colonies that failed to grow were picked from the original crotonate plates with sterile pasteur pipets, inoculated into 10 ml each of crotonate medium and incubated until turbid. The cultures were then diluted and plated onto crotonate plates and the entire screening procedure was repeated. This procedure was repeated for a third time and the isolates were cultured for further study.

High pressure liquid chromatography. Samples of culture or assay buffer were sampled anaerobically, injected into N<sub>2</sub> purged serum vials, acidified with 1% (v/v) concentrated H<sub>2</sub>SO<sub>4</sub>, and frozen until analysis. Thawed samples were centrifuged at 16,000 x g to remove precipitated protein and 20 µl of the supernatant were chromatographed on a 25 cm x 4.6 mm i.d. C<sub>18</sub> reverse phase column (Supelco, Bellefonte, PA). The mobile phase was 90% 0.02 N H<sub>2</sub>SO<sub>4</sub>-10% methanol at a flow rate of 0.8 ml/min. The detector wavelength was 268 nm. When necessary samples were diluted with 0.02 N H<sub>2</sub>SO<sub>4</sub> prior to analysis.

Protein. Protein concentration was determined by the method of Bradford (2) with bovine serum albumin as the standard.

Chemicals. Gallic acid and pyrogallol were purchased from the Aldrich Chemical Co., Milwaukee, WI. Phloroglucinol dihydrate was purchased from the Chemical Dynamics Corp., S. Plainfield, NJ. Ethylmethane sulfonate was purchased from the Sigma Chemical Co., St. Louis, MO. 1,2,3,5-benzenetetrol was a gift from A. Brune. Dihydrophloroglucinol was synthesized by reduction of phloroglucinol with sodium borohydride (19), acidified to pH 1-2 with HCl and extracted into ethylacetate. Ethylacetate was removed at room temperature on a rotary evaporator and dihydrophloroglucinol was recovered by redissolving the residue in deionized water.

## RESULTS

Plating efficiency and antibiotic susceptibility. In order to obtain mutant strains it was necessary to develop a method for reproducible plating of the organism. A correlation curve relating culture absorbance versus cfu showed that a net  $A_{520\text{nm}}$  of 0.5 resulted in approximately  $8.3 \times 10^8$  cfu/ml. Exposure to 1.5% (v/v) EMS for 15 minutes resulted in approximately 50% killing. Inhibition of growth by ampicillin in broth cultures was affected by the type of growth substrates in the medium (Fig. 2A and 2B). Five  $\mu\text{g/ml}$  ampicillin did not affect growth while concentrations of 50  $\mu\text{g/ml}$  or greater inhibited growth. However, when gallate plus formate were the growth substrates, inhibition of growth by ampicillin at concentrations of 50 to 250  $\mu\text{g/ml}$  was overcome after incubation for 8 to 12 hours. With crotonate as the substrate, inhibition by ampicillin was not overcome. Additional experiments showed that a second addition of ampicillin about eight hours after inoculation into medium containing gallate plus formate continued to prevent growth (unpublished results), ruling out the possibility that growth was due to the selection of antibiotic resistant mutants. Gallate may react slowly with ampicillin making it inactive as an antibiotic.

Mutant isolation. Of approximately 1900 colonies randomly picked from plates containing crotonate as the growth substrate, 19 did not grow when



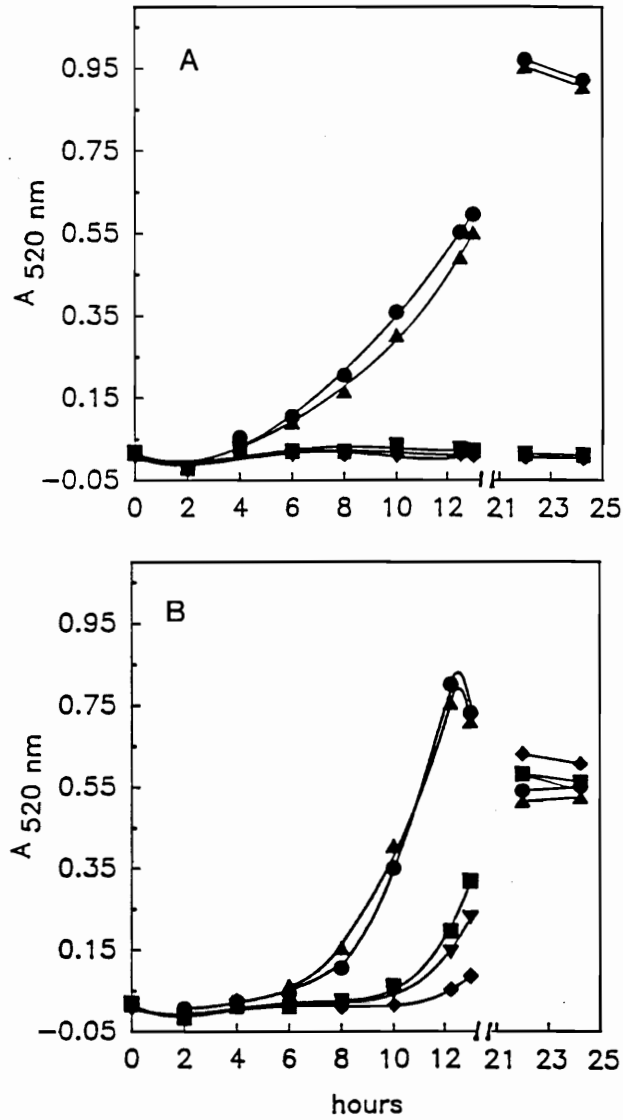


FIG. 2. Effect of ampicillin concentration on growth of *E. oxidoreducens*. A. Growth on 100 mM crotonate. B. Growth on 20 mM each gallate plus formate. The ampicillin concentrations were 0  $\mu\text{g/ml}$ , ● ; 5  $\mu\text{g/ml}$ , ▲ ; 50  $\mu\text{g/ml}$ , ■ ; 100  $\mu\text{g/ml}$ , ▼ ; 250  $\mu\text{g/ml}$  ◆ .

placed on plates containing gallate plus formate. Twelve of these were rescreened which yielded five strains unable to grow on gallate which were further characterized by: i) the ability to grow on aromatic substrates plus formate, ii) accumulation of aromatic intermediates while growing on crotonate in the presence of gallate plus formate, and iii) enzyme assays.

Growth on aromatic intermediates. The inoculum was 10% (v/v) for all strains and came from log phase cultures with crotonate as the growth substrate. The wild type strain grew on gallate, pyrogallol and phloroglucinol with formate in the medium as previously reported (15), however, it failed to grow on dihydrophloroglucinol. Therefore, the mutant strains were not tested for growth on this intermediate. Krumholz and Bryant (15) noted some inhibition of *E. oxidoreducens* growth when the pyrogallol concentration was 10 mM or greater. Growth of the wild type strain lagged approximately 5 hours on 20 mM gallate plus formate and 10-15 hours on 10 mM pyrogallol plus formate. No lag was seen for growth on 10 mM phloroglucinol plus formate. Relative to the wild type strain, all of the mutant strains failed to grow on gallate plus formate (Table 1), while two strains grew on pyrogallol plus formate and phloroglucinol plus formate. Strain C15-41-23 grew on 5 mM but not 10 mM pyrogallol. Three other strains did not readily grow on any of the aromatic substrates when compared to the wild type strain. Eventually,

Table 1. Growth of the wild type and mutant strains in broth culture on aromatic substrates plus an equimolar concentration of formate.

Strain	Growth <sup>a</sup> Substrates		
	Gallate <sup>b</sup>	Pyrogallol <sup>c</sup>	Phloroglucinol <sup>c</sup>
Wild type	+	+	+
C14-17-16	-	+	+
C15-41-23	-	+ <sup>d</sup>	+
C21-21-7	-	-	-
C15-45-35	-	-	-
C6-24-18	-	-	-

<sup>a</sup> +, increase in culture absorbance > twice that of inoculated control media containing no growth substrates; -, no increase in culture absorbance above the control.

<sup>b</sup> 20 mM.

<sup>c</sup> 10 mM unless noted otherwise.

<sup>d</sup> 5mM.

however, after incubation of the mutant strains on aromatic substrates for greater than 30 hours, growth did occur, suggesting the presence of a population of revertants in the cultures. To test this hypothesis, mutant strain C6-24-18 was inoculated into media containing either gallate plus formate or phloroglucinol plus formate and incubated for a prolonged period until growth was detected. These cultures were then used as inocula for fresh media containing aromatic substrates with formate. The cultures showed the wild type growth pattern described above indicating that revertants had been selected for and had become enriched upon prolonged incubation in the presence of aromatic substrates.

Growth of the wild type and mutant strains on crotonate plus gallate and formate is shown in Fig. 3. Strains C21-21-7 and C15-45-35 grew slowly with generation times of 6.5 and 13.2 hours respectively and produced low final culture densities. Strains C6-24-18, C14-17-16 and C15-41-23 had intermediate growth rates with respective generation times of 3.4, 3.8 and 4.8 hours and reached final culture densities similar to that of the wild type. The wild type had a generation time of 1.7 hours which is similar to the value of 1.5 hours calculated from the data from Fig. 2B for the wild type when grown on gallate plus formate only. The generation time for the wild type strain growing on crotonate was 2.2 hours (Fig. 2A).

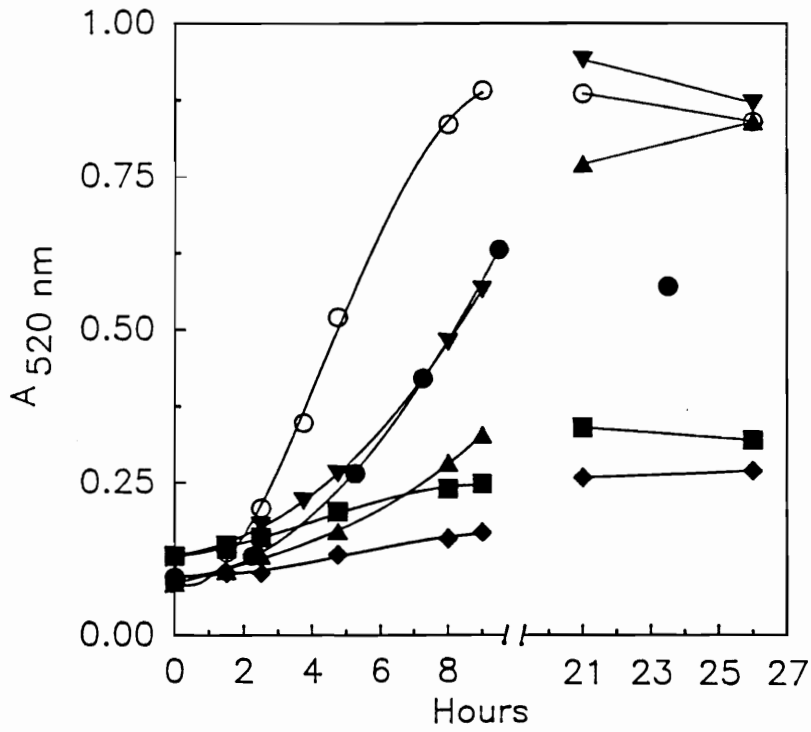


FIG. 3. Growth of wild type and mutant strains of *E. oxidoreducens*. Growth on 100 mM crotonate, 15 mM gallate, and 15 mM formate. Wild type, ○. Mutant strains: C14-17-16, ▼; C21-21-7, ■; C15-45-35, ◆; C15-41-23, ▲; C6-24-18, ●.

Accumulation of intermediates. Fig. 4 shows the results of HPLC analysis of the spent media after growth had ceased in the cultures shown in Fig. 3. As noted by Krumholz and Bryant (15), uninoculated media contained a small amount of pyrogallol (0.42 mM) due to gallate decarboxylation that occurred during sterilization. The wild type strain metabolized all of the gallate and pyrogallol and accumulated phloroglucinol to a concentration of 2.9 mM. Sampling of the cultures at earlier time points showed the same pattern of accumulation for all strains except the wild type (data not shown). In the latter case, pyrogallol but no phloroglucinol was detected while the culture was actively growing. The accumulation of phloroglucinol after growth had ceased was probably due to insufficient formate as a source of electrons for phloroglucinol reduction. Some of the reducing potential may have been diverted away from phloroglucinol reduction to biosynthesis during growth. Strains C14-17-16 (and C15-41-23, not shown) did not metabolize gallate and no intermediates, including the pyrogallol that was initially present, were detected. Strain C6-24-18 metabolized some of the gallate and accumulated pyrogallol. Strains C21-21-7 (and C15-45-35, not shown) metabolized less of the gallate and accumulated pyrogallol and dihydrophloroglucinol. Overall recoveries of gallate plus the detected intermediates in spent media after growth of the mutant strains was >90%.

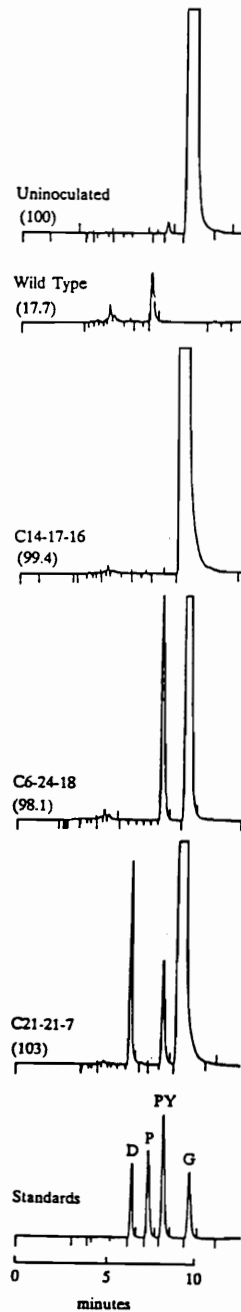


FIG. 4. Analysis by HPLC of spent media after growth of mutant and wild type strains of *E. oxidoreducens*. Grown on 100 mM crotonate and 15 mM each gallate plus formate. Samples were withdrawn after approximately 24 hours of growth from the cultures shown in Fig. 3. Recovery of gallate and its metabolites is shown in parentheses and is expressed as a percentage of the initial concentration. D, 0.15 mM dihydrophloroglucinol; P, 5.0 mM phloroglucinol; PY, 5.0 mM pyrogallol; G, 0.21 mM gallic acid.

Enzyme activities. Results of assays of cell extract for the enzymes known to be involved in the initial reactions of the proposed pathway are shown in Table 2. Formate dehydrogenase activity was found in all strains. Much of the variability among the strains was probably due to the extreme oxygen sensitivity of this enzyme (Section II). Gallate decarboxylase activity in cell extract from strain C14-17-16 and C15-41-23 was not detected. However, when cell extract from the wild type strain was added to the assay cuvetts for these strains decarboxylase activity was found at the expected rate, ruling out the possibility that unfavorable assay conditions caused the failure to detect activity. No decarboxylase activity was detected when extracts from the two strains were added to the same assay cuvet. Phloroglucinol reductase activity was detected in all strains, with strains C14-17-16 and C6-24-18 having relatively lower levels of activity. This enzyme is not oxygen sensitive. Dihydrophloroglucinol hydrolase activity was barely detectable in strains C21-21-7 and C15-45-35. Activity in cell extract of the wild type was four-fold higher for cells grown on crotonate plus gallate and formate than for crotonate grown cells. Transhydroxylase activity appeared to be inducible and was barely detectable in wild type or mutant strains grown on crotonate only. This enzyme is not oxygen sensitive, (3, 17). Strains C21-21-7 and C6-24-18 had



Table 2. Enzyme activities of cell extracts from the wild type and mutant strains of *E. oxidoreducens*.

Strain	Growth Substr.*	Dehydro- genase	Decarboxylase	Trans- hydroxylase <sup>c</sup>	Reductase	Hydrolase
Wild type	G,F	1.55 ± .11	0.203 ± .005	0.78 ± .01	11.6 ± .40	0.93 ± .06
Wild type	C,G,F	0.36 ± .02	0.032 ± .002	0.29 ± .01	15.4 ± .40	2.51 ± .01
C14-17-16	C,G,F	1.46 ± .06	< .004	0.37 ± .01	2.71 ± .28	0.49 ± .03
C21-21-7	C,G,F	1.76 ± .06	0.036 ± .003	1.29 ± .05	18.7 ± .92	0.01 ± .01
C6-24-18	C,G,F	0.56 ± .04	0.059 ± .005	1.17 ± .03	3.59 ± .06	0.59 ± .05
Wild type	C	0.42 ± .02	0.110 ± .016	< .02	11.3 ± .76	0.62 ± .08
C15-41-23 <sup>b</sup>	C	0.52 ± .00	< .004	0.02 ± .01	9.02 ± .38	0.89 ± .04
C15-45-35 <sup>b</sup>	C	1.01 ± .07	0.077 ± .002	0.05 ± .01	7.52 ± .14	0.01 ± .01

Values are the mean ± 1 SD, n=3, expressed as μmol/min/mg protein.

\* C, 100 mM crotonate; G, 10 mM gallate; F, 10 mM formate.

<sup>b</sup> Growth was insufficient to provide cell mass for cell extract preparation when grown on C,G,F containing medium.

<sup>c</sup> Activity was determined with the standard spectrophotometric assay as described in Materials and Methods.

the highest activities yet accumulated pyrogallol in the medium to concentrations of 4.3 and 10.0 mM respectively.

In order to determine if the low transhydroxylase activity in extract from crotonate grown cells was due to the absence of the necessary enzymes, cell extract was incubated with pyrogallol plus DMSO as well as pyrogallol plus 1,2,3,5-benzenetetrol. The presence of either DMSO or the 1,2,3,5-benzenetetrol have been shown to be required for the conversion of pyrogallol to phloroglucinol in cell extracts of *P. acidigallici* (3) and *E. oxidoreducens* (17). Cell extracts of the wild type strain grown on 50 mM each gallate plus formate had no detectable transhydroxylase activity when incubated with pyrogallol alone (data not shown). When 1.0 mM benzenetetrol was present in the assay, pyrogallol was immediately converted to phloroglucinol, which accumulated at a rate of 0.91  $\mu\text{mol}/\text{min}$  per mg protein (Fig. 5A). When 10 % (v/v) DMSO was present, 1,2,3,5-benzenetetrol was synthesized at an initial rate of 0.46  $\mu\text{mol}/\text{min}$  per mg, while the rate of phloroglucinol formation was initially low but increased to 0.93  $\mu\text{mol}/\text{min}$  per mg after 10 minutes when the benzenetetrol concentration had reached approximately 1.3 mM (Fig. 5B). These results indicate that 1,2,3,5-benzenetetrol is a co-substrate for the transhydroxylase reaction and that DMSO is involved in the formation of benzenetetrol as suggested for *P. acidigallici* by Brune and Schink (Fig. 1C).

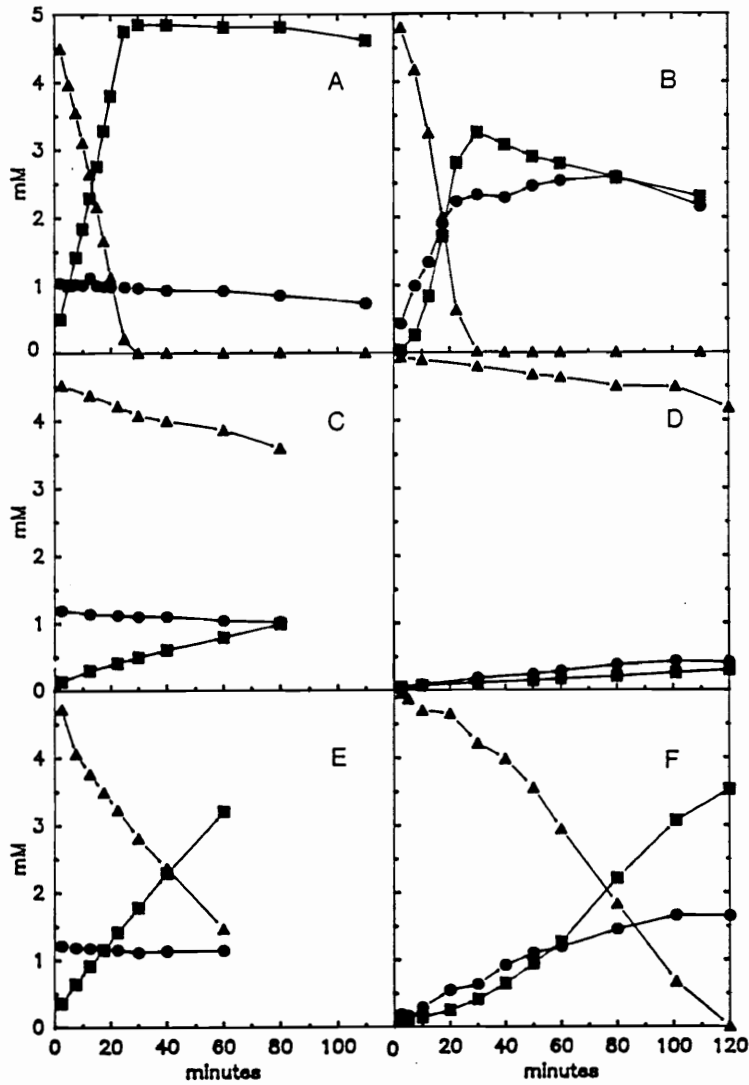


FIG. 5. Conversion of pyrogallol to phloroglucinol and 1,2,3,5-benzenetetrrol by cell extract from the wild type strain of *E. oxidoreducens*. Phloroglucinol, (■); pyrogallol, (▲). The cells were grown on: A and B, 50 mM each gallate plus formate; C and D, 100 mM crotonate; E and F, 100 mM crotonate plus 10 mM each gallate and formate. A, C, and E, with 1 mM 1,2,3,5-benzenetetrrol (●); B, D, and F, with 10% (v/v) DMSO. The cell extract protein concentrations were 0.21 mg/ml for A, B, C and D and 0.36 mg/ml for E and F.

These authors found the highest rates for transhydroxylase activity in *P. acidigallici* when the benzenetetrol concentration was 1.5 mM, consistent with the present results for *E. oxidoreducens*. When the same assays were performed with extract from the wild type strain grown on crotonate only, phloroglucinol accumulated at a rate of 0.050  $\mu\text{mol}/\text{min}$  per mg protein in the presence of 1,2,3,5-benzenetetrol (Fig. 5C), which was 18-fold lower than the value for cells grown on gallate plus formate. These results suggest regulation of transhydroxylase activity by the growth substrate. With DMSO present in the assay, the rate of phloroglucinol accumulation was 0.0093  $\mu\text{mol}/\text{min}$  per mg protein (Fig. 5D), which was 5-fold lower than the value with 1,2,3,5-benzenetetrol present. This lowered rate was probably due to the low rate of formation of 1,2,3,5-benzenetetrol required for the transhydroxylase reaction. When assays were performed with cell extract of the wild type strain grown on crotonate plus gallate and formate, the rate of phloroglucinol formation in the presence of 1,2,3,5-benzenetetrol was 0.14  $\mu\text{mol}/\text{min}$  per mg protein (Fig. 5E). With DMSO the rate was 0.12  $\mu\text{mol}/\text{min}$  per mg after the 1,2,3,5-benzenetetrol concentration reached about 1.3 mM (Fig. 5F). These activities were 7 to 8-fold less than the activity for extracts from cells grown with gallate plus formate only. These results further support that transhydroxylase activity is regulated by the growth substrate, but repression by crotonate or induction by

aromatic substrates cannot be distinguished with these data. These results do not indicate whether or not phloroglucinol and 1,2,3,5-benzenetetrol are produced by the same enzyme. Both activities were unaffected when cell extract was exposed to air for 30 min prior to assay. Krumholz and Bryant (17) partially purified an enzyme from E. oxidoreducens that catalyzed the conversion of pyrogallol to phloroglucinol in the presence of DMSO or 1,2,3,5-benzenetetrol. Further studies with the purified enzyme or with appropriate mutants are needed to determine if both activities are catalyzed by the same enzyme.

The function of DMSO in the formation of 1,2,3,5-benzenetetrol from pyrogallol is not known but it may act as a non-physiological electron acceptor (3). Nicotinic acid hydroxylase purified from Clostridium barkeri was specific for NADP as the electron acceptor for the conversion of nicotinic acid to 6-hydroxynicotinic acid (13). Attempts to substitute other potential electron acceptors in place of DMSO or 1,2,3,5-benzenetetrol in assays with E. oxidoreducens cell extract were unsuccessful. Neither NAD (1.0 mM), NADP (0.25 mM), phloroglucinol (2.0 mM) nor 2,3,5-triphenyltetrazolium chloride (1.0 mM) functioned in the place of DMSO for formation of 1,2,3,5-benzenetetrol from pyrogallol. Phenazinemethosulfate and methylene blue were

rapidly reduced by pyrogallol in the absence of cell extract and the rate was not accelerated when cell extract was added.

Cell extract from strain C6-24-18 grown on crotonate in the presence of gallate and formate catalyzed the conversion of pyrogallol to phloroglucinol, in the presence of DMSO, at rates similar to those found for the wild type strain grown on gallate plus formate. However, strain C6-24-18 accumulated pyrogallol to a concentration of 10 mM when grown on crotonate plus gallate and formate. This result seems to indicate that the physiological analog of DMSO is not functional in this strain. If the physiological electron acceptor for 1,2,3,5-benzenetetrol formation was deficient during growth, conversion of pyrogallol to phloroglucinol in cell extract with added DMSO or 1,2,3,5-benzenetetrol would be observed at the expected rates. However, the failure of this strain to grow on phloroglucinol as well as pyrogallol and gallate suggests that this strain is blocked at a step farther along in the pathway. Additional studies with this strain are needed to clarify these results.

## DISCUSSION

Difficulties with the growth of strict anaerobes on solid media has retarded efforts to study the genetics of these organisms. E. oxidoreducens

gave consistently high plating efficiencies when the procedures were done in an anaerobic glove bag and when incubated in anaerobe jars with sulfide added to ensure reducing conditions. The rapid growth rate and high cell yield have also enhanced the study of the degradative pathway for aromatic compounds in this organism.

The use of EMS to induce specific mutations in the pathway for gallic acid degradation combined with ampicillin enrichment for the mutants was an effective method for the production and isolation of mutant strains. The screening procedure was effective for the identification of mutant strains and resulted in the selection of few false positives.

Strains C14-17-16 and C15-41-23 appear to have a defect in gallate decarboxylase because i) enzyme activity was not detected in these strains; ii) gallate was not degraded during growth on crotonate; and iii) they failed to grow on gallate but were capable of growth on phloroglucinol and pyrogallol. It is unlikely that both strains are identical, owing to the low probability of isolating the same strain more than once following EMS mutagenesis (18) and the minimal growth that was allowed to occur between treatment with the mutagen and inoculation onto solid media. No decarboxylase activity was detected when cell extracts from the two strains were mixed.

Strains C21-21-7 and C15-45-35 appear to be defective in dihydrophloroglucinol hydrolase, the ring cleavage enzyme. The two strains were isolated during separate experiments and are not derived from the same mutagenic event. Activity was not reconstituted when cell extracts from both strains were mixed. These strains failed to grow on any of the aromatic substrates, which confirms the necessity of this enzyme in the pathway. Accumulation of intermediates during growth on crotonate plus gallate and formate confirms that pyrogallol and dihydrophloroglucinol are metabolites of gallate degradation. The stoichiometric recovery of gallate and its intermediates rules out major alternative pathways for the metabolism of these compounds. Phloroglucinol was not detected and if present was below 25-50  $\mu$ M. Winter et al. (6) suggested that phloroglucinol is complexed by media components; however, we found a 95.4% recovery of phloroglucinol from uninoculated media incubated at 37°C for 28 hours. Phloroglucinol was detectable in spent media after growth of the wild type strain on crotonate and gallate with no added formate (unpublished results, also see ref 16). These results suggest that the reversible reductase step (10) is driven to near completion in the forward direction with formate present by the reductive pressure placed on the reaction by formate dehydrogenase (also see Section V).



No specific defect for strain C6-24-18 could be shown. Accumulation of a high concentration of pyrogallol during growth on crotonate plus gallate and formate is consistent with a defect in the transhydroxylase reaction; however, strain C6-24-18 had significant levels of the transhydroxylase and all other enzymes tested. This strain did express 1,2,3,5-benzenetetrol forming activity as shown by HPLC analysis of cell extracts incubated with pyrogallol and DMSO (unpublished results). The use of 10% DMSO in the transhydroxylase assay is artificial; thus, the enzyme may not be active in this strain if the physiological counterpart to DMSO is not present. In that case, strain C6-24-18 should have grown on phloroglucinol, the next intermediate in the proposed pathway; however, the mutant failed to grow on this substrate. A likely explanation for these results is that this strain has a defective enzyme in the pathway after the hydrolysis of dihydrophloroglucinol. The accumulation of pyrogallol may indicate a link between the transhydroxylase reaction and another essential reaction involved later on in the pathway.

Expression of the transhydroxylase activity was regulated in response to the growth substrate. All of the enzymes except for the transhydroxylase were constitutively expressed in the wild type strain during growth on crotonate (Table 2 and ref 16). Regulation of transhydroxylase activity has not been previously reported. Activity was very low when wild type cells were grown on

crotonate only, but activity was 7-fold greater in extracts of cells grown on crotonate plus gallate and formate and 18-fold greater in extracts of cells grown on gallate plus formate alone. It is possible that formate (or  $H_2$ ) is involved in regulation of the transhydroxylase since neither was added to crotonate media. Induction by the electron donor required for ring reduction would ensure that sufficient reducing potential was available prior to conversion of pyrogallol to phloroglucinol by the transhydroxylase. Otherwise reduction of pyrogallol to phloroglucinol in the absence of hydrogen or formate as a source of electrons would result in the depletion of NADPH which is needed for biosynthesis. Concentrations of formate and hydrogen are maintained at very low levels in the rumen due to competition for these substrates by methanogens and other anaerobes (14). Therefore regulation of the transhydroxylase activity in E. oxidoreducens may be involved in maintaining the redox balance of the cell.

Guyer and Hegeman (9) used nitrosoguanidine to obtain several classes of Rhodopseudomonas palustris mutants that were unable to grow anaerobically on benzoate. Characterization of the mutants provided some of the first evidence for the reductive pathway of anaerobic aromatic degradation. Additional studies with mutant strains of E. oxidoreducens will help to further elucidate the metabolic capabilities of this unique microorganism. The decarboxylase mutants should be useful for studying gallate uptake. Thayer

and Wheelis (22) used a mutant strain of Pseudomonas putida that could not metabolize benzoate to show that uptake was mediated by an inducible active transport system capable of accumulating this substrate 150-fold against a concentration gradient. In contrast Harwood and Gibson (11) and Geissler et al. (8) have described a mechanism for benzoate uptake in R. palustris that enhances diffusion into the cell by maintaining a low intracellular benzoate concentration via thioesterification to benzoyl-CoA. Isolation of mutants defective in the complex reactions involved in the conversion of pyrogallol to phloroglucinol, and other aromatic hydroxylation reactions, would be very useful for determining the number of enzymes involved and for the identification of the hypothesized electron acceptor involved in 1,2,3,5-benzenetetrol formation. These mutants should be easily identified by their failure to grow on pyrogallol and ability to grow on phloroglucinol.

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SECTION VIII: DEGRADATION OF GALLATE TO NON-AROMATIC  
PRODUCTS BY CELL EXTRACT OF EUBACTERIUM OXIDOREDUCENS

SUMMARY

Gallate was completely degraded to non-aromatic products by cell extract of Eubacterium oxidoreducens plus formate, NADP and 1,2,3,5-benzenetetrol. Dihydrophloroglucinol was the only intermediate detected and it rapidly disappeared after the gallate was depleted from the reaction mixture. Thus, dihydrophloroglucinol hydrolysis appeared to be the rate limiting reaction of the first several steps of the pathway in cell extract. When 1,2,3,5-benzenetetrol was deleted from the reaction mixture, gallate was stoichiometrically converted to pyrogallol which was not further degraded. When formate was deleted from the reaction mixture, gallate was stoichiometrically converted to phloroglucinol which was not further degraded. These results are consistent with the hypothesized biochemical pathway for gallate degradation by E. oxidoreducens (5). However, it appears that the step which converts pyrogallol to phloroglucinol involves 1,2,3,5-benzenetetrol as a catalyst for the reaction as described for P. acidigallici (2) rather than as a direct intermediate as proposed by Krumholz and Bryant (4).

## INTRODUCTION

A pathway has been proposed for the degradation of gallate by E. oxidoreducens (see Section VII, Fig. 1A). In P. acidigallici the pathway appears to involve the same steps and intermediates for the conversion of gallate to non-aromatic intermediates, except that formate or hydrogen are not required to supply electrons for the reduction of phloroglucinol to dihydrophloroglucinol (2, 6, 7). Krumholz and Bryant (4) have partially purified the enzyme(s) responsible for the conversion of pyrogallol to phloroglucinol and have proposed that pyrogallol is hydroxylated to produce 1,2,3,5-benzenetetrol which is then dehydroxylated to form phloroglucinol. However, Brune and Schink (2) have shown that the benzenetetrol is not a direct intermediate of the reaction in P. acidigallici but instead the compound is involved in an intermolecular transhydroxylation which converts 1,2,3,5-benzenetetrol to phloroglucinol and pyrogallol to 1,2,3,5-benzenetetrol (see Section VII, Fig. 1B).

This study was conducted to determine the requirements for the degradation of gallate to non-aromatic products by cell extract of E. oxidoreducens and to examine the role of 1,2,3,5-benzenetetrol in the proposed pathway of this organism.



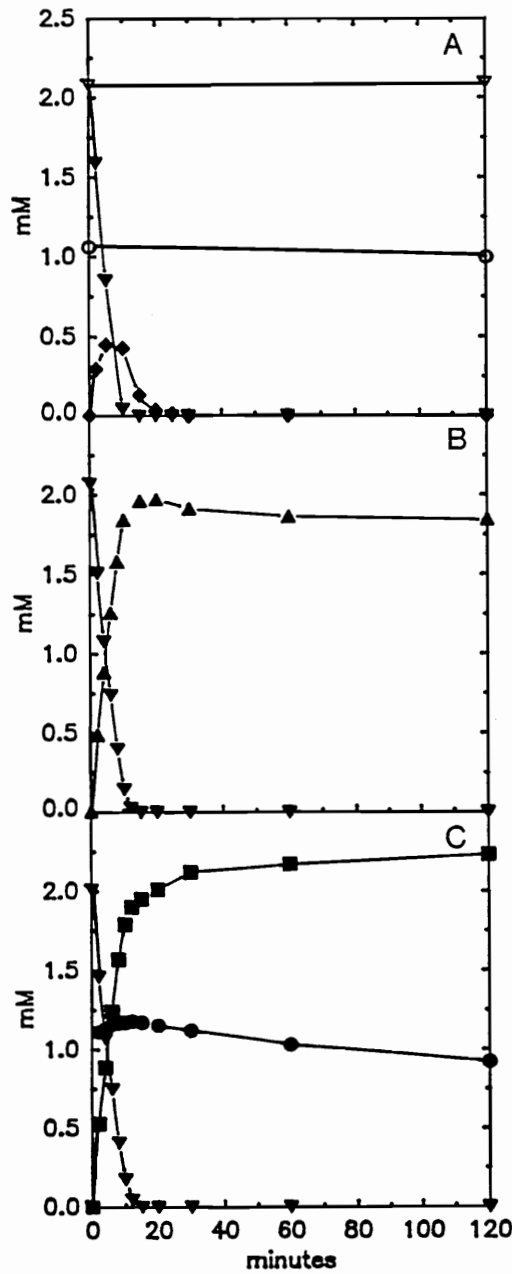


FIG. 1. Degradation of gallate by cell extract of *E. oxidoreducens*. A. With formate (4 mM initially) and 1,2,3,5-benzenetetrol, (~1 mM initially). B. With formate and without 1,2,3,5-benzenetetrol. C. Without formate and with 1,2,3,5-benzenetetrol. Symbols:  $\nabla$ , Gallate;  $\bullet$ , 1,2,3,5-benzenetetrol;  $\blacklozenge$ , dihydrophloroglucinol;  $\blacktriangle$ , pyrogallol;  $\blacksquare$ , phloroglucinol. Open symbols represent concentrations in the control vial which contained gallate, formate, 1,2,3,5-benzenetetrol and boiled cell extract.

## MATERIALS AND METHODS

Cell extract. All procedures were performed anaerobically. Five g wet weight of cells grown in a 10 L fermentor on 50 mM each gallate plus formate (Section IV) were suspended in 45 ml of 50 mM potassium phosphate buffer, pH 7.2, containing 2 mM 2-mercaptoethanol and 10 mM sodium azide. The cells were pelleted by centrifugation at  $12,100 \times g$  at  $4^{\circ}\text{C}$  for 15 minutes and the wash procedure was repeated. The cells were resuspended in 10 ml of the same buffer and broken by two passages through a chilled French pressure cell at  $20,000 \text{ lb/in}^2$ . Deoxyribonuclease I (Sigma) was added and the crude cell extract was centrifuged at  $18,800 \times g$  at  $4^{\circ}\text{C}$  for 20 minutes. The supernatant was frozen as small pellets in liquid  $\text{N}_2$  until use. The cell extract protein concentration was  $20.1 \text{ mg/ml}$  as determined by the method of Bradford (1) with bovine serum albumin as the standard.

Gallate transformation. Cell extract,  $0.435 \text{ mg/ml}$  final concentration, was incubated anaerobically under nitrogen at  $37^{\circ}\text{C}$  in 50 mM potassium phosphate buffer, pH 7.2, containing 2 mM 2-mercaptoethanol. The complete reaction mix also contained 4.0 mM sodium formate, 2.0 mM sodium gallate,  $250 \mu\text{M}$  NADP and approximately 1.0 mM 1,2,3,5-benzenetetrol in a total volume of 3.0 ml. For comparison, the benzenetetrol was omitted from one reaction and sodium formate was omitted from another. After initiation of the

reactions by addition of cell extract, 200  $\mu$ l samples were periodically withdrawn with a gas tight syringe, injected into anoxic sample vials that contained 2  $\mu$ l concentrated sulfuric acid and frozen until analysis.

HPLC analysis. Samples were thawed and placed on ice. Just prior to analysis, each sample was centrifuged for 5 minutes at 16,000 x *g* to remove precipitated protein and 20  $\mu$ l of the supernatant were immediately injected onto a 4.6 mm x 25 cm C<sub>18</sub> reverse phase column (Supelco, Bellefonte, PA). The mobile phase consisted of 10% methanol-90% 0.02N sulfuric acid pumped at 0.8 ml/min. Eluting peaks were detected with a variable wavelength absorbance detector set at 268 nm.

Enzyme assays. Specific activities of enzymes in cell extract were determined using the procedures previously described (Section VII). Transhydroxylase activity was determined using the HPLC method with 10% (v/v) dimethyl sulfoxide (Section VII). The gallate decarboxylase assay was modified so that the initial gallate concentration was 2.0 mM in a total volume of 2.0 ml. After initiation of the reaction by addition of 10  $\mu$ l of cell extract, 0.1 ml were periodically removed and diluted to 2.0 ml with 50 mM potassium phosphate buffer, pH 7.2, and the absorbance at 259 nm was observed.

Chemicals. 1,2,3,5-benzenetetrol was a gift from A. Brune and was estimated to have a purity of 88% based on a molar absorbance coefficient of  $1.9 \text{ M}^{-1} \text{ cm}^{-1}$  (A. Brune, personal communication).

## RESULTS

The specific activities of the enzymes involved in the transformation of gallate to non-aromatic products are shown in Table 1. With the exception of the higher formate dehydrogenase activity, the values are very close to those previously determined for cell extract from the same batch of cells prepared without sodium azide (Section VII). Therefore, inclusion of sodium azide in the wash and breakage buffers had no effect on the activity of these enzymes. However, formate dehydrogenase activity was significantly higher for cell extract prepared with azide, suggesting that even though strict anaerobic procedures were followed in preparation of the extract, some oxygen inactivation of formate dehydrogenase occurs when extracts are prepared without sodium azide.

When cell extract was incubated with gallate, formate, NADP and 1,2,3,5-benzenetetrol, gallate rapidly disappeared within the first 10 minutes and dihydrophloroglucinol accumulated transiently (Fig. 1A). Pyrogallol and phloroglucinol were not detected. A control reaction with boiled cell extract

Table 1. Enzyme activities in cell extract of E. oxidoreducens.

Enzyme	Specific Activity (U/mg) <sup>a</sup>
Gallate decarboxylase	0.310
Transhydroxylase	0.816
Phloroglucinol reductase	12.0
Dihydrophloroglucinol hydrolase	0.681
Formate dehydrogenase	4.23

<sup>a</sup> 1 unit = 1  $\mu$ mol/min.

showed little change in the concentration of gallate and 1,2,3,5-benzenetetroI after 120 minutes of incubation. When 1,2,3,5-benzenetetroI was omitted, the rate of disappearance of gallate was unaffected, but pyrogallol accumulated stoichiometrically at the same rate that gallate disappeared (Fig. 1B). No other intermediates were detected. The rate of gallate disappearance was also unaffected when formate was omitted from the reaction (Fig. 1C). However, a stoichiometric increase in phloroglucinol was observed which accumulated at the same rate that gallate was depleted. The 1,2,3,5-benzenetetroI concentration showed only a slight decrease after 120 minutes.

## DISCUSSION

The specific activities determined for the enzymes in cell extract of *E. oxidoreducens* suggest that gallate decarboxylation would be the rate limiting step during the conversion of gallate to non-aromatic products. However, when cell extract was incubated with gallate, formate, NADP, and 1,2,3,5-benzenetetroI, gallate was rapidly degraded and dihydrophloroglucinol accumulated until the gallate was depleted. Under these conditions, the rate of gallate disappearance was 0.57 U/mg which is similar to, but still lower than,

the specific activity determined for dihydrophloroglucinol hydrolase.

Accumulation of dihydrophloroglucinol was probably due to a low subsaturating dihydrophloroglucinol concentration that resulted in an enzyme activity that was lower than the value that was determined with a substrate concentration of 1.0 mM used to assay enzyme activity.

Gallate was stoichiometrically converted to pyrogallol when 1,2,3,5-benzenetetrol was omitted from the reaction. This result is in agreement with previous studies showing the dependence of the pyrogallol conversion reaction on this compound (2, 4 and Section VII). The one to one relationship between gallate disappearance and pyrogallol appearance supports the proposed pathway for gallate degradation in which gallate decarboxylation is the first reaction (5). Accumulation of phloroglucinol in the absence of formate also supports the proposed pathway in which formate supplies electrons for the reduction of phloroglucinol. No pyrogallol was detected however, suggesting that the conversion of pyrogallol to phloroglucinol is thermodynamically favorable. Brune and Schink (2) have determined that the  $K_{eq} = 475$  to  $500$  ( $\Delta G' = -15.4$  to  $-15.5$  kJ/mol) for the conversion of pyrogallol to phloroglucinol by *P. acidigallici*.

E. oxidoreducens had a minimum generation time of 1.8 h and a maximum cell yield of 7.69 g protein/mol gallate consumed when grown on gallate and formate (3). These values require a calculated minimum specific activity of 0.83  $\mu\text{mol}/\text{min}$  per mg protein for the enzymes involved in the degradation of gallate. The specific activities of all the enzymes assayed except gallate decarboxylase are sufficient to support a 1.8 h generation time. Gallate decarboxylase activity may be higher in vivo, however. The specific activities for dihydrophloroglucinol hydrolase are approximately the same, are approximately 2-fold higher for gallate decarboxylase and phloroglucinol reductase and are approximately 10-fold higher for formate dehydrogenase than previously reported values for E. oxidoreducens cell extract (5). The specific activity for the transhydroxylase is similar to the 1 U/mg cell extract protein reported for P. acidigallici (2).

1,2,3,5-benzenetetrol was required for the conversion of pyrogallol to phloroglucinol and formate was required to supply the electrons for reduction of phloroglucinol to dihydrophloroglucinol. Cell extract from E. oxidoreducens converts gallate to non-aromatic products when 1,2,3,5-benzenetetrol and formate are present. Gallate decarboxylase, transhydroxylase, formate



dehydrogenase, phloroglucinol reductase and dihydrophloroglucinol hydrolase are the enzymes responsible for catalyzing the reactions.

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## SECTION IX. SUMMARY AND CONCLUSIONS

The key step, ring reduction, in the pathway for the anaerobic, aromatic degradation of aromatic compounds by Eubacterium oxidoreducens was studied. Phloroglucinol reductase was purified from cell extract to homogeneity. Characterization of the pure enzyme showed that the protein is an  $\alpha_2$  homodimer with a native molecular weight of 78,000 and a subunit molecular weight of 33,000. No metals or cofactors other than NADPH were found, suggesting a hydride transfer mechanism. The enzyme is specific for phloroglucinol and NADPH as substrates and the reverse reaction produces phloroglucinol and NADPH from dihydrophloroglucinol and NADP. The equilibrium constant for the reaction at pH 7.2 and 30°C is 9.6 indicating that phloroglucinol reduction is thermodynamically favorable but is not highly exergonic. At pH 7.2 and 30°C,  $V_{\max}$  in the forward direction is 640  $\mu\text{mol}/\text{min}$  per mg and the  $K_m$  for phloroglucinol is 600  $\mu\text{M}$ . The  $K_m$  for phloroglucinol decreased with increasing pH while  $V_{\max}$  increased over the pH interval of 6.8 to 7.6 and then decreased at pH 8.0. It is likely that ionization of both the substrate and enzyme amino acids are involved in substrate binding and catalysis. Inhibition experiments suggested that cysteine and histidine functional groups are involved. The dianion of phloroglucinol exists as the keto tautomer in solution and the carbonyl carbon may be the position on the ring

that is reduced by a hydride equivalent from NADPH. The enzyme mechanism may involve induction of formation of the keto dianion through substrate ionization. Dihydrophloroglucinol is the product of phloroglucinol reduction by NADPH in the enzyme catalyzed reaction and by sodium borohydride. The  $^1\text{H}$  NMR spectra for dihydrophloroglucinol produced by both methods were identical. The structure of dihydrophloroglucinol is similar to that of dihydroresorcinol which is a competitive inhibitor of the reverse reaction. Either  $\text{H}_2$  or formate served as the source of electrons for phloroglucinol reduction.

Four different mutant strains of *E. oxidoreducens* were isolated and characterized. Two strains lacked gallate decarboxylase activity and were unable to degrade or to grow on gallate. Two additional strains lacked dihydrophloroglucinol reductase activity and did not grow on any of the aromatic substrates that supported growth of the wild type strain. These mutants also accumulated dihydrophloroglucinol and pyrogallol in the medium when grown on crotonate in the presence of gallate and formate.

Cell extract from *E. oxidoreducens* catalyzed the conversion of gallate to non-aromatic products in the presence of formate and 1,2,3,5-benzenetetrol. Enzyme specific activities were sufficient to support the previously reported growth rate and cell yield (2). When formate was eliminated from the

reaction, gallate was stoichiometrically converted to phloroglucinol. When 1,2,3,5-benzenetetrol was eliminated from the reaction, gallate was stoichiometrically converted to pyrogallol. The conversion of pyrogallol to phloroglucinol by cell extract was dependent on 1,2,3,5-benzenetetrol or high concentrations of dimethyl sulfoxide (DMSO), as previously reported (3). 1,2,3,5-benzenetetrol acted as a co-substrate for the conversion of pyrogallol to phloroglucinol in cell extract of *E. oxidoreducens* as previously shown for *Pelobacter acidigallici* (1). DMSO apparently acted as an artificial electron acceptor for the conversion of pyrogallol to 1,2,3,5-benzenetetrol, and was then involved in a transhydroxylation that resulted in the conversion of pyrogallol to phloroglucinol. Of the five enzyme activities assayed, only the conversion of pyrogallol to phloroglucinol was induced by growth on aromatic substrates.

Formate dehydrogenase was found in the soluble fraction of cell extract, as was phloroglucinol reductase. Formate dehydrogenase was typical of those described for most strict anaerobes in that it had a low  $K_m$  for formate relative to the formate dehydrogenase from aerobes, it was very sensitive to exposure to air and activity was protected by sodium azide.

The results of this study support the previously proposed pathway for anaerobic, aromatic degradation by *E. oxidoreducens* except that the proposed pyrogallol-phloroglucinol isomerase reaction (3) appears to be an intermolecular

transhydroxylation as shown for P. acidigallici (1). No alternative pathways or fates for the aromatic growth substrates were detected as shown by the quantitative recovery of intermediates in studies with mutants.

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