Université de Montréal

Isolation of a New Anaerobic Bacterium Transforming Phenol to Benzoate and Purification of the 4-Hydroxybenzoate Decarboxylase

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Isolation of a New Anaerobic Bacterium Transforming Phenol to Benzoate and Purification of the 4-Hydroxybenzoate Decarboxylase

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SUMMARY

Aromatic compounds are the second largest group of natural products. Some of them are mutagenic, teratogenic and/or carcinogenic. Especially, phenol and related compounds have a commercial importance since they are part of the aromatic compounds that are most widely used in modern world. Microbial degradation of aromatic compounds is the best solution of those contamination problem and more acceptable way up to now.

Anaerobic microbial degradation of aromatic compounds has received much attention over the past decade. This is mainly due to the fact that anaerobic biodegradation is more advantageous than aerobic biodegradation on an economic point of view. Once the aromatic substrate is anaerobiocally transformed to a simple aromatic intermediate, such as phenol or benzoic acid, the reduction occurs and converts the aromatic ring to an alicyclic ring which is then hydrolytically cleaved and gradually mineralized.

A new strain carboxylating phenol from a methanogenic consortium has been isolated under anaerobic conditions during this study. It is the first pure culture isolated from a methanogenic consortium for its ability to carboxylate phenol to 4-hydroxybenzoate and dehydroxylate 4-hydroxybenzoate to benzoate. This was achieved by treating a heat-treated methanogenic consortium with antibiotics followed by dilution to eliminate the four *Clostridium* strains which was without effect on the phenol transformation activity. Isolated strain 6 transformed phenol or 4-hydroxybenzoate in presence of proteose peptone. The new strain 6 was

characterized. It is a flagellated rod, 0.6 μm width and a Gram-variable bacterium. It has a doubling time of 10 to 11 hours in the presence of phenol. The result of 16S rRNA sequence analysis showed that strain 6 was most similar to that of *Clostridium* species, with homology ranging from 80 to 86%.

The purification and characterization of a reversible 4-hydroxybenzoate decarboxylase from a coculture constituted of strain 6 and 7 is also described in this thesis. The reversible activity of the 4-hydroxybenzoate decarboxylase was responsible for the carboxylation of phenol to 4-hydroxybenzoate. The molecular mass of the enzyme was estimated to 420 kDa and consisted of four identical subunits of 119 kDa. The optimum temperature for decarboxylation had a wide range between 15-45°C and the optimum pH was 5.0-6.5. The pI of the enzyme was 5.6. The enzyme also catalyzed the decarboxylation of 3,4-dihydroxybenzoate. The K_m value obtained for 4-hydroxybenzoate was 4.3 mM and V_m was 139 μmol/min/mg at pH 6.5 and 37°C. No stimulation of the activity was observed uponaddition of ATP. Phenol phosphorylation was not implicated in phenol or 4-hydroxybenzoate transformation. The N-terminal amino acid sequence of the purified enzyme was shown to be very similar to a pyruvate-flavodoxin oxidoreductase (*nif*J gene product).

These results give a better understanding of the microorganisms and of the enzyme implicated in a first step of phenol transformation. This knowledge is

valuable for the eventual optimization of the anaerobic treatment of phenolic compounds.

Key words: Isolation, characterisation, phenol, carboxylation, *Clostridium*-like bacteria, purification, 4-hydroxybenzoate decarboxylase, coculture, anaerobic conditions.

RÉSUMÉ

Les activités industrielles et agricoles, en plus des sources biologiques, introduisent une grande variété de composés phénolés dans l'environnement. Plusieurs de ces substances sont considérées comme des polluants prioritaires étant donné leur production en grande quantité, leur toxicité, leur résistance à la dégradation et à leur accumulation dans les sédiments. Le phénol est l'un des composés organiques les plus utilisés dans le monde. De plus, il est un important intermédiaire de dégradation de plusieurs composés phénolés.

Les connaissances sont limitées en ce qui concerne la dégradation anaérobie de ces composés. Notamment au niveau des microorganismes impliqués et des mécanismes régissant leur action. Ces microorganismes agissent habituellement en consortium et n'ont que très rarement été isolés en culture pure. Comparativement aux procédés aérobies, les procédés anaérobies sont avantageux surtout du point de vue économique. Il existe un réel besoin de mieux connaître les microorganismes anaérobies et les enzymes impliquées dans la dégradation des composés phénolés, plus particulièrement du phénol afin d'éventuellement mettre au point des traitements plus efficaces.

A partir d'un consortium de bactéries sporulantes transformant le phénol en acide benzoïque en conditions anaérobies, des antibiotiques ont été utilisés dans le but d'éliminer les souches non essentielles. La souche de *Clostridium ghonii* a été inhibée par le chloramphénicol (10 µg/mL) alors que la souche 3 de *C. hastiforme* et la souche de *C. glycolicum* ont été inhibées par la clindamycine (20 µg/mL) et ce,

sans affecter la transformation du phénol. Des observations au microscope électronique de la sous-culture résultante ont révélé la présence de deux bacilles différents : une souche 2 de *C. hastiforme* (1 μm de largeur) qui était dominante et une souche 6 (0.6 μm de largeur) non identifiée qui n'était pas détectée sur milieu solide. L'utilisation de la bacitracine (0.5 U/mL) a permis de changer le ratio des souches en faveur de la souche 6. La dilution de cette culture par un facteur de l'ordre de 10^{-6} a permis d'éliminer la souche 2 de *C. hastiforme* d'après les observations effectuées à l'aide d'un microscope électronique. De plus, la souche 6 était la seule à croître sur milieu solide au sang Columbia confirmant l'élimination de l'autre souche. La souche 6 isolée transforme le phénol en acide benzoïque et l'acide 4-hydroxybenzoïque en phénol et acide benzoique en présence de protéose peptone. Ces deux activités sont inductible par le phénol et l'acide 4-hydroxybenzoique. Cette souche anaérobie est un bâtonnet, Gram variable, flagellé avec un temps de dédoublement de 10 à 11 heures en présence du phénol. En dépit du fait qu'elle a été isolée d'un consortium résistant à la chaleur, nous n'avons pas observé de spores dans les cultures de la souche 6. Elle possède une composition en acides gras semblable à celle de *C. hastiforme*. Cependant, la souche 6 n'hydrolyse pas la gélatine et ne produit pas d'indole. La séquence du rRNA 16S de la souche 6 est très similaire à celle de certaines espèces de *Clostridium,* avec une homologie allant de 80 à 86 %. La relation évolutive de la souche 6 avec différents groupes de *Clostridium* et des espèces reliées aux *Clostridium* a révélé qu'elle n'émergait pas d'aucun de ces groupes. La souche 6 appartient fort probablement à une nouvelle espèce étroitement associée aux espèces du genre *Clostridium*.

La purification et la caractérisation partielle d'une enzyme 4-hydroxybenzoate décarboxylase sensible à l'oxygène provenant d'une coculture constituée de la souche 6 apparentée au *Clostridium* et d'une souche 7 non identifiée ont été réalisées. Cette purification a été réalisée à l'aide de différentes colonnes chromatographiques. La procédure développée était la suivante : l'extrait brut a d'abord été passé sur une colonne de DEAE-Séphacel, la fraction active résultante a été chargée sur une colonne Macro-Prep t-butyl HIC, puis sur une colonne hydroxyapatite, par la suite sur une colonne Protéine Pak, DEAE-5PW et finalement sur deux colonnes de filtration moléculaire en série de type Protéine Pak 300 SW. L'enzyme a été purifiée par un facteur de 6 et possédait une activité spécifique de 760 µmol/min/mg-protéine. Approximativement 0.9 % de l'activité 4-hydroxybenzoate décarboxylase présente dans l'extrait brut a été récupérée dans la préparation purifiée. La masse moléculaire de l'enzyme a été estimée par filtration sur gel à 420 kDa. Une bande unique de 119 kDa a été obtenue sur SDS-PAGE suggérant une structure homotétramérique. La température optimale pour la décarboxylation variait de 15 à 45°C et le pH optimal était compris entre 5.0 et 6.5. Le point isoélectrique de l'enzyme était de 5.6. Cette enzyme catalyse aussi la décarboxylation du 3,4-dihydroxybenzoate. La valeur du K_m obtenu pour le 4-hydroxybenzoate était 4.3 mM et le V_m était de 139 µmol/min/mg à pH 6.5 et 37°C. Environ 50 % de l'activité enzymatique a été éliminée après une incubation d'une heure en présence d'air à 4°C et l'activité était complètement perdue après 48 heures. De l'autre côté, l'activité n'a pas diminué après 7 jours à 4°C sous conditions

anaérobies. L'activité de l'enzyme a diminué d'environ 50 % en présence de 5 mM EDTA. Par contre, elle a été restorée et même augmentée de 100 % par l'addition de Mg^{++}, Mn^{++}, Zn^{++} ou Ca^{++}. Cependant, l'addition de Fe^{++} a entraîné la restoration de seulement 20 % de l'activité. Aucune stimulation de l'activité a été observée avec 1, 2 ou 5 mM ATP. Le phénol est carboxylé par une voie différente de celle utilisant d'abord la phosphorylation du phénol. Tous les composés *p*-hydroxyl testés ont inhibé l'activité carboxylase à des degrés divers. La plus forte inhibition ayant été observée avec le *p*-hydroxypyridine. Ceci suggère que le groupement *p*-hydroxyl est important pour l'activité de l'enzyme. La séquence d'acides aminés N-terminal de l'enzyme était très similaire à la pyruvate-flavodoxine oxidoréductase (produit du gène *nifJ*) d'*Enterobacter agglomerans* (95% d'homologie) et de *Klebsiella pneumoniae* (76% d'homologie). L'enzyme catalyse aussi les réactions inverses suivantes : la carboxylation du phénol en acide 4-hydroxybenzoique et du cathécol en acide 3,4-dihydroxybenzoique. Les caractéristiques de l'enzyme suggèrent que cette 4-hydroxybenzoate décarboxylase est différente de d'autres décarboxylases.

Cette étude a permis d'obtenir une meilleure compréhension des microorganismes et de l'enzyme impliquée dans la première étape de transformation du phénol. Cette connaissance est importante pour le développement éventuel d'un procédé anaérobie plus efficace pour le traitement des composés phénolés polluants.

Mots clés : Isolement, caractérisation, phénol, carboxylation, bactéries apparentées au *Clostridium*, purification, décarboxylase de l'acide 4-hydroxybenzoïque, coculture, conditions anaérobies.

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LIST OF ABBREVIATIONS

ATCC: American Type Culture Collection

BHI: Brain Heart Infusion broth

BSTFA : N, O-bis (Trimethylsilyl) Trifluoroacetamide

CFA: cellular fatty acid

CUF: Colony forming units

EDTA: Ethylenediaminetetraacetic acid

DTT: dithiothreitol

$\Delta G^{o'}$: standard free energy

IEF: isoelectric focusing

kDa : kilodalton

k_{cat} : the catalytic constant

K_{eq} : the equilibrium constant

K_m : the Michaelis constant

PBS: Phosphate Buffered Saline

PCR : Polymerase chain reaction

PMSF :phenylmethylsulfonyl fluoride

psi : pound per square inch

SDS-PAGE : sodium dodecyl sulfate-polyacrylamide gel electrophoresis

V_{max} : the maximal velocity of reaction at saturating substrate concentrations

vol : volume

wt : weight

DEDICATION

Dedicated

to

my parents,

my family and friends,

and every one along the way.....

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I. GENERAL INTRODUCTION

Today, there is a great concern regarding the occurrence of aromatic compounds in the environment. Especially, phenol and related compounds have a great commercial importance since they are part of the aromatic compounds that are most widely used (Fedorak and Hrudey 1988). Many aromatic compounds are considered as priority pollutants by the U.S. Environmental Protection Agency (Babich *et al.* 1981; EPA 1979a; EPA 1979b). Microorganisms play a significant role in the degradation of aromatic compounds. Biodegradation is a natural process and treatments based on this process to eliminate these pollutants are often complete. Thus, they are more acceptable than chemical and physical treatments.

The microbial degradation of aromatic compounds under aerobic conditions has been studied and is well documented. However, under anaerobic conditions, the degradation of these compounds has been less well study, but it has been receiving much more attention recently (Harwood and Gibson 1997; Elder and Kelly 1994; Fuchs *et al.* 1994; Haggblom 1992; Young and Haggblom 1991).

Degradation of phenol to benzoate by methanogenic consortia has been well studied recently (see review Londry and Fedorak 1992). However, methanogenic consortia are difficult to characterize because there are multiple interactions in the consortia for growth and degrading function (Kumaran and Shivaraman 1988; Slater and Lovatt 1984). Many researchers have tried without success to isolate pure cultures responsible for the degradation of phenol from methanogenic consortia. Knoll and Winter (1989) failed to isolate a pure culture responsible of the

carboxylation of phenol because the phenol-degrading bacteria were in a syntrophic relation with methanogens. Béchard *et al.* (1990) have found that nonsyntrophic microorganisms were responsible in their consortium for the carboxylation of phenol since inhibition of the methanogens had no effect on this activity. Létourneau *et al.* (1995) have shown that spore-forming bacteria were responsible for the carboxylation of phenol. Their heat-treated coculture contained at least five different strains of bacteria. Four of them were identified as *Clostridium* sp. and the other were not identified. Genthner *et al.* (1989c; 1989d) have characterized an anaerobic phenol-degrading methanogenic consortium. It contained five Gram-negative cell types. Zhang *et al.* (1990a; 1990b; 1994) have isolated in pure culture, *Clostridium hydroxybenzoicum* sp. nov. from a methanogenic consortium. This bacterium can decarboxylate 4-hydroxybenzoate to phenol but can not further metabolize phenol. Until now, there has been no report on the isolation from a methanogenic consortium of a pure culture able to transform phenol to benzoate.

For the enzymatic study of phenol degradation under anaerobic conditions, most of the work has been done with cell-free extracts or partially purified enzymes (Gallert and Winter 1992; Lack and Fuchs 1992; Lack *et al.* 1991; Tschech and Fuchs 1989) because the activity is very sensitive to oxygen. Also pure cultures are difficult to isolate and grow very slowly. Much experimental evidence indicate that the degradation of phenol occurs via carboxylation (see review Fuchs *et al.* 1994; Schink *et al.* 1992). The 4-hydroxybenzoate decarboxylase or reverse phenol carboxylase activity has been reported using the facultative anaerobe *Klebsiella*

pneumoniae (syn. *aerogenes*) (Grant and Patel 1969), mixed rumen populations (Martin 1982), the denitrifying *Pseudomonas* strain K172 (Lack and Fuchs 1994; 1992; Lack *et al.* 1991; Tschech and Fuchs 1989), an obligate anaerobic phenol-degrading coculture (Gallert and Winter 1992) and the obligate anaerobe, *C. thermoaceticum* (Hsu *et al.* 1990). So far, there is only one report on the purification and characterization of a 4-hydroxybenzoate decarboxylase. This was achieved by He and Wiegel (1995) with *C. hydroxybenzoicum* isolated from a methanogenic consortium. It is an oxygen-sensitive reversible enzyme with six identical subunits of 57 kDa each and a molecular mass of 350 kDa.

The present study had two main objectives. First, in order to better understand the microorganisms involved in the carboxylation of phenol and allow the improvement and development of more efficient treatment processes, we planned to isolate a pure strain of bacteria, which is responsible for the carboxylation of phenol to benzoate under anaerobic conditions. This isolated strain was obtained from the heat-treated coculture (Létourneau *et al.* 1995), which came from a methanogenic consortium. The characterization of this isolated strain was done using electron microscopy, microbial identification methods, biochemical tests, and 16S rRNA sequence analysis. Second, in order to further study the mechanism of phenol degradation, we planned to purify and characterize the phenol carboxylase or 4-hydrobenzoate decarboxylase from our culture. The purification of the enzyme was done by liquid chromatography and HPLC (High Pressure Liquid Chromatography) with different ion-exchange, affinity chromatography, and size-

exclusion columns under anaerobic condition. The purified enzyme was characterized by using Gel-filtration, SDS-PAGE, enzyme assay with different substrates, and N-terminal amino acid sequence analysis.

II. LITERATURE REVIEW

1. Microbial degradation of aromatic compounds

1.1. Aromatic compounds

Aromatic compounds constitute a class of organic molecules that are of major importance in the recycling of global carbon. Petroleum, peat and coal deposits, lignin, plant phenolics, and proteins contain a large aromatic fraction (Haddock and Ferry 1990). In addition, a variety of xenobitics, man-made aromatic pollutants, enter into the biosphere as herbicides, insecticides, detergents and industrial waste products. Some of these compounds are mutagenic, teratogenic and/or carcinogenic (Thakker *et al.* 1985; Keith and Telliard 1979).

1. 2. Anaerobic degradation of aromatic compounds

Anaerobically, the aromatic ring is not oxidized but reduced. In the absence of oxygen, substituted aromatic compounds appear to be more easily degraded than nonsubstituted ones (Bouwer and Zehnder 1993). Current knowledge suggests that benzene is recalcitrant under all anaerobic conditions, whereas substituted aromatic compounds are degraded, depending on the functional group and the terminal electron acceptor present.

1. 2. 1. Biochemistry

Once the chemical contaminants have been released into the environment, if rapid loss and degradation does not occur, they may eventually find their way into anaerobic regions of the soil, sediment, or subsurface where they can be degraded

(Young and Haggblom 1989). A comparison of energy yields of aerobic and anaerobic biomass degradation (here exemplified by glucose) demonstrates convincingly why anaerobic microorganisms are primarily applied :

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

$$\Delta G_o' = -2870 \text{ kJ/mol}$$

$$C_6H_{12}O_6 \rightarrow 3CO_2 + 3CH_4$$

$$\Delta G_o' = -390 \text{ kJ/mol}$$

The lower energy resulting from anaerobic biodegradation (only approximately 15 percent of that obtained by aerobes according to the equation) explains the small amount of biomass formed during this process (Schink 1990). This helps to reduce sludge production; a problem that becomes more and more difficult to solve in densely populated areas. Moreover, products such as methane or ethanol, acids, and so forth can be formed that might be of interest for either energy generation or solvent production purposes (Schink 1988). However, the low energy yield of anaerobic compared with aerobic biodegradation also implies some drawbacks. Low growth rates and yields make an anaerobic microbial population less flexible in adapting to changing environmental conditions, changes in substrate availability, or recovery after shock loads of, for example, toxic compounds. For these reasons, the application of anaerobic bacteria to waste treatment was considered for a long time to be less efficient than aerobic processes. In fact, many compounds are even better degraded without oxygen than in the presence of oxygen (Schink 1988). This field of study has been extensively documented (Fuchs *et al.* 1994; Evans and Fuchs 1988; Berry *et al.* 1987; Sleat and Robinson 1984; Young

1984; Evans 1977). It is now clear that a wide variety of aromatic compounds can be completely degraded by bacteria in the absence of oxygen (Colberg and Young 1995; Elder and Kelly 1994; Fuchs *et al.* 1994; Gibson and Harwood 1994; Schink *et al.* 1992).

1. 2. 2. Biodegradation Steps

Anaerobic biodegradation of aromatic compounds proceeds in three steps as shown in Figure 1.

First, chemically inert compounds need to be activated by reactions such as carboxylations, anaerobic hydroxylations, and CoA thioester formation of aromatic acids. Furthermore, the enormous diversity of natural and synthetic aromatic compounds has to be directed into a few central intermediates, which are suitable for a reductive attack on the aromatic nucleus. The enzymes of these pathways, often encoded by a plasmid genes, are more or less specific for certain growth substrates and are generally induced by the corresponding substrate (Heider and Fuchs 1997).

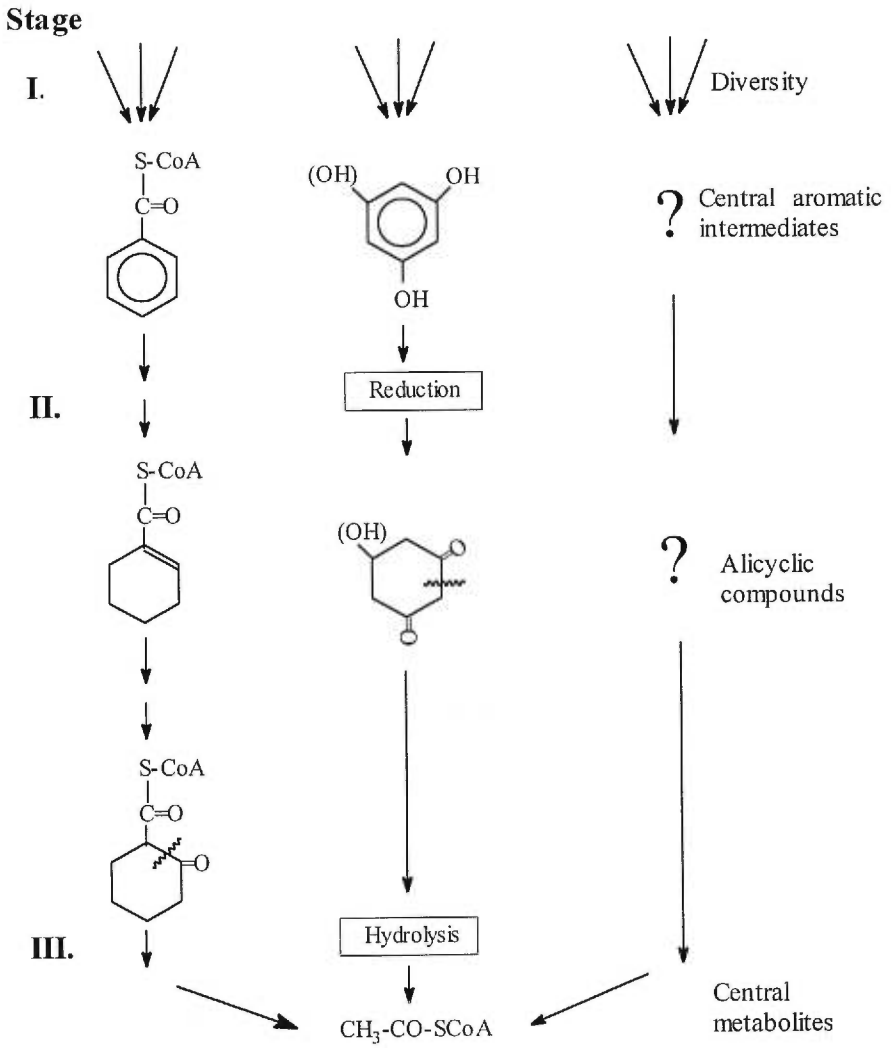


Fig. 1. Simplified scheme of anaerobic metabolism of aromatic compounds in three steps (Fuchs *et al.* 1994).

Second, the central aromatic intermediates are attacked enzymatically by reductases. The most common central intermediate in anaerobic aromatic metabolism is benzoyl-CoA. (rather than benzoate) (Fuchs *et al.* 1994). The resulting alicyclic compounds are easily converted by β-oxidation to 3-oxo-compounds, or the reduction directly provides the 3-oxo-compound. The 3-oxo-compounds have been shown or postulated to become hydrolytically cleaved. Enzymes of the *ortho*-cleavage pathway are normally chromosomally coded (Harayama and Timmis 1989).

Third, the non-cyclic compounds are transformed into central metabolites using rather conventional pathways. The products of C_6, C_6-C_1 and C_6-C_2 compounds are probably three acetyl-CoA and CO_2.

1. 2. 3. Channelling reactions

The conversion of different aromatic structures into a few central reactive aromatic intermediates (channelling reactions) has to serve two functions: to maximize the chemical reactivity of the aromatic nucleus while minimizing the number of enzymes required to deal with hundreds of similar compounds and to oxidize all of them to acetyl-CoA and CO_2. Central aromatic intermediates recognized so far are benzoyl-CoA, 4-hydroxybenzoyl-CoA, resorcinol, phloroglucinol and possibly others (Fig. 1 and Fig. 2). Peripheral metabolic pathways of these intermediate compounds is illustrated in Figure 2 (Brackmann and Fuchs 1993).

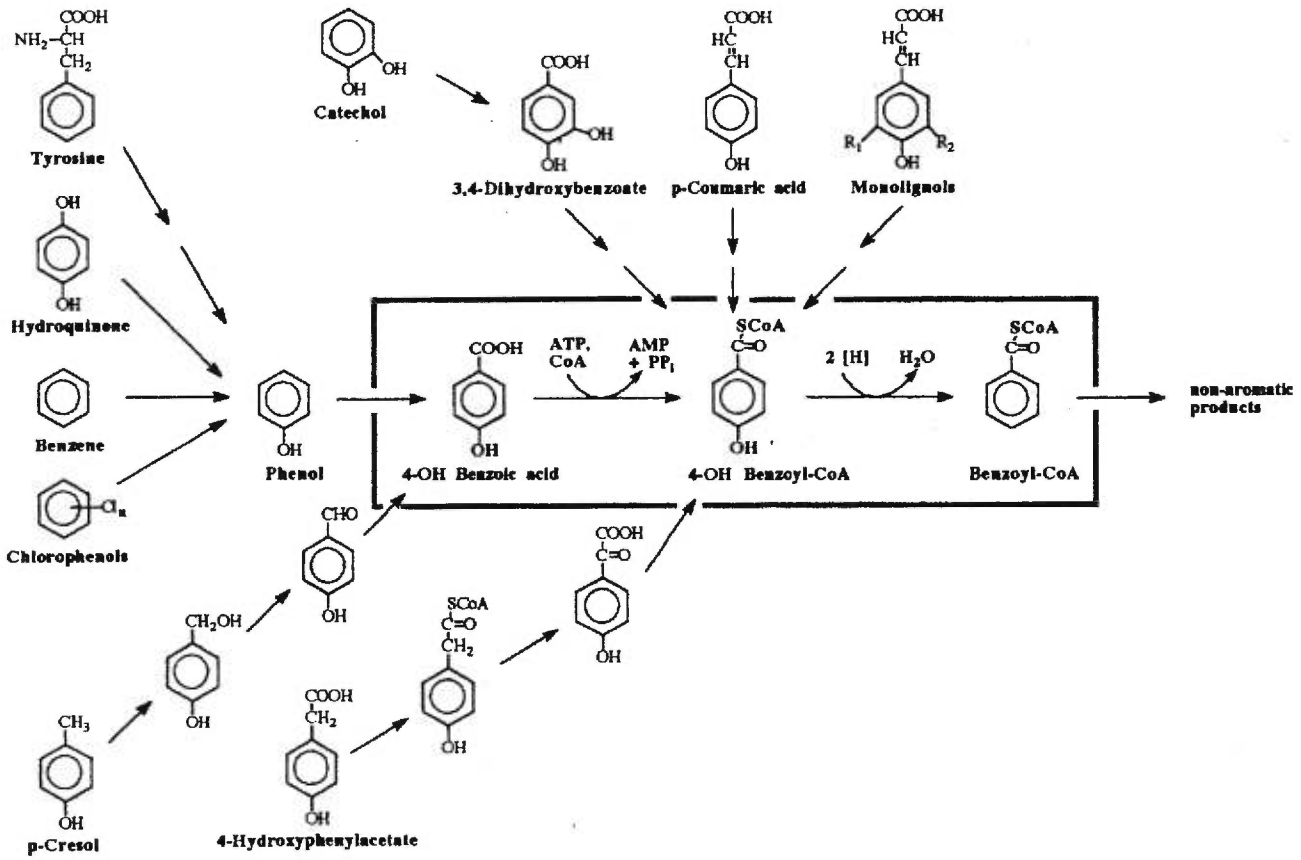
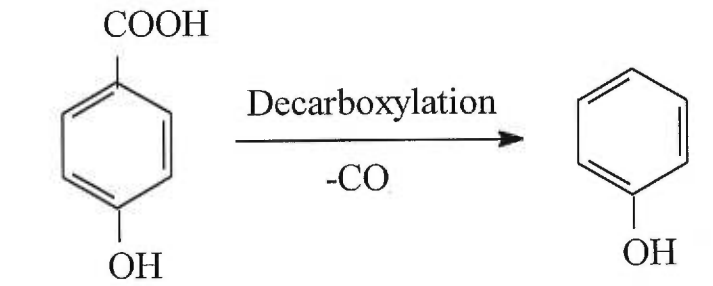


Fig. 2 Scheme illustrating the role of 4-hydroxybenzoyl-CoA as an intermediate in the degradation of various phenolic compounds and of the reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA, the central aromatic intermediate (Brackmann and Fuchs 1993).

The catalytic reaction participates in channelling phenol, 4-hydroxybenzoate, *p*-cresol, 4-hydroxyphenylacetate and other aromatics into the central intermediate benzoyl-CoA prior to reduction of the aromatic ring. Some examples of these reactions are described as follow :

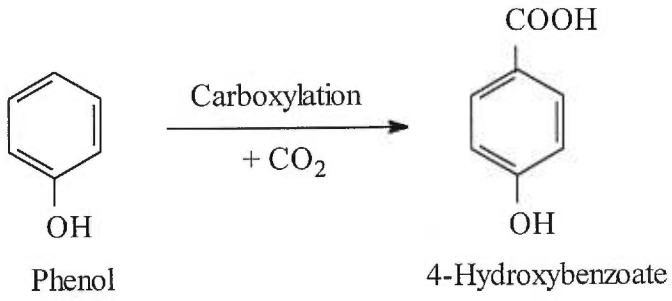
A. Decarboxylation



4-Hydroxybenzoate Phenol

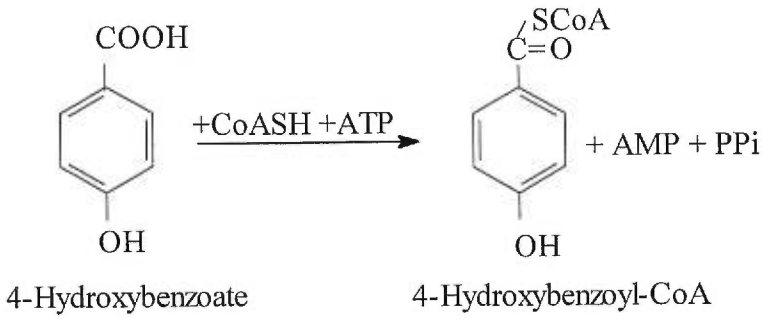
The decarboxylation of aromatic acids is common in transformations which are not part of a degradation pathway. There are numerous reports in the literature like the decarboxylation of 4-hydroxybenzoate to phenol (Gallert and Winter 1992 and 1994; Zhang and Wiegel 1994; He and Wiegel 1995), gentisate to hydroquinone, or protocatechuate to catechol (Fuchs *et al.* 1994). Hus *et al.* (1990) reported that an aromatic-dependent decarboxylase plays an important role in the food chain since they remove organic acids or provide CO_2 for acetogenesis. In general, the decarboxylation of aromatic acids with a hydroxyl function *para* to the carboxyl group is a chemically favoured reaction and has been observed in biological systems.

B. Carboxylation



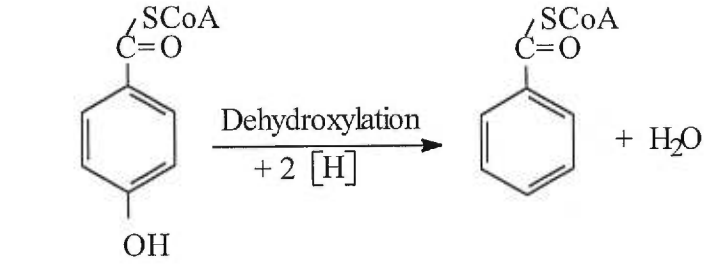
Carboxylation of the aromatic ring is the first step in the degradation of phenol (Tschech and Fuchs 1987; 1989; Dangel *et al.* 1991), of *o*-cresol and possibly of other phenolic compounds substituted in *ortho*-position (Bisaillon *et al.* 1991b; Rudolphi *et al.* 1991). Also *m*-cresol seems to be carboxylated in some microorganisms (Roberts *et al.* 1990; Ramanand and Suflita 1991). Phenol carboxylation has been studied in denitrifying bacteria (Tschech and Fuchs 1987; 1989; Lack *et al.* 1991; Lack and Fuchs 1992), in sulphate reducing bacteria (Bak and Widdel 1986), and phenol-degrading consortia (Genthner *et al.*1991; Bisaillon *et al.* 1991a; Gallert and Winter 1992).

C. Coenzyme A thioester formation



A thioester bond is more reactive than an oxyester bond, because the electron resonance interaction between the sulfur atom and the carbonyl group is diminished as compared with the oxygen-carbonyl delocalization in ordinary esters (Villemur 1995). Benzoate and analogues, if they are not decarboxylated, are converted into their coenzyme A thioesters by soluble, relatively specific, inducible coenzyme ligases before they are further metabolized (Hutber and Ribbons 1983). No exception to this rule is known so far. CoA ligases of benzoate (Geissler *et al.* 1988; Altenschmidt *et al.* 1991), 2-aminobenzoate (Altenschmidt *et al.* 1991), phenylacetic acid (Martinez-Blanco *et al.* 1990; Mohamed and Fuchs 1993), and 4-hydroxybenzoate (Fogg and Gibson 1990; Biegert *et al.* 1993) have been studied in more detail.

D. Reductive dehydroxylation



4-Hydroxybenzoyl-CoA Benzoyl-CoA

The reductive dehydroxylation of aromatic hydroxyl functions, notably with hydroxyl *para* to a carboxyl group, has been known for a long time (Scheline *et al.* 1960; Szewzyk *et al.* 1985; Grbic-Galic 1986). The only case studied in some detail

is the reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA by 4-hydroxybenzoyl-CoA reductase (dehydroxylating) (Glockler *et al.* 1989; Brackmann and Fuchs 1993). This reaction plays an important role in the metabolism of phenol, 4-hydroxybenzoate, *p*-cresol and 4-hydroxyphenylacetate (Dangel *et al.* 1991).

1. 3. Aerobic degradation of aromatic compounds

Under aerobic conditions, aromatic substrates are metabolized by a variety of bacteria, with ring fission accomplished by mono- and dioxygenases. Molecular oxygen is essential for these enzymes to function since it is incorporated into the reaction products. In aerobic metabolism, oxygen activates the ring. Aerobic biodegradation of many classes of aromatic compounds is common and proceeds through the key intermediate, catechol (Bouwer and Zehnder 1993). The aromatic ring of the catechol is opened by a further reductive reaction by either an *ortho*- or *meta*-fission. Halogenated aromatic compounds are most often degraded in this manner and the chlorine is, in general, eliminated after ring cleavage by mechanisms that are not yet fully understood (Reineke and Knackmuss 1988). There are examples where at least one chlorine is eliminated hydrolytically. The resulting phenol is further oxidized by a mono-oxygenase to a catechol (Thiele *et al.* 1987). Therefore, the final steps and end products also differ between aerobic and anaerobic degradation. A comparison of aerobic and anaerobic aromatic metabolism is presented in Table. 1.

Table 1. Comparison of anaerobic and aerobic pathways of degradation of aromatic compounds

	Anaerobic	Aerobic
Channelling reactions	$+H_2O$, $+2H^+$, $+CO_2$, $+H_2O$, +CoA, +ATP	$+ O_2$
Central intermediates	Benzoyl-CoA 4-Hydroxybenzoate Resorcinol Phloroglucinol	Catechol Protocatechuate Gentisate
Properties of central intermediates	easy to reduce (hydrogenate)	easy to oxidize (cleave)
Attack at the ring	2 or $4H^+$ ($+ H_2O$)	O_2
Ring cleavage	Hydrolysis of 3-oxo compound	Oxygenolysis of aromatic
Pathway to central metabolites	β-Oxidation, e. g. → Glutaryl-CoA → Acetyl-CoA	3-Oxoadipate pathway, e. g. → Succinate + Acety-CoA

(Fuchs *et al.* 1994).

Aerobic biodegradation has not been fully reviewed here since our research objective was to study anaerobic biodegradation. Additional information on aerobic biodegradation of aromatic compounds can be found in reviews of Evans (1969), Dagley (1971), Cerniglia (1992), Haggblom (1992), and Mohn and Tiedje (1992).

2. Metabolisms of anaerobic degradation of aromatic compounds

Anaerobically, aromatic compounds can be degraded by six different types of metabolism: photometabolism, denitrification, sulfate-reduction, iron reduction, fermentation, and methanogenic fermentation. Each of these will be discussed separately with emphasis on metabolism under methanogenic conditions since this study was done with a methanogenic consortium.

2.1. Photometabolism

For the phototrophic purple nonsulfur bacteria (i.e., *Rhodospirillaceae*), organic compounds can serve as the major source of electrons and carbon for cellular components (Berry *et al.* 1987). The path of electron flow in anoxygenic photosynthesis is cyclic and establishes an electrochemical proton gradient across an energy transducing membrane. This electrochemical proton gradient is dissipated by a proton-translocating ATPase in order to produce ATP. Electrons are withdrawn from the cycle and provide reducing power by the reduction of NAD (Jackson 1988).

Several species of the family *Rhodospirillaceae* (purple non-sulfur bacteria) have been found to metabolize aromatic compounds anaerobically in the presence of light (Pfennig and Truper 1989). The purple non-sulfur photosynthetic bacteria occur in all aquatic environments (Pfennig 1967) and soils, thus photometabolism could play a significant role in the dissimilation of simple aromatics in these environments.

2.2. Denitrification

Nitrate-reducing bacteria couple the oxidation of organic compounds with water to the exergonic reduction of nitrate via nitrite to N_2 or, less often, to NH_3. Energy is derived mainly from electron transport phosphorylation during nitrate respiration, and cell carbon is derived from breakdown products of the organic substrates – including aromatic compounds. Most of nitrate-reducing bacteria are facultative anaerobes and will preferentially carry out aerobic respiration (Gottschalk 1986); the enzymes for denitrification are formed only under anaerobic conditions or conditions of low oxygen tension. It has been well studied in *Pseudomonas* (see review Bakker 1977; Fuchs *et al.* 1994). The spectrum of aromatic substrates for anaerobic growth of a denitrifying *Pseudomonas* strain K172 was examined by Fuchs *et al.* (1994). Fourteen aromatic compounds including benzoic acid, 3- or 4-hydroxybenzoate, phenol, *p*-cresol, and toluene can be completely oxidized to CO_2 and serve as source of carbon for strain K172.

Bacteria carrying out active denitrification inhabit a variety of anoxic environments, including soil, sludge and lakes (Jeter and Ingraham 1981; Schlegel 1988). So the potential for anaerobic dissimilation of aromatics via nitrate reduction probably exists in all these environments.

2. 3. Sulfate-reduction

The growth of sulfate-reducing bacteria is supported by a form of anaerobic respiration where sulfate acts as a terminal electron acceptor and is reduced to hydrogen sulfide. Some sulfite-reducing bacteria can utilize relatively few electron donors, which they oxidize to the level of acetate, whereas a second group have the ability to utilize a wider range of organic acids, including aromatic acids, and generate CO_2 (Gottschalk 1986). In each case the reduction of sulfate is coupled to ATP synthesis. Sulfate-reduction occurs predominate in decomposing sediments in aquatic and terrestrial anoxic environments, where there are sufficient levels of sulfates (Elder 1994).

Until recently there has been little information on the anaerobic degradation of aromatics via sulfate reduction. Mountfort & Bryant (1982) and Widdel & Pfennig (1984) have provided evidence that sulfate reducers are able to use aromatic compounds as the sole source of carbon and electrons. The metabolism of aromatic compounds by pure cultures of sulfate reducing bacteria was reviewed by Widdel (1988).

2. 4. Iron reduction

Iron III-reducing bacteria obtain energy for growth by the complete oxidation of organic compounds to CO_2 with Fe^{+++} as the sole electron acceptor. During this process of dissimilatory Fe^{+++} reduction, Fe^{++} is excreted. Bacteria actively reducing Fe^{+++} occur predominantly in anaerobic aquatic sediments, submerged soils and ground water (Lovely 1991). The dissimilatory Fe^{+++} reducing bacterium, GS-15, is the first microorganism so far isolated in pure culture and in defined anaerobic media that can oxidize toluene, phenol, and *p*-cresol (Lovely and Lonergan 1990). *p*-Hydroxybenzoate was a transitory extracellular intermediate of phenol and *p*-cresol metabolism but not of toluene metabolism.

2. 5. Fermentation

Many microorganisms that inhabit anoxic environments obtain their energy for growth through fermentation of organic carbon. Fermentation is a process that can be carried out in the absence of light by facultative or obligate anaerobes. Growth-sustaining energy results from substrate-level phosphorylation reactions (Berry *et al.* 1987). In fermentation, organic compounds serve not only as electron donors but also as electron acceptors. Most natural aromatic compounds that consist of carbon, oxygen, hydrogen and/or nitrogen can be fermented under anaerobic conditions (Schlegel 1988).

Schink and Pfennig (1982) isolated five strains of strictly anaerobic bacteria from limnic and marine mud samples on a medium of mineral salts, trace elements,

and vitamins with gallic acid or phloroglucinol as sole carbon source. The five isolates fermented gallic acid, pyrogallol, 2,4,6-trihydroxybenzoate, or phloroglucinol. Using cell-free extracts, Schink & Pfennig (1982) also obtained experimental evidence which suggested that this initial step in phloroglucinol fermentation was a reduced nicotinamide adenine dinucleotide phosphate-dependent reduction to dihydrophloroglucinol. This same reaction has also been proposed by Patel *et al.* (1981). In 1986, Krumholz and Bryant (1986a; 1986b) isolated two strictly anaerobic chemoorganotrophs from the rumen. One cleaved methyl-ether linkages of substituted monobenzenoids. The other one degraded gallate, pyrogallol, and phloroglucinol to acetate, butyrate, and occasionally CO_2; it required either formate or hydrogen as an electron donor to catabolize these aromatic substrates.

2. 6. Methanogenic fermentation

Methanogenic bacteria are obligate anaerobes that are found in diverse anaerobic ecosystems and that produce methane as part of their energy metabolism. Pure cultures of methanogenic bacteria are able to use only a few simple substrates for growth, e.g. acetate, formate, methanol, and CO_2; hydrogen is used as the electron donor for CO_2 reduction. All methanogenic consortia must therefore rely on syntrophic associations with fermentors that degrade complex organic compounds into suitable substrates for the methanogens (Evans and Fuchs 1988).

Chmielowski *et al.* (1965) have screened 17 phenolic compounds to determine whether they could be fermented by methanogenic cultures. Eight of these

(phenol, *p*-cresol, resorcinol, pyrogallol, phloroglucinol, *o*-, *m*-, *p*-hydroxybenzoate) were degraded to methane and carbon dioxide. Healy and Young (1978; 1979) showed that 11 different aromatic compounds (including 9 phenolics) derived from lignin hydrolysis could be fermented to methane by mixed populations and studied the degradation of phenol and catechol to methane and CO_2 by adapted consortia from sewage. Smolinski and Suflita (1987) have also described cresol metabolism in anoxic water under methanogenic conditions.

Knoll and Winter (1987) have observed the formation of benzoate from phenol under methanogenic conditions; the metabolism may have proceeded via 4-hydroxybenzoate. Kobayashi *et al.* (1989) found that methanogenic degradation of phenol and benzoate was proceed via the following path: phenol $\rightarrow$ benzoate $\rightarrow$ cyclohexane carboxylate $\rightarrow$ fatty acids $\rightarrow$ CO_2 and CH_4 in acclimated sludge. Béchard et al. (1990) have shown that phenol was degraded by their methanogenic consortium. They found that phenol was completely degraded to benzoate then to acetate, and finally to CH_4 and CO_2. This consortium could also transform the other phenols with *ortho*-substitutions such as chloro-, hydroxyl-, amino-, or carboxyl- (Bisaillon *et al.* 1993). Zhang and Wiegel (1990a) have shown that 4-chlorophenol is dechlorinated to phenol, phenol is carboxylated to benzoate, and benzoate is degraded via acetate to methane and CO_2. At least five different organisms are involved sequentially in their consortium. Mikesell and Boyd (1986) have described the mineralization of pentachlorophenol. Genthner *et al.* (1989a; 1989b) also characterized four methanogenic consortia which could degrade chloroaromatic

compounds. In another investigation involving lignin-derived aromatic compounds, Kaiser and Hanselmann (1982) have observed the mineralization of 3,4,5-trisubstituted aromatic compounds to CO_2 and CH_4 by a methanogenic consortium obtained from a lake sediment enriched with syringic acid.

3. Metabolic interactions between anaerobic bacteria in methanogenic environments

3. 1. Food chain

Methanogenic degradation of aromatic compounds requires the cooperation of several groups of bacteria and the methanogens serve only as the terminal organism of a metabolic food chain (Ferry and Wolfe 1976). Figure 3 shows the microbial groups involved in the flow of carbon from complex compounds to methane and is based on the model described by McInerney and Bryant (1981). Complex compounds are broken down to soluble products by enzymes that are produced by fermentative bacteria (Fig. 3, group 1). These products are then fermented primarily to short-chain fatty acids, H_2, and CO_2. Fatty acids longer than acetate are metabolized to acetate by the hydrogen-producing (proton-reducing) acetogenic bacteria (Fig. 3, group 2). The net effect of the first two groups is conversion of the polymeric substrate to acetate, H_2 and CO_2. H_2 and CO_2 can be converted to acetate by H_2-oxidizing acetogens (Fig. 3, group 3) or to CH_4 by CO_2-reducing (H_2-oxidizing) methanogens (Fig. 3, group 4). Acetate can be split into CH_4 and CO_2 by aceticlastic methanogens (Fig. 3, group 5).

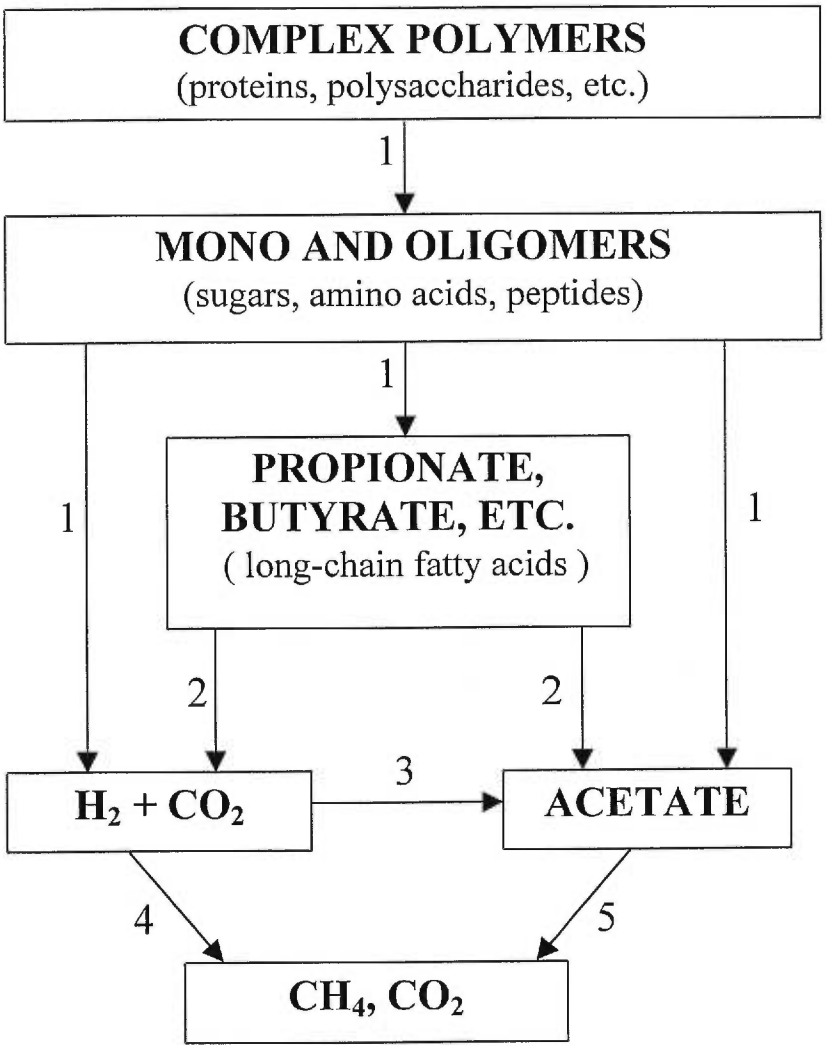


Fig. 4 Microbial groups involved in the flow of carbon from polymeric materials to methane in an anaerobic bioreactor.

Group 1, fermentative bacteria; group 2, hydrogen-producing acetogenic bacteria; group 3, hydrogen-consuming acetogenic bacteria; group 4, CO_2-reducing methanogens; group 5, aceticlastic methanogens. (Zinder, 1984)

It is generally accepted that two-thirds or more of the methane produced in an anaerobic degradation is derived from acetate, whereas nearly all of the remainder is derived from CO_2 reduction (Smith *et al.* 1980).

3. 2. Syntrophic associations

Syntrophism is a special case of symbiotic cooperation between two metabolically different types of bacteria, which depend on each other for degradation of a certain substrate, typically for energetic reasons (Schink 1997). The small amount of energy available in methanogenic conversion forces the microorganisms to be involved into a very efficient cooperation. They exhibit a metabolic activity that neither one could accomplish on its own. Such cooperation is called a syntrophic relationship. The term "syntrophy" should be restricted to cooperations in which both partners depend on each other to perform the metabolic activity observed and in which the mutual dependence can not be overcome by simply adding a cosubstrate or any type of nutrient (Schink 1997). This type of relationship has caused severe problems in the cultivation of such bacteria and the isolation of pure cultures from methanogenic environments (Stams 1994).

Syntrophic degradations have been described for a number of compounds including aromatic compounds (Dolfing 1988; McInerney 1988; Schink *et al.* 1992). In some mixed microbial systems, the bacteria degrading phenol have demonstrated syntrophic cooperations with methanogens (Knoll and Winter 1987; Londry and Fedorak 1992). Syntrophic degradation of monohydroxybenzoates (Tschech and

Schink 1986), catechol and hydroquinones (Szewzyk and Schink 1989), and aminobenzoate (Schnell and Schink 1992) have been described as well. Xenobiotic aromatic compounds may also be degraded by syntrophic associations. For example, the anaerobic degradation of 3-chlorobenzoate has been fully studied. For detailed information see the review of Mohn and Tiedje (1992). In this consortium, four bacterial species are involved in the degradation of 3-chlorobenzoate, a bacterium which converts 3-chlorobenzoate to benzoate, and three organisms which are required for benzoate degradation (Shelton and Tiedje 1984).

On the contrary, Béchard *et al.* (1990) and Zhang and Wiegel (1990a; 1990b) have shown that inhibition or elimination of the methanogens did not influence the phenol carboxylating activity of their consortia. They suggested that methanogens were not involved in the carboxylation of phenol. Moreover, they have shown that the bacteria responsible for the degradation of benzoate are not essential for the transformation of phenol (Zhang and Wiegel 1990a; 1990b; Létourneau *et al.* 1995). All these results suggest that their carboxylating microorganisms are nonsyntrophic.

4. Microbial degradation of phenols under anaerobic conditions

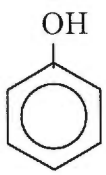
The remainder of this review will be limited to phenolic compounds, mainly phenol, since the present study is related to the biodegradation of these compounds.

4. 1. Source of phenol in the environment

Phenolic compounds contain one or more hydroxyl groups attached to an aromatic nucleus. They may also contain other functional groups such as alkyl, amino, carboxyl, halo, methoxy, nitro, etc. (Fedorak and Hrudey 1988).

At parts per billion (ppb) levels, phenolics cause taste and odor problems in drinking waters and may taint the flavor of fish taken from contaminated waters. At parts per million (ppm) concentrations, these compounds can be toxic to aquatic life. The toxicity of phenol to aquatic biota has been reviewed by Babich and Davis (1981). Phenolic compounds found in the environment may originate from a variety of sources. Plants produce simple phenols, including hydroxyquinone, gallic acid, salicylic acid, protocatechuic acid, and *p*-hydroxybenzoic acid; and more complex phenols, such as the flavonoids; and complex phenolic polymers, like lignins, catechol melanins, and flavolans (Harborne 1980).

Phenol is one of the most widely used aromatic compounds in the world. It is not only a frequent environmental contaminant, but also a common metabolite in the anaerobic metabolism of many compounds (Londry and Fedorak 1992).



Chemical configuration of phenol

Phenols released into the environment from industrial processes are of greater concern than plant phenols because of their high concentration, potential toxicity, and recalcitrance. The worldwide industrial production of phenol appeared to be fairly constant throughout the 1980s, and the U.S.A. is the most important producer. For example, in 1993, 1700 million kilograms of phenol were synthesized in the U.S. (Anonymous 1993). Also countless quantities of phenol and its derivatives are produced as industrial by-products such as phenolic resins, alkylphenols, bisphenol A, and all 17 possible chlorinated phenols, which are used in wood-preserving preparations (National Research Council of Canada 1982) or produced during the bleaching process in pulp and paper mills (Lindstrom and Nordin 1976; Kovacs *et al.* 1984).

4. 2. Phenol biodegradation pathways

Phenol, with its single hydroxyl substituent, is far less easily reduced directly than are aromatics with multiple hydroxyls. The hydroxyl substituent has a strong inductive effect on the π-electron system and thus affects the symmetry and stability of the aromatic ring structure much more than a methyl or carboxyl substituent. Kobayashi *et al.* (1989) have reviewed the proposed pathways for phenol degradation under methanogenic conditions. Two main metabolic routes have been observed. One is a reductive pathway; the other is a carboxylation pathway.

4. 2. 1. Reductive pathway

Bakker (1977) has demonstrated that phenol could be degraded by a mixed culture by using nitrate as the terminal electron acceptor. *n*-Caproic acid was the only intermediate identified, and it was postulated that the aromatic ring was reduced yielding cyclohexanone prior to ring fission. Balba and Evans (1980) added [U-^{14}C] phenol to a catechol-adapted methanogenic consortium. They found labeled cyclohexanone, 2-hydroxycyclohexanone, adipate, succinate, propionate, and acetate in the culture extracts. These results confirmed the reductive pathway hypothesized by Bakker (1977). A pathway for phenol degradation, which proceeds through complete ring saturation to cyclohexanol, conversion to cyclohexanone, and subsequent ring cleavage was suggested and is illustrated in Figure 4a (Evans 1977; Young 1984). However, there have been only a few reports on the early intermediates being found. Indeed several investigators have added cyclohexanol or cyclohexanone to phenol-degrading anaerobic cultures and failed to detect utilization of these intermediates (Bak and Widdel 1986; Tschech and Fuchs 1987; Kobayashi *et al.* 1989; Béchard *et al.* 1990).

4. 2. 2. Carboxylation pathway

A second degradation pathway of phenol has been reported by Neufeld *et al.* (1980), based on the work of Chmielowski *et al.* (1965). In this pathway, phenol is first carboxylated to benzoate before reduction and cleavage of the aromatic ring (Fig. 4b). This pathway will be reviewed in detail since it is the one used by our methanogenic consortium.

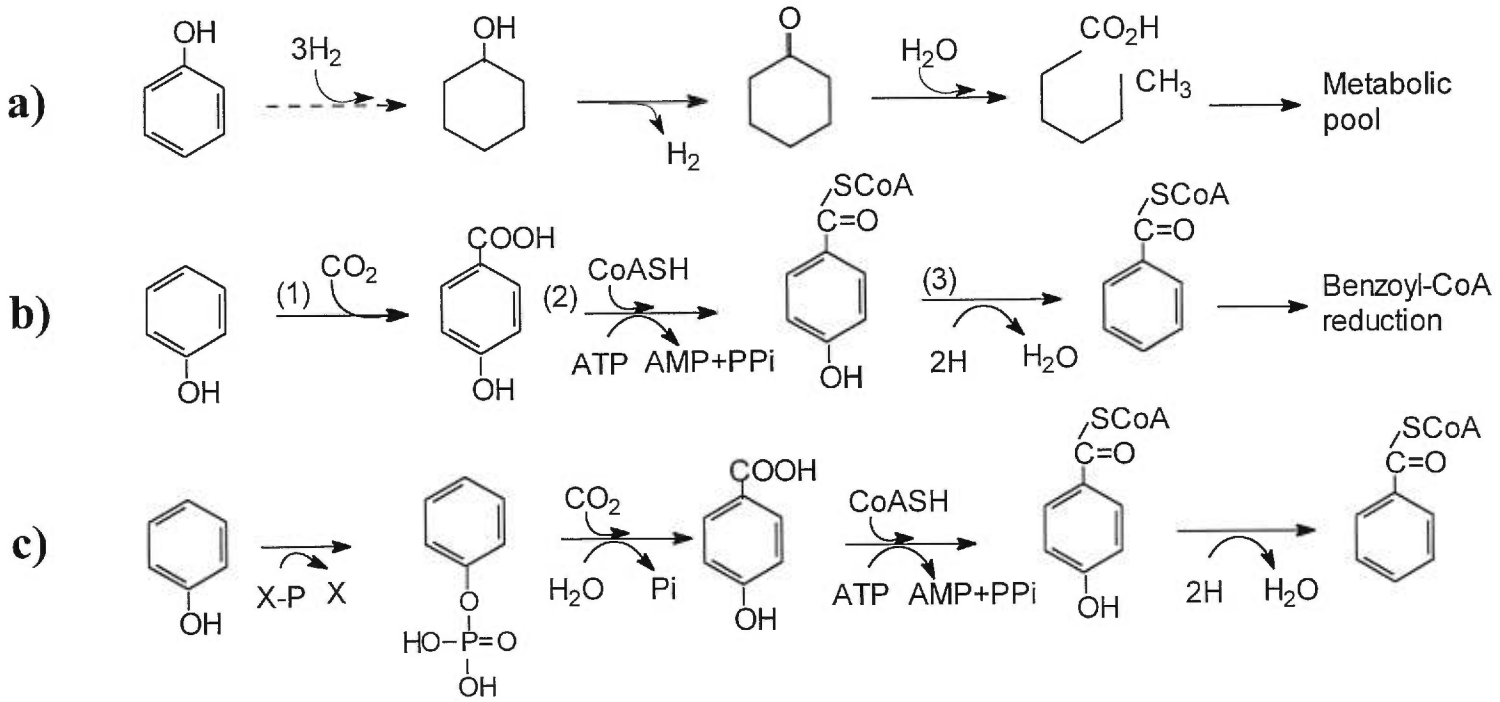


Fig. 4 Proposed pathways in the anaerobic degradation of phenol.

a) Reductive pathway (Balba and Evans, 1980) ; b) Carboxylation pathway (Neufeld et al. 1980). (1) Phenol carboxylase, (2) 4-Hydroxybenzoate-CoA ligase, (3) 4-Hydroxybenzoyl-CoA reductase. c) Carboxylation pathway in the denitrifying *Pseudomonas* sp. K172 (Lack and Fuchs 1994).

Several recent investigations have indicated that phenol degradation is initiated by carboxylation rather than insertion of other hydroxyl groups. Since phenol lacks a carboxylic group, it can not be activated by coenzyme A; a prerequisite which turns out to be essential for complete ring reduction in the benzoate pathway. Carboxylation of phenol has been detected in denitrifying bacteria (Tschech and Fuchs 1987; 1989) and methanogenic consortia (Knoll and Winter 1987; Kobayashi *et al.* 1989; Bisaillon *et al.* 1991a; Genthner *et al.* 1991).

Much evidence supports the carboxylation of phenol. Knoll and Winter (1987) used an atmosphere of 80% H_2 and 20% CO_2 to stimulate feedback inhibition in a phenol-degrading methanogenic consortium. This led to the accumulation of an intermediate that was identified as benzoic acid. Benzoic acid was also formed in subsequent experiments under an atmosphere of 80% N_2 and 20% CO_2. These results indicated that the metabolic pathway used was not a reductive one. Radiolabeled benzoic acid was detected when unlabeled phenol and $^{14}CO_2$ were added to the methanogenic consortium (Knoll and Winter 1987). This clearly showed the involvement of CO_2 in the metabolism of phenol under methanogenic conditions and is consistent with the results of study by Fedorak and Hrudey (1988), who hypothesized that the phenol-degrading bacteria in a methanogenic consortium may require CO_2.

Kobayashi *et al.* (1989) established a phenol enriched methanogenic consortium. They obtained essentially the same results as Knoll and Winter (1987).

Under an atmosphere of 80% H_2 and 20% CO_2, phenol was depleted from the medium and benzoic acid accumulated, whereas under an atmosphere of 80% N_2 and 20% CO_2, benzoic acid was observed as a transient intermediate during phenol degradation. In addition, cyclohexane carboxylate, 1-cyclohexene carboxylate and caproate were easily degraded in 5 days, while cyclohexanol, cyclohexanone, 1,2-cyclohexanediol, 1,2-cyclohexanedione, 2-methylcyclohexanone and pimelate were not degraded after 23 days. These results suggested that their consortium degraded phenol via carboxylation to benzoate, and not via a reducing pathway under methanogenic conditions.

Béchard *et al.* (1990) also enriched a methanogenic consortium, in which benzoic acid accumulated as phenol disappeared and cyclohexanol, cyclohexanone, propionate, butyrate, isobutyrate, valerate, and isovalerate were not detected as phenol degradation intermediates. These results suggested that their consortium is using the carboxylating pathway to transform phenol.

Genthner *et al.* (1990) have fed 2- and 3-fluorinated phenols to their consortium and obtained the corresponding benzoic acids by carboxylation and dehydroxylation (Fig. 5). Using the 4-hydroxybenzoate analog 6-hydroxynicotinic acid, 4-hydroxybenzoate was detected in phenol-transforming cultures (Genthner *et al.* 1991). These results are in agreement with the carboxylating pathway.

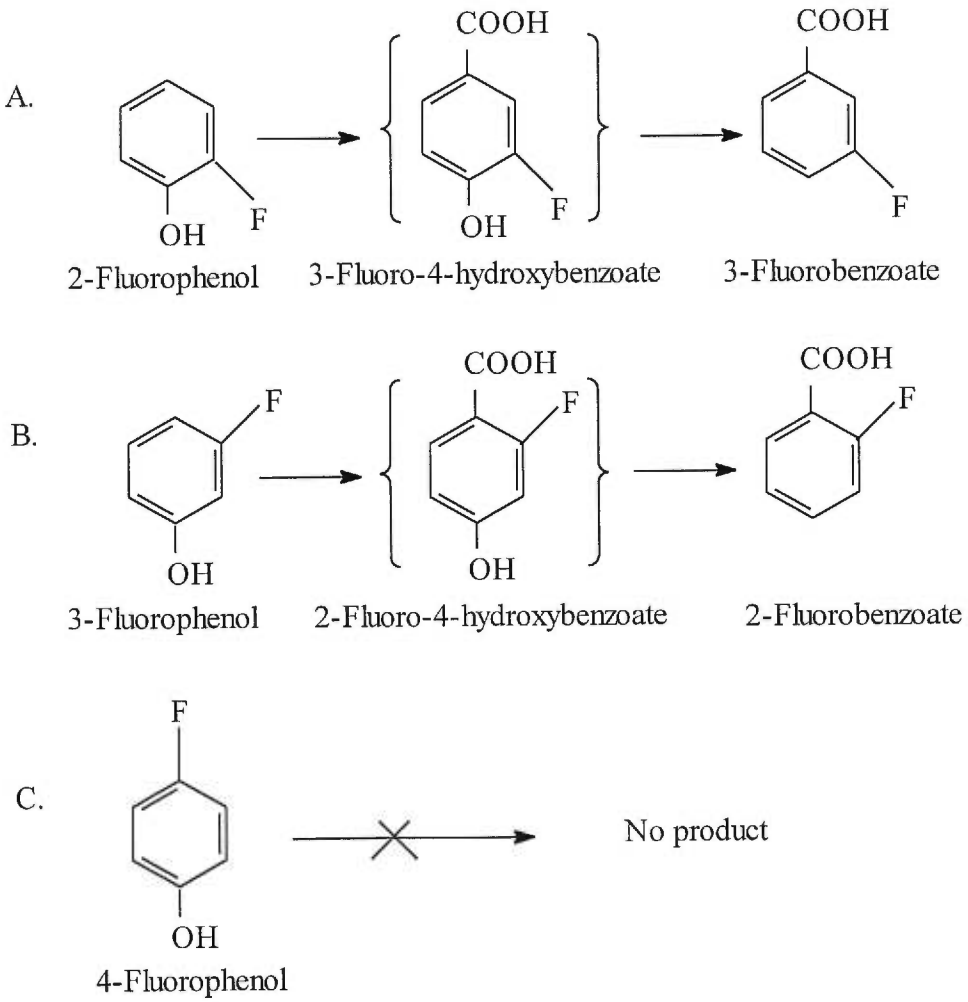


Fig. 5. Products which result from the transformation of monofluorophenols to monofluorobenzoates via *para*-carboxylation.

Compounds in the brackets are intermediates that have not been detected (Genthner *et al.* 1989).

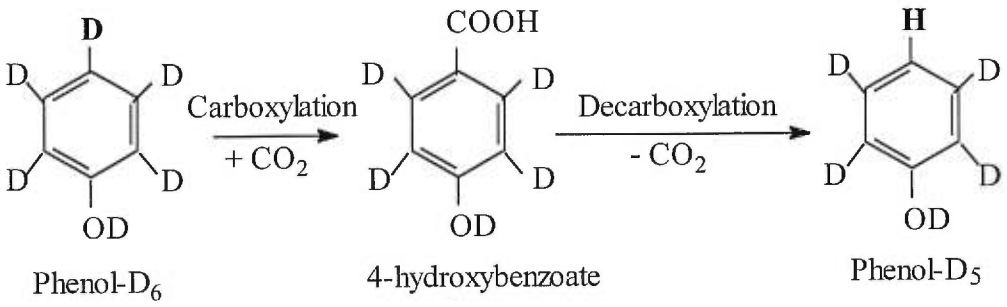
[1-^{13}C] Phenol was carboxylated to [4-^{13}C] benzoate by an anaerobic phenol transforming enrichment consortium, which did not form 4-hydroxybenzoate as a free intermediate but converted 4-hydroxybenzoate first to phenol and then to benzoate (Zhang *et al.* 1990). A strictly anaerobic defined mixed culture catalyzing deuterated phenols revealed that carboxylation was exclusively in the C-4-position (Gallert *et al.* 1991). It is to be expected that 4-hydroxybenzoate is an intermediate in all fermenting bacteria and that benzoate is not formed directly. Benzoate formation from phenol would require carboxylation followed by dehydroxylation (Knoll and Winter 1987; Kobayashi *et al.* 1989; Zhang *et al.* 1990; Béchard *et al.* 1990; Genthner *et al.* 1990; Gallert *et al.* 1991; Bisaillon *et al.* 1991)

Tschech and Fuchs (1987; 1989) reported the first evidence of phenol oxidation in denitrifying bacteria via phenol carboxylation to 4-hydroxybenzoate. First, the hypothetical intermediate of phenol degradation, cyclohexanone could not be detected in the phenol degrading cultures nor were cyclohexanone-derivatives degradated. Second, denitrifying bacteria enriched and isolated under anaerobic conditions with cyclohexanol or cyclohexanone were not able to degrade phenol anaerobically. Their experiments have shown that carboxylation of the aromatic ring is the first step in degradation of phenol under denitrifying conditions. Phenol was carboxylated to 4-hydroxybenzoate, followed by coenzyme A activation and dehydroxylation to eliminate the hydroxy substituent to form benzoyl-CoA (Fig 4b). Lack and Fuchs (1992) found that the denitrifying *Pseudomonas* K172 catalyzes the carboxylation of phenylphosphate to 4-hydroxybenzoate and phosphate.

Futhermore, they presented the evidence that phenol phosphorylation to phenylphosphate is the activating step for carboxylation in their strain (Lack and Fuchs 1994). *In vitro* the carboxylating enzyme was inactive with phenol; only phenylphosphate was readily carboxylated as indicated in Fig. 4c.

4. 2. 2. 1. Position of carboxylation

Using monofluorophenols, Genthner *et al.* (1989a) have shown that transformation of phenol to benzoate occurred via *para*-carboxylation followed by dehydroxylation under methanogenic conditions (Fig. 5). The transformation pattern observed with the three isomers of fluorophenol could only result from carboxylation at the position *para* to the phenolic hydroxyl group. 4-Fluorophenol was not transformed since the *para* position was blocked. Using gas chromatography/mass spectroscopy (GC/MS) analysis of deuterated phenol D/H-exchange, Gallert *et al.* (1991) showed that the D/H-isotope exchange was restricted almost exclusively to position 4 of phenol (more than 97 %), and no significant double D/H-exchange from phenol-D_6 was measured (Fig. 6). This proves that the C4-position of phenol is the reaction site for carboxylation. All the isolated methanogenic consortia known (Zhang *et al.* 1990; Gallert *et al.* 1991; Bisaillon *et al.* 1993) and the denitrifying bacteria of Tschech and Fuchs (1989) carboxylate phenol in the *para*-position to the hydroxyl group.



Phenol-D_6 4-hydroxybenzoate Phenol-D_5

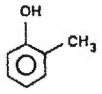
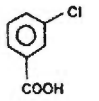
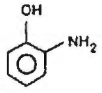
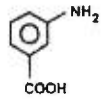
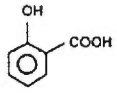
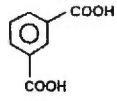
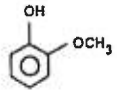
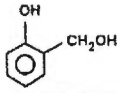
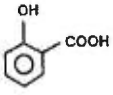
Fig. 6. Phenol-D_6 was carboxylated and decarboxylated to phenol-D_5 in cell-free extracts of a phenol-degrading mixed culture.

The bold letters is the position of D/H-exchange.

4. 2. 2. 2. Spectrum of carboxylating activities

Since anaerobic digestion is a potential method for treating phenolic compounds, determining the spectrum of the carboxylating activity is important. The spectrum for carboxylation and dehydroxylation of phenolic and related compounds of their methanogenic consortium was determined in the presence of phenol by Bisaillon *et al.* (1993). Phenols with *ortho*-substitutions (methyl-, chloro-, fluoro-, bromo-, hydroxyl-, amino-, or carboxyl-) were transformed to *meta*-substituted benzoate (Table 2). However, *meta*- and *para*- substituted phenols (cresols, fluorophenols, and chlorophenols) were not transformed. It was suggested that the substitution position is important. Phenol was most rapidly metabolized, followed by catechol, 2-cresol, 2-fluorophenol, 2-aminophenol, 2-chlorophenol, 2-hydroxybenzoic acid, and 2-bromophenol.

Table 2. Transformation potential of the anaerobic consortium against a variety of phenolic and related compounds in the presence of phenol

Added to the culture medium		Detected in the culture medium		
Chemical	Structure	Chemical	Structure	Reaction
Phenol		Benzoic acid		Carboxylation–dehydroxylation
2-Cresol		3-Methylbenzoic acid		Carboxylation–dehydroxylation
Catechol		3-Hydroxybenzoic acid		Carboxylation–dehydroxylation
2-Fluorophenol		3-Fluorobenzoic acid		Carboxylation–dehydroxylation
2-Chlorophenol		3-Chlorobenzoic acid		Carboxylation–dehydroxylation
2-Bromophenol		3-Bromobenzoic acid		Carboxylation–dehydroxylation
2-Aminophenol		3-Aminobenzoic acid		Carboxylation–dehydroxylation
2-Hydroxybenzoic acid		Isophthalic acid		Carboxylation–dehydroxylation
Anisole		Phenol		O-Demethylation
2-Methoxyphenol		Catechol		O-Demethylation
2-Hydroxybenzyl alcohol		2-Hydroxybenzoic acid		Oxidation

(Bisaillon *et al.* 1993).

m-Cresol seems to be carboxylated by some microorganisms, such as the sulfate-reducing bacterial enrichment of Ramanand and Suflita (1991) and the methanogenic consortium of Roberts *et al.* (1990). Genthner *et al.* (1989a) also observed that some phenols with *meta* substitution position could be carboxylated and dehydroxylated (Fig. 5).

4. 2. 2. 3. Cometabolism

Cometabolism has been used to denote a situation where one microorganism can transform a compound without utilizing it as a source of carbon or energy for growth (Kumaran and Shivaraman 1988).

Jeannin (1986) suggested that phenol was carboxylated by cometabolism in their methanogenic consortium. She found that addition of glucose to the medium enhances phenol carboxylation and the growth of *Clostridium.* Kobayashi *et al.* (1989) also observed that the addition of peptone as a supplemental nutrient to the culture stimulated the methanogenic degradation of phenol. Zhang and Wiegel (1990b) have shown that yeast extract was required for growth of their phenol carboxylating bacteria, and only high concentrations of casein hydrolysate or tryptone could substitute for it. The major carbon source for the growth of their bacteria was amino acids.

Béchard *et al.* (1990) suggested that carboxylation of phenol by their consortium is accomplished by cometabolism under a methanogenic conditions.

Proteose peptone was essential for this transformation to occur. One of the components of proteose peptone or some degradion products might be the carbon and energy source for growth of the phenol carboxylating microorganisms in their consortium. Contrary to the results of Jeannin (1986), glucose did not appear to be implicated. In a subsequent study, Bisaillon *et al.* (1991a) found that yeast extract or a mixture of tryptophan and lysine could replace proteose peptone without affecting the carboxylating activity. This combination of amino acids needed for carboxylation of phenol might indicate that a *Clostridium* sp. carrying out a Stickland reaction was involved (Stickland 1934). A characteristic feature of the Stickland reaction is that single amino acids are not decomposed appreciably, but appropriate pairs are decomposed very rapidly. One member of the pair is oxidized while the other is reduced (Doelle 1975). It is also possible that the carbon and energy sources for the carboxylating bacteria might not be these amino acids but one of their degradation intermediates or a secondary metabolite secreted by one of the bacteria in the consortium. Identification of this substance might aid in the isolation of the carboxylating microorganisms from the methanogenic consortium.

4. 2. 2. 4. Induction of degradative activities

Lack *et al.* (1991) have found that phenol carboxylation activity of denitrifying *Pseudomonas* sp. was induced when cells were grown with phenol, but not with 4-hydroxybenzoate. Comparison of SDS/PAGE protein patterns of *Pseudomonas* sp. cells grown on phenol or 4-hydroxybenzoate revealed several additional protein bands in phenol-grown cells. The residual activity in cells grown

on 4-hydroxybenzoate was less than 1% of the activity in cells grown on phenol (Lack *et al.* 1991). This suggests that phenol rather than 4-hydroxybenzoate acted as an inducer.

The delayed transformation of phenol in cultures inoculated with the consortium previously grown in the absence of phenol revealed that phenol carboxylating-dehydroxylating activity is induced by phenol (Bisaillon *et al.* 1991b). This is further supported by the results they obtained with chloramphenicol, which block *de novo* protein synthesis. The lack of transformation of phenol in the presence of chloramphenicol by cells previously grown in the absence of phenol is due to the inhibition of the synthesis of inducible enzymes (Bisaillon *et al.* 1993).

As observed by Genthner *et al.* (1990), phenol enhanced the rate of 2-fluorophenol transformation to 3-fluorobenzoate and extends the spectrum of carboxylation to 2-cresol. Bisaillon *et al.* (1993) also found that phenol stimulated the rate of carboxylation of 2-cresol whereas 2-cresol delayed the transformation of phenol. These results suggested that phenol is an inducer of the carboxylating activity.

Zhang and Wiegel (1990b) reported the induction of the decarboxylating activity of their methanogenic consortium by 4-hydroxybenzoate. Decarboxylation activity towards 3,4-dihydroxybenzoate was also inducible by 4-hydroxybenzoate or 3,4-dihydroxybenzoate. However, no comparable induction was observed in the

presence of benzoate, phenol, or the individual aromatic amino acids, phenylalanine, tyrosine and tryptophane.

4. 3. Carboxylation and decarboxylation

The carboxylation of phenol and the decarboxylation of 4-hydroxybenzoate have been first reported by Grant and Patel (1969) with the facultative anaerobic *Klebsiella pneumoniae* (syn. *aerogenes*). It also occurs with mixed rumen populations (Martin, 1982), denitrifying *Pseudomonas* (Tschech and Fuchs 1989), an obligate anaerobic phenol-degrading coculture (Gallert and Winter 1992), ferric-, sulfate-reducing microorganisms (Londry and Fedorak 1992), and *Clostridium* (Hsu *et al.* 1990; Zhang and Wiegel 1994).

4. 3. 1. Under methanogenic conditions

When phenol is degraded, 4-hydroxybenzoate is difficult to detect. No hydroxybenzoate intermediate could be observed by Knoll and Winter (1987; 1989) because reductive dehydroxylation of 4-hydroxybenzoate occurs immediately in their mixed consortium (Fig. 7). Direct measurement of phenol carboxylation to 4-hydroxybenzoate was impossible due to a very active reversible decarboxylation activity in cell-free extracts. This was proven by using mass spectrometry analysis of benzoate formed from deuterated phenol (Fig. 6) and $^{14}CO_2$ test (Gallert *et al.* 1991).

Genthner *et al.* (1991) found that 4-hydroxybenzoate was detected in their phenol-degrading consortium using a structural analog of 4-hydroxybenzoate or elevating initial concentrations of phenol or benzoate. This phenol-degrading consortium completely transformed 4-hydroxybenzoate to phenol via decarboxylation. They also found that decarboxylation was more rapid than carboxylation in their methanogenic consortia.

Zhang and Wiegel (1994) reported that the decarboxylation of 4-hydroxybenzoate and 3,4-dihydroxybenzoate are reversible reactions in whole-cell suspensions and in cell extracts of their *C. hydroxybenzoicum*. Furthermore, the apparent equilibrium constant for the reactions 4-hydroxybenzoate$^-$ + H_2O == phenol + HCO_3^- and 3,4-dihydroxybenzoate$^-$ + H_2O == catechol + HCO_3^- were determined to be K_{eq}=11.4 and 5.05, respectively. These values indicate that the formation of the decarboxylation product would be strongly favored. This could explain why no or only trace amounts of 4-hydroxybenzoate were detected in the phenol-degrading enrichment cultures supplemented with low concentrations of phenol. Generally, the reversible activities of phenol carboxylation and 4-hydroxybenzoate decarboxylation are found universally under methanogenic conditions (Knoll and Winter 1989; Béchard *et al.* 1990; Genthner *et al.* 1990; Bisaillon *et al.* 1991a; Londry and Fedorak 1993).

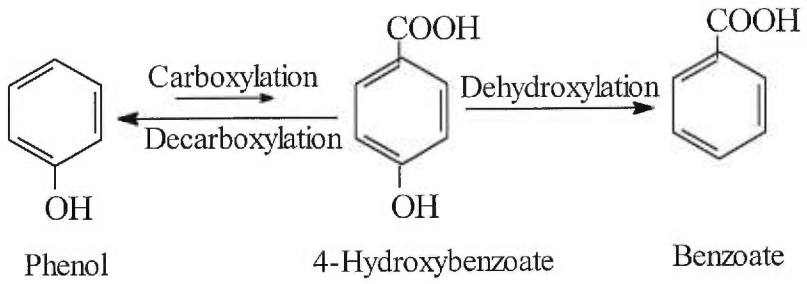


Fig. 7 Transformation of phenol to benzoate via 4-hydroxybenzoate under methanogenic conditions.

4. 3. 2. Under nitrate-reducing conditions

There have been few studies on the carboxylation of phenol under nitrate-reducing conditions. Tschech and Fuchs (1989) found that isotopic exchange between $^{14}CO_2$ and the carboxyl group of 4-hydroxybenzoate is reversible in a denitrifying *Pseudomonas* K172 grown with phenol. If the cells were grown with 4-hydroxybenzoate, there was no isotopic exchange between $^{14}CO_2$ and the carboxyl group of 4-hydroxybenzoate. Consequently, Lack and Fuchs (1992; 1994) presented the evidence that phenol phosphorylation to phenylphosphate is the first step in their phenol-degrading strain, because this carboxylating activity needs phenol phosphorylation to activate phenol (Fig.4c). Actually, only phenylphosphate can be carboxylated to 4-hydoxybenzoate as described in the carboxylation pathway.

4. 3. 3. Under ferric- and sulfate-reducing conditions

Lovely and Lonergan (1990) described the carboxylation of phenol to 4-hydroxybenzoate by an isolate, designated GS-15, which used Fe^{+++} as its terminal

electron acceptor. 4-Hydroxybenzoate was detected as a transient intermediate in this culture.

Bak and Widdel (1986) described a new sulfate reducer, *Desulfobacterium phenolicum* that degraded phenol and 4-hydroxybenzoate. Although the pathway was not studied, 4-hydroxybenzoate could be used, which suggested that phenol might be metabolized via *para*-carboxylation. *Desulfobacterium anilini* isolated by Schnell and Schink (1991) required CO_2 to metabolize phenol and aniline. The intermediates of phenol metabolism were not studied, however, aniline was *para*-carboxylated to aminobenzoate.

5. Microorganisms implicated in phenol carboxylation and 4-hydroxybenzoate decarboxylation under methanogenic conditions

5. 1. Consortia

Kobayashi *et al.* (1989) acclimated a methanogenic consortium from sludge. It degraded phenol via carboxylation. However, they did not identify the microorganisms in their consortium.

Genthner *et al.* (1989d) characterized an anaerobic consortium that mineralize phenol via its transformation to benzoate. It contained five Gram-negative cell types: coccobacilli, often in pairs; rods similar to *Methanospirillum* sp. and *Methanothrix* sp.; small rods with rounded ends; and large ovoid cocci. Most

probable number analysis of the original consortium indicated that it contained 1.1 X 10^7 cells/mL of benzoate-degrading bacteria and 1.1 X 10^8 cells/mL of phenol-degrading bacteria. At the 10^{-8} dilution, phenol was transformed to benzoate without mineralization of benzoate (Genthner *et al.* 1989c). The strain responsible for this transformation was never isolated by these authors.

Knoll and Winter (1987) reported the selection and improvement of an anaerobic phenol-degrading consortium from sewage sludge. Benzoate was formed from phenol, CO_2 and H_2. They failed to isolate a pure culture degrading phenol since an obligate syntrophic interdependence was observed for the two different rod-shaped bacteria and the methanogen during feeding with either 4-hydroxybenzoate, phenol or benzoate (Knoll and Winter 1989). Their consortium consisted of a short and a long rod-shaped bacterium, a low number of *Desulfovibrio* cells and the methanogen *Methanospirillum hungatei*. From feeding experiments, they concluded that the long rod-shaped bacterium could be the carboxylating microorganism and it would require an as yet unknown cosubstrate or cofactor, probably cross-fed by the short, motile rod. The short motile rod-shaped bacterium grew only in syntrophy with methanogens and benzoate was degraded to acetate, CO_2 and methane in their consortium.

Beaudet *et al.* (1986) isolated an anaerobic consortium with the ability to transform phenol and *p*-cresol under methanogenic conditions. Béchard *et al.* (1990) enriched this methanogenic consortium, which can degrade phenol via carboxylation

to benzoate (Bisaillon *et al.* 1991a). The consortium was composed of various microbiological forms dominated by Gram-negative rods in a 20-day-old culture. Gram-positive coccobacilli, many spores, and free flagellas were also observed. Minor changes in this mixed population were seen if the incubation period of the culture was shortened. Phenol-carboxylating microorganisms were evaluated to about $\geq 1 \times 10^8$ cells/mL by using diluted inocula since this activity still existed even when the inoculum was diluted as much as 8^{-9}. Using plate count, anaerobic bacteria were estimated to 2×10^6 cfu/mL and facultative bacteria to 7×10^2 cfu/mL. Aerobic Gram-negative bacteria were not found in this consortium (Béchard *et al.* 1990). Using electron microscopy, Bisaillon *et al.* (1991a) observed that this consortium growing in the presence of phenol and proteose peptone had seven different morphological bacterial types. Replacement of proteose peptone with tryptophan and lysine reduced the morphological types to six. When the consortium was inhibited by 2-bromoethanosulfonic acid (BESA), the long-chain of large rods were diluted out suggesting that they were methanogens. Thus, only five morphological types remained in the consortium and they were cocci, long-rods, pointed-end coccobacilli and two types of short rods. Using this consortium, Létourneau *et al.* (1995) have shown that spore-forming bacteria are implicated in the carboxylation of phenol because it remained active after treatment at 80°C for 15 min. They isolated five macroscopically different strains on Columbia blood agar from their heat-treated consortium. Four of these were *Clostridium.* The other one was associated to the genus *Fusobacterium.* None of the strains isolated was able to carboxylate phenol alone. They suggested two possibilities: 1) the carboxylating

microorganism does not grow on solid medium or grows more slowly than the five isolated strains; 2) the carboxylating microorganism is one of those five isolated strains but the proper conditions to show its activity were not found.

5. 2. Pure cultures

In 1990, Zhang and Wiegel (1990a) isolated a consortium degrading 2,4-dichlorophenol from freshwater sediment. After dechlorination, phenol was carboxylated to benzoate, and benzoate was degraded via acetate to methane and CO_2. At least five different groups of microorganisms were involved in this process. They isolated these groups and obtained different cultures exhibiting the respective activities. They found that the microorganisms of their consortium were not syntrophic. It is thus different from the syntrophic consortium obtained by Knoll and Winter (1989). The microorganisms of group 3, which contained two types of rods and a *Methanospirillum hungatei*-like organism, carboxylated phenol to benzoate without further degradation of benzoate. However, this culture still contained the microorganisms responsible for the decarboxylation of 4-hydroxybenzoate to phenol. From group 3, they isolated and characterized a sporeforming, strict anaerobic bacterium, *C. hydroxybenzoicum* strain JW/Z-1^T, capable of decarboxylating 4-hydroxybenzoate to phenol (Zhang and Wiegel 1990a; 1990b; Zhang *et al.* 1994). It is a nonmotile, Gram-positive type, spore-forming, amino acid-utilizing, strictly anaerobic, and slightly curved rod that is 0.35-0.67 X 2.5-5.1 μm in size. Sporulating cells elongate to between 5 and 8 μm long and produce

terminal swollen sporangia. The phenol produced is not further metabolised by this strain. Thus phenol was the final product of this pure culture.

This is the first microorganism isolated in pure culture from a methanogenic consortium with the capacity to decarboxylate 4-hydroxybenzoate. Up to now, no pure microorganism has been isolated from a methanogenic consortium for its ability to carboxylate phenol or transform phenol to benzoate.

6. Enzymatic studies of phenol carboxylase and 4-hydroxybenzoate decarboxylase under anaerobic conditions

6. 1. Cell-free extracts from mixed cultures

The first report on a 4-hydroxybenzoate decarboxylase activity was presented by Grant and Patel in 1969. They found an oxygen sensitive 4-hydroxybenzoate decarboxylase activity from the facultative anaerobe *Klebsiella pneumoniae* (syn. *aerogenes*). This activity in the cell extract had an optimum pH of 6.0, an activation energy of 25,500 cal/mol at 28-38°C, and a K_m of 4 mM (Grant and Patel 1969).

Gallert *et al.* (1991) used a cell-free extract from a strictly anaerobic, define methanogenic mixed culture to study phenol carboxylation activity. They found that phenol carboxylation and 4-hydrobenzoate decarboxylation were reversible by using deuterated phenol and GC/MS (see Figure 6). Direct measurement of phenol

carboxylase activity in the cell-free extract was impossible due to a very active interfering reverse decarboxylase activity. In this extract, the carboxylase activity could only be measured by means of D/H-exchange tests (Gallert *et al.* 1991). Two 4-hydroxybenzoate decarboxylase activities and one phenol carboxylase activity were found in this cell-free extract (Gallert *et al.* 1991; Gallert and Winter 1992).

The optimum pH of the phenol carboxylase was 6.5 in phosphate buffer. Addition of 5 mM of the metal chelator EDTA to the test assay reduced the phenol carboxylase activity by 60%. If Mg^{++}, Mn^{++} or Zn^{++} was added, the inhibitory effect of EDTA could be reversed. Ca^{++} had not effect on restoration of phenol carboxylase activity. The requirement of K^+ for carboxylase activity was demonstrated indirectly with the K^+ chelator valinomycin (Gallert *et al.* 1991). The specific activity of the carboxylase was 1-2 μmol/min/mg-protein. The K_m for phenol was 0.125 mM (Gallert and Winter 1992).

Both decarboxylase activities were loosely membrane-associated. Addition of 0.2% of cetyltrimethylammonium bromide liberated the decarboxylase activities from the membranes. Both decarboxylase activities required K^+ but had different optimum pH (6.4 and 7.8) and ionic strength dependencies (Gallert and Winter 1992). The specific activities of the two enzymes for 4-hydroxybenzoate were 960 μmol/min/mg-protein at pH 6.4 and 760 μmol/min/mg-protein at pH 7.8, respectively. The partial loss of activity of both decarboxylases with EDTA could be compensated by Zn^{++}. The K_m values of the decarboxylases for 4-hydroxybenzoate

were 2.5 and 0.5 mM, respectively. In addition to 4-hydroxybenzoate, both decarboxylase activities transformed only 4-hydroxybenzoate derivatives that did not contain an *ortho*-substituent such as 3,4-dihydroxybenzoate, 3,4,5-trihydroxybenzoate, 3,5-dimethoxy-4-hydroxybenzoate and 3-chloro-4-hydroxybenzoate.

6.2. Cell extracts from pure culture

Phenol carboxylase has been well studied in cell-free extract of a denitrifying *Pseudomonas* strain K 172, which catalyzes an isotope exchange between $^{14}CO_2$ and the carboxyl-group of 4-hydroxybenzoate under anaerobic conditions (Tschech and Fuchs 1989; Lack *et al.* 1991; Lack and Fuchs 1992; 1994). The carboxylase requires Mn^{2+} and K^+ for activity (Tschech and Fuchs 1989). The activity was induced by phenol, but not by 4-hydroxybenzoate. The pH optimum was 7.0 with a specific activity of 100 nmol $^{14}CO_2$ exchange to carboxyl-group/min/mg-protein (Lack *et al.* 1991). The carboxylase activity is very sensitive to oxygen, with a half-life time in air of 30 seconds. Molecular mass is 280 kDa, which consists of three different subunits. It was not active with phenol; only phenylphosphate was the substrate of the carboxylase as mentioned before (Fig 4c). This carboxylase is *para*-specific (Fuchs *et al.* 1994).

6. 3. Purified enzymes

Recently, He and Wiegel (1995) have purified and characterized an oxygen-sensitive reversible 4-hydroxybenzoate decarboxylase from the obligate anaerobe, *C. hydroxybenzoicum* strain JW/Z-1^T. The molecular mass of this enzyme was 350 kDa and consisted of six identical subunits of 57 kDa. The purified enzyme specific activity was 8.29 μmol/min/mg-protein. The temperature optimum for the decarboxylation was approximately 50°C, and the optimun pH 5.6-6.2. The pI of the enzyme was 5.1. About 50 % of the activity was lost after an exposition to air for two hours at 5°C. In crude extracts, the activity had a half-life of less than 6 hours at 4-7°C under anaerobic conditions, but the purified enzyme was stable for more than 24 hours at 11-40°C, pH 6.0. Addition of Zn^{++} (as the sulfate salt) almost completely abolished the activity. The enzyme also catalyzed decarboxylation of 3,4-dihydroxybenzoate to catechol. The apparent K_m and k_{cat} values obtained for 4-hydroxybenzoate were 0.40 mM and 3.3 X 10^3 min^{-1}, and for 3,4-dihydroxybenzoate were 1.2 mM and 1.1 X 10^3 min^{-1}, respectively, at pH 6.0 and 25°C. The *p*-hydroxyl group of hydroxybenzoate appears to be essential for binding to the enzyme because it only decarboxylates benzoate derivatives that contain a hydroxyl group in that position. *p*-Hydroxybenzoates substituted (hydroxyl, methyl or halogen) in the *meta*-position are decarboxylated but not these substituted in the *ortho*-position. The enzyme catalyzed the reverse reactions: the carboxylation of phenol to 4-hydroxybenzoate and of catechol to 3,4-dihydroxybenzoate. The carboxylation did not require ATP. The N-terminal amino acid sequence showed some similarity with

the uroporphyrinogen decarboxylase. This is the first report on the purification of a 4-hydroxybenzoate decarboxylase or a corresponding phenol carboxylase.

Subsequently, He and Wiegel (1996) purified and characterized an oxygen-sensitive reversible 3,4-dihydroxybenzoate decarboxylase from the same strain (*C. hydroxybenzoicum*). The estimated molecular mass of the enzyme was 270 kDa with five identical subunits 57 kDa. The temperature and pH optima were 50°C and 7.0, respectively. The K_m and k_{cat} for 3,4-hydroxybenzoate were 0.6 mM and 5.4 x 10^3 min^{-1}, respectively, at pH 7.0 and 25°C. The half-life of the activity was 1.5 hours at 55°C. The enzyme catalyzed the reverse reaction, that is, the carboxylation of catechol to 3,4-dihydroxybenzoate. In contrast to the 4-hydroxybenzoate decarboxylase from the same strain (He and Wiegel 1995), this enzyme exhibited a remarkable substrate specificity. It did not decarboxylate 4-hydroxybenzoate or other benzoate derivatives. The addition of KCl, $MnCl_2$, or EDTA did not influence the activity. However, 2 mM $ZnSO_4$ decreased the activity by 25 %, and 0.5 mM $HgCl_2$ completely destroyed the activity. ATP did not affect the activity. The N-terminal amino acid sequence is different from the 4-hydroxybenzoate decarboxylase from the same strain.

A comparison of the properties of a few hydroxybenzoate decarboxylases is listed in Table 3. Contrary to the preceding enzymes reviewed the one produced by some *Pseudomonas* and fungi were not sensitive to oxygen.

Table 3. Comparison of properties of hydroxybenzoate decarboxylases

Decarboxylase	Organism	Pure enzyme	M.W. (kDa)	Subunits (kDa)*	K_m (mM)	Optimum pH	Optimum T °C	Oxygen sensitivity	Other substrate	Reference
4-hydroxybenzoate decarboxylase	*Clostridium hydroxybenzoicum*	Yes	350	6X57	0.40	5.6 - 6.2	50	Yes	3,4-Dihydroxybenzoate 3-F-4-hydroxybenzoate	He and Wiegel, 1995
4-hydroxybenzoate decarboxylase	*Klebsiella pneumoniae*	No	ND	ND	4	6.0	43	Yes	3,4-Dihydroxybenzoate 2,5-Dihydroxybenzoate 3,4,5-Trihydroxybenzoate	Grant and Patel, 1969
4-hydroxybenzoate decarboxylase	Methanogenic consortium	No	ND	ND	2.5	6.4	41	Yes	4-OH-Benzoate 3,4-Dihydroxybenzoate	Gallert and Winter, 1992
		No	ND	ND	0.5	7.8	3.7	Yes	3,4,5-Trihydroxybenzoate *et al.*	
4-hydroxybenzoate decarboxylase	*Clostridium thermoaceticum*	No	ND	ND	3.33	6.0	50	Yes	4-Hydroxybenzoate 3,4-Dihydroxybenzoate *et al.*	Hsu *et al.*, 1995
3,4-Dihydroxybenzoate decarboxylase	*Clostridium hydroxybenzoicum*	Yes	270	5X57	0.59	7.0	50	Yes	Not found	He and Wiegel, 1996
2,3-Dihydroxybenzoate decarboxylase	*Trichosporon cutaneum*	Yes	66.1	2X36.5	0.037	7.7	ND	No	2,3,5-, 2,3,6-Trihydroxybenzoate	Anderson and Dagley, 1981
	Aspergillus niger	Yes	120	4X28	0.34	5.2	42	No	Not found	Klamath *et al.*, 1987
	Aspergillus oryzae	Yes	150	4X38	0.42	5.2	ND	No		Santha *et al.* 1996
4,5-Dihydroxybenzoate decarboxylase	*Pseudomonas testosertoni*	Yes	150	4X38	0.011	7.5	ND	No	4-hydroxybenzoate	Nakasawa and Hayashi, 1978
	Pseudomonas fluorescens	Yes	420	6X66	0.01	6.8	ND	No		Pujar and Gibson, 1985

* : Number of subunits X subunit mass. ND: Not detected.

III. PAPER 1

Isolation and Characterization of a New Bacterium Carboxylating Phenol to Benzoic Acid under Anaerobic Conditions.

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ABSTRACT

A consortium of spore-forming bacteria transforming phenol to benzoic acid under anaerobic conditions was treated with antibiotics to eliminate the four *Clostridium* strains which were shown unable to accomplish this reaction in pure culture and coculture. *C. ghonii* was inhibited by chloramphenicol (10 μg/ml) whereas *C. hastiforme* (strain 3) and *C. glycolicum* were inhibited by clindamycin (20 μg/ml), without the transformation of phenol being affected. Electron microscopic observations of resulting liquid subcultures revealed the presence of two different bacilli: a dominant *C. hastiforme* (strain 2) (width 1 μm) and an unidentified strain 6 (width 0.6 μm) which was not detected on solid medium. Bacitracin (0.5 U/ml) changed the ratio of the strains in favour of strain 6. *C. hastiforme* strain 2 was eliminated from this culture by dilution. The isolated strain 6 transformed phenol to benzoic acid and 4-hydroxybenzoic acid to phenol and benzoic acid in the presence of proteose peptone. Both of these activities are inducible. This strain is a gram-variable flagellated rod with a doubling time of 10 -11 h in the presence of phenol. It has a cellular fatty acid composition like that of *Clostridium hastiforme*. However, strain 6 does not hydrolyse gelatin nor produce indole. The 16S rRNA sequence of strain 6 was found to be most similar to that of some *Clostridium* species, with homology ranging from 80 to 86%. The evolutionary relationships of strain 6 to different groups of *Clostridium* and *Clostridium*-related species revealed that it does not emerge from any of these groups. Strain 6 most likely belongs to a new species closely related to *Clostridium* species.

Key words : Isolation, characterization, carboxylation, phenol, anaerobic, *Clostridium*-like bacteria

INTRODUCTION

The transformation of phenol to benzoic acid by methanogenic consortia has been the subject of many studies. However, this transformation is carried out by bacterial consortia which have been only partially characterized. From the results of feeding experiments and kinetic studies, Knoll and Winter (16) suggested that a long nonmotile, gram-negative rod was responsible for the carboxylation of phenol to benzoic acid by their methanogenic consortium in their study. Sharak-Genthner *et al.* (23) found five gram-negative cell types of different shapes in their phenol carboxylating methanogenic consortium. Zhang and Wiegel (28) have shown that their phenol carboxylating consortium grown in the presence of yeast extract was composed of two types of rods and an organism resembling *Methanospirillum hungatei.*

Zhang and Wiegel (27, 29) have isolated from their consortium *Clostridium hydroxybenzoicum,* which decarboxylated 4-hydroxybenzoic acid to phenol but could not further metabolize phenol. However, with resting cell suspensions and cell extracts of this strain, Zhang and Wiegel observed reversible conversion of 4-hydroxybenzoic acid and phenol (30). This is the only microorganism isolated from a methanogenic consortium that was shown to be able to carboxylate phenol. Recently, Létourneau *et al.* (17) have shown the involvement of spore-forming bacteria in the carboxylation of phenol in their methanogenic consortium. However, none of the strains isolated was

able to carboxylate phenol in pure culture or coculture, nor could the strains decarboxylate 4-hydroxybenzoic acid.

The difficulty in working with a methanogenic consortium arises from the fact that these bacteria are part of interdependent trophic groups, which makes the isolation of one particular strain more problematic. Under denitrifying conditions, *pseudomonads* responsible for the carboxylation of phenol were more readily isolated (24). Also, *Desulfobacterium phenolicum* was shown to metabolize phenol and 4-hydroxybenzoate under sulfate reducing conditions (1).

The present study focused on the isolation of the carboxylating microorganism in subcultures of the heat treated anaerobic consortium of Létourneau *et al.* (17). This was performed by using different antibiotics to eliminate unnecessary strains. Different microbiological and biochemical tests were done together with 16S rRNA sequence analysis to characterize this strain.

MATERIALS AND METHODS

Microorganisms

The anaerobic consortium of Létourneau *et al.* (17) heated at 80°C for 15 min was used in this study. It was maintained by serial transfers in minimal medium of Boyd *et al.* (6) supplemented with 1.6 to 2.1 mM phenol (Baker Chemical Co., Phillipsburg, N.J.) and 0.05% (wt/vol) proteose peptone no 3 (Difco Laboratories, Detroit, Mich.) using a 12.5% (vol/vol) inoculum as described previously (4). The four *Clostridium* strains (*C. ghonii* strain 1, *C. hastiforme* strains 2 and 3, *C. glycolicum* strain 5) isolated by Létourneau *et al.* (17) from the anaerobic consortium were used and maintained by successive transfers on Columbia Blood Agar. Strain 6, which was isolated in the present study, was maintained in Boyd's minimal liquid medium supplemented with phenol and 0.5% (wt/vol) proteose peptone. In all experiments, duplicate cultures were incubated at 37° C.

Antibiograms

Antibiograms were done with the four *Clostridium* strains isolated by Létourneau *et al.* (17) from the heated consortium. These strains were inoculated on Columbia Blood Agar to obtain confluent growth. Before incubation, a series of discs (Becton and Dickerson Microbiology System, Maryland) containing different antibiotics were placed on the surface of the solid media. These were incubated in an anaerobic jar for 48 hours before the sensitivity of the strains was determined.

Isolation of the carboxylating strain

On the basis of the results of the antibiograms, different concentrations of chloramphenicol (Sigma Chemical Co., Missisauga, Canada) were tested in liquid cultures to eliminate unnecessary strains from the consortium. Control cultures without antibiotics were used. Counts (CFU per milliliter) on Columbia Blood Agar following ten fold serial dilutions of the cultures were done after 72 h of incubation to determine the activity of the antibiotics on the strains. Clindamycin phosphate (Sigma) was also used because some *Clostridium* strains have been reported to be resistant to this antibiotic (8). It was added at different concentrations up to 100 μg/ml to subcultures of the culture treated with 10 μg chloramphenicol per millilitre. The resulting subcultures of the culture treated with 20 μg clindamycin per mL were then incubated with different concentrations of bacitracin.

Solid (1% [wt/vol] agar) and semisolid (0.4% agar) Boyd's media were supplemented with phenol (2 mM) and proteose peptone (5% [wt/vol]) and cysteine (0.5 g/L) had been substituted for Na_2S. A 10^{-6} dilution of the culture treated with 0.5 U of bacitracin per ml was used as an inoculum for these media. The strain 6 maintenance liquid culture originated from a colony growing in the semisolid medium.

Electron microscopic observations

Electron microscopic observations were done with a model 7100 (Hitachi, Tokyo, Japan). The microorganisms were first fixed with 1 to 2% glutaraldehyde and

then negatively stained with 2% phosphotungstate (3). In the bacitracin experiment, the proportion of each of the strains present was determined by estimating at least 20 randomly selected fields for each culture.

Activities of the isolated strain

Strain 6 from the maintenance culture was inoculated (12.5% [vol/vol]) in the liquid medium supplemented with 0.5% (wt/vol) proteose peptone and 2 mM phenol or 4-hydroxybenzoic acid. The test compounds and their metabolites were periodically analyzed. Also, 10-fold dilutions were plated on Boyd's solid medium supplemented with phenol (2mM) and proteose peptone (0.5% [wt/vol]) to follow the growth of strain 6 in these cultures. In some cultures with phenol, proteose peptone was omitted from the liquid medium and replaced by yeast extract (0.5% [wt/vol]) (Gibco, Grand Island, N.Y.) or a mixture of twenty one amino acids (0.024% [wt/vol] each) (Sigma).

The inducibility of the carboxylating activity of strain 6 was determined by a procedure similar to the one previously described for the original consortium (5). After growth of the strain with and without phenol in Boyd's medium supplemented with proteose peptone, the cells from both cultures were centrifuged, washed, and resuspended in fresh medium containing phenol (2 mM) with and without chloramphenicol (250 μg/ml) (Sigma). A similar experiment was done with 4-hydroxybenzoic acid (Sigma) to determine the inducibility of the decarboxylating activity of strain 6.

Analytical methods

The test compounds and their metabolites in all the cultures were periodically analyzed in duplicate by gas chromatography (GC). Extracts from acidified culture fluids were concentrated, derivatized, and analyzed by GC using a 25-m HP-5 capillary column (Hewlett Packard, Arondale Pa.) as described previously (4). Metabolites were identified by GC-mass spectrometry with an Ion Trap 800 (Finnigan, San Jose, Calif.) mass spectrometer, using a GC column similar to the one described above (4).

Characterization of strain 6

Strain 6 was characterized with phenotypic and cellular fatty acid (CFA) composition analyses. All biochemical tests were performed as described by Holdeman *et al.* (13). The strain was grown in prereduced, anarobically sterilized medium (PRAS) containing Tween (Carr-Scarborough, Decatur, Ga) and CFAs were extracted as described by Moore *et al.* (18). The samples were run on the Microbial Identification System (MIS, Newark, Del.) and analyzed against the commercial data library Moore ver 3.8.

Extraction of bacterial total DNA

Liquid cultures of strain 6 and of *C. hastiforme* strain 2 were centrifuged at 5000 x g for 10 min. The pellets were washed with phosphate-buffered saline (PBS) and centrifuged. Strain 6 cells that grew on the supplemented solid medium were recovered,

washed with PBS, and centrifuged. Bacterial pellets were resuspended in TEN (50 mM Tris-HCl [pH 8.0], 20 mM EDTA, 150 mM NaCl) containing lysosyme (5 mg/mL) (Boehringer Mannheim, Laval, Canada) and incubated 30 min at 37°C. Sodium dodecyl sulfate and β-mercaptoethanol were added to a final concentration of 0.5% (wt/vol) and 1% (vol/vol), respectively. The suspensions were frozen at -70°C in dry ice-ethanol for 10 min and thawed at 65° C. This step was repeated twice. The extracts were incubated at 45°C for 2 to 3 h with proteinase K (50 μg/mL) (Boehringer Mannheim). DNA was extracted once with phenol (saturated with Tris-HCl pH 8.0), once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated with ethanol and resuspended in TE (1 mM Tris-HCl [pH 8.0], 0.1 mM EDTA). DNA was treated with 1 μg of RNase A (Boehringer Mannheim) for 15 min at 37°C.

PCR amplification of the 16S rRNA

Primers (5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGAT-CCAGCCGCA-3') representing the extremities of all eubacterial 16S rRNA genes (7) were synthesized with a Gene Assembler Plus (Pharmacia, Baie d'Urfé, Canada). PCRs were carried out in the Gene ATAQ controller (Pharmacia) with 50-μl reaction mixtures containing 100 ng of total DNA, deoxynucleoside triphoshates (200 μM each), *Pfu* DNA polymerase buffer (20 mM Tris HCl [pH 8.75], 10 mM KCl, 10 mM $(NH4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg of bovine serum albumin per ml), 10 pmole

of each primer, and 2,5 U of *Pfu* DNA polymerase (Stratagene, La Jolla, Ca). This mixture was heated at 80° C for 2 min before the addition of the DNA sample. The PCRs were started at 94°C for 5 min and 55°C for 5 min, followed by 30 cycles of 72°C for 2 min, 94°C for 40 s, and 55°C for 1 min. This was followed by one cycle at 72°C for 10 min.

Cloning and sequencing of the 16S rRNA gene of strain 6

The PCR product for strain 6, a unique fragment of 1.5 kb, was treated with proteinase K (Boehringer Mannheim) and the large fragment of *Escherichia coli* DNA polymerase (Pharmacia) as described by Hitti and Bertino (12). This fragment was cloned in a Bluescript vector (Stratagene) at the *SmaI* site as described by Sambrook *et al.* (21).

Several clones containing a 1.5 kb insert were isolated, and two of them were sequenced completely. Different parts of the 1.5-kb insert were subcloned into the Bluescript plasmid vector at appropriate restriction sites, using *E. coli* MV1190 as a bacterial host. Single-stranded DNA were generated with M13K07 helper phage (21). Both strands of the 16S sequences were sequenced by the dideoxynucleotide method (22).

DdeI restriction endonuclease (Pharmacia) was used as recommended by the supplier. Electrophoretic agarose gels were made in 40 mM Tris-acetate (pH 7.2), 1 mM EDTA. In some experiments, *Sau 3A1* restriction endonuclease was also used.

Phylogenetic study

The FASTA and PILEUP programs (Genetics Computer Group Inc.) were used to find 16S rRNA sequences similar to that of strain 6. Phylogenetic analysis were carried out with different programs on the PHILIP package 3.5 (10). The 16S sequences of 15 *Clostridium* species and of *E. coli* were aligned with the 16S sequence of strain 6 by using the PILEUP program. The 1,522 unambiguously alignable nucleotide sites from the 17 sequences were used to do a pairwise comparison using the DNADIST program with the Kimura 2-parameter (15). A distance matrix representing the number of substitutions per site for each pairwise comparison was generated. The FITCH program was used to derive the best phylogenetic tree. A bootstrap of 500 replicates was also derived with the SEQBOOT program. A distance matrix for each replicate was calculated with the DNADIST program with the Kimura 2-parameter and the FITCH program and was used to generate the best tree for each replicate. The CONSENSE program was used to derive the consensus tree. The neighbor-joining method or the unweighted pair-group means analysis method to derive the best phylogenetic tree from the distance matrix, and the parsimony (DNAPARS program) and the maximum-likelihood (DNAML program) methods were also used.

RESULTS

Antibiograms

All the strains were resistant to streptomycin (10 μg), neomycin (5 μg), and gentamycin (10 μg) and sensitive to chloramphenicol (30 μg) and bacitracin (10 U). However, the susceptibilities to erythromycin (15 μg), ampicillin (10 μg), penicillin (10 U) and tetracycline (50 μg) varied from strain to strain (data not shown).

Isolation of the carboxylating strain

At a concentration of 10 μg chloramphenicol per ml, most of the phenol in the liquid medium was transformed after 36 days of incubation but at a higher concentration (100 μg/mL) no transformation was observed. In each of these cultures *C. ghonii* was eliminated and the three other strains were not inhibited by the antibiotic.

The resulting subculture from the culture treated with chloramphenicol (10 μg/ml) transformed phenol in presence of clindamycin (up to 100 μg/mL). However, *C. hastiforme* 3 and *C. glycolicum* were inhibited after 15 days of incubation with ≥ 5 μg clindamycin per ml. Only *C. hastiforme* 2 grew in these cultures. The addition of clindamycin (250 μg/mL) slowed or inhibited phenol transformation without affecting *C. hastiforme* 2. Pure cultures of this strain did not carboxylate phenol nor decarboxylate 4-hydroxybenzoic acid.

Phenol (1.9 mM) was completely transformed to benzoic acid in the liquid media inoculated with a 10^{-6} dilution of the clindamycin (20 μg/ml) treated culture. These cultures showed only *C. hastiforme* 2 colonies when inoculated on Columbia blood agar. However, electron microscopic observations of these cultures revealed the presence of two different rod shaped bacteria (Fig. 1A). *C. hastiforme* 2, which grows on solid medium, is the larger bacillus (1 μm wide). An unidentified bacillus (strain 6) with a width of about 0.6 μm, which was not observed on solid medium, was also present. The ratio of the strains in a 21-day-old culture favored *C. hastiforme* 2 by approximately 5 to 1.

Bacitracin at a concentration as low as 0.5 U/ml inhibited the transformation of phenol by the clindamycin (20 μg/ml) subculture. This treatment changed the ratio between strain 6 and *C. hastiforme* 2 so that strain 6 became dominant. When this culture was diluted to 10^{-6} and inoculated in the minimal liquid medium supplemented with 0.5% (wt/vol) proteose peptone, *C. hastiforme* 2 was not present in the resulting culture, as confirmed by electron microscopy (Fig. 1B) and by the absence of growth on Columbia blood agar. Strain 6 was the only bacillus observed in these cultures and phenol was transformed to benzoic acid.

When these cultures were inoculated on minimal solid medium supplemented with 0.5% (wt/vol) proteose peptone and phenol (2mM), a single-colony morphology

was observed after 4 days of incubation. The colonies were small, grey, and up to 0.5 mm in diameter. Strain 6 was also able to grow on Columbia blood agar but only if the inoculum came from the solid medium described above and not from the liquid medium. However, this growth was slower than on the supplemented minimal solid medium. Phenol was transformed when the entire biomass growing on the supplemented minimal solid medium was recovered and inoculated in the liquid medium, but not when a single colony was used as the inoculum.

Semi-solid medium was similarly inoculated. Some of the colonies growing in this medium transformed phenol when inoculated in the liquid medium. These cultures were replicated and gave rise to strain 6 maintenance cultures.

Restriction endonuclease analysis of the 16S r RNA

DNA fragments of 1.5 kb were obtained from the amplification by PCR of the 16S ribosomal sequence of strain 6 and *C. hastiforme* 2 grown in their respective maintenance cultures. The patterns observed for these fragments after digestion with restriction endonuclease *DdeI* were different (Fig. 2, lane 1 and 4) suggesting that these strains are different. Similar DNA fragments were also obtained from strain 6 growing on the supplemented minimal solid medium, and in the supplemented minimal liquid medium after inoculation from the solid medium. The patterns observed for these fragments after digestion with *DdeI* endonuclease were identical to that obtained for strain 6 grown in the maintenance culture (Fig. 2, lane 1 to 3). Similar results were

obtained after digestion with restriction endonuclease *Sau 3A1* (data not shown). This confirms that the bacterial cells obtained from the different cultures were from strain 6.

Activities of the isolated strain

In Boyd's liquid medium supplemented with 0.5% (wt/vol) proteose peptone, strain 6 grew from 10^4 to 10^7 CFU/ml in 7 days and completely transformed phenol to benzoic acid after 14 days of incubation (Fig. 3A). In this culture, the doubling time of strain 6 in the log phase of growth was 10 to 11 h. In the absence of proteose peptone, growth and carboxylating activity were not observed. Yeast extract (0.5% [wt/vol]) or a mixture of 21 amino acids (0.024% [wt/vol] each) could efficiently replace proteose peptone. Strain 6 was able to transform 4-hydroxybenzoic acid to phenol and benzoic acid after only 4 days of incubation in the presence of proteose peptone (Fig. 3B). Under these conditions, its doubling time was only 5 to 6 h.

The resuspended cells from cultures grown with 4-hydroxybenzoic acid exhibited a faster transformation of 4-hydroxybenzoic acid than the cells from cultures grown without this compound (Table 1). In the presence of chloramphenicol, only the cells previously grown with 4-hydroxybenzoic acid transformed this compound. Similar results were obtained in the phenol experiment (data not shown).

Characterization of strain 6

Strain 6 is a gram-variable motile anaerobic bacillus. It grows on supplemented brain heart infusion agar after 2 days of incubation under anaerobic conditions but not aerobically, in CO_2 or under microaerophillic conditions. Colonies were circular, slightly convex, grey, opaque, and mottled. Strain 6 has a CFA composition like that of *C. hastiforme* with a similarity index of 0.33 to 0.635 after growth in PRAS containing Tween. However, it can be differentiated from this species since indole is produced and gelatin is not hydrolysed. Carbohydrates (fructose, glucose, lactose, maltose, mannitol, mannose, melibiose, raffinose, ribose, salicin, starch , sucrose, xylose) are not fermented even when supplemented with serum, which is consistent with *C. hastiforme* as well as a number of other species of *Clostridium*. Esculin was not hydrolysed, milk and meat were not digested, nitrate was not reduced, oxidase, catalase, lecithinase and lipase were not produced and hemolysis was not observed. Electron microscopic observations revealed that the bacilli were flagellated (Fig. 1B) and their aspect was consistent with gram-positive bacteria. Spores were not observed but when grown on Wagenaar and Dack medium the extremities of some cells were distended. The maintenance culture was not heat resistant (70° C for 10 min) but strain 6 and 2 cocultures (from the 20 μg clindamycin per ml treated culture) were resistant to a treatment of 80° C for 15 min.

Phylogenetic study of strain 6

The sequencing of two clones revealed that each had an insert of 1,514 nucleotides corresponding to 16S rRNA genes of strain 6. These two sequences were identical except for seven differing nucleotides clustered in two short regions (positions 64 to 68 and 88 to 93). Another clone was sequenced at this particular region and the same differences were found, suggesting that the two sequences are from different 16S rRNA genes. No identical 16S rRNA sequence was found in gene banks. However, 34 of the 50 most similar sequences were from *Clostridium* species, with identity ranging from 80 to 86%. Several progressive, pairwise alignments with the strain 6 16S rRNA sequence and sequences of single representatives of different families and genera of bacterial species (19) were made with the PILEUP program. These analysis also showed that strain 6 was closely related to *Clostridium* species.

The evolutionary relationship of strain 6 to different *Clostridium* and *Clostridium*-related species was determined by 16S rRNA sequence analysis. The species chosen for this analysis are representative of different groups or clusters of *Clostridium* and *Clostridium*-like species based on the classification of Collins *et al.* (9). Pairwise comparisons of the sequences were made to determine the evolutionary distance between each pair of sequence (Fig. 4). The best phylogenetic tree was derived from these distances (Fig. 5). This analysis showed that strain 6 does not emerge from cluster I and II, which represent the main core of the *Clostridium genus* (9). It does not also emerge from other clusters, as it branches deeply into the tree. Similar results were

obtained following analysis with other phylogenetic methods (parsimony, maximum likelihood). Strain 6 is most likely a new *Clostridium*-like species that is not related to any clusters of *Clostridium* or *Clostridium*-like species.

DISCUSSION

Strain 6, the phenol-carboxylating microorganism, was isolated by its resistance to heat (17) chloramphenicol, clindamycin and its lower sensitivity to bacitracin. To our knowledge, there is only one other microorganism (*C. hydroxybenzoicum*) able to carboxylate phenol that has been isolated from a methanogenic consortium (27, 29). Our experimentation focused on liquid culture since Létourneau *et al.* (17) had shown that none of the strains isolated on solid medium from the heated consortium were able to transform phenol in pure culture or coculture.

The strategy of using antibiotics to obtain a carboxylating microorganism in pure culture was based on the fact that *Clostridium* spp. are known to vary widely in their susceptibility to some antibiotics (8). *C. ghonii, C. glycolicum* and *C. hastiforme* 3 are not implicated in the carboxylation of phenol, since they were eliminated from the consortium by chloramphenicol and clindamycin without this reaction being affected. Those species are known to be susceptible to these antibiotics (8). Moreover, *C. hastiforme* 2 is not the carboxylating microorganism, since it was resistant to a high concentrations of clindamycin (250 μg/ml) while phenol transformation was inhibited under these conditions, suggesting the presence of an unknown microorganism. Electron microscopic observations confirmed this hypothesis. *C. hastiforme* 2 was more susceptible to bacitracin than strain 6 and thus it was eliminated by dilution.

Like the *C. hydroxybenzoicum* strain isolated by Zhang *et al.* (27, 29) and the *C. thermoaceticum* ATCC 39073 strain studied by Hsu *et al.* (14), the strain we isolated was able to transform 4-hydroxybenzoic acid to phenol. However, our strain was the only one to further metabolize phenol produced from this reaction. *C. hydroxybenzoicum* showed some carboxylating activity only when resting cell suspensions or cell extracts were in the presence of a high concentration of phenol (10 mM) (30). A long nonmotile, gram-negative rod presumably possessing both these carboxylation and decarboxylation activities was observed by Knoll and Winter (16) in feeding experiments with their methanogenic consortium, but it was not isolated. The nitrate-reducing *Pseudomonas sp.* described by Tschech and Fuschs (24, 25) can accomplish both reactions. Several other anaerobic bacteria, including *Desulfobacterium phenolicum* (1), are able to metabolize 4-hydroxybenzoic acid and phenol.

Strain 6 carboxylates phenol by cometabolism, as previously shown for the original consortium (2). Amino acids or their degradation intermediates are probably the carbon and energy sources of this strain, as suggested elsewhere(3). *C. hydroxybenzoicum* is also known as an amino acid-utilizing microorganism (27).

The data obtained with strain 6 confirm previous results presented for the original consortium on the inducibility of the carboxylating activity by phenol (5). They also add the finding that the decarboxylating activity is inducible. *C.*

hydroxybenzoicum also produces an inducible enzyme that catalyzes the decarboxylation of 4-hydroxybenzoic acid (27). This enzyme was purified and shown to also catalyze the reverse reaction, namely, the carboxylation of phenol to 4-hydroxybenzoic acid (11). The purification of the phenol carboxylating enzyme of strain 6 should reveal if there is one enzyme with both activities or two different enzymes.

By its CFA composition, strain 6 was shown to be related to *C. hastiforme*, but it differs from this species in other properties (hydrolysis of gelatin and production of indole). This carboxylating strain, devoid of spores and of heat resistance, also differs from characteristics generally observed in *Clostridium* spp. However, the heat stability (80°C, 15 min) and ethanol resistance of the carboxylating microorganism present in the original consortium have been repeatedly observed and Létourneau *et al.* (17) concluded that spore-forming microorganisms were involved. In addition, strain 6 and 2 cocultures survived a similar heat treatment. Such a discrepancy has previously been observed by Utkin *et al.* (26) for a dechlorinating activity between their enrichment culture and the purified organism. Some *Clostridium* spp. are known to sporulate rarely even if special media or conditions are used. Thus, spore demonstration is sometimes difficult. For example, fresh isolates of *C. spiroforme* from humans may not form spores or survive at 70°C for 10 min (8). It is possible that some factors which are present only in the consortium culture or coculture are needed for the sporulation of strain 6.

No 16S ribosomal sequences that were identical or nearly identical to the 16S sequence of strain 6 were found in Genbank. The phylogenetic analysis revealed that the carboxylating microorganism is closely related to the genus *Clostridium*. However, this genus has been recognized to be very heterogenous. Several *Clostridium* species have been found to be phylogenetically related to other spore-forming and non-spore-forming genera. Collin *et al.* (9) proposed a new classification of the *Clostridium* genus. They grouped together several *Clostridium* and *Clostridium*-like species as clusters on basis of phenotypic criteria and phylogenetic analyses. Clusters I and II would make the core of the *Clostridium* genus and the other clusters would form new families and genera. Strain 6 does not emerge from cluster I and II, and, under the proposed system, it would not be considered as a true *Clostridium* species. Strain 6 also does not emerge from other clusters, although it may be classified in the vicinity of clusters III and IV. Species in these two clusters are polysaccharolytic *Clostridium* spp., such as *C. thermocellum, C. thermiditis, C. cellubioparum, C. papyrosolvens, C. aldrichii, C. cellulotycum* and *C. thermolactum* (20). However, since strain 6 was shown asaccharolytic, these species differ from it physiologically. Also, because strain 6 branches deeply in the phylogenetic tree, it is more likely that it is not related to any particular clusters of *Clostridium* spp.

Strain 6 is also different from the newly isolated *C. hydroxybenzoicum* of Zhang *et al.* (27) (Fig.5). The isolated carboxylating strain most likely belongs to a new *Clostridium*-like species. This would explain why strain 6 could not be classified in

any known species on the basis of its characteristics. In order to confirm this hypothesis, experiments to attempt to induce sporulation and heat resistance in strain 6 are under way in our laboratory.

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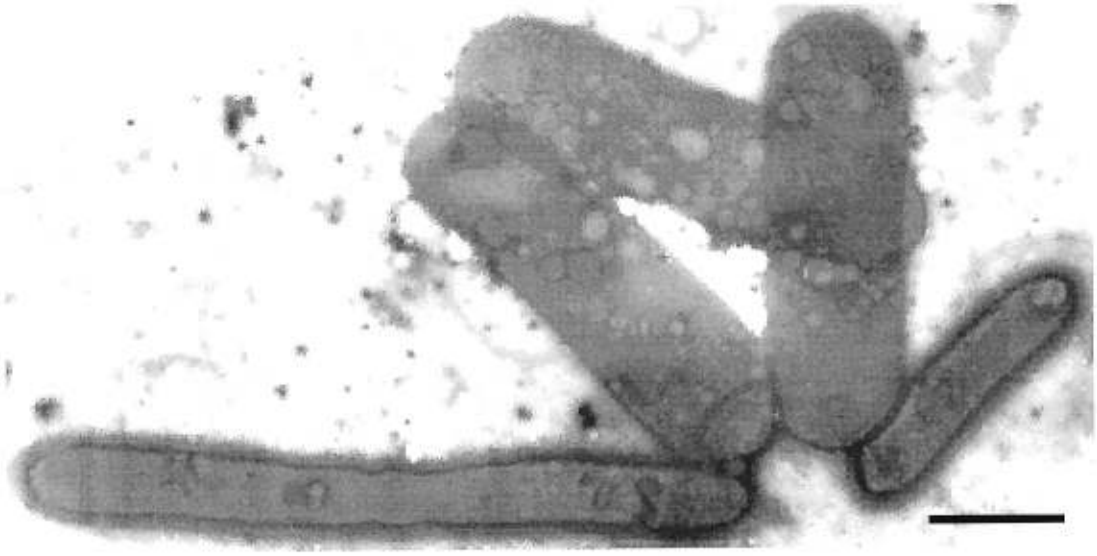
TABLE 1 : Transformation of 4-hydroxybenzoic acid to phenol and benzoic acid by 4-hydroxybenzoic acid-induced and -noninduced cultures of strain 6 in the presence and absence of chloramphenicol.

	Concentration (mg/l)[a]											
	4-Hydroxybenzoic acid Induced culture						Noninduced culture					
Days of incubation	Without chloramphenicol			With chloramphenicol			Without chloramphenicol			With chloramphenicol		
	4-OHB	P	B	4-OHB	P	B	4-OHB	P	B	4-OHB	P	B
0	326	2	2	338	2	2	357	1	6	327	2	3
3	28	153	38	303	19	2	377	2	2	337	2	3
8	1	126	126	280	40	5	270	6	70	342	2	3
11	11	103	161	252	52	2	5	145	104	332	2	3
18	12	53	256	247	66	2	1	116	168	340	2	3
24	ND[b]	ND	ND	216	85	6	2	92	225	355	3	<1

[a] 4-OHB, 4-hydroxybenzoic acid; P, phenol; B, benzoic acid. [b] ND, not determined.

A)



B)

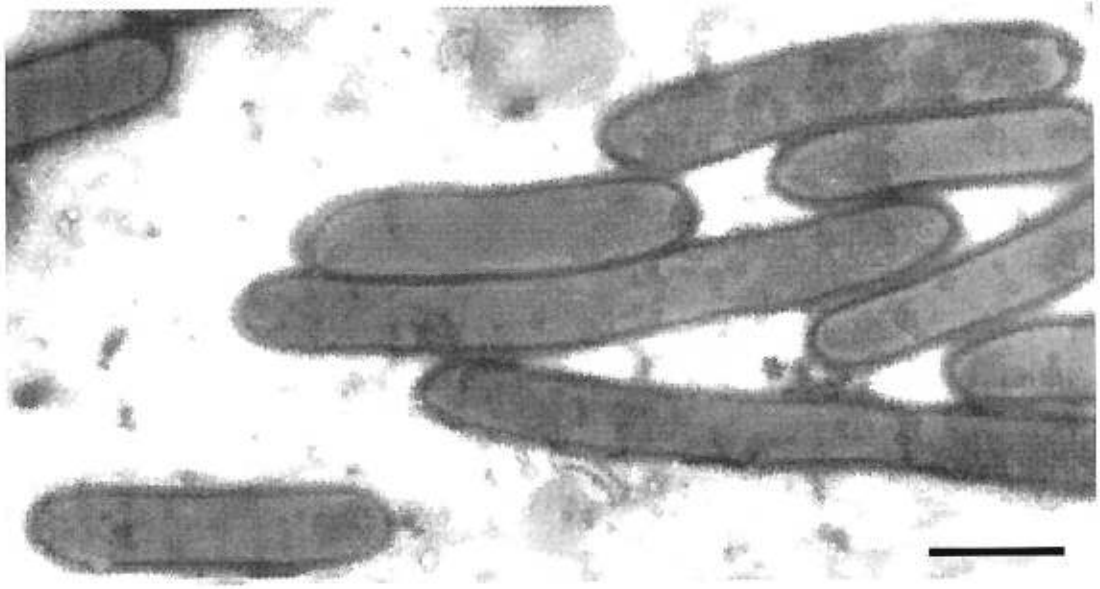


Fig. 1: Transmission electron micrographs of the cells from the culture inoculated with the 10^{-6} dilution of the clindamycin-treated culture, showing *C. hastiforme* 2 and strain 6, with widths of 1 and 0.6 μm, respectively (A) and the maintenance culture, showing only strain 6 (B). (Bars = 1 μm).

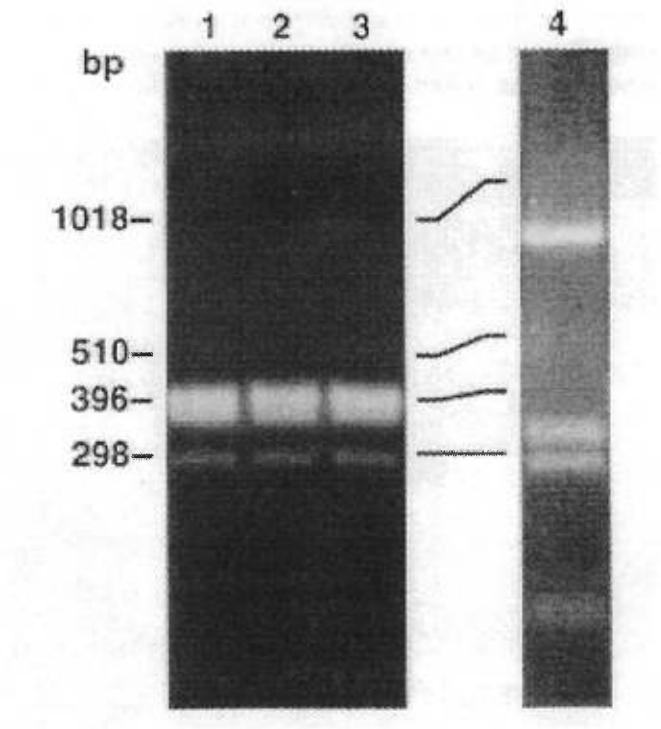


Fig. 2. Restriction endonuclease analysis of 16S ribosomal DNA. 16S ribosomal sequences from total DNA of different bacterial strains were amplified by PCR. *DdeI* restriction digestion patterns of strain 6 (lanes 1 to 3) and of *C. hastiforme* 2 (lane 4) are shown. Lane 1, culture grown in maintenance liquid medium, with the carboxylating activity conserved; Lane 2, culture grown on solid medium and having lost the carboxylating activity; Lane 3, culture grown in the liquid medium after a passage on solid medium and having lost the carboxylating activity. DNA were fractionated on a 1.8% agarose gel and visualized with ethidium bromide.

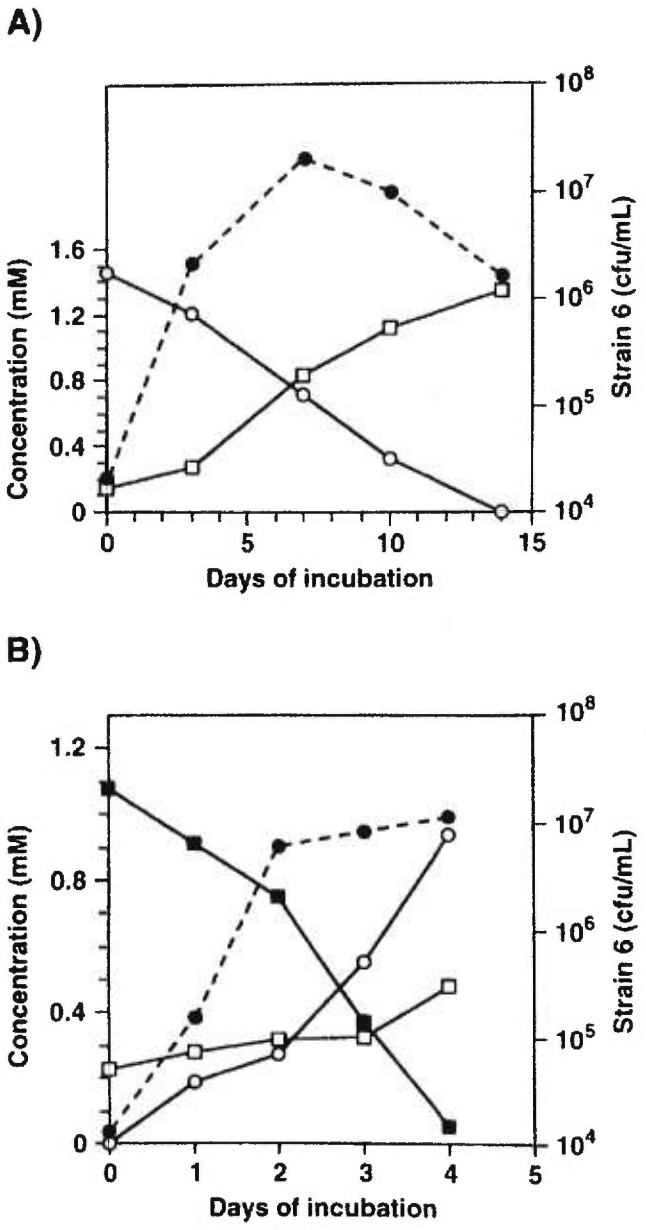


Fig. 3. Kinetics of phenol (A) and of 4-hydroxybenzoic acid (B) transformation by strain 6 in Boyd's medium supplemented with 0.5% (wt/vol) proteose peptone.

○, phenol; ■, 4-hydroxybenzoic acid; □, benzoic acid; ●, CFU per milliliter.

1-*C. hydroxybenzoicum*															
2-*C. ghoni*	0.186														
3-*C. thermocellum*	0.208	0.224													
4-*C. thermiditis*	0.222	0.239	0.096												
5-*C. thermolacticum*	0.202	0.230	0.100	0.137											
6-Strain 6	0.200	0.233	0.167	0.151	0.177										
7-*C. cellulosi*	0.214	0.264	0.151	0.172	0.171	0.187									
8-*C. butyricum*	0.203	0.228	0.178	0.200	0.220	0.193	0.219								
9-*C. proteolyticum*	0.202	0.209	0.182	0.197	0.215	0.201	0.196	0.102							
10-*C. putrificum*	0.206	0.217	0.178	0.200	0.203	0.182	0.205	0.105	0.100						
11-*C. pfennigii*	0.190	0.220	0.171	0.189	0.195	0.183	0.206	0.177	0.159	0.169					
12-*Th. brockii*	0.219	0.220	0.193	0.212	0.192	0.224	0.215	0.236	0.233	0.240	0.212				
13-*C. thermoaceticum*	0.189	0.200	0.170	0.196	0.173	0.210	0.197	0.220	0.213	0.217	0.193	0.129			
14-*C. ferridus*	0.193	0.208	0.151	0.189	0.156	0.186	0.176	0.165	0.164	0.166	0.165	0.170	0.164		
15-*D. autralicum*	0.251	0.259	0.220	0.253	0.214	0.274	0.253	0.282	0.276	0.288	0.261	0.193	0.171	0.211	
16-*T. xylanolyticum*	0.214	0.226	0.185	0.203	0.215	0.219	0.211	0.224	0.216	0.229	0.200	0.155	0.158	0.170	0.219
17-*E. coli*	0.314	0.308	0.266	0.298	0.270	0.282	0.288	0.287	0.288	0.296	0.302	0.281	0.280	0.271	0.328	0.301
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Fig. 4. Evolutionary distances between strain 6 and related *Clostridium* and *Clostridium*-like species. T. *Thermoanaerobacterium*; Th. *Thermoanaerobacter*; D. *Desulfotomaculum.*

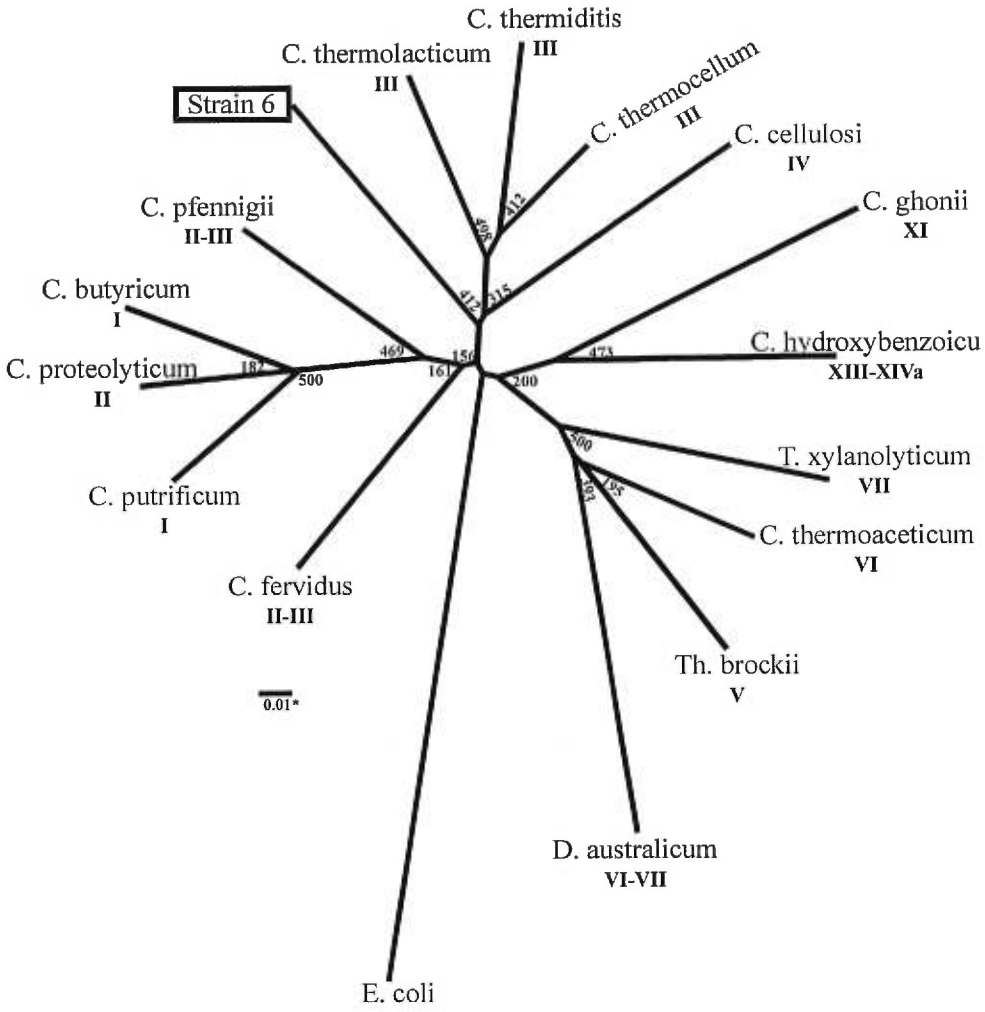


Fig. 5. Phylogenetic tree based on 16S rRNA gene sequences of strain 6 and related *Clostridium* and *Clostridium*-like species. Species whose sequences were used for comparison (and Genbank accession number) were as follows: *Clostridium proteoliticum* (X73448), *Clostridium thermiditis* (X71854), *Clostridium pfennigii* (X77838), *Clostridium putrificum* (X73442), *Clostridium fervidus* (L09187), *Clostridium thermolacticum* (L09176), *Clostridium butyricum* (X68176), *Clostridium thermocellum* (L09173), *Clostridium cellulosi* (L09177), *Thermoanaerobacter brockii* (L09165), *Clostridium hydroxybenzoicum* (L11305), *Clostridium ghonii* (X73451), *Thermoanaerobacterium xylanolyticum* (L09172), *Desulfotomaculum australicum* (M96665), *Clostridium thermoacticum* (M59121), and *E. coli* (J01695). Roman numerals indicate the cluster in which the *Clostridium* or the *Clostridium*-like species belong. The tree was derived from the FITCH program on the PHILIP package, using the distance in Fig. 4 with the *E. coli* 16S rRNA gene as an outgroup species. A bootstrap analysis of the same sequences was done with the SEQBOOT program with 500 replicates. The distances were calculated from each replicate with the DNADIST program using the Kimura 2-parameter option. The FITCH program derived the best tree for each replicate. The CONDENSE program was used to derived the consensus tree. The numbers at the forks indicate the number of times the group consisting of the species originating from that fork occurred among 500 trees.
*, Evolutionary distance (in nucleotide substitutions per site).

IV PAPER 2

Purification and Characterization of a 4-Hydroxybenzoate Decarboxylase from an Anaerobic Coculture

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ABSTRACT

An oxygen-sensitive 4-hydroxybenzoate decarboxylase from a coculture constituted of *Clostridium*-like strain 6 and an unidentified strain 7 was purified and partially characterized. The molecular mass of the enzyme estimated by gel filtration was 420 kDa. A single band of 119 kDa was obtained on SDS-PAGE suggesting a homotetrameric structure. The temperature optimum for the decarboxylation had a wide range between 15-45°C and the optimum pH was 5.0-6.5. The pI of the enzyme was 5.6. The enzyme also catalyzed the decarboxylation of 3,4-dihydroxybenzoate. The K_m value obtained for 4-hydroxybenzoate was 4.3 mM and the V_{max} was 139 μmol/min/mg at pH 6.5 and 37°C. The 50% of enzyme activity was lost after one hour in the presence of air at 4°C and the activity was lost completely after 48 hours. The activity did not decrease after seven days at 4°C under anaerobic conditions. The activity of the enzyme decreased by 50% in the presence of 5 mM EDTA and it was restored and even enhanced by the addition of Mg^{++}, Mn^{++}, Zn^{++} or Ca^{++}. No stimulation of the activity was observed with 1 to 5 mM ATP. The N-terminal amino acid sequence showed 95 and 76 % homology with the pyruvate-flavodoxin oxidoreductase (*nif*J gene product) from *Enterobacter agglomerans* and *Klebsiella pneumoniae,* respectively. The enzyme also catalyzed the following reverse reactions: carboxylation of phenol to 4-hydroxybenzoate and catechol to 3,4-dihydroxybenzoate. These characteristics suggest that this 4-hydroxybenzoate decarboxylase is different from other decarboxylases.

Keywords: Purification, characterization, 4-hydroxybenzoate decarboxylase, coculture, *Clostridium,* phenol carboxylation, anaerobic conditions.

INTRODUCTION

Phenolic compounds are widespread polluting substances from agricultural and industrial processes. Some of these compounds are considered as priority pollutants, since many are toxic or carcinogenic even in small concentration (2, 7, 27). Numerous investigations have shown that anaerobic bacteria can degrade phenolic compounds efficiently (3, 6, 8, 26, 33). Decarboxylation and carboxylation are important channeling reactions in the anaerobic degradation of phenolic compounds (9, 36).

Phenol is an important intermediate in the anaerobic degradation of many phenolic compounds. The carboxylation of phenol to 4-hydroxybenzoate was shown in methanogenic consortia (10, 12, 24). It has also been shown that 4-hydroxybenzoate can be decarboxylated to phenol (22, 35). Gallert and Winter (11) have partially characterized two 4-hydroxybenzoate decarboxylase activities and a phenol carboxylase activity found in cell-free extracts of a defined methanogenic consortium. He and Wiegel (14) are the only one to have purified and characterized a 4-hydroxybenzoate decarboxylase. This enzyme produced by *Clostridium hydroxybenzoicum* was oxygen sensitive and also catalyzed the reverse reaction, that is, the carboxylation of phenol. However, this strain was not able to further metabolize phenol. Li *et al.* (23) were the only one to claim the isolation of a strain carboxylating phenol from a methanogenic consortium. This strain, named 6, was shown to belong to a new species closely related to *Clostridium* species. It could

also decarboxylate 4-hydroxybenzoate to phenol and this reaction was even more rapid than carboxylation. Recently, Letowski *et al.* (personal communication) have shown that this culture was a coculture since an unidentified strain 7 was found in low concentration. Phenol degradation was possible only when both strains were present in the culture. Thus, no degradation was observed in pure culture of strain 6 or strain 7.

In this paper, the purification and characterization of the 4-hydroxybenzoate decarboxylase produce by the coculture is presented. This enzyme also had phenol carboxylase activity.

MATERIALS AND METHODS

Microorganisms and culture conditions

The coculture constituted of *Clostridium*-like strain 6 and an unidentified strain 7 was anaerobically grown in a mineral medium (4) supplemented with 2 mM phenol and 0.5 % (wt/vol) proteose peptone as already described (23). The coculture was incubated under anaerobic conditions in the dark at 37°C. The cells were harvested by centrifugation (11, 000 x g, 10 min) under anaerobic conditions in which approximately 70% of the phenol in the coculture were transformed (12 days) and they were stored at -80°C.

Preparation of crude extracts

Frozen cell paste was thawed and resuspended in 4 volumes of 100 mM potassium phosphate buffer (pH 6.5) containing RNase (1 mg/100 ml), DNase (1mg/100 ml), PMSF (phenylmethylsufonyl fluoride) (1 mg/100 ml) and 2 mM DTT (dithiothreitol). The suspended cells were passed through a French-Press using a pressure of 20, 000 psi (pound per square inch) under anaerobic conditions. The crude extract was clarified by centrifugation at 88, 500 x g for 60 min at 4°C.

Purification

All the purification steps were performed under anaerobic conditions. Some steps were carried out in an anaerobic chamber (Bactron II, Sheldon Manufactory

Inc. Oreg). All solutions were sparged with O_2-free N_2 and contained 2 mM DTT. The following steps were used to obtain the purified enzyme.

Step1. DEAE-Sephacel column. The crude extracts resulting from 24 L of culture were applied to a column (2 x 7 cm) of DEAE-Sephacel (Pharmacia) which was previously equilibrated with 50 mM potassium phosphate buffer, pH 6.5, and containing 0.05 M NaCl. The proteins were eluted from the column with a three-step increasing NaCl gradient (0.05, 0.15 and 1M) in the same buffer. The 4-hydroxybenzoate decarboxylase activity was eluted in the buffer containing 0.15 M NaCl.

Step2. Macro-Prep t-butyl HIC column. The active fraction from the previous step was adjusted to 2 M ammonium sulfate by adding ammonium sulfate crystals. The preparation was loaded onto a Macro-Prep t-butyl HIC (Bio-Rad) column (1.5 x 6 cm) previously equilibrated with 100 mM potassium phosphate buffer, pH 6.5, containing 2 M ammonium sulfate. The enzyme was eluted from the column with a step gradient of 1.5 M ammonium sulfate. The fractions containing 4-hydroxybenzoate decarboxylase activity were desalted on a gel-filtration P6 column (2.5 x 11 cm) (Bio-Rad). This fraction was used as semi-purified enzyme.

Step3. Hydroxyapatite column. The pooled fractions of the last step were loaded onto a hydroxyapatite (Bio-Rad) column (1.5 x 6 cm) which was previously equilibrated with 100 mM potassium phosphate buffer, pH 6.5. The column was

washed with 30 mL of the same buffer. The activity of the 4-hydroxybenzate decarboxylase was eluted with 250 mM potassium phosphate buffer, pH 6.5.

Step4. DEAE-5PW HPLC column. The fraction containing 4-hydroxybenzoate decarboxylase activity was desalted and its buffer was changed by using a gel-filtration P6 (Bio-Rad) column (2.5 x 11 cm) equilibrated with 20 mM potassium phosphate buffer pH 8.0. The pooled fractions were loaded onto a Protein Pak, DEAE-5PW column (8 x 75 mm) (Waters) which was previously equilibrated with 20 mM potassium phosphate buffer, pH 8.0 by using a Water 650, Advanced Protein Purification systems. The proteins were eluted with a 60 min linear NaCl gradient (0 to 0.6 M) at a flow rate of 1.5 ml/min. The 4-hydroxybenzoate decarboxylase was eluted in buffer containing 0.27 M NaCl.

Step5. Gel-filtration column. The active fractions from the DEAE-5PW step were pooled and a volume of 0.5 ml was applied on two serial Protein Pak 300 SW gel-filtration columns (8 mm x 30 cm) (Waters) which were equilibrated with 50 mM potassium phosphate buffer, pH 6.5, containing 0.1 M NaCl. The flow rate used was 0.6 ml/min and the 4-hydroxybenzoate decarboxylase was eluted at a retention time of 30 min.

Enzyme assay

Assays were performed anaerobically at 37°C in a serum bottle containing 1.0 ml of a solution composed of 0.5 ml of 200 mM potassium phosphate, pH 6.5,

containing 200 mM $NaHCO_3$, 2 mM $MnCl_2$•H2O, 2 mM $MgCl_2$•H_2O, 4 mM DTT and 8 mM substrate (4-hydroxybenzoate or phenol-D_6) and 0.5 ml of enzyme preparation. After 10 or 15 min. of incubation, the reaction was terminated by immersion in boiling water for 10 min. The decarboxylase activity was measured from the concentration of phenol produced as determined by using HPLC, a Nova-Pak C18 reverse-phase column (3.9 X 150 mm) (Waters) with a water-acetonitrile gradient containing 0.1% acetic acid. The phenol was monitored at 260 nm and the flow rate was 2 ml/min. After injection of 100 µl of sample in the column, acetonitrile concentration increased from 20 to 60 % in the first 4 min and afterward this concentration was maintained for 1 min. Phenol was eluted at a retention time of about 2.3 min. The carboxylase activity was determined by the amount of phenol-D_4 (D/H-exchange) produced from phenol-D_5 (uniformly deuterated phenol) (11). A gas chromatograph model 3500 (Varian) coupled to a mass spectrometer Ion Trap 800 (Finnigan) was used for this determination. The initial column temperature of 70°C was kept for 1 min and it was increased to 125°C at a rate of 10°C/sec. After 7 min at this temperature, it was increased to 165°C at a rate of 4°C/sec, then to 210°C at a rate of 8°C/sec and to 310°C at a rate of 20°C/sec and kept at this last temperature for 4 min.

One unit of activity was defined as the amount of enzyme producing 1 µmol of phenol per min under our conditions.

Decarboxylation and carboxylation of other substrates

The decarboxylation of different hydroxybenzoate substrates was determined by measuring the amount of product generated. When phenol was the expected product, its concentration was determined by HPLC as described previously. This procedure was modified when other products were expected. In the case of catechol and pyrogallol, the concentration of acetonitrile increased from 10 to 30 % in the first 8 min with a flow rate of 2 ml/min. and monitoring was done at 270 nm while for resorcinol and hydroquinone, it was 0 to 40 % and 290 nm.

The carboxylation of catechol was estimated by determining the concentration of 3,4-dihydroxybenzoate produced. A HPLC method similar to the one previously presented for catechol was used.

Inhibition of the carboxylase activity

Several *p*-hydroxyl compounds were tested for their ability to inhibit the carboxylase reaction. The enzymatic assay was performed as previously described except that 2 mM of phenol-D_6 was used as substrate in the presence of 2 mM of a potential inhibitor.

Protein determination

Protein concentrations were determined by the method of Lowry (25) by using bovine albumin as a standard.

Estimation of molecular mass

Purity and molecular mass of 4-hydroxybenzate decarboxylase were determined by SDS-PAGE (Bio-Rad) and Gel-filtration. The samples were run in a 10% SDS-PAGE mini-gel by following the Laemmli procedure (21) using a high-molecular-mass electrophoresis calibration kit (Pharmacia) containing myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), serum albumin (66 kDa) and ovalbumin (45 kDa). Proteins after electrophoresis were stained with silver (Bio-Rad) and their apparent molecular weight was determined by comparison of the R_f values with the one of the standard proteins. The gel-filtration was carried out in two serials Protein Pak 300 SW column (8 x 300 mm) (Waters) which were equilibrated with 50 mM potassium phosphate buffer, pH 6.5, containing 0.1 M NaCl and with a flow rate of 0.6 ml/min. The native enzyme molecular mass was measured by locating the point on the standard curve, which corresponds to the K_{av} value for the protein of unknown molecular weight. The Gel-filtration markers were bleu dextran (2000 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and bovine serum albumin (67 kDa) (Pharmacia).

Determination of the isoelectric point

The isoelectric point was evaluated by isoelectric focusing gel electrophoresis. It was performed on PhastSystem from Pharmacia LKB using PhastGel IEF 3-9 calibration kit (Pharmacia).

N-terminal sequence

N-terminal sequencing by automated Edman degradation was performed with the 03RBLOT program on a 470A Gas-Phase sequencer from Applied Biosystems Inc. (ABI). The protein was electroblotted to a polyvinylidene difluoride membrane and the 119 kDa protein was excised and loaded in a vertical cross-flow reaction cartridge (ABI). Phenylthiohydantoin amino acids (PTH-aas) derivatives were determined by comparison with standards (PTH Standards, ABI) analysed on-line with a 120A PTH Analyser HPLC system from ABI.

RESULTS

Purification of 4-hydroxybenzoate decarboxylase

The 4-hydroxybenzoate decarboxylase was purified by using several chromatographic columns. The results obtained through the different purification steps are summarized in Table 1. The enzyme was purified 6 fold and had a specific activity of 760 U/mg. Approximately 0.9% of the 4-hydroxybenzoate decarboxylase activity in the crude extract was recovered. This low activity recovered in the purified preparation was probably due to the sensitivity of the enzyme to oxygen. The purified enzyme showed a single band by SDS-PAGE analysis (Fig.1). The enzyme molecular mass of the subunit was estimated to be 119 kDa. The molecular mass of the native enzyme was estimated to be 420 kDa by Gel-filtration. These results suggest that the enzyme is a homotetrameric structure.

General catalytic properties

The semi-purified enzyme decarboxylated 4-hydroxybenzoate optimally at a pH between 5.0 to 6.5 (Fig.2). A fast decrease of the activity was observed at lower and higher pHs. The optimal temperature of the partially purified enzyme had a wide range between 15 to 45° C (Fig. 3a). The activity of the enzyme decreased rapidly over 50°C. The decarboxylase activity was stable at temperatures lower than 40°C and it decrease rapidly at higher temperatures (Fig. 3b). The activity was lost completely after 30 min incubation at 60°C.

The activity of the enzyme was oxygen-sensitive. About 50 % of the decarboxylase activity of the semi-purified enzyme was lost after one hour in the presence of air at 4 ^{o} C and the residual activity was lost completely after 48 hours (data not shown). On the contrary, the activity did not decreased after seven days under anaerobic conditions at 4^{o}C.

The isoelectric point of the purified enzyme was shown to be 5.6 by comparing the band positions of the enzyme with standard markers.

The K_m value derived from Lineweaver-Burk plots with the semi-purified decarboxylase at 37^{o}C and pH 6.0 was 4.3 mM for 4-hydroxybenzoate and the V_m was 139 μmol/min/mg.

Substrate specificity

The purified enzyme also decarboxylated 3,4-dihydroxybenzoate but this reaction was much slower since only 10% transformation was obtained after 20 hours of incubation as compare with 30% transformation of 4-hydroxybenzoate in 3 hours. This enzyme can not transform the other hydroxybenzoates tested such as 2-hydroxybenzoate, 3-hydroxybenzoate, 2,3-dihydroxybenzoate, 2,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, 2,3,4-trihydroxybenzoate, and 3,4,5-trihydroxybenzoate.

Effects of metal ions on the enzyme activity

Capturing the divalent ions by addition of 5 mM EDTA reduced 4-hydroxybenzoate decarboxylase activity by nearly 50% (Table 2). This suggests that no metal ion is essential for the enzyme activity. However, the activity was restored and even enhanced (over 100%) by addition of Mg^{++}, Mn^{++}, Zn^{++} or Ca^{++}. In the case of Fe^{++}, it could restore only about 20 % of the enzyme activity.

ATP requirement and phenol phosphorylation

The activity did not require ATP since addition of 1 to 5 mM ATP did not enhance the decarboxylase activity (data not shown). Phenylphosphate was not transformed when incubated with the semi-purified decarboxylase and it was not found during the transformation between phenol and 4-hydroxybenzoate (Table 3). These results suggest that phenol and 4-hydroxybenzoate are the direct substrates for this enzyme and phenol phosphorylation does not occur during phenol carboxylation.

Carboxylation of phenol and catechol

The purified enzyme also catalyzed the reverse reaction, which is the carboxylation of phenol to 4-hydroxybenzoate. Carboxylation is slower than decarboxylation and 4-hydroxybenzoate was not detected. GC/MS was used to detect phenol-D_5 exchange to phenol-D_4. The D_5 phenol is itself produced by rapid D/H exchange of the hydroxyl deuterium of D_6 phenol. In the carboxylation reaction, the deuterium at the 4 position is replaced by a carboxylic function. After

decarboxylation an hydrogen atom from the medium replace the carboxylic group, so the measure of the rate of D/H exchange from D_5 to D_4 phenol can be used to monitor carboxylation activity even in the presence of a larger decarboxylation activity (11). The carboxylation of catechol was also observed after an incubation of over two hours. 3,4-Dihydroxybenzoate produce was detected by HPLC.

All *p*-hydroxyl compounds tested were shown to inhibit significantly the carboxylase activity (Table 4). The strongest inhibition was observed with *p*-hydroxypridine. These results suggest that the *para*-hydroxyl group is important for an effective binding of the substrate to the carboxylase/decarboxylase enzyme.

N-terminal amino acid sequence.

An N-terminal amino acid sequence for the first 24 residues of the purified enzyme subunit was determined and compared for sequence similarities using the National Centre for Biotechnology Information basic BLAST search (Non-redundant GenBank CDS Database). Among all known sequences, some similarities were observed with some pyruvate-flavodoxin oxidoreductases (*nifJ* gene product) (Table 5). The highest similarity (95% in 21-residue overlap) was obtained with the sequence from *Enterobacter agglomerans* followed by the one from *Klebsiella pneumoniae* (76% identity in 21-residue overlap). It was also similar, but to a lesser extent, to pyruvate-ferredoxin oxidoreductases produced by *Entamaeba histolitica* (65% identity in 20-residue overlap) and *Desulfovibrio africanus* (57% identity in 21-residue overlap). There is no significant similarity with the 4-hydroxybenzoate

and the 3,4-hydroxybenzoate decarboxylases of *Clostridium hydroxybenzoicum* and any other decarboxylases.

DISCUSSION

A new 4-hydroxybenzoate decarboxylase was purified from a coculture isolated from a methanogenic consortium. It can be differentiated from the 4-hydroxybenzoate decarboxylase purified from *Clostridium hydroxybenzoicum* on the basis of its N-terminal amino acid sequence, molecular mass (420 kDa vs 350 kDa), number of subunits (4 x 119 kDa vs 6 x 57 kDa), K_m (4.3 mM vs 0.4 mM) and the effect of Zn^{++} (stimulation vs inhibition) (14). However, these two enzymes share some similarities since both are oxygen sensitive, catalyze the reverse reaction, are able to decarboxylate 3,4-dihydroxybenzoate and did not require ATP.

Because the cell yield of the anaerobic coculture is low under the conditions used and the enzyme is sensitive to oxygen, a limited amount of purified and active enzyme was obtained. This is the reason why some characteristics were determined with the semi-purified enzyme instead of the purified enzyme. However, the most important properties were determined with the purified enzyme.

Since no other 4-hydroxybenzoate decarboxylase was purified, only a partial comparison is possible with crude extracts showing this type of activity. The 4-hydroxybenzoate decarboxylase activities from the methanogenic consortium of Gallert and Winter (11) showed a similar response to metal ions as our enzyme since they were enhanced in the presence of Zn^{++}. However, these partially characterized decarboxylase activities appear different from ours since they showed no

carboxylase activity. In their consortium, the decarboxylase activities were loosely membrane associated, while the phenol carboxylase activity was soluble. Decarboxylase activity specific for hydroxybenzoates with a hydroxyl group in the *para* position has already been reported for the thermophilic *Clostridium thermoaceticum* (16) and *C. hydroxybenzoicum* (14). The phenol carboxylating enzyme of a denitrifying *Pseudomonas* K172 strain is different from ours since it was inactive on phenol and 4-hydroxybenzoate but active on phenylphosphate. Contrary to our enzyme, in this system phenol phosphorylation is the first step of the anaerobic phenol degradation (19, 20).

Similarity of the N-terminal amino acid sequence of our enzyme was observed with some pyruvate-flavodoxin oxidoreductases produced by some nitrogen-fixing microorganisms. The reasons to explain these similarities are unknown; however, these enzymes share some common features. These oxidoreductases are inactivated by oxygen and implicated in a decarboxylation reaction (34). In the presence of Coenzyme A, they decarboxylate pyruvate to acetyl CoA and CO_2, and flavodoxin is reduced. Our purified enzyme also showed a low decarboxylating activity of pyruvic acid (4 mM), α-*keto*-glutaric acid (4 mM) and phenyl pyruvic acid (4 mM) since 10% transformation of these compounds was obtained after 20 hours of incubation (data not shown). However, we cannot exclude the possibility that this low activity could be due to a residual protein in trace amount in our purified preparation. Grant and Patel (13) have already reported that the crude extract from the facultative *Klebsiella pneumoniae* (syn. *aerogenes* known

as a nitrogen fixing microorganism) can decarboxylate 4-hydroxybenzoate. Their enzyme had a similar optimum pH (6.0), optimum temperature (43°C) and K_m (4.0 mM) as our 4-hydroxybenzoate decarboxylase. It is probable that all of these enzymes share a common point in their evolution.

The 4-hydroxybenzoate decarboxylase we purified is also different from the oxygen sensitive 3,4-dihydroxybenzoate decarboxylase of *C. hydroxybenzoicum* that was purified by He and Wiegel (15). They have different molecular mass, number of subunit and no significant similarity was observed between their N-terminal amino acid sequence. Other hydroxybenzoate decarboxylases have been purified, like the 4,5-dihydroxyphtalate decarboxylase from *Pseudomonas* (28, 30), the 2,3-dihydroxybenzoate decarboxylase from the fungus *Aspergillus niger* (17), *Aspergillus oryzae* (32) and from the yeast *Trichosporon cutaneum* (1). Our purified enzyme also differs in its properties from these oxygen sensitive enzymes.

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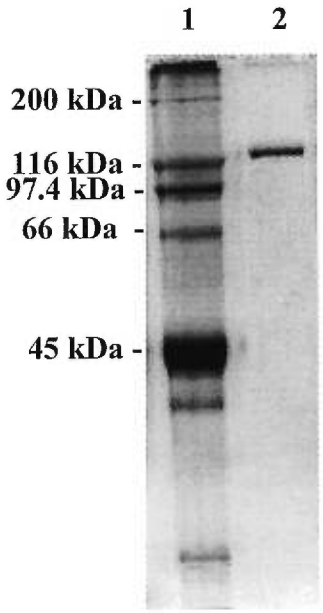

Fig. 1. SDS-PAGE (10 %) of the purified 4-hydroxybenzoate decarboxylase from the anaerobic coculture.. Lane 1: standard protein markers; Lane 2: the purified 4-hydroxybenzoate decarboxylase (2 μg).

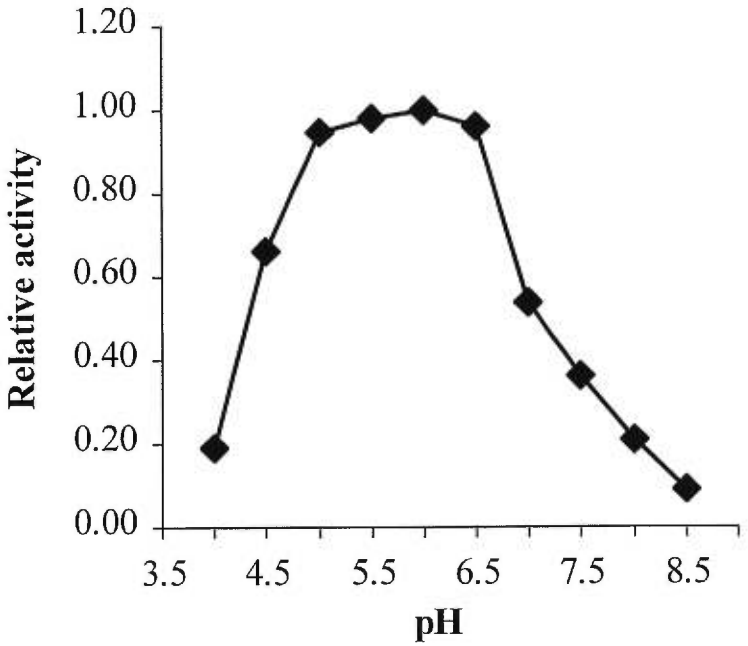

Fig. 2. Effect of pH on the activity of 4-hydroxybenzoate decarboxylase. The enzyme assay was performed in triplicate as described in Materials and Methods section except that the pH of the potassium phosphate buffer varied from 4.0 to 8.5.

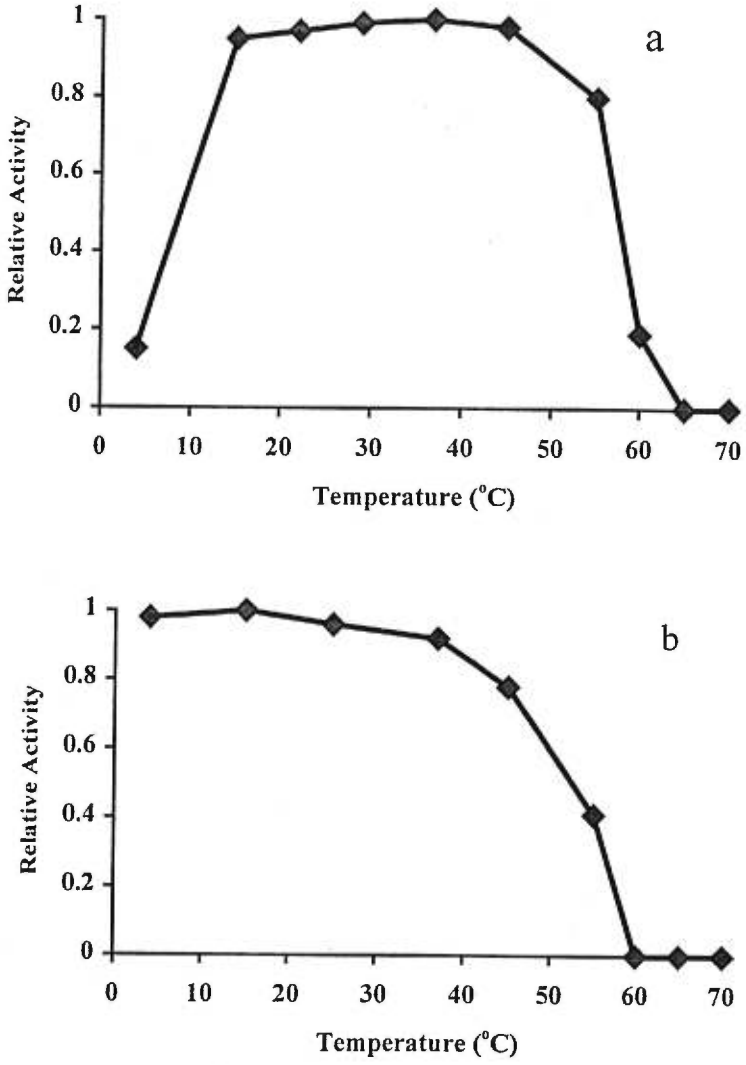

Fig. 3. Effect of temperature on 4-hydroxybenzoate decarboxylase. a) Optimal temperature of the activity. The enzyme assay was performed as described in Materials and Methods section except that the temperatures indicated were used. **b) Thermostability.** The enzyme was preincubated at indicated temperatures for 30 min and then tested at 37°C. All of the tests were performed in triplicate.

Table 1. Purification of 4-hydroxybenzoate decarboxylase from an anaerobic coculture.

Activity was determinated by the procedure described in Materials and Methods.

Step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Purification fold	Activity yield (%)
Cell-free extract	309	38250	123.2	1	100
DEAE-Sephacel	78.75	17850	223.7	1.8	46.7
T-Butyl (HIC)	10.95	5175	472.6	3.8	13.5
Hydroxyapatite	7.44	3720	500.0	4.1	9.7
DEAE-5PW	2.0	1200	718.6	5.8	3.1
Gel-filtration	0.5	360	759.5	6.1	0.9

Table 2. Effect of EDTA and metal ions on the 4-hydroxybenzoate decarboxylase

Condition	Relative activity
Standard substrate*	1.89
Standard substrate + 0.25 mM EDTA	1.19
Standard substrate + 5 mM EDTA	1.00
Standard substrate + 5 mM EDTA + 10 mM $MgCl_2$	2.77
Standard substrate + 5 mM EDTA + 10 mM $MnCl_2$	2.53
Standard substrate + 5 mM EDTA + 10 mM $ZnCl_2$	2.36
Standard substrate + 5 mM EDTA + 10 mM $CaCl_2$	2.17
Standard substrate + 5 mM EDTA + 10 mM $FeCl_2$	1.19

*: Standard substrate was 100 mM potassium phosphate buffer, pH6.5, containing 4 mM 4-hydroxybenzoate, 100 mM $NaHCO_3$ and 2 mM DTT. The enzyme assay was performed in triplicate as described in Materials and Methods.

Table 3. Test for phenol phosphorylation.

Substrate (1 mM)	Enzyme (μl)	Phenol (mM)	Phenylphosphate (mM)	4-Hydroxybenzoate (mM)
Phenylphosphate	0	0	1	0
Phenylphosphate	100	0	1	0
Phenol	0	0.86	0	0
Phenol	100	0.51	0	0.40
4-Hydroxybenzoate	0	0	0	0.97
4-Hydroxybenzoate	100	0.57	0	0.33

Enzyme assays were performed as in Materials and Methods except that different substrates were used as indicated. 4-Hydroxybenzoate and phenylphosphate were determined by HPLC analysis.

* : The semi-purified enzyme preparation was used in this test.

Table 4. Effect of inhibitors on carboxylation of phenol.

Inhibitors	Relative activity
None	1
p-hydroxypyridine	0.15
p-hydroxybenzyl alcohol	0.75
p-hydroxybenzoic hydrazide	0.87
p-hydroxybenzamide	0.58
p-hydroxybenzaldehyde	0.38

Phenol–D_6 (2 mM) was used as substrate. Assays were performed as Materials and Methods except that different inhibitors (2 mM) were added.

Table 5. Comparison of N-terminal amino acid sequence of 4-hydroxybenzoate decarboxylase with other enzymes.

Enzyme	Organism	Sequence*	% of ** Identity	References
4-hydroxybenzoate decarboxylase	Coculture	XgKMKTMDGNTAaXYVSYAFTdv ||||||||||| ||||||||		This work
Pyruvate-flavodoxine oxidoreductase	*Enterobacter agglomerans*	KMKTMDGNTAAAYVSYAFTDV |||||||| |||::|||||:|	95	(17)
Pyruvate-flavodoxine oxidoreductase	*Klebsiella pneumoniae*	KMKTMDGNAAAAWISYAFTEV |...||| |||::||| .:|	76	(4)
Pyruvate-ferredoxin oxidoreductase	*Entamoeba histolytica*	MQSVDGNQAAAYVSYALSDV | . ||| | |:|.||:|:|	65	(31)
Pyruvate-ferredoxin oxidoreductase	*Desulfovibrio africanus*	KMMTTDGNTATAHVAYAMSEV	57	(29)
3,4-hydroxybenzoate decarboxylase	*Clostridium hydroxybenzoicum*	MNKVTDLRSAIELLKTIPGQLIETNXDV		(14)
4-hydroxybenzoate decarboxylase	*Clostridium hydroxybenzoicum*	(A/M)KVYRDLREFLEVLXQXGXLI		(13)

* : Amino acids in small capital letter were determined as the most probable.

** : Percentage of identity between the 4-hydroxybenzoate decarboxylase of the coculture and the four oxidoreductases.

V. GENERAL DISCUSSION

Comparison of strain 6 and *C. hydroxybenzoicum*

Up to now, *C. hydroxybenzoicum* (Zhang *et al.* 1990; 1994) and strain 6 (This thesis, PAPER1) have been the only two microorganisms isolated from methanogenic consortia based on their ability to carboxylate phenol or decarboxylate 4-hydroxybenzoate. The comparison of these two strains reveals many differences. Firstly, *C. hydroxybenzoicum* is a 4-hydroxybenzoate-decarboxylating microorganism. The phenol produced from this reaction is not further metabolized by this strain. Thus, phenol is the final product in this pure culture. Strain 6 is the first microorganism isolated from a methanogenic consortium for its ability to transform phenol to benzoate via carboxylation and dehydroxylation. Secondly, phylogenetic analysis based on 16S rRNA gene sequences of strain 6 and *C. hydroxybenzoicum* showed that they are significantly different (This thesis, PAPER1-Fig.5). *C. hydroxybenzoicum* is closely related to *C. purinolyticum* and *C. acidiurici*. It could be classified in the vicinity of clusters XIII and XIV. Strain 6 does not emerge from any *Clostridium* clusters, although it may be classified in the vicinity of clusters III and IV. These two strains also differ with respect to other characteristics such as motility, sporulation and so on (This thesis, PAPER1; Zhang *et al.* 1990 and 1994).

Strategy for isolation of strain 6

Generally, a successful isolation of a strain depends on the exploitation of specific properties of the desired microorganism. In the case of clostridia, anaerobic incubation in a suitable liquid medium is itself often sufficient for effective

enrichment, and isolation may be achieved simply by subsequent plating on solid media (Hobbs *et al.* 1971). However, no single method can be relied upon to isolate all the varieties of bacterial strains. Therefore, a knowledge of the bacterium is a prerequisite for their successful isolation. In our case, the heat-treated consortium (Létourneau *et al.* 1995) contained fast growing strains, which did not allow strain 6 to grow on the solid medium. Thus, it was not possible to proceed by plating on solid media to isolate the active strain 6. The use of antibiotics to isolate strain 6 is a strategy based on the fact that *Clostridium* sp. are known to vary widely in their susceptibility to these substances (Cato *et al.* 1986). The resistance to antimicrobials is also a criterion used for the identification of anaerobic clostridia. Although there has been little or no change in the susceptibility patterns of some agents such as chloramphenicol and metronidazole, significant resistance has developed to others such as clindamycin and penicillin (Rosenblatt 1991). In this study (This thesis, PAPER1), three strain of clostridia were eliminated from the consortium by chloramphenicol and clindamycin. Using bacitracin combined with dilutions, *C. hastiforme* 2 was eliminated. Purity of the isolated culture was assessed based on the fact that only one type of colony growed on solid medium and only one microbiological form was observed by optical and electron microscopy. This pure culture showed no phenol-degrading activity when a single-colony was inoculated in a fresh liquid medium. However, it was active when the inoculum came from a liquid culture. Therefore strain 6 probably needs some unknown cofactors produced in liquid medium to transform phenol. The consortium was stable in liquid medium, because it maintained its phenol degrading activity following several transfers.

These results suggest that antibiotics can be used for the isolation of a desired strain, which can not be seen on solid medium when grown in coculture with fast growing strains.

Strain 6 and coculture

According to microbiological standard procedures, we believed that strain 6 culture was pure since no other strain was observed by optical and electron microscopy in the liquid medium and only one type of colony grew on solid medium. After maintaining this culture for one year, it has changed dramatically in the summer of 1997. We found that it contained two bacterial strains, a small long rod, strain 6 and a large electron dense short rod, named strain 7. The ratio between strain 6 and strain 7 in the coculture was evaluated as 10:1 in favor of strain 6. We could not easily eliminate strain 7 in short time because it can not grow on solid medium and has some related with strain 6. Strain 6 grows on solid medium, but as mentioned before, a single-colony inoculated in liquid medium did not show phenol-degrading activity. Due to this situation and in order to save time, we decided to purify the enzyme from the coculture, which was still very active in transforming phenol to benzoate.

We think that there are two possible explanations for this dramatic change in our culture. One is that the pure culture of strain 6 became contaminated with strain 7 during manipulation. Another would be an adaptation to changes in some growth conditions such as using a new package of proteose peptone, which allowed the

concentration of strain 7 to increase from non-detectable to 10^7cells/ml. We believed that we had isolated an active pure culture in 1996 based on the observation of only one morphological form by optical and electron microscopy and only one colony type growing on solid medium.

The determination of purity based on the fact that only one type of colony grows on the solid medium is generally accepted, but there are exceptions (Krieg 1984). One of these is when the contaminant does not grow on solid medium. Another criterion of purity is morphology. Microorganisms from a pure culture generally exhibit a high degree of morphological similarity (Krieg 1984). However, one exception to this criterion is when the contaminant is present in a non-detectable concentration. Taking into account of these exceptions, we can not be entirely sure of the purity of strain 6.

Isolation of a pure culture from a methanogenic consortium is more difficult, because of the interactions between the microorganisms in this association. For example, the microorganisms may exchange specific nutrients, remove growth-inhibitory products, and so on. Knoll and Winter (1987) failed to isolate a pure culture degrading phenol since an obligate syntrophic interdependence was observed. In Wiegel's laboratory a situation similar to ours has happened (personal communication). They found that the pure culture *C. hydroxybenzoicum* (Zhang *et al.* 1990; 1994) became a coculture of two bacterial strains. But they do not know if this new strain came from a contamination or from the original culture.

Utility of PCR amplification of 16S rRNA gene sequences for classification and identification of anaerobic bacteria

The sequence of nucleic acids in the bacterial genome contains a wealth of information, some of which is especially suitable for identifying individual species. The genes for the 16S and 23S rRNAs are particularly suitable as targets for identifying most microorganisms. With the exception of viruses, rRNA genes are found in all organisms. To fulfill their roles in protein biosynthesis, rRNA molecules contain several functionally different regions, some of which have conserved sequences and others of which are highly variable (Bottger 1996).

Ribosomal nucleic acids are thus considered phylogenetically meaningful molecules, which provide a record of evolution. When bacteria evolved into what we call domains, divisions, classes, families, genera, and species, these events were imprinted in the sequence of rRNAs. These imprints or molecular signatures form another basis for identifying microorganisms. Molecular signatures involve the primary sequence as well as secondary structure characteristics of ribosomal genes. Moreover, the evolutionary distance between organisms can be inferred from their rRNA sequence differences (Staley and Krieg 1984). For identification purposes, hypervariable regions are particularly useful because of relatively high differences between species but relatively low variability within a species (Bottger 1996).

The use of PCR has significant potential in the identification of bacteria. The technique allows for sensitive detection of a given DNA fragment in a complex mixture of molecules (Hofstad 1994). The 16S rRNA gene sequencing analytical

procedure combined with PCR is a fast and accurate method of classification and identification of a newly isolated bacterium (Bottger 1996). Strain 6 was identified successfully using these techniques. The evolutionary distances and phylogenetic tree based on 16S rRNA gene sequences clearly showed that strain 6 is a new *Clostridium*-like species and is not related to any particular cluster of *Clostridium* sp. (This thesis, PAPER1).

The N-terminal amino acid sequence of the 4-hydroxybenzoate decarboxylase

The N-terminal amino acid sequence of our 4-hydroxybenzoate decarboxylase was shown to be very similar to that of pyruvate-flavodoxin oxidoreductases produced by nitrogen-fixing microorganisms. This suggests a possible duplication and divergence of a common ancestral gene. In fact, they share some common features like oxygen sensitivity and decarboxylating activity. In the presence of Coenzyme A, pyruvate-flavodoxin oxidoreductases decarboxylate pyruvate to acetyl CoA and CO_2, and flavodoxin is reduced (Williams 1987). They have probably evolved from a common protein in their evolution (Clarke 1984).

Our purified enzyme also showed a low decarboxylating activity towards pyruvic acid (4 mM), α-*keto*-glutaric acid (4 mM) and phenyl pyruvic acid (4 mM), with 10% transformation of these compounds after 20 hours of incubation (This thesis, PAPER2). Betz and Clarke (1972) found that if an enzyme has a weak activity for a compound other than its optimal substrate, it might be possible to

obtain an "improved enzyme" by a suitable selection method. The reason for the low decarboxylating activity of pyruvic acid and the other compounds, might be that strain 6 was selected with phenol, but not with pyruvic acid.

Purified enzyme

The specific activity of the enzyme was purified only 6 times and approximately 0.9% of the 4-hydroxybenzoate decarboxylase activity in the crude extract was recovered, partly due to the fact that the enzyme is sensitive to oxygen and a limited amount of purified enzyme was obtained. Another reason was the difficulty to obtain a large amount of bacterial cells since the anaerobic bacteria grow slowly. Consequently, some characteristics of the enzyme were tested with the semi-purified enzyme. However, the most important properties were tested with the purified enzyme (This thesis, PAPER2).

ATP requirement and phenol phosphorylation

Lack and Fuchs (1992; 1994) found that the actual substrate used in *para*-carboxylation is not phenol itself in the denitrifying *Pseudomonas* strain K172 and in other facultatively or strictly anaerobic microorganisms. They demonstrated that the initial step of phenol degradation in whole cells is phenol phosphorylation to phenylphosphate. Therefore, phenylphosphate must be the actual substrate of the carboxylating enzyme, which is Mn^{++}-dependent. This system resembles the phosphotransferase system (PTS) in many Gram-negative bacteria (Saier *et al.* 1977).

Phenylphosphate carboxylation could not be found in our coculture (This thesis, PAPER2). Our experimental results clearly showed that phenylphosphate was not transformed when incubated with the semi-purified decarboxylase and it was not found during the transformation between phenol and 4-hydroxybenzoate (This thesis, PAPER2-Table 3). No metal ion is essential for our 4-hydroxybenzoate decarboxylase activity (This thesis, PAPER2-Table 2). These results suggest that phenol and 4-hydroxybenzoate are the direct substrates for this enzyme and phenol phosphorylation does not occur during phenol carboxylation. Similar results were obtained by Gallert and Winter (1993). They showed that no ^{14}C-labelled phenylphosphate could be detected from ^{14}C-labelled phenol in their phenol-degrading consortium. These authors demonstrated that phenylphosphate may not necessarily be an intermediate in all organisms carboxylating phenol anaerobically, at least not in methanogenic conditions. Our results showed for the first time, by using enzyme assay, that there are at lest two different pathways for phenol carboxylation.

In addition, we found that the 4-hydroxybenzoate decarboxylase did not require ATP since addition of 1 to 5 mM ATP did not affect the activity (This thesis, PAPER2). This indicated that the process of 4-hydroxybenzoate decarboxylation did not require ATP. Gallert and Winter (1993; 1994) even found that addition of 4-hydroxybenzoate to cell suspensions of their mixed culture resulted in a rapid

increase of the cellular ATP level while 4-hydroxybenzoate was decarboxylated, suggesting that energy is generated during 4-hydroxybenzoate decarboxylation.

Enzyme Nomenclature

The first 4-hydroxybenzoate decarboxylation activity was reported by Grant and Patel (1969) from *Klebsiella aerogenes*. It was classified as a 4-hydroxybenzoate decarboxylase (EC 4.1.1.61) in *Enzyme Nomenclature* (Webb 1992). The phenol carboxylase activity from *Pseudomonas* strain K172 (Tschech and Fuchs 1989) was given the same EC number as 4-hydroxybenzoate decarboxylase in *Enzyme Nomenclature* (Webb 1992). However, phenol carboxylase and 4-hydroxybenzoate decarboxylase catalyze different reactions. We suggest that it is better to subclassify EC 4.1.1.61 into the following three subclasses: A: 4-hydroxybenzoate decarboxylase, without the reversible phenol carboxylase activity (Gallert and Winter 1992; Grant and Patel 1969); B: phenol carboxylase, without the reversible 4-hydroxybenzoate decarboxylase activity (Gallert and Winter 1992; Lack and Fuchs 1994); C: 4-hydroxybenzoate decarboxylase with reversible phenol carboxylase activity. At this point, the reversible 4-hydroxybenzoate decarboxylase purified by He and Wiegel (1995) and our purified reversible 4-hydroxybenzoate decarboxylase (This thesis, PAPER2) could be classified as EC 4. 1. 1. 61-C.

Improving the phenol biodegrading capacity of bacteria

The use of microorganisms isolated from subsurface environments would be advantageous, because these organisms are already adapted to subsurface

conditions. But there are several problems that need to be overcome in biodegradative processes for the rapid degradation of phenol. For instance, the enzymes, which are responsible for the degradation of these compounds, may not be produced in very high concentrations. Many reasons might explain this such as the low levels of the substrates that are available to the microorganisms or that the operons involved are induced primarily by downstream metabolites rather than the initial substrates. These obstacles can be overcome by application of genetic engineering to construct new strains with biodegradative enzymes under the control of promoters that can be regulated by inexpensive and nontoxic external factors. After having determined the N-terminal amino acid sequence of the 4-hydroxybenzoate decarboxylase, we could synthesize a DNA probe for detecting genes involved in catabolism of phenol. Recombinant DNA technology could allow the construction of a bacterial strain with improved degradative efficiency or expanded catabolic capacity. Little has been reported on the genetic manipulation of anaerobic strains for improving the efficiency of biodegradation. One possible strategy is simple over-expression of the enzymes involved. Two candidate plasmids, pMMb66EH (Furste et al.1986) and pNM185 (Mermod *et al.* 1986), could be used as vehicles for transferring genes encoding recruited enzymes into selected bacteria because they can be maintained in a wide variety of microorganisms.

It is now accepted that the microorganisms inhabiting anoxic environments make a significant contribution to the overall turnover of aromatic compounds. Anaerobic degradation has an enormous potential in solving the environmental

problem of pollution with organic wastes. The present study has increased the knowledge of anaerobic biodegradation of phenolic compounds which should aid in the eventual development of an optimal anaerobic treatment of phenolic compounds.

VI. GENERAL CONCLUSION

In order to better understand the anaerobic biodegradation of phenol, this study focused on the degradation of this compound under methanogenic conditions. For the first time a pure phenol-carboxylating strain, strain 6, was isolated from a methanogenic consortium. Using different antibiotics, we successfully eliminated the other clostridia, which are not responsible for phenol carboxylation. This overcame the difficulty that strain 6 was not seen on solid medium when faster growing strains were present. Strain 6 can transform phenol to benzoate via *para*-carboxylation followed by dehydroxylation. Phylogenetic studies based on 16S rRNA gene sequences suggest that strain 6 is a new species closely related to *Clostridium* species.

4-Hydroxybenzoate decarboxylase, which is an oxygen sensitive enzyme, was successfully purified and characterized. This enzyme can also carboxylate phenol to 4-hydroxybenzoate. This is the second report on the purification of a 4-hydroxybenzoate decarboxylase under methanogenic conditions. He and Wiegel (1995) have previously purified a 4-hydroxybenzoate decarboxylase from *Clostridium hydroxybenzoicum.* However, the characteristics of the two purified enzymes are different in many respects. Obviously, they are two isozymes of 4-hydroxybenzoate decarboxylase.

Our results also showed that there are different anaerobic pathways that metabolize phenol via *para*-carboxylation to 4-hydroxybenzoate in methanogenic consortium and in denitrifying *Pseudomonas.* Under methanogenic conditions,

contrary to denitrifying conditions, phenol phosphorylation does not occur during phenol carboxylation and phenol was directly carboxylated to 4-hydroxybenzoate.

More studies on anaerobic phenol degradation are needed in order to understand better this process. As well, further research concerning the basic microbiology, biochemistry and molecular biology of anaerobic digestion is required since even after the rapid increase in research efforts in this field during the last decade, it is still, in many respects, as a "black box". This is due to the difficulty of isolating pure cultures from methanogenic consortia and of purifying oxygen-sensitive enzymes.

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