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METABOLIC DIVERSITY IN AROMATIC COMPOUND UTILIZATION BY ANAEROBIC MICROBES

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■ **Abstract** A vast array of structurally diverse aromatic compounds is continually released into the environment due to the decomposition of green plants and as a consequence of human industrial activities. Increasing numbers of bacteria that utilize aromatic compounds in the absence of oxygen have been brought into pure culture in recent years. These include most major metabolic types of anaerobic heterotrophs and acetogenic bacteria. Diverse microbes utilize aromatic compounds for diverse purposes. Chlorinated aromatic compounds can serve as electron acceptors in dehalorespiration. Humic substances serve as electron shuttles to enable the use of inorganic electron acceptors, such as insoluble iron oxides, that are not always easily reduced by microbes. Substituents that are attached to aromatic rings may serve as carbon or energy sources for microbes. Examples include acyl side chains and methyl groups. Finally, aromatic compounds can be completely degraded to serve as carbon and energy sources. Routes by which various types of aromatic compounds, including toluene, ethylbenzene, phenol, benzoate, and dihydroxylated compounds, are degraded have been elucidated in recent years. Biochemical strategies employed by microbes to destabilize the aromatic ring in preparation for degradation have become apparent from this work.

CONTENTS

INTRODUCTION

About 25% of the Earth's biomass is composed of compounds that have a benzene ring as the main structural constituent. Green plants synthesize the overwhelming proportion of these aromatic compounds and assemble them to form lignin, a polymer that is highly stabilized by a complexity of ether and other linkages. The processes that lead to lignin depolymerization and the ultimate breakdown of the aromatic compounds generated during depolymerization are poorly understood. In addition to natural sources, human ingenuity and activities have added a plethora of new aromatic chemicals to the environment. Although the input of these xenobiotic compounds is less in total amount than that of plant materials, their novel structures pose major challenges to the microbial communities that are the major recyclers of natural products. Concerns about environmental pollution have resulted in many attempts to define conditions under which aromatic compound degradation can occur, as this would help in the development of strategies for accelerating rates of biodegradation.

Many of the aromatic nucleus-containing materials that are released into the environment by humans or by the degradation of plant material find their way into anaerobic sediments. This has prompted studies of aromatic compound degradation in the absence of oxygen, where the already well-understood aerobic reactions catalyzed by mono- or dioxygenases, or the peroxidases that initiate attack on lignins cannot occur. The past decade has seen major advances in our understanding of the range and rates at which aromatic compounds of varying degrees of complexity can be degraded in natural environments that are devoid of oxygen. More importantly, a number of bacterial strains that degrade aromatic compounds in pure culture have recently been isolated, opening the door to more detailed physiological, biochemical, and molecular genetic studies. Many of these microbes and the compounds they degrade are listed in Table 1, which is provided as supplementary material on the web (follow the Supplemental Material link in the online version of this chapter or at http://www.annualreviews.org). Several factors have facilitated these isolations. Among the most significant is the recognition that a broader range of electron acceptors can be used for anaerobic respiration than had been previously

thought. The use of nitrate and sulfate for this purpose has been known for a long time, but the significance of Fe(III) reduction and chlorate respiration has only recently been recognized. Ferric iron is widespread and abundant in most environments, and bacteria that can benefit from using this compound as a terminal electron acceptor are widely distributed (77). Chlorate used as a bleaching agent, disinfectant, and component of rocket propellants has been a significant anthropogenic addition to natural environments only for a small number of decades. Even so, it is possible to isolate strains from many habitats that use chlorate, as well as nitrate, as electron acceptors in the absence of oxygen (22). Use of inert carriers such as heptamethylnonane (93) or Amberlite XAD7 (3, 85) to mediate the slow release of toxic organic compounds into growth media has enabled the cultivation of microbes on substrates that may be tolerated over only a narrow concentration range. Bacteria thought to grow only as obligate syntrophs in coculture with a hydrogen-utilizing microbe such as a methanogen were found to grow in pure cultures on crotonate (6, 57). Finally, many small improvements in handling and culturing strict anaerobes have made the long incubations required for slow-growing cultures technically feasible.

Important new concepts have emerged recently. These include recognition that interspecies transfers of the kind first defined for hydrogen by Wolin and colleagues (56) occur with other metabolic intermediates, including acetate, formate, and probably many others in natural ecosystems. Also that some reactions thought to be the province of organic chemists can be carried out by enzymes. Examples include the biological Birch reduction involved in anaerobic benzoate ring reduction and a Kolbe-Schmidt-type reaction for aromatic dehydroxylation reactions. In addition there is an appreciation that enzyme radical chemistry is critical in overcoming the stability of aromatic as well as of alkyl hydrocarbons, as demonstrated by the proposed reaction mechanisms for initial attack on ethylbenzene and toluene.

This review considers the physiological roles that aromatic compounds play in the lives of facultatively or obligately anaerobic microorganisms, and how these lead to the partial or complete degradation annually of large quantities of biomass and industrial by-products. This viewpoint naturally restricts our focus to studies with isolated pure strains or with defined cocultures. We have concentrated on work published during the past five years and restricted the range of aromatic compounds considered almost entirely to monocyclic compounds. Many excellent reviews on various aspects of the anaerobic utilization of aromatics give more detailed accounts, especially of the biochemistry (47, 52, 53, 77, 78, 97, 115).

AROMATIC COMPOUNDS AS ELECTRON ACCEPTORS AND SHUTTLES

General Considerations

Many microorganisms can utilize substituted and complex aromatic compounds in the absence of oxygen in ways that do not perturb the benzene nucleus itself (Figure 1). Aromatic compounds can participate in energy metabolism by serving

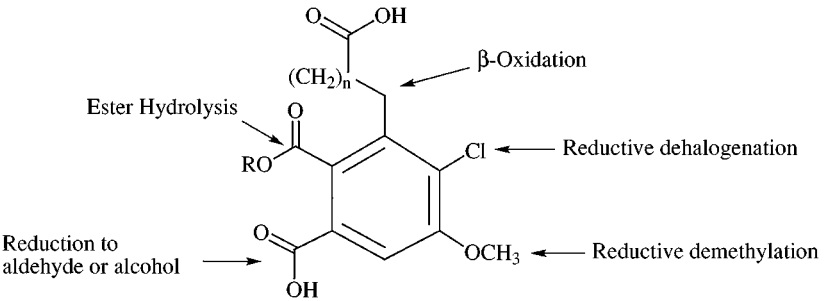

Figure 1 Commonly encountered attacks on aromatic ring substituents that are mediated by microbes.

as electron acceptors, generally with accompanying modifications of ring substituents. An example would be the use of chlorinated aromatic compounds as electron acceptors in dehalorespiration. Aromatic compounds can also serve as electron shuttles. For instance, extracellular quinones can bridge the activities of different kinds of microorganisms by transferring electrons from the respiratory chain of one microorganism to an insoluble electron acceptor or even to another microorganism (81). This provides a mechanism for extending the possibilities for interspecies transfer to the level of a single electron.

Humic Substances

These are complex mixtures of partial lignin degradation products that can be extracted from soils of many kinds and that contain a number of quinonoid compounds. Lovley et al. (80) showed that humic substances could serve as electron acceptors for natural sediment populations and for pure cultures of *Geobacter* or *Shewanella* species. Amounts of acetate that were well in excess of the Fe(III) content of the medium were oxidized when humic substances were present. EPR measurements showed that quinonoid molecules in the mixture were reduced. Subsequently, it has been found that humic substances could be replaced by the convenient analog anthraquinone disulfonate (AQDS).

Humics can serve as electron acceptors. In natural situations, however, it is likely that they more commonly serve as electron shuttles. Microbes reduce quinones to hydroquinones, which in turn react spontaneously to reduce insoluble iron oxides. The oxidized quinone can then shuttle back to the microbe to be reduced again (Figure 2). Substantially higher rates of respiration, and therefore growth, are thus achieved in the presence of humic substances. The Lovley group (81) has also emphasized that humic substances can be reduced by a number of facultatively anaerobic heterotrophic bacteria such as *Paracoccus denitrificans* under anaerobic conditions. This opens the way for ferric iron in the environment to act as terminal electron acceptor for microorganisms that lack the ability to reduce Fe(III) directly (104). Reduced humic substances or anthrahydroquinone disulfonate (the product

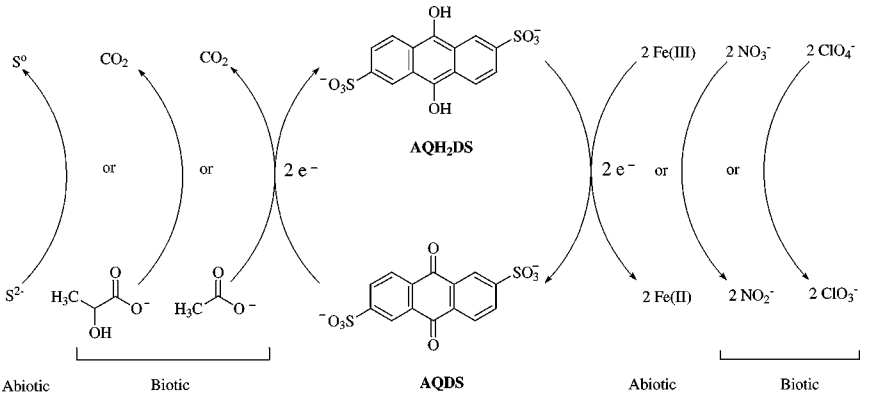

Figure 2 Use of the humic substance analog anthraquinone disulfonate (AQDS) as an electron shuttle to mediate the reduction of inorganic compounds. This enables the oxidation of organic or inorganic compounds by microbes.

of AQDS reduction) can also be used as electron donor for reduction of other terminal electron acceptors such as nitrate, fumarate, arsenate, or selenate (81) (Figure 2). These and related studies suggest that naturally occurring aromatic derivatives can play an important role in interspecies distribution of reducing equivalents.

Compounds produced by the microorganisms themselves may also facilitate electron transfer to abundant but poorly soluble potential acceptors. An intriguing report by Newman & Kolter (88) demonstrated that mutants of *Shewanella putrefaciens* that were unable to reduce humic substance or AQDS differed from the wild type in failing to secrete a small redox-active menaquinone derivative, which was required to mediate the reduction.

Dehalogenation

Public concern about environmental contamination, particularly by halogenated industrial products, has led to extensive investigations of the fates of such compounds in anaerobic environments. Many studies have shown that reductive dehalogenation of aromatic as well as aliphatic compounds occurs fairly rapidly in anaerobic sediments (116), and a large number of pure cultures of anaerobes that carry out reductive dehalogenations have been isolated (54).

Understandably, major emphasis has been placed on the dehalogenation reactions themselves, and less attention has been given to analyzing in detail the advantages a microorganism might derive from catalyzing these reactions. Dehalogenations without further conversions of products are carried out by many different groups of strictly anaerobic and facultatively anaerobic microbes. Under anaerobic conditions, dehalogenations are almost invariably reductive, and consequently haloaryl as well as haloalkyl compounds can serve as electron acceptors for the process of dehalorespiration. Usually those bacteria that have this potential

grow more rapidly with alternative electron acceptors such as nitrate or sulfate, or even by fermentation of a readily usable substrate such as pyruvate. It is only when they are required to use a more reduced carbon substrate, for example lactate or acetate, and are also constrained by the availability of potential electron acceptors that their ability to use halogenated compounds as an electron acceptor becomes evident. The isolates that can dehalogenate aromatic compounds appear to have moderately relaxed substrate specificity, but a few highly specialized isolates, such as strain 2CP-1 (23) and several *Desulfitobacterium* strains (54), that dehalogenate only haloaromatic substrates have been described.

Some strictly anaerobic bacteria, such as *Desulfitobacterium frappieri* (16), that dechlorinate a range of aromatic chlorobenzoates or phenols also use other electron acceptors such as sulfite, thiosulfate, or nitrate when the carbon and energy source is pyruvate. A strain of *Desulfovibrio* TBP-1 grows anaerobically on lactate with a number of mono- or dibromophenols in place of oxidized sulfur compounds, but it is unable to use chlorinated or fluorinated or iodinated phenols (17). A strong preference for the positions that are dehalogenated has been evident in many, but not all, isolates. *Desulfomonile tiedgei* (26) preferentially catalyzes *m*-dechlorinations of substituted benzoates, while *Desulfitobacterium dehalogenans* (108, 109) reacts almost exclusively with chlorophenols that have *o*-substituents. *D. frappieri*, however, dechlorinates aromatic compounds with substituents in the para, meta, and ortho positions (16). The number of different compounds that are dehalogenated anaerobically in enrichment sediment samples is much larger than the number of compounds found to be dehalogenated by pure cultures. One must expect therefore that many additional dehalogenating strains or defined consortia of strains exist and have yet to be brought into captivity.

The discovery that halogenated compounds could replace sulfate as electron acceptors opened the possibility that such reactions might contribute to the microorganisms' energy metabolism as well as redox balance. The enzymes involved in dehalogenation of tetrachlorethylene have been extensively investigated, leading to the suggestion that a short electron transport chain is involved, which can develop a proton gradient (54). Energy conservation accompanying dehalogenation of aromatic compounds does not appear to be universally possible. It has, however, been convincingly demonstrated in a number of strains, in the first place during the use of 3-chlorobenzoate by *Desulfomonile tiedgei* [(29, 84); see (54) for a full review of the evidence]. Careful comparisons of growth yields in cultures of *D. dehalogenans* with limited carbon source, or with electron donors such as H_2 or formate, indicated that the increment generated from dehalorespiration is fairly modest (110). Moreover, energy was conserved from dehalogenation only when substrate-level phosphorylation was precluded. The major physiological advantage of reductive dehalogenation is likely to arise less from the generation of an electrochemical gradient than from the use of chlorinated compounds as electron acceptors. This frees cells from having to use any of the available carbon as electron acceptors in fermentation reactions. However, the enzymes that carry

out dehalorespiration of aromatic derivatives resemble aliphatic dehalogenases in being membrane-bound, and a comparable energy conservation system based on vectorial proton translocation has not been excluded.

The enzymes that catalyze dehalogenation reactions have repeatedly been found to be inducible by growth with halogenated aromatics, as in the very thorough study of Utkin et al. (108). Dehalogenases active with haloaromatics have been purified from *D. tiedgei* (89) and *Desulfitobacterium hafniense* (20), *D. dehalogens* (111), and *Desulfitobacterium chlororespirans* (64). Whereas the *D. tiedgei* 3-chlorobenzoate dehalogenase is a heme enzyme, all other reductive dehalogenases that have been purified are corrinoid-containing iron-sulfur proteins. The purified enzymes from *Desulfitobacterium* species all require a hydroxyl group ortho to the chlorine substituent in order for dehalogenation to occur. As indicated above, activities of whole cells suggest, however, that aromatic dehalogenases with different substrate specificities exist but have not yet been purified.

AROMATICS AS SOURCES OF ENERGY AND/OR CARBON

General Considerations

Microorganisms able to use the entire molecule of aromatic compounds as sources of carbon for growth, or as both carbon and energy sources, in the absence of oxygen are distributed among almost all groups of the Bacteria. Until this year, no Archaea with this ability had been described, although a strain of *Haloferax* could use phenylpropionic acid aerobically (41). Lovley's group (106) has demonstrated that the strictly anaerobic and hyperthermophilic archeon *Ferroglobus placidus*, originally isolated by others by incubating under nitrate-reducing conditions (45), can utilize a number of aromatic compounds, provided Fe(III) is added as an electron acceptor in place of nitrate. Molar growth yield measurements clearly indicate that the aromatic ring itself is attacked. Complete mineralization of aromatic molecules under anoxic conditions appears to be a trait restricted to prokaryotes (48), although fungi may carry out superficial attacks on complex molecules.

The rate of growth and the growth yield that is achieved by a particular microbe when it utilizes an aromatic compound as a source of both carbon and energy depends not only on the biochemistry of the degradation pathway but also on the fate of the reducing equivalents released. Clearly the use of any outside acceptor for electrons increases the proportion of substrate carbon that can be used for biosynthesis. The greatest energy conservation with a given substrate is obviously to be expected when nitrate or chlorate is the final electron acceptor (NO_3^-/NO_2^-, +430 mV; ClO_4^{2-}/ClO_3^{2-}, +1190 mV). Use of ferric iron (Fe^{III}/Fe^{II}, (~0–400 mV)) [see (104)] as an electron acceptor also allows considerable energy conservation. The midpoint potential of the SO_4^{2-}/SO_3^{2-} couple is only −250 mV, so the potential for energy conservation when sulfate is the electron acceptor is much more limited. Fermentative bacteria are even more tightly constrained

energetically. So far, the only strains of fermentative bacteria that have been obtained in pure culture are those that use simple aromatic substrates, such as benzoate, as carbon and energy sources. These fermentative strains tend to be restricted to a syntrophic existence when they are growing on aromatic compounds. Typically, complete biodegradation becomes energetically feasible only when accompanying methanogens or sulfate-reducing bacteria promptly use metabolic end products such as hydrogen that are generated during aromatic compound breakdown. Photosynthetic bacteria avoid many of the constraints imposed on their nonphotosynthetic counterparts, since all their energy is derived from light and aromatic compounds are degraded to form intermediary metabolites such as acetyl-CoA, which are subsequently used in biosynthetic reactions. Carbon and energy sources are thus uncoupled from each other.

Biochemical Strategies for Aromatic Substrate Degradation

The outline and much of the biochemical details of anaerobic attack on an array of simple and substituted aromatic compounds are now known. The substituents attached to more complex aromatics may serve as carbon and energy, or even as nitrogen sources, for microorganisms that are unable to attack the benzene ring (Figure 1). These reactions may serve the additional purpose of making an attack on the ring feasible for those bacteria that can perform complete mineralization but cannot remove some blocking substituents. Benzoyl-CoA emerges as the most common intermediate in the degradation of a diversity of aromatic molecules that are halogenated, methoxylated, or have carbon side chains. Benzoyl-CoA is also a frequent intermediate in the degradation of monohydroxylated aromatic substrates and of some dihydroxylated compounds such as catechol (97).

A few common themes are recognizable in many of the pathways that have been studied in detail. One is that aromatic compounds that are destined to enter the benzoyl-CoA degradation pathway must either carry a carboxyl group (i.e., be an aromatic acid) to begin with or they must be carboxylated to form an aromatic acid in one of the initial metabolic steps. Reactions of this kind initiate attack on phenol, *o*-cresol (2-methylphenol), catechol, and hydroquinone (47, 97). All of the intermediates of the reductive benzoate degradation pathway are CoA thioesters, and intermediates in the funneling pathways that lead to benzoyl-CoA formation are themselves frequently thioesterified at a carboxyl group with coenzyme A (47). Initial attack on aryl hydrocarbons requires condensation with fumarate, followed by β-oxidation reactions that lead to formation of a thioesterified carboxyl group (115) (Figure 3).

Aromatic compounds with two or more hydroxyl groups are less stabilized by the resonance of the benzene ring and are more easily degraded (Figure 4). The degradation of these compounds does not necessarily include carboxylation as an initial step, and further hydroxylations or rearrangements provide alternative ways to decrease the stability of the ring (97). In fact, trihydroxylated aromatic acids, such as gallate, undergo a decarboxylation reaction prior to further degradation (70).

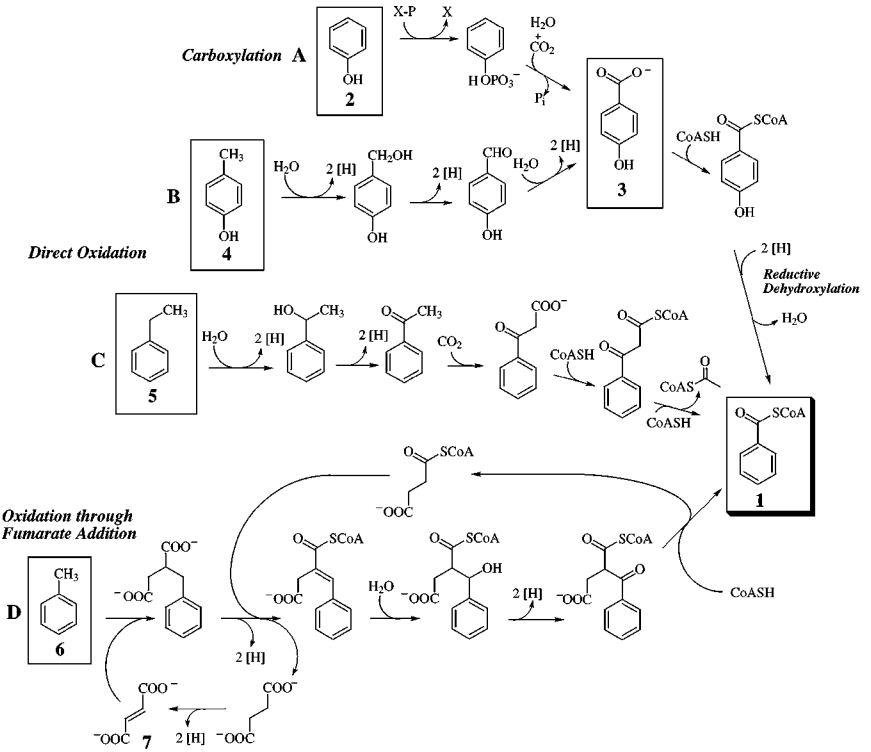

Figure 3 Representative routes of carboxylation and direct oxidation that are followed by bacteria in the conversion of phenol [2] and 4-cresol [4] to 4-hydroxybenzoate [3] and then to benzoyl-CoA [1] as a central intermediate. Ethylbenzene [5] is directly oxidized to benzoyl-CoA, and toluene [6] is oxidized to benzoyl-CoA following fumarate [7] addition.

REACTIONS THAT REMOVE RING SUBSTITUENTS AND BENEFIT THE MICROBE

Many microbes utilize aromatic ring substituents to their advantage while leaving the benzene ring untouched. Such modifications include acyl side chain removal, demethoxylation, and ester hydrolysis (Figure 1).

Acyl Side Chains

The side chains of phenylalkanoates, such as cinnamate, are readily degraded by β-oxidation, yielding acetyl groups for biosynthesis or for energy. Benzoate may appear transiently in enrichments (51) or in pure cultures of the phototroph *Rhodopseudomonas palustris* growing on phenylalkanoates (35), or it may accumulate as an end product (25, 44).

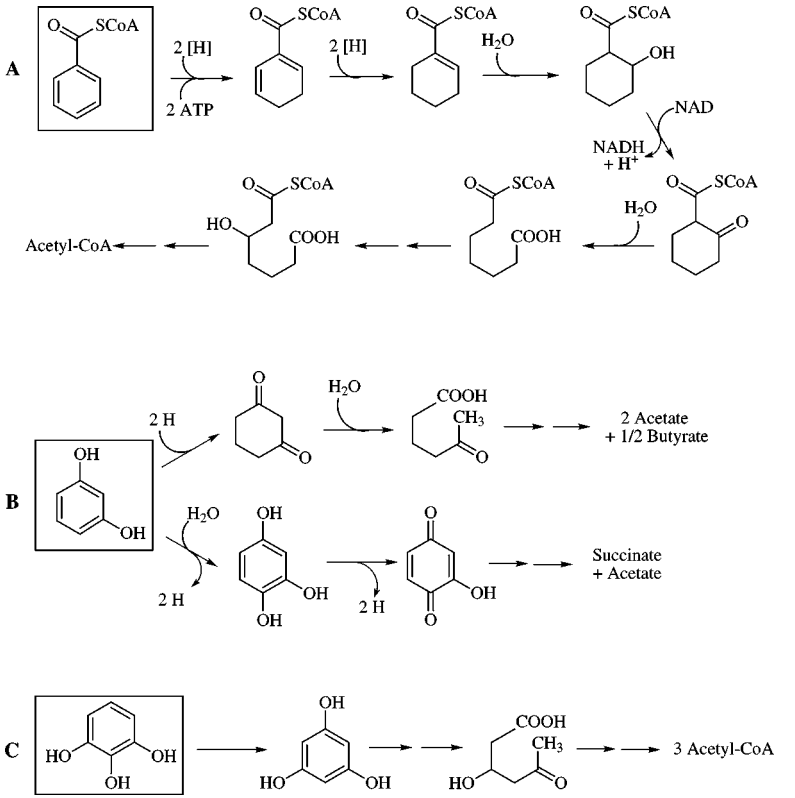

Figure 4 Anaerobic ring cleavage pathways. (*A*) Benzoyl-CoA pathway in *R. palustris*. A slightly different pathway is used by *T. aromatica*. Monohydroxylated and some dihydroxylated aromatic compounds are processed though benzoyl-CoA. (*B*) Dihydroxylated compounds such as resorcinol can be degraded either oxidatively or reductively by routes that do not involve benzoyl-CoA. (*C*) Initial steps in the degradation of trihydroxybenzene.

Aromatic Esters

Aromatic esters are hydrolyzed by enzymes from various sources. Among anaerobic fungi found in the rumen, such as *Neocallimastix* strain MC-2, *p*-coumaroyl, and feruloyl, esterases play an important part in the degradation of plant cell walls (14, 15). The physiological value of these enzymes to the producer lies in improving access to the xylanases that serve as the fermentable substrates for their growth. The aromatic ring does not appear to be attacked by these microorganisms.

The mammalian urine constituent hippurate (benzoylglycine) can be used anaerobically as well as aerobically by a range of microorganisms, among them nonsulfur purple photosynthetic bacteria. Whereas *Rhodobacter capsulatus* uses only the glycine resulting from hydrolysis by its hippuricase, as indicated by both recovery

of benzoate from the culture supernatant and the molar growth yield of the culture, *R. palustris* uses both hydrolysis products (82).

Methoxylated Compounds

Methoxylated aromatic molecules are major components of lignins. A number of acetogenic and other bacteria can use the methyl group of phenylmethyl ethers for synthesis of acetic acid. The CH_3 portion is removed by reactions involving corrinoid and tetrahydrofolate cofactors, but the phenolic derivative of the reactions is not used (27, 65, 66, 114).

Aromatic Nitrogen Sources

Nitrotoluenes are readily reduced either partially or completely in anaerobic environments to corresponding amino compounds, which are considerably more stable. There is evidence that anaerobic enrichments of microbes can use these products as nitrogen source, but the identity of the microorganisms that benefit in this way has not yet been established (39, 71).

MOBILIZING THE BENZENE RING FOR COMPLETE DEGRADATION

As noted above, benzoyl-CoA enters a central pathway of ring reduction and cleavage that allows for the complete degradation of diverse aromatic compounds to acetyl-CoA and carbon dioxide under anaerobic conditions. Many natural and synthetic aromatics either lack the carboxyl group that is necessary for benzoyl-CoA to be ultimately formed or they carry ring substituents that must first be removed. Some examples of modifications of aromatic compounds that occur to prepare them for entry into central ring degradation pathways follow, and are shown in Figure 3.

Amino Groups

Anthranilate (2-aminobenzoate) supports anaerobic growth of denitrifying bacteria as sole carbon and nitrogen source (18). In *Thauera aromatica*, the molecule is first thioesterified and subsequently reductively deaminated to benzoyl-CoA (76). Aniline serves as carbon and nitrogen source for both sulfate-reducing (98) and denitrifying strains (61). The molecule is first carboxylated to 4-aminobenzoate, converted to the CoA thioester, and then reductively deaminated as in *T. aromatica* (61, 99).

Halogen Groups

Monohalogenated benzoates can be completely degraded and serve as carbon sources for a variety of anaerobes, for example, some denitrifiers of the *Thauera/Azoarcus* group (46, 102). Some but not all strains of the phototrophic bacterium

R. palustris also degrade chlorinated benzoates (112). A strain (RCB100) of *R. palustris* that can grow with 3-chlorobenzoate as sole carbon source forms 3-chlorobenzoyl-CoA as an initial intermediate, and this is then dehalogenated to yield benzoyl CoA (31). Most laboratory strains of *R. palustris* are unable to degrade 3-chlorobenzoate, but enrichments usually yield 3-chlorobenzoate-degrading strains (31). Moreover, strains that do not degrade 3-chlorobenzoate initially will often acquire this ability upon extended incubation with 3-chlorobenzoate as the sole utilizable carbon source. This suggests that just one or a few mutations may suffice for phototrophs to acquire the ability to convert chlorinated benzoates to benzoyl-CoA. A similar phenomenon may contribute to the long lags commonly observed in establishing nonphotosynthetic dehalogenating enrichments.

Phthalates

Phthalates are aromatic dicarboxylates that are widely used industrially. Various isomers have repeatedly been shown to be degraded under nitrate-reducing, sulfate-reducing, and methanogenic conditions, but few pure cultures have been studied. A denitrifying *Pseudomonas*-like strain accumulated benzoate transiently (90, 91) during growth on phthalate, indicating that one of the carboxyl groups was removed before the ring was degraded. The enzymology of phthalate degradation has not been defined.

Hydroxylated Compounds

At least four distinct pathways for the degradation of hydroxylated aromatic compounds have been described (97). The specific route taken appears to be dictated in large part by the number of hydroxyl groups and their relative positions on the ring. Compounds with a single hydroxyl group are processed through the benzoyl-CoA pathway, following introduction of a carboxyl group when this is not already present. Phenol carboxylation by *T. aromatica* has been extensively studied. Phenylphosphate, formed using an unidentified phosphoryl donor, is carboxylated to give 4-hydroxybenzoate, and this is thioesterified and then reductively dehydroxylated (72, 73). Rapid progress is being made in defining the genes and enzyme mechanisms involved (19). It is not yet clear whether the energy-requiring carboxylation reaction is also used by bacteria that have more restricted energy budgets, but phenol utilization under methanogenic or sulfate-reducing conditions has commonly been observed (5, 42).

The reversible 4- and 3,4-hydroxybenzoate carboxylases/decarboxylases from *Clostridium hydroxybenzoicum* (49, 50, 55, 118) have strikingly different properties from the phenol carboxylase of *T. aromatica*. *Cl. hydroxybenzoicum* uses amino acids for growth and does not completely degrade either phenol or 4-hydroxybenzoate. Thus it is not yet clear what physiological advantage this bacterium may derive from carrying out the carboxylation/decarboxylation reaction.

The pathways and the bacteria that can use aromatic substrates bearing two or more hydroxyl groups or a hydroxyl group in addition to other substituents have been comprehensively reviewed (97) and are only superficially described here (Figure 4). Hydroquinones and catechols are often fed into the benzoate pathway in sulfate-reducing bacteria and fermenters, using carboxylation reactions that have not been studied in detail. Dihydroxylated compounds can also be degraded, particularly by denitrifying bacteria, by routes that do not include benzoyl-CoA as an intermediate. When more than one hydroxyl is present, destabilization of the aromatic ring may be achieved by tautomerization, sometimes after adding another hydroxyl group. Tautomerized intermediates may then react either oxidatively or reductively (Figure 4), and subsequent ring opening is easily achieved. Although detailed studies have only been carried out with nitrate-reducing, sulfate-reducing, and some fermentative bacteria, it is probable that similar metabolic steps will be found in both iron and chlorate reducers. The availability and nature of the electron acceptor(s) used by the cultures govern the end products of the degradations.

Aromatic Hydrocarbons

As with halogenated compounds, potential toxicity and widely distributed contamination have prompted a plethora of studies on aromatic hydrocarbon degradation. Strategies used by anaerobic bacteria of various physiological types for the degradation of the mono aromatic compounds benzene, toluene, ethylbenzene, and xylene (BTEX) have been examined (53). So far, all studies indicate that the initial attack on these compounds is geared to converting them to benzoyl-CoA. Specific, inducible enzymes catalyze several unusual reactions in the sequences of reactions leading to benzoyl-CoA formation.

TOLUENE Of the BTEX components, toluene appears to be most readily degraded in anaerobic microcosms established from contaminated, or even pristine, sites (53, 79, 92, 95, 103). Benzylsuccinate, which appears extracellularly in generally small quantities ($\sim$1%) during toluene degradation, was at first believed to be a metabolic dead-end by-product (40). Benzylsuccinate is formed by the condensation of toluene with fumarate and this reaction is now recognized as the initial step in toluene degradation in strains of *Thauera* and *Azoarcus*, as well as a sulfate-reducing strain (7, 8, 10, 67). Benzylsuccinyl-CoA is then converted by β-oxidation reactions to form benzoyl-CoA (Figure 3). Many of the enzymes catalyzing toluene degradation have been purified and the corresponding genes cloned (1, 24, 74, 75). It is becoming clear that addition of fumarate is a general reaction used to activate hydrocarbons, and it occurs also in the initial reactions with other methylated aromatics such as xylenes (7, 9, 37, 67), *m*- and *p*-cresol (86, 87), and methyl naphthalene (3). It also has a counterpart in the first reaction undergone by alkanes (69, 94). So far, it appears that fumarate is the only cellular metabolite that can initiate the reaction sequence.

ETHYLBENZENE Although less rapidly removed from contaminated sites, ethylbenzene can yet serve as carbon and energy sources for nitrate- and sulfate-reducing bacteria (93, 96), provided steps are taken to keep its concentration in the growth medium low. Maximum growth rates are low; doubling times of 30–48 h seem to be common. Ethylbenzene is oxidized to 1-phenylethanol and then converted in a series of reactions to benzoyl-CoA (Figure 3). The steps leading to benzoyl-CoA formation have been defined, and the initiating enzyme, which converts ethylbenzene to 1-phenylethanol, has been purified, characterized, and the corresponding gene cloned (58, 59, 62, 63).

BENZENE Benzene has been shown to disappear from contaminated soils and aquifers with accompanying reduction of ferric iron, nitrate, or sulfate, and stable anoxic microcosms in which benzene was oxidized have also been studied (2, 53, 78). Despite intense efforts, pure cultures of benzene-degrading bacteria have not yet been obtained from such enrichments, which has precluded biochemical studies. This frustrating state of affairs is likely to change shortly. Coates et al. (21) have found that two strains of *Dechloromonas*, isolated under different conditions, could grow with benzene as the sole carbon source when nitrate was supplied as electron acceptor. Cells transferred to fresh benzene-containing medium grew as well as the original culture, and the doubling times appeared to be only a small number of hours. These striking observations provide obvious opportunities for elucidating the physiology and biochemistry of benzene degradation under anaerobic conditions.

NAPHTHALENE Naphthalene-degrading bacteria have been obtained in pure culture (43), but most work on mobilization of naphthalene and related compounds has been done with stable enrichments of microbes (3, 83, 105). There is evidence that a carboxyl group is added to the 2-carbon of one ring of naphthalene by a reaction(s) that has not been studied in detail (83, 117). The uncarboxylated ring is then reduced by a series of reactions that may be analogous to the benzoyl-CoA reduction outlined below. 2-Methylnaphthalene degradation has been shown to be initiated by fumarate addition (3).

BENZOATE REDUCTION

Benzoate is the aromatic compound that is most commonly degraded by anaerobic bacteria, and the processes involved have been more thoroughly studied than any other aspect of anaerobic aromatic compound degradation. Benzoate degradation pathways have been worked out in the phototroph *R. palustris* and the denitrifier *T. aromatica* K172 (Figure 4). Both are initiated by conversion of benzoate to benzoyl-CoA by specific CoA ligases (47), and this product is attacked reductively to give a cyclohexadienecarboxyl-CoA intermediate (13). Detailed studies with the four-subunit-enzyme benzoyl-CoA reductase from *T. aromatica* indicate that two

ATP are consumed in each two-electron reduction reaction (107). The subsequent steps of ring modification and ring cleavage differ in *T. aromatica* and in *R. palustris*, but each pathway eventually leads to the formation of 3-hydroxypimelyl-CoA, which is degraded to give three molecules of acetyl-CoA and one carbon dioxide. Genes encoding enzymes of anaerobic benzoate degradation have been cloned from both *R. palustris* and *T. aromatica*, and the gene sequences reflect the observed similarities and differences in the pathways used by each organism (47).

Whether the limited energy resources of benzoate-utilizing syntrophs and sulfate-reducing bacteria could support the same reductive enzymology as is utilized by the phototrophs and denitrifiers has been questioned (47, 101). In the syntrophic-fermenting bacteria, the generation of three ATP by substrate-level phosphorylation from conversion of benzoate to three molecules of acetate would be negated by the initial investment of three ATP (one for benzoate activation by a CoA ligase and two for benzoyl-CoA reduction). It has been suggested that some of the initial energy investment could be spared if benzoyl-CoA were reduced to the level of cyclohex-1-ene-carboxylate or cyclohexanecarboxylate by the concerted addition of four or six reducing equivalents in an ATP-independent reaction that has yet to be demonstrated. Evidence that is consistent with this idea is the observation that *Syntrophus aciditrophicus* transiently releases significant quantities (at least 20% of benzoate used) of cyclohexanecarboxylate into its growth medium during benzoate degradation when grown in a defined coculture with *Methanospirillum hungatei* (36). Cyclohexadienecarboxylate, the expected two-electron reduction product, was not released during benzoate degradation. Measurements of some appropriate enzyme activities (36), suggests that the steps following benzoyl-CoA ring reduction are the same in *S. aciditrophicus* and in *R. palustris* (34, 47).

In all syntrophs examined so far, benzoate is activated to its CoA derivative by an ATP-consuming ligase rather than by a CoA transferase. A proton-translocating pyrophosphatase that can theoretically recover one third of the ATP that is expended when benzoate is converted to benzoyl-CoA, pyrophosphate, and AMP, has been sought and identified in *Syntrophus gentianae* (100) and should be expected also in *S. aciditrophicus*.

Benzoate degradation intermediates appear to exist as CoA thioesters in *S. aciditrophicus* (36), and release of the free-acid forms of these compounds into the external medium thus requires that the CoA moiety first be cleaved off. This seems tantamount to the loss of a high-energy bond and accumulation of such a large amount of cyclohexanecarboxylate in the culture medium during benzoate degradation by *S. aciditrophicus* is therefore surprising. These considerations suggest that cyclohexanecarboxylate formation by syntrophs must confer some advantage to cells. The possibility that cyclohex-1-ene-carboxylate or its CoA derivative serves as an electron acceptor for *S. aciditrophicus* cells growing in pure culture on benzoate suggests one advantage and has recently received experimental support (38). Product analyses indicate that reducing equivalents generated during the fermentation of one molecule of benzoate to acetate and carbon dioxide are used to reduce a second benzoate molecule to cyclohexanecarboxylate. Pure cultures of *S.*

aciditrophicus do not utilize cyclohexanecarboxylate that is produced during benzoate fermentation. The observation that cyclohexanecarboxylate is both produced and then reutilized by cocultures of the syntroph and a hydrogen-utilizing microbe may reflect that *S. aciditrophicus* is physiologically biased toward a fermentative mode of growth on benzoate, even when a hydrogen-utilizing partner is present.

INSIGHTS FROM GENOMICS AND MOLECULAR APPROACHES

The use of traditional genetic approaches has lagged behind traditional biochemical investigations as a means of elucidating pathways of anaerobic aromatic compound degradation. This is partly because many of the microbes that have been used in studies are difficult to grow routinely as colonies on solid media. Also, relatively little effort has been put toward the development of genetic systems for use in those microbes that do grow well on plates. Molecular approaches have been successfully applied to *R. palustris* to identify regulatory genes involved in aromatic compound degradation (28) and to construct mutants for purposes of verifying proposed routes of degradation (34). Recently, an *Azoarcus* sp. strain T mutant with a disruption in one of the genes encoding benzylsuccinate synthase was constructed and found to be defective in growth on both toluene and *m*-xylene (1). This simple experiment provided the valuable information that the same enzyme that initiates toluene degradation initiates *m*-xylene degradation.

Genes encoding dozens of enzymes involved in various anaerobic aromatic degradation pathways have been sequenced. Clones carrying genes of interest have been identified by complementation of mutants (24), by immunoscreening with antisera raised against purified proteins (30), or most commonly, by using nucleotide probes that were designed from N-terminal amino acid sequences of purified enzymes. Inspection of gene sequences sometimes reveals features that are important for catalytic function but that were not immediately obvious from studies of purified enzymes. Based on sequence analysis it seemed probable that pyrogallol-phloroglucinol transhydroxylase, 4-hydroxybenzoyl-CoA reductase, and ethylbenzene dehydrogenase were molybdenum-containing enzymes (4, 28, 58, 62). Inferences about the mechanisms that govern efficient catalysis by benzoyl-CoA reductase, 4-hydroxybenzoate-CoA reductase, and benzylsuccinate synthase that were gleaned from gene sequence analysis have been confirmed by experimental studies (11, 12, 68).

As a final example, the N-terminal amino acid sequences of proteins extracted from two-dimensional polyacrylamide gels led to the identification of a cluster of genes from *T. aromatica* that encodes phenol-inducible proteins (19). The gene sequences suggest that the phenylphosphate carboxylation reaction may be mechanistically similar to 3-octaprenyl-4-hydroxybenzoate carboxy-lyase, an enzyme that catalyzes a decarboxylation reaction in ubiquinone biosynthesis. The reversible 4-hydroxybenzoate carboxylase from *Cl. hydroxybenzoicum* also bears

some similarity to the same biosynthetic enzyme (55). Although phenylphosphate carboxylase and 4-hydroxybenzoate decarboxylase appear to be dissimilar enzymes, gene sequences suggest that the enzymes may have more in common than is apparent from a superficial comparison of their properties.

Molecular biology has revolutionized studies of microbial ecology by providing tools to examine the presence of specific genes, and therefore metabolic capabilities, in all sorts of soil and water environments. Typically, nucleotide primers specific to genes encoding particular enzymes are used to amplify DNA that has been extracted from environmental samples using polymerase chain reactions. This approach has not yet been widely applied to study aromatic compound degradation in anoxic environments, as there are as yet only a few gene sequences available from different organisms for any given enzyme. Without a set of related genes in hand, it is difficult to know which regions of the gene are the most highly conserved, and therefore the best suited for primer design. This situation is likely to change soon as more examples of gene sequences for enzymes like benzylsuccinate synthase, benzoyl-CoA reductase, and ethylbenzene dehydrogenase become available. Recently, primers specific to reductive dehalogenases have been developed and used to amplify reductive dehalogenase-like genes from dechlorinating microbial consortia (113).

At this writing the whole genome sequence of just one organism that can completely degrade aromatic compounds in the absence of oxygen is available; that of the phototroph *R. palustris*. The genome of the reductive dechlorinating bacterium *Desulfitobacterium hafniense* has also been sequenced (60). However, since it is now possible to sequence microbial genomes in a matter of weeks, one can anticipate that in the next few years the sequences of anaerobic aromatic degrading microbes representative of each metabolic type (denitrifiers, sulfate reducers, and Fe(III) reducers) will become available. Comparative genomics can be expected to yield valuable insights into aspects of aromatic compound degradation that are universal and aspects that are specific to a particular metabolic group. Approaches of functional genomics, such as DNA microarray analysis and whole-cell mass spectrometry, which take advantage of whole genome sequence information, provide the means for developing a comprehensive picture of the complete biodegradation potential of an individual microbial species. It should also be possible to determine how multiple degradation pathways are functionally coordinated at the level of a single microbe to operate simultaneously.

Another advantage of having the nucleotide sequences of large fragments of DNA is that one can more easily identify and study regulatory genes, transporters, and other ancillary genes that are important or essential for degradation but that do not directly contribute to catalysis. Several regulatory genes that control anaerobic benzoate and 4-hydroxybenzoate degradation were identified by examining a benzoate degradation gene cluster from *R. palustris* and by creating site-directed mutants to verify functions that were suspected based on nucleotide sequences (32, 33). A fragment of DNA encoding the ortho-chlorophenol reductive dehalogenase from *Desulfitobacterium dehalogenans* includes a gene

for an integral membrane protein that may act as a membrane anchor for the dehalogenase (111).

FUTURE CHALLENGES AND PROSPECTS

We now have a better understanding of the microbial world's anaerobic degradation potential as a result of studies carried out in the past decade. Starting with initial observations of aromatic compound disappearance in anoxic sediments, metabolically diverse microbes have steadily been brought into captivity as pure cultures. These have served as the experimental subjects for a range of physiological and biochemical studies aimed mainly at elucidating the enzymatic basis for various aromatic compound degradation pathways. The number of microbes that are present in pure cultures is nevertheless still relatively small, and there is no reason to suppose that the full range of microorganisms that participate in anaerobic decomposition of aromatic compounds is known. In particular, defined groups of microorganisms that normally participate in a complex web of metabolite transfers between different species have only occasionally been cultivated.

To date, humans have tended to study aspects of biodegradation that are of interest to humans. Hence the strong emphasis on microbes and enzymes involved in BTEX degradation. Relatively little attention has been given to the microbial decomposition of green plant material in anaerobic environments. It may be useful practically to try to understand biodegradation from the point of view of the participating microbes. How does the possession and utilization of selected aromatic degradation pathways contribute to the success of that organism in the physical and biological environment in which it lives? With the possible exception of some halorespirers, aromatic substrates are among the less favored substrates for anaerobic bacteria. Degradation enzymes are induced only under appropriate conditions of absence of oxygen and presence of substrates, and the growth rate supported is almost always substantially lower than in the presence of more favored carbon and energy sources. Measurements of standing concentrations and fluxes of as large a number as possible of potential nutrients should help us understand factors that control the rates of aromatic compound degradation. We also need sensitive approaches for coaxing microbes to tell us more about how they perceive their environment. With the availability of complete genome sequences, differential displays of gene expression using microarrays can be used to provide insights into the effects of environmental changes. Such technical developments can be expected to have major impacts on our basic understanding of the degradation of aromatic substances in the absence of oxygen, as well as on the ability to manipulate the outcome of natural processes.

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CONTENTS