Anaerobic Metabolism of Aromatic Compounds

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Aromatic compounds comprise a wide variety of natural and synthetic compounds that can serve as substrates for bacterial growth. So far, four types of aromatic metabolism are known. (1) The aerobic aromatic metabolism is characterized by the extensive use of molecular oxygen as cosubstrate for oxygenases that introduce hydroxyl groups and cleave the aromatic ring. (2) In the presence of oxygen, facultative aerobes use another so-called hybrid type of aerobic metabolism of benzoate, phenylacetate, and anthranilate (2-aminobenzoate). These pathways use coenzyme A thioesters of the substrates and do not require oxygen for ring cleavage; rather they use an oxygenase/reductase to dearomatize the ring. (3) In the absence of oxygen, facultative aerobes and phototrophs use a reductive aromatic metabolism. Reduction of the aromatic ring of benzoyl-coenzyme A is catalyzed by benzoyl-coenzyme A reductase. This Birch-like reduction is driven by the hydrolysis of 2 ATP molecules. (4) A completely different, still little characterized benzoyl-coenzyme A reductase operates in strict anaerobes, which cannot afford the costly ATP-dependent ring reduction.

Key words: **aromatic metabolism; benzoyl-CoA pathway; anaerobes; toluene; phenol; benzoate; oxygenases; benzoyl-CoA reductase**

Introduction

Aromatic compounds comprise a wide variety of natural and synthetic compounds that can serve as substrates for bacterial growth (FIG. 1). The interest in cellulose degradation has fostered scientific interest in the degradation of the aromatic copolymer lignin in lignocellulose. Whereas lignin is metabolized mainly by fungi, bacterial metabolism is thought to be limited to the metabolism of low-molecular-weight aromatic compounds. The diversity of aromatic metabolism is larger than previously thought. So far, besides the well-studied aerobic type, four types of aromatic metabolism are known. The distribution of these pathways depends on the availability of oxygen, on the availability of alternative electron acceptors for anaerobic respiration, and also on the rapid fluctuation of oxic and anoxic conditions.

The well-studied aerobic aromatic metabolism is characterized by the extensive use of molecular oxygen as cosubstrate for oxygenases, which introduce hydroxyl groups to facilitate the oxidative cleavage of the ring. Most importantly, the aromatic ring is cleaved by dioxygenases.

Under microaerobic conditions, facultative aerobes use another so-called hybrid type of aerobic metabolism of benzoate, phenylacetate, and anthranilate (2-aminobenzoate). This metabolism does not require oxygen for ring cleavage. All intermediates of these pathways are coenzyme A (CoA) thioesters. Dearomatization is catalyzed by an oxygenase/reductase acting on benzoyl-CoA, phenylacetyl-CoA, and 2-aminobenzoyl-CoA, respectively, followed by an oxygen-independent ring cleavage.

In anoxic water, groundwater, sediments and parts of soil, aromatic compounds are metabolized by facultative aerobes and phototrophs in the absence of oxygen in a purely reductive rather than oxidative process. Essential to anaerobic aromatic metabolism is the replacement of all oxygen-dependent steps by an alternative set of reactions and the formation of different central intermediates. These pathways involve a series of unprecedented enzymes. Notably, two-electron reduction of the aromatic ring of benzoyl-CoA is driven by the hydrolysis of two molecules of adenosine triphospate (ATP). The cyclic, nonaromatic product formed becomes hydrolytically opened and finally is oxidized to three molecules of acetyl-CoA.

Another reductive metabolism is found in strict anaerobes. Here again, benzoyl-CoA is a central

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Dedicated to Lars Ljungdahl.

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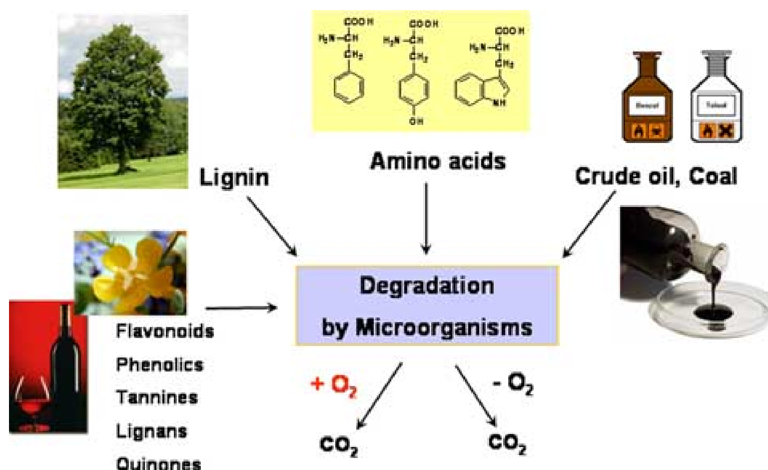


FIGURE 1. Sources of aromatic compounds in nature and man-made aromatic compounds derived from fossil material.

intermediate of aromatic metabolism. However, when one considers growth of strict anaerobes on benzoate, an energetic problem becomes evident: Facultative aerobes, such as denitrifyers and phototrophs, spend four ATP equivalents to activate benzoate as CoA thioester (two ATP equivalents) and to reductively dearomatize the ring (another two ATP equivalents). Their energy metabolism, anaerobic respiration, such as nitrate respiration, yields many more than four ATP equivalents per one benzoate metabolized; phototrophs conserve energy by photophosphorylation. In contrast, strict anaerobes gain fewer than four ATP equivalents out of one benzoate, which is metabolized via three molecules of acetyl-CoA plus one CO_2. Yet, they still require two ATP equivalents for benzoyl-CoA formation. However, they cannot spend another two ATP equivalents for the reductive dearomatization of benzoyl-CoA, because otherwise their energy metabolism would be energy-consuming rather than energy-providing. The outlines of this postulated new principle of benzoyl-CoA reduction are just emerging.

Most of the novel enzymatic reactions have counterparts in organic chemistry ("Chemistry-inspired Biology"). The chemical principles are modified according to biological constraints, for example, the limits of the redox potential of the cellular electron carriers, the use of water as solvent at relatively moderate temperature, or the low ambient substrate concentrations.

This short review gives an overview of the different strategies, with a focus on the anaerobic pathways. It does not cover the classic aerobic pathways and other important aspects, such as transport, regulation of enzymes, transcriptional control, genetic organization, distribution of the pathways, and ecological, evolutionary, and applied aspects.

Aerobic Aromatic Metabolism

The use of molecular oxygen in the cleavage of the aromatic ring and in hydroxylation reactions in general is widely distributed in nature, notably when inert or recalcitrant compounds and chemical bonds need to be attacked. Substrates, whose metabolism normally requires molecular oxygen, include aromatic, hydrocarbon, and ether compounds. Oxygenases and oxygen as cosubstrate in hydroxylation reactions have been known since the seminal work of Hayaishi (FIG. 2).[1] In the case of aromatic metabolism, the large variety of substrates is channeled via peripheral (upper) pathways into a few central intermediates. These peripheral pathways make extensive use of oxygen, which is required by monooxygenases and dioxygenases/reductases. The aromatic ring of the central intermediates contains two phenolic hydroxyl groups next to each other, or one hydroxyl group next to a carboxyl. Examples are catechol (1,2-dihydroxybenzene), protocatechuate (3,4-dihydroxybenzoic acid), and gentisate (2,5-dihydroxybenzoic acid). These free intermediates are substrates of ring cleaving dioxygenases of the central (lower) pathways. Ring cleavage may occur between the two hydroxyl groups (ortho cleavage) or next to one of the hydroxyl groups (meta clavage). This metabolism was established decades ago.[2–6]

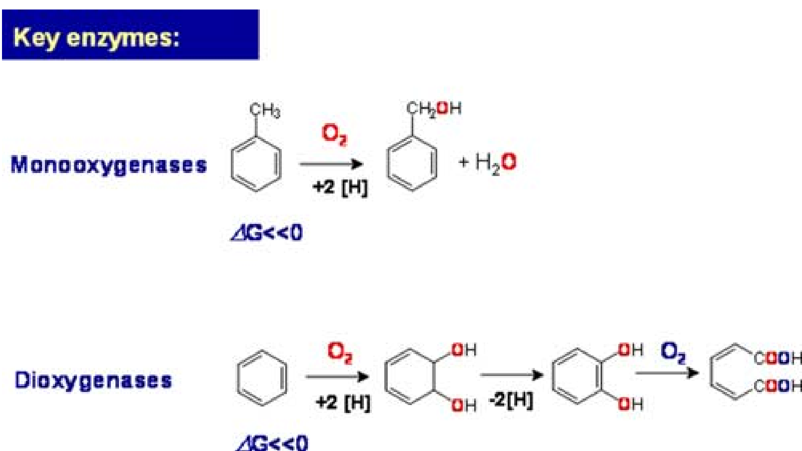


FIGURE 2. Aerobic monooxygenase and dioxygenase reactions in aromatic metabolism. Introduction of phenolic hydroxyl groups and ring cleavage both depend on molecular oxygen.

Aromatic Metabolism Under Microaerobic Conditions: The Hybrid Pathways

A few substrates have been recognized to be metabolized by facultative aerobes in a different way. These so-called hybrid pathways still make use of oxygen to introduce hydroxyl groups, as in the classic aerobic pathways. At the same time, the aromatic ring is reduced and CoA thioesters are used, as in the anaerobic metabolism (see later in the chapter). Ring cleavage also does not require oxygen. The new hybrid pathway of benzoate is shown in FIGURE 3.

Denitrifying facultative aerobes (and possibly others) convert phenylacetate,[7–14] benzoate,[15–18] and 2-aminobenzoate (anthranilate)[19–24] to their CoA thioesters first. Bacteria may even exclusively metabolize phenylacetate via this new principle; the more conventional pathway—via ring hydroxylation to homogentisate (2,5-dihydroxyphenylacetate) and ring cleavage of homogentisate—appears to be restricted to fungi. Benzoyl-CoA and phenylacetyl-CoA are then converted by dioxygenases/reductases to the corresponding nonaromatic *cis*-dihydrodiols. Normally, such intermediates would be rearomatized by oxidation, yielding dihydroxylated aromatic products. In this case, CoA thiosterification of the carboxyl group helps to promote the subsequent cleavage of the aromatic ring, whereby oxygen is not required. The postulated pathway of phenylacetyl-CoA degradation has not been solved yet, but the principle seems to be similar to the benzoate case (see FIG. 3). In the case of anthranilic acid, 2-aminobenzoyl-CoA is converted by a monooxygenase/reductase to a monohydroxylated nonaromatic intermediate. The subsequent ring cleavage reaction has not been studied yet.

These CoA thioester–dependent pathways may be advantageous under fluctuating oxic/anoxic conditions. The pathways allow flexibility and rapid adaptation to fluctuating oxygen levels, since both oxic and anoxic situations require substrate CoA thioesters. If the classic pathway of, for example, benzoate would operate, the shift from anoxic to oxic conditions would result in the accumulation of benzoyl-CoA, since the ring-reducing enzyme benzoyl-CoA reductase of the anoxic pathway is oxygen-sensitive and therefore would be inactive (see later in the chapter). All cellular CoA would be trapped in this dead-end product, since benzoate-CoA ligase is oxygen-insensitive and would still operate under oxic conditions. CoA depletion ultimately would be lethal. In addition, benzoyl-CoA oxygenase/reductase, the enzyme that dearomatizes benzoyl-CoA, has a high affinity for oxygen, and therefore can operate under microaerobic conditions. The energy spent in CoA thioester formation is not lost, but is later regained in the form of acetyl-CoA. Furthermore, the CoA ester intermediates may be less toxic than some intermediates of the classic pathways, notably those of some meta cleavage routes. Also, CoA thioester formation indirectly facilitates the transport of the aromatic acids.

Anaerobic Aromatic Metabolism by Phototrophs and by Facultative Aerobes with Anaerobic Respiration

Pioneering work on the anaerobic aromatic metabolism was done by Charles W. Evans (1911–1988)

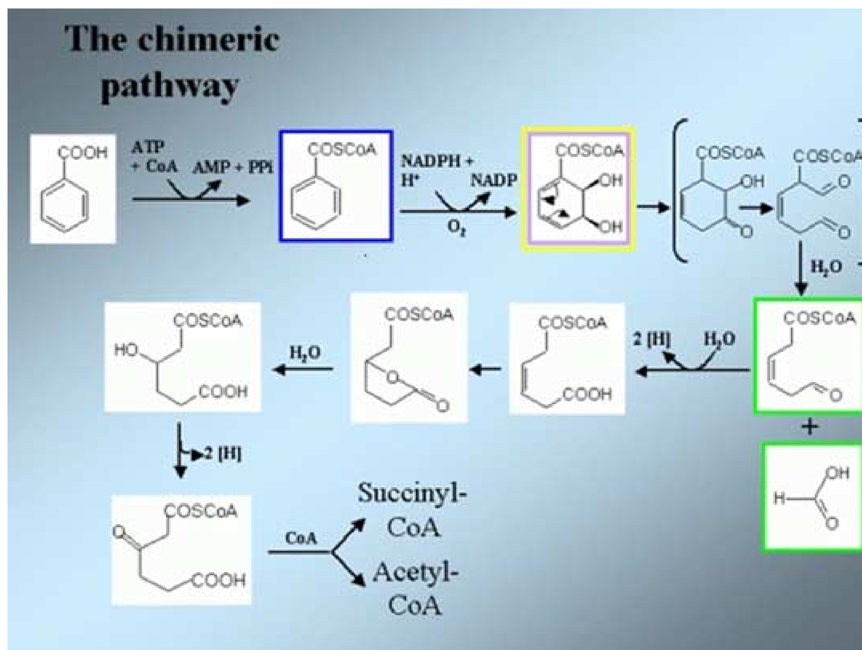


FIGURE 3. Hybrid (chimeric) aerobic pathway of benzoate metabolism carried out by some denitrifying bacteria. The reactions involve principles of aerobic and anaerobic aromatic metabolism.

and his group.[25,26] Several reviews have covered the state of the art.[27–42] Representative bacterial species include the phototrophic bacterium *Rhodopseudomonas palustris* and the denitrifying bacteria *Thauera aromatica, Azoarcus evansii, Azoarcus* strain EbN1 (renamed *Aromatoleum* sp.), and *Magnetospirillum* sp. These bacteria metabolize aromatic (and sometimes hydrocarbon) compounds anaerobically under phototrophic or denitrifying conditions. The energy yields (ATP) by metabolism in these bacteria is high, as compared to strict anaerobes such as iron-reducing, sulfate-reducing, or syntrophic-fermenting bacteria. Still, the aromatic molecule catabolism is performed anaerobically.

The aromatic substrates are mostly handled as CoA esters, and the peripheral pathways yield completely different central intermediates, such as benzoyl-CoA, resorcinol (1,3-dihydroxybenzene) or phloroglucinol (1,3,5-trihydroxybenzene), among others. In contrast to the aerobic pathways, where phenolic hydroxyl groups are introduced by oxygenases, phenolic hydroxyl groups are often reductively removed. The aromatic ring is reduced, affording alicyclic compounds. Whereas ring reduction in phloroglucinol can be accomplished by using pyridine nucleotides as reductant, the reduction of resorcinal already requires ferredoxin as reductant. The reduction of benzoyl-CoA, however, cannot be accomplished by ferredoxin alone; rather, ring reduction in addition requires the hydrolysis of one ATP per one electron transferred. The nonaromatic ring of such ring-reduction products is hydrolytically opened. Beta-oxidation yields acetyl-CoA as the final product.

To illustrate the new principles, the metabolism of phenol, toluene, and benzoate will be considered in more detail. These substrates are metabolized via the benzoyl-CoA pathway (for a description of the less sophisticated resorcinol and phloroglucinol pathways, see Refs. 33–35 and 38). Each of these pathways has its metabolic constraint (FIG. 4). Phenol carboxylation is unfavorable due to its unfavorable equilibrium constant. Toluene metabolism is difficult, since breaking C-H bonds by withdrawing a hydrogen atom from the hydrocarbon is energetically unfavorable. Reduction of the aromatic ring requires an enormous activation energy, because the transfer of the first electron requires an extremely low redox potential.

Anaerobic Phenol Metabolism

Anaerobic Phenol Metabolism in Facultative Aerobes

In *T. aromatica* and other facultative aerobes phenol is metabolized via transformation to benzoyl-CoA, whose aromatic ring then becomes reduced in the

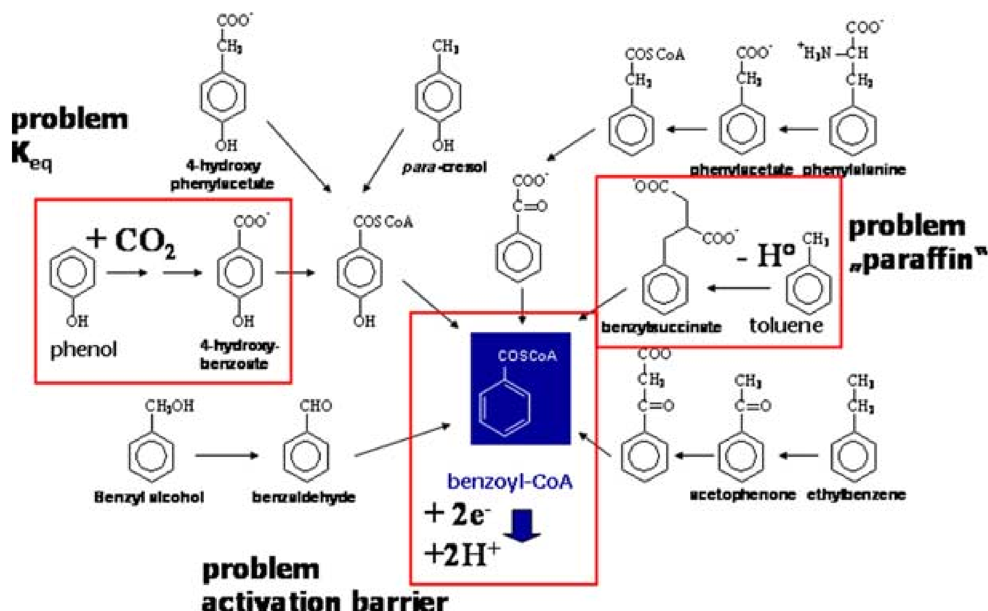


FIGURE 4. Anaerobic metabolism of aromatic compounds: three cases, three problems. Peripheral reactions in anaerobic aromatic metabolism. The conversion of phenol, toluene, and benzoyl-CoA are highlighted by *boxes*.

central benzoyl-CoA pathway. The peripheral phenol pathway leading to benzoyl-CoA obviously requires a carboxylation step, a CoA thioester activation of the carboxyl group, and a reductive removal of the phenolic hydroxyl group (FIG. 4). Such reactions have their counterparts in organic synthesis, for example, the Kolbe–Schmitt synthesis (phenol carboxylation) and the Birch reduction (reductive benzene ring dearomatization and the reductive dehydroxylation of phenols). The Kolbe–Schmitt synthesis (FIG. 5) requires high concentrations of phenol and CO_2. This process is well-studied and requires a phenolate anion as nucleophilic substrate, the electrophilic CO_2 as second substrate, as well as K^+ or Na^+ as cocatalyst.[43,44] Taking into account the concentrations of phenol and CO_2/bicarbonate under natural conditions, the carboxylation of phenol would yield 4-hydroxybenzoate at very low concentrations. If the assumed equilibrium constant is correct (FIG. 5), and given the assumed low concentrations of phenol and CO_2, theoretically fewer than one molecule 4-hydroxybenzoate per cell would result in the reaction equilibrium. This energetically unfavorable situation can be drastically improved by using ATP to phosphorylate the incoming phenol in an irreversible reaction (however, see later in the section for anaerobes that use an ATP-independent phenol carboxylation system). The product phenylphosphate, however, is a poor substrate (a poor nucleophile) for the electrophilic attack of CO_2 in the subsequent carboxylation step. Therefore, special care has to be taken to dephosphorylate phenylphosphate in the course of the carboxylation reaction without releasing phenol (which otherwise would result in a futile recycling of phenol coupled to ATP hydrolysis).

The first two steps in phenol metabolism in the denitrifying bacterium *T. aromatica* are phosphorylation of phenol to phenylphosphate by ATP, catalyzed by phenylphosphate synthase,[45–49] and subsequent carboxylation of phenylphosphate to 4-hydroxybenzoate with release of phosphate, catalyzed by phenylphosphate carboxylase.[49–51] At least 15 genes are phenol-induced and cotranscribed. They include the genes coding for seven purified proteins that are required for phenol carboxylation to 4-hydroxybenzoate.[51,52] The function of at least seven other genes of the phenol operon is unknown.

Phenylphosphate Synthase

The molecular and catalytic features of phenylphosphate synthase (FIG. 6) (E.C. 2.7.9.-) resemble those of phosphoenolpyruvate synthase (E.C. 2.7.9.2), albeit with interesting modifications. The reaction follows a

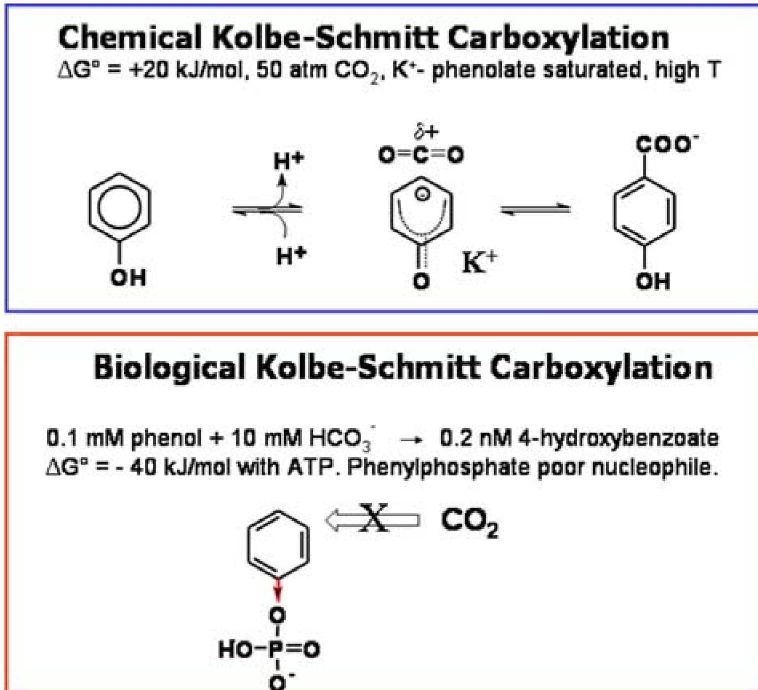


FIGURE 5. Comparison of the energy demands of chemical versus biological phenol carboxylation. Under natural conditions, low substrate and low temperature may limit carboxylation activity.

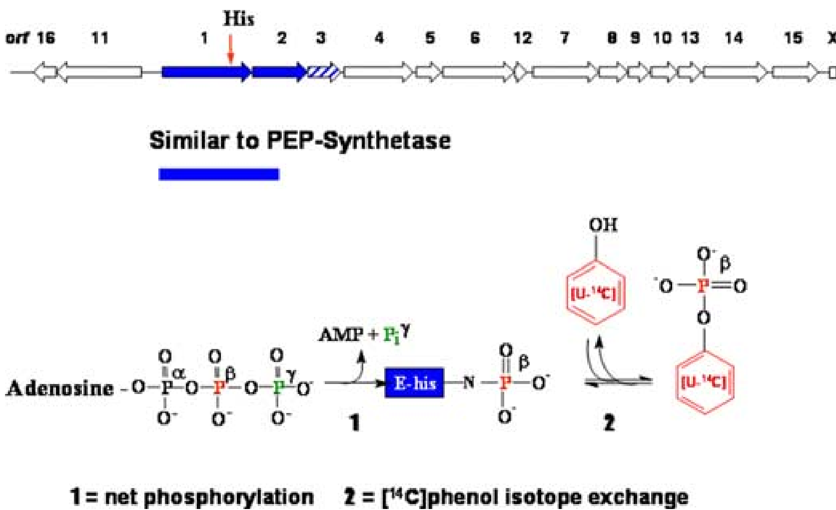
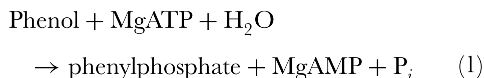


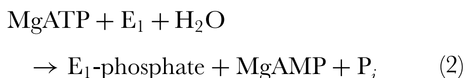
FIGURE 6. Schematic presentation of phenylphosphate synthase (or synthetase) reaction and of the genes involved. For details, see the text.

Ping-Pong mechanism, and is described by Equation 1 (referred to as the *net phosphorylation reaction*). The whole reaction (Eq. 1) is understood as the sum of Equation 2 and Equation 3. In the course of net phenol phosphorylation, the enzyme becomes phosphorylated by ATP in an essentially irreversible step (Eq. 2). The phosphorylated enzyme E_1 subsequently transforms phenol to phenylphosphate in a reversible reaction (Eq. 3).

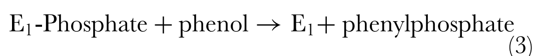
Consistent with this mechanism, the enzyme also catalyzes an exchange of free [^{14}C]phenol and the phenol moiety of phenylphosphate (Eq. 4, referred to as *phenol exchange reaction*). This suggests that enzyme E_1 becomes phosphorylated by phenylphosphate in the course of this phenol exchange reaction (Eqs. 5 + 6).



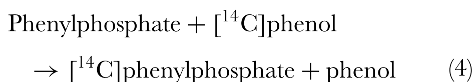
$$\text{Phenol} + \text{MgATP} + H_2O \rightarrow \text{phenylphosphate} + \text{MgAMP} + P_i \quad (1)$$



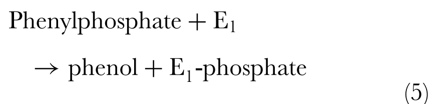
$$\text{MgATP} + E_1 + H_2O \rightarrow E_1\text{-phosphate} + \text{MgAMP} + P_i \quad (2)$$



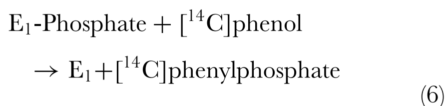
$$E_1\text{-Phosphate} + \text{phenol} \rightarrow E_1 + \text{phenylphosphate} \quad (3)$$



$$\text{Phenylphosphate} + [^{14}C]\text{phenol} \rightarrow [^{14}C]\text{phenylphosphate} + \text{phenol} \quad (4)$$



$$\text{Phenylphosphate} + E_1 \rightarrow \text{phenol} + E_1\text{-phosphate} \quad (5)$$

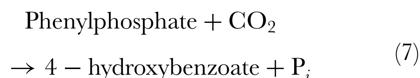


$$E_1\text{-Phosphate} + [^{14}C]\text{phenol} \rightarrow E_1 + [^{14}C]\text{phenylphosphate} \quad (6)$$

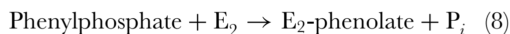
Phenylphosphate synthase E_1 consists of three proteins whose genes are located adjacent to each other on the phenol operon.[48,53] Protein 1 (ORF1, 70 kDa) resembles the central part of phosphoenolpyruvate synthase, which contains a conserved histidine residue. It alone catalyzes the exchange of free [^{14}C]phenol and the phenol moiety of phenylphosphate (Eq. 4, which is the sum of Eqs. 5 and 6), but not the phosphorylation of phenol (Eq. 1). It interacts with the substrate phenol and transfers the phosphoryl group from the phosphorylated protein 1 to the substrate (Eq. 3). Phosphorylation of phenol requires protein 1, MgATP, and another protein 2 of 40 kDa, which resembles the N-terminal part of phosphoenolpyruvate synthase. Protein 2 (ORF2) catalyzes the phosphorylation of protein 1 (Eq. 2). The combination of proteins 1 + 2 affords the net phosphorylation reaction (Eq. 1). The phosphoryl group in phenylphosphate is derived from the β-phosphate group of ATP. It is suggested that protein 2 intermediately transfers a pyrophosphate group from ATP to the conserved histidine of protein 1, from which γ-phosphate is released. The overall reaction is stimulated severalfold by another protein 3 (ORF3, 24 kDa). The exact role of this protein is unknown; it may have a regulatory function since it has some similarity to adenosine monophosphate (AMP) binding proteins and contains a cystathionine beta-synthase (CBS) domain.

Phenylphosphate Carboxylase

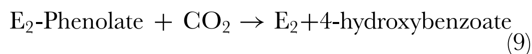
Phenylphosphate synthase makes use of ATP to render the endergonic phenol carboxylation process unidirectional, even under the very low ambient concentrations of phenol (K_M 0.04 mM phenol) and of CO_2. At the same time, however, the electron-withdrawing phosphoryl group makes phenylphosphate a poor substrate for an electrophilic attack by CO_2. Hence, the subsequent phenylphosphate carboxylase (FIG. 7) (E.C. 4.1.1.-) E_2 is expected to exhibit special features.[49–51] The enzyme requires divalent metal ions (Mg^{2+} or Mn^{2+}) as well as K^+, and catalyzes the carboxylation of phenylphosphate to 4-hydroxybenzoate (Eq. 7) (referred to as *net carboxylation reaction*). The actual substrate is CO_2 rather than bicarbonate. Enzyme E_2 follows a Ping-Pong mechanism. The presumed E_2-phenolate intermediate is formed in an exergonic reaction from phenylphosphate (Eq. 8), followed by the reversible carboxylation reaction (Eq. 9). Consistent with this proposal, the enzyme also catalyzes an exchange of free $^{14}CO_2$ and the carboxyl group of 4-hydroxybenzoate (Eq. 10) (referred to as *CO_2 exchange reaction*), which is the sum of Equations 11 and 12. Free ^{14}C-phenol does not exchange with the phenol moiety of phenylphosphate.



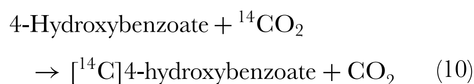
$$\text{Phenylphosphate} + CO_2 \rightarrow 4-\text{hydroxybenzoate} + P_i \quad (7)$$



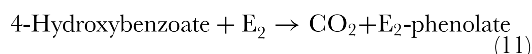
$$\text{Phenylphosphate} + E_2 \rightarrow E_2\text{-phenolate} + P_i \quad (8)$$



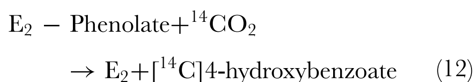
$$E_2\text{-Phenolate} + CO_2 \rightarrow E_2 + 4\text{-hydroxybenzoate} \quad (9)$$



$$4\text{-Hydroxybenzoate} + {}^{14}CO_2 \rightarrow [^{14}C]4\text{-hydroxybenzoate} + CO_2 \quad (10)$$



$$4\text{-Hydroxybenzoate} + E_2 \rightarrow CO_2 + E_2\text{-phenolate} \quad (11)$$



$$E_2 - \text{Phenolate} + {}^{14}CO_2 \rightarrow E_2 + [^{14}C]4\text{-hydroxybenzoate} \quad (12)$$

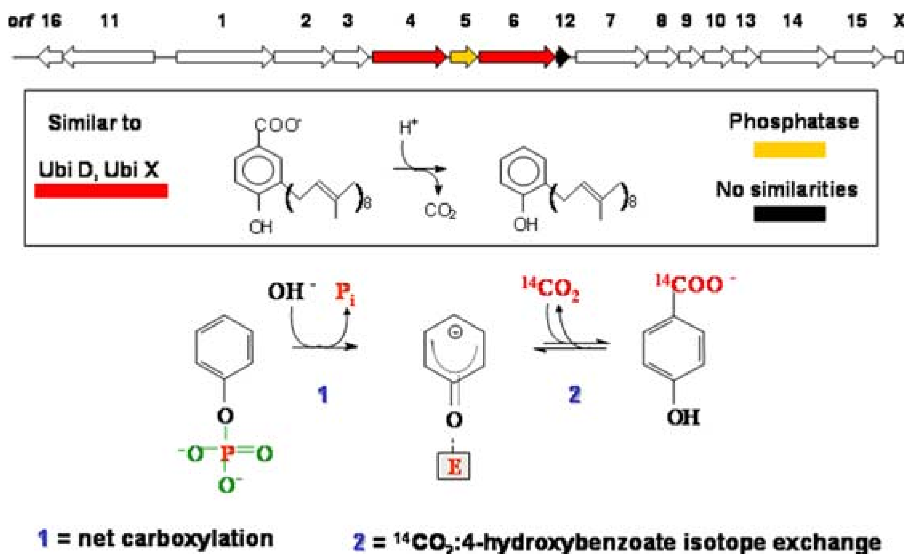


FIGURE 7. Schematic presentation of phenylphosphate carboxylase reaction, of the genes involved, and of similar enzymes and reactions. For details, see the text.

Phenylphosphate carboxylase consists of four proteins whose genes are located adjacent to each other on the phenol gene cluster.[51,52] Three of the subunits (αβγ, 54, 53, and 10 kDa) are sufficient to catalyze the CO_2 exchange reaction (Eq. 10, which is the sum of Eqs. 11 and 12), but not the net phenylphosphate carboxylation (Eq. 7). Phenylphosphate carboxylation is restored when the 18-kDa (δ) subunit is added. This 18-kDa phosphatase subunit alone also catalyzes a very slow hydrolysis of phenylphosphate. The 54- and 53-kDa subunits show similarity to UbiD, 3-octaprenyl-4-hydroxybenzoate carboxy lyase, which catalyzes the decarboxylation of a 4-hydroxybenzoate derivative in ubiquinone (*ubi*) biosynthesis.[51] The 18-kDa subunit belongs to a hydratase/phosphatase protein family. The 10 kDa is unique. The function of the remaining seven other genes of the phenol gene cluster, two genes related to *ubiD* and *ubiX*, respectively, is completely unknown.

The genomes of several bacteria contain genes related to phenol metabolism. Examples are *Magnetospirillum magnetotacticum*; *Magnetospirillum* sp. are among the dominant phenol-degrading denitrifyers.[54,55] The recent sequencing of the genome of *Azoarcus* strain EbN1,[56] which is closely related to *T. aromatica*, revealed a gene cluster very similar to the one found in *T. aromatica*. Phenol metabolism by iron-reducing or sulfate-reducing bacteria has not been studied in detail.

Anaerobic Phenol Metabolism in Strict Anaerobes

Enzymes related to the UbiD- and UbiX-like proteins are involved in phenol or hydroxybenzoate metabolism in strict anaerobes. The 54-kDa 4-hydroxybenzoate decarboxylase from *Clostridium hydroxybenzoicum*,[57,58] and subunits of various proven or putative vanillic acid (3-methoxy-4-hydroxybenzoic acid) decarboxylases from *E. coli*, *Bacillus subtilis*,[59] and *Streptomyces* sp.[60] show similarity with UbiD and UbiX. In some anaerobes these enzymes may function as decarboxylases, yielding phenolic compounds from the corresponding phenolic acids.[61] This reaction serves as a CO_2 source for acetogenic bacteria. However, clostridia appear to use the energetically unfavorable ATP-independent phenol carboxylation reaction even in phenol metabolism.[62–66] This follows from the time course of phenol consumption and product formation by whole cells and from the observation that ^{13}C labeled benzoate is formed from ^{13}C-labeled phenol; also, fluorinated phenolic compounds give rise to fluorinated benzoic acids. Obviously, these bacteria encounter higher phenol and CO_2 concentrations in their natural habitat. These substrates are formed there by other bacteria that decarboxylate hydroxybenzoic acids. Furthermore, effective consumption of 4-hydroxybenzoate in phenol-grown cells may indeed lower its concentration dramatically, thus enabling these anaerobes to live with phenol.

4-Hydroxybenzoate-CoA Ligase and 4-Hydroxybenzoyl-CoA Reductase (Dehydroxylating)

The product of phenol carboxylation, 4-hydroxybenzoate, is converted to its CoA thioester by a specific CoA ligase.[67,68] The dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA is essential for phenol metabolism, as the following enzyme in the pathway, benzoyl-CoA reductase, does not accept the *para*-hydroxylated compound as a substrate for mechanistic reasons. In contrast, benzoyl-CoA reductase can reduce the *ortho*- and *meta*-isomers of monohydroxylated benzoyl-CoA analogues.[69,70]

The reaction catalyzed by 4-hydroxybenzoyl-CoA reductase is shown in FIGURE 4. The enzyme from *T. aromatica* has a molecular mass of 270 kDa and consists of three subunits of 82 (a), 35 (b), and 17 kDa (c), suggesting an $(abc)_2$ composition.[71] The enzyme contains two [2Fe–2S] clusters, a [4Fe–4S] cluster, a flavin adenine dinucleotide (FAD), and a molybdopterin-cytosine dinucleotide cofactor per abc-trimer.[72] The genes coding for the three subunits of 4-hydroxybenzoyl-CoA reductase were identified in *T. aromatica*,[73] and in the phototrophic bacterium *R. palustris*.[74] The structure of 4-hydroxybenzoyl-CoA reductase confirmed that the enzyme belongs to the xanthine oxidase family of molybdenum enzymes.[75,76] The Mo-atom is coordinated by two sulfur atoms from the dithiolene group of the molybdopterin, by an oxo and a water ligand. A fifth ligand is most probably a sulfur atom, which, however, was artificially replaced by an oxo-ligand. Further structural and spectroscopic details of 4-hydroxybenzoyl-CoA reductase and comparisons with other members of the xanthine oxidase family have been discussed elsewhere.[77]

4-Hydroxybenzoyl-CoA reductase is the only member of this family whose function is to catalyze the reduction of substrate; the reversibility of this reaction could not be demonstrated. Buckel and Keese (1995) discussed the essential role of the thiol ester functionality for the reductive dehydroxylation reaction.[78] In analogy to the related process of benzene-ring reduction, they suggested that a ketyl radical anion is transiently formed. The electrochemical properties of the redox centers of 4-hydroxybenzoyl-CoA reductase also appear to be suited for a low-potential redox chemistry: a low potential reduced ferredoxin serves as electron donor; in addition, a unique [4Fe-4S] cluster and a Mo-cofactor with an unusually low redox potential are present.[79] In principle, the proposed catalytic cycle of 4-hydroxybenzoyl-CoA reductase runs counterclockwise to the one of xanthine oxidase members.[80]

Anaerobic Toluene Metabolism

In spite of their chemical inertness, hydrocarbons are degraded by microorganisms in the complete absence of oxygen. As all known aerobic hydrocarbon degradation pathways start with oxygen-dependent reactions, hydrocarbon catabolism in anaerobes must be initiated by novel biochemical reactions. Under anoxic conditions, a variety of reactions seems to be employed to overcome the activation barrier of different hydrocarbons. Examples include oxygen-independent hydroxylation, as employed in ethylbenzene metabolism, fumarate addition to methyl or methylene carbons in toluene or alkane degradation, and only recently discovered reactions, such as methylation of naphthalene or anaerobic methane oxidation via reverse methanogenesis.[80] All these reactions have in common a high-energy requirement of cleaving C-H sigma bonds in alkanes (FIG. 8).

Benzylsuccinate Synthase

The initial step in toluene degradation consists of the radical addition of fumarate to the methyl group of toluene, yielding (*R*)-benzylsuccinate, catalyzed by the glycine radical enzyme benzylsuccinate synthase (FIG. 9).[80–84] It consists of a large, glycyl-radical carrying subunit of 97 kDa and two very small subunits (8.5 and 6.5 kDa) of unknown function. The enzyme-bound radical is thought to abstract a hydrogen atom from the methyl group of toluene, generating a benzyl radical intermediate to which fumarate is added.[80] This process affords a benzylsuccinyl radical, which then abstracts the hydrogen atom from the enzyme to form benzylsuccinate; the enzyme radical is thereby regenerated. The formation of the active enzyme in the radical form requires activation by an activase enzyme, which uses *S*-adenosylmethionine and an electron donor as cosubstrates. A chaperon-like protein may be required for the assembly or activation of the system. These proteins are coded on a single operon. This unique metabolic capability has been discussed recently (see Refs. 80 and 85–87).

Oxidation of Benzylsuccinate to Benzoyl-CoA and Succinate

Benzylsuccinate is converted by a kind of beta-oxidation, which is initiated by CoA transfer from succinyl-CoA by a specific CoA transferase, forming 2-(*R*)-benzylsuccinyl-CoA. All enzymes of this peripheral pathway are encoded by a second operon. The reaction cycle is complete when succinate is oxidized to fumarate. Thus, the overall pathway brings about a six-electron oxidation of the methyl group of toluene to the carbonyl group of benzoyl-CoA. The reduced

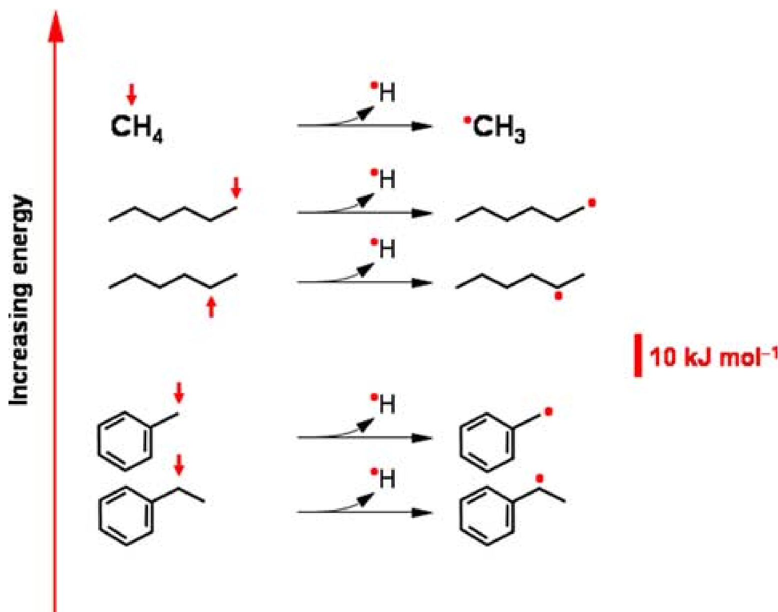


FIGURE 8. Energy requirement for C-H bond cleavage. Illustration of the comparative energy needs to homolytically cleave the C-H bond in various hydrocarbons by withdrawing a hydrogen atom via a radical mechanism. (After Friedrich Widdel, Bremen.)

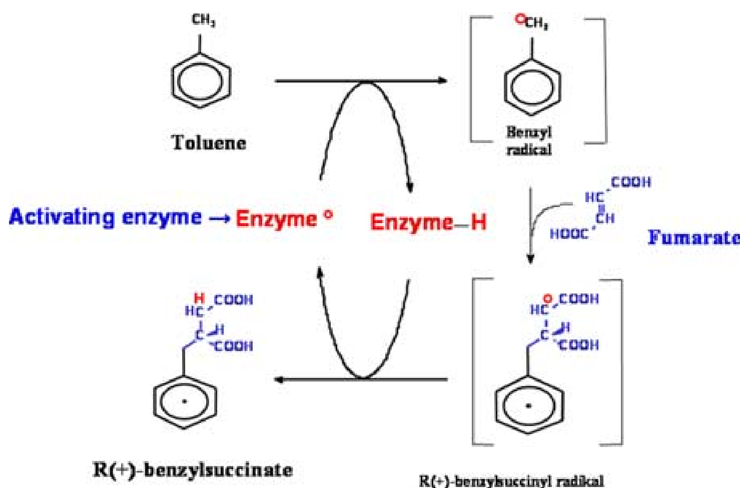


FIGURE 9. Proposed mechanism of benzylsuccinate synthase. Enzymatic attack of toluene by radical addition of fumarate. The same principle applies to many other hydrocarbons.

electron carriers are reoxidized in the course of the anaerobic respiration.

Anaerobic Benzoyl-CoA Reduction, and following Oxidation, to Acetyl-CoA

As can be seen from the scheme of the peripheral anaerobic metabolism (FIG. 4), quite different aromatic compounds, such as phenol, toluene, ethylbenzene, phenylacetate, some cresols, or benzyl alcohol, are all converted to benzoyl-CoA. Benzoyl-CoA reduction is the key step in the central metabolism of these and other, though not all, aromatic compounds.[25,26,88,89] As indicated earlier, resorcinol or phloroglucinol can be reduced more easily, and the metabolism of various

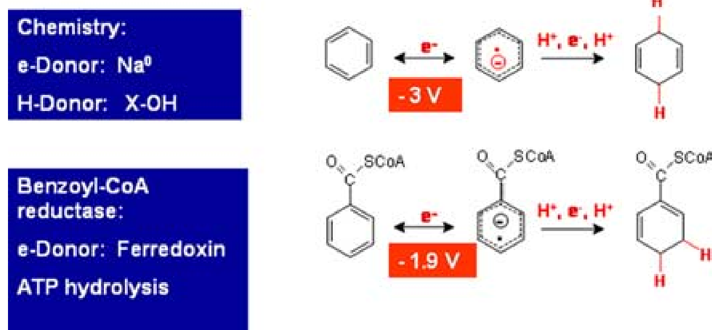


FIGURE 10. Birch reduction of the aromatic ring in benzene and enzymatic benzoyl-CoA reduction. Effect of the CoA thioester group of benzoyl-CoA on the redox potential of the first electron transfer reaction.

di- and trihydroxylated aromatic compounds proceeds via these intermediates.[34,35,38,39]

Benzoyl-CoA Reductase

The reduction of benzoyl-CoA is catalyzed by benzoyl-CoA reductase,[90,91] which has only been studied in some detail in the denitrifying bacterium *T. aromatica*. The genes for the benzoyl-CoA pathway were first detected in *R. palustris* and later in *T. aromatica*.[92,93] Benzoyl-CoA reduction seems to follow a Birch mechanism (FIG. 10), that is, a sequential transfer of single electrons and protons.[94] The product of the two-electron reduction of benzoyl-CoA is cyclohex-1,5-diene-1-carbonyl-CoA.[95] This reduction reaction is greatly facilitated by the use of the CoA thioester, because the thioester group lowers the redox potential difference of the first electron transfer step by almost one volt. This strong effect explains why the anaerobic pathways use CoA thioesters throughout, because at the end benzoyl-CoA rather than benzoate is needed for ring reduction. The formation of CoA thioesters is coupled to ATP hydrolysis to AMP and pyrophosphate, which normally is followed by hydrolysis of pyrophosphate. This drives the reaction forward and supports the transport of the substrate by keeping its intracellular pool concentration low. Whether the conjugated cyclic 1,5-diene is the general product of dearomatizing reductases from other organisms is not clear; in benzoyl-CoA reductase from *R. palustris,* a four-electron reduced monoene species (cyclohex-1-ene-1-carbonyl-CoA) is formed.[36]

The oxygen-sensitive benzoyl-CoA reductase from *T. aromatica* contains three [4Fe-4S] clusters as sole redox centers.[91,96,97] It has a modular composition: the 49- and 29-kDa subunits each contain one ATP-binding site, and a single [4Fe-4S] cluster is coordinated by two cysteine residues of each subunit. This arrangement is considered as the electron activation module. The other two subunits contain two [4Fe-4S] clusters; they are assigned to the aromatic ring-reduction module.

Benzoyl-CoA reductase couples the transfer of electrons from the electron donor, reduced ferredoxin, to the substrate benzoyl-CoA, to the hydrolysis of ATP[91,98]; one molecule of ATP is hydrolyzed per one electron transferred to the aromatic ring (i.e., two ATP are required to reduce benzoyl-CoA) (FIG. 11). In the catalytic cycle of ATP-dependent electron transfer, ATP hydrolysis yields a high-energy enzyme-phosphate linkage[99]; hydrolysis of the latter enables electron transfer to the substrate. During catalysis, two different ATP-dependent switches are involved in electron transfer to the substrate. The "nucleotide binding-switch" induces conformational changes in the vicinity of a special [4Fe-4S] cluster. The cluster serves as primary acceptor for electrons transferred from the external donor, which is reduced ferredoxin. In addition, binding of nucleotides switches the substrate-binding pocket into an open state, which enables benzoyl-CoA binding.[98] The "enzyme-phosphate hydrolysis" switch induces a low-spin/high-spin ($S = 7/2$) transition of a [4Fe-4S] cluster. This transition is assigned to a substantial lowering of the redox potential of the cluster.[97]

The proposed "Birch-like" reduction mechanism of benzoyl-CoA reductase was probed by kinetic and computational studies using a number of benzoyl-CoA analogues.[100] The redox potential for the one-electron reduction of benzoyl-CoA is -1.9 V (as determined

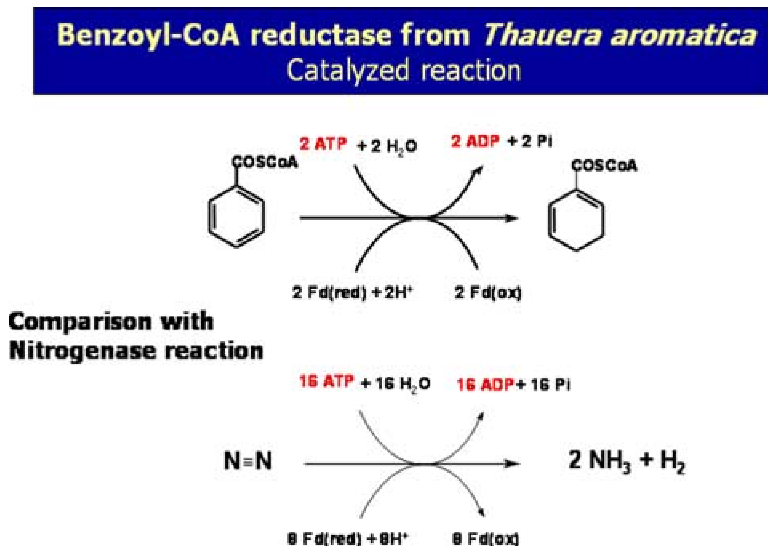


FIGURE 11. Benzoyl-CoA reductase reaction and comparison to the nitrogenase reaction. Both reactions require ATP for electron transfer, though with a different stoichiometry of ATP per electron.

for the benzoic acid *S*-methylthiol ester),[95] which is far from the potential of the electron donor. However, a partial protonation of the carbonyl-oxygen of benzoyl-CoA could increase this redox potential considerably. In addition, the first electron transfer could be proton assisted, which would further facilitate the reaction. These steps may complement the action of ATP hydrolysis, which is thought to lower dramatically the redox potential of the reduced-electron transferring group. Single-turnover studies[97] and studies with ^{33}S- and ^{57}Fe-labeled enzymes suggested the presence of a sulfur-centered species, most probably a disulfide radical anion formed by two cysteine residues in close proximity of an active-site [4Fe–4S] cluster.

Following Oxidation of Cyclohex-1,5-diene-1-carbonyl-CoA to Acetyl-CoA

The oxidation of the product of benzoyl-CoA reductase, cyclohex-1,5-diene-1-carbonyl-CoA, follows a kind of beta-oxidation, including a hydrolytic opening of the alicyclic ring (FIG. 12). Finally, three molecules of acetyl-CoA and one molecule of CO_2 are formed. Denitrifying bacteria assimilate part of the acetyl-CoA into cell material, and most of the acetyl-CoA is oxidized completely to CO_2; they gain energy by electron-transport phosphorylation, using nitrate as an electron acceptor (anaerobic respiration). Phototrophs do not oxidize acetyl-CoA, but rather use it as a carbon source for biosynthesis; they obtain energy from photosynthesis.

Anaerobic Aromatic Metabolism in Strict Anaerobes

In strict anaerobes, benzoyl-CoA is again a central intermediate of aromatic metabolism. However, when one considers growth of strict anaerobes on benzoate, an energetic problem becomes evident: Facultative aerobes gain many more than four ATP equivalents from one benzoate metabolized than strict anaerobes and can therefore spend four ATP equivalents to activate benzoate as CoA thioester (two ATP equivalents) and to reductively dearomatize the ring (another two ATP equivalents). In contrast, strict anaerobes gain fewer than four ATP equivalents (probably three ATP equivalents) out of one benzoate, which is metabolized via three molecules of acetyl-CoA plus one CO_2. Yet, they still require two ATP equivalents for benzoyl-CoA formation. Clearly, they cannot spend another two ATP equivalents for the reductive dearomatization of benzoyl-CoA, because otherwise their energy metabolism would be energy consuming rather than energy providing (see FIG. 13). Consequently, they must use a less costly mechanism of ring reduction. In any case, benzoate needs to be activated to benzoyl-CoA by an ATP-dependent benzoate-CoA ligase (AMP plus PP_i forming). The sulfate-reducing bacterium *Desulfococcus multivorans*, when cultivated on benzoate and sulfate, requires selenium and molybdenum for growth, whereas growth on nonaromatic compounds does not require those trace elements (FIG. 14). In extracts of cells grown on benzoate in the presence

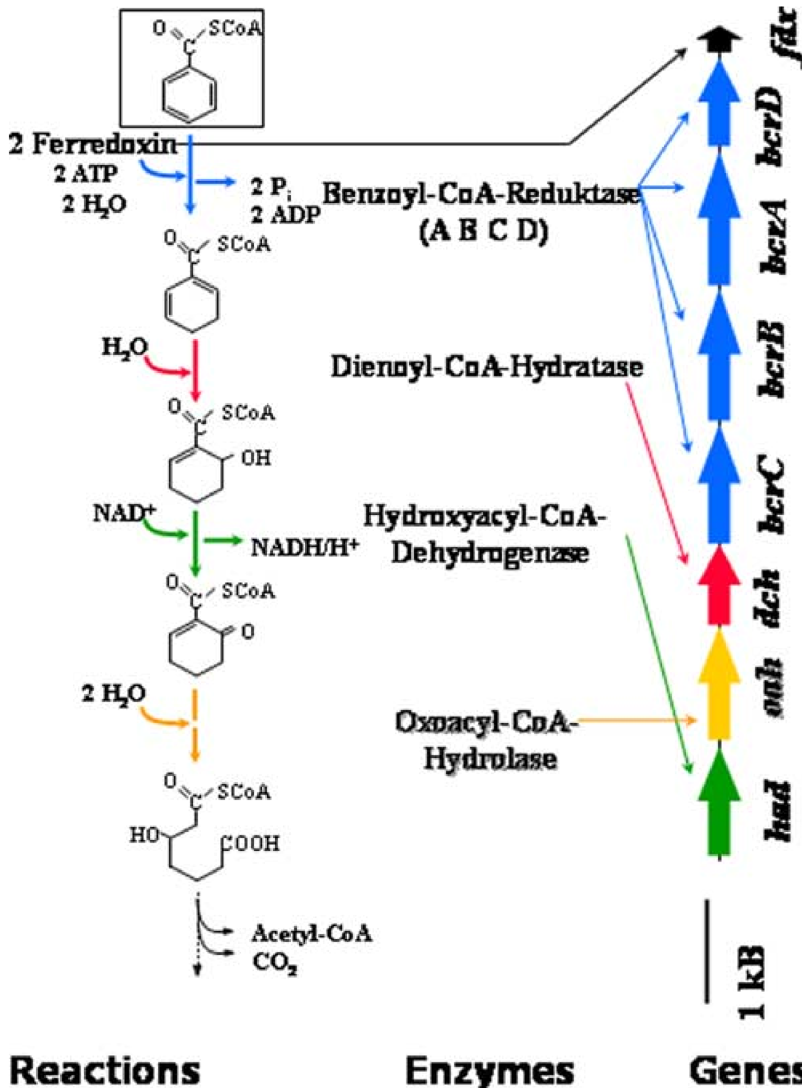


FIGURE 12. The anaerobic benzoyl-CoA pathway via two-electron reduction of benzoyl-CoA to cyclohex-1,5-diene-1-carbonyl-CoA, as studied in *T. aromatica*. The pathway in *R. palustris* differs in that the aromatic ring is reduced in a four-electron reduction, yielding cyclohex-1-ene-1-carbonyl-CoA, with corresponding modification of the subsequent steps.

of [^{75}Se]selenite, three radioactively labeled proteins with molecular masses of 100, 30, and 27 kDa were found. The 100- and 30-kDa selenoproteins were 5- to 10-fold induced in cells grown on benzoate compared to cells grown on lactate. These results suggest that the dearomatization process in *D. multivorans* is not catalyzed by the ATP-dependent Fe-S enzyme benzoyl-CoA reductase (as in facultative aerobes), but rather involves unknown molybdenum- and selenocysteine-containing proteins.[101]

Studies of the obligate anaerobic iron-reducing bacterium *Geobacter metallireducens* uncovered the genes coding for anaerobic benzoate metabolism. They are organized in two clusters comprising 44 genes. Induction of representative genes during growth on benzoate was confirmed by a reverse-transcription polymerase chain reaction. The results obtained suggest that benzoate is activated to benzoyl-CoA, which is then reductively dearomatized. However, in *G. metallireducens* (and most likely in other strict anaerobes) the process of reductive dearomatization of the benzene ring appears to be catalyzed by a set of completely different protein components comprising putative molybdenum- and selenocysteine-containing enzymes.[102]

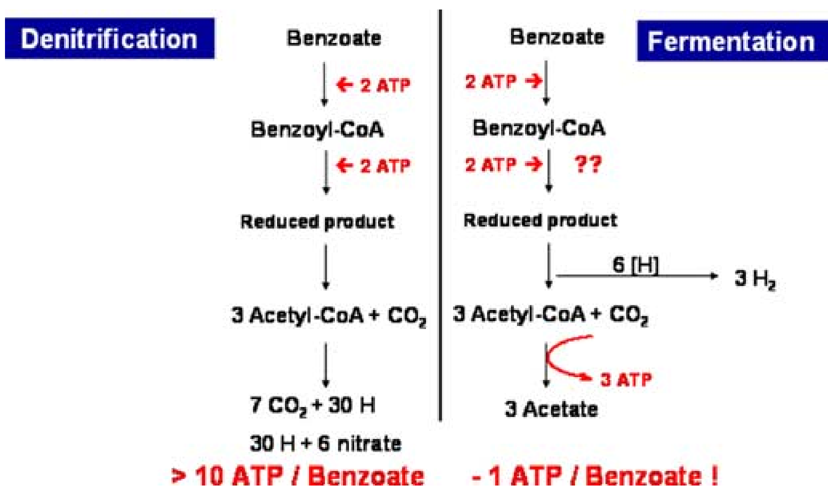


FIGURE 13. Energetic comparison of benzoate catabolic pathways in a denitrifying and a fermenting bacterium. Obviously, activation of benzoate and ATP-driven ring reduction consumes more energy than is gained in fermenting bacteria in the subsequent processes. This suggests an ATP-independent ring reduction in strict anaerobes.

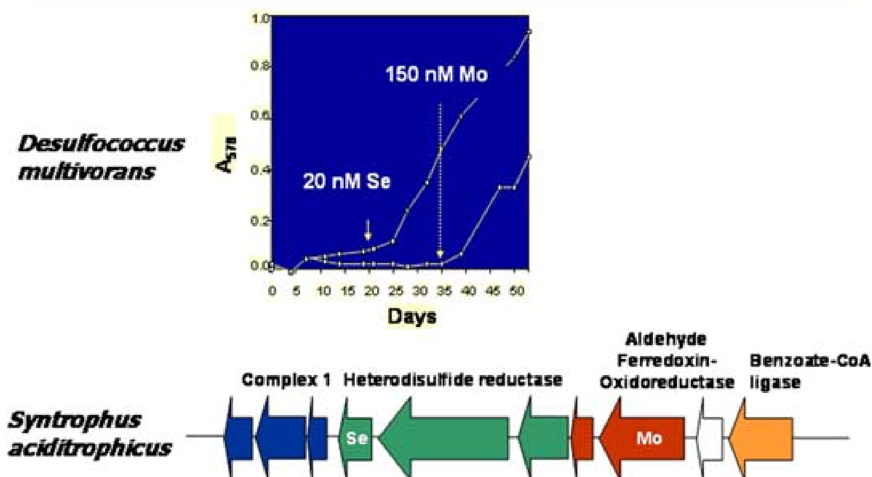


FIGURE 14. Molybdenum and selenium dependence of anaerobic growth of *D. multivorans* on benzoate and sulfate, and the gene cluster postulated to code for benzoate metabolism in *S. aciditrophicus*.

Surprisingly, the product of this new type of ring reduction is also cyclohex-1,5-diene-1-carbonyl-CoA, which must be formed by two-electron reduction of the aromatic ring. This follows from the fact that all genes coding for enzymes, which convert cyclohex-1,5-diene-1-carbonyl-CoA to three molecules of acetyl-CoA plus one molecule of CO_2, are present and the enzymes are active in those strict anaerobes; they are even highly similar to those found in denitrifying bacteria. This applies to *G. metallireducens* (iron reducing), *Syntrophus aciditrophicus* (fermenting), and probably also to *D. multivorans* (sulfate reducing).[103]

Perspective

Our view of aromatic metabolism has been widened by studying bacteria that do not live at high oxygen

tensions. The metabolic diversity has been exemplified by considering the fate of benzoate under different conditions. The classic pathways using ring-cleaving dioxygenases represent just one principle to cope with the problem of breaking the stable aromatic ring. There are other options found by (facultative) aerobes to attack such molecules, even though they still use molecular oxygen to introduce hydroxyl groups at the ring. Interestingly, they use benzoyl-CoA as substrate for hydrolytic ring cleavage. This allows metabolic flexibility and rapid adaptation to fluctuating oxygen levels, since both oxic and anoxic types of metabolism use benzoyl-CoA as an intermediate. Under anoxic conditions, all oxygen-dependent steps need to be replaced by reductive steps. Depending on the energy yield of their metabolism, anaerobes use an ATP-driven or an ATP independent reduction of benzoyl-CoA. Our understanding of the different metabolic strategies is just at the beginning, as are the studies of the enzymes and reaction mechanisms. Their biotechnological potential has not been used. Many other aspects, such as regulation of the new pathways and their evolution, need to be addressed.

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Conflict of Interest

The author declares no conflicts of interest.

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