11 Hydrocarbon Degradation Coupled to Metal Reduction

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Abstract: It is known that hydrocarbon degradation can be coupled to dissimilatory microbial Fe(III) reduction under anaerobic conditions. Of the phylogenetically diverse Fe(III)-reducing organisms isolated and characterized only the *Geobacter* species have been shown to be capable of hydrocarbon oxidation, and even then, these organisms could only utilize simple monoaromatic hydrocarbons such as toluene. Analysis of the *Geobacter metallireducens* genome suggests that the route of aromatic hydrocarbon degradation in Fe(III) reducing bacteria mimics that found in facultative anaerobes with a few exceptions. Similar to facultative anaerobes, parent aromatic hydrocarbons are converted through an upper pathway to benzoyl-CoA. However, benzoyl-CoA appears to be reduced to cyclohexa-1,5-dienecarboxy-CoA by a novel protein complex. This novel benzoyl-CoA reductase (BCR) is hypothesized to contain proteins that have similarities to those found in hydrogenases, heterodisulfide reductases, NADH: Ubiquinone oxidoreductases, aldehyde/ketone: ferredoxin oxidoreductases, and selenium-containing proteins. Unlike other organisms, the method of activation is not ATP dependant, and may occur by a novel process. Here we discuss possible methods of activation, along with a mechanism detailing the steps of benzoate dearomatization and differences in benzoyl-CoA reductases from *G. metallireducens* and *Thauera aromatica*, a well-characterized hydrocarbon-oxidizing facultative anaerobe.

1 Introduction

Several previous studies have demonstrated the hydrocarbon biodegradative capacity of dissimilatory Fe(III) reducing bacteria (Lovley and Lonergan, 1990; Lovley et al., 1994, 1996; Anderson et al., 1998; Anderson and Lovley, 1999; Rooney-Varga et al., 1999; Coates et al., 2001; Kunapuli et al., 2007) and various aromatic hydrocarbons have been shown to be biodegraded in sediments in which Fe(III) was the terminal electron acceptor (Lovley et al., 1994, 1996; Anderson et al., 1998; Anderson and Lovley, 1999; Kunapuli et al., 2007). Microbial community studies demonstrated that these sediments were often enriched in organisms of the family Geobacteraceae (Rooney-Varga et al., 1999). However, there was no direct evidence to show that members of this family of organisms are capable of hydrocarbon metabolism other than toluene degradation. In contrast, stable isotope analysis of a benzene-degrading Fe(III)-reducing enrichment culture obtained from soil of a former coal gasification site in Gliwice, Poland, suggested that hydrocarbon degradation was mediated by a novel syntrophic interaction between members of the gram-positive clostridial *Peptococcaceae* and the *Desulfobulbaceae* species within the delta subclass of the Proteobacteria (Kunapuli, Lueders et al., 2007). Of all the known phylogenetically diverse Fe(III)-reducing bacteria only *Geobacter metallireducens* and *G. grbiciae* are capable of hydrocarbon degradation in pure culture and even then their metabolism is limited to toluene (Lovley and Lonergan, 1990; Coates et al., 2001).

2 Hydrocarbon Degradation by *G. metallireducens*

The degradation of aromatic compounds including toluene by *G. metallireducens* can be inferred through its available genome sequence. Computational annotation indicates that the organism has genes that enable the degradation of phenol, p-cresol, benzyl alcohol, benzaldehyde, toluene, and 4-hydroxybenzoate (Butler et al., 2007). Similar to facultative

anaerobes, it is believed that benzoyl-CoA is a common intermediate in the degradation of all listed aromatic compounds under Fe(III)-reducing conditions. In addition, many of the genes involved in the processing of these compounds are found on the "aromatic island"; a region of genes in the *G. metallireducens* genome that are believed to be involved in aromatic hydrocarbon degradation (Butler et al., 2007).

Biochemical analysis on the enzyme responsible for the conversion of toluene to benzylsuccinate, an activating step necessary for toluene conversion to benzoyl-CoA, revealed the initial activation is mediated by a glycine-radical-dependent enzyme called benzyl succinate synthase (BSS) (Leuthner et al., 1998). A similar type reaction is observed in toluene-degrading denitrifying bacteria, sulfate reducing bacteria, phototrophic bacteria, and methanogenic consortia (Chakraborty and Coates, 2003). As in *T. aromatica* (Leuthner et al., 1998), BSS was identified in *G. metallireducens* by its activity, the radical catalyzed stereospecific addition of fumurate to toluene to produce (R)-benzylsuccinate (Kane et al., 2002). In addition, *bssA* and *bssB*, genes for the subunits of this enzyme complex, were identified using southern blot hybridization (Kane et al., 2002). The C-terminal region of BssA from *Thauera aromatica* str. K172 corresponded strongly to 411 residues found in the BssA from *G. metallireducens* (Kane et al., 2002). Most notably, this subunit was found to contain a conserved cysteine and glycine. The cysteine is believed to participate in the formation of a radical on the amino acid glycine (Kane et al., 2002). This essential glycine-radical abstracts a hydrogen atom from toluene forming a benzyl radical. This enzyme bound benzyl radical adds to the double bond of the co-substrate fumarate, which is also bound at the active site. The resulting benzylsuccinyl radical abstracts the hydrogen from the aforementioned glycine residue, thus regenerating the glycine radical that initiated the reaction (Kane et al., 2002). The BSS from *G. metallireducens* appears to follow this model reaction as d8-toluene (toluene deuterated at all positions) was converted to d8-benzylsuccinate, suggesting the initial deuterium atom removed from toluene to form the benzyl radical was subsequently added back to the benzylsuccinyl radical to form benzylsuccinate (Kane et al., 2002).

3 Conversion of Benzoyl-CoA to Cyclohexa-1,5-Dienecarboxy-CoA

As outlined above, a central metabolite of aromatic degradation in Fe(III) reducing bacteria is benzoyl-CoA. Benzoyl-CoA is formed by benzoyl-CoA ligase (Egland et al., 1995; Schuhle et al., 2003). This enzyme hydrolyzes ATP to AMP and PP_i while ligating benzoate to coenzyme A through a thioester linkage (Egland et al., 1995). While an energetically intensive step (it uses two equivalents of ATP per unit benzoate), it is consistently seen in all strictly anaerobic bacteria that oxidize aromatics, as well as facultative anaerobes (Elder and Kelly, 1994; Fuchs, 2008).

Analysis of the upregulated proteome of *G. metallireducens* in the presence of benzoate showed that one such upregulated protein, called BamY, had greater than 50% sequence identity to benzoyl-CoA ligases from *T. aromatica*, *Azoarcus evansii*, and *Magentospirillum magnetotacticum* (Wischgoll et al., 2005). The gene for this protein was cloned and overexpressed in *Escherichia coli* (Wischgoll et al., 2005). Using a spectrophotometric assay for carboxylic acid coenzyme A ligases, overexpressed BamY was found to have a specific activity of 16 μmol min^{-1} mg^{-1} for benzoate. This value was higher than that reported for other anaerobes, and proved the existence of this enzyme in Fe(III) reducing organisms (Wischgoll et al., 2005).

The next step in the aromatic degradation pathway appears to be a two electron reduction of the aromatic ring. In *T. aromatica* this step is performed by the enzyme benzoyl-CoA reductase (BCR) (Boll and Fuchs, 1995; Boll et al., 1997). BCR is composed of four subunits (bcrA-D) that contain 3 [4Fe-4S] clusters and two ATP-binding domains (Boll et al., 1997; Breese et al., 1998; Boll et al., 2000; Boll et al., 2001) (also see *Fig. 1*). Similar to nitrogenase, BCR couples electron transfer to its substrate with ATP hydrolysis (Boll et al., 1997; Mobitz et al., 2004). In this reaction ATP binds to BCR changing the enzyme from a "closed" to an "open" state (Mobitz et al., 2004). In the "open" state, a unique [4Fe-4S] cluster is reduced by soluble ferredoxin, and benzoyl-CoA enters the enzyme's substrate-binding pocket (Mobitz et al., 2004). Following these two events, the ATP bound to the enzyme is hydrolyzed. This ATP hydrolysis serves as an "activating" step changing the spin state of the reduced [4Fe-4S] cluster from S = 1/2 to S = 7/2 (Boll et al., 2000). In addition, this hydrolysis also presumably lowers the redox potential of this cluster to the −1 V redox potential required for the reduction of benzoyl-CoA to a dienoyl-CoA radical anion (Boll, 2005; Fuchs, 2008).

It appears the two electron reduction of benzoyl-CoA occurs in *G. metallireducens* (Peters et al., 2007). A study by Wischoll et al. showed that 44 genes upregulated during benzoate degradation in *G. metallireducens* included a putative cyclohexa-1,5-dienecarboxy-CoA dehydratase (bamR). This enzyme hydrolyzes the product of benzoyl-CoA reductase, cyclohexa-1,5-dienecarboxy-CoA, indicating that the initial two electron reduction occurs (Peters et al., 2007). In addition, whole cell lysate from *G. metallireducens* was shown to have the ability to oxidize cyclohexa-1,5-dienecarboxy-CoA, but not cyclohexa-1-ene-carbonyl-CoA (a metabolite formed when phototrophs oxidize benzoyl-CoA by a presumably different pathway (Harwood et al., 1999)). Further, when the bamR gene product is overexpressed in *E. coli*, it was capable of oxidizing cyclohexa-1,5-dienecarboxy-CoA to 6-OH-cyclohexanoyl-CoA (Peters et al., 2007). This reaction is characteristic of the pathway exemplified by *T. aromatica*.

4 A Unique Benzoyl-CoA Reductase in *Geobacter*

Unexpectedly, no transcript bearing any similarity to benzoyl-CoA reductase is upregulated during growth on benzoate (Butler et al., 2007). Although one gene in the *G. metallireducans* genome bears some significant similarity to one of the subunits in benzoyl-CoA reductase (Hosoda et al., 2005), it was not upregulated during growth on benzoate (Butler et al., 2007). Furthermore, *G. metallireducens* whole cell lysate cannot reduce benzoyl-CoA in the presence of ATP and added reductants (Wischgoll et al., 2005). Therefore it appears that *G. metallireducens* reduces benzoyl-CoA to cyclohexa-1,5-dienecarboxy-CoA with a novel enzyme. This alternative pathway may exist as a method to save ATP (Fuchs, 2008) as obligate anaerobes only acquire a maximum of four ATPs per molecule of benzoate oxidized (Fuchs, 2008). This, in combination with the fact that *G. metallireducens* hydrolyzes two molecules of ATP to ligate benzoate to coenzyme A (Wischgoll et al., 2005), suggests that there is a limited net energy gain from benzoate metabolism. Therefore, it may make sense for the organism to utilize an alternative benzoyl-CoA reductase that does not expend ATP. Analysis of the 44 upregulated genes in *G. metallireducens* in the presence of benzoate indicated that many previously uncharacterized enzymes were expressed (Wischgoll et al., 2005). Some of these enzymes appeared to contain selenium and tungsten or molybdenum (Wischgoll et al., 2005). The presence of selenium in an enzyme essential to this process has previously been indicated with other strictly anaerobic bacteria oxidizing hydrocarbons (Peters et al., 2004;

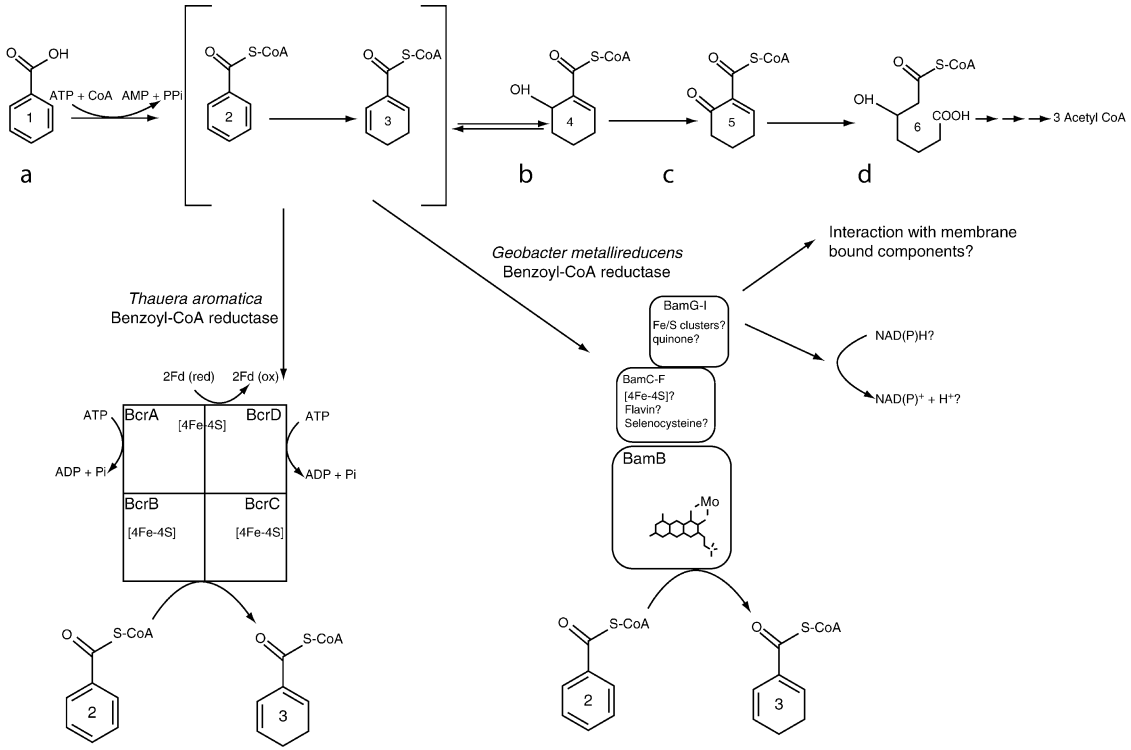

Figure 1

A diagram showing the steps of dearomatization of benzoate in *Geobacter metallireducens*. The step catalyzed by a different enzyme complex compared to *T. aromatica* is indicated. The numbers 1–6 refer to intermediates in the dearomatization of benzoate. They are 1 – benzoate; 2 – benzoyl-CoA; 3 – cyclohexa-1,5-diene-1-carbonyl-CoA; 4 – 6-hydroxylcyclohex-1-en-1-carbonyl-CoA dehydrogenase; 5 – 6-oxocyclohex-1-ene-1-carbonyl-CoA; 6 – 3-hydroxypimelyl-CoA. The letters A–D refer to enzymes involved in the benzoate dearomatization process. They are A – benzoyl-CoA ligase; B – cyclohexa-1,5-diene-1-carbonyl-CoA dehydratase; C – 6-hydroxylcyclohex-1-en-1-carbonyl-CoA; and D – 6-oxocyclohex-1-ene-1-carbonyl-CoA hydratase.

Wischgoll et al., 2005). In 2004, it was found that *D. multivorans*, a sulfate reducing bacteria whose hydrocarbon degradation pathway may bear similarity to the pathway seen in *G. metallireducens*, has an absolute requirement for molybdenum and selenium to degrade benzoate (Peters et al., 2004). It was also found that two proteins with molecular weights of 30 and 100 kDa were upregulated in *D. multivorans* in the presence of benzoate and could be labeled with ^{75}Se (Peters et al., 2004). This selenium remained even after denaturing the proteins with SDS, prior to analysis on a polyacrylamide gel. The tenacity of this label suggests that the selenium is most likely incorporated into these polypeptides as selenocysteine.

One of the polypeptides upregulated when *G. metallireducens* is incubated with benzoate, BamF, is similar to VhuD (Wischgoll et al., 2005; Butler et al., 2007), a protein in methanogens that binds selenocysteine. VhuD has been implicated in the formation of a tight complex between soluble hydrogenases and heterodisulfide reductases (Stojanowic et al., 2003). Additional polypeptides produced in the presence of benzoate have similarities to subunits of heterodisulfide reductases, soluble hydrogenases, and NADH:Ubiquinone oxidoreductases (BamC-I) (Wischgoll et al., 2005). The protein BamC has a strong similarity to soluble hydrogenases that contain three [4Fe-4S] clusters, but the whole cell lysate of *G. metallireducens* does not have any hydrogenase properties. In addition, no large subunit containing the NiFe center required for proton reduction is present in the *G. metallireducens* genome (Wischgoll et al., 2005). The proteins BamD-E contain Fe/S clusters and flavins, and are similar to heterodisulfide redutases (Wischgoll et al., 2005). It could be that BamC-E form a tight complex that transfers electrons to a potential catalytic subunit (*Fig. 1*). This tight complex would be anchored by the BamF protein, functioning similarly to VhuD (Wischgoll et al., 2005). The catalytic subunit in this case may be formed by BamB. The gene for BamB is upregulated 36-fold in the presence of benzoate (Butler et al., 2007). This gene is similar to molybdenum containing aldehyde:ferredoxin oxidoreductases, and formaldehyde:ferredoxin oxidoreductases (Wischgoll et al., 2005; Butler et al., 2007). These enzymes typically contain a *bis*molybdo(tungsto)pterin mononucleotide cofactor and a [4Fe-4S] cluster (Chan et al., 1995; McMaster and Enemark, 1998). The motifs for these cofactors are present in BamB (Wischgoll et al., 2005), implying that they may be involved in the reduction of benzoyl-CoA (see *Fig. 1*). This implication appears to fit with present data that molybdenum is a required cofactor for the oxidation of benzoate in *G. metallireducens* (Wischgoll et al., 2005). In addition, molybdenum biosynthetic genes are upregulated in the presence of benzoate in *G. metallireducens* (Butler et al., 2007). Other proteins expressed in the presence of benzoate include BamG-I, which bear similarity to NAD(P)H:Ubiquinone oxidoreductases (Wischgoll et al., 2005; Butler et al., 2007). It is entirely possible that the BamG-I complex oxidizes NAD(P)H and transfers these electrons to the BamB subunit through the BamC-F complex (see *Fig. 1*). These electrons would be used to reduce benzoyl-CoA to cyclohexa-1,5-dienecarboxy-CoA.

5 Activation of Benzoyl-CoA in *G. metallireducens*

The challenging question with this hypothesis remains the method of activation. NAD(P)H has redox potential of −340 mV, while the redox potential of the dienoyl CoA radical anion that results from a single reduction of benzoyl-CoA has a redox potential of approximately −1 V. This disparity in redox potential of over 600 mV can be accomplished by coupling the electron transfer to an activation step. In the benzoyl-CoA reductase from *T. aromatica* this

activation step occurs by the hydrolysis of ATP. However, experiments with whole cell lysates of *G. metallireducens* indicate that ATP and reductants alone cannot stimulate the reduction of benzoyl-CoA (Wischgoll et al., 2005). Further none of the aforementioned polypeptides have ATP-binding domains (Wischgoll et al., 2005), indicating a different method of activation may take place. One possible solution is that this reduction of benzoyl-CoA is driven by some membrane potential (Wischgoll et al., 2005). In addition, the benzoyl-CoA reductase from *G. metallireducens* may have an alternative mechanism of stabilizing the dienoyl-CoA radical that forms following an electron transfer event. This different mechanism may decrease the energy required to activate the enzyme prior to this reduction.

6 Catabolism of Cyclohexa-1,5-Dienecarboxy-CoA

Following the reduction of benzoyl-CoA in *T. aromatica*, the resulting cyclohexa-1,5-dienecarboxy-CoA is converted to 3-hydroxypimelyl-CoA. The enzymes required for this conversion; cyclohexa-1,5-diene-1-carbonyl-CoA dehydratase, 6-hydroxylcyclohex-1-en-1-carbonyl-CoA dehydrogenase, and 6-oxocyclohex-1-ene-1-carbonyl-CoA hydratase all have homologues in the *G. metallireducens* genome (Butler et al., 2007). All of these genes are upregulated in the presence of benzoate (Butler et al., 2007). Presumably, the resulting 3-hydroxypimelyl-CoA enters the β-oxidation pathway, where it is converted to acetyl-CoA subunits. Acetyl-CoA subunits are transformed into CO_2 and NADH by the tricarboxylic acid cycle. A genomic analysis also shows that the enzymes for this cycle change slightly in the presence of benzoate.

One additional adaption by *G. metallireducens* may generate more ATP per unit benzoate. Acetate: succinyl-CoA transferase, which functions in the *G. sulfurreducens* to transfer coenzyme A from succinyl-CoA to acetate, is downregulated in *G. metallireducens* 2.2-fold in the presence of benzoate (Butler et al., 2007). However, a homologue of succinyl-CoA synthetase was found to be upregulated and appears to convert succinyl-CoA to succinate, coupled to a substrate level phosphorylation (Butler et al., 2007). One of three copies of this gene is found in the "aromatic island" in the *G. metallireducens* sequence, and is upregulated 8- to 32-fold in the presence of benzoate. This upregulation of succinyl-CoA synthase appears prudent as acetyl-CoA is already formed from β-oxidation, and there is no need to acquire coenzyme A groups from succinyl-CoA. This is an interesting modification that generates additional molecules of ATP during the degradation of benzoate.

7 Research Needs

Key enzymes involved in processing benzene remain unknown, although it appears clear that benzene oxidation is coupled to Fe(III) reduction in the environment by some unknown organism. Isolating a pure culture or well-characterized consortia that can oxidize benzene coupled to Fe(III) reduction would be a large contribution to this field.

Future directions for this field involve a complete characterization of genes involved in benzoyl-CoA reduction. The absence of homologues to the benzoyl-CoA reductase protein complex indicates that a novel enzyme system is involved in the reduction of benzoyl-CoA. The necessary requirement of both selenium and molybdenum in benzoate degradation implies this enzyme complex may contain these atoms. Proteins related to hydrogenases,

heterodisulfide reductases, NADH:Ubiquinone oxidoreductases, Aldehyde/ketone:ferredoxin oxidoreductases, and a selenium-containing protein have all been implicated as possible components in this novel complex. Another key question regarding this enzyme complex concerns a method of activation of electrons entering the complex from physiological sources.

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