Benzene and Beyond: Mechanisms of Novel Anaerobic Aromatic Degradation Pathways in Geobacter daltonii

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BENZENE AND BEYOND:
MECHANISMS OF NOVEL ANAEROBIC AROMATIC DEGRADATION PATHWAYS IN

*Geobacter daltonii*

by

ALISON KANAK

Under the Direction of Kuk-Jeong Chin

ABSTRACT

Petroleum spills causes contamination of drinking water with carcinogenic aromatic compounds including benzene and cresol. Current knowledge of anaerobic benzene and cresol degradation is extremely limited and it makes bioremediation challenging. *Geobacter daltonii* strain FRC-32 is a metal-reducing bacterium isolated from radionuclides and hydrocarbon-contaminated subsurface sediments. It is notable for its anaerobic oxidation of benzene and its unique ability to metabolize *p*, *m*, or *o*-cresol as a sole carbon source. Location of genes involved in aromatic compound degradation and genes unique to *G. daltonii* were elucidated by genomic analysis using BLAST.
Genes predicted to play a role in aromatic degradation cluster into an aromatic island near the start of the genome. Of particular note, *G. daltonii* has two copies of the *bss* genes, which are responsible for the first step in anaerobic toluene oxidation. This bacterium is unique among the family *Geobacteraceae* and other toluene degraders in this aspect. The α subunits have 74% identity to one another. The remaining genes in each operon are not identical. *BssA* was upregulated when *G. daltonii* was grown on benzene and toluene while the *grlA* was upregulated during growth on *m*-cresol. Toluene was accumulated during degradation of benzene by cell lysate. Cells grown with benzene and toluene exhibited a similar protein profile compared to cells grown with benzoate. These results indicate that benzene is converted to toluene and further degraded via the toluene pathway.

Both the *bss* and *grl* operons were predicted to have sigma54-dependent promoters. This was confirmed using 5’ RACE and sequence analysis. *E. coli* transformed with the *bss* operon were able to grow in the presence of toluene but lost this capability when sigma 54 was knocked out. Growth was restored with complementation of sigma 54. The sigma 54-dependent signaling system *bamVW* was upregulated in the presence of all aromatic compounds tested. These results suggest that the *bss* operon is regulated via sigma 54-dependent mechanisms. This study significantly contributes to anaerobic aromatic gene regulation which is crucial in effective oil spill bioremediation.

INDEX WORDS: Benzene, Anaerobic, Geobacter
BENZENE AND BEYOND:
MECHANISMS OF NOVEL ANAEROBIC AROMATIC DEGRADATION PATHWAYS IN

GEOBACTER DALTONII

by

ALISON KANAK

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2014
BENZENE AND BEYOND:
MECHANISMS OF NOVEL ANAEROBIC AROMATIC DEGRADATION PATHWAYS IN
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Electronic Version Approved: 7-21-14

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August 2014
ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr Kuki Chin, for all of her help and guidance during my research. I also thank my committee for serving as invaluable sources of wisdom and advice while completing this work. To my husband, Jason Kanak, there are not words that express my gratitude for your support and understanding throughout my graduate career. None of it could have been completed without your help. You held me through the tears, celebrated with my triumphs, and never minded late nights and weekends spent in the lab. Thank you so much for your years of support. Finally, thank you to my fellow PhD student, Ryan Perry. You have always understood and always had an open ear.
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1 INTRODUCTION

Bioremediation and significance of the present study

Widespread use of petroleum in developed countries often results in the contamination of natural environments, as occurred after the 2010 Deepwater Horizon oil spill (DWH) off the Gulf of Mexico coast. The explosion resulted in the loss of 11 lives as well as the release of an estimated $6.7 \times 10^5$ mT of Macondo well 252 Crude oil into local waters and caused an estimated $100-200$ million of lost revenue in local fisheries (1). The DWH is considered the largest accidental marine oil spill in the history of petroleum industry. This contamination results in a desperate need for finding economic means for its removal. Benzene, toluene, ethylbenzene, and xylene isomers (BTEX), cresols and polycyclic aromatic hydrocarbons (PAHs) are found in high quantities in contaminated sites, including the DWH site (2-4). Due to their higher water solubility than other organic compounds monoaromatics spread to and pollute nearby groundwater and sediments. Since groundwater is often a vital source for obtaining drinking water, technologies to remove petroleum hydrocarbons from polluted areas are critically necessary.

There are currently multiple methods by which BTEX can be eliminated from groundwater including physical, chemical, and biological removal (5). Bioremediation is an environmentally sound approach that is both more efficient and less costly than other more acerbic methods (6). The continued pollution of our environment has tangible costs to human health as many human diseases have preventable environmental contributors. Bioremediation is the neutralization of environmental contaminants via the utilization of living organisms. These organisms range from bacteria to archaea to plants with the strategies employed being as diverse as the organisms themselves. There are three classes of bioremediation: in situ, biofiltration, and
bioreactors. *In situ* bioremediation relies on stimulation of the natural environment to promote degradative processes. Techniques include bioaugmentation, or the introduction of an organism to a contaminated site for remediation, and biostimulation, the stimulation of growth conditions of organisms already present at the contaminated site. Processes can involve the biodegradation of contaminants as well as the adsorption of contaminants into cells for later removal. While other strategies exist for remediating contaminated sites bioremediation provides a natural approach. Because of this it requires less equipment, labor, or energy which translates to it being less costly than other remediation approaches. Bioremediation is also appealing as it creates few waste byproducts. Bioremediation is by no means a novel approach to remediating contaminants, however it is evolving as new technologies are developed for remediating more and more challenging pollutants (7).

Microorganisms or microbial products are frequently used in bioremediation strategies to degrade waste or concentrate it for removal. In the case of organic pollutants microbes are able to catabolize the waste, taking it from a high energy substance to a low energy one. This can occur through novel xenobiotic pathways or also through co-metabolism in which natural microbial catabolic enzymes degrade wastes as well. Microbes are also capable of concentrating metals for more easy removal. Microbes extract metals from the environment both via direct transport and secretion of metal-sequestering compounds such as siderophores. The siderophore-metal complex is then absorbed into the cell and utilized. The focus of this work is to understand an organism present at contaminated sites so as to stimulate its growth and natural degradative properties (7).
Anaerobic benzene-oxidizing microorganisms

Because a lot of the contaminants needing to be removed are buried deep within the soil, understanding anaerobic degradation of organic pollutants has become much more important in recent years. Anaerobic degradation of benzene involves reduction of the very stable benzene ring, which is a more complex process than oxygenation. It is known that benzene is rapidly degraded and completely oxidized to carbon dioxide under iron-reducing, sulfate-reducing, and methanogenic conditions (3, 8, 9). No universal pathway has currently been demonstrated in anaerobic benzene degradation. Recent studies have shown that in the archaeon *Ferroglobus placidus* benzene is first activated via carboxylation and conversion to benzoate while in the anaerobe *Geobacter metallireducens* benzene is activated via hydroxylation and conversion to phenol (10, 11).

Family Geobacteraceae and anaerobic respiration

Under iron-reducing conditions the most prevalent family of bacteria found are the family *Geobacteraceae* (12). For example, they were found to represent 85% of the active microorganisms oxidizing acetate in an Fe(III)-reducing rice paddy soil (13). *Geobacter* species are part of the family *Geobacteraceae* within the Bacteria domain, phylum *Proteobacteria*, class *Deltaproteobacteria*, and order *Desulfuromonadales*. These bacteria are Gram-negative, non-spore-forming, curved rod-shaped cells capable of motility (14-18). Twenty seven published species of the family *Geobacteraceae* have been reported to date (19). Many of the members of this family are obligate anaerobes capable of heavy metal reduction with oxidation of acetate (20-22).
These organisms play an important role in the biogeochemical cycling and the microbial ecology of sediments by reducing insoluble Fe(III) and releasing soluble Fe(II). *Geobacter* species have also been found to play a role in the reduction of U(VI) in sediments resulting in the differential migration of this species and other radionuclides. Because of their ability to couple oxidation of organic compounds to the reduction of Fe(III), *Geobacter* species occupy key niches in anaerobic environments. Another factor could be their “remarkably low” maintenance energy requirement. This allows them to live in such varied environments as petroleum-contaminated aquifers, groundwater contaminated with landfill leachate, sediments contaminated with organic acids and radionuclides such as uranium and technetium as well as environments contaminated with arsenates and chlorinated compounds. These organisms can also be found on the surface of electrodes. In addition to iron, some of the family *Geobacteraceae* can also reduce Mn(IV), U(VI), Tc(VII), Pu(VI), elemental sulfur and humic substances. Some are able to utilize hydrogen or ethanol as electron donors in addition to small organic acids or aromatic compounds (13).

In order to study the physiology and genetics of the family *Geobacteraceae* it is imperative to obtain pure cultures. The goal is to have isolates that are good representatives of this group that are abundant in the environment of interest. The first species discovered was *Geobacter metallireducens*, and was isolated from freshwater sediment by Lovley and Philips (1988) (11). This organism has served as the focus for much *Geobacter* study and subsequent knowledge. *Geobacter sulfurreducens* is another model organism that has been extensively studied physiologically and genetically and was isolated from hydrocarbon-contaminated surface sediments (10, 13). Coppi and colleagues established a genetic system for *G. sulfurreducens* (23). *G. sulfurreducens* is the first member of the *Geobacter* genus which was able to be
genetically manipulated. Because of this it has been the source of most of the knowledge about physiology and gene regulation, in family Geobacteraceae. G. sulfurreducens was also the first to be found to utilize hydrogen as an electron donor. One laboratory strain of G. sulfurreducens, KN400, was designed to study growth of these organisms on electrodes. This organism produces a greater amount of current than others and also reduces Fe(III) oxides much more rapidly. This strain is also motile, whereas other lab strains have lost this ability (13).

One of the unique features of Geobacter species is their ability to utilize a wide variety of electron acceptors. Soluble electron acceptors such as nitrate, fumarate, and chlorinated compounds can be reduced intra-cellularly. These organisms are also capable of utilizing a number of external electron acceptors such as soluble (ferric citrate) and insoluble iron (ferrihydrite) and manganese. Fe(III) is the most common electron acceptor for Geobacter species and it can be present in the form of crystalline hydroxides and phyllosilicates within clay fractions. Fe(III) oxides are rarely encountered in the environment and so serve as poor electron acceptors during lab cultivation. Smaller particle sizes of Fe(III) oxides may accelerate reduction. In addition to insoluble Fe(III) compounds Geobacter species can also be cultivated using soluble chelated forms of Fe(III) such a ferric citrate. However, these cells appear to have different physiologies than their insoluble Fe(III)-reducing counterparts. Geobacter species have also been found to be able to pass electrons to graphite electrodes, thus using them as terminal electron acceptors and harvesters of electricity. Geobacters were the first organisms found to be able to sustain growth coupling oxidation of organic matter with electron transfer to electrodes. These organisms are so adept at this process that they are frequently the most abundant organisms found after starting with mixed inoculum. Geobacter species are also able to transfer electrons to other organisms if other electron acceptors are not available. This is accomplished
by the interspecies hydrogen transfer between the syntrophic species or possible the interspecies transfer of electrons (13).

Acetate is a key extracellular intermediate of the degradation of organic matter anaerobically. It is a conserved ability for *Geobacter* species to be able to oxidize acetate as an electron donor and carbon source via the TCA cycle. In addition to acetate these organisms can also oxidize other short fatty acids, alcohols, hydrogen, and aromatic compounds as electron donors. *Geobacter* species are frequently found in petroleum-contaminated environments where aromatic hydrocarbons are being removed. *G. metallireducens* was the first *Geobacter* species found to degrade aromatic compounds such as toluene and has also been implicated in the anaerobic degradation of other aromatic compounds as well. Common to any *Geobacter* species capable of degrading any aromatic compound is the ability to also degrade benzoate. Pathways involved in anaerobic aromatic degradation have been predominantly studied in *G. metallireducens*. All monoaromatic pathways converge on a Benzoyl-CoA intermediate. *Geobacter* species utilize a class II benzoyl Co-A reductase that is not ATP-dependent like others are. The eight gene cluster BamBCDEFGHI is believed to code for this reductase and this protein is believed to be membrane-bound. Additional reactions further oxidize benzoyl CoA to 3-hydroxypimelyl-CoA and eventually acetyl-CoA and CO₂. The pathways for anaerobic toluene and phenol degradation elucidated in *G. metallireducens* are similar to that in the facultative anaerobic bacterium *Thauera aromatica* (13, 24).

*Geobacter* species utilize complex gene regulatory pathways in order to optimally respond to their local environment. Bacteria rely on the use of multiple sigma factors as an upstream means of regulating gene expression with each sigma factor recognizing different promoter elements. *Geobacter* species are no exception to this. One regard where *Geobacters*
differ from other bacteria is the heavy use of the “alternative sigma factor”, sigma 54. Deletion of this gene is lethal to *Geobacter* species and so is implicated in regulating a wide variety of *Geobacter* genes. The presence of 151 putative transcription factors and an uncommonly large number of two-component signaling cascades demonstrates the diversity of signals perceived by *G. sulfurreducens*. The transcription factor HgtR, for example, is induced when hydrogen is the sole electron donor and represses genes involved in classical central metabolism such as citrate synthase. HgtR is involved in the sigma 54 regulatory networks and is thought to be an enhancer binding protein for this sigma. The genes BamVW are an example of a two-component regulatory system that is also part of the sigma 54 network. BamVW are coded in one operon, not always observed in *Geobacter* species, and are induced in the presence of aromatic compounds such as benzoate and *p*-cresol. Most of the domains in the sensor proteins of a two-component system in *Geobacters* are unique or do not share much homology with other bacterial systems (13).

*Geobacter* species are motile. It aids in the acquisition of external nutrients in sediments and chemotaxis. Interestingly, *Geobacter* species have multiple chemotaxis regulatory systems whereas *E. coli* only has one. Some of these systems regulate flagellar motility while others are responsible for regulating extracellular protein expression and cytochromes. These bacteria code a very high number of chemoreceptor genes as well which are randomly located throughout the genome (13).

*Geobacter daltonii* strain FRC-32

A member of the genus *Geobacter*, *Geobacter daltonii* strain FRC-32, was isolated from the radionuclides and hydrocarbon-contaminated subsurface of the U.S. Department of Energy...
Oak Ridge Field Research Center (ORFRC), Oak Ridge, Tennessee (25). Like other Geobacteraceae, cells of *G. daltonii* are Gram-negative, non-spore-forming and curved rods. Due to the large number of cytochromes present, also characteristic of its genus, *G. daltonii* cultures take on a distinctive pink color during cultivation and form biofilms when growth conditions are optimal. Its optimal growth temperature is 30°C and growth is best when cultures are not agitated.

*G. daltonii*’s genome is larger than those of other Geobacter species at 4.3 kb in length (22). This could be due to gene duplications present within its genome. For example, *G. daltonii* encodes multiple copies of benzoate oxidation genes as well toluene oxidation genes. The organization of aromatic degradation genes in *G. daltonii* is similar to *G. metallireducens* in that both group these genes together into aromatic islands. Both of these organisms also have their toluene degradation genes farther away from the large island. However, whereas *G. metallireducens* has its aromatics island close to the midpoint of the genome *G. daltonii*’s aromatics island is close to the start of the genome. According to 16S rRNA gene sequence analysis, *G. daltonii* is most closely related to *G. uraniireducens*, however, it is unique from this organism in its ability to degrade aromatic compounds (22).

**Anaerobic oxidation of aromatic compounds**

Benzoate is the simplest of the aromatic compounds that *G. daltonii* can degrade. The pathway for its degradation has been studied extensively in anaerobes. Benzoate is first activated to Benzoyl CoA via an ATP-dependent benzoate-CoA ligase (26). This reaction also releases AMP and PPi. The ligase is coded by the *bamY* gene in *G. metallireducens* and *G. daltonii* also has this gene (27). Following this activation the classical benozyl CoA degradation
pathway proceeds. This pathway involves linearization of the stable aromatic ring and subsequent beta oxidations. While dearomatization is achieved in other anaerobes by the benzoyl CoA reductase this enzyme has not yet been identified in *Geobacteraceae* (27) and therefore some alternate strategy must be employed. Following dearomatization is a series of beta oxidation reactions resulting in the production of an aliphatic C$_7$-dicarboxyl-CoA compound (26) (Fig. 1).

![Figure 1](image-url)

Figure 1. Anaerobic degradation pathway of aromatic compound benzoate (adapted from Carmona *et al.* (26))

Phenol is a substrate formed by various natural processes as well as environmental contamination. The pathway involved in degradation of phenol has been determined in *G. metallireducens*. Phenol is first converted to phenylphosphate by the phenylphosphate synthase (*pps*). This enzyme is made of three subunits and transfers a phosphoryl group from ATP to the hydroxyl group of phenol. Next, phenylphosphate carboxylase (*ppc*) catalyzes the conversion of phenylphosphate to 4-hydroxybenzyl alcohol (Fig. 2). The *pps* and *ppc* operons are both found in the *G. daltonii* genome.
The first step of anaerobic toluene degradation is the enzymatic addition of toluene’s methyl group to the double bond of fumarate, resulting in benzylsuccinate biosynthesis (28). This step is catalyzed by a radical enzyme called benzylsuccinate synthase (bss). An acetyl CoA group is then added to the hydroxyl group to form benzylsuccinyl CoA. This compound is then further oxidized to E-phenylitaconyl CoA and Hydroxymethylphenyl succinyll CoA until eventually being oxidized again to form benzoyl CoA. This compound then undergoes beta oxidation and ring cleavage (Fig. 3) and eventually becomes CO$_2$ and acetyl CoA via traditional anaerobic mechanisms (29).

Multiple primary activation steps have been proposed for benzene degradation. Conversion to benzoate via carboxylation (30, 31), to phenol via hydroxylation (30), and to toluene via methylation are all proposed initial steps in the pathway (10, 11, 32). To predict
whether one of these pathways is used during anaerobic benzene oxidation by *G. daltonii* transcript levels were measured for key genes in each pathway. This approach has been successful previously with other metabolic pathways in other *Geobacter* species both in pure culture and in the environment (33, 34).

It has been reported that all three cresol isomers can be degraded anaerobically in contaminated environments (35). Also, apparent from previous studies is that each of these isomers is degraded by different metabolic pathways. In *G. metallireducens* *p*-cresol is first converted to *p*-hydroxybenzyl alcohol and then eventually is degraded via the classical benzoate degradation pathway (36). This same pathway is also observed in denitrifiers (Fig. 4) (7).

![Figure 4. Anaerobic degradation pathway of *p*-cresol (adapted from Carmona et al.)](image)

The accumulation of 3-hydroxybenzoate degradation intermediates during the anaerobic degradation of *m*-cresol is also observed in denitrifying bacteria and this compound is then coadenylated and linearized (Fig. 5) (13, 22).
The o-cresol pathway also does not appear to funnel into the benzoate degradation pathway like the p-cresol pathways does. Under methanogenic conditions a consortia produces metabolites indicative of carboxylation para to the hydroxyl group followed by subsequent dehydroxylation and formation of methylbenzoic acid (37) and under denitrifying conditions a Pseudomonas-like strain S100 proceeds through a 3-methylbenzoyl CoA intermediate (Fig. 6) (38, 39).

No organism to date has been reported to be capable of degrading all three cresol isomers.
Transcriptional Regulation in bacteria and G. daltonii

The initiation of transcription in bacteria requires the recognition of a specific promoter and the melting of that region to provide a single stranded DNA template for RNA synthesis. The presence of multiple sigma factors within bacteria lends a level of transcriptional control as these sigma factors direct the RNA polymerase to appropriate promoters with great specificity. While both sigma 70 and sigma 54 direct RNA polymerase, this is the extent of similarity in their actions (Fig. 7A). Sigma 70 typically contacts its target promoter after it has already bound the polymerase while sigma 54 binds to promoters before recruiting the polymerase. Sigma 70 does not need additional accessory proteins to initiate transcription while sigma 54 is reliant on accessory proteins, termed enhancer binding proteins, to initiate transcription in a manner similar to eukaryotic RNA transcription. These proteins aid in the start of transcription by facilitating the opening of the DNA at the promoter. While this is achieved spontaneously by the sigma 70 holoenzyme, sigma 54 binds its promoter so tightly that formation of an open complex can only occur by hydrolyzing ATP (38). These activators bind upstream of the target promoter, typically around 80-150bp away. These regions are termed upstream activator sequences or enhancer sites. Because these regions are so far away from the promoter, DNA bending is required for the activator to interact with the holoenzyme. To accomplish this integration host factor is often recruited. The location of the promoter sequence also differs for each sigma factor. Rather than identifying the classical -10/-35 promoter associated with sigma 70, sigma 54 recognizes a -12/-24 promoter sequence. There is also a much lower degree of sequence conservation in the sigma 54 promoter. A consensus of TGGCA—N7—TGC(t/a)(t/a) is recognized for sigma 54 with GG at -24 and GC at -12 being the most critical sites to maintain function (40-43). Mutation of either the GC or spacing between the -12 and -24 elements entirely eliminates transcription (38).
composition of the sigma factors differs from each other also. Whereas sigma 70 has 4 conserved regions, sigma 54 only has three. Region I is typically involved in binding of the -12 region of the promoter as well as the activator protein. Region II is highly variable amongst bacterial species both in length and necessity for sigma activity. Region III is responsible for promoter recognition and binds to the majority of the promoter, including the -24 element.

The effector binding proteins typically have three domains to their structure. The N-terminal region is termed the “regulatory” domain and plays a part in signal perception. The central domain is the location of the ATPase activity of the activator as well as binding to sigma 54. This region is critical to the activator function. Finally, the C-terminal domain contains a helix-turn-helix responsible for DNA binding. While the central domain is conserved across activator proteins, either of the flanking regions varies amongst bacteria. The enhancer binding protein typically functions as hexamer or heptamer ring (38) (Fig. 7B).

Also, known as the “alternate” sigma factor, sigma 54 is known to regulate genes involved in alternative metabolisms, and has been found to regulate aromatics related genes in Geobacter species as well as Pseudomonas putida (44, 45). It also plays a role in the regulation of virulence, biofilm formation, and quorum sensing genes in other bacteria (41, 46). Immediately upstream of the operons encoding the bss genes in G. daltonii and oppositely oriented are genes annotated as a “Sigma 54-specific transcriptional regulator” (Geob_2441 and Geob_2451; Fig. 8). Due to their proximity and orientation, these regulators may be responsible for regulating the transcription of the bss operons. The G. daltonii genome also contains the genes bamVW, which have been demonstrated to be activated during degradation of toluene and p-cresol in G. metallireducens (44). The py promoter recognized by bamV was also demonstrated to be sigma54-dependent for transcriptional activation. Considering the role
played by sigma 54 in other aromatics pathways and the proximity of sigma-54 type transcriptional regulators, it is possible this sigma is involved in regulating transcription of the \textit{bss} operons.

A

B
Figure 7. A. Diagram showing differences between the classical sigma 70 and the alternative sigma 54. B. Diagram showing action of effector binding proteins, IHF, and sigma 54 (adapted from Bush et al.) (38)

Figure 8. Diagram showing coding and upstream regions of bss (Geob_2442-Geob_2450) and grl (Geob_2431-Geob_2441) genes in G. daltonii. Dark purple arrows are grl genes and light purple arrows are bss genes.

Aims of this study

Aim 1: How does G. daltonii activate the benzene ring for further degradation?
Before the stable benzene ring can be cleaved open for further utilization it must be activated via the addition of a functional group. Multiple primary activation steps have been proposed for benzene degradation. The anaerobic mechanism of activation of the stable benzene ring was different between G. metallireducens and F. placidus, which are two known anaerobic benzene-oxidizing prokaryotes. Because no single pathway was determined for anaerobic
benzene oxidation it is uncertain which mechanism will be utilized by \textit{G. daltonii} when oxidizing benzene anaerobically. The present study aimed to determine how \textit{G. daltonii} activates benzene and which degradation pathway will subsequently be used. In order to determine which pathway is utilized by \textit{G. daltonii} this study \textbf{1) Examined expression of genes uniquely critical to the phenol, benzoate, and toluene pathways during growth of \textit{G. daltonii} on benzene, 2) Measured formation of downstream metabolites formed during anaerobic degradation of benzene by \textit{G. daltonii}, and 3) Compared protein profiles of cells grown using toluene, benzoate, or benzene as the sole carbon source.}

\textbf{Aim 2: Are the \textit{bss} operons in \textit{G. daltonii} regulated via sigma 54 dependent mechanisms?} The \textit{bss} operons in \textit{G. daltonii} are preceded by genes annotated as sigma 54 type transcriptional regulators. Analysis of the upstream regions of \textit{bss} with bioinformatics programs also fails to identify the classical -35/-10 sequence of TTGACA/TATAAT typically observed in promoters regulated by the vegetative sigma, sigma 70. Sigma 54 has been demonstrated to help regulate expression of toluene degradation in \textit{Pseudomonads} as well as benzoate degradation in \textit{G. metallireducens}. Because other aromatic operons are controlled via sigma 54 dependent mechanisms, it is possible that the toluene degradation genes in \textit{G. daltonii} are as well. \textbf{In order to determine the role of sigma 54 in regulation of \textit{bss} the present study: 1) Defined the promoter regions of \textit{bssI} and 2) Tested whether the absence of sigma 54 halts transcription of the \textit{bss} operon in \textit{E. coli}.}
2 MATERIALS AND METHODS

Chemicals, gases and source of bacterium

All chemicals used in this study were of analytical grade and obtained from Sigma-Adlrich (St. Louis, MO), Acros Organics (New Jersey, US) and Thermo Fisher Scientific (Waltham, MA).

Gases including N\textsubscript{2} (99.999\%), CO\textsubscript{2} (99.995\%), N\textsubscript{2}/CO\textsubscript{2} (80:20 [vol:vol]) and H\textsubscript{2} (99.999\%) were supplied by Airgas (Radnor, PA).

*Geobacter daltonii* strain FRC-32 (22) was originally enriched and isolated from anoxic subsurface sediments which were contaminated with radionuclides and hydrocarbon waste. The strain is deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) under number DSM 22248 and the Japan Collection of Microorganisms (JCM) under number JCM 15807. The strain has been subcultured in the laboratory since its isolation, and was kindly provided for the present study from Dr. Kostka laboratory (Georgia Institute of Technology, Atlanta, GA).

Cultivation technique

Medium preparation and cultivation of *G. daltonii* strain FRC-32 were carried out as described previously (22, 47) with some modification as described below.
Medium composition and preparation

Preparation of stock solutions

Trace element - SL 10

<table>
<thead>
<tr>
<th>Salt</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl (25%; 7.7 M)</td>
<td>10 mL</td>
</tr>
<tr>
<td>FeCl₂ x 4 H₂O</td>
<td>1.5 g</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>70 mg</td>
</tr>
<tr>
<td>MnCl₂ x 4 H₂O</td>
<td>100 mg</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6 mg</td>
</tr>
<tr>
<td>CoCl₂ x 6 H₂O</td>
<td>190 mg</td>
</tr>
<tr>
<td>CuCl₂ x 2 H₂O</td>
<td>2 mg</td>
</tr>
<tr>
<td>NiCl₂ x 6 H₂O</td>
<td>24 mg</td>
</tr>
<tr>
<td>Na₂MoO₄ x 2 H₂O</td>
<td>36 mg</td>
</tr>
<tr>
<td>Deionized water</td>
<td>990 mL</td>
</tr>
</tbody>
</table>

First, FeCl₂ was dissolved in HCl, then was diluted in deionized water. Remaining salts were dissolved and the final volume was made up to 1 L. The solution was bubbled with N₂ gas and headspace was filled with N₂ gas. Solutions were stored in serum bottles and sealed with butyl rubber stoppers and aluminum caps at 4°C.
Vitamin solution

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>2 mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>2 mg</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
<td>10 mg</td>
</tr>
<tr>
<td>Thiamine-HCl x 2 H₂O</td>
<td>5 mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>5 mg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>5 mg</td>
</tr>
<tr>
<td>D-Ca-pantothenate</td>
<td>5 mg</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>5 mg</td>
</tr>
<tr>
<td>Lipoic acid</td>
<td>5 mg</td>
</tr>
</tbody>
</table>

Salts were dissolved in deionized water (ca. 800 mL) and adjusted to a final volume of 1000 mL. Solutions were bubbled with N₂ gas and anaerobically filter-sterilized through nitrocellulose membrane (pore size 0.2 µm) and transferred to sterile anaerobic serum bottles. Bottles were sealed with butyl rubber stoppers and aluminum caps and stored in the dark at 4°C.

Sodium bicarbonate solution

<table>
<thead>
<tr>
<th>Sodium bicarbonate</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50.0 mL</td>
</tr>
</tbody>
</table>
Salt was dissolved in water (ca. 40 mL) and a final volume of 50 mL was adjusted. The solution was bubbled with CO₂ gas, sealed with a butyl rubber stopper and aluminum cap, autoclaved, and stored at room temperature.

Selenite-Tungstate solution

\[
\begin{align*}
Na_2SeO_3 \times 5H_2O & \quad 3 \text{ mg} \\
Na_2WO_4 \times 2H_2O & \quad 4 \text{ mg} \\
NaOH & \quad 0.5 \text{ g} \\
\text{Distilled water} & \quad 1 \text{ L}
\end{align*}
\]

Salts were dissolved in water (ca. 800 mL) and final volume was adjusted to 1L. The solution was bubbled with N₂ gas, sealed with a butyl rubber stopper and aluminum cap, autoclaved, and stored at 4°C.

Cysteine hydrochloride solution

\[
\begin{align*}
\text{Cysteine hydrochloride} & \quad 17.56 \text{ g} \\
\text{Distilled water} & \quad 100.0 \text{ mL}
\end{align*}
\]

Salts were dissolved in water (ca. 80 mL) and final volume was adjusted to 100 mL. The solution was bubbled with N₂ gas, sealed with a butyl rubber stopper and aluminum cap, autoclaved, and stored at 4°C.
Preparation of freshwater medium

Salt Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>0.2 g</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.25 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.0 g</td>
</tr>
<tr>
<td>MgCl₂ x 6 H₂O</td>
<td>0.4 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5 g</td>
</tr>
<tr>
<td>CaCl₂ x 2 H₂O</td>
<td>0.1 g</td>
</tr>
<tr>
<td>* Fumarate (NaC₄H₄O₄)</td>
<td>1.6 g</td>
</tr>
<tr>
<td>* Nitrate (NaNO₃)</td>
<td>0.085 g</td>
</tr>
</tbody>
</table>

Distilled water 930 mL

* Either fumarate or nitrate was added as electron acceptor according to specific experimental purpose unless ferric citrate was required as electron acceptor.

Salts were dissolved in water and transferred to serum bottles. The solution was bubbled with N₂/CO₂ anaerobically (80%:20% [vol:vol]) and pressurized to 5-10 psi. The serum bottles were sealed with butyl rubber stopper and aluminum cap, and were autoclaved. Solutions were stored at room temperature.
Trace element (1 ml), sodium bicarbonate (50 ml), vitamin (10 ml), selenite-tungstate (1 ml), and cysteine hydrochloride (2 ml) solutions are added to the sterile, cooled salt solution (930 mL) in the sequence as indicated. Final pH of the medium was adjusted to 7.2-7.3.

**Preparation of organic substrates**

Aromatic compound solution

<table>
<thead>
<tr>
<th>Compound</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>108.5 µL</td>
</tr>
<tr>
<td>Toluene</td>
<td>90 µL</td>
</tr>
<tr>
<td>Benzoate</td>
<td>0.1 mL</td>
</tr>
</tbody>
</table>

For the preparation of organic substrates, 10 mL of 2,2,4,4,6,8,8-Heptamethylnonane (HMN, Acros Organics only) was bubbled with N₂ gas and the serum bottle was sealed with a butyl rubber stopper, aluminum cap, and laboratory tape. This solution was then autoclaved. The appropriate volume of HMN was removed anaerobically and replaced with an equal volume of organic substrate. Solutions were vortexed for 20 min to dissolve the organic solutes. Solutions were stored at room temperature in the dark. The concentration of the stock solution of benzene, toluene and benzoate was 100 mM, 100 mM and 1M, respectively.

Cresol solution

<table>
<thead>
<tr>
<th>Cresol</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-cresol</td>
<td>100 µL</td>
</tr>
<tr>
<td>p-cresol</td>
<td>0.1 g</td>
</tr>
<tr>
<td>m-cresol</td>
<td>0.1 g</td>
</tr>
</tbody>
</table>
A volume of 10 mL of distilled water was bubbled with N₂ gas, autoclaved, and the serum bottle was sealed with a butyl rubber stopper and aluminum cap. Solutions were then autoclaved. Appropriate volume of water was removed and replaced with the cresol compound. Solutions were vortexed for 10 min to dissolve all cresol evenly. The concentration of the stock solution of o-cresol, p-cresol and m-cresol was 100 mM, 100 mM and 100 mM, respectively.

**Preparation of iron medium**

Ferric citrate 13.7g

Deionized water 1.0 L

Approximately 50% volume of water was heated to near boiling. Ferric citrate was added and allowed to dissolve. The solution was then cooled to room temperature and pH adjusted to 6.0 using NaOH. The solution was bubbled with N₂/CO₂ anaerobically (80:20 [vol:vol]). The serum bottles were sealed with butyl rubber stopper and aluminum cap, and were autoclaved. Solutions were stored at room temperature.

Trace element (1 ml), sodium bicarbonate (50 ml), vitamin (10 ml), selenite-tungstate (1 ml), and cysteine hydrochloride (2 ml) solutions are added to the sterile, cooled salt solution (930 mL) in the sequence as indicated.

**Cultivation**

Cells were grown at 30°C in the dark with minimal agitation in serum bottles sealed with butyl rubber stoppers and an aluminum seal. Bicarbonate-buffered freshwater minimal media
Cells were grown with either 1 mM benzene, 1 mM toluene, 1 mM \( p-, o-, \) or \( m- \) cresol as a sole carbon source, and either 10 mM fumarate or 1 mM nitrate as the sole electron acceptor.

**Physiological adaptations**

*G. daltonii* strain FRC-32, which has been initially grown on acetate as carbon source and fumarate as electron acceptor, was adapted to various organic substrates such as benzoate, toluene, benzene, \( p-, o-, \) or \( m- \) cresol and different electron acceptors such as fumarate, nitrate or ferric citrate. Cells were transferred for at least 5 passages to allow adaptation to the respective adaptation conditions.

**Monitoring growth**

The growth of *G. daltonii* was monitored by measuring optical density changes at 600 nm, loss of aromatic compounds, succinate formation (in cultures grown on fumarate as electron acceptor), loss of nitrate (in cultures grown on nitrate as electron acceptor), and Fe(II) formation (in cultures grown on ferric citrate as electron acceptor).

Growth was monitored carefully to determine accurate time points (middle log phase) for harvesting the cells for total RNA extraction and other experiments. Incubation and measurements of substrate and products were carried out as described above.

**Genomic analysis of anaerobic aromatic compound degradation genes**

The genomes of *Geobacter metallireducens* (NC_007517) and *G. daltonii* (NC_011979) were compared using the basic local alignment search tool (BLAST) of the National Center for
Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi) (49). The relative locations and similarities of aromatics degradation genes were compared as well as similarities between these genes amongst species using the KEGG database (50) and BLAST of the NCBI non-redundant protein database. Genes within the aromatic-degrading gene islands in the G. daltonii genome were analyzed by BLAST, LAlign, and ClustalW (51, 52) for homology to other organisms and functional assessment. Genes without similarities to other Geobacteraceae species or known aromatic-degrading prokaryotes were notated as “unique”. These genes were categorized into functional segments based on proximity to one another within the genome. These segments were analyzed for GC content using a script in Python (https://www.python.org) coded for this study. Using a Python script that searched for locations of classical sequences the genome was screened for the presence of insertion sequences, inverted repeats, and transposons.

Cell lysis and analysis of cellular lysate activity

All procedures were carried out anaerobically in an anaerobic chamber (Coy Laboratory, Grass Lake, MI). G. daltonii cultures in middle log phase were centrifuged at 4500 rpm for 20 min at 2°C. Resulting pellet was resuspended in 200 µL of lysis buffer consisting of 50 mM HEPES pH7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM benzamidine, and 1 mM DTT and agitated with glass beads for 1 min. After centrifugation lysate supernatant was collected and analyzed for enzymatic activity.

Anaerobic lysis buffer, 9.8 mL, was stored in 20 mL serum bottles sealed with butyl rubber stoppers and pressurized to 5 psi. Aromatic substrates, either benzene or toluene, were added to the lysis buffer to a final concentration of 1 mM and the solution was shaken vigorously for 1 min. Either 0.1 mL of lysis buffer or 0.1 mL of cell lysate was then added to the system.
Reactions were halted by exposure to oxygen. Aromatic metabolites were then extracted using an equal volume of ethyl acetate and the aqueous phase was tested for these metabolites by HPLC.

**Analytical methods**

Quantification of organic substrates

Cells were harvested anaerobically from middle log phase cultures and lysed (see Lysate section above). Forty microliters of cell lysates were separated in a Supelco LC-PAH column, 25cm x 4.6mm filled with 5µm silica particles using a Beckman Gold High Performance Liquid Chromatograph (HPLC) (Pasadena, CA). Samples were eluted using acetonitrile in water (60:40 [vol:vol]) at a flow rate of 0.7 mL/min for 20 min. Benzene, toluene, p-cresol, and m-cresol were detected at 254 nm and o-cresol was detected at 270 nm. Karat 62 software was used to analyze chemical peaks.

Quantification of fumarate, nitrate and succinate

Cells (1 mL) were harvested anaerobically from middle log phase cultures and filtered using Nalgene 0.2 µm PTFE syringe filters. Cell filtrate (0.5 mL) was injected into a Dionex ICS-2000 Ion Chromatograph (IC) (Thermo Scientific Dionex, Canton, GA) equipped with anionic resin column and eluted with 5.6% 1M KOH at a flow rate of 1.5mL/min for 20 min. Dionex Chromeleon software was used to analyze peak area for nitrate, fumarate, and succinate.
Detection of lysate metabolites

In order to determine metabolites of benzene degradation Thin Layer Chromatography (TLC) was performed. Silica TLC plates, 0.25 cm F254 (Sorbent Technologies, Norcross, GA) were spotted with products of lysate reactions and eluted with 1:10 ethyl acetate: glacial acetic acid and then visualized with iodine.

Separation of total cellular protein by SDS-PAGE

Cells were lysed by boiling in TruSep SDS Sample Buffer (NuSep, French Forest Australia) for 20 min. Proteins were separated in a 4%-20% gradient SDS Nusep pre-cast polyacrylamide gel and visualized under ultraviolet light.

Total RNA extraction

Total RNA was extracted from *G. daltonii* cultures in middle log phase using the method described by Chin and colleagues (53) with some modifications. The complete procedure was performed at 2°C or on ice. A volume of 100 mL of cells were harvested by centrifugation for 25 min and the cell pellets were resuspended in 0.6 mL of TM buffer [50 mM Tris-HCl (pH 7.0), 20 mM MgCl$_2$ in DEPC-treated water]. The suspension was transferred to a tube containing 0.5 g of 0.1 mm diameter glass beads. The tubes were beaten by ballistic cell destruction for 1 minute at 2,500 rpm using a mini-bead beater (Biospec Products; Bartlesville, OK). Cell debris and glass beads were concentrated by centrifugation for 3 min. The pellet was resuspended in 0.6 ml phenol-saturated lysis buffer [50 mM Tris/HCl (pH 7.0), 50 mM EDTA, 1% (w/v) sodium dodecyl sulfate (Life Technologies-Ambion), and 6% water-saturated phenol. After an additional round of bead beating at the same conditions, the tubes were exposed to centrifugation for 5 min
and the supernatant was pooled with the supernatant of the first round of bead-beating. A volume of 0.6 ml phenol (pH 4.3) was added to the pooled supernatant, the tubes were vortexed for 40 seconds and exposed to centrifugation for 5 min. The supernatant was extracted with phenol-chloroform-isoamylalcohol (pH 7.8-8.2) [25:24:1 (v/v/v)] and chloroform-isoamylalcohol [24:1 (v/v)]. The aqueous phase was transferred to a fresh 2 ml tube containing 10% volume of 3 M sodium acetate (pH 5.2), and filled with 100% cold ethanol up to a final volume of 2 ml.

Samples were incubated at -80°C for 1 hour and then centrifuged for 1 hour. The supernatant was added to 0.5 mL 70% ethanol and spun for 10 min. Following removal of ethanol the remaining pellet was dried by desiccation, resuspended in 20 µL of nuclease-free water and treated with TURBO DNase (Life Technologies-Ambion, Grand Island, NY) according to manufacturer’s instructions to remove any DNA. DNA contamination was checked with agarose gel electrophoresis following reverse transcription-polymerase chain reaction (RT-PCR) by performing control experiments in which no reverse transcriptase was added to the isolated RNA before the PCR step. RNA concentration was determined by absorption at 260 nm with a Biophotometer (Eppendorf, Hamburg, Germany) and NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Purified RNA was stored at -80°C.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

PCR primers were designed using the PrimerQuest software (Integrated DNA Technologies, Coralville, IA). The primers used in this study are detailed in Table 1. Degenerate primers directed at citrate synthase genes were used to identify DNA contamination in RNA samples. Reactions were carried out in 0.2mL thin-walled PCR tubes with total reaction
volumes of 20 μL containing final concentrations of 20 mM Tris-HCl, 10 mM KCl, 10 mM NH₄SO₄, 2 mM MgSO₄, 0.1% TritonX, 200 μM of each dNTP, 0.1 mL of 10X BSA, 1 μM of each primer, and 2U Ampli Taq Gold polymerase. For amplification of genes, 1 μg of template cDNA was used. The following program was used: 95°C for 5 min followed by 43 cycles of 96°C for 40 sec, 52°C for 1 min, 72°C for 30 sec and a final incubation of 72°C for 10 min. Amplification products were visualized on a 1.5% agarose gel and stained with ethidium bromide.

The cDNA synthesis was carried out in 0.2 mL thin-walled PCR tubes with total reaction volumes of 20 μL containing final concentrations of 200 μM of each dNTP, 1 μM of gene-specific reverse primers, 2U Multiscribe reverse transcriptase (Life Technologies-Applied Biosystems, Grand Island, NY), and 10 μM RNase inhibitor, and 500 ng of total RNA. The following program was used: 25°C for 10 min, 37°C for 60 min, and 85°C for 5 sec.
Table 1: Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bsaA</td>
<td>YP_002537902</td>
<td>ACTGGCGATGCTACGTCGCA</td>
<td>ACCTGGGGCGCTGAAATCTGA</td>
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<td>bsaD</td>
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<td>grlA</td>
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<td>ATCTGGCTCATAACGGCGCTC</td>
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<td>133</td>
</tr>
<tr>
<td>grlD</td>
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<td>bannA</td>
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<tr>
<td>lbcL</td>
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<td>AACATGCCGAGTACGCG</td>
<td>CGGACAGATGAGCCGATATT</td>
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<tr>
<td>Bsa1RaceL</td>
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<td>N/A</td>
<td>TTGTGGCGACCACGCGGAAATGGGAGTAGGGAC</td>
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<td>RaceUT*</td>
<td>N/A</td>
<td>C CGCCAATTCCTCTCTTAGATGGG</td>
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<tr>
<td>RaceUTa</td>
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<tr>
<td>bamW</td>
<td>YP_002535620</td>
<td>CACAGCGATGTCCGTTATC</td>
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<td>191</td>
</tr>
</tbody>
</table>

*primers RaceU and RaceUT were kindly provided by the Wilson laboratory, Georgia State University, Atlanta, GA.

**Cloning and sequencing**

One Shot Top10 *E. coli* (Life Technologies Invitrogen, Carlsbad, CA) were transformed with the *pCR2.1Topo* vector containing the amplicon of interest. Cells were thawed for a few minutes on ice and incubated on ice for 5 min with vector. Cells were then heat shocked at 42°C
for 30 sec and immediately transferred to ice. Then, 250 µL S.O.C. medium was added and cells were incubated at 37°C while shaking vigorously for 1 hour. Cells were spread on antibiotic-selective plates and grown overnight at 37°C. Clones were grown in the presence of X-gal and IPTG and selected by color.

Those colonies which were white were picked for PCR verification of insert. These cells were then inoculated in 2 mL of LB media and grown overnight at 37°C shaking vigorously. The vector was then isolated from cells. Briefly, tubes were centrifuged at 10,000 rpm for 5 min at room temperature. Supernatant was discarded and the pellet was resuspended in 100 µL chilled GTE buffer (50 mM Glucose, 25 mM Tris-HCl, 10 mM EDTA). Also, 200 µL of 0.2 M NaOH and 1% SDS was added as well as 150 µL potassium acetate (100 mL 5 M potassium acetate, 57.5 mL glacial acetic acid, 142.5 mL distilled water). Tubes were incubated on ice for 10 min and occasionally mixed by inversion. Tubes were then centrifuged for 20 min at 20,000 rpm at room temperature and the supernatant was recovered. To the supernatant 280 µL of 100% chilled Isopropanol was added and these were mixed by inversion. Samples were incubated at -20°C for 30 min and centrifuged for 15 min at 13000 rpm at room temperature. Supernatant was discarded and the pellet was washed with cold 75% ethanol then centrifuged for 10 min at 13000 rpm at room temperature. The ethanol was discarded and pellet was dried by desiccation. This pellet was then resuspended in nuclease-free water. The sample was then sequenced by the Georgia State University Department of Biology Core Facilities to confirm accurate PCR amplification.
Real-time Reverse Transcription-PCR quantification (qPCR)

cDNAs were amplified with gene specific primers and the resulting amplicons were purified, quantified, and prepared for serial dilution then stored at -20°C. These dilutions were used as calibration standards for real-time PCR. qPCR reactions were carried out in 0.2 mL thin-walled optical PCR tubes with total reaction volumes of 20 μL. The protocol with SYBR Green PCR Master Mix (Life Technologies-Applied Biosystems, Grand Island, NY) was followed per manufacturer’s instructions. Primers were carefully designed to prevent formation of primer dimers during amplification and these cycles were followed by a dissociation curve to verify no dimers or non-specific amplification was present. If more than a single peak was observed on the dissociation curve, the concentration of primer and MgCl₂ was optimized. An amount of primer and MgCl₂ that forms no primer dimers and gives optimal amplification was used for qPCR assays of all the samples. The temperature profile used was as follows: an initial activation step at 50°C for 5 min and denaturation at 98°C for 40 s, followed by 40 cycles of denaturation at 98°C for 40 s, annealing at primer specific temperature (Table 1) for 32 sec, and elongation at 65°C for 32 s, with a final extension step at 65°C for 10 min.

The size of the PCR products was checked with agarose gel electrophoresis, and the specificity of PCR products was verified by sequence analysis of the clone library. qPCR analysis of the cDNA was carried out with the Applied Biosystems 7500 Real-Time PCR system (Life Technologies, Grand Island, NY) using 7500 Real-Time PCR System Sequence Detection Software (Version 1.3.1). The precision as well as the reproducibility of quantification were carefully optimized, and PCR products were checked for their correct lengths as described previously (53).
Identification and analysis of Sigma 54-dependent genes

Sigma 54 promoter identification

Regions upstream of the bss and grl operons were analyzed for the presence of the sigma54 promoter consensus using the PromScan (http://molbiol-tools.ca/promscan/) software.

Rapid amplification of 5’ cDNA ends (5’ RACE)

RNA was treated with 0.1 M DTT and incubated at 42°C for 2 min to eliminate secondary structure. First strand synthesis was carried out using Reverse Transcriptase as described previously. Primers reverse complimentary to the region near the start sight of bssD were used for first strand synthesis. See Table 1 for primer sequences. cDNA was purified by incubating with 1 M NaOH at 65°C for 20 min and cleaned with a GeneJet PCR Purification kit (Thermo Scientific-Fermentas, Pittsburgh, PA) per manufacturer’s instructions. DNA was tailed by incubating 5 pmol DNA with Terminal Transferase (New England Biolabs, Ipswitch, MA), 10x TdT Buffer (New England Biolabs, Ipswitch, MA), 2.5 mM CoCl$_2$ and 2 mM dCTP for 30 min at 37°C. The reaction was halted by incubating at 70°C for 10 min. For second strand synthesis the RACEUT and Race1 long primers were used with the following recipe: 20 mM TrisHCl, 10 mM KCl, 10 mM NH$_4$SO$_4$, 2 mM MgSO$_4$, 0.1% TritonX, 200 μM of each dNTP, 0.1 mL of 10X BSA, 1 μM of each primer, and 2U Ampli Taq Gold polymerase (Life Technologies-Applied Biosystems, Grand Island, NY). Reactions were carried out in thin-walled PCR tubes with the following program: 95°C for 5 min followed by 43 cycles of 96°C for 40 sec, 54°C for 1 min, 72°C for 30 sec and a final incubation of 72°C for 10 min. This step was repeated, replacing the RACEUT primer with RACEU, to obtain high enough DNA
concentrations for future steps. Amplification products were separated on a 1.5% agarose gel and stained with ethidium bromide. Products were cleaned with a GeneJet PCR Purification kit (Thermo Scientific Fermentas, Pittsburgh, PA) per manufacturer’s instructions and sequenced.

**Sigma 54 regulation of bss genes**

The *bbsD, bssA, bssB, bssC,* and Geob_2441 were inserted into the pCR Topo2.1 vector (Life Technologies-Invitrogen, Carlsbad, CA) following amplification with AmpliTaq Gold polymerase (*bssI*). RpoN− *E. coli* strains were purchased from the *E. coli* Genetic Stock Center at Yale University (New Haven, CT). One Shot Top10 *E. coli* (Life Technologies Invitrogen, Carlsbad, CA) and RpoN− were transformed with the *bssI* vector. Cells were thawed for a few minutes on ice and incubated on ice for 5 min with vector. Cells were then heat shocked at 42°C for 30 sec and immediately transferred to ice. Then, 250 mL S.O.C. medium was added and cells were incubated at 37°C while shaking vigorously for 1 hour. Cells were spread on antibiotic-selective plates and grown overnight at 37°C. Clones were grown in the presence or absence of toluene and assayed for the presence of growth.
3 RESULTS

Growth characteristics of *G. daltonii* strain FRC-32

*G. daltonii* FRC-32 can grow with various aromatic compounds including benzoate, toluene, benzene, m-, o-, and p-cresol. Toluene and benzene were toxic when amended at a high concentration (2 mM). Therefore, these aromatic hydrocarbons were supplied in a hydrophobic carrier phase with 2,2,4,4,6,8,8-*Heptamethylnonane* (HMN) to allow continuous supply with low concentrations (1 mM) because the direct addition to the culture was known to be inhibitory for growth (54). Also, cultures were tested for growth on toluene and benzene without HMN as a carrier in order to test if *G. daltonii* utilizes HMN as substrate. Good growth with toluene and benzene without HMN was observed (data not shown).

Various electron acceptors such as fumarate, nitrate and ferric citrate were tested for *G. daltonii* cultivation. *G. daltonii* was grown using 1 mM nitrate as an electron acceptor. Higher concentrations, up to 5 mM, were tested. However, these were all toxic and inhibitory to growth. Ferric citrate was tested as an electron acceptor at either 60 mM or 30 mM. Growth was observed when benzoate was used as the substrate (data not shown). However, significant growth was not observed when toluene or benzene were provided to the culture with ferric citrate as the electron acceptor. This is possibly due to the stressful nature of the combination of aromatic hydrocarbons and ferric citrate. *G. daltonii* culture was able to only grow using one of these at a time. Molecular genetic experiments for the present study were performed on cultures grown with 10 mM fumarate as an electron acceptor.

After establishing the growth conditions, *G. daltonii* FRC-32 was adapted to the respective aromatic substrates by subsequent transfer for at least five passages. A detailed growth
curve of the adapted cultures was then monitored by measuring increases in optical density, substrate loss and product formation in order to determine optimal time points for harvesting cultures. At mid-log phase cells were harvested for further genomic and metabolite analyses, so that misleading results in the gene expression study originating from growth phase effects were avoided.

These cultures grew to an average OD$_{600}$ of 0.1-0.3. This growth required 7-60 days depending upon the substrate provided. While *G. daltonii* is able to utilize multiple electron acceptors, it did not correspond with equal energy output. For example, more energy, and thus more rapid growth, was accomplished when this organism was grown utilizing nitrate as a sole electron acceptor than Fe(III) or even fumarate.

*Genomic analysis of anaerobic aromatic compound degradation genes*

In order to identify which regions of the *G. daltonii* genome might code for enzymes involved in anaerobic degradation of aromatic compounds, particularly benzene, genes within the reported aromatic compound-degrading gene island of *G. metallireducens* (55) were compared to genes within *G. daltonii* using BLAST analysis. As observed in the *G. metallireducens* genome (Fig. 9A), many genes predicted to play a role in aromatic compound degradation clustered into a large aromatic degrading gene island from Geob_0095 to Geob_0255 in the *G. daltonii* genome (Fig. 9B). This island spans a region of 165 genes and roughly 194 kbp. While most of the genome has a GC content of 53.5%, this region has a GC content of 56.3%. In addition to the main aromatic degrading gene island, both organisms have smaller gene clusters at other locations in the genome. The smaller cluster containing genes
Geob_2420-Geob_2451 in *G. daltonii* and has a GC content of 56.65% and spans a region of 32 genes and approximately 39 kbp.

**A**  
*Geobacter metallireducens*

**B**  
*Geobacter daltonii*

**Figure 9: Locations of predicted aromatic compound degradation genes in Geobacter species.** Genes predicted to play a role in aromatic compound degradation in *G. metallireducens* and *G. daltonii* were mapped with black bars indicating presence of genes associated with aromatic compound degradation.

Because anaerobic benzene oxidation is such a unique characteristic of *G. daltonii*, its genome was scanned for genes that shared no homology to other anaerobic aromatic-degrading bacteria. Once identified, these genes were grouped into functional blocks (Fig. 10) based on physical proximity within the genome, and functions were predicted if possible based on homology and existing annotation (Table 2).
Figure 10: Location of “unique” genes in the *G. daltonii* genome. Genes that are predicted to have “hypothetical” or “unknown” function that are proximal to one another.

Table 2. Predicted function of the unique genes

<table>
<thead>
<tr>
<th>Functional Block</th>
<th>Genes</th>
<th>Predicted Function</th>
<th>Notes of Interest</th>
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<td>DNA restriction/repair</td>
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<td>DNA Recombination</td>
<td>Multiple resolvase proteins</td>
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<td>3100-3130</td>
<td>RNA modification, DNA repair</td>
<td>Flanking inverted repeats, Components of ICE</td>
<td>52.0</td>
</tr>
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</table>

* All of the *G. daltonii* proteins from submitted annotation were blasted against other Geobacteraceae species for which complete genome sequences were available and annotated. Genes which were present in *G. daltonii*, but not found in any other Geobacteraceae species were considered as unique. The algorithm was BlastP, and the e-value cut-off score was 1e-20.
One of the largest segments, segment 1 is located close to the large aromatic-degrading gene island close to the start of the genome. At 55.9% GC, this region has a different GC content from the rest of the genome. This region contains multiple glycosylases and glycosyl transferases as well as dehydratases and cyclases. Because of these observations, this region is predicted to encode genes involved in biosynthesis pathways. Also unique to this region is that it is flanked by insertion sequences which could be used for genetic movement. This bares interest as it has been speculated that large mobile genetic elements, sometimes called ICE regions, are the mechanism by which the ability to degrade aromatics spread from one organism to another. An example of this is Tn4371, a 55 kbp region that confers the ability to degrade biphenyls to its host (56). Another region in *G. daltonii* with characteristics similar to ICE/integrated transposons is segment 7. This region has genes for DNA recombination as well as competence proteins and a recombinase. Segment 15 is an anomaly in that most of the genes in this region seem to code for a toxin/antidote system. This block also has a vastly different GC content from the rest of the genome at 48.7%. Finally, segment 18 is a region with mostly hypothetical proteins. However, it is flanked by inverted repeats and transposons as seen with ICEs. None of the regions of unique genes fell within close proximity to either of the aromatic degrading gene islands identified.

The large aromatic-degrading gene cluster from Geob_0095-Geob_0253 includes genes for utilization of phenol, which is a proposed intermediate in anaerobic oxidation of benzene. The first step in the anaerobic degradation of phenol is the addition of a phosphate group from ATP by the enzyme phenylphosphate synthase (*pps*). Phenylphosphate is carboxylated with CO₂ by phenylphosphate carboxylase (*ppc*), yielding 4-hydroxybenzoate. Coenzyme A is added to
the carboxyl group by an unknown enzyme, and the hydroxyl group of 4-hydroxybenzoyl-CoA is eliminated by reduction to form benzoyl CoA (57). The remainder of the pathway consists of reduction and opening of the aromatic ring, catalyzed by the bam gene products, beta-oxidation to acetyl-CoA, and oxidation to CO₂ through the TCA cycle. Expression levels of pps and ppc genes have been examined to detect activation of this pathway within anaerobic phenol-degrading organisms and could be used to determine whether they are involved in degradation of benzene by G. daltonii (58).

The smaller aromatic-degrading gene cluster from Geob_2420-Geob_2451 contains genes involved in toluene oxidation, another proposed route for anaerobic benzene degradation. The first step in anaerobic toluene degradation is the enzymatic addition of fumarate to toluene’s methyl group, resulting in the formation of R-benzylsuccinate (59). The enzyme benzylsuccinate synthase is a duplex of three subunits (α, β and γ), forming a heterohexamer (60); it is activated by an accessory protein, BssD, which generates a glycyl radical within the 98 kDa alpha subunit (61). Bss must be maintained in strictly anaerobic conditions as O₂ will cause irreversible cleavage of the protein (62). Subsequent steps are catalyzed by the bbs (beta oxidation of benzylsuccinate) gene products (Fig. 11A). Coenzyme A is transferred from succinyl-CoA to the alpha-carboxyl group of R-benzylsuccinate to form R-benzylsuccinyl-CoA. This compound is oxidized to E-2-benzylidenesuccinyl-CoA, hydrated to [2-hydroxy(phenyl)methyl]succinyl-CoA, oxidized to benzoylsuccinyl-CoA, and cleaved thiolytically to succinyl-CoA plus benzoyl-CoA. Benzoyl-CoA undergoes reduction and opening of the aromatic ring, catalyzed by the bam gene products, followed by beta oxidation and entry into the TCA cycle as acetyl-CoA which is finally converted to CO₂ (63). Upregulation of bss or bbs genes during anaerobic degradation of
benzene could be an evidence for use of the toluene pathway during anaerobic benzene degradation.

**Figure 11: bbs and bss gene clusters in *G. daltonii*.** Organization of anaerobic toluene oxidation genes within the genome of *G. daltonii*. White arrows indicate genes without predicted aromatic compound degradation functions according to NCBI annotations. Yellow arrows indicate genes predicted to be responsible for Bss formation and activation. Green arrows indicate similar predicted sigma 54-dependent transcriptional regulators. Spaces within arrows indicate predicted non-coding regions between genes and/or operons.

Of particular interest is the observation that *G. daltonii* has two adjacent operons encoding glycl radical-like genes (Fig. 11B). No other anaerobic toluene-oxidizing prokaryotes at present have been identified which code two copies of Bss. Also, it is interesting that these copies lie within completely independent operons, each with its own divergently transcribed gene encoding a transcriptional regulator and with different genes downstream. These copies only share 76% amino acid identity to one another. The *bbs* genes, which are responsible for the conversion of benzylsuccinate to benzoyl-CoA, are present only once within the genome.
However, the bam genes, predicted to encode electron transfer proteins associated with benzoyl-CoA reductase, are present in two copies each, organized into two operons. One of these operons is headed by a single bamB gene (encoding benzoyl-CoA reductase proper), and the other by two paralogs of bamB. Between the two operons are seven genes, of which Geob_0225 encodes a transporter of the major facilitator superfamily with homology to a benzoate transporter of Acinetobacter sp. strain ADP1 (64). Another of the intervening genes, Geob_0224, encodes a homolog of 2,3-dihydroxybenzoate adenyllyltransferase from Bacillus subtilis (1, 27), which may function as 2,3-dihydroxybenzoate-coenzyme A ligase in G. daltonii. If indeed G. daltonii is capable of degrading 2,3-dihydroxybenzoate, the pair of BamB-paralogous proteins and their associated electron transfer proteins may function in the reductive elimination of hydroxyl groups from 2,3-dihydroxybenzoyl-CoA, converting it to benzoyl-CoA for further degradation.

The gene cluster Geob_2442-Geob_2451 was named Bss (benzylsuccinate synthase) while the gene cluster Geob_2433-Geob_2441 was named Grl (glycyl radical-like). These clusters were compared to each other as well as the Bss cluster in G. metallireducens. The organization of the Grl cluster most similarly resembles the Bss cluster found in G. metallireducens. Both clusters first encode a gene (Geob_2433/Gmet_1533) for a flavin-binding oxidoreductase. However, in lieu of the Gmet_1535 gene of the G. metallireducens Bss cluster that encodes an outer membrane protein very similar to the toluene transporters of Ralstonia pickettii and Pseudomonas putida (65), the Geob_2434 gene in Grl encodes an outer membrane protein closely related to those encoded by Geob_0136/Gmet_2107 in the phenol degradation gene clusters and Geob_0117/Gmet_2127 in the p-cresol degradation gene clusters (66). According to the information, it can be hypothesized that one or both benzylsuccinate synthase-
like complexes of *G. daltonii* might be involved in degradation of a hydroxyl-substituted toluene such as *o*-cresol or *m*-cresol as a substrate. Assuming that the remaining enzymes of the toluene degradation pathway (which act on the methylsuccinate moiety) can accommodate a hydroxylated aromatic ring, the end product would be 2-hydroxybenzoyl-CoA or 3-hydroxybenzoyl-CoA, plausible substrates for the BamB-paralogous reductase described above. Other than this gene, all genes in Grl and the *G. metallireducens* Bss cluster are predicted to be the same, encoding a metal-binding protein of the VWFA superfamily with high-sequence-identity homologs in *Thauera aromatica*, *Aromatoleum aromaticum* and deltaproteobacterial strain NaphS2, an ATPase homologous to the putative chaperone BssE (TutH) required for toluene metabolism by *T. aromatica* (67, 68). The benzyllsuccinate synthase enzyme and its activator, BssD follows. All of these Bss operons are divergently transcribed with genes encoding sigma-54-dependent transcriptional regulators, which probably act as activators for these operons. Similarly to Grl and the *G. metallireducens* Bss operon, in *G. daltonii* Bss begins with BssCAB and BssD. However, the remaining Bss genes have homologs in *G. metallireducens* that are located outside the toluene degradation gene cluster.

The Bss cluster in *G. metallireducens* is adjacent to genes Gmet_1543 to Gmet_1556, which correspond to genes Geob_2569 to Geob_2552 of *G. daltonii*, separated from the Bss cluster by an island of 121 genes (Geob_2452 to Geob_2550) and roughly 102 kbp. This island is very interesting because it encodes multiple sets of proteins that resemble assembly factors for a vanadium-iron nitrogenase, yet the structural genes for this type of nitrogenase are not present in the *G. daltonii* genome. *G. daltonii*, like other *Geobacter* species, possesses ancestral genes for a molybdenum-iron nitrogenase and its assembly factors, Geob_2573 to Geob_2591. The assembly factors encoded by this island in *G. daltonii* may act in pathways analogous to the
synthesis of the molybdenum-iron cofactor of nitrogenase (69), but the end products may be various iron-sulfur-metal clusters within enzymes that catalyze difficult reactions other than nitrogen fixation. The location of the island next to Bss suggests that these reactions might serve to activate recalcitrant aromatics such as benzene.

For the first step in iron-sulfur-metal cluster synthesis, extraction of sulfur atoms from cysteine, the island encodes two cysteine desulfurases (Geob_2507, Geob_2526) and the rest of the genome encodes two more (Geob_1980, Geob_3049). The enzyme that assembles the sulfur atoms into iron-sulfur clusters is encoded by Geob_3050 outside the island. The third step, the merging of two 4Fe-4S clusters with a ninth sulfur atom and an unidentified small atom X, is carried out by NifB (Geob_2584) for the nitrogenase cofactor and presumably by three similar enzymes encoded by the island (Geob_2468, Geob_2497, Geob_2529). In many species, NifB includes an iron-sulfur cluster-binding oxidoreductase domain of the radical SAM type, which in Geobacter species is found as a separate protein (Geob_2591); thus, a plausible source of the ninth sulfur atom is S-adenosylmethionine (SAM), which may also donate its methyl group to become atom X. A very similar radical SAM domain protein encoded by Geob_2456 in the island may function together with the three NifB-like enzymes. The island encodes several supernumerary proteins for assimilation of sulfur into both cysteine and SAM: periplasmic sulfate-binding protein (Geob_2453, redundant with Geob_1651), cysteine synthase (Geob_2459 and Geob_2530, redundant with Geob_1403), and O-acetyl-L-homoserine sulphydrylase (Geob_2488, Geob_2499, redundant with Geob_0796, Geob_1890). There is also a gene for selenide, water dikinase (Geob_2508, redundant with Geob_1420), which could be an indication that selenium is a component of some of the iron-sulfur clusters synthesized by the island.
The 8Fe-9S-X cluster assembled by each NifB-like protein is expected to be transferred to a scaffold protein homologous to NifEN (Geob_2581) or a heterodimer of VnfE and VnfN in the case of a vanadium-iron nitrogenase, where the iron atom at one apex of the cluster may be displaced by a different metal before the cluster is inserted into the active site of an enzyme. The island encodes four homologs of VnfE (Geob_2460, Geob_2466, Geob_2500, Geob_2539) and five homologs of VnfN (Geob_2461, Geob_2467, Geob_2491, Geob_2501, Geob_2540), suggesting that *G. daltonii* can synthesize at least five different clusters. Although these proteins are clearly homologs, they are not most closely related each other according to the BLAST analysis output, suggesting that their evolutionary divergence is older than their acquisition by *G. daltonii*. The island encodes numerous metal transporters (Geob_2474, Geob_2475, Geob_2476 for nickel uptake, Geob_2512 for magnesium uptake, Geob_2552, Geob_2553, Geob_2554, Geob_2555, Geob_2556 for a heavy metal) and efflux pumps (Geob_2462, Geob_2477, Geob_2478, Geob_2487, Geob_2562, Geob_2563, Geob_2564 for a metal), and other ABC transporters, for which the number of periplasmic substrate-binding proteins (Geob_2470, Geob_2532, Geob_2533, Geob_2534, Geob_2536) and membrane proteins (Geob_2489, Geob_2490, Geob_2493, Geob_2494, Geob_2535, Geob_2537) exceeds the number of ATP-binding proteins (Geob_2492, Geob_2524, Geob_2531), suggesting adaptability to transport different solutes. These transporters may provide the VnfE and VnfN scaffolds with a variety of trace metals to insert into iron-sulfur clusters.

Displacement of a cluster’s apical iron atom with molybdenum, which requires ATP hydrolysis and electron transfer to molybdate, is catalyzed by the NifH protein (Geob_2574), which also transfers reducing equivalents to nitrogenase during nitrogen fixation. The island encodes two homologs of NifH (Geob_2455 and Geob_2458), which may reduce different
metals and provide the energy to insert them into iron-sulfur clusters, and may also transfer electrons to the enzymes that bind these clusters. The molybdenum atom is ligated by homocitrate, which is synthesized by the Geob_2573 gene product; the island-derived clusters may or may not contain homocitrate. Consistent with a role of these clusters in activation of benzene by methylation to toluene, the island encodes three proteins involved in methyl group transfer: methyl-Co(III) 5-hydroxybenzimidazolylcobamide-binding protein (Geob_2541), UroD-like decarboxylase/methyltransferase (Geob_2542), and methylcobamide--coenzyme M methyltransferase (Geob_2543).

An anaerobic sulfate-reducing Deltaproteobacterial strain NaphS2, which was isolated from anoxic marine sediment, can anaerobically degrade naphthalene (70). It is reasonable to predict that dearomatization of naphthalene might occur by mechanisms relevant to dearomatization of benzene. Therefore, genes that were upregulated in NaphS2 during growth on naphthalene, versus benzoate, as its substrate were compared to the G. daltonii genome in order to identify potential anaerobic benzene oxidation genes. Most of the genes which shared homology with G. daltonii were housekeeping genes related to protein translation. In addition to these, the two organisms shared genes for an ATPase and benzoate degradation genes. However, there were some genes in NaphS2 which corresponded to aromatic-degrading genes in G. daltonii. Among these were a fumarate reductase, sensor histidine kinase, and BbsF genes within the smaller aromatic-degrading gene cluster. Finally, both organisms share genes for the Clp protease. This protein has been indicated in a wide range of genetic regulation mechanisms within multiple organisms, including regulation of aromatic degradation (71).
Utilization of benzene as an electron donor by *G. daltonii*

*G. daltonii* was able to grow using benzene as a sole carbon source and an electron donor with fumarate as the electron acceptor (Fig. 12B). The growth was accompanied by the disappearance of benzene and formation of succinate, the product of fumarate reduction (Fig. 12A). In a *G. daltonii* culture grown on benzene and fumarate the electron equivalents recovered in succinate production measured was commensurate with the electron equivalents calculated from consumed benzene with a calculated electron recovery of 121.2%. In order to test if *G. daltonii* utilizes fumarate as a substrate some cultures were incubated only with fumarate but without addition of benzene. Without benzene *G. daltonii* did not grow and no succinate production was observed during the incubation. *G. daltonii* also grew with benzene in the presence of nitrate as an electron acceptor (Fig. 12D), consuming both benzene and nitrate (Fig. 12C). In *G. daltonii* culture grown on benzene and nitrate the electron equivalents recovered in nitrate loss measured was corresponding with the electron equivalents calculated from the amount of benzene consumed with a calculated electron recovery of 108.6%. *G. daltonii* reached a lower optical density during growth with nitrate versus fumarate as its terminal electron acceptor. This might be due to the lower availability of electron acceptor in nitrate cultures as low concentrations must be used in order to avoid toxicity. Concurrent with this hypothesis, *G. daltonii* achieved twice the optical density when grown with 2 mM nitrate rather than 1 mM nitrate while oxidizing benzene (data not shown). *G. daltonii* also grew more rapidly in the presence of nitrate than in the presence of fumarate, reaching middle log phase five days after inoculation rather than 30 days. This has also been observed in *G. metallireducens* (data not shown) and could be due to nitrate having a higher redox potential than fumarate.
Figure 12: *G. daltonii* utilizes benzene as a sole carbon source. A. Anaerobic benzene oxidation is coupled to reduction of fumarate as electron acceptor and production of succinate. B. Growth, measured as optical density at 600 nm, of *G. daltonii* on benzene as a sole carbon source and fumarate as electron acceptor. C. Anaerobic benzene oxidation is coupled to the reduction of nitrate as electron acceptor. D. Growth, measured as optical density at 600 nm, of *G. daltonii* on benzene as a sole carbon source and nitrate as electron acceptor. Data are the means ± standard deviations of triplicate experiments.

Benzene-dependent responses in *G. daltonii*

To test the possibility that *G. daltonii* degrades benzene via methylation, the toluene pathway was examined for multiple reasons. First, this is the only organism known to encode two copies of the Bss complex. It has also been shown that addition of toluene to enrichment cultures accelerates benzene degradation (72), possibly because the presence of toluene induces the expression of enzymes of the toluene degradation pathway that would be useful for the degradation of benzene through toluene. Expression of the *bssA* and *grlA* genes as well as
upstream possible transcriptional regulators, *tf1* and *tf2*, was tested under multiple growth conditions in *G. daltonii*. When the organism was grown with benzene as its sole substrate, expression of both *bssA* and *grlA* was observed by RT-PCR (Fig. 13). In order to obtain a clearer picture of differential gene regulation, these experiments were repeated using quantitative Real Time PCR. When *G. daltonii* was grown with either toluene or benzene as its substrate *bssA* was highly expressed, roughly three fold over what the expression observed when the organism was grown with benzoate as a carbon source (Fig. 14A). During growth with either benzene or toluene *grlA* was expressed roughly three fold lower than the baseline observed when cells were grown with benzoate. This difference suggests that the *bss* genes may encode the sole or primary benzylsuccinate synthase of *G. daltonii*, and the *grl* genes may act on substrates other than toluene. Neither proposed transcription factor was upregulated in response to the presence of benzene or toluene in the growth media.

![Figure 13: Relative expression of bssA and grlA genes in G. daltonii during anaerobic oxidation of benzene and toluene.](image)

Agarose gel of RT-PCR products (133bp and 119 bp) formed with *bssA*- and *grlA*-specific primers, respectively, using cDNA. Lanes L: 100 bp DNA ladder; +: positive control- *G. daltonii* genomic DNA; -:negative control-RT-PCR reaction mix only without cDNA template; 1: *G. daltonii* grown on benzene. Gene amplified was *bssA1*; 2: *G. daltonii* grown on toluene. Gene amplified was *bssA1*; 3: *G. daltonii* grown on benzene. Gene amplified was *grlA*; 4: *G. daltonii* grown on toluene. Gene amplified was *grlA*. 
Genes encoding components of the glycl-radical enzyme benzylsuccinate synthase (bssA or grlA) for the toluene pathway, phenylphosphate carboxylase (ppcB) for the phenol pathway and benzoate CoA ligase (bcl) for the benzoate pathway as well as an enzyme common to all three pathways 6-oxocyclohex-1-ene-1-carbonyl-CoA hydratase (bamA) was tested using qPCR. The gene bamA was expressed at an identical level for all three carbon sources tested. Even though growth using phenol as a substrate has not been demonstrated for G. daltonii it does possess a cluster of genes resembling phenol degradation genes. Expression of the gene encoding phenylphosphate carboxylase, ppcB, was examined and was strongly downregulated in cells grown with benzene or toluene, exhibiting a three-fold reduction in expression in comparison to cells grown with benzoate (Fig. 14A).
Figure 14: Relative expression abundance of anaerobic aromatic degradation genes in *G. daltonii*. Expression of genes predicted to be in aromatic degradation pathways was measured using quantitative RealTime RT-PCR. Genes were measured in terms of copy number per µg of total RNA. Fold change is calculated by dividing expression of test substrate by expression of benzoate. Expression of various genes in cultures grown on either benzene (blue bars) or toluene (green bars) is shown. *PpsA* and *ppcB* are predicted to be involved in the pathway for phenol degradation. *BssA1* and *grlA* are predicted to be in the pathway for toluene degradation. *Bcl* is predicted to play a role in benzoate degradation. *BamA* is a gene indicative of use of the Benzoyl CoA pathway predicted to be utilized during degradation of any aromatic compound. *Tfl* and *tf2* are predicted as Sigma 54-like transcription regulators.

The protein profiles of *G. daltonii*, grown using different substrates, were examined in order to ensure that alterations of gene expression could be observed at the protein level using SDS-PAGE. Cells grown with benzoate expressed several proteins that are not observed in cells grown on toluene and benzene (Fig. 15). Cells grown on toluene and cells grown on benzene also expressed proteins of the same size. Of note, cells grown on benzene and toluene expressed a protein roughly 230 kDa in size that was not observed in cells grown on benzoate. This is roughly the predicted size of benzylsuccinate synthase. Taken together, these results show that
cells grown on benzene and toluene exhibit similar protein profiles that differ from the profile observed for cells grown on benzoate.

Figure 15: Cells grown with either benzene or toluene exhibit similar protein profiles. Cells were boiled 20 min in NuSep loading buffer and visualized on a NuSep SDS-PAGE gel. Lanes 1: Cells were grown on benzoate; 2: Cells were grown on benzene; 3: Cells were grown on toluene; +: Pure BSA was used as a loading control; L: High molecular weight ladder. Arrow indicates band of approximate size of Bss protein.

Metabolites of anaerobic benzene and toluene oxidation by G. daltonii

Because Bss is an unstable enzyme, the ability of cell lysate to sustain benzene degradation was tested by observing the loss of benzene over time using HPLC. Lysate harvested from cells grown with toluene as substrate was successful in oxidizing toluene as evidenced by
the significant loss in concentration over the course of 20 min (data not shown). With lysate harvested from cells grown with benzene as electron donor and fumarate as electron acceptor, after 5 min a loss of benzene was observed along with the generation of a small amount of toluene. However, after this initial conversion, the process slowed dramatically. The addition of fumarate to the reaction 10 minutes after its initiation facilitated the further degradation of benzene and accumulation of toluene equal to 95% of the original concentration of benzene (Fig. 18A). There are at least two possible explanations for this phenomenon. The addition of fumarate may have altered the redox potential of the reaction mix such that conversion of benzene to toluene was easier to achieve. Alternatively, fumarate could have acted as a co-substrate for Bss and thus shifted the reaction such that more downstream products were formed and thus more reactant (toluene) could be produced. In order to determine the role of fumarate in the reaction, lysate harvested from cells grown with benzene as substrate and nitrate as electron acceptor was used. Again, there was sharp decline in benzene concentration accompanied by a low amount of toluene formation. Upon addition of fumarate, there was no additional activity observed (Fig. 16B). The addition of nitrate to the reaction resulted in the continued degradation of benzene coupled to formation of toluene equal to approximately 90% of the original concentration of benzene in the system (Fig. 16C). Altogether, these results suggest that conversion of benzene to toluene requires a terminal electron acceptor and does not require fumarate specifically to react with toluene.
Anaerobic degradation of benzene whole cell lysate of *G. daltonii* is coupled to formation of toluene. Cells were lysed anaerobically and lysate was added to 1 mM benzene. Samples were taken every minute and aromatic metabolites were measured with HPLC. Benzene (blue circles) and toluene (green squares) were observed. A. Cells lysed were grown using fumarate as an electron acceptor. At 10 minutes (black arrow) 1 µM fumarate was added to the reaction. B. Cells lysed were grown using nitrate as an electron acceptor. At 10 minutes (black arrow) 1µM fumarate was added to the reaction. C. Cells lysed were grown using nitrate as an electron acceptor. At 10 minutes (black arrow) 1µM nitrate was added to the reaction.

Anaerobic oxidation of cresols by *G. daltonii*

Because glycine radical-like enzymes typically utilize a methyl group in their active site, it is possible that a cresol compound could be the substrate used by grlA. Therefore, the ability of *G. daltonii* to oxidize any of the three cresol isomers was tested. As can be seen in the Figure 17A, *G. daltonii* can grow very well using any of the cresol isomers as a carbon source. Cultures reached middle log phase after approximately 3 days, a relatively rapid growth time for *G. daltonii* when utilizing aromatic substrates as a carbon source, and a maximal OD600 of
approximately 0.1. All samples without inoculum maintained an \( \text{OD}_{600} \) of approximately zero. Figure 17B demonstrates that the growth of this organism correlated to the loss of cresol isomers in the growth media. Cresols maintained a steady concentration throughout the course of the experiment in the absence of \( G. \text{daltonii} \) and dramatically declined in the presence of culture.

Genes that have been proposed to be part of the various cresol degradation pathways were tested for induction using qPCR in the presence of the three cresol isomers. Genes encoding glycine radical-like enzymes were also tested. The \( pcmT \) gene, proposed to be part of the \( p \)-cresol pathway, was only substantially upregulated in the presence of \( m \)-cresol. The presence of \( m \)-cresol also resulted in upregulation of the \( grlA \) gene, previously suspected to contribute to toluene degradation in this organism (Fig. 18). None of the other genes tested exhibited remarkable upregulation in response to any of the cresols as compared to gene expression during the growth on benzoate.

**Figure 17:** \( G. \text{daltonii} \) utilizes cresols as a sole carbon source. A. Growth, measured as optical density at 600nm, of \( G. \text{daltonii} \) is coupled to use of cresols as a carbon source. B. Cresol degradation is coupled to the growth of cells.
Figure 18: Relative expression of proposed cresol degradation genes in *G. daltonii*. Expression of genes predicted to be in aromatic compound degradation pathway was measured using quantitative Real Time RT-PCR. Genes were measured in terms of copy number per µg of total RNA. Fold change is calculated by dividing expression of test substrate by expression of benzoate. *pcmT* is predicted to be a part of the *p*-cresol degradation pathway, *hbcL* is predicted to be a part of the *m*-cresol degradation pathway, and *mbdA* is predicted to be a part of the *o*-cresol degradation pathway.

The expression of the *bss* and *grl* operons was measured in response growth using a cresol compound or naphthalene as the sole carbon source. Growth on *m*-cresol resulted in an upregulation in *grlA*, the only compound shown to do so thus far. The predicted transcriptional regulators were not upregulated in response to any of these compounds. Downregulation was observed in genes that are the part of the phenol and benzoate degradation pathway in response to all of these compounds (Fig. 18).
Figure 19: Relative expression of aromatic compound degradation genes in *G. daltonii*. Expression of genes predicted to be in aromatic compound degradation pathway was measured using quantitative Real Time RT-PCR. Genes were measured in terms of copy number per µg of total RNA. Fold change is calculated by dividing expression of test substrate by expression of benzoate. *PpcB* is predicted to be in the pathway for phenol degradation. *BssA1* and *grlA* are predicted to be in the pathway for toluene degradation. *Bcl* is predicted to play a role in benzoate degradation. *BamA* is a gene indicative of use of the Benzoyl CoA pathway predicted to be utilized during degradation of any aromatic compound. *Tf1* and *tf2* are predicted as Sigma 54-like transcription regulators.

**Proposed regulation of anaerobic aromatic compound degradation genes in *G. daltonii***

Directly upstream and in opposite orientation of the glycyl-radical like operons are genes annotated as “sigma 54-related transcriptional regulators” (Fig. 11B). These genes are 75% identical to sigma 54 enhancer binding proteins found in *G. sulfurreducens* and so could represent a sigma 54-dependent regulation system for these operons in *G. daltonii*. The presence of sigma 54-like elements proximal to these operons also indicates a high level of regulation of the gene products as sigma 54 regulons are highly regulated. The use of sigma 54 to regulate
these genes also marks them as highly unique. To date, those operons regulated by sigma 54 are capable of unique functions in many different bacteria.

Using the program PromScan (73), the *G. daltonii* genome was scanned for possible sigma 54 promoters. Among others, regions upstream of both Bss and Grl were identified as containing the sigma 54 consensus sequence. Analysis of these regions indicated the presence of the mandatory GG and GC elements at -12 and -24 as well as other less highly conserved elements (Fig. 8). This indicates that sigma 54 could be the sigma factor which is used to activate these operons, as observed with other aromatic degradation gene operons in other *Geobacteraceae* species. The method 5' RACE was used to determine whether the *in silico* predicted promoters were in fact the transcriptional start sites of these genes. Multiple trial conditions were optimized to provide a correct sequence (Fig. 20). The transcriptional start site was confirmed to be that predicted by the computer.

Figure 20: 5'RACE amplicons after three rounds of RT-PCR. Lanes +: *G. daltonii* genomic DNA with bssAFR primers; – : genomic DNA with no primers; 1-3: amplicons of 5' RACE tailing and PCR amplification with bssRACElong and RACEU primers. Arrow indicates expected size of RACE amplicon.
In order to determine physiological evidence of involvement of sigma 54 in the regulation of the *bss* genes, mutation of sigma 54 in *G. daltonii*, resulting in a “knock out” clone, would be a classical genetic approach. Such knock outs have previously been shown to be lethal in other *Geobacter* species. Therefore, an alternative system was used in the present study. *E. coli* strain Top10, similar to *E. coli* strain K12/DH10B, was transformed with the pCRTOPO2.1 vector ligated with the *bss* genes. These cells were then exposed to toluene in a contained anaerobic environment. *E. coli* cultures containing the *bss* genes were able to grow after 7 days, forming colonies. *E. coli* cultures without *bss* genes were never able to grow. *E. coli* cells without a functional sigma 54 were also unable to grow in the presence of toluene (Fig. 21). However, this strain was able to grow aerobically on LB medium (data not shown). The complementation of the *rpoN* gene from *G. daltonii* combined with *bss* restored the ability to grow in the presence of toluene (Fig 21D).

**Figure 21:** *E. coli* clones grown in the presence of toluene. A: wild type *E coli* with *bss* genes B: wild type *E coli* without *bss* genes C: Sigma 54- *E. coli* with *bss* D: Sigma 54- *E. coli* with *bss* and *rpoN*

The expression of *bamV* (Geob_0144) and *bamW* (Geob_0145) was measured using qPCR because expression of these genes were demonstrated previously in *G. metallireducens* to be a part of the sigma 54 regulatory network. As shown in Figure 22, both genes are upregulated
under all conditions when compared to growth with acetate as a negative control. All conditions also resulted in an upregulation of relatively equal magnitude.

**Figure 22:** Relative expression of the sigma 54 two component regulatory system in *G. daltonii*. Expression of genes involved in sigma 54 dependent regulation of aromatic degradation was measured using quantitative Real Time RT-PCR. Genes were measured in terms of copy number per µg of total RNA. Fold change was calculated by dividing expression of substrate over expression of acetate. *BamV* (blue bars) response regulator and *bamW* (red bars) sensor kinase.
4 DISCUSSION

Anaerobic activation of toluene and benzene in G. daltonii

Aerobic mechanisms of BTEX degradation have been demonstrated for a wide variety of organisms, which successfully degrade these compounds through diverse pathways encoded on TOL plasmids. Even among the aerobes, activation of the benzene ring is a complex mechanism. Aerobic benzene activation proceeds through rapid hydroxylation of benzene to either phenol or 1,4-diphenol via either mono- or di-oxygenases. These processes produce reactive oxygen species that must be neutralized by either superoxide dismutase or hydratases in order to avoid DNA damage (74). Since anaerobic microorganisms cannot utilize oxygen, benzene activation must proceed through a different mechanism using other electron acceptors.

Although knowledge of anaerobic benzene catabolism is limited, anaerobic degradation of other aromatic compounds has been well characterized. In G. metallireducens many pathways involved in anaerobic oxidation of aromatic compounds have been elucidated (55). This organism is known to anaerobically degrade phenol, p-cresol, benzyl alcohol, benzaldehyde, 4-hydroxybenzoate, toluene, and benzoate. Anaerobic phenol degradation involves conversion to phenylphosphate, carboxylation to 4-hydroxybenzoate and eventual conversion to benzoyl-CoA. Anaerobic toluene degradation involves conversion to benzylsuccinate and eventual conversion to benzoyl-CoA. This same toluene pathway has been demonstrated in multiple other organisms capable of anaerobic toluene degradation such as Thauera aromatica (62). Each of these pathways has been experimentally demonstrated and linked to genetic regulation. Of note, this organism is capable of degrading downstream intermediates of the phenol pathway, but not others.
For many years the inability to cultivate a pure culture capable of anaerobic benzene degradation has limited investigation into the mechanism of anaerobic benzene metabolism. Elucidation of the mechanism has also been hampered by the presence of contradictory metabolites within contaminated sediments. However, recent research has revealed several microorganisms capable of degrading benzene anaerobically, each utilizing a different pathway. An anaerobic, hyperthermophilic, Fe(II)-oxidizing archaeum, *F. placidus* apparently carboxylates benzene, converting it to benzoate and utilizing the existing genes in this pathway to finish its degradation (58). In addition to this, *G. metallireducens*, a member of the family *Geobacteraceae*, has been demonstrated capable of degrading benzene anaerobically. This organism oxidizes benzene by utilizing the phenol pathway through an apparent hydroxylation of benzene (11). Finally, *G. daltonii* also is capable of degrading benzene anaerobically in pure culture. The down-regulation of genes involved in the phenol oxidation, *ppcB*, or the benzoate oxidation pathways, *bamA*, which was found in the present study (Fig. 14), rules out the utilization of these pathways during anaerobic benzene degradation by *G. daltonii*.

When lysate-based anaerobic benzene degradation was measured the catalytic loss of benzene surprisingly plateaued (Fig. 16). The reaction was continued by the addition of fumarate or nitrate as electron acceptor. The addition of fumarate or nitrate to the reaction resulted in the degradation of the remaining benzene present, as evidenced by its subsequent consumption. Benzene degradation was coupled to toluene accumulation in both cases. These results suggest that conversion of benzene to toluene is energetically challenging. However, the reason that toluene is accumulating, rather than being further degraded and mineralized to CO$_2$, is not clear. It is possible that some cofactor or substrate required for one of the participating enzymes is limiting. Or it is also possible that the redox potential of the cell lysate might not be
representative of what is observed in intact cells. Thus a downstream reaction might now carry a positive delta G and no longer be energetically favorable. This could cause accumulation of upstream substrates, and thus toluene. This could potentially be tested with the addition of a redox compound, such as succinate, to the reaction and observation of whether toluene ceases to accumulate. It is also possible that disruption of the cellular membrane has disrupted the physical location of enzymes needed for them to fully complete the pathway. One of the participants in the reaction may be membrane-bound and therefore is inactive once the membrane has been destroyed to make the cell lysate.

*Regulation of genes involved in anaerobic oxidation of aromatic compounds and Sigma 54 in G. daltonii*

The duplication of glycyl-radical genes in *G. daltonii* initially led to the hypothesis that one copy would be upregulated when this organism degraded toluene and the other copy would be upregulated during benzene degradation (Fig. 11). The two copies of *bssA* are 76% identical in amino acid sequences, similar enough to share similar activities but also unique enough to possibly have divergent capabilities as was observed with the *omcB* and *omcC* genes in *G. sulfurreducens* (75). The Grl operon has the most similarity to the Bss operon in *G. metallireducens* with respect to the genes within it. The Bss operon is distinctly different. This operon has multiple genes with unknown functions predicted and lacks the predicted fumarate reductase typically observed in Bss operons. However, rather than both pathways being active, only the *bssA* gene was upregulated and it was upregulated whether the carbon source utilized was benzene or toluene, indicative that the toluene pathway is utilized during anaerobic benzene degradation in *G. daltonii*. It is notable that not all genes within the operon have stoichiometric amounts of mRNA present in the cell. Both *bssD* and *bssE* exhibited higher transcript levels
than bssA using qPCR (data not shown). This might presumably be due to an uncharacterized post-transcriptional regulatory mechanism.

**Anaerobic cresol oxidation in G. daltonii**

If grlA is not used to degrade either benzene or toluene, why has *G. daltonii* expended the energy required to maintain this operon and its preceding transcriptional regulator? One possibility is that while grlA is similar to benzylsuccinate synthase its substrate is something else entirely. For example *p*-, *o*- or *m*-cresol are structurally similar to toluene and could be the substrate for this enzyme rather than toluene. Therefore, growth on these substrates by *G. daltonii* was tested (Fig. 17). *G. daltonii* was able to grow using all three cresol isomers as a carbon source. The presence of any cresol as the sole carbon and energy source in the media resulted in growth of *G. daltonii* with the classical lag, log, and stationary phases evident. These growth patterns are directly correlated with loss of the cresol isomer measured in the cultures. While the trend is more challenging to observe in the loss of *o*-cresol, this is most likely due to the difficulty in measuring this isomer with HPLC rather than an inability of the organism. Most HPLC measurements of *o*-cresol use flow rates as low as 0.1mL min⁻¹ (76). This flow rate exceeds the lower limits of the HPLC machine used in this study, however.

All the previously characterized organisms have only been able to degrade one or two of the cresol isomers (26); therefore, the degradation of all three cresol isomers should be a highly unique capability. Also, unique to the organism appears to be the genes involved in the cresol degradation pathways. Previous studies with family member *Geobacter metallireducens* indicated that the degradation of *p*-cresol proceeds through a hydroxylation of the methyl group by the enzyme *p*-cresol methylhydroxylase and formation of p-hydroxybenzoate which is then
activated by the addition of a coenzyme A (CoA) group and eventually feeds into the benzoyl-CoA pathway (26). These modifications are coded by p-cresol modification (pcm) genes (36). This same strategy is employed by denitrifiers such as *Thauera aromatica* (77). The *G. daltonii* genome contains genes predicted to be part of this pcm suite within its large aromatics island (Geob_0103-Geob_0126). However, the growth of *G. daltonii* on p-cresol did not upregulate the pcmT gene tested according to qPCR (Fig. 18). Rather it was expressed at relatively the same level regardless of whether p-cresol or benzoate was the substrate provided. Instead, p-cresol had a slightly greater effect on the expression of the grlA gene, indicating the activation of this compound by the addition of fumarate to the methyl group. This mechanism of p-cresol activation has also been observed in an anaerobic sulfate-reducer, *Desulfobacterium cetonicum* (78).

Degradation of m-cresol is proposed to proceed through the production of a 3-hydroxybenzoate intermediate which is activated by the addition of a CoA group by the aromatic CoA ligase coded by the hbcL gene (26, 79). The sequence for this gene in *T. aromatica* was compared to genes in the *G. daltonii* genome using BLAST and a gene within the aromatics island was returned, Geob_0245, and primers targeting this gene were designed. However, these genes were not significantly upregulated in *G. daltonii* cultures grown on m-cresol compared to cultures grown on other cresols or benzoate (Fig. 18). Interestingly, the grlA gene was greatly upregulated in *G. daltonii* cultures grown on m-cresol (Fig. 19). These results indicate that m-cresol, like p-cresol, is possibly activated by the addition of fumarate to the methyl group and further degraded in *G. daltonii*. This pathway has been demonstrated to be used to degrade m-cresol in *D. cetonicum*, also as it was observed with p-cresol (80).
The anaerobic degradation of \( o \)-cresol is proposed to follow a different pathway. This compound is predicted to follow through a 3-methyl benzoyl CoA degradation pathway rather than the usual benzoyl CoA pathway predicted for degradation of other aromatic compounds by the family \textit{Geobacteraceae}. This is predicted to proceed through a carboxylation of the hydroxyl group and formation of a 4-hydroxy-3-methylbenzoate intermediate by the gene \textit{mbdA} which is then activated via CoA addition and reductive dehydroxylation. The 3-methyl benzoyl CoA reductase was found to be similar to the benzoyl CoA reductase typically observed in \textit{Geobacteraceae} (26). The BLAST analysis of the \textit{G. daltonii} genome elucidated a gene in the large aromatics island, Geob_0167, which is next to a gene predicted to be a benzoyl CoA reductase by the NCBI database and has homology to the \textit{mbdA} gene. Transcript levels of this gene did not exhibit significant upregulation by any of the cresol isomers. Also, \( o \)-cresol did not induce upregulation of any of the genes tested with qPCR (Fig. 18 and 19). The lack of knowledge about \( o \)-cresol degradation is common. It is not currently known whether obligate anaerobic prokaryotes capable of degrading this aromatic compound do so through modification of the methyl group, the hydroxyl group, or some other position on the aromatic ring. Degradation of the aromatic compound needs much further testing and analysis for conclusions to be made as to the intermediates formed and pathways utilized in its anaerobic degradation.

Conclusions and future studies

The results obtained from the present study clearly suggest that \textit{G. daltonii} catabolizes benzene via an unknown mechanism that employs the toluene pathway. It is observed both genetically and metabolically. The first gene in the toluene degradation pathway, \textit{bssA}, is highly upregulated while genes utilized in the phenol and benzoate pathways are downregulated during
anaerobic oxidation of benzene when compared to benzoate (Fig. 14). Toluene is generated during the degradation of benzene by cell lysate to quantities equivalent to the amount of oxidized benzene (Fig. 16). Also, cells grown on toluene and cells grown on benzene showed similar protein profiles visualized with SDS-PAGE that are markedly different from the profile observed for cells grown with benzoate (Fig. 15).

What are the roles of genes within the Grl operon? Answers to this question could provide insights into how these pathways may be accelerated both in the laboratory and in situ, resulting in greater bioremediation of contaminated sites.

Another question is what is the exact mechanism involved in converting benzene to toluene. The use of a radical is always observed when side chains are enzymatically added to benzene (81). How is the radical supplied in this anaerobic bacterium and how is it neutralized to prevent damage to the cell? One possibility is the use of radical SAM enzymes as they have been shown to be capable of activating inert C-H compounds and also of creating an environment to allow radical methylations (82). Alternatively, the utilization of metalloproteins containing extensive iron-sulfur structures which are capable of forming a radical may be employed. These proteins are located very near to the Bss operon, and a suite of genes could possibly encode such a protein. Geob_2507-Geob_2582 is a region which should be investigated with regards to possible FeMoCo assembly. This enzyme works with nitrogenases, and could contain genes capable of catalyzing the radical methylation of benzene (83).

A very interesting finding from the present study provided a significant insight about the possible regulation of the bss and grl operons. It was initially hypothesized that the transcriptional regulators directly upstream of each operon would regulate the operon they were physically closest to. However, gene expression analysis during the anaerobic oxidation of
benzene and toluene demonstrated that *tf2* is upregulated under similar conditions as the Bss operon, rather than the Grl operon (Fig. 14). This result indicates that *tf2* is possibly responsible for Bss regulation. Because this gene is annotated as a sigma 54-like transcriptional regulator it is likely that sigma 54 is responsible for the regulation of this operon.

The role of sigma 54 in the regulation of aromatics degradation has been demonstrated in other *Geobacter* species (84). The prediction of a sigma 54 consensus sequence upstream of these glycyl radical operons supports the hypothesis that they will be regulated in the sigma 54 network. This prediction was tested using 5' RACE analysis and the transcriptional start was confirmed to be the start of the predicted consensus sequence (Fig. 20). This result demonstrates that these operons are regulated via sigma 54-dependent mechanisms.

To test this *in vivo*, *E. coli* were transformed with the *bss* genes. The transformed cells formed colonies in the presence of toluene and wild type cells did not form any colony (Fig. 21). The ability of transformed *E. coli* to grow and form colonies suggests that the *bss* genes are actively transcribed and the proteins made to allow enzymatic conversion of toluene, which is toxic, to benzylsuccinate, which is not toxic. This finding also confirmed that the use of an artificial system such as the *E. coli* sigma 54 is useful and applicable to regulation studies in *G. daltonii* and could be used for other purposes in future. It is particularly beneficial as a system to manipulate *G. daltonii* genetically has not been developed yet. Sigma 54- *E. coli* were also transformed with the *bss* genes. These cells were unable to grow in the presence of toluene. This result suggests that the *bss* genes are no longer active and toluene is no longer converted to benzylsuccinate, presumable due to the loss sigma 54. However, growth was restored when these cells were complemented with the *rpoN* gene *in trans* (Fig 21). This loss in transcription
correlated to the loss of sigma 54 along with successful complementation indicates that the \textit{bss} operon is regulated via a sigma 54-dependent mechanism.

\textit{BamV} and \textit{bamW} have been demonstrated to be a two component signaling system in \textit{G. sulfurreducens} and have also been shown to be part of the sigma 54 network in that organism (44). Sigma 54 two component signaling systems have also been observed in flagellar synthesis in \textit{Campylobacter jejuni} and extracellular lipase production by \textit{Pseudomonas alcaligenes} (85, 86). The histidine kinase, \textit{bamV}, was analyzed using the SMART database. It is predicted to contain two transmembrane domains, one at the N-terminus and another approximately 60 amino acids away from its signal sensing domain. Additionally, \textit{bamV} was predicted to partner with the following genes: Geob_0145, Geob_0741, Geob_0809, Geob_1898, Geob_2158, Geob_2162, Geob_2736, Geob_3388, Geob_3534, and Geob_3555. None of these genes cluster in the aromatics island. However, they could be genes of interest with regards to the regulation of other aromatics degradation pathways as the BamVW system is involved in aromatic signaling. The BamVW genes were active in the presence of benzoate and \textit{p}-cresol in \textit{G. sulfurreducens}. Due to their implication in sigma 54 regulatory mechanisms the expression of these genes was tested in \textit{G. daltonii} under a variety of conditions. \textit{BamV} and \textit{bamW} were both upregulated when \textit{G. daltonii} was grown on a variety of aromatic compounds compared to growth on acetate (Fig. 22). This result indicates that this signaling system may be the universal sensor for any aromatic in \textit{G. daltonii}.

All of these findings from the present study support the hypothesis that the \textit{bss} genes in \textit{G. daltonii} are regulated via sigma 54-dependent mechanisms. The promoter upstream matches the sigma 54 consensus sequence. When sigma 54 is deleted the \textit{bss} genes are no longer functional. The proven sigma 54 signaling system, \textit{bamVW}, is upregulated when \textit{G. daltonii} is
grown in the presence of aromatic compounds. These results suggests that sigma 54 is critical to
*G. daltonii* degrading aromatic compounds. Without sigma 54, anaerobic oxidation of aromatic
compounds in *G. daltonii* would be hindered and it could be entirely lethal to the organisms as
has been observed in other *Geobacter* species (84).
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