

BURKHOLDERIA FUNGORUM STRAIN RIFLE: GROWTH ON AND
INTERACTION WITH URANIUM

by

NICOLE MICHELE KORIBANICS

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ABSTRACT OF THE DISSERTATION

Burkholderia fungorum strain Rifle: Growth On and Interactions with Uranium

By NICOLE MICHELE KORIBANICS

Dissertation Director:

Professor Lee Kerkhof

A novel strain of *Burkholderia fungorum* was isolated from the Department of Energy's Rifle Integrated Field-Scale Subsurface Research Challenge Site in Rifle Colorado which can utilize uranium as a terminal electron acceptor under anaerobic conditions. The Rifle strain demonstrated growth with uranyl acetate as the terminal electron acceptor (1-10 μM) in a dose-dependent manner concomitant with decreases in soluble uranium. Each 1 μM increase of uranium initiated an increase of $\sim 0.5 \times 10^6 \pm 1.3 \times 10^5$ cells/ml, concurrent with a shift in uranium solubility from 90% soluble to 70-97% insoluble at the end of the time course. Cultures exposed to 5-10 μM uranium did not grow, but exhibited significantly decreased levels of soluble uranium at time point zero (78-18%), indicating either an additional cellular precipitation/detoxification mechanism in response to increased uranium concentrations or a media solubility threshold.

To identify the capability of *Burkholderia fungorum* strain Rifle to precipitate elevated levels uranium, cultures were grown aerobically in the absence and presence of

uranium (0-200 μ M). Cultures grown in the presence of uranium exhibited variable lag phases, indicating a toxic response to uranium. However, the fraction of uranium which remained within the soluble fraction exhibited no difference over the course of growth, excluding bio-precipitation as mechanism of toxicity resistance under aerobic conditions.

To identify possible genes of interest in regards to the anaerobic reduction of uranium or the overall toxicity resistance mechanism, the genome of *Burkholderia fungorum* strain Rifle was sequenced. The draft genome was assembled and annotated for prospective gene functions. A subset of seven genes sharing homology with genes of known uranium reducing species was earmarked for redox reaction capabilities. Additionally, genes associated with heavy metal toxic response were tested for up-regulation by transcription analysis using cultures shifted from uranium (-) to uranium (+) conditions. Reverse Transcriptase-PCR analysis demonstrated no variation in transcriptional level from day 0 of uranium transition to day 3 compared to the expression levels of the maleate isomerase indicating that while all select genes chosen were constitutively expressed.

The nucleotide sequencing reads for these strains were also submitted for functional annotation of sequencing reads via mifaser to generate an enzymatic enrichment profile for these strains. This profile revealed that the Rifle strain exhibited enrichment for c-class cytochrome and P-type ATPase functions. An in silico proteome was also generated to identify genes of functionally similar phenotype, including a number which belong to a different genus, using Fusion phenotypic classification. Comparisons for proteins of similar function identified 42 proteins shared across genus, most with clearly defined functions but including 4 hypothetical proteins of interest.

However, no gene was identified which was up-regulated by exposure to uranium.

Further study of the remaining hypothetical proteins is warranted to identifying the genes involved with uranium respiration/detoxification in *B. fungorum* Rifle.

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Chapter 1

Introduction

The remediation of environmental sites contaminated with uranium from industrial operations is a costly problem of global interest. Uranium is a naturally occurring radionuclide that can exist as several isotopes, three of which are stably found within nature (IUPAC 1998). Approximately 99% of naturally occurring uranium is isotope U-238, which is the most stable of the isotopes, and has a low radioactivity, while a much smaller fraction is the fissile U-235 isotope (NRC 1983). During the process of harvesting uranium for the US nuclear arsenal, mine and mill tailings were deposited in unlined retention ponds and contributed to contamination of ground and drinking water (Riley et al. 1992; Abdelouas et al. 1999; Akob et al. 2008). Naturally occurring uranium in general does not pose a significant threat of radiotoxicity in drinking water, and the depleted uranium byproduct upon U-235 harvesting at mines is approximately 30-40% less radioactive than naturally occurring uranium (NRC 1983; Craft et al. 2004, Mathews et al. 2009; Bleise et al. 2003). However, uranium is also classified as a heavy metal and chemical toxicity is of greater concern (Markich 2002; Burkart 1988; Burkart 1991). The chemical speciation and complexation of the uranium plays a significant role in its mobility and toxic reactivity, and so the focus for the past few decades has been in understanding the natural attenuation of uranium using indigenous microorganisms to alleviate the problem of toxic metal pollution caused by industrial processes (Francis & Dodge 2008; Kovacova & Sturdik 2002). A more in-depth understanding of the mechanisms employed during microbial transformation of uranium is critical for the development of comprehensive and effective remediation strategies.

There are two features that determine the mobility of uranium in the environment, chemical speciation and redox state. The naturally occurring speciation of uranium compounds in the environment are generally oxides which are solid, and tend towards stability and insolubility over a wide range of environmental conditions, but other compounds including those utilized during uranium enrichment and processing at nuclear mining sites are much more mutable (Bleise et al., 2003). Under oxidizing conditions the much more mobile U (VI) redox state is common, which has an affinity for clay minerals, iron oxides, and organics (Davis et al. 2004; Andersson et al. 2001; Koch-Steindl & Prohl 2001). While this sorption would normally keep the oxidized uranium ions immobile through association with sediment components, U(VI) remains soluble in the presence of carbonate or organic ligands like humic acids, when there are competing cations such as calcium, and at pH levels > 6.5 (Choppin 1999; Siegal 2003; Fox et al. 2013; Singer et al. 2009a/b/c; Singer et al. 2012a/b; Ginder-Vogel et al. 2006; Ulrich et al. 2011).

As part of the U.S. Department of Energy's Subsurface Biogeochemical Research Program, three Integrated Field Research Challenge Projects were established with the purpose of providing research teams with environmental samples and field scale experimental investigations into the mobility of environmental contaminants in the subsurface. One of the sites at a uranium mine tailings site in Rifle Colorado showed slow natural immobilization of uranium radionuclide contamination. Work at this site was specifically focused on the processes controlling the mobility of uranium in the subsurface, with special regards to the microbial, geochemical, and hydrological aspects (DOE 2013). The chemical speciation that evolved from the leachate of this mine

included many soluble U (VI) compounds, including high concentrations of aqueous Ca-uranyl-carbonate complexes (Grenthe et al. 1992; Singer et al. 2009; Brooks et al. 2003; Fox et al. 2013). The uranium speciation at the site was therefore shown to likely limit abiotic immobilization of hexavalent uranium during bioremediation efforts at the field site. It is through isolation of a novel strain of bacteria from the sediment of this site that all the following scientific work was made possible.

BIOTIC REDUCTION OF URANIUM

Mobility of uranium in the subsurface is controlled by the redox state, wherein soluble U(VI) transitions to insoluble U(IV) (Wall & Krumholz 2006). Reduction renders the ions into a less bioactive and therefore less toxic form (Glasauer et al. 2001; Valls et al. 2005). Initially, uranium reduction was believed to be entirely abiotic in nature, and was attributed to surface interactions with Fe(II) within redoximorphic soils under reducing conditions which are now studied in conjunction with biotic reduction (Behrends & Van Cappellen 2005; Latta et al. 2012). This concept changed when biotic reduction of uranium was attributed to an iron reducing bacterium *Geobacter metallireducens* (Lovley, 1991). Biotic reduction of uranium via microbial metabolic pathways appears to occur at a rate greater than abiotic uranium immobilization, making bioreduction of toxic uranyl radionuclides solubilized in groundwater an attractive bioremediation prospect (Gorby & Lovley 1992; Lovley et al. 1991). Following the initial discovery of the capability of bacterial species to biotically reduce uranium, many diverse species have been shown to possess the capacity (Table 1.1).

The reduction of a toxic metals is a common trait amongst bacteria exposed to high metal concentrations, resulting in decreased mobility and solubility or less volatile and reactive ions (Lovley 1993). Metal reduction has been found in sulfate-reducing bacteria, both as a secondary byproduct of growth and direct enzymatic metal reduction (Fude et al. 1994; Labrenz et al. 2000; De Luca et al. 2001). Furthermore, some dissimilatory metal reducing bacteria can utilize the energy generated during the reduction of heavy metals to sustain growth (Nealson & Saffarini 1994). Amongst the bacterial strains exhibiting uranium reduction capabilities, *Shewanella oneidensis*, *Desulfotomaculum reducens*, and *Carboxydotherrmus ferrireducens* are capable of generating energy sufficient to sustain growth (Blakeney et al. 2000; Khijniak et al. 2005; Tebo & Obraztsova 1998-for review see Wall & Krumholz, 2006). These species belong to various classes of the phyla Proteobacteria and Firmicutes.

Geobacter metallireducens GS 15 and *Shewanella putrefaciens* were reported to be capable of utilizing uranium as a terminal electron acceptor in early studies (Lovley et al. 1991). In the years to follow, numerous other *Geobacter* strains and species have been discovered to reduce uranium (see Table 1.1). However successful reduction is limited to anaerobic environments with organisms utilizing respiratory reduction, or the reduction being a secondary byproduct of another anaerobic process. Once reduced, the uranium exists primarily as uraninite (UO_2) which is mostly stable under anoxic conditions, but chemical U (IV) phases can vary depending on the chemical constituents of the environment (Bargar et al. 2013). However, exposure to an oxic environment could easily lead to re-oxidation of the uranium. Thus reductive precipitation may be a

temporary solution if the redox conditions are not sustained, and is only reliable under anaerobic conditions.

In terms of energetics, the reduction of U (VI) to U (IV) under neutral pH generates a similar energy yield to the reduction of iron, yet growth with uranium as a primary electron acceptor yields a lower biomass than predicted based on yield calculations (Sanford et al. 2007). Numerous works have shown that biomass yield as a function of ATP generated is not constant, as ATP is also utilized in maintenance functions not directly related to growth, such as protection of cellular components from toxic by-products and environmental contaminants (for review see Russell & Cook 1995). In essence this indicates the possibility that there might be some universal components to the mechanisms that reduce uranium, which cannot generate sufficient energy to satisfy the growth requirements for some organisms under certain conditions. Additionally, the fact that low level uranium reduction has been reported in so many varied species implies a wider distribution of reductive capability than is currently reported (for review see Wall & Krumholz 2006). The use of techniques, such as stable isotope probing, can identify specific community members responding to an environmental stimulus, while other studies done at Rifle, CO identified additional microorganisms which were actively synthesizing ribosomes and incorporating acetate in response to uranium amendment (Chang et al. 2005; Kerkhof et al. 2011; McGuinness et al. 2015). These findings suggest that there are many prokaryotic species capable of uranium reduction not yet characterized, suggesting enrichment cultures from contaminated sites may yield additional novel strains of interest.

OTHER MECHANISMS OF TOXICITY RESISTANCE

According to the Center for Disease Control's Agency for Toxic Substances and Disease Registry (ATSDR) heavy metals are chemically toxic substances. A number of elements such as copper, nickel, zinc, uranium, etc. are classified as heavy metals. Many of these elements are necessary micronutrients for biological systems. However, at higher levels they exhibit similar chemotoxic effects as other non-essential heavy metals. Uranium is not an element necessary for life functions at all, and all ions regardless of radioactivity exhibit identical chemotoxic effects (ATSDR, 2013). Cells are exposed to oxidative stress when uranium species are introduced, resulting in damage to membranes, proteins, and nucleotide components (Pourahmad et al. 2006; Miller et al. 2002).

Bacterial species employ a number of mechanisms in responding to other toxic heavy metals, some of which are unsuitable for universal bioremediation purposes as they do not result in immobilization of the ions. An example of this documented for other heavy metals includes the internal alteration of cellular components to resist the toxic interactions, such as permanent mutations or post-translational modifications that remove sensitive residues, or a general up-regulation of gene expression to compensate for damaged components (Rouch et al. 1995). Another commonly employed mechanism which can occur without permanently modifying the ions is efflux systems, which remove the toxic ions from the cell internal space (Nies & Silver 1995; Bruins et al. 2000; Apell 2003). Likewise, if the mechanism employed is an impermeability barrier then the internal cellular components are never exposed to uranium, and avoid the toxic

effect without truly interacting with the ions. These mechanisms protect the individual cell from damage but do not benefit the environment.

Alternative mechanisms for resisting the toxic effects of heavy metal ions affect overall mobility. While rare in bacteria, metal-chelating proteins known as metallothioneins have been studied in cyanobacteria, which can remove metals from solution when secreted into the environment (Robinson et al. 2001). More common detoxification mechanisms include chemical transformation and sequestration or transport (Silver et al. 1989; Silver 1996). External sequestration can include binding to cell surface proteins or extracellular polysaccharides, oftentimes accompanied by a chemical transformation. Chemical transformations which result in the immobilization of uranium are examples of biomineralization, wherein radionuclides are complexed with microbially produced sulfide, phosphate, or reduced iron (Labrenz et al. 2000; Macaskie et al. 1992; Jeong & Macaskie 1999; Banfield et al. 2000). Under anaerobic conditions sorption and accumulation sometimes accompany the reduction process. The reduction of uranium by *Geobacter* species has been coupled with the extracellular surface complexation of uranium ions to the cell pili, leading to a biotic sequestration through sorption (Cologgi et al. 2011). Internal sequestration can likewise be mediated by binding to membrane bound proteins or polysaccharides or can involve localizing the ions to the periplasm where interactions with cellular components can be minimized. This has also been recognized in both *Geobacter* and *Shewanella* species, where uraninite deposits have been measured both outside the cell and accumulated in the periplasm of the cells concurrent with uranium reduction linked to c-type cytochrome activity (Marshall et al. 2006; Shelobolina et al. 2007).

While reductive processes are limited to anaerobic conditions, redox independent mechanisms are critical for remediation under aerobic conditions. *Citrobacter* species have been characterized for their ability to generate an exocellular lipopolysaccharide associated with a phosphatase that is responsible for the biomineralization and accumulation of uranium (Macaskie et al. 1992; Macaskie et al. 1994; Macaskie et al. 2000). Diverse isolates from uranium contaminated sites, such as various *Bacillus* species, having been characterized as uranium accumulators as well (Selenska-Pobell et al. 1999; Panak et al. 2002). A *Rahnella* sp. strain Y9602 and a *Bacillus* sp. strain Y9-2, which were isolated from the IFRC Oak Ridge site, were shown to express non-specific phosphatases which resulted in precipitation of uranium via a surplus of reactive phosphate (Martinez et al. 2007). Species like *Acidithiobacillus ferrooxidans* have been identified for their complexation capabilities (Merroun et al. 2003a/b) while others such as *Pseudomonas aeruginosa* are also implicated in biomineralization, wherein chemical complexation causes uranium to become insoluble (Choudhary & Sar 2011). These transformations are not limited by the redox state of the environment, but like many reduction processes have been linked to enzymatic functions.

ENZYMATIC BASIS FOR URANIUM INTERACTIONS

The cellular capabilities of microorganisms to interact with their environment are encoded by their genome and expressed primarily as proteins or the byproducts of enzymatic function (Corbin & Rannels 1980; Juodka 1980). It has long been understood that bacteria are useful for bioremediation purposes. Bioremediation is generally defined as the use of biological systems to alleviate hazardous levels of contamination via

transformation, destruction, or reduction by a microorganism, and is highly preferred over alternative methods as it results in a more complete detoxification in situ without destruction of the environment or the extensive costs associated with physical or chemical clean ups (Timmis & Pieper 1999; Barton et al. 1996). While studies have been performed to successfully identify the components of mechanisms for other heavy metals such as mercury, lead, zinc, copper, silver and chromium (Ji & Silver 1995) the exact genes responsible for the mechanisms behind cellular interactions with uranium remain poorly understood.

While universal mechanisms for uranium-microbe interactions have not been identified, some enzymes have been implicated. For the enzymatic reduction of uranium, c-type cytochromes have been reported to function in reductive process with some strains (Lovley 1993; Lovley et al. 1993 a/b; Lloyd et al. 2003; Marshall et al. 2006, Williams et al. 2013). However, variations in electron acceptor and reductive capabilities suggest multiple mechanisms may be involved. Knock-out work with outer membrane c-type cytochromes in *Geobacter sulfurreducens* indicates that a range of these cytochromes can contribute to uranium reduction, as the uranium reduction capacity of the strain did not significantly decrease until five of the most common c-type cytochrome genes were deleted (Orellana et al. 2013). Phosphatase enzyme activity has been linked to biomineralization and bioaccumulation processes for uranium (Macaskie et al. 1992; Beazley et al. 2007; Lloyd & Macaskie 2000), and phosphatase activity is key in active transport using efflux mechanisms for toxicity resistance in regards to other heavy metals (Gillan 2016). The identification of similar functional proteins of interest in novel

microbial strains would be beneficial to future studies into the mechanisms by which microbe-uranium interactions are catalyzed.

The traditional *in vivo* biochemical and physiological studies employed for the elucidation of proteins can be cost prohibitive, and in some cases even hundreds of experiments may only unveil between 5 and 20% of the organism's functional potential (Garrity 2001). The utilization of computational methods to identify genes of interest for a particular mechanism is therefore intuitive. Traditional classification of prokaryotic species relies on phylogenetic relatedness between nucleotide sequences, but while this can track evolutionary relatedness it does not necessarily correspond with phenotypic expression as the functional capabilities of a protein are dependent on amino acid sequence, which can on occasion suffer from significant phenotypic variation due to a relatively low degree of nucleotide sequence variation. When two organisms share a functional ability, it stands to reason that they must also share a genomic feature which expresses in such a fashion as to bring that capability into being. As an organism's functional capabilities is defined by its proteome complement, the development of phenetic approaches to microorganism classification, utilizing functional similarities rather than phylogenetic relatedness, illuminates new approaches to identifying genes/proteins of interest (Sokal 1986). With this concept in mind, evaluation of enzymatic features which are functionally significant may be more accurately assessed via sequence comparisons of amino acids rather than nucleotides. To streamline the identification of genes likely to be involved in a process(es) of interest, proteome analysis and comparison provides more meaningful targets of interest than nucleic acid homology. An overlap comparison of the genomic features of a number of strains known to perform

similar functions would allow for the identification of proteins of interest, both amongst those already annotated and those with only hypothetical functions, thus providing the targets for a focused study on the mechanisms involved.

OBJECTIVES

The purpose of this dissertation was to identify a bacterial strain capable of reducing uranium, and to characterize its capabilities in regards to uranium reduction. Sediment samples from a uranium contaminated site were used in a series of experiments in which the specific research objectives were to:

1. Isolate a bacterial strain capable of growth utilizing uranium as a terminal electron acceptor under anaerobic conditions.
2. Determine if uranium reduction was dependent on anaerobic conditions or if strain capabilities included the aerobic bio-precipitation of uranium.
3. Map the genome of the identified strain to illuminate the gene content.
4. Identify genomic features key to the strain ability to reduce uranium and resist the toxic effects.

Through the completion of this thesis study a novel microorganism capable of generating energy for growth from the reduction of uranium was isolated and characterized. The application of computational methods for mining genome data has great potential for the identification of gene(s) involved in uranium respiration and other interactions, but requires some refinement to be fully effective, with the primary goal of understanding and fine tuning bioremediation efforts.

Table 1.1. Bacterium capable of reducing uranium.

Highlighted cells contain strains capable of generating energy sufficient for growth.

Organism	Citation
<i>Anaeromyxobacter dehalogenans</i> strain 2CP-C	Sanford et al. 2007
<i>Bacillus</i> sp. dwc-2	Li et al. 2017
<i>Carboxydotherrnus ferrireducens</i> *	Khijniak et al. 2005; Slobodkin et al. 2006
<i>Cellulomonas flaigena</i> ATCC 482a, sp. WS01, WS18, ES5, ES6	Sani et al. 2002; Sivaswamy et al. 2011
<i>Clostridium sphenoides</i> ATCC 19403, <i>Clostridium acetobutylicum</i> DSM 792	Francis et al. 2004; Bernier-Latmani et al. 2010
<i>Cupriavidus metallidurans</i> CH34	Llorens et al. 2012
<i>Deinococcus radiodurans</i> R1	Fredrickson et al. 2000
<i>Desulfomicrobium norvegicum</i> DSM 1741	Lovley et al. 1993
<i>Desulfosporosinus orientis</i> DSM 765, sp. P3 S	Suzuki et al. 2004
<i>Desulfotomaculum reducens</i> MI-1	Tebo and Obraztsova 1998, Bernier-Latmani et al. 2010
<i>Rhodopseudomonas palustris</i>	Llorens et al. 2012
<i>Desulfovibrio alaskensis</i> strain G20, <i>Desulfovibrio baarsii</i> DSM 2075, <i>Desulfovibrio desulfuricans</i> ATCC 29577, <i>Desulfovibrio sulfodismutans</i> DSM 3696, <i>Desulfovibrio vulgaris</i> Hildenborough ATCC 29579, <i>Desulfovibrio</i> sp. UFZ B 490	Payne et al. 2002; Lovley 1993; Lovley & Phillips 1992b; Pietzsch & Babel 2003; Pietzsch et al. 1999
<i>Geobacter metallireducens</i> GS-15, <i>Geobacter sulfurreducens</i> , <i>Geobacter bemedjiensis</i> , <i>Geobacter lovleyii</i> , <i>Geobacter uraniireducens</i> Rf4	Lovley et al. 1991; Jeon et al. 2004; Cologgi et al. 2014; Anderson et al. 2003
<i>Pseudomonas putida</i> , <i>Pseudomonas</i> sp. CRB5	Barton et al. 1996; McLean & Beveridge 2001
<i>Pyrobaculum islandicum</i>	Kashefi & Lovley 2000
<i>Saccharomyces cerevisiae</i>	Wang et al. 2017
<i>Salmonella subterranea</i> sp. nov. strain FRC1	Shelobolina et al. 2004
<i>Shewanella alga</i> BrY 14, <i>Shewanella oneidensis</i> MR-1, <i>Shewanella putrefaciens</i> strain 200, sp. RCR17	Caccavo et al. 1992; Truex et al. 1997; Lovley et al. 1991; Blakeney et al. 2000; Bernier-Latmani et al. 2010; Abdelvand et al. 2017
<i>Thermoanaerobacter</i> sp.	Roh et al. 2002
<i>Thermus scotoductus</i>	Kieft et al. 1999
<i>Veillonella alcalescens</i>	Woolfolk & Whiteley 1962

CHAPTER II

Spatial Distribution of an Uranium-Respiring Betaproteobacterium at the Rifle, CO Field Research Site¹

Abstract:

The Department of Energy's Integrated Field-Scale Subsurface Research Challenge Site (IFRC) at Rifle, Colorado was created to address the gaps in knowledge on the mechanisms and rates of U (VI) bioreduction in alluvial sediments. Previous studies at the Rifle IFRC have linked microbial processes to uranium immobilization during acetate amendment. Several key bacteria believed to be involved in radionuclide containment have been described; however, most of the evidence implicating uranium reduction with specific microbiota has been indirect. Here, we report on the cultivation of a microorganism from the Rifle IFRC that reduces uranium and appears to utilize it as a terminal electron acceptor for respiration with acetate as electron donor. Furthermore, this bacterium constitutes a significant proportion of the subsurface sediment community prior to biostimulation based on TRFLP profiling of 16S rRNA genes. 16S rRNA gene sequence analysis indicates that the microorganism is a betaproteobacterium with a high similarity to *Burkholderia fungorum*. This is, to our knowledge, the first report of a betaproteobacterium capable of uranium respiration. Our results indicate that this microorganism occurs commonly in alluvial sediments located between 3-6 m below ground surface at Rifle and may play a role in the initial reduction of uranium at the site.

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Introduction

The leaching of ore minerals from mining and mill sites has long been a serious problem, resulting in acid mine drainage and polluted run-off to surface and ground waters. One example of ore-processing contamination is subsurface uranium plumes that infiltrate groundwater (Williams et al. 2011; Newsome et al. 2014). Natural attenuation of soluble, oxidized uranium can occur when hexavalent uranium is reduced and precipitated, but the process can be slow due to conditions where suitable endogenous electron donors are limiting. Therefore, recent research efforts have turned to field amendments of electron donors, such as acetate or ethanol, to stimulate microbial uranium (VI) reduction and bring the soluble concentration below safe drinking water standards in a timely manner (Senko et al. 2002; Anderson et al. 2003; Istok et al. 2004; North et al. 2004; Peacock et al. 2004; Long et al. 2015). A principal study site for uranium bioremediation research is the Integrated Field-Scale Subsurface Research Challenge Site (IFRC) in Rifle, Colorado (USA), which is situated near a historic vanadium/uranium mill that operated from the 1920's to the 1960's (Fig 2.1). Extensive research into the in situ bioreduction of uranium has been conducted at the Rifle IFRC (Williams et al. 2011; Anderson et al. 2003; Holmes et al. 2002; Chang et al. 2005; Vrionis et al. 2005; Wilkins et al. 2009; Kerkhof et al. 2011; Bargar et al. 2013), with field experiments repeatedly demonstrating that groundwater uranium concentrations could be decreased below the U.S. Environmental Protection Agency's (EPA) drinking water standard of 0.126 μM by adding acetate to the subsurface (Williams et al. 2011; Senko et al. 2002; Finneran et al. 2002). The initial decrease in aqueous uranium concentrations occurred concurrently with iron reduction, suggesting that an iron

reducing bacterium may be involved in uranium reduction. Substantial shifts in the bacterial community have been documented at the Rifle site following acetate amendment, notably increases in *Geobacter*-like species (Anderson et al. 2003; Holmes et al. 2002; Vrionis et al. 2005; Wilkins et al. 2009). In addition, *Geobacter uraniireducens* and *G. sulfurreducens* have been isolated from the site or its near vicinity and have been shown to be capable of U(VI) reduction, although these bacteria do not utilize uranium as a terminal electron acceptor (Shelobolina et al. 2007; Shelobolina et al. 2008). In contrast, laboratory experiments (including those using Rifle sediments) demonstrated uranium reduction associated with sulfate-reducing bacteria (Spear et al. 2000; Moon et al. 2010). Likewise, stable isotope probing methods (SIP) have identified a variety of bacteria in the Rifle subsurface which utilize the acetate amended to the groundwater (Williams et al. 2011; Chang et al. 2005; Kerkhof et al. 2011), but these active bacteria have not been directly linked to uranium reduction. Unfortunately, none of these studies have conclusively demonstrated growth by a bacterium present at the Rifle site using U(VI) as a terminal electron acceptor or that a particular bacterium capable of uranium reduction is widespread and abundant at the site prior to acetate amendment.

This study was initiated to determine if a microorganism capable of growth on uranium could be isolated from Rifle samples. Using uranyl acetate as an electron acceptor at low concentrations ($<10\ \mu\text{M}$) and acetate as an electron donor, a bacterium, designated strain Rifle, was isolated that could grow on U(VI). The growth of the culture was associated with the reduction of uranium, as evidenced by a partitioning from the soluble into the particulate phase. 16S ribosomal RNA gene analysis indicated the isolated strain was closely related to *Burkholderia fungorum* (Coenye et al. 2001). The

spatial distribution of *Burkholderia fungorum* strain Rifle at the field site was assessed by TRFLP profiling of sediments collected during well installation by employing a diagnostic 4-bp cutter (*MnII*) and a more definitive 6-bp cutter (*EagI*). The different terminal restriction fragments (TRF) associated with this strain were detected in subsurface sediments at the site prior to acetate amendment. The highest relative contribution to the overall profiles occurred at 3–5 meters depth. Since this microorganism is widely distributed at Rifle and capable of growth using uranium, it may be responsible for a portion of the U(VI) reduction observed during biostimulation with acetate. In addition, understanding the role this microorganism plays in the process of natural immobilization of uranium within the Rifle aquifer can help to elucidate processes inhibiting natural flushing of the aquifer and contributing to the problem of uranium plume persistence at this site and others like it (Campbell et al. 2011; Alessi et al. 2014; Qafoku et al. 2014)

Methods

Field site

The Rifle IFRC experimental Plot C consists of a gallery of injection (4 cm diameter) /monitoring (10 cm diameter) wells installed in 2007 (Fig 2.1). Fifty cm thick core sections were collected at 3–6 m below ground surface, bagged, sparged with nitrogen in the field, and transferred to an anaerobic glove bag. Subsamples of sediment (10 g) from the various monitoring wells (D01-D12) were collected in the glove bag and immediately frozen at -80°C in the field prior to transport to the laboratory and processing. A detailed description of the site is found in Williams et al., 2010.

Isolation of a bacterium capable of growth on uranium

Frozen sediment (1 g) from well D01 was thawed and spiked with 200 μ M sodium acetate and 2 μ M uranyl(VI) acetate as the sole electron acceptor in a modified minimal carbonate salts media under anaerobic conditions (Knight et al. 2002; Fennell et al. 2004). Modifications included decreasing the NaCl concentration to 2.5 g/L, decreasing the bicarbonate to 1 g/L, and reducing the vitamin and trace salts concentrations by a factor of two. Enrichment cultures were incubated at room temperature for 14 days before transfer. Once slight turbidity was observed in the enrichment, an aliquot was diluted 1:10 with fresh media containing 200 μ M sodium acetate and 2 μ M uranyl acetate. With each transfer of the enrichment, 1 ml of liquid culture was filtered and extracted for TRFLP analysis (McGuinness et al. 2006). The isolation transfers (7x) were repeated until the enrichment culture consisted of a single 16S rRNA gene TRFLP peak.

Testing of aerobic growth and identification of the isolated strain

The enrichment was screened for the potential of aerobic growth on Tryptic Soy Agar medium. After streaking onto plates, a large number of colonies with a uniform morphology were obtained. Following 5 rounds of colony purification, an isolate designated strain Rifle was screened by TRFLP again to ensure purity. The 16S rRNA gene from the strain was amplified via PCR using the primers 27F (5'-AGA GTT TGA TCC TGG CTC AG3') and 1525R (5'-AAG GAG GTG WTC CAR CC-3'), following protocols described previously (Sakano & Kerkhof 1998). The amplicon was sequenced

and analyzed via BLAST to detect closely related sequences (99% similarity: 1493/1503 bp identity-to *B. fungorum* strain KN-08). The isolate's 16S rRNA gene (Genbank accession number-KP212894) was used to re-construct a phylogenetic tree with 1366 bp of unambiguously aligned sequence of strain Rifle and 24 closely matching taxa using Geneious software (Guindon & Gascuel 2003).

Verification of growth on uranium as an electron acceptor

In order to demonstrate that the isolate was capable of growth on acetate using U(VI) as a terminal electron acceptor, a dosage experiment was established to track changes in cell number associated with increasing concentrations of uranyl acetate. We began by inoculating strain Rifle grown on acetate and uranium (<5 ml) into 1-liter anaerobic minimal media with 200 μ M sodium acetate. A schematic of the transfer apparatus that allowed for the anaerobic transfer of aliquots to replicate microcosms is shown in Fig. 2.2 This design allowed for the continuous sparging of all microcosms during the transfer procedure to minimize oxygen contamination. Initially, subsamples (9 ml) were anaerobically transferred under a headspace of N₂ /CO₂ [70:30] to triplicate 10 ml bottles. After these no-uranium amendment control cultures were established, the culture was supplemented with 1 μ M uranyl acetate and triplicate 9 ml cultures were established for the time course incubations (i.e. 0, 11, and 24 days; n = 9). The culture was then amended with an additional 1 μ M uranyl acetate, bringing the concentration to 2 μ M and dispensed as above. Further amendments of 3 μ M, 5 μ M, and 10 μ M uranyl acetate were done to complete the experimental treatments. All cultures were incubated under anaerobic conditions (T0, T11, T24 days). Cell numbers were determined by

collecting 1 ml of culture from each replicate microcosm at a given uranium amendment and time point ($n = 3$), preserving with 40 μL of 25% glutaraldehyde, staining with 1% SYBR gold for 15 min, and collected by filtration onto 0.22 μm GE polycarbonate black filters (GE Water & Process Technologies, Trevose, PA USA). Cells were enumerated in 10–15 microscopic fields via fluorescent microscopy using a BH2-RFCA microscope (Olympus, Japan).

Measurement of uranium by mass spectroscopy

For assessment of uranium concentration in the soluble and particulate fractions within the replicate microcosms, 2 ml of culture at each time point were filtered through 0.025 μm filters (Millipore Corp, Billerica, MA, USA) in an anaerobic chamber (Coy Laboratory Products, Grass Lakes, MI, USA). This soluble fraction (filtrate) was acidified by addition of 0.2 ml nitric acid (70%) and stored in glass vials until analysis. The particulate fraction (filter and the uranium adhering to the walls of the culture vials) were collected by dissolving the filter in 3 ml nitric acid and heating 250°C for 3 hours, or by rinsing the empty culture vials with 3 ml of nitric acid and vortexing for 2 minutes. The uranium concentration was determined by iCAPQ ICP-MS (Thermo Fisher Scientific Inc., Waltham, MA, USA) using Indium as an internal standard. Three iterations per sample were analyzed and RSD percentages averaged 3.6%. A commercial standard (High Purity Standards, Charleston, SC, USA) was used to generate a standard curve for uranium mass based on the instrument signal (counts per second; $r^2 = 0.9955$).

Mapping of the Rifle strain at the study site

Bacterial community composition at the Plot C study site was assessed by 16S rRNA gene TRFLP analysis of DNA extracted from sediment samples collected in 2007. Triplicate nucleic acid extracts were generated from the subsampled sediment (0.25 g) from each core location (n = 12) at 3-6m below ground surface using a high EDTA/phenol/chloroform purification procedure (Sakano & Kerkhof 1998; Corredor et al. 2004). Amplification of 16S rRNA genes employed the bacterial forward primer 27F (6'-FAM-5'-AGA GTT TGA TCC TGG CTC AG-3') and a universal reverse primer 1100R (5'-GGG TTG CGC TCG TTG-3'). Cycling parameters were 30 cycles of 94°C for 1 min, 55°C for 0.5 min, and 72°C for 1:10 min, followed by a final extension at 72°C for 10 min. Twenty ng of PCR product were digested for 6 hours with *MnII* endonuclease and analyzed on an ABI 310 genetic analyzer to visualize the overall bacterial community profile of the sample, and to identify the presence/abundance of the Rifle strain (McGuinness et al. 2006). Verification that samples positive for the strain Rifle *MnII*-peak (166 bp) represented the *Burkholderia fungorum* strain Rifle isolate was done by digesting a subset of the amplicons using the endonuclease EagI (6-bp cutter) that yields a 215 bp terminal restriction fragment diagnostic of the strain.

Results

Isolation of a bacterium capable of growth on uranium

The Rifle IFRC experimental Plot C consists of a gallery of injection (4 cm diameter) /monitoring (10 cm diameter) wells installed in 2007 (Fig 2.1). Sediment

samples from the well gallery were used to establish enrichment cultures on U(VI) minimal media. Colony purification on tryptic soy agar in air yielded a facultative anaerobic isolate with a TRF of 166 bp using *MnII*. To verify whether this microorganism could grow on U(VI) as a terminal electron acceptor under anaerobic conditions, a dose-dependent experiment was established using increasing uranyl acetate concentrations (1–10 μM) with a uniform amount of acetate as electron donor (Fig 2.2). The initial inoculum contained $0.45 \pm 0.08 \times 10^6$ cells/ml. After 24 days in the uranium amendments, the control (0 μM , i.e. no uranium amendment) displayed a slight increase in cell numbers to $0.6 \pm .14 \times 10^6$ cells/ml (Fig 2.3). Each 1 μM uranium increment up to 3 μM yielded an increase of $0.5 \pm .13 \times 10^6$ cells/ml. For the 5 and 10 μM amendments, lower cell numbers were observed, suggesting toxicity at the higher uranium dose.

Biomass increase linked with insolubilization of uranium

Verification that the increase in cell number of the pure culture was linked to the reduction of uranium was done by quantifying the fraction of uranium in both the soluble and insoluble pools. Uranium in the cultures spiked to 3 μM shifted from ~90% soluble at the T0 time point for all 3 concentrations to 70–97% insoluble by the end of the 24 day incubations (Fig 2.4). A mass balance indicated that 93–102% of the added uranium could be accounted for in the soluble/insoluble pools. Interestingly, the bacterium also reduced uranium as efficiently at 5 and 10 μM concentration (up to 97%-Fig 2.5). However, the percent of soluble uranium at the T0 sampling time point was 78% for the 5 μM and 18% for the 10 μM treatments. Presumably, the bacteria were stimulated by the

prior exposure to uranium during transfer and began reducing the radionuclide before the T0 samples could be collected.

Verification of uranium reduction

To ensure that the U(VI) was reduced (rather than adhering to cells or abiotically precipitating with phosphates in the media) a separate growth experiment with 2 μ M uranyl acetate was conducted as described above. However, subsamples from this time course were filtered through 0.025 μ m filters in air and not in the anaerobic chamber. The results demonstrate the uranium is largely in the soluble fraction (>90%) when exposed to oxygen (Fig 2.6) and verify that the U(VI) is reduced to U(IV) during growth by strain Rifle.

Identification and mapping of the Rifle strain at the study site

To determine the identity of strain Rifle, 16S rRNA sequence analysis was performed. BLAST results indicated the Rifle isolate was closely related to members of the *Burkholderia* genus. A phylogenetic tree of strain Rifle and other bacterial taxa places the Rifle strain within the *Burkholderia* cluster, closest to *B. fungorum* (Fig 2.7). The spatial distribution of *Burkholderia fungorum* strain Rifle within the field gallery was determined by TRFLP analysis of 16S rRNA genes in sediments recovered during well installation. These sediments were not field amended with acetate and represent the microbial community prior to perturbation to stimulate uranium reduction through organic carbon injection. A 166 bp TRFLP peak in 16S rRNA gene profiles from the site using *MnII* was considered diagnostic of *Burkholderia fungorum* strain Rifle in the

various samples. The contribution of OTU 166 to the overall bacterial community is presented in Fig 2.8. The relative abundance of *Burkholderia fungorum* strain Rifle ranged from 0–15% in the community profiles within these sediments. The 166 peak was most abundant in the upper part of the soil column (3 and 4 m depth) and the signal generally diminished at lower depths (6 m). The highest contribution of the 166 bp peak to the overall community (>15%) was in well D03 at 3 m. The patchy nature of the *Burkholderia fungorum* strain Rifle distribution can be seen in many wells where the relative contribution to the microbial community could vary from <15% to <1% within 1 to 2 m. To test whether the 166 bp peak might represent more than one 16S rRNA gene, a second set of enzyme digests of the fluorescent amplicons was performed using a 6-bp cutter (EagI), yielding a 215bp TFLP peak for *B. fungorum* strain Rifle. This analysis produced the appropriate sized TRF for all samples analyzed and the EagI 215 bp peak area was 1–50% of the original *MnII* 166 peak area.

Discussion

The reduction of soluble U(VI) to less soluble U(IV) species is an essential step in the redox immobilization of this toxic radionuclide in contaminated groundwater.

Initially, uranium reduction was believed to be entirely abiotic in nature, with organic and inorganic compounds acting as reducing agents. This notion changed in the early 1990's when biotic uranium reduction was described for a dissimilatory Fe(III)-reducing bacterium (Lovley et al. 1991). Since then, numerous bacteria have been found which are capable of reducing uranium (Wall & Krumholz 2006). Of these bacteria, only *Anaeromyxobacter dehalogenans*; *Carboxydotherrnus ferrireducens*, *Desulfotomaculum*

reducens, *Geobacter metallireducens*, and *Shewanella putrefaciens* have been reported to grow using U(VI) as a terminal electron acceptor (Lovley et al. 1991; Wall & Krumholz 2006; Tebo & Obraztsova 1998; Blakeney et al. 2000; Khijniak et al. 2005; Sanford et al. 2007). More often, a resting cell assay at 200–10,000 μM is used to assess uranium reducing potential and over 30 bacteria have been described that reduce U(VI) to U(IV) but are not necessarily capable of growth on uranium (Wall & Krumholz 2006; Prakash et al. 2010). In addition, mixed cultures have demonstrated uranium reduction under co-metabolic conditions with terminal electron acceptors including sulfate (Boonchayaanant et al. 2008) or iron (Shelobolina et al. 2007; Lovley et al. 1991; Lovley et al. 1992; Brooks et al. 2003; Nevin et al. 2005).

Because so few of the known bacteria capable of growth on uranium have been detected at the Rifle IFRC site, much of the effort has focused on the role of *Geobacter*-like organisms during the course of acetate amendment and bioremediation. Yet, stable isotope probing experiments indicated that a variety of bacterial species are stimulated by the amendment of acetate at Rifle (Williams et al. 2011; Chang et al. 2005; Kerkhof et al. 2011). Interestingly, one of the major bacteria discovered at Rifle that incorporates ^{13}C -acetate also displayed a 166 bp peak (using *Mnl* I) in the active community profiles (Kerkhof et al. 2011). This earlier report identified the 166 bp peak as an alphaproteobacterium from clone libraries. Here, we suggest that the 166 bp peak in the unamended sediment could also be *Burkholderia fungorum* strain Rifle. This result is consistent with our digestion of the TRFLP amplicons using *Eag*I, suggesting that 1–50% of the *Mnl* I 166 bp peak area as some other microorganisms (such as the previously described alphaproteobacterium).

Furthermore, a next generation 16S rRNA gene analysis of samples taken from the Rifle site indicated that *Burkholderiales* comprised nearly 30% of the subsurface microbial community prior to field amendment (Miller et al. 2013). *Burkholderia*-like microorganisms have also been shown to be a substantial proportion of the microbial community in uranium reducing enrichments from the Oak Ridge, TN IFRC site (North et al. 2004; Moreels et al. 2008; Vishnivetskaya et al. 2010). As with Rifle, the beta-proteobacteria detected after enrichment at Oak Ridge were not thought to play a role in uranium reduction. However, the fact that *Burkholderia fungorum*. strain Rifle grows on U(VI) is not entirely surprising as this genus is well documented for its wide variety of metabolic capabilities. For example, *Burkholderia* species have been found to be capable of anaerobic growth on nitrate, including *B. fungorum* (Andreolli et al. 2011; Chaudhary et al. 2012), *B. xenovorans* (King 2006) and *B. pseudomallei* (Hamad et al. 2011). In addition, many members of the genus have been isolated based on the ability to degrade a broad range of carbon compounds (Mueller et al. 1997; Wang et al. 2001; Denef et al. 2004; Ali et al. 2012; Tanase et al. 2013; Kotik et al. 2013; Plangklang & Reungsang 2013). This metabolic flexibility by *Burkholderia* species has been attributed to the large differences in their multiple genomes, varying as much as 2 Mbp between strains (Konstantinidis & Tiedje 2004; Chain et al. 2006). Interestingly, a preliminary study of the terminal electron acceptor preferences by *B. fungorum* strain Rifle indicated only oxygen and uranium are respired on M9 media with dextrose as an electron donor (Fig 2.9). This study suggested *B. fungorum* strain Rifle does not grow on iron, in contrast to all other known bacteria capable of growth on uranium which are also capable of utilizing iron as a terminal electron acceptor. These results imply a genomic comparison of our

isolate with other related *Burkholderia* species that cannot grow on uranium should provide additional insight into the genes and mechanisms of biotic uranium reduction in this genus and may represent a novel respiratory pathway for radionuclide reduction.

Conclusion

In conclusion, *Burkholderia fungorum* strain Rifle is the first microorganism isolated specifically from the Rifle IFRC site that is capable of growth on U(VI), and exhibits an similar to growth yields on uranium as calculated and reported in Sanford et al. 2007. Previously, members of the *Burkholderiaceae* were not suspected to play a role in uranium reduction and immobilization. Given the long period of uranium contamination at the site (>60-years), even slow rates of natural uranium reduction by *Burkholderia fungorum* strain Rifle and similar microorganisms could be expected to immobilize a potentially significant pool of uranium within the aquifer and can lead to uranium plume persistence. Such immobilization has the potential to impede the groundwater compliance strategy for Rifle and similar mill tailings impacted sites, which rely on natural flushing of the aquifer by low uranium groundwater to remove residual contamination. This study suggests that the ability to respire uranium could be widespread among facultative anaerobes and testing to date is often performed under conditions that could be toxic to many microorganisms. Furthermore, our findings highlight how integrated research at DOE field sites can lead to the discovery of novel metabolic capabilities in different microorganisms and to new ideas for promoting biostimulation and uranium reduction at these contaminated sites.

Figure 2.2. Schematic of the sparging and distribution apparatus

Experimental apparatus for distributing a uniform inoculum for the various uranium additions while maintaining anaerobic conditions.

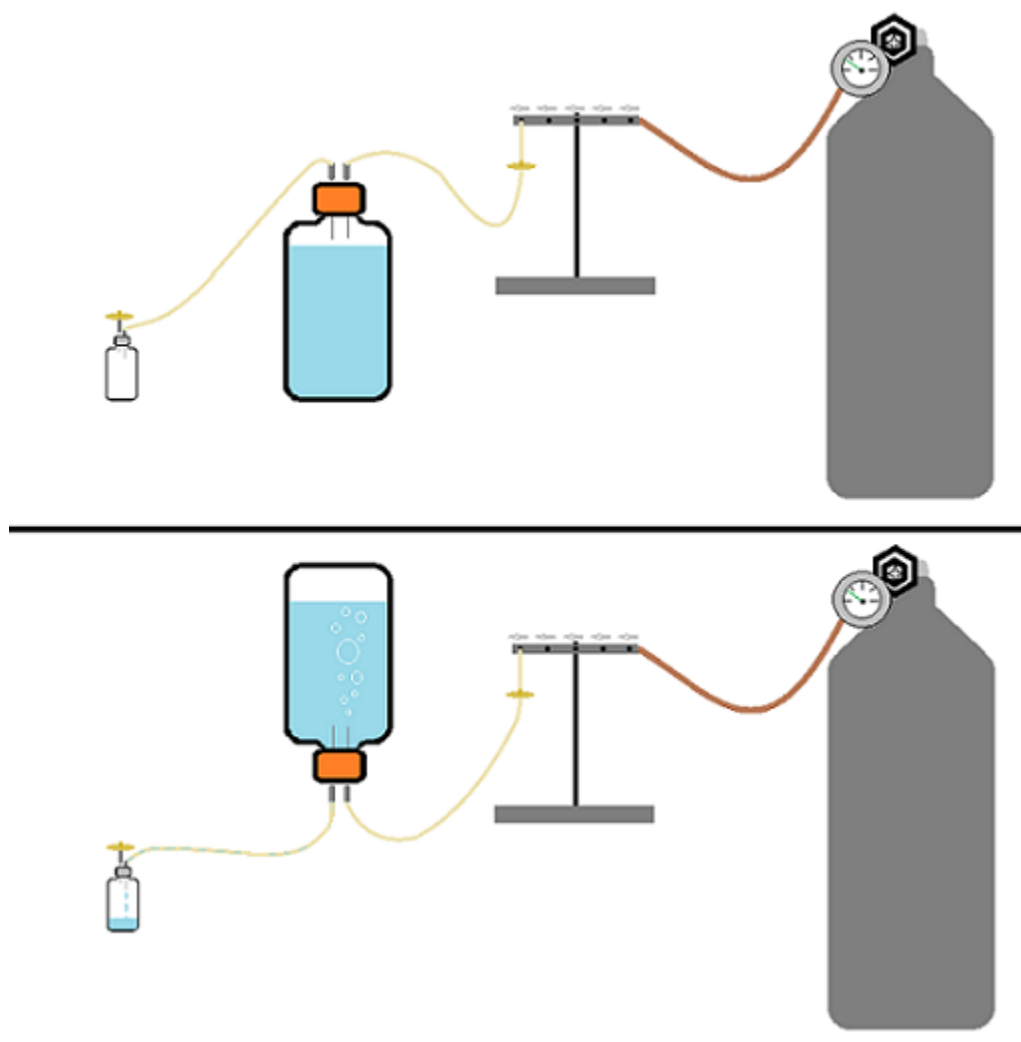


Figure 2.3. Changes in cell number in replicate microcosms with the various uranyl acetate additions (0–10 μM) after a 24-day incubation.

Error bars indicate the variability (SD) in the cell counts for each replicate.

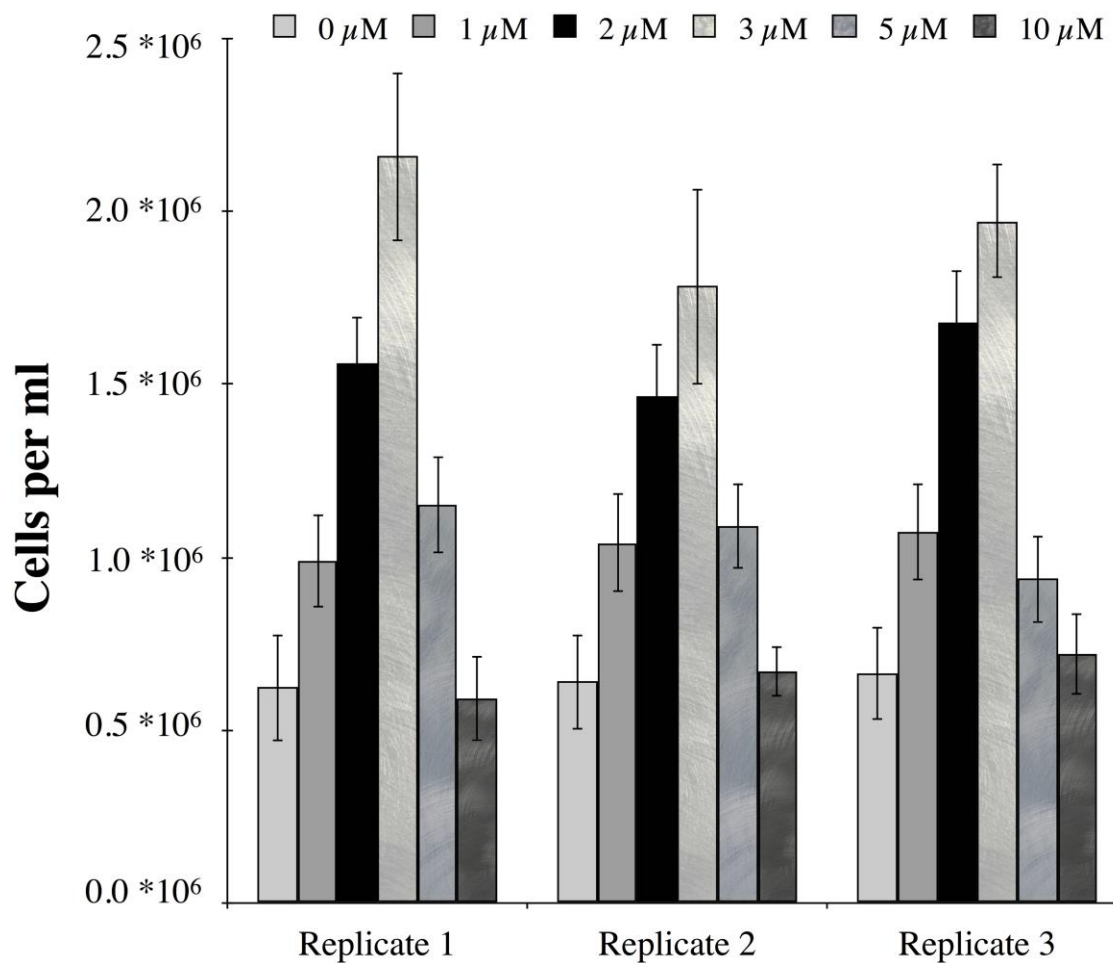


Figure 2.4. Average proportion of soluble and insoluble uranium over time with uranyl acetate addition (0–3 μM) measured by ICP-mass spectrometry.

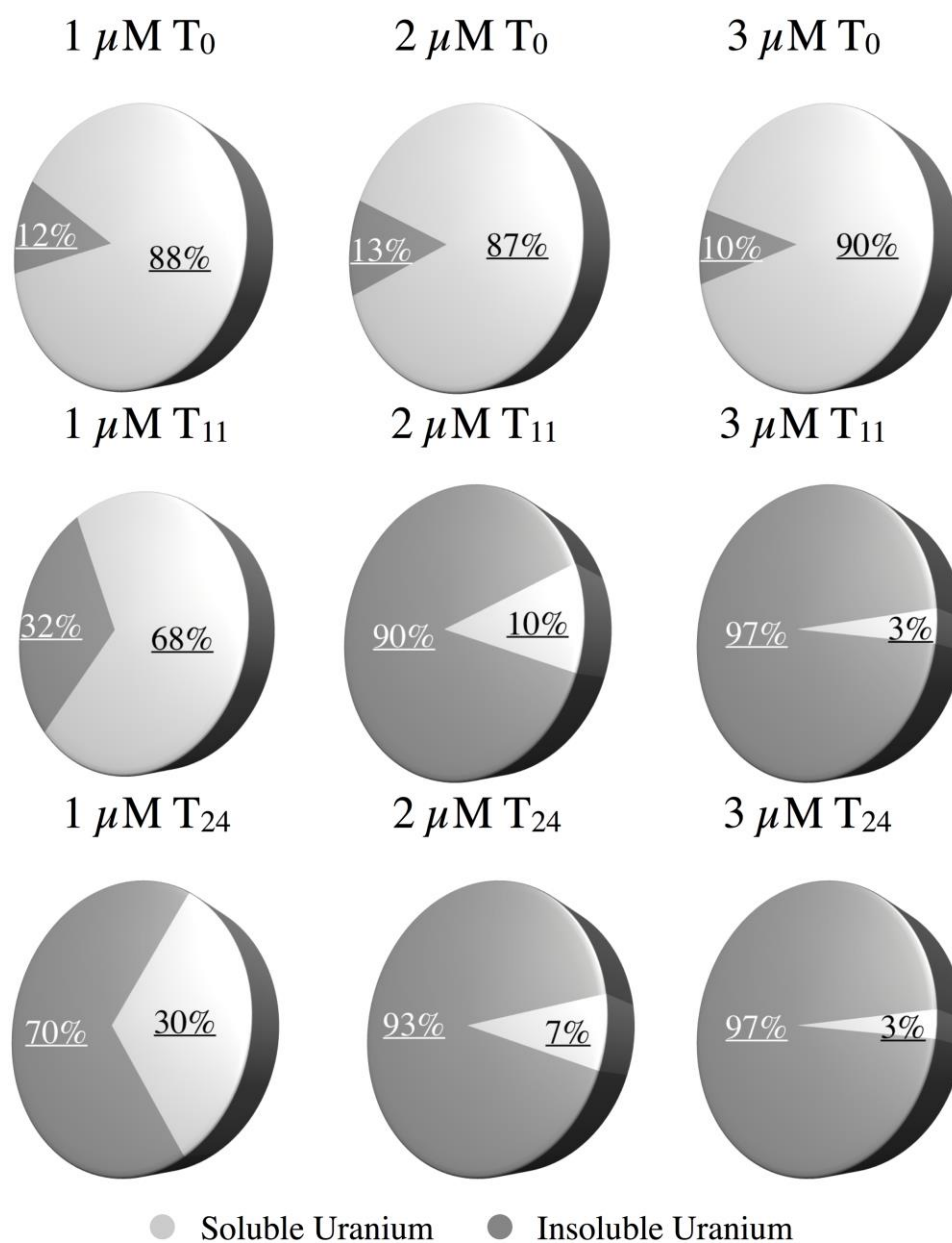


Figure 2.5. Average proportion of soluble and insoluble uranium over time with uranyl acetate addition (5 and 10 μM) measured by ICP-mass spectrometry.

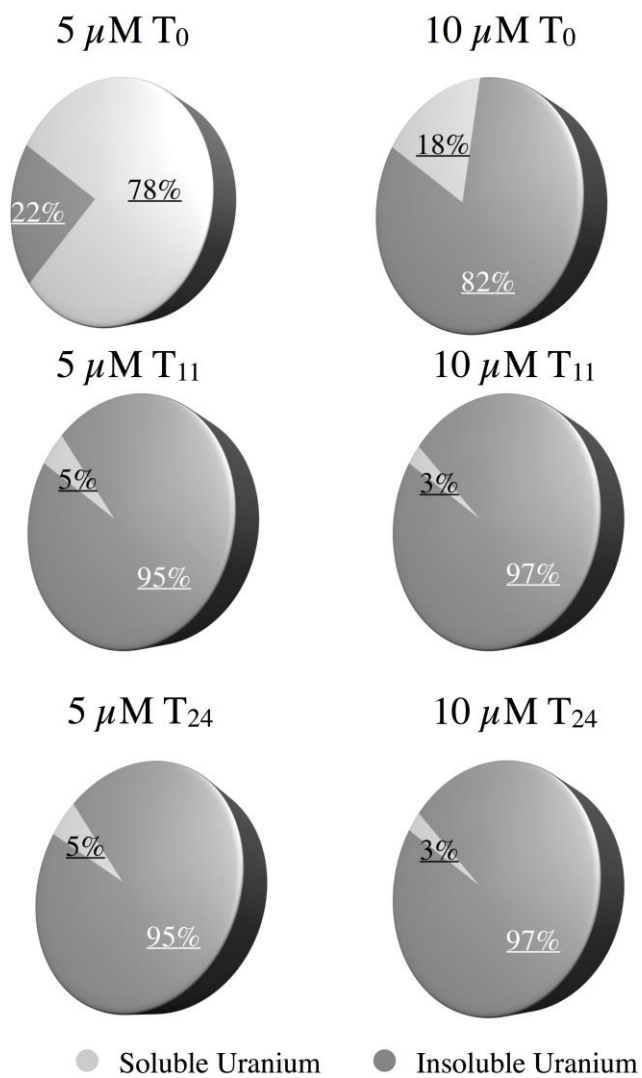


Figure 2.6. Uranium solubility when anaerobic culture samples are filtered under oxidizing conditions.

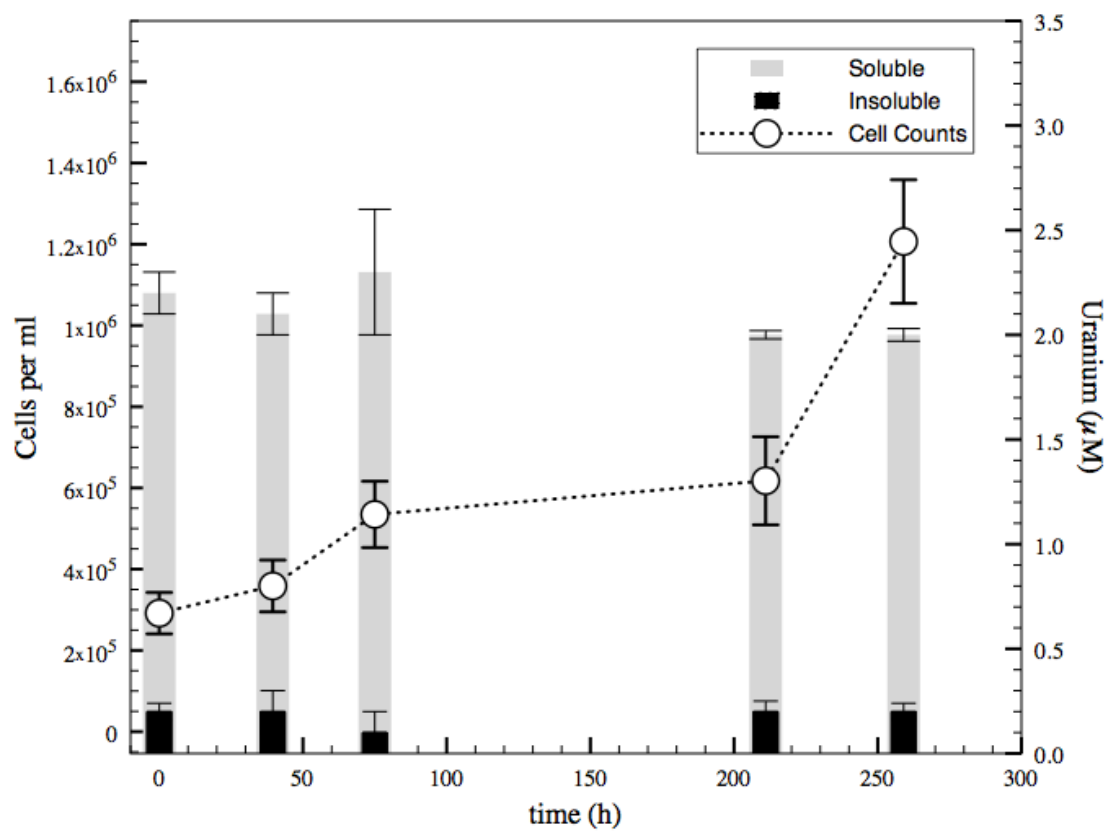


Figure 2.7. Maximum likelihood phylogenetic tree re-construction of *Burkholderia* type strains using 1366 bp of unambiguously aligned sequence of the 16S rRNA gene.

Burkholderia fungorum strain Rifle is indicated

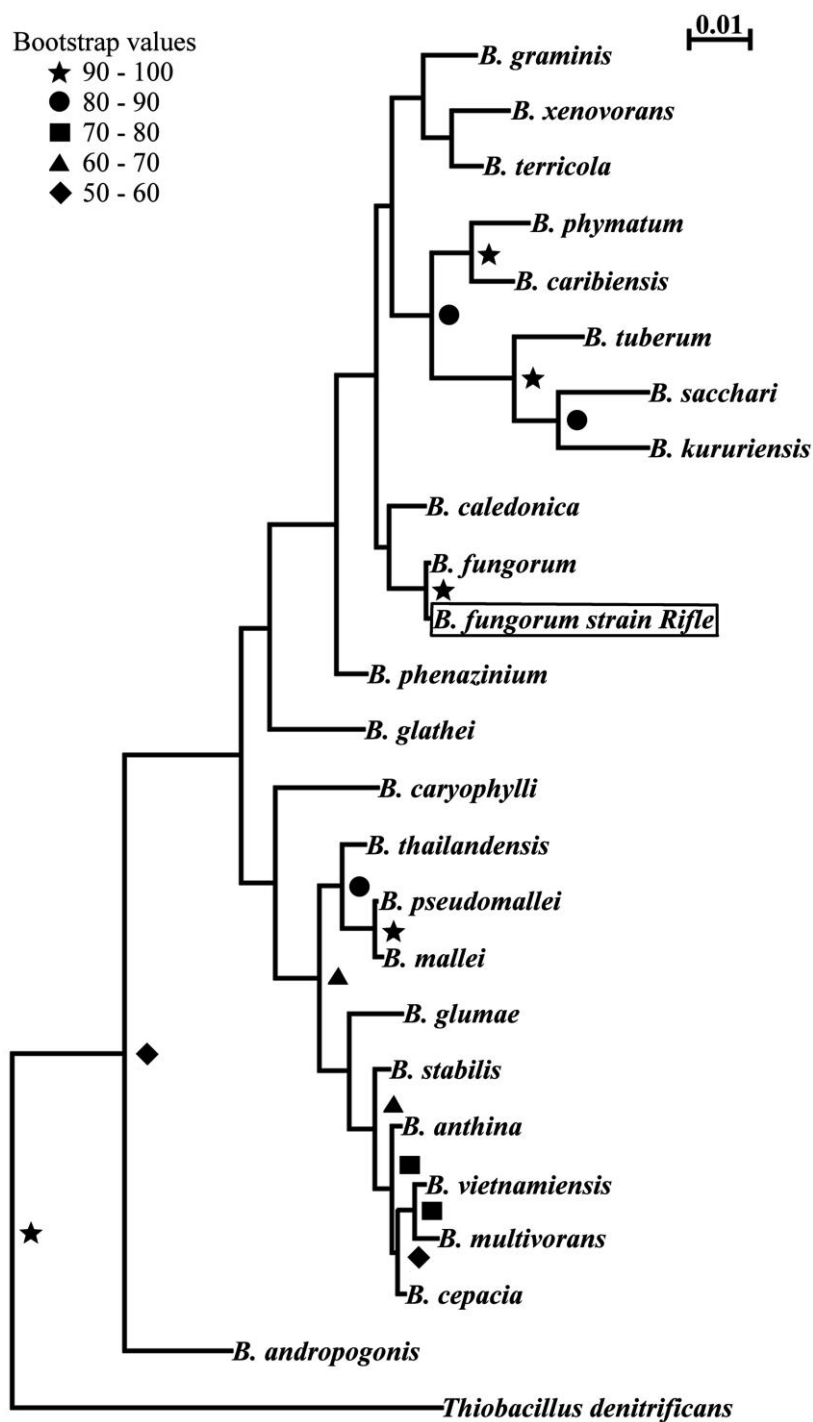


Figure 2.8. Spatial distribution of *Burkholderia fungorum* strain Rifle based on percent contribution to overall microbial community using the 166 bp MnlI peak in the bacterial TRFLP profile.

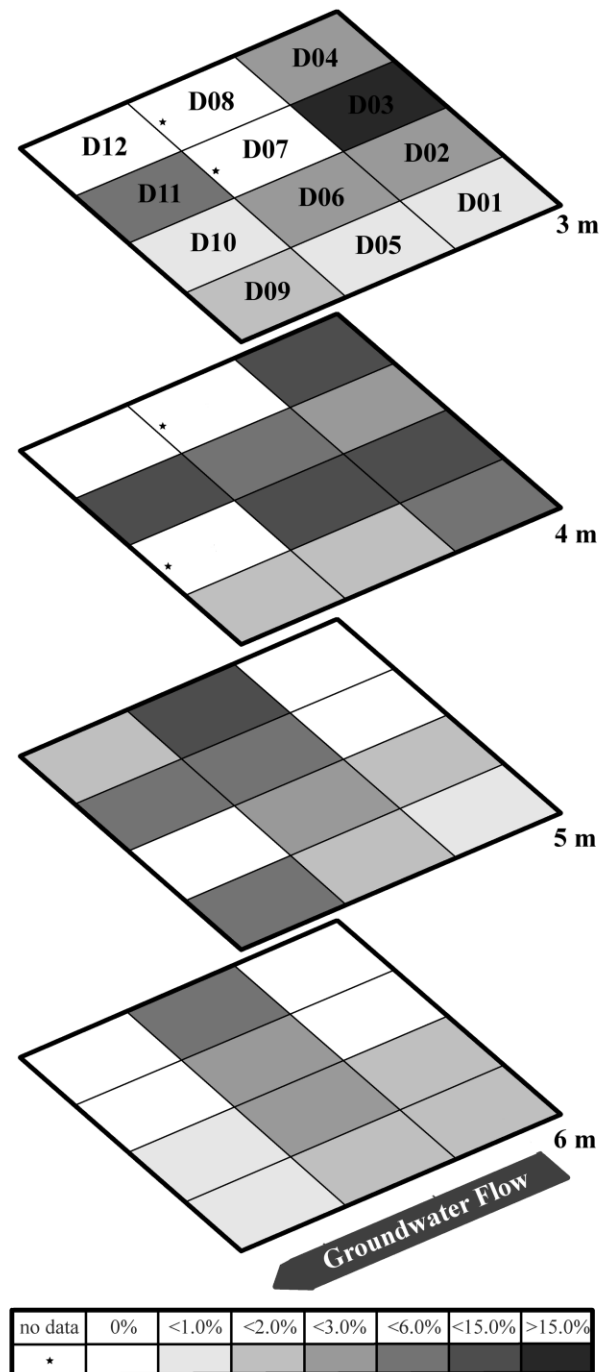
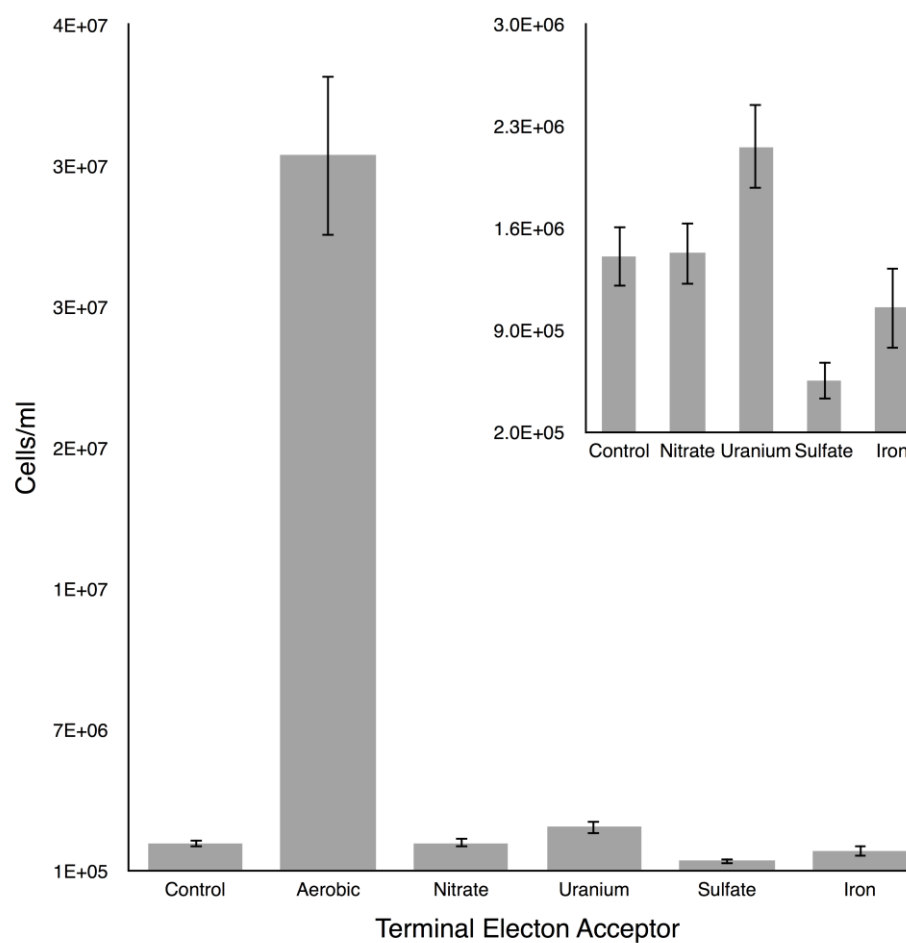


Figure 2.9. Changes in cell number in replicate microcosms with various terminal electron acceptors after a 5-day incubation.

Cells were grown in M9 media with 0.4% dextrose as an electron donor and the terminal electron acceptor indicated. Error bars indicate the variability (SD) in the cell counts for each microcosm. The inset is the anaerobic incubations on a different scale.



CHAPTER III

Mechanisms of toxicity resistance to uranium

by *Burkholderia fungorum* strain Rifle

Abstract:

Bioremediation of the environment to remove toxic contaminants is a topic of much interest. For heavy metals, such as uranium, bioremediation methods are limited to immobilization or chemical conversions into a less toxic or less bioavailable form. This approach relies on the metabolic capabilities of living microorganisms to sequester or convert toxic substances to their less hazardous forms. The facultative microorganism, *Burkholderia fungorum* strain Rifle, is capable of reducing uranium during growth under anaerobic conditions. Furthermore, during growth, uranium was found to be converted from a soluble to a particulate form, reducing its bioavailability, even before cellular growth could be detected at the highest uranium amendments (10 μ M; Chapter 2). This study was conducted to test if there was bioprecipitation of uranium when dense aerobic cultures were exposed to this radionuclide and to determine if *B. fungorum* Rifle exhibited a toxic response during aerobic growth. Aerobic cultures in a dilute LB medium demonstrated delayed growth rates in response to the increasing levels of uranium. However, the same culture density was achieved after this lag period, suggesting a cellular resistance mechanism to toxicity. In order to test the ability of aerobically grown cultures to precipitate uranium, the cultures were fractionated into soluble and insoluble fractions by centrifugation/filtration and analyzed for uranium concentration. The proportion of uranium in each fraction was found to not vary between abiotic and biotic

treatments, indicating that the mechanism whereby *Burkholderia fungorum* strain Rifle resisted the toxic effects of uranium did not immobilize uranium.

Introduction

The primary cause of contamination of groundwater and soil by heavy metals is due to industrial processes, such as mining operations (Bhagure & Mirgane 2011). Uranium is a naturally occurring heavy metal element which is ubiquitous in nature, and most often occurs as reduced, immobile uraninite (UO_2) or pitchblende ($\text{U}_3\text{O}_8^{2+}$) complexes (Finch & Murakami 1999; Bleise et al. 2003). However, once these minerals are exposed to air, they easily become oxidized and soluble (Bleise et al. 2003). Reduced uranium can also be oxidized to U(VI) in the presence of NO_2^- , Fe(III), or dissolved oxygen, resulting primarily in aqueous uranyl (UO_2^{2+}) complexes (Wu et al. 2007; Senko et al. 2002, Senko et al. 2005; Wan et al. 2005). Uranium mobility, like many toxic metals, is entirely dependent on these chemical speciations and the oxidation state of the ion (Wersin & Bruno 1999; Wersin et al. 1994; Suzuki & Banfield 1999). Complexation interactions with other subsurface materials and precipitation reactions with environmental constituents can also play a role in how mobile uranium remains in a given environment (Kim & Czerwinski 1996; Zeh et al. 1997; Finch & Murakami 1999; Clark et al. 1995). While natural attenuation can occur, biotic mechanisms show a higher effectiveness at removal of low metal concentrations than physical or chemical methods (Lloyd & Macaskie 2000). The removal of these soluble and highly mobile complexes from the groundwater is the main focus of most bioremediation efforts.

The primary concern with heavy metals, such as uranium, is the damage to DNA and lipids through the formation of free radicals, and the disruption of protein functions by interfering with tertiary bonding (Flora et al. 2008). A number of resistance mechanisms have evolved to allow microorganisms to survive heavily contaminated

environments (Fig. 3.1). Some cellular mechanisms exclude uranium ions from interacting with intracellular components, either by establishing a permeability barrier or by active transport outside the cell using efflux pumps to lower intracellular concentrations (Silver 1996). While these exclusion mechanisms protect the cell from damage, the soluble uranium in the environment remains unchanged. A different resistance mechanism, the microbial reduction of U(VI) into the less reactive and insoluble U(IV), has also been the focus of many bioremediation studies (Lovley et al. 1991; Wade & DiChristina 2000; Bernier-Latmani et al. 2010; Sharp et al. 2011). Due to the low pH, high nitrate concentrations, and environmental constituents like carbonates and calcium, uranium reduction may be limited in some environments (Brooks et al. 2003; Finneran et al. 2002; Choppin 1999; Wu et al. 2006). Under these conditions alternative mechanisms to remove uranium from the groundwater are required for bioremediation purposes. Of the variety of adaptations and strategies for surviving uranium toxicity, there are several which serve to immobilize uranium, including biosorption to the cell surface (Tsezos & Volesky 1981; Macaskie et al. 2000), intracellular and extracellular accumulation (Merroun et al. 2002; Choudhary & Sar 2011; Lovley & Phillips 1992 a/b; Lovley 1993), and biomineralization (Macaskie et al. 1994; Shelobolina et al. 2009; Beazley et al. 2011) all generally result in immobilization of the uranium. Other mechanisms such as the production of metabolic products, like ligands or anions, can cause precipitation (Ehrlich 1997) and the modification of internal components to decrease sensitivity (Rouch et al. 1995) exist for some metals. However, only sorption, accumulation, mineralization, and reduction have been documented for uranium to date (Nancharaiah et al. 2006; Chabalala & Chirwa 2010). For the purpose of

bioremediation in oxygenated environments, biomineralization is the more feasible strategy to immobilize the uranium.

This study was initiated to determine whether *Burkholderia fungorum* strain Rifle can bioprecipitate soluble uranium during aerobic growth or if other mechanism(s) are used to resist the toxic effect of uranium. The *Burkholderia fungorum* strain utilized in this study was isolated from The Department of Energy's Integrated Field-Scale Subsurface Research Challenge Site (IFRC) at Rifle, Colorado and was chosen for its ability to convert uranium to an insoluble form under anaerobic reduction conditions (Chapter 2; Koribanics et al. 2015). While uranyl ions can sorb strongly to particulates within the environment in the absence of certain stabilizing elements such as carbonate, in the presence of other environmentally common ligands (e.g. humic substances, calcium, and magnesium) sorption levels decrease significantly (Andersson et al. 2001; Koch-Steindl & Prohl 2001; Davis et al. 2004; Siegal 2003; Newsome et al. 2014). At the Rifle field site, previous studies have indicated that biotic precipitation seems to be the most viable option for immobilizing uranium due to environmental conditions (Grenthe et al. 1992; Singer et al. 2009; Brooks et al. 2003; Fox et al. 2013). Under anaerobic conditions *Burkholderia fungorum* strain Rifle appears to generate growth energy via the reduction of uranium, indicating that it contains the capability of biochemical transformation via reduction, but under aerobic conditions the reduced uranium re-oxidizes. To assess the ability of *Burkholderia fungorum* strain Rifle to bioprecipitate uranium under aerobic conditions, cultures were grown in media with

concentrations sufficient to induce a growth lag, and the soluble concentration of uranium post growth initiation was measured via ICP-OES.

Methods

Aerobic growth in the presence of uranium

The Rifle strain was grown in a modified 25% Luria-Bertani Broth (LB) containing 2.5 g/L NaCL, 2.5 g/L tryptone, and 1.25 g/L yeast extract at 25 °C on an orbital shaker. The culture was harvested during exponential growth phase and diluted and gently homogenized via vortex to a starting OD of 0.07 at 600 nm as measured via spectrophotometer (Aminco DW-2a). This starting culture was further diluted 1:150 with sterile media and aliquoted into 9mL volumes, which were amended in triplicate with sterile uranyl acetate to the following final concentrations: 0, 1, 3, 10, 25, 50, 100, and 200 μ M. A separate sample of sterile media was amended with 200 μ M but without inoculum to provide an abiotic uranium precipitation control. Sterile modified minimal carbonate salts media was prepared as described in Chapter 2 and amended with 1, 2, 3, 100, and 200 μ M uranium to assess the abiotic precipitation thresholds in more fully. These test tubes were incubated at 25 °C on a tube rotator. Biomass was measured over the course of 52 h via optical density (600 nm) to track growth rate.

ICP-OES assessment of uranium

0.5 mL of 100 μ M uranium samples was processed at time of inoculation and at 24 hours post-inoculation by separating the phases in a Beckman Optima ultracentrifuge (Palo Alto, CA) at 48,000 x g in a TLA 120 rotor for 25 minutes, and

the top 400 μL was designated the soluble fraction. The soluble fraction was diluted in sterile water and 70% nitric acid for a final concentration of 2% nitric acid, and analyzed via ICP-OES (Thermo Fischer Scientific) to determine the concentration of uranium ions still soluble.

Uranium-media interaction assay

To determine if the time of uranium exposure to the media components could affect *B. fungorum* Rifle growth, cells were re-inoculated into media as shown in Fig. 3.2. Inoculated test tubes were amended in duplicate as follows: 1 mL of sterile water added to 9 mL of sterile 25% LB broth for a final concentration of 0 μM uranyl acetate, to function as the no-uranium growth control. 1 mL of sterile 2 mM uranyl acetate stock added to 9 mL of sterile 25% LB broth amended at the time of cell inoculation to serve as the uranium toxicity control. 1 mL of sterile 2mM uranyl acetate stock added to 9 mL of sterile 25% LB broth, incubated for 1 week for a final starting concentration of 200 μM uranyl acetate, to function as an abiotic media interaction control. Cultures were incubated at 25 °C on an orbital shaker. Growth was measured via optical density for 24 hours. 0.5 mL was processed at time of inoculation and at 24 h post-inoculation by ultracentrifugation and the top 400 μL was designated the soluble fraction and analyzed via spectrometry as previously described to determine the concentration of uranium ions still soluble.

Genome analysis

Burkholderia fungorum strain Rifle was grown to turbidity aerobically on 50% LB media for at 25 °C, spun into a pellet via centrifugation. Genomic DNA was extracted via a high EDTA/phenol/chloroform purification procedure (Sakano & Kerkhof 1998; Corredor et al. 2004) followed by agarose gel electrophoresis to determine concentration. Purity was determined by terminal restriction length polymorphism analysis of fragments generated from the 16S rRNA gene using the restriction enzyme *Mnl* I. The genomic DNA was commercially sequenced by Illumina (Mr. DNA, Shallowater, TX). Genomic DNA was also sequenced via MinION sequencer in an attempt to close the genome using longer reads (Chapter 4). Sequence reads were assembled via SPADES and Geneious assembly programs, and contigs were submitted to the RAST-SEED rapid annotation pipeline for analysis (Kearse et al. 2012; Aziz et al. 2008; Overbeek et al. 2014; Brettin et al. 2015).

Results

Growth rates when exposed to increasing uranium concentrations

In order to determine whether uranium inhibited growth of *B. fungorum* Rifle, aerobic culture tubes were established and inoculated in the presence of 0-200 μ M uranyl acetate. The control culture (0 μ M uranium) initially showed evidence of growth at 11 hours post-inoculation, and exhibited a logarithmic increase in OD for the duration of the remaining time points with slight dips, presumably from temporary low oxygen tension during growth. (After vortexing and increased agitation via increased rotation on the shaker, the cultures resumed their prior growth rate). Similarly, cultures grown in the

presence of 1-25 μM exhibited growth patterns identical to the control culture. Cultures exposed to 50 μM uranium exhibited a longer lag phase by approximately 2 hours, but from 3 h forward exhibited typical logarithmic growth rates. Cultures exposed to 100 μM uranium were delayed an additional 8 hours, with increase in OD by 21 h post-inoculation, while cultures exposed to 200 μM uranium did not initiate growth until 29 h, indicating significant toxic interactions with uranium affecting growth under aerobic conditions at uranium concentrations $> 50 \mu\text{M}$ (Fig 3.3).

Assessment of the soluble uranium fraction within biotic and abiotic 200 μM uranium samples showed that ~70% of the uranium was removed from solution under both biotic and abiotic conditions, and the final soluble uranium fraction remained static throughout growth. These results indicated that media interactions rather than bioprecipitation by *B. fungorum* Rifle were affecting the solubility of uranium (Fig. 3.4). A series of abiotic media controls were sampled, and the soluble uranium fraction assessed to verify these results (Fig. 3.5). LB media amended with 200 μM uranium also precipitated ~60-70% of the uranium within the first 24 hours, while the anaerobic modified minimal carbonate media used in Chapter 2 would abiotically precipitate ~90-97% of the uranium within the first 24 hours depending on amendment concentration. Interestingly, samples amended with 1-3 μM uranium remained soluble, which correlated with previous growth findings of *B. fungorum* Rifle under anaerobic conditions (Chapter 2).

Media effects on uranium and growth

To assess whether growth delay could be attributed entirely to media interactions with the uranium, cultures were grown in the presence of pre-conditioned and freshly amended media to compare growth capabilities. Media without uranium amendment exhibited evidence of growth at 24 hours post inoculation with an OD of 0.08 which increased to an average maximum of OD of 1.0 within 30 hours of growth initiation. Media which was allowed to equilibrate with uranium for a week prior to inoculation showed a marked difference in initiation of growth of culture compared to media which was amended with uranium at the time of inoculation. The abiotic media interaction control samples exhibited evidence of growth at 44 h with an OD of 0.3 that increased to a final average OD of 1. The biotic samples amended with uranium at the time of inoculation did not show visible evidence of growth until 52 h post inoculation with an OD of 0.06, but once initiation of growth was achieved the culture reached a final OD of 1 (Fig. 3.6).

Regardless of culture amendments, the solubility of uranium in the modified LB showed no appreciable difference under either biotic or abiotic conditions (Fig. 3.7). Preliminary results indicate that the natural components of LB interact with uranyl acetate in the presence or absence of a bacterial culture to reduce the solubility, causing a degree of uranium precipitation. While calculated initial amended concentrations of uranyl acetate was 200 μM , at the time of cellular interaction the soluble fraction of uranium measured $\sim 95\mu\text{M}$ in media freshly amended with uranium, while media allowed to abiotically interact with uranium for a week pre-inoculation exhibited a starting solubility of $\sim 65\mu\text{M}$. At 24 h post inoculation when control cultures were initiating

logarithmic growth phase the soluble uranium fraction remained $\sim 64.5 \mu\text{M}$ in cultures grown in media incubated abiotically prior to inoculation. Cultures amended with uranium at the time of inoculation showed a decrease to $\sim 71 \mu\text{M}$ at 24 h post inoculation.

Presence of heavy metal resistance genes.

To test whether *B. fungorum* Rifle contained heavy metal resistance genes associated with other mechanisms of detoxification (e.g. metal efflux pumps), a draft genome was obtained and submitted to RAST for annotation [6666666.250044 - Burkholderia fungorum str.Rifle2]. This genome consists of 8.73Mb assembled into 17 contigs with a 61.6% G+C content, containing 8,123 candidate proteins, of which $\sim 30\%$ are hypothetical proteins of unknown or theoretical function. Among the genes predicted, 44% could be assigned to prospective cellular function subsystems based on RAST annotation. Approximately 50% of the 214 genes assigned to the Stress Response Subsystem were identified as responding to oxidative stress, and about 70% of the 203 genes assigned to cellular defense functions were associated with the resistance to various toxic heavy metals. Included amongst the functions were several reductases and efflux systems, including over 70 efflux pump homologs (Fig. 3.8).

Discussion

To viably serve as a biotic agent of bioremediation, a strain of bacteria would need to utilize a uranium toxicity resistance mechanism which resulted in the immobilization of uranium, including A-C mechanisms from Fig. 3.1.

Reduction/chemical modification, complexation, or extracellular chelation would result

in speciation with lower solubility, causing a precipitation of uranium within the media. Biosorption or internal sequestration would also result in the removal of uranium from solution along with the cellular biomass. Therefore, any biotic mechanism utilized by *Burkholderia fungorum* which would be useful for aerobic treatment of low concentration of uranium would yield an increase in insoluble uranium over the length of the experiment.

Prior studies of uranium reduction under anaerobic conditions by *Clostridium*, *Desulfotomaculum*, and *Shewanella* species (Bernier-Latmani et al. 2010; Junier et al. 2009) utilized media components that exhibited some interactions with uranium at higher concentrations under aerobic conditions, as seen by a portion of the uranium co-precipitating with LB components during this study. Our results clearly show that interactions between uranyl acetate and the LB media components affect the overall solubility of the uranyl ions. Because the precipitation of uranium in LB is relatively instantaneous, *B. fungorum* Rifle was not deemed capable of bioprecipitation under aerobic conditions.

The presence of a lag in growth initiation indicates that the cellular components are not inherently resistant to uranium via a genomic modification, such as a permanent mutation which reduces the sensitivity of cellular components to a toxin. Although growth of *B. fungorum* Rifle in media pre-conditioned with uranium exhibited a reduced lag (Fig. 3.6), the culture components did not remove all chemotoxic effects. These results suggest *B. fungorum* Rifle does not utilize an impermeability barrier (Fig. 3.1.E) as the mechanism by which uranium toxicity is resisted.

Therefore, the most likely mechanism of resistance for uranium toxicity is an efflux pump. Investigation of genomic features involved in the resistance of toxic resistance to heavy metals other than uranium identified over 70 genes homologous for various types of transmembrane transport proteins, including several variations on P-type ATPase efflux pumps. In silico annotation assigned multiple genes as functioning as efflux pumps for various heavy and toxic metals based on sequence homology, but with variations in gene length exceeding 30 base pairs in some cases. This is indicative of variable protein products which are theorized to perform the same or similar functions, but which might apply to a different target. In conclusion, the exact method of uranium resistance for *B. fungorum* Rifle under aerobic conditions remains unknown. The utilization of efflux pumps to create a localized microcosm of decreased uranium concentration within the intracellular space seems most likely given the results, but utilization of a non-precipitous chelator or chemical modification cannot be excluded based on current studies.

Figure 3.1. Diagrammatic representation of possible mechanisms of uranium resistance.

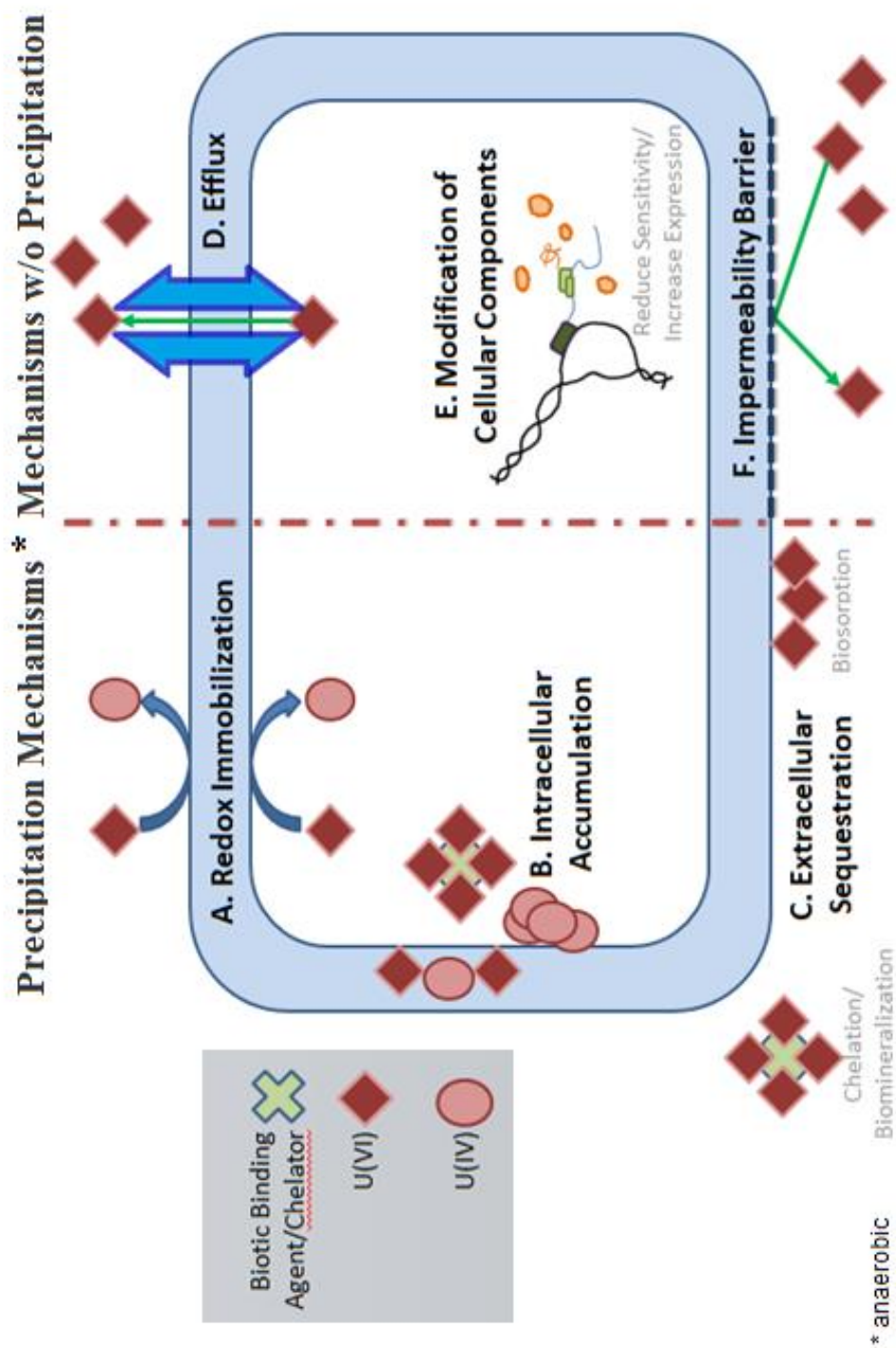


Figure 3.2. Experimental design for assessing effect of media interactions on growth rate.

Experimental Design

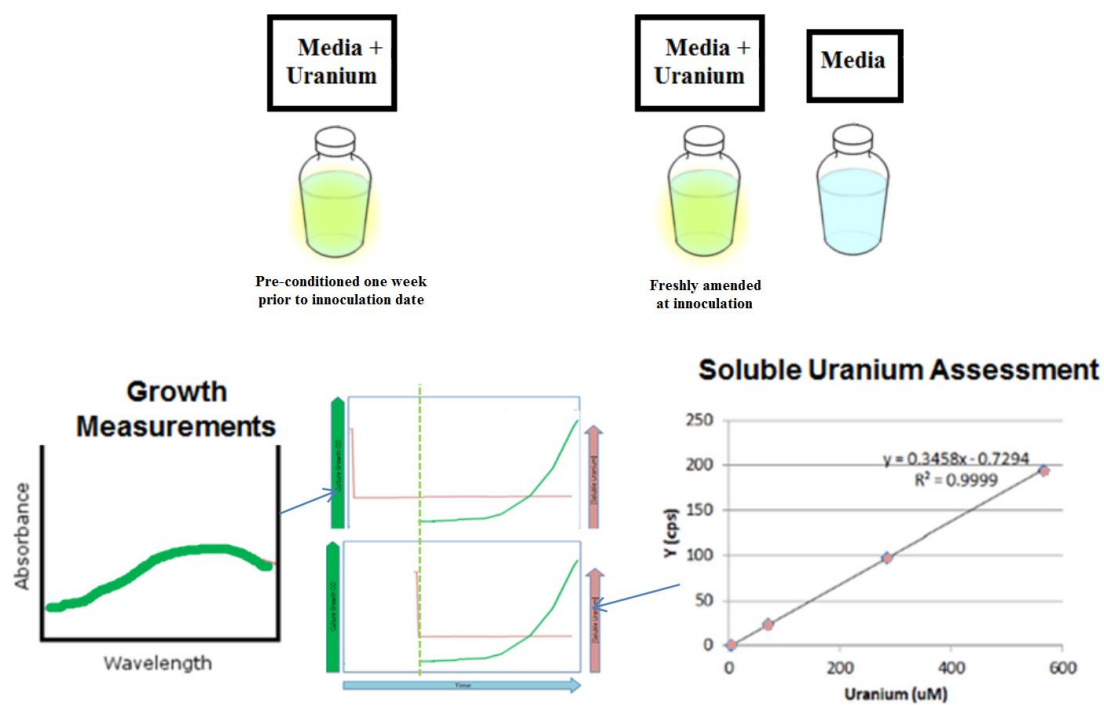


Figure 3.3. Effect of increasing uranium concentrations on growth over time.

Error bars indicate the variability (SD) in the OD reading for each replicate

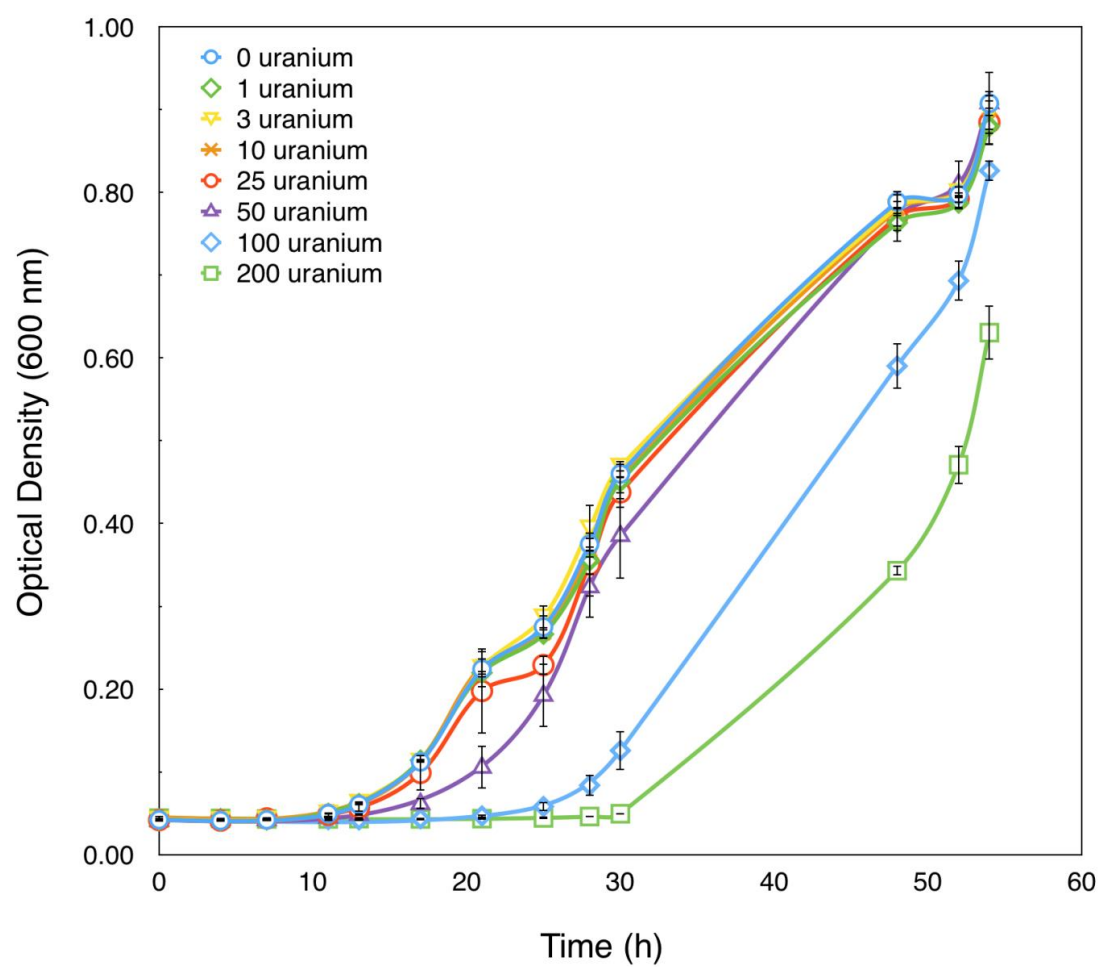


Figure 3.4. Soluble uranium in modified LB media over 21 hour incubation.

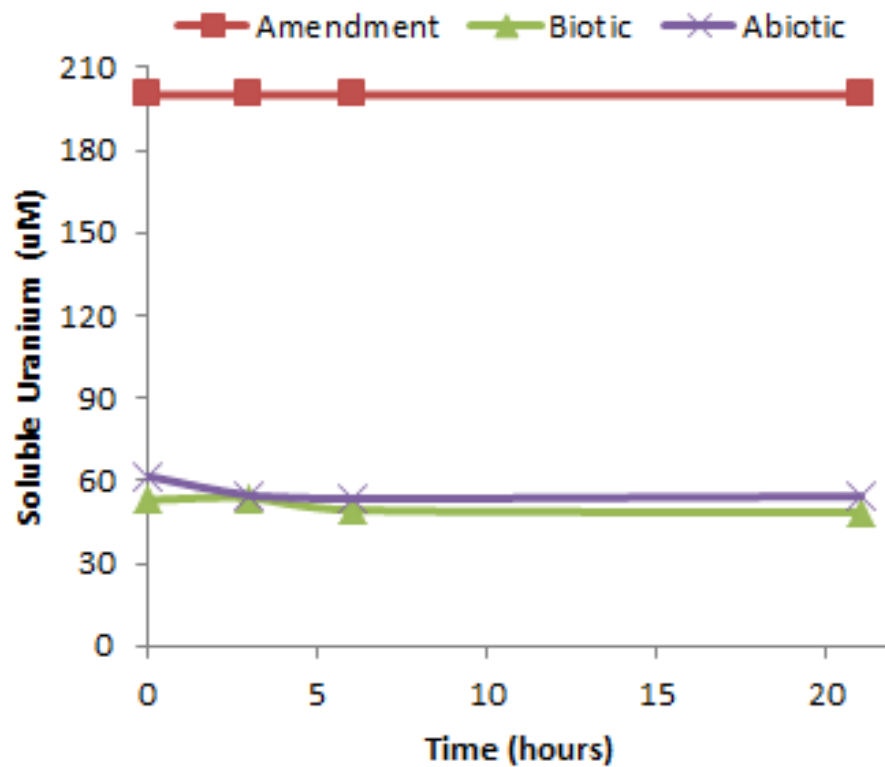


Figure 3.5. Soluble uranium concentrations in media after 24 hour abiotic incubations.

Inset is the low concentration anaerobic media samples on a different scale.

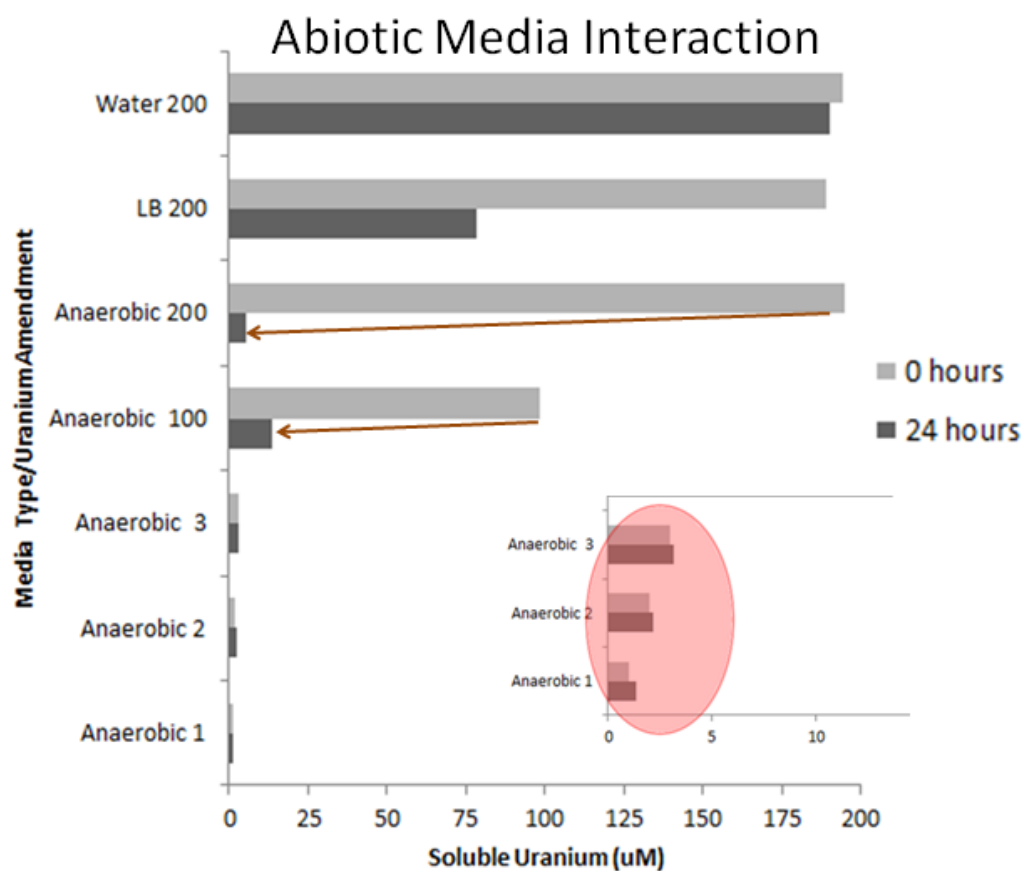


Figure 3.6. Effects of media conditioning with uranium on growth capabilities.

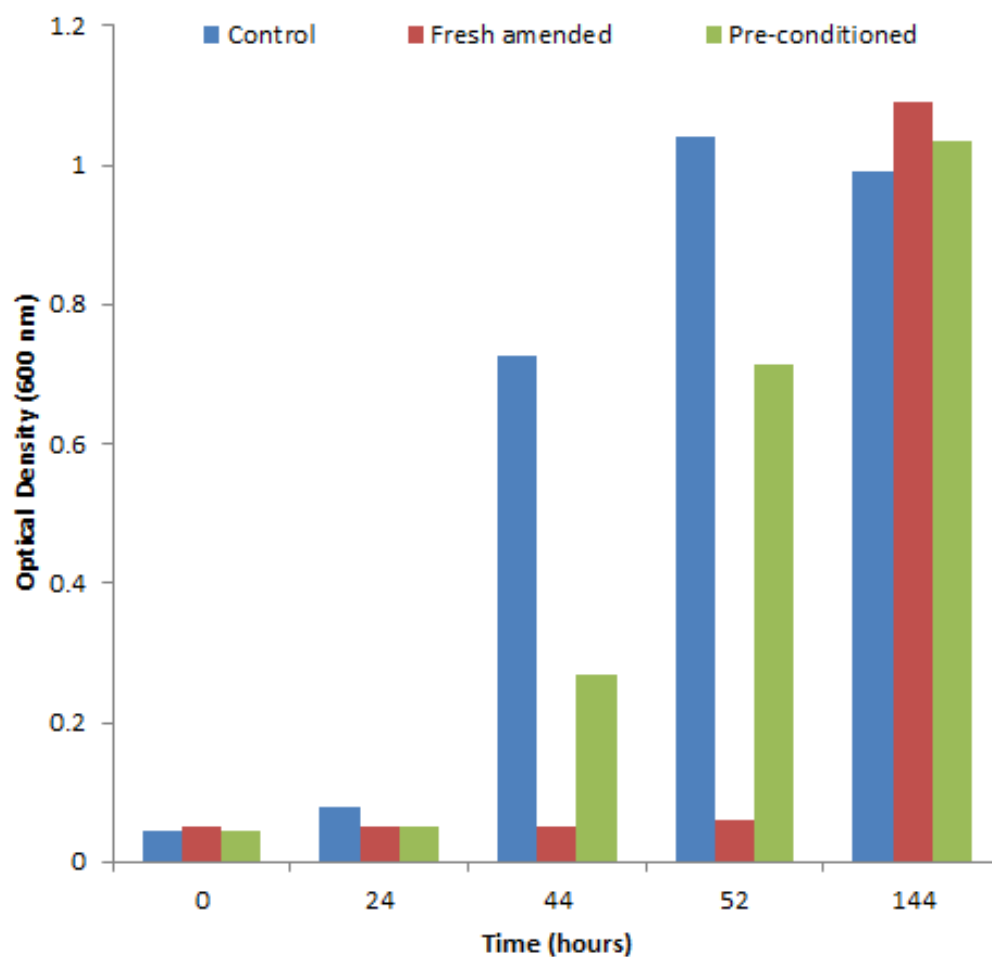


Figure 3.7. Uranium solubility during growth analysis of effect of pre-conditioning

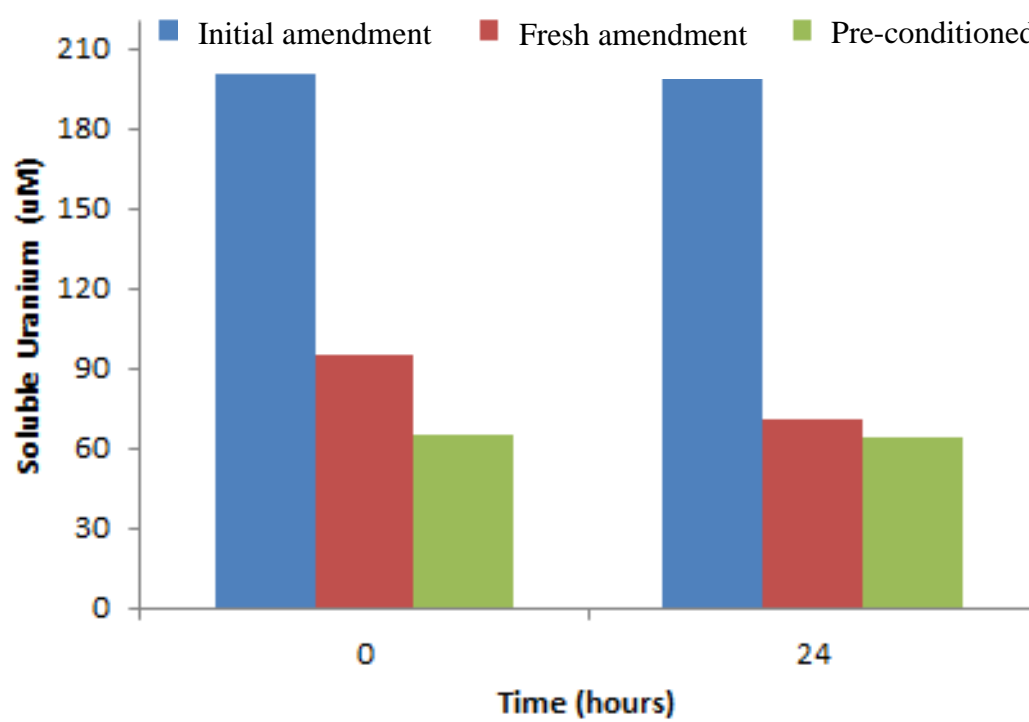
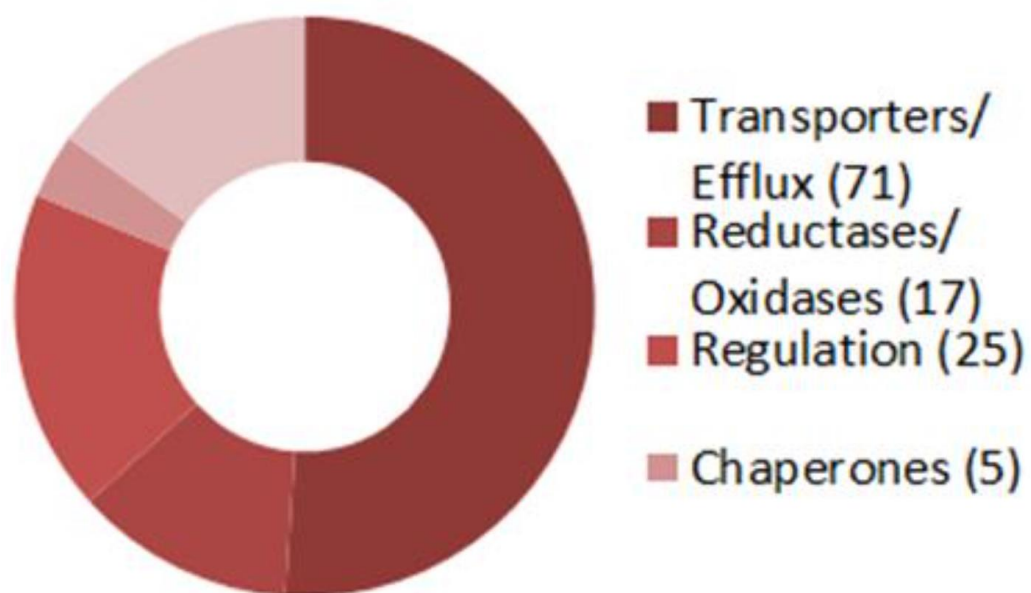


Figure 3.8. Breakdown of genes annotated with heavy metal resistance functions.



CHAPTER IV

Utilization of traditional annotation and functional proteome analysis in the evaluation of *Burkholderia fungorum* strain Rifle functional interactions with uranium

Abstract:

Burkholderia fungorum strain Rifle has been shown to reduce uranium under anaerobic conditions. However, little is known about the enzymes used to respire or to detoxify uranium in this strain when exposed to this radionuclide. For this study, a draft genome of *B. fungorum* Rifle was obtained to screen for enzymes involved in uranium interactions (respiration, biomineralization, or metal efflux). The draft genome consists of 8.73 Mb assembled into 17 contigs with a 61.6% G+C content, containing 8,123 candidate proteins, of which approximately 30% are hypothetical proteins of unknown or theoretical function. Two bioinformatic approaches were used to identify gene targets. One approach searched for examples of horizontal gene transfer from known uranium reducing bacteria. In a second method, a theoretical proteome for *B. fungorum* Rifle was generated *in silico* using based on similarity to the annotated genome of its closest relative (*B. fungorum* strain ATCC BAA-463). This proteome was utilized to identify functionally related bacteria via Fusion microorganism classification. The unassembled genomic sequences for these and an additional 4 strains confirmed to have significant interactions with uranium were submitted to functional annotation of sequencing reads to an enzymatic enrichment profile. Comparative analysis of genomic features for *B. fungorum* strain Rifle to other organisms known to have significant interactions with uranium resulted in the identification of a number of possible features of interest. Some

of these targets (n=7) were tested for transcriptional activation when *B. fungorum* Rifle was transferred from uranium (-) aerobic media to uranium (+) anaerobic media. All targets tested were found to be constitutively expressed and were not deemed to be involved in uranium respiration, with n=9 targets remaining to be tested. Further computational analysis and regulation studies are warranted on the gene targets identified by mi-faser and fusiondb.

Introduction

Most cellular functions are defined by proteins expressed within a cell and their interactions with the constituents of the environment. By definition a proteome is the subset of proteins present within a cell at a particular time under certain conditions, and are theoretically invaluable in the study of functions (Wasinger et al. 1995). However, many cellular processes require very few proteins numerically to function, and often these may be lost due to low expression in extracted proteomes (Derry 2004; Raser & O'Shea 2005). While transcriptome analysis can provide a general overview of which genes are being transcribed at a given moment, the relationships between protein expression and mRNA levels are poorly understood, and likely to vary from gene to gene based on a variety of differing expression and degradation factors (Pratt et al. 2002; Wang et al. 2009).

Advances in whole genome sequencing have smoothed the way for the development of a number of predictive databases and programs for the theoretical annotation of prospective gene coding regions (CDS) (Heather & Chain 2016; Yandell & Ence 2012). Yet, the annotations provided by these databases are largely theoretical, as the experimental validation of the majority of these is limited (Schnoes et al. 2009; Green & Karp 2005; Galperin & Koonin 2010). The function of proteins is in part controlled by their three dimensional shape and often only a few key residues. Proteins which show a high degree of amino acid similarity may perform identical functions, functions with small variations in a target or substrate, or be functionally unrelated depending on the location of the differences within the amino acid sequence. To date, annotated functions are largely assigned based on homology to genes with presumed

known functions documented in the International Nucleotide Sequences Database Consortium (INSDC) (Benson et al. 1993; Sayers et al. 2010; Kaminuma et al. 2010; Leinonen et al. 2006).

In this study, two possible approaches were considered to facilitate the identification of target proteins that may be involved in uranium respiration, by narrowing the pool of possible target proteins (Fig. 4.1). The first approach compares the genome of *B. fungorum* strain Rifle to the sequences of phylogenetically similar strains which do not possess the ability to grow on uranium, and focuses on any genetic elements exclusive to *B. fungorum* strain Rifle. This approach assumes that multiple related *Burkholderia* strains are incapable of growing on uranium. The second approach examines the overlapping genome sections shared among strains capable of growth on uranium. Either method should theoretically reduce the potential proteome volume, allowing for a targeted assessment of genomic features involved in the functional interactions with uranium. Based on the lack of strains which are conclusively demonstrated not to be capable of uranium reduction, approach two was favored heavily, though elements of approach one were employed during fusionDB analysis.

In this study, a computational sequence driven analysis was employed to identify possible proteins of interest in regards to cell interactions with uranium. The genome of *Burkholderia fungorum* strain Rifle, a beta-proteobacterium capable of growth utilizing uranium as a terminal electron, was sequenced and assembled. This sequence data was mined for gene motifs of interest using the schematic in Fig. 4.2. Prospective gene motif targets include reductive and toxicity resistance/sequestration elements, including reductase like genes, cytochrome gene motifs, membrane transporter motifs, and

structural elements involved in pili formation (for review see Wall & Krumholz 2006, Newsome et al. 2014). The potential proteins were screened for lateral gene transfer from known uranium reducers. Genes identified via KEGG annotation in this way were theorized to have potential redox or detoxification activity and become potential targets for transcriptional assays. Fourteen potential sequences were identified, and of these 7 were tested for up-regulation of transcription. Initial findings show all the target genes tested as being constitutively expressed, and thus unlikely to be directly involved in uranium reduction or detoxification.

Proteome analysis pipelines developed by the Bromberg lab were utilized, e.g. FusionDB (<http://services.bromberglab.org/fusiondb/>) and mi-faser (<http://services.bromberglab.org/mifaser/>). FusionDB is a web interface for mapping new microbial genomes to a functional spectrum of reference bacteria, while the mi-faser pipeline is a super-fast and accurate method for annotation of molecular functionality encoded in metagenomic sequences without the need for assembly or gene finding. FusionDB was employed to identify functional neighbors and assess protein functions shared between the Rifle isolate and other microorganisms known to be capable of uranium reduction. However, mi-faser (<http://services.bromberglab.org/mifaser/>) was used off-label to test if sequence data from the Rifle strain could yield profiles of enriched functional proteins that may provide additional target genes for further analysis.

The result indicated 63 prospective genes identified via these computational approaches. Unfortunately, only a few of these target genes (n=4) could be tested by RT-PCR analysis to empirically demonstrate up-regulation when shifted to anaerobic conditions and uranium exposure. All RT-PCR results were negative for up-regulation.

Additional efforts to characterize the remaining target genes or a global transcriptional analysis is required to elucidate the genes involved in uranium respiration or detoxification for *B. fungorum* strain Rifle.

Methods

Genome contig assembly and annotation

Genomic DNA of aerobically grown *Burkholderia fungorum* Rifle was commercially sequenced by Illumina (Mr. DNA, Shallowater, TX) and via MinION. The contigs were assembled by SPADes and the Geneious assembler and annotated to the phylogenetically similar *Burkholderia fungorum* ATCC BAA-463 at 80% similarity. Genomic assembly via mauve alignment to assess genome similarity and rearrangements was also performed. These contigs were annotated using traditional annotation programs such as RAST (Aziz et al. 2008) and Geneious v 7.2, and assessed for homologous functions as defined by the KEGG database (Kanehisa et al. 2012) to strains of bacteria known to be capable of similar interactions with uranium. In addition, alternative assessments were utilized to identify possible orthologs or genes transferred horizontally.

Identification of possible targets through KEGG comparison

Comparison of annotated CDS sequences to the KEGG database was used to identify genes and any gene annotation with known uranium reducing species as the closest relative based on sequence similarity was chosen as possible horizontal gene transfer (Table 4.1). Primers were generated for each gene, and tested on DNA extracted from the isolate to verify production of a fragment with the correct length. Dilute DNA

extract was amplified for each primer set using the protocols described in Sakano & Kerkhof 1998 with the following cycling parameters: 35 cycles of 94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 0.5 min, followed by a final extension at 72°C for 10 min. Lengths for each amplicon were verified by running on 4% metaphor gels for 3 hours at 70V in chilled 1X TAE buffer.

Growth of *B. fungorum* Rifle

Burkholderia fungorum strain Rifle was grown aerobically on 25% LB, then pelleted and washed with modified minimal carbonate salts media as described previously (Chapter II), and re-suspended in 100 mL of sterile minimal carbonate salts media which was sparged gently with sterile N₂/CO₂ gas mixture (Koribanics et al. 2015). This inoculum was diluted into 1L of sterile anoxic media and aliquoted into six 100 mL anaerobic vials, whose headspace had been purged with argon. Three of the vials were amended to a final concentration of 3 µM uranium while the other 3 vials were amended with the same volume of sterile anoxic water to maintain media concentrations and cell density. Immediately after amendment and at day 1, 3, and 6, four mL aliquots of each vial were taken under anaerobic purge and spun down in a 4 °C centrifuge to pellet biomass before freezing. Biomass pellets were extracted via high EDTA/phenol/chloroform purification procedure with nuclease free reagents (Sakano & Kerkhof 1998; Corredor et al. 2004). Samples were precipitated with ethanol and resuspended in the same volume of nuclease free water to preserve RNA concentrations. Samples were treated with Turbo DNA-free kit (Life technologies; Grand Island, NY) at

37°C for 20 minutes to digest DNA. Samples were then tested for transcript regulation using reverse transcription PCR.

RT-PCR testing of up-regulation

RNA amplifications were performed using Two-Step RT-PCR Kit (Invitrogen) with each of the priming sets using the following parameters: 1 cycle of 30 minutes at 55°C for reverse transcription followed by 35 cycles of 94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 0.5 min, and a final extension at 72°C for 10 min. Each sample was also analyzed without reverse transcriptase to verify amplification was only of RNA transcripts. Quantification of amplicon relative concentration was compared to a control gene amplicon (housekeeping: maleate isomerase) using ethidium bromide fluorescence in 4% metaphor gels (Kerkhof 1997).

FusionDB analysis

A theoretical complete proteome of 6540 unique protein sequences was generated using the Geneious annotation software (<http://www.geneious.com>, Kearse et al., 2012) and submitted to the FusionDB classification service for functional analysis (<http://services.bromberglab.org/fusiondb/>, Suhre & Claverie 2004). Based on functional similarity, subsets of phylogenetically similar and dissimilar strains were chosen for comparisons between the isolate and a subset of strains known to significantly interact with uranium (Table 4.2). Using the database generated protein profiles, functionally based pan-proteomes were generated between each test organism and *B. fungorum* Rifle. Within the pan-proteome of these strains, proteins identified to be present in all uranium

reducers were targeted as probable proteins of interest and investigated using BLAST (Altschul et al. 1990; Altschul et al. 1997).

mi-faser analysis

The raw sequence reads for the isolate *Burkholderia fungorum* Rifle and 4 known uranium reducers (*Desulfotomaculum reducens* MI1, *Geobacter uraniireducens* Rf4, *Anaeromyxobacter dehalogenans* 2CPC, and *Shewanella oneidensis* MR1- see Table 1.1) were submitted to mi-faser for mapping via algorithmic functional annotation of sequencing reads (<http://services.bromberglab.org/mifaser/>). This metagenomic analysis tool uses the functional annotation of sequence reads algorithm (FASER) to map reads to functions defined and encoded in a reference database (Sharpton 2014). The enzymes identified as enriched for each strain were compared to identify enzyme enrichment across the genus, which would theoretically identify functional enzymes of interest. Representative protein sequences from the most enriched enzyme commission numbers in the classes of interest were assessed via amino acid BLAST homology for functional significance in regards to uranium interactions.

Results

Traditional genome assembly and annotation – KEGG targets

Final assembly yielded 17 contigs comprised of 8.735 Mb of sequence with 61.6% GC content. Annotation revealed 8123 prospective coding sequences with 78 RNAs, 5684 annotating to known functions, and the remaining 2439 sequences remaining hypothetical (Fig. 4.3). Among these CDS, 6540 unique protein sequences

were generated by Geneious annotation and translation software to serve as the theoretical proteome. To check sequence reassembly, the contigs were aligned into a scaffold based on *B. fungorum* strain ATCC BAA-463. Relatively large segments of sequence similarity support accurate assembly of the sequencing reads. Sequence homology to the KEGG genome database identified 8 possible genes of interest based on similarity to sequences belonging to known uranium reducers and 6 based on annotation assignments related to the functions previously implicated (Table 4.1) and compared their expression to an eighth housekeeping gene (maleate isomerase). When rt-PCR was performed on these genes, constitutive expression was observed (Fig. 4.5).

Functional classification of the strain

FusionDB identified the functional similarities between the generated theoretical proteome and the proteomes of a number of bacterial species, with the maximum similarity recognized in the database as 60%. Two phylogenetically similar strains (*Burkholderia xenovorans* LB400 and *Burkholderia mallei* ATCC 23344) and two phylogenetically dissimilar strains (*Variovorax paradoxus* S110 and *Achromobacter xylosoxidans* A8) were chosen for comparison in addition to 5 strains known to interact with uranium in various ways (*Desulfotomaculum reducens* MI1, *Geobacter uraniireducens* Rf4, *Anaeromyxobacter dehalogenans* 2CPC, *Shewanella oneidensis* MR1, and *Cupriavidus metallidurans* CH34). While *Burkholderia xenovorans* LB400 showed the highest degree of functional similarity at 59%, both *Variovorax paradoxus* S110 (51%) and *Achromobacter xylosoxidans* A8 (52%) had higher degrees of functional relation to our isolate than the other *Burkholderia* strain chosen (*Burkholderia mallei*

ATCC 23344, 45%). The strains chosen for their ability to interact with uranium varied from 44% (*Cupriavidus metallidurans* CH34) to 19% similarity (*Desulfotomaculum reducens* MI1). Figure 4.6 displays a visualization of the functional proteome analysis, displaying how phylogenetically unrelated species can share a higher functional similarity, though whole genome similarity analysis shows a higher correlation at the Class level (Fig. 4.7).

Based on the concept of shared functions deriving from similar proteins, analysis of the proteins identified as similar across all known uranium reducers narrowed the field of interest to 42 proteins (Fig. 4.8). While the majority of these are identified via annotation to known functions with little relation to heavy metal interactions, one identifies as the arsenic reductase gene, and four are hypothetical proteins. When the amino acid sequences of the hypothetical proteins were analyzed via Psi-BLAST (Fig. 4.9), the system identified domains which indicate that the hypothetical proteins likely display ABC-type transporter permease abilities, exonuclease repair function, secretion/pilin function, and one with a putative conserved domain hypothesized to deal with transamidase function.

mi-faser analysis

Functional annotation of the raw sequencing reads identified between 159 (*Geobacter uraniireducens*) and 570 (*Burkholderia fungorum* Rifle) enzymes that were enriched. The relative fraction of each enzyme commission number for the isolate is mapped in Figure 4.10. When cross-genus comparisons were made, 69 enzymes were

found to be present in all 5 strains, but only 21 were members of EC classes I and III, for which known enzymatic functions relating to uranium interactions belong.

Representative protein sequences from the most enriched EC numbers for classes of interest (1.17.2.1 nicotinate dehydrogenase and 3.6.3.21 P-type ATPase) were Blasted against the annotated genome of *Burkholderia fungorum* Rifle. While in the case of nicotinate dehydrogenase the exact enzyme was not located amongst the annotation, many enzymes with functionally similar domains were identified, comprising a series of genes whose functions are best classified as c-type cytochromes. The homology analysis for the P-type ATPase enzymatic function once more identified a number of genes that shared domains functionally serving as P-type ATPase transmembrane transport, including several heavy metal efflux pumps (data not shown).

However, it should be noted that while the fusionDB analysis assigned the complete theoretical proteome to 3,865 distinct functions, the mi-faser results only encompassed 570 defined enzyme functions. Analysis was redirected to single isolate analysis of the enriched functions identified in *B. fungorum* strain Rifle. The sequence data for the enzyme commission numbers 1.17.2.1 (nicotinate dehydrogenase) and 3.6.3.21 (polar-amino-acid-transporting ATPase) were retrieved from Genbank and blasted against the assembled genome. No exact matches were uncovered, but multiple near matches were uncovered. It was notable that the majority of the genes identified shared conserved sequence domains responsible for key functions within the enzyme. So the enrichment for nicotinate dehydrogenase 1.17.2.1 can essentially be expressed as an enrichment in c-type cytochromes, which have been implicated in uranium reduction in other organisms (Blakeney et al. 2000; Lloyd et al. 2003; Lovley et al. 1993 a/b). The

majority of the hits for the polar-amino-acid-transporting ATPase 3.6.3.21 sequence identified as heavy metal transporting P-type ATPases, which are known components of the toxicity resistance mechanisms for other heavy metals and may function similarly for uranium (Apell 2003). Statistical analysis may prove that mi-faser analysis functions more smoothly to identify enrichment for conserved functional domain motifs rather than enzymes as a whole when applied to single organism raw reads.

Discussion

High through-put sequencing of genomes is becoming a widespread hallmark of the scientific studies of new species, but the ability to accurately infer biologically relevant information from the sequence data is still somewhat lacking, especially for prokaryotic species. Annotation algorithms rely on biological databases to define the theoretical gene function of predicted coding sequences. One popular biological repository and pathway database is the Kyoto Encyclopedia of Genes and Genomes (KEGG), which relies on pathway data manually drawn, organized, and approved from literature based on model organisms, and which has an API allowing scientists and even other databases and programs to extract information about genes and pathways (Kanehisa & Goto 2000; Kanehisa et al. 2012; Kanehisa et al. 2017). While convenient, this type of annotation can be misleading for a number of reasons. Annotation using KEGG Orthologs is based entirely on sequence similarity, but as sequence similarity does not automatically confer functional similarity depending on the amount and locations of amino acid differences. Unfortunately, current methods rely too greatly on computational models rather than truly descriptive biological analysis.

The potential genes from *B. fungorum* Rifle chosen via this annotation mechanism were selected based on sequence homology to genes from bacteria known to interact with uranium, based on annotations which indicated that the gene was of completely unknown function (hypothetical or uncharacterized proteins), or identified as functionally involved in pathways with functions similar to the functions being studied (prospective heavy metal transport ATPase, phosphatase activity). Targeted transcriptome analysis failed to show any expression based variability between cultures unexposed and exposed to uranium, indicating constitutive expression for the selected proteins. However, transcript levels alone cannot be used to infer a direct relationship to protein levels or functionality. For example, operon regulator proteins are expressed constitutively, but can be active or inactive functionally based on the presence or absence of a signaling molecule. Similarly, a passive component of the cell wall or a multi-function gene product may be expressed at all times, and confer a functional interaction with uranium as a side benefit. For these reasons the genes identified via KEGG annotation pathways cannot be eliminated as possible targets and warrant further studies.

While the fusionDB algorithm was initially intended to phenetically classify organisms based on functional similarity, it may also be useful for extracting functionally relevant proteome overlaps amongst species. Utilizing the *in silico* genome annotation, a theoretical complete proteome was generated for the strain which encompasses all direct protein sequences *possible* for the cell to express under all conditions. This theoretical proteome was used to identify prokaryotic species with the highest degree of functional similarity by querying the fusionDB database. The database uses a recently developed program designed to exploit molecular whole genome functionality for classification

using FuSiON (functional-repertoire similarity-based organism network), which offers a consistent and objective function-based measure of organism similarity and shows high correlation with environmental niche diversification (Zhu et al. 2015; Zhu et al. 2016). Using these profiles, the pan-proteome was generated to show those proteins were present in all uranium reducers. The overlap/exclusion analysis of these pan-proteomes was used to identify proteins of probable interest. The program consistently groups of microorganisms based on molecular functionality, which reflects the environment for which that microbe is adapted (Zhu et al. 2015; Zhu et al. 2016). The genome of *B. fungorum* strain Rifle appears to have 8,123 CDS, but only 6,540 unique protein sequences are annotated, and among these sequences fusionDB only assigns 3,865 functions. By generating functionally relevant protein profiles the program is designed to provide a phenotype assessment which would provide more meaningful results in regards to the studies of a specific function (e.g. the ability to bioremediate uranium) than classical phylogenetic based assessments. In general, this is supported by our findings, as it is clearly visible that members of the genus *Burkholderia* are not always the most functionally similar to our strain despite a high degree of evolutionary relatedness. The products of their genomes predispose them to be suited to slightly different functional tasks and environmental niches. However, the use to which this study put the database was an overlap-exclusion analysis to identify proteins of functional similarity between dissimilar organisms which share a capability of interest. Using the proteomes defined for each prokaryote by the fusiondb system it was found that a total of 435 proteins were common among all ten species, and 42 proteins which were common to the 5 strains verified to have a functional interaction with uranium, but which show variable

expressivity in the other strains. These 42 proteins were initially targeted based on the assumption that the capability to reduce or resist uranium is not universal, but without experimental validation further analysis could extend into the 435 proteins shared across all genus involved. While the names assigned to each protein are based on classical annotation, generalized functions based on enzymatic classes tend to be reliable, so further studies would primarily focus on proteins with enzyme commission numbers indicating roles in redox reactions (EC 1) and active transport (EC 3). Analysis of the 4 hypothetical proteins identified isolated several conserved domains which could implicate them in the function of interest, in particular ABC permease capabilities such as those found in export proteins. Further computational analysis and knock-out studies are warranted. It should be noted these targeted investigations of potential target are preliminary and other approaches for the identification and study of possible genomic features involved in uranium interactions have not been exhausted.

Table 4.1. Gene targets chosen via traditional annotation based on homology to genes present in known uranium reducing species and their primer sequences

Gene of Interest Identifier	Forward Primer (sense)	Reverse Primer (antisense)	Amplicon length	Source Organism of KEGG homology	Y/N Successful Amp from DNA extract
maleate isomerase *	<u>GAAGAAGGTGGTGAAGGAAGAG</u>	<u>CCATGATCGCAACCAGACA</u>	119	Burkholderia sp. Y123	Y
hypothetical gme	<u>CACGTACTGGGCAACGATTA</u>	<u>ACAATCAGCGAGACGCATAG</u>	136	Geobacter metallireducens	Y
hypothetical2 gme	<u>GCGATGAGCCTTGGTTAICT</u>	<u>CCACAATCAGCGACCAATAAAG</u>	121	Geobacter metallireducens	Y
Sefir domain protein (gur)	<u>ACCGCGAAGTTCATTCTATAA</u>	<u>CCACAATCAGCGACCAATAAAG</u>	125	Geobacter uratireducens	Y
HipA (geb)	<u>GACAGACCCATGCGTAACTT</u>	<u>CAAGAAACCGGTCTCTACATC</u>	106	Geobacter sp. M18	Y
transposase (gen)	<u>CTCCTCCGTTTCTCCTTTGTT</u>	<u>GCGATTAGATCAGGATACCTT</u>	126	Geobacter sp.	Y
HMT P-type ATPase	<u>CAGGCGCTGATGAGTATGA</u>	<u>GCGCTCTTCTTCGTCATAGG</u>	123	Acidobacterium capsulatum	Y
hypothetical spl	<u>AACGCGCTCGGCTAATT</u>	<u>TGTCGGAATTGTGTCGATT</u>	102	Shewanella pealeana	Y
CopG DNA-binding domain	<u>CAACAGCATGCGGAATTGG</u>	<u>AGTGGAAGTTTGGCAATCGT</u>	119	Geobacter sp. M18	N
Hypothetical sbp	<u>TCCGATCATGAGCCATTGATTA</u>	<u>CGCCTGATATCCGTGAAAAGT</u>	105	Shewanella baltica OS223	Y
HMT transport/detox	<u>TACGAATGCGATCCGTGAAC</u>	<u>CGTCGATGGCGCTCTTTAAT</u>	116	Burkholderia phytofirmans	Y
HMT response regulator	<u>CITATCTGAAGAAAGGCGCTGGA</u>	<u>CGATCACGTCGTACTCTTCTTC</u>	105	Burkholderia xenovorans LB4	Y
Nitrate reductase subunit	<u>GCAAGCGGATGGAGAAGAT</u>	<u>CTGCACATCGTCGAAATCTTG</u>	101	Acidovorax sp.	Y
C-class cytochrome biogenesis	<u>CGGCTTCTGCTGGGATT</u>	<u>GAATGAACAGGCCGCCATA</u>	128	Oligotropha carboxidovorans	Y
Class I cytochrome c housekeeping gene*	<u>GATTGGCATTCCTCTGCTTATTG</u>	<u>CTGCCGACGTCGTGATAAA</u>	98	Ralstonia eutropha JMP134	Y

Table 4.2. Proteins of interest generated by gene homology and proteome comparisons between *Burkholderia fungorum* strain Rifle and other known uranium reducers.

A. Traditional annotation and KEGG homology were employed to identify possible targets of horizontal gene transfer from known uranium reducers based on gene sequence similarities.

B. FusionDB generated pan-proteome between the proteome of *B. fungorum* and other known reducers identified shared functional proteins. Mi-faser analysis provided enrichment profiles for comparison, to potentially identify functionally significant genes of interest regardless of evolutionary providence.

A.

Annotation/KEGG	
Chosen for:	ID
<i>Geobacter metallireducens</i>	hypothetical gme
<i>Geobacter metallireducens</i>	hypothetical2 gme
<i>Geobacter uraniireducens</i>	Sefir domain protein (gur)
<i>Geobacter</i> sp. M18	HipA (geb)
<i>Geobacter</i> sp.	transposase (gem)
functional annotation	HMT P-type ATPase
<i>Shewanella pealeana</i>	hypothetical spl
<i>Geobacter</i> sp. M18	CopG DNA-binding domain protein
<i>Shewanella baltica</i> OS223	Hypothetical sbp
functional annotation	HMT transport/detox
functional annotation	HMT response regulator
functional annotation	Nitrate reductase subunit
functional annotation	C-class cytochrome biogenesis
functional annotation	Class I cytochrome c

B.

FusionDB		m-faser	
Protein ID	Annotation	E.C. Number	Enzyme Function
C_653	D-tyrosyl-tRNA(Tyr) deacylase	1.1.1.23	histidinol dehydrogenase
C_634	Deoxyuridine 5'-triphosphate nucleotidohydrolase (EC 3.6.1.23)	1.1.1.94	glycerol-3-phosphate dehydrogenase [NAD(P)+]
C_622	FIG00928228: hypothetical protein	1.17.1.8	4-hydroxy-tetrahydronicotinamide reductase
C_415	UDP-N-acetylmuramoylalanine-D-glutamate--2,6-diaminopimelate ligase (EC 6.3.2.13)	1.2.1.11	aspartate-semialdehyde dehydrogenase
C_287	Leucyl-tRNA synthetase (EC 6.1.1.4)	1.2.1.41	glutamate-5-semialdehyde dehydrogenase
C_1180	LSU m5C1962 methyltransferase RlmI	1.3.8.11	cyclohexane-1-carboxyl-CoA dehydrogenase
C_465	beta-lactamase-like protein	1.4.3.16	L-aspartate oxidase
C_1391	Iron-sulfur cluster-binding protein	1.8.1.4	dihydrolipoyl dehydrogenase
C_4	FIG00834007: hypothetical protein	3.1.1.61	protein-glutamate methyltransferase
C_45	COG1278: Cold shock proteins	3.1.26.12	ribonuclease E
C_710	INTEGRAL MEMBRANE PROTEIN (Rhomboid family)	3.1.26.3	ribonuclease III
C_970	3-polypropenyl-4-hydroxybenzoate carboxy-lyase (EC 4.1.1.-)	3.1.4.52	cyclic-guanilate-specific phosphodiesterase
C_941	3-polypropenyl-4-hydroxybenzoate carboxy-lyase UbiX (EC 4.1.1.-)	3.2.1.23	β-galactosidase
C_566	Oligopeptide transport system permease protein OppB (TC 3.A.1.5.1)	3.4.21.53	endopeptidase La
C_1183	Glycolate permease	3.5.4.33	tRNA(adenine34) deaminase
C_514	2-isopropylmalate synthase	3.6.3.14	H ⁺ -transporting two-sector ATPase
C_76	Anthranilate synthase, aminase component (EC 4.1.3.27) / Anthranilate synthase, amidotransferase component (EC 4.1.3.27)	3.6.3.31	polyamine-transporting ATPase
C_784	Glutamyl-tRNA reductase (EC 1.2.1.70)	3.6.3.32	quaternary-amine-transporting ATPase
C_145	Indole-3-glycerol phosphate synthase (EC 4.1.1.48) / Phosphoribosylanthranilate isomerase (EC 5.3.1.24)	3.6.3.39	lipopolysaccharide-transporting ATPase
C_204	TRAP dicarboxylate transporter, DctM subunit	3.6.3.54	Cu ⁺ -exporting ATPase
C_222	D-allulose-6-phosphate 3-epimerase (EC 5.1.3.-), row_id 1016	3.6.4.12	DNA helicase
C_283	3-isopropylmalate dehydratase large subunit (EC 4.2.1.33)		* does not include EC classes 2 and 4-6
C_302	Sulfate transport system permease protein CysT		
C_331	GTP-binding and nucleic acid-binding protein YchF		
C_375	hypothetical protein		
C_376	Methyltransferase corrinoid protein Dhaf_2146		
C_483	Glutamate racemase (EC 5.1.1.3)		
C_506	Fumarate hydratase class I, aerobic (EC 4.2.1.2)		
C_875	FIG01076780: hypothetical protein		
C_545	Tryptophan synthase alpha chain (EC 4.2.1.20)		
C_476	Amidophosphoribosyltransferase (EC 2.4.2.14)		
C_459	Geranylgeranyl pyrophosphate synthetase (EC 2.5.1.29)		
C_374	DNA primase (EC 2.7.7.-)		
C_372	Dihydrofolate synthase (EC 6.3.2.12) / Folylpolyglutamate synthase (EC 6.3.2.17)		
C_363	YgjD/Kae1/Qri7 family, required for N6-threonylcarbamoyl adenosine t(6)A37 modification in tRNA		
C_289	Phosphoribosylanthranilate isomerase (EC 5.3.1.24) / Tryptophan synthase beta chain (EC 4.2.1.20)		
C_231	ErkK/YbiS/YcfS/YnhG family protein		
C_174	Exodeoxyribonuclease IX (EC 3.1.11.-)		
C_149	Ethanolamine utilization protein similar to PduL		
C_1265	Arsenical-resistance protein ACR3 / Arsenate reductase (EC 1.20.4.1)		
C_1059	Formate dehydrogenase O alpha subunit (EC 1.2.1.2)		
C_178	Transketolase (EC 2.2.1.1)		

Table 4.3. Transcriptional regulation screen of target genes

Genes of Interest	Kegg similar	Ura	No Ura
hypothetical gme	<i>Geobacter metallireducens</i>	+	+
hypothetical2 gme	<i>Geobacter metallireducens</i>	+	+
Sefir domain protein	<i>Geobacter uraniireducens</i>	+	+
HipA	<i>Geobacter</i> sp. M18	+	+
transposase gem	<i>Geobacter</i> sp.	+	+
HMT P-type ATPase	<i>Acidobacterium capsulatum</i>	+	+
hypothetical spl	<i>Shewanella pealeana</i>	+	+
maleate isomerase*	<i>Burkholderia</i> sp. YI23	+	+
* housekeeping gene			

Figure 4.1. Approaches to distill protein complement to relevant portions.

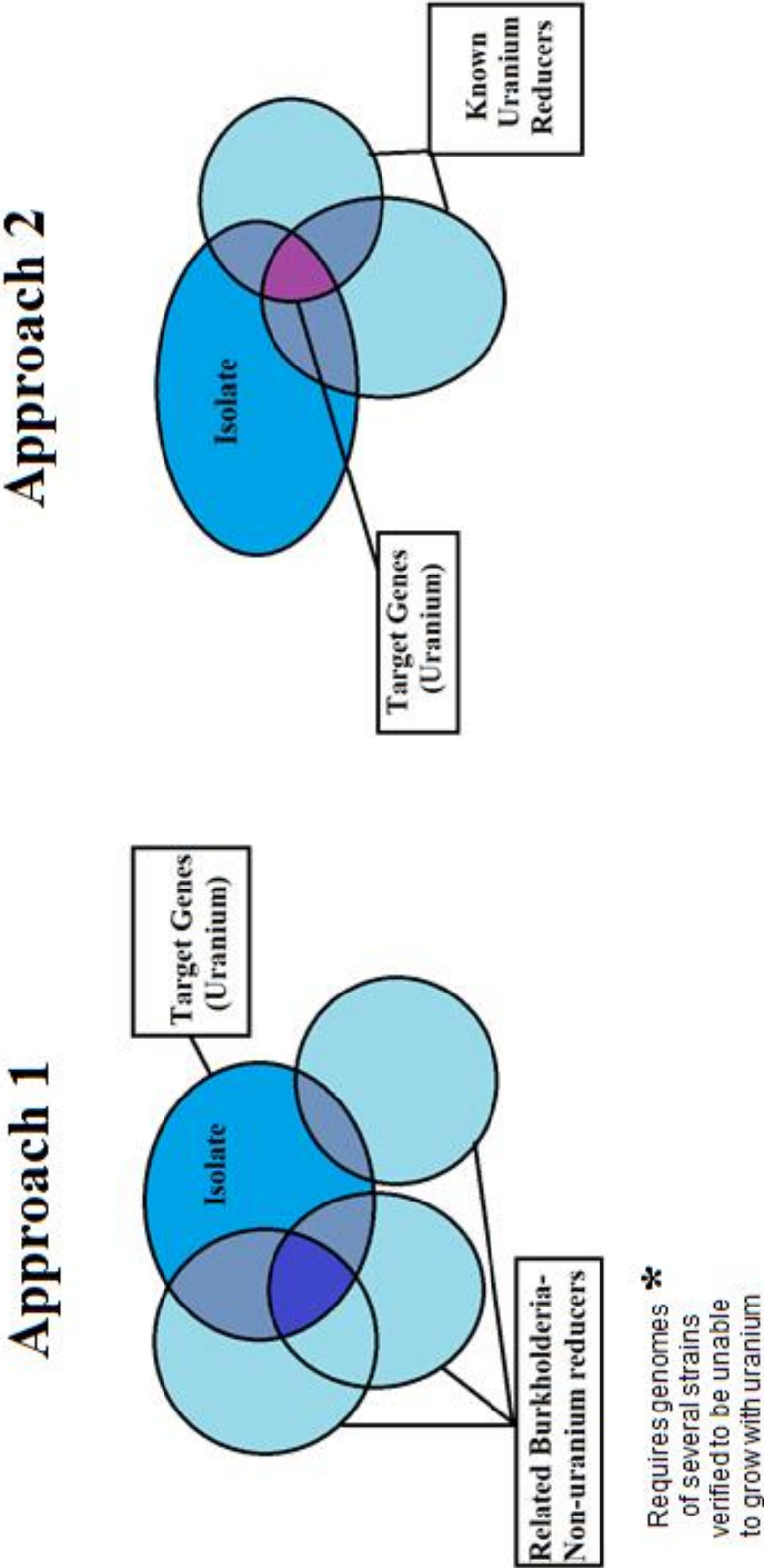


Figure 4.2. Schematic of genome data mining approaches.

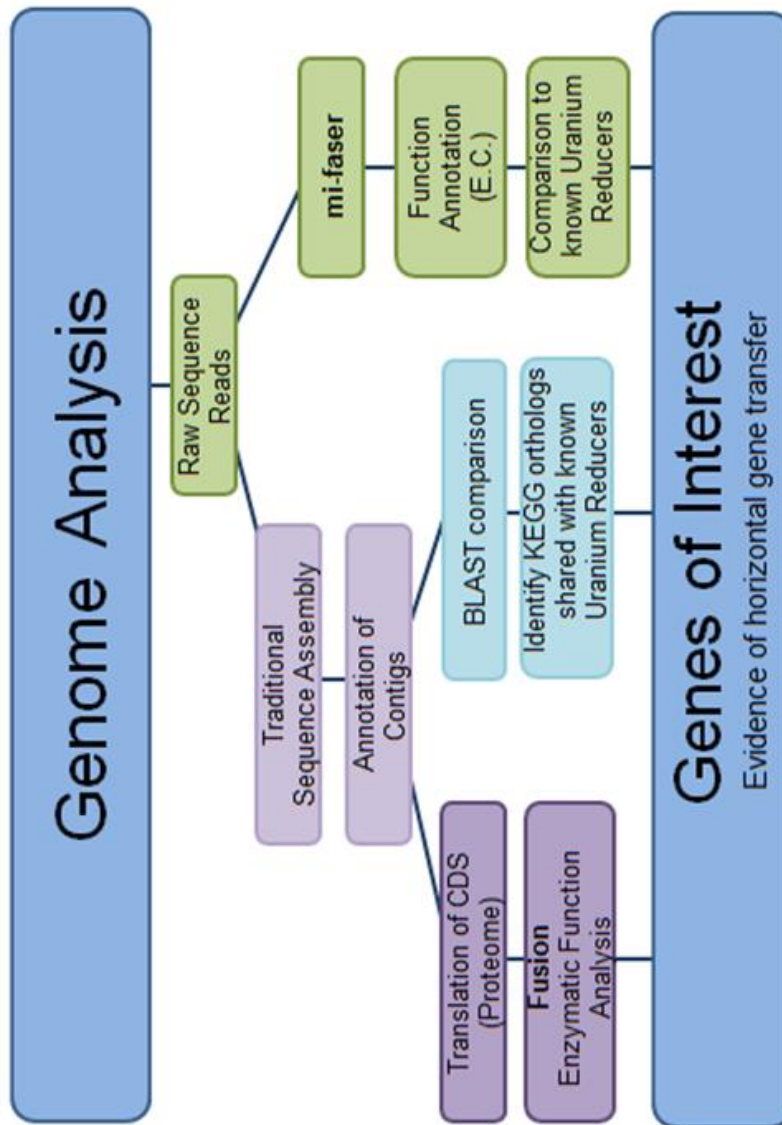


Figure 4.3. Representation of a subset of the assembled and annotated genome of *Burkholderia fungorum* strain Rifle

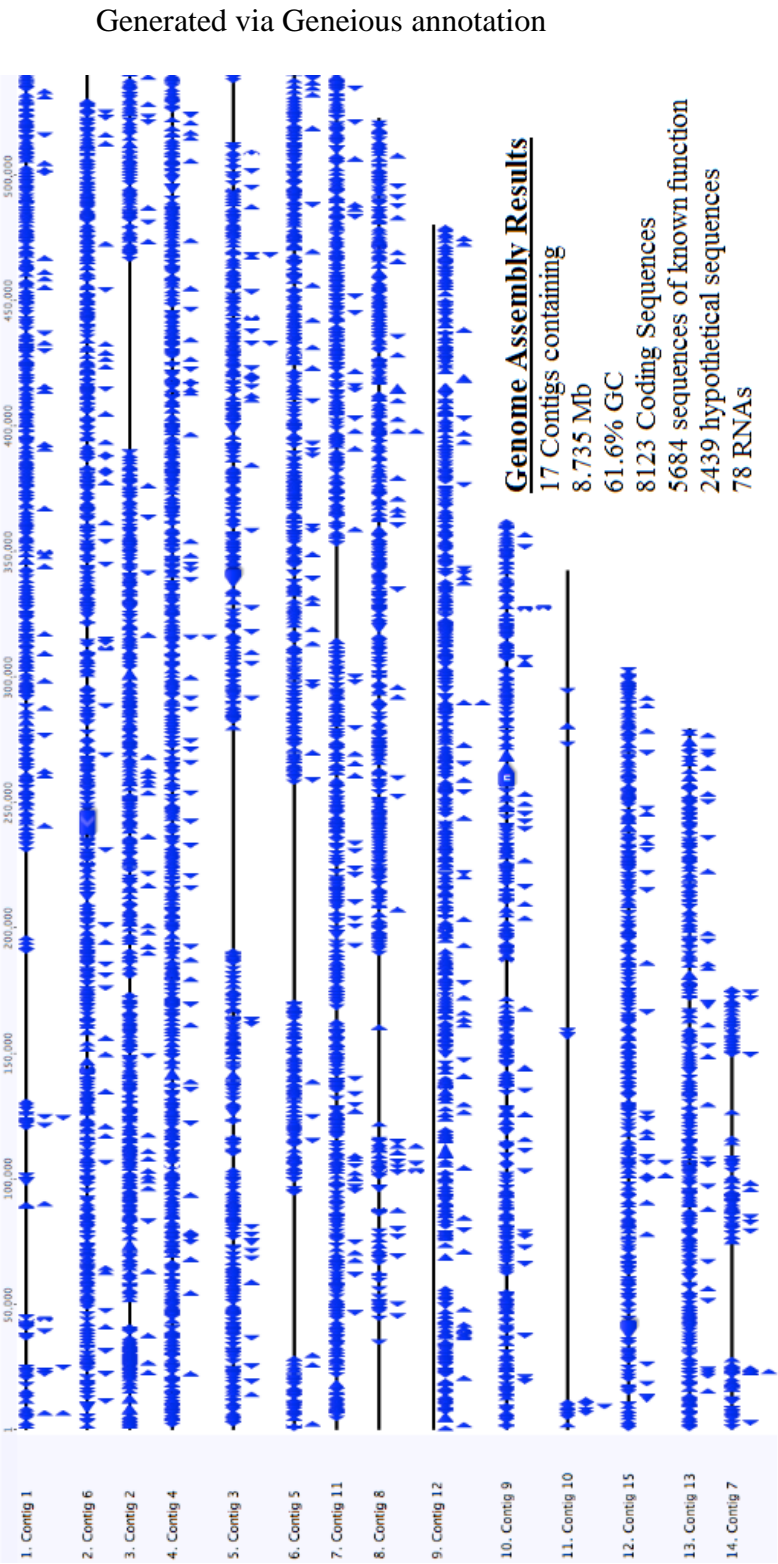


Figure 4.4. Mauve alignment of *B. fungorum* Rifle contigs against *B.fungorum* strain ATCC BAA-463.

LOD of 104

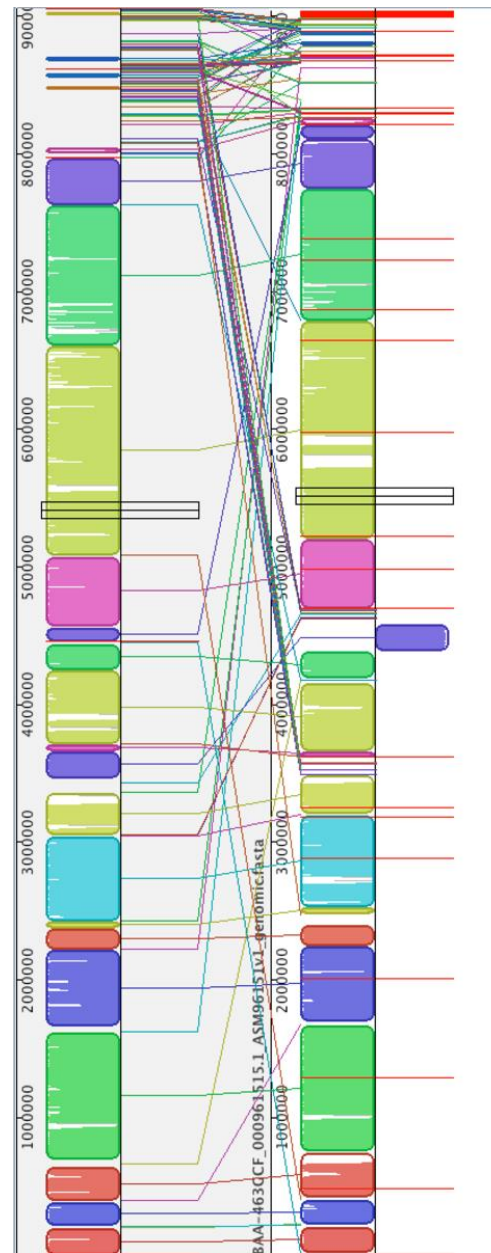


Figure 4.5. Electrophoresis gel showing rt-PCR product of genes identified as possible targets of interest based on gene sequence homology to genes of known uranium reducers.

Samples were extracted at Day 3 post transition to anaerobic conditions. Lanes marked U were exposed to uranium, lanes marked N were left unamended. Refer to Table 4.1 for gene identifiers.

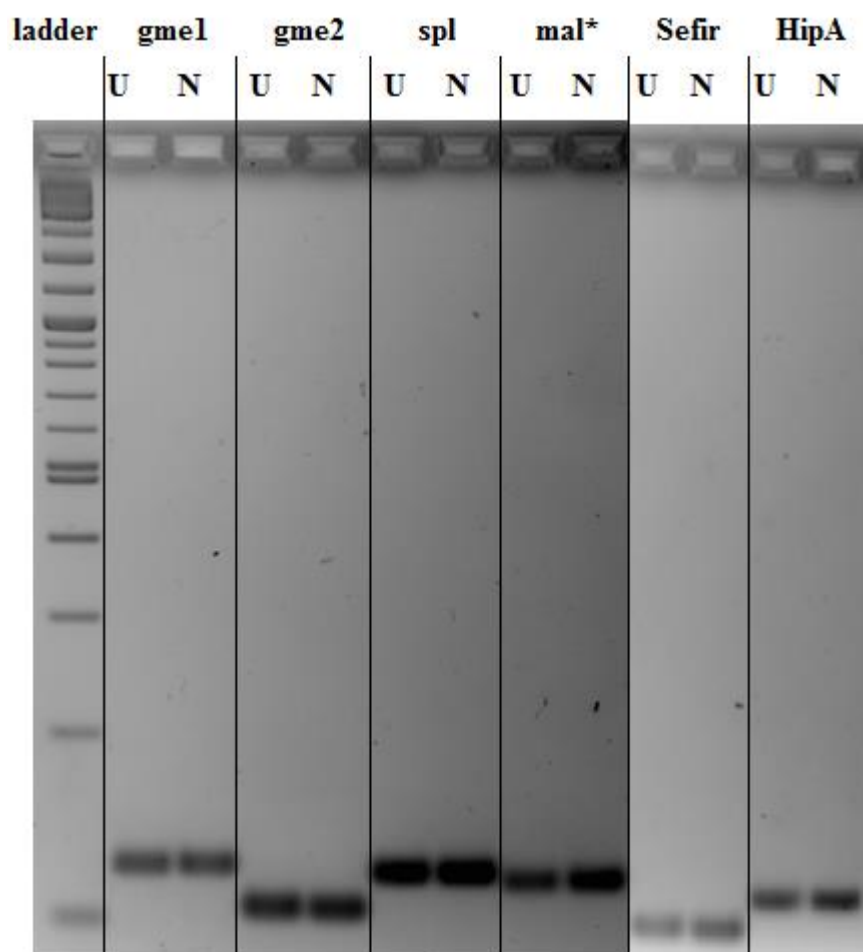


Figure 4.6. Multi-strain functional proteome analysis comparing *Burkholderia fungorum* Rifle to an array of proteomes for closely related strains, unrelated strains, and known uranium reducers.

Closely related species include *Burkholderia mallei* and *Burkholderia xenovorans*. Unrelated strains include *Achromobacter xylosoxidans* and *Variovorax paradoxus*. Known uranium reducing strains include *Desulfotomaculum reducens*, *Geobacter uraniireducens*, *Anaeromyxobacter dehalogenans*, *Shewanella oneidensis*, and *Cupriavidus metallidurans*.

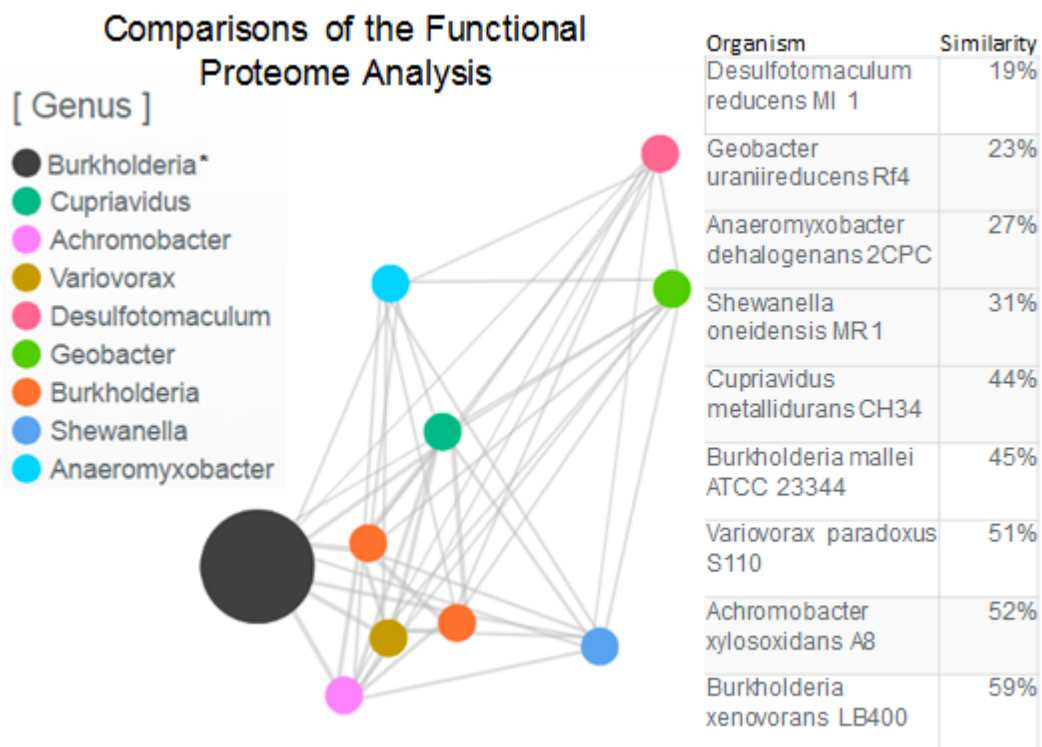
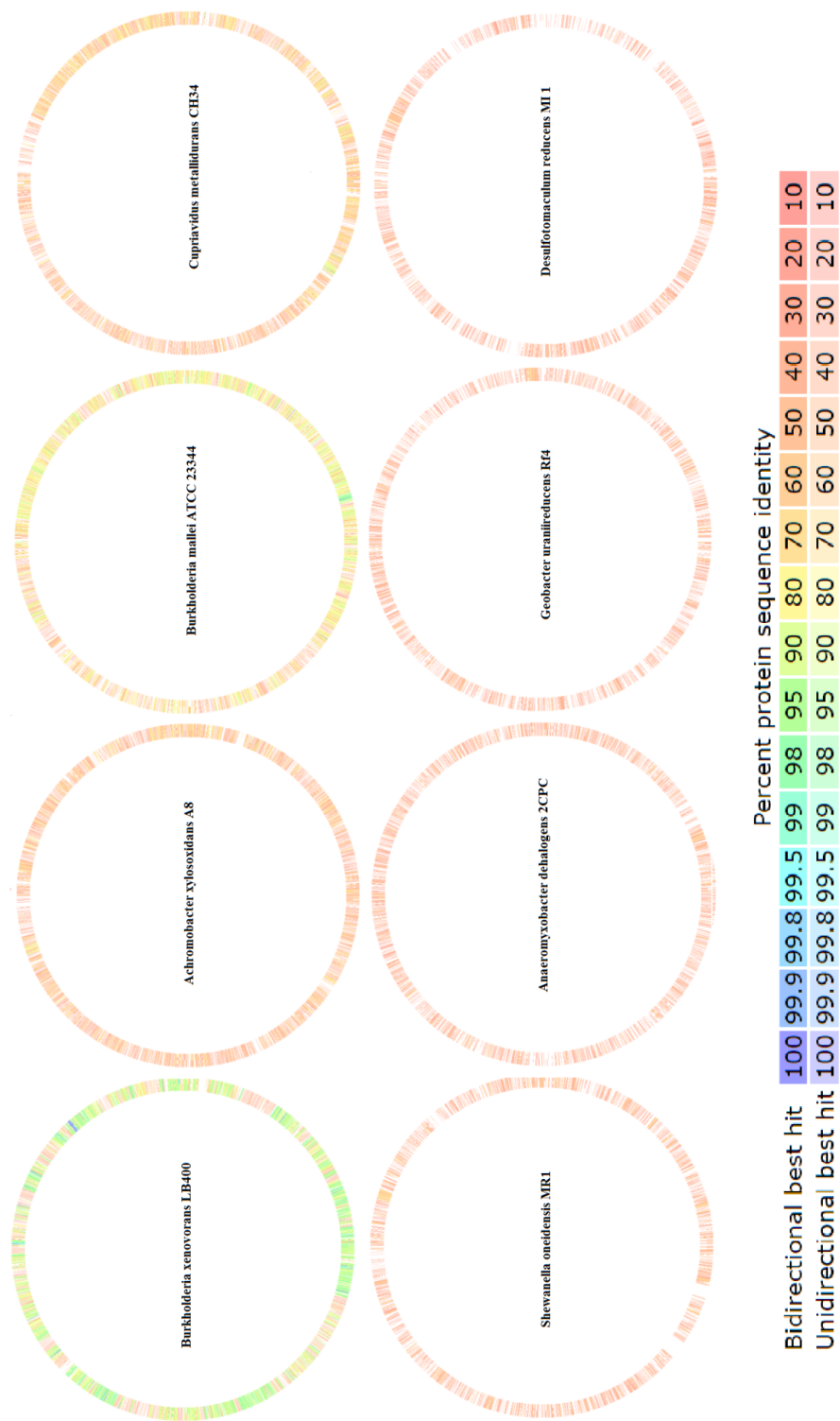


Figure 4.7. Protein sequence homology for all CDS throughout the genome of Burkholderia fungorum Rifle with genomes of example organisms.



Desulfotomaculum reducens M1, *Geobacter uraniireducens* Rf4, *Anaeromyxobacter dehalogenans* 2CPC, and *Shewanella oneidensis* MR1

Cupriavidus metallidurans CH34

Burkholderia mallei ATCC 23344 and *Burkholderia xenovorans* LB400

Achromobacter xylosoxidans A8 and *Variovorax paradoxus* S110

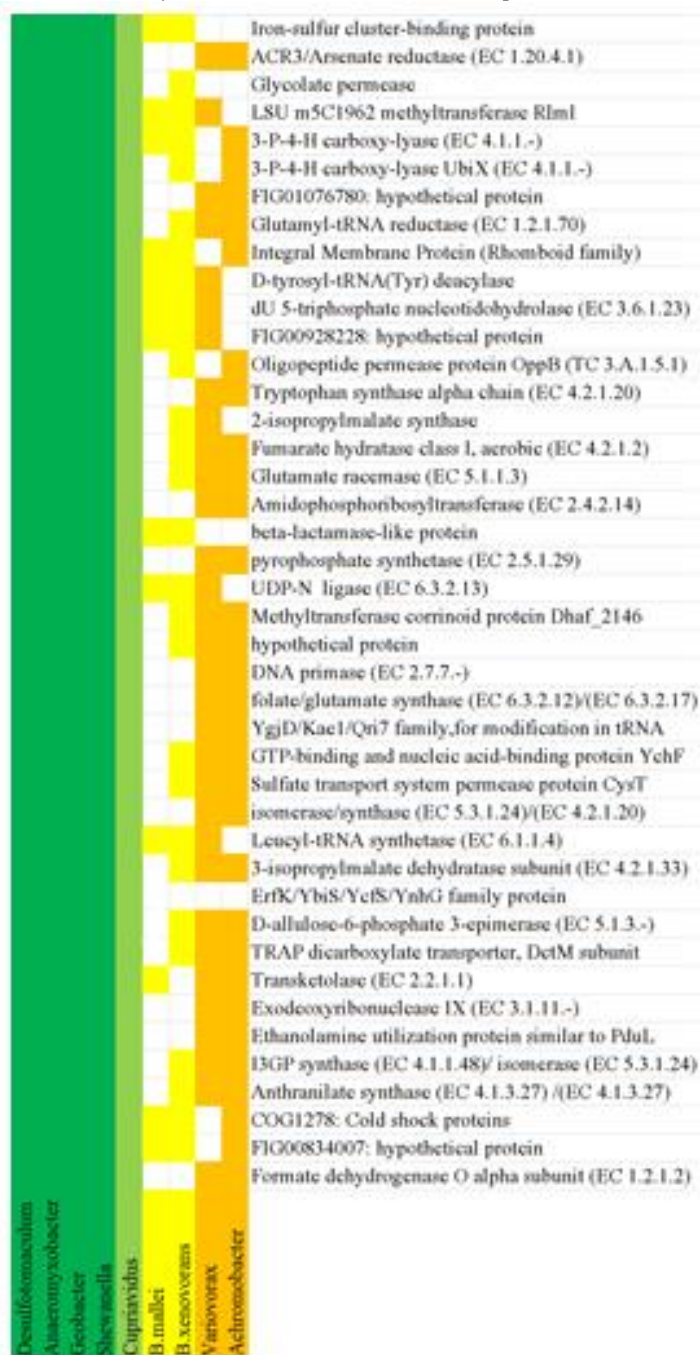


Figure 4.9. BLAST analysis of hypothetical proteins targets identified via FusionDB pan-proteome analysis for Burkholderia fungorum Rifle for conserved domain motifs.

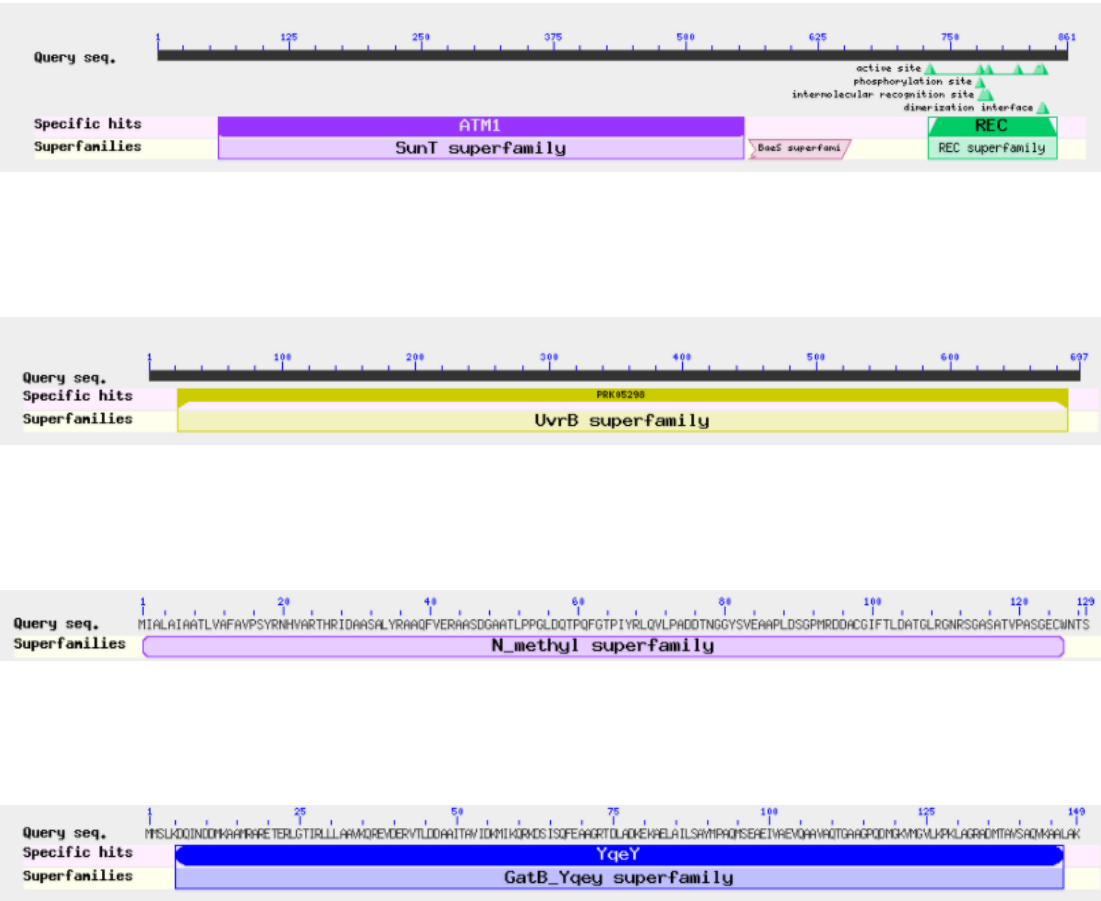
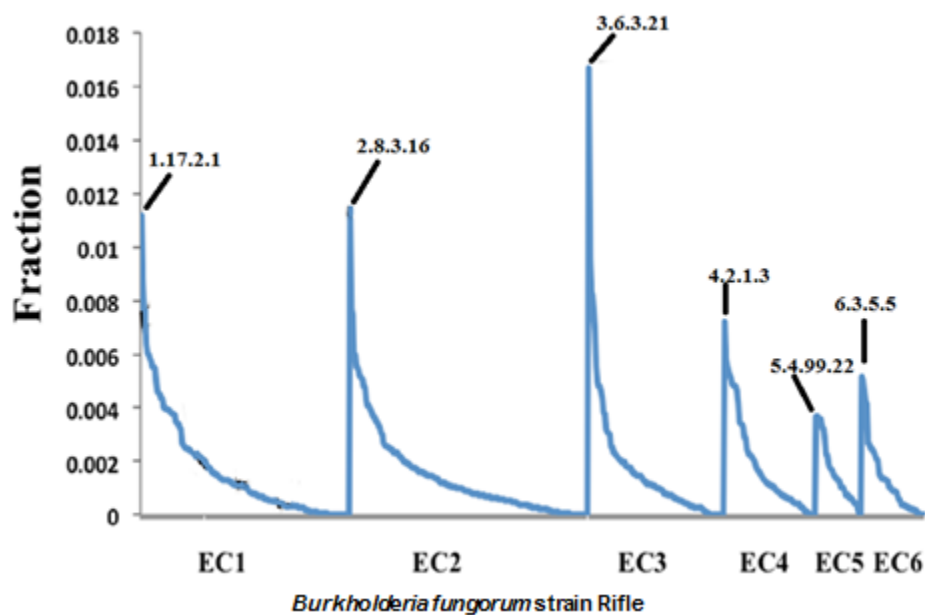


Figure 4.10. Enzymatic enrichment profile for *Burkholderia fungorum* Rifle generated by mi-faser.



CHAPTER V

Summary and Future Directions

A comprehensive understanding of the mechanisms behind the microbial interactions with toxic substances is essential for the effective development of bioremediation strategies. The employment of bioremediation to treat sites contaminated with the radionuclide uranium is a promising concept that focuses on preventing transport via groundwater movement. Stimulating the activity of the native bacterial community often proves an efficient *in situ* method to immobilize the uranium, with field trials verifying the success at specially designated field research sites (Barton et al. 1996; Anderson et al. 2003; Istok et al. 2004; Wu et al. 2006; Williams et al. 2011). It has been demonstrated that microbial processes have direct impact on uranium behavior in the environment (for review see Newsome et al. 2014). Elucidation of the mechanism(s) responsible for the electron transfer to uranium is a key goal to improving bioremediation, via mineralizing or sorption. This thesis was initiated to isolate a novel prokaryote capable of reducing uranium, with the goal of identifying genes for the microbe-radionuclide interactions.

Testing of oxidation state by XANES

Initial studies at the Rifle, CO IFRC identified microorganisms utilizing acetate which was shown to lower soluble uranium concentration in groundwater (Williams et al. 2010; Kerkhof et al. 2011). Subsequent studies utilized rRNA content to determine those microorganisms growing with uranium addition as a terminal electron acceptor (McGuinness et al. 2015). This led to efforts to isolate the various strains shown to grow

via the anaerobic reduction of uranium in the field and the recovery of *B. fungorum* strain Rifle. Although we observed increases in cell count and insolubilization of uranium at 1-5 μM concentrations, we could not rigorously prove speciation.

To assess the oxidation state of uranium under anaerobic growth conditions, the *B. fungorum* Rifle isolate was inoculated into modified minimal carbonate salts media amended with 200 μM sodium acetate and 13 μM uranyl acetate additions in 6 cumulative aliquots over a month under anaerobic conditions. For assessment of the insoluble uranium fraction, 240 mL of culture was separated by centrifugation (16,000 x g) in 2 mL aliquots for 90 seconds per subset in an anaerobic chamber and composited together into a single cell pellet (Coy Laboratory Products, Grass Lakes, MI, USA). The pellet was deposited into the slot of a poly(tetrafluoroethylene) sample holder, and sealed with X-ray transparent Kapton tape (Suda & Haraya 1997). Additionally, an aerobically grown inoculum was homogenized and transitioned into fresh 1% LB media, sparged and aliquoted into three treatment subsets. One aerobic treatment was amended with 300 μM calcium carbonate, one was incubated with 60 mg of sterile coralline sands, and the last was left untreated. The purposes of these amendments were to vary the initial complexation of the uranium ions before XANES analysis. These samples were allowed to incubate for 12 hours to eliminate any residual oxygen. Samples were amended to 200 μM uranium and 50 mL volumes collected and composited at three days post amendment.

All samples were heat sealed in mylar bags and transported with chemical oxygen scrub packs to the national synchrotron-radiation light source research facility (Argonne National Laboratory, Illinois, USA). Oxidation state was determined using Uranium

XANES analysis. Analysis of the X-ray absorption spectra of pellets prepared from cultures incubated anaerobically in modified minimal carbonate salts media showed that the uranium precipitate was comprised of oxidized U(VI) (data not shown). Similarly, all cultures incubated in LB also showed the uranium precipitate to be in the oxidized form (Figure 5.3). While these results supported the supposition that an alternative process than uranium reduction was not source of uranium insolubilization at concentrations exceeding 10 μ M uranium, the oxidized U(VI) in the anaerobic growth samples presented a dilemma that made XFAS analysis for chemical composition of precipitated uranium problematic. The results were ultimately inconclusive, since we could not assess if oxidation occurred during sample processing or shipment.

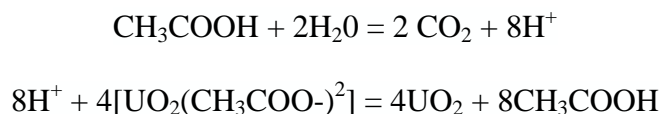
Colorimetric assays to determine uranium concentration

Prior to sample processing for the XANES analysis a piroxicam assay was performed to verify that centrifugation in the sub-oxic Coy chamber was sufficient to capture the insoluble uranium fraction. Piroxicam is a non-steroidal anti-inflammatory drug found to chelate U (VI) to form a yellow complex in 1,4-dioxan-water media that absorbs at 390nm, allowing for a spectrophotometric method to determine U (VI) concentrations (Lutfullah et al. 2009). When 2 mM uranyl acetate stock solution was used to create a serial dilution in water, then mixed with in a 1:3 ratio with a piroxicam solution made with methanol (final concentration of 0.032% piroxicam), a visible colorimetric spectrum was observed, though attempts to quantify spectrophotometrically failed, as the complex rapidly precipitated (Figure 5.4). Nevertheless, the generation of

yellow precipitate visible to the naked eye at concentrations as low as 25 μM provided a useful assay for our purposes. In order to generate sufficient signal, the XANES samples required concentration in volumes exceeding feasible filtration within the confines of the Coy Chamber, so preliminary 2 mL samples were centrifuged as described above. One subset of samples was washed twice with sterile anoxic water in the chamber, and the wash fluid saved for testing before the pellet was resuspended in oxic sterile water. Another subset was washed with sterile oxic water in the hood, then resuspended. When each of these was treated with piroxicam, the growth sample washed with sterile anaerobic water showed no color evolution in either wash, but demonstrated a yellow precipitate in the resuspended pellet. The sample washed with oxic water showed color evolution in the wash supernatant, indicating the presence of a reduced uranium particulate (data not shown). These results indicate that the anaerobic growth samples prepared for XANES analysis at the Argonne Laboratory underwent oxidation at some point during processing or transport.

Growth yields

Post publication of chapter 2 in PlosONE, growth yield calculations were re-examined for *Burkholderia fungorum* strain Rifle based on the following balanced redox reactions:



For every μmole of uranium reduced, 0.25 μmole of acetate is oxidized. This generates 3×10^6 femtograms of carbon per μmole of uranium reduced. Based on the

dimensions of 1.5 μm long and 0.5 μm diameter based on microscopy, and in accordance with the dimensions generally reported for members of this genus (i.e. $0.5\text{--}1 \times 1.5\text{--}4 \mu\text{m}$ as reported by Palleroni 2015) a cellular volume of $0.29 \mu\text{m}^3$ per cell was calculated, assuming minimal alteration in cell volume over the course of the 24 day growth period (personal observation). Based on the $63 \text{ fg C}/\mu\text{m}^3$ mean carbon content as reported based on the C:volume ratio of multiple aquatic and cultured bacteria, an approximate value of 18.3 fg/cell was used for our calculations (Fagerbakke et al. 1996). When this conversion factor was applied to the cell yields for 1, 2, and 3 μM concentration cultures, a total mass of 9.2×10^6 , 1.8×10^7 , and 2.7×10^7 femtograms of carbon per mL respectively were required to yield the cell increases reported. Based on the proportion of uranium reduced in each, 4.2×10^6 , 1.1×10^7 , and 1.75×10^7 femtograms of carbon per mL was generated by the reduction of uranium. Assuming 100% incorporation the carbon generated, the cell number increases reported are too high by a factor of 2. One possibility for this overproduction is oxygen contamination, wherein the culture flasks became microaerobic/suboxic rather than fully anaerobic during the experiment, allowing for some degree of biomass increase with oxygen reduction. Alternatively, the conversion factor used to calculate cells/mL for the fluorescent microscope could be incorrect by a factor of 2.

The cell counts reported in previous sections of this chapter use the units cells/mL, based on μMolar concentrations of uranium. Sanford et al. 2007 also reports all biomass increases in cells/ μmole of uranium reduced. This included 7.7×10^6 to 8.6×10^6 cells per μmole of uranium for *Anaeromyxobacter dehalogenans* as measured experimentally by the authors, and calculated the growth yield for several other species based on the

reports of other publications. Converting μMolar concentrations per mL into μmoles , the biomass increases for *Burkholderia fungorum* Rifle can be enumerated as 5.2×10^8 to 7.1×10^8 cells per μmole of uranium reduced. While this is two orders of magnitude greater than that reported for *Anaeromyxobacter dehalogenans* by Sanford et al., it is comparable to the calculated yield of 2.3×10^8 cells per μmole of uranium for *Shewanella putrefaciens* as calculated based on the Wade and DeChristina 2000 study. While cell yields can vary across species based on metabolic capabilities, cell volume/composition, and energy requirements (Fagerbakke et al. 1996, He & Sanford 2004), Sanford et al. reported the possibility of an enumeration bias depending on the method employed, based on comparisons of biomass yield for the same species in independent studies. Specifically, when yield was calculated for *Shewanella putrefaciens* in a separate study performed by Lovley et al. 1991, it was reported as 6.5×10^6 cells per μmole of uranium, a 35X difference from the Wade & DiChristina yields. Based on this it is reasonable to assume that an enumeration error based on the cell count method utilized might account for the discrepancy.

Additional genomic assessment of general anaerobic capabilities

Most members of the genus *Burkholderia* are primarily strict aerobes, relying on respiratory metabolism with oxygen as a terminal electron acceptor, but some species exhibit nitrate respiration under anaerobic conditions (Palleroni 2015). In the interest of assessing the general anaerobic capabilities of *Burkholderia fungorum* strain Rifle, the genome was evaluated for a number of genomic features associated with anaerobiosis. The RAST (<http://rast.nmpdr.org/>) pipeline was used for preliminary annotation of the

genome for the presence of key anaerobiosis genes as shown below. Once target genes of interest were discerned, the amino acid sequence from the target was retrieved from Genbank (<https://www.ncbi.nlm.nih.gov/genbank/>) and compared to the translated CDS sequences from the *Burkholderia fungorum* Rifle genome via BLAST to assess protein similarity and possible function.

Acetate oxidation pathways

Acetate oxidation via the citric acid cycle has been characterized under anaerobic conditions in *Geobacter sulfurreducens* (Galushko & Schink 2000). *Burkholderia fungorum* Rifle has a functioning system of enzymes for the citric acid cycle based on RAST annotation, as well as functioning citrate synthase, isocitrate dehydrogenase, and malate dehydrogenase, which have shown to be required in this study. While it does have acetate kinase, preliminary investigation of the draft genome did not definitively identify a phosphotransacetylase, though *B. fungorum* Rifle does have an enzyme annotating as propionyl-CoA:succinyl-CoA transferase, which has been shown to also function as succinyl-CoA:acetate CoA-transferase in some eukaryotes (Saz et al. 1996). Also present are two copies of a functional acetyl-coenzyme A synthetase (EC 6.2.1.1) enzyme which has been shown to reversibly convert acetate to acetyl CoA for inclusion in the TCA cycle in *Escherichia coli* (Kumari et al. 2000) and function in acetate oxidation in *Acetobacter* sp (Saeki et al. 1999). If *Burkholderia fungorum* strain Rifle is oxidizing acetate via the TCA cycle, further enzymatic studies are warranted to identify the exact pathway functions.

Alternative studies have found that acetate oxidation can also happen via the CO Dehydrogenase/Acetyl Coenzyme A Pathway (Hattori et al. 2005). *Burkholderia fungorum* strain Rifle has a functioning copy of some of the necessary genes, but lacks a complete system and a number of anaerobiosis specific forms. The formate dehydrogenase O operon is present though, which in *Escherichia coli* strains has been shown to be membrane bound and ubiquitously expressed regardless of the presence of oxygen, independent of the FNR regulation and responsible for the ability to rapidly respond to anaerobiosis and begin nitrate respiration (Abaibou et al. 1995). The carbon monoxide dehydrogenase subunit genes are present, but not homologous to those in *Carboxydotherrnus hydrogenoformans* (WP_011342982.1), an anaerobic bacteria which was characterized for the distinct [Ni-4Fe-5S] Ni-Fe-S configuration found in anaerobic versions of the enzyme (Dobbek et al. 2001), indicating it likely utilizes the aerobic version of this enzyme and is unlikely to be capable of utilizing this pathway for acetate oxidation unless one of the paralog versions without usual motifs utilizes an alternative configuration for anaerobic function.

Global regulation of anaerobiosis

In response to alteration in oxygen levels, microorganisms employ regulatory sensors to respond to changes in redox conditions (Green & Paget 2004). The response regulatory systems for anaerobiosis have been studied extensively in *Escherichia coli*, including 2-component systems such as the Arc A/B and Nar X/L systems and activator/repressor proteins such as FNR (Allen 1993). FNR is a transcription factor well studied in *Escherichia coli* which has been shown to control the regulation of numerous

genes and operons in response to oxygen deprivation (Sawers et al. 1988, Constantinidou et al. 2006). It is 250 residues long, with the majority of the protein at the C-terminal end being highly similar in sequence and function to the catabolite activator protein (Shaw et al. 1983). Within the N-terminal 30 amino acids unique to the FNR therein lies a specific consensus sequence rich in cysteines which is responsible for forming an oxygen sensitive iron-sulfur cluster (Kiley & Beinert 1999). FNR has also been identified in *Shewanella* species (Cruz-Garcia et al. 2011) and homolog ANR in *Pseudomonas aeruginosa* (Schreiber et al. 2007).

These homologous FNR and ANR regulators have been shown to contain some overlap in their specificity for anaerobically inducible promotor sequences, but had distinct specificities (Winteler & Haas 1996). Blast homology of the FNR protein sequences for FNR from *Escherichia coli* str. K-12 substr. MG1655 (NP_415850.1), and *Shewanella oneidensis* MR-1 (NP_717946.2), and the ANR of *Pseudomonas aeruginosa* (NP_250235.1), identifies the protein of closest homology to be a transcriptional regulator from the CRP/FNR family, but it lacks the critical N-terminal sequence to function as an FNR-like oxygen sensitive transcriptional regulator, making this mechanism unlikely in this strain. Finally, studies of other *Burholderia* species identified a fixLJ system for oxygen sensing and gene regulation (Schaefer et al. 2017). With two component oxygen sensor histidine kinase fixL (NP_769400.1) and response regulator fixJ (NP_435915.1) function to regulate expression in response to oxygen levels. BLAST homology uncovered a number of functionally similar genes in *B. fungorum* Rifle, but these genes cannot be positively identified as oxygen sensing systems without experimental data.

Alternate anaerobic respiratory components

Oxireductases are enzymes which catalyze the transfer of electrons, and are critical for respiratory processes. Cytochromes are heme-proteins which function as part of the electron transport chain, ultimately contributing to the generation of ATP. They are classified based on their reduced state energy absorption with c-type cytochromes absorbing at approximate 550 nm. The cytochrome c family are water soluble electron-transfer proteins which bind their heme groups with thioether bonds and function in a variety of redox processes. C3 cytochromes are Class III c-type cytochromes with multiple hemes but low redox potential, ranging from 0 to -400 mV, which transfer electrons to a variety of lower energy electron acceptors (Pereira & Xavier 2006).

The annotated genome of *Burkholderia fungorum* Rifle contains several respiratory reductases classified as anaerobic (total of 30 CDS annotated), including arsenate reductase regulator (ArsR), ferredoxin, and ferric reductases, and a variety of cytochromes, including the oxireductases and dehydrogenases with c-type cytochrome motifs identified during mi-faser analysis as functionally similar to nicotinate dehydrogenase. Other *Burkholderia* strains capable of sustaining anaerobic growth utilize nitrate reduction. For example, experiments utilizing sealed cultures in LB broth with *Burkholderia pseudomallei* showed that fully aerated media was depleted within 3 hours, and that by 9-12 hours post-sealing (6-7 generations) the cultures ceased to grow and showed persistence with a decline over the next year (Hamad et al. 2011).

Nitrate Reduction

Investigation of *Burkholderia fungorum* strain Rifle's genome indicated the presence of NarGHJI, and though it lacked specific genes recognized as NarR and NarK, it did show upstream functional nitrate/nitrite transporters, sensors, and regulators with sequence similarity to the NarX protein (NP_415740.1) and the transporter sequence generated by NarK (NP_415741.1). In place of a single NarK gene there appears to be a tandem repeat of the transporter gene, and the two copies vary from one another in length by 27 amino acids. Figure 5.4a shows the operon configuration of this strain. The nitrate/nitrite sensor is just downstream of a second, the two of which have similarity to the NarXL sensors, a configuration is similar to the *Pseudomonas aeruginosa* configuration wherein the first is anaerobically induced by Anr and Dnr regulators (Schreiber et al. 2007). While Dnr (NP_249218.1) is present, the Anr analog is the same CRP/FNR-like protein which was identified by FNR homology, and still lacked that presumably critical n-terminal region (24 amino acids). These findings suggest *Burkholderia fungorum* strain Rifle contains a complete Nar operon.

Sulfate Reduction

Other anerobic respiratory pathways assessed included sulfate reductase capabilities. Anaerobic dimethyl sulfoxide reductase has been well studied in *Escherichia coli* (Sambasivarao & Weiner 1991, Bilous et al. 1988) and consists of three subunits which were found in *Burkholderia fungorum* strain Rifle, shown in Figure 5.4b. It has adenylylsulfate reductase beta (YP_010067.1) subunit homologs, but the downstream gene which has some homology to adenylylsulfate reductase alpha (YP_010068.1) also

has additional nonspecific domains which caused the RAST annotation software to identify it as a putative reductase. One of the nonspecific blast domains identified by RAST was *aprA*, the adenylylsulfate reductase alpha. While the exact mechanisms of anaerobic regulation and function are ambiguous, the genome appears to contain a number of features indicative of anaerobic functional capabilities. Further study into what global response regulator if any responds to anoxic conditions in this strain are needed.

In conclusion, this thesis project focused on isolation of a novel prokaryotic strain capable of growth on uranium, and investigated possible methods of identifying genomic features of interest for future study in regards to microbe-uranium interactions. The isolation of a previously unexpected strain belonging to a class not known for uranium reduction capabilities emphasizes how widespread the function is within prokaryotes. We report no discernable biomineralization by the strain, but future experiments are warranted to evaluate the presence of uranium efflux pumps which function under aerobic conditions to resist the toxic effects of uranium. Finally, we assessed several methods to compare the genomic features of *B.fungorum* strain Rifle and compare them to the genomic repertoire of several other known uranium reducers in order to perform an overlap analysis to identify features likely to have functional relevance to the reduction of uranium. The exact proteins involved in the reduction of uranium are yet to be determined, but a library of gene targets for future studies was generated. While mi-faser presents a novel strategy to identify general function classes for which a pure strain genome is enriched, it is poorly suited to the identification of exact genes responsible for specific functions, and excludes any hypothetical proteins with entirely novel or unknown functions. FusionDB provided a much more promising method for functional analysis of

genomes. Further studies are called for to assess the capabilities of this bioinformatics tool to isolate genome features of interest. The next step regards assessment of the genes identified through knockout studies. These findings may advance the study of uranium bioremediation, and this method of identifying genes of interest would, if refined, benefit the scientific community in general.

Figure 5.1. Scanning electron microscope photograph of *B. fungorum* strain Rifle.

No evidence of uranium sorption was observed, though particulate debris was found to contain the radionuclide.

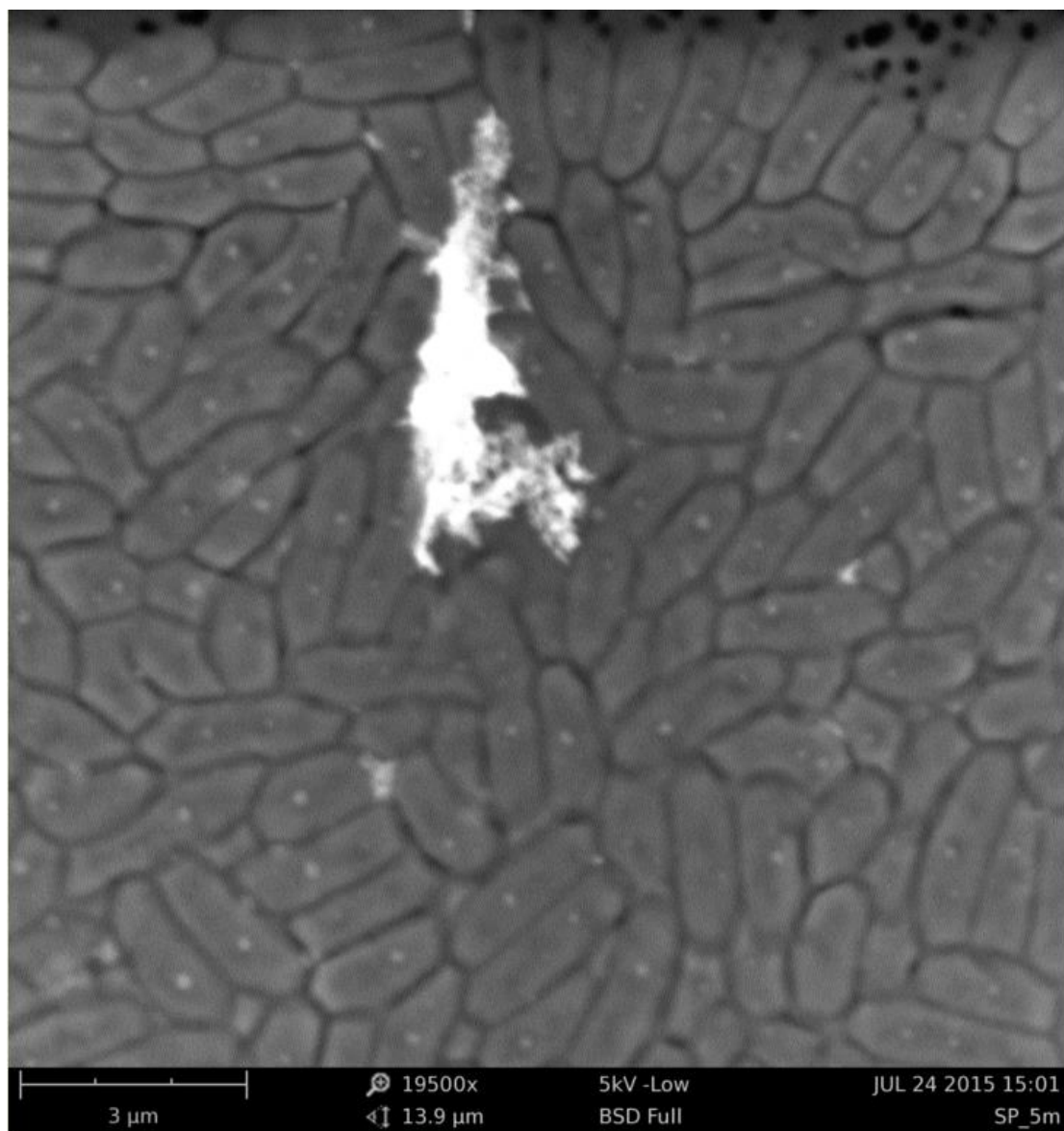


Figure 5.2. XANES Analysis of insoluble uranium fraction under anaerobic conditions.

Sample A is culture in LB alone, Sample T culture incubated with 300 μM calcium carbonate, and Sample R samples incubated with 1.2 mg/ml sterile coralline sands.

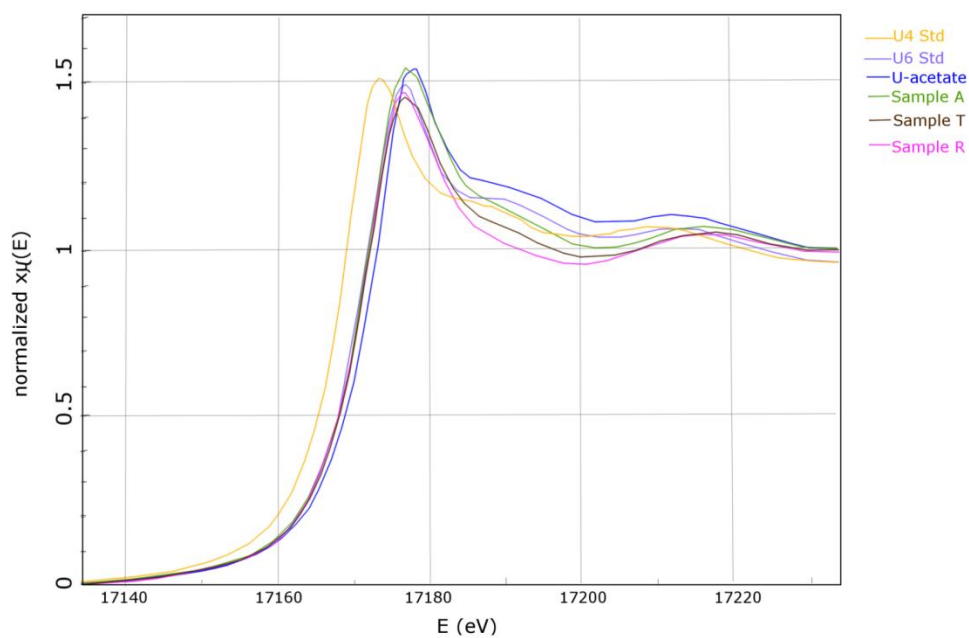


Figure 5.3. Visualization of piroxicam-uranium assay.

Uranium concentrations range from 0-150 μM .

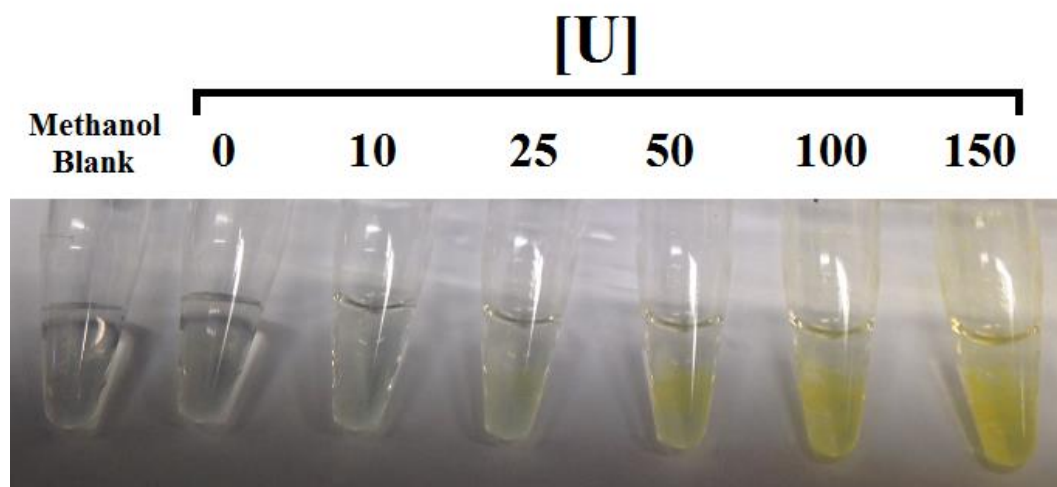
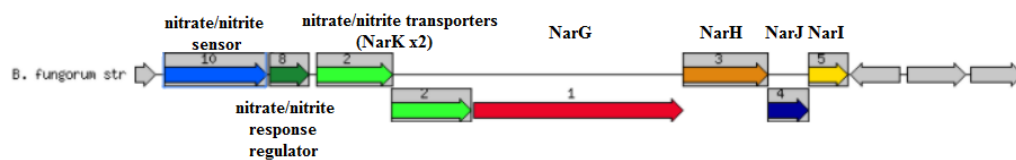
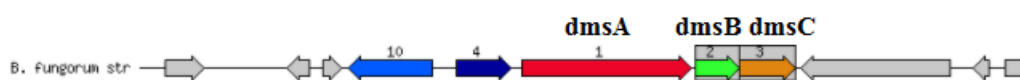


Figure 5.4. Diagram representations of nitrate and sulfate reduction genes

A



B



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